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Orginial Submission

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GRN 000009

Quest INTERNATIONAL

October 19, 1998

Quest International
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Alan M. Rulis, Ph.D. Director
Office of Premarket Approval (HFS-200)
Center for Food Safety & Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, DC 20204

Re: GRAS Notification for Polyglycerol Polyricinoleic Acid (PGPR)

Dear Dr. Rulis:

Quest International, by the enclosed GRAS Notification letter and supporting documents, notifies you of its intention to introduce into interstate commerce the generally recognized as safe ("GRAS") substance, polyglycerol polyricinoleic acid (PGPR), for use as an emulsifier in chocolate. This substance has been approved in the European Union under the E-number E476 and is marketed outside of the United States under the tradename Admul WOL.

PGPR has unique and highly advantageous properties when added to chocolate at amounts up to 0.3%. These properties offer the chocolate manufacturer a remarkable degree of control over the flow properties of molten chocolate.

Because Quest International has determined that the use of PGPR as an emulsifier in chocolate is GRAS, it is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act. The data and information supporting Quest International's GRAS determination are available to the Food and Drug Administration, upon request.

Very truly yours,

Don Boudreaux, Ph.D.
Technical Director, Quest Foods North America

Encl.

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A member of ICI Group

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QST- 005

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October 13, 1998

Alan M. Rulis, Ph.D. Director
OFFICE OF PREMARKET APPROVAL (HFS-200)
CENTER FOR FOOD SAFETY & APPLIED NUTRITION
FOOD AND DRUG ADMINISTRATION
200 C Street, S.W.
Washington, D.C. 20204

RE: GRAS Notification for Polyglycerol Polyricinoleic Acid ("PGPR") Exemption Claim

Dear Dr. Rulis:

Pursuant to proposed 21 C.F.R. § 170.36(c)(1), Quest International hereby claims that polyglycerol polyricinoleic acid ("PGPR") is Generally Recognized as Safe ("GRAS") for use as an emulsifier in chocolate; therefore, it is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act. PGPR is authorized for use in the European Union under the E-number E476 and is marketed outside the United States under the tradename Admul WOL.™

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36(c)(1)(i) *The name and address of the notifier.*

Quest International
5115 Sedge Boulevard
Hoffman Estates, IL 60192

Proposed § 170.36(c)(1)(ii) *The common or usual name of notified substance.*

The common or usual name for polyglycerol polyricinoleic acid is "PGPR."

Proposed § 170.36(c)(1)(iii) *Applicable conditions of use.*

PGPR is to be used in the food industry as an emulsifier, primarily in chocolate applications. PGPR has unique and highly advantageous properties when added to chocolate at amounts up to 0.3%. These properties offer the chocolate manufacturer a remarkable degree of control over the flow properties of molten chocolate. PGPR is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

A member of ICI Group

Alan M. Rulis, Ph.D. Director
Food and Drug Administration
October 13, 1998

Quest INTERNATIONAL

Proposed § 170.36(c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures, specfically generally available and accepted published and unpublished scientific data, information, methods, and principles, as well as the conclusions of an independent panel of experts convened by Quest International, qualified by their individual training and experience to review the data supporting the safety of PGPR as an emulsifier in chocolate. The panel was provided with scientific and technical data on the manufacture, application, exposure, and safety of PGPR. The panel also was provided with a comprehensive search of the scientific literature on PGPR and related products. Following a critical review and analysis of the material on PGPR, the panel of experts concluded that the available data clearly support the safety of the use of PGPR at a maximum rate of 0.3% in chocolate. The panel of experts further concluded that PGPR is GRAS for this use.

Subsequent to the review and conclusion of the expert panel, 5 articles documenting the safety of PGPR were published in the September/October 1998 issue of the peer-reviewed journal, Food and Chemical Toxicology (Volume 36, Number 9/10).

Proposed § 170.36(c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The data and information that are the basis for this GRAS determination and notification are available to the Food and Drug Administration for review and copying upon request.

Very truly yours,

Angel Diaz de Leon
President and CEO,
Quest Foods North America

Encl.

000165

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GRAS NOTIFICATION FOR POLYGLYCEROL POLYRICINOLEIC ACID (PGPR)

October 8, 1998

000166

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QST

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TAB 1

Quest
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COMMON OR USUAL NAME FOR POLYGLYCEROL POLYRICINOLEIC ACID (PGPR)

October 8, 1998

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1. COMMON OR USUAL NAME FOR POLYCLYCEROL POLYRICINOLEIC ACID

1.1 Introduction

Polyglycerol polyricinoleic acid is an important water in oil emulsifier that is manufactured from the interesterification of castor oil fatty acids with polyglycerol. The polyglycerol is mainly composed on di-, tri- and tetraglycerol with no more than 10% polyglycerols equal to or higher than heptaglycerol. The castor oil fatty acids are mainly composed of 80-90% ricinoleic acid.

As discussed in greater detail in other sections of this report, Quest has determined that the use of polyglycerol polyricinoleic acid as an emulsifier in chocolate is generally recognized as safe. Currently Quest markets polyglycerol polyricinoleic acid outside the United States under the tradename Admul WOL™.

According to the section 403(I)(2) of the Federal Food, Drug, and Cosmetic Act and 21 C.F.R. § 101.4, a food product must list the common or usual name of each ingredient in the food. Quest recognizes, in this GRAS notification, that the common or usual name of polyglycerol polyricinoleic acid is "PGPR."

1.2 PGPR is an appropriate "common or usual name"

Quest agrees with the marketplace that PGPR is an appropriate common or usual name for polyglycerol polyricinoleic acid. It is appropriate because the letters clearly represent the essential portions of the substance's name. P (Poly) G (glycerol) P (poly) R (ricinoleic acid, also known as ricinoleate). It is also a distinct name such that consumers would quickly recognize it on a product's label and not confuse it with other multi-syllabic polyglycerol ingredients. Because PGPR does not contain any vowels, it is also readily apparent that PGPR abbreviates a longer name.

This proposal is not unusual. For example, Mitsubishi Chemical Corp. has recently filed a food additive petition for sucrose esters of fatty acid in which the petitioner proposes "SOE" as the common or usual name for this product. 63 Fed. Reg. 46465 (Sept. 1, 1998). Additionally, many of FDA's regulations acknowledge acronyms and abbreviations as common or usual names, e.g., "BHA" (21 C.F.R. § 172.110), "BHT" (21 C.F.R. § 172.115), "EDTA" (21 C.F.R. § 172.120), "TBHQ" (21 C.F.R. § 172.185), and "THBP" (21 C.F.R. § 172.190). Therefore, Quest concurs with the marketplace that "PGPR" is an appropriate common or usual name for polyglycerol polyricinoleic acid.

TAB 2

Quest
INTERNATIONAL

SUMMARY OF POLYGLYCEROL POLYRICINOLEIC ACID (PGPR) COMPOSITION AND USE IN CHOCOLATE

August 28, 1997

000172

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QST-

1. THE USE OF POLYCLYCEROL POLYRICINOLEIC ACID (PGPR) IN CHOCOLATE

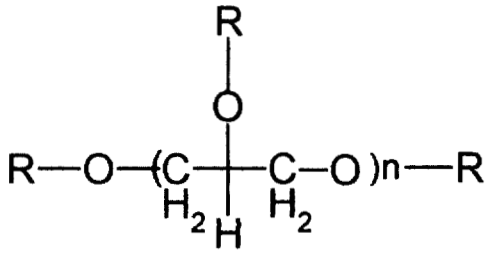
1.1 Introduction to polyglycerol polyricinoleic acid (PGPR)

Admul WOL is a Quest tradename for Polyglycerol Polyricinoleate (PGPR). Quest has for many years manufactured and marketed this surfactant. Admul WOL and PGPR will be used interchangeably in this report. PGPR has unique and highly advantageous properties when added to chocolate. It offers the chocolate manufacturer a remarkable degree of control over the flow properties of molten chocolate and gives the possibility for substantial savings in cocoa butter usage.

Polyglycerol polyricinoleate is an important water in oil emulsifier and is manufactured from the interesterification of castor oil fatty acids with polyglycerol. The polyglycerol is mainly composed of the di-, tri and tetraglycerol with no more than 10% polyglycerols equal to or higher than heptaglycerol. The castor oil fatty acids are mainly composed of 80-90% ricinoleic acid. PGPR is thus a mixture of these partial esters. PGPR is a viscous light brown coloured liquid and has the EC Nr. E476.

Synonyms :
Partial polyglycerol esters of polycondensed fatty acids of castor oil, Glyceran esters of condensed castor oil fatty acids,
Polyglyceryl ricinoleate.

General Structure :



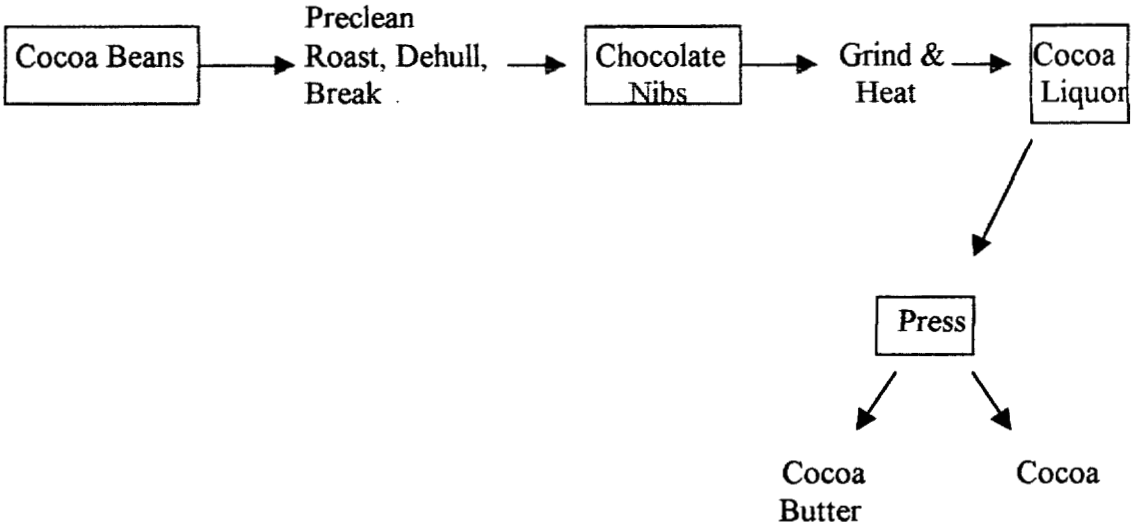
R = H or polyricinoleic acid ester , n = 2-6

PGPR meets the following specifications:

Acid Value	: max. 6
Iodine Value	: 72 - 103
Saponification Value	: 170 - 210
Moisture	: max. 1%
Refractive Index at 65°C	: 1.4630 - 1.4665
OH Value	: 80 – 100

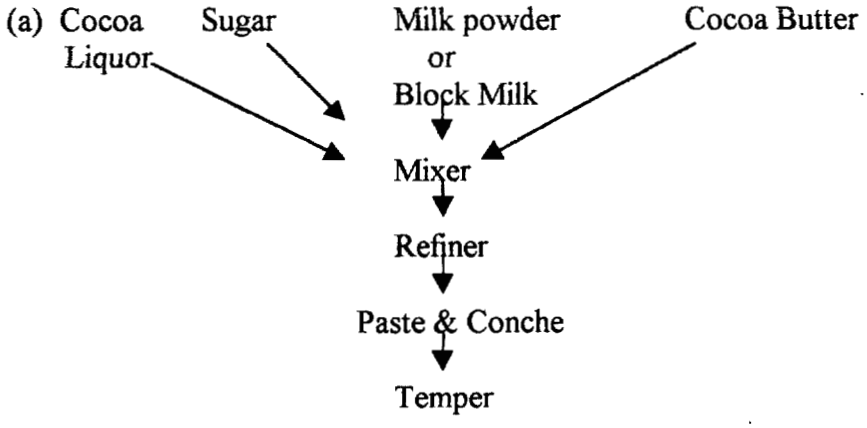
1.2 Method of Chocolate Manufacture

In summary the steps used in processing cocoa beans are as follows:



Thus after a preliminary cleaning and roasting stage the beans are dehulled and broken up to yield "Nibs". Nibs are blended to give the correct colour and flavour. Nibs are ground and heated to release and liquefy the cocoa butter held in the nibs celluar structure. The cocoa liquor is then used directly for chocolate manufacture or separated into cocoa and cocoa butter.

The precise method of processing depends to some extent on the size of the manufacturer. The principle steps used to manufacture chocolate are outlined below:



The key processes in chocolate manufacture are:

(i) Mixing

Straight forward operation.

(ii) Refining

In this process the chocolate mix is worked between polished steel rollers to reduce the particle size of the sugar, cocoa and milk particles to between roughly 15-30 μ. The optimum particle size will vary depending on the type chocolate produced.

(iii) Pasting

In this operation the chocolate flake from the refining stage is worked into a fluid paste, often with the addition of extra cocoa butter and surfactants before transfer to the conching operation.

(iv) Conching

The precise action at this stage is not well understood. The refined chocolate is kneaded gently under heavy granite rollers in the presence of air for long periods (10-24 hours for milk and 24-96 hours for dark chocolate). The effect of this is to greatly improve the flavour of the chocolate by removing harsh notes and this process also removes residual traces of moisture thus reducing the viscosity somewhat. Additional cocoa butter and lecithin are added at this stage to reduce the viscosity of the chocolate. PGPR is typically added at the end of conching to reduce the rheological yield value of the chocolate. High shear mixing is commonly employed in this operation.

(v) Tempering

Cocoa butter exists in a number of polymorphic forms and the nature of the crystalline form depends on the cooling procedure of the fat. The β form has the desirable high melting point characteristics. The Chocolate must be cooled such that the β crystals are formed uniformly throughout the cocoa butter matrix. If this procedure is not carried out correctly, and the fat in the chocolate is produced in one of the less stable crystalline forms on storage, a slow transition to the β form takes place with resultant discolouration and poor texture typically called bloom.

1.3 Proportions of Molten Chocolate

The processing of chocolate takes place in the liquid state and it is important to consider the factors which affect the flow properties of chocolate in this physical form.

(a) General

For most pure liquids there is a direct relationship between the force difference applied to unit areas of two adjacent layers in a liquid and the velocity gradient across those layers as a result of this force. These liquids are termed Newtonian and in mathematical terms this is expressed: Force applied per unit area = (constant) x (velocity gradient between layers
(shear rate). The constant is called the coefficient of viscosity and given the symbol η.

Some solutions of long molecules, molecules showing a high degree of interaction, or suspensions do not behave in this manner and the coefficient of viscosity of such solutions changes with the force applied to the solution.

(b) Chocolate

Molten chocolate consists of a liquid matrix of cocoa butter. Suspended in the liquid fat (cocoa butter and milk fat) is a high proportion of solid particles, e.g. sugar crystals, ground cocoa material and in the case of milk chocolate, non-fat milk solids. Because of the presence of these solids, liquid chocolate does not behave as a true liquid and exhibits non-Newtonian or plastic flow properties.

Such a material is said to exhibit plasticity and no flow occurs until the applied shear stress exceeds a limiting value called the "yield stress" or "yield value". The relationship is not linear and hence the apparent coefficient of viscosity (slope of the shear stress vs. shear rate curve) changes as the shear stress is increased.

The viscosity of liquid chocolate is very much greater than that of the liquid fat component alone. The flow of the chocolate is greatly dependent on the concentration of solid particles and the ease with which these are able to move past each other within the liquid phase. The precise nature of the interactions between the solid particles and the liquid phase is not fully understood Several factors seem to be responsible for increasing viscosity, for example:

(i) Attractive interactions between the hydrophilic solid particles.

(ii) Agglomeration of particles which occlude liquid fat hence reducing the proportion of continuous fat phase.

(iii) Absorption of liquid fat into solid particles.

It should be mentioned that traces of moisture also markedly increase the yield value of molten chocolate. This is thought to be due to swelling of the hydrophilic particles, which also reduces the relative volume of the continuous oil phase.

Clearly the chocolate manufacturer is concerned with the flow properties of his molten chocolate. On the one hand he requires a material which can be refined, conched and generally transported with a minimum of handling difficulties and energy requirements. A highly viscous system would be unsuitable. On the other hand cocoa butter is his most expensive raw material. He will wish to keep his cocoa butter content to the minimum and maintain good flow properties and a consistent quality product. These two considerations are contradictory and the manufacturer must optimize on the minimum cocoa butter content which will also give sufficient fluidity for easy processing. The manufacturer wants to control and manipulate viscosity and yield value for various applications. PGPR is an excellent emulsifier to assist in these capabilities.

Other factors that are important to define the final rheological properties of chocolate include final use of the chocolate.

(i) Chocolate used for moulding, a relatively low yield value and viscosity are desirable; the chocolate should flow well into the mould and take the shape of the mould allowing ready release of air bubbles and enrobing any filling material, e.g. dried fruit or nuts.

(ii) Chocolate intended for enrobing confectionery products reqires both a low yield value and a low viscosity to allow a thin even coating over the products.

(iii) Chocolate used for decoration a low viscosity allows easy flow of the mix. Higher yield is desirable such that when the shear stress is removed the chocolate will hold its shape and not flow under gravity.

These requirements can be met in chocolate systems by the correct choice of ingredients.

1.4 Effect of Surfactants on Molten Chocolate

(a) Lecithin

The addition of lecithin to chocolate has a marked effect on the viscosity of the chocolate at a given concentration of cocoa butter. Chocolate with a viscosity suitable for moulding or enrobing can be prepared with lower cocoa butter content if lecithin is present. It has been largely shown that it is primarily the interaction between the lecithin and the sugar particles which is responsible for the drop in viscosity obtained by the addition of this particular surfactant.

High additions of added lecithin can lead to increased rather than cleared viscosity. A level of 0.5% is considered optimum. A 0.5% addition of lecithin is generally recognized to give up to 5% savings of cocoa butter depending on the application.

(b) PGPR

In contrast to the effect shown with lecithin, the addition of PGPR to chocolate causes only a minor change in the plastic viscosity. However, PGPR has a pronounced effect on the yield value of the chocolate. Concentrations of PGPR up to 1% reduce the yield value to zero. Lower concentration of PGPR will result in a significant reduction in the yield values. The exact effect on the yield value will also depend on the type of chocolate and concentration of solid particles.

Thus PGPR reduces the force needed to start a mass of molten chocolate flowing while having a relatively minor affect on the plastic viscosity of the chocolate.

The addition of either PGPR or lecithin causes a reduction in the apparent viscosity.

In summary, lecithin appears to reduce principally the plastic viscosity of chocolate or in simplified terms reduce the force needed to keep liquid chocolate flowing once it has started moving. PGPR on the other hand has its greatest influence on yield stress. In simple terms it reduces the force required to start a mass of liquid chocolate moving. Both these effects reduce the apparent viscosity or increase the fluidity of the system.

In practice, chocolate manufacturers use lecithin to reduce the plastic viscosity of the chocolate and the addition of PGPR will reduce the yield value and give a chocolate melt which is more workable than can be obtained by either ingredient alone.

The optimum ratio of lecithin and PGPR on yield value has been determined by Banford et al (Confectionery Production, June 1970). In milk chocolate, 0.5% lecithin and 0.2% PGPR gave a greater reduction in yield value than either emulsifier used separately at 0.7%. It should be recognized that too much lecithin creates problems in chocolate making.

PGPR/lecithin blends of course have greatest applications for moulding and enrobing chocolate while for decorating chocolate one would not use PGPR since a high yield value is desirable.

PGPR will also be beneficial when used in couverture chocolate for "wet" products, e.g., ice cream. It increases tolerance to moisture pick up and counteracts the corresponding viscosity increase. The mechanism of this action is not clearly defined. However, it is probably because of PGPR's excellent "W/O" emulsifying characteristics which disperse water drops in the fat phase. This prevents them being absorbed into the hydrophilic solids and the associated swelling of the particles.

When to Add Surfactants

It is reported that the processes of refining and conching tends to destroy the potency of lecithin added to chocolate for the purposes of reducing viscosity. Since the main advantage of using surfactants is the saving of cocoa butter it is suggested that advantages can be gained from adding surfactant at different stages in the chocolate manufacturing process. Thus some lecithin is frequently added at the refining stage followed by addition of extra surfactant as late as possible in the final conching stage as a means of obtaining maximum viscosity reduction. Typically if total lecithin addition is 0.5% then 0.2% should be added before refining and 0.3% late in the conching stage. Similarly,
PGPR would be added at the later stage in the process.

In practical terms, the availability to the chocolate manufacturer of both lecithin and PGPR means that he has a remarkable degree of control over the flow properties of his molten chocolate.

2. SMALL SCALE MANUFACTURE OF PGPR

As summarized in Food and Chemical Toxicology, PGPR is prepared in four stages: preparation of the castor oil fatty acids, condensation of the castor oil fatty acids, preparation of polyglycerol, and partial esterification of the condensed castor oil fatty acids with polyglycerol. (Wilson, R. et. al. 1998. Overview of the Preparation, Use and Biological studies on Polyglycerol Polyricinoleate (PGPR). Food and Chemical Toxicology 31(9/10): 711-718.)

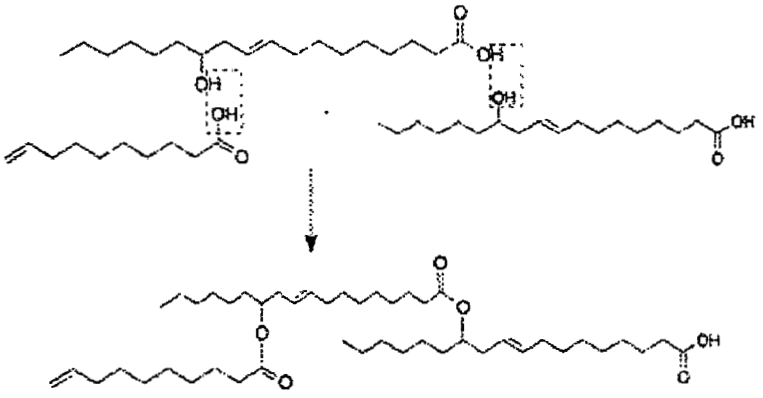
2.1 Preparation of castor oil fatty acids

The starting material is castor oil which contains as its main fatty acid component ricinoleic acid (80-90%). Other fatty acids present are oleic acid (3-8%), linoleic acid (3-7%), and stearic acid (0-2%). Since the ricinoleic acid is the essential component, determination of the hydroxyl value of the oil is valuable as a check on possible contamination. The castor oil is hydrolysed in an autoclave by water and steam at 400 psi pressure without any added catalyst. The resulting fatty acids are removed from glycerol by water washing. They are thought to contain a little unconverted, combined glycerol but the amount is not known. It is possible that there may be as much as 10% glycerides in this product. A determination of the free fatty acid content of the product, assuming the mean equivalent weight of the original acids, yields a value of about 62-63%. This product is a normal commercial material which is sold as "split seconds castor fatty acids".

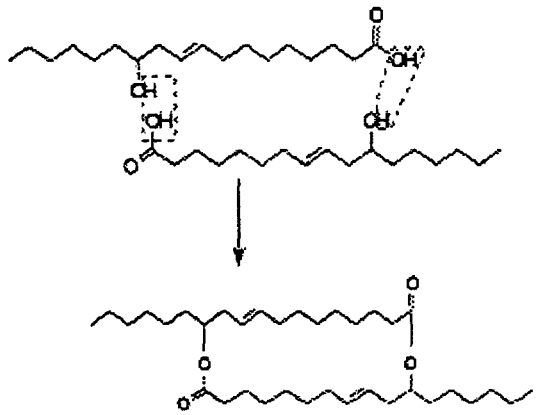
2.2 Condensation of castor oil fatty acids

On heating castor oil fatty acids alone to about 200°C the ricinoleic acid may react in a number of ways.

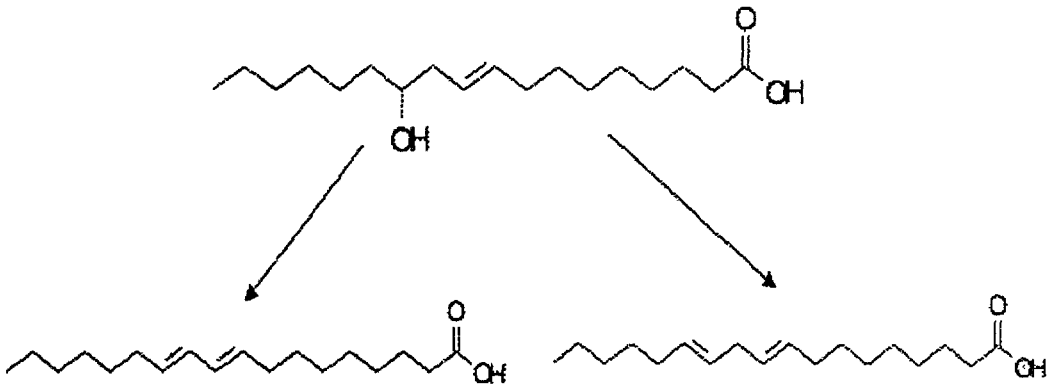
(a) Linear Esterification



(b) Cyclic Esterification



(c) Dehydration



Simple linear esterification (scheme a) is the desired reaction but cyclic esterification (scheme b; a chain terminating process) is theoretically possible. However there is no evidence of the presence of this type of cyclic compound in the condensed castor oil fatty acids. Dehydration has been shown to occur to only a small extent.

Linear esterification ensues even on drying the castor oil fatty acids at temperatures as low as 120°C in vacuo. The condensation reaction is ordinarily carried out at about 200-210°C under a 28" vacuum. The reaction is carried out in vacuo to prevent oxidation and favour the formation of a light-coloured final product. Results of a laboratory experiment involving this reaction at 200°C and 2 cm Hg for 9 hours is illustrated in Table 1.

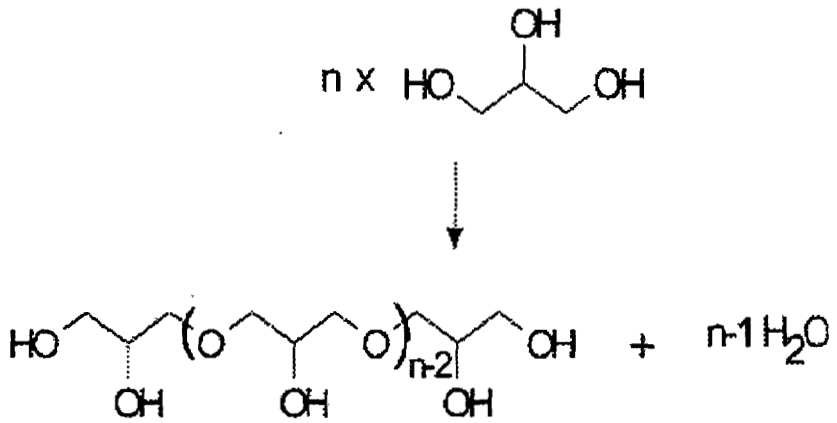
Table 1 Condensation of castor oil fatty acids at 200°C in vacuo (2 cm Hg) with CO_2 stirring

Time	Acid Value
Original castor oil fatty acids	182.0
Brought to 200°C	162.8
After 1 hour	103.0
After 2 hours	82.3
After 4 hours	58.3
After 5 hours	50.3
After 6 hours	44.8
After 7 hours	43.2
After 9 hours	35.5
Final colour 1/2" Lov.	1.1R+3.5Y

2.3 Preparation of Polyglycerol

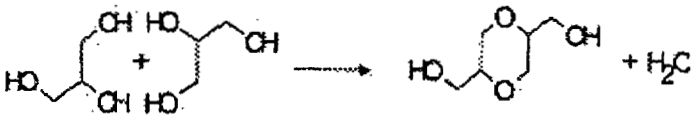
Glycerol is heated at temperatures above 200°C in the presence of a small amount of alkali, when two or more molecules condense with loss of water, forming a mixture of products containing varying numbers of glycerol residues. Carbon dioxide is bubbled through the reaction vessel to prevent oxidation. Unchanged glycerol is removed at the

end of the reaction. The terminal 1 :3-hydroxy groups of glycerol are more reactive than the 2-hydroxy group, so that in general the "polyglycerols" are formed by the overall reaction.



The detailed mechanism of the reaction is not known but it has been suggested (Hauschild and Petit, 1956) that glycidol **HOCH₂ CH. CH₂**
O
is an important intermediate.

In addition to the straight-chain "polyglycerols" there is some evidence that small amounts of cyclic by-products may be formed in the reaction mixture as a result of condensation between the 1-hydroxy group of one glycerol molecule and the 2-hydroxy group of another, the simplest case of which is the formation of compound (I):



(I)

This product (I) is a solid, mp 96°C, that has been isolated in very small amounts from reactions of this type

2.4 Partial Esterification of Condensed Castor Oil Fatty Acids with Polyglycerol

The final stage of the preparation involves heating an appropriate mixture of the condensed castor oil fatty acids and polyglycerol. The resulting product is a very complex mixture of chemical individuals, most of them containing in the molecule one or more condensed fatty acid groups, one or more ester links, usually one or more ether linkages and several free hydroxyl groups, in addition to small amounts of free glycerol.

3. LARGE SCALE MANUFACTURE OF WOL

3.1 Condensation of the Castor Oil Fatty Acids

The condensation of the castor oil fatty acids on a manufacturing scale is carried out as follows:

The castor oil fatty acids as prepared in Section 2.1 are heated to 205-210°C under a 28" vacuum and an atmosphere of carbon dioxide. This condition is maintained with a maximum reflux temperature of 50°C. Samples are taken at hourly intervals and tested for their free fatty acid content until a titration of 12.5 cc is obtained using N/5 caustic soda on a 4 g sample. This represents and acid value of 35.0; and is equivalent to an average of about five fatty acid residues per molecule of the condensed product. The period of heating is usually about 8 hours, and thereafter polyglycerol is added immediately.

3.2 Preparation of Polyglycerol

Polyglycerol is prepared by heating glycerol under specific conditions and then distilling off unreacted glycerol. Chemically pure glycerol (4480 lb) is introduced into the reactor and heated by external gas burners together with an internal closed steam coil, and carbon dioxide is passed into the vessel. The agitator is started, and when the temperature has been reached 75°C caustic potash (481b; in the form of a strong aqueous solution) is sucked in. At 170°C the steam is shut off, and the vacuum applied and regulated to 18". Cooling water to the reflux condenser is adjusted to give a maximum reflux temperature of 90°C. The gas heating is continued until the temperature reaches 240°C; these conditions are maintained throughout the reaction period. The reaction is continued until the refractive index of the mixture at 50°C has been raised to 1.4815; this usually takes about 24 hours. The batch is then allowed to cool to below 190°C. Cooling is aided by (a) blowing air on to the outside of the vessel, and (b) putting full cooling water on the reflux and increasing vacuum to 20-24".

When the product has cooled to 190°C the carbon dioxide is shut off, and the steam valve to the reactor is carefully opened to give steam agitation without priming.

The maximum vacuum obtainable is then applied. Cooling water is shut off and drained from the reflux Distillation now commences and unpolymerized glycerol is distilled from the reaction product at a rate of approximately 100 Ib/h for approximately 10 hours.

Vessel samples are taken hourly and their refractive index at 50°C determined. Distillation is stopped when the refractive index of the polyglycerol remaining in the reactor has reached 1.4885. The overall yield of polyglycerol is approximately 62%.

3.3 Partial Esterification of the Condensed Castor Oil Fatty Acids with Polyglycerol

The reaction of polyglycerol with the condensed castor oil fatty acids takes place immediately following the preparation of the latter (as described in Section 3.2) and in the same vessel whilst the charge is still hot. Polyglycerol, 171 lb (equivalent to 9% of the charge weight) is added to the condensed castor oil fatty acids prepared from 1,900 lb of castor fatty acids. The esterification reaction is also carried out at 205-210°C under 28" vacuum and in an atmosphere of carbon dioxide. This process is continued until a sample withdrawn from the reaction mixture is found to have an acid value of 2 or less; this takes 5-8 hours. The vacuum is released by admitting carbon dioxide and the reaction mixture is transferred to a wash vessel via a cooler. To the material cooled to 96°C is added ½ lb caustic soda dissolved in 12-3/4 lb of water, the addition being made slowly with continuous stirring for at least 15 minutes. The product is strained through muslin direct to the packages.

TAB 3

Quest
INTERNATIONAL

REPORT OF THE EXPERT PANEL ON THE SAFETY OF POLYGLYCEROL POLYRICINOLEIC ACID (PGPR)

December 8, 1995

000188

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THE SAFETY OF POLYGLYCEROL-POLYRICINOLEIC ACID EMULSIFIER (ADMUL WOL®) FOR USE IN FOOD

Quest International invited an independent panel of recognized experts qualified by their individual scientific training and experience to evaluate the safety, using appropriate scientific procedures, of ADMUL WOL under the conditions of its intended use in food as an emulsifier in chocolate. The Panel members included Professors Joseph F. Borzelleca, Lloyd J. Filer, Bert N. LaDu, and Michael W. Pariza. Resumes of the Panel members appear as Appendix 1. A comprehensive search of the scientific literature on ADMUL WOL and related products was conducted by Quest International and the results were made available to the Expert Panel. Quest International also provided to the Expert Panel the material identified in Appendix 2. This included information on the manufacture, applications, exposure, and safety of ADMUL WOL. The members of the Expert Panel made use of other information deemed appropriate or necessary. Panel members individually reviewed the materials submitted and other materials deemed appropriate or necessary and conferred by telephone. The Expert Panel was convened in Chicago, Illinois on 11 August 1995. At this meeting, oral presentations were made by Quest International and Unilever.

Polyglycerol-polyricinoleic acid is marketed by Quest International under the trade name ADMUL WOL as an emulsifier in foods. ADMUL WOL has been used in the production of chocolate for more than 30 years and is approved for use in many countries outside the United States. ADMUL WOL is approved in the European Union under the E-number E 476.

Manufacture of ADMUL WOL

ADMUL WOL production is summarized in the document "Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL) : Part 1. Preparation and Chemical Nature" which appears in Appendix 2. Only food grade materials are used in the production of ADMUL WOL. In summary, the production consists of

1-Preparation of castor oil fatty acids.
2-Condensation of castor oil fatty acids.
3-Preparation of polyglycerol.
4-Partial esterification of the condensed castor oil fatty acids with polyglycerol.

Analytical data comparing production lots of ADMUL WOL indicate that a constant composition can be maintained by controlling the refractive index and hydroxyl value of the product. ADMUL WOL (E 476) is a viscous liquid that does not crystalize even at temperatures below 0°C and has the following composition (meets the following specification):

hydroxyl value	80-100
acid value	6 mg KOH/g
refractive Index @ 65°C	1.4630-1.4665
color	Brown
heavy metals* (as Pb)	Not more than 10 mg/kg
arsenic*	Not more than 3 mg/kg
lead*	Not more than 5 mg/kg
mercury*	Not more than 1 mg/kg

(*Heavy metal specification is in proposed EU specification for ADMUL WOL).

Quantitative analysis of the ADMUL WOL present in food involves the extraction of the fat from the food and the determination of the amount of ricinoleic acid present by GLC. Details of the analytical methods are included in the supporting document "Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL) : Part 1. Preparation and Chemical Nature" (Section 8. Determination of WOL in Chocolate) which appears in Appendix 2.

Applications of ADMUL WOL

It is well documented that ADMUL WOL is an effective emulsifier which forms stable water-in-oil emulsions even at very high water contents and it decreases the viscosity and the yield stress of molten chocolate. These latter effects are similar to those produced by lecithin but the flow properties are altered in a totally different way; optimum effects are obtained with ADMUL WOL/lecithin mixtures. The best synergistic effect for many purposes is obtained with a blend of 0.25% ADMUL WOL and 0.5% lecithin. This eliminates most of the difficulties associated with the production of low-fat chocolate. The use of ADMUL WOL also results in the molten chocolate having better viscosity stability, reduced sensitivity to high temperatures, and shorter processing times. By modifying the way in which the fat phase crystallizes during the final stage of manufacture, the ADMUL WOL also improves the appearance, texture, and keeping qualities of the resultant chocolate. In coating for ice cream, ADMUL WOL has the further advantage of producing viscosity reduction in the presence of small quantities of water, which would otherwise lead to excessive thickening. ADMUL WOL was stable in milk chocolate stored at room temperature for 21 months.

Consumption

ADMUL WOL is used in chocolate at levels up to 0.3%.
Based on the National Food Consumption Survey (NFCS) for chocolate candy for the years 1991-1992, the potential intake of the 90th percentile group (males aged 12-19) would be 336 mg/day for a 70 kg human.

AGE	GENDER	WEIGHT	INTAKE CHOCOLATE (GRAMS)		INTAKE ADMUL WOL (MPK)	
years		kg	MEAN	90TH	MEAN	90TH
1-2	M&F	12	23	49	4.8	10.2
3-5	M&F	18	--	55	--	7.6
12-19	M	60	58	112	2.4	4.7
50-59	M	77	54	66	1.8	2.1
50-59	F	65	52	100	2.0	3.8

The above estimates are likely to be greatly in excess of those ever likely to be actually achieved. It seems highly unlikely that one to two year-old children eat on average 23 grams of chocolate per day. It should be noted that the sample size of one to two year-olds in the survey was small; dietary recall records are highly inaccurate, and chocolate candy is not always bar chocolate but may be an enrobed confection. Under these circumstances, the consumption of cocoa butter or ADMUL WOL would not be at 0.3% of the weight of the confection, but less than that. Furthermore, these figures are based on the consumption by chocolate eaters, assuming the content of the ADMUL WOL to be the maximum content of 0.3%, in all the chocolate products consumed. The figures must be considered very generous over-estimates of the actual projected daily consumption of ADMUL WOL by any age group if it is added as an emulsifying agent to chocolate and chocolate couverture products.

Safety Evaluation

The Expert Panel reviewed the toxicological studies (10 volumes) identified in Appendix 2 and summarized in the document attached as Appendix 3 and identified as "Summary of Unilever Biological Studies on a Polyglycerol-polyricinoleic Acid Emulsifier (ADMUL WOL)." Extensive toxicological studies were conducted in rodent and non-rodent species. These studies included acute, subchronic (90 days, rats and chickens), chronic (1 year, rats),

carcinogenicity (2 years, rats; 80 weeks, mice), reproduction (3 generation rats), biodisposition (absorption, distribution, metabolism and excretion in rats, mice, and humans). The toxicity of ADMUL WOL is very low. Enlarged livers and kidneys were seen in rats and mice that received ADMUL WOL as a dietary admixture at a level of 5% (the only level tested) for 104 and 80 weeks respectively. Special studies demonstrated that the enlargement was due to hypertrophy and not hyperplasia and was reversible. ADMUL WOL was not carcinogenic. Enlarged livers and kidneys were not seen in rats, mice and chickens exposed to ADMUL WOL at levels up to 2% for up to 90 days but were seen at levels of 3% and above.

The No Observed Adverse Effect Level (NOAEL) from the most sensitive study, the multigeneration reproduction study, was 1.5% (equivalent to 750 mg/kg bw/ day), the only level tested. JECFA reviewed the available data on ADMUL WOL and established an Acceptable Daily Intake (A.D.I.) of 0-7.5 mg/kg bw/day based on the reproduction data and use of a 100 fold safety factor. This information appears in "Seventeenth Report of the Joint FAO/WHO Expert Committee on Food Additives", Wld. Hlth Org. Techn. Rep. Ser., 1974, No. 539; FAO Nutrition Meetings Report Series, 1974, No.53.

Availability and Use of ADMUL WOL

The Codex Commission of Food Additives fully endorsed the inclusion of ADMUL WOL in the Draft Standards for "Chocolate", "White Chocolate" and the "Composition of Flavored Chocolate" up to a maximum level of 5 g/kg. In 1976, the Codex Alimentarius Commission in Rome adopted the Draft Standard and it has been circulated as the "Recommended International Standard for Chocolate". The emulsifiers listed in the Standard include polyglycerol polyricin-oleate (ADMUL WOL) up to a maximum level of 5 g/kg. Under this standard, ADMUL WOL is permitted in all 15 member states of the EEC, Argentina, Australia, Canada, India, Kenya, New Zealand, South Africa, Switzerland, and Zambia.

CONCLUSIONS

Following critical review and analysis of available information on ADMUL WOL, the Expert Panel concluded that the available data support the safety of the proposed uses of ADMUL WOL as an emulsifier in the manufacture of chocolate and chocolate couverture. The expert Panel further concluded that ADMUL WOL is GRAS for these uses.

Expert Panel on the GRAS Status of
Polyglycerol-Polyricinoleic Acid Emulsifier (ADMUL WOL)

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APPENDIX 1. - Resumes of the Expert Panel

[Note: The resumes of the Expert Panel are maintained in Quest International's PGPR master file and are available for review.]

APPENDIX 2. - Documentation Review by the Expert Panel in Support of the Safety of ADMUL WOL.

Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Synopsis.
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 1. Preparation and Chemical Nature
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 2. Acute Toxicity Tests
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 3. Sub-Acute Toxicity in Rats
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 4. 90-Day Rat Test
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 5. 90-Day Chicken Test
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 6. Liver and Kidney Enlargement
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 7. 7A Indirect Studies
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 7. 7B Direct Studies
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 8. Chronic Toxicity
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 9. Carcinogenicity
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Part 10. Human Studies
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Appendices I - XXXIV
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Appendices XXXV - LXII
Glyceran Esters of Condensed Castor Oil Fatty Acids (WOL):
Appendices LXIII – C

[Note:The documents reviewed by the Expert Panel are maintained in Quest International's PGPR master file and are available for review.]

APPENDIX 3. Summary of Unilever Studies on a Polyglycerol-Polyricnoleic Acid Emulsifier (ADMUL WOL)

[Note: The Summary of Unilever Studies on a Polyglycerol-Polyricnoleic Acid Emulsifier reviewed by the Expert Panel is maintained in Quest International's PGPR master file and is available for review.]

APPENDIX 4. ADMUL WOL Summary Document Provided to the Expert Panel

[Note: The ADMUL WOL Summary Document Provided to the Expert Panel is maintained in Quest International's PGPR master file and is available for review.]

TAB 4

[redacted]

Quest
INTERNATIONAL

ANALYSIS AND STABILITY POLYGLYCEROL POLYRICINOLEATE (PGPR) (ADMUL WOL™ EMULSIFIER)

JUNE 26, 1997

000199

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1. Introduction 1
   1.1 Description of PGPR 1
   1.2 Product Specification 2

2. Analytical Methods to Characterize Emulsifiers / PGPR 3
   2.1 Physical Measurements 3
   2.2 Chemical Measurements 4
   2.3 Chromatography 5
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3. Stability of PGPR 11
   3.1 Pure Product 11
   3.2 In Chocolate Confectionery Products 12

4. General Conclusion 13

5. Bibliographic References 14

Annex Part 1 - Admul WOL Product Information Sheet

Annex Part 2 - Standard Methods of Analysis

Annex Part 3 - Viscosity Values of Chocolate containing PGPR

1. INTRODUCTION

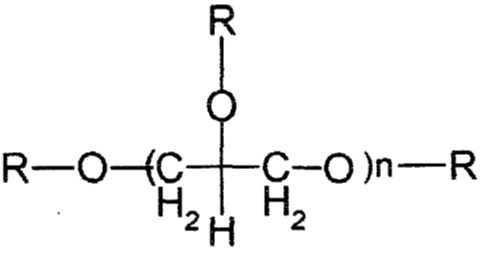
The purpose of this document is to review known analytical methodologies for testing the emulsifier PGPR. Analytical methods to characterize PGPR have provided data on its stability as a pure product and in chocolate confectionery applications. An extensive search for analytical methodologies and testing protocols was carried out covering the last three decades. References are made to internal Quest (formerly PPF) R&D reports, Unilever R&D reports, as well as publicly cited information from Nestlé, patents, and litterature such as the Swiss Foodstuffs Manual. The most important findings with respect to product characterization and stability are reported here.

1.1 Description of PGPR

The Quest tradename for Polyglycerol Polyricinoleate (PGPR) is Admul WOL™. This product is an important water in oil emulsifier and is manufactured from the interesterification of castor oil fatty acids with polyglycerol (1). The polyglycerol is mainly composed of the di-, tri and tetraglycerol with no more than 10% polyglycerols equal to or higher than heptaglycerol. The castor oil fatty acids are mainly composed of 80-90% ricinoleic acid. PGPR is thus a mixture of these partial esters.
PGPR is a viscous light brown coloured liquid and has the EC Nr. E476 (2).

Synonyms :
Partial polyglycerol esters of polycondensed fatty acids of castor oil,
Glyceran esters of condensed castor oil fatty acids,
Polyglyceryl ricinoleate.

General Structure :



R = H or polyricinoleic acid ester , n = 2-6

1.2 Product Specification

The Quest product information sheet (5Z00493 dated 17/07/97), cf. annex part 1, lists the following specifications:

Acid Value	: max. 6
Iodine Value	: 72 - 103
Saponification Value	: 170 - 210
Moisture	: max. 1%
Refractive Index at 65°C	: 1.4630 - 1.4665
OH Value	: 80 - 100

2. ANALYTICAL METHODS TO CHARACTERIZE EMULSIFIERS / PGPR

Food emulsifiers are a particularly difficult class of compounds to characterize. This is due to their complex composition, often containing a mixture of hundreds of individual chemical compounds. As mentioned, PGPR is produced from the interesterification of a polyglycerol with castor oil fatty acids. The great variety of individual compounds arises from several aspects notably :

- the different degree of esterification of the polyglycerol,
- by the different chain-lengths of the fatty acids,
- by the degree of unsaturation,
- and by the positional isomerism of the fatty acids esterified with the polyglycerol's hydroxy groups.

When confronted by the analysis of PGPR in a foodstuff such as chocolate it also has to be taken into account that at an application level of for example 0.25%, the actual amounts of individual chemical compounds is even far less. The analysis is all the more difficult when some of these compounds are also similar or present in the food itself. As well, many of the individual compounds do not exhibit UV absorption or contain any specific functional groups that could allow them to be detected. Even with modern analytical instrumentation, the complete analysis of PGPR is extremely complex. In many instances certain compounds are used to detect for PGPR in food products, eg. methyl ricinoleate. PGPR can be diffrentiated from other types of emulsifiers such as propylene glycol esters, polysorbates and sorbitan esters on the basis that these types contain products that do not normally occur in food, example: propane-1,2-diol characteristic of propylene glycol emulsifiers (3).

Analytical methods for fats and oils can be found in the Official Methods and Recommended Practices of the American Oil Chemists Society (4), and the IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives (5).

2.1 Physical Measurements

Here, it is intended to mention the routine quality control tests that are performed for PGPR production at Quest Zwijndrecht NL. Methods for these tests can be found in annex part 2.

Moisture

This is performed by Karl-Fisher titration for the exact water content. cf. standard method of analysis code 144 in annex.

Refractive Index
This is used for identification and as a complimentary test to determine the grade of purity. cf. standard method of analysis code 136 in annex.

Viscosity
As stated, PGPR is used in chocolate as it modifies the yield value and improves the viscosity of the chocolate. Viscosity is an important measurement. although not routine quality control on PGPR emulsifier production. For interest, yield values of PGPR at different percentages in chocolate are given in annex part 3 (6). When needed, viscosity can be measured in PGPR based on a standard method of analysis used for another emulsifier DATEM, cf. method code 173 in annex part 2. The Haake viscometer used in this method can accurately measure yield values and plastic viscosity. It can easily detect less then 0.1% differences in emulsifier content in chocolate.

2.2 Chemical Measurements

Again the purpose here is to mention the routine quality control tests that are performed for PGPR production at Zwijndrecht. Methods for these tests can be found in annex part 2.

Iodine Value
This value is related to the emulsifier's number of double bonds, both cis and trans. It can also be related to it's resistance to autoxidation. cf. standard method of analysis code 123 in annex.

Saponification Value
Saponification is related to the molecular weight expressed as the number of mg KOH required to saponify 1 g of sample. cf. standard method of analysis code 141 in annex.

OH Value
The standard method of analysis coded 122 in annex makes the distinction that all OH- groups are titrated and the result does not differentiate between OH- groups in the fatty acid chains and those present in the mono- or diglycerides. Thus, partially hydrolysed emulsifiers, which is the case of PGPR, may show a high hydroxyl content without containing hydroxy acids.

Acid Value
This is a measurement of the free fatty acids not chemically bound to an alcohol group. In emulsifiers, the acid value can also be used to determine if the refining and deodorizing step in production has been efficient or if a high level of citric acid could be present. cf. standard method of analysis code 148 in annex.

2.3 Chromatography

Over the last three decades several reports have been made concerning the analysis of PGPR. A compilation of classical GC, HPLC and TLC methods for emulsifiers can be found in the Swiss Foodstuffs Manual (1). The fatty acids of emulsifiers have mainly been analysed by GC via their esters, (7)(8). HPLC using size exclusion chromatography (3) has been applied and reversed phase RP-HPLC has also been used for some specific fatty acids (9). In this last method, the lack of a chromophore in free fatty acids is overcome by derivitizing into the phenacyl esters. An interesting method has been published using silica gel minicolumns with TLC (10). The most recent developments to fatty acid analysis have been the application of Capillary Electrochromatography (CEC) (11).

Gas Chromatographic Analysis
Like monoglycerides, direct GC analysis on emulsifiers is not possible due to their high boiling points. Thus, it is necessary to derivitize the free hydroxy groups into more volatile compounds. A general procedure from Quest Zwijndrecht is performed using MSTFA (N-methyl N-trimethylsilyl trifluoro acetamide). The Quest standard method of analysis of the polyglycerol composition of polyglycerol or PGPR is given in the annex part 2, method code 193, along with a typical gas chromatogram.

In a study conducted at the Unilever Research Laboratory Colworth House UK (12), batch to batch variations of the ricinoleic acid content of three samples of PGPR were measured. Analysis was performed after alkali hydrolysis and methyl esterification. Silyation was also performed to improve GC detection. The results of this study showed the fatty acid composition and % ricinoleic acid in PGPR, table 1.

Table 1 : Percentage Fatty Acid composition of total and percentage ricinoleic acid in PGPR. 16:0 = palmitate, 18:0 = stearate, 18:U = 18 carbon unsaturates, OH18:1 = ricinoleate (12, hydroxy oleate), diOH18:0 = 9,10 dihydroxy stearate (natural erythro, cis form).

Sample	16:0	18:0	18:U	OH18:1	diOH18:1	% ricinoleic acid
ricinoleic acid	0.7	0.6	5.1	92.8	0.8	—
polyricinoleic	0.7	0.9	5.1	92.6	0.7	—
PGPR 49-8-25	0.6	1.1	5.3	92.3	0.7	55.1
PGPR 37-8-25	0.7	1.2	5.3	92.1	0.7	54.1
PGPR 35-8-24	0.7	1.4	5.6	91.7	0.6	53.5

The above data were corrected for recoveries using a standard of methyl heptadecanoate which was also used as the internal standard for the calculation of the ricinoleic acid content.

In 1979 an extensive study was conducted by Unilever Research Colworth House UK in order to develop an analytical method for PGPR (13). This was used to study the effect of storage on the emulsifier content of milk chocolate. The method that was developed represents a standard analytical procedure for the determinantion of PGPR. Using this method the analysis of PGPR in milk chocolate can be determined with a relative precision of +/- 16.6%. Stability results in chocolate are presented in this report 3.2. The method will be described here, as follows.

The method is composed of 5 steps :

- Extraction of lipids
- Hydrolysis of lipids
- Methylation of fatty acids
- Preparative TLC for the isolation of the hydroxy FAME's
- GC analysis of the hydroxy FAME's

Methyl 2-hydroxy docosanoate was chosen as an internal standard added during the hydrolysis step. This was chosen as being similar to the analytes and is easily separated from the ricinoleate peak on the gas chromatogram. It is also commercially available.

Step 1 - Extraction Procedure of Lipids

In dry ice (-80°C) the chocolate sample is ground as finely as possible. Approximately, 10 +/- 0.01 g of chocolate is placed in a 150ml beaker. Then 10g of Kisselguhr is added to the beaker and mixed. The mixture is placed into a Soxhlet extraction tube and plugged with glass wool. Soxhlet extraction is performed using 150 ml chloroform for approx. 5-6 hours. In a rotory evaporator the chloroform is removed under vacumn at 50°C to constant weight. The percentage lipid present in the chocolate is determined from the weight of the extract.

Step 2 - Hydrolysis Procedure of Lipids and Internal Standard

A fresh solution of the standard is prepared, 80 mg Methyl 2-hydroxy docosanoate in 10 ml ether. 1ml of the standard solution is pipetted into a 100 ml round bottom flask. Thus, the flask contains 8 mg of standard. 500 mg of the lipid extract from step 1 along with anti-bumping granules and 20 ml of 1.0M methanolic KOH is added to the flask. The mixture is then refluxed for 2 hours using a heating mantle. After this, 80 ml of chloroform is placed into the flask and shaken vigourously.

The resulting solution is transfered to a separatiing funnel and NaCl solution is added (100ml containing 3ml of concentrated hydrochloric acid). After shaking and settling, an aqueous phase separates which should be acidic, tested with pH paper. The chloroform phase is recovered and the aqueous phase is re-extracted twice using 50 ml chloroform. To the entire chloroform extract dry magnesium sulphate is added until slight excess. This is then filtered and evaporated to dryness at 50°C using the rotary evaporator.

Step 3 - Methylation Procedure of the Fatty Acids

The fatty acid extract from step 2 is dissolved in 20 ml of a 2% methanol/ether solution and derivitization performed using diazomethane, added dropwise until a yellow colour persists. Great care must be taken when using this derivitization agent. The mixture is allowed to stand for 1 hour and the solvent is then evaporated under a stream of nitrogen. The subsequent residue is dissolved in 1.5 ml chloroform containing the methylated fatty acid esters for chromatographic separation.

Step 4 - Preparative TLC for the isolation of the hydroxy FAME's

TLC plates are prepared in the conventional way by prewashing precoated TLC plates in chloroform in the TLC tank so that the solvent front is 1 cm from the top of the plate. The top is marked and allowed to dry. The chloroform solution from step 3 is applied 2 cm from the lower edge and developed in the tank using chloroform. In order to obtain an adequate yield of FAME's for the GC analysis, several applications should be made to the plate. Phosphomolybdic acid spray is applied to the plate and placed in an oven at 140°C/15minutes. The hydroxymethyl esters are revealed as a narrow dark band below a larger upper black spot attributed to cocoa butter methyl esters all on a yellow background. The plates can be viewed under a UV lamp at 365nm. The hydroxy acid methyl esters are sometimes bounded by an upper blue fluorescent band and a lower cream fluorescent band. Fluorescent bands may not be observed if solutions are too dilute. The central dark zone is scraped off into a sintered funnel and the methyl esters are extracted using hot chloroform (4 x 10 ml). The chloroform extract is then evaporated to dryness using a gentle stream of nitrogen.

Step 5 - GC analysis of the hydroxy FAME's

The hydroxy fatty methyl esters from step 4 are analysed by gas chromatography. In the original report, packed GC coloumns were used. The analyst is refered to the recommended GC conditions of the GC supplier. The analysis of FAME's is well known and most GC equipment suppliers have standard application notes. Examples from Supelco Inc. USA are:

- 37 Component FAME Mix on Omegawax 250 Column (Capillary GC)
- 37 Component FAME Mix on PAG Column (Capillary GC)
- 37 Component FAME Mix on SP-2380 Column (Capillary GC)
- 37 Component FAME Mix on SP-2560 Column (Capillary GC)

For quantitative determinations, standard solutions of methyl ricinoleate and methyl 2-hydroxydocosanoate in toluene are made up (weight ratios 1:2, 1:1, 2:1 etc.), generally 5 concentration levels are used to draw a calibration curve, each point being the average of three injections. The standard solutions are injected and response factors of methyl ricinoleate and methyl 2-hydroxydocosanoate determined by plotting peak area ratio against weight ratio.

The methyl esters are injected and the peak area ratios of methyl ricinoleate and methyl 2-hydroxydocosanoate are recorded. Weight ratios can be determined from the response factors.

An alternative method is to perform the analysis on silylated hydroxy fatty acid methyl esters. This involves making up standard solutions as before and converting them into silyl derivatives. Response factors are obtained for calculation of the weight ratios. Silylation can be performed using either (i) hexamethyldisilazine/ trimethylchlorosilane / pyridine, or (ii) n-trimethylsilylimidazole.

The calculation of % PGPR in chocolate is made with the following formula :

% PGPR in chocolate = W1 x W2/W3 x 100/W4 x W5

where,

W1 = weight ratio of methyl ricinoleate/methyl 2-hydroxy-docosanoate (step 5).
W2 = weight of standard used (step 2) in mg.
W3 = weight of extracted fat used (approx. 500 mg, step 2).
W4 = % of ricinoleate present in PGPR (see below).
W5 = % of lipid extracted from chocolate (step 1).

Ideally, the ricinoleate content, W4, should be determined on the PGPR used in the manufacture of chocolate under test by the method described, except that the isolation step of the fat and PGPR (step 1) is omitted, and W3 becomes - weight of hydrolysed PGPR used (approx. 10 mg).

In the case that the PGPR used by the chocolate manufacturer may not be available, it is recommended that the ricinoleate content of PGPR (W4) be taken as 73%.

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Determination of the Precision of Analysis

Using the above method a series of measurements were carried out in order to determine the method's precision for PGPR analysis in Chocolate. An experimental milk chocolate was made containing 0.267% PGPR, as calculated from the recipe. The chocolate was extracted six times and each extract analysed randomly and at least twice. A total of nineteen determinations were made from the six extracts. From the results the mean value for PGPR content was calculated at 0.277% with a standard error of 0.0248. As well, the recovery of PGPR was found to be 65 - 70%. The relative precision for PGPR determination in chocolate is given as +/- 16.6%.

2.4 NMR

According to the same 1986 study conducted at Unilever Research Laboratory (12), nuclear magnetic resonance spectroscopy was used to characterize three different batches of PGPR. Batch codes were (49-8-26),(37-8-25),(35-8-24) dated 12/12/85. The purpose of the study was to determine batch to batch variations of production at Zwijndrecht NL. High resolution 13C NMR found the three batches to be essentially similar.

The experiments were conducted by relating all shifts to the terminal methyl shift (100% intensity) at 14.035 ppm. This is common to medium and long chain fatty acids characteristic of all PGPR emulsifiers. The three batches were compared on this basis and it is stated that while minor differences were apparent, there were no differences which would be significant in terms of the gross composition.

3. STABILITY OF PGPR

For many years, PGPR has been successfully used in chocolate confectionery as a viscosity improver (14) and in the baking industry as a pan release agent (15). It has been stated to be synergistic to lecithin and has proved very useful in modifying the yield value of high viscosity chocolates (6), cf.annex part 3. Most analytical studies have only been concerned with the characterization of the pure product and results of stability studies in final products is not prevalent in literature. Indeed, any knowledge on PGPR stability is most likely to be within the confines of industrial confidentiality. One patent has been found where good storage and heat stability of the pure product was cited (16). This was a 1995 Henkel patent for cosmetic and pharmaceutical applications.

3.1 Pure Product

Stability tests of physical and chemical parameters listed below, table 2, were performed between 1986 to 1988 over a 32 month period. This was carried out at Quest Zwijndrecht. PGPR was held at a constant temperature of 15°C in a 200 kg drum. No significant change can be observed from these results. Indeed the stability of PGPR for these parameters is shown to be excellent.

Table 2: Stability test of physical and chemical parameters of PGPR at 15°C

Time / months	0	10	18	27	32
Acid Value	2.6	2.7	2.8	2.5	2.1
Iodine Value	92	91	88	90	86
Saponification Value	185	183	183	186	188
Refractive Index 65°C	1.4639	1.4639	1.4638	1.4640	1.4639
Hydroxy Value	86	84	85	86	88
Peroxide Value	1.3	1.7	1.2	2.5	3.5

Another observation was reported by a Quest customer who had a sample stored over several years. The peroxide value of this sample was 4.7. Fresh samples are generally 0 - 1.0. However, for this same sample the acid value was only 1.2.

3.2 In Chocolate Confectionery Products

Using the GC analytical procedure mentionned previously, 2.3, the analysis of PGPR was carried out in storage trials of three milk chocolates, Hershey, Cadbury and a URL Colworth sample (13). Results are reported on table 3.

Table 3: Stability Test of PGPR in Chocolate

Chocolate	PGPR content %	1 month	4 months	5 months	9 months	12 months	16 months
Hershey Milk	0.360	0.37	–	0.39	–	0.33	–
Cadbury Milk	0.188	0.20	–	0.18	–	0.20	–
URL Colworth	0.267	0.28	0.26	–	0.29	–	0.24

The Hershey sample was dated Sept. 1976 and purchased on the UK market on 30/11/77. The Cadbury sample came from a US moulding dated 27/10/77. The URL Colworth sample had the code 140 3050 batch A2 x 35. A known concentration of PGPR was added to the chocolates: Hershey chocolate contained 0.360%; Cadbury chocolate contained 0.188%; and the URL chocolate contained 0.267%. The Hershey and Cadbury chocolates were spiked with the PGPR by adding it to the molten chocolate and stirring for one hour.

The chocolates were analysed using the methylation procedure previously described, part 2.3. The differences observed between PGPR content after storage and the actual amounts added to each of the chocolates are in all cases within experimental error. As the method for determining PGPR relies on the determination of methyl ricinoleate, it is impossible to draw conclusions that PGPR has not been hydrolysed on storage but only that the ricinoleate species (esters and free acid) have not been converted into non-ricinoleate compounds.

4. GENERAL CONCLUSION

An extensive overview of analytical methodologies applied to PGPR has been presented with specific attention given to gas chromatographic analysis. The cited methodology for the determination of ricinoleate ester has been used to study the storage stability of chocolate containing PGPR over a 16 month period. Stability data of physical and chemical parameters of PGPR over 32 months have also been presented. Under the test conditions employed, stability for both ricinoleate ester, physical and chemical parameters are shown to be excellent.

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ANNEX PART 1 - Admul WOL Product Information Sheet

ADMUL WOL 1403

Product Code: 5Z00493 H55MS

General Description

ADMUL WOL 1403 is a mixture of the partial esters of polyglycerol with linearly interesterified castor oil fatty acids.
The polyglycerol moiety is predominantly di-, tri- and tetraglycerol.

Physical Form	: liquid
Appearance	: viscous, light brown coloured liquid
EC nr	: E 476

Application

Recommended in the following applications:
- viscosity modifier in chocolate and cocoa products
- water-in-oil emulsifier in tin greasing emulsions
- water-in-oil emulsifier in low and very low fat spreads and dressings

Specification

Acid Value	: max. 6
Iodine Value	: 72 - 103
Saponification Value	: 170 - 210
Moisture	: max. 1 %
Refractive Index at 65 C	: 1.4630 - 1.4665
OH Value	: 80 - 100

Packaging & Storage

ADMUL WOL 1403 is packed in 200 kg open head drums.
The product is also available in 20 kg plastic containers (32 containers per pallet) and 50 kg open head drums (16 drums per pallet).
It is advised that the product is stored under dry and cool (below 25 °C) conditions.

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ANNEX PART 1 - Admul WOL Product Information Sheet

ADMUL WOL 1403 (5Z00493) (05/27/97)

01.IDENTIFICATION

Manufacture	: Quest International Nederland B.V., Lindtsedijk 8, 3336 LE Zwijndrecht The Netherlands. Telephone 078-6109920
Product name	: **ADMUL WOL 1403**
Chemical name	: Polyglycerol esters of interesterified ricinoleic acid.
Quest code	: 5Z00493
Uses	: Food emulsifier, release agent.

02.COMPOSITION

Polyglycerol-polyricinolate, E 476	: 100 %

03.HAZARD INFORMATION

Health hazards	: No harmfull effects have been identified
Physical-chemical hazards	: None identified.
Environmental hazards	: None identified.

04.FIRST AID MEASURES

Ingestion	: Rinse mouth with water. Drink at least 250ml of milk or water
Skin contact	: Remove contaminated clothing. Wash affected areas with plenty of water and soap. In case of persisting irritation or any sign of tissue damage seek medical help
Eyes contact	: Flush eyes with copious amounts of water. If irritation persists, seek medical help.

05.FIRE FIGHTING MEASURES

Extinguishing media	: CO2, foam, dry chemical.
Special measures	: None.
Special protective equipment	: None.

06.ACCIDENT RELEASE MEASURES

Absorb the material in a porous inert material such as earth, sand or vermiculite.
Collect and dispose of in accordance with local regulations.

Large spillages should be contained by the use of sand or other inert materials, transferred to a suitable vessel and disposed of in accordance with local regulations.

07.STORAGE AND HANDLING PROCEDURES

Storage	: Store in a cool, dry place Keep away from heat and flame.
Shelf-life	: 9 months from date of manufacture in unopened original Quest packaging
Special precautions	: none.

ANNEX PART 1 - Admul WOL Product Information Sheet

08.PERSONAL PROTECTION

General precautions	: Handle in accordance with good occupation hygiene and safety practices. Avoid contact with skin and eyes.
Ventilation	: General.
Eye protection	: Safety glasses.
Skin protection	: Gloves.

09.PHYSICAL AND CHEMICAL PROPERTIES

Appearance	: viscous, light brown liquid
Odour	: neutral
Flash point (cc)	: > 200 °C
Boiling point	: decomposes
Vapour pressure	: < 5mBar at 25 °C
Specific gravity	: 0.95 (20 °C)
Solubility (water)	: practically insoluble in water, but forms emulsion
Other data	: none

10.STABILITY AND REACTIVITY

Stability	: Stable under normal conditions.
Materials to avoid	: Strong oxidising agents.
Hazardous combustion or decomposition products	: Not to be expected.
Hazardous polymerisation	: Will not occur.

11.TOXICOLOGICAL INFORMATION

Polyglycerol polyricinolate has been studied in acute, subacute and chronic studies using various species. Acute toxicity is low (L.D.50 over 10g/kg BW). The enlargement of the liver and kidneys observed in long terms tests was not accompanied by any lesions detectable by histopathology.

No significant adverse departures from control patterns in a number of biochemical parameters were detected in a study using human volunteers who ingested the material at rates of 5 and 10g/day for two weeks. An ADI of 0-75 mg/kg BW has been set for this material (WHO Food additives Series, no.5, 1974, p246-253).

12.ECOLOGICAL INFORMATION

No ecological data are available for this product.

13.DISPOSAL PROCEDURES

Waste disposal method	: Sanitation, landfill, or incineration in accordance with local regulations
Treatment of empty containers	: As usual

14.TRANSPORT INFORMATION

CEFIC-code	: Not assigned.
UN-number	: Not assigned.
Hazard label nr.	: Not assigned.
ADR Class	: Not assigned.

ANNEX PART 1 - Admul WOL Product Information Sheet

Kemler-code	: Not assigned.
IMDG-code	: Not assigned.

15.REGULATORY INFORMATION

Label information	: Not classified as dangerous.

16.OTHER INFORMATION

E-number	: E 476
US.FDA.nr.	: not available

HBAS-3HGB9Q

ANNEX PART 2 - Standard Methods of Analysis

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 144 Rev.: 1
	Page: 1 of 4
WATER CONTENT by KARL FISCHER TITRATION	Supersedes: Q 44

1. SCOPE AND FIELD OF APPLICATION

This method is applicable for the determination of the water content of emulsifiers and oils and fats.

2. DEFINITION

The water content, expressed as percentage by weight, is the quantity of water calculated from the iodine, reacted under the conditions of this method.

3. PRINCIPLE

The water of the sample is titrated by means of the Karl Fischer method, using a modified, pyridine free reagent. This reagent consists of sulphur-dioxide, iodine and a base. The water of the sample reacts with the sulphur-dioxide and the iodine of the reagent and the water content is calculated from the amount of iodine consumed.
The end-point of the titration is detected electrometrically by means of the "dead-stop" end-point technique.

4. REAGENTS AND PRODUCTS

Methanol - Anhydrous, p.a.
Chloroform - Anhydrous, p.a.
Karl Fischer solution Merck 9241 - (Concentrated solvent).
Karl Fischer solution Merck 9233 - Reagent.
Sodium tartrate standard - Dihydrate, p.a.

Solvent mixture - Mix equal volumes of anhydrous methanol, chloroform and Karl Fischer solution 9241.

5. APPARATUS

Karl Fischer Titrator - Metrohm type 633 with dosimat 645with double platinum electrode and titration vessel, which can be heated up to 65°C. (or equivalent apparatus).
Syringe 2 ml - Without a needle, prepared (if necessary) to add the sample to the titration vessel.
Syringe 25 µl - with needle.
Weighing spoon with protective tube.

STANDARD METHOD
OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 144 Rev.: 1
	Page: 2 of 4
WATER CONTENT by KARL FISCHER TITRATION	Supersedes: Q 44

6. PROCEDURE

Sample preparation

Liquid samples and solid samples in powder or flake form should be well homogenized. Other solid samples should be heated at a temperature of approximately 10°C above their melting point and then homogenized. (Note 1.)

Determination

Add 30 ml of the solvent mixture into the titration vessel, which must be dry and clean.
Close the switch in the indicator circuit and titrate immediately and quickly the Karl Fischer reagent to the end-point. (Repeat if necessary).

In case of liquid samples: Add 1 g of the sample to the titration vessel by means of a syringe which is weighed before and after use. (Note 1)

In case of (homogenized) solid samples: Use a weighing spoon to introduce the sample into the titration vessel.
Titrate the contents with the Karl Fischer reagent and record the volume of reagent used. (Note 2)

Standardization

Determine the water content of the sodium tartrate, 2 H2O (W%) by drying in an oven at 105°C to constant weight. It should be approx. 15.65%
Add 30 ml solvent to the titration vessel and titrate with the Karl-Fischer reagent to the end-point.
Weigh approximately 250 mg of the sodium tartrate standard into the titration vessel, noting the exact weight used.
Titrate immediately with the Karl Fischer reagent to the end-point. Record the volume of reagent used. (Note 3)

7. CALCULATION

Calculate the factor (F) of the reagent with the formula:

$$F = \frac{m \times W}{100 \times a}$$ (calculate to 2 decimals).

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 144 Rev.: 1
	Page: 3 of 4
WATER CONTENT by KARL FISCHER TITRATION	Supersedes: Q 44

Where:
m = the mass of the weighed sodium tartrate standard in mg.
W = the water content (in %) of the sodium tartrate.
a = ml. of reagent used to titrate the standard.

Calculate the water content of the sample with the formula:

$$(\%)\ \text{Water} = \frac{a \times F}{10 \times m}$$

Where:
a = ml reagent used to titrate the sample.
F = standardization factor for reagent. (in mg water / ml).
m = mass of the weighed sample in grams.

8. ACCURACY

When water content is < 1 %, it must be reported to 2 decimals. Repeatability: 0.02 %

When water content is > 1 %, it must be reported to 1 decimal. Repeatability approx. 5 % of the value found.

9. REMARKS

Note 1 : For most emulsifiers 1 g will do. For oils and fats, containing 0.04% or less, 10 g of sample is preferred.

Note 2 : In case of hard fat or hard emulsifiers it is advantageous to heat the reaction vessel with water or oil to approx. 65°C.
When a heating device is not available, proceeds as follows:
Weigh a quantity of the sample in a graduated flask and dissolve in a suitable solvent. Make up to volume and add a quantity, containing "m" grams of the sample, to the titration vessel. Perform a blank to correct for the water present in the solvent.

Note 3 : For daily standardization of the reagent, direct addition of pure water by means of a micro-syringe can also be applied. The factor F is then calculated by the formula:
F= µl of pure water / ml of reagent used.It has been proved that the variation in the factor F is within ± 0.02 (mg/ml) when determined in this way.

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 144 Rev.: 1
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WATER CONTENT by KARL FISCHER TITRATION	Supersedes: Q 44

10. REFERENCES

Merck, Karl Fischer leaflets.
UMA A.IIc.15
FAO FOOD AND NUTRITION PAPER 5 Rev.1

UNCONTROLLED DOCUMENT

This method is up to date at: 23 July, 1997
It will not be replaced by a new version automatically

Approved by: W.L. Punt. date: 04-03-1994
Authorized by: H. Bas. date: 14-03-1994

STANDARD METHOD
OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 136 Rev.: 1
	Page: 1 of 2
REFRACTIVE INDEX	Supersedes: Q 36

1. SCOPE AND FIELD OF APPLICATION

The determination of the refractive index of a oil, fat or emulsifier is used for their identification as well as for the establishment of their grade of purity as a complemen-tary test.

2. DEFINITION

The refractive index is defined as the ratio of the velo-
city of light of defined wave lenght (the mean D-line of sodium in air to its velocity in the liquid sample. The refractive index is expressed as N_D^t, where t stands for temperature in °C and D represents the D-line of sodium light. (Note 1)

3. PRINCIPLE

The refractive index of a liquid sample is measured by means
of a suitable refractometer at constant temperature and using a monochromatic light source, emitting monochromatic light by means of a sodium vapour lamp.

4. REAGENTS AND PRODUCTS

Used for cleaning.
Cellulose swabs - Pur-Zellin 143 212/2, ex Hartmann.
Solvent - Hexane or acetone.

5. APPARATUS

Abbe refractometer - Bellingham + Stanley Limited, type 60/70. (or equivalent)
Water bath - Thermostatically controlled and equipped with a circulation pump, capable of maintaining the temperature to within ± 0.2 °C.

6. PROCEDURE

Set the temperature of the thermostatted water bath to the desired temperature and allow to stabilize for at least 15
min. Control the temperature of the water issued from the refractometer.
Lower the movable half of the prism to a horizontal position
Clean the two prism surfaces by wiping them with a swab. (Use some solvent if necessary).
Place 2 or 3 drops of the clear and dry sample (Note 2) on the prism.
Fix the movable half of the prism to the fixed half.
Allow the instrument to stabilize for a few minutes.
Read the refractive index of the sample ditectly on the scale by setting the boundary line between the dark and light field exactly on the point of intersection of the cross lines.

STANDARD METHOD
OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 136 Rev.: 1
	Page: 2 of 2
REFRACTIVE INDEX	Supersedes: Q 36

Estimate the fourth decimal.
Clean the instrument immediately after reading.

7. EXPRESSION OF RESULTS

The results should be reported in 4 decimals and expressed as: $N_D^{x°C}$ = 1.xxxx
For instance: N_D^{65}= 1.4622

Another allowed expression for oils, fats and emulsifiers is: N_D^{65}= 622
(The three decimals coming after 1.4)

8. ACCURACY

The Repeatability of the determination is: 0.0002 units.

9. REMARKS

Note 1 : The B + S refractometer is used with white light.
Readings relative to the Sodium D-line are generated by the instrument.

Note 2 : Samples that are not clear may be dried over anhydrous sodium sulphate and filtered through a fluted filter paper.

10. REFERENCES

UMA A.IIIb.20
Food Chemical Codex III

UNCONTROLLED DOCUMENT

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STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 173 Rev.: 2
	Page: 1 of 3
VISCOSITY of DATEM	Supersedes: 173 rev. 1

1. SCOPE AND FIELD OF APPLICATION

This method serves for the determination of the viscosity of DATEM by means of a Haake rotation viscometer.

2. DEFINITION

The viscosity is a measure of a material's resistance to flow and is expressed as mPa.s, determined under the con-
ditions of the test.

3. PRINCIPLE

The viscosity is determined by a rotation viscosimeter.
The DATEM to be tested is placed in the space between two cylinders, one placed inside the other. When one of these cylinders is rotated, the liquid ondergoes a tangential force from which the viscosity can be calculated.

4. REAGENTS AND PRODUCTS

5. APPARATUS

Viscometer - Haake Viscotester VT 181, provided with a temperature jacket and a frequency converter.
Heating Circulator
Viscosity sensor system SV II FL.

6. PROCEDURE

Heat the sample to about 60 °C, and stabilize the sample for half an hour at 40 °C. (Note 1).
Homogenize the sample and carefully pour the sample into the cup to the required level, avoiding aeration.
Load the cup into the temperature jacket of the viscotester, onto the SV II FL-bob is already fixed.
Allow the sample to stay for half an hour at the temperature of interest. (Note 2)
Set the frequency converter to attenuation number 8.
Switch the viscotester on and rotate the bob for half a minute. Switch the attenuation number to 4 and rotate until the reading is stable. (Note 3)
Note the reading and attenuation number.

7. CALCULATION

Calculate the viscosity with the formula:

Viscosity (in mPa.s) = F x U x S. (Note 4)

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 173 Rev.: 2
	Page: 2 of 3
VISCOSITY of DATEM	Supersedes: 173 rev. 1

Where:
F = factor for the viscosity sensor system.(Given by Haake)
U = Attenuation number. (speed factor)
S = Scale deflection. (reading)

For the viscosity sensor system SV II FL, the formula may be simplified:

Viscosity (in mPa.s) = 49.5 x U x S.

Report viscosity and temperature of measurement.

8. ACCURACY

Sufficient agreement in results between different laboratories can only be expected if they use the same instrument
under strictly identical conditions. To compare different samples of the same product, e.g. comparison of a sample with a "standard" value in process control, acceptable results are obtained. The difference between the results of a determination carried out in duplicate at the same time, or within the same day, by one and the same person must not be more than 5% of the value found.

9. REMARKS

Note 1 : The ADMUL DATEM 1913 should be tested at 20 °C, without any preheating. So start the determination
with: Homogenize the sample.....
Semi-liquid samples, which contain solid particles, should be stabilized as described.

Note 2 : Most samples of (semi-Liquid) DATEM are determined at 25 °C.

Note 3 : If the reading exceeds 150, switch to a higher at- tenuation number. (Best readings are between 50 and 125)

Note 4 : Viscosity is expressed as mPa.s (or in Pa.s) The expression in P (poises) or cP (centipoises) are incorrect, but they may still be used. (P and cP are old cgs units)
Factors for converting cgs units to SI units are:

1 P = 0.1 Pa.s
1 cP = 1 mPa.s

Note 5 : DATEM behave as (almost) Newtonian fluids. This means that the viscosity is a linear relationship
between shear rate and shear stress. (So is inde-
pendent to attenuation number of the viscotester)
This should be checked for samples, other than those known to be Newtonian.

10. REFERENCES

UMA A.IIIc.25

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 173 Rev.: 2
VISCOSITY of DATEM	Page: 3 of 3
	Supersedes: 173 rev. 1

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STANDARD METHOD
OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 141 Rev.: 1
SAPONIFICATION VALUE of oils, fats and emulsifiers	Page: 1 of 2
	Supersedes: Q 42

1. SCOPE AND FIELD OF APPLICATION

This method serves for the determination of the saponification value of oils, fats and (most) emulsifiers. (Note 1)

2. DEFINITION

The saponification value, which is related to the molecular weight of the sample, denotes the number of mg potassium hydroxide, which is required to saponify 1 g. of the sample.

3. PRINCIPLE

The saponification value is determined by completely saponifying the sample with a known amount of potassium hydroxide, of which the excess is determined by titration.

4. REAGENTS AND PRODUCTS

Hydrochloric acid - 0.5 M aqueous solution, accurately standardized
Potassium hydroxide - 0.5 M solution in ethanol
Phenolphtalein - 1% solution in ethanol

5. APPARATUS

The usual laboratory equipment.

6. PROCEDURE

Weigh, to the nearest 0.1 mg, approx. 4 g of the sample into a 300 ml conical flask. (Note 2)
Add, accurately measured, 48.00 ml. 0.5 M ethanolic potassium hydroxide solution. Add a few anti-bumping granules and
attach a reflux condenser to the flask. Heat, and as soon as the ethanol boils, occasionally shake the flask until the sample is completely dissolved.
Boil the solution for one hour.
Add a few drops of phenolphtalein indicator and slowly titrate the hot soap solution obtained with 0.5 M aqueous hydrochloric. (a ml)
Carry out a blank determination upon the same quantity of
potassium hydroxide solution at the same time and under the same conditions. (b ml)

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 141 Rev.: 1
SAPONIFICATION VALUE of oils, fats and emulsifiers	Page: 2 of 2 Supersedes: Q 42

7. CALCULATION

Calculate the saponification value (SV) with the formula:

$$\text{Saponification value} = \frac{56.1 \times t \times (b - a)}{m}$$

Give the result correct to 1 mg/g.

Where:

t = molarity of the hydrochloric acid used.

a = ml. 0.5 M hydrochloric acid used in the test.

b = ml. 0.5 M hydrochloric acid used in blank.

m = the mass of the weighed sample in grams.

8. ACCURACY

The difference between the results of a determination carried out in duplicate at the same time, or within the same day, by one and the same person must not be more than 1% of the value found.

9. REMARKS

Note 1 : This method may be used for most emulsifiers. Some emulsifiers, however, need different methods. For Glycerol Lacto Palmitate (=GLP) use method nr 151; for Stearoyl Lactylic Acid esters and their salts (=SSL\CSL) use method nr 161 and for DATEM (=Diacetyl Tartaric Acid esters of Mono- and Digly-cerides) use method nr 171.

Note 2 : Since the coefficient of expansion of ethanol is relatively great, a blank should always be carried out at the same time, ensuring that the temperature of the ethanolic potassium hydroxide solution during measuring is the same for the blank and the determination.

10. REFERENCES

FCC III, p 159.

UNCONTROLLED DOCUMENT

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Authorized by: H. Bas. date: 13-06-1994

STANDARD METHOD
OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 122 Rev.: 1
HYDROXYL VALUE of Emulsifiers	Page: 1 of 3
	Supersedes: Q 22

1. SCOPE AND FIELD OF APPLICATION

The method given in this instruction is applicable to the determination of the hydroxyl value of emulsifiers, oils and fats. (Note 1).

2. DEFINITION

The hydroxyl value is the number of mg of potassiumhydroxide necessary to neutralize the amount of acetic acid, that is combined with 1 g of the sample by acetylation.

3. PRINCIPLE

The hydroxyl-compounds are converted into their acetates with acetic acid in pyridine. The pyridine promotes a quick and complete termination of the acetylation.
The excess of acetic anhydride is destroyed with water and determined as acetic acid by titration, together with the acetic acid, formed in the reaction.

4. REAGENTS AND PRODUCTS

Pyridine - pure.
Acetic anhydride. - pure.
Acetylating mixture. - Dissolve 25 ml acetic anhydride in the pyridine in a 100 ml volumetric flask, dilute to volume with pyri-dine and mix. (Note 2)
Ethanol - 96 % v/v; neutralised to phenolphtalein end-point.
Potassium hydroxide - 0.5 M solution in ethanol, accurately standardized.
Phenolphtalein - 1 % solution in ethanol.
Distilled water.

5. APPARATUS

Acetylation flasks (Note 3).
Oil, water or steam bath, kept at 95 - 100 °C.
Condensers.

6. PROCEDURE

Weigh accurately the appropriate amount of sample, according to the expected hydroxyl value, into the acetylation flask.
(Note 3).
Add an accurately measured amount of the acetylating mixture. The quantities to be taken are shown below. (Note 4.
Perform a blank determination simultaneously in the same way and with the same amount of acetylating agent.

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Hydroxyl value to be expected	Approx. amount of sample to be weighed.(g)	Accuracy of weighing (mg)	Volume of acetylating agent to be added (ml).
10 - 100	2.0	10	5
100 - 150	1.5	1	5
150 - 200	1.0	1	5
200 - 250	0.75	1	5
250 - 300	1.2	1	10
300 - 400	1.0	1	10
> 400	0.5	1	10

Mix and place the flasks in the bath, kept at 95 - 100 °C, under reflux condensers.
The flask must not be immersed more than 1 cm deep in the bath.
After 1 hour, remove the flask from the bath, cool and add 1 ml of distilled water (Note 5) through the condenser and shake. (Note 6)
Place the flask again in the bath for 10 minutes in order to convert the anhydride to acetic acid quantitatively and to decompose anhydrides of fatty acids or mixed anhydrides which may be formed.
Allow the flask with contents to cool to room temperature, add 5 ml (neutralised) 96% ethanol, rinsing the condenser and the neck of the flask, and titrate with 0.5 M ethanolic potassium hydroxide solution (Note 7), using phenolphtalein as indicator. (Note 8)

7. CALCULATION

Calculate the hydroxyl value with the formula:

$$\text{Hydroxyl value} = \frac{(b - a) \times t \times 56.1}{m} + AV$$

Where:
a = ml. of 0.5 M potassium hydroxide used for the sample.
b = ml. of 0.5 M potassium hydroxide used for the blank.
t = molarity of the potassium hydroxide solution used.
m = the mass of the weighed sample in grams.
AV= Acid Value, determined according to Quest standard method of analysis 148 rev. 1.

8. ACCURACY

The difference between the results of a determination carried out in duplicate at the same time, or within the same day, by one and the same person must not be more than 1 mg KOH /g + 5 % of the value found.

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9. REMARKS

Note 1	:	This method for the determination of the hydroxyl content does not differentiate between OH-groups in the fatty acid chains and those present in mono- or diglycerides. Hence partially hydrolysed fats and emulsifiers may show a high hydroxyl value, without containing hydroxy acids.
Note 2	:	The acetylating agent must be kept in the dark, free from moisture and from carbon dioxide and other acidic vapours. If, owing to exposure to light, a slight discoloration occurs the solution may still be used.
Note 3	:	A 100-ml glass-stoppered Erlenmeyer flask will do.
Note 4	:	The excess of acetylating reagent should be at least 85 % of the blank value.
Note 5	:	When 10 ml of acetylating reagent was used, add 2 ml of distilled water.
Note 6	:	If the solution goes turbid, add a little pyridine to clear it.
Note 7	:	The hydroxyl value of some emulsifiers, like polyglycerol polyricinoleate (PGPR), is difficult to be determined. The end-point of the titration should be approached as fast as possible and is reached when the pink colour does not disappear at once, but last for 1 or 2 seconds. The determination of the hydroxyl value of PGPR can only be performed well, by a skilled person
Note 8	:	If the liquid is dark brown, use alkali-blue 6B indicator. (2 % solution in 96 % v/v ethanol).

10. REFERENCES

UMA A.IIc.5
NEN 6342.
FAO Food and Nutrition Paper 5 rev.2 (page190)

UNCONTROLLED DOCUMENT

This method is up to date at: 23 July, 1997
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Approved by: W.L. Punt. date: 03-12-1995
Authorized by: H. Bas. date: 04-12-1995

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 148 Rev.: 1
ACID VALUE of emulsifiers	Page: 1 of 2 Supersedes: Q 48

1. SCOPE AND FIELD OF APPLICATION

This determination of the acid value is, not only, applicable to most emulsifiers, but also to oil and fat. Some types of emulsifier, like DATEM, GLP, SSL and CSL, are analyzed by a different method.

2. DEFINITION

The acid value is the number of mg potasium hydroxide, which is required to neutralize 1 g of the sample.

3. PRINCIPLE

The acids are neutralized with potassium hydroxide.

4. REAGENTS AND PRODUCTS

Solvent - Mix equal volumes of ethanol and cyclohexane.
Potassium hydroxide - 0.1 M solution in ethanol, accurately standardized.
Phenolphthalein - 1% solution in ethanol.

5. APPARATUS

The usual laboratory equipment.

6. PROCEDURE

Weigh, to the nearest 1 mg, approx. 10 g of the sample into a 200 ml conical flask. (note 1)
Dissolve, by gently heating in a waterbath, in 50 ml. of the solvent, which has been neutralized shortly before use by titration with 0.1 M potassium hydroxide solution, using phenolphthalein as indicator.
Titrate, while still warm, with 0.1 M potassium hydroxide solution. (Titrate to the first, overall, colour change, which lasts at least 10 seconds.)

7. CALCULATION

Calculate the acid value (AV) with the formula:

$$\text{Acid Value} = \frac{56.1 \times t \times a}{m}$$

Express the results as indicated under "accuracy"

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Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 148 Rev.: 1
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ACID VALUE of emulsifiers	Supersedes: Q 48

Where:
t = molarity of the potassium hydroxide solution used.
a = ml. 0.1 M potassium hydroxide used for the sample.
m = the mass of the weighed sample in grams.

8. ACCURACY

The accuracy is about 2% of the value determined.
The acid values obtained should be expressed as follows:
Acid values less than 1, to the nearest 0.01
Acid values higher than 1 to the nearest 0.1

9. REMARKS

Note 1 : Liquid samples and solid samples in powder or flake form should be well homogenized. Other solid samples should be heated at a temperature of approximately 10°C above their melting point and then be homogenized.

10. REFERENCES

FAO FOOD AND NUTRITION PAPER 5 REV. 1 (page 175.)
UMA A.IIIb. 1

UNCONTROLLED DOCUMENT

This method is up to date at: 23 July, 1997
It will not be replaced by a new version automatically

Approved by: W.L. Punt. date: 23-03-1994
Authorized by: H. Bas. date: 28-03-1994

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL

Food Ingredients Division

Title:	Code: 123 Rev.: 2
IODINE VALUE (IV) (Wijs' method)	Page: 1 of 4 Supersedes: 123 rev. 1

1. SCOPE AND FIELD OF APPLICATION

This method for the determination of the iodine value based on the Wijs procedure is applicable to oil, fat and oil- and fat-related products. (like emulsifiers. Note 1)

2. DEFINITION

The iodine value denotes the percentage by weight of halogen calculated as iodine (I), absorbed under the conditions of the test (Wijs' test).

3. PRINCIPLE

The product to be investigated is treated with an iodine monochloride solution (Wijs' solution). After addition of the halogen has taken place, the excess of iodine monochloride is determined by titration with a sodium thiosulphate solution.

4. REAGENTS AND PRODUCTS

Potassium iodine - 10% aqueous solution, free from iodine and iodate.
Sodium thiosulphate - 0.1 mol/l aqueous solution, accurately standardized.
Solvent - cyclohexane. (Note 2)
Starch indicator - stir 10 g soluble starch and 1.25 g sali-cylic acid in 30 ml water and pour the mixture into 1 litre boiling water.
Keep the solution just boiling during 3 min and cool. The addition of the salicylic acid makes the solution stable a long time.
Wijs' solution - c(ICl)=0.1 mol/l. Merck 9163 or equivalent.
Note 3.
SDS solution - 1% aqueous solution of Sodium Dodecyl Sulfate, freshly prepared. Merck 822050 or equivalent. (only recommanded for the IV determination of monoglycerides).

5. APPARATUS

The usual laboratory equipment.

6. PROCEDURE

The amount of sample to be weighed depends on the iodine value and is given in the table below (Note 4).

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Title:	Code: 123 Rev.: 2
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IODINE VALUE (IV) **(Wijs' method)**	Supersedes: 123 rev. 1

Iodine value	Amount of substance to be weighed (g.)	Weigh to the nearest (mg.)
0-5	3.0 (Note 5)	10
5-20	1.0	1
20-60	0.34	0.1
60-80	0.25	0.1
80-130	0.15	0.1
>130	0.10	0.1

Dissolve the weighed amount of substances in a conical flask in 30 ml solvent. Add 25.00 ml Wijs' solution from a bu rette. Close the flask, mix and allow to stand in the dark at about 20°C for 2 h.
After standing, add 20 ml potassium iodide solution and approximately 150 ml water. (Note 6).
Titrate with thiosulphate solution with shaking and add starch indicator towards the end of the titration.
Make a blank determination with the same quantities of reagents at the same time and under the same conditions. Note7

7. CALCULATION

Calculate the iodine value (IV) with the formula:

$$\text{Iodine Value} = \frac{12.69 \times t \times (b - a)}{m}$$

Where:
t = molarity of the thiosulphate solution used.
a = ml. 0.1 M thiosulphate solution used in test.
b = ml. 0.1 M thiosulphate solution used in blank.
m = the mass of the weighed sample in grams.

Express the results as follows:
< 100 to the first decimal place.
> 100 to half units.

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Title:	Code: 123 Rev.: 2
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IODINE VALUE (IV) **(Wijs' method)**	Supersedes: 123 rev. 1

8. ACCURACY

Repeatability: 0 - 25 : approx. 0.3 units.
25 - 100 : approx. 0.5 units.
>100 : approx. 1.0 units.

9. REMARKS

Note 1 : This method is applicable to (almost) all emulsifiers. i.e. glycerolmonoesters, Datem (even those with calciumacetate, calciumphosphate and carbonate as anti-caking agents) etc.

Note 2 : Cyclohexane is the solvent of choice. When substances are insoluble in this solvent, one might try cyclohexane:acetic acid 4:1 v:v or carbon tetrachloride. Sometimes it is possible to use the Wijs' solutions the solvent, thus omitting the addition of solvent at all. Solvents should not contain any oxidizable substances. This can be tested by shaking 1 to 2 ml of the solvent with a small amount of sulfuric acid and a droplet of a saturated potassium bichromate solution. No green discoloration may be observed.

Note 3 : In the solution absolutely no iodine trichloride should be left, since this will make the solution unstable and may cause side-reactions.

Note 4 : After the reaction, at least 70% of the halogen initially present should remain.

Note 5 : When determining the IV of Hymono's it is recommended not to exceed 1 g. of material, even when the IV is < 5.

Note 6 : Because of the strong emulsifying properties of Hymono's, the water can best be replaced by the same quantity of freshly prepared SDS solution. also in the blank, an equal quantity of the same solution should be added.)

Note 7 : Owing to the large coefficient of expansion of the reagent it is imperative that the volumes of Wijs' solution used in the test and the blank determination shall be measured at the same temperature.

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Title:	Code: 123 Rev.: 2
IODINE VALUE (IV) (Wijs' method)	Page: 4 of 4 Supersedes: 123 rev. 1

10. REFERENCES

ISO 3961.
UMA A.IIIb.14.

UNCONTROLLED DOCUMENT

This method is up to date at: 23 July, 1997
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Approved by: W.L. Punt. date: 17-10-1996
Authorized by: H. Bas. date: 17-10-1996

STANDARD METHOD
OF ANALYSIS

Quest
INTERNATIONAL
Food Ingredients Division

Title:	Code: 193	Rev.: 1
Polyglycerol-composition of (poly-)glycerol or (poly-)glycerol esters of fatty acids.	Page: 1 of 3	
	Supersedes: Q 93	

1. SCOPE AND FIELD OF APPLICATION
This method serves for the determination of the composition of polyglycerol(-esters), as well for the determination of Di-, and Triglycerol in monoglycerides.

2. DEFINITION
The polyglycerol-composition, determined by this method, is expressed as percentage of mass of each compound, calculated by "area normalization". (i.e. Total mass of all (poly-)glycerols is regarded to be 100 %).

3. PRINCIPLE
The sample is hydrolysed with hydrochloric acid. The liberated (poly-)glycerols are extracted, esterified with MSTFA, and analyzed by gas-chromatography.

4. REAGENTS AND PRODUCTS
Ethanol containing 5 % conc. Hydrochloric acid. - Make 16 ml of 32 % HCl up to 100 ml with ethanol.
Hexane - Analytical grade.
Pyridine - Analytical grade.
MSTFA - N-Methyl-N-trimethylsilyl-trifluoroacetamide)

5. APPARATUS
Magnetic stirrer with hot plate.
Rotary evaporator.
Sealable vials of 10 ml.

6. PROCEDURE
A sample of the product (approx. 100 mg) in 1.0 ml ethanol containing 5% conc. HCl is heated at 70°C for 2-3 hrs. in a sealed vial with continuous agitation.
The ethanolic mixture is cooled, diluted with 1.0 ml distilled water and extracted 4 times with 2.0 ml hexane.
The hexane phase is rejected and the residual fat-free aqueous ethanol layer evaporated to dryness at 70°C under reduced pressure.
The dried sample prepared above is dissolved in 1.0 ml dry pyridine and converted to the silylethers by the addition of 0.5 ml. MSTFA. Heat this mixture on a water bath and allow to stand for 5 minutes at ambient.
The sample is ready for analysis by GC.

000240

Suggested GC conditions: (Note 1)
Column: 1.0 m x 2 mm. I.D. glass packed with 3 % OV-101 on 100 -120 mesh Chromosorb W-HP.
Carrier gas: Nitrogen 15 ml/min.
Oven Temp.: 80°C ---> 200°C at 10°C/min., then 200°C --->

STANDARD METHOD
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Quest
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Title:	Code: 193	Rev.: 1
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350°C at 5°C/min.
Injector: 325°C.
Detector: 350°C. F.I.D.

7. CALCULATION
Calculate the composition with the formula:

$$\% a = \frac{\text{Area } a \times Fa}{\Sigma \text{ Area} \times F} \times 100$$

Where:
Area a = Area of component a , taken from the dataprocessor.
Fa = Response factor (or correction factor) for component a.
Σ Area x F = Sum of all individual areas multiplied by their corresponding correction factors.

8. ACCURACY
The difference between the results of two determinations carried out on the same day by the same operator using the same apparatus for the same sample and for constituents present in excess of 5 % should not exceed a relative figure of 3 % of the determined value, with an absolute value of 1 % For components present in amounts of less than 5 % , the repeatability diminishes progressively as the content is reduced.

9. REMARKS
Note 1: The GC-conditions suggested in this method are for analyses on packed-columns. The method can also be performed on capillary columns.
Note 2: Sometimes it is not necessary to determine the response factors. (for example for determining the polyglycerols equal or greater than heptaglycerol acc. to the EEC-specs., which are area %-related.) In the other cases calculate response factor as described below. (Note 3).
Note 3: The area % , calculated by an integrator, are not directly proportional to the percentages by weight. (i.e. different compounds have different detector responses.) That is why it is necessary to determine correction factors for each individual compound. Once established, response factors are valid for a long time. The easiest way to check if the are still valid is running a check sample with known composition. When a new check sample has to be prepared, or when the check sample gives incorrect figures, response factors have to be recalculated by running

STANDARD METHOD OF ANALYSIS

Quest INTERNATIONAL
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reference mixtures.
Response factors may be calculated by the formula:

$$Fa = \frac{\text{weight a}}{\text{Area a}}$$

If we establish the response factors for all components, we can set one factor equal to 1.00 and calculate the others relative to that one.

Note 4: When glycerol or polyglycerol has to be analysed, one can avoid the hydrolysation. The sample can then be converted to the silylethers directly by adding pyridine and MSTFA.

10. REFERENCES

FAO Food and Nutrition Paper 5 Rev.1

UNCONTROLLED DOCUMENT

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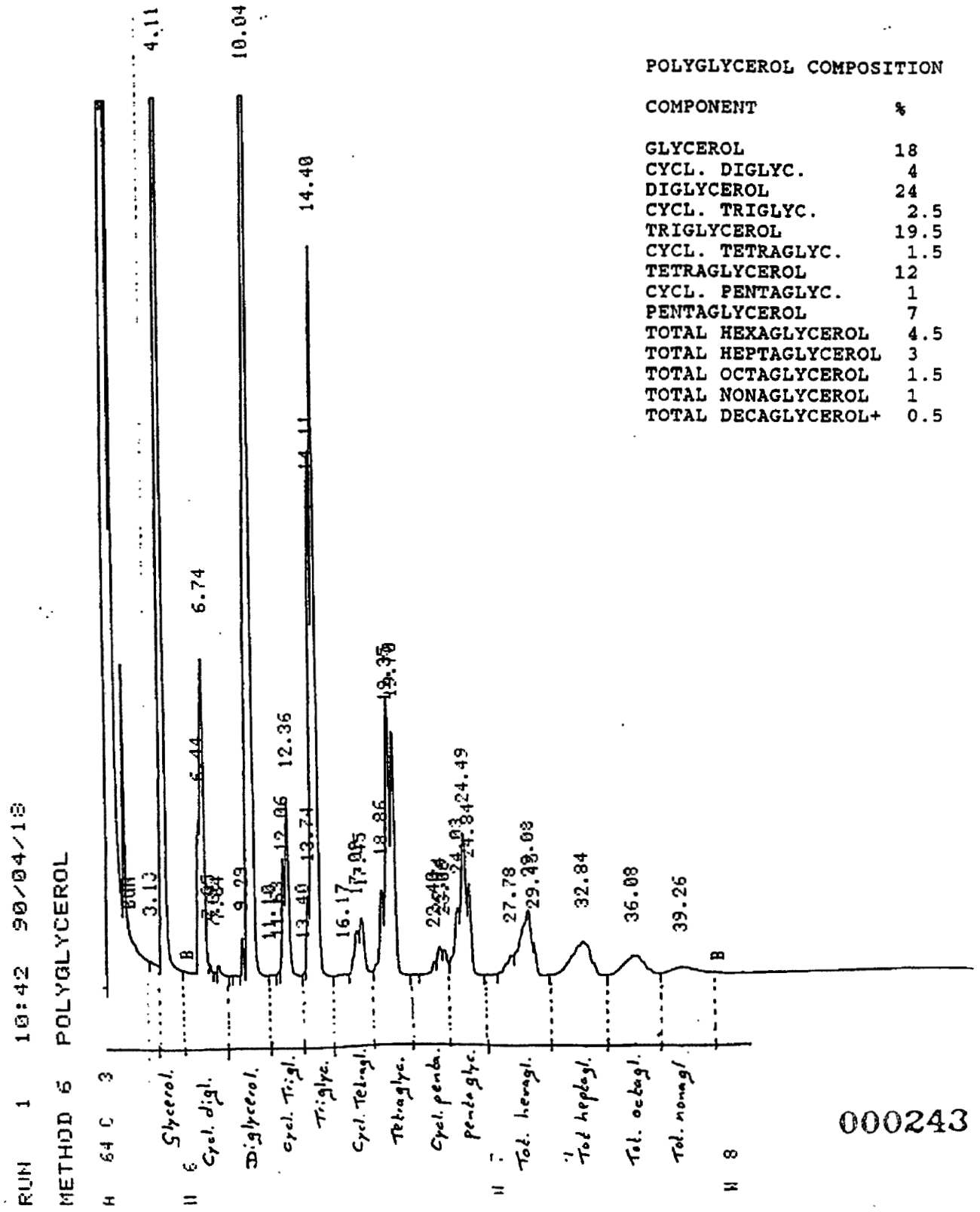
Approved by: W.L. Punt.	date: 9-02-1995
Authorized by: H. Bas.	date: 14-02-1995

PERKIN ELMER CORP. PART NO. VE 16-2038

Polyglycerol composition.



POLYGLYCEROL COMPOSITION

COMPONENT	%
GLYCEROL	18
CYCL. DIGLYC.	4
DIGLYCEROL	24
CYCL. TRIGLYC.	2.5
TRIGLYCEROL	19.5
CYCL. TETRAGLYC.	1.5
TETRAGLYCEROL	12
CYCL. PENTAGLYC.	1
PENTAGLYCEROL	7
TOTAL HEXAGLYCEROL	4.5
TOTAL HEPTAGLYCEROL	3
TOTAL OCTAGLYCEROL	1.5
TOTAL NONAGLYCEROL	1
TOTAL DECAGLYCEROL+	0.5

ANNEX I PART 3 - Viscosity Values of Chocolate containing PGPR

Table 1: Viscosity Yield Values In dark Chocolate (1)

Lechithin %	Plastic Viscosity (poise)	Yield Value (dynes/cm²)	PGPR %	Plastic Viscosity (poise)	Yield Value (dynes/cm²)
0.3	18.5	155	0.1	12.5	151
0.7	17.1	221	0.2	14.8	82
1.3	12.4	285	0.5	14.9	13

Table 2: Viscosity Yield Values in Milk Chocolate Containing 0.5% Lecithin (1)

PGPR added %	Plastic Viscosity (poise)	Yield Value (dynes/cm²)
nil	15.3	72
0.1	15.2	64
0.2	15.6	56
0.3	17.4	30
0.4	16.4	26

TAB 5

Pages 000246 - 000255 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

TAB 6

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TAB 7

Summary of Biological Studies on Polyglycerol-Polyricinoleic Acid (PGPR) Emulsifier (Admul WOL™)

October 8, 1998

000275

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SUMMARY OF BIOLOGICAL STUDIES ON A POLYGLYCEROL-POLYRICINOLEIC ACID (PGPR) EMULSIFIER

ABSTRACT

Extensive published and unpublished toxicological studies have been conducted at Unilever Research Laboratories in the United Kingdom, on the polyglycerol ester of the polycondensed fatty acids from castor oil (PGPR). These studies reflect the state of the art of toxicology and toxicological test guidelines in place at the time.

The safety assessment studies are summarized in the following report and include:

A. Acute Toxicity Tests
B. Subchronic/Chronic Toxicity Studies
C. Multigeneration Reproduction Study
D. Carcinogenesis Evaluation
E. Metabolism - Rodents
F. Clinical Evaluations - Humans

PGPR is an emulsifier that is approved for food use in the European Union and is widely used in Europe in the manufacture of various chocolate products. Due to recent interest in the use of this emulsifier in the United States, a review of the safety support for PGPR has been prepared for consideration by a panel of scientists qualified by scientific training and experience to evaluate the safety of food ingredients. Additionally, articles specifically evaluating the safety of PGPR have been published in the September/October 1998 issue of the peer-reviewed Journal, Food and Chemical Toxicology (1-5).

The data presented herein demonstrates that PGPR is generally recognized as safe (GRAS).

A. ACUTE TOXICITY

1. Acute Oral Toxicity In The Rat And The Rabbit

a. Acute Oral Toxicity in the Rat

The acute oral toxicity of the polyglycerol-polyricinoleic acid emulsifier was evaluated by administration of a single oral dose of 20ml/kg (approx. 20 g/kg) to 55 weanling male and female Colworth rats weighing between 77 and 152 grams.

Each of the 22 weanlings in the control group was intubated with a similar dosage of groundnut oil. Due to the high viscosity of PGPR both materials were maintained at 37°C prior to dosing. Other than slight diarrhoea in 42 rats dosed with PGPR and 2 rats dosed with groundnut oil no other untoward toxic effects were observed for any of the parameters measured. These included food consumption and weight gain during the 21 day observation period, mortality, gross examination of the viscera at autopsy and absolute organ weights. The results of the test indicate that the PGPR emulsifier possesses an LD_{50} of greater than 20 g/kg of body weight in the rat.

b. Acute Oral Toxicity in the Rat

To investigate the potential cumulative toxic effect of the PGPR emulsifier, single oral doses of 10 ml/kg (approx. 10 g/kg) were administered daily for five consecutive days to six male and six female weanling rats weighing from 56 to 87 grams. Two control groups, one receiving groundnut oil and another physiological saline, were dosed in a similar manner, and in all instances the solutions were maintained at 37°C during administration. Gross observation of all animals for 14 days after the final dosing, for mortality, food consumption and body weight change failed to reveal any significant toxic effects which could be attributed to treatment with the test materials.

c. Intraperitoneal Injection in the Rat

The test was initiated to investigate the response to a single intraperitoneal (IP) injection of PGPR emulsifier in Colworth weanling rats maintained on restricted and unrestricted diets. Thirty-two weanlings approximately 48 days of age and averaging 100 grams in weight were divided into two groups; one group of 24 animals was maintained on a restricted diet (5 g/day), while the remaining eight animals were provided with food *ad libitum*. After four weeks the 24 animals on the dietary stress regimen were divided into three groups of eight animals; the first remaining untreated, while the second and third groups received a single IP injection (2.0 ml/100g rat) of either groundnut oil or PGPR respectively. At this time two additional groups of eight weanlings (42 days of age) provided with food *ad libitum*, were also treated with either groundnut oil or PGPR as described. An injection of 2.0ml/l00g rat of PGPR administered in a 60% aqueous suspension provided a dosage of approximately 12 g/kg of body weight.

No significant toxic effects were observed during the 14 day observation period, and gross examination of the viscera at sacrifice did not reveal any abnormal findings. Evaluation of the liver and kidney relative organ weights revealed no findings which were considered significant, since the differences in liver weights observed between the rats maintained on the restricted and unrestricted diets was attributed to the differences in age. The results of the test indicate that restriction of diet had no untoward effect on the ability of the rat to absorb and metabolize exaggerated dosages of either PGPR or groundnut oil.

d. Acute Oral Toxicity in the Rabbit

The acute oral toxicity of the PGPR emulsifier was evaluated by administration of a single oral dose of 10ml/kg of a 50% PGPR in groundnut oil solution (approx. 5 g/kg) to four New Zealand white rabbits weighing between 1.74 to 2.10 kilograms. Each of the four rabbits in the control group was intubated with a similar dosage of groundnut oil. As in other tests the materials were maintained at 37°C due to the high viscosity of the emulsifier. No evidence of adverse toxic effects, which could be attributed to treatment with PGPR, were observed for any of the parameters measured. These included food consumption and weight gain during the 21 day observation period, mortality, gross observation of the viscera at autopsy and absolute organ weights. The results indicate that PGPR possesses an LD_{50} of greater than 5.0 g/kg of body weight in the rabbit.

2. Biological Effects Of PGPR Emulsifier. The Acute Oral Toxicity In Chickens, Rabbits, Guinea Pigs and Mice

The acute oral toxicity of the PGPR emulsifier was investigated in several species including the chicken, rabbit, guinea pig and the mouse. Three males and three females of each species, excepting the chicken where six males were used, were administered a single oral dose of PGPR which was considered the maximum possible volume tolerated by each species. Dosages for chickens, rabbits, guinea pigs and mice were 30, 25, 30 and 100 ml/kg respectively. Each of the six control animals for each species received similar dosages of groundnut oil. Four deaths occurred during the study in the PGPR - treated groups (two rabbits, one guinea pig and one mouse) but these were confirmed at necropsy to be unrelated to treatment with the test material. The results of the tests indicated that highly exaggerated concentrations of PGPR were without toxic effect in each of the species examined.

B. SUBCHRONIC/CHRONIC TOXICITY STUDIES

1. Thirty And forty-five week feeding tests on Polyglycerol-Polyricinoleic Acid Emulsifier (PGPR)

Two dietary feeding trials of 30 and 45 weeks duration were initiated to investigate the potential adverse effects of an exaggerated dosage of polyglycerol-polyricinoleic acid emulsifier (PGPR) on the normal body metabolism of the rat (1). In each of the feeding studies 24 male and 24 female Wistar rat littermates were randomly divided among the three test groups of 16 rats. Each of the test groups was maintained on purified diets where the normal fat content of 10% by weight was replaced with 9% PGPR and 1% groundnut oil. For comparison, two control diets containing either 1 or 10% groundnut oil were also evaluated.

Parameters of response common to both studies included gross observation of appearance and behaviour, food consumption, body weight gain, bromsulphthalein (BSP) liver function test, specific gravity of urine samples as a measure of kidney function, absolute organ weights and gross and microscopic examination of selected tissue samples. Also included in the 30 day study were faecal analyses for excreted fats during the fifth week and an evaluation of the effects of restricted and unrestricted diets on body fat composition on animals sacrificed during the eleventh week.

After 45 weeks the diets were modified by completely excluding wheat germ oil and increasing the levels of groundnut oil to 9% or 18% at the expense of starch. Faecal analyses were conducted for seven days to determine the effects of PGPR on the digestibility of fats and protein. Haematological evaluation of blood samples obtained prior to sacrifice included red blood cell (RBC) and haemoglobin counts, packed cell volume and erythrocyte fragility tests. Also evaluated were the effects of PGPR on *in vitro* plasma lipid clearance and plasma turbidity, and determination of chylomicron (emulsified fat particles) concentration in the blood to investigate the route of PGPR absorption. The data obtained in this study indicate that ingestion of a diet containing 9% PGPR for 30 and 45 weeks in the rat did not result in any untoward systemic effects on growth, food consumption, liver and kidney function, haematological evaluations or gross and microscopic examination of selected tissues including the liver, spleen, testis, kidney, adrenal and pituitary.

Evaluation of absolute organ weights revealed no differences among group means for the kidneys, adrenals, pituitary, spleen or testes. Although the livers of several rats in the PGPR - treated groups were significantly enlarged, liver function and histology did not indicate any toxic effects. It seemed reasonable to attribute that the hepatic enlargement was due to the need to metabolize a fatty acid (ricinoleic acid) of atypical chemical structure as similar effects were seen in a separate study with rats fed castor oil.

On the basis of the results obtained at dietary levels of 90,000ppm (9%) PGPR emulsifier in both the 30 and 45 week subacute studies was without significant toxic effect in the rat.

2. Subchronic feeding study in the rat - effect of PGPR. Emulsifier on liver weight

A 13 week feeding study was initiated to investigate the occurrence of liver enlargement in rats fed exaggerated dietary levels of PGPR emulsifier, and to establish a level at which it does not occur.

One hundred littermate males and females were randomly divided into five test groups each containing 10 males and 10 females. Each group was supplied *ad libitum* with purified diets containing either 0, 1, 2, 4 or 8% PGPR for 13 weeks. Parameters of response included weekly body weight gain and food consumption, mortality, urinalysis biweekly during weeks 1 and 4 and once during weeks 2, 5, 6 and 9 to 13, gross observation of the viscera at autopsy and absolute organ weights for the liver, kidneys and adrenals

Evaluation of the body weight gain and food consumption data revealed no significant differences among group means. None of the test animals died during the course of the study. Urine samples obtained during weeks 1 to 6 in the 4 and 8%. PGPR - treated groups revealed positive reactions to Ketostix reagent strips, but were negative during weeks 9 to 13. Animals in both the 1 and 2% PGPR-treated groups showed negative responses throughout the test. Further investigations were conducted to ascertain whether the positive reactions were due to acetone bodies or a metabolite of PGPR. Since PGPR urine gave positive reactions after extraction with ether, benzene or chloroform, and also after freeze-drying and the residue dissolved in water, but not after distillation, it was concluded that the Ketostix reaction is due to a metabolite of PGPR.

Statistical evaluation of the kidney and adrenal organ weights revealed no significant differences among any of the groups tested. Analysis of liver weights revealed enlargement occurred predominantly in females at the 4 and 8% dietary PGPR levels. Significantly enlarged livers were also observed for males in the 4% PGPR- treated group, and to a lesser extent at the 8% dietary level.

No significant differences were observed among the control and the 1 and 2% PGPR-treated groups.

3. Subchronic feeding study in the rat - hepatic pathology of rats fed PGPR emulsifier

The study was initiated to investigate whether or not liver enlargement in rats fed exaggerated dietary levels of PGPR emulsifier is a transient effect, and to examine further the conclusion that enlargement is probably due to adaptive hypertrophy without toxic generation.

Twenty-four male and twenty-four female Colworth Wistar Rats, approximately 6 weeks old, were randomly divided into 4 groups and fed the following diets *ad libitum*.

	Dietary Levels		
Group	**Initial age (weeks)**	**First Week**	**Second Week**
A	6	Purified diet containing 2% groundnut oil and 18% PGPR	Stock diet (Spital)
B	6	Purified diet containing 20% groundnut oil	Stock diet (Spital)
C	6	Stock diet (Spital)	Purified diet containing 2% groundnut oil and 18% PGPR
D	6	Stock diet (Spital)	Purified diet containing 20% groundnut oil

After the second week all animals were sacrificed and the viscera examined for gross abnormalities. In all instances the livers were exercised, weighed and sectioned for microscopic examinations. The results obtained revealed that dietary feeding of 18% PGPR for one week resulted in liver weights which were still significantly greater than the groundnut oil controls one week later. However, subsequent studies of longer duration have shown that liver enlargement induced by highly exaggerated levels of PGPR is a transient effect in mice and chickens.

Histological examination of hepatic parenchymal cells did not reveal any significant pathological changes. Animals fed PGPR during the second week possessed small quantities of neutral fat in the periportal parenchymal cells as did males fed groundnut oil, differing only in distribution which may reflect different metabolic pathways. This effect was not seen in animals fed either PGPR or groundnut oil for one week and a stock diet during the second week indicating that this hepatic change is reversible, and in fact, may be due to the feeding of a 20% dietary level of fat.

Microscopic examination of parenchymal cell nuclei also failed to reveal any degenerative change or increased mitotic activity. Results obtained by the nuclear counting technique revealed that as hepatic weight increased the number of nuclei visible per unit area of liver decreased, suggesting that enlargement is due to an increased metabolic workload induced hypertrophy (as seen when castor oil itself is fed to rats) and not a hyperplastic (proliferative) change which could be associated with liver cell damage.

4. Subchronic toxicity study - Investigations into the nature of liver enlargement in rats fed emulsifier PGPR

This study was conducted to investigate the exact nature of PGPR induced liver enlargement. Thirty two male weanling Colworth albino rats were randomly divided into four test groups and maintained on the following diets.

	Dietary Levels		
Group	**Initial Age (Days)**	**First Week**	**Second Week**
A	21	Purified diet containing 10% groundnut oil	Purified diet containing 18% PGPR emulsifier and 2% groundnut oil
B	21	Purified diet containing 10% groundnut oil	Purified diet containing 18% castor oil and 2% groundnut oil
C	21	Purified diet containing 10% groundnut oil	Purified diet containing 20% groundnut oil
D	21	Stock diet (Spital)	Stock diet (Spital)

All animals in groups A, B and C were "conditioned" to the purified diet during the first week, and then provided with the test diets ad libitum during the second week. Body weights were recorded initially and after the first and second weeks. Food consumption data were obtained twice weekly. At the conclusion of the test all animals were anesthesized with ether and exsanguinated. The liver and kidneys were then excised from each animal, weighed, examined and prepared for histological evaluation. Both the liver and kidneys were analysed for RNA and DNA content, total moisture, total solids and total nitrogen.

At the end of the second week animals maintained on diets containing either 18% PGPR or castor oil showed somewhat smaller weight gains than did the 20% groundnut oil-treated and Spital control groups. Food and water intakes were also higher for the Spital-fed controls than observed for the groups receiving purified diets.

Results obtained for the liver and kidney organ weights revealed that dietary levels of 18% PGPR or castor oil both resulted in an increase in relative liver weight, but had no effect on relative kidney weight.

Further investigation revealed that liver enlargement was not accompanied by an increase in DNA content indicating that an increase in cell number (hyperplasia) had not occurred. However, a change in cell composition was observed. Increases in the liver cell content of water and particularly nitrogen (cell protein) were observed for rats fed either PGPR or, to a lesser extent, castor oil, and were attributed to work hypertrophy. The RNA/DNA ratios were also increased, but were not indicative of marked hypertrophy. No significant adverse effects were observed during a similar examination of the kidneys.

PGPR Induced liver hypertrophy resulting from adaptation to increased functional requirements was not considered an effect of toxicological significance. Since the liver cells, by enlargement, can apparently meet the functional demands of a highly exaggerated level of PGPR without proliferation (hyperplasia) it appears that the PGPR emulsifier should not present any hazard to humans even at levels greatly in excess of the estimated daily exposure.

5. Subchronic toxicity study - Effects of diet and feeding cycle on liver enlargement in the mouse

A short term feeding study was initiated to investigate the potential effects of restricted and unrestricted diets containing exaggerated levels of PGPR emulsifier in the mouse. Two hundred and eighty male and female C 57 BL mice, 6 to 8 weeks old, were randomized into 7 dietary groups of 20 males and 20 females. The seven dietary groups included levels of either 5, 10 or 15% PGPR emulsifier in purified diet, 5, 10, or 15% groundnut oil in purified diet, and a single group received Spital (stock diet). Each of the groups was further divided (10 males and 10 females) and received the respective diets either *ad libitum* or restricted to seven hours per day (9 a.m. to 4 p.m.). A record of body weight gain and food consumption was obtained for each group during the study. During the fourteenth day of the test five male and five female mice from each feeding schedule were sacrificed. All remaining animals were fed the diet containing 10% groundnut oil for an additional two weeks (stock diet fed as before).

All mice fed the diets *ad libitum* showed comparable weight gains with the exception of smaller gains observed for males in the 15% PGPR-treated group. Weight gains observed for animals maintained on restricted diets were smaller than those observed for animals fed *ad libitum*, but the gains were greater in the groundnut oil-treated groups than in the PGPR groups. Food consumption data revealed no significant differences among group means obtained for either dietary schedule.

Analysis of relative liver weights revealed that enlargement occurred in all of the PGPR-treated groups following either *ad libitum* or restricted feeding for two weeks, but the effect was slightly greater in mice maintained on the restricted dietary schedule. Evaluation of the data obtained for relative kidney weights did not reveal any differences among groups receiving either restricted or unrestricted dietary levels of PGPR or groundnut oil.

Statistical evaluation of the relative liver weights from mice fed a purified diet containing 10% groundnut oil for the additional two weeks revealed no evidence of liver enlargement indicating that the liver hypertrophy observed in this study was transient. Metabolic overload induced liver hypertrophy of a transient nature was not considered an adverse finding.

6. Subchronic toxicity in the mouse - Evaluation of emulsifier PGPR - Effect of dietary level on liver enlargement

This short term subacute feeding study was initiated to determine the maximum no-effect level of PGPR and also to investigate the potential influence of high dietary levels of fat on liver enlargement.

C57 BL mice (100 males and 100 females) 6 to 8 weeks old were randomized into 18 dietary treatment groups of 5 males and 5 females, and one control group of 10 male and 10 female mice. In this two week test animals in the control group were fed stock diet (Spital) *ad libitum* while each test group was provided with either PGPR or groundnut oil at one of the following dietary levels; 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0%.

Study observations included body weight gain, food consumption, gross examination of the viscera at autopsy and liver and kidney organ weights.

Analysis of the body weight data did not reveal any significant differences among groups fed either PGPR or groundnut oil. Food consumption was slightly greater for mice fed the 0.5 to 4.0% PGPR diets than observed for groundnut oil-treated groups at the same dietary levels.

Statistical analysis of the relative liver organ weights revealed that male and female mice provided with diets containing 3% and greater levels of PGPR had significantly heavier livers than mice in the non-treated control (Spital) group. Significant increases in liver weight were also observed for males fed 4, 6 and 8% and females fed 3% and greater levels of PGPR when compared to mice provided with identical levels of groundnut oil. No significant differences among group means were observed for the relative kidney weights. No significant adverse effects were observed at the 2% PGPR dietary level.

7. Subchronic toxicity test - Effects of dietary feeding PGPR to chickens

A ninety day feeding study was initiated to investigate the potential effects of PGPR in a non-rodent species (1). One hundred sixty male and female three week old chickens were equally divided into 10 groups of 8 cockerels and 8 pullets and provided with the following diets *ad libitum*.

Group	Dietary Levels	Duration of Treatment (weeks)
A	Basic diet* (Control)	13
B	Basic diet (Control)	17
C	Basic diet containing 15% PGPR	13
D	Basic diet containing 15% PGPR followed by basic diet (Control)	13 4
E	Basic diet containing 10% PGPR	13
F	Basic diet containing 5% PGPR	13
G	Basic diet containing 2% PGPR and 3% groundnut oil	13
H	Basic diet containing 15% groundnut oil	13
I	Basic diet containing 10% groundnut oil	13
J	Basic diet containing 5% groundnut oil	13

*BOCM baby chick mash

All animals were weighed and food consumption was measured once per week. Plasma glutamate-pyruvate amino-transferase (GPT) and plasma glutamate-oxaloacetate amino-transferase (GOT) levels were obtained for 4 males and 4 females during the sixth and thirteenth weeks of treatment for all groups except B and D which were evaluated during the fifteenth and seventeenth weeks of treatment. Haematological evaluations included packed cell volume (PCV), haemoglobin, mean corpuscular haemoglobin concentration (MCHC) and erythrocyte sedimentation rate for animals sacrificed after 13 weeks. Several vital organs including the liver, heart, spleen, kidneys and gonads were excised from all animals at sacrifice, weighed and sectioned for microscopic examination.

Analysis of the body weight data revealed that chickens provided with diets containing 10 or 15% of either PGPR or groundnut oil showed smaller weight gains than the control groups. Since the smallest weight gains were observed in the groundnut oil-treated groups the effect is

probably due to the high levels of dietary fat and not related specifically to treatment with PGPR. Evaluation of the food consumption data revealed no differences in the amount of food consumed between the control and PGPR- treated groups. Four of the test animals (one male and one female in group G and two males in group J) were sacrificed during the tenth week of the study due to ill-health unrelated to treatment with the test materials.

Analysis of plasma enzyme (GPT and GOT) levels, and haematological parameters revealed no adverse findings which could be attributed to treatment with the PGPR emulsifier.

Liver enlargement occurred at the 15% PGPR dietary level, but not at the 10% PGPR or lower levels. Significant kidney enlargement was also observed at 5, 10 and 15%, but not at the 2% dietary PGPR level. Both liver and kidney enlargement was reversible when chickens fed 15% PGPR for 13 weeks were placed on the non PGPR control diet for an additional 4 weeks, although a complete return to normal kidney weight had not been reached for the male chickens after 4 weeks. Reversible liver and kidney enlargement which is due to hypertrophy is thought to be a normal functional response to an increased hepatic or renal workload.

The significantly decreased spleen weights in cockerels fed either 15% PGPR or groundnut oil, and the elevated heart weights in hens fed 10 or 15% of either PGPR or groundnut oil were attributed to the high concentrations of dietary fat and are not considered to be specific effects of the fatty acid emulsifier.

Microscopic examination of selected tissue slides failed to reveal any significant morphological effects which could be attributed to ingestion of PGPR.

C. MULTIGENERATION REPRODUCTION (WITH COMBINED CHRONIC TOXICITY PHASES) STUDY IN RATS FED PGPR EMULSIFIER

A combined chronic toxicity/multigeneration feeding study was initiated to investigate the potential adverse effects of PGPR on reproduction and following ingestion on a chronic basis (3). The first generation parents were selected from five litters of Colworth Wistar albino weanling rats weighing from 32 to 51 grams, and divided into two groups including a control (11 males and 17 females) and a 1.5% dietary PGPR-treated group (6 males and 13 females). Breeding was conducted continuously in pairs until each female had produced five litters or it became evident that breeding had ceased. In all instances the first litters were discarded after weaning and second generation breeders were selected (2 males, 2 females) from each of the second and fourth litters. By selecting from two first generation litters the number of animals was increased to 52 of each sex in the control and 32 of each sex in the PGPR-treated groups. By selecting the third generation breeders in a similar manner the control and PGPR groups were increased to 92 and 44 rats of each sex respectively. The large control groups were considered necessary due to the variations in breeding results which can be considerable within a normal colony.

In addition to the rats required for breeding, 12 littermate pairs of male PGPR weanlings were selected from each generation, equally divided by litter into two groups, and provided *ad libitum* with either the 1.5% PGPR diet or a control diet for six weeks. The breeding parents

of each generation were maintained on the diets for approximately one year for the chronic toxicity segment of the study.

Observations for each of the three generations included, conception success, number of litters per dam, average litter size, averaged weaning weights of males and females, litters per group showing 100% survival and total survival (%) at day 21. In the chronic toxicity segment of the study determinations for the first, second and third generation breeders maintained on the diets for approximately one year included gross observation of appearance and behaviour, mortality and average weight gain from weaning to either 65 days in males or to the time of mating in females. Gross observation of the viscera at sacrifice and microscopic examination of selected tissues, including the liver, kidney, adrenal, spleen, testis, gastro-duodenal junction and small intestine, were conducted only on the third generation breeders. Parameters examined in each of the six week feeding trials were identical to those given above for the first and second generation breeders.

Data obtained in the multigeneration portion of the study are presented in the following table:

	1st Generation		2nd Generation		3rd Generation	
Parameter	PGPR	Non-Treated Control	PGPR	Non-Treated Control	PGPR	Non-Treated Control
Number of dams	13	17	32	52	44	92
% Dams failing to breed, (Conception Success)	0	0	15.6	15.0	15.9	17.0
Average no. of litters per dam	4.6	4.7	3.2	4.4	4.0	3.5
% Dams producing 5 litters	84.6	76.0	40.6	50.0	70.4	52.0
Average size litter	7.35	8.35	7.24	7.22	7.45	7.03
% Survival at Day 21	78.9	79.0	44.0	54.0	82.8	70.0
% Litters showing 100% survival at Day 21	64.0	57.0	22.0	38.0*	67.0*	53.0
Avg. wt. of weanling males (g)	37.3	37.8	33.7	33.7	35.4	36.8
Avg. wt. of weanling females (g)	36.2	36.1	30.8	32.5	33.6	36.4
% Weanling males	48.0	48.0	57.0*	48.0	50.0	46.0

*Significantly different from the control group at $p<0.05$

Statistical evaluation of the data obtained provided no evidence of any adverse effect of PGPR on reproductive performance or development of the offspring. In general, breeding performance for both the control and PGPR-treated groups appeared to be more successful in the first and third generations. Differences observed In the second generation included a significantly higher proportion of males in the PGPR litters, and a greater number of control litters showing 100% survival at weaning when compared with the treatment group. However, data obtained for the third generation revealed that the number of PGPR litters showing 100% survival was significantly greater than the control group and a smaller proportion of males was observed for both groups. The similarity observed between the data obtained for the first and third generations failed to provide any evidence of a cumulative effect due to long-term ingestion of the PGPR emulsifier over three generations.

Littermate males selected from the first, second, and third generation weanlings and maintained on either the control or 1.5% PGPR diets for six weeks failed to reveal any significant differences in weight gain or any differences in appearance and behaviour between groups. None of the test animals died during the three studies and gross examination of the viscera at sacrifice did not indicate any signs of systemic toxicity. Cysticercus fasciolaris (intermediate stage of the cat tape worm) was observed in the livers of 14 to 16% of the rats of both groups, and was probably due to contamination of the diet during preparation. Bladder plugs were also observed in approximately 50% of the males in both groups. These plugs (solidified prostatic and seminal secretions) apparently did not interfere with urine secretion, and have been known to occur in sexually mature males with little opportunity to breed.

Third generation breeders (83 control and 23 PGPR-treated rats) maintained on the diets for more than one year were sacrificed and examined for the chronic toxicity portion of the study to investigate the potential systemic effects of long-term ingestion of PGPR. Gross examination of all animals at sacrifice failed to reveal any significant findings which could be attributed to treatment with the test material. Examination of carcass and abdominal fat revealed no variations in distribution, colour or consistency. Cysticercus was observed in several livers (white cyst, 5-10mm diameter) of both groups possibly due to contamination of the diet during preparation. Other findings such as bladder plugs (as observed in the six week feeding trials), diaphragmatic hernia, peritonitis, pyometra, testicular degeneration and nephritis occurred in several instances in both groups.

Histopathological examination of selected tissues excised at sacrifice from 31 control and 21 PGPR-treated rats did not indicate any significant distribution of abnormal tissue morphology. Examination of the livers revealed slight cellular infiltration and small focal areas of parenchymal degeneration occurring equally in both groups. A mixed cell sarcoma was observed in one control male and was apparently associated with the presence of Cysticercus in the liver. In the PGPR-treated group no evidence of hyperplastic activity was observed in the livers exhibiting Cysticerci.

Glomerular changes which appeared to be associated with arteriosclerosis were frequently observed in the kidneys, but the severity and distribution of these findings was unrelated to the treatment. No evidence of any other morphological changes were observed in any of the other tissues examined.

Based on the data obtained in the combined chronic toxicity/multigeneration feeding study ingestion of PGPR, at a dietary level of 1.5% did not elicit any adverse effect on reproductive capacity or development of the offspring during three generations of continuous exposure. In addition, ingestion of PGPR on a chronic basis provided no evidence of any significant morphological changes which could be related to the test material.

D. CARCINOGENESIS

I. Investigation of the carcinogenic potential of PGPR by dietary administration in the rat and the mouse

In order to investigate the carcinogenic potential of PGPR several studies were initiated in the rat and the mouse. These included dietary administration of 5% PGPR on a chronic basis to rats and mice for 2 years and 80 weeks respectively (5).

Carcinogenic potential in the rat

Feeding study

In the first study 60 male and 60 female Colworth Wister rats (aged 32 to 42 days) were equally divided by sex into two groups and provided *ad libitum* with purified diets containing 5% of either PGPR or groundnut oil for 2 years.

Parameters of response included gross observation of appearance and behaviour, weekly weight gain and food consumption, survival, liver function (bromsulphthalein excretion) and kidney function (specific gravity) tests at 83 and 103 weeks, haematological evaluations at 80 and 104 weeks including red blood cell counts, haemoglobin determinations, packed cell volume, mean corpuscular haemoglobin concentration, mean corpuscular volume, red cell fragility, prothrombin time, percent reticulocytes and total and differential white blood cell counts. The incidence of abnormal growths observed during the study and at autopsy was also recorded. At the conclusion of the study absolute and relative organ weights were obtained for the liver, heart, kidneys, testes, thyroid and pituitary, and a complete gross and microscopic evaluation of tissue pathology was conducted.

Analysis of the data obtained for weekly body weight gain and food consumption, haematological evaluations and liver and kidney function tests failed to reveal any significant untoward signs of systemic toxicity.

Total survival for males and females was higher in the groundnut oil control group (73.3%) than in the PGPR-treated group (60%). However, if the animals which succumbed due to either cardiac puncture to obtain blood samples or intestinal obstruction (hair ball) are

excluded, the percentage of rats either sacrificed due to ill-health (primarily respiratory infection) or dying spontaneously in the PGPR-treated group was not statistically different from the control value. Statistical evaluation of relative organ weights revealed that the kidneys of males, and both the livers and kidneys of females treated with PGPR-were significantly heavier than the control values. Analysis of the livers from rats fed PGPR indicated that approximately 66%, 15% and 0.3% of the increased weight was due to water, protein and fat respectively. These values were similar to the water:protein:fat ratios obtained for livers and kidneys of both the control and PGPR-treated groups. It was therefore assumed that liver and kidney enlargement is due to adaptive hypertrophy. Histopathological examination of selected tissues, including the liver and the kidney, failed to reveal any untoward signs of systemic toxicity which could be attributed to treatment with PGPR.

The number of primary tumours (primarily pituitary and thyroid adenomas) observed during the course of the study totalled 22 in the groundnut oil control group and 24 in the PGPR-treated group. In a separate study males and females of the Colworth Wistar strain were shown to be sensitive to small doses (0.5mg in groundnut oil) of 20-methylcholanthrene a known carcinogen, when given twice per week for 66 weeks.

Analysis of body fat excised at autopsy from males in both groups revealed only slight differences in fat composition for rats fed PGPR and were therefore not considered to be of particular significance.

Carcinogenic potential in the mouse

Studies similar to those described for the rat were conducted to determine the carcinogenic potential of PGPR in the mouse.

Feeding study

In the first study of 80 weeks duration, 100 male and 100 female Colworth C57BL mice (aged 6 to 8 weeks) were equally divided by sex into four groups and provided *ad libitum* with either (1) stock diet (non-treated control), (2) purified diet containing 5% groundnut oil, (3) purified diet containing 5% PGPR, or (4) the purified diet alone. The fourth group also received 0.5 mg of 9,10-dimethyl-1, 2-benzanthracene (DMBA) in groundnut oil by gavage once weekly to demonstrate the sensitivity of this strain to small doses of a known carcinogen.

Parameters of response included gross observation of appearance and behaviour, weekly body weight gain and food consumption, survival, haematological evaluations at 80 weeks including red blood cell counts, haemoglobin levels and total and differential white blood cell counts. The incidence of abnormal growths observed during the study and at autopsy were also recorded. At the conclusion of the study absolute organ weights were obtained for the liver, spleen, kidneys, heart and gonads, and a complete gross and microscopic evaluation of tissue pathology was conducted.

Statistical analysis of body weight gain and food consumption data failed to reveal any significant untoward effects which could be attributed to ingestion of the PGPR emulsifier.

No significant differences for survival to 80 weeks were observed between the control and PGPR-treated group. All animals provided with the purified diet and treated with DMBA died or were sacrificed due to the onset of tumours by the 58th week for females and the 66th week for males. Evaluation of haematological parameters revealed no significant differences between the groundnut oil control or the PGPR-treated group. The only difference observed was a significantly lower white blood cell count for the PGPR group when compared to the non-treated control group.

Analysis of absolute organ weights revealed that the liver and kidneys of female mice in the PGPR-treated group were significantly heavier than those of mice in the groundnut oil control group. It was also observed that livers from both male and female mice fed the PGPR and groundnut oil control diets were significantly heavier than those of the mice in the non-treated control group. Based on these findings and the results obtained from histopathological examination, it was concluded that enlargement was probably due to adaptive hypertrophy without toxic degeneration. No significant differences were observed among group means for the spleen, testes or heart organ weights.

Examination of body fat excised at sacrifice revealed no evidence of polyglycerol in the PGPR-treated group, but ricinoleic acid (<0.1%) was observed suggesting that fatty acids from the emulsifier are utilized to some degree in the mouse.

Histopathological examination of selected tissue samples failed to reveal any significant incidence of abnormal morphology which could be attributed to treatment with the test material.

Instances of neoplasia occurred at a high frequency in the carcinogen (DMBA)-treated group. The results indicated that the C57BL strain is sensitive to small doses of a known carcinogen. The incidence and types of tumours observed in the non-treated control, groundnut oil control and PGPR-treated groups were not considered to be significantly different indicating that the PGPR emulsifier is not a carcinogen in the mouse.

E. METABOLISM STUDIES - RODENTS

I. Studies on the digestion, absorption and metabolism of castor oil

The study was initiated to examine several specific aspects of the metabolism of castor oil *in vitro* and in the rat, and to show that the oil, and its principal component ricinoleic acid, are metabolized like the more common oils and fatty acids (i.e. olive oil and oleic acid). The first three experiments were conducted to determine the rate of absorption and distribution of ricinoleic acid in the rat. Each of the following tests was conducted on male young adult Sprague-Dawley rats weighing from 100 to 200 grams. Castor oil (medicinal grade) was supplied by the Baker Castor Oil Co, Bayonne, N J, and GLC (gas liquid chromatography) analysis gave the following fatty acid composition: 90.0% ricinoleic, 4.7% linoleic, 3.2% oleic, 1.0% stearic, 1.0% palmitic, and 0.1% palmitoleic.

Four fasted and four non-fasted rats were administered 1.0 ml of castor oil by oral intubation and the chyle collected from the thoracic duct for 24 hours. The chyle was then extracted with 2:1 chloroform/methanol for estimation of total lipids and for GLC analysis. The amount of ricinoleic acid absorbed, as calculated from the combined procedures, was significantly greater for the rats fed *ad libitum* (mean 24.2%) than observed for the fasted animals (mean 6.8).

In a second experiment seven weanling rats were provided *ad libitum* with a diet containing 20% castor oil, and a similar group was maintained on an olive oil supplemented diet. During the 4th and the 8th weeks an epididymal fat pad was removed from each animal in both groups and examined for fatty composition by GLC. The fat pads of olive oil-treated rats contained no ricinoleic acid, but the fat pads of the castor oil-treated group revealed that the maximum degree of incorporation is approximately 10%. Analyses of other tissues showed similar amounts of ricinoleic acid in perinephric and subcutaneous fat and in muscle, but not in the liver or the brain. Hydroxystearic acid was observed in the faeces of several animals examined and its presence has been attributed to hydrolysis of ricinoleic acid by intestinal bacteria.

In a similar study, biopsy of epididymal fat demonstrated that withdrawal of castor oil from the diet results in a rapid disappearance of ricinoleic acid from adipose tissue. Within 48 hours after the removal of castor oil from the diet neither ricinoleic nor hydroxystearic acid could be detected in the faeces, suggesting that the disappearance of the fatty acid from adipose tissue is due to metabolic degradation rather than excretion.

In an *in vitro* experiment the hydrolysis of castor oil was compared with that of olive oil by: (1) direct titration of the free fatty acid after pancreatic lipase hydrolysis, and (2) GLC analysis of the composition of the free fatty acids following hydrolysis of a mixture of equal volumes of the oils. Both the titration data and especially the GLC analysis showed that castor oil undergoes a more efficient enzymatic hydrolysis.

Preliminary studies have shown that the maximum enzyme activity resided in the microsomal fraction of the rat gut mucosal extract, and that this fraction provides the optimum substrate conditions for fatty acid activation. Based on this, activation by coenzyme A (Co A) and adenosine triphosphate (ATP), which is the first step in the conversion of a free fatty acid to glyceride, was measured for both ricinoleic and oleic acid.

Mucosal scrapings (rat intestine) were homogenized, placed in a centrifuge and the microsomes collected and resuspended in phosphate buffer. Fatty acids were prepared as potassium salts in a 5% aqueous solution of lipid free bovine albumin. The reaction system contained 0.5 ml of suspended enzyme, 0.5 ml 2N hydroxylamine, 20 μM ATP, 30 μM cysteine, 50 μM NaF, 20μM $MgCl_2$ and 1 mg Co A, and was incubated at 37°C for 30 minutes. Fatty acid and Co A were omitted from the control.

Colorimetric measurement of hydroxamic acid was used as an index of fatty acid activation. The results obtained in this study indicate that ricinoleic acid is less readily activated to its fatty acyl Co A than is oleic acid, and may account for the rapid accumulation of free ricinoleic acid within the intact gut. In another experiment polyethylene cannulae were inserted into the

thoracic ducts and into the duodenum through a puncture wound in the anterior surface of the stomach near the pyloric antrum of two rats. After an overnight fast each rat received either 0.6 or 0.2 ml of castor oil at a timed interval. After 45 minutes the animal given the larger dose exhibited diarrhoea and was immediately sacrificed. The second animal, which showed no signs of diarrhoea was also sacrificed after 45 minutes.

The large and small intestines of each animal were washed, homogenized and extracted in 1:1 acetone/ethanol. Chyle collected during the 45 minute test period was measured and extracted in 2:1 chloroform/methanol. Total livid estimation and GLC analyses performed on the extracts, revealed that a greater amount of the smaller dose of castor oil (0.2 ml) was absorbed, as indicated by a greater percentage of ricinoleic acid in the intestinal mucosal lipids and the considerable quantity of fatty acid in the chyle. The animal which exhibited the laxation effect contained no ricinoleic acid in the lipids of the large intestine. This evidence suggests that the laxative effect of castor oil is related to the dosage since it is easily hydrolysed, but the activation and absorption of the free acid is not as efficient, resulting in the rapid increase of ricinoleic acid and its mineral salts.

The results of the preceding experiments indicate that the principal and active fatty acid of castor oil (ricinoleic) is remarkably similar to other fatty acids: following hydrolytic release in the gut it is activated by mucosal enzymes, esterified in the intestinal epithelial cell and discharged into the chyle. Thereafter it is deposited in adipose tissue, and released in a normal manner, apparently undergoing oxidative degradation.

2. Indirect studies of the absorption and metabolism of Polyglycerol-Polyricinoleic Acid Emulsifier (PGPR)

To investigate if PGPR is utilised as a nutrient rats were fed 9% PGPR for 9 weeks and then placed on a restricted non-fat diet (equivalent to 18 kcal/day) for 17 days. A number of animals were killed and examination of the distribution of body fat revealed that PGPR had no effect on the normal gross distribution of body lipids which had been metabolised during the depletion period. Subsequent feeding of PGPR to the remaining rats gave complete restoration of body weight in 14 days. This demonstrated that PGPR would not adversely effect normal fat metabolism and that the emulsifier is utilised as a nutrient.

Additional studies confirmed that feeding 9%PGPR to rats had no effect on the absorption or utilisation of vitamin A and no effect on the digestibility of groundnut oil or protein.

PGPR had no effect on either the in vitro lipolysis of fat or on the in vitro clearing of lipaemic plasma. Ingestion of PGPR by the rat did not increase plasma turbidity (lipaemia), and did not induce chylomicronaemia indicating that PGPR is absorbed directly into the blood and transported to the liver (similarly to medium chain triglycerides) and not via the lymphatic system as is the case for groundnut oil.

3. Biological effect of emulsifiers for edible fats. An auto-radiographic study of the distribution of Carbon-14-Polyglycerol in mice

PGPR is prepared by condensing polyricinoleic acid (polymerised castor oil fatty acids) with polyglycerol. Thus PGPR consists of several acid polymers linked to a poly-glycerol chain.

The effectiveness of a whole body autoradiographic technique, to study the distribution of labelled PGPR, emulsifier in the mouse, was demonstrated after gastric intubation of 14C-polyglycerol (2).

In this experiment six adult male C57 black mice were administered a single oral dose of 14C-polyglycerol (activity 10.98µc/dose) by gastric intubation and two each were sacrificed after 1, 2 and 24 hours. The two mice in the latter group were placed in a Jencon's "Metabowl" and the urine and expired CO_2 monitored for 14C activity. After sacrifice all animals were frozen to minus 78°C and sectioned by a "tape section" technique at minus 15°C. The tapes were then superimposed on X-ray film (Gevaert Dentus film) for 14 days at minus 15°C. Blackening of the film emulsion over tissue sites was indicative of the presence of 14C-polyglycerol.

After examination of the film it was observed that the bulk of the polyglycerol was present in the stomach after one hour, but was already passing into the small and large intestine. After 2 hours the majority of the dose was present in the intestinal tract. Metabolic studies of the two mice kept for 24 hours revealed that 14C activity was present in the urine and expired CO_2 at this time. Autoradiographs of these animals revealed only slight blackening of the film over the stomach and intestinal tract. No evidence of 14C activity was observed over other tissue sites.

The results obtained in this experiment indicated that the bulk of the 14C-polyglycerol was excreted. However, evidence has been obtained that some of the labelled material was absorbed, and the absorbed materials were: (1) either totally expired or excreted in the urine and not deposited in other tissues, or (2) some of the labelled material was deposited in body tissues but the technique was not sensitive enough to detect sites of low activity.

Should the second interpretation of the data be correct, the autoradiographic technique can be used to observe the passage of labelled material through the alimentary canal during digestion, but it would not be sensitive enough to provide adequate data necessary to determine fully the metabolic fate of the labelled PGPR emulsifier after absorption.

4. The metabolism of l-14C-Glycerol, l4C Polyglycerol and l4C (Polyglycerol) PGPR in the rat

In this study only the polyglycerol part of the PGPR polymer was examined. For this evaluation glycerol-1-14C (Radiochemical Center, Amersham) was used to synthesize l4C - polyglycerol which was then condensed with polyricinoleic acid to form the 14C (polyglycerol) PGPR. The metabolic pathways of each of the three isotopically labelled compounds were monitored in male Colworth Wistar rats averaging 250-300 grams in weight (2).

In the first study 100μc of Glycerol-1-14C was diluted to 5ml with glycerol and again to 10ml with water and 1.0 ml of the solution was administered to each of six rats by gastric intubation. The animals were immediately placed into metabolic cages. The animals were provided with food and water *ad libitum* prior to and during the experiment.

A flow rate of 3 liters/min was maintained and the expired CO_2, urine and faeces were evaluated for 14C activity. After 48 hours the animals were sacrificed and tissue samples of liver and epididymal fat were excised for 14C analysis.

After 48 hours 84.3% of the original dose (9.6μc) was recovered; 68.5% in expired $C0_2$, 12.1% in the urine and 3.7% in the faeces. These results indicate that glycerol is rapidly absorbed from the gut and extensively metabolized. The large proportion of the labelled isotope expired as $14CO_2$ indicates that glycerol is utilized as an energy source. The mean activities in the liver and epididymal fat were 8.2 and 5.9μc/g of tissue, respectively.

The metabolic fate of 14C polyglycerol was also investigated by the same procedure with a total recovery of 88% in 48 hours. Analyses for 14C activity revealed that the results were completely different from those obtained for glycerol-l-l4C. More than one-half of the administered 14C was found in the faeces (52%) and was attributed to the overloading of the intestinal tract with glycerol to cause laxation in the test animals. Some of the glycerol polymers were absorbed as indicated by 30.5% of the activity recovered in the urine. The 14C activity recovered from expired CO_2 (5.5%) was probably due to the metabolism of the 9% free glycerol-1-14C present in the 14C polyglycerol sample.

When the experiment was repeated using a smaller dose (8,89μc) of 14C polyglycerol with a higher specific activity to examine the pattern of excretion without overloading the intestine, a greater percentage of the activity was found in the faeces (62%), while the amount excreted in the urine (25%) had decreased. No difference in the amount of expired $14CO_2$ was observed. The dissimilar faecal/urine 14C ratios were attributed to the differences in concentration of the two samples of polyglycerol, the lower concentration of polyglycerol (higher specific activity) did not lead to laxation and greater absorption was possible.

In a third experiment 14C polyglycerol (6.67μc) was administered by gastric intubation in a ground dietary slurry. The results obtained indicated that since the 14C activity in the excreta (30.5% in urine, 49% in faeces) in this experiment, was similar to the data obtained for animals treated with an aqueous solution of 14C polyglycerol, the faecal 14C activity was not

due to an overloading effect. In this experiment, the polyglycerol used contained about 10% free glycerol as indicated by the recovery of 8% expired $14CO_2$.

Analysis of the results obtained in these experiments indicate that about 30% of the glycerol polymers are absorbed from the intestine, but the lower glycerol polymers are preferred. The presence of $14CO_2$ in the expired air, indicative of use as an energy source, is most likely due to the presence of free glycerols and not metabolism of polyglycerols. Chromatographic analyses of urinary and faecal 14C revealed that polyglycerols were excreted unchanged. Based on these results it was concluded that polyglycerols are not metabolized in the rat and that the ether linkage between the two glycerol units render the compound inert. The residues of 14C activity found in the liver and epididymal fat suggests some retention by these tissues, but the activities were too low for chromatographic analysis. However, the data obtained did indicate a turnover of the labelled material.

The metabolic fate of 14C (polyglycerol) PGPR was investigated in the rat by gastric intubation of 1.0 ml of the materials as a 50% aqueous emulsion, thereby providing a dosage of 3.9μc/rat. A flow rate of 3 liters/min was maintained in each of the cages, and expired CO_2 was monitored for 36 hours. Urine and faeces were collected at 24 hour intervals for four days. Each of the six animals was provided with food and water *ad libitum*.

A total of 70% of the administered dose was recovered after four days. Chromatographic analysis of the urine revealed that, as in previous studies, the 14C activity was concentrated in the lower glycerol polymers ie. diglycerol and triglycerol. Analysis of the faeces by thin layer chromatography revealed that approximately 85% of the 14C recovered was free polyglycerols suggesting that the PGPR, polymer was being broken down in the intestine. The data indicated however, that approximately 10% of the 14C found in the faeces was either undigested or partially digested PGPR. It was assumed that the administered emulsion had separated in the stomach and formed globules of PGPR, which could not be completely digested.

In another experiment 14C (polyglycerol) PGPR (0.432μc) was administered in an aqueous dietary slurry. Analysis of expired $C0_2$, urine and faeces revealed 14C recoveries of 8, 31 and 54% respectively, and are similar to the results obtained in the 14C polyglycerol experiments. Chromatographic analysis showed that the PGPR polymer was completely broken down to liberate the polyglycerols. Thus, PGPR is digested in the rat liberating polyglycerols, some of which are absorbed and excreted unchanged in the urine (lower polymers) and others which were egested in the faeces (higher polymers). The fate of polyricinoleic acid molecules which were also released during digestion of PGPR was investigated in a separate study.

5. The metabolism in the rat of Oleic Acid-l-14C and Stearic Acid-l-14C when condensed with Ricinoleic Acid and incorporated into the PGPR Polymer

The metabolic fate of the polyricinoleic acid portion of PGPR was investigated by introducing stearic-l-14C and oleic-l-14C acids into the polymer (2). As indicated earlier castor oil contains both stearic acid and oleic acid, therefore an addition of trace amounts of l4C oleic and stearic acids would not affect the overall composition of PGPR.

All animals used in the following experiments were male Colworth Wistar rats weighing from 250-300 grams in weight. During each experiment a flow rate of 3 liters/min was maintained in the metabolism cages and CO_2 absorbing traps. Expired CO_2 was monitored for 14C activity and the separated urine and faeces were collected at 24 and 48 hours. Liver and epididymal fat were assayed for 14C activity by saponification of weighed samples in 80% aqueous ethanol containing 10% NaOH. The acidified samples were then extracted three times with 2:1 chloroform/methanol. 14C activity was determined in the faeces by a combustion technique and in expired CO_2, urine, liver and fat extractions by liquid scintillation.

First, glycerol trioleate-1-14C (13.5μc) was administered by gastric intubation of 0.5ml of the test material in groundnut oil. Analyses for expired CO_2, urine and faeces showed recoveries of 52.3, 2.0 and 8.3% respectively. The mean values obtained for 14C analysis of the lipid extracts of liver and epididymal fat were 5.34 and 33.16 mμc/g respectively. The results show that approximately one-half of the administered dose was expired in the CO_2 indicating that the fatty acids were absorbed and metabolized as a source of energy. The material recovered in the faeces was probably due to either poor absorption or 14C metabolites excreted in the bile. Urinary 14C was also considered to be due to 14C metabolites. The large amount 14C activity in the depot fat (33mμc/g) showed that the test material had been deposited and the amount of $14CO_2$ expired at the termination of the study indicated that the deposited material was being slowly turned over.

In a similar experiment l4C (Oleic) PGPR (9.4μc) was administered as a 50% aqueous emulsion by gastric intubation in a 1.0ml volume. Analyses of expired CO_2, excreta and selected tissues were conducted as previously described.

The amount of 14C activity found in the expired CO_2 indicated that the non-hydroxy fatty acids were being hydrolysed from the ricinoleic acid polymer. Results obtained during faecal analysis differed from the previous experiment in that more than half the administered dose was excreted as undigested or partially digested PGPR ie. polyricinoleic acid still bound to the polyglycerol.

Gastric intubation of 14C (stearic) PGPR (9.93μc) in 1.0ml of a 50% aqueous emulsion to two groups of rats maintained for 21 days on stock diets containing 5% of either groundnut oil or PGPR, showed similar results, as observed in the table below:

Treatment		% Recovery			14C Activity (mμc/g)	
		Expired CO_2	Urine	Faeces	Liver	Epididymal Fat
1.	Pre-fed diet containing 5% groundnut oil	18.9	0.8	55.2	26.8	11.6
2.	Pre-fed diet containing 5% PGPR	16.1	0.8	49.4	25.8	28.8

The only difference observed was a higher residual activity in the epididymal fat of the PGPR pre-fed animals. Similar results were obtained for faecal 14C activity, which upon analysis showed approximately 75% of the activity to be undigested or partially digested PGPR However, the presence of $14CO_2$ indicated that to some extent polyricinoleic acid was being metabolized as an energy source.

In a separate experiment it was observed that an aqueous emulsion of PGPR pipetted into 0.1 N HCl at 37°C disassociated after a few minutes and formed globules of the test material, Therefore the large amounts of faecal 14C activity associated with undigested or partially digested PGPR was attributed to the method of administration.

In two separate experiments 14C (stearic) PGPR was administered in the diet to 15 young adult Colworth Wistar rats, and intubated as a stock diet slurry to 6 fasted and 6 non-fasted rats weighing from 250 to 300 grams in weight. For comparison, stearic acid -1-14C was also administered as a dietary slurry. Analysis of expired $C0_2$, excreta and selected tissues for each experiment after 48 hours are listed below:

Treatment		% Recovery			14C Activity (mμc/g)	
		Expired CO_2	Urine	Faeces	Liver	Epididymal Fat
1.	Intubation of a dietary slurry containing 10.74μc of stearic acid-1-14C/rat	38.7	1.5	4.7	-	-
2.	Intubation of a dietary slurry containing 10.493μc of 14C (stearic) PGPR/rat (non-fasted)	24.7	1.7	14.8	46.1	82.9
3.	Intubation of a dietary slurry containing 10.493μc of 14C (stearic) PGPR/rat (fasted)	29.9	1.5	16.3	34.1	76.2
4.	Dietary administration of 10.89μc 14C (stearic) PGPR/rat	11.2	0.8	9.9	15.1	303.1

Results obtained for stearic acid-l-14C intubated in a dietary slurry indicated that the stearic acid was absorbed, some deposited in the fatty tissue, a larger portion metabolized directly giving rise to $14CO_2$ and a small amount excreted in the urine. The presence of approximately 5% of the dose in the faeces indicated that some of the 14C material would also be expected in the faeces of PGPR - treated rats.

No differences in 14C recovery were observed between fasted and non-fasted rats receiving 10.493μc of 14C (stearic) PGPR in a ground diet slurry by gastric intubation. The low recovery values obtained for faecal analyses indicated that the digestion and absorption of 14C (stearic) PGPR was complete. As revealed by the amount of 14C recovered in the expired CO_2 the stearic acid-l-14C, incorporated into the polymer, was oxidized.

Based on the low recovery of faecal 14C in the final experiment it would appear that the ideal situation for digestion, absorption and metabolism would be the administration of l4C PGPR in the diet. A comparison of the results obtained in this study with the data obtained after intubation of a dietary slurry revealed a large discrepancy between the amounts of 14C recovered in the expired CO_2 and depot fat. An explanation for these differences was based on the observation that the three meal per day training period, which allowed dietary administration of exact amounts of 14C-PGPR, induced metabolic stress. Thus when placed in metabolic cages and provided with stock diet *ad libitum*, fat was laid down at a higher than normal rate during the first 24 hours, which would also account for the low recovery of 14C in the expired CO_2 and liver.

Overall conclusions based on the results obtained in the metabolism experiments are that the PGPR polymer is digested in the alimentary canal of the rat, liberating free polyglycerols and the polyricinoleic acid which is either absorbed as such or digested and the free acids absorbed. Evidence has been obtained indicating that the non-hydroxy fatty acids in PGPR present as the terminal entities of polyricinoleic acid are hydrolysed from the polymer and metabolized in a similar manner to other dietary fatty acids.

6. <u>The metabolism in the rat of 12-^{3}H-Ricinoleic Acid and 9-10-^{3}H Ricinoleic Acid when condensed to Polyricinoleic Acid and incorporated into PGPR</u>

The following experiments were conducted to determine the metabolic fate of the polyricinoleic entity of PGPR. Ricinoleic acid labelled with tritium (l2-3H and 9-10-3H) was incorporated into the PGPR polymer and samples administered by gastric intubation to 12 adult male Colworth Wistar rats as a component of a dietary slurry (2).

The first experiment was conducted to investigate the metabolism of l2-3H (ricinoleic) PGPR in the rat. Immediately after dosing (14.0μc) all animals were placed in metabolism cages and provided with food and water *ad libitum*. A flow of 1.5 liters/min of air was maintained through the cages and H_20 traps during the experiment, and animals were sacrificed in groups of 3, after 3, 6, 12 and 24 hours. For each group terminal urine, faeces and expired H_20 were monitored for 3H activity. Also monitored were excised samples of liver, epididymal fat, brain, heart blood and gastrointestinal tissue and contents. All samples were analysed by liquid scintillation counting in a Packard 4000 series instrument.

Analysis of the gastrointestinal contents after 24 hours revealed that less than 1% of the labelled material was present, and only approximately 5% of the dose was found in the faeces indicating efficient absorption. Examination of the stomach and intestinal tissues after 24 hours showed 3H activities of <0.5% and <0.1% of the administered dose, respectively. The blood lipid 3H level remained at a low level throughout the experiment indicating rapid transport of the absorbed material. Approximately 5% of the administered dose was deposited in the epididymal fat of which only 30% was hydroxy fatty acid. The presence of 10% 3H activity in the urine as tritiated water and metabolites, and the recovery of volatile 3H in the expired air indicated that ricinoleic acid was metabolized The presence of 3H in the aqueous phase of all tissues analyzed also provides evidence of the metabolic conversion of the labelled material.

The results obtained in this experiment showed that 12-3H-PGPR is hydrolysed in the gut to ricinoleic acid, ricinoleic acid polymer and polyglycerols. There is also an indication that ricinoleic acid is extensively metabolized and only a small percentage of the dose is deposited in the depot fat as ricinoleic acid. However, further investigation as to the nature of the 3H was necessary since hydrolysis in the intestine could have liberated the tritium from the ricinoleic acid molecule, thus the results obtained may indicate not ricinoleic acid, but some other 3H material.

In order to determine the fate of l2-3H-PGPR during digestion several *in vitro* experiments were conducted. In the first *in vitro* experiment the intestinal tracts of 3 adult male Colworth Wistar rats were excised and samples of 3H-PGPR were incubated in separately prepared preparations of intestinal mucosa and intestinal contents at 37°C for 3 hours. Extraction of both preparations revealed only trace amounts of 3H in the aqueous phase while analysis of the lipid phases revealed that digestion had taken place. The concentrations of liberated ricinoleic acid/ricinoleic acid polymer released from PGPR in the preparations of intestinal mucosa and contents were 6 and 10% respectively.

In a second in vitro experiment the pancreas from each of the 3 rats was excised, homogenized and 3H-PGPR was added and incubated at 37°C for 2 hours. The results indicated that no tritium was liberated in the aqueous phase, however, 12 to 15% of the PGPR was digested to ricinoleic acid and ricinoleic acid polymers. A similar experiment performed with crude Hog Pancreatic Lipase (Sigma Chemical) revealed that up to 40% of the 3H activity was present as free ricinoleic acid and polymer after 2 hours.

The results obtained from the in vitro experiments show that the action of lipase removed the ricinoleic acid polymer from PGPR. The polymer is further digested to free ricinoleic acid without involvement of the l2-3H portion of the molecule. Based on these results it was also assumed that metabolism occurred by β oxidation.

The metabolic fate of 9-l0-3H (ricinoleic) PGPR (12.82μc) was investigated in 12 adult male Colworth Wistar rats by the procedures outlined in the first experiment. The results obtained were similar to those obtained for l2-3H (ricinoleic) PGPR, as indicated by the overall low recovery of 3H in the lipid phase of extracted tissue samples after 24 hours. The presence of 3H ricinoleic acid and polymer in the intestinal contents indicated that digestion of PGPR had

taken place, and the 10% of the labelled dose recovered in the faeces, (mainly ricinoleic acid polymer) was indicative of efficient absorption. The recovery of 3H in the urine and expired H_2O showed the metabolic conversion of ricinoleic acid.

The blood lipid tritium level remained low throughout the experiment indicating the rapid transport and metabolism of the material. After 24 hours analyses of selected tissues revealed less than 1% of the administered dose incorporated in the lipid phase, and approximately 75% of the tritium in the aqueous phase of the tissue hydrolysate.

The results obtained in both experiments indicated that the PGPR polymer is extensively digested in the small intestine of the rat to a mixture of hydroxy fatty acids and ricinoleic acid polymers. The hydroxy fatty acid is absorbed and metabolized so that after 24 hours only traces (<5%) of the absorbed material was present in the lipid phase of the selected tissues examined.

The results also suggest that the digestion, absorption and metabolism of PGPR emulsifier is similar to castor oil (triglyceride of ricinoleic acid) in that the compound is hydrolysed and ricinoleic acid is absorbed from the small intestine, and metabolized by oxidative degradation.

F. CLINICAL EVALUATION - MAN

I. Investigation of the effects of dietary administration of PGPR in humans

Under the supervision of Dr J C Eaton, PGPR emulsifier was fed to human volunteers (students of Glasgow University) over a period of two weeks at the Metabolic Unit of Glasgow Royal Infirmary from March of 1964 until August of 1965 (4). All 19 volunteers (8 males and 11 females) were regarded as healthy, and with the exception of two females (aged 66 and 64 years), all volunteers were young adults. Two volunteers were maintained for each three week treatment period in a metabolic unit which was equipped for sample collection (urine and faeces) and had a special dietetic kitchen.

PGPR was introduced into soups (5g/pint), cakes (10g/cake or 25g/portion) and mint flavoured toffee bars (5g/bar). The soups and cakes were supplied as a dry mix and were freshly prepared each day in the hospital kitchens. Toffee bars were prepared at Unilever Research Labs, Colworth House.

The three week feeding study was designed on the basis of a fat-balance experiment where endogenous fat excretion was established during a control period (1 week) and then during dietary administration of PGPR at 5 and 10g per day during the second and third weeks respectively. The diets were designed to maintain constant intakes of fat (150g/day) with a flexible level of carbohydrates to suit individual requirements.

Blood samples were taken twice per week during each of the three test periods for the following biochemical tests; serum albumin and globulin, serum protein electrophoresis, and as tests of liver function; thymol turbidity, serum bilirubin, total and free cholesterol, serum cholinesterase and serum glutamic pyruvic transaminase (SGPT).

Urine samples were collected quantitatively during each of the three weekly test periods and analyzed for creatinine as a measure of the glomerular filtration rate. This served as a renal function test.

Faeces were also collected quantitatively during the three week test period and analyzed for the presence of endogenous fat to investigate the potential effects of PGPR on digestion and intestinal absorption of fats. Total faecal nitrogen determinations were conducted and are also indicative of digestion and absorption disturbances in the alimentary tract.

Fractionations of the serum proteins are useful to detect liver and kidney dysfunction and certain malignancies, as detected by decreased albumin and simultaneously increased globulin levels.

Fractionation was conducted by two methods; chemical separation, which differentiates between albumin and globulins, and paper electrophoresis, which further splits the globulin fraction into 4 to 5 subfractions.

With the exception of three female volunteers, serum concentrations of albumin and globulin did not differ during the control and test periods and were within the normal ranges of from 3.5 to 5.9g/l00ml and 1.2 to 3.2g/l00 ml respectively. Two of the three women showed subnormal albumin levels during both the control and test periods, and the subnormal level observed for the: third woman (64 years) was considered to be a function of age. In all instances results obtained by paper electrophoresis were considered normal.

Results obtained in the thymol turbidity test, in all cases, did not exceed the normal turbidity values for thymol of up to 4.0 McLagan units indicating that there was no increase in serum globulin.

In all but three subjects serum bilirubin values did not exceed the normal level of up to 0.8mg/l00ml. In one subject hyperbilirubinemia was considered due to a subclinical liver disorder. In the two other subjects hyperbilirubinemia improved after administration of PGPR, but was probably due to the environment and a balanced diet.

Total and free cholesterol determinations were conducted to investigate whether or not the ingestion of PGPR causes lipaemia or a liver parenchymal disorder diagnosed by a decrease in the total cholesterol/free cholesterol ratio. Four of the females revealed abnormally high levels of total cholesterol and serum cholesterol levels observed for three males were also unusually high, but in all instances were unrelated to treatment with the PGPR emulsifier.

Increased serum glutamate-pyruvate transaminase (SGPT) and decreased cholinesterase enzyme activity are specific indicators of abnormal liver cell pathology. Analysis of the data obtained for SGPT determinations in all instances, were within the normal range of up to 35 Sigma-Frankel units. Four of the volunteers (2 males, 2 females) showed abnormally low cholinesterase values during the study, however no correlation was observed between the depressed values and ingestion of PGPR.

Results obtained for all volunteers indicated that creatinine clearance was not effected by the consumption of exaggerated dosages of the PGPR emulsifier. Total faecal fat and nitrogen analyses, in most instances, were within the normal ranges of up to 6g/day and between 1 and 3g/day respectively. Deviations from these ranges were observed during the study, but were not attributed to the consumption of the test material.

The results obtained in this study support the extensive safety data obtained in the animal studies.

CONCLUSION

Extensive tests and published literature on PGPR and related products show that PGPR is pharmacologically non-toxic. It is digested to 98% and is utilised as a source of energy superior to starch and nearly equivalent to groundnut oil. PGPR is unlikely to interfere with normal fat metabolism or utilisation of fat-soluble vitamins. Despite the intimate relationship with fat metabolism, there is no evidence of any adverse effects on such vital processes as growth and reproduction. The biological effect of PGPR is that of a nutrient. Lengthy carcinogenic tests have demonstrated freedom from any carcinogenic hazard. Limited human studies showed that digestion takes place with tolerance to large doses and the absecnce of clinical symptoms. It is concluded that the use of PGPR in chocolate and chocolate couverture is generally recognized as safe.

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TAB 8

Quest
INTERNATIONAL

Summary of Legislative Approvals for Polyglycerol Polyricinoleate (PGPR) Admul WOL™

August 13,1997

000306

Quest International
5115 Sedge Blvd.
Hoffman Estates, IL 60192
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Toxicology

FAO/WHO Codex Alimentarious Commission

The joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1973 on the basis of no effect level determined in long-term animal feeding studies, established an ADI of 0-7.5 mg/kg body weight for "polyglycerol esters of interesterified ricinoleic acid".

Subsequently in 1975, the Codex Commission of Food Additives fully endorsed the inclusion of this substances in the Draft Standards for "Chocolate", "White Chocolate" and the "Composite and Flavoured Chocolate" up to a maximum level of 5 g/kg. it appears as number 6.29 on the CAC List A(1) of additives, which had been cleared toxicologically for use in food by JECFA. Finally, in 1976 the Codex Alimentarius Commission in Rome adopted the Draft Standard and it has now been circulated as the "Recommended International Standard for Chocolate" CAC/RS87-1976. In it, on page 7, can be found in the list of emulsifiers, 4, 2, 4 polyglycerol polyricinoleat up to a maximum level of 5 g/kg.

Legislation

EEC

The Council Directive of the 18 June 1974 and subsequent amendments on the approximation of the laws of the member states relating to Emulsifiers, Stabilisers, Thickeners and Gelling Agents for use in foodstuffs placed partial Polyglycerol Esters of Polycondensed Fatty Acids of castor oil in Article 2(a). Member states were therefore able to authorise their use in foodstuffs.

This directive has now been replaced by a new directive. The European Parliament and Concil Directive No 95/2/EC which had to be taken into all member states laws by 25 September 1996. After this date polyglycerol polyricinoleate was allowed in low and very low fat spreads and dressings at a maximum level of 4 g/kg and in cocoa based confectionery including chocolate at 5 g/kg in all 15 member states, ie France, Italy, Spain, Portugal, Luxembourg, Belgium, Austria, Germany, Greece, Denmark, Eire, UK, Sweden, Finland and Holland. Although some of these countries have not issued new national regulations as yet it is now permitted in all member states.

Legislation in Non-EU Countries

Switzerland

The Swiss authorities have stated "polyricin oleate de polyglycerol is permitted up to a level of 5 g/kg as an emulsifier in the manufacture of chocolate, white chocolate, couverture and filled chocolate. Also acceptable in tin greasing".

Canada

The Canadian Food and Drugs Regulations, table IV Item no. PI8 allows "polyglycerol esters of interesterified castor oil fatty acids" in milk chocolate and sweet chocolate up to a total of 0.5% of emulsifying agents. In addition it is permitted in chocolate coatings - maximum 0.25%.

Argentina

An amendment passed in 1988 permits the use of polyglycerol polyricinoleate in chocolate up to a maximum level of 0.5%.

Zambia

Food and Drugs Regulations 1976 permit the use of polyglycerol esters of interesterified castor oil fatty acids in chocolate.

Kenya

Food, Drugs and Chemical substances (Food Labelling, Additives and Standards) Regulations permit the addition of polyglycerol esters of interesterifed castor oil fatty acids to milk and sweet chocolate.

Australia

Regulations state that polyglycerol esters of interesterified ricinoleic acid may be added to chocolate, milk chocolate, compound chocolate, confectioner's chocolate, chocolate coating and white chocolate to a maximum level of 0.4%.

New Zealand

Polyglycerol polyricin oleate was originally permitted in couverture chocolate up to limit 0.125%. The legislation was subsequently changed to bring it into line with Australian standards.

South Africa

The South African Regulations permit the use of polyglycerol polyricinoleate in milk chocolate, white chocolate, chocolate vermicelli and flake at a maximum level of 5000 mg/kg.

India

The Indian Regulations allow polyglycerol esters of interesterified ricinoleic acid in bakery products and in chocolate to 0.2% by weight.

Hungary

The Hungarian legislation allows PGPR in chocolate up to a level of 4 g/kg.

Kuwait

Polyglycerol polyricinoleate is allowed in margerine and chocolate up to 5 g/kg.

Malaysia

The Malaysian Food Regulations permit the use of polyglycerol polyricinoleate.

Saudi Arabia

Saudi Arabian Regulations permit PGPR in certain types of chocolate up to 5 mg/kg.

Turkey

PGPR is allowed in chocolate coatings at 3 g/kg max.

Chili

PGPR is allowed in chocolate at levels up to 10 g/kg.

Updated: 13 August 1997
AJH/LJW/J1450

TAB 9

Quest
INTERNATIONAL

BIBLIOGRAPHY OF PUBLISHED LITERATURE ON POLYGLYCEROL POLYRICINOLEIC ACID (PGPR) AND RELATED PRODUCTS

October 8, 1998

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Quest International
5115 Sedge Blvd.
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QST

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End Submission

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Quest INTERNATIONAL

October 19, 1998

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Novel Ingredients Branch, HFS-206
Division of Product Policy
Office of Premarket Approval
Center for Food Safety & Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Re: GRAS notification for Polyglycerol Polyricinoleic Acid ("PGPR")

Dear Dr. Anderson:

On behalf of Quest International ("Quest"), I would like to inform you that Quest has provided a GRAS Notification for polyglycerol polyricinoleic acid ("PGPR") to Dr Rulis.

As you may recall, Quest filed a draft notification in November 1997. I have enclosed copy of the transmittal letter and the of the GRAS Notification that was provided to Dr. Rulis.

Very truly yours,

Don P. Boudreaux, Ph.D.
Technical Director, North America

Encl.

1998 OCT 21 A 11:56

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QST- 005

GRN 000009

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Quest INTERNATIONAL

November 16, 1998

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Dr. Linda Kahl
Office of Premarket Approval (HFS-206)
Center for Food Safety & Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, DC 20204

Re: GRAS Notification for Polyglycerol Polyricinoleic Acid (PGPR)

Dear Dr. Kahl:

Quest International filed GRAS Notification materials supporting our introduction into interstate commerce of the generally recognised as safe ("GRAS") substance, polyglyceride polyricinoleic acid (PGPR), with your office on October 19, 1998. I am now providing you with electronic versions of the submission in *WORD* format. I understand that your office will use the WORD document to facilitate the review of the submission.

Quest has not yet reached a decision on your request that we provide an *html* version of the notification material. I will inform you of our decision regarding the *html* documentation when the question is resolved.

Thank you for your consideration of our notification and I look forward to your decision.

Very truly yours,

Don Boudreaux, Ph.D.
Technical Director, Quest Foods North America

Encl.

000316

[redacted]

A member of ICI Group

QST- 005

Reference List for Industry Submission, GRN 000009

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000246 - 000255	Joint FAO/WHO Committee (JECFA)	Listing For Polyglycerol Polyricinoleic Acid (PGPR)	NA	NA	NA
000257 - 000271	Joint FAO/WHO Food Standards Programme -- Codex Alimentarius Commission	Codex Alimentarius Listing for Polyglycerol Polyricinoleic Acid (PGPR)	1997	Codex Alimentarius: Recommended International Standard for Chocolate	NA
000272- 000273	NA	FAO Sales Agents And Booksellers	NA	NA	NA

NA- Not applicable