#99
SOY BEAN OIL, HYDROGENATED VOL IV
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X27

Soy Bean Oil Hydrogenated-Volume 4 #99 12/27/73

VOLUME 4

# GRAS MONOGRAPH SERIES
# SOY BEAN OIL, HYDROGENATED

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

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## PART 45—OLEOMARGARINE, MARGARINE

Sec.
45.1 Oleomargarine, margarine; identity; label statement of optional ingredients.
45.2 Liquid oleomargarine, liquid margarine; identity; label statement of optional ingredients.

### § 45.1 Oleomargarine, margarine; identity; label statement of optional ingredients.

(a) Oleomargarine, margarine is the plastic food prepared with one or more of the optional fat ingredients named in subparagraph (1) (i), (ii), (iii), and (iv) of this paragraph, as follows:

(i) (i) The rendered fat or oil, or stearin derived therefrom (any or all of which may be hydrogenated), of cattle, sheep, swine, or goats, or any combination of two or more of such articles.

(ii) Any vegetable food fat or oil, or oil or stearin derived therefrom (any or all of which may be hydrogenated), or any combination of two or more of such articles.

(iii) Any combination of ingredients named in subdivisions (i) and (ii) of this subparagraph, in such proportion that the weight of the ingredients named in subdivision (i) either equals the weight of the ingredients named in subdivision (ii), or exceeds such weight by a ratio of not greater than 9 to 1.

(iv) Any combination of ingredients named in subdivisions (i) and (ii) of this subparagraph, in such proportion that the weight of the ingredients named in subdivision (ii) exceeds the weight of the ingredients named in subdivision (i) by a ratio not greater than 9 to 1.

(2) One of the articles designated in subdivisions (i), (ii), (iii), (iv), (v), (vi), (vii), (viii), or (ix) of this subparagraph is intimately mixed with the fat ingredient or ingredients. The ingredients named in subdivisions (i), (ii), (iii), (iv), (v), (vi), and (vii) of this subparagraph are pasteurized and then may be subjected to the action of harmless bacterial starters. The term "milk" as used in this subparagraph means cow's milk.

(i) Cream.

(ii) Milk.

(iii) Skim milk.

(iv) Liquid sweet cream buttermilk.

(v) Any combination of dry or condensed sweet cream buttermilk and water with a total solids content of not less than 8.5 percent.

(vi) Any combination of nonfat dry milk and water, in which the weight of the nonfat dry milk is not less than 10 percent of the weight of the water.

(vii) Any mixture of two or more of the articles named in subdivisions (i), (ii), (iii), (iv), (v), and (vi) of this subparagraph.

(viii) In case only of the fat ingredient designated in subparagraph (1)(ii) of this paragraph, any combination of finely ground soybeans and water, in which the weight of the finely ground soybeans is not less than 10 percent of the weight of the water. The finely ground soybeans are subjected to a heat treatment before or after mixing with the water. The soybeans may or may not be dehulled.

(ix) Water in lieu of any of the foregoing articles of this subparagraph. Congealing is effected, either with or without contact with water, and the congealed mixture may be worked.

(3) In the preparation of oleomargarine one or more of the following optional ingredients may also be used:

(i) Artificial coloring. For the purposes of this subdivision provitamin A shall be deemed to be artificial coloring.

(ii) Sodium benzoate or benzoic acid or a combination of these, in a quantity not to exceed 0.1 percent of the weight of the finished product.

(iii) Vitamin A (with or without any accompanying vitamin D and with or without vitamin D concentrate), in such quantity that the finished oleomargarine contains not less than 15,000 United States Pharmacopeia units of vitamin A per pound, as determined by the method prescribed in the Pharmacopeia of the United States for total biological vitamin A activity. The vitamin A potency prescribed may be furnished by fish liver oil; by concentrates of vitamin A or its fatty acid esters from animal sources; by synthetic vitamin A or its fatty acid esters; by mixtures of synthetic vitamin A or its fatty acid esters with harmless substances formed during the synthesis of the vitamin A, if the vitamin A or its fatty acid ester constitutes not less than 50 percent of the mixture; by provitamin A; or by any combination of two or more of these. For the purposes of this subdivision the term "fatty acid" may include acetic acid.

(iv) Any safe and suitable artificial flavoring substance that imparts to the food a flavor in semblance of butter. Such artificial flavoring substances are deemed to be safe if they are not food additives as defined in section 201(s) of the Federal Food, Drug, and Cosmetic Act; or, if they are food additives as so defined, they are used in conformity with regulations established pursuant to section 409 of the act.

(v) (a) Lecithin, in an amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(b) Monoglycerides or diglycerides of fat-forming fatty acids, or a combination of these, in an a... not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(c) Such monoglycerides and diglycerides in combination with the sodium sulfo-acetate derivatives thereof, in a total amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(d) A combination of (a) and (b) of this subdivision, in which the amount of neither exceeds that above stated; or

(e) A combination of (a) and (c) of this subdivision, in a total amount not exceeding 0.5 percent of the weight of the finished oleomargarine.

The weight of the diglycerides in each of ingredients (b), (c), (d), and (e) of this subdivision is calculated at one-half actual weight.

(vi) Butter.

(vii) Salt.

(viii) Citric acid incorporated in the fat or oil ingredient used.

(ix) Isopropyl citrates incorporated in the fat or oil ingredient used, in an amount not to exceed 0.02 percent by weight of the finished oleomargarine.

(x) Stearyl citrate incorporated in the fat or oil ingredient used, in an amount not to exceed 0.15 percent by weight of the finished oleomargarine.

(xi) Potassium sorbate, in an amount not to exceed 0.1 percent by weight of the finished oleomargarine.

(xii) Calcium disodium EDTA (calcium disodium ethylenediaminetetraacetate), in an amount not to exceed 75 parts per million by weight of the finished oleomargarine.

(xiii) BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene), or a combination of these, incorporated in any animal fat ingredient permitted by subparagraph (1)(i) of this paragraph in an amount not to exceed 0.02 percent by weight of such animal fat content.

The finished oleomargarine contains not less than 80 percent fat, as determined by the method prescribed in "Official Methods of Analysis of the Association of Official Agricultural Chemists," 7th Edition, page 259 under "Indirect Method," section 15.111. [Ed. note, 10th edition, 1965, p. 242, sec. 15.132.]

(b) The name of the food for which a definition and standard of identity is prescribed by this section is "oleomargarine" or "margarine." The presence







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of optional ingredients, provided for in paragraph (a) of this section, in the finished margarine shall be declared as set forth in this paragraph.

(1) Fat ingredients shall be declared first in the ingredient statement by the name of the specific fat or oil or stearin used. Where combinations of fat ingredients are used, the names shall be arranged in order of decreasing predominance. If any fat ingredient is hydrogenated, the ingredient statement shall include the word "hydrogenated" or "hardened" at such place or places in the list of fats as to indicate which fats are hydrogenated; for example, "corn oil, hardened soybean oil." If any animal fat ingredient contains an ingredient provided for in paragraph (a)(3)(xiii) of this section, the statement "with _____ added as (a) preservative(s)" or "with _____ added to retard rancidity" shall appear at such place or places in the list of fats as to indicate which animal fats contain these ingredients. The blank is to be filled in with "BHA" and/or "BHT" as appropriate; for example, "beef fat with BHA and BHT added as preservatives".

(2) Immediately following the listing of fat ingredients, other optional ingredients used shall be named in the order of decreasing predominance.

(i) The optional ingredients butter, salt, water, cream, milk, skim milk, sweet cream buttermilk, dried sweet cream buttermilk and water, condensed sweet cream buttermilk and water, nonfat dry milk and water, ground soybeans and water, lecithin, mono- or diglycerides, sodium sulfo-acetate derivatives of mono- or diglycerides shall each be declared by those terms.

(ii) Artificial colors shall be declared by the statement "Artificially colored" or "Artificial coloring added" or "With added artificial coloring."

(iii) Artificial flavors shall be declared by the statement "Artificially flavored" or "Artificial flavoring added" or "With added artificial flavoring."

(iv) Margarine that contains the optional ingredients citric acid, isopropyl citrate, stearyl citrate, or calcium disodium EDTA shall be labeled by the statement "_____ added as a preservative" or "_____ added to protect flavor." Margarine that contains the optional ingredients sodium benzoate or benzoic acid shall be labeled by the statement "_____ added as a preservative" or "_____ as a preservative" or "With added _____ as a preservative." Margarine that contains the optional ingredient potassium sorbate shall be labeled by the statement "_____ added as a preservative" or "_____ added to retard mold growth." The blank in each of the statements in this subdivision is filled in with the common name of the preservative ingredient used.

(v) Vitamin A shall be declared by the statement "Vitamin A added" or "With added vitamin A." Vitamin D shall be declared by the statement "Vitamin D added" or "With added vitamin D." Margarine containing added vitamin A, vitamin D, or both, is subject to the regulations for foods for special dietary use promulgated under the provisions of section 403(j) of the act.

(vi) Where two or more optional ingredients named in paragraph (a)(3) of this section are used the words "Added" or "With added" need appear only once, either at the beginning or end of the list of such ingredients declared.

(3) Wherever the name "oleomargarine" or "margarine" appears on the label so conspicuously as to be easily seen under customary conditions of purchase, the words and statements prescribed in this section, showing the ingredients used shall immediately and conspicuously precede or follow, or in part precede and in part follow, such name, without intervening written, printed, or other graphic matter.

(Secs. 401, 701, 52 Stat. 1046, as amended, 1055, as amended; 21 U.S.C. 341, 371) [20 F.R. 9615, Dec. 20, 1955, as amended at 23 F.R. 7473, July 23, 1958; 29 F.R. 2323, Feb. 12, 1964; 31 F.R. 5433, Apr. 6, 1966; 33 F.R. 17752, Nov. 28, 1968; 36 F.R. 977, Jan. 21, 1971; 36 F.R. 2400, Feb. 4, 1971]

## § 45.2 Liquid oleomargarine, liquid margarine; identity; label statement of optional ingredients.

(a) Liquid oleomargarine, liquid margarine conforms to the definition and standard of identity and to the requirements for label declaration of optional ingredients prescribed for oleomargarine by § 45.1, except that it is in liquid rather than plastic form.

(b) The name of the food for which a definition and standard of identity is prescribed by this section is "liquid oleomargarine" or "liquid margarine."

(Secs. 401, 701, 52 Stat. 1046, 1055 as amended, 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371) [31 F.R. 5434, Apr. 6, 1966]

J. Am. Oil Chem. Soc. 46(9): 498-500, 1969 041

# Removal of Copper From Hydrogenated Soybean Oil[1]

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## Abstract

Hydrogenation with a copper-chromite catalyst at 170 C, 30 psi, increased the copper content of a refined, bleached soybean oil from 0.02 to as much as 3.8 ppm. Removing residual copper from soybean oil is essential to the successful use of copper catalysts for selective hydrogenation. Various methods were examined to remove this copper, including alkali refining, bleaching, acid washing, citric acid treatment and cation-exchange resin treatment. Properly conducted, each of the methods except alkali refining gives 95% or higher removal of copper introduced during hydrogenation. Ion exchange appears to be the most economical, but addition of about 0.01% citric acid during deodorization may be needed to inactivate traces of unremoved copper. Soybean oil hydrogenated with a copper-chromite catalyst, bleached or treated with an ion-exchange resin and deodorized with 0.01% citric acid added had low AOM peroxide values and acceptable flavor scores after eight days at 60 C which indicate that removal of residual copper from the oil should be adequate for the production of stable oils low in linolenic acid content.


## Introduction

In processing soybean oil for edible use, the practice generally accepted is to avoid contact of the oil with equipment constructed of copper or copper alloys. If not removed or deactivated, minute amounts of copper dissolved in the oil from such contact can cause the oil to have poor oxidative and flavor stabilities. As little as 0.03 ppm of copper, in active form, has been shown by Evans et al. (4) to reduce significantly the flavor stability of soybean oil. Small amounts of copper and other metals found in crude vegetable oils are largely removed during refining and bleaching (9). These metals are apparently obtained from the seed during oil extraction.

Copper catalysts, such as the Adkins type (1), promote selective linolenate reduction during partial hydrogenation of soybean oil [Moulton, Moore and Beal, Manuscript in preparation (3,5,7,8)]. These findings have focused attention on a need to remove small amounts of copper from the oil. To produce a flavor-stable soybean salad oil low in linolenate content by copper-catalyzed hydrogenation, effective removal of trace amounts of copper is necessary. Five methods of copper removal were examined to compare their effectiveness and to consider their practicality.

## Materials and Methods

Commercially refined, bleached soybean oils were used for all tests. The oils were partially hydrogenated in a 1 gal autoclave with a commercial copper-chromium catalyst to iodine values (IV) of 115–120. IVs were calculated from fatty acid composition data from GLC and by using the theoretical IVs for the pure acid glycerides (9). The partially hydrogenated oils were filtered with about 2% of Hy-Flo SuperCel to remove the catalyst and obtain a clear oil.

The copper content of the hydrogenated oils varied from 0.3 to 3.8 ppm. Increase in copper appeared to be related to free fatty acid content of the oil, type and amount of catalyst used, and time of hydrogenation. The copper content of hydrogenated oils and of oils treated in various ways after hydrogenation was determined by ashing 100 to 200 g samples and analyzing for copper colorimetrically. Samples of oil were weighed into acid-washed porcelain evaporating dishes, which were placed on hot plates at low heat. As the oil samples started to decompose, the temperature was gradually raised but kept below the point where autoignition would occur. After the samples were converted to a dry, carbonaceous material, the evaporating dishes were placed in a furnace at 500 C for 16 hr and a light gray to white ash resulted. Ashing at temperatures higher than 500 C resulted in loss of copper. The ash was dissolved in dilute (1–4) nitric acid and the solution was diluted to 25 ml in a volumetric flask. Copper was then determined for a suitable aliquot of the solution by the diethyldithiocarbamate method of Andrews and Stringer (2). The method was checked by analyzing oil samples containing added copper. Samples containing 5.0, 1.0, 0.20 and 0.04 ppm copper (added as copper nitrate) analyzed 5.0, 0.75, 0.15 and 0.036 ppm by ashing at 500 C and colorimetric analysis, 4.68, 0.91, 0.16 and 0.036 ppm by low temperature dry ashing and colorimetric analysis (6), and 4.43, 0.837, 0.167 and 0.042 ppm by neutron activation analysis (6).

### Copper Removal Treatments and Results

Either alkali refining plus bleaching or bleaching alone has been used to remove nickel from nickel-hydrogenated vegetable oils. Refining alone is not generally recommended because of the difficulty of washing out traces of soap left in the oil. Bleaching after refining removes substantially all traces of soap.

To study the effectiveness of refining, bleaching and other treatment methods for removing traces of copper from soybean oil, an oil containing 0.08% free fatty acid and 0.04 ppm copper was partially hydrogenated with a commercially available copper-chromium catalyst. The hydrogenated oil contained 3.4 ppm copper as shown in Table I.

To refine this oil, 200 g was vigorously mixed for 15 min at 25 C with 2 g of sodium hydroxide dissolved in 50 g of water. The resulting emulsified

TABLE I
Removal of Copper From Hydrogenated Soybean Oil by Alkali Refining or Bleaching

| Treatment | Copper content, ppm | Copper removed, % |
|---|---|---|
| Hydrogenated soybean oil | 3.4 | .... |
| Refine, 1% NaOH (on oil) in 4% solution | 1.5 | 60 |
| Bleach | | |
| ½% Super Filtrol | 0.52 | 86 |
| 1% Super Filtrol | 0.23 | 93 |
| 2% Super Filtrol | 0.20 | 93 |
| 3% Darco G-60 activated carbon | 0.13 | 97 |

[1] Presented at AOCS Meeting, Chicago, October 1967.
[2] No. Utilz. Res. Dev. Div., ARS, USDA.

TABLE II
Removal of Copper From Hydrogenated Soybean Oil by Acid Extraction

| Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Acid | Strength, % | Amount, g solution/100 g oil | Mixing time, min | No. of extractions | Copper content, ppm | Copper removed, % |
| Original soybean oil | | | | | 0.036 | ... |
| Hydrogenated soybean oil, Cu-Cr catalyst | | | | | 8.80 | ... |
| Citric | 2.0 | 85 | 15 | 1 | 1.10 | 71 |
| $H_2SO_4$ | 5.0 | 5 | 20 | 1 | 1.30 | 66 |
| $H_2SO_4$ | 5.0 | 50 | 5 | 1 | 0.53 | 86 |
| HCl | 1.0 | 16 | 1 | 3 | 0.17 | 95 |
| HCl | 2.5 | 15 | 1 | 3 | 0.19 | 95 |
| $H_2SO_4$ | 2.5 | 15 | 1 | 3 | 0.15 | 96 |
| $H_2SO_4$ | 5.0 | 5 | 20 | 3 | 0.17 | 95 |
| $H_2SO_4$ | 5.0 | 50 | 20 | 3 | 0.21 | 94 |

mixture was centrifuged without heating to separate the phases and the aqueous layer was removed. The refined oil contained 1.5 ppm copper. This amount corresponds to a removal of about 60% of the copper in the hydrogenated oil. Other refining conditions were not examined.

To bleach the oil, 200 g of oil was heated to about 80 C. Bleaching clay or carbon was added and the temperature was raised to 105–110 C and held 15 min after which the oil was filtered and analyzed for copper. Bleaching with ½% clay was more effective for removing traces of copper than refining with 1% sodium hydroxide. Activated carbon and activated bleaching earth were, within experimental error, about equally effective for removing copper (Table I).

Washing the oil with dilute aqueous acid has been described as an effective method of copper removal (8). Results of several tests of acid washing are shown in Table II.

For these tests 200 g samples of soybean oil partially hydrogenated with a Cu-Cr catalyst and containing 3.8 ppm of copper were vigorously mixed with aqueous acid for several minutes by means of a magnetic stirrer. The resulting mechanical emulsion was centrifuged and the oil layer was washed with 10% distilled water, centrifuged and analyzed for copper. A single wash with a 2% citric acid solution (65 g/100 g oil) with 15 min mixing removed only about 70% of the copper. One wash with a 5% sulfuric acid solution (5 g/100 g oil) for 20 min was about equally effective. A larger amount of sulfuric acid was somewhat more effective. Three consecutive treatments with either 1.0% or 2.5% solutions of hydrochloric acid or a 2.5% and 5% solution of sulfuric acid removed about 95% of the copper. These data indicate that continuous countercurrent washing with a dilute acid should remove copper effectively. However, acid washing must be followed by very thorough water washing because traces of acid left in the oil adversely affect the flavor of the oil after it is deodorized.

The complexing effect of citric acid for trace amounts of iron and copper in soybean oil is widely employed commercially in producing a stable deodorized oil. Usually the metal complex is left in the oil after this treatment because it is extremely small in amount and gelatinous in nature, but it may be separated from the oil by filtration. Some oil processors accomplish this by filtering the oil after it is deodorized.

TABLE III
Removal of Copper From Hydrogenated Soybean Oil by Citric Acid Chelation

| Treatment | | | |
|---|---|---|---|
| Amount of citric acid, % of oil | Method of separation | Copper content, ppm | Copper removed, % |
| Hydrogenated soybean oil | | 3.4 | ... |
| 0.01 | Centrifugation | 2.7 | 21 |
| 0.1 | Centrifugation | 1.3 | 62 |
| 0.1 | Centrifugation + filtration | 0.22 | 93 |
| 1.0 | Centrifugation + filtration | 0.04 | 99 |

In tests summarized in Table III an aqueous solution of citric acid was added to hydrogenated soybean oil, and the oil was then heated to about 125 C under high vacuum to remove the water, passing steam through the oil to promote mixing and to prevent bumping. The oil was cooled under vacuum. In the first two tests of this series, the oil was treated with 0.01% and 0.1% citric acid (1% aqueous solution) and the water then removed under vacuum. The oil was centrifuged (15 min, 900 g), decanted and analyzed. These treatments gave only partial removal of copper. In the third and fourth tests in which the oil was both centrifuged and filtered after addition of citric acid solution and dehydration, almost all copper was removed. These and other data indicate that between 0.1% and 1.0% citric acid is required to remove copper from an initial level of 3.4 ppm.

The copper removed from the oil was accounted for quantitatively in two tests. In each test, 100 g of an oil, found to contain 2.4 ppm copper by ashing the oil, was treated with 1% citric acid in 10 ml distilled water, vacuum dried and filtered as described above. The flask in which the oil was dried was thoroughly rinsed with hexane and absolute ethanol. The solution was placed in a beaker and the solvent evaporated. The filter paper was added to the beaker and its contents ashed. Analysis of the ash (two samples) showed 2.8 and 2.5 ppm copper in the oil.

Treatment of acetone or hexane solutions of vegetable oils with cation-exchange resins reported is partially effective for removing metals (10,11) but no reports have been found describing the use of such resins in a vegetable oil-aqueous system.

Results obtained by using cation-exchange resins in the hydrogen form with a water-soybean oil system are summarized in Table IV. The oil used in this series contained less copper, 1.2 ppm, than oils used in previous studies; however, the ion-exchange resin treatment was highly effective when only 1% resin (dry basis) was used. This amount of resin has several hundred times the exchange capacity required to remove the copper. Oil was mixed with one-fifth its weight of water to which a weighed quantity of resin had been added. The oil-water-resin system was stirred for 15 min at room temperature and centrifuged to recover oil for analysis. It was found that adding the resin to the oil before adding the water frequently resulted in poor copper removal, as did the use of extremely high rates of agitation. Preferential wetting of the resin by the oil appeared to be responsible.

Portions of two resins, one Dowex 50, the other Amberlite IR-120, were used in copper removal tests. Each resin was then washed with a detergent solution, rinsed on a Buchner funnel, washed with 15% HCl and then with distilled water until the effluent from the filter was neutral. These regenerated resins were used to remove copper from a partially hydrogenated soybean oil containing 0.80 ppm copper. For 15 min 200 g of oil, 40 g distilled water and 2 g resin (dry basis) were rapidly mixed. Both treated oils contained less than 0.08 ppm copper.

TABLE IV
Removal of Copper From Hydrogenated Soybean Oil by Cation-Exchange Resin

| Treatment | Resin used, % of oil | Copper content, ppm | Copper removed, % |
|---|---|---|---|
| Hydrogenated soybean oil | .. | 1.2 | .... |
| Resin (Dowex) | | | |
| 50–X8, 100–200 mesh | 1 | 0.043 | 96 |
| 50–X8, 100–200 mesh | 2 | 0.027 | 98 |
| 50–X8, 100–200 mesh | 3 | 0.030 | 97 |
| 50–X4, 50–100 mesh | 1 | 0.027 | 98 |
| 50–X4, 50–100 mesh | 2 | 0.046 | 96 |
| 50–X4, 50–100 mesh | 3 | 0.034 | 97 |

Several 1 gal batches of refined, bleached soybean oil were partially hydrogenated in the presence of a copper-chromium-barium catalyst and treated with 1% cation-exchange resin and 20% water for 15 min at room temperature. After treatment, respective batches contained 0.02, 0.03, 0.04, 0.06 and 0.02 ppm copper. The original unhydrogenated oil analyzed 0.06 ppm copper but analyses were not made on the hydrogenated oils before treatment.

Analyses of our samples by the Wisconsin Alumni Research Foundation showed that hydrogenation of soybean oil with a copper-chromium-barium catalyst increased the amount of all three metals in the oil. The oil samples were ashed at the Northern Laboratory, and the ash was analyzed by emission spectroscopy at Wisconsin. For example, a soybean oil containing 0.01 ppm barium, 0.046 ppm copper and <0.017 ppm chromium before hydrogenation contained 2.46 ppm barium, >0.85 ppm copper and 0.83 ppm chromium after hydrogenation. After a sample of the hydrogenated oil was treated 15 min with 1% resin and 20% water, it contained 0.5 ppm barium, 0.059 ppm copper and 0.049 ppm chromium.

## Results and Discussion

Based on the data secured and economic considerations, a preliminary comparative evaluation may be made of the five methods which we examined for removing copper from soybean oil. Alkali refining under the conditions investigated is not adequate for copper removal, although removal achieved by refining followed by bleaching may be adequate. Treatments with citric acid by the method described above, or by the ion-exchange method, have been shown to lower the copper content of a soybean oil partially hydrogenated with a copper-bearing catalyst to about 0.1 ppm or lower. Bleaching and washing with dilute acid were slightly less effective, but properly conducted countercurrent acid washing should remove a greater amount of copper than our acid-washing results show. Bleaching entails a loss of oil, but the other methods of treatment are not expected to. Consideration must be given to other factors, such as reagent cost, equipment cost, heating requirements and waste disposal in the selection of the preferred method.

### Oil Stability

One gallon of commercially refined, bleached soybean oil was hydrogenated to an IV (calculated from GLC data) of 118.0 in the presence of a commercial copper-chromium catalyst. The linolenate content was 0.7% as determined by alkali isomerization. After removal of the catalyst, one-half of the oil was bleached 15 min at 110 C with 1% Super Filtrol, and the other half was treated with 1% Dowex 50–X4, 100–200 mesh, and 20% distilled water for 15 min at room temperature. The original oil contained 0.06 ppm copper and the hydrogenated oil 0.29 ppm copper. Both the bleached and cation-exchange resin-treated oils contained 0.11 ppm copper.

TABLE V
Taste Panel Evaluation of Partially Hydrogenated Soybean Oil

| Samples | Hydrogenated soybean oil | | | |
|---|---|---|---|---|
| | Cation resin treated | | Bleached | |
| | Flavor score | Peroxide value | Flavor score | Peroxide value |
| Storage conditions | | | | |
| Initial | 7.7 | 0.0 | 7.8 | 0.0 |
| Stored 4 days at 60 C | 8.5 | 0.6 | 6.1 | 0.7 |
| Stored 8 days at 60 C | 6.0 | 5.5 | 6.4 | 3.0 |
| AOM peroxide value | 3.9 | | 4.9 | |
| Copper, ppm | 0.11 | | 0.11 | |

Two samples of the oil, variously treated, were deodorized in the laboratory with 0.01% citric acid added at 125 C during the cooling period; these consisted of partially hydrogenated, resin-treated oil and partially hydrogenated, bleached oil. Taste panel flavor and oxidative stability results for these oils are summarized in Table V. The initial flavor scores for the partially hydrogenated, deodorized oils were typical of good quality oils. After both four and eight days' storage at 60 C, the hydrogenated oils received about the same average flavor scores and were still in the range considered acceptable by the taste panel. They also had low peroxide values after four and eight days' storage and low AOM peroxide values. Comparison of flavor scores for the bleached and the resin treated oils indicates that the resin and bleaching treatments were about comparable with respect to their effect on flavor stability.

Of the several methods available for removing copper from partially hydrogenated soybean oils, the cation-exchange resin treatment in an oil-aqueous system appears very promising from overall considerations.

ACKNOWLEDGMENT

Gary List deodorized oil samples for taste panel evaluation and Lloyd Carlson assisted with hydrogenations.

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PART TWO

Baker's Dig. 24(2): 31-34, 1950

## Some Considerations in

# Commercial Pie Baking

By Lou Bisno
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* * * * * * * * * * * * * * * * * *

**Production**

**In this second of a series of three articles on commercial pie baking, the author discusses pie shortenings, miscellaneous small ingredients, dough mixing procedures, and raw materials control.**

* * * * * * * * * * * * * * * * * *

SHORTENING, constituting the greatest factor of cost in the dough formula is highly significant in the structure, texture, and appearance of the finished crust, as well as in its capacity to stand up under the conditions to which pies are ordinarily subjected. It may be classified into three principal groups—animal, vegetable, and blends of the two.

### Lard

Among the animal shortenings, lard has been used over a long period of time. It has excellent shortening characteristics due to its wide range of plasticity and its tendency to spread in a manner resulting in thin flakes in the crust. It is an extremely variable product, however, from the standpoints of the range in melting points found in the commercial products, as well as those of flavor and keeping qualities. Variations in these properties result from the differences in the type of feed used for the hogs, the body source of the fat rendered, and the conditions under which the lard is rendered.

The finest lard is "leaf lard" derived from the fat surrounding the kidneys, and has an iodine number not greater than 60, the softer lards having higher iodine numbers. Back lard is next in quality. Neutral lard is rendered at 40° - 50° F. Most of the commercial lards on the market are steam rendered under pressure. These steam lards usually are obtained from the fat of the entire hog, excluding the leaf fat. Low temperature rendering favors low acidity and mildness in flavor; at the higher temperatures, while the yield is increased, the free fatty acid content is raised, and the flavor impaired. When hogs are fed an undue proportion of cottonseed meal, the melting point of the fat is lowered, and the iodine number increased. Leach, in "Food Inspection and Analysis", taking the data from Richardson and Farey, reports iodine numbers of 93.9 and 94.0 in lard from "oily hogs", while corresponding values of lard from normal hogs, quoted from Dennstedt and Voigtlander, are given as 61.5 to 68.8.

In addition to the above considerations, much variation in consistency derives from the conditions under which the lard is filled into the selling container; particularly is this true in the case of the larger containers such as barrels or drums. If the lard is warm or grained out when filled into containers, large crystals develop, and an excessively soft consistency results. When using such lard in dough-making, a dough is formed which is soft and difficult to handle.

Others than lard, beef-fat products may be used in shortening blends. These include edible tallows, embracing oleo stock as well as oleo-oil and oleo-stearine, the latter two being obtained by pressing the oleo stock. High-grade products in this category may contribute a flavor which is characteristic and pleasant. Oleo-oil, being liquid at ordinary temperatures, presents some problems in use; when price considerations warrant, however, it may be used in the form of blended shortening.

Butter or oleomargarine are used principally in the rolled-dough types for which a rather firm shortening is required, although they can also be used in a mixture with other shortenings in the more usual types of pie-dough.

From the practical standpoint better results in quality and uniformity may be expected when the higher grades of lard are used. Where products of particularly high melting point are required, as under high summer temperatures, hydrogenated lards may be used. These have exceptional stability under such conditions from the standpoints of both shortening and crusts.

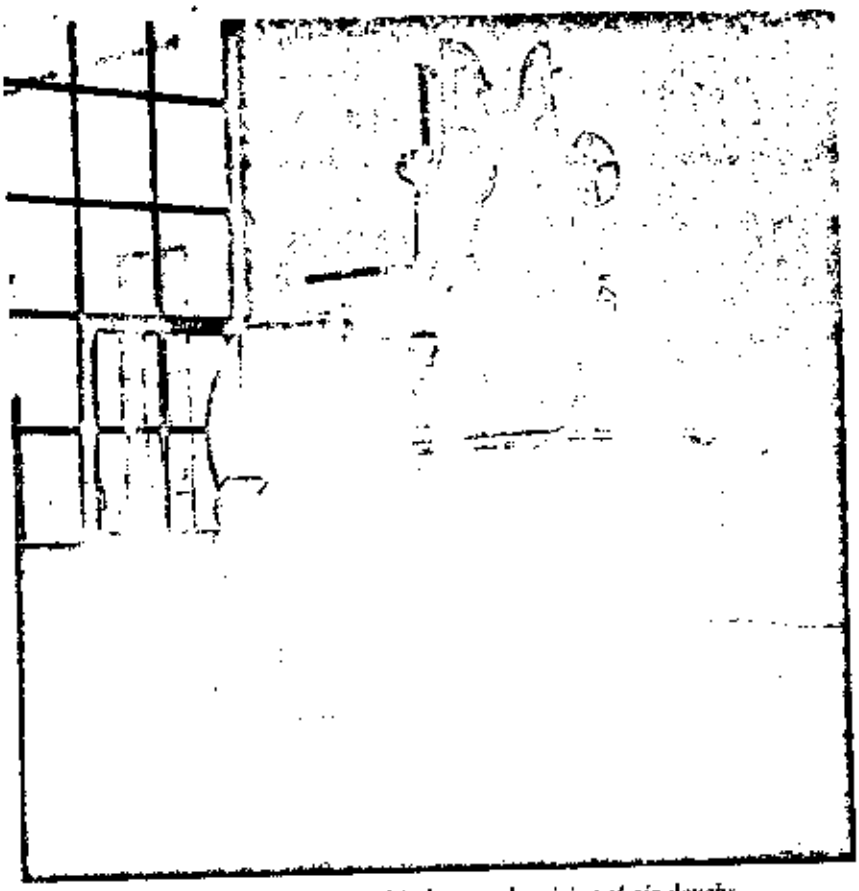
### Vegetable Shortenings

In the vegetable shortenings we find the oils—cottonseed, soy bean, corn, peanut, coconut, sesame, as well as several others. From the baker's standpoint they may be used in the form of the refined, bleached, and deodorized oils, as hydrogenated products, or as blends of the two. Since coconut oil is quite hard and brittle at lower temperatures, its value as a shortening is poor. Cottonseed and soy bean oils are the two principal vegetable oils used in this country.

In view of the fact that these oils are liquid at the ordinary temperatures, and that relatively high shortening percentages are required in pie-dough, crusts made from such shortenings exclusively are too soft and too prone to excessive soaking for commercial use. For this reason hydrogenated products are used. In hydrogenation, both the firmness of the shortening, and the plastic range permit of considerable control. When it is considered that the oils used may be hydrogenated to different degrees, and that any number of blends using only hydrogenated oils, or those mixed with unhydrogenated oil may be made, it is not hard to visualize that countless products with varying firmness and workability under different temperature ranges may be produced.

### Basis of Selection

In determining the adaptability of a given shortening to pie-making in a specified locality, not only must the temperature and humidity of that locality be considered, but the shop conditions as well as the type of crust also must be borne in mind. When we take into account the fact that in Chicago and Los Angeles, for example, the average January temperatures are 29° F. and 56° F. respectively, it is quite apparent that shortenings of differing softening and melting points must be used to meet these conditions. Of extreme but seldom recognized importance is the variation in temperature in the storage and dough-



*Artofex dough mixer used in large scale mixing of pie doughs.*

making departments of pie bakeries, as well as the consistency of the shortenings as affected by those variations.

Vegetable shortenings may be made from an oil, such as cottonseed or soybean, and a relatively low percentage of solid hydrogenated vegetable fat. Thus, a typical formula is 84% cottonseed oil and 16% cottonseed oil which has been hydrogenated to the 48° titer plant. The essential method of preparing the compound shortening is the rapid cooling of the proper mixture after refining, bleaching and deodorizing the oils used, after which it is run into the containers in which it is sold. In the cooling process air is incorporated with the shortening. As filled into the containers it is normally of a soft, pasty consistency. After standing, however, it sets to a considerably firmer mass. The texture may be modified by the extent to which air is incorporated. Such a compound, without incorporation of air would tend to have a salve-like consistency and appearance. With an excess of air whipped into it, a short, cheese-like texture results. In attempting to cream such a shortening at ordinary temperatures, a likely result would be the breaking up into small lumps. When the proper percentage of air is incorporated, however, a plastic mass is produced, which handles well.

If, with increasing temperatures, as in the change from winter to spring, or from spring to summer, such shortening shows too soft characteristics, the percentage of hard stock is increased. Several methods are available for obtaining better resistance to high temperatures. Among these are the following:

(1) Using a hard stock oil which has been hydrogenated to a greater degree than the previous.

(2) Using a greater proportion of the hard stock.

(3) Using a blend of oils, each of which has been hydrogenated to different degrees or under different conditions.

(4) Blending with emulsifying glycerides.

Modifications of formula in the ways indicated affect the final melting points as well as the rate of softening with various temperature changes. Thus, in the classification (3) above, greater stability occurs than in the two preceding groups. Since, at any temperature, the firmness of the pie crust in which such shortenings are used will depend largely upon the firmness of the shortening at that temperature, the selection of the shortening will enable a measure of control of the crust characteristics.

In addition to the purely animal and purely vegetable shortening products, there are available the various compounds containing both types, such as oleo-stearine compound and oleo margarine.

### Importance of Proper Storage

While the formula of the shortening blend is of prime importance, the conditions under which the product is filled into the containers also has a bearing upon the blend's consistency. Shortening producers ordinarily control both the temperature of the product and the percentage of air introduced at the time of filling. In view of the better controls possible in the case of vegetable shortenings than in the case of lards, greater uniformity exists in the former. In either case, however, changes in consistency may occur between the time the product sets up in the selling container and when it is used. If, for instance, the shortening is stored at 60° F. before delivery to the user, and the baker stores it in a room either at a definite temperature different from this one, or in one in which there are no temperature controls, there will be a change in consistency which will increase with time. In using such shortening, variations in the doughs may occur, resulting in problems in handling and baking. When, in attempting to change the temperature of shortening in a drum, for example, the latter is moved from one room into another of considerably higher or lower temperature, approximately two weeks may be required for the entire contents to reach the new temperature level. If, during the period in such storage, while the temperature of the shortening is changing, only part of the contents of a particular drum be used for pie-dough, the balance or part of it being used on successive days, variations will be found in the texture and handling of the dough, as well as in the baked product. Thus, for uniform results, the shortening should be held and used at a constant temperature.

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### Temperature

***Changes in shortening temperature result in variations in texture and handling of dough as well as in the baked product.***

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While salt in pie-dough is used primarily from the flavor standpoint, variation in the percentage used has a slight effect upon the dough consistency. From the practical standpoint, this is not important, however.

Cerelose is used to impart color to the baked crust. While milk, principally in the powdered non-fat form, has been used for years, and is still being used by some bakers for this purpose, it has the objectionable feature of resulting in soaking bottoms under summer or otherwise hot conditions. Heating the reconstituted milk, then cooling before use, tends to reduce this problem.

## Absorption

Water varies considerably in different parts of the country, and in some cases in different sections of a city, depending upon the source of supply. In one case, the water supply in the city had been at a pH of under 7.0 at the time when considerable trouble developed in certain products during the course of baking. Compensations were then made to change the pH to a higher figure, which eliminated the problems. Later when the water supply had been changed and several sources were being used, the variations in pH ranged from 6.9 to 8.9.

Since the water absorption capacity of flours will vary with pH as well as the mineral content of the water, both in respect to kind and amount, it is apparent that water, although taken for granted as a constant, may cause problems, compensations or partial compensations for which may be made in other materials at high cost. As a matter of fact, changing the pH of a dough may, on the one hand, eliminate a tendency for shrinkage during baking, and, on the other, may make a firmer, drier baked crust.

## Plant Layout

A typical plant layout for the close control of dough involves the following:

Temperature and humidity-controlled room for flour storage, with capacity for thirty-days' supply.

Refrigerated room for storing the shortening at the required temperature for a period sufficient to bring it to the proper condition for use.

Dough room, controlled as to temperature and humidity, provided with:

Automatic scale-tank for weighing the sifted flour, the flour bin and screening equipment being either in this room or the flour-storage area.

Artofex dough mixer unit whose bowl is located directly under the outlet from the scale tank.

Scale for weighing the shortening, the platform of which is at the level of the top of the dough bowl.

Water-meter for measurement of the water used in the dough. The water used may be run through a cooler.

Table and scale for salt, cerelose, etc.

In the three barrel Artofex mixers provision is made for mechanically dumping the dough into the troughs. Since these units produce about 800 pounds of dough per batch, they are used only where the production requirements are of the order of 10,000 to 12,000 pounds per shift of six and one-half hours. Depending on several conditions the number of batches produced per hour may range from two to four. Thus in the machine described, a capacity of 2000 pounds per hour is a conservative figure.

## Mixing Procedures

The essential procedure in making pie dough is a light mixing of the flour and shortening, after which the water, together with the salt and coloring agent are added. The dough is then lightly mixed until it just holds together, after which it is allowed to stand in a cool room for several hours before use. The actual mixing procedure is varied, depending upon the type of crust desired. Three principal types will be considered, the fine-grained, moderately flaky, and the rolled doughs, which are of the long-flake variety.

The general procedure in producing the fine-grained type of crust involves a fairly complete mixing of flour and shortening to the extent that the flour particles are quite well covered, resulting in a pasty condition. The water, the percentage of which is approximately 25% based upon the weight of flour, is then added together with the salt and cerelose, and lightly worked in. As finished, the mixed dough has a wet, grainy appearance.

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### Mixing

***The degree of initial mixing given to the flour and shortening determines the character of the crust, i.e., whether it will be fine-grained, moderately flaky, or of the long-flake type.***

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Completion of the water absorption occurs during the standard period which should be three to four hours at least in a room at approximately 60° F. Such a dough requires a flour somewhat stronger than that used for the ordinary dough type.

For the intermediate flake size, a softer type flour, together with more water, may be used than for the small flake. In this case, however, the flour is only partly covered with the shortening which may exist in very small

*Pie dough mixer shown in dump position.*

lumps, or lightly flaked throughout the mix. In either case, after the addition of the water together with the salt and coloring agent, the dough as mixed holds together better after mixing, and feels considerably drier than in the case of the small-flake type. Overmixing is to be avoided, although it is ordinary practice in a number of shops to mix the dough to the point that it can be immediately used. Better results are obtained, however, with less mixing after the water addition, and several hours' standing time for the dough.

The representative dough type of the third classification is the rolled dough. In this case a fairly firm shortening is used, broken into small pieces approximately one inch diameter and one-fourth inch thick. The firmness should be such that the lumps are not broken down during the mixing of flour and shortening. A typical formula is as follows:

280 lbs. pie flour
210 lbs. shortening
8 lbs. salt
1½ lbs. baking powder
130 lbs. water

The flour and firm shortening are lightly mixed, after which the water containing the other materials of the formula in solution is added and lightly mixed in until the dough just barely holds together. It should be fairly dry. Small batches are then rolled to a thickness of approximately two inches, following which the dough is folded twice, producing a six inch thickness containing three layers. The rolling and folding are twice repeated, after which the dough is again rolled to two inch thickness and allowed to rest in the ice box for several hours, or overnight, before use. Such a crust has large flakes and shows considerable "spring" in baking.

Because of the great number of factors involved, pie dough constitutes one of the biggest problems in the production department of the pie bakery. A formula, which at the time of first use produces excellent results may, a week later, prove exceedingly troublesome, possibly because of changes of temperature of the raw materials, of the dough room, or of the outside. Frequently, variations in the raw materials themselves are responsible. When a combination of two or more changed conditions occurs at the same time, then the problem frequently gets out of hand. Conditions such as falling crust, soaking bottoms, off-flavor, blistering of bottoms, particularly in custard pies may be related to such combinations. Thus, a fallen crust in juiced apple pies might be due to the shortening being too warm as used, or to the unbaked pie standing too long in a warm location before baking. A change in the type of flour or shortening might be responsible, or a variation in the amount of water used in the dough formula. These are among the many possibilities to be considered. Since the factors are so many, and in large bakeries the problems of supervision are so vital, the necessity of standardization in the raw materials and in the storage and production conditions is apparent. Such standardization involves the necessary checks of the materials as purchased and used.

## Raw Materials Control

Examination of the raw materials as purchased and received involves both the quantities and the quality. Considering such items as fresh apples, the weights may be obtained in the case of truckloads or carloads by substracting the tareweight of the truck and containers from the gross weight of the load. In this case, however, since considerable variation is not infrequent among the containers, principally second-hand boxes or crates, it is advisable to obtain the tare on all of the containers. If this be not practical, then a sufficient sampling may result from taking twenty to twenty-five containers out of a lot of 500. In the absence of a certified total weight of the load, a number of the individual boxes or baskets may be emptied and the average net weight per container determined from these figures. After a number of months in cold storage weight losses up to 5% may be found due simply to moisture evaporation.

It frequently happens that a sample # 10 can of such a product as canned pineapple is submitted for purchase, upon the basis of which a deal is made. Upon receipt of the lot it is found that the average does not come up to the sample. Since it is practically impossible to find one can in a lot which is thoroughly representative, two alternative conditions of purchase may be made:—

(1) The average net and drained weights, and quality factors to come up to a definite standard, with maximum allowable deviations noted.

(2) The initial sampling be made on, say, one dozen cans, the results of the examination to be used as a basis for the entire purchase lot.

In the case of cold-pack products, in thirty-pound or fifty-pound cans, sufficient variation in net weights sometimes occurs to warrant taking the net weights on a number of individual cans.

In reference to the weights of materials, both as purchased and used, the importance of periodic checking of the scales and meters used cannot be overemphasized. It is highly desirable to have several standard weights, say 20 lb., 50 lb., and 100 lbs., on hand at all times for testing the larger scales. Scales should be adapted to the quantities to be weighed. Thus, if one ounce of a spice is to be weighed out, the use of a scale accurate to only one-fourth ounce introduces a large percentage of error. In such a case, a scale accurate to one-sixteenth ounce should be used. Because in all cases the accuracy of weights depends upon the condition of the scales and weights at the time when they are in use, the weights themselves as well as the moving parts of the scale should be clean at all times. Since, in practice, the weights and beams tend to accumulate dirt and incrustation, dial-type scales of high accuracy are preferable to the beam type.

## COLLOIDAL PROPERTIES OF DOUGH

*(Continued from page 30)*

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## Estrogen-like Activity in Vegetable Oils and Mill By-products

*Abstract*. By the immature female mouse bioassay technique, an increased uterine weight was observed when certain vegetable oils were fed or injected. By-products from the milling of cereals were also capable of eliciting a uterine response.

In the course of evaluation of the estrogenic potency of the coumarin derivative coumestrol (*1*), administered subcutaneously in vegetable oil solution, it was observed that the vegetable oils employed as carriers were themselves capable of eliciting a measurable response in the test animals. Estrogenic activity has been previously reported in wheat germ oil (*2*) and coffee oil (*3*). We have now found that a number of the commonly used vegetable oils are capable of producing estrogen-like responses in mice.

The bioassay employed in our laboratory for measuring estrogenic activity when estrogen is administered orally has been recently described (*4*). In our subcutaneous injection studies, the estrogen, dissolved in a suitable vegetable oil carrier, was administered for four successive days at the rate of 0.1 ml of oil solution per day; the mouse was killed on the 5th day, and the uterine weight was determined as described previously (*4*).

The vegetable oils employed in these studies included samples of commercially available salad oils as well as some unrefined or semirefined samples. Samples of mineral and cod-liver oils were also included for comparison. For oral administration, the oils or cereal fractions were uniformly mixed into the basal feed mixture at levels equivalent to 10 or 20 percent of the diet. The animals were fed in groups of five, and the results presented (Tables 1–3) represent the averages for five animals. Successive repetitions of the test gave very similar results.

The results of oral administration of the oils are summarized in Table 1. Control mice fed no oil beyond that normally present in the stock diet had an average uterine weight of about 9.5 mg at the end of the test period. Some of the oils tested at the 10-percent level of the diet produced uterine weights more than double this value, while others produced lesser effects. Cottonseed, mineral, and castor oil had no effect. The crude coffee oil and tung oils proved toxic, and the mice died before the test was completed. When the oils were administered at the 10-percent level of the diet, each mouse consumed a total of 1 gm of the oil during the period of the assay. Corn oil was slightly more effective when injected (Table 2) than when administered orally. Olive oil was also effective when it was injected subcutaneously.

As has been pointed out by Biggers (*5*), uterine response to oral or subcutaneous administration of a substance is not necessarily indicative that the substance is an estrogen. A true estrogen is also capable of producing vaginal cornification. Levin *et al.* (*2*) were able to demonstrate that a concentrate obtained from wheat germ oil by the Girard-Sandulesco reaction (*6*) produced vaginal cornification in mature, ovariectomized rats.

Since oils from cereal sources showed activity, it was decided to check unextracted cereal products. When wheat bran, wheat germ, rice bran, or rice polish were fed by incorporating them in the diet at the 10-percent level, uterine weight responses very similar to those obtained with the oils resulted (Table 3).

The findings recorded in this report may be related to observations of growth stimulation in chicks fed high levels of certain fats (*7*). This hypothesis is strengthened by the observations of Carew and Hill (*8*) that diethylstilbestrol stimulated chick growth when it was added to a high fat ration but did not when it was added to a low fat ration, it being assumed that the corn oil used did not supply enough estrogen for maximal response (*9*).

Table 1. Mouse uterine weight response to various oils administered orally. The oils were fed at the 10-percent level unless otherwise indicated.

| Kind of oil | Level fed (%) | Mean uterine wt. (mg) |
|---|---|---|
| None (control diet) | | 9.5 |
| Mineral oil | 10 | 8.6 |
| Castor oil, refined | 10 | 9.4 |
| Cottonseed oil, refined | 10 | 10.1 |
| Safflower oil, refined | 10 | 13.6 |
| Wheat germ oil, refined (sample 1) | 10 | 13.6 |
| Cod-liver oil, refined | 10 | 13.9 |
| Corn oil, refined | 10 | 14.2 |
| Corn oil, refined | 20 | 15.8 |
| Linseed oil | 10 | 14.6 |
| Wheat germ oil (sample 2) | 10 | 15.0 |
| Peanut oil, refined | 10 | 15.9 |
| Olive oil, refined | 10 | 16.7 |
| Soybean oil, refined (sample 1) | 10 | 16.8 |
| Soybean oil, refined (sample 2) | 10 | 17.7 |
| Coconut oil, crude | 10 | 19.0 |
| Rice bran oil, refined | 10 | 22.5 |
| Rice bran oil, crude | 10 | 23.1 |

Table 2. Mouse uterine weight response to vegetable oils injected subcutaneously. Four 0.1-ml injections were given in each case, unless otherwise indicated.

| Material | Total vol. (ml) | Mean uterine wt. (mg) |
|---|---|---|
| None | | 9.9 |
| 0.9% Saline | 0.4 | 10.3 |
| Corn oil (sample 1) | 0.4 | 13.2 |
| Corn oil (sample 1)* | 0.8 | 18.2 |
| Corn oil (sample 2) | 0.4 | 16.8 |
| Corn oil (sample 3) | 0.4 | 15.1 |
| Olive oil | 0.4 | 12.5 |

* Eight 1-ml injections.

Table 3. Mouse uterine weight response to cereal fractions fed at the 10-percent level.

| Fraction | Mean uterine wt. (mg) |
|---|---|
| No supplement | 9.5 |
| Wheat bran | 14.3 |
| Wheat germ | 25.6 |
| Rice bran | 18.5 |
| Rice polish | 21.3 |

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# Effect of Saturated and Unsaturated Fatty Acids on the Desaturation *in Vitro* of Palmitic, Stearic, Oleic, Linoleic, and Linolenic Acids*

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## SUMMARY

**Quantitative oxidative desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids to palmitoleic, oleic, octadeca-6,9-dienoic, γ-linolenic, and octadeca-6,9,12,15-tetraenoic acids, respectively, by liver microsomes of rats was studied by gas-liquid radiochromatography after incubation of the acids in a medium containing adenosine triphosphate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, $MgCl_2$, coenzyme A, glutathione, nicotinamide, NaF, and NaCN in oxygen at pH 7 and 35°.**

**The rates of conversion of oleic into octadeca-6,9-dienoic, linoleic into γ-linolenic, and linolenic into octadeca-6,9,12,15-tetraenoic acids were measured, and Lineweaver-Burk plots showed the existence of competitive reactions among the three acids. Saturated acids were ineffective in altering the percentage of desaturation of the acids, whereas polyunsaturated acids of 20 and 22 carbon atoms generally activated the conversion of the three acids. Docosahexaenoic acid decreased the percentage of desaturation of oleic, linoleic, and linolenic acids. The unsaturated acids of 18 carbons apparently competed for the same oxygenase, and the order of affinity was linolenic, linoleic, and oleic acids. Substrate fatty acids and products after the incubation were shown to be mainly esterified to choline and ethanolamine glycerophosphatides.**

Polyunsaturated fatty acids are now known to belong to different series not interconvertible in animals. The acids of each series are individualized by the position of the first double bond proximal to the methyl group and are synthesized in animals from the first acid of the family through elongation and desaturation. Additional double bonds are added in the direction of the carboxyl group. The principal series is that derived from oleic, linoleic, and linolenic acids. Oleic acid, following a sequence of steps, is converted into octadeca-6,9-dienoic, eicosa-5,8,11-trienoic, and docosa-7,10,13-trienoic (1) acids; linoleic is transformed into γ-linolenic, arachidonic, docosa-7,10,13,16-tetraenoic (2), and docosa-4,7,10,13,16-pentaenoic acids, while linolenic acid is elongated and desaturated to eicosa-5,8,11,14,17-pentaenoic, docosa-7,10,13,16,19-pentaenoic, and docosa-4,7,10,13,16,19-hexaenoic acids (3).

Although they are not interconvertible, dietary experiments indicated that the amount of polyunsaturated acids of each series accumulated in animal tissues depends on the presence and amount of the acids of the other series. For example, eicosa-5,8,11-trienoic (4) and docosa-7,10,13-trienoic (1) acids synthesized from oleic acid in the fat-deficient rat are rapidly eliminated from the tissues by incorporation of either linoleic and arachidonic acid or linolenic, eicosa-5,8,11,14,17-pentaenoic, and docosa-4,7,10,13,16,19-hexaenoic acids (5) in the diet. The reciprocal effect was also shown (6). These interrelationships have been attributed by Dhopeshwarkar and Mead (6), Marco *et al.* (7), Machlin (8), Bozian and Coniglio (9), Edwards and Marion (10), and Mohrhauer and Holman (11) to metabolic competition among the unsaturated acids of the different families. This so-called competitive inhibition may be located at the steps involving desaturation, elongation, or esterification. However, dietary experiments are too crude to resolve this problem, and we have therefore reduced the number of variables by studying *in vitro* the relative effects of unsaturated acids of 18, 20, and 22 carbon atoms on the microsomal desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids.

## EXPERIMENTAL PROCEDURE

*Preparation of Microsomes*—Livers from male white rats of the Institute strain maintained either on a full or semisynthetic fat-deficient diet (12) were used. After the rats were killed, the livers were washed in the cold with a solution of 0.15 M KCl, 5 mM $MgCl_2$, 1.5 mM glutathione, 62 mM phosphate buffer (pH 7), and 0.25 M sucrose in 0.1 mM EDTA, minced, and homogenized with the same solution (4:1, v/w). The homogenate was centrifuged at 800 × *g* for 10 min, and the supernatant solution was recentrifuged at 8,000 × *g* for 30 min, and, after decanting, at 140,000 × *g* for 1 hour and 20 min. The pellets were suspended

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‡ Members of the Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

in the homogenizing solution (2:1, v/v), and aliquots containing 5 mg of protein, estimated by the biuret method (13), were assayed for the enzymatic desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids.

*Labeled Acids*—Palmitic-1-$^{14}$C (29.6 mC per mmole) and linolenic-1-$^{14}$C (10 mC per mmole) acids were purchased from Calbiochem. All the other labeled acids were obtained from the Radiochemical Centre, Amersham, England. Stearic-1-$^{14}$C (37.1 mC per mmole) and oleic-1-$^{14}$C acids (29.4 mC per mmole) were 99% radiochemically pure, the latter having 3% *trans* isomer. Linoleic acid (24.7 mC per mmole) was 91% radiochemically pure and had 8% *cis,trans*-unconjugated acid.

*Assay for Oxidative Desaturation*—In animals, fatty acid desaturation has repeatedly been proven to be effected by microsomal enzymes. The first step involves conversion to the CoA derivative through activation with ATP. The fatty acyl-CoA derivatives so formed are desaturated in an ATP-independent reaction in which oxygen and NADPH or NADH are also necessary. In order to obtain a desaturating system free from elongating and oxidizing capacity, the microsomes were incubated in a medium containing suitable inhibitors. After several tests the medium of Gellhorn and Benjamin (14), without added α-glycerol phosphate, was adopted. Ten millimicromoles of each of the acids tested, dissolved in 10 μl of propylene glycol, were incubated with 5 mg of microsomal protein and 0.1 ml of supernatant in a solution containing, in micromoles: ATP, 3; CoA, 0.2; NADPH, 0.8; NADH, 1.8; magnesium chloride, 15; GSH, 4.5; nicotinamide, 1; sodium fluoride, 125; sodium cyanide, 3; phosphate buffer (pH 7) 125, in 0.15 M potassium chloride and 0.25 M sucrose. The total volume was 3 ml. After the addition of the microsomal suspension, the flasks were gassed with oxygen in the cold for 1 min and incubated in a Dubnoff metabolic shaker at 37° for 20 min. The incubation was stopped by the addition of 2 ml of 10% methanolic KOH. Myristic acid (1 mg) was added and, after 10 min of saponification at 85°, the acidified solution was quantitatively extracted with petroleum ether (b.p. 30–40°). Fatty acids were esterified by treatment with methanolic 3 N HCl for 3 hours at 60°.

The composition of the mixture of methyl esters was determined by quantitative gas-liquid chromatography in a Pye apparatus with ionization detector. The samples were analyzed in columns packed with 10% polyethylene glycol adipate on Chromosorb W (100 to 120 mesh) at 195° or 10% diethylene glycol succinate on Chromosorb W (60 to 80 mesh) at 165° or 22% Apiezon L in Chromosorb W (100 to 120 mesh) treated with hexamethyldisilazane at 206°. The distribution of radioactivity in the fatty acids was determined by preparative gas-liquid radiochromatography with the same kinds of columns specified above. A Pye radiochromatograph with proportional counter was used. The apparatus was set with the integrator at 1000 counts per deflection. The counter efficiency detection of $^{14}$C was 70%. The recovery of the radioactivity in individual incubations was 85 to 100% by means of the outlined procedure.

*Structure of Fatty Acids*—Labeled acids synthesized by the microsomal preparation were separated and purified by preparative gas-liquid chromatography in 10% diethylene glycol succinate on Chromosorb W (60 to 80 mesh) at 164°. The structure and position of labeling were determined by reductive ozonolysis (15) followed by gas-liquid radiochromatography with the same column conditions. Standards (5 mg) of methyl arachidonate and methyl docosa-4,7,10,13,16,19-hexaenoate were also ozonized to get aldehyde esters of 5 and 4 carbon atoms and to facilitate the identification of the expected aldehyde esters containing 6 carbon atoms.

*Assay for Competitive Reactions*—In order to find any competitive effect of other saturated and unsaturated acids upon the desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids, similar amounts of palmitic (>99% pure), stearic (>99% pure), oleic (>99% pure), linoleic (>99% pure), linolenic (>99% pure), arachidonic (>90% pure), eicosa-5,8,11,14,17-pentaenoic (>90% pure), docosa-7,10,13,16-tetraenoic (>99% pure), docosa-7,10,13,16,19-pentaenoic (>99% pure), and docosa-4,7,10,13,16,19-hexaenoic (>90% pure) acids were added to the incubation medium. Except for docosatetraenoic and docosapentaenoic acids, all of these acids were purchased from the Hormel Institute. Docosa-7,10,13,16-tetraenoic and docosa-7,10,13,16,19-pentaenoic acids were separated by preparative gas-liquid chromatography in 10% polyethylene glycol adipate in Chromosorb W (100 to 120 mesh) from bovine adrenal glands (16). Percentage of conversion was studied by gas-liquid radiochromatography as described above.

Competitive effects among oleic, linoleic, and linolenic acids were also recognized by measuring the rate of desaturation of increasing concentrations of the labeled acids during the first 4 min of incubation in the same system in the presence of the tested acids. The number of molecules converted was measured by gas-liquid radiochromatography. Approximate initial velocities were calculated and Lineweaver-Burk plots were constructed from the data (see Figs. 7 and 8).

*Distribution of Radioactivity in Lipids*—The distribution of labeled linoleic and stearic acids and their products of desaturation in the microsomal lipids was determined after 20 min of incubation in the same system. The incubation was stopped by pouring the contents of the flasks into 60 ml of chloroform-methanol (2:1, v/v) containing 0.5 ml of 0.25 M sulfuric acid and 1 mg of myristic acid. After shaking, 12 ml of 0.37% KCl were added and the aqueous layer was removed. The lower layer was dried, and the lipids were separated by thin layer chromatography on plates (20 × 20 cm) covered with Silica Gel G (0.5 mm thick). Chloroform-methanol-water (65:25:4, v/v) was used as the developing solvent. Standards of phosphatidylcholine and ethanolamine, fatty acids, cardiolipin, and triglycerides were run at the same time in order to identify the lipids. The surface of the plate covered by the spots of the sample was marked in 1-cm squares, and each such square was removed and directly esterified with methanolic 3 N HCl. The esters were taken up in chloroform and washed three times with water. The aqueous fraction was used for phosphorus determination by Bartlett's (17) technique, and an aliquot of the esters was counted in a Nuclear-Chicago gas flow counter while the remaining material was analyzed in the gas-liquid radiochromatograph. The distribution of label in the different lipids and the proportion of labeled acids in the main fractions were studied by this procedure.

*Assay for Acyl-CoA Desaturation*—Oleyl-1-$^{14}$C-CoA desaturation to octadeca-6,9-dienoic acid in the presence of linoleyl-CoA was also studied. Linoleyl-CoA and labeled oleyl-CoA were prepared as described by Kornberg and Pricer (18) by incubation of the free acids and CoA with guinea pig microsomes. Oleyl-CoA (10 mμmoles, 10.5 mC per mmole) was incubated at 37° for 1 hour with 5 mg of microsomal protein in a medium containing, in millimicromoles: NADPH, 200; NADH, 200; EDTA, 10; and phosphate buffer (pH 7.2), 30, in a total volume of 0.5 ml. Mi-

crosomes were separated in the usual way from rats fed a complete diet.

## RESULTS AND DISCUSSION

The differential gas-liquid radiochromatograms of the esterified microsomal fatty acids obtained after 20 min of incubation with labeled stearic, oleic, linoleic, and linolenic acids are shown in Fig. 1. They show the conversion of stearic into octadecenoic acid, oleic into octadecadienoic acid, linoleic into octadecatrienoic acid, and linolenic into octadecatetraenoic acid. No other labeled acid was found in our experimental conditions except for the experiments with stearic-1-$^{14}$C acid. When enough radioactive stearic acid was incubated, about 2% of labeling could be detected in the octadecadienoic peak. This acid would be derived from oleic acid synthesized in turn from stearic acid. Microsomal oxygenases have been shown to convert stearic into oleic acid (19), while conversion of oleic into octadeca-6,9-dienoic acid by the same subcellular fraction has been shown by Holloway, Peluffo, and Wakil (20). Linoleic desaturation to γ-linolenic acid was shown by Nugteren (21), and in our experiment the octadecatrienoic acid synthesized from linoleic-1-$^{14}$C acid was also proven to be γ-linolenic acid. By reductive ozonolysis and direct gas-liquid radiochromatography it was possible to localize the radioactivity in the 6-carbon aldehyde methyl ester. None was found in the other products of cleavage.

As far as we know, desaturation of linolenic to octadeca-6,9,12,15-tetraenoic acid *in vitro* has not been described in the literature. However, Klenk and Oette (3) and Klenk and Mohrhauer (22) have found that linolenic and octadeca-6,9,12,15-tetraenoic acids administered to fat-deficient rats are

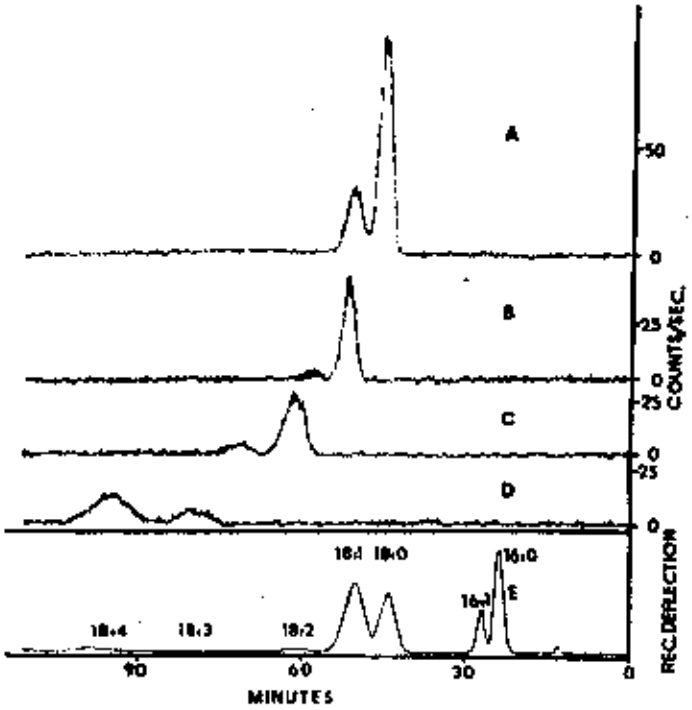


FIG. 1. Differential gas-liquid radiochromatograms after 20 min of incubation of 10 μmoles of *A*, stearic-1-$^{14}$C; *B*, oleic-1-$^{14}$C; *C*, linoleic-1-$^{14}$C, and *D*, linolenic-1-$^{14}$C acids. Microsomal protein 5 mg from the livers of fat-deficient rats was incubated at 35° in $O_2$ under the conditions described in the text. *E*, the simultaneous gas-liquid chromatogram of microsomal lipids.

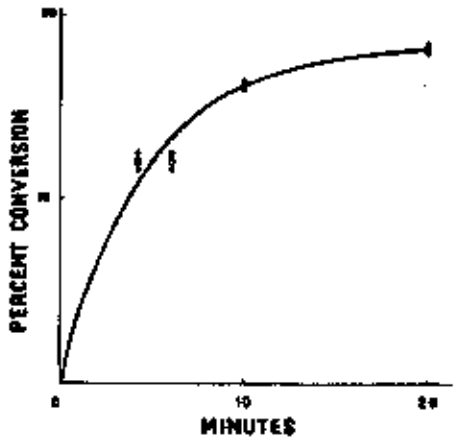


FIG. 2. Time course and percentage of linoleic-1-$^{14}$C acid converted into γ-linolenic-1-$^{14}$C acid by liver microsomes from rats fed a fat-free diet. Labeled linoleic acid (10 μmoles) was incubated at 35° in $O_2$ with 5 mg of microsomal protein. The conditions of incubation are described in the text. Limits of variation in two experiments are indicated.

similarly converted into eicosa-5,8,11,14,17-pentaenoic and docosa-4,7,10,13,16,19-hexaenoic acid. Octadecatetraenoic acid synthesized by our preparation of liver microsomes from linolenic-1-$^{14}$C acid was shown to be the octadeca-6,9,12,15-tetraenoic acid by reductive ozonolysis and gas-liquid radiochromatography. As expected, the labeling was found only in the 6-carbon aldehyde ester that can be produced only by ozonolysis of a double bond added to the linolenic acid in positions 6 and 7.

Desaturation of palmitic acid to palmitoleic and approximately 5% hexadecadienoic acid was also found under the same experimental conditions.

Although sufficient chain length still exists in all of the unsaturated acid molecules synthesized in our system to introduce a further double bond maintaining the divinyl rhythm, no acid with a double bond in carbon 3 and carbon 4 was found. The absence of this type of unsaturation is very probably related to polar effects due to the proximity of the carboxyl group. The other possibility that would lead to similar results is a change in the position of the double bond from carbon 3 and carbon 4 to carbon 2 and carbon 3 followed by β oxidation. Migration of double bonds from carbon 2 and carbon 3 to carbon 3 and carbon 4 has been shown by Bloch (19) to occur in the anaerobic pathway of synthesis of oleic acid by bacteria. However, this explanation must be discarded because of the lack of β-oxidative capacity of our incubation system.

The percentage of desaturation of the acids tested depended on the time of incubation. Fig. 2 shows the percentage of conversion of linoleic into γ-linolenic acid as a function of time of incubation. The conversion initially increased with time and then became approximately constant after 20 min. Similar curves were found for the other acids and are analogous to the findings of Gellhorn and Benjamin (14) for stearic desaturation. The highest percentages of desaturation found for palmitic, stearic, oleic, linoleic, and linolenic acids were 60, 41, 6, 21, and 70%, respectively, after 20 min of incubation with 5 mg of microsomal protein obtained from rats fed a fat-deficient diet for at least 4 months. However, the percentage of conversion of the acids depends on the dietary and hormonal conditions (23) of the animals. For example, 10 rats fed a fat-deficient diet for 8

TABLE I

*Effect of fatty acids on oxidative desaturation of palmitic, stearic, oleic, linoleic, and linolenic acids*

Labeled acids, 10 mμmoles each, were incubated with equivalent amounts of the test acids and with 5 mg of microsomal protein obtained from the livers of fat-deficient rats by using the conditions described in the text. Incubation was for 20 min at 35° in oxygen. Results are expressed as percentage of the conversion of the labeled acid without additions, and are the means of at least two experiments.

| Acids added | Relative conversion | | | | |
|---|---|---|---|---|---|
| | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
| | % | % | % | % | % |
| None | 100 | 100 | 100 | 100 | 100 |
| 16:0[a] | | 100 | 100 | 99 | 10 |
| 18:0 | 104 | | 99 | 99 | 99 |
| 18:1 | 117 | 104 | | 90 | 92 |
| 18:2 (ω 6)[b] | 117 | 100 | 56 | | 87 |
| 18:3 (ω 3) | 105 | 109 | 45 | 72 | |
| 20:4 (ω 6) | 119 | 111 | 113 | 102 | 106 |
| 20:5 (ω 3) | 115 | 112 | 110 | 102 | 110 |
| 22:4 (ω 6) | | | 100 | 105 | |
| 22:5 (ω 3) | | 112 | 99 | 102 | 100 |
| 22:6 (ω 3) | 114 | 91 | 73 | 92 | 96 |

[a] In the abbreviations of fatty acids, the number preceding the colon represents the number of carbon atoms in the chain; the number following the colon represents the number of double bonds.

[b] The number following ω indicates the position of the last double bond, counting the double bonds from the terminal methyl group.

months showed 20.6 ± 1.5%[1] conversion of linoleic into γ-linolenic acid, whereas 33.4 ± 2.5% was found for 4 animals maintained on a full diet for 7 weeks and the fat-deficient diet supplemented with 5% protein for the last week.

The effect of equivalent amounts of palmitic, stearic, oleic, linoleic, linolenic, arachidonic, eicosa-5,8,11,14,17-pentaenoic, docosa-7,10,13,16-tetraenoic, docosa-7,10,13,16,19-pentaenoic, and docosa-4,7,10,13,16,19-hexaenoic acids on the oxidative desaturation of labeled palmitic, stearic, oleic, linoleic, and linolenic acids is shown in Table I. Only unsaturated fatty acids of similar chain length significantly depressed the conversion of oleic, linoleic, and linolenic acids into the corresponding, more unsaturated acids. The depressive effect increased with the unsaturation of the acid tested, higher for linolenic and lower for oleic. This order of depressive power is coincident with the sequence found by Hohman (24) in the inhibition of eicosa-5,8,11-trienoic acid synthesis by fat-deficient rats after supplementation of the diet with linoleic or linolenic acid. In consequence, the very low percentage of eicosatrienoic acid found in the rat fed a complete diet may be the result of a decreased synthesis of octadeca-6,9-dienoic acid due to the presence of linoleic and linolenic acids in the diet. This result, however, does not eliminate the existence of other steps in the synthesis of eicosa-5,8,11-trienoic acid that may show regulatory effects.

Under the conditions of the experiments the polyunsaturated acids of 20 and 22 carbon atoms, belonging either to the linoleic or the linolenic family, did not significantly depress the desaturation of oleic, linoleic, and linolenic acids, except for the case of docosahexaenoic acid. Furthermore, some of them even increased the conversion in some way. Therefore, the displacement of eicosa-5,8,11-trienoic acid from the tissues of fat-deficient rats by arachidonic acid (4) cannot be attributed to an inhibitory effect at the step of octadeca-6,9-dienoic acid synthesis but to another specific effect, possibly at the level of eicosa-8,11-dienoic acid desaturation or phospholipid synthesis.

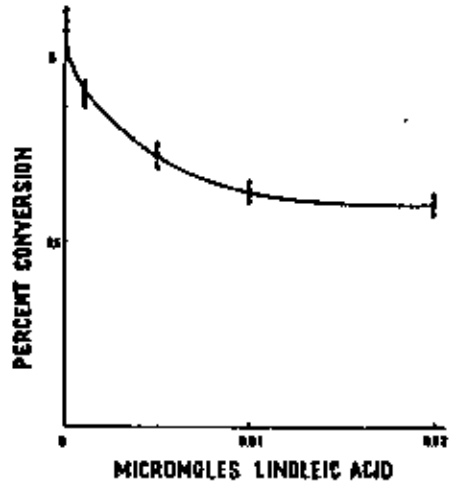


FIG. 3. Linoleic acid dependence on oleic-1-$^{14}$C acid conversion into octadeca-6,9-dienoic acid. Oleic-1-$^{14}$C acid (10 mμmoles) was incubated for 20 min at 35° in $O_2$ with increasing amounts of linoleic acid and 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

Both palmitic and stearic acids, which were shown to be highly desaturated, when incubated as labeled acids together with linoleic-1-$^{14}$C and linolenic-1-$^{14}$C acids did not exhibit a depressing effect. The oxidative desaturation of palmitic and stearic acids was also not depressed by equivalent amounts of any of the unsaturated acids tested, whether they were of a similar or a different chain length. These results would indicate that the inhibitory effects are apparently not related either to an inadequate supply of CoA or reduced pyridine nucleotide, or to the mere presence of free fatty acids. The same or very closely related oxygenases would desaturate oleic, linoleic, or linolenic acids, but they would be different from those which desaturate palmitic acid, stearic acid, and the acids of 20 and 22 carbon atoms. Palmitic oxygenase would also be different from stearic oxygenase.

Incubation of labeled oleic and linoleic acid with increasing amounts of linoleic and linolenic acids, respectively (Figs. 3 and 4), showed a relation between the depressive effect and the concentration of the acids added. However, the effect was not linear, and became asymptotic when it reached approximately half of the maximal conversion. This kind of relationship would indicate that when higher amounts of acids are added other factors may play an important role in the desaturation of the labeled acids, in addition to a possible competitive effect. Similar results were also found (Fig. 5) when labeled linolenic acid was incubated with increasing amounts of linoleic and oleic acids.

Equivalent amounts of arachidonic and eicosa-5,8,11,14,17-pentaenoic acids incubated with labeled oleic, linoleic, or linolenic acids (Table I) seemed to increase somewhat the desaturation of any of the three acids. When increasing amounts of the

[1] Standard deviations of the mean.

two polyunsaturated acids were incubated with 10 mμmoles of labeled linoleic acid a significant increase in the synthesis of γ-linolenic acid was found (Fig. 6). The peak corresponded approximately to 50 mμmoles of the added arachidonic or eicosapentaenoic acid and decreased with higher amounts of the acids. This unexpected result is difficult to explain and requires further research. It may be caused by a direct activating effect of arachidonic or eicosapentaenoic acids on the desaturation, or may be produced through an appropriate change in the composition of the lipids that are bound to the oxygenases. This has been shown by Holloway and Peluffo (25) to be important in determining the specific activity of the enzymes.

The mutual inhibitory effects of oleic, linoleic, and linolenic acids appear to be caused by competition reactions (Figs. 7 and 8). Lineweaver-Burk plots showed convergent lines which intersected at the ordinate when increasing amounts of linoleic-1-$^{14}$C acid were incubated for 4 min in the absence and presence of a constant level of oleic or linolenic acid. Similar results were also obtained when increasing amounts of either linolenic-1-$^{14}$C or oleic-1-$^{14}$C acids were incubated with constant concentrations of oleic-1-$^{14}$C or linolenic-1-$^{14}$C acids, respectively (Fig. 8).

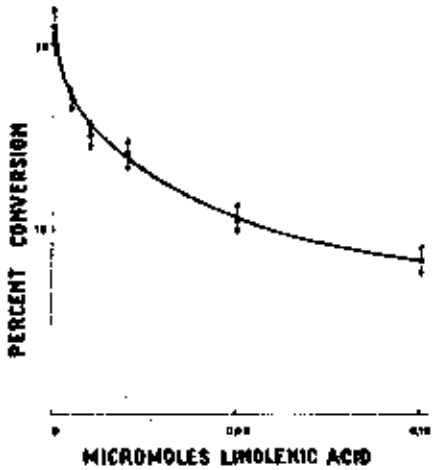


FIG. 4. Linolenic acid dependence on linoleic-1-$^{14}$C acid conversion into γ-linolenic acid. Linoleic-1-$^{14}$C acid (10 mμmoles) was incubated for 20 min at 35° in $O_2$ with 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

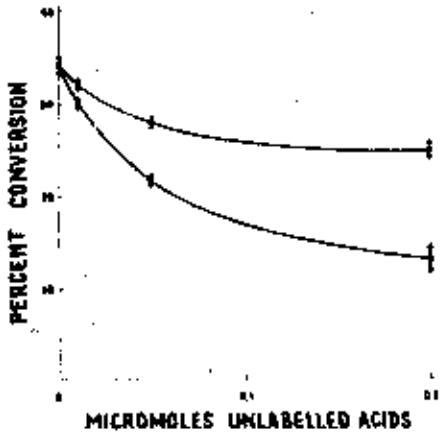


FIG. 5. Linoleic acid and oleic acid dependence on linolenic-1-$^{14}$C acid conversion into octadecatetraenoic acid. Linoleic-1-$^{14}$C acid (10 mμmoles) was incubated for 20 min at 35° in $O_2$ with linoleic (■) or oleic (●) acids and 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

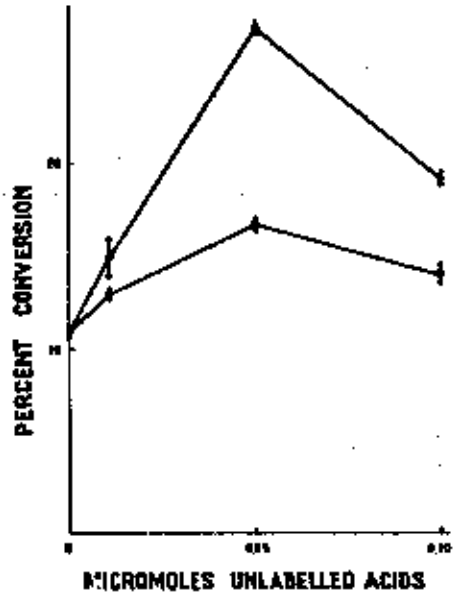


FIG. 6. Effect of arachidonic and eicosa-5,8,11,14,17-pentaenoic acids upon the conversion of linoleic-1-$^{14}$C acid into γ-linolenic acid. Linoleic-1-$^{14}$C acid (10 mμmoles) was incubated for 20 min at 35° in $O_2$ with increasing amounts of arachidonic (●) or eicosa-5,8,11,14,17-pentaenoic (■) acids and 5 mg of microsomal protein from rats fed a fat-free diet under the conditions described in the text. Limits of variation in two experiments are indicated.

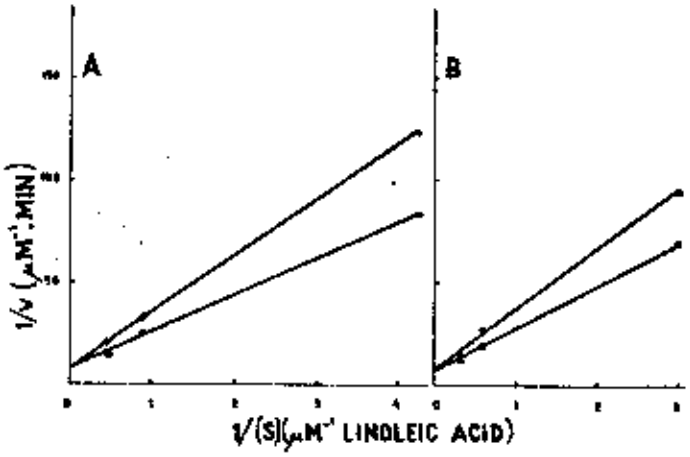


FIG. 7. Lineweaver-Burk plots for linoleic-1-$^{14}$C acid incubation. *A*, in the presence (●) and absence (■) of 1.58 μM linolenic acid. *B*, in the presence (●) and absence (■) of 33.3 μM oleic acid. Velocities were measured for the first 4 min of incubation at 35° in $O_2$ under the conditions described in the text. Each tube contained 5 mg of microsomal protein from rats fed a fat-free diet.

Approximate $K_m$ and $K_i$ values were calculated (Table II) from the graphs, but the enzymatic system tested was too crude to assure that they measure the affinity of the enzyme for the substrate. The $K_i$ was used for the Michaelis constants for the unlabeled acids tested; $K_i$ values were calculated from the graphs in the usual way from the $K_m$ values calculated in the same experiment. They may not be real "inhibitor constants" but constants of a simultaneous reaction of the enzyme with another substrate. The maximal velocities calculated (Table II, Ex-

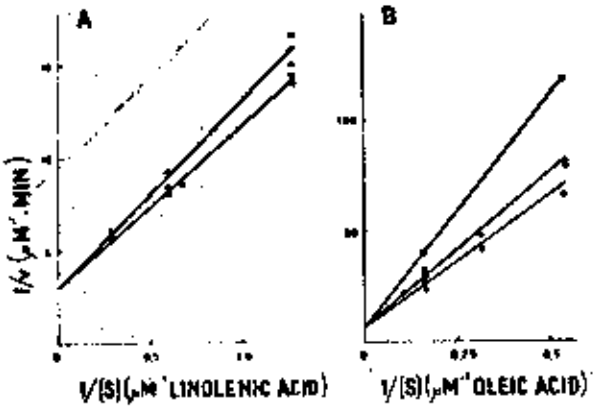


FIG. 8. Lineweaver-Burk plots for: *A*, linolenic-1-$^{14}$C acid incubation in the presence (▲) and absence (●) of 6.13 μM oleic-1-$^{14}$C acid; *B*, oleic-1-$^{14}$C acid incubation in the absence (●) and presence of 0.78 μM (▲) and 3.33 μM (■) linolenic-1-$^{14}$C acid. Velocities were measured for the first 4 min of incubation at 35° in $O_2$ under the conditions described in the text. Each tube contained 5 mg of microsomal protein from rats fed a fat-free diet.

TABLE II

*Approximate Michaelis constants of oleic, linoleic, and linolenic oxidative dehydrogenation*

The $K_m$ values were calculated from Lineweaver-Burk plots. The velocities were measured for the first 4 min of incubation of the labeled acids with 5 mg of microsomal protein of fat deficient rats at 35°. Conditions are as described in the text.

| Labeled acids incubated | Experiment | Maximal velocities | $K_m$ for substrate | $K_i$ | |
|---|---|---|---|---|---|
| | | | | Oleic | Linolenic |
| | | *mμmole/min* | *μ × 10⁻⁴* | *μ × 10⁻⁴* | |
| Linoleic | 6 | 0.16 | 3.2 | | 3.3 |
| Linoleic | 7 | 0.37 | 8.0 | 87 | |
| Linoleic | 8 | 0.32 | 0.9 | | |
| Linolenic | 12 | 0.32 | 2.9 | 54 | |
| Oleic | 12 | 0.23[a]<br>0.22[b] | 27 | | 5.9[a]<br>4.3[b] |

[a] Calculated from Fig. 8 when the inhibitory effect of 0.78 μM linolenic acid was tested.

[b] Calculated from Fig. 8 when the inhibitory effect of 3.33 μM linolenic acid was tested.

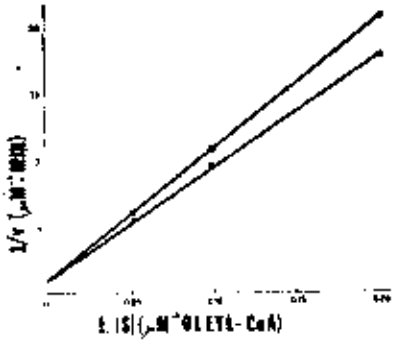


FIG. 9. Lineweaver-Burk plots for oleyl-1-$^{14}$C-CoA incubation in the presence (●) and absence (■) of 10 mμmoles of linoleyl-CoA. Increasing concentrations of oleyl-1-$^{14}$C-CoA were incubated with 5 mg of microsomal protein in a solution containing, in millimicromoles: NADPH, 200; NADH, 200; EDTA, 10; and phosphate buffer (pH 7.2), 30, in a volume of 0.5 ml. Velocities were measured from the conversion attained along the first 1 min of incubation at 37° in $O_2$.

periment 12) were higher for linolenic than for oleic acid, whereas $K_m$ values were lower for linolenic than for oleic acid. Both results coincide in provoking the same effect, the displacement of oleic products of desaturation by linoleic products when both acids are added together. This result is in harmony with those obtained from experiments *in vivo* in which both acids were administered together (5).

The rates measured in our enzymatic system are the result of several reactions, including acylation of the substrate with CoA, desaturation of the acid, and esterification of substrate and newly synthesized acids. The slowest reaction undoubtedly controls the over-all rate of the reaction, and the competition found in our

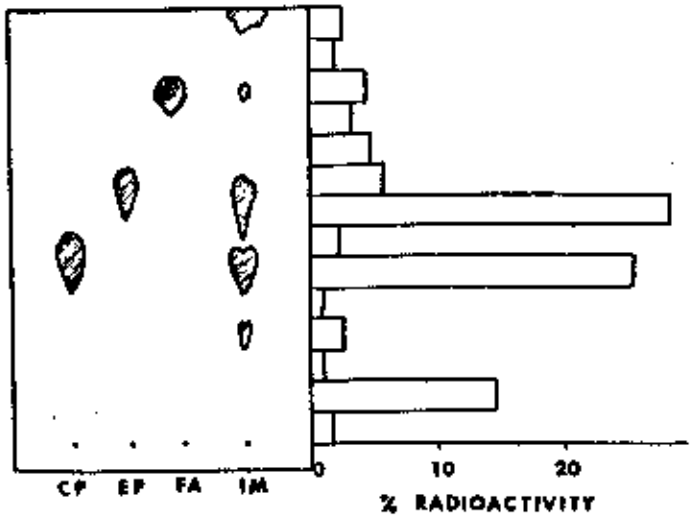


FIG. 10. Thin layer chromatogram and radioactivity distribution in the microsomal lipids after the incubation of 10 mμmoles of linoleic-1-$^{14}$C at 35° for 20 min in $O_2$ under the conditions described in the text. Each tube contained 5 mg of microsomal protein from rats fed a fat-free diet. Total lipids were chromatographed in chloroform-methanol-water (65:25:4, v/v). Chromatogram stained with iodine. *CP*, choline glycerophosphatide; *EP*, ethanolamine glycerophosphatide; *FA*, free fatty acids; *IM*, lipids of incubated microsomes.

TABLE III

*Effect of α-glycerol phosphate and lysolecithin on oxidative desaturation of linoleic and stearic acids*

Labeled acids, 0.01 μmole each, were incubated with 5 mg of microsomal protein for 20 min at 35° in oxygen. Conditions were as described in the text. Results are expressed as percentage of the conversion of the labeled acid without additions.

| Addition | Relative conversion | |
|---|---|---|
| | Linoleic-1-$^{14}$C | Stearic-1-$^{14}$C |
| *μmoles* | % | % |
| None | 100 (±3)[a] | 100 (±3) |
| α-Glycerol phosphate | | |
| 0.01 | 92 (±3) | 101 (±3) |
| 0.10 | 96 (±2) | |
| 1.00 | 82 (±7) | 100 (±8) |
| Lysolecithin | | |
| 0.01 | 82 (±22) | 120 (±8) |
| 0.10 | 98 (±10) | |
| 1.00 | 20 (±20) | 97 (±16) |

[a] Maximal deviation of the mean of at least two experiments.

system would correspond to this reaction. There is sufficient NADPH and CoA in our system to discard these cofactors as limiting factors. Acylation of CoA may also be discarded, since saturated acids of 16 and 18 carbons and unsaturated acids of 20 carbons do not inhibit the desaturation of oleic, linoleic, and linolenic acids. This conclusion was also definitely proved when the competition of linoleyl-CoA with labeled oleyl-CoA was tested (Fig. 9) and convergent lines which intersected at the ordinate were obtained again.

Esterification of substrate fatty acids and products by our microsomal system was shown by separation of the lipids by thin layer chromatography followed by determination of their content of radioactive fatty acids (Fig. 10). After incubation, some labeled linoleic and γ-linolenic acids were found as free acids, but they were preponderantly bound to choline and ethanolamine glycerophosphatides. Moreover, in our experimental conditions, esterification of fatty acids does not appear to be a limiting factor because α-glycerophosphate which has been proven to increase the synthesis of fatty acids (14) by regulating fatty acid esterification (26), and lysolecithin, which is a good acceptor of unsaturated fatty acids (27), do not increase the rate of desaturation (Table III).

Consequently, the experiments described above suggest that the competitive phenomena found may probably be located at the desaturating step. The $K_m$ and $K_i$ values calculated for oleic, linoleic, and linolenic acids would measure roughly their oxidative dehydrogenation, and it is interesting to note that $K_m$ and $K_i$ values for oleic acid are similar. The same similarity was also found for linolenic acid. When 3.33 μM linolenic-1-$^{14}$C and 6.13 μM oleic-1-$^{14}$C acid were added together to 5 mg of microsomal protein, the total rate of reaction for the first 4 min of incubation was 0.18 μM per min, which is less than the sum of the rates of the reactions measured separately, 0.21 μM per min. This criterion of identity of enzymes (28), together with previous results, provides some evidence that the same oxygenase and the same active center desaturate oleic, linoleic, and linolenic acids. The other oxygenases that desaturate stearic and palmitic acids, and very probably the acids of 20 and 22 carbons, would be different. However, when a more than 20-fold excess of oleic or linoleic acids was included in the system a decrease was found in the desaturation of both saturated acids, indicating that the oxygenases that desaturate saturated acids may have a similar active center able to react with oleic or linoleic acid.

The competitive reactions shown among the unsaturated acids of 18 carbons undoubtedly play an important role in the regulation of the lipidic composition of the tissues, together with similar competitions that can be predicted to exist among the unsaturated acids of 20 and 22 carbons, respectively. They may explain at least some of the effects found in dietary experiments in which different fatty acids are supplied to the animals, but many problems still remained unsolved. Other factors, such as elongation, oxidative and hydrolytical reactions, phospholipid and triglyceride synthesis, hormones, and even enzyme synthesis at the level of RNA or DNA, cannot be discarded.

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# A STUDY OF THE POTENTIAL APPLICATIONS OF OLFACTORY RESEARCH IN MAN

MARCH 1973

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# FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments of topics in the biomedical sciences. These reports are based upon comprehensive literature reviews and the perceptive observations of knowledgeable scientists engaged in work in the field. Although LSRO reports are recognized by FASEB as contributions to societal needs and most LSRO consultants are members of FASEB constituent societies, the reports do not necessarily reflect the views of the members of its six constituent societies. However, the report has been reviewed for policy matters by the LSRO Advisory Committee, which includes representatives of each constituent society.

This technical report was prepared for the Life Sciences Office, Office of the Chief of Research and Development, Department of the Army, by the staff of the Life Sciences Research Office, FASEB, in accordance with the provisions of U.S. Army Contract No. DAHC19-72-C-0010.

We acknowledge the contributions of the numerous investigators and consultants who have assisted with this study. The report reflects the opinions expressed by participants in an *ad hoc* study group that met at Beaumont House, FASEB, on September 19-20, 1972, and other consultants. The report has been reviewed by these scientists and a judicious attempt has been made to incorporate the different viewpoints and opinions.

The listing of the consultants' names in Section XII must not be construed as indicating that they are authors of the report or that they endorse the conclusions or recommendations. The authors accept full responsibility for the contents of this report.

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## SUMMARY

This study provides a comprehensive review of the feasibility of developing and utilizing the chemical senses of the soldier. While biosensors and odor-sensing devices can supplement, they cannot replace the potential performance of the normal human olfactory system. There is no current program in the Army to train soldiers to use their chemical senses. Experience in other countries suggests that a soldier trained to recognize olfactory cues is more effective in the field, more responsive to hazards, and less likely to give olfactory information to the enemy than one whose chemical senses are underutilized.

Practical testing and training methods suited to the Army's needs have been lacking in the past, and basic knowledge of the mechanisms of olfaction and taste is incomplete. However, elements of a suitable technology have been identified in agencies such as the National Bureau of Standards and in certain industries. This technology appears suitable for further development within the Army research and development community. This report concludes that it is feasible for the Army to use these methods in determining classifications of personnel that require normal acuity of the chemical senses and to institute training programs.

In addition, regular testing for normal acuity of the chemical senses may uncover early cases of some illnesses, indicate extensive exposures to ionizing radiation, and categorize personnel as either suitable or unfit for certain Army duties. If administered at recruitment, these tests can be used later to document disability claims for service-related losses of olfaction and taste.

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# I. THE PROBLEM

Visual and auditory proficiency are generally recognized as important requirements for the soldier, and are not only related to efficient performance in the military environment but also may be essential for survival. The military significance of these sensory modalities has been reviewed recently (Carr and Fisher, 1970, 1971, Carr *et al.*, 1969, Fisher *et al.*, 1969). On the other hand, acuity of the "chemical senses," olfaction and taste, has been less widely recognized as important, and the only available review of the subject related to Army interests was concerned mainly with animal biosensors (Smith and Coates, 1965). Another trend has been to substitute instruments for odor detection in the field without considering the performance potential of man.

Although some of the basic mechanisms of the neurophysiology of olfaction and taste are unknown, human skill in the recognition of odors and tastes can be enhanced by training and may be used efficiently by the soldier (Woods, 1968). The Army has sponsored major work related to olfaction and taste on nutrition, diet, food components, and acceptability of rations, but there has been little in-house military research on the potential contribution of the chemical senses to military performance.

When men are incapable of detecting odors of fire, toxic gases, or spoiled foods, this must obviously be a handicap to performance and even to survival. Most men are capable of detection, yet are often untrained in odor recognition. Failure to identify odors that characterize machine malfunctions, enemy operations, states of the environment or other situations could mean the difference between success or failure of a military mission. In general, any underutilization of sensory responses to environmental stimuli detracts from the quality of human performance. Woods (1968) has indicated that olfactory acuity is required for certain military duties in the British and Commonwealth armies. In contrast, in the United States Army, no military duties are specified as requiring a normal level of acuity and the chemical senses are not even tested.

A major requirement of this study was to identify aspects of the military environment in which normal acuity of olfaction and taste are important and factors that contribute to losses of these senses. In the past, a major difficulty has been inadequate testing methods to evaluate

acuity of olfaction and taste. Although many testing methods continue to be directed toward elucidation of basic mechanisms rather than evaluation of human capabilities, recent technical advances indicate that the latter objective should now be attainable.

## II. SCOPE OF THE STUDY

Preliminary discussions with research investigators in universities and Army laboratories revealed many facets of the problems of understanding the mechanisms of human olfactory and taste systems, the nature of loss of acuity, recognition and detection levels, environmental and genetic influences, and analytical techniques employed in assessment of acuity in man.

From this background an agenda was developed for an *ad hoc* review group meeting convened at Beaumont House, Federation of American Societies for Experimental Biology, on 19-20 September 1972 (see Section XII-A). These reviews of the subject provided insight into the basic and applied aspects of olfactory and taste research and the Army programs related to these areas. The scope of this report emerged from these reviews.

The study includes a synopsis of the current limits of scientific and technical knowledge, especially of olfaction, and a more detailed assessment of opinions on how this information may be applied to Army needs. It was not deemed necessary to review current in-house Army research programs on food acceptability, biosensors, or odor-sensing devices. The study stresses the practical applications of human sensory acuity in olfaction and taste, rather than fundamental concepts and theories that are available in the literature.

# III. COMPONENTS OF THE HUMAN OLFACTORY AND TASTE SYSTEMS

## A. OLFACTION

### 1. Air Passages

The nose is a paired organ, like the eyes but unlike the mouth (Figure 1). The olfactory system is exquisitely sensitive; in nose-breathing, only 3 percent of the inspired air passes over the olfactory epithelium (Stuiver, 1958). When the nasal epithelium is either dry or congested the airflow is abnormal and olfactory function is further diminished. The rate of flow and the temperature of the air are important (Schneider and Wolf, 1960). When exhaled air passes through the mouth it may pick up odorants that affect responses of the olfactory epithelium to inhaled odorants.

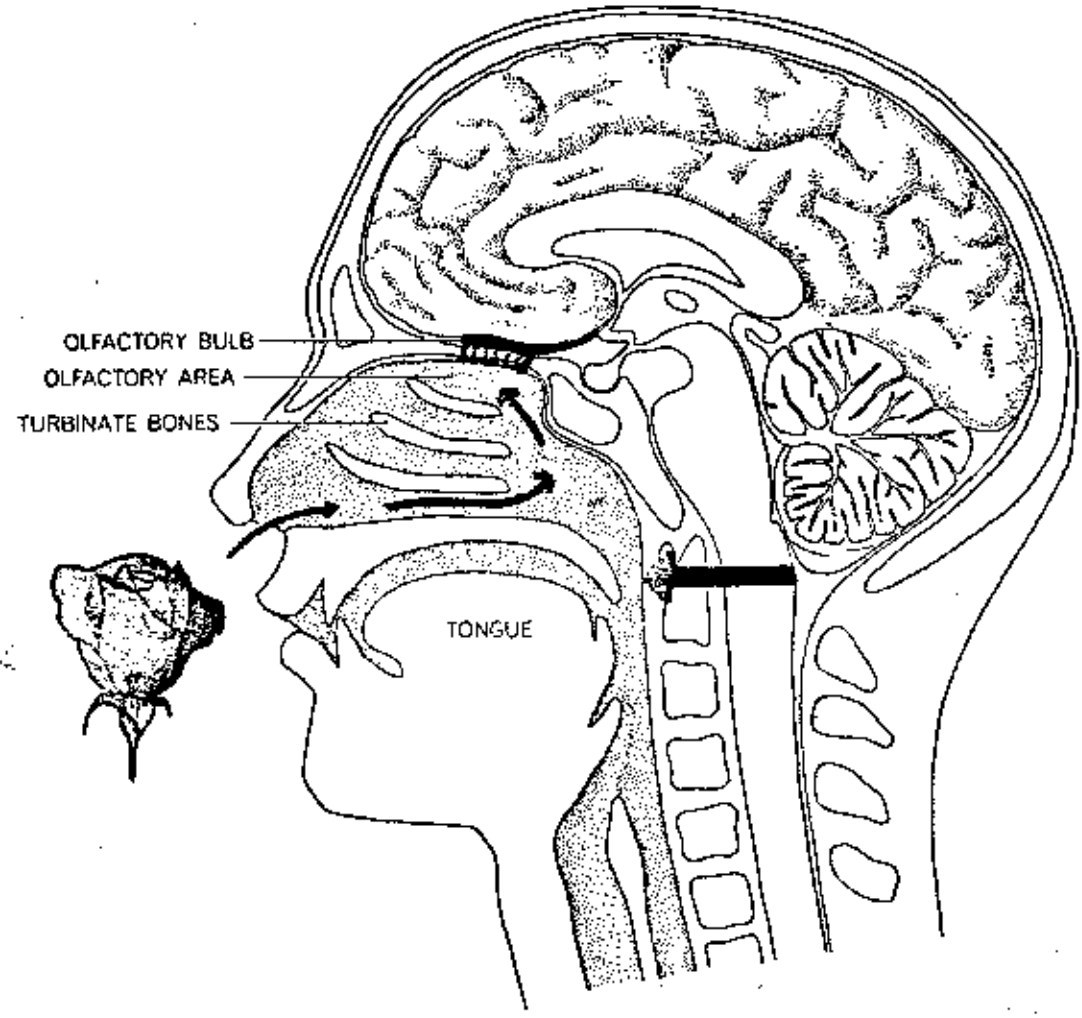
### 2. Olfactory Epithelium

A small area of the mucous membrane at the apex of the nose contains odor-sensitive cells (Figures 1 and 2). This epithelium contains pigments (Takagi, 1969, Moulton, 1971). In animals, some of these pigments are carotenoids. Carotenoids are precursors of vitamin A and losses of smell occur in vitamin A deficiency. But since vitamin A deficiency affects virtually all membranes, and carotenoids are absent from the pigments in human olfactory epithelium, Moulton and Beidler (1967) have concluded that these pigments probably have little to do with olfaction in man.

### 3. Olfactory Receptor Cells

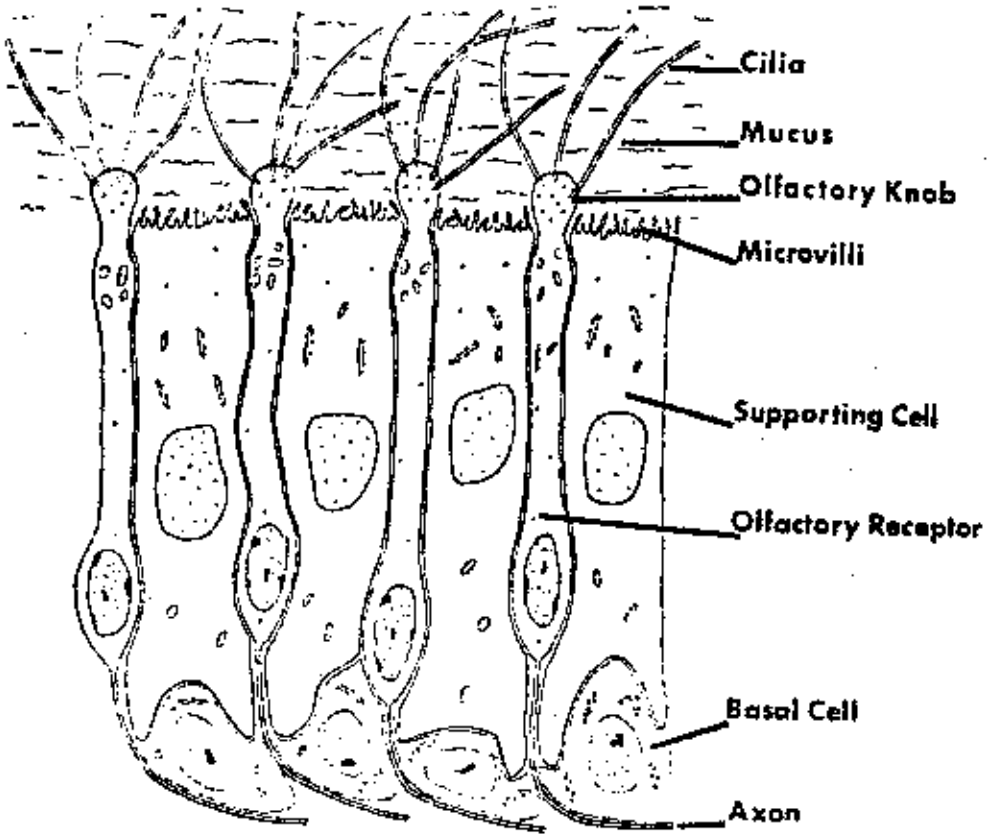
Estimates of cell numbers vary. Allison and Warwick (1949) estimated that the olfactory nerves of the rabbit contain millions of primary fibers, each presumably connected to a cell body. From electrophysiological responses, Beets (1971) has estimated 10 million cells, each able to conduct 10 impulses/sec, giving a system capacity of 100 million inputs/sec, comparable to the input capacity from the retina of the eye.

FIGURE 1




Human anatomy of olfaction, showing the right nasal air passage with olfactory area (epithelium) and bulb. Similar structures on the left are not shown. Left and right air passages enter the single air duct at the throat (large arrow). Reproduced from Amoore, Johnston, and Rubin (1964) with permission of the copyright holder, Scientific American, Inc.

FIGURE 2




Human anatomy of olfaction, showing the structure of the olfactory epithelium. The olfactory receptor cells are nerve cells distributed among the supporting, mucous-producing cells. Not shown are trigeminal nerve endings which occur between the supporting cells and, like other tactile nerve endings, do not reach the surface of the epithelium. Reproduced from Moulton and Beidler (1967) with permission of the American Physiological Society and the authors. For further anatomical detail see Schneider, 1967.

The cilia of the olfactory nerve cells protrude above the epithelial surface (Figure 2). Receptor molecules are believed to be proteins in the outer membranes of the olfactory cells including the cilia. The number of cilia per cell is known for several species (Moulton and Beidler, 1967), but the number of receptor molecules per cell is unknown. Most authorities agree that the receptor cells including their axons are continually replaced; thus, each cell is functional for only part of its life. This turnover might explain the diversity of the estimates of input capacity from the olfactory epithelium.

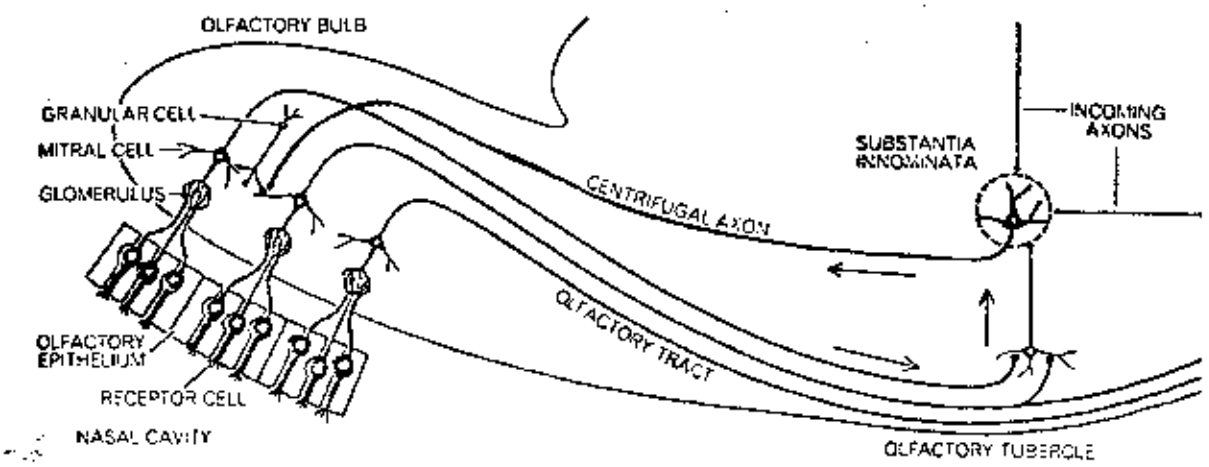
4. Olfactory Nerve Connections

Fibers from the receptor cells track back to left and right olfactory bulbs (Figures 2 and 3). "Accessory" olfactory bulbs and vomeronasal organs are found in lower animals but are absent or vestigial in man. The role of the bulb may be analogous to that of the horizontal cell layer of the retina. Each bulb contains a large number of glomeruli. Allison and Warwick (1949) have estimated that each glomerulus collects about 26,000 primary nerve fibers, and provides one nerve fiber to the higher brain centers; this is a large input reduction. From each bulb about 2,000 channels go back to the higher centers, some to the same side, others to the opposite, as with the optic nerves (Stone, 1969). Responses evoked by odorants have been detected electrophysiologically in the 1st, 5th, 7th, 9th and 10th cranial nerves.

Trigeminal nerve endings, usually associated with pain or irritation, also are stimulated by impulses arising in the olfactory epithelium; some of these fibers enter the glomeruli, where they interconnect with the olfactory nerves (Amoore *et al.*, 1964).

Channels from the olfactory bulbs are distributed in the general area of the prepyriform cortex (Figure 3). The interconnecting pathways in the brain have not been fully delineated (Stone, 1969), but from psychophysical data there is little doubt that they extend to the reticular cortex, thalamus, hypothalamus (MacLeod, 1971), and other areas in the brainstem and midbrain. These observations suggest that there are interconnections between brainstem pathways and the endocrine system.

FIGURE 3




Nerve pathways of the rat olfactory system. Primary nerves from the epithelium enter glomeruli in the olfactory bulb, where they synapse with fewer nerves that go deeper into the brain (right side of diagram). The brain sends other nerves ("centrifugal axon") back to the olfactory bulb, and it is thought that by this pathway the brain can modify sensory input from the olfactory bulb. Reproduced from Heimer (1971) with permission of the copyright holder, Scientific American, Inc.

## B. TASTE

### 1. Anatomy of Taste Receptors

The primary organs of taste are the taste buds. The salivary glands contribute to taste by secreting fluid in which solid tastants are dissolved; undissolved molecules cannot be tasted. Locations that exhibit differential sensitivity to specific tastants (e.g., Figure 4) have been demonstrated by experiments in which sensations in parts of the mouth were blocked by local anesthetics (Henkin, 1971, Murray and Murray, 1970). Taste buds contain receptor cells associated with nerve endings (Figure 5); unlike olfactory receptors, these are not nerve cells and do not have fully developed cilia. Taste buds are renewed every 10-10.5 days if innervated; if the nerve supply is severed, the taste bud dies and does not regenerate until the nerve grows back (Beidler, 1970). The number of taste buds varies widely between animal species (Table 1), and cannot be correlated with taste acuity in animals or man (Kare, 1971).

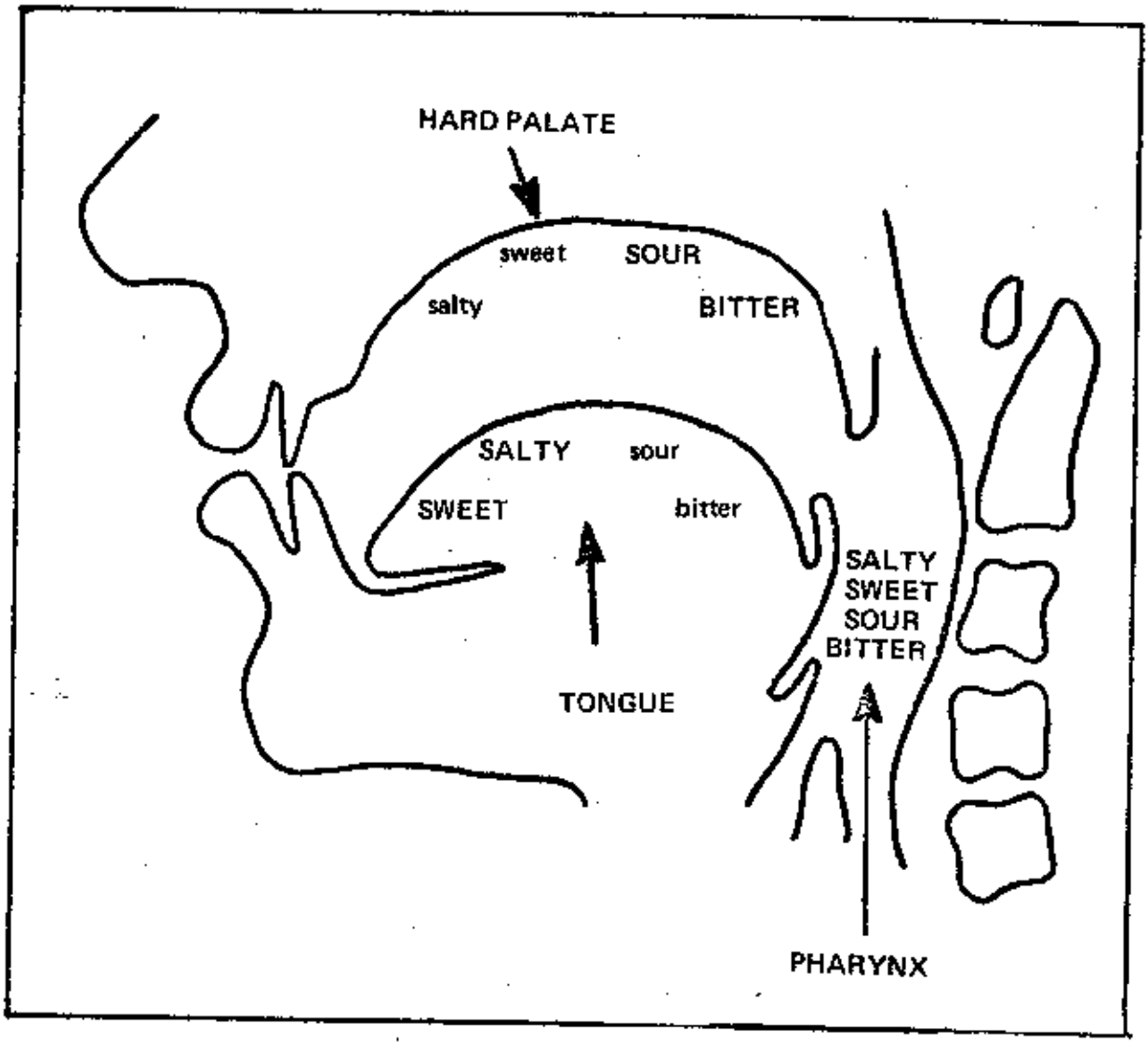
Receptor molecules are thought to be proteins of the receptor cell membranes, because all taste sensations diminish after proteolytic enzymes are held in the mouth (Giroux and Henkin, 1971). It has been suggested that they might be lipoproteins with weak binding capacities. Others believe that the shape of the binding site is more important than the identity of the protein (Amoore *et al.*, 1964, Mazur *et al.*, 1969). However, proteins that bind dissolved tastant molecules and initiate taste sensations have not been identified. Dastoli and Price (1966) have isolated a protein from the sweet-sensitive areas of the tongue. Price and Hogan (1969) reported this protein had dehydrogenase activity, but its structure is not known.

### 2. Taste Nerve Connections

The taste bud of a rat may contain about 200 nerve fibers, which make contact with most of its cells, but only about 50 fibers enter the base of the bud (Beidler, 1969, 1970). The reduction factor is 4:1; in man the cell numbers may be smaller, but the reduction factor is thought to be of the same order (Pfaffmann, 1959).

The afferent fibers from the taste buds in the anterior two-thirds of the tongue join the lingual nerve, and then are included in the chorda tympani nerve, which leaves the lingual nerve, passes through

FIGURE 4




Taste sensitivity of the normal human tongue, palate and pharynx in terms of the "primary" tastes. Each area perceives all four tastes, and differences are relative; capital letters indicate high sensitivity, lower-case letters lower sensitivity. (Redrawn from Henkin, 1970a).

FIGURE 5

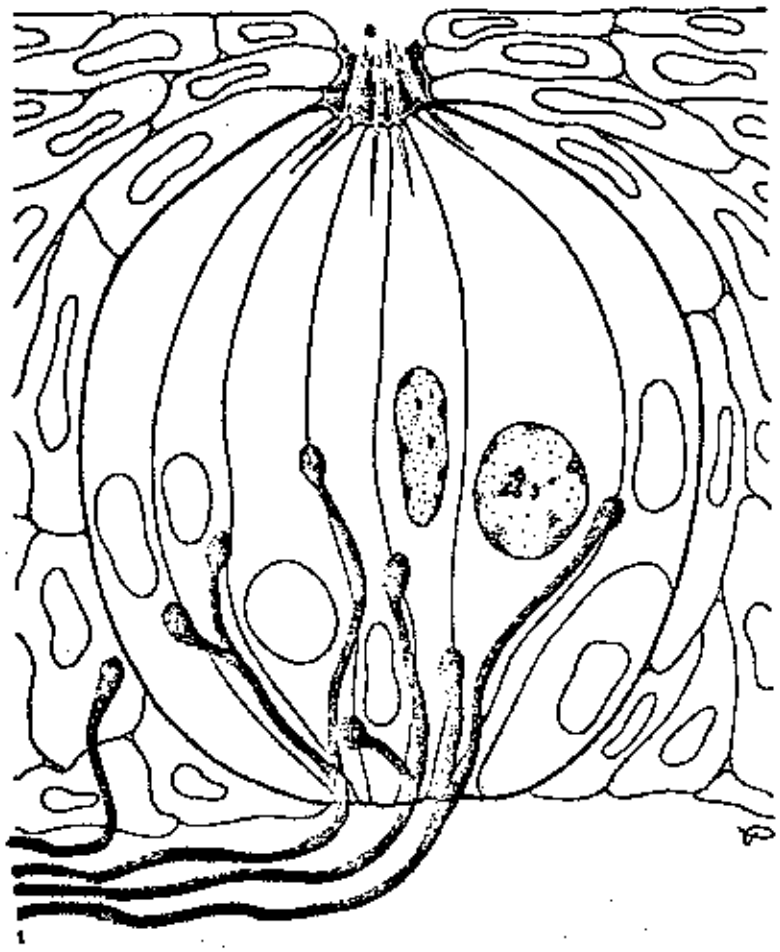


Diagram of a taste bud. The nerves are outside of the cells. Some cells are slender, and others broad, as illustrated in the two cells with detailed nuclei; this difference may or may not be meaningful. Reproduced from Murray and Murray (1970) with permission from the copyright holder, Longman Group, Ltd.

TABLE 1

NUMBERS OF TASTE BUDS IN VARIOUS ANIMALS AND MAN

| | | | |
|---|---|---|---|
| Snake | 0 | Kitten | 473 |
| Chicken | 24 | Bat | 800 |
| Pigeon | 37 | Human | 9,000 |
| Bullfinch | 46 | Pig and Goat | 15,000 |
| Starling | 200 | Rabbit | 17,000 |
| Duck | 200 | Calf | 25,000 |
| Parrot | 350 | Catfish | 100,000 |

Estimates by many investigators published between 1906 and 1961. (Adapted from Kare, 1971).

the middle ear, and enters the brain as part of the 7th cranial nerve (Pfaffmann, 1959, Zotterman, 1971, Sato, 1971). Taste nerves from other parts of the mouth and palate join the 7th, 9th and 10th cranial nerves, which enter the medulla (Pfaffmann, 1959).

Taste areas in the brain are believed to be diffuse, and closely correlated with the somatosensory system (Pfaffmann, 1959). The hindbrain, thalamus and hypothalamus are probably involved (Halpern and Nelson, 1965, Halpern, 1967), but the pathways remain obscure (Burton and Benjamin, 1971).

## C. OTHER RELATED SENSORY INPUT

The neuroanatomy of olfaction may be analogous to that of vision; however, the neuroanatomy of taste appears more analogous to that of touch. Both the olfactory and taste-sensitive areas are innervated for transmission of pain sensations, but the innervations differ. Irritation (leading to sneezing) is integrated into the trigeminal system. Touch, leading to texture recognition, is separate from taste but is important in the identification of foods. Although taste fibers pass through the middle ear, there is no anatomical evidence that auditory stimuli interact with taste sensations within the chorda tympani nerve. However, middle ear damage or other closed head injuries often lead to chorda tympani destruction and subsequent abnormal taste responses. Also middle ear surgery often affects taste responses (Benjamin *et al.*, 1965).

It is evident that all senses interact, but the pathways are largely unknown. Brush and Halpern (1970) have noted that efferent nerve impulses are involved. In addition, the associating functions of memory and vivid recall are factors in these interactions.

# IV. BASIC MECHANISMS OF OLFACTION AND TASTE

## A. OLFACTION

### 1. Odorants - Substances Perceived by Olfaction

All odorants are volatile, are thought to have molecular weights of 300 or less, and with a few exceptions, contain 1 to 2 functional atomic groups. Aggregates of small molecules or macromolecules may be odorless. Beets (1971) considered that much published information on compounds under 140 molecular weight was unreliable. It is likely that these issues are related to the purity of the compounds tested.

Nonmolecular stimuli such as X-rays and ionizing radiation can be "smelled." The nature of this effect is unknown. Passage of X-rays through air is known to generate ozone and peroxides; several investigators (Smith and Tucker, 1969, Garcia and Koelling, 1971) have suggested that these substances are generated by interactions at the surface of the olfactory epithelium. Cooper (1968) has shown that the surgically exposed nasal epithelium of anesthetized rabbits can be stimulated by soft β-rays. Olfactory bulb neurons responded only when the rays were directed at the olfactory mucosa.

### 2. Odor Receptor Function

Current theories about how substances interact with chemoreceptors have been reviewed by Moulton and Beidler (1967), and Dravnieks (1967). These theories are based on the principle of a three dimensional "fit" between molecules (Laffort, 1969). A number of pure substances has been found to be consistent with this concept (Table 2). Amoore (1971) has proposed that an odorant initiates impulses when it alights on a specific protein of the receptor cell membrane.

While there is considerable evidence that a single molecule of some odorants can excite one receptor, the probability of a single inhaled molecule reaching a receptor site is quite low. There are other theories of impulse initiation.

TABLE 2

FIFTY PURE SUBSTANCES WITH MOLECULAR CHARACTERISTICS THAT HAVE BEEN CORRELATED WITH RESPONSES OF THE OLFACTORY SYSTEMS

| | |
|---|---|
| Ethane | Formic acid |
| Butane | Acetic acid |
| Pentane | Propionic acid |
| Heptane | Butyric acid |
| Octane | Pentanoic acid |
| Nonane | Hexanoic acid |
| Hendecane | Heptanoic acid |
| | Octanoic acid |
| Methanol | Nonanoic acid |
| Ethanol | Decanoic acid |
| Propanol | Dodecanoic acid |
| Butanol | |
| Pentanol | Acetaldehyde |
| Hexanol | Acetone |
| Heptanol | Allylic alcohol |
| Octanol | Ammonia |
| Decanol | Bromine |
| Dodecanol | Carbon disulfide |
| | Chloroform |
| Ethyl acetate | Dichloro-1-2-ethane |
| Ethyl butyrate | Isopropyl alcohol |
| Ethyl hexanoate | Propenal |
| Ethyl octanoate | Pyradine |
| Ethyl nonanoate | Hydrogen sulfide |
| Ethyl decanoate | Ethane thiol |
| Ethyl hendecylate | Ethyl sulfide |
| | Benzene |

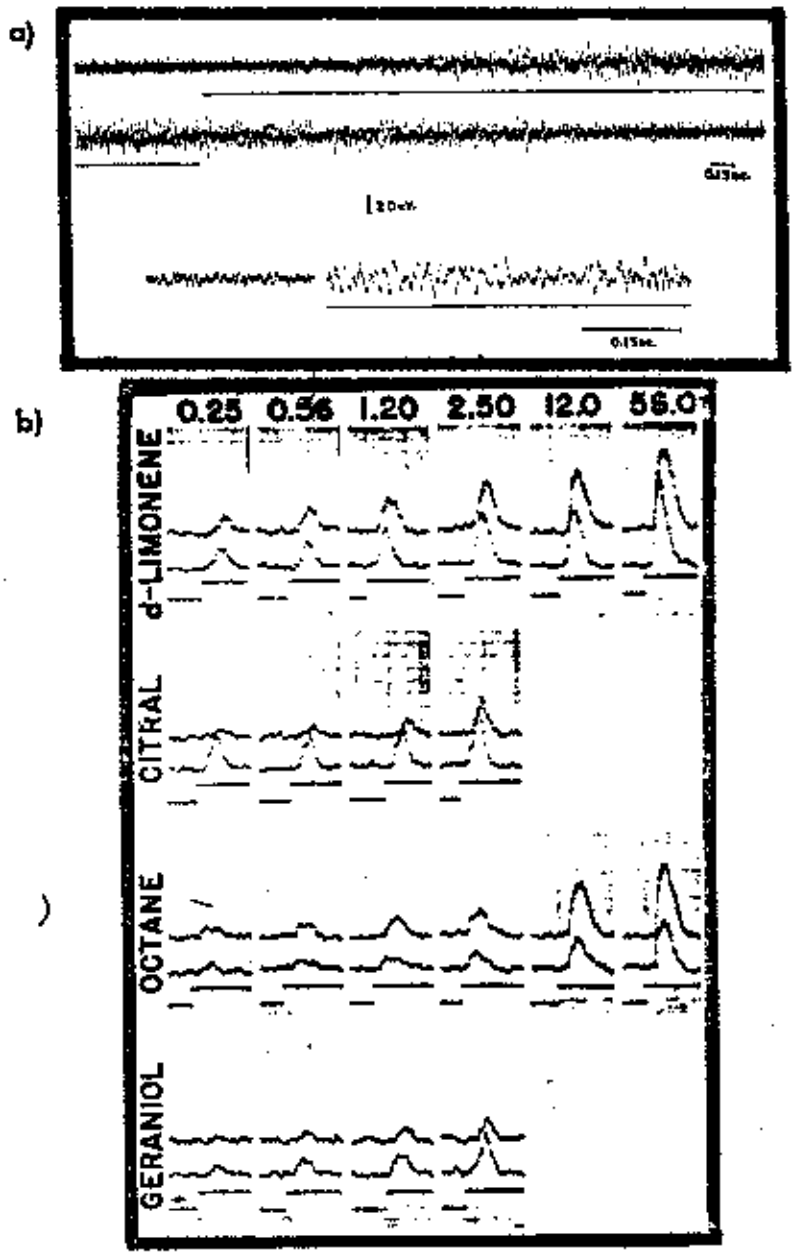
(Modified from Laffort, 1969).

The "chromatographic" theory was proposed first by Adrian (Moulton and Tucker, 1964) and is being studied by Mozell (1971). Mozell has shown that odorant molecules are repeatedly adsorbed onto the olfactory mucosa and revolatilized, a process that is mimicked by gas-chromatography. The tenacity of the mucosa for an odorant is a major, but not the only determinant of migration rate across the entire mucosa, which can vary from less than 2 to more than 270 seconds. Because most odorants are mixtures, each shows typical "spatial and temporal" migration spectra which, from electrophysiological measurements, are reversed when the air flows in the opposite direction (Figure 6). The absence of quality differences in records of nerve impulses taken from single receptors was a major reason for originally hypothesizing that odorants were somehow separated spatially and temporally.

The arrest mechanism for odorant molecules by receptors is unknown. Beets (1971) suggested that an odorant molecule is held by the receptor molecule until the former is degraded; on the other hand, Davies (1969, 1970) believed that the odorant "penetrates or punctures" the receptor cell, allowing sodium to enter and potassium to escape. The biochemical mechanism by which the nerve impulses are initiated is also unknown, but a recent finding that cyclic AMP (adenosine 3', 5'-monophosphate) is involved (Kurihara and Kayama, 1972) suggests that the mechanism is the same as that by which hormones and other circulatory solutes trigger nerve impulses. Receptor responses have been observed following intravenous injection of some odorants, suggesting also that receptor sites may not be limited to the exposed, ciliated parts of the receptor cells. However, there is evidence that these effects may be due to volatilization of the substances into the alveoli of the lungs and then exhalation through the nose.

To account for the logical inference that specificity resides in the receptor cell membrane, and also for the degrees of specificity observed subjectively, limitations on specificity have been proposed (Cain, 1969, Dravnieks and Laffort, 1972). Thus, theoretically a binding site may attract a range of different odorant molecules, an odorant molecule may bind at a range of different sites, and a receptor cell may contain many different sites and may bind more than one molecule at a time.

FIGURE 6




Electrophysiological tracings from a frog olfactory nerve: a) Examples of raw data; b) Electronically summated raw data of neural discharges recorded from the lateral (upper tracing of each pair) and medial (lower tracing) branches of the olfactory nerve of the frog. The time differences and relative sizes of the peaks are part of the evidence for the "chromatographic" theory of odor reception. (Supplied by Mozell).

### 3. Olfactory Nerve Responses

The transmission of olfactory nerve impulses is better understood from both the anatomy and electrophysiological recordings when they are interpreted by electronic summations (Figure 6). Such recordings may be made in animals from single neurons, nerve branches, or the olfactory bulb. Thus it is known that the bulb either summates or to some extent analyzes receptor impulses before transmitting them centrally. The chromatographic theory was inferred from electronic summations of responses recorded simultaneously in the lateral and medial branches of the primary olfactory nerve. Trigeminal nerve responses to odorants differ from olfactory responses and in some cases are more sensitive (e.g., to phenyl ethyl alcohol), but the sensations are difficult to separate subjectively (Moulton and Beidler, 1967).

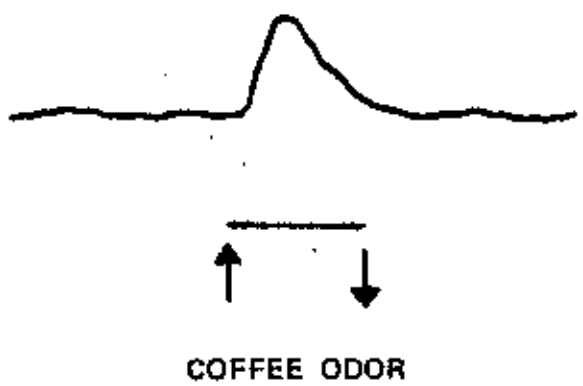
Although the details of the trigeminal inputs into the olfactory bulb (Smith and Tucker, 1969) and the efferent inputs from the cortex that evidently influence transmissions by the bulb (Moulton and Beidler, 1967) have not been fully elucidated, it is clear that the bulb has selective functions and that bulbar lesions may diminish, distort or prevent the perception of odors.

### 4. Detection Thresholds

The presence of most odorants is detected at concentrations lower than are needed for their identification. The latter is called the *recognition threshold* and the former is the *detection threshold.*

Detection thresholds can be established in animals and man by the electro-olfactogram (EOG) (Figure 7), as revealed by Ottoson in 1956. He defined the EOG as "*the monophasic negative potential evoked by odors in the sensory region of the nasal mucosa*" as measured by simultaneous recordings from the mucosa under well-controlled conditions of stimulus (Ottoson, 1971). EOG responses vary in amplitude, while "unit" responses (all-or-nothing) are found in the nerves that leave the bulb (Takagi, 1969). Increases of odorant concentrations above certain levels are not detected olfactorily, although some increases may be signaled by the trigeminal nerves; these are perceived as changes in odor quality with increasing concentrations. However, the significance of EOG data in relation to the existence of true thresholds in man is controversial.

FIGURE 7




Typical human EOG (electro-olfactogram), from the olfactory epithelium. The peak response is very brief. Coffee contains at least 500 different volatile substances, all of which may contribute to the odor (Wick, 1965). Redrawn from Osterhammel *et al.*, 1969 .

In man, detection thresholds, estimated by sniffing tests, differ among people and fluctuate over time, so much so that the range of normality is usually expressed in log units of odorant concentration. (Amoore, 1968). In a population, the highest normal threshold may be $10^3$ of the lowest, and the range in 95 percent can be 10-fold above and below the mean (Amoore, 1968, Berglund *et al.*, 1971). The direction of an odor source can sometimes be detected within 7-10 degrees (Moulton and Beidler, 1967), especially if the subject is free to move his head or if the odorant stimulates the trigeminal nerves (Schneider, 1967).

5. Odor Recognition Thresholds

The inferred location of recognition mechanisms is the prepyriform cortex (Adey, 1970). Recognition is studied indirectly, by its behavioral effects, which currently are better understood than biochemical or electroencephalographic data. The involvement of memory is implicit (Engen, 1972); however, other sensory input and psychomotor responses are "unwanted variables" that cannot be excluded. Engen (1970) has proposed three forms of mental judgement: discrimination, scaling, and recognition itself. Discrimination, or perception that odors differ, appears to be described by the psychophysical power function (quantum steps) proposed by Stevens (1972). Scaling, or categorization, includes a quantitative element. To the subject, recognition is identification beyond doubt.

Tests comparing odorants are usually more reliable than tests of single odorants alone. Observations suggest that memory generally becomes confused after identification of about 16 single odorants in sequence. Furthermore, behaviorally obtained thresholds were elevated by the addition of thresholds for psychomotor responses, and complicated by "training" effects of repeated test performance. Thus observed recognition thresholds tend to vary more than do detection thresholds. Beets (1971) suggested that, all factors considered, human acuity for odor recognition may be, at its best, no less than canine acuity.

As odorant concentrations increase, there comes a point above which either increases will not be recognized or the odor quality will appear to change. These upper thresholds have been relatively little studied but could be relevant to recognition of dangers, especially when dangerous concentrations are above the upper recognition threshold.

Most odors are generated by mixtures of odorants, and most odorants are hard to obtain in chemically pure form. When this is done, the recognized odor can sometimes be attributed to some impurity or contaminant. Thus, the question of whether all pure odorants are "primary" remains unanswered, and for all of the above reasons it is difficult to utilize any current listing of "primary" odors (Table 3) (Schneider, 1967). The concept of primary odors is derived by analogy from primary tastes and colors. The analogy is in doubt owing to the differences in anatomy and physiology among these three sensory systems.

## B. TASTE

### 1. Taste Receptor Function

All tastants are soluble (Beidler, 1954); even quinine ethylcarbonate has been demonstrated experimentally to be tastable (Faull *et al.*, 1971). This material, originally believed tasteless and insoluble has been shown to be very slightly soluble in saliva. Vastly more fluid bathes the taste areas than the olfactory epithelium, and there is no evidence of "chromatographic" migration of tastants (Davies 1971). Larger molecules can be tasted than can be smelled and there seems to be no grounds for hypothesizing penetration of taste receptor cells. It is emphasized that, unlike olfactory receptors, taste receptor cells are not nerve cells. Support is growing for the proposal that taste receptor molecules are proteins with one or more sites that bind and react with tastant molecules so as to generate nerve impulses (Beidler, 1954, 1971).

Stereochemistry is a guide to taste with some but not all tastants. Sugars and some amino acids can be ranked for sweetness (Tables 4 and 5), and the "glucophore" appears to be some functional group of atoms within the molecule (Kier, 1972).

### 2. The Primary Tastes

Most workers provisionally accept the theory, formulated by Fick in 1864 (Andersen, 1970), of 4 species of receptors corresponding to 4 "primary" sensations: sweet, bitter, salty, and sour (Figure 8). In 1817, Park had proposed "warm" and "cool" as primary taste sensations; however, these additions are not universally accepted although these 2 sensations are compatible with the 4 primary sensations (Dzendolet, 1969, Sato *et al.*, 1969). The 4 primary sensations are interrelated

TABLE 4

RELATIVE SWEETNESS OF VARIOUS SUGARS IN SOLUTION*

| | |
|---|---|
| β-D-Fructose | 100-175 |
| Sucrose | 100 |
| α-D-Glucose | 40-79 |
| β-D-Glucose | <α-D-Glucose |
| α-D-Galactose | 27-67 |
| α-D-Mannose | 59 |
| β-D-Lactose | 48 |
| β-D-Maltose | 32-46 |
| β-D-Galactose | ** |
| α-D-Lactose | 16-38 |
| Raffinose | 23 |
| Stachyose | ** |
| β-D-Mannose | Bitter |

*Compared with arbitrary value of 100 for sucrose.
**Value undetermined.

Table adapted from Shallenberger and Acree (1971).

TABLE 3

SCHEMA PROPOSED FOR CLASSIFICATION OF PRIMARY ODORS

| Linnaeus 1756 (7) | Zwaardmaker 1895 (30) | Henning 1915 (6) | Crocker & Henderson 1927 (4) | Amoore 1952 (7) | Schultz 1964 (9) | Wright & Michels 1964 (8) | Harper et al. 1968 (44) | Amoore 1969 (added) |
|---|---|---|---|---|---|---|---|---|
| | Almond | | | | Spicy | | Almond | |
| | Amber | | | | | | Animal | |
| | Aniseed | | | | | Benzo-thiazole | | |
| | Bed-Bug | | | | | | | |
| | Bromine | | | | | | | |
| | Burnt | Burnt | Burnt | | Burnt | Affective | Burnt | |
| | Camphor | | | Camphor | | | Camphor-Mothballs | |
| Hircine | Caproic | | Caprylic | | | | Sweaty | |
| Nauseous | Carrion | | | | | | Sickly | |
| | Cat-Urine | | | | | | | |
| | Cinnamon | Spicy | | | | Spice | Spicy | |
| | Citrous | Fruity | | | | Citral | Citrous | |
| Aromatic | Clove | | | | | | Aromatic | |
| | Ethereal | | | Ethereal | Etherish | | Etherish-Solvent | |
| | Fecal | | | | | | Fecal | |
| | Fishy | | | | | | Ammonia-Fishy | |
| | Fruity | | | | | Hexyl-acetate | Fruity | |
| | Jasmine | Flowery | | Floral | | | Floral | |
| Alliaceous | Leek | | | | | | Garlic | |
| | Lily | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Minty | | | Minty | | | Minty | |
| Ambrosial | Musky | | | Musky | | | Musk | |
| Repulsive | Narcotic | | | | | | | |
| Fragrant | Orange-Blossom Phenolic | | Fragrant | | Fragrant | | Fragrant | |
| | | | | | | | Carbolic | |
| | Rosy | | | | | | | |
| | Thyme | | | | | | | |
| | Vanilla | | | | Sweet | | Vanilla-Sweet | |
| | Violet | | | | | | Soapy | |
| | Waxy | | | | | | Soapy | |
| | | Resinous | | | | Resinous | Resinous-Paint | |
| | | Foul | | Putrid | Sulfurous | Unpleasant | Putrid-Sulfurous | |
| | | | Acid | | | | Acid | |
| | | | | | Oily | | Oily | |
| | | | | | Rancid | | Rancid | |
| | | | | | Metallic | | Metallic | |
| | | | | | | | Meaty | |
| | | | | | | | Moldy | |
| | | | | | | | Grassy | |
| | | | | | | | Bloody | |
| | | | | | | | Cooked-Vegetable | |
| | | | | | | | | Sandal |
| | | | | | | | | Watery |
| | | | | | | | | Urinous |

Modified from Amoore (1969), who has reduced the 118 proposed odor classifications to 44 "primary" odor descriptions. See Amoore (1969) for a more complete discussion and the original references.

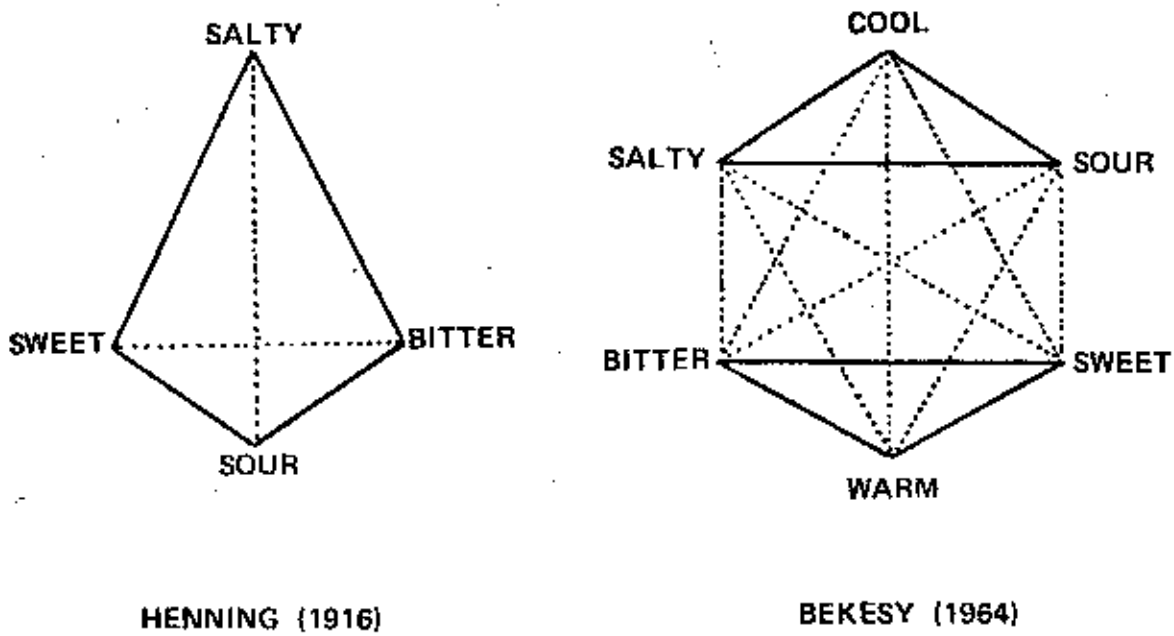
TABLE 5

TASTES OF SOME AMINO ACIDS*

| Amino Acid | Form of Structure D- | L- |
|---|---|---|
| Alanine | sweet | sweet |
| Asparagine | sweet | tasteless |
| Glutamic acid | mixed | tasteless |
| Glycine | sweet | sweet |
| Histidine | sweet | tasteless |
| Hydroxyproline | - | sweet |
| Isoleucine | sweet | bitter |
| Leucine | sweet | bitter |
| Methionine | sweet | tasteless |
| Phenylalanine | sweet | bitter |
| Proline | - | sweet |
| Serine | sweet | sweet |
| Threonine | sweet | sweet, bitter |
| Tryptophane | sweet | tasteless |
| Tyrosine | sweet | bitter |
| Valine | sweet | sweet, bitter |

*Data from Shallenberger and Acree (1971), who note that according to Mazur *et al.* (1969) the dipeptide, or joined pair of amino acids, L-aspartyl-L-phenylalanine methylester is 100-200 times sweeter than sucrose although singly, both amino acids taste bitter.

FIGURE 8




Two proposed relationships between the primary tastes in man. Left: the 4-taste system proposed by Henning in 1916, cited by Pfaffmann *et al.* (1971). Right: the 6-taste system proposed by von Békésy in 1964, cited by Sato (1971), who found nerve responses to all 6 "tastes" in hamsters but somewhat different relationships between them, interpreted as interspecies differences.

(Figure 8), either at the receptor level or, more likely, at a cognitive level in the brain (Frank and Pfaffmann, 1969, Pfaffmann *et al.*, 1971). The opposed pairs, sweet-bitter, salty-sour, warm-cool, were inferred from after-tastes that could not be attributed to breakdown products in the mouth (Pfaffmann *et al.*, 1971). Most workers accept the 4-taste classification as convenient and recognize that the underlying biological systems are more complex.

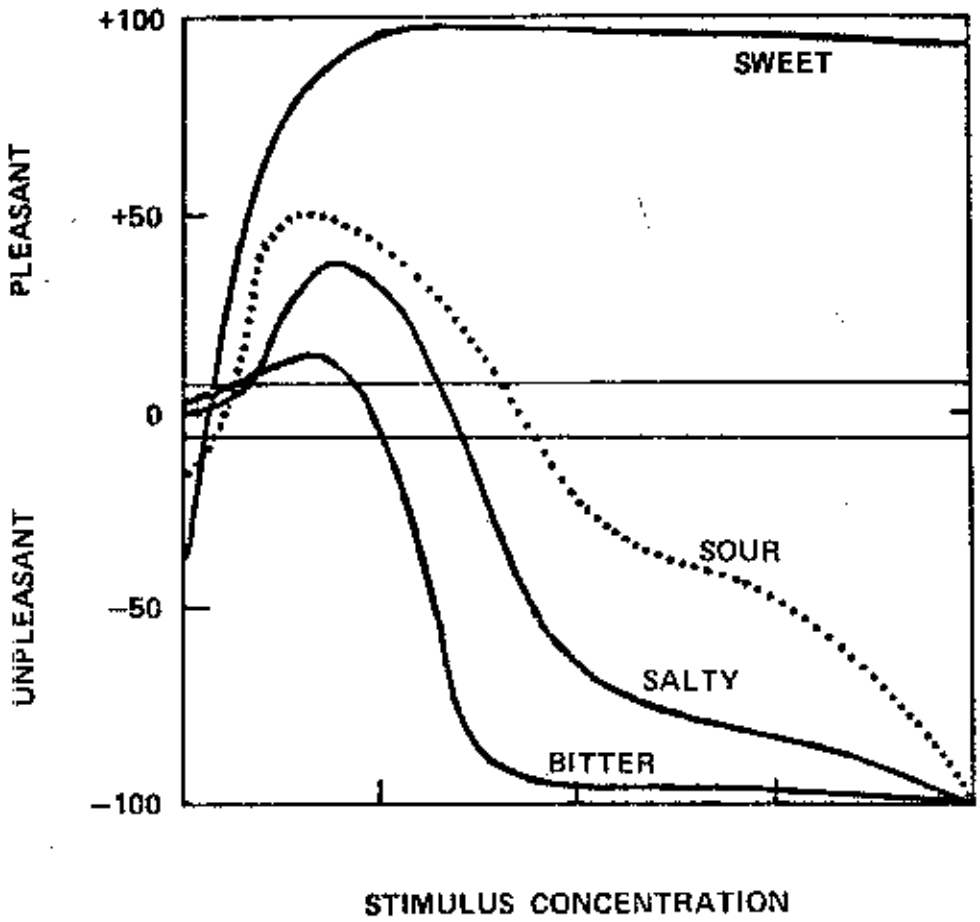
This theory is supported by some electrophysiological data, but supporting stereochemical data are at best equivocal. Some amino acids taste sweet, but others do not; and not all acids taste sour. Very weak sodium chloride solutions taste sweet. There are electrophysiological indications that some taste buds contain more than one species of receptor, while others do not. Perhaps such tastants are bound at more than one type of site. Some shifts of taste quality indicate merely that a tastant is a mixture. The "taste" of electrical current has been debated since 1754; whether it reflects nerve stimulation, electrolysis of saliva components, or both, is still undecided (Bujas, 1971).

### 3. Taste Nerve Responses

Chemical mediators are probably involved in the generation of responses, as in other sensory modalities (Kurihara and Kayama, 1972). Responses are monitored electrophysiologically in animals, from single fibers or nerve trunks, and thus detection thresholds are estimated (Doetsch *et al.*, 1969).

Unlike the olfactory system and its paired organ, the olfactory bulb, the innervation of taste appears to have few peripheral neural ganglia for sensory analysis. The taste bud does not seem comparable with the olfactory glomerulus; this suggests two problems for future study. The first is whether the primary taste sensations result from local dominance patterns in the transmission of taste information by the many nerve fibers, rather than from receptor cell differences. The second concerns the possible evolution of taste sense by a localized development of chemoreceptivity in epithelial cells associated with tactile nerve endings. Compared with olfaction, the perception of taste obviously is more restricted by the receptors' microenvironment, cell type, cell turnover, and the need to transfer responses to an associated nerve instead of transmitting them directly.

FIGURE 9




Human subjective judgments of "pleasant" or "unpleasant" sensations in response to increasing concentrations of cane sugar ("sweet"), tartaric acid ("sour"), sodium chloride ("salty"), and quinine sulfate ("bitter"). The maximum concentrations were: sugar 40%, acid 1.12%, NaCl 10%, quinine 0.004% (weight/volume). The minima were all zero. "Pleasant" and "unpleasant" are separated by a neutral zone. Redrawn from Pfaffmann *et al.* (1971), who reproduced the figure from Engel.

## 4. Taste Detection and Recognition Thresholds

Detection thresholds are measured electrophysiologically in animals and there is limited work on their subjective assessment in man. Thresholds in man involve some degree of identification, first in terms of the primary taste, and with increasing intensity, in terms of recognition of the tastant, involving memory and usually other factors as well. It seems likely that chemically pure tastants that stimulate only taste receptors can evoke only recognition of primary tastes (Figure 8). Tactile input, indicating texture, accompanies taste input, but is not integrated peripherally as is trigeminal input.

Recognition is enhanced by practice, and the amount of practice is largely determined by the hedonic effects of tastants (Figure 9). For example, people tend to repeat "pleasant experiences." The mechanism of hedonic effects is important but obscure (Pfaffmann *et al.*, 1971). Enhanced sucrose preference is inducible, and is evident in a vast majority of the U.S. population. The role of taste mechanisms in sugar-seeking appears limited to detection and recognition; thus, sugar preference may have its basis in physiological mechanisms other than taste *per se* (Gentile, 1969, Nachman and Cole, 1971). Contrary opinions have been expressed (Bartoshuk *et al.*, 1971). This is an area in which basic research is urgently needed.

# V. FACTORS INFLUENCING THE CHEMICAL SENSES

## A. OLFACTION

The variability of sensory responses to olfactory stimuli reflects a large number of independently varying factors, such as stimulus, receptor, and subjective factors. On the other hand, this variability may have been exaggerated by tests designed for basic research rather than for screening, because observation of the different stages of a response requires the elimination of stabilizing factors normally present, e.g., efferent impulses from the brain to the olfactory bulb.

Olfaction can be altered in various ways, including anosmia (absence), hyposmia (decrease), hyperosmia (enhancement), parosmia (distortion), dysosmia (unpleasant distortion), and phantosmia (hallucination.) Such states may be transient or permanent, and may arise within the subject or be related to his environment.

### 1. Stimulus Factors

Seldom if ever is the air so pure that only one odorant is present. Odorant concentrations vary, and different odorants have different potencies. An odor may be masked by others present either at the same time or shortly beforehand. If a stimulus is too brief, the odorant is not recognized; if too prolonged, adaptation occurs. The time and intensity factors in recognition of repeated or inconstant stimuli are complex and poorly understood. Odorants, or concentrations of an odorant, that stimulate the trigeminal nerves, add further complications. Similarity between odors may hinder identification, especially when odorants are presented singly or under conditions precluding comparison.

Some odorants and odorless gases may actively distort the perception of others, an effect that is better known in taste and requires study in olfaction. A face mask may affect inhaled air by adding or removing odorants. Intense input from other senses can mask olfactory input (Stone and Pangborn, 1968). Sensory responses are affected by the temperature, pressure, and humidity of the inhaled air, and by its flow rate and turbulence; these factors are partly under the subject's control.

2. Receptor Factors

Other determinants include the condition of the olfactory epithelium, and the amount and pH of the mucus. Factors such as blood endocrine levels and the degree of vasodilation may affect receptor sensitivity. The functional activity of the nasal mucous membranes has an important influence on acuity. Best acuity is seen when the membrane is moderately active (moderately swollen and red), poor acuity is associated with low membrane activity (shrunken and pale) and acuity is lost when the membrane is in a hyperfunctioning state (marked swelling, redness, and wetness) (Schneider and Wolf, 1960).

3. Subjective Factors

A normal, healthy person may associate an odor with particular visual, auditory or tactile sensations, absence of which will modify his detection or recognition threshold for that odor. The presence of other sensations may induce phantosmia, the hallucination that an odor is also present. Other sensations can also distract a person from recognizing an actual odor (Stone and Pangborn, 1968). On the other hand, odor may confirm or deny suspicions raised by other sensory input, or may raise suspicions for other sensory input to confirm or dismiss. Engen (1972) has emphasized that expectation strongly influences judgments of odors.

Odor discrimination can be taught; thus acuity can be enhanced by practice, and self-training can be directed by appetitive or aversive effects of odorants. However, these effects are not uniformly predictable and their mechanisms are obscure. Children are very tolerant of smells (Engen and Moskowitz, 1972), and thus aversive effects may be unique to adult humans. Examples of such aversive odors are onions, smoke and mercaptan. The food and perfume industries capitalize on supposed hedonic effects of odors. Human pheromones (sex attractants) are thought to exist, probably in the apocrine system, though none have yet been identified (Comfort, 1971).

A number of physiological variables influence olfactory acuity. Olfactory response is thought to vary with age and may vary by sex according to some workers (Moulton and Beidler, 1967, Venstrom and Amoore, 1968). Schneider (1967) has reported that females are generally more sensitive, and are most sensitive when ovulating. This has been attributed to cyclical effects of certain hormones, including

an androgen, 5-α-androst-16-en-3-one (Comfort, 1971) and estrogens (Moulton and Beidler, 1967). Circadian fluctuations of acuity can be ten-fold (Stone and Pryor, 1967). Acuity is influenced by exercise (Stone *et al.*, 1967), but may not be affected by smoking (Venstrom and Amoore, 1968) nor by alcohol consumed in moderation. Woods (1968) holds that smoking, alcohol, and diet markedly affect olfactory acuity.

Variations of acuity in naive subjects during preliminary tests should be expected, but the processes of long-term and short-term adaptation differ, and the variations are large. All persons adapt to the constant presence of an odorant (Moncrieff, 1968). Schneider and Costiloe (1969) have shown that the same subject whose pattern of adaptation is determined in the laboratory at intervals, may vary from rapid adaptation to no adaptation, and most often the latter occurs in a setting of psychosocial stress. Food deprivation may be one such stress, but reports that people are more sensitive when hungry are not unanimous (Moulton and Beidler, 1967). Henkin *et al.* (1972) have reported that olfactory acuity is diminished when tissues are depleted of zinc, copper, or vitamin A. The steroid hormones secreted by the gonads and adrenal cortex are influential. Women who are estrogen deficient have relatively low olfactory acuity which increases when they are given estrogens and decreases when they are given androgens (Schneider *et al.*, 1958). Hyperosmia is associated with Addison's disease (adrenocortical insufficiency) and with cystic fibrosis of the pancreas, and hyposmia with gonadal dysgenesis in females. Many stresses that alter the output of these hormones are believed to affect olfactory acuity directly.

Connective tissue disorders, e.g., Sjogren's syndrome (associated often with rheumatoid arthritis), diminish olfactory acuity (Henkin *et al.*, 1972), as do atrophic rhinitis, and any injuries in the 1st, 5th, 9th, or 10th cranial nerves, such as may result from trauma or surgery to the head (Henkin *et al.*, 1968). Several causes of secondary anosmia or hyposmia have been briefly reviewed by Schneider (1972). Males *et al.*, (1972) have noted evidence for a relationship between olfactory performance and pituitary function. When sensitivity to particular odorants is lost, the symptom may be parosmia (Amoore, 1969, 1971), which is usually noticed when it affects a subject's scale of hedonic values.

General anosmias of various origins are estimated to occur in about 0.2 percent of the U.S. population, and it is believed that they are seldom reported (Amoore, 1968). The view that all albinos are

anosmic has been proved false (Moulton and Beidler, 1967). Anosmias to specific substances (Table 6) are thought to arise by mutations of specific receptor proteins; however, very few such anosmias have been studied in detail. Anosmias or hyposmias for the musky odor typical of urine or sweat are relatively common (Amoore, 1969, Brown *et al.*, 1968).

## B. TASTE

### 1. Stimulus Factors

Temperature influences the potency of tastants, which tends to be greatest at 30-35° C (Pfaffmann *et al.*, 1971). Refrigeration of foods diminishes the potency of most tastants. Dentures that cover the hard palate diminish the acuities for sour and bitter tastes (Henkin and Christiansen, 1967).

A number of substances distort the tastes of other substances, because they do not affect all the receptor types equally. For instance, anesthetics applied to the tongue block taste sensations in the order: bitter>salty>sour>sweet (Kare and Henkin, 1969). Some antibiotics reportedly cause dysgeusia (Henkin, 1970a). Two vegetable extracts have recently attracted attention: "miracle-fruit" and gymnemic acid.

Miracle-fruit *(Synsepalum dulcificum)* contains a basic glycoprotein of about 44,000 *M* with L-arabinose and D-xylose. When applied to the mouth, it may cause acids to taste sweet for over three hours without altering thresholds for sour, salty or bitter tastes. It is hypothesized that the glycoprotein binds to the epithelium near the sweet receptors so that acids act on these as well as on their "own" receptors (Kurihara, 1971).

The leaves of *Gymnema sylvestre* contain gymnemic acids of which one, known as $A_1$, contains a molecule each of gymnemagenin and glucuronic acid. When gymnemic acid $A_1$ is applied to the tongue it suppresses sweet taste for up to 30 minutes. Other taste judgments are also modified (Meiselman and Halpern, 1970). The nerve responses are blocked, which could mean merely that the receptors are blocked. However, there are additional biological effects, so that the mechanism of action must be described as unknown at present (Kurihara *et al.*, 1969, Kurihara, 1971).

TABLE 6

REPUTED SPECIFIC ANOSMIAS

| | |
|---|---|
| Acetic acid | Hibiscolide |
| Adamantane | Hydrogen cyanide |
| Allicin | Indole |
| Allyl isothiocyanate | Idoform |
| $\Delta^{4,16}$-Androstadien-3-one | Ionone |
| $\Delta^{16}$-Androsten-3-ol | Isobutyraldehyde |
| Anisic aldehyde | Isobutyric acid |
| Anisole | Isovaleric acid |
| Benzene | Menthol |
| Benzyl alcohol | d, l-Menthone |
| Benzyl salicylate | Methional |
| t-Butyl carbinol | Methyl cyclopropyl ketone |
| n-Butyl mercaptan | Methyl mercaptan |
| t-Butyl mercaptan | Musk xylol |
| Caproic acid | Naphthalene |
| Cedryl acetate | PEME carbinol |
| 1, 8-Cineole | Pentadecanolactone |
| Cinnamaldehyde | Phenylacetic acid |
| Cyclotene | Phenylethyl alcohol |
| p-Dichlorobenzene | Phenyl isocyanide |
| Dimethyl disulfide | Phenyl isothiocyanate |
| Ethylene brassylate | 1-Propenylsulfenic acid |
| Ethylenedichloride | Putrescine |
| Eugenol | Salicylaldehyde |
| Farnesol | Skatole |
| Formic acid | Thiophane |
| Geranial | Thymol |
| Geraniol | Trichloroethylene |
| 2-Heptanone | γ-Undecalactone |
| $\Delta^{9}$-Hexadecenolactone | Vanillin |
| Hexylamine | Versalide |

Substances to which some people are reputedly anosmic as listed by Amoore, 1969.

Other vegetable extracts, e.g., from *Discoreophyllum cumminsii* and *Thaumatococcus daniellii*, have been found to contain proteins that taste very sweet but do not actively distort the reception of other tastes (Morris and Cagan, 1972).

General enhancement of taste acuity is claimed for substances such as monosodium glutamate, the 5'-nucleotides, and maltol (Kermode, 1972). Their mechanisms of action are obscure.

2. Subjective Factors

Carbohydrate-active adrenal corticosteroid hormones inhibit taste acuity; when more of them are secreted, acuity diminishes, and *vice versa*. Thus, a progressive taste loss may signify an adrenocortical tumor, or a progressive gain may signify a case of Addison's disease (Henkin, 1970b). By contrast, estrogens promote taste acuity. Stresses that affect secretion of these hormones will influence taste acuity accordingly. It has been noted that acuity increased with rising levels of plasma ascorbic acid, which were influenced by corticosteroid secretion and, in females, by the estrous cycle. Furthermore, the capacity of taste receptors to initiate nerve impulses appears to be governed by blood pressure (Hellekant, 1970).

Dependence of taste acuity on adequate tissue levels of zinc and copper was reported after examination and manipulation of these levels in patients presenting with ageusia (Henkin and Bradley, 1969). However, other workers have found few abnormalities of taste acuity in patients presenting with zinc deficiency. Further studies are needed in which the selection of subjects will be a controlled variable.

Taste thresholds are thought to be influenced by hunger and satiety, but the mechanisms are obscure; nevertheless, taste recognition can directly stimulate processes of digestion (Kare, 1969). Both detection and recognition of tastes are influenced by other sensory input. For instance, the sight and smell of a meal elicits salivation as well as changes in insulin levels not related to modifications in the level of blood glucose (Engen, 1972, Goic *et al.*, 1969).

On the other hand, other sensory input may have masking or distracting effects that diminish taste acuity (Stone and Pangborn, 1968). Many observations are difficult to interpret, because behavioral responses of animals differ between species and tend not to correspond either with electrophysiological responses or with human subjective

judgments of taste. Nevertheless, taste preferences are thought to result from diet selection in man and animals, and to reinforce that selection rather than to determine it in the first place.

A recent preliminary report (Lum *et al.*, 1971) suggests that taste acuities may vary slightly with age and between the sexes, and do vary consistently with the amino acid composition and pH of the saliva. Inherited ageusias have been little studied except for insensitivity to bitter compounds with an N-C=S group, such as phenylthiocarbamide (PTC). The incidence of this is correlated with that of goiter (Sunderland and Cartwright, 1968); nevertheless, a Mendelian genetic pattern is accepted (Fischer and Griffin, 1964, Henkin, 1970b, Kalmus, 1971).

Traumatic head injuries, in which some of the taste nerves lose their function, can result in distortions of taste sensations (parageusia, dysgeusia) (Henkin *et al.*, 1971, 1972).

# VI. TECHNIQUES FOR STUDIES IN MAN

Both olfaction and taste are tested by methods developed for basic research into the mechanisms of those senses (Engen, 1971). Electrophysiological observations describe stimuli transmitted by the nerves to which electrodes are applied but do not indicate perception of those stimuli by the conscious brain. Perception is estimated subjectively by volunteers. An indirect measure of perception, behavior, includes responses to the sense tested and to other sensory input, as well as variables and thresholds pertaining to the mechanisms of the behavioral response. A subject responds differently before and after he has learned the study techniques (Cain and Engen, 1969).

However, this LSRO study is concerned only with techniques available for practical evaluation of human olfactory and taste acuities by screening populations who are not volunteers, not with tests that are used only in basic research. Methods that can be used for setting and maintaining standards and for interpretation of results of screening tests are of prime importance.

## A. OLFACTION

### 1. Standards and Criteria

Perhaps the most important difficulty has been simultaneous standardization of both background and test stimuli, because it is the difference between these that must be detected and recognized. Odorant-free air probably cannot be achieved or maintained; however, the composition of air can be monitored by analysis.

Methods available for analyzing air for odorants include gas chromatography (GC), mass spectrometry (MS), and plasma chromatography (PC). Each method gives its own pattern of results, that is, it senses some odorants and some odorless gases, fails to sense others, and senses some more acutely than others. Some workers use combinations of these methods, and the relative merits appear to depend on the situation and purposes of the analyses. Many odors are complex; e.g., Wick (1965) showed that coffee contained over 500 volatiles,

and Young *et al.* (1970) demonstrated the analytical problems by synthesizing a "bourbon" with a gas-liquid chromatogram similar to that of real bourbon but with dissimilar odor and taste.

Analysis of air samples for dilute vapors can sometimes be facilitated by preconcentration methods (Dravnieks *et al.*, 1971). The GC-MS combination is sensitive for detection of traces of contaminants in larger samples of gaseous, liquid, or solid materials. Such techniques would have contributed greatly to a recent study on the social and economic impact of odors in the community (Copley Corp., 1971), where not a single air sample was analyzed. However, the preparation of air samples for analysis by GC or MS may be a problem outside the well-equipped laboratory. Here the PC method has the advantage that air samples do not require preparation.

The PC method is relatively new, and its instrumentation (Franklin GNO Corp., 1972) is still under development. Recent research at the National Bureau of Standards suggests that air from different sources may be simultaneously and continuously monitored by one instrument, and this could be useful for screening situations in the military environment. The degrees of analytical specificity and sensitivity (Cram and Chesler, 1973) seem comparable with those obtainable by GC or MS, and combinations of PC with these methods are currently being explored.

Evaluation of human olfactory acuities by these methods will require the assembly of many standards: identity, quality and intensity of test odors; controlled test conditions; information about subjects; and interpretation of results. Knowledge from basic research will need to be reassessed before application to evaluation of human olfactory acuities (Engen, 1971).

2. Choice of Odors

In selecting odorants for tests, preference should be given to those that are chemically pure and with known olfactory effects (Table 2). When study goals require the use of less well-defined odorants, the limits of the information to be obtained should be recognized. A balance will be struck between the number of odorants and the number of concentrations used. At the present time, approximately 20-30 subjects can be screened per day by double-blind techniques.

### 3. Threshold Determinations

The utility of threshold studies is highly controversial. One common definition of "threshold" is the lowest concentration detected in 50 percent of responses. There are always many false positive and negative responses; the same applies to recognition thresholds, and to upper thresholds above which concentration increases are not recognized. It may be that studies on lower thresholds are more useful in a training situation than for simple evaluations.

### 4. Testing of Humans

Techniques rely primarily on subjective responses to odorants delivered to the nasal passages under relatively standard conditions of temperature, pressure, flow rate, and humidity of the air carrying the odorant (Schneider *et al.*, 1963, Amoore, 1968). Though it is difficult to standardize the airflow over the olfactory epithelium, this is desirable for determining differences in olfactory acuity of the two sides of the nose (Schneider and Schmidt, 1966, Schneider, 1972). In practice, subjects tend to determine for themselves the airflow rate that gives them the greatest response. To minimize self-adaptation, some investigators use intervals between successive stimuli of at least 7 seconds, while others find 5 to 10 minutes necessary.

The importance of routinely examining subjects for gross unilateral or bilateral intranasal conditions, such as atrophic rhinitis, or polyps, has been emphasized (Schneider, 1972). Furthermore, it is obvious that valid test results will not be obtained during or immediately after respiratory tract infections, especially influenza.

Behavioral responses and subjective judgments contain extraneous variables that often detract from the validity of olfactory test results (Mozell, 1969). Much of the early work was conducted without emphasis on standardized test conditions. Standardized psychophysical testing techniques are being developed that are said to interfere minimally with the assessment of odor detection and recognition (Cain and Engen, 1969). These include statistical procedures such as factor analysis and "frequency component hypothesis" (Hughes *et al.*, 1969), that may be expected to assist in evaluating the responses of screened subjects who are not research volunteers.

## B. TASTE

Acuity in the primary tastes is tested with standard substances: sucrose (sweet), quinine (bitter), sodium chloride (salty), dilute hydrochloric acid (sour) (Sato *et al.*, 1969). Temperature of the tastant is important (Stone *et al.*, 1965). To test the detection and recognition thresholds, prepared solutions are applied to the tongue or are held in the mouth for a set time and then swallowed or ejected (McBurney, 1969). Tongue application avoids unevenness of dilution in saliva, but also neglects the taste areas of the hard palate and pharynx. The perceived intensity of taste is compared with the concentration of tastant. However, perception includes many subjective variables (Powers *et al.*, 1971) and some workers prefer not to use primary taste judgments to evaluate human acuities.

Foods seldom if ever contain only single tastants and their analysis by taste seldom corresponds with instrumental or chemical analysis (Powers, 1970, 1972). Consistent with the relatively minor role of taste in diet selection, panelists in the food industry who judge the potential acceptability of foods before marketing are not screened for acuity in the primary tastes (Hirsh, 1973). Food processing techniques have diminished the usefulness of taste as an indicator of spoilage or contamination, but awareness may still be valuable; often there is an intensity change in only one of the primary sensations. For sensory evaluation of taste acuities, behavioral controls can minimize many errors in volunteer research subjects, and may be effective in the comprehensive screening of nonvolunteers. Such studies are needed; little is known about standardization of screening methodologies for taste acuity.

Electrophysiological studies of taste acuity in man are seldom practical, although in a few cases the results have corresponded well with subjective test data (Zotterman, 1971). The methods tend to interfere with responses, and the results need complex statistical treatment in order to be interpreted (Beets, 1971).

# VII. SIGNIFICANT ASPECTS OF HUMAN OLFACTION AND TASTE IN THE MILITARY ENVIRONMENT

## A. OLFACTION

The need for acuity in perception of odors is evident from the emphasis that the military has placed on developing sensory devices and biosensors. Yet, the greater part of this need can probably be fulfilled more adequately, reliably, and economically by man. In a recent report to the U.S. Air Force, Gestetland (1972) stated:

> "No broad-spectrum detection and identification system for air born chemical substances exists which begins to approach the performance of the vertebrate nose with respect to discriminability, sensitivity, dynamic range and variability of substances detectable."

Instruments do give objective information. However, they require operators and servicing personnel. They have poor directional sensitivity and the ranges of their responses are different from those of man. In addition, there are relatively few substances for which instrumental response spectra have been determined.

Therefore, while instruments and biosensors, such as dogs, will continue to have specialized roles in detection of explosives, illicit drugs, and intruders, and while instruments should become useful in standardization of personnel tests and testing situations, these roles do not substitute for human olfactory performance.

Olfactory acuity seems potentially important in soldiers selected for certain duties, e.g., point duty on patrol. This criterion for selection is seldom used and it seems to be generally unrecognized. Olfactory training is known to enhance aspects of performance and survival of military personnel (Woods, 1968). Enemy weapons, personnel, bivouacs, propellants, arson and chemical agents are examples of odor sources that personnel can be trained to recognize. This training could provide a "vocabulary" of odors and a repertoire of behavioral responses.

It should be noted that man has the ability to mask out many unacceptable odors, particularly those unfamiliar to him and encountered for the first time in foreign lands. Unfortunately, these are the very odors that he should be trained to recognize as they are often peculiar to the enemy. Failure of timely recognition may result in needless casualties.

Though machine-operation odors may mask odors from other sources, machine dysfunctions often give off other odors that can be recognized by the operator before breakdown occurs, e.g., an electrical short circuit or overheating of a power unit. Because sight, hearing, and touch often are fully employed in the operation of military machines, it has been suggested that specific olfactory cues (e.g., amyl acetate) might be built into some machines to give early warning of certain critical dysfunctions.

Personnel who are untrained in the use of their olfactory capabilities will tend to give olfactory information to the enemy and to be unaware that the enemy is using it. Anecdotal information suggests that indigenous military forces may be able to map the deployment of troops by the odors of their personal deodorants and insect repellants. Odors emanating from a work area or a specific military operation may give critical intelligence to a trained enemy agent. The element of surprise in military field operations can be squandered through lack of utilization of the olfactory sense.

Tactical uses of odorants have not been explored to any great extent. Odors typical of certain military preparations might be used to deceive enemy intelligence. An enemy who is trained or experienced might do likewise, and untrained personnel would have little immediate defense.

An improved knowledge of the effects of odors on behavior could be useful to the military. For instance, appetitive or aversive effects of odors on the responses of soldiers to food in mess-halls should be recognized. Specific odor signals could be used judiciously to alert personnel to the presence of hazards, e.g., damage to packaged foods. Personnel with defective olfactory acuity could be allocated to jobs in unavoidably malodorous environments. If personnel were regularly screened for olfactory acuity, options such as these would be available.

Sudden or progressive alterations of olfactory acuity (gains, losses or distortions) can signify hidden trauma or early cases of certain diseases. Examples include the endocrine disorders, anterior fossa tumors, head trauma, the phantosmia of incipient schizophrenia, and exposure to high-intensity X-rays or ionizing radiation. Olfactory testing could result in early referral of such cases for specific medical diagnosis and thus permit early treatment. In contrast, the present practice is to refer a soldier complaining of olfactory or taste loss to either a neurologist or a psychiatrist without ever verifying the original complaint.

A permanent loss of olfaction is rated as a 10% disability for compensation purposes (Veterans Administration, 1964). Claims of this type are being prepared in increasing numbers, especially by personnel who may have had service-connected head trauma. Ideally, claims for service-related olfactory losses should be based on the degree of olfactory competence established at the time of induction.

## B. TASTE

Taste is an important guide to recognition of spoiled foods. The use of sauces is thought to have originated in order to mask food spoilage. Kermode (1972) has noted that numerous substances are added to foods to diminish the rate of spoilage and suggested that additives may also diminish the role of taste in recognizing food spoilage. Military personnel abroad have problems of discrimination with unfamiliar foods, in which spoilage may be present. Spoiled foods often result in gastrointestinal casualties which could be minimized by appropriate training in taste recognition and by screening to identify ageusic and hypogeusic individuals.

Taste enhancers, aversants, or distorters might be introduced into an enemy's food supply to induce, respectively, inordinate appetite, rejection, or suspicion. The purpose would be to disrupt the planned use of rations. However, the technical problems may be formidable. Because an enemy might adopt this approach, training of personnel in detection and recognition of tastes may be a desirable protective measure.

Service-related loss of taste is rated like loss of olfaction, as a 10% disability (Veterans Administration, 1964). Claims that such losses are service-related are being prepared in increasing numbers and will be difficult to evaluate in personnel who were not previously tested.

# VIII. TESTING AND TRAINING OF ARMY PERSONNEL IN THE CHEMICAL SENSES

The current techniques of sensory evaluation in olfaction and taste, other than for basic research on their mechanisms, were developed about 35 years ago, and there have been few recent advances (Hirsh, 1973). The methods of personnel training that have evolved from basic research are non-uniform. Industrial corporations with products identified by aroma and taste have of necessity developed techniques of testing and training. Because much of this information is empirical, it is not readily available in the published literature. However, this body of knowledge and experience is applicable to the Army's needs.

In a pilot program for personnel evaluation and training, the following appear to be the initial steps and priorities.

## A. OLFACTION

### 1. Requirements

A preliminary list of odors that ought to be recognized by trained personnel would be compiled, together with the estimated concentrations at which recognition should become easy. Critical concentrations above which increases should be recognized, will be important in some cases. Situations requiring normal olfactory acuity, e.g., tasks involving "fieldcraft" or machine operation, should be surveyed and subcategorized according to (a) whether or not parallel input would be available from other senses such as vision and audition, and (b) whether recognition of the odors would contribute to assessment of a situation or would demand a specific behavioral response, e.g., to take cover. At this stage feasibility in terms of human olfactory acuity would not be considered.

### 2. Standards and Criteria

The next step would be to estimate the extent to which these requirements could be met by both instruments and trained human subjects. These estimates would set provisional standards and

criteria for a pilot program to evaluate and train personnel in odor recognition and discrimination. At this stage the effects of different diets, stresses, and fatigue on odor recognition in the military environment could be assessed.

3. Evaluation of Personnel

Personnel would be screened according to these standards and methods, both before and after training. Primarily screening data would be used to assist in future selection of personnel for specific military duties. In addition these data would be useful: (a) as part of the recruiting process, to assist in detecting disease conditions that might disqualify a recruit, (b) as a component of a soldier's continuing medical record, and (c) for reference in case of a future claim for service-related loss of olfaction. During the pilot program, screening data could be used to evaluate the feasibility of the provisional standards and criteria, and to evaluate the design and the execution of the training program.

## B. TASTE

Initially personnel could be screened for acuity in the four accepted primary tastes and for discrimination between compound tastes. As with suggested olfactory studies, screening and training methodologies developed for industrial use might be adopted or adapted to meet Army needs. Screening would be carried out before and after training, and the uses of the screening data would be analogous.

# IX. SUGGESTIONS FOR FUTURE EMPHASIS

The following suggestions for future emphasis have been developed during the course of this study. The order of presentation does not imply an order of priority.

1. The olfactory and taste acuities of Army personnel should be assessed and aspects of the military environment in which these acuities are significant should be catalogued. Although techniques for these purposes have been inadequate in the past, this no longer seems to be the case (See Section VI & VII).

2. The usefulness of the chemical senses can be developed in regular personnel training programs. The olfactory component of this training could include recognition of the presence of personnel, recognition by operations and maintenance personnel of various aspects of machine operation and malfunction, and training of combat personnel in detection and security related to field missions (See Section VIII).

3. Self-protection of personnel against environmental hazards from volatile substances can be improved by training in olfactory detection and discrimination. Similarly, hazards from eating unfamiliar or spoiled foods can be minimized by training in taste detection and discrimination. In addition, specific odor producing substances might be used in packaging or in foods to provide olfactory or taste clues to food spoilage (See Section VI & VII).

4. The standards and criteria used for evaluating human sensory performance in civilian environments may not always be appropriate to military situations. Thus the Army will need to determine its own general and special requirements in terms of odors and tastes, their critical

concentrations, and significant thresholds for recognition by personnel. These determinations and the preparation of the standards and criteria could be conducted by the Army utilizing existing research personnel and facilities. (See Section VI).

5. A number of resources are available for the determination of standards and criteria that the Army will need. These include Army laboratories and other government agencies in which instruments or biosensors are being developed to detect military explosives or personnel. For example, the National Bureau of Standards is studying plasma chromatography instrumentation for the chemical analysis of air. Plasma chromatography avoids the need for sample preparation that is inherent in other analyses and has potential for multiple sampling that should be useful for simultaneous monitoring of background and stimulus during Army personnel testing programs (See Section VI, A).

6. In developing techniques for training and evaluation of personnel in the use of their chemical senses, the potential contributions of industrial laboratories should be explored. Industries whose products are identified by aroma and taste have, of necessity, developed evaluation techniques that appear to be directly relevant to many of the Army's needs. Relatively little of this information has been reported compared to the quantity of literature published on basic research (See Section VI, B).

7. There is a need to explore the effects of odors on behavior and the modification of human behavior in the military environment. The Army has an opportunity to investigate and utilize the behavioral consequences of individual variability in detection, discrimination, and responses to odorants and tastants (See Section IV & V).

8. There is a need to explore alterations of sensory performance in personnel exposed to X-rays or

ionizing radiation. Alterations of olfactory and taste sensations may be useful early signs of exposure or of inadequate radiation protection. Human patients undergoing radiation therapy or X-ray diagnosis would be suitable subjects for such observations (See Section IV & VII).

9. There is a need to study the effects of drugs of abuse and therapeutic drugs on the chemical senses. This may be of particular concern to the Army if drug use compromises a man's olfactory or taste acuity and subsequently his performance.

10. The relationships of hyposmias and hypogeusias to nutritional deficiencies of zinc and other trace elements are controversial. Because zinc deficiency might be found in nutritionally deficient inductees and may follow regular heavy consumption of alcohol, these uncertainties should be resolved (See Section V).

11. Because service-related losses of the senses of olfaction and taste are rated as disabilities, testing procedures are required to check the validity of complaints. Evaluation of olfactory and taste acuities at induction would assist in documentation of subsequent claims for service-related losses (See Section VII).

12. While instruments and biosensors have specialized roles in odor detection, these do not substitute for human olfactory capabilities. At present, detection devices and biosensor programs are being developed without reference to the performance potentialities of the man. Increased emphasis on the use of the human olfactory system is required (See Section VII & VIII).

13. When the soldier knows that his full performance capabilities are recognized and used, morale tends to be high. On the other hand, when a soldier's personal capabilities are not fully used, he has less pride in himself. Substitution of detection devices and

biosensors for the chemical senses of man underestimates his capabilities. A program to develop the full capabilities of soldiers to exercise their senses of olfaction and taste in the military environment would tend to enhance their morale and potentially, their performance.

14. This study suggests that there is a need to designate some office or laboratory within the Army research and development community as a center for collation of projects involving the chemical senses and odor-sensing devices.

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# XI. GLOSSARY

| | |
|---|---|
| Ageusia . . . . . | Lack of a sense of taste, for all tastants ("general") or for particular tastants ("specific"). |
| Anosmia . . . . . | Lack of a sense of smell, for all odorants ("general") or for particular odorants ("specific"). |
| Cilia . . . . . | Hair-like projections of some cells. |
| Biosensor. . . . . | An animal trained to signal its responses to sensory stimuli, e.g., a dog trained to communicate to man the recognition of certain odors. |
| Dysgeusia. . . . . | Unpleasantly distorted taste sense; some workers prefer the term "cacogeusia." |
| Dysosmia . . . . . | Unpleasantly distorted sense of smell; some workers prefer the term "cacosmia." |
| Glomerulus . . . . . | A cluster of small structures, tubules or, in the present context, the nerve fibers in the olfactory glomerulus. |
| Hypergeusia. . . . . | Overly sensitive taste sense; can be general or specific. |
| Hyperosmia. . . . . | Overly sensitive sense of smell; can be general or specific. |
| Hypogeusia . . . . . | Abnormally diminished sense of taste, general or specific. |
| Hyposmia . . . . . | Abnormally diminished sense of smell, general or specific. |

| | |
|---|---|
| Odorant | Substance perceived by olfaction. |
| Olfaction | The sense of smell. |
| Olfactory bulb | A nerve center intermediate between the receptors for olfaction in the nose, and the brain itself. |
| Parosmia | Altered sense of smell, without implying pleasant or unpleasant. See dysosmia. |
| Parageusia | Altered sense of taste, without implying pleasant or unpleasant. See dysgeusia. |
| Phantosmia | Hallucination that an odor is present when in fact it is not. Phantogeusia means the same for taste. |
| Tastant | Substance perceived by taste. |

# XII. SCIENTIFIC CONSULTANTS

ON

# A STUDY OF THE POTENTIAL APPLICATIONS OF OLFACTORY RESEARCH IN MAN

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13. ABSTRACT
This study provides a comprehensive review of the feasibility of developing and utilizing the chemical senses of the soldier. While biosensors and odor-sensing devices can supplement, they cannot replace the potential performance of the normal human olfactory system. Experience in other countries suggests that a soldier trained to recognize olfactory cues is more effective in the field, more responsive to hazards, and less likely to give olfactory information to the enemy than one whose chemical senses are underutilized. Practical testing and training methods suited to that Army's needs have been lacking in the past, and basic knowledge of the mechanisms of olfaction and taste is incomplete. However, elements of a suitable technology have been identified. This technology appears suitable for further development within the Army research and development community. This report concludes that it is feasible for the Army to use these methods in determining classifications of personnel that require normal acuity of the chemical senses and to institute training programs. Regular testing for normal acuity of the chemical senses may uncover cases of some illnesses, indicate extensive exposures to ionizing radiation, and categorize personnel as either suitable or unfit for certain Army duties. If administered at recruitment, these tests can be used later to document disability claims for service-related losses of olfaction and taste.

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| 14 KEY WORDS | LINK A ROLE | LINK A WT | LINK B ROLE | LINK B WT | LINK C ROLE | LINK C WT |
|---|---|---|---|---|---|---|
| Chemical Senses | | | | | | |
| Acuity | | | | | | |
| Evaluation | | | | | | |
| Testing | | | | | | |
| Training | | | | | | |
| Odorants | | | | | | |
| Odor | | | | | | |
| Detection | | | | | | |
| Recognition | | | | | | |
| Standards and Criteria | | | | | | |
| Olfaction | | | | | | |
| Taste | | | | | | |
| Detection | | | | | | |
| Recognition | | | | | | |
| Standards and Criteria | | | | | | |

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Joint FAO/WHO Food Standards Programme
CODEX ALIMENTARIUS COMMISSION

CAC/RS 19-1969

# RECOMMENDED INTERNATIONAL GENERAL STANDARD FOR EDIBLE FATS AND OILS

## NOT COVERED BY INDIVIDUAL CODEX STANDARDS



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## INTRODUCTION

The FAO/WHO Codex Alimentarius Commission was established to implement the Joint FAO/WHO Food Standards Programme. Membership of the Commission comprises those Member Nations and Associate Members of FAO and/or WHO which have notified the Organizations of their wish to be considered as Members. By 1 May 1970, 75 countries had become Members of the Commission. Other countries which participate in the work of the Commission or of its subsidiary bodies in an observer capacity are expected to become Members in the near future.

The purpose of the Joint FAO/WHO Food Standards Programme is to protect the health of consumers and to ensure fair practices in the food trade; to promote coordination of all food standards work undertaken by international governmental and non-governmental organizations; to determine priorities and initiate and guide the preparation of draft standards through and with the aid of appropriate organizations; to finalize standards and after acceptance by governments, publish them in a Codex Alimentarius either as regional or world-wide standards.

The Commission has held seven sessions, the most recent being in April 1970. At the sixth session, the Commission adopted as a recommended standard to be sent to all Member Nations and Associate Members of FAO and/or WHO a world-wide General Standard for Edible Fats and Oils not covered by International Codex Standards (1). Members of the Commission are requested to notify the Secretariat of the Codex Alimentarius Commission - Joint FAO/WHO Food Standards Programme, FAO, Rome - of their acceptance of the recommended standard in accordance with the acceptance procedure for Codex Commodity standards contained in paragraph 4 of the General Principles of the Codex Alimentarius. An extract from the General Principles containing this procedure is included in the Appendix to this publication. The full text of the General Principles of the Codex Alimentarius is contained in the Second Edition of the Commission's Procedural Manual (Ref. No. CX 8/7 - 1969). Member Nations and Associate Members of FAO and/or WHO which are not Members of the Commission are

(1) A complete list of standards for fats and oils adopted by the Codex Alimentarius Commission and sent to governments for acceptance is given on page 20.

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also invited to notify the Secretariat if they wish to accept the recommended standard. The recommended standard will be published in the Codex Alimentarius as a world-wide Codex standard when the Commission determines that it is appropriate to do so in the light of acceptances received.

The methods of analysis and sampling referred to in this standard are not necessarily intended to replace routine methods of analysis and sampling, but are for acceptance and use as international referee methods of analysis and sampling by governments for the settlement of international disputes.

Specifications of identity and purity have been drawn up for the food additives provided for in this standard by the Joint FAO/WHO Expert Committee on Food Additives. These are being further elaborated by the Codex Alimentarius Commission into internationally recognized specifications for food additives. The statement "Not limited" should be taken to mean that the amount of food additive should be used in accordance with good manufacturing practices.

# RECOMMENDED INTERNATIONAL GENERAL STANDARD FOR EDIBLE FATS AND OILS NOT COVERED BY INDIVIDUAL CODEX STANDARDS



CAC/RS 19-1969

## RECOMMENDED INTERNATIONAL GENERAL STANDARD FOR EDIBLE FATS AND OILS NOT COVERED BY INDIVIDUAL CODEX STANDARDS

### 1. SCOPE

This standard applies to edible oils, fats and mixtures thereof, including those that have been subjected to processes of modification but not including oils and fats which must be subjected to such processes in order to render them suitable for human consumption. This standard does not apply to any oil or fat which is the subject of a specific Codex commodity standard and is designated by a specific name laid down in such standards.

### 2. DESCRIPTIONS

2.1 Edible Fats and Oils means foodstuffs composed of glycerides of fatty acids of vegetable, animal or marine origin. Fats of animal origin must be produced from animals in good health at the time of slaughter and be fit for human consumption as determined by a competent authority recognized in national legislation. They may contain small amounts of other lipids such as phosphatides, of unsaponifiable constituents and of free fatty acids naturally present in the fat or oil.

2.2 Virgin Fats and Oils means edible fats and oils obtained by mechanical procedures and the application of heat only. They may have been purified by washing, settling, filtering and centrifuging only.

### 3. ESSENTIAL COMPOSITION AND QUALITY FACTORS

3.1 Raw Materials

Edible fats and/or oils or mixtures thereof.

3.2 Colour

Characteristic of the designated product.

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3.3 Odour and Taste

Characteristic of the designated product and free from foreign and rancid odour and taste.

3.4 Acid Value

| | |
|---|---|
| Virgin fats and oils | not more than 4 mg KOH/g fat or oil |
| Non-virgin fats and oils | not more than 0.6 mg KOH/g fat or oil |

3.5 Peroxide Value — not more than 10 milliequivalents of peroxide oxygen/kg fat or oil

4. FOOD ADDITIVES

4.1 Colours

The following colours are permitted for the purpose of restoring natural colour lost in processing or for the purpose of standardizing colour, as long as the added colour does not deceive or mislead the consumer by concealing damage or inferiority or by making the product appear to be of greater than actual value:

| | Maximum level of use |
|---|---|
| 4.1.1 Beta-carotene | Not limited |
| 4.1.2 Annatto (*) | Not limited |
| 4.1.3 Curcumin (*) | Not limited |
| 4.1.4 Canthaxanthine | Not limited |
| 4.1.5 Beta-apo-8'-carotenal | Not limited |
| 4.1.6 Methyl and ethyl esters of beta-apo-8'-carotenoic acid | Not limited |

(*) Temporarily endorsed.



4.2 Flavours

Natural flavours and their identical synthetic equivalents, except those which are known to represent a toxic hazard, and other synthetic flavours approved by the Codex Alimentarius Commission are permitted for the purpose of restoring natural flavour lost in processing or for the purpose of standardizing flavour, as long as the added flavour does not deceive or mislead the consumer by concealing damage or inferiority or by making the product appear to be of greater than actual value. (*)

4.3 Emulsifiers

The following are permitted but only in fats and oils not specifically designated with the name of the plant or animal from which they originate:

| | Maximum level of use |
|---|---|
| 4.3.1 Mono- and diglycerides of fatty acids | Not limited |
| 4.3.2 Mono- and diglycerides of fatty acids esterified with the following acids: acetic, acetyltartaric, citric, lactic, tartaric and their sodium and calcium salts | 20 g/kg of the emulsifiers specified under 4.3.2 to 4.3.11 inclusive, individually or in combination |
| 4.3.3 Lecithins and components of commercial lecithin | |
| 4.3.4 Polyglycerol esters of fatty acids | |
| 4.3.5 Esters of fatty acids with polyalcohols other than glycerol: Sorbitan monopalmitate, Sorbitan monostearate, Sorbitan tristearate | |

(*) Temporarily endorsed.

| | Maximum level of use |
|---|---|
| 4.3 Emulsifiers (Cont.) | |
| 4.3.6 1,2 propylene glycol esters of fatty acids | ) |
| 4.3.7 Sucrose esters of fatty acids (including sucroglycerides) (*) | ) |
| 4.3.8 Stearoyl lactylic acid and its calcium salt (*) | ) |
| 4.3.9 Polyglycerol esters of interesterified ricinoleic acid (*) | ) |
| 4.3.10 Polyoxyethylene (20) sorbitan monostearate | ) |
| 4.3.11 Polyoxyethylene (20) sorbitan monoöleate | ) |
| 4.4 Antioxidants | |
| 4.4.1 Propyl, octyl, and dodecyl gallates | 100 mg/kg individually or in combination |
| 4.4.2 Butylated hydroxytoluene (BHT) Butylated hydroxyanisole (BHA) | 200 mg/kg individually or in combination |
| 4.4.3 Any combination of gallates with BHA or BHT, or both | 200 mg/kg, but gallates not to exceed 100 mg/kg |
| 4.4.4 Natural and synthetic tocopherols | Not limited |
| 4.4.5 Ascorbyl palmitate ) | 200 mg/kg individually or in combination |
| 4.4.6 Ascorbyl stearate ) | |
| 4.4.7 Dilauryl thiodipropionate | 200 mg/kg |

(*) Temporarily endorsed.



| 4.5 Antioxidant Synergists | Maximum level of use |
|---|---|
| 4.5.1 Citric acid and its sodium salt | Not limited |
| 4.5.2 Isopropyl citrate mixture ) | 100 mg/kg individually or in combination |
| 4.5.3 Phosphoric acid (*) ) | |
| 4.6 Anti-foaming Agent | |
| Dimethyl polysiloxane (dimethyl silicone) singly or in combination with silicone dioxide (*) ) | 10 mg/kg |
| 4.7 Crystallization Inhibitor | |
| Oxystearin (*) | 1250 mg/kg |

| 5. CONTAMINANTS | | Maximum level |
|---|---|---|
| 5.1 Matter volatile at 105°C | | 0.2% m/m |
| 5.2 Insoluble impurities | | 0.05% m/m |
| 5.3 Soap content | | 0.005% m/m |
| 5.4 Iron (Fe) | (Virgin oil | 5 mg/kg |
| | (Non-virgin oil | 1.5 mg/kg |
| 5.5 Copper (Cu) | (Virgin oil | 0.4 mg/kg |
| | (Non-virgin oil | 0.1 mg/kg |
| 5.6 Lead (Pb) | | 0.1 mg/kg |
| 5.7 Arsenic (As) | | 0.1 mg/kg |

6. HYGIENE

It is recommended that the product covered by the provisions of this standard be prepared in accordance with the appropriate Sections of the General Principles of Food Hygiene recommended by the Codex Alimentarius Commission (Ref. No. CAC/RCP 1-1969).

(*) Temporarily endorsed.

## 7. LABELLING

In addition to Sections 1, 2, 4 and 6 of the General Standard for the Labelling of Pre-packaged Foods (Ref. CAC/RS 1-1969) the following specific provisions apply:

### 7.1 The Name of the Food

7.1.1 The name designated for the product conforming to the definition at 2.1 of the standard shall be such as to give a true indication of the nature of the fat or oil, and not to mislead the consumer. Names such as edible oil and salad oil which do not indicate a plant or animal source may be used without further qualification.

7.1.2 Where an oil has been subject to any process of esterification or to processing which alters its fatty acid composition or its consistency the specific name of the oil shall not be used unless qualified to indicate the nature of the process.

7.1.3 The designation virgin fat or virgin oil may only be used for individual fats or oils conforming to the definition at 2.2 of this standard.

### 7.2 List of Ingredients

7.2.1 A complete list of ingredients shall be declared on the label in descending order of proportion.

7.2.2 A specific name shall be used for ingredients in the list of ingredients except that class titles may be used in accordance with sub-section 3.2 (c)(ii) of the General Standard for the Labelling of Pre-packaged Foods.

### 7.3 Net Contents

The net contents shall be declared in accordance with sub-section 3.3 (a) of the General Standard for the Labelling of Pre-packaged Foods.

### 7.4 Name and Address

The name and address of the manufacturer, packer, distributor, importer, exporter or vendor of the product shall be declared.



### 7.5 Country of Origin

7.5.1 The country of origin of the product shall be declared if its omission would mislead or deceive the consumer.

7.5.2 When the product undergoes processing in a second country which changes its nature, the country in which the processing is performed shall be considered to be the country of origin for the purposes of labelling.

## 8. METHODS OF ANALYSIS AND SAMPLING

The methods of analysis and sampling referred to hereunder are international reference methods.

### 8.1 Determination of Acid Value ($I_A$)

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.1.2. Acid Value ).

Results are expressed as the number of mg KOH required to neutralize 1 g oil or fat.

### 8.2 Determination of Peroxide Value ($I_P$)

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.13 Peroxide Value).

Results are expressed as milliequivalents active oxygen/kg fat or oil.

### 8.3 Determination of Matter Volatile at 105°C

According to the IUPAC (1964) method (IUPAC Standard Methods for the analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.C.1.1 Moisture and Volatile Matter).

Results are expressed as % m/m.

8.4 Determination of Insoluble Impurities

According to the IUPAC (1964) method (IUPAC Standard Methods for the Analysis of Oils, Fats and Soaps, 5th Edition, 1966, II. C. 2 Impurities).

Results are expressed as % m/m.

8.5 Determination of Soap Content

According to the FAO/WHO Codex Alimentarius Method (FAO/WHO Codex Alimentarius Methods of Analysis for Edible Fats and Oils, CAC/RM 13-1969, Determination of Soap Content).

Results are expressed as % m/m sodium oleate.

8.6 Determination of Iron (*)

According to the FAO/WHO Codex Alimentarius Method (FAO/WHO Codex Alimentarius Methods of Analysis for Edible Fats and Oils, CAC/RM 14-1969, Determination of Iron Content).

Results are expressed as mg iron/kg.

8.7 Determination of Copper (*)

According to the AOAC (1965) method (Official Methods of Analysis of the AOAC, International Union of Pure and Applied Chemistry Carbamate Method, 24.023 - 24.028).

Results are expressed as mg copper/kg.

8.8. Determination of Lead (*)

According to the AOAC (1965) method, after complete digestion, by the colorimetric dithizone determination procedure (Official Methods of Analysis of the AOAC, 1965, 24.053 (and 24.008, 24.009, 24.043 j, 24.046, 24.047 and 24.048) ).

Results are expressed as mg lead/kg.

8.9 Determination of Arsenic

According to the colorimetric silver diethyldithiocarbamate method of the AOAC (Official Methods of Analysis of the AOAC, 1965, 24.011 - 24.014, 24.016 - 24.017, 24.006 - 24.008).

Results are expressed as mg arsenic/kg.

(*) Might be replaced by Atomic Absorption Spectrophotometry in the future.



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- Supplement 1966 to the above


# ACCEPTANCE PROCEDURE FOR CODEX COMMODITY STANDARDS

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## EXTRACT OF THE GENERAL PRINCIPLES OF THE CODEX ALIMENTARIUS

## ACCEPTANCE OF CODEX COMMODITY STANDARDS

"4.A. A Codex standard may be accepted by a country in accordance with its established legal and administrative procedures in respect of distribution of the product concerned, whether imported or home-produced, within its territorial jurisdiction in the following ways:

i) Full acceptance

a) Full acceptance means that the country concerned will ensure that a product to which the standard applies will be permitted to be distributed freely, in accordance with (c) below, within its territorial jurisdiction under the name and description laid down in the standard, provided that it complies with all the relevant requirements of the standard.

b) The country will also ensure that products not complying with the standard will not be permitted to be distributed under the name and description laid down in the standard.

c) The distribution of any sound products conforming with the standard will not be hindered by any legal or administrative provisions in the country concerned relating to the health of the consumer or to other food standard matters except for considerations of human, plant or animal health which are not specifically dealt with in the standard.

ii) Target acceptance

Target acceptance means that the country concerned indicates its intention to accept the standard after a stated number of years and will meanwhile not hinder within its territorial jurisdiction the distribution of any sound products conforming with the standard by any legal or administrative provisions relating to the health of the consumer or to other food standard matters except for considerations of human, plant or animal health which are not specifically dealt with in the standard.



iii) Acceptance with minor deviations

Acceptance with minor deviations means that the country concerned gives full acceptance as defined in paragraph 4. A(i) to the standard with the exception of minor deviations which are recognized as such by the Codex Alimentarius Commission; it being understood that a product complying with the standard as qualified by such minor deviations will be permitted to be distributed freely within the territorial jurisdiction of the country concerned. The country concerned will further include in its declaration of acceptance a statement of such deviations, the reasons for them, and also indicate:

a) whether products fully conforming to the standard may be distributed freely within its territorial jurisdiction in accordance with paragraph 4. A(i);

b) whether it expects to be able to give full acceptance to the standard and, if so, when.

B. A country which considers that it cannot accept the standard in any of the ways mentioned above should indicate:

i) whether products conforming to the standard may be distributed freely within its territorial jurisdiction;

ii) in what ways its present or proposed requirements differ from the standard, and, if possible, the reasons for these differences.

C. i) A country which accepts a Codex standard according to one of the provisions of 4. A is responsible for the uniform and impartial application of the provisions of the standard as they apply to all home-produced and imported products distributed within its territorial jurisdiction. In addition, the country should be prepared to offer advice and guidance to exporters and processors of products for export to promote understanding of and compliance with the requirements of importing countries which have accepted a Codex standard according to one of the provisions of 4. A.

ii) Where, in an importing country, a product claimed to be in compliance with a Codex standard is found not to be in compliance with that standard, whether in respect of the label accompanying the product or otherwise, the importing country should inform the competent authorities in the exporting country of all the relevant facts and in particular the details of the origin of the product in question (name and address of the exporter), if it is thought that a person in the exporting country is responsible for such non-compliance."

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RECOMMENDED INTERNATIONAL STANDARDS FOR FATS AND OILS

CAC/RS 19-1969 Recommended International Standard for Edible Fats and Oils not covered by individual Codex Standards

CAC/RS 20-1969 Recommended International Standard for Edible Soya Bean Oil

CAC/RS 21-1969 Recommended International Standard for Edible Arachis Oil

CAC/RS 22-1969 Recommended International Standard for Edible Cottonseed Oil

CAC/RS 23-1969 Recommended International Standard for Edible Sunflowerseed Oil

CAC/RS 24-1969 Recommended International Standard for Edible Rapeseed Oil

CAC/RS 25-1969 Recommended International Standard for Edible Maize Oil

CAC/RS 26-1969 Recommended International Standard for Edible Sesameseed Oil

CAC/RS 27-1969 Recommended International Standard for Edible Safflowerseed Oil

CAC/RS 28-1969 Recommended International Standard for Lard

CAC/RS 29-1969 Recommended International Standard for Rendered Pork Fat

CAC/RS 30-1969 Recommended International Standard for Premier Jus

CAC/RS 31-1969 Recommended International Standard for Edible Tallow

CAC/RS 32-1969 Recommended International Standard for Margarine

CAC/RS 33-1970 Recommended International Standard for Olive Oil, Virgin and Refined, and for Refined Olive Residue Oils

CAC/RS 34-1970 Recommended International Standard for Edible Mustardseed Oil

Joint FAO/WHO Food Standards Programme
CODEX ALIMENTARIUS COMMISSION

CAC/RS 32-1969

# RECOMMENDED INTERNATIONAL STANDARD FOR MARGARINE

FOOD AND AGRICULTURE ORGANIZATION
OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

Issued by the Secretariat of the
Joint FAO/WHO Food Standards Programme, FAO, Rome

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## INTRODUCTION

The FAO/WHO Codex Alimentarius Commission was established to implement the Joint FAO/WHO Food Standards Programme. Membership of the Commission comprises those Member Nations and Associate Members of FAO and/or WHO which have notified the Organizations of their wish to be considered as Members. By 1 May 1970, 75 countries had become Members of the Commission. Other countries which participate in the work of the Commission or of its subsidiary bodies in an observer capacity are expected to become Members in the near future.

The purpose of the Joint FAO/WHO Food Standards Programme is to protect the health of consumers and to ensure fair practices in the food trade; to promote coordination of all food standards work undertaken by international governmental and non-governmental organizations; to determine priorities and initiate and guide the preparation of draft standards through and with the aid of appropriate organizations; to finalize standards and after acceptance by governments, publish them in a Codex Alimentarius either as regional or world-wide standards.

The Commission has held seven sessions, the most recent being in April 1970. At the sixth session, the Commission adopted as a recommended standard to be sent to all Member Nations and Associate Members of FAO and/or WHO a world-wide Standard for Margarine. Members of the Commission are requested to notify the Secretariat of the Codex Alimentarius Commission - Joint FAO/WHO Food Standards Programme, FAO, Rome - of their acceptance of the recommended standard in accordance with the General Principles of the Codex Alimentarius. An extract from the General Principles containing this procedure is included in the Appendix to this publication. The full text of the General Principles of the Codex Alimentarius is contained in the Second Edition of the Commission's Procedural Manual (Ref. No. CX 8/7 - 1969). Member Nations and Associate Members of FAO and/or WHO which are not Members of the Commission are also invited to notify the Secretariat if they wish to accept the recommended standard. The recommended standard will be published in the Codex Alimentarius as a world-wide Codex standard when the Commission determines that it is appropriate to do so in the light of acceptances received.

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The methods of analysis and sampling referred to in this standard are not necessarily intended to replace routine methods of analysis and sampling, but are for acceptance and use as international referee methods of analysis and sampling by governments for the settlement of international disputes.

Specifications of identity and purity have been drawn up for the food additives provided for in this standard by the Joint FAO/WHO Expert Committee on Food Additives. These are being further elaborated by the Codex Alimentarius Commission into internationally recognized specifications for food additives. The statement "not limited" should be taken to mean that the amount of food additive should be used in accordance with good manufacturing practice.

# RECOMMENDED INTERNATIONAL STANDARD FOR MARGARINE

# RECOMMENDED INTERNATIONAL STANDARD FOR MARGARINE

## 1. SCOPE

This standard will not apply to any product which contains less than 80% fat and is not labelled in any manner which implies, either directly or indirectly, that the product is margarine.

## 2. DESCRIPTION

### 2.1 Product Definition

Margarine is a food in the form of a plastic or fluid emulsion, which is mainly of the type water/oil, produced principally from edible fats and oils, which are not or are not mainly derived from milk.

### 2.2 Other Definitions

2.2.1 Edible fats and oils means foodstuffs composed of glycerides of fatty acids of vegetable, animal or marine origin. Fats of animal origin must be produced from animals in good health at the time of slaughter and be fit for human consumption as determined by a competent authority recognized in national legislation. They may contain small amounts of other lipids such as phosphatides, of unsaponifiable constituents and of free fatty acids naturally present in the fat or oil.

2.2.2 Pre-packed means packed or made up in advance, ready for retail sale in a container.

## 3. ESSENTIAL COMPOSITION AND QUALITY FACTORS

### 3.1 Raw Materials

3.1.1 Edible fats and/or oils, or mixtures of these, whether or not they have been subjected to a process of modification.

3.1.2 Water and/or milk and/or milk products.

3.2 Minimum Fat Content 80% m/m of the product

3.3 Maximum Water Content 16% m/m of the product

3.4 Additions

The following substances may be added to margarine :

3.4.1 Vitamins: Vitamin A and its esters
Vitamin D
Vitamin E and its esters
Other vitamins

Maximum and minimum levels for Vitamins A, D and E and other Vitamins should be laid down by national legislation in accordance with the needs of each individual country including, where appropriate, the prohibition of the use of particular Vitamins.

3.4.2 Sodium chloride

3.4.3 Sugars 1/

3.4.4 Suitable edible proteins

| 4. FOOD ADDITIVES | Maximum level of use |
|---|---|
| 4.1 Colours | |
| 4.1.1 Beta-carotene | Not limited |
| 4.1.2 Annatto (*) | Not limited |
| 4.1.3 Curcumin (*) | Not limited |

(*) Temporarily endorsed.

1/ 'Sugars' means any carbohydrate sweetening matter.



| | Maximum level of use |
|---|---|
| 4.1.4 Canthaxanthine | Not limited |
| 4.1.5 Beta-apo-8'-carotenal | Not limited |
| 4.1.6 Methyl and ethyl esters of Beta-apo-8'-carotenoic acid | Not limited |
| 4.2 Flavours | |
| 4.2.1 Natural flavours and their identical synthetic equivalents, except those which are known to represent a toxic hazard (*) | Not limited |
| 4.2.2 Other synthetic flavours approved by the Codex Alimentarius Commission (*) | Not limited |
| 4.3 Emulsifiers | |
| 4.3.1 Mono- and diglycerides of fatty acids | Not limited |
| 4.3.2 Mono- and diglycerides of fatty acids esterified with the following acids: acetic, acetyltartaric, citric, lactic, tartaric and their sodium and calcium salts | 10 g/kg |
| 4.3.3 Lecithins and components of commercial lecithin | Not limited |
| 4.3.4 Polyglycerol esters of fatty acids | 5 g/kg |
| 4.3.5 1,2-propylene glycol esters of fatty acids | 20 g/kg |

(*) Temporarily endorsed.

| | Maximum level of use |
|---|---|
| 4.3.6 Esters of fatty acids with polyalcohols other than glycerol: | 10 g/kg |
| Sorbitan monopalmitate<br>Sorbitan monostearate<br>Sorbitan tristearate | |
| 4.3.7 Sucrose esters of fatty acids (including sucroglycerides) (*) | 10 g/kg |
| 4.4 Preservatives | |
| 4.4.1 Sorbic acid and its sodium, potassium and calcium salts<br>4.4.2 Benzoic acid and its sodium and potassium salts | 1000 mg/kg individually or in combination, expressed as the acids |
| 4.5 Antioxidants | |
| 4.5.1 Propyl, octyl, and dodecyl gallates<br>4.5.2 Butylated hydroxytoluene (BHT)<br>Butylated hydroxyanisole (BHA) | 100 mg/kg individually or in combination |
| 4.5.3 Natural and synthetic tocopherols | Not limited |
| 4.5.4 Ascorbyl palmitate<br>4.5.5 Ascorbyl stearate | 200 mg/kg individually or in combination |
| 4.6 Antioxidant Synergists | |
| 4.6.1 Isopropyl citrate mixture | 100 mg/kg |
| 4.7 Other Additives | |
| 4.7.1 Citric and lactic acids and their potassium and sodium salts | Not limited |

(*) Temporarily endorsed.



| | Maximum level of use |
|---|---|
| 4.7.2 L-tartaric acid and its sodium and sodium-potassium salts | Not limited |
| 4.7.3 Sodium hydrogen carbonate, sodium carbonate, sodium hydroxide | Not limited |

| 5. CONTAMINANTS | Maximum level |
|---|---|
| 5.1 Iron (Fe) | 1.5 mg/kg |
| 5.2 Copper (Cu) | 0.1 mg/kg |
| 5.3 Lead (Pb) | 0.1 mg/kg |
| 5.4 Arsenic (As) | 0.1 mg/kg |

## 6. HYGIENE

It is recommended that the product covered by the provisions of this standard be prepared in accordance with the appropriate Sections of the General Principles of Food Hygiene recommended by the Codex Alimentarius Commission (Ref. No. CAC/RCP 1-1969).

## 7. PACKAGING

Margarine when sold by retail shall be pre-packed and may be sold in a pack of any shape.

## 8. LABELLING

In addition to Sections 1, 2, 4 and 6 of the General Standard for the Labelling of Pre-packaged Foods (Ref. CAC/RS 1-1969) the following specific provisions apply:

### 8.1 The Name of the Food

The product shall be designated margarine and all products designated as margarine shall conform to this standard.

8.2 List of Ingredients

A complete list of ingredients shall be declared on the label in descending order of proportion in accordance with sub-sections 3.2 (c) of the General Standard for the Labelling of Pre-packaged Foods.

8.3 Net Contents

The net contents shall be declared by weight in either the metric ("Système International" units) or avoirdupois or both systems as required by the country in which the product is sold.

8.4 Name and Address

The name and address of the manufacturer, packer, distributor, importer, exporter or vendor of the product shall be declared.

8.5 Country of Origin

8.5.1 The country of origin of the product shall be declared if its omission would mislead or deceive the consumer.

8.5.2 When the product undergoes processing in a second country which changes its nature, the country in which the processing is performed shall be considered to be the country of origin for the purposes of labelling.

8.6 Labelling Prohibitions

8.6.1 No reference shall be made to the presence of milk fat or butter in margarine except in a complete list of ingredients.

8.6.2 No reference shall be made, other than in a complete list of ingredients, to the presence of any vitamin in margarine unless the name and quantity of the vitamin is stated on the label.

9. METHODS OF ANALYSIS AND SAMPLING

The methods of analysis and sampling described hereunder are international referee methods.



9.1 Estimation of Milk Fat Content (*) CAC/RM 15-1969

Milk fat content in margarine is estimated by the determination of the following values which are obtained by specifically defined empirical methods:

Reichert-Meissl-Wollny value (R)
Polenske value (P)
Kirschner value (K)

9.1.1 Determination of (R) and (P) values

According to the IUPAC (1964) method (IUPAC Standards Methods for the Analysis of Oils, Fats and Soaps, 5th Edition, 1966, II.D.9, Determination of Soluble and Insoluble Volatile Acids) and the AOAC (1965) method (Official Methods of Analysis of the AOAC, 1965, 26.032 - 26.033).

Note I: Both the methods of IUPAC and AOAC are necessary because of the extremely empirical nature of the methods depending on regional deviations.

Note II: Determination of (R) and (P) values are not sufficient for the evaluation of milk fat content in margarine, which also involves the determination of (K) values as in 9.1.2.

9.1.2 Determination of (K) value (Based on British Standards Institution Method).

9.1.2.1 Principle of the method

The Reichert Value (R) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-soluble volatile fatty acids distilled from 5 g of the oil or fat under the precise conditions specified in the method.

The Polenske Value (P) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-insoluble volatile fatty acids distilled from 5 g of the oil or fat under the precise conditions specified in the method.

(*) It is intended to replace the methods described below by gas chromatographic methods in the near future.

The Kirschner Value (K) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-soluble volatile fatty acids which form water-soluble silver salts distilled from 5 g of the oil or fat under the precise conditions specified in the method.

Margarine fat is saponified. The fatty acids are then liberated from the soap and distilled in a standard apparatus under specified conditions. The water-insoluble fatty acids are filtered off, dissolved in ethanol and titrated. (P) is calculated from the amount of alkali required. The water-soluble fatty acids are then titrated with barium hydroxide solution, and (R) is calculated from the amount of the solution required in the titration. The neutralized solution is then treated with silver sulphate. After separation of the insoluble silver salts by filtration, the filtrate containing the water-soluble silver salts is acidified, distilled in the standard apparatus under the same specified conditions and titrated to determine (K). The percentage of milk fat in margarine fat is then calculated according to a formula based on (K) and (P). This process does not determine the total quantities of volatile fatty acids, soluble and insoluble in water, present in combination in the fat. The amounts of these acids actually determined by the process are dependent on strict adherence to the dimensions of the apparatus and the details of procedure.

9.1.2.2 Reagents

The reagents used shall be of recognized analytical reagent quality.

9.1.2.2.1 Glycerol

9.1.2.2.2 Sodium hydroxide, 50% m/m solution. Dissolve sodium hydroxide in an equal mass of water and store the solution in a bottle protected from carbon dioxide. Use the clear portion free from deposit.

9.1.2.2.3 Sulphuric acid, dilute. Dilute approximately 25 ml of concentrated sulphuric acid to 1 litre and adjust until 40 ml neutralize 2 ml of the 50% sodium hydroxide solution.,



9.1.2.4 Pumice powder, ground, passing through a 60 mesh British Standard (B.S.) test sieve and remaining on a 120 mesh B.S. test sieve (*) or equivalent (**).

9.1.2.2.5 Phenolphthalein indicator, 0.5% solution in 95% (v/v) ethanol.

9.1.2.2.6 Ethanol, 95% (v/v), neutralized to phenolphthalein immediately before use.

9.1.2.2.7 Sodium hydroxide, approximately 0.1 N solution, accurately standardized.

9.1.2.2.8 Barium hydroxide, approximately 0.1 N solution, accurately standardized.

9.1.2.2.9 Silver sulphate, powdered.

9.1.2.3 Apparatus

9.1.2.3.1 Graduated cylinder of 100 ml capacity, complying with B.S. 604, or equivalent.

9.1.2.3.2 Pipette of 50 ml capacity, complying with B.S. 1583 ('one-mark bulb pipettes') Class B, or equivalent.

9.1.2.3.3 Distillation apparatus, as shown in Fig. 1. The constituent parts are as follows:

- Flat-bottomed boiling flask (Polenske) of borosilicate glass. The volume contained to the bottom of the neck is 310 ± 10 ml.

(*) British Standard 410, 'Test sieves'.
(**) These sieves correspond respectively to ISO sieves, the meshes of which are made by so weaving wire as to form square openings of 250 μm x 250 μm and of 125 μm x 125 μm (ISO Recommendation R 565).

- Still-head, of glass tubing of wall thickness 1.25 ± 0.25 mm and conforming to the shape and dimensions shown in Fig. 2. A rubber stopper, fitted below the bulb of the longer arm of the still-head, and used for connecting it to the flask, shall have its lower surface 10 mm above the centre of the side hole of the still-head

- Condenser of glass tubing. The wall thickness of the inner tube shall be 1.0 ± 0.2 mm and that of the outer jacket and of the wider part at the top 1.25 ± 0.25 mm.

- Receiver, consisting of a flask complying with B.S. 675 ("Sugar flasks") or equivalent carrying two graduation marks on the neck, one at 100 ml and the other at 110 ml.

- Asbestos board. An asbestos board, 120 mm in diameter and 6 mm in thickness, with a circular hole about 65 mm in diameter, used to support the flask over the burner. During distillation the Polenske flask shall fit snugly into the hole in the board so as to prevent the flame from impinging on the surface of the flask above the hole. A new asbestos board may be conveniently prepared by levelling the edge of the hole, soaking in water, moulding the edge with a flask, and drying.

- Bunsen burner. The burner should be sufficiently large to allow the distillation to be completed in the time specified. The apparatus is supported on a retort stand, not shown in the diagramme.

9.1.2.4 Procedure

9.1.2.4.1 Hydrolysis and distillation

Weigh 5 ± 0.01 g of the fat into a Polenske flask. Add 20 g of glycerol and 2 ml of the 50% sodium hydroxide solution. Protect the burette containing the latter from carbon dioxide, and wipe its nozzle clean from carbonate deposit before withdrawing solution for the tests; reject the first few drops withdrawn from the burette. Heat the flask over a naked flame, with continuous mixing, until the fat, including any drops adhering to the upper parts of the flask, is saponified, and the liquid becomes perfectly clear; avoid overheating during this saponification. Cover the flask with a watch glass.



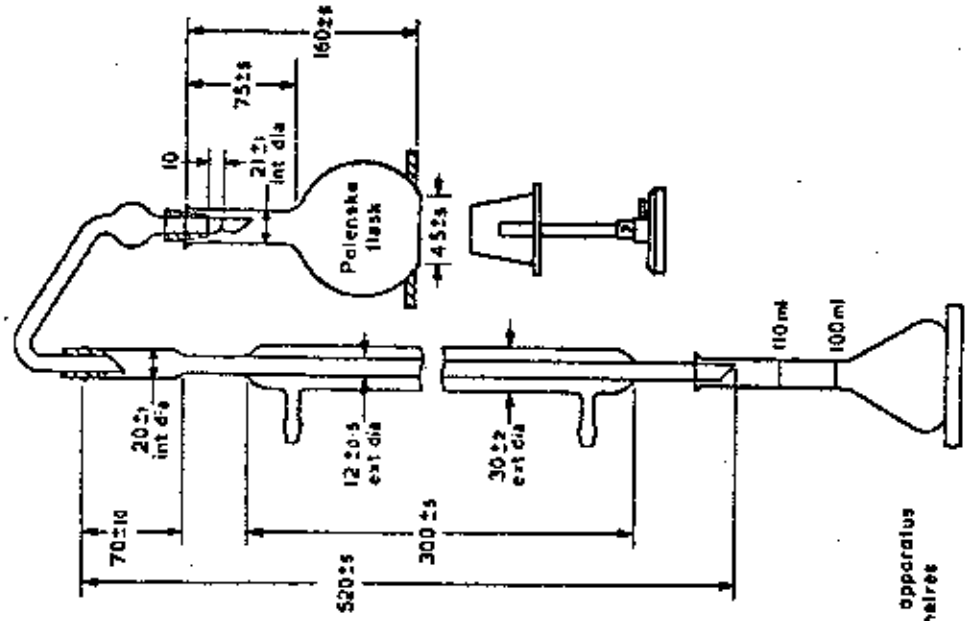


Fig. 1 - Polenske distillation apparatus
Dimensions in millimetres

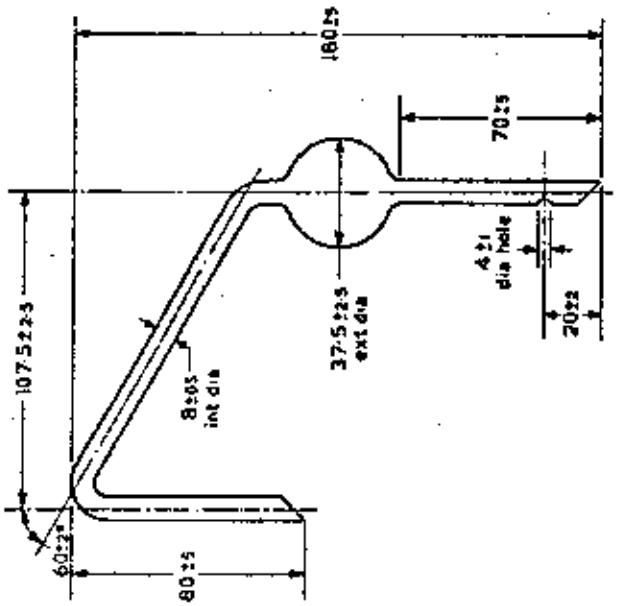


Fig. 2 - Still-head for Polenske distillation apparatus
Dimensions in millimetres

Make a blank test without fat, but using the same quantities of reagents and following the same procedure, again avoiding over-heating during the heating with sodium hydroxide; such over-heating would be indicated by darkening of the solution.

Measure 93 ml of boiling distilled water, which has been vigorously boiled for 15 minutes, into a 100 ml graduated cylinder. When the soap is sufficiently cool to permit addition of the water without loss, but before the soap has solidified, add the water, draining the cylinder for 5 seconds, and dissolve the soap. If the solution is not clear (indicating incomplete saponification) or is darker than light yellow (indicating over-heating) repeat the saponification with a fresh sample of the fat.

Add 0.1 g of powdered pumice, followed by 50 ml of the dilute sulphuric acid, and connect the flask at once with the distillation apparatus. Heat the flask without boiling its contents, until the insoluble acids are completely melted, then increase the flame and distil 110 ml in between 19 and 21 minutes. Keep the water flowing in the condenser at a sufficient speed to maintain the temperature of the issuing distillate between 18°C and 21°C.

When the distillate reaches the 110 ml mark, remove the flame and replace the 110 ml flask by a cylinder of about 25 ml capacity, to catch drainings. Close the 110 ml flask with its stopper, and without mixing the contents place it in water at 15°C for 10 minutes so as to immerse the 110 ml mark. Remove the flask from the water, dry the outside and invert the flask carefully, avoiding wetting the stopper with the insoluble acids. Mix the distillate by four or five double inversions, without violent shaking. Filter through a dry 9 cm open-texture filter paper (a Whatman No. 4 or 41 or a Barcham Green No. 801 paper or equivalent is suitable) which fits snugly into a funnel. Reject the first runnings and collect 100 ml in a dry flask, cork the flask and retain the filtrate for titration as under 9.1.2.4.2 (a). The filtrate should be free from insoluble fatty acids.

Where liquid, insoluble fatty acids pass through the filter, receive the filtrate in a separating funnel, and after separation draw off the lower (aqueous) layer, leaving behind insoluble acids which have risen to the surface. Add these to the main bulk of the insoluble acids.



Detach the still-head and wash the condenser with three successive 15 ml portions of cold distilled water, passing each washing separately through the cylinder, the 110 ml flask, the filter and the separating funnel, nearly filling the paper each time and draining each washing before filtering the next. Discard the washings.

Dissolve the insoluble acids by three similar washings of the condenser, the cylinder, and the filter with 15 ml of neutralized ethanol, collecting the solution in the 110 ml flask and draining the ethanol after each washing. Cork the flask, and retain the solution for titration, as at 9.1.2.4.2 (b).

9.1.2.4.2 Determinations (*)

(a) Reichert, or soluble volatile acid, value (R)

Pour 100 ml of the filtrate containing the soluble volatile acids into a previously dried titration flask, add 0.1 ml of phenolphthalein indicator and titrate with the barium hydroxide solution until the liquid becomes pink, rinsing the 100 ml flask with the nearly neutralized liquid towards the end of the titration. Note the actual volume of barium hydroxide solution used; drain the 100 ml flask into the titration flask, close with a cork and continue as at 9.1.2.4.2 (c).

(b) Polenske, or insoluble volatile acid, value (P)

Titrate the alcoholic solution of the insoluble volatile acids after addition of 0.25 ml of phenolphthalein indicator with the 0.1 N barium hydroxide solution until the solution becomes pink.

(c) Kirschner value (K)

Add 0.5 g of finely powdered silver sulphate to the neutralized solution from (a) above. Allow the flask to stand in the dark for one hour with occasional shaking and filter the contents in the dark through a dry filter. Transfer 100 ml of the filtrate to a dry Polenske flask, add 35 ml of cold distilled water, recently boiled for 15 minutes, 10 ml of the dilute sulphuric acid and a loosely-wound 5 mm coil of 30 cm of aluminium wire (about 1 mm thick or 18 to 20 Standard Wire Gauge) or 0.1 g of pumice powder.

(*) See Note under 9.1.1.

Connect the flask with the standard apparatus and repeat the process as described above, i.e. the distillation of 110 ml in from 19 to 21 minutes, the mixing (but without the cooling for 10 minutes), and the filtration and the titration of 100 ml of the filtrate with the barium hydroxide solution.

9.1.2.5 Calculation and expression of results

Reichert value (R) = $1.10\,(T_1 - T_2)$

Polenske value (P) = $T_3 - T_4$

$$\text{Kirschner value (K)} = \frac{121\,(100 + T_1)\,(T_5 - T_6)}{10\,000}$$

where

$T_1$ = ml of 0.1 N barium hydroxide solution used for sample under 9.1.2.4.2 (a).

$T_2$ = ml of 0.1 N barium hydroxide solution used for blank under 9.1.2.4.2 (a).

$T_3$ = ml of 0.1 N barium hydroxide solution used for sample under 9.1.2.4.2 (b).

$T_4$ = ml of 0.1 N barium hydroxide solution used for blank under 9.1.2.4.2 (b).

$T_5$ = ml of 0.1 N barium hydroxide solution used for sample under 9.1.2.4.2 (c).

$T_6$ = ml of 0.1 N barium hydroxide solution used for blank under 9.1.2.4.2 (c).

Milk fat content in margarine is estimated according to the following formula:

$$\text{Percentage m/m milk fat in margarine fat} = \frac{K - 0.1P - 0.24}{0.244}$$

where K = Kirschner value
P = Polenske value



Note I: Correction factors are to be used, if necessary, for the Reichert and Polenske values due to the fact that both values have been found to be low when determined at low barometric pressures of $\rho$ mm Hg, such as may occur at high altitudes. The following factors may be applied to values determined at a barometric pressure of $\rho$ mm Hg, to convert them to the values determined at normal pressure (2).

$$\text{Corrected Reichert value} = \frac{(\text{observed value} - 10)\log 760}{\log \rho} + 10$$

$$\text{Corrected Polenske value} = \text{observed value} \times \frac{760 - 45}{\rho - 45}$$

Note II: The referee methods of determining (P) values are those indicated under 9.1.1 In the case of discrepancies between (P) values determined according to 9.1.2.5 and those determined according to 9.1.1, these latter values are to be used in the calculation of butter fat content.

Note III: Tables and graphs relating to Polenske values with palm kernel oil and coconut oil contents and Kirschner values with butter fat contents were reported by K.A. Williams (2).

9.1.2.7 Literature references

(1) British Standard 684: 1958, Methods of Analysis of Oils and Fats, pages 65-71.

(2) Kirkham (1920) Analyst, 44, 293

(3) Williams, K.A. (1949) Analyst, 74, 508

9.2 Determination of Fat Content CAC/RM 16-1969

(Direct method of determination of total fat content in margarine. Based on the Official Netherlands Food Analysis Method).

9.2.1 Principle of Method

A preliminary treatment will dilute hydrochloric acid is followed by extraction with light petroleum.

9.2.2 Reagents and Materials

9.2.2.1 Hydrochloric acid, 25% d = 1.126

9.2.2.2 Light petroleum, freshly distilled, boiling range 40° to 60°

9.2.2.3 Pumice, pieces of, diameter 0.9 to 1.0 mm

9.2.3 Apparatus

9.2.3.1 Liquid/liquid continuous 'ether' extractor as illustrated in Fig. 1 or equivalent, with fat determination flask.

9.2.3.2 Electrically heated drying oven adjusted to operate at 102° ± 1°C.

9.2.3.3 Conical flask (type Philipps beaker or equivalent) of 100 ml, with spout and perfect rim.

9.2.4 Procedure

Melt about 50 g of the product in a wide-necked bottle of suitable size with a close-fitting stopper at about 40°C and cool whilst shaking vigorously until the mass is homogeneous and viscous. Introduce about 10 g accurately weighed, into the pre-weighed conical flask. Add 20 ml of 25% hydrochloric acid and five pieces of pumice and boil very gently while swirling continuously until all non-fatty substances are dissolved. Allow to cool to about 30°C.

Introduce five pieces of pumice into the fat-determination flask and dry the flask for 1 hour in the drying oven at 102° ± 1°C. Allow the flask to cool to room temperature in a dessicator, weigh the flask to the nearest 0.1 mg and fit it to the extraction apparatus (9.2.3.1).

Transfer the contents of the conical flask into the extractor's bulb and rinse the flask alternatively with tepid water and light petroleum, transferring each time the liquid into the extractor's bulb until the bulb is filled with the aqueous liquid to half level. Rinse again the conical flask with light petroleum until enough light petroleum running through the bulb of the extraction apparatus makes the fat-determination flask filled to about 2/5, and heat the flask for 4 hours.

Check the completion of the extraction using a second fat-determination flask and extracting during a further period of 1 hour with a fresh portion of solvent. The increase in mass should not exceed 0.01% of the sample mass.



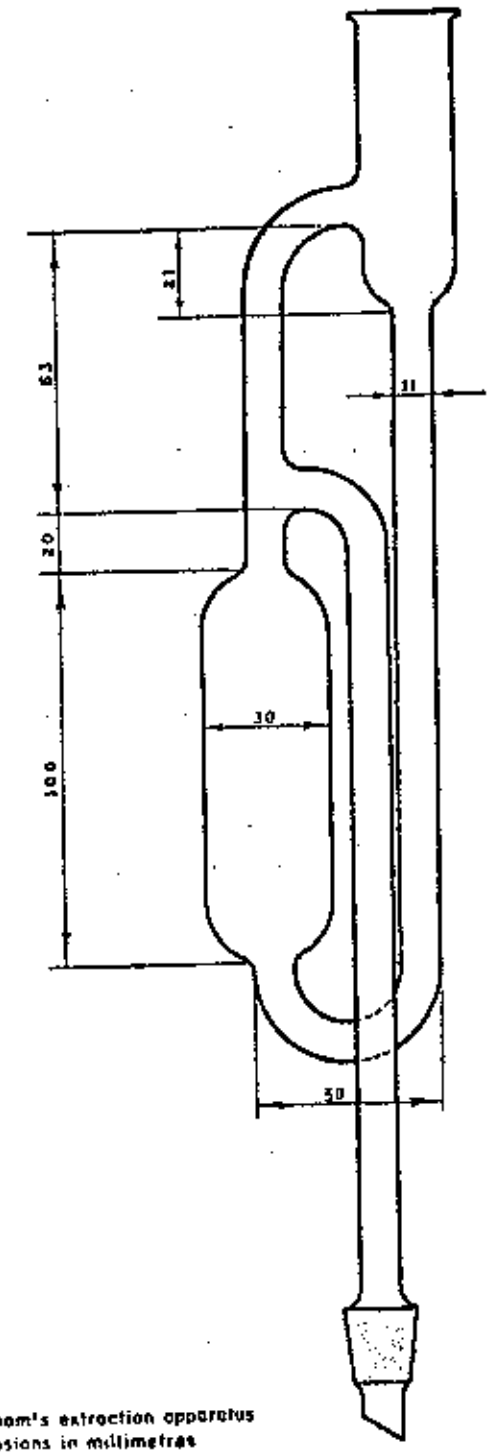


Fig. 1 - Smetham's extraction apparatus
Dimensions in millimetres

After extraction, remove the fat-determination flask from the extraction apparatus and distill off the solvent on a water-bath. Evaporate the last traces of the solvent (when desired) on the water-bath, using a current of nitrogen. Dry the flask for 1 hour in drying oven at 102° ± 1°C, allow to cool to room temperature in a dessicator and weigh to the nearest 0.1 mg.

Repeat drying and weighing every half hour until two successive weighings do not differ by more than 1 mg.

9.2.5 Calculation and Expression of Results

Results are expressed as % m/m fat content, calculated as follows:

$$\% \text{ m/m fat content} = \frac{\text{mass of light petroleum extract (g)}}{\text{original sample mass (g)}} \times 100$$

9.2.5.1 Repeatability

The difference between duplicate determinations (obtained simultaneously or in rapid succession by the same analyst) does not exceed 0.2% of the fat content.

9.2.6 Literature References

Smetham, A; Analyst, 17 (1892) 44-45
Van Ginkel, J.G. and Roos, J.B.; the Analysis of Butter and Cheese in H.A. Bockenogen "The Analysis and characterization of Oils, Fat and Fat products", Vol. 1, pp. 191-193, Interscience Publishers.

9.3 Determination of Water Content CAC/RM 17-1969

(To be developed)

9.4 Determination of Vitamin A Content

According to the AOAC (1965) method (Official Methods of Analysis of the AOAC, 1965, 39.001 - 39.007, Chemical Methods, Vitamin A in Margarine).

Results are expressed as microgrammes retinol (Vitamin A - alcohol) per kg.

9.5 Determination of Vitamin D Content

According to the AOAC (1965) method (Official Methods of Analysis of the AOAC, 1965, 39.116 - 39.129 Vitamin D).

Results are expressed as IU Vitamin D per kg.



9.6 Determination of Vitamin E (Tocopherols) Content CAC/RM 18-1969

9.6.1 Scope: Tocopherols which are present in foods are known for their two main properties:

(a) in nutrition (Vitamin E activity)

(b) in preventing oxidization of foods (antioxidant activity)

The determination of Vitamin E, by chemical method, involves the determination of tocopherols, either natural or synthetic. Results are expressed as the quantity of each ( $\alpha$ , $\beta$ , $\gamma$ , etc.) tocopherol and may also be expressed as the quantity of total tocopherols. The method is applicable to oils and fats and is suitable for determining tocopherols in micro-amounts. It is also applicable to fat-soluble extracts of other biological materials. The tocopherols are sufficiently stable to involve no special hazards or difficulties when one of the several normal methods of fat extraction is used. Several such methods, applied particularly to tocopherol determination have been described (2) (4) (8).

9.6.2 Principle of Method: The method is based on the Emmerie-Engel method (5). It involves the determination of all seven tocopherols present in the material. After removal of interfering substances, the tocopherols are treated with a mixture of ferric chloride and 2:2'-dipyridyl in ethanol solution. Tocopherols reduce the iron to the ferrous state; it then combines with the dipyridyl to form a red colour, which is measured in a spectrophotometer or electric colorimeter. The method may involve all, or most of, the seven stages enumerated below with their purposes:

(a) Solvent extraction of a representative sample with a lipid solvent for quantitative seperation of all tocopherols present, along with other fatty material

(b) Saponification of the lipoid extract and seperation from it of the unsaponifiable matter. The method is based on that of Tosic and Moore (9)

(c) Seperation of most of the steroid material by freezing from solution in methanol

(d) Column chromatography on floridin earth to remove carotenoids and residual steroids (6)

(e) Two dimensional paper chromatography (7) to separate the tocopherols into five zones, and individual elution

(f) Treatment of the eluates with the Emmerie-Engel reagent, the reading of the extinctions at 520 nm in a suitable instrument (4). The concentration of reagents is such that all the tocopherols give maximum or almost maximum colour in the short time (2 minutes) before measurement is made

(g) Calculation of tocopherol equivalents from extinctions by using the established factor for each tocopherol.

The seven stages enumerated above may not all be necessary. The most obvious omission is of stage (a) when the sample to be examined is a clean oil or fat. Stage (c) is only necessary for materials of low potency. Stage (d) can be omitted during the analysis of samples containing more than 5 to 10 mg of tocopherols per g of oil.

9.6.3 Reagents

The reagents used shall be of recognized analytical reagent quality.

9.6.3.1 Pyrogallol solution, 5% m/v in ethanol - Prepare this solution daily for each assay.

9.6.3.2 Potassium hydroxide solution - Dissolve 160 g of potassium hydroxide in 100 ml of distilled water.

9.6.3.3 Diethyl ether - Note: Normally, ether is stabilized by adding to it a small amount of a reducing substance that prevents peroxide formation. The reducing substance does not affect tocopherol analysis; it is removed during washing after saponification or during floridin earth purification. If, however, the ether has been in the laboratory for a long time, it may contain peroxides. These may be removed by the method of Werner (11). Shake 500 ml of ether for 6 minutes with a mixture of 4 g of silver nitrate in 30 ml of water and 2 g of sodium hydroxide in 50 ml of water, allow the phases to separate, decant and use the ether without distillation.

9.6.3.4 Ethanol, absolute - Purify by distillation in an all-glass apparatus over potassium permanganate and potassium hydroxide (1 and 2 g, respectively, per litre of ethanol).



9.6.3.5 Ethanol, diluted - Add 75 parts by volume of absolute ethanol to 25 parts by volume of distilled water.

9.6.3.6 Floridin earth XS - Specially selected for use in tocopherol determination. "Floridin earth XS for the determination of Vitamin E", (made by British Drug Houses Ltd.) or equivalent.

9.6.3.7 Stannous chloride

9.6.3.8 Hydrochloric acid, d = 1.18

9.6.3.9 Benzene

9.6.3.10 Cyclohexane

9.6.3.11 Zinc oxide

9.6.3.12 Ammonium hydroxide, d = 0.880

9.6.3.13 Ammonium carbonate

9.6.3.14 Sodium fluorescein - 0.1% m/v solution in water

9.6.3.15 Papers - Whatman No. 1 grade "for chromatography", or equivalent, in sheets 51.2 cm x 46.4 cm (22 1/2 inches x 18 1/4 inches).

9.6.3.16 Liquid paraffin solution - 3% m/v solution of liquid paraffin, British Pharmacopoeia, or equivalent, in light petroleum, boiling range 50° to 80°C.

9.6.3.17 Ferric chloride solution - Ferric chloride hexahydrate, 0.2% m/v in ethanol. Being extremely liable to photochemical reduction, this solution must be prepared directly in the amber bottle in which it is to be stored. The bottle should not be exposed even to moderate daylight and is best masked by a covering of black paper or black paint.

9.6.3.18 Dipyridyl solution - 0.5% m/v solution of 2:2'-dipyridyl in ethanol.

9.6.3.19 Phenolphthalein, ethanolic solution, 1% m/v, neutralized before use.

9.6.4 Apparatus

9.6.4.1 Columns for chromatography

As described under 9.6.5.3.1

9.6.4.2 Bi-dimensional chromatography apparatus

As described under 9.6.5.4.3.

9.6.4.3 Special glassware: 3.5 ml automatic pipette, Trenner pipettes, Agla micrometer syringes, chromatographic tubes for floridin earth columns and glass atomizers for spraying chromatograms (e.g. from V.C. Ward, Church Street, Little Shelford Cambridge), or equivalent may be used.

9.6.4.4 Spectrophotometer or photoelectric colorimeter suitable for reading extinctions at 520 nm.

9.6.5 Procedure

9.6.5.1 Saponification of the oil or the fat

Weigh accurately approximately 1 g of the oil or the fat into a round-bottomed flask (50 to 150 ml is a suitable size). Add 4 ml of the pyrogallol solution, and heat in a water bath, using a cold-finger condenser or a small reflux condenser fitted with a ground-glass joint. When the mixture boils, remove the condenser, and add 1 ml of the potassium hydroxide solution. Replace the condenser, immerse the flask in the boiling-water bath, and heat under vigorous reflux for 3 minutes, with occasional shaking.

Remove the flask from the water bath, cool the contents, and add 20 ml of distilled water. Extract the unsaponifiable matter with diethyl ether three times, using about 25 ml for each extraction. If the phases fail to separate quickly and sharply, add 1 or 2 ml of ethanol. If emulsions persist, consult Notes on Procedure 9.6.7.1. Wash the combined ether extracts with 20 ml portions of distilled water until neutral to phenolphthalein, avoiding vigorous shaking at first. Three or four washes are usually necessary. Transfer the ether solution to a round-bottomed flask, and remove the ether by evaporation in a



current of nitrogen, or under reduced pressure, while warming the solution on the water bath. Dry the residue, if necessary, by adding a little absolute ethanol and benzene and re-evaporating. Do not dry the solution with magnesium sulphate or sodium sulphate, because these chemicals may contain traces of iron. Dissolve the dry residue either in 5 ml of benzene for step 9.6.5.3 (or in methanol for step 9.6.5.2 if it is required to remove sterols).

9.6.5.2 Removal of Sterols

It is rarely necessary to remove sterols before chromatography. However, with extracts of certain materials, poor separation on paper may be due to the presence of sterols.

Dissolve the unsaponifiable residue in boiling methanol and transfer the solution to a 15 ml centrifuge tube, using, in all, 12 ml of solvent. Cool to about -10°C in a suitable bath, remove the precipitate by centrifugation in a previously cooled centrifuge cup, and pour the supernatant liquid into a flask. Redissolve the sterol precipitate in 5 ml of hot methanol, and repeat the process twice. Evaporate the combined methanol solutions to dryness under reduced pressure. Dissolve the residue in 5 ml of benzene.

9.6.5.3 Floridin earth separation

9.6.5.3.1 Apparatus - Columns are prepared in the usual type of chromatographic absorption tube, 12 to 14 mm internal diameter and about 160 to 200 mm long. Tubes must not have smaller dimensions than those indicated.

9.6.5.3.2 Preparation of column - Mix 5 g of floridin earth and 0.5 g of stannous chloride with 20 ml of hydrochloric acid, and bring to the boil. With rapid stirring, pour the slurry into a chromatographic tube in one continuous operation, so that the earth settles with an even gradation. After the liquid has passed through, wash the column successively with five portions, each of 5 ml, of ethanol, and then with five portions, each of 5 ml, of benzene.

9.6.5.3.3 Column chromatography - Pass the benzene solution from step 9.6.5.1 (or step 9.6.5.2) through the prepared floridin earth column. Elute the column with seven portions, each of 5 ml, of benzene, the complete elution taking not less than 45 minutes. Nitrogen under pressure or gentle suction may be used to hasten both the preparation of the columns and the chromatography, provided the latter process takes not less than 45 minutes. If total reducing substances are to be determined, an aliquot may be taken at this stage. It is safe to store the benzene solution overnight in a refrigerator.

9.6.5.4 Paper chromatography

9.6.5.4.1 Preparation of zinc-ammine solution - Dissolve 16 g of zinc oxide and 25 g of ammonium carbonate in a mixture of 150 ml of ammonium hydroxide and 600 ml of distilled water. Add 5 ml of sodium fluorescein solution. This zinc-ammine solution is indefinitely stable in a stoppered bottle.

9.6.5.4.2 Preparation of zinc carbonate impregnated paper - Because solvents move faster along the machine direction of filter-paper than across it, all the papers should be similarly orientated. The procedure described below ensures the most evenly coated papers with the minimum handling of the working portion; the position of the written data gives a clue to the machine direction of the paper. Cut the large sheets of paper into four equal parts, as indicated in Fig. 1. Pour the zinc-ammine solution into a large dish. Immerse each sheet separately in the solution to within about 5 cm of one short edge, and hang all the sheets to drain at room temperature for about an hour. The dry margin allows the use of clips for supporting the papers. Dry the papers, hanging vertically, at 95° to 100° for 3 hours in an oven with or without forced air circulation. Remove the dried papers, cut a 20-cm square from the coated portion of each sheet, and write the date close to the edge GH. The prepared papers may be stored for a few weeks in a thoroughly dry place, but they are soon spoiled by humidity.

Rule each paper lightly in pencil, as shown by the thin lines in Fig. 1. Punch holes in the corners, if necessary, for use on a frame.



Figure 1

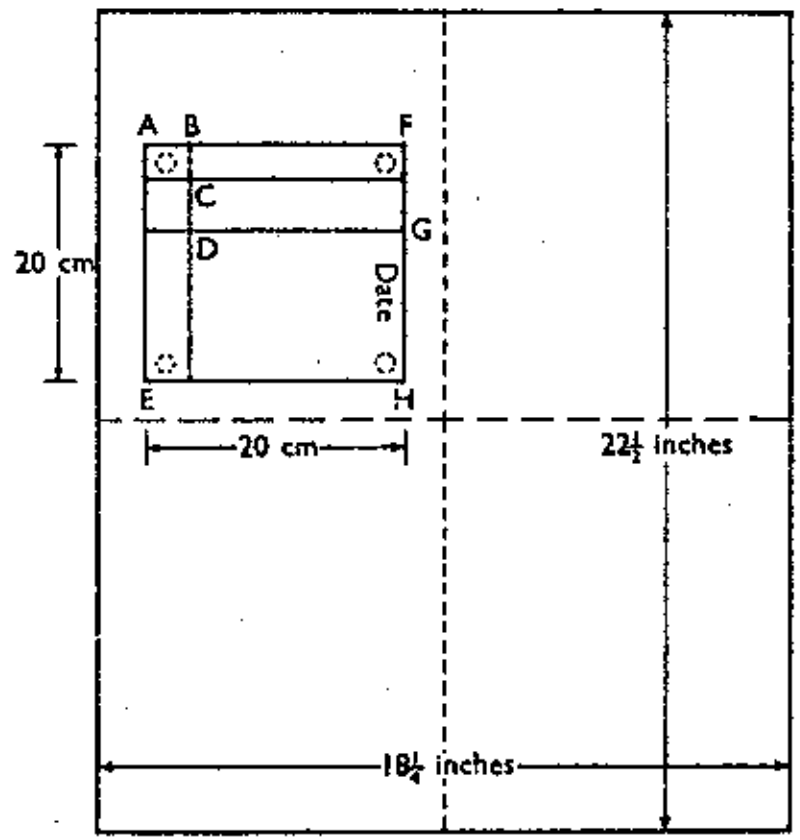


Fig. 1. Dimensions of zinc carbonate impregnated paper. Care should be taken to preserve the machine direction of the paper (as indicated on the package). AB = BC = 2 cm. CD = 3 cm. The letters need not be copied; they are inserted here only for explanatory purposes

Note I: Some batches of chromatographic paper have contained reducing material that raised the blank value. This may be removed by continuous extraction of the paper with hot methanol in a Soxhlet apparatus or by extraction and decantation in a measuring cylinder.

Note II: Zinc carbonate paper of sufficient activity can be prepared by drying in the atmosphere overnight (providing the relative humidity is not too high) instead of in an oven, but oven drying gives the more active paper.

9.6.5.4.3 Apparatus - Chromatography in both dimensions is done by the ascending-solvent method, and should be carried out in a glass tank large enough to hold the 20-cm x 20-cm papers in a vertical position. It is advisable to use a separate tank for each dimension. Alternatively, both runs may be carried out in the same tank; the necessary change of solvent must then be made before running the second dimension. The inside walls of each tank must be lined with a sheet of filter-paper, saturated with the mobile phase. The tanks must have gas-tight lids, and they should be shielded from strong light.

When less than four papers are run together, and provided that the chromatograms are to be examined later under ultra-violet light (method (a) of section 9.6.5.4.6 below), they may be supported in each tank by glass rods, arranged so that the papers hang evenly, with their edges dipping into the solvent. It is, however, more convenient to use some sort of two-dimensional chromatographic frame for carrying the papers, and this is recommended. If spot location is to be by spraying (method (b) of section 9.6.5.4.6 below), or if four or more papers are to be run together, such a frame is essential. Its use enables the papers to be manipulated easily and simultaneously during the various stages of the chromatographic separation, and ensures that the chromatograms are uniform and reproducible. Such frames have been described (3) and (10) and are suitably made of aluminium. Commercial frames are also available.

9.6.5.4.4 First dimension chromatography - Evaporate the solution from step 9.6.5.3.3 to dryness under reduced pressure or in nitrogen. Dissolve the residue in 0.5 to 3 ml of benzene in a stoppered flask, to give a solution containing 0.5 to 2.0 mg of tocopherol per ml, if possible (polar solvents



must not be used for preparing this solution). Set the tocopherol solution, by means of discrete additions of 10 to 20 $\mu$l as a narrow band across the starting line CD (Fig. 1), so that, in all, 5 to 30 $\mu$g of each tocopherol are present. The total solution set should not contain more than 100 $\mu$g of total tocopherols. Use three replicate papers for each test sample and a fourth paper as a blank. The additions should be made from a fine-tipped glass capillary, calibrated by weight, from a 10-$\mu$l blood pipette, from a Trenner pipette with a curved tip or from an Agla micrometer syringe (see 9.6.4.3). It is important to apply the solution as a regular band.

Into the first chromatographic tank pour enough 30% v/v benzene in cyclohexane to produce a depth of about 1/2 to 1 cm. Support test and blank papers with the dates at the top and their edges AE just immersed in the solvent. Leave the papers in the tank until the solvent front has migrated about 15 cm. This usually takes about 1 hour.

9.6.5.4.5 Second-dimension chromatography - If no frame is being used, remove the papers individually from the first tank and dip each one in the liquid paraffin solution, with edge EH at the bottom, to within 1/2 cm of the line DG. If a frame is used, remove it with the papers from the first tank, and, with edges EH at the bottom, immerse the assembly in a bath of the liquid paraffin solution to within 1/2 cm of the line DG. Remove the papers from the paraffin solution, drain, and allow the light petroleum to evaporate in the air. Then place the papers in the second tank with edge AF just immersed in the 75% ethanol as the mobile phase. Run the papers for 2 to 3 hours or until the front has migrated to within 4 or 5 cm from the top of the papers.

Remove the papers from the tank, and dry them by means of a current of nitrogen or air.

9.6.5.4.6 Removal of spots from chromatograms - Remove the papers from the frame, if one is used. Use either of two methods for spot location and removal for analysis. Method (a) is much to be preferred, but method (b) can be used when no ultra-violet lamp is available. Even when method (a) is used, it is still desirable to spray one of the papers as in the first part of method (b) in order to confirm the position of reducing spots.

(a) Examine the dry papers in a dark room under ultra-violet light; the lamp should not be nearer to the paper than 10 cm. Tocopherol spots are visible as dark patches on a fluorescing background, less than 1μg being easily detected (See Fig. 2). Mark the spots by ringing with a hard pencil as quickly as possible with a slight margin round each spot. Remove the paper, and cut out the marked spots with sharp scissors, cutting within the pencilled lines. With the minimum of handling, roll the cut-out pieces of paper into cylinders and drop each one separately into a numbered test-tube (measuring about 10 cm x 1.5 cm and fitted with a ground-glass stopper) containing 3 ml of ethanol and 0.5 ml of the dipyridyl solution. Mark the blank chromatogram correspondingly, and cut out the blank areas similarly.

Note I: A suitable lamp for this purpose is one emitting light with a waveband around 254 nm. A new lamp should be run for 2 or 3 hours before being put into use for ultra-violet location of tocopherols on paper. Other sources of ultra-violet illumination have been found suitable.

Note II: When large numbers of tubes are to be filled, it is convenient to use 3.5 ml of 0.07% m/v 2:2'-dipyridyl solution instead of 0.5 ml of 0.5% m/v dipyridyl solution plus 3 ml of ethanol. This solution is conveniently dispensed from a 3.5-ml automatic pipette.

(b) Spray one of the test papers heavily from a fine glass atomizer with a mixed reagent (equal parts of the ferric chloride solution and the dipyridyl solution). Tocopherols appear as red spots, which should be ringed with a hard pencil. If no ultra-violet lamp is available use these spots to locate tocopherols on the assay papers. Remove the sprayed spots and a generous margin by cutting outside the pencilled rings, and use the resulting series of holes as a template for marking another test paper, and a blank paper. Alternatively, trace the sprayed paper on to a clean sheet of thin paper, and use the latter as a template. Having marked the tocopherol positions, cut out the areas from both test and blank papers, and drop them into tubes containing dipyridyl solution, as in (a) above.



Figure 2

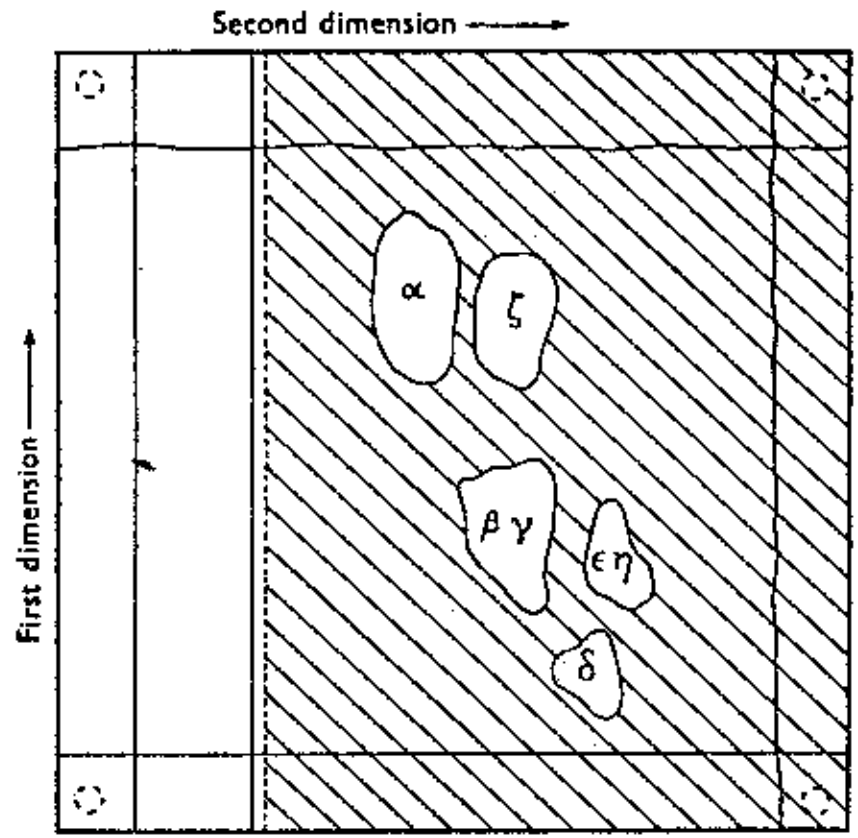


Fig. 2. Reproduction, one-third full size, from a paper, marked to show the positions of tocopherol zones after two-dimensional chromatography. The shaded area shows the part of the paper impregnated with paraffin after running in the first dimension. The wavy lines show approximate levels reached by the solvent fronts

9.6.5.5 Positions of the spots on the chromatogram

The seven tocopherols separate as shown in Fig. 2. Depending on the material being analysed, one spot or several spots may be visible. Although by this method, $\beta$ - $\gamma$ tocopherol are inseparable, as are $\varepsilon$ - and $\eta$ - tocopherol, instructions for their distinction are given under Note below. Other spots appearing on the papers are normally distinguished from the tocopherols by their positions. It must be emphasized that, depending on the degree of purification of the sample and the relative proportions of the different tocopherols present, some distortion of the movements of the spots may occur. Absolute R and $R_F$ values may be disregarded, however; provided separation occurs according to the general pattern shown in Fig. 2, the next stage may be carried out. If separation is unsatisfactory, or recognition difficult, consult Notes on procedure (9.6.7).

Note: Detection of $\gamma$ -, $\eta$ -and $\delta$ -tocopherol by diazotised o-dianisidine

Sodium carbonate - a 5% m/v solution in water.

Diazotised o-dianisidine - Dissolve 0.5 g of o-dianisidine dihydrochloride in 60 ml of water. Add 6 ml of hydrochloric acid, d = 1.18, and then 12 ml of 5% m/v aqueous sodium nitrite solution. Mix thoroughly. After 5 minutes add 12 ml of 5% m/v aqueous urea solution.

Spray one of the test papers (it is advisable to run an extra paper if the presence of $\gamma$ -, $\eta$ - or $\delta$ -tocopherol is suspected) heavily with the sodium carbonate and then with the diazotised o-dianisidine from a fine glass atomiser. The tocopherols may be identified by the resultant colours: $\gamma$ -, blue-green changing to indigo; $\eta$ -, brownish purple changing to purple; $\delta$ -, reddish brown changing to purple. The colours may be stabilised by washing the papers in water after spraying.

The presence of colour in either the $\gamma$- or the $\eta$ - position does not exclude the presence of $\beta$ - or $\varepsilon$ -tocopherol, respectively.



9.6.5.6 Colorimetric determination

Gently swirl the stoppered test-tubes, each containing 3.5 ml of ethanolic dipyridyl solution (9.6.3.18) and the slip of paper, for a few seconds. Transfer to a darkened room, and carry out the rest of the determination under dim artificial light. To one of the solutions add 0.5 ml of the ferric chloride solution (9.6.3.17) from a fast-delivery pipette, shake the stoppered tube for a moment, and then transfer the contents to a glass spectrophotometer cell 1 cm in path length. Exactly two minutes after adding the ferric chloride, measure the extinction at 520 nm against a similar cell containing ethanol. Repeat this procedure with each tube. Subtract the reading of each "blank" tube from the reading obtained from the corresponding "test" tube. This gives the "net extinction" reading. Multiplication of the net extinction reading by the spectrophotometric factor for the tocopherol being measured gives directly the number of micrograms of tocopherol in the cut-out spot.

9.6.5.7 Blanks

The tocopherol-containing solution has been indirectly measured against a corresponding blank solution. The highest accuracy can only be obtained, therefore, if it can be safely assumed that the blank reaction of the mixed reagents is identical in both "blank" and "test" tubes. The "blank" has two components, namely (i) that derived from the residual reducing material in the paper, and (ii) the reaction between the dipyridyl and traces of ferrous iron in the ferric chloride. Component (i) amounts to an extinction of about 0.010; component (ii) can be reduced to about 0.050 by suitable precautions, such as preparing and keeping the ferric chloride solution in the dark and the observance of scrupulous cleanliness in the use of the pipettes for ferric chloride. Ferric chloride is converted to the ferrous state photochemically; hence the solution should not be exposed to daylight in transparent glass. The total blank value can be reduced to about 0.060 to 0.080. Blanks that give extinctions of over 0.100 are likely to be unstable and should be suspect. If high blank values continue, and other forms of contamination have been eliminated, it is best to prepare a new ferric chloride solution in a dark bottle previously cleaned with chromic acid solution, thoroughly rinsed with distilled water and dried.

9.6.6 Calculation and expression of results

The amount of each tocopherol, in microgramme per gramme of product is calculated as follows:

$$\mu g \text{ tocopherol} = \frac{D \times F \times V \times 1000}{v \times M}$$

where:

M = mass in grammes of sample taken
V = volume in millilitres of benzene used to prepare solution for paper chromatography
v = volume in microlitres spotted on paper
D = net extinction
F = spectrophotometric factor for 4 ml

The recommended factors F are:

α -tocopherol 98
β -tocopherol 96
γ -tocopherol 90
δ -tocopherol 75
ε -tocopherol 98
ζ -tocopherol 94
η -tocopherol 88

The β - and ε -tocopherol are determinable when spots in their respective positions give no colour on spraying with the dianisidine reagent (See Note under 9.6.5.5). However, if the dianisidine reaction shows the presence of γ - or η - tocopherol the analyst may either assume that β - and ε - tocopherol are absent or attempt a differential analysis of the four substances by chromatographic separation of nitroso-derivatives, such as has been described by Marcinkiewicz and Green (7). Factors for γ - and η -tocopherol should be used even though β - or ε -tocopherol, or both, may be present but masked.

Results are expressed as milligrammes of each tocopherol per kilogramme of the product.



9.6.7 Notes on Procedure

Some difficulties may be encountered in the carrying out of the various phases of the analysis. They are covered in the following descriptions and suggestions thereto are given:

9.6.7.1 Saponification and extraction

After the saponification has been completed, the extraction of the tocopherols into ether must be carried out as rapidly as possible, the aim being to achieve clean, fast separation of the two phases at every stage. Sometimes emulsions are formed; they can usually be dispersed by adding a few millilitres of ethanol. If they persist, it may be because the directions have not been followed closely enough; the proportions of reagents are somewhat critical, and too much ethanol may even encourage the formation of emulsions. Vigorous shaking at any stage is unnecessary and should be avoided, especially as some products are rich in emulsifying agents.

9.6.7.2 Floridin earth chromatography

Occasionally a batch of floridin earth may be found with too slow a percolation rate for column chromatography. This fault is difficult to remedy; in practice it is simplest to procure another batch.

Carotenoids, retinol and its derivatives, sterols and some other substances are adsorbed on floridin, producing a green, blue or red band. Sometimes, when the eluate is yellow or the coloured band reaches the bottom of the column, subsequent paper chromatography may be unsatisfactory. This may be because the column was developed too fast; or the ethanol used in its preparation contained water; or possibly the column was overloaded, too large a sample of oil having been saponified. The "activity" or adsorptive power of the column depends chiefly on the fineness of the particles and the extent of dehydration produced by washing with ethanol. The conditions recommended in the method described here give an active column, suitable for removing most normal amounts of interfering substances. It may occasionally be found if an extremely active batch of floridin is used, that even 35 ml of benzene is insufficient to elute all the δ -tocopherol, which is the most strongly adsorbed tocopherol; if so, increase the amount of benzene to 40 or even 45 ml.

9.6.7.3 Paper chromatography

Exploratory techniques - An analyst first practising the method will find it a good plan to chromatograph first a solution of α-tocopherol and then a solution containing a known mixture of tocopherols. Reference solutions are best prepared in benzene and, for convenience, should contain about 1 mg of each tocopherol per ml. They may be kept in a refrigerator without appreciable deterioration.

In analysing a specimen of oil whose tocopherol content cannot be approximately guessed it is often advisable in step 9.6.5.4.4 to apply a different amount of the benzene solution to each of two or three papers. When the developed chromatograms are scanned, the one with the optimum amount of each tocopherol can be recognised easily after a little practice. It is sometimes useful to survey an unknown solution by a preliminary run on a single-dimensional zinc carbonate paper strip or, more rarely, to run a preliminary paraffin-coated strip. These preliminary runs give information about the tocopherols present and their separations. It must be emphasized that either single-dimensional procedure is for guidance only.

The use of tracers - Sometimes a lipid is encountered containing uncommon proportions of tocopherols or excessive amounts of unusual impurities. Such material may produce a distorted paper chromatogram, on which the spots are sufficiently displaced to make difficult the identification of tocopherols by position alone. If this occurs, a duplicate paper, to which small amounts of α-, γ- or δ-tocopherol have been added to act as tracers, may be of use. It should be prepared as follows: Add from a micro-pipette, along the starting line CD of chromatographic paper, about 10 μg of each tocopherol being investigated. The solutions of tocopherols in benzene, described above, are convenient for this purpose, 10 μl being a satisfactory amount to add to the paper. Then add the test solution in the usual fashion. It is important that the tracers be added first, to ensure their even solubility in the small amount of test solution. Prepare a test paper, omitting the tracers, and run both papers together. The tracer tocopherols will migrate to the same positions as those in the test solution when they are identical with them.



Unsatisfactory chromatograms - If the developed papers are still damp with aqueous solvent, observation of the spots under ultra-violet light is unsatisfactory.

If the tocopherols seem to have migrated too far in the first dimension or if they are poorly separated, it may be because the papers are stale; the zinc adsorbent deteriorates in time, especially in a humid atmosphere. If the tanks are shallow and are not lined with filter paper, excessive evaporation of solvent from the front may occur; this causes fast running and poor separation of bands. Alternatively, the solvent in the first tank may need modifying. It has been found that, under the conditions that obtain in some laboratories, 25% of benzene in cyclohexane is sufficient. If the tocopherols do not appear to have migrated enough, it may be necessary to increase the benzene to 35%. It was considered that 0.5% of ethanol in cyclohexane produces as good chromatograms as do the benzene-cyclohexane mixtures, but this percentage is somewhat critical and difficult to maintain in the tank. If the migration of the tocopherols in the second dimension is grossly abnormal, the solvent in the second tank may be at fault; it should be changed.

If papers show ill-defined spots of unexpected shapes or in the "wrong" places, it may well be that the papers have been overloaded, possibly with tocopherol, but more probably with other lipid materials. Spots that absorb ultra-violet light and do not reduce ferric chloride, as well as spots that fluoresce, are not tocopherols. If large amounts of interfering substances are observed on the paper, saponification may have been incomplete or the treatment with floridin earth ineffectual or the oil one requiring the "removal of sterols" step.

When lipids of very low potency (say, less than 0.05 mg of total tocopherols per g) are being analysed, two further difficulties in the paper chromatography are occassionally encountered, particularly if the lipids have been extracted from certain animal tissues. Even when purification according to the recommended method (including the "separation of sterols" step) has been performed, the residual tocopherol fraction may contain substantial amounts of hydrocarbons and other inert material. Because of the low tocopherol content of the sample, the fraction has to be added to the starting line of the paper in such an amount that not only does the first dimension become clogged for several centimetres along its length, but also an

immiscible barrier may form at the paraffin boundary along the line DG. Tocopherols may be trapped in the barrier; if this happens, no separation will take place. Sometimes the phenomenon leads to streaking over a large area of the paper. One device that has proved successful in dealing with many of these difficult materials is a modification of the floridin earth purification. After normal preparation of the column, including the wash with five portions of 5 ml benzene, pass 25 ml of light petroleum (40° to 60°C) through the column. Dissolve the tocopherol residue from step 9.6.5.1 (or, more usually with these difficult materials, step 9.6.5.2) in 5 ml of light petroleum, and add the solution to the top of the column. Develop the column with 25 ml of light petroleum. Discard the filtrate, which contains many of the interfering substances, and elute the tocopherols with the normal 30 ml of benzene.

9.6.7.4 Spectrophotometric factors

The factors given in the recommended method have been obtained as a result of careful study in several laboratories, and it is unlikely that the individual analyst will want to determine them for himself. The determination of tocopherols by the ferric chloride-dipyridyl oxidation method is affected slightly by conditions that vary from laboratory to laboratory; the recommended procedure has eliminated most of these effects as causes of variation. Those that remain - due to variations in temperature, humidity and purity of reagents - are not normally under control; in any event, the factors are not likely to vary by more than 2% between different laboratories. If the analyst should wish, for the most accurate work, to determine any factor in his own laboratory, he should procure a sufficiently pure specimen of the tocopherol concerned and carry out some determinations daily for a while at two or three different levels of tocopherol. In practice, he is most likely to be interested in the accurate determination of α-tocopherol.

9.6.7.5 General Precautions

The tocopherols are reasonably stable substances. They are most vulnerable to oxidation in the presence of traces of alkali, and the necessity for care and speed during the saponification stage has already been stressed. Strong light, abnormally high temperatures and the presence of ozone or ammonia in the air have all been found to affect the tocopherols



during paper chromatography. Chromatograms should not be left in the dry state for longer than necessary. For useful information on paper chromatography, including hints on cleanliness in handling papers, consult Mitchell (12).

If an analysis cannot be completed in a day, it is advisable to interrupt the procedure after saponification and extraction, or after floridin treatment, and to store the benzene solution in a refrigerator.

9.6.8 Literature references


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9.7 Determination of sodium chloride content CAC/RM 19-1969

9.7.1 Principle of method

After melting the margarine by adding boiling water, the chlorides in the mixture are titrated with a standard solution of silver nitrate, using potassium chromate as indicator, according to the Mohr procedure.

9.7.2 Reagents

The reagents used shall be of recognized analytical reagent quality.

9.7.2.1 Silver nitrate solution, 0.1 N, standardized.

9.7.2.2 Potassium chromate solution 5% (m/v) in distilled water.

9.7.3 Apparatus

9.7.3.1 Analytical balance

9.7.3.2 Conical flasks, capacity 250 ml

9.7.3.3 Burette, graduated to 0.1 ml

9.7.4 Procedure

9.7.4.1 Preparation of the sample

Soften sample in closed sample container by warming in water bath kept at as low a temperature as practicable in order not to break the emulsion. Shake sample container at frequent intervals during the softening process to thoroughly mix sample. Remove sample container from water bath and shake vigorously at frequent intervals until sample has cooled to a thick, creamy consistency. A mechanical shaker may be used.

9.7.4.2 Blank test

Do a blank determination using the same reagents in the same amounts and according to the same procedure as described under 9.7.4.3, excluding the sample.



9.7.4.3 Determination

Weigh, to the nearest 0.01 g approximately 5 g of the sample into the conical flask. Carefully add 100 ml of boiling distilled water. Allow to stand for 5-10 min., swirling occasionally, while cooling to 50-55°C (titration temperature). Add 2 ml of the potassium chromate solution (9.7.2.2). Mix by swirling. While swirling continuously, titrate with the silver nitrate solution (9.7.2.1) until the colour change to orange-brown persists for 30 sec.

9.7.5 Calculation and expression of results

9.7.5.1 The sodium chloride content (expressed as percentage m/m NaCl) is:

$$\frac{5.85\ N\ (V_1 - V_0)}{m}$$

where:

$N$ = normality of the silver nitrate solution.

$V_1$ = number of ml of silver nitrate solution used in 9.7.4.3.

$V_0$ = number of ml of silver nitrate solution used in 9.7.4.2.

$m$ = mass, in grammes, of sample used.

Round off the result to the nearest 0.01%.

9.7.5.2 Repeatability

The difference between results of duplicate determinations (results obtained simultaneously or in rapid succession by the same analyst) should not exceed 0.02 sodium chloride for 100 g of the product.

9.7.6 Literature references

Report of the Tenth Session of the Joint FAO/WHO Committee of Government Experts on the Code of Principles concerning Milk and Milk Products (Ref. No. SP-10/105, 10th Session, September 1967, Appendix IV-D).

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# ACCEPTANCE PROCEDURE FOR CODEX COMMODITY STANDARDS

## EXTRACT OF THE GENERAL PRINCIPLES OF THE CODEX ALIMENTARIUS

### ACCEPTANCE OF CODEX COMMODITY STANDARDS

"4.A. A Codex standard may be accepted by a country in accordance with its established legal and administrative procedures in respect of distribution of the product concerned, whether imported or home-produced, within its territorial jurisdiction in the following ways:

i) Full acceptance

a) Full acceptance means that the country concerned will ensure that a product to which the standard applies will be permitted to be distributed freely, in accordance with (c) below, within its territorial jurisdiction under the name and description laid down in the standard, provided that it complies with all the relevant requirements of the standard.

b) The country will also ensure that products not complying with the standard will not be permitted to be distributed under the name and description laid down in the standard.

c) The distribution of any sound products conforming with the standard will not be hindered by any legal or administrative provisions in the country concerned relating to the health of the consumer or to other food standard matters except for considerations of human, plant or animal health which are not specifically dealt with in the standard.

ii) Target acceptance

Target acceptance means that the country concerned indicates its intention to accept the standard after a stated number of years and will meanwhile not hinder within its territorial jurisdiction the distribution of any sound products conforming with the standard by any legal or administrative provisions relating to the health of the consumer or to other food standard matters except for considerations of human, plant or animal health which are not specifically dealt with in the standard.

iii) Acceptance with minor deviations

Acceptance with minor deviations means that the country concerned gives full acceptance as defined in paragraph 4. A(i) to the standard with the exception of minor deviations which are recognized as such by the Codex Alimentarius Commission; it being understood that a product complying with the standard as qualified by such minor deviations will be permitted to be distributed freely within the territorial jurisdiction of the country concerned. The country concerned will further include in its declaration of acceptance a statement of such deviations, the reasons for them, and also indicate:

a) whether products fully conforming to the standard may be distributed freely within its territorial jurisdiction in accordance with paragraph 4. A(i);

b) whether it expects to be able to give full acceptance to the standard and, if so, when.

B. A country which considers that it cannot accept the standard in any of the ways mentioned above should indicate:

i) whether products conforming to the standard may be distributed freely within its territorial jurisdiction;

in what ways its present or proposed requirements differ from e standard, and, if possible, the reasons for these differences.

C. i) A country which accepts a Codex standard according to one of the provisions of 4. A is responsible for the uniform and impartial application of the provisions of the standard as they apply to all home-produced and imported products distributed within its territorial jurisdiction. In addition, the country should be prepared to offer advice and guidance to exporters and processors of products for export to promote understanding of and compliance with the requirements of importing countries which have accepted a Codex standard according to one of the provisions of 4. A.

ii) Where, in an importing country, a product claimed to be in compliance with a Codex standard is found not to be in compliance with that standard, whether in respect of the label accompanying the product or otherwise, the importing country should inform the competent authorities in the exporting country of all the relevant facts and in particular the details of the origin of the product in question (name and address of the exporter), if it is thought that a person in the exporting country is responsible for such non-compliance."

Committee on Specifications 1972

Food Chemicals Codex

Committee on Food Protection,

National Academy of Sciences,

National research Council,

Washington, D. C.

# Flavor Evaluation of Copper-Hydrogenated Soybean Oils[1]

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## Abstract

The use of copper catalyst to reduce selectively the linolenate in soybean oil improves its flavor stability. As previously shown, the copper must be removed or properly inactivated to obtain an oil of high initial quality. In oven and heat tests, odor and flavor development in the hydrogenated soybean oil samples correlate surprisingly well with actual levels of linolenate, but there were some differences in overall responses among cottonseed oil, copper-reduced (0.0% linolenate) and nickel-reduced (3.0% linolenate) soybean oils. The taste panel generally scored the last three oils in the following order: cottonseed oil, copper-reduced and nickel-reduced soybean oil.


## Introduction

The use of copper-chromite and copper-oxide-supported catalysts for hydrogenating soybean oil has been extensively investigated at the Northern Regional Research Laboratory and elsewhere (1–10). These studies have established that copper catalysts have a selectivity as high as 15 or more for linolenate, where selectivity is the ratio of the rate of hydrogenation of linolenate divided by the rate of hydrogenation of linoleate. Some of these reports indicate that flavor or oxidative stability was improved.

Okkerse et al. (5) showed that oils degummed, refined, bleached and dried, reduced with copper, and refined with phosphoric acid had good stability. These Dutch workers found that 14 of 20 tasters preferred soybean oil hydrogenated with copper catalyst to unhydrogenated oil initially and 19 of 19 after the oil was stored two weeks at 15 C in the dark. They also scored two samples of copper-reduced oil equal to a refined sunflower seed oil after one day in diffuse daylight, and one sample slightly higher and the other not quite so high as the refined sunflower oil after three days. Under similar aging conditions, other Dutch workers (4) scored a copper-reduced oil containing 0.7% linolenate slightly higher than one having 2.0%; both reduced soybean oils scored higher than a sunflower seed oil of 0.1% linolenate. Beal et al. (11) reported that copper-reduced oils, bleached or resin-treated to remove copper and deodorized with citric acid, had good initial quality. Scores of 7.7 and 7.8 initially dropped only to 6.0 to 6.5 on aging these oils for four and eight days at 60 C. Kuwata and coworkers (10) hydrogenated soybean oil with a copper-chromite catalyst to give different levels of linolenate and reported that such oils had good stability during long storage. Since Koritala (12) found a copper catalyst based on a copper-ammonium complex had high activity and selectivity for linolenate over linoleate, we studied the flavor stability of oils hydrogenated with this catalyst. Work with copper-chromite-reduced oils is also reported. Different levels of linolenate were evaluated for flavor score initially, after four and eight days at 60 C, after heating to 150, 170 and 200 C and with room odor tests. Similar comparisons of 0.0% linolenate oils were made with nickel hydrogenated-winterized soybean oil and cottonseed oil.

## Methods and Materials

Degummed and alkali-refined soybean oils, cottonseed salad oil, and a nickel-reduced-winterized soybean oil were obtained from commercial suppliers. Degummed oils were alkali-refined and bleached; alkali-refined oils were bleached; bleached oils were deodorized in all-glass laboratory apparatus as previously described (13,14). When desired, the oils were redeodorized with antioxidants and antifoam agents added during the last stages of deodorization. Hydrogenations were carried out according to the procedures of Koritala and Dutton (6,7,12).

A typical hydrogenation with copper-oxide or copper-chromite catalyst was effected as follows: A laboratory refined and bleached soybean oil (3 liters) was heated for 2 hr at 175 C in the presence of a catalyst and an initial hydrogen pressure of 30 psi. The catalyst from copper-ammonium complex (12) contained 3.3 g as cupric oxide deposited on 50 g of silica gel. The oil was filtered, bleached with 1% activated clay (Superfiltrol) and deodorized as described previously (14).

Peroxide values were determined by a modification of the Wheeler method (15) and the active oxygen method (AOM) values were obtained by determining peroxides after 8 hr under AOM conditions (16).

Fatty ester analyses were performed by gas liquid chromatography (GLC), and linolenate was determined by alkali-isomerization (6). Compositions of the original and hydrogenated oils are given in Table I. The analyses of the cottonseed salad oil by GLC and alkali-isomerization were 0.6 myristate, 16.8 palmitate, 0.6 palmitoleate, 2.5 stearate, 22.0 oleate, 56.7 linoleate and 0.9% linolenate. Calculated IV was 119.8. Although the linolenate content suggests that this cottonseed oil might have been contaminated with a small amount of soybean oil, the palmitoleate, stearate and oleate contents suggest it was not. All flavor evaluations were made by our research taste panel. Various sample preparations and evaluating techniques were used. Some evaluations were conducted with samples aged at 60 C for four and eight days in bottles two thirds full (17). Others were carried out with samples heated rapidly to 150, 170 and 200 C, cooled to 55 C and evaluated in the same manner as the four- and eight-day samples. Finally, room odor tests were carried out by a procedure that will be more fully described later (Evans et al., unpublished). From 150 to 300 cc of oil samples were heated to 380 F (193 C) in an open deep-fat fryer in two new relatively odor-free laboratory rooms. Members of the taste panel were asked to enter the rooms and to score and describe the oil odors.

In these room odor tests, a flavor intensity value (FIV) was used to quantitize the flavor and odor of the oils. FIV equals the intensity-weighted summations of the flavor responses divided by the number of tasters: FIV = [(number of weak responses) + 2 (number of medium responses) + 3 (number of strong-

[1] One of 10 papers to be published from the Symposium "Hydrogenation," presented at the AOCS Meeting, New Orleans, April 1970.
[2] No. Utilz. Res. Dev. Div., ARS, USDA.

TABLE I

Composition of Soybean Oils (wt. %)

| Component | Original oils | | Copper reduced from A | | | Copper chromite reduced from B | | Nickel-reduced-winterized | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | | | | | | From B | Commercially |
| Palmitic | 10.2 | 10.6 | 9.6 | 10.3 | 9.7 | 10.6 | 10.5 | 10.5 | 8.6 |
| Stearic | 4.6 | 4.6 | 4.2 | 4.7 | 4.1 | 4.9 | 5.0 | 5.2 | 3.9 |
| Monoene | 26.5 | 23.3 | 30.9 | 32.2 | 51.1 | 38.4 | 29.5 | 49.0 | 46.3 |
| Diene | 51.8 | 52.8 | 53.2 | 51.4 | 35.1 | 45.6 | 51.7 | 38.5 | 38.1 |
| Triene | 7.8 | 8.4 | 2.0 | 1.3 | 0.0 | 0.1 | 2.7 | 3.8 | 3.0 |
| IV | 131.4 | 132.4 | 123.4 | 119.6 | 104.7 | 113.3 | 122.5 | 111.0 | 113.7 |

responses]/n, where n is the number of tasters. Limits of the flavor intensity values will, of course, be from 0.0 to 3.0.

## Results and Discussion

Early tests demonstrated that soybean oil hydrogenated to low linolenate content with copper-chromite as a catalyst was more stable than the original oil. In Table II note that the flavor scores for the copper-reduced oil were highly significantly above the control oil at four and eight days. Copper-chromite catalyst was used in preparation of the copper-reduced oils reported in Tables II-IV. Flavor responses shown are typical of soybean oils subjected to these conditions.

If the hydrogenations were stopped at about 3.0% linolenate—a value comparable to that obtained commercially for nickel hydrogenated-winterized soybean oil—winterization of the copper-reduced oil was unnecessary, but no superiority in flavor stability was achieved. Note in Table III that the copper-reduced oil with 2.7% linolenate has flavor scores, AOM values and flavor responses comparable to the nickel-reduced and winterized oil prepared by engineers at the Northern Laboratory. When copper-reduced oil with 0.1% linolenate was compared organoleptically with the nickel-reduced oil with 3.3% linolenate, flavor scores indicated that we did achieve superior stability in the copper oil but failed to achieve equal initial quality. Table IV shows that the copper-reduced oil of 0.1% linolenate content was just as stable in the 8 hr AOM test as the nickel oil and that copper-reduced oil scored higher after aging for four days at 60 C.

### Comparison of Soybean Oils Low in Linolenate

Evaluations were made of hydrogenated oils prepared with Koritala's recently announced copper catalyst (12). It combines high selectivity with high reactivity and stability. Practical tests were designed with the knowledge that removal of trace amounts of catalysts before deodorization was desirable (5,11) and that reuse of catalyst was required. Oil samples were prepared with catalysts previously used once (second use) and twice (third use) and the resulting oils had 1.3 and 2.0 linolenate, respectively. The catalysts were still active and useful after four hydrogenations.

Bleaching the oil before hydrogenation prolongs the activity of the copper catalysts (8). We confirmed that bleaching the oil after hydrogenation improves initial flavor and flavor stability and also lowers copper content (11). If copper is not removed by bleaching or is not inactivated before or during early stages of deodorization, the initial flavor quality of the oil is lowered; results are given in Table V. Data show that this oil has excellent oxidative stability because treatment with citric acid apparently inactivates the copper without removing it, but initial flavor suffers.

Examination of data in Table VI indicates that all these oils had excellent initial flavor, as well as oxidative and flavor stability. It was necessary to subject them to prolonged exposure in accelerated storage at 60 C or to test them as cooking oils to create significant differences among them. No significant difference in flavor scores was found after four days at 60 C, but after eight days at 60 C the copper-reduced oils were significantly higher in flavor score even though the original oil had performed excellently. The copper-reduced oils were rated at flavor scores of about 6, which we generally believe to be acceptable.

One test that is frequently used to evaluate cooking oils is the heat test. The oil is heated to a given temperature, cooled to 55 C and evaluated by the taster or taste panel. In tests with oil heated to 170 C, all the copper-reduced oils were significantly higher in flavor score than the original oil sample. Tests with oils heated to 200 C showed that the copper sample with the 1.3% linolenate scored significantly higher than the copper samples with 2.0% linolenate and highly significantly above the original oil. Lowering linolenate value from 7.8% to 2.0% made a significant improvement and further reduction to 1.3% gave additional improvement.

In related tests, when soybean oils were hydrogenated with a commercial copper-chromite catalyst to linolenate contents of 0.4%, 1.4% and 5.5%, flavor scores and oxidative stabilities were inversely related to linolenate content. Initially, all oils scored 7.4 to 7.9, but after eight days at 60 C, the respective flavor

TABLE II

Comparison of Copper-Chromite-Reduced Soybean Oil With Unhydrogenated Control

| Storage 60 C, days | Flavor scores and significance[a] | | |
|---|---|---|---|
| | Control, Ln 8.4% | Sig. | Copper reduced, Ln 0.1% |
| 0 | 7.5(0.0) | + | 7.5(0.0) |
| 4 | 4.7(3.0) | ** | 6.7(0.5) |
| 8 | 4.2(11.0) | ** | 6.1(3.2) |
| AOM, 8 hr, PV | 19.0 | | 3.4 |
| | Flavor responses | | |
| 0 | Buttery | | Buttery |
| 4 | Rancid, buttery, grassy | | Buttery, beany |
| 8 | Pahty, rancid, grassy | | Buttery, rancid |

[a] The (+) indicates no statistical significance at the 5% level; (*) denotes significance at the 5% level; (**) shows significance at the 1% level; Ln represents linolenate; values in parentheses are peroxide values, milliequivalents per kilo.

TABLE III

Comparison of Copper-Chromite-Reduced With Nickel-Reduced-Winterized Soybean Oil at Comparable Linolenate Contents

| Storage 60 C, days | Flavor scores and significance[a] | | |
|---|---|---|---|
| | Copper reduced,[b] Ln 2.7% | Sig. | Nickel reduced,[b] Ln 3.3% |
| 0 | 8.0(0.6) | + | 8.4(0.0) |
| 4 | 5.7(0.9) | + | 6.2(2.1) |
| AOM, 8 hr, PV | 5.5 | | 4.6 |
| | Flavor responses | | |
| 0 | Buttery | | Buttery |
| 4 | Rancid | | Rancid |

[a] See footnote a, Table II.
[b] Copper-chromite and nickel-reduced oils. The nickel-reduced oil was winterized.

TABLE IV

Comparison of Copper-Chromite-Reduced Oil of Low Linolenate Content With Nickel-Reduced-Winterized Soybean Oil

| Storage 60 C, days | Flavor scores and significance[a] | | |
|---|---|---|---|
| | Copper reduced,[b] Ln 0.1% | Sig. | Nickel reduced,[b] Ln 3.3% |
| 0 | 7.5(0.0) | * | 8.4(0.0) |
| 4 | 7.1(0.5) | * | 5.8(2.2) |
| AOM, 8 hr, PV | 3.4 | | 4.6 |
| | Flavor responses | | |
| 0 | Buttery, beany | | Buttery, beany |
| 4 | Buttery, rancid | | Grassy |

[a] See footnote a, Table II.
[b] See footnote b, Table III.

scores were 6.1, 5.7 and 4.2. Peroxide values after 8 hr under AOM conditions were 7.0, 8.0 and 15 and were positively related to their linolenate contents of 0.4%, 1.4% and 5.5%, respectively. Data compiled from some 38 different samples, however, showed little correlation between the peroxide values in the AOM tests and iodine values or linolenate content. Apparently other factors had more effect on the level of peroxide achieved in the AOM test than did linolenate content or iodine value. This work with oils reduced by copper-chromite, as well as the work with the copper catalyst from the ammonium complex, suggested that additional tests with oil containing a minimum of linolenate were desirable.

The same general flavor evaluations were obtained for oil in which the linolenate was substantially eliminated (Table VII). The initial qualities of this oil and the original unhydrogenated oil were excellent; flavor scores of both approached 8. Treatments more severe than four days at 60 C were again necessary to achieve differences. If peroxide value is a criterion, the oxidative stability of these oils was approximately the same. Thus the peroxide values of the copper-reduced and unhydrogenated oil were equal, but the flavor score of the copper-reduced oil was highly significantly above the unhydrogenated oil after eight days at 60 C. Consequently, the flavors of the copper-reduced oil must be different. A similar observation holds for oils from the heat test at 200 C. Accordingly, consideration of flavor responses was definitely indicated.

The flavor responses for unaged oils were generally reported as buttery with FIVs ranging from 0.8 to 1, nutty, from 0.2 to 0.4 and beany 0.0 to 0.2, respectively. The results of several tests on unhydrogenated soybean oil and copper-reduced oils containing 0.0%, 1.3% and 2.0% linolenate are included in the following compilation. In both the copper-reduced and the unhydrogenated oils after four days at 60 C, buttery responses had not dropped much with FIVs ranging from 0.3 to 0.9, but rancid values had increased up to 0.7 and beany values up to 0.5. Grassy FIVs increased up to 0.4 and appeared at the higher levels

TABLE V

Effect of Bleaching on Copper-Reduced Oils

| Condition | No bleach plus citric acid | | Bleach plus citric acid |
|---|---|---|---|
| | Flavor scores and significance[a] | | |
| Initial | 6.9(0.0) | ** | 8.4(0.0) |
| Aged eight days at 60 C | 6.1(6.3) | + | 6.9(3.6) |
| Heat to 200 C | 2.7(2.1) | * | 3.4(2.4) |
| Copper (ppm) after hydrogenation[b] | | | |
| Before bleaching | 0.22 | | 0.22 |
| After bleaching | | | 0.01 |
| After deodorization | 0.15 | | 0.01 |

[a] See footnote a, Table II.
[b] Copper catalyst from copper ammonium complex (12).

Table VI

Comparison of Copper-Reduced Oils With Low Linolenate Contents

| Condition | Original plus citric acid | | Copper-reduced oils: Third use of catalyst | | Copper-reduced oils: Second use of catalyst |
|---|---|---|---|---|---|
| | Flavor scores and significance[a] | | | | |
| Initial | 8.1(0.0) | + | 8.3(0.0) | + | 8.4(0.0) |
| Aged 60 C, eight days | 5.0(1.0) | * | 6.8(0.5) | + | 6.8 |
| | | | (*) | | |
| Heat test, 170 C | 3.8(2.7) | * | 4.7(2.7) | + | 4.4(3.0) |
| | | | (*) | | |
| Heat test, 200 C | 2.3(3.9) | + | 2.6(3.8) | * | 3.4(2.4) |
| | | | (**) | | |
| Linolenate content | 7.8 | | 3.0 | | 1.8 |
| AOM, 8 hr, PV | 10.2 | | 4.0 | | 4.2 |

[a] See footnote a, Table II; copper catalyst from copper ammonium complex (12).

in unhydrogenated oils. After eight days at 60 C, the buttery responses were about equal in all samples at 0.5 FIV. Rancid values were high for the unhydrogenated oil at 1 to 1.9 but were substantially lower for the all-copper-reduced oils at 0.5 to 0.6. Painty and grassy responses were also higher at 0.4 to 0.6 for unhydrogenated oil in comparison to 0.1 to 0.4 values for most of the copper-reduced oils with none in the 0.0% linolenate sample.

The flavor responses of oils heated to 150, 170 and 200 C and cooled to 55 C before tasting showed definite patterns. The copper-reduced oils at 150 C had buttery FIVs of 0.8 and 0.9 and rancid 0.1 to 0.5; unhydrogenated oils had lower buttery responses ranging from 0.3 to 0.5 with high rancid responses from 0.5 to 0.8. As the temperature increased to 170, grassy responses increased in unhydrogenated oils ranging from 0.6 to 1.0, whereas in the copper-reduced oils (0.0% linolenate) they were 0.0 to 0.5. Rancid responses were relatively high for all samples with FIVs ranging from 0.7 to 1.0. The big difference in responses was found in painty where FIVs for unhydrogenated oil ranged from 0.8 to 1.1 and for the copper-reduced oils from 0.2 to 0.3. The unhydrogenated oils also had higher values for fishy and grassy responses.

Heat tests at 200 C gave rather convincing evidence that differences appear to be associated with linolenate content. Figure 1 shows unhydrogenated oils with high painty responses of 1.5 to 1.6 with a reduction in FIVs that follows linolenate content. Fishy responses were also high for the unhydrogenated oils at FIVs 0.5 to 0.8; this flavor was negligible with copper-reduced oils. Rancid responses were remarkably close together at 1.2 to 1.4 for all oils.

Another dramatic difference in flavor responses came in our room odor tests on soybean oils containing

TABLE VII

Comparison of Copper-Reduced Oil Having 0.0% Linolenate With Unhydrogenated Oil-Flavor Scores and Significance

| Condition | Copper-reduced oil | Sig.[a] | Original oil |
|---|---|---|---|
| Initial | 7.7(0.0) | + | 7.6(0.0) |
| 60 C, four days | 6.5(0.8) | + | 6.2(0.7) |
| 60 C, eight days | 5.7(6.2) | ** | 4.2(6.2) |
| Heat test, 150 C | 6.3(0.7) | * | 4.2(0.9) |
| Heat test, 170 C | 4.2(3.1) | + | 3.7(2.8) |
| Heat test, 200 C | 3.8(3.6) | ** | 2.3(2.5) |
| AOM, 8 hr, PV | 5.0 | | 5.6 |
| Triene, % | 0.0 | | 7.8 |

[a] See footnote a, Table II.

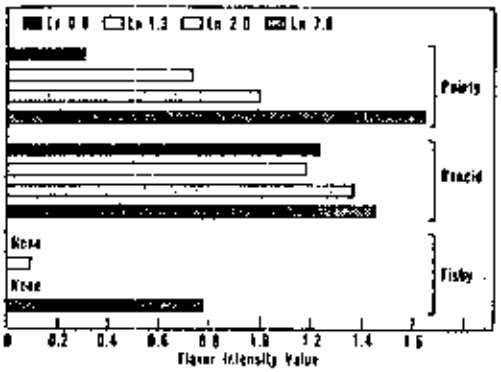


FIG. 1. Flavor responses as measured by flavor intensity values of soybean oils containing from 0.0% to 7.8% linolenate (Ln). Oils were heated to 200 C and then cooled to 55 C before tasting.

0.0% linolenate. Figure 2 presents the results of these tests in FIVs for hot-oil, rancid and fishy responses. Hot oil is a response apparently associated with heated oil and popcorn fryers. The copper-reduced oil with 0.0% linolenate content was high in hot-oil responses and low in rancid and fishy. The unhydrogenated soybean oil was the reciprocal-high rancid and fishy responses and low hot-oil responses.

In another room odor test, a commercial nickel-reduced-winterized soybean oil containing antioxidants gave FIVs of 0.8 for hot oil, 0.4 for fishy and 0.3 for rancid. Copper-reduced soybean oil and unhydrogenated soybean oil without added antioxidants or silicone gave respective responses of hot oil, 0.8 and 0.3; rancid, 0.4 and >0.2; and fishy, 0.2 and 1.2.

#### Cottonseed Oil vs. Copper-reduced and Nickel-reduced Soybean Oils

After establishing that copper-reduced oils gave higher flavor scores and responses different from those of unhydrogenated soybean oil, a direct comparison with cottonseed salad oil and nickel-reduced-winterized soybean oil was undertaken. Cottonseed oil containing 0.1% of Tenox 6 and 5 ppm of methyl silicone, copper-reduced soybean oil (0.0% linolenate) with the same additives, and a sample of a commercial nickel-reduced-winterized soybean oil containing similar additives were chosen. We believe the samples were representative of better quality if not the best quality of each type of oil. Room odor and heat tests were run (Table VIII, Fig. 3 and 4).

These three oils showed comparatively good initial flavor and flavor stability. Buttery and bland responses were characteristic with FIVs of 0.9 to 1.0 and of 0.3 to 0.4, respectively. Heating to 150 C and then smelling and tasting the cooled oil resulted in no significant difference in scores and no major differences in odor and flavor responses. Buttery was still a major response and rancid contends for the highest FIVs. At 170 C, cottonseed oil was given a higher flavor score than either of the others and highly significantly above copper-reduced oil. Rancid responses were high for the cottonseed and copper-reduced oils at FIVs of 0.9 and 1.2, but painty responses appeared in the copper-reduced and nickel-reduced oils having FIVs of 0.4 and 0.3, respectively. Although heating to 170 C appeared to have a greater oxidative as well as flavor effect on the copper-reduced oil than on the nickel-reduced oil (see peroxide values), further heating to 200 C had a much greater effect on the nickel-reduced than on the copper-reduced or cottonseed oils.

In the heat test at 200 C, scores for cottonseed oil were significantly above the other two oils when tasted

TABLE VIII

Comparison of Cottonseed Oil With Copper-Reduced and Nickel-Hydrogenated-Winterized Soybean Oils: Heat and Room Odor Tests—Scores and Significance*

| Condition | Cottonseed | | Copper reduced | | Nickel reduced |
|---|---|---|---|---|---|
| Initial | | | | | |
| Odor | 8.0 | + | 7.5 | + | 7.8 |
| | | | (+) | | |
| Flavor | 8.1 | + | 7.4 | + | 7.4 |
| | | | (+) | | |
| Heat test, 170 C | | | | | |
| Odor | 6.1(1.7) | + | 5.1(1.6) | + | 5.6(0.8) |
| | | | (+) | | |
| Flavor | 6.8 | ** | 4.0 | + | 4.6 |
| | | | (+) | | |
| Heat test, 200 C | | | | | |
| Odor | 4.7(0.3) | + | 3.9(0.4) | | |
| | 5.6(0.8) | | | * | 4.4(1.0) |
| | | | 5.1(0.8) | ** | 3.9(0.8) |
| Flavor | 3.9 | ** | 3.1 | | |
| | 4.6 | | | * | 3.3 |
| | | | 3.9 | ** | 2.8 |
| Room odor | 6.4 | + | 5.9 | | |
| | 7.1 | | | * | 6.0 |
| | | | 6.1 | + | 5.7 |
| AOM, 8 hr, PV | 2.9 | | 2.0 | | 1.1 |

* See footnote a, Table II.

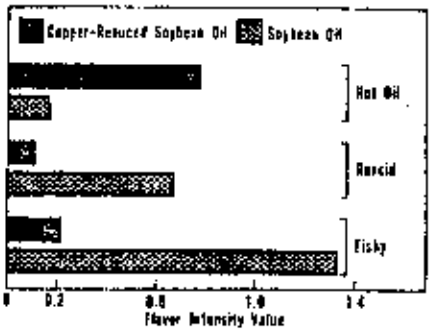


FIG. 2. Odor responses as measured by flavor intensity values of soybean oils containing 0.0% and 7.8% linolenate. Oils heated to 193 C in a fry pan and room odors described.

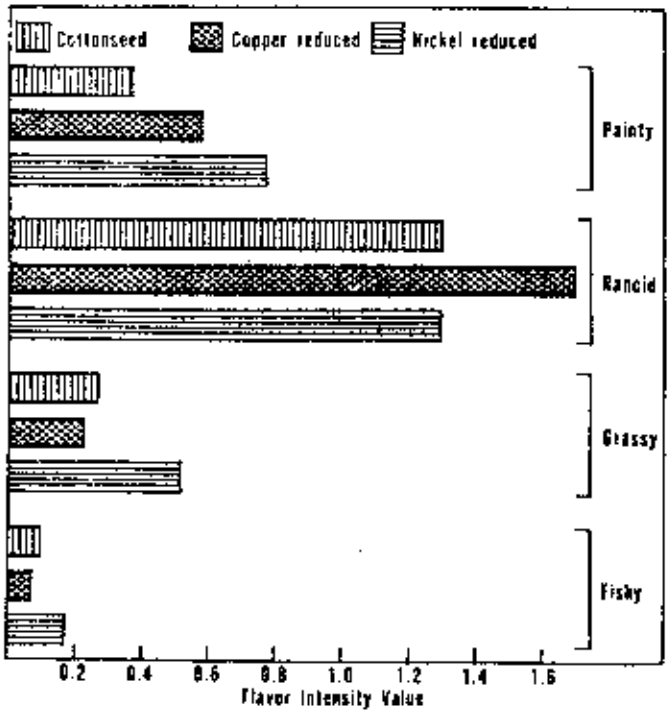


FIG. 3. Flavor responses as measured by flavor intensity values of cottonseed, copper-reduced soybean and nickel-reduced-winterized soybean salad oils. Oils heated to 200 C and cooled to 55 C for tasting.

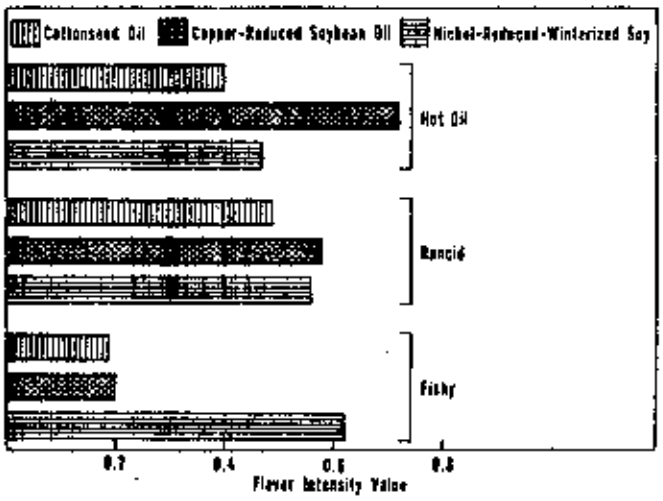


FIG. 4. Odor responses as measured by flavor intensity values of cottonseed, copper-reduced soybean and nickel-reduced-winterized soybean salad oils. Oils heated to 193 C in a fry pan and room odors noted.

but were not significantly higher than the copper-reduced oil when smelled. The copper-reduced oil and cottonseed oil were scored significantly or highly significantly above the nickel-reduced oil when either tasted or smelled.

In room odor tests, scores for cottonseed oil were significantly higher than the nickel-reduced oil but not significantly above the copper-reduced oil. The latter scored higher than the nickel-reduced oil but not significantly.

Figure 3 shows the important flavor responses given by panel members for the three oils subjected to the heat test at 200 C. The cottonseed oil was low in painty, fishy and grassy responses as measured by FIVs. Compared to cottonseed, copper-reduced oil was slightly higher in painty responses but slightly lower in both grassy and fishy. The nickel-reduced oil was the highest of the three in painty, grassy and fishy responses as measured by FIVs.

Figure 4 gives FIV results on room odor tests. These flavor values are the average of three different odor tests in which the three oils were tested one against the other, i.e., cottonseed vs. copper-reduced, cottonseed vs. nickel-reduced, and copper-reduced vs. nickel-reduced. In the copper-reduced oil, hot-oil and rancid responses overshadow the low fishy responses. Results were the same with cottonseed oil, but none of these responses were as high as with the copper-reduced oil. Although hot oil and rancid responses were high, fishy responses were slightly higher at FIVs of 0.6 for nickel-reduced oil.

In studies with flavor intensity values, we have generally considered that a value should be above 0.25 to have much significance or have at least one fourth of the panel report the response. The hot oil and rancid responses are above this level in all three oils. Only the nickel-reduced oil and unhydrogenated oil were above this value for fishy responses in the room odor tests.

Private reports from Europe indicate that some refiners believe that nickel-reduced-winterized soybean oil does give fishy odors in a room when the oil is heated to frying temperatures. Our results confirm their reports.

One can ask why copper-reduced oil of 0.0% linolenate is not scored equal to cottonseed. The answer is probably the presence of certain isolinoleates that remain in the oil. Analysis (18) shows that these copper-reduced oils contain as much as 5.2% to 6% of isolinoleate when the original oil contained 7.3 to 8.0 linolenate. Although lowering linolenate content from about 8% to 0.0% substantially improves soybean oil for use as a salad and cooking oil, the effect of isolinoleate remains.

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# Analytical Method for Determining Copper in Edible Shortenings and Oils

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## Abstract

A reliable, practical method was developed for the quantitative determination of sub-ppm levels of copper in edible shortenings and oils. Copper is quantitatively extracted with a boiling solution of 0.01% EDTA in 25% HCl. The extract was analyzed with 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (Bathocuproine), a highly specific and exceptionally sensitive reagent for the colorimetric determination of copper. An average recovery of 99.8% was obtained on 22 samples containing added oil soluble copper standards. A standard deviation of 0.002 ppm was established at the 0.05 ppm level during routine application of this method to a variety of shortenings and oils.


## Introduction

Quantitative analysis of copper in edible shortenings and oils is necessary, both in quality control and experimental development. Several methods are in the literature, but are either impractical or not reliable for everyday laboratory use. The method developed and described in this report is not only precise and accurate, but applicable to high volume analyses of copper in shortenings and oils with a minimum of time and equipment. While a single sample can be analyzed in 3 hr, six samples can easily be run by an analyst in one day.

List et al. (7) adopted a low-temperature dry asher, an apparatus in which oxygen plasma is generated in a radio frequency field under high vacuum, for ashing glyceride oils. The residues were analyzed by a colorimetric procedure using zinc dibenzyldithiocarbamate as a reagent. The relative error they reported of a single determination was ±13% and that of the mean of duplicate determinations was ±9%. They used pure copper nitrate as the reagent for preparing oils containing known levels (0.04–5.0 ppm) of copper. A sample commonly employed required 60–72 hr to be ashed in their system. For lower levels of copper, 15 g of sample were needed, so three to five residues had to be combined before assay. They showed levels of copper in unhydrogenated, commercially processed soybean oils varying from 0.03–0.10 ppm. Labuza and Karel (6) described a direct solvent extraction procedure for copper and iron in fatty acids and fatty methyl esters. The application of this method to shortenings and oils was not demonstrated. A recent symposium (8) included discussions on several analytical techniques for copper in edible oils. Atomic absorption spectrophotometry was shown to have a lower limit of 0.05–0.10 ppm when measuring copper concentration directly in fats. With several instrument modifications, limits of 0.01–0.05 ppm copper in fat have been reached.

Hogdahl and Melson (5) determined copper in two samples of hydrogenated fat by neutron activation analysis. A sample of fat was irradiated for 24 hr and the radioactive copper was extracted with 6 N HCl from a toluene solution of the irradiated fat. The gross gamma activity was measured with a NaI(Tl) well-type scintillation crystal. A colorimetric measurement (as copper dibenzyldithiocarbamate) was also made and the absolute amount of copper in the sample calculated. The sensitivity reported is about 1–3 ppm with a reproducibility of ±7% when 2 g of fat are used.

Two papers describe determination of copper in dairy products. Cranston and Thompson (2) reduced the pH of milk with perchloric acid until copper-protein complexes were broken. After filtering off fat and precipitated protein, the copper was then concentrated by an ion exchange procedure and determined polarographically. The amounts of copper recovered were erratic, since varying quantities of sugars, salts and casein markedly influenced the polarographic method. This condition was corrected by using an internal standard. Willis (10) found it necessary to extract butter and butteroil with nitric acid and to analyze the extract for copper by atomic absorption spectroscopy. Results at concentrations of <0.1 ppm were quite scattered and appear to be the lower limit of this method.

Van De Bogart and Beinert (9) published an excellent review and technique paper for copper analysis of biological materials by wet ashing, evaporation of excess acid, reduction, neutralization, color development and concentration of the colored complex by extraction with a small amount of organic solvent.

There is a wide choice of colorimetric reagents for copper. Some of the more common ones, their properties and drawbacks are shown in Table I. The reagent of choice is Bathocuproine (2,9-dimethyl-4,7-

TABLE I
Color Reagents for Copper

| Reagent | Solvent | λ Max, nm | Molar absorbance | Disadvantages |
|---|---|---|---|---|
| Diethyldithiocarbamate | Water | 440 | 8,000 | Unstable in acid |
| | Isoamyl alcohol | 440 | 12,700 | Light sensitive |
| | $CCl_4$ | 435 | 14,700 | |
| Dibenzyldithiocarbamate | $CCl_4$ | 435 | 16,200 | Interference from $Fe^{+3}$ and $Ni^{+}$ |
| Dithizone | $CCl_4$ | 508 | 24,600 | Not specific for copper alone |
| 2,2'-Biquinoline (Cuproine) | Isoamyl alcohol | 546 | 5,220 | Low sensitivity |
| 2,9-Dimethyl-1,2-phenanthroline (Neocuproine) | Isoamyl alcohol | 454 | 7,950 | Low sensitivity |
| 2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline (Bathocuproine) | n-hexanol | 479 | 14,160 | |

TABLE III
Copper Analysis by Extraction

| | Copper added to vegetable oil ppm | µg | Copper found[a] µg | Recovery, % |
|---|---|---|---|---|
| NBS Standard[b] | | | | |
| | 0.002 | 1.00 | 0.93 | 93.0 |
| | 0.010 | 1.00 | 1.00 | 100.0 |
| | 0.010 | 1.00 | 0.95 | 95.0 |
| | 0.010 | 1.00 | 1.05 | 105.0 |
| | 0.010 | 5.00 | 4.80 | 96.0 |
| | 0.010 | 5.00 | 4.35 | 87.0 |
| | 0.014 | 7.00 | 6.48 | 92.5 |
| | 0.040 | 4.00 | 4.20 | 105.0 |
| | 0.050 | 4.97 | 4.90 | 98.6 |
| | 0.050 | 4.97 | 4.90 | 98.6 |
| | 0.050 | 5.00 | 4.99 | 99.8 |
| | 0.050 | 5.00 | 5.10 | 102.0 |
| | 0.050 | 5.00 | 5.20 | 104.0 |
| | 0.100 | 9.95 | 9.60 | 96.5 |
| | 0.22 | 2.00 | 2.10 | 105.0 |
| | 0.22 | 2.00 | 1.85 | 92.5 |
| | 0.22 | 2.00 | 2.00 | 100.0 |
| Cupric stearate | | | | |
| | 0.01 | 1.00 | 1.20 | 120.0 |
| | 0.01 | 1.00 | 1.05 | 105.0 |
| | 0.02 | 2.00 | 1.95 | 97.5 |
| | 0.03 | 3.00 | 3.00 | 100.0 |
| | 0.07 | 7.00 | 7.10 | 101.4 |
| | | | Average | 99.8 |

[a] Corrected for initial copper levels in samples used. Copper metal, 400 to 475 µg, well over limits normally encountered can be determined by this procedure.
[b] NBS Standard, Bis(1-phenyl-1,3-butanediono) copper (II) (oil soluble material containing 16.5% Cu), from National Bureau of Standards.

After the flame went out, the residue was placed in a muffle furnace at the indicated temperature until the carbon was gone. The ash was then analyzed for copper by the colorimetric procedure described.

Extracting copper with 0.01% EDTA in 25% HCl was found to give a quantitative extraction of copper from shortenings and oils. The extracts were analyzed for copper by the colorimetric procedure described. These data are presented in Table III. An average recovery of 99.8% added oil soluble copper standards was obtained with a standard deviation of 0.002 ppm established at the 0.05 ppm level during routine application of this method to a variety of shortenings and oils.

Using 25% HCl alone without added EDTA resulted in low recovery (88–90%) of added copper. Two somewhat similar methods are available for determining trace metals in petroleum distillates. Barney II (1) extracted an oil with sulfuric acid and then with a mixture of hydrochloric acid, acetone and water. The extract was analyzed by spectrographic analysis. Sulfuric acid chars edible oil so it cannot be used. Hackett (4) extracted mineral oils with alcoholic hydrochloric acid and colorimetrically determined the copper by extraction of the 2,2-diquinol complex with chloroform. The method operates satisfactorily when more than 10 µg copper are present. EDTA was not needed for a quantitative extraction of copper in mineral oils but was needed for edible shortenings and oils.

Less than 90% of added copper was extracted from shortenings and oils with 25% HCl alone. The addition of EDTA leads to a quantitative extraction of copper from shortenings and oils. Postulating the role of EDTA in assuring successful extractions of copper from shortenings and oils is beyond the scope of this report. Little, if any, information is available concerning the role of EDTA in highly acid media and at the elevated temperature used in the heated system. One is that the EDTA becomes increasingly deprotonated, the extent of which is not known, making it a better chelating agent. The other is that a copper-EDTA complex would be less stable or more likely to dissociate at high temperatures.

The method as written is particularly applicable to the determination of copper in shortenings and oils at low levels (0.002–0.10 ppm), but could also be applied for analysis of copper in other media. It seems quite apparent that the isolation method could be extended to other metals (e.g., nickel in oil hydrogenation catalyst).

ACKNOWLEDGMENTS

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# SELECTIVE HYDROGENATION OF LINOLENATE GROUPS IN SOYA-BEAN OIL

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SINCE the Second World War much biochemical and biological research work has been done on the influence of the fatty acid composition of fats on cardiovascular abnormalities. The results of this work have convinced many investigators that higher levels of polyunsaturated fatty acids in fats are able to prevent or inhibit the appearance of these abnormalities. Consequently, in the past few years margarines with a high polyunsaturated fatty acid content have been marketed.

For the production of such margarines fatty oils with a high content of polyunsaturated fatty acids are needed. One of the most attractive oils for this purpose is soya-bean oil, since it contains about 8 per cent linolenic acid and 50 per cent linoleic acid. It is generally accepted, however, that taste and keeping quality of soya-bean oil are unsatisfactory so that large quantities cannot be incorporated in compositions of margarine. There is evidence that linolenic acid is—directly or indirectly—responsible for the poor flavour stability of soya-bean oil[1]. Consequently, it is generally believed that by preferential elimination of linolenic acid from soya-bean oil an oil of good taste and keeping quality and of high nutritive value can be obtained[2]. Linolenic acid can be eliminated completely by hardening soya-bean oil in the presence of a nickel catalyst. However, then most of the linoleic acid is converted into less unsaturated acids so that the biological value of the oil is lowered. Therefore, many investigations have been devoted to a more selective hydrogenation of linolenic acid present in soya-bean oil, in which process most of the natural linoleic acid—9 *cis*, 12 *cis*-octadecadienoic acid—should also be saved.

The hydrogenation of the linolenic acid ($L_N$) and linoleic acid ($L$) in soya-bean oil to octadecenoic acid ($O$) may be represented by the following equation (consecutive first-order reactions):

$$\underset{\text{(triene)}}{L_N} + H_2 \xrightarrow{k_N} \underset{\text{(diene)}}{L} + H_2 \xrightarrow{k_L} \underset{\text{(monoene)}}{O}$$

In this article the selectivity of a catalyst for the hydrogenation of the linolenic compounds ($S_{LN}$) is defined in the usual way, namely, as the ratio of the rate constants of the linolenic and linoleic acid hydrogenation[3] ($S_{LN} = k_N/k_L$). The value of $S_{LN}$ can be calculated from the fatty acid compositions of the original oil and of the oil obtained after a certain hardening time by applying the formula for the kinetics of consecutive first order reactions[4].

In the United States, where an important part of the world stock of soya-bean oil is produced, great interest is shown in the selective hydrogenation of linolenic acid in soya-bean oil[5-11]. Johnston *et al.*[5] investigated the selectivity characteristics of commercially available hydrogenation catalysts (nickel, palladium and platinum on different carriers). In hydrogenating a linoleate-linolenate equimixture they found $S_{LN}$-values between 1·33 for platinum on carbon at 25° C and 2·71 for nickel catalyst at 140° C. Willard and Martinez[6] tried to establish the hydrogenation conditions under which the natural linolenic acid (9 *cis*, 12 *cis*, 15 *cis*-octadecatrienoic acid) is converted into the natural linoleic acid (9 *cis*, 12 *cis*-octadecadienoic acid) virtually without formation of other unsaturated fatty acids. They investigated different types of catalysts, including reduced nickel oxide, reduced nickel oxide poisoned with cupric acetate, copper chromite and palladium lead catalysts. In all hydrogenations performed only minor amounts of 9 *cis*, 12 *cis*-octadecadienoic acid were formed. Starting from soya-bean oil, Rinsz and Weber[10-12] tried to hydrogenate linolenic acid present in this oil preferentially. The reaction conditions were varied within wide limits. They investigated nickel, platinum, palladium and rhodium catalysts. The maximal value of $S_{LN}$ found was 2·9 for a palladium on carbon catalyst.

In conclusion, the values for $S_{LN}$ reported up to now in the literature do not exceed about 3·0.

Our experiments, in which we compared the properties of nickel and copper catalysts in hydrogenating soya-bean oil, yielded the surprising result that linolenic acid is hydrogenated much more preferentially in the presence of copper catalysts than in the presence of nickel catalysts. The $S_{LN}$-values for a nickel catalyst found by us varied between 2·0 and 2·5, which is in good agreement with results already published. On the other hand, the $S_{LN}$-values for copper catalysts varied between 7 and 10, indicating the very high selectivity of these catalysts.

The experiments were carried out in the following way: Refined soya-bean oil was hydrogenated using 0·2 per cent copper (as copper on gulo catalyst) at 185° C under 5 atm. gauge pressure or with 0·1 per cent nickel (as nickel on gulo catalyst) at 185° C under 1 atm. gauge pressure. Samples were taken at different stages of the hydrogenation. After filtration and thorough removal of copper traces the samples were analysed for iodine value and fatty acid composition. For this purpose the hydrogenated samples were converted into the methyl esters and analysed by gas liquid chromatography. The amount of *trans* fatty acids was determined by means of infra-red spectroscopy. The selectivity ($S_{LN}$) was calculated according to the method already mentioned[4]. The analytical results are given in Table 1. The content of

Table 1. ANALYTICAL RESULTS OF SOYA-BEAN OIL HYDROGENATED WITH A NICKEL (NOS. 1–5) OR A COPPER (NOS. 6–9) CATALYST

| No. | Iodine value | Composition* (wt. %) C 18:3 | C 18:2 | C 18:1 | C 18:0 | C 16:0 | Trans fatty acid content (wt. %) | $S_{LN}$ |
|---|---|---|---|---|---|---|---|---|
| 0† | 132·3 | 8·0 | 52·0 | 24·0 | 4·0 | 11·5 | 0 | — |
| 1 | 120·0 | 4·5 | 41·5 | 35·0 | 5·5 | 12·0 | [illegible] | 2·5 |
| 2 | 111·4 | 4·0 | 37·5 | 39·5 | 5·0 | 12·0 | 12·0 | 2·0 |
| 3 | 103·2 | 3·0 | 32·0 | 45·0 | 6·0 | 12·0 | [illegible] | 2·0 |
| 4 | 94·5 | 2·0 | 28·0 | 52·5 | 6·0 | 11·0 | [illegible] | 2·0 |
| 5 | 94·7 | 0·7 | 23·5 | 58·0 | 7·0 | 11·0 | 23·3 | 2·5 |
| 6 | 127·0 | 5·5 | 53·0 | 26·0 | 4·0 | 11·5 | 3·0 | 8·0 |
| 7 | 120·9 | 2·7 | 51·5 | 31·5 | 3·5 | 11·0 | 7·0 | 8·5 |
| 8 | 115·8 | 1·5 | 49·5 | 35·0 | 4·0 | 11·0 | 9·0 | 7·0 |
| 9 | 112·1 | 0·7 | 41·5 | 40·0 | 4·0 | 11·0 | 13·0 | 8·0 |

* C 18:3 linolenic acid, C 18:2 linoleic acid, C 18:1 oleic acid, C 18:0 stearic acid, C 16:0 palmitic acid—as determined by gas liquid chromatography.

† Original oil.

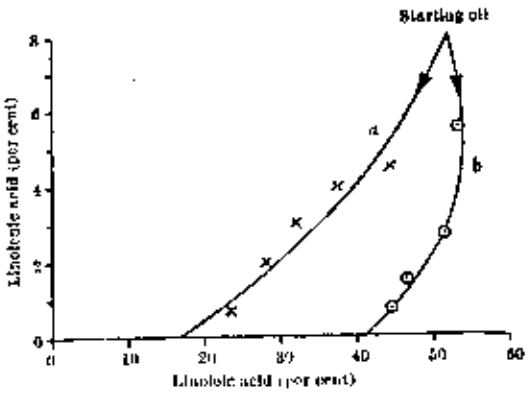


Fig. 1. Percentage of linolenic acid as a function of the percentage of linoleic acid in soya-bean oil hydrogenated with (a) nickel catalyst; (b) copper catalyst. Hydrogenation temperature 165° C; gauge pressure of $H_2$: (a) 1 atm; (b) 5 atm.

linolenic acid as a function of the percentage of linoleic acid is plotted in Fig. 1.

From the introduction it is clear that it is important to know how much of the linoleic acid as found by gas liquid chromatography is still in its natural 9 *cis*, 12 *cis* state. During hydrogenation *trans*-isomers and positional isomers of the natural linoleic acid are invariably formed. Therefore the isotope dilution method[10] was used for determining the content of natural linoleic acid in soya-bean oil hydrogenated with a copper catalyst. In Table 2 the fatty acid composition and the amount of natural linoleic acid for the hydrogenated products are given.

From the experimental results it appears that: (1) When soya-bean oil is hydrogenated with a copper catalyst linolenic acid is eliminated much more preferentially than when the hydrogenation is carried out with a nickel catalyst ($S_{Le}$ for copper catalyst is about 8, $S_{Le}$ for nickel catalyst about 2). (2) If linolenic acid present in soya-bean oil is removed completely by hydrogenation, about 41 per cent linoleic acid remains when a copper catalyst is used and only about 17 per cent when nickel is applied. (3) The amount of natural linoleic acid (9 *cis*, 12 *cis*) found in soya-bean oil hydrogenated with copper catalyst is about 75–85 per cent of the total amount of linoleic acid isomers, as determined by gas liquid chromatography. (4) As to whether a copper or a nickel catalyst is used does not influence the amount of *trans* fatty acids formed during hydrogenation of soya-bean oil under the reaction conditions applied in our experiments.

Table 2. FATTY ACID COMPOSITION AND AMOUNT OF NATURAL LINOLEIC ACID IN SOYA-BEAN OIL HYDROGENATED WITH A COPPER CATALYST

| Iodine value | Composition (wt. %) from gas liquid chromatography C 18 : 3 | C 18 : 2 | C 18 : 1 | C 18 : 0 | C 16 : 0 | Content of natural linoleic acid (wt. %) | % Natural linoleic acid of gas liquid chromatography linoleic acid |
|---|---|---|---|---|---|---|---|
| 138·5* | 7·5 | 51·0 | 26·5 | 3·5 | 11·5 | 50·7 | 100 |
| 111·3 | Trace | 44·5 | 39·5 | 4·0 | 11·0 | 34·4 | 77 |
| 112·8 | Trace | 40·0 | 38·5 | 4·0 | 11·0 | 30·9 | 67 |
| 114·2 | 0·5 | 40·5 | 37·0 | 4·0 | 10·5 | 28·0 | 63 |
| 119·2 | 2·0 | 50·0 | 32·5 | 3·5 | 11·5 | 36·4 | 73 |

* Original oil.


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# The 2-Thiobarbituric Acid Reagent for Determination of Oxidative Rancidity in Fish Oils

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## Abstract

A method has been devised for the quantitative determination of malonaldehyde in oxidized fish oils by means of the 2-thiobarbituric acid reagent in alcoholic solution. TBA numbers and peroxide values have been determined at intervals during hydroperoxidation of pilchard oil. The curves follow the same general pattern but the numerical relationship between them depends upon the temperature of oxidation.

In alcoholic solution, the reaction between thiobarbituric acid and malonaldehyde is carried out in the dark, because sunlight causes a decrease in optical density of the TBA-malonaldehyde complex at 532 mμ with the appearance of a second maximum at 452 mμ. The nature of the compounds responsible for absorption at these two wave-lengths is discussed.


## Introduction

In recent years the Kohn and Liversedge color reaction with 2-thiobarbituric acid (TBA) has been widely used to estimate oxidative rancidity in fatty materials (1,2,4,5,12,17,18,20,22).

The TBA reagent determines the amount of malonaldehyde arising from oxidative decomposition of unsaturated fatty acids with more than one double bond (9,14). Oxidative rancidity is frequently expressed in terms of TBA number or milligrams of malonaldehyde per kilogram of material, measured by comparing the optical density of the pink TBA-malonaldehyde reaction product with that of standards prepared from 1,1,3,3-tetraethoxypropane (TEP) which gives rise to malonaldehyde on acid hydrolysis.

The TBA-reaction has been successfully applied to the measurement of rancidity in dairy (2–4,12,20), fishery (1,13,22) and meat (17,18) products. Experimentally the procedure requires dispersion of the test material in a water medium containing the TBA reagent, but Schmidt (9) has pointed out that the existence of a two-phase system can lead to errors in the case of oils and fats. We have confirmed this in measuring the oxidative rancidity of pilchard oil by the method of Yu and Sinnhuber (22), which is eminently satisfactory for materials such as fish meal. Applied to pilchard oil, the method gave results which sometimes differed by as much as 100% on the same sample. Addition of a detergent to the two-phase system of water and oil as suggested by Biggs and Bryant (2) gave turbid solutions which could not be read in the spectrophotometer.

Removal of malonaldehyde from the oil by steam distillation was tried according to the method of Sidwell et al. (12) and Tarladgis et al. (17), but was found to be unreliable in application to pilchard oil. The method requires distillation of only a certain percentage of the malonaldehyde during an arbitrarily chosen time, but in the case of fish oils with high peroxide values the amount in the distillate was not proportionate to the total amount in the sample, and duplicate estimations sometimes differed by as much as 50%.

Since previous methods did not appear to be satisfactory, a modified experimental procedure was devised to determine the TBA numbers of oxidized fish oils with a standard deviation of 10%. It was found that difficulties arising from the existence of a two-phase system during condensation between malonaldehyde and TBA could be overcome by carrying out the reaction in a monophase system of ethyl alcohol. The standard calibration curve in aqueous-alcoholic medium was identical to that obtained by Sinnhuber and Yu (13) in aqueous medium alone.[1] TBA numbers for fish meal were identical in both aqueous and alcoholic solution, but in the case of fish oils only the alcoholic medium afforded reproducible results.

In aqueous-alcoholic solution there is a marked influence of light on the absorption spectrum of the TBA-malonaldehyde reaction product. The maximum at 532 mμ disappears with the simultaneous appearance of a peak at 452 mμ. This observation has been investigated, and a study has been made of the relationship between peroxide value and TBA number during oxidation of pilchard oils at room temperature and at 75C.

## Experimental

### Reagents and Apparatus

*TBA Reagent.* This was prepared in a black-painted vessel. Sodium citrate buffer (25 ml) according to Kohn and Liversedge (5) was added to a solution of thiobarbituric acid (0.5 g) in warm ethyl alcohol (50 ml). Crystals of sodium citrate deposited on standing but this did not affect the quality of the reagent which was used within 4 hr of preparation.

*Ethyl Alcohol and Methyl Alcohol.* These solvents were purified according to the method of Stout and Schuette (16).

*Alcoholic-HCl (0.6 N).* Dry hydrogen chloride gas was passed through ethyl alcohol until the required weight increase was achieved.

*1,1,3,3-Tetraethoxypropane (TEP).* The TEP was supplied by K & K Laboratories, Jamaica, N. Y. and was used as an approximately $2 \times 10^{-4}$ molar solution in 40% ethyl alcohol.

*Epihydrin Aldehyde and Glyceraldehyde.* These chemicals were supplied by Stuart Patton of the Pennsylvania State University.

All other chemicals were analytical reagent grade.

[1] The calibration curve given by Sinnhuber and Yu (18) refers to "molar concentration TEP ($\times 10^{6}$)" and not "molar concentration TEP ($\times 10^{5}$)" as printed.

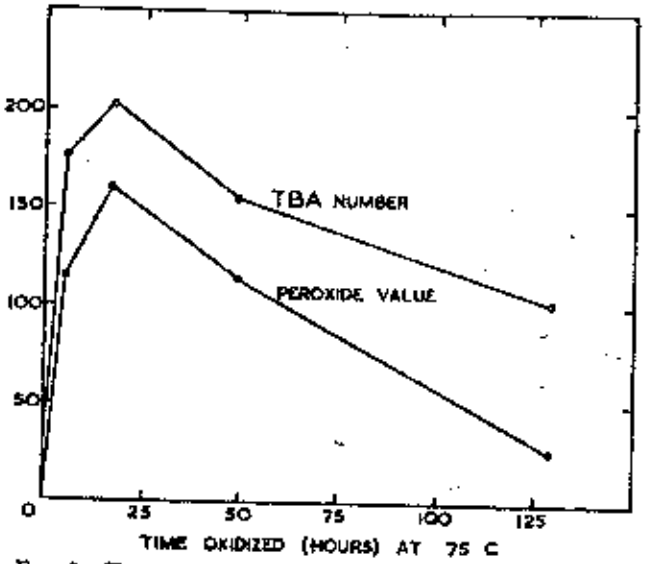



FIG. 2. Change in TBA number and peroxide value during oxidation of pilchard oil at 75C.

tion between alcoholic-TBA and malonaldehyde was carried out in the dark, the product had a pink color with an absorption maximum at 532 mμ. However, if the reaction was carried out in daylight, an orange-brown product was obtained with two absorption maxima, one at 532 mμ and the other at 452 mμ. The orange-brown solution became pink after storage in the dark for 70 hr and the 452 mμ maximum disappeared with a slight increase in absorption at 532 mμ. These effects are illustrated in Figure 3.

B) TBA undergoes a condensation to yield a colored compound without addition of malonaldehyde. Thus solution of 0.04% TBA in a mixture of 4 parts 0.6 N HCl and 1 part 0.6 N alcoholic-HCl, turned yellow in sunlight with development of an absorption peak at 452 mμ. This maximum disappeared after 48 hr storage in the dark and a slight increase in absorption was observed at 532 mμ (Fig. 4).

The same phenomenon was observed when methyl

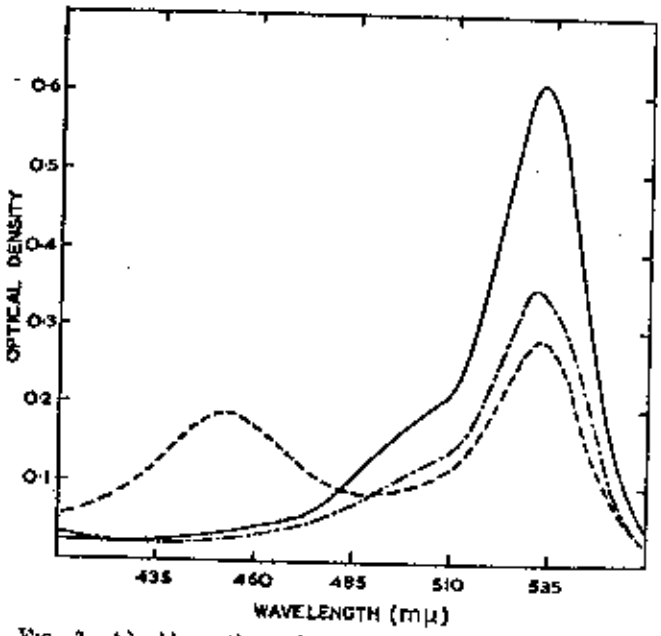



FIG. 3. A) Absorption of the derivative obtained by reaction between alcoholic-TBA and malonaldehyde in the dark (———). B) Absorption of the derivative obtained by reaction between alcoholic-TBA and malonaldehyde in sunlight (— — —). Absorption after storage of product B for 70 hr in the dark (—·—·—). The maximum at 452 mμ disappears with an increase in absorption at 532 mμ.

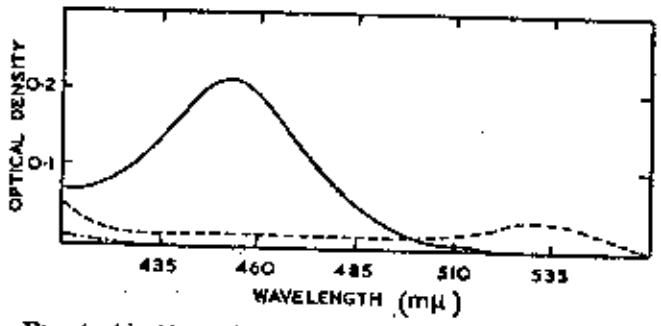



FIG. 4. A) Absorption of 0.04% TBA in a solution of 1 part 0.6 N alcoholic-HCl and 4 parts 0.6 N HCl (—·—·—). B) Absorption of solution A after exposure to sunlight (———). C) Absorption of solution B after 48 hr storage in the dark (— — —).

alcohol was used in place of ethyl alcohol, but no color was produced when TBA in aqueous solution was exposed to sunlight.

C) *Reaction of TBA with Epihydrin Aldehyde and Glyceraldehyde.* Patton (8) has shown that epihydrin aldehyde and glyceraldehyde react with TBA in aqueous solution to give a yellow product which has an absorption maximum at 456 mμ.

This reaction was compared with the development of an absorption peak at 452 mμ on exposure of an alcoholic solution of TBA to sunlight (see B above).

Epihydrin aldehyde and glyceraldehyde were condensed in the dark with TBA in aqueous-alcoholic solution to give a yellow compound with an absorption maximum at 455 mμ (Fig. 5). On continued storage in the dark the absorption maximum shifted to 485 mμ (Fig. 5).

D) *Reaction of TBA with Formic Acid.* Schmidt (10) has shown that formic acid reacts with TBA in aqueous solution to give a yellow derivative with an absorption maximum at 452 mμ.

A compound with the same absorption maximum at 452 mμ is formed by reacting TBA and formic acid in a mixture of ethyl alcohol and water (1:4) away from the influence of light (Fig. 6). A decrease in absorption at 452 mμ with a corresponding increase of

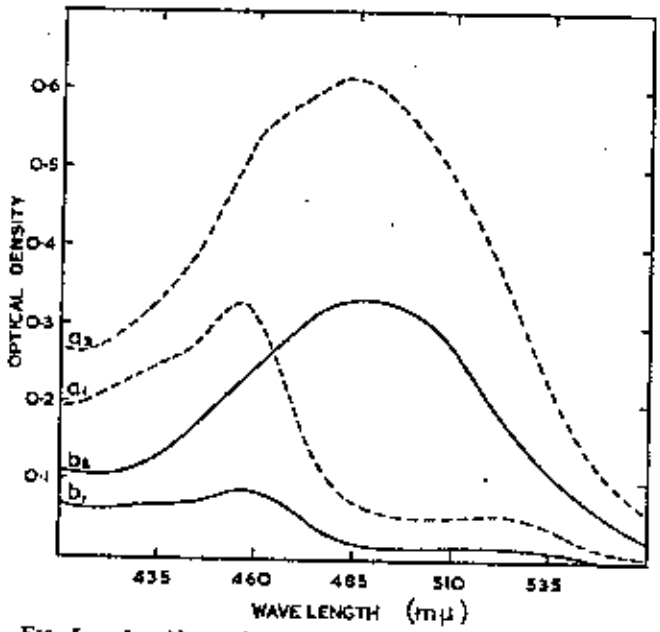



FIG. 5. a.1. Absorption of the derivative obtained by reaction between TBA and glyceraldehyde. a.2. Absorption of the TBA-glyceraldhyde derivative after 48 hr storage in the dark. b.1. Absorption of the derivative obtained by reaction between TBA and epihydrin aldehyde. b.2. Absorption of the TBA-epihydrin aldhyde derivative after 48 hr storage in the dark.

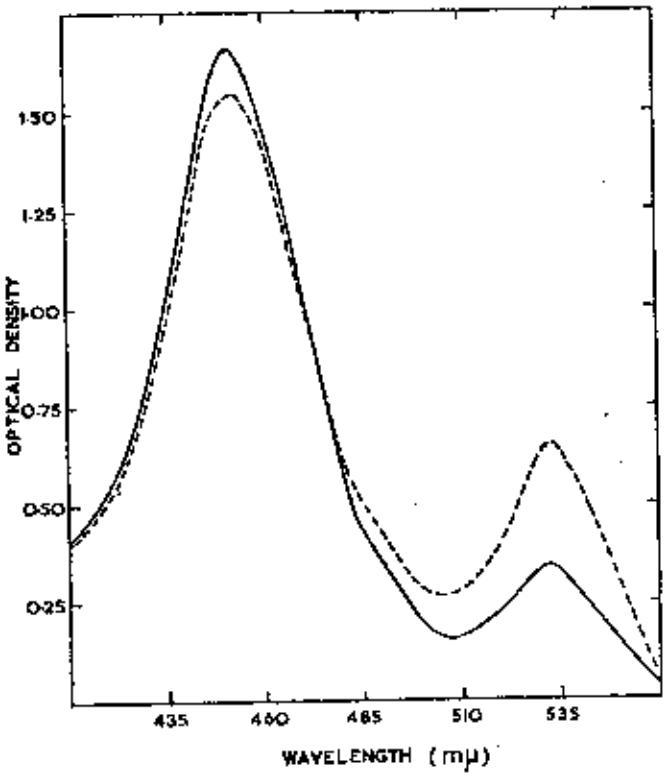



FIG. 6. A) Absorption of the TBA-formic acid derivative after 96 hr storage in the dark (———). B) Absorption of the TBA-formic acid derivative after 144 hr storage in the dark (— — —).

absorption at 532 mμ is shown after 96 hr and 144 hr storage in the dark (Fig. 6).

## Discussion

The peroxide value of oils is a useful criterion of decomposition during the early stages of oxidation, but is of lesser value once the peak of the oxidation curve has been passed. The TBA number has been shown to be a useful quality index for assessment of rancidity in the early stages of oxidation (9), but no definite information could be found on the relationship between peroxide value and TBA number during continued hydroperoxidation of oils.

Several unsuccessful attempts were made to determine the TBA number of oxidized oils according to published methods. For example the procedure of Yu and Sinnhuber (22) and the distillation methods of Sidwell et al. (12) and Tarladgis et al. (17), all gave erratic results when applied to fish oil.

A new method was therefore devised to overcome errors inherent in the existence of a two-phase system between oil and water during reaction between malonaldehyde and TBA reagent. By carrying out the determination in a monophase system of ethyl alcohol it was found that the recovery of malonaldehyde added to sunflower oil was approximately 100% and that the standard deviation was less than 10% of the arithmetic mean for a series of 7 to 9 determinations on fish oils with different peroxide values (Table II).

TBA numbers determined in alcohol solution were compared with peroxide values at various intervals during oxidation of pilchard oil at 20–25C and at 75C. The curves followed the same general pattern at both temperatures (Figs. 1 and 2) but the maximum TBA number and peroxide value were less at 75C than at 20–25C. This would indicate that not only hydroperoxides, but also malonaldehyde or its precursors, are more rapidly decomposed at the higher temperature. Were it not for the fact that the malonaldehyde formed by oxidative degradation is itself labile, the TBA number might be expected to show a continued increase beyond the peak of the peroxide value curve. Since this is not the case [cf. Schmidt (9)], it would seem that the TBA number cannot be regarded as a more useful criterion of rancidity than peroxide value after the initial stages of oxidation.

Comparison of Figures 1 and 2 shows that the relationship between TBA number and peroxide value depends upon the temperature of oxidation. For example, an oil having a peroxide value of 160 may have a TBA number of 480 or 200 depending upon whether it was oxidized at 20–25C or at 75C.

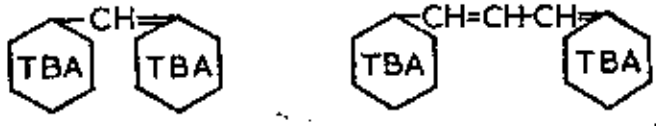
The measurement of TBA number in alcoholic medium introduces a complication which is not encountered in aqueous medium. The reaction must be carried out in the dark because daylight reduces the intensity of the TBA-malonaldehyde peak at 532 mμ and causes the appearance of a second maximum at 452 mμ (Fig. 3).

A product with an absorption maximum at 452 mμ can be obtained by the action of sunlight on an alcoholic solution of TBA alone. The yellow derivative absorbing at 452 mμ is converted to a pink compound with a peak at 532 mμ during storage in the dark (Fig. 4).

A yellow compound formed by reaction between TBA and epihydrin aldehyde or glyceraldehyde shows an absorption maximum at 455 mμ but is converted to a compound with a peak at 485 mμ after 48 hr storage in the dark (Fig. 5). The compound cannot therefore be identical with the reaction product of sunlight on TBA in alcoholic solution which is converted to a substance with a maximum at 532 mμ after storage in the dark (Fig. 4).

Formic acid, on the other hand, reacts with TBA to give a product which has an absorption maximum at 452 mμ and is converted to a substance with a peak at 532 mμ after storage in the dark (Fig. 6). Reaction between TBA and formic acid therefore yields a compound which is similar to, if not identical with, that formed by the action of sunlight on TBA in alcoholic solution.

Schmidt (10) has proposed structure I ($\lambda_{max}$ 452 mμ) for the reaction product of TBA with formic acid, and structure II ($\lambda_{max}$ 532 mμ) for the condensation product of TBA with malonaldehyde.




I ($\lambda_{MAX}$ 452 mμ) II ($\lambda_{MAX}$ 532 mμ)

Thus sunlight irradiation of an alcoholic solution of TBA (Figs. 4 and 6) probably gives rise to compound I. The reaction could possibly take place through a free radical mechanism.

Compound II may be identical with the compound formed when an alcoholic solution of TBA is exposed to sunlight and subsequently stored in the dark (Fig 4). If this is the case, it is possible that compound II is formed from compound I in aqueous alcoholic solution when stored away from light (Fig. 4).

Methine dyes such as compounds I and II apparently undergo changes in structure when in solution

This is evidenced by the TBA reaction products with epihydrin aldehyde or glyceraldehyde (Fig. 5) and with formic acid (Fig. 6), although the phenomenon has also been observed by Landucci et al. (6,7) in the case of various aldehydes present in gelatin. The mechanism of these reactions does not appear to be understood and requires further investigation.

Shepherd (11), Sinnhuber et al. (14) and Schmidt (9) have shown that certain pyrimidines are capable of forming the same pigment as malonaldehyde on reaction with TBA. The mechanism is thought to involve hydrolysis of the pyrimidine ring to produce an oxyacrolein intermediate (9) which is tautomeric with malonaldehyde and therefore undergoes condensation to produce compound II. Although TBA itself has a pyrimidine structure, it is substituted in the 4 and 6 positions and would therefore be unlikely to give rise to malonaldehyde on hydrolysis (9). No color is formed on exposure of an aqueous acid solution of TBA to sunlight, but if hydrolysis were to occur, the expected product would be malonic acid which would not give the same compound as malonaldehyde in any reaction with TBA.

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# Measurement of the Consistency of Plastic Vegetable Fats

## A Standard Micropenetration Technique

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### Introduction

It is noteworthy that there is no standard and generally accepted method for evaluating the highly important property of consistency in plastic fats. It is still customary to describe the hardness or softness of fats in terms of their melting points, setting points, titers, etc. These characteristics are more or less related to consistency, but singly or combined, they fail by a good deal to give an adequate picture of the body of a fat over the entire range of temperatures at which it is to be stored or used.

Among shortening manufacturers it has become rather general practice to employ some mechanical device for testing the body of commercial products after the latter have been solidified and filled into packages. However, as recently pointed out by Rich (4), there is nothing approaching standardization of methods or instruments among the different manufacturers. The instruments in use, moreover, are adapted only to the examination of relatively large samples of material.

Experience has shown that the initial solidification of a fat must be carried out very rapidly, so that the solidified crystals are small, if the later consistency of the fat is to be definite and predictable at any given temperature. Rapid chilling is attained as a matter of course in the chill rolls or votators used in solidifying commercial products. However, it is extremely difficult to solidify a large sample of fat quickly in the laboratory. Consequently, the above-mentioned devices are unsuitable for the examination of experimental or partly finished products.

The consistency of a plastic product must necessarily be measured according to an empirical procedure and expressed in arbitrary units. Hence any method is suitable for its determination if it gives reproducible results over a reasonable range of values. From the standpoint of simplicity and ease of manipulation of the instrument, the penetration technique of consistency measurement has much to recommend it. This is particularly true if the penetrating device is actuated simply by the force of gravity, as it is, for example, in the Goddard-Hewes (2) penetrometer, which employs a falling needle.

Freyer (1) has devised a micro-form of the falling needle-type of penetrometer. The sample required by the Freyer instrument is very small, and is solidified with sufficient rapidity by being simply poured into a small hole drilled in a chilled copper block.

The apparatus and technique described by Freyer were admittedly only suggestive, and were not standardized in all particulars. However, with proper standardization they are capable of yielding results of rather remarkable precision. One of the authors has, over a period of years, had a considerable amount of experience in the use of a modified Freyer micropenetrometer to check the consistency of hydrogenated cottonseed oil products. The results of this experience fully justify the statement that, in the case of these products at least, the micropenetration curve of a fat (penetrations plotted against temperature) may be considered as definite and characteristic as its melting point, solidification point, or titer. Furthermore, there is a close correlation between the micropenetrations and the actual consistency of the finished product, as determined by macropenetrations or other methods.

Unfortunately, the micropenetrometers previously used, by the authors and others, have not been of a design that can be readily reproduced, so that it has been difficult or impossible to obtain comparable results with the instruments in use in different laboratories. Particular difficulty is experienced in attempting to standardize the penetrating needle, inasmuch as ordinary darning needles are customarily used, and these vary considerably in length, weight and taper, even amongst needles of the same nominal size.

The work reported here had the following objects: (a) The design of an improved micropenetration apparatus, capable of being reproduced in all essential details. (b) Investigation of the various factors influencing the consistency of micro-solidified fat samples, in order to determine standard test conditions applicable to different kinds of fat. (c) The recording of micropenetrometer data on a variety of fat products, including commercial shortenings and margarines.

### Theory of Plasticity in Fats

Plastic fats, such as lard, shortenings, butterfat and margarine oils, are composed of a solid phase of fat crystals intimately mixed with a liquid phase of fluid oil. Body is given to the fat by the tendency of the solid particles to interlock. Technically, the fat is plastic so long as this interlocking effect is sufficient to enable it to resist completely small deforming stresses. With the application of increased stresses, a point is reached where the structure of the product abruptly yields, and plastic flow occurs. The consistency of a fat—its relative hardness or softness—is simply a matter of the stress or load required to cause plastic flow.

The internal strength of the material, or its capacity for resisting such stress, is determined by the number of points of contact between the crystal particles. If the crystals are very small, there will be more points of contact and the fat will be firmer than if the crystals are relatively large. With crystals of a given size, the consistency will be determined

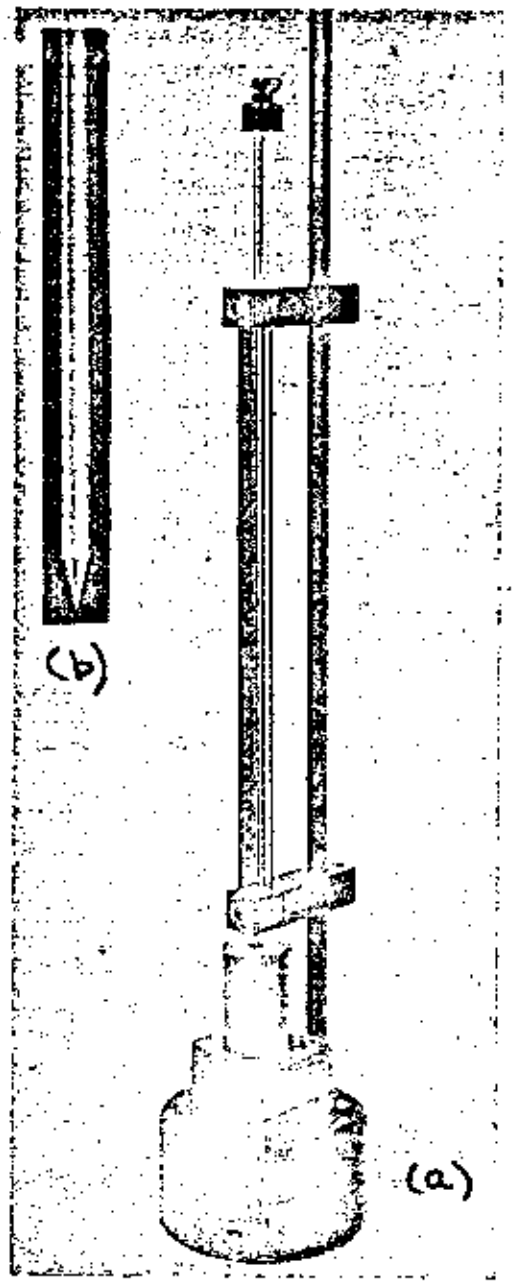


Fig. 1. Photograph of (a) micropenetrometer, and (b) point of micropenetrometer needle (enlarged).

by the number of crystals, or in other words, the relative proportions of fat existing in the solid and liquid states. These proportions are in turn determined primarily, but not entirely, by the temperature of the fat.

There are two factors which prevent the proportion of solids in a fat from being purely a function of temperature. One of these is the phenomenon of polymorphism, or the ability of fat crystals to exist in unstable modifications of low melting point. In order to insure that the crystals of a fat are small and uniform, it is necessary to form the crystals rapidly, by chilling the fat strongly. Unfortunately, rapid chilling also favors the formation of unstable crystal forms. The unstable forms are transformed irreversibly to higher melting forms of greater stability at temperatures below their melting point, but the transformation requires an appreciable time. The other factor which may lead to abnormal proportions

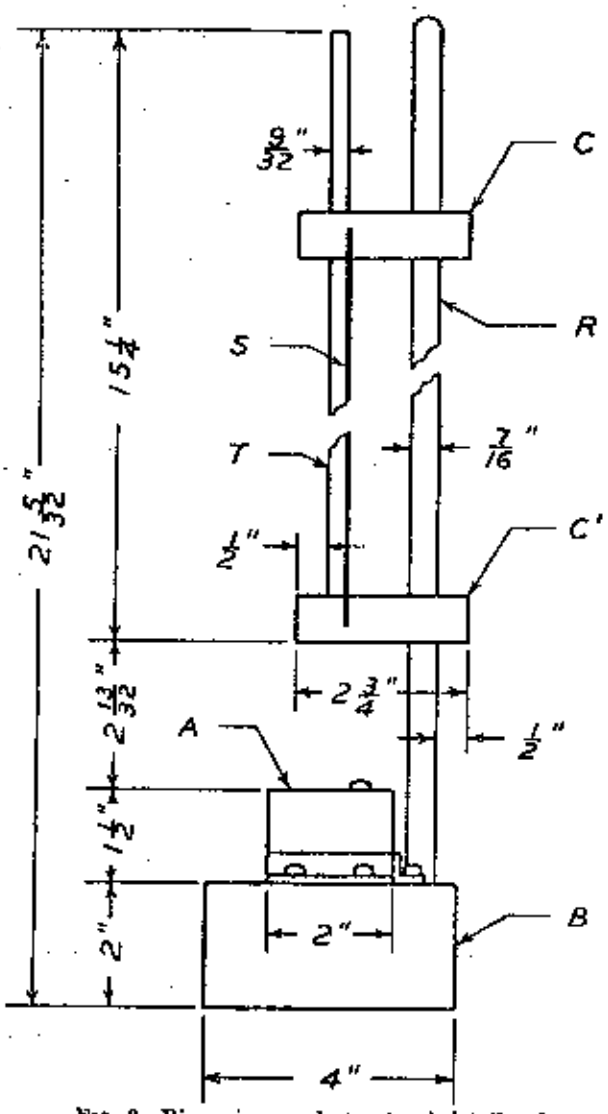


Fig. 2. Dimensions and structural details of micropenetrometer.

of solids and liquids is the pronounced tendency of fats to supercool in passing from a higher to a lower temperature. A fat which is in a supercooled condition contains, of course, an abnormally low proportion of the solid phase.

All of the foregoing considerations must be taken into account in the formulation of a standard micropenetration technique.

### The Micropenetrometer

The design and construction of the micropenetrometer are shown in Figures 1 and 2.

The base, *B*, of the instrument is turned from a 4-inch billet of machinable cast iron, and the removable sample block support *A* is cut from a 2-inch square bar of mild steel. The top and bottom faces of both the base and sample block support must, of course, be exactly parallel. Two fillister-head machine screws are set in the top face of the sample block support to fix the position of the sample blocks with respect to the support. The inner sides of the machine screw heads are flattened so that they will bear solidly against the sides of the sample blocks when the latter are placed in the instrument. Three guides fashioned from ½x½x⅛ inch angle iron are fastened to the base with heavy machine screws to

maintain the moveable sample block support in a fixed position with respect to the other members of the instrument. The guides, which are slotted, are adjustable on the base. When the instrument is first assembled they are so adjusted that when the needle is dropped into the sample under test, its point will strike the surface of the latter 1.0 to 1.5 mm. from its outer edge.

The penetrometer needle is directed in its fall by a glass capillary tube *T* of 1.5-1.75 mm. bore. Care must be taken to select a straight piece of tubing and to mount it exactly perpendicular to the top face of the base *B*. The tube and the steel millimeter scale *S*, immediately behind it, are held in position by a single set screw in each of the front end faces of the clamps, *C* and *C'*. The clamps are made from ¾-inch square bar stock. Larger set screws in the other end faces fix the supports on a cold rolled steel rod *R*. The scale *S* is graduated in 0.5-mm. divisions. A needle release is made from a binder clip, a machine screw and two nuts. The distance that the needle drops measures 330 mm. from the point of the needle to the surface of the sample.

The cylindrical blocks in which the samples are solidified are turned from 1½-inch billets of bearing bronze. The finished blocks are 1⅜ inches in diameter, 2 inches high, and have a vertical hole in the top surface 5/16 inch in diameter and 1⅝ inches deep. The outside dimensions of all blocks must be identical, in order that the solidified samples will be in the proper position when the blocks are placed in the penetrometer. The holes in the blocks must be accurately bored and centered. Each block is stamped with an identifying number.

The penetrometer needles were made from number 18½ music wire. If a length of this wire is stretched, and then heated and quenched in the stretched condition, perfectly straight sections can be obtained. This treatment does not affect the diameter of the wire appreciably; the original diameter is 0.042 inch, and the diameter of the finished needles will be about 0.0419 inch. The ends of the straightened sections are ground to a 30° conical point with the aid of a small jig and power grinder. The points are then specially tempered and given a final grinding with a fine oil stone. The needles are then cut to the proper length and ground to a square surface on the unsharpened end.

The finished needles weighed 0.5000 gram each, were 1.06 mm. (0.0419 inch) in diameter, and were 73.6 mm. long. The points were sufficiently hard that when the needles were dropped through the instrument into a bronze surface two or three times there was no evidence of blunting. The three needles prepared were found to give identical penetrations.

The temperature of the tempering bath used in connection with the instrument must be carefully controlled if precise and reproducible results are to be obtained. The bath used in the present work consisted of a 12-quart insulated earthen crock, stirred by an electric motor. It was heated by means of a 500-watt knife-type heater controlled through a vacuum tube relay by a mercury filled thermoregulator. The temperature, which was read with a low titer thermometer, could be maintained within 0.05° C. of that desired.

Before the needle is dropped into the tempered sample, the surface of the latter is scraped off even with the sample block by means of a spatula or other sharp straight edge. The scraping edge is inclined so that it makes an angle of about 45° with the block surface on the side *toward* which it is traveling. Very hard samples must be cut away carefully, in small portions, in order to avoid cracking and pitting the cut surface. The heat capacity of the blocks is sufficient to maintain the temperature of the sample reasonably well while the determination is being made, but if the room temperature is markedly different from that of the sample, the determination should be made promptly. If the fat is relatively firm, several successive penetration measurements can easily be made on the sample contained in a single sample block. At higher penetrations the entrance of the needle is inclined to disturb the entire sample, and succeeding measurements will be erroneously high. It is important for the needle to strike 1.0 to 1.5 mm. from the edge of the sample hole, as the hardness of the sample varies with its distance from the chilling surface.

Penetrations are recorded in terms of one-tenth millimeter units. At penetrations of 100 or below, duplicate measurements will usually agree within about 2 units. At penetrations approaching the extreme range of the instrument (about 375) the difference between duplicate determinations may be as much as 5 units.

### Solidification and Tempering of the Samples

In all cases the fat to be examined was first melted by heating to about 70° C. The bronze blocks were immersed in a tray of cracked ice and water to almost their entire depth until they were thoroughly chilled. When the melted sample had cooled to about 50° C., it was poured into the chilled blocks. Care was taken to have a drop of fat projecting above the block surface, to provide for shrinkage in the fat on cooling. It was found that the temperature of the fat when poured into the blocks was not critical, and could be varied within reasonable limits without appreciable effect on the micropenetrations.

Since it is convenient to allow the samples to chill overnight, most of the tests were carried out with a chilling time of approximately 16 hours in ice water. A chilling period of this duration is apparently sufficient to produce a condition of equilibrium in the fat. In some of the experiments, in order to investigate the feasibility of accelerating the test, the chilling period was considerably shortened.

At the end of the chilling period the solidified samples were immediately transferred to a thermostatically controlled water bath for tempering to the proper temperatures for the actual penetration measurements. The usual procedure was to place the entire series of blocks in the bath at the lowest temperature employed (10° C.), and then increase the temperature of the bath step-wise, removing a block for measurement after a proper tempering period at each successively increasing temperature. In this way each sample examined was not only tempered at the temperature of examination, but was also subjected to corresponding tempering periods at each of the previous lower temperature levels. In some of the experiments, however, the samples were transferred directly from the ice bath to a water bath operating at the temperature at which the penetration was to be determined.

### Measurements on All-Hydrogenated Products

A popular brand of all-hydrogenated vegetable oil shortening was tested under the different conditions ed in Table 1. After a chilling period of 16 hours, was found that virtually identical penetrations

TABLE 1

Micropenetrations of an All-Hydrogenated Type Vegetable Oil Shortening Chilled and Tempered Under Different Conditions.

| Test No. | AH-1 | AH-2 | AH-3 | AH-4 | AH-5 | AH-6 |
|---|---|---|---|---|---|---|
| Chilling time, hrs. | 16 | 16 | 16 | 1 | 16 | 16 |
| Tempering time, min. at each temp. | 15 | 60 | 60 | 30 | 30 | 30* |
| Micropenetrations, mm./10: | | | | | | |
| at 10.0°C. | 15 | 15 | 16 | 17 | 15 | 18 |
| 12.5°C. | .... | .... | .... | .... | 19 | .... |
| 15.0°C. | 22 | 25 | 25 | 23 | 24 | 28 |
| 17.5°C. | .... | .... | .... | .... | 28 | .... |
| 20.0°C. | 39 | 42 | 42 | 39 | 41 | 45 |
| 22.5°C. | .... | .... | .... | .... | 62 | .... |
| 25.0°C. | 86 | 92 | 94 | 86 | 97 | 96 |
| 27.5°C. | .... | .... | .... | .... | 133 | .... |
| 30.0°C. | 185 | 181 | 183 | 171 | 178 | 191 |
| 32.5°C. | .... | .... | .... | .... | 243 | .... |
| 35.0°C. | 329 | 324 | 319 | 305 | 317 | 333 |

* Transferred directly from ice to water bath at temperature at which penetration was determined.

were obtained with tempering times of 30 and 60 minutes. The penetrations were inclined to be slightly lower at the lower temperatures with a tempering time of 15 minutes, and were also slightly lower at all temperatures when the chilling period was reduced to 1 hour. Slightly higher penetrations were obtained when the samples were transferred directly from the ice bath to water at the temperature at which they were to be examined, rather than being brought to this temperature gradually. Reducing the temperature intervals from 5.0° to 2.5° C. had no appreciable effect on the consistency of the samples examined with a chilling time of 16 hours and a tempering time of 30 minutes at each temperature.

It is apparent that in fats of the all-hydrogenated type variations in consistency due to polymorphism are not much in evidence, and that the procedure followed in tempering the samples is not, as a consequence, extremely critical.

### Measurements on Blended Type Products

The fat selected as a representative blended product consisted of a mixture of 11 percent hydrogenated cottonseed oil with an iodine value of 12.5 and 89 percent unhydrogenated cottonseed oil with an iodine value of 108.6. Detailed results of the examination of this fat under different conditions are recorded in Table 2. With this fat, the micropenetrations were to a large extent dependent upon the conditions under

TABLE 2

Micropenetrations of Blended Type Vegetable Oil Shortening Chilled and Tempered Under Different Conditions.

| Test No. | BS-1 | BS-2 | BS-3 | BS-4 | BS-5 | BS-6 |
|---|---|---|---|---|---|---|
| Chilling time, hrs. | 16 | 16 | 16 | 16 | 16 | 1 |
| Tempering time, min. at each temp. | 30 | 60 | 120 | 240 | 30* | 30 |
| Micropenetrations, mm./10: | | | | | | |
| at 10°C. | 30 | 30 | 30 | 31 | 30 | 35 |
| 15°C. | 37 | 42 | 42 | 43 | 39 | 36 |
| 20°C. | 61 | 70 | 76 | 85 | 62 | 50 |
| 25°C. | 113 | 118 | 125 | 131 | 108 | 91 |
| 30°C. | 176 | 175 | 184 | 179 | 165 | 145 |
| 35°C. | 253 | 236 | 222 | 203 | 275 | 232 |
| 40°C. | 300 | 272 | 243 | 218 | Too soft | 313 |

* Transferred directly from ice to water bath at temperature at which penetration was determined.

which the fat was tempered. After an overnight chilling period, the samples became progressively softer at the lower temperatures and firmer at the higher temperatures as the tempering time was increased from 30 minutes to 60, 120, and finally to 240 minutes.

Whereas a tempering time of 30 minutes sufficed to bring the consistency of the hydrogenated fat to a constant value, it is apparent that a comparable consistency cannot be attained in the case of the blended fat with any practicable tempering time. The conditions chosen for blended products must, therefore, be arbitrarily selected. Any conditions adopted for chilling and tempering hydrogenated fats would appear to be suitable also for blended products.

The progressive stiffening of the blended fat at the higher temperatures with increased tempering time is quite evidently due to polymorphic transformations. Some manifestation of polymorphism in such fats is to be expected from the relatively simple nature of their solid glycerides, and is in line with common experience in the solidification of blended shortenings in commercial equipment.

Obviously, any set of standard solidifying and tempering conditions must be rather carefully adhered to in examining blended fats, if consistent results are to be obtained.

### Standard Conditions for Chilling and Tempering the Samples

The foregoing results suggest that suitable conditions for conducting a standard micropenetration test would include an overnight chilling period of approximately 16 hours, followed by tempering periods of 30 minutes each at successively increasing temperatures at intervals of 5° C. All of the micropenetration determinations reported in subsequent portions of this article were made under these conditions.

### Effect of Hydrogenation on the Consistency of Vegetable Oils

An indication of the rate at which the consistency of vegetable oils change with hydrogenation is furnished by the data represented in Figures 3, 4, 5, and 6. In Figure 3 are plotted the micropenetrations of a laboratory hydrogenated peanut oil at varying iodine values. Similar data for hydrogenated cottonseed and soybean oils are shown in Figures 4 and 5, respectively.

In Figure 6 the interpolated micropenetrations of the different hydrogenated oils at a temperature of 22.5° C. are plotted against their iodine values. It will be noted that the maximum deviation of micropenetration values from the smooth curves is about 2 units, which is approximately the limit of accuracy of the method. This corresponds to a reduction in iodine value of about 0.5 unit. The micropenetration method may thus be said to be sensitive to an alteration in the consistency of the fat equivalent to less than 1 unit change in iodine value.

The three hydrogenated oils are comparable with one another, inasmuch as they were hydrogenated under identical conditions and with the same catalyst. The micropenetration data illustrate the general principle that the hardness of a hydrogenated oil at a given iodine value is in direct proportion to the iodine value of the original oil. The original iodine values of

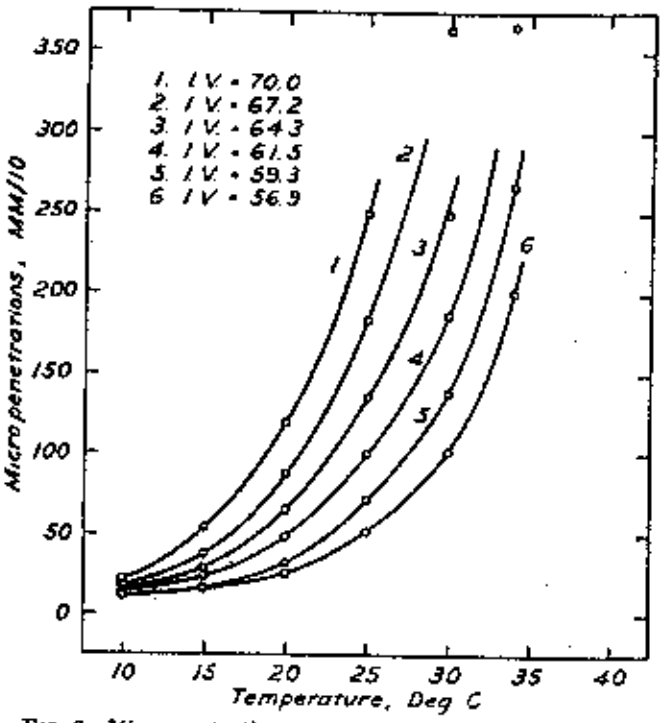


FIG. 3. Micropenetration curves, peanut oil hydrogenated to varying degrees of hardness.

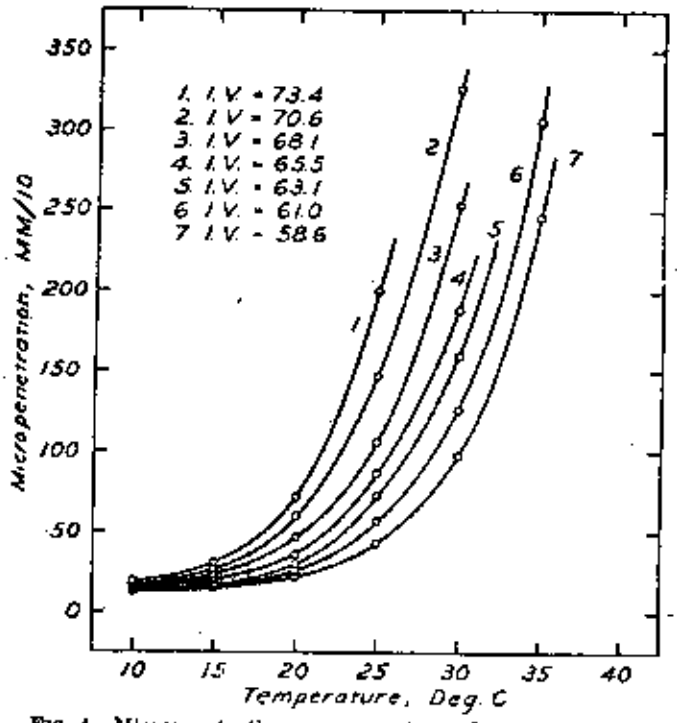


FIG. 4. Micropenetration curves, cottonseed oil hydrogenated to varying degrees of hardness.

the peanut, cottonseed, and soybean oils were 93.2, 108.6, and 133.3, respectively.

### Consistency vs. Hard Fat Content of Blended Products

A series of blended fats were prepared from cottonseed oil with an iodine value of 108.6 and hydrogenated cottonseed oil with an iodine value of 12.5. In this series the content of hydrogenated oil was varied from 8 to 13 percent, in increments of 1 percent. The micropenetration curves of this series of fats are reproduced in Figure 7. In Figure 8 the micropenetrations at 22.5° C. are plotted against the hard fat contents of the mixtures. It will be seen that the micropenetration method is sensitive to an addition of hard fat of less than 0.5 percent.

### Consistency of Commercial Products

The micropenetrations of the fat from a number of commercial fat products of different types are listed in Table 3.

### A Quick Micropenetration Technique for the Control of Hydrogenated Products

A rapid modification of the standard micropenetration method may be used for controlling the

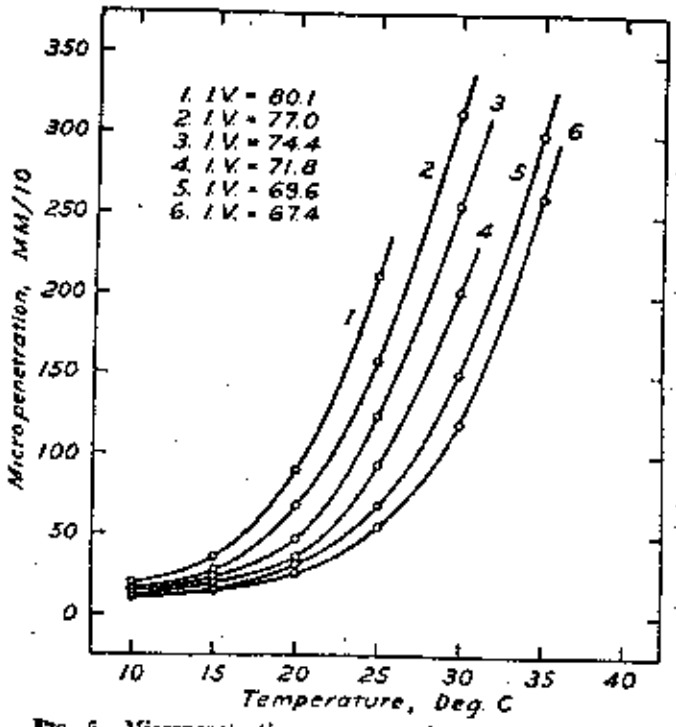


FIG. 5. Micropenetration curves, soybean oil hydrogenated to varying degrees of hardness.

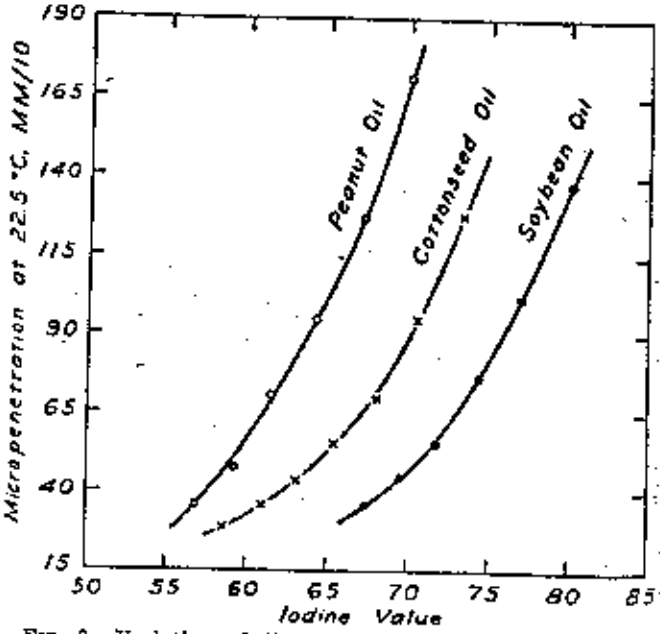


FIG. 6. Variation of the consistency of various oils with hydrogenation as measured by micropenetrations at 22.5°C. by the standard method.

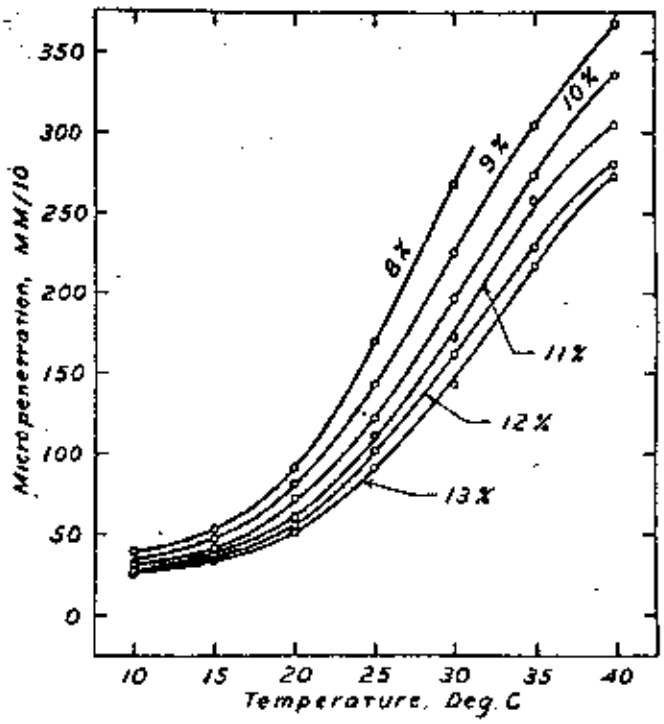


FIG. 7. Micropenetration curves, cottonseed oil containing varying percentages of hard oil.

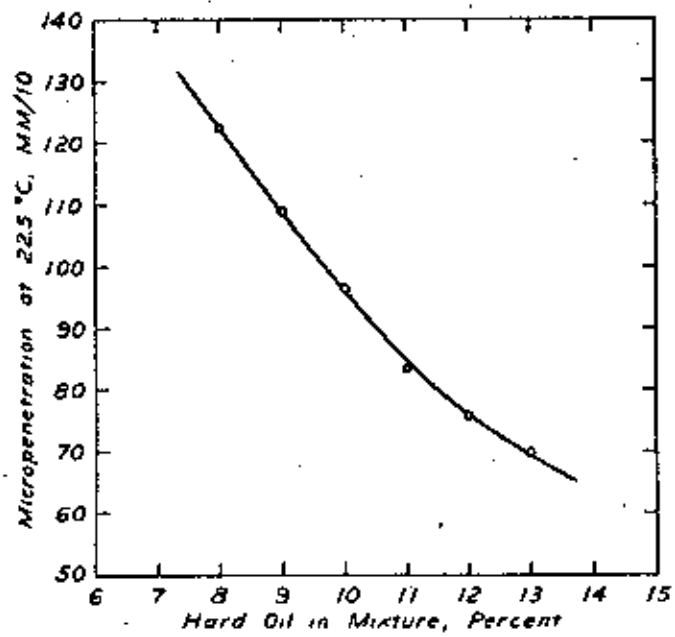


FIG. 8. Variation of the consistency of cottonseed oil with the addition of hard oil, as measured by micropenetrations at 22.5°C. by the standard method.

hydrogenation process. It has been found that consistent micropenetrations can be obtained on hydrogenated oils of the approximate consistency of shortening or margarine, in the temperature range of 20 to 25° C. if the chilling and tempering times are reduced to 15 minutes each.

The so-called "quick" micropenetrations thus obtained are not identical with the regular micropenetrations, but they bear a definite relationship to the latter. The relation found between regular and quick micropenetrations at 22.5° C. in the case of different hydrogenated oils is shown graphically in Figure 9. The variations which occur in the quick micropenetrations at 22.5° C., as the different oils are hydrogenated, are shown in Figure 10.

In practice, it will be found that the position of a curve representing micropenetrations vs. iodine value will vary somewhat from one hydrogenation run to another, even with careful control of the variables of temperature, pressure, concentration of catalyst, etc., during hydrogenation. Differences in the consistency of products hydrogenated at different times are apparently due to unavoidable variations in the nature of the catalyst and the composition of the raw oil. Therefore, if plots are made of a number of different hydrogenations of the same kind of oil, a family of curves will result, similar in appearance to those of Figures 6 or 10.

The procedure to be followed in applying the quick micropenetration method to hydrogenation control may be best illustrated by a specific example. Let it be supposed that cottonseed oil is being hydrogenated to produce a shortening of the all-hydrogenated type. The average consistency of good shortenings of this type at 22.5° C. is equivalent to a regular micropenetration of about 65, which corresponds in turn to a quick micropenetration of 55. The oil is hydrogenated until its iodine value approaches but does not reach the approximate value desired in the final product. Hydrogenation is then interrupted, and a sample is drawn and its quick micropenetration at 22.5° C. determined. At the same time its iodine value is also determined by a quick method (3). The point corresponding to this penetration and iodine value is plotted amidst the family of curves referred to previously. After this is done it will be readily apparent just how much farther the hydrogenation

TABLE 8

Micropenetrations of Fat From Various Plastic Commercial Products.
(Determined under standard conditions, chilled overnight and held 30 minutes at each temperature.)

| Sample No. | S-1 | S-2 | S-3 | S-4 | S-5 | S-6 | M-1 | M-2 | B-1 |
|---|---|---|---|---|---|---|---|---|---|
| Product | Shortening | Shortening | Shortening | Shortening | Shortening | Shortening | Margarine | Margarine | Butter |
| Brand | Spry | Crisco | Dexo | Jewel | Vegetola | Sroco | Parkay | Nucoa | Cloverbloom |
| Type | All-hydrog. veg. oils | All-hydrog. veg. oils | All-hydrog. veg. oils | Blended animal and veg. oils | Blended animal and veg. oils | All-hydrog. veg. oils | All-hydrog. veg. oils | All-hydrog. veg. oils | ..... |
| Iodine value | 72.2 | 74.1 | 75.4 | 87.2 | 74.6 | 67.9 | 72.9 | 72.3 | 83.2 |
| Micropenetrations, mm./10: | | | | | | | | | |
| at 10°C. | 15 | 15 | 16 | 26 | 21 | 14 | 12 | 13 | 11 |
| 15°C. | 25 | 24 | 25 | 41 | 31 | 19 | 18 | 19 | 17 |
| 20°C. | 42 | 45 | 48 | 71 | 56 | 37 | 35 | 41 | 43 |
| 25°C. | 92 | 101 | 102 | 124 | 112 | 83 | 87 | 117 | 145 |
| 30°C. | 181 | 212 | 203 | 196 | 172 | 186 | 252 | 337 | 367 |
| 35°C. | 324 | 336 | 315 | 270 | 258 | 253 | Too soft | Too soft | Too soft |

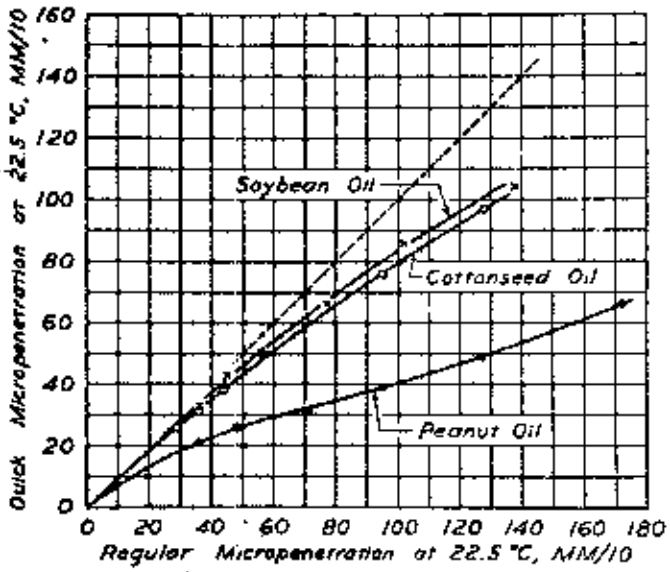


FIG. 9. Relationship between regular (standard) micropenetrations and quick micropenetrations of different hydrogenated oils at 22.5°C.

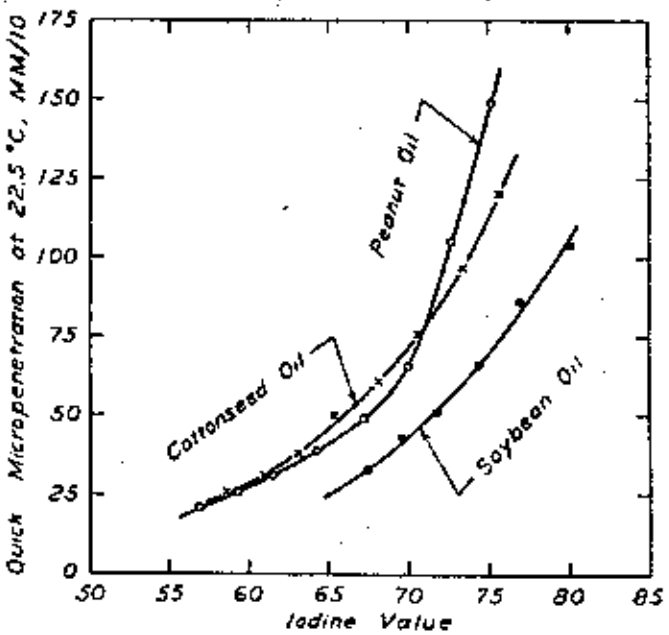


FIG. 10. Variation of the consistency of various oils with hydrogenation, as measured by micropenetrations at 22.5°C. by the quick method.

must be continued to reach the penetration desired in the final product.

Suppose that the iodine value and quick micropenetration of the sample are found to be 72.0 and 85, respectively. Reference to the curve for cottonseed oil in Figure 10 will reveal that the iodine value must be further reduced to about 66.7 in order for it to have a quick micropenetration of 55. Hydrogenation is then resumed until the oil has absorbed an amount of hydrogen equivalent to a reduction in iodine value of 72.0 to 66.7. By the careful application of this technique it is possible to hydrogenate oil to any desired consistency with a high degree of accuracy.

## Summary

1. An improved micropenetrometer is described, by means of which it is possible to measure the consistency of fats with a high degree of precision.

2. The intelligent use of a micropenetration method requires some consideration of the theory of plasticity in fats. This theory is briefly discussed.

3. The influence of various factors on the consistency of solidified fats has been investigated, and as a result of this investigation a standard technique for making micropenetration tests is proposed.

4. Micropenetration data are recorded on cottonseed, peanut, and soybean oils hydrogenated to different degrees, on cottonseed oil blended with various proportions of highly hydrogenated oil, and on various commercial samples of shortening and margarine.

5. A quick micropenetration method, applicable as a control in the hydrogenation of fat products, is described.

## Acknowledgment

The authors are indebted to Miss Maizie Caravella for the determination of the iodine values reported in this article.

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Gilbar

THE HEART AND SOYA BEAN OIL

By now both the public and the profession must be thoroughly confused by the variety of methods advocated for preventing coronary attacks and their recurrence. Fat, particularly saturated fat, sugar, sloth and stress have all been incriminated and preventive measures have been directed accordingly, with pretty equivocal results.

To study the role of saturated fats in preventing recurrence of myocardial infarction, a Medical Research Council committee has made a controlled dietary study lasting for two to seven years and involving 393 male patients who had recovered from a first infarction. The object of the trial was to discover whether replacement of saturated fat in the diet by a mainly unsaturated fat, soya bean oil, three ounces a day, would reduce the incidence of second infarctions. This measure had been shown to cause a satisfactory fall in serum cholesterol values, and indeed in the present trial and in a 1966 Oslo trial with similar conditions the regimen certainly achieved this result if nothing else, maximum fall being around 22%.

Unfortunately, there was no indication from the results that mortality was reduced by this diet either in the London or the Oslo trials, though there is a faint hint that the incidence of non-fatal re-infarction may have been reduced. Since, moreover, the soya bean oil regimen is somewhat tedious and difficult to arrange, the present trial has shown no particular reason for adopting it. However, this is not to say that the regimen has no virtues in preventing myocardial infarction in coronary candidates who have as yet [illegible] no attack.

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# The Hydrogenation of Soybean Oil

## Experimental Investigation of Its Application to Lard-Substitute Production

BY AUGUSTUS H. GILL AND YU M. MA.

ALTHOUGH there are four varieties of soybean, only one, the yellow, or soybean as it is universally called, is used in the soybean mills. The mills, or soybean oil-pressing plants, rank foremost in the manufacturing industry in Manchuria which comprises the three Provinces: Fengtien, Kirin and Heilungkiang, having 20 million inhabitants and an area of 365,000 square miles. The output of the oil in 1923 was about 500,000 tons. In Dairen, the center of soybean mills, 390 tons of oil are produced daily.

Since soybean oil is between peanut and cottonseed oils in the uses and the qualities, and no hydrogenation plant is established in China, the commercial hydrogenation of this oil, especially for edible purposes, should be developed, to better feed the 400 million Chinese, as well as their neighbors of non-farming nations.

The oil used had the following characteristics:

| | |
|---|---|
| Specific gravity at 15.5° | 0.925 |
| Free fatty acid calculated as oleic acid | 0.57% |
| Iodine number | 140.0 |

The catalyst is 'poisoned' by free fatty acids and moisture in oil. The latter is removed by drying and the former with caustic soda solution.

The oil was agitated with an electrical stirrer and 80 percent of the theoretical amount of caustic soda solution (16° Be.) were added. After 30 minutes the oil was heated in a water bath to 50° until the emulsion was "broken." It was then allowed to settle and was filtered through filter paper. The oil thus obtained was dried in the same cup for ten minutes at 120°, being agitated during the operation and then being filtered again. It was stored for hydrogenation.

The hydrogenation took place in a bronze autoclave holding about 600 cc., provided with a stirrer and oil bath for heating.

### Preparation of the Catalyst

There are two distinct methods of preparing the catalyst, one being known as the dry process and involving nickel salts in the dry state, and the other being known as the wet process, according to which the catalyst is prepared by reduction in oil. The latter process has been proved simpler and more reliable.

The catalyst was prepared by grinding nickel formate, the most commonly used material in the wet process, in the oil, and heating the oil emulsion thus obtained in the autoclave, hydrogen being passed in to displace the air in the autoclave until the reduction was complete. After the air had been completely displaced, (usually in five minutes), the burners were lighted and so regulated that the temperature was raised gradually and kept at 240°. When the bubbles of hydrogen became even and regular, usually after three to four hours, the nickel formate was considered to be completely reduced. The catalyst was now ready for use. It was newly prepared for every hydrogenation.



### Hydrogenation

The oil to be hydrogenated was poured into the autoclave, the air displaced as before and the cap bolted on. The burners were lighted and regulated so as to get a definite temperature, and the valve admitting hydrogen was regulated so as to get a definite pressure indication on the gauge. Stirring was begun and after a definite time a sample of 20 to 60 cubic centimeters was withdrawn with a pipette, and filtered through a hot water funnel. The process from air-displacing to sample-filtering was repeated until several samples had been taken.

The conditions under which the oil was hydrogenated and the iodine numbers of the samples were as follows:

Run 1. Temperature 170°C., pressure 40 pounds, 100 revolutions per minute, 0.6% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 60 | 106.0 |
| 2 | 120 | 95.7 |
| 3 | 180 | 87.3 |
| 4 | 240 | 79.0 |
| 5 | 300 | 75.8 |
| 6 | 340 | 72.0 |
| 7 | 370 | 66.8 |

Run 2. Temperature 180°C., pressure 40 pounds, 100 r.p.m., 1.00% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 60 | 83.9 |
| 2 | 120 | 72.7 |
| 3 | 180 | 66.4 |
| 4 | 240 | 62.9 |
| 5 | 300 | 56.8 |

Run 3. Temperature 170°C., pressure 2 pounds, 406 r.p.m., 1.00% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 60 | 2.78 |
| 2 | 70 | 2.54 |

Run 4. Temperature 170°C., pressure 20 pounds, 406 r.p.m., 1.00% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 15 | 18.3 |
| 2 | 20 | 18.0 |
| 3 | 25 | 17.3 |

Run 5. Temperature 160°C., pressure 10 pounds, 400 r.p.m., 0.40% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 15 | 45.0 |
| 2 | 30 | 42.2 |
| 3 | 45 | 37.9 |

Run 6. Temperature 150°C., pressure 10 pounds, 400 r.p.m., 0.40% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 10 | 78.2 |
| 2 | 20 | 66.6 |
| 3 | 30 | 50.2 |

Run 7. Temperature 150°C., pressure 10 pounds, 320 r.p.m., 0.40% catalyst.

| Sample | Elapsed Time Minutes | Iodine No. |
|---|---|---|
| 1 | 30 | 82.1 |
| 2 | 60 | 56.6 |
| 3 | 90 | 43.9 |
| 4 | 120 | 40.5 |

### The Effect of Pressure

The working pressure[1] of hydrogenation of cottonseed oil is below 40 pounds. No higher pressure seems to be necessary for hydrogenation of soybean oil. Moore[2] has pointed out that the hydrogenation rate of cottonseed oil is approximately proportional to the pressure. This fact will hold true for soybean oil.

[1] Rogers "Industrial Chemistry" (1926) 764.
[2] Jour. I. and E. Chem. 9, 459 (1917).

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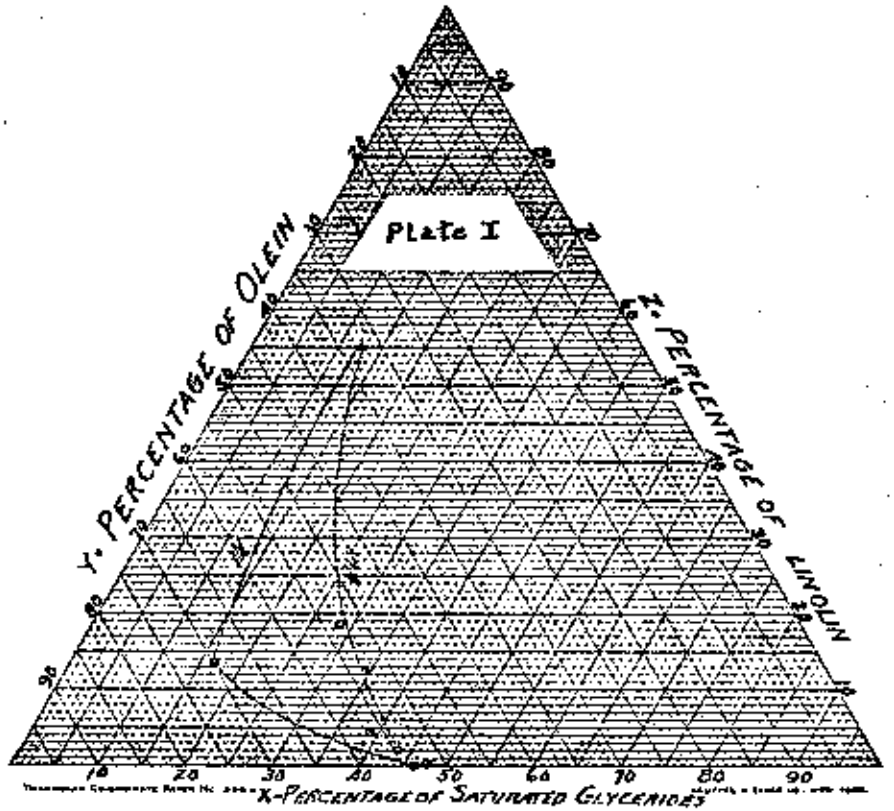


*Graphs showing progress of reaction in hydrogenation of soybean oil*

### The Effect of the Amount of Catalyst

As in practically all catalytic reactions, the hydrogenation rate[3] is increased as the percentage catalyst is raised. The fact[4] that no more than one percent of catalyst is necessary, is applicable to the hydrogenation of soybean oil, for no more than one percent of catalyst was used in the experiments and the results were satisfactory.

### The Composition of Fats from Runs 6 and 7

The fats were analyzed in order to know how the reactions progressed in the course of the hydrogenation process. The Gusserow-Varrentrapp lead-salt-ether method was used here. It is in principle a separation of two groups of lead salts of fatty acids based on the difference in solubility of the two groups in ethyl ether. The detailed procedure of analysis has been fairly well mapped out by Tortelli and Ruggieri[5] and others.

The composition of the fats, together with the iodine numbers of the fats and the liquid fatty acids, were as follows:

[3] Rogers, ibid.
[4] Moore, ibid.

[5] Lewkowitsch Analysis Oils, Fats and Waxes, I 560 (1921).



| | | Saturated Glycerides x | Per Cent Olein y | Linolin z | Fat A | Iodine Number Liquid Acids B |
|---|---|---|---|---|---|---|
| Run 6 | (1) | 28.5 | 53.5 | 18.0 | 78.2 | 114.1 |
| | (2) | 34.2 | 53.7 | 12.1 | 66.6 | 100.0 |
| | (3) | 43.4 | 54.5 | 2.1 | 50.2 | 92.7 |
| Run 7 | (1) | 16.5 | 70.0 | 13.5 | 82.1 | 103.5 |
| | (2) | 39.0 | 56.2 | 4.8 | 56.6 | 97.3 |
| | (3) | 46.3 | 53.7 | 0.0 | 43.9 | 86.2 |
| | (4) | 47.5 | 52.5 | 0.0 | 40.5 | 80.3 |

Graphs I and II, plate 1, show the composition of the fats during hydrogenation; taking the composition of the oil as x, (saturated glycerides) 12.5% y, (olein) 33.4% z, (linolin) 54.1%.

Both the figures show that linolin changed into saturated glycerides and, more quickly, into olein. The linolin disappeared when the saturated glycerides reached 45.6 percent, then olein changed into saturated glycerides very slowly. The agitation was higher in run six than in run seven, so the reaction was quicker in the former than in the latter.

### The Melting Points of Fats from Run 7

As the hydrogenated fat is to be used for edible purposes, its melting point should approximate that of lard. It was determined with the Mulliken capillary tube apparatus[6]. The melting points of the fats, together with the corresponding iodine numbers were as follows:

| | | | | |
|---|---|---|---|---|
| Melting points °C | 18.5 | 35.0 | 39.1 | 46.9 |
| Iodine numbers.. | 82.1 | 56.6 | 43.9 | 40.5 |

The melting points of lard, "Crisco," and "Snowdrift" were determined with the same apparatus. They are 40.4°, 40.7°, and 42.3° respectively; 40° is taken as the melting point of the commercial hydrogenated soybean fat.

### Conclusions

(1) An increase of temperature, pressure, agitation or amount of catalyst will increase the hydrogenation rate.

(2) In high agitation, at 320 r.p.m. or above, the change of 10° in temperature between 150° to 180° produces little or no effect.

(3) The hydrogenation rate is approximately proportional to the pressure, the amount of catalyst, and one half of the speed of agitation.

(4) No more than one per cent of catalyst is necessary.

(5) The course of the reactions of hydrogenation of soybean oil is the same as with cottonseed oil.

(6) The commercial hydrogenation of soybean oil can be a reality, for it is easily hydrogenated. As in run seven the time for obtaining the fat of 40° melting point was only one and one-half hours, although the time may be different on the commercial scale.

[6] Woodman "Food Analysis" (1915) p. 154.

United States Bureau of Mines, Reno, Nev., has recently finished a study of native clays to be used as substitutes for fullers' earth in bleaching oils. The raw clays studied resemble sepiolite, and the clays requiring acid treatment resemble bentonite.

# Physico-Chemical Changes in Artificial Fat Emulsions during Storage

## Studies of the Hydrolysis and its Physiological Effects

IVAN HÅKANSSON

Acta Chem. Scand. 20: 2267-2281. 1966

*The Department of Nutrition and Food Hygiene, National Institute of Public Health, Stockholm, Sweden*

Artificial soybean oil emulsions intended for intravenous administration have been studied, with particular reference to their spontaneous hydrolysis. In kinetic studies in buffered systems, the reaction-rate constant has been determined at varying temperature and pH. In the pH range studied, a hydrolysis minimum is found at about pH 7. Since the addition of electrolytes impairs the stability of the emulsion, buffered emulsions cannot be prepared commercially. Consequently, the course of hydrolysis has also been studied in unbuffered systems, in which the liberation of free fatty acids (FFA) is found to be rapidly accelerated at high temperatures, due to the catalytic action of the hydronium ions.

Since the concentration of FFA liberated on hydrolysis is hitherto the only certain parameter of the toxicity, toxicity studies have been made with emulsions stored for long periods at high temperatures. $LD_{50}$ in the rabbit is determined, and found to be 0.21 mequiv. FFA/kg body weight.

Long-term tolerance tests have been made in the dog, with administration of 9 g of fat/kg body weight and day, representing the total caloric requirement, for 28 consecutive days. Emulsions (Intralipid 20 %) stored up to 3 years are found to be well tolerated.

Clinical studies have been made with emulsions (Intralipid 10 % and 20 %) stored up to 24 months at temperatures ranging from + 4 to + 20°C. The low incidence of toxic reactions noted is in good agreement with corresponding results on infusion of newly prepared emulsions.

The possibility of supplying the organism with large quantities of calories by the parenteral route has been of considerable clinical interest for several decades. Consequently, numerous fat emulsions for intravenous use have been produced and tested.





One of these, a soybean oil emulsion,* has been subjected to comprehensive studies in both animals and man.[1-3] This emulsion has, for example, proved to have low toxicity in long-term tolerance tests in the dog,[4-6] when up to 9 g of fat/kg body weight and day were given. Moreover, its rate of elimination from the blood stream of the dog was shown to be similar to that of the chylomicrons.[7] The emulsion has also been used as a substrate in lipoprotein-lipase reactions.[8]

Certain studies of the stability of the artificial soybean oil emulsion have been reported previously.[9] An account is given in the present paper of studies of some physico-chemical changes in the emulsion, which occur as a result of long-term storage at varying temperature. It is demonstrated, *e.g.*, that buffered emulsions have a hydrolysis minimum at about pH 7. In connexion with this finding, the physico-chemical changes in the emulsion have been studied with respect to their physiological effects when infused intravenously in the rabbit, dog and man.

## MATERIAL AND METHODS

### Composition of emulsions used and physico-chemical analyses

*Fat emulsions.* 1. Commercial soybean oil emulsions (Intralipid 10 % and Intralipid 20 %); the compositions are shown in Table 1. They were stored at + 4, + 12, + 20, and + 40°C for up to 3 years.

*Table 1.* Composition of fat emulsions used.

| | Intralipid 10 % | Intralipid 20 % |
|---|---|---|
| Soybean oil | 100 g | 200 g |
| Egg-yolk phospholipids | 12 g | 12 g |
| Glycerol (85—89 % anhydrous) | 25 g | 25 g |
| Sterile water to | 1000 ml | 1000 ml |
| pH adjusted to 6.0—6.5 | | |

2. Nine emulsions with 20 % soybean oil and otherwise the same composition as in Table 1, except for pH. They were prepared from the same stock emulsion in such a way that all emulsions, before sterilization, had a pH within the range 4—11 and with a pH difference of 0.8—1 unit. These emulsions were stored at + 80°C for 11 days and at + 40°C for 240 days, respectively.

3. Four emulsions with 20 % soybean oil and otherwise the same composition as in Table 1 were buffered by the addition of a sodium phosphate buffer corresponding to a final concentration of 0.05 M. Their pH was then adjusted to 5.8, 6.3, 6.8, and 7.3, respectively. pH was adjusted with a 0.1 M solution of either sodium hydroxide or hydrochloric acid.

All emulsions were filled into 100 or 500 ml bottles, which were sterilized by autoclaving. They were then stored in the dark at varying temperatures. Samples for testing were withdrawn at fixed intervals.

*Determination of free fatty acids (FFA).* Dole's method[10] was used, in the modification of Trout *et al.*[11] pH was determined with a glass-electrode pH meter (Radiometer, Copenhagen, Denmark, Model 25 SE).

* Intralipid ®, Vitrum AB, Stockholm, Sweden.

*Determination of particle size.* A Zeiss Standard microscope with phase-contrast equipment was used. The magnification of the objective was 100 × and that of the eyepiece 20 ×. The eyepiece was provided with a micrometer whose smallest scale division corresponded to 0.5 μ. The emulsion was diluted 1:50 (20 % emulsion) or 1:25 (10 % emulsion) with a 50 % aqueous solution of propylene glycol according to Levius and Drommond.[11] One drop of the mixture was placed on a slide under a cover-glass. The number of particles with a diameter of ≥ 0.5 μ was counted in the rectangle formed by the micrometer scale of the eyepiece (35 – 40 particles). Ten such rectangles were counted in each analysis.

*Kinetic studies of hydrolysis.* These were made on emulsions buffered with sodium phosphate. To determine the pH range within which the hydrolysis minimum in this system was situated, four emulsions with different pH were used, *i.e.*, pH 5.8, 6.3, 6.8, and 7.3. Samples were taken at fixed intervals from the emulsions stored at + 4, + 20, + 40, and + 60°C for long periods, for the determination of the FFA concentration.

## Animal experiments

*Acute toxicity.* This was studied in white rabbits of both sexes, weighing about 2.5 kg. The emulsion (8 ml/kg body weight) was injected into the marginal vein of the ear. The animals were observed for 24 h. However, those that did not survive generally died immediately, or within less than 5 min after the injection.

*Chronic toxicity.* These studies were made in mongrel dogs. After a control period of at least 4 weeks, daily infusions were given for 28 consecutive days. During the infusion, each dog was placed in a Pavlov stand. The amount of fat administered was 9 g/kg and day, representing the total caloric requirement in the dog (about 80 cal/kg and day). The infusion rate was adjusted so that the infusion lasted for 4 h. During the whole experimental period, the dogs were allowed to eat and to drink water *ad libitum*.

To study the chemical and physiological changes in the blood, two samples of venous blood were taken before starting the infusion period, and thereafter once a week. All the samples were taken in the morning, directly before starting the infusion, with the exception of the first one, which was taken a few days before. The haemoglobin and haematocrit were determined, as well as the serum triglycerides, phospholipids, cholesterol, total protein, albumin, and globulin. The serum triglycerides and phospholipids were determined with the methods described by Carlson,[12,13] and serum cholesterol according to Sperry and Webb.[14] Total protein in serum was determined by Kjeldahl's method, and electrophoretic separation of the serum proteins was performed according to Köiw *et al.*[15]

Furthermore, the dogs were weighed once a week, and their rectal temperature was measured 15 min before starting the infusion, as well as 2, 3, 4, and 5 h after its beginning. The urine was also analysed once a week for the presence of glucose, protein, and ketone bodies. The dogs' appetite, general condition, and behaviour were noted.

On the morning of the day after the 28th infusion, 3 of the 4 dogs were killed by injection of Veterinary Nembutal * (1.5 ml/kg body weight). Autopsy was then performed and specimens of different organs were taken for sectioning. Special staining procedures, as described by Meyer *et al.*[17] and Thompson and co-workers,[18-20] were used to demonstrate intravenous fat pigment in unstained and paraffin-embedded sections of the liver and spleen. The histopathological studies were carried out under supervision of Professor Anna-Lisa Obel, Department of Anatomy and Pathology, State Veterinary Medical Institute, Stockholm.

## Clinical studies in man

Emulsions (Intralipid 10 % and 20 %) subjected to physico-chemical analyses, as well as to tests in animals, were used in clinical studies in man. They were stored at + 4, + 12, and + 20°C, and were tested every 6 months. The emulsions were administered as intravenous drip infusions to patients in whom indications for parenteral nutrition existed. Each infusion lasted for about 4 h. The occurrence of any side reactions — such as nausea, vomiting and rigors — was studied, as well as any rise in temperature in

* Abbot Laboratories Ltd., Queenborough, Great Britain.





connexion with infusion. The temperature was recorded once an hour before, during and after each infusion, in all for 10 – 14 h. These infusions were carried out under the supervision of Assistant Professor Oscar Schuberth, Department of Surgery, St Görans Sjukhus, Stockholm.

## RESULTS

In the following, the results refer to the commercially available soybean oil emulsion (Intralipid 20 %), unless it is stated that specially prepared emulsions were used.

*Changes in FFA concentration and pH* are demonstrated in Fig. 1. After 11 weeks' storage at + 4°C, the FFA concentration was 1.4 times the initial value (0.6 mequiv./1000 ml). After the same period and storage at + 20°C, the concentration had increased to 2.4 times the initial value; at + 40°C the value was 10-fold and at + 60°C 90-fold. The liberation of FFA during storage for 24 months at + 4, + 12, + 20, and + 40°C is illustrated in Fig. 2.

As a result of the liberation of FFA with temperature and time, there was a marked fall in pH. The changes in pH occurring during storage for 11 weeks at the four specified temperatures were most marked at the beginning of the period (Fig. 1). It is also evident from Fig. 1 that the fall in pH was remarkably large during the processes of homogenization and sterilization.

*Changes in particle size.* In freshly prepared emulsions, the optical diameter of all particles was less than 1 μ, and most of them were smaller than 0.5 μ. Fig. 2 shows the changes in size of the particles as a result of storage of the emulsions for 24 months at + 4, + 12, and + 20°C. In each case, the results represent the mean value of 8 batches. It can be seen that the increase in

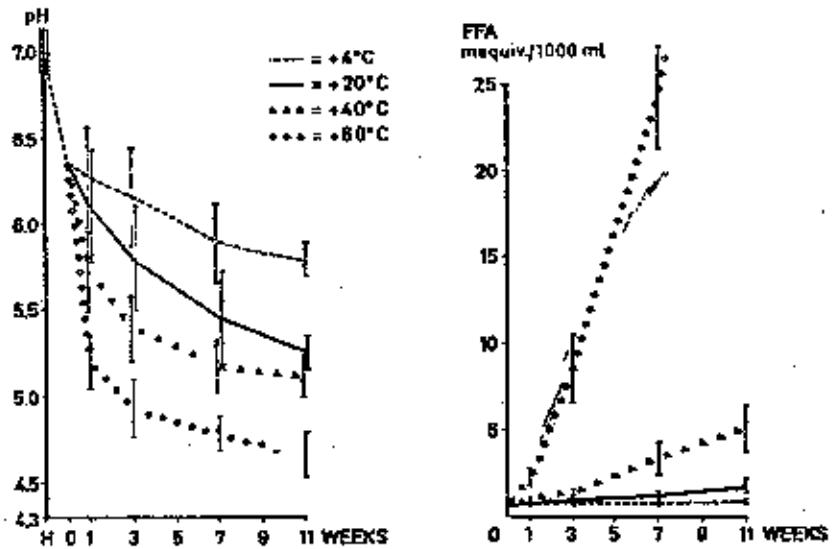


*Fig. 1.* Changes in pH and release of FFA during storage of soybean oil emulsion (Intralipid 20 %) at four different temperatures. H = pH during homogenization. Mean of 4 batches.

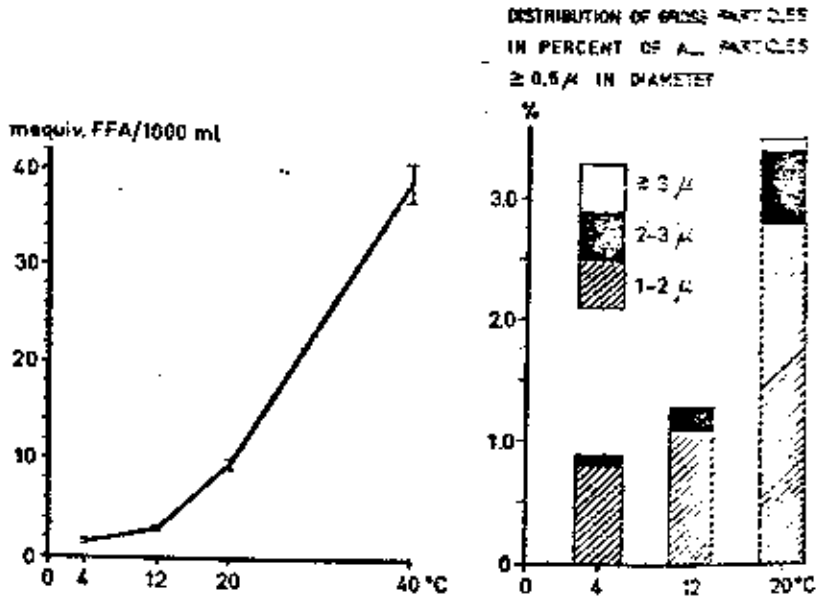


*Fig. 2.* Changes in FFA and particle size in soybean oil emulsion (Intralipid 20 %) after storage for 24 months at various temperatures. All particles in fresh emulsions have a diameter ≤ 1 μ. Mean of 8 batches.

particle size was dependent on the temperature. Thus, particles with a diameter exceeding 3 μ were present only in emulsions stored at + 20°C.

*pH-dependence of hydrolysis.* Nine emulsions with varying pH, prepared from the same stock emulsion, were used for this study. In Fig. 3, the FFA concentration is plotted against the final pH of the emulsions. After storage at + 80°C for 11 days and at + 40°C for 240 days, the emulsions whose pH was between 6 and 7 showed a minimum of hydrolysis.

It can be seen in Fig. 3 that hydrolysis was catalyzed by both hydronium and hydroxyl ions. Another matter of interest in this connexion was demonstration of the toxicity of the free fatty acids in the rabbit. Thus, at FFA concentrations exceeding 20 mequiv./1000 ml emulsion (representing a FFA dose of 0.16 mequiv./kg body weight), injection had a lethal effect in the majority of cases.

*Kinetic mechanism of hydrolysis.* Since the hydronium ion concentration in the buffered emulsions was practically constant, the reaction could be regarded as a pseudo first-order reaction in the pH range studied. In view of the fact that hydrolysis was followed in only a small part of its course, Guggenheim's method[21] was used to determine the reaction-rate constants. According to this method, the rate constants can be determined without knowledge of the total course of hydrolysis, using the following equations:

$$v' - v = (v_\infty - v_0)\ (1 - e^{-k\tau})\ e^{-kt} \qquad (1)$$





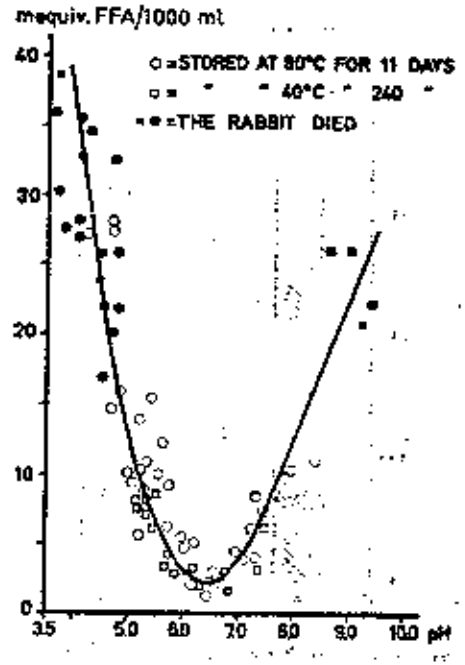


*Fig. 3.* Effect of pH on FFA liberation in soybean oil emulsions (20 %) at two different temperatures. Nine emulsions with different pH were used. The concentration of FFA is plotted against the final pH. The lethal effect of the FFA in the emulsions injected in rabbits is denoted by black circles and squares.

which is logarithmized and transformed into

$$\ln\ (v' - v) + kt = \ln\ (v_\infty - v_0)\ (1 - e^{-k\tau}) \qquad (2)$$

in which $k$ = the reaction-rate constant, $t$ = the time of the determinations, $\tau$ = a constant time interval, and $v$ and $v'$ = the concentration of FFA at time $t$ and $t + \tau$, respectively. $v_0$ and $v_\infty$ represent the FFA concentration at zero time and infinite time, respectively. Log $(v' - v)$ was plotted against time, after which $k$ was calculated from the graphic representation, as seen in Fig. 4. The reaction-rate constants calculated from Fig. 4 with Briggsian logarithms are listed in Table 2.

*Table 2.* Rate constants for the hydrolysis of buffered fat emulsions at various temperatures and pH, calculated on the basis of the curves in Fig. 4.

| °C | pH = 5.8 | pH = 6.3 | pH = 6.8 | pH = 7.3 |
|---|---|---|---|---|
| | $k \times 10^3$ day$^{-1}$ | $k \times 10^3$ day$^{-1}$ | $k \times 10^3$ day$^{-1}$ | $k \times 10^3$ day$^{-1}$ |
| 4 | 0.78 | 0.60 | 0.41 | 0.96 |
| 20 | 2.41 | 1.73 | 1.33 | 2.82 |
| 40 | 11.9 | 8.10 | 6.50 | 14.1 |
| 60 | 36.3 | 27.7 | 24.1 | 45.8 |

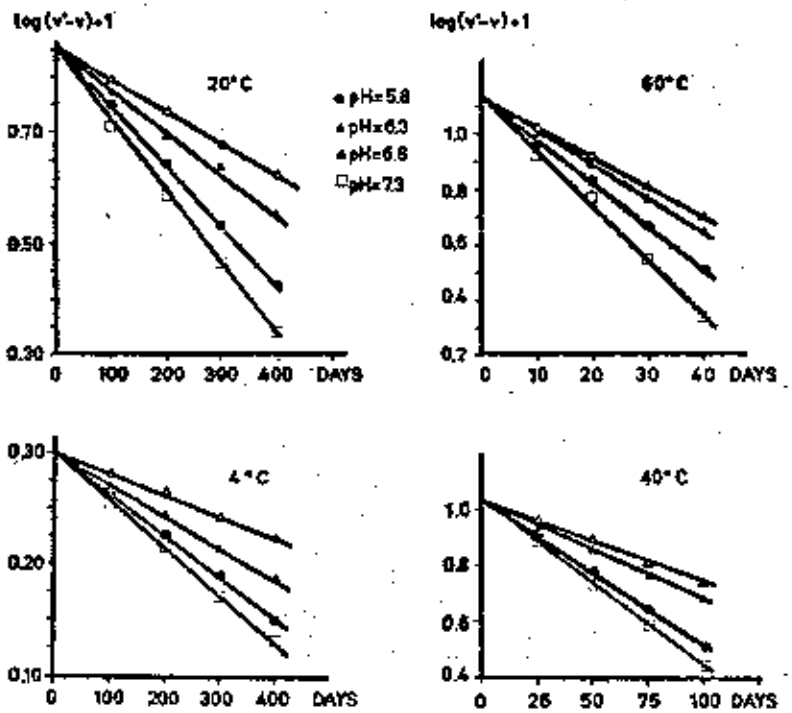


*Fig. 4.* Hydrolysis of buffered soybean oil emulsions (20 %) at four different temperatures and pH.

In Fig. 5, the reaction-rate constants expressed as p*k* are plotted against pH. The treatment of the course of hydrolysis as a pseudo first-order reaction seems to be valid within the experimental accuracy.

*Acute toxicity.* Emulsions with the composition shown in Table 1 (Intralipid 20 %) kept for long periods at + 4, + 20, and + 40°C, were used to study the

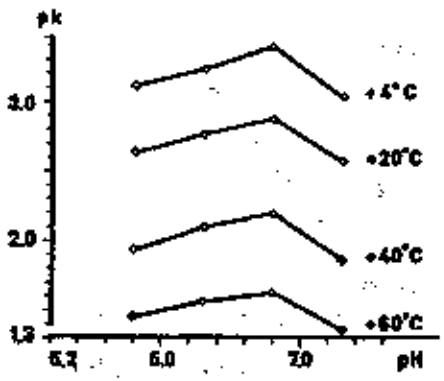


*Fig. 5.* Rate constants of the hydrolysis expressed as p*k* of buffered soybean oil emulsions (20 %) plotted against pH.





acute toxicity in rabbits with regard to lethal effects. Samples were taken after storage up to 24 months. Before the toxicity studies the amount of FFA was determined. The relation between the FFA concentration in the emulsion and the lethal effect is shown in Fig. 6. At a concentration of 15—20 mequiv./1000 ml emulsion (0.12—0.16 mequiv. FFA/kg body weight), the mortality exceeded 30 %. At a FFA concentration below 15 mequiv./1000 ml, on the other hand, the mortality was nil.

In order to determine the $LD_{50}$ value of free fatty acids in the emulsion, 114 rabbits were divided into 5 groups, with at least 20 rabbits in each. The

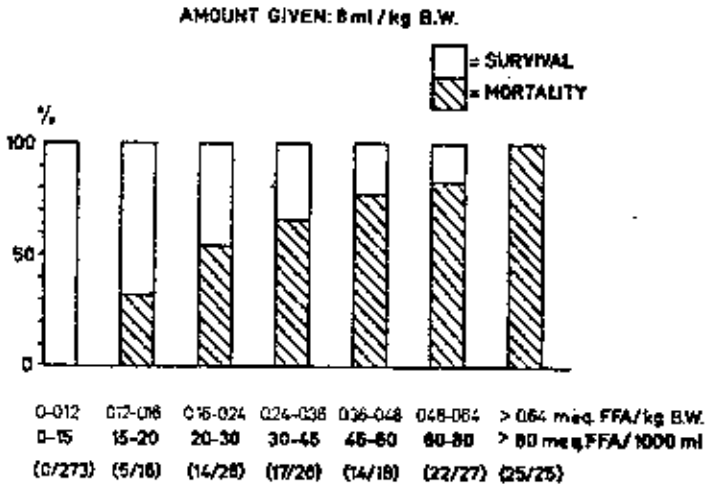


*Fig. 6.* Toxicity of aged soybean oil emulsions in the rabbit. Abscissa: Range of FFA concentration in mequiv./kg body weight and in mequiv./1000 ml emulsion. Bracketed numerals: mortality rate. Ordinate: % mortality.

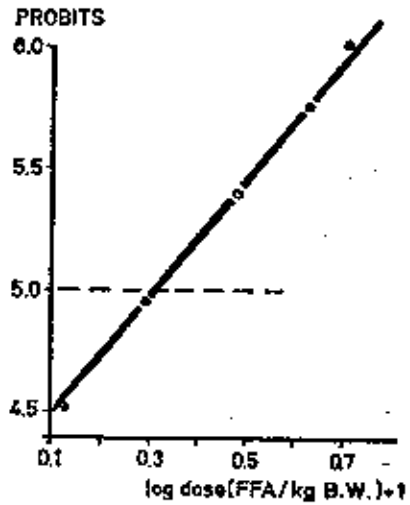


*Fig. 7.* Dose-mortality curve in the rabbit of aged soybean oil emulsion. Probits are plotted against log dose (mequiv. FFA/kg body weight) after injection of emulsion in a dose of 8 ml/kg body weight.

FFA concentration in the stored emulsions given to the respective groups was 17.3, 24.7, 38.1, 53.3, and 66.5 mequiv./1000 ml. $LD_{50}$ was determined graphically, after the percentage mortality had been converted into probits using the method of Bliss.[22] In Fig. 7 the probits are plotted against log dose (mequiv. FFA/kg body weight). With a dose of emulsion amounting to 8 ml/kg body weight, $LD_{50}$ for FFA in the emulsion was found to be 0.21 mequiv./kg body weight, corresponding to a FFA concentration in the emulsion of 26.3 mequiv./1000 ml.

*Long-term tolerance tests.* The FFA concentration, pH and particle size in the tested emulsions (Intralipid 20 %) stored under varying conditions are shown in Table 3. The results of the long-term tolerance test in dogs with

*Table 3.* FFA concentration and pH of aged emulsions (Intralipid 20 %) stored under various conditions. 9 g fat (45 ml)/kg body weight and day given for 28 consecutive days to 4 dogs.

| Dog No. | Storage | | FFA mequiv./1000 ml | pH |
|---|---|---|---|---|
| | Time months | Temp. °C | | |
| 34 | 6 | 20 | 1.65 | 5.19 |
| 41 | 12 | 20 | 4.60 | 4.97 |
| 68 | 36 | 4 | 2.90 | 5.14 |
| 71 | 36 | 12 | 6.10 | 4.90 |

9 g of fat/kg body weight and day for 28 consecutive days are depicted in Fig. 8. Slight anaemia appeared in all the dogs, with a fall in both haemoglobin and haematocrit to about 80 % of the initial value. The two dogs given emulsion stored for 36 months at + 4 and + 12°C showed a somewhat greater decrease in haemoglobin concentration than that in the other two, which had got emulsions stored at + 20°C for 6 and 12 months, respectively.

Apart from the triglyceride concentration in dog 34 (emulsion stored for 6 months at + 20°C), the concentration of all serum lipids determined rose during the infusion period. This rise showed an increase with storage temperature and time. For example, dog 71 (emulsion stored for 36 months at + 12°C) showed an almost 6-fold increase in both triglycerides and phospholipids during the 4-week period, and the cholesterol level increased 4.5 times.

As far as total serum protein was concerned, a moderate increase was recorded in 3 of the 4 dogs. The exception was dog 41 (emulsion stored for 12 months at + 20°C); a decrease of 10 % occurred during the infusion period. An increase in serum albumin was found only in dog 34 (emulsion stored for 6 months at + 20°C). In the other three, the serum albumin concentration fell by 7 % (dog 41), 14 % (dog 68) and 7 % (dog 71). A distinct increase in serum globulin concentration was noted in the two dogs (68 and 71) given emulsion stored for 36 months at + 4 and + 12°C, respectively.

During the whole experimental period, all the dogs were in good condition, had a normal coat and exhibited normal reactions. On no occasion did the





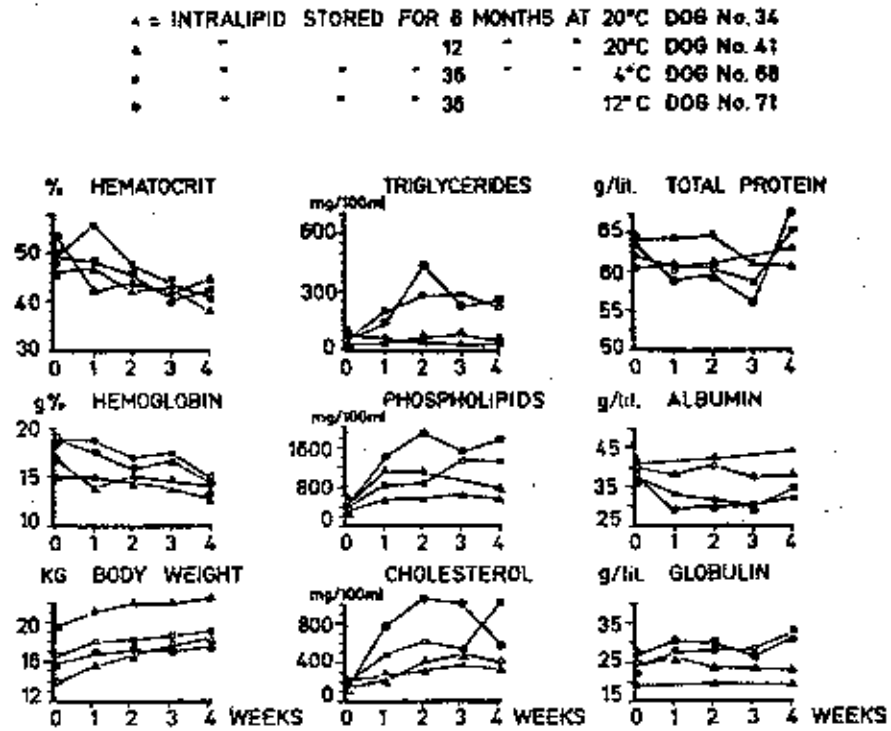


*Fig. 8.* Effects of aged Intralipid 20 % on the haematocrit, haemoglobin, body weight serum lipids and serum proteins in the dog.

urine of any of the dogs contain glucose, protein, or ketone bodies. A rise in temperature exceeding 0.5°C during the infusion was recorded on only one occasion (20th infusion in dog 41 with a rise of 0.6°C). The dogs' appetite was good, and all gained in weight during the experimental period. The gain amounted to 15 %, 35 %, 13 %, and 10 % of the initial weight (dogs in the order shown in Fig. 8). All the changes in the blood constituents are also recorded in Fig. 8.

Autopsy was performed on three of the four dogs used. Table 4 lists the histopathological changes that can be attributed to the administration of Intralipid 20 % after lengthy storage. Intravenous fat pigment was observed in the cells of the reticuloendothelial system of the liver, spleen, lymph nodes, and bone marrow. Slight erythrophagia and centrilobular necrosis of the liver were observed in one dog. Generalized jaundice was present in two dogs and fat in degenerated liver cells in one.

*Clinical studies in man.* Table 5 shows the results from 8 series of infusion of Intralipid 10 % and Intralipid 20 % after 18 months' storage at + 12 and + 20°C, as well as after 24 months' storage at + 4, + 12, and 20°C. On each occasion of infusion recorded in the Table, 4 batches each of Intralipid 10 % and Intralipid 20 % were used. The number of infusions of emulsions stored for the

*Table 4.* **Histopathological changes in dogs given aged fat emulsions (Intralipid 20 %) in doses of 9 g fat/kg and day for 28 consecutive days.**

| Dog No. | Storage conditions | Pathological changes |
|---|---|---|
| 41 | 12 months at + 20°C | Intravenous fat pigment in cells of reticuloendothelial system of liver and lymph nodes. Fat in degenerated centrilobular liver cells. |
| 68 | 36 months at + 4°C | Large amount of intravenous fat pigment in cells of reticuloendothelial system of liver, spleen, lymph nodes and bone marrow. Centrilobular necroses in the liver. Generalized jaundice. Signs of erythrophagia. |
| 71 | 36 months at + 12°C | Large amount of intravenous fat pigment in cells of reticuloendothelial system of liver, spleen, lymph nodes and bone marrow. Generalized jaundice. |

relevant time (18—24 months) and temperature (+ 4, + 12, and + 20°C), the quantity of fat administered, the FFA concentration and pH of the various emulsions can also be inferred from Table 5. It also lists the rises in temperature divided into four groups, in which all infusions with a temperature rise of less than 0.6°C are collected in the first group. Increases limited by temperature rises of 0.6—1.1°C and 1.1—1.6°C, respectively, form the second and third groups, while a temperature rise exceeding 1.6°C constitutes the fourth. In addition, other reactions — such as nausea, vomiting and rigors — observed during and after infusion have also been noted. In a total of 122 infusions recorded, *ca.* 2 g of fat/kg body weight have been given on 97 occasions. A temperature rise of less than 1.1°C has been recorded at 109 infusions. Of these 65 showed an increase not exceeding 0.6°C. In 114 out of 122 infusions no "other reactions" have been reported.

In Intralipid 10 % after storage for 18 months at + 20°C, the mean FFA concentration was 8.30 ± 0.52 mequiv./1000 ml emulsion, while Intralipid 20 % stored for 24 months at + 20°C showed a mean concentration of 9.90 ± 0.36 mequiv. FFA/1000 ml. Consequently, the patients given 2 g of fat/kg body weight of Intralipid 10 % received a FFA dose of around 0.17 mequiv./kg body weight. In view of the variations in FFA concentration in the four batches of Intralipid 10 % used, and the fact that the amount infused exceeded 20 ml/kg body weight in certain cases, a dose of 0.20 mequiv./kg body weight was given in a few infusions.

## DISCUSSION

As a result of the spontaneous hydrolysis occurring in the commercial fat emulsions (Intralipid 10 % and 20 %), the concentration of free fatty acids (FFA) increases with time and temperature of storage. This, in turn, produces a fall in pH. Since hydrolysis is catalyzed by hydronium ions, the reaction rate gradually increases with a rising concentration of these ions. However,





*Table 5.* Clinical studies of aged Intralipid. Mean of four batches each of 10 % and 20 % emulsion. Other reactions include nausea, vomiting, rigors etc.

| Emulsion: Intralipid % | Infusions (total No.) | Storage | | pH | FFA mequiv./1000 ml | No. of infusions with amount of fat given (g/kg BW) | | | | No. of infusions with rise in temperature expressed as °C | | | | No. of infusions with other reactions recorded as | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Temp. °C | Time months | | | 0.9—1.3 | 1.3—1.8 | 1.8—2.5 | | ≤0.6 | 0.6—1.1 | 1.1—1.6 | ≥1.6 | none | slight | moderate |
| 10 | 13 | 12 | 18 | 6.11 | 2.72 | 2 | 7 | 4 | | 3 | 6 | 0 | 1 | 12 | 0 | 1 |
| 20 | 23 | 12 | 18 | 6.12 | 2.67 | 0 | 5 | 18 | | 8 | 7 | 8 | 8 | 23 | 0 | 1 |
| 10 | 12 | 20 | 18 | 4.78 | 8.30 | 0 | 0 | 12 | | 7 | 5 | 0 | 0 | 12 | 0 | 0 |
| 20 | 23 | 20 | 18 | 4.81 | 6.08 | 2 | 4 | 17 | | 13 | 9 | 0 | 1 | 23 | 0 | 0 |
| 10 | 10 | 12 | 24 | 4.84 | 8.14 | 1 | 1 | 8 | | 7 | 3 | 0 | 0 | 10 | 0 | 0 |
| 20 | 12 | 4 | 24 | 5.15 | 1.24 | 1 | 0 | 11 | | 6 | 6 | 0 | 0 | 11 | 1 | 0 |
| 20 | 16 | 12 | 24 | 4.86 | 2.78 | 0 | 0 | 16 | | 11 | 2 | 4 | 1 | 13 | 3 | 0 |
| 20 | 13 | 20 | 24 | 4.72 | 9.00 | 0 | 2 | 11 | | 10 | 3 | 0 | 0 | 11 | 2 | 0 |
| Total | 122 | | | | | 6 | 10 | 97 | | 65 | 44 | 7 | 8 | 114 | 6 | 2 |

in alkaline hydrolysis — whose reaction rate is higher than that of the acidic hydrolysis — the reverse applies in unbuffered emulsions. The reaction rate decreases rapidly as hydrolysis proceeds, because the pH of the emulsion approaches the neutral point. In buffered emulsions, particularly at a pH around 6.8, the rate of hydrolysis has a minimum. Buffering substances other than sodium phosphate were tested with similar results.

A gradual increase in particle size was demonstrated after long-term storage of emulsions at varying temperatures. Although this kind of instability increases with temperature, it is probably not primarily dependent on the increased concentration of FFA. Thus, buffered emulsions with a pH around the neutral point show a low FFA concentration but impaired stability, with increasing particle size and creaming as a result. Another measure of the physical stability of the emulsions was obtained by applying the same principles as those described by Singleton *et al.*[22] The resistance of the emulsions to mechanical shock was studied with respect to such factors as particle size and creaming, after shaking in a shaking apparatus. All emulsions containing electrolytes then proved to be highly unstable. The instability increased with rising ionic strength, appeared within a relatively large pH range, and varied somewhat with different electrolytes. In the oil/water system studied with phospholipids as emulsifiers, it is likely that valency of the ions, ionic strength and pH of the emulsion are of importance for the interaction with the phospholipids, which can be characterized as ampholytes.

It is evident from the studies described in this paper that the FFA concentration of the emulsion was, in fact, the only one of the parameters studied that could be correlated to the increased toxicity in the rabbit. The toxicity did not seem to be influenced by a final pH in the range of 3.5—9.5, despite the borderline values being somewhat extreme from the physiological point of view, nor by any unknown hydrolysis products that might have been present at these pH values, or the fact that an increase of the particle size was demonstrated at storage for a long time at higher temperatures. Injection of 8 ml of 20 % emulsion, representing 1.6 g of fat/kg body weight and a FFA concentration below 0.12 mequiv./kg body weight, proved to be non-lethal in every case.

Using the procedure described, $LD_{50}$ in the rabbit was determined, and found to be equal to a FFA dose of 0.21 mequiv./kg body weight, corresponding to 26.3 mequiv./1000 ml emulsion. This is in agreement with the results obtained by Orö and Wretlind [24] in mice. Among the findings of these authors were that $LD_{50}$ of stearic acid was about 0.08 mequiv./kg body weight, whereas that of palmitic acid was about 0.22 mequiv./kg. The mechanism responsible for the lethal effect of FFA on infusion is unknown, but some relevance can perhaps be ascribed to the fact that small doses of saturated FFA cause massive thrombosis in the dog.[25]

The haematological changes differed in the dogs given 9 g of fat/kg/day, in the form of Intralipid 20 % stored for long periods under varying conditions. For example, two dogs given emulsion stored for 36 months showed a marked increase in serum lipids compared to those given emulsion stored for 6 and 12 months, respectively. With respect to serum albumin concentration, a relatively marked decrease was recorded in the two former dogs. However, apart from the serum cholesterol in one of them, a normalization in the concentration of both serum lipids and albumin took place towards the end of the infusion period.





As far as the histopathological changes are concerned, centrilobular necrosis of the liver was observed in one of the two dogs given emulsion stored for 36 months, and generalized jaundice was present in both. Moreover, there was a larger amount of intravenous fat pigment in these dogs than in the others. Because of the large doses that have been given for a long time and in view of the few dogs involved, no definite conclusions can be drawn from these results. In addition to that, emulsions stored up to 16 months at temperatures between + 12 and + 20°C have been used in similar experiments without observing the above discussed findings at sectioning.

The primary aim of the present study was to ascertain whether the emulsions could be stored for long periods at varying temperatures without the appearance of toxic effects. Another aim was to find one or several parameters that could be used, in a simple way, to determine whether such emulsions could be used clinically. Clinical tests were therefore made with emulsions stored under varying conditions. The results of infusions in man showed a low incidence of toxic reactions, of about the same order of magnitude as those reported by Schuberth [26] in his collocation of 1405 infusions of Intralipid.

Infusion of Intralipid stored for 18 months was associated with a larger number of relatively marked rises in temperature than was infusion of that stored for 24 months (Table 5). This apparent anomaly is explained by the fact that in the latter case the patients were selected, so that any reactions could be ascribed, as far as possible, to the infusions. This did not apply to the patients given Intralipid stored for 18 months.

Although the tolerance in the rabbit is sometimes regarded to correspond to that in man, it is perhaps hazardous to establish the toxicity of the FFA concentration in the emulsions in man on the basis of that in the rabbit. A comparison shows, however, that some of the emulsions tested clinically after storage at + 20°C for 18 months had a mean FFA concentration of 8.30 ± 0.52 mequiv./1000 ml. This implies that in the few infusions in man in which the dose of fat on infusion of Intralipid 10 % exceeded 2 g/kg body weight, the FFA dose was about 0.2 mequiv./kg. However, the marked differences in administration — in a few seconds in the rabbit, and in 4 h in man — have to be noted. This apparently good tolerance to FFA in man might be due to the rapid rate at which free fatty acids are eliminated from the blood stream.[27] Consequently, there will not be a high plasma concentration of FFA during the 4-h infusion period.

To sum up, the following conclusions can be drawn from the present study. The FFA concentration of the emulsion seems to be the most important parameter responsible for its toxicity on storage. Moreover, the toxicity of FFA in the rabbit under the conditions described might be used as a criterion of the clinical usefulness. Finally, the emulsion can, with a satisfactory safety margin, be stored up to 2 years at a temperature of + 4 to + 8°C.

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# LX. A NOTE ON THE DIGESTION OF FATS BY PANCREATIC LIPASE

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FATS have for some time been recognized as basal dietary constituents, though even now it is not definitely established whether or not these constituents should be regarded as essential food factors. That animals eat a variety of fats, both of animal and vegetable origin, is widely recognized and there is evidence that man can digest a number of fats other than those which are normally included in his diet.

Special consideration was given to this type of food when a Committee of the Royal Society [1918–19] considered the possibility of utilizing fatty acids as food. Burns & Sharpe [1918–19] concluded that the fatty acids of whale oil were as thoroughly digested and absorbed as was the hardened oil from which they were prepared. Gardner & Fox [1919] found that cocoabutter could be satisfactorily utilized by man. Barbour [1934], working with rats, showed that peanut oil or cottonseed oil could be effectively metabolized when it constituted 20% of the diet. Langworthy & Holmes [1917, 1], from experiments on man, found that olive oil, cottonseed oil, peanut oil, coconut oil, sesame oil and cocoabutter could safely be included in his diet, but concluded that the limit of tolerance was less for cocoabutter than for the other fats studied. With animal fats these observers [1915] found the coefficient of digestibility of butter to be 97·1%, of lard 97·3%, of beef fat 93·1% and of mutton fat slightly less, 87·6%; for cream the figure was 96·9% [1917, 2].

In the experiments just quoted the subjects were left entirely free as to the amount of food they consumed and wide variations were noted. For instance, with beef fat the average amount taken per day was 100 g. and with mutton fat only 53 g. This work demonstrated that various fats could be adequately digested and assimilated by man, but afforded no information as to the respective rates of digestion of the fats. Weber & King [1935] found that the α- and β-monoglycerides of lauric, myristic, palmitic and stearic acids were hydrolysed with equal facility by pancreatic lipase, but on the whole there appears to be little experimental evidence as to the comparative rates of digestion of different fats.

## EXPERIMENTAL

Various methods for emulsifying fats have been used by different workers. A method in general use involves the use of alcohol, but other methods have been suggested such as the use of gum acacia or neutral sodium oleate as emulsifying agents [Weinskin & Wynne, 1935–36]. Such methods have no direct application to digestion in man and it was desirable to use the fat in a form in which it could be taken as food. At first an emulsion in water [Plimmer, 1933] was tried, but this method could not be used because it was applicable only to some and not to all fats. Reconstituted creams in which various fats were substituted for butter were next tried and this method proved satisfactory.

*Preparation of creams*

The creams used in these experiments were made with a well-known type of machine and it was found that once the machine was "run in" a standard product could always be obtained. It had been shown previously [Hartwell, 1937] that the best emulsification was obtained with the proportion of 57 g. butter to 105 ml. milk: the cream so made contains approximately 33 % of fat. With other fats which contained little or no water, 47 g. fat were added to 105 ml. milk and 10 ml. water. When creams were kept overnight they were placed in a refrigerator, previous experiments [Hartwell, 1937] having shown that keeping them in this way made no difference to their rate of digestion. Such creams were allowed to stand for half an hour after being made, so that the layer of air bubbles which came to the top could be removed. If this precaution were not taken, a thick crust formed which rendered an even mixing of the cream impossible.

Beef, mutton and bacon fats were prepared in the following way. Any lean was removed and the fat was cut into small pieces and heated in a gas oven for ½–¾ hr., after which the liquid fat was poured off, allowed to cool and used for the experiments.

In making the creams the fat was melted and the milk was heated to boiling point; the machine was also heated.

Butter cream was made by pumping the mixture once through the machine. With all the other fats the mixture was pumped through three times to ensure complete emulsification, because it was found that some fats (especially olive oil and lard) were apt to oil out on top if only put through once. Some preliminary experiments were made with creams which had been put through the machine six and nine times. These creams, however, digested at the same rate as those which had been put through only three times, therefore it seemed unnecessary to take this extra precaution. Microscopic examination showed that all the creams consisted of very small fat globules, and no differences in the sizes of the fat droplets could be detected.

*Method of experiment*

The commercial form of pancreatic substance used as a source of lipase was that found by Cilliard [1931] to be suitable for this type of work. In a typical experiment 50 ml. cream, 175 ml. distilled water and 25 ml. of 2·4 % pancreatic substance (the exact strength depending on the activity of the sample) were used.

The cream was measured in a 50 ml. graduated cylinder, and washed into a flask with the distilled water, the pancreatic substance being added last. The reagents were mixed cold, not warmed separately as is usual in digestion experiments.

The sample of lipase used was active at room temperature (19–19·5°), and in some cases digestion started so rapidly that the initial stages would not have been recorded had the reagents been at 38° when mixed.

Immediately after mixing 20 ml. were taken for titration, further 20 ml. samples being removed every 15 min. To the 20 ml. digestion mixture were added approximately 150 ml. boiling distilled water and 2 ml. phenolphthalein and the whole was titrated hot with 0·1 *N* NaOH. The flasks were placed in a large water bath and the temperature was maintained at 38–39°.

Butter cream was used as a standard for comparison and control experiments were made with milk and with boiled pancreas substance and cream. The fats

used were almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, castor oil, cocoabutter, coconut oil, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, palm kernel oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil.

Blank titrations of the creams alone gave 0·25–0·3 ml. of 0·1 *N* NaOH for butter. The other creams ranged from 0·2 to 0·3 ml. except beef oleo, premier jus and castor oil which gave slightly higher values, 0·4–0·45 ml. The slightly higher figure for the first titrations of some creams is due to the rapid start of digestion. Palm oil had an appreciably higher value, 1·8 ml., and owing to its yellow colour, it was difficult to judge the end point of the titration.

## Results and comments

Butter cream was taken as a standard for comparison and each experiment included three or four of the other creams. These were arranged in different combinations and at least three experiments were made with each.

A comparison of lard, fat from smoked bacon and fat from green bacon showed that there was no difference in the rates of digestion of these three fats. Fat prepared from beef suet and from scrap beef fat also gave similar digestion curves, as did mutton suet fat and scrap mutton fat, so it was concluded that it was unnecessary to use more than one type of fat from any animal.

When it was found that beef and mutton fats were digested so slowly when compared with butter fat, it was thought possible that heating the fats might have had some detrimental effect. In order to see if this supposition were true two samples of butter were heated in a gas oven, one sample at the same time as some beef suet, and the other sample with some mutton suet. The digestion of the fat in creams made from these heated butters showed no alteration in rate, therefore it was unlikely that heating the beef and mutton fats was the cause of the slower digestion.

The majority of the fats used were digested by lipase much more slowly than was butter fat, three only showing a more rapid rate of digestion. Coconut oil was digested more rapidly than any other fat: three separate samples were used and all gave similar digestion curves. Palm kernel oil also showed a rapid rate of digestion, but not so rapid as the coconut oil. Castor oil was digested more rapidly than butter, but the curve obtained with this fat was of rather a different type from the other curves.

Almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, cocoabutter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil were digested at a much slower rate than butter and all at approximately the same rate. This is in agreement with the experiments of Gardner & Fox [1919] who found that cocoabutter was rather less digestible than butter. Palm oil digested slowly, but could hardly be compared with the others, owing to the difficulty in judging the end point because of the orange colour of the fat itself. The experiments, however, served to demonstrate that palm kernel oil belongs to the group of fats which are digested slowly by pancreatic lipase.

That coconut oil is digested so much more quickly than butter may be of dietetic interest in that this oil is said to be used for the making of cream buns. It seems possible that if margarines contain this fat they may be more rapidly digested than butter. Except in the case of castor oil, the rate of digestion runs parallel with the saponification value of the fats. Thus of the fats used, coconut oil has the highest saponification value [Bolton, 1928] and is the most rapidly

digested. According to Lewkowitsch [1923] palm kernel oil is very nearly related in its chemical composition to coconut oil and its digestion curve (Fig. 1) is nearer to that of coconut oil than that of any other fat. Wright & Mitchell [1921] state that in butter and coconut oil fatty acids of low molecular weight

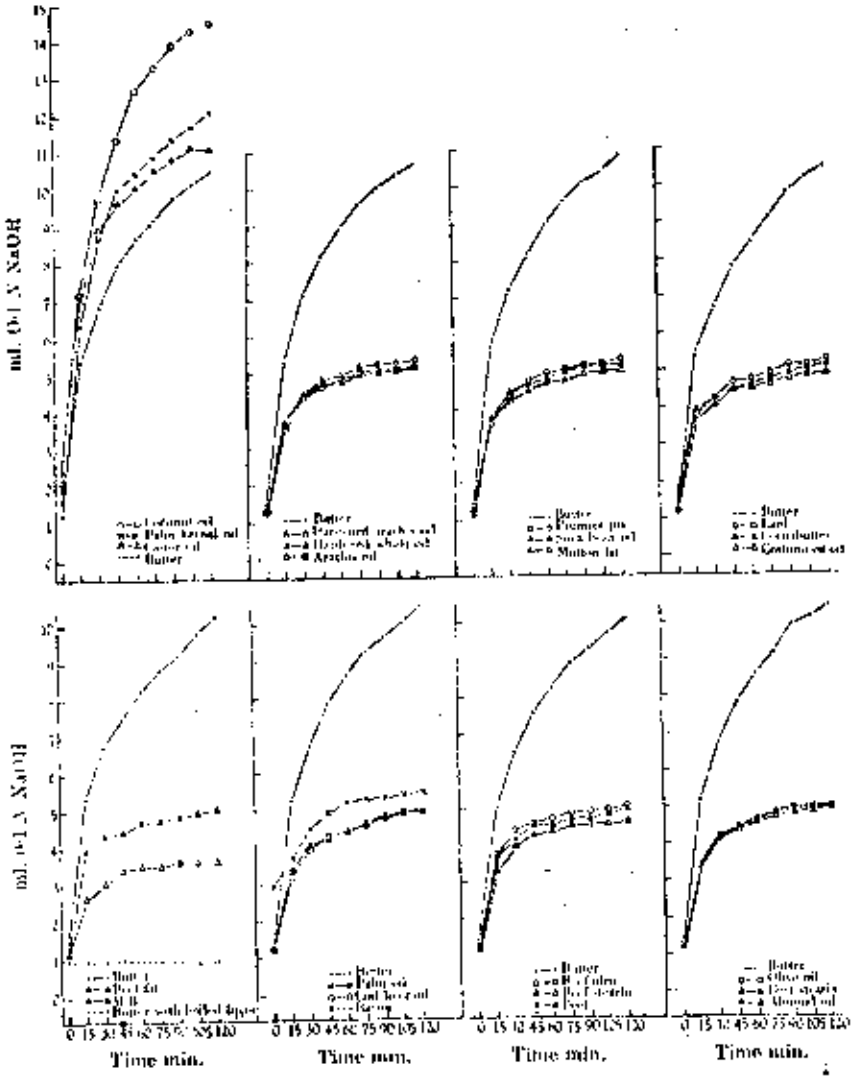


Fig. 1.

are present "in some notable extent", while as a general rule natural oils and fats rarely yield fatty acids of that description. It is possible that this may be correlated with the more rapid rate of digestion of coconut oil and butter fat by lipase. Evans *et al.* [1931] put coconut oil first in order of fats able to spare vitamin B. If, as suggested by Burr *et al.* [1932] linoleic is an essential fatty

acid, then coconut oil which contains 1% (Chemists' Year Book 1934) and is rapidly digested, may prove a more valuable food than has hitherto been supposed.

## Summary

1. Coconut oil was digested by pancreatic lipase more rapidly than any other fat.

2. Palm kernel oil and castor oil were digested more rapidly than butter fat, while almond oil, arachis oil, bacon fat, beef fat, beef oleo, beef stearin, cocoabutter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, premier jus, soya bean oil, hardened whale oil and hardened arachis oil were digested appreciably more slowly, and at approximately equal rates.

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# THE EFFECT OF COD-LIVER OIL ON TUMOR GROWTH[1]

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## INTRODUCTION

Sugiura and Benedict (1925) fed cod-liver oil and various vegetable oils and found them without influence on the percentage of takes obtained or on the rate of growth of the Flexner-Jobling rat carcinoma or the Bashford and Twort mouse carcinoma. Preliminary experiments in connection with the effect of dietary fats on growth and composition of tumors (Haven, 1935) had indicated that cod-liver oil inhibits the growth of a rat carcinosarcoma. This fact is further substantiated in the present paper.

## EXPERIMENTAL

*Tumor:* The tumor used throughout the subsequent experiments was rat carcinosarcoma 256 obtained from the Institute of Cancer Research, Columbia University. Young male rats weighing from 70 to 100 gm. were inoculated subcutaneously in the groin by the trocar method. In each of the experiments all the rats were inoculated from the same tumor.

*Diets:* The diet used was Diet Number 262 (Sinclair, 1931) having the following percentage composition:

| | Casein | Cane Sugar | Salt Mixture * | Fat | Yeast |
|---|---|---|---|---|---|
| Per 100 gm. | 21.8 | 41.0 | 4.8 | 21.3 | 11.1 |
| Per 100 cal. | 18.0 | 34.0 | | 40.0 | 8.0 |

* McCollum, E. V., and Simmonds, N.: J. Biol. Chem. 33: 63, 1918.

The fats used were cod-liver oil of iodine number 157 and, for comparison, coconut oil of iodine number 9. When coconut oil was fed, vitamins A and D were supplied by dissolving the unsaponifiable matter from 5 gm. of cod-liver oil in each 100 gm. of fat.

In each of the experiments half of the rats were placed on the cod-liver oil diet and half on the coconut oil diet on the day of inoculation and kept on this diet until the tumors were removed.

*Measurement of Tumor Growth:* The method of measurement of tumor growth involved weighing the tumor at the time of removal from the animal which, in the majority of cases, was from five to seven weeks after inoculation. To permit comparison of the growth of a tumor of one age with that of a

[1] The material presented in this paper is taken from a thesis submitted by the author to the faculty of the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. (Received for publication, January 1936.)





tumor of another age, the growth tendency of each tumor has calculated by means of a formula of Bierich and Lang (1933) involving the weight of the tumor in grams upon its removal from the rat and its age in days from the date of inoculation, namely, $Wt. = a.t^2$; a (growth tendency of the tumor) $= \frac{\text{weight}}{\text{time}^2} \times 10{,}000$.

TABLE I: *Comparative Growth Tendency of Rat Carcinosarcoma 256 on Coconut Oil and Cod-Liver Oil Diets*

| Experiment | Dietary Fat | No. Animals | Average Growth Tendency |
|---|---|---|---|
| 1 | Coconut | 4 | 112 |
| | Cod-liver | 5 | 84 |
| 2 | Coconut | 9 | 145 |
| | Cod-liver | 3 | 117 |
| 3 | Coconut | 5 | 151 |
| | Cod-liver | 4 | 104 |
| 4 | Coconut | 3 | 172 |
| | Cod-liver | 5 | 128 |
| 5 | Coconut | 1 | 125 |
| | Cod-liver | 3 | 75 |
| 6 | Coconut | 3 | 130 |
| | Cod-liver | 7 | 115 |
| 7 | Coconut | 12 | 189 |
| | Cod-liver | 13 | 130 |
| 8 | Coconut | 7 | 203 |
| | Cod-liver | 10 | 169 |
| av. | Coconut | 44 | 164 |
| | Cod-liver | 50 | 124 |

TABLE II: *Average Residual Body Weights, Grouped According to Tumor Age*

| Dietary Fat | Tumor Age (days) | No. Animals | Body Wt. (gm.) | Tumor Wt. (gm.) | Residual Body Wt. (gm.) |
|---|---|---|---|---|---|
| Coconut | 30–38 | 15 | 199 | 22.5 | 177 |
| | 39–48 | 15 | 199 | 31.3 | 168 |
| | 49–57 | 14 | 204 | 38.8 | 165 |
| Cod-liver | 28–38 | 21 | 197 | 14.4 | 183 |
| | 39–48 | 21 | 188 | 26.3 | 162 |
| | 49–75 | 8 | 211 | 27.8 | 183 |

*Results:* Table I shows the average growth tendency of rat carcinosarcoma 256 on coconut and cod-liver oil diets as determined in seven different experiments carried out over the course of a year. The fluctuations in growth energy exhibited by a transplantable tumor account for the fact that the average growth tendency for coconut oil tumors in several of the experiments is below that for the cod-liver oil tumors in other experiments. It is significant, however, that in each experiment, the average growth tendency of the tumors in animals on the cod-liver oil diet is lower than that of the tumors in animals on the coconut oil diet. The average tumor growth tendency of the 50 animals on the cod-liver oil diet was 76 per cent of that of the 44 animals on the coconut oil diet.

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In Table II the animals on each diet have been grouped according to tumor age. Comparison of the average tumor weight in each age group on the coconut oil diet with that in the corresponding group on the cod-liver oil diet reveals the poorer tumor growth on the cod-liver oil diet without the use of the formula for calculation of growth tendency. The residual body weight is obtained by subtracting the average tumor weight of each group from the average body weight.

## Discussion

Hartwell (1927) and Harris and Moore (1928, 1929) reported that the incorporation of 12 to 15 per cent cod-liver oil in the diet inhibited the growth of rats. Such inhibition of growth of the host animal might account for the

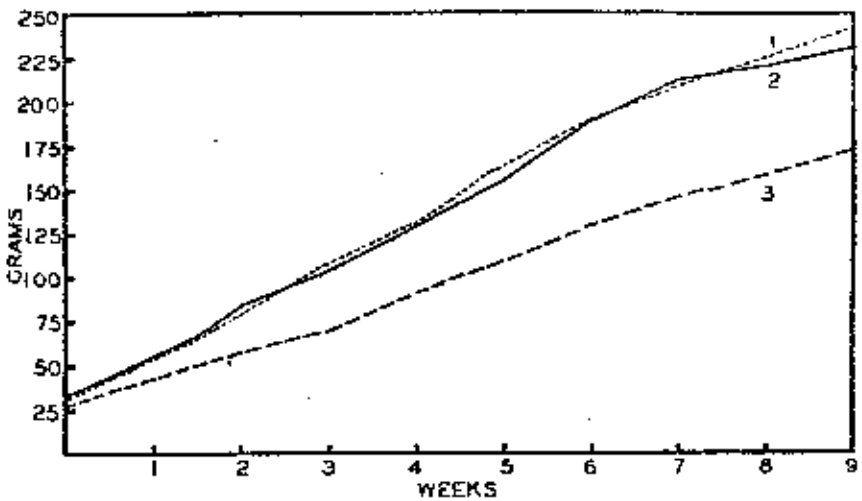


Fig. 1. Chart Showing the Increase in the Body Weight of Uninoculated Male Rats Placed on Diets at Weaning

Ordinate = Body weight in gms. Abscissa = Time in weeks from weaning. Curve 1, coconut oil diet; Curve 2, cod-liver oil diet; Curve 3, normal male rat growth curve (Donaldson: The Rat, Ed. 2, 1924, p. 175).

differences in tumor growth on the two diets. However, when the values for the body weights of the rats minus the weights of the tumors or residual body weights on the two diets are compared (Table II), they are found to be essentially the same for each tumor age group. Thus the cod-liver oil diet has retarded the growth of the tumor as such rather than exerting an effect on the tumor by a deleterious effect on the host animal.

Further confirmation of this point was obtained by growth studies of uninoculated rats on the two diets. In Fig. 1 are given the composite growth curves of rats on coconut oil and on cod-liver oil diets. Twelve young male rats were divided into two equal groups at weaning so that the rats in one group were litter mates of those in the other group. One group of rats was placed on the coconut oil diet, the other on the cod-liver oil diet. Comparison of the growth curve of the coconut oil group (Curve 1) with that of the cod-



liver oil group (Curve 2) reveals the fact that growth on the two diets was practically identical.

The possibility that the difference in tumor growth on the two diets might be due to a difference in vitamin content of the diets must be borne in mind. The coconut oil diet was adequate in respect to vitamins A and D due to addition of the unsaponifiable fraction of cod-liver oil. However, the cod-liver oil diet provided more of these vitamins than the supplemented coconut oil diet. Excess vitamin A was found by Ihara (1931) to promote the growth of a rat carcinoma. The results of our experiments would lead to the opposite conclusion. Vitamin D, on the other hand, has been reported by Sumi (cited by Shear, 1933) to exert no perceptible influence on the growth of a transplanted rat tumor. The inhibitory effect of cod-liver oil on tumor growth might be explained by the fact that vitamin D increases the "net absorption" of calcium, and calcium has been reported by many investigators to exert a slight retarding effect on tumor growth (Shear, 1933). In one experiment using the cod-liver oil diet alone, the addition of calcium gluconate to the diet of half of the tumor animals produced a slight acceleration rather than an inhibition of tumor growth.

Aside from vitamin content, coconut and cod-liver oil differ in their degree of unsaturation or iodine numbers. It is improbable that this difference would account for the effect on tumor growth, since linseed oil of iodine number 172 (cod-liver oil = 157) has been found to be devoid of any inhibitory effect (unpublished data). A third difference between coconut and cod-liver oil is in the length of chain of their constituent fatty acids, coconut oil being composed largely of short-chain acids and cod-liver oil of long-chain acids. In fact, cod-liver oil contains an abundance of $C_{20}$ and $C_{22}$ acids, which are absent from both coconut and linseed oils. It is possible that these long-chain acids might exert an inhibitory effect on tumor growth.

## Summary

1. The rate of growth of rat carcinosarcoma 256 on a cod-liver oil diet is notably less than on a coconut oil diet.

2. The difference in tumor growth on the two diets is not due to differences in growth of the host animals since the growth curves of uninoculated rats on the two diets have been shown to be essentially the same and normal.

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# Nutritional and metabolic interrelationships between fatty acids[1]

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THE INFLUENCE of dietary components upon the composition of depot fat of animals has been known for generations. It is common knowledge that swine fed starchy cereals develop a hard fat, whereas peanut-fed swine develop a liquid lard. The interrelationships between composition of dietary fat and the composition of animal fats have been studied repeatedly by empirical feeding trials involving different fats and, more recently, by more precisely controlled nutritional experiments, and this information already comprises a large literature. The transition from practical trial to detailed knowledge has encompassed several decades and has moved in proportion to the precision of the analytical methods available. In recent years the availability of gas chromatographic methods has made possible the detailed study of the composition of complex fats, and simultaneously the advent of methods of preparation of pure fatty acids on a large scale has made possible nutritional study involving single fatty acids and combinations of pure fatty acids. The result of these developments is that at present one can resolve the effects of dietary fat into several effects of dietary fatty acids. It has been possible to study the metabolism of single fatty acids and to study the influences of each fatty acid upon the metabolism of others. This field of study is still in infancy and the results available thus far encompass only a few of the numerous fatty acids found in foodstuffs. An attempt will be made here to treat the interrelationships thus far known to exist between polyunsaturated acids (PUFA) and between these, monoenoic acids, and the saturated acids.

## INTERRELATIONSHIPS BETWEEN POLYUNSATURATED FATTY ACIDS

Shortly after Burr and Burr (3) described the essential fatty acid (EFA) syndrome, Nunn and Smedley-MacLean (25) investigated the fatty acids of the liver of EFA-deficient rats. They discovered that although the highly unsaturated acids of livers of normal rats reacted with bromine to produce octabromoarachidic acid, the liver unsaturated fatty acids from deficient rats yielded polybromides with a lower bromine content. This they deduced to be due to the presence in the deficient animal of what they called dihydroarachidonic acid. This trienoic acid was found to increase sharply in several tissues of EFA-deficient rats by Rieckehoff et al. (31), using the alkaline isomerization procedure developed in the 1940's for analysis of polyunsaturates. Later, Fulco and Mead (10) found this acid to be 5,8,11-eicosatrienoic acid and showed it to be a metabolite derived from oleic acid. The appearance of this trienoic acid in deficient animals and its suppression by the administration of linoleate probably constitutes the first observations of an interrelationship between specific fatty acids.

The development of alkaline isomerization permitted study of the fate of dietary PUFA in the animal. Rieckehoff et al. (31) showed that the PUFA composition of the lipids of several organs of the rat were related to the dietary fat. For example, the feeding of corn oil to rats increased markedly the tetraenoic acid content of tissue lipids, and feeding cod liver oil altered the composition of tissue lipids in quite a different way. The deposition of pentaenoic and hexaenoic acids was so marked that the lipids of the heart approached cod liver oil in respect to its content of those acids. This study was extended by Widmer and Hofman (36) to a study of the changes occurring when single unsaturated fatty acids were fed to rats receiving no other fat. Their results indicated that the tetraenoic acid, arachidonic acid, was formed from dietary linoleate and that pentaenoic and hexaenoic acids appeared in the animal when linolenate was fed. Thus, the suspected relationship of linoleate and arachidonate was confirmed and the relationship of linolenate to pentaenoic and hexaenoic acids was indicated. The study also revealed that linoleate and linolenate were not interconverted, for the metabolites from one did not appear when the other was the only dietary fatty acid.

The metabolic relationships suggested by nutritional and compositional studies have been confirmed by elegant studies in which radioactive fatty acids were used. By this means the metabolic conversion of linoleic to arachidonic acids has been proven by Steinberg et al. (32). The comparable conversions of linolenate to

[1] This work was supported in part by Research Grant AM-04724 from the National Institutes of Health.

apentaenoic and docosahexaenoic acids have been in as well (16, 33, 36). Some steps in the metabolic sequences remain to be confirmed, but the general outlines of the interconversions are now well known.

Much of the nutritional experimentation which led to the knowledge of the essentiality or nonessentiality of individual acids and their isomers and to the metabolic conversions of polyunsaturated acids in animals was by means of comparisons of nonsupplemented animals with animals supplemented with single dose levels. This approach does not allow description of how tissue lipid composition varied with changes in the composition of diet. The usefulness of the latter approach was inadvertently revealed through an experiment in our laboratory designed to be a multiple comparison of supplements fed at single dose levels. The interpretation of the results was very confusing until it was realized that the several supplements could be considered as multiple dose levels of linoleate (12). This realization has led to a program of study of dose-response curves for several single pure unsaturated fatty acids and for combinations of them. These studies shall be the major portion of the discussion to be presented here.

Despite the use of three levels of total dietary fat and three quite different dietary fats, the influence of dietary linoleate upon the content of dienoic, trienoic, and tetraenoic acids in the lipids of supplemented rats was found to follow exponential curves (19). In order to combine the responses of trienoic acid and tetraenoic acids in a single expression related to deficiency and in order to remove variation due to differences in extraction and handling of lipids, the ratio of triene to tetraene in the tissue lipids was plotted against dietary linoleate. Triene and tetraene have the inherent advantage that neither is a significant component of common dietary fats, and both are metabolic products, one from oleate, the other from linoleate. When this was done, the curve described a hyperbolic function, one leg of which represented high values in deficiency and the other low values in well-supplemented animals. The curve was used to set the minimum requirement for linoleate, which lies between 1 and 2% of calories for rats (12), swine (11), and infants (13).

The dose-response curves have also been obtained for linoleate, linolenate, and arachidonate fed as single pure supplements, and the curves have been described for liver (20), heart (21), brain (22), and erythrocytes and adipose tissue (23). The family of curves for heart polyunsaturates in linoleate-supplemented rats is shown in Fig. 1. In this study the conversion of linoleate to 20:4ω6 and 22:5ω6 was clearly demonstrated. Likewise, linolenate is converted primarily to 20:5ω3, 22:5ω3, and 22:6ω3.[1] The nonconvertibility of members of the linoleate family to members of the linolenate family and vice versa was also confirmed conclusively.

[1] The notation used here is required to distinguish the various isomers of PUFA and indicate relationships. The first number indicates chain length, the second indicates the number of double bonds, and the number following the omega indicates the position of the last double bond, counting from the terminal methyl groups.

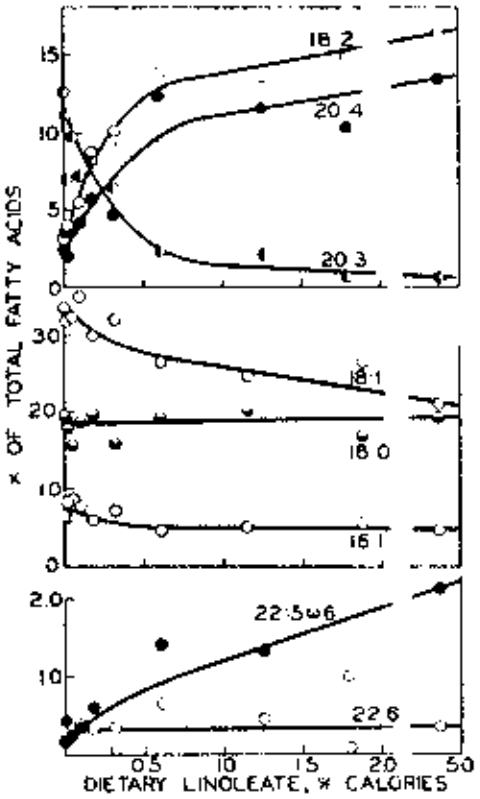


FIG. 1. Relationship of fatty acid composition of heart lipids to level of dietary linoleate in the rat (21).

The study also indicated that the PUFA of rat tissues belongs principally to the linoleate, linolenate, and oleate families although other minor groups are present.

In these several studies a depression of metabolites of linoleate by high levels of dietary linolenate is also indicated. Depression of PUFA of one family by feeding members of another family has been observed several times previously (5, 9, 14, 17, 28, 29, 31, 36), but the significance of the observations was not then apparent. Dhopeshwarkar and Mead (7) postulated an inhibition of the metabolism of linoleate by oleate, and Marco et al. (19) and Macklin (18) observed a depression of arachidonate by dietary linolenate and postulated a competitive inhibition between linolenate and linoleate in their metabolisms. Bazan and Coniglio (1, 2), Edwards and Marion (8), and Mohlhauer and Holman (20) have postulated that linoleate, linolenate, and arachidonate competed with oleate metabolism to depress the synthesis of eicosatrienoic acid.

Once the competitive inhibition in the metabolism of PUFA was recognized, experiments planned for the study of the phenomenon were necessary. A repetition of the observations of Marco et al. and Macklin, in which linolenate was the antagonist of linoleate metabolism, were studied first, by using purified linoleate and linolenate in appropriate ranges of dietary intake (24). At three levels of dietary linoleate (0.2, 0.3, and 0.6% of calories) the metabolism of linoleate to arachidonate

was significantly and consistently depressed by increasing levels of dietary linolenate. Figure 2 shows the effect of level of linolenate intake upon the metabolites of linoleate measurable in liver total fatty acids. In addition to the depression of arachidonate content of liver lipids, an equally significant depression of the content of 22:5ω6 was also observed. The effect upon 20:3ω6 was minimal, but the accumulation of 18:2ω6 itself, as reported by Machlin, is clearly seen. These results indicate that the higher metabolites of linoleate are inhibited most and that linoleate itself accumulates under conditions of inhibition.

In a short-term study of the inhibition of arachidonate metabolism by linolenate, similar results have been observed (Rahm and Holman, unpublished data). In EFA-deficient rats which were fed a constant level of

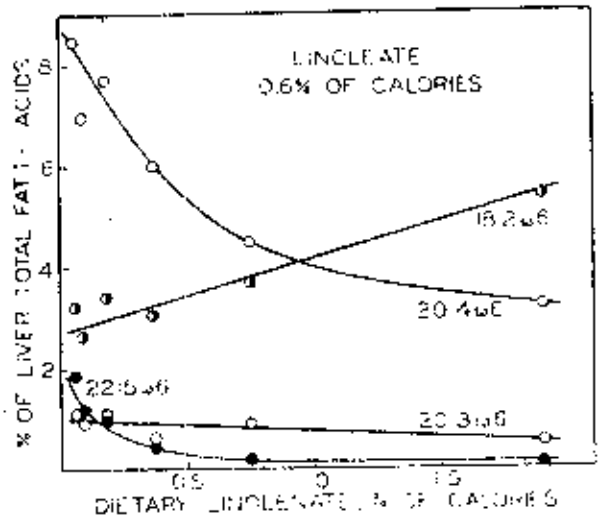


FIG. 2. Effect of dietary linolenate upon metabolism of linoleate (24).

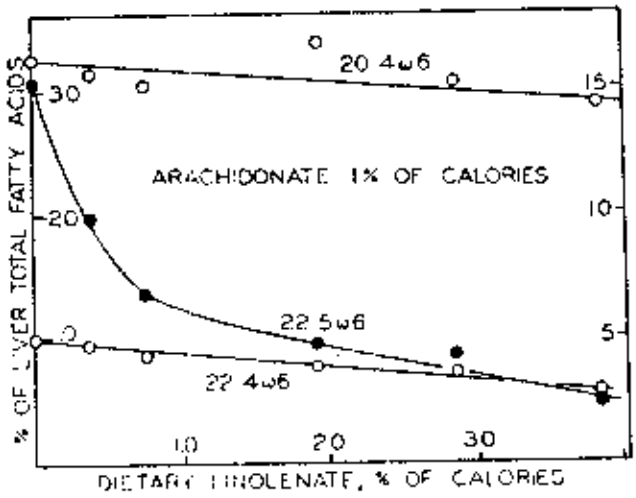


FIG. 3. Effect of dietary linolenate upon metabolism of arachidonate as demonstrated in a short-term experiment. Rahm and Holman, unpublished data.

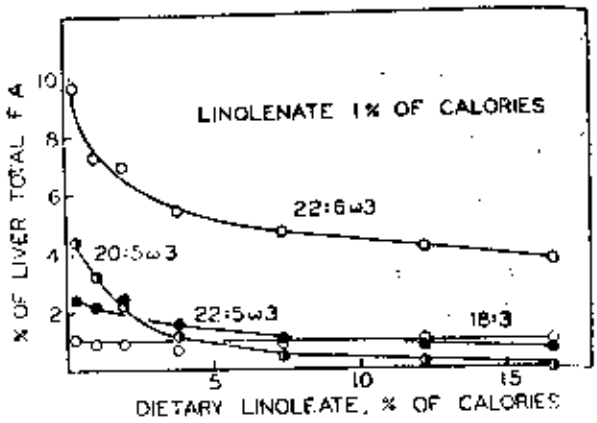


FIG. 4. Effect of linoleate upon metabolism of linolenate. (Rahm and Holman, unpublished data).

arachidonate for 5 days, a second supplement of linolenate caused inhibition of its conversion to 22:4ω6 and 22:5ω6. These results are shown in Fig. 3. In this study arachidonate did not accumulate as a consequence of inhibition of its metabolism. The study revealed that inhibition at the later steps of metabolism is not due only to inhibition at earlier steps. Linolenate apparently competes at more than one step in the metabolic chain.

If this phenomenon is indeed competitive inhibition, then inhibition of linolenate metabolism by linoleate should be demonstrable. This had been indicated fortuitously in one of the earlier studies (30). Therefore, an experiment was planned to test this possibility. Linolenate was fed at a constant level of 1% of calories to all groups of weanling male rats, and the groups were given different levels of dietary linoleate. The effect upon the metabolites of linolenate is shown in Fig. 4. The concentration of linolenate itself seems unaffected by levels of linoleate which cause striking depressions in the concentrations of the metabolites of linolenate. All three of the measurable linolenate metabolites, 20:5ω3, 22:5ω3, and 22:6ω3, were inhibited by dietary linoleate. Thus, the inhibition of linoleate metabolism by linolenate is paralleled by the inhibition of linolenate metabolism by linoleate. The inhibition is competitive.

The competitive phenomena observed between the linoleate and linolenate families of polyunsaturates are not mere dilution effects. If that were so, the appearance of, for example, linolenate and its metabolic products in the lipids of a tissue should diminish the concentrations of the metabolites of linoleate in a proportional manner. This is not the case, for as dietary linolenate is increased, linoleate itself increases, whereas 20:4ω6 and 22:5ω6 decrease exponentially in a parallel fashion, and 20:3ω6 decreases only slightly in a linear fashion (Fig. 2). The same conclusion can be drawn from an examination of the successive precursor:product ratios for the metabolite sequences (15).

A comparison of the curves in Figs. 2, 3, and 4 suggests that perhaps the chain-lengthening steps in the metabolism are inhibited to a different degree than are

...dehydrogenation steps. The information is not complete on this point, and the relative effects of inhibition of individual steps in the chain cannot be deduced from steady state concentrations. Nevertheless, the steady state concentrations of those substances formed by dehydrogenation steps respond to dietary inhibition in a sharply exponential manner, whereas those formed by chain lengthening respond weakly in a seemingly linear fashion. This point should be investigated with labeled fatty acids.

### MONOENE-POLYENE RELATIONSHIPS

Similar attempts have been made to describe the competition between oleate and linoleate (Rahm, Mohrhauer, and Holman, unpublished results). Groups of rats were fed either 0, 0.2, or 1.0% of calories of methyl linoleate, and subgroups were fed levels of methyl oleate varying from 1 to 7.6% of calories. The effects of 100 days of such dietary treatment upon the content of 20:4ω6 and 22:5ω6 in heart and liver lipids was minimal. However, when the effects upon 20:3ω9 and 20:4ω6 were combined in the 20:3–20:4 ratio, the inhibition by oleate is apparent (Fig. 5). At the zero level of dietary linoleate, the residual 18:2 in the deficient animals is variable and sufficiently low to permit a heightening of the triene:tetraene ratio by supplements of oleate. At 0.2% of calories of linoleate, the effect is imperceptible, but at 1% of linoleate calories the effect is not exerted.

Dhopeshwarkar and Mead reported that dietary

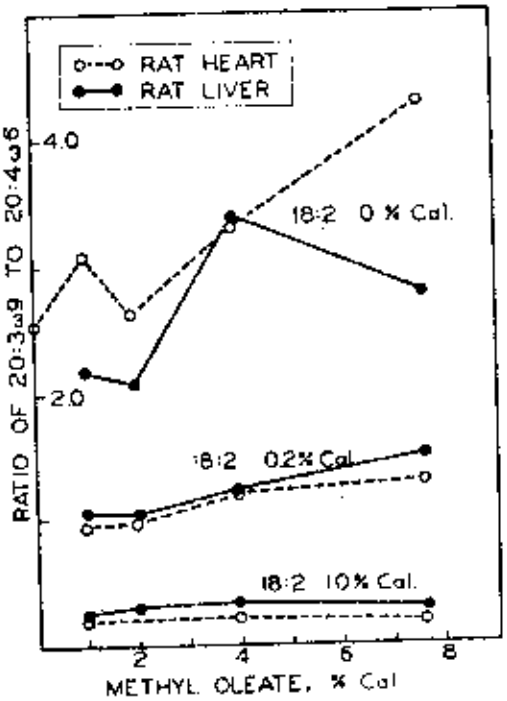


FIG. 5. Inhibition of linoleate metabolism by oleate as revealed by an examination of the triene:tetraene ratio in heart and liver lipids of the rat. (Rahm and Holman, unpublished data.)

TABLE 1. *Dietary levels of fatty acids required to induce 50% inhibition of metabolic reactions*

| Inhibited Reaction | Inhibitor | Calories | Reference |
|---|---|---|---|
| 18:2ω6 → 22:5ω6 | 18:1ω9 | [illegible] | |
| 18:3ω3 → 22:6ω3 | 20:2ω6 | 5.4 | |
| 18:2ω6 → 22:5ω6 | 18:3ω3 | [illegible] | 143 |
| 20:4ω6 → 22:5ω6 | 18:3ω3 | [illegible] | |
| 18:1ω9 → 20:3ω9 | 18:2ω6 | [illegible] | 144 |
| 18:1ω9 → 20:3ω9 | 20:4ω6 | [illegible] | 197 |
| 18:1ω9 → 20:3ω9 | 18:3ω3 | [illegible] | 191 |

oleic acid induced an EFA deficiency (57). A similar study from this laboratory has shown that 10% of olive oil as sole dietary fat raises the triene:tetraene ratio in liver lipids and that more stainable fat was observed in the livers and the kidneys of the animals (62). These effects are consistent with marginal EFA deficiency. Tinsley (135) has observed that in rats fed pork diets which varied in oleate content, the fatty acid composition of the rat liver lipids varied in relation to the oleate intake. Moreover, there was a strong correlation between the oleate level in the liver lipids of the rats and the ratio of arachidonate to linoleate (product:precursor ratio). This suggests that the level of dietary oleate has an influence on the conversion of linoleate to arachidonate. Tinsley recently has reported (134) that as the proportion of dietary oleate increased to about 7% of the diet (about 15% of calories), the triene:tetraene ratio was observed to increase fourfold in heart phospholipids and liver mitochondria without any observable gross changes in the animals' performance.

The antagonistic effects of oleate upon linoleate metabolism appear to be minimal effects, produced only by high dietary levels of oleate or high oleate:linoleate ratios. Attempts in this laboratory to induce suppressions of linoleate metabolites of the order observed in inhibitions between linoleate and linolenate have thus far failed, although triolein has been fed at levels as high as 22% of calories. The only observable effects of oleate at dietary levels less than this limit are changes in the triene:tetraene ratio. The development of changes in composition indicative of EFA deficiency was stimulated only by very high ratios of dietary oleate to linoleate at comparatively low absolute levels of linoleate. This is demonstrated in Fig. 5.

The relative competitive abilities of oleate, linoleate, arachidonate, and linolenate are shown in Table 1. From the appropriate dose response curves for final metabolite shown in Figs. 2–4, and from other data from this laboratory, the levels of dietary inhibitor were chosen at which 50% inhibition of the overall reaction occurred. In the instance of oleate, the value was found by extrapolation from a 15% inhibition at 21.6% of calories (Rahm and Holman, unpublished data). The data clearly indicate that oleate is the weakest inhibitor, that is, very high dietary levels are required to exert its effect. Linolenate is the most active inhibitor. Only 0.1% of calories of linolenate are required to inhibit the metabolism of linoleate to 50%, whereas in the converse 3.2% of calories of linoleate are required.

When a series of natural polyunsaturated acids was

fed at 0.8% of calories as sole supplements to a fat-free diet, the suppression of the conversion of oleate to 20:3ω9 was somewhat in proportion to the degree of unsaturation (29). This is shown in Table 2. The inhibition exhibited by the natural ω6 and ω3 acids and the relatively low activities of 19:2ω6 suggests that the terminal structure may not be the definitive feature in the metabolic competitive inhibition. There is no strict correlation between EFA activity and 20:3ω9 suppression.

In the metabolism of oleate, linoleate, linolenate, and also other minor families of fatty acids, the fatty acids can undergo either chain lengthening or dehydrogenation, and either reaction can proceed first. This is illustrated in Fig. 6 for the metabolism of linoleate. In the metabolic mechanism depicted, all of the products of linoleate shown have been found in nature. Moreover, all have been found by gas chromatography in one tissue, in beef testis tissue, and all except 18:3ω6 in sufficient quantity for isolation and proof of structure by ozonolysis (Holstetter and Holman, unpublished

TABLE 2. *Suppression of metabolism of oleate and relieving of dermal signs of EFA deficiency by 0.8% of calories of several polyunsaturated acids**

| Fatty Acid | Suppression of 20:3ω9, % | Relief of Dermatitis, % |
|---|---|---|
| 18:2ω6 | 65 | 100 |
| 18:3ω3 | 81 | 77 |
| 20:4ω6 | 91 | 100 |
| 20:5ω3 | 92 | 69 |
| 22:6ω3 | 88 | 35 |
| 19:2ω6 | 28 | 65 |

* Data from (29).

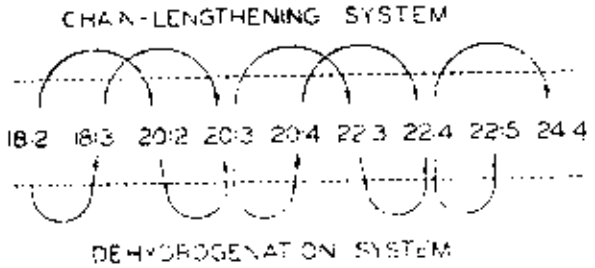


FIG. 6. Metabolism of linoleate, showing major possible pathways of chain elongation and dehydrogenation. All metabolites depicted have been isolated from testis tissue.

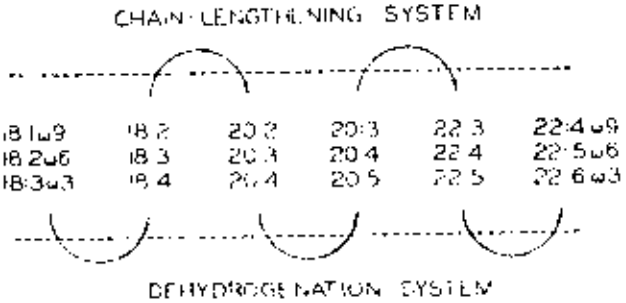


FIG. 7. Competitive metabolism of oleate, linoleate, and linolenate.

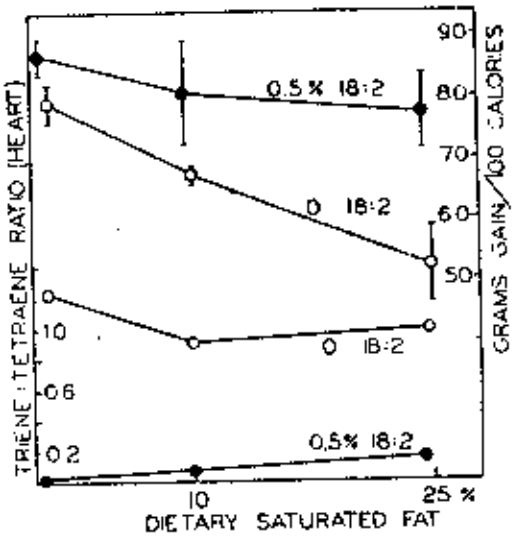


FIG. 8. Influence of dietary saturated fat upon caloric efficiency and triene:tetraene ratio of heart in rats fed diets containing zero or 0.5% linoleate (27).

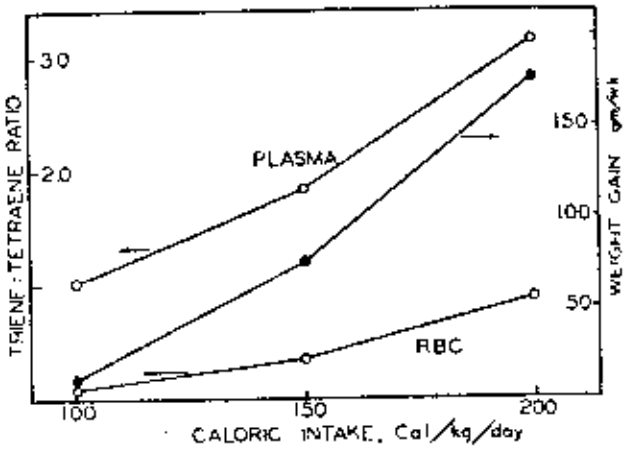


FIG. 9. Relationships between total calories, growth, and plasma triene:tetraene ratio in dogs. From data of Patil and Hansen (26) and Wiese et al. (37).

data). The competitive inhibition which exists between oleate, linoleate, and linolenate is depicted for alternate dehydrogenation and chain lengthening along the principal pathway in Fig. 7. Members of all three families exist in the metabolic pool at each step in the sequence, and competition may be exerted between these and between these and other members of the sequences. In the absence of linoleate and linolenate (EFA deficiency), the conversion of endogenous 18:1ω9 to 20:3ω9 and 22:3ω9 becomes dominant. When linoleate is present, its metabolic products are formed and suppress those of oleate. When linolenate is also present, its conversion takes precedence, and the metabolism of linoleate is damped. Thus, the composition of the tissue PUFA is dependent upon the dietary supplies of their precursors and is regulated by competitive inhibitions of their metabolisms.

### ...TIONSHIPS BETWEEN SATURATED AND UNSATURATED ACIDS

In addition to the competitions between unsaturated fatty acids, dietary saturated fatty acids likewise exert influences upon the polyunsaturated acids of tissues. When the content of saturated fat in the diet is increased and linoleate is held constant, the weight gain of animals is decreased. Figure 8 shows this relationship for rats fed a diet containing either zero or 0.5% linoleate and varying amounts of hydrogenated coconut oil (27). In the linoleate-free diet, caloric efficiency decreased sharply as saturated fat was increased. The same was true for the diet containing the marginal level of linoleate, but the decrease was less. The triene:tetraene ratio of the groups fed linoleate-free diets was consistently high and did not respond to additional nutritional insult. However, at the minimal linoleate level, increased saturated fat produced a small but consistent increase in the triene:tetraene ratio.

The response quoted above for saturated fat may well be merely a response to caloric intake for, the diets fed were not isocaloric nor pair fed. An examination of data of Patil and Hansen (26) and of Wiese et al. (37), gathered in a study on dogs, reveals that the triene:tetraene ratio of the plasma lipids increases as caloric intake and consequent growth are increased (see Fig. 9). A correlation between the dermatitis of fat-deficient rats and their growth has been noted previously (4). The probable explanation for this effect is that when stores of essential fatty acid are limited, the growing organism depletes its supplies for the formation of new cells more rapidly than the organism whose growth is retarded. That is, on diets in which EFA content is marginal, a low caloric intake and low growth induces less severe deficiency than high intake and rapid growth.

From the various evidences reviewed here it is clear that the metabolism of unsaturated acids is influenced by the concentrations and kinds of other fatty acids present in the diet and in the metabolic pool. The metabolic conversions of polyunsaturated acids are dependent not only upon the concentrations of the various precursors, but also upon the concentrations of other families of polyunsaturated acids which act as competitive inhibitors. Thus, the concept of balanced diet must include a consideration of balanced concentrations of the several polyunsaturated, monounsaturated, and saturated fatty acids.

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# The validity of the results obtained when a stream-splitting device is used before the gas-liquid chromatography detector

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We have previously reported using several different approaches to the radioassay of components in the effluent of a gas-liquid chromatographic column (1–4). In this work we found it convenient to record the mass of the components by the use of ionization detectors because of their simplicity and high sensitivity, and particularly because of their relative insensitivity to small temperature changes. Each of the ionization detectors, however, has at least one of several features that might cause difficulty. Different ionization detectors might destroy the sample, or dilute it with large volumes of other gases, or contaminate it with radioactivity, or provide sufficient penetrating radiation to make operation of a sensitive radiation detector in the close vicinity difficult. Most of these difficulties might be avoided by dividing the effluent of the column in a proportion similar to that of the effluent is directed to the mass detector while the other is used for the radioassay.

A device for dividing the effluent is easily constructed. A procedure we have used several times has been to drill a 1/16-in. diameter hole along the axis of a 1-in. long, 1/4- to 1/2-in. diameter stainless steel cylinder; and a 1/16 in. diameter hole in the side of the cylinder to meet the axial hole. A stainless-steel tube is silver soldered into each of the three orifices of the T connection thus formed. A 1/16-in. o.d., thin-walled, stainless-steel hypodermic tube soldered in one end of the cylinder forms the gas inlet. Two lengths of capillary tubing, 0.010 in. i.d., 1/16 in. o.d., one 1 in. long, the other 8 in. long, are used as outlets. The lengths of the outlets are generally chosen to be in inverse ratio to the division desired but final adjustment is made by constricting either tube slightly using diagonal cutting pliers. The rates of carrier gas flow in each limb are then determined and the quantity of radioactivity assayed is corrected, if desired, for the small fraction used for mass detection.

The validity of the assumption that when the carrier gas is divided in a given ratio, each of the individual components being analyzed will likewise be divided in the same ratio or a constant ratio is open to question. The resistance to flow offered by each outlet tube is a function of the flow rate of gas in the tube and the viscosity of the gas as well as of the cross-sectional area of the tube or of the constrictions. Since the viscosity of the gas as well as the flow rate might undergo changes during the course of the elution of a component, the fraction sent through each outlet might vary. It was interesting, therefore, to evaluate the magnitude of the resulting errors.

A stream-splitting device was constructed as described. It was adjusted until the flows through the exit tubes were in the ratio of approximately 10:1. The device was then placed in a detector oven of a gas chromatograph. Its inlet was connected to the outlet of a gas-liquid chromatography column (glass, 4 mm i.d., 5 ft long, filled with Chromosorb W [Johns-Manville Corp., NYC], 80%, coated with ethylene glycol adipic polyester, 10%). Each outlet was connected to two heated inlet of separate Packard Instrument Co. Model 830 gas fraction collectors. Each of the two fractions of the effluent was thus passed through similar cartridges containing anthracene crystals coated with silicone oil for collection and radioassay of components (1). No mass detector was used in these experiments since the column had been used previously at similar pressures and temperatures and its retentivity for methyl esters was known. A series of 5-µl samples of

TABLE 1. [illegible] FLOW BY THE STREAM-SPLITTER DEVICE WITH THE DIVISION OF $C^{14}$-METHYL LAURATE VAPOR [illegible] GAS*

| Sample No. | Carrier Gas Flow (cc/min) A | B | Splitting Ratio A:B | Methyl Laurate Radioactivity (cpm) A | B | Splitting Ratio A:B |
|---|---|---|---|---|---|---|
| 1 | 6.6 | 53.5 | 1:8.1 | 7,926 | 63,801 | 1:8.05 |
| 2 | 6.6 | 55.5 | 1:8.4 | 7,882 | 67,172 | 1:8.52 |
| 3 | 6.6 | 55.5 | 1:8.4 | 7,370 | 61,648 | 1:8.36 |
| 4 | 6.6 | 55.5 | 1:8.4 | 7,656 | 65,031 | 1:8.48 |
| 5 | 6.6 | 55.5 | 1:8.4 | 7,967 | 66,074 | 1:8.30 |
| 6 | 6.5 | 53.6 | 1:8.2 | 8,047 | 65,312 | 1:8.12 |
| 7 | 6.5 | 53.6 | 1:8.2 | 7,923 | 64,507 | 1:8.16 |
| 8 | 6.5 | 53.6 | 1:8.2 | 7,716 | 62,094 | 1:8.14 |
| 9 | 6.5 | 53.6 | 1:8.2 | 8,615 | 67,054 | 1:7.80 |
| 10 | 6.5 | 53.6 | 1:8.2 | 7,570 | 65,580 | 1:8.66 |
| 11 | 6.5 | 53.6 | 1:8.2 | 7,963 | 66,399 | 1:8.33 |
| 12 | 8.5 | 63 | 1:7.4 | 7,801 | 59,975 | 1:7.60 |
| 13 | 9.7 | 70.6 | 1:7.4 | 8,946 | 67,214 | 1:7.55 |
| 14 | 12 | 86 | 1:7.2 | 9,530 | 68,511 | 1:7.20 |

* Each injection consisted of 5 µl of a solution of $C^{14}$-methyl laurate in trimethyl pentane. Injections 1–6 contained less than 1 µg of methyl laurate; 7–11 contained 1 mg; 12–14 show the effect of increasing the total flow of gas. Assayed by liquid scintillation counting at the same voltage, 5 µl contained 76,000 cpm (100,000 dpm).

than 1 µg of methyl ester was injected. Following each injection, the entire effluent of each limb of the stream splitter was collected in a single cartridge. A second series of samples, containing the same amount of radioactivity plus 1 mg of carrier methyl laurate in the 5 µl, was then injected into the column, fractionated, and collected similarly. The flow rate through each tube was determined in triplicate both before and after each analysis using a soap bubble flowmeter. The average value was used. The radioactivity in each cartridge was determined by scintillation counting as described previously (1).

The results are tabulated in Table 1. Within the limits of accuracy of the flow measurements and radioassay, no difference was detected at any of the flow rates tested between the division of carrier gas flows and the division of components, whether the component was low in total mass or whether the concentration of methyl esters in carrier gas was close to the upper limit for this type of column. It should be noted that the ratio of the flow rates in the outlet limbs of the stream divider did not exceed 10:1. A difference between the ratio of carrier gas division and the ratio of component division might be expected to be more evident if the gas stream were divided 100:1 [illegible] have not determined this.

The device that was used offered sufficient resistance to flow in both limbs so that insertion or removal of an anthracene-filled vial in one limb did not alter the flow in the other limb. This property is necessary if the stream-dividing device is to be used followed by any equipment that itself offers a small resistance to flow. As shown in the table, the fraction flowing through each limb was changed by changing the total flow. This finding suggested that the flow through each limb should be determined under actual operating conditions. Since changing the total flow rate of gas changed the splitting ratio, a change in flow rate during the course of an analysis thus is a potential source of error. A significant change in flow rate might be produced by a change in the temperature of the column during the analysis unless care is taken to avoid large changes in flow.

The effect of change in the composition of the gas from argon to argon plus low concentration of methyl laurate to argon plus higher concentrations of methyl laurate was insufficient to produce a measurable change in splitting ratio. It was thought unlikely, therefore, that other methyl esters of fatty acids or other compounds analyzed by gas chromatography when present in ordinarily expected concentrations in the gas would produce a sufficiently greater change in viscosity to produce a different splitting ratio, and we have not explored this possibility.

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# notes on methodology

## Chromatography of unusual lipids on thin layers of magnesium oxide

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SUMMARY The chromatographic behavior of some minor components of natural lipids was studied using layers of magnesium oxide and of commercial adsorbents containing magnesium oxide. The lipids investigated included wax esters, cholesteryl esters, diester waxes, and esters, ethers and ether-esters of ethanediol and glycerol. Pronounced differences were found in the patterns of separation of certain lipids on magnesium oxide as compared to silica gel and Florisil. The procedures described afford means for the detection of unusual lipids in natural materials, and their isolation.

SUPPLEMENTARY KEY WORDS adsorbents · silica gel · Florisil · detection of minor lipids · wax esters · cholesteryl esters · diol lipids · diester waxes · sterols · long-chain alcohols · alkoxylipids

It is well known that in adsorption chromatography the sequence of elution of compounds differing in the types and numbers of functional groups depends to some extent on the nature of the adsorbent (1, 2). In the lipid field, silica gel is the adsorbent used most commonly, and hence the chromatographic behavior of various lipid classes on this adsorbent is well established (3–6). However, certain separations have not been accomplished using silica gel. This has prompted us to investigate the potential of other adsorbents.

We have found that magnesium oxide is especially suitable for the separation of unusual lipids. Magnesium oxide has been employed previously as such (7, 8) or in admixture with other adsorbents (9–12) for the fractionation of alicyclic lipids. To the best of our knowledge, only two reports have appeared on its use in the fractionation of aliphatic lipids (8, 13). However, in the latter studies it has been stated that magnesium oxide does not offer any advantage over silica gel.

The present communication records the application of thin layers of magnesium oxide, and of mixed adsorbents containing magnesium oxide, in the fractionation of classes of lipids which cannot be resolved satisfactorily by thin-layer chromatography on silica gel. Specifically, experimental conditions are described for the separation of wax esters from cholesteryl esters, diesters of ethanediol from triglycerides, and sterols from long-chain alcohols and diglycerides. Furthermore, the chromatographic behavior of alkyl and alk-1-enyl ethers of ethanediol and glycerol, and their acyl derivatives, on layers of magnesium oxide as well as commercial adsorbents containing magnesium oxide is compared with that on layers of Silica Gel G and Florisil.

*Reference Substances.* Refined soybean oil and rapeseed oil were purchased locally. Diesters of ethanediol were prepared by esterifying ethanediol with the total fatty acids derived from soybean and rapeseed oils, respectively. Wax esters, cholesteryl esters, diester waxes, and partial glycerides were prepared following established procedures (14). Syntheses of long-chain alkyl and dialkyl ethers of ethanediol (15) and glycerol (16, 17), trialkyl ethers of glycerol (17) as well as of ether-esters of ethanediol and glycerol (15), and of alk-1-enyl ether-esters of ethanediol (18) were described previously. Synthetic alk-1-enyl ether-esters of glycerol (19) were gifts of Dr. R. Gigg, London, England.

*Adsorbents.* Three different charges of pure magnesium oxide (article no. 28306, Serva Entwicklungslabor, Heidelberg, Germany) were used. According to the supplier, the distribution of particle sizes was 64%, 75%, and 80%, respectively, between 35 and 100 μ. Magnesium oxide for thin-layer chromatography (article no. 5864, E. Merck A.G., Darmstadt, Germany) was used for comparison.

Anasil B (containing 10% plaster of Paris as binder) and Anasil S (without binder) (Analabs Inc., North Haven, Conn.) are preparations of silica gel that are known to contain 15% magnesium oxide.

Silica Gel G (E. Merck A.G.) was used without further treatment.

Florisil (article no. 21528, Serva Entwicklungslabor) was pulverized in a ball mill to yield a product that could be spread as a thin layer.

*Preparation of Layers.* Batches of five glass plates, 20 × 20 cm, were coated with adsorbent layers, 0.25 mm in thickness, unless stated otherwise, using a commercial applicator (Shandon Scientific Co., London, England).

Magnesium oxide, 30 g, was slurried with 55 ml of water. After coating with this slurry, the plates were allowed to stand for 15 min at room temperature, and the layers were activated at 95°C for 30 min.

Layers of Anasil B were prepared from slurries of 30 g of adsorbent with 65 ml of water; for Anasil S, 30 g of adsorbent was slurried with 70 ml of water. Layers of both Anasil B and Anasil S were activated at 130°C for 2 hr.

Layers of Silica Gel G were prepared from slurries of 30 g of adsorbent with 60 ml of water and activated at 130°C for 2 hr.

Florisil layers were made from slurries of 50 g of pulverized adsorbent and 67 ml of water. After partial drying and setting at room temperature for 30 min, the layers were activated at 100°C for 1.5 hr.

*All of the adsorbent layers were used immediately after activation and subsequent cooling to room temperature.*

*Chromatography.* Known amounts of lipids, dissolved in benzene, were spotted onto the adsorbent layers, and the plates were developed by the ascending technique in rectangular jars lined with filter paper. The solvent systems used are recorded in the legend of Fig. 1. After development, the chromatograms were heated for 10–15 min at 95–100°C to remove residual solvents.

*Detection.* On layers of magnesium oxide, lipid fractions usually were detected by spraying with a 0.02% aqueous solution of rhodamine 6G (20) and viewing the chromatogram in UV light (240 or 260 nm). The conventional procedures of charring with aqueous mineral acids were not suitable for layers of magnesium oxide, as they tended to crack and peel off during heating. This difficulty was largely overcome by adopting the following procedure: The chromatograms were sprayed with a freshly prepared 0.1% solution of potassium dichromate in concentrated sulfuric acid up to a point where the lipid fractions became just visible due to wetting of the layer. Subsequently the chromatograms were covered with a glass plate and placed in an oven at 95°C for 10 min. The covering plate was then removed and the chromatogram was heated at 210°C for 20 min.

On layers of Anasil B, Anasil S, and Silica Gel G, lipid fractions were detected by spraying with a saturated solution of potassium dichromate in 70% aqueous sulfuric acid and charring at 210°C for 20 min.

For the charring of lipids on layers of Florisil, a 0.1% solution of potassium dichromate in concentrated sulfuric acid was employed as spray reagent; charring was carried out at 210°C for 20 min.

*Results.* The separation patterns of various ester lipids on layers of magnesium oxide, and other adsorbents, are compared in Fig. 1.

It is obvious that wax esters and cholesteryl esters are separated more effectively on magnesium oxide than on any of the other adsorbents. Furthermore, on magnesium oxide as well as on the two types of Anasil, the diester wax, 1,2-dihexadecanoyloxy-hexadecane, is well resolved from wax esters and from ethanediol diesters, whereas on Silica Gel G and on Florisil these separations are not satisfactory. The differences in the distribution of particle sizes in the various lots of magnesium oxide have hardly any effect on the separation of the lipids studied. The chromatographic behavior of lipids on the two types of Anasil is essentially identical.

Fig. 1 shows that on layers of magnesium oxide ethanediol diesters migrate ahead of triglycerides, and that the two lipid classes are clearly separated. Similar resolution is achieved on layers of Anasil B and Anasil S. This point is of particular interest, as small amounts of esters of ethanediol and other diols reportedly occur in nature, together with esters of glycerol (21, 22). It is known that adsorption chromatography on silica gel does not resolve the esters of ethanediol from those of glycerol (21, 23). We have found that by using thin-layer chromatography on magnesium oxide, it is possible to detect as little as 0.001% of ethanediol diesters in mixture with triglycerides.

Model mixtures of compounds representing these two classes of lipids have been separated recently by gel permeation chromatography on columns of Sephadex LH-20 (24).

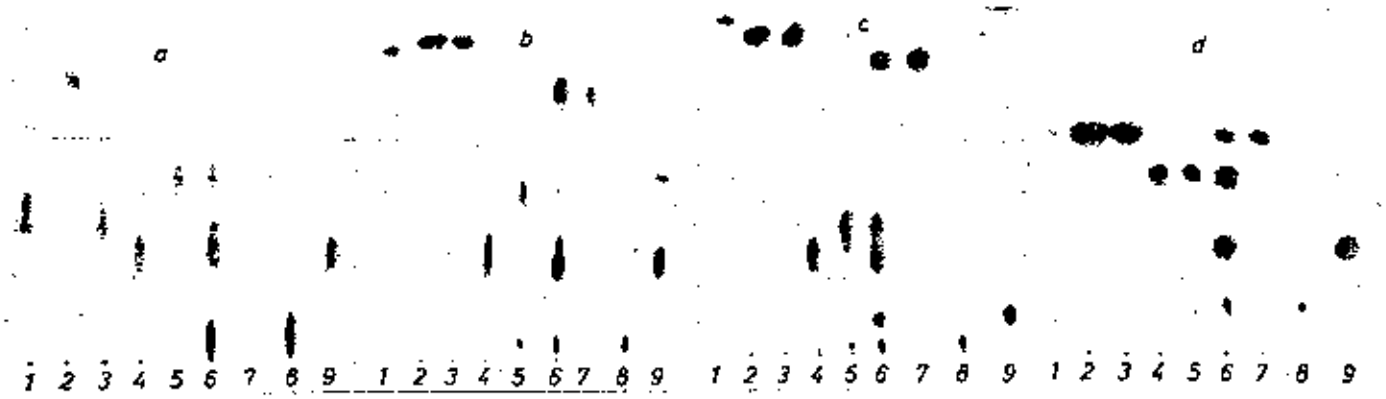


FIG. 1. Chromatographic behavior of ester lipids. Adsorbents: *a*, magnesium oxide; *b*, Anasil B; *c*, Silica Gel G; *d*, Florisil. Solvents: *a* and *d*, hexane–diethyl ether–ethyl acetate 60:40:1; *b* and *c*, hexane–diethyl ether 85:15 and 90:10, respectively. *1*, cholesteryl esters; *2*, wax esters; *3*, mixture of *1* and *2*; *4*, triglycerides of soybean oil; *5*, diesters of ethanediol derived from the fatty acids of soybean oil; *6*, mixture of *4*, *5*, *7*, *8*, and *9*; *7*, diester waxes (diesters of long-chain 1,2-alkanediols); *8*, diglycerides; *9*, long-chain *prim.* alcohols. Amount of each substance, 20 μg. Detection by charring.

It is known that mixtures of long-chain alcohols, 'yerides, and sterols cannot be resolved by adsorp- chromatography on silica gel (25, 26) nor by gel permeation chromatography on Sephadex LH-20 (27). We have found that on layers of Silica Gel G as well as Florisil using hexane–diethyl ether 60:40 as developing solvent, long-chain alcohols can be separated as a class from sterols, e.g., cholesterol and β-sitosterol. Using the same solvent system on Silica Gel G, long-chain alcohols are not resolved from diglycerides, whereas on Florisil, diglycerides and sterols migrate together. Thus, a mixture containing these three lipid classes is not separated completely on either of the two adsorbents. In contrast, a distinct separation of these three lipid classes is achieved on layers of magnesium oxide using hexane–diethyl ether–ethyl acetate 50:50:1 as developing solvent; long-chain alcohols migrate ahead of sterols and the latter ahead of diglycerides. It should be noted, however, that on magnesium oxide, using the solvent systems described, long-chain alcohols are not resolved from triglycerides.

It is evident from the foregoing discussion that the order of migration rates of the various ester lipids on layers of magnesium oxide is similar to that observed on Silica Gel G. In contrast, the order of elution of long-chain ethers of ethanediol and glycerol on magnesium ide is different from that observed on Silica Gel G. nus, on layers of magnesium oxide, with hexane–diethyl ether–ethyl acetate 90:10:1 as solvent, diethers of ethanediol migrate ahead of the triethers of glycerol, and alkyl ethers of ethanediol migrate ahead of 1,2-dialkyl ethers of glycerol. The same is true for the ether-esters of ethanediol and glycerol when chromatographed with hexane–diethyl ether–ethyl acetate 70:30:1. On layers of Silica Gel G, with hexane–diethyl ether 90:10 as solvent, the aforementioned ethanediol-derived alkoxylipids migrate behind the corresponding glycerol-derived compounds.

Alk-1-enyl diacyl glycerols and alkyl diacyl glycerols, which are well resolved on layers of Silica Gel G, migrate together on magnesium oxide, and the same is true for the corresponding diol lipids.

Anasil B is as suitable as Silica Gel G for the resolution of alk-1-enyl ether-esters and alkyl ether-esters of ethanediol and glycerol. Thus, in the separation of alkoxylipids the properties of Anasil B are similar to those of Silica Gel G, whereas in the chromatography of ester lipids, as mentioned above, Anasil B and Anasil S resemble magnesium oxide.

Neutral lipid fractions resolved by thin-layer chromatography on magnesium oxide can be eluted from the adsorbent with water-saturated diethyl ether. The magnesium oxide can be dissolved in 2 N aqueous sulfuric acid, and the lipids, with the exception of lipids containing alk-1-enyl moieties, can be recovered with hexane or benzene. However, gravimetric analyses of eluted fractions are rather impractical, as the capacity of magnesium oxide is considerably less than that of silica gel. The amounts available are sufficient for colorimetric analyses, e.g., by the hydroxamic acid method (28).

The preparation of methyl esters for their further gas–liquid chromatographic analysis is possible by reacting the lipids with methanol–sulfuric acid in benzene (29) without prior elution from the magnesium oxide.

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# Hydrogenation of Linolenate. XII. Effect of Solvents on Selectivity[1]

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## Abstract

Selectivity of heterogeneous catalysts for hydrogenation of linolenate over linoleate is increased by the presence of certain polar solvents. A ratio of specific reaction rate constants for linolenate to linoleate of 4 was obtained with a 5% palladium-on-alumina catalyst when dimethyl formamide (DMF) was used as the solvent. This high selectivity of DMF was independent of temperature and catalyst concentration. Other solvents that improved selectivity include furfural, acetonitrile, tetramethyl urea and trimethyl phosphate.


## Introduction

THE CAUSE FOR CERTAIN undesirable flavors in soybean oil has been attributed to the presence of linolenic ester groups (1). A cooking or salad oil of increased stability can be obtained from soybean oil by a two-step process: a) selective hydrogenation, to reduce linolenate from its original 7–9% to 2–3%, and b) winterization, to remove the solid high-melting fatty acid esters formed during hydrogenation (2). Commercially available catalysts have selectivity ratios ($k_{linolenate}/k_{linoleate}$) of 1.8–2.9 (3,4). A ratio of 4 or greater, as calculated on an analog computer (5), is required to reduce the linolenate content in a liquid soybean oil to 2% or less without substantial loss of linoleate or increase in high melting glycerides, and such ratios increase the yield of liquid oil during winterization or may eliminate the need for that processing step.

When evaluating solvents for the selective extraction of linolenic acid-containing triglycerides from soybean oil, the possibility was suggested of hydrogenating in a two-phase oil-solvent system. By suspending a catalyst in the selective solvent layer, it was hoped to reduce preferentially the more unsaturated triglycerides and improve selectivity. As reduction proceeded, an equilibrium would be maintained between oil and solvent phases; thus during the hydrogenation the more unsaturated triglycerides from the oil phase would tend to move into the solvent layer and the more saturated triglycerides produced in the solvent phase would tend to migrate into the oil phase. Hydrogenating in the presence of solvents has been covered in Marvott's patent (6), and the concept of hydrogenating in a selective solvent phase has been disclosed in another (7). However, the purpose of the second patent was to reduce the polyunsaturated esters to monounsaturated esters without producing any saturated esters, whereas our study is concerned with increasing the hydrogenation rate of linolenate to linoleate.

## Experimental

### Materials

Solvents evaluated were of the purest grade commercially available and were used without further

TABLE I

Hydrogenation of Linseed; Safflower Oil Mixture
(Temp. 25C, atmospheric pressure, 0.06% of 5% Pd-on-alumina catalyst)

| Sample | Time of hydrogenation, min. | Composition, % | | | IV Calcd | trans % | Selectivity ($k_{Le}/k_{Lo}$) |
|---|---|---|---|---|---|---|---|
| | | Ol | Lo | Le | | | |
| Starting material | — | 16.2 | 37.8 | 36.7 | 175.5 | — | — |
| No solvent | 70 | 34.6 | 41.2 | 14.2 | 136.3 | 14.4 | 1.65 |
| 6 Volumes DMF + 1 Volume oil | 16 | 31.5 | 49.6 | 9.7 | 136.4 | 13.0 | 3.98 |

[1] Presented at AOCS meeting in Chicago, 1964.
[2] A laboratory of the No. Utilization Res. & Dev. Div., ARS, USDA.

TABLE II

Effect of Temperature on Selective Hydrogenation in Dimethyl Formamide
(0.06% of 5% Pd-on-$Al_2O_3$ catalyst, DMF/oil = 4)

| Temp., C | Selectivity ($^{K}Le/^{K}Lo$) | *trans*, % |
|---|---|---|
| 15 | 3.3 | 9.2 |
| 25 | 3.9 | — |
| 30 | 3.7 | 13.2 |
| 40 | 3.9 | 14.3 |
| 50 | 3.8 | 15.9 |
| 60 | 3.8 | 17.8 |

purification except for acetonitrile, hexane and methoxyethanol, which were distilled, and furfural, which was freshly distilled under reduced pressure and stabilized with hydroquinone. Peroxides were removed from 1,2-dimethoxyethane by passage through alumina followed by distillation over sodium.

### Hydrogenations

Catalytic reductions were performed in glass manometric apparatus as described previously (3). Linseed and safflower oils were combined in such proportions as to yield a mixture containing equal amounts of linolenate (Le) and linoleate (Lo). The mixture (1 ml) hydrogenated with solvent and a 5% palladium-on-alumina catalyst was compared to a similar hydrogenation without any solvent. Hydrogenation was stopped after adding 1 mole of hydrogen per mole of Le. Use of the equimixture and this experimental design facilitate a more accurate determination of selectivity ratio (8). Selectivity ratio ($^{K}Le/^{K}Lo$) was determined from the fatty acid compositions of the linseed-safflower oil mixture before and after hydrogenation (8).

### Analytical Methods

Methyl esters were prepared by refluxing the oil mixture with 0.5% sodium methoxide and a great excess of methanol for 1 hr. The percent *trans* was determined from infrared absorption of the methyl esters at 10.36 $\mu$ region by using methyl elaidate as the standard (3). Fatty acid compositions were determined by gas-liquid chromatography (GLC) of the methyl esters. A Pye argon gas chromatograph equipped with a 4 ft × ¼ in. glass column, packed with 14% EGSS-X on Gas-Chrom P, 100–120 mesh (Organosilicon polyester packing obtained from Applied Science Laboratories, Inc., State College, Pa.) and with a radium D ionization detector was operated at 180C with an argon gas flow of 45-ml per min. The area under each peak was determined from an electronically integrated curve.

## Results and Discussion

Analyses of the hydrogenated linseed-safflower oil mixture with and without dimethyl formamide (DMF) are given in Table I. The addition of solvent doubled selectivity without a concomitant increase in *trans* content. The rate of hydrogenation was considerably increased.

Figure 1 illustrates the effect of increasing the amount of DMF on selectivity. As little as 1 ml DMF per milliliter oil gave nearly maximal selectivity. Increasing the solvent above 5 ml decreased selectivity; the optimum was from 3 to 5 ml. The claim of no upper limit to the amount of solvent used (7) for selective hydrogenation of polyunsaturated esters does not hold for selective reduction of linolenate.

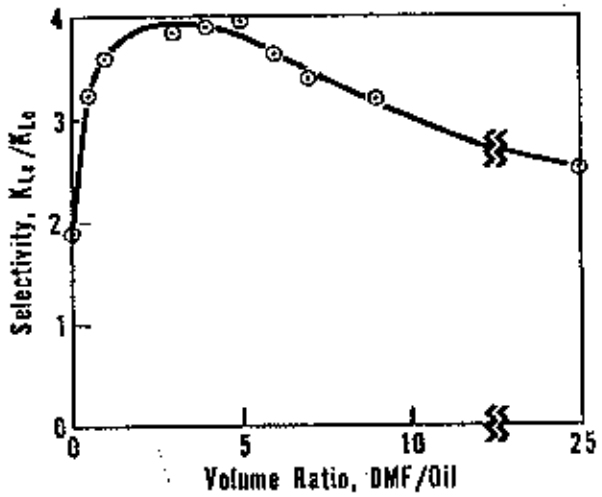


FIG. 1. Effect of increasing dimethyl formamide on selective hydrogenation of a linseed:safflower oil mixture.

The relationship between selectivity and temperature of hydrogenation is given in Table II. Below room temperature selectivity decreased, whereas above room temperature it remained essentially constant. The *trans* content increased with increase in temperature of hydrogenation, an observation which is in agreement with previous reports (9–12). Above 50C

TABLE III

Effect of Catalyst Concentration on Selectivity
(temp. 25C; DMF/oil = 4)

| Catalyst concn., % Pd | Pd on $Al_2O_3$, % Pd | Selectivity ($^{K}Le/^{K}Lo$) |
|---|---|---|
| 0.1 | 5.0 | 3.6 |
| 0.05 | 5.0 | 3.8 |
| 0.01 | 0.5 | 4.0 |
| 0.005 | 0.5 | 3.8 |

TABLE IV

Effect of Solvents on Selectivity of 5% Pd on $Al_2O_3$

| Solvent | Ml solvent/ml oil | Hydrogenation temp., C | Rate of hydrogenation ml/min | *trans*, % | Selectivity ($^{K}Le/^{K}Lo$) |
|---|---|---|---|---|---|
| None | | 25 | 0.42 | 13.0 | 1.9 |
| DMF (DuPont, tech. grade) | 3 | 25 | 1.78 | 13.8 | 3.6 |
| DMF (Matheson, Coleman and Bell, chem. grade) | 4 | 25 | 1.78 | — | 3.0 |
| Diethyl formamide (spectrograde) | 2 | 25 | 2.82 | 16.7 | 3.2 |
| Dimethyl acetamide | 3 | 25 | 1.06 | — | 2.5 |
| Furfural | 2 | 40 | 1.11 | 34.0 | 3.6 |
| Trimethyl phosphate* | 3 | 100 | 0.27 | 83.0 | 3.3 |
| Tetramethyl urea | 2 | 25 | 1.60 | 11.0 | 3.4 |
| Acetonitrile | 4 | 50 | 4.99 | 10.5 | 3.2 |
| Propionitrile | 2 | 25 | 3.20 | — | 2.6 |
| Ethoxy propionitrile | 3 | 25 | 1.18 | — | 2.5 |
| Butyrolactone | 1 | 30 | 1.00 | 16.7 | 2.0 |
| 1,2-Propanediol cyclic carbonate | 1 | 30 | 1.10 | 15.8 | 2.2 |
| N-Methyl 2-pyrrolidinone | 1 | 25 | 0.74 | 14.4 | 2.5 |
| Methoxyethanol | 4 | 30 | 2.20 | — | 2.2 |
| 1,2-Dimethoxyethane (peroxides) | 2 | 30 | 2.24 | 23.3 | 4.0 |
| 1,2-Dimethoxyethane (peroxide free) | 2 | 30 | 5.36 | 17.8 | 2.3 |
| Tetraethylene glycol dimethyl ether | 1 | 25 | 0.85 | — | 1.8 |
| tert-Butyl alcohol | 1 | 25 | 1.42 | — | 1.8 |
| Acetone | 3 | 25 | 4.05 | 25.5 | 2.2 |
| Diethyl carbonate | 3 | 30 | 2.87 | 12.9 | 2.3 |
| Dimethyl carbonate | 3 | 30 | 8.43 | 15.7 | 2.0 |
| Dimethyl malonate | 1 | 30 | 1.41 | 14.4 | 1.9 |
| Diethyl oxalate | 1 | 30 | 3.35 | 14.4 | 1.9 |
| Methyl formate | 3 | 0 | 1.79 | 11.4 | 1.9 |
| Trimethyl borate | 1 | 30 | 4.04 | 14.4 | 1.5 |
| Hexane (tech. distilled) | 2 | 25 | 3.01 | 18.8 | 2.0 |

*Hydrogenation was carried out with a commercial nickel catalyst.

ie oil was completely miscible with the solvent, but selectivity was the same, contrary to our original hypothesis of two-phase hydrogenation. Thus a polar solvent does not need to form a two-phase system to improve selectivity. Though this observation refutes our earlier hypothesis, the experimental application of this concept remains unaffected.

Table III lists the selectivities at various catalyst concentrations. Within experimental error, selectivity remained constant over a wide range of catalyst concentrations.

In an effort to find other solvents useful for selective hydrogenation, a survey was made; the results are shown in Table IV. All solvents were tested with 5% palladium-on-alumina catalyst at room temperature and atmospheric pressure, except for trimethyl phosphate which was used with a commercial nickel catalyst at 100C. Hydrogenation with methyl formate was performed at 0C. As can be seen from Table IV, highly polar solvents like DMF, furfural and tetramethyl urea improved selectivity, whereas less polar and nonpolar solvents were not beneficial despite an increase in hydrogenation rates. Furfural and trimethyl phosphate produced large amounts of *trans* isomers possibly because of the presence of catalyst poisons in the solvents. If 1,2-dimethoxyethane containing peroxides was used, a high selectivity of 4.0 was obtained. A high *trans* content was also produced, which is probably indicative of the peroxides acting as selective poisons (13). After removal of peroxides from the solvent, selectivity dropped to a nearly normal value. The *trans* was still slightly higher possibly due to traces of peroxides which are removed with difficulty. With acetone, the high *trans* content also indicates poisons; but selectivity is not improved. It appears that selectivity can often be improved by poisoning the catalyst. However, since the *trans* content did not increase with DMF or tetramethyl urea, it might be inferred that these two solvents act by a process other than catalyst poisoning. The two-phase hydrogenation hypothesis proposed earlier is not a necessary prerequisite for improving selectivity since the same high selectivity was obtained even when there is only one phase. A more likely explanation is that of McQuillin et al. (14), namely, the solvent influences the polarity of the catalyst surface so that the highly unsaturated molecules are adsorbed on the catalyst surface in preference to the less unsaturated triglycerides.

A technical grade DMF gave a selectivity of 3.6; a chemical grade, 3.0. The spectro grade DMF used gave higher values than either of the other two samples. These differences may reflect the extent of impurities present in the solvent.

Selectivities achieved, when applied to soybean oil, should produce a liquid oil of greater flavor stability. Since hydrogenations are carried out at low temperatures, the flavor stability may further be improved by the suppression of many side reactions that normally accompany a high-temperature hydrogenation.

ACKNOWLEDGMENT

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# Analysis of Methyl Octadecenoate and Octadecadienoate Isomers by Combined Liquid–Solid and Capillary Gas–Liquid Chromatography

SIR: Liquid–solid chromatography on silver nitrate-impregnated adsorbents is frequently used for the separation of unsaturated fatty acid methyl esters according to their geometry (*cis* and *trans*) or degree of unsaturation (monoene, diene, triene, etc.) (*1, 6, 16*). Capillary gas–liquid chromatography is used extensively for the study of complex mixtures of isomeric esters, particularly the methyl octadecenoates (*11, 18*) and octadecadienoates (*10, 12, 15*). However, almost all reported analyses of fatty acid methyl esters on capillary columns have been confined to the separation of positional isomers of a given geometry or geometrical isomers at a given bond position. We have applied a combination of the above chromatographic techniques to the analysis of partially hydrogenated vegetable oils. The successful analysis of samples containing such a multiplicity of isomeric fatty acids has not been reported previously.

In the present work liquid–solid chromatography on silver nitrate–silica gel was used to separate monoenoic fatty acid methyl esters into *trans* and *cis* isomer fractions and dienoic methyl esters into several distinct fractions depending upon geometry and bond position. These fractions were then analyzed by infrared spectrometry, capillary gas chromatography, and oxidative cleavage to give a fairly complete picture of the fatty acid composition of partially hydrogenated fats and oils. A similar combination of methods was recently used by the authors to analyze a much less complex mixture of internal olefins (*8*).

## EXPERIMENTAL

**Preliminary Treatment of Sample.** Methyl esters of the fatty acids were prepared from the fat or oil by base-catalyzed alcoholysis according to procedures previously described (*7, 8*). For oils containing appreciable dienoic fatty acids, the monoenoic and dienoic methyl esters were first separated by liquid–solid chromatography of their mercury derivatives (*8*). The unsaturated ester fractions thus isolated were then further separated by chromatography on silver nitrate–silica gel as described below. For samples of low or negligible diene content the methyl esters can be chromatographed directly on silver nitrate–silica gel.

**Liquid–Solid Chromatography.** The technique used here was a modification of that reported by DeVries (*5, 6*). Silica gel impregnated with about 30% silver nitrate was used in 39- × 2-cm. columns containing 75 to 80 grams of adsorbent. The adsorbent was sometimes mixed with Celite 545 filter aid in 20:1 or 30:1 ratio to facilitate solvent flow. Sample sizes averaged about 1.0 gram for saturated-monoenoic methyl ester mixtures and 0.65 gram for dienoic methyl esters. The chromatography required 20 to 48 hours depending upon the complexity of the sample. Petroleum ether–benzene mixtures were used to elute the various components: 80:20 for the saturated esters, 70:30 for the *trans* monoenoic esters, and 55:45 for the *cis* monoenoic esters. For dienoic ester mixtures, the elution was started with 60:40 petroleum ether–benzene, followed by 50:50, 40:60, and 30:70 mixtures. The final dienoic ester fraction was eluted with 90:10 benzene–ethyl ether. The composition of these fractions will be discussed later. Ethyl ether was used to strip the column of the non-ester components. The "valleys"

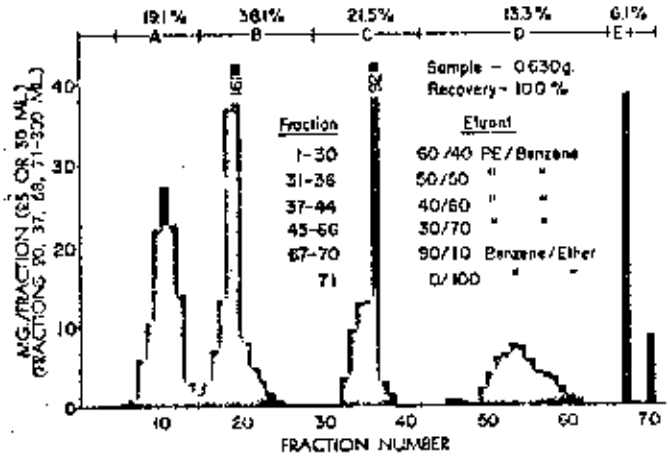


**Figure 1. Liquid–solid chromatography of $C_{18}$ dienoates from hydrogenated corn oil**

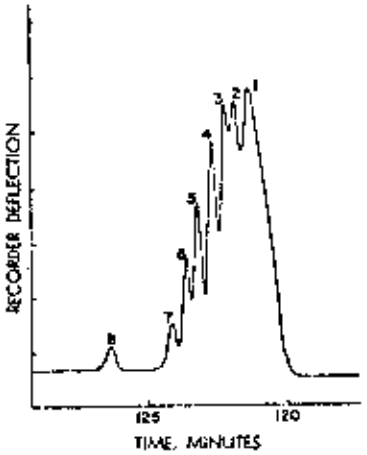


**Figure 2. Gas chromatographic separation of *trans* $C_{18}$ monoenoate isomers**

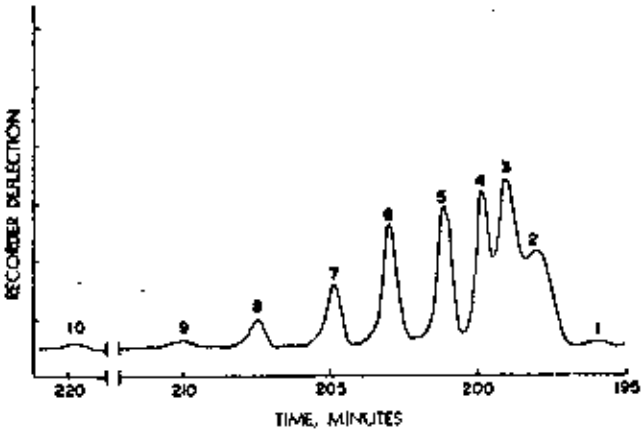


**Figure 3. Gas chromatographic separation of *cis* $C_{18}$ monoenoate isomers**

between the major fractions were determined by taking numerous small individual fractions. Prior knowledge of the composition of the sample, either by a preliminary chromatographic run or other independent data, is of help in determining which fractions should be composited to keep coelution to a minimum. Solvent was added to all individual fractions immediately after obtaining the initial weight to minimize oxidation before making the composites.

The adsorbent was prepared in a manner similar to that described for silver nitrate–alumina (*8*). Davison Grade 923 100–200 mesh silica gel was used as received. The silver nitrate–silica gel was dried overnight at 120° C. rather than at the 180° C. used for the alumina. The final adsorbent usually contained 19 to 21% silver and 1 to 2% moisture (weight loss after three hours at 180° C.). Best results were obtained with adsorbents containing 1.6% moisture.

**Gas–Liquid Chromatography.** All gas chromatographic analyses were performed on 150-foot by 0.01-inch capillary columns (pancake configuration) in a Perkin-Elmer Model 226 chromatograph. The *trans*-octadecenoate isomers were analyzed on an XF-60 coated capillary under programmed temperature conditions; 100–170° C. at 0.5° C. per minute. The *cis*-octadecenoate and all the octadecadienoate isomers were analyzed on a polyphenyl ether coated column (OS-138, Perkin-Elmer Corp.); the former at 100–180° C. at 0.5° C. per minute and the latter at 140–180° C. at 0.5° C. per minute. The inlet pressure was 20–30 p.s.i.g. and all other conditions were as noted previously (*8*). Peak areas were determined by the peak height times width at half height method.

Samples of the *cis* and *trans* isomers of methyl 6-, 9-, and 11-octadecenoates and the geometric isomers of methyl 9,12-octadecadienoate were run to aid in peak identification.

**Oxidative Cleavage.** Details of the periodate permanganate oxidation and subsequent programmed temperature gas chromatography of the products as well as typical chromatograms have been reported (*9*). Mono- and dicarboxylic acid cleavage fragments ($C_4$ and up) are recovered and determined quantitatively (as their methyl esters) by this method.

**Infrared *trans* Determinations.** Analytical and instrument conditions employed in the infrared method for *trans* unsaturation have been described (*3*). A calibration curve prepared from binary mixtures of methyl elaidate and methyl oleate was used. Because of the limited sample available some of the infrared *trans* values were obtained on weights as low as 20 mg. (in a 1-ml. volume).

## RESULTS AND DISCUSSION

Methyl esters were prepared from a mixed cottonseed–soybean oil which had been hydrogenated to an iodine value of 48. The methyl esters were chromatographed directly on silver nitrate–silica gel to give a clear separation of the methyl esters into saturated (46.6790), *trans* monoenoic (37.9%), *cis* monoenoic (13.3%) esters and dienoic esters plus non-ester components (2.2%). The *trans* monoenoic ester fraction analyzed 96% *trans*, the *cis* monoenoic ester fraction <1% *trans*. Oxidative cleavage and capillary gas chromatography were run on these unsaturated ester fractions as discussed below.

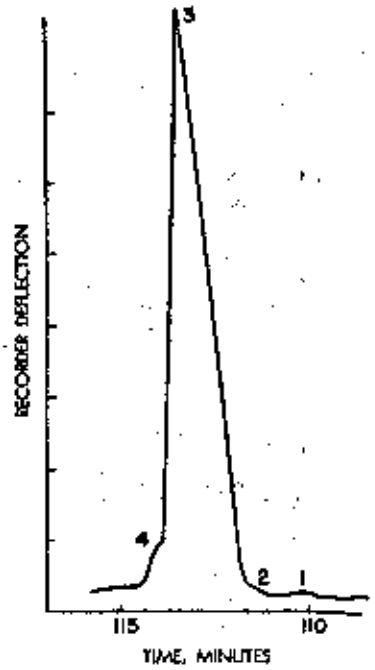


**Figure 4. Gas chromatographic separation of $C_{18}$ dienoate isomers—fraction A**

Methyl esters were also prepared from a corn oil which had been hydrogenated to an iodine value of 100. These methyl esters were carried through a preliminary separation of their mercury derivatives by chromatography on alumina. The dienoic esters thus isolated (31.3% of the original esters) were then separated by silver nitrate–silica gel chromatography into five distinct fractions as shown in Figure 1. Table I gives the infrared *trans* content of each fraction and the major types of dienoic esters which were eluted in the various fractions. It can be seen that the strength of the silver ion complexes, and hence the order of elution from the column, depends on the number of methylene groups between the double bonds as well as the geometry of the double bonds. The deviation from 100% *trans* values for Fractions B and D is believed due to lower absorptivities (based on peak height alone) for dienoic esters containing one *trans* double bond. Similar low absorptivities have been observed by others (*14*). The width of the absorption band, however, is visibly

**Table I. Major Methyl Octadecadienoate Classes Separated (Figure 1) by Liquid–Solid Chromatography**

| Fraction | % IR *trans* | No. of $CH_2$ groups between double bonds | Major components |
|---|---|---|---|
| A | 136 | 0 | 20% conjugated dienes |
| | | 1 | 75% *trans,trans* dienes |
| B | 91 | 1 | *cis,trans* dienes |
| C | 58 | 1 | 70% *cis,cis* dienes |
| | | 2 | 30% *trans,trans* dienes |
| D | 86 | 2 | *cis,trans* dienes |
| E | 19 | 2 | *cis,cis* dienes |

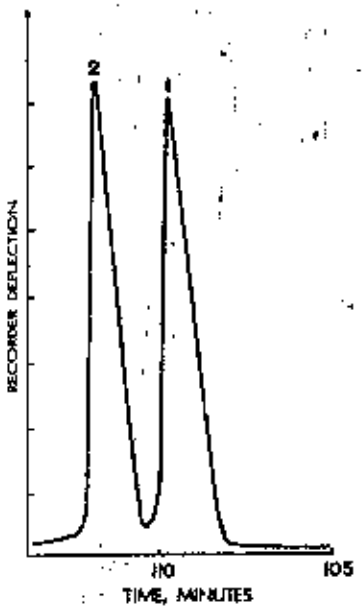


Figure 5. Gas chromatographic separation of $C_{18}$ dienoate isomers—fraction B

greater for these dienoic esters than that given by a corresponding amount of methyl elaidate. It has been known for some time in these laboratories (4) that methyl *trans,trans*-9,12-octadecadienoate gives an absorptivity based on peak height of only 1.8 times that of methyl elaidate while the integrated area of the adsorption band approaches 2.0 times that given by methyl elaidate. It appears the same phenomenon holds for *cis, trans* isomers having one and two methylene groups between the double bonds (Fractions B and D). The detailed composition of the various fractions of Figure 1 and Table I is discussed in the following sections.

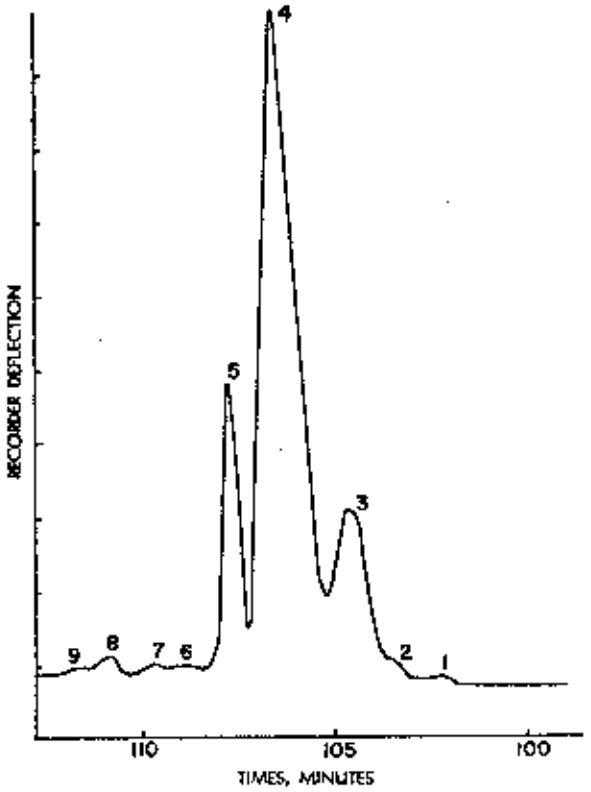


Figure 7. Gas chromatographic separation of $C_{18}$ dienoate isomers—fraction D

The capillary gas–liquid chromatographic separations of the various geometric isomer fractions are shown in Figures 2–8. Where possible, identifications were made by comparision to the standard samples noted previously. The order of elution and comparison to oxidative cleavage data on the same fractions were also used as bases of identification.

A comparison of capillary gas-liquid chromatography and oxidative cleavage data for both monoenoates and dienoates is given in Tables II and III. All data in the tables are on a fraction basis rather than on an original sample basis. Results are reported to the nearest whole number for components above 10% and to the nearest 0.5% for components below 10% because of the limitations of the analytical methods used (partial resolution in gas chromatography, numerous steps in cleavage method, etc.).

In Table II, the spread between $\Delta^4$-$\Delta^{11}$ isomer values by the two methods is attributable to the construction of the

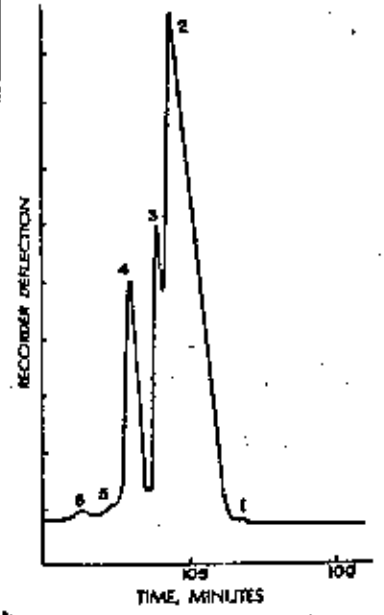


Figure 6. Gas chromatographic separation of $C_{18}$ dienoate isomers—fraction C

Table II. Comparison of Gas–Liquid Chromatography and Oxidative Cleavage Results

Methyl Octadecenoate Isomers from Hydrogenated Cottonseed-Soybean Oil

| | *cis* Fraction—Figure 3 | | | | *trans* Fraction—Figure 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Isomer | Oxidative cleavage, % | | GLC, % | Peak no. | Oxidative cleavage, % | | GLC, % | Peak no. |
| $\Delta^4$ | 0.5 | | | | 0.5 | | | |
| $\Delta^5$ | 1.0 | | | | 1.0 | | | |
| $\Delta^6$ | 3.0 | 21 | 20 | 2 | 2.5 | 37 | 39 | 1 |
| $\Delta^7$ | 5.5 | | | | 5.0 | | | |
| $\Delta^8$ | 11 | | | | 11 | | | |
| $\Delta^9$ | 18 | | 22 | 3 | 17 | | | |
| $\Delta^{10}$ | 16 | | 14 | 4 | 18 | | 17 | 2 |
| $\Delta^{11}$ | 15 | | 15 | 5 | 18 | | 14 | 3 |
| $\Delta^{12}$ | 14 | | 14 | 6 | 12 | | 12 | 4 |
| $\Delta^{13}$ | 8.0 | | 7.5 | 7 | 8.5 | | 9.0 | 5 |
| $\Delta^{14}$ | 4.0 | | 4.0 | 8 | 5.0 | | 5.0 | 6 |
| $\Delta^{15}$ | 2.5 | | 1.5 | 9 | 2.0 | | 2.5 | 7 |
| $\Delta^{16}$ | 1.0 | | 1.0 | 10 | 1.0 | | 1.5 | 8 |
| Unknown | ... | | 0.5 | 1 | | | | |

partially resolved peaks required for area measurement (see Figures 2 and 3). Attempts to improve the separation of the $\Delta^{4}$-$\Delta^{8}$ *cis* and $\Delta^{4}$-$\Delta^{8}$ *trans* isomers were unsuccessful. Polyphenyl ether and butanediol succinate columns did not split out the $\Delta^{4}$-$\Delta^{8}$ *trans* isomers and lost the separation of the $\Delta^{13}$ and $\Delta^{14}$ *trans* isomers. The resolution of the $\Delta^{4}$-$\Delta^{8}$ *cis* isomers as a group, from the $\Delta^{8}$ isomer could be obtained only on the polyphenyl ether column. The use of a 5A molecular sieve precolumn did not improve the resolution. Derivatization of the double bonds was considered, but no derivatives were found which could be prepared quantitatively and quickly while not yielding a mixture of products.

In Table III, the high purity of Fraction B is confirmed by the absence of the $\Delta^{9,12}$ *cis,cis* and *trans,trans* isomers. This lends support to the apparent low absorptivity of the *trans* bond in the infrared spectra which was noted above. The order of elution of the *cis,trans* and *trans,cis* isomers was assumed on the basis of observed shorter retention times for *cis* isomers of a given position. The data in Table III indicate the major octadecadienoate isomers in the partially hydrogenated corn oil to be the geometric isomers of both methyl 9,12-octadecadienoate and of those positional isomers involving a one carbon migration of the double bond ($\Delta^{8,12}$, $\Delta^{9,13}$).

Resolution of the dienoate isomers was not improved by chromatography on an XE-60 column or by increasing column residence times by modifying the programming conditions.

When the monoenoate and dienoate fractions are analyzed by capillary gas-liquid chromatography without prior silver nitrate–silica gel chromatographic separation (as in the case of samples from a preparative gas chromatographic separation), quantitative data can be obtained only when bond wandering and *trans* isomer formation has been minimized—i.e., a lightly hydrogenated oil. When this condition does not exist, the chromatograms can be utilized as qualitative fingerprints.

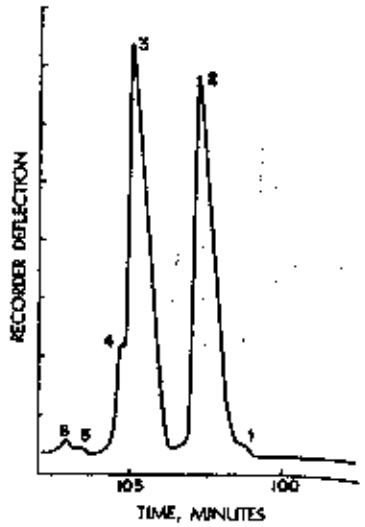


**Figure 8. Gas chromatographic separation of $C_{18}$ dienoate isomers—fraction E**

**Table III. Comparison of Gas–Liquid Chromatography and Oxidative Cleavage Results**

Methyl Octadecadienoate Isomers from Hydrogenated Corn Oil

| Fraction | Isomer | Oxidative cleavage, % | GLC, % | Peak no. |
|---|---|---|---|---|
| A (Figure 4) | $\Delta^{9,12}$ *t,t* | 68 } 71 | 71 | 3 |
| | $\Delta^{8,12}$ *t,t* | 2.5 | | |
| | $\Delta^{10,12}$ *t,t* | 2.0 | 2.0 | 4 |
| | $\Delta^{9,11}$ conj. | 14 | 14[a] | |
| | $\Delta^{12,13}$ conj. | 9.5 | 9.5[a] | |
| | other dienes | 3.5 | 3.5 | 1, 2 |
| B (Figure 5) | $\Delta^{9,12}$ *c,t* | } 95 | 52 | 1 |
| | $\Delta^{9,12}$ *t,c* | | 48 | 2 |
| | other dienes | 5.0 | ... | |
| C (Figure 6) | $\Delta^{9,12}$ *c,c* | 69 } 71 | 71 | 2 |
| | $\Delta^{8,12}$ *c,c* | 1.0 | | |
| | $\Delta^{10,12}$ *c,c* | 1.0 | | |
| | $\Delta^{9,12}$ *t,t* | 13 | 11 | 3 |
| | $\Delta^{8,12}$ *t,t* | 13 } 14 | 15 | 4 |
| | $\Delta^{7,12}$ *t,t*?[b] | 0.5 | | |
| | $\Delta^{8,14}$ *t,t*? | 0.5 | 1.5 | 5 |
| | $\Delta^{9,14}$ *t,t*? | 0.5 | 1.0 | 6 |
| | other dienes | 1.5 | <0.5 | 1 |
| D (Figure 7) | $\Delta^{9,12}$ *c,t-t,c* | 45 | [c] | |
| | $\Delta^{8,12}$ *c,t-t,c* | 37 | | |
| | $\Delta^{9,14}$ *c,t-t,c* | 4.0 | | |
| | $\Delta^{8,14}$ *c,t-t,c* | 4.0 | | |
| | $\Delta^{7,12}$ *c,t-t,c* | 4.0 | | |
| | $\Delta^{10,14}$ *c,t-t,c* ? | 1.5 | | |
| | $\Delta^{9,16}$ *c,t-t,c* ? | 1.5 | | |
| | $\Delta^{8,12}$ *c,t-t,c* ? | 1.5 | | |
| | $\Delta^{7,14}$ *c,t-t,c* ? | 0.5 | | |
| | other dienes | 1.0 | | |
| E (Figure 8) | $\Delta^{9,12}$ *c,c* | 45 | 49 | 3 |
| | $\Delta^{8,12}$ *c,c* | 39 | 43 | 2 |
| | $\Delta^{9,14}$ *c,c* | 3.0 } 16 | 8.0[d] | 1, 4, 5, 6 |
| | $\Delta^{8,14}$ *c,c* | 3.0 | | |
| | $\Delta^{7,12}$ *c,c* | 2.5 | | |
| | $\Delta^{7,14}$ *c,c*? | 2.5 | | |
| | $\Delta^{10,14}$ *c,c*? | 1.5 | | |
| | $\Delta^{9,16}$ *c,c*? | 1.0 | | |
| | $\Delta^{8,16}$ *c,c*? | 1.0 | | |
| | other dienes | 1.0 | | |

[a] Conjugated diene results not shown because of excessive length of time required for elution under conditions used.

[b] The presence of these isomers is consistent with both the cleavage pattern and the assumption of minimum migration of double bonds from the original $\Delta^{9,12}$ position. However, since several of the isomers give common cleavage fragments, absolute proof of such low levels of these components is lacking.

[c] Because of the probable presence of two peaks for each positional isomer, no reasonable interpretation of the chromatogram could be made.

[d] Because of the multiplicity of isomers present, further reasonable interpretation of the chromatogram could not be made.

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# TRANSACTIONS AND COMMUNICATIONS

OCTOBER 16, 1936

## ANTIOXIDANTS AND THE PRESERVATION OF EDIBLE FATS

By COLIN H. LEA

In common with other antioxidative processes the oxidation of an unsaturated fat exposed to the air may be either accelerated or retarded by the presence of traces of foreign substances termed "pro- or anti-oxidants." It is now believed that substances of the latter class are present in plant and animal tissues and, being to some extent extracted with the fat, are responsible for the initial lag phase or "induction period" which in the majority of fats precedes the stage of rapid oxidation. Little progress has yet been made towards the isolation and identification of these natural anti-oxidants, and at present the stabilisation of oils by direct addition of small quantities of substances of known composition appears to be more promising.

Numerous aromatic phenols and amines have been shown to possess in varying degree the property of retarding atmospheric oxidation of unsaturated organic compounds, but such substances are undoubtedly toxic when taken in any appreciable quantity. Only recently have substances of a definitely non-toxic character been proposed as antioxidants, and in practically every case the evidence so far obtained as to their usefulness is conflicting. Lecithin has been stated to possess protective properties,[1] and to be inactive.[2] The observation that lecithin and cyanide are powerful antioxidants in presence of a metallic catalyst (cobalt)[3] is, as shown in the present paper, by no means a certain indication that they are active in absence of the metal. Carotene has been stated to accelerate[4, 5, 6] and to retard[7, 8] oxidation. Maleic acid (which is reputed to possess a toxicity similar to that of oxalic acid[9]) has been described as a powerful antioxidant[9, 10] and as completely inactive.[2, 11]

Since the completion of a number of the experiments described in the present report, two papers containing references to some of the substances here described have been published. Greenbank and Holm[6] found antioxidant properties, though less marked than with maleic acid, for several organic acids including tartaric and citric. They state, however, that the salts are ineffective, and that protection is much reduced in presence of water. Olcott,[11] on the other hand, failed to obtain any protective effect whatever from employment of these acids. Some of the contradictory results mentioned above are undoubtedly due to different experimental conditions, but the whole situation is somewhat confusing.

### *Estimation of the efficiency of antioxidants*

In practically the whole of the published work on pro- and anti-oxidants the protective effect has been measured by addition of the substance to the pure, dry fat. The majority of foods, however, contain fat in contact with an aqueous phase, and information regarding protection from oxidation under these more complicated conditions is entirely lacking. That anti-oxidants do not necessarily behave similarly under the two sets of conditions is shown by the fact that polyphenolic substances such as pyrogallol, which for dry fats are among the most powerful antioxidants known, are of little use in presence of water, particularly at a slightly alkaline $p_H$.

Measurement of the efficiency of antioxidants has almost invariably been made at some fairly high temperature (usually 70–100°), or in presence of light or a metallic salt as accelerator. In the present paper it has been considered more satisfactory to work at ordinary temperatures (20°) in presence of an aqueous phase and in absence of light. Oxidation has been followed by determination of peroxide-oxygen content by the method described in previous papers,[12] using 0·5 g. of fat and 20 ml. of 2 : 1 mixture of glacial acetic acid and chloroform. The ratio of the induction periods in absence and in presence of the substance has been used as a "protection factor" to measure the efficiency of the substance as an antioxidant. A negative protection factor indicates that the substance accelerates oxidation, and a factor of 1·0 or −1·0 that it has no effect.

### EXPERIMENTAL

This investigation was originally undertaken with the idea that antioxidants might prove of value in the curing of bacon, a foodstuff in which rancidity arising from atmospheric oxidation of the unsaturated constituents of the fat is the chief obstacle to successful

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storage for prolonged periods.[13] For this reason in earlier experiments the rate of oxidation of lard in contact with a synthetic pickle of composition, sodium chloride 26·0 g., potassium nitrate 3·5 g., potassium nitrite 0·03 g., distilled water to 100 ml., was investigated. Later the work developed along more general lines.

Chemically, the aromatic amines and polyhydric phenols provide some of the best known inhibitors for the autoxidation of unsaturated organic compounds, though by no means all such substances are active. It therefore seemed possible that some of the aliphatic amino- and polyhydroxy-compounds might also possess this power in some degree.

*Oxidation of lard at 20° in presence of hydroxy- or amino-compounds*

Lard extracted in the laboratory from the leaf fat of a freshly killed animal was used. The fat was pipetted into small flat-bottomed glass dishes to a depth of 1·5 mm., allowed to solidify, and covered with pickle to a depth of 3·75 mm. Six dishes were stored in a stoppered glass jar at a constant temperature of 20° and removed one by one for examination. The aqueous layer was poured off, the fat dissolved in peroxide-free ether, washed free from all traces of nitrite, and dried over sodium sulphate, after which the solvent was removed under vacuum at 50° and the fat extracted for peroxide content. In this, as in subsequent experiments, care was taken to prevent contamination with traces of

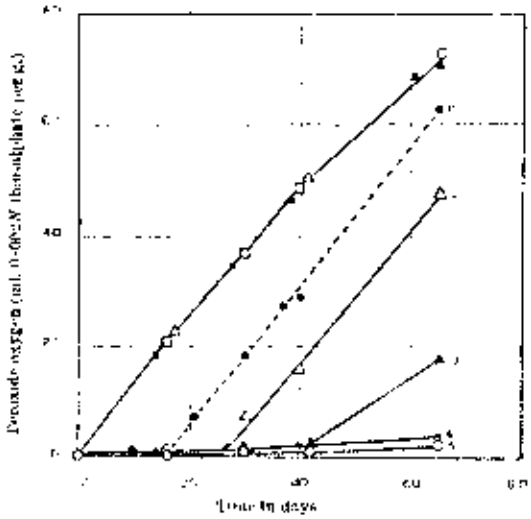


FIG. 1

*The oxidation of lard in pickle containing 5% of hydroxy-compound. (1) Acetic, citric, or tartaric acid. (2) Glycerol. (3) Glucose. (4) Sodium tartrate. (5) Sodium citrate. (P) Control.*

oxidised fat, which clings tenaciously to glass apparatus and tend to increase the rate of oxidation of subsequent samples. Treatment with hot, concentrated sodium hydroxide solution prior to the customary washing with chromic acid sufficed to eliminate trouble from this cause.

[13] C. H. Lea, J.S.C.I., 1931, 50, 313 T; 1933, 52, 57 T.

The resulting curves (Fig. 1) show that all three of the acids examined eliminated the induction period, but that some of the other substances, notably the tartrate and citrate, possessed quite marked protective properties. The quantity of antioxidant used (5% of the aqueous phase) is, however, too high for most practical purposes.

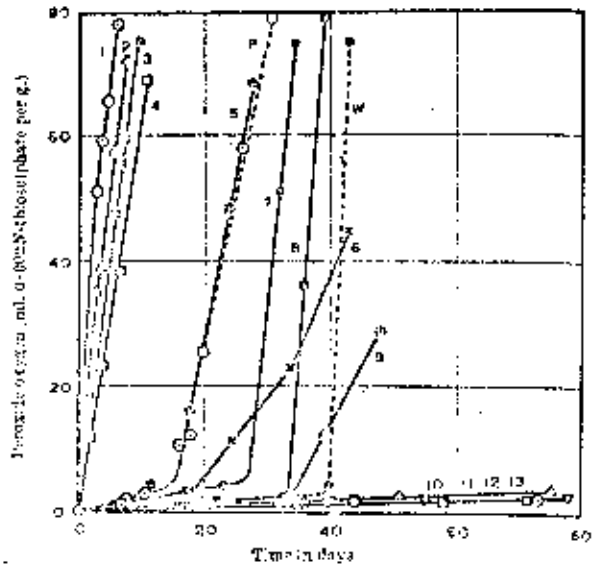


FIG. 2

*The oxidation of lard emulsified with pickle containing 2% of hydroxy- or amino-compound. (1) Lactic acid. (2) Oleic acid. (3) Tartaric acid. (4) Succinic acid. (5) Mannitol. (6) Glucose. (7) Sodium malate. (8) Ammonium lactate. (9) Sodium tartrate. (10) Glycine. (11) Sodium citrate. (12) Asparagine. (13) Sodium malonate. (P) Control. (W) Water.*

A second series of samples was therefore put down with a smaller proportion of active substance, the melted fat in this case being emulsified with 15% of its volume of synthetic pickle containing 2% (0·3% calculated on the fat) of antioxidant, and solidified before separation could occur. The thickness of the fat layer was again 1·5 mm., and the temperature 20°. The results are summarised below.

| | |
|---|---|
| Pro-oxidants (P.F. −20) | Succinic, tartaric, citric, and lactic acids. |
| Inactive (P.F. 1·0) ... | Mannitol. |
| Weak antioxidants (P.F. 1·0—2·0) | Glycerol, glucose, sucrose, fructose, sodium malonate, sodium succinate. |
| Moderate antioxidants (P.F. 2·0—3·0) | Sodium malate, ammonium lactate, sodium tartrate, sodium glycollate, sodium lactate. |
| Powerful antioxidants (P.F. > 4·5) | Glycine, asparagine, sodium citrate, sodium malonate. |

Fig. 2 conveys some idea of the enormous range in rate of oxidation covered by these figures.

These two experiments proved the existence of a wide variety of water-soluble substances capable of acting as inhibitors for the autoxidation of lard, but

closer study of the results indicated that some disturbing factor was present. It was observed, for example, that while smooth and fairly reproducible oxidation curves could be obtained in many cases, in others, particularly those of water or pickle alone, duplicate experiments gave erratic results or parallel curves with varying induction periods.

Investigations on the mechanism of yellowing in lard, also in progress at this time,[14] indicated that traces of alkali markedly accelerated the oxidation of lard stored in thin films in glass bottles. These data, in conjunction with the unexpected pro-oxidant effect of all the acids tested, now directed attention to the question as to whether the rate of oxidation of the fat might not be influenced by the $p_H$ of an aqueous phase in contact with it.

### *Effect of $p_H$ on the oxidation of lard*

In a preliminary trial, uniform thin films (0·4 mm.) of freshly rendered lard were spread on microscope slides immersed to a depth of 1 cm. in pickle contained in jars which were stored at 20° under atmospheres containing different partial pressures of carbon dioxide. The results are tabulated below.

| Atmosphere | $p_H$ | Induction period (days) |
|---|---|---|
| Air | 7·6 | 12 |
| Air 98·7% carbon dioxide 1·3% | 6·5 | 32 |
| Air 50% carbon dioxide 50% | 4·8 | 21 |
| Air 50% nitrogen 50% | 7·8 | 11 |

Oxidation of the sample in air diluted with 50% of nitrogen was quite as rapid during the induction period as that of the control in air, but later the rate became decidedly less. Owing to the presence of the aqueous phase oxygen pressures above the fat would be quite small—of the order of 0·0005 and 0·001 atmosphere in the two cases.

These figures indicate a considerable sensitivity towards $p_H$ of the aqueous phase, but cannot be considered as more than a rough guide to the quantitative effect. The experiments were therefore repeated using standard buffer systems to cover a wider $p_H$ range. Under these conditions a considerable range of oxidation rates was observed,[15] but it was now apparent that the nature of the buffer salts was having some influence on the oxidation rate. Later work showed this to be due to the fact that phosphate possesses marked antioxidant properties,[16] whilst borate is a very weak pro-oxidant.

In subsequent experiments, therefore, the mixed phosphate, borate, phenylacetate buffer system (in water) of Prideaux and Ward[17] was used, by means of which it was possible to cover the range from $p_H$ 2 to 10 simply by variation of the concentration of $Na^+$ or $Cl^-$, neither of which at the low total concentration employed is likely to affect the oxidation in any marked degree. Under these conditions several series of curves were obtained for different specimens of fat spread on slides and immersed in buffer in the manner already described. In Fig. 3 the initial rate of oxidation, considered in this case as the reciprocal of the number of days required by the fat to reach 5 ml. of 0·002N-thiosulphate per gram (induction period), is plotted against $p_H$. In all cases a sharp decrease in stability occurred as the solution became alkaline. The actual magnitude of the change, however, varied considerably from sample to sample, being greater in the water-extracted and charcoal-treated lards, which contain less of the natural antioxidant, than in the untreated ether-extracted specimens. When nitrite was present (0·03 g. per 100 ml.) stability also decreased sharply as the $p_H$ fell below 5. This behaviour, which accounts for the marked accelerating effect of acids on the oxidation of

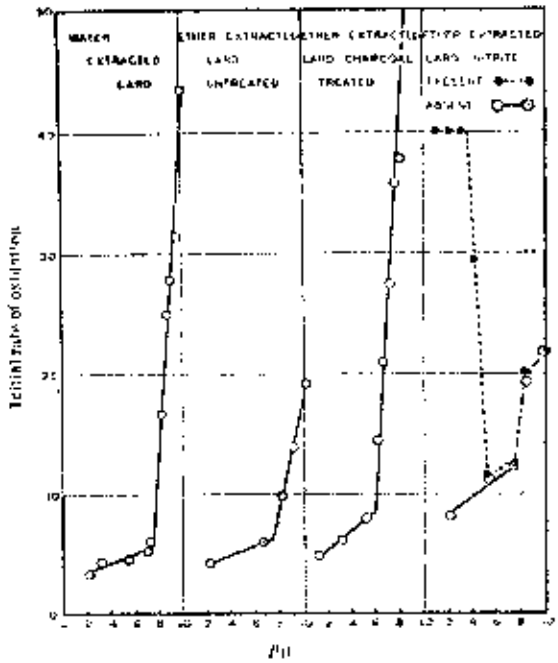


FIG. 3

*The effect of $p_H$ on the oxidation of lard in glass*

lard in pickle, already observed, is presumably due to a powerful pro-oxidant effect of free nitrous acid. Colorimetric determination by the Hosvay method did, in fact, show that the nitrite radical quickly disappeared from those solutions in which rapid oxidation occurred, but remained undestroyed for long periods in the others.

The similarity of these curves to the known variation in solubility of glass with change in $p_H$, coupled with the results of other experiments on the catalytic effect of minute traces of copper, now suggested that the accelerating effect of an alkaline $p_H$ might possibly be due to dissolution of copper from the glass, rather than to any direct action on the fat. The area of glass (which was of the ordinary "soft" variety) exposed to the liquid in each jar was fairly large—of the order of 1·3 sq. cm. per ml. of fluid.

To test this hypothesis, the interiors of small Pyrex beakers were completely covered with a thin film of

[14] C. H. Lea, J.S.C.I., 1934, 53, 152 T.

[15] C. H. Lea, Report of the Food Investigation Board for the Year 1934, p. 41.

[16] C. H. Lea, J.S.C.I., 1934, 53, 328 T.

[17] J.C.S., 1924, 125, 426.

charcoal-treated lard (average thickness 0·04 mm.), and filled with buffer solution. To one series, cyanide (0·001% as HCN) was added in order to inhibit the effect of any copper already present in the chemicals used. Under these conditions the fat oxidised rapidly and rather erratically, but now the $p_H$ effect (Table I, Expt. 1) appeared to be much reduced or, in case of the cyanide, almost eliminated. The difference in rate between the two series seemed to point to the presence of copper in the buffers, and analysis showed 0·2 and 0·5 parts of copper and iron respectively per million of solution.

TABLE I

*The initial rate of oxidation* of lard in buffer without contact with glass*

| Experiment 1 | | | Experiment 2 | | |
|---|---|---|---|---|---|
| $p_H$ | Cyanide absent | Cyanide present | $p_H$ | Cyanide absent | Cyanide present |
| 2·2 .... | 25 | .. | 1·9 .... | 6 | 4 |
| 3·9 .... | 26 | 10 | 3·3 .... | 6 | 5 |
| 4·8 .... | 25 | 10 | 4·1 .... | 6 | 5 |
| 6·2 .. | 19 | 11 | 5·1 .... | 4 | 6 |
| 7·1 . | 26 | 11 | 6·5 .... | 5 | 6 |
| 8·1 .... | 33 | 13 | 7·2 .... | 5 | 7 |
| 9·1 .... | 33 | 11 | 8·5 | 5 | 5 |
| 10·0 .... | 41 | 10 | 10·1 .... | 6 | 5 |

* Reciprocal of the number of days required to reach 25 and 5 ml. per g. respectively in experiments 1 and 2.

The boric, phenylacetic, and phosphoric acids used were therefore purified until the buffer made up in water distilled twice from Pyrex contained less than 0·005 part of copper per million. Water-extracted lard was used without charcoal treatment. The results (Table I, Expt. 2) showed that now the fat oxidised in presence and absence of cyanide at approximately the same rate, presumably indicating complete removal of significant amounts of copper. The spread of the points in both of these experiments is unusually high, owing partly to the thinner and much less uniform films obtainable by this technique, and partly to the increasing effect of chance contamination, as the amount of catalyst hitherto controlling the oxidation rate was reduced.

From consideration of these data it appears that, although the rate of oxidation of lard in glass vessels is very sensitive to the $p_H$ of an aqueous phase in contact with it, the effect is in fact a secondary one due probably to dissolution of copper from the glass, and does not take place when contact between aqueous phase and glass is prevented.

### *Effect of antioxidants in buffered solution*

It was apparent at an early stage in the experiments described above that if reliable comparisons were to be made between the efficiencies as antioxidants of a series of substances in aqueous solution in contact with films of lard and with glass, it would be necessary to maintain the $p_H$ at some fixed value throughout, or alternatively, as the later data suggested, to prevent contact between aqueous phase and glass.

Actually much of the later work was carried out in a borate buffer adjusted accurately to $p_H$ 7·0 by means of the glass electrode, after addition of the substance under examination. At this $p_H$ dissolution of the glass is relatively slight, and possible traces of copper derived from this source would in any case be the same for both antioxidant solution and control. Fig. 4, obtained by use of the slide technique already described, is, however, of interest in that it shows that antioxidants of the type under investigation retain their activity even in an alkaline solution, but it is obvious that the alkaline $p_H$

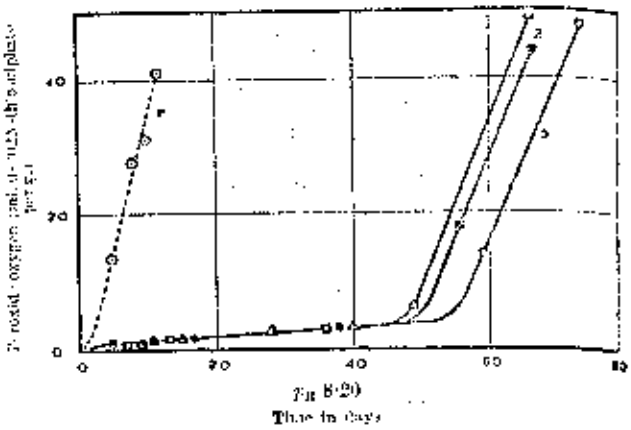


FIG. 4

*The effect of antioxidants (1%) in borate buffer at $p_H$ 8·2. (P) Pickle control. (1) Glycine. (2) Citric. (3) Malonic.*

has greatly reduced stability in the control solution, probably owing to dissolution of copper from the glass.

Fig. 5 and Table II give results obtained in a similar manner from charcoal-treated, ether-extracted lard at $p_H$ 7·0 with several antioxidants at three concentrations.

TABLE II

*The effect of concentration on the activity of inhibitors*

| Substance | Protection factors 0·01% | 0·1% | 1·0% |
|---|---|---|---|
| Buffer at $p_H$ 7·0 .... | 1·0 | 1·0 | 1·0 |
| Buffer + acetate .... | 1·3 | 2·1 | 3·1 |
| Buffer + malonate .... | 2·0 | 2·5 | 4·7 |
| Buffer + phosphate | 1·7 | 3·0 | 7·5 |
| Buffer + succinate .... | 4·3 | > 8·0 | > 8·0 |
| Buffer + phthalate | 6·6 | > 8·0 | > 8·0 |
| Buffer + citrate .... | 7·7 | > 8·0 | > 8·0 |
| Buffer + glycine .... | > 8·0 | 7·3 | — |

the latter calculated, as in all other cases, as the free acid. Protection factors shown in the table as > 8·0 varied from 8·0 to 11·0, the precise figure possessing little significance owing to the growth of moulds in some of the solutions after prolonged storage. Two other samples of lard showed much similar protection factors for 0·01% of antioxidant, the figures being: for urea —1·1, —1·1; phosphate 1·0, 1·1; citric 1·5, 1·7; and glycine 1·8, 2·2.

### *Pro-oxidant effect of copper and iron*

It is well known that a number of metals are able to function as accelerators in various oxidative reactions. Cobalt, vanadium, copper, manganese, iron, and lead, for example, are all capable of accelerating the atmospheric oxidation of unsaturated fatty acids, cobalt, manganese, and lead finding commercial application as driers for paints. Copper and iron are the metals most likely to be of importance in the commercial oxidation of the fat of foodstuffs, and here again, through their

[illegible] on [illegible] fats has repeatedly been demonstrated, but little information is available as to the effect of the metals when present in a system containing both fat and aqueous phase.

To investigate the effect of metallic salts in aqueous solution, a slightly different technique was employed. The fat, in this case herring oil extracted by ether from the fresh muscle, was dissolved (10%) in carbon tetrachloride, and pipetted into flat-bottomed Pyrex crystallising dishes to a depth of 4 mm., beneath a 2-cm. layer of buffer at $p_H$ 7·0 containing the metal in solution.* Glass-distilled water, and salts recrystallised until the

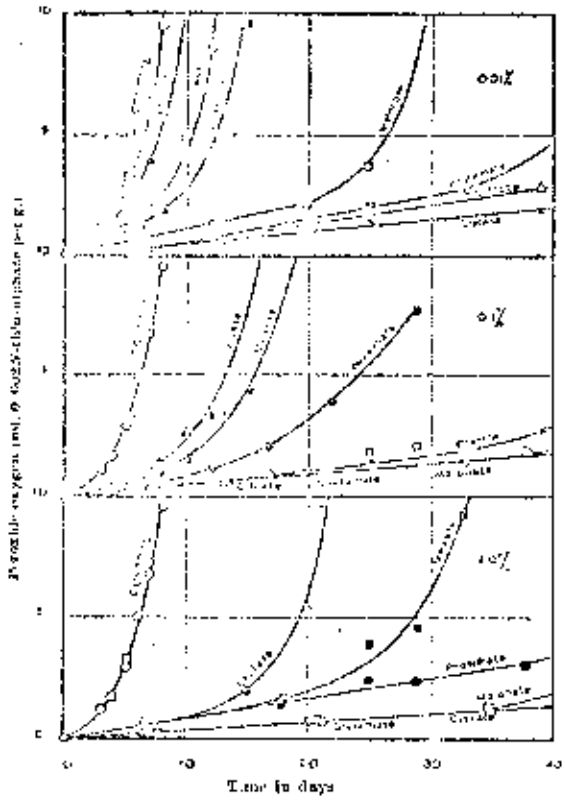


FIG. 5

*The effect of antioxidants (0·01, 0·1, and 1·0%) in borate buffer at $p_H$ 7·0*

control buffer and sodium chloride solutions at double concentration failed to give any reaction for copper, were used.† The presence of the solvent considerably retarded oxidation of the oil, but this does not affect the result.

The observed rates of oxidation (Fig. 6) indicate that the presence of one part of copper in ten million parts of the solution in contact with the fat increased the rate of oxidation of the latter to about 2·5 times its original value. Iron present at ten times the concentration was only about half as effective. The effect of copper was detectable even at a dilution of one part in one hundred million of solution.

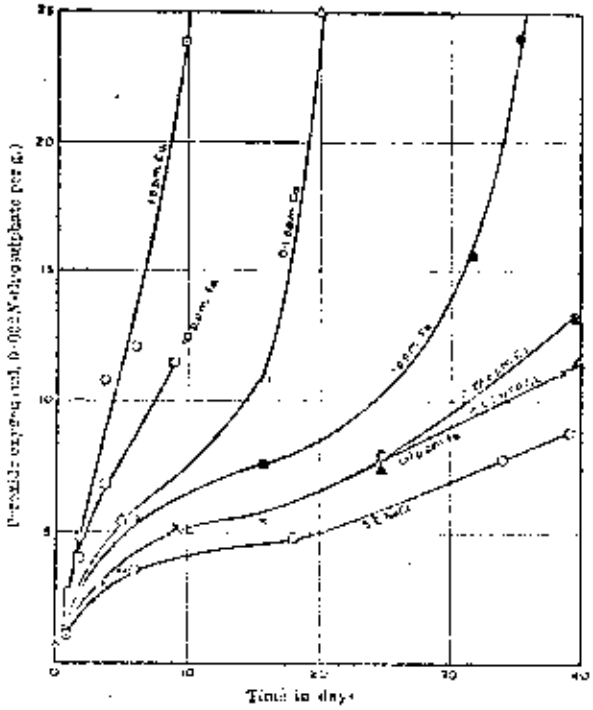


FIG. 6

*The effect of metals on the oxidation of herring oil at 20°*

Several experiments with films of lard immersed in borate buffer at $p_H$ 7·0 containing 5 parts of iron per million of solution proved that the metal under these

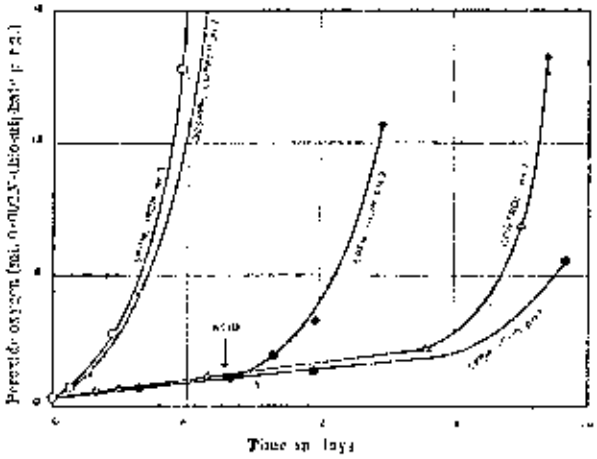


FIG. 7

*The effect of $p_H$ on the pro-oxidant activity of iron*

conditions was completely inactive. In acid solution, on the other hand, it appeared to possess about one twentieth of the activity of copper (Fig. 7). The $p_H$ at

* In [illegible] solutions containing iron it was necessary to add a little [illegible] in order to prevent precipitation of the metal.

† [illegible] the limit of sensitivity of the method employed for [illegible] of copper [illegible] dithiocarbonate with extraction by carbon tetrachloride is of the order of 0·01 part per million, [illegible] in experiments described as with "copper-free" reagents contained less than 0·005 p.p.m. of this metal.

which iron loses its activity (apparently owing to precipitation of the hydroxide) was not determined, nor were any tests made of the activity of the metal in the presence of protein.

Sodium chloride at a concentration of 5% not only failed to accelerate oxidation, but actually slightly retarded it (Fig. 6). This observation is in agreement with other experiments in which 26% of sodium chloride buffered at various $p_H$ values also failed to produce any appreciable acceleration. The earlier result in which an increased rate of oxidation was observed in presence of sodium chloride[11] was probably due either to the effect of traces of alkali in the salt on the $p_H$ of the unbuffered solution or to the presence of metallic impurities.

*Action of antioxidants in complete absence of copper*

The majority of the results so far reported concerning the effect of antioxidant substances in aqueous solution on the oxidation of lard were obtained in presence of traces of copper derived from the distilled water, buffer salts, or glass vessels. The amounts of active metal involved were extremely small but, in view of the data already presented, could not be ignored. Experiments with buffered solutions had attempted to overcome this difficulty by ensuring that control and antioxidant solution should always be under the same conditions as regards copper content. The possibility, however, still remained that these antioxidants might be acting indirectly via the copper rather than directly on the fat; in fact, it seemed reasonable to suspect that some or all of the substances under investigation might perhaps function as antioxidants only by inhibiting the positive catalytic action of copper.

In order to test this hypothesis it was necessary to oxidise the fat in contact with a copper-free aqueous phase in presence and absence of antioxidant. The method used was similar to that already described for investigation of the effect of $p_H$. The bottom and 1 cm. of the wall of small Pyrex beakers were covered with a thin film of fat, and the "copper-free" buffer solution containing the substance under investigation and adjusted to $p_H$ 7·0 was added in quantity just sufficient to cover the fat. Films produced in this way were very much less uniform in thickness than those obtained by means of the glass-slide technique previously used. At 20°, however, oxidation is relatively slow, and the rate is not very sensitive to variation in thickness of the film.

In addition to lard, freshly extracted herring oil transformed into a solid of suitable consistency by admixture with half its weight of the fully hydrogenated oil was used. The latter, after treatment several times with boiling dilute acid, gave negative tests for both iron and copper. The data listed in Table III were obtained from films of average thickness 0·325, 0·179, and 0·175 mm. at 30°, 20°, and 20° respectively. In experiments 2 and 3 the oil was boiled with dilute acid in an inert atmosphere for some time before use, in order to destroy or remove as much as possible of the natural antioxidant.

As was to be expected from its higher degree of unsaturation (iodine value about 136), the herring oil

TABLE III

*The effect of antioxidants on the oxidation of herring oil in presence and in absence of copper ($p_H$ 7)*

| Solution | Protection factors Expt. 1 | Expt. 2 | Expt. 3 |
|---|---|---|---|
| Buffer at $p_H$ 7·0 | 1·0 | 1·0 | 1·0 |
| Buffer + copper 0·2 p.p.m. | — | −7·7 | −3·1 |
| Buffer + cyanide 0·001 *M* | — | −1·1 | −1·1 |
| Buffer + copper + cyanide | — | −1·1 | — |
| Buffer + albumin 1% | 2·0 | 1·7 | — |
| Buffer + albumin 1% + copper | — | 1·7 | — |
| Buffer + albumin 10% | 2·5 | — | — |
| Buffer + triethanolamine 0·1% | — | — | 2·0 |
| Buffer + triethanolamine + copper | — | — | 1·0 |
| Buffer + glycine 0·01% | — | — | 2·5 |
| Buffer + glycine 0·1% | — | — | 2·5 |
| Buffer + glycine 0·1% + copper | — | — | 2·5 |
| Buffer + iron 5 p.p.m. | — | 1·0 | 1·1 |
| Buffer + iron + cyanide | — | 1·0 | — |
| Buffer + iron + albumin | — | −1·1 | — |

oxidised much more rapidly than lard under similar conditions, and without any very sharply defined induction period. For this reason the length of time required for the controls to reach 15 ml. of 0·002 *N*-thiosulphate per gram (calculated on the herring oil present) was taken as the unit (its actual magnitude was 4·0, 4·5, and 3·25 days), and the protection factors listed in Table III were obtained by comparison with this.

These data prove that cyanide in the absence of copper or similar metal does not possess antioxidant properties but may, if anything, slightly accelerate oxidation*. It does, however, completely inhibit the pro-oxidant influence of copper (and presumably also of iron). Absence of any retardation by cyanide can therefore be used as a criterion of the absence of significant quantities of these metals. Results of similar experiments with water-extracted, acid-treated lard are given in Table IV.

TABLE IV

*The effect of antioxidants on the oxidation of lard in "complete" absence of copper (temp. 20°)*

| Solution | Induction period* | Protection factor |
|---|---|---|
| Buffer at $p_H$ 7·0 | 5·6 | 1·0 |
| Buffer + diethanolamine 0·01% | 7·1 | 1·3 |
| Buffer + " 0·1% | 14·0 | 2·5 |
| Buffer + phosphate 0·1% | 16·7 | 3·0 |
| Buffer + malonate 0·1% | 16·8 | 3·0 |
| Buffer + citrate 0·01% | 23·6 | 4·3 |
| Buffer + phosphite† 0·1% | 25·0 | 4·5 |
| Buffer + pyrophosphate 0·1%, Buffer + citrate 0·1%, Buffer + glycine 0·01% | ca. 31·0 | ca. 5·5 |

* Days to reach 6 ml. per g.
† Contains also 0·01% phosphate.

It appears from these figures that the antioxidant effect of the substances tested is not dependent on the presence of copper in the solution, but that they are capable of retarding oxidation in absence of significant quantities of active metal. The fact that some of these antioxidants are also capable of completely inhibiting the effect of small amounts of copper (presumably by formation of very slightly ionised compounds, e.g., with proteins, or to incorporation of the metal in complex ions) indicates that in presence of traces of active metal

* The difference between −1·1 or 1·1·0 is, in this case, of the same order as the experimental error.

the protection factor found for substances of this type might be greatly increased (Fig. 4).

When the rate of oxidation of the oil was measured in presence of both protein and cyanide, the protective effect of the protein was found to be reduced. For example, 0·001 M-cyanide reduced the protection factor for 1% albumin from 2·0 to 1·4 and from 1·7 to 1·0 in two cases. Similar results were obtained with gelatin. This effect may perhaps be due to combination of the cyanide at antioxidant active centres of the protein molecule.

*Effect of large amounts of copper*

The results presented above indicate that the pro-oxidant effect of copper at a concentration of 0·2 p.p.m. (calculated on the aqueous phase, 0·6 p.p.m. on the fat) is completely inhibited by a number of antioxidants including protein. Milk, which has the approximate composition fat 3·5%, protein 4%, carbohydrate 5%, and water 87%, is known to develop an oxidised flavour

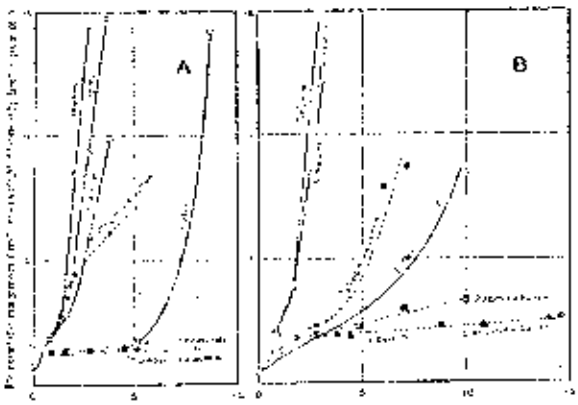


FIG. 8

*The pro-oxidant effect of copper in presence of protein, (A) in water, (B) in borate buffer at pH 7·0*

in about 24 hours at 0—5° when 1·5 p.p.m. of copper is present.[18] Similarly butter (fat 80%, protein 0·7%, water 13%) containing 2 p.p.m. of copper develops incipient tallowiness in about 6 days at room temperature.[19] The aqueous phases of tallowy milk and butter would therefore contain at least 1·7 and 15 p.p.m. of copper respectively, in addition to 4—5% of protein. Calculated on the fat these figures correspond to 40 and 2·5 p.p.m., and to percent quantities of metal much higher than those used in the present experiments, where the relative proportions of fat, protein, and water, but not the area of surface of the fat exposed, approximated to those in milk.

Experiments similar to those already described, but using an aqueous phase containing 4% of albumin and 0·4, 2·0, and 15·0 p.p.m. of copper (as lactate), were therefore carried out. The fat was water-extracted, acid-treated lard, and the temperature 20°. It was necessary to duplicate the samples, using water in one case and buffer in the other, since a large proportion of the metal precipitated from buffer solutions containing 15 p.p.m. of copper. On the other hand, the presence of borate was essential when readings extended beyond 4—5 days in order to inhibit the growth of micro-organisms. The results (Fig. 8) show that although protein completely inhibits the pro-oxidant action of small amounts (0·4 p.p.m.) of copper, larger quantities of the metal (15 p.p.m.) powerfully accelerate oxidation in spite of the presence of the protein.

The rate of oxidation after conclusion of the induction period was slightly greater in presence of the larger amounts of copper, but the acceleration produced, except perhaps at very low concentrations, did not increase in proportion to the amount of copper added. It is possible that in this case the limiting rate of oxidation, as fixed by diffusion of oxygen into the fat, was being approached, but this explanation cannot account for the failure of the large amount of copper to reduce the induction period below about 1·7 days. Much more rapid oxidation has been observed for solidified herring oil under identical physical conditions.

*Action of water-soluble antioxidants in presence of very little water*

The majority of the experiments on the effect of water-soluble antioxidants were carried out in presence of a volume of the aqueous phase large by comparison with the fat. In only one case (Fig. 2) was the proportion of water present relatively small (15% v. v., of the same order as in butter), and in this, reduction of the volume of water by 200—300 times had apparently failed to impair the activity of the antioxidants used. Some natural fats, however, occur in the tissue in presence of still smaller amounts of water, and commercial lards contain only 0·2—0·3%. It seemed doubtful whether antioxidants of the water-soluble type would be effective under such conditions.

Aqueous solutions of several antioxidants, adjusted approximately to pH 6 but unbuffered, were therefore prepared of such concentration that addition of 0·25% of the solution to the fat (water-extracted, acid-treated lard) gave a concentration of 0·02% of antioxidant in the fat. Under these conditions the antioxidants were still effective in stabilising the lard (Table V).

TABLE V

*The effect of water-soluble antioxidants in presence of very little water (temp. 32°)*

| Added to the lard | Induction period* | Protection factor |
|---|---|---|
| Dry lard | 6·7 | — |
| Water 0·25% | 6·4 | 1·0 |
| Water 0·25% + albumin 0·02% | ca. 7·7 | 1·4 |
| Water 0·25% + phosphate 0·02% | 10·6 | 2·0 |
| Water 0·25% + glycine 0·02% | 20·7 | 3·8 |
| Water 0·25% + pyrophosphate 0·02% | 22·0 | 4·1 |
| Water 0·25% + citrate 0·02% | 22·0 | 4·1 |

* Days to reach 4 ml. per g.

*The combined effect of two antioxidants*

The results of experiments already described had shown that, under the conditions used, a concentration of antioxidant was soon reached beyond which further increase gave no increase in protection; in fact in case

[18] W. L. Davies, Milk Ind., 1934, 18, 55.

[19] W. L. Davies, "The Chemistry of Milk," London, 1936, p. 63.

of glycine concentrations greater than 0·01% usually gave inferior results. On the other hand some, at least, of the antioxidants tested were capable of prolonging the induction period of the fat beyond that due to the natural antioxidant (Table VI). The data given for acid-treated lard in Table VII show that citrate plus

TABLE VI

*The effect of antioxidants in aqueous solution* on the oxidation of ether-extracted, untreated lard at 32°*

| Solution | Induction period† | Protection factor | Induction period‡ | Protection factor |
|---|---|---|---|---|
| Water ... ... | 7—11 | 1·0 | 9—13 | 1·0 |
| Glycine 0·01% ... | 16 | 1·8 | 19 | 1·7 |
| Pyrophosphate 0·01% ... ... | 18 | 2·0 | 22 | 2·0 |
| Citrate 0·01% | 20 | 2·2 | 24 | 2·2 |

* $p_H$ 6 but unbuffered. † Days to 4 ml. per g. ‡ Days to 6 ml. per g.

TABLE VII

*The combined effect of two antioxidants on lard at 20°*

| Solution | Induction period* | Protection factor |
|---|---|---|
| Buffer at $p_H$ 7·0 ... ... ... | 6 | 1·0 |
| Buffer + albumin 4% ... ... ... | 14—20 | 2·8 |
| Buffer + glycine 0·02% ... ... | 15 | 2·5 |
| Buffer + pyrophosphate 0·02% ... | 19 | 3·2 |
| Buffer + citrate 0·02% ... ... | 23 | 3·8 |
| Buffer + albumin + citrate ... ... | 15 | 2·6 |
| Buffer + albumin + pyrophosphate | 14—19 | 2·7 |
| Buffer + citrate + glycine ... ... | 28 | 4·7 |
| Buffer + glycine + pyrophosphate ... | 31 | 5·2 |

* Days to 5 ml. per g.

glycine, or glycine plus pyrophosphate, protected the fat considerably more efficiently than did either component of the pair alone. Unfortunately the same was not true when one of the substances was protein, neither citrate nor pyrophosphate increasing the protective effect over the normal figure for the protein alone. These results must be confirmed with higher concentrations of antioxidant and extended to cover other substances, before it can be assumed that non-toxic antioxidants of the types studied are in general ineffective in presence of protein.

## DISCUSSION

The majority of the experiments described here have been carried out in presence of a borate buffer at $p_H$ 7·0. This, even in the "copper-free" condition, acts as a very weak pro-oxidant, reducing, for example, the induction period of an untreated lard (at 20°) from 27 days in water to 22 days in buffer. Measurements of the efficiency of antioxidants in water confirmed those obtained in buffer, but were more erratic, even when contact with glass was minimised by deposition of the fat on the walls of the beaker (Table VI). The case in which only 0·25% of water was present was exceptional in giving curves as smooth as in the buffer experiments.

Treatment of lard by (*a*) heating with 2½% by volume of 30% sodium hydroxide at 100° for 8 minutes, (*b*) boiling with 8% hydrochloric acid under steam for 1 hour, or (*c*) heating with 2% of absorbent charcoal at 100° under nitrogen for 15 or 30 minutes, all reduced the induction period (measured at 100° by a method previously described[16]) to approximately half its previous value. The maximum effect obtained by boiling several other samples of lard and two of herring oil with dilute hydrochloric acid was a similar decrease, the effect in case of lard being often much less than this. Hilditch and Sleightholme,[20] on the other hand, reduced the induction period of olive oil at 100° from 260 to 20 minutes by this means. It follows that the properties of antioxidants present in different natural fats vary considerably. Even the numerous samples of leaf lard used in the present experiments varied both in susceptibility to oxidation and in ease of stabilisation by antioxidants.

Several substances were found to produce a protective effect over and above that due to the natural antioxidant. Sometimes the peroxide-oxygen content increased steadily throughout the induction period, first at a constant rate, and then more rapidly as the induction period drew to an end. In other cases the peroxide value rose rapidly at first, slowed down again or even remained practically constant for a more or less prolonged period, and then rose again as rapid oxidation set in (Figs. 6 and 8). This behaviour could be due either to an initial oxidation of some labile constituent of the fat, or, perhaps more probably, to attainment of a temporary equilibrium between the rates of formation of peroxide and of its reduction by the antioxidant. In the latter process the antioxidant itself would be destroyed and rapid oxidation of the fat would follow.

Since the samples containing added antioxidants began to oxidise rapidly at approximately but not exactly the same peroxide value, the protection factors obtained by comparison of the times required for the stabilised and unstabilised samples to reach 4, 5, or 6 ml. per g. give only an approximate comparison of the activities of antioxidants. These figures also fail to take into account the depressing action still occasionally in evidence after the end of the induction period proper (*e.g.*, curve 9, Fig. 2), and when this is present the protection factor gives an estimate of efficiency which is too low.

The water-soluble antioxidants examined appear to retain some of their activity on the alkaline side of the neutral point, but if tested on lard in glass the $p_H$ effect already described will result in an increased rate of oxidation as compared with the same antioxidant in acid solution, although the protection factor obtained may actually be higher (Fig. 4). These observations possibly explain the insistence on an acid reaction in the patents cited below. A second reason is that the salts of the various antioxidants are probably insoluble, or much less soluble in the dry fat than the corresponding acids.

### *Relationship between antioxidant activity and structure*

Of the organic substances examined, polyhydroxy-compounds such as glycerol $C_3H_5(OH)_3$ and the sugars—glucose $C_6H_{12}O(OH)_5$, fructose $C_6H_{12}O(OH)_5$, and sucrose $C_{12}H_{22}O_3(OH)_8$—were all active in 2% aqueous solution (0·3% calculated on the fat). They were not tested at higher dilutions. Mannitol $C_6H_8(OH)_6$ was inactive, but the sample used contained 8 p.p.m. of copper (0·16 p.p.m. on the aqueous solution), which probably accounts for the apparent discrepancy.

* J.S.C.I., 1936, 55, 39 T.

With one or more carboxyl groups present in the molecule, even one hydroxyl group is effective. Glycollic $OH \cdot CH_2 \cdot CO_2H$, lactic $CH_3 \cdot CH(OH) \cdot CO_2H$, and malic $CO_2H \cdot CH_2 \cdot CH(OH) \cdot CO_2H$, acids all possessed marked protective properties. In the case of malonic acid $CH_2(CO_2H)_2$, the two carboxyl groups plus a reactive methylene group produce a moderately powerful antioxidant, although succinic acid, in which the carboxyl groups are further separated and the methylene groups inactive, does not confer protection. A combination of two or more carboxyl groups with one or more hydroxyl groups as in tartaric $CO_2H \cdot CH(OH) \cdot CH(OH) \cdot CO_2H$, or even more so in citric acid $CO_2H \cdot CH_2 \cdot C(OH)(CO_2H) \cdot CH_2 \cdot CO_2H$, produces a powerful antioxidant.

Aliphatic amino-acids appear to be even more effective than the corresponding hydroxy-compounds. Glycine $NH_2 \cdot CH_2 \cdot CO_2H$, with only one amino- and one carboxyl group, is of the same order of efficiency as citric acid. Aspartic acid $CO_2H \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$, glutamic acid $CO_2H \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$, and asparagine $CO_2H \cdot CH(NH_2) \cdot CH_2 \cdot CO \cdot NH_2$, are all powerful antioxidants. All of these substances are effective at concentrations as low as 0·01%. In some cases increasing the concentration in the aqueous phase above this value increased the protective effect (Table II), in others (*e.g.*, glycine) it did not.* Proteins (albumin and gelatin) were found to possess quite marked antioxidant properties, which suggests that natural oxidation inhibitors may in some cases be of a protein nature. Di- and tri-ethylamine $N(H)(C_2H_5 \cdot OH)_2$ and $N(C_2H_5 \cdot OH)_3$ possessed some activity, but less than might have been anticipated from their structure.

Among inorganic substances, phosphoric acid $PO(OH)_3$ is active and boric acid $B(OH)_3$ practically inactive. No data appear to have been published on the use of phosphoric acid as an antioxidant, but since its activity was observed,[13] patents covering the use of ortho- and meta-phosphoric acid, sulphuric acid, and their salts having an acid reaction have appeared.[21] The author finds pyrophosphoric acid $P_2O_3(OH)_4$ considerably more active than the ortho-acid, particularly at low concentrations. Phosphite $HPO(OH)_2$ also seems to be better than phosphate. The use of acid phosphites for the prevention of rancidity in cereal products, flours, etc., has been patented.[22] Phosphite has the advantage of being a reducing agent which on oxidation yields phosphoric acid, itself an antioxidant. Phosphites, however, are rather unstable. Several commercial samples examined, after removal of the large amount of copper originally present, were found to contain only about 20% of phosphite, the remainder being phosphate.

Until quite recently it was generally assumed that a substance to be an antioxidant must also be a reducing agent, or at least a fairly readily oxidisable substance. Instances, however, have been accumulating for autoxidative reactions outside the field of fat chemistry in which this relationship does not hold, and phosphoric and pyrophosphoric acids, as shown in the present paper, are similar exceptions in the case of fats.

The majority of the antioxidants examined are non-toxic substances suitable for addition to foodstuffs. Pyrophosphoric or citric acid or their salts, or glycine, for example, might be incorporated into lard, margarine, or shortening fats. Glutamic acid and protein, though fairly effective, are probably less suitable because of their tendency to encourage microbial spoilage. On the basis of the results given in Table VII it seems doubtful whether addition of antioxidants of the type here investigated to fat-water systems already containing an appreciable quantity of soluble protein, will produce any marked stabilising effect. This point requires further investigation.

## SUMMARY

The effects of water-soluble substances on the rate of oxidation of lard in contact with an aqueous phase have been examined. It has been found that:

(1) The oxidation of lard in glass vessels in contact with water is much more rapid at an alkaline than at an acid $p_H$. It is suggested that this effect is due to dissolution of copper from the glass.

(2) At $p_H$ values below 5 nitrite is a powerful pro-oxidant. Its action at a concentration of 0·03% is not inhibited by 5% of various antioxidants.

(3) Copper and iron in aqueous solution accelerate the oxidation of lard. Copper is approximately 20 times as active as iron, its effect being perceptible at concentrations as low as 0·01 part per million. Iron in neutral or alkaline solution, *i.e.*, colloidal ferric hydroxide, is inactive.

(4) Aliphatic polyhydroxy-compounds such as glycerol and the sugars are weak antioxidants.

(5) Aliphatic hydroxy-acids, *e.g.*, lactic and glycollic, the ethanolamines, and malic acid are moderate, and polybasic hydroxy-acids, *e.g.*, tartaric and citric, powerful, antioxidants. Malonic acid is also effective.

(6) Aliphatic amino-acids, *e.g.*, glycine, aspartic and glutamic acids, and asparagine, are all powerful antioxidants. Protein has also considerable antioxidant activity and may be partly responsible for the stabilisation of crude natural oils and fats.

(7) The pro-oxidant effect of copper at low concentrations (up to approximately 1 p.p.m. under the conditions here employed) is completely inhibited by protein, cyanide, and other antioxidants. At higher concentrations of the metal, even 1% of protein fails to prevent a powerful acceleration of oxidation.

(8) Orthophosphoric acid is a fairly good, and pyrophosphoric a very good, antioxidant. Phosphorous acid is also effective.

(9) These water-soluble antioxidants still inhibit oxidation when the water content of the fat is as low as 0·25%.

(10) They are active at $p_H$ 7, certainly in more acid and probably in more alkaline solutions. In the latter

* These conclusions apply to the two-phase system. Soluble antioxidants added directly to the fat in some cases prolong the induction period for a time roughly proportional to the initial amount added.[15]

[21] I. W. E. Co., U.S.P. 1,982,997, 1934; U.S.P. 1,993,152, 1935, A. S. Richardson, F. C. Vibrans, and J. T. R. Andrews, U.S.P. 1,993,184, 1935.

[22] G. Co., U.S.P. 1,938,000, 1933.

[23] H. B. Cornell, R. S. Olcott, and H. A. Mattill, Ind. Eng. Chem., 1935, 27, 724.

case the effect obtained in glass vessels will be reduced by (1).

(11) Under the conditions of these experiments the more powerful of the antioxidants examined were effective at concentrations as low as 0·01%, producing an extension of the induction period, measured at 20° in complete absence of copper, of from 2 to 6 times. The magnitude of the factor obtained varied with the sample of fat, and in presence of traces of copper was sometimes as high as 20. For some of the substances used, increase in the concentration in the aqueous phase beyond 0·01% produced an increased effect; for others it did not. These inhibitors produce an effect over and above that of the natural antioxidants, and in two cases examined an appreciable advantage was secured by using a mixture of two antioxidants. This, however, did not appear to be the case when protein was one component of the mixture.

(12) It has been shown that a number of these antioxidants do not function by inhibiting the pro-oxidant effect of traces of copper. An exception is cyanide, which has no antioxidant properties in the absence of active metal. Whether or not copper and other pro-oxidants function only by destroying natural antioxidants is not known.

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# The Evolution of Vitamins in the Technology of Dietary Oils and Fatty Materials

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Summary

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## Introduction

Natural fatty materials contain what are commonly called the lipsoluble vitamins, among which the most important are vitamins A, D and E (Table 1). The first two are found in fish liver and also in butter, but the study of these products is not within the scope of this paper.

Table 1

Liposoluble vitamins

| Nature | Sources |
|---|---|
| Vitamin A ..... | Fish liver oil<br>Butter<br>Animal fat (?)<br>Olive oil (?) |
| Vitamin B ..... | Rice bran oil (?) |
| Vitamin D ..... | Fish liver oil<br>Wheat germ oil |
| Vitamin E ..... | Germ oil (corn wheat)<br>Soy bean oil |
| Vitamin K ..... | Soy bean oil (?) |
| Provitamin A ..... | Palm oil |
| <u>Essential Fatty Acids</u> | |
| Linoleic acid ..... | Soybean oil and sunflower seed oil |
| Arachidonic acid (Vitamin F) .... | Animal fat |

Animal fats (tallow, lard) are virtually lacking in vitamins, but it must be remembered that they do contain essential fatty acids which are of great interest in the nutritional field (arachidonic acid) and which are not found in vegetable oils (2). These limits being drawn, we propose to study the latter which may contain vitamin E in oils of grains, and provitamin A in the pulp of the fruits of palm trees (in oil form).

# I

## Vegetable Oils as a Source of Tocopherol

Vitamin E or tocopherol is widely found in vegetable oils. (We are not considering the cases of wheat germ or corn oils which must be considered more as dietetic products. [Table II].

Liquid vegetable oils thus appear to be our principal food source of vitamin E (1). It is therefore important to know what becomes of them in the course of various technical operations. The vegetable oils are extracted from grains, from the pulp or cores of oily fruits by grinding, pressure, centrifugation or dissolution in a solvent. This last method designated more exactly as "extraction" is now generally used in all grain oil-works. It has the advantage of giving a higher yield which is of importance to industry, but it also has the advantage of enriching the oils with liposoluble vitamins (as in the case of soybean oil, for example).

Numerous studies have been done on the amounts of tocopherol contained in various food products and on the techniques of quantitative determination (8). The old method of Emmerie-Engel has undergone many modifications to increase the accuracy. Other methods have been developed which enable (12) (23) (28) discrimination to be made between the different tocopherols $\alpha$, $\beta$, $\gamma$ and $\delta$ in which the vitamin E effect will decrease in the indicated order while the protective antioxygen effect will vary in the reverse order (11) (14) (33).

Among these studies, the important work of W. Lange (19), whose figures have been quoted in many publications (3) (9) (30), should be pointed out. Soybean oil is the most rich in tocopherol (16) (25) (33), outside of wheat germ or corn oil which are considered dietetic products:

the amount currently approaches the value of 200 mg per 100 g (Tables III and IV). Then follow cotton seed and sunflower seed oils with average amounts established between 110 and 140 mg (11) (16) (18). The methods of determination of the various tocopherols show cotton seed oil to be particularly rich in α tocopherol (11). Considerably below these numbers are peanut oils, colza oils and palm oils which may contain up to 50 mg per 100 g (11) (16) (18).

Table II

Amounts of tocopherols in raw vegetable oils

(in mg/100 g)

[According to (19)]

| Nature | Total | α | β | γ | δ |
|---|---|---|---|---|---|
| Soybean oil ..... | 152<br>212<br>168 | 20 | | 98 | 50 |
| Cotton seed oil ..... | 110<br>120 | 76 | | 34 | |
| Sunflower seed oil ..... | 140 | 10 | | 130 | |
| Colza oil ..... | 55 | | | | |
| Palm oil ..... | 56<br>44 | 30 | | 26 | |
| Peanut oil ..... | 52 | 30 | | 22 | |
| Olive oil ..... | 3<br>8<br>20 | | | | |
| Coco oil ..... | 3 | | | | |
| Summer butter ..... | 4 | | | | |
| Winter butter ..... | 1.7 to 3 | | | | |
| Egg yolk ..... | 2 | | | | |
| Wheat germ oil ..... | 550 | | | | |
| Corn seed oil ..... | 250 | | | | |

Table III

Amounts of tocopherol (1) in soybean oil extracted (2) from different varieties of grain.

| Variety | P. 100 extracted oil | Amounts in mg/100 g |
|---|---|---|
| Lincoln ..... | 15.3 | 236 |
| Richland ..... | 15.9 | 203 |
| Illine ..... | 15.3 | 225 |
| Dunfield ..... | 14.6 | 203 |
| Ogden ..... | 13.4 | 169 |
| Armyedo ..... | 17.7 | 280 |
| Delsta ..... | 16.7 | 217 |
| Average ..... | 15.9 | 215 |

(1) Evaluated by the Emmerie-Engel method modified by Rawlings and Colleagues (Jaocs, 1948, 25, 25).
(2) Extraction is done with petroleum ether after drying the grain for 4 hours at 130°C.

Table IV

Comparison of the amounts of tocopherol in raw soybean oil according to the method of extraction.

[From Rawlings and colleagues, (Jaocs, 1948, 25, 25)]

| Procedure | Amount in mg/100 g |
|---|---|
| Extraction (Hexane) ..... | 172 |
| Pressure (Expeller) ..... | 180 |
| Extraction and Removal of mucilage ..... | 176 |

Coming far behind is olive oil (8) (26) with average amounts of 10 mg and coco oil which contains only a few milligrams per 100 g.

It must be taken into consideration that butter, as well as eggs, meat and cereals which comprise the basis of our diet are extremely poor in tocopherol (16) (17) (27). By contrast, wheat germ oil or corn oil is

very rich in tocopherol (20), the former having 500 mg per 100 g and the second often surpassing amounts of 200 mg.

## II

## Refining

### A. Necessity of Refining

Most extracted vegetable oils are not edible in raw form: peanut oil has a taste of fresh peanuts, soybean oil has a bean flavor and colza oil smells like cabbage. Even unprocessed olive oil is not universally appreciated.

Besides, the commercial usages of oils require that they generally have a liquid yellow gold appearance which is demanded by the consumer. There is therefore the necessity of a real improvement in their flavor and appearance. Also, and this is of prime importance for the trademark, it is necessary that they can be preserved for a sufficient period of time, in other words, that they not become rancid in stocking or in the store. It has been shown that if vegetable oils possess natural anti-oxidant properties or precisely stated, the tocopherols which protect them from oxidation, they also contain oxidizing agents which accelerate the attack by the oxygen in the air. Among these substances, it is necessary to mention metallic traces and especially the presence of free fatty acids. These are formed by the action of the lipolytic enzymes which hydrolyze the triglycerides at the time of extraction and which may be particularly active under certain favorable conditions. The industrial preparation of the sterilization of palm fruits for oil at the time of picking, has as a consequence, in destroying the enzymes, the effect of producing palm oils

of weak acidity, while without this precaution the free acidity could in a few days surpass 50 p. 100.

It has been shown that the free fatty acids are much more oxidizable than the triglycerides, and that because of this fact, they may play the role of initiator of auto-oxidation and quite rapidly cause the oil to become rancid. It is therefore necessary to eliminate them. All these considerations lead to the necessity of refining.

B. Refining (Table V)

The refining of oils has been the subject of a great number of publications which we cannot mention here (10) (29) (31), and we will not go into the details of the procedures. Refining is comprised of the following standard operations:

Table V — Refining of Oils

| Operations | Products Used | Operating Conditions | Purpose |
|---|---|---|---|
| Removal of mucilage | Water<br>Phosphoric Acid<br>Acetic anhydride | 70-80° Ordinary pressure | Elimination of phosphatides |
| Neutralization | Alkaline Washing 10-20° B | 70-80° Ordinary pressure | Elimination of free fatty acids in the form soap |
| Deacidification (*) | Water vapor | 220-240° at reduced pressure | By distillation |
| Decolorization | Activated soil<br>Activated charcoal | 80-120° | Elimination of colored pigments + traces of soap |
| Deodorizing | Heated water vapor | 180-230° at reduced pressure | Elimination of odorous products (natural + resulting from an alteration) |
| Hydrogenation | Technical operations:<br>Hydrogen catalyst with nickel base | 175-195° pressure 1 to 40 kg. | Saturation of the double bonds |
| Chilling | None | 10° | Elimination of solid products (triglycerides and wax) |

(*) This operation usually takes place after decolorization.

(1) Removal of mucilage is still called "degumming", and its purpose is to eliminate the water soluble phosphatides and to prevent them from precipitating in the oil giving it a clouded appearance. To achieve this effect, water at 70-80° is most often used, and the operation lasts for 45 minutes in discontinuous process. The mucilage which is formed is eliminated by centrifugation. In certain cases and for the purpose of more complete removal of mucilage, the operation is done in the presence of

phosphoric acid. Even strong acid (nitric, for example) and acetic anhydride have been used for soybean oil (15) (21).

It must be added that the operation of removing the mucilage is equally for the purpose of facilitating later operations, that is to say, the alkaline neutralization where it is essential to reduce to a minimum the losses from the neutral oil, and especially for the operation of continuous deacidification where it is essential to completely eliminate mucilage in order to avoid dirtying the machinery.

(2) Neutralization — The purpose of this operation is to eliminate the free fatty acids in the form of soap precipitate (soap stock or neutralization precipitates). To obtain this effect, the oil with the mucilage removed is heated by washing with caustic soda of concentration between 14 to 32° Be (Baume) (average of 20° Be), and then, sodium carbonate is added. The operation takes place at a temperature between 70 and 90°C and lasts for anywhere between 15 minutes to two hours. The soap can be separated by centrifugation which is always a continuous process.

The neutralization is followed by washing the oil with saline solution, and then with pure water for the purpose of eliminating all traces of soap. Then the drying of the oil is done, an operation which is essential before decolorization or deacidification. To do this, the oil is warmed in a vacuum of 100 mm/g at 90-100°. Dripping apparatuses are actually used. The operation lasts 30 to 40 minutes. Often in the course of the operation, a little citric acid is added which plays a synergetic anti-oxidant role. It should be noted that the soap precipitation carries with it a certain amount of colored pigments and because of this, there is a noticeable "clearing" of the oil.

(3) Deacidification — This involves a physical neutralization. This method was developed in conjunction with the continuous processes. The elimination of the fatty acids takes place by evaporation or distillation in the presence of heated water vapor. The operation is done on a thin film and under a vacuum of several millimeters of Hg at high temperatures: 220 to 240°C. This operation has the advantage of not necessitating any chemical reagent. Furthermore, it is combined with the deodorizing process. It has the disadvantage of requiring the oils to have the mucilage completely removed in order to avoid soiling the contact surfaces.

(4) Decolorization or bleaching. The purpose of this operation is to eliminate the colored pigments in the oils, in particular, the chlorophylls and the carotenes which give the oils a dingy appearance. The oil is shaken and treated with activated acid soil, or less infrequently with activated charcoal, or sometimes with a mixture of the two adsorbants. The discontinuous operation lasts from 20 to 40 minutes. It is done under reduced pressure of about 100 mm/Hg and at temperatures from 80 to 120°. The oil is then filtered through a layer of diatomaceous soil in a filter-press or a pressure filter (at 70-80° under 3 atmospheres, for example).

It must be added that another purpose of this operation is to eliminate the last traces of soap and to identify certain impurities, oxidized acids and metallic traces, which are harmful to the preservation of the oil.

In the case where the elimination of the free fatty acids is done by physical means by using their greater volatility in the presence of water vapor, the order of the physical decolorization-neutralization operations is reversed in order to have oils as pure as possible at the time when they go into the deacidification apparatus.

(5) Deodorizing — The purpose of this is to eliminate the sapid products which give the oil "taste". These products create the unique flavor which comes from the beginning of the oxidation of the oil. The oil is sprayed with warm and deaerated water vapor, and often a small amount of citric acid is added. The operation is done at 180-220° under reduced pressure of 2 to 18 mm/Hg. The process lasts from 4 to 8 hours as with the deacidification process with which it is combined. The operation may be done continuously with a dripping apparatus of a thin layers in rows. This procedure has the advantage of decreasing the time of the operation which may be reduced to 40 minutes.

This operation has often been criticized because it makes the oil lose all flavor giving it a neutral or "flat" taste. However, it offers to the consumer the guarantee of a standard product of good quality because it is not possible in this case to hide the changes marking the beginning of the process of becoming rancid, which are immediately detectable. Finally, as we have said in most cases, it is an essential operation, the aim of which is to make the oil consumable. Sight must not be lost of the fact that the aim of industry is to satisfy the greatest number of consumers.

Let us add in conclusion, that the oil is submitted to drying under reduced pressure and to purification by a cellulose pocket filter to give it its limpidity and "sparkle".

It appears that it is especially during the deodorizing process that the losses of the tocopherols are the most significant given their relatively much weaker volatility than the triglycerides and the fatty acids.

They are found in significant quantities in the distillates or the by-products of the deodorizing process (24). This loss is estimated at about 5 p. 100 (5).

## III

## Other Technical Operations

A. Freezing (demargarination or winterization)

The purpose of this operation is to separate the crystallizable elements at low temperatures (glycerides, sterols, waxes, etc.); the precipitation of these elements when the product is stored would be harmful to the limpidity of the oil.

This operation takes place at low temperature (<10°) and with slow agitation in order to promote the formation and growth of the crystals. It is followed by filtration through a filter-press or under pressure.

B. Hydrogenation (or stiffening)

This may be considered as the opposite of the preceding operation and has for its purpose the raising of the melting point of the oil or fat. This may be selective, in order words, an effort is first made to saturate the most unsaturated chains which give the oil a certain instability and may be the cause of a change in the taste (reversion). The operation is done under pressure of from 1 to 40 atmospheres in the presence of catalytic nickel and the hydrogen is added under strong turbulence at temperatures from 175 to 195°. The operation is followed by a refining to eliminate all traces of the nickel and the acidity which may have been formed. It should be noted that this operation may cause the essential fatty acids to disappear. (1)

IV

## Influence of the Technical Operations on the Amounts of Tocopherol in Vegetable Oils (Tables VI, VII, VIII)

Let us say first of all that the experts who have studied what becomes of tocopherol during the course of the refining operations were very affirmative. M. R. Feron (9) expressed it thus: "Agreement on this point is virtually unanimous: the tocopherols undergo with nearly no damage, the operations of refining and hydrogenation".

This conclusion results from the study of the figures indicated in the table. A systematic study was done in the laboratory of a large American company on the refining of cotton seed oil, and the results showed that neither the successive operations of refining, nor the hydrogenation brought about a loss of tocopherol greater than 10 p. 100. The Institute for the Study of Fats is interested in this problem. In the course of a study on the refining of soybean oil, we followed the evolution of the tocopherols during the different stages of the refining process. We found here that the loss of tocopherols was on the order of 20 p. 100 (17) (Table IX).

Table VI

Amounts of tocopherols in refined vegetable oils
(in mg/100 g)

[From (19)]

| Nature | Total | α | β | γ | δ |
|---|---|---|---|---|---|
| Soybean oil ..... | 125 to 175 | | | | |
| | 190 | | | | 57 |
| | 168 | 20 | | 98 | 50 |
| | 140 | 10 | | 130 | |
| Cotton seed oil ..... | 110 | 76 | | 34 | |
| | 90 | 56 | | 34 | |
| | 87 | 49 | | 37.7 | |
| Peanut oil ..... | 51 | | | | |
| | 48 | | | | |

The oil technology has evolved during the past twenty years towards the adoption of continuous processes. These processes have the advantage of a more limited reaction time and because of this have beneficial effects on the amounts of vitamins. This results from the comparison between the continuous processes obtained by the use of centrifuges and the discontinuous ones by the threshing machine or even by a laboratory method. Tocopherol, being particularly sensitive to oxidation in an alkaline milieu, the advantage of the use of continuous processes which limit the reaction time, is obvious. (Table VII)

Table VII

Comparison of the losses of tocopherols according to the method of alkaline refining

[From Rawlings and colleagues, (Jaocs, 1948, 25, 25)]

| Method | Amounts in mg/100 g | | Loss in % |
|---|---|---|---|
| | Raw | Refined | |
| By centrifugation (in the factory) ... | 187 | 185 | 1.1 |
| | 198 | 190 | 4 |
| | 193 | 189 | 2.1 |
| | 193 | 192 | 0.5 |
| | 211 | 199 | 5.7 |
| Average ..... | | | 2.7 |
| By threshing-machine (in the factory) ... | 191 | 167 | 12.6 |
| | 188 | 168 | 10.6 |
| | 186 | 161 | 13.4 |
| Average ..... | | | 12.6 |
| By agitation (in the laboratory) ..... | 189 | 148 | 21.7 |
| | 178 | 156 | 12.4 |
| | 195 | 161 | 17.4 |
| | 191 | 162 | 15.1 |
| Average ..... | | | 16.7 |

Table VIII

Influence of refining and hydrogenation on the amount of tocopherols in cotton seed oil

(From the work of the Proctor and Gamble laboratories, W. Lange; Jaocs, 1950, 419).

| Operation | Amounts of tocopherols in mg/100 g |
|---|---|
| Raw oil ..... | 102.8 |
| Refining: | |
| Removal of mucilage in water ..... | 102 |
| Neutralization with soda ..... | 101 |
| Decolorization and filtration ..... | 100.7 |
| Deodorizing ..... | 95.9 |
| Hydrogenation: | |
| Refined, but not deodorized ..... | 100.7 |
| Hydrogenation ..... | 90.3 |
| Deodorizing ..... | 97.6 |

Table IX

Evolution of minor constituents during the course of the refining of soybean oil

[Experiments done in a factory by Iterg Service of Technical Assistance (7)].

| Stage of Refining | Constituents | | | | | |
|---|---|---|---|---|---|---|
| | Water p.100 | Soap ppm of NaOH | Insaponifiables p.100 | Sterols ppm | Tocopherols ppm | Oxidized acids p.100 |
| Raw oil from the extractor | 0.11 | — | 0.86 | 430 | 925 | -- |
| "Pre-degummed" and dried oil ..... | 0.12 | -- | 0.83 | 300 | 890 | -- |
| Neutralized oil ..... | 0.15 | — | 0.90 | 370 | --- | -- |
| Oil washed with 1% NaOH at 20°B ..... | 0.60 | 894 | 0.81 | 340 | --- | -- |
| Oil washed with water .... | 0.57 | 13 | -- | --- | --- | -- |
| Oil after drying ..... | 0.07 | 23 | 0.82 | 410 | --- | -- |
| Oil after decolorization . | 0.02 | 0 | 0.84 | 320 | 765 | -- |
| Oil after deodorizing .... | 0.01 | — | 0.60 | 300 | 715 | 0.12 |

V

Carotene in Palm Oil

The presence of vitamin A in vegetable oils has not been established with certainty in spite of a number of important studies on this subject, especially on olive oil and avocado oil (13).

By contrast, a vitamin A factor exists in palm oil: carotene, whose transformation into vitamin A has been demonstrated in living organisms(20). In fact, the presence of carotene in significant quantities (0.15 to 0.05%) gives palm oil its orangish red appearance and generally exists in the form of three isomers α, β and γ. But it is the β carotene which has the greatest vitamin potential and which predominates (4).

Considerable improvements have been made in the production of palm oils, especially in West Africa (Ivory Coast and Dahomey) following the work of the Research Institute for oils and oil-seeds (6). Oils may be produced having weak acidity, whose refinement causes no particular problems. This generally consists of neutralization eventually followed by a limited treatment with soil, then by deodorizing.

For palm oils which are especially rich in carotene, refinement under managed conditions allows the maximum preservation of carotene pigments, especially when the oils are to be used in margarine. On the other hand, the operation of hydrogenation destroys the colored pigments of the oil but does not seem to affect the anti-oxidant products such as the tocopherols.

Numerous French and foreign patents have been filed for the separation of carotenes from palm oil or the obtaining of enriched fractions. Preferential adsorption with decolorizing soil is used, then molecular distillation after the addition of alcohol and freezing. Concerning this last method, we have shown to the IRHO which operates under strictly determined conditions, that it allows for the separation of the solid, slightly colored glyceride elements and a liquid oil very colored and rich in carotene, these two fractions each having diverse applications. Industry uses them to enrich margarine with vitamin A, and as a food coloring, thus giving these products an agreeable appearance while at the same time increasing the nutritional quality (9) (32).

Industry utilizing fats (except for margarine) (Table X) has been up to now little concerned with what happened to the vitamins during the course of the technical operations, the purpose of which has been to present the consumer with a product of pleasant taste and appearance, able to

be well preserved. But, as we have seen, these improvements did not adversely affect the amount of vitamins, and the introduction of modern techniques, in particular, the development of continuous processing, has had the effect of increasing the vitamin potential of oils.

The supply of provitamin A does not pose any problem in normal diets, but vitamin E coming principally from vegetable oils -- and as much as this vitamin is essential to human nutrition -- it may be wondered whether the amounts ingested are sufficient.

Let us look, for example, at some statistical results. Table XI shows according to a study by the U. S. Department of Agriculture the average levels of ingestion of α tocopherols in human nutrition. It is stated that oils and margarine in the United States contain nearly 60% of the total requirement of Vitamin E. The amount of 31.3 mg seems to be largely sufficient if a daily minimum requirement of 14 mg is accepted.

Table X

Finished industrial products destined for sale in the United States prepared from mixtures of hydrogenated oils

(From information from the Proctor and Gamble Company)

| Type of product | Amounts of tocopherols (in mg/100 g) |
|---|---|
| Vegetable fat (*) tradename "Crisco" ..... | 104.2 |
| -- -- "Primex" ..... | 98.4 |
| -- -- "Swetex" ..... | 101 |
| Primary substances for margarine ..... | 100.5 |

(*) Products designated in the U. S. under the name of "shortening". Jaocs. 1950, p. 419.

Table XI

Average ingestion of α tocopherol in normal diet

(From a study by the U. S. D. A.)

| Product consumed | Average quantity | Average amount of α tocopherols | Ingested tocopherols |
|---|---|---|---|
| | g/day | mg/100 g | mg/day |
| Butter ..... | 50 | 1.5 | 0.7 |
| Oil and margarine ..... | 30 | 30 | 12 |
| Bread and food dough ..... | 200 | 0.5 | 1 |
| Meats and fish ..... | 200 | 0.5 | 1 |
| Potatoes ..... | 150 | 0.5 | 0.7 |
| Eggs ..... | 60 | 0.7 | 0.4 |
| Milk products ..... | 500 | 0.5 | 2.5 |
| Green vegetables ..... | 140 | 0.2 | 0.3 |
| Dried peas and beans, nuts .... | 20 | 1 | 0.2 |
| Tomatoes, lemons ..... | 130 | 0.3 | 0.4 |
| Fruits ..... | 300 | 0.3 | 1 |
| Coffee, tea, cocoa ..... | 25 | 0.5 | 0.1 |
| Sugar and syrups ..... | 130 | 0 | 0 |
| Total ingested tocopherols (average in mg/day) .....21.3 | | | |

Table XII

Visible consumption per inhabitant per year of oils and fatty materials in the countries of the Common Market in 1960 and the daily calculated amount of absorption of tocopherol

(From figures given by the IASC Congress, 1962)

| | Butter | Margarine | Fats | Oil | Tocopherols ingested per day |
|---|---|---|---|---|---|
| | kg | kg | kg | kg | mg |
| Low countries .... | 4.65(0) | 20 (50) | 3.6(0) | 1.5 (50) | 22 |
| Belgium and Luxembourg ..... | 9 (0) | 11.7(50) | 2 (0) | 2.85(50) | 16.5 |
| West Germany ..... | 8.4 (0) | 11 (50) | 6.7(0) | 2.9 (30) | 15 |
| France ..... | 7.3 (0) | 2.3(50) | 3.3(0) | 6.4 (35) | 10 |
| Italy ..... | 1.5 (0) | 1.1(50) | 3.6(0) | 10 (5) | 3 |
| Common Market .. | 5,85(0) | 6.5(50) | 4.5(0) | 5.6 (30) | 13.5 |

(In parentheses, estimated minimum amounts of tocopherols.)

Table XII concerns the countries of the Common Market. We have taken some liberty with the actual amounts of tocopherols for the purpose of arriving at approximate values which could constitute the minima. Disregarding the amount of butter and fat of animal origin, it can be seen that the principal sources of vitamin E are the oils and margarine, the latter being able to furnish in countries of high consumption more than 90% of the vitamin supply. On the other hand, in countries of low consumption, this vitamin supply appears to be clearly below the minimum requirement.

In certain cases, should table oils be enriched with vitamin E? It is a fact that most of them currently being consumed have only average amounts (peanut, colza), and others have relatively small amounts (olive). It will always be possible to blend these with oils having large amounts of tocopherols (soybean for example). But I believe that in this domain the final say is with the nutritionists.

## Discussion

Mr. R. Francois -- Concerning the different forms of tocopherols, there is an interest in preserving this factor in food. We may wonder whether certain products, such as margarine, should not be enriched with tocopherols.

Mr. Loury -- Margarine may actually be an important source of tocopherols for human consumption. We have proposed the addition of tocopherol or enriched extracts of tocopherols such as those obtained by the molecular distillation of soybean oil, to margarines prepared from the oils of fatty materials, poor in tocopherols. This addition would have the advantage of enhancing the liposoluble elements without the disadvantage of the starches which are actually used.

Mr. Dupaigne -- I am not competent in the field of fatty materials. Nevertheless, aside from all judicial or economic considerations, if the diet of the French people is alittle weak in tocopherols, couldn't the nutritional quality of butter be improved by adding tocopherols in order to contribute to the reduction of surpluses? Couldn't the cost of this addition be significantly reduced by the savings that will automatically result from the partial using up of the surpluses?

Mr. Loury -- You will certainly agree that the improvement of the nutritional quality of butter cannot bring a remedy for the using up of surpluses. It is essentially a product poor in tocopherols and interest should be in increasing the amount. In some countries, it is permitted to mix butter and margarine, but this practice is prohibited in France.

Mr. A. Francois -- I simply want to remind us that oils and fats are not the only foods which furnish tocopherols. Evans discovered antisterility properties of vitamin E in the rats, by using diets with and without fresh vegetables such as lettuce. The daily supply is thus probably higher than that which was calculated by Mr. Loury, when one takes into account the consumption of fruits and vegetables.

The National Research Council just fixed 30 mg per day as the recommended supply, because it estimates that this quantity is necessary since the diet of Americans is rich in poly-unsaturated fatty acids (more than 35/g day. Some authors [Bunnell, Keating, Quaresimo, Parman, Amer. J.

Besides, Mr. Loury has emphasized that "the commercial uses of oils demand that they generally present an appearance of a yellow gold liquid, perfectly limpid, as demanded by the consumer". I would like to know whether this question has been the subject of objective studies in France. Furthermore, is it established that the French consumer wants to be able to be confident about the appearance of the oil at the time when he buys it, and that this requires the use of transparent bottles? Since amber-colored glass assures better preservation of the product than clear glass, has the acceptability of this really been tested and under what conditions?

Mr. Loury -- The deodorizing process theoretically follows the laws for water vaporization: temperature plays an essential role and the concern is to operate at the highest possible temperature during the shortest time.

In any case, the losses of tocopherols in present industrial operations do not surpass 20 p. 100.

As for the very important question in the commercial field, that of presentation, I confess to you my incompetence, and I do not know if systematic studies have been done on the subject. I have the feeling that a yellow gold oil would sell better.

However, in some countries, bottles of brown glass are used, for example in the United States.

In France, we seem to be oriented towards bottles of uncolored and transparent plastic materials.

Perhaps, it is a question of fighting against habit by the education of the consumer.

Clin. Nutr., 1965: 17 (1-10)], contend that this supply is not generally satisfactory.

Mr. Loury -- I have the impression that nutritionists are for from being in agreement on the quantity of tocopherols which should be present in normal diets, but the figures which I have indicated show well that the by far the most important sources remain vegetable oils and their manufactured derivatives such as margarine, while the other sources, in my opinion, remain negligible.

Mr. Terroine -- I must point out that the role of tocopherols was discovered by the comparison of diets poor in this vitamin with diets exactly the same except for the addition of lettuce or lucern-grass.

Mr. Loury -- Actually lettuce, like several other green vegetables contains tocopherol but in general, in small quantities. Lucern-grass and certain varieties of cabbages have relatively greater quantities of tocopherols, but I remain convinced that the principal supply is furnished by vegetable oils and their derivatives.

Mr. Causeret -- Mr. Loury has indicated the average losses of tocopherols due to the deodorizing of soybean oil. Can we say what the influence of temperature is on these losses? For example, how much do they rise when the oil is kept at between 180 and 200°C for several hours, or at about 230°C during a shorter time?

Mr. Raoul -- Are we concerned about the undesirable presence of unsaturated acids of the linoleic series in certain oils such as colza?

Mr. Loury -- It is indisputable that the presence of linoleic acid is detrimental to the good preservation of oil because of its great oxidizability, but fortunately, vegetable oils contain anti-oxidant elements -- such as tocopherols -- which for a time prevent changes by oxidizing, and generally, this time greatly exceeds that of current consumption.

If there are certain precautions for the use of colza oil in nutrition, there are several reasons for them, and I don't think linoleic acid is alone responsible.

Although I lack competence in the subject, I have the feeling that it cannot be demonstrated that the absorption of this acid is harmful to the organism.

On this subject, we must look at the works of Professor Jacquot and his colleagues.

ANN. NUT. ALIM.; 1970, 24, B 227 - B 246

# L'ÉVOLUTION DES VITAMINES DANS LA TECHNOLOGIE DES HUILES ET CORPS GRAS ALIMENTAIRES

par

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## INTRODUCTION

Les matières grasses naturelles contiennent ce qu'il est convenu d'appeler les vitamines liposolubles parmi lesquelles il faut mentionner les plus importantes : vitamines A, D et E (tableau I). On trouve les deux premières dans les huiles de foie de poisson et également dans le beurre, mais l'étude de ces produits n'entre pas dans le cadre de cet exposé.

TABLEAU I

*Vitamines liposolubles*

| Noms | Sources |
|---|---|
| Vitamine A | Huile de foie de poisson. Beurre. Graisses animales (?). Huile d'olive (?). |
| Vitamine B | Huile de son de riz (?). |
| Vitamine D | Huile de foie de poisson. Huile de germe de blé. |
| *Vitamine E* | Huile de germe (maïs, blé). *Huile de soja.* |
| Vitamine K | Huile de soja (?). |
| *Provitamine A* | *Huile de palme.* |
| ***Acides gras essentiels :*** | |
| Acide linoléique | Huile de soja et de tournesol. |
| Acide arachidonique (Vitamine F) | Graisses animales. |

D'autre part les corps gras d'origine animale (suif, saindoux) sont pratiquement dépourvus de facteur vitaminique mais il faut rappeler qu'ils contiennent des acides gras essentiels d'un grand intérêt sur le plan nutritionnel (acide arachidonique) que l'on ne trouve pas dans les huiles végétales (2). Ces limites étant fixées, nous envisagerons l'étude de ces dernières qui peuvent contenir la vitamine E dans les huiles de graines et la provitamine A dans l'huile de la pulpe des fruits du palmier à huile.



## I

## LES HUILES VÉGÉTALES COMME SOURCE DE TOCOPHÉROL

La vitamine E ou tocophérol est largement répandue dans les huiles végétales. (Nous n'envisageons pas le cas des huiles de germe de blé ou de maïs qui doivent être considérées plutôt comme des produits diététiques. Tableau II.)

Les huiles végétales fluides apparaissent donc comme étant la source principale de notre alimentation en vitamine E (1). Il est par conséquent important de connaître leur devenir au cours des différentes opérations technologiques qu'elles subissent. Les huiles végétales sont extraites de graines, de la pulpe ou des noyaux de fruits oléagineux par broyage, pression, centrifugation ou dissolution dans un solvant. Cette dernière méthode désignée plus particulièrement par « extraction » s'est maintenant généralisée à toutes les huileries de graines. Elle offre l'avantage d'un meilleur rendement ce qui évidemment intéresse l'industriel mais elle s'est trouvée avantageuse également pour l'enrichissement des huiles en vitamines liposolubles (cas des huiles de soja par exemple).

De nombreuses études ont paru sur les teneurs en tocophérol contenu dans divers produits alimentaires, ainsi que sur les techniques de dosage (8). L'ancienne méthode d'Emmerie-Engel a subi de nombreuses modifications de manière à en augmenter la précision. D'autres méthodes ont été mises au point qui permettent (12) (23) (28) de faire une discrimination entre les divers tocophérols α, β, γ et δ dont l'action vitaminique E décroîtrait dans l'ordre indiqué alors que l'action protectrice antioxygène varierait dans l'ordre inverse (11) (14) (33).

Parmi ces études il faut signaler l'important travail de W. LANGE (19) dont les chiffres ont été souvent repris dans beaucoup de publications (3) (9) (29). Les huiles de soja sont, en dehors des huiles de germe de blé ou de maïs considérées comme produits diététiques, les plus riches en tocophérol (16) (29) (33) : leur teneur approche couramment la valeur de 200 mg pour 100 g (tableaux III et IV). Viennent ensuite les huiles de coton et de tournesol dont les teneurs moyennes s'établissent entre 110 et 140 mg (11) (16) (18). Les méthodes de dosage des divers tocophérols indiquent une richesse particulière en α tocophérol pour les huiles de coton (11). Assez loin de ces chiffres on trouve ensuite les huiles d'arachide, de colza et de palme qui peuvent atteindre jusqu'à 50 mg pour 100 g (11) (16) (18).

TABLEAU II

*Teneur des huiles végétales brutes en tocophérols*

*(en mg/100 g)*

(D'après [19]).

| Nature | Total | α | β | γ | δ |
|---|---|---|---|---|---|
| Huile de soja | 152<br>212<br>168 | 20 | | 98 | 50 |
| Huile de coton | 110<br>120 | 76 | | 34 | |
| Huile de tournesol | 140 | 10 | | 130 | |
| Huile de colza | 55 | | | | |
| Huile de palme | 56<br>44 | 30 | | 26 | |
| Huile d'arachide | 52 | 30 | | 22 | |
| Huile d'olive | 3<br>8<br>20 | | | | |
| Huile de coco | 3 | | | | |
| Beurre d'été<br>Beurre d'hiver<br>Jaune d'œuf | 4<br>1,7 à 3<br>2 | | | | |
| Huile de germe de blé<br>Huile de germe de maïs | 550<br>250 | | | | |

TABLEAU III

*Teneur en tocophérol (1) des huiles de soja extraites (2) des différentes variétés de graines*

| Variété | p. 100 huile extraite | Teneur en mg/100 g |
|---|---|---|
| Lincoln | 15,3 | 236 |
| Richland | 15,9 | 203 |
| Illiné | 15,3 | 225 |
| Dunfield | 14,6 | 203 |
| Ogden | 13,4 | 169 |
| Amiyedo | 17,7 | 230 |
| Delsta | 16,7 | 217 |
| *Moyenne* | *15,9* | *215* |

(1) Évalué par la méthode d'EMMERIE-ENGEL modifiée par RAWLINGS et coll. (*Jaocs*, 1948, 25, 25).

(2) L'extraction est faite à l'éther de pétrole après séchage des graines 4 h à 130 °C.

TABLEAU IV

*Comparaison des teneurs en tocophérol des huiles de soja brutes suivant le mode d'extraction*

[D'après RAWLINGS et coll., (*Jaocs*, 1948, 25, 25)].

| Procédé | Teneur en mg/100 g |
|---|---|
| Extraction (Hexane) | 172 |
| Pression (Expeller) | 180 |
| Extraction et démucilagination | 176 |

Très loin derrière viennent les huiles d'olive (8) (26) qui présentent des teneurs moyennes de 10 mg et les huiles de coco (25) qui ne contiennent que quelques milligrammes pour 100 g.

Il faut d'autre part considérer que le beurre, de même que les œufs, la [illegible] et les céréales qui forment la base de notre alimentation sont extrêmement pauvres en tocophérol (16) (17) (27). Par contre les huiles de germe de blé ou de maïs sont très riches en tocophérol (20), les premières atteignent 500 mg pour 100 g et les secondes dépassent souvent la teneur de 200 mg.

## II

## LE RAFFINAGE

### A. Nécessité du raffinage

La plupart des huiles végétales extraites sont inconsommables à l'état brut. L'huile d'arachide a un goût de cacahuète fraîche, l'huile de soja présente une saveur de haricot et l'huile de colza sont le cheux. Même les huiles d'olive vierges ne sont pas appréciées de tous les consommateurs.

D'autre part, la commercialisation des huiles exige qu'elles présentent généralement l'aspect d'un liquide jaune d'or parfaitement limpide, ce que demande la clientèle. La nécessité apparaît donc d'une amélioration profonde de leur saveur et de leur aspect. Il faut en outre, et ceci est primordial pour la marque de fabrique, qu'elles aient une durée suffisante de conservation c'est-à-dire qu'elles ne rancissent pas au stockage ou en magasin. Or on a montré que si les huiles végétales possèdent des antioxygènes naturels dont précisément les tocophérols qui les protègent de l'oxydation, elles contiennent des prooxygènes qui accélèrent l'attaque par l'oxygène de l'air. Parmi ces substances, il faut citer les traces métalliques et surtout la présence d'acides gras libres. Ceux-ci sont formés par l'action d'enzymes lipolytiques qui hydrolysent les triglycérides au moment de l'extraction et qui peuvent être particulièrement actifs dans certaines conditions favorables. La mise en route industrielle de la stérilisation des fruits du palmier à huile dès la récolte a eu pour conséquence, en détruisant ces enzymes, de pouvoir produire des huiles de palme à faible acidité alors que sans cette précaution l'acidité libre peut dépasser en quelques jours 50 p. 100.

Or on a montré que les acides gras libres sont beaucoup plus oxydables que les triglycérides et qu'ils peuvent de ce fait jouer le rôle d'initiateur d'autoxydation et provoquer assez rapidement le rancissement de l'huile. Il est donc nécessaire de les éliminer. Toutes ces considérations aboutissent à la nécessité du raffinage.

### B. Le raffinage (tableau V)

Le raffinage des huiles a fait l'objet d'un trop grand nombre de publications pour que nous puissions les citer ici (10) (29) (31) et nous n'entrerons pas dans le détail des procédés. Rappelons qu'il comporte les opérations classiques suivantes :



| Opérations | Produits utilisés | Conditions opératoires | But |
|---|---|---|---|
| Démucilagination | Eau.<br>Acide phosphorique.<br>Anhydride acétique. | 70-80° pression ordinaire. | Élimination des phosphatides. |
| Neutralisation | Lessive alcaline de 10° à 20° B. | 70-80° pression ordinaire. | Élimination des acides gras libres sous forme de savon. |
| Désacidification (*) | Vapeur d'eau. | 220-240° sous vide. | Par distillation. |
| Décoloration | Terre activée.<br>Charbon actif. | 100-120°. | Élimination des pigments colorés et des traces de savon. |
| Désolorisation | Vapeur d'eau surchauffée. | 180-250° sous vide. | Élimination de produits odorants (naturels et résultant d'une altération). |
| | | *Opérations technologiques :* | |
| Hydrogénation | Hydrogène et catalyseur à base de nickel. | 175°-195° pression 1 à 40 kg. | Saturation des doubles liaisons. |
| Trigellisation | Néant. | 10°. | Élimination des produits concrets (triglycérides et cires). |

(*) Cette opération n'intervient bien après la décoloration.

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## LE RAFFINAGE

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| Opérations | Produits utilisés | Conditions opératoires | But |
|---|---|---|---|
| Démucilagination | Eau.<br>Acide phosphorique.<br>Anhydride acétique. | 70-80° pression ordinaire. | Élimination des phosphatides. |
| Neutralisation | Lessive alcaline de 16° à 20° B. | 70-80° pression ordinaire. | Élimination des acides gras libres sous forme de savon. |
| Désacidification (*) | Vapeur d'eau. | 220-240° sous vide. | Par distillation. |
| Décoloration | Terre activée.<br>Charbon actif. | 80-120°. | Élimination des pigments colorés et des traces de savon. |
| Désodorisation | Vapeur d'eau surchauffée. | 180-210° sous vide. | Élimination de produits odorants (naturels et résultant d'une altération). |
| | | *Opérations technologiques :* | |
| Hydrogénation | Hydrogène et catalyseur à base de nickel. | 175°-195° pression 1 à 40 kg. | Saturation des doubles liaisons. |
| Frigélisation | Néant. | 10°. | Élimination des produits cuits (triglycérides et cires). |

(*) Cette opération a généralement lieu après la décoloration.

1° La démucilagination

La démucilagination est encore appelée « dégommage » et a pour but d'éliminer les phosphatides hydratables et d'empêcher que ceux-ci précipitent ensuite dans l'huile en leur donnant un aspect trouble. On utilise à cet effet le plus souvent l'eau à 70-80° et l'opération dure 15 minutes en marche discontinue. Les mucilages qui se sont formés sont éliminés par centrifugation. Dans certains cas et dans le but de réaliser une démucilagination plus complète, on opère en présence d'acide phosphorique. On a même préconisé les acides forts (nitrique par exemple) et l'anhydride acétique pour les huiles de soja (15) (21).

Il faut ajouter que l'opération de démucilagination a également pour but de faciliter les opérations ultérieures, c'est-à-dire la neutralisation alcaline où il est essentiel de réduire au maximum les pertes en huile neutre entraînée, et surtout pour l'opération de désacidification en continu où il est essentiel d'éliminer complètement les mucilages pour éviter l'encrassement des appareils.

2° La neutralisation.

Cette opération a pour but d'éliminer les acides gras libres sous forme d'un précipité de savon (soap-stock ou pâtes de neutralisation). A cet effet on traite l'huile démucilaginée par une lessive de soude caustique dont la concentration peut varier de 14 à 32° Bé (en moyenne 20° Bé) et éventuellement avec addition de carbonate de sodium. On opère entre 70 et 90 °C et la durée de l'opération peut varier de 15 minutes à 2 heures. Le savon peut être séparé par centrifugation ce qui a toujours lieu en procédé continu.

La neutralisation est suivie de lavages de l'huile par des solutions salines, puis par de l'eau pure dans le but d'éliminer les traces de savon. Ensuite a lieu le séchage de l'huile, opération essentielle avant la décoloration ou la désacidification. A cet effet on chauffe l'huile sous un vide de 100 mm/g à 90-100°. On utilise actuellement des appareils à ruissellement. L'opération dure de 30 à 40 minutes. On ajoute souvent un peu d'acide citrique au cours de cette opération, lequel joue le rôle de synergique des antioxygènes. Il est à noter que la précipitation du savon entraîne également une certaine proportion de pigments colorés et qu'on aboutit de ce fait à un notable « éclaircissement » de l'huile.

3° La désacidification.

Il s'agit d'une neutralisation physique. Cette méthode s'est développée parallèlement avec les procédés continus. L'élimination des acides gras a lieu ici par évaporation ou distillation en présence de vapeur d'eau surchauffée. On opère sur film mince et sous un vide de quelques millimètres



de Hg et à température élevée : 220° à 240 °C. L'opération a l'avantage de ne nécessiter aucun réactif chimique. Elle se confond en outre avec la désodorisation. Elle a l'inconvénient de nécessiter des huiles parfaitement démucilaginées pour éviter l'encrassement des surfaces de contact.

4° La décoloration ou blanchiment.

Cette opération a pour but d'éliminer les pigments colorés des huiles en particulier les chlorophylles et les caroténoïdes qui leur donnent un aspect sombre. On traite l'huile sous agitation par une terre activée acide plus rarement par du charbon actif et quelquefois par un mélange de ces deux adsorbants. L'opération en marche discontinue dure de 20 à 40 minutes. On opère sous un vide de 100 mm/Hg environ et à des températures de 80 à 120°. L'huile est ensuite filtrée sur une couche de terre de diatomées au filtre-presse ou au filtre sous pression (à 70-80° sous 3 atmosphères par exemple).

Il faut ajouter que cette opération a également pour but d'éliminer les dernières traces de savon et de fixer certaines impuretés, acides oxydés et traces métalliques, nuisibles à la bonne conservation de l'huile.

Dans le cas où l'élimination des acides gras libres est réalisée par voie physique en utilisant leur plus grande volatilité en présence de vapeur d'eau, l'ordre des opérations décoloration-neutralisation physique est inversé toujours dans le but d'obtenir une huile aussi épurée que possible au moment du passage dans les appareils de désacidification.

5° La désodorisation.

Elle a pour but d'éliminer les produits sapides qui donnent du « goût » à l'huile. Ces produits forment la saveur *sui generis* ou proviennent d'un début d'oxydation de l'huile. On insuffle dans l'huile de la vapeur d'eau surchauffée et désaérée et on ajoute souvent une petite quantité d'acide citrique. On opère à 180-220° sous un vide de 2 à 18 mm/Hg. L'opération dure de 4 à 8 heures comme pour la désacidification avec laquelle elle se confond. On peut opérer en marche continue avec un appareil à ruissellement en couche mince sur colonne. Ce procédé a l'avantage de diminuer le temps de l'opération qui peut être réduit à 40 minutes.

Cette opération a souvent été critiquée car elle fait perdre à l'huile toute saveur pour lui donner un goût neutre ou « plat ». Cependant elle offre au consommateur la garantie d'un produit standard de bonne qualité car il n'est pas possible dans ce cas de dissimuler un début d'altération par rancissement, lequel serait immédiatement décelable. Enfin comme nous l'avons dit dans la plupart des cas, c'est une opération essentielle et dont le but est de rendre l'huile consommable. Il ne faut pas perdre de vue que le but de l'industriel est de satisfaire le plus grand nombre de consommateurs.

Ajoutons que pour terminer, l'huile est soumise à un séchage sous vide et à une clarification sur filtre à poche de cellulose pour lui donner sa limpidité et son scintillant.

Il apparaît que c'est surtout à la décolorisation que les pertes en tocophérols sont les plus importantes étant donné sa volatilité relativement beaucoup plus faible que celle des triglycérides et acides gras. On en retrouve effectivement en quantité importante dans les distillats ou « échappés de désodorisation » (24). Cette perte peut s'évaluer environ à 5 p. 100 (5).

## III

## AUTRES OPÉRATIONS TECHNOLOGIQUES

A. *La frigellisation* (démargarination ou winterisation).

Le but de cette opération est de séparer les éléments cristallisables à basse température (glycérides, stérols, cires, etc.) dont la précipitation au stockage nuirait à la limpidité de l'huile.

Cette opération a lieu à basse température (< 10°) et sous agitation lente de manière à favoriser la formation et la nourriture des cristaux. Elle est suivie d'une filtration au filtre-presse ou sous pression.

B. *L'hydrogénation* (ou durcissement).

Elle peut être considérée comme l'opération inverse de la précédente et a pour but d'élever le point de fusion d'une huile ou d'une graisse. Elle peut être sélective, c'est-à-dire qu'on s'efforce tout d'abord de saturer les chaînes les plus insaturées qui donnent à l'huile une certaine instabilité et peuvent être la cause d'une altération du goût (réversion). On opère sous pression de 1 à 40 atm. en présence de nickel catalytique et l'hydrogène est admis sous forte turbulence à des températures de 175 à 195°. L'opération est suivie d'un raffinage destiné à éliminer les traces de nickel et l'acidité ayant pu se former. Il est à noter que cette opération peut faire disparaître les acides gras essentiels (1).



## IV

## INFLUENCE DES OPÉRATIONS TECHNOLOGIQUES SUR LA TENEUR DES HUILES VÉGÉTALES EN TOCOPHÉROL (tableaux VI, VII, VIII).

Disons tout de suite que les spécialistes qui ont examiné le devenir du tocophérol au cours des opérations de raffinage sont particulièrement affirmatifs. M. R. Fenton (9) s'exprime ainsi : « L'accord sur ce point est pratiquement unanime : les tocophérols subissent à peu près sans dommage les opérations de raffinage et d'hydrogénation. »

Cette conclusion ressort de l'examen des chiffres que nous indiquons dans le tableau. Une étude systématique a été faite dans le laboratoire d'une grande société américaine sur le raffinage d'une huile de coton d'où il ressort que ni les opérations successives de raffinage, ni l'hydrogénation n'apportent de perte en tocophérol supérieure à 10 p. 100. L'Institut des corps gras s'est intéressé à ce problème. Au cours d'une étude sur le raffinage de l'huile

TABLEAU VI

*Teneur des huiles végétales raffinées en tocophérols*

*(en mg/100 g)*

[D'après (19)].

| Nature | Total | α | β | γ | δ |
|---|---|---|---|---|---|
| Huile de soja | 125 à 175 | | | | |
| | 190 | | | | 57 |
| | 168 | 20 | | 98 | 50 |
| | 140 | 10 | | 130 | |
| Huile de coton | 110 | 76 | | 34 | |
| | 90 | 56 | | 34 | |
| | 87 | 49 | | 37,7 | |
| Huile d'arachide | 51 | | | | |
| | 43 | | | | |

de soja nous avons suivi en particulier l'évolution du tocophérol au cours des différentes étapes du raffinage (voir tableau VI). On constate que la perte en tocophérol est ici de l'ordre de 20 p. 100 (7) [tableau IX].

La technologie des huiles a évolué ces vingt dernières années vers l'adoption de procédés continus. Ces procédés présentent l'avantage d'un temps réactionnel plus limité et sont par conséquent bénéfiques pour les teneurs en vitamines. C'est ce qui ressort de la comparaison entre les procédés continus par emploi de centrifugeuses et discontinus par batteuse ou même par une méthode de laboratoire. Le tocophérol étant particulièrement sensible à l'oxydation en milieu alcalin on voit l'avantage de l'emploi de procédés continus qui limite le temps réactionnel (tableau VII).

TABLEAU VII

*Comparaison des pertes en tocophérol d'après la méthode de raffinage alcalin*

(D'après RAWLINGS et coll., (*Jaocs*, 1948, 25, 25).

| Méthode | Teneur en mg/100 g | | Perte en % |
|---|---|---|---|
| | Brute | Raffinée | |
| Par centrifugation (en usine).... | 187 | 185 | 1,1 |
| | 198 | 190 | 4 |
| | 193 | 189 | 2,1 |
| | 193 | 192 | 0,5 |
| | 211 | 199 | 5,7 |
| *Moyenne*..... | | | *2,7* |
| Par batteuse (en usine)..... | 191 | 167 | 12,6 |
| | 188 | 168 | 10,6 |
| | 186 | 161 | 13,4 |
| *Moyenne*..... | | | *12,6* |
| Par agitation (en laboratoire).... | 189 | 148 | 21,7 |
| | 178 | 156 | 12,4 |
| | 195 | 161 | 17,4 |
| | 191 | 162 | 15,1 |
| *Moyenne*..... | | | *16,7* |



TABLEAU VIII

*Influence du raffinage et de l'hydrogénation sur la teneur en tocophérol d'une huile de coton*

(D'après un travail des laboratoires de la Société PROCTER et GAMBLE, W. LANGE; *Jaocs*, 1950, 419).

| Opération | Teneur en tocophérol en mg/100 g |
|---|---|
| Huile brute..... | 102,8 |
| *Raffinage :* | |
| Démucilaginée à l'eau..... | 102 |
| Neutralisée à la soude..... | 101 |
| Décolorée et filtrée..... | 100,7 |
| Désodorisée..... | 95,9 |
| *Hydrogénation :* | |
| Raffinée mais non désodorisée..... | 100,7 |
| Hydrogénée..... | 90,3 |
| Désodorisée..... | 97,6 |

TABLEAU IX

*Évolution des constituants mineurs au cours du raffinage de l'huile de soja*

(Essais effectués en usine par le Service d'assistance technique de l'ITERG [7]).

| Stade du raffinage | Constituants | | | | | |
|---|---|---|---|---|---|---|
| | Eau p. 100 | Savon ppm de NaOH | Insaponifiables p. 100 | Stérols ppm | Tocophérols ppm | Acides oxydés p. 100 |
| Huile brute sortie extracteur..... | 0,11 | – | 0,86 | 430 | 925 | – |
| Huile pré-dégommée et séchée..... | 0,12 | – | 0,83 | 300 | 890 | – |
| Huile neutralisée..... | 0,15 | – | 0,90 | 370 | – | – |
| Huile lavée avec 1 %, NaOH 20 °B... | 0,60 | 894 | 0,81 | 340 | – | – |
| Huile lavée à l'eau..... | 0,57 | 13 | – | – | – | – |
| Huile après séchage..... | 0,07 | 23 | 0,82 | 410 | – | – |
| Huile après décoloration..... | 0,02 | 0 | 0,84 | 320 | 765 | – |
| Huile après désodorisation..... | 0,01 | – | 0,60 | 300 | 715 | 0,12 |

## V

## LE CAROTÈNE DE L'HUILE DE PALME

La présence de vitamine A dans les huiles végétales n'a pu être démontrée avec certitude malgré de nombreux et importants travaux sur ce sujet, en particulier sur les huiles d'olive et d'avocat (13).

Par contre un facteur vitaminique A existe dans les huiles de palme : le carotène dont la transformation en vitamine A a été démontrée dans les organismes vivants (20). En fait, le carotène dont la présence en quantité notable (0,15 à 0,05 p. cent) donne à l'huile de palme sa couleur rouge orangé y existe généralement sous forme des trois isomères α, β et γ. Mais c'est le β carotène qui présente le potentiel vitaminique le plus élevé qui prédomine (1).

Des améliorations considérables ont été apportées dans la production des huiles de palme, plus spécialement en Afrique Occidentale (Côte-d'Ivoire et Dahomey) à la suite des travaux de l'Institut de Recherche pour les huiles et oléagineux (6). On peut produire actuellement des huiles à faible acidité dont le raffinage ne pose pas de problèmes particuliers. Celui-ci consiste généralement en une neutralisation éventuellement suivie d'un traitement limité à la terre puis d'une désodorisation.

Pour les huiles de palme particulièrement riches en carotène un raffinage dans des conditions ménagées permet de préserver au maximum la teneur en pigments caroténoïdes surtout lorsque les huiles sont destinées à la margarinerie. Par contre l'opération d'hydrogénation détruit les pigments colorés de l'huile mais n'apparaît pas toucher les produits antioxygènes tels que les tocophérols.

De nombreux brevets français et étrangers ont été déposés pour la séparation des carotènes de l'huile de palme ou l'obtention de fractions enrichies. On a utilisé l'adsorption préférentielle sur terre décolorante, la distillation moléculaire après alcoolyse et la frigellisation. En ce qui concerne cette dernière méthode, nous avons montré à l'IRHO que pratiquée dans des conditions bien déterminées, elle permet de séparer une fraction glycéridique concrète peu colorée et une huile fluide très colorée et enrichie en carotène, ces deux fractions pouvant avoir chacune des applications diverses (22). L'huile de palme constitue une source importante et économique en carotène. L'industrie les utilise dans ce but pour enrichir les margarines en facteur vitaminique A et comme colorant alimentaire donnant ainsi à nos produits



un aspect agréable en même temps que des qualités nutritionnelles appréciées (9) (32).

L'industrie des corps gras (sauf la margarinerie) [tableau X] s'est jusqu'à présent peu préoccupée du devenir des vitamines au cours des opérations technologiques, son principal souci étant l'amélioration du rendement et aussi du résultat de ces opérations, dans le but de présenter au consommateur un produit de goût et d'aspect agréables et de bonne conservation. Mais, nous l'avons vu, ces améliorations ne portent aucunement préjudice à la teneur en vitamines et l'introduction des techniques modernes, en particulier le développement des procédés continus, a eu pour conséquence une augmentation du potentiel vitaminique des huiles.

L'apport en provitamine A ne pose pas de problème dans les conditions d'une alimentation normale, mais par contre la vitamine E provenant principalement des huiles végétales — et si tant est que ce facteur vitaminique soit essentiel en nutrition humaine — on peut se demander si la teneur ingérée est suffisante.

Voyons par exemple quelques résultats statistiques. Le tableau XI nous montre d'après une enquête de l'U.S. Department of Agriculture, l'ingestion moyenne d'α tocophérol en alimentation humaine. On constate que les huiles et la margarine aux États-Unis apportent près de 60 p. 100 de la ration vitaminique E totale. Celle-ci de 31,3 mg apparait comme largement suffisante si l'on admet un minimum de 14 mg nécessaire quotidiennement.

TABLEAU X

*Produits finis industriels destinés à la vente au détail aux États-Unis, préparés à partir des mélanges d'huiles hydrogénées*

(D'après les données de la Société PROCTER et GAMBLE)

| Nature du produit | Teneur en tocophérol (en mg pour 100 g) |
|---|---|
| Graisse végétale (*) de marque « Crisco » | 104,2 |
| — — « Primex » | 98,4 |
| — — « Swetex » | 101 |
| Matières premières pour margarine | 100,5 |

(*) Ces produits sont désignés aux États-Unis sous le nom de « shortening ». *Jacs*, 1950, p. 419.

TABLEAU XI

*Ingestion moyenne d'α tocophérol en alimentation normale*

(D'après une enquête de l'U. S. D. A. aux États-Unis).

| Produit consommé | Quantité journalière | Teneur moyenne en α tocophérol | Tocophérol ingéré |
|---|---|---|---|
| | g/jour | mg/100 g | mg/jour |
| Beurre | 50 | 1,5 | 0,7 |
| Huile et margarine | 39 | 30 | 12 |
| Pain et pâtes alimentaires | 200 | 0,5 | 1 |
| Viandes et poissons | 200 | 0,5 | 1 |
| Pommes de terre | 150 | 0,5 | 0,7 |
| Œufs | 60 | 0,7 | 0,4 |
| Produits laitiers | 500 | 0,5 | 2,5 |
| Légumes verts | 110 | 0,2 | 0,3 |
| Pois et haricots secs, noix | 20 | 1 | 0,2 |
| Tomates, citrons | 130 | 0,3 | 0,4 |
| Fruits | 300 | 0,3 | 1 |
| Café, thé, cacao | 25 | 0,5 | 0,1 |
| Sucre et sirops | 130 | 0 | 0 |
| Tocophérol total ingéré (moyenne en mg/jour) | | | 21,5 |

TABLEAU XII

*Consommation visible par habitant et par an des huiles et matières grasses des pays du marché commun en 1960 et teneur journalière calculée d'absorption en tocophérol*

(D'après des chiffres donnés au Congrès IASC, 1962).

| | Beurre | Margarine | Graisses | Huile | Tocophérol ingéré par jour |
|---|---|---|---|---|---|
| | kg | kg | kg | kg | mg |
| Pays-Bas | 4,65 (0) | 20 (50) | 3,6 (0) | 1,5 (50) | 22 |
| Belgique et Luxembourg | 9 (0) | 11,7 (50) | 2 (0) | 2,85 (50) | 16,5 |
| Allemagne Ouest | 8,4 (0) | 11 (50) | 6,7 (0) | 2,9 (30) | 15 |
| France | 7,3 (0) | 2,3 (50) | 3,3 (0) | 6,4 (35) | 10 |
| Italie | 1,5 (0) | 1,1 (50) | 3,6 (0) | 10 (5) | 3 |
| MARCHÉ COMMUN | 5,85 (0) | 6,5 (50) | 4,5 (0) | 5,6 (30) | 13,5 |

(Entre parenthèses, teneur moyenne en tocophérol).



Le tableau XII intéresse les pays du Marché Commun. Nous avons pris quelque liberté avec les teneurs réelles en tocophérol dans le but d'arriver à des valeurs approximatives qui pourraient constituer des minima. En négligeant la teneur du beurre et des graisses d'origine animale on voit que l'apport principal en vitamine E est constitué par les huiles et la margarine, cette dernière pouvant fournir dans les pays de forte consommation plus de 90 p. 100 de l'apport vitaminique. Par contre dans les pays de faible consommation cet apport vitaminique apparaît comme nettement au-dessous du minimum.

Y a-t-il lieu dans certains cas d'enrichir les huiles de table en vitamine E? Il est de fait que la plupart d'entre elles, de consommation courante, n'ont que des teneurs moyennes (arachide, colza), d'autres des teneurs assez faibles (olive). Il sera toujours possible de faire des coupages avec des huiles à forte teneur en tocophérol (soja par exemple). Mais je crois que dans ce domaine la parole est aux nutritionnistes.

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## DISCUSSION

M. R. François. — En ce qui concerne les différentes formes de tocophérol, il y a intérêt à conserver ce corps dans les aliments. On peut se demander si on ne pourrait pas enrichir certains produits en tocophérol, par exemple la margarine.

M. Loury. — La margarine peut être en effet une source intéressante de tocophérol pour l'alimentation humaine. Nous avons proposé l'adjonction de tocophérol ou d'extrait enrichi en tocophérol tels qu'on peut les obtenir par distillation moléculaire des huiles de soja, aux margarines préparées à partir des huiles ou de corps gras, pauvres en tocophérol. Cette addition avait également l'avantage de fournir un révélateur liposoluble ne présentant pas l'inconvénient de la féculle actuellement employée.

M. Dufaigne. — Je suis incompétent dans l'ensemble des corps gras. Néanmoins, en dehors de toute considération juridique ou économique, si l'alimentation du Français est un peu faible en tocophérol, ne pourrait-on améliorer la qualité nutritionnelle du beurre en lui ajoutant du tocophérol afin de contribuer à en réduire les excédents? Le coût de cette addition pourrait-il réduire notablement celui qui résultera automatiquement de la résorption partielle de ces excédents?

M. Loury. — Vous comprendrez certainement que l'amélioration de la qualité nutritionnelle du beurre ne puisse apporter un remède à la résorption des excédents. C'est effectivement un produit pauvre en tocophérol et il aurait intérêt à en augmenter sa teneur. Dans certains pays d'ailleurs, on autorise les mélanges beurre et margarine, mais cette pratique est interdite en France.

M. A. François. — Je voudrais simplement rappeler que les huiles et graisses ne sont pas les seuls aliments fournisseurs de tocophérol. Evans a découvert les propriétés antistérilité de la vitamine E chez le Rat, à l'aide de régimes contenant ou ne contenant pas de végétaux frais tels que la laitue. L'apport quotidien est donc probablement plus élevé que celui qui a été calculé par M. Loury compte tenu de la consommation de fruits et légumes.

Le National Research Council vient de fixer à 30 mg par jour l'apport recommandé, car il estime que cette quantité est nécessaire en raison du fait que le régime des Américains est riche en acides gras poly-insaturés (plus de 25 g/jour). Certains auteurs [Bunnell, Keating, Quaresimo, Parran *Amer. J. Clin. Nutr.*, 1965, **17**, (1-30)] estiment que cet apport n'est généralement pas satisfait.



M. Loury. — J'ai l'impression que les nutritionnistes sont loin d'être d'accord sur la quantité de tocophérol qu'il faut introduire dans un régime alimentaire normal, mais les chiffres que j'ai indiqués montrent bien que la source de loin la plus importante reste certaines huiles végétales et leurs dérivés manufacturés tels que la margarine, les autres sources restant, à mon avis, négligeables.

M. Tenhoine. — Je dois rappeler que le rôle du tocophérol a été découvert par comparaison de régimes pauvres en cette vitamine avec les mêmes régimes mais additionnés de laitue ou de luzerne.

M. Loury. — Effectivement la laitue de même que de nombreux légumes verts contiennent du tocophérol mais en général en petites quantités. La luzerne et certaines variétés de choux en sont relativement plus riches, mais je reste persuadé que l'apport principal est fourni par les huiles végétales et leurs dérivés.

M. Causeret. — M. Loury a indiqué les pertes moyennes de tocophérols dues à la désodorisation de l'huile de soja. Peut-il nous dire quelle est l'influence de la température sur ces pertes? Par exemple, à combien s'élèvent-elles, selon que l'huile est portée entre 180 et 200 °C pendant plusieurs heures, ou aux environs de 230 °C pendant un temps plus court?

D'autre part, M. Loury a souligné que « la commercialisation des huiles exige qu'elles présentent généralement l'aspect d'un liquide jaune d'or parfaitement limpide, ce que demande la clientèle ». J'aimerais savoir si, en France, cette question a fait l'objet d'études objectives. En outre, est-il établi que le consommateur français tient à pouvoir s'assurer de l'aspect de l'huile au moment même où il l'achète, ce qui exige l'emploi de bouteilles transparentes? Puisque le verre ambré assure une meilleure conservation du produit que le verre blanc, a-t-on vraiment essayé d'en tester l'acceptabilité, et dans quelles conditions?

M. Loury. — La désodorisation suit théoriquement les lois de l'entraînement à la vapeur d'eau : la température joue donc un rôle essentiel et on a intérêt à opérer le plus haut possible pendant un temps plus court.

De toute façon, dans une opération industrielle courante, la perte en tocophérol ne dépasse pas 20 p. 100.

Quant à la question très importante sur le plan commercial de la présentation, je vous avouerai mon incompétence et j'ignore si elle a fait l'objet d'études systématiques. On a l'impression qu'une huile jaune d'or se vendra mieux.

Cependant, dans certains pays on utilise des bouteilles en verre brun, aux Etats-Unis par exemple.

En France, on semble s'orienter vers la bouteille en matière plastique incolore et transparente.

Il y a peut-être là matière à lutter contre l'habitude par une éducation du consommateur.

M. Racoul. — S'inquiète-t-on de l'apport indésirable des acides insaturés de la série linolénique de certaines huiles comme celle de colza?

M. Loury. — Il est indiscutable que la présence d'acide linolénique est préjudiciable à la bonne conservation de l'huile étant donné sa très grande oxydabilité mais fort heureusement les huiles végétales contiennent des anti-oxygènes — dont le tocophérol — qui les préservent un certain temps de l'altération oxydante et généralement ce temps dépasse largement celui d'une consommation courante.

Si une certaine prévention existe contre l'huile de colza en nutrition, il a des causes multiples et je ne pense pas que l'acide linolénique soit si responsable.

Sans être compétent en la matière, j'ai l'impression qu'on n'a pu démontrer que l'absorption de cet acide soit nuisible à l'organisme.

Il faudrait voir, à ce sujet, les travaux du Pr Jacquot et de ses collaborateurs.

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# Analysis of Arsenic and Bromine in Marine and Terrestrial Oils

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## ABSTRACT

Samples of marine and terrestrial oils of both plant and animal origin have been analyzed for arsenic and bromine content. Two oil samples (cod liver oil and oil extracted from mackerel tallets) were fractionated on silica gel columns and bromine was determined in the different fractions. The results obtained indicate that lipid soluble bromine and arseno organic compounds are characteristic components of marine animal and marine plant oils (seaweed). The results also show that the bromine is not localized in any particular compound or type of compounds. The bromine-containing compounds seem to be relatively stable, but the arseno-containing compounds are not. When oils containing arsenic and bromine were saponified, some of the arsenic and bromine compounds were found in the fatty acid fraction while others appeared in the water soluble fraction.


## INTRODUCTION

It has been shown in previous work (1-3) that lipids extracted from various marine organisms contain arsenic and bromine as organic compounds. In analyses made on lipids from whale and fish, the contents of these elements were found to vary between 3-25 ppm for arsenic and 1-50 ppm for bromine. It has also been shown that arsenic is present as two or more lipid soluble arseno organic compounds in herring (4). Bromine and arsenic are likewise found in the lipid phase of fresh water fish (5,5). Bromine is also present in lipid extracts from fresh and salt water algae, 20-150 ppm and 10-150,000 ppm respectively (3). Arsenic was not determined in these samples.

The results obtained so far indicate that the presence of arseno and bromine organic compounds is characteristic of oils produced from marine and fresh water fish. The purpose of this work was to analyze oils from other species of both terrestrial and marine origin in order to see if this assumption has a more general validity. In addition to analyzing these oils some of which both arsenic and bromine were present, were saponified so that the fatty acids could be isolated and analyzed separately.

It was also of interest to ascertain whether bromine was present as one or more specific bromine organic compound. Such a study was undertaken by fractionating some of the oil samples and studying the different fractions. By calculating the bromine as parts per million (µg Br/g oil), it should be possible to obtain an indication as to whether this element was present as one or a few discrete compounds, i.e., present in one or a few fractions, or whether it is more distributed in several or all of the different compounds in the oil.

## EXPERIMENTAL PROCEDURE

### Methods

The production and saponification of the oils were carried out using conventional methods. In some samples where little material was available and where the yield of oil was low, the saponification was carried out directly on the raw material.

Analyses for bromine and arsenic were made using neutron activation. Such analyses may be made without chemical treatment of the sample (oil and fatty acid) after the activation, providing that after activation they do not contain trace elements that will interfere with the detection of the induced radioactivity of the arsenic and bromine. Among elements which do interfer, sodium should be emphasized. The content of sodium and other inorganic ions may, however, be reduced by washing with distilled water. When little material is available, a solution of the material in a suitable solvent may be washed. Some solvents appear to have an affinity to inorganic ions, and may carry the ions into the oil phase where they are left behind when the solvent is evaporated. Chloroform seems to be such a solvent, and should be avoided (unpublished results obtained in the author's laboratory). Among the solvents which may be used are diethylether and toluene. By this method it is also possible to analyze small amounts of material (milligram amounts) without taking any special precautions. Transfer of the samples before and after the neutron activation should be accomplished with the aid of a suitable solvent. Further details concerning activation analyses of oils are discussed elsewhere (3,4,6).

The fractionation of the two oil samples was made on silica gel columns according to the directions given by Barron and Hanahan (7).

### Materials

The raw materials used for the production of the oil samples were obtained from the local fish and meat markets and also through the kind assistance of other laboratories

TABLE I

Eluting Agent and Characterization of the Main Fractions From Chromatography of Fish Lipids on Silicic Acid

| Fraction | Eluting agent | Amount, ml | Characterization of main components |
|---|---|---|---|
| A | Hexane (H) | 150 | Hydrocarbons |
| B | 15% Benzene in H | 400 | Sterol esters |
| C | 5% Diethylether in H | 1330 | Triglycerides and free fatty acids |
| D | 15% Diethylether in H | 950 | Free sterol |
| E | 30% Diethylether in H | 600 | Diglycerides |
| F | 50% Diethylether in H | 600 | Diglycerides + monoglycerides |
| G | Diethylether (H) | 600 | Monoglycerides |
| H | 3% Methanol in D | 250 | Phospholipids ? |
| I | 5% Methanol in D | 150 | Phospholipids |
| J | 80% Methanol in D | 250 | Phospholipids |
| K | Methanol | 100 | Phospholipids |

TABLE II

Bromine and Arsenic in Oil and Fatty Acids Extracted from Marine Fishes and Invertebrates

| Sample | | | Locality | Br, ppm | | As, ppm | |
|---|---|---|---|---|---|---|---|
| | | | | Oil | Fatty acid | Oil | Fatty acid |
| Capelin | *Mallotus villosus* | whole fish | Northern Norway | 9.2 | 9.1 | 12.1 | 6.3 |
| Herring | *Clupea harengus* | whole fish | Western Norway | 5.8 | 2.2 | 13.8 | 9.2 |
| Herring | *Clupea harengus* | mature whole fish | Skagerak | 2.6 | 2.7 | 19.3 | 12.1 |
| Mackerele | *Scomber scomber* | fillet | Southern Norway | 2.8 | 3.1 | 8.2 | 4.1 |
| Mackerel | *Scomber scomber* | liver | Southern Norway | 16.5 | 8.5 | 13 | 6.2 |
| Cod[a] | *Gadus morrhua* | liver | Western Norway | 34 | 12 | 8.4 | 6.1 |
| Cod[a] | *Gadus morrhua* | liver | Western Norway | 36 | 9.2 | 9.9 | 7.2 |
| Cod | *Gadus morrhua* | liver | Western Norway | 26 | 6.8 | 10 | 6.0 |
| Plaice | *Pleuronectes platessa* | fillet | Southern Norway | 17.3 | 7.6 | 6.1 | 5.2 |
| Scallop | *Pecten maximum* | whole animal | Northern Norway | 15 | 7.8 | 4.8 | 1.9 |
| Squid | *Ommatostrephes sagittatus* | whole animal | Northern Norway | ... | 3.9 | ... | 0.7 |
| Starfish | *Asterias rubens* | whole animal | Oslo fjord Norway | 14.6 | 6.4 | 9.1 | 7.5 |
| Shrimp | *Pandalus borealis* | whole animal | Oslo fjord Norway | 17 | 13.0 | 10.1 | 4.8 |
| Lobster | *Homarus vulgaris* | whole animal | Southern Norway | 50 | ≈3 | 4.7 | ≈3 |
| Mussel | *Mytilus edulis* | whole animal | Western Norway | 137 | 35 | 18 | 22 |
| Snail | *Littorina littorea* | whole animal | Western Norway | 204 | 82 | 84 | 32 |

[a]From the same locality.

Some of the vegetable raw oils were factory produced.

### Treatment of the Samples

All samples were homogenized in a mechanical blender, and extracted with two volumes of chloroform-methanol 2:1. The extractions were carried out for 2-4 hr at 50 C on a waterbath, under reflux. The extract was then filtered, and the extraction was continued with a similar amount of solvent for another 2-4 hr. The two extracts were combined, the chloroform phase was removed in a separatory funnel, and finally removed under reduced pressure at 35-40 C. If a sufficient amount of oil was available, the oil phase was washed twice with distilled water. Usually it was necessary to add magnesium sulphate as a deemulsifying agent. The oil was then centrifuged. Where the oil yield was small, the oil was dissolved in toluene or diethylether and washed with water. After two washing processes the organic phase was separated by centrifuging and the solvent evaporated.

The saponification of the oils was performed in an alcoholic potassium hydroxide solution (2 N KOH in $C_2H_5OH$). The solution was subsequently made acidic with sulphuric acid and extracted with diethylether. The ether solution was then washed with water and the ether evaporated. Some seaweed samples with a low oil content were saponified directly. The fatty acids were separated as described for the other samples.

### Fractionation of Oil

Oil produced from mackerel fillets and from cod liver were used for the fractionation experiments. The silica gel (Merck AG, Darmstadt, 0.2-0.5 mesh) used as solid phase was prepared in the following way. The gel was first dried at 110 C and then treated with the following solvents: diethylether, 15% benzene in *n*-hexane, and *n*-hexane. About 90 g of gel was added to a column with diameter 2.5 cm. Approximately 20 mg oil per gram of gel was used. The different eluting agents used and the amounts of each are given in Table I. The absorption of the eluate at 254 mμ was obtained with an Uvicord-Unit (LKB, Sweden). From the elution curves obtained, and information given by Barron and Hanahan (7), the eluate was devided into different fractions and the solvents were removed by evaporation.

### Neutron Activation

The oil and fatty acid samples were neutron activated in quartz ampoules. The quartz ampoules were first washed with warm nitric acid and rinsed with distilled water. Standards (PA Chemicals; Merck AG, Darmstadt) of ammonium bromide and arsenic dissolved in hydrochloric acid (0.1 N) were neutron activated under the same conditions as the samples. The activation took place in the nuclear reactor JEEP 2, Kjeller, Norway, with a neutron flux of approximately $5 \times 10^{12}$ n/cm$^2$ sec for 2 hr. The quartz ampoules were cooled to -196 C before opening in order to reduce the pressure from volatile compounds formed during the irradiation. The samples were then transferred to inactive glass vials. The standards were treated in the same way.

TABLE III

Bromine and Arsenic in Oil and Fatty Acids Extracted From Seaweed

| Sample[a] | Br, ppm | | As, ppm | |
|---|---|---|---|---|
| | Oil | Fatty acid | Oil | Fatty acid |
| *Laminaria digitata* | 566 | 640 | 221 | 36 |
| *Laminaria saccharina* | 496 | 477 | 185 | 7.5 |
| *Laminaria hyperborea* | 368 | 385 | 197 | 10 |
| *Ascophyllum nodosum* (1968) | 61 | 27 | 7.8 | 5.2 |
| *Ascophyllum nodosum* (1969) | 56 | 25 | 49 | 23 |
| *Fucus vesiculosus* | 43 | 44 | 35 | 5.1 |
| *Fucus Serratus* | 40 | 39 | 27 | 6.1 |
| *Furcus spiralis* | 12 | 19 | 5.7 | 5.0 |
| *Pelvetia canaliculata* | 34 | 41 | 10.8 | 7.3 |

[a]The samples were collected at the west coast of Norway.

TABLE IV

Bromine in Oil Extracted From Birds, Terrestrial Mammals and Vegetables

| Sample | | Locality | Bromine, ppm |
|---|---|---|---|
| Hare | *Lepus timidus* | Inland Norway | <0.1 |
| Reindeer | *Rangifer tarandus* | | 0.12 |
| Elk | *Alces alces* | | <0.01 |
| Cow | *Bos taurus* | | <0.05 |
| Wood-grouse | *Tetrao urogallus* | | 0.01 |
| White-grouse | *Lagopus lagopus* | | 0.03 |
| Lesser black-backed gull | *Larus fuscus* | West coast Norway | 1.0 |
| Common gull | *Larus canus* | | 0.6 |
| Herring gull | *Larus argentatus*[a] | | 11.0 |
| Herring gull | *Larus argentatus*[a] | | 13.2 |
| Soybean oil | | | 0.06 |
| Olive oil[b] | | | 0.03 |
| Peanut oil | | | 0.09 |
| Linseed oil[b] | | | 0.12 |
| Cocoa fat | | | 0.18 |

[a]Traces of arsenic present.
[b]Commercially produced raw oil.

### Registration of the Samples

After neutron activation, measurement of the induced activity in the samples was made with the aid of a 2 x 2 in. sodium iodide detector coupled to a multichannel γ-spectrometer. The samples were not treated chemically before these measurements. Some of the samples contained traces of sodium and the registration of their activity was therefore postponed for 4-8 days to ensure that the activity from sodium-24 would not disturb the registration of the induced bromine and arsenic activity. The sensitivity for the analysis of bromine and arsenic when using this method is approximately 0.005-0.01 μg and 0.002-0.005 respectively. The sensitivity is reduced when the registration after activation is postponed, as activities from other radioactive isotopes increase the "background" activity.

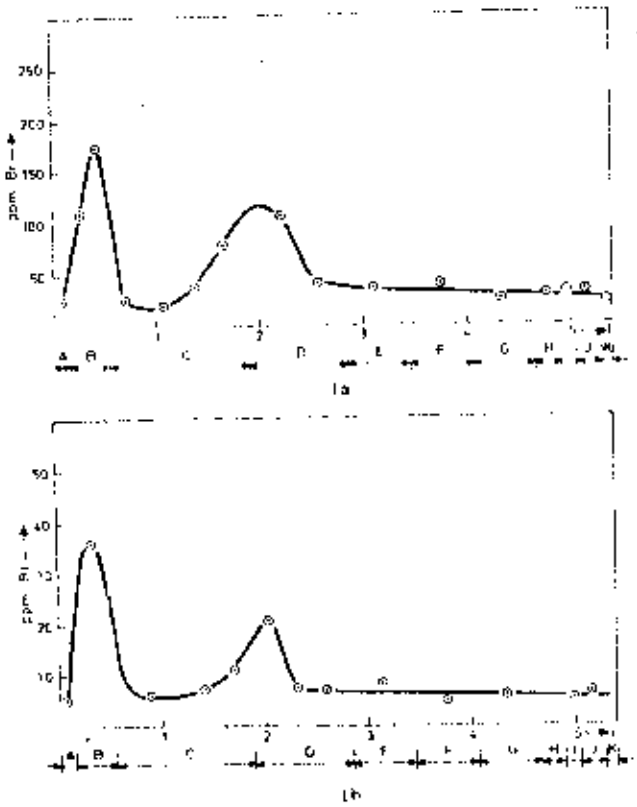


FIG. 1. The Bromine content in different fractions of cod liver oil (a) and oil extracted from mackerel fillet (b) fractionated in a silica gel column (see Table I for characterization of fractions).

## RESULTS AND DISCUSSION

The results of the bromine and the arsenic analysis in the different oils and in the corresponding fatty acids are shown in the Tables II-IV. They confirm the earlier findings that lipid soluble arseno and bromine organic compounds are characteristic of oils of marine origin. This conclusion applies to lipids extracted from fish, different invertebrates, seaweed and algae. The amount of bromine in vegetable oils and in some oils extracted from terrestrial animals is significantly lower than in marine oils, and it is probably present as inorganic bromine which has not been removed from the oils by the washing process.

The only oil from organisms of terrestrial origin in which bromine was detected came from seabirds, and the bromine-organic components were here partly present in somewhat reduced amounts. Fish constitutes the main part of the diet of these birds and is most likely the source of the bromine-containing compounds found in them. The analytical results indicate both a gradual reduction in the content of bromine organic compounds through the feed chain from algae to fish and to seabirds, and that at least some of these components are quite stable. No arsenic was found in any of the terrestrial oils, except for some traces in the oil from seabirds.

When the arsenic content in marine oils is compared with the arsenic content in the corresponding fatty acids, the results show that at least two groups of arseno organic compounds are present in the marine oils. One type consist of an arsenic-containing acid which follows the fatty acid during the saponification process while the other type is converted to a water soluble compound during this process.

It is interesting to note that the lipid phase in seaweed also contains arseno organic compounds as do the lipid from the marine animals studied. The amount following the fatty acids in a saponification is, however, relatively lower than for the other marine oils studied. These compounds are probably formed from inorganic arsenic absorbed from the sea water, synthesizing first a water soluble arseno organic compound. Compared with the total amount of arsenic present in seaweed (30-110 ppm) (8,9), the amount of arsenic found in the oil represents a relatively small amount of the total arsenic present in the seaweed. The yield of extractable lipids here is usually from 3-6%. However it should be noted that the contents of arsenic particularly in oil extracted from some *laminaria* species exceeds that in any other marine raw material analyzed.

The analysis of bromine in the marine oils and in the

corresponding fatty acids shows that this element also is localized in the fatty acid fraction, but that the content is somewhat lower here than in the oils. This would indicate that the bromine organic compounds are not formed solely by an addition of bromine to double bonds in the fatty acids.

The results of the bromine analysis in the fractions from the two oils fractionated on a silica-gel column (see Figs. 1a and b) give nearly the same picture of the bromine distribution in the different fractions, although the absolute amount of bromine is considerably higher in the cod liver oil than in the mackerel oil. Both diagrams show that the highest bromine concentration is located in fractions B and C-D. Fraction B (15% benzene in hexane) contains mainly cerolesters while the fractions C-D (5-15% diethylether in hexane) contains triglycerides, free fatty acids and sterols. It is also possible that the bromine may be present in other compounds which are eluted under these conditions, but which are present in smaller amounts and not found in this study. Although the results indicate that the bromine is randomly added to the main components of the oil, there may be one or more specific bromine organic compound located in fractions B and C-D.

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Mr. Shistad contributed technical assistance

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# Analysis of Complex Lipid Mixtures by Thin-Layer Chromatography and Complementary Methods

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THIN-LAYER ADSORPTION chromatography on silicic acid according to Stahl (11) has been used for the resolution of lipid extracts of blood (12), acetylated lipids (3,4), and fats, oils, and waxes (7). The present contribution is concerned with further applications of this technique to lipid separations. In addition, the preparation and some applications of "siliconized chromatoplates" for the fractionation of classes into their constituents by reversed-phase partition chromatography in conjunction with thin-layer chromatography are also described.

## Experimental

*Preparation of Chromatoplates.* Thin layers (250 to 275 μ) of silicic acid on glass (20 x 20 cm.) were prepared according to Stahl (11), using "Silica Gel G"[2] a fine grade (60μ or about 250 mesh) of silica gel containing 1% of plaster of Paris. "Silica Gel G" and the apparatus for its application were purchased from C. Desaga G.m.b.H., Heidelberg, Germany.

Siliconized chromatoplates were obtained by slowly immersing the silicic acid plates at room temperature into a solution of 5% silicone[3] ("Dow Corning 200 fluid," viscosity 10 cs.) in diethyl ether. Plates may be used immediately after evaporation of the solvent.

*Solvents and Indicators.* Thin layers of silicic acid were developed with mixtures of petroleum ether, B.P. 60-70°C., diethyl ether, and acetic acid.

Separation within classes of lipids on siliconized chromatoplates was achieved with systems of acetonitrile-acetic acid-water or acetic acid-water. After having been sprayed with 0.2% 2',7'-dichlorofluorescein[5] in ethanol, all lipids on untreated silicic acid films were manifested as a bright yellow-green fluorescence by ultraviolet light.[6] Unsaturated lipids were detected as brown spots on a light yellow background with iodine vapors.

Dichlorofluorescein was found to be unsatisfactory for locating the position of lipids on siliconized chromatoplates because the entire plates fluoresced. The indicators α-cyclodextrin-iodine however revealed monochain saturated lipids as white spots on a purple background (8). Unsaturated lipids were detected in the usual manner, with iodine vapors alone. The substances were amenable to further fractionation since no evidence was found which suggested that the indicators reacted chemically with the lipids to any marked extent.

*Separation of Lipids into Classes.* Complex lipid mixtures may be fractionated rapidly into classes of compounds by thin-layer chromatography on silicic acid. Figure 1 demonstrates the different migration rates of a variety of lipids, ranging from hydro-

[1] Supported in part by the U. S. Public Health Service National Institutes of Health Research Grant 5817, and the National Science Foundation.

[2] E. Merck A. G., Darmstadt, Germany.

[3] "Silica Gel G" may be obtained in the U.S.A. from Terra Chemicals Inc., 500 Fifth avenue, New York 16, N. Y., or from C. A. Brinkmann and Company Inc., 115 Cutter Mill road, Great Neck, L. I., N. Y. The later firm is also the U. S. representative of C. Desaga G.m.b.H., Heidelberg, Germany.

[4] Dow Corning Corporation, Midland, Mich.

[5] Eastman Kodak Company, Rochester 3, N. Y.

[6] "Mineralight," Ultra-Violet Products Inc., San Gabriel, Calif.

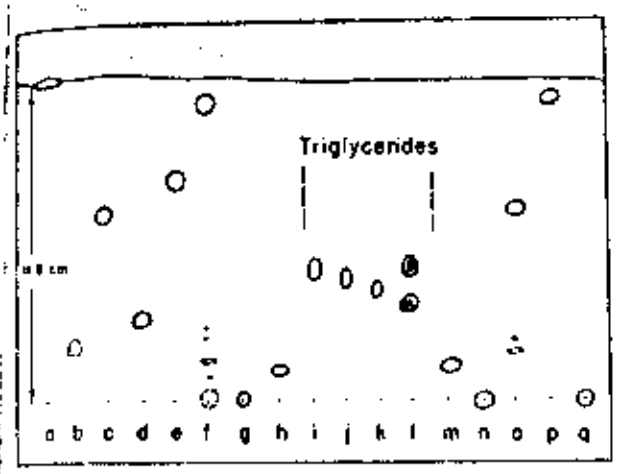


FIG. 1. Thin-layer adsorption chromatography of lipid classes on silicic acid.

Solvent system: 90 volumes of petroleum ether, B.P. 60-70°C., 10 volumes of diethyl ether, 1 volume of acetic acid.
Development time: 40 min.
Indicator: Dichlorofluorescein, 0.2% in ethanol.
Amounts: 20 γ, each.
a—Hexadecene-9, b—Oleyl alcohol, c—Oleylaldehyde, d—Oleic acid, e—Methyl oleate, f—Cholesteryl oleate, g—Monoolein, h—Diolein, i—Triolein, j—Trilinolein, k—Trilinolenin, l—Triarachidin, m—Cholesterol, n—Setachyl alcohol, o—Chlorophyllone, p—Oleyl oleate, q—Dioleoyl lecithin.

carbons to phosphatides. The separation of these substances on silicic acid is effected by virtue of their differences in polarity, which depend primarily upon the type and number of functional groups in the molecules. Subfractionation of triglycerides derived from fatty acids of widely different chainlengths (1) and from unsaturated acids of the $C_{18}$-series (i,j,k, is also presented. Homologous compounds, such as triglycerides, are only slightly separated from each other. Unsaturation however has a more pronounced effect on the subfractionation of lipid classes. The spots of triolein, trilinolein, and trilinolenin demonstrate the diminution in migration rate because of increasing unsaturation. These subfractionations, although perceptible with pure compounds, usually are not sufficient to interfere with the separation of classes. Larger amounts of a substance result in a slightly increased migration rate. This increased rate and the undetermined factors account for a lack of close reproducibility of $R_f$ values. The pattern of separation of a mixture however is reproducible and reliable.

Polar substances, such as monoglycerides, glycerylethers, and phospholipids, which do not migrate under the conditions stated in Figure 1, were fractionated with a more polar solvent. A mixture of 30 volumes of petroleum ether, 70 volumes of diethyl ether, and 1 volume of acetic acid served to segregate monoolein or setachyl alcohol, both $R_f$ 0.27, from dioleoyl lecithin, which remains at the starting point.

*Resolution Within Classes on Siliconized Chromatoplates.* Classes of lipids were resolved into their constituents by reversed-phase partition chromatography on siliconized silicic acid plates. Figure 2 demonstrates the separation of reference mixtures and of a $C_{18}$-fraction from a distillation of methyl esters derived from menhaden oil. By comparison of the spots with those of reference compounds the components of this fraction were assumed to be octadecanoic, octadecenoic, octadecadienoic, octadecatrienoic, and octadecatetraenoic acids. After iodine vapors were used for staining, the unsaturated esters were scratched off the plate and eluted from the adsorbent with diethyl ether. The identity of the polyunsaturated fatty acids was confirmed by their ultraviolet spectra after alkaline isomerization. A constituent of unknown structure was evident in the chromatogram, and comparison with reference compounds, alkaline isomerization, and gas-liquid chromatography all failed to reveal the identity of this substance.

As in chromatography on siliconized paper (8), and on columns of silicone-Celite (2), aqueous acetic acid yielded favorable separations of lipids on thin layers of siliconized silicic acid. The system of 85 volumes of acetic acid and 15 volumes of water was used to separate $C_{18}$-acids from each other within 3 to 4 hrs. at room temperature. The corresponding methyl esters were resolved under the same conditions. Acids and esters of chainlengths other than $C_{18}$ were separated in solutions of acetic acid and water in different proportions.

In reverse-phase partition chromatography at ambient temperatures the rate of migration of a lipid with one double bond is about equal to that for a saturated analog shorter by two methylene groups. Consequently palmitic acid is not separated from oleic acid. Such pairs were resolved by low-temperature chromatography (10) on siliconized chromatoplates. Palmitic acid, $R_f$ 0.0, and oleic acid, $R_f$ 0.1, were separated in the solvent system of 40 volumes of formic acid, 40 volumes of acetic acid, and 20 volumes of water within 8 hrs. at 4-6°C.

Saturated and unsaturated acids or methyl esters were also resolved by the use of solvents in which part of the acetic acid was replaced by peracetic acid (5). By this procedure saturated substances were still separated from each other on siliconized chromatoplates in 3 to 4 hrs. at room temperature while all unsaturated compounds migrated to the solvent front as oxygenated derivatives. A mixture of 10 volumes of peracetic acid,[7] 75 volumes of acetic acid, and 15 volumes of water served as the developing solvent.

[7] Berry Chemical Division, Food Machinery and Chemical Corporation, Buffalo 7, N. Y.

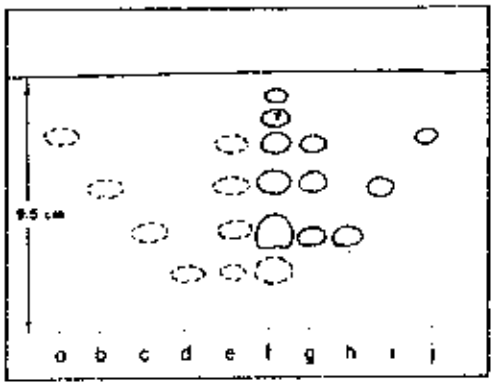


FIG. 2. Reverse-phase partition chromatography of methyl esters on a siliconized chromatoplate.

Solvent system: 70 volumes [illegible] 30 volumes acetic acid, 5 volumes of water.
Development time: [illegible] min.
Indicators: [illegible] by a cobaltous [illegible] spray, eluted and [illegible]
Amounts: 20 γ of individual compounds.
a—Methyl [illegible] b—Methyl myristate, c—Methyl palmitate, d—Methyl stearate, e—Mixture of chlorinated methyl esters, f—[illegible] derived from [illegible] g—Mixture of unsaturated [illegible] esters, h—Methyl oleate, i—Methyl linoleate, j—Methyl linolenate.

*Combined Methods.* Adsorption chromatography of lipid mixtures yields classes of compounds that may be further resolved by standard methods of partition chromatography. In our experience thin-layer chromatography gives separations that are more discrete and more rapid than those obtained by column chromatography. Thin-layer adsorption chromatography on silicic acid is an ideal supplement and offers a desirable preliminary for other separation techniques on a microscale, such as paper chromatography and gas-liquid chromatography.

Lipid classes, after having been isolated on silicic acid, were scratched off the plate and eluted from the adsorbent. All substances presented in Figure 1 were eluted with diethyl ether except the phospholipid, which was partially recovered with methanol. The appropriate eluant for lipids may be determined by chromatographing these substances with different solvents on silicic acid. Solvents that carry a compound with the solvent front are suitable as eluants for that substance.

Thin-layer adsorption chromatography on silicic acid was used for the purification of small amounts of lipids. About 10 mg. of impure linolenyl aldehyde, for example, were dissolved in diethyl ether, and the solution was applied along one side of a plate, 2 cm. from the edge. The aldehyde was separated within 20 min. from linolenic acid, monoenoic-2, and nonadecatrienol-1,2 with the solvent system of 95 volumes of n-hexane and 5 volumes of diethyl ether at room temperature. A narrow vertical strip of the chromatogram was sprayed with dichlorofluorescein solution. All contaminants were located close to the starting-point. The pure aldehyde, $R_f$ 0.65, was scratched off the plate and eluted from the adsorbent with diethyl ether. Thin-layer chromatography of the recovered aldehyde indicated that it was pure.

The combination of thin-layer chromatography with reversed-phase paper chromatography is illustrated by the following example. Batish liver oil was saponified and acidified, and the glyceryl ethers were separated as a class from the fatty acids, Vitamin A, sterols, and hydrocarbons. The system of 70 volumes of petroleum ether, 30 volumes of diethyl ether, and 1 volume of acetic acid was used as the developing solvent. The substances were located with dichlorofluorescein. After elution from the adsorbent with diethyl ether, the glyceryl ethers were resolved into their constituents by chromatography on silicon-ized paper with the solvent system of 60 volumes of tetrahydrofuran and 40 volumes of water at 30 C. for 18 hrs. (6). Batyl alcohol, $R_f$ 0.38, and selachyl alcohol, $R_f$ 0.45, were identified as the major constituents of this fraction.

Fatty acids were analyzed by combining thin-layer chromatography with gas-liquid chromatography. A Beckman "GC-2 Gas Chromatograph"[2] was used in this investigation. Methyl esters of fatty acids derived from menhaden oil were separated from the unsaponifiable constituents by thin-layer adsorption chromatography on silica gel with a mixture of 90 volumes of petroleum ether and 10 volumes of diethyl ether. The esters appeared, after exposure to iodine vapors, as two spots, $R_f$ 0.81 and 0.89, which were eluted with diethyl ether and analyzed by gas chromatography (4). Saturated constituents were found mainly in the upper spot whereas highly unsaturated esters were enriched in the lower. The gas-chromatogram of the total methyl ester fraction agreed in all details with a chromatogram obtained from a large-scale preparation of these methyl esters.

Many vegetable oils and some waxes and bacterial lipids contain hydroxylated acids that are altered during gas-liquid chromatography (9) so that in analysis by this method alone is difficult and often unsatisfactory. Classes of hydroxylated acids may be separated by thin-layer chromatography from the nonhydroxylated acids, and the latter may be eluted from the chromatogram and analyzed by gas-liquid chromatography. About 10 mg. of the total acidic fraction from castor oil, for example, were separated into dihydroxystearic acid, ricinoleic acid, and nonhydroxylated acids with the solvent system of 70 volumes of petroleum ether, 30 volumes of diethyl ether, and 1 volume of acetic acid. The nonhydroxylated acids, which comprise about 10% of the total, were eluted, esterified with diazomethane, and further resolved into their constituents by gas-liquid chromatography. Palmitic, stearic, and three unsaturated $C_{18}$-acids were identified as major components of this fraction.

In all kinds of column chromatography one depends upon analysis of the eluant, and substances remaining on the column are not detected. In thin-layer chromatography ("open columns") and in paper chromatography all components can be visualized and measured.

## Conclusion

Further work has been done on the application of thin-layer adsorption chromatography to the fractionation of complex lipid mixtures into classes.

New methods, the use of siliconized silicic acid plates and the application of thin-layer adsorption chromatography combined with the complementary techniques of gas-liquid chromatography and paper chromatography, are presented for the resolution of classes of lipids into their constituents.

In contrast to such elaborate conventional techniques as column chromatography, analyses using the methods reported in this paper can be performed rapidly in large numbers on a routine basis.

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[2] Beckman Scientific and Process Instruments Division, Fullerton, Calif.

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Part III

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# Thin-Layer Chromatography of Lipids

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A METHOD for chromatographic adsorption analysis on thin layers of adsorbent was described as early as 1938 by Izmailov and Shraiber (68). A decade later Williams (226) used a layer of adsorbent between horizontal glass plates, the upper one containing a small hole through which samples and solvents were introduced. Meinhard and Hall (120) developed "surface chromatography" on a powdered adsorbent, fixed rigidly to a microscope slide by means of a suitable binder. At the same time, Kirchner and Keller (81) described a procedure for impregnating filter paper with silicic acid. In 1951 Kirchner, Miller, and Keller (82) extended the use of adsorption chromatography to glass strips, 0.5 x 5.25 in., or sheets coated with a layer of adsorbent which contained starch or plaster of Paris as binding agent. Miller and Kirchner (125) developed an apparatus for coating glass strips with the adsorbent more rapidly than could be accomplished by hand. The same group of workers applied the new technique extensively to the separation and identification of terpenes and oxygenated terpenes and to their isolation from essential oils (82, 83, 84, 85, 86, 122, 123, 124).

Through the work of Kirchner, Miller, and Keller the chromatostrip method became well known and it was also used by other workers for the analysis of terpenes (21, 51, 67, 90, 142). With this method Vannier and Stanley (212) were able to detect the addition of grapefruit oil to lemon oil. These workers (193) and Bernhard (12) applied the chromatostrip technique to the detection and isolation of substituted coumarins from lemon oil. Stanley and Vannier (192) used chromatostrips for the quantitative estimation of biphenyl in citrus fruits. Onoe (143) utilized the same technique for the fractionation of 2,4-dinitrophenyl hydrazones of aliphatic aldehydes; Fukushi and Obata (45) analyzed azulenes; and Maruyama, Onoe, and Goto (115) separated organic peroxides.

A modification and extension of the chromatostrip method was developed by Reitsema (157, 158). He prepared chromatoplates, 5 x 7 in., to characterize various essential oils by chromatography. The use of chromatoplates instead of narrow strips permits running reference compounds alongside the mixtures of unknowns. Rigby and Bethune (159) used chromatoplates for separating and characterizing hop oils, and Wagner (218) analyzed phenols similarly.

Further application of the chromatostrip and chromatoplate techniques was reported by Jagoni and Wortmann (91, 92), who developed a method for the detection of fat-soluble pigments. These techniques were also used by Mottier (131, 132, 133, 134, 135), who carefully described methods for the analysis of food colors and of amino acids. Demole (34) separated phytol and isophytol, and Röchelmeyer (161) described the fractionation of alkaloids on thin layers of adsorbent.

It is amazing that the elegant technique of chromatographing on open columns, i.e., on thin layers of an adsorbent, was not applied to other lipids after it had become such a prominent method in terpene research.

[1] The author's work is being supported by the U. S. Public Health Service, National Institutes of Health Research Grant 5817, by The Bureau of Commercial Fisheries, Fish and Wildlife Service, U. S. Department of the Interior (Contract No. 14-17-008-127), by The National Science Foundation, and by The Hormel Foundation.

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Actually the method remained in obscurity until 1958 when Stahl (182, 183, 184) described equipment and procedures for the preparation of chromatoplates, and demonstrated the potential usefulness of thin-layer chromatography in the fractionation of substances other than terpenes. Thin-layer chromatography (TLC) has suddenly gained recognition and the technique is now being applied to the analysis of a great variety of substances. Simultaneously the method itself has been developed further and supersedes all other separation techniques in many laboratories, especially those engaged in lipid research.

Adequate equipment for work with thin-layer chromatography, such as spreaders for the uniform coating of glass plates, and standardized adsorbents, have become commercially available.

Several reviews on the application of TLC have appeared recently (35, 36, 73, 130, 168, 188, 190, 232, 233). Vioque (213) and Fontell, Holman and Lambertsen (44) discussed specific applications of TLC for the analysis of lipids. Brenner and Pataki (18) studied some theoretical aspects of TLC. (See also 40, 117, 234.)

The present review provides detailed descriptions of apparatus and techniques used in TLC. Applications of the method to lipids and related substances are summarized briefly.

Lipid fractions resolved by TLC may be isolated and further analyzed by complementary methods, such as paper chromatography (PC) and gas-liquid chromatography (GLC). Examples of the application of TLC in conjunction with PC and GLC are presented here.

## Apparatus and Techniques

*The Applicator and Other Equipment.* The uniform coating of glass plates with a thin layer of powdered material requires the use of a special applicator.

At present there are three types of applicators on the market.[2,3,4] The equipment designed by Stahl (182, 183, 186) is the oldest and most widely used, and therefore it is described in detail.

A plastic board 22 x 113 cm., with retaining ledges 1.8 cm. wide along a short and a long side (Fig. 1), is placed on a laboratory bench so that the long edge faces the worker. The short retaining ledge is now on the right side of the board. A glass plate, 5 x 20 cm., and five carefully cleaned square glass plates of uniform thickness, 20 x 20 cm., and another plate, 5 x 20 cm., are placed closely together in a row, on the board. A drop of water under each plate will prevent the glasses from sliding on the board. The applicator (Fig. 1) is placed on the small plate in the "open" position. The operator should grasp the applicator with both hands and move it slowly, in one stroke, across the plates to assure that they are well aligned. In the author's laboratory it was found useful to attach a piece of cork about 0.5 cm. high with adhesive tape to the leading edge of the applicator's guide bar. This permits one to move the applicator more smoothly across the row of plates.

A slurry of adsorbent is made by thoroughly mixing in a mortar 25 g. of Silica Gel G, a commercial

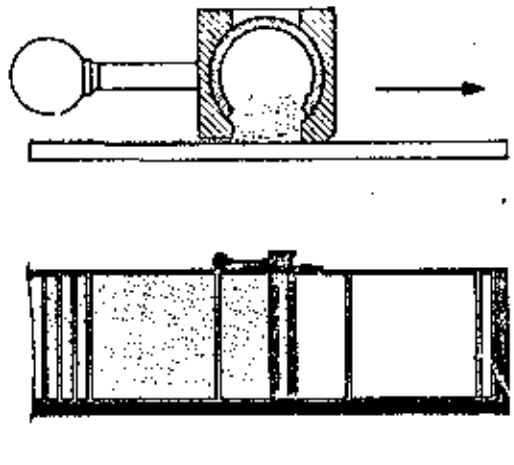



FIG. 1.

preparation containing calcinated calcium sulfate (or another adsorbent containing the same binding material[5,6]), with 50 ml. of distilled water. The slurry must be of uniform consistency and free of air bubbles. The time required for preparing the slurry should not exceed 1–1½ min. The mixture is poured into the applicator and the lever turned 180° in the direction of the red arrow on top of the instrument, to permit the slurry to run out of the slit as the applicator is moved smoothly across the row of glass plates on the board. The entire operation, from the addition of water to the silica gel until the spreading of the thin layer, must be finished in about 4 min. before the slurry hardens. The chromatoplates are air-dried for 10–20 min. to allow the binder to set and then are placed in a rack and activated by heating in an oven to 110–120°C. for 1–2 hr. The adsorbent layer thus produced is about 250–275 μ thick and very uniform in appearance in both reflected and transmitted light. According to Stahl (186) the activity of the adsorbent layer corresponds to grades II to III of the Brockmann activity scale (20). The chromatoplates may be kept in a storage cabinet to protect them from dust and laboratory fumes.

The adsorbent layer absorbs water from the air and thus slowly becomes deactivated. Adsorbents of different activities produce different rates of migration of various substances during chromatography. Therefore, $R_f$ values usually are not highly reproducible in thin-layer adsorption chromatography. The pattern of separation, however, is constant and reproducible.

Plates, whose efficiency of separation is affected as a result of too much moisture, may be reactivated by heating. In very humid laboratories it is advisable to store the activated chromatoplates in a desiccator over freshly activated silica gel. Seiler and Seiler (174), working on the separation of inorganic cations, recommend further drying of the air-dry chromato-

[2] C. Desaga G.m.b.H., Hauptstrasse 60, Heidelberg, Germany; U. S. representative: C. A. Brinkmann and Company, Inc., 115 Cutter Mill Road, Great Neck, Long Island, New York.

[3] Camag A. G., Homburger Str. 24, Muttenz, B.L., Switzerland; U. S. representative, Kensington Scientific Corporation, 1717 Fifth Street, Berkeley 10, California.

[4] Research Specialties Co., 200 South Garrard Blvd., Richmond, California.

[5] E. Merck, A. G., Darmstadt, Germany; U. S. representative: Terra Chemicals Inc., 500 Fifth Avenue, New York 36, New York.

[6] Fluka, A. G., Buchs, S. G., Switzerland; U. S. representative: Gallard-Schlesinger Chemical Manufacturing Corp., 1061 Franklin Avenue, Garden City, New York.

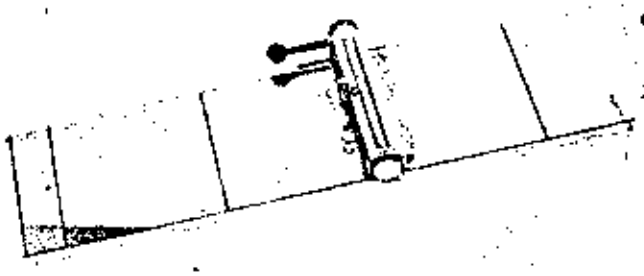


FIG. 2. Applicator "Model S" according to Stahl. Desaga G.m.b.H.[2]

plates over calcium chloride in a desiccator overnight. Thereafter the plates should be stored in a cabinet.

Recently a versatile model of Stahl's applicator has been produced which permits one to obtain uniform thin layers of any desired thickness between 250 μ and 2 mm. This applicator, Model S, as shown in Figure 2, is commercially available.[2] Stahl's new spreader may find application especially in preparative work where the use of plates of higher capacity is desired.

A rather different applicator for the preparation of chromatoplates has been designed by Mutter and Hofstetter (137). This relatively new apparatus[3] and its use have been adequately described by Wollish, Schmall, and Hawrylyshyn (232). They have given detailed instructions for the building of a similar instrument, as well as auxiliary equipment, in any small machine shop. The instrument is shown in Figure 3. It produces uniform layers of adsorbent in adjustable thicknesses on glass plates, 3.5 x 4.5 in.

An important advantage of this instrument, according to Wollish *et al.* (232), is that the glass plates used need not be uniform in thickness; thus one can easily buy inexpensive window glass cut to size in any glass shop.

Another new applicator for thin-layer chromatography has just become commercially available.[4] It is a simplified version of Stahl's original applicator, as shown in Figure 4. It yields fine layers about 275 μ thick. Layers of different thickness cannot be produced with this apparatus.

In many cases inexpensive, self-made applicators may be adequate, such as those described by Barbier, Jäger, Tobias, and Wyss (7), Černy, Joska and Lábler (25), Machata (98), Miller and Kirchner (125), Mottier (134), and Vioque (213). The author, however, believes that the purchase of a commercial applicator, standardized and uniform glass plates, and commercially available adsorbents is a good investment because they will save the many hours of experimentation that would be required to build a satisfactory instrument and produce a suitable adsorbent.

Peifer (145) has recently developed a method for TLC on lantern slides, 8 x 10 cm., on microscopic slides, 2.5 x 7 cm., and even on microscope cover slips, 2.5 x 3 cm. The glass plates are covered with adsorbent by dipping them into a suspension of Silica Gel G in chloroform or chloroform-methanol. The microchromatoplates may be used after drying over a hot plate for 1–3 min. This rapid and simple technique does not require the use of applicators, glass plates of specific dimensions, or prolonged drying periods. Therefore, it will become a useful modification of TLC even though it is not suited for resolving complex mixtures or larger samples.

#### Adsorbents, Stationary Phases for Partition Chromatography, and Ion Exchangers

*Silica Gel (Silicic Acid).* This adsorbent is most frequently used for the separation of neutral and acidic lipids (234) in columns (29), on impregnated cellulose paper (163), or on impregnated glass fiber paper (54). Fine grade (about 80 μ or 200 mesh) silica gel, containing up to 15% calcinated calcium sulfate (gypsum, plaster of Paris) as a binder, is widely used in TLC. Such preparations may be purchased as "Silica Gel G for thin-layer chromatography according to Stahl."[2,5] If very fine layers are desired, the commercial product should be run through a 200-mesh sieve before use. Silica Gel G is slurried with distilled water in a ratio 1/2, w/v, and applied to the glass plates as described above.

For special purposes it may be necessary to remove small amounts of ferric ion from the adsorbent. Seiler and Seiler (174) accomplished this by washing 500 g. of Silica Gel G with 1000 ml. of concentrated hydrochloric acid-water, 1/1, v/v. After removing of the acid by decanting, the adsorbent was washed with three to four portions of 1000 ml. of water, and filtered through a Büchner funnel. After drying in a stream of air the adsorbent was washed with 300 ml. of benzene and then dried in an oven at 120°C. for 1–2 hr. Because the acid treatment removes a great part of the calcium sulfate, it is necessary to add 2 g. of this material to the 500 g. of washed adsorbent before preparing the chromatoplates.

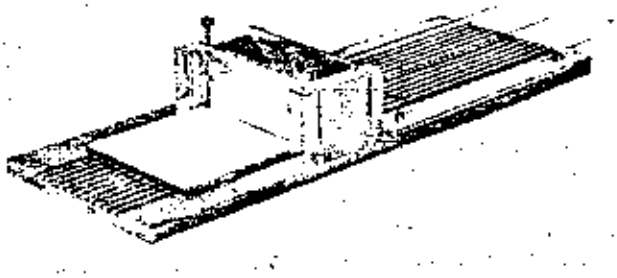


FIG. 3. Applicator according to Mutter and Hofstetter. Camag A.G.[3]

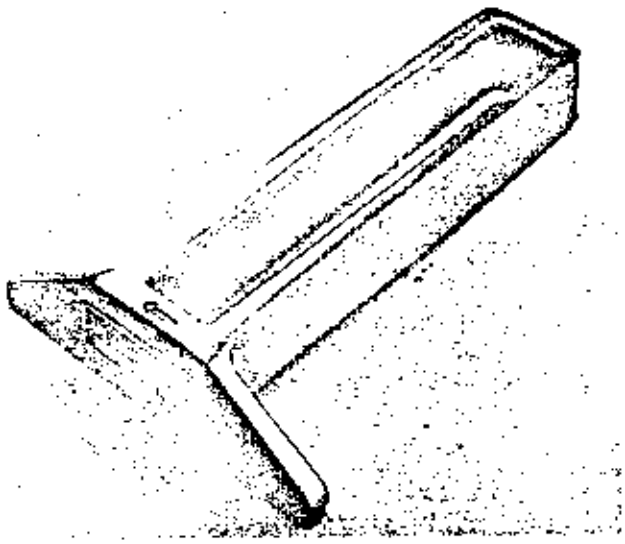


FIG. 4. Applicator of Research Specialties Co.[4]

A product closely resembling the commercial materials may be obtained by thoroughly mixing; e.g. Mallinkrodt Silica Gel,[1] 200 mesh, (154) with 10–15% calcinated sulfate of the same grain size (145). Once (143) prepared thin layers of silicic acid on glass strips from a slurry of 23 g. of silica gel, 200 mesh, and 30 ml. of a 2.5% solution of polyvinyl alcohol to which a few drops of ethanol were added to prevent foaming.

Several workers have recommended solvents other than water for preparing slurries of silicic acid. Müller and Honerlagen (136) claim that more uniform layers are obtained if 16 g. of Silica Gel G is mixed with 30 g. of acetone. Peifer (145) and Peifer, Muesing and Janssen (146) suspended 35 g. of Silica Gel G in 100 ml. of either chloroform or a mixture of chloroform-methanol, 3/2, v/v, for the preparation of microchromatoplates by the dipping technique. The use of nonaqueous solvents results in shorter drying periods for the wet chromatoplates. The adsorbent layer does not adhere very well to the glass and therefore, it may be necessary to expose the plates to steam (145).

It may be advantageous, especially in preparative work, to chromatograph on thin layers of powdered silica gel which do not contain calcinated calcium sulfate or other binders. Such loose layers can easily be obtained by applying the dry adsorbent with any spreader which will produce layers of any specific thickness from 0.5 to 2 mm.

Hernandez, Hernandez, Jr., and Axelrod (57) have described procedures for activating and standardizing silicic acid preparations used for chromatographing so that all Brockmann activity grades are obtainable and reproducible with this adsorbent. In TLC it is usually not necessary to know the activity of the adsorbent layer, as long as satisfactory separations can be obtained. Since the $R_f$-values are not reproducible in TLC, they may be referred to the position of standard dyes on the chromatogram. Stahl (183) recommends developing a mixture of 50 mg. each of p-dimethyl amino benzene (butter yellow), sudan red G, and indophenol, in 50 ml. of benzene,[2] concurrently with the samples to be separated. However, this test mixture can be applied only when benzene, methylene chloride, chloroform, or solvent mixtures of similar eluting power are used. Azulene is used with less polar solvents, such as petroleum hydrocarbon, toluene, or carbon tetrachloride.

Seher (171) has found that constant $R_f$ values are obtained on Silica Gel G plates if the adsorbent is conditioned by developing the plate with chloroform before the sample itself is chromatographed.

*Modified Silicic Acid Layers.* For the separation of acidic compounds, Stahl (185) prepared acidic silicic acid chromatoplates, by using aqueous 0.5 *N* oxalic acid solution instead of water in making the silicic acid slurry. Peifer (145) and Peifer, Muesing, and Janssen (146) added 2.5 ml. of concentrated sulfuric acid to 100 ml. of chloroform-methanol, 3/2, v/v. This mixture was slurried with 35 g. of Silica Gel G, as described above, for the preparation of microchromatoplates. The sulfuric acid in the adsorbent layer has the added advantage that the spraying of the plates after chromatography can be circumvented. The microchromatoplates may be placed on a hot plate right after developing, and the sulfuric acid in the adsorbent suffices for charring the organic matter in the individual spots.

Stahl (185) also described the preparation of alkaline chromatoplates. He used aqueous 0.5 *N* KOH solution instead of water in making the slurry of Silica Gel G. The use of such alkaline silica gel layers has not yet been described for TLC of lipids. This author assumes that such plates might be suitable for achieving separations of the kind described by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (162) on silicic acid-silicate-water columns.

Winterstein, Studer, and Rüegg (229) separated carotenals on chromatoplates that were prepared from a slurry of 5 g. Silica Gel G and 20 g. calcium hydroxide in 50 ml. of water.

Honegger (63), in his work on thin-layer ionophoresis and combined thin-layer ionophoresis-TLC, described the use of adsorbent layers containing sodium citrate buffer. Honegger prepared chromatoplates with a slurry of 25 g. of Silica Gel G (or Kieselguhr G, or Aluminum Oxide G) in 50 ml. of 0.1 M sodium citrate buffer of pH 3.3. Spraying of chromatoplates with buffer solution also yielded useful layers. Nürnberg (141) added a buffer to the developing solvent instead of incorporating it into the adsorbent layer.

Mangold and Kammereck (111) used chromatoplates of Silica Gel G containing about 10% ammonium sulfate for the separation of phospholipids and strongly acidic fatty acid derivatives. Such weakened plates were prepared from a slurry of 25 g. of Silica Gel G in a solution of 2.5 g. ammonium sulfate in 60 ml. of water. This slurry has to be applied to the glass plates very quickly, as it hardens even faster than do slurries of plain Silica Gel G.

Adsorbent layers containing acids, bases, or salts do not adhere well to the glass plates and tend to flake off. It is recommended that such plates be dried at room temperature for several hours.

*Diatomaceous Earth (Kieselguhr).* This adsorbent may be purchased already mixed with calcinated calcium sulfate as Kieselguhr G, Merck, for thin-layer chromatography according to Stahl.[25] Diatomaceous earth may be useful for separations of polar lipid where a very weak adsorbent is desired. It is applied like Silica Gel G, as a slurry in water, 25 g. per 50 ml. Stahl and Kaltenbach (191) used it for the fractionation of sugars by TLC.

*Aluminum Oxide (Alumina).* This adsorbent is rarely used for the chromatography of complex lipid mixtures because it causes hydrolysis of ester linkages and isomerization of double bonds (14, 15, 204, 205, 206). Still, alumina is superior to any other adsorbent for the chromatographic fractionation of the A-vitamins, also of hydrocarbons and other classes of lipids, especially of basic ones. It is often used for chromatographing sterols (138).

Two brands of alumina for TLC are commercially available: Aluminum Oxide G Merck, for thin-layer chromatography according to Stahl,[5] and Fluka Alumina for coated-glass chromatography.[6] Both preparations contain around 5% of calcinated calcium sulfate as a binder. The product manufactured by Merck is slurried with water in a ratio of 25 g./50 ml., whereas Fluka Alumina is mixed in a ratio of 20 g./65 ml. of water. The manufacturer of the latter material states that a high activity stage, approximately corresponding to Brockmann activity grade II (20), is obtained by heating the chromatoplates for at least 4 hr. to 200–220°C. The plates are cooled and stored

[1] Mallinckrodt Chemical Works, St. Louis 7, Missouri.

in a desiccator containing alumina of Brockmann activity grade I (Fluka Alumina with moisture indicator[6]). If plates with an adsorbent layer of lower activity (III or IV) are desired, the plates are dried at a temperature of 150–160°C. for 4 hr. In any case, the activated plates must be stored for at least 24 hr. over alumina of the corresponding Brockmann grade for setting the required activity, according to the producer's[6] specifications.

Alumina may also be obtained by mixing, e.g., Alcoa Activated Alumina[7] 200 mesh with 5% of calcinated calcium sulfate of the same grain size (108).

Heřmánek, Schwarz, and Čekan (56) applied a layer, about 0.6 cm. thick, of plain alumina powder to glass plates using Mottier's procedure (134) and they separated a great many steroids on such chromatoplates. Černy, Joska, and Lábler (25) used loose alumina layers, 3–4 mm. thick, for TLC of steroids also.

Stahl (189) has recently described a convenient technique for determining the activity of alumina according to the Brockmann scale (20). The gadget and the test dyes required for this procedure are now commercially available.[2]

*Other Adsorbents.* All adsorbent materials used in column chromatography may also be applied in TLC, either as a loose layer of powdered material (153), or, with a binder, as a solid film (121). Kirchner, Miller, and Keller (82) have already described the preparation of thin layers of magnesium oxide, magnesium carbonate, calcium hydroxide, calcium carbonate, dicalcium phosphate, Anex, Filtrol, Florisil, Talc, and Starch. These workers used starch (5%) or calcinated calcium sulfate (20%) as a binding agent.

Florisil,[9] which has recently been adapted for the fractionation of complex lipid mixtures on columns (24), appears to be especially promising. Peifer (145) has described a procedure for coating microchromoplates with a thin layer of this adsorbent.

Sodium carbonate (156), charcoal (62), and sugar (75) have been used in paper and column chromatography of lipids but the author is not aware of any applications of these adsorbents in TLC.

*Cellulose.* The preparation and use of chromatoplates covered with cellulose was first described by Teichert, Mutschler, and Röchelmeyer (203) who separated alkaloids. Several brands of cellulose powder for TLC are now commercially available.[10,11,12] The oldest product of this kind[10] contains calcinated calcium sulfate as a binder. Only 8 g. of this material, slurried with 55 ml. of water, suffice for coating 5 standard size square plates with a fine layer of cellulose. The coated plates are dried in air for a few minutes, and then in an oven for ½ hr. at 100°C. Plain cellulose powder[12] can also be applied as thin layers on glass plates (145).

To this reviewer's knowledge, cellulose chromatoplates have not yet been used successfully in the lipid field. They might become very helpful for the separation of lipids from non-lipids by adapting the procedures described by Lea and Rhodes (95) and by Westley, Wren, and Mitchell (224).

[6] Aluminum Ore Company, East St. Louis, Illinois.
[7] Floridin Co., Tallahassee, Florida.
[10] Exscorna G.M.B.H., Maine, Germany; U. S. representative: C. A. Brinkmann and Company, Inc. (see 2).
[11] Macherey, Nagel & Co., Düren, Rhld., Germany; U. S. representative: C. A. Brinkmann and Company, Inc. (see 2).
[12] H. Reeve Angel & Co., Ltd.; U. S. representative: H. Reeve Angel & Co., Inc., 52 Duane Street, New York 7, New York.

Teichert, Mutschler, and Röchelmeyer (203) impregnated cellulose chromatoplates with a 20% solution of formamide in acetone. Such plates may be useful for TLC of phospholipids (219).

*Layers for Reversed-Phase Partition TLC.* Thin layers of Silica Gel G can be impregnated with silicone (101), undecane (79) or higher paraffins (80, 229) or with squalene (26). They are then hydrophobic and can be used for the resolution of a lipid class into its individual constituents by reversed-phase partition TLC. Malins and Mangold (101) coated thin layers of Silica Gel G by slowly immersing the plates at room temperature into a 5% solution of silicone (Dow Corning 200 fluid viscosity 10 cs)[13] in diethyl ether. Such a silicone solution is now also commercially available in an aerosol package[4] for impregnating chromatoplates by spraying. Winterstein, Studer, and Rüegg (229) dipped Silica Gel G plates for 2 min. into a 5% solution of paraffin oil in petroleum hydrocarbon. The same procedure was later applied by Kaufmann and Makus (79) and by Kaufmann, Makus, and Deicke (80). These authors also used undecane as a temporary impregnating agent. In all these procedures it is important that the chromatoplates to be impregnated be of the same temperature as the silicone or paraffin solutions and, also, that the plates be immersed into these solutions very slowly. Disregarding these precautions will lead to crumbling of the thin layers. The impregnated plates may be used immediately after evaporation of the solvent.

The author has impregnated thin layers of cellulose[10] and of diatomaceous earth[5] with silicone. Such plates, however, proved to be inferior to impregnated Silica Gel G plates for lipid separations.

Polyethylene powder (Hostalen S)[14] as used in columns (231) has also been used successfully for the preparation of chromatoplates which yield favorable separations of fatty acids or their methyl esters. Hostalen S, 20 g. 250 mesh, is slurried with 50 ml. of aqueous ethanol (96%), 1/1, v/v, and applied to glass plates with a commercial spreader. The chromatoplates are dried at room temperature for several hours and are then ready for use. Davídek and Davídková (33) have recently reported the preparation of thin layers of polyamide on glass plates, and the separation of flavonoids on such chromatoplates. Stahl (188) mentioned the use of Perlon as a stationary phase in TLC.

*Ion Exchangers.* Only one publication has appeared so far on the use of ion exchangers in TLC. Randerath (155) used Eteola Cellulose[12] bound to the glass plates by collodion to separate various purine and pyrimidine nucleotides. Undoubtedly, materials like the various modified cellulose ion exchangers applied on columns by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (162) will also be useful if applied in the TLC technique.

Ion exchange chromatography has so far found little application in the lipid domain. It can be expected that the current wide use of TLC will also lead to extensive utilization of ion exchange materials for the separation of polar lipids.

*Various Coating Materials.* Urea has been used for separating straight-chain from branched-chain fatty acid in columns (62) and on paper (3). It can be expected that similar separations are possible on

[13] Dow Corning Corp., Midland, Michigan.
[14] Farbwerke Hoechst A. G., Frankfurt-M., Germany.

glass plates which are coated with a thin layer of urea or another clathrate-forming material. Molecular sieves should be tried also.

### The Sample[15]

Neutral lipids such as fats, oils, and waxes are applied to the plates with a micropipette or better, with a microsyringe, 10–50 λ,[16,17] as 0.1–1% solutions in petroleum hydrocarbon, b.p. 60–70°C. More polar solvents such as diethyl ether should be avoided as they effect chromatographic separations at the point of application which results in wider spots and poor resolution. Phospholipids, sulfolipids, glycolipids, and other very polar compounds are applied as chloroform or chloroform-methanol solutions, 1/1, v/v. Mercuric acetate adducts of unsaturated lipids *(vide infra)* are also dissolved in chloroform.

The samples must always be applied as single spots, not in a streak across the plate, as is frequently done in paper chromatography, because the developing solvent will not migrate across such a cut in the adsorbent layer. To avoid irregularities in migration of the solvent, the chromatoplates should always be developed against the direction of application of the layer. To achieve optimal resolutions the spots should not spread more than about 0.5 cm. in diameter. This is accomplished by applying the solutions in several small portions in a stream of nitrogen. Several firms[2,3,4] offer special transparent template with side rails that permit covering a chromatoplate without touching the adsorbent layer. Such a template facilitates spotting of samples; it may also be used to draw a line across the plate, 10–15 cm. from the starting points, to mark the level to which the solvent will be allowed to travel. The use of the template as designed by Stahl is schematically illustrated in Figure 5.

In adsorption chromatography, between 10 and 20 different samples, 50–500 γ of each, may be applied on one plate in a row of spots along one side of a plate, about 2 cm. from the edge. Several mg. of one complex lipid mixture may be fractionated on one plate by applying its solution along one side of a plate. Even up to 50 mg. of less complicated mixtures may be separated on one plate if the constituents are widely different in polarity, such as fatty acids and their methyl esters.

Only 1–10 mg. of lipids or lipid derivatives like mercuric acetate adducts of unsaturated lipids can be separated on one plate through partition chromatography by the thin-layer technique.

### Selection of Solvents

Lipids may be separated by adsorption into classes of compounds, and classes may be resolved by reversed-phase partition chromatography. Fractionation is also possible according to degree of unsaturation. The choice of solvents depends on the type of separation desired. Solvent mixtures, rather than individual solvents, are usually applied, but the mixture should be kept as simple as possible for better reproducibility. Also, multicomponent systems tend to produce secondary solvent fronts, due to demixing during chromatography. A binary or ternary mixture of solvents can always replace a developing system consisting of five or six components.

*Separating Lipids Into Classes.* Brockmann and Volpers (19) found that a solvent elutes organic compounds from adsorbent columns according to the type and number of functional groups in these compounds. Hydrocarbons leave the column first, followed by ethers, ketones, alcohols, and acids, in this sequence. Within each class, saturated compounds are eluted before unsaturated ones. Polyunsaturated compounds with isolated double bonds are more easily eluted than are those containing a conjugated system of double bonds; *cis*-compounds are more easily eluted than their *trans*-isomers.

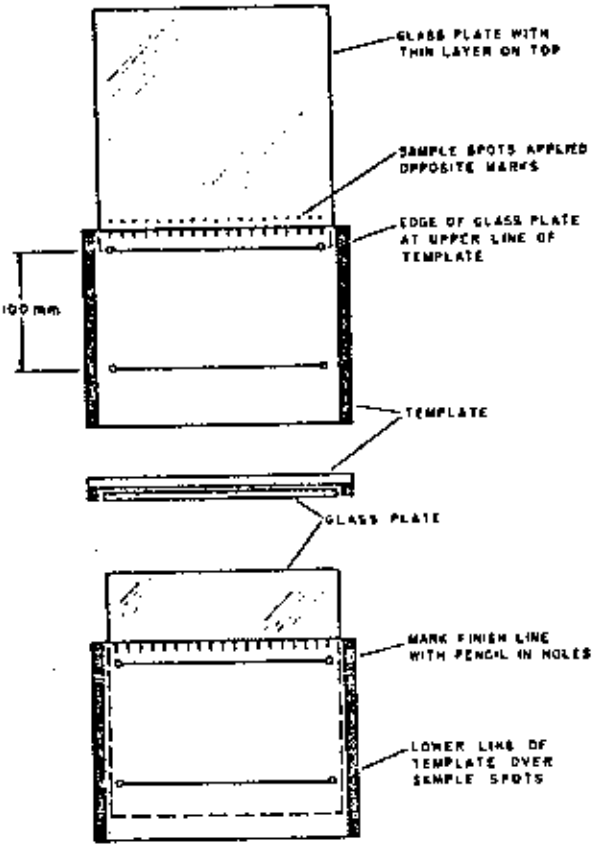



FIG. 5. Use of the template.[1]

These rules apply also in TLC: hydrocarbons migrate further on a chromatoplate than do ethers, ketones, or alcohols, and within each spot the saturated components of a class are concentrated in the front part of this spot. These subfractionations are quite pronounced with, e.g., the methyl esters of fatty acids derived from fish oils, where great differences exist in chain length and degree of unsaturation of the individual constituents. However, they never interfere with separation according to classes.

Trappe (204, 205, 206) established a series of solvents of increasing elution power. This Eluotropic Series of Solvents was extended, and other series were described, e.g., by Strain (199), and by Knight and Groennings (88). The tabulation of eluotropic series of solvents (Table 1) is very helpful in choosing suitable solvents for adsorption chromatography on columns or on thin layers. Izmailov and Shraiber (68) and other workers (145, 183) have described a spot

[15] Lipid standards for TLC may be obtained from The Hormel Foundation, Austin, Minnesota.

[16] Hamilton Company, Inc., Whittier, California.

[17] Disposable self-filling, self-measuring dilution micropipettes (Gerarde Pipettes) are recommended for work with radioactive lipids. "Cooperie," Becton Dickinson, Rutherford, New Jersey.

test for choosing a suitable solvent for chromatography: drops of a solution of the sample to be fractionated are placed on a thin layer of the adsorbent to be used and different solvents are applied to these spots. The various solvents will yield concentric rings of the constituents of the mixture and the solvent effecting the clearest separation by this test is also most suitable for chromatographing the mixture in a larger scale on a chromatoplate or on a column.

TABLE I

Eluotropic Series of Solvents

| Trappe (204) | Strain (199) | Knight and Groenwinkel (86) |
|---|---|---|
| Light petroleum | Light petroleum 30–50° | Heptane |
| Cyclohexane | Light petroleum 50–70° | Diisobutylene |
| Carbon tetrachloride | Light petroleum 70–100° | Benzene |
| Trichlorethylene | Carbon tetrachloride | Isopropyl chloride |
| Toluene | Cyclohexane | Diisopropyl ether |
| Benzene | Carbon disulfide | Diethyl ether |
| Dichloromethane | Anhydrous diethyl ether | Ethyl acetate |
| Chloroform | Anhydrous acetone | sec. Butyl alcohol |
| Diethyl ether | Benzene | Ethyl alcohol |
| Ethyl acetate | Toluene | Water |
| Acetone | Esters of organic acids | Acetone |
| n-Propanol | 1,2-Dichloroethane | Methanol |
| Ethanol | Alcohols | Pyruvic acid |
| Methanol | Water | |
| | Pyridine | |
| | Organic acids | |
| | Mixtures of acids or bases, water, alcohols, or pyridine | |

Mixtures of two or three solvents of different polarities may be chosen for optimum resolution, instead of a single solvent. Schroeder (169) found mixtures of petroleum hydrocarbon with diethyl ether to be especially useful. This solvent pair is now preferred in column chromatographic separations of natural lipid mixtures according to classes of compounds (e.g. 42, 58, 118, 119, 181) and also in TLC (e.g. 107, 108, 126, 149). Ethyl acetate may be used to replace diethyl ether in such mixtures.

Classes of non-polar lipids, such as hydrocarbons, ethers, cholesteryl esters, and alkyl esters are best separated, as classes, by TLC with nonpolar solvents such as petroleum hydrocarbons, carbon tetrachloride or benzene or mixtures of two solvents containing largely one of these solvents (Table II). More polar lipids are resolved with mixtures of petroleum hydrocarbon and diethyl ether in different ratios (Table II). The same solvent mixtures with 1–2% of glacial acetic acid serve for resolving complex mixtures of natural lipids (Table II). The addition of acetic acid is essential if free fatty acids are present, in order to prevent their streaking.

Very polar nitrogenous lipids of industrial importance, like amines, amides, and nitriles, are well resolved with a mixture of chloroform-methanol-aqueous ammonia. Chloroform is equilibrated, at 20°C., with aqueous ammonia, 10/1, v/v, and 97 volumes of this ammoniacal chloroform and 3 volumes of methanol are used as the developing solvent system (111).

Acidic lipids, such as sulfates and sulfonates, phosphates and phosphonates, are separated by TLC on Silica Gel G containing 10% of ammonium sulfate, with mixtures of chloroform-methanol-aqueous sulfuric acid. Methanol containing 5% of 0.1 N sulfuric acid is mixed with chloroform, depending upon the polarity of the lipids to be resolved (111). Typical solvent systems are chloroform-acidic methanol-sulfuric acid, 97/3 and 8/2, v/v. The latter solvent mixture is especially suitable for the separation of natural phospholipids, such as lecithins, cephalins, and sphingomyelins on modified Silica Gel G plates. This acidic system will split, however, any aldehydogenic phospholipids.

Solvent systems for the separation of phospholipids, sulfolipids, and glycolipids on Silica Gel G plates are listed in Table II. Typical solvent systems for the separation of steroids, of bile acids, and of triterpenoic acids are listed in the same table.

*Solvents for Fractionating Classes by Reversed-Phase Partition TLC.* The solvent systems used in chromatography on silicone-impregnated paper or on paraffin-impregnated paper can also be applied in reverse-phase partition TLC. Chakrabarty (26) has used the same developing solvents on silicic acid plates that had been impregnated with squalane.

TABLE II

Solvents for Thin-Layer Chromatography of Lipids on Silica Gel[a]

| | References |
|---|---|
| **Fats, Oils, and Waxes, Oxygenated Acids and Their Esters** | |
| Tetralin-Hexane, 1/1, 1, 3 | (80) |
| Petroleum hydrocarbon[b]-diethyl ether, 95/5, 90/10, 80/20, 50/50 | (101,108,149) |
| Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, 80/20/1, 70/30/2 | (101,108,125) |
| Diethyl ether | (79) |
| Diisopropyl ether | (79,80) |
| Diisopropyl ether-acetic acid, 100/1.5 | (79,80) |
| n-Propanol-conc. aqueous ammonia, 2/1, followed by chloroform-benzene, 3/2, followed by carbon tetrachloride | (65,223) |
| **Phospholipids, Sulfolipids, and Glycolipids** | |
| Chloroform-methanol-water, 70/22/3, 65/30/5, 65/25/4, 60/35/8 | (164,219,220) |
| Chloroform-methanol-aqueous ammonia, 3/1 with 40 ml. of conc. aqueous ammonia/liter; 1/3 with 40 ml. of conc. aqueous ammonia/liter | (30,64,111) |
| Chloroform-methanol-aqueous sulfuric acid | |
| Chloroform-acidic-methanol (containing 5% of 0.1 N sulfuric acid), 97/3, 4/1 | (111)[c] |
| n-Propanol-water, 7/3 | (89) |
| n-Propanol-aqueous ammonia (12%), 4/1 | (72,223) |
| n-Propanol-acetic acid-aqueous ammonia, 7/2 | (72) |
| n-Propanol-aqueous ammonia (12%), 4/1, followed by ethylene chloride-methanol, 49/1, followed by chloroform-acetic acid (96%), 99/5 | (72) |
| n-Butanol-pyridine-water, 3/2/1 | (87) |
| **Steroids** | |
| Benzene, toluene, cyclohexane, hexane, dichloroethane | (23,31,56)[d] |
| Benzene-ethyl acetate, 9/1, 2/1 | (31) |
| Benzene-ethanol, 49/1, 95/5, 9/1 | (35) |
| Cyclohexane-benzene, 4/1, 1/1 | (31) |
| Cyclohexane-ethyl acetate, 19/1, 17/3, 9/1, 7/3 | (7,31) |
| Diethyl ether | (56)[d] |
| **Bile Acids** | |
| Upper phase of the system, toluene-acetic acid-water 10/10/1, 5/5/1 | (48) |
| n-Butanol-acetic acid-water, 10/1/1 | (46) |
| **Triterpenoic Acids and Esters** | |
| Benzene, cyclohexane, methylene chloride, diisopropyl ether, ethyl acetate, butyl acetate | (207,210) |
| Cyclohexane-toluene, 4/1, saturated with formic acid | (210) |
| Diethyl ether-hexane, 1/1 | (214) |
| Diisopropyl ether-acetone, 5/2 | (210) |
| Diisopropyl ether-acetone, 5/2, with 5% of pyridine | (210) |
| Ethyl acetate-methanol-diethyl amine, 14/1/3 | (210) |
| Chlorobenzene-acetic acid, 9/1 | (210) |
| Methylene chloride-pyridine, 7/2 | (210) |

[a] All ratios are v/v.
[b] Usually petroleum hydrocarbon, b.p. 60–70°C. (mainly hexane).
[c] On silica gel G containing 10% of ammonium sulfate.
[d] On alumina.

Suitable solvent systems for reversed-phase partition PC or TLC of a given class of lipids can be chosen after studying the behavior of this lipid in adsorption chromatography (111). Typical solvent systems are given in Table III.

Compounds differing by two methylene groups and one double bond, such as palmitic and oleic acids, or palmitonitrile and oleonitrile, myristonitrile and linoleonitrile, lauronitrile and linolenonitrile, overlap in reversed-phase partition chromatography. Such critical pairs are separated by low temperature chromatography. Palmitic and oleic acids may be resolved with a mixture of acetic acid-formic acid-water, 2/2/1,

TABLE III

Solvents for Reversed-Phase Partition Thin-Layer Chromatography

| Solvents[a] | Ratios v/v | Stationary phase[b] | Lipid classes fractionated | Ref. |
|---|---|---|---|---|
| Methanol | ..... | Higher paraffin | Carotenals | (229) |
| Acetic acid-water | 24/1 | Undecane | Fatty acids | (79) |
| ..... | 3/1 | Silicone | Fatty acids and their methyl esters | (101) |
| ..... | 17/3 | Silicone | Fatty acids and their methyl esters and aldehydic cores derived from lecithins | (101) (153) |
| ..... | 17/3 | Squalane | Fatty acids and their methyl esters | (26) |
| Acetic acid-acetonitrile | 1/1 | Undecane | Fatty acids | (79) |
| Acetic acid-acetonitrile-water | 2/14/5 | Silicone squalane | Methyl-esters of fatty acids | (101) (26) |
| Methyl ethyl ketone-acetonitrile | 7/3 | Higher paraffin | Cholesteryl esters of fatty acids | (80) |
| Chloroform methanol-water | 5/15/1 | Undecane | Diglycerides | (79) |
| Acetone-acetonitrile | 7/3 | Undecane | Triglycerides | (79) |
| Acetone-ethanol-water | 6/1/3 | Polyethylene | Methyl esters of fatty acids | ..... |
| Acetic acid formic acid-water[c] | 2/2/1 | Silicone | Fatty acids saturated/unsaturated | (101) |
| Acetic acid-peracetic acid-water | 15/2/3 | Silicone | Fatty acids saturated/unsaturated | (101) |

[a] All solvent mixtures must be saturated with the stationary phase.
[b] Silica Gel G served as a carrier for the stationary phases. Only polyethylene was applied directly to the plates.
[c] Chromatographed at 4–6°C. (All others at room temperature.)

v/v/v, at 4–6°C., on siliconized Whatman #1 paper[12] (165) or on a siliconized Silica Gel G plate (101). An alternative method of resolving critical pairs is to chromatograph with oxidizing solvents (101, 105, 107). Saturated and unsaturated nitriles can be resolved on siliconized paper or on a siliconized chromatoplate with solvent systems consisting of 75 volumes of acetic acid and 25 volumes of water, or 70 volumes of acetonitrile and 30 volumes of water. A mixture of 65 volumes of acetic acid, 10 volumes of peracetic acid[13] and 25 volumes of water, or of 70 volumes of acetonitrile, 10 volumes of perhydrol, and 20 volumes of water separates saturated nitriles from one another, whereas all unsaturated lipids are quantitatively oxidized and migrate in bulk to the solvent front.

*Solvents for Separating Lipids According to Unsaturation.* Saturated and unsaturated lipids can be separated by chromatography after reacting the latter with mercuric acetate in methanol. A procedure for forming acetoxymercuri-methoxy compounds of unsaturated lipids is described later in this text.

Saturated and the acetoxymercuri-methoxy derivatives of unsaturated lipids, e.g., hydrocarbons, methyl esters, or nitrils, are resolved by TLC on Silica Gel G with a mixture of petroleum hydrocarbon-diethyl ether, 4/1, v/v. The derivatives of the unsaturated lipids are then fractionated according to the degree of unsaturation, regardless of chain length, in a solvent consisting of n-propanol-glacial acetic acid, 100/1, v/v. After isolation by TLC, the original monounsaturated, diunsaturated, and triunsaturated lipids can each be recovered by treating their complexes with ethanolic hydrochloric acid.

### Developing

Chromatostrips and chromatoplates are usually developed at room temperature in ascending technique, i.e., they are placed on edge in a covered jar containing a 0.5–1 cm. layer of solvent which then travels up the length of the plate until it reaches a pre-set line. Jars for use with standard chromatoplates are commercially available.[3,4,19] Several chromatoplates may be simultaneously developed in one jar. To obtain straight solvent fronts, the adsorbent should be scraped off the edges of the plates before chromatography, and the chromatoplates should not touch the sides of the jar. For the same reason, it is advantageous to line the inside of the jars with filter paper which acts as a wick to assure that they are uniformly saturated with the solvent vapors (185). This gives a straight solvent front, rounder spots and it shortens the developing time by about ⅓. It may be noted that chromatography in lined jars requires somewhat more polar solvent than in plain jars. For example, to obtain the same kind of separation one will have to use a system of petroleum hydrocarbon, b.pt. 60–70°C., diethyl ether, glacial acetic acid, 80/20/1, on a jar lined with filter paper, but the same solvents in a ratio of 90/10/1, v/v/v, in a jar that does not contain such a wick. Generally the separations achieved in a saturated chamber are not quite so sharp as those obtained in plain chambers.

Chromatoplates may also be developed step-wise, running the same solvent once or twice or different solvent systems consecutively in the same direction (multiple development). Stahl (184) has utilized Matthias' wedge strip technique (116) in TLC, by scratching a row of wedge-tipped strips in the adsorbent layer.

Dhopeshwarkar and Mead (38), also Chakrabarty (26), have used a gradient-elution scheme for TLC of lipids. The developing solvent mixture is continuously being diluted with a more polar solvent. This technique permits fractionation of lipids of widely different polarity on a single plate.

Brenner and Niederwieser (17) extended the scope of TLC by developing a simple arrangement which forces solvents to run over. This method was applied to the separation of substances that could not be separated otherwise. The same technique should be useful for collecting fractions from an overflowing chromatoplate. An apparatus for use with chromatostrips has been devised by Stanley, Ikeda, and Cook (194).

Chromatoplates may be run in two-dimensional technique. A single sample is placed in a corner of the plate, and the plate is then developed, consecutively, in two directions with two different solvent systems. Using the same solvent in both directions yields little improvement in resolution but indicates alteration of components if the resulting spots are not uniformly on a line bisecting the plate. For example, in TLC of tissue lipids it can be observed that the phospholipid fraction remains where it had been after developing in the first dimension (211). Stahl (187) has applied two-dimensional TLC for studying chemical reactions on the plate of the compounds fractionated by chromatography in the first direction (Separation-Reaction-Separation; SRS Technique). Miller and Kirchner (124) had already described carrying out oxidations, reductions, dehydration, and hydrolysis reactions, and formation of derivatives on the plate. Kaufmann and Makus (79) have performed adsorption chromatography and reversed-phase partition chromatography

[12] Beckman Chemical Division, Food, Machinery and Chemical Corp., Dunsdale 7, New York.

[13] A. H. Thomas Company, Vine Street at Third, Philadelphia 5, Pennsylvania.

of a sample, consecutively, on the same plate. The lipid mixture was first fractionated according to classes by adsorption chromatography. Then the unused major portion of the chromatoplate was impregnated, and classes were resolved into individual compounds by reversed-phase partition chromatography in the second direction.

The possibility of reacting parts of a complex mixture in the course of chromatography has been illustrated by chromatography in oxidizing solvents (101, Table III).

Chromatoplates may be developed horizontally. This is mandatory if loose layers of adsorbent, free of binding agent, are applied (e.g., 25, 56, 91, 92, 131, 153). It is also used in radial development, i.e., Circular-TLC. The sample is placed in the center of a plate and developed by slowly dripping solvent onto it from a pipette (91, 92, 226) or by supplying solvent from a reservoir through a wick (184). Chakrabarty (26) has found centrifugally accelerated TLC useful.

### Visualization and Identification of Lipids

Most indicators used in PC of lipids (103, 113) may also be applied in TLC, provided washing of the chromatograms is not required. These indicators are usually non-destructive, thus permitting the individual fractions to be eluted from the plates for further fractionation and identification. In addition, corrosive spray reagents, such as oxidizing acids, may be used for charring organic matter on the chromatoplates over a hot plate. Such indicators have the great advantage that all compounds that may not be detected by more specific reagents are visualized unless they are volatile. The author prefers chromic sulfuric acid solution. With this agent, unsaturated lipids appear as light brown spots on a white background, even before heating. Heating the plates yields further information on the chemical nature of the various compounds as different colors appear at increasing temperatures.

A list of indicators used in TLC of lipids is presented in Table IV.

The consecutive use of two or several indicators on one chromatoplate increases the probability that no substance remains undetected. The author prefers using iodine vapors on one plate, and after evaporation of the iodine (5 min.), dichlorofluorescein, followed by chromic sulfuric acid solution (111). The dichlorofluorescein on the plate does not interfere with the subsequent charring process, neither is silicone on reversed-phase plates charred when heated with chromic-sulfuric acid. Dichlorofluorescein, however, cannot be used on reversed-phase layers, as the whole plate fluoresces when sprayed with this indicator (101).

Fluorescent chemicals may be incorporated in the adsorbent layer for visualizing spots in U.V. light after chromatography. Reitsema (157) added about 0.004% of Rhodamine 6 G to his chromatoplates, Stahl (183) used 0.04% sodium fluorescein, Cerny, Joska, and Lábler (25) mixed morin into loose layers of alumina. Kirchner, Miller, and Keller (182), following a suggestion by Sease (170), incorporated about 0.75% of each zinc silicate and zinc cadmium sulfide as fluorescent agents into silicic acid layers on glass. The reviewer adds 1% of the commercial Luminescent Chemical #601[29] to Silica Gel G plates, in

[29] Eastman Kodak Company, Rochester 3, New York.
[30] "Mineralight," Ultra-Violet Products Inc., San Gabriel, California.
[31] E. I. du Pont de Nemours & Co. (Inc.), Photo Products Department, Wilmington 98, Delaware.

TABLE IV
Indicators Used in Thin Layer Chromatography of Lipids

| Reagent | Color of spot | Substances visualized | Sensitivity | Ref |
|---|---|---|---|---|
| Daylight or U.V. light | Various colors, white background | Natural compounds. Fluorescent substances | 0.1–10 γ | (37) (225) (328) |
| Fluorescent dye or mineral in adsorbent layer | Various colors, colored background | Aromatic derivatives of lipids. Conjugated unsat. fatty acids | 1 γ | (47) (82) (157) |
| Radioisotopes | An autoradiograph is taken | All radioactive labeled materials | <0.1 γ | (107) (112) (195) |
| Iodine vapors or iodine, 1% in methanol[a] | Brown, white or yellow background | All unsaturated lipids, also some saturated nitrogenous lipids | <1 γ | (101) (107) (108) |
| α-Cyclodextrin, 1% in aqueous ethanol, 30% followed by iodine vapors | White, purple background | Monochain lipids | 5 γ | (101) (107) |
| 2',7'-Dichlorofluorescein, 0.2% in ethanol, 96%[a] | Green, purple background in U.V. light | Nonpolar lipids, saturated and unsaturated | 1–5 γ | (101) (108) (111) |
| Rhodamine B, 0.05% in ethanol, 96% | Purple, rose background | Most lipids | 1 γ | (79) (80) (220) |
| Bromothymol blue 0.04 gr. in 100 ml. 0.01 N aqueous NaOH | Yellow on blue background | Most lipids | 0.1–1 γ | (53) (71) (72) |
| Fluorescein-bromine 0.01% sodium fluorescein in water followed by bromine vapors | Yellow, pink background, best in U.V. light | Unsaturated compounds | 1 γ | (4) (82) (163) |
| Wet hydroxylamine impregnated paper, followed by 5% ferric chloride in 0.5 N aqueous HCl | Purple, white background | Esters | 5–10 γ | (35) (40) |
| Antimony trichloride or antimony pentachloride or stannic chloride. Saturated solution in chloroform or carbon tetrachloride | Various colors in daylight and U.V. light | Aliphatic lipids, steroids | 1–10 γ | (7) (31) (222) |
| 10% Phosphoric acid 40% in water | Gray-blue | Tocopherol | ..... | (221) |
| Phosphomolybdic acid, 5–20% in ethanol, 96% | Blue, white, or yellow-green background after heating to 110°C. | Neutral lipids. Antioxidants | <0.5 γ 1 γ | (7) (60) (171) |
| Aqueous sulfuric acid, 1%, 5%, or conc. sulfuric acid, or saturated chromic-sulfuric acid solution | Various colors during heating, eventually gray or black | Most nonvolatile organic compounds | <1 γ | (82) (112) (128) |
| Ninhydrin[a] 0.2% in n-butanol-aqueous acetic acid 10% (95:5) | Red purple, white background after heating to 105°C. | Aminophospholipids | 10 γ | (184) (219) (220) |
| Dragendorff reagent a) 17 g basic bismuth nitrate in 100 ml aqueous acetic acid (20%) b) 40 g potassium iodide in 100 ml water. Reagent: 20 ml. of a) with 5 ml. of b) and 70 ml. of water | Orange or red-orange | Choline-phosphatides | 10–20 γ | (184) (219) (220) |
| Diphenylamine 20 ml. 10% diphenylamine in ethanol, 96%, plus 100 ml. conc. HCl and 80 ml. acetic acid | Blue-gray, light gray background | Glycolipids | 4–10 γ | (37) (219) (220) |
| 2,4-Dinitrophenylhydrazine, 0.5% in 2 N-HCl | Yellow to orange | Aldehydes and ketones | 1–5 γ | (7) (157) (158) (183) |
| Potassium iodide-starch, 5 ml. 4% KOH with 20 ml. acetic acid, after 5 min.: 1% aqueous starch solution | Blue, white background | Peroxides | ..... | |
| Ammonium thiocyanate 0.2 g. in 15 ml. acetone and 0.4 g. ferrous sulfate in 10 ml. water | Red and red-brown, white background | Peroxides | ..... | (163) |
| 2,6-Dichlorquinone chloroimide 1% in ethanol | Various colors, white background | Antioxidants | 1 γ | (171) |
| 3-Diphenylcarbazone 0.1% in ethanol, 95% | Purple and lilac, light rose background | Mercuric acetate adducts of unsaturated lipids | 10–20 γ | (110) |

[a] Available as aerosol packages.

TLC of derivatives of short-chain alcohols, aldehydes and ketones, acids, amines and mercaptans.

Thin-layer chromatograms of radioactively labeled lipids are recorded by taking autoradiographs on x-ray film, as described below. Still, it is to be emphasized that chemical indicators should always be used also. Application of both photographic and chemical detection methods yields information regarding both radiochemical and chemical purity of a substance.

#### Documentations of Chromatograms

Because thin-layer chromatograms are relatively small, they can readily be copied by tracing them with a pencil directly into a laboratory journal.

Thin-layer chromatograms can be preserved if a stable indicator was used for visualizing the individual spots. This may be done by covering the chromatoplates with a glass plate of the same dimensions; the two plates protecting the adsorbent layer are sealed along the edges with tape. Barrollier (9) recommended dipping chromatoplates into a dilute solution of label glaze. The adsorbent layer is firmly held together by the label glaze and can be pulled off the glass as a film. The reviewer has found Fisher Label Glaze[23] suitable for Barrollier's procedure. This technique requires, of course, that an indicator be used which is insoluble in the solvent used for diluting the label glaze. Meinhard and Hall (120) demonstrated that the adsorbent layer can be stripped off on adhesive tape and pasted into a laboratory journal.

Black and white or color photography of chromatoplates is easily done in daylight or in U.V. light. Bürki and Bolliger (23) recommend Sylvania F8T5/BLB, Blacklite Blue tubes[24] and a yellow filter for taking black and white photographs in U.V. light. A photograph of a thin-layer chromatogram taken in daylight is shown in Figure 10.

Chromatoplates may be copied by exposing them to sheet film or photographic paper in an enlarger. Seher (e.g. 171) and Morris, Holman, and Fontell (e.g. 126) prepared photostats[25] of chromatoplates (Fig. 11). This technique has been applied also for quantitative analysis (74, 172, 173). It is simple and rapid, but it has the disadvantage that small or weakly stained bigger spots are easily lost. Hefendehl (55) recommended spraying the chromatoplates with a solution of paraffin in ether (1/1, w/v) to render them transparent. These plates yield better photostats than do untreated ones.

Chromatoplates containing radioactively labeled substances yield fine x-ray prints (107, 112). Autoradiographs are obtained by exposing the plates in the dark to No-Screen Medical X Ray Safety Film[26] for a few hours to one week, depending upon the type of radiation and the activities of the resolved compounds. The films are developed with Supermix Developer[26] for 4 to 6 min., and they are fixed for 30 min. with Acid Fixer.[26] Autoradiographs of commercial preparations of $C^{14}$-labeled lipids are presented in Figures 18 and 19.

#### Elution and Recovery of Lipids

One of the striking features of TLC is the high capacity of the plates. It is possible to separate by adsorption TLC up to 50 mg. of a lipid mixture on a single chromatoplate, 20 x 20 cm., coated with a thin layer of Silica Gel G of 250–275 $\mu$ thickness. About 5 mg. of lipid may be separated on such a plate by partition and reversed-phase partition TLC. Lipids, after having been isolated by TLC, may be scraped off the plates and extracted from the carrier material. The appropriate eluans for lipids may be determined by chromatographing these substances with different solvents on plates. Solvents that carry a compound with the solvent front are suitable as eluans for that substance (101). Care must be taken, however, that the eluans does not dissolve large amounts of the calcium sulfate, and thus aqueous solvents cannot be used.

A novel technique of recovering substances from a chromatoplate is to sublime the material from the adsorbent layer onto a cooled glass plate held 1 mm. above it (39).

TLC has been used to analyze commercial preparations of lipids labeled with $C^{14}$, $H^3$, or $I^{131}$, and for the purification and isolation of milligram amounts of compounds (112). Winterstein, Studer, and Rüegg (229) isolated unusual carotenoids by TLC, stating that no other method allowed the preparation of some of these materials (230).

With polar lipids, the yields of recovered material are usually very poor. This may be due to the presence of calcinated calcium sulfate in the solid adsorbent layer; and if so, better yields may be expected with loose layers of plain adsorbents. The use of sugar (75) as an adsorbent may be helpful. Even though it has a rather low capacity, its solubility in water may facilitate recovery of lipids. Thicker layers of adsorbents may be applied to preparative work. However, the thickness of the coating should not exceed 1 mm. to avoid distortion by uneven migration rates on the surface and inside the adsorbent layer.

The yields are improved by using the less polar derivatives of lipids containing free functional groups. Thus, alcohols and acids are more easily recovered as acetates and methyl esters, respectively (112). The nonpolar derivatives are especially important in TLC with isotopically labeled lipid derivatives *(vide infra)* where quantitative recovery is crucial.

Recovering various substances from a chromatostrip (194) or from a chromatoplate (17) is also possible by letting the developing solvent run over.

Esters of unusual fatty acids are often found by GLC in mixtures of methyl esters derived from natural lipids. Curves from such analyses can be correctly interpreted only if the sample subjected to GLC was free of compounds that are not methyl esters of fatty acids. The absence of such contaminants is not routinely checked. It appears to be necessary to analyze each sample of methylation products by TLC prior to GLC for substances other than methyl esters. Pure methyl esters may be isolated by TLC (211).

#### Quantitative Thin-Layer Chromatography

The methods that have been suggested for the quantitative evaluation of thin-layer chromatograms are summarized in Table V.

Few of the above methods are exact and the better ones are particularly impractical. Four of these methods involve elution of the various lipids from the plate. A suitable eluans for a given lipid is found by

[23] Fisher Scientific Company, 633 Greenwich Street, New York 14, New York.
[24] Sylvania Electric Products Inc., 500 Fifth Avenue, New York, New York.
[25] Copease Corporation, 425 Park Avenue, New York, New York.
[26] General Electric X Ray Department, Milwaukee 1, Wisconsin.

TABLE V

Methods of Quantitative Thin-Layer Chromatography

| Method | Compounds analyzed | Range of sample size (per spot) | Error % | Ref. |
|---|---|---|---|---|
| Eluting, weighing | Neutral lipids from feces and fecaliths | 20–50 mg. (per plate) | semi-quant. | (225) |
| Eluting and U.V. spectrophotometry | Esters of p-hydroxybenzoic acid | 20–70 γ | 2–4 | (47) |
| Eluting, colorimetry of hydroxamic acid-Fe complexes | Methyl esters of fatty acids and of oxygenated acids, mono-, di-, and triglycerides | 0.1–1 mg. | ± 5 | (216) |
| Eluting with aqueous sulfuric acid, colorimetry after heating | Bile acids | 10–60 γ | ± 3 γ/ml. | (44) |
| Eluting and radiometry | Labeled derivatives of acids, hydroxy and amino compounds | 0.1–100 γ | ± 5 | (107, 113) |
| Charring followed by photodensitometry | Mono-, di-, and triglycerides | 2–30 γ | ± 5 | (150) |
| | Glycerides and "aldehydic cores" derived from unsaturated glycerides | 2–30 γ | ± 5 | (149) |
| | Lecithins and "aldehydic cores" derived from unsaturated lecithins | 2–30 γ | ± 5 | (152) |
| Measuring of spot area | Tocopherols | 30–100 γ | ± 5 | (172,173) |
| | Industrial lipids | 30–130 γ | ± 5 | (74) |
| Mineralization, colorimetry of phosphate | Phospholipids | 0.2–25 γ | ± 3 | (52) |

chromatographing it in various polar solvents as described in the preceding paragraphs.

Semiquantitative analysis by weighing eluted material is possible only with nonpolar lipids that can easily be recovered from the adsorbents. The same limitations apply to the hydroxamic acid method; however, the colorimetric determination is much more exact than weighing of small amounts. In view of its general applicability to a wide range of compounds having various functional groups (28) the method will, in the reviewer's opinion, become a preferred procedure for the quantitative evaluation of thin-layer chromatograms. So far, the hydroxamic acid method has been applied only to the quantitative analysis of esters of common and oxygenated fatty acids derived from seed oils. Extension of the method to the analysis of mono-, di-, and triglycerides has been suggested (216). The method described for the elution and quantitative colorimetry of bile acids with sulfuric acid may be of general applicability. The isotopic derivative technique is, in the author's experience, very precise, one reason being that the lipids are separated as derivatives which are more easily quantitatively recovered than are the more polar original compounds.

Direct quantitative determination of lipids on the plates would be desirable, especially if a method could be found that would not involve destruction of the compounds measured. Most methods suggested so far require the use of destructive reagents for visualizing the lipids on the plate, or derivatization before chromatography. Photodensitometry after charring of the lipid materials as applied by Privett and Blank (149), Privett, Blank, and Lundberg (150) gives good results in a range of 5–35 γ of lipid This method has been most extensively applied by the last-mentioned authors, and has yielded valuable information. The spots are measured in a Photovolt photodensitometer Model 520A, 52-C,[27] with a stage attached for semi-automatic plotting of curves. The areas under the densitometer curves are directly proportional to the amount of sample in a range of about 5–30 μg. of carbon (Fig. 14). This applies to saturated compounds. Different classes of saturated lipids give different curves. Also, unsaturated lipids are more deeply stained than are the saturated members of the same class. Therefore, individual standard curves must be prepared for all compounds that do not give spots of the same densitometrically determined response (Fig. 14) (149). Mixtures of known composition should be run alongside the sample to be analyzed. The densitometer mentioned is easily adapted to fit square plates. Complex mixtures of natural lipids containing both saturated and unsaturated fatty acid moieties can be measured only if the whole mixture is measured after catalytic hydrogenation. Unfortunately, hydrogenation often involves not only the desired saturation of the double bonds, but changes the functional groups, and thus also the pattern of the mixture to be analyzed.

The method of measuring spot areas on photostats of chromatoplates supplements the photodensitometric method, as it can be applied to amounts of 30–130 γ of lipid per spot. In the author's laboratory this method has been found useful for the analysis of synthetic lipid mixtures with an error of ± 5% (174).

### Preparation of Radioactively Labeled Lipid Derivatives

The isotopic derivative technique, i.e., the method for detecting and analyzing components of mixtures after reaction of their functional groups with a radioactively labeled reagent, has so far found little application in the lipid field, due to lack of efficient fractionation procedures. TLC allows the rapid separation of complex lipid mixtures and thus constitutes an ideal auxiliary tool for the analysis of lipids by the isotopic derivative technique.

Fatty acids may be labeled by esterifying them with radioactive diazomethane, whereas lipids containing hydroxy or amino groups are labeled by acetylation with radioactive acetic anhydride. p-Tolylsulfonyl methyl-$C^{14}$-nitrosamide (198), a precursor for the preparation of "hot" diazomethane, and acetic anhydride-1-$C^{14}$ and acetic anhydride-$H^3$ are all commercially available. The latter two reagents are available in license-exempt packages.

Radioactive diazomethane, $C^{14}H_2N_2$, is prepared by reacting a solution of 10 mg. (0.05 millimoles) of p-tolylsulfonyl-methyl-$C^{14}$-nitrosamide, specific activity of 0.6 millicuries per millimole[28] in 1 ml. diethyl ether with 2 ml. of an ice-cold solution of 0.1 g. of NaOH in ethanol-water, 10/1, v/v, in a micro gas generator. Diazomethane and ether are distilled in a stream of nitrogen by heating the reaction vessel to 60–70°C. The diazomethane is collected in two test tubes in series, each containing 1–2 ml. of ether which is maintained at 0–5°C. The diazomethane solutions are combined at the end of the distillation and aliquots are added to solutions of 2–20 mg. fatty acids (0.01–0.1 millimoles) in diethyl ether-methanol 9/1, v/v (166, 198). Excess diazomethane is avoided in order to conserve this expensive reagent and also to prevent the formation of polymethylenes that may interfere

[27] Photovolt Corporation, 95 Madison Avenue, New York 16, New York.

[28] New England Nuclear Corporation, 575 Albany Street, Boston 18, Massachusetts.

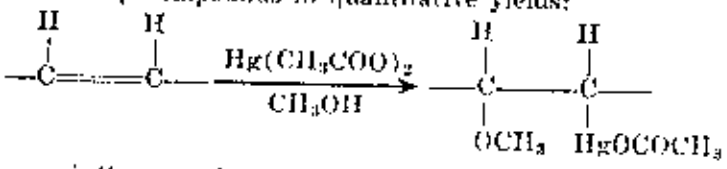
in subsequent analyses. The diazomethane can also be distilled directly into a solution of the fatty acids be esterified in diethyl ether–methanol, 9/1, v/v 5).

Lipids (10–20 mg.) containing hydroxy or amino groups may be labeled by reaction with a 1/10 solution of acetic-1-$C^{14}$ anhydride, specific activity of 0.6 millicuries per millimole[29] in pyridine. The reaction is conducted in a 5 x 150 mm. sealed tube at 100°C. for 30–60 min., using about 20% excess of reagent. After cooling, the tubes are opened, the reaction mixture is diluted with 10 ml. *N*-sulfuric acid, the acetylated lipids are extracted with diethyl ether, washed with water, and dried over sodium sulfate. Monoglycerides and diglycerides undergo acetolysis to a small extent; and therefore, if quantitation of lipids containing ester-bonds is desired, this method must be applied with caution (107).

Reaction with radioactive ketene (2) may yield cleaner acetylation products.

#### Preparation of Mercuric Acetate Addition Compounds

Unsaturated lipids react with mercuric acetate in methanol, at room temperature, to give acetoxymercuri-methoxy compounds in quantitative yields:




*cis*-Compounds react ten times faster than their *s* isomers (70). The original lipid may be regenerated by cleaving the addition compound with hydrochloric acid.

Chromatography of acetoxymercuri-methoxy compounds of unsaturated lipids on silicic acid effects their fractionation according to degree of unsaturation.

The acetoxymercuri-methoxy compounds of lipids are best obtained by the method published by Jantzen and Andreas (70) for the preparation of adducts of methyl esters of unsaturated fatty acids. This procedure is described here:

The reagent is a solution of 14 g. mercuric acetate in 250 ml. methanol, 2.5 ml. water, and 1 ml. glacial acetic acid. About 25 to 50 ml. of this solution is added to 1 g. of esters and allowed to react in a stoppered flask in the dark at room temperature. After 24 hr., the methanol is evaporated at <30°C. *in vacuo* or by a stream of nitrogen, and the dry residue is taken up with 50 ml. chloroform. This chloroform solution is washed with five 25-ml. portions of water to remove excess mercuric acetate, and then dried with sodium sulfate. Saturated lipids do not react with mercuric acetate, unless they contain a reactive functional group.

The unsaturated lipids are recovered after chromatography by shaking the silicic acid containing the adducts in a test tube, with 10 ml. methanol and 0.5 ml. conc. HCl. After most of the adsorbent has settled, the supernatant is decanted and filtered. The residual silicic acid is treated once more with methanol-hydrochloric acid. The combined filtrates of the two extractions are diluted with 25 ml. water and extracted consecutively, once with 25 ml., and four times with 10 ml. of diethyl ether. The combined ether extracts are washed with water, dried over sodium sulfate, and evaporated.

[29] Radioactive Acetic Anhydride, either labeled with $C^{14}$ or $H^3$, is available from several companies. The author has used preparations of the Pitker X-Ray Corporation, 27 South Broadway, White Plains, New York.

### Applications

In the lipid field, the following types of separations have been performed by TLC:

*Fractionations according to classes of compounds (Fig. 6).* Long-chain hydrocarbons, alcohols, aldehydes, acids, monoglycerides, diglycerides, triglycerides, and other lipids are separated as classes according to the type and number of functional groups, irrespective of chain lengths and degree of unsaturation, by adsorption TLC on silicic acid. Subfractionations within classes are perceptible but generally do not interfere with class separations.

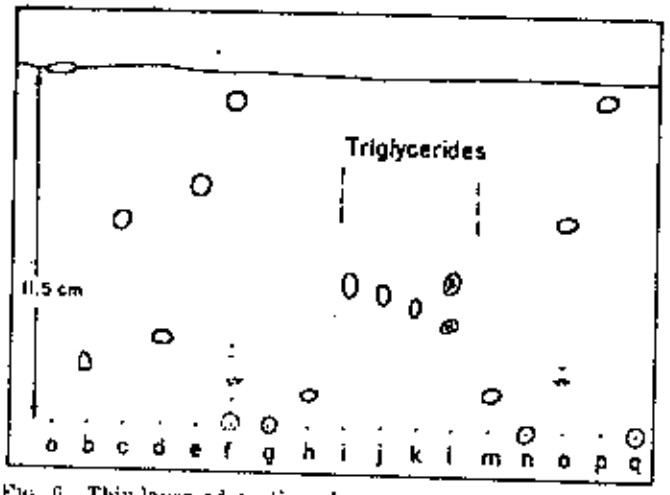



FIG. 6. Thin-layer adsorption chromatography of lipid classes on Silica Gel (101). Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. Indicator: Dichlorofluorescein. Amounts: 20 γ each. a) octadecene-9, b) oleylalcohol, c) oleylaldehyde, d) oleic acid, e) methyl oleate, f) cholesteryl oleate, g) monoolein, h) diolein, i) triolein, j) trilinolein, k) trilinolenin, l) tricaproin (α) and tristearin (β), m) cholesterol, n) selachyl alcohol, o) selachyl diolein, p) oleyl oleate, q) dioleoyl lecithin.

*Fractionations within homologous series (Fig. 7 and 8).* a) Compounds of low chain lengths ($C_1$–$C_6$) are separated within classes by TLC of their derivatives. Alcohols, for example, are separated as 3,5-dinitrobenzoates on silicic acid. With the same adsorbent, aldehydes and also ketones are fractionated as 2,4-dinitrophenyl hydrazones, and acids are separated as anilides.

b) Long-chain compounds ($C_{12}$–$C_{20}$ lipids) are fractionated within classes by reversed-phase partition TLC on, e.g., siliconized silicic acid. Higher fatty acids, for example, are separated according to chain length and unsaturation.

Overlapping critical partners, such as palmitic and oleic acids, are well separated by chromatography at low temperatures, by chromatography with oxidizing solvents, or by the following technique:

*Fractionation according to degree of unsaturation (Fig. 9).* Saturated and various unsaturated members of a homologous-vinylogous series are separated according to their degree of unsaturation by TLC of the mercuric acetate addition compounds formed with unsaturated lipids. As an example, mixtures of methyl esters of naturally occurring fatty acids are rapidly and quantitatively separated by TLC, in the

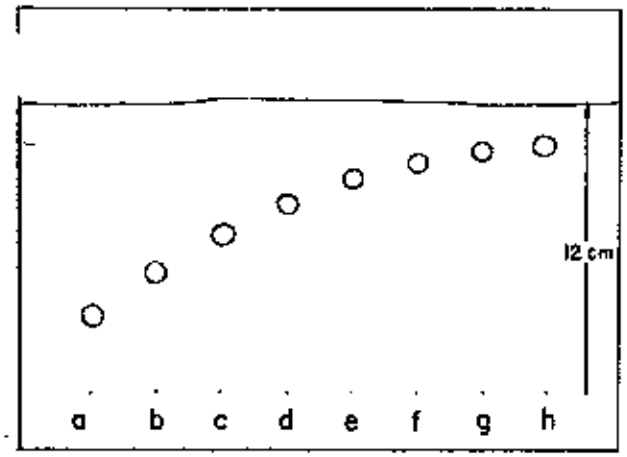



FIG. 7. Thin-layer adsorption chromatography of derivatives of short-chain compounds of a homologous series. Solvent: Petroleum hydrocarbon-diethyl ether, 70/30, v/v. Development time: 40 min. Indicator: 1% of "Fluorescent Chemical No. 604" in the silica gel layer. Observed in U.V. light. Amounts: 5 γ, each. 2,4-dinitrophenyl derivatives of a) methyl amine, b) ethyl amine, c) propyl amine, d) butyl amine, e) amyl amine, f) hexyl amine, g) heptyl amine, h) octyl amine.

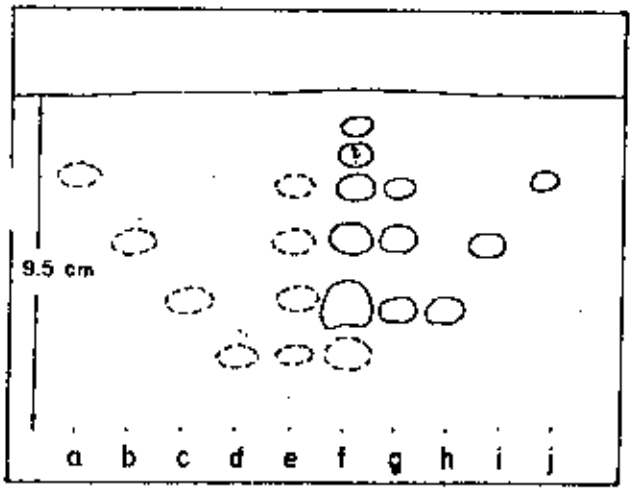



FIG. 8. Reversed-phase partition chromatography on a siliconized chromatoplate (101). Solvent: Acetonitrile acetic acid-water, 70/10/25, v/v/v. Development time: 40 min. Indicators: Iodine (solid lines), followed by α-cyclodextrin-iodine (dotted lines). Amounts: 20 γ of individual compounds. a) methyl laurate, b) methyl myristate, c) methyl palmitate, d) methyl stearate, e) mixture of saturated methyl esters, f) $C_{18}$-Fraction derived from menhaden oil, g) mixture of unsaturated $C_{18}$ methyl esters, h) methyl oleate, i) methyl linoleate, j) methyl linolenate.

form of their acetoxymercuri-methoxy derivatives, into saturated esters, monoenoates, dienoates, and trienoates. The original esters can be recovered after separation of their addition compounds.

*Fractionation of positional isomers and fractionation of* cis-trans *isomers is also possible in certain cases (128, 117) (Fig. 11).*

The fractions obtained by one of these methods are amenable to further resolution by either or several of the other techniques, or by complementary methods. Combinations of adsorption-TLC with reversed-phase PC and GLC have proven to be especially useful. Lipids which cannot be resolved by adsorption chromatography (TLC) may well be separated by partition chromatography (PC, GLC).

**Fats, Oils and Waxes**

Procedures for the fractionation of neutral lipids—naturally occurring crude vegetable fats, marine oils, and insect waxes, and their hydrolysis products, such as fatty acids, and also alcohols, glyceryl ethers and other nonsaponifiables have been described. Adsorption-TLC effects sharp separations of chemically very similar classes of lipids, such as long-chain alkyl esters, steryl esters, and polyenol esters of fatty acids; even triglycerides and glyceryl ether diesters may be resolved.

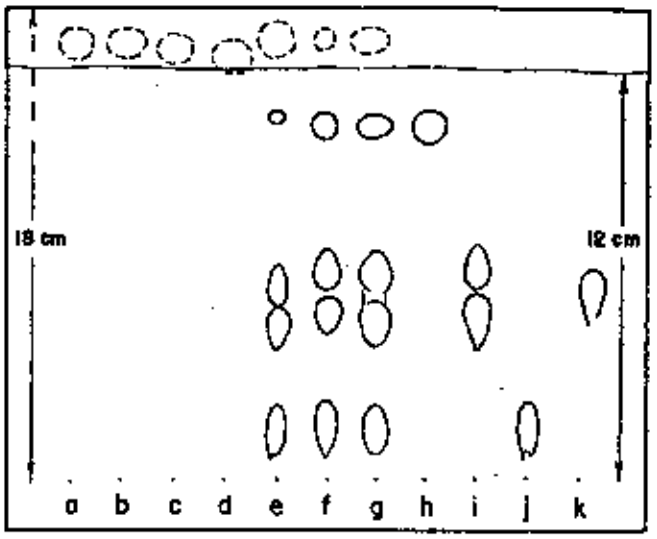



FIG. 9. Thin-layer chromatography of mercuric acetate adducts on Silica Gel (See 110). First solvent: Petroleum hydrocarbon-diethyl ether, 4/1, v/v. Second solvent: n-Propanol-acetic acid, 100/1, v/v. Development times: First Solvent, 1.5–2 hours, Second Solvent, 3–4 hours. Indicators: s-diphenyl carbazone (solid lines), followed by iodine (dotted lines). Amounts: 20 γ of esters, 50–100 γ of mercuric acetate adducts. a) methyl stearate, b) methyl oleate, c) methyl linoleate, d) methyl linolenate; e–j) Mercuric acetate adducts of e) $C_{18}$ fraction from *Chlorella*, f) $C_{18}$ fraction from *Chlorella*, g) total methyl esters from *Chlorella Pyrenoidosa*, h) methyl oleate, i) methyl linoleate, j) methyl linolenate, k) mercuric acetate.

Among the vegetable lipids analyzed are jojoba oil, castor oil, olive oil, oiticica oil (108, 112), and wheat (140). The following animal lipids have been investigated: lanolin, liver oils of soupfin shark, basking shark, and ratfish, also fur seal blubber oil (108) and dogfish liver oil (102) (Fig. 10).

The last-mentioned publication refers to work of Malins on the analysis of the constituent fatty acids of each of the triglyceride and the glyceryl ether diester fractions of dogfish liver oil. This commendable work would not have been possible by any other means than TLC and GLC.

Weicker (222), Hulmstock and Weicker (65), and van Dam (32) applied TLC to the analysis of human serum lipids. Williams, Sharma, Morris, and Holman (225) analyzed human feces and fecaliths by TLC and GLC. The same two techniques have also been applied, consecutively, to the analysis of human tissue lipid extracts, by Mangold and Tuna (109). The total lipids of human serum and of the following tissues were analyzed: bone marrow, liver, kidney, perinephric fat, spleen, and atheromatous plaques of the aorta. TLC was used to obtain lipid classes, the fatty acid

patterns of which were then determined by GLC of the methyl esters. Lipid classes not previously found in man were detected in several human tissues. Some of these lipids, such as methyl esters of fatty acids, are most likely artefacts produced during extraction. Others, like glyceryl ether diesters, must be regarded as genuine constituents of human tissues (211). Stein and Stein (195) used TLC in studying the selective *in vivo* labeling of epididymal fat of the rat with radioactively labeled fatty acids. Dhopeshwarkar and Mead (38), and Fulco and Mead (46) used TLC extensively in nutritional and metabolic investigations.

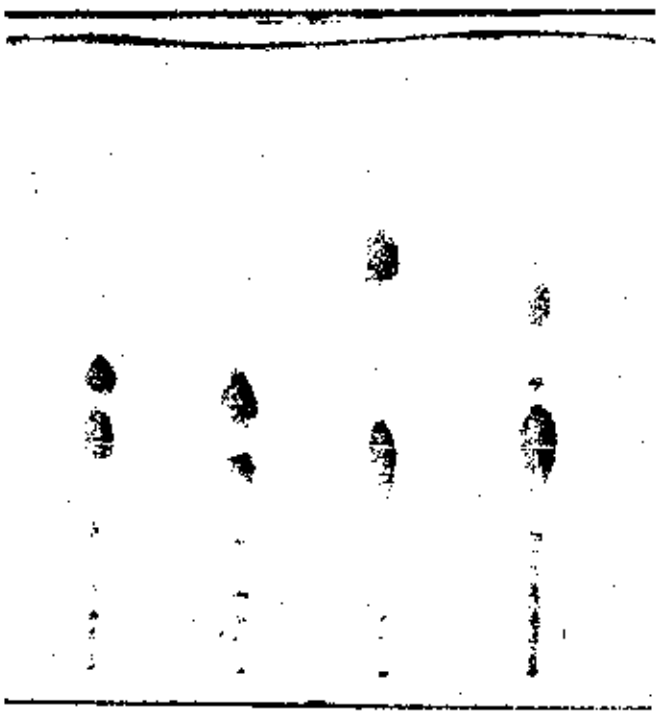


FIG. 10. Fractionation of shark liver oils on silica gel. Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Indicator: Iodine, reproduced by photographing. Amounts: 300 γ, each. Liver oils of a) Dogfish, b) Ratfish, c) Basking shark, d) Soupfin shark. The chromatoplate was developed in saturated atmosphere.

Naturally occurring oxygenated fatty acid were studied by Morris, Holman, and Fontell (126, 128), and by Morris, Hayes, and Holman (129). These workers found two isomeric acids, namely 9-hydroxy-*trans*-10-*cis*-12- and 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic acids, and also at least three distinct epoxy acids in various seed oils (Fig. 11). Vioque, Morris, and Holman (215) found *trans*-9,10-epoxystearic acid in orujo oil. Chalvardjian, Morris, and Holman (27) applied TLC in nutritional studies with epoxy acids. TLC of oxygenated fatty acids was recently also reported by Kaufmann and Makus (79) and by Applewhite, Diamond, and Goldblatt (4).

A method, based on TLC, for the quantitative determination of mono-, di-, and triglycerides in mixtures has been reported by Privett, Blank, and Lundberg (150) (Fig. 12). These workers could quantitatively estimate as little as 0.1% of tripalmitin in monopalmitin.

Procedures for the quantitative analysis of α- and β-monoglycerides after splitting of the former with periodate, and for the analysis of 1,2- and 1,3-diglycerides were also presented. These new methods yielded exact results with synthetic mixtures and were then further applied to the analysis of monoglyceride preparations from safflower oil, menhaden oil, and lard. Periodate titration and molecular distillation served for checking the accuracy of these analyses.

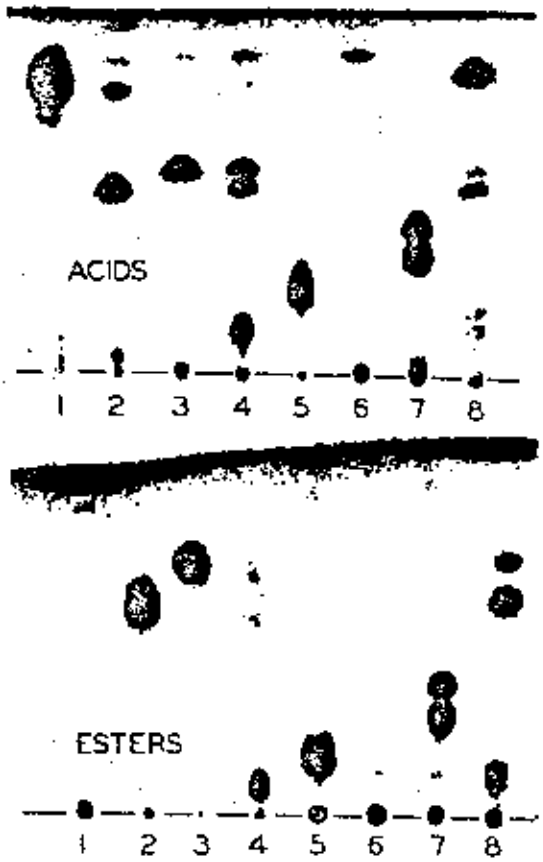



FIG. 11. Thin-layer chromatography of oxygenated fatty acids and their methyl esters (128). Solvent: Petroleum hydrocarbon-diethyl ether, 9/1, v/v (for esters). Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v (for acids). Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v. Reproduced by photocopying. Amounts: About 50 γ, each. 1) palmitoleic and oleic, 2) *cis*-9:10 epoxystearic, 3) *cis*-12:13-epoxyoleic, 4) *cis cis*-9:10, 12:13-diepoxystearic plus two monoepoxy impurities, 5) 12-hydroxyoleic, 6) *threo*-12,13-dihydroxyoleic, 7, *threo*-12,13-chlorohydroxyoleic and *threo*-13,12-chlorohydroxyoleic, 8) *Artemisia absinthium* mixed acids and mixed esters.

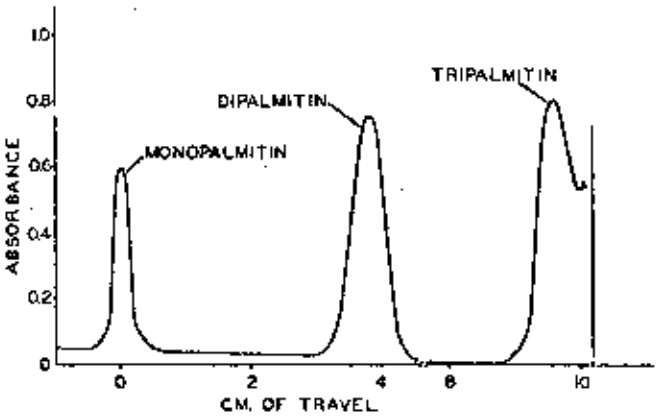
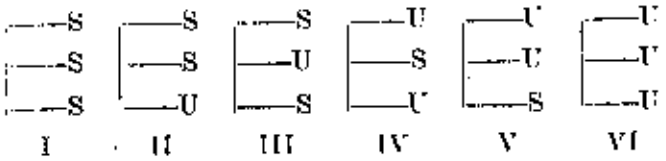


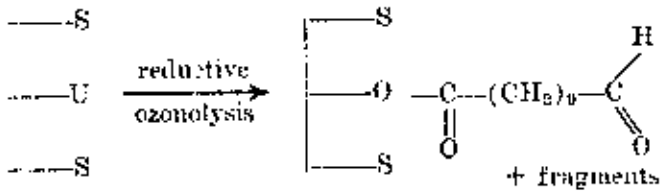

FIG. 12. Densitometric analysis of a mixture of mono-, di-, and tripalmitin (150). Solvent: Petroleum hydrocarbon-diethyl ether, 7/3, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v.

Privett and Blank (149) have published an elegant method for the determination of mono-, di-, and triglycerides varying in fatty acid constituents in regard to unsaturation. It is based upon reductive ozonolysis of these types of compounds, followed by separation and quantification of the glyceryl residues by TLC. This ozonization-reduction-thin-layer chromatographic method (149) of analysis permits analyzing the four possible types of monoglycerides, six of the seven diglyceride types, and four of the six triglyceride types. Since this method has such wide potential applications, it is explained here in more detail, with the example of the structural analysis of triglycerides.

There are six types of triglycerides, *viz.*,




S and U in these models denote saturated (S) and unsaturated (U) fatty acid moieties. Reductive ozonolysis will leave triglycerides of type I untouched, whereas all other triglyceride types will yield glyceryl residues—aldehydic cores—of the kind demonstrated with a triglyceride of type III:




The saturated fatty acid constituents of a type III triglyceride are not affected by ozonolysis, whereas the long chain of the unsaturated acid is ruptured to yield a structure as depicted above, i.e., the unsaturated-disaturated triglyceride has become a compound of a polarity which is widely different from that of the unchanged fully saturated triglyceride (type I). Consequently, the aldehydic cores resulting from triglycerides of type III and from the other triglycerides containing one or more unsaturated fatty acid moieties, are easily resolved, as classes, by TLC on Silica Gel G. An example of such separations is presented in Figure 13. Saturated and unsaturated triglycerides give very different standard curves, but the aldehydic cores resulting from unsaturated triglycerides by reductive ozonolysis yield identical curves as saturated triglycerides, if correction is made for the different percentages of fissioned carbon lost in the various types of compounds. The quantitative evaluation of chromatograms is demonstrated in Figure 14.

Privett (151) utilized TLC in studies on the autoxidation of unsaturated fatty acids and esters. Figure 15 serves as an example to illustrate the kind of separations possible.

TLC has been used to check the course of synthesizing procedures and the purities of final products (49, 50, 94, 100, 217). In checking by means of adsorption-TLC it is not necessary to start a sequence of reactions with an individual compound, say oleic acid, as is necessary with reversed-phase paper partition chromatography (106). The use of adsorption-TLC permits working with a lipid class, e.g., a mixture of fatty acids derived from fish oils (49, 50).

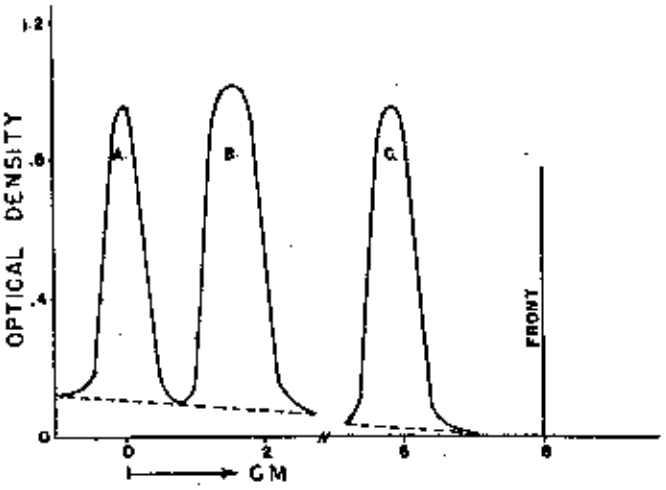



FIG. 13. Densitometric analysis of the aldehyde cores derived from A. Triolein, B. Palmitodiolein, C. Oleodipalmitin (149). Solvent: Petroleum hydrocarbon-diethyl ether, 65/35, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v.

Methods for analyzing polar lipids of industrial importance, such as amines, amides, and nitrils—also alkyl sulfates and sulfonates, alkyl phosphates and phosphonates—have recently been worked out (111).

Several investigators have applied TLC to check the efficiency of other separation techniques (59, 129, 211). Morris, Holman, and Fontell (127) have found, by the use of TLC, that the methyl esters of some long-chain fatty acids are altered during GLC. Conjugated trienoates undergo *cis-trans* isomerization. The esters of vicinally unsaturated hydroxy acids are dehydrated and unsaturated hydroperoxides are similarly altered to more highly unsaturated derivatives. Mangold and Kammereck (110) fractionated mixtures of the methyl esters of saturated fatty acids and the acetoxymercuri-methoxy compound of unsaturated ones by TLC into groups, according to the degree of unsaturation in the original esters (Fig. 9). Each group of esters was recovered and after cleavage of the addition compounds, it was resolved into its components by GLC, according to chain length. Pairs of esters which are not resolved by GLC are well separated by TLC of their adducts and *vice versa*. The successive application of TLC and GLC results in more reliable identification of the various compounds

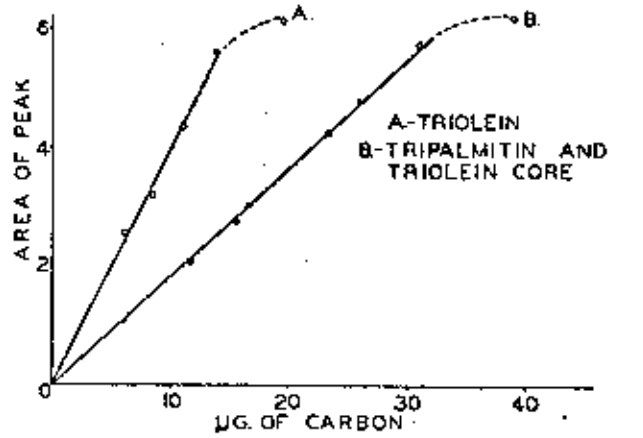



FIG. 14. Standard curves, thin-layer chromatographic analysis of triolein, tripalmitin, and the aldehydic core derived from triolein (corrected for fissioned carbon) (149).

recorded in GLC. In addition, this combination of methods yields more trustworthy quantitative analyses than does GLC alone.

### Phospholipids, Sulfolipids, and Glycolipids

Application of TLC in this domain have been especially fruitful (30, 52, 64, 71, 72, 87, 89, 111, 164, 219, 220, 223). The solvents applied are usually the same as those used for resolving phospholipids by chromatography on silicic acid-impregnated paper (113) or on columns of silicic acid (95, 113). The capacity of the chromatoplates is much less with such polar developing solvents than with mixtures of petroleum hydrocarbon and diethyl ether.

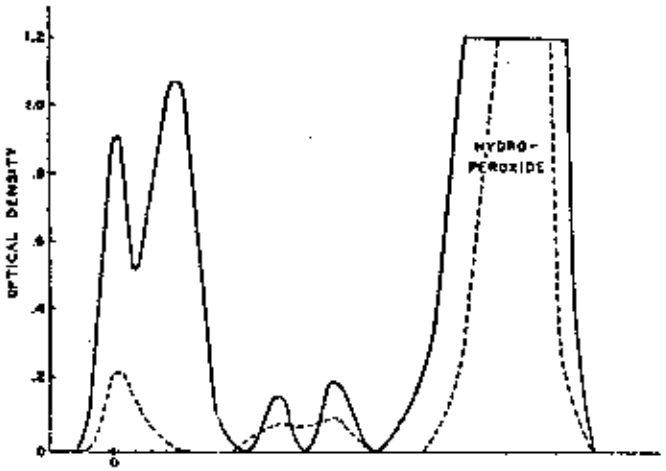



FIG. 15. Densitometric analysis of a chromatogram of autoxidized methyl linolenate (151). Solvent: Petroleum hydrocarbon-diethyl ether, 4/1, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid 1/1, v/v. Unchanged methyl linoleate migrates to the solvent front.

Figure 16 shows a chromatogram of various phospholipids, as presented by Wagner (219) and Wagner, Hörhammer, and Wolff (220).

Privett and Blank (152) have extended their ozonization-reduction-thin-layer chromatographic method by applying it to lecithins. The four types of lecithins can be distinguished and quantitatively determined. In this case the aldehydic cores derived by reductive ozonolysis are separated by reversed-phase partition TLC on siliconized Silica Gel G plates (101).

Figure 17 shows photodensitometer curves obtained from chromatograms of aldehydic cores derived from various lecithin preparations as obtained by Privett and Blank (152).

TLC has become an important tool in research concerned with the structure of gangliosides (87, 89, 219, 220). With this method Kuhn, Wiegandt, and Egge (89) isolated four crystalline gangliosides from beef brain, each containing one mole of galactosamine and three moles of glucose plus galactose.

### Steroids and Bile Acids, Triterpenoic Acids

Linford (97) reported that sterols are chemically altered when they are chromatographed on columns of dry silicic acid, and he recommended that sterols should be separated by partition chromatography on silicic acid containing between 35 and 40% of water. Other workers (e.g., 15, 204) had previously observed that sterols are altered when chromatographed on alumina. Reference is made to a comprehensive discussion of the problem by Neher (138).

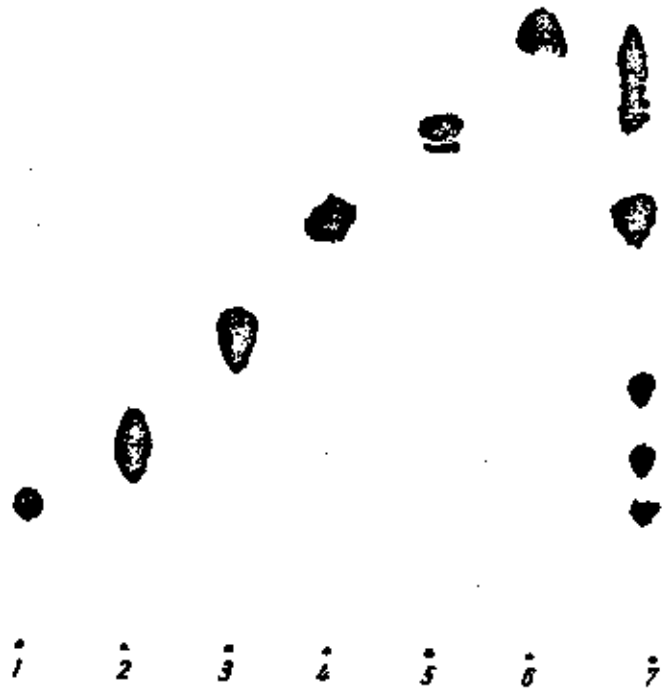



FIG. 16. Separation of phospholipids on Silica Gel. (219, 220). Solvent: chloroform-methanol-water, 65/25/4, v/v/v. Development time: 2 hours. Indicators: Rhodamin B and Dragendorff reagent. Amounts: 50–100 γ, each. 1) lysolecithin, 2) sphingomyelin, 3) lecithin, 4) cephalin, 5) cerebroside, 6) cardiolipid, 7) mixture.

Several recent publications described the use of TLC for resolving steroids by adsorption or partition chromatography on silicic acid; alumina was used as an adsorbent also (1, 7, 25, 31, 32, 69, 100, 139, 201, 202, 221).

Especially remarkable is the resolution of fifteen different cholesteryl esters by two-dimensional reversed-phase partition TLC on paraffin-impregnated Silica Gel G plates, as reported by Kaufmann, Makus, and Deicke (80).

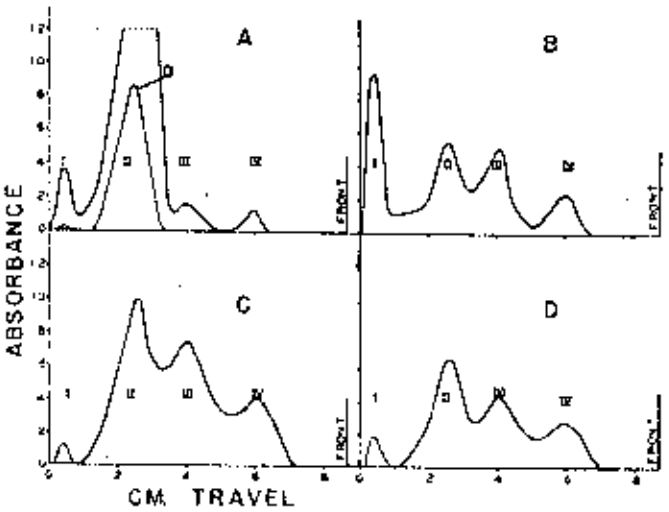



FIG. 17. Densitometric analysis of the aldehydic cores derived from A) egg lecithins, B) beef spinal cord lecithins, C) soybean lecithins, D) wheat lecithins (152). I. Disaturated lecithins, II. aldehydic cores derived from a saturated-α-unsaturated lecithins, III. aldehydic cores derived from α-unsaturated-β-saturated lecithins, IV. aldehydic cores derived from diunsaturated lecithins. Solvent: acetic acid-water, 85/15, v/v on siliconized Silica Gel G.

Waldi and Munter (221) developed an early pregnancy test which is based upon a semiquantitative determination of pregnandiol in the urine.

Gänshirt, Koss, and Morianz (48) worked out a simple and rapid method for the quantitative determination of bile acids by adsorption TLC. The bile acids were eluted from the plates with aqueous sulfuric acid (65%) and they were quantitatively determined by colorimetry of the red solutions that formed upon heating. The above authors encountered a standard error of only ± 3% in analyses of mixtures of cholic, deoxycholic, and glycodeoxycholic acids, and of glycocholic, taurocholic, and taurodeoxycholic acids (48). TLC of bile acids was also reported by Hofmann (60).

TLC has been extensively applied in work with triterpenoic acids by Tschesche and his co-workers (207, 208, 209, 210). Vioque and Morris (214) reported the isolation of two triterpenoic acids from orujo oil. Stahl (186) demonstrated the use of TLC for the fractionation of tars and naturally occurring balsams and rosins.

#### Fat-Soluble Vitamins

Winterstein and Hegedüs (227, 228) and Winterstein, Studer, and Rüegg (229) have applied both adsorption and reversed-phase partition TLC to the analysis of vitamin A and the corresponding aldehyde, viz., retinene and other polyenals $C_{20}$–$C_{10}$. Winterstein and Hegedüs (227, 228) prepared deeply colored rhodanine derivatives of these aldehydes on the plates, and with this very sensitive indicator technique they were able to detect as little as 0.03 γ of an aldehyde on a plate. The ubiquitary occurrence of retinene in a plant kingdom and its presence in liver was demonstrated. A thorough review on this work has already appeared (230).

Mangold and Malins (108) demonstrated vitamin A esters in various fish oils and free vitamin A in the nonsaponifiable fractions therefrom. Both vitamin A and its ester can readily be recognized by the blue color they yield by heating with sulfuric acid.

Janecke and Maas-Goebels (69) investigated the behavior of vitamins $D_2$ and $D_3$ under the conditions usually applied for their assay and they concluded that the D-vitamins are less stable than had generally been believed.

Methods for the quantitative analysis of the E-vitamins were published by Seher (171, 172, 173). The β and γ tocopherols could not be resolved by TLC, but both were well separated from α and from γ tocopherols.

Stoffel and Martius (196) and Billeter and Martius (13) have used TLC in studying the metabolism of K-vitamins. Martius (114) has summarized the results of these investigations in a recent review.

#### Food Coloring Agents

TLC was applied for analyzing vitamin A, carotenes, and other food coloring agents by Lagoni and Wortmann (91, 92) and also by Mottier (131, 132, 134). Isler, Rüegg, and Schudel (66) have presented a most interesting survey on the use of synthetic carotenoids as food-coloring agents, and in this review they also discussed in detail applications of TLC and other methods in analyzing such substances.

Stanley and Vannier (192) used chromatostrips for detecting biphenyl.

#### Antioxidants, Prooxidants

Methods for the analysis of synthetic and natural antioxidants by TLC have been described by Seher (171, 172, 173). Complex mixtures of antioxidants in commercial preparations and in oils have been analyzed by two-dimensional TLC (17), mixtures of tocopherols have been quantitatively determined (172, 173). Naturally occurring quinones, which are of significance in this connection, may also be detected by TLC (6). Procedures for quantitative determination of p-hydroxy benzoates, widely used preservatives, have been described by Gänshirt and Morianz (47).

Organic peroxides have been separated by Maruyama, Onoe, and Goto (113) and by Stahl (183). Reference is made again to the work of Privett (151) on the use of TLC for the analysis of autoxidation products (Fig. 15), and to the publications by Seiler and Seiler (174, 175), Seiler and Rothweiler (176) and Seiler and Kaffenberger (177) on the separation by TLC of inorganic cations and anions, many of which are prooxidants.

#### Minor Constituents of Fats

Because of its sensitivity, TLC is ideal for detecting minor lipid constituents, such as naturally occurring steroids, diterpenoids, triterpenoids, including saponins, aromatic rosins, and fat-soluble vitamins, or additives such as food coloring agents, antioxidants, and insecticides (8, 187).

Methods for analyzing non-lipid material in fats are scarce. The author believes that TLC can be effective in identifying carbohydrates (191, 235), amino alcohols and amino acids (16, 133), as well as short-chain alcohols and aldehydes (37, 143), acids, amines (209), mercaptans and other odoriferous compounds, plasticizers (144), and trace amounts of metals (174, 175, 176).

#### Thin-Layer Chromatography in Lipid Radio-Chemistry

Methods for analysis of trace amounts of lipids by the use of the isotopic derivative technique in conjunction with TLC and PC have been described by the author (107). The lipid substances were labeled by reacting their functional groups with $C^{14}$-labeled diazomethane—$H^3$-labeled diazomethane cannot be used (10, 96)—or with $C^{14}$- or $H^3$-labeled acetic anhydride. "Hot" methyl esters, acetylated long-chain alcohols, monoglycerides, diglycerides, glyceryl ethers, amines, and hydroxy ethyl amides of higher fatty acids were fractionated from each other as classes by adsorption-TLC. Their ratios were determined by radiometry. Consecutively, each of the classes of labeled lipid derivatives was further resolved by reversed-phase paper partition chromatography. The ratios of the components of each class were then determined by scanning their respective activities with a radio chromatograph or by evaluating autoradiograms in a photodensitometer. Mangold, Kammereck, and Malins (112) have recently presented an analysis of the fatty acids of castor oil, using these methods.

The same authors have analyzed commercial preparations of radioactively labeled lipids and found that most of them are very impure (112, see also, e.g., 93). Because their indiscriminate use must lead to erroneous results, TLC should be used for purifying such "hot" lipids prior to their application in chemical and metabolic studies (112). Figure 18 shows an

autoradiograph of various commercial fatty acids labeled in the carboxyl group with $C^{14}$, and Figure 19 represents an autoradiograph of a chromatogram of cholesterol-4-$C^{14}$. The radioactive sterol preparation was applied in a row across the plate to permit recovering the pure cholesterol. The isolation of $C^{14}$-labeled phytol from the nonsaponifiable fraction of the alga *Chlorella pyrenoidosa*, grown in an atmosphere of radioactive carbon dioxide (104), was also reported (112).

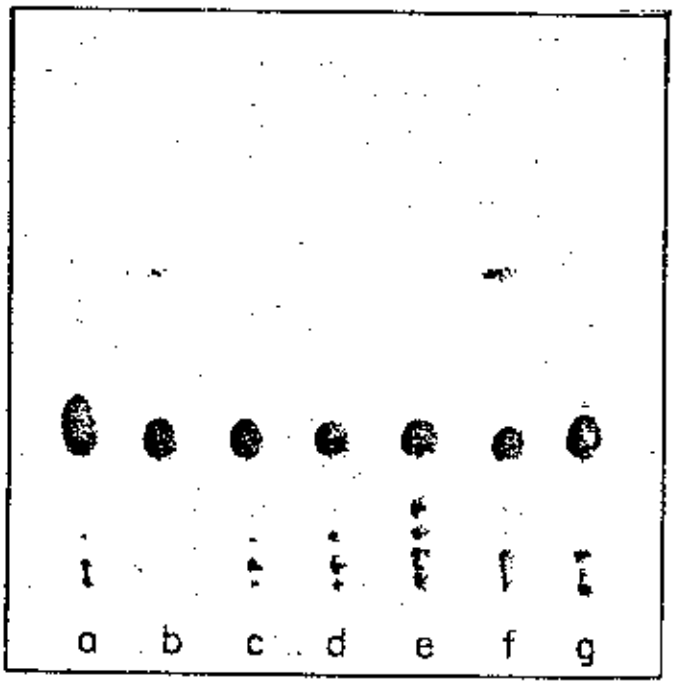



FIG. 18. Autoradiograph of a chromatoplate of commercial fatty acids-1-$C^{14}$. Solvent: petroleum hydrocarbon-diethyl ether-acetic acid, 90:10/1, v/v/v. Development time: 40 min. a) lauric acid, b) myristic acid, c) palmitic acid, d) stearic acid, e) oleic acid, f) linoleic acid, g, linolenic acid.

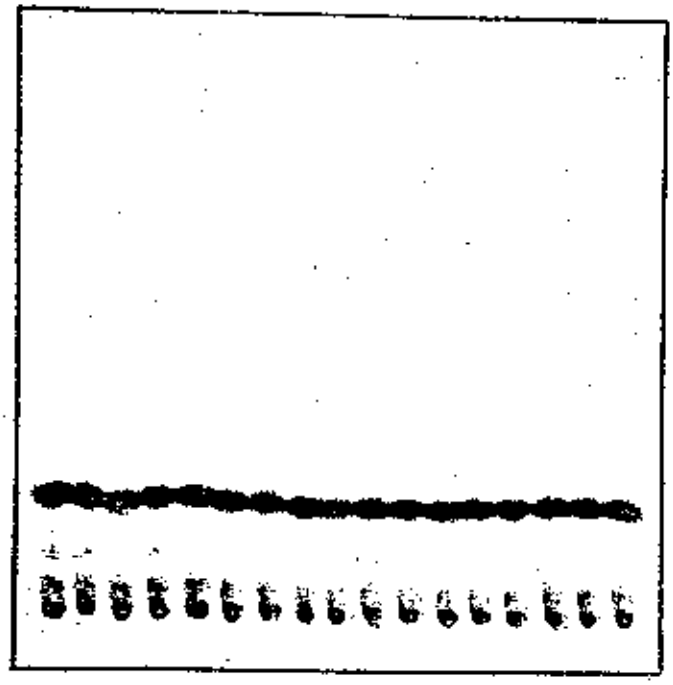


FIG. 19. Autoradiograph of a chromatoplate of cholesterol-4-$C^{14}$. Solvent: petroleum hydrocarbon-diethyl ether-acetic acid, 70:30:2, v/v/v. Development time: 40 min.

## Summary and Discussion

The technique of thin-layer chromatography and its numerous uses in the lipid field have been summarized. The most significant advantages of TLC over existing methods of lipid analysis are, in the reviewer's opinion, the following:

*Simplicity:* The equipment required can be handled by inexperienced workers.

*Speed:* Complex lipid mixtures can be separated in 30 min. Less complicated samples can often be resolved in 5 min. All other chromatographic methods require hours or days to achieve similar separations.

*Efficiency:* Thin-layer chromatography yields separations which are much sharper than the fractionations obtained by column or paper chromatography.

*Sensitivity:* In many instances, the sensitivity of the detection methods used in paper chromatography are increased one hundred fold when applied on thin-layer chromatograms, because in the latter the individual spots are more discrete and not as diffuse as on paper chromatograms.

Another advantage of thin-layer chromatography is that several indicators may be applied consecutively, on one plate, also corrosive spray reagents, such as chromic-sulfuric acid may be used to char all organic material on the chromatoplate. In other chromatographic techniques frequently the indicator employed for the detection of spots is blind in regard to one component.

*Capacity:* Up to 50 mg. per plate can be separated and isolated by thin-layer chromatography, whereas only microgram amounts are available, in a reasonable time, through paper chromatography.

Thin-layer chromatography is fully exploited only when it is used in conjunction with other methods. Especially rewarding is the combined use of thin-layer chromatography with the following methods:

- Gas-Liquid Chromatography
- Paper Chromatography
- Column Chromatography
- Radioactive Tracers
- Derivatization, i.e., the application of chemical reactions prior to chromatography.

Although thin-layer chromatography has been widely applied to lipid analysis and preparation, its usefulness is certainly not yet exhausted.

Refinements of TLC are being designed and new techniques are being developed. Honegger (63) has described ionophoresis on chromatoplates which he combined with TLC in two-dimensional technique for the resolution of amines and amino acids. He found this combination of methods in some respects superior to the separation of amines and amino acids on paper.

Only relatively small amounts of material can be separated on a plate by reversed-phase partition TLC. It may be useful to arrange several impregnated chromatoplates in a block, in a manner similar to that for large-scale paper chromatography recently described by Hall (53).

The isotopic derivative technique involves fractionation of the labeled derivatives on a microscale. TLC is especially applicable to the resolution of such complex lipid mixtures. TLC should be applied in combination with existing methods of labeling sterols (5, 11, 61) and other hydroxy and amino compounds (107, 112) with acetic anhydride-1-$C^{14}$ (or -2-$H^3$), or

with 3-chloro-anisoyl chloride-3-$Cl^{36}$ (179), or with 4-iodobenzoylchloride-$I^{131}$ (197). Several publications describe procedures for labeling acids and their analysis as methyl-$C^{14}$ esters after esterification with radioactive diazomethane-$C^{14}$ (10, 105, 112, 167, 178, 198), or 4-chloroaniline-$Cl^{36}$ (180). Unsaturated fatty acids were labeled by addition of radio-iodine $I^{131}$ (22, 76, 77). The speed of TLC will be appreciated in work with short-lived isotopes, especially if apparatus can be constructed which will perform automatic advancement of the chromatoplate and graphic or numerical recording of the radioactivity, as in the quantitative evaluation of paper strips and two-dimensional paper chromatograms (148).

Autoradiography of paper chromatograms (43) has in recent years become a prominent method in studies utilizing radioactive tracers (160). Because of its high capacity, adsorption-TLC will certainly also find wide application in isotope studies.

Strickland and Benson (e.g., 200) have been very successful in using neutron activation of phospholipids and sulfolipids on paper chromatograms. It would be desirable to develop techniques for neutron activation TLC analysis. As both calcium and sulfur atoms are activated, calcinated calcium sulfate cannot be applied, and starch will have to be used as a binder. For similar reasons, plastic plates will have to be used instead of glass.

Lipids are well protected against autoxidation if they are chromatographed on silicone-impregnated paper (165). Mahadevan (99) has investigated to what extent unsaturated lipids autoxidize during adsorption TLC, and even with the most sensitive methods he could not find any sign of alteration of unsaturated lipids during chromatography. He demonstrated, though, that TLC is well suited for rapidly purifying oxidized lipids.

This review is concluded with a hint as to the potentials of TLC as a teaching method. The chromatoplate shown in Figure 10 was prepared by a high school student on the first day of his adventures with TLC. It is obvious that this method will find wide application in the training of students.

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CHAPTER 17

Noboru Matsuo

# Nutritional Effects of Oxidized and Thermally Polymerized Fish Oils

The fish oils obtained from sardines, cuttlefish, mackerel, etc. are extremely unstable to oxidation and heating, since they contain larger amounts of the glycerides of the highly unsaturated fatty acids. Recent research made it clear that autoxidized or thermally polymerized fish oil shows acute toxicity as compared with other oils and fats.

This paper deals with the toxicity of autoxidized fish oil and of thermally polymerized fish oil, with special reference to the results of the research the author conducted primarily on cuttlefish oil.

## TOXICITY OF AUTOXIDIZED FISH OILS

Detailed studies were made by Ozaki (1926A, 1926B, 1927A, 1927B, 1932) on the nutritive values of various kinds of fatty acids. He reached the conclusion that highly unsaturated fatty acids are toxic and that such toxicity is attributable to the presence of many double bonds. Accordingly, the toxicity of fish oils was thought to be due to the highly unsaturated fatty acids contained in the fish oils.

It was later made clear that autoxidized oil destroys vitamins (Holman 1941; Kaunitz *et al.* 1952) and inhibits some kinds of enzymatic actions (Bernheim *et al.* 1952; Ottolenghi *et al.* 1955). Kaneda *et al.* (1953, 1954A, 1954B), of the Higashi Laboratory of the Tokai Regional Fishery Research Institute, fed rats the ethyl esters of highly unsaturated fatty acids separated from sardines, taking strict care to prevent autoxidation, and reported that no toxicity was found, but that when the esters were once autoxidized simply by being exposed to air, they became toxic. This fact was confirmed with highly unsaturated fatty acids separated from cuttlefish oil, and an attempt was made to locate the origin of toxicity in the autoxidized products, with reference to the biological effect of the toxic substances and their relationship to proteins, amino acids, enzymes, etc.

Oils, especially fish oils, which contain such highly unsaturated fatty acids in the form of glycerides, as shown in Table 63, are unstable to autoxidation and heating.

### Toxicity of Autoxidized Ethyl Ester of Highly Unsaturated Fatty Acids

The properties of the cuttlefish oil used are shown in Table 64.

The highly unsaturated fatty acids were separated by the soda acetone

TABLE 63

HIGHLY UNSATURATED FATTY ACIDS DERIVED FROM FISH OILS

| Molecular Formula | Fatty Acid | Number of Double Bonds | Source |
|---|---|---|---|
| $C_{18}H_{32}O_2$ | Moroctic acid | 4 | Sardine oil |
| $C_{20}H_{32}O_2$ | Arachidonic acid | 4 | Sardine and whale oil |
| $C_{20}H_{34}O_2$ | Clupanodonic acid | 5 | Sardine oil |
| $C_{21}H_{34}O_2$ | Scolidonic acid | 5 | Tuna oil |
| $C_{22}H_{34}O_2$ | Shibinic acid | 5 | Tuna oil |
| $C_{24}H_{34}O_2$ | Nisinic acid | 6 | Sardine oil |
| $C_{22}H_{40}O_2$ | Thynnic acid | 6 | Tuna oil |

TABLE 64

PROPERTIES OF RAW CUTTLEFISH OIL

| $d_4^{15}$ | Iodine Value (Wijs) | Acid Value | Saponification Value | Non-saponifiable matter, Per Cent |
|---|---|---|---|---|
| 0.9305 | 182.5 | 2.03 | 189.3 | 2.19 |

TABLE 65

PROPERTIES OF THE FRACTIONS OF HIGHLY UNSATURATED FATTY ACIDS (ETHYL ESTERS)

| Fraction No. | Temperature, °C./2 mm. Hg | Yield, Per cent | Yield, Per cent of Raw Oil | Iodine Value, Wijs | $n_D^{20}$ | Saponification Value |
|---|---|---|---|---|---|---|
| 1 | −180[1] | 10.8 | 3.0 | 232.3 | 1.4652 | 179.2 |
| 2 | 180–192 | 16.0 | 4.4 | 276.3 | 1.4700 | 176.0 |
| 3 | 192–205 | 21.3 | 5.8 | 320.0 | 1.4771 | 168.7 |
| 4 | 205–215 | 20.0 | 5.5 | 358.7 | 1.4823 | 163.9 |
| 5 | 215–227 | 19.5 | 5.4 | 384.9 | 1.4861 | 160.2 |
| 6 | 227– | 5.0 | 1.4 | 386.1 | 1.4902 | 155.7 |
| 7 | Residue[2] | 7.3 | 2.0 | — | — | — |

[1] Temp. of the first drop of the distillate: 140°C.
[2] Solid at room temperature.

process (Toyama and Tsuchiya 1925) as shown in Fig. 71. The properties of fractions of ethyl ester of highly unsaturated fatty acids are shown in Table 65.

Raw cuttlefish oil was saponified with an ethanolic solution of sodium hydroxide, mixed with acetone, left overnight at 0° to −10°C. and filtered. From the filtrate, which contained acetone-soluble sodium salts of highly unsaturated acids, acetone was distilled off and its residue was decomposed by dilute hydrochloric acid.

The free acids were dissolved in petroleum ether (b.p. below 65°C.); the solution was washed several times with water and dried over anhydrous sodium sulfate. Petroleum ether was then distilled off, and highly unsaturated acids were left as the residue.



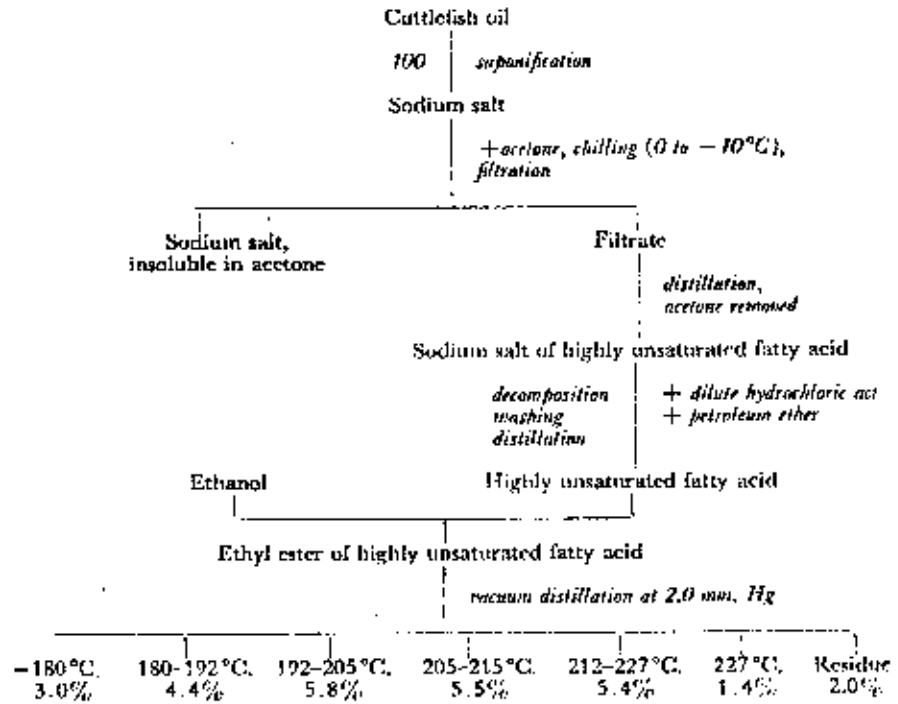



FIG. 71. SEPARATION OF HIGHLY UNSATURATED FATTY ACIDS BY SODA-ACETONE METHOD

Since the sample contained some unsaponifiable matter which was present in the original oil, it was desirable to remove as much of it as possible. In order to accomplish this, the sample was dissolved in twice its volume of absolute ethanol containing 2.5 per cent hydrochloric acid, and heated on a water bath for about three hours. By this process the ethyl esters of the acids were prepared. The ethyl esters were fractionated in a vacuum at 2 mm. of Hg at various temperatures. The fraction with a b.p. 205° to 215°C. was chiefly used for the experiment. This fraction had a light yellow color and no odor. Since these esters are liable to be rapidly oxidized when they are in contact with air, special precautions were taken throughout the process.

The container of the esters was always filled with carbon dioxide gas and was kept in dry ice during the experiment. An aliquot of the sample thus prepared was put in a petri dish, and was brought into contact with air at room temperature. As the oxidation proceeded, the esters developed a light yellow color. At a certain point of oxidation the reaction was stopped and the ester was again kept in dry ice to avoid further change.

Ethyl ole[illegible] was used as control. Some chemical properties of the esters and of their oxidized compounds are shown in Table 66 together with those of oleic acid ester for comparison.

TABLE 66

ANALYSIS OF THE SAMPLES USED IN EXPERIMENT

| | $d_4^{15}$ | $n_D^{25}$ | Iodine Value | Saponification Value | Non-saponifiable Matter |
|---|---|---|---|---|---|
| Ethyl esters of highly unsaturated fatty acids | — | 1.4823 | 358.70 | 163.90 | Trace |
| Oxidized esters of highly unsaturated fatty acids | 0.9568 | — | 264.82 | 201.49 | Trace |
| Ethyl oleate | 0.8822 | — | 82.96 | 187.69 | 0 |

Rats of nearly the same size and weight (about 60 gm.) were divided into three groups, each comprising four rats. The first group was given the feed containing five per cent of unsaturated fatty acid esters in basal diet, that of the oxidized esters to the second group, and that of oleic acid ester, which was considered as the control, to the third. The composition of the basal diet is given in Table 67.

TABLE 67

COMPOSITION OF BASAL DIET[1]

| | Per Cent |
|---|---|
| Starch (rice powder) | 60 |
| Casein (ether extracted) | 9 |
| McCollum salt mixture | 3 |
| Yeast | 3 |
| Liver oil | One drop a day |
| Ester | 5 |

[1] Total amounts of diet per day was 10 gm. (from 1st to 5th day), 12.5 gm. (from 6th to 10th day), 15 gm. (from 11th to 30th day).

The experimental results are recorded in Table 68 and also illustrated in the accompanying graph (Fig. 72) and in the photographs (Fig. 73, 74, and 75) (Matsuo 1954A). It is apparent that the rats fed on the diet containing unsaturated fatty acid ester showed as satisfactory a growth as those fed on the oleic acid ester diet, but it is remarkable that all rats fed on the oxidized ester diet not only showed retarded growth but died within a few days after the beginning of the experiment. It was also noticed in this group that the white fur had turned brown.

The effect of oxidized esters in causing mild or chronic intoxication was investigated. Mature rats were fed diets containing five per cent oxidized esters, not daily but at frequent intervals.



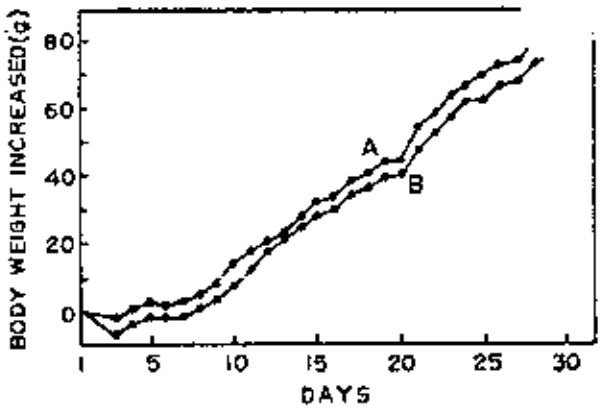



FIG. 72. THE CURVE OF AVERAGE BODY WEIGHT INCREASE

A (Control): Average body weight increment of the rats fed on the diet containing five per cent of ethyl oleate. B: That of rats fed on the diet containing five per cent of the ethyl ester of highly unsaturated fatty acids.

One of the typical examples is illustrated in Fig. 76. The appearance of the animal was quite peculiar. Fur on the face was depilated, the mouth and rear legs were swollen, the remaining fur changed into brown color, and hemorrhages were seen here and there on the skin.

Kaunitz *et al.* (1952) observed that rats developed a flavin-deficiency when rancid lard was given, but it seems that the toxic symptoms in the present case are too acute to be ascribed solely to riboflavin deficiency.

TABLE 68

FEEDING RECORD OF RATS FED UNSATURATED FATTY ESTERS

| | | Increase of Body Weight, Gm. | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10th Day | | 20th Day | | 30th Day | |
| Kind of Ester Fed | Sex | | Average | | Average | | Average |
| Ethyl oleate | ♀ | +11.0 | +13.1 | +34.0 | +43.3 | + 61.0 | +82.1 |
| | ♂ | +14.0 | | +51.2 | | +113.5 | |
| | ♀ | +15.0 | | +40.5 | | + 70.8 | |
| | ♀ | +12.5 | | +47.5 | | + 83.0 | |
| Ethyl ester of unsaturated fatty acid | ♂ | + 7.7 | + 6.3 | +41.0 | +38.1 | + 85.0 | +75.8 |
| | ♀ | + 7.0 | | +38.5 | | + 77.0 | |
| | ♂ | + 7.0 | | +44.5 | | + 75.0 | |
| | ♀ | + 3.2 | | +28.5 | | + 66.0 | |
| Oxidized form of the above ester | ♀ | − 3.8 (died on 6th day) | | | | | |
| | ♀ | − 5.0 (died on 7th day) | | | | | |
| | ♀ | − 5.0 (died on 8th day) | | | | | |
| | ♂ | − 8.5 (died on 3rd day) | | | | | |

FIG. 73. THE RAT FED ON THE DIET CONTAINING FIVE PER CENT OF ETHYL OLEATE

Control, on the 27th day after the start of the experiment.

FIG. 74. THE RAT FED ON THE DIET CONTAINING FIVE PER CENT OF THE ETHYL ESTER OF HIGHLY UNSATURATED ACID

On the 27th day after the start of the experiment.

## Peroxide Formation and Its Relation to Toxicity

Since it seemed most likely that the toxicity is related to the formation of peroxide by the autoxidation of unsaturated fatty acid, the amount of peroxide was determined.

The determination of peroxide was carried out following the Wheeler's method as improved by Nakamura (1937): A suitable amount of the sample, usually 0.1 to 0.5 gm., was weighed accurately into a conical flask

FIG. 75. THE RAT FED ON THE DIET CONTAINING FIVE PER CENT OF THE OXIDIZED ETHYL ESTER OF HIGHLY UNSATURATED ACID

On the 4th day after the start of the experiment.

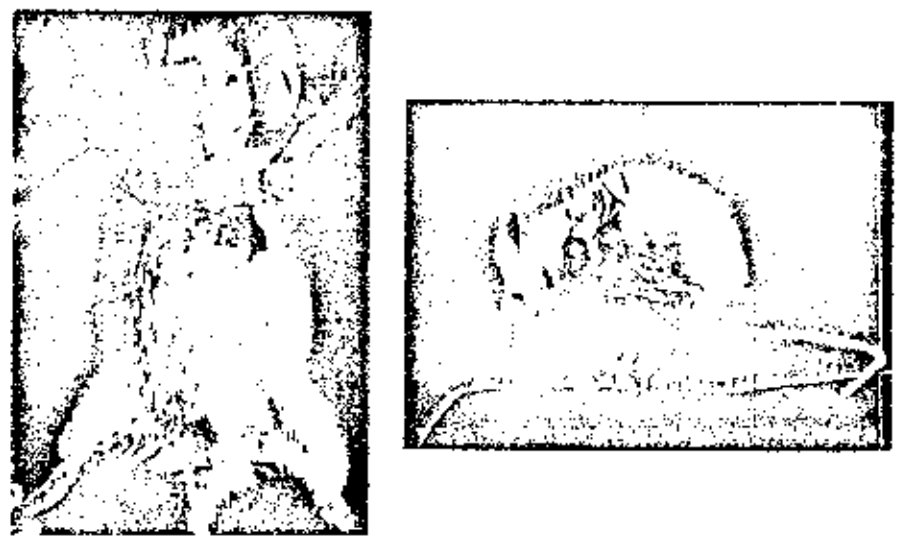


FIG. 76. TYPICAL EXAMPLE OF EFFECT OF FEEDING OXIDIZED ESTERS TO MATURE RATS

Same animal in both photographs.

of 300-ml. capacity; mixed with 20 ml. of carbon tetrachloride, 30 ml. of glacial acetic acid, and 1 ml. of saturated potassium iodide solution. After shaking for one minute, 100 ml. of distilled water were added and the free iodine liberated in the solution was titrated with 0.01N thiosulfate solu-

tion. One ml. of this thiosulfate solution corresponds to 0.00008 gm. of oxygen.

The acid ester used here was prepared from cuttlefish oil and had the properties shown in Table 69.

TABLE 69

PROPERTIES OF ETHYL ESTER OF HIGHLY UNSATURATED FATTY ACID

| $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Non-saponifiable Matter, Per Cent | Peroxide Value, Mg./100 Gm. |
|---|---|---|---|---|
| 1.4815 | 342.1 | 168.6 | Trace | 2 |

About 50 gm. of this ester was poured into a petri dish and subjected to autoxidation by contact with air at room temperature (2° to 18°C.). Measurement of peroxide was made at a few days' interval over 200 days. The results are illustrated in Fig. 77.

It is interesting to note that during the initial two weeks the formation of peroxide was almost negligible and then increased abruptly. On the 42nd day, it reached the maximum, showing a peroxide value of 4,400 and an iodine value of 208. Then it decreased gradually until the peroxide value became 1,200 and the iodine value 120. In the course of this autoxidation the ester became gradually viscous and yellowish brown. The toxicity of samples corresponding to various phases of autoxidation was surveyed by feeding the rats a diet containing five per cent of the oxidized oil.

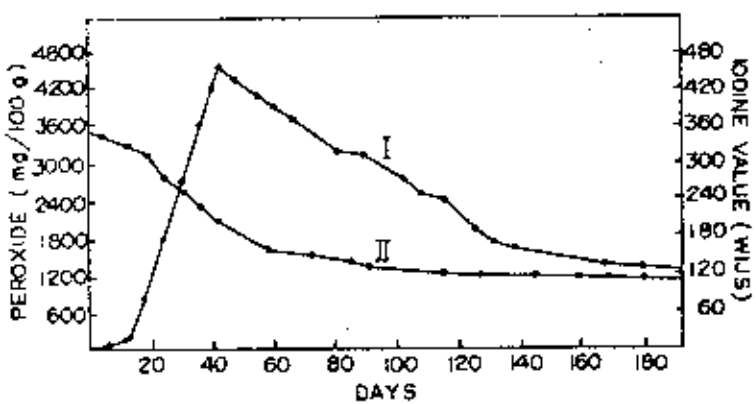



FIG. 77. THE CHANGE OF PEROXIDE (I) AND IODINE VALUES (II) OF THE FATTY ACID ESTER EXPOSED TO AIR AT ROOM TEMPERATURE (2-18°C.)



When the process of autoxidation was allowed to proc 30°C., the formation of peroxide was accelerated, but its maximu... amount was much reduced compared with that at room temperature (Fig. 78).

At 100°C. the effect of heat was even more apparent, the maximum amount of peroxide being only 240 in this case (Fig. 79). Further, it was noticed that at higher temperatures the decomposition of the peroxide formed occurred more rapidly and thoroughly.

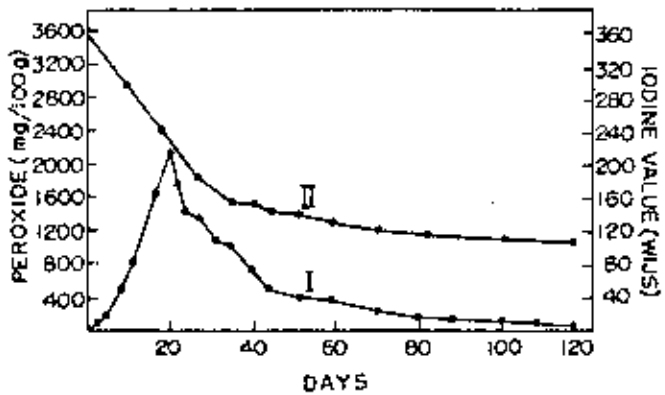



FIG. 78. THE CHANGE OF PEROXIDE (I) AND IODINE VALUES (II) OF THE FATTY ACID EXPOSED TO AIR AT 30°C.

Experiments using rats showed that the toxicity is proportional to the amount of peroxides. In the case of ethyl linolenate and ethyl linoleate, their autoxidized products were also found to be toxic.

When peroxides were removed from the autoxidized product prior to feeding, the rats grew well. As for cuttlefish oil itself, the rats grew normally when they were given unoxidized oil, while autoxidized oil showed toxicity (Figs. 80 and 81).

From the experimental results mentioned above, it may be concluded that the toxic action of fish oil is due to the peroxide formed by autoxidation (Matsuo 1954B).

Meanwhile, no toxicity appeared when peroxide content was 100 mg./100 gm. or less. Crampton *et al.* (1953) are of the same opinion on this point.

### Toxicity of Autoxidized Ester Applied to Back Skin of Rats

Autoxidized ethyl ester of highly unsaturated fatty acid (peroxide value: 1,700 mg./100 gm.) was applied in an amount of 1 gm. once each day to a depilated skin area of about 2.5 to 3 cm. in diameter on the back of rats weighing about 60 gm. each. It was found, in this case too,

that all rats died in a few days. Fig. 82 gives the curves depicting the change in the body weight of the rats.

Another experiment was conducted by applying the original ester (not oxidized) to the back skin of rats, expecting that such ester will change into the oxidized ester by undergoing gradual autoxidation and show similar toxicity although it may take a longer time. The result was just as expected. It was found that all rats died in 14 to 19 days in this case, while they died in 7 to 10 days in the case of applying the autoxidized

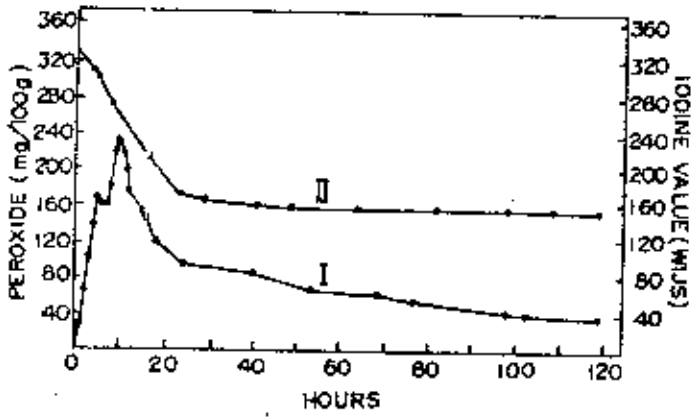



Fig. 79. The Change of Peroxide (I) and Iodine Values (II) of the Fatty Acid Ester Exposed to Air at 100°C.

ester. Curves showing the change in the body weight of the rats in this experiment are given in Fig. 83.

It is notable that toxicity is quite remarkable when the esters are applied on the back skin of the animal, which eventually caused death (Matsuo 1957A).

### Effect of Autoxidized Ester on Proteins, Amino Acids, and Enzymes

In order to ascertain the origin of the toxicity, the effect of autoxidized ethyl ester of highly unsaturated fatty acids on proteins, amino acids, and enzymes was examined.

### Effect of the Autoxidized Ester on Protein

A mixture of 40 ml. of a two per cent aqueous solution of the ovoalbumin and 5 ml. of the ethyl ester of highly unsaturated fatty acid or its autoxidized product, showing a peroxide value of about 1,800 mg./100 gm., was left at 37°C., with occasional shaking (Fig. 84). The solution to which the original ester (not oxidized) was added showed little noticeable change. The solution to which autoxidized ester was added,



on the other hand, gradually turned yellowish brown. As time passed, the precipitate increased and became more deeply colored. At suitable intervals of time, the precipitate was filtered off and washed thoroughly in water. To the filtrate and the washings, the same volume of a ten per cent aqueous solution of trichloroacetic acid was added, producing a second precipitate.

The appearance of the precipitate I is shown in Fig. 85.

Precipitate II was obtained when an equivalent volume of ten per cent trichloroacetic acid was added to filtrate I, which was obtained by standing at 37°C. and then filtering the ovoalbumin solution to which original

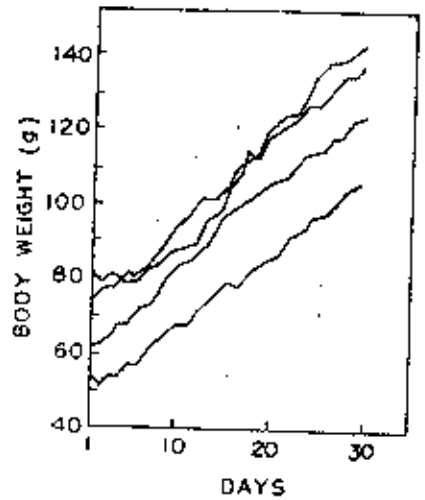



Fig. 80. Growth of Rats Fed on Diet Containing 20 Per Cent Raw Cuttlefish Oil.

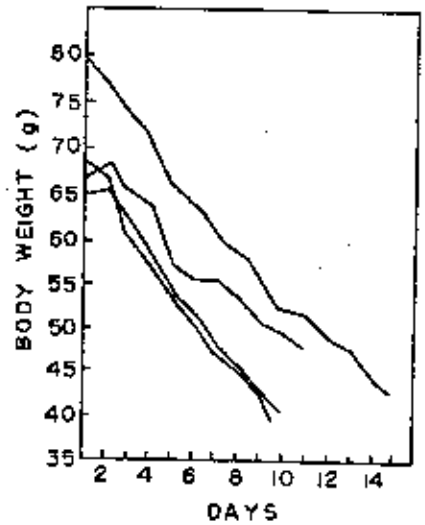



Fig. 81. Growth of Rats Fed on Basal Diet Containing 20 Per Cent Autoxidized Cuttlefish Oil.

Peroxide value: 912 mg./100 gm.; all rats died at the end of the curves

ester (not oxidized) was added. On the other hand, there was no precipitation when ten per cent trichloroacetic acid was added to the same volume of filtrate I, which was obtained by standing for over 100 hours at 37°C. and then filtering the ovoalbumin solution to which autoxidized ester had been added. This means that protein, when left for a long time, has been completely denatured and precipitated by the autoxidized ester.

Fig. 86 represents the change in the percentage of the nitrogen content in precipitate I for all nitrogen of the ovoalbumin solution (40 ml.) plotted against time.

When an autoxidized ethyl ester of highly unsaturated fatty acid was added to the aqueous solution of ovoalbumin, almost all the ovoalbumin was denatured and precipitated in about 100 hours. When the original (not oxidized) ester was added, the nitrogen content of the precipitate 1 was only 6 to 7 per cent. When autoxidized ester was added to the aqueous solution of ovoalbumin, the ratio of the quantity of nitrogen contained in filtrate II to that contained in 40 ml. of the original aqueous solution of the ovoalbumin, as shown in Fig. 87, remained below one per cent, irrespective of a lapse of time.

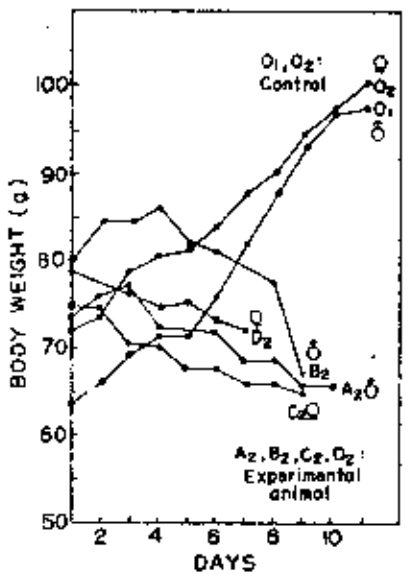



Fig. 82. Growth of Rats When Autoxidized Ethyl Ester of Highly Unsaturated Fatty Acid Was Applied to Their Depilated Back Skin Once Every Day

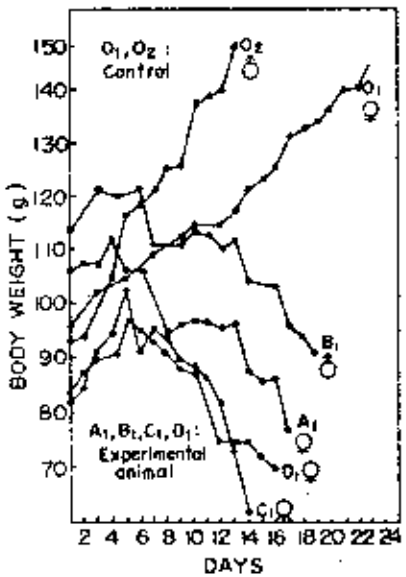



Fig. 83. Growth of Rats When Original Ester (Not Oxidized) Was Applied to Depilated Back Skin Once Every Day

Terminal amino acid was more or less oxidized, but it became clear that the greater part of ovoalbumin precipitates by forming an adduct with the autoxidized ester (Matsuo 1957B).

### The Effect of Autoxidized Fatty Acid Ester on Amino Acids

The autoxidized ethyl ester of highly unsaturated fatty acid shows a notable action on protein as mentioned. In order to investigate its action on amino acids, experiments were conducted using glycine, glycylglycine, and cystine. Cystine, for example, was oxidized into cysteic acid by

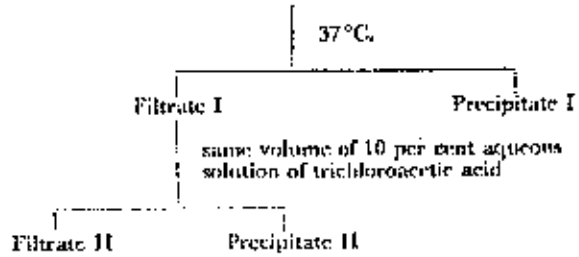
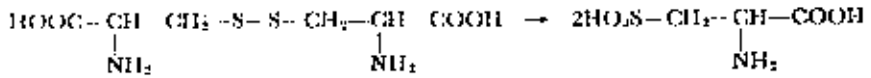



Fig. 84. Reaction of Ovoalbumin with Unsaturated Fatty Acid Esters

Forty ml. of two per cent aqueous solution of ovoalbumin plus five ml. of the ethyl ester of highly unsaturated fatty acid or its autoxidized product.

autoxidized ester as shown in the following formula with the —S—S— bond ruptured (Matsuo 1957B):




### Effect of Autoxidized Ester on Enzymes

As proteins are markedly denatured by autoxidized ester, it is natural to expect that enzymes will also be affected to a greater extent. To confirm this point, experiments were conducted on succinodehydrogenase, salivary amylase, and sweet potato amylase (Matsuo 1957C).

Succinodehydrogenase.—Three rats weighing 132, 202, and 234 gm., respectively, were fed a basal diet containing five per cent of autoxidized ethyl ester of highly unsaturated fatty acid (peroxide value: about 1,800 mg./100 gm.) for a few days. The animals were killed when they became critically weak.

Succinodehydrogenase activity of their muscle and liver was measured and compared with that of the control rats. The results are shown in Table 70, where the time required for decolorization of methylene blue is given.

Table 70

Comparison of Succinodehydrogenase Activities of Muscle and Liver of Rats Fed with Autoxidized Ethyl Ester of Highly Unsaturated Fatty Acid with Those of Control Rats

| Rat No. | | I | II | III | Control I | Control II |
|---|---|---|---|---|---|---|
| Time required for decolorization of methylene blue | Muscle | 15′20″ | 17′0″ | 17′20″ | 8′0″ | 8′40″ |
| | Liver | 16′30″ | 17′20″ | 16′40″ | 7′10″ | 7′0″ |

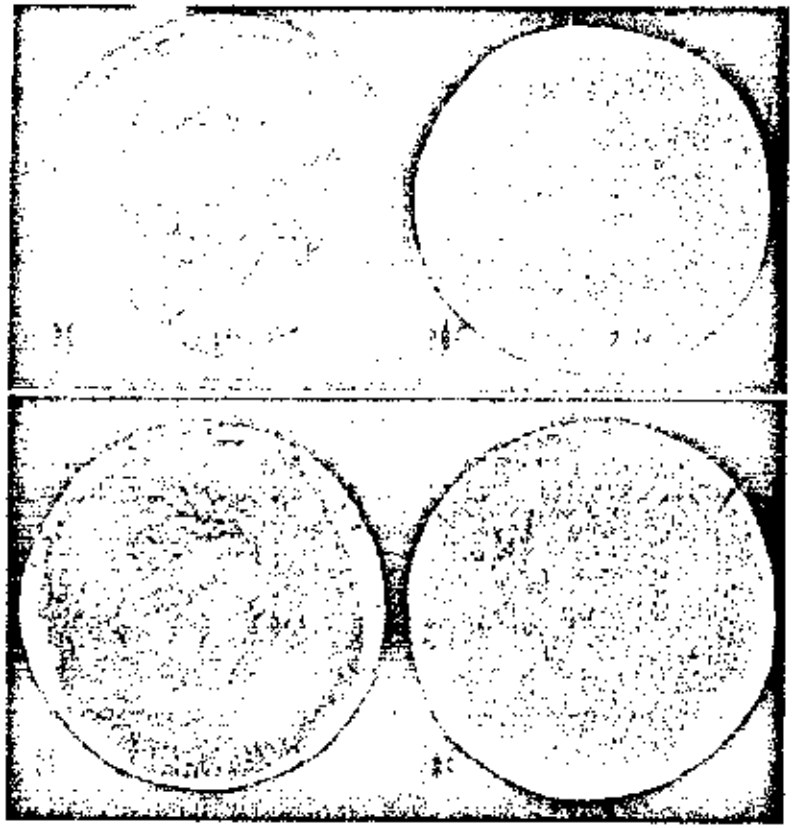


Fig. 85. Denaturation of Protein by Autoxidized Ethyl Ester of Highly Unsaturated Fatty Acid

Upper figure after 51 hrs.
Lower figure after 75 hrs.

**Salivary Amylase.**—Three kinds of solutions of the following compositions (in ml.) were prepared:

| | Buffer Solution pH 6.81 | Saliva | Oxidized Ester (Peroxide Value: 2152) | Original Ester | Water |
|---|---|---|---|---|---|
| I | 20 | 5 | 5 | — | — |
| II | 20 | 5 | — | 5 | — |
| III | 20 | 5 | — | — | 5 |

The above-mentioned mixtures were thoroughly shaken and kept at 37 C. At regular intervals, 0.5 ml. of a one per cent starch solution was added to each 2.5 ml. aliquot of these mixtures, left for 30 minutes at



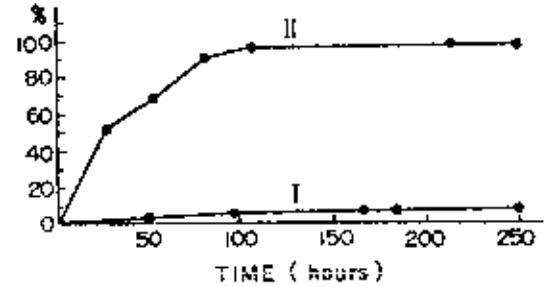



Fig. 86. Nitrogen Content of Precipitate I

I: Ovoalbumin solution plus ethyl ester of highly unsaturated fatty acid. II: Ovoalbumin solution plus autoxidized ethyl ester of highly unsaturated fatty acid.

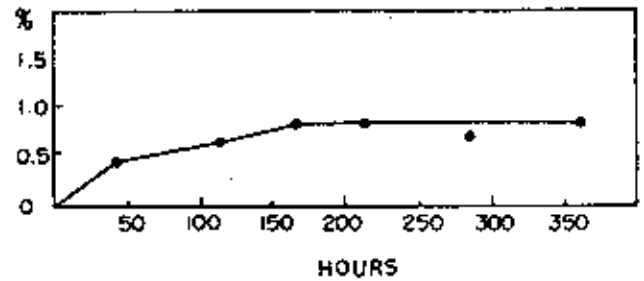



Fig. 87. Nitrogen Content in Filtrate II When Autoxidized Ester is Added

37°C., and each solution was subjected to examination by the iodine-starch reaction.

In the case of II and III, in which original (not oxidized) ester or distilled water was added, as shown in Table 71, salivary amylase activity was noticed even after a lapse of 20 hours. When oxidized ester (peroxide value 2,152 mg./100 gm.) was added, on the other hand, disappearance of the activity of salivary amylase was evident 22 hours after the mixing.

The same results were obtained with sweet potato amylase.

**Pathological Studies.**—An attempt was then made to determine whether autoxidized ethyl esters of highly unsaturated fatty acid are as toxic to rabbits as they are to rats.

Once a day, 0.8 ml. of autoxidized ester was given orally to four healthy rabbits each weighing about 1.5 kg. As shown in Table 72, all these rabbits died. Upon autopsy, it was found that the mucous membrane of the stomach was seriously affected and in one a stomach ulcer had formed (Fig. 88).

TABLE 71

SCHEMATIC REPRESENTATION OF LAPSE OF TIME AND IODINE-STARCH REACTION WHEN 5 ML. OXIDIZED ESTER OR ORIGINAL ESTER IS MIXED WITH 5 ML. SALIVA AND LEFT AT 37°C.

| Time Elapsed, Hours | I | II | III |
|---|---|---|---|
| 10 | − | − | − |
| 14 | − | − | − |
| 18 | − | − | − |
| 22 | + | − | − |
| 26 | + | − | − |

Plus and minus signs represent positive and negative iodine starch reaction, respectively.

TABLE 72

FEEDING RECORD OF RABBITS FED AUTOXIDIZED ESTERS

| | Sex | Initial Weight, Gm. | Final Weight, Gm. | Wt. Decreased, Gm. | |
|---|---|---|---|---|---|
| $A_1$ | ♀ | 1500 | 1290 | −210 | Died on 5th day |
| $A_2$ | ♀ | 1570 | 1350 | −220 | Died on 12th day |
| $A_3$ | ♂ | 1500 | 1520 | + 20 | Died on 15th day |
| $A_4$ | ♂ | 1720 | 1575 | −145 | Died on 5th day |

The intestines were found to be less pliable and apt to break when stretched.

An autopsy on the rabbits revealed that fairly severe deterioration and destruction had occurred in the mucous membranes of the stomach and intestine. This finding suggests that the unsaturated fatty acids, when autoxidized, become a protein poison.

Detailed pathological examination of the internal organs of the rats fed with autoxidized ester is being made by Professor Ogata and others (1955) in the Department of Pathology, Faculty of Medicine, Tokushima University, Japan.

## Toxicity of Thermally Polymerized Fish Oils

Sakurai *et al.* (1951) conducted research on the nutritive value of whale oil and found that the polymerized oil of the fin whale (*Balaenoptera physalus*) showed considerable toxicity when rats were fed a diet containing this oil at a level of 20 per cent. In this case, the rats died in about three weeks, but if the percentage content is reduced to below ten per cent they grew well, utilizing the oil as a caloric source without developing any undesirable symptoms.

Crampton *et al.* (1951A, 1951B, 1951C, 1953, 1956) reported that heat-polymerized oil is toxic to rats despite the extremely low peroxide content. Kaunitz *et al.* (1955, 1956A, 1956B) also announced that the oil obtained by heating cottonseed oil at 95°C. for 200 to 300 hours was toxic to rats.

FIG. 88. INSIDE OF THE STOMACH OF A RABBIT WHICH DIED AFTER TAKING 0.8 ML. OF THE AUTOXIDIZED ESTER ONCE EVERY DAY

The coats of the stomach are ulcerous at the points marked a and b.

Johnson *et al.* (1956, 1957A, 1957B) and Perkins and Kummerow (1959A, 1959B) also reported on the physiological effects of feeding polymerized oils.

Judging from the mechanism of heat polymerization, it is believed that the degree of unsaturation of oil is most closely related to polymerization. Since fish oil is more unsaturated than vegetable oil, it may polymerize more easily. Examinations were therefore made to see whether such a product from refined cuttlefish oil is toxic to rats.

## Toxicity of Thermally Polymerized Cuttlefish Oil

The cuttlefish oil used in this experiment is a light yellow, transparent liquid, whose properties are shown in Table 73.

Fig. 89 gives the growth curves of rats fed the basal diet shown in Table 74. All curves show normal growth. When the experiment was over, the

TABLE 73

PROPERTIES OF RAW CUTTLEFISH OIL

| $n_D^{20}$ | Iodine value, Wijs | Saponification Value | Acid Value | Peroxide value, Mg./100 Gm. | Molecular weight, Rast |
|---|---|---|---|---|---|
| 1.4847 | 194.6 | 180.2 | 3.31 | 0 | 680 |

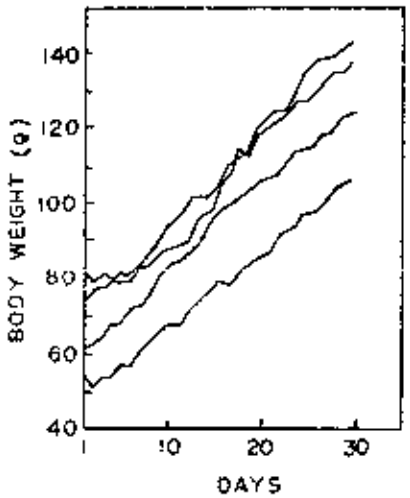



FIG. 89. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT RAW CUTTLEFISH OIL

peroxide value of the cuttlefish oil used was measured. The increase was found to be extremely small.

TABLE 74

COMPOSITION OF BASAL DIET

| | Per Cent |
|---|---|
| Starch (rice powder) | 65 |
| Casein (ether extracted) | 9 |
| McCollum salt mixture | 3 |
| Yeast | 3 |
| Liver oil | One drop a day |
| Sample oil | 20 |

Raw cuttlefish oil was heated at 250°C. for ten hours in an oil bath in a stream of carbon dioxide. Some properties of the polymerized oil are shown in Table 75.

Animal experiments were conducted using rats each weighing about 60 to 80 gm. They were fed the same basal diet but containing 20 per cent of

TABLE 75

PROPERTIES OF THE POLYMERIZED OIL HEATED AT 250°C. FOR TEN HOURS

| #% | Iodine Value, Wijs | Saponification Value | Acid Value | Peroxide Value, Mg./100 Gm. | Molecular Weight, Rast |
|---|---|---|---|---|---|
| 1.4947 | 121.9 | 171.1 | 2.42 | 0 | 1320 |



polymerized oil (Table 75). All the rats succumbed wi[illegible] days after the start of the experiment (Fig. 90).

When rats were fed on a basal diet containing five per cent of thermally polymerized oil, no particular toxicity appeared, though the nutritive value of the oil was somewhat lower than the original cuttlefish oil.

Extensive research under way in Japan and abroad demonstrated the intra- and inter-molecular cyclic compounds are formed in the course of thermal polymerization.

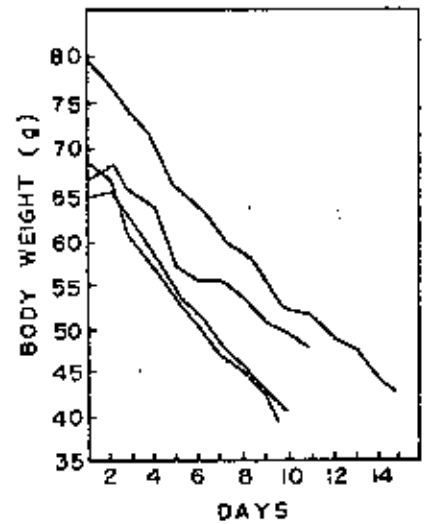



FIG. 90. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF POLYMERIZED OIL HEATED AT 250°C. FOR TEN HOURS

The ethyl ester was prepared by ethanolysis of the oil thermally polymerized in a carbon dioxide stream at 250°C. for ten hours. An animal experiment using rats was conducted on straight-chain and cyclic esters separated from this ethyl ester by the urea-adduct method. The results confirm those reported by Crampton and others, that when rats were given 20 per cent of straight-chain ester they grew well (Fig. 91), while feeding with 20 per cent of cyclic ester resulted in marked loss of weight. In the latter case, all the rats died in 2 to 7 days (Fig. 92). Similar results were obtained when only ten per cent was fed (Matsuo 1957C).

The properties of the straight-chain and cyclic esters are shown in Table 76.

Experiments using cuttlefish oil which had been thermally polymerized in air at 250°C. for ten hours give similar results (Matsuo 1958).

TABLE 76

PROPERTIES OF STRAIGHT-CHAIN AND CYCLIC ESTERS

| | $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Acid Value | Peroxide Value, Mg./100 Gm. | Molecular Weight, Rast |
|---|---|---|---|---|---|---|
| Straight-chain ethyl ester | 1.4624 | 75.1 | 170.7 | 0.36 | 0 | 330 |
| Cyclic ethyl ester | 1.5059 | 175.2 | 148.3 | 1.30 | 0 | 380 |

## Toxicity of Thermally Polymerized Ethyl Linolenate

For the purpose of elucidating the properties and structure of cyclic products, experiments were designed to investigate the toxicity or thermally polymerized ethyl linolenate (Matsuo 1959A).

**Separation of Linolenic Acid from Linseed Oil.**—Refined linseed oil was saponified by the ordinary method and the separated fatty acid was dis-

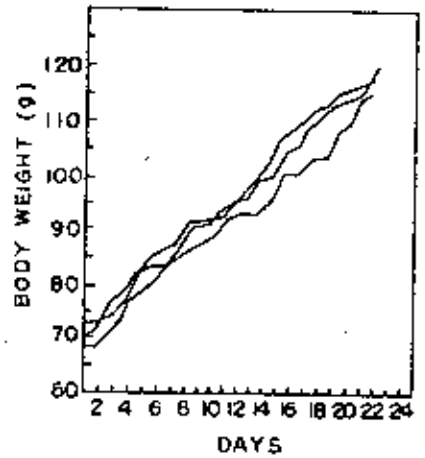



FIG. 91. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF STRAIGHT CHAIN ESTER

solved in ether. Bromine was dropped into the cooled ether solution. The precipitated bromide was collected by filtration, washed in cold ether, and dried. For purification, the bromide was dissolved in a large quantity of benzene and the solution was boiled on a hot water-bath. The hexabromide of linolenic acid was obtained as a white precipitate on cooling. The precipitate was collected, washed in cold ether, and dried. The hexabromide thus obtained was treated with zinc, ethanol, and hydrochloric acid to effect simultaneous debromination and esterification.



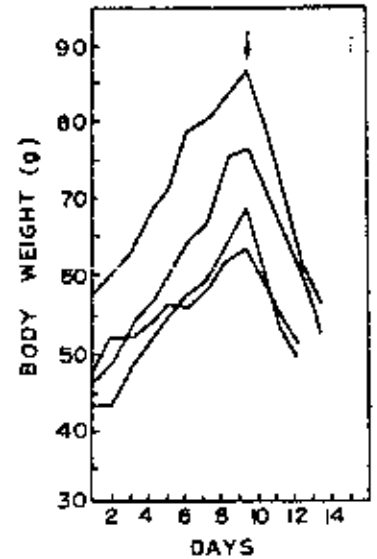



FIG. 92. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF CYCLIC ESTER

All rats died at the end of the curves.

The ester mixture was distilled in vacuum (1.5 mm. Hg) and the fraction boiling at 167° to 170°C. was used in this experiment. The properties of ethyl linolenate and linseed oil are shown in Table 77.

TABLE 77

PROPERTIES OF LINSEED OIL AND ETHYL LINOLENATE

| | $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Acid Value | Molecular Weight, Rast |
|---|---|---|---|---|---|
| Linseed oil | 1.4833 | 191.8 | 186.9 | 0 | — |
| Ethyl linolenate | 1.4688 | 242.5 | 183.1 | 1.0 | 302 |

**Thermal Polymerization of Ethyl Linolenate and Separation of Cyclic Monomer.**—Thermal polymerization of ethyl linolenate was carried out by heating at 250°C. in a stream of carbon dioxide for 40 hours. During the process, the iodine value decreased from 242.5 to 157.5, as shown in Fig. 93.

Straight-chain ester and cyclic esters of the polymerized ethyl linolenate were separated by the urea-adduct method. The yields of the straight-chain ester and the cyclic ester were 21.2 per cent and 63.3 per cent

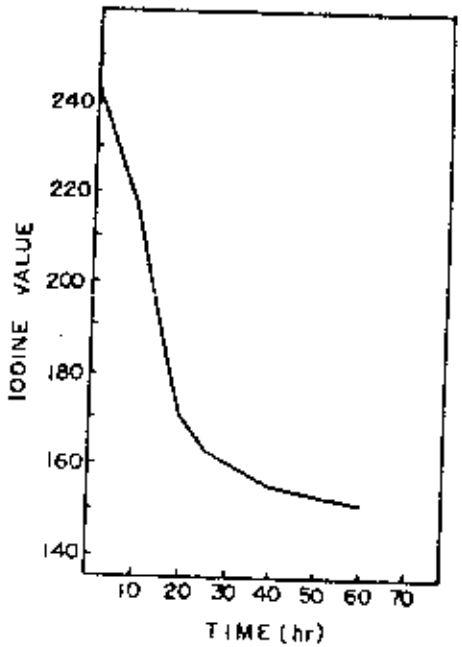



FIG. 93. CHANGE IN IODINE VALUE OF ETHYL LINOLEATE DUE TO THERMAL POLYMERIZATION

respectively. By vacuum distillation, the latter was separated into a distillate (153 to 175°C./1.8–2.0 mm. Hg) and a residue. The distillate is transparent, light yellow, and its yield was 24.7 per cent of the total cyclic ester. The residue is somewhat viscous, transparent, reddish brown, and its yield was 70.8 per cent.

**Animal Experiments with the Various Esters.**—Rats, each weighing about 60 gm. were fed the basal diet (Table 74) containing, respectively, 20 per cent of each of the sample esters shown in Table 78.

TABLE 78

PROPERTIES OF ESTERS

| | $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Acid Value | Molecular Weight, Rast |
|---|---|---|---|---|---|
| Ethyl linolenate | 1.4688 | 242.5 | 183.1 | 1.0 | 302 |
| Polymerized ethyl linolenate | 1.4838 | 157.5 | 170.1 | 6.5 | |
| Straight-chain ester | 1.4692 | 201.5 | 177.4 | 0.4 | |
| Cyclic ester | 1.4891 | 142.5 | 168.6 | 7.3 | |
| Distilled ester | 1.4751 | 159.4 | 171.0 | 7.4 | 293 |
| Residual ester | 1.4948 | 135.8 | 168.5 | 8.2 | |



***Ethyl Linolenate.***—The rats fed a diet cont[illegible] ethyl linolenate showed healthy growth as indicated in Fig. 94.

***Polymerized Ethyl Linolenate.***—As shown in Fig. 95, the rats became gradually thinner and died in about 15 days.

***Distilled and Residual Cyclic Esters.***—When rats were fed a basal diet containing 20 per cent of distilled cyclic ester, extremely acute toxicity

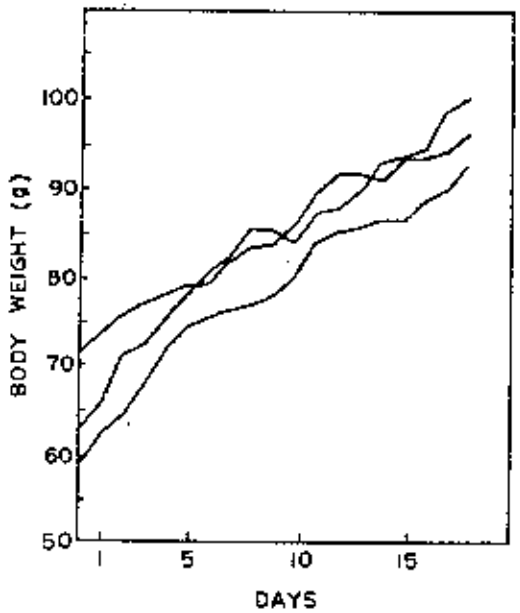



FIG. 94. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF ETHYL LINOLENATE

appeared and all rats died in three or four days. However, no particular change was noticed in the appearance of the rats. In the case of feeding a basal diet containing 20 per cent of residual cyclic ester, toxicity was considerably less and there was some increase in the weight of the rats, although they had diarrhea (Fig. 96).

From these observations, it was apparent that distilled cyclic ester was severely toxic.

**Properties of Various Esters.**—The properties of the esters which were fed are shown in Table 78.

From the iodine value (242.5) of ethyl linolenate (theoretical value: 247.4), it is assumed that the linolenate contained about six per cent of ethyl linoleate, if the contaminant was only ethyl linoleate. The saponification value of this ethyl linolenate was 183.1 (theoretical value: 183.0)

and its molecular weight, 302 (theoretical value: 308). The molecular weight of the distilled cyclic ester was 293. From its boiling point, the main component of this cyclic ester is considered to be a cyclic monomer. Since its iodine value was 159.4 it seems that the original linolenate with three double bonds lost one as a result of cyclization. Iodine value of ethyl linolenate with two double bonds is 166.0.

**Infrared Absorption Spectra of Ethyl Linolenate and Distilled Cyclic Esters.**—The infrared absorption spectra of ethyl linolenate and the distilled cyclic ester, as measured with the Baird B-type spectrophotometer, are shown in Fig. 97.

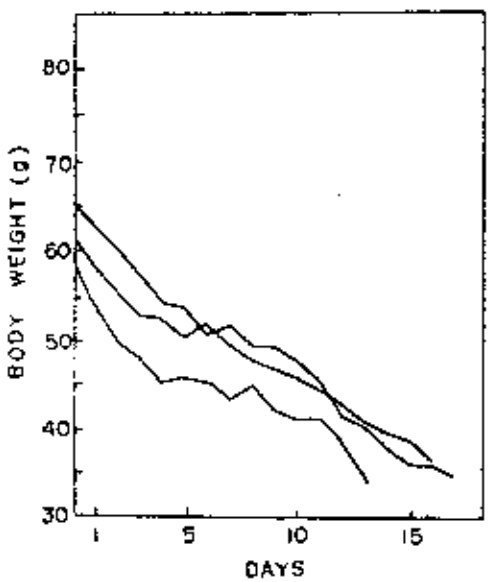



FIG. 95. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF POLYMERIZED ETHYL LINOLENATE

All rats died at the ends of the curves

The absorption band at about 14 $\mu$ for ethyl linolenate is considerably intense and attributable to cis double bonds.

Linolenic acid has naturally cis double bonds. When it is separated by the bromination method, however, isomerization takes place to some extent and an absorption band of 10.3 $\mu$ (970 cm.$^{-1}$) attributable to trans double bonds appears. When the absorption band near 6 $\mu$ was examined with increased concentration of the sample, a small absorption was noticed. This absorption is also considered to be attributable to cis double bonds.



When the infrared absorption spectrum (Fig 97–II) of the distilled cyclic ester is compared with that (Fig. 97–I) of ethyl linolenate, it is found that the absorption attributed to cis double bonds is considerably weaker. This seems to be due to the disappearance of some double bonds. A new absorption band was observed at 15.2 $\mu$ (660 cm.$^{-1}$). Such absorption was noticed also in the cyclic monomer separated from the prod-

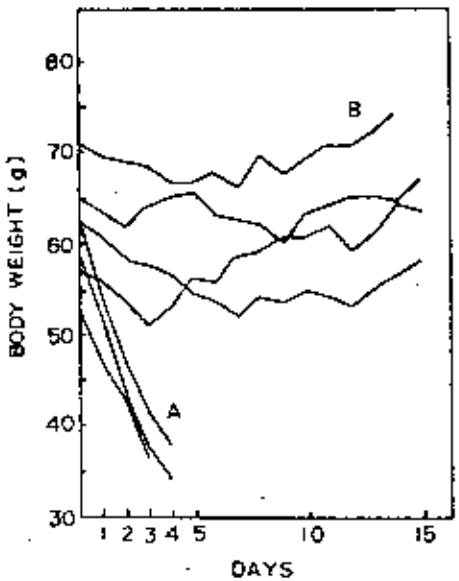



FIG. 96. GROWTH OF RATS FED ON BASAL DIET CONTAINING 20 PER CENT OF DISTILLED CYCLIC ESTER (A) AND RESIDUAL ESTER (B)

All rats died at the end of the A curve.

uct obtained by thermal polymerization of the highly unsaturated fatty acid from cuttlefish oil (Matsuo 1960A).

Cyclohexene shows a band at 670 cm.$^{-1}$ (14.97 $\mu$) (MacDonald 1956). The bands at 663 and 671 cm.$^{-1}$ (15.1 and 14.9 $\mu$) are characteristic of $\Delta^2$ - and $\Delta^3$ -steroids respectively (Jones and Herling 1954), and cis $\alpha$ -ionone (Buchi and Yang 1955) also absorbs in this region. Therefore, this absorption band is considered to be attributable to the formation of a cyclohexene ring resulting from cyclization. This absorption band was also ascertained by MacDonald (1956) as the monomeric, non-urea-adduct-forming material from the ethanolysis of heated linseed oil.

Absorption due to trans double bonds became somewhat greater, but no significant change was observed. Examination of the absorption sy

tra near 6 μ observed with the higher concentration of the sample showed the presence of two small bands. It seems that the new absorption band at about 6.1 μ (1650 cm.⁻¹) originates from the cyclic compound.

In the experiment on highly unsaturated fatty acid separated from fish oil, cyclic compounds also showed toxicity, and the cyclic monomer, separated in vacuum distillation, was the main source of toxicity.

From the result of the experiment on ethyl linolenate, it can be concluded that the source of toxicity is a cyclic monomer and that the main

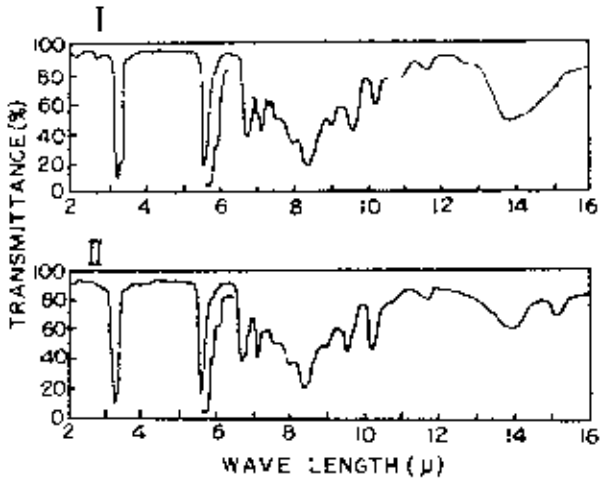



FIG. 97. INFRARED ABSORPTION SPECTRA OF ETHYL LINOLENATE (I) AND DISTILLED CYCLIC ESTER (II)

component of this cyclic monomer is a compound having a cyclohexene ring.

## Toxicity of Cyclic Compound of β-Eleostearic Acid

In order to investigate this point, a compound having a cyclohexene ring in the molecule was prepared and its toxicity was examined (Matsuo 1960B).

**Preparation of Pure β-Eleostearic Acid.**—Tung oil indicated in Table 79—i, was isomerized by the method of Hoffman *et al.* (1957). About 500 gm. of tung oil was mixed with 1 ml. of saturated potassium iodide solution, the mixture was thoroughly stirred, kept air-tight, and exposed to the sunlight to accelerate isomerization. Then the oil was saponified with alkali as usual, and fatty acid was then obtained. This was washed in distilled water, dissolved in ethanol, and allowed to stand overnight at −15°C. The precipitated β-eleostearic acid was collected by filtration and dried in vacuum. The melting point of the acid was 70° to 71°C.



and its infrared absorption spectrum, as shown in Fig. 98-a, was exactly the same as that reported by Hoffman *et al.* (1957).

The ethyl ester of β-eleostearic acid was prepared as usual and distilled in vacuum. The fraction collected at 193° to 196°C./2 mm. Hg showed the properties indicated in Table 79—ii, which were almost comparable to the theoretical values. The infrared absorption spectrum of this ester is shown in Fig. 98-b.

TABLE 79

PROPERTIES OF TUNG OIL AND DERIVATIVES

| No. | Sample | $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Acid Value | Molecular Weight, Rast |
|---|---|---|---|---|---|---|
| i | Tung oil | 1.5063 | 173.7 | 194.2 | 5.4 | — |
| ii | Ethyl β-eleostearate | 1.4892 | 165.5 (166.0) | 182.2 (183.0) | 2.0 | 301 (306) |
| iii | Ethyl β-eleostearate acrolein adduct | 1.4847 | 139.8 (140.3) | 153.6 (154.7) | 1.6 | 367 (362) |

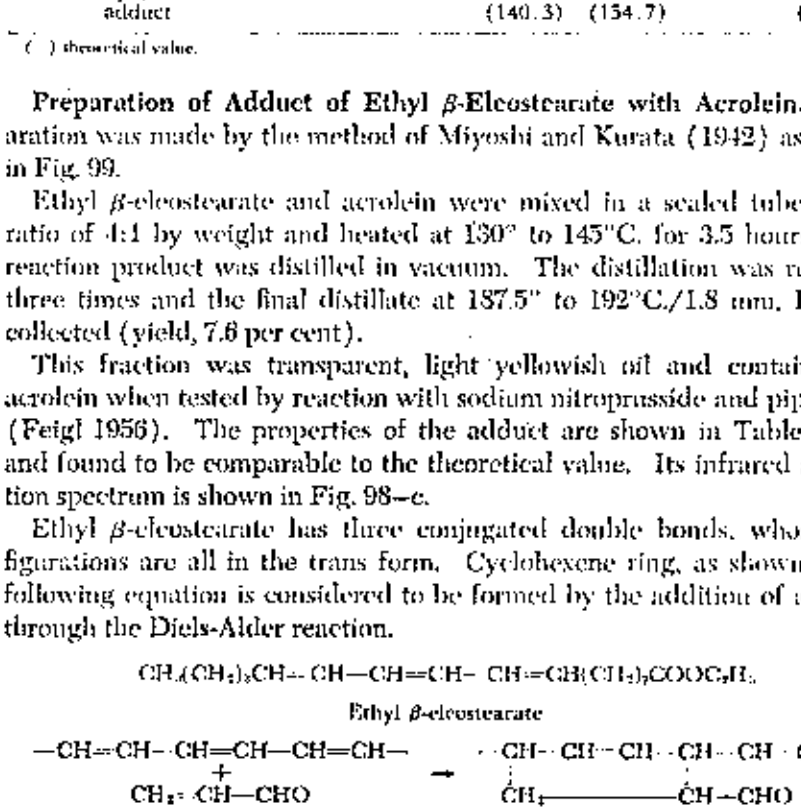
( ) theoretical value.

**Preparation of Adduct of Ethyl β-Eleostearate with Acrolein.**—Preparation was made by the method of Miyoshi and Kurata (1942) as shown in Fig. 99.

Ethyl β-eleostearate and acrolein were mixed in a sealed tube in the ratio of 4:1 by weight and heated at 130° to 145°C. for 3.5 hours. The reaction product was distilled in vacuum. The distillation was repeated three times and the final distillate at 187.5° to 192°C./1.8 mm. Hg was collected (yield, 7.6 per cent).

This fraction was transparent, light yellowish oil and contained no acrolein when tested by reaction with sodium nitroprusside and piperidine (Feigl 1956). The properties of the adduct are shown in Table 79—iii, and found to be comparable to the theoretical value. Its infrared absorption spectrum is shown in Fig. 98—c.

Ethyl β-eleostearate has three conjugated double bonds, whose configurations are all in the trans form. Cyclohexene ring, as shown in the following equation is considered to be formed by the addition of acrolein through the Diels-Alder reaction.




When the infrared absorption spectrum of ethyl β-eleostearate-acrolein adduct is compared with that of ethyl β-eleostearate, the following

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ferences are observed. The strong absorption at 10.1 $\mu$ (990 cm.$^{-1}$) due to the conjugated trans-trans double bond, which was found in ethyl $\beta$-eleostearate, almost disappeared as a result of the addition of acrolein, and an absorption at 10.3 $\mu$ (970 cm.$^{-1}$) due to trans double appeared.

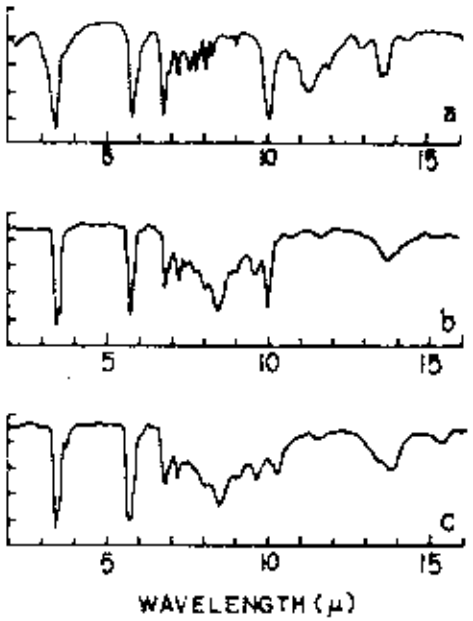


FIG. 98. INFRARED ABSORPTION SPECTRA

(a) $\beta$-Eleostearic acid, (b) ethyl $\beta$-eleostearate, (c) ethyl $\beta$-eleostearate-acrolein adduct.

The absorption at 15.2 $\mu$ (660 cm.$^{-1}$) was not found in the spectrum of the original ester. This absorption band was also observed by MacDonald (1956). This absorption can be attributed to the cyclohexene ring. Another absorption, due to the aldehyde radical, was observed at 3.7 $\mu$ (2700 cm.$^{-1}$).

TABLE 80

COMPOSITION OF BASAL DIET

| | Per Cent |
|---|---|
| Starch (rice powder) | 75 |
| Casein (ether extracted) | 9 |
| McCollum salt mixture | 3 |
| Yeast | 3 |
| Liver oil | One drop a day |
| Sample | 10 |



These differences seem to be caused by the addition of acrolein to ethyl $\beta$-eleostearate as shown in the above formula.

From the properties and infrared absorption spectrum, the product seems to be the adduct of one molecule of acrolein with one molecule of ethyl $\beta$-eleostearate.

**Toxicity of Ethyl $\beta$-Eleostearate-Acrolein Adduct to Rats.**—Rats, each weighing 60 to 70 gm., then fed a basal diet (Table 80) containing ten per cent of ethyl $\beta$-eleostearate, grew normally.

Rats, each weighing about 60 gm., were fed the same basal diet (Table 80) but containing ten per cent of the adduct of ethyl $\beta$-eleostearate and acrolein. In this experiment, as shown in the growth curves given in Fig.

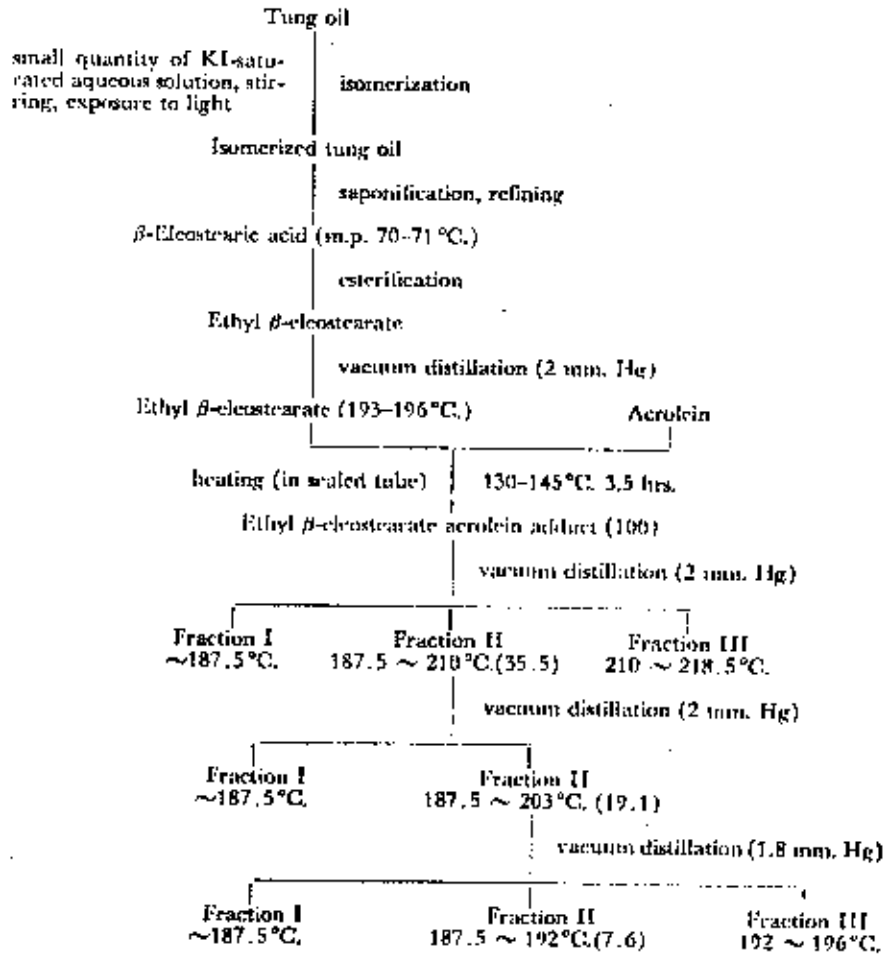


FIG. 99. PREPARATION OF ETHYL $\beta$-ELEOSTEARATE-ACROLEIN ADDUCT

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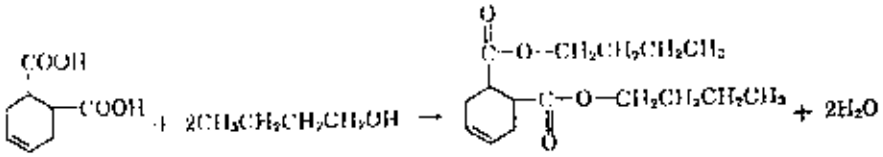
100, all rats died in 4 to 6 days after showing an abrupt decrease in body weight.

### Toxicity of the Cyclic Compound

To obtain further evidence, an experiment was conducted on a compound synthesized from cyclohexene dicarboxylic acid and butanol (Matsuo 1959B). 4-Cyclohexene-1,2-dicarboxylic acid and butanol were reacted by the usual method to form an ester as follows:




Mol. wt. 282

The ester thus obtained was distilled in vacuum twice after being washed thoroughly in water, and finally the distillate at 160 to 165°C./3 mm. Hg was collected. It is a colorless, transparent liquid, and showed the properties listed in Table 81, which are very close to the theoretical values. This ester resembled the cyclic monomer obtained from ethyl linolenate.

TABLE 81

PROPERTIES OF DIBUTYL 4-CYCLOHEXENE-1,2-DICARBOXYLATE

| $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Acid Value | Peroxide Value, Mg./100 Gm. |
|---|---|---|---|---|
| 1.4593 | 90.1<br>90.1[1] | 398.7<br>398.0[1] | 1.2 | 0 |

[1] Theoretical value.

In an experiment in which rats, each weighing about 60 gm., were fed a basal diet containing 20 per cent of the ester, the rats lost weight quickly as shown in Fig. 101, and all died within eleven days.

From these experiments, it is recognized that the cyclic monomer produced by heat treatment of unsaturated fatty acid is a substance responsible for toxicity to animals.

Rats were fed for more than a year on a basal diet containing five to ten per cent of fish oil (cuttlefish oil) thermally polymerized in a stream of carbon dioxide gas at 250°C. for ten hours. Pathological studies on these rats are being made by the present author with the cooperation of Professor Ogata of the Department of Pathology, Faculty of Medicine, Tokushima University.



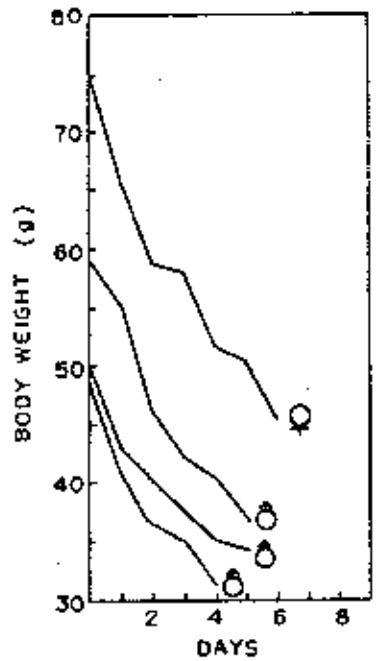



FIG. 100. GROWTH CURVES OF RATS FED ON BASAL DIET CONTAINING TEN PER CENT OF ADDUCT OF ETHYL β-ELEOSTEARATE AND ACROLEIN

All rats died at the end of the curves.

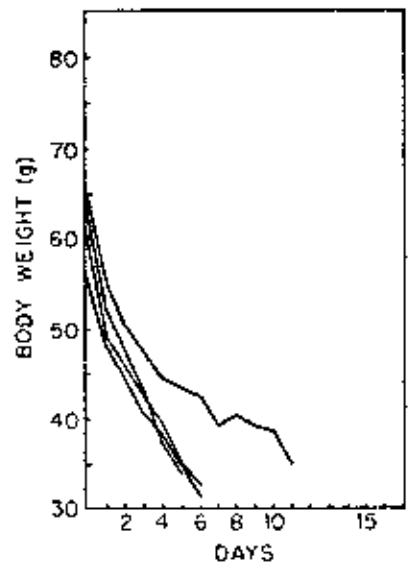



FIG. 101. RATS FED ON 20 PER CENT OF DIBUTYL CYCLOHEXENE DICARBOXYLATE

All rats died at the end of the curves.

## SEBORRHEA

The author would like to add a few words with regard to seborrhea. When rats are fed with a large amount of sperm oil, seborrhea is developed and an oily secretion comes out of their skin, making the rats look as if taken out of an oil bath. This fact was discovered by Sahashi

TABLE 82

PROPERTIES OF OLEYL OLEATE AND SPERM OIL

| | $n_D^{20}$ | Iodine Value, Wijs | Saponification Value | Acid Value |
|---|---|---|---|---|
| Oleyl oleate | 1.4612 | 88.6<br>(95.5) | 113.8<br>(105.3) | 4.9 |
| Sperm oil | 1.4640 | 86.7 | 126.4 | 0.5 |

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(1933) some time ago and was further studied by Somekawa (1933, 1938) and Kaneda (1954C, 1957).

Fig. 102 shows an example of rats with seborrhea (Matsuo 1957D). When rabbits were fed with 15 per cent sperm oil or oleyl oleate having the properties as shown in Table 82, it was also clear that severe seborrhea

Fig. 102. Example of Rat's Seborrhea

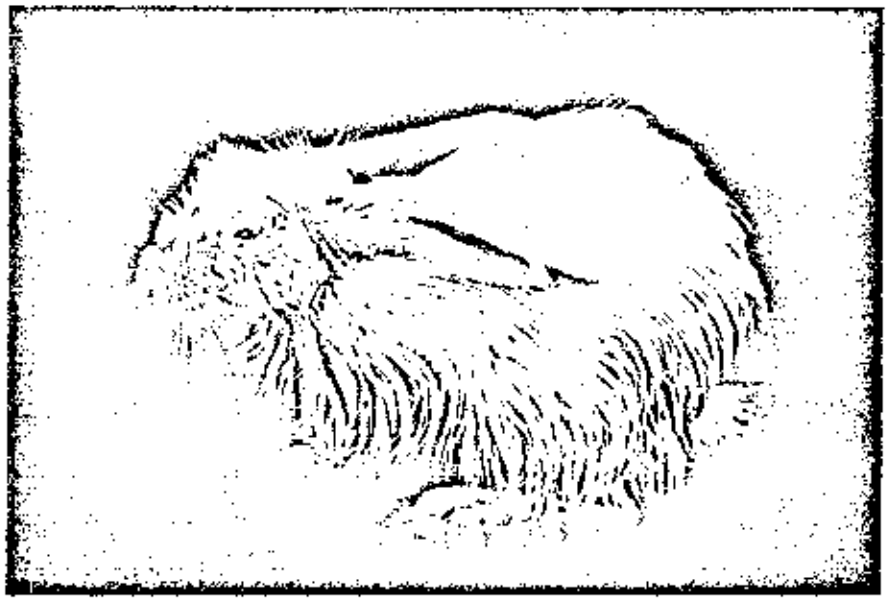


Fig. 103. Rabbit Presenting Symptoms of Seborrhea Due to Feeding with 15 Per Cent Oleyl oleate

27th day after start of experiment.



developed and they died in about a month. Figs. 103 and 104 show rabbits with seborrhea (Matsuo 1960C).

The saturated monohydroxy alcohol esters of oleic acid were prepared (Table 83), and fed to rats at a level of 15 per cent of the feed for the purpose of clarifying the relationship between the number of carbon atoms

Fig. 104. Rabbit Presenting Symptoms of Seborrhea Due to Feeding with 15 Per Cent Sperm Oil.

27th day after start of experiment.

and development of seborrhea. Up to the carbon number of 28, there were no symptoms of seborrhea. When dodecyl oleate ($C_{17}H_{33}COOC_{12}H_{25}$) and tetradecyl oleate ($C_{17}H_{33}COOC_{14}H_{29}$) were used, insignificant development of seborrhea was observed.

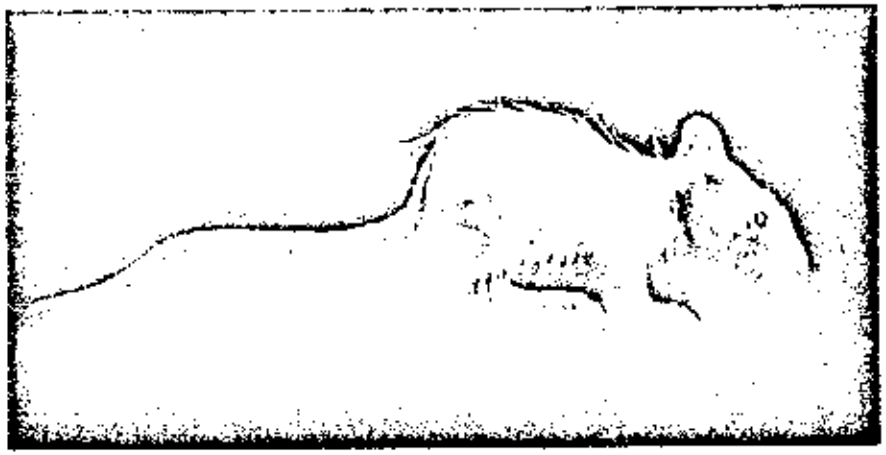


FIG. 105. RAT PRESENTING SYMPTOMS OF SEBORRHEA DUE TO FEEDING WITH 15 PER CENT 4-ETHYL-2-METHYL-4-UNDECYLOLEATE

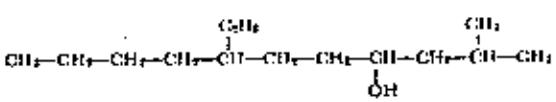
28th day after start of experiment.

TABLE 83

PROPERTIES OF OLEIC ACID ESTERS

| Ester | Formula | $n_D^{20}$ | Iodine Value | Saponification Value | Acid Value | Melting Point |
|---|---|---|---|---|---|---|
| Ethyl oleate | $C_{17}H_{33}COO-C_2H_5$ | 1.4546 | 84.8 (81.9)[1] | 180.9 | 3.9 | |
| Amyl oleate | $-C_5H_{11}$ | 1.4545 | 71.6 (72.0) | 161.0 (159.1) | 4.1 | |
| Octyl oleate | $C_8H_{17}$ | 1.4529 | 63.4 (64.3) | 142.9 (142.1) | 1.5 | |
| Decyl oleate | $-C_{10}H_{21}$ | 1.4525 | 60.2 (60.0) | 136.8 (132.7) | 2.0 | |
| Dodecyl oleate | $-C_{12}H_{25}$ | 1.4550 | 55.3 (56.3) | 126.0 (124.4) | 0.6 | |
| Tetradecyl oleate | $-C_{14}H_{29}$ | 1.4540[2] | 53.6 (53.0) | 120.9 (117.2) | 4.8 | 20.0 ~22.0 |
| Cetyl oleate | $-C_{16}H_{33}$ | 1.4540[2] | 50.0 (52.1) | 111.8 (110.7) | 3.1 | 31.0 ~32.5 |
| Octadecyl oleate | $-C_{18}H_{37}$ | 1.4540[2] | 47.5 (47.6) | 105.9 (104.9) | 3.9 | 32.0 ~33.5 |
| Oleyl oleate | $-C_{18}H_{35}$ | 1.4664 | 91.4 (95.5) | 106.9 (105.3) | 2.3 | 31.0 ~32.5 |
| Tetradecyl oleate | $-C_{14}H_{29}$[3] | 1.4539 | 54.2 (53.0) | 120.4 (117.2) | 6.2 | - |

[1] Numbers in parentheses are theoretical values. [2] $n_D^{40}$.
[3] $C_{14}H_{29}OH$: 7-ethyl-2-methyl-4-undecanol.





When cetyl oleate ($C_{17}H_{33}COOC_{16}H_{33}$) or other compounds of larger carbon number were fed, there was a marked development of seborrhea. The average rate of increase in the weight of rats generally decreased with an increase in carbon number (Table 84). For instance, when rats were fed 15 per cent of the ester of 7-ethyl-2-methyl-4-undecyl oleate, which is a saturated monohydric alcohol with a side chain of oleic acid, a particularly remarkable development of seborrhea was noticed.

TABLE 84

OCCURRENCE OF SEBORRHEA

| Ester | Formula | Occurrence of Seborrhea | Average Body Weight Increased, Gm./22 Days |
|---|---|---|---|
| Ethyl oleate | $C_{17}H_{33}COO-C_2H_5$ | ○ ○ ○ ○ ○ | 60.1 |
| Amyl oleate | $-C_5H_{11}$ | ○ ○ ○ ○ ○ | 60.3 |
| Octyl oleate | $-C_8H_{17}$ | ○ ○ ○ ○ ○ ○ ○ | 53.8 |
| Decyl oleate | $C_{10}H_{21}$ | ○ ○ ○ ○ ○ ○ ○ ○ ○ | 50.4 |
| Dodecyl oleate | $-C_{12}H_{25}$ | ◐ ○ ○ ◐ ◐ ○ ○ ○ ○ ◐ ○ ◐ ○ | 37.4 |
| Tetradecyl oleate | $C_{14}H_{29}$ | ◐ ◐ ◐ ◐ ◐ | 20.6 |
| Cetyl oleate | $-C_{16}H_{33}$ | ● ● ● ● ● | 9.0 |
| Octadecyl oleate | $C_{18}H_{37}$ | ● ● ● ● ● x | 15.0 |
| Oleyl oleate | $-C_{18}H_{35}$ | ● ● ● ● ● x x | −6.0 |
| Tetradecyl oleate | $C_{14}H_{29}$[1] | ● ● ● ● ● ● ● ● x x x | −17.5 |

○ Seborrhea not produced.
◐ Seborrhea slightly produced.
● Seborrhea produced.
[1] $C_{14}H_{29}OH$: 7-ethyl-2-methyl-4-undecanol.
x Died.

Fig. 105 shows the appearance of a rat with acute symptoms after feeding 4-ethyl-2-methyl-4-undecyl oleate (Matsuo 1960C).

## DISCUSSION

**M. E. Stansby.**—At the very beginning of your paper in the second sentence, you state "Research conducted recently made it clear that autoxidized or thermally polymerized fish oil shows acute toxicity as compared to other oils and fats." My question is, particularly with reference to the autoxidized part of it, is it not true that other oils and fats if oxidized to the same peroxide value, as you used in your experiments, would be expected to show similar results.

**N. Matsuo.**—Regarding fish oils, because they have a greater content of unsaturated fatty acids compared with the other oils, also show greater toxicity after autoxidation. I agree that if there is autoxidation in the other fat it will also show the toxicity, but this was not studied.

**J. E. Halver.**—Have you conducted any longer term feeding studies with lower levels of these autoxidized oils?

N. Matsuo.—Chronic feeding trials have been under way for the period of one year, but the data hasn't been analyzed yet. Chronic studies of three months' duration were carried out.

**A. L. Tappel.—In Japan, I understand, you have many fish products that, for example, are dried under rather severe conditions and so you do get thermal polymerization and peroxidation. Is there a human dietary problem because of the consumption of these products?**

N. Matsuo.—Most dried fish is heated or cooked before being consumed and the peroxides would therefore be decomposed.

**H. Kaunitz.—There has been repeated reports from Spain and particularly from Japan, that the lipid polymerization of fish oil does not render it unpalatable. There was one report I think by Osaki, but I may be mistaken, that when he took the oil of a fish called the plaice and strongly polymerized it and then fed it to rats, that there was no difference in growth of those rats compared to fresh oils. Do you have any experience with this?**

N. Matsuo.—I have not seen this literature.

**L. J. Machlin.—This is more in the nature of a comment than a question. I noticed that in most of the diets only nine per cent casein was used, and as Dr. Kummerow pointed out that as you increase the protein level you get some protection. Kaunitz used 30 per cent casein. I would suspect that the results are exaggerated in these studies.**

N. Matsuo.—I used twelve per cent casein and it gave similar results.

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# The Role of Tocopherol Content in the Comparative Stability of Chicken and Turkey Fat

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## INTRODUCTION

It is well known that turkey fat is much less stable than chicken fat. This difference in stability assumes economic importance to the extent that it is reflected in the seriously shorter frozen storage life of turkeys, and in the relative unacceptability in foods of rendered turkey fat compared to chicken fat. Investigations on the fundamental causes for this difference in stability have been made at this laboratory in order to provide a basis for the development of methods to increase shelf life of frozen poultry, particularly turkeys.

Of the two recognized chemical factors governing the resistance of natural fats to rancidification, *i.e.*, content of fatty acids of varying degrees of unsaturation and content of natural antioxidants, fatty acids have received more attention than antioxidants in most of the previous studies on poultry fat. Studies by Hilditch *et al.* (1931), Cruickshank (1934), Kummerow *et al.* (1950), Chu and Kummerow (1950), and Klose *et al.* (1951, 1952) have shown that the fatty acid composition of the depot fat in poultry, and its corresponding stability, are directly related to the amount and fatty acid composition of the ingested fat. Nutter *et al.* (1943) compared certain chemical and physical constants of depot fats from chickens and turkeys fed unspecified and presumably different commercial diets, and found no differences that were consistent enough or large enough to explain the great difference in stability between commercial chicken fat and turkey fat. They suggested the possibility that the inferior stability of turkey fat is due to a lower content of natural antioxidants, such as tocopherols. Cridlle and Morgan (1947, 1951) fed tocopherols to turkeys at dietary levels of 0.01, 0.1, 1 or 2% for 2.5 to 35 days before slaughter and found corresponding measurable increases in tocopherol content of the tissues and storage life of the frozen carcasses.

Objectives of the work reported here were to compare chickens and turkeys maintained on the same diet for their contents of fatty acids and tocopherol in the depot fat, in order to determine the cause of inferior stability of turkey fat. Comparative efficiencies of deposition of added dietary tocopherol exhibited by chickens and turkeys were also sought. This study thus concerns the major role that tocopherol content plays in establishing fat stability for the two species and provides quantitative evidence of the much greater efficiency of deposition of tocopherol by the chicken compared to the turkey.



## EXPERIMENTAL METHODS

Chickens and turkeys were purchased from a commercial source and were held in wire cages and fenced concrete yards respectively for the various feeding periods. The basal diet for both species was identical, a 16 percent protein turkey growing mash. Fresh batches of the tocopherol-supplemented diets were mixed from the same lot of a basal diet at biweekly intervals. All groups were furnished feed and water *ad libitum*.

At the end of the various feeding periods, the birds were starved overnight, shocked to prevent struggling, bled with an outside neck cut, scalded at 127°F., picked by machine, and immediately eviscerated. The gizzard fat (fat surrounding the gizzard), skin fat (fat in and attached to the skin, down to the meat), dark meat (legs and thighs), and light meat (breast) were removed and stored separately by groups in sealed polyethylene bags at −30°F.

In preparation for analysis, the frozen tissue was cut into approximately one-half-inch cubes and dried *in vacuo* from the frozen state. The dried gizzard fat (tissue) was warmed to about 60°C. and the liquid fat was then expressed in a small hydraulically operated, stainless-steel press. Dried skin was either pressed out in the same manner or extracted in an all-glass Soxhlet extractor for 3.5 to 4 hours with redistilled petroleum ether (b.p. 40-50°C.). Dried meat samples were extracted with petroleum ether. Solvent was removed from the various extracts by heating on a hot-water bath under the vacuum of a water aspirator, with a stream of dry nitrogen passing through the extract. All fat samples were finally filtered through Whatman No. 2 filter paper and stored in completely filled bottles at −30°F.

Tocopherol content of the fats was determined essentially by the method of Quaife and Harris (1948) with the Emmerie-Engel reagent. The hydrogenation step was eliminated after it was established by analyses with and without hydrogenation that, for the depot fats involved, contribution by carotenoids or other extraneous reducing substances to the apparent tocopherol content was negligible. Carotenoids were also roughly measured by determining their absorbancy at 450 mμ, and their contribution to the color developed with the Emmerie-Engel reagent, from which it was estimated that carotenoids in no case contributed more than 2 percent of the total apparent tocopherol value. Induction periods for oxygen uptake of the fats were determined under an atmosphere of oxygen at 70°C. in a Barcroft-Warburg constant volume manometric apparatus, as described by Johnston and Frey (1941) but with modified flasks. Fatty acid composition of the fats was determined by the method of Brice *et al.* (1945, 1952), and Herb *et al.* (1951, 1952) with a Cary Recording Spectrophotometer Model 11. Poultry fats contain appreciable amounts of lower homologues of oleic acid (palmitoleic) and also small percentages of pentaenoic acids and hexaenoic acids contributed by fish products in the diet. Hence, the applied spectrophotometric methods can not be expected to give strictly quantitative results, and this fact should be taken into

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consideration in the interpretation of the data. In the fatty acid analyses for experiment B, an estimate of the percentage of acids with five or more double bonds, expressed as clupanodonic acid, was made. Calculations were based on the absorption constants reported by Herb *et al.* (1951, 1952) for docosapentaenoic acid. This estimate necessarily involves broad assumptions, particularly that the compound(s) absorbing at 340 mμ, contributed mainly by dietary fish oil, can be considered to be the same as, or have the same absorption characteristics as, docosapentaenoic acid isolated from adrenal lipids by Herb *et al.* (1951). Consequently the results should be interpreted in a comparative rather than an absolute manner. Inclusion of clupanodonic acid into the analytical scheme did not significantly alter the comparative relations between chickens and turkeys for any of the other fatty acids. Iodine numbers were obtained by Wijs method with a one-half hour reaction period in the dark. Peroxide values were determined by a modification of the Wheeler iodometric method (Pool *et al.*, 1950) and carbonyl (aldehyde) values by the method of Pool and Klose (1951).

Organoleptic evaluation of birds that had been stored in polyethylene bags at 0–10°F. for nine months were made by an eight-member trained taste panel. Half birds were roasted without seasoning in a horizontally rotating electric oven at 300°F. to an internal temperature of 180°F. in the breast. Light meat, dark meat, skin fat, and pan drippings were scored for degree of rancid off-flavor, 0 signifying no off flavor and 10 maximum rancidity (inedible). Because of inherent flavor differences between chicken and turkey meat, no direct comparisons were made between species, but rather comparisons were made between basal and tocopherol-supplemented groups within species.

## EXPERIMENT AND RESULTS

As a preliminary experiment, small numbers of chickens, turkeys, ducks, geese and pigeons were obtained from commercial farms, slaughtered, and the depot fat surrounding the gizzard analyzed for its fatty acid composition. The results, summarized in Table 1, indicate a considerable variability between lots within species for either chickens or turkeys, and a general overlapping of values between species. It was evident that in order to establish whether or not species differences exist, it would be necessary to maintain the two species on the same diet for a reasonable length of time prior to slaughter and analysis. Previous results with turkeys (Klose *et al.*, 1952) had indicated that a fairly rapid equilibration took place between composition of dietary fat and that of carcass depot fat, so the two experiments to be described below were conducted with feeding periods ranging from five to ten weeks.

*Experiment A.* Twelve New Hampshire cockerels, twenty-six weeks of age, and twelve Beltsville White toms, sixteen weeks of age were obtained from the same commercial source and were fed the same diet (a 16% protein turkey growing mash) for six weeks. At this time six birds of each species were slaughtered, while the remaining birds were fed for an additional three weeks before slaughtering. Gizzard fat, skin fat obtained by pressing, skin fat by extraction, and dark meat fat were analyzed for crude tocopherols and fatty acids, and the induction periods determined (Table 2).

The much greater stability (induction period) of chicken fat over turkey fat was positively correlated with the higher content of tocopherols in the chicken fat. Differences in fatty acid content between the species were small and probably of no great significance in establishing the over-all difference in stability. In fact, the difference in diene (linoleic) acid content is in a direction tending to counteract rather than to augment the difference in stability. In general, there were no appreciable differences between six and nine week feeding periods with respect to fatty acid contents, and the differences in the case of tocopherol content and induction period appear to rule out any possibility that the values for the two species would have become closer on prolonged feeding of the same diet. Gizzard fat and skin fat were for the most part quite similar. Fat extracted from the dark meat contained a much greater concentration of tetraene fatty acids and tocopherol than did the gizzard or skin fat, and the corresponding induction periods were much longer than those for the gizzard and skin fats.

*Experiment B.* Thirty-two New Hampshire female chickens, ten weeks of age, and the same number of seventeen week-old, Broad Breasted Bronze female turkeys were obtained from a commercial source. Four representative birds from each species were slaughtered immediately and processed as described above for experiment A. Of the remaining twenty-eight birds for each species, fourteen were fed the basal diet (16% protein turkey growing mash) and fourteen were fed the basal diet plus 0.1% tocopherol, added as d-alpha tocopherol acetate. After five weeks on these diets, six birds from each of the four groups were slaughtered and processed as described above;

TABLE 1.—*Summary of results for fatty acid composition for different species of poultry*

| Species | No. of lots | Iodine No. | Saturated Acids (%) | Oleic acid (%) | Linoleic acid (%) | Linolenic acid (%) | Arachidonic acid (%) |
|---|---|---|---|---|---|---|---|
| Chicken | 4 | 63–80 | 28–31 | 47–51 | 14–18 | 0.7–1.0 | 0.3–0.5 |
| Turkey | 6 | 73–79 | 28–33 | 39–51 | 13–21 | 0.8–1.3 | 0.2–0.7 |
| Duck | 1 | 87 | 27 | 42 | 24 | 1.3 | 0.20 |
| Goose | 1 | 67 | 30 | 57 | 8 | 0.4 | 0.05 |
| Pigeon | 1 | 82 | 23 | 56 | 17 | 0.7 | 0.14 |



TABLE 2.—*Experiment A. Composition and stability of fat from male chickens and turkeys fed the same diet*

| Sample | Feeding time (wks.) | Iodine No. | Saturated acids (%) | Monoene "oleic" acids (%) | Diene "Linoleic" acids (%) | Triene "Linolenic" acids (%) | Tetraene "Arachidonic" acids (%) | Tocopherol (mg.) | Induction period (hrs.) |
|---|---|---|---|---|---|---|---|---|---|
| Chicken | | | | | | | | | |
| Gizzard Fat (Pressed) | 6 | 83.3 | 37 | 32 | 25 | 1.8 | 0.4 | 120 | 80 |
| | 9 | 86.6 | 36 | 30 | 28 | 1.7 | 0.6 | 140 | 70 |
| Skin Fat (Pressed) | 6 | 86.0 | 35 | 31 | 27 | 1.8 | 0.6 | 110 | 80 |
| | 9 | 86.3 | 36 | 31 | 27 | 1.8 | 0.6 | 90 | 50 |
| Skin Fat (Extracted) | 6 | 87.2 | 35 | 32 | 27 | 1.7 | 0.7 | 100 | 60 |
| | 9 | 86.5 | 35 | 32 | 27 | 1.6 | 0.7 | 130 | 100 |
| Dark Meat (Extracted) | 6 | 93.6 | 31 | 36 | 25 | 0.6 | 3.3 | 400 | 150 |
| | 9 | 93.1 | 31 | 35 | 26 | 0.7 | 2.5 | 280 | 220 |
| Turkeys | | | | | | | | | |
| Gizzard Fat (Pressed) | 6 | 82.2 | 37 | 32 | 23 | 1.8 | 0.7 | 21 | 26 |
| | 9 | 81.1 | 36 | 37 | 21 | 1.7 | 0.6 | 14 | 17 |
| Skin Fat (Pressed) | 6 | 85.2 | 35 | 33 | 24 | 2.2 | 0.6 | 22 | 21 |
| | 9 | 82.8 | 35 | 38 | 21 | 1.8 | 0.6 | 13 | 13 |
| Skin Fat (Extracted) | 6 | 84.5 | 36 | 32 | 24 | 2.1 | 0.7 | 23 | 23 |
| | 9 | 82.7 | 35 | 38 | 21 | 1.9 | 0.6 | 15 | 14 |
| Dark Meat (Extracted) | 6 | 94.2 | 32 | 35 | 23 | 0.5 | 4.3 | 180 | — |
| | 9 | 89.7 | 32 | 39 | 20 | 0.2 | 4.1 | 100 | (26) |

Table 3.—*Experiment B. Composition and stability of gizzard fat from female chickens and turkeys on a normal and a tocopherol supplemented diet*

| Diet | Feeding period (wks.) | I. No. | Saturated acids (%) | Monoene "Oleic" acids (%) | Diene "Linoleic" acids (%) | Triene "Linolenic" acids (%) | Tetraene "Arachidonic" acids (%) | Pentaene "Clupanodonic" acids (%) | Tocopherol (γ/gm.) | Induction period (hrs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| *Chickens* | | | | | | | | | | |
| Basal | 0 | 84.1 | 34 | 35 | 26 | 1.6 | 0.4 | 0.4 | 35 | 42 |
| Basal + "Tocopherol"* | 5 | 81.3 | 35 | 35 | 23 | 2.4 | 0.2 | 0.2 | 68 | 66 |
| Basal | 10 | 78.7 | 34 | 40 | 19 | 2.3 | 0.2 | 0.2 | 29 | 46 |
| Basal + Tocopherol | 10 | 80.8 | 34 | 38 | 21 | 2.4 | 0.2 | 0.2 | 400 | 126 |
| *Turkeys* | | | | | | | | | | |
| Basal | 0 | 77.5 | 35 | 39 | 19 | 2.1 | 0.1 | 0.5 | 10 | [illegible] |
| Basal | 5 | 80.6 | 35 | 37 | 21 | 2.3 | 0.1 | 0.8 | 50 | [illegible] |
| Basal + Tocopherol | 5 | 79.8 | 35 | 37 | 21 | 2.3 | 0.1 | 0.2 | 50 | [illegible] |
| Basal | 10 | 79.0 | 34 | 40 | 19 | 2.4 | 0.1 | 0.5 | 1 | [illegible] |
| Basal + Tocopherol | 10 | 79.9 | 34 | 40 | 19 | 2.3 | 0.1 | 0.5 | 80 | [illegible] |

* d-α-Tocopherol acetate added to basal diet at level of 0.1%.

the remaining eight birds from each group were maintained on the diets for an additional five weeks before processing. Two birds from each experimental condition of species—diet-feeding period were set aside at −30°F. for fat analyses, while the remainder were stored in evacuated polyethylene bags at +10°F. for nine months before subjecting to chemical and organoleptic analysis.

Results of the analyses of the fresh gizzard fat for experiment B are summarized in Table 3. Conclusions drawn from experiment A were confirmed. Induction periods and tocopherol contents were positively correlated, and were markedly greater in chickens than in turkeys. The effect of tocopherol supplementation was pronounced, with the greatest relative increase taking place in the first five weeks. There was no tendency for the tocopherol contents for the two species to approach each other on continued feeding of the same diet. Also, fatty acid contents did not change appreciably with time, and for the most part were the same for the two species. The apparent content of fatty acids with greater than four double bonds (e.g. clupanodonic acid) was higher in turkeys than in chickens, while the reverse was true for fatty acids containing four double bonds. However, the significance or effect on stability of

Table 4.—*Effect of tocopherol supplementation on development of peroxides in frozen storage at +10°F. for 9 months*

| | Peroxide values* (Milliequivalents/kg. raw fat) | | | | | |
|---|---|---|---|---|---|---|
| Supplementation period | 5 weeks | | | 10 weeks | | |
| Location of fat sample | Back | Neck | Visceral | Back | Neck | Visceral |
| *Chickens* | | | | | | |
| Basal | 2.5 | — | — | 2.7 | 9 | 25 |
| Basal + Tocopherol | 0.6 | — | — | 0.9 | 9 | 27 |
| *Turkeys* | | | | | | |
| Basal | 2.6 | 19 | 120 | 0.6 | 5 | 88 |
| Basal + Tocopherol | 1.4 | 8 | 32 | 0.5 | 4 | 16 |

* Each value is mean of determinations for 4 birds.



Table 5.—*Effect of tocopherol supplementation on development of carbonyl compounds in frozen storage at +10°F. for 9 months*

| | Carbonyl values* (Millimoles/kg. raw fat) | | | | | |
|---|---|---|---|---|---|---|
| Supplementation period | 5 weeks | | | 10 weeks | | |
| Location of fat sample | Back | Neck | Visceral | Back | Neck | Visceral |
| *Chickens* | | | | | | |
| Basal | 0.09 | — | | 0.11 | 0.21 | 0.46 |
| Basal + Tocopherol | 0.09 | — | — | 0.08 | 0.11 | 0.23 |
| *Turkeys* | | | | | | |
| Basal | 0.11 | 0.50 | 0.37 | 0.13 | 0.19 | 2.16 |
| Basal + Tocopherol | 0.12 | 0.15 | 0.63 | 0.11 | 0.13 | 0.30 |

* Each value is mean of determinations for 4 birds.

these small absolute differences is not known.

Data on development of rancidity during 9 months storage at +10°F. are presented in Table 4 for peroxides, in Table 5 for carbonyl compounds, and in Table 6 for organoleptic scores of the cooked birds. Samples of depot fat available in the chicken carcasses were in several cases too small for accurate analysis. Although the 9 months period proved to be too short for development of serious rancidity in either species, with a few exceptions the samples from tocopherol supplemented birds had less peroxides, carbonyls, and rancid off flavor than their unsupplemented counterparts. In general the differences due to tocopherol were more pronounced and significant in turkeys than in chickens. The decreasing stability of back, neck and visceral fat in that order has been reported previously (Pool *et al.*, 1950).

*Supplementary Experiments with Synthetic Mixtures of Fat and Tocopherol.* Induction periods were determined for turkey fat having a natural tocopherol content of 10 micrograms per gram, and for samples of this same fat to which various amounts of α-tocopherol were added. Results in Table 7 indicate the marked response to additional tocopherol and the diminishing effect of successive additions.

Table 6.—*Effect of tocopherol supplementation on frozen storage stability as measured by roasted flavor (storage for 9 months at +10°F.)*

| | Panel mean scores* of roasted carcass | | | | | |
|---|---|---|---|---|---|---|
| Supplementation period | 5 weeks | | | 10 weeks | | |
| Type of sample tasted | Light meat | Dark meat | Skin | Light meat | Dark meat | Skin |
| *Chickens* | | | | | | |
| Basal | 0.91 | 1.44 | 2.28 | 0.72 | 0.97 | 1.31 |
| Basal + Tocopherol | 0.59 | 0.91 | 1.97 | 0.53 | 1.00 | 1.81 |
| *Turkeys* | | | | | | |
| Basal | 1.12 | 1.97 | 2.66 | 0.81 | 1.31 | 2.05 |
| Basal + Tocopherol | 0.31** | 1.44 | 1.66* | 0.72 | 0.62 | 1.00** |

* Scoring on scale of 0 to 10, with 0 signifying no detectable rancidity and 10 signifying extreme rancidity. Values represent direct comparisons only for Basal vs. Basal + Tocopherol. Each value is mean of 32 determinations (8 judges × 4 samples).

* Significant difference between basal and basal + tocopherol at 5% level.

** Significant difference between basal and basal + tocopherol at 1% level.

TABLE 7.—*Effect of tocopherol additions on stability of turkey fat*

| Tocopherol | | | |
|---|---|---|---|
| Naturally Present γ/gm. | α Tocopherol Added γ/gm. | Total Amount Present γ/gm. | Induction Period (Hours) |
| 10 | | 10 | 19 |
| 10 | 20 | 30 | 45 |
| 10 | 40 | 50 | 58 |
| 10 | 60 | 70 | 67 |

In another experiment, the induction period of a typical turkey fat was determined before and after stripping of its tocopherol content by molecular distillation. A sample of the stripped fat was restored to its original tocopherol content by the addition of pure α tocopherol, and the induction period again determined. A comparison of the resulting induction periods in Table 8 shows that even at relatively low tocopherol contents, the major portion of the induction period of turkey fat is attributable to tocopherol content.

TABLE 8. *Effect of tocopherol removal on stability of turkey fat*

| Treatment | Tocopherol Removed γ/gm. | Tocopherol Added α-tocopherol γ/gm. | Induction period (hours) |
|---|---|---|---|
| None | — | — | 19 |
| Distillation at 200° C. (10 Min., at 1 Micron) | 9.0 | — | 4 |
| Distillation at 200° C. (10 Min., at 1 Micron) | 9.0 | 9.0 | 18 |

## DISCUSSION

The general relationships between tocopherol content and stability, and between efficiencies of tocopherol deposition in the two species, do not appear to be greatly influenced by sex or by appreciable differences in age of the experimental birds. General over-all agreement in fatty acid composition between species is apparent in both experiments A and B. Small differences between the values for any particular fatty acid or group of fatty acids should be interpreted with caution. Bird to bird variation, including variation in degree of fattening or finish, may be responsible for most of the differences. Values for "eicosapentaenoic" acid and "arachidonic" acid should be considered only as approximate or indicative, because they have been calculated on rather broad assumptions regarding the optical absorbancies (after alkali isomerization) of fatty acids in poultry depot fat which have four or more double bonds.

Compared with the other types of fat studied, the dark meat extract had a many fold greater amount of fatty acids with four or more double bonds, which suggests that we were dealing with a more complex mixture containing appreciable amounts of phospholipids in addition to simple triglycerides.

Variability in tocopherol content and induction period observed between basal groups of either chickens or turkeys in experiment B (basal at beginning, basal after 5 weeks, and basal after 10 weeks) is indicative of the over-all experimental error involved in these measurements. It is fortunate that the effects of tocopherol supplements are so great as to be obviously significant in the face of such large variability.

A complete explanation of the greater efficiency of deposition of tocopherol in the chicken compared to the turkey is not apparent from existing data. An additional, cooperative experiment with chickens and turkeys during the entire rapidly growing period, with short term tocopherol supplementation just prior to slaughter, has been completed and reported in a companion paper.

## SUMMARY

Chickens and turkeys were maintained on the same diet, with and without tocopherol supplements, for various periods and the results were compared with respect to fatty acid composition, tocopherol content, and stability of fat. With birds on a natural dietary level of tocopherol, the efficiency of deposition of tocopherol in the carcass fat was much greater for the chicken than for the turkey, and stabilities were in corresponding order. A dietary supplement of 0.1% tocopherol for periods of five and ten weeks increased the tocopherol content and stability (induction period) of carcass fat in both species, but the superiority of chicken over turkey was maintained or enhanced. The stabilizing effect of tocopherol supplements was confirmed by chemical and organoleptic tests on carcasses stored at 0°F. for nine months.

There was no appreciable difference in the fatty acid composition of depot fats between chickens and turkeys fed the same diet. It was concluded that tocopherols play a major role in establishing the stability of poultry fat. Additional measurements of induction periods of turkey fat before and after removal of tocopherol confirmed this conclusion.

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# POSITIONAL DISTRIBUTION OF FATTY ACIDS IN FISH AND OTHER ANIMAL LECITHINS

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## SUMMARY

Phosphatidyl choline (lecithin) fractions were prepared from tuna, salmon, and menhaden muscle, from egg yolk, and from rat and beef liver. Hydrolysis by snake-venom phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) was used to determine the positional distribution of the fatty acids. The fatty acid composition of the hydrolyzed fatty acids ($\beta$ position) and those remaining with the phosphatidyl moiety ($\alpha'$ position) were separately determined by gas-liquid chromatography of the methyl esters. 36–86 mole % of the fatty acids esterified in the $\alpha'$ position were saturated; 91–99 mole % of the fatty acids in the $\beta$ position were unsaturated. Palmitic and stearic acids were the predominant saturated acids in the $\alpha'$ position. Some similarity was noted in the $\beta$-position distribution of linoleic, arachidonic, eicosapentaenoic, docosatetraenoic, docosapentaenoic, and docosahexaenoic acids. This observation tends to support the hypothesis of the interchangeability of linoleic and arachidonic acids with "non-essential" polyenoic acids.

## INTRODUCTION

This work was initiated as an extension of studies on the nature of the phospholipids of tuna[1] and menhaden[2]. It is known that phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) of snake venom specifically hydrolyzes the $\beta$ position of both synthetic and naturally occurring phospholipids[3–8], and that these fatty acids are primarily unsaturated[4]. The fish lecithins are characterized by large amounts of highly unsaturated fatty acids[1,2], hence it appeared of interest to determine the positional distribution of these fatty acids in them.

During the course of this investigation BROCKERHOFF *et al.*[9] described the specific distribution of fatty acids in the phospholipids and triglycerides of several marine organisms: cod (*Gadus callarias*), scallop (*Platopectin magellaricus*) and lobster (*Homarus americanus*). The present paper contains our results obtained by similar means on lecithins isolated from albacore tuna (*Thunnus alalunga*), menhaden (*Brevoortia tyrannus*), and salmon (*Oncorhynchus tshawytscha*) muscle, and from beef liver, rat (Long–Evans) liver, and chicken egg yolk.

## EXPERIMENTAL PROCEDURE

### A. Purification of lecithins

Tissues were homogenized in chloroform–methanol (1:1, v/v) solution. Lipids were extracted, and fractionated by column chromatography on silicic acid as described previously[1]. The lecithin fractions showed only one component with the thin-layer silicic acid chromatography system of VOGEL *et al.*[10]. The product used in each case contained the bulk of the lecithin fraction, to avoid fractionation with regard to the fatty acid content. Ester[11], phosphorus [12] and plasmogen contents[13] were determined for each lecithin. These, together with the specific optical rotation in chloroform solution, and the ester/phosphorus ratios are set forth in Table I. During the preparation of the fractions trace amounts of hydroquinone were added as an antioxidant, and all samples were stored under $N_2$.

### B. Liberation of fatty acids by phospholipase A (ref. 4)

30 mg of each lecithin were dissolved in peroxide-free diethyl ether to give a final concentration of 20 $\mu$moles/ml. Lyophilized venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) (Ross Allen's Reptile Institute, Silver Spring, Florida) was dissolved in a solution of 0.1 M Tris buffer, 20 mM $CaCl_2$ and 2 mM EDTA (tetrasodium salt) to give a final concentration of 2.0 $\mu$g of venom per $\mu$l. The buffered enzyme was adjusted to pH 7.4. To each ml of the etheral solution of lecithin, 10 $\mu$l of the enzyme solution was added. The mixture was shaken vigorously for 30 sec and allowed to stand at room temperature for 2 h. 10-$\mu$l aliquots were taken at fixed time intervals to check the progress of the reaction by chromatographing the aliquot directly in the thin-layer system mentioned above. The reaction was complete by this test at the end of 2 h. The ether solution was evaporated to dryness under $N_2$ and the residue dissolved in a minimum of solvent (0.5% methanol in chloroform). The resulting solution was transferred to a 12 mm × 155 mm column containing 5 g of silicic acid equilibrated in the same solvent. The free fatty acids liberated during the reaction were eluted from the silicic acid column by an additional 40 ml of solvent. Elution of unreacted lecithin (trace amounts) was accomplished by the addition of 40 ml of 25% methanol in chloroform to the column. The remaining reaction product, lysolecithin, was eluted from the column with 90 ml of 85% methanol in chloroform. Each of the fractions isolated showed only a single spot corresponding to an authentic sample when subjected to chromatography on the thin-layer silicic acid system. Approx. 90% of the amount used was recovered.

### C. Preparation and analysis of methyl esters of fatty acids

Methyl esters of the fatty acids liberated during the reaction were prepared from the first silicic acid chromatography fraction by reaction with 3% (v/v) conc. $H_2SO_4$ in anhydrous methanol for 2 h at boiling, followed by extraction with light petroleum and washing with water.

The fatty acids remaining esterified to the lysolecithin fraction were methanolized in 5 ml of chloroform–methanol (1:1, v/v) to which was added 1.0 ml of 0.5 N sodium methoxide[14]. The reactants were cooled to 0° before reaction, mixed rapidly, allowed

to stand in the cold for 20 min, then allowed to warm to room temperature, neutralized with 2.5 ml of 3% HCl in anhydrous methanol and evaporated to dryness. The residue was suspended in water and extracted with light petroleum.

Methyl esters from the various fractions were analyzed by gas–liquid chromatography. An Aerograph Hy–Fi (Wilkens Instrument Co., Walnut Creek, Calif.) flame-ionization instrument was used. It was equipped with a 366-cm, 3-mm O.D. glass column which had been treated with dichlorodimethylsilane and packed with 1% EGSS-X on 100–120-mesh hexamethyldisilizane-treated Gas-Chrom P (Applied Science Laboratories, State College, Pa.). The separation was conducted at 190° with $N_2$ (40 ml/min) as the carrier gas. The instrument was calibrated with authentic standards and National Institute of Health standard mixtures of fatty acid methyl esters.

## RESULTS AND DISCUSSION

The data of Table I illustrate the nature of the preparations. Although all lecithin preparations showed only one spot by thin-layer chromatography and corresponded to the expected infrared absorption spectra, the ester/phosphorus ratios are lower for the tuna and menhaden lecithin samples than can be accounted for by their content of plasmalogen. They probably therefore contained small amounts of non-lecithin lipid material. Analyses for the other lecithin preparations gave the expected results within the limits of precision of the methods used. By using the total fraction from the silica-gel column chromatography, it was hoped to avoid the initial fractionation of lecithins noted by RHODES AND LEA[15].

TABLE I

CHEMICAL AND PHYSICAL PROPERTIES OF ISOLATED LECITHINS

| *Source* | *Specific rotation* ($\alpha_D^{25}$) *°* | *P content* (%) | *Ester content* (*μequiv. per 100 mg*) | *Ester/P ratio* | *Mole % plasmalogen* |
|---|---|---|---|---|---|
| Salmon muscle | +4.4 | 3.60 | 2.06 | 1.78 | 5.5 |
| Tuna muscle | −3.6 | 3.94 | 2.22 | 1.75 | 8.7 |
| Menhaden muscle | −5.0 | 3.70 | 2.28 | 1.92 | 5.8 |
| Egg yolk | +6.6 | 3.76 | 2.40 | 2.04 | 1.5 |
| Rat liver | −6.1 | 3.59 | 2.13 | 1.84 | 3.8 |
| Beef liver | +6.0 | 4.00 | 2.61 | 2.02 | 3.8 |

The specific distribution of fatty acids in the lecithins examined is presented in Table II and summarized in Table III. It is apparent from Table III that, regardless of the origin of the lecithin, the $\beta$ position is predominantly unsaturated and that the distribution of saturated and unsaturated fatty acids in the $\beta$ position is very nearly the same in all samples while the mole per cent of saturated fatty acids in the $\alpha'$ position varies considerably. Egg-yolk, menhaden-muscle and rat-liver lecithins were similar in the degree of saturation of the $\alpha'$ position (80%) while beef liver was of an intermediary value (60%) and salmon and tuna muscle of the lowest degree of saturation (35%). Palmitic acid was the predominant saturated fatty acid in the $\alpha'$ position. Indeed, palmitic and stearic acids represented the highest positional enrich-

TABLE II

SPECIFIC DISTRIBUTION OF THE PRINCIPLE FATTY ACID FROM THE ISOLATED LECITHINS

Identification is based on relative retention times of authentic standards of the fatty acid methyl ester or on the relative retention times of homologs followed by hydrogenation and rechromatography. The notation in the first column includes the number of carbon atoms followed by the number of double bonds, "tr" denotes trace amounts; *e.g.*, < 0.1%.

| Tentative identification of fatty acids | Weight per cent of specific fatty acid isolated from | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Salmon | | | Menhaden | | | Tuna | | | Egg | | | Rat liver | | | Beef liver | | |
| | Total | α' | β | Total | α' | β | Total | α' | β | Total | α' | β | Total | α' | β | Total | α' | β |
| 14:0 | 0.7 | 1.9 | 0.6 | 0.5 | tr | 1.3 | 0.7 | tr | 1.0 | tr | 2.3 | 0 | 0.1 | 0 | 0.4 | 0.2 | 0.7 | 0.3 |
| 14:1 | | | | tr | tr | tr | | tr | 1.8 | tr | 0 | tr | | | | | | |
| 16:0 | 22 | 37 | 10 | 45 | 78 | 0.2 | 19 | 36 | 5.0 | 34 | 61 | 4.8 | 16 | 32 | 2.1 | 11 | 19 | 1.2 |
| 16:1 | 8.0 | 1.4 | 3.8 | tr | 0 | tr | 2.1 | tr | 2.3 | 2.2 | 0 | 1.7 | 0.6 | tr | 1.1 | 1.7 | 2.9 | 1.7 |
| 18:0 | 2.0 | 2.7 | tr | 3.2 | 5.9 | 0.9 | 5.0 | 7.9 | 1.0 | 14 | 25 | 2.4 | 27 | 50 | 0.9 | 23 | 46 | tr |
| 18:1 | 6.7 | 8.2 | 23 | 7.4 | 2.3 | 13 | 7.6 | 6.4 | 16 | 35 | 9.5 | 59 | 5.6 | 3.5 | 6.6 | 18 | 19 | 17 |
| 18:2 | tr | tr | tr | 1.0 | tr | 2.4 | 0.7 | tr | 2.2 | 13 | 2.4 | 26 | 12 | 5.2 | 24 | 8.9 | 3.1 | 16 |
| 18:3 | tr | tr | tr | tr | tr | tr | 0.8 | tr | 1.1 | tr | 0 | tr | 0.3 | tr | 0.7 | 1.1 | 0.5 | 1.8 |
| 20:4 | 1.0 | 0.6 | 0.3 | 2.2 | tr | 0.9 | 2.7 | 1.3 | 5.0 | 2.0 | 0 | 6.3 | 26 | 4.4 | 45 | 8.0 | 2.0 | 18 |
| 20:5 | 12 | 14 | 17 | 13 | 1.7 | 29 | 7.1 | 8.4 | 15 | 0 | 0 | 0 | 1.5 | 4.2 | 2.6 | 0.8 | tr | 2.3 |
| 22:1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.5 | 2.3 | 15 |
| 22:4 | tr | tr | tr | tr | tr | tr | tr | 0 | 0.5 | 0 | 0 | 0 | tr | tr | tr | 2.5 | 1.0 | 6.9 |
| 22:5 | tr | tr | tr | tr | tr | tr | tr | tr | tr | 0 | 0 | 0 | tr | tr | 1.8 | 6.1 | 2.5 | 14 |
| 22:6 | 43 | 33 | 46 | 28 | 12.5 | 42 | 55 | 39 | 48 | 0 | 0 | 0 | 11 | tr | 14 | 2.1 | tr | 5.6 |

TABLE III

RELATIVE DISTRIBUTION OF SATURATED AND UNSATURATED FATTY ACIDS IN THE $\alpha'$ AND $\beta$ POSITIONS OF LECITHIN

| *Source* | *Position* | *Mole % saturated fatty acids* | *Mole % unsaturated fatty acids* |
|---|---|---|---|
| Salmon muscle | $\alpha'$ | 38 | 62 |
| | $\beta$ | 9 | 91 |
| Menhaden muscle | $\alpha'$ | 81 | 19 |
| | $\beta$ | 7 | 93 |
| Tuna muscle | $\alpha'$ | 36 | 64 |
| | $\beta$ | 4 | 96 |
| Egg yolk | $\alpha'$ | 86 | 14 |
| | $\beta$ | 6 | 94 |
| Rat liver | $\alpha'$ | 82 | 18 |
| | $\beta$ | 3 | 97 |
| Beef liver | $\alpha'$ | 66 | 34 |
| | $\beta$ | 1 | 99 |

ment of any of the fatty acids found. In the fish lecithins, docosahexaenoic acid was the other major fatty acid; however, in the egg-yolk, rat- and beef-liver materials, lower unsaturated fatty acids completed the balance of fatty acids.

Only small amounts of arachidonic acid were found in the fish lecithins; oleic, eicosapentaenoic and docosahexaenoic acids were the prevalent fatty acids in $\beta$ position. Beef-liver lecithin contained some of the fatty acids normally encountered in marine organisms. Docosamonoenoic acid was tentatively identified only in the beef sample and was primarily esterified to the $\beta$ position. Trace amounts of odd chain length and branch-chained fatty acids were also detected in the beef sample but were not positively identified. These fatty acids are possibly the products of rumen bacteria.

The fatty acid composition of the lecithin of egg yolk shows good correlation with that reported by other workers; *e.g.*, in the $\alpha'$ position, palmitic acid 61%, stearic acid 25%, oleic acid 10%, compared to palmitic acid 65%; stearic acid 33%, oleic acid 1% (ref. 5), and palmitic acid 55%, stearic acid 36%, and oleic acid 6% (ref. 16); the correlation is also good for the $\beta$ position. In the more complex lecithins containing a greater proportion of higher polyenoic acids, two difficulties are of importance: first, the quantitative problem of gas-liquid chromatography, and second, contamination with plasmalogen (compare Table I).

The difficulty with quantitative gas chromatography is illustrated by the data in Table II. Particularly in the fatty acid analyses of the fish-lipid fractions, there are discrepancies in the amounts of total compared to the amounts in the $\alpha'$ and $\beta$ position. Since careful repetition failed to resolve this difficulty, we are inclined to conclude that it represents an inherent weakness of the methods or equipment. Previous investigators more often than not do not include data for total fatty acids so that direct comparisons are not possible. However DE TOMAS *et al.*[17] have recently published an analysis of phosphatidylethanolamine fatty acids from rats fed diets deficient in essential fatty acids in which similar discrepancies appear.

RAPPORT AND FRANZL[18] report that plasmalogens are attacked by phospholipase A and hence the liberated fatty acids will also contain the fatty acids esterified to the contaminating plasmalogen. No scheme is currently available to differentiate between the lecithin $\beta$ position and that of plasmalogen. In the plasmalogen-containing lecithins this error would be expected to decrease the apparent recovery of fatty acids not associated with the plasmalogen and to increase the apparent recovery of those associated with the plasmalogen. Should the pattern of fatty acids of plasmalogen follow that of lecithin, triglycerides and other phospholipids having a highly unsaturated $\beta$ composition[18-22], then one would expect to recover a larger amount of unsaturated and a smaller amount of saturated fatty acids than the theoretically calculated amount.

With the above reservation in mind, it is of interest to relate the current data with that of others and with the schemes of biosynthesis proposed for phosphatidyl choline. First, regarding the distribution of the polyenoic acids of the "linolenic acid family" vs. those of the "linoleic acid family", the latter being considered "essential fatty acids", it is apparent that the primary representative of the linolenic-group, docosahexaenoic acid, is primarily esterified to the $\beta$ position of both the marine and terrestrial lecithins in which it occurs and hence follows the classical distribution of arachidonic and linoleic acid. Linolenic, eicosapentaenoic, docosatetraenoic and docosapentaenoic acids are also similarly distributed. This distribution is then in agreement with the suggestion of KLENK AND BROCKERHOFF[23], RICHARDSON *et al.*[24], and KLENK AND EBERHAGEN[25] that the so-called essential fatty acids are not absolute requirements in the structure and function of cellular membranes, but that other unsaturated fatty acids may substitute. The enzymatic studies of LANDS AND MERKL[26] would also support such a thesis. Secondly, as was mentioned previously, the most marked positional enrichment of a given fatty acid observed was that of palmitic acid for the $\alpha'$ position.

Dietary effects will influence the relative proportion of saturated and unsaturated fatty acids esterified to the $\alpha'$ position. RHODES[27], for example, found an increase in the unsaturation of egg-yolk lecithin, when the laying hens were fed cod-liver oil. Yet even in those lecithins having the highest $\alpha'$-position unsaturation, the enrichment of palmitic acid for the $\alpha'$ position was of the same order as that of arachidonic and docosahexaenoic acid for the $\beta$ position (consider salmon and tuna: palmitic acid: ratio of $\alpha'/\beta = 4-7$; arachidonic acid: ratio $\beta/\alpha' = 0.5-4$; docosahexaenoic acid: ratio $\beta/\alpha'$ = about 1.5). BROCKERHOFF *et al.*[9] also found more palmitic acid esterified to the $\alpha'$ position in cod-muscle and cod-liver, scallop-muscle and lobster-liver lecithin than that found in the $\alpha'$ position of the triglycerides isolated from the same tissue.

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# Chain Elongation of Linoleic Acid and Its Inhibition by Other Fatty Acids *in Vitro**

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## SUMMARY

**Linoleic acid-1-$^{14}$C was incubated with subcellular fractions of rat liver to study the mechanism of polyunsaturated fatty acid synthesis. The most effective system for the chain elongation of linoleic acid to eicosadienoic acid was liver microsomes plus malonyl coenzyme A with cofactors, NADPH, and ATP. Under anaerobic conditions, the chain elongation reaction could be separated quantitatively from the dehydrogenation reaction which otherwise required the same system. The chain elongation of linoleic acid was inhibited by saturated fatty acids of which myristic and pentadecanoic acids were most effective. Mono-unsaturated and polyunsaturated fatty acids also act as inhibitors. From consideration of reaction kinetics and inhibition by other fatty acids, the conversion of linoleic acid to γ-linolenic acid seems preferred over the conversion of linoleic acid to homolinoleic acid.**

The metabolism of polyunsaturated fatty acids has been studied mainly by feeding experiments with rats, and thus the over-all reactions are well known. Linoleic acid, 18:2ω6,[3] for example, is converted to 20:4ω6 either via 18:3ω6 and 20:3ω6 (1) or via 20:2ω6 and 20:3ω6 (2). Factors governing the preference between pathways in the tissue remain to be determined.

Several attempts have been made to find the location of the enzymes catalyzing chain elongation and dehydrogenation; it is possible that they may be studied separately.

Wakil (3) reported a system located in mitochondria of rat liver which catalyzed the chain elongation of polyunsaturated fatty acids and which was activated by pyridoxamine phosphate. Stoffel (4) described a system which elongated linoleic acid to eicosadienoic acid and which required acetate and bicarbonate. The system was located in the cytoplasm of rat liver, whereas the dehydrogenation of linoleic acid occurred mainly in a microsomal fraction. Later Stoffel and Ach (5) found that the supernatant of the microsome preparation had merely catalyzed the formation of malonyl coenzyme A from acetate and bicarbonate, and that the chain elongation was actually activated by microsomes. Nugteren (6, 7) confirmed that the chain elongation enzyme is mainly present in the microsomes. The microsomal system not only elongated but also dehydrogenated polyunsaturated fatty acids; $^{14}$C-labeled linoleic acid thus yielded 18:3ω6, 20:2ω6, 20:3ω6, and 20:4ω6.

In the present report we describe a system in which the enzymatic activity responsible for the lengthening of the chain of linoleic acid and other polyunsaturated fatty acids is retained but in which the dehydrogenation activity is suppressed. Experiments are described which compare the activities of several cell constituents and which specify the optimum reaction conditions. We also describe the inhibition of the reaction 18:2ω6 → 20:2ω6 by many common fatty acids, which shows at the subcellular level inhibitions in the metabolism of polyunsaturated fatty acids which have been predicted in experiments with whole animals (8-10).

## EXPERIMENTAL PROCEDURE

*Preparation of Subcellular Fractions from Rat Liver*—Weanling male rats of the Sprague-Dawley strain were kept on a fat-free diet as described previously (11), except that the protein content of the diet was increased from 18 to 30%, replacing part of the sucrose. Rats kept on this regime develop liver lipids which are poor in polyunsaturated acids. Rats, at 3 to 5 months of age, were killed by drawing the blood from the heart during ether anesthesia. By this method the liver obtained was pale and yielded blood-free subcellular fractions. The livers were quickly removed and cooled in 0.05 M potassium phosphate buffer, pH 7, which contained 0.25 M sucrose (isotonic), 0.15 M KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 1.5 mM glutathione. The livers were then sliced and homogenized in the same solution (1:4, w/v) with the use of a Potter-Elvehjem homogenizer at 0° (cold room). The fractional centrifugation was carried out according to the procedures described by Siekevitz (12) for microsomes (105,000 × *g*, Spinco ultracentrifuge) and Hogeboom

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[3] The number preceding the colon indicates number of carbon atoms, the number following it denotes number of double bonds; and the number following the ω indicates the position of the last double bond counting from the terminal methyl group.

(13) for mitochondria (17,000 × *g*). The microsomal and mitochondrial pellets were resuspended in a solution containing 0.25 M sucrose, 0.15 M KCl, and 0.1 mM EDTA. The protein contents of the suspensions were determined by the biuret reaction (14). Fatty acids were incubated with aliquots of these suspensions. Subcellular fractions were prepared in the morning, and the incubations were carried out in the afternoon of the same day.

*Electron Micrographs of Microsome Preparations*[2]—Electron micrographs were prepared to judge the uniformity and morphology of the microsomal preparation obtained by fractional centrifugation. A sample of the microsomal pellet was suspended in a freshly filtered solution of 0.2% ammonium acetate in quartz-distilled water and sedimented at 105,000 × *g* for 60 min. The pellet was then resuspended in the same solution and diluted 1:10,000 and 1:50,000. The particles were sedimented on Formvar-covered copper grids (400 mesh) in a Sorvall RC2 centrifuge with the SV rotor at 30,000 × *g* for 20 min. The specimens were examined in a Siemens Elmiscopy 1 electron microscope and photographed on Kodak projection slides, 0.5 × 9.0 cm.

*Preparation and Assay of Malonyl-CoA*—Malonyl-CoA was synthesized by transacylation of *S*-malonyl-*N*-decanoyl-cysteamine with reduced coenzyme A by a modification (14) of the method described by Eggerer and Lynen (15). Batches of 12 to 20 μmoles of malonyl-CoA were prepared and stored frozen at pH 5. The solutions were stable for several weeks. However, for most of the experiments a fresh preparation was used. The malonyl-CoA concentration was determined by the delayed nitroprusside reaction as described by Lynen (16) and by the hydroxamic acid test as described by Stadtman (17).

*Linoleic Acid (1-$^{14}$C)*—This compound, with a specific activity of 37.8 mC per mmole, was purchased from Nuclear Chicago. Its radiopurity was determined after dilution (1:27, w/w) with cold linoleic acid (Lipid Preparations Laboratory of the Hormel Institute). Gas-liquid chromatography (18, 19) of an esterified sample showed that all of the radioactivity was associated with linoleic acid. Thin layer chromatography of the free fatty acid and autoradiography of the plate showed that only traces of radioactivity, probably due to autoxidation products, remained at the starting point. The content of *trans* double bonds of the fatty acid was approximately 17%, as determined by infrared spectrometry. The content of *trans* double bonds was not taken into consideration for computation of concentrations and yields, because the metabolic conversions of *cis-trans*, and *trans-trans* 18:2ω6 acids have not been elucidated fully.

Medium chain and long chain fatty acids were purchased from the Lipid Preparations Laboratory of the Hormel Institute. γ-Linolenic acid was synthesized via the trienoic acid in this laboratory.[3] The acids 18:3ω6, 20:4ω6, 20:5ω3, and 22:6ω3 were purified shortly before use by thin layer chromatography on Silica Gel G impregnated with silver nitrate. Gas-liquid chromatography of the respective methyl esters showed that the purified acids were at least 99% pure.

*Incubation*—Microsomal protein, 10 mg, and 0.3 to 0.6 μmole of malonyl-CoA were placed in Warburg flasks with 120 μmoles of potassium phosphate buffer at pH 7.0, 0.1 μmole of reduced CoA, 0.2 μmole of NADPH, 10 μmoles of ATP, and 15 μmoles of $MgCl_2$ (5, 20). In each case the malonyl-CoA was added in at least a 10% M excess over the fatty acid to be incubated. The oxidizing capacity of the system was suppressed by 3 μmoles of KCN and 125 μmoles of KF (21). The volume of the incubation mixture was adjusted to 3 ml with a solution of 0.25 M sucrose, 0.15 M KCl, and 0.1 mM EDTA.

The substrate and inhibitor fatty acids (1 mg) were dispersed in twice their molar amount of 0.1 N $NH_4OH$ and made up to 1 ml with 1% Triton WR 1339 (Ruger Chemical Company, Irvington on Hudson, New York). Aliquots of this micellar solution were inserted into the side arms of Warburg flasks.

In anaerobic incubations, air was evacuated and the flasks were flushed with argon (99.995%) at least three times. After gassing with argon, the flasks were tipped and the side arms were rinsed with the incubation mixture. The flasks were shaken in a Dubnoff metabolic shaking incubator at 37°. The reaction was stopped by pouring the content of each flask into a precooled (−70°) test tube fitted with a ground joint. The contents were quickly frozen and lyophylized overnight. The dry residue was refluxed with 15 ml of a 5% solution of HCl in methanol for 2 hours in the same tube. The methyl esters were extracted with three portions of light petroleum hydrocarbon which were combined, washed free of acid, and dried over anhydrous $Na_2SO_4$. This procedure yields a more complete extraction of fatty acids from the incubation mixture than conventional saponification and re-esterification. Therefore it was not necessary to dilute the ester mixture with cold carrier for purposes of handling.

*Analysis of Fatty Acid Mixtures*—The chain elongation of $^{14}$C labeled linoleic acid to $C_{20}$ acids was observed qualitatively and quantitatively. The mixture of methyl esters obtained from the incubation slurry was analyzed by radio-gas-liquid chromatography by means of a gas chromatograph with radiation ionization detector. The columns employed were aluminum tubing, 300 × 0.6 cm, packed with 20% ethylene glycol succinate plus 2% phosphoric acid on Gaschrom P (Applied Science Laboratories). Temperatures were 180° at the column and 230° at the inlet, in the detector cell, and at the outlet system. Argon flow was 60 to 80 ml per min. The apparatus was equipped with an outlet that allowed collection of the effluent directly into a scintillation counter vial, as described previously (18, 19). In each initial experiment, fractions of the effluent were collected in intervals of 1 to 5 min in order to coordinate the radioactivity with peaks on the chromatographic chart (see Fig. 1). For routine analyses the relevant peak was collected in one vial. Radioactivity was determined in a Packard dual channel scintillation counter. The scintillation solution contained 5 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyl oxazolyl)]-benzene (Packard Instrument Company) in 1 liter of redistilled toluene.

The rate of conversion of linoleic to $C_{20}$ acids was calculated from the ratio of the radioactivity in the $C_{20}$ acid to the total radioactivity collected from a gas liquid chromatography run. This method of scanning radioactivity has a distinct advantage over "radio-gas chromatographs" which employ a flow through cell with ratemeter. It is more sensitive because the radioactivity can be counted independently of the flow rate. Therefore the specific activity of the substrates used in biologica

[1] We appreciate the help of Dr. H. Jenkin of this laboratory in the preparation of the electron micrographs and of Miss Donna Rika, Department of Microbiology, University of Minnesota.

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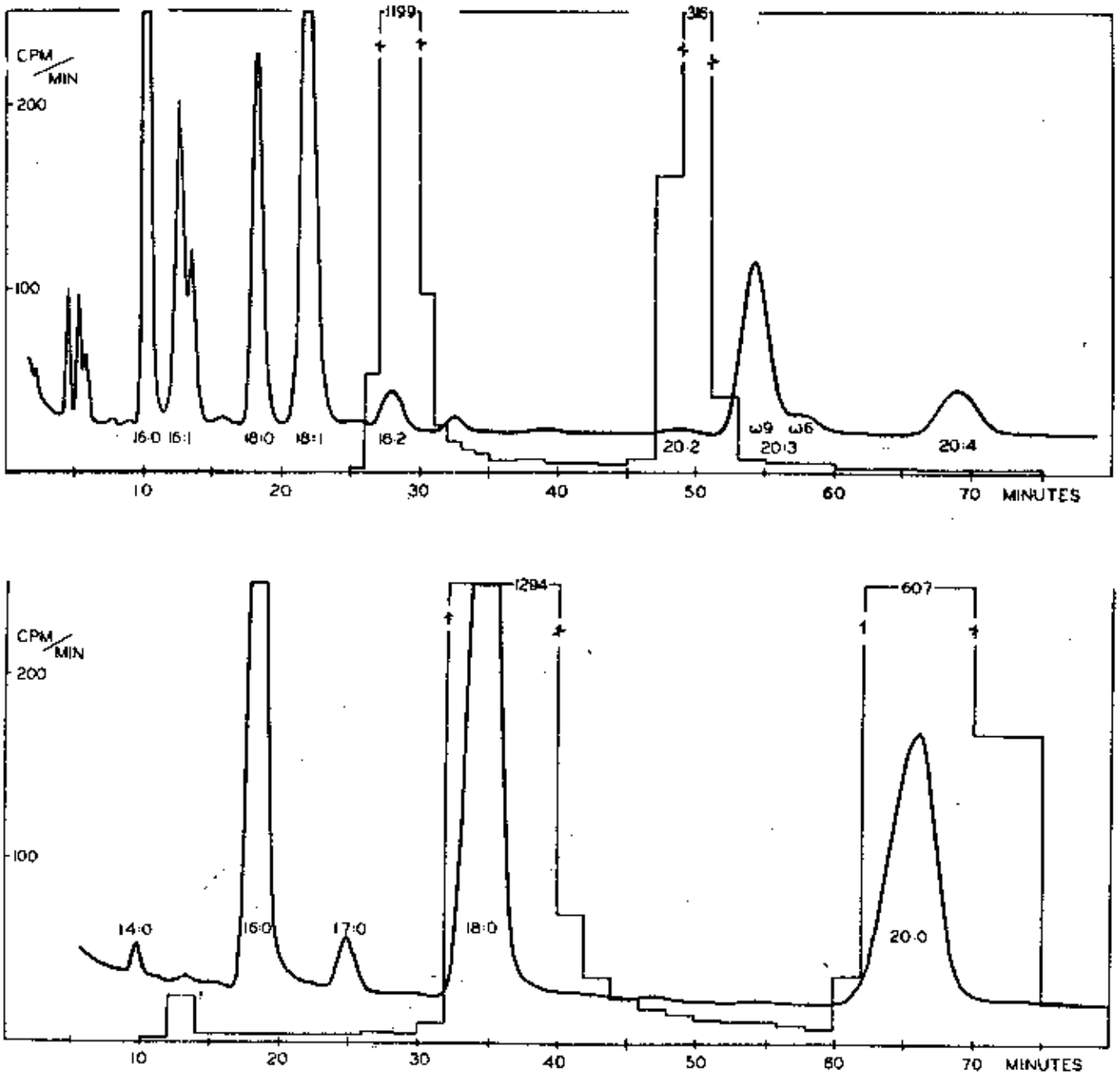



FIG. 1. *(upper)*, gas-liquid chromatogram of the fatty acid mixture obtained from an anaerobic incubation of 1-$^{14}$C-18:2ω6 with liver microsomes (10 mg) from fat-deficient rats for 20 min at 37° with the cofactors described under "Experimental Procedure." Only the portion of the chromatogram is given that is of interest for the chain elongation of 18:2ω6 to 20:2ω6. The *step diagram* depicts the radioactivity in counts per minute that is associated with the gas-liquid chromatographic effluent collected in 1 min. *(lower)*, gas-liquid chromatogram of a hydrogenated fatty acid mixture obtained under the same conditions as described above.

experiments can be much lower than that required for ratemeter counting.

*Identification of Fatty Acids.* Tentative identification of the fatty acids was made via the equivalent chain lengths (22) determined on authentic fatty acid esters. Authentic 20:2ω6, which was synthesized from linoleyl methanesulfonate (23) and sodium malonate (24), confirm the chain length of the metabolic product; the ester mixture was hydrogenated, and the saturated fatty acids were separated by gas-liquid chromatography and identified by internal standards (Fig. 1). Final proof of identity of the principal product 20:2ω6, was furnished by ozonizing the mixture of $C_{20}$ acids obtained by gas-liquid chromatography of esters from the incubation mixture, and reducing the ozonides with triphenylphosphine (Eastman Kodak) followed by scanning of radioactivity of the resulting mixture of aldehydes and aldehyde esters separated by gas-liquid chromatography (25) under the same conditions as for methyl esters. The peak of radioactivity coincided with the retention time of the authentic $C_{11}$-aldehyde ester produced by ozonization and reduction of Δ-11-eicosenoic acid, 20:1ω9 (Lipid Preparation Laboratory of The Hormel Institute).

*Incorporation of Substrate into Lipids.*—To investigate the incorporation of the substrate fatty acid and its chain-elongated product into various lipids, the incubation was carried out as

described above. The lyophilized residue was extracted three times with 10 ml of chloroform-methanol (2:1) by shaking in a separatory funnel. The extract was washed with 0.9% NaCl solution and dried over $Na_2SO_4$. Aliquots of the lipid extract were analyzed on thin layer plates with the solvent systems, petroleum ether-diethyl ether-acetic acid (90:10:1) and chloroform-methanol-water (65:25:4). The lipid classes were identified by the use of standards of phosphatidyl choline and ethanolamine and the thin layer chromatography standard no. 2 from the Lipid Preparations Laboratory of The Hormel Institute. Autoradiograms of the plates were produced on Kodak No-Screen medical x ray film. The distribution of radioactivity was measured by densitometry of the autoradiograms and by scraping off the spots from the plates and counting of these fractions in suspension according to Snyder and Stephens (26).

*Assay for Inhibition of Chain Elongation*—The same basic incubation procedure was applied as described above, but varying amounts of the competing fatty acids in concentrations similar to that of 18:2ω6 were incubated together with linoleic acid. The saturated acids tested included even and odd fatty acids from 8:0 to 22:0. The unsaturated acids were 18:1ω9, 18:3ω6, 18:3ω3, 20:4ω6, 20:5ω3, and 22:6ω3. The inhibitory effect of these fatty acids was measured by determining the decrease in yield of 20:2ω6. To measure initial reaction velocity, the incubation was interrupted after 6 min, before the substrate concentration had changed appreciably. From these data Lineweaver-Burk plots were constructed and Michaelis-Menten constants were calculated.

## RESULTS AND DISCUSSION

*Chain Elongation of Linoleic Acid*—A chromatogram obtained from incubation of liver microsomes of fat-deficient rats with $^{14}$C-linoleic acid is shown in Fig. 1 (*upper half*). Appreciable amounts of radioactivity are associated only with the substrate fatty acid, 18:2ω6, and with the first product of chain elongation, 20:2ω6. Confirmation of its chain length was provided by hydrogenation of the total fatty acid mixture, and by radio

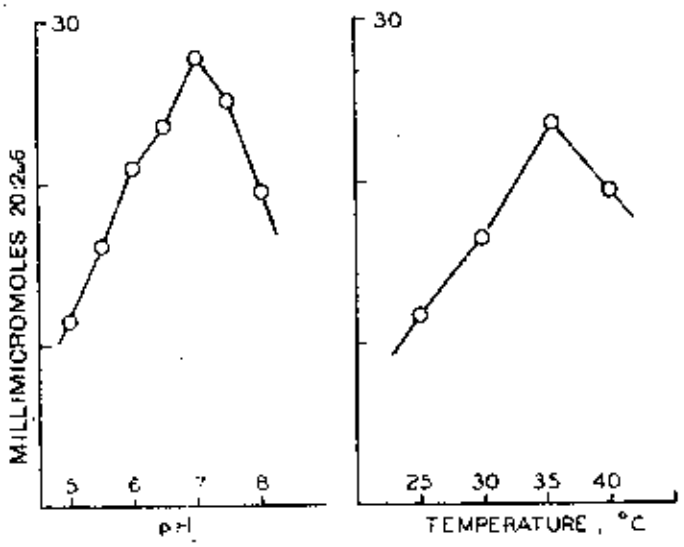



FIG. 2. pH and temperature optima for the conversion of linoleic to eicosadienoic acid by liver microsomes from fat-deficient rats. At varying pH, temperature was 36°; at varying temperatures, pH was 6.5; 450 nμmoles of the radioactive acid were incubated with 10 mg of protein and 0.6 μmole of malonyl-CoA. Other cofactors were as described under "Experimental Procedure."

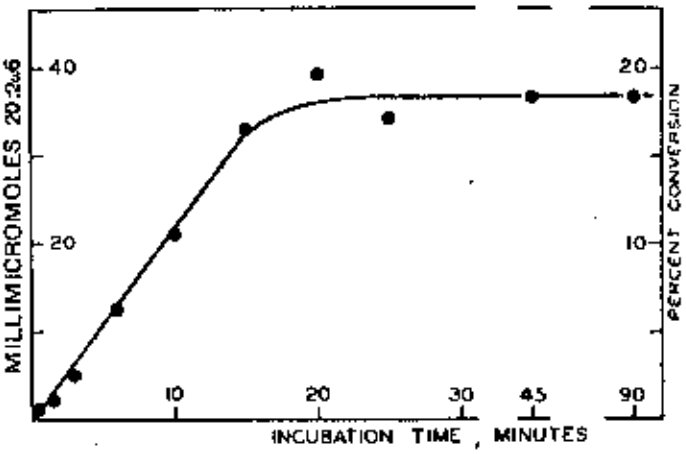



FIG. 3. Time dependence and yield of the chain elongation of linoleic (18:2ω6) to eicosadienoic acid (20:2ω6) by liver microsomes from fat-deficient rats. 1-$^{14}$C-18:2ω6, 200 nμmoles, was incubated at 37° with 10 mg of microsomal protein, 0.6 μmole of malonyl-CoA, and the other cofactors as described under "Experimental Procedure."

gas-liquid chromatographic analysis. The chromatogram of this preparation is shown in Fig. 1 (*lower half*). The only peaks which were radioactive were $C_{18}$ and $C_{20}$. Thus the conversion of a $C_{18}$ acid to a $C_{20}$ acid is demonstrated. The location of the first double bond at position 11 in the 20:2 was shown by ozonolysis and reductive cleavage of the ozonides. Radioactivity was associated only with a $C_{11}$-aldehyde ester.

The optimum conditions for the chain elongation of linoleic acid were found to be near pH 7 and 35° (Fig. 2), and all subsequent incubations were carried out under these conditions. The yields of 20:2ω6 were proportional to time up to 20 min, after which they were constant (Fig. 3).

In the presence of oxygen, liver microsomes catalyze the dehydrogenation of linoleic acid to γ-linolenic acid (20, 27). In the presence of oxygen and malonyl-CoA the reactions 18:2ω6 → 20:2ω6, 18:2ω6 → 18:3ω6, and 20:2ω6 → 20:3ω6 → 20:4ω6 all took place. Chain elongation of 18:3ω6 to 20:3ω6 acid may occur also. In our experiments under aerobic conditions, the chain elongation reaction constituted about 50% of the over-all conversions (Table I). The rigid exclusion of oxygen increased the yield of longer chain products when malonyl-CoA was present. In many experiments we succeeded in completely eliminating the dehydrogenation reaction, so that the only reaction observed was chain elongation (Table I, "Anaerobic 2").

The substances necessary for the chain elongation of linoleic acid are listed in Table II. There is minimal conversion of linoleic acid in the absence of malonyl-CoA, NADPH, or ATP. All other components of the system used in this study served to prevent side reactions, or helped to preserve the structural integrity of the subcellular particles employed. Whereas malonyl-CoA and NADPH are directly involved in the chain elongation cycle (28), ATP is required for the formation of the CoA ester of linoleic acid, and need not be present if the CoA ester of linoleic acid is used as a substrate (5, 20). Only catalytic amounts of CoA are required for the formation of the fatty acid CoA esters, since the CoA is set free at the end of the elongation reaction and may return to the cycle. These small amounts are introduced with the malonyl-CoA and the liver microsomes. Boiling the microsomal preparation for 5 min destroyed most

TABLE I

*Separation of chain elongation and dehydrogenation reactions of linoleic acid (18:2ω6) by microsomal protein*

1-$^{14}$C-Linoleic acid was incubated in the amounts indicated with 10 mg of protein, 0.3 μmole of malonyl-CoA, 0.1 μmole of reduced CoA, 2 μmoles of NADPH; 10 μmoles of ATP, 15 μmoles of $MgCl_2$, 4.5 μmoles of GSH, and 125 μmoles of potassium phosphate buffer (pH 7) in 3 ml of a solution containing $10^{-4}$ M EDTA, 0.15 M KCl, and 0.25 M sucrose at 37°.

| | Substrate 18:2ω6 | Incubation time | Product of conversion | | | | Chain elongation[a] |
|---|---|---|---|---|---|---|---|
| | | | 18:3ω6 | 20:2ω6 | 20:3ω6 | 20:4ω6 | |
| | mμmoles | min | mμmoles | | | | % conversion |
| Aerobic | 200 | 10 | 6.0 | 17.4 | 8.6 | 8.4 | 52 |
| | 200 | 20 | 8.0 | 20.4 | 7.6 | 11.0 | 51 |
| Anaerobic[b] 1 | 200 | 10 | 1.2 | 30.0 | 5.8 | 2.0 | 77 |
| | 200 | 20 | 1.2 | 38.0 | 9.0 | 2.0 | 78 |
| Anaerobic[b] 2 | 50 | 20 | <0.1 | 10.7 | <0.1 | <0.1 | 96 |
| | 100 | 20 | <0.1 | 22.1 | <0.1 | <0.1 | 98 |

[a] 20:3 represents one elongation plus one dehydrogenation, and 20:4 represents one elongation plus two dehydrogenations. Therefore, elongation as percentage of total conversions = 100 [(20:2 + 20:3 + 20:4)/(18:3 + 20:2 + 2(20:3) + 3(20:4))].

[b] The amount of dehydrogenation products varied from incubation to incubation, probably in relation to differences in microsomal preparation and efficiency in oxygen removal.

of its activity, an indication that the chain elongation reaction is catalyzed by an enzyme system.

Mitochondria effected a low rate of conversion which did not change after their further purification (Table III). Therefore, a catalytic action of this fraction cannot be ruled out. However, it is definitely not the major site of the enzymes for the chain elongation, as previously suggested (3). The same is true for the supernatant of the microsomal fraction. Even after a very rigorous sedimentation at high speed for a long time, some activity could be observed in the cytoplasmic fraction, but it cannot be considered the primary site of chain elongation. This is contrary to a previous report (4). The postmicrosomal pellet which most likely consists of some microsomes and ribosomes has minimal activity. We agree with Nugteren (6, 7) that the endoplasmic reticulum (microsomes) must be regarded as the major site for the chain elongation of polyunsaturated fatty acids in rat liver. Purification of the microsomal fraction by resuspension and resedimentation did not change the rate of conversion of 18:2ω6 to 20:2ω6. Thus the addition of supernatant to the microsomal fraction represented no advantage. The uniformity of the microsome fraction was checked by preparing electron micrographs. Only particles of similar size could be observed. Mitochondria were not present. Some of the microsomal fragments aggregated, forming lumps of fun to eight of the normal pieces, which could not be separated by ultrasonic vibration.

*Incorporation of Substrate and Chain-elongated Fatty Acids into Lipids of Liver Microsomes.* The substrate and the product of the chain elongation system were incorporated into the lipids of liver microsomes. The lipid classes were separated by thin layer chromatography, and the radioactivity of the fractions was determined after the bands were scraped off into vials containing scintillation solution and Cab-O-Sil (26). The highest radioactivity was found in the phosphatidyl cholines (37%), followed by 32% in the triglycerides. Only 15% was found in the phosphatidyl ethanolamines, 10% in the free fatty acids, and 6% in other polar lipids. In comparable studies of dehydrogenation, Brenner and Peluffo (20) found almost no radioactivity in the triglycerides, only a small amount in the free fatty acids, and the rest of the radioactivity in the polar lipids. Within the phospholipids, the phosphatidyl ethanolamines were found to contain slightly more of the labeled acids than the phosphatidyl cholines.

*Inhibition of Chain Elongation of Linoleic Acid by Other Fatty Acids*—The inhibition of the conversion of linoleic acid to arachidonic acid by linolenic acid was first observed in nutritional experiments with chickens and rats (9, 29), and this effect has been studied by a number of investigators (10, 30, 31). Recently Brenner and Peluffo (20) have shown that inhibition occurs at the dehydrogenation step of the conversion of linoleic to arachidonic acids by liver microsomes of fat-deficient rats.

TABLE II

*Cofactor dependence of chain elongation of 1-$^{14}$C-linoleic acid (18:2ω6) to eicosadienoic acid (20:2ω6) by microsomal protein*

| Cofactors | Product of conversion | | |
|---|---|---|---|
| | 18:3ω6 | 20:2ω6 | 20:3ω6 |
| | mμmoles | | |
| Complete system[a] | 1.0 | 21.9 | 0.9 |
| Boiled 5 min | 5.6 | 0.7 | 0.0 |
| Minus malonyl-CoA | 2.0 | 0.9 | 0.4 |
| Minus NADPH | 2.7 | 2.2 | 0.6 |
| Minus ATP | 0.7 | 0.1 | 0.3 |
| Minus reduced CoA | 1.0 | 28.6 | 0.6 |

[a] The complete system contained: 10 mg of microsomal protein; 200 mμmoles of 18:2ω6; 0.3 μmole of malonyl-CoA; 0.1 μmole of reduced CoA; 2 μmoles of NADPH; 10 μmoles of ATP; 15 μmoles of $MgCl_2$; 4.5 μmoles of GSH; 125 μmoles of potassium phosphate buffer; EDTA, $10^{-4}$ M; KCl, 0.15 M; sucrose, 0.25 M; pH 7; volume, 3 ml; incubation time, 20 min; temperature, 37°; anaerobic.

TABLE III

*Chain elongation of linoleic acid (18:2ω6) by various subcellular fractions of liver from fat-deficient rats*

Protein, 10 mg, was incubated with 100 mμmoles of 1-$^{14}$C-18:2ω6, 0.3 μmole of malonyl-CoA, and the other cofactors as described under "Experimental procedure" at pH 7, temperature 37°, for 20 min. Total volume was 3 ml.

| Subcellular fraction | Centrifugal force | 20:2ω6 Produced | |
|---|---|---|---|
| | | Experiment 1 | Experiment 2 |
| | | mμmoles | |
| Mitochondria | | | |
| Crude | 17,000 × *g* | 3.2 | 3.6 |
| Purified | | 4.7 | 5.3 |
| Microsomes | | | |
| Crude | 105,000 × *g* | 20.4 | 19.7 |
| Purified | | 20.8 | 23.6 |
| Postmicrosomal pellet | 165,000 × *g* | 4.9 | 4.9 |
| Supernatant | | 3.2 | 3.3 |

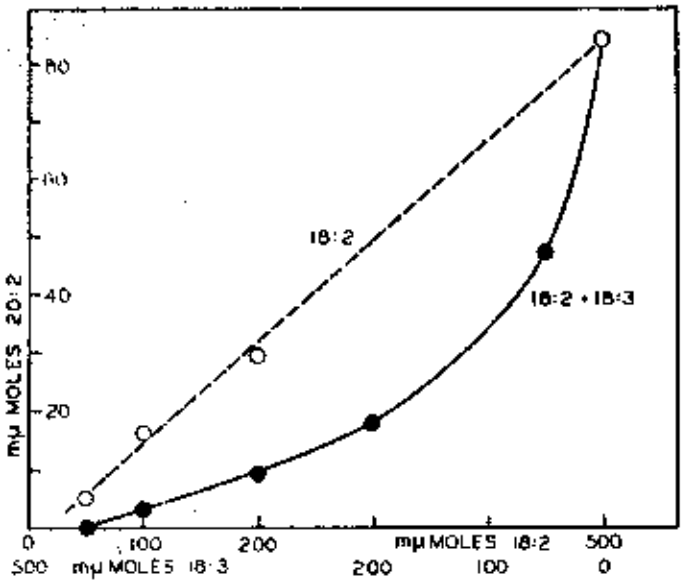



FIG. 4. Biosynthesis *in vitro* of eicosadienoic acid (20:2ω6) in response to increasing amounts of linoleic acid (1-$^{14}$C-18:2ω6) as substrate and inhibition of this conversion by linolenic acid (18:3ω3). Each incubation contains 10 mg of microsomal protein and 0.6 μmole of malonyl-CoA. The other cofactors are described under "Experimental Procedure." Incubation time, 90 min at 35°. The *dotted line* shows the amount of 20:2ω6 synthesized when 1-$^{14}$C-18:2ω6 is the only substrate. The *solid curve* shows the suppressed synthesis of 20:2ω6 when 1-$^{14}$C-18:2ω6 is incubated simultaneously with 18:3ω3.

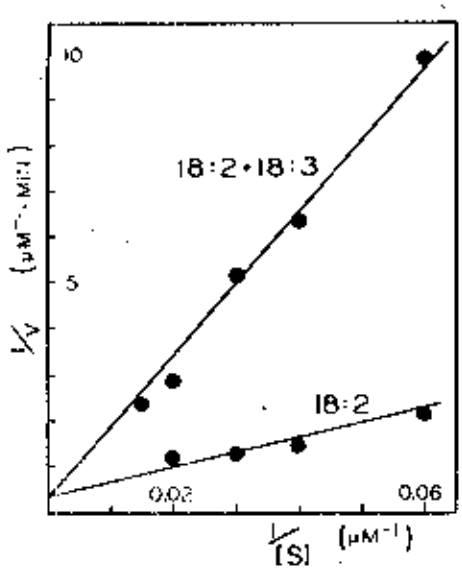



FIG. 5. Lineweaver-Burk plot for the chain elongation of linoleic (18:2ω6) to eicosadienoic acid (20:2ω6) by rat liver microsomes (10 mg). 1-$^{14}$C-Linoleic acid (50 to 200 nanomoles) was incubated simultaneously with and without 150 nanomoles of linolenic acid for 6 min at 37°, pH 7.0, anaerobically. Cofactors are as described under "Experimental Procedure."

We have shown that the chain elongation step of the conversion of linoleic to arachidonic acids is also inhibited by linolenic acid (Fig. 4). The sum of the amounts of linoleic and linolenic acids was constant in this experiment, and was less than the capacity of the microsomal protein, as shown by the linearity of yield and substrate concentration (Fig. 4, *dotted line*). The yield of 20:2ω6 was smaller in all cases in which 18:3ω3 was added than when linoleate was the only substrate. This inhibition has been verified in several experiments.

The Lineweaver-Burk plots (Fig. 5) of the conversion, 18:2ω6 → 20:2ω6, with and without linolenic acid as inhibitor, were derived from experiments interrupted after only 6 min of incubation time. As in the case of the dehydrogenation reaction (20), the *straight lines* for both the uninhibited and the inhibited reactions intersect the *vertical axis* at about the same point. The Michaelis-Menten constants for the reactions were $K_m$ = 3.3 × 10$^{-6}$ and $K_i$ = 12.1 × 10$^{-6}$. $K_i$ is about 4-fold higher than the constant for the dehydrogenation reaction derived by Brenner and Peluffo (20). It should be kept in mind, however, that computing a reaction constant for a multienzyme reaction is not very meaningful. Therefore, in our further investigations of substrate inhibition we did not emphasize kinetic considerations.

The chain elongation of linoleic acid is not only inhibited by linolenic acid, but by a large number of saturated and unsaturated fatty acids of different chain lengths. Table IV lists the series of saturated fatty acids from $C_8$ to $C_{22}$ and all major unsaturated fatty acids occurring in rat liver lipids which have been incubated together with radioactive linoleic acid. In all cases the test level of inhibitor was twice the concentration of 18:2ω6. The conversion 18:2ω6 → 20:2ω6 was measured by the usual procedure. Since the yields were somewhat different in the several experiments needed to study all of these

TABLE IV

*Inhibition of chain elongation of linoleic (18:2ω6) to eicosadienoic acid (20:2ω6) by fatty acids*

1-$^{14}$C-18:2ω6, 100 nanomoles, was incubated simultaneously with 200 nanomoles of the nonradioactive inhibitor fatty acid, 10 mg of microsomal protein, 0.4 μmole of malonyl-CoA, and other cofactors as described under Experimental Procedure at pH 7, 37°, for 20 min. Total volume, 3 ml.

| Inhibitor | Product 20:2ω6* | | % Inhibition |
|---|---|---|---|
| | nanomoles | | |
| Octanoic acid | 8:0 | 27.9 | 7 |
| Nonanoic acid | 9:0 | 25.2 | 16 |
| Decanoic acid | 10:0 | 20.4 | 32 |
| Dodecanoic acid | 12:0 | 14.4 | 52 |
| Tridecanoic acid | 13:0 | 14.4 | 52 |
| Tetradecanoic acid | 14:0 | 11.1 | 63 |
| Pentadecanoic acid | 15:0 | 9.9 | 67 |
| Hexadecanoic acid | 16:0 | 13.5 | 55 |
| Heptadecanoic acid | 17:0 | 19.8 | 34 |
| Octadecanoic acid | 18:0 | 25.2 | 16 |
| Nonadecanoic acid | 19:0 | 28.8 | 4 |
| Eicosanoic acid | 20:0 | 27.3 | 9 |
| Heneicosanoic acid | 21:0 | 30.0 | 0 |
| Docosanoic acid | 22:0 | 30.0 | 0 |
| Octadecenoic acid | 18:1ω9 | 15.9 | 47 |
| Octadecatrienoic acid | 18:3ω3 | 8.4 | 72 |
| Octadecatrienoic acid | 18:3ω6 | 10.2 | 66 |
| Eicosatetraenoic acid | 20:4ω6 | 22.2 | 26 |
| Eicosapentaenoic acid | 20:5ω3 | 13.5 | 55 |
| Docosahexaenoic acid | 22:6ω3 | 17.0 | 43 |

* The results are taken from several experiments with different conversion rates. Therefore, the amount of 20:2ω6 has been computed based on a conversion rate of 30% for control experiments without inhibitor.

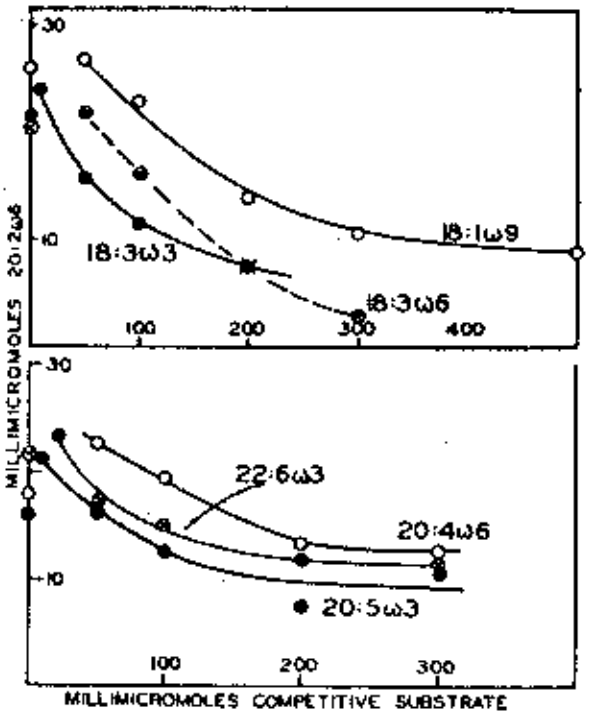



FIG. 6. Inhibition of the conversion by microsomal protein of linoleic to eicosadienoic acid by increasing amounts of other unsaturated fatty acids. 1-$^{14}$C-18:2ω6 (100 mμmoles) was incubated simultaneously with the nonradioactive inhibitor, 10 mg of microsomal protein, and the cofactors described under "Experimental Procedure," at pH 7, anaerobically for 20 min. Competitive substrates (*upper*): ●, linolenic acid (18:3ω3); ○, oleic acid (18:1ω9); ⊗, γ-linolenic acid (18:3ω6). Competitive substrates (*lower*): ○, arachidonic acid (20:4ω6); ●, eicosapentaenoic acid (20:5ω6); ⊗, docosahexaenoic acid (22:6ω3).

acids, the results were adjusted by computing the amount of 20:2ω6 formed, assuming 30% conversion in the uninhibited reaction.

The saturated fatty acids all inhibit the chain elongation of linoleic acid, and $C_{14}$ and $C_{16}$ acids are the best inhibitors. Expressed in terms of competitive inhibition, they themselves may be elongated. From $C_{14}$ to $C_{22}$ the degree of inhibition decreases, $C_{21}$ and $C_{22}$ acids being ineffective. Thus the most effective inhibitors are those acids which, after chain elongation, produce the most common and abundant saturated fatty acids of animal tissue, $C_{18}$, $C_{20}$, and $C_{22}$.

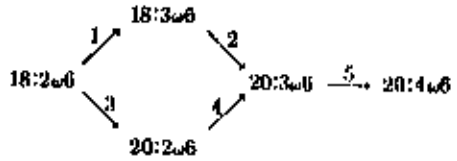
Among the unsaturated fatty acids tested, 18:3ω6 and 18:3ω3 are by far the best inhibitors. The relatively high efficiencies of 22:6ω3 as inhibitor may be product inhibition, because 22:6ω3 is the last product in the sequence of chain lengthening and dehydrogenation in its family of acids.

The inhibition curves obtained with the unsaturated fatty acids are shown in Fig. 6. The yield of 20:2ω6 decreases with increasing amounts of the inhibitor fatty acids in a similar manner for all acids tested. The most dramatic decrease was observed for 18:3ω6, which belongs to the linoleic acid family, and which has the same chain length as the substrate, but has one more double bond.

The conversion rates for 18:2ω6 → 20:2ω6 in these experiments were slightly higher in those samples which contained a small amount of the inhibitor fatty acid than they were for the blanks which contained linoleic acid only (*points* on the *vertical axis* in Fig. 6). It therefore appears that the inhibitors have a stimulating effect at low concentrations. Brenner and Peluffo (20) observed a similar slight increase in yield of the dehydrogenation products when polyunsaturated fatty acids were added to the reaction mixture.

Quite different mechanisms seem to be functioning in the inhibition of the chain elongation reaction and in the dehydrogenation reaction. All 20 fatty acids tested, except 21:0 and 22:0, inhibited the chain lengthening of linoleic acid to some extent. In the case of the desaturation reaction, only 18:1ω9, 18:3ω3, and 22:6ω3 inhibited the conversion of 18:2ω6 to 18:3ω6 (20, 22). Therefore, the enzyme systems for the dehydrogenation must be much more substrate-specific than are those for chain elongation. The latter enzyme system elongates both saturated and unsaturated fatty acids (5).

The metabolic conversions of linoleic acid are shown in the following scheme.




The abilities of various fatty acids to inhibit the dehydrogenation of 18:2ω6 (Reaction 1) and its chain lengthening (Reaction 3) are not equal. For instance, the concentration of 18:3ω3 required to inhibit Reaction 1 by 50% is about 10 times the concentration of the substrate (20), whereas the concentration required to inhibit Reaction 3 by 50% is about equal to the substrate concentration. This suggests that in the presence of fatty acids, such as occur in a living system, the pathway 1-2-5 may be preferred over the pathway 3-4-5.

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# Spectrophotometric Estimation of Soybean Oil in Admixture With Cottonseed and Peanut Oils[1]

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IT is customary in southern cotton oil mills to process various oilseeds, including soybeans and peanuts, in the same equipment as is used for processing cottonseed. This practice necessarily provides numerous opportunities for one type of oil to become contaminated with another. In one such instance a tank car containing a mixture of cottonseed and soybean oil was shipped to a refiner and was used in the production of shortening without due allowance being made for the unsuspected presence of soybean oil in the original oil. There is always a possibility of accidental, and sometimes unavoidable, contamination from incomplete draining and cleaning the oil troughs, settling tanks, pipe lines, and other processing and handling equipment during conversion from one oilseed to another. Because of the possibility of accidental admixture of soybean with cottonseed and peanut oils and as a means for checking the composition of intentional blending of such oils, a method for estimating the percentage of soybean oil in such admixtures is desirable.

Cottonseed oil may be detected by the Halphen test and peanut oil by its content of higher saturated fatty acids. However, the composition of soybean oil is such that it does not permit its detection or estimation by distinctive color tests or by ordinary chemical methods, especially if it is present in only small proportions. The most distinctive characteristic of soybean oil is its content of linolenic acid which is present to the extent of about 7% in commercial lots. This acid has not been found in cottonseed or peanut oil.

The spectrophotometric measurement of the small but measurable ultraviolet absorption of the triene conjugation, produced by alkali isomerization of linolenic acid or soybean oil, appeared to be the most promising method for the determination of the presence of this oil in mixtures of cottonseed and peanut oils. Mitchell, Kraybill, and Zscheile (4) have described the use of such measurements to determine quantitatively the linolenic and linoleic acid content of various oils. The present authors have established a correlation between the spectrophotometrically measured optical densities of triene conjugation produced by alkali isomerization and the percentage composition of binary oil mixtures. This correlation

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can be applied to the quantitative determination of soybean oil in admixtures with cottonseed, peanut, and similar vegetable oils.

## Experimental

The experimental procedure consisted of two steps: the alkali isomerization of the oil mixtures and the spectrophotometric measurement of the optical densities of the isomerized oils at the wavelength position of maximum absorption of the triene conjugation, namely 268 mμ. A modification of the procedure, as described by Mitchell, Kraybill, and Zscheile (4), was used for the alkali isomerization. If a mixture of soybean and cottonseed oils contains 5% soybean oil, which in turn contains about 7% linolenic acid, then the linolenic acid content of the mixture is only about 0.3%. Therefore, it was necessary to modify the procedure of Mitchell *et al.* to adapt it to the measurement of the small quantities of linolenic acid present in such mixtures.

1. The absorption of ultraviolet light by the reagent or blank, when handled by the method of Mitchell *et al.*, was found to be too high to permit measuring absorption of the weak triene conjugation. This limitation was overcome by preparing the reagent and conducting the isomerization under an atmosphere of nitrogen purified by passage through an electrically heated tube containing copper oxide previously reduced by hydrogen. With this modification oxidation of the ethylene glycol in the presence of the alkali is prevented, and a reagent is obtained which is almost completely transparent to ultraviolet light in the region of the triene conjugation. The optical densities of the ethylene glycol-potassium hydroxide reagent, measured through 1 cm. cells against distilled water, are: 220 mμ, 1.89; 232 mμ, 0.357; 250 mμ, 0.092; 268 mμ, 0.063.

2. The entire isomerization was conducted in alkali resistant glassware to avoid the formation of the troublesome white precipitate, described by Mitchell *et al.*, which slowly forms when Pyrex glassware is used.

3. Since the alkali resistant glass would not stand the thermal shock of cooling in cold water, the tubes when removed from the hot isomerization bath were rapidly cooled in a stream of air. To allow for the somewhat slower rate of cooling the time of isomerization was decreased from 25 to 22 minutes.

4. In order to measure the triene conjugation of mixtures containing very small percentages of soybean oil, an investigation was made of the effect of sample size and final dilution of the isomerized oils. On the basis of the results obtained it was found possible to increase the sample size from 0.1 gram to 0.3 gram and to maintain the final dilution at 50 ml., as compared to the 250 ml. recommended by Mitchell *et al.* This modification of the method effected a fifteen-fold increase in concentration and, coupled with the first modification, considerably increased the sensitivity of the method for determining linolenic acid content.

## Analytical Procedure

*Preparation of Reagent.* Seventy ml. of ethylene glycol (E. K. Co.) are placed in a 250-ml. flat bottom flask of Kimble "Resistant" or Corning "Alkali Resistant" glass. The flask is fitted with a cork stopper having an inlet tube for introducing purified nitrogen, an outlet tube, and a thermometer. The inlet tube extends just below the stopper. The outlet tube, which extends to about 10 mm. above the surface of the liquid, is bent so that the emerging vapor stream is directed downward into a graduate where the condensate may be collected. The thermometer bulb is placed below the surface of the ethylene glycol.

A slow stream of purified nitrogen is passed into the flask which is heated on a hot plate until the ethylene glycol reaches a temperature of about 195° C. Four or five ml. are allowed to distill over and condense in the graduate. The hot plate is then removed and the ethylene glycol is allowed to cool to about 100° C. The stopper is raised while 4.5 grams of KOH in pellet form are quickly added. After replacing the stopper, the ethylene glycol is again heated on the hot plate to about 195° C. until a total of 10 ml. of condensate is collected. It is then allowed to cool to room temperature under an atmosphere of nitrogen and is ready to be used.

By this procedure a clear colorless reagent, which has very little absorption in the ultraviolet region, is prepared.

The final alkali concentration is 7.5 grams of 85 per cent potassium hydroxide per 100 ml. of ethylene glycol as recommended by Mitchell *et al.* (*loc. cit.*). The nitrogen purification is conveniently accomplished by passing tank nitrogen through a heated combustion tube filled with copper oxide wire which has been previously reduced with hydrogen. The heater is constructed as follows: A standard length of combustion tubing is wrapped with a thin sheet of asbestos 50 cm. long, leaving a 1 cm. gap the length of the tube for observation. Over this asbestos are wound 100 turns of 1/16" x 0.0045" Chromel A ribbon with turns spaced about 5 mm. apart. The tube is then laid in a section of magnesia pipe covering which is arranged so that the top half may be lifted to permit observation of the condition of oxidation of the copper in the tube.

*Isomerization of Sample.* The isomerization is carried out in approximately 25 x 150 mm. "Alkali Resistant" glass test tubes.[3] Ten ml. of the ethylene glycol-potassium hydroxide reagent are pipetted into each tube, which is fitted with a two-hole cork stopper carrying an inlet tube for purified nitrogen and a straight glass tube about 15 cm. long which serves as an air condenser. The gas inlet tube is extended just through the stopper while the condensing tube is allowed to extend to within about 2 cm. of the surface of the ethylene glycol-potassium hydroxide reagent.

The tubes containing the reagent are kept under an inert atmosphere during heating, isomerization, and cooling by passing a slow stream of purified nitrogen into the gas inlet tube and allowing it to escape through the air condenser. The tubes containing the reagent are placed in a constant temperature oil bath at 180° C. and left for at least 20 minutes to allow the reagent to reach this temperature. One of the tubes of reagent serves as a blank. The stoppers of the other tubes may be raised long enough for a 0.1 to 0.3 gram sample of oil to be dropped in.

The oil samples are weighed in micro-beakers made from "Alkali Resistant" glass tubing. The tubes are shaken three times at one-minute intervals. After twenty-two minutes, the tubes are removed from the bath and quickly cooled by a strong jet of air. It was found that the tubes could be cooled in this manner from 180° C. to 60° C. in about three minutes.

After cooling to room temperature, the samples are transferred to 100-ml. volumetric flasks with the aid of about 30 ml. of 99 per cent ethyl alcohol. They are usually held in a refrigerator overnight as recommended by Mitchell *et al.* (*loc. cit.*), although tests show that this delay in the measurement of the absorption spectra is not necessary when the alkali resistant glassware is used. Although no evidence of precipitation has been observed, as an added precaution, the samples are filtered before absorption measurements are made. Further dilution with 99 per cent specially purified ethyl alcohol is usually necessary, depending on the absorption of the sample. The optical densities of the specially purified alcohol, measured through 1 cm. cells against distilled water, are: 220 mμ, 0.214; 232 mμ, 0.111; 250 mμ, 0.022; 268 mμ, 0.002.

*Spectrophotometric Measurements.* The spectrophotometric measurements required by the method can be made with either a photographic or a photoelectric instrument. Both instruments were used in this work. This photographic instrument consisted of a high-intensity ultraviolet light source adapted for absorption work in this laboratory (5), a Hilger "Spekker"-type photometer, and a Bausch and Lomb medium quartz spectrograph. The photoelectric instrument used was the Beckman quartz spectrophotometer. By means of either of these instruments the optical density (E) of the isomerized oil mixtures at 268 mμ is measured. From this value the linolenic acid content and the soybean oil content of the mixture are calcu-

[3] Since test tubes of these dimensions made of "Alkali Resistant" glass are not commercially available at the present time, these tubes were made from the necks of long-necked "Alkali Resistant" glass flasks.

lated using equations derived by the methods described under "Discussion."

## Discussion

Figure 1 (A and B) illustrates the effect of alkali isomerization upon the ultraviolet absorption of a crude soybean oil. For all the spectrophotometric measurements described in this paper the oils before isomerization were measured in cyclohexane solution with this pure hydrocarbon in the solvent cell. The cyclohexane was purified by methods previously described (3). The isomerized oils were measured in the alcohol-ethylene glycol reagent, with the blank in the solvent cell. The characteristic absorption due to triene conjugation with a maximum at 268 mμ in the spectrum of the isomerized oil is absent in the oil before isomerization. Figure 1 (C and D) shows the effect of an identical treatment on the absorption of a crude cottonseed oil. The cottonseed oil has a measurable general absorption throughout this region which decreases in magnitude with increasing wave length. A comparison of Figure 1 (B) with Figure 1 (D) illustrates the relative effect of alkali isomerization upon the ultraviolet absorption of a crude soybean oil and a crude cottonseed oil.

Figure 1 also illustrates these same effects on a refined soybean and a refined cottonseed oil. From these curves a simple method for the qualitative detection of at least 3 to 4% of soybean oil in cottonseed oil can be readily made. It is necessary only to measure the optical density of the isomerized mixture at 260 mμ and again at 268 mμ. If soybean oil is present in the mixture, the optical density at 268 mμ will be considerably greater than the measurement at 260 mμ (for 268 mμ is a position of maximum absorption of the triene system while 260 mμ is near a position of minimum absorption). If, however, no soybean oil is present in the mixture, the optical density reading at 260 mμ will be greater than the reading at 268 mμ, since the general absorption of the cottonseed oil throughout this region is decreasing rapidly with increasing wave length. (See Table 1.)

TABLE 1

Relative Extinction Coefficients of Vegetable Oils

| Oil Sample | Extinction Coefficient | |
|---|---|---|
| | α at 260 mμ | α at 268 mμ |
| Crude soybean oil | 0.069 | 0.070 |
| Crude soybean oil after isomerization | 3.34 | 4.04 |
| Crude cottonseed oil | 0.076 | 0.066 |
| Crude cottonseed oil after isomerization | 0.35 | 0.18 |
| Refined soybean oil | 0.16 | 0.14 |
| Refined soybean oil after isomerization | 3.37 | 4.06 |
| Refined cottonseed oil | 0.16 | 0.14 |
| Refined cottonseed oil after isomerization | 0.42 | 0.27 |
| Crude peanut oil | 0.017 | 0.016 |
| Crude peanut oil after isomerization | 0.28 | 0.18 |
| Refined peanut oil | 0.10 | 0.11 |
| Refined peanut oil after isomerization | 0.31 | 0.31 |

Usually, however, a quantitative, rather than a qualitative, method for the detection of soybean oil in cottonseed oil is required. Absorption at 268 mμ is due to the presence of the chromophore containing three double bonds in conjugate positions. In the case of soybean oil this chromophore is produced by the isomerization of linolenic acid in the oil during saponification. No similar absorption was expected for isomerized cottonseed oil as chemical tests have not shown this oil to contain linolenic acid. However, it was observed that isomerized cottonseed oil had a definite, though small, absorption at 268 mμ and the absorption of known mixtures of the oils when plotted

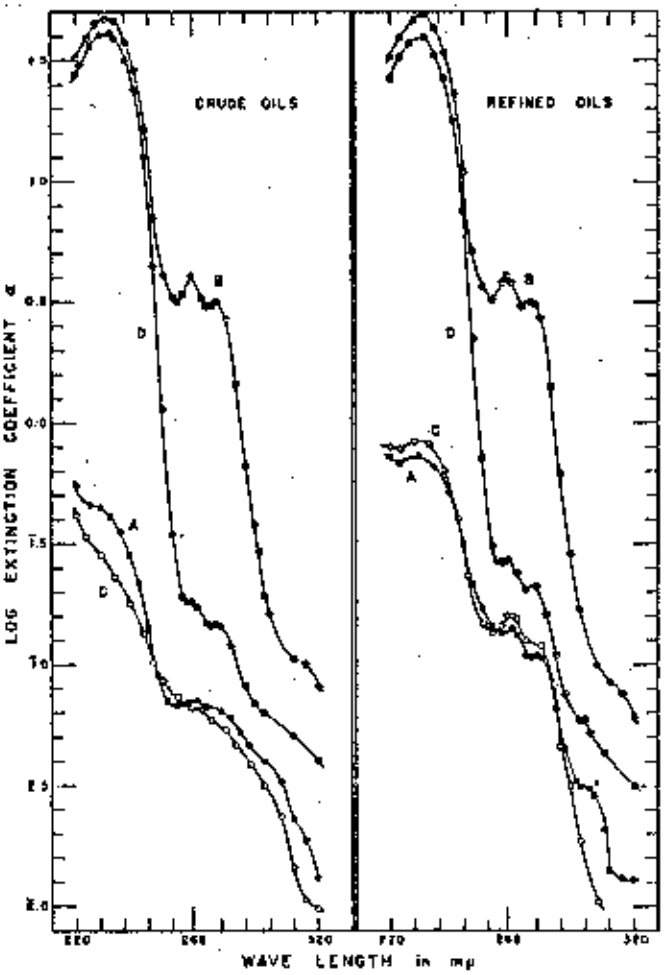


FIG. 1. Ultraviolet absorption of crude and refined soybean and cottonseed oils before and after isomerization.

A. Soybean oil before isomerization.
B. Soybean oil after isomerization
C. Cottonseed oil before isomerization.
D. Cottonseed oil after isomerization.

against the amounts of soybean oil gave a curvilinear instead of the straight line relationship required by the Beer-Lambert law. This absorption constitutes an interference which must be taken into account in applying the principles of spectrophotometry to the determination under consideration.

The most common explanation for the failure of the Beer-Lambert law to apply to a given solution is the presence of an interfering chromophore. The observed behavior is explainable on the basis of an interfering absorption of rather low magnitude which is always included in the optical density measurements. When the soybean oil content of the mixture is high, the contribution of this interfering absorption is relatively negligible. However, when the concentration of soybean oil is low, the contribution of the interfering absorption becomes a major factor in the measurements. This interfering absorption is due either to a small quantity of linolenic acid in the cottonseed oil or to the presence in this oil of some other material which produces triene conjugation upon alkali isomerization.

The character of the low-magnitude absorption is shown by Figure 2. The curves to the left in this figure were obtained by replotting, throughout the limited region of triene conjugation only, the extinction coefficients of the crude and refined cottonseed

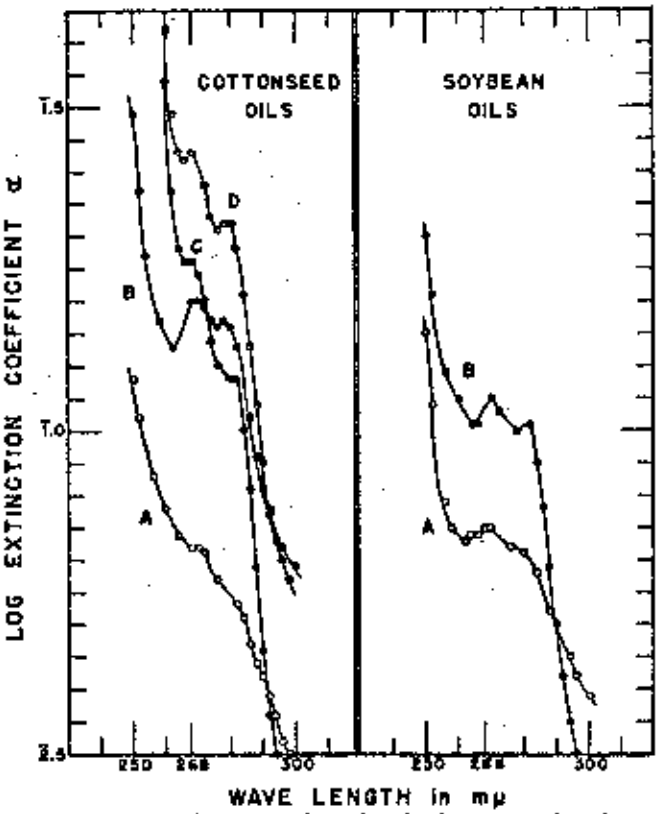


FIG. 2. Absorption of crude and refined cottonseed and soybean oils in region of triene conjugation.
A. Crude oil.
B. Refined oil.
C. Isomerized crude oil.
D. Isomerized refined oil.

oils, both before and after isomerization. The expanded ordinate makes possible a study of the nature of the absorption of the cottonseed oils at 268 mμ. In all four curves the characteristic absorption of triene conjugation is detectable although in the crude oil before isomerization its presence can be observed by only a slight shoulder in the absorption curve at 268 mμ. The refined oil has an appreciably greater absorption in the region of triene conjugation, and isomerization increases this absorption in both the crude and the refined oil. It should be noted that all the absorption values are low. The extinction coefficient range of Figure 2 is only from $\alpha = 0.03$ to $\alpha = 0.4$. The triene conjugated material is, therefore, present in the maximum case of the refined isomerized oil, only to the extent of a few tenths of a per cent. Figure 2 also shows that both the crude and the refined soybean oil have very small but detectable absorption at 268 mμ before isomerization. Again the amount present has increased with the refining processes.

Extinction values for the isomerized soybean oils are, of course, beyond the range covered in Figure 2. Table 1 shows the value of the extinction coefficients at 268 mμ for each of these particular oils. These values vary with the nature and history of the particular oil. From the values for the cottonseed oil it is apparent that for a precise method of determining soybean oil content from optical density measurements, a means of correcting the measurements for absorption due to triene conjugation unrelated to soybean oil content is required.

Several mixtures of a crude soybean oil and a crude cottonseed oil, with gradually increasing proportions of the former, were prepared. Each mixture, and each individual oil, was isomerized, after which the optical density was measured and the linolenic acid content calculated, using the empirical extinction coefficient of Mitchell *et al. (loc. cit.)*. These calculated values were then plotted against the percentage composition of the mixtures. The results, listed in Table 2, show that the pure cottonseed oil had an absorption at 268 mμ equivalent to the absorption of about 0.3% of linolenic acid. This absorption of a cottonseed oil at 268 mμ, expressed in terms of the amount of linolenic acid which would have an equal absorption, will hereafter be called "the apparent linolenic acid content of cottonseed oil."* The linolenic acid content of the soybean oil used was 7.7%. In the mixture containing 5% soybean oil having a 7% linolenic acid content, the linolenic acid contributed by the soybean oil, approximately 0.3%, is just about equal to the apparent linolenic acid contributed by the cottonseed oil. Hence, in such a mixture, direct measurements of optical density should give a value about 100% too high. This effect was noted, for mixtures containing 5% or less soybean oil, in first attempts at correlation between direct measurement of optical density and per cent soybean oil.

TABLE 2

Data Used to Correlate Percent Linolenic Acid, Calculated from Optical Density, with Percent of Soybean Oil in Admixtures with Cottonseed Oil

| Soybean Oil | Apparent Linolenic Acid* | Soybean Oil | Apparent Linolenic Acid* |
|---|---|---|---|
| *Percent* | *Percent* | *Percent* | *Percent* |
| 0** | 0.37 | 49.14 | 3.89 |
| 5.86 | 0.77 | 70.80 | 5.43 |
| 14.45 | 1.34 | 78.02 | 5.94 |
| 17.28 | 1.53 | 91.43 | 6.95 |
| 24.35 | 2.17 | 100.00 | 7.68 |
| 37.20 | 3.05 | | |

* All linolenic acid determinations are averages from three density measurements at 268 mμ.
** Cottonseed oil alone.

TABLE 3

Soybean Oil Content of Soybean-Cottonseed Oil Mixtures from Percent Linolenic Acid—Percent Soybean Oil Relationship and Probable Accuracy in Various Soybean Oil-Cottonseed Oil Mixtures

| Sample | Soybean Oil in Sample | Soybean Oil Found: Range for Determinations* | Soybean Oil Found: Average | Soybean Oil Found: Deviation from Amount Present | Predicted Range in Values of Soybean Oil in Mixtures** |
|---|---|---|---|---|---|
| | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| 1 | 4.92 | 3.89- 4.13 | 3.91 | —1.0 | 1.55- 4.75 |
| 2 | 9.1 | 8.00- 8.02 | 8.02 | —1.1 | 5.01- 9.10 |
| 3 | 8.1 | 9.67- 9.71 | 9.69 | +0.59 | 5.51- 9.50 |
| 4 | 15.1 | 15.8 -15.8 | 15.8 | +0.70 | 12.4 -18.0 |
| 5 | 15.1 | 15.4 -15.7 | 15.6 | +0.45 | 11.8 -17.2 |
| 6 | 25.0 | 25.3 -25.6 | 25.4 | +0.45 | 20.6 -28.0 |
| 7 | 50.8 | 49.1 -50.8 | 49.7 | —0.60 | 41.8 -54.2 |
| 8 | 75.25 | 74.2 -75.1 | 74.7 | —0.60 | 61.8 -79.5 |

* Range for two to four determinations.
** Predicted range of values of soybean oil which would be found due to variations in the linolenic acid content of different soybean oils or "apparent linolenic acid" content of different cottonseed oils comprising the mixtures. See Figure 5.

Values for the linolenic acid content of the various mixtures are in excellent agreement with the straight line drawn between the points representing the apparent linolenic acid content of the cottonseed oil and the true linolenic acid content of the soybean oil, as shown by the solid line in Figure 3. As a satisfactory

* Although cottonseed and peanut oils have never been shown to contain linolenic acid, it is probable that the absorption of these oils at 268 mμ after alkali isomerization is due to the presence of 0.2 to 0.4 percent of linolenic acid in these oils.

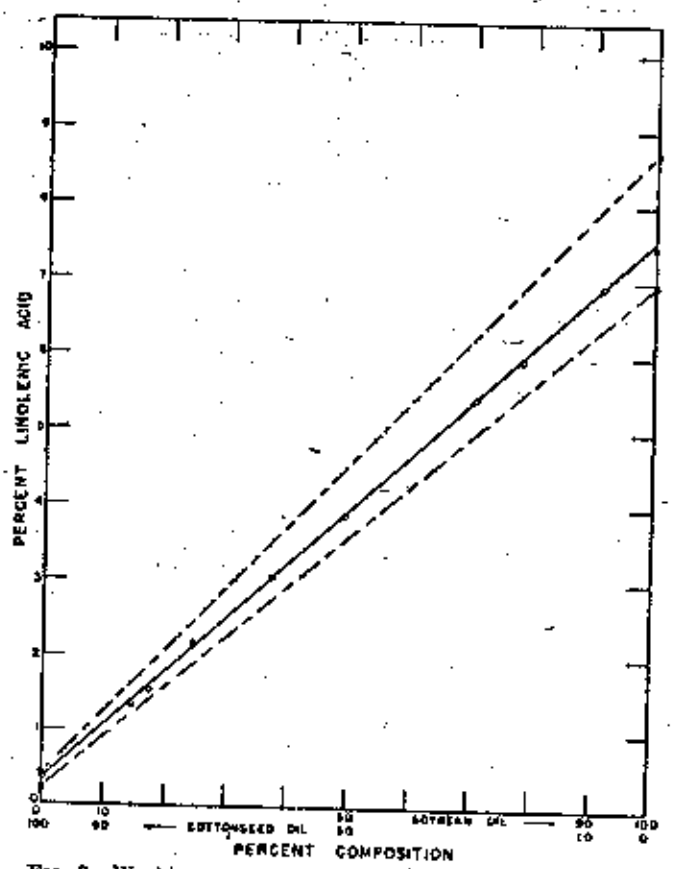


FIG. 3. Working curves for determination of soybean oil in mixtures of cottonseed and soybean oils.

working curve was obtained, it was tested by use in the analysis of a series of "unknowns." The results from this working curve are shown in Table 3. With a known cottonseed oil and a known soybean oil, it is possible to determine the percentage of soybean oil in mixtures to within ± 1%. Calculation of the results from measured optical densities can be reduced to computations from two equations. The percentage of linolenic acid can be computed by use of the Beer's law equation:

(1) Percentage of linolenic acid = E/53.7 x 100/cl. where E is the measured optical density at 268 mμ and 53.7 is the empirical extinction coefficient for alkali isomerized linolenic acid as reported by Mitchell *et al. (loc. cit.)*, c is the concentration of the unknown oil sample in the solution measured expressed in grams per liter and l is the length of the absorption cell in centimeters. With this value for the percentage of linolenic acid, the percentage of soybean oil in the mixture can be computed using the equation of the empirically determined working curve, as illustrated by the compositional diagram of Figure 3:

(2) Percentage of soybean oil =

$$\frac{(\text{Per cent linolenic acid} - 0.33)}{0.0735}$$

where 0.33 is the average value of "y" intercept, and 0.0735 is the slope of the line. Equations (1) and (2) can be combined to the single relationship:

$$(3)\ \text{Percentage of soybean oil} = \frac{100\ E - 17.72\ cl.}{3.947\ cl.}$$

The accuracy of the method will depend, in large part, upon the accuracy with which the constants of the straight-line equation are obtained. These constants will, in turn, depend upon the variations in the apparent linolenic acid content of various cottonseed oils and the linolenic acid content of various soybean oils.

In order to evaluate the magnitude of possible error in the method due to variations in either the apparent linolenic acid content of cottonseed oils or the true linolenic acid content of soybean oils, the ranges for such values were determined for a number of different crude, refined, and edible cottonseed and soybean oils. The results of some of these measurements are shown in Table 4. The absorption of the cottonseed oils corresponded to an apparent linolenic acid content of from 0.2 to 0.4 per cent. The commercial soybean oils varied in linolenic acid content from 6.8 to 8.7%.

TABLE 4

Linolenic Acid Content of Various Oils from Optical Density Measurements at 268 mμ

| Oil Number | Type of Oil | Iodine Number | Linolenic Acid | Apparent Linolenic Acid * |
|---|---|---|---|---|
| | | (Wijs) | Percent | Percent |
| S-1 | Soybean, crude | 131.8 | 7.47 | |
| S-2 | Soybean, refined | 134.0 | 7.64 | |
| S-3 | Soybean, refined | 134.1 | 7.98 | |
| S-4 | Soybean, crude | 132.2 | 7.14 | |
| S-5 | Soybean, crude | 134.5 | 8.71 | |
| S-6 | Soybean, refined | 132.8 | 6.97 | |
| S-7 | Soybean, crude | 129.7 | 6.08 | |
| C-1 | Cottonseed, crude | 111.4 | | 0.33 |
| C-2 | Cottonseed, refined | 103.7 | | 0.42 |
| C-3 | Cottonseed, crude | 107.4 | | 0.37 |
| C-4 | Cottonseed, refined | 107.8 | | 0.33 |
| C-5 | Cottonseed, refined | 103.9 | | 0.50 |
| C-6 | Cottonseed, refined | 105.3 | | 0.28 |
| P-1 | Peanut, crude | 94.8 | | 0.25 |
| P-2 | Peanut, refined | 94.4 | | 0.40 |
| P-3 | Peanut, crude | 92.1 | | 0.36 |

* Optical density at 268 mμ expressed in equivalent percent of linolenic acid.

To evaluate the maximum error which could be caused by the measured variation in the cottonseed oils and soybean oils, the soybean oil having the highest linolenic acid content and the cottonseed oil having the highest apparent linolenic acid content were selected and used for plotting another working curve. A similar curve was plotted from the lowest values for each oil. These two curves are shown by the broken lines in Figure 3. From these curves it is possible to calculate the maximum range over which the soybean oil content of the "unknowns" already measured could vary due to differences in the soybean oils and cottonseed oils which could compose the mixtures. Results from some of these calculations are shown in the last column of Table 3.

If the soybean oil content of the mixture is less than 25%, the maximum range with extreme variation in the constituent oils would be from 2 to 3%. Above 25%, this variation increases rapidly so that when more than this proportion is present in the mixture, accurate estimation of the percentage of soybean oil is impossible, due to the range over which the linolenic acid content of various soybean oils present as components in the mixtures may vary. Below 25%, results which should not vary more than 2 to 3% can be obtained. These variations are the possible extremes. Statistically, it might reasonably be expected that the error in the average mixture would be about midway between the extreme and the probable error when using the ideal curve for the particular mixture—that is, from ± 1 to 2%.

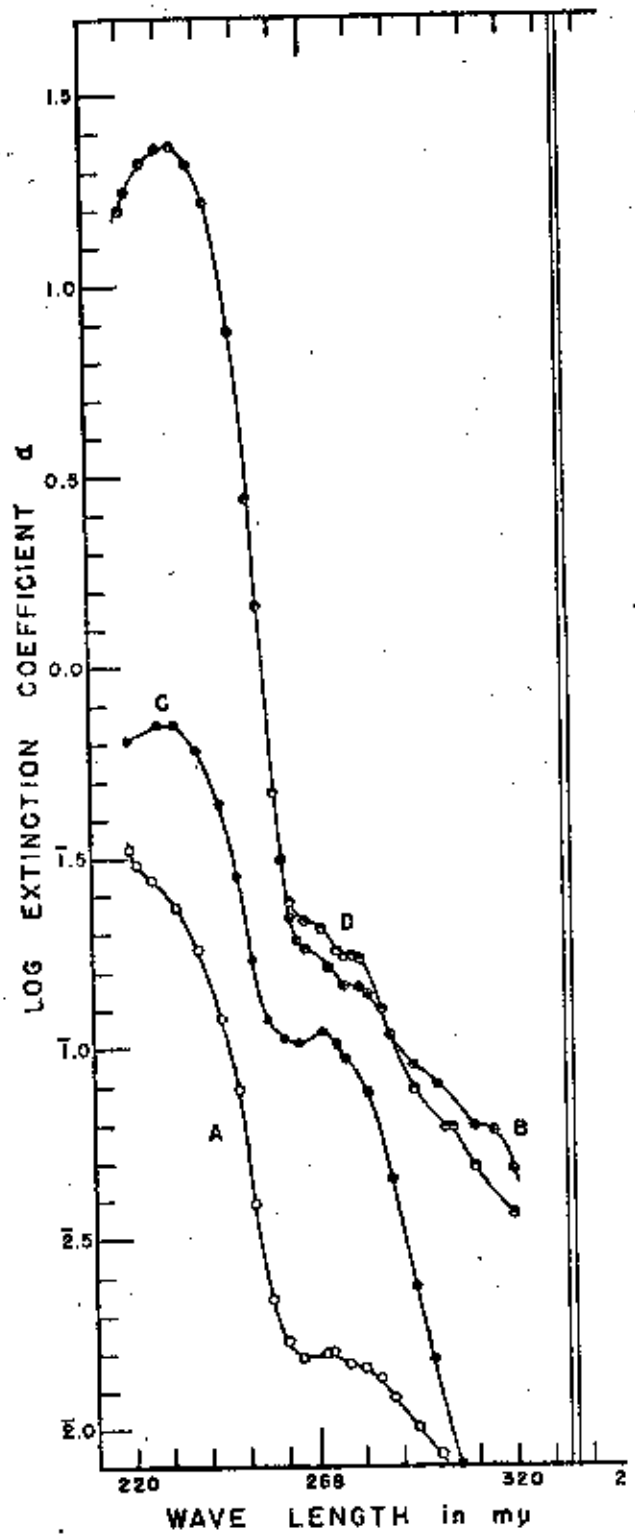


FIG. 4. Ultraviolet absorption of crude and refined peanut oils before and after alkali isomerization.

A. Crude oil before isomerization.
B. Crude oil after isomerization.
C. Refined oil before isomerization.
D. Refined oil after isomerization.

In order to further test the magnitude of error incurred when measuring mixtures of soybean and cottonseed oils having different linolenic and apparent linolenic acid contents, samples containing mixtures of different oils were prepared, isomerized, and measured spectrophotometrically. The results, in Table 5, show that the actual variations are for the most part within the predicted limits.

Two modifications of the method of Mitchell *et al.* *(loc. cit.)* have been proposed to increase the accuracy of the determination of linolenic acid. Brode, Patterson, Brown, and Frankel (2) suggest a method for correction for diene and tetraene conjugation which interferes with triene measurement, and Brice and Swain (1) propose a method for correction for general background absorption. Neither of these corrections is required for the measurement of the percentage of a particular soybean oil in admixture with a particular cottonseed oil because the magnitude of the interfering absorption is the same in the mixtures used to prepare the working curve and in the unknown mixtures analyzed. These types of corrections need be considered only as variation in the character of the various soybean and cottonseed oils encountered in various mixtures appreciably changes the magnitude of interfering absorption. However, as the magnitude of both of these corrections is very small (1, 2), variations in their magnitude, due to variations in the composition of different mixtures, will be negligible. Hence, only some method, such as the one proposed, of prorating the measured triene conjugation between the true linolenic acid contributed by the soybean oil and the apparent linolenic acid contributed by the cottonseed oil will appreciably improve the accuracy in the determination of the percentage of soybean oil in admixture with cottonseed oil. If, however, a linolenic acid value of the mixture is desired as a criterion of the economic value, use of the corrections as proposed by both Brode, Patterson, Brown, and Frankel (2) and by Brice and Swain (1) should permit a somewhat more accurate estimation of this value.

Measurements of peanut oils showed that they resemble cottonseed oils in that they have an absorption at 268 mμ equivalent to from 0.2 to 0.4% linolenic acid (Table 4, P-1, P-2, and P-3). The absorption of both crude and refined peanut oils before and after isomerization is shown in Figure 4. From these curves, and from the value of the extinction coefficients in Table 1, it will be seen that, like the cottonseed oils, the refined peanut oil had a higher absorption than the crude oil and that isomerization increased the value of the general absorption at 268 mμ (again due, probably, to increase in diene conjugation) without appreciably increasing the triene conjugation. It is possible, therefore, to determine peanut oil in admixture with soybean oil by the method described for cottonseed-soybean oil mixtures. Results of a few determinations are included in Table 5.

## Conclusion

A spectrophotometric method has been described for the determination of soybean oil in admixture with cottonseed oil. The method provides a simple and rapid means of detecting gross adulteration of one oil with another and permits an accurate determination of linolenic acid for use as a criterion of the economic value of an oil mixture and as a guide in oil processing.

The factor limiting the precision of the method is variation in composition of the cottonseed and soybean oils in the mixtures to be analyzed. Variations in composition affect the proportion of measured

TABLE 5

Evaluation of Soybean Oil Content of Various Mixtures of Soybean Oils with Cottonseed Oils and Peanut Oils

| Oils in Mixtures* | | | Soybean Oil in Mixture | Linolenic Acid in Mixture (Calculated) | Linolenic Acid Found | Soybean Oil Found | Deviation of Soybean Oil Found from Amount Present |
|---|---|---|---|---|---|---|---|
| Soybean | Cottonseed | Peanut | | | | | |
| | | | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| S-3 | C-4 | | 4.9 | 0.71 | 0.68 | 4.8 | —0.1 |
| S-3 | C-4 | | 10.0 | 1.10 | 1.02 | 9.4 | —0.6 |
| S-2 | C-2 | | 7.1 | .93 | .98 | 8.8 | +1.7 |
| S-3 | C-6 | | 15.0 | 1.40 | 1.47 | 15.5 | +0.5 |
| S-6 | C-4 | | 3.1 | .54 | .84 | 6.9 | +3.8 |
| S-8 | C-6 | | 9.9 | .90 | 1.14 | 11.0 | +1.1 |
| S-1 | | P-1 | 7.3 | .78 | .85 | 7.1 | —0.2 |
| S-3 | | P-2 | 10.2 | 1.18 | 1.12 | 10.8 | +0.6 |
| S-6 | | P-2 | 15.9 | 1.32 | 1.50 | 15.9 | +0.3 |
| S-4 | C-3 | | 7.0 | .75 | .82 | 6.7 | —0.3 |
| S-5 | C-3 | | 5.0 | .62 | .60 | 6.4 | +1.4 |
| S-4 | | P-3 | 8.0 | .56 | .70 | 5.0 | +2.0 |
| S-5 | | P-3 | 5.0 | .76 | .88 | 6.8 | +1.8 |

* See Table 4 for description of oils.

triene conjugation, due to the linolenic acid content of the soybean oil and the apparent linolenic acid content of the cottonseed oil. Thus, for unknown mixtures only average value corrections can be made for apparent linolenic acid content and the accuracy of a particular analysis will depend upon how well the composition of the oils in the particular mixture follows those of the average mixture.

The method described can be extended to mixtures other than those of soybean and cottonseed oils. Thus, soybean oil may be determined in admixture with a peanut oil. In general, any oil which has an unsaturated fatty acid capable of producing triene conjugation upon alkali isomerization can be determined in the presence of any other oil containing no appreciable quantity of unsaturated fatty acids which can produce triene conjugation by such treatment.


## Acknowledgment

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# DETERMINATION OF OXYGEN IN OILS

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## Introduction.

Perhaps the most important reason for the deterioration of a fat is the sorption of oxygen from the air and its reaction initially with highly unsaturated glycerides in the fat.

In this context sorption is used as a comprehensive term
for adsorption = accumulation of gas on the liquid surface
for absorption = solution of gas in the liquid and
for desorption = liberation of gas from the liquid.

Adsorption and solution may be physical or chemical. The sorption process between oxygen and oil includes physical solution of oxygen, diffusion and a chemical reaction.

Insight into the oxygen sorption process and the oxygen sorption isotherms therefore forms the basis for the understanding of the changes that can occur in a fat through the contact between air and fat.

Our aim has been to develop a simple and reliable method for measuring the amount of oxygen that is found in an oil or taken up during the various stages of processing in the factories. In this paper an account is given for the principal results achieved.

## Apparatus

The determination of oxygen dissolved in oil is carried out by means of a Beckman pH-meter model 76 with an oxygen adapter, which consists of a silver anode and a gold cathode protected by a teflon membrane.

When a voltage of 0.8 volt is connected between the anode and cathode all oxygen passing the membrane at the cathode is reduced and produces a current directly proportional to the quantity of oxygen in the sample. The electrode measures a unit proportional to the partial pressure of oxygen in the gas phase. The diffusion of oxygen into the electrode through the membrane is dependent on the temperature. By the cathode a certain consumption of oxygen takes place. The shape of the reaction vessel, the location of the electrode, and the flow rate of the oil round the electrode are therefore of great importance.

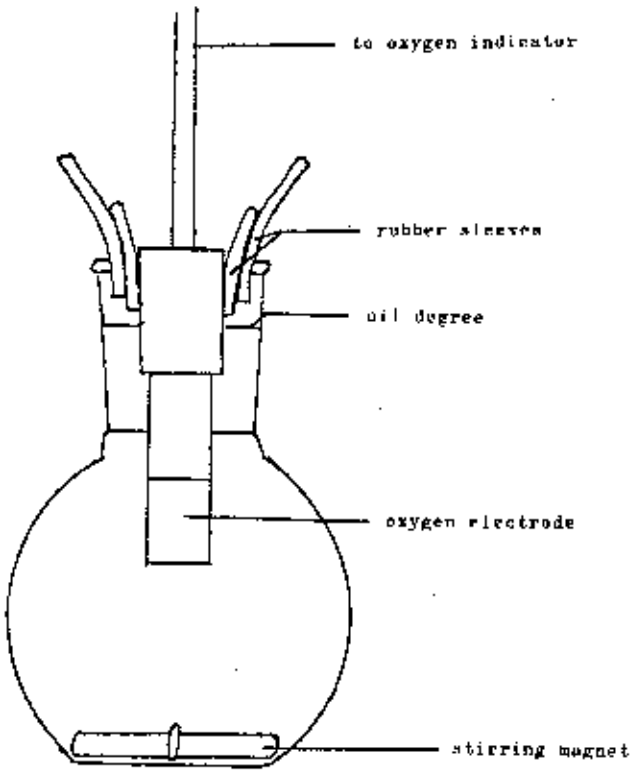


Figure 1.
The vessel used for the measurements in the laboratory.



The instrument was calibrated by keeping the electrode in the air and adjusting the value 160 torr (the partial pressure of oxygen in air). The instrument has an automatic temperature compensation and the electrode can be used for measurements in solutions up to + 50°C. It is of course a disadvantage that direct measurements cannot be made at higher temperatures, as many interesting applications are found in oils at high temperatures.

We have also tested Radiometer equipment Model 27. This instrument works according to the same principle and there is no vital difference in the practical possibilities for measuring oxygen. Higher absolute values of oxygen content may possibly be obtained with Radiometer instrument.

Through leading air into various oils the saturation value attainable for oxygen from air was determined. For all vegetable oils studied this value was 143 (142-8) torr read from the instrument calibrated as described above. For fish oil the saturation value was approx. 130. We assume this to be due to a greater rate of the chemical reaction than the absorption rate of oxygen. Other experiments also indicate this. We have not investigated, why the saturation value was not 160. To check that 143 is the value attained by an oil in equilibrium with air, we have carried out experiments in which we led oxygen gas into the oil, until the measured oxygen value of the oil was about 200 torr. The oil was then stored in contact with air and attained the value 143. This is shown in figure 2. The figure also shows that the saturation value turned out to be the same within the temperature interval of 20-50°C.

## The dependence of the sorption rate on various factors.

The dependence of the sorption rate of oxygen on various factors has also been studied. Different refined vegetable oils absorb oxygen at different rates under constant conditions.

Soybean oil and peanut oil for example are more rapidly saturated than rapeseed oil, mustard oil, coconut oil and cotton oil. This is probably due to differences in the coefficients of permeability,

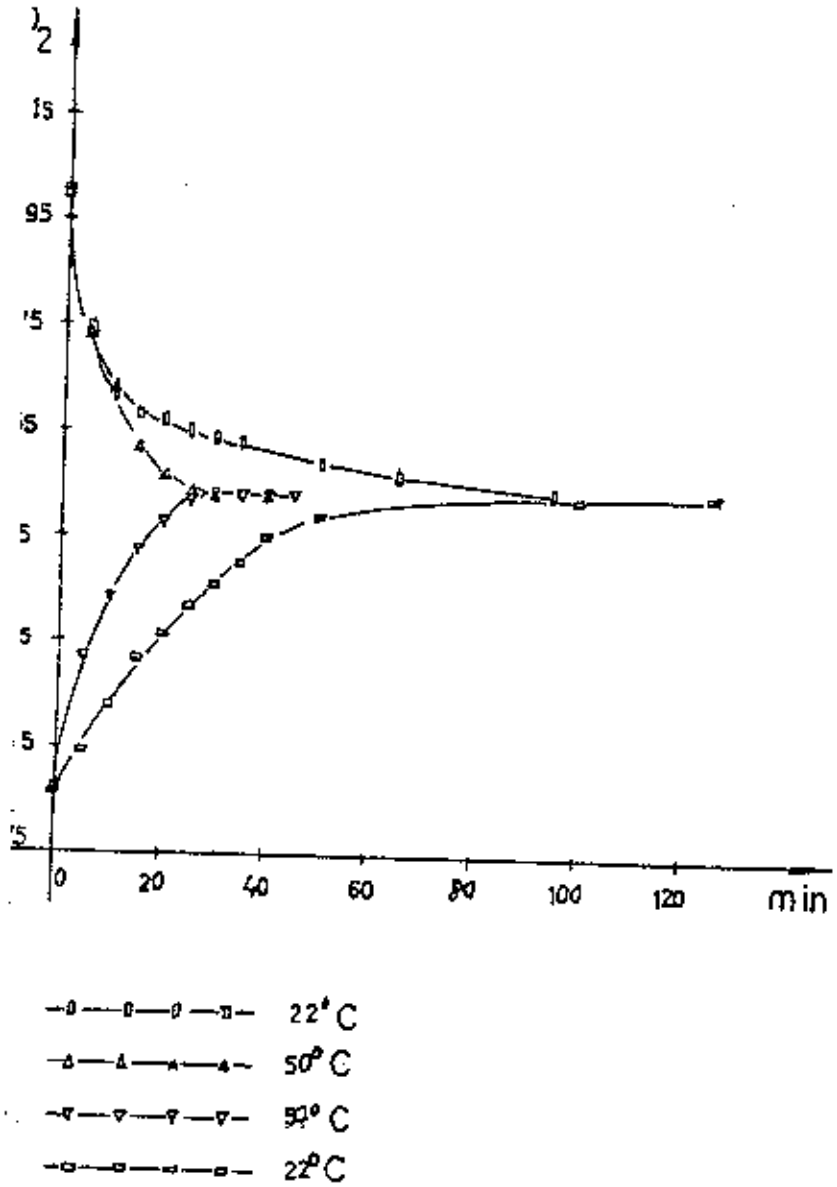


Figure 2.



solubility and diffusion from oil to oil. It is otherwise known that the solubility of oxygen from air is practically the same for different oils, about 3.5 - 4 mg $O_2$/100 ml at + 20°C.

Different treatments have an effect on the sorption rate. Refined oils for instance absorb oxygen more rapidly than the corresponding raw oils. The same applies to hydrogenated oils. This should be seen in connection with what is already known, namely that isomerized oils dissolve more oxygen than the natural oils.

The sorption rate increases greatly at increasing temperatures as seen in figure 2.

The saturation value as previously shown, does not, however, seem to depend on the temperature within the interval studied. This does not mean that the quantity of oxygen absorbed is the same.

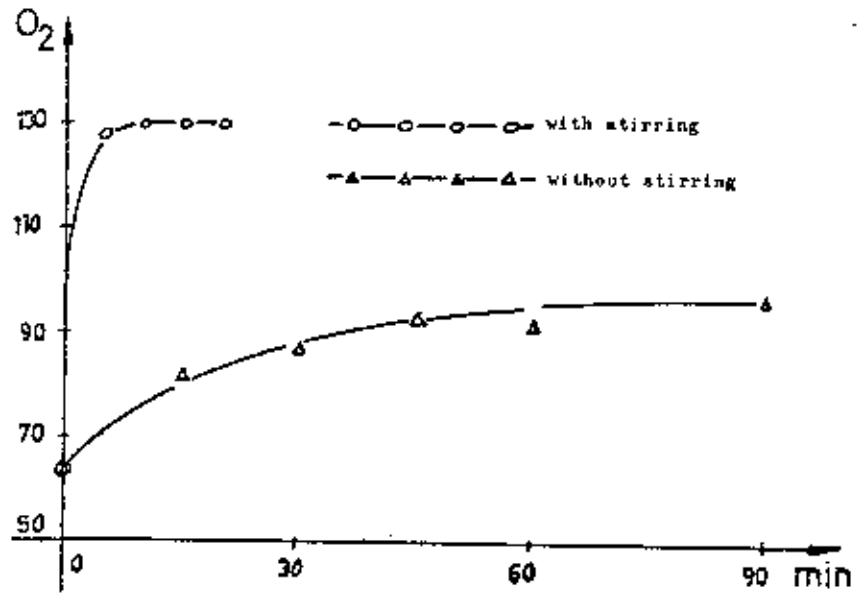


Figure 3.

The solubility of the oxygen is increased at higher temperatures. The sorption rate depends to a very great extent on the contact surface between air and oil. Figure 3 shows the absorption of oxygen in two samples from the same oil. The one sample has not been touched, whereas the second has been stirred.

The higher the ratio between the contact surface and volume of an oil sample (short diffusion distance), the more rapid the sorption of oxygen. The sorption rate depends on the difference in the partial pressures. The greater the difference of partial pressures, the faster the sorption rate of oxygen. The sorption isotherm (sorption of $O_2$ in relation to the partial pressure of $O_2$) is a straight line at constant temperature. The figure 4 has been taken from a newly published German paper (1).

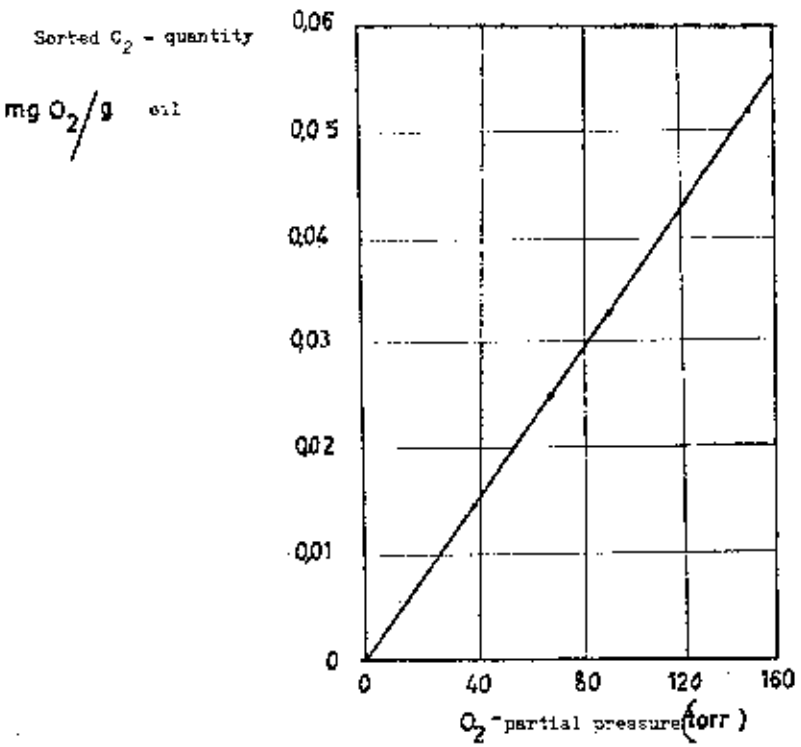


Figure 4.



From previous studies it is known that the form of the curve reveals whether the sorption is purely physical or whether it includes both physical and chemical sorption. The physical sorption gives a very straight and steep beginning to the oxygen sorption curve which later flattens out, whereas a chemical sorption is characterized by a slight rise in the latter part of the curve.

According to our investigations all vegetable oils give rise to a purely physical sorption. In fish oils the situation is more complicated and a strong chemical sorption can be detected. Hintze and others (1) have shown that the physical sorption takes place rapidly (1) 0.055 mg $O_2$/g oil in a natural soybean oil in air at 20°C. Thereafter, no sorption takes place for 25 days, but when a very

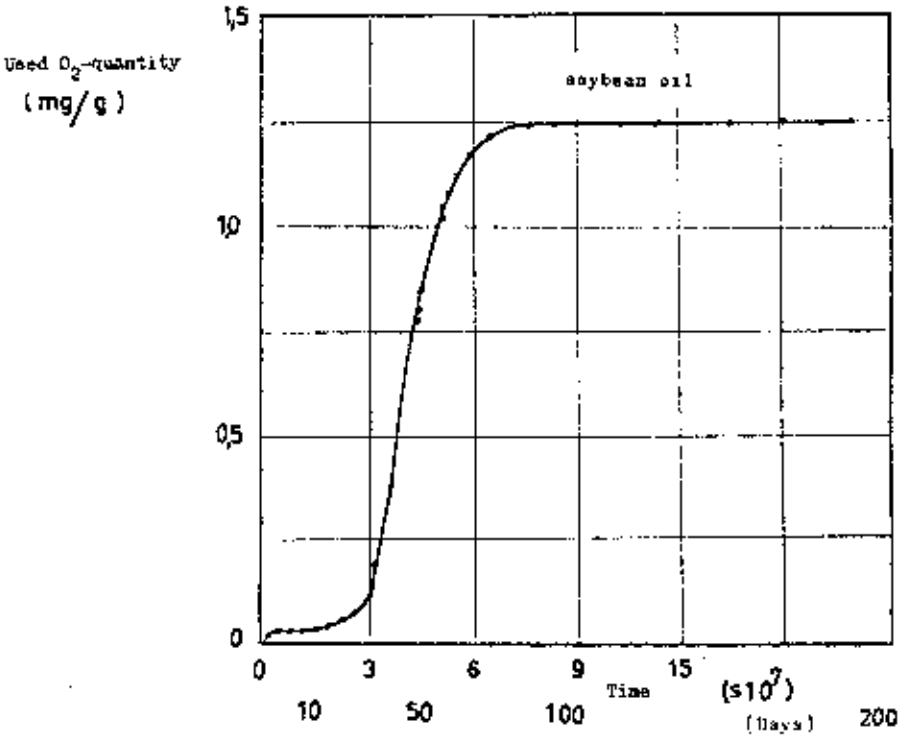


Figure 5.

strong oxygen consumption starts, caused by the chemical reaction between the oil and oxygen. This stops after about 80 days when the oil is completely oxidized, and no further sorption takes place.

## The connection between the sorption of oxygen and the oxidation of the oil

The mechanism for the oxidation of natural fats is complicated, especially because one must take into account effects from natural or synthetic antioxidant systems. With low oxygen content ($PO_2$ 100-200 torr) in the liquid phase and in an equilibrium between the gas phase and the liquid phase, it may be assumed that the auto-oxidation rate is proportional to the partial pressure of oxygen. This does not apply in rapidly auto-oxidated systems, such as, for example, marine oils, for which the auto-oxidation rate is primarily dependent on the dissolution rate for oxygen, i.e. on the partial

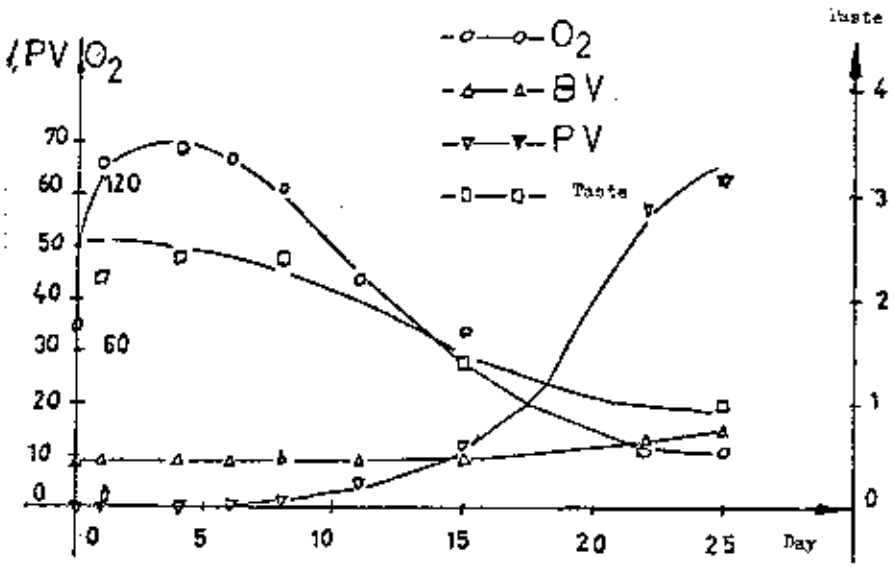


Figure 6.



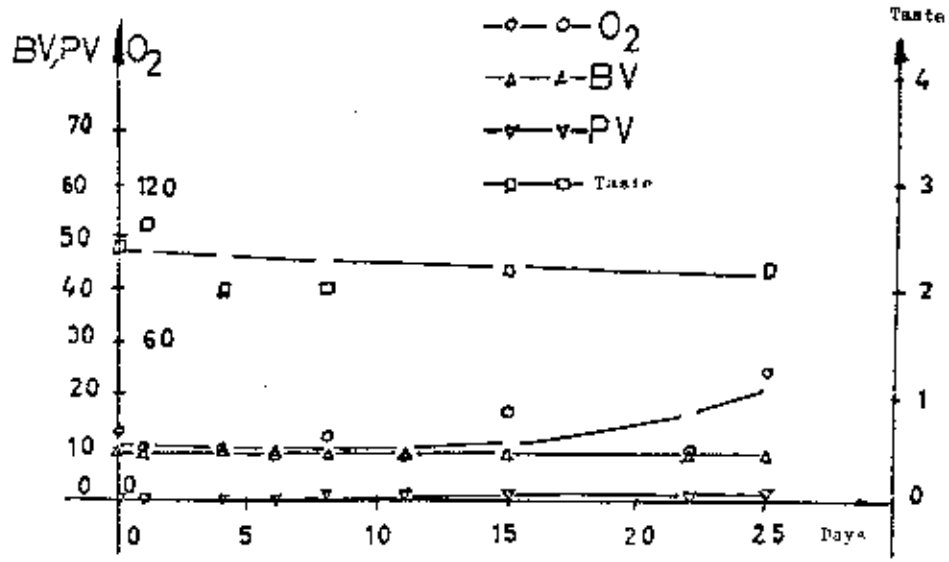


Figure 7.

pressure and the contact surface. Existing possibilities might be the use of a protective gas or vacuum during the processing and storage of oils.

Figures 6 and 7 illustrate the dramatic differences in quality that can be obtained. Figure 6 shows the oxidation process in a rapeseed oil that has been kept in contact with air at + 50°C.

Figure 7 presents data for a similar oil that has been protected by nitrogen gas.

Vacuum was also applied, but no substantial differences were found in the quality of the oil compared with the experiments with protective gas. Unfortunately it is difficult to carry out many treatments of the oil in the absence of air. How far the protection is to be carried is therefore mainly a technical and economic question.

The laboratory tests have, however, shown the way.

Literature reference:

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# Standardization of Nuclear Magnetic Resonance Measurement of Solids in Fats and Shortenings

J. Am. Oil Chem. Soc. 44: 397-399, Jul. 1967

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## Abstract

An investigation of factors that influence the measurement of solids in fats and shortenings by nuclear magnetic resonance was directed toward standardization of the method. The aim was to select abbreviated tempering conditions that would yield results comparable with those existing at temperature equilibrium. Conditions were found which reduced the time and gave an accuracy that made the method suitable for both research and control purposes.


## Introduction

REPORTS ON THE APPLICATION of nuclear magnetic resonance (NMR) measurements to the determination of solids in fats and shortenings (1–4), have been concerned primarily with showing the solids content of samples at selected temperatures and pointing out the superiority of these measurements over other techniques for evaluating solids content. Although investigators have standardized the testing for their own purposes, little or no information is available on the factors necessary for obtaining comparable results in different laboratories. These factors involve instrument calibration, appropriate chilling temperature and time, and tempering time that will give results comparable with equilibrium conditioning in as short a time as possible. This report will deal with the effect of these various factors on the determination of solids and selection of the conditions which, in a minimum time, give results identical with or close to the equilibrium values.

## Measurement by Nuclear Magnetic Resonance

For this purpose a low resolution NMR instrument, Schlumberger NMR Analyzer Model 104,[1] was employed. The first step in the application of an instrument of this type is the establishing of appropriate settings for the various parameters, based upon characteristics of the material to be tested and upon the size of sample to be employed. The conditions employed for the reported data were:

| | |
|---|---|
| Sample size | 4.5 to 5.5 grams |
| Instrument settings | |
| Sweep time | 1 minute |
| Sweep constant | 1 second |
| R.F. selector | 40 df |
| Sweep amplitude selector | 0.5 gauss |
| Sensitivity selector | X 500 |
| Modulation amplitude selector | 0.1 gauss |
| Integrator settings | |
| Read-out multiplier | 2 |
| Threshold, mv | 0.1 |
| Signal multiplier | 5 |

In preparing a sample for testing, a procedure must be followed which is reproducible. The principal steps are heating the sample to a selected starting temperature, chilling to a point where the solids formed are greater than they will be at the testing temperature, and tempering in a bath with proper temperature control for a period that will give equilibrium or conditions close to those existing at equilibrium.

After appropriate instrument settings and a satisfactory procedure for preparing the sample have been established, the next step is calibration of the instrument.

## Instrument Calibration

A calibration curve can be established from a sufficient number of measurements on a series of liquid oils, which will give a reliable average value for the liquid oils at the desired testing temperature. The calibration curve for the selected temperature is prepared by plotting the average of the series of measurements on the liquid oil as 0% (zero) solids and zero NMR measurement as 100% solids and then drawing a straight line between the two points. Measurements on a solid fat are not necessary as a completely solid fat will measure zero. Samples of vegetable oil hydrogenated to a low iodine value may measure 0 to 1 mv/g at room temperature but, on cooling, will measure zero, indicating the presence of a small amount of liquid at room temperature.

Measurements made on a series of oils at 0, 28, and 60C indicated that soybean, cottonseed, and peanut oil were similar and that safflower oil gave slightly lower NMR values, also that these values vary with temperature. Typical measurements are given in Table I. Thus, in preparing calibration curves, one must take into account both the testing temperature and the type of oil. Since soybean and cottonseed oils are the most typical oils processed, the calibration curves for different temperatures given in Figure 1 are based on the average values for these oils over the range of 0 to 60C. Values below 0C are taken from an extrapolation of the data to −15C. This extrapolation is in line with data for safflower oil, which were carried to −10C without solids appearing in the sample, as evidenced by the NMR measurements and the visual clarity of the sample.

In the past it has been the practice to set up calibration curves which were based on measurements made of known blends of completely hydrogenated oils with liquid oil. These have followed a straight line connecting the NMR value for the liquid oil (0% solids) and zero NMR value (100% solids), within the limits of the method, when the measurements were made in the region (0 to 30C), where the hydrogenated oil has not gone into solution appreciably.

TABLE I

Typical N-M-R Measurements on Liquid Vegetable Oils

| Temperature | Safflower oil | Soybean oil | Cottonseed oil | Peanut oil |
|---|---|---|---|---|
| 0C | 65.2 | 55.6 | 61.6 | 64.5 |
| 28C | 60.7 | 61.5 | 62.3 | 61.8 |
| 60C | 56.5 | 57.6 | 57.4 | 57.6 |

[1] This model has been replaced by Varian Associates as Model PA-7 Process Analyzer.

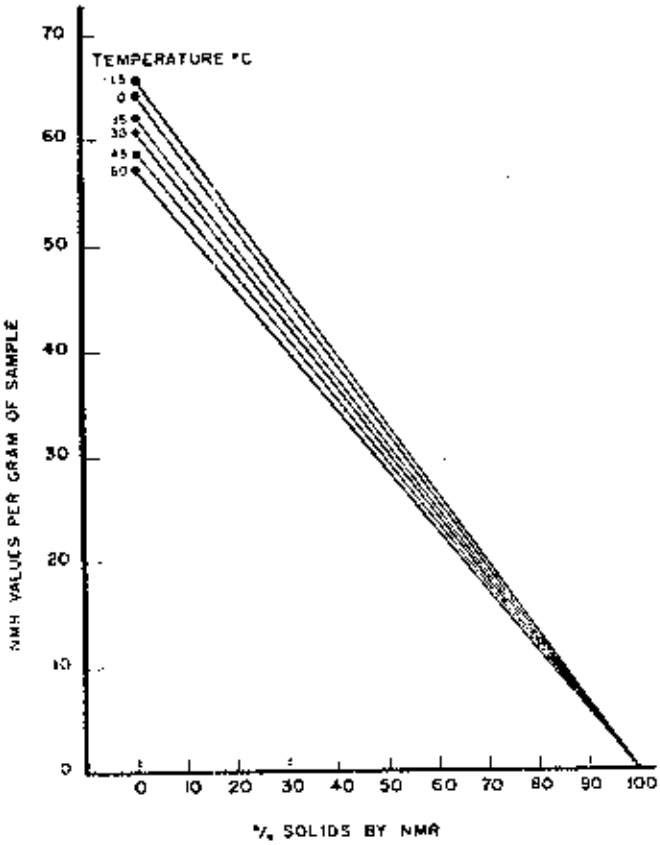


Fig. 1. Calibration graphs showing the effect of temperature.

This new approach to the preparation of a calibration graph saves time and increases the accuracy of the measurement of solids at a selected temperature.

## Chilling and Tempering

In previous reports by the authors, samples were chilled at 0C and for a time were comparable with that used in the Solids Fat Index (SFI) method (3) because subsequent measurements were going to be compared with SFI data. Subsequent work has revealed that chilling at 0C for the short periods selected was not adequate to solidify all the material that would be solid at 10C. This was evidenced by the increase in solid content of certain samples with time, when held in the 10C bath after chilling at 0C.

To attain results in a short time which will be comparable with those at equilibrium, it is necessary to approach the equilibrium conditions from the solid side. To accomplish this the sample must be chilled to a point where more solids are formed than will be present at the tempering temperature. If chilling is to be done rapidly, so that the method is suitable for routine testing, a very low temperature bath must be used. A carbon dioxide-acetone bath maintained at below −60C was the simplest and most convenient means for chilling the samples. Samples heated to 70C and placed in the carbon dioxide-acetone bath were chilled to −30C in 10 min. At −30C all of the glycerides solid at 0C and some or most triglyceride liquid at 0C will be in the solid state. Samples chilled as low as −40C attained the temperature of the tempering bath in 15 min as measured by a thermocouple in the center of the sample. This provided conditions satisfactory for obtaining an excess of solids prior to tempering. The temperature attained in the sample during tempering appeared to offer conditions that would be close to equilibrium after 30 min of tempering.

Tests made to show the effect of 5-, 10-, and 15-min chilling in the −60C bath, followed by tempering at 10C for 20, 30, and 50 min and for 16 hr, are shown graphically in Figure 2 for a lard and a tallow. Previous tests on these two fats after chilling at 0C had indicated inadequate chilling. The results in Figure 2 indicate that 10 min of chilling in the −60C bath, followed by 30 min of tempering, would probably be satisfactory for routine testing and give results comparable with those at equilibrium (16 hr of tempering).

## Measurement Tempered at 10 to 37.8C

In routine tests at a variety of temperatures the instrument will be maintained at a constant room temperature. If the samples are colder than room temperature, melting of the solids will occur with time. If the samples are warmer than room temperature, solids will form with time. Under these conditions it may be appropriate to deviate from the normal practice of taking the average of the first two sweep measurements. The operating procedure that will give the best results, based on tests made on 15 shortenings and five hydrogenated vegetable oils in which the samples were heated to 70C, chilled 10 min in the −60C bath, then measured after 30 min and 16 hr of tempering, are summarized in Table II. All measurements were based on the average of the first and second sweep except when indicated otherwise.

Under the conditions illustrated in Table II the precision of the method, as expressed by the standard

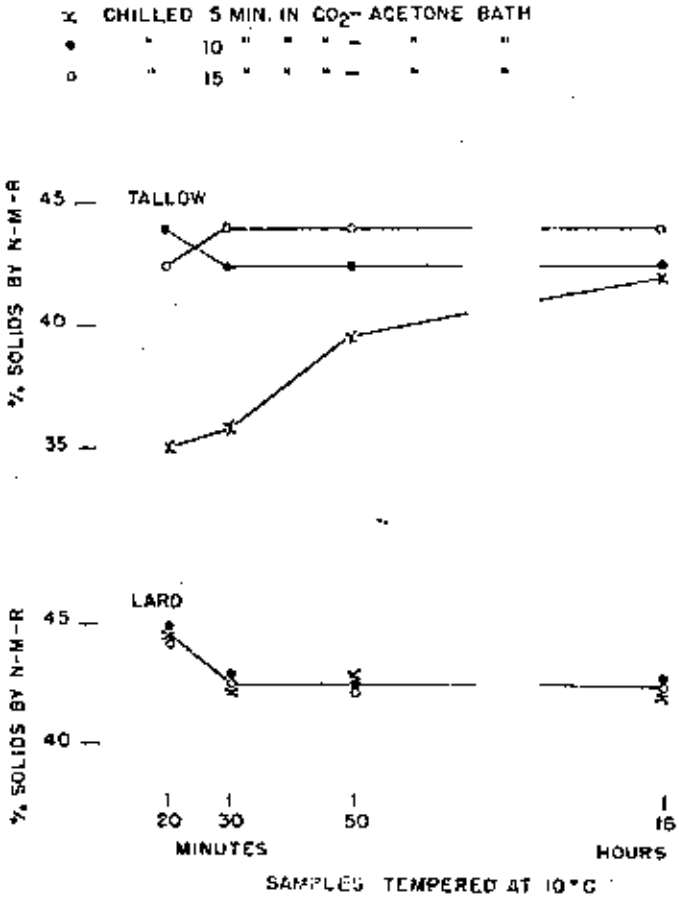


Fig. 2. Effect of chilling and tempering time on solids found when tempered at 10C.

TABLE II

Summary of Data for the Determination of Solids in 16 Shortenings and Five Hydrogenated Vegetable Oils after 30 Minutes and 16 Hours of Tempering

| | Tempering Temperature, °C | | | | |
|---|---|---|---|---|---|
| | 10 | 21.1 | 26.7 | 33.3 | 37.8 |
| Tempered 30 minutes | 50.3[a] | 33.4 | 23.7 | 11.2 | 10.3 |
| Tempered 16 hours | 50.4 | 31.1 | 22.6 | 11.2 | 9.2 |

[a] Average of the second and third sweep used for calculating the solids content

deviation, is approximately ±1.0%, and the 95% confidence limits for a single value (average of two weeps) is approximately ±2.0%.

The precision of the method and accuracy of the results obtained after 30 min of tempering are satisfactory for routine analysis, control analysis, and many research studies. Greater accuracy can be attained as required, by appropriate changes in chilling, tempering, and/or increasing the number of determinations on the same sample.

The conditions reported in this paper covering instrument calibration, chilling, tempering, and measurements have simplified the preparation of a calibration graph without sacrificing accuracy and reduced the time required for a test to a minimum while at the same time retaining accuracy. The testing parameters set forth should make it possible to obtain comparable results in different laboratories which would be only slightly more variable than those within a laboratory, for example, than those reported in this paper.

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# Chemical Reactions Involved in the Catalytic Hydrogenation of Oils. II. Identification of Some Volatile By-Products[1,2]

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## Abstract

Gas chromatography of the volatile by-products produced during the catalytic hydrogenation of an autoxidized soybean oil with a peroxide number of 11.2 meq/kg yielded approx 41 peaks. Twenty-one of the gas chromatographic fractions were collected and rechromatographed, and their chemical identity studied with micro-IR and mass spectrophotometry. These volatile by-products, according to peak area of gas chromatogram, showed a predominance of hydrocarbons and alcohols.

The fractions which have been identified are *n*-octane, *n*-nonane, *n*-decane, *n*-heptadecane, *n*-hexanol, *n*-heptanol, *n*-decanol, *n*-hexanal, *n*-decanal and 3-nonanone. Among the gas chromatographic fractions collected, *n*-decanol has an odor most reminiscent of that of catalytic hydrogenation.


## Introduction

THE IMPORTANCE OF THE chemical characterization of the volatile by-products of hydrogenation and the scarcity of literature in this field were discussed previously (1). The present paper reports an investigation of the isolation, fractionation and chemical identification of some volatile by-products which are produced during catalytic hydrogenation of a soybean oil with a peroxide value of 11.2 meq/kg.

## Experimental

*Volatile By-Products of Hydrogenation.* The volatile by-products isolated from 9.5 gal of hydrogenated soybean oil as previously described (1), were used for the present investigation.

*Gas Chromatography.* The ether solution of the volatile by-products was fractionated with a Beckman GC-2A gas chromatograph. The temp was linearly programmed with a Thermotrac Temperature Programmer from 45-200C in 30 min and then maintained at 200C for the remainder of the chromatography. An 8-ft aluminum column, ¼ in. I.D. packed with 15% Ucon Polar 50HB 280X on 80/100 mesh Chromosorb W (Acid washed), was used at a helium flow rate of 80 ml/min.

*Collection of Gas Chromatographic Fractions.* The gas chromatographic fractions were collected with a fraction collector designed and built in our laboratories (2). This fraction collector can be used to collect 18 fractions from one run. The gas chromatography was repeated 10 times. Each fraction was accumulatively collected in one cold trap. In this investigation, two runs of collection were made. In the first, 10 fractions were collected; in the second, 18 fractions. Since some of the fractions collected were duplicate peaks, a total of only 21 different gas chromatographic fractions were collected and studied.

*Identification of Gas Chromatographic Fractions.* The gas chromatographic fractions were identified by their IR spectra with the use of Sadtler standard spectra. The identifications were then confirmed by comparison of retention times with known compounds. The IR spectra of the gas chromatographic fractions in carbon tetrachloride solution were determined with a Beckman IR-8 spectrophotometer using ultra-micro cavity cells of 0.1-mm path length and beam condenser (Connecticut Instrument Corp.). An attenuated grid and variable wedge cell (W-1, Connecticut Instrument Corp.) were used in the reference beam to compensate for the adsorption due to solvent. By this technique, an IR spectrum could be obtained from 0.2 mg of sample.

When IR spectrum alone was insufficient for the chemical characterization of a gas chromatographic fraction, its mass spectrum was determined with a Bendix time-of-flight mass spectrometer.

## Results and Discussion

Gas chromatography of the volatile compounds which were produced from soybean oil (PV, 11.2 meq/kg) during catalytic hydrogenation at 180C with 0.125% nickel catalyst (1) yielded approx 41 peaks (Fig. 1). The gas chromatogram is considerably different from the one published in our previous paper because of temp programming and the use of a more coned solution. Carbowax 20M and Carbowax 1000 were also tried as the stationary phase but did not give as good resolution as Ucon Polar 50 HB 280X. As shown by the Arabic number on the chromatogram, 21 of these gas chromatographic fractions were collected. Each of the collected fractions was then rechromatographed under the original conditions to insure that it was not contaminated by its neighboring peaks. Each fraction yielded only one symmetrical peak.

The volatile by-products of hydrogenation according to peak area of gas chromatogram, showed a predominance of hydrocarbons and alcohols. As determined by the IR spectra of the 21 collected gas chromatographic fractions (Fig. 1), 10 were hydrocarbons (fractions 2,3,4,6,7,8,14,16,17 and 20), three were alcohols (fractions 10,13 and 18), two were aldehydes (fractions 5 and 15), one was a ketone (fraction 12), three were other carbonyl compounds (fractions 1,9

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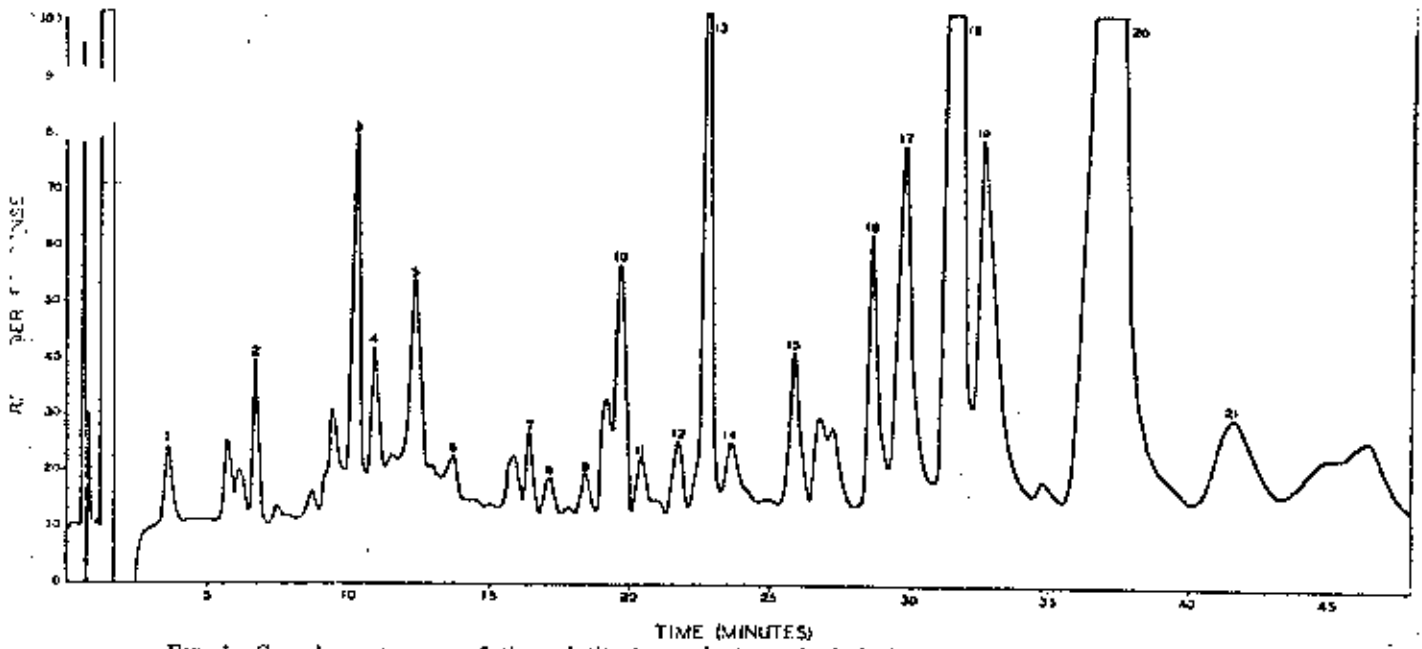


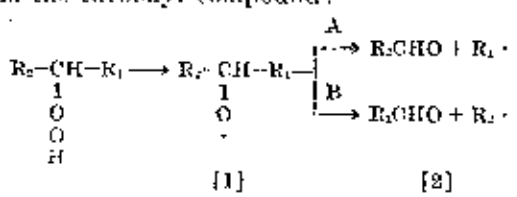

FIG. 1. Gas chromatogram of the volatile by-products evolved during hydrogenation of soybean oil.

nd 11), and two could not be classified (fractions 19 and 21). These compounds are considerably different from the decomposition products of autoxidative deg-dation. The latter are predominantly carbonyl com-unds while the former are predominantly hydro-carbons and alcohols.

Ten compounds have been identified (Table 1), viz. ·, *n*-nonane, *n*-decane, *n*-heptadecane, *n*-hexa-ptanol, *n*-decanol, *n*-hexanal, *n*-decanal and 3-non...one. Among them, *n*-decanal has an odor most reminiscent of that of catalytic hydrogenation accord-g to a panel of four members. In a previous paper this series, it has been reported that saturated alde-hydes and ketones may also contribute to the char-acteristic hydrogenation flavor (1).

The mechanism for the formation of by-products by talytic hydrogenation is difficult to postulate be-cause many variables, such as temp, pressure, catalyst and oil may each affect the reactions involved. Since e refined and bleached oils used for this experiment ..ve a peroxide number of 11.2 meq/kg, a likely source of the volatile by-products could be hydro-peroxides.

The generally accepted mechanism for the formation .. carbonyl compounds by decomposition of hydro-peroxides of unsaturated fatty esters is based upon Farmer's theory (3,4) which involves a homolytic straction of a hydrogen atom to give the alkoxyl radical [1] which undergoes carbon-to-carbon fission to form the carbonyl compound:




[1] [2]

$R_1$ = ester end
$R_2$ = hydrocarbon end

The formation of *n*-hexanal and *n*-decanal could occur by a type A cleavage. The formation of alcohols and hydrocarbons could be explained by a type B cleavage. For example, a type B cleavage from the hydroperoxide of reduced oleate could lead to *n*-decanol or *n*-decane by combining the alkyl radical with hydroxyl or hydrogen radicals respectively. Since it is well known that peroxides are readily reduced to alcohols, if the above scheme is applicable, one would have to assume that the thermal decomposition of the hydroperoxide occurs more rapidly than reduction.

TABLE I

Gas Chromatographic Fractions Identified

| Fraction number | Identified as |
|---|---|
| 1 | Acetone (tentative) |
| 2 | *n*-octane |
| 3 | *n*-nonane |
| 5 | *n*-hexanal |
| 6 | *n*-decane |
| 10 | *n*-hexanol |
| 12 | 3-nonanone (by mass spectrum) |
| 13 | *n*-heptanol |
| 15 | *n*-decanal |
| 16 | *n*-decanol |
| 20 | *n*-heptadecane (by mass spectrum) |

The formation of 3-nonanone could be predicted by the mechanism as suggested by Smouse and Chang (5) through the reaction of a carbonyl free radical originated from an aldehyde and a free ethyl radical. It is also possible that 3-nonanone is produced by catalytic hydrogenation of vinyl hexyl ketone. Fross (6) has reported that vinyl amyl ketone can originate from the autoxidation of linoleate. The *n*-heptadecane may be formed through decarboxylation of a free fatty acid under the conditions of hydrogenation.

The present investigation indicates that a major predominant by-product of catalytic hydrogenation is hydrocarbon. Its formation may also yield relatively high mol wt carbonyl esters as shown by [2], where R in this formula could conceivably be a triglyceride group. Difunctional compounds of this type would not be expected to be volatile under conditions of deodorization and would therefore remain in the hydrogenated oil. Studies on the nonvolatile constituents remaining in the oil are currently in progress.

ACKNOWLEDGMENTS

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Lancet, 2:877, Oct. 19, 1968

## SOYA-BEAN OIL IN MYOCARDIAL INFARCTION

SIR,—Dr. Leitner (Oct. 5, p. 781) may be interested to know that studies aiming at primary prevention of coronary heart-disease (i.e., prevention among a population initially free from manifestations) by partial dietary replacement of non-essential with essential fatty acids have been running for several years. The Anti-Coronary Club Project in New York, which is not properly controlled, was started in 1957 and has reported favourable results.[1] The controlled trial at the Los Angeles Veterans Administration Center was started in 1959.[2] The National Diet-Heart Study in the U.S.A. has recently reported its results.[3]

At the International Nutrition Congress in Hamburg two years ago the results of the Finnish trial were reported, and a full account of the results in men was published six months ago.[4] In this study, which started in 1958, middle-aged men in a mental hospital had their diet altered so that the main effect was almost totally to replace the milk fat in the original diet with vegetable oils, mostly soya-bean oil. In a second mental hospital no intentional change in the diet was made. The incidence of coronary heart-disease, assessed on the basis of either electrocardiographic changes or coronary-heart-disease mortality, was significantly lower in the experimental hospital; the authors concluded "that the lower incidence in the experimental group was primarily due to the cholesterol-lowering special diet".

Oxford. HUGH SINCLAIR.

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# PROBLEMS DISCOURAGING USE OF FISH OIL IN AMERICAN MANUFACTURED SHORTENING AND MARGARINE

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p. 629

SUGGESTED KEY WORDS

Fish oil

Shortening

Margarine

Hydrogenation

Monoglycerides

Diglycerides

Flavor, reversion of

## INTRODUCTION

Twenty-five years ago more than 100 million pounds of fish oil, primarily pilchard (i.e., California sardine) oil, were used in manufacture of American produced margarine and shortening; today no American fish oil is so used. Prices of fish oils often fluctuate widely. When this has occurred, American fish oil manufacturers have felt that had they had access to margarine and shortening uses as an outlet for their product, the price fluctuations would have been less severe. In all other countries the principal use of fish oils is in the edible oil field. Yet there are a variety of problems which would have to be overcome before such markets could be available to the American fish oil industry. In this article, the historical developments which have brought about the situation here are traced and the problems which would have to be overcome to alleviate the situation are considered.

## PRE-1920 PERIOD

The chemistry of preparing hydrogenated fats from triglycerides containing unsaturated fatty acids was worked out and reported between 1897 and the first few years of the twentieth century. By 1910, with the chemical basis of the process well documented, industrial research was under way toward perfecting methods for efficient manufacture of hydrogenated fat for use in edible products. Fish oils were among the first oils considered in this early work. E. Bedford and C. E. Williams (3) were among the first in this field, filing on December 11, 1911 for a patent on methods for hydrogenating fish oils. The first U. S. patent on methods for hydrogenating fish oil was granted to Vilbushevitch in 1912 (14).

ABSTRACT

Prior to 1950 California sardine oil was a component of many margarines and shortenings of American manufacture with about 140 million pounds of fish oil used per annum at peak years. With the disappearance of commercial quantities of California sardines after 1950, fish oil dropped out of use for edible purposes in the United States. In intervening years various circumstances such as adoption of margarine standards of identity without provision for the use of fish oils has discouraged use of other American fish oils (e.g., menhaden oil) in domestic production of edible fats. Of the various problems - uniformity of quality, legal requirements, public image, and price considerations - the latter problem will be the most difficult to overcome before fish oils can be in demand for such uses. Satisfaction of legal and sanitation requirements can be met only by costly improvements in manufacturing facilities for preparation of the fish oil, yet the main inducement for use of fish oils is their availability at competitively low cost.

During the decade 1910 to 1920, applied research brought the hydrogenation industry in European countries from a tiny endeavor to almost a full-grown operation. The nucleus for the later gigantic Unilever Company began with research operations and a very small output at a small concern started in 1911 at Joseph Cross Field near Warrington, England. By 1916 this concern, renamed Unilever, was, according to Lassen (8), already using fish oils.

Although by 1920 the use of hydrogenated fish oils in Europe was a fair-sized industry, operations had yet to get under way in this country. Even the use of vegetable oils for hydrogenation lagged far behind that of most European countries. Failure to use fish oil in margarine as early as was done in Europe was due, at least in part, to the fact that margarine itself was not as important a food commodity here as in Europe. Repressive legislative made sale of margarine illegal in some states, or in others subject to such restrictive practices that the margarine could only be sold as a white, colorless product.

Although hydrogenated fish oil was not manufactured in the United States during the decade ending in 1920, during World War I fish oil for edible use was included in demonstrations of fish cookery conducted by the Bureau of Fisheries.

## 1920 TO 1951 PERIOD

Shortage of vegetable oils in the United States during World War I had focused attention upon the possibility of using fish oil in margarine or shortening. Considerable research and experimentation was carried out during the first part of the decade starting in 1920. Research by

Richardson, Knuth, and Milligan (10) investigated the types of fatty acids produced during the hydrogenation of American species of fish oils, especially menhaden. Meantime, applied research on California sardine oil was under way at two hydrogenation plants in the Los Angeles area. This oil was the type which looked the most promising of any American fish oils to use for hydrogenation. California sardines had become a major source of fish during World War I. Markets for sardine oil had not been firmly established and much of it was going into the low-priced outlet for use as soap. Furthermore, California law prohibited the taking of sardines for other than food use so that only the waste from sardine canneries could be processed into oil if it was to be used for industrial purposes. If, however, a market for use of sardine oil could be developed where the oil was used in an edible product, it was argued (1) that whole sardines could be legally rendered. These possibilities spurred research to find wider markets for sardine oil, especially in the field of an edible oil. By 1925, 120,000 pounds of California sardine oil were hydrogenated and used in margarine. By 1928, the annual use of sardine oil in margarine had risen to 15 million pounds, and in 1936, a peak use in this way of 40 million pounds was reached.

An indirect use of fish oil in shortening accounted for a much larger annual consumption than showed up in published statistics for use of fish oil in edible products. This use involved the preparation of hydrogenated mono- and diglycerides from sardine oil by partial glycerolysis and hydrogenation. Mixtures of mono- and diglycerides have been added to shortening for many years (2) (12) in the preparation of superglycerinated shortening. This product gets its name from the process of preparing the

mono- and diglycerides by heating the oil with glycerine. The resulting mixture of mono- and diglycerides is added at a level of 2.5 to 3 percent to make the superglycerinated shortening. Such shortening is much in demand in the baking industry, especially for the preparation of cakes. Its use permits a higher ratio of sugar to flour to be used in cake without resulting in a deterimental texture. Superglycerinated shortenings are widely used in bakeries and for general household use. Their application to deep-fat frying is not successful owing to lowering of smoke temperature.

A very large volume of mono- and diglycerides was made from California sardine oil at a hydrogenation plant at Tacoma, Washington, during the 1930's and 1940's. This product was used not only in this country but also was shipped abroad, and the volume of sardine oil used reached at one time a peak of about 100 million pounds per year.

The last year that California sardine oil was available was 1951. After that year, the fish disappeared almost completely. During the late 1940's when a shortage of sardine oil first occurred, the large glycerolysis operation supplemented its oil supply with herring oil from Alaska and British Columbia. The supply of this and other oils on the Pacific Coast was not sufficient to sustain this production after complete disappearance of the California sardine. Furthermore, the increasing demand for hydrogen in the Tacoma area for the manufacture of ammonia left too little for other uses. As a result of these factors, this large-scale glycerolysis-hydrogenation operation ceased in 1951, as did also the use of fish oil as a triglyceride source for margarine and shortening manufacture in the United States.

PERIOD SINCE 1951

In the years immediately following 1951, no fish oil whatever was made into shortening or margarine in this country. Principal reasons for this situation stem from changes in the U.S. Food and Drug regulations. The standards of identity promulgated for margarine did not permit the use of fish oil. While there was no such restriction on use of fish oil in shortening, new sanitary requirements for raw materials going into food products precluded the use of the ordinary commercial fish oil for this purpose. All existing plants for manufacture of fish oil were far from meeting the new sanitation requirements for manufacture of ingredients of food products. The only case of which we know where fish oil was included in an American manufactured shortening since 1951 occurred during 1967. A large manufacturer of shortening used fish oil for a short time in their product marketed here. The oil used was herring oil imported from Iceland and manufactured in a plant having adequate sanitation for processing of food for human consumption. The inclusion of fish oil was discontinued because of the apprehension that inclusion of the required labeling statement which indicated presence of a marine oil would reduce the volume of sale of the product.

Since 1951 fish oils have continued to be used in increasing volume by most countries other than the United States. Most European countries are using considerable quantities in this way, especially northern European countries such as Great Britain and the Scandinavian countries. Japan also uses much hydrogenated fish oil, and this practice is beginning in South America, especially in Peru and Chile. Canada, prior to 1950, had legal restrictions which prevented hydrogenated fish oil from being

used in this way. Since that time, however, these restriction have been removed. Canada uses much domestic herring oil and some imported menhaden oil in their margarine and to a lesser extent in shortening.

In the period since 1951, the margarine and shortening industry has made the transition from the situation where cottonseed and coconut oils were the principal sources for their production to one where soybean oil is now the principal source. This situation should be of interest to the fishing industry. Soybean oil resembles fish oil in that it contains considerable quantities of $\omega$-3 fatty acids. These fatty acids cause a serious reversion problem. During storage, there is a tendency for the original, somewhat unpleasant flavor which had been removed by hydrogenation and other refining to return. Nevertheless research has largely overcome this problem.

PROBLEMS IN USE OF FISH OIL IN SHORTENING IN THE UNITED STATES

There is a variety of reasons given by different individuals in the American shortening industry for the failure of fish oil to be looked upon as a highly desirable raw material source. In this section these reasons will be examined and evaluated.

A. Reversion

Early (prior to 1952) users of fish oil in this country mentioned flavor reversion as a major problem which was at that time a greater one than when vegetable oils were employed. Before considering reversion problems, a discussion will be given on the nature of reversion.

Two kinds of flavor reversion occur with hydrogenated oils. One of these results in the return of the flavor of the original oil itself.

Soybean oil has a "green" or "grassy" flavor. It is sometimes described as "beany." This flavor is generally disliked. Immediately after hydrogeneration and other refining steps are completed, this type of flavor is absent. It may return, however, after prolonged storage, especially when the product is shortening. Shortening is stored at room temperature for a year or longer, whereas margarine is held under refrigeration for, at most, only a few months. Furthermore, margarine contains flavor components, e.g., diacetyl, which may mask small degrees of flavor reversion.

Similar reversion can occur with fish oil except that instead of a "grassy" or "beany" flavor, the typical "fishy" flavor returns. A slight "fishy" flavor in a shortening would probably be considered to be a worse defect than a slight "grassy" or "beany" flavor.

There is an entirely different kind of flavor reversion that can occur with any hydrogenated oil. The process of hydrogenation results in a flavor peculiar to the hydrogenation process itself. This flavor is variously described as a "hardening" flavor, a "tallowy" flavor, a "candle-like" flavor, or a "cardboard" flavor. This flavor disappears as a result of refining steps which follow hydrogenation. It can, however, revert upon storage. These flavors and their reversion properties occur alike with hydrogenated soybean oil, hydrogenated fish oil, or in fact with any hydrogenated oil.

Hannewijk (4) ascribes the return of this "hardening" flavor to a very slight increase in bound aldehyde content which occurs upon storage. Bound aldehydes form as a result of oxidation so that this form of flavor reversion is also one involving oxidation. Hannewijk (4) also describes a very odd situation with regard to this type of flavor

reversion. The reverted flavor is sometimes not noticed when the product is tasted indoors, but when the taster goes outdoors and inhales fresh air (even as long as a half-hour after tasting), he experiences "an offensive taste in the throat." The hardening type of flavor in hardened soybean oils, linseed oil, and marine oils according to Hannewijk (4) is caused by the presence of 6-trans-nonenal, an aldehyde oxidation product which has a taste threshold value of only 0.0003 parts per million.

Since both types of flavor reversion result from oxidation, the degree of unsaturation of the fish oil (and changes that occur during hydrogenation) is important in determining the stability against flavor reversion. This makes hydrogenated sardine oil more vulnerable to reversion than hydrogenated menhaden oil. Very likely the severe difficulties experienced with flavor reversion in shortening made with California sardine oil could be much less acute in shortening made from hydrogenated menhaden oil.

Flavor reversion has been an equally difficult problem with soybean oil. The very extensive research carried out the past 20 years on soybean oil reversion has overcome most of these problems. Because both soybean oil and fish oil have similar types of fatty acids which are both highly polyunsaturated and of the $\omega$-3 type, there is good reason to believe that experience of American shortening manufacturers with soybean oil can be adapted to overcoming similar problems with fish oils. This has certainly been the case with handlers of fish oil in margarine and shortening abroad. There is actually a feeling abroad that from the standpoint of reversion, fish oils are perhaps easier to handle than soybean oil.

B. Miscellaneous Technical Problems

There are a number of miscellaneous problems which potential users of fish oil mention as giving difficulties. One of these is the statement that the quality of fish oils is less uniform than is that of most other oils. By quality is generally meant such factors as free fatty acid content and color. The variation also extends sometimes to composition, e.g., iodine value may vary from batch to batch.

From the standpoint of hydrogenation costs, the content of sulfur compounds in fish oil is sometimes excessive. High sulfur content poisons the hydrogenation catalyst, requiring more to be used. This problem is one of considerable current concern to hydrogenators, and at the Eleventh World Congress, IEF, several papers discussed it. Mørk (9) described extensive research on the mechanism of the action of sulfur in fish oils as it affected catalyst poisoning and Urdahl et al. (13) showed that fish oils prepared from very freshly caught fish contained considerably less sulfur than did those made from spoiled fish. Recent legislation in Denmark requires all fish, including those to be used for fish oil production, to be iced with at least 20% of their weight of ice, and research is under way to develop mechanical icing equipment for use aboard vessels which would make it economically feasible to ice fish destined for fish meal and oil production (5).

Most problems of uniformity can be overcome by more careful quality control or by blending of different batches of oil.

C. Public Image of Fish Oil

The image of fish oil in the mind of the public varies from one region of the world to another. In many countries a statement on the

label of margarine or shortening that the product contains oil of marine origin causes little or no difficulty. There is a strong belief by many that such a statement on an American product would cause strong buyer resistance. Perhaps this situation stems partly from the buildup of the idea that vegetable oils in a shortening are far superior to animal fat. This situation, of course, developed years ago when the then newly introduced hydrogenated vegetable oil shortenings provided superior resistance against oxidation and resulting rancidity than was possessed by lard. Even today many shortenings stress the wording "100% vegetable oils."

When sardine oil was added to shortening in the United States prior to 1952, it was common to use it only in a second-grade product under a different label of a given manufacturer. There is considerable belief in the American shortening industry that such a practice would be necessary today should fish oil be used. Use of fish oil in only second-grade shortenings would have an adverse effect on the price that could be paid for the fish oil.

There is one possible way to avoid this problem. In past years when a mono- and diglyceride product was added to a shortening, the additive was classified as a chemical, and there was no requirement to indicate the source of components of added chemicals. If this is still the case, it should be possible to add hydrogenated mono- and diglycerides prepared from fish. While only 2.5 to 3 percent of a superglycerinated shortening can be of this mono- and diglyceride mixture, the volume of shortening of this kind is so great that a very large proportion, if not all, of the American menhaden oil production would be required to saturate the market.

D. Price

The principal, perhaps the only reason that the American shortening industry might be interested in using hydrogenated fish oil in its product is the factor of price. Yet the reason the fish oil industry wants to develop markets for their product in shortening is to stabilize price -- hopefully to increase it substantially.

The steps necessary to improve American produced fish oil to a point where it can be used in shortening -- improving the manufacturing plants with respect to sanitation and making the general quality of the oil more uniform -- will raise the cost of production. Probably this will increase the selling price of the fish oil. Is there still room between the price the shortening industry is paying for its raw material and this increased manufacturing cost of fish oil, to add a further price increment to improve the profit margin of the fish oil industry and still leave a price attractive to the shortening industry? The answer to this question may be one of the most critical factors in determining whether fish oil will again be used by the American shortening industry.

E. Legal Restrictions

When American fish oil started to be used during the 1920's as a component of shortening and margarine, there were no legal restrictions concerning its use, and this situation continued for many years. Today, and since use of fish oils in the United States for edible purposes has all but disappeared, new requirements established by the Food and Drug Administration have been adopted relating to the sanitation conditions of the plant in which the food component is prepared. Unfortunately few, if any, fish oil plants in this country meet such requirements for

preparation of a component of food for human consumption. If fish oil is to be used in food in this country, it is quite universally agreed that most plants will have to be modified to meet required standards.

Since the early days when American margarine manufacturers used fish oil in their product, a standard of identity has been set up for margarine by the Food and Drug Administration. Because fish oil was not included in these standards, it cannot now be used in margarine. While it is possible to change standards of identity, such changes require extensive hearings with new evidence submitted, and at best are very time-consuming and lengthy.

There is some fear on the part of the fishing industry that the Food and Drug Administration might take some administrative action toward barring the use of fish oil in shortening on some other grounds. These might relate to the question of whether hydrogenated fish oils are a safe or desirable component of a shortening, or whether, from esthetic grounds, a product made from whole fish is desirable. Fish oils were used in this country extensively for many years up to 1952 in both shortening and margarine. Today hydrogenated fish oils are used more than ever before in northern European countries, in Canada, in Japan, and in some South American countries. In no place has either of the two above-mentioned reasons ever been used to bar use of hydrogenated fish oils in shortening.

Another problem in using marine oils, particularly menhaden oil, for edible products has recently arisen as a result of Codex Alimentarius standards (recommended but not yet adopted) for international trading purposes, where the amounts of iron and arsenic are to be limited

to 0.1 parts per million. In menhaden oil each of these trace elements is often present at levels above those limits. Research on the trace element, sulfur, as previously mentioned has shown that use of the freshest possible fish will diminish the amount of this element. The same may be true for iron and especially for arsenic, and restriction of production of menhaden oil to use of only fresh raw material might overcome this problem. This might mean that the fish would need to be iced at sea as is being done in Denmark for herring and certain other species, which would increase production costs considerably.

Since recent reports of the adverse dietary effects of certain components of peanut oil such as erucic acid, e.g., Kritchevsky et al. (7), upon fatty infiltration of the myocardium with subsequent fibrotic changes in experimental animals, the question as to whether hydrogenated herring or other fish oil might pose similar problems has concerned manufacturers of edible products containing such fish oil. Currently, a large cooperative investigation on this matter between governmental laboratories in Canada and Norway (6) is under way. Until results of this or of similar investigations have been concluded, we cannot yet assess whether such factors may be a further deterrent to wider use of fish oils in edible food products. Other nutritional aspects of fish oils have been reviewed by Stansby (11).

In contacts with individuals outside the fishing industry who might be concerned about the use of hydrogenated fish oils in shortening, of the five main potential sources of problems discussed above--reversion, miscellaneous technical problems, public image, price, and legal

restrictions--the last mentioned of these was the one which seemed to be of least concern, on the assumption that the fish oil would, in any case, have to be made in plants possessing adequate sanitation. The matter of price of the fish oil would appear to be the most critical in determining the possibility for use of hydrogenated fish oils in edible fats of American manufacture.

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# Pesticide Levels in Fish of the Northeast Pacific

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During the past year, a program was initiated to monitor the pesticide levels of edible fish in the Pacific Northwest. The residues monitored were DDT and its metabolites, DDE and TDE. The species sampled were anchovy (Engraulis mordax), Dungeness crab (Cancer magister), English sole (Parophrys vetulus), hake (Merluccius productus), ocean perch (Sebastodes alutus), starry flounder (Platichthys stellatus), true cod (Gadus macrocephalus), and yellowtail rockfish (Sebastodes flavidus)[1]. Since only minimal information about pesticides in fishery products appears in the literature, our results may prove useful to others in the field.

## Experimental Methods

The samples were extracted (1) and analyzed (2) according to standard methods. The sample size was adjusted to keep the fat content below 2 g. Except for individual analyses of anchovies, all fish samples weighed at least 20 g. Although the florisil PR[2] was activated at 145°, part of the endrin still appeared in the 94+6 fraction. Dieldrin, however, was completely recovered in the 85+15 fraction. Since the endrin GLC peak was well separated from the other peaks, its presence in the 94+6 eluate was ignored. A Wilkens 600D gas chromatograph with a tritium detector and a 5'x1/8" glass column containing 2% SE-30 on HMDS treated 80/100 mesh Chromosorb W at 195° was used for quantitation. The identity of peaks was confirmed using p-values (3).

The reported values for DDT include both o,p'- and p,p'-DDT. They were obtained by comparison with technical DDT, in order to better approximate the toxicologically significant amount of organochlorine compounds present.

In general, a standard error of about 15% can be assumed.

## Results

The data are tabulated. All samples included in an individual entry are from the same catch of fish. Where male and female samples are listed sequentially, they are from a single catch. The inclusion of anchovy and hake as edible species derived from the current use of meal from them for livestock feeding and the projected production of marine protein concentrates from species

[1]Oysters were intentionally omitted from this study because they are under monthly surveillance as part of the National Pesticide Monitoring Program initiated by Philip A. Butler, U.S. Bureau of Commercial Fisheries Biological Laboratory, Gulf Breeze, Florida.

[2]Trade names referred to in this publication do not imply endorsement of commercial products.



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TABLE

Pesticide Residues in Various Species of Fish and Shellfish

| Species | Source | Sex | Number of fish analyzed[a] | Residues (ppm.) DDE | TDE | DDT |
|---|---|---|---|---|---|---|
| Anchovy | Grays Harbor, Wash. | | 24 | 0.074 | 0.074 | |
| | | | 19 | 0.074 | 0.098 | |
| | | | 4[a] | 0.058-0.172 | 0.073-0.244 | |
| Crab | Destruction Island, Wash. | M | 1 | 0.039 | 0.011 | --- |
| | Ilwaco, Wash. | M | 4[a] | 0.027-0.040 | 0.017-0.021 | trace-0.013 |
| English sole[b] | Blaine, Wash. | F | 3[a] | 0.009-0.016 | 0.009-0.016 | 0.010-0.019 |
| | | Immature | 1 | 0.053 | 0.071 | 0.058 |
| Hake | Saratoga Passage, Wash. | F | 10 | 0.058 | 0.047 | 0.090 |
| | " " | M | 12 | 0.042 | 0.030 | 0.043 |
| | Port Susan, Wash. | F | 11[a] | 0.038-0.083 | 0.030-0.090 | 0.065-0.147 |
| | " " " | M[c] | 5[a] | 0.057-0.111 | 0.062-0.074 | 0.101-0.223 |
| | Near Cape Foulweather, Ore. | | 12 | 0.074 | 0.068 | 0.143 |
| Hake meal | Near Aberdeen, Wash. | | | 0.267 | 0.027 | 0.080 |
| Ocean perch | Hecate Strait, B.C. | | d | 0.012 | trace | 0.013 |
| Starry flounder[b] | Blaine, Wash. | F | 1 | 0.018 | 0.026 | 0.013 |

TABLE (continued)

| Species | Source | Sex | Number of fish analyzed[a] | Residues (ppm.) DDE | TDE | DDT |
|---|---|---|---|---|---|---|
| True cod[b] | Blaine, Wash. | Unknown | 2 | 0.005-0.006 | 0.006-0.007 | 0.004 |
| Yellowtail rockfish | | | | | | |
| Fillets | Hecate Strait, B.C. | F | 8 | 0.017 | Trace | 0.004 |
| Remains[e] | " " " | F | 8 | 0.042 | 0.006 | 0.021 |
| Fillets | " " " | M | 18 | 0.030 | Trace | 0.014 |
| Remains[e] | " " " | M | 18 | 0.076 | 0.009 | 0.051 |
| Fillets | Ilwaco, Wash. | F | 13 | 0.119 | 0.022 | 0.048 |
| Remains[e] | " " | F | 13 | 0.256 | 0.055 | 0.104 |
| Fillets | " " | M | 14 | 0.092 | 0.028 | 0.036 |
| Remains[e] | " " | M | 14 | 0.416 | 0.092 | 0.194 |

[a]Composites, unless a range of values is given.

[b]Fillets only.

[c]Different catch from preceding entry.

[d]16 fillets.

[e]Fish after removal of fillets.

not current [illegible]nsumed directly. In comparing the data for hake meal with that of whole fish, the difference in water content must be considered (hake meal 4-6%; whole fish 75-80%).

Analysis of DDT in anchovy is complicated by a GLC peak with a retention time only slightly lower than that of DDT. The interference had a p-value in acetonitrile/hexane of 0.71 compared to DDT 0.38 in this laboratory or 0.35 in the literature (3). The presence of a small amount of DDT could be recognized by a shoulder on the leading side of the interference peak but, since only about 0.010 ppm. appeared to be involved, further analysis was dropped.

Since o,p'-DDT overlapped with p,p'-TDE in the GLC quantitation on SE-30, the presence of large amounts of DDT required correction of the apparent TDE concentration for o,p'-DDT included. Correction was achieved by using the p,p'-DDT concentration and the ratio 21:70 of o,p'- to p,p'-DDT in technical DDT (4) and, therefore, in nature.

The 85+15 chromatographic fraction caused gross contamination of the detector and the facilities for cleanup were not regularly available. Consequently, the 85+15 pesticide extracts from fish were merely examined cursorily. At random about one out of three samples was quantitated. The dieldrin and endrin content were generally insignificant. Occasionally a sample would show up to 0.006 ppm. of one of these compounds. p-Value analysis was not, however, performed so the presence of these compounds is scarcely confirmed.



## Discussion

The drainage of pesticides through the Columbia [illegible]r and Puget Sound provides an obvious source of contamination of significant toxicological consideration to the fishing industry and the consuming public of the Pacific Northwest. Evidence from fish collected in California waters (5) indicates that pesticide levels in that area have not been alarmingly high, but no information about Pacific Northwest specimens has been publicly available. To keep abreast of the current situation a survey of the more common local species has been undertaken.

As the data indicate the level of pesticide residues in marine products is substantially below the currently acceptable FDA tolerance for beef (7 ppm.). As might have been anticipated, fillets contain substantially lower pesticide levels than the remaining carcasses. The pesticide levels in fillets undoubtedly represent an even more accurate estimate of the human ingestion level than do whole-fish data, although the latter are of more direct biological interest.

The most striking results are those for yellowtail rockfish. A distinctly larger amount of pesticides occurs in the fish from Ilwaco, Washington, at the mouth of the Columbia River than in those from Hecate Strait, British Columbia, where no major river is entering the ocean. Obviously the agricultural run-off from Washington and Oregon is reaching the ocean fish nearby.

## Acknowledgment

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# RE-ESTERIFICATION OF FATS WITH GLYCEROL

By Tomotarō Tsuchiya and Gorokū Akiyama

Although the production of di- and mono-glycerides by re-esterification ("Umesterung") of fats with glycerol and their application have frequently been described in the patent literature, few work has hitherto been published which deals with the influence of various experimental conditions upon the re-esterification of fats with glycerol. The authors have carried out a series of experiments on the re-esterification of fats by heating with glycerol and determined the influence of various conditions upon the course of the reaction. The results are summarized bellow.

1. Experiments have been carried out using hardened fish oil with the object of determining the effect of reaction temperature, duration of heating, and amount of glycerol used upon the course of the reaction, and it has been shown that the reaction product with highest acetyl value is obtained when hardened fish oil with 25~100% its weight of glycerol is heated for 1~2

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hours at 270~280°C. But the highest acetyl value obtained in the experiments is considerably lower than the calculated value for the mono-glycerides of hardened fish oil fatty acids, and consequently the reaction product contains di-glycerides, possibly with unchanged tri-glycerides in addition to mono-glycerides.

2. From the results of the experiments with hardened soy-bean oil, tallow, cocoanut oil, olive oil and castor oil it has been shown that also these oils when heated with glycerol undergo re-esterification to a marked degree as in the case of hardened fish oil.

3. Further experiments have been carried out with hardened fish oil by using alkali carbonates, alkali hydroxides, magnesium oxide, lime and zinc oxide as catalyst, and it has been found that the re-esterification is facilitated by the addition of the above mentioned substances and the reaction temperature could be lowered to a certain extent.

4. Also in the cases of hardened soy been oil, tallow, cocoanut oil, olive oil, castor oil, sperm oil and hardened sperm oil, the reaction is facilitated by the addition of sodium carbonate. In the cases of sperm oil and hardened sperm oil, the fatty acids esters of higher alcohols which form the main constituents of these oils undergo re-esterification to a marked degree, thus leading to the formation of free higher alcohols. The reaction temperature which is necessary to obtain a reaction product of high acetyl value, is, in the cases of sperm oil and hardened sperm oil, more or less higher than in the cases of ordinary fatty oils.

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# (106) The Relationship between Thiocyanogen Value and Iodine Value in the Course of Oil-hardening

By Sei-ichi Ueno

(Received April 30, 1939)

The author has previously published several reports on the progress of hydrogenation of various oils. In this experiment sardine oil is subjected to hydrogenation under the same conditions as adopted in the practical oil-hardening on a large scale. The catalyst is the reduced nickel supported on kieselguhr, using in the ratio of 0.5% metallic nickel to oil. The procedure of hydrogenation is just the same as described before, employing both the ordinary pressure and a little higher pressure at about 180°C. With the progress of the hydrogenation small portions of reaction products are drawn out from the hydrogenation vessel at intervals of 1 hour, and the iodine value and thiocyanogen value of these samples are examined. The results are given as follows:

**Exp. 1—Hydrogenation at 5—7 atm. p.**

| Lapse of Time hours | Iodine Value | Thiocyanogen Value | Melting Point |
|---|---|---|---|
| (Original Oil) | 176.1 | 95.0 | — |
| 1 | 163.6 | 92.4 | — |
| 2 | 138.8 | 91.5 | — |
| 3 | 105.5 | 74.2 | — |
| 4 | 80.4 | 72.2 | — |
| 5 | 68.8 | 54.5 | 26.8—28.6 |

| Lapse of Time hours | Iodine Value | Thiocyanogen Value | Melting Point |
|---|---|---|---|
| 6 | 42.9 | 34.9 | 36.3—37.5 |
| 7 | 22.1 | 17.9 | 44.4—45.8 |
| 8 | 9.2 | 5.8 | 50.8—51.8 |
| 9 | 7.0 | 2.7 | 54.8—55.7 |
| 10 | 6.3 | 2.5 | 55.3—56.0 |
| 11 | 6.0 | 2.4 | 55.4—56.1 |
| 12 | 5.8 | 2.3 | 55.4—56.1 |
| 13 | 5.5 | 2.2 | 55.5—56.2 |

**Exp. 2—Hydrogenation at 5—7 atm. p.**

| | | | |
|---|---|---|---|
| (Original Oil) | 180.3 | 89.0 | — |
| 1 | 175.1 | 85.3 | — |
| 2 | 158.7 | 84.7 | — |
| 3 | 123.0 | 82.1 | — |
| 4 | 92.7 | 74.1 | 28.4—30.2 |
| 5 | 74.1 | 52.3 | 31.2—36.7 |
| 6 | 61.5 | 44.8 | 38.9—40.3 |
| 7 | 52.5 | 41.0 | 42.3—43.6 |
| 8 | 39.0 | 25.2 | 45.6—47.1 |

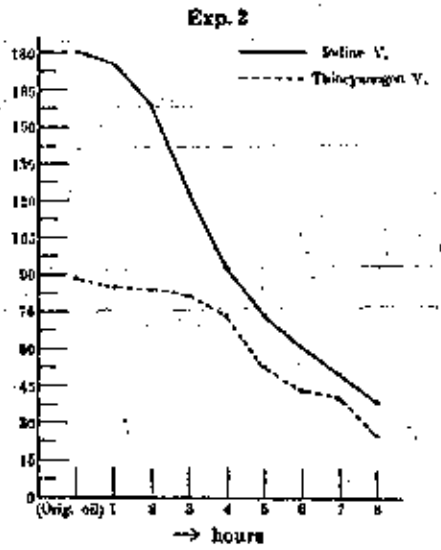
**Exp. 3—Hydrogenation at ordinary pressure**

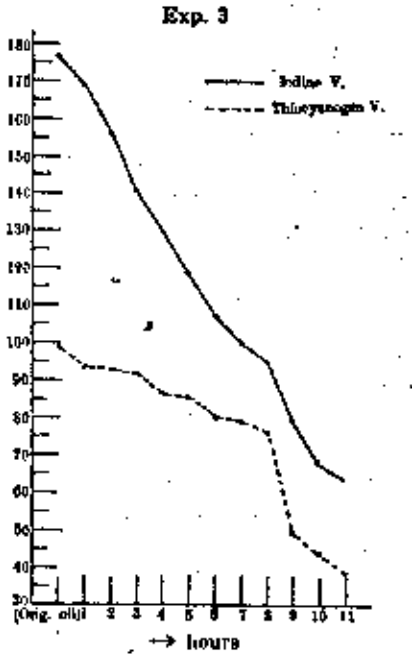
| | | | |
|---|---|---|---|
| (Original Oil) | 176.9 | 95.8 | — |
| 1 | 168.3 | 93.5 | — |
| 2 | 156.7 | 92.9 | — |
| 3 | 140.8 | 92.0 | — |
| 4 | 129.5 | 86.6 | — |
| 5 | 118.4 | 85.4 | — |
| 6 | 107.0 | 80.4 | — |
| 7 | 99.9 | 79.0 | 28.9—30.6 |
| 8 | 93.1 | 76.2 | 29.4—31.3 |
| 9 | 79.1 | 49.7 | 34.7—36.4 |
| 10 | 67.3 | 43.7 | 38.5—39.9 |
| 11 | 63.6 | 38.9 | 39.1—40.4 |

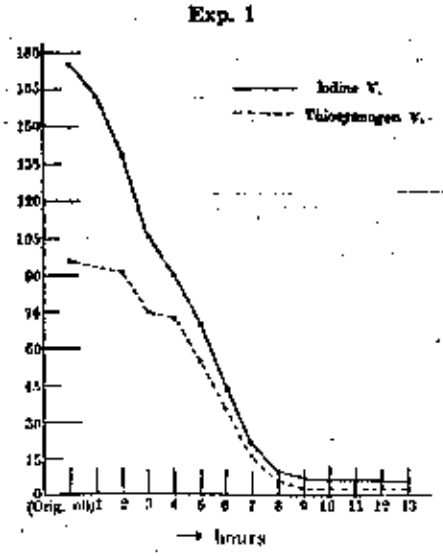
**Exp. 4—Hydrogenation at ordinary pressure**

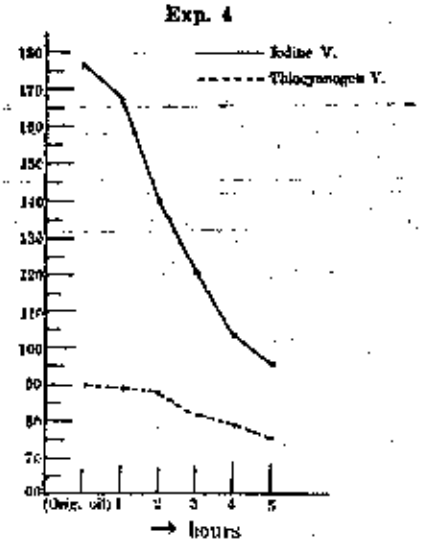
| | | | |
|---|---|---|---|
| (Original Oil) | 170.7 | 90.2 | — |
| 1 | 167.6 | 88.9 | — |
| 2 | 140.0 | 87.8 | — |
| 3 | 126.8 | 81.6 | — |
| 4 | 103.1 | 79.8 | — |
| 5 | 95.9 | 75.2 | 33.6—35.3 |

The diagrams in figs. 1—4 constructed from the data of this experiment show the relationship between thiocyanogen value and iodine value during the hydrogenation course of the sardine oil. From




these curves it will be easily seen that the sardine oil shows a rapid decrease in thiocyanogen value at the stage of the hydrogenation process where the iodine value falls to 70—80. This fact is considered to indicate the selective and step-wise character of the hydrogenation process.

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# Toxicity of Topically Applied Fats and Oils.* (32030)

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During the course of experiments which involved the use of high fat diets to produce vitamin E deficiency in chicks, a potentially lethal dermatitis was observed. When fed otherwise adequate diets containing about 20% of a vegetable oil, the chicks suffered a high rate of mortality within a period of approximately 2 weeks. The feathers soon became saturated with oil and preliminary experiments showed that the dermal lesions and eventual death resulted from external contact with the fat and not from consumption of the diet. Similar observations have been reported previously(1,2,3) but the mechanism by which topical fat causes death has not been described.

This investigation was undertaken to provide information regarding the etiology of this syndrome. The results show that the degenerative changes in the skin induced by lipid allows infiltration of microorganisms and it is probable that infection is the actual cause of death.

*Methods.* Groups of 4-day-old White Leghorn chicks were maintained in electrically heated wire-mesh cages and fed a practical-type, corn-soybean meal diet. Topical application of fats, oils and related substances was achieved by massaging the material into the feathers and skin of the neck and breast area. This treatment was performed once every 48 hours unless otherwise noted.

In later trials when it became obvious that the syndrome was complicated by microbial infection, precautions were taken to reduce environmental contamination. Cages were steam cleaned daily and feed, spread in 2-3 inch layers, was autoclaved at 121°C for 15 minutes. One group (A) was treated daily with corn oil (3 ml on the breast area). Group B was washed daily with an anionic detergent solution (1% Alconox), followed by drying under a forced air heater and treatment with corn oil. Group C was treated in the same manner as those in group B except the corn oil contained benzalkonium chloride (0.3%), chlortetracycline (0.5%), chloramphenicol (0.25%) and nyrostatin (1.0%). A fourth control group (D) was washed with Alconox but was not treated with oil.

Tissues, including liver, spleen, heart, kidney and skin from the breast, inner surface of the thigh and wing-web area, were fixed sectioned and stained with hematoxylin and eosin. Based on gross and microscopic observations, lesions were classed as none, mild, moderate or severe. Photomicrographs illustrating the various degrees of damage are shown in Fig. 1-4. The mild category (Fig. 2) included skin that showed slight hyperkeratosis or thickening of the cornified layer, occasional lymphocytes in the dermis with few or no bacteria. A moderate lesion (Fig. 3) describes skin with at least 2-fold thickening of the corneum and some ulceration, mild congestion of the dermis, inflammation of feather follicles and presence of many bacteria on the skin surface and in contact with ulcerated epithelium. A lesion classed as severe (Fig. 4) refers to skin with extensive hyperkeratosis and ulceration of the epithelium, vascular congestion, fibrosis and many lymphocytes in the dermis, and presence of numerous bacteria, even under the keratin layer.

*Results and discussion.* White Leghorn chicks were used because preliminary observations showed them to be more susceptible to topical fat than broiler breeds. It was further noted that chicks became less susceptible with age and, as might be expected, mortality was directly related to the proportion of the body covered with fat. All vegetable oils tested including soybean oil, corn oil, olive oil and hydrogenated cottonseed oil produced similar symptoms. When the oils were applied to chicks at 2 weeks of age, the skin became hyperemic by the second or third day, and by the fourth day there was severe skin damage, characterized grossly by blisters, erythema, edema and a serous exudate. Death ensued in [illegible] t 40% of the birds within 5 days. The [illegible] of those that survived developed a crusty dermatitis by the seventh day and the chicks became dehydrated in appearance. Microscopically, the

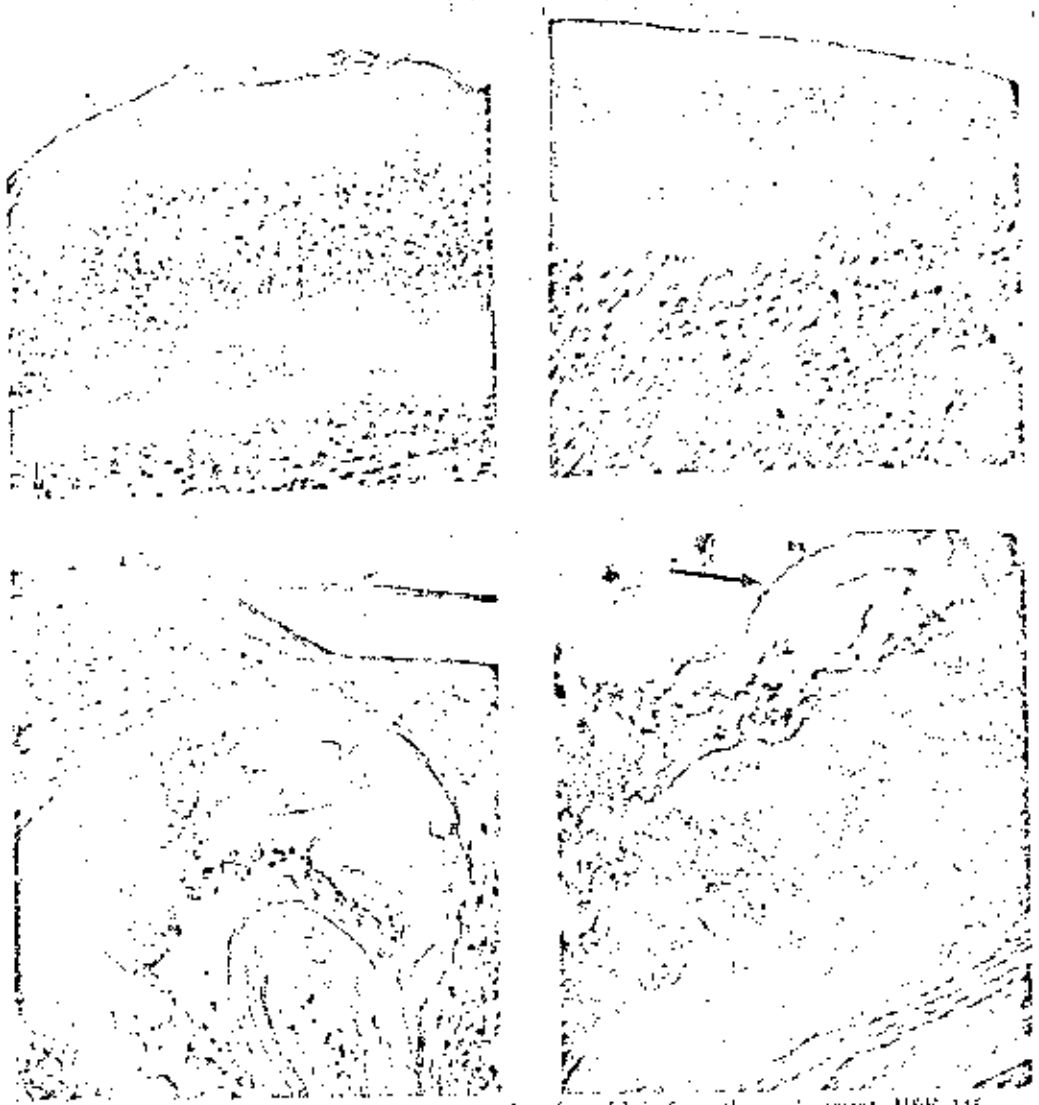

FIG. 1. Normal skin with a small amount of surface debris from the environment. H&E 145.
FIG. 2. Mild thickening of epidermis and slight vascular congestion characteristic of mild dermatitis. H&E 145.
FIG. 3. Moderate dermatitis with epithelium thickened 3 to 4 times, keratin layer thickened, and accumulation of bacteria and debris within a feather follicle. H&E 145.
FIG. 4. Severe ulceration of the skin in which epidermis has been replaced by masses of bacteria and cell debris except for a detached fragment of the thickened, poorly cornified stratum corneum (arrow). H&E 145.

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TABLE I. Dermatitis and Mortality Resulting from Topical Application of Various Substances to the Chick.

| Substance applied* | Initial age (days) | Skin lesions, 1 days | Mortality,‡ 5 days |
|---|---|---|---|
| Corn oil | 4 | severe | 2/2 |
| *Idem* | 11-15 | " ‖ | 3/5 |
| Corn oil (2×/day) | 1 | moderate | 6/2 |
| *Idem* | 17 | " | 0/2 |
| Corn oil + tyrothricin† | 1 | mild | 0/2 |
| Hydrogenated vegetable oil | 14-15 | severe‖ | 1/6 |
| Soybean oil | 11-15 | " ‖ | 2/6 |
| Olive " | 14-15 | " | 0/2 |
| Mineral " | 4 | moderate-severe | 2/8 |
| Dimethylpolysiloxane (Dow Corning Fluid 200) | 1-5 | none | 1/6 |

* Applications to breast and neck every 48 hr except where noted.
† Tyrothricin and chlortetracycline were added to corn oil at the level of 0.5%, each.
‡ Mortality is indicated by number of deaths over initial number of chicks.
§ Killed for histological examination prior to 5 days.
‖ Hematocrits at this time ranged from 10-51%; normal range is 32-35%.

skin showed ulceration, thickening of the keratin layer, epithelial degeneration and the presence of bacterial colonies and fungi in the epithelial debris. Treatment of chicks at 4 to 6 days of age resulted in higher mortality accompanied by similar lesions; in addition, a narcotic like effect was observed. Within a few minutes after application of fat, the chicks became drowsy and developed an unsteady gait or showed loss of balance. The narcosis was usually apparent for a period of 4 to 5 hours. A similar reaction was evoked by other lipids, such as glycerol, silicone fluid and propylene glycol, which had no detrimental effect upon the skin.

The presence of micro-organisms in and on the skin suggested that infection was contributing to both skin lesions and mortality. Consequently, antibiotics were incorporated into the oils before application so as to determine the effect of oil when the microbial population was depressed. The results of a typical experiment involving various oils and corn oil containing tyrothricin and chlortetracycline are summarized in Table I. Application of corn oil at 48-hour intervals produced severe skin lesions and high mortality. More frequent application was less harmful, probably because the twice daily treatment inhibited microbial growth as did the antibiotics. The mitigating effect of antibiotics upon skin lesions supports the concept that microbial infection plays a major role in both the dermatitis and mortality. Without antibiotics, all oils, including mineral oil, produced severe skin lesions and the chicks had high hematocrits suggesting dehydration and hemoconcentration. However, keeping the chicks' skin and feathers coated with another lipid-soluble lipid, dimethylsiloxane (Dow Corning Silicone Fluid 200), did not produce skin lesions or cause high mortality. This may have been due to the high molecular weight of the silicone fluid or to the fact that is is not closely related chemically to tissue lipids.

With the evidence that microbial infection aggravated the effect of topical lipids, additional experiments were performed using the sanitation procedures described under methods. The results of one experiment are shown in Fig. 5. The early deaths in groups B and C were accidental and not associated with skin lesions. Surviving chicks in group B, which were treated with corn oil after daily washing with detergent, showed lesions rated as moderate to severe. Their kidneys showed considerable degenerative change. Only one chick in group C died as a result of skin damage and the survivors had a mild dermatitis with slight epithelial thickening. The livers were darker and more swollen than the chicks treated for shorter periods of time. Clearly the presence of antibiotics increased the survival time and decreased the severity



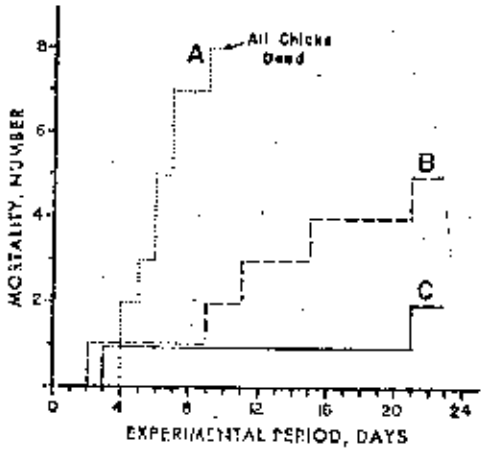


FIG. 5. Mortality of 9-day old chicks (8 per group) treated daily as described in text. Group A, treated with corn oil; B, washed with 1% Alconox and treated with corn oil; C, washed with 1% Alconox and treated with corn oil containing 0.5% chlortetracycline, 0.25% chloramphenicol, 1% nystatin and 0.5% benzalkonium chloride.

of the skin lesion but they did not afford complete protection.

Available evidence indicates that the primary toxic effect of topically applied fats arises from their penetration of the epithelium and injury of the lipid cell structures. Butcher (4) noted that daily application of olive oil, or mineral oil to the skin of young rats caused acanthosis and parakeratosis in 5 to 8 days. The granulosum cells remained larger than normal and mitosis was increased. In later studies(5) it was shown that linoleic acid rapidly penetrates rat epithelium and was found as a film on capillary linings within 10 minutes after application. Using the guinea pig, Hoekstra and Phillips(6) found that various fats and mineral oils induced degenerative skin changes by penetration into the cells of the skin. The most potent compound studied, n-hexadecane produced marked reddening of the skin followed by thickening and desquamation similar to that observed in the chicks.

The results presented here indicate that topical fat injury in the chick is complicated and intensified by microbial infection associated with the initial skin damage. It seems likely that bacterial toxins are the immediate cause of death. The reduced severity of the skin lesions when antibiotics are present suggests that at least part of the damage that has been attributed to fat is actually due to microbial toxins.

*Summary.* A potentially lethal dermatitis observed in chicks fed a high fat (20% vegetable oil) diet can be attributed to the external application of the oil. Topical application of both vegetable and mineral oils to young chicks produced a severe hyperkeratosis and high mortality within a few days. Mortality was largely prevented and skin damage substantially alleviated by including antibiotics in the oil and minimizing the number of environmental microbes. At least part of the topical fat toxicity syndrome can be attributed to secondary microbial infection.

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# Autoxidation of Fish Oils. II. Changes in the Carbonyl Distribution of Autoxidizing Salmon Oils[a,b]

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SUMMARY

**The carbonyl compounds of autoxidizing salmon oil were qualitatively and quantitatively analyzed at predetermined levels of oxidation. The 2-thiobarbituric acid (TBA) number, peroxide values, and total carbonyls were determined in addition to the individual volatile and Girard-T-insoluble carbonyls. The $C_5$-$C_{12}$ alkanals, $C_6$-$C_{12}$ alk-2-enals, and the $C_8$-$C_{10}$ alk-2,4-dienals composed the major part of the monocarbonyl fractions. The shorter-chain carbonyls were predominant, with methanal and propanal in the highest concentration. It appears that the Girard T reagent, as employed in the investigation, degrades the precursors of the volatile carbonyl fraction.**

**The hydroperoxides were readily destroyed in unpolymerized oil by the Girard T reaction conditions, but as polymerization proceeded the yield of hydroperoxides increased and that of the Girard-isolable monocarbonyls and malonaldehyde decreased. Polymerization of the salmon oil stabilized a large portion of the measurable hydroperoxides toward the degradative effects of the Girard and TBA reactions.**

Carbonyl compounds have been implicated in the flavor deterioration of lipids (Lea and Swedsodi, 1953) and in the rusting or browning reaction of fishery products (Nonaka, 1957). Work on the chemical definition of carbonyls in autoxidizing salmon oil has been carried out by Yu *et al.* (1961), and a wealth of information has been accumulated for other lipids in recent years (Day and Lillard, 1960; Lillard and Day, 1961; Gaddis *et al.*, 1960, 1961a; Forss *et al.*, 1960a,b,c).

The carbonyl compounds have been classified as volatile, or free, and nonvolatile, or bound (Gaddis *et al.*, 1960). The volatile fraction has been defined for many lipids, and it is usually less than 3% of the total measurable carbonyls (Lillard and Day, 1961; Gaddis *et al.*, 1960). The volatile monocarbonyls consist largely of alkanals, alk-2-enals, alk-2,4-dienals, and traces of vinyl ketones (Stark and Forss, 1962), and if the lipid is thermally oxidized, methyl ketones may occur (Crossley *et al.*, 1962). The remainder of the volatile carbonyl fraction has been reported as dicarbonyls in salmon oil (Yu *et al.*, 1961) and in lard (Gaddis *et al.*, 1960), but chemical definition of the mixture has not been reported.

Gaddis *et al.* (1960) distinguished between volatile and free carbonyls by finding that a portion of the free carbonyls were not accountable in the volatile fraction. The free carbonyls were measured by the Pool and Klose (1951) procedure. Recent work indicates, however, that their interpretation may be incorrect because of inherent errors in the original Pool and Klose method. Keith and Day (in press) modified the procedure to enable measurement of the major monocarbonyl classes, thus eliminating the quantitative error in the original method. They found that the volatile monocarbonyl fraction in milk fat was comparable to the free monocarbonyl fraction measured by the modified Pool and Klose procedure.

The nonvolatile (bound) carbonyl fraction has not been chemically elucidated, but it may be defined as that portion of the

[a] Technical paper no. 1607, Oregon Agricultural Experiment Station.

[b] This investigation was supported in part by PHS Research Grant EF-182 from the National Institutes of Health, Toxicology Section.

total measurable carbonyls that is not distillable or detectable by the Pool and Klose procedure. The fraction appears to be largely derived from the degradation of precursors by the reagent used in measuring it. Gaddis *et al.* (1960) attempted to define the fraction more clearly by using the Girard T reagent. They employed a procedure that caused degradation of the carbonyl precursors, and the carbonyls were isolated as the water-soluble hydrazones. They found that the volatile portion of the Girard-T-isolable compounds was at least superficially similar to the free volatile carbonyls, but they also found nonvolatile monocarbonyls and dicarbonyls.

Both the volatile and bound carbonyl fractions of salmon oil are of practical significance. The volatile fraction appears to be largely responsible for the rancid odor (Yu *et al.*, 1961), and, from available evidence, it is logical to assume that the "so-called" nonvolatile fraction, which exists as carbonyl precursors, is the origin of the volatile fraction. Both fractions undoubtedly are implicated in the rusting of frozen salmon and the browning of fish meal. Since the extent to which the salmon oil or the oil-containing products are oxidized varies so widely, the work reported herein was designed to study changes in the carbonyl composition of the oil at different stages of oxidation. An attempt was made to extend the qualitative identification of the volatile carbonyl fraction reported on previously by Yu *et al.* (1961) and to characterize the nonvolatile fractions. Attention was also directed to ascertaining the effect of oxidation on both the total and relative quantities of the various carbonyl compounds in the two fractions.

## METHODS

**Solvents.** Hexane, chloroform, benzene, and ethylene chloride were purified by the procedure of Schwartz and Parks (1961). Absolute ethanol was refluxed for 3 hr with 2,4-dinitrophenyl-(DNP)hydrazine and trichloracetic acid, followed by distillation. Equilibrated hexane was prepared by shaking hexane with excess acetonitrile, and the acetonitrile layer removed.

**Column-chromatography packing.** Analytical-grade Celite was dried 18 hr at 160°C. Celite 545 was dried 24 hr at 160°C. Seasorb 43 (Fisher Scientific) was activated for 48 hr at 400°C. The iodine adsorption number of the Seasorb was 58. The iodine adsorption number of the Seasorb was determined by weighing to the nearest 0.01 g a 4-g sample of Seasorb 43. One hundred ml of 0.0500*N* iodine in carbon tetrachloride was added, and the sample was shaken for 15 min. After settling for 5 min, 25 ml of the clear solution was pipetted into a flask containing 50 ml of 0.03*N* KI in 75% ethanol. The solution was titrated with standard 0.05*N* sodium thiosulfate (detailed procedure available from Food Machinery Corporation, New York, New York). The silicic acid-Celite 545 mixture was prepared by using five parts of 100-mesh silicic acid with one part Celite, and the mixture was dried 12 hr at 200°C.

**Oxidation of salmon oil.** Fresh salmon oil, edible grade, was oxidized by maintaining it under positive oxygen pressure in a sealed bottle at room temperature. The oil was shaken daily and analyzed periodically for peroxide value and 2-thiobarbituric acid (TBA) number. The fresh oil and four samples taken at predetermined levels of oxidation were subjected to carbonyl analysis.

**Peroxide value.** Peroxides, determined by the AOCS (1959) method, are expressed as mg of peroxide per kg of oil.

**Thiobarbituric acid number.** The procedure of Sinnhuber and Yu (1958) was used to measure the mg of malonaldehyde per kg of oil.

**Total carbonyl values.** Total saturated and unsaturated carbonyls were determined by the method of Henick *et al.* (1954).

**Isolation of volatile carbonyl compounds.** The volatile carbonyls were isolated by means of the reduced-pressure distillation apparatus of Day and Gilbert (1960) except that 100-g samples were used and 100 ml of distillate were collected. The distillate was reacted with an equal volume of 5*N* HCl, saturated with DNP-hydrazine. The DNP-hydrazones of the monocarbonyls were isolated by extraction with hexane. The extracts were combined and the hexane removed under reduced pressure. The bis-DNP-hydrazones of the dicarbonyls were subsequently isolated from the reaction mixture by benzene extraction.

**Isolation of water-soluble Girard T carbonyl derivatives.** The Girard T reagent was used to isolate the carbonyls from salmon oil. Reaction conditions were selected to give the maximum yield of carbonyl derivatives. The procedure was essentially as outlined by Gaddis *et al.* (1960) except the reflux time was increased to 3 hr. One hundred grams of oil, 10 g of Girard T reagent, 2 g of Dowex 50 cation-exchange resin, and 200 ml of carbonyl-free ethanol were refluxed 3 hr. The mixture was cooled, 2 L of distilled

water were added, and the oil was removed by extraction with petroleum ether. Sodium chloride was added during the extraction step to help break the emulsion and to facilitate removal of final traces of oil. The water-soluble hydrazones were converted to DNP-hydrazones by reacting equal volumes of the aqueous solution and 5*N* HCl saturated with DNP-hydrazine. The reaction was allowed to proceed 12 hr at room temperature, after which the DNP-hydrazones were isolated by extraction with chloroform. Upon removal of the chloroform, the DNP-hydrazones of monocarbonyls were separated from bis-DNP-hydrazones by exhaustive extraction with hexane. The procedure as outlined gave an average yield of 57% of the estimated total carbonyls in unpolymerized salmon oil.

**Quantitative analysis of DNP-hydrazones.** The DNP-hydrazones were separated into classes with the Seasorb Celite-ethylene chloride procedure of Schwartz *et al.* (1960). The quantity of DNP-hydrazones represented in each class was determined by measuring the absorbance of chloroform solutions at 358, 373, and 380 mμ for alkanals, alk-2-enals, and alk-2,4-dienals, respectively, and calculating the millimoles of carbonyl using molar absorptivity indices of 22,500, 27,500, and 37,500 for the respective classes.

Each of the DNP-hydrazone mixtures obtained from the class separation was chromatographed by the column-partition method of Corbin *et al.* (1960). The column was modified in that no water was used during preparation. The eluate from the column was collected in 10-ml fractions, and the absorbance of each fraction was measured at the wavelength of maximum absorption for the class of DNP-hydrazones being separated. The data, when plotted in the manner described by Corbin *et al.* (1960), depicted the typical chromatograms in which each peak represented a specific number of a DNP-hydrazone class. The area under each peak was determined with a planimeter, and these values were used to calculate the percentage of each compound in each carbonyl class. Since the total carbonyl concentration of each class had been determined in a previous step, the amount of each carbonyl was easily calculated from the percentage composition data.

**Quantitative analysis of bis-DNP-hydrazones.** Excess DNP-hydrazine present in the crude bis-DNP-hydrazone mixtures was removed by the resin exchange procedure of Schwartz *et al.* (1962). The bis-DNP-hydrazones were separated on the silicic acid-Celite column of Wolfrom and Arsenault (1960). All of the chromatographic bands gave positive tests for the bis-DNP-hydrazones of vicinal dicarbonyls. The absorbance of each fraction, in chloroform, was measured at 393 mμ, and the moles of carbonyl were calculated using a molar absorptivity value of $4.3 \times 10^4$.

**Qualitative analysis of DNP-hydrazones.** Evidence to establish the identification of DNP-hydrazones was based on the following criteria. Class separation of the DNP-hydrazones as described by Schwartz *et al.* (1960) and confirmed in our laboratory was effective in separating the various hydrazone classes of the monocarbonyls. The chain length of compounds in each class was ascertained as described by Corbin *et al.* (1960) through preparation of reference chromatograms from authentic DNP-hydrazones. The chromatographically resolved compounds were checked by determining absorption maxima, and chain lengths were confirmed by comparing chromatographic behavior with authentic derivatives on paper chromatograms. Melting-point analyses were conducted on questionable derivatives.

## RESULTS AND DISCUSSION

Table 1 shows gross changes in the salmon oil during autoxidation. The oil was checked periodically for the TBA number and peroxide values. Aliquots were taken for detailed analyses at the points indicated in the table.

The quantity of Girard-isolable monocarbonyls reached a maximum during the oxidation period and the subsequent decrease in yield was accompanied by visible evidence of polymerization of the oil. The percent Girard-isolable monocarbonyls of the total estimated carbonyls decrease from 89% in Sampling 3 to less than 4% in Sampling 5. Peroxide values for Samplings 1 and 5, after treatment of the oil by the Girard procedure, were 0 and 153.5, respectively. This means that in advanced stages of oxidation, polymerization appears to stabilize a substantial portion of the hydroperoxides towards the degradative effects of the Girard reaction. The decrease in yield of Girard-isolable monocarbonyls with the concomitant stabilization of hydroperoxides also suggests that hydroperoxide degradation effected by the Girard reaction accounts for a large portion of the carbonyls isolated by this procedure. This is true also for the Girard isolable dicarbonyls even though the quantity in this fraction increased as oxidation proceeded. The maximum yield (3% of the total carbonyls) was obtained for Sampling 3.

Table 1. Gross changes in salmon oil during oxidation.

| | Sample no. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Days' storage | 0 | 16 | 31 | 66 | 163 |
| TBA no | 8.35 | 350 | 1102 | 2024 | 1472 |
| Peroxide value | 4.0 | 62.6 | 171 | 224 | 358 |
| Total carbonyls* | 9.5 | 52.6 | 110.8 | 312.3 | 569.8 |
| Volatile monocarbonyls* | 0.096 | 0.176 | 0.562 | 1.463 | 2.057 |
| Girard-isolable* monocarbonyls | 4.056 | 3.820 | 98.670 | 77.888 | 19.209 |
| Volatile dicarbonyls* | 0.039 | 0.047 | 0.289 | 0.308 | 0.890 |
| Girard-isolable* dicarbonyls | 0.194 | 1.114 | 3.309 | 5.972 | 8.250 |

* mM/kg of oil.

The above conclusions may be questioned because the quantity of Girard-isolable monocarbonyls is lower in Sampling 2 than in Sampling 1 while the peroxide value is much higher in Sampling 2. A possible cause for this discrepancy may be the poor recovery of the monocarbonyls at one of the several steps in the isolation procedure. The data in Table 3 show that methanal and propanal are the compounds accounting for the decrease in yield. These compounds, along with ethanal, are the most difficult to recover because of their limited solubility in the solvents employed.

The decrease in TBA number, while not simultaneous with the decrease in Girard-isolable monocarbonyl fraction, also appeared to be related to stabilization of hydroperoxides. Previous investigators (Day, 1960; Day and Lillard, 1960; Simhuber *et al.*, 1958; Patton and Kurtz, 1955) have reported that only a small percentage of the total measurable malonaldehyde exists free in oxidizing lipids, and Day (1960) has suggested that the remaining malonaldehyde results from degradation of hydroperoxides during the analysis.

Tarladgis and Watts (1960) observed that malonaldehyde production and the rate of oxygen absorption reached a maximum simultaneously in oxidizing fatty acids and that the rancid-odor intensity decreased concomitantly with the TBA number. In the present study, no attempt was made to evaluate odor intensity quantitatively. However, it appeared to increase throughout the oxidation period. The observed increase in volatile carbonyls (Table 1) and the reported high correlation between flavor intensity of oxidizing fat and volatile monocarbonyls (Lillard and Day, 1961) lend credence to this point.

Table 2 shows qualitative and quantitative composition of the volatile monocarbonyl fractions of the salmon oil. In earlier work, Yu *et al.* (1961) obtained evidence for tentative identification of a number of methyl ketones. The ketones were not found in the present work except as contributed by contaminated solvents. It is believed that the methyl ketones found by Yu *et al.* (1961) came from the same source. Compounds found in this investigation that were not reported by Yu *et al.* (1961) were the $C_9$, $C_{10}$, $C_{11}$, $C_{12}$ alkanals; $C_{12}$ alk-2-enal; $C_6$, $C_8$, $C_9$ alk-2,4-dienals.

Substantial amounts of volatile monocarbonyls were found in the fresh oil, and the total quantities continued to increase during autoxidation. Of possible significance was the observed coincidence of the TBA number maximum and the peak concentration for a large number of the alkanals. In fact, only hexanal continued to increase during oxidation, and if quantitative data were available for the $C_9$, $C_{10}$, $C_{11}$ and $C_{12}$ alkanals of Sampling 4 (Table 2) it is probable that the total alkanal value would show a maximum at that point.

The volatile alkanal concentration increased at an early stage whereas no substantial increase was apparent in the unsaturated carbonyls until after the 16th day of oxidation. The large increase in the

Table 2. Volatile monocarbonyl compounds from autoxidizing salmon oil (mM/kg).

| | Sample no. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Alkanals | | | | | |
| n-Dodecanal | .0029 | .0017 | ..... | ..... | .0126 |
| n-Undecanal | .0023 | .0022 | .0058 | .0709 | .0194 |
| n-Decanal | .0022 | .0018 | .0102 | .0705 | .0675 |
| n-Nonanal | ..... | .0019 | .0058 | .1292 | .0606 |
| n-Octanal | .0015 | .0016 | .0087 | .1212 | .0720 |
| n-Heptanal | .0020 | .0023 | .0109 | .0758 | .0755 |
| n-Hexanal | .0024 | .0043 | .0310 | .2234 | .2549 |
| n-Pentanal | .0025 | .0097 | .0358 | .1794 | .0569 |
| n-Butanal | ..... | .0036 | .0375 | .0698 | .0274 |
| n-Propanal | .0079 | .0421 | .1287 | ..... | .1875 |
| n-Ethanal | .0168 | .0062 | .0621 | ..... | .1600 |
| n-Methanal | .0111 | .0720 | .1335 | ..... | .0514 |
| Total | .0516 | .1494 | .4700 | .9400 | 1.0517 |
| Alk-2-enals | | | | | |
| Dodec-2-enal | .0008 | .0006 | .0010 | .0031 | .0107 |
| Undec-2-enal | .0011 | .0006 | .0008 | .0041 | .0101 |
| Dec-2-enal | .0016 | .0015 | .0018 | .0139 | .0275 |
| Non-2-enal | .0010 | .0010 | .0025 | .0291 | .0407 |
| Oct-2-enal | .0012 | .0013 | .0019 | .0272 | .0173 |
| Hept-2-enal | .0023 | .0022 | .0025 | .0595 | .0891 |
| Hex-2-enal | .0024 | .0030 | .0205 | .0724 | .2463 |
| Pent-2-enal | .0054 | .0034 | .0262 | .0451 | .2386 |
| But-2-enal | ..... | ..... | .0146 | ..... | ..... |
| Total | .0159 | .0135 | .0717 | .2543 | .7412 |
| Alk-2,4-dienals | | | | | |
| Dec-2,4-dienal | .0022 | .0010 | .0008 | .0193 | .0080 |
| Non-2,4-dienal | .0012 | .0004 | ..... | .0120 | .0627 |
| Oct-2,4-dienal | .0025 | .0011 | .0023 | .0225 | .0458 |
| Hept-2,4-dienal | .0227 | .0102 | .0143 | .1704 | .1005 |
| Hex-2,4-dienal | ..... | .0009 | .0029 | .0441 | .0471 |
| Total | .0285 | .0135 | .0204 | .2688 | .2641 |

alkanals is accountable primarily by methanal and propanal, both of which could arise from the oxidation of terminal unsaturated fatty acids and the fatty acids with a double bond between the third and fourth carbons from the methyl end of the chain. These acids are very common in menhaden oils (Stoffel and Ahrens, 1961), and it is conceivable they would occur in salmon oil.

Table 3 shows qualitative and quantitative data for the Girard-isolable monocarbonyls. The qualitative composition of the various classes is comparable to that of the fraction isolated by distillation (Table 2). The Girard procedure was not as sensitive or consistent as the distillation procedure in detecting the compounds occurring at lower concentrations in the oil. For some unknown reason, some of the compounds were not detectable at consecutive sampling periods. This is especially noticeable for Sampling 4, in which the longer-chain members of each class were conspicuously absent. A recheck of the sample failed to reveal the missing compounds. To check this point further, a second sample of fresh salmon oil was oxidized to a comparable TBA number (2125) and the analysis of the Girard monocarbonyls again did not reveal the missing compounds.

When the individual monocarbonyls in the volatile fraction (Table 2) are compared with those of the Girard-isolable fraction (Table 3), in practically all cases the rela-

Table 3. Monocarbonyl compounds from autoxidizing salmon oil isolated with the Girard T reagent (mM/kg).

| | Sample no. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Alkanals | | | | | |
| n-Dodecanal | ..... | ..... | ..... | ..... | .2883 |
| n-Undecanal | ..... | ..... | ..... | ..... | .4580 |
| n-Decanal | ..... | ..... | .3619 | ..... | 1.2045 |
| n-Nonanal | ..... | ..... | .4891 | ..... | .8991 |
| n-Octanal | ..... | ..... | .4206 | ..... | 1.0603 |
| n-Heptanal | ..... | .0328 | .8706 | .4669 | 1.0008 |
| n-Hexanal | .1253 | .1669 | 3.8345 | 1.7714 | 2.7326 |
| n-Pentanal | .1473 | .3086 | 6.5442 | 1.7714 | .7548 |
| n-Butanal | ..... | ..... | 10.7700 | 11.9812 | .6109 |
| n-Propanal | 1.8208 | 1.2328 | 30.5690 | 24.8687 | 2.4767 |
| n-Ethanal | .2598 | .7253 | 11.8360 | 9.4270 | .6785 |
| n-Methanal | 2.0433 | 1.0537 | 32.1440 | 18.3666 | 2.0696 |
| Total | 4.3960 | 3.5800 | 97.8200 | 68.6600 | 14.2240 |
| Alk-2-enals | | | | | |
| Dodec-2-enal | ..... | .0065 | .0056 | ..... | .0063 |
| Undec-2-enal | ..... | .0045 | .0048 | ..... | .0208 |
| Dec-2-enal | ..... | .0076 | .0286 | ..... | .0444 |
| Non-2-enal | ..... | .0056 | ..... | ..... | .0679 |
| Oct-2-enal | ..... | .0140 | .0413 | .0755 | .1160 |
| Hept-2-enal | ..... | .0118 | .1255 | .3186 | .4804 |
| Hex-2-enal | ..... | .0101 | .3440 | 1.2440 | .4917 |
| Pent-2-enal | ..... | .0109 | .0564 | 2.2347 | .5808 |
| But-2-enal | ..... | ..... | .0330 | .2953 | .0798 |
| Total | ..... | .0710 | .6391 | 4.6680 | 1.8912 |
| Alk-2,4-dienals | | | | | |
| Dec-2,4-dienal | .0101 | .0206 | .0047 | ..... | .1234 |
| Non-2,4-dienal | ..... | ..... | .0152 | .1352 | .1898 |
| Oct-2,4-dienal | .0101 | .0422 | .0116 | .5407 | .1234 |
| Hept-2,4-dienal | .0103 | .1669 | .1625 | 2.8585 | 2.2965 |
| Hex-2,4-dienal | ..... | ..... | .0174 | 1.0356 | .3606 |
| Total | .0605 | .2297 | .2113 | 4.5700 | 3.0936 |

tive amounts in the carbonyl classes are comparable for both fractions. Apparently the conditions of the Girard reaction cause degradation of the volatile carbonyl precursors in the oil.

A portion of the ethanal shown in Table 3 may have resulted from dehydrogenation of the ethanol used in the Girard reaction, in the manner described by Gaddis *et al.* (1961b). The amount could not have been significant, however, because the ethanal concentration closely followed the trend of the other compounds in the mixture during oxidation of the oil.

Methanal and propanal formed a large percentage of the alkanal fraction of the Girard carbonyls, and, again, the most feasible origin of these compounds would be as mentioned for the volatile fractions. An additional mechanism that may help account for methanal has been described by Bell *et al.* (1951), in which decomposition of the primary alkoxy radical could give rise to methanal and the alkyl radical:

$$R - CH_2O \cdot \rightarrow R \cdot + CH_2O$$

Relatively large amounts of methanal were also reported in oxidizing milk fats by Lillard and Day (1961). The decrease in methanal and propanal may be associated with the destruction of the fatty acid precursors. Similarly, the drop in the alk-2-enals and alk-2,4-dienals in advanced stages of oxidation is undoubtedly related to oxi-

Table 4. Volatile dicarbonyl compounds from autoxidizing salmon oil (mM/kg).

| Fraction | Sample no. 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | .0019 | .0150 | .0742 | .0213 | .2211 |
| 2 | .0345 | .0065 | .0550 | .1455 | .1982 |
| 3 | .0027 | .0098 | .0631 | .0923 | .0734 |
| 4 | ..... | .0164 | .0906 | .0491 | .3968 |

Table 5. Dicarbonyl compounds from autoxidizing salmon oil isolated with Girard T reagent (mM/kg).

| Fraction | Sample no. 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | .0542 | .4307 | .6341 | .7991 | 4.7725 |
| 2 | .0511 | .2193 | 1.000 | 1.2045 | 1.9545 |
| 3 | .0890 | .4636 | 1.039 | 1.3229 | 1.5225 |
| 4 | ..... | ..... | .6159 | 1.7729 | ..... |
| 5 | ..... | ..... | ..... | .8727 | ..... |

dative destruction of the aldehydes and the polyunsaturated fatty acids.

The quantitative data for the bis-DNP-hydrazones are presented in Tables 4 and 5. Positive identification of these fractions is not complete. Recent work in our laboratories has indicated that the Wolfrom and Arsenault (1960) procedure does not give complete separation of mixtures of dicarbonyl derivatives such as isolated from oxidizing fats and oils. The percentage of volatile dicarbonyls in the volatile carbonyl fraction ranged from 17 to 35%. There was not a consistent trend in changes of the relative amounts of individual fractions in either the volatile or Girard-isolable dicarbonyls during oxidation of the salmon oil.


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# Quantitative determination of phosphorus in soybean lipids

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SUMMARY Black and Hammond's method for determining lipid phosphorus has been modified for use with oils containing small amounts of phosphorus. The minimum amount of P that can be determined is 3 nmoles, with a coefficient of variation SD of 2%.

SUPPLEMENTARY KEY WORDS perchloric acid · acid molybdate reagent · stannous chloride · phospholipids

Of the many published methods for determining lipid phosphorus (1-8), many are unsuitable for this determination in soybean lipids, which contain a small quantity of phospholipids and about 10 times as much triglyceride. Prolonged heating during the digestion of the necessarily large amounts of oil leads to loss of acid and of reproducibility in the colorimetry (7). We have therefore modified the method of Black and Hammond (5), the main characteristic of which is preliminary oxidation of the lipids by nitric acid to facilitate the subsequent digestion by perchloric acid. The modifications include reducing the scale, use of a modified Kjeldahl flask, and neutralization of excess perchloric acid with ammonia instead of evaporation.

*Materials.* Reagent grade $KH_2PO_4$ and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ were recrystallized. Technical grade nitric acid (d = 1.407) and perchloric acid (d = 1.655) were distilled to remove impurities. Other chemicals were reagent grade. Volumetric glassware was calibrated by standard methods. All calibrations and specific gravity determinations were performed at 20 ± 0.02°C. Solutions were prepared in twice-distilled water. The molybdate reagent, a 1:3 (v/v) mixture of 10% ammonium molybdate and 10 N $H_2SO_4$ (d = 1.39), remained stable

for several months. The $SnCl_2$ solution was prepared in a 10 ml volumetric cylinder by the solution of 190 mg of $SnCl_2 \cdot 2H_2O$ in 0.15 N $H_2SO_4$; after the addition of two drops of 4% $CuSO_4 \cdot 5H_2O$, the solution was brought to volume with 0.15 N $H_2SO_4$. The lipid solution was prepared by the extraction of freshly-ground soybean seeds first with butanol and then with chloroform-methanol 2:1.

*Procedure.* For the construction of the calibration curve, 2–8 ml aliquots of standard $KH_2PO_4$ solution (1 μg P/ml, freshly diluted from a stock solution containing 100 μg P/ml) were pipetted into 10-ml glass-stoppered volumetric flasks, followed by addition of water to a volume of about 8 ml and 0.2 ml of the molybdate reagent. After the solution had been shaken, it was brought to volume with water and thoroughly mixed. A drop of the freshly prepared $SnCl_2$ solution was added to each flask at 3-min intervals, and after exactly 45 min the absorbance A of each solution was read at 720 nm (9) against a water reference in a cell of 3.5 ml capacity with a light path of 0.998 cm. The values were corrected for a reagent blank, and P concentrations, corrected for the glassware calibration, were used to plot a standard curve.

The digestion of lipid phosphorus (5) was carried out with single and double volumes of solution, every test being conducted in replicate. An amount of lipid extract containing 10–20 mg of lipid was pipetted into a modified Kjeldahl flask (Fig. 1). The sample size could be reduced to about 1 mg, in which case the necessary dilutions were modified accordingly. The flask was stoppered with the modified stopper (Fig. 1), and the solvents were evaporated in boiling water for 5–10 min under a stream of air. Two drops of nitric acid were added, and the flask was heated on the thermostated block heater, care being taken to avoid charring (the temperature inside the flask was 175°C). The heating was continued until the vigorous brown foaming had subsided and a homogeneous dark liquid was formed. The flask was cooled, 0.3 ml of perchloric acid was added, and the flask was heated for 2–3 min to 175°C with continuous stirring. After the appearance of heavy white fumes, the flask was heated at 200°C for a further 15–30 min until the mixture was completely decolorized. The flask was cooled, and 0.5 ml of aqueous ammonia (d = 0.914) was added. A rubber stopper was inserted at position 7 (Fig. 1), and the flask was heated at 100°C under reduced pressure (water pump) until the liquid had evaporated off and no odor of ammonia remained. 25 ml of water was added to the flask, and 2 ml of the resultant solution (in replicate) was pipetted into a 10 ml volumetric flask. The phosphorus content was estimated as described above. Solvents used in the procedure were sufficiently free of phosphorus to give blank values of zero. The accuracy of the method was evaluated by the recovery of inorganic phosphorus (0.5 ml of the stock phosphate solution being added to occasional samples before digestion).

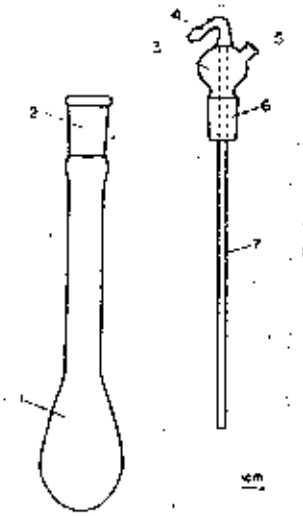


FIG. 1. Modified Kjeldahl flask for digestion of lipids without loss of phosphate. *1*, Kjeldahl flask (volume, 30 ml); *2*, female standard joint No. 14.5; *3*, stopper, pierced by glass tube; *4*, outlet tube for connection with the water pump; *5*, hole for rubber stopper; *6*, male standard joint No. 14.5; *7*, vapor exit tube.

*Results.* The calibration curve for the lipid phosphorus assay was constructed from the data in Table 1. The color of the reduced phosphomolybdate obeyed the Lambert–Beer law in the range of 2–8 μg of P. The sD was calculated by the method of Gänshirt and Polderman (10). The relative sD (average coefficient of variation) for successive estimations was 0.56%. The sensitivity of the quantitative method was evaluated using the molar extinction coefficient at 720 nm (11). As derived from the data of Table 1, $\epsilon_{720}$ = 25,200. This value was used to calculate the minimum amount of phosphorus (M) that can be determined by the spectrophotometric procedure described. Calculation by the method referred to in

TABLE 1. CALCULATION OF STANDARD DEVIATION IN THE SPECTROPHOTOMETRIC ASSAY OF INORGANIC PHOSPHORUS

| | P, μg/10-ml* | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| | $A \times 10^3$ | | | | | | |
| $x$ | 167 | 243 | 323 | 400 | 478 | 554 | 632 |
| | 166 | 243 | 325 | 399 | 478 | 561 | 634 |
| | 164 | 247 | 325 | 400 | 478 | 558 | 633 |
| $\bar{x}$ | 165 | 244 | 324 | 400 | 478 | 558 | 633 |

* Actually in μg × 1.017, the correction factor for the glassware calibration.

Designations: $x$, single measurements of absorbance obtained successively by a single experimenter; $\bar{x}$, arithmetic mean. $m$ is number of P solutions of different concentrations (=7) and $S$ = pooled standard deviation (ref. 10) = 0.0018, relative sD (average coefficient of variation) = $10^3 S/m\Sigma\bar{x}$ = 0.56%.

TABLE 2 CALCULATION OF STANDARD DEVIATION IN ASSAY OF PHOSPHORUS IN SOYBEAN LIPIDS*

| Test No. | A† | B† | $x_i$ |
|---|---|---|---|
| 1 | 3.62 | 3.61 | 99.7 |
| 2 | 3.46 | 3.55 | 102.6 |
| 3 | 3.27 | 3.31 | 101.2 |
| 4 | 3.26 | 3.29 | 100.9 |
| 5 | 3.57 | 3.48 | 97.5 |
| 6 | 3.26 | 3.38 | 103.7 |
| 7 | 3.32 | 3.37 | 101.5 |
| 8 | 3.03 | 2.98 | 98.3 |
| 9 | 3.01 | 2.97 | 98.6 |
| 10 | 2.96 | 2.95 | 99.7 |
| 11 | 3.09 | 3.01 | 97.4 |
| 12 | 1.89 | 1.93 | 102.1 |

* The data were obtained by two experimenters at different times.

† Arithmetic mean of four measurements.

Designations: A, μg P in 1 volume of the lipid extract; B, μg P in 2 volumes of the same extract divided by 2; $x_i = 100\ B/A$; $n = 12$; $\bar{x} = \Sigma x_i/n = 100.3$; $S = [\Sigma(x_i - \bar{x})^2/(n-1)]^{1/2} = 2.06$. Relative SD (coefficient of variation) $= 100\ S/\bar{x} = 2.05\%$.

Gorchakov's manual (11) gives $M = 2.77 \times 10^{-7}$ moles of P. The recovery of inorganic phosphorus after the lipid digestion was 102%.

In order to evaluate the accuracy and precision of the method, single and double volumes of the soybean lipid solutions were wet ashed and the P content in the inorganic residues was measured (Table 2, columns 2 and 3). The estimated absolute values of the phosphorus concentrations varied from test to test since the content of phospholipids in the extracts obtained under different conditions differed slightly and since various volumes of extracts were used for digestion in successive experiments. Therefore, absolute (11) and relative (10) standard deviations were calculated using relative $x_i$ values obtained as shown in Table 2. It was found that the coefficient of variation in the assay of lipid phosphorus after digestion (2.05%) was somewhat higher than that in the estimation of inorganic phosphorus (0.56%). In our samples of soybean lipids the phosphorus content was 0.6-0.7%.

*Discussion.* The method of Black and Hammond (5) has been modified as follows: the sample volume was reduced 10-fold, and the amount of oxidants ($HNO_3$ and $HClO_4$) was decreased from 4- to 5-fold. This allowed digestion without glass beads that have been used (5) to achieve less vigorous boiling. Use of a powerful oxidant (perchloric acid) and high temperature (over 200°C) at the terminal stage of digestion made it possible to carry out reliable digestion of lipid material for up to 30 min without any loss of orthophosphate.

Stannous chloride is a powerful reducing agent (5), but reproducible results with it can be obtained only if the pH of the solution is accurately maintained and if the time interval between the addition of stannous chloride and measurement of the absorbance is strictly observed. With other reducing agents (1-4, 6-8, 12, 13) the acidity of the solution is usually created by the acid used for the digestion. If perchloric acid serves as the oxidizing agent, it cannot be used for accurate acidification of the medium because of the inevitable losses of acid during lipid digestion. Black and Hammond (5) attempted to solve this problem by evaporating perchloric acid in a current of compressed air that passed through the Kjeldahl flask as it was heated. In our experiments we failed to achieve complete removal of the acid when a stream of inert gas was passed through the hot flask. Connection of the heated flask (175°C) with the water pump (7) resulted in a better evaporation of perchloric acid, but this method proved to be unsuitable because of a significant loss of phosphate. We therefore removed excess perchloric acid from the reaction mixture by neutralization with aqueous ammonia followed by the vacuum distillation of water and excess ammonia at a moderate temperature (100°C). The acidity necessary for color development was obtained by an addition of a precise amount of the molybdate reagent. As shown in Table 2, this method assures complete retention of phosphorus in the Kjeldahl flask, and the ammonium salt formed after neutralization does not affect the colorimetric determination. The sensitivity of the proposed method compares favorably with other modern methods of phosphorus assay (2, 4, 12, 13), and the method can be used for the determination of microamounts of phospholipids after their analytical separation by TLC.

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