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On the knowledge of the antimicrobial action of sorbic acid

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The effect of sorbic acid on aspergillus niger van Tieghem and other mold fungi

by Eva Maria Lukas (1)

(with 9 figures in the text)

As it was described in detail in the first report (Rehm and Lukas, 1963), sorbic acid has been used for some time on the basis of its antimicrobial action as an inhibitor against bacteria, yeasts and particularly against mold fungi.

It is to a great extent unknown how sorbic acid acts on the individual development stages of mold fungi. Whether, for example, the germination is greatly inhibited or whether other stages of the vegetative development are also impaired. This question was to be investigated on aspergillus niger as a model organism in connection with the investigations of possible morphological changes of the fungus by sorbic acid.

The results of Melnick, Luckmann and Gooding (1954) showed that sorbic acid is decomposed in sublethal concentrations by penicillium roqueforti and mixed cultures of mold fungi. In the absence of glucose in the lightly acid region, methyl ketones may be formed, which were also found in the decomposition of other unsaturated fatty acids under certain test conditions (Thaler and Eisenlohr (1941). On the basis of these results it is assumed that sorbic acid - just as in the animal organism (Deuel, Calbert, Anisfeld, McKeehan and Blunden 1954; Deuel, Alfin-Slater, Weil and Smith (954) - can be converted like other unsaturated fatty acids by mold fungi to CO_2 and H_2O.

An exact knowledge of the decomposition of sorbic acid by microorganisms and of the consequences of this effect is therefore necessary for the proper application of sorbic acid as an inhibitor.

In addition to the above mentioned questions, the decomposition of sorbic acid by mold fungi, particularly by aspergillus niger, was to be

investigated more fully and characterized for these reasons.

Methodology

1. Fungus material and cultivation

The tests were carried out substantially with A. niger. This fungus was selected as a test organism because it is a common food spoiler, and because it is relatively insensitive to high sorbic acid concentrations (Rehm and Lukas, 1963; Rehm 1961).

For some tests we also included various mold fungi and a yeast strain in the investigations. A compilation is found in table 10.

As a liquid nutrient substrate was used Czapek-Dox nutrient solution (5% glucose, 0.1% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4$) + 0.1% yeast extract.

After sterilization the respective amounts of sorbic acid or other inhibitors were added to this nutrient solution and dissolved under stirring. After the inhibitors had been added, the pH-value was standardized with the glass electrode, and the solution was sterilized for eactly 15 minutes in streaming vapor. Tests had shown that no decomposition of volatilization of the inhibitors takes place. Subsequently the solution was distributed sterile into the respective test vessels, (Erlenmeyer flasks, Fernbach flasks or penicillin flasks).

The inoculation was effected - depending on the objective of the test - with the following development stages of A. niger:

1. Ungerminated conidia from a vigorously shaken (½ hour) conidia suspension in Czapek-Dox nutrient solution. 0.05 ml with about 900,00 conidia per 10 ml nutrient solution were added to the test solutions.
2. Slightly germinated conidia: The conidia suspension was incubated for 10 hours at 30 degC. Under the microscope we found incipient formation of germ tubes. Inoculation with 0.05 ml per 10 ml nutrient solution.
3. Well germinated conidia: (incubation period 20 hours at 30 degC). Under the microscope good formation of germ tubes with incipient branches. Inoculation with 0.05 ml per 10 ml nutrient solution.

4. Mycelium: (incubation period 30 hours at 30 degC) From the mycelium cover- which was macroscopically well visible at that time - small mycelium floccules (about 0.5 qcm) were inoculated into the test solution (10 m) with the inoculating needle.

2 different methods were used to test the action of sorbic acid on the conidia-germination of A. niger.

Method 1: 10 ml nutrient solution with graded sorbic acid content were inoculated with 0.105 ml of a conidia suspension of A. niger, shaken and incubated at 30 degC. Sterile samples were taken in corresponding time intervals and the number of germinated conidia was determined by microscopic counting of at least 1000 conidia (10 parallels each).

Method 2: An agar slant culture of A. niger was washed with 10 ml nutrient solution of graded sorbic acid content and shaken for 30 minutes. Of these conidia suspensions one drop was placed sterile on a hollow-ground object slide (at least 10 parallel samples) and incubated in a moist chamber at 30degC. After corresponding time intervals, up to 10,000 conidia were counted.

In the tests with a solid nutrient substrate we used Czapek-Dox agar. The corresponding amounts of inhibitor were dissolved hot in nutrient agar, the pH-value was standardized by spotting on indicator paper and sterilizing for 15 minutes in streaming vapor.

Then 10 ml of this nutrient substrate were poured sterile into Petri dishes and inoculated either by two crossed inoculating lines along the diameter of the plates or by puncture.

The following inhibitors were used in the tests: sorbic acid p.a.(Merck)- after recrystallization of the commercial product with ethanol - benzoic acid p.a. (Merck) sodium formiate p.a. (Merck) and ethyl ester of p-hydroxybenzoic acid p.a. (Merck).

Other methodical data will be found in the text.

2. Analytical determinations

Dry weight determinations of Mycelium: The mycelia (Including conidia)

were sucked off, washed repeatedly with distilled water and dried at 99 degC to weight contancy. The sorbic acid content was determined in the culture solution as the difference compared to uninoculated control solutions according to the physical-chemical determination method indicated by Harden and Visser (1958). In order to eliminate as far as possible the partly very high blank values in this method, the distillation solutions were alkalized with soda solution and concentrated to 50 ml.

In addition, blank test samples of the same nutrient solution, but without sorbic acid, were also determined in order to eliminate as far as possible non-specific absorptions. (With the Czapek-Dox nutrient solution the values were between 0.5 and 0.75 mg/100 ml).

The accuracy of the method was calculated from 20 determinations:

$$s = \frac{(x - M)^2}{n(n-1)} = \pm 0.59 \text{ mg/100 ml}$$

x - m = deviation of the individual values from the mean value

n = number of determinations

Results

I. Effect of sorbic acid on germination and development of A.niger

As indicated in the formulation of the problem, we wanted to investigate first whether sorbic acid in sublethal concentrations can influence the germination and the mycelial growth of A.niger.

These tests were carried out at a pH-value of 4.5

1. Effect on conidial germination

In order to investigate the effect of sorbic acid on A.niger, we used two different methods (method I and method II, for details see methodology). Of these, method I corresponds more to the natural conditions, for example, the spoiling of foods, but the microscopic counting of the conidia was made more difficult by the relatively low spore density. In addition, small microcolonies may be formed in this method on the boundary surface liquid/glass, which could have an adverse effect on the germination tests and not yield a realistic picture.

For these reasons it seemed advisable to repeat the tests with another method (method II), where a high spore density and a uniform germination of the conidia was ensured.

The results obtained with method I are shown in fig. 1a.

With increasing sorbic acid concentration, the germination of the inoculated conidia of A.niger is more and more delayed. While 82% of the controls have germinated after 10 hours, this stage is only achieved after 24 hours in samples with 40 mg/100 ml sorbic acid. Apparently, however, not all inoculated conidia are equally inhibited in their growth by sorbic acid. With very high sorbic acid concentrations (80 mg/100 ml sorbic acid) macroscopically visible small mycelia may form on the boundary surface liquid/glass, though the major part in the nutrient solution itself (more than 99%) still shows no germination, The closer the sorbic acid concentrations come to the limiting inhibition concentration, the fewer of the inoculated conidia germinate even after prolonged test periods.

As it can be seen from fig. 1b, the results with method II are partly considerably different.

Only with 20 mg/100 ml sorbic acid do the samples of the two methods not differ substantially, while with higher sorbic acid concentrations the delay of the germination of the inoculated conidia in the hollow-ground slides is considerably reduced - compared to method I. With method II it is not possible either to verify the inhibition of the germination of a part of the conidia.

These results show that the effect of sorbic acid on the germination of the conidia of A.niger is partly changed, depending on the testing method. The action of sorbic acid may possibly be influenced by various factors, for example, by the amount of the germs with which the test solutions are inoculated, the oxygen conditions during the growth of the fungi, the absolute amounts of sorbic acid present in the nutrient solutions etc.

This different action of sorbic acid on the germination of the conidia, which is particularly pronounced close to the limiting inhibition concentration, causes the frequently varying limiting inhibition concentrations as

shown in the following table 1.

Table 1 Different development of A.niger in the presence of sorbic acid in liquid nutrient substrate after incubation for 10 days (1)

n-number	MD mg/100 ml	notes
47	100	80 mg/100 ml: in18 batches no growth
84	80	60 mg/100 ml: in 72 batches no growth
2	110	100 mg/100 ml: in 1 batch no growth

MD =maximum dose

(1) These results are no longer changed even after prolonged incubation periods (3 to 4 weeks).

It is therefore not possible to indicate exactly the limiting inhibition concentration of sorbic acid toward A.niger. At pH 4.5 it is about between 80 and 100 mg/100 ml sorbic acid. The above mentioned inhibition of the germination of a part of the inoculated conidia of A. niger can also be observed on solid nutrient substrate. Fig. 2 shows the results.

Fig. 1a and 1b. Influence of sorbic acid on the germination of A.niger.

o_____o = no addition of sorbic acid

o-----o = 20 mg/100 ml sorbic acid

0-.-.-.o = 40 mg/100 ml

x.....x = 60 mg/100 ml

X.....X = 80 mm/100 ml

Q = swelling and incipient germination of the conidia (less than 1%)

M = a few small macroscopically visible mycelia floccules have formed on the liquid surface.

Fig. 2 Formation of conidia ofA.niger on sorbic acid-containing nutrient agar.

a = no addition of sorbic acid

b = 40 mg/100 ml sorbic acid

c and d = 60 mg/100 ml sorbic acid

e and f = 80 mg mg/100 ml sorbic acid

Up to a sorbic acid concentration of 40 mg/100 ml, a uniform fungus growth can be observed. Starting from the inoculation lines, the plate is completely overgrown with the fungus (see fig. 2b).

At concentrations of 60 and 80 mg/100 ml sorbic acid, only a few colonies are formed along the inoculation line (see fig. 2c - 2f). This can be accounted for by the fact that only few conidia can germinate at these sorbic acid concentrations, while the majority of the inoculated spores remain ungerminated in the substrate.

2. Effect of sorbic acid on various development stages of A.niger

In order to observe the effect of sorbic acid on the vegetative development of A.niger we made test series with different sorbic acid concentrations and inoculated with different development stages of the fungi.

The results of these tests are compiled in table 2.

Table 2 Limiting inhibition concentration of sorbic acid toward different development stages of A.niger at a pH-value of 4.5

inoculating material	limiting inhibition conc. mg/100 ml	sorbic acid portion present in the MD undissociated mg/100 ml.	dissociated mg/100 ml
ungerminated conidia	100	64.62	35.38
slightly germinated conidia	110	71.08	38.92
well germinated conidia	135	87.24	47.76
mucelia	150	96.93	53.07

As development was evaluated in the tests if growth appeared in at least one of four parallel samples. The tests were reproduced three times.

With increasing development of the inoculating material, the limiting inhibition concentration of sorbic acid was gradually increased. While in ungerminated conidia 100 mg/100 ml sorbic acid are sufficient to cause

an inhibition, 150 mg/100 ml are required in the full-grown mycelium. It can thus be assumed that the inhibition intensity of sorbic acid toward A.niger decreases gradually with increasing development of the fungus.

Table 2 shows in addition the undissociated and dissociated sorbic acid portions present in the MD, because at this pH-value (4.5) the anti-microbial action of sorbic acid is caused substantially by the undissociated sorbic acid portions. Here too, we see that the fungus tolerates in the slightly germinated state higher concentrations of undissociated sorbic acid molecules than in the ungerminated state.

Since the findings so far relate to the relatively early development stages of A.niger, our next objective was to investigate to what extent the later growth of A.niger can be influenced by sorbic acid. To this end we investigated the influence of sorbic acid on the weight of the mycelium and on the diameter of the colonies (on solid substrate).

Fig. 3a and 3b show the results.

In the liquid nutrient substrate, the growth of the fungus is at first completely inhibited for a certain time, and then sets in spontaneously and continues at about the same rate as in the controls. Only with very high sorbic acid concentrations (80 mg/100 ml) does the development of the fungus slowly start, since only a few conidia can germinate here, as mentioned above.

Fig. 3a and 3b Influence of sorbic acid on the growth of A.niger in the liquid (fig.3a) and in the solid (fig.3b) nutrient substrate (tests repeated 10 times). For legend see fig. 1.

*B = at this sorbic acid concentration the fungus developed only in two out of ten parallel samples

The mean errors of the determinations are also entered in the diagrams.

On solid nutrient substrate the inhibition of the germination of A. niger is not so pronounced. Here the development of the fungus already start after 27 hours at 40 mg/100 ml sorbic acid (in liquid substrate after 36 hours),

while at 60 mg/100 ml this stage is reached after 36 hours (in the liquid medium after 48 hours), (see fig. 3b).

In most cases the mycelia growth rate of the fungus is not reduced by sorbic acid and the transition from the logarithmic to the stationary growth phase takes place after a certain mycelium weight has been attained, independent of the sublethal sorbic acid concentrations in the nutrient solution.

3. Effect of sorbic acid on the morphology of A.niger

In the tests we observed frequently in the acid pH-region at relatively high sorbic acid concentrations macroscopically visible changes in the formation of the mycelium of A.niger.

Up to about 40 mg/100 ml sorbic acid, the fungus forms closed mycelium covers with good formation of conidia, which do not differ from the controls. But the more the sorbic acid doses approach the limiting inhibition concentrations, (60 to 80 mg/100 ml) the more frequently do the macroscopically as well as microscopically; visible changes appear in the mycelium of the fungus:

1. partly no closed mycelium covers are formed any more, only a few individual colonies which differ from the colonies by their lumpy form (see fig.4).
2. In the mycelium lumps, which appear mainly at the boundary surface liquid/glass, less in the cover center of the mycelium, sclerotia (diameter ab. 1 mm) are formed which have a reddish brown color and which show in microscopic sections the characteristic apparent parenchyma cells.

Fig. 4 Macroscopically visible morphological mycelium changes of A.niger by sorbic acid

1 = without sorbic acid; 2 - with sorbic acid (80 mg/100 ml).

Sclerotia appear in some strains of the aspergillaceae - occasionally also in A.niger strains (Peters and Rippel-Baldes 1950). but they are not wide-spread. The strain of A.niger van Tieghem used in this study does not have this property under normal conditions.

3. If a macroscopically visible lumping of the mycelium with sclerotia

appears, microscopic examination of the mycelium also show morphological changes of the fungus hyphae (see fig. 5). Shorter and blistery swollen hyphae appear then which can only be interpreted on the basis of the present experiences as nonspecific involution-forms because similar changes can also appear by the influence of alkali-ions. (Fiedler 1959) antibiotics (Julius 1952), caffeine phosphates (Mueller and Stapp, 1925), some preservatives (Rehm and Stahl 1960) and others.

Fig.5 Microscopically visible morphological changes of A.niger by sorbic acid. Only hyphae from the underside of the mycelium cover which were in direct contact with the sorbic acid-containing nutrient solution were examined in the tests. a) no addition of sorbic acid; b) addition of 80 mg/100 ml sorbic acid.

Fungistatic or fungicidal action of sorbic acid

It was extablished so far in the tests that the conidia of A.niger are inhibited in their development by sorbic acid. Not clarified, however, was the question whether and to what extent the conidia, which were hindered in their germination by certain sorbic acid concentrations, were still capable of development or not. For this reason we transferred conidia which showed no growth after 10 days in a sorbic acid-containing nutrient solution, into a sorbic acid-free nutrient solution and incubated them for another 10 days at 30 degC. The result of these tests is shown in table 3.

Up to a sorbic acid concentration of 60 to 80 mg/100 ml, only a fungistatic action of sorbic acid can be expected, because after 10 days of action by sorbic acid the conidia are a still capable of development in all samples. Starting from about 90 mg/100 ml sorbic acid, partial fungicidal action appears already, which increases with increasing sorbic acid concentration. But these sorbic acid concentrations are still partly under the MD (80 to 100 mg/100 ml) for other conidia of A.niger, which could thus still develop in such sorbic acid-containing nutrient solutions. Only starting from 120 mg/100 ml sorbic acid does a clear fungicidal action appear

for all conidia of A.niger.

These results were not changed substantially in the inoculation with full grown mycelia. But here somewhat higher sorbic acid concentrations (ab. 135 mg/100 ml) had to be used in order to achieve an absolutely lethal effect. The undissociated sorbic acid portions likewise listed in table 3 - which have at this pH-value of 4.5 a substantially antimicrobial effect - show that up to about 50 mg/100 ml only an inhibition of the development appears, while above 50 mg/100 ml an increasing destruction of the cells can be observed.

Table 3 Germination of conidia after the action of sorbic acid has stopped

	undissociated mg/100 ml	dissociated mg/100 ml	3
60 - 80	38.77 - 51.70	21.23 - 28.30	all conidia germinated
90 - 100	58.16 - 71.08	31.84 - 38.92	conidia germinated only partly
120	77.54	42.46	no germination of conidia

1) - sorbic acid concentration which had a germination-inhibiting effect, mg/100 ml

2) respective sorbic acid portion

3) germination of conidia in sorbic acid-free nutrient solution

5. Resistance build up of A.niger against sorbic acid

It is known that even prolonged cultivation of E.coli can not produce in sublethal sorbic acid concentrations any marked resistance against sorbic acid (Lueck and Rickerl, 1959; Rehm 1961). This question has not yet been investigated for A.niger, and it was interesting to know whether and to what extent resistance buildup against sorbic acid can be expected in this fungus.

In order to achieve gradual habituation to sorbic acid, the fungus was cultivated for a few passages on low sorbic acid concentrations. Only then where the conidia inoculated into such high sorbic acid concentration that a fungus development could still be achieved (fig.6).

With subthreshold amounts of sorbic acid (40 to 60 to 80 mg/100 ml) a good development of the fungus is still observed over 10 to 14 passages. After five passages on sublethal sorbic acid concentrations (see fig.6), the fungus can still be cultivated over 9 passages in a concentration of 100 mg/100 ml sorbic acid - at which the major part of the conidia are normally inhibited or killed in the first generation already.

Growing the fungus in nutrient solution with 120 mg/100 ml sorbic acid was only possible after 4 passages in 100 mg/100 ml. In this concentration the fungus was completely inhibited after 4 passages, after initial good development.

After several passages on100 and 120 mg/100 ml sorbic acid, a development of A.niger could be achieved once at 130 mg/100 ml. But after one passage already, the conidia were no longer capable of development.

As it can be seen from the investigations, the degree of adaptation of A.niger is low within the tested 14 passages (factor of maximum resistance increase = 1.3 - 1.6) and shows little constancy.

Fig.6 Resistance development of A.niger against sorbic acid

a = cultivation of A.niger inthe indicated sublethal sorbic acid concentrations

b = successive growth of A.niger in the indicated sorbic acid concentrations

c = resistance buildup of A.niger against sorbic acid

W = growth limit of grown non-resistant conidia of A.niger.

II. Characterization of the sorbic acid decomposition

The investigations have given us so far an insight into the effect of sorbic acid on conidia germination, mycelium growth and morphology of A.niger.

Our next goal is to investigate the decomposition of sublethal amounts of sorbic acid by this fungus. Since the pH-value of the nutrient medium plays an important role in the antimicrobial action of sorbic acid (Rehm and Lukas 1963), the tests were carried out both at a pH-value of4.5

(undissociated acid portion = 64.62%) and at a pH-value of 7.5 (undissociated acid portion = 0.18%).

1. Methodical prerequisites for demonstrating the decomposition of sorbic acid

The decomposition of sorbic acid was to be determined quantitatively by the disappearance of the sorbic acid introduced earlier into the nutrient solution. It was therefore necessary to determine first if it is possible at all to recover sorbic acid quantitatively in the test solutions.

Due to its structure as a polyunsaturated organic compound, an auto= oxidative decomposition of sorbic acid can set in, (Sabalitschika and Marx, 1959); on the other hand, there is a possibility that sorbic acid forms with the existing substrate compounds which can no longer be determined with the applied method. The investigations were carried out mostly in a synthetic nutrient solution (Czapek-Dox) under exactly defined conditions, which were always maintained later (see methodology).

The results are compiled in table 4.

Table 4 Detection of sorbic acid in Czapek-Dox nutrient solution

added amount of sorbic acid mg/100 ml	-recovered amount of sorbic acid (mg/100 ml) after 2 days	10 days	30 days
20	201.	19.93	18.50
60	60.0	60.0	60.5
100	100.0	100.0	100.5

After 10 days the added amount of sorbic acid can be determined quantitatively (within the error margin of ± 0.59 gm/100 ml). After 30 days the samples with relatively llow sorbic acid concentrations (20 mg/100 ml) show beginning decomposition, and about 7.5% or the added sorbic acid can no longer be detected. Amounts of sorbic acid of 60 and 100 mg/100 ml can be recovered quantitatively even after this long test period (see table 4).

It can thus not be expected that the results are falsified by self-decomposition or inactivation of the sorbic acid, because all other investi-

gations were partly carried out in much shorter test periods.

Table 5 Detection of sorbic acid in the inoculated nutrient solution

added amount of sorbic acid mg/100 ml	recovered (1) amount of sorbic acid after inoculation with ungerminated conidia of A.niger, 100 mg/100 ml
100	99.5
80	80.6
60	59.9
40	39.60
20	19.40

1) These data relate only to samples which had been examined before the incipient germination of the inoculated conidia of A.niger.

Another factor which could possibly simulate a false result in an adsorption of the sorbic acid on the conidia of A.niger. For this reason sorbic acid-containing nutrient solutions were inoculated with conidia of the fungi (900,000 conidia per 10 ml nutrient solution) and the sorbic acid content in the samples was determined before the incipient germination (checked microscopically).

As it can be seen from Table 5, the sorbic acid is not adsorbed here on the inoculated spores or at least not so firmly that it could no longer be detected completely. We have thus found that no false results can be expected by the above indicated factors under certain test conditions.

2. Decomposition of sorbic acid on inoculation with ungerminated conidia of A.niger at a pH-value of 4.5

Fig. 7 shows the result of the investigation of the sorbic acid decomposition by A.niger at a pH-value of 4.5 after different development periods.

With incipient development on A.niger, the sorbic acid disappears extremely fast from the nutrient substrate. With an initial concentration of 20 mg/100 ml sorbic acid, the decomposition starts after 10 hours and is completed after 27 hours, while at 40 mg/100 ml, the periods are between 13 and 46 hours and at 60 mg/100 ml between 36 and 84 hours.

Fluctuation in time are due to the above described different sensitivity

of the inoculated conidia material because the sorbic acid can only be removed from the nutrient substrate when the inoculated conidia begen to germinate. This can be the case after different test periods (fig.7).

With high sorbic acid concentration close to the MD, the time variability of the sorbic acid decomposition increases (see fluctuations of the single determinations at 60 mg/100 ml) because condia can only develop sporadically in the sorbic acid-containing solution nutrient. But as soon as some conidia begin to germinate, these are sufficient to completely remove from the nutrient substrate sorbic acid concentrations which are lethal for most of the inoculated conidia. If there is no germination of the fungus, the sorbic acid is not attacked.

Fig.7 Decomposition of sorbic acid by A.niger with a pH of 4.5

Fig. 8 relation between sorbic acid decomposition and length of germ tubes of A.niger. For explanation of drawing see fig. 7.

Q = swelling and incipient formation of germ tubes (length under 1 mu m) of the conidia. The figure contains the mean values and the mean errors of at least 100 measurements. The time variability of the sorbic acid decomposition had a disturbing effect in the tests, particularly in reproductions of the findings. For this reason it seemed advisable to find a reference quantity which takes better into account these biologically caused differences in the results. Since the decomposition of sorbic acid sets in with incipient germination, and is to a great extent completed when the mycelium is visible, it was tried to use the length of the germ tubes as a reference quantity. Fig. 8 shows the germ tube lengths of the fungus conidia after various test periods, measured in microscopically in the culture solutions.

There is thus a relation between the sorbic acid decomposition and the length of the germ tubes. With a germ tube length of about 6 mum, 49%, in a later development stage (11 mum) 75% of the sorbic acid added are no longer detectable. As soon as the germ tubes have reached a length of 30 to 35 mum,

the sorbic acid has to a great extent disappeared from the nutrient solution.

These results are principally not changed by the absolute sorbic acid concentrations if they are under the MD of the fungus. Since the germination of the conidia depends on the time with different sorbic acid concentrations, a certain relation can also be derived between decomposition time and sorbic acid concentration. With increasing sorbic acid content, the time interval increases which is required for the complete decomposition. The rate at which the sorbic acid is decomposed by the fungus is in all sorbic acid concentrations about 1.2 mg/100 ml per hour (see table 6).

Table 6 Rate of sorbic acid decomposition with different sorbic acid concentrations (pH 4.5).

applied sorbic acid concentration, mg/100 ml	period of sorbic acid decomposition, hours	rate of sorbic acid decomposition mg/100 ml per hour
20	16	1.20
	15	1.25
	16	1.20
40	33	1.21
	34	1.18
	30	1.33
60	48	1.25
	54	1.11

Apart from inhibiting the germination - which influences the start of the sorbic acid decomposition - the sorbic acid may also cause a delay in very early development stages of A.niger. Particularly the induction phase of the fungus seems to be affected here, because, as soon as the sorbic acid has disappeared from the nutrient substrate, a normal further development and formation of conidia is observed, unless the sorbic acid concentrations are so high that morphological changes are already occurred.

3. Decomposition of sorbic acid by different development stages of A.niger at a pH of 4.5

It was shown that A.niger does not go beyond a certain development stage before the sorbic acid has disappeared from the nutrient substrate.

This observation raises the following questions. Can the fungus remove the sorbic acid from the substrate only in very early development stages or is it possible for A.niger to attack the sorbic acid in a later development stage too? Should this be the case, it remains to determine whether only a limited portion of the acid is decomposed by the fungus or whether a continuous decomposition can be achieved, for example, by the contiguous addition of sublethal sorbic acid concentrations.

In order to clarify these questions, Fernback Flasks (450 ml), which contained each 100 ml nutrient solution with 40 mg/100 ml sorbic acid content, were incubated for 48 hours at 30 degC after the inoculation. Subsequently an aliquot portion of the culture solution (25 ml) was removed sterile and new nutrient solution (25 ml) with a corresponding nutrient content was added, so that the end concentration of the entire culture solution was again 40 mg/100 ml. After each 24 hours the removal and addition resp. was repeated, as explained above.

Table 7 Decomposition of sorbic acid by different development stages of A.niger

1 -	2	3 a	3 b	4	5
1. addition	40	swelling of inoculated conidia	good germination of conidia; length of germ tubes ab.35 mum	34	1.18
2nd addition	80	good germination of conidia; length of germ tubes ab. 35 mum	mycelium cover not quite closed with incipient sporulation	24	1.65
3rd addition	120	mycelium cover not quite closed with incipient sporulation	unchanged cover closed with very good sporulation	24 each	1.65
4 to 16th	160-640	mycelium cover closed with very good sporulation	unchanged	24 each	1.65

1 - continuous addition of doses of 40 mg/100 ml sorbic acid; 2 - total concentration of sorbic acid added, mg/100 ml; 3 - development stages of fungus; a - at beginning of sorbic acid decomposition; b - after completion of sorbic acid decomposition. 4 - time up to which all sorbid acid has been decomposed, in hours; 5 - rate of sorbic acid decomposition, mg/100 ml per hour. The results of these tests are indicated in table 7.

The fungus is capable of decomposing the sorbic acid in all tested growth phases. Thus a continuous decomposition of 640 mg/100 ml sorbic acid - an amount which is 6.4 times higher than the MD of the fungus - could be achieved in the tests within 20 days (16 additions of 40 mg/100 ml sorbic acid). The upper limit is probably much higher, because the tests were stopped after the 16th addition.

The development of the fungus is not visibly influenced by the continuous addition of sublethal doses of sorbic acid apart from the lumping of the mycelium and the formation of sclerotia, which also appear with a single addition of sorbic acid.

The rate at which the sorbic acid is removed from the culture substrate has increased in the later development stages of A.niger from about 1.20 mg/100 ml per hour (inoculation with ungerminated conidia) to about 1.70 mg/100 ml per hour. It is possible that a sorbic acid concentration which prevents to a great extent the germination of the conidia no longer has such a pronounced inhibiting effect for a full-grown mycelium cover.

4. Decomposition of sorbic acid by A.niger at pH 7.5

In the first report (Rehm and Lukas 1964) the influence of the pH value on the antimicrobial action of sorbic acid was discussed in detail.

Here we were interested in the question whether and to what extent the effect of a microbial sorbic acid decomposition can be influenced by varying the hydrogen-ion concentration of the nutrient substrate (fig.8).

Table 8 Decomposition of sorbic acid by A.niger at different pH values

pH value of nutrient solution	amount of sorbic acid decomposed completely after 10 days mg/100 ml
3.00	35
4.50	60
6.00	450 1)
7.00	2500
7.50	3000

1) Here we did not test the maximum sublethal sorbic acid concentration.

As the results show, the decomposed sorbic acid concentrations vary at differentpH-values. The course of the sorbic acid absorption at a pH 7.5 can be seen from fig. 9. Extremely high sorbic acid concentrations can be removed by A.niger from the nutrient substrate at this pH.

Since a time variability appears here at high sorbic acid concentrations - similar as at pH 4.5 - two parallel determinations (2500 mg/100 ml sorbic acid) are shown in fig. 9 (I and II).

The germination of the inoculated conidia and the development of the fungus are delayed by sorbic acid in the alkaline pH-range. But after a certain time the fungus forms a closed dense mycelium cover on the surface of the culture solution, in contrast to pH 4.5, though sorbic acid is still contained in the nutrient solution. An exception are the samples with a very high addition of sorbic acid (2500 mg/100 ml) where the development of the fungus is greatly inhibited (see fig. 9).

Fig. 9 Decomposition of sorbic acid by A.niger at pH 7.5

o - - - 0 = 1000 mg/100 ml sorbic acid

o -.-.-.-0 = 1500 mg/100 ml

x.....x = 2500 mg/100 ml

M = some small mycelium floccules have formed on the liquid surface

SM = good mycelium formation, but not the entire liquid surface is covered with mycelia

MC = closed mycelium cover with incipient sporulation

SMC = dense closed mycelium cover with very good sporulation

x = the numbers on the curves indicate the length of the germ tubes of the germinating conidia.

The inhibiting effect of sorbic acid on the development of the fungus is reduced in the alkaline pH-range, because the fungus is not stimulated to form sclerotia despite very high absolute sorbic acid concentrations, and the rate at which the sorbic acid is removed from the nutrient substrate is about three to four times higher than at pH 4.5. About 3.2 to 4.5 mg sorbic acid/100 ml are decomposed per hour. This may be due to the enzyme activity of the fungus, which is increased at this pH, because the optimum pH of most enzymes, is in the weakly acid and neutral region (Karlson 1961).

An important factor which can influence the antimicrobial action of sorbic acid is the presence of other preservatives in the substrate, and it was to be investigated whether the sorbic acid can also be decomposed in sublethal doses by A.niger in their presence.

As combination partners for sorbic acid we selected a compound each with known (Rehm and Stahl 1960), additive (benzoic acid), antagonistic (ethyl ester of p-hydroxybenzoic acid) and synergistic tendency (sodium formiate).

As it can be seen from table 9, the decomposition of sorbic acid is not influenced by the presence of different benzoic acid concentrations, if the percentual decompositions of both mixing partners are under 100%. With incipient development of A.niger, the sorbic acid disappears from the nutrient subtrate.

Table 9 Decomposition of sorbic acid by A.niger in the presence of other preservatives (pH 4.5)

sorbic acid(1) + benzoic acid		decomposition of sorbic acid after 7 days in %
my/100 ml	mg/100 ml	
20	125	100
60	50	100
60	125	0

sorbic acid + sodium formiate		decomposition of sorbic acid after 7 days in %
mg/100 ml	mg/100 ml	
20	2500	97.5-100
60	1000	100
80	500	0

sorbic acid + ethylester		decomposition of sorbic acid after 10 days in %
mg/100 ml	mg/100 ml	
20	35	90.5-96.5
40	35	98.5
60	40	0

1) Absolute limiting inhibition concentration of:

sorbic acid = 100 mg/100 ml (pH = 4.5)

benzoic acid = 250 mg/100 ml (pH = 4.5)

sodium formiate = 5000 mg/100 ml (pH = 4.5)

ethyl ester of p-hydroxybenzoic acid = 35 mg/100 ml (pH ; 4.5)

With an additive tendency there thus seems to be no interrelation between the mechanisms of action of the two mixing partners which could disturb the decomposition of sorbic acid.

Sodium formiate, combined with sorbic acid, shows in most concentration ranges a synergistic tendency. If the percentual concentrations of the four mixing partners are not sufficient to produce inhibition, the sorbic acid is removed here to o from the nutrient substrate with beginning development of A.niger.

If the sorbic acid is combined with the ethyl ester of p-hydroxybenzoic acid, the sum of the percentual concentrations of both partners which are sufficient to produce inhibition, is in most concentration ranges above 100%.

Here too, the sorbic acid is decomposed by A.niger. Partly a weak

development of the fungus may even occur after the sorbic acid has disappeared from the nutrient substrate, though the second combination partner (ethyl ester) remains in lethal concentrations in the substrate. At the time of the complete sorbic acid decomposition, a few small mycelia have formed on the liquid surface (diameter about 0.5 cm) . After another 18 days the size of these mycelia, which now show partly scarce sporulation - has about doubled. But if the relative ethyl ester concentrations are under 100%, the fungus can develop much better (after 18 days a dense closed well-sporulating mycelium cover has formed). It can thus be assumed that the development of A.niger is greatly reduced though not completely stopped in the presence of lethal ethyl ester concentrations - if an initial development is enhanced by sorbic acid (as an antagonist).

5. Decomposition of sorbic acid by other micro-organisms

Finally the question was to be clarified whether the decomposition of sorbic is wide-spread among fungi or whether this capacity is limited only to a few species. As mentioned above, penicillium roqueforti and mixed cultures of mold fungi are said to be able to remove sorbic acid from the substrate (Meilnick, Luckmann and Gooding, 1954).

Various mold fungi and a yeast strain were tested for their capacity to decompose sorbic acid. Table 10 shows the result.

All tested fungi are more sensitive to sorbic acid than the A.niger strain which had been used as a test object in the previous tests. Thus very different sorbic acid concentrations are necessary for inhibiting species of the same genus.

All tested fungus strains had completely decomposed the sorbic acid after 7 days. Saccharomyces cerevisiae attacks sorbic acid only in a very late development stage, and even than the decomposition is very slow and incomplete, compared to mold fungi.

Table 10 Decomposition of sorbic acid by various fungi at a pH 4.5 (temperature 30 degC)

name of speciel	a	b	c
aspergillus oryzae(Ahlburg) Cohn	20-50 1)	10	100.0
Penicillium expansum(Link) Thom	40	20	99.5
Penicillium spec. 19/1	40-60 1)	20	98.8
Alternaria spec. 17/1	10	5	97.5
Trichoderma ligorum(Tode) Harz	60-80 1)	50	100.0
Mucor racemosus Fresenius	10	5	97.0
Rhizopus nigricans Ehrenberg	20	10	96.0
Saccharomyces cerevisiae (Backerhefe)	> 50	50	5.0
(baker's yeast)		20	37.5 2)

a - limiting inhibition concentration mg/100 m

b - amount of sorbic acid applied mg/100 ml

c - decomposition of sorbic acid after 7 days in %

1) These tests were carried out on solid nutrient agar (Czapek-Dox), all others in liquid nutrient substrate.

2) The decomposition of sorbic acid was determined only after 10 days.

Discussion

The results of the first part of the study have shown that the inhibiting action of sorbic acid causes in sublethal concentrations among other a delay of the germination of A.niger. Furthermore it was found that the conidia show a different sensitivty to sorbic acid so that different inhibition concentrations had to appear.

The delay of the germination can be caused by physical- chemical conditions, for example, by the fact that the permeability of the conidia membrane is substantially changed by sorbic acid. Since various other similarly acting inhibitors are said to influence the membrane, it is logical to assume here similar effects (Wyss. 1948; Hotchkiss 1946; Gale and Taylor 1947; Mitchell and Crowe 1947).

The size and age of the conidia of A.niger in a culture vary frequently, and one could see in this fact the cause for the different sensitivity to sorbic acid.

Sorbic acid, as an unsaturated fatty acid, can be adsorbed to a great extent on the conidia so that we have to figure with higher absolute concentrations on the membrane than exist in the solution.

Starting from certain concentrations, the conidia are damaged irreversibly and are no longer viable, and they can hardly be grown with a high resistance to sorbic acid. This argues in favor of the assumed relatively nonspecific mechanism of action which is seen in the blocking of SH-groups of various ferments (Whitaker 1959; Morgan and Friedemann 1938). But possibly only an exclusive action on the conidia membranes has taken place, which has prevented complete germination and which manifests itself now in a fungicidal sorbic acid action.

Even more difficult to evaluate is the question of the action of sorbic acid if we investigate the effect on vegetative cells. Here the great decomposition effect appears which has been characterized in the second part and which is caused by early development stages, which makes the interpretation of the antimicrobial action difficult. The greater insensitivity of the mycelia to sorbic acid compared to the conidia of the fungus is probably simulated only by the continuous decomposition of the sorbic acid. If it were possible to eliminate this decomposition, the sensitivity of the vegetative cells of the fungi would probably be substantially changed.

The decomposition effect of sorbic acid has raised a number of new questions which could not be answered in the present investigations, for example: Is sorbic acid decomposed within the mycelia or in the substrate: What are the decomposition stages of the sorbic acid like? Is sorbic acid included in the metabolism of A.niger as a normal fatty acid or is it merely detoxified by a certain mechanism, such as benzoic acid with glycocoll in other organisms? These and other questions will be investigated in the next report.

Both actions, decomposition and microbial action, are possibly interrelated insofar as the decomposition is absolutely weaker in greatly inhibiting regions of the sorbic acid, for example, at pH 4.5, than in those regions where sorbic acid hardly shows any microbial action,because dissociated ions are primarily present here, for example, at pH 7.5.

For the practical application of sorbic acid as a preservative the very rapid decomposition as well as the decomposition by mycelia in the first germination stage are of great importance, since slightly germinated conidia of mold fungican easily get into foods which must be preserved with sorbic acid and which can then decompose the sorbic acid. Whether this is possible in foods must be investigated. The relatively frequent decomposition of sorbic acid among the mold fungi is also of importance here.

In the investigation of the decomposition of sorbic acid we found the interesting case in combination investigations that an antagonist (in this case sorbic acid) disappears in the source of the development of the microorganism from the substrate and the other combination partner (ethyl ester or p-hhdroxybenzoic acid) is now present in lethal concentrations. If A.niger still has developed further - though slowly - though the concentrations of the ethyl ester were normally lethal, this is probably due to the fact that larger amounts of ethyl ester were adsorbed on the mycelia and thus were not subject to their action. In a previous development stage an inhibition of the fungus would certainly have to be expected. Naturally other explanations are not excluded, for example, a greater insensitivity of older development stages of A.niger against the ethyl ester, compared to the sensitivity of the conidia.

The effects of sorbic acid on the morphology of the fungus must be considered to a great extent non-specific, as mentioned above.

Summary

The investigations of the antimicrobial action of sorbic acid against A.niger brought the following results:

1. The inhibiting effect of sorbic acid is directed substantially against the germination of the conidia and can cause in sublethal concentrations among others a delay of the germination of the fungus. The conidia show a varying sensitivity to sorbic acid.

2. Starting from certain sorbic acid concentrations, the conidia are damaged irreversibly and are no longer capable of germination; they can not be grown with a high resistance to sorbic acid.

3. The inhibiting effect of sorbic acid on the vegetative cells is much weaker (up to 50%) compared to that on the conidia. This is probably due to the fact that the sorbic acid is extremely rapidly decomposed with incipient germination of the conidia and can no longer be detected in the substrate by macroscopically visible mycelium formation.

4. The effect of sorbic acid on the morphology of the fungus (formation of sclerotia and involution forms)is to a great extent non-specific.

5. The decomposition of sorbic acid is not impaired by pH variations of the nutrient substrate nor by the presence of other preservatives.

6. Decomposition of sorbic acid was also found in various other mold fungi. In saccharomyces cerevisiae the decomposition of sorbic acid was slow and incomplete.

We are indebted to Dr.Habil. H.J. Rehm for suggesting this study and for his valuable advice.

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(Translated by Carl Demrick Associates,Inc/IE/t)

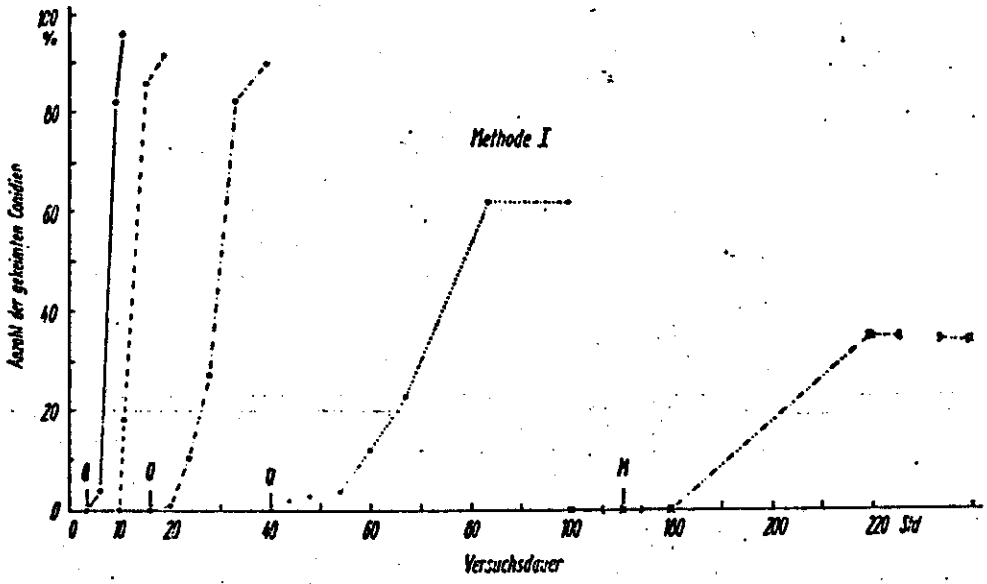


Figure 1a

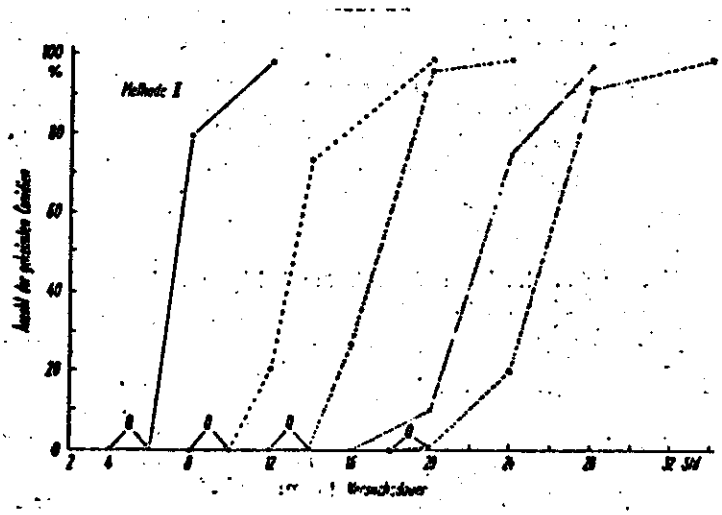


Figure 1b

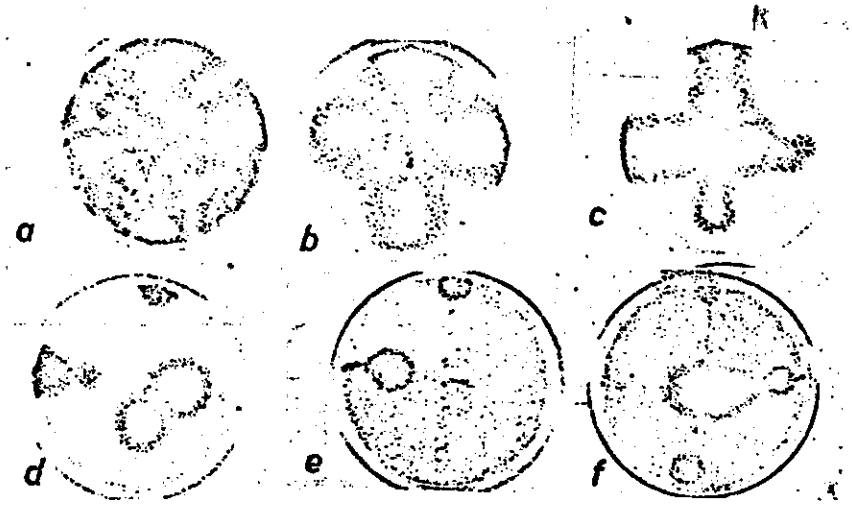


Figure 2

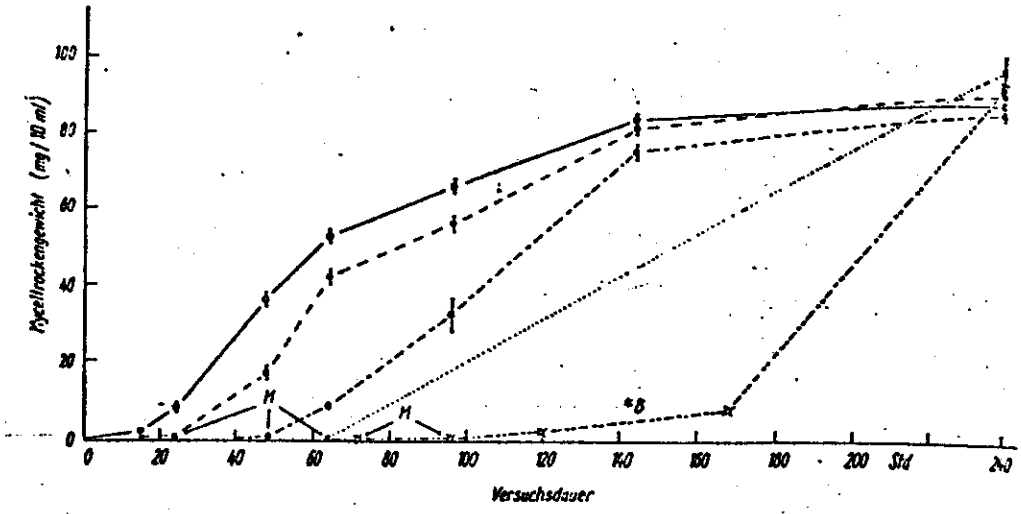


Figure 3a

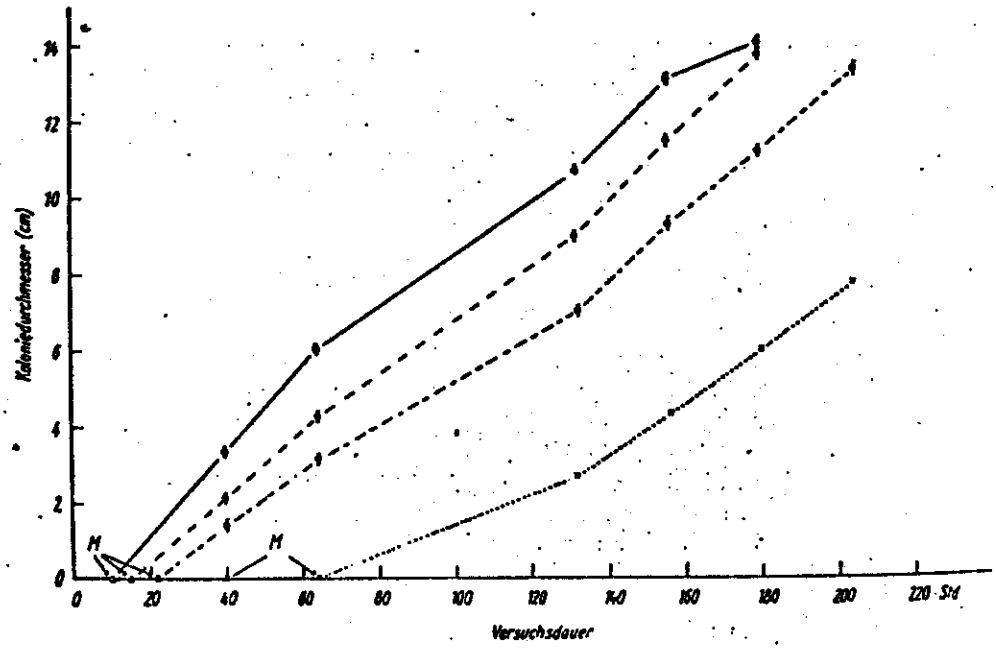


Figure 3b

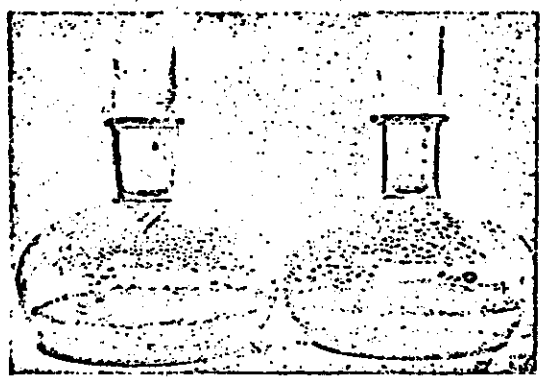
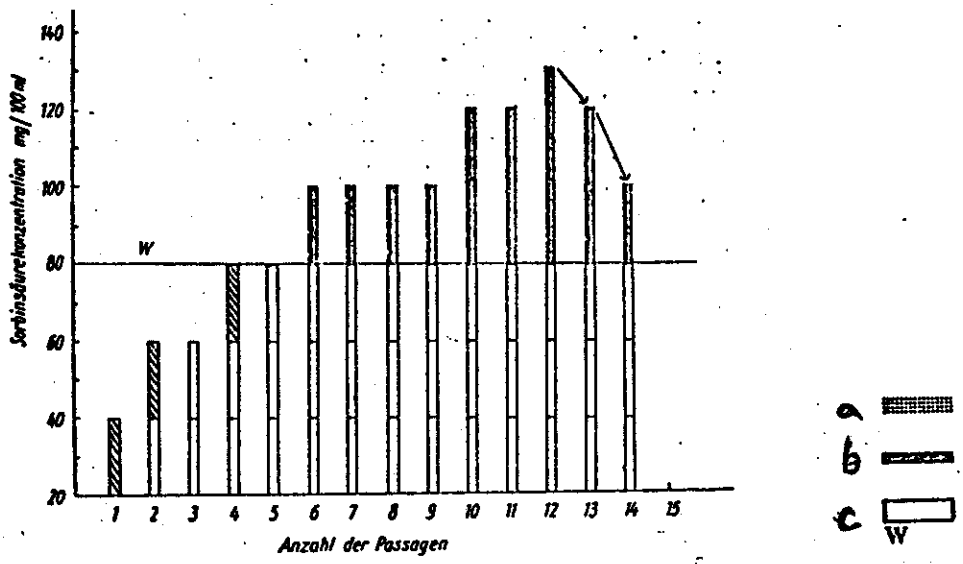


Figure 4

a b

Figure 5



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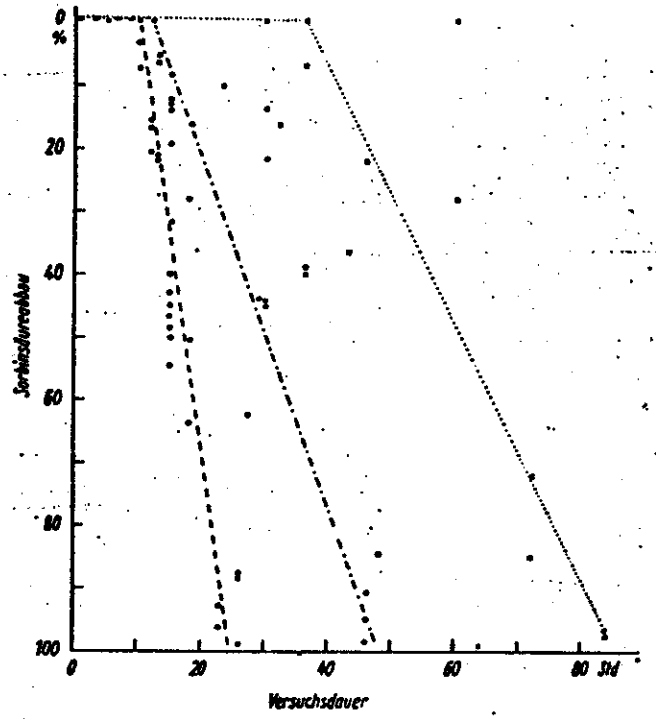

Figure 7

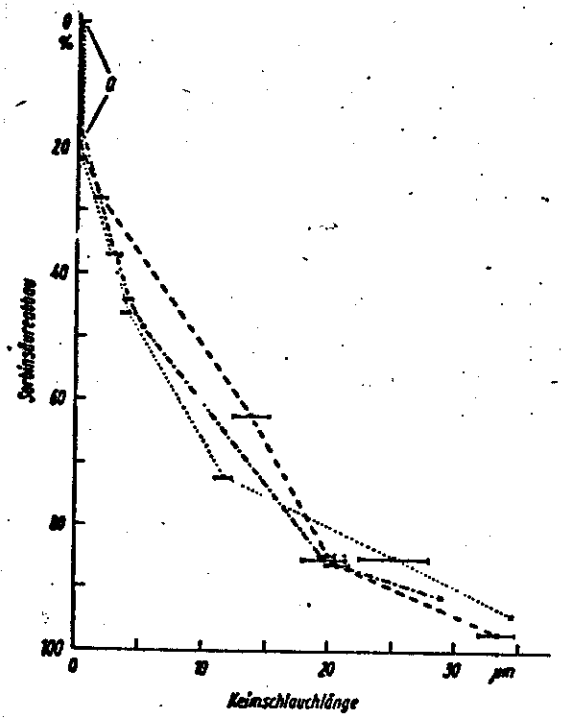

Figure 8

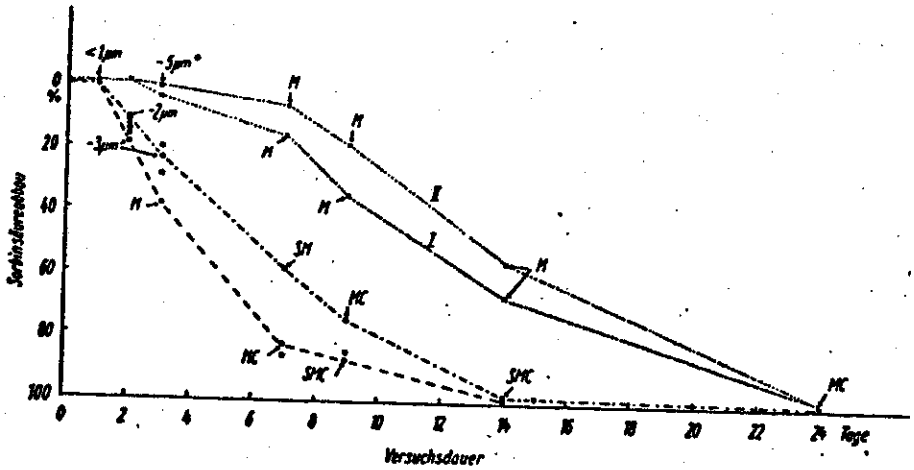

Figure 9

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The successive irradiations induced also an increase of the yield of citric acid (Maximum by the 3rd or 4th Irradiation). Further irradiations induced a constant decrease of the yield of Citric Acid, the slowliest after 23 h irradiation. By irradiations with larger doses of UV-rays the yield of Citric Acid is high, f. e. by 26 h irradiation 321% and 292% in ratio to the initial yield of the strain *Aspergillus niger* X_1. *Aspergillus niger* after irradiation did not produce oxal acid.

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Anschrift der Verfasser:

Irena Tumidalska, Olga Ilnicka und Jerzy Sożyński, Wroclaw, Polen, Wyższa Skola Ekonomiczna, Katedra Mikrobiologii, ul. Komandorska 118/120.

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[Aus der Deutschen Forschungsanstalt für Lebensmittelchemie, München]

Zur Kenntnis der antimikrobiellen Wirkung der Sorbinsäure

II. Mitteilung

Die Wirkung der Sorbinsäure auf *Aspergillus niger* van Tieghem und andere Schimmelpilze

Eva-Maria Lukas [1])

Mit 9 Abbildungen im Text

Wie in der 1. Mitt. (Rehm und Lukas 1963) ausführlich dargestellt wurde, wird die Sorbinsäure seit einiger Zeit auf Grund ihrer antimikrobiellen Wirkung als Hemmstoff gegen Bakterien, Hefen und besonders gegen Schimmelpilze angewandt.

Es ist dabei weitgehend unbekannt, wie die Sorbinsäure auf die einzelnen Entwicklungsstadien der Schimmelpilze wirkt, ob z. B. die Keimung stark gehemmt wird oder ob auch verschiedene Stadien der vegetativen Entwicklung geschädigt werden. An *Aspergillus niger* als Modellorganismus sollten diese Fragen, verbunden mit der Untersuchung eventueller morphologischer Veränderungen des Pilzes durch Sorbinsäure, untersucht werden.

Die Ergebnisse von Melnick, Luckmann u. Gooding (1954) konnten zeigen, daß die Sorbinsäure in subletalen Konzentrationen durch *Penicillium roqueforti* und Mischkulturen von Schimmelpilzen abgebaut wird. Hierbei kann in Abwesenheit von Glukose im schwach sauren Gebiet eine Methylketonbildung auftreten, wie sie auch beim Abbau anderer ungesättigter Fettsäuren unter bestimmten Versuchsbedingungen nachgewiesen worden ist (Thaler u. Eisenlohr 1941). Auf Grund dieser Ergebnisse wird angenommen, daß die Sorbinsäure — ähnlich wie im tierischen Organismus (Deuel, Calbert, Anisfeld, Mc Keehan u. Blunden 1954; Deuel, Alfin-Slater, Weil u. Smith 1954) — wie andere ungesättigte Fettsäuren durch Schimmelpilze zu CO_2 und H_2O veratmet werden kann.

Für eine gerichtete Anwendung der Sorbinsäure als Hemmstoff sind daher die genaue Kenntnis des Sorbinsäureabbaus durch Mikroorganismen sowie die Folgeerscheinungen dieses Effektes notwendig.

[1]) Die Arbeit stellt einen Auszug aus der Dissertation „Untersuchungen über die Einwirkung subletaler Sorbinsäuremengen auf *Aspergillus niger* van Tieghem" dar. Technische Hochschule München 1963.

Neben der schon erwähnten Fragestellung sollte aus diesen Gründen der Sorbinsäureabbau durch Schimmelpilze, besonders durch *Aspergillus* (A.) *niger* näher untersucht und charakterisiert werden.

Methodik

1. Pilzmaterial und -züchtung

Die Versuche wurden im wesentlichen mit *A. niger* durchgeführt. Dieser Pilz wurde deshalb als Testorganismus ausgewählt, weil er als Lebensmittelverderber weit verbreitet und relativ unempfindlich gegen hohe Sorbinsäurekonzentrationen ist (Rehm u. Lukas 1963; Rehm 1961).

Für einige Versuche wurden außerdem verschiedene Schimmelpilze und ein Hefestamm in die Untersuchungen einbezogen. Eine Zusammenstellung findet sich in Tab. 10.

Als flüssiges Nährsubstrat diente Czapek-Dox-Nährlösung (5% Glucose, 0,1% KH_2PO_4, 0,2% $NaNO_3$, 0,05% $MgSO_4$, 0,05% KCl, 0,001% $FeSO_4$) + 0,1% Hefeextrakt.

Nach dem Sterilisieren wurden dieser Nährlösung die jeweiligen Mengen an Sorbinsäure bzw. anderen Hemmstoffen zugesetzt und unter Rühren in Lösung gebracht. Nach Zugabe der Hemmstoffe wurde der pH-Wert mit der Glaselektrode eingestellt und die Lösung genau 15 min im strömenden Dampf sterilisiert. Versuche hatten ergeben, daß hierbei keine Zersetzung oder Verflüchtigung der verwendeten Hemmstoffe stattfindet. Anschließend wurde die Lösung steril in die betreffenden Versuchsgefäße (Erlenmeyerkolben, Fernbachkolben oder Penicillinkolben) verteilt.

Die Beimpfung erfolgte — je nach Versuchsziel — mit den folgenden Entwicklungsstadien von *A. niger*:

1. Ungekeimte Konidien: Aus einer gut geschüttelten (½ Std.) Konidienaufschwemmung in Czapek-Dox-Nährlösung wurden den Versuchslösungen 0,05 ml mit etwa 900 000 Konidien für jeweils 10 ml Nährlösung zugesetzt.
2. Schwach gekeimte Konidien: Die Konidienaufschwemmung wurde 10 Std. bei 30° C bebrütet. Mikroskopisch zeigte sich beginnende Keimschlauchbildung. Beimpfung mit 0,05 ml für je 10 ml Nährlösung.
3. Gut gekeimte Konidien: (Bebrütungszeit 20 Std. bei 30° C.) Mikroskopisch zeigte sich gute Keimschlauchbildung mit beginnenden Verzweigungen. Beimpfung mit 0,05 ml für je 10 ml Nährlösung.
4. Mycel: (Bebrütungszeit 30 Std. bei 30° C.) Von der — zu diesem Zeitpunkt makroskopisch gut sichtbaren — Myceldecke wurden mit der Impfnadel kleine Mycelflocken (etwa 0,5 cm²) in die Versuchslösungen (10 ml) eingeimpft.

Zur Prüfung der Wirkung der Sorbinsäure auf die Konidienkeimung von *A. niger* wurden 2 verschiedene Methoden angewandt.

Methode I: Jeweils 10 ml Nährlösung mit abgestuftem Sorbinsäuregehalt wurden mit je 0,05 ml einer Konidienaufschwemmung von *A. niger* beimpft, geschüttelt und bei 30° C bebrütet. In entsprechenden Zeitabständen wurden steril Proben entnommen und die Anzahl der ausgekeimten Konidien durch mikroskopische Auszählung von mindestens 1000 Konidien (jeweils 10 Parallelen) ermittelt.

Methode II: Mit jeweils 10 ml Nährlösung mit abgestuftem Sorbinsäuregehalt wurde je eine Schrägagarkultur von *A. niger* abgeschwemmt und 30 min geschüttelt. Von diesen Konidienaufschwemmungen wurde je ein Tropfen steril auf einen hohlgeschliffenen Objektträger gegeben (mindestens 10 Parallelproben) und in einer feuchten Kammer bei 30° C bebrütet. Nach entsprechenden Zeitabständen wurden bis zu 10 000 Konidien ausgezählt.

Bei den Versuchen mit festem Nährsubstrat wurde Czapek-Dox-Agar verwendet. Die entsprechenden Hemmstoffmengen wurden in der Wärme im Nähragar gelöst, der pH-Wert durch Tüpfeln auf Indikatorpapier eingestellt und 15 min im strömenden Dampf sterilisiert.

Dann wurden je 10 ml dieses Nährsubstrates steril in Petrischalen ausgegossen und entweder strichförmig (2 Impfstriche in Kreuzform längs des Durchmessers der Platten) oder im Stich beimpft.

Folgende Hemmstoffe wurden bei den Untersuchungen verwendet: Sorbinsäure p. a. (Merck) — nach Umkristallisation des Handelspräparates mit Äthanol — Benzoesäure p. a. (Merck), Natriumformiat sicc. p. a. (Merck) und Äthylester der p-Hydroxybenzoesäure p. a. (Merck).

Weitere methodische Angaben finden sich im Text.

2. Analytische Bestimmungen

Myceltrockengewichtsbestimmungen: Die Pilzmycelien (einschließlich Konidien) wurden abgesaugt, mehrfach mit destilliertem Wasser gewaschen und bei 99° C bis zur Gewichtskonstanz getrocknet.

Der Sorbinsäuregehalt wurde in der Kulturlösung, als Differenz gegenüber unbeimpften Kontrollösungen, nach einer von Hardon und Visser (1958) angegebenen physikalisch-chemischen Bestimmungsmethode ermittelt. Um die bei dieser Methode teilweise sehr hohen Blankowerte weitgehend zu eliminieren, wurden die Destillationslösungen mit Sodalösung alkalisiert und auf 50 ml eingeengt.

Außerdem wurden Blindproben von gleicher aber sorbinsäurefreier Nährlösung mitbestimmt, um unspezifische Absorptionen weitgehend auszuschalten. (Bei Czapek-Dox-Nährlösung lagen die Werte zwischen 0,5 und 0,75 mg/100 ml.)

Die Genauigkeit der Methode wurde aus 20 Bestimmungen errechnet:

$$s = \frac{(x - M)^2}{n(n-1)} = \pm\, 0{,}59 \text{ mg/100 ml}$$

x — M = Abweichung der Einzelwerte vom Mittelwert
n = Zahl der Bestimmungen

Ergebnisse

I. Wirkung von Sorbinsäure auf Keimung und Entwicklung von *A. niger*

Wie in der Problemstellung angegeben wurde, sollte zunächst untersucht werden, ob die Sorbinsäure in subletalen Konzentrationen die Keimung und das Mycelwachstum von *A. niger* beeinflussen kann.

Diese Versuche wurden bei einem pH-Wert von 4,5 durchgeführt.

1. Wirkung auf die Konidienkeimung

Zur Untersuchung der Sorbinsäurewirkung auf *A. niger* wurden zwei verschiedene Methoden angewandt (Methode I und Methode II, Einzelheiten hierüber vgl. Methodik). Von diesen entspricht die Methode I mehr den natürlichen Verhältnissen, z. B. dem Verderb von Lebensmitteln, aber durch die relativ geringe Sporendichte wurde die mikroskopische Auszählung der Konidien erschwert. Zudem können sich bei dieser Methode an der Grenzfläche Flüssigkeit/Glas kleine Mikrokolonien ausbilden, die möglicherweise die Keimungsversuche ungünstig beeinflussen und kein reales Bild ergeben können.

Aus diesen Gründen erschien es zweckmäßig, die Versuche mit einer anderen Methode (Methode II) zu wiederholen, bei der eine hohe Sporendichte und eine weitgehend gleichmäßige Keimung der Konidien gewährleistet war.

Die mit Methode I erzielten Ergebnisse zeigt Abb. 1 a.

Mit zunehmenden Sorbinsäurekonzentrationen wird die Keimung der eingeimpften Konidien von *A. niger* immer mehr verzögert. Während die Kontrollen

nach 10 Std. zu 82% ausgekeimt sind, wird dieses Stadium bei Proben mit 40 mg/100 ml Sorbinsäure erst nach 34 Std. erreicht. Anscheinend werden aber nicht alle eingeimpften Konidien durch Sorbinsäure gleichmäßig in ihrer Entwicklung gehemmt. Bei sehr hohen Sorbinsäurekonzentrationen (80 mg/100 ml Sorbinsäure) können sich nach einiger Zeit an der Grenzfläche Flüssigkeit/Glas makroskopisch sichtbare kleine Myceelien ausbilden, obwohl in der Nährlösung selbst noch der größte Teil (> 99%) der eingeimpften Konidien keine Keimung zeigt. Je mehr sich die Sorbinsäurekonzentrationen der Grenzhemmkonzentration (III) nähern, desto weniger der eingeimpften Konidien keimen auch nach längeren Versuchszeiten noch aus.

Wie die Abb. 1 b zeigt, sind mit Methode II die Ergebnisse zum Teil deutlich verändert.

Nur bei 20 mg/100 ml Sorbinsäure unterscheiden sich die Proben bei beiden Methoden nicht wesentlich voneinander, dagegen ist bei höheren Sorbinsäurekonzentrationen die Keimungsverzögerung der eingeimpften Konidien in den hohlgeschliffenen Objektträgern — verglichen mit Methode I — wesentlich abgeschwächt. Auch die Keimungshemmung eines Teiles der Konidien kann mit Methode II nicht bestätigt werden.

Diese Ergebnisse zeigen also, daß die Wirkung der Sorbinsäure auf die Konidienkeimung von *A. niger*, je nach Versuchsmethodik, zum Teil verändert wird. Möglicherweise kann die Wirksamkeit der Sorbinsäure durch verschiedene Faktoren, z. B. durch die Menge der Keime, mit der die Versuchslösungen beimpft werden, die Sauerstoffverhältnisse während der Anzucht des Pilzes, die absolut in den Nährlösungen vorliegenden Sorbinsäuremengen usw., beeinflußt werden.

Diese unterschiedliche Einwirkung der Sorbinsäure auf die Konidienkeimung, die besonders stark in der Nähe der Grenzhemmkonzentration wird, verursacht die oft unterschiedlichen Grenzhemmkonzentrationen, wie die folgende Tab. 1 zeigt.

Tabelle 1

Unterschiedliche Entwicklung von *A. niger* in Gegenwart von Sorbinsäure in flüssigem Nährsubstrat nach 10 tägiger Bebrütung[1])

n-Zahl	HD mg/100 ml	Bemerkungen
47	100	80 mg/100 ml: In 18 Ansätzen kein Wachstum
84	80	60 mg/100 ml: In 72 Ansätzen kein Wachstum
2	110	100 mg/100 ml: In 1 Ansatz kein Wachstum

[1]) Auch nach längeren Bebrütungszeiten (3 bis 4 Wochen) werden diese Ergebnisse nicht mehr verändert.

Es ist also nicht möglich, die Grenzhemmkonzentration von Sorbinsäure gegen *A. niger* genau anzugeben. Sie liegt bei pH 4,5 etwa zwischen 80 und 110 mg/100 ml Sorbinsäure. Auch auf festem Nährsubstrat läßt sich die eben erwähnte Keimungshemmung eines Teiles der eingeimpften Konidien von *A. niger* beobachten. Abb. 2 zeigt die Ergebnisse.

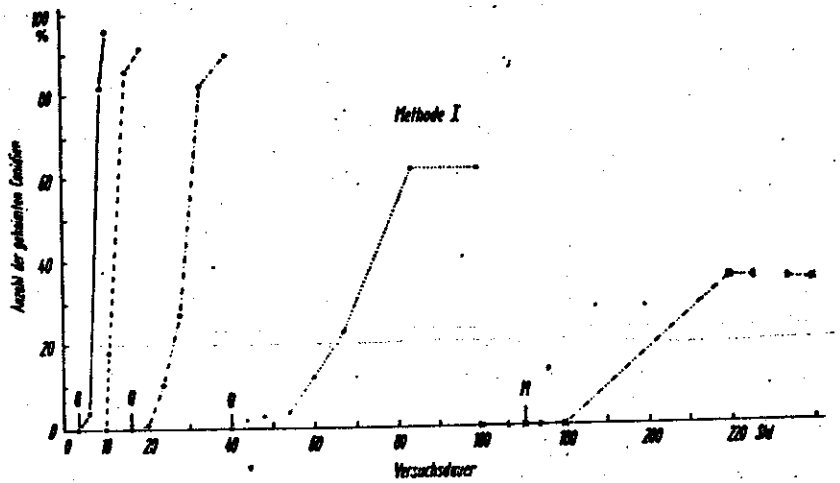


Abb. 1a

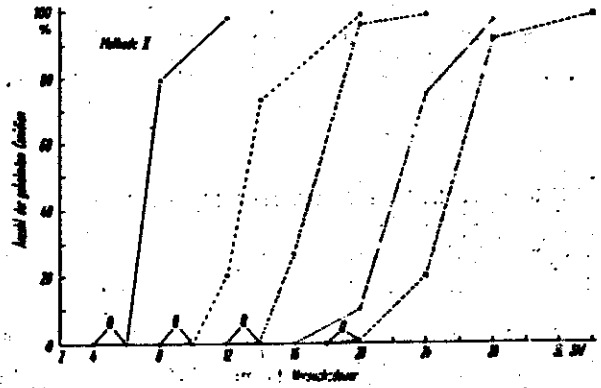


Abb. 1b

Abb. 1 a und 1 b. Einfluß der Sorbinsäure auf die Keimung von *A. niger*
o —— o = Kein Sorbinsäurezusatz
o - - - - o = 20 mg/100 ml Sorbinsäure
o · · · · o = 40 mg/100 ml Sorbinsäure
x . . . . x = 60 mg/100 ml Sorbinsäure
X · · · · X = 80 mg/100 ml Sorbinsäure
Q = Quellung und beginnende Keimung der Konidien (< 1%)
M = An der Flüssigkeitsoberfläche haben sich einige kleine makroskopisch sichtbare Myceelflocken ausgebildet

Bis zu einer Sorbinsäurekonzentration von 40 mg/100 ml kann ein einheitliches Pilzwachstum beobachtet werden. Von den Impfstrichen ausgehend wird die Platte vollkommen von dem Pilz überwachsen (vgl. Abb. 2 b).

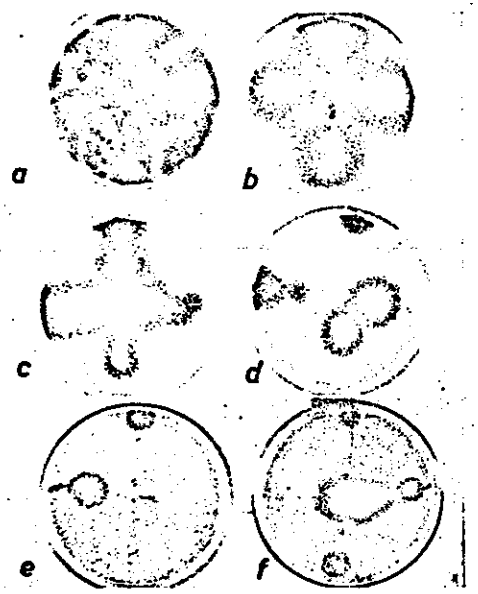


Abb. 2. Koloniebildung von *A. niger* auf sorbinsäurehaltigem Nähragar
a = Kein Sorbinsäurezusatz
b = 40 mg/100 ml Sorbinsäure
c und d = 60 mg/100 ml Sorbinsäure
e und f = 80 mg/100 ml Sorbinsäure

Bei Konzentrationen von 60 und 80 mg/100 ml Sorbinsäure entwickeln sich nur noch einige Kolonien längs des Impfstriches (vgl. Abb. 2 c—2 f). Dies läßt sich damit erklären, daß bei diesen Sorbinsäurekonzentrationen nur noch wenige Konidien auskeimen können, während die Mehrzahl der eingeimpften Sporen ungekeimt im Substrat liegenbleibt.

2. Wirkung der Sorbinsäure auf verschiedene Entwicklungsstadien von *A. niger*

Zur Beobachtung der Wirkung der Sorbinsäure auf die vegetative Entwicklung von *A. niger* wurden Versuchsreihen mit abgestuftem Sorbinsäuregehalt angesetzt und mit unterschiedlichen Entwicklungsstadien des Pilzes beimpft (vgl. Methodik).

Die Ergebnisse dieser Versuche sind in Tab. 2 zusammengestellt.

Tabelle 2

Grenzhemmkonzentration von Sorbinsäure gegen verschiedene Entwicklungsstadien von *A. niger* bei einem pH-Wert von 4,5

Impfmaterial	Grenzhemmkonzentration mg/100 ml	In der HD vorliegender Sorbinsäureanteil: Undissoziiert mg/100 ml	Dissoziiert mg/100 ml
Ungekeimte Konidien	100	64,62	35,38
Angekeimte Konidien	110	71,08	38,92
Gut gekeimte Konidien	135	87,24	47,76
Mycel	150	96,93	53,07

Als Entwicklung wurde bei den Versuchen gewertet, wenn sich bei mindestens einer von vier Parallelproben Wachstum zeigte. Die Versuche wurden dreimal reproduziert.

Mit zunehmender Entwicklung des Impfmaterials wird die Grenzhemmkonzentration von Sorbinsäure allmählich erhöht. Während bei ungekeimten Konidien 100 mg/100 ml Sorbinsäure genügen, um eine Hemmung zu verursachen, sind bei ausgebildetem Mycel hierzu 150 mg/100 ml notwendig. Es ist also anzunehmen, daß die Hemmungsintensität der Sorbinsäure gegen *A. niger* mit zunehmender Entwicklung des Pilzes allmählich abgeschwächt wird.

Zusätzlich sind in Tab. 2 die in der HD vorliegenden undissoziierten und dissoziierten Sorbinsäureanteile angegeben, denn bei diesem pH-Wert (4,5) wird die antimikrobielle Wirksamkeit der Sorbinsäure im wesentlichen durch die undissoziierten Säureanteile hervorgerufen. Auch hierbei zeigt sich, daß der Pilz in angekeimtem Zustand höhere Konzentrationen undissoziierter Sorbinsäuremoleküle verträgt als bei ungekeimten Konidien.

Da sich bis jetzt die Aussagen auf relativ frühe Entwicklungsstadien von *A. niger* bezogen haben, sollte als nächstes untersucht werden, inwieweit der spätere Wachstumsverlauf von *A. niger* durch Sorbinsäure beeinflußt werden kann. Hierzu wurde der Einfluß der Sorbinsäure auf das Mycelgewicht und auf den Koloniedurchmesser (auf festem Substrat) untersucht.

Abb. 3 a und 3 b zeigen die Ergebnisse.

In flüssigem Nährsubstrat wird das Pilzwachstum durch Sorbinsäure zunächst für eine gewisse Zeit völlig gehemmt, um dann spontan einzusetzen und mit etwa der gleichen Geschwindigkeit wie in den Kontrollen abzulaufen. Nur bei sehr hohen Sorbinsäurekonzentrationen (80 mg/100 ml) kommt die Entwicklung des Pilzes erst allmählich in Gang, da hier, wie schon erwähnt, nur einige Konidien auskeimen können.

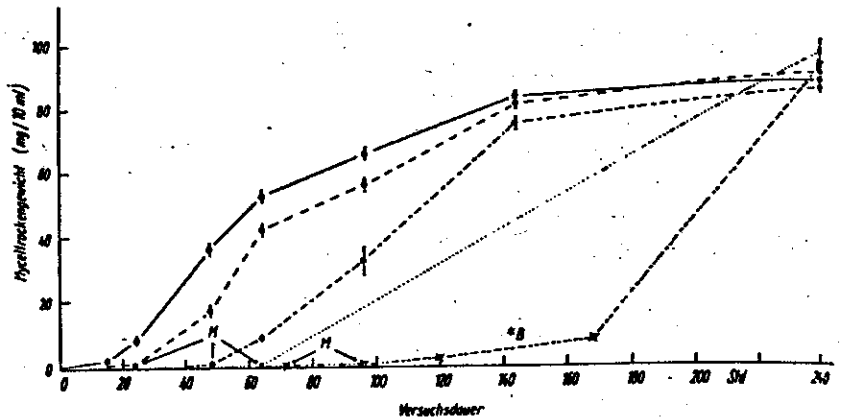


Abb. 3a

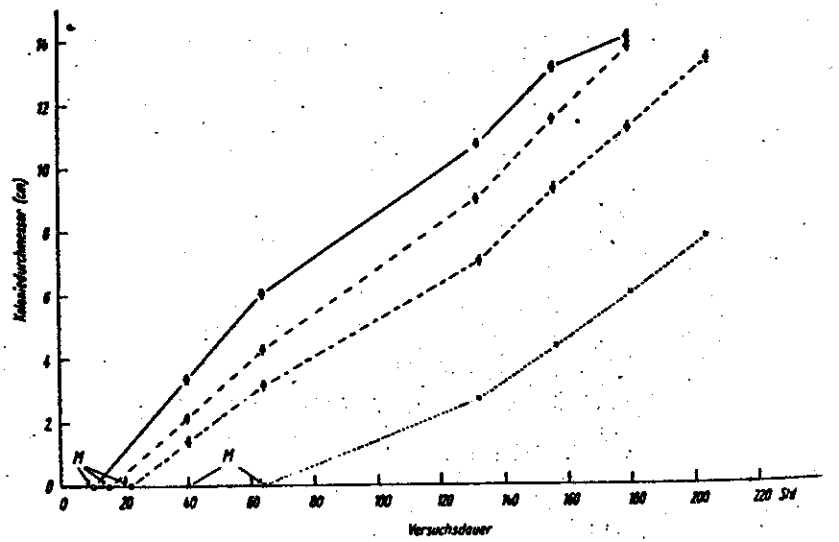


Abb. 3b

Abb. 3 a und 3 b. Einfluß der Sorbinsäure auf den Wachstumsverlauf von *A. niger* in flüssigem (Abb. 3 a) und festem (Abb. 3 b) Nährsubstrat (zehnfache Versuchswiederholung). Zeichenerklärung vgl. Abb. 1
*B = Bei dieser Sorbinsäurekonzentration entwickelte sich der Pilz nur noch in zwei von zehn Parallelproben
In die Diagramme sind die mittleren Fehler der Bestimmungen eingezeichnet

Auf festem Nährsubstrat tritt die Keimungsverzögerung von *A. niger* nicht in dem Maße hervor. Hier setzt die Entwicklung des Pilzes bei 40 mg/100 ml Sorbinsäure schon nach 27 Std. ein (in flüssigem Substrat nach 36 Std.), während bei 60 mg/100 ml dieses Stadium nach 36 Std. (in flüssigem Medium nach 48 Std.) erreicht wird (vgl. Abb. 3 b).

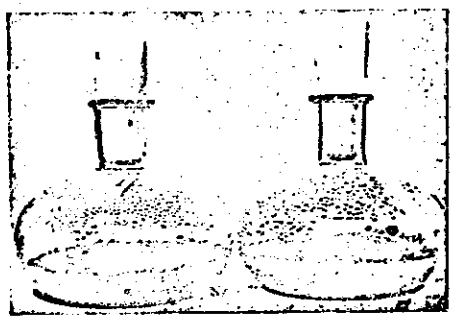
In den meisten Fällen wird die Mycelzuwachsrate des Pilzes durch Sorbinsäure nicht erniedrigt und der Übergang von der logarithmischen zur stationären Wachstumsphase erfolgt nach Erreichen eines bestimmten Mycelgewichtes, unabhängig davon, welche subletalen Sorbinsäurekonzentrationen in der Nährlösung vorliegen.

3. Wirkung der Sorbinsäure auf die Morphologie von *A. niger*

In den Versuchen wurden im sauren pH-Bereich häufig bei relativ hohen Sorbinsäurekonzentrationen makroskopisch sichtbare Veränderungen in der Mycelbildung von *A. niger* beobachtet.

Bis etwa 40 mg/100 ml Sorbinsäure bildet der Pilz geschlossene Myceldecken mit guter Konidienbildung, die sich von den Kontrollen nicht unterscheiden. Je mehr sich aber die Sorbinsäuredosen der Grenzhemmkonzentration nähern (60 bis 80 bis 100 mg/100 ml), desto häufiger treten die folgenden makroskopisch sowie mikroskopisch sichtbaren Veränderungen im Mycel des Pilzes auf:

1. Es bilden sich teilweise keine geschlossenen Myceldecken mehr aus, sondern nur einige Einzelkolonien, die sich durch ihre verklumpte Form von den Kontrollen unterscheiden (vgl. Abb. 4).
2. In den Mycelklumpen, die hauptsächlich an der Grenzfläche Flüssigkeit/Glas — seltener in der Mitte der Myceldecke — auftreten, werden Sklerotien (Durchmesser ∞ 1 mm) gebildet, die eine rötlichbraune Färbung besitzen und in mikroskopischen Schnitten die für sie charakteristischen Scheinparenchymzellen zeigen.



2 1

Abb. 4. Makroskopisch sichtbare morphologische Mycelveränderungen von *A. niger* durch Sorbinsäure
1 = Ohne Sorbinsäure
2 = Mit Sorbinsäure (80 mg/100 ml)

Ein weiterer Faktor, der möglicherweise ein falsches Ergebnis vortäuschen könnte, ist eine Adsorption der Sorbinsäure an die Konidien von *A. niger*. Deshalb wurden sorbinsäurehaltige Nährlösungen mit Konidien des Pilzes (900 000 Konidien pro 10 ml Nährlösung) beimpft und vor beginnender Keimung (mikroskopisch überprüft) in den Proben der Sorbinsäuregehalt ermittelt.

Wie aus Tab. 5 ersichtlich ist, wird hierbei die Sorbinsäure an die eingeimpften Sporen nicht oder zumindest nicht so fest adsorbiert, daß sie nicht mehr vollständig nachgewiesen werden könnte. Es hat sich somit gezeigt, daß unter bestimmten Versuchsbedingungen durch die angeführten Faktoren keine Fehlergebnisse zu erwarten sind.

2. Sorbinsäureabbau bei Beimpfung mit ungekeimten Konidien von *A. niger* bei einem pH-Wert von 4,5

Abb. 7 zeigt das Ergebnis der Untersuchung des Sorbinsäureabbaus durch *A. niger* bei einem pH-Wert von 4,5 nach verschiedenen Entwicklungszeiten.

Mit beginnender Entwicklung von *A. niger* verschwindet die Sorbinsäure außerordentlich schnell aus dem Nährsubstrat. Bei einer Anfangskonzentration

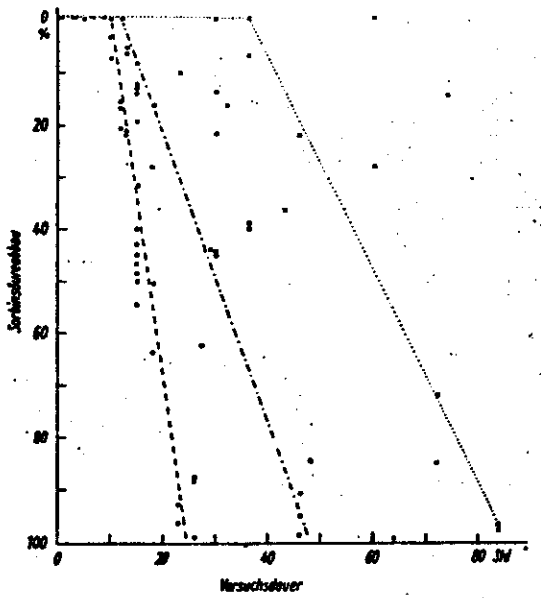


Abb. 7. Sorbinsäureabbau durch *A. niger* bei einem pH von 4,5
o - - - o = 20 mg/100 ml Sorbinsäure
o - · — o = 40 mg/100 ml Sorbinsäure
x · · · x = 60 mg/100 ml Sorbinsäure

von 20 mg/100 ml Sorbinsäure beginnt der Abbau nach 10 Std. und ist nach 27 Std. beendet, während bei 40 mg/100 ml die Zeiten zwischen 13 und 46 Std. und bei 60 mg/100 ml zwischen 36 und 84 Std. liegen.

Schwankungen im zeitlichen Verlauf des Sorbinsäureabbaus beruhen auf der schon beschriebenen unterschiedlichen Empfindlichkeit des eingeimpften Konidienmaterials, denn die Sorbinsäure kann nur dann aus dem Nährsubstrat entfernt werden, wenn die eingeimpften Konidien zu keimen beginnen. Dies kann nach verschiedenen Versuchszeiten der Fall sein (Abb. 7).

Bei hohen, nahe der HD liegenden Sorbinsäurekonzentrationen verstärkt sich die zeitliche Variabilität des Sorbinsäureabbaus (vgl. Schwankungen der Einzelbestimmungen bei 60 mg/100 ml), denn hier können sich nur vereinzelte Konidien in der sorbinsäurehaltigen Nährlösung entwickeln. Sowie aber einige Konidien zu keimen beginnen, genügen diese, um Sorbinsäurekonzentrationen, die für den größten Teil der eingeimpften Konidien schon letal wirken, vollkommen aus dem Nährsubstrat zu entfernen. Unterbleibt eine Keimung des Pilzes, so wird die Sorbinsäure nicht angegriffen.

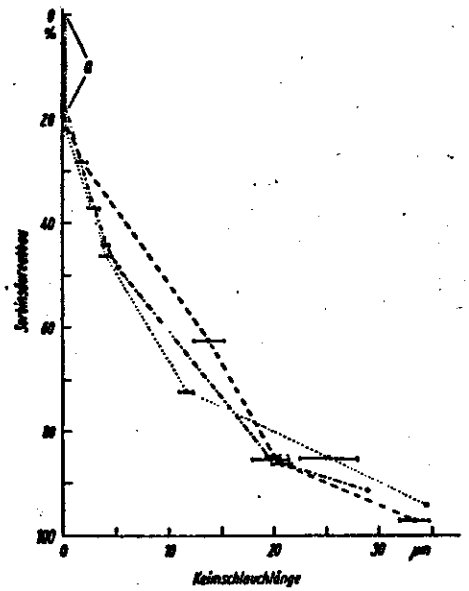
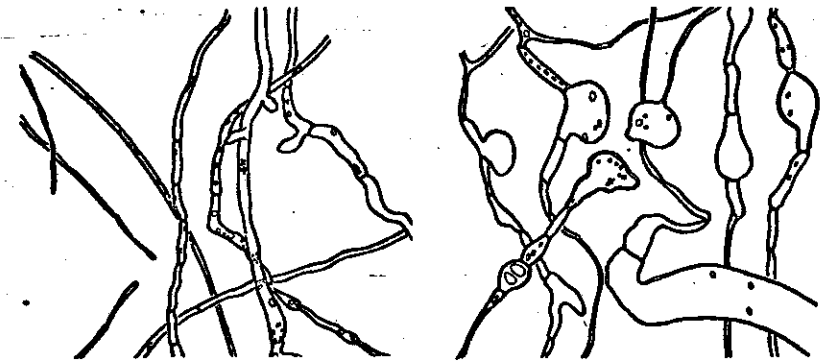


Abb. 8. Beziehungen zwischen Sorbinsäureabbau und Länge der Keimschläuche von *A. niger*. Zeichenerklärung vgl. Abb. 7
Q = Quellung und beginnende Keimschlauchbildung (Länge unter 1 µm) der Konidien
In der Abbildung sind die Mittelwerte und mittleren Fehler aus mindestens 100 Messungen angegeben

Sklerotienbildung tritt bei einigen Stämmen der Aspergillaceen — gelegentlich auch bei *A. niger*-Stämmen (PETERS u. RIPPEL-BALDES 1950) auf, ist aber nicht allgemein verbreitet (THOM u. RAPER 1945). Der in dieser Arbeit verwendete Stamm von *A. niger* van Tieghem besitzt unter normalen Bedingungen diese Eigenschaft nicht.

3. Wenn eine makroskopisch sichtbare Verklumpung des Myceels mit Sklerotienbildung auftritt, so zeigen auch mikroskopische Myceluntersuchungen morphologische Veränderungen der Pilzhyphen (vgl. Abb. 5). Es treten dann verkürzte und blasig angeschwollene Hyphenformen auf, die sich nach den bisherigen Erfahrungen wohl nur als unspezifische Involutionsformen deuten lassen, denn ähnliche Veränderungen können durch den Einfluß von Alkaliionen (FIEDLER 1959), Antibiotica (JULIUS 1952), Koffein, Phosphaten (MÜLLER u. STAPP 1925), einiger Konservierungsstoffe (REHM u. STAHL 1960) u. a. auftreten.



Kein Sorbinsäurezusatz — 80 mg/100 ml Sorbinsäurezusatz

Abb. 5. Mikroskopisch sichtbare morphologische Veränderungen von *A. niger* durch Sorbinsäure. Bei den Versuchen sind nur Hyphen von der Unterseite der Myceldecke untersucht worden, die in unmittelbarem Kontakt mit der sorbinsäurehaltigen Nährlösung standen

4. Fungistatische oder fungicide Wirkung der Sorbinsäure

Bei den bisherigen Versuchen hatte sich gezeigt, daß die Konidien von *A. niger* durch Sorbinsäure in ihrer Entwicklung gehemmt werden. Nicht geklärt wurde aber die Frage, ob und in welchem Umfang die Konidien, die durch bestimmte Sorbinsäurekonzentrationen an der Keimung gehindert wurden, nach Überimpfung in sorbinsäurefreie Nährlösung noch entwicklungsfähig waren oder nicht. Deshalb wurden Konidien, die nach 10 Tagen in sorbinsäurehaltiger Nährlösung kein Wachstum zeigten, in sorbinsäurefreie Nährlösung übertragen und weitere 10 Tage bei 30° C bebrütet. Das Ergebnis dieser Versuche zeigt Tab. 3.

Bis zu einer Sorbinsäurekonzentration von 60 bis 80 mg/100 ml ist nur mit einer fungistatischen Wirkung der Sorbinsäure zu rechnen, denn nach 10tägiger Sorbinsäureeinwirkung sind die Konidien noch in allen Proben entwicklungsfähig.

Ab etwa 90 mg/100 ml Sorbinsäure tritt teilweise schon eine fungicide Wirkung auf, die sich mit zunehmenden Sorbinsäurekonzentrationen verstärkt. Diese Sorbinsäurekonzentrationen liegen aber noch teilweise unter der HD (80 bis 100 mg/100 ml) für andere Konidien von *A. niger*, die sich also noch in solchen sorbinsäurehaltigen Nährlösungen entwickeln konnten. Erst ab 120 mg/100 ml Sorbinsäure tritt für alle Konidien von *A. niger* eine eindeutige fungicide Wirkung ein.

Diese Ergebnisse werden im wesentlichen auch bei Beimpfung mit ausgebildeten Myceelien nicht verändert. Allerdings müssen hier etwas höhere Sorbinsäurekonzentrationen (∾ 135 mg/100 ml) aufgewendet werden, um eine absolute Abtötung zu erreichen. Die ebenfalls in Tab. 3 angegebenen undissoziierten Sorbinsäureanteile — die bei diesem pH-Wert von 4,5 im wesentlichen antimikrobiell wirksam sind — zeigen, daß bis etwa 50 mg/100 ml nur eine Entwicklungshemmung, über etwa 50 mg/100 ml aber eine zunehmende Abtötung der Zellen eintreten kann.

Tabelle 3

Konidienkeimung nach Beendigung der Einwirkung von Sorbinsäure

Sorbinsäurekonzentration, die keimhemmend gewirkt hatte mg/100 ml	Hierzu gehöriger Sorbinsäureanteil:		Konidienkeimung in sorbinsäurefreier Nährlösung
	Undissoziiert mg/100 ml	Dissoziiert mg/100 ml	
60— 80	38,77—51,70	21,23—28,30	Sämtliche Konidien keimten
90—110	58,16—71,08	31,84—38,92	Konidien keimten nur noch teilweise
120	77,54	42,46	Keine Konidienkeimung

5. Resistenzbildung von *A. niger* gegen Sorbinsäure

Von *Escherichia coli* ist bekannt, daß auch eine längere Anzucht in subletalen Sorbinsäurekonzentrationen keine wesentliche Resistenz gegen Sorbinsäure hervorrufen kann (LÜCK u. RICKERL 1959; REHM 1961). Für *A. niger* ist diese Frage noch nicht untersucht worden und es interessierte, ob und wieweit bei diesem Pilz eine Resistenzbildung gegen Sorbinsäure zu erwarten ist.

Um eine allmähliche Gewöhnung an Sorbinsäure zu erreichen, wurde der Pilz einige Passagen auf geringen Sorbinsäurekonzentrationen angezüchtet. Erst dann wurden die Konidien in so hohe Sorbinsäurekonzentrationen überimpft, daß gerade noch eine Pilzentwicklung erreicht werden konnte (Abb. 6).

Bei unterschwelligen Sorbinsäuremengen (40 bis 60 bis 80 mg/100 ml) zeigt sich über 10 bis 14 Passagen immer eine gute Entwicklung des Pilzes. Nach 5 Passagen auf subletalen Sorbinsäurekonzentrationen (vgl. Abb. 6) kann der Pilz noch bei einer Konzentration von 100 mg/100 ml Sorbinsäure — bei der normalerweise schon in erster Generation der größte Teil der Konidien gehemmt oder abgetötet wird — 9 Passagen weitergezüchtet werden.

Den Pilz in Nährlösung mit 120 mg/100 ml Sorbinsäure zu züchten, gelang erst nach 4 Passagen in 100 mg/100 ml. In dieser Konzentration war der Pilz nach anfangs guter Entwicklung aber nach 4 Passagen wieder vollkommen gehemmt.

Nach mehreren Passagen auf 100 und 120 mg/100 ml Sorbinsäure konnte auch bei 130 mg/100 ml einmal eine Entwicklung von *A. niger* erreicht werden. Aber schon nach einer Passage waren hier die Pilzkonidien nicht mehr entwicklungsfähig.

Wie die Untersuchungen zeigen, ist also der Grad der Anpassung bei *A. niger* innerhalb der geprüften 14 Passagen gering (Faktor der maximalen Resistenzsteigerung = 1,3—1,6) und wenig konstant.

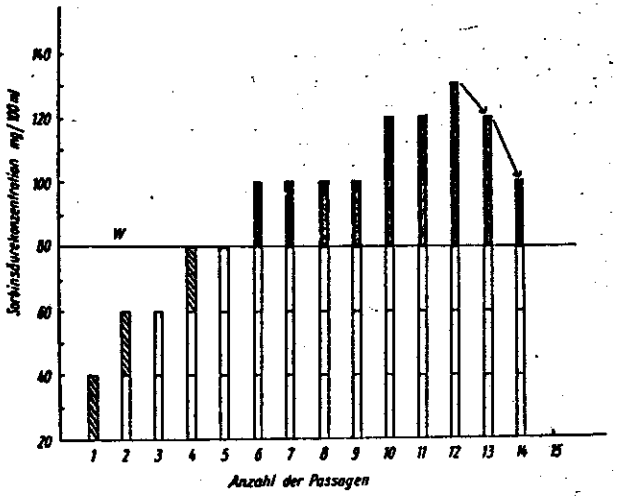


Abb. 6. Resistenzentwicklung von *A. niger* gegen Sorbinsäure
a = Anzucht von *A. niger* in den angegebenen subletalen Sorbinsäurekonzentrationen
b = Weiterzucht von *A. niger* in den angegebenen Sorbinsäurekonzentrationen
c = Resistenzbildung von *A. niger* gegen Sorbinsäure
W = Wachstumsgrenze nicht resistent gezüchteter Konidien von *A. niger*

II. Charakterisierung des Sorbinsäureabbaus

Die Untersuchungen gaben bis jetzt einen Einblick in die Wirkung der Sorbinsäure auf Konidienkeimung, Mycelwachstum und Morphologie von *A. niger*.

Als nächstes soll nun der Abbau subletaler Sorbinsäuremengen durch diesen Pilz untersucht werden. Die Versuche wurden, da dem pH-Wert des Nährmediums eine wesentliche Rolle bei der antimikrobiellen Wirksamkeit der Sorbinsäure zukommt (Rehm u. Lukas 1963), sowohl bei einem pH-Wert von 4,5 (undissoziierter Säureanteil = 64,62%), als auch bei einem pH-Wert von 7,5 (undissoziierter Säureanteil = 0,18%) durchgeführt.

1. Methodische Voraussetzungen zum Nachweis eines Sorbinsäureabbaus

Der Abbau von Sorbinsäure sollte quantitativ durch ein Verschwinden der vorher in die Nährlösung eingegebenen Sorbinsäuremenge bestimmt werden. Daher

war zunächst festzustellen, ob es überhaupt möglich ist, Sorbinsäure in den Versuchslösungen quantitativ wiederzufinden.

Auf Grund ihrer Struktur als mehrfach ungesättigte organische Verbindung kann einerseits eine autoxydative Zersetzung der Sorbinsäure eintreten (Sabalitschka u. Marx 1959); andererseits besteht die Möglichkeit, daß die Sorbinsäure mit dem vorhandenen Substrat Verbindungen bildet, die sich mit der angewandten Bestimmungsmethode nicht mehr erfassen lassen. Die Untersuchungen wurden zum größten Teil in synthetischer Nährlösung (Czapek-Dox) vorgenommen und unter genau definierten Bedingungen durchgeführt, die auch später immer eingehalten wurden (vgl. Methodik).

Die Ergebnisse zeigt Tab. 4.

Tabelle 4
Nachweis der Sorbinsäure in Czapek-Dox-Nährlösung

Zugegebene Sorbinsäuremenge mg/100 ml	Wiedergefundene Sorbinsäuremenge (mg/100 ml) nach: 2 Tagen	10 Tagen	30 Tagen
20	20,1	19,93	18,50
60	60,0	60,0	60,5
100	100,0	100,0	100,5

Nach 10 Tagen kann in allen Proben die zugesetzte Sorbinsäuremenge quantitativ (innerhalb der Fehlergrenze von ± 0,59 mg/100 ml) nachgewiesen werden. Nach 30 Tagen zeigen Proben mit relativ geringen Sorbinsäurekonzentrationen (20 mg/100 ml) eine beginnende Zersetzung, und etwa 7,5% der zugesetzten Sorbinsäure sind hier nicht mehr nachweisbar. Sorbinsäuremengen von 60 und 100 mg/100 ml können auch nach dieser relativ langen Versuchszeit quantitativ wiedergefunden werden (vgl. Tab. 4).

Es ist also nicht zu erwarten, daß eine Verfälschung der Ergebnisse durch Eigenzersetzung oder Inaktivierung der Sorbinsäure eintritt, denn alle weiteren Untersuchungen wurden zum Teil in wesentlich kürzeren Versuchszeiten durchgeführt.

Tabelle 5
Nachweis der Sorbinsäure in beimpfter Nährlösung

Zugegebene Sorbinsäuremenge mg/100 ml	Wiedergefundene[1]) Sorbinsäuremenge nach Beimpfung mit ungekeimten Konidien von *A. niger* mg/100 ml
100	99,5
80	80,6
60	59,0
40	39,60
20	19,40

[1]) Diese Angaben beziehen sich nur auf Proben, die noch vor beginnender Keimung der eingeimpften Konidien von *A. niger* untersucht worden waren.

Nährlösung (25 ml) mit entsprechendem Sorbinsäuregehalt zugegeben, so daß die Endkonzentration der gesamten Kulturlösung wiederum 40 mg/100 ml betrug. Nach jeweils 24 Std. wurde die Entnahme bzw. Zugabe, wie erläutert, wiederholt.

Die Ergebnisse dieser Versuche sind in Tab. 7 angegeben.

Der Pilz ist in allen geprüften Wachstumsphasen in der Lage, die Sorbinsäure abzubauen. So konnte in den Versuchen innerhalb von 20 Tagen (16malige Zugabe von 40 mg/100 ml Sorbinsäure) ein kontinuierlicher Abbau von 640 mg/100 ml Sorbinsäure — eine Menge, die etwa um das 6,4fache über der HD des Pilzes liegt — erreicht werden. Die obere Grenze liegt wahrscheinlich noch wesentlich höher, denn die Versuche wurden nach der 16. Zugabe abgebrochen.

Die Entwicklung des Pilzes wird durch die kontinuierliche Zugabe subletaler Sorbinsäuremengen — abgesehen von der auch bei einmaliger Sorbinsäurezugabe teilweise auftretenden Myceiverklumpung und Sklerotienbildung — nicht sichtbar beeinflußt.

Die Geschwindigkeit, mit der die Sorbinsäure aus dem Kultursubstrat entfernt wird, hat sich bei späteren Entwicklungsstadien von *A. niger* von etwa 1,20 mg/100 ml pro Std. (bei Beimpfung mit ungekeimten Konidien) auf etwa 1,70 mg/100 ml pro Std. erhöht. Es ist möglich, daß eine Sorbinsäurekonzentration, die das Auskeimen der Konidien weitgehend unterbindet, für eine ausgebildete Myceldecke keine so ausgeprägte Hemmwirkung mehr besitzt.

4. Sorbinsäureabbau durch *A. niger* bei einem pH-Wert von 7,5

In der 1. Mitt. (Rehm u. Lukas 1964) wurde der Einfluß des pH-Wertes auf die antimikrobielle Wirkung der Sorbinsäure ausführlich dargelegt.

Hier interessierte die Frage, ob und wieweit durch eine Veränderung der Wasserstoffionenkonzentration des Nährsubstrates der Effekt eines mikrobiellen Sorbinsäureabbaus beeinflußt werden kann (Abb. 8).

Tabelle 8

Sorbinsäureabbau durch *A. niger* bei unterschiedlichen pH-Werten

pH-Wert der Nährlösung	Nach 10 Tagen vollständig abgebaute Sorbinsäuremenge mg/100 ml
3,00	35
4,50	60
6,00	450[1])
7,00	2500
7,50	3000

[1]) Hier wurde nicht die höchste subletale Sorbinsäurekonzentration geprüft.

Wie diese Ergebnisse zeigen, sind die abgebauten Sorbinsäurekonzentrationen bei verschiedenem pH-Wert sehr unterschiedlich. Der Verlauf des Sorbinsäureabbaus bei einem pH-Wert von 7,5 ist aus Abb. 9 zu ersehen. Es können bei diesem pH außerordentlich hohe Sorbinsäurekonzentrationen durch *A. niger* aus dem Nährsubstrat entfernt werden.

Da hier — ähnlich wie bei pH 4,5 — bei hohen Sorbinsäurekonzentrationen eine zeitliche Variabilität des Sorbinsäureabbaus auftritt, sind in Abb. 9 zwei Parallelbestimmungen (2500 mg/100 ml Sorbinsäure) eingezeichnet worden (I und II).

Die Keimung der eingeimpften Konidien und die Entwicklung des Pilzes werden durch Sorbinsäure im alkalischen pH-Bereich verzögert. Nach einiger Zeit bildet der Pilz aber — im Gegensatz zu pH 4,5 — eine dichte geschlossene Myceldecke an der Oberfläche der Kulturlösung aus, obwohl noch Sorbinsäure in der Nährlösung vorhanden ist. Eine Ausnahme bilden Proben mit sehr hohem Sorbinsäurezusatz (2500 mg/100 ml), bei denen die Pilzentwicklung sehr stark gehemmt wird (vgl. Abb. 9).

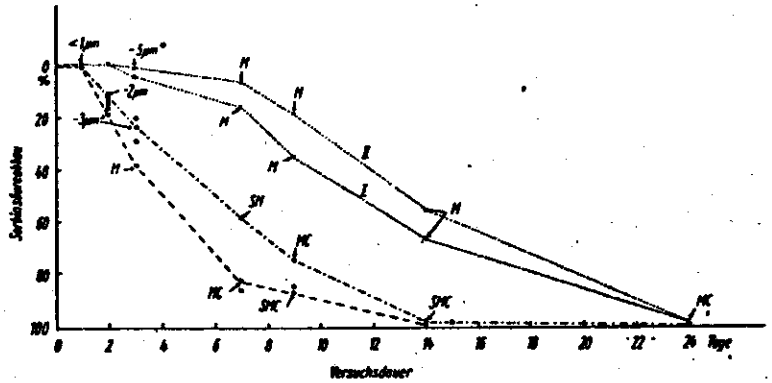


Abb. 9. Sorbinsäureabbau durch *A. niger* bei einem pH-Wert von 7,5

o - - - - o = 1000 mg/100 ml Sorbinsäure
o · · · · o = 1500 mg/100 ml Sorbinsäure
x · · · · x = 2500 mg/100 ml Sorbinsäure
M = An der Flüssigkeitsoberfläche haben sich einige kleine Myceiflocken gebildet
SM = Gute Myceibildung, jedoch ist nicht die gesamte Flüssigkeitsoberfläche mit Myceilen bedeckt. Keine Sporulation
MC = Geschlossene Myceldecke mit beginnender Sporulation
SMC = Dichte geschlossene Myceldecke mit sehr guter Sporulation
x = Die Zahlen an den Kurven geben die Länge der Keimschläuche der keimenden Konidien an

Die Hemmwirkung der Sorbinsäure auf die Entwicklung des Pilzes ist im alkalischen pH-Gebiet verringert, denn trotz sehr hoher absoluter Sorbinsäurekonzentrationen wird der Pilz nicht zu einer Sklerotienbildung angeregt, und die Geschwindigkeit, mit der die Sorbinsäure aus dem Nährsubstrat entfernt wird, ist gegenüber einem pH-Wert von 4,5 etwa drei- bis vierfach erhöht. Es werden etwa 3,2 bis 4,5 mg Sorbinsäure/100 ml in einer Stunde abgebaut. Möglicherweise ist hierfür eine bei diesem pH-Wert gesteigerte Enzymaktivität des Pilzes verantwortlich, denn das pH-Optimum der meisten Enzyme liegt im schwach sauren und neutralen Bereich (Karlson 1961).

Tabelle 10

Sorbinsäureabbau durch verschiedene Pilze bei einem pH-Wert von 4,5 (Temperatur 30° C)

Artname	Grenzhemmkonzentration mg/100 ml	Applizierte Sorbinsäuremenge mg/100 ml	Sorbinsäureabbau nach 7 Tagen in %
Aspergillus oryzae (Ahlburg) Cohn ...	20—50[1])	10	100,0
Penicillium expansum (Link) Thom ..	40	20	99,5
Penicillium spec. 19/1	40—60[1])	20	98,8
Alternaria spec. 17/1	10	5	97,5
Trichoderma lignorum (Tode) Harz ...	60—80[1])	50	100,0
Mucor racemosus Fresenius	10	5	97,0
Rhizopus nigricans Ehrenberg	20	10	96,0
Saccharomyces cerevisiae (Bäckerhefe) .	>50	50	5,0
		20	37,5[2])

[1]) Diese Versuche wurden auf festem Nähragar (Czapek-Dox), alle anderen in flüssigem Nährsubstrat durchgeführt.

[2]) Der Sorbinsäureabbau wurde erst nach 10 Tagen bestimmt.

Diskussion

Die Ergebnisse des ersten Teiles der Arbeit haben gezeigt, daß die Hemmwirkung der Sorbinsäure in subletalen Konzentrationen u. a. eine Keimungsverzögerung von *A. niger* hervorruft. Weiterhin war festgestellt worden, daß die Konidien unterschiedlich empfindlich gegen Sorbinsäure sind und dadurch unterschiedliche Hemmkonzentrationen auftreten mußten.

Die Keimungsverzögerung kann evtl. durch physikalisch-chemische Bedingungen verursacht werden, z. B. dadurch, daß sich die Permeabilität der Konidienmembran durch Sorbinsäure wesentlich verändert. Da man auch verschiedenen anderen, ähnlich wirkenden Hemmstoffen eine Wirkung auf die Membran zuschreibt, liegt es hier nahe, analoge Einwirkungen anzunehmen (Wyss 1948; Hotchkiss 1946; Gale u. Taylor 1947; Mitchell u. Crowe 1947).

Größe und Alter der Konidien von *A. niger* in einer Kultur sind oft sehr unterschiedlich, und man könnte hier die Ursache für eine unterschiedliche Empfindlichkeit gegen Sorbinsäure sehen.

Die Sorbinsäure, als ungesättigte Fettsäure, kann möglicherweise stark an die Konidien adsorbiert werden, so daß man direkt an den Membranen mit höheren absoluten Konzentrationen rechnen muß, als in der Lösung vorhanden sind.

Von bestimmten Konzentrationen ab werden die Konidien irreversibel geschädigt und sind nicht mehr lebensfähig, sie lassen sich auch kaum wesentlich resistent gegen Sorbinsäure züchten. Dies spricht für den angenommenen relativ unspezifischen Wirkungsmechanismus, den man in einer Blockierung von SH-Gruppen verschiedener Fermente sieht (Whitaker 1959; Morgan u. Friedemann 1938). Möglicherweise kann aber auch lediglich eine ausschließliche Wirkung auf

die Konidienmembranen stattgefunden haben, die eine Keimung vollständig verhindert hat und sich uns als fungicide Sorbinsäurewirkung zeigt.

Noch schwieriger wird die Frage der Sorbinsäureeinwirkung zu beurteilen sein, wenn man die Wirkung auf vegetative Zellen untersucht. Hier tritt der im zweiten Teil charakterisierte, schon durch früheste Entwicklungsstadien verursachte starke Abbaueffekt auf und erschwert die Deutung der antimikrobiellen Wirkung. Die größere Unempfindlichkeit der Mycelien, im Vergleich zu den Konidien des Pilzes, gegen Sorbinsäure wird wahrscheinlich nur durch den fortlaufenden Abbau der Sorbinsäure vorgetäuscht. Wenn es gelänge, diesen Abbau auszuschalten, würde die Empfindlichkeit der vegetativen Zellen des Pilzes wahrscheinlich wesentlich verändert sein.

Der beobachtete Abbaueffekt von Sorbinsäure hat eine Reihe neuer Fragen aufgeworfen, die in den vorliegenden Untersuchungen nicht beantwortet werden konnten, z. B.: Wird die Sorbinsäure innerhalb der Mycelien oder im Substrat abgebaut? Wie sind die Abbaustufen der Sorbinsäure beschaffen? Wird die Sorbinsäure in den Stoffwechsel von *A. niger* als normale Fettsäure einbezogen oder lediglich durch einen bestimmten Mechanismus entgiftet, wie z. B. die Benzoesäure mit Glykokoll in anderen Organismen? Diese und weitere Fragen sollen in der nächsten Mitteilung untersucht werden.

Beide Wirkungen, Abbau und antimikrobielle Wirkung, sind insofern auch dadurch eventuell miteinander verbunden, daß in stark hemmenden Bereichen der Sorbinsäure, z. B. beim pH von 4,5, der Abbau absolut wesentlich schwächer vor sich geht als in solchen Bereichen, in denen Sorbinsäure kaum noch eine antimikrobielle Wirkung hat, weil hier vorwiegend dissoziierte Ionen vorliegen, z. B. beim pH von 7,5.

Für die praktische Anwendung der Sorbinsäure als Konservierungsstoff ist der sehr schnelle Abbau sowie der Abbau durch Mycelien im ersten Keimungsstadium von großer Bedeutung, da angekeimte Konidien von Schimmelpilzen leicht in solche Lebensmittel gelangen können, die mit Sorbinsäure konserviert werden müssen und dann die Sorbinsäure abbauen können. Ob dies im Lebensmittel möglich ist, muß noch untersucht werden. Auch die relativ große Verbreitung des Sorbinsäureabbaus unter den Schimmelpilzen ist hier von Bedeutung.

Bei der Untersuchung des Sorbinsäureabbaus hatte sich der für Kombinationsuntersuchungen interessante Fall ergeben, daß ein Antagonist (in diesem Falle Sorbinsäure) im Laufe der Mikroorganismenentwicklung aus dem Substrat verschwindet und der andere Kombinationspartner (Äthylester der p-Hydroxybenzoesäure) nun in letalen Konzentrationen vorliegt. Wenn sich *A. niger* trotzdem noch — wenn auch langsam — etwas weiterentwickelt hatte, obwohl die Konzentrationen des Äthylesters normalerweise letal waren, so liegt dies wahrscheinlich daran, daß größere Mengen von Äthylester an die Mycelien adsorbiert und dadurch ihrer Wirkung entzogen wurden. In einem früheren Entwicklungsstadium wäre sicher eine Hemmung des Pilzes zu erwarten gewesen. Natürlich sind andere Esklärungen nicht ausgeschlossen, z. B. eine größere Unempfindlichkeit älterer Entwicklungsstadien von *A. niger* gegen den Äthylester im Vergleich zur Empfindlichkeit der Konidien.

Die Einwirkungen der Sorbinsäure auf die Morphologie des Pilzes sind — wie schon erwähnt — als weitgehend unspezifisch anzusehen.

5. Sorbinsäureabbau durch *A. niger* in Gegenwart anderer Konservierungsstoffe

Ein wichtiger Faktor, der die antimikrobielle Wirkung der Sorbinsäure beeinflussen kann, ist die Anwesenheit anderer Konservierungsstoffe im Substrat, und es sollte untersucht werden, ob auch in deren Gegenwart die Sorbinsäure in subletalen Dosen durch *A. niger* abgebaut werden kann.

Als Kombinationspartner für Sorbinsäure wurde je eine Verbindung mit bekannter (REHM u. STAHL 1960) additiver (Benzoesäure), antagonistischer (Äthylester der p-Hydroxybenzoesäure) und synergistischer Wirkungstendenz (Natriumformiat) ausgewählt.

Wie Tab. 9 erkennen läßt, wird durch die Anwesenheit unterschiedlicher Benzoesäurekonzentrationen der Sorbinsäureabbau nicht beeinflußt, wenn die prozentualen Konzentrationen beider Mischpartner unter 100% liegen. Mit beginnender Entwicklung von *A. niger* verschwindet die Sorbinsäure aus dem Nährsubstrat.

Tabelle 9

Sorbinsäureabbau durch *A. niger* in Gegenwart anderer Konservierungsstoffe (pH 4,5)

Sorbinsäure[1]) mg/100 ml	+ Benzoesäure mg/100 ml	Sorbinsäureabbau nach 7 Tagen in %
20	125	100
60	50	100
60	125	0

Sorbinsäure mg/100 ml	+ Natriumformiat mg/100 ml	Sorbinsäureabbau nach 7 Tagen in %
20	2500	97,5—100
60	1000	100
80	500	0

Sorbinsäure mg/100 ml	+ Äthylester mg/100 ml	Sorbinsäureabbau nach 10 Tagen in %
20	35	90,5—96,5
40	35	98,5
60	40	0

[1]) Absolute Grenzhemmkonzentration von:
Sorbinsäure = 100 mg/100 ml (pH = 4,5)
Benzoesäure = 250 mg/100 ml (pH = 4,5)
Natriumformiat = 5000 mg/100 ml (pH = 4,5)
Äthylester der p-Hydroxybenzoesäure = 35 mg/100 ml (pH = 4,5)

Also scheinen sich bei additiver Wirkungstendenz keine Wechselbeziehungen zwischen den Wirkungsmechanismen der beiden Mischpartner einzustellen, die eine Störung des Sorbinsäureabbaus verursachen können.

Natriumformiat, mit Sorbinsäure kombiniert, weist in den meisten Konzentrationsbereichen eine synergistische Wirkungstendenz auf. Reichen die prozentualen Konzentrationen beider Mischpartner für eine Hemmung nicht aus, so wird auch hier mit beginnender Entwicklung von *A. niger* die Sorbinsäure aus dem Nährsubstrat entfernt.

Wird die Sorbinsäure mit dem Äthylester der p-Hydroxybenzoesäure kombiniert, so liegt die Summe der prozentualen Konzentrationen beider Partner, die für eine Hemmung ausreichen, in den meisten Konzentrationsbereichen über 100%.

Auch hier wird die Sorbinsäure durch *A. niger* abgebaut. Teilweise kann sogar noch nach dem Verschwinden der Sorbinsäure aus dem Nährsubstrat eine schwache Weiterentwicklung des Pilzes eintreten, obwohl der zweite Kombinationspartner (Äthylester) in letalen Konzentrationen im Substrat zurückbleibt. Im Zeitpunkt des vollständigen Sorbinsäureabbaus haben sich an der Flüssigkeitsoberfläche einige kleine Myceelien ausgebildet (Durchmesser etwa 0,5 cm). Nach weiteren 18 Tagen hat sich die Größe dieser Myceelien — die nun teilweise eine spärliche Sporulation aufweisen — etwa verdoppelt. Liegen dagegen die relativen Äthylesterkonzentrationen unter 100%, kann sich der Pilz wesentlich besser weiterentwickeln (nach 18 Tagen hat sich eine dichte, geschlossene, sehr gut sporulierende Myceldecke gebildet). Es ist also anzunehmen, daß die Entwicklung von *A. niger* in Gegenwart letaler Äthylesterkonzentrationen — wenn durch Sorbinsäure (als Antagonisten) eine anfängliche Entwicklung begünstigt wird — zwar stark zurückgedrängt, aber nicht vollständig unterdrückt wird.

5. Sorbinsäureabbau durch andere Mikroorganismen

Abschließend sollte die Frage geklärt werden, ob ein Sorbinsäureabbau unter Pilzen allgemein verbreitet ist oder ob sich diese Fähigkeit nur auf einige Arten beschränkt. Wie erwähnt, sollen *Penicillium roqueforti* und Mischkulturen von Schimmelpilzen die Sorbinsäure aus dem Substrat entfernen können (MELNICK, LUCKMANN u. GOODING 1954).

Verschiedene Schimmelpilze und ein Hefestamm wurden auf ihre Fähigkeit, Sorbinsäure abzubauen, geprüft. Tab. 10 zeigt das Ergebnis.

Alle untersuchten Pilze sind empfindlicher gegen Sorbinsäure als der *A. niger*-Stamm, der in den bisherigen Versuchen als Testobjekt verwendet worden ist. Es sind also für eine Hemmung von Arten der gleichen Gattung zum Teil sehr unterschiedliche Sorbinsäurekonzentrationen notwendig.

Alle geprüften Schimmelpilzstämme haben nach 7 Tagen die Sorbinsäure beinahe restlos abgebaut. *Saccharomyces cerevisiae* greift erst in einem sehr späten Entwicklungsstadium die Sorbinsäure an und auch dann verläuft ein Abbau — verglichen mit Schimmelpilzen — sehr langsam und unvollständig (Tab. 10).

Die zeitliche Variabilität des Sorbinsäureabbaus machte sich in den Versuchen, vor allem bei Reproduktionen der Befunde, störend bemerkbar. Aus diesem Grunde erschien es zweckmäßig, nach einer Bezugsgröße zu suchen, die diese biologisch bedingte Streuung der Ergebnisse besser berücksichtigt. Da der Sorbinsäureabbau mit beginnender Keimung der Konidien einsetzt und bei sichtbarer Myceibildung weitgehend beendet ist, wurde versucht, die Länge der Keimschläuche als Bezugsgröße zu verwenden. Abb. 8 zeigt die in den Kulturlösungen mikroskopisch ausgemessenen Keimschlauchlängen der Pilzkonidien nach verschiedenen Versuchszeiten.

Es besteht demnach eine Beziehung zwischen dem Sorbinsäureabbau und der Länge der Keimschläuche. Bei einer Keimschlauchlänge von etwa 6 μm sind 49%, in einem etwas späteren Entwicklungsstadium (11 μm) 75% der zugegebenen Sorbinsäuremenge nicht mehr nachweisbar. Sowie die Keimschläuche eine Länge von 30 bis 35 μm erreicht haben, ist die Sorbinsäure zum größten Teil aus der Nährlösung verschwunden.

Prinzipiell werden diese Ergebnisse durch die absoluten Sorbinsäurekonzentrationen — wenn diese unter der HD des Pilzes liegen — nicht verändert. Da die Keimung der Konidien bei unterschiedlichen Sorbinsäurekonzentrationen aber abhängig von der Zeit ist, kann auch eine gewisse Beziehung zwischen Abbauzeit und Sorbinsäurekonzentration abgeleitet werden. Mit steigenden Sorbinsäuremengen nimmt die Zeitspanne zu, die zum vollkommenen Abbau benötigt wird. Die Geschwindigkeit, mit der die Sorbinsäure durch den Pilz abgebaut wird, beträgt bei allen geprüften Sorbinsäurekonzentrationen etwa 1,2 mg/100 ml pro Stunde (vgl. Tab. 6).

Tabelle 6

Geschwindigkeit des Sorbinsäureabbaus bei unterschiedlichen Sorbinsäurekonzentrationen (pH 4,5)

Applizierte Sorbinsäurekonzentration mg/100 ml	Zeitraum des Sorbinsäureabbaus Std.	Geschwindigkeit des Sorbinsäureabbaus mg/100 ml pro Std.
20	16	1,20
	15	1,25
	16	1,20
40	33	1,21
	34	1,18
	30	1,33
60	48	1,25
	54	1,11

Möglicherweise verursacht die Sorbinsäure außer einer Keimungsverzögerung — die den Beginn des Sorbinsäureabbaus beeinflußt — auch noch eine Verzögerung sehr früher Entwicklungsstadien von *A. niger*. Vor allem scheint hier die Induktionsphase des Pilzes betroffen zu sein, denn sowie die Sorbinsäure aus dem Nährsubstrat verschwunden ist, findet eine normale Weiterentwicklung und Konidienbildung des Pilzes statt, wenn nicht so hohe Sorbinsäurekonzentrationen vorliegen, daß schon morphologische Veränderungen aufgetreten sind.

3. Sorbinsäureabbau durch verschiedene Entwicklungsstadien von *A. niger* bei einem pH-Wert von 4,5

Es wurde gezeigt, daß *A. niger* ein gewisses Entwicklungsstadium nicht überschreitet, bevor die Sorbinsäure aus dem Nährsubstrat verschwunden ist.

Aus dieser Beobachtung ergeben sich die folgenden Fragestellungen: Kann der Pilz nur in sehr frühen Entwicklungsstadien die Sorbinsäure aus dem Substrat entfernen, oder ist es *A. niger* auch in einem späteren Entwicklungsstadium möglich, die Sorbinsäure anzugreifen? Sollte dies der Fall sein, bleibt zu klären, ob hierbei nur ein begrenzter Teil der vorhandenen Sorbinsäure durch den Pilz abgebaut wird oder ob z. B. durch laufende Zugabe subletaler Sorbinsäurekonzentrationen ein kontinuierlicher Abbau erreicht werden kann.

Zur Klärung dieser Fragen wurden Fernbachkolben (450 ml), die je 100 ml Nährlösung mit 40 mg/100 ml Sorbinsäuregehalt enthielten, nach Beimpfung 48 Std. bei 30° C bebrütet. Anschließend wurde ein aliquoter Teil der Kulturlösung (25 ml) steril entnommen und neue

Tabelle 7

Sorbinsäureabbau durch verschiedene Entwicklungsstadien von *A. niger*

Laufende Zugabe von jeweils 40 mg/100 ml Sorbinsäure	Insgesamt zugegebene Sorbinsäurekonzentration mg/100 ml	Entwicklungsstadium des Pilzes: Zu Beginn des Sorbinsäureabbaus	Entwicklungsstadium des Pilzes: Nach Beendigung des Sorbinsäureabbaus	Zeit, bis zu der sämtliche Sorbinsäure abgebaut wurde Std.	Geschwindigkeit des Sorbinsäureabbaus mg/100 ml pro Std.
1. Zugabe	40	Quellung der eingeimpften Konidien	Gute Keimung der Konidien; Keimschlauchlänge etwa 35 μm	34	1,18
2. Zugabe	80	Gute Keimung der Konidien; Länge der Keimschläuche etwa 35 μm	Nicht ganz geschlossene Myceldecke mit beginnender Sporulation	24	1,65
3. Zugabe	120	Nicht ganz geschlossene Myceldecke mit beginnender Sporulation	Geschlossene Myceldecke mit sehr guter Sporulation	24	1,65
4. bis 16. Zugabe	160—640	Geschlossene Myceldecke mit sehr guter Sporulation	Unverändert	Jeweils 24	1,65

Zusammenfassung

Die Untersuchungen über die antimikrobielle Wirkung der Sorbinsäure gegen *Aspergillus niger* erbrachten folgende Ergebnisse:

1. Die Hemmwirkung der Sorbinsäure richtet sich im wesentlichen gegen die Konidienkeimung und kann in subletalen Konzentrationen u. a. eine Keimungsverzögerung des Pilzes hervorrufen. Hierbei sind die Konidien gegen Sorbinsäure unterschiedlich empfindlich.
2. Von bestimmten Sorbinsäurekonzentrationen ab werden die Konidien irreversibel geschädigt und sind nicht mehr keimungsfähig; sie lassen sich auch nicht wesentlich gegen Sorbinsäure resistent züchten.
3. Die Hemmwirkung der Sorbinsäure auf die vegetativen Zellen ist im Vergleich zu der auf die Konidien wesentlich schwächer (bis zu 50%). Dies beruht wahrscheinlich darauf, daß die Sorbinsäure mit beginnender Konidienkeimung außerordentlich schnell abgebaut wird und bei makroskopisch sichtbarer Myceibildung im Substrat nicht mehr nachweisbar ist.
4. Die Einwirkung der Sorbinsäure auf die Morphologie des Pilzes (Sklerotienbildung und Involutionsformen) ist weitgehend unspezifisch.
5. Der Sorbinsäureabbau wird weder durch pH-Veränderungen des Nährsubstrates noch durch die Gegenwart anderer Konservierungsstoffe beeinträchtigt.
6. Bei verschiedenen a n d e r e n Schimmelpilzen wurde ebenfalls ein Sorbinsäureabbau nachgewiesen. Bei *Saccharomyces cerevisiae* verlief ein Sorbinsäureabbau nur langsam und unvollständig.

Summary

The antimicrobial action of sorbic acid was investigated with the following results: Sorbic acid inhibits the germination of conidia of *Aspergillus niger*. There was no succeed in getting strains of *A. niger* which were resistant against sorbic acid.

Sorbic acid is very quickly decomposed by *A. niger* in the earliest steps of development. Sorbic acid was decomposed by other moulds too.

Herrn Dr. habil. H.-J. R e h m gilt mein aufrichtigster Dank für die Anregung zu dieser Arbeit sowie für seine zahlreichen wertvollen Hinweise und Ratschläge.

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Anschrift der Verfasserin:

Eva-Maria Lukas, München 42, Gotthardstr. 35

The inhibition of interferon production by raphanin at low concentrations indicated that some metabolic processes of the host cell are intensely injured even by such low concentrations. According to MOLNÁR [9] the effect of raphanin is based on the inhibition of thiol enzymes. Inhibition of the multiplication of some viruses appears to be attributable to this phenomenon. On the basis of the present results it may be assumed that the reproduction of DNA viruses is more sensitive to the inhibition of SH-containing enzymes than the reproduction of RNA viruses.

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Address of the author:

István Rosztóczy

Institute of Microbiology, University Medical School, Dóm tér 10, Szeged, Hungary

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THE EFFECT OF SORBIC ACID ON THE GROWTH OF THE YEAST PROCANDIDA (CANDIDA) ALBICANS

By

T. DEÁK and E. K. NOVÁK

National Institute of Public Health (Director: T. BAKÁCS), Budapest

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Summary. The inhibitory effect of potassium sorbate on the growth of *Procandida albicans* in different incubation systems has been examined. Determination of the growth rate constant, lag phase and final population density has shown that independently of the composition of the medium the inhibitory effect of sorbate is stronger under anaerobic than under aerobic conditions. The decreased inhibition at aerobic incubation was attributed to a detoxication of sorbate by the yeast culture. The degree of this aerobic detoxication varied with the composition of the medium. Cysteine and glutathione exerted an effect quite opposite to that expected on the basis of the literature; instead of antagonizing the growth inhibitory effect of potassium sorbate, they increased the degree and duration of inhibition by decreasing the detoxication of the inhibitory agent.

Sorbic acid (2,4-hexadienic acid) is a fungistatic substance particularly effective against yeasts and moulds. It is widely used in food industry [4, 7—9] and there are several data concerning its concentration inhibiting the growth of yeasts [1, 2, 11, 13]. However, no systematic examinations have been carried out as to the kinetics of its growth inhibitory effect. In the present paper some studies into this problem are described.

Materials and methods

Sorbic acid was used as potassium sorbate (Farbwerke Hoechst A. G.). Determination of potassium sorbate concentration in the cultures and control measurement of the added substance were performed as described by LUCKMANN and MELNICK [6] and MELNICK and LUCKMANN [10].

Pc. albicans strain 85/1957 was isolated in our laboratory and maintained on CHILLAC's molasses agar slants [3]. Cultures obtained on the same medium after 48 hours incubation were suspended in saline, set to a density corresponding to $5-8 \times 10^6$ cells per ml and used as inocula. Growth was examined in 4 different media: 1. Sabouraud glucose broth with organic nitrogen source (Witte peptone) adjusted to pH 5.6 and supplied with a yeast extract as a vitamin source (1000 g baker's yeast cake mixed with 1200 ml water, autoclaved at 121 °C for 30 minutes; 0.025 ml of this was added to 10 ml medium before inoculation); 2. Medium 1 without yeast extract; 3. Synthetic medium with inorganic nitrogen source: glucose, 40 g; $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; distilled water to 1000 ml; pH 5.6; yeast extract as described above; 4. Medium 3 without yeast extract.

Aerobic and anaerobic cultivation was performed under shaking and under static conditions, respectively. Ten ml amounts of the media were distributed into T-shaped tubes and aerated on a home-made tilting shaker set at 60 r.p.m. and a vertical amplitude of 5 cm at the end of tubes. As before each photometric measurement the test tubes were shaken, until a constant optical density had been reached, the anaerobic cultures were in reality only semi-anaerobic ones. Both aerobic and semi-anaerobic incubations were carried out at 22 ± 2 °C.

The degree of growth was determined turbidimetrically in a model FEK-M photoelectric colorimeter by placing the test tubes in cuvettes filled with distilled water as described by VAS [15]. Optical density was expressed as population density by the use of a calibration curve prepared by direct counting in a Bürker chamber. By plotting the logarithm of population density against time, the growth rate constant (k) was calculated from the slope of the straight part of the curve. The effect of different experimental conditions was always measured by comparing the growth rate constant of the individual tubes to that of the control culture and was expressed by the ratio of the value of the respective culture and of the control (relative k). In examining the kinetics of growth, on the basis of HINSHELWOOD's suggestion [5] the changes of the duration in the lag phase and in the final population density were also taken into consideration. The importance of these factors has been emphasized also by VAS [14]. The duration of lag phase was estimated by extrapolating graphically the straight part of the growth curve, drawing a line at population density 8×10^6/ml and reading the interval between zero time and the time indicated by the intersection of the two lines. The final population density was calculated from photometric readings performed for aerobic conditions after 72 hours and for semi-anaerobic ones after 96 hours.

Results

The effect of potassium sorbate on the growth of *Pc. albicans* was examined in 8 experiments representing different combinations of the following three factors: 1. mode of incubation (aerobic shaken or semi-anaerobic

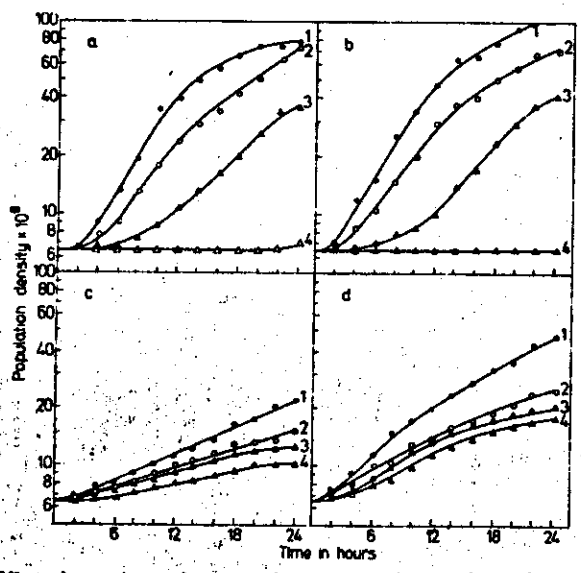

Fig. 1. Effect of potassium sorbate at various concentrations on the aerobic growth of *Pc. albicans*. *a* — Sabouraud glucose medium; *b* — Sabouraud glucose medium supplemented with yeast extract; *c* — synthetic medium; *d* — synthetic medium supplemented with yeast extract. 1 — control; 2 — 250 μg sorbate/ml; 3 — 500 μg sorbate/ml; 4 — 1000 μg sorbate/ml

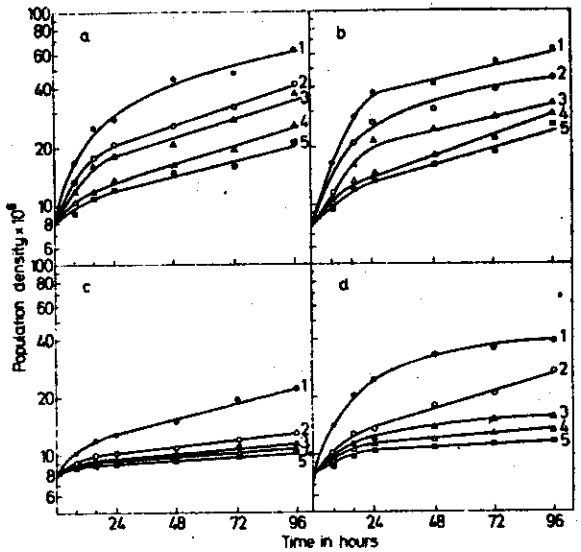

Fig. 2. Effect of potassium sorbate at various concentrations on the anaerobic (semi-anaerobic) growth of *Pc. albicans*. *a*, *b*, *c* and *d*: see Fig. 1. 1 — control; 2 — 100 μg sorbate/ml; 3 — 150 μg sorbate/ml; 4 — 200 μg sorbate/ml; 5 — 250 μg sorbate/ml

static cultures); 2. growth in the presence of organic or inorganic nitrogen source (Sabouraud glucose broth and synthetic medium); 3. growth in the presence and absence of vitamins (yeast extract). On the basis of preliminary experiments the sorbate concentrations applied were 250, 500 and 1000 μg/ml for shaken and 100, 150, 200 and 250 μg/ml for static cultures. The results of growth experiments in these systems are shown in Figs. 1 and 2.

Although the characteristics of the growth of *Pc. albicans* seem to vary with the incubation system, the effect of sorbate could be properly evaluated by taking into account the relative values of the parameters.

Changes in the relative k values in aerobic cultures as a function of different sorbate concentrations are presented in Fig. 3. The dose-response curve was definitely steeper for cultures growing in the presence of organic nitrogen, and the effect of sorbate was not appreciably influenced by the presence or absence of vitamins. When the cultures were incubated under anaerobic conditions (Fig. 4), neither the nitrogen nor the vitamin source exerted a significant effect on the results.

Potassium sorbate was found to prolong the lag phase in aerobic cultures (Fig. 5). The inhibitor, especially at the highest concentration used, retarded

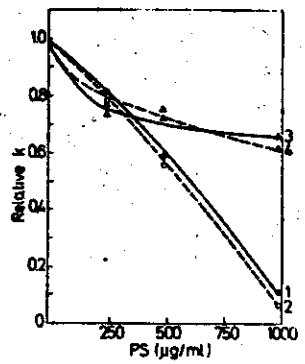

Fig. 3. Effect of potassium sorbate concentration on growth rate of *Pc. albicans* in aerobic culture. 1 — Sabouraud glucose broth; 2 — Sabouraud glucose broth supplemented with yeast extract; 3 — synthetic medium; 4 — synthetic medium supplemented with yeast extract

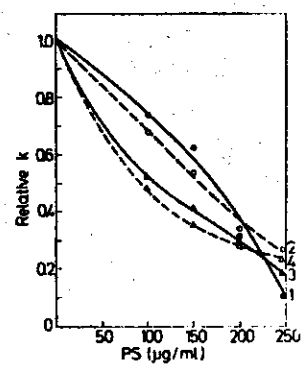

Fig. 4. Effect of potassium sorbate concentration on growth rate of *Pc. albicans* in anaerobic (semi-anaerobic) cultures. The curves are as in Fig. 3

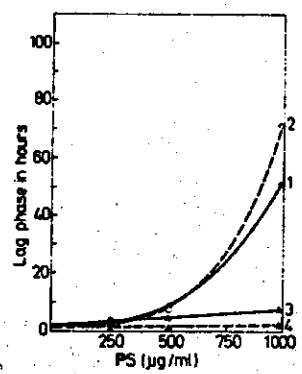

Fig. 5. Effect of potassium sorbate concentration on the lag phase in aerobic culture. The curves are as in Fig. 3

the start of growth in the organic nitrogen medium more definitely than in the synthetic medium. The effect of sorbate on the lag phase in anaerobic cultures could not be evaluated because, in order to avoid a frequent shaking of the cultures, results were read at 8 and later at 12-hour intervals.

As to final population density, sorbate at 1000 µg/ml concentration exerted a drastic inhibitory effect in Sabouraud glucose broth (Fig. 6). In synthetic medium the dose-response curve was linear and there was a definite decrease in the inhibitory effect when the medium had been supplemented with vitamins. As most of the anaerobic cultures showed a more or less definite growth after 96 hours, the differences in final population density could not be evaluated. However, the growth curves (Fig. 2) clearly indicated that in synthetic media in the presence of sorbate at concentrations of 150 µg/ml or more the population density did not change considerably after 24 hours, and was inversely proportional to the sorbate concentration.

After longer incubation periods, although after prolonged lag phases, a definite growth started even at higher sorbate concentrations. Sorbate concentration was therefore checked not only at the start but also at the end of the experiments. In anaerobic cultures there was no change in the amount of sorbate; in aerobic cultures, however, as it could be expected from the changes in the growth curves, the initial sorbate concentration decreased considerably (Table I).

Table I

Decrease of potassium sorbate concentration in aerobic cultures of Pc. albicans

Initial potassium sorbate concentration 1000 µg/ml

Medium	Decrease of potassium sorbate concentration, per cent	
	72 hours	96 hours
Synthetic	43	74
Synthetic + vitamin source	80	100
Sabouraud	—	30
Sabouraud + vitamin source	—	15

Since potassium sorbate inhibits SH-enzymes in vitro and this inhibition can be antagonized with cysteine and other thiols [16, 17], it has been supposed that the same was true for the effect of the agent on growth in vivo. The experiments were performed with a moderately inhibiting sorbate concentration (500 µg/ml) and equimolar (3.33 *mM*) cysteine or reduced glutathione (GSH) concentration; GSH was applied also at 10 and 100 times lower concentrations (Fig. 7). In contrast to the expected result, GSH at 0.33 *mM* concentration or over increased the inhibitory effect of sorbate considerably or even up to the total inhibition. Cysteine at 3.33 *mM* concentration exerted an effect identical with the corresponding GSH level. In accordance with the

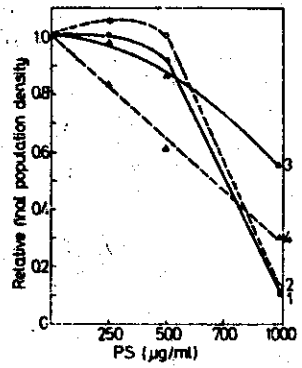

Fig. 6. Effect of potassium sorbate on the final population density in aerobic culture. The curves are as in Fig. 3

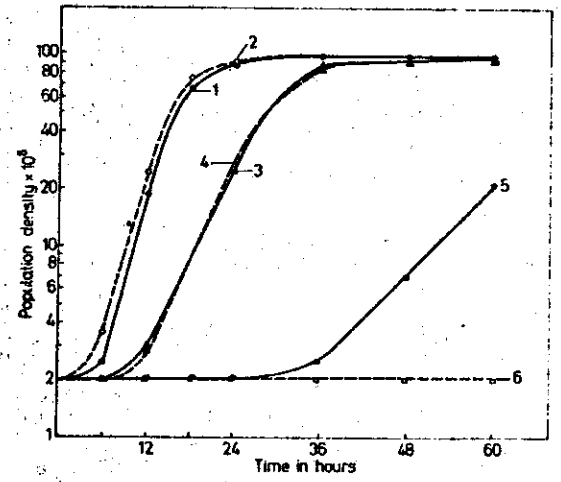

Fig. 7. Synergic effect of potassium sorbate and reduced glutathione (GSH) on the growth of *Pc. albicans*. Aerobic cultures in Sabouraud glucose broth. 1 — control; 2 — GSH controls without sorbate (0.033, 0.33 and 3.33 *mM* GSH); 3 — 3.33 *mM* (500 μg/ml) sorbate; 4 — 3.33 *mM* sorbate + 0.033 *mM* GSH; 5 — 3.33 *mM* sorbate + 0.33 *mM* GSH; 6 — 3.33 *mM* sorbate + 3.33 *mM* GSH

increased growth inhibitory effect in the presence of these thiols, the more the cultures were inhibited, the higher was their sorbate content, even after 48 to 60 hours of incubation (Table II).

Table II

Effect of reduced glutathione (GSH) and cysteine on the decrease of potassium sorbate concentration in aerobic cultures of Pc. albicans

Sabouraud glucose broth cultures; initial potassium sorbate concentration, 500 μg/ml (3.33 *mM*)

Thiol	Decrease of potassium sorbate concentration, per cent	
	48 hours	60 hours
Nil	100	100
0.033 *mM* GSH	100	100
0.33 *mM* GSH	42	57
3.33 *mM* GSH	—	—
3.33 *mM* cysteine	—	—

Discussion

It may be concluded that independently of the vitamin content and nitrogen source of the medium, the anaerobic growth of *Pc. albicans* was definitely inhibited at small concentrations of sorbate. In anaerobic cultures 250 μg/ml concentration decreased the growth rate by about 80%; in aerobic cultures the decrease at the same concentration was only 20%. At higher sorbate concentrations the degree of inhibition differed according to the nitrogen source. In peptone (Sabouraud) medium 1000 μg/ml sorbate caused about 90%, in ammonium sulphate (synthetic) medium about 35% inhibition. The degree of inhibition was not influenced by the presence or absence of yeast extract applied as a vitamin source. In peptone medium the lag phase was considerably prolonged with the increase of the sorbate concentration. The final population density in the presence of the inhibitory agent at 1000 μg/ml concentration was the lowest in peptone medium. Supplementing of the medium with vitamins influenced the final population density only in synthetic medium.

These results should be interpreted in the light of the changes in sorbate concentration. Sorbate determinations during incubation indicated that under aerobic conditions the concentration of the inhibitory agent gradually decreased. In peptone medium the decrease was considerably less than in synthetic medium and thus the effective sorbate concentration was higher in the former medium throughout cultivation. Thus at a given sorbate concentration the changes observed in the growth of the micro-organism are associated with the degree of the decrease in sorbate concentration which is influenced by the composition and aeration of the medium. Before discussing the effect

of glutathione in detail, it should be noted that the stronger and more durable inhibition shown in peptone medium may have been due to the presence in the peptone of cysteine, glutathione or other substances exhibiting a similar effect. It should be emphasized that sorbate concentration was found to decrease only in aerated tubes. This finding explains the fact that in anaerobic cultures even lower sorbate concentrations exerted a lasting inhibitory effect and, on the other hand, as the concentrations of sorbate remained practically unaltered, there was no difference between the various media as to the inhibitory effect of the agent. Accordingly, in aerobic cultures the composition of the medium is not directly responsible for differences in the inhibition, because it does not influence directly the effect of sorbate but affects only the rate of the decrease in sorbate concentration.

It may be assumed that the decrease in the concentration, or sometimes even the total disappearance of sorbate is associated with the aerobic life activities of the yeast. This process may consist in a "detoxication" of such a degree which already caused an alteration in the sorbate molecule involving the loss of its inhibitory effect and adsorption maximum at 262 mμ. Elucidation of this problem requires further experiments.

According to the data based on experiments in vitro it may be supposed that the effect of sorbate in vivo is associated with its inhibitory action on SH-enzymes. It seemed therefore justified to expect that in analogy to the elimination of enzyme inhibition in vitro, the growth inhibitory effect of the agent can also be antagonized with GSH or cysteine. In contrast, these SH-compounds were found to increase and prolong the inhibitory effect of sorbate. Determinations of the inhibitor in the cultures indicated that this effect of thiols was an indirect one, as it involved only a delaying or hindering of the process of sorbate detoxication. The observations indicate partly that the effect of sorbate in vivo differs in mechanism from its effect on enzymes in vitro, and partly that the presence of GSH in the system is unfavourable for the microbial detoxication of sorbate. The present data are insufficient for a more detailed discussion of the problem; it should, however, be added that our observations are supported by REHM [12], who found a synergism with cysteine in the inhibitory effect of sorbic acid.

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Address of the authors:

TIBOR DEÁK
Department of Food Technology and Microbiology, University of Horticulture, Ménesi út 45, Budapest XI, Hungary

ERVIN K. NOVÁK
Laboratory of Mycology, National Institute of Public Health, Gyáli út 2–6, Budapest IX, Hungary

.. G. L. MARSH

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INHIBITION OF SULFHYDRYL ENZYMES WITH SORBIC ACID

JOHN R. WHITAKER,
Department of Food Technology,
University of California, Davis

(Manuscript received May 31, 1958)

Gooding *(11)* found that certain of the α, β -unsaturated monocarboxylic aliphatic acids, including sorbic and crotonic acids, are good fungistatic agents for foods and food wrappers. Since then a great deal of work has been done on the toxicity, purity, method of determination, fate, and the use of sorbic acid as a fungistatic agent in such foods as cheese, fish, pickles, gum and sugar solutions, citrus products, fruit purees and pharmaceutical preparations. These investigations have been reviewed by Melnick, Vahlteich, and Hackett *(18)*. Deuel and Alfin-Slater *(3)* reviewed the advantages of using sorbic acid rather than sodium benzoate in foods. Emard and Vaughn *(4)* and York and Vaughn *(31)* have investigated the selective nature of sorbic acid on the catalase negative lactic acid bacteria and clostridia.

Some postulations have been presented to explain the mechanism of the inhibition of microbial growth by sorbic acid *(5, 17)* but no experimental work has been published on this. Mukherjee *(21)* presented data to show that in the presence of inhibitory amounts of cyanide, butyric acid was oxidized to crotonic acid up to a certain point but after this point was reached the oxidation could not continue further because of inhibition of the enzyme by the accumulated crotonic acid. He found that cyanide was a selective inhibitor of the oxidation of butyric acid to ketones by its action on the enzyme system responsible for converting crotonic acid to β-hydroxy butyric acid. The cyanide had no effect on the dehydrogenases which convert butyric acid to crotonic acid. On the basis of this work, Melnick, Luckmann and Gooding *(17)* postulated that sorbic acid inhibited microbial growth and reproduction through the mechanism of inhibition of the dehydrogenase system.

The present investigation was undertaken to determine if sorbic acid is an inhibitor of sulfhydryl enzymes. Evidence is presented to show that sorbic acid is a sulfhydryl enzyme inhibitor, and it is postulated that it may inhibit the growth of microorganisms by inhibition of the myriad sulfhydryl enzymes present in the living cell.

EXPERIMENTAL

Enzymes. The ficin used was a commercial preparation obtained from Merck. The ficin was dissolved in water, let stand at 0°C. overnight and then centrifuged at 70,000 x g. and 0° C. to remove any insoluble material that might be present. Crystalline alcohol dehydrogenase from yeast and crystalline aldolase from rabbit muscle obtained from Worthington Biochemical Corporation were used. The urease, obtained from Nutritional Biochemicals Corporation, was not a crystalline preparation.

Inhibitors. Sorbic acid, obtained from Union Carbide Chemicals Company, was recrystallized 3 times from water before use. The irradiated sorbic acid was prepared by radiating a saturated solution of sorbic acid at 45° C. for 12 hours with an ultraviolet light (Mineralite, Ultraviolet Prod., Inc., South Pasadena, California) using a wavelength of 2540A. The concentration of sorbic and irradiated sorbic acid was determined by the method of Melnick and Luckmann *(16)*. The maleic acid was prepared from a tech-

nical grade preparation (Nutritional Biochemicals Corporation) by decolorizing with charcoal and recrystallizing 3 times from a water solution.

Inhibition. It has been shown that the inhibition of a sulfhydryl enzyme by maleic acid is a time reaction *(10, 12, 20)*. Figure 1 shows that this is true of sorbic and irradiated sorbic acids also. Therefore, the enzyme was allowed to react for one hour at room temperature (24° C.) with the inhibitors before the activity was determined.

Determination of enzymatic activity. The milk-clotting activity of ficin was determined by the method of Balls and Hoover *(1)* modified as described in a previous publication *(30)*. The esterase activity of ficin was measured by the potentiometric method of Schwert, Neurath, Kaufman and Snoke *(24)* modified to fit the requirements of ficin. The reaction was carried out at pH 6.50 and 35.0° C. using 6.7×10^{-4} molar phosphate buffer and 1.7×10^{-2} molar *p*-toluene sulfonyl-L-arginine methyl ester HCl in a total of 3.0 ml. reaction mixture.

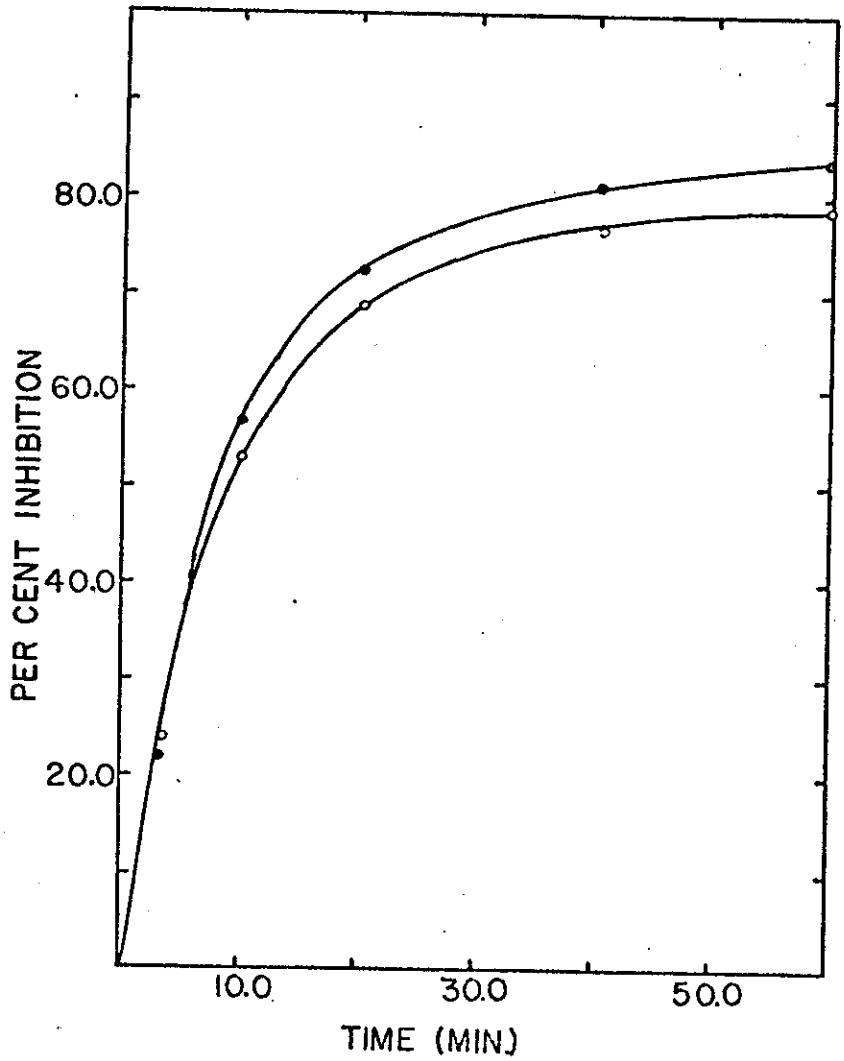

Figure 1. The rate of inhibition of the milk-clotting activity of ficin by sorbic and irradiated sorbic acids. Ficin, at a concentraton of 1.21 mg. per ml. was reacted with 2.82×10^{-5} M sorbic acid (0) and 1.41×10^{-5} M irradiated sorbic acid (●).

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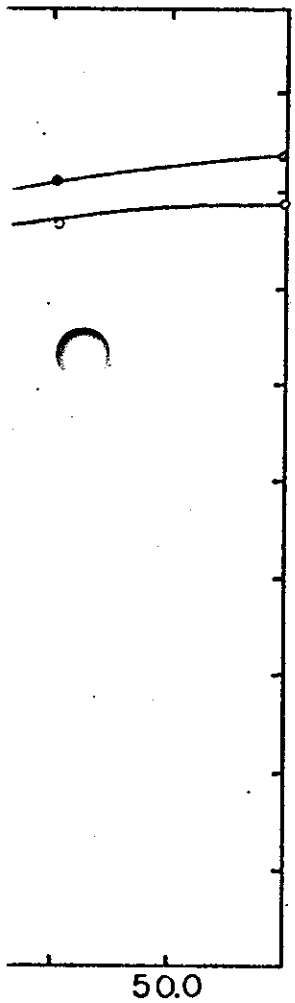

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iated sorbic acid (●).

Alcohol dehydrogenase activity was determined by the method of Racker *(22)*. Aldolase activity was determined by the method of Sibley and Lehninger *(25)* modified according to Swenson and Boyer *(27)*. The enzyme was allowed to react with the substrate for 10 minutes rather than 30 minutes and the reaction was carried out at 35.0° C. rather than 38.0° C. The urease activity was determined by the method of Sumner *(26)*.

RESULTS

The inhibition of ficin activity with sorbic and maleic acids. Table 1 shows that maleic acid, sorbic acid and irradiated sorbic acid inhibited both the milk-clotting and the esterase activities of ficin under the conditions used here. Krishnamurti and Subrahmanyan *(14)* have also found that maleic acid inhibits ficin activity. Sorbic acid and irradiated sorbic acid were found to be approximately 51 and 83 times more effective, respectively, than the same amount of maleic acid for the inhibition of the milk-clotting activity of commercial ficin.

Sorbic acid and irradiated sorbic acid were found to be equally effective and about 20 times more effective than maleic acid in inhibiting the esterase activity of ficin (Table 1). It should be noted that the inhibitors were present in a concentration 6 times greater in this experiment as compared with the experiments on the milk-clotting activity during the reaction of enzyme and substrate. The effect of dilution and of substrate concentration on the enzyme-inhibitor complex formed after the inhibition period has not been investigated. Table 1 shows that cysteine did not reverse the inhibition under the conditions used here. It is surprising that the irradiated sorbic acid is not more effective than the sorbic acid in reducing the esterase activity of ficin since it is about twice as effective in reducing the milk-clotting activity.

The inhibition of alcohol dehydrogenase activity. Table 2 shows that the activity of alcohol dehydrogenase is inhibited by these acids. Irradiated sorbic acid was found to be only slightly more effective than sorbic acid and these were found to be only two to three times more effective than maleic acid. While maleic acid was just as effective an inhibitor of both ficin and alcohol dehydrogenase, sorbic acid and irradiated sorbic acid were only one-fourth to one-sixth as effective against alcohol dehydrogenase as against ficin (compare Tables 1 and 2). The reason for this is not known. However, one should take note that the alcohol dehydrogenase and milk-clotting assays are carried out at pH 8.5 and 5.2, respectively. The difference in pH may well produce a greater effect on the dissociation constant of the sorbic acid-enzyme complex than it does on the maleic acid-enzyme complex. This remains to be shown experimentally.

It was found that the addition of cysteine, added one hour after the inhibitor and allowed to react for 15 minutes before the assay for activity, did not reactivate the maleic acid-inhibited enzyme; in fact, further activity was lost. But the cysteine did partially reactivate the sorbic acid- and irradiated sorbic acid-inhibited enzyme in contrast to its action on inhibited ficin. The sorbic acid-inhibited enzyme was found to be reactivated more than the irradiated sorbic acid-inhibited enzyme.

The inhibition of aldolase and urease activity. These enzymes were not inhibited to a great extent by the concentrations of inhibitors used (Table 2). Aldolase activity appeared to be slightly inhibited at the higher concentration of sorbic and irradiated sorbic acids but the urease activity was not affected. It has been found by Tsen *(29)* that aldolase is much more resistant to inhibition by sodium selenite, cupric chloride and *p*-chloromercuribenzoate than is alcohol dehydrogenase. Fischer *(7)* reported that urease is inhibited by many of the same compounds, such as allyl isothiocyanate, chloropicrin, iodoacetamide, chloroacetophenone, chloroacetone, β, β-diethyl sulfone and bromopicrin, which inhibit papain activity but that much larger concentrations of these materials must be used. Thus, the sluggish nature of the sulfhydryl group apparently involved in the active centers of aldolase and urease would explain why these enzymes were not inhibited by maleic and sorbic acids in this experiment. Perhaps higher concentrations of the inhibitors would produce an appreciable inhibition of these sulfhydryl enzymes also.

DISCUSSION

Maleic acid has been shown to be an inhibitor of a number of sulfhydryl enzymes such as malic dehydrogenase *(23)*, glutamic dehydrogenase *(13)*, succinic dehydrogenase *(20, 28)*, ficin *(14)*, papain *(10, 20)* glyoxalase *(20)*

TABLE 1

Inhibition of the milk-clotting and esterase activities of ficin by sorbic and maleic acids.
(Reacted for one hour at room temperature (24° C.) and pH 5.0 with inhibitor.)

Maleic acid			Sorbic and irradiated sorbic acids				
Inhibitor concentration [1] (millimolar)	Activity lost (per cent)		Inhibitor concentration [1] (millimolar)	Activity lost (per cent)			
	Milk-clotting	Esterase		Milk-clotting		Esterase	
				Sorbic acid	Irradiated sorbic acid	Sorbic acid	Irradiated sorbic acid
1.47	7.05	11.8	0.0294	− 1.12	8.99	—	—
2.94	12.8	18.8	0.0735	3.85	12.5	14.1	17.4
5.88	19.1	31.9	0.147	15.6	29.2	17.4	22.5
8.82	—	35.7	0.294	30.8	49.8	31.4	36.1
11.8	30.0	—	0.588	—	—	45.1	50.2
11.8 + 12.5 millimolar cysteine	33.6	—	0.294 + 12.5 millimolar cysteine	31.4	44.2	—	—
Calculated I_{50} (molar)	2.5×10^{-2}	1.3×10^{-2}		4.9×10^{-4}	3.0×10^{-4}	6.0×10^{-4}	5.9×10^{-4}

[1] Concentration of inhibitor during reaction of inhibitor and enzyme and also during the reaction of enzyme and substrate in the determination of esterase activity. It was 0.17 of this in the presence of substrate during the milk-clotting activity determination.

TABLE 2

Inhibition of alcohol dehydrogenase, aldolase and urease by sorbic and maleic acids.

(Reacted for one hour at room temperature (24° C.) and pH 7.0 with the inhibitor.)

Enzyme	Inhibitor concentration [1] (millimolar)	Activity lost (per cent)		
		Maleic acid	Sorbic acid	Irradiated sorbic acid
Alcohol Dehydrogenase (ADH)	1.47	7.81	31.2	26.0
ADH	2.94	13.0	28.6	33.9
ADH	5.88	20.8	47.4	52.6
ADH	8.82	28.6	60.5	68.3
ADH	13.2	44.8	—	—
ADH	8.82 + 12.5 millimolar cysteine	36.8	10.9	42.5
Aldolase	8.82	− 1.24	1.24	1.65
Aldolase	13.2	− 1.24	5.76	7.00
Urease	8.82	− 0.68	0.34	2.04
Urease	11.8	2.04	− 0.68	− 0.34
ADH	Calculated I_{50} (molar)	1.5×10^{-2}	6.5×10^{-3}	5.5×10^{-3}

[1] Concentration of inhibitor during reaction of inhibitor with enzyme. It was 0.17, 0.22 and 0.50 of this value for alcohol dehydrogenase, aldolase and urease, respectively, during the reaction with the substrate.

and to inhibit the growth and reproduction of cells *(8, 9, 15)*. Morgan and Friedmann *(19, 20)* have shown that maleic acid and sulfhydryl compounds react to form stable compounds. They isolated the addition compounds with thioacetic acid, cysteine and glutathione and showed that they reacted with maleic acid to form α-thiosuccinic acid derivatives, RSCH-(CO_2H)-CH_2-CO_2H. While maleic acid, the *cis* isomer, is an efficient blocking agent of the sulfhydryl group, fumaric acid, the *trans* isomer, has not been found to be reactive against this group.

On the basis of the similarity in the structure of sorbic acid, CH_3-CH = CH-CH = CH-COOH, and maleic acid, HOOC-CH = CH-COOH, and the similarity in their action on ficin, alcohol dehydrogenase, aldolase and urease it is suggested that sorbic acid inhibits these enzymes by the same mechanism as does maleic acid; i.e. it forms a thiohexenoic acid derivative, CH_3-CH = CH-RSCH-CH_2CO_2H. Because of the two double bonds in sorbic acid it can exist in 4 isomeric forms: *trans, trans; trans, cis; cis, trans;* and *cis, cis.* In order for the sorbic acid to react as postulated above it would have to be the *cis, cis* or *cis, trans* isomer. It is thought that the commercial sorbic acid is largely of the *trans, trans* configuration since this would be the most stable form. On the basis of this assumption and the fact that fumaric acid can be converted to the *cis* isomer, maleic acid, by ultraviolet irradiation *(6)* the sorbic acid was irradiated as described in the experimental section. The irradiated sorbic acid was found to be a better inhibitor of ficin than was sorbic acid. The final proof of the postulated mechanism of inhibition of sulfhydryl enzymes by sorbic acid, however, must await the synthesis of the *cis, trans* isomer and its reaction with thioacetic acid, cysteine or glutathione.

The inhibition of the sulfhydryl enzyme systems of microorganisms could certainly explain why many of these organisms cannot grow and reproduce

in the presence of sorbic acid. Some of the important sulfhydryl enzymes found in microorganisms are hexokinase, galactokinase, 6-phosphogluconic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, carboxylase, coenzyme A-linked pyruvic acid oxidase, alcohol dehydrogenase, TPN-alcohol dehydrogenase, potassium-activated yeast aldehyde dehydrogenase, TNP-linked aldehyde dehydrogenase, aldehyde dehydrogenase, butyryl coenzyme A dehydrogenase, acetate kinase, succinic dehydrogenase, amino acid reductases, histidase, tryptophanase, aspartase, DPNH cytochrome C reductase and ribonuclease. This is not a complete list and differences would undoubtedly be found in the rates of reaction of these enzymes with sorbic acid. For an excellent review on the thiol groups of biological importance the reader is referred to the work of Barron *(2)*. In view of the many sulfhydryl enzymes in microorganisms and the demonstrated effectiveness of sorbic acid in inhibiting these enzymes it is not surprising that their growth is inhibited. What is more surprising is that certain bacteria can grow quite well on media containing sorbic acid *(4, 31)*. Apparently, these organisms have a mechanism for metabolizing or detoxifying the sorbic acid.

SUMMARY

Sorbic acid has been shown to be an inhibitor of the sulfhydryl enzymes ficin and alcohol dehydrogenase at concentrations of 10^{-4} molar and is more efficient than maleic acid. Aldolase and urease were not significantly inhibited by sorbic and maleic acids under the conditions used in this work. Irradiated sorbic acid was found to be a better inhibitor of ficin than sorbic acid.

It is suggested that the mechanism of inhibition of the sulfhydryl enzymes by sorbic acid and similar α, β-unsaturated acids is similar to the one found for maleic acid. It is further postulated that this is the mode of inhibition of the growth and reproduction of microorganisms.

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...IVE GRAS SURVEY — NAS/NRC 1972

...E 19. PART A — POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
SODIUM THIOSULFATE NAS 0209	24 BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	**********	.000010	**********
			12-23 MO.	**********	.000020	**********
			2-65+ YR.	.003250	.009440	.016250
SODIUM THIOSULFATE NAS 0209	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	**********	.000010	**********
			12-23 MO.	**********	.000020	**********
			2-65+ YR.	.003250	.009440	.016250
SODIUM TRIPOLYPHOSPHATE NAS 0210	10 MEAT PRODS(R)	16	0-5 MO.	3.423090	9.024810	5.309120
			6-11 MO.	64.416330	173.644020	101.413440
			12-23 MO.	93.979380	161.507610	147.955340
			2-65+ YR.	243.972960	404.858190	384.097260
SODIUM TRIPOLYPHOSPHATE NAS 0210	11 POULTRY(R)	*	0-5 MO.	1.875000	5.625000	2.500000
			6-11 MO.	14.625000	49.500000	19.500000
			12-23 MO.	24.750000	69.000000	33.000000
			2-65+ YR.	48.375000	123.000000	64.500000
SODIUM TRIPOLYPHOSPHATE NAS 0210	13 FISH PRODS(R)	*	0-5 MO.	.400200	1.200600	.499980
			6-11 MO.	5.202600	19.609800	6.499740
			12-23 MO.	21.610800	54.027000	26.998920
			2-65+ YR.	49.624200	123.661800	61.997520
SODIUM TRIPOLYPHOSPHATE NAS 0210	20 GELATIN PUD(R)	*	0-5 MO.	2.755600	8.720060	2.755600
			6-11 MO.	17.635840	53.450540	17.635840
			12-23 MO.	19.013540	46.299080	19.013540
			2-65+ YR.	28.107120	72.334500	28.107120
SODIUM TRIPOLYPHOSPHATE NAS 0210	48 SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	.037500	**********
			12-23 MO.	**********	.075000	**********
			2-65+ YR.	.037500	.187500	.050000
SODIUM TRIPOLYPHOSPHATE NAS 0210	ALL CATEGORIES ********** ********** **********	21	0-5 MO.	8.453390	22.570170	11.144700
			6-11 MO.	101.879770	296.249960	145.049020
			12-23 MO.	159.353020	330.503690	226.968400
			2-65+ YR.	370.117380	724.041990	538.751920
SORBIC ACID NAS 0211	01 BAKED GOODS(R)	10	0-5 MO.	.502360	1.194300	1.423240
			6-11 MO.	6.741160	13.747720	10.632440
			12-23 MO.	14.464300	23.832970	22.813700
			2-65+ YR.	36.412880	54.080520	57.431920

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TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
SORBIC ACID NAS 0211	04 FATS OILS(R)	4	0-5 MO.	.275550	.275550	.275550
			6-11 MO.	1.543080	4.133250	1.543080
			12-23 MO.	3.471930	6.613200	3.471930
			2-65+ YR.	9.644250	17.414760	9.644250
SORBIC ACID NAS 0211	05 MILK PRODS(R)	*	0-5 MO.	.321300	.239000	.353700
			6-11 MO.	3.712100	17.855950	4.087200
			12-23 MO.	3.242750	10.376800	3.569750
			2-65+ YR.	2.350250	7.175700	2.587250
SORBIC ACID NAS 0211	06 CHEESE(R)	5	0-5 MO.	**********	.147080	**********
			6-11 MO.	3.971160	14.266760	5.121630
			12-23 MO.	11.472240	32.651760	14.795820
			2-65+ YR.	13.825520	34.710880	17.830860
SORBIC ACID NAS 0211	07 FROZN DAIRY(R)	*	0-5 MO.	.100000	.410000	.120000
			6-11 MO.	.990000	2.640000	1.140000
			12-23 MO.	1.440000	3.380000	1.728000
			2-65+ YR.	2.560000	6.170000	3.072000
SORBIC ACID NAS 0211	14 PRCCSD VEGS(R)	*	0-5 MO.	.368900	1.106700	.369740
			6-11 MO.	6.324000	14.756000	6.338400
			12-23 MO.	10.276500	17.200550	10.295900
			2-65+ YR.	22.397300	37.733200	22.448500
SORBIC ACID NAS 0211	15 COOKM RELSH(R)	*	0-5 MO.	**********	.010000	**********
			6-11 MO.	.080000	.220000	.400000
			12-23 MO.	.280000	.760000	1.400000
			2-65+ YR.	.880000	2.120000	4.400000
SORBIC ACID NAS 0211	16 SOFT CANDY(R)	*	0-5 MO.	2.800000	28.000000	2.800000
			6-11 MO.	30.800000	95.200000	30.800000
			12-23 MO.	49.000000	130.200000	49.000000
			2-65+ YR.	81.200000	246.400000	81.200000
SORBIC ACID NAS 0211	17 CONF FROST(R)	*	0-5 MO.	**********	.005000	**********
			6-11 MO.	.005000	.010000	.010000
			12-23 MO.	.010000	.035000	.020000
			2-65+ YR.	.015000	.040000	.030000
SORBIC ACID NAS 0211	18 JAM JELLY(R)	*	0-5 MO.	**********	.150000	**********
			6-11 MO.	3.350000	11.150000	6.700000
			12-23 MO.	1.500000	5.600000	3.000000
			2-65+ YR.	2.850000	8.850000	5.700000

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
SORBIC ACID NAS 0211	19 SWEET SAUCE(R)	6	0-5 MO.	.139170	.185550	.258330
			6-11 MO.	.417510	1.438090	.774990
			12-23 MO.	1.206140	3.525640	2.238860
			2-65+ YR.	3.154520	8.303610	5.859480
SORBIC ACID NAS 0211	20 GELATIN PUD(R)	*	0-5 MO.	.006800	.009180	.007200
			6-11 MO.	.043520	.131920	.046080
			12-23 MO.	.046920	.114240	.049680
			2-65+ YR.	.069360	.178500	.073440
SORBIC ACID NAS 0211	23 BEV TYPE I(R)	*	0-5 MO.	.000240	.000360	.000240
			6-11 MO.	.002270	.007770	.002270
			12-23 MO.	.005420	.016250	.005420
			2-65+ YR.	.010400	.027770	.010400
SORBIC ACID NAS 0211	27 GRAVIES(R)	*	0-5 MO.	.087040	.261120	.121600
			6-11 MO.	1.218560	3.394560	1.702400
			12-23 MO.	3.132440	8.878080	4.377400
			2-65+ YR.	7.224320	18.539520	10.092800
SORBIC ACID NAS 0211	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	**********
			6-11 MO.	.700000	1.150000	**********
			12-23 MO.	.400000	1.700000	**********
			2-65+ YR.	.450000	.750000	**********
SORBIC ACID NAS 0211	ALL CATEGORIES ********** ********** **********	28	0-5 MO.	5.001360	31.992850	5.729600
			6-11 MO.	59.259060	180.102020	69.298400
			12-23 MO.	99.949640	244.890440	116.770680
			2-65+ YR.	163.044000	442.502660	220.376900
SORBITOL NAS 0212	01 BAKED GOODS(R)	7	0-5 MO.	473.757700	627.032250	522.024780
			6-11 MO.	3539.248700	7217.837900	3899.832180
			12-23 MO.	7594.057250	12512.776900	8367.750150
			2-65+ YR.	19117.516600	28397.593900	21065.235240
SORBITOL NAS 0212	04 FATS OILS(R)	*	0-5 MO.	1.130150	1.130150	1.146700
			6-11 MO.	6.328840	16.952250	6.421520
			12-23 MO.	14.239890	27.123600	14.448420
			2-65+ YR.	39.855250	71.425480	40.134500
SORBITOL NAS 0212	05 MILK PRODS(R)	*	0-5 MO.	.740340	.548400	303.883920
			6-11 MO.	8.555040	41.143710	3511.547520
			12-23 MO.	7.471950	23.910240	3066.976600
			2-65+ YR.	5.415450	16.534260	2222.854600

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIET, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE -- SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** (AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
POTASSIUM PHOSPHATE DI NAS 0157	19 SWEET SAUCE(R)	*	0-5 MO.	1.095000	1.460000	1.095000
			6-11 MO.	3.285000	11.315000	3.285000
			12-23 MO.	9.490000	27.740000	9.490000
			2-65+ YR.	24.820000	65.[illegible]	24.820000
POTASSIUM PHOSPHATE DI NAS 0157	20 GELATIN PUD(R)	*	0-5 MO.	3.000000	[illegible]	[illegible]
			6-11 MO.	15.200000	58.200000	28.[illegible]
			12-23 MO.	20.700000	50.400000	27.600000
			2-65+ YR.	30.600000	78.750000	40.[illegible]
POTASSIUM PHOSPHATE DI NAS 0157	23 BEV TYPE I(R)	*	0-5 MO.	.760000	1.[illegible]	[illegible]
			6-11 MO.	7.[illegible]	24.700000	[illegible]
			12-23 MO.	17.[illegible]	51.[illegible]	25.[illegible]
			2-65+ YR.	33.[illegible]	82.[illegible]	48.[illegible]
POTASSIUM PHOSPHATE DI NAS 0157	25 INST DAIRY(R)	9	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	19.[illegible]	[illegible]	[illegible]
			12-23 MO.	11.[illegible]	47.[illegible]	[illegible]
			2-65+ YR.	12.527010	28.[illegible]	25.[illegible]
POTASSIUM PHOSPHATE DI NAS 0157	ALL CATEGORIES **********	12	0-5 MO.	18.[illegible]	17.[illegible]	[illegible]
			6-11 MO.	121.[illegible]	[illegible]	[illegible]
			12-23 MO.	197.[illegible]	[illegible]	[illegible]
			2-65+ YR.	[illegible]	[illegible]	[illegible]
POTASSIUM SORBATE NAS 0158	01 BAKED GOODS(R)	14	0-5 MO.	2.149140	2.844450	2.459000
			6-11 MO.	16.[illegible]	32.[illegible]	[illegible]
			12-23 MO.	24.465450	[illegible]	[illegible]
			2-65+ YR.	56.[illegible]	124.[illegible]	[illegible]
POTASSIUM SORBATE NAS 0158	04 FATS OILS(R)	12	0-5 MO.	.680950	.[illegible]	.[illegible]
			6-11 MO.	3.811000	10.[illegible]	4.[illegible]
			12-23 MO.	8.574930	16.[illegible]	[illegible]
			2-65+ YR.	23.815250	43.010760	25.278750
POTASSIUM SORBATE NAS 0158	05 MILK PRODS(R)	4	0-5 MO.	1.066500	.700000	2.[illegible]
			6-11 MO.	12.324000	59.264750	30.[illegible]
			12-23 MO.	10.763750	34.444000	[illegible]
			2-65+ YR.	7.201250	23.[illegible]	[illegible]
POTASSIUM SORBATE NAS 0158	06 CHEESE(R)	8	0-5 MO.	**********	.1[illegible]	**********
			6-11 MO.	4.092660	14.703260	5.[illegible]
			12-23 MO.	11.823240	33.650760	15.[illegible]
			2-65+ YR.	14.246520	35.772280	19.126130

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
POTASSIUM SORBATE NAS 0158	07 FROZN DAIRY(R)	6	0-5 MO.	.050500	.207050	.098900
			6-11 MO.	.479750	1.333200	.939550
			12-23 MO.	.727200	1.706900	1.424160
			2-65+ YR.	1.292800	3.115850	2.531640
POTASSIUM SORBATE NAS 0158	08 PROCSD FRUT(R)	9	0-5 MO.	2.743200	7.354630	4.782070
			6-11 MO.	30.229660	75.237300	52.737900
			12-23 MO.	58.720220	116.564690	102.420660
			2-65+ YR.	69.051710	146.275280	120.441230
POTASSIUM SORBATE NAS 0158	09 FRUIT ICES(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.195000	.520000	.210000
			12-23 MO.	.390000	1.365000	.420000
			2-65+ YR.	.455000	1.625000	.490000
POTASSIUM SORBATE NAS 0158	10 MEAT PRODS(R)	*	0-5 MO.	.002200	.005000	.002300
			6-11 MO.	.041400	.111600	.002100
			12-23 MO.	.060400	.168800	.090800
			2-65+ YR.	.156600	.260200	.235200
POTASSIUM SORBATE NAS 0158	13 FISH PRODS(R)	*	0-5 MO.	.042800	.128400	.042800
			6-11 MO.	.556400	2.097200	.556400
			12-23 MO.	2.311200	5.776000	2.311200
			2-65+ YR.	5.307200	13.225200	5.307200
POTASSIUM SORBATE NAS 0158	14 PROCSD VEGS(R)	*	0-5 MO.	.707280	2.121840	1.178660
			6-11 MO.	12.124800	28.291200	20.207800
			12-23 MO.	19.702800	32.989560	32.834100
			2-65+ YR.	42.942000	72.344640	71.561600
POTASSIUM SORBATE NAS 0158	15 CONDM RELSH(R)	5	0-5 MO.	**********	.030030	**********
			6-11 MO.	.240240	.660660	.438720
			12-23 MO.	.840840	2.282280	1.595520
			2-65+ YR.	2.642640	6.366360	4.829920
POTASSIUM SORBATE NAS 0158	16 SOFT CANDY(R)	10	0-5 MO.	.108420	1.084200	.115720
			6-11 MO.	1.192620	3.686280	1.272920
			12-23 MO.	1.897350	5.041530	2.025100
			2-65+ YR.	3.144180	9.540960	3.335880
POTASSIUM SORBATE NAS 0158	17 CONF FROST(R)	8	0-5 MO.	**********	.082620	**********
			6-11 MO.	.082620	.165240	.083330
			12-23 MO.	.165240	.578340	.166660
			2-65+ YR.	.247860	.660960	.249990

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
POTASSIUM SORBATE NAS 0158	18 JAM JELLY(R)	4	0-5 MO.	**********	.177180	**********
			6-11 MO.	3.957020	13.176330	6.395820
			12-23 MO.	1.771800	6.614720	2.863800
			2-65+ YR.	3.366420	10.453620	5.441220
POTASSIUM SORBATE NAS 0158	19 SWEET SAUCE(R)	13	0-5 MO.	.160640	.229120	.199620
			6-11 MO.	.506520	1.744680	.598860
			12-23 MO.	1.463280	4.277280	1.730040
			2-65+ YR.	3.627040	10.074120	4.524720
POTASSIUM SORBATE NAS 0158	20 GELATIN PUD(R)	8	0-5 MO.	11.609800	15.673230	11.673300
			6-11 MO.	74.302720	225.231120	74.726400
			12-23 MO.	80.107620	195.044640	80.564400
			2-65+ YR.	118.419960	304.757250	119.095200
POTASSIUM SORBATE NAS 0158	22 SNACK FOODS(R)	*	0-5 MO.	**********	.030000	**********
			6-11 MO.	.120000	.330000	.330000
			12-23 MO.	.330000	.930000	.930000
			2-65+ YR.	.390000	1.110000	1.040000
POTASSIUM SORBATE NAS 0158	23 BEV TYPE I(R)	12	0-5 MO.	.392400	.580600	.853440
			6-11 MO.	3.711450	12.703950	8.072120
			12-23 MO.	8.861700	26.560750	19.273520
			2-65+ YR.	17.004000	45.403950	36.952400
POTASSIUM SORBATE NAS 0158	24 BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	**********	.020970	**********
			12-23 MO.	**********	.041940	**********
			2-65+ YR.	6.815250	19.795680	8.388250
POTASSIUM SORBATE NAS 0158	27 GRAVIES(R)	*	0-5 MO.	.048940	.146820	.064130
			6-11 MO.	.685160	1.906660	.897820
			12-23 MO.	1.761640	4.991880	2.308630
			2-65+ YR.	4.062020	10.424220	5.322770
POTASSIUM SORBATE NAS 0158	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.910000	1.495000	1.000000
			12-23 MO.	.520000	2.210000	.600000
			2-65+ YR.	.585000	.975000	.675000
POTASSIUM SORBATE NAS 0158	48 SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	.001000	**********
			12-23 MO.	**********	.002000	**********
			2-65+ YR.	.001000	.005000	.001000

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TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
POTASSIUM SORBATE NAS 0158	ALL CATEGORIES ********** ********** **********	83	0-5 MO.	19.770760	32.322090	24.[illegible]
			6-11 MO.	165.624440	405.691480	226.[illegible]
			12-23 MO.	245.242860	548.282050	342.[illegible]
			2-65+ YR.	412.304020	887.837350	552.371020
POTASSIUM SULFATE NAS 0159	23 BEV TYPE I(R)	*	0-5 MO.	.236880	.355320	.355440
			6-11 MO.	2.240490	7.668990	3.361070
			12-23 MO.	5.349540	16.038750	8.027020
			2-65+ YR.	10.264800	27.408990	15.402460
POTASSIUM SULFATE NAS 0159	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.236880	.355320	.355440
			6-11 MO.	2.240490	7.668990	3.361070
			12-23 MO.	5.349540	16.038750	8.027020
			2-65+ YR.	10.264800	27.408990	15.402460
DL-PROLINE NAS 0161	10 MEAT PRODS(R)	*	0-5 MO.	.022000	.055000	.110000
			6-11 MO.	.414000	1.110000	2.070000
			12-23 MO.	.604000	1.038000	3.020000
			2-65+ YR.	1.560000	2.602000	7.840000
DL-PROLINE NAS 0161	15 CONDM RELSH(R)	*	0-5 MO.	**********	.002000	**********
			6-11 MO.	.016000	.044000	.080000
			12-23 MO.	.056000	.152000	.280000
			2-65+ YR.	.176000	.424000	.800000
DL-PROLINE NAS 0161	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.022000	.050000	.110000
			6-11 MO.	.430000	1.160000	2.150000
			12-23 MO.	.660000	1.190000	3.300000
			2-65+ YR.	1.744000	3.026000	8.720000
PROPIONIC ACID NAS 0163	01 BAKED GOODS(R)	17	0-5 MO.	.255000	.337500	.409020
			6-11 MO.	1.905000	3.885000	3.855620
			12-23 MO.	4.087500	6.735000	6.[illegible]
			2-65+ YR.	10.290000	15.285000	16.505160
PROPIONIC ACID NAS 0163	04 FATS OILS(R)	*	0-5 MO.	.012100	.012100	.037250
			6-11 MO.	.067760	.181500	.208000
			12-23 MO.	.152460	.290400	.469350
			2-65+ YR.	.423500	.764720	1.303750
PROPIONIC ACID NAS 0163	06 CHEESE(R)	4	0-5 MO.	**********	.001170	**********
			6-11 MO.	.031590	.113400	.203040
			12-23 MO.	.091260	.259740	.586560
			2-65+ YR.	.105980	.276120	.706880

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Sorbic Acid. *2,4-Hexadienoic acid;* 2-propenylacrylic acid. $CH_3CH{=}CHCH{=}CHCOOH$; mol wt 112.12. $C_6H_8O_2$; C 64.27%, H 7.19%, O 28.54%. May be obtained from berries of the mountain ash, *Sorbus aucuparia* L., *Rosaceae* where it occurs as the lactone, called parasorbic acid: Hofmann, *Ann.* 110, 129 (1859). Synthesis by condensing crotonaldehyde and malonic acid in pyridine soln: Doebner, *Ber.* 33, 2140 (1900); Allen, Van Allan, *Org. Syn.* coll. vol. 3, 783 (1955); by condensing crotonaldehyde and ketene in the presence of boron trifluoride; Hagemeyer, Jr., *Ind. Eng. Chem.* 41, 768 (1949). Prepn from 1,1,3,5-tetraalkoxyhexane: Parker, MacLean, U.S. pat. 2,921,090 (1960 to Celanese). Several other syntheses: Fernholz, Mundlos, Ger. pat. 1,049,852 (1959 to Farbwerke Hoechst); U.S. pat. 3,021,365 (1962 to Farbwerke Hoechst). Purification: Fernholz, Ger. pat. 1,044,803 (1958 to Farbwerke Hoechst). The *trans,trans*-isomer is usually obtained and is the commercial product.

Needles from water, mp 134.5°. Should be stored at temps below 40°. bp 228° (decompn). Vapor pressure at 20° = <0.01 mm, at 143° = 50 mm. Flash pt 260°F (127°C). K at 25° = 1.73×10^{-5}. Solubility in water at 30° = 0.25%, at 100° = 3.8%, in propylene glycol at 20° = 5.5%, in abs ethanol or methanol = 12.90%, in 20% ethanol = 0.29%, in glacial acetic acid 11.5%, in acetone 9.2%, in benzene 2.3%, in carbon tetrachloride 1.3%, in cyclohexane 0.28%, in dioxane 11.0%, in glycerol 0.31%, in isopropanol 8.4%, in isopropyl ether 2.7%, in methyl acetate 6.1%, in toluene 1.9%.

Also used as the sodium salt. Prepn: Horn, Fernholz, Ger. pat. 1,045,390 (1958 to Farbwerke Hoechst).

USE: Mold and yeast inhibitor. Fungistatic agent for foods, especially cheeses. To improve the characteristics of drying oils. In alkyd type coatings to improve gloss. To improve milling characteristics of cold rubber. LD_{50} orally in rats: 7.36 g/kg. The use of calcium sorbate is described in U.S. pat. 3,139,378 (Gooding, 1964 to Corn Products Co.). *See also* Potassium Sorbate. *Human Toxicity:* Practically nontoxic. May cause slight skin irritation.

Potassium Sorbate. *Sorbic acid potassium salt;* 2,4-hexadienoic acid potassium salt; potassium 2,4-hexadienoate; BB powder. $CH_3CH{=}CHCH{=}CHCOOK$; mol wt 150.22. $C_6H_7KO_2$; C 47.97%, H 4.70%, K 26.03%, O 21.30%. Prepd from sorbic acid and KOH: Probst, Oehme, U.S. pat. 3,173,948 (1965 to Hoechst AG).

Crystals, d_{20}^{25} 1.363. Dec above 270°. Solubility in water at 20° = 58.2%; in alcohol 6.5%.

USE: As mold and yeast inhibitor, like sorbic acid, especially where greater sol'y in water is desirable. *Human Toxicity:* Low toxicity. May cause mild irritation of skin.

No.	Name	Synonym	Formula	Ref.	Mol. Wt.
5604	**Scopoletin**	7-OH-6-MeO-coumarin	$C_{10}H_8O_4$	XVIII-99	192.17
5605	**Sebacic acid**	decandioic acid	$(CH_2)_8(CO_2H)_2$	II-718	202.25
5606	nitrile	.....	$(CH_2)_8(CN)_2$	II-720	164.25
5607	**Selene-phenol**	.....	$C_6H_5{\cdot}SeH$	VI-345	157.07
5608	urea	.....	$NH_2{\cdot}CSe{\cdot}NH_2$	III-227	123.02
5609	**Semicarbazide**	.....	$NH_2{\cdot}NH{\cdot}CO{\cdot}NH_2$	III-98	75.07
5610	hydrochloride	.....	$NH_2CON_2H_3{\cdot}HCl$	III-100	111.53
5611	**Serine (*dl*)**	.....	$HO{\cdot}CH_2{\cdot}CHNH_2{\cdot}CO_2H$	IV-512	105.09
5612	**Serine (*d* or *l*)**	.....	$C_3H_7O_3N$	IV-505	105.09
5613	***iso*-Serine (*dl*)**	.....	$NH_2{\cdot}CH_2{\cdot}CHOH{\cdot}CO_2H$	IV-503	105.09
5614	***iso*-Serine (*d* or *l*)**	.....	$C_3H_7O_3N$	IV-503	105.09
5615	**Silico-acetic acid**	.....	$C_2H_3{\cdot}SiO{\cdot}OH$	IV-629	76.13
5616	benzoic acid	.....	$C_6H_5{\cdot}SiO{\cdot}OH$	XVI-911	138.20
5617	**Silicon methyl**	.....	$(CH_3)_4Si$	IV-625	88.23
5618	phenyl trichloride	.....	$C_6H_5{\cdot}SiCl_3$	XVI-911	211.55
5619	tetraethyl	siliconenane	$(C_2H_5)_4Si$	IV-625	144.33
5620	**Silver† fulminate**	.....	$Ag_2C_2O_2N_2$	I-722	299.77
5621	**Sodium‡ acetanilide**	.....	$CH_3CON(Na){\cdot}C_6H_5$	XII-237	157.15
5622	ethyl	.....	$C_2H_5{\cdot}Na$	*IV-618	52.05
5623	glycerolate	.....	$NaC_3H_7O_3$	I-511	114.08
5624	mercaptide	.....	$C_2H_5{\cdot}S{\cdot}Na$	I-341	84.12
5625	**Sorbic acid**	hexadienoic acid	$CH_3(CH{:}CH)_2CO_2H$	II-483	112.13
5626	**Sozoiodolic acid**	2,6-diiodophenol-4-sulfonic acid	$I_2(OH)C_6H_2{\cdot}SO_3H{\cdot}3H_2O$	XI-245	480.03
5627	Na salt	sozoiodol-sodium	$C_6H_3O_4I_2SNa{\cdot}2H_2O$	XI-245	483.98
5628	Hg salt	.....	$C_6H_2O_4I_2SHg$	*XI-56	624.54
5629	Zn salt	.....	$(C_6H_3O_4I_2S)_2Zn{\cdot}6H_2O$	XI-245	1023.38
5630	**Starch‡**	.....	$(C_6H_{10}O_5)_x$	.....	(162.14)
5631	**Stearic acid**	octadecanoic acid	$CH_3(CH_2)_{16}CO_2H$	II-377	284.47
5632	ammonium salt	amm. stearate	$C_{17}H_{35}CO_2NH_4$	*II-171	301.52
5633	aldehyde	octadecanal	$CH_3(CH_2)_{16}CHO$	I-718	268.49
5634	amide	stearamide	$C_{17}H_{35}{\cdot}CO{\cdot}NH_2$	II-384	283.50
5635	anhydride	.....	$(C_{17}H_{35}CO)_2O$	II-384	550.96
5636	anilide	stearanilide	$C_{17}H_{35}CO{\cdot}NHC_6H_5$	XII-257	359.60
5637	chloride	stearyl chloride	$C_{17}H_{35}{\cdot}CO{\cdot}Cl$	II-384	302.93
5638	nitrile	heptadecyl cyanide	$C_{17}H_{35}{\cdot}CN$	II-384	265.49
5639	**Stearolic acid**	octadecynoic acid	$C_8H_{17}C{:}C(CH_2)_7{\cdot}CO_2H$	II-495	280.45
5640	**Stearone**	.....	$C_{35}H_{70}O$	I-720	506.95
5641	**Stearoxylic acid**	.....	$CH_3(CH_2)_7(CO)_2{\cdot}(CH_2)_7CO_2H$	III-761	312.45
5642	**Stilbene (*trans*)**	diPh-ethylene	$C_6H_5{\cdot}CH{:}CH{\cdot}C_6H_5$	V-630	180.25
5643	**Stilbene (*cis*)**	diPh-ethylene	$C_6H_5{\cdot}CH{:}CH{\cdot}C_6H_5$	V-633	180.25
5644	**Stovaine‖**	benzoyl-EtdiMe-amino-*iso*-propanol HCl	$C_6H_5CO_2C(C_2H_5){\cdot}(CH_3)CH_2N(CH_3)_2{\cdot}HCl$	IX-175	271.79
5645	**Strophanthidin**	.....	$C_{23}H_{32}O_5{\cdot}\frac{1}{2}H_2O$	.....	413.52
5646	**Styrene**	phenyl-ethylene	$C_6H_5{\cdot}CH{:}CH_2$	V-474	104.15

† See also inorganic compounds.
‡ See also No. 3443.
‖ Free base, liquid, b. p. 149[25mm], s. organic solvents.

Salt of amber 5553
Salysal 5585
Sandoptal 1938
Saponrubrin, cf. glcde.
Saponotoxin, cf. glcde.
Sarcine, cf. alkd.
Saxin 5579
Scammonin, cf. glcde.
Schaeffer acid 4498, 4507
Scholikopf's acid 4543
Scopolamine, cf. alkd.
Secaline, cf. alkd.

No.	Name	Specific Gravity	Melting Point °C.	Boiling Point °C.	Solubility in 100 Parts: Water	Alcohol	Ether
5604	[illegible]	.....	204	subl.	v. sl. s.	s. h.; i. bz.	s. h. ac.
5605	pr. HNO_3	1.207^{0}_{4}°	134.5	294.5[100mm]	0.1 c.; 2 h.	v. s.	v. s.
5606	col. lq.	.....	7-9	199-200[15mm]	.....	.....	.....
5607	oil	1.487^{15}°	.....	183.6	v. sl. s.	v. s. CCl_4	v. s.
5608	pr./aq.	.....	200 ± d.	.....	10^{15}°	3^{15}°	0.6^{100}°
5609	pr./al.	.....	96	.....	v. s.	v. s.	i.
5610	pr./aq. al.	.....	173 d.	.....	v. s.	i. abs.	i.
5611	pr./aq.	.....	246 d.	.....	4^{20}°	i.	i.
5612	col. pr.	.....	228 d.	.....	33^{25}°	i.	.....
5613	mn.	.....	248	.....	1.5^{20}° s. h. aq.	v. sl. s.	v. sl. s.
5614	col. cr./aq.	.....	199-200 d.	.....	25^{20}°	.....	.....
5615	amor. pd.	.....	.....	.....	i.	i. h. aq. Na_2CO_3	s. conc. KOH
5616	glass/et.	.....	−99(−102)	.....	i.	s. KOH	s.
5617	lq.	0.648^{12}_{4}°	ign. in air	26.6	i. H_2SO_4	.....	.....
5618	lq.	$1.326^{12.5}_{4}$°	.....	201.5	d.	d.; s. chl.	s.
5619	col. lq.	0.768^{12}_{4}°	.....	154.7	i.	.....	.....
5620	wh. nd./aq.	.....	.....	expl.	0.02^{100}	s.	i. HNO_3
5621	cr. pd.	.....	.....	.....	d. h.	.....	.....
5622	wh. pd.	.....	ign. in air	.....	d.	i. bz.	.....
5623	wh. pd.	.....	d. 245	.....	d.	s.	i.; i. CS_2
5624	cr./al.	.....	.....	.....	v. s.; d. h.	v. s.; d. h.	.....
5625	nd./aq.	.....	134.5	228 d.	s. h.	v. s.	v. s.
5626	col. mn. pr.	.....	120 (anh.); d. 190	$-3H_2O$, 100	s.	s.	s.
5627	col. cr.	.....	.....	.....	16	6	i.
5628	or. pd.	.....	.....	.....	0.05^{100}	i.; 13.3 aq. 15% NaCl	i.; s. aq. KI
5629	col. nd.	.....	.....	.....	5	30	i.
5630	wh. amor.	1.50^{210}	d.	.....	i.	i.	i.
5631	mn./CS_2	$0.847^{69.3}$°	69.6	291^{110mm}	0.03^{250}; s. bz.; s. act.	2^{20}°; 100^{300}	6^{15}°; s. CCl_4
5632	waxy	0.89	73-5	d.	v. sl. s.	s. h.	i.; i. bz.
5633	lf.	.....	38	251-2[100mm]	.....	.....	s.
5634	col. cr.	.....	108-9	250-1[12] sl. d.	i.	s. h.	s. h.
5635	col. cr.	0.855^{69}_{4}°	72	.....	i.; s. chl.	0.02^{200} abs.	0.2^{15}°
5636	nd./al.	.....	94-5	153.5^{10mm}	i.; s. act.	s.; s. chl.	v. s.; s. bz.
5637	col. cr.	.....	23	215^{15} sl. d.	.....	.....	.....
5638	col. cr.	0.8184^{41}_{4}°	41	274.5^{100mm}	i.	.....	.....
5639	pr./al.	.....	48	260	i.	s. h.	s.
5640	lf./lg.	0.798^{93}_{4}°	88.4	345^{12mm}	i.	sl. s. h.	sl. s. h.
5641	yel. pl.	.....	86	.....	sl. s. lg.	s. h.	s.
5642	mn./al.	0.970^{125}_{15}°	124	306-7	i.	0.9^{170} abs.	7.9^{140}
5643	yel. oil	.....	1	145^{11mm}	.....	.....	.....
5644	nd./al.	.....	175	.....	50; 2 chl.	22 abs.	i.; i. act.
5645	lf./aq. al.	.....	170-6°	.....	sl. s.; s. bz.	s.; s. chl.	s.; i. pet.
5646	col. lq.	0.906^{200}	−30.6	145.2	v. sl. s.	∞	∞

* Anhyd., m. p. 235°.
Selenium ethyl 2207
Selenium methyl 2553
Semicarbazidam 4573
Semicarbazide 3619
Semicarbazide 5617
Serine 4165
Serine 5316
Serine reagent 1967
Siliconenane 5619
Sinalbine, cf. glcde.
Sinapine, cf. alkd.
Sinigrin, cf. glcde.
Sinigroside, cf. glcde.
Six-O-six 5592
Skatole 4264
Skatole-acetic acid 3867
Skatole-carboxylic acid 3865
Smilacin, cf. glcde.
Smilagenin (pseudo) 5602
Sneezing gas 2712
Sobrerone 5320
Sodium acetone bisulfite 64
Solanidine, cf. alkd.
Solanine, cf. alkd.
Solid green base 5762
Solid violet 3353

243a

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The TAS method for rapid separation of pesticides and preservatives

by Egon Stahl*)

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Summaries in English and German

Special clean-up methods are generally necessary to isolate preservatives and pesticides from foodstuffs and other samples. The substances in question are present in the various samples in low concentrations, mostly 0.01 - 0.1%. Enrichment by extraction with various highly polar solvents and/or steam distillation are here the classical methods. They require frequently more time and material than the subsequent qualitative identification. While particularly in the latter field, thin-layer- and gas chromatography brought important progress in terms of time savings and increased reliability of the results, preliminary studies, that is, the analytical process proper, were still necessary. For the detection of the most common preservatives we start, for example, from 40 - 60 g test sample and work on it for a half day or day or longer. Since a considerable part of the organic insecticides, herbicides, fungicides and preservatives are distillable, subliminable or steam-volatile. The TAS method recently described by us offers itself as an aid in the rapid separation with direct transfer of the test substance to a glass plate with or without a TC layer (1,2,3).

TAS is the abbreviation of thermicro-, separation-, transfer- and application method for substances according to Stahl.

Principle of the standard method

The sample to be tested (1-500 mg, mostly 10-50mg) is placed in the special glass cartridge. The latter can be rapidly sealed with a retaining- and sealing clip (RS-clip) and is placed with it in the TAS stove heated to a predetermined temperature (50 - 350 degC) (fig.1). The volatile substances issue in the form of a fine vapor jet and arrive thus directly on the glass plate held in front of the capillary point. The stay period of the cartridge in the stove is as a rule only 30 - 90 seconds). Then the cartridge is removed. The collecting plate is pushed ahead by 1.5 - 2 cm and the next cartridge is inserted.

Fig. 1 Front view of the TAS stove with TC plate. In the center is the stove block proper with the round thermometer, to the left the temperature control, to the right the deposit grooves. In the foreground two TAS cartridges (Manufacturer: DESAGA Heidelberg).

In addition to the above described "standard method" a number of variants were tried out which yielded additional application possibilities (4). They refer primarily to the charging and design of the cartridges.

Technique for separating pesticides and preservatives

The techniques are explained on the basis of fig. 2.

a) For the preliminary test, where a substance can be distilled or sublimated from the sample, a tightly fitting thin-walled test tube (3) with 20-50 mg of the sample (4) is placed in the cold TAS stove (2). Then the oven is heated, in contrast to the standard method. We observe whether and at what temperature a condensate (1) is formed on the projecting cold part of the test tube. This preliminary test can be extended by placing special reagent paper strips into the opening of the test tube. Within the framework of this study we are interested among others in the detection of volatile acids, for example, sulfur-

ous acid, formic acid etc; here a moistened pH-indicator tape is used. But other substances can also be identified with such "test papers."

b) Here a "propellent" in the form of a solid (8) is added to the sample (4) in the cartridge. These solids can be of an organic or inorganic nature, and they should evolve a certain amount of vapor at the present stove temperature. For such an "ultra-micro-steam distillation" we use preferably up to a temperature of 220 degC 50 - 100 mg corn starch (it should be standardized to a water content of 15 or 20%), also moistened "blue gel" (pink with 20% water content). If basic vapors are desired, $CaCl_2 \cdot 8HN_3$ can be used among others.

c) In this technique 5 - 20 mu l of a suitable solvent are injected with a micro-syringe (9) through the sealing diaphragm into the hot cartridge. This way the "vapors from the sample" contained in the interior of the cartridge are blown onto the layer. We canalso introduce a weak gas current, for example, N_2, with a velocity of flow of 3-4 ml/min. (see fig. 2d).

e) Here the cartridge is filled loosely with quartz wool (10), asbestos fibers (10), small glass balls, or coarse sand grains. The liquid sample, for example, 100 mu l, is injected into the central part of the cartridge and the solvent is "distilled off" at a low temperature. The interesting substances can then be expelled from the residue by increasing the stove temperature.

f) This technique is useful in the case of semi-solid samples, like mayonnaise, marmelade, aqueous canned foods, fresh fruit dishes, etc. To this end a "trough" (fig. 3b) a "weighing boat" is prepared from aluminum foil. The sample (12) is introduced with a spatula and then the "boat" is pushed at the handle (11) in horizontal position into the cartridge. After closing with the RS-clip, we proceed in known manner.

Fig. 2a - t TAS cartridge and some of the techniques possible with it. (for details to a-f see text)

Production of the weighing trough or of the boat

A 10 x 50 mm strip of thin aluminum foil is formed to a trough by pressing it against a glass rod of 5 mm thickness (e.g. the 10 mu l blood sugar pipette). On the glass rod one end of the rough can be turned inside about 2 mm and the other end pressed together so that a trough-shaped structure is formed. The details of the production can be seen from fig. 3.

Fig. 3a-c Production of a weighing trough (b) of of a boat (c) for insertion into the TAS cartridge of a piece of aluminum foil (a).

other techniques are described elsewhere (1,4)

After the good experience with the direct charging of the active substances of drugs which contain essential oils (2), tests were made among others to separate new growth regulators (morphotactines) (5). On the basis of synthetic and natural mixtures, we will now describe the experimental conditions for the thermomicro-separation of additional substances.

A. Preservatives

A Compilation of the "Detection and determination of preservatives in foodstuffs" is found in Diemair and Postel 1967 (6). Our tests showed that most of the substances listed here can be separated with the TAS method. As mentioned above, the other identification method such as drop reactions, thin-layer chromatography (TLC) spectroscopic identification etc. have already been studied sufficiently. For this reason we will confine ourselves to indicating only the TAS conditions determined by us for the substances and some practical application possibilities. Thin-layer chromatography is discussed if a considerable improvement of the known state of the art was achieved with regard to separation or detection. In the connection we also want to mention the possibility of the direct recording of spectral of a substance spot by means of the chromatogram-spectral photometer developed by us (7-10). The combination

TAS -- TL^C --UV.IR (200 - 2000 mm)

offers today new possibilities in the ultramicroanalysis of these substances.

I. Diphenyl, diphenylamine and o-phenylphenol

The above mentioned substances are used preferably to preserve citrus fruits. Mostly the fruits are wrapped in thin paper which is impregnated with one or several of these compounds. The concentration in the paper is between 0.5 and 5 mg per 100 qcm. These lipophilic compounds have a high vapor pressure and evaporate relatively fast, they therefore arrive in considerable amounts in the lipid-rich fruitdish. The separation and detection from paper and fruit dish are therefore of interest.

a) TAS method and TLC for testing wrapping papers

In the conventional concentrations it suffices if a strip of 1 cm width and 10 cm length is cut off from the "wrapping paper" to be tested (weight 20-25 mg), folded together, and inserted into a TAS cartridge with a small spatula. In the positive finding, the quantities to be expected are between 50 and 500 mu g, that is, even a tength of the paper surface would be sufficient if a major part has not already evaporated. At a temperature of the TAS stove of 200 degC, the stay period of the cartridge is 90 + 90 sec. that is, after 1½ minutes the TLC plate is pushed to the next starting point. This way we are sure that the bulk was deposited on the TLC plate in the first 90 seconds. The distance: cartridge point - TLC layer is 0.5 - 1 mm. The TLC is effected under standard conditions (11,12). As a layer is used silica gel GF-254. The development is effected with benzene at chamber saturation once every 10 cm. For comparison, 2 mu 1 (= 2 mu g) of a 0.1% solution of the substances in question are applied. After the TLC, we recognize in the chromatogram in the short-waved UV (254 nm) the substances by their fluorescence extinction. Other detections are found in Diemair and Postel (6). The approximate hRf-values are: o-phenylphenol 35, diphenylamine 70 and diphenyl 80;

hexamethylene tetramine and thiourea, if any, are found at the start with this flow medium. Thiourea travels with methanol water (80 + 20), while hexamethylene tetramine still remains in the range of the starting point. It should be added that hexamethylene tetramine and thiourea can be detected easily in the chromatogram with iodine platinate reagent (no. 140 in 13). They are recognized as white cones on a brown background.

b) TAS method and TLC for testing the citrus furit dish

The TLC is effected as described above. For the TAS method, a cube of about 3 qmm that is, a piece weighing about 50 mg is stamped out with the spatula from the citrus fruit dish, the latter is introduced into a TAS cartridge and its exact weight is determined. To remove the bulk of the water, the cartridge closed with the RS-clip is placed in the TAS stove heated to 200 degC and is "heated" for 2 minutes (starting point 1). The TLC plate is moved to the left by 2 cm and left in this position, that is, in starting point 2, for another 3 minutes. Subsequently the cold cartridge can be weighed to determine the original water content (+ essential oils).

The foregoing procedure resulted from series tests which are represented as examples for the determination of the optimum conditions in fig. 4a and b. In test I (Fig. 4a), the stove temperature was 160 degC and every minute the TLC plate was moved by 1 cm to the left.

We see from fig. 4a that a "batch" of diphenyl arrives at starting point 1, but then practically nothing passes over from the 2nd to the 6th minute. In this time we observe relatively large "water spots." Only then, that is, after the 6th minute, up to the 12th minute, the bulk of the diphenyl appears. Increased time-savings are achieved by increasing the stove temperature to 200 degC. Fig. 4b shows that after a first "heating period" of 2 minutes, the bulk of diphenyl is expelled between the 3rd and the 5th minute.

Fig. 4a and b Diphenyl from ab. 50 mg preserved fresh citrus fruit dish a) TAS at 160 degC and after 1 minute each change of starting point of the TLC plate. V = reference solution (diphenyl, top; diphenylamine; o-phenylphenol bottom b) at 200 degC (for details see text)

II Admissible and inadmissible preservatives from preparations

The 4-hydroxybenzoic esters (= PHB esters) and sorbic acid are frequently used in concentrations of 0.05 - 0.2% for the preservation of numerous foods and medicinal preparations. They can be used legally in many countries for this purpose. In addition, a number of other preservatives are known which may no longer be used in West Germany, for example, dehydracetic acid, salicylic acid, benzoic acid, p-chlorobenzoic acid, etc.

After it had been found in preliminarytests according to method a (fig. 2a) that all the above mentioned esters and acids can be used for the separation by means of the TAS method, the corresponding model mixtures were prepared.

To a preservative-free mayonnaise were added per 1.0 g:

a) 1.2 mg Nipasol M and 2.5 mg each of sorbic-and benzoic acid (fig.5,no.11)

b) 1.0 mg each of dehydracetic acid and p-chlorobenzoic acid (fig.5, no. 12)

In addition commercial meat salad (that is, its sauce) was examined which had been preserved with benzoic acid and sorbic acid according to the label (fig. 15, no. 10).

Corresponding tests for the above mentioned samples yielded the following TAS conditions:

Weighed-in portion: 10-20 mg in aluminum trough (fig.3b), temperature 200 degC, stay period: 90 seconds.

In the case of hydrous samples, such as mustard, preserved horseradish, preserved paprika, "evaporation" of the bulk of the water is advisable, as it was described for the citrus fruit dishes.

Fig. 5 TLC of preservatives (1-9) after their separation from samples (10-12) with the TAS method. 1. thiourea (hRf .0), 2 Nipagin M (hRf 5), 3 Nipasol M (hRf 10), 4 dehydracetic acid (hRf 15-20), 5 - salicylic acid (hRf 35-40), 6 - benzoic acid (hRf 45-55) 8 - p-chlorobenzoic acid (hRf 50-55); 9 - diphenyl (hRf 85-95), diphenylamine (60-65), o-phenylphenol (3-545), 10 - meat salad with 6 and 7 TAS, 11 - mayonnaise with 3,6 and 7 TAS, 12 - mayonnaise with 4 and 8 TAS.

Fig. 5 shows a TL chromatogram of the preservatives separated from samples by means of the TAS method. We worked according to the standard method. As a layer was used silica gel GF-254, and as a flow medium hexane acetic acid 96% DAB 6 (85+15). 10 cm were developed once at chamber saturation. For comparison 1 mu 1 of a 0.1 - 0.5% solution of all substances were applied, also the three colored test mixture. All substances are easily recognizable in the short-waved UV light on the basis of their fluorescence extinction.

B. Pesticides

From the great number of substances to be considered here (14), we selected only a few known substances from different groups as practical examples.

I. Insecticides

a) Plant insecticides

In the first report we already dealt with the separation of volatile vegetable ingredients, particularly the essential oils from drugs (2,3). The TAS method brought here considerable progress. Other tests showed that the well known and widely used pyrethrins can be identified easily and rapidly this way. For the drug analysis we obtained the following conditions, which can also be applied to extracts and preparations:

TAS for pyrethrine from blossoms: Weighed-in portion 10 tubular bloesoms (ab. 3-5 mg), expanding agent 50 mg blue gel (pink with 20% water), temperature 220 degC, stay period: 90 sec. For the subsequent TLC we could likewise

go back to previous experiences (15,16). For separating the particularly important pyrethrins I (pyrethrin I, cinerin I and jasmolin I) the development was effected GF-254 8 cm high with hexane/acetic ethyl ester (90+10) at normal saturation, that is, without KS; For the detection, 2.4 -DNPH-reagent (no. 76 A in (13)) was sprayed on and heated for 5 minutes to 100 degC.

TAS for volatile alkaloids: The alkaloid nicotine used as an insecticide can also be separated and detected both from tobacco and from preparations:

TAS: 20 mg tobacco, 240 degC, 40 sec.

The TLC is effected on silica gel CF 254 with chloroform/methanol/ammonia solution 25% (90 + 9 + 1). Detection: short-waved UV light and subsequent Dragendorff-reagent no. 90 in (13)).

Similar volatile alcohols, like coniin, but others too, such as atropine, morphine and codeine proved accessible to the method (17).

b) Synthetic insecticides

From the group of the "chlorinated hydrocarbons." We selected DDT (dichlorodiphenyl trichloroethane), hexachorocyclohexane (= gamma.HCH, lindane, gammexane) and aldrine (hexachlorohexahydrodimethanonahphthalene) and prepared with potato starch diluting 1:1000. The further tests show that the interesting substances can be separated directly from these samples under the following conditions (fig. 6).

Fig. 6 TLC of some insecticides. Applied: L = as solution and T = from a sample with the TAS method; G = mixture in solution; F = color test solution.

TAS: 20 mg sample (see above) + 50 mg starch (expanding agent standardized to 20% water content); temperature 200 degC; stay period 60 sec.

Note: If kieselguhr was used as a "diluent," partial decomposition set in, which had already been observed in the morphactins. (5). As tests with sea and sand have shown, decomposition is hardly likely with corresponding soil

samples. Weighed-in portions of up to 200 mg and more can be filled into the TAS cartridges.

The subsequent TLC was effected according to the standard method. As a layer was used silica gel GF-254 and as a flow medium heptane/glacial acetic acid (95:5) (18). T he detection of DDT and aldrine was effected by observation in the short-waved UV light (fluorescence extinction). Subsequently the o-dianisidine-UV-treatment (13, p.618) was carried out, and the substances became thus visible as more or less intensive green zones.

TLC Herbicides

As practical examples was prepared sea sand triturations 1:1000 of the following substances: phenoxacetic acid, naphthalene-acetic acid, 2,4-dichlorophenoxy-acetic acid, 2,4,5-trichlorophenoxy-acetic acid in order to test the application possibility of the TAS method. First it had been found that starch or kieselguhr are not suitable for this purpose; the above mentioned herbicides could not be separated from starch triturations with the TAS method. The compounds are no longer stable at temperatures of about 200 degC, but can be transferred undercomposed at 150 and 180 degC by way of "ultramicro-steam distillation" to the TLC plate. The following working conditions were obtained.

TAS for non-chlorinated acids

weighed-in portion: 20 mg of the 1:1000 trituration. Expanding agent: 25 mg "blue gel" (pink with 20% water), temperature 180 degC stay period 90 sec. TLC: silica gel GF-254, acetic ethyl ester/acetic acid 96% DAB 6 (98.5 + 1.5) 1 x 10 cm at chamber saturation. Detection: Antimony-V-chloride reagent (13) 120 degC, 10 min. (fig.7).

Fig. 7 TLC of phenoxy-(pheno.) and naphthalene-acetic acid (naptha). Applied L = as solution and T - with the TAS method; G = mixture in solution; F = color test solution.

TAS for chlorinated acids

TAS for chlorinated acids

Weighed-in portion: 40 mg of the 1:1000 trituration. Expanding agent 100 mg "blue gel" (pink with 20% water), temperature 150 degC, stay period 90 sec. The TLC was effected as described above. For detection we used the o-dianisidine-UV reaction as with the insecticides. A pink coloration is obtained.

Application range, thermostability and "quantitative" separation

Far more problems than were assumed at first can be solved with the TAS method. An indication of the number of substances in question is obtained from the gas chromatography (CG). All solid or liquid compounds successfully chromatographed there can be separated directly from non-liquid samples with the TAS method and be subjected to TLC. Beyond that, the method of "ultramicro-steam distillation" expands the application range. The comparison of the two "thermomicro methods" (GC with TAS) also dissipates the doubts that the TAS method offers only limited possibilities because of the thermolability of the substances. In the TAS method we work with an "all glass system" and a short stay period. But this must not disguise the fact that in a number of substances only "cold" separating methods can be used, because of their lability and/or their too low vapor pressure. But in the latter case we can frequently produce more volatile derivatives which are partly more thermostable. The apparent disadvantage of a "decomposition" of substances by the effect of heat can also be utilized with advantage analytically, as it is done routinely in the method of the pyrolysis CG of plastics. The TAS oven permits studies for the "low temperature pyrolysis" coupled directly with TLC. An interesting example is the deliberate splitting of some glycosides, for example, of picrocrocin to safrannal (19) and of arbutin to hydroquinone (20) directly in the drug or of the proazulenes to chamazules.

Every analyst is engaged in the question of the quantitative side of a new method, because he knows how difficult it is, particularly in extraction

methods to arrive at absolute values. But in chromatography more or less great losses occur in the separation system, which are mostly unknown. We are at present investigating on model mixtures the degree of yield. As expected, it differs considerably, depending on the substance and the "carrier material." But based on present experiences, the qualitative questions predominate in our case too, and losses are gladly accepted in view of the simple and rapid method. In any case it will be determined first whether a substance to be detected exists in the sample before the mostly very time-consuming quantitative determination methods are applied. Particularly for this preliminary investigation the TAS is very useful, as the agove described tests show.

Finally we want to mention additional application possibilities of the method for testing foods, stimulants and objects of use. On the basis of the present results it is possible to separate perfumes and aromatic substances as well as anti-oxidants and other auxiliary substances from oils, fats, soaps, salves, pomades and sticks. But in the investigations of tooth pastes, shoe polishing creams, ice cream, pudding powder and dairy products too, application possibilities can be seen. Another field is the detection of plasticizers and other auxiliary substances in objects of use. WE must not forget the separation of wood protectives and other "impregnating agents."

It can already be predicted today that the TAS method will become a useful and simple tool in the laboratory, just as TLC is today. Both methods supplement each other very favorably.

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(Translated by Carl Demrick Associates, Inc/IE/t)

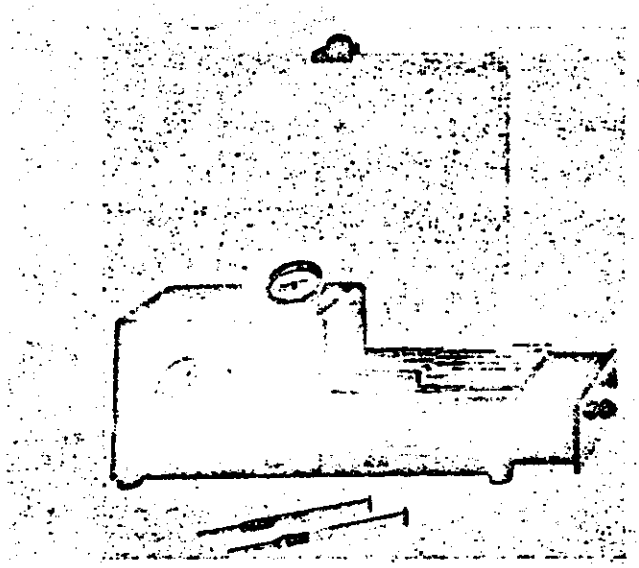

Figure 1

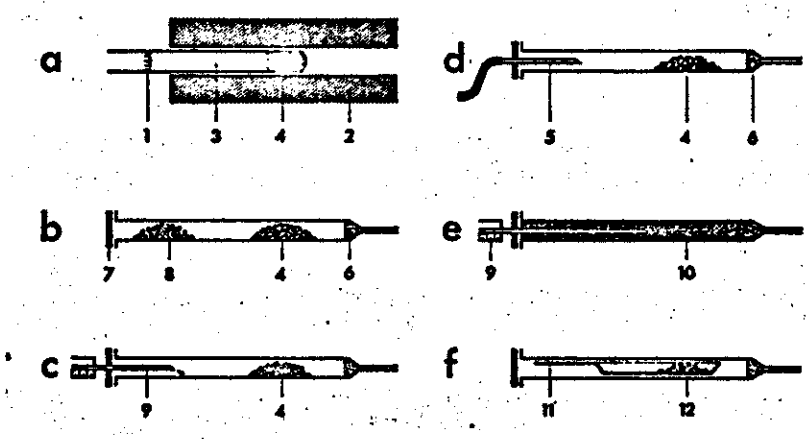

Figure 2

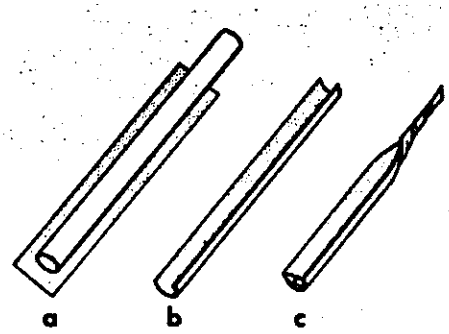

Figure 3

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TAS-Verfahren zur schnellen Abtrennung von Pesticiden und Konservierungsstoffen

EGON STAHL*

Mitteilung aus dem Institut für Pharmakognosie und Analytische Phytochemie der Universität des Saarlandes

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The TAS-Method for Rapid Seperation of Pesticides and Preservatives

Summary

The principles of the new thermomicroseperation method, the function of the TAS-stove and the techniques applied are described. Using modell-mixtures and commercial samples, the possibilities for rapid detection of preservatives, e.g. diphenylderivates, PHB-esters, sorbic and other acids, are demonstrated. From the pesticides some insecticides, e.g. pyrethrine, DDT, γ-HCH and some aromatic acids effective as herbicides are taken and analyzed. By a comparison with gaschromatography the advantages and disadvantages of the new method are discussed by the author together with additional possibilities of application in the field of food science and technology.

Zusammenfassung

Einleitend werden die Grundzüge des neuen Thermomikro-Abtrenn- und Auftrageverfahrens sowie die Funktion des TAS-Ofens geschildert und bewährte Arbeitstechniken zusammengestellt. Anhand von Modellgemischen und Handelsproben sind dann die Möglichkeiten zum schnellen Nachweis von Konservierungsstoffen, z. B. von Diphenylderivaten, PHB-Estern, Sorbin- und anderen Säuren aufgezeigt. Aus dem Gebiet der Pesticide wurden einige Insecticide, u. a. Pyrethrine, DDT, γ-HCH, sowie einige der herbicid wirkenden aromatischen Säuren herausgegriffen und die Abtrenn- und DC-Bedingungen ermittelt.

Zahlreiche weitere Anwendungsmöglichkeiten auf dem Gebiet der Analytik von Lebens-, Genußmitteln und Gebrauchsgegenständen sind abschließend zusammengestellt, nachdem zuvor im Vergleich mit der Gaschromatographie auf die Vor- und Nachteile der TAS-Methode eingegangen wurde.

Zur Isolierung von Konservierungsmitteln und Pesticiden aus Lebensmitteln und anderen Proben sind zumeist spezielle Vorreinigungsverfahren ("clean-up") notwendig. Die in Betracht kommenden Stoffe liegen in den verschiedenartigen Proben in geringer Konzentration — zumeist 0,01—0,1% — vor. Eine Anreicherung durch Extraktion mit verschieden stark polaren Lösungsmitteln und/oder Wasserdampfdestillation sind hier die klassischen Verfahren. Sie erfordern häufig einen größeren Zeit- und Materialaufwand, als die sich daran anschließende qualitative Identifizierung. Während gerade auf dem letzteren Gebiet die Dünnschicht- und Gaschromatographie einen wichtigen Fortschritt im Hinblick auf Zeitersparnis und vergrößerte Sicherheit der Resultate brachte, mußten die Vorarbeiten, d. h. der eigentliche Analysengang, nach wie vor durchgeführt werden. Zum Nachweis der gebräuchlichsten Konservierungsstoffe geht man z. B. von 40—60 g Untersuchungsprobe aus und arbeitet daran etwa einen halben bis einen Tag und länger. Da nun ein nicht unerheblicher Teil der organischen Insecticide, Herbicide, Fungicide und Konservierungsstoffe destillierbar, sublimierbar oder wasserdampfflüchtig ist, bietet sich das von uns kürzlich beschriebene TAS-Verfahren als Hilfsmittel zur schnellen Abtrennung mit direktem Transfer der Untersuchungssubstanz auf eine Glasplatte mit oder ohne DC-Schicht an (1, 2, 3).

TAS ist die Abkürzung von *T*hermomikro-, *A*btrenn-, Transfer- und Auftrage-verfahren für *S*ubstanzen nach STAHL.

* Herrn J. FUCHS danke ich für die sorgfältige experimentelle Durchführung der Versuche.

Sorbic Acid 428

Prinzip des Normalverfahrens

Die zu untersuchende Probe (1—500 mg, zumeist 10—50 mg) wird in die Spezial-Glaspatrone gebracht. Diese ist mit einer Halte- und Abdichtungsklammer (= HD-Clip) schnell verschließbar und wird hiermit in den auf eine vorbestimmte Temperatur (50—350° C) erhitzten TAS-Ofen (Abb. 1) gesteckt. Die flüchtigen Substanzen treten nun in Form eines feinen Dampfstrahles aus und gelangen so direkt auf die unmittelbar vor der Capillarspitze gehalterte Glasplatte. Die Verweilzeit der Patrone im Ofen beträgt in der Regel nur 30—90 sec. Danach wird die Patrone herausgenommen, die Auffangplatte 1,5—2 cm weitergeschoben und die nächste Patrone eingesteckt.

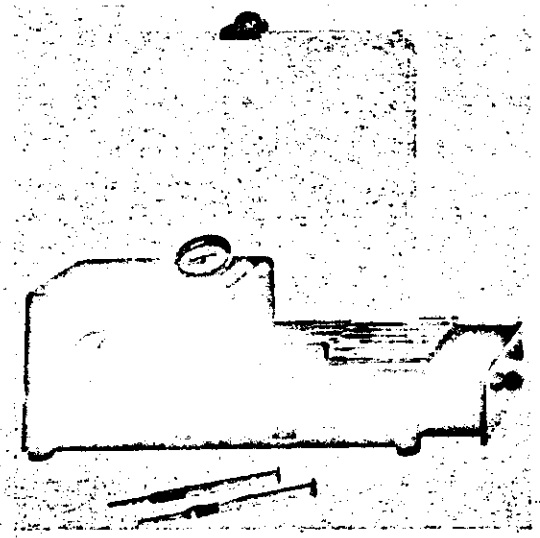

Abb. 1. *Vorderansicht des TAS-Ofens mit DC-Platte.* In der Mitte der eigentliche Ofenblock mit dem Rundthermometer, links die Temperaturregelung, rechts die Ablagerillen. Im Vordergrund zwei TAS-Patronen. (Hersteller Fa. DESAGA, Heidelberg)

Neben diesen geschilderten „Normalverfahren" wurden eine Reihe von Varianten erprobt, die weitere Anwendungsmöglichkeiten ergaben (4). Sie beziehen sich vorzugsweise auf die Beschickung und Ausbildung der Patrone.

Technik für die Abtrennung von Pesticiden und Konservierungsmitteln

Die Techniken sind an Hand der Abb. 2 erläutert.

Zu a) Zur *Vorprüfung*, ob aus der Probe eine Substanz herausdestillierbar oder -sublimierbar ist, steckt man in den kalten TAS-Ofen (2) ein genau passendes dünnwandiges Reagensgläschen (3) mit 20—50 mg der Probe (4). Im Gegensatz zum Normalverfahren wird erst danach angeheizt. Man beobachtet, ob sich und bei welcher Temperatur ein Kondensat (1) am herausragenden kalten Teil des Reagensgläschens niederschlägt. Diese Vorprobe kann erweitert werden, indem man in die Öffnung des Röhrchens spezielle Reagenspapierstreifen steckt. Im Rahmen dieser Arbeit interessiert u. a. der Nachweis flüchtiger Säuren, z. B. schweflige Säure, Ameisensäure usw.; hier wird man einen angefeuchteten pH-Indicatorpapierstreifen verwenden. Aber auch manche anderen Stoffe lassen sich bekanntlich mit solchen „Prüfpapieren" erkennen.

Zu b) Hier wird zur Probe (4) ein „Treibmittel" in Form eines Feststoffes (8) in die Patrone gegeben. Diese Feststoffe können organischer oder anorganischer Natur sein, und sie sollen bei eingestellter Ofentemperatur eine bestimmte Menge Dampf entwickeln. Für eine derartige „Ultramikro-Wasserdampfdestillation" verwenden wir bis zu einer Temperatur von 220 C gerne 50—100 mg Maisstärke (sie soll auf einen Wassergehalt von 15 oder 20% eingestellt sein), ferner befeuchtetes „Blaugel" (rosa mit 20% Wassergehalt). Sind basische Dämpfe erwünscht, kann u. a. $CaCl_2 \cdot 8\,NH_3$ verwendet werden usw.

Zu c) Bei dieser Technik werden mit einer Mikroinjektionsspritze (9) durch die Abdichtungsmembran 5—20 μl eines geeigneten Lösungsmittels in den heißen Patronenraum gespritzt. Auf diese Weise werden die im Patronenraum befindlichen „Dämpfe aus der Probe" auf die Schicht geblasen. Man kann auch einen schwachen Gasstrom, z. B. N_2, mit einer Strömungsgeschwindigkeit von 3—4 ml/min einleiten (vgl. Abb. 2d).

Zu e) Hier ist die Patrone lose mit Quarzwolle (10), Asbestfasern (10), kleinen Glaskugeln oder groben Sandkörnern gefüllt. Die flüssige Probe, z. B. 100 µl werden in den mittleren Teil der Patrone injiziert und das Lösungsmittel bei niedriger Temperatur „abdestilliert". Aus dem Rückstand können dann durch Erhöhen der Ofentemperatur die interessierenden Stoffe ausgetrieben werden.

Zu f) Diese Technik ist beim Vorliegen halbfester Proben, wie Mayonnaise, Marmelade, wasserhaltige Konserven, frische Fruchtschalen usw. nützlich. Man stellt sich hierzu aus Aluminiumfolie eine „Rinne" (Abb. 3b) oder ein „Wägeschiffchen" her; bringt die Probe (12) mit dem Spatel ein, wägt und schiebt danach das „Schiffchen" am Griff (11) in waagerechter Stellung in die Patrone. Nach dem Verschließen mit dem HD-Clip verfährt man in bekannter Weise.

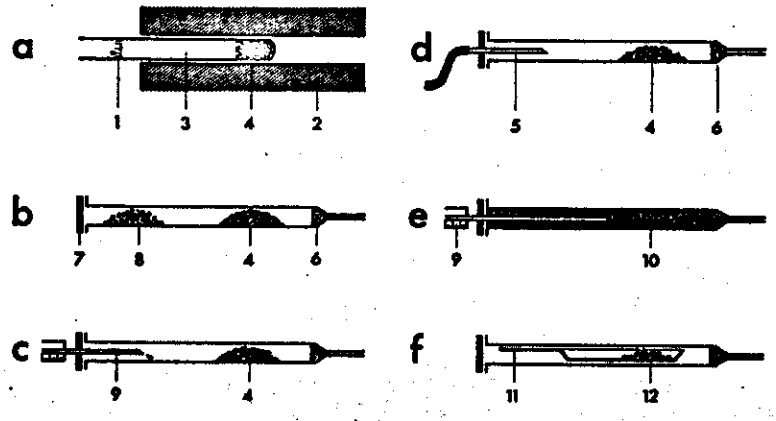

Abb. 2a—f. *TAS-Patrone und einige der hiermit möglichen Arbeitstechniken* (Einzelheiten zu a—f im Text)

Herstellung der Wägerinne bzw. des Schiffchens

Ein 10×50 mm-Streifen aus dünner Aluminiumfolie wird durch Andrücken an einen 5 mm dicken Glasstab (z. B. die 10 µl-Blutzuckerpipette) zu einer Rinne geformt. Bereits am Glasstab kann das eine Ende der Rinne ca. 2 mm nach innen zu umgebördelt und das andere Ende zusammengedrückt werden, so daß ein trogförmiges Gebilde entsteht. Die Einzelheiten der Herstellung sind aus der Abb. 3 zu entnehmen.

Weitere Techniken sind a. a. O. beschrieben (1, 4).

Nach den guten Erfahrungen bei der Direktaufgabe der Wirkstoffe von Drogen, die ätherische Öle enthalten (2), wurden u. a. Versuche zur Abtrennung von neuen Wachstumsregulatoren (Morphaktine) durchgeführt. (5). Im folgenden werden nun an Hand von künstlichen und natürlichen Gemischen die experimentellen Bedingungen zur Thermomikro-Abtrennung weiterer Substanzen mitgeteilt.

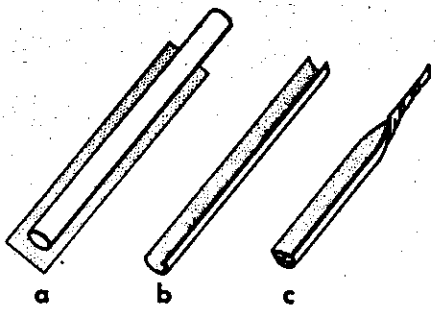

Abb. 3a—c. *Herstellen einer Wägerinne (b) oder eines Schiffchens (c) zum Einschieben in die TAS-Patrone aus einem Stück Aluminiumfolie (a)*

A. Konservierungsstoffe

Eine Zusammenstellung über „Nachweis und Bestimmung von Konservierungsstoffen in Lebensmitteln" gaben DIEMAIR u. POSTEL 1967 (6). Unsere Versuche zeigten, daß sich die meisten der dort angeführten Substanzen mit dem TAS-Verfahren

abtrennen lassen. Wie bereits einleitend erwähnt, sind die weiteren Identifizierungsmethoden, wie z. B. Tüpfelreaktionen, Dünnschicht-Chromatographie, spektroskopische Identifizierung usw. bereits hinreichend bearbeitet. Deshalb begnügen wir uns, nur die von uns ermittelten TAS-Bedingungen für die Substanzen und einige praktische Anwendungsbeispiele anzugeben. Auf die DC gehen wir ein, wenn eine beachtenswerte Verbesserung des bisher Bekannten in bezug auf Trennung oder Nachweis erzielt wurde. In diesem Zusammenhang sei aber auf die Möglichkeit der direkten Aufnahme von Spektren eines Substanzfleckes mittels des von uns konzipierten Chromatogramm-Spektralphotometers (7—10) hingewiesen. Die Kombination

TAS → DC → UV-IR (200—2000 nm)

bietet neue Möglichkeiten in der Ultramikroanalytik dieser Stoffe.

I. Diphenyl, Diphenylamin und o-Phenylphenol

Die genannten Stoffe gebraucht man vorzugsweise zur Haltbarmachung von Citrus-Früchten. Zumeist werden die Früchte in dünnes Papier eingewickelt, das mit einem oder mehreren dieser Verbindungen getränkt ist. Die Konzentration im Papier liegt im Bereich 0,5 bis 5 mg pro 100 cm². Diese lipophilen Verbindungen haben einen hohen Dampfdruck und verflüchtigen sich relativ schnell, sie gelangen somit auch in erheblicher Menge in die lipidreiche Fruchtschale. Daher sind die Abtrennung und der Nachweis aus Papier und Fruchtschale von Interesse.

a) TAS-Verfahren und DC zur Untersuchung von Einwickelpapieren

Bei den üblichen Konzentrationen genügt es, wenn man von dem zu untersuchenden „Einwickelpapier" einen 1 cm breiten und 10 cm langen Streifen abschneidet (Gewicht: 20—25 mg), zusammenlegt und in eine TAS-Patrone mit einem kleinen Spatel einschiebt. Bei positivem Ausfall liegen die zu erwartenden Mengen zwischen 50 und 500 µg, d. h. es würde also auch ein Zehntel der Papierfläche genügen, falls nicht schon ein Großteil abgedampft ist. Bei einer Temperatur des TAS-Ofens von 200° C beträgt die Verweilzeit der Patrone 90 + 90 sec, d. h. nach $1^1/_2$ min verschiebt man die DC-Platte zum nächsten Startpunkt. Man hat so eine Kontrolle, daß in den ersten 90 sec die Hauptmenge auf die DC-Platte aufgedampft wurde. Der Abstand Patronenspitze — DC-Schicht beträgt 0,5—1 mm. Die DC erfolgt unter Standardbedingungen (11, 12). Als Schicht dient Kieselgel GF_{254}. Man entwickelt mit Benzol bei Kammersättigung einmal 10 cm. Zum Vergleich trägt man von einer 0,1%igen Lösung der in Betracht kommenden Substanzen je 2 µl (= 2 µg) auf. Nach der DC erkennt man beim Betrachten des Chromatogramms im kurzwelligen UV (254 nm) die Substanzen an ihrer Fluorescenzlöschung; weitere Nachweise findet man bei DIEMAIR u. POSTEL (6). Die hRf-Richtwerte sind: o-Phenylphenol 35, Diphenylamin 70 und Diphenyl 80, evtl. vorhandenes Hexamethylentetramin und Thioharnstoff liegen bei diesem Fließmittel am Start. Thioharnstoff wandert mit Methanol-Wasser (80 + 20), während hiermit Hexamethylentetramin noch im Bereich des Startpunktes bleibt. Nachzutragen bleibt, daß sich Hexamethylentetramin und Thioharnstoff auf dem Chromatogramm sehr gut mit Jodplatinat-Reagens (Nr. 140 in 13) nachweisen lassen. Man erkennt sie hiermit als weiße Zonen auf braunem Grund.

b) TAS-Verfahren und DC zur Untersuchung der Citrusfruchtschale

Die DC erfolgt wie vorstehend beschrieben. Für das TAS-Verfahren stanzt man sich mit dem Spatel einen kleinen ~ 3 mm³-Würfel, d. h. ein ca. 50 mg schweres Stückchen, aus der Fruchtschale aus, bringt dieses in eine TAS-Patrone und bestimmt ihr genaues Gewicht. Zur Entfernung der Hauptmenge des Wassers wird die mit

HD-Clip verschlossene Patrone in den auf 200° C erhitzten TAS-Ofen gesteckt und zunächst 2 min „ausgeheizt" (Startpunkt 1). Danach verschiebt man die DC-Platte 2 cm nach links und beläßt in dieser Stellung, d. h. auf Startpunkt 2 weitere 3 min. Anschließend kann die erkaltete Patrone zur Ermittlung des ursprünglichen Gehalts an Wasser (+ äther. Öl) gewogen werden.

Die vorstehende Arbeitsanweisung ergab sich aus Reihenversuchen, die als Beispiele für die Ermittlung der optimalen Bedingungen in den Abb. 4a u. b wiedergegeben sind. Im 1. Versuch (Abb. 4a) betrug die Ofentemperatur 160° C und jede Minute wurde die DC-Platte um 1 cm nach links verschoben.

Man erkennt aus der Abb. 4a, daß zwar innerhalb der 1. Minute ein „Schub" Diphenyl auf den 1. Startpunkt gelangt, dann aber von der 2. bis 6. Minute praktisch nichts übergeht. In dieser Zeit beobachtet man relativ große „Wasserflecke". Erst danach, d. h. von der 6. Minute an bis zur 12., erscheint die Hauptmenge des Diphenyls. Eine erhebliche Zeitersparnis erzielt man durch Erhöhung der Ofentemperatur auf 200° C. Die Abb. 4b zeigt, daß nach einer ersten „Ausheizzeit" von 2 min die Hauptmenge Diphenyl zwischen der 3. und 5. Minute ausgetrieben wird.

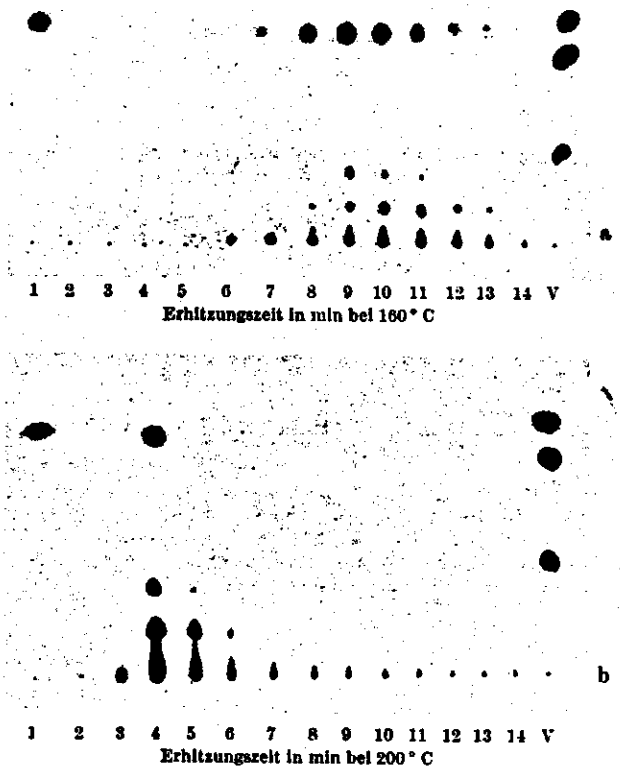

Abb. 4a u. b. *Diphenyl aus ca. 50 mg konservierter, frischer Citrusfruchtschale.* a) TAS bei 160° C und nach jeweils 1 min Wechsel des Startpunktes der DC-Platte. V = Vergleichslösung (Diphenyl, oben; Diphenylamin; o-Phenylphenol, unten). b) bei 200° C (Einzelheiten s. Text)

II. Zulässige und nichtzulässige Konservierungsstoffe aus Zubereitungen

Die 4-Hydroxybenzoesäure-Ester (= PHB-Ester) und die Sorbinsäure werden zur Konservierung zahlreicher Nahrungsmittel und Arzneizubereitungen zumeist in Konzentrationen zwischen 0,05—0,2% häufig gebraucht. Sie sind hierzu auch in

zahlreichen Staaten gesetzlich zugelassen. Daneben sind eine Reihe weiterer Konservierungsstoffe bekannt, die in der BRD nicht mehr verwendet werden dürfen, z. B. Dehydracetsäure, Salicylsäure, Benzoesäure, p-Chlorbenzoesäure usw.

Nachdem in Vorversuchen nach Verfahren a (Abb. 2a) festgestellt war, daß alle vorstehend genannten Ester und Säuren für eine Abtrennung mittels des TAS-Verfahrens in Betracht kommen, wurden entsprechende Modellgemische hergestellt.

Zu einer konservierungsmittelfreien Mayonnaise wurden auf 1,0 g
a) 1,2 mg Nipasol M und je 2,5 mg Sorbin- und Benzoesäure (Abb. 5 Nr. 11),
b) je 1,0 mg Dehydracetsäure und p-Chlorbenzoesäure (Abb. 5 Nr. 12) zugesetzt.
Ferner wurde ein aus dem Handel stammender Fleischsalat (d. h. dessen Sauce) untersucht, der laut Angabe mit Benzoe- und Sorbinsäure konserviert war (Abb. 5 Nr. 10).

Entsprechende Versuche ergaben für die vorstehenden Proben folgende TAS-Bedingungen:

Einwaage: 10—20 mg in Aluminiumrinne (Abb. 3b), Temperatur: 200° C, Verweilzeit: 90 sec.
Beim Vorliegen wasserhaltiger Proben, wie z. B. Senf, Dosen-Meerrettich, Paprikakonserven, ist ein „Abdampfen" der Hauptmenge des Wassers zweckmäßig, wie dies schon bei den Citrusfruchtschalen beschrieben wurde.

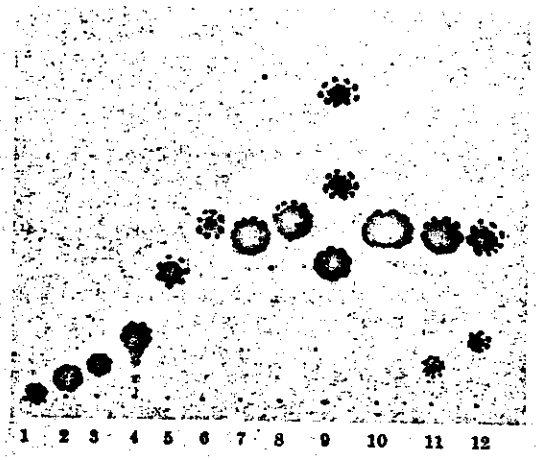

Abb. 5. *DC von Konservierungsstoffen (1—9) nach ihrer Abtrennung aus Proben (10—12) mit dem TAS-Verfahren.* 1 Thioharnstoff (hRf 0), 2 Nipagin M (hRf 5), 3 Nipasol M (hRf 10), 4 Dehydracetsäure (hRf 15—20), 5 Salicylsäure (hRf 35—40), 6 Benzoesäure (hRf 45—55), 7 Sorbinsäure (hRf 45—55), 8 p-Chlorbenzoesäure (hRf 50—55), 9 Diphenyl (85—95), Diphenylamin (60—65), o-Phenylphenol (35—45), 10 Fleischsalat mit 6 und 7 TAS, 11 Mayonnaise mit 3, 6 und 7 TAS, 12 Mayonnaise mit 4 und 8 TAS

Die Abb. 5 zeigt ein Dünnschicht-Chromatogramm der mittels des TAS-Verfahrens aus Proben abgetrennten Konservierungsstoffe. Es wurde nach der Standard-Methode (11, 12) gearbeitet. Als Schicht diente Kieselgel GF_{254} und als Fließmittel Hexan-Essigsäure 96% DAB 6 (85 + 15). Es wurde bei Kammersättigung einmal 10 cm entwickelt. Zum Vergleich wurden von allen Substanzen 1 µl einer 0,1—0,5%igen Lösung aufgetragen, ferner das dreifarbige Testgemisch. Alle Substanzen sind im kurzwelligen UV-Licht auf Grund ihrer Fluorescenzlöschung gut erkennbar.

B. Pesticide

Aus der großen Zahl der hier zu berücksichtigenden Substanzen (14) wurden nur einige bekannte Stoffe aus verschiedenen Gruppen als Anwendungsbeispiele ausgewählt.

I. Insecticide

a) Pflanzliche Insecticide

Bereits in der ersten Mitteilung beschäftigte uns die Abtrennung flüchtiger Pflanzeninhaltsstoffe, insbesondere der äther. Öle aus Drogen (2, 3). Das TAS-Verfahren brachte hier einen erheblichen Fortschritt. Weitere Versuche ergaben, daß sich auch die bekannten und viel verwendeten Pyrethrine auf diesem Wege schnell und einfach erkennen lassen. Für die Drogenanalyse ergaben sich folgende Bedingungen, die sich leicht auf Extrakte und Zubereitungen übertragen lassen:

TAS für Pyrethrine aus Blüten: Eingabe 10 Röhrenblüten (ca. 3—5 mg), Treibmittel 50 mg „Blaugel" (rosa mit 20% Wasser). Temperatur 220° C, Verweilzeit 90 sec. Zur anschließenden DC konnte ebenfalls auf frühere Erfahrungen zurückgegriffen werden (15, 16). Zur Trennung der besonders wichtigen Pyrethrine I (= Pyrethrin I, Cinerin I und Jasmolin I) wurde auf Kieselgel GF_{254} zweimal 8 cm hoch mit Hexan/Essigsäureäthylester (90 + 10) bei Normalsättigung, d. h. ohne KS entwickelt. Zum Nachweis wurde 2,4-DNPH-Reagens (Nr. 76 A in (13)] aufgesprüht und 5 min auf 100° C erhitzt.

TAS für flüchtige Alkaloide: Auch das als Insecticid verwandte Alkaloid Nicotin läßt sich sowohl aus Tabak als auch aus Zubereitungen wie folgt abtrennen und nachweisen:

TAS: 20 mg Tabak, 240° C, 40 sec.

Die DC erfolgt auf Kieselgel GF_{254} mit Chloroform/Methanol/Ammoniaklösung 25%ig (90 + 9 + 1). Nachweis: kurzwelliges UV-Licht und anschließend Dragendorff-Reagens Nr. 90 in (13)].

Ähnlich flüchtige Alkaloide wie Coniin, aber auch manche andere, wie z. B. Atropin, Morphin und Codein zeigten sich dem Verfahren zugänglich (17).

b) Synthetische Insecticide

Aus der Gruppe der „chlorierten Kohlenwasserstoffe" wurden DDT (= Dichlordiphenyltrichloräthan), Hexachlorcyclohexan (= γ-HCH, Lindan, Gammexan) und Aldrin (Hexachlorohexahydrodimethanonaphthalin) ausgewählt und mit Kartoffelstärke Verdünnungen 1 : 1000 hergestellt. Die weiteren Versuche zeigten, daß man aus diesen Proben die interessierenden Substanzen direkt unter folgenden Bedingungen abtrennen kann (Abb. 6):

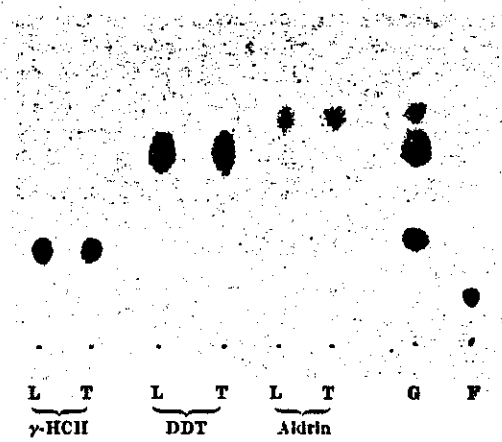

Abb. 6. *DC einiger Insecticide.* L = als Lösung und T = aus einer Probe mit dem TAS-Verfahren aufgetragen; G = Gemisch in Lösung; F = Farbtestlösung

TAS: 20 mg Probe (s. oben) + 50 mg Stärke (Treibmittel auf 20% Wassergehalt eingestellt); Temperatur 200° C; Verweilzeit 60 sec.

Anmerkung: Bei Verwendung von Kieselgur als „Verdünnungsmittel" trat bei DDT eine partielle Zersetzung ein, wie wir dies bereits bei den Morphaktinen beobachteten (5). Wie

Versuche mit Seesand zeigten, ist bei entsprechenden Bodenproben eine Zersetzung kaum zu befürchten. Es können Einwaagen bis 200 und mehr mg in eine TAS-Patrone gefüllt werden.

Die anschließende DC erfolgte nach der Standardmethode. Als Schicht diente Kieselgel GF_{254} und als Fließmittel Heptan/Eisessig (95 + 5) (18). Der Nachweis von DDT und Aldrin erfolgte durch Betrachten im kurzwelligen UV-Licht (Fluorescenzlöschung). Anschließend wurde die o-Dianisidin-UV-Behandlung (13; daselbst S. 618) durchgeführt und so wurden die Substanzen als mehr oder weniger intensiv grüne Zonen sichtbar.

II. Herbicide

Als Anwendungsbeispiele wurden Seesandverreibungen 1 : 1000 folgender Substanzen hergestellt: Phenoxyessigsäure, Naphthalinessigsäure, 2,4-Dichlorphenoxyessigsäure, 2,4,5-Trichlorphenoxyessigsäure, um die Anwendungsmöglichkeit des TAS-Verfahrens zu prüfen. Zuvor hatte sich gezeigt, daß sich hier Stärke oder Kieselgur nicht eignen; aus Stärkeverreibungen konnten die genannten Herbicide nicht mit dem TAS-Verfahren abgetrennt werden. Die Verbindungen sind bei Temperaturen um 200° C nicht mehr stabil, lassen sich aber bei 150 bzw. 180° C unzersetzt auf dem Wege der „Ultramikrowasserdampfdestillation" auf die DC-Platte transferieren. Es ergaben sich folgende Arbeitsbedingungen:

TAS für die nichtchlorierten Säuren

Einwaage: 20 mg der 1 : 1000-Verreibung, Treibmittel 25 mg „Blaugel" (rosa mit 20% Wasser), Temperatur 180° C, Verweilzeit 90 sec. DC: Kieselgel GF_{254}, Essigsäureäthylester/Essigsäure 96%ig DAB 6 (98,5 + 1,5) 1 × 10 cm bei Kammersättigung. Nachweis: Antimon-V-chlorid-Reagens (13) 120° C, 10 min (Abb. 7).

TAS für die chlorierten Säuren

Einwaage 40 mg der 1 : 1000-Verreibung, Treibmittel 100 mg „Blaugel" (rosa mit 20% Wasser). Temperatur 150° C, Verweilzeit 90 sec. Die DC erfolgte wie vorstehend beschrieben. Als Nachweis wurde die o-Dianisidin-UV-Reaktion wie bei den Insecticiden mit Erfolg verwandt. Man erhält hier eine rosa Färbung.

Abb. 7. *DC von Phenoxi- (Pheno.) und Naphthalinessigsäure (Napht.).* L = als Lösung und T = mit dem TAS-Verfahren aufgetragen; G = Gemisch in Lösung; F = Farbtestlösung

Zur Anwendungsbreite, Thermostabilität und „quantitativen" Abtrennung

Weit mehr Probleme als man zunächst annahm, lassen sich mit dem TAS-Verfahren lösen. Einen Anhaltspunkt über die Zahl der in Betracht kommenden Substanzen ergibt sich aus der Gaschromatographie (GC). Alle dort erfolgreich chromatographierten festen oder flüssigen Verbindungen lassen sich aus nichtflüchtigen Proben mit dem TAS-Verfahren direkt abtrennen und der DC zuführen. Darüber hinaus erweitert das Verfahren der „Ultramikrowasserdampfdestillation" den Anwendungsbereich. Der Vergleich beider „Thermomikromethoden" (GC mit TAS) zerstreut auch das Bedenken, daß infolge der Thermolabilität der Substanzen das TAS-Verfahren nur eng begrenzte Möglichkeiten bietet. Man arbeitet ja bei der TAS-Methode mit einem „Ganzglassystem" und kurzer Verweilzeit. Das darf jedoch nicht darüber hinwegtäuschen, daß bei einer Reihe von Substanzen infolge ihrer Labilität und/oder ihres zu geringen Dampfdruckes nur „kalte" Trennverfahren angewandt werden können. Allerdings kann man im letzteren Falle oft leichter flüchtige Derivate herstellen, die z. T. auch thermostabiler sind. Im übrigen läßt sich der scheinbare Nach-

teil einer „Zersetzung" von Substanzen durch Hitzeeinwirkung auch analytisch vorteilhaft verwenden, wie es beim Verfahren der Pyrolyse-GC von Kunststoffen routinemäßig betrieben wird. Der TAS-Ofen ermöglicht Studien zur „Niedertemperaturpyrolyse" in direkter Kopplung mit der DC. Ein interessantes Beispiel ist die gezielte Spaltung einiger Glykoside, so z. B. des Pikrocrocins zu Safranal (19) und des Arbutins zu Hydrochinon (20) direkt in der Droge — oder der Proazulene zu Chamazulen.

Jeden Analytiker beschäftigt die Frage nach der quantitativen Seite eines neuen Verfahrens, weil er weiß, wie schwer es ist, gerade bei Extraktionsverfahren zu absoluten Werten zu kommen. Aber auch bei der Chromatographie treten im Trennsystem mehr oder weniger hohe Verluste auf, die zumeist unbekannt sind. Wir untersuchen z. Z. an Modellgemischen den Grad der Ausbeute. Erwartungsgemäß ist er je nach Substanz und „Trägermaterial" recht verschieden. Beim augenblicklichen Stand des Erfahrungsmaterials überwiegen jedoch auch bei uns die qualitativen Fragestellungen und man nimmt Verluste im Hinblick auf die Einfachheit und Schnelligkeit gerne in Kauf. In jedem Falle wird man zunächst feststellen, ob ein nachzuweisender Stoff in der Probe vorhanden ist, bevor man die zumeist aufwendigen quantitativen Bestimmungsverfahren anwendet. Gerade für diese Voruntersuchung ist — wie die verschiedenen beschriebenen Versuche zeigen — das TAS-Verfahren nützlich.

Abschließend sei noch auf weitere Anwendungsmöglichkeiten des Verfahrens zur Untersuchung von Lebens-, Genußmitteln und Gebrauchsgegenständen hingewiesen: Auf Grund der bisherigen Ergebnisse gelingt die Abtrennung von Duft- und Aromastoffen sowie von Antioxidantien und weiteren Hilfsstoffen aus Fetten, Ölen, Seifen, Salben, Pomaden und Stiften. Aber auch bei der Prüfung von Zahnpasten, Schuhcremes, Speiseeis, Puddingpulver und Milcherzeugnissen lassen sich Anwendungsmöglichkeiten erkennen. Ein weiteres Gebiet ist der Nachweis von Weichmachern und sonstigen Hilfsstoffen in Gebrauchsgegenständen; auch die Abtrennung von Holzschutz- und anderen „Imprägnierungsmitteln" sei hier nicht vergessen.

Schon heute läßt sich voraussagen, daß das TAS-Verfahren zu einem nützlichen und einfachen Hilfsmittel im Labor werden wird, wie es die DC heute ist. Beide Verfahren ergänzen sich aufs beste.

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Differential Determination of Several Kinds of Preservatives by Using Solvent Extraction Method and Ion Exchange Resin. *1

(The manuscript was received by January 28, 1966)

by Tetsuo Inoue, *2 Taro Kawamura *2, Masaya Kamijo *2, Yoko Sato *2, Saburo Sugaya *3, Shogo Ochiai *3 and Tadayoshi Nakaoka *3

(For a brief English synopsis, refer to the original text on p.204)

I. Introduction

In the previous report (1), we reported on the method for the separatory (differential) identification (determination) of several kinds of preservatives contained in food by the water vapor distillation method.

Regarding the method for extraction by using a solvent, there are cases where there is created a marked emulsification phenomenon in those foods which use an emulsification agent (emulsifier) or those foods which contain a large amount of protein or sugar, thereby making it extremely difficult to carry out the operation. According to the solvent extraction method, further, all of the substances which are soluble in an organic solvent used are extracted, with a result that, in many cases, the substance which has been extracted secondarily makes the qualitative determination of the target substance difficult.

Therefore, we imagined the cases where several acidic preservatives such as benzoic acid (BA), paraoxybenzoic acid esters (PHBA-E), sorbic acid (SOA), salicylic acid (SA) and dehydroacetic acid (DHA), etc. co-exist with saccharin, and discovered that it is approximately possible to carry out a differential determination of these by using ion exchange resin in the conventional solvent extraction method. This finding will be reported in the following report:

(Figure 1 Apparatus)

The skeleton of this method is as follows: As shown in Figure 2, the neutral and basic substances are first removed by using ether in alkalinity

in the case of these sample solutions containing various preservatives. After this, extraction was carried out by using ether in acidity. From this extraction solution, PHBA-E was separated from the other preservatives by using a one per cent sodium bicarbonate. Then, carbon tetrachloride was added to the residue obtained after the distillation of ether, thereby removing the unsoluble saccharin. The preservatives from which saccharin had been removed were separated into SA and other preservatives by using ion exchange resin of Amberlite CG 400-1. A test solution was prepared for each fraction and the ultraviolet part absorption absorbance measuring method was carried out as in the previous report.

II. Experimental Method

(Apparatus)

1. Ion Exchange Resin Chromatographic Tube:

A chromatographic tube, whose inner diameter was one centimeter and length was 30 centimeters, with a cock being equipped at the lower terminal, was charged with 4.5 milliliters of Amberlite CG 400-1 type (of the -Cl form) which was prepared in accordance with the ordinary method, to a height of five centimeters.

2. Automatic Recording Spectrophotometer

(Reagent)

1. Buffer Solution: Fifty (50) milliliters of a 2M potassium chloride and 10.6 milliliters of 2N hydrochloric acid were mixed and water was added to this mixture to make the total volume 200 milliliters.

(Figure 2 . Procedure of analytical method of the preservatives in food.)

2. Elution Solution: 0.1N hydrochloric acid, 0.1N hydrochloric acid 60 per cent ethanol solution.

(Test Operation)

1. Preparation of the test solution:

Liquid Samples: From 50 to 100 grams of a sample was taken in accordance with the content of a preservative and, if necessary, water was added to make the entire volume 100 to 150 milliliters. This was used as a sample solution.

Solid Samples: A sample was finely out or ground and mashed. In conformity with the content of a preservative, from 30 to 50 grams of the same was taken to a triangle flask of 200 milliliters. One hundred (100) milliliters of water was added to the same. This was made alkaline by using a 10 percent sodium hydroxide solution. It was heated over water bath and, after it was left for a while, it was filtered. The residue was cleaned with a small amount of water and the cleaning solution was added to the filtration solution to make a sample solution.

2. Preparation of Sample Solutions

The sample solution was taken into a separatory funnel and neutralized with 10 per cent hydrochloric acid or a 10 per cent sodium hydroxide solution. Then, three milliliters of a 10 per cent sodium hydroxide solution was added to make it alkaline, sodium chloride was saturated, agitated three times with ether, the ether was removed and, after the water layer was made sufficiently acidic with 10 per cent hydrochloric acid, extraction was carried out three times using 50 milliliters of ether.

The ether layer is added, cleaning was carried out twice with 10 milliliters of water, and extraction was carried out three times with 30 milliliters of a one per cent sodium bicarbonate solution. The ether layer was dehydrated with anhydrous sodium sulfate and the other is removed.

Two milliliters of a 10 per cent sodium hydroxide solution and three milliliters of water were added to the residue, a reflux cooler was attached, boiling was carried out for a period of 15 minutes on a metal net equipped with asbestos, it was cooled, five milliliters of 10 per cent hydrochloric acid was added to it to make it acidic and extraction was carried out three

times with 40 milliliters of ether. The ether layer was added to the same and cleaning with water was carried out twice and the ether was removed. From two to three milliliters of a 0.1N sodium hydroxide was added to dissolve the residue. After it was neutralized with 0.1N hydrochloric acid, water was added to make the volume 100 milliliters and this was used as test solution A.

Next, the water layer which was extracted by using a one per cent sodium bicarbonate solution was added. After it was made acidic with 10 per cent hydrochloric acid, the sodium chloride was saturated, extraction was carried out three times with ether, and the ether layer was cleaned twice with 10 milliliters of water, dehydration was carried out with anhydrous sodium sulfate and the ether was removed.

Five milliliters of carbon tetrachloride was dissolved in this residue and filtration was carried out. The insolubles were cleaned with a small amount of carbon tetrachloride and the cleaning solution was added to the filtration solution. The total amount was made exactly 10 milliliters. Five milliliters of the same was taken and, while air was being blown lightly, the carbon tetrachloride was removed. From one to two milliliters of a 0.1N sodium hydroxide solution was added to the residue so as to dissolve the same. After it was neutralized with 0.1N hydrochloric acid, water was added to the same to make the amount 100 milliliters. This was used as test solution B.

When SA co-exists in test solution B, the following operation was further carried out:

The chromatographic tube shown in Figure 1 was charged with Amberlite CG 400- (of the -Cl type) and 50 milliliters of test solution B was caused to flow down at the rate of one milliliter per minute. Then, the same was cleaned with 50 milliliters of water. When the dropping of the cleaning solution ended, elution was immediately carried out at the same flow-rate by using 250 milliliters of 0.1N hydrochloric acid and this was used as test solution C.

Next, elution was similarly carried out by using 50 milliliters of a 0.1N hydrochloric acid 60 per cent ethanol solution and this was used as test solution D.

3. Qualitive Determination

Test solutions A and B or test solutions C and D were taken and were diluted to such a concentration as would be convenient to a measurement. Ten milliliters of the same was taken and one milliliter of the buffer solution was added. By using an automatic recording spectrophotometer, one milliliter of the buffer solution was added to 10 milliliters of water to be used as a control, and its ultraviolet absorption was measured. In the case of test solutions C and D, however, their respective elution solutions were used as controls.

In the presence of PHBA-E, anmaximum absorption was observed in the neighborhood of 256 m(mu) in test solution A. In the individual presence of DHA, SOA, BA and SA, further, the maximum absorptions were observed at the wave lengths of 308 m(mu) and 225 m(mu) in the case of DHA, at the wave length of 265 m(mu) in the case of SOA,at the wave length of 230 m(mu) in the case of BA and in the neighborhood of 303 m(mu) and 236 m(mu) respectively in test solution B.

When more than two co-exist, a synthesized wave is observed in conformity with the respective contents. In the case of test solution C, the absorptions of DHA, SOA and BA appear and, in the case of test solution C, only the absorption of SA was observed.

4. Determination

Regarding test solutions A and B, 10 milliliters of the same was taken. Two milliliters of a buffer solution and water were added to make the total volume 20 milliliters. By using a buffer solution which had been diluted to 10 times, if necessary, the absorbance in the neighborhood of the absorption

maximum wave length for each preservative was measured on the basis of those whose concentrations were diluted to a concentration suitable for the measurement.

On the other hand, the content of a preservative was calculated from the absorbance obtained from the standard solution of each preservative. Regarding the test solutions C and D, they were diluted to a concentration which was suitable for a measurement by using each elution solution for the measurement of the absorbances.

On the other hand, a similar operation was carried out by using ion exchange resin in the case of the standard solution of each preservative and the content of the preservative was calculated from the absorbance which was thus obtained.

III Experimental Result and Observations:

In the current experiment main emphasis was placed on the separation of saccharin by carbon tetrachloride and the separation between salicylic acid and the other preservatives by using ion exchange resin.

1. On the Separation of Saccharin:

Regarding the separation of saccharin by carbon tetrachloride, saccharin is extremely difficultly soluble (translator's note: in carbon tetrachloride?) while the preservatives are soluble in carbontetrachloride as stated in the previous report (2). Table 1 shows the solubility of 0.5 grams of a preservative and saccharin in 10 milliliters of carbon tetrachloride.

(Table 1 Solubility of several preservatives and saccharin in carbon tetrachloride.)

In addition, saccharin and preservatives are dissolved in carbon tetrachloride and filtered. The infrared absorption spectra regarding this filtration solution were measured. As a result, it was found that the absorptions of preservatives appeared but the absorption of saccharin was not observed. This was indicative of the fact that saccharin was not soluble in

carbon tetrachloride.

2. Comparison of Two Kinds of Ion Exchange Resins

An examination was made by using Amberlite CG 4B-1 type (100 to 200 mesh) and Amberlite CG 400-1 type (100 to 200 mesh) for ion exchange chromatography purposes.

(i) Amberlite CG 4 B-I type

As shown in Table 2 and Figure 3, there were cases where ion exchange was incomplete in this resin. In addition, there were many cases where the peaks of the elution curves overlapped each other. Accordingly, it is believed that this resin is not suitable for the separation of preservatives.

(Table 2 Exchange and Elute Rates of Preservatives)

(Figure 3 Recovery of several preservatives eluted with 0.1N HCl by ion-exchange resin (Amberlite CG 4B-I).)

(ii) Amberlite CG 400-I Type

All preservatives were exchanged 100 per cent. As shown in Table 2 and Figure 4, almost 100 percent elution was observed by 0.1N hydrochloric acid. In other words, there was 100 elution in the range between zero and 60 milliliters in the case of DHA, in the range between 30 and 100 milliliters in the case of SOA and in the range between 60 and 250 milliliters in the case of BA. In the case of SA, there gradually starts elution in the neighborhood of 220 milliliters, with approximately 90 per cent being eluted in approximately 300 milliliters. However, PHBA-E is not eluted.

From the above examination, it was believed that the CG- 400-I type was suitable for the separation of preservatives. Therefore CG-400-I type was used in the following experiments.

3. Examination of Elutes:

(i) Concentration of Hydrochloric Acid

An examination was made at various concentrations including 0.02N, 0.1N,

0.2N, 0.5N and 1N. However, no marked change was observed in the elute due to the difference in the concentration of hydrochloric acid.

(Figure 4 Recovery of several preservatives eluted with 0.1N HCl by ion-exchange resin (Amberlite CG 400-I).)

(ii) Simultaneous Use of Ethanol Solution

The elution of SA requires 300 milliliters in the case of 0.1N hydrochloric acid and, therefore, it lacks practicability in terms of time required. An examination was carried out to see to it that this elution be carried out quickly. As shown in Figure 5, it is seen that the elution of SA is easily carried out by using 50 milliliters of a 0.1N hydrochloric acid 60 per cent ethanol solution instead of 300 milliliters of a 0.1N hydrochloric acid.

(Figure 5 Eluting result of SA by 0.1N HCl with 60 percent ethanol.)

(iii) Length of the Resin Layer

An examination was carried out on 10 centimeters (double the length) and 15 centimeters (triple the length) in order to observe the differential state of the preservatives and the charged length of resin. The result obtained is shown in Figure 6. As the length of the layer becomes greater, the separation between the preservatives themselves becomes better, with complete separation being made from DHA and BA and almost complete separation being also made from SOA and BA. However, a large amount of the elution solution (elute) is also required and the elution time also becomes longer, thereby making it useless in terms of practicability. Even in the co-existence of BA, SOA and DHA, it is possible to carry out a differential determination at a considerable accuracy by a measurement in the ultraviolet range. Therefore, the length of five centimeters is considered to be sufficient. (Refer to Figure 4).

(Figure 6 Effect of column length.)

(iv) Rate of Dropping:

The state of the separation of DHA, SOA, and BA was examined when the length of the resin was five centimeters and the rate of the dropping of the elute was 1/2 (0.5 milliliters per minute). The result, which is shown in Figure 7, reveals the fact that separation is not particularly better than in the case where the rate of dropping was one milliliter per minute (refer to Figure 4).

(Figure 7 Effect of flow-rate, 0.5 milliliters per minute)

4. Elution Test on PHBA-E

In this method, there was a fractionation during the course of extraction operation in PHBA-E, with a result that there is no particular problem. An examination of the elution state relative to Amberlite CG 400-I type shows that, in the case of the PHBA-E -ethyl, -propyl, and -butyl which were used, there was observed no elution in 200 milliliters of the 0.1N hydrochloric acid, 100 milliliters of 0.5N hydrochloric acid and 100 milliliters of 1N hydrochloric acid. In the case of -butyl, elution takes place as shown in Figure 8 in the 10 per cent, 30 per cent, 50 per cent and 60 percent butanol solutions.

(Figure 8 Eluting result of PHBA-Bu by ethanol solutions.)

In the case of a 0.1N hydrochloric 60 per cent ethanol solution, elution takes place up to 20 milliliters. When SA and PHBA-E co-exist, it is better to elute the PHBA-E alone with a 60 per cent ethanol solution in which no SA at all had been eluted and then to elute SA with a 0.1N hydrochloric acid 60 per cent ethanol solution.

5. Differential Test on Mixed Preservatives:

The standard products of preservatives were mixed on the basis of the

examinations of (i) and (iv) in 3. The result of an analysis of these samples is shown in Table 3. As for the differentiating method, a column whose inner diameter was one centimeter was used (refer to Figure 1), the refinement of Amberlite CG 400-I type was carried out in accordance with the normal method to make it into the -Cl form and this was crammed to the lengths of five centimeters. After those which were mixed with from ont to 10 milligrams of DHA, SOA, BA and SA were ion-exchanged, each elute solution was used to carry out elution at the rate of one milliliter per minute.

By using 0.1N hydrochloric acid, a fraction including up to 60 milliliters (1) and a fraction including the range between 60 and 250 milliliters were prepared. By using a 0.1N hydrochloric acid 60 per cent ethanol solution, a fraction of 50 milliliters was taken. If required, the respective elute for each fraction was diluted and the absorbance was measured at the absorption maximum wave length of each preservative. In other words, absorbances were obtained at the following locations:

In (I) at the wave length of 303 m(mu) DHA) and at the wave length of 265 m(mu) (SOA).

In (II), at the wave length of 265 m(mu) (SOA) and at the wave length of 230 m(mu) (BA), and

In (III), at the wave length of 303 m(mu) (SA),

and the contents were obtained from the calibration curve which had already been prepared and, since BA and SOA co-exist in (II), the absorbance of BA was corrected for the reason which was described in the previous report (1).

(Table 3 Recovery of mixed several preservatives by ion exchanger method.)

An examination of the blind absorptions of various fractions shows that, when the wave length is shorter, it is more likely that the effect is received. As the concentration of the target substance is high and the absorbance is measured by diluting it 10 times to 100 times, there is no effect of the blind

absorption for practical purposes.

6. Removal of Obstructing Substances by Amberlite CG 400-I Type.

When preservatives which are contained in some kinds of lactic acid bacteria drinks, baked beans, jam and soy bean jam, etc. are separated by fusing the water-vapor distillation method, perfume and other substances, originating in the food are distilled out in the distillation solution, with a result that the ultraviolet absorption of the preservative is obstructed, thereby making it difficult to determine the presence or absence of the preservative. In this case, this obstacle is removed by extraction operation in the water-vapor distillation method. Instead of this extraction operation, it is possible to employ the Amberlite 400-I type.

Figure 9 shows the ultraviolet absorption spectra of the distillate of soy bean jam containing DHA and SOA and the ultraviolet absorption spectra of the same as treated with Amberlite CG 400-I type.

(Figure 9 U. V. Spectra of distillate obtained from "Miso" containing DHA or SOA and eluate treated with ion-exchange resin (Amberlite CG 400-I).)

Figure 10 shows a comparison between the ultraviolet absorption spectra of the distillate obtained by the water vapor distillation of the "gyoza" bark containing 0.016 grams per kilogram of DHA and the same which was concentrated to 70 milliliters by an Amberlite CG 400-I type treatment, Accordingly, the ion exchange operation can be used for the concentration of the distillate.

(Figure 10 U.V. spectra of distillate obtained from "Gyoza" containing 0.016 grams per kilogram of DHA and eluate treated by ion-exchange resin (Amberlite CG 400-I).)

IV Result of Examination of Items Purchased on the Market

The result of an analysis of preservatives contained in juices and pickles, etc. according to this invention is shown in Table 4. In the foods of this kind, the SA content is generally small. Therefore, the differentiation operation to separate SA from other preservatives by means of ion exchange resin was not carried out this time.

V. Conclusion

(Table 4 Experimental Results of Juices and Pickles Purchased on the Market.)

The solvent extraction of preservatives and the method for the differential determination using ion exchange resin were examined in the case of those foods which contained such acidic preservatives as DHA, SOA, BA, SA and PHBA-E and saccharin. By placing emphasis upon the separation of saccharin from the preservatives and the separation of SA from the other preservatives, the following result was obtained:

(1) A preservative and saccharin can be almost completely separated by utilizing the difference in solubility from carbon tetrachloride.

(2) By using Amberlite CG 400-I type, it is possible to separate SA from DHA, BA and SOA. If the length of the resin layer is increased, it is possible to separate between DHA and BA and between SOA and BA.

(3) The use of Amberlite CG 400-I type can be a substitute for the operation of removing obstacles of the ultraviolet absorption spectra observed in the distillate at the time when the water vapor distillation method is used. It is also effective as a step for the concentration of the preservatives in a test solution by the water vapor distillation method.

This report was published at the 10th assembly of the Food Hygiene Society of Japan which was held on October 27, 1965, in Tokyo.

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*1. A Study on the Preservatives in Food, Tetsuo Inoue, Taro Kawamura, Masaya Kamijo, and Yoko Sato (Report No. 7).

A study on the Analysis of Food Additives (Report No. 11), Saburo Sugano, Shogo Ochiai and Tadayoshi Nakaoka.

*2. Yokohama Municipal Public Health Laboratory, 2-102 Nakamura Cho, Minami Ku, Yokohama.

*3. Kanagawa Prefectural Public Health Laboratory, 52-2 Nakao Cho, Hodogaya Ku, Yokohama.

1) Saburo Sugano and Tetsuo Inoue, et al.: Journal of Food Hygiene, 7, 72 (1966).

2) Tetsuo Inoue, Taro Kawakami, et al.; Journal of Food Hygiene, 5, 368 (1964).

(Translated by Carl Demrick Associates,Inc/RO/t)

Table 1. Solubility of Several Preservatives and Saccharin in CCl_4

Sample	Soluble mg in CCl_4 10 ml	Solubility
Dehydroacetic acid(DHA)	349 mg	3.5%
Sorbic acid (SOA)	107 mg	1.1%
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Ethyl-*p*-hydroxybenzoate (PHBA-Et.)	13 mg	0.1%
Propyl-*p*-hydroxybenzoate (PHBA-Pr.)	31 mg	0.3%
Butyl-*p*-hydroxybenzoate (PHBA-Bu.)	>500 mg	> 5.0%
Saccharin	< 1 mg	0

Table 2. Exchange and Elute Rates of Preservatives

Preservatives (mg) \ Resins		Amberlite CG 4B-I		Amberlite CG 400-I	
		Exchange rate (%)	Elute rate (%)	Exchange rate (%)	Elute rate (%)
DHA	3	100	*1 60	100	*1 100
SOA	10	100	*1 100	100	*1 100
BA	10	100	*1 100	100	*1 100
SA	1	100	*1 70	100	*1 90 *2 100
PHBA-Et.	1	20	*1 20	100	*3 100
PHBA-Pr.	1	70	*1 66	100	*3 100
PHBA-Bu.	1	80	*1 65	100	*3 100

*1 by 0.1 N HCl
*2 by 0.1 N HCl with 60% Ethanol
*3 by 60% Ethanol soln.

Table 3. Recovery of Mixed Several Preservatives by Ion Exchanger Method

Elutes \ Mixed preservatives	DHA 5 mg	SOA 10 mg	BA 10 mg	SA 1 mg
0.1N HCl 0~60 ml	4.9 mg 4.9 〃 5.0 〃 4.9 〃	9.0 mg 9.0 〃 9.0 〃 9.0 〃		
0.1N HCl 61~250 ml		1.7 mg 1.5 〃 1.5 〃 1.5 〃	9.5 mg 9.5 〃 9.7 〃 9.8 〃	
(0.1N HCl 251~550 ml)				(0.90 mg 0.88 〃 0.84 〃 0.85 〃)
0.1N HCl with 60% Ethanol 50 ml				0.91 mg 0.88 〃 1.00 〃 0.97 〃
Total	4.9 mg 4.9 〃 5.0 〃 4.9 〃	10.7 mg 10.5 〃 10.5 〃 10.5 〃	9.5 mg 9.5 〃 9.7 〃 9.8 〃	0.91 mg 0.88 〃 1.00 〃 0.97 〃
Means	4.9 mg	10.6 mg	9.6 mg	0.94 mg
Recovery (%)	98%	106%	96%	94% (87%)

Table 4. Experimental Results of Market Juices and Pickles

Sample	DHA g/kg	SOA g/kg	BA g/kg	SA g/kg	PHBA g/kg
Orange juice (1)	0.026	negative	negative	negative	negative
〃 (2)	negative	〃	0.015	〃	〃
Pine juice	0.01	〃	0.017	〃	〃
Melon juice	0.07	〃	0.025	〃	〃
Orange conc. juice	0.055	〃	0.28	〃	0.015
Lemon conc. juice	0.015	〃	0.09	〃	0.006
Strawberry syrup	0.063	〃	0.25	〃	0.014
Melon syrup	0.34	〃	0.218	〃	negative
Cucumber soaked in "Soy sauce"	negative	0.56	negative	〃	〃
Vegetables soaked in "Soy sauce"	〃	0.30	〃	〃	〃
"Hana-rakkyo"	〃	0.12	〃	〃	〃
Ginger root soaked in "Miso"	〃	0.38	〃	〃	〃

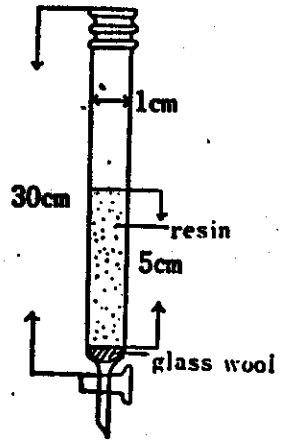

Fig. 1. Apparatus
Ion-exchange resin: Amberlite CG 400-I type (anion exchange resin, Cl-type)
Elute: 0.1 N HCl, 0.1 N HCl with 60% Ethanol, Flow Rate: 1 ml/min, Temperature: room temperature

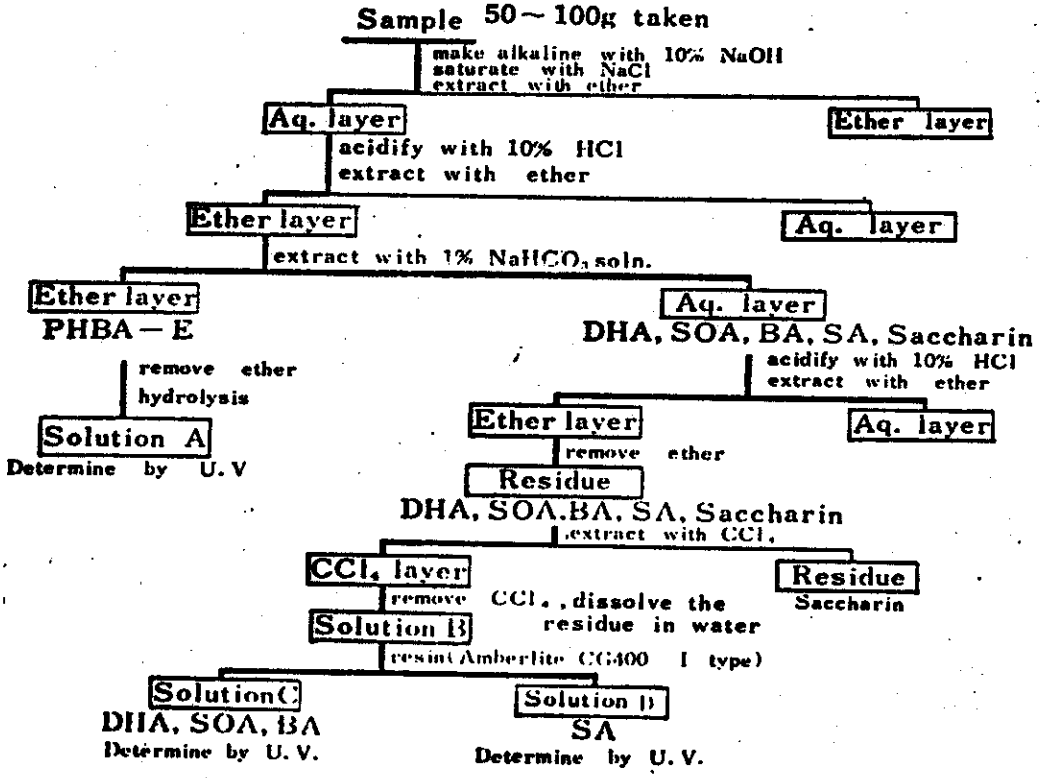

Fig. 2. Procedure of analytical method of the preservatives in food.

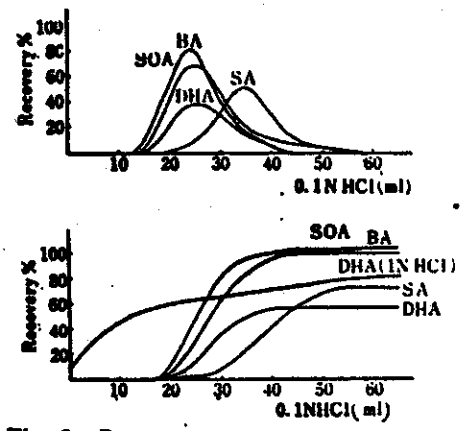

Fig. 3. Recovery of several preservatives eluted with 0.1N HCl by ion-exchange resin (Amberlite CG 4B-I)

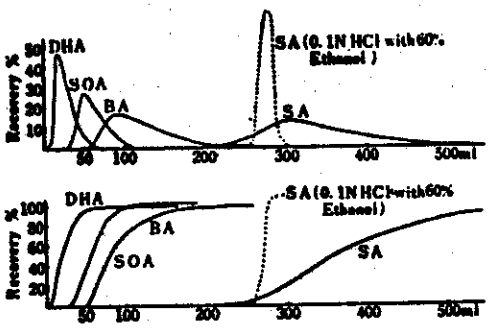

Fig. 4. Recovery of several preservatives eluted with 0.1N HCl by ion-exchange resin (Amberlite CG 400-I)

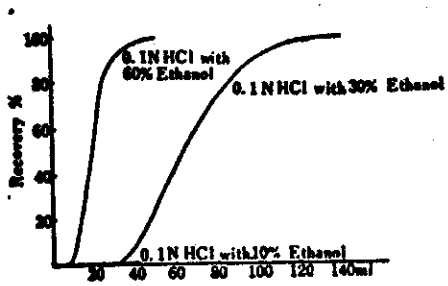

Fig. 5. Eluting result of SA by 0.1N HCl with 60% ethanol

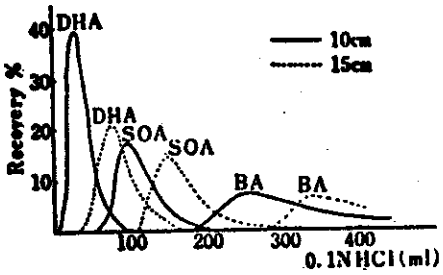

Fig. 6. Effect of column length

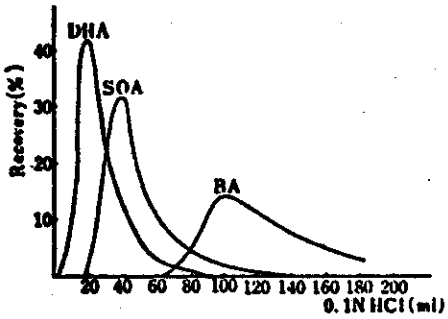

Fig. 7. Effect of flow rate, 0.5 ml/min

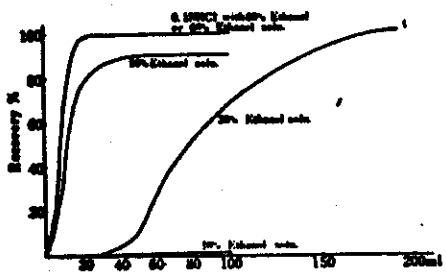

Fig. 8. Eluting result of PHBA-Bu. by ethanol solutions

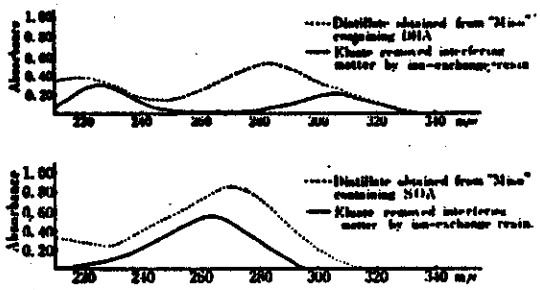

Fig. 9. U.V. spectra of distillate obtained from "Miso" containing DHA or SOA and eluate treated by ion-exchange resin (Amberlite CG 400-I)

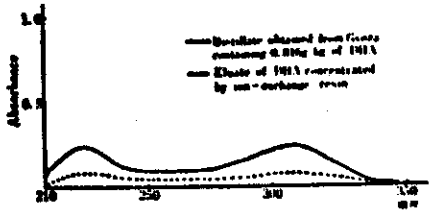

Fig. 10. U.V. spectra of distillate obtained from "Gyōza" containing 0.016 g kg of DHA and eluate treated dy ion-exchange resin (Amberlite CG 400-I)

溶媒抽出法およびイオン交換樹脂応用による数種の保存料の分離定量[*1]

(昭和41年1月28日受理)

井上哲男[*2] 河村太郎[*2] 上條昌弥[*2]
佐藤洋子[*2] 菅野三郎[*3] 落合昭吾[*3]
中岡正吉[*3]

Differential Determination of Several Kinds of Preservatives in Foods by Extraction and Ion Exchange Resin Methods

Tetsuo INOUE[*2], Taro KAWAMURA[*2], Masaya KAMIJO[*2], Yōko SATŌ[*2], Saburo KANNO[*3], Shogo OCHIAI[*3], and Tadayoshi NAKAOKA[*3]

([*2]Yokohama Municipal Public Health Laboratory: 2-102, Nakamura-cho, Minami-ku, Yokohama; [*3]Kanagawa Prefectural Public Health Laboratory: 52-2, Nakao-cho, Hodogaya-ku, Yokohama)

For the purpose of the differential determination of the preservatives in foods, the combination method of the procedures of solvent extraction, ion exchange resin and ultraviolet absorption spectra was studied.

As the preservatives five kinds of substances were taken up, namely, benzoic acid (BA), *p*-hydroxybenzoic acid esters (PHBA-E), sorbic acid (SOA), dehydroacetic acid (DHA) and salicylic acid (SA).

Characteristic points of this method were as follows:

(1) Saccharin was removed from the mixture of preservatives by CCl_4 extraction.

(2) SA was separated from the other preservatives by ion exchange resin.(Amberlite CG 400-I)

(3) Ion exchange resin (Amberlite CG 400-I) was useful to remove the interference accompanied with foods.

This method was applied to the market foods, for example, juices or pickles, and the results obtained were reported.

(Received January 28, 1966)

I まえがき

われわれは前報[1]において，水蒸気蒸留法による食品中の数種の保存料の分離定性定量法について報告した．

溶媒による抽出法は，タンパク質や糖質を多く含む食品および乳化剤を使用している食品では，はなはだしい乳化現象を起こし，操作がきわめて困難となる場合が少なくない．また溶媒抽出法では使用する有機溶媒に可溶の物質はすべて抽出され，二次的に抽出された物質が目的物の定性定量を困難にする場合もしばしばある．

そこでわれわれは安息香酸 (BA)，パラオキシ安息香酸エステル類 (PHBA-E)，ソルビン酸 (SOA)，サリチル酸 (SA)，デヒドロ酢酸 (DHA) 等数種の酸性保存料およびサッカリンが共存する場合を想定し，従来の溶媒抽出法にイオン交換樹脂を利用することにより分離定量

[*1] 井上哲男，河村太郎，上條昌弥，佐藤洋子：食品中の防腐剤に関する研究（第7報）
菅野三郎，落合昭吾，中岡正吉：食品添加物の分析に関する研究（第11報）

[*2] 横浜市衛生研究所：横浜市南区中村町2-102
[*3] 神奈川県衛生研究所：横浜市保土ケ谷区中尾町52-2

がほぼ可能であることがわかったので，以下に報告する．

本法の骨子はFig.2に示すように各保存料を含む試料溶液をはじめアルカリ性でエーテルを用い中性，塩基性物質を除いたのち，つぎに酸性でエーテル抽出を行ないこの抽出液から，1%炭酸水素ナトリウム溶液を用いてPHBA-E を他の保存料と分離したのち，エーテル留去残留物に四塩化炭素を加えて不溶のサッカリンを除去し，サッカリンを除いた保存料を Amberlite CG 400-I 型イオン交換樹脂を用いて SA と他の保存料とに分離し，それぞれのフラクションについて試験溶液を調製し，以下前報のように紫外部吸光度測定法を行なう．

II 実験方法

〔装　置〕

1. イオン交換樹脂クロマト管：内径1cm，長さ30cm，下端に活栓コック付のクロマト管に常法に従い精製した Amberlite CG400-I 型(-Cl 形)約4.5 ml を5cmの高さに充填したもの．

2. 自記分光光度計

〔試　薬〕

1. 緩衝液：2M塩化カリウム溶液50 ml と2N塩酸10.6 ml を混合し，水を加えて200 ml とする．

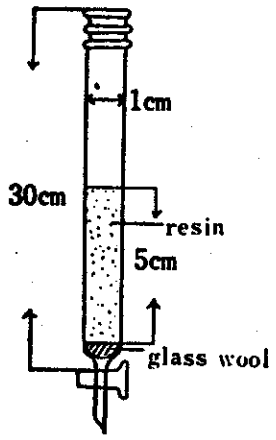

Fig. 1. Apparatus
Ion-exchange resin: Amberlite CG 400-I type (anion exchange resin, Cl-type)
Elute: 0.1 N HCl, 0.1 N HCl with 60% Ethanol, Flow Rate: 1 ml/min, Temperature: room temperature

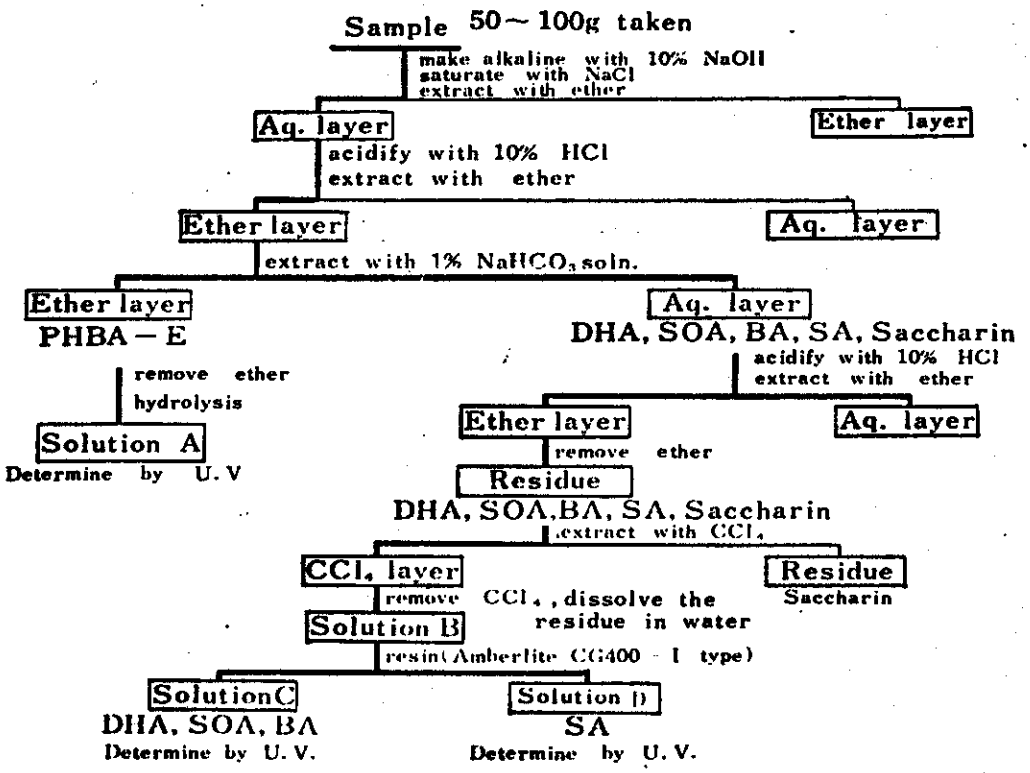

Fig. 2. Procedure of analytical method of the preservatives in foods

2. 溶離液：0.1N塩酸，0.1N塩酸60%エタノール溶液

〔試験操作〕

1. 試料溶液の調製

液体試料：保存料の含量に応じ試料50～100gをとり，必要あれば水を加えて全量を100～150 ml とし試料溶液とする．

固体試料：試料を細切またはすりつぶし，保存料の含量に応じその30～50gを200 mlの三角フラスコにとり，水100 ml を加え，10%水酸化ナトリウム溶液でアルカリ性とし，水浴上で加温してしばらく放置したのちろ過する．残留物は少量の水で洗い洗液をろ液に合し試料溶液とする．

2. 試験溶液の調製

試料溶液を分液ロートにとり10%塩酸または10%水酸化ナトリウム溶液で中和したのち，あらためて10%水酸化ナトリウム溶液3 ml を加えてアルカリ性とし，塩化ナトリウムを飽和させ，エーテル50 ml で3回振りエーテル層を除く．つぎに水層を10%塩酸で十分に酸性にしたのちエーテル50 ml で3回抽出する．エーテル層を合し水10 ml で2回水洗したのち1%炭酸水素ナトリウム溶液30 ml で3回抽出する．エーテル層は無水硫酸ナトリウムで脱水しエーテルを留去する．残留物に10%水酸化ナトリウム溶液2 ml および水3 ml を加え還流冷却器を付し，石綿付金網上で15分間煮沸したのち，冷却し，10%塩酸5 ml を加え酸性としエーテル40 ml で3回抽出する．エーテル層を合し水10 ml で2回水洗しエーテルを留去する．残留物に0.1N 水酸化ナトリウム溶液2～3 ml を加えて溶かし，0.1N塩酸で中和したのち水を加えて100 ml とし，これを試験溶液Aとする．

つぎに，1%炭酸水素ナトリウム溶液で抽出した水層を合わせ，10%塩酸で酸性としたのち塩化ナトリウムを飽和させ，エーテル50 ml で3回抽出し，エーテル層を水10 ml で2回洗い，無水硫酸ナトリウムで脱水しエーテルを留去する．この残留物に四塩化炭素5 ml を加えて溶かし，ろ過し，不溶物を少量の四塩化炭素で洗い，洗液はろ液に合し，全量を正確に10 ml とする．その5 ml をとり軽く送風しながら四塩化炭素を除去する．残留物に0.1N水酸化ナトリウム溶液1～2 ml を加えて溶かし，0.1N塩酸で中和したのち，水を加えて100 ml としこれを試験溶液Bとする．

試験溶液B中にSAが共存するときはさらにつぎの操作を行なう．

Fig. 1に示すクロマト管に精製した Amberlite CG 400-I 型（-Cl 形）を詰め，これに試験溶液B 50 ml を1 ml/min で流下させ，続いて水50 ml で水洗する．洗液の滴下が終ったならば直ちに0.1N塩酸250 ml を用いて同じ流速で溶出を行ないこれを試験溶液Cとする．ついで，0.1N塩酸60%エタノール溶液50 ml を用い同様に溶出を行ないこれを試験溶液Dとする．

3. 定　性

試験溶液AおよびBまたはC，Dをとり，測定に適した濃度に希釈し，その10 ml をとり緩衝液1 ml を加え自記分光光度計を用い，水10 ml に緩衝液1 ml を加えたものを対照とし紫外部吸収を測定する．ただし，C，Dについてはそれぞれの溶離液を対照とする．

PHBA-E が存在するときには試験溶液Aで波長256 mμ 付近に極大吸収を認める．また，DHA，SOA，BA，SA がそれぞれ単独で存在するときは試験溶液Bで，DHA は波長308 mμ，225 mμ，SOA は波長265 mμ，BA は波長230 mμ，SA は波長303 mμ，236 mμ 付近にそれぞれ極大吸収を認める．これらが二者以上共存するときにはそれぞれの含量に応じた合成波を認める．試験溶液Cでは DHA，SOA，BA の吸収が，試験溶液Dでは SA のみの吸収が認められる．

4. 定　量

試験溶液AおよびBについては，その10 ml をとり緩衝液2 ml および水を加えて全量を20 ml とし，必要に応じて10倍に薄めた緩衝液を用いて測定に適した濃度に薄めたものにつき，各保存料の吸収極大波長付近における吸光度を測定する．一方，各保存料の標準溶液から得られた吸光度から保存料の含量を算出する．試験溶液CおよびDについては測定に適した濃度に各溶離液を用いて薄め吸光度を測定する．一方，各保存料の標準溶液についてイオン交換樹脂を用いて同様に操作して得た吸光度から保存料の含量を算出する．

III 実験結果および考察

本実験においては四塩化炭素によるサッカリンの分離とイオン交換樹脂によるサリチル酸と他の保存料との分離に主眼をおいて行なったものである．

1. サッカリンの分離について

四塩化炭素によるサッカリンの分離についてはすでに著者らが前報[3]で述べたように保存料が四塩化炭素に溶解するのに対しサッカリンはきわめて難溶である．Table 1 は保存料およびサッカリン0.5g について四塩

Table 1. Solubility of Several Preservatives and Saccharin in CCl_4

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Ethyl-*p*-hydroxybenzoate (PHBA-Et.)	13 mg	0.1%
Propyl-*p*-hydroxybenzoate (PHBA-Pr.)	31 mg	0.3%
Butyl-*p*-hydroxybenzoate (PHBA-Bu.)	>500 mg	> 5.0%
Saccharin	< 1 mg	0

化炭素10 ml に対する溶解度をみたものである．

また，サッカリンおよび保存料を四塩化炭素に溶かしろ過したろ液についての赤外吸収スペクトルを測定してみたところ保存料の吸収は現われるが，サッカリンの吸収は見られずサッカリンの四塩化炭素に対する不溶性を示している．

2. 二種のイオン交換樹脂の比較

イオン交換クロマトグラフ用 Amberlite CG 4B-I型（100～200メッシュ）および Amberlite CG 400-I 型（100～200メッシュ）について検討した．

(i) Amberlite CG 4B-I 型

本樹脂については Table 2 および Fig. 3に示すようにイオン交換の不完全なものがあり，また溶出曲線のピークが重なるものが多く，100% 溶出の困難なものがある．したがってこの樹脂は保存料の分離には不適当と思われる．

Table 2. Exchange and Elute Rates of Preservatives

Preservatives (mg) \ Resins		Amberlite CG 4B-I		Amberlite CG 400-I	
		Exchange rate (%)	Elute rate (%)	Exchange rate (%)	Elute rate (%)
DHA	3	100	*1 60	100	*1 100
SOA	10	100	*1 100	100	*1 100
BA	10	100	*1 100	100	*1 100
SA	1	100	*1 70	100	*1 90 *2 100
PHBA-Et.	1	20	*1 20	100	*2 100
PHBA-Pr.	1	70	*1 66	100	*2 100
PHBA-Bu.	1	80	*1 65	100	*2 100

*1 by 0.1 N HCl
*2 by 0.1 N HCl with 60% Ethanol
*3 by 60% Ethanol soln.

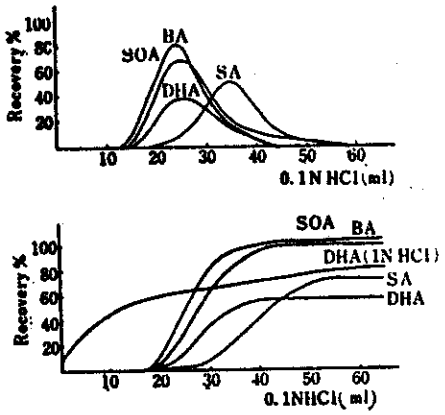

Fig. 3. Recovery of several preservatives eluted with 0.1N HCl by ion-exchange resin (Amberlite CG 4B-I)

(ii) Amberlite CG 400-I 型

いずれの保存料も100%交換され，Table 2, Fig. 4に示すように0.1N塩酸によってほぼ100%溶出される．すなわち，DHA は 0～60 ml，SOA は30～100 ml，BA は60～250 ml あたりにかけてほぼ 100%溶出する．SA は220 ml あたりから徐々に溶出がはじまり約300 ml 中にほぼ90%溶出するが PHBA-E は溶出してこない．

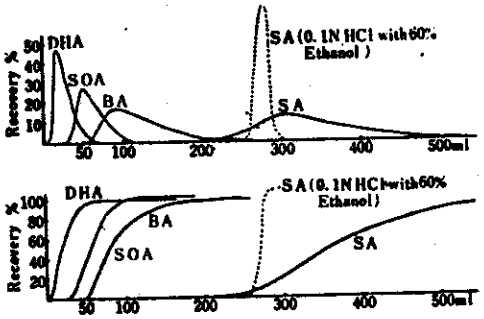

Fig. 4. Recovery of several preservatives eluted with 0.1N HCl by ion-exchange resin (Amberlite CG 400-I)

以上の検討から保存料の分離には CG 400-I 型が適当と思われるので以下の実験は CG 400-I 型を用いて行なうことにした．

3. 溶離液についての検討

(i) 塩酸濃度

0.02N，0.1N，0.2N，0.5N，1Nの各濃度について検討したが塩酸の濃度差による溶出の著しい変化は認め

られない.

(ii) エタノール溶液の併用

SA の溶出は0.1N塩酸では300 ml を要し時間的に実用性を欠くので，この溶出をすみやかに行なう点について検討した．Fig. 5 に示すように SA は 0.1N 塩酸300 ml を用いる代りに0.1N塩酸60%エタノール溶液50 ml を用いると容易に溶出が行なえることがわかる.

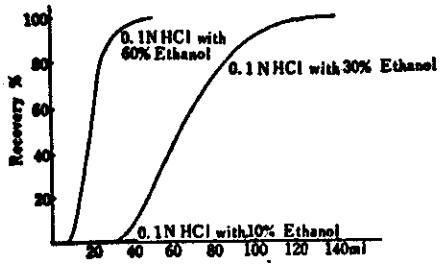

Fig. 5. Eluting result of SA by 0.1N HCl with 60% ethanol

(iii) 樹脂層の長さ

樹脂の充塡の長さと保存料相互の分離状態をみるため2倍の長さの10cm および3倍の長さの15cm について検討したのが Fig. 6 である．層の長さが長くなるほど保存料相互の分離はよくなり，DHA と BA とは完全に分離でき，SOA と BA もほとんど分離できる．しかし同時に溶離液も多量を要し，溶出時間も長くなり実用性にかける．BA, SOA, DHA は共存しても紫外部吸収測定でかなりの精度で分別定量が可能であるから5cm の長さでも十分であると考える (Fig. 4).

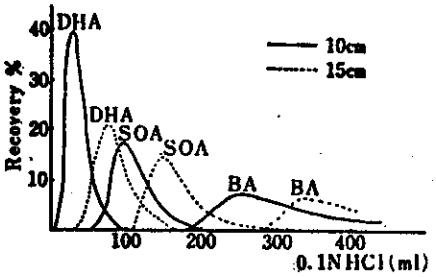

Fig. 6. Effect of column length

(iv) 滴下速度

樹脂の長さ5cm の条件において溶出液の滴下速度を½(0.5 ml/min) にしたときの DHA, SOA, BA の分離状態をみた．その結果は Fig. 7 に示すように滴下速度1 ml/min (Fig. 4) と比べてとくに分離がよくならない.

4. PHBA-E の溶出試験

PHBA-E は本法の場合，抽出操作の途中で分別しているのでとくに問題はないが，Amberlite CG 400-I 型

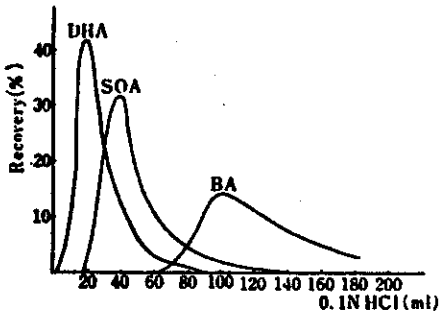

Fig. 7. Effect of flow rate, 0.5 ml/min

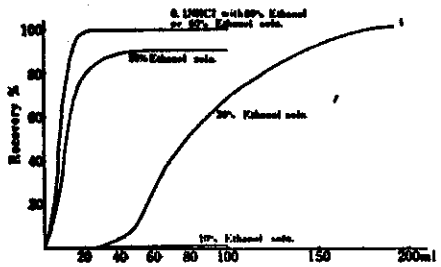

Fig. 8. Eluting result of PHBA-Bu. by ethanol solutions

についての溶出状態をみると，使用した PHBA-E の-エチル，-プロピル，-ブチルではともに 0.1N塩酸200 ml, 0.5N塩酸100 ml, 1N塩酸100 ml 中には溶出しないが，-ブチルの場合10%，30%，50%，60%エタノール溶液では Fig. 8 のように溶出する.

また，0.1N塩酸60%エタノール溶液でも 20 ml までに溶出するので SA と PHBA-E とが共存しているときは，はじめに SA を全く溶出しない 60% エタノール溶液で PHBA-E のみを溶出させたのち 0.1N塩酸60%エタノール溶液で SA を溶出させればよい.

5. 混合保存料についての分離試験

3. の (i)～(iv) の検討に基づいて保存料の標準品を混合した試料を分析した結果を Table 3 に示す．分離法は内径1cm のカラム (Fig. 1) を用い，常法に従って精製し -Cl 形とした Amberlite CG 400-I 型を5cm の長さに詰め，DHA, SOA, BA および SA の1～10mg を混合したものをイオン交換させたのち各溶離液を用いて 1 ml/min の速度で溶出を行なう．すなわち 0.1N 塩酸を用いて 60 ml までのフラクション (I) と 60～250 ml までのフラクション (II) に分取し，ついで0.1N塩酸60%エタノール溶液を用いて50 ml のフラクション (III) をとる．各フラクションにつき必要に応じてそれぞれの溶離液で希釈し，各保存料の吸収極大波長で吸光度を測定する．すなわち，

（I）においては
　波長 308 mμ(DHA)，　波長 265 mμ(SOA)
（II）においては
　波長 265 mμ(SOA)，　波長 230 mμ(BA)
（III）においては　波長 303 mμ(SA)

での吸光度を求め，あらかじめ作成した検量線から含量を求め，（II）は BA と SOA とが共存しているので前報[1]で述べたような理由から BA の吸光度を補正して求めた．

Table 3. Recovery of Mixed Several Preservatives by Ion Exchanger Method

Elutes \ Mixed preservatives	DHA 5 mg	SOA 10 mg	BA 10 mg	SA 1 mg
0.1N HCl 0～60 ml	4.9 mg 4.9 〃 5.0 〃 4.9 〃	9.0 mg 9.0 〃 9.0 〃 9.0 〃		
0.1N HCl 61～250 ml		1.7 mg 1.5 〃 1.5 〃 1.5 〃	9.5 mg 9.5 〃 9.7 〃 9.8 〃	
(0.1N HCl 251～550 ml)				(0.90 mg 0.88 〃 0.84 〃 0.85 〃)
0.1N HCl with 60% Ethanol 50 ml				0.91 mg 0.88 〃 1.00 〃 0.97 〃
Total	4.9 mg 4.9 〃 5.0 〃 4.9 〃	10.7 mg 10.5 〃 10.5 〃 10.5 〃	9.5 mg 9.5 〃 9.7 〃 9.8 〃	0.91 mg 0.88 〃 1.00 〃 0.97 〃
Means	4.9 mg	10.6 mg	9.6 mg	0.94 mg
Recovery (%)	98%	106%	96%	94% (87%)

各フラクションの着吸収をみると波長が短いほど影響を受けやすいが目的物質の濃度が高く10倍あるいは100倍希釈で吸光度を測定するので実際には着吸収の影響はほとんどない．

6. Amberlite CG 400-I 型による妨害物質の除去

ある種の乳酸菌飲料，煮豆，ジャム，みそなどに含有する保存料を水蒸気蒸留法を用いて分離するとき，留液中に同時に香料その他食品由来の物質が留出し保存料の紫外部吸収が妨害され保存料の有無を判定しかねることがある．この場合，水蒸気蒸留法では抽出操作でこの妨害を除いているが，この抽出操作の代りに Amberlite CG 400-I 型を用いることもできる．Fig. 9 は DHA および SOA を含有するみその留液について紫外部吸収スペクトル，およびこれの Amberlite CG 400-I 型処理による紫外部吸収スペクトルを示す．

Fig. 10 は DHA 0.016 g/kg を含有するぎょうざの皮について水蒸気蒸留を行ない，ここに得た留液とこの留液210 ml を Amberlite CG 400-I 型処理により 70 ml に濃縮したものについて試験溶液を調製し，両者の紫外部吸収スペクトルを比較したものである．したがってイオン交換操作は留液の濃縮操作として用いることもできる．

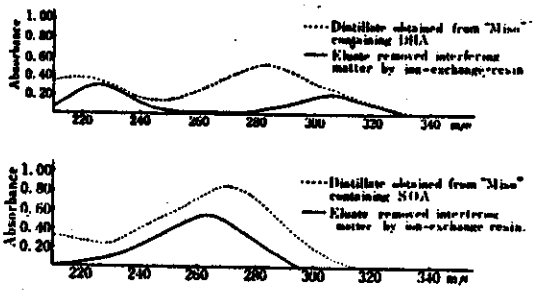

Fig. 9. U.V. spectra of distillate obtained from "Miso" containing DHA or SOA and eluate treated by ion-exchange resin (Amberlite CG 400-I)

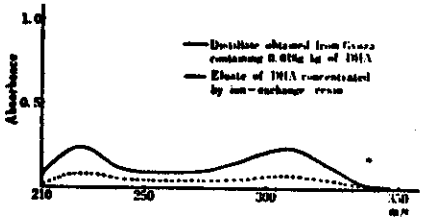

Fig. 10. U.V. spectra of distillate obtained from "Gyōza" containing 0.016 g kg of DHA and eluate treated dy ion-exchange resin (Amberlite CG 400-I)

Ⅳ 市販品についての検査成績

本法でジュース，漬物類中の保存料を分析した結果は Table 4 である．この種の食品には SA を含有することが少ないので今回はイオン交換樹脂による SA と他の保存料との分離操作はとくに行なわなかった．

Ⅴ むすび

DHA, SOA, BA, SA および PHBA-E の酸性保存料およびサッカリンを含む食品について保存料の溶媒抽出およびイオン交換樹脂を用いる分離定性定量法を検討した．とくにサッカリンの保存料からの分離 および SA と他の保存料との分離に主眼をおき以下の結果を得た．

(1) 保存料とサッカリンとは四塩化炭素に対する溶解度の差でほぼ完全に分離できる．

(2) Amberlite CG 400-I 型を用いれば SA を DHA, BA, SOA と分離できる．また，樹脂層の長さを長くすれば DHA と BA との分離および SOA と BA との分離も可能である．

(3) Amberlite CG 400-I 型を用いれば水蒸気蒸留法の際，留液中にみられる紫外部吸収スペクトルの妨害除去操作に代えられる．また，水蒸気蒸留法による試験溶液中の保存料濃度の濃縮操作としても有効である．

本報告は第10回日本食品衛生学会(昭和40年10月27日，東京）において発表．

文 献

1) 菅野三郎，井上哲男，他：食衛誌．7，72(1966)．
2) 井上哲男，河村太郎，他：食衛誌．5，368(1964)．

Table 4. Experimental Results of Market Juices and Pickles

Sample	DHA g/kg	SOA g/kg	BA g/kg	SA g/kg	PHBA g/kg
Orange juice (1)	0.026	negative	negative	negative	negative
〃 (2)	negative	〃	0.015	〃	〃
Pine juice	0.01	〃	0.017	〃	〃
Melon juice	0.07	〃	0.025	〃	〃
Orange conc. juice	0.055	〃	0.28	〃	0.015
Lemon conc. juice	0.015	〃	0.09	〃	0.006
Strawberry syrup	0.063	〃	0.25	〃	0.014
Melon syrup	0.34	〃	0.218	〃	negative
Cucumber soaked in "Soy sauce"	negative	0.56	negative	〃	〃
Vegetables soaked in "Soy sauce"	〃	0.30	〃	〃	〃
"Hana-rakkyo"	〃	0.12	〃	〃	〃
Ginger root soaked in "Miso"	〃	0.38	〃	〃	〃

Application of thin layer chromatography to the investigation of antifermentatives in foods

III Quantitative determination

by M. Covello and O. Schettino

Institute of Pharmaceutical and Toxicological Chemistry, University of Naples

(for summary in English see p.337 of Italian publication)

In undertaking a study for the purpose of investigating the antifermentatives in preserved foods (1), we had brought out the difficulties the analyst is faced with due to the variety of products of this kind that have come into common use, or that may be new in this field (2). Such difficulties, it was noted, are increased by the lack of sufficiently explicit laws. All this is evident if one takes into account some general considerations.

The investigation of the antifermentatives, or, more generally, of the additives in preserved foods, can be carried out by pursuing two different objectives:

1) a purely qualitative ascertainment of the presence of the additive and its identification;

2) quantitative determination of the identified additive.

An investigation of the first type can suffice when the use of the additive, the presence of which is found in the food, is expressly prohibited by law. But it is indispensable to resort to quantitative determination when its use is permitted within certain limits.

The existence of a clear regulation regarding the use of additives represents, however, the better guide for giving the analyst a criterion of evaluation about the analytical techniques to be used in each particular case.

It had, in fact, been pointed out repeatedly in Italy that it was necessary to have, following the example of other countries, a positive list of the additives that may be used in the preparation and preservation of food

preserves, both by the big food packing industry and by the artisan type producers. This need was recognized by the legislator who, in a recent decree (3), faced up to the problem by determining which chemical substances may be used in the preparation of food products. The same decree fixed the applicable cases and the maximum doses to be used and established the physico-chemical characteristics and the purity requirements thereof.

Some antifermentatives, consisting of sublimable substances, have been the subject of our above-mentioned investigations. These, precisely, are included among those whose use is permitted under the new provisions (see Table 1).

Table 1

Foods for which the use of sublimable antifermentatives is provided in D.M. 19 January 1963 - G. U. No. 64 of March 7, 1963 and respective minimum doses and concentrations. Chromatographic values referred to the solvent isopropyl alcohol - ethyl alcohol - conc. NH_3 - water (5:2:0.5:1)

Food product	Permitted antifermentative	Max.dose	Conc.	R_f
Fats and oils (excluding olive oil)	Sorbic acid and its Na, K and Ca salts	500 mg/kg	98-99%	0.77
Cheese	Sorbic acid and its Na, K and Ca salts	1000 mg/kg	98-99%	0.77
Pasta filling	Sorbic acid and its Na, K and Ca salts	1000 mg/kg	98-99%	0.77
Fish semi-preserves	Sorbic acid and its Na,K and Ca salts	1000 mg/kg	98-99%	0.77
	Benzoic acid and its Na, K and Ca salts	1500 mg/kg	74-99.5%	0.65
	Ethyl p-oxy-benzoate	1000 mg/kg	99.5%	0.76
	Propyl p-xoy-benzoate	1000 mg/kg	99.5%	0.74
Mayonnaise	Sorbic acid and its Na,K and Ca salts	1000 mg/kg	98-99%	0.77
	Ethyl p-oxy-benzoate	1000 mg/kg (on the fat)	99.5%	0.76

	Propyl p-xoy-benzoate	1000 mg/kg	99.5%	0.74
Sweets	Sorbic acid and its Na,K and Ca salts	2000 mg/kg	98-99%	0.77
Packaged bread	Sorbic acid and its Na.K and Ca salts	2000 mg/kg	98-99%	0.77
Dried fruit	Sorbic acid and its Na,K and Ca salts	1000 mg/kg	98-99%	0.77
Nonalcoholic beverages	Benzoic acid and its Na,K and Ca salts	500 mg/l	74-99.5%	0.65
	Ethyl p-oxy-benzoate	500 mg/l	99.5%	0.76
	Propyl p-oxy-benzoate	500 mg/l	99.5%	0.74
Popular sweet wines	Ethyl p-oxy-benzoate	250 mg.l	99.5%	0.76
	Propyl p-oxy-benzoate	250 mg/l	99.5%	0.74

In this paper we report on the latest studies conducted in the testing for preservatives in foods, for the purpose of establishing quantitative standards of the analytical process which we had previously examined only qualitatively (1, 2).

The technique of quantitative determination here proposed was tested on all sublimable antifermentatives already studied. Inview of the above considerations, it seemed to us desirable to focus our attention on the products which the law permits the use of, in particular applying the method to practical cases.

EXPERIMENTAL PART

The purpose pursued in the qualitative analysis proposed and illustrated in the preceding paper was to develop a simple and fast technique, characteristics of obvious usefulness in analyzing commercial products. This objective prompted us, when it cam to a quantitative application of the process, to leave aside overly complicated methods and to try, instead, to use a rapid photodensitometric technique. Already in our preliminary report we ruled out the possibility of obtaining exactly reproducible quantitative results by using crystalline sublimates obtainable by development of the antifermentatives according to the technique here proposed.

Repeated tests have shown, in fact, that such a sublimate represents only an aliquot, though an important one, of the chromatographed product, nor has it been possible, on the other hand, to set that fraction in a regular and reproducible numerical ratio. We arranged, therefore to carry out a double chromatography for each sample, assigning the first chromatogram for the qualitative detection of the antifermentative and the second for its quantitative determination.

The literature on antifermentative research (4-13) offers a wide choice of processes, applicable to the small quantities of substance used in chromatography. They involve, in general, the quantitative elution of the product from the chromatogram and its evaluation with methods which avail themselves mainly of spectrophotometric analysis. Such techniques, undoubtedly efficient and susceptible of giving fairly precise results, have the disadvantage of being laborious and delicate and, in short, requiring much time for their execution.

It was therefore thought desirable to avoid any long manipulation of the chromatogram and to evaluate directly the spot obtained by proper development of the substance under examination. For this purpose we used an integrating recording photodensitometer (the Joyce Chromoscan). The results of this experimentation, set forth in detail below, are preceded by a brief re-examination of the chromatographic part, in order better to clarify the various phases of the process.

a) Chromatographic separation of the antifermentative

For purposes of identification we have used (see the cited papers for particulars) the chromatographic technique on thin layer of silica gel G. Merck, of the thickness of 250 μ.

As rigid support in the preparation of thin layers for development by sublimation we have always used metal plates, size 5 x 20 cm or 20 x 20 cm. For the quantitative determination, instead, it was necessary to use chromium-

plated metal or glass plates, 2.5 x 20 cm, so that they could be introduced in the slide of the densitometer. These plates were used for a single deposition of substance.

Thesolution under examination, obtained by extraction with ether from the food product, was deposited in the form of round spots 2 cm from the edge of the above plates, being extremely careful to maintain the diameter of the spots within minimum limits (approx. 2 mm). For the subsequent desnitometric measurement, in fact, it is preferable that the size of the spots after chromatography should be rather small. Besides, for the purposes of reproducibility and comparability, the depositions should cover areas as nearly equivalent as possible.

As stated before, the chromatography is carried out on a duplicate sample, and it is advisable, for this purpose, to perform two different extractions on two samples of the food product exactly measured, rather than dividing the extract obtained from a single sample.

b) Quantitative determination

In carrying out the measurements we used the "Chromoscan Joyce" automatic integration recording photodensitometer, establishing the following operative parameters:

Ratio of movement - recording/sample 1:1

Filters	standard
Slit	10 x 0.5 mm
Cam	No. 5-034/A

The standard sample holder mounted on this instrument consists of a rectangular frame, size 2.7 x 21 cm, with a window of 1.9 x 20.4 cm.

These dimensions, as mentioned before, imposed on us the use of plates of a width limited to about 2.5 cm for the execution of the chromatograms.

The length of the slit, 10 mm, did not constitute a limit to the size of the measurable chromatographic spot which, if of a diameter greater than 10 mm,

was examined in two stages, according to the diagrams of figures 1 and 2. In this case the total value of the measurement was calculated by adding up the areas of the peaks or, more simply, the numerical values proportional to the integral of the two partial areas into which the spot had been subdivided, furnished by the integrator.

Fig. 1 1 Zone of the chromatogram analyzed by the light ray; 2 Slit 3 Movement of the sample holder 4 Sample holder 5 Record 6 Indication of the integrator

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Fig. 2 (Legend for 1, 2, 3 as in Fig 1) 4 Graphic recording, Zone I; Zone II, 5 Indication of the integrator

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As an alternative of this procedure a single examination of the spot might be made, analyzing the central portion thereof, as described by Greig et al. (14). This faster and fairly reliable process, as observed by these authors, is, however, linked to a subjective appraisal of the zone of the spot to be selected for the measurement, and therefore may lead to inconsistant results. We therefore decided that it was best always to keep to the above indicated criterion in our measurements.

In the evaluation of the spot, the photodensitometric technique adopted for the determination of the antifermentatives takes account of its area as well as of the intensity of the coloration developed with a suitable reagent. These two quantities, while they are a function of the quantity of substance under examination, are influenced by several contingent factors, at equality of composition of the developing solvent, such as the degree of moisture of the thin layer, the degree of saturation of the atmosphere of the chromatographic chamber, the temperature, etc.; it follows that the execution of the calibration curve, for the purpose of establishing absolute values for the indications of the instrument, would be of little use.

Table 2 Trials of chromatic developing

Antifermentative	Chromogenic reagent			
	Ferric chloride 2%	Millon's reagent	Bromocresol green	Copper acetate	2-4-Dinitro-phenyl hydrazin
Solicylic acid	Violet spot *** (o)	Brown spot**	Yellow spot **	Yellow spot **	
Sorbic acid	Yellow spot*		Yellow Spot**	Blue spot***	Gray spot*** (o)
Dehydroacetic a.	Yellow spot*** (o)		Yellow spot**		
Cinnamic acid	Yellow spot labile*		Yellow spot*** (o)	Green spot*	
P-chlorobenzoic acid			Yellow spot ** (o)		
Benzoic acid	Pink spot*	Violet red spot *** (o)	Yellow spot **		
P-oxybenzoic	Yellow spot*	Red spot *** (o)	yellow spot**	Blue spot*	
Nipagin		Red spot *** (o)		Blue spot *	
Nipasol		Red spot *** (o)		Blue spot *	

Legend: * coloration little evident; ** coloration evident; *** coloration intense; (o) reaction to be preferred in the developing.

We preferred, therefore, to adopt a procedure of direct control by sample substances. In practice, the spot of the substance under examination was compared with that obtained by the chromatography of a known quantity of the same compound. Of it, in fact, a solution of known strength was prepared, of which, by means of a "Chemetron" microsyringe, an exactly measured quantity

was deposited on a 2.5 x 20 cm plate. Said plate was developed simultaneously with that containing the spot of the ether extract of the product under examination. We were thus assured of the uniformity of the greater part of the variables.

After development of the two spots obtained, the measurement in the Chromoscan was carried out, and from the direct ratio of the two values obtained the unknown value was obtained.

The total procedure may therefore be considered divided into two distinct parts, namely:

1) Execution of the qualitative test on a first sample of the product under examination, according to the known technique, in order to identify the nature of the antifermentative possibly present in the product;

2) Execution of a second chromatogram, on a second, exactly measured sample, intended for the quantitative determination. This second chromatograph is carried out, as stated before, on 2.5 x 20 cm plates.

The identification of the antifermentative, rendered possible by the first operation, will permit establishing which substance must be used for the comparison, and selecting the proper reagent for the development of the second chromatogram.

c) Limits of applicability and reproducibility of the method

Before applying the above described method for the determination of antifermentatives present in food preserves, it was desired to check its sensitivity, besides the limits of precision and reproducility. It was therefore applied repeatedly, using solutions of known strength of the various products studied, which were deposited on the plates in variable quantities, comparing the spots after chromatography. In this phase also different developers were tested, to find the most suitable ones for well defined and stable spots for each of the antifermentatives. The results of this experimentation are

reported in Table 2, with indication of the developers that proved most suitable for the purpose.

The sensitivity of the method, understood as minimum quantity of substance capable of giving, with a suitable chromogenic developer, a spot of such intensity and size as to furnish a measurable indication on the Chromoscan, proved satisfactory for all products examined. From the data listed in Table 3 it can be seen, in fact, that for some substances it has been possible to evaluate clearly 1 gamma of product deposited, and in any case the minimum limit was never greater than 10 gamma.

Table 3 Systematic error in series of determinations, made after chromatography, of the ether extracts of the antifermentatives obtained from measured volumes of solutions thereof of known strength.

Antifermentative	Min.quant. detectable (gamma)	Mean systematic error for quantities of substance comprised between	
		1-10 gamma	10-50 gamma
Sorbic acid	1	7.3%	6.5%
dehydroacetic acid	4	8.1%	7.0%
Cinamic acid	5	8.7%	8.1%
Salicylic acid	1	6.2%	5.1%
P-chlorobenzoic a.	10	-	8.4%
Benzoic acid	1	7.6%	6.8%
Poxybenzoic a.	1	6.9%	5.7%
Methyl p-oxybenzoate	2	7.2%	6.3%
Ethyl p-oxybenzoate	2	7.4%	6.2%
Propyl p-oxybenzoate	2	7.5%	6.1%
Butyl p-oxybenzoate	2	7.2%	6.2%

The maximum quantity of substance that can be used in the process is determined by the limits of applicability of thin layer chromatography. Anyway, well defined spots are still obtained with 100 gamma of substance and more.

As to the precision and reproducibility of the method, it has been observed that the best results are obtained when the quantity of sample substance, deposited on the comparison chromatogram, is of the same order of magnitude as that of the unknown sample under examination.

In practice, therefore, the procedure has been as follows: For the purposes of standardization of the operations, two intervals are considered, the first comprising quantities of unknown substance ranging from 1 to 10 gamma and the second for quantities between 10 and 50 gamma. In the first case the chromatographic spot is compared with that obtained by depositing on the comparison plate 2 gamma of the same substance, taken from a standard solution of known strength. The calculation of the unknown quantity x is then carried out on the basis of the proportion:

$$X : 2 = \text{alpha}_x : \text{alpha}$$

from which

$$x = \frac{2\ \text{alpha}_x}{\text{alpha}}$$

where alpha_x and alpha represent the area of the peak of the graph obtained on the Chromoscan or the respective numerical indication of the integrator, respectively for the unknown quantity and for the reference quantity.

Analogously, in the second case, the comparison is carried out with the spot obtained by depositing 20 gamma of substance, then calculating the unknown quantity z of the equation

$$z = \frac{20\ \text{alpha}_z}{\text{alpha}}$$

where alpha_z and alpha indicate the areas of the peaks respectively of the unknown and of the known quantities.

In Table 3 are listed the results obtained in the experimentation conducted according to the above stated criteria.

d) Application to some practical cases.

The applicati on of the process to some practical cases has confirmed the results obtained in the above experimentation, bringing out the flexibility of the method which has proved valuable in research on antifermentatives in food products of various kinds.

There were examined, in fact, a wine, a can of tomatoes, and a non-alcoholic beverage. To these products, in which preliminary tests had ascertained the absence of antifermentatives, were added exactly weighed quantities of the preservatives under examination. Leaving the chromatographic operations described before unchanged in all cases, the extractive techniques were adapted, from case to case, to the particular nature of the food product under examination.

1) Testing for antifermentatives added to a wine. 50 cc of wine admixed with 0.01 mg of nipagin were subjected to defecation by addition of 0.5 g of $CaCO_3$ and 1 cc of Carrez's reagent (mixture of equal volumes of potassium ferrocyanide solution 15% and zinc acetate 30%) stirring well. This is filtered after standing for half an hour, then the precipitate is carefully washed, collecting the filtrate and the wash waters in a 100-cc graduated flask. On completed washing and filling up, one takes 2 cc of the solution obtained, which are charged in a 10-cc cylindrical separating funnel, adding a few drops of HCl at 20% and extracting three times with ether (2 cc each time). The other phases, collected in a small crystallizer, are allowed to evaporate completely, then taking up the residue with a drop of NaOH 10%, being careful to wash with said drop the entire bottom of the vessel and its walls to make sure of the salification and dissolution of the entire antifermentative extracted. The drop of solution thus obtained is deposited on the chromatographic plate with the aid of a capillary, speeding up the

evaporation of the water with a stream of hot air.

Thereafter washing is repeated two or three times with one drop of water at a time, depositing also the wash waters and always taking care to keep the spot as small as possible. Then the chromatographic developing is carried out with the solvent indicated in Table 4.

Table 4 Determination of methyl para-oxybenzoate added to a red wine in the proportions of 0.2 mg per liter

Test no.	Quant. of wine subjected to extraction (cc)	Quantity of antifermentative		Error %
		Theory (gamma)	Found (gamma)	
1	5	1	0.89	10.5
2	10	2	1.84	8.1
3	20	4	3.70	7.5
4	50	10	9.31	6.9

The development with Millon's reagent (1 cc of mercury dissolved in 2 cc of concentrated HNO_3 and diluted with 4 cc of water) is carried out according to the technique proposed by Durant (15), modified in the sense that the plate, sprayed with reagent until it is well wetted, is then dried in an oven at 105°C for 5 minutes. This intense heating is made possible by the inertia of the thin layer toward nitric acid, as compared with the filter paper employed by Durant.

After cooling, the chromatogram is again sprayed moderately with the same reagent; in this phase the spots appear at once clearly, with red-violet coloration on white ground.

In Table 4 are reported the results of tests of this type with indication of the experimental errors found.

2) Testing for antifermentatives added to a non-alcoholic beverage. A commercial non-alcoholic beverage was added with 100 mg/l of benzoic acid.

On aliquots of this mixture the benzoic acid was determined, either by depositing directly the solution as such, or by preliminarily extracting the antifermentative with ether, after acidification with dilute HCl and then depositing the ether extract as indicated for the wine. From the results reported in Table 5 we found it preferable to deposit the solution directly, as we observed no interferènce from the substances contained in the beverage on the chromatographic separation of the benzoic acid.

Table 5 Determinations of benzoic acid, added to a non-alcoholic beverage in the proportions of 100 mg/liter

	Test No.	Volume of beverage subjected to extraction or deposited directly (Cc)	Quantity of antifermentative Theory (gamma)	Found (gamma)	Error %
Det. made by depositing the ether extract of the drink	1	0.1	10	9.29	7.1
	2	0.5	50	4.67	6.6
	3	1.0	100	94.30	5.7
Detn.made by depositing the drink as such directly	1	0.1	10	9.31	6.9
	2	0.5	50	46.95	6.1
	3	1.0	100	94.90	5.1

3) Testing for antifermentatives added to a can of tomatoes. There was used a concentrated preserve known to be free of antifermentatives. To a weighed quantity thereof solicylic acid was added so as to obtain a concentration equal to 0.1% by weight. Then, exactly weighed quantities of the preserve thus treated were taken, which were added with NaOh at 10%(5 cc/g) and left to digest for several hours.

They were then filtered and the residue was washed with distilled water, collecting filtrate and wash waters in graduated flasks. Then, after acidification, extraction with ether was carried out, as already indicated in the case of the wine.

In order to avoid the losses deriving from the operations of extraction with ether, some tests were carried out also by depositing directly on the plate the alkaline solution obtained in the lixiviation of the canned product. The results, shown in Table 6, were decidedly satisfactory, as no interference was noted in the separation and definition of the salicylic acid spot from the substances present in solution.

Table 6 Determination of salicylic acid added to a can of tomatoes in the proportions of 0.1% by weight.

	Quantity of antifermentative			
	Test No.	Theory (gamma)	Found (gamma)	Error %
Detn. made by depositing the ether extract of the waters from alkaline lixiviation of the canned product	1	20	18.74	6.3
	2	50	46.95	6.1
	3	75	71.47	4.7
Detn. made by depositing the alkaline lixiviation waters directly	1	20	18.82	5.9
	2	50	47.25	5.5
	3	75	71.85	4.2

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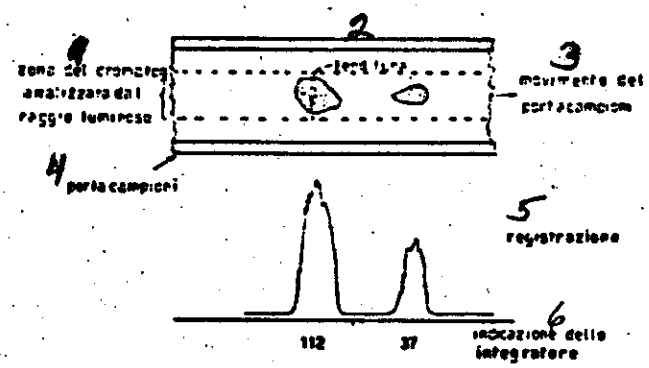
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112
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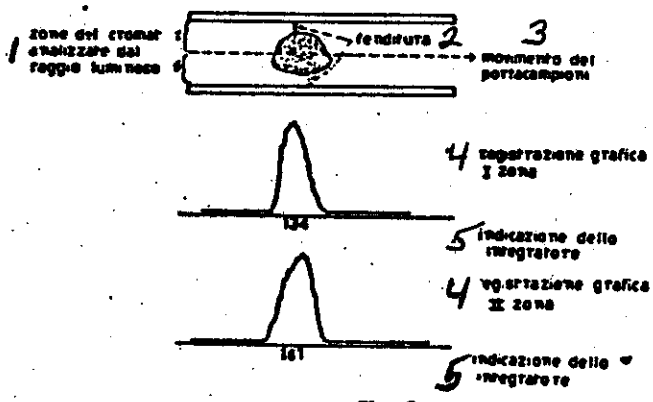
Fig. 1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134
5
indicazione dello
integratore
4
registrazione grafica
II zona
161
5
indicazione dello
integratore

Fig. 2

Sodium Sorbate 88

Applicazione della cromatografia su strato sottile alla ricerca degli antifermentativi negli alimenti

Nota III - Determinazione quantitativa

M. COVELLO - O. SCHETTINO

Istituto di Chimica Farmaceutica e Tossicologica dell'Università di Napoli

Applicazione della cromatografia su strato sottile alla ricerca degli antifermentativi negli alimenti. Nota III - Determinazione quantitativa.

Nel quadro del piano di ricerche che si va svolgendo nel nostro Istituto sul tema degli additivi alimentari, si è ritenuto opportuno estendere le ricerche nel settore dei conservativi, da noi iniziate con lo studio puramente qualitativo di un certo numero di antifermentativi, allo scopo di conferire una finalità quantitativa al procedimento analitico.

Si è adottata, a tal fine, una tecnica fotodensitometrica atta ad eseguire misure quantitative mediante la diretta valutazione dell'area e della intensità delle macchie ottenute mediante cromatografia su strato sottile delle sostanze in esame.

I risultati ottenuti applicando ad una serie di casi pratici il procedimento proposto hanno dimostrato una buona riproducibilità dei valori analitici, con errori sistematici di entità non rilevante.

Queste caratteristiche, integrate dalla semplicità operativa e dalla rapidità di esecuzione, rendono la tecnica proposta particolarmente adatta al lavoro di «routine» di un laboratorio di controllo dei prodotti alimentari.

Application de la chromatographie sur couche mince à la recherches des antifermentatifs dans les produits alimentaires. Note III. Détermination quantitative.

Dans le cadre du programme de recerche concernant les additifs alimentaires qu'on est en train de réaliser dans notre Institut, on a jugé nécessaire d'étendre les recherches dans le secteur des conservatifs, recherches que nous avion commencées en étudiant au point de vue purement qualitatif un certain nombre d'antifermentatifs. Le but de cette nouvelle étude est celui de conférer un caractère quantitatif au procédé analytique.

A cet effet on a employé une technique photodensitométrique permettant de faire des mesures quantitatives par l'évaluations directe de l'aire ainsi que de l'intensité des tâches obtenues à l'aide de la chromatoraphie sur couche mince des substances soumises à examen.

Les résultats obtenus dans une série de cas pratiques, employant le procéssus proposé, ont démontré une reproductibilité satisfaisante des valeurs analytiques avec des erreurs systématique peu importantes.

Grâce à ces caractéristiques complétées par la simplicité opérative ainsi que par la rapidité de l'exécutions, la tecnique proposée est particulièrement convenable pour le travail de «routine» dans le laboratoires de contrôle des produits alimentaires.

Application of TLC to the search of antifermentatives in food products - Note III: quantitative determination.

In the research program which is being carried out at our Institute on the subject of food additives, it has been judged opportune to extend the investigation to the preservatives field, which has been started by us with the purely qualitative study of a certain number of antifermentation products, with the intent of imparting a quantitative scope to the analytical procedure.

A photodensimetric technique has been adopted to this purpose, suitable to perform quantitative measurements through direct evaluation of the area and intensity of spots obtained by TLC of the substances being examined.

The results attained by applying the suggested procedure to a number of practical cases, have demonstrated a good reproducibility of the analytical data, with almost negligible systematic errors.

These features in addition to the operating facility and rapidity of execution, make the proposed tecnique particularly suitable for «routine» work in a control laboratory for food products.

Ueber die Anwendung der Dünnschicht-Chromatographie bei Untersuchungen der Antifermente in Lebensmitteln. III Bericht: quantitative Bestimmung.

Im Rahmen des Forschungsprogramms, das in unserem Instiut zum Thema der Lebensmittelzusätze durchgeführt wird, wurde es für zwechmäßig angeschen, die Forschungen auf das Gebiet der Konservierungsmittel auszudehnen, auf dem wir bereits rein qualitative Ansätze über eine gewisse Anzahl von Antifermenten vorliegen hatten, um der Analysen methode einen qualitativen Zweck zu geben. Um dies zu erreichen wurde eine photodensitometrische Verfahrensweise befolgt, die quantitative Messungen durch die direkte Bewertung von Fläche und Stärke der bei der Dünnschicht-Chromatographie der zu prüfenden Substanzen erhaltenen Flecken ermöglicht. Die bei der proktischen Anwendung der vorgeschlagenen Arbeitsweise erhalenen Ergebnisse haben eine gute Reproduzierbarkeit der analytischen Werte bewiesen, wohei die systematischen Fehler von unbedentender Größeordnung waren. Diese Eigenschaften, ergänzt durch die einfache Arbeitsweise.

Nell'intraprendere uno studio avente per oggetto la ricerca degli antifermentativi negli alimenti conservati (1), ponevamo in rilievo le difficoltà che si oppongono all'analista in conseguenza della varietà di prodotti del genere entrati nell'uso comune o, eventualmente, di nuovo impiego in questo campo (2). Tali difficoltà, si osservava, risultano aggravate in assenza di leggi sufficientemente esplicite in proposito. Tutto ciò risulta evidente se si tiene conto di alcune considerazioni di carattere generale.

La ricerca degli antifermentativi o, più in generale, degli additivi aggiunti agli alimenti conservati, può essere condotta orientando l'indagine verso due distinte finalità:

1) accertamento puramente qualitativo della presenza dell'additivo e sua identificazione;
2) determinazione quantitativa dell'additivo individuato.

Ci si può limitare ad un'indagine del primo tipo qualora l'uso dell'additivo, la cui presenza viene riscontrata nell'alimento, sia espressamente vietato dalla legge. E' invece indispensabile ricorrere alla de-

TABELLA 1

Alimenti per cui è previsto l'impiego di antifermentativi sublimabili nel D.M. 19 gennaio 1963 - G.U. n. 64 del 7 marzo 1963 e relative dosi minime e titoli. Valori cromatografici riferiti al solvente alcool isopropilico-alcool etilico NH_3 conc.-acqua (5 : 2 : 0,5 : 1)

Prodotto alimentare	Antifermentativo consentito	Dose massima	Titolo	R_f
Grassi ed oli (escluso l'olio di oliva)	Acido sorbico e suoi sali di Na, K e Ca.	500 mg/Kg	98-99%	0,77
Formaggi	Acido sorbico e suoi sali di Na, K e Ca.	1000 mg/Kg	98-99%	0,77
Ripieno di paste alimentari	Acido sorbico e suoi sali di Na, K e Ca.	1000 mg/Kg	98-99%	0,77
Semiconserve ittiche . . .	Acido sorbico e suoi sali di Na, K e Ca.	1000 mg/Kg	98-99%	0,77
	Acido benzoico e suoi sali di Na, K e Ca.	1500 mg/Kg	74-99,5%	0,65
	Etile p.ossi benzoato.	1000 mg/Kg	99,5%	0,76
	Propile p.ossi benzoato.	1000 mg/Kg	99,5%	0,74
Maionese	Acido sorbico e suoi sali di Na, K e Ca.	1000 mg/Kg	98-99%	0,77
	Etile p.ossi benzoato.	1000 mg/Kg (sul grasso)	99,5%	0,76
	Propile p.ossi benzoato.	1000 mg/Kg (sul grasso)	99,5%	0,74
Prodotti dolciari	Acido sorbico e suoi sali di Na, K e Ca.	2000 mg/Kg	98-99%	0,77
Pane in cassetta	Acido sorbico e suoi sali di Na, K e Ca.	2000 mg/Kg	98-99%	0,77
Frutta secca	Acido sorbico e suoi sali di Na, K e Ca.	1000 mg/Kg	98-99%	0,77
Bibite analcoliche	Acido benzoico e suoi sali di Na, K e Ca.	500 mg/litro	74-99,5%	0,65
	Etile p.ossi benzoato.	500 mg/litro	99,5%	0,76
	Propile p.ossi benzoato.	500 mg/litro	99,5%	0,74
Vini amabili sfusi	Etile p.ossi benzoato.	250 mg/litro	99,5%	0,76
	Propile p.ossi benzoato.	250 mg/litro	99,5%	0,74

terminazione quantitativa nel caso che il suo uso sia consentito entro limiti determinati.

L'esistenza di una chiara regolamentazione relativa all'impiego degli additivi rappresenta, pertanto, la migliore guida atta a fornire all'analista un criterio di valutazione circa le tecniche analitiche da impiegare caso per caso.

In effetti già da tempo, in Italia, veniva da più parti segnalata la necessità di poter disporre sulla falsariga di quanto già da tempo realizzato in altri paesi, di un elenco positivo degli additivi impiegabili ai fini dell'allestimento e della conservazione delle riserve alimentari, sia ad opera della grande industria conserviera che dei produttori a carattere artigianale. Tale esigenza è stata fatta propria dal legislatore che, con un recente decreto (3), affrontava il problema determinando quali sostanze chimiche potessero essere impiegate nella preparazione di prodotti alimentari. Lo stesso decreto ne fissava i casi e le dosi massime di impiego e ne stabiliva le caratteristiche fisico-chimiche ed i requisiti di purezza.

Alcuni antifermentativi, costituiti da sostanze sublimabili, sono stati oggetto delle nostre ricerche precedentemente menzionate. Essi rientrano, per l'appunto, tra quelli il cui uso è consentito in base alle nuove disposizioni (vedi Tab. 1).

Nella presente nota riferiamo sugli ulteriori studi condotti nel settore della ricerca dei conservativi negli alimenti, aventi lo scopo di conferire una finalità quantitativa al procedimento analitico del quale in precedenza avevamo esaminato il solo aspetto qualitativo (1, 2).

La tecnica di determinazione quantitativa che qui si propone è stata sperimentata su tutti gli antifermentativi sublimabili già studiati. Peraltro, alla luce delle considerazioni che precedono, ci è sembrato opportuno polarizzare la nostra attenzione sui prodotti di cui la legge permette l'impiego, e ciò specialmente in fase di applicazione del metodo a dei casi pratici.

PARTE SPERIMENTALE

La finalità da noi perseguita nella tecnica analitica qualitativa, proposta ed illustrata nelle precedenti note, era quella della semplicità e della rapidità operativa, caratteristiche di evidente utilità in analisi volte al controllo di prodotti commerciali. Tale finalità ci ha spinto, in sede di applicazione quantitativa del procedimento, a scartare metodologie eccessivamente complesse per tentare, invece, la utilizzazione di una rapida tecnica fotodensitometrica. Già nella nostra nota preliminare si escludeva la possibilità di ottenere risultati quantitativi esattamente riproducibili utilizzando i sublimati cristallini ottenibili nella rivelazione degli antifermentativi secondo la tecnica ivi proposta.

Ripetute prove ci hanno dimostrato, infatti, che tale sublimato rappresenta soltanto un'aliquota, sia pure importante, del prodotto cromatografato, né d'altra parte si è riusciti ad inquadrare in un regolare e riproducibile rapporto numerico tale frazione. Si prospettò, perciò, l'opportunità di eseguire una doppia cromatografia per ciascun campione, destinando un primo cromatogramma alla rivelazione qualitativa dell'antifermentativo ed il secondo ad una sua eventuale determinazione quantitativa.

La letteratura esistente nel campo della ricerca degli antifermentativi (4-13) permette di scegliere tra numerosi procedimenti, applicabili alle piccole quan-

tità di sostanza messe in gioco nella cromatografia. Essi implicano, in genere, l'eluizione quantitativa del prodotto dal cromatogramma e la sua valutazione con metodologie che si avvalgono prevalentemente della analisi spettrofotometrica. Tali tecniche, indubbiamente efficaci e suscettibili di dare risultati abbastanza precisi, presentano l'inconveniente di essere laboriose e delicate e, in definitiva, di richiedere un tempo apprezzabile per l'attuazione.

Si è perciò ritenuto opportuno evitare ogni lunga manipolazione del cromatogramma, valutando direttamente l'entità della macchia ottenuta mediante opportuna rivelazione della sostanza in esame. Ci siamo serviti, a questo scopo, di un fotodensitometro integratore a registrazione (il Chromoscan Joyce). Ai risultati di questa sperimentazione, esposti dettagliatamente più sotto, si fa precedere un breve riesame della parte cromatografica, allo scopo di chiarire meglio le varie fasi del procedimento.

a) - *Separazione cromatografica dell'antifermentativo.*

Ai fini dell'identificazione si è impiegata (vedi per i particolari le note citate) la tecnica cromatografica su strato sottile di gel silice G Merck, dello spessore di 250 μ.

Come supporto rigido nella preparazione di strati sottili da destinare alla rivelazione per sublimazione si sono impiegate sempre lastre metalliche, di formato cm. 5 x 20 o cm. 20 x 20. Per la determinazione quantitativa, invece, si sono dovute impiegare lastrine, di metallo cromato o di vetro, di cm. 2,5 x 20 onde poterle introdurre nella slitta del densitometro. Tali lastrine venivano utilizzate per una sola deposizione di sostanza.

La soluzione in esame, ottenuta per estrazione con etere dal prodotto alimentare, veniva deposta in forma di macchie tondeggianti a 2 cm. dal margine delle suddette lastre, ponendo la massima cura nel mantenere entro limiti minimi il diametro delle macchie stesse (~ 2 mm). E' preferibile, infatti, per la successiva misura densitometrica, che l'estensione delle macchie dopo la cromatografia, risulti piuttosto limitata. Inoltre, ai fini della riproducibilità e della confrontabilità, è opportuno che le deposizioni coprano delle aree per quanto possibile equivalenti.

La cromatografia, come già detto sopra, va eseguita su un duplice campione ed è consigliabile, a questo scopo, eseguire due estrazioni distinte su due campioni del prodotto alimentare esattamente misurati, anzichè operare una suddivisione dell'estratto ottenuto da un unico campione.

b) - *Determinazione quantitativa.*

Nell'esecuzione delle misure si è impiegato il fotodensitometro a registrazione e ad integrazione automatica «Chromoscan Joyce» fissando i seguenti parametri operativi:

Rapporto di movimento - registrazione/campione 1:1

Filtri	standard
Fenditura	mm 10 x 0.5
Camma	N. 5-034/A

Il portacampioni standard montato su questo strumento è costituito da un telaio rettangolare di cm 2,7 x 21, recante una finestra di cm 1,9 x 20,4.

Queste dimensioni, come si è detto, ci hanno imposto l'uso di lastrine di larghezza limitata a circa cm. 2.5 per l'esecuzione dei cromatogrammi.

La lunghezza della fenditura, 10 mm, non ha costituito un limite all'estensione della macchia cromatografica misurabile che, se di diametro superiore ai 10 mm, veniva esaminata in due riprese, secondo gli schemi di figg. 1 e 2. In questo caso il valore totale

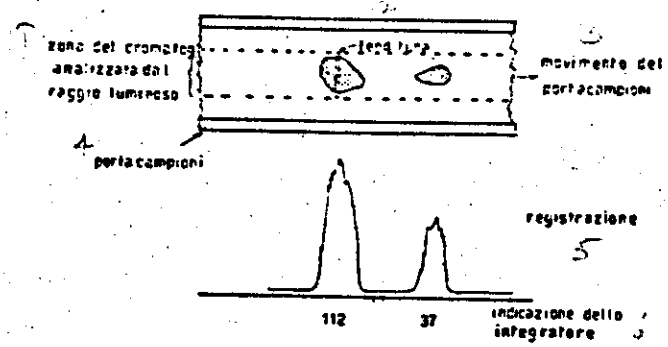

Fig. 1

della misura veniva calcolato sommando le aree dei picchi o, più semplicemente, i valori numerici proporzionali all'integrale delle due aree parziali in cui la macchia era stata suddivisa, forniti dall'integratore.

Come alternativa a tale procedimento si potrebbe eseguire un unico esame della macchia, analizzandone la parte centrale, come prospettato da Greig ed altri (14). Questa procedura, più rapida ed abbastanza attendibile, come osservato dai citati AA., è però legata ad un apprezzamento soggettivo della zona della macchia da prescegliere per la misura, e conseguentemente può condurre a risultati discordanti. Ci è sembrato opportuno, perciò, attenerci sempre nelle nostre misure al criterio precedentemente indicato.

La tecnica fotodensitometrica adottata per la determinazione degli antifermentativi tiene conto, nella valutazione della macchia, sia della sua area che dell'intensità della colorazione sviluppata con adatto reattivo. Queste due grandezze, pur essendo funzione della quantità di sostanza in esame, vengono influenzate

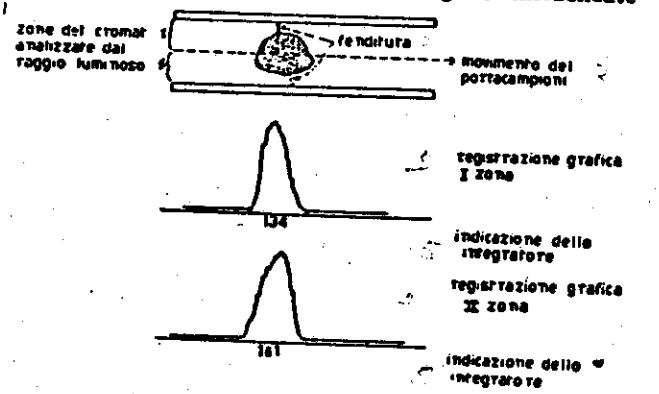

Fig. 2

da diversi fattori contingenti, a parità di composizione del solvente di sviluppo, quali il grado di umidità dello strato sottile, il grado di saturazione dell'atmosfera della camera cromatografica, la temperatura, ecc.; ne consegue che la esecuzione della curva di taratura, al fine di stabilire dei valori assoluti in corrispondenza delle indicazioni dello strumento, presenterebbe scarsa utilità.

TABELLA 2

Saggi di rivelazione cromatica.

Antifermentativo	Reattivo cromogeno				
	Cloruro ferrico al 2%	Reattivo di Millon	Verde di bromocresolo	Acetato di rame	2-4-Dinitrofenilidrazina
Acido salicilico	Macchia violetta *** (*)	Macchia bruna **	Macchia gialla **	Macchia gialla **	
Acido sorbico	Macchia gialla *		Macchia gialla **	Macchia azzurra ***	Macchia grigia *** (*)
Acido deidroacetico	Macchia gialla *** (*)		Macchia gialla **		
Acido cinnamico	Macchia gialla labile *		Macchia gialla *** (*)	Macchia verde *	
Acido p.clorobenzoico			Macchia gialla ** (*)		
Acido benzoico	Macchia rosa *	Macchia rosso violacea *** (*)	Macchia gialla **		
Acido p.ossibenzoico	Macchia gialla *	Macchia rossa *** (*)	Macchia gialla **	Macchia azzurra *	
Nipagina		Macchia rossa *** (*)		Macchia azzurra *	
Nipasolo		Macchia rossa *** (*)		Macchia azzurra *	

Leggenda: * colorazione poco evidente; ** colorazione evidente; *** colorazione intensa; (*) reazione da preferirsi nella rivelazione.

Si è preferito, perciò, adottare un procedimento di controllo diretto mediante sostanze campione. In pratica, la macchia della sostanza in esame veniva confrontata con quella ottenuta mediante la cromatografia di una quantità nota del medesimo composto. Di questo, infatti, si preparava una soluzione a titolo noto della quale si deponeva, mediante microsiringa «Chemetron», una quantità esattamente misurata su una lastrina di cm 2,5 x 20. Detta lastrina veniva sviluppata contemporaneamente a quella contenente la macchia dell'estratto etereo del prodotto in esame. In tal modo ci si garantiva l'uniformità della maggior parte delle variabili.

Dopo rivelazione delle due macchie ottenute si eseguiva la misura al Chromoscan e, dal rapporto diretto dei due valori ottenuti, si risaliva al valore incognito.

Pertanto la schematica complessiva del metodo si può considerare divisa in due parti distinte e precisamente:

1) esecuzione dell'indagine qualitativa su un primo campione del prodotto in esame, secondo la tecnica già nota, al fine di identificare la natura dell'antifermentativo eventualmente presente nel prodotto;
2) esecuzione di un secondo cromatogramma, su un secondo campione esattamente misurato, da destinare alla determinazione quantitativa. Questa seconda cromatografia va eseguita, come già detto, su lastrine di cm 2,5 x 20.

L'identificazione dell'antifermentativo, resa possibile dalla prima operazione, permetterà di stabilire quale sostanza debba essere impiegata per il confronto, e di scegliere il reattivo opportuno per la rivelazione del secondo cromatogramma.

c) - *Limiti di applicabilità e di riproducibilità del metodo.*

Prima di applicare il metodo suddescritto per la determinazione di antifermentativi presenti in conserve alimentari, si è voluto controllarne la sensibilità, oltre i limiti di precisione e di riproducibilità. Lo si è pertanto applicato ripetutamente impiegando soluzioni a titolo noto dei vari prodotti studiati, che venivano deposti sulle lastrine in quantità variabili, confrontando fra di loro le macchie dopo cromatografia. In questa fase si sono sperimentati anche diversi rivelatori, ricercando i più adatti a dare macchie ben definite e stabili per ciascuno degli antifermentativi. Nella tabella 2 si riportano i risultati di detta speri-

mentazione, con l'indicazione dei rivelatori dimostratisi più adatti all'impiego.

La sensibilità del metodo, intesa come quantità minima di sostanza capace di dare, con opportuno rivelatore cromogeno, una macchia di intensità e grandezza tali da fornire una indicazione misurabile al Chromoscan è risultata soddisfacente per tutti i prodotti esaminati. Si può osservare, infatti, dai dati elencati in Tabella 3 che per alcune sostanze è stato

TABELLA 3

Errore sistematico in serie di determinazioni, eseguite dopo cromatografia, degli estratti eterei degli antifermentativi ottenuti da volumi misurati di soluzioni a titolo noto degli stessi.

Antifermentativo	Quant. minima rivelab. (γ)	Errore sistematico medio per quant. di sostanza comprese fra	
		1-10 γ	10-50 γ
Acido sorbico	1	7,3%	6,5%
Acido deidroacetico	4	8,1%	7,0%
Acido cinnamico	5	8,7%	8,1%
Acido salicilico	1	6,2%	5,1%
Acido p.clorobenzoico	10	—	8,4 %
Acido benzoico	1	7,6%	6,8%
Acido p.ossibenzoico	1	6,9%	5,7%
Metile p.ossibenzoato	2	7,2%	6,3%
Etile p.ossibenzoato	2	7,4%	6,2%
Propile p.ossibenzoato	2	7,0%	6,1%
Butile p.ossibenzoato	2	7,2%	6,2%

possibile valutare nettamente 1 γ di prodotto deposto, ed in ogni caso il limite minimo non è risultato mai superiore ai 10 γ.

La quantità massima di sostanza impiegabile nel procedimento trova i suoi limiti nei limiti stessi di applicabilità della cromatografia su strato sottile. Si ottengono, comunque, ancora macchie ben definite con 100 γ di sostanza ed oltre.

Per quanto riguarda la precisione e la riproducibilità del metodo, si è osservato che i risultati migliori si ottengono quando la quantità di sostanza campione, deposta sul cromatogramma di confronto, è dello stesso ordine di grandezza di quella del campione incognito in esame.

In pratica, pertanto, si è operato come segue: ai fini della standardizzazione delle operazioni, si considerano due intervalli, il primo comprendente quantità di sostanza incognita che vanno da 1 a 10 γ ed il secondo per quantità comprese fra 10 e 50 γ. Nel primo caso la macchia cromatografica viene confrontata con quella ottenuta deponendo sulla lastrina di confronto 2 γ della medesima sostanza, prelevati da una soluzione standard a titolo noto. Il calcolo della quantità incognita x viene quindi eseguita in base alla proporzione:

$$X : 2 = \alpha_x : \alpha$$

da cui

$$x = \frac{2\alpha_x}{\alpha}$$

in cui α_x ed α rappresentano l'area del picco del grafico ottenuto al Chromoscan o la relativa indicazione numerica dell'integratore, rispettivamente per la quantità incognita e per quella di riferimento.

Analogamente nel secondo caso, il confronto viene eseguito con la macchia ottenuta deponendo 20 γ di sostanza, calcolando poi la quantità incognita z della relazione

$$z = \frac{20\,\alpha_z}{\alpha}$$

in cui α_z ed α indicano le aree dei picchi rispettivamente della quantità incognita e di quella nota.

Nella Tabella 3 sono elencati i risultati ottenuti nella sperimentazione condotta secondo i criteri sopra indicati.

d) - *Applicazione ad alcuni casi pratici.*

L'applicazione del procedimento ad alcuni casi pratici ha confermato i risultati ottenuti nella sperimentazione su riferita, ponendo in rilievo l'elasticità del metodo stesso che è risultato valido nella ricerca degli antifermentativi in prodotti alimentari di vario genere.

Sono stati presi in esame, infatti, un vino, una conserva di pomodoro ed una bibita analcolica. A tali prodotti, in cui saggi preliminari avevano accertata l'assenza di antifermentativi, venivano aggiunte quantità esattamente pesate dei conservanti in esame. Ferme restando, in tutti i casi, le operazioni cromatografiche già descritte, si sono adattate, caso per caso, le tecniche estrattive secondo la natura particolare del prodotto alimentare in esame.

1) *Ricerca di antifermentativi aggiunti ad un vino.* 50 cc. di vino addizionati di mg 0,01 di nipagina si sottopongono a defecazione mediante aggiunta di 0,5 g di $CaCO_3$ e di 1 cc di reattivo di Carrez (miscela di volumi eguali di soluzione di ferrocianuro potassico al 15% e di acetato di zinco al 30%) agitando bene. Dopo mezz'ora di riposo si filtra, lavando poi accuratamente il precipitato e raccogliendo il filtrato e le acque di lavaggio in palloncino tarato da 100 cc. Ultimato il lavaggio si porta a volume e si prelevano 2 cc della soluzione ottenuta, che si portano in imbuto separatore cilindrico da 10 cc, si acidifica con poche gocce di HCl al 20% e si estrae per tre volte con etere (2 cc per volta). Le fasi eteree, riunite in un piccolo cristallizzatore, vengono lasciate evaporare completamente, riprendendo poi il residuo con una goccia di NaOH al 10% ponendo cura di lavare con detta goccia tutto il fondo del recipiente e le sue pareti in modo da assicurarsi della salificazione e soluzione di tutto l'antifermentativo estratto. La gocciolina di soluzione così ottenuta si depone sulla lastrina cromatografica con l'ausilio di un capillare, accelerando l'evaporazione dell'acqua con una corrente di aria calda.

Successivamente si lava ancora per due o tre volte con una goccia di acqua alla volta, deponendo anche le acque di lavaggio e ponendo sempre cura nel mantenere la macchia nelle dimensioni minime possibili. Indi si esegue lo sviluppo cromatografico con il solvente indicato in Tabella 4.

La rivelazione con reattivo di Millon (1 cc di mercurio disciolto in 2 cc di HNO_3 conc. e diluito con 4 cc di acqua) viene eseguita secondo la tecnica proposta da Durant (15), modificata nel senso che la lastra, spruzzata col reattivo fino a buona umidificazione, viene poi asciugata in stufa a 105°C per 5 minuti.

TABELLA 4

Determinazione di metile-paraossibenzoato aggiunto ad un vino rosso nelle proporzioni di mg 0,2/litro.

Prova N.	Quant. di vino sottoposta ad estrazione (cc)	Quantità di antifermentativo		Errore %
		Teorica (γ)	Trovata (γ)	
1	5	1	0,89	10,5
2	10	2	1,84	8,1
3	20	4	3,70	7,5
4	50	10	9,31	6,9

Questo intenso riscaldamento è reso possibile dalla inerzia dello strato sottile nei confronti dell'acido nitrico, rispetto alla carta da filtro impiegata dal Durant.

Dopo raffreddamento il cromatogramma viene di nuovo spruzzato moderatamente con lo stesso reattivo; in questa fase le macchie si rivelano subito nettamente, con colorazione rosso-violacea su fondo bianco.

Nella Tabella 4 sono riportati i risultati di prove di questo tipo con l'indicazione degli errori sperimentali riscontrati.

2) *Ricerca di antifermentativi aggiunti ad una bibita analcolica.* Una bibita analcolica del commercio è stata addizionata di 100 mg/l di acido benzoico. Su aliquote di tale miscela si è proceduto alla determinazione dell'acido benzoico, sia deponendo direttamente la soluzione tal quale, che eseguendo preliminarmente l'estrazione dell'antifermentativo con etere, dopo acidificazione con HCl dil. e deponendo successivamente l'estratto etereo come indicato per il vino. I risultati riportati nella Tabella 5 ci hanno indotto a considerare preferibile la diretta deposizione della

TABELLA 5

Determinazioni di acido benzoico, aggiunto ad una bibita analcolica nelle proporzioni di mg 100/litro.

	Prova N.	Volume della bibita sottop. ad estrazione o deposto dirett. (cc)	Quantità di antifermentativo		Errore %
			Teorica (γ)	Trovata (γ)	
Det. eseguite deponendo l'estratto etereo della bibita	1	0,1	10	9,29	7,1
	2	0,5	50	4,67	6,6
	3	1,0	100	94,30	5,7
Det. eseguite deponendo direttamente la bibita tal quale	1	0,1	10	9,31	6,9
	2	0,5	50	46,95	6,1
	3	1,0	100	94,90	5,1

soluzione, non essendosi osservata alcuna interferenza da parte delle sostanze contenute nella bibita sulla separazione cromatografica dell'acido benzoico.

3) *Ricerca di antifermentativi aggiunti ad una conserva di pomodoro.* Si è impiegata una conserva concentrata sicuramente esente da antifermentativi. Ad una quantità pesata di questa si è aggiunto dell'acido salicilico, in modo da ottenere una concentrazione pari allo 0,1% in peso. Si sono, poi, prelevati quantitativi esattamente pesati della conserva così trattata, che sono stati addizionati di NaOH al 10% (cc 5/g) e lasciati digerire per qualche ora.

Trascorso tale tempo si è filtrato e si è lavato con acqua distillata il residuo, raccogliendo filtrato ed acque di lavaggio in palloncini tarati. Indi si è proceduto all'estrazione con etere, previa acidificazione, come già indicato nel caso del vino.

Allo scopo di evitare le perdite derivanti dalle operazioni di estrazione con etere, sono state eseguite anche alcune prove deponendo sulla lastra direttamente la soluzione alcalina ottenuta nella lisciviazione della conserva. I risultati, esposti nella Tab. 6,

TABELLA 6

Determinazioni di acido salicilico aggiunto ad una conserva di pomodoro nelle proporzioni dello 0,1% in peso.

	Prova N.	antifermentativo Quantità di		Errore %
		Teorica (γ)	Trovata (γ)	
Det. eseguite deponendo l'estratto etereo delle acque provenienti dalla lisciviazione alcalina della conserva.	1	20	18,74	6,3
	2	50	46,95	6,1
	3	75	71,47	4,7
Det. eseguite deponendo direttamente le acque alcaline di lisciviazione.	1	20	18,82	5,9
	2	50	47,25	5,5
	3	75	71,85	4,2

sono stati decisamente soddisfacenti, non essendosi notata alcuna interferenza nella separazione e definizione della macchia dell'acido salicilico ad opera delle sostanze presenti in soluzione.

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Gli impianti italiani per l'estrazione dell'olio dalle olive (*) - II

Sistemi tradizionali e sistemi nuovi (**)

C. CAROLA - E. FEDELI - G. JACINI

Stazione sperimentale oli e grassi, Milano - Centro nazionale per la lipochimica del C.N.R. - Milano

A5) Pressatura

Nonostante l'introduzione di recenti tecniche di disoleazione (impianti Corteggiani, Primolia, Sinolea, Elettrolia) nelle quali si fa a meno — totalmente o parzialmente — della fase di pressatura, non si prevede una scomparsa a breve scadenza delle presse discontinue, idonee ad essere impiegate anche nei piccoli e medi oleifici, numerosi in Italia.

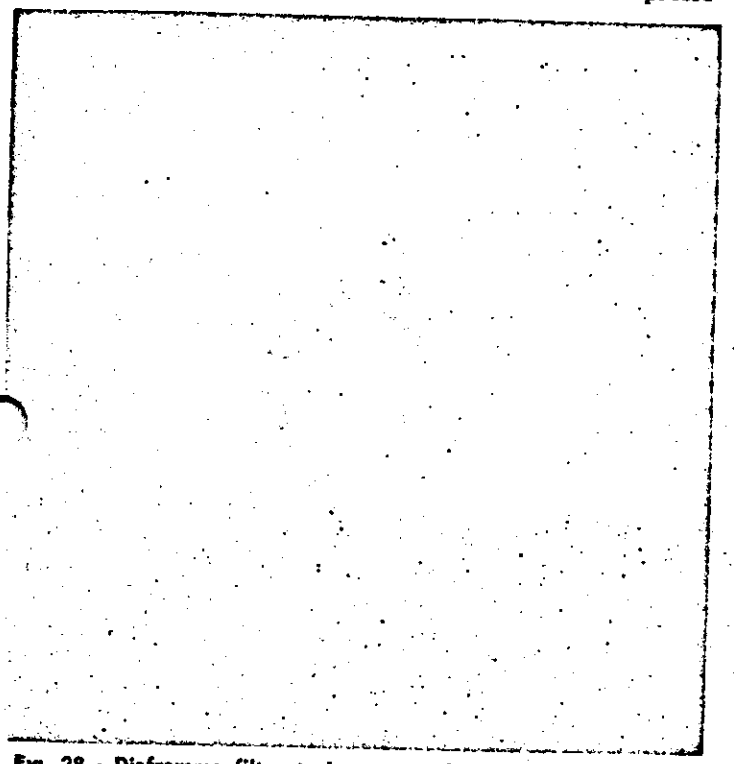

Fig. 28 - Diaframma filtrante in tessuto di cocco della SIDOP.

Nella descrizione degli impianti per pressatura, si reputa opportuno considerare successivamente:

a) i diaframmi
b) i sistemi di caricamento delle presse
c) le pompe idrauliche
d) le presse
e) le macchine per staccare la sansa dai diaframmi delle presse
f) costo della pressatura e durata optimum della stessa.

A5 a) - *I diaframmi*

I dischi filtranti piani (o fiscoli) (diaframmi che si interpongono fra strato e strato di pasta di oliva, onde permettere un più regolare svolgimento dell'operazione di pressatura) possono essere fabbricati con materiali diversi: tessuto di cocco (8) (fig. 28), crine animale (tipo Perogio), lana, canapa (13), sparto, tunisino, ginestra (rafforzati con raggi e bordi di filo metallico), acciaio inossidabile, capelli cinesi, fibre artificiali (movil, polietilene, polipropilene) ecc. Un buon diaframma deve essere abbastanza robusto da non rompersi alle pressioni di esercizio delle presse; deve essere indeformabile; non assorbire eccessivamente l'olio; essere facilmente lavabile; non deve alterare sensibilmente l'olio (caratteristiche organolettiche, acidità, numero di perossidi); deve soprattutto avere una notevole resistenza all'abrasione, in modo da permettere un numero di pressature il più alto possibile. In effetti in ogni pressatura lavorano molte decine di diaframmi, il cui costo complessivo è notevole (si è al di là delle 100.000 lire). E' ovvia l'importanza che sul bilancio dell'oleificio ha la spesa per la sostituzione dei diaframmi danneggiati.

Recentemente Carocci Buzi ha sperimentato dia-

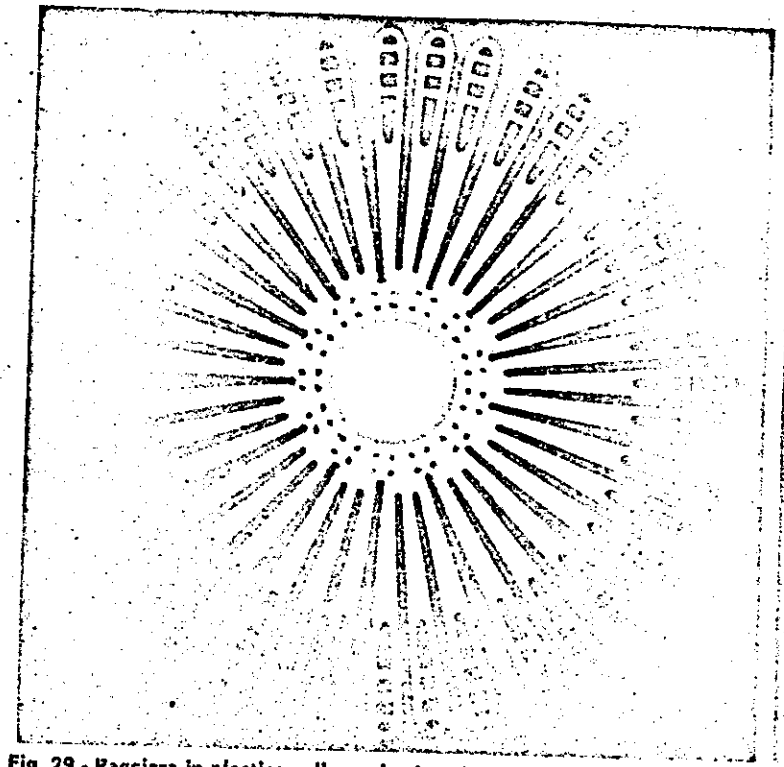

Fig. 29 - Raggiera in plastica sulla quale viene intrecciato il tessuto filtrante.

(*) Lavoro eseguito con contributo dell'Ufficio attività di ricerca di interesse industriale del Consiglio Nazionale delle Ricerche. Cogliamo l'occasione per rinnovare i nostri ringraziamenti per l'autorizzazione a pubblicare.

(**) La Nota I è stata pubblicata sul n. di giugno 1964 della presente Rivista, pag. 290.

...ystallization" succeeded only after ...ly freezing the solution in liquid ... The amounts of the two prepara... ...overed are 0.8 mg (Nos. 4a and 7) and ...Nos. 1, 2, 3, 4, 5, 6, and 8), repre... ...0.9 and 6.6 ppm of the starting ... respectively. Further efforts to ...each component have thus far been ...ful.

...V maximum for the first prepara... ...aks 4a and 7) is 242 mμ; for the ...reparation (Peaks 1, 2, 3, 4, 5, 6, ... is 255 mμ (Fig. 4). These prepara... ...e not been tested for biological ...From earlier reports (1–3), the sec... ...aration (UV max., 255) should be ...toxic than the first (UV max., 242).

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PRESERVATIVES

Determination of Benzoates and Hydroxybenzoates in Foods

By SALVATORE J. PINELLA, ANTHONY D. FALCO, and GEORGE SCHWARTZMAN (Food and Drug Administration, 850 Third Ave., Brooklyn, N.Y. 11232)

A method has been devised for the qualitative and quantitative determination of benzoates and hydroxybenzoates in foods. The qualitative procedure is based on the TLC separation of benzoic acid, the hydroxybenzoates, and sorbic acid, using Kieselguhr G-silica gel GF 254 as the absorbent and hexane:acetic acid as the mobile solvent. The separated preserving acids are identified under UV radiation and by specific chromogenic reagents. Benzoic acid in foods was determined quantitatively by steam distillation, extraction, and TLC separation. The benzoic acid was removed from the chromatographic plate, extracted with ethanol, and determined in the UV region with a recording spectrophotometer and 5 cm micro cells. These procedures should be subjected to collaborative study.

Benzoic acid, generally as the sodium or potassium salt, has been used for many years in the food industry to retard yeasts and molds. Hydroxybenzoates and similar compounds have been developed and are now being used as preservatives, alone or in combination with benzoic acid and/or benzoates.

It is difficult to qualitatively confirm the presence of benzoic acid following the extraction from foods by 27.002.[1] Neither the $FeCl_3$ test, 27.003, nor the Mohler test, 27.004, is specific for benzoic acid; salicylic acid also gives a positive reaction. With 13.044, 4th paragraph, benzoic acid gives a positive reaction and *p*-hydroxybenzoic acid, sorbic acid, salicylic acid, and the parabens give negative reactions. This method is sensitive to 200 μg of benzoic acid. Benzoic acid can also be identified by infrared or ultraviolet spectroscopy and thin layer chromatography (TLC). For IR spectroscopy, a disk is made from a mixture of 1 mg residue and 200 mg KBr. The resulting IR spectrum is easily distinguishable from the other preserving acids. With a recording UV spectrophotometer (250–350 mμ) and 1 cm cells, a 5 mg/100 ml solution in various solvents, such as ethyl ether, chloroform, ethanol, and sodium hydroxide, will give a characteristic curve and an absorbance of ca 0.400. The use of 5 cm micro cells with a 2.5 ml capacity makes this identification more sensitive. With TLC, 20 μg of benzoic acid can be detected; its R_f value is not affected by the usual food interferences, such as colors, flavors, or caramelization products. Kieselguhr G-silica gel GF 254 is used as the absorbent and hexane:acetic acid as the mobile solvent. The spots are identified under shortwave UV light.

The AOAC quantitative methods for benzoic acid are a titrimetric method, 27.005–27.006, and a spectrophotometric procedure, 27.007–27.009. Both these methods have shortcomings: the former does not indicate whether other preserving acids are present, and the latter is affected by interfering substances in foods. In addition, the AOAC extraction procedure is not adequate for all foods, e.g., emulsion problems are encountered with direct chloroform extraction of most foods.

We used steam distillation to isolate the standard preserving acids and to eliminate the emulsion-causing agents; the distillate was then extracted with a chloroform-ether shakeout. When the acids were quantitatively determined by UV and identified by IR, recoveries ranged from 94.7–98.8%. Steam distillation was used to determine the benzoic acid content of various food products. Products containing large quantities of sugar interfered in the UV assay. Caramelization occurs with carbohydrates (polyhydroxycarbonyl compounds, such as reducing sugars

[1] All official methods cited refer to *Official Methods of Analysis*, 10th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1965.

and sugar acids) at high temperatures. Unsaturated polymers result from caramelization and are accelerated by the presence of carboxylic acids and the salts of these acids, phosphates, and metallic ions, which are common in foods (1). These impurities will distort the UV spectrum for benzoic acid. Additional cleanup procedures (alkaline-chloroform/alkaline-ether shake-outs) did not remove these interferences. Unspiked jams and caramelized sugar gave UV spectra at or about the maximum for benzoic acid when the distillation procedure was followed. A method which would be applicable to all foods and remove caramel interference was desired. TLC not only eliminated the interferences but also was very sensitive for the detection of benzoic acid.

The preserving acids were qualitatively identified on TLC plates developed with hexane-acetic acid solvent and examined under shortwave UV light. (*See* Table 1.) Benzoic and sorbic acids have about the same R_f values but can be differentiated by direct bromination of the chromatogram (2). Bromination of sorbic acid yields bromocaproic acid. Benzoic acid is not affected by bromination and forms one spot; the other preserving acids do brominate and form two spots. (*See* Table 1 and Figs. 1 and 2.)

Bromination not only distinguishes between the two acids but also lowers the R_f of sorbic acid in relation to benzoic acid. Thus, this technique can be used to eliminate sorbic acid interference in the quantitative

Table 1. R_f values for benzoates and hydroxybenzoates with different TLC solvent systems[a]

Compound	Hexane-Acetic Acid		tert.-Amyl alcohol-tert.-Butanol-NH_3-Acetone
	Brominated[b]	Non-Brominated	
Benzoic acid	.65	.80	.38
Sorbic acid	.37	.72	.31
Salicylic acid	.27	.53	.60
p-Hydroxybenzoic acid	.19	.09	.21
Methyl-*p*-hydroxybenzoate	.33	.10	.79
Ethyl-*p*-hydroxybenzoate	.43	.13	.82
Propyl-*p*-hydroxybenzoate	.49	.17	.86

[a] With an acetone-isopropyl alcohol-12% NH_3 system all the compounds listed, except *p*-hydroxybenzoic acid with an R_f of .82, moved with the solvent front.

[b] With the exception of benzoic acid, two spots were formed on the TLC plate; the R_f given is for the higher spot.

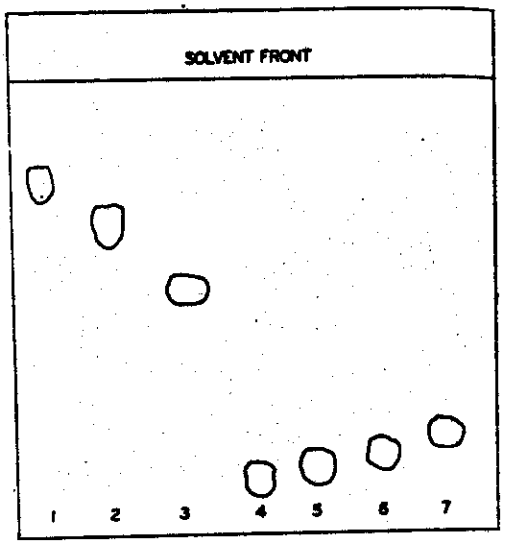

Fig. 1—TLC chromatogram of 100 μg of non-brominated preservatives on kieselguhr G-silica gel GF 254, using hexane:acetic acid system. (1) Benzoic acid, (2) sorbic acid, (3) salicylic acid, (4) *p*-hydroxybenzoic acid, (5) methyl-*p*-hydroxybenzoate, (6) ethyl-*p*-hydroxybenzoate, and (7) propyl-*p*-hydroxybenzoate.

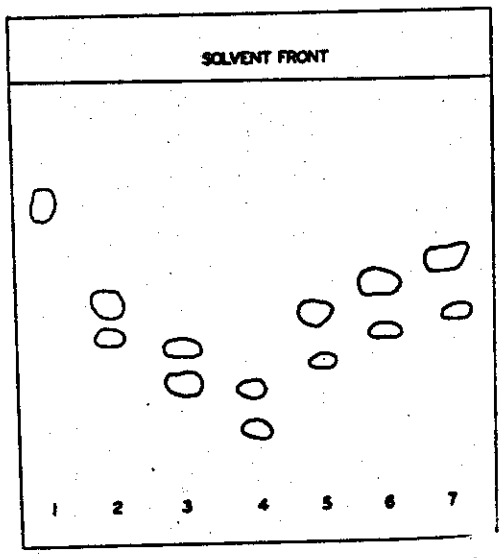

Fig. 2—TLC chromatogram of 100 μg of brominated preservatives on kieselguhr G-silica gel GF 254, using hexane:acetic acid system. (1) Benzoic acid, (2) sorbic acid, (3) salicylic acid, (4) *p*-hydroxybenzoic acid, (5) methyl-*p*-hydroxybenzoate, (6) ethyl-*p*-hydroxybenzote, and (7) propyl-*p*-hydroxybenzoate.

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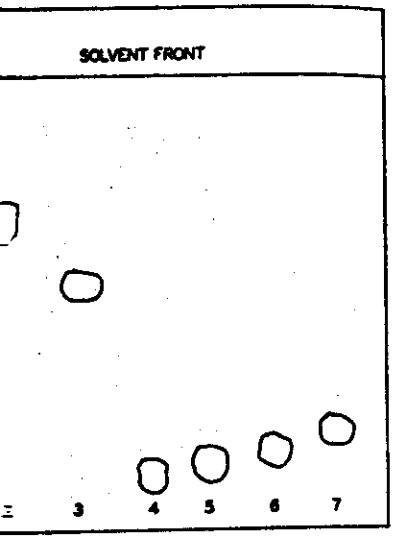
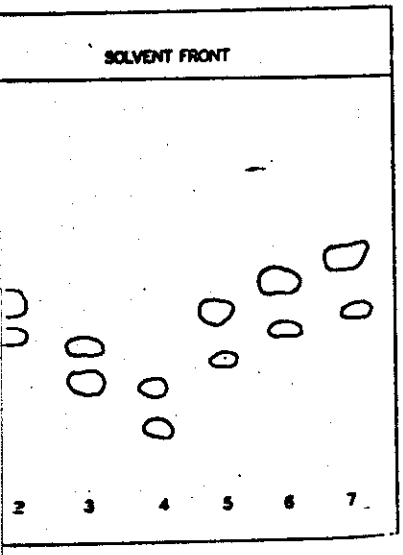


LC chromatogram of 100 μg of non- preservatives on kieselguhr G-silica gel ing hexane:acetic acid system. (1) Ben- (2) sorbic acid, (3) salicylic acid, (4) nzoic acid, (5) methyl-p-hydroxybenzoate, -p-hy enzoate, and (7) propyl-p- droxybenzoate.



LC chromatogram of 100 μg of bre- reservatives on kieselguhr G-silica gel ing hexane:acetic acid system. (1) Ben- . (2) sorbic acid, (3) salicylic acid, (4) nzoic acid, (5) methyl-p-hydroxyben- thyl-p-hydroxybenzote, and (7) propyl-p- hydroxybenzoate.

analysis of benzoic acid and also to identify sorbic acid.

For quantitative determination, benzoic acid was spotted on kieselguhr G-silica gel GF 254 TLC plates, which were then developed, air-dried, and observed under UV. After the spot was identified, the entire area was quantitatively removed from the plate and extracted with ethanol. This solution was analyzed by UV spectrophotometry, using 5 cm micro cells (2.5 ml capacity). The UV spectrum for benzoic acid was clearly observed with no distortions. Benzoic acid standards were distilled and extracted by this technique, and compared to non-treated standards. Recoveries of 99% were obtained. Benzoic acid standard spikes on a variety of foods (liquids, solids, and semi-solids) gave excellent recoveries (*see* Table 2).

Table 2. Benzoic acid recoveries in various foods[a]

Product	mg Added	Recovered mg	Recovered %
Ginger ale	48.9	48.5	99.1
Tea concentrate[b]	50.5	48.8	96.6
Apricot preserves	47.9	47.9	100.0
Grape jam	48.5	48.3	99.6
Blue cheese spread[b]	50.8	44.8	88.2
Ketchup	47.5	47.0	98.8
Herring in tomato sauce	55.1	56.3	102.1
Sardines in tomato sauce	49.4	47.7	96.4
Coffee sirup concentrate[b]	44.3	43.6	98.3
Pickled beets[c]	46.9	51.9	110.7
Mayonnaise-pickled relish spread	49.2	48.2	98.0
Buttered sirup[b]	44.3	40.7	91.9

[a] 50 g portions used for each product.
[b] Contained sodium benzoate.
[c] Contained 0.004% propyl-*p*-hydroxybenzoate.

METHOD

Apparatus and Reagents

Use reagent grade chemicals unless otherwise specified.

(a) *Steam distillation apparatus.*—*See* Fig. 3 for arrangement. (*1*) Connecting tube only, with flask joint ₮ 34/45 and condenser joint ₮ 24/40 (JD 1710); (*2*) Kjeldahl flask, 800 ml capacity with outer joint ₮ 34/45 (JF 6030); (*3*) condenser, 30 cm with outer joint ₮ 24/40 at top and drip tip at delivery end (JC 6400); (*4*) steam generator, *see* 18.016(a) and Fig. 18:1; (*5*) powerstats (9-521); and (*6*) Glas-Col heating mantle, 500 ml (11-472-10V4). Items *1–3* cite Scientific Glass Apparatus Co., Inc., catalogue numbers; items *5* and *6* cite Fisher Scientific Co. catalogue numbers.

(b) *Ultraviolet recording spectrophotometer and accessories.*—Recording between 250 and 350 mμ; with 5 cm micro cells and cell adapter (Pyroull Mfg. Co., Westwood, N.J., Nos. 5009, 5009a).

(c) *Thin layer chromatographic equipment and absorbents.*—Standard TLC equipment; kieselguhr G and silica gel GF 254 (Brinkmann Instruments, Inc., Westbury, N.Y.).

(d) *Solvent systems.*—(*1*) Hexane:acetic acid (96:4); (*2*) tert.-amyl alcohol:tert.-butanol: NH_3:acetone (40:40:10:10); (*3*) acetone:isopropyl alcohol:12% NH_3 (150:50:50); and (*4*) 10% bromine in chloroform.

(e) *Spray reagents.*—(*1*) 0.1% Rhodamine 6G (trianisoline) in ethanol; (*2*) 3% H_2O_2; (*3*) saturated potassium dichromate solution (10 g $K_2Cr_2O_4$ in 50 ml concentrated H_2SO_4); (*4*) saturated thiobarbituric acid solution; (*5*) 2% $FeCl_3$ solution; (*6*) 20% thymol in ethanol; and (*7*) methyl red-bromthymol blue reagent (dissolve 200 mg each of methyl red and bromthymol blue in 100 ml formaldehyde and dilute to 500 ml with ethanol).

(f) *Sodium hydroxide T.S.*—*See* USP, XVII, 1965, p. 1080.

(g) *Magnesium sulfate.*—$MgSO_4.7H_2O$, USP.

(h) *Qualitative standard solutions.*—Prepare separate solutions of benzoic acid, sorbic acid, salicylic acid, *p*-hydroxybenzoic acid, methyl-*p*-hydroxybenzoate, ethyl-*p*-hydroxybenzoate, propyl-*p*-hydroxybenzoate containing 10 mg/ml in ethanol.

Quantitative Procedure

Preparation of Plates

In a 250 ml ₮ Erlenmeyer flask, mix 10 g each of kieselguhr G and silica gel GF 254. Add 45 ml distilled water and shake for 30 sec. Set applicator at 0.25 mm and apply mixture; coat five glass plates. Air-dry for 10 min. and oven-dry in a forced draft at 100°C for 1 hr.

Sample Preparation

Liquids.—Weigh accurately ca 50-60 g sample directly into 800 ml Kjeldahl flask. Continue as described under "*Semisolids* . . ." beginning with "Add 200 g $MgSO_4.7H_2O$."

Solids.—Weigh accurately ca 40 g sample into a Waring Blendor. Add 100 ml water (or more if necessary) and blend until a homogeneous mixture is obtained. Quantitatively

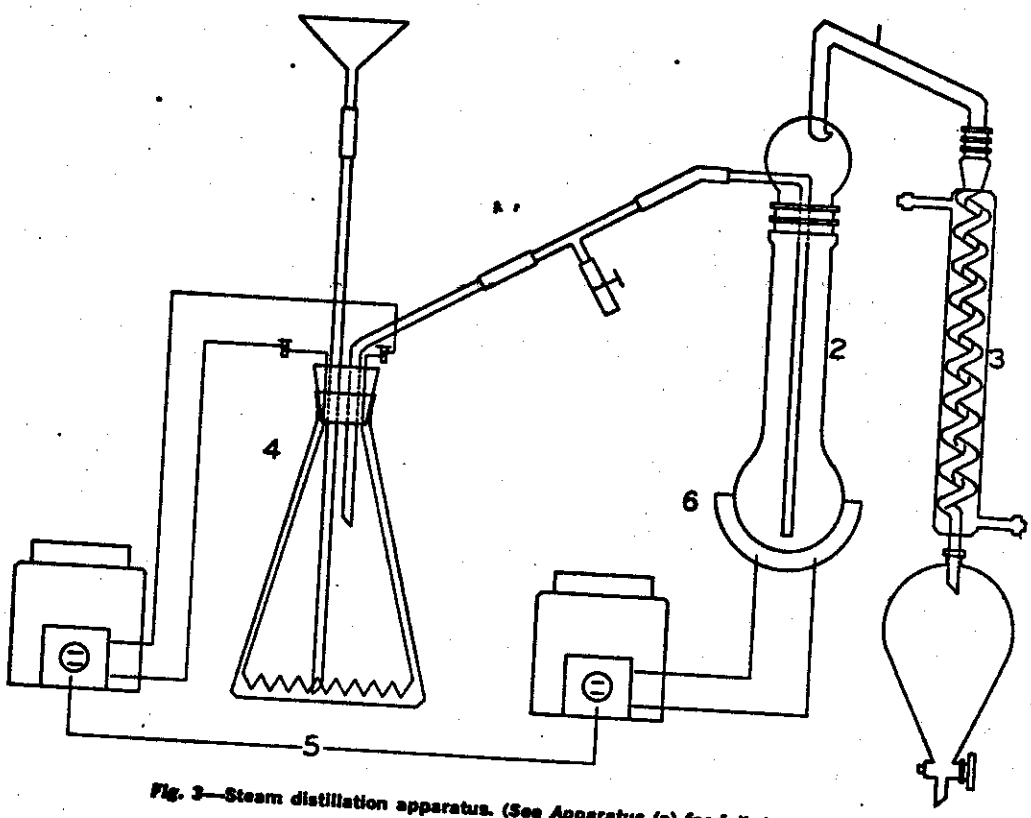
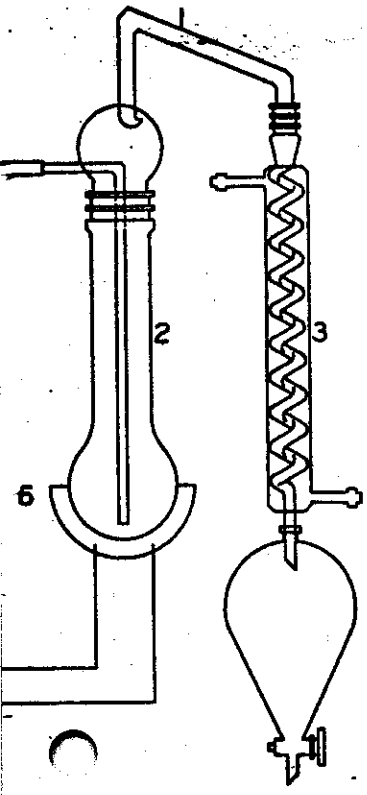


Fig. 3—Steam distillation apparatus. (See *Apparatus* (a) for full description.)

transfer to 800 ml Kjeldahl flask with small portions of water. Continue as described under "*Semisolids* . . ." beginning with "Add 200 g $MgSO_4.7H_2O$."

Semisolids and solid-liquid mixtures.—Blend entire unit sample in a Waring Blendor to a homogeneous mixture. Quantitatively transfer ca 50-60 g sample (accurately weighed) to an 800 ml Kjeldahl flask with small portions of water, if necessary. Add 200 g $MgSO_4.7H_2O$ and 25 ml phosphoric acid (3). Wash down neck of flask with water until total volume is 350-375 ml. Steam distill sample directly into a 1 L separatory funnel containing 50 ml NaOH T.S. Collect 725-750 ml distillate in a *minimum* time of 90 min. by adjusting powerstats. (*Note:* Distillation rate is very critical. Our drop time was 50 drops/20 sec.) Rinse condenser with ca 20 ml water. Acidify distillate to litmus with 20 ml concentrated HCl. Extract with one 100 ml and four 50 ml portions of $CHCl_3$-ether (2+1). Shake each extract vigorously for at least 1 min. and collect extracts in 600 ml beaker. Evaporate combined $CHCl_3$-ether extracts carefully on a steam bath, using a *gentle* current of air, to ca 25 ml, washing down the sides of the beaker periodically with $CHCl_3$-ether. *Do not let extracts go to dryness.* Transfer to a 50 ml volumetric flask. Wash beaker with small portions of $CHCl_3$-ether and transfer to volumetric flask. Dilute to volume with $CHCl_3$-ether for TLC analysis.

Spot 100 µl sample solution, using a 50 µl Hamilton syringe twice, with gentle air draft on prepared plate. Also, spot 100 µl of a 50 mg/50 ml standard solution of benzoic acid. Sample(s) and standard should be spotted ca 1" from bottom edge of plate and 1½" apart, beginning 1" in from side edge. Place plate in chromatographic chamber containing 250-300 ml mobile solvent (*n*-hexane:acetic acid (96:4)) and develop chromatogram for a distance of 10 cm. Remove and air-dry for 5 min. Observe under UV shortwave radiation (2540Å). Sample and standard benzoic acid appear as dark blue-purple spots on a light fluorescent background. With a pencil, circle the spots ½" from outer edge of each spot. This gives the exact location of each acid when plate is removed from the UV radiation. Scrape encircled area with a steel spatula.



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shing down the sides of the beaker with $CHCl_3$-ether. *Do not let exo dryness.* Transfer to a 50 ml ask. Wash beaker with small por- HCl_3-ether and transfer to volu- Dilute to volume with $CHCl_3$- C analysis.

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onto a piece of Glassine paper and carefully transfer to a 10 ml volumetric flask. From an unused portion of the plate, scrape off an area approximately equal to that used for the sample into a 10 ml volumetric flask to serve as a blank. To each, add 7 ml 95% ethanol, stopper, and shake for 30 sec. Dilute to volume, transfer to a centrifuge tube, and centrifuge in a clinical centrifuge at high speed for 5 min. Decant clear supernatant solutions into 5 cm micro cells and compare on a recording spectrophotometer from 310 to 250 mµ against a blank.

Calculate benzoic acid or sodium benzoate concentration as follows:

(1) $(A_{spl}/A_{std}) \times$ (concn std in g per 50 ml/wt spl in g per 50 ml) $\times 100 =$ % benzoic acid

(2) % Benzoic acid $\times 1.180 =$ % Na benzoate

Qualitative Procedure

From final 50 ml sample extract obtained for UV quantitative analysis, spot at least 100 µg sample on a prepared plate. To separate primarily benzoic and sorbic acids, overspot each sample and standard with 5 µl 10% bromine-$CHCl_3$ solution. (Bromination is not necessary to determine acids other than benzoic and sorbic acids.) Develop chromatogram for a distance of 15 cm in a chromatographic chamber containing 250 ml hexane:acetic acid. Air-dry for 5 min. and observe under UV 2540Å (shortwave) radiation prior to specific color reactions. The acids appear as blue spots on a fluorescent background. If brominated, all the preservatives show up as two spots with the exception of benzoic acid, which appears as a single spot. (*See* Table 1 and Figs. 1 and 2.)

Specific Color Reactions

Rhodamine 6G-3% H_2O_2 (4).—Intensively spray chromatogram with 0.1% Rhodamine 6G. By visual examination, benzoic acid appears as a pink spot, sorbic acid as light purple, salicylic acid as dark purple, *p*-hydroxybenzoic acid as pink, and the methyl, ethyl, and propyl-*p*-hydroxybenzoates as light pink. Under UV shortwave radiation (2540Å), benzoic and sorbic acids are light brownish purple spots; salicylic and *p*-hydroxybenzoic acids and the parabens are dark brownish purple spots. Overspray with 3% H_2O_2. Visually, benzoic acid is pink; sorbic, salicylic, and *p*-hydroxybenzoic acids are rose; and the parabens are pink. Under UV, benzoic acid is light pink, sorbic acid is purple, salicylic and *p*-hydroxybenzoic acids are dark purple, and the parabens are rose-colored.

Potassium dichromate.—Carefully spray with a saturated solution of potassium dichromate in H_2SO_4 and heat at 110°C for 15 min. in oven. All preservatives appear brownish green on a yellow-green background.

Thiobarbituric acid (4).—Intensively spray with a saturated solution of thiobarbituric acid. Sorbic acid is a light pink spot and salicylic acid is purple; the other compounds do not react.

2% $FeCl_3$ (4).—With this reagent salicylic acid becomes purple, while all the others do not react.

3% H_2O_2–2% $FeCl_3$ (4).—Intensively spray with 3% H_2O_2 and heat at 90°C for 5 min. in oven. Overspray with 2% $FeCl_3$. Benzoic acid is a pale purple spot, sorbic acid a pale yellow spot, salicylic acid a deep purple spot, *p*-hydroxybenzoic acid a yellow spot, and the parabens do not give a reaction.

Thymol-H_2SO_4 (5).—Intensively spray with 20% thymol and heat at 90°C for 10 min. Overspray with H_2SO_4 (1 + 9). Sorbic acid is detected as a blue-green spot, *p*-hydroxybenzoic acid and the parabens are detected as pink spots; the others do not react.

The presence of salicylic acid can be established more conclusively with the tert.-amyl alcohol-tert.-butanol-NH_3-acetone solvent system, developing for 10 cm, and spraying with the methyl red-bromythymol blue reagent (6). All the parabens show up as rose-purple spots on a pink-orange background; however, the parabens do not show up until after a half hour. (*See* Table 1.)

Similarly, the presence of *p*-hydroxybenzoic acid is further substantiated by the use of the acetone-isopropyl alcohol-12% NH_3 system with a 10 cm development distance. *p*-Hydroxybenzoic acid exhibits a deep rose color and an R_f of about .82 when sprayed with the same methyl red-bromthymol blue reagent. The other parabens, however, move with the solvent front.

Results and Discussion

This study indicates that the quantitative procedure described is very sensitive, reliable, and practicable for routine laboratory use. The recoveries for the foods fortified with benzoates were in the 96.4–102.1% range. Blank recoveries for these products were negative. With foods containing declared benzoates, only one product to which

benzoic acid was added, a blue cheese spread, gave recovery with appreciable deviation. Since it was a thick emulsion, the low recovery may have resulted from improper blending. Two different portions of the products were analyzed; the benzoate content found prior to fortification was subtracted from recovery values. Benzoic acid recoveries from food products are given in Table 2.

A product containing another preservative, propyl paraben, was spiked with benzoic acid; the former did not interfere with the recovery of benzoic acid. The over-all recovery for benzoic acid in the foods examined was 98.3%.

Method 13.044, 4th paragraph, for the qualitative determination of benzoic acid, was studied at various concentrations. Ether solutions of *p*-hydroxybenzoic acid, the parabens, salicylic acid, sorbic acid, and benzoic acid were tested at concentrations of 1.0, 0.5, and 0.2 mg. At all levels of concentration, only benzoic acid gave positive pink or red coloration. The other preserving acids gave negative reactions. A blank consisting of all reagents and solutions used in the test was run with the standards.

Recommendations

It is recommended that the methods reported above be submitted for collaborative study and that further work be continued on these acids.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D, and were accepted by the Association. See *This Journal*, 49, 172–175 (1966).

This report of the Associate Referee, S. J. Pinella, was presented at the Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

Sulfur Dioxide Determination by Modified Monier-Williams Method

By JOEL J. THRASHER (Food and Drug Administration, 900 Madison Ave., Baltimore, Md. 21202)

An alternative trapping system for the two U-tubes now incorporated in the modified Monier-Williams method for total sulfurous acid, 27.079, is recommended. This system is easy to use, permits the titration of distilled SO_2 during the determination, and is cheaper and more durable than the U-tube system. The nonapplicability of 27.079 to dried onions, leeks, and cabbage is also discussed.

Alternate Trapping System for Modified Monier-Williams Method for Sulfur Dioxide

This report proposes an alternative trapping system for the two U-tubes now incorporated as Fig. 27:1 in 27.079. The inconveniences of the current apparatus have been pointed out in previous reports (1, 2).

The alternative trapping system offers distinct advantages over the U-tube system. In addition to being efficient, it is distinctly easier to use, it permits the titration of distilled SO_2 during the determination (not possible with the U-tube system), and it is cheaper and more durable than the U-tube system. The apparatus suggested here (Fig. 1) consists of the two lower components of the pesticide evaporative apparatus, known as the Kuderna-Danish evaporative concentrator (Kontes Catalog TG-20, K-57000,

COMBINED EXTRACTION AND PAPER CHROMATOGRAPHY OF FOOD PRESERVATIVES

LIBRARY OF LOAN SERVICE

J. RAJAMÄ AND P. MÄKELÄ

Laboratory for Food Research and Technology, The State Institute for Technical Research, Otaniemi (Finland)

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If a foodstuff is applied direct on paper for the chromatographic separation of food preservatives, food components interfere with the migration, and by masking the spots on the chromatogram, make this rather difficult to interpret. For this reason, a preliminary extraction step usually precedes paper chromatography of ether-soluble preservatives. A large number of extraction funnels and other items of glassware are needed for the extraction, if several analyses are to be made simultaneously. Further trouble is occasioned by emulsions; these are formed easily and can make the funnel extraction of fruit juices and jellies a rather difficult matter.

The purpose of this study is to find a remedy for these difficulties. Use is made of the well-known effect—normally detrimental in chromatographic separation—of compounds migrating with the solvent front if the solvent is too powerful. This phenomenon can be regarded as a kind of extraction on paper, as it permits the direct

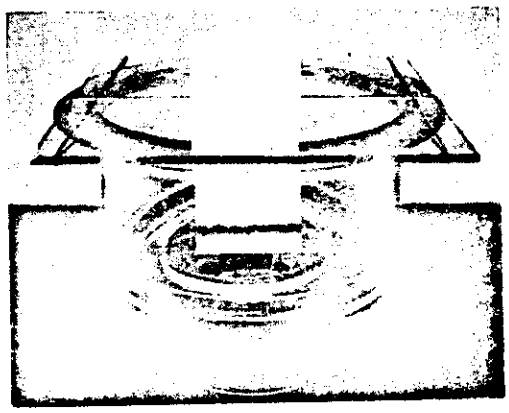

Fig. 1. Extraction vessel.

separation of ether-soluble compounds from interfering substances on chromatographic paper. The procedure is simple and rapid and does not call for any complicated apparatus. The extracting solvent concentrates the preservatives in a narrow line, well suited as a starting line for chromatography on the same paper. Moreover, some preservatives can be separated quickly and efficiently during the course of the extraction, with the help of arresting reagents. A modification of the method has been developed for fat-containing foods.

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EXPERIMENTAL

Apparatus and materials

Extraction vessel (see Fig. 1). The lower part of a desiccator is used for this purpose. The arrangement allows easy regulation and change of the extraction distance. The rim of the extraction vessel should be smooth so that a saturated atmosphere is maintained within the vessel, otherwise ether evaporates from the paper before it reaches the slit between the glass plates.

Bromination chamber (see Fig. 2). This is an arrangement which resembles the extraction vessel, but a shallow dish (height about 4 cm) replaces the desiccator. Only the lowest part of the paper (starting line) comes inside the chamber through a slit between the glass plates.

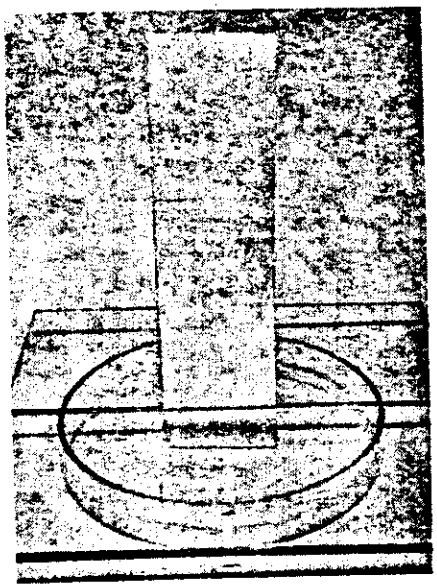

Fig. 2. Bromination chamber.

Chlorination chamber. A 2-l decanter glass used for this purpose is covered with a saran-membrane attached by means of a rubber band. Chlorine is led from a chlorine generation bottle into the chamber through a plastic tube.

U.V.-lamp. The maximum wavelength was 254 nm.

Chromatographic paper. Whatman 3 MM, or a corresponding thick paper, is cut into strips 5 cm in width; the length is varied from 15 to 30 cm according to the needs of the analysis.

Reagents

Extraction. Ethyl ether.

Impregnating solution: 1 part of 0.5 *N* sodium hydroxide, aqueous solution and 2 parts of acetone. The acetone is added immediately before use.

Alkaline "arresting reagent": a saturated sodium bicarbonate solution.

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Chromatography. Solvent system: isoamyl alcohol, ethanol and (1/10 diluted) ammonium hydroxide (6:3:2).

Identification reagents. Potassium permanganate, 0.05 % solution, 1 *N* with respect to sulphuric acid.

Manganese dioxide, for the development of chlorine gas.

Hydrochloric acid, concentrated and 1 *N*.

Sodium hypochlorite: an 11 % commercial solution, diluted to 1/15 with water.

Extraction procedure

Low fat foods. A juice of low sugar concentration (10 to 20 %) can be applied as such on to the paper by pipetting 50 to 100 μl across a strip of chromatographic paper as a broad band at a distance of about 1 cm from the end of the paper. The material does not need concentration in a narrow area as in ordinary chromatographic separation; it is spread across the paper quickly, without any intermittent drying. Juices with a higher sugar content, as well as jellies and jams, should be diluted with the same or double the amount of water.

In other cases, a sample of homogenized and diluted material, corresponding to 50 to 100 mg of the original product, is transferred to the paper as described above. Application has also been effected by weighing about 50 to 100 mg of the food in a small decanter glass, diluting with double the amount of water, and transferring the total weighed and diluted material to the paper with the aid of a glass capillary.

After the sample has been applied to the paper, it is left to equilibrate in the air for a short period (3 to 7 min), so that the moisture becomes equalized at the starting line; however, it must not be allowed to dry. The presence of moisture is essential if the extraction is to succeed. With new material for analysis, some experimentation may be needed to determine the correct equilibration time. The extraction is also dependent on a certain degree of acidity, but most food materials are sufficiently acidic to make the addition of acid seldom necessary.

The strip is now pressed between the edges of two glass plates so that the lower end of the paper (about 5 to 8 cm) reaches through the slit between the plates into a dish, filled with ethyl ether, in the extraction vessel (Fig. 1). The ether is adsorbed through the paper, and evaporates on the slit, concentrating the ether-soluble material as a narrow line in the solvent front (Fig. 3). The extraction is complete within 15 min, and the paper is removed from the extraction vessel.

The evaporation line may serve as a starting line for the chromatographic separation. The paper strip is cut off one to two centimetres below the evaporation line, and the paper is transferred into a chromatographic solvent. *p*-Hydroxybenzoic acid, and its esters, are separated from sorbic and benzoic acid within an hour; 30 min may be adequate if the sample is small enough. The solvent system was described by Joux[1].

Fat-containing foods. About 50 mg of the homogenized food is weighed into a small decanter glass. A material with a medium fat content, such as fish, is mixed with double the amount of water; a fatty substance, such as margarine, is mixed with double the quantity of butanol. If the acidity of the material is insufficient, acidifi-

cation with hydrochloric acid may be necessary. The weighed and mixed material is transferred to the paper strip as described above.

The paper is placed into the extraction vessel as above, but instead of the preservatives being extracted to the solvent evaporation front, they are now arrested by alkaline arresting reagents and thus separated from the fat, which is extracted by the ether over the alkaline area up to the evaporation front. Two types of arresting reagents are used: sodium bicarbonate for free acids, and sodium hydroxide for esters.

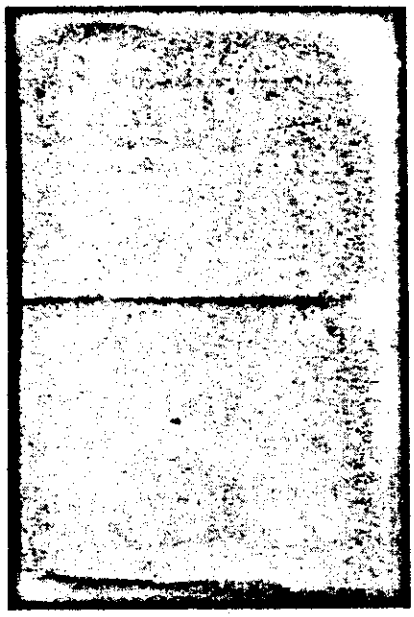

Fig. 3. The line of sorbic and benzoic acids photographed in U.V. light on the ether evaporation front.

Sorbic, benzoic and *p*-hydroxybenzoic acids are arrested in a line of saturated sodium bicarbonate which had previously been streaked across the paper with a glass capillary at a distance of 2 to 3 cm from the start line where the food material is applied. Fat, and such neutral substances as the esters of *p*-hydroxybenzoic acid, are extracted through the bicarbonate line to the solvent front. The distance between the evaporation line and the starting line should be at least 10 cm, to leave space for paper chromatography. The long migration distance, and the need to extract an appreciably large amount of fat to the evaporation line, make a longer extraction time necessary —at least half an hour, or even more. When the extraction is completed, the paper is cut off between the foodstuff and the bicarbonate lines, and transferred to the chromatographic solvent, which directly dissolves out benzoic and sorbic acids from the bicarbonate line; *p*-hydroxybenzoic acid is retained by the bicarbonate, however.

Chromatography of PHB esters. The efficient separation of the *p*-hydroxybenzoates from each other and from free acids is possible by a single direct ether extraction. After the food has been applied to the paper as described above, the strip is dipped into a freshly prepared mixture of aqueous sodium hydroxide and acetone,

which has been added to ensure uniform impregnation. The lower border of the alkaline region is one to two cm from the foodstuff line, so that ether may collect the preservatives into a narrow line before the alkaline region where the separation take place, is reached. With foods of moderate fat content, the paper is transferred into the ether extraction vessel 5 to 7 minutes after dipping into the impregnation solution; with fats, the equilibrating time is even shorter (1 to 3 min). The migration of the esters is slower on wet paper; this facilitates the separation from the fats. An acidic milieu on the extraction line is required also in this case.

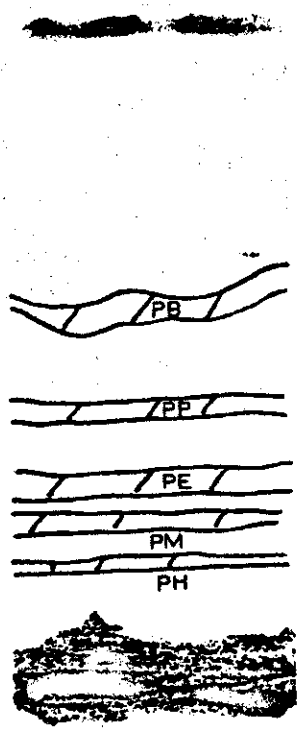

Fig. 4. 50 mg of fish homogenate (the broad dark line at the start) have been extracted with ether for 30 min on NaOH impregnated paper. Concentrations of 12% NaCl and 12% sugar, pH 6.2. To the material has been added *p*-hydroxybenzoic acid (PH) and methyl, ethyl, propyl and butyl *p*-hydroxybenzoates, PM, PE, PP and PB respectively, 0.02% of each compound. The extracted fat shows as a dark line on the upper part of the chromatogram marking the evaporation front. The preservatives have been outlined with pencil in U.V. light.

While the PHB-esters are separated from each other as distinct lines in the alkaline region, the fats are extracted to the evaporation line; acid substances, such as benzoic, sorbic and *p*-hydroxybenzoic acid, are arrested at the lower border of the alkaline region (see Fig. 4).

Detection and identification

All the acids and esters mentioned above are detectable as dark blue bands in short wave, ultra-violet light. This is the simplest method of detection, and makes quantitative determination possible after elution.

Most of these compounds are distinguishable by differences in the migration rate, either in the chromatographic solvent, or simply after the ether extraction. However, two pairs of compounds could not be resolved: the butyl and benzyl *p*-hydroxybenzoates migrated at the same speed in the extraction–separation procedure for esters; and benzoic and sorbic acids had R_F values which were too close, so that separation within a short period of time was impracticable in the chromatography of these acids. However, these acids can be distinguished by their differences in reactivity with the visualization reagents.

Permanganate reaction. Sorbic acid is detectable as a white band against a pink background after the paper has been sprayed with 0.05 % potassium permanganate in 1 *N* sulphuric acid. Benzoic acid does not decolorize potassium permanganate under the same conditions. PHB and PHB esters are also discernible as white bands on the chromatogram, although they are distinguished by their different migration rates. The pink colour of the background disappears within a few minutes, but if desired, the spraying may be repeated a number of times.

Chlorination. For detection of the possible presence of benzoic acid in addition to sorbic acid, the chromatogram is cut lengthwise into two parts, and one half is put into a chamber containing chlorine gas for five minutes. Sorbic acid is no longer visible in U.V. light after the chlorination. Benzoic acid does not undergo any change, and is also visible after the chlorine treatment as a blue band in U.V. light. The chlorine is generated in a reaction bottle by the addition of concentrated hydrochloric acid to manganese dioxide, and the application of gentle warmth. The gas is led to the chlorination chamber through a plastic tube.

The chlorine treatment should be carried out while the paper is still wet after chromatography. If the paper has dried, it should be wetted by a fine water spray.

Bromination. Bromination has the same effect as chlorination, but this treatment must be effected before the chromatography. Immediately after applying the foodstuff to the paper, it is pressed between the edges of the two glass plates, so that the lowest part of the paper strip, with the still moist line of food, reaches into a shallow glass dish filled with bromine vapour—generated by a small drop of bromine at the bottom of the dish (Fig. 2). Five minutes of this bromine treatment is adequate to ensure disappearance of the U.V. absorption of sorbic acid.

Hypochlorite treatment. Disappearance of the dark blue band of sorbic acid in U.V. light is instantly achieved by spraying with 0.1 *N* sodium hypochlorite solution, to which has been added immediately before spraying two drops of concentrated hydrochloric acid per 10 ml. A similar effect is obtained by spraying first with 0.1 *N* sodium hypochlorite solution, and then with 1 *N* hydrochloric acid. Benzoic acid remains stable during this treatment.

DISCUSSION

Experiments have been made with fruit and berry material, such as juices, jams and jellies, and with foods of medium fat content, such as herring preserves. Margarine was studied as an example of fat.

To date, the preservatives studied have been: benzoic and sorbic acids, *p*-hydroxybenzoic acid and methyl, ethyl, butyl, propyl and benzyl *p*-hydroxybenzoates. After 15 min extraction on paper a chromatographic run lasting half an hour was capable of separating these compounds into three groups: (I) *p*-hydroxybenzoic acid (PH); (II) benzoic and sorbic acids (BH + SH); and (III) *p*-hydroxybenzoates (PM).

This short running time is feasible by virtue of the favourable form of the new starting line caused by evaporation of the ether front, and the absence of interfering agents.

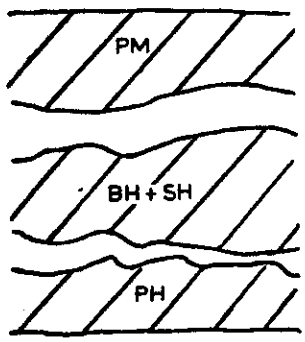

Fig. 5. Separation of extracted preservatives after a ½ h chromatographic run. The compounds have been outlined with pencil in U.V. light.

Half an hour was sufficient for the simultaneous extraction and chromatographic separation of *p*-hydroxybenzoates from non-fatty foods and foods of medium fat content on sodium hydroxide impregnated paper. One hour, or occassionally $1^1/_2$ h, was required for separation from fats, such as margarine.

Stress is laid upon the critical nature of the degree of moisture of the starting line and the impregnated paper—if the paper is dried too extensively after impregnation, the esters migrate too fast, and the lines become so diffuse that the substances are not detectable, or may even be pushed into the evaporation line. Conversely, if the paper is too wet, the separation of the esters from each other may not be achieved within a reasonable length of time. An increasing amount of fat calls for less drying time, and consequently a longer extraction time.

In Fig. 6, the PHB esters were isolated from margarine, and separated by three successive 30 min runs with ether. Drying time in air after impregnation was 1 minute.

The chromatography of acid substances, and separation of PHB esters, has been effected on a single paper as follows: food material was applied to a strip of paper 25 cm in length as described above. A line of bicarbonate was drawn across the paper, 2 cm from the starting line. Fats and *p*-hydroxybenzoates were extracted by ether over a distance of 14 cm to the evaporation front. The paper was cut 2 cm below the evaporation front. The lower part of the strip was transferred to the isoamyl alcohol solvent mixture for chromatography of the acids, and the upper part was impregnated with sodium hydroxide solution and subjected to ether extraction for separation of the PHB esters.

Observations on the identification treatments

The method of generating bromine vapour is very simple, but the treatment is practicable only when conducted before the chromatographic stage; the background of the paper is dark coloured, due to adsorbed bromine, in U.V. light, and some hours are required before bromine has totally evaporated. In the arrangement employed here, the effect is limited to the starting line. This treatment is well suited to the quantitative determination of benzoic acid in the presence of sorbic acid. The examination of U.V. spectra after chromatography and elution has shown that under the conditions

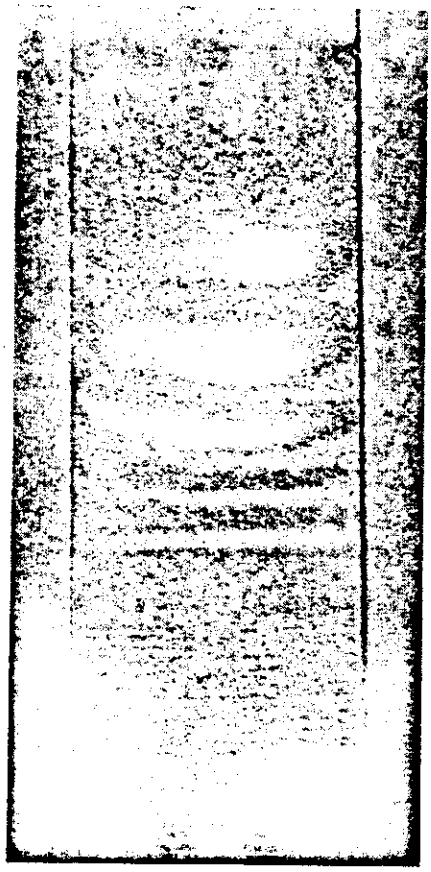

Fig. 6. The separated compounds are in a similar sequence to those in Fig. 4. The extracted fat shows as a broad light band in the upper part of the chromatogram. The compounds have been made visible by permanganate spraying.

stated sorbic acid undergoes complete reaction in 5 min, whereas benzoic acid is practically unaffected[2].

Chlorine generation is slightly more complicated than that of bromine, although the advantage here is that chlorination can be effected after chromatography. Chlorine does not impart a colour to the paper such as would hamper U.V. observations. Chlorination may be introduced before or after chromatography—and the same thing applies to hypochlorite treatment.

Treatment with hypochlorite has the advantage that ordinary spraying techniques can be applied. The spraying must not be too generous, as benzoic acid is easily eluted from the chromatogram, and may thus become undetectable. Pre-chromatographic treatment of sorbic acid has been effected not only by spraying, but also by streaking hypochlorite solution on both sides of the line containing sorbic acid, so that the reactant is brought to the action site by adsorption. The reaction has also been effected by directly mixing hypochlorite solution with the food to be analysed. The small amount of hydrochloric acid added to hypochlorite is insufficient to make the solution acidic, but activates the reagent so that it is capable of reacting with sorbic acid; in fact, if too much acid is added, the hypochlorite loses effectiveness in a very short time.

Some foodstuffs may yield interfering reaction products with the sorbic acid decomposition reagents, and thus it might be advisable to carry out the pre-chromatographic treatments not on the extraction line, but on the ether evaporation front, or on the alkaline arresting line.

CONCLUSIONS

It seems to be a reasonable assumption that modifications of this technique of extraction combined with chromatography on paper should also be suitable for other analytical applications, in cases where preliminary extraction before chromatography is necessary.

ACKNOWLEDGEMENT

This study was supported by a research grant from the Food Research Foundation in Finland.

SUMMARY

Food preservatives—sorbic, benzoic, *p*-hydroxybenzoic acid and *p*-hydroxybenzoates—have been extracted from foods directly on to chromatographic paper with ethyl ether. Paper chromatographic separation of the extracted compounds was effected on the same paper.

Methyl, ethyl and propyl *p*-hydroxybenzoates have been separated from each other during ether extraction and from the critical pair, the butyl and benzyl *p*-hydroxybenzoates, on a paper impregnated with sodium hydroxide. An examination was made of the reactions which distinguish sorbic and benzoic acids when both are present.

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Talanta, 1967, Vol. 14, pp. 981 to 983. Pergamon Press Ltd. Printed in Northern Ireland

Determination of sorbic acid with sodium chlorite

(*Received* 8 *December* 1966. *Accepted* 14 *March* 1967)

THE diene structure of sorbic acid suggests the possibility of its determination by a method similar to that used to measure the degree of unsaturation of fats and fatty acids. Such attempts included those by Maurel and Touyé,[1] who tried to use iodine monobromide (Hanus method) or iodine trichloride (Wijs method) in a way resembling the determination of iodine number in fats, but were not satisfied with the results obtained. Spanyár and Sándor,[2] using a bromate–bromide mixture, obtained a good quantitiative bromination in useful analytical conditions, and proposed a method applicable to complex foods, with a maximum error of ±5%.

Using a similar procedure but another halogenation mixture, we have developed a new method for the determination of aldrin (1,2,3,4,10,10-hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-1,4,5,8-dimethanonaphthalene), the halogen being added to the "reactive" double bond in the unchlorinated ring and the excess being determined iodimetrically or potentiometrically.[3,4]

Starting from these and other results we have tried to use sodium chlorite in hydrochloric acid medium for the determination of sorbic acid (hexa-2,4-dienoic acid).

EXPERIMENTAL

Reagents

Sodium chlorite, 0·1*N*. Dissolve 2·25 g of anhydrous sodium chlorite (*p.a.*) in doubly distilled water and make just alkaline with sodium hydroxide. The solution is stable for several months if kept in the dark and at low temperature (refrigerator). The solution is standardised with 0·1*N* thiosulphate, in sulphuric acid medium in the presence of potassium iodide, starch being used as indicator. The solution can be diluted if required.

Procedure

Take a 5- or 10-ml aliquot, containing 2–25 mg of sorbic acid, in an iodine flask or a well-stoppered conical flask. From a burette add 10 ml of sodium chlorite solution and then rapidly add 6 ml of 8*M* hydrochloric acid from a graduated cylinder. Swirl the flask and close it with a ground-glass stopper.

The solution gradually becomes yellow–green, reaching an apparently constant colour in a few seconds. After 30–90 sec add 5 ml of 10% potassium iodide solution rapidly but carefully down the flask walls. To prevent loss of iodine agitate the closed flask gently until the solution becomes homogeneous. Then mix the solution well, wash down the walls with distilled water and immediately titrate to pale-yellow, add 1–2 ml of starch solution and continue the titration until the solution becomes colourless.

The quantity of sorbic acid in the aliquot titrated is $56{\cdot}1\, f(V_1 - V_2)$ mg where V_1 and V_2 are the volumes of $f\,N$ thiosulphate consumed by a blank and the sample respectively. More dilute thiosulphate (0·01 or 0·05*N*) may be used if desired.

RESULTS AND DISCUSSION

The reaction on which the determination is based appears to be chlorination at the 2 and 5 positions leading to a double bond at the 3,4 position:

$$HClO_3 + 3HCl = 2H_2O + 2Cl_2$$

$$CH_3{-}CH{=}CH{-}CH{=}CH{-}COOH + Cl_2 \rightarrow CH_3{-}CHCl{-}CH{=}CH{-}CHCl{-}COOH$$

A similar reaction forms the basis of the Spanyár and Sándor method,[2] though in the original work its mechanism is not discussed by the authors. Maurel and Touyé,[1] in trying to apply this method to the determination of pure sorbic acid obtained recoveries of about 50%. In contrast, we obtained a good recovery. The explanation of this contradiction most probably lies in the fact that the earlier authors considered that addition took place at both double bonds. Some results for pure sorbic acid solutions are shown in Table 1.

TABLE I.—DETERMINATION OF SORBIC ACID

Sorbic acid		Difference, *mg*
Taken, *mg*	Found, *mg*	
2·50	2·47	−0·03
2·50	2·57	+0·07
2·50	2·52	+0·02
5·00	4·94	−0·06
5·00	5·16	+0·16
6·00	6·08	+0·08
7·50	7·39	−0·11
7·50	7·50	nil
10·00	10·28	+0·28
12·50	12·45	−0·05
12·50	12·70	−0·20
15·00	15·03	+0·03
15·00	14·83	−0·17
17·50	17·62	+0·12
17·50	17·48	−0·02
20·00	20·21	+0·21
25.00	25·31	+0·31
25·00	25·00	+0·00

The precision and reproducibility are influenced by the conditions of halogenation. To ensure quantitative addition an excess of reagent is necessary. In the conditions specified, reaction is complete in 30–90 sec. The rate of the reactions is determined by the excess of sodium chlorite added. The end of the reaction is indicated by cessation of the colour change to yellow–green. Prolongation of the reaction beyond this time is neither necessary nor useful, because it allows loss of chlorine. Premature addition of iodide, before the yellow–green colour is obtained, gives low results, and so may too small an excess of chlorite reagent. If a large excess of chlorite is used, the reaction is rapid, the yellow colour appears in 4–6 sec and iodide must be added immediately to prevent loss of chlorine. The speed of addition is greater than that of bromine or iodine.

Interferences

A few substances which are used for food conservation and can exist together with sorbic acid in foods have been tested for possible interference. Benzoic, citric and formic acids do not affect the determination; salicylic acid consumes a small quantity of reagent, and sulphite interferes. The effects of maleic and fumaric acids were not investigated.

TABLE II.—DETERMINATION OF SORBIC ACID IN VEGETABLE PRODUCTS*

Extraction method	Sorbic acid added,† *mg*	Sorbic acid found, *mg*	Difference, *mg*	Recovery, %
Ether extraction	2·50	2·35	−0·15	94
	10·00	9·31	−0·79	93
	15·00	14·01	−0·99	93
	20·00	21·80	+1·80	109
Chloroform extraction	5·00	4·52	−0·48	90
	10·00	9·00	−1·00	94
	15·00	16·30	+1·30	107
Steam-distillation and ether extraction	10·00	8·50	−1·50	85
	15·00	14·00	−1·00	93
	15·00	13·28	−1·72	88

* Tomato juice, wine and fruit pulps.
† Added before extraction.

Determination of sorbic acid in food

The sorbic acid must first be extracted from the food sample with solvents or steam to give a solution of the required concentration. We tried three schemes for extraction. The first was the ether extraction at pH 1–2 used by Spanyár and Sándor,[2] followed by stripping of the sorbic acid with an alkaline aqueous phase. We also tried evaporating the ether extract at room temperature and redissolving the residue in 5 ml of 0·5% sodium hydroxide solution. The same procedure was used with a chloroform extraction[5] but persistent emulsions appeared, especially when alkaline solutions were used.

We also used the Sudario[6] and the Schmidt[7] methods based on steam-distillation of sorbic acid. The principal disadvantage was the need to collect a large quantity of distillate to ensure the quantitative removal of the sorbic acid, and the final solution was too dilute to be used directly and had to be concentrated either by evaporation (after it had been made alkaline) or by extraction.

The results obtained were influenced by the extraction method used and the relative error was never less than 6%. However, the procedure can be successfully used for the determination of sorbic acid in various samples and has the advantage of considerable rapidity.

Department of Inorganic Chemistry
University of Bucharest
Splaiul Independentei 89
Bucharest, Rumania

P. Spacu
H. Dumitrescu

Summary—A new method for the determination of sorbic acid is proposed, using sodium chlorite in hydrochloric acid medium as reagent. In these conditions the hydrochloric acid is oxidized to chlorine which adds on to the double bonds of the sorbic acid. The addition is rapid (30–90 sec) and for this reason the determination is more rapid then others similar to it. Sorbic acid has been determined in foods by this method, after a preliminary solvent extraction or steam-distillation.

Zusammenfassung—Eine neue Methode zur Bestimmung von Sorbinsäure mit Natriumchlorit in Salzsäure als Reagens wird vorgeschlagen. Unter diesen Bedingungen wird Salzsäure zu Chlor oxidiert, das sich an die Doppelbindungen der Sorbinsäure addiert. Die Addition geht schnell (30–90 sec); aus diesem Grund geht die Bestimmung schneller als andere, ähnliche Methoden. Auf diese Weise wurde Sorbinsäure in Lebensmitteln nach vorausgehender Extraktion oder Wasserdampfdestillation bestimmt.

Résumé—On propose une nouvelle méthode de dosage de l'acide sorbique, en utilisant comme réactif le chlorite de sodium en milieu acide chlorhydrique. Dans ces conditions, l'acide chlorhydrique est oxydé en chlore qui s'additionne sur les doubles liaisons de l'acide sorbique. L'addition est rapide (30–90 sec) et, pour cette raison, le dosage est plus rapide que les autres qui lui sont similaires. On a dosé l'acide sorbique par cette méthode dans des aliments, après extraction par solvant ou entraînement à la vapeur préliminaires.

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Journal of Food Hygiene (Shoku Ei Shi) Vol. 5, No.5 October 1964, pp. 368-372 Studies on the Preservatives in Food (Report No. 3)

On the Qualitative Determination of Sorbic Acid by Infrared Absorption Spectra. by Tetsu Inoue, Taro Kawamura, Masaya Kamijo and Michiko Asakura (Yokohama Municipal Public Health Laboratory: 2-102, Nakamura-cho, Minamiku, Yokohama).

(For a brief English synopsis, refer to the original text on p.268)

Introduction:

As the method for the qualitative determination of sorbic acid contained in food, the method using ultraviolet absorption spectra is currently used. The absorption maximum of sorbic acid in the ultraviolet region is located at one place. When obstructing substances are mixed, moreover, the absorption maximum of sorbic acid is shifted or covered up, thereby making it impossible to carry out the qualitative determination sufficiently.

In the previous report (1, 2), the present authors pointed out that an obstructing substance is distilled out if burning takes place at the time of distillation. As the improved methods, they presented, in the same report, a method for the removal of obstructing substances in the distillation solution and the dialytical method which is convenient for the treatment of a large number of detection bodies. In order to make these methods more accurate, we examined the qualitative determination of sorbic acid by using infrared absorption spectra. Some facts which were discovered will be described in the following report:

I. Experimental Method

1. Apparatus Used:

A Shimatsu Self-recording infrared Absorption Spectrophotometer, IR-27A type.

2. Standard Solution of Sorbic Acid

The sorbic acid pruchased on the market was recrystallized from alcohol and that which had a melting point between 133 and 135 degC was dissolved in carbon tetrachloride to make a solution of from two to 10 milligrams per milliliter.

3. Method for the Preparation of Test Solution:

From five to 10 grams (an amount which corresponded to five to 10 milligrams of sorbic acid) of a sample which had been finely cut or ground was placed in a collodion film and 20 milliliters of a dialytical auxiliary solution was added to the same, while mixing was carried out. Next, the upper end of the film was tightly sealed and the same was suspended in a wide-mouthed bottle of 300 milliliters to which 200 milliliters of distilled water had been added in such a manner that the water surface in the film and the water surface in the bottle might be approximately of the same height. While the same was being stirred from time to time, it was left at normal temperature for a period of 20 to 24 hours for the purpose of carrying out dialysis (2).

Next, from 100 to 200 milliliters of the dialytical solution was taken into a separatory funnel of from 300 to 500 milliliters. After adding five milliliters of 10 per cent hydrochloric acid and from 30 to 60 grams of sodium chloride, its extraction was carried out by using approximately the same amount of ether. The water layer was separated and two more extractions were carried out with ether in an amount half as large. The ether layer was added, cleaning was carried out by using from 10 to 20 millilitersof distilled water. The cleaning solution was discarded and the ether solution was dehydrated with anhydrous sodium sulfate. Afterward, the ether was distilled away and the residue was lightly dried by air blowing. It was dissolved in exactly one to 2 milliliters of carbon tetrachloride so as to make a test solution.

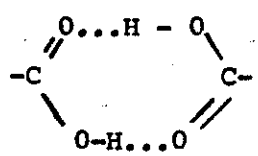
4. Measurement Operation

Judging from its structural formula of CH_3-CH=CH-CH+CH-COOH sorbic acid

is expected to show characteristic absorptions based on a methyl group, a carboxylic group and a conjugate double bond. Regarding the standard solution of sorbic acid, the infrared absorption spectra were measured. As a result, Figure 1 was obtained.

(Figure 1 Infrared spectra of sorbic acid.)

At 2,960 centimeters^{-1}, there was observed an absorption of (gamma)OH due to $-CH_3$ and there was observed an absorption of (gamma)OH at 3,000 to 2,500 centimeters^{-1}. There was observed a strong absorption of (gamma)C=O based on -COOH at 1,690 centimeters^{-1}. At 1,640 centimeters^{-1} and 1,620 centimeters^{-1}, there was observed an absorption of (gamma) C=C due to the conjugate double bond. All of the absorptions which were observed at 1,415 centimeters^{-1}, 1,325 centimeters^{-1} and 1,260 centimeters^{-1} are believed to be due to the coupling of C-O elongation and contraction and angle variation within the O-H surface as constituted by the dimer of -COOH (3) or



The absorption at 1,000 centimeters^{-1} is believed to be the $(\text{delta})_{CH}$ of a conjugate double bond.

As stated above, sorbic acid shows considerably peculiar infrared absorption spectra. Therefore, as a method for the determination of sorbic acid based on the infrared absorption spectra, it is possible to carry out an accurate determination by comparing the absorptions of the sample solution at 2,960 centimeters^{-1}, 1,690 centimeters^{-1}, 1,640 centimeters^{-1}, 1,620 centimeters^{-1}, 1,415 centimeters^{-1}, 1,325 centimeters^{-1}, 1,260 centimeters^{-1} and 1,000 centimeters^{-1} with the sorbic acid standard solution.

The reason why the solution method was adopted in our experiment lies in the fact that sorbic acid is satisfactorily dissolved in carbon tetra-

chloride and that the solution method was considered to be better than the tablet method because the amounts of sorbic acids contained in foods are too small.

As the solvents to be used in the solution method, we examined chloroform and carbon disulfide in addition to carbon tetrachloride. It was found that carbon tetrachloride, which shows little obstacles by the absorption of the solvent itself, is best suited as a solvent for sorbic acid.

2. On the Method of Determination

In the case of infrared spectra, the rule of Lambert-Beer becomes valid within a certain range of concentrations between the intensity of the absorption and the concentration of the component as in the case of ultraviolet and visible range absorption spectra. By using the standard solution of sorbic acid, therefore, the carbon tetrachloride solutions whose sorbic acid concentrations are 2, 4, 6, 8 and 10 milligrams per milliliter were prepared, the infrared absorption spectra of these were measured and the 1,690 centimeters^{-1} and 1,000 centimeters^{-1} where absorptions are comparatively large and where the obstacle by other food additives is comparatively small were selected as the key bands of sorbic acid. On this basis, it is believed better to measure the absorbances and prepare the calibration curves in accordance with the base-line method. (Refer to Figure 2)

(Figure 2 , Calibration curve for sorbic acid at each wave number).

3. Effect of Food Additives Other than Sorbic Acid

In a measurement of sorbic acid contained in foods, the effect of other food additives and natural substances will have to be taken into consideration. Therefore, the degree of solubility in carbon tetrachloride and infrared absorption spectra of several kinds of food additives which seem to be contained together in many cases were examined.

(1) Solubility in Carbon Tetrachloride

Since the permissible amount of sorbic acid to be contained in food is comparatively high, it does not receive much of the effect of other food additives. However, the solubility of several food additives which are believed to be contained in food at high frequency in carbon tetrachloride was examined.

As for its method, a 0.5 per cent carbon tetrachloride solution of each of these food additives was prepared and the infrared absorption spectra of this solution (a saturated solution in the case of a difficultly soluble solution) was measured.

As shown in Table 1, nitrofurazone, nitrofuryl acrylic acid amide, saccharine, dulcin, and cyclamic acid were all found hardly soluble, with no absorption being shown in the infrared region. On the other hand, benzoic acid, salicylic acid, dehydroacetic acid, paraoxybenzoic acid esters, and methyl naphthoquinone were dissolved in carbon tetrachloride and showed absorptions.

Among the salicylic acids and paraoxybenzoic acid esters, ethyl ester alone was not completely dissolved as far as the outside appearance was concerned. Since it showed some absorption, it is believed to be partially soluble.

(Table 1 Solubility in CCl_4.

Accordingly, in the infrared absorption analysis of sorbic acid, the effects of nitrofurazone, saccharin, dulcin and cyclamic acid which are not soluble in CCl_4 can all be removed by the solution method.

(2) On the Infrared Absorption of Food Additives Other Than Sorbic Acid

The effect (or the absence of its) in the case where sorbic acid coexists with a substance which is dissolved in carbon tetrachloride and which shows an infrared absorption spectrum is as follows:

Figure 3 (a) through (g) shows a comparison between the infrared absorption spectra of benzoic acid and salicylic acid, etc. which are soluble in carbon tetrachloride and the absorptions shown at the time when these substances coexist with sorbic acid. From these Figures, an examination was made on the effects of the other food additives at the characteristic wave numbers of sorbic acid. The result obtained is shown in Table 2.

(Figure 3-a Infrared spectra of benzoic acid and benzoic acid plus sorbic acid)

(Figure 3-b Infrared spectra of salicylic acid and salicylic acid plus sorbic acid)

(Figure 3-c Infrared spectra of dehydroacetic acid and dehydroacetic acid plus sorbic acid)

(Figure 3-d Infrared spectra of methyl naphthoquinone and methyl naphthoquinone plus sorbic acid)

(Figure 3-e Infrared spectra of ethyl (p_-hydroxybenzoate and ethyl (p_-hydroxybenzoate plus sorbic acid)

(Table 2 Interference of various preservatives at each wave number.)

From the above Table, it is observed that it is the benzoic acid alone that obstructs the absorption at 1,690 centimeters^{-1} of sorbic acid. Likewise, it is the dehydroacetic acid alone which obstructs the absorption at 1,000 centimeters^{-1}. If the wave numbers of these two can be used, therefore, it is possible to carry out a determination of sorbic acid even when these additives co-exist. If, in this case, other means such as chromatography, etc can be used for the determination of a substance which co-exists, the determination will become all the more accurate.

In actuality, however, the use of benzoic acid, salicylic acid, dehydroacetic acid and paraoxybenzoic acid esters is prohibited in those foods

where the use of sorbic acid is permitted. Therefore, their effect is believed to be almost non-existent. Since the permissible amount of various preservatives such as methyl naphthoquinone, etc. in foodstuffs is in the range between 1/5 and 1/10 of the amount of sorbic acid, it is not believed that their absorption will have a serious effect upon the absorption of sorbic acid, provided that the standard amounts of these additions are strictly adhered to. In fact, the infrared absorption spectrum of a solution containing methyl naphthoquinone in an amount which is 1/10 of the amount of sorbic acid was hardly any different from the absorption of sorbic acid alone.

(Figure 3-f Infrared spectra of propyl p-hydroxybenzoate and propyl p-hydroxybenzoate plus sorbic acid)

(Figure 3-g Infrared spectra of butyl p-hydroxybenzoate and butyl p-hydroxybenzoate plus sorbic acid)

In addition, in the case of baked beans, fish meat sausages and smoked cuttlefish, etc. which did not contain any sorbic acid, a blank test was carried out by using the test solutions which were obtained in accordance with the method of preparing test solutions as described above. As is shown in Figure 4, the result obtained shows the fact that almost no absorption is shown in the key band and, therefore, it is possible to remove the blank test values obtained by using the obstructing substances as measured in the case of ultraviolet absorptions.

(Figure 4 Infrared spectra of CCl_4 and blank test solution obtained from foods)

III COnclusion

The determination of sorbic acid based on the infrared absorption spectra can be used as means for qualitative determination since its spectra are extremely highly characteristic as compared with the ultraviolet absorptions. If its characteristic absorptions are used, it is possible to carry out a

determination even in the case where other additives are co-existing. One difficulty in infrared analysis is the fact that it is not possible to carry out a satisfactory measurement unless the concentration of the test solution is made considerably higher than in the ultraviolet absorptions.

In the case of sorbic acid, however, since the permissible amount to be added to food is large, it is believed that the disadvantage in this respect is not as great as it appears.

References:

1) Tetsuo Inoue, Taro Kawamura, Shoya Kamijo and Rinko Asakura: Chemistry of Hygiene, 10(1), 26 (1964).

2) Tetsuo Inoue, Taro Kawamura, Shoya Kamijo and Rinko Asakura: Chemistry of Hygiene, 10 (1), 47 (1964).

3) Kaji Nakanishi: "Infrared Absorption Spectrum Determination and Practice thereon," (1962) Nankodo Co.

(Translated by Carl Demrick Associates,Inc/RO/t)

Table 1. Solubility in CCl_4

Soluble substance	Insoluble substance
Benzoic acid	Nitrofurazone
Salicylic acid	Nitrofurylacrylamide
Dehydroacetic acid	
Ethyl *p*-Hydroxybenzoate	Saccharin
Propyl *p*-Hydroxybenzoate	Dulcin
Butyl *p*-Hydroxybenzoate	
Methyl naphthoquinone	Sodium Cyclamate

Table 2. Interference of Various Preservatives in Each Wave Number

Kinds of preservatives \ Wave number	1690 cm^{-1}	1640 cm^{-1}	1415 cm^{-1}	1260 cm^{-1}	1000 cm^{-1}
Benzoic acid	×	○	×	×	○
Salicylic acid	○	×	×	○	○
Dehydroacetic acid	○	×	×	×	×
Ethyl *p*-Hydroxybenzoate	○	○	○	×	○
Propyl *p*-Hydroxybenzoate	○	○	○	×	○
Butyl *p*-Hydroxybenzoate	○	○	○	×	○
Methyl naphthoquinone	○	×	○	×	○

○ no interference
× interference

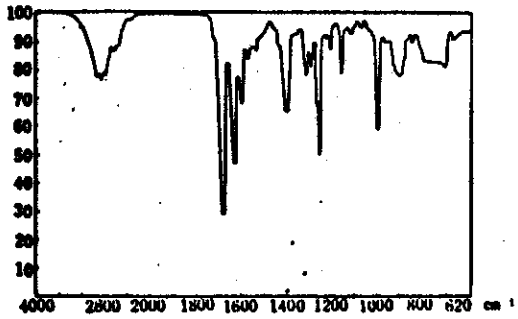


Fig. 1. Infrared spectra of sorbic acid
I.R: Sobic acid (KRS 6 0.1 mm, CCl_4 10 mg/ml)

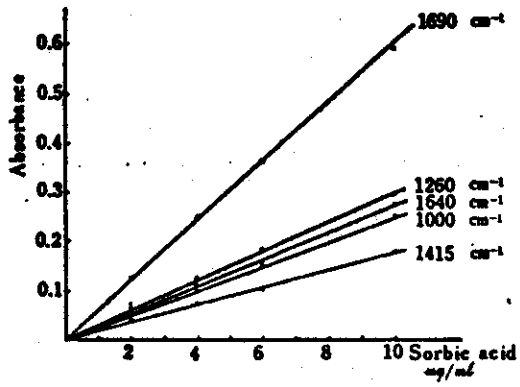


Fig. 2. Calibration curve for sorbic acid each wave number

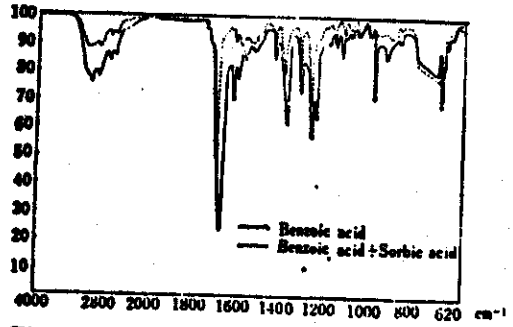


Fig. 3-a. Infrared spectra of benzoic acid and benzoic acid+sorbic acid

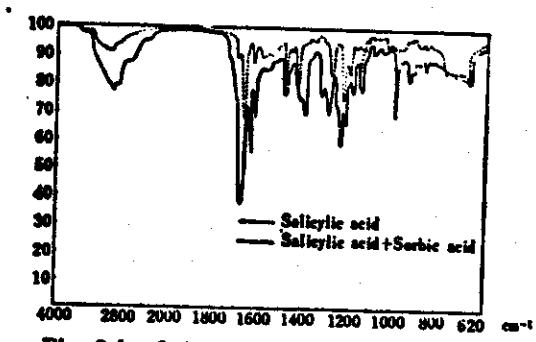


Fig. 3-b. Infrared spectra of salicylic acid and salicylic acid+sorbic acid

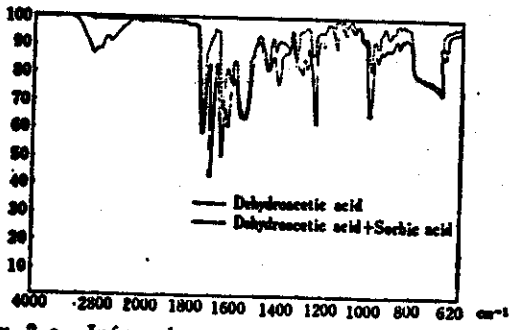


Fig. 3-c. Infrared spectra of dehydroacetic acid and dehydroacetic acid+sorbic acid

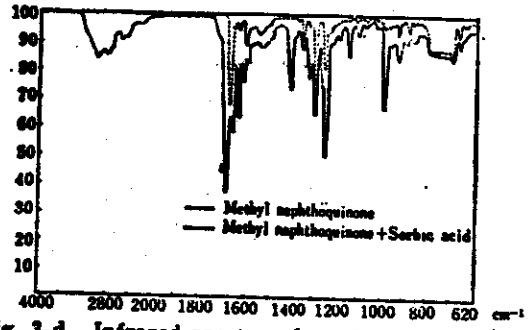


Fig. 3-d. Infrared spectra of methyl naphthoquinone and methyl naphthoquinone +sorbic acid

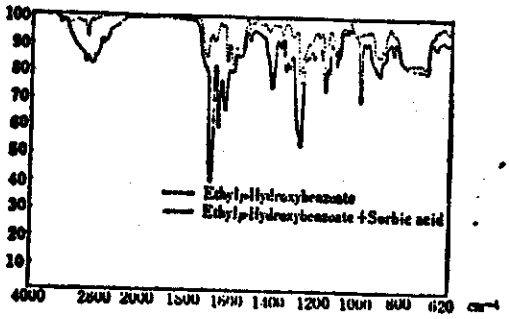


Fig. 3-e. Infrared Spectra of ethyl *p*-hydroxybezoate and ethyl *p*-hydroxybenzoate +sorbic acid

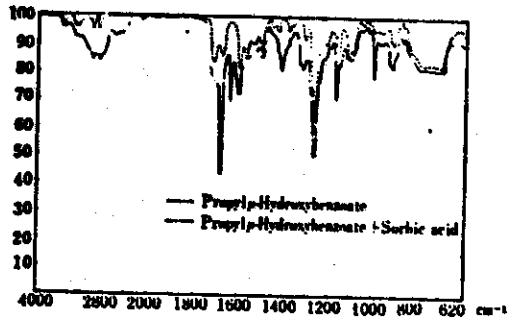


Fig. 3-f. Infrared spectra of propyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate +sorbic acid

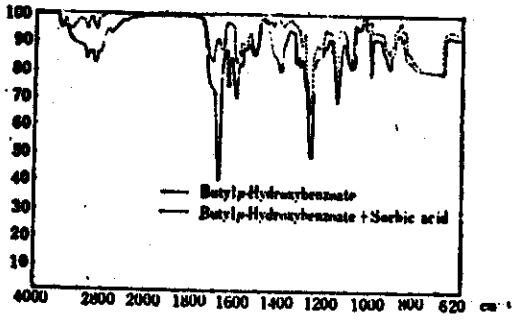


Fig. 3-g. Infrared spectra of butyl *p*-hydroxybenzoate and butyl *p*-hydroxybenzoate +sorbic acid

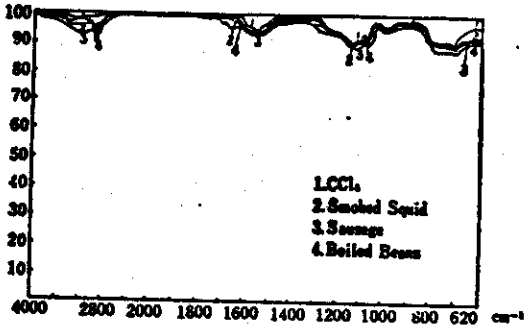


Fig. 4. Infrared spectra of CCl_4 and blank test solution obtained from foods

食品中の防腐剤に関する研究（第3報）

赤外部吸収スペクトルによるソルビン酸の定性定量法について

（昭和39年7月2日受理）

井上哲男* 河村太郎*
上条昌弥* 浅倉倫子*

Studies on the Preservatives in Foods (III)
Analysis of Sorbic Acid by Infrared Spectra

Tetsuo INOUE, Taro KAWAMURA, Masaya KAMIJO, and Michiko ASAKURA

(Yokohama Municipal Public Health Laboratory: 2-102, Nakamura-cho, Minami-ku, Yokohama)

Sorbic acid in foodstuffs has been determined UV-spectrometrically. However, the acid shows only one absorption band in UV region and its shift and being masked by interfering substances reduce the accuracy and precision of the analysis.

In the present report, studies on the infrared spectrometric analysis of the acid are described. The results obtained are as follows:

1. Characteristic absorption of sorbic acid in carbon tetrachloride in IR-region provides an IR spectrometric method of analysis of the acid.
2. For the quantitative analysis, 1690 cm^{-1} and 1000 cm^{-1} are useful as key bands for the acid.
3. The interfering effects of food additives and of natural substances on the analysis have, also, been investigated.

(Received July 2, 1964)

まえがき

食品中のソルビン酸の定性定量法として，現在おもに紫外部吸収スペクトル法が利用されているが，ソルビン酸の紫外部における吸収極大は1個所であり，しかも妨害物質が混在する場合は，ソルビン酸の吸収極大がずれたり，陰ぺいされるために，十分な定性定量を行ないにくくなる．この点について著者らは前報[1)2)]において，蒸留の際に焦げると妨害物質が留出することを指摘するとともに，その改良法として留液中の妨害物質の除去法および多数の検体を処理する場合に都合のよい透析法について報告したが，これらの方法をより一層確実なものとするために，赤外部吸収スペクトルによるソルビン酸の定性定量法について検討を行ない，二，三の知見を得たので以下に報告する．

I 実験方法

1. 装置

島津自記赤外分光光度計 IR—27A形

2. ソルビン酸標準溶液

市販ソルビン酸をアルコールから再結晶し融点133～135°のものを，四塩化炭素に溶かし，2～10 mg/ml の溶液を作る．

3. 検液の調製法

細切またはすりつぶした試料5～10 g（ソルビン酸5～10 mg に対応する量）をコロジオン膜に入れ，これに透析補助液20 ml を試料とよく混和させながら加える．つぎに膜の上端を糸で密封し，あらかじめ200 ml の蒸留水を加えた300 ml の広口ビン中に膜中の水面と，ビン中の水面がほぼ同じ高さになるように懸垂し，20～24時間ときどき揺り動かしながら常温に放置し，透析を行なう[3)]．

つぎに，透析液100～200 ml を300～500 ml の分液ロ

* 横浜市衛生研究所：横浜市南区中村町2—102

ートにとり，10%塩酸5 ml，塩化ナトリウム30～60 gを加え約同量のエーテルで抽出する．水層を分離し，さらに半量のエーテルで2回抽出を行なう．エーテル層を合し，10～20 ml の蒸留水で水洗し，洗液を捨てエーテル液を無水硫酸ナトリウムで脱水したのちエーテルを留去し残渣を軽く送風乾燥させたのち，正確に1～2 ml の四塩化炭素に溶解し，試験溶液とする．

4. 測定操作

KRS-6 のセル板に厚さ 0.1 mm のスペーサーをはさんだ揮発液体用セルに注射筒を用いて標準溶液または試験溶液を注入し，これを試料光束側に置き，別に四塩化炭素のみを注入したセルを基準光束側に置き，赤外部吸収スペクトルを測定する．

II 実験結果および考察

1. 定性法について

ソルビン酸はその構造式 $CH_3-CH=CH-CH=CH-COOH$ よりリメチル基，カルボキシル基および共役二重結合に基づく特性吸収が出現することが予想される．ソルビン酸標準溶液について赤外部吸収スペクトルを測定した結果は Fig. 1 のようである．すなわち，2,960 cm^{-1} において $-CH_3$ による ν_{CH} の吸収，3,000～2,500 cm^{-1} において ν_{OH} の吸収が認められる．つぎに 1,690 cm^{-1} においては $-COOH$ に基づく $\nu_{C=O}$ の強い吸収がみられ，1,640 cm^{-1} と 1,620 cm^{-1} に共役二重結合による $\nu_{C=C}$ の吸収が認められる．1,415 cm^{-1}，1,325 cm^{-1}，1,260 cm^{-1} の吸収はいずれも $-COOH$ の2量体

$$-C\left\langle\begin{matrix}O\cdots H-O\\ O-H\cdots O\end{matrix}\right\rangle C-$$

のつくる面についての O-H 面内変角および C-O 伸縮とがカップリングしたものと考えられる[3]．また1,000 cm^{-1} における吸収は共役二重結合の δ_{CH} と思われる．以上のごとくソルビン酸はかなり特徴のある赤外部吸収スペクトルを示すので，赤外線分析によるソルビン酸の定性法としては，試料溶液の 2,960 cm^{-1}，1,690 cm^{-1}，1,640 cm^{-1}，1,620 cm^{-1}，1,415 cm^{-1}，1,325 cm^{-1}，1,260 cm^{-1} および1,000 cm^{-1} における吸収をソルビン酸標準溶液と比較することにより正確に行なうことができる．

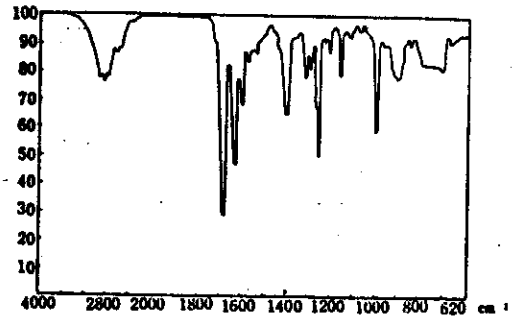


Fig. 1. Infrared spectra of sorbic acid
IR: Sobic acid (KRS-6 0.1 mm, CCl_4 10 mg/ml)

なお，本実験に溶液法を採用した理由は，ソルビン酸が四塩化炭素によく溶けることおよび食品中のソルビン酸を測定するにはその量が微量であるため錠剤法よりも溶液法の方が取扱いに適しているためである．また溶液法に用いる溶媒として四塩化炭素の他に，クロロホルム，二硫化炭素についても検討を行なったが，溶媒自身の吸収による妨害の少ない四塩化炭素がソルビン酸の溶媒として適していることがわかった．

2. 定量法について

赤外部吸収スペクトルの場合も，紫外，可視部吸収スペクトルの場合と同様に，吸収の強さと成分の濃度との間には一定濃度の範囲で Lambert-Beer の法則が成立する．そこでソルビン酸標準溶液を用いて，ソルビン酸濃度が2，4，6，8，10 mg/ml の四塩化炭素溶液を調製し，おのおのの赤外部吸収スペクトルを測定し，比較的吸収が大きく，しかも後述するように，他の食品添加物による妨害の少ない波数の1,690 cm^{-1} および1,000 cm^{-1} をソルビン酸の Key band として選び，吸光度を測定し base-line 法により検量線を作成するのがよいと考える (Fig. 2)．

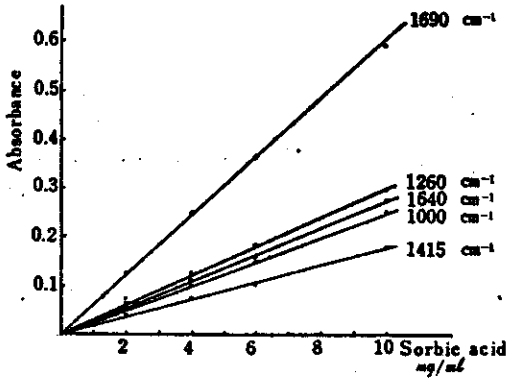


Fig. 2. Calibration curve for sorbic acid each wave number

3. ソルビン酸以外の食品添加物の影響

食品中のソルビン酸を測定する場合，他の食品添加物または天然物による影響を考慮しなければならない．それで共存の可能性が多い食品添加物数種について四塩化炭素に対する溶解度ならびに赤外部吸収スペクトルについて検討した．

(1) 四塩化炭素に対する溶解度

ソルビン酸の食品に対する許容量は比較的高いので，他の食品添加物による影響をそれほど受けないが，共存すると思われる数種の食品添加物について四塩化炭素に対する溶解度を調べた．

その方法は，これらの食品添加物の0.5%四塩化炭素溶液を調製し，この溶液（難溶性のものについてはその

飽和溶液）について赤外部吸収スペクトルを測定した．結果は Table 1 に示すようにニトロフラゾーン，ニトロフリルアクリル酸アミド，サッカリン，ズルチン，サイクラミン酸はほとんど不溶で，赤外部に全く吸収を示さなかった．一方安息香酸，サリチル酸，デヒドロ酢酸，パラオキシ安息香酸エステル類，メチルナフトキノンは四塩化炭素に溶解し，吸収を示した．サリチル酸およびパラオキシ安息香酸エステル類のうちエチルエステルだけは外観的には完全に溶解しないが，若干の吸収を示すので一部溶解するものと思われる．

Table 1. Solubility in CCl_4

Soluble substance	Insoluble substance
Benzoic acid	Nitrofurazone
Salicylic acid	Nitrofurylacrylamide
Dehydroacetic acid	Saccharin
Ethyl *p*-Hydroxybenzoate	Dulcin
Propyl *p*-Hydroxybenzoate	Sodium Cyclamate
Butyl *p*-Hydroxybenzoate	
Methyl naphthoquinone	

したがって，ソルビン酸の赤外線分析において，四塩化炭素に不溶のニトロフラゾーン，ニトロフリルアクリル酸アミド，サッカリン，ズルチン，サイクラミン酸などの影響は溶液法によって除去しうる．

(2) ソルビン酸以外の食品添加物の赤外部吸収について

四塩化炭素に溶け赤外部吸収スペクトルを示す物質とソルビン酸が共存した場合の影響の有無についてはつぎのとおりである．Fig. 3 a～g は四塩化炭素に可溶の安息香酸，サリチル酸などの赤外部吸収スペクトルとこれらの物質とソルビン酸が共存した場合の吸収を比較したものであり，これらの図からソルビン酸の特性波数において他の食品添加物の影響の有無を表にまとめたのが Table 2 である．この表よりソルビン酸の1,690 cm⁻¹における吸収を妨害するのは安息香酸のみであり，同様に

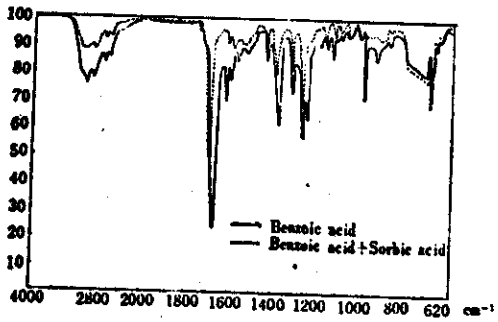


Fig. 3-a. Infrared spectra of benzoic acid and benzoic acid+sorbic acid

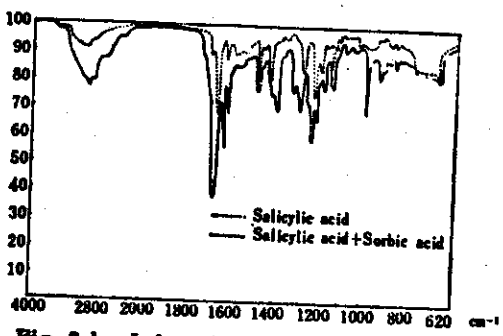


Fig. 3-b. Infrared spectra of salicylic acid and salicylic acid+sorbic acid

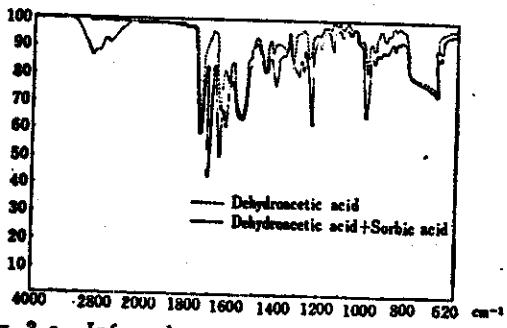


Fig. 3-c. Infrared spectra of dehydroacetic acid and dehydroacetic acid+sorbic acid

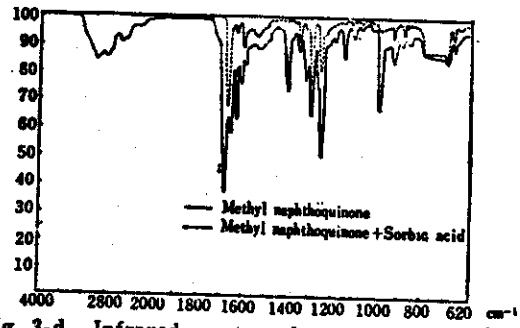


Fig. 3-d. Infrared spectra of methyl naphthoquinone and methyl naphthoquinone +sorbic acid

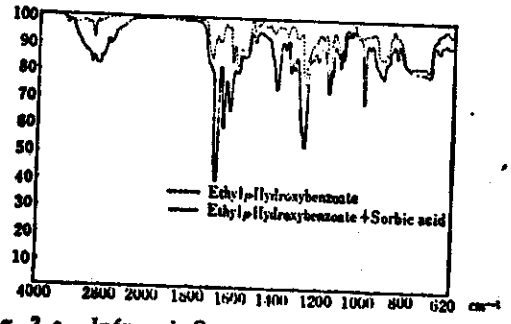


Fig. 3-e. Infrared Spectra of ethyl *p*-hydroxybezoate and ethyl *p*-hydroxybenzoate +sorbic acid

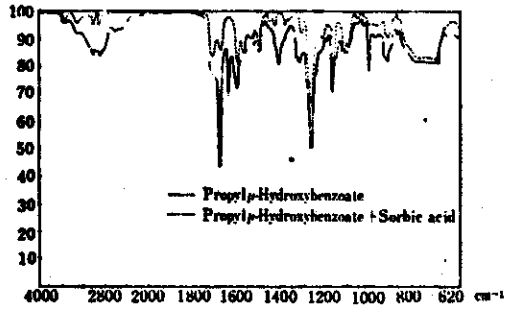


Fig. 3-f. Infrared spectra of propyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate +sorbic acid

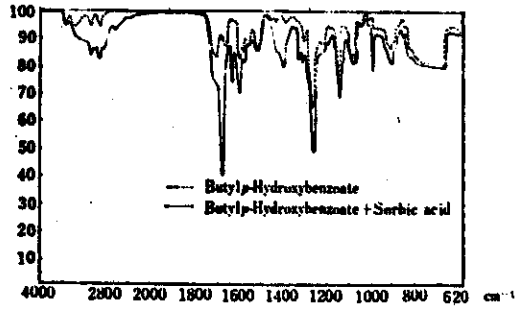


Fig. 3-g. Infrared spectra of butyl *p*-hydroxybenzoate and butyl *p*-hydroxybenzoate +sorbic acid

Table 2. Interference of Various Preservatives in Each Wave Number

Kinds of preservatives \ Wave number	1690 cm⁻¹	1640 cm⁻¹	1415 cm⁻¹	1260 cm⁻¹	1000 cm⁻¹
Benzoic acid	×	○	×	×	○
Salicylic acid	○	×	×	○	○
Dehydroacetic acid	○	×	×	×	×
Ethyl *p*-Hydroxybenzoate	○	○	○	×	○
Propyl *p*-Hydroxybenzoate	○	○	○	×	○
Butyl *p*-Hydroxybenzoate	○	○	○	×	○
Methyl naphthoquinone	○	×	○	×	○

○ no interference
× interference

1,000 cm⁻¹において吸収を妨害するのはデヒドロ酢酸のみであることがわかる．したがってこの両者の波数を利用すれば，これらの添加物が共存する場合でもソルビン酸の定量は可能である．この場合，別の手段たとえばクロマトグラフィーとか紫外部吸収スペクトルなどにより共存する物質が判明すれば定量は一層確実なものとなる．

しかし，実際には安息香酸，サリチル酸，デヒドロ酢酸，パラオキシ安息香酸エステル類は，ソルビン酸の使用を認めている食品への使用が禁じられているので，これらの影響はほとんどないものと考えられる．またメチルナフトキノンをはじめ各防腐剤の食品への使用許容量がソルビン酸に比較して1/5～1/10であるため添加基準量が守られていれば，これらの吸収がソルビン酸の吸収に重大な影響を及ぼすとは考えられない．実際にソルビン酸の$^1/_{10}$の量のメチルナフトキノンを含む溶液の赤外部吸収スペクトルは，ソルビン酸のみの吸収とほとんど変らなかった．

また，ソルビン酸を全く含まない煮豆，魚肉ソーセージ，イカのクン製品につき，前に述べた検液の調製法に従って操作して得た試験溶液を用いて空試験を行なった結果は Fig. 4 に示すように Key band において，ほとんど吸収を示さないので，紫外部吸収の場合に測定された妨害物質による盲検値を除去することができる．

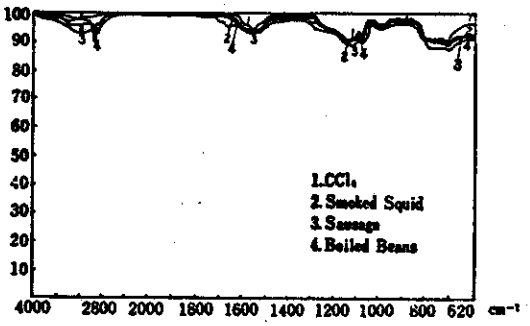


Fig. 4. Infrared spectra of CCl_4 and blank test solution obtained from foods

Ⅲ むすび

赤外部吸収スペクトルによるソルビン酸の定性定量は紫外部吸収に比べ，そのスペクトルはきわめて特徴があるので，定性の手段として用いることができる．すなわち，その特性吸収を利用すれば他の添加物が共存する食品においても定量が可能である．ただ赤外線分析における難点は，紫外部吸収などより試料溶液の濃度をかなり高くしないと十分な測定ができないことである．しかしソルビン酸の場合食品への使用許容量が多いのでこの点

における不利はさほどないと考える.

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4-アミノアンチピリンによるクロルフェノール類の定量 (第1報)

食品中の Trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide の定量

(昭和37年10月10日受理)

近藤竜雄* 川城巌*

Determination of Chlorophenols with 4-Aminoantipyrine (I)
Determination of Trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide in Foods

Tatsuo KONDO and Iwao KAWASHIRO

(National Institute of Hygienic Sciences:
2-chome, Tamagawayoga-machi, Setagaya-ku, Tokyo)

Determination of trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide in food was studied and the colorimetric method was established. Its principle is based on the oxidative condensation of 2,4,5-trichlorophenol, produced from trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide in food by steam distillation, with 4-aminoantipyrine in pH 7.8 phosphate buffered medium to yield an indophenol type pigment.

Residual trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide in the fruit of *Citrus natsudaidai* HAYATA which had been soaked in 10 v/v % ethanolic aqueous solution containing 500 ppm of it for one hour, was determined according to the authors' method.

3~10 days after soaking, the amount of residual trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide was not detected in the fruit (except peel and seed), but 16~22 ppm of it was found in the peel of *Citrus natsudaidai* HAYATA.

(Received October 10, 1962)

いわゆるヨーカビン(Trimethyl-lauryl-ammonium-2,4,5-trichlorophenoxide, 以下 T.T と略称) は昭和29年頃より, つくだ煮その他の防腐剤としての指定申請が厚生省食品衛生課に対しなされたが, このものの毒性に関し種々論議が行なわれ現在なお懸案中のものである. ところが昨年より本品を表面塗布による果実防腐剤として使用する可否について, 新たなる局面が展開されてきたため, 行政当局より果実に付着残留した本品の検出定量法の研究を要請され, その検討を行なった. 著者らはフェノール側から定量することを企て食品中に存在するT.T からリン酸酸性で水蒸気蒸留することによって2,4,5-トリクロルフェノール (以下2,4,5-T と略称) を分離し, なお存在する定量妨害物を水酸化ナトリウムアルカリ性でエーテル抽出することによって除去し, 2,4,5-Tの単離を試みた. クロルフェノール類の定量法にはニトロソフェノール法[1], IR[2][3], UV, ガスクロマト

* 国立衛生試験所食品部：東京都世田谷区玉川用賀町2-203

CHROM. 5662

Gas chromatographic determination of preservatives in food

The determination of preservatives in food involves the extraction of the preservative from the food. Two methods of isolation are generally used, depending on the properties of the preservative, *viz.* steam distillation, where the preservative is separated from the food sample and then extracted from the distillate by an organic solvent[1-3], and direct extraction of the preservative from the food sample by an organic solvent[4-9].

The procedures used for the subsequent determination of the preservatives vary from spectrophotometric methods in the visible, UV and IR regions to thin-layer and gas chromatographic methods. As the gas chromatographic methods[3,7,9,10] seem to be the most practical, we have used these methods for some years. Here again we must differentiate between injection of the preservative without any modification on to polar columns[8,9] and injection after conversion into an ether or an ester[4,6,11,12]. We have tried injecting the free acids on to polar columns[5], as NISHIMOTO[7] did, but found the method rather difficult because the standard curves varied with time, as was also reported by NOBE AND STANLEY[8]. For that reason we switched over to conversion to silyl ethers[10].

Experimental

Preparation of samples. For the preparation of samples of marmalades, mustard, mayonnaise, sardines in oil, canned tunny and margarine 10 g of the food and 60 g sand were thoroughly mixed in an erlenmeyer flask. After the addition of 3 ml 25% H_2SO_4, the mixture was extracted three times with 30 ml ether by agitating the ground-glass stoppered flask vigorously for at least 1 min. The preservatives were then extracted from the ether by two extractions with 20 ml 0.1 *N* NaOH. (After each NaOH addition 10 ml saturated NaCl solution were added.) The aqueous layer was neutralised with HCl (1:3) to the end point of the Methyl Red indicator. The preservatives were then extracted from the acid aqueous phase with one 100-ml and four 50-ml portions of chloroform. (After the first addition of chloroform another 10 ml saturated NaCl solution were added.) The extracts thus obtained were passed through a funnel containing anhydrous Na_2SO_4. A washing procedure as described below for beer and wine can also be recommended. After concentration of the combined chloroform extracts in a Kuderna–Danish evaporator on a water-bath to approximately 5 ml, the lower receiving tube of the Kuderna–Danish evaporator was directly heated on a water-bath at 60° and concentrated under a current of air to approximately 1 ml. One millilitre of internal standard (4 mg/ml methyl gallate in pyridine) was then added. The 2 ml were concentrated to approximately 1 ml and 0.2 ml N,O-bis(trimethylsilyl)acetamide (BSA) was added. This mixture was heated under reflux for 15 min at 60°. The solutions were then ready for gas chromatographic analysis.

For the preparation of beer and wine samples 3 ml 25% H_2SO_4 were added to 20 ml beer or wine and the mixture was extracted three times with 30 ml ether by agitating the stoppered flask vigorously for at least 1 min. The ethereal layer was then washed with 50 ml water. After the washing step the preservatives were extracted as described above.

Silanisation. The silanisation methods tested were: (a) the method of SWEELEY *et al.*[13] — hexamethyldisilazane and trimethylchlorosilane; (b) the method of BROBST AND LOTT[14] — hexamethyldisilazane and trifluoroacetic acid; (c) trimethylsilylimidazole; and (d) N,O-bis(trimethylsilyl)acetamide (BSA)[15,16]. Some difficulties were experienced owing to the amount of water in the final extract. Therefore, under the conditions used, the best silanisation reagents were those described in the method of BROBST AND LOTT[14] and BSA. Trifluoroacetic acid also causes trouble as it corrodes the detector electrodes. With some detectors, for a fortnight after sample injection the electrodes were completely corroded. The best results were obtained with BSA.

The most attractive points of the silanisation process were: (a) the ease with which derivatives of all the preservatives examined (benzoic and sorbic acid and the propyl, ethyl and methyl esters of *p*-hydroxybenzoic acid) were obtained; (b) the very reproducible standard curves obtained day after day; and (c) the high stability of the silyl ethers (the same solution injected the following day giving nearly the same results).

Isolation of the preservatives. The esters of *p*-hydroxybenzoic acid are not very steam volatile, so the steam distillation method can be used only for the quantitative isolation of sorbic and benzoic acid (Fig. 1). Fig. 1 shows that the amount of ester left in the residue in the flask is nearly equal to the amount in the distillate. On the other hand, several methods based on extraction with an organic solvent[4-9] give good results on some kinds of food but very bad recoveries on other kinds of food. The extraction with ether as proposed by GROEBEL[6] is indeed satisfactory for fatty food, but not for food such as marmalade. Another problem is caused by the extraction of the preservatives from the ether solution with 1 *N* NaOH solution. This concentration of NaOH causes hydrolysis of the esters, especially the propyl and ethyl esters of *p*-hydroxybenzoic acid. This was easily demonstrated by the supplementary peak

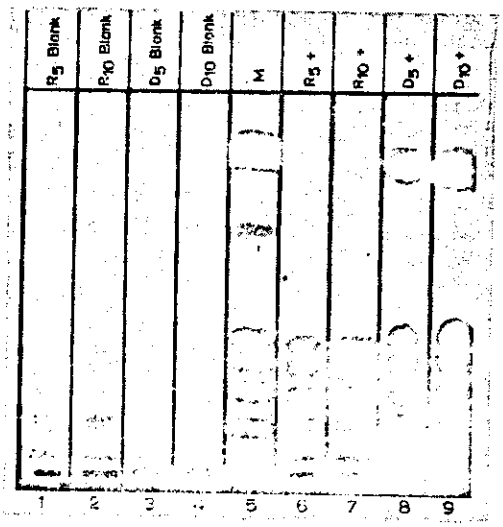
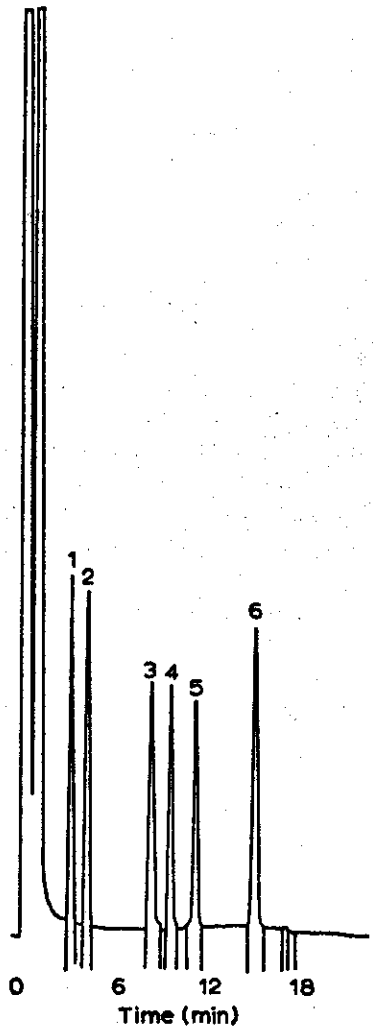


Fig. 1. Thin-layer chromatogram of a mixture of sorbic and benzoic acid and esters of *p*-hydroxybenzoic acid. D_5 and D_{10} refer to 5 and 10 μl of the distillate, and R_5 and R_{10} to 5 and 10 μl of the residue, respectively. + = Sample spiked with mixture M, a mixture of nine preservatives added to the sample before steam distillation.

of *p*-hydroxybenzoic acid, and the diminishing peak area of the propyl and ethyl esters.

Gas chromatography. A 1-ml standard solution containing 0.5, 2, 4, 6 and 8 mg/ml of benzoic and sorbic acid and the propyl, ethyl and methyl esters of *p*-hydroxybenzoic acid, respectively, was prepared, to which 1 ml of internal standard was added. This mixture was subjected to gas chromatography (Fig. 2).

Fig. 3 shows a standard curve for sorbic acid from which it is possible to read the amount of preservative in 10 g of sample directly.



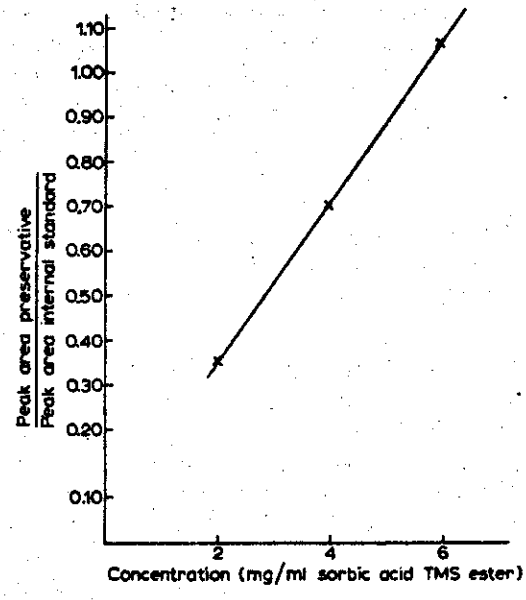


Fig. 2. Gas chromatogram of the TMS derivatives of five preservatives. Column: 3 m 3% SE-30 on 100–120-mesh Aeropak. Temperature programmed from 90° to 290° at a rate of 8°/min. Detector and injector temperatures: 300° and 290°, respectively. Carrier gas: nitrogen, flow-rate approximately 30 ml/min. FID detector. Quantitation with a digital integrator. 1 = Sorbic acid; 2 = benzoic acid; 3 = methyl, 4 = ethyl and 5 = propyl ester of *p*-hydroxybenzoic acid; 6 = internal standard (the TMS derivative of methyl gallate).

Fig. 3. Standard curve for sorbic acid.

Results

The recoveries for the preservatives in the samples tested were very good, *i.e.* at least 85 %, except for the propyl and ethyl esters of *p*-hydroxybenzoic acid in mustard, which gave lower recoveries (57 and 76 %, respectively). Even the addition of 80 or 100 g of sand did not increase the recovery. The best results were in general obtained with a sample of 10 g, 60 g sand and three extractions with 30 ml ether. Without the addition of sand, samples such as marmalade gave very low recoveries because of the bad penetration of the solvent in the sample.

I wish to thank Dr. L. A. HOODLESS of the Laboratory of the Government Chemist, London, for his suggestions. Grateful acknowledgement is made to Mr. E. BAERT for the practical experiments.

Institute of Hygiene and Epidemiology,
14 Jul. Wytsmanstreet, 1050 Brussels (Belgium)

J. A. W. GOSSELÉ

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CHROM. 4219

CHROMATOGRAPHY OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS AND COLUMNS

KINZO NAGASAWA AND HISAE YOSHIDOME
Faculty of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo (Japan)

AND

RYUZO TAKESHITA
National Institute of Public Health, 1-39, Shibashirokane-Daimachi, Minato-ku, Tokyo (Japan)

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SUMMARY

The separation of eight kinds of food preservative by one- and two-dimensional chromatography on polyamide layers was investigated. A comparison was made between the detection limits on polyamide and silica gel layers, and the superiority of the polyamide thin layers was confirmed.

The separation of the food preservatives on a polyamide column with two types of elution system was established. The recovery of these compounds was satisfactory.

INTRODUCTION

After establishing the efficacy of polyamide in chromatography[1-7], it has been applied extensively to column and thin-layer chromatography where it was found that the separation was very much influenced by the hydrogen bond formed between the HO-group in phenolic compounds and the –CONH-group in polyamide[1,2,8].

This paper deals with the separation of food preservatives (aromatic acids with and without a phenolic HO–group and its alkyl esters, and some other conventional preservatives) by column and thin-layer chromatography using polyamide as the chromatographic medium.

EXPERIMENTAL

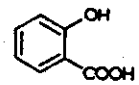
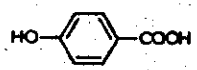
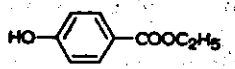
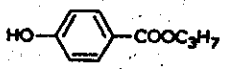
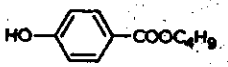
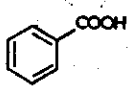
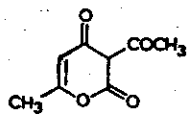
Materials

Polyamide powder from two sources, Polyamide Woelm TLC and Wakō Polyamide B-10* were used for thin-layer chromatography while for the column chromatography only Polyamide Woelm was used.

* "Wakō Polyamide B-10" contains 10% anhydrous calcium sulfate as a binder (Wakō Pure Chemical Industries, Ltd., Nihonbashi-honcho, Chuō-ku, Tokyo, Japan).

TABLE I

WAVE LENGTHS AND MOLAR ABSORBANCY INDICES OF FOOD PRESERVATIVES AT THEIR UV ABSORPTION MAXIMA

Sample		*λmax (mμ) and ε × 10⁻³*					
		In water		*In 1% (w/w) NH_3*		*In H_2O–AcOH–MeOH (5.5:0.5:4, v/v)*	
Salicylic acid		232 298	6.66 3.37			305[a]	3.60[a]
p-Hydroxybenzoic acid		251	11.8	280	17.5	256	15.0
Ethyl *p*-hydroxybenzoate		256	15.0	297	23.4	256	15.9
n-Propyl *p*-hydroxybenzoate		256	15.0	297	24.1	256	15.9
n-Butyl *p*-hydroxybenzoate		256	15.5	297	24.2	256	15.9
Benzoic acid		227.5	9.18			—[b]	—[b]
Sorbic acid	CH_3—CH=CH·CH=CH·COOH	260	23.6			262	29.3
Dehydroacetic acid		227.5 306	14.5 12.1			308	14.1

[a] λ max and its molar absorbancy of salicylic acid were measured in H_2O–AcOH–MeOH (5.5:0.5:4).
[b] λ max and its molar absorbancy of benzoic acid could not be measured in this medium.

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Solvent systems for thin-la

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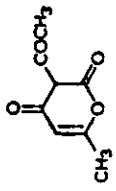
Sorbic acid	$CH_3—CH=CH·CH=CH·COOH$	260	2.6	227.5 306	14.5 12.1	262 308	20.1 14.1
Dehydroacetic acid							

a λ max and its molar absorbancy of salicylic acid were measured in H_2O–AcOH–MeOH (3:1:6).
b λ max and its molar absorbancy of benzoic acid could not be measured in this medium.

The food preservatives used in this experiment are of a specially refined grade. The chemical and optical characteristics of the preservatives are as listed in Table I.

Preparation of the polyamide chromatoplates

(a) One gram of Polyamide Woelm TLC was mixed with 12 ml of ethanol to give a homogeneous suspension, which was spread evenly with a suitable applicator on a glass plate (20 × 20 cm) to a thickness of 0.25 mm. The plate was dried in air at room temperature and stored in a desiccator.

(b) Fourteen grams of Wakō Polyamide B-10 were made into a homogeneous suspension by mixing it with 56 ml of distilled water. The chromatoplate was prepared in the same way as (a).

Solvent systems for thin-layer chromatography

The solvent systems (A–F) used in the experiments are listed in Table II.

Application of samples and development of chromatoplates

For one-dimensional chromatography, 0.5 μl of an 0.2 % (w/v) ethanolic solution of each preservative was applied except for dehydroacetic acid and benzoic acid where 1.5 μl of a sample of the same concentration was used. Sample application was made on a line 3 cm from the lower edge, and the sample was developed by the ascending method.

In order to ensure the equilibration of the vapor in the chamber, the inside was lined with filter paper soaked in the solvent.

The development was continued until the solvent reached 12 cm from the point of sample application.

For two-dimensional chromatography, 1 μl of an 0.2 % (w/v) ethanolic solution of the samples was applied; as above increased amounts (2 μl) of dehydroacetic acid and benzoic acid were applied. Samples were spotted at a point 3 cm from both edges.

Detection of the spots on the chromatoplates

The spots can be seen under UV light as dark areas against a bright background, but for confirmation's sake *p*-hydroxybenzoic acid, its esters, and salicylic acid were detected as reddish violet spots by spraying with diazotized sulfanilic acid reagent; and dehydroacetic acid, sorbic acid and benzoic acid gave reddish spots against a yellow background with Methyl Red reagent.

The composition of the reagents used are as follows:

Diazotized sulfanilic acid. One gram of sulfanilic acid, dissolved in 8 ml of conc. HCl and 100 ml of distilled water, is added to the same volume of 0.69 % $NaNO_2$ solution.

Methyl Red. A mixture of 10 ml of 0.1 % ethanolic solution of Methyl Red and 20 ml of 0.1 *M* phosphate buffer (pH 7.0).

Preparation of the polyamide column and application of samples

Forty-five grams of Polyamide Woelm were washed with distilled water, methanol and chloroform, successively. After drying in air, the washed polyamide powder was suspended in the same solvent as that used for elution, and the powder was packed into a glass column (1.7 × 50 cm) to form a column bed of 45 cm height. The samples

TABLE II

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS

Symbol	*Components*	*Ratio (v/v)*
A	MeOH–H_2O	6:4
B	Acetone–H_2O	5:5
C	MeOH–AcOH–H_2O	5:1:4
D	MeOH–conc. NH_3–H_2O	2:1:7
E	AcOH–H_2O	3:7
F	*n*-Hexane–AcOH	8:2

TABLE III

R_F VALUES OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS

(*a*) *R_F values on polyamide (Woelm)*

Sample	*Solvent system*					
	A	*B*	*C*	*D*	*E*	*F*
Salicylic acid	0.05	0.05	0.18	0.56	0.14	0.25
p-Hydroxybenzoic acid	0.13	0.18	0.30	0.96	0.16	0.04
Ethyl *p*-hydroxybenzoate	0.32	0.41	0.38	0.64	0.16	0.11
n-Propyl *p*-hydroxybenzoate	0.27	0.37	0.32	0.55	0.10	0.15
n-Butyl *p*-hydroxybenzoate	0.22	0.32	0.29	0.44	0.07	0.19
Benzoic acid	0.14	0.21	0.46	0.85	0.23	0.56
Sorbic acid	0.20	0.29	0.56	0.92	0.32	0.64
Dehydroacetic acid	0.18	0.25	0.73	0.79	0.54	0.45

(*b*) *R_F values on polyamide (Wakō B-10)*

Sample	*Solvent system*					
	A	*B*	*C*	*D*	*E*	*F*
Salicylic acid	0.12	0.16	0.25	0.75	0.29	0.40
p-Hydroxybenzoic acid	0.31	0.34	0.53	0.95	0.33	0.06
Ethyl *p*-hydroxybenzoate	0.53	0.63	0.61	0.76	0.34	0.15
n-Propyl *p*-hydroxybenzoate	0.49	0.58	0.56	0.68	0.25	0.21
n-Butyl *p*-hydroxybenzoate	0.44	0.54	0.52	0.58	0.18	0.28
Benzoic acid	0.34	0.33	0.66	0.91	0.43	0.62
Sorbic acid	0.43	0.44	0.72	0.93	0.55	0.68
Dehydroacetic acid	0.45	0.40	0.83	0.86	0.73	0.59

(each 10 μmoles) were dissolved in 5 ml of the same solvent and applied on the top of the column. The elution was performed at a flow rate of 0.22–0.25 ml per min, and each 3.5 ml fraction was collected. The concentration of the food preservatives in the fractions was determined by measuring the absorbancy at its λ max* in the medium used for elution.

* In the case of benzoic acid, the absorbancy was measured at 240 mμ, as benzoic acid has no absorption maximum in H_2O–AcOH–MeOH (5.5:0.5:4).

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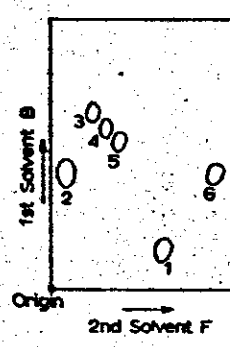

Fig. 1. Two-dimensio
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0.23	0.56
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0.54	0.45

E	F
0.29	0.40
0.33	0.06
0.34	0.15
0.25	0.21
0.18	0.28
0.43	0.62
0.55	0.68
0.73	0.59

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RESULTS AND DISCUSSION

Thin-layer chromatography

The solvent systems suitable for the separation of the food preservatives on polyamide layers are summarized in Table II. They are classified into two types, solvents A–E and solvent F, according to the polarity of the constituent solvents. Tables III a and b show the R_F values of the food preservatives in the above solvent systems.

It may generally be concluded from these tables that the Woelm Polyamide possesses stronger adsorption properties than the Wakō Polyamide B-10.

Hydrogen bonding effects can be observed from the R_F values of salicylic acid and *p*-hydroxybenzoic acid which are known to form an intra-molecular hydrogen bond and inter-molecular hydrogen bond, respectively.

Comparison of the differences in the R_F values of these compounds in any two types of solvent A–E or one of these and solvent F shows that a complete separation could be obtained by two-dimensional development in either of the following combinations:

Solvent B–Solvent F
Solvent B–Solvent E

Of all the possible combinations of the solvent systems listed in Table II, these two combinations gave the best distribution of spots on the chromatograms (Fig. 1).

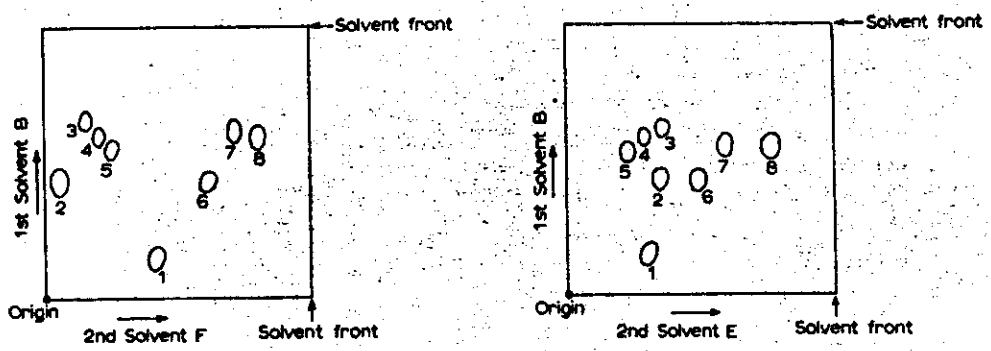

Fig. 1. Two-dimensional chromatograms of food preservatives on polyamide layers. 1 = salicylic acid; 2 = *p*-hydroxybenzoic acid; 3 = ethyl *p*-hydroxybenzoate; 4 = *n*-propyl *p*-hydroxybenzoate; 5 = *n*-butyl *p*-hydroxybenzoate; 6 = benzoic acid; 7 = sorbic acid; 8 = dehydroacetic acid.

One of the characteristics of the polyamide thin layers is to give a bright background in transmitted UV light, and this phenomenon provides a highly sensitive method of detection of UV-absorbing substances on the chromatoplate[9,10]. As shown in Table I, all the food preservatives tested absorb the UV light, thus they are all detectable as dark spots under transmitted UV light. In Table IV, a comparison is made between the detection limit of the spots on polyamide layers and on silica gel layers. It can be seen from Table IV that the detection of these substances is more sensitive on polyamide layers than on silica gel layers. With regard to other methods of detection of the spots, the Methyl Red reagent and the diazotized sulfanilic acid reagent can be used on the polyamide layers as well as on silica gel layers (see EXPERIMENTAL).

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TABLE IV

DETECTION LIMITS OF FOOD PRESERVATIVES BY UV ABSORPTION ON POLYAMIDE LAYERS AND SILICA GEL LAYERS

Sample	*Polyamide in µg*	*Silica gel*[a] *in µg*
Salicylic acid	0.1	0.5
p-Hydroxybenzoic acid	0.25	5–10
Ethyl *p*-hydroxybenzoate	0.25	5–10
n-Propyl *p*-hydroxybenzoate	0.25	5–10
n-Butyl *p*-hydroxybenzoate	0.25	5–10
Benzoic acid	3[b]	50[b]
Sorbic acid	0.25	5–10
Dehydroacetic acid	2[b]	25–50[b]

[a] Fluka Silica gel (Fluka AG, Chemische Fabrik, Switzerland) was used.
[b] The low sensitivities of both these compounds are due to the large discrepancy between their absorption maxima and the wave length (2537 Å) of the UV light (see Table I).

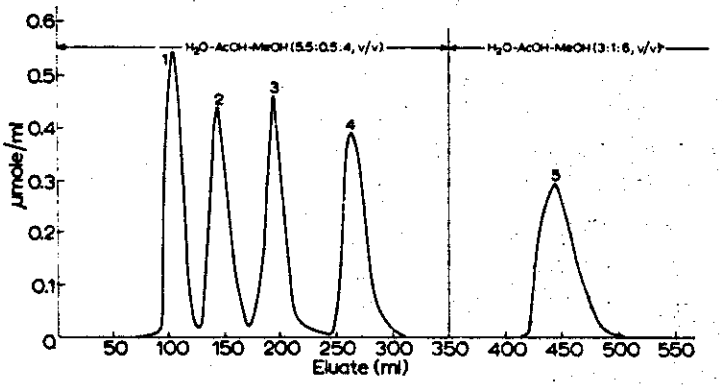

Fig. 2. Separation of food preservatives by polyamide column chromatography. Samples: 1 = dehydroacetic acid; 2 = sorbic acid; 3 = benzoic acid; 4 = *p*-hydroxybenzoic acid; 5 = salicylic acid; each 10 µmoles applied. Column: Polyamide Woelm for column use; 1.7 × 45 cm.

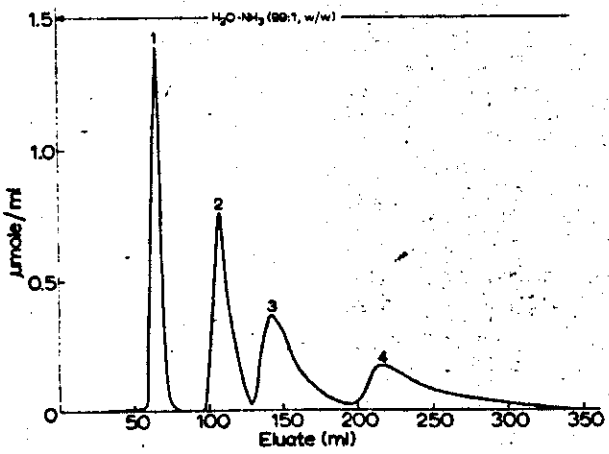

Fig. 3. Separation of food preservatives by polyamide column chromatography. Samples: 1 = *p*-hydroxybenzoic acid; 2 = ethyl *p*-hydroxybenzoate; 3 = *n*-propyl *p*-hydroxybenzoate; 4 = *n*-butyl *p*-hydroxybenzoate; each 10 µmoles applied. Column: Polyamide Woelm for column use; 1.7 × 45 cm.

Column chromatography

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CONCLUSION

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Berlin, 1962, p. 383.
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Column chromatography

Suitable conditions for the separation of the food preservatives on column chromatography have been established as shown in Figs. 2 and 3. A mixture of salicylic acid, *p*-hydroxybenzoic acid, benzoic acid, sorbic acid and dehydroacetic acid was separated on a polyamide column by stepwise elution using two elution systems: (1) H_2O–AcOH–MeOH (5.5:0.5:4) and (2) H_2O–AcOH–MeOH (3:1:6) successively (Fig. 2). The recovery of the samples in this separation system was satisfactory, as is shown: dehydroacetic acid 95.5 %, sorbic acid 96.5 %, benzoic acid 101.2 %, *p*-hydroxybenzoic acid 100.1 %, salicylic acid 103.2 %. An elution procedure using water containing NH_3 (1 %, w/w) was selected for the separation of a mixture of *p*-hydroxybenzoic acid and its alkyl esters. Separation was attained as shown in Fig. 3 and recovery was also good: *p*-hydroxybenzoic acid 92.0 %, ethyl *p*-hydroxybenzoate 96.0 %, *n*-propyl *p*-hydroxybenzoate 98.3 %, *n*-butyl *p*-hydroxybenzoate 91.0 %.

Because neither of the elution media above used has any absorption in the UV, the concentration of each preservative in the fractions was directly determined by measuring the absorbance at the λ max (see Table I).

CONCLUSION

Eight kinds of food preservative, which include five phenolic hydroxyl compounds, one enolic hydroxyl compound and two carboxylic compounds, were separated on polyamide layers. From the correlation of the mobility of the samples and the solvents used, it was noticed that some hydrogen bonding forces have a definite effect on the interaction between samples having phenolic hydroxyl groups and the polyamide molecule, and that the polarity of the solvents exerts a considerable influence on the hydrogen bonding. The detection of the spots on the polyamide layers by the UV absorption method is 5–20 times more sensitive than for those on silica gel layers. This confirms that polyamide layer chromatography is suitable for the micro-analysis of food preservatives.

The conditions for the separation of these substances on a polyamide column were established. The simultaneous separation of all the samples was not possible, but by combining two types of elution system, *viz.*, a H_2O–AcOH–MeOH system and a H_2O–NH_3 system, good separations in two groups of samples were attained. The recovery of the samples separated on the polyamide column was also satisfactory.

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Quantitative determination of sorbic acid in wine

By Ch. Junge and Ch. Spadinger

For the detection and determination of sorbic acid in wine, the thiobarituric acid reaction according to Schmidt (1) is specific, highly sensitive and hardly susceptible to trouble. The members of the wine analysis committee of the Federal Board of Health (among others Rebelein, Postel, Hess and Indinger) have therefore suggested within the framework of the studies for amending and supplementing the "General Administrative Regulations for the investigation of wine and similar alcoholic beverages as well as fruit juices" to provide a method for the qualitative detection and for the quantitative determination for inclusion in the said administrative regulations.

In the determination methods generally used in practice, which are based either on the principle of the thiobarbituric reaction or on the UV-measurement of sorbic acid in the distillate (2), quantities below 20 mg/l sorbic acid can not be determined quantiatively in a reliable manner, because, on the one hand, the amounts of sugar and alcohol extracted make the satisfactory determination of small amount of sorbic acid difficult in the thiobarbituric acid reaction, while other substances passing over into the distillate make the UV-measurement more difficult. We have therefore tried to develop a quick and reliable method for the determination of sorbic acid in wine on the basis of the thiobarbituric acid and to find an extracting agent which determines the sorbic acid quantitatively, but excludes alcohol and sugar as far as possible. Like Woidich and co-workers (3), we arrived at the result that a mixture of ether-petroleum ether (I:1) meets these requirements best. According to the procedure indicated below, we found for 25% by volume alcohol an apparent sorbic acid content of about 1 mg/l; 200 g/l invert sugar simulate about 0.5 mg/l and 1.5 g/l hydroxymethyl furfurol simulate about 1 mg/l sorbic acid. Alcohol, sugar and

hydroxymethyl furfurol react with a yellow-brown color whose absorption maximum is at 495 mm.

Extraction of sorbic acid from aqueous solutions and from wine is possible quantitatively (see tables 1 and 2).

Table 1. Extraction of Sorbic Acid From Aqueous Solutions.

mg/l sorbic acid	E	mu g/2 ml	dilution	sorbic acid found mg/l	%
2.5	0.140	1.8	10/25	2.3	92
5.0	0.315	4.1	10/25	5.1	102
7.5	0.455	5.9	10/25	7.4	99
10.0	0.630	8.1	10/25	10.1	101
12.5	0.775	10.0	10/25	12.5	100
25.0	0.185	2.4	5/100	24.0	96
50.0	0.730	9.4	5/50	47.0	94
75.0	0.570	7.4	5/100	74.0	99
100.0	0.305	4.0	5/250	100.0	100
125.0	0.380	4.9	5/250	122.0	98

Table 2.

Wine	1	E	"g/2 ml	2	F	3	4	%	5	6
1966 White Bordeaux	-	0.235	3.1	10/25	1.25	-	3.9	-	red viol.	pos.
1966 White Bordeaux	4	0.465	6.0	10/25	1.25	7.9	7.5	95	red viol.	pos.
1966 Haute Sauternes	-	0.080	1.1	10/25	1.25	-	1.4	-	weak brown	neg.
1966 Haute Sauternes	20	0.650	8.4	10/50	2.5	21.4	21.0	98	red viol.	pos
1962 Sylvaner	-	0.050	0.7	10/25	1.25	-	0.9	-	practically color less	neg.
1962 Sylvaner	10	0.640	8.3	10/25	1.25	10.9	10.4	96	red viol.	pos.
Greek Muscatel	-	0.100	1.3	10/25	1.25	-	1.6	-	weak red	neg.
Greek Muscatel	16	0.560	7.2	5/25	2.5	17.6	18.0	102	prac. clear red viol.	pos.
Tarragona	-	0.110	1.5	10/25	1.25	-	1.9	-	brown	neg.
Tarragona	118	0.455	5.9	5/200	20.0	120.	118.	98	red viol.	pos
Malaga	-	0.145	1.9	10/25	1.25	-	2.4	-	yell. brown	neg.
Malaga	8	0.605	7.8	10/25	1.25	10.4	9.8	94	red viol.	pos
Malaga	40	0.620	8.0	5/50	5.0	42.	40.	95	red viol.	pos.
Span Red Wine	-	0.055	0.8	10/25	1.25	-	1.0	-	almost clea	pos.
Germ. White Wine	-	0.030	0.4	10/25	1.25	-	0.5	-	colorless	neg.

Table Con't

Germ. Wine	White	20	0.625	8.1	5/25	2.5	20.5	20.2	98	red viol.	pos

As it can be seen from table 2, attention must also be given to the resulting color in the determination of sorbic acid, since small amounts of existing sugars and alcohols yield a slight brown coloration, which can only simulate, however, a total of less than 3 mg sorbic acid according to our findings. If the TLC-detection was negative, the color of the measuring solution in the thiobarbituric acid reaction was not red violet in the wines tested by us, but almost colorless to brownish.

In connection with the TLC-detection of various preservatives by Woidich and coworkers (3) we set up the following procedure for the quantitative determination of sorbic acid, where a preceding TLC-detection is necessary to evaluate the amount of sorbic acid to be expected.

Experimental Part.

A. Detection of Sorbic Acid (3).

B. Determination of Sorbic Acid.

Principle; After ether-petroleum ether-extraction the sorbic acid is oxidized with potassium dichromate to malonic dialdehyde, which forms with thiobarbituric acid a red-colored condensation product whose color intensity is measured at 530 mm.

Apparatus and Auxiliary Devices.

Electrophotometer with filter gravity center at 530 mm or spectral photometer

cuvettes: 10 mm

rotary evaporator

thermostat

water bath

separating funnel with short outlet tube: 100 ml

test tubes with ground section

measuring pipettes: 0.5 ml

full pipettes: 1, 2, 5, 10, 20, 30, 40, 50 ml

measuring cylinder: 50 ml

measuring flasks: 25 to 250 ml, depending on the amount of sorbic acid expected.

Reagents.

2 n-sulfuric acid

0.1 n-soda lye

diethyl ether p.a. and petroleum benzine (petroleum ether), boiling range to 40°C, are mixed in a ratio of 1:1 for the analysis.

sulfate of potassium chromate solution: equal parts of an aqueous 0.05 n-potassium-dichromate solution and 2 n-sulfuric acid are mixed.

0.2% thiobarbituric acid solution (g/v): 200 mg 2-thiobarbituric acid were dissolved in dist. water at 60 to 80°C. The solution must be completely clear, otherwise it must be filtered over a blue band filter. It necessary it must be produced fresh.

Execution of Determination.

5.0 ml (for amounts under 10 mg/l sorbic acid 10.0 ml) of the liquid to be tested are acidified with 5 ml 2 n-sulfuric acid and shaken vigorousl three times with 30 ml diethyl ether-petroleum benzine. The combined dieth ether-petroleum-benzine solutions are washed once with 30 ml dist. water. After adding 2 ml 0.1 n-soda lye, the solution is evaporated to dryness in the rotary evaporator at 30°C. The residue is taken up in dist. water and, depending on the amount of sorbic acid to be expected on the basis of the TLC-detection, filled up with dist. water up to the mark in a measuring flask which contains 0.1 ml 2 n-sulfuric acid.

Dilution Table.

Amount of sorbic acid to be expected, mg/l	Size of measuring flask ml
20	25
40	50

Table con't

80	100
160	200
200	250

The mostly slightly turbid solution is filtered over a blue band filter.

Since sorbic acid is sensitive to light, the photometric determination is made immediately thereafter.

To this end 2.0 ml of the clear solution, which should not contain more than 10 mu g sorbic acid, are poured into a test tube with ground section; 1.0 ml of the sulfate of potassium dichromate solution and 200 ml thiobarbituric acid solution are added, and the mixture is kept for exactly 10 minutes in a boiling water bath after the 10 minutes, the test tube is removed and cooled for 10 minutes in moving water (thermostat) at about 20°C.

The resulting red-violet coloration is measured against a blank value at 530 mm assumed in the same manner with 2.0 ml dist. water. The color is constant for over 1 hour. A weak brown coloration, if any, caused by small amounts of alcohol or sugar, does not interfere with the determination.

Establishing the Calibration Curve (see fig. 1).

134 mg potassium sorbate (corresponding to 100 mg sorbic acid) are dissolved with dist. water to 1 liter. 20.0 ml of this solution are filled in a 200 measuring flask with dist. water up to the mark.

Figure 1. Measuring conditions: 530 mm, 1 cm cuvettes, PMQ II.

10.0, 20.0, 30.0, 40.0 and 50.0 ml of this dilution are filled in 100 ml measuring flasks with dist. water up to the mark. 2.0 ml of these dilutions, which contain 2, 4, 6, 8 and 10 mu g sorbic acid, are subjected to the measurement in the above indicated manner.

The extinction values obtained, plotted against the sorbic acid conter

corresponding to 2.0 ml of the dilution used, yield a straight line which passes through the zero point.

Calculation

The content of sorbic acid is taken from the calibration curve and must be indicated in mg per liter (mg/l), taking into account the dilution.

Summaries in German, English and French.

(Translated by Carl Demrick Associates, Inc./IE)

Bibliography

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3) Z. Lebensmittel-Untersuch. u. -Forsch. 133, 317 (1967).

Quantitative determination of sorbic acid in wine

By Ch. Junge and Ch. Spadinger

For the detection and determination of sorbic acid in wine, the thiobarituric acid reaction according to Schmidt (1) is specific, highly sensitive and hardly susceptible to trouble. The members of the wine analysis committee of the Federal Board of Health (among others Rebelein, Postel, Hess and Indinger) have therefore suggested within the framework of the studies for amending and supplementing the "General Administrative Regulations for the investigation of wine and similar alcoholic beverages as well as fruit juices" to provide a method for the qualitative detection and for the quantitative determination for inclusion in the said administrative regulations.

In the determination methods generally used in practice, which are based either on the principle of the thiobarbituric reaction or on the UV-measurement of sorbic acid in the distillate (2), quantities below 20 mg/l sorbic acid can not be determined quantiatively in a reliable manner, because, on the one hand, the amounts of sugar and alcohol extracted make the satisfactory determination of small amount of sorbic acid difficult in the thiobarbituric acid reaction, while other substances passing over into the distillate make the UV-measurement more difficult. We have therefore tried to develop a quick and reliable method for the determination of sorbic acid in wine on the basis of the thiobarbituric acid and to find an extracting agent which determines the sorbic acid quantitatively, but excludes alcohol and sugar as far as possible. Like Woidich and co-workers (3), we arrived at the result that a mixture of ether-petroleum ether (1:1) meets these requirements best. According to the procedure indicated below, we found for 25% by volume alcohol an apparent sorbic acid content of about 1 mg/l; 200 g/l invert sugar simulate about 0.5 mg/l and 1.5 g/l hydroxymethyl furfurol simulate about 1 mg/l sorbic acid. Alcohol, sugar and

hydroxymethyl furfurol react with a yellow-brown color whose absorption maximum is at 495 mm.

Extraction of sorbic acid from aqueous solutions and from wine is possible quantitatively (see tables 1 and 2).

Table 1. Extraction of Sorbic Acid From Aqueous Solutions.

mg/l sorbic acid	E	mu g/2 ml	dilution	sorbic acid found mg/l	%
2.5	0.140	1.8	10/25	2.3	92
5.0	0.315	4.1	10/25	5.1	102
7.5	0.455	5.9	10/25	7.4	99
10.0	0.630	8.1	10/25	10.1	101
12.5	0.775	10.0	10/25	12.5	100
25.0	0.185	2.4	5/100	24.0	96
50.0	0.730	9.4	5/50	47.0	94
75.0	0.570	7.4	5/100	74.0	99
100.0	0.305	4.0	5/250	100.0	100
125.0	0.380	4.9	5/250	122.0	98

Table 2.

Wine	1	E	"g/2 ml	2	F	3	4	%	5	6
1966 White Bordeaux	-	0.235	3.1	10/25	1.25	-	3.9	-	red viol.	pos.
1966 White Bordeaux	4	0.465	6.0	10/25	1.25	7.9	7.5	95	red viol.	pos.
1966 Haute Sauternes	-	0.080	1.1	10/25	1.25	-	1.4	-	weak brown	neg.
1966 Haute Sauternes	20	0.650	8.4	10/50	2.5	21.4	21.0	98	red viol.	pos
1962 Sylvaner	-	0.050	0.7	10/25	1.25	-	0.9	-	practically color less	neg.
1962 Sylvaner	10	0.640	8.3	10/25	1.25	10.9	10.4	96	red viol.	pos.
Greek Muscatel	-	0.100	1.3	10/25	1.25	-	1.6	-	weak red	neg.
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cuvettes: 10 mm

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full pipettes: 1, 2, 5, 10, 20, 30, 40, 50 ml

measuring cylinder: 50 ml

measuring flasks: 25 to 250 ml, depending on the amount of sorbic acid expected.

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diethyl ether p.a. and petroleum benzine (petroleum ether), boiling range to 40°C, are mixed in a ratio of 1:1 for the analysis.

sulfate of potassium chromate solution: equal parts of an aqueous 0.05 n-potassium-dichromate solution and 2 n-sulfuric acid are mixed.

0.2% thiobarbituric acid solution (g/v): 200 mg 2-thiobarbituric acid were dissolved in dist. water at 60 to 80°C. The solution must be completely clear, otherwise it must be filtered over a blue band filter. It necessary, it must be produced fresh.

Execution of Determination.

5.0 ml (for amounts under 10 mg/l sorbic acid 10.0 ml) of the liquid to be tested are acidified with 5 ml 2 n-sulfuric acid and shaken vigorously three times with 30 ml diethyl ether-petroleum benzine. The combined diethy ether-petroleum-benzine solutions are washed once with 30 ml dist. water. After adding 2 ml 0.1 n-soda lye, the solution is evaporated to dryness in the rotary evaporator at 30°C. The residue is taken up in dist. water and, depending on the amount of sorbic acid to be expected on the basis of the TLC-detection, filled up with dist. water up to the mark in a measuring flask which contains 0.1 ml 2 n-sulfuric acid.

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To this end 2.0 ml of the clear solution, which should not contain more than 10 mu g sorbic acid, are poured into a test tube with ground section; 1.0 ml of the sulfate of potassium dichromate solution and 200 ml thiobarbituric acid solution are added, and the mixture is kept for exactly 10 minutes in a boiling water bath after the 10 minutes, the test tube is removed and cooled for 10 minutes in moving water (thermostat) at about 20°C.

The resulting red-violet coloration is measured against a blank value at 530 mm assumed in the same manner with 2.0 ml dist. water. The color is constant for over 1 hour. A weak brown coloration, if any, caused by small amounts of alcohol or sugar, does not interfere with the determination.

Establishing the Calibration Curve (see fig. 1).

134 mg potassium sorbate (corresponding to 100 mg sorbic acid) are dissolved with dist. water to 1 liter. 20.0 ml of this solution are filled in a 200 measuring flask with dist. water up to the mark.

Figure 1. Measuring conditions: 530 mm, 1 cm cuvettes, PMQ II.

10.0, 20.0, 30.0, 40.0 and 50.0 ml of this dilution are filled in 100 ml measuring flasks with dist. water up to the mark. 2.0 ml of these dilutions, which contain 2, 4, 6, 8 and 10 mu g sorbic acid, are subjected to the measurement in the above indicated manner.

The extinction values obtained, plotted against the sorbic acid conten

corresponding to 2.0 ml of the dilution used, yield a straight line which passes through the zero point.

Calculation

The content of sorbic acid is taken from the calibration curve and must be indicated in mg per liter (mg/l), taking into account the dilution.

Summaries in German, English and French.

(Translated by Carl Demrick Associates, Inc./IE)

Sorbic acid 210

Aus dem Max von Pettenkofer-Institut des Bundesgesundheitsamtes
Leiter: Erster Direktor und Professor Dr. R. Franck

Quantitative Bestimmung der Sorbinsäure im Wein

Von Ch. Junge und Ch. Spadinger

Zum Nachweis und zur Bestimmung der Sorbinsäure ist die Thiobarbitursäurereaktion nach *Schmidt* [1]) spezifisch, sehr empfindlich und kaum störanfällig. Von den Mitgliedern der Weinanalysenkommission des Bundesgesundheitsamtes (u. a. *Rebelein, Postel, Hess* und *Indinger*) wurden daher im Rahmen der Arbeiten zur Änderung und Ergänzung der „Allgemeinen Verwaltungsvorschrift für die Untersuchung von Wein und ähnlichen alkoholischen Erzeugnissen sowie von Fruchtsäften" vorgeschlagen, diese Methode zum qualitativen Nachweis und auch zur quantitativen Bestimmung für die Aufnahme in die o. a. Verwaltungsvorschrift vorzusehen.

Bei den in der Praxis allgemein angewendeten Bestimmungsverfahren, die entweder auf dem Prinzip der Thiobarbitursäurereaktion oder auf der UV-Messung der Sorbinsäure im Destillat [2]) beruhen, können Mengen unter 20 mg/l Sorbinsäure nicht einwandfrei quantitativ erfaßt werden, da einerseits mitextrahierte Zucker- und Alkoholmengen bei der Thiobarbitursäurereaktion, andererseits bei der UV-Messung andere in das Destillat übergehende Stoffe die einwandfreie Bestimmung geringer Sorbinsäuremengen erschweren. Wir waren daher bemüht, ein schnelles und sicheres Verfahren zur Bestimmung der Sorbinsäure im Wein auf der Grundlage der Thiobarbitursäurereaktion zu entwickeln und hierfür ein Extraktionsmittel zu finden, das die Sorbinsäure quantitativ erfaßt, Alkohol und Zucker aber weitgehend ausschließt. Wie schon *Woidich* und Mitarbeiter [3]), kamen wir zu dem Ergebnis, daß ein Gemisch aus Äther-Petroläther (1:1) diese Forderungen am besten erfüllt. Nach der u. a. Vorschrift fanden wir für 25 Vol.%igen Alkohol einen scheinbaren Sorbinsäuregehalt von etwa 1 mg/l; 200 g/l Invertzucker täuschen etwa 0,5 mg/l und 1,5 g/l Hydroxymethylfurfurol täuscht etwa 1 mg/l Sorbinsäure vor. Alkohol, Zucker und Hydroxymethylfurfurol reagieren mit gelbbrauner Farbe, deren Absorptionsmaximum bei 495 nm liegt.

Die Ausschüttelung der Sorbinsäure aus wäßrigen Lösungen und aus Wein gelingt quantitativ (siehe Tabelle 1 und 2).

Tabelle 1

Extraktion der Sorbinsäure aus wäßrigen Lösungen

mg/l Sorbinsäure	E	µg/2 ml	Verdünnung	gefundene Sorbinsäure mg/l	%
2,5	0,140	1,8	10/25	2,3	92
5,0	0,315	4,1	10/25	5,1	102
7,5	0,455	5,9	10/25	7,4	99
10,0	0,630	8,1	10/25	10,1	101
12,5	0,775	10,0	10/25	12,5	100
25,0	0,185	2,4	5/100	24,0	96
50,0	0,730	9,4	5/50	47,0	94
75,0	0,570	7,4	5/100	74,0	99
100,0	0,305	4,0	5/250	100,0	100
125,0	0,380	4,9	5/250	122,0	98

Wie aus der Tabelle 2 ersichtlich, ist bei der Bestimmung der Sorbinsäure auch auf die entstehende Färbung zu achten, da geringe Mengen an noch vorhandenen Zuckern und Alkohol eine schwache Braunfärbung ergeben, die aber nach unseren Feststellungen in der Gesamtheit nur weniger als 3 mg/l Sorbinsäure vortäuschen können. War der dünnschichtchromatographische Nachweis negativ, ist in allen von uns un-

Tabelle 2

Wein	Zugesetzte Sorbinsäure mg/l	E	µg 2 ml	Verdünnung	F	Sorbinsäure theoret. mg/l	gefundene Sorbinsäure mg/l	%	Farbe der Meßlösung	Dünnschichtchromatograph. Nachweis
1966er weißer Bordeaux	—	0,235	3,1	10/25	1,25	—	3,9	—	rotviolett	positiv
1966er weißer Bordeaux	4	0,465	6,0	10/25	1,25	7,9	7,5	95	rotviolett	positiv
1963er Haute Sauternes	—	0,080	1,1	10/25	1.25	—	1,4	—	schwach braun	negativ
1966er Haute Sauternes	20	0,650	8,4	10/50	2,5	21,4	21,0	98	rotviolett	positiv
1962er Sylvaner	—	0,050	0,7	10/25	1,25	—	0,9	—	fast farblos	negativ
1962er Sylvaner	10	0,640	8.3	10/25	1,25	10,9	10,4	96	rotviolett	positiv
Griech. Muskateller	—	0,100	1,3	10/25	1,25	—	1,6	—	fast farblos, schwach rötlich	negativ
Griech. Muskateller	16	0,560	7,2	5/25	2,5	17,6	18,0	102	rotviolett	positiv
Tarragona	—	0,110	1,5	10/25	1,25	—	1,9	—	bräunlich	negativ
Tarragona	118	0,455	5,9	5/200	20,0	120	118	98	rotviolett	positiv
Malaga	—	0,145	1,9	10/25	1,25	—	2,4	—	gelbbraun	negativ
Malaga	8	0,605	7,8	10/25	1,25	10,4	9,8	94	rotviolett	positiv
Malaga	40	0,620	8,0	5/50	5,0	42	40	95	rotviolett	positiv
Span. Rotwein	—	0,055	0,8	10/25	1,25	—	1,0	—	fast farblos	negativ
deutscher Weißwein	—	0,030	0,4	10/25	1.25	—	0,5	—	farblos	negativ
deutscher Weißwein	20	0,625	8,1	5/25	2,5	20,5	20,2	98	rotviolett	positiv

tersuchten Weinen auch die Farbe der Meßlösung bei der Thiobarbitursäure-Reaktion nicht rotviolett, sondern fast farblos bis bräunlich gewesen.

In Verbindung mit dem dünnschichtchromatographischen Nachweis verschiedener Konservierungsmittel von *Woidich* und Mitarbeiter[3]) stellten wir die folgende Vorschrift zur quantitativen Bestimmung der Sorbinsäure auf, wobei der vorherige dünnschichtchromatographische Nachweis zur Abschätzung der zu erwartenden Sorbinsäuremenge erforderlich ist.

Experimenteller Teil

A. *Nachweis der Sorbinsäure*[3])

B. *Bestimmung der Sorbinsäure*

Prinzip: Nach Äther-Petroläther-Extraktion wird die Sorbinsäure durch Kaliumdichromat zum Malonsäure-Dialdehyd oxydiert, der mit Thiobarbitursäure ein rotfarbiges Kondensationsprodukt bildet, dessen Farbintensität bei 530 nm gemessen wird.

Geräte und Hilfsmittel

Elektrophotometer mit Filterschwerpunkt bei 530 nm oder Spektralphotometer
Küvetten: 10 mm
Rotationsverdampfer
Thermostat
Wasserbad
Scheidetrichter mit kurzem Abflußrohr: 100 ml
Reagensgläser mit Schliff
Meßpipetten: 0,5 ml
Vollpipetten: 1, 2, 5, 10, 20, 30, 40, 50 ml
Meßzylinder: 50 ml
Meßkolben: 25 bis 250 ml, je nach der zu erwartenden Menge Sorbinsäure

Reagenzien:

2 n-Schwefelsäure
0,1 n-Natronlauge
Diäthyläther z. A. und Petroleumbenzin (Petroläther), Siedebereich bis 40° C, z. A. werden im Verhältnis 1:1 gemischt.
Schwefelsaure Kaliumchromatlösung: gleiche Teile einer wäßrigen 0,05 n-Kaliumdichromatlösung und 2 n-Schwefelsäure werden gemischt.
0,2 %ige Thiobarbitursäurelösung (g/v): 200 mg 2-Thiobarbitursäure werden in dest. Wasser bei 60 bis 80° C gelöst. Die Lösung muß vollkommen klar sein, andernfalls ist die Lösung über ein Blaubandfilter zu filtrieren. Sie ist bei Bedarf frisch herzustellen.

Durchführung der Bestimmung

5,0 ml (bei Mengen unter 10 mg/l Sorbinsäure 10,0 ml) der zu untersuchenden Flüssigkeit werden mit 5 ml 2 n-Schwefelsäure angesäuert und 3 mal mit je 30 ml Diäthyläther-Petroleumbenzin kräftig geschüttelt. Die vereinigten Diäthyläther-Petroleumbenzinlösungen werden einmal mit 30 ml destilliertem Wasser gewaschen. Nach Zusatz von 2 ml 0,1 n-Natronlauge wird die Lösung im Rotationsverdampfer bei 30° C zur Trockne eingedampft. Der Rückstand wird in destilliertem Wasser aufgenommen und, je nach dem aufgrund des dünnschichtchromatographischen Nachweises zu erwartenden Gehalt an Sorbinsäure in einem Meßkolben, in dem sich 0,1 ml 2n-Schwefelsäure befindet, mit destilliertem Wasser zur Marke aufgefüllt.

Verdünnungstabelle

zu erwartender Gehalt an Sorbinsäure mg/l	Meßkolbengröße ml
20	25
40	50
80	100
160	200
200	250

Die meist leicht trübe Lösung wird über ein Blaubandfilter filtriert.

Da Sorbinsäure lichtempfindlich ist, wird die photometrische Bestimmung gleich anschließend durchgeführt.

Hierzu werden 2,0 ml der klaren Lösung, die nicht mehr als 10 µg Sorbinsäure enthalten soll, in ein Reagensglas mit Schliff gegeben; 1,0 ml der schwefelsauren Kaliumdichromatlösung und 2,0 ml Thiobarbitursäurelösung werden zugefügt und die Mischung genau 10 Minuten in einem siedenden Wasserbad gehalten. Nach Ablauf der 10 Min. nimmt man das Reagensglas heraus und kühlt 10 Min. lang in bewegtem Wasser (Thermostat) bei etwa 20° C ab.

Die entstandene rotviolette Färbung wird gegen einen mit 2,0 ml destilliertem Wasser in gleicher Weise angesetzten Blindwert bei 530 nm gemessen. Die Farbe ist über 1 Stunde konstant. Eine eventuell auftretende schwache Braunfärbung, die durch geringe Mengen Alkohol oder Zucker hervorgerufen wird, stört die Bestimmung nicht.

Aufstellung der Eichkurve (siehe Abb. 1)

134 mg Kaliumsorbat (entsprechend 100 mg Sorbinsäure) werden mit destilliertem Wasser zu 1 Liter gelöst. 20,0 ml dieser Lösung werden in einem 200 ml-Meßkolben mit destilliertem Wasser zur Marke aufgefüllt.

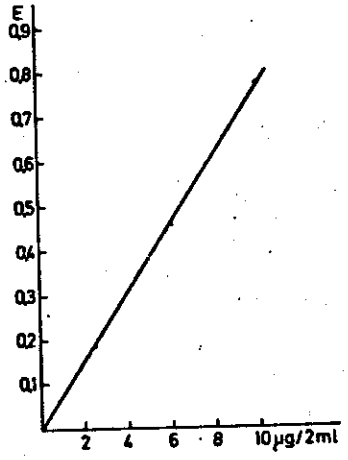

Abb. 1. Meßbedingungen: 530 nm, 1-cm-Küvetten, PMQ II.

Je 10,0; 20,0; 30,0; 40,0 und 50,0 ml dieser Verdünnung werden in 100 ml-Meßkolben mit destilliertem Wasser zur Marke ergänzt. 2,0 ml dieser Verdünnungen, die 2, 4, 6, 8 und 10 µg Sorbinsäure enthalten, werden in der oben angegebenen Weise der Messung unterworfen.

Die erhaltenen Extinktionswerte, gegen die, den angewandten 2,0 ml der Verdünnung entsprechenden Sorbinsäuregehalt aufgetragen, ergeben eine Gerade, die durch den Nullpunkt geht.

Berechnung

Der Gehalt an Sorbinsäure wird der Eichgeraden entnommen und ist, unter Berücksichtigung der Verdünnung, in Milligramm in 1 Liter (mg/l) anzugeben.

Zusammenfassung

Eine spektrophotometrische Bestimmung der Sorbinsäure wird beschrieben, die auf dem Prinzip der Thiobarbitursäurereaktion nach *Schmidt*[1]) beruht. Durch Äther-Petroläther-Extraktion der Sorbinsäure aus der zu untersuchenden Flüssigkeit werden nur so geringe Mengen an Zucker und Alkohol mitextrahiert, daß sie die exakte Messung des rotfarbigen Kondensationsproduktes des aus der Sorbinsäure entstandenen Malonsäure-Dialdehyds mit Thiobarbitursäure nicht beeinflussen. Die Empfindlichkeit der Bestimmung beträgt 1 mg/l Sorbinsäure.

Summary

A spectrophotometrical analysis of sorbic acid is described here, based in principle on the thiobarbituric acid reaction according to *Schmidt*[1]). Through the ether/light petroleum extraction of the sorbic acid from the test liquid,

only negligible quantities of sugar and alcohol are carried along that will not affect exact measuring of the red-coloured condensate of malonic acid dialdehyde formed from the sorbic acid with thiobarbituric acid. Reaction sensitivity is 1 mg/l of sorbic acid.

Résumé

L'article décrit une détermination spectrophotométrique de l'acide sorbique fondée sur le principe de la reaction à l'acide thiobarbiturique d'après *Schmidt* [1]). Par extraction de l'acide sorbique par éther et éther de pétrole, on extrait, à partir du liquide à examiner, seulement de petites quantités de sucre et d'alcool ne pouvant influencer le dosage exact du produit de condensation du dialdéhyde de l'acide malonique, formé à partir de l'acide sorbique avec l'acide thiobarbiturique de couleur rouge. La sensibilité de la réaction s'élève de 1 mg l d'acide sorbique.

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Anschrift der Verfasser:
Dr. Charlotte Junge und *Christl Spadinger*
Max von Pettenkofer-Institut des Bundesgesundheitsamtes
1 Berlin 45
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Pectin research in the sixties

Von A. G. J. Voragen and W. Pilnik

Department of Food Science, Agricultural University, Wageningen, Netherlands

Introduction.

Pectic substances are part of all higher plant tissues. They are mainly deposited in the primary cell wall during the early stages of growth when the area of the wall is increasing. Meristematic and parenchymous tissues are therefore particularly rich in pectic substances. They occur predominantly in a water-insoluble form which is called proto-pectin. The texture of fruits and vegetables on growing, ripening and storage is strongly influenced by the amount and nature of the pectin present. Important changes — desirable and undesirable ones — of the properties of fruit and vegetable products during storing and processing are related to pectic changes. Native or added pectic enzymes may play a dominant role in these phenomena.

Pectins form gels under certain circumstances and this property has made them a very important food additive for the jam, jelly and marmalade as well as for the confectionery industry. Pectin is also used in medical preparations because of its pharmacological activities. From this it may be clear that pectin research captures the interest of plant physiologists, fruit growers, food technologists, pectin manufacturers, pharmacologists and nutritionists.

The following article deals with the most important results of research on pectic substances and pectic enzymes during the past decade.

Literature on pectic substances and pectic enzymes.

While the 50's have seen several important reviews and two books, there has appeared only one but very good book in the sixties (*Doesburg*)[20]). This book deals with the knowledge on chemical, colloidal and plant physiological nature of pectic substances. A shorter summary ist given by *Neukom*[84]). Structural and physiological aspects have been summarized by *Worth*[119]). The industrially important gel formation properties of pectin were discussed by *Solms*[109]) and *Harvey*[34]). The knowledge on protopectin has been reviewed by *Joslyn*[46]). The role and application of pectic enzymes in fruit and vegetable were subject of publications from *Reed*[100]) and *Mayer*[70]).

Pectin.

Molecular Structure:

From studies on pectin of all sources it has become clear that pectin is not a homopolysaccharide but that it has a chain structure of axial-axial α-(1–4)linked D-galacturonic acid units, containing blocks of L-rhamnose rich regions[9, 108, 7, 29]) with mainly arabinose, galactose and xylose as side chains[56, 72, 9, 122, 37, 43, 7, 29, 106]). The chemistry and properties of the native water-insoluble pectic substances, called proto-pectin were studied and reviewed by *Joslyn*[46]), *Joslyn* and *Deuel*[48]), *Gould* et al.[32]) and *Doesburg*[20]). Proto-pectin emerges as a mere term to designate high molecular galacturonides which are chemically and mechanically enmeshed with other cell wall constituents.

Properties:

By studying the instability of pectin in alkaline solutions *Albersheim, Neukom* and *Deuel*[1]) observed a β-elimination cleavage of the glycosidic linkages. *Slavíčková*[107]) and *Bock* and *Lange*[13]) studied the saponification of pectin with ammonium hydroxide. It appears that this saponification is accompanied by amidation and that this increases Ca-sensibility[59]). *Pilnik* and *MacDonald*[94]) compared the stability of pectin with other commercial jelling and thickening agents. *Pilnik*[93]) and *Smith* and *Bryant*[105]) studied the gelling properties of pectin preparations of different degree of esterification under varying circumstances, and found that setting time increases with decreasing esterification down to a certain point from which on the medium determines setting behaviour. *Madi D. Janse*[44]) studied gel strength and gelling temperature of low methoxyl pectin under various circumstances. She found that the setting temperature does not depend on cooling rate. All factors which increase gel strength also cause a higher setting temperature. There is no hysteresis in thermal gel-sol transformation. Setting behaviour of LM-pectin was also very thoroughly elucidated by *Ehrlich*[23]). *Van der Lijn*[66]) studied the gel strength of gels formed by the diffusion of $CaCl_2$ in a 0,5 % LM-pectin solution in the presence of different NaCl concentration. Higher doses of NaCl peptize the gels. As these Ca-diffusion gels have a Ca-concentration gradient their strength also varies accordingly. On single measurement (extrusion force) with the Allo-Kramer Shear Press allows the determination of gel strength as function of Ca-concentrations. *Schweiger*[101]) found that the binding of divalent ions involves the carboxyl and the hydroxyl groups. It was also indicated that both inter- and intra-molecular chelates are formed. This was confirmed by *Kohn* and *Furda*[53]).

French publication, Industr. alim. agr., 1962, pages 99-101.

Practical method of characterization and assessment of sorbic acid in wine

by G. Guimberteau, Agronomic and Oenologic Station of Bordeaux

The anti-yeast properties of sorbic acid are utilized more and more in the practice of food and beverage preservation. Research on this antiseptic in technical or control laboratories poses a difficult problem. The author indicates a simple and fast chromatographic method within the reach of a laboratory with limited equipment.

Since October 15, 1959, sorbic acid is authorized in France for wine preservation in a dose limited to 200 mg per liter. The total sulphurous acid content of the wines treated must not exceed 250 mg per liter for the 1958 harvest; 350 mg for the wines of 1959, and 450 mg for the wines of 1960. In addition, some countries do not allow the importation of wines treated with sorbic acid. These various limitations, therefore, mean that both the user and the official laboratory are required to make continuing tests for this antiseptic; hence it is necessary to have a fast and reliable method.

Critical study of the proposed test problems

The analytical problem is not the same in every case of practice and control; we may be required to make a precise measurement of the sorbic acid content of a wine, but very often it is simply necessary to note its presence. The various quantitative methods proposed generally require extensive apparatus and fussy operations, and are therefore, within the scope only of laboratories with a wide range of equipment. In a widely documented recent work, Jacquin (1) described the various analytical methods; they are rather numerous at the present time, but most of them lack precision. The most classic method is the measurement of the absorption of the distillate of a wine in ultraviolet at 2560 Ang. This absorption for a wine, even free of sorbic acid and even after purification by extraction, is always sensitive and can give the illusion of the presence of 10 to 20 mg of sorbic acid per liter. Volatile substances in the wine interfere, therefore, and it is not only a question

of sulphurous acid, which intervenes to a small extent only, and would in any event be easy to eliminate. Colorimetric methods often have the same defect; for example the very sensitive Schmidt method (2), which uses the red coloration yielded, with thiobarbituric acid, by the malonic dialdehyde obtained by chromic oxidation of the solution of sorbic acid, yields a faint but definite coloration when we apply it to wines which have never received sorbic acid, a coloration also representing an error of several tens of mg per liter.

The methods utilizing paper chromatography, although less precise from the quantitative point of view, generally are quite specific, and we followed this line in our search for a simple and practical method.

Tanner and Rentschler (3) pointed out the following chromatographic method.

30 mu liter of the wine to be tested is deposited on Whatman No. 4 paper. We operate an ascending chromatography in a solvent with the following composition: n-propanol, 3 parts; ethyl acetate, 1 part; 25% ammonia, 1 part; distilled water, 1 part. The chromatography lasted one hour. The chromatogram is dried without exceeding 60°C. It is then examined under ultra-violet radiation from a special lamp emitting at 250 - 260 m mu. The presence of sorbic acid, at an Rf of 0.72 - 0.75, is detected by a strong, deep violet fluorescence. The authors estimate the sensitivity threshold at 10 mg per liter.

We applied this method to a Bordeaux wine to which increasing quantities of sorbic acid were added: 0 - 25 - 50 - 75 - 100 - 125 - 150 - 175 and 200 mg per liter. Examination of the chromatograms enabled us to make the following observations:

The deep violet fluorescence is perceptible for a wine containing 25 mg per liter of sorbic acid, but it is clear only for 50 mg. The spots are rather irregular and it is not possible to estimate the quantity of sorbic acid with certainty. We observe a phenomenon of retention of sorbic acid due to the presence of annoying substances such as the mineral salts and the glu-

cides of the wine. It is therefore indispensable to operate a separation of the sorbic acid in a less complex solution.

According to the results of this test, and knowing that in a pure solution it is possible with this method to detect 1 mu g of sorbic acid per spot, we tried to obtain, in a simple manner, a purified solution of this antiseptic which could be found in the wines. The object of this purification is to obtain sharper chromatograms, making for better sensitivity, and the possibility of estimating the sorbic acid by comparison with the control solutions.

Chromatographic technique adopted

Equipment needed: Whatman No. 4 chromatography paper; two crystallizers serving as chromatography tank; special lamp emitting radiation at 2540 Ang (Quartzlampen Pl 320, 20 W) (fig. 1).

Analytical technique

In a test tube we place 20 ml of wine, we add 1 ml of sulphuric "at (to) the third", and 5 ml of sulphuric ether. We agitate several times carefully, by inversion. The sorbic acid passes into the ethered phase. We deposit 25 mu liter of the ethered solution on the Whatman No. 4 chromatography paper, taking care to space the spots by 3 cm. Evaporation is very rapid. The paper is then wound in a cylinder and kept rigid by fastening; it is placed vertically in a crystallizer, its base being immersed 5 mm in the solvent recommended by Tanner and Rentschler. We allow the solvent to rise by capillary action to between 12 and 15 cm, which takes about thirty minutes. The chromatogram is then dried and examined by irradiation at 2540 Ang. The spots are sharp, very regular. We easily detect 5 mg per liter of sorbic acid.

An estimate can be made by comparing the spots with those obtained from a range of solutions of sorbic acid of known titer, prepared in 12° alcohol, or, too, from a white wine to which increasing doses of sorbic acid were added.

To avoid gross errors, it can be well to add to the wine we are analyzing, a known quantity of sorbic acid, and operate a simultaneous chromatography. It should be noted that a photographic record of the spots can be obtained very simply by apposition of a photographic paper (light R 6/5) on the chromatogram, and exposure for 5 seconds under radiation at 2540 Ang. This is how we obtained the reproduction opposite (fig. 2).

Fig. 1 Equipment required for characterization of a sorbic acid: crystallizer serving as chromatography tank and special lamp emitting at 2540 Ang., under the radiation of which the chromatograms are examined.

Fig. 2. Photographic reproduction, as a negative in ultra-violet light of spots corresponding to increasing doses of sorbic acid, relating to the method of operation indicated with proportions of 25 to 175 mg per liter (right to left).

Remark

The solvent used beforehand can be replaced more simply by a solution of citric acid acid at 6 g per liter. In this case the Rf of the sorbic acid is close to 0.90 - 0.92, the spots obtained are somewhat wider spread, but this in no way prevents a good estimation of the antiseptic. We know that citric acid at 6 g per liter is the solvent used for detection of diglucoside of malvidol (4). We can therefore use the same equipment in the detection or sorbic acid (3). Since technical laboratories are already equipped for malvoside research (the character of wines from hybride vines), the method described will therefore be very easy for them to apply. The lamp emitting at 2540 Ang. also can reveal the fluoroescence characteristic of malvoside in a more sensitive manner than even the Wood light (5).

Note also that Biol and Foulonneau (6), with the same object in mind, have just reported a chromatographic method using, as solvent, ammoniacal

butanol, but it takes eight hours (and even 15 to 20 hours to separate the sorbic acid from the salicylic acid) and seems not as simple, in our opinion, as the method of separation indicated here.

Fig. 3. Simple chromatography device which can be used for studying either malvoside or sorbic acid.

Resume

We offer a method for studying sorbic acid utilizing distribution chromatography on paper, and the revelation under ultra-violet light at 2540 Ang. which, with the aid of a small amount of easily operated equipment, permits characterizing this antiseptic and estimating the quantities added to wines for their preservation.

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Industr. alim. agr. 1962

Sorbic Acid 162

Méthode pratique de caractérisation et d'estimation de l'acide sorbique dans les vins

par G. GUIMBERTEAU

Station agronomique et œnologique de Bordeaux

LES propriétés anti-levure de l'acide sorbique sont mises de plus en plus à profit dans la pratique de la conservation des aliments et des boissons. La recherche de cet antiseptique dans les laboratoires techniques ou de contrôle pose un problème difficile. L'auteur indique une méthode chromatographique simple et rapide, à la portée d'un laboratoire ne disposant que d'une installation assez modeste.

L'acide sorbique est autorisé en France, pour la conservation des vins à la dose limite de 200 mg par l, depuis le 15 octobre 1959. La teneur en acide sulfureux total des vins traités ne doit pas dépasser 250 mg par l pour les vins de la récolte 1958 ; 350 mg pour les vins de 1959 et 450 mg pour les vins de 1960. D'autre part, quelques pays n'admettent pas l'importation de vins traités à l'acide sorbique. Ces diverses limitations imposent donc, aux usagers comme aux laboratoires officiels, le dosage courant de cet antiseptique ; il est nécessaire de pouvoir disposer d'une méthode rapide et sûre.

ETUDE CRITIQUE DES PROBLEMES DE DOSAGE PROPOSEES

Le problème analytique n'est pas le même dans tous les cas de la pratique et du contrôle ; on peut avoir à déterminer avec précision la teneur en acide sorbique d'un vin, mais très souvent il suffit simplement de caractériser sa présence. Les diverses méthodes quantitatives proposées demandent généralement un appareillage onéreux, des manipulations minutieuses et ne sont donc de ce fait qu'à la portée des laboratoires disposant de moyens importants. Dans un travail très documenté, JACQUIN (1) a fait récemment le point des différentes méthodes analytiques ; elles sont à l'heure actuelle assez nombreuses, mais la plupart d'entre elles manquent de spécificité. La méthode la plus classique est la mesure de l'absorption du distillat d'un vin dans l'ultra-violet à 2560 Å. Or cette absorption pour un vin pourtant exempt d'acide sorbique, et même après purification par extraction, est toujours sensible et peut laisser croire à la présence de 10 à 20 mg d'acide sorbique par l. Des substances volatiles du vin interfèrent donc et il ne s'agit pas seulement de l'acide sulfureux qui n'intervient que pour une faible part et qui serait d'ailleurs facile à éliminer. Les méthodes colorimétriques présentent souvent le même défaut ; par exemple la méthode très sensible de SCHMIDT (2), qui utilise la coloration rouge que donne, avec l'acide thiobarbiturique, la dialdéhyde malonique obtenue par oxydation chromique de la solution d'acide sorbique, donne une coloration faible, mais nette, lorsqu'on l'applique à des vins n'ayant jamais reçu d'acide sorbique, coloration représentant également une erreur de quelques dizaines de mg par l.

Les méthodes utilisant la chromatographie sur papier, si elles sont moins précises au point de vue quantitatif, présentent généralement une bonne spécificité et nous avons cherché dans cette voie la mise au point d'une méthode simple et pratique.

TANNER et RENTSCHLER (3) avaient indiqué la méthode chromatographique suivante.

80 µl du vin à essayer sont déposés sur papier Whatman n° 4. On effectue une chromatographie ascendante dans un solvant de composition suivante : n-propanol, 3 parties ; acétate d'éthyle, 1 partie ; ammoniaque à 25 %, 1 partie ; eau distillée, 1 partie. La chromatographie dure une heure. Le chromatogramme est séché sans dépasser 60° C. Il est ensuite examiné sous le rayonnement ultra-violet d'une lampe spéciale émettant à 250 - 260 mμ. La présence d'acide sorbique, à un Rf de 0,72 - 0,75, est décelée par une forte fluorescence violet foncé. Les auteurs estiment le seuil de sensibilité à 10 mg par l.

Nous avons appliqué cette méthode à un vin de Bordeaux additionné de quantités croissantes d'acide sorbique : 0 - 25 - 50 - 75 - 100 - 125 - 150 - 175 et 200 mg par l. L'examen des chromatogrammes nous a permis de faire les observations suivantes.

La fluorescence violet foncé est perceptible pour un vin contenant 25 mg par l d'acide sorbique, mais elle n'est vraiment nette que pour une teneur de 50 mg. Les spots sont assez irréguliers et il n'est pas possible d'estimer la quantité d'acide sorbique avec certitude. On observe un phénomène de rétention de l'acide sorbique dû à la présence de substances gênantes telles que les sels minéraux et les glucides du vin. Il est donc indis-

pensable d'opérer une séparation de l'acide sorbique en solution moins complexe.

D'après les résultats de cet essai et sachant qu'en solution pure, il est possible de déceler par cette méthode 1 µg d'acide sorbique par spot, nous avons essayé d'obtenir de manière simple une solution purifiée de cet antiseptique pouvant se trouver dans les vins. Le but de cette purification est d'obtenir des chromatogrammes plus nets, permettant une meilleure sensibilité et la possibilité d'une estimation de l'acide sorbique par comparaison avec des solutions témoins.

TECHNIQUE CHROMATOGRAPHIQUE ADOPTEE

Matériel nécessaire : Papier à chromatographie Whatman n° 4 ; deux cristallisoirs servant de cuve à chromatographie ; lampe spéciale émettant un rayonnement à 2340 Å (Quartzlampen Pl 320, 20 W) (fig. 1).

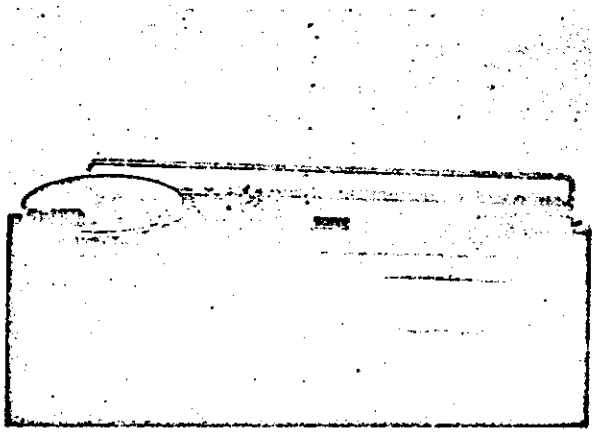

Figure 1

Matériel nécessaire à la caractérisation de l'acide sorbique : cristallisoirs servant de cuve à chromatographie et lampe spéciale émettant à 2340 Å, sous le rayonnement de laquelle on examine les chromatogrammes.

Technique analytique. — Dans un tube à essai, on place 20 ml de vin, on ajoute 1 ml d'acide sulfurique au tiers et 5 ml d'éther sulfurique. On agite plusieurs fois avec précaution, par retournement. L'acide sorbique passe dans la phase éthérée. On dépose 25 µl de la solution éthérée sur le papier à chromatographie Whatman n° 4, en prenant soin d'espacer les spots de 3 cm. L'évaporation est très rapide. Le papier est ensuite enroulé en cylindre et maintenu rigide par agrafage ; il est placé verticalement dans un cristallisoir, sa base plongeant de 5 mm dans le solvant préconisé par TANNER et RENTSCHLER. On laisse le solvant monter par capillarité de 12 à 15 cm, ce qui demande trente minutes environ. Le chromatogramme est ensuite séché et examiné par irradiation à 2540 Å. Les spots sont nets, très réguliers. On décèle facilement 5 mg par l d'acide sorbique.

Une estimation peut être effectuée en comparant les spots à ceux que l'on obtient à partir d'une gamme de solutions d'acide sorbique de titres connus, préparées dans l'alcool à 12° ou encore à partir d'un vin blanc additionné de doses croissantes d'acide sorbique. Pour éviter des erreurs grossières, il peut être bon d'ajouter au vin que l'on analyse une quantité connue d'acide sorbique et d'effectuer simultanément une chromatographie. Il est à noter qu'un enregistrement photographique des spots peut être obtenu très simplement par apposition d'un papier photographique (Lumière R 6/5) au chromatogramme et exposition pendant 5 secondes sous rayonnement de 2540 Å. C'est de cette façon qu'a été obtenue la reproduction ci-contre (fig. 2).

REMARQUE. — Le solvant utilisé précédemment peut être remplacé plus simplement par une solution d'acide citrique à 6 g par l. Dans ce cas, le Rf de l'acide sorbique est voisin de 0,90 - 0,92, les spots obtenus sont un peu plus étalés, ce qui n'empêche nullement

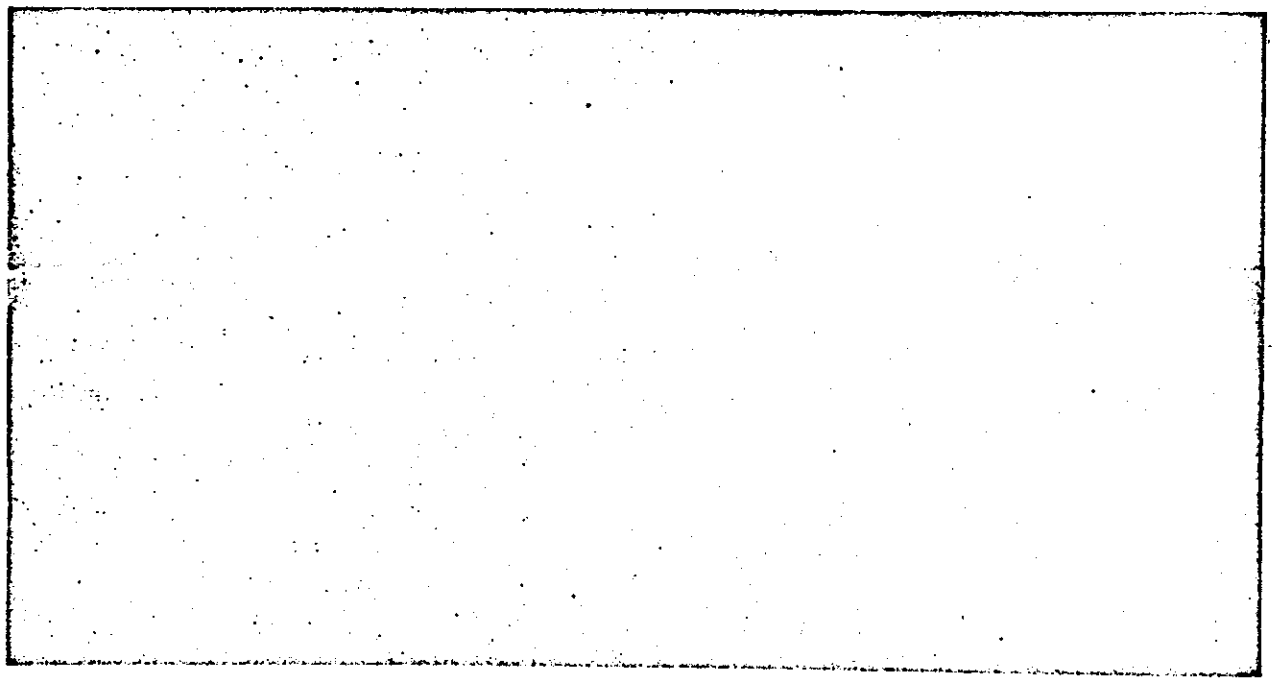

Figure 2

Reproduction photographique en négatif dans l'ultra-violet des spots correspondant à des doses croissantes d'acide sorbique, se rapportant avec le mode opératoire indiqué à des teneurs de 25 à 175 mg par l. (de droite à gauche).

une bonne estimation de l'antiseptique. On sait que l'acide citrique à 6 g par l. est le solvant utilisé pour la détection du diglucoside du malvidol (4). On peut donc employer à la caractérisation de l'acide sorbique le même matériel (fig. 3). Les laboratoires techniques étant déjà équipés pour la recherche du malvoside (caractère des vins issus de cépages hybrides), la méthode décrite sera donc pour eux très facile à mettre en œuvre. La lampe émettant à 2540 Å permet également de mettre en évidence la fluorescence caractéristique du malvoside, d'une manière plus sensible même que la lumière de WOOD (5).

Signalons encore que BIOL et FOULONNEAU (6) viennent, dans le même but, d'indiquer une méthode chromatographique, utilisant comme solvant le butanol ammoniacal, mais qui demande huit heures (et même quinze à vingt heures pour séparer l'acide sorbique de l'acide salicylique) et que nous semble moins simple que le mode opératoire indiqué ici.

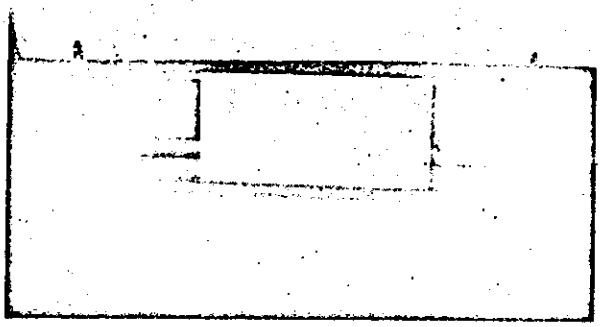

Figure 3

Dispositif simple de chromatographie pouvant être utilisé soit pour la recherche du malvoside soit pour celle de l'acide sorbique.

RESUME. — Nous donnons une méthode de recherche de l'acide sorbique utilisant la chromatographie de partage sur papier et la révélation sous lumière ultra-violette à 2540 Å, qui permet, à l'aide d'un matériel restreint et de manipulations simples, de caractériser cet antiseptique et d'estimer les quantités ajoutées aux vins pour en assurer la conservation.

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Partie B. — DOCUMENTATION BIBLIOGRAPHIQUE ET SERVICE D'INFORMATION

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We are convinced that there are today less than ever cogent technical or economical reasons for the introduction of new wine preservatives, in addition to sulfurous acid. The admission (even of so-called harmless) new substances delays the progress in the hygiene and technology of wine preparation.

Corresponding to a taste trend and enhancing it at the same time, more and more residual sugar-containing wines are today on the market. Securing their stability and protection then against post-fermentation requires increased attention on the part of the wine producers. This can be conveniently bypassed by a chemical preservation method.

Anybody skilled in the art knows the problems of the adaptation and resistance of micro-organisms to chemical substances of all kinds. A preservative alone therefore does not mean permanent protection. What has been said about the admission of new preservatives is thus verified.

The number of chemical preservatives has not diminished. In addition to these that are already known, new ones appear and want to take their place in the market. At the same time, however, the temptation to use these preservatives in wines, particularly under the new conditions of consumption, does not cease. The controlling chemist is always confronted with new analytical problems.

Under these conditions the qualitative detection of chemical wine preservation gains in importance. Microbiological methods are particularly suitable for this purpose. They are the more reliable, the less the wine to be tested has to be subjected to a preliminary treatment.

A number of microbiological methods have already been described in the literature. They can be divided into the following groups:

1. Volumetric methods
2. Acidimetric methods

3. Colorimetric methods

4. Methods for controlling the yeast growth.

The volumetric methods generally use a fermentation test by means of simple Einhorn saccharimeters (Hansen (1955), Tarantola (1955), Mossel (1956), Koch (1956), Beziers (1957) or similar simple glass - (Garoglio and Stella (1955)) instruments. Kiermeir (1953) suggested the use of the manometric Warburg technique.

Without special treatment of the material to be tested, this method is particularly suitable for low-alcohol (less than 5% by volume) or alcohol-free fruit juices. For this use in the control of chemically preserved wines many authors suggest steam- or vacuum distillation (beziers 1957), others ether-extraction (Garoglio and Stella, 1953) of the beverages. We see great disadvantages therein, like change or less or non-detection of the preservatives.

The acidimetric methods determine the growth of certain test organisms on the basis of their acid formation after a varying preliminary treatment of the beverage to be tested (extraction, neutralization) (Galeslot (1953) Baetsle et al (1955)). The test organisms used are mostly bacteria. The main disadvantage of these methods is seen by us in the above mentioned preliminary treatment of the wines.

The colorimetric methods use the appearance of color changes of added reagents caused by the test organisms used (Kuhn, Jerchel 1941; Mecca 1952); the colorimetric determination of the coloring substance formed can be effected only after an extraction (Kiermeier 1953). The above described methods appear to us cumbersome or not sensitive enough.

The control of the yeast growth can be effected in the liquid medium directly with the counting chamber (Saller, 1956) or indirectly with the naphelometer (Clerck et al, 1954). The increase of the cells compared to the control permits conclusions upon the existence of preservatives. In the

solid medium a dense yeast growth is produced. A chemical preservation manifests itself by the appearance of inhibiting zones whose width depends on the concentration (agar diffusion test).

In our own method we control the yeast colonies and the formation of inhibition zones resp. in a suitable agar medium which we fill into small glass tubes and over which the wine is poured after solidification.

This study was made possible with funds from the Schweiz.Rebbaufonds. Our special gratitude goes to the responsible authorities.

II. Material and method

Material

Yeast strain: sacch. cerevisiae of the type "Johannisberg" yeast wine from our collection.

Agar medium: Difco-agar 1% with addition of 10% white grape juice (spec. gravity 1.065).

Standardization of the pH-value to 4.5 with KOH. Filled into test tubes (18 ml each), subsequent sterilization in the steam pot for 25 minutes.

Test tubes: glass tubes of 3 mm diameter and about 80 mm length

Syringes: One 5 ml and one 1 ml syringe

Preservatives: We used in our tests the following preservatives and concentrations of the stock solutions:

10 mg/l actidione

10 g/l potassium sorbinate

10 g/l p-oxy benzoic propyl ester in 40% alcohol

0.1 ml/l = ab. 0.15 monobromacetic ethyl ester in 20% alcohol.

Method

The yeast culture is prepared in white grape juice at 25 degC. centrifuged after 1-3 days, and suspended in sterile tap water. By means of a counting chamber (Thoma chamber) a starting suspension is prepared with 1.10? ml

yeast cells. 2 ml therefore are added to 18 ml liquid agar kept exactly at 45degC and are mixed thoroughly. A suspension with about 100 yeast cells per ml is thus obtained. The test tubes are filled halfway (about 0.5 ml).

After solidification the wine to be tested (about 0.1 ml) is placed without air bubble on the agar surface. Over night the wine diffuses at 8 to 10 degC into the agar. The next day the incubation starts. at 25 degC. We recommend the preparation of at least 5 parallel samples beforeeach test.

Time required

Preparations: 4 hours, diffusion about 16 hours, incubation 40 hours, total about 60 hours. The evaluation consists in determining and measuring inhibition zones of the yeast growth, if any.

III. Results

Determination of the zero value

The applicability of the method had to be tried out first on unpreserved wines. These can contain by nature varying amounts of alcohols and tannins. With fungus-iffested raw material, we have to figure with the presence of certain antibiotic substances (e.g. botrycin (Ribereau-Gayon et al. 1952). Besides, sulfurous acid may be present as a permissible preservative. All these factors were tested for their effect in the tube test.

We tested more than 40 wines with guaranteed origin from various domestic and foreign wine regions. Among them were also wines which were not yet ready for consumption with extremely high tanning contents. We also tested various wines with contents of up to 35 mg/l free sulfurous acid.

The results can be summarized to the effect that inhibition zones (zero values) of more than 3 mm were not found in each case. On the basis of our tests such an amount would have to be deducted in case of doubt if an inhibition is observed before and conclusions can be drawn. In our experience, inhibition zones with a value not more than 3 mm, secured by sufficient repetitions indicated with great probability a chemical preseruation.

These cases must be considered at least suspicious. Inhibition values of an average of 5 mm and more indicate certainly a chemical preservation.

Model tests with different preservatives.

The effects of concentration series of different preservatives could be tested in model tests. The values obtained provide valuable data about the usefulness and sensitivity of the method. The experiences gathered with various preservatives are compiled in tables 1 - 4.

1. Tests with actidione (cycloheximide)

This anitibotic produced from streptomyces griseus is characterized by its specific action against the growth of many (not all) yeasts, but it is practically ineffective against a fermentation in progress.

The use of this preservative for the preservation of wine was discussed by Ribereau Gayon et al. (1958), but also by other authors and was seriously considered for practical use. With certain wine yeasts the above mentioned authors obtained in grape must a total inhibition with additions of 10 g/l. Due to the alcohol content, the effective addition in wines was reduced to 200 - 300 gamma/l. Our experiences with various actidione-concentrations in wines with alcohol contents between 9 and 12% by volume, pH-values of 3.4 - 3.8, and concentrations of free sulfurous acid of less than 35 mg/l, are complied in table 1. In order to check these experimentally found values, we tried to determine the size of the inhibitions mathematically according to the formula by Cooper and Woodman (1946). Our values obtained this way are in good agreement with the experimental values, which seems to us to indicate the usefulness of this formula.

Table 1

acticione concentr. (gamma/l)	0	50	100	200	300	400	500	600
inhibition zones								
mean values in (mm)	0.55	2.0	4.0	8.0	10.5	13.0	14.0	15.0
extreme values of the inhibition zones.(mm)	0-3.0	0-3.5	2.0-7.5	3.5-12.0	6.5-16.0	8.0-13.5	9.5-15.0	10.5-16.0

2. Tests with potassium sorbinate

Sorbic acid is a preservative with a great fungicidal action, which is considered harmless to man and which is now widely used in practice. In order to overcome its poor solubility, its salts are mostly used. For certain uses it is tolerated in many countries. In France sorbic acid is already permitted as a wine preservative in doses of up to 200 mg/l. According to Saller (1957) 40 - 70 mg/l are already sufficient to protect wines against post fermentation under certain conditions. Raible and Busch (1957) determined for sodium sorbinate in malt wort a limiting value of 480 mg/l at pH 4.1 - 4.2. Here a clear fungicidal action was already observed. At a concentration of 320 mg/l, however, the yeast cells still continue to grow. This value is reduced in wine to 200 to 300 mg/l, due to the alcohol content. Boehringer (1956/6) reports that 200 mg/l sorbic acid (as potassium sorbate) are not sufficient to preserve wines.

Our own experiences with potassium sorbate are compiled in table 2.

Table 2

potassium sorbate(mg/l)	0	50	100	200	300	400	500
inhibition zones mean values in (mm)	0.9	2.2	3.94	7.03	8.35	10.6	118
extreme values of the inhibition zones (mm)	0-4.0	0.5-5.0	2.0-5.0	4.5-9.0	6.0-11	8.0-14	11-11

3. Tests with p-oxybenzoic propyl ester

The esters of p-oxybenzoic acid, which were thoroughly studied by

Sabilitschka, have long been used for preservation purposes. He compares (1959) the action of the propyl ester (Nipasol M) to that of sorbic acid and finds under certain test conditions a four times greater effect of the ester, of which 200 - 300 mg/l prevent the yeast growth. For the preservation of wines Ribereau-Gayon and Peynaud (1958) indicate similar values.

Our tests are compiled in table 3.

Table 3

p-oxybenzoic propyl ester (mg/l)	0	50	100	200	300	500
inhibition zones mean values in (mm)	0.47	0.85	1.81	3.69	6.95	10.4
extreme values of the inhibition zones (mm)	0-2.0	0-1.5	0-5.0	1-6.5	5-10.5	7-11.5

4. Tests with monobromoacetic ethyl ester

Monobromoacetic ethyl ester (antibiotin) is a strong preservative and disinfectant, as well as an enzyme toxin. It is rather unstable. Even small amounts are highly effective in fruit juices and particularly in wines. This effects increases considerably from methyl ester to glycol ester. In tests by Oberto (1955) it was found that 15 mg/l monobromoacetic acid only throttled the fermentation to a complete stop. Hansen (1955) indicated similar values. For wine preservation Ribereau-Gayon and Peynaud (1960) indicate concentrations of 2-10 mg/l.

Our tests showed that monobromoacetic ethyl ester is the most effective of all preservatives tested (see fig. 1).

The test results compiled in table 4 show that very small additions to the wine produce clear inhibition zones in the growth of the yeasts.

Table 4

monobromoacetic ethyl ester (mg/l)	0	0.75	1.5	3.0	4.5	7.5	15.0
inhibition zones mean values in (mm)	1.19	4.28	9.64	13.95	18.48	22.0	27.55
extreme values of the inhibition zones	0-3.5	1.5-7.0	6.5-12.0	10.5-17.0	16-21.0	18.5-24.5	26-28.5

Fig. 1 Model test of a red wine preserved with monobromoacetic ethyl ester

The dark zone in the upper part of the tubes is caused by the red wine color which diffused into the agar.

From left to right:

inhibition zone in mm	1	3	9	13.5	20	21.5
tested concentration in mg/l	0	0.75	1.5	3.0	4.5	7.5

The wine tested was a Veltliner (1954) with a high tannin content. The measured inhibition zones provide information about the practice of reading the results. Note the size of the colonies in the inhibition range.

IV. Discussion

With the above described tube method we collected in several hundreds of tests experiences in the detection of the chemical preservation of wines. The method is so advanced that it works with a satisfactory constancy and permits a reliable evaluation. By covering the agar with the double amount of wine, (ca. 0.3 ml) the sensitivity of the method can be considerably increased. The zero values increase likewise.

In order to be able to make comparisons between several tests, the age of the yeasts and the density of the suspension should be the same. Important for the success is also a good distribution of the yeasts in the agar.

Fig. 1 shows that the actual yeast content per ml is much less than was to be expected on the basis of the counting with the Thoma chamber. This must

be due to the high melting temperature (45 degC) of the agar.

The figures in table 1 - 4 show that the tested preservatives differ enormously in their effect on the yeast growth under our test conditions. Where there is a suspicion of chemical preservation of a wine, the method can provide reliable clues in most cases. The tested preservatives can already be detected in concentrations which would not yet insure positive preservation of a wine (see table 5).

Table 5 Evaluation of the efficiency of the method

preservative	dose recommended for the preservation of wine (mg/l)	concentration to be determined reliably with the method(mg/l)
actidione	0.1 - 0.3	0.125
potassium sorbinate	200 - 480	150
p-oxybenzoic propyl ester	200 - 500	250
monobromoacetic ethyl ester	2 - 10	1.5

In the evaluation of the result we can not rely on the measured inhibition zones alone, The size of the yeast colonies must also be taken into consideration. A preservation can be definitely recognized by the fact that the top colonies are much smaller. Such a finding is of particular importance if only relatively small inhibition zones are observed.

The extreme values of the inhibition zones shown in table 1-4 seem at first sight to be very far apart and to make the usefulness of the method doubtful. But this is due to the necessary low yeast concentrations per ml and their distribution in the solid nutrient medium (see fig. 1). The method makes it easy to make a large number of parallel tests. The determined mean value of the inhibition zones, combined with the evaluation of the size of the colonies, permits a reliable evaluation of the investigated case.

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* This article also appears in English translation in American Journal of Ecology, 1962.

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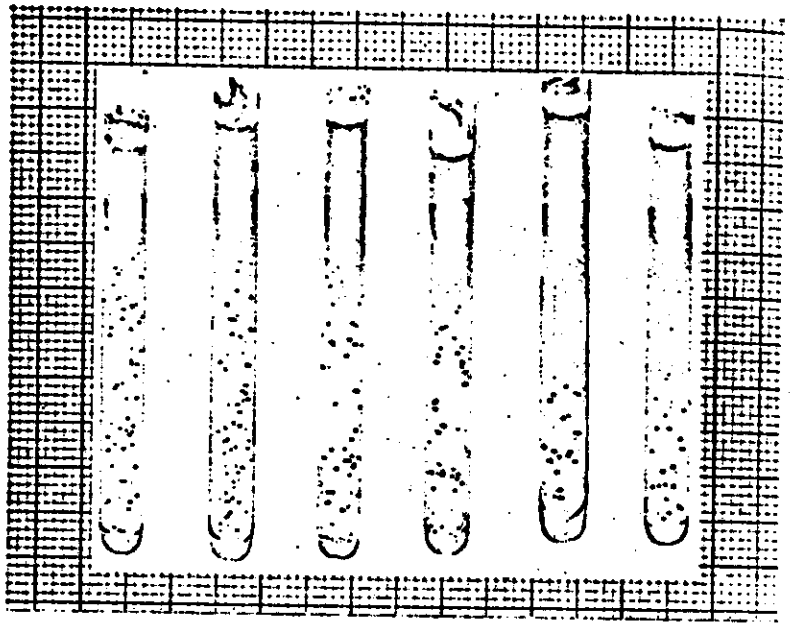

Figure 1

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A microbiological method for the qualitative detection of the chemical preservation of wines.*

by H. Luethi and T. Bezzegh

From the Swiss Federal Testing Bureau for fruit culture, viniculture and horticulture in Waedenswil

I. Introduction

Sulfurous acid is permitted in most countries as a chemical preservative in wines. Its unpleasant side effects on the consumer have been known for decades. The numerous efforts to partly substitute or reduce the hitherto tolerated maxima are therefore understandable. In the course of the last years several countries have revised their regulations and have reduced the tolerances for sulfurous acid.

For years the necessary scientific foundations have been said for the stabilization of wines. Short time-high temperature heating, pasteurization or sterilizing filtration permit to eliminate the micro-organisms and at the same time to reduce the enzymatic oxidation processes. They are also applicable and effective in small-scale operations. The importance of sulfurous acid is thus automatically reduced. Modern plants use only insignificant additions and observe an increase in the sale of wines.

Under these conditions it is not understandable why some countries permitted new preservatives for the preparation of wines (e.g. sorbic acid) or why they are considering their admission. The chemical compounds in question are used primarily to provide additional protection against undesired micro organisms (yeasts, acetic acid bacteria, lactic acid bacteria). The above mentioned effective physical methods and the general use of sulfurous acid are thus to be augmented by a convenient chemical preservation method.

Potassium Sorbate 576

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Ueber eine mikrobiologische Methode zum qualitativen Nachweis der chemischen Konservierung von Weinen*

Von *H. Lüthi* und *T. Bezzegh*
Eidg. Versuchsanstalt für Obst-, Wein- und Gartenbau, Wädenswil

I. Einleitung

Die schweflige Säure wird in den meisten weinbautreibenden Ländern als einziges chemisches Konservierungsmittel erlaubt. Ihre unangenehmen Nebenwirkungen auf den Konsumenten sind seit Jahrzehnten bekannt. Die zahlreichen Bestrebungen zum teilweisen Ersatz oder zur Reduktion der bisher tolerierten Maxima sind daher verständlich. Im Verlaufe der jüngsten Zeit haben mehrere Länder ihre Verordnungen revidiert und die Toleranzen für schweflige Säure reduziert.

Seit Jahren sind die notwendigen wissenschaftlichen Grundlagen zur Stabilisierung der Weine geschaffen worden. Die Hochkurzzeiterhitzung, Pasteurisation oder Entkeimungsfiltration erlauben eine Ausschaltung der Mikroorga-

* Diese Arbeit erscheint in englischer Uebersetzung auch im American Journal of Enology 1962.

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nismen und gleichzeitig eine beliebige Reduktion der enzymatischen Oxydationsvorgänge. Sie sind auch im Kleinbetrieb anwendbar und wirksam. Damit reduziert sich von selber die Bedeutung der schwefligen Säure. Moderne Betriebe kommen heute mit unbedeutenden Zusätzen davon aus und beobachten einen Fortschritt im Absatz der Weine.

Unter diesen Umständen ist es unverständlich, daß einige Länder neue Konservierungsmittel für die Weinbereitung zuließen (z. B. Sorbinsäure), oder daß sie deren Zulassung in Erwägung ziehen. Die in Frage stehenden chemischen Verbindungen sollen in erster Linie zusätzlichen Schutz gegen unerwünschte Mikroorganismen (Hefen, Essigbakterien, Milchsäurebakterien) bieten. Neben die erwähnten und wirksamen physikalischen Methoden und die allgemein übliche Verwendung von schwefliger Säure soll also eine bequeme chemische Konservierung gestellt werden.

Wir sind davon überzeugt, daß heute weniger denn je zwingende technische oder volkswirtschaftliche Gründe vorliegen, um neben der schwefligen Säure neue Weinkonservierungsmittel einzuführen. Die Zulassung (auch sogenannt harmloser) neuer Stoffe verzögert den Fortschritt in der Hygiene und Technologie der Weinbereitung.

Einer neuen Geschmacksrichtung entsprechend und diese zugleich fördernd sind heute mehr und mehr restzuckerhaltige Weine auf dem Markt. Die Sicherung ihrer Haltbarkeit und der Schutz gegen Nachgärungen fordert vermehrte Aufmerksamkeit von den Betrieben. Durch die Zulassung einer chemischen Konservierung kann sie bequem umgangen werden.

Jeder Fachmann kennt die Probleme der Anpassung und Resistenz von Mikroorganismen gegenüber chemischen Substanzen aller Art. Ein Konservierungsmittel allein bedeutet daher keinen Dauerschutz. Das über die Zulassung neuer Konservierungsmittel Gesagte wird dadurch bekräftigt.

Die Zahl der chemischen Konservierungsmittel ist nicht kleiner geworden. Neben bereits bekannten treten unbekannte auf und wollen ihren Platz auf dem Markte erobern. Gleichzeitig stirbt leider die Versuchung zum Einsatz solcher Mittel gerade unter den neuen Konsumbedingungen bei Weinen nicht aus. Der kontrollierende Chemiker sieht sich immer vor neuen analytischen Problemen.

Unter diesen Umständen gewinnt der qualitative Nachweis einer chemischen Weinkonservierung an Bedeutung. Dazu sind mikrobiologische Methoden besonders geeignet. Sie sind um so zuverlässiger, je weniger der zu untersuchende Wein einer Vorbehandlung unterzogen werden muß.

In der Literatur sind bereits eine Anzahl mikrobiologische Methoden beschrieben worden. Sie lassen sich in folgende Gruppen einteilen:

1. Volumetrische Methoden
2. Acidimetrische Methoden
3. Kolorimetrische Methoden
4. Methoden zur Kontrolle des Hefewachstums

Die *volumetrischen Methoden* benützen gewöhnlich einen Gärtest mit Hilfe einfacher Einhorn-Saccharimeter [Hansen (1955), Tarantola (1955), Mossel (1956), Koch (1956), Beziers (1957)] oder ähnliche einfache Glas- [Garoglio und Stella (1955)] Instrumente. Kiermeier (1953) schlug die Verwendung der manometrischen Warburg-Technik vor.

Ohne spezielle Behandlung des Untersuchungsmaterials eignet sich diese Methode vor allem für alkoholarme (weniger als 5 Vol. %) oder alkoholfreie Fruchtsäfte. Zu ihrer Anwendung bei der Kontrolle chemisch konservierter Weine schlagen verschiedene Autoren eine Wasserdampf- oder Vakuumdestillation [Beziers (1957)], andere eine Aetherextraktion [Garoglio und Stella (1953)] der Getränke vor. Wir sehen darin bedeutende Nachteile, wie Veränderung bzw. Verlust oder Nichterfassen der Konservierungsmittel.

Die *acidimetrischen Methoden* bestimmen nach verschiedener Vorbehandlung des zu untersuchenden Getränkes (Extraktion, Neutralisation) das Wachstum bestimmter Testorganismen anhand ihrer Säurebildung [Galesloot (1953), Baetslé et al. (1955)]. Die verwendeten Testorganismen sind meistens Bakterien. Den Hauptnachteil dieser Methoden erblicken wir in der erwähnten Vorbehandlung der Weine.

Die *kolorimetrischen Methoden* benützen das durch die verwendeten Testorganismen verursachte Auftreten von Farbveränderungen zugesetzter Reagenzien (Kuhn, Jerchel 1941; Mecca 1952); wobei die kolorimetrische Bestimmung des gebildeten Farbstoffes nur nach einer Extraktion vorgenommen werden kann (Kiermeier 1953). Die beschriebenen Methoden erscheinen uns umständlich oder zuwenig empfindlich.

Die *Kontrolle des Hefewachstums* kann man im flüssigen Medium direkt mit Zählkammer (Saller, 1956) oder indirekt mit Nephelometer (Clerck et al. 1954) vornehmen. Die Zellvermehrung verglichen mit der Kontrolle ermöglicht es auf das Vorhandensein von Konservierungsmitteln zu schließen. Im festen Medium wird ein dichtes Hefewachstum erzeugt. Eine chemische Konservierung äußert sich durch das Auftreten von Hemmungsöfen, deren Breite von der Konzentration abhängt (Agar-Diffusionstest).

In unserer eigenen Methode kontrollieren wir die Hefe-Kolonien bzw. die Hemmzonebildung in einem geeigneten Agarmedium, welches wir in kleine Glasröhrchen einfüllen und nach dem Erstarren mit dem zu prüfenden Weine überschichten.

Die Arbeit wurde aus Mitteln des schweizerischen Rebbaufonds ermöglicht. Den verantwortlichen Behörden gebührt an dieser Stelle ein besonderer Dank.

II. Material und Methode

Material:

Hefestamm: Sacch. cerevisiae vom Typus der «Johannisberg»-Weinhefe aus unserer Sammlung.

Agar-Medium: Difco-Agar 1 % mit Zusatz von 10 % weißem Traubensaf (spez. Gewicht 1,065).

Einstellen des pH-Wertes auf 4,5 mit KOH. Abgefüllt in Reagensgläse (je 18 ml), anschließend Sterilisieren im Dampftopf 25 Minuten.

Prüf-Röhrchen: Glasröhrchen von 3 mm Durchmesser und etwa 80 mm Länge.

Injektionsspritzen: Eine 5-ml- und eine 1-ml-Spritze.

Konservierungsmittel: Zu unseren Testversuchen verwendeten wir folgend Konservierungsmittel und Konzentrationen der Stammlösungen:

10 mg/l Actidion
10 g/l Kalium-Sorbinat
10 g/l p-Oxybenzoesäure-propylester in 40 % Alkohol
0,1 ml/l = ca. 0,15 Monobromessigsäure-aethylester in 20 % Alkohol

Methode:

Die Hefekultur wird in weißem Traubensaft bei 25° C angesetzt, nach 1— Tagen abzentrifugiert und in sterilem Leitungswasser suspendiert. Mit Hil einer Zählkammer (Thomakammer) wird eine Ausgangs-Suspension mit 1 · 1 ml Hefezellen hergestellt. 2 ml davon werden zu 18 ml flüssigem, auf gen 45° C gehaltenem Agar gegeben und gut durchgemischt. Dadurch entsteht ei Suspension mit etwa 1000 Hefezellen pro ml. Damit werden die Prüfröhrch bis zur Hälfte (etwa 0,5 ml) gefüllt.

Nach dem Erstarren wird der zu prüfende Wein (etwa 0,1 ml) ohne Lu blase auf die Agaroberfläche gebracht. Ueber Nacht läßt man den Wein 8—10° C in den Agar hineindiffundieren. Anderntags beginnt die Bebrüt bei 25° C. Wir empfehlen von jeder Untersuchung mindestens 5 Parallelp ben anzufertigen.

Zeitbedarf:

Vorbereitungen 4 Stunden, Diffusion etwa 16 Stunden, Bebrütung 40 St den, total etwa 60 Stunden. Die *Auswertung* besteht in der Feststellung Messung von eventuell auftretenden Hemmzonen des Hefewachstums.

III. Ergebnisse

Bestimmung des Nullwertes

Die Verwendbarkeit der Methode mußte zuerst an nichtkonservierten W nen geprüft werden. Solche können von Natur aus stark variierende Me von Alkohol und Gerbstoffen aufweisen. Bei verpilztem Rohmaterial muß ner mit der Anwesenheit gewisser antibiotisch wirkender Stoffe [z. B. Bot cin (Ribéreau-Gayon et al. 1952)] gerechnet werden. Außerdem ist als erl tes Konservierungsmittel die schweflige Säure in Betracht zu ziehen. Alle Faktoren wurden auf ihre Auswirkung im Rörchenversuch geprüft.

Wir untersuchten mehr als 40 Weine mit verbürgter Herkunft aus verschiedensten in- und ausländischen Weinbaugebieten. Darunter befanden sich noch nicht konsumfertige Weine mit extrem hohen Gerbstoffgehalten. Ferner wurden verschiedene Weine mit Gehalten bis zu 35 mg/l freier schwefliger Säure mit an schweflige Säure adaptierten und nicht adaptierten Hefen untersucht.

Die Resultate lassen sich dahin zusammenfassen, daß wir in keinem Fall Hemmzonen (Nullwerte) von mehr als 3 mm feststellen konnten. Auf Grund unserer Versuche wäre in Zweifelsfällen von einer beobachteten Hemmung ein Betrag von dieser Höhe abzuzählen, bevor ein Schluß gezogen werden darf. Nach unserer Erfahrung lassen Hemmzonen mit einem durch genügende Wiederholungen gesicherten Wert von mehr als durchschnittlich 3 mm mit sehr großer Wahrscheinlichkeit auf eine chemische Konservierung schließen. Solche Fälle müssen zum mindesten als verdächtig bezeichnet werden. Hemmwerte von durchschnittlich 5 mm und mehr deuten mit *Sicherheit* eine chemische Konservierung an.

Modellversuche mit verschiedenen Konservierungsmitteln

In Modellversuchen ließen sich die Wirkungen von Konzentrationsreihen verschiedener Konservierungsmittel prüfen. Die auf diese Weise erhaltenen Werte geben wertvolle Anhaltspunkte über die Brauchbarkeit und Empfindlichkeit der Methode. Die mit verschiedenartigen Konservierungsmitteln gewonnenen Erfahrungen sind in den Tabellen 1—4 zusammengestellt worden.

1. Versuche mit Actidion (Cycloheximid)

Dieses aus Streptomyces griseus hergestellte Antibioticum zeichnet sich durch seine spezifische Wirkung gegen das Wachstum vieler (nicht aller) Hefen aus, gegenüber einer in Gang befindlichen Gärung ist es dagegen praktisch wirkungslos.

Die Anwendung dieses Mittels zur Weinkonservierung wurde vor allem durch Ribéreau Gayon et al. (1958), aber auch von andern Autoren diskutiert und ernsthaft für die Praxis in Erwägung gezogen. Bei bestimmten Weinhefen erzielten die erwähnten Autoren in Traubenmosten eine totale Hemmung mit Zusätzen von 10 mg/l. Infolge des Alkoholgehaltes reduzierte sich bei Weinen der wirksame Zusatz auf Mengen von 200—300 γ/l. Unsere Erfahrungen mit verschiedenen Actidion-Konzentrationen in Weinen mit Alkoholgehalten zwischen 9 und 12 Vol. %, pH-Werten von 3,4—3,8 und Konzentrationen an freier schwefliger Säure von weniger als 35 mg/l sind in Tabelle 1 zusammengestellt.

Um diese experimentell gefundenen Werte zu kontrollieren, wurde versucht, nach der Formel von Cooper und Woodman (1946) die Größe der Hemmzonen auch mathematisch zu ermitteln. Unsere auf diese Weise erhaltenen Werte stimmten mit den experimentell gefundenen eine gute Uebereinstimmung, worin wir einen Beweis für die Brauchbarkeit dieser Formel erblicken.

Tabelle 1

Actidion-Konzentrat. (γ/l)	0	50	100	200	300	400	500	600
Hemmungszonen Durchschnittswerte in (mm)	0,55	2,0	4,0	8,0	10,5	13,0	14,0	15,0
Extremwerte der Hemmzonen (mm)	0 – 3,0	0 – 3,5	2,0– 7,5	3,5– 12,0	6,5– 16,0	8,0– 13,5	9,5– 15,0	10,5 16,0

2. *Versuche mit Kalium-Sorbinat*

Sorbinsäure ist ein für den Menschen als harmlos bezeichnetes Konservierun mittel mit starker fungistatischer Wirkung, welches in neuerer Zeit vielfach Eingang in die Praxis fand. Zur Ueberbrückung ihrer schlechten Löslichk werden meistens ihre Salze verwendet. Für bestimmte Zwecke werden sie zahlreichen Ländern toleriert. In Frankreich ist Sorbinsäure in einer Dosieru bis maximal 200 m/gl schon als Weinkonservierungsmittel zugelassen word Nach Saller (1957) genügen 40—70 mg/l, um Weine unter bestimmten Bed gungen vor einer Nachgärung zu schützen. Raible und Busch (1957) haben Natrium-Sorbinat in Malzwürze den Grenzwert von 480 mg/l bei pH 4,1— ermittelt. Hier wurde schon eine deutliche abtötende Wirkung beobachtet. einer Konzentration von 320 mg/l dagegen nehmen die Hefezellen noch Dieser Wert reduziert sich im Wein infolge des Alkoholgehaltes auf 200— mg/l. Böhringer (1959/60) berichtet, daß 200 mg/l Sorbinsäure (als Kali Sorbat) zur Konservierung von Weinen nicht genüge.

Unsere eigenen Erfahrungen mit Kalium-Sorbinat sind in Tabelle 2 zu mengefaßt.

Tabelle 2

Kalium-Sorbinat (mg/l)	0	50	100	200	300	400
Hemmungszonen Durchschnittswerte in (mm)	0,9	2,2	3,94	7,03	8,35	10,6
Extremwerte der Hemmzonen (mm)	0 – 4,0	0,5– 5,0	2,0– 5,0	4,5– 9,0	6,0– 11	8,0– 14

3. *Versuche mit p-Oxybenzoesäure-propylester*

Die von Sabalitschka eingehend untersuchten Ester der p-Oxybenzoe haben seit langem Eingang in die Konservierungspraxis gefunden. Er verg (1959) die Wirkung des Propylesters («Nipasol-M») mit jener der Sorbin und findet unter bestimmten Versuchsbedingungen eine viermal bessere kung des Esters, von welchem 200—300 mg/l die Hefevermehrung unterd

:en. Für Weinkonservierung geben Ribéreau-Gayon und Peynaud (1958) ähn-
:che Werte an.

Unsere Versuche sind in Tabelle 3 zusammengefaßt.

Tabelle 3

p-Oxybenzoesäure-propylester (mg/l)	0	50	100	200	300	500
Hemmungszonen Durchschnittswerte in (mm)	0,47	0,85	1,81	3,69	6,95	10,4
Extremwerte der Hemmzonen (mm)	0 – 2,0	0 – 1,5	0 – 5,0	1 – 6,5	5 – 10,5	7 – 11,5

4. *Versuche mit Monobromessigsäure-aethylester*

'onobromessigsäure-aethylester («Antibiotin») ist ein kräftig wirkendes Kon-
'rvierungs- und Desinfektionsmittel sowie Enzymgift. Es ist ziemlich unbestän-
:. Bereits sehr kleine Mengen sind in Fruchtsäften und besonders in Weinen
n großer Wirkung. Diese nimmt vom Methylester zum Glycolester stark zu.
i Testversuchen von Oberto (1955) ergab sich, daß 15 mg/l Monobromessig-
ure die Gärung nur drosselte und eine Konzentration von 40 mg/l nötig war,
n einen vollkommenen Gärstopp zu erzeugen. Hansen (1955) hat auch ähn-
he Werte bekanntgegeben. Für die Weinkonservierung geben Ribéreau-
ayon und Peynaud (1960) Konzentrationen von 2—10 mg/l an.

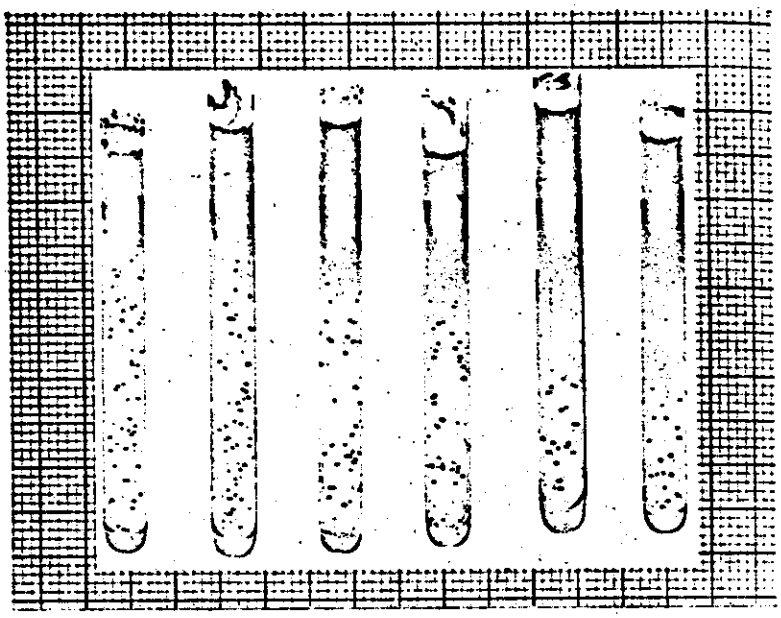
Unsere Versuche zeigten, daß Monobromessigsäure-aethylester von allen ge-
üften Mitteln die größte Wirkung hat (siehe Abb. 1).

Die in Tabelle 4 zusammengestellten Versuchsresultate zeigen, daß schon
ir kleine Zusätze zu Wein deutliche Hemmzonen im Wachstum der Hefen
ursachen.

Tabelle 4

Monobromessigsäure-aethylester (mg/l)	0	0,75	1,5	3,0	4,5	7,5	15,0
Hemmungszonen Durchschnittswerte in (mm)	1,19	4,28	9,64	13,95	18,48	22,0	27,55
Extremwerte der Hemmzonen	0 – 3,5	1,5 – 7,0	6,5 – 12,0	10,5 – 17,0	16 – 21,0	18,5 – 24,5	26 – 28,5

Abbildung 1



Modellversuch eines mit Monobromessigsäure-aethylester konservierten Rotweines

Die dunkle Zone im oberen Teil der Gläschen ist auf die in den Agar hinein diffu-dierte Rotweinfarbe zurückzuführen.

Von links nach rechts:

Hemmzone in mm	1	3	9	13,5	20	21,5
Geprüfte Konzentration mg/l	0	0,75	1,5	3,0	4,5	7,5

Bei dem Versuchswein handelt es sich um einen Veltliner (1954) mit hohem Gerbst-gehalt. Die gemessenen Hemmzonen geben Auskunft über die Praxis der Ablesung v Resultaten. Man beachte die Größe der Kolonien im Hemmungsbereich.

IV. Diskussion

Mit der beschriebenen Röhrchenmethode sind in mehreren hundert Ver-chen Erfahrungen zum Nachweis einer chemischen Konservierung von Wei-gesammelt worden. Die Methode ist so weit ausgearbeitet, daß sie mit einer b-friedigenden Konstanz arbeitet und eine zuverlässige Beurteilung erlaubt. Du-die Ueberschichtung des Agars mit der doppelten Weinmenge (ca. 0,2 ml) k-die Empfindlichkeit der Methode noch deutlich erhöht werden. Auch die N-werte werden dabei größer.

Um Vergleiche zwischen mehreren Versuchen zu ermöglichen, soll auf gleiches Hefealter und gleich dichte Suspensionen geachtet werden. Wichtig für den Erfolg ist ferner die gute Verteilung der Hefen im Agar.

Aus Abbildung 1 ist zu sehen, daß der tatsächliche Hefegehalt pro ml wesentlich kleiner ist, als er auf Grund der Auszählung mit der Thoma-Kammer hätte erwartet werden müssen. Dies muß auf die hohe Schmelztemperatur (45° C) des Agars zurückgeführt werden.

Die Zahlen in den Tabellen 1—4 zeigen, daß die untersuchten Konservierungsmittel sich in ihrer Auswirkung auf das Hefewachstum unter unseren Versuchsbedingungen außerordentlich stark unterscheiden. Wo Verdacht auf chemische Konservierung eines Weines vorliegt, vermag die Methode wohl in den meisten Fällen sichere Anhaltspunkte zu geben. Die untersuchten Konservierungsmittel sind schon in Konzentrationen nachweisbar, welche noch keine sichere Konservierung eines Weines gewährleisten würden (vgl. Tabelle 5).

Tabelle 5

Beurteilung der Leistungsfähigkeit des Verfahrens

Konservierungsmittel	Für die Weinkonservierung empfohlene Dosis (mg/l)	Mit der Methode sicher erfaßbare Konzentration (mg/l)
Actidion	0,1 — 0,3	0,125
Kalium-Sorbinat	200 — 480	150
p-Oxybenzoesäure-propylester	200 — 500	250
Monobromessigsäure aethylester	2 — 10	1,5

Bei der Beurteilung des Resultates ist nicht nur auf die gemessene Hemmungszone allein abzustellen. Gleichzeitig sollen auch die Größen der Hefekolonien in Betracht gezogen werden. Ein Konservierungseffekt zeigt sich stets mit Sicherheit darin, daß die obersten Kolonien deutlich kleiner sind. Eine solche Feststellung ist besonders von Bedeutung, wenn nur relativ kleine Hemmzonen zu beobachten sind.

Die in Tabelle 1—4 gezeigten Extrem-Werte der Hemmzonen scheinen auf den ersten Blick sehr weit auseinander zu liegen und die Brauchbarkeit der Methode in Zweifel zu ziehen. Sie erklären sich aber aus der notwendigen geringen Hefekonzentration pro ml und ihrer Verteilung im festen Nährmedium (siehe Abb. 1). Die Methode gestattet leicht, eine größere Zahl von Parallelversuchen auszuführen. Der dabei ermittelte Durchschnittswert der Hemmzonen, kombiniert mit der Beurteilung der Koloniengröße, gestattet eine sichere Beurteilung des untersuchten Falles.

Zusammenfassung

Es wird eine mikrobiologische Methode zum qualitativen Nachweis der chemische Weinkonservierung beschrieben. Sie beruht auf der Kontrolle des Wachstums und de Größe von Hefekolonien in Agar-Röhrchen.

Es wird zunächst das Verhalten zahlreicher nichtkonservierter Weine geprüft. Mi den Konservierungsmitteln Actidion, Kaliumsorbinat, p-Oxybenzoesäure-propylest und Monobromessigsäure-aethylester werden Modellversuche durchgeführt und ih Resultate beschrieben. Die dabei festgestellten Hemmungszonen werden miteinande verglichen und der Schluß gezogen, daß die Methode eine für die Praxis genügend Empfindlichkeit besitzt. Sie erlaubt für die untersuchten Konservierungsmittel berei den Nachweis kleinerer Konzentrationen, als sie für die Praxis in Frage kämen. D Methode ermöglicht eine direkte Verwendung des zu untersuchenden Weines und b nötigt keine Vorbehandlung desselben. Sie ist einfach und läßt sich routinemäßig gleich zeitig für viele Proben durchführen.

Résumé

Description d'une méthode microbiologique pour la détection des agents conserv teurs dans le vin reposant sur le contrôle de la croissance de colonies de levure (Sacc cerevisiae du type de la levure de vin «Johannisberg») et sur leur grandeur dans d éprouvettes contenant de l'agar additionnée de jus de raisin blanc.

Des essais à blanc ont été faits avec de nombreux vins non conservés ou conserv avec du SO_2; d'autres essais ont été faits avec des vins additionnées d'actidione, sorbate de potassium, d'ester propylique de l'acide p-hydroxybenzoique et de l'es éthylique de l'acide monobromacétique. La méthode est sensible, simple et prése l'avantage de ne pas nécessiter un traitement préalable du vin; elle convient bien p des essais en série.

Summary

Description of a simple and sensitive microbiological method for the detection presentatives in wine based on their inhibiting effect upon the growth of yeast and size of yeast colonies in agar tubes. The preservatives examined were actidione, po sium sorbate, the propyl ester of p-hydroxybenzoeic acid and the ethyl ester of mo bromacetic acid. An advantage of this method is that it does not require any prelimin treatment of the wine.

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Sur la teneur des vins en zinc

Par *J. Vogel* et *J. Deshusses*
(Laboratoire cantonal de chimie, Genève)

Selon l'article 347, alinéa 4, de l'Ordonnance fédérale réglant le commerce s denrées alimentaires, du 26 mai 1936, les vins contenant des substances xiques, des sels solubles de zinc entre autres, doivent être considérés comme sibles à la santé et, par voie de conséquence, être exclus de la consommation. Cependant, il est établi que toutes les denrées tant d'origine végétale qu'ani-le contiennent normalement du zinc. Le vin n'échappant pas à cette règle, est nécessaire de fixer la teneur des vins en «zinc normal». Les vins qui tiendraient plus de zinc que cette teneur normale seraient dès lors considérés me étant accidentellement souillés par des sels de zinc et les dispositions de ticle précité pourraient alors leur être appliquées sans difficultés.

Mais nous ne disposons, aujourd'hui encore, que de fort peu de documents

of the reported values and should be verified by comparing estimations of the unhydrogenated and hydrogenated fractions. For trace esters (< 0.1%), a larger variation is allowable within the sensitivity of the method.

(*k*) It is difficult, if not impossible, to prevent baseline drift with dual columns in temperature programming. Initially, it is difficult to match the packing densities and flow rates precisely and, after use, retention times cannot be matched by changing flow rates.

(*l*) One of the most important points is to anticipate the amounts of esters present and change programming rates when necessary so they are effective.

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PRESERVATIVES AND ARTIFICIAL SWEETENERS

Rapid Determination of Sorbic Acid in Orange Juice

By KERMIT M. FLOYD (Food and Drug Administration, 60 Eighth St., N.E., Atlanta, Ga. 30309)

In the rapid procedure described here, orange juice is screened for the presence of sorbic acid by measuring the UV absorbance of an alcoholic solution of the juice. The sorbic acid is isolated by solvent extraction, measured quantitatively by UV spectrophotometry, and finally identified by paper chromatography.

Standards have been established for orange juice which do not permit the use of sorbic acid as a preservative (1). However, because of the perishable nature of orange juice, some manufacturers have used sorbic acid to extend its shelf life. Thus, a rapid method for the screening, identification, and quantitative measurement of sorbic acid is necessary for regulatory purposes.

Several methods have been reported for the determination of sorbic acid in foods. Isolation of sorbic acid by steam distillation (2–6) is usually a long procedure, gas chromatographic methods (7–11) require a spe-

cial column, and the paper chromatographic method of Höyem (12) requires 18 to 20 hours for identification and quantitation.

During the search for a fast and simple method, an extraction procedure, analogous to that developed by Stanley for benzoic acid (13), was investigated; however, collaborators reported that troublesome emulsions formed when chloroform or ether were used as extractants. In addition, ethyl ether is less desirable as a spectrophotometric solvent because it is volatile and flammable. Other solvents were therefore tested and a mixture of ether and petroleum ether (according to Sigrido (10)) was found suitable since emulsification was kept comparatively low. The difficulties caused by ether as the photometric solvent were overcome by reextracting the sorbic acid into dilute aqueous base and measuring it in that solution. The combination of extraction and a photometric determinative step proved to be satisfactory because several samples can be analyzed in a short time, and no unusual equipment is required.

In the procedure described here, juice is rapidly screened for the presence of sorbic acid; if present, the sorbic acid is isolated, quantitatively measured, and finally confirmed by paper chromatography.

A somewhat modified version of the method of Vandercook and Rolle (14) for determination of total polyphenolics has been used for the rapid screening procedure. In this method the orange juice is diluted with alcohol, the precipitated pectins are filtered out, and the UV absorption of the juice is measured. An absorbance spectrum characteristic for orange juice is found in the absence of sorbic acid. If sorbic acid is present, no point of inflection is noted in the 273–277 mμ region and excessive increase in absorbance is noted at about 255 mμ (Fig. 1). As little as 0.005% sorbic acid may cause this behavior.

For quantitation, the orange juice is acidified with hydrochloric acid and the sorbic acid is extracted into an ether-petroleum ether mixture. The extract is carried through sodium sulfate for complete destruction of emulsion and subsequently the sorbic acid is reextracted into aqueous sodium hydroxide. This reextraction separates interfering substances, e.g., colorants which are left in the organic phase. The sorbic acid is determined quantitatively by UV spectrophotometry in the weakly basic solution; *see* Fig. 2.

For identification, the paper chromatographic method of Höyem (12) was modified in order to shorten the development time but consequently it became unselective. Therefore, the spot test of Copius-Peereboom and Beekes (15) was used for specificity.

A paper chromatographic method for identification was selected in preference to TLC because no time is needed to prepare chromatographic plates and a paper chromatogram serves as a permanent record.

Sorbic acid was separated from plant materials by descending paper chromatography. A spot due to sorbic acid was detected specifically by spraying the chromatogram first with a potassium dichromate solution and then with a 2-thiobarbituric acid solution.

Sorbic acid is oxidized to an intermediate malonaldehyde by potassium dichromate. The malonaldehyde reacts with 2-thiobarbituric acid to produce a characteristic pink color. Both sorbic and benzoic acids are visible on the chromatogram under shortwave UV light and their R_f values are very close. Since benzoic acid does not react with 2-thiobarbituric acid, the color reaction differentiates the acids.

METHOD

Apparatus

(a) *Chromatographic tank.* — 9 × 9 × 3½", Mitchell type, stainless steel, for descending paper chromatography.

(b) *Filter paper.*—Whatman No. 1, 8 × 8" sheets.

Reagents

(a) *Chromatographic solvent.*—Shake a mixture of *n*-butanol, NH_4OH, and H_2O (5:2:3) in a separatory funnel. Place bottom layer in a beaker located in the tank; use top layer as the mobile phase.

(b) *Sorbic acid solution.*—Approximately 1 mg/ml in $CHCl_3$.

(c) *Sorbic acid standard solution.*—Dissolve 0.100 g sorbic acid or an equivalent amount of potassium sorbate and dilute to 1 L with

ction separates interfering sub-
colorants which are left in the
e. The sorbic acid is determined
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asic solution; *see* Fig. 2.
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METHOD

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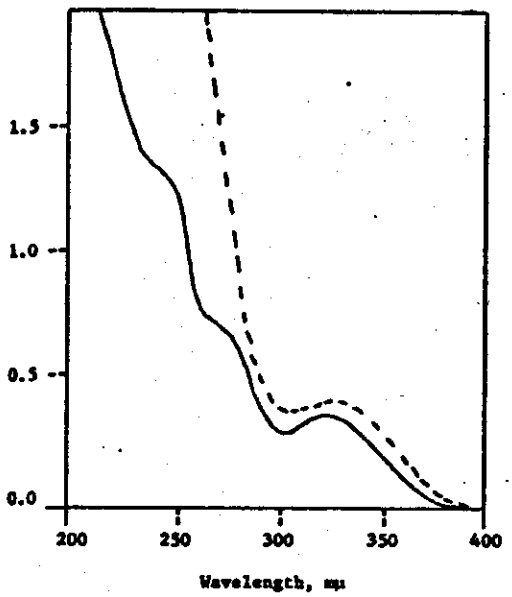

Fig. 1—UV spectra of orange juice in alcohol, with (---) and without (—) sorbic acid.

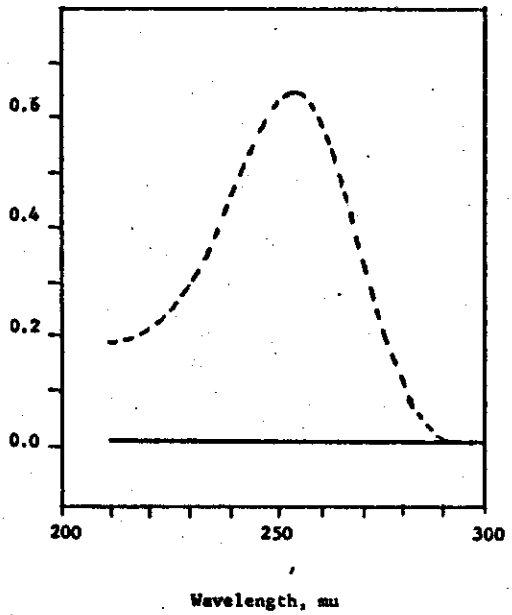

Fig. 2—Orange juice extracted into 0.001*N* sodium hydroxide, with (---) and without (—) sorbic acid.

0.001*N* NaOH; dilute 25.0 ml of that solution to 1 L with 0.001*N* NaOH (1 ml = 0.0025 mg sorbic acid).

Sample Preparation

(a) *Orange juice concentrate.*—Mix thoroughly to insure a uniform sample and determine soluble solids with a refractometer so the sorbic acid content can be related to the concentrate by calculation. Dilute with distilled water to ca 11.8% soluble solids and proceed as in (b), beginning with " . . . filter through a pad . . .".

(b) *Single strength orange juice.*—Mix thoroughly to insure a uniform sample and filter through a pad of absorbent cotton or centrifuge to remove pulp.

Screening Procedure

Place 5.0 ml prepared sample into a 100 ml volumetric flask and fill with alcohol to mark. Mix well and filter through fine paper. Measure the UV spectrum of the filtrate with a recording spectrophotometer from 400 to 220 mμ.

If the spectral curve does not look normal for orange juice (no point of inflection at ca 273–277 mμ and excessive absorbance at ca 255 mμ), dilute 2 ml filtrate to 10 ml or more with alcohol to reveal the sorbic acid peak at ca 255 mμ.

Determination

Quantitative.—Put 50.0 ml sample into a 125 ml separatory funnel and add 2 ml HCl. Extract with three 50 ml portions of ether-petroleum ether mixture (1 + 1), shaking first extraction mixture gently for 3 min. Drain each extract, including small quantities of emulsion, into glass-stoppered Erlenmeyer flask containing 10 g anhydrous Na_2SO_4.

Shake combined ether extracts with Na_2SO_4 and decant into a 250 ml separatory funnel. Rinse Na_2SO_4 with three 10 ml portions of the ether mixture, and combine with original extracts. Next, extract with four 50 ml portions of ca 0.01*N* NaOH, and collect extracts in a 250 ml volumetric flask. Heat flask on a steam bath under gentle air stream to remove traces of ether, cool, and dilute to volume with 0.01*N* NaOH; dilute 5.0 ml of this solution to 50.0 ml with H_2O, and record the spectra of the sample and standard solution from 300 to 220 mμ in 1 cm silica cells against 0.001*N* NaOH. Calculate % sorbic acid in juice by comparison of sample and standard absorbances at ca 255 mμ.

Qualitative.—Transfer remainder of solution from the 250 ml volumetric flask to a separatory funnel, acidify with HCl against litmus paper, and extract with 50 ml $CHCl_3$. Filter through cotton and concentrate to ca 5 ml.

Place 10 μl sorbic acid soln (b) and 10 μl sample solution along the starting line drawn 7.5 cm from the top edge of paper. Place paper

in tank, add mobile phase, and allow to develop for ca 10 cm. Remove chromatogram, mark solvent front, and air-dry in a hood. View chromatogram under short-wavelength UV light. Outline blue spots with a pencil. (Approximate R_f values for sorbic and benzoic acids are 0.50 and 0.43, respectively.)

Spray chromatogram lightly with 10% aqueous $K_2Cr_2O_7$ and let dry; spray with saturated aqueous 2-thiobarbituric acid solution. Sorbic acid appears as a pink spot.

Results and Discussion

This procedure is rapid and accurate; recoveries of sorbic acid ranged from 94 to 103%. Representative results are shown in Table 1.

No interferences or false absorbances have been noted in blank determinations on orange juice free of sorbic acid. See Fig. 2.

Conformity to Beer's Law for sorbic acid in 0.001*N* sodium hydroxide from 0.5 to 4 μg/ml in the final dilution by this procedure (0.0025 to 0.02% in the juice) is shown by the standard curve in Fig. 3.

Acknowledgment

The author would like to acknowledge with grateful appreciation the assistance of Grayson R. Rogers, Atlanta District, Food and Drug Administration, who performed the collaborative check and was the chief critic of this method during its development.

Table 1. Recoveries of sorbic acid added to orange juice or water solutions

Product	Sorbic Acid, mg		
	Added	Recovered	% Recovery
Water solution	5.0	5.0	100
Commercial orange juice	5.0	4.7	94
	3.7	3.6	97
	3.7	3.6	97
	3.0	3.0	100
	3.0	3.1	103
Authentic orange juice concentrate[a]	10.0	9.6	96
	7.5	7.4	99
	5.0	5.1	102
	2.5	2.5	100
Authentic orange juice	10.0	9.6	96
	7.5	7.2	96
	5.0	4.9	98
	2.5	2.5	100

[a] Concentrate diluted to about 11.8% soluble solids.

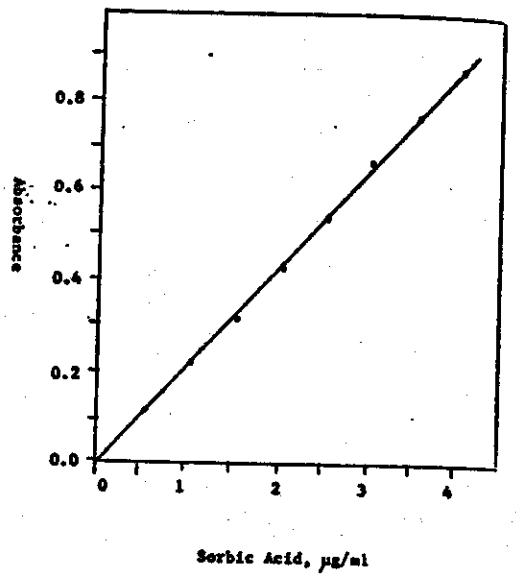

Fig. 3—Sorbic acid in 0.001*N* sodium hydroxide.

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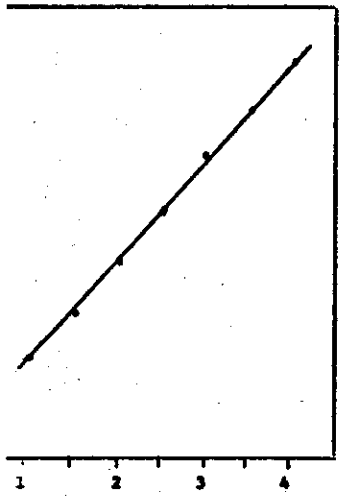

Sorbic Acid, μg/ml

acid in 0.001N sodium hydroxide.

ve check and was the chief method during its development.

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Preservatives: A Review of Methods of Analysis

By PIETER L. SCHULLER and EDUARD VEEN (Laboratory of Chemical Analysis of Foods, Institute of Public Health, Utrecht, The Netherlands)

This article presents a review of the most important methods of analysis which have been published during the last decade for the common preservatives sulfur dioxide, sorbic acid, nitrate and nitrite, and benzoic acid.

Part I: Sulfur Dioxide

Several methods are available for determining sulfur dioxide in liquid and solid food. The fact that so many methods and modifications of methods have been proposed indicates that no entirely satisfactory general procedure has yet been devised. Literature on the determination of sulfur dioxide is extensive, confused, and sometimes contradictory, and it largely reflects the different problems presented by various foods that contain interfering substances.

When sulfur dioxide is used as a preservative, part of it combines with naturally occurring components of the food such as aldehydes, ketones, sugars, polysaccharides, aminosugars, reductone, dehydroascorbic acid, α-ketoglutaric acid and polyphenols (1) and part of it remains free in the inorganic state as sulfurous acid and bisulfite ions. It is widely recognized that only free sulfur dioxide is effective against microorganisms. Consequently there are two analytical problems: the determination of free and of total sulfur dioxide.

Qualitative Determination

The qualitative determination is important only where the use of sulfur dioxide is forbidden in certain foods, e.g., meat. Several methods have been proposed. In the first group, sulfur dioxide is reduced by zinc (2) or titanous chloride (3); the hydrogen sulfide generated by the sulfur dioxide is detected with lead acetate, or potassium iodate (4) and starch. In the second group, sulfur dioxide decolorizes a colored solution, e.g., pararosaniline as proposed by Paland (5) or malachite green by Kaplan (6). After a collaborative study, the method of Kaplan was adopted as official, first action by the AOAC. In addition, spot tests for sulfites have been proposed: one by Senise (7) based on the induced oxidation of cobalt azide solution, a second by Nemets and Grzhivo (8) using mercuric nitrate as reagent, and a third by Luis and Sà (9) based on the reduction of Cu (II) to Cu (I) followed by precipitation as Cu (I) Hg (II) iodide.

Quantitative Determination

The quantitative methods of analysis can be divided into two main groups: those that require distillation and those that do not require distillation.

This review was presented at the second meeting of the Codex Committee on methods of analysis and sampling of the Joint FAO/WHO Codex Alimentarius Commission, Sept. 19–24, 1966, in Berlin, West Germany as a source document for deliberation at the international level to assist in a choice of methods of analysis for these preservatives.

PRESERVATIVES AND ARTIFICIAL SWEETENERS

Rapid Screening Procedure for Determination of Benzoic Acid and Sorbic Acid in Fruit Beverages

By W. M. GANTENBEIN and A. B. KARASZ (New York State Food Laboratory, Department of Agriculture and Markets, 1220 Washington Ave., Albany, N.Y. 12226)

A rapid and accurate method, requiring one extraction and UV analysis, has been developed for simultaneous detection of benzoic and sorbic acids in fruit beverages. Quantitative measurements of benzoic and sorbic acids are based on the strong absorbances at 225 and 250 mμ, respectively. Recoveries of benzoic and sorbic acids from orange juice and apple juice at levels normally present were adequate.

One of the routine tasks of this laboratory is the detection of undeclared preservatives in fruit beverages; the most commonly suspected preservatives are benzoic and sorbic acids. Since a large number of samples must be examined to find the relatively few violations, there is a need for a rapid screening test.

The official AOAC spectrophotometric method, 27.008, for benzoic acid in beverages requires 15 consecutive extractions and is not suitable for processing a large number of samples. In the official method the benzoic acid is estimated from the absorption of the extract at 272 mμ where benzoic acid has an absorption maximum. To obtain a suitably high absorption at the commonly occurring benzoic acid concentrations, a 10 ml beverage sample is analyzed; however, the extract from this size sample contains enough of a UV absorbing material so that correction is required.

The proposed rapid screening method for benzoic acid is based on the fact that, in addition to the absorption maximum at 272 mμ and the two adjacent lesser ones at 282 and 265 mμ (1), benzoic acid also has a maximum at 225 mμ (2); the absorptivity at this wavelength is about ten times greater than that at 272 mμ, as shown in Fig. 1. Consequently, measuring the absorption at 225 mμ permits the use of a much smaller sample: 1.0 ml is sufficient. This small volume can be dissolved in ethyl ether, which then can be dried by adding petroleum ether and anhydrous sodium sulfate. Purification by extraction is not needed, as the concentration of UV-absorbing materials in the small volume of fruit beverage taken for analysis is quite low.

This rapid test will also reveal the presence of sorbic acid, which absorbs strongly at 250 mμ (Fig. 2). Sorbic acid may be quantitatively measured by its absorption at 250 mμ.

We have not encountered beverage samples containing both benzoic and sorbic acids. Such a combination would have absorption maxima at 250, 225, and 207 mμ; *see* Fig. 3. A rough estimate of the amount of sorbic acid may still be made, since the absorption of benzoic acid at 250 mμ is at a minimum. The quantitation of benzoic acid in such a mixture

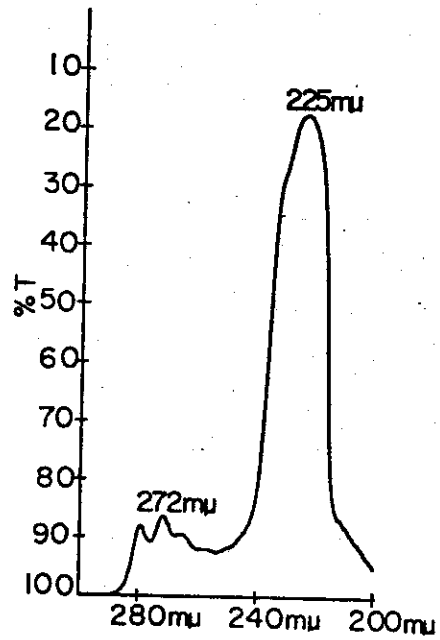

FIG. 1—UV spectrum of 0.10% benzoic acid in orange juice.

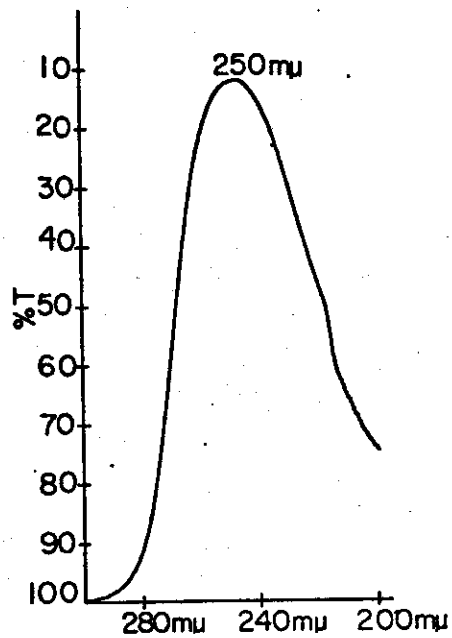

FIG. 2—UV spectrum of 0.04% sorbic acid in orange juice.

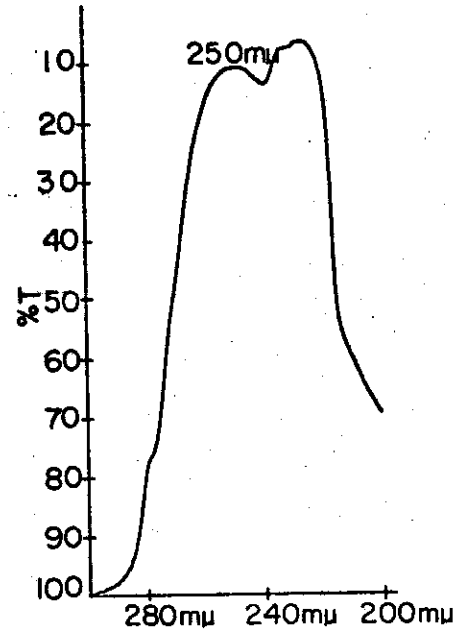

FIG. 3—UV spectrum of 0.10% benzoic acid and 0.04% sorbic acid in orange juice.

requires separation of the two acids by thin layer or gas chromatography.

In the application of this method we have not encountered any interferences. However, fumaric acid has been approved as an acidulant in beverages and it has an absorption maximum at 225 mμ. Fumaric acid can be distinguished from benzoic acid, since it does not exhibit either a triple peak at 282, 272, and 265 mμ or a shoulder at 233 mμ. We have not found fumaric acid in any of the fruit beverages we have examined.

In non-fruit beverages many UV-absorbing materials, such as vanillin and other flavors, may be expected, so the method has to be applied to such samples with caution.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.* — Beckman DB, or equivalent, with recorder.

(b) *Hydrochloric acid.*—1 + 1.

(c) *Benzoic acid solutions.*—(*1*) *Stock solution:* Dissolve 0.5900 g sodium benzoate in water and dilute to 100 ml. (*2*) *Standard solutions:* Dilute 4.0, 8.0, 12.0, 16.0, and 20.0 ml stock solution to 100 ml with water.

(d) *Sorbic acid solutions.*—(*1*) *Stock solution:* Dissolve 0.6700 g potassium sorbate in water and dilute to 200 ml. (*2*) *Standard solutions:* Dilute 2.0, 4.0, 8.0, 12.0, and 16.0 ml stock solution to 100 ml.

Preparation of Standard Curve

Pipet 1.0 ml of each standard solution into glass-stoppered Erlenmeyer flask and acidify with 0.5 ml HCl (1 + 1). Add 40 ml ethyl ether, 40 ml petroleum ether, and ca 5 g anhydrous sodium sulfate separately and shake after each addition. Filter into 100 ml volumetric flask, and wash Erlenmeyer flask and filter three times with ether; dilute to volume with ether. Prepare blank by treating 1.0 ml water in same manner. Record absorbance of the standard solutions against blank, using 1 cm cells.

For benzoic acid, plot concentration against absorbance at 225 mμ. For sorbic acid, plot concentration against absorbance at 250 mμ. These plots are linear for benzoic acid up to 1.0 mg and for sorbic acid up to 0.4 mg in 100 ml ether solution, as shown in Fig. 4, and obey the Beer-Lambert law.

Determination

Analyze 1.0 ml beverage as described above for the standard. From the absorbance of sample, find in standard plot corresponding concentration of preservative in 1.0 ml sample. Multiply

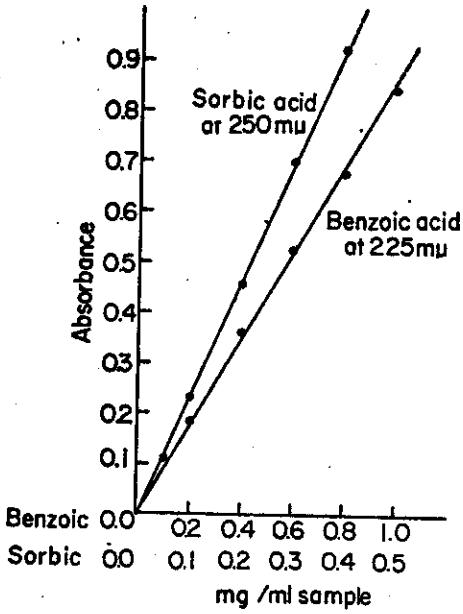

FIG. 4—Calibration curves for benzoic acid and sorbic acid.

this value by 100 and report result as mg preservative/100 ml beverage.

If concentration of preservative exceeds that of standard plot, dilute beverage with water and repeat determination.

Results and Discussion

The Food Additives regulations (3) permit the use, with declaration, of 0.1% benzoic and sorbic acids. The actual amounts used in commercial practice generally range between 0.04 and 0.1%. Sorbic acid usually is found near or below the lower value and benzoic acid closer to 0.1%.

We spiked authentic orange and apple juices prepared in the laboratory with sorbic and benzoic acids at the levels indicated by the above considerations. The highest spike for benzoic acid was 0.1% and for sorbic acid 0.04%. The sorbic acid range was kept lower partly because of its use as a preservative at a lower concentration and partly because a greater concentration yields excessive light absorption and the solution must be diluted as explained in the *Method*.

The results (Table 1) indicate that at the preservative concentrations generally used in juices the recoveries are reasonably good. At the lowest concentrations examined the recoveries were high; however, these are below the effective preservative levels and are not likely to be encountered.

Table 1. Recovery of benzoic and sorbic acids from fruit juices

Benzoic Acid, mg/ml			Sorbic Acid, mg/ml		
Added	Found	% Rec.	Added	Found	% Rec.
Orange Juice					
0.200	0.255	127.5	0.040	0.057	142.5
0.400	0.395	98.8	0.100	0.120	120.0
0.600	0.540	90.0	0.200	0.210	105.0
0.800	0.730	91.2	0.300	0.300	100.0
1.00	0.835	83.5	0.400	0.395	98.8
0.200	0.240	120.0	0.200	0.222	110.0
0.600	0.547	91.1	0.300	0.310	103.0
1.00	0.820	82.0	0.400	0.418	104.5
Apple Juice					
0.200	0.220	110.0	0.050	0.060	120.0
0.300	0.280	93.2	0.100	0.112	112.0
0.400	0.360	90.0	0.150	0.142	94.6
0.500	0.480	96.0	0.200	0.205	102.5
0.600	0.600	100.0	0.250	0.250	100.0
0.750	0.710	94.6	0.300	0.312	103.9
0.800	0.780	97.5	0.350	0.345	98.5
1.000	0.950	95.0	0.400	0.412	103.0

The high values found at the low preservative concentration are undoubtedly due to the background absorption of the juice which assumes greater significance at this level although, under the conditions of this test, the UV absorption of fruit juices is rather small, as it may be seen from typical absorption curves obtained for an authentic orange juice (Fig. 5, top) and an authentic apple juice (Fig. 5, bottom).

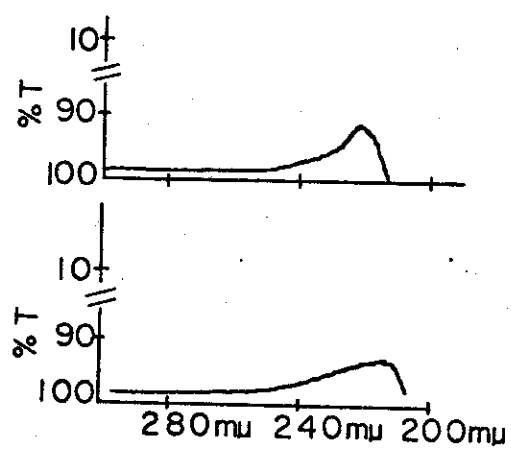

FIG. 5—UV absorption spectra of authentic orange juice (top) and authentic apple juice (bottom).

The absorption curves in Figs. 1–3 were obtained by spiking authentic orange juice with the preservatives indicated. These curves are characteristic and easily recognized. The benzoic acid peak at 225 mμ is not symmetrical but shows a noticeable shoulder at 235 mμ.

Summary

The method described is a rapid screening method for analyzing a large number of samples. It was primarily developed for beverages where undisclosed preservatives have been used. Compared to the official method, which requires 15 extractions and is time-consuming, this method is rapid and accurate for levels of preservatives normally present. In addition it is capable of detecting both sorbic and benzoic acids.

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FLAVORS AND NONALCOHOLIC BEVERAGES

Differentiation of Certain Types of Cassias and Cinnamons by Measurement of Mucilaginous Character

By WILLIAM H. STAHL, JEAN N. SKARZYNSKI, and WILLIAM A. VOELKER
(McCormick & Co., Inc., 204 Wight Ave., Cockeysville, Md. 21030)

A simple method has been refined to differentiate Korintji and Batavia cassias from cassias of different geographical origin and also from true cinnamons. A certain particle-size fraction of the spice is slurried in an isopropyl alcohol-water mixture, heated to 80°C, transferred to a 100 ml graduated cylinder, shaken, and allowed to settle over a 2 hr period. The height of the column of sediment is measured. Korintji and Batavia give average column heights of 85.0 ml with a standard deviation of 6.7, and Seychelle cinnamon and Saigon cassia give an average height of 25.3 ml with a standard deviation of 4.1.

It has been recognized for many years that certain cassias have mucilaginous substances present that, when ground and suspended in water, become highly gelatinous and stringy. While this property does not significantly affect the flavor character, the presence of large quantities of these mucilaginous materials makes the cassia unsuitable for certain baking and other applications.

The literature contains three methods which claim to distinguish true cinnamon from cassia. In 1952 Dequeker (1) reported a quantitative microscopic method in which he measured the area of fibers per gram from length and width. He found that cinnamon measured 92.5 sq. cm, cassia measured 13.1 sq. cm, and the ratio of one to the other was 7:1. Betts (2) in 1965 developed a thin layer chromatographic analysis for the essential oil from these species; he found eugenol in cinnamon oil, but not in cassia oil. The mucilaginous characteristic has also been claimed to distinguish cinnamon from cassia. Dutta (3) in 1961 reported a chemical method based on an alcoholic extraction of the defatted powder, followed by an acid precipitation of the mucilage. Unfortunately, Dutta used only *Cinnamomum zeylanicum* (Ceylon), which gave values in the range 1.9 to 2.1%, and *Cinnamomum cassia* (geographical origin unspecified), which gave values in the range 9.6 to 10.9%.

All of the above methods are either long and tedious (Dequeker and Dutta) or require special technical skill (Betts). In 1961, a relatively

(Italian publication, pp. 360-4)

Photometric determination and identification of sorbic acid in butter

A. Alessandro, V. Dal Piaz, E. Tendi

As is known, sorbic acid is one of the chemical additives approved by Italian legislation in force for the preparation and conservation of food substances. The addition of 500 mg of sorbic acid per kg of product is permitted in butter.

For the identification and quantitative analysis of this preservative various techniques have been tried and developed, most of them based on steam extraction,; later the qualitative and quantitative determinations were made by gravimetric (1), volumetric (2, 3, 4, 5), colorimetric (6, 7, 8, 9, 10, 11), spectrophotometric (12, 13), chromatographic (14, 15, 16, 17, 18) and gas chromatographic (19, 20) methods.

The various photometric methods are nearly all based on the oxidation of sorbic acid, separated by steam distillation, with potassium bichromate in a medium rendered acid by sulfuric acid; the malonic aldehyde formed is made to react with 2-thiobarbituric acid.

However, the results are not always very satisfactory, either because in the distillation there already occurs a loss of at least 4% of sorbic acid (12), or because of the difficulty to regulate the oxidation process perfectly.

The purpose of the present project has been to study the known analytical techniques and to develop a rapid method of quantitative analysis easy to carry out.

The extraction of the preservative from the butter is carried out, rather than by steam distillation, more simply by solubilizing the fatty substance in ethyl ether, in the presence of trichloroacetic acid; from the ether solution obtained the sorbic acid is then extracted in the form of sodium salt, with aqueous solution of sodium hydroxide. The reaction is carried out on the alkaline phase, acidifying first with sulfuric acid and then adding ferrous

ammonium sulfate (Mohr's salt), 2-thiobarbituric acid, and potassium persulfate; the cherry red color develops as the solution obtained is maintained in an ultrathermostat at 72°C for 5 minutes.

In our experiments we have found that to obtain results as nearly concordant as possible it is necessary always to operate at the same temperature.

The presence of the ferrous salt, which acts as catalyst in the oxidation stage, makes the reaction more sensitive, faster, and more reproducible.

The extinction/concentration diagram was prepared in an interval from 5 to 100 mcg. The reaction, with an absorption maximum at 532 millimicron, follows the law of Lambert-Beer, and the error obtained in the various determinations ranges around 5%.

The identification of sorbic acid in butter can be carried out, besides with the reaction described above, by substituting phloroglucinol for the 2-thiobarbituric acid; with it the test is much more sensitive and faster because already in the cold, in the presence of some few mcg of sorbic acid, a characteristic red color appears with an absorption maximum at about 558 millimicron.

EXPERIMENTAL PART

Reagents

1) Sulfuric acid diluted 1:3.

2) Aqueous solution of ferrous ammonium sulfate 0.15%.

3) Aqueous solution of 2-thiobarbituric acid 0.30%.

4) Aqueous solution of potassium persulfate 0.15%.

Solutions 2) and 3) are prepared each time, before use.

Extinction-concentration ratio

To prepare the calibration curve 10 mg of sorbic acid are dissolved in 100 ml of an alkaline solution obtained from 20 g of butter without preservative, and following the extraction process described below in the quantitative analysis technique.

One takes 0.05 to 1 ml, corresponding to 5-100 mcg of sorbic acid, and the reaction with 2-thiobarbituric acid is performed. The extinction values at 532 millimicron are measured in the spectrophotometer, and from these the calibration curve is obtained as a function of the concentration.

Figure 1 Extinction-concentration ratio

Quantitative determination

Twenty grams of butter, placed in a flask, are added with 10 g of trichloroacetic acid and 100 ml of ethyl ether, and heated for a few minutes on a waterbath. After cooling, the ether phase is decanted and the part insoluble in ether is treated twice more, first with 80 ml and then with 50 ml of ethyl ether. The decanted ether phases, collected in a separating funnel, are extracted with sodium hydroxide 1.16 N, making two extractions with 20 ml each and then one with 10 ml.

From the combined alkaline extracts the residual traces of ether are removed, by heating for 30 minutes at 50-60°C on an electric heater; immediately thereafter the product is put to cool in a refrigerator for about 30 minutes. Lastly it is filtered on paper and washed with 50 ml of sodium hydroxide 0.025 N until 100 ml of filtrate are obtained. On this last solution the photometric analysis is carried out.

Sampling about 0.5 ml, it is brought to 1 ml with the control (solution obtained with the above described procedure, from butter in a good state of preservation and definitely free from sorbic acid) and there are added 1 ml of dilute sulfuric acid (1:3), 1 ml of Mohr's salt solution 0.15%, 1 ml of 2-thiobarbituric acid 0.3%, 1 ml of potassium persulfate 0.15% and 6 ml of distilled water. Heat in an ultrathermostat at 72°C for 5 minutes and measure the extinction at 532 millimicron.

The control test is carried out on 1 ml of the alkaline solution obtained from the extraction of the sorbic acid-free butter, with the reagents and methods already described.

Determination of sorbic acid (mg%) in butter

Sample number	Quantity added	Quantity found	Error %
1	50	48	- 4
2	45	46	+ 2.2
3	30	31.5	+ 5
4	25	23.5	- 6
5	35	34	- 2.8

Qualitative determination

Reagents

1) Sulfuric acid diluted 1:3.

2) Aqueous solution of ferrous ammonium sulfate 0.5%.

3) Acetic solution of phloroglucinol 5%.

4) Aqueous solution of potassium persulfate 0.5%.

Solutions 2) and 3) are prepared each time before use.

One takes about 1 ml of the alkaline extract obtained as described, adding 1.5 ml of sulfuric acid 1:3, 1 ml of Mohr's salt solution, 0.5 ml of phloroglucinol solution and 0.2 ml of potassium persulfate solution. In the presence of sorbic acid, already in the cold and after brief agitation, there appears a cherry-red color, which becomes more evident by heating for about 1 minute on the waterbath at 60°C.

Conclusion

The tested analytical method permits the determination of sorbic acid in butter, with fairly satisfactory results. The direct extraction of the preservative with the use of solvents may be regarded as very fast compared to steam distillation; the presence of the F^{++} ion increases the sensitivity of the reaction with 2-thiobarbituric acid and makes it more reproducible and stable.

The identification test obtained with phloroglucin, besides being sensitive and rapid, has proved to be specific and easy to carry out.

(For Summary in English and Bibliography see p. 364).

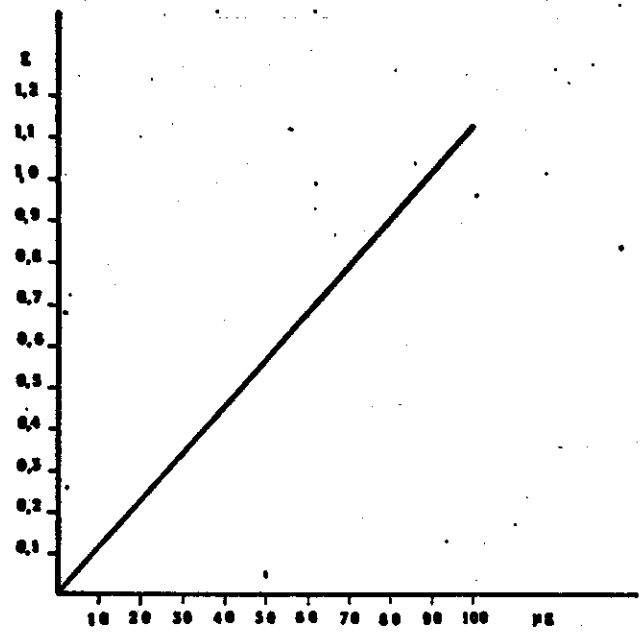

Figure 1

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Sorbic acid 6

SCUOLA DI SANITÀ MILITARE
Comandante: Magg. Gen. Med. M. Cappelli
ISTITUTO DI CHIMICA
Direttore: Ten. Col. Chim. Farm. Prof. A. Alessandro

DOSAGGIO FOTOMETRICO E RICONOSCIMENTO DELL'ACIDO SORBICO NEL BURRO

A. Alessandro V. Dal Piaz E. Tendi

L'acido sorbico, come è noto, è uno degli additivi chimici consentiti dalla vigente legislazione italiana riguardante la preparazione e la conservazione delle sostanze alimentari. Al burro è permessa l'aggiunta di mg 500 di acido sorbico per kg di prodotto.

Per il riconoscimento ed il dosaggio di questo conservante sono già state sperimentate e messe a punto diverse tecniche basate, nella maggior parte dei casi, sull'estrazione in corrente di vapore; successivamente le determinazioni qualitative e quantitative vengono effettuate per via gravimetrica [1], volumetrica [2, 3, 4, 5], colorimetrica [6, 7, 8, 9, 10, 11], spettrofotometrica [12, 13], cromatografica [14, 15, 16, 17, 18] e gascromatografica [19, 20].

I varî metodi fotometrici si basano quasi tutti sulla ossidazione dell'acido sorbico, separato per distillazione in corrente di vapore, con bicromato di potassio in ambiente acido per acido solforico; l'aldeide malonica formatasi viene fatta reagire con l'acido 2-tiobarbiturico.

I risultati non sono però sempre molto soddisfacenti, sia perchè nella distillazione si ha già una perdita di almeno il 4% di acido sorbico [12], sia per le difficoltà di regolare perfettamente il processo di ossidazione.

Scopo del presente lavoro è stato quello di studiare le tecniche analitiche già note e di mettere a punto un metodo di dosaggio rapido e di facile esecuzione.

L'estrazione del conservante dal burro, anzichè mediante distillazione in corrente di vapore, viene effettuata più semplicemente solubilizzando la sostanza grassa in etere etilico, in presenza di acido tricloroacetico; successivamente dalla soluzione eterea ottenuta l'acido sorbico viene estratto, sotto forma di sale sodico, con soluzione acquosa di idrossido di sodio. Sulla fase alcalina separata, viene effettuata la reazione, acidificando prima con acido solforico e aggiungendo poi solfato ferroso ammonico (sale di Mohr), acido

2 - tiobarbiturico e potassio persolfato; la colorazione rosso - ciliegia si sviluppa mantenendo la soluzione ottenuta, in ultratermostato a 72°C per 5 minuti.

Nelle nostre esperienze abbiamo constatato che per avere risultati il più possibile concordanti tra di loro è necessario operare sempre alla stessa temperatura.

La presenza del sale ferroso, che agisce da catalizzatore nella fase dell'ossidazione, rende la reazione più sensibile, più rapida e riproducibile.

Il diagramma estinzione/concentrazione è stato costruito in un intervallo da 5 a 100 μg. La reazione, con un massimo di assorbimento a 532 mμ, segue la legge di Lambert - Beer e l'errore ottenuto nelle varie determinazioni si aggira intorno al 5%.

Il riconoscimento dell'acido sorbico nel burro, oltre che con la reazione già descritta, si può effettuare sostituendo all'acido 2 - tiobarbiturico la floroglucina; con quest'ultima sostanza il saggio è molto più sensibile e più rapido poichè già a freddo, in presenza di pochi μg di acido sorbico, compare una colorazione rossa caratteristica con un massimo di assorbimento a circa 558 mμ.

PARTE SPERIMENTALE

Reattivi.

1) Acido solforico diluito 1:3.

2) Soluzione acquosa di solfato ferroso ammonico 0,15%.

3) Soluzione acquosa di acido 2 - tiobarbiturico 0,30%.

4) Soluzione acquosa di potassio persolfato 0,15%.

Le soluzioni 2) e 3) vanno preparate, di volta in volta, prima dell'uso.

Rapporto estinzione - concentrazione.

Per la preparazione della curva di taratura si sciolgono 10 mg di acido sorbico in 100 ml di una soluzione alcalina ottenuta partendo da 20 gr di burro privo di conservante, e seguendo il processo di estrazione descritto subito dopo nella tecnica di dosaggio.

Si prelevano da 0,05 ad 1 ml, corrispondenti a 5 - 100 μg di acido sorbico, e si esegue la reazione con acido 2 - tiobarbiturico. Si misurano allo spettrofotometro i valori di estinzione a 532 mμ e da questi si ricava la curva di taratura in funzione della concentrazione.

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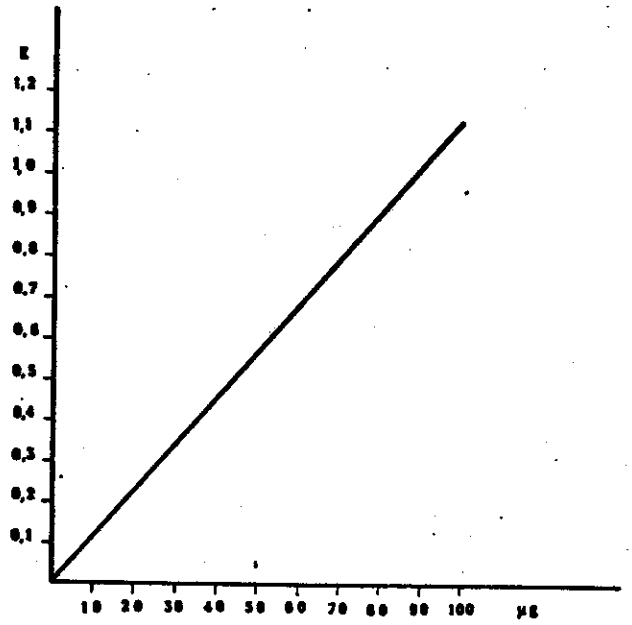
RAPPORTO ESTINZIONE - CONCENTRAZIONE



Determinazione quantitativa.

Venti grammi di burro, posti in bevuta, vengono addizionati di 10 gr di acido tricloroacetico, di 100 ml di etere etilico e scaldati per qualche minuto a b. m. Quindi si raffredda, si decanta la fase eterea e la parte insolubile in etere viene, ancora, trattata per due volte consecutive, prima con 80 ml e poi con 50 ml di etere etilico. Le fasi eteree decantate, riunite in imbuto separatore, vengono estratte con idrossido di sodio 1,16 N, effettuando due estrazioni con 20 ml per volta ed una successiva con 10 ml.

Dagli estratti alcalini riuniti vengono allontanate le tracce di etere residue, per riscaldamento di 30 minuti a 50°-60°C su mantello elettrico; subito dopo si pone a raffreddare in frigorifero per circa 30 minuti. Infine si filtra su carta e si lava con 50 ml di idrossido di sodio 0,025 N fino ad ottenere 100 ml di filtrato. Su quest'ultima soluzione si esegue il dosaggio fotometrico.

Si prelevano 0,5 ml circa, si portano ad 1 ml col bianco (soluzione ottenuta col procedimento già descritto, partendo da un burro in buono stato di conservazione e sicuramente esente da acido sorbico) e si aggiungono 1 ml di acido solforico diluito (1:3), 1 ml di soluzione di sale di Mohr allo 0,15%, 1 ml di acido 2-Tiobarbiturico allo 0,3%, 1 ml di potassio per-

solfato allo 0,15% e 6 ml di acqua distillata. Si riscalda in ultratermostato a 72°C per 5 minuti e si misura l'estinzione a 532 mμ.

La prova in bianco si esegue su 1 ml della soluzione alcalina ottenuta dall'estrazione del burro esente da acido sorbico, con i reattivi e le modalità già descritte.

DETERMINAZIONE DELL'ACIDO SORBICO (mg%) NEL BURRO

Campione numero	Quantità aggiunta	Quantità trovata	Errore %
1	50	48	− 4
2	45	46	+ 2,2
3	30	31,5	+ 5
4	25	23,5	− 6
5	35	34	− 2,8

DETERMINAZIONE QUALITATIVA.

Reattivi.

1) Acido solforico diluito 1:3.
2) Soluzione acquosa di solfato ferroso ammonico 0,5%.
3) Soluzione acetica di floroglucina 5%.
4) Soluzione acquosa di potassio persolfato 0,5%.

Le soluzioni 2) e 3) vanno preparate di volta in volta prima dell'uso.

Si preleva 1 ml circa dell'estratto alcalino ottenuto come già descritto, e si aggiungono 1,5 ml di acido solforico 1:3, 1 ml di soluzione di sale di Mohr, 0,5 ml di soluzione di floroglucina e 0,2 ml di soluzione di potassio persolfato. In presenza di acido sorbico, già a freddo e dopo breve agitazione, si manifesta una colorazione rosso-ciliegia, che diviene più evidente riscaldando per circa 1 minuto a b. m. a 60°C.

CONCLUSIONE

Il metodo di dosaggio sperimentato, permette di determinare l'acido sorbico nel burro, con risultati abbastanza soddisfacenti. L'estrazione diretta del conservante mediante l'impiego di solventi, è da considerarsi molto rapida rispetto alla distillazione in corrente di vapore; la presenza dello ione

is urged to investigate its copyright status.

Fe^{++}, aumenta la sensibilità della reazione con acido 2-tiobarbiturico e la rende più riproducibile e stabile.

Il saggio di riconoscimento ottenuto con la floroglucina, oltre che sensibile e rapido, si è dimostrato specifico e di facile esecuzione.

RIASSUNTO. — Gli AA. descrivono un dosaggio fotometrico dell'acido sorbico nel burro, basato sull'impiego dell'acido 2-tiobarbiturico in presenza di solfato ferroso ammonico. La reazione di riconoscimento viene effettuata con floroglucina.

RÉSUMÉ. — Les AA. décrivent un dosage photométrique de l'acide sorbique dans le beurre, par l'emploi de l'acide thiobarbiturique-2 en presence de sulfate de fer et d'ammonium (ferreux). L'identification est effectuée par un réactif à la phloroglucine.

SUMMARY. — The AA. describe a photometric test for dosage of sorbic acid in butter, based on the use of 2-thiobarbituric acid in the presence of iron ammonium sulphate (ferrous). The identification is effectued with phloroglucine reagent.

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Ceskoslovenska Hygiena (Czechoslovak Hygiene), 13, No. 6 (1968).

POLAROGRAPHIC DETERMINATION OF SORBIC ACID IN FOODS

Z. Malkus, Institute of Hygiene, Prague

(The Russian and English summaries preceding the article were not translated)

Sorbic acid (the trans-trans form of 2,4-hexadiene carboxylic acid, also called 2-propenylacrylic acid), as well as its potassium, sodium, or calcium salts are, as it is known, recently used chemical conservation agents. The significant fungistatic effect of sorbic acid, as well as its inhibitory effect mainly toward the catalase-positive microorganisms has been well-known for some time (1--4). These effects are of great interest to the food-processing industry. As the other preservatives of weakly acidic nature, sorbic acid is effective only in the form of the free, undissociated acid. Thus, its activity is very strongly dependent on the pH of the medium in which it is being used.

The health aspects of sorbic acid, particularly its acute and chronic toxicity, its metabolism and tolerance were the subject of numerous toxicological studies that have led to an adequate understanding of these areas (5-8). Based on the studies and results obtained to date, this material is being used on a world-wide basis as an additive to various foods, or to food raw materials (9) because it is a tolerable chemical preservative. From the physiological point of view, this additive is more acceptable than the previously used chemical preservatives, such as sulfur dioxide, benzoic acid, etc. (7). Sorbic acid, added to a number of foods in the amounts from 0.05 to 0.25% is useful in the stabilization of some cosmetic products as well as for impregnating food packaging materials. A certain disadvantage arising from the use of sorbic acid is due to its effects on the taste of foods. A negative effect on the taste of some foods may be observed at concentrations as low as 0.1%.

The approval of the use of sorbic acid as a chemical preservative in several European countries, as well as its widespread use during the last 5 to 10 years, resulted in numerous analytical studies of this material. Thus, not only qualitative tests for the presence of sorbic acid appeared in recent

More methods were published based on the colorimetric principle. Diemair and Franzen (14) described a determination based on the color-forming reaction with benzothiazole which gives rise to a pentacyanine-type dye. Hardon and Visser (36) used the reaction with hydroquinone and sulfuric acid, whereas Schmidt (37) published a very original method based on the reaction of the oxidation product of sorbic acid, i.e., of malonodialdehyde, with 2-thiobarituric acid and developed this procedure for the determination of sorbic acid in wine. A colorimetric method involving the use of resorcinol and sorbic acid was described by Lemieszekova-Chodorowska (39). This method was used by Maurel and Touyé (40) for the determination of beta-nephthol. All of these methods and particularly Schmidt's method have been many times evaluated for use with various types of foods and were found to be very useful (41-43). A number of studies were also devoted to chromatographic methods involving quantitative evaluation on paper (18-20,44) as well as on thin layers (21-24,25). Gas chromatography (26-28) proved to be a useful method for the detection as well as for the quantitative determination of sorbic acid in the presence of other preservatives during the last several years. One of the most recent rapid and specific methods involving infrared spectroscopy was described by Calloway and Schwarzmann (46) who recommended this method for the detection as well as for the quantitative determination in various foods.

As far as the isolation of sorbic acid from foods is concerned, the various authors have basically used only two methods, i.e., the steam distillation method originally published by Melnick and Luckmann (29) and extraction with organic solvents (35). In practice, the stem distillation procedure has been much more widely accepted and Schmidt (37,38) proposed suitable apparatus for this technique.

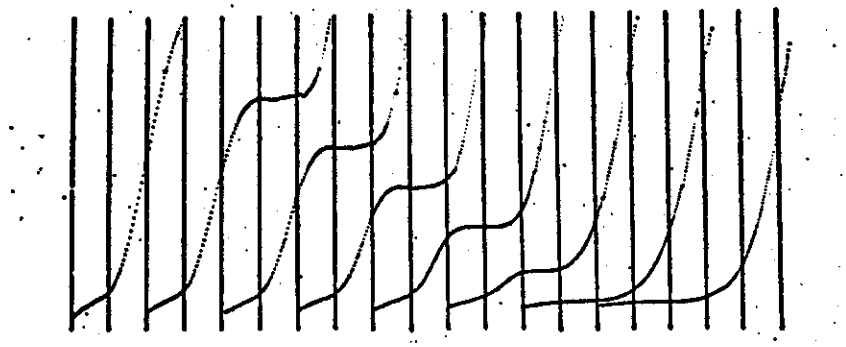

Fig. 2. Calibration polarogram for sorbic acid in tetramethylammonium hydroxide solution (0.05 M). Sensit. 1/20, Act.: 4 V; sorbic acid concentration from $1 \cdot 10^{-4}$ to $5 \cdot 10^{-3}$ M

Although the published methods are suitable and useful in most cases, the objective of the present study was an investigation of the polarographic behavior of sorbic acid and the subsequent development of a method based on this electrochemical technique for the simultaneous detection as well as determination, which would by its specific nature constitute a useful addition to the methods for determining of this preservative which is being widely used at the present time.

Polarographic study of sorbic acid received relatively little attention up to the present time. The first mention of this subject dates back to the year 1932 when Schwaer (47) described the reducibility of some unsaturated acids, including sorbic acid, at the dropping mercury electrode. Likewise, the report by Hanc and Santavy (48) contains a mention of the possible polarographic reduction of sorbic acid. Polarography of sorbic acid was studied more thoroughly by Fornasari (49) who determined its behavior in certain supporting electrolytes and also determined the half-wave potential values.

Starting from the available results, particularly from Fornasari's work, we carried out a more detailed study of the polarographic behavior of sorbic acid and attempted to develop on the basis of the results obtained a polarographic method for the determination of sorbic acid in some types of foods.

EXPERIMENTAL PART

For the polarographic studies we used analytical reagent-grade sorbic acid produced by n. p. (People's Enterprise) Aroma (Czechoslovakia), which was twice recrystallized from water and had an mp 133.5°C.

For the preparation of supporting electrolytes, i.e., of the Britton-Robinson solution, stable phosphate solution, lithium chloride, tetramethylammonium hydroxide, and tetramethylammonium bromide, we used analytical-grade reagents throughout. The solutions were prepared by dissolving the corresponding weighed-out amount of reagent in distilled water. The purity of the tetraalkylammonium salt solutions was controlled polarographically.

In this study we employed a polarograph of the Heyrovsky type, model V-301 A equipped with photographic recorder and having a galvanometer sensitivity of $3 \cdot 10^{-9}$ A/mm. The dropping mercury electrode used had the constants t = 2.9 sec and m = 5.03 mg/sec at the reservoir height of 62 cm. The polarography was carried out in a Kalousek flask with a separate calomel electrode (saturated). The temperature was 21 ± 1°C. The half-wave potential values were determined with the aid of the E/2 of thallium.

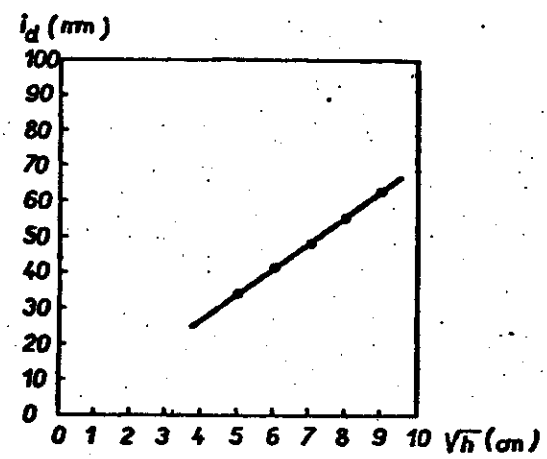

Fig. 3. Graph of the dependence of the polarographic wave height (i_d) on the square root of the polarographic reservoir height ($\sqrt{h}$)

Oxygen was removed from the solutions with a stream of nitrogen (the remaining oxygen traces were removed by passing through a trap containing an alkaline pyrogallol solution).

The value of the constant (K) was calculated from the Ilkovic equation

$$K = \frac{i_d}{c \cdot m^{2/3} \cdot t^{1/6}}$$

where i_d is the wave height, c is the concentration, and m and t are capillary constants.

Initially we examined the polarographic behavior of pure aqueous sorbic acid solutions (of $1 \cdot 10^{-3}$ to $2 \cdot 10^{-4}$ M concentrations) in the following basic electrolytes: in the Britton-Robinson buffer solution, in the phosphate buffer solution, as well as in the 0.1 N LiOH, 0.1 N H_2SO_4, and 0.1 N HCl solutions. In all of the above-mentioned solutions, we were unable to determine the polarographic activity of sorbic acid. For this reason we investigated the following electrolytes: 0.1 N LiCl as well as 0.05 M, 0.1 M, and 1 M solutions of tetramethylammonium hydroxide and tetramethylammonium chloride. In all of these electrolytes, we were able to determine polarographic waves corresponding to sorbic acid, but the most highly developed and analytically usable waves were obtained in tetramethylammonium hydroxide as well as in tetramethylammonium bromide solutions at the concentrations of 0.1 M and 0.05 M. For this reason, we subsequently studied sorbic acid in these two electrolytes. In both cases, we determined the half-wave potentials with the aid of thallium. In order to eliminate the effect of the apparatus hysteresis on the half-wave potential position, we determined two curves, i.e. in the direction of increasing as well as of decreasing potential in each case.

Fig. 2 indicates the polarographic waves due to thallium and sorbic acid in a solution of tetramethylammonium bromide (0.05 M).

The half-wave potentials measured in the above solutions are listed in Table 1, which indicates that the half-wave potential of sorbic acid solution in tetramethylsammonium bromide solution is by about 500 mV more positive than the E/2 in the tetramethylammonium hydroxide solution. In the above supporting electrolytes derived from the tetraalkylammonium salts, we determined a satisfactory proportionality between the polarographic wave-height and the sorbic

acid concentration in the range from 10^{-4} to 10^{-2} M. Fig. 2 shows the calibration polarogram of sorbic acid in the supporting electrolyte of 0.05 M tetramethylammonium hydroxide. Table 2 contains the calculated values of the constant K as well as the measured values of the half-wave potential for various sorbic acid concentrations in 0.05 M tetramethylammonium hydroxide solution. Fig. 3 shows the dependence of the wave-height on the reservoir height.

Based on the experimental results indicating that sorbic acid may be determined polarographically in a suitable electrolyte solution, we carried out experiments on the determination of this material in some food samples. Its direct determination in foods was impossible due to obvious reasons and, hence, we used known isolation

TABLE 1. Half-Wave Potential Values (E/2) of Sorbic Acid in Various Electrolytes

Electrolyte	E/2 (vs. SCE)
0.1 M LiCl	(-1.60 V)
0.1 M $N(CH_3)_4Br$	-1.55 V
0.05 M $N(CH_3)_4BR$	-1.57 V
0.05 M $N(CH_3)_4OH$	-2.13 V

procedures for sorbic acid involving, on one hand, steam distillation, and, on the other hand, extraction with organic solvents (dichloromethane and chloroform). The steam distillation was carried out in the presence of magnesium sulfate. The polarographic determination in distillates was carried out in such a manner that an aliquot, if necessary after evaporation, was mixed with a 1 M tetramethylammonium hydroxide solution to give a resulting supporting electrolyte concentration of 0.05 M. Extractions with organic solvents were, after evaporation, dissolved in the electrolyte and the polarographic curves were measured on these solutions. These experiments, except the determination of sorbic acid in coated wrapping paper, did not give reproducible results. In many cases, the determination was subject to interference by polarographically

TABLE 2 Values of the Constants K and of the Half-Wave Potentials (E/2) for Various Sorbic Acid Concentrations (c) in 0.05 M Solution of $N(CH_3)_4OH$

E/2 (mV)	c (mmol)	k
-2.14	0.09	2.32
-2.12	0.44	2.36
-2.12	0.89	2.30
-2.13	1.32	2.32

active materials, which were also removed by distillation, or extracted from the foods that were analyzed, or which were formed during the distillation at elevated temperatures. For these reasons, we carried out another series of experiments in which sorbic acid was first removed from the samples by steam distillation and the resulting distillate was then extracted after acidification with chloroform and, after careful evaporation of the solvent, the residue was dissolved in the supporting electolye, and the polarographic curve was determined.

TABLE 3 Results of Sorbic Acid Determination in Various Samples.

Type of sample	Determined (mg/kg) Polarographically	Colorimetrically	Error, %
Foundation cream	74	65	+ 13.8
Foundation cream	110	124	-11.3
Foundation cream	243	266	-8.7
Almond meats	172	155	+11.0
Pickled cucumbers	48	55	-12.7
Pickled cucumbers	142	165	-13.9
Pickled cucumbers	120	106	+13.2
Canned vegetables	92	78	+17.9
Press oil	138	127	+8.7
Coated paper	413	364	+13.5
Coated paper	514	546	-5.9
Coated paper*	660	604	+9.3

* Colorimetric result = 100%

Evaluation was carried out by the standard addition method. This technique yielded good results in model experiments with the addition of various amounts of sorbic acid (50 to 200 mg per sample), in which 87 to 104.2% of the added amount was found.

The following procedure proved to be the most advantageous one for the determination of sorbic acid: A 50 to 100-g sample is, after the addition of water (about 100 ml). magnesium sulfate (2 g), and sulfuric acid (5 ml,steam-distilled and the distillate is collected in an acidified chloroform solution (10 ml of hydrochloric acid is mixed with 40 ml of freshly distilled chloroform). Upon collecting 300 to 400 ml, the distillate is transferred into a separatory funnel and, after shaking for 1 minute,the layers are allowed to separate. The lower chloroform layer is separated and the aqueous phase is then extracted five more times with 25-ml portions of chloroform and the extracts are combined in a porcelain dish. The combined extracts are evaporated on a water bath to a volume of about 5 ml and finally to dryness at room temperature. To the dry residue is added 5 to 10 ml of 0.05 M tetramethylammonium hydroxide solution and the polarographic curve is then determined at a suitable galvanometer sensitivity. The evaluation is carried out by the standard addition method.

Using the above method, sorbic acid was determined in 12 different samples and the results are compared with the colorimetric determinations according to Schmidt (37). The results are listed in Table 3.

DISCUSSION AND CONCLUSIONS

The experiments that we carried out indicate that sorbic acid may be reduced at the dropping mercury electrode under certain conditions. The constant values of K and the half-wave potentials of sorbic acid at various concentrations (Table 2) indicate that the polarographic method is usable in the two recommended supporting electrolyte solutions, i.e., in tetramethylammonium bromide and tetramethylammonium hydroxide, for the qualitative as well as for

the quantitative sorbic acid analysis. The linear dependence between the half-wave height and the concentration was verified for the 10^{-4} to 10^{-2} M concentration range. The half-wave potential value for sorbic acid in tetramethylammonium bromide solution is by about 500 mV more positive than in tetramethylammonium hydroxide solution, which is advantageous fromthe analytical point of view. As far as the character of sorbic acid reduction at the dropping mercury electrode is concerned, Fornasario showed in her study that we are dealing with an irreversible process, which involves in an alkaline solution a two-electron reduction corresponding to the reaction sorbic acid $\longrightarrow$ dihydrosorbic acid.

Fig. 3 shows the linear dependence between the wave height and the square root of the reservoir height. This plot indicates that a diffusion-controlled electrode process takes place during the reduction of sorbic acid.

Table 3 indicates that the developed polarographic method yields results that are comparable to the colorimetric method. The advantage of the polarographic method is due to its specificity as well as due to the fact that it may be used for the simultaneous identification (from the position of the half-wave potential and quantitative determination (height of the polarographic wave).

The determination is not subject to the interference by other common preservatives, such a benzoic acid, esters of p-hydroxybenzoic acid, sulfur dioxide, etc.

SUMMARY

The polarographic behavior of sorbic acid was studied in detail, suitable electrolytes were identified, the conditions for the determination of sorbic acid were determined, and the half-wave potentials were measured. On the basis of the present study, a polarographic method was developed for the determination of sorbic acid in some foods. This method includes prior isolation of sorbic acid by steam distillation and the determination itself is carried out in tetramethylammonium hydroxide, or tetramethylammonium bromide solution.

The method was successfully tested on 12 different types of samples and the results obtained were compared with the corresponding values obtained on the same samples by Schmidt's colorimetric method. An agreement between the results derived by the respective methods was observed.

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Sorbic Acid 277

ČESKOSLOVENSKÁ HYGIENA — 13 — 6 — 1968

POLAROGRAFICKÉ STANOVENÍ KYSELINY SORBOVÉ V POTRAVINÁCH

Полярографическое определение сорбиновой кислоты в пищевых продуктах

The Polarographic Determination of Sorbic Acid in the Food

Z. Malkus

Ústav hygieny, Praha

Выводы: В эксперименте изучалось полярографическое поведение сорбиновой кислоты, установлены оптимальные электролиты и условия для ее определения и измерены величины потенциалов полуволн. На основании проведенных экспериментов был разработан полярографический метод определения сорбиновой кислоты в некоторых пищевых продуктах, после предварительной перегонке с водяным паром, в растворе гидроокиси тетраметиламмония, или тетраметиламмоний бромида. Метод был успешно проверен в 12 разных образчиках и полученные результаты сравнивались с данными, полученными в тех же образчиках колориметрическим методом по Шмидту. Сравнение показало согласие результатов обоих методов.

Čs. Hyg., 13, 1968, 6 : 351—358.

Summary: The polarographic behaviour of sorbic acid was experimentally investigated. The author found suitable electrolytes as well as the conditions for the determination of the acid and the values of half — wave potentials. On the basis of the performed study, a polarographic method for the determination of sorbic acid in some kinds of food after the preliminary distillation with steam, in the solution of tetramethylammoniumhydroxide or tetramethylammoniumbromide. The method was successfully checked with 12 different kinds of food samples and the obtained results compared with the values, obtained with the same samples by the colorimetric method according to Schmidt. The agreement of the results was stated.

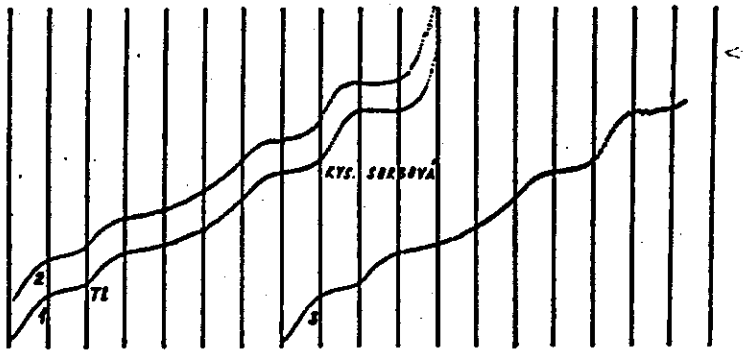
Čs. Hyg., 13, 1968, 6 : 351—358.

Kyselina sorbová (trans-trans forma kyseliny 2,4-hexadienkarboxylové, jinak také kyselina 2-propenylakrylová), respektive její draselná, sodná nebo vápenatá sůl, patří, jak známo, k novějším chemickým konzervačním činidlům. Význačný fungistatický efekt kyseliny sorbové i její inhibiční působení zejména proti katalázapozitivním mikroorganismům, je již delší dobu dobře znám[1-4] a v popředí velkého zájmu potravinářského průmyslu. Podobně jako jiné konzervační látky slabě kyselého charakteru, je kyselina sorbová účinná jen ve formě volné nedisociované kyseliny. Její působení tedy velmi závisí na pH prostředí, ve kterém je používána.

Také zdravotnické otázky kyseliny sorbové, zejména pak akutní a chronická toxicita, její metabolismus a snášenlivost, byly již četnými toxikologickými studiemi dostatečně prozkoumány[5-8]. Tato látka je na základě dosud získaných výsledků a poznatků dnes již ve světě široce využívána jako přísada do řady potravin, respektive potravinářských surovin[9], neboť je — jako trpěné chemické konzervační činidlo — z fyziologického hlediska mnohem přijatelnější než některé dříve nebo dosud používané chemické konzervační látky, jako je kysličník siřičitý, kyselina benzoová aj.[7] Kyselina sorbová je přidávána do celé řady potravin v množství

od 0,05 do 0,25 %, uplatňuje se při stabilizaci některých kosmetických výrobků i pro impregnaci potravinářských obalových materiálů. Určitou nevýhodou kyseliny sorbové při aplikaci v potravinářství jsou její smyslové vlastnosti. Negativní ovlivnění chuti některých potravin lze pozorovat již v koncentraci od 0,1 %.

Povolení kyseliny sorbové jako chemické konzervační látky v řadě evropských zemí a její široké využívání v posledních 5 — 10 letech, mělo za následek také četné studie týkající se analytiky této látky. Tak se v posledních letech objevovaly často metody nejen na důkaz kyseliny sorbové, ale i metody na její stanovení v některých druzích potravin, založené na různých principech.



Graf 1. Polarogram kyseliny sorbové a thalia v roztoku 0,05 M $N(CH_3)_4Br$. Koncentrace kyseliny sorbové $4{,}46 \cdot 10^{-4}$ M, citlivost 1/30; 180 mV/absc, křivka 1 a 3: směrem stoupajícího napětí, křivka 2: směrem klesajícího napětí

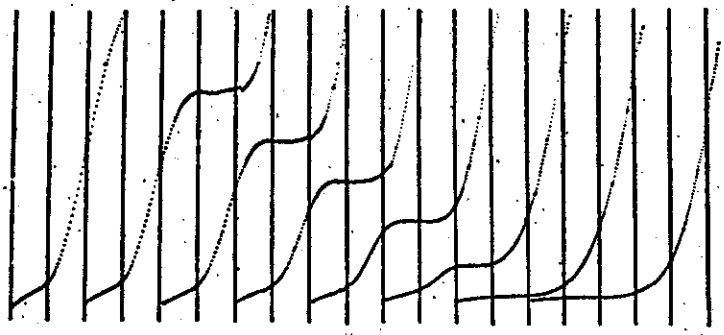
Pro důkaz kyseliny sorbové byly navrženy četné barevné reakce, například s použitím nitrozobenzenu[10], karbo-di-p-dimetyl-aminofenylimidu[11], chloridu železitého a Schiffova činidla[12], kyseliny p-diazobenzensulfonové[13], benzthiazolu[14] aj. Byl rovněž popsán důkaz pomocí frakcionované mikrosublimace[15] a mikrobiologický důkaz kyseliny sorbové[16 17]. Řada těchto postupů však nemá přísně specifický charakter. Proto se v současné době považuje za nejspolehlivější důkaz pomocí papírové[18–20] nebo tenkovrstvé chromatografie[21–24], eventuálně elektroforeticky[25] s následovným využitím barevných reakcí při detekci nebo sledování absorpce v UV-oblasti. Vhodný je také důkaz pomocí plynové chromatografie[26–28].

Pokud jde o stanovení kyseliny sorbové bylo rovněž v literatuře popsáno mnoho metod založených na různých principech. Jedna z prvních, kterou popsal Melnick a Luckmann[29], využívá světelné absorpce kyseliny sorbové v UV-oblasti spektra. Tuto metodu mnohokrát prostudovali a modifikovali další autoři[30–34]. Jiná metoda pocházející od Spanyara a spol.[35] spočívá na principu adice brómu na dvojné vazby kyseliny sorbové a titračním stanovení nezreagovaného halogenu.

Více metod bylo uveřejněno na principu kolorimetrickém. Diemair a Franzen[14] popsali stanovení pomocí barevné reakce s benzthiazolem, při kterém vzniká barvivo pentacyaninového charakteru. Hardon a Visser[36] využili reakce s hydrochinonem a kyselinou sírovou a Schmidt[37] uveřejnil velmi originální metodu založenou na principu reakce oxidovaného produktu kyseliny sorbové — dialdehydu kyseliny malonové — s kyselinou 2-thiobarbiturovou a upravil ji také pro stanovení ve víně[38]. Kolorimetrickou metodu s využitím rezorcínu a kyseliny sorbové popsala dále Lemieszekova-Chodorovska[39]; Maurel a Touyé[40] použili pro fotometrické stanovení β-naftolu. Všechny tyto metody, zejména pak postup podle Schmidta, byly již mnohokrát prověřeny pro různé druhy

potravin a dobře se osvědčily[41–43]. Řada prací byla také věnována chromatografickým metodám s kvantitativním vyhodnocením, a to jak na papíře[18–20 44], tak také na tenkých vrstvách[21–24 45]. V posledních letech se pro důkaz a stanovení kyseliny sorbové vedle dalších konzervačních látek uplatnila také plynová chromatografie[26–28]. Jednu z posledních rychlých a specifických metod pomocí infračervené spektroskopie popsali Calloway a Schwarzmann[46] a doporučují ji jak pro důkaz, tak i pro stanovení v různých potravinách.

Pokud jde o izolaci kyseliny sorbové z potravin, různí autoři v podstatě použili jen dva způsoby: destilace s vodní párou, původně uveřejněná Mel-



Graf 2. Kalibrační polarogram kyseliny sorbové v rozboru 0,05 M tetrametylamoniumhydroxidu. Citl. 1/20, Ak.: 4 V, koncentrace kyseliny sorbové v rozmezí 1.10^{-4}–5.10^{-3} M

nickem a Luckmannem[29], a extrakce organickými rozpouštědly[35]. V praxi daleko širší uplatnění nalezl postup pomocí destilace, pro nějž také Schmidt[37 38] navrhl vhodnou aparaturu.

I když uveřejněné metody jsou ve většině případů dobře použitelné a vyhovující, cílem této práce bylo prostudování polarografického chování kyseliny sorbové a na tomto elektrochemickém principu vypracování metody, která svým charakterem — možný současný důkaz i stanovení, specifičnost — by přispěla k rozšíření metod na stanovení tohoto dnes již značně rozšířeného konzervačního prostředku.

Polarografii kyseliny sorbové bylo až dosud věnováno poměrně málo pozornosti. První zmínka pochází již z roku 1932, kdy Schwaer[47] popsal redukovatelnost některých nenasycených kyselin, mezi jinými také kyseliny sorbové na rtuťové kapkové elektrodě. Rovněž ve sdělení Hanče a Šantavého[48] je uvedena zmínka o možnosti polarografické redukce kyseliny sorbové. Více se polarografií kyseliny sorbové zabývala Fornasari[49], která prostudovala její chování v některých nosných elektrolytech a stanovila rovněž hodnoty půlvlnových potenciálů.

Podle dosavadních poznatků, zejména podle práce Fornasari, jsme provedli bližší studii polarografického chování kyseliny sorbové a pokusili se vypracovat na základě dosažených výsledků polarografickou metodu na stanovení kyseliny sorbové v některých potravinách.

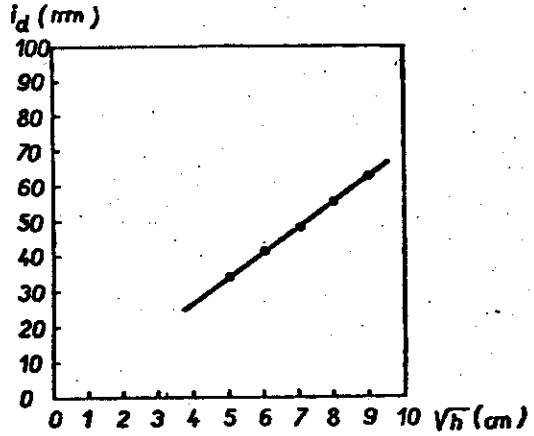
Experimentální část

Pro polarografické studium kyseliny sorbové byl použit preparát čistoty p. a. výrobek n. p. Aroma (ČSSR), 2krát rekrystalovaný z vody, o bodu tání 133,5 °C.

Při přípravu nosných elektrolytů — Brittonův-Robinsonův roztok, ústojný fosfátový roztok, chlorid litný, hydroxid litný, tetrametylamoniumhydroxid a

tetrametylamoniumbromid — bylo použito vesměs preparátů čistoty p. a. Roztoky byly připraveny rozpuštěním příslušné navážky v destilované vodě. Kontrola čistoty roztoků tetraalkylsolí byla provedena polarograficky.

Pro studium bylo použito polarografu systém Heyrovský-model V-301 A s fotografickým záznamem, s citlivostí galvanometru $3 . 10^{-9}$ A/mm. Použitá rtuťová kapková eletroda měla konstanty t = 2,9 s, m = 5,03 mg/s při výšce rezervoáru 62 cm. Polarografováno bylo v Kalouskově nádobce s oddělenou nasycenou kalomelovou elektrodou. Teplota 21 °C ± 1. Hodnoty půlvlnového potenciálu byly stanoveny pomocí E/2 thalia.



Graf 3. Graf závislosti výšky polarografické vlny (i_d) na druhé odmocnině z výšky rezervoáru ($\sqrt{h}$)

Kyslík byl z roztoku odstraňován proudem dusíku (poslední zbytky kyslíku byly absorbovány předřazeným alkalickým roztokem pyrogalolu).

Pro výpočet hodnoty konstanty (K) bylo použito Ilkovičovy rovnice

$$K = \frac{i_d}{c . m^{2/3} . t^{1/6}} ,$$

kde i_d je výška vlny, c — koncentrace, m a t — konstanty kapiláry.

Nejprve bylo vyzkoušeno polarografické chování čistého vodného roztoku kyseliny sorbové (koncentrace 1.10^{-3} až 2.10^{-4}M) v těchto základních elektrolytech: v Brittonově-Robinsonově ústojném roztoku, ve fosfátovém ústojném roztoku a dále v roztocích 0,1N LiOH, 0,1 N H_2SO_4 a 0,1 N HCl. Ve všech uvedených elektrolytech nebyla zjištěna polarografická aktivita kyseliny sorbové. Proto byly dále zkoušeny tyto elektrolyty: 0,1 N LiCl a roztoky tetrametylamoniumhydroxidu a tetrametylamoniumbromidu v koncentracích 0,05 M; 0,1 M a 1 M. Ve všech těchto elektrolytech byly zjištěny polarografické vlny kyseliny sorbové, přičemž nejvyvinutější, analyticky použitelné vlny byly zaznamenány v elektrolytech tetrametylamoniumbromidu a tetrametylamoniumhydroxidu v koncentracích 0,1 M a 0,05 M. Proto jsme dále věnovali pozornost studiu kyseliny sorbové jen ve dvou výše uvedených elektrolytech. V obou byly stanoveny hodnoty půlvlnového potenciálu pomocí thalia (pro odstranění vlivu hysterze přístroje na polohu půlvlnového potenciálu byly registrovány vždy dvě křivky, a to směrem stoupajícího a klesajícího napětí). Na grafu 1 jsou na polarogramu patrny vlny thalia a kyseliny sorbové v roztoku 0,05 M tetrametylamoniumbromidu.

Změřené půlvlnové potenciály ve zkoumaných elektrolytech jsou uvedeny v tabulce 1, z které je patrno, že půlvlnový potenciál kyseliny sorbové v roztoku

tetrametylamoniumbromidu je asi o 500 mV pozitivnější než E/2 v roztoku tetrametylamoniumhydroxidu. V uvedených základních elektrolytech tetraalkylsolí byla zjištěna dobrá proporcionalita výšky polarografické vlny a koncentrace kyseliny sorbové v rozmezí 10^{-4}—10^{-2} M. Na grafu 2 je uveden kalibrační polarogram kyseliny sorbové v základním roztoku 0,05 M tetrametylamoniumhydroxidu. V tabulce 2 jsou uvedeny vypočítané hodnoty konstanty K a změřené hodnoty půlvlnového potenciálu, pro různé koncentrace kyseliny sorbové v roztoku 0,05 M tetrametylamoniumhydroxidu. Na grafu 3 je uvedena závislost výšky vlny na výšce rezervoáru.

Tab. 1. Hodnoty půlvlnového potenciálu (E/2) kyseliny sorbové v různých elektrolytech

Elektrolyt	E/2 (proti NKE)
0,1 M LiCl	(−1,60 V)
0,1 M $N(CH_3)_4Br$	−1,55 V
0,05 M $N(CH_3)_4Br$	−1,57 V
0,05 M $N(CH_3)_4OH$	−2,13 V

PLEASE CHANGE COMMAS TO DECIMAL POINTS

Na základě experimentálního zjištění, že kyselinu sorbovou je možno ve vhodném elektrolytu polarograficky stanovit, byly provedeny pokusy s jejím stanovením v některých vzorcích potravin. Její přímé stanovení v potravinách nebylo ze zřejmých důvodů možné, a proto bylo použito známých izolačních postupů kyseliny sorbové z potravin, a to jednak destilace s vodní párou po okyselení a za přísady síranu hořečnatého, jednak extrakce pomocí organických

Tab. 2. Hodnoty konstanty K a půlvlnového potenciálu (E/2) pro různé koncentrace kyseliny sorbové (c) v roztoku 0,05 M $N(CH_3)_4OH$

E/2 (mV)	c (mmol)	K
−2,14	0,09	2,32
−2,12	0,44	2,36
−2,12	0,89	2,30
−2,13	1,32	2,32

rozpouštědel (dichlórmetan, chloroform). Polarografické stanovení v destilátu bylo prováděno tak, že jeho alikvotní část, eventuálně po odpaření byla smíchána s 1 M roztokem tetrametylamoniumhydroxidu tak, aby výsledná koncentrace základního elektrolytu byla 0,05 M. Extrakty pomocí organických rozpouštědel byly po odpaření rozpuštěny v elektrolytu a zaznamenány polarografické křivky. Tyto pokusy až na stanovení kyseliny sorbové v natíraném obalovém papíru nedávaly dobře reprodukovatelné výsledky, mnohdy bylo stanovení znemožněno polarograficky aktivními látkami, které byly vydestilovány, popřípadě spoluextrahovány ze zkoumaných potravin, nebo které při destilačním postupu za vyšších teplot vznikaly. Byla proto provedena série dalších pokusů, při nichž byla kyselina sorbová nejprve vydestilována ze vzorků s vodní párou, z okyseleného destilátu pak vyextrahována chloroformem a po opatrném odpaření rozpouštědla a rozpuštění odparku v základním elektrolytu zaznamenána polarografická křivka.

Tab. 3. Výsledky stanovení kyseliny sorbové v různých vzorcích

(1) Druh vzorku	(2) Stanoveno (mg/kg)		(5) Chyba v % (*)
	(3) polarograficky	(4) kolorimetricky	
(6) Fondantový krém	74	65	+13,8
Fondantový krém	110	124	−11,3
Fondantový krém	243	266	− 8,7
(7) Mandlová hmota	172	155	+11,0
(8) Konzervované okurky	48	55	−12,7
Konzervované okurky	142	165	−13,9
Konzervované okurky	120	106	+13,2
(9) Konzervovaná zelenina	92	78	+17,9
(10) Tuk na lisy	138	127	+ 8,7
(11) Natíraný papír	413	364	+13,5
Natíraný papír	514	546	− 5,9
Natíraný papír	660	604	+ 9,3

(12) * výsledek kolorimetrického stanovení 100 %.

Vyhodnocení bylo prováděno metodou standardního přídavku. Tímto postupem byly získány dobré výsledky v modelových pokusech s přidáním různých množství kyseliny sorbové (50—200 mg ve vzorku), kdy bylo dosaženo 87—104, 2 % z výsledku.

Jako nejvýhodnější se ukázal tento postup pro polarografické stanovení kyseliny sorbové: 50—100 g vzorku se po přidání vody (asi 100 ml), síranu hořečnatého (2 g) a kyseliny sírové (5 ml) destiluje s vodní párou, a destilát se jímá do okyseleného roztoku chloroformu (10 ml kyseliny chlorovodíkové se smíchá se 40 ml čerstvě předestilovaného chloroformu). Po nadestilování 300—400 ml se destilát převede do třepací baňky obsahu 1 l, přidá se 10 ml kyseliny chlorovodíkové a po jednominutovém protřepání se ponechají vrstvy oddělit. Spodní choloroformová vrstva se odpustí a vodní fáze se vytřepe ještě pětkrát s 25 ml chloroformu a výtřepky se spojí a převedou do porcelánové misky. Spojené výtřepky se odpaří na vodní lázni na objem asi 5 ml a později úplně do sucha (za normální teploty). K suchému odparku se přidá 5—10 ml 0,05 M roztoku tetrametylamoniumhydroxidu a zaznamená se polarografická křivka při vhodné citlivosti galvanometru. Vyhodnocení se provede metodou standardního přídavku.

Za použití uvedeného postupu byla kyselina sorbová stanovena ve 12 různých vzorcích a výsledky porovnány s hodnotami, zjištěnými kolorimetrickou metodou podle Schmidta[37]. Výsledky jsou uvedeny v tabulce 3.

Diskuse a závěr

Jak vyplývá z provedených experimentů podléhá kyselina sorbová za určitých podmínek elektroredukci na rtuťové kapkové elektrodě. Z konstantních hod-

not K a půlvlnových potenciálů kyseliny sorbové při jejích různých koncentracích (tab. 2) je zřejmé, že polarografická metoda je dobře použitelná v doporučených dvou základních elektrolytech — tetrametylamoniumbromidu nebo tetrametylamoniumhydroxidu — pro kvalitativní i kvantitativní analýzu kyseliny sorbové. Lineární závislost mezi koncentrací a výškou vlny byla zjištěna v mezích 10^{-4}—10^{-2} M. Hodnota půlvlnového potenciálu kyseliny sorbové v roztoku tetrametylamoniumbromidu je asi o 500 mV pozitivnější než v roztoku tetrametylamoniumhydroxidu, což je z hlediska analytického výhodnější. Pokud jde o charakter redukce kyseliny sorbové na rtuťové kapkové elektrodě, prokázala již ve své práci Fornasariová, že jde o ireverzibilní elektrodový proces, při kterém v alkalickém prostředí dochází k dvouelektronové redukci odpovídající procesu: kyselina sorbová → kyselina dihydrosorbová.

Z grafu 3, kde byla zjištěna lineární závislost mezi výškou vlny a druhou odmocninou z výšky rezervoáru vyplývá, že při redukci kyseliny sorbové jde o elektrodový děj řízený difúzí.

Z tabulky 3 je dále zřejmé, že vypracovaná polarografická metoda dává srovnatelné výsledky s metodou kolorimetrickou. Výhoda polarografické metody je v její specifičnosti a dále v tom, že může být současně proveden nejen důkaz (poloha půlvlnového potenciálu), ale i stanovení (výška polarografické vlny). Metoda je dostatečně citlivá a spolehlivá. Její nevýhodou například proti kolorimetrickým metodám je však větší pracnost spolu s časovou náročností.

Při stanovení neruší běžné konzervační látky, jako je kyselina benzoová, estery kyseliny p-oxybenzoové, kysličník siřičitý aj.

Souhrn

Experimentálně bylo blíže prostudováno polarografické chování kyseliny sorbové, zjištěny vhodné elektrolyty, podmínky pro její stanovení a změřeny hodnoty půlvlnových potenciálů. Na podkladě provedeného studia byla vypracována polarografická metoda na stanovení kyseliny sorbové v některých potravinách, po předcházejícím vydestilování s vodní párou, v roztoku tetrametylamoniumhydroxidu nebo tetrametylamoniumbromidu. Metoda byla s úspěchem přezkoušena na 12 různých druzích vzorků a dosažené výsledky porovnány s hodnotami, stanovenými v těchže vzorcích kolorimetrickou metodou podle Schmidta. Byla konstantována shoda těchto výsledků.

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SORBIC ACID AS A FUNGISTATIC AGENT FOR FOODS. III. SPECTROPHOTOMETRIC DETERMINATION OF SORBIC ACID IN CHEESE AND IN CHEESE WRAPPERS *

DANIEL MELNICK AND FREDERICK H. LUCKMANN
Research Laboratories, The Best Foods, Inc., Bayonne, N. J.

(Manuscript received July 16, 1953)

In 1945, Gooding described *(1)* a process for inhibiting growth of molds in or on food products by adding to the food or to the wrapper an alpha-beta unsaturated fatty acid. Sorbic acid, with the formula $CH_3 - CH = CH - CH = CH - COOH$, is one of the unsaturated fatty acids shown to be effective. Because of the conjugated double bonds, sorbic acid absorbs light in the ultraviolet region of the spectrum. Sorbic acid is steam distillable and this distillation is more effective from a saturated magnesium sulfate solution. Advantage of these properties of sorbic acid was taken in the development of a spectrophotometric method for the determination of the fatty acid *(6)*. In the present study, the spectrophotometric method was adapted to the determination of sorbic acid in cheese and cheese wrappers.

EXPERIMENTAL PART

Background for the spectrophotometric test. In Figure 1 are plotted the ultraviolet absorption curves of sorbic acid; these show the influence of pH on the position of the absorption maximum. Rather than plot extinction coefficients, the spectrophotometric findings have been plotted in terms of extinction ratios, i.e. the ratio of the extinction coefficient (or absorbancy) at a given wave-length to that at the absorption maximum. By this method of plotting, ready comparison can be made between the absorption curve of any sample, regardless of dilution of the test material, and that of a product of known purity, for example, pure sorbic acid in water.

It will be noted that both curves for sorbic acid in Figure 1 are sharply defined with an absorption maximum at 256 mμ with distilled water and at 262 mμ with acidulated water as the respective solvents. The extinction coefficients are very large, a desirable feature in that the spectrophotometric method enjoys a high degree of sensitivity. The absorption curve moves to the right as the pH of the medium decreases, but the extinction coefficient is affected to a very small degree. Thus, in the region of pH 4.0-5.9, blanketing the pH of distillates obtained in analyzing cheese for sorbic acid, the absorbancy need be determined only at the wave length maximum and sorbic acid concentration read from a reference chart. Correction for pH variations of the test solution is not required.

Figure 2 gives a typical reference chart for converting absorbancy readings into concentration of sorbic acid. The method is very sensitive; quantities as low as 0.001 mg. of sorbic acid per ml. can be determined with an error no greater than ± 1%. Beer's law is followed over the entire range of spectrophotometric readings.

The assay method. It is expected that sorbic acid will be used on the inside of cheese wrappers in an amount up to 5.0 g. of sorbic acid per 1,000 sq. in. of wrapper. The highest ratio of wrapper to cheese exists in the most popular commercial package (3¼×3½×1⅛ in.) used in packaging one-half pound of cheese. Such cheese would contain up to 0.2 g. of sorbic acid per half-pound *(5)*, assuming complete migration of the sorbic acid into the cheese and complete retention of the sorbic acid.

* This paper was presented in part at the Thirteenth Annual Meeting of the Institute of Food Technologists, Boston, Massachusetts, June 24, 1953.

ATIC AGENT FOR FOODS.

TERMINATION OF SORBIC
CHEESE WRAPPERS *

ERICK H. LUCKMANN
e Best Foods, Inc.,
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July 16, 1953)

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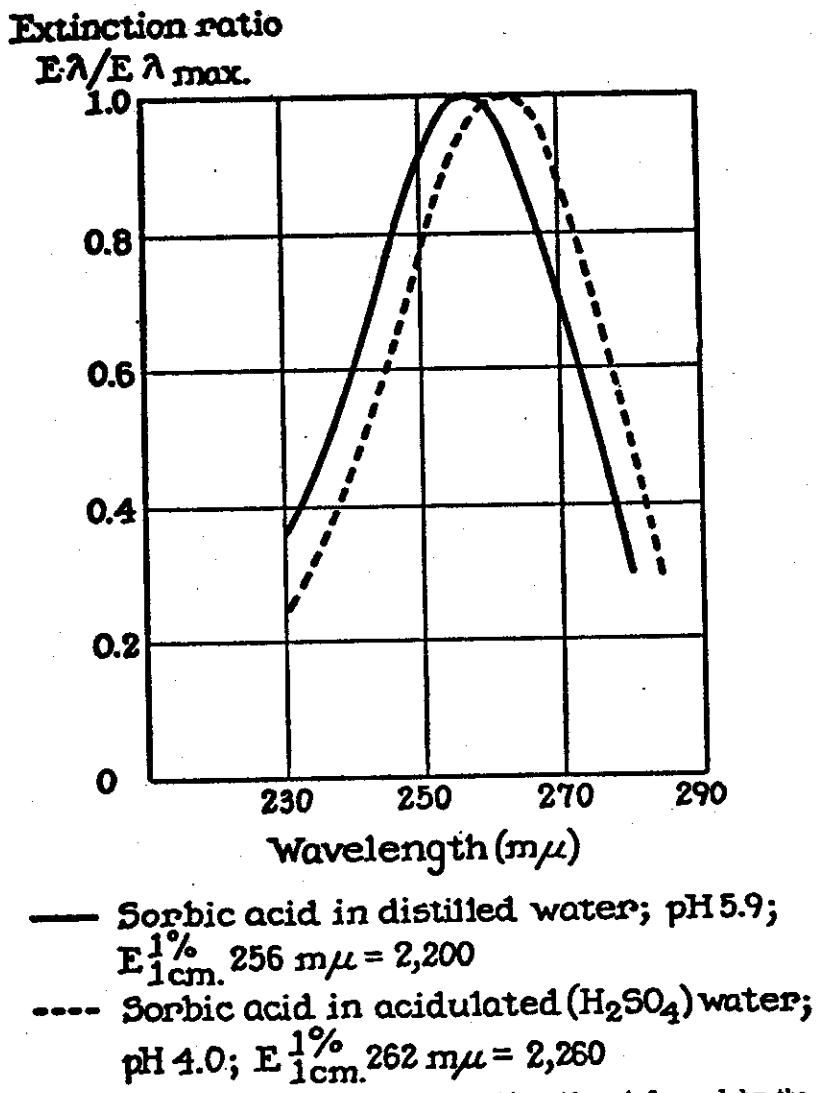

Figure 1. Ultraviolet absorption curves of sorbic acid as influenced by the pH of the solution.

To a sample of cheese, finely cut and containing preferably 0.75 to 2.25 mg. of sorbic acid, in a 500-ml. distilling flask equipped with a side inlet tube are added 50 g. of magnesium sulfate and 50 ml. of distilled water. An adaptor with ground glass joints connects the distilling flask with a Friedrich's condenser. This equipment should be of amber glass or, if clear glass is used, the distillations should be carried out in subdued light. The contents of the flask are heated with a Bunsen burner and approximately 40-45 ml. of distillate collected in the receiving flask. The *distilling* flask is then cooled by surrounding it with cracked ice and water. Another 50-cc. portion of distilled water is added through the side inlet tube, and after closing the inlet tube, another 40-45 ml. of distillate are collected. After washing the condenser with hot (90°C.) distilled water, the combined distillates are made to a volume of 500 ml. The absorbancy of this solution is determined at 235, 245, 258, 260, 262, 270, and 280 mμ on the Beckman Spectrophotometer using quartz cells and the hydrogen discharge lamp versus

distilled water as a blank, i.e. to set the spectrophotometer at 100% transmission for each of the wave length readings. The pH of the solution used in determining the absorbancy readings will be 4.8 ± 0.3 (maximal deviation). Due to this pH the absorption maximum of sorbic acid is at 260 mμ (see Figure 1).

The shape of the ultraviolet absorption curve will identify the material measured as sorbic acid. Absorbancy at 260 mμ is converted to mg. of sorbic acid per ml. of distillate (see Figure 2); calculations based upon weight of cheese sample taken for analysis and the dilution factor give the quantity of sorbic acid in the cheese.

The same method is applicable to the analysis of cheese wrappers treated with sorbic acid as a dusting powder or as a solution or suspension in the wax coating. However, the pH of the distillates from the wrappers is somewhat less acid (pH of 5.0 ± 0.1) than those from cheese, so that maximal light absorption occurs at 258 mμ. The same reference curve (see Figure 2) may be employed for converting absorbancy to sorbic acid concentration. The differences in absorbancy values due to variations in pH of the medium are very small, in the order of ± 1.5% over the pH range of 4.0 to 5.9 (see Figure 1).

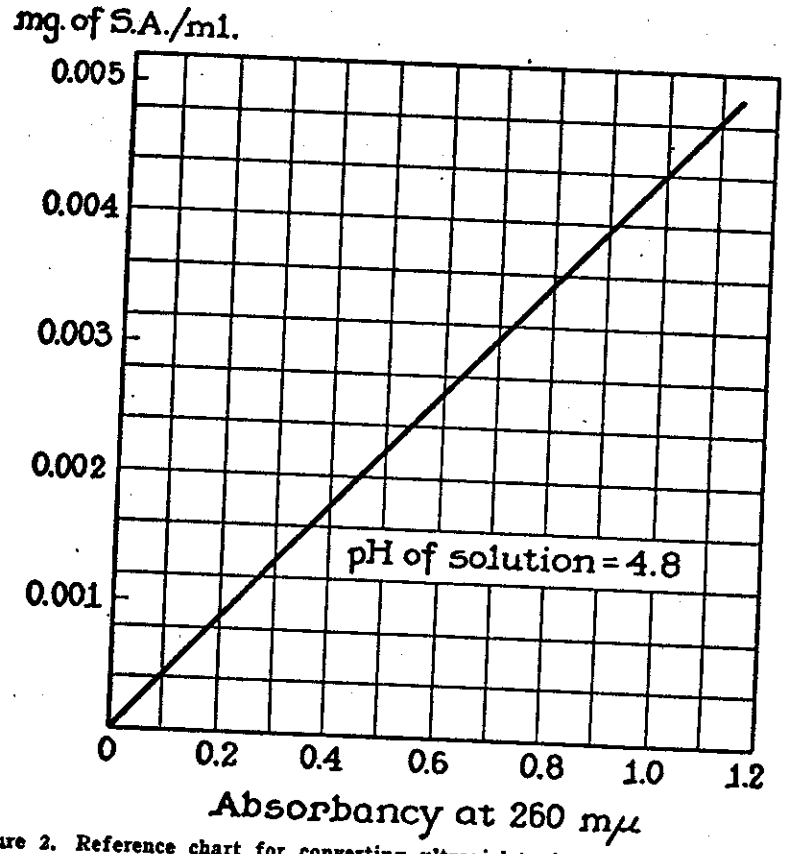

Figure 2. Reference chart for converting ultraviolet absorbancy into sorbic acid concentration.

RESULTS AND DISCUSSION

Reliability of the assay method. In the present study, a number of different cheeses and cheese products were subjected to sorbic acid analyses before and after supplementation with the fatty acid. The sorbic acid was completely and uniformly dispersed in each product (first softened) about 15 minutes before the analysis.

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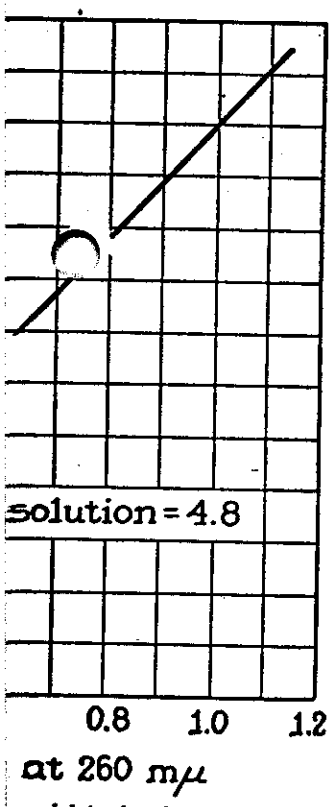

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CUSSION

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The test samples comprised Natural American, Natural Brick, Blue, Natural Swiss, Sliced Processed American, and Smoked Provolone Salami Cheese. These samples are representative of cheese varieties of different degrees of firmness, cheese cured internally with bacterial starters, cheese that had been surface cured, cheese with mold added in addition to the bacterial starters, cheese with controlled gas formers in the bacterial starters, pasteurized cheese emulsified with additional skim milk solids, and smoked cheese masticated and pressed before internal curing. These samples, supplied by Mr. Donald P. Smith of Milprint, Inc., West de Pere, Wisconsin, blanket the varieties of cheeses and cheese products to be packed in a wrapper containing added sorbic acid.

In Table 1 are presented the results of the tests on the control and supplemented cheese samples. It will be apparent that excellent recoveries of added sorbic acid are obtained in all cases, varying from 89 to 96%,

TABLE 1

Applicability of assay method for sorbic acid in cheese

Cheese	pH[a]	Sorbic acid content per ½ lb. of cheese			
		Apparent in control[b]	Apparent in supplemented cheese	Recovery[c]	
		mg.	*mg.*	*mg.*	%
Natural American	5.58	5.7	190.0	184.3	92.1
Natural Brick	6.10	4.8	182.0	177.2	88.6
Blue	6.38	4.8	184.0	179.2	89.6
Natural Swiss	6.10	5.7	198.0	192.3	96.1
Sliced Processed American	6.38	4.5	190.0	185.5	92.7
Smoked Provolone Salami	6.72	5.7	190.0	184.3	92.1
Average	6.21	5.2	189.0	183.8	91.9

[a] Ten g. of cheese macerated in 50 cc. of warm distilled water, cooled and made to volume of 100 ml., filtered, pH measured on the Beckman pH meter.
[b] Irrelevant absorption since none of the samples exhibited a curve typical of sorbic acid, i.e. no maximum at 260 mμ.
[c] Theoretical of 200 mg. per ½ lb. of cheese in each case. Approximately 1.5 g. of cheese were taken for the analysis, since such a sample size contained about 1.3 mg. of expected sorbic acid, a value falling in the 0.75 to 2.25 mg. range specified in the method. The distillate of the control unsupplemented cheese of the same size sample was diluted to only one-fourth volume in order to gain greater precision in the spectrophotometric measurements.

with an average of 92%. Small losses of sorbic acid of about 5% are unavoidable in carrying out the test. The cheese products contain no appreciable quantities of interfering substances; the apparent sorbic acid figures for the blank unsupplemented cheese samples due to irrelevant absorption are in this series less than 3% of the true values following sorbic acid addition.

In Figure 3 are plotted the absorption curves for the distillates of each of the cheese samples before and after sorbic acid supplementation. The distillates containing sorbic acid exhibit absorption maxima at 260 mμ; the pH of the distillates are 4.8 ± 0.3 (maximal deviation). This absorption maximum falls between 256 and 262 mμ obtained for pure sorbic acid solutions at pH 5.9 and 4.0, respectively (see Figure 1).

Also included are the curves for the supplemented samples after correction for the irrelevant absorption. The absorption curves obtained in

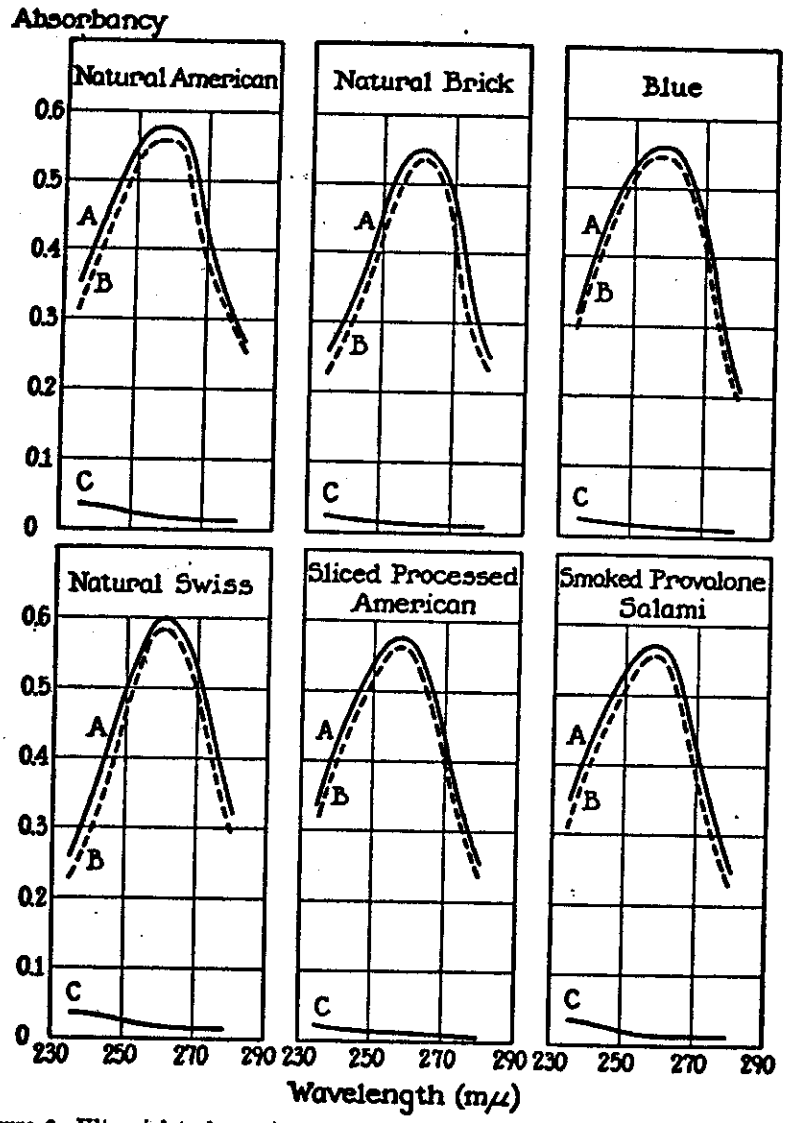
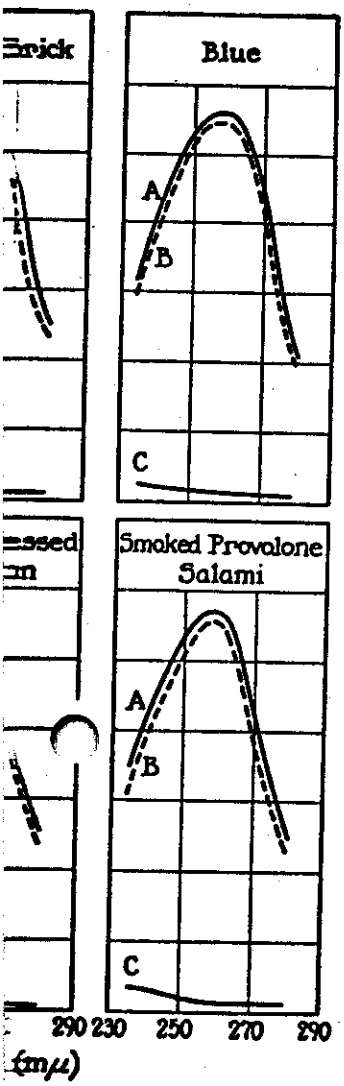


Figure 3. Ultraviolet absorption curves of distillates from cheese samples before and after supplementation with 200 mg. of sorbic acid per one-half pound. Distillate from 1.50 g. of cheese brought to a volume of 500 ml.; A = cheese supplemented with sorbic acid; C = unsupplemented control cheese; and B = curve for A corrected for C.

testing the blank unsupplemented cheese products are plotted in terms of the same common dilution of distillates containing sorbic acid; actually, four-fold concentrations of the blank solutions were employed in the spectrophotometric tests in order to gain greater precision. It is immediately apparent on visual examination that the absorption curves for sorbic acid derived from cheese are identical with those for pure sorbic acid.

Two distillations are called for in the section of this paper describing the assay method. Each distillate comprises a volume of 40 to 45 ml. From



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pure aqueous solutions 83 to 88% of the sorbic acid is recovered in the first distillate; the second distillation raises the yield to 95 to 100%. From cheese samples, the respective values are 80 to 85% and 89 to 96%. No further increases in the recovery values are obtained on subsequent distillations. It is assumed that the unavoidable loss of sorbic acid is due to oxidation *(3)*.

In the assay of cheese samples, an aliquot containing preferably 0.75 to 2.25 mg. of sorbic acid is employed. In most cases, this will require the testing of 1.5 to 5.0 g. of cheese. Obviously in a given test the per cent irrelevant absorption at 260 mμ, due to components in the cheese other than sorbic acid, will be inversely related to the amount of sorbic acid present; it is necessary to test samples of greater size when the sorbic acid concentration is low. Illustrative data covering this point are presented in Table 2. The findings obtained in the present recovery experiments and in the migration studies to be reported in a following paper *(2)* were employed in drawing up this compilation.

TABLE 2

Illustrative data showing relationship of sample size to the contribution of irrelevant absorption at 260 mμ in the spectrophotometric determination of sorbic acid

Cheese	Sample taken for test	Sorbic acid content per ½ lb. cheese			Irrelevant absorption in relation to that due to sorbic acid
		Apparent in control	Apparent in supplemented cheese	True	
	g.	*mg.*	*mg.*	*mg.*	%
Natural American	1.5	5.7	190.0	184.3	3.1
	3.0	5.0	100.0	95.0	5.3
	5.0	4.2	51.4	47.2	8.9
	5.0	5.2	27.4	22.2	23.4
Natural Brick	1.5	4.8	182.0	177.2	2.7
	3.0	5.7	116.0	110.3	5.2
	5.0	3.9	61.1	57.2	6.8
Blue	1.5	4.8	184.0	179.2	2.7
	5.0	3.3	3.3	0.0	∞
Natural Swiss	1.5	5.7	198.0	192.3	3.0
	5.0	4.8	42.0	37.2	13.0
Sliced Processed American	1.5	4.5	190.0	185.5	2.4
	5.0	5.7	63.4	57.7	9.9
Smoked Provolone Salami	1.5	5.7	190.0	184.3	3.1
	5.0	5.4	30.8	25.4	21.2

It will also be noted from the data in Table 2 that (a) irrelevant absorption at 260 mμ due to components in the cheese other than sorbic acid is very small, of the order of 3.3 to 5.7 mg. of apparent sorbic acid per ½ pound of cheese, (b) size of sample taken for the test has no significant effect on the apparent sorbic acid content of the unsupplemented cheeses when expressed on a unit weight basis (*viz.* per ½ pound of cheese), and (c) variations in the apparent sorbic acid content between different cheese varieties are exceedingly small and of the same order of magnitude as

between cheese samples of the same variety, less than 2 mg. per ½ pound of cheese. The irrelevant absorption by all the blank cheese samples exhibited no absorption maximum in that part of the spectrum where readings are made.

In research studies, such as those covering the migration *(2)* and fate of sorbic acid *(4)* in packaged cheese, the apparent sorbic acid figures should be and were corrected for irrelevant absorption attributable to components in the cheese other than sorbic acid. For all practical purposes, however, reliable sorbic acid estimates are obtainable in the absence of blank cheese samples, the small positive error due to irrelevant absorption being neutralized by the small negative error due to unavoidable analytical loss of sorbic acid. According to the ruling of the U. S. Food and Drug Administration, sorbic acid in cheese shall not exceed 0.10%. At this maximal sorbic acid concentration, the irrelevant absorption would amount to only about 3% of that due to sorbic acid present. In commercial operations sorbic acid will be applied to wrappers in concentrations approximating for the most part 2.5 g. per 1,000 sq. in. of wrapper *(5)*. Such a wrapper can furnish sorbic acid to the outer ¼ inch of cheese in concentration of about 0.056% *(5)*. This latter value is equivalent to 127 mg. of sorbic acid per ½ lb. of cheese and at this concentration irrelevant absorption amounts to only about 5% of that due to the sorbic acid present (see Table 2). At the lowest anticipated use level of 1.25 g. of sorbic acid per 1,000 sq. in. of wrapper, irrelevant absorption may amount to about 10% of that due to the sorbic acid present. At lower concentrations of sorbic acid, the potential positive error due to the omission of a blank control cheese from the test may be as much as 20 to 25%, but there is little interest in such small sorbic acid concentrations, *viz.* 0.01% in the cheese or 23 mg. per ½ pound of product. It is of no practical significance whether the sorbic acid concentration is 28 mg. (apparent and true) or 23 mg. (true) per ½ pound of cheese. These practical considerations lead to the recommendation that a test conducted on blank control cheese be omitted in routine plant operations.

That sorbic acid degradation products do not interfere with the analytical test will be demonstrated in subsequent reports covering migration *(2)*, oxidation *(3)*, and mold metabolism *(4)* of sorbic acid during the storage of the same cheese samples in wrappers impregnated with sorbic acid.

Recoveries of 90 to 100% of sorbic acid are regularly obtained in analyses of wax-coated cellophane wrappers treated with the fatty acid. No irrelevant absorption is obtained in testing the distillates obtained from the untreated wrappers.

SUMMARY

A spectrophotometric method for the estimation of sorbic acid in cheese and in cheese wrappers has been presented. The sorbic acid in the test sample is distilled at atmospheric pressure along with the water in the test system; this distillation is rendered more efficient by the presence of added magnesium sulfate. The sorbic acid in the distillate is identified by its characteristic absorption curve, and concentration calculated from the

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The spectrophotometric procedure has been shown to be a precise and specific method for the estimation of sorbic acid in cheese products of all varieties and in cheese wrappers. In practical operations it can be applied to the analysis of cheese or wrapper in the absence of blank unsupplemented samples.

Acknowledgment

The technical assistance of Mr. William Ferren of The Best Foods Laboratory in the course of these studies is greatly appreciated.

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DAIRY PRODUCTS

Quantitative Determination of Sorbic Acid in Cheddar Cheese by Gas-Liquid Chromatography

By D. E. LACROIX and N. P. WONG (Dairy Products Laboratory, Eastern Marketing and Nutrition Research Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C. 20250)

A rapid method is described for the extraction and quantitative determination of sorbic acid in Cheddar cheese. Cheddar cheese is impregnated onto a Celite 545 column, extracted with acetonitrile, and analyzed for sorbic acid by gas-liquid chromatography, using a 7.5% ethylene glycol adipate + 2% H_3PO_4 column. Average recovery values for known amounts of sorbic acid were 107%.

Present methods for the determination of sorbic acid in Cheddar cheese are not suitable for performing screening analyses of a large number of samples. Sorbic acid is removed by steam distillation of the sample, followed by solvent extraction and spectrophotometric determination of the sorbic acid in the distillate (1–5). Besides being time-consuming and requiring extensive apparatus, this procedure is not entirely satisfactory, since the spectrophotometric assay gives high blank values and/or is subject to interference by other preservatives which may be present, especially benzoic acid (1, 4, 6).

Attempts have been made to overcome spectrophotometric interference by utilizing differences in absorption maxima at various wavelengths (6–8). Calloway and Schwartzman (9) eliminated benzoic acid interference by measuring the infrared absorption of sorbic acid at 10 nm in chloroform and obtained good recoveries with a variety of samples.

The acetonitrile extraction procedure of Wong and Parks (10), followed by gas-liquid chromatography (GLC) of the extract, provides a simple method for the determination of sorbic acid in Cheddar cheese. Two unique advantages of this method are that the cheese fat is not extracted and that GLC analyses can be performed directly on the extract. GLC analyses of sorbic acid in other foods have been performed (11–13), although to our knowledge none has been adapted to Cheddar cheese. Therefore, it would appear that determination of sorbic acid by GLC would eliminate the disadvantages of high blank values and absorptive interference of other preservatives which may be present, since sorbic and benzoic acids are easily separated by GLC.

METHOD

Apparatus and Reagents

(a) *Diatomaceous earth.*—Celite 545, or equivalent.

(b) *Acetonitrile.*—Redistill before use.

(c) *Sorbic acid.*—Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.

(d) *Sorbic acid standard solution.*—2 μg/μl in acetone.

Gas Chromatography

Barber-Colman Model 5000 gas chromatograph equipped with hydrogen flame ionization detector was used. GLC parameters: 6′ × 4 mm id stainless steel coiled column containing 7.5% ethylene glycol adipate (EGA) + 2% H_3PO_4 on 90–100 mesh Anakrom ABS (Analabs, Inc., N. Haven, Conn. 06473). Operating conditions: column 165°C and injection port and flame ionization detector 280°C, nitrogen carrier gas 115 ml/min.

Determination

Prepare cheese for extraction according to procedure of Wong and Parks (10). To 20 g finely shredded Cheddar cheese, add 25 g Celite 545 and grind thoroughly. Transfer resulting homogeneous mass to 1.6 × 40 cm column and pack firmly, using packing rod. Rinse mortar with 25 ml acetonitrile; add rinse to column. Let acetonitrile flow into cheese-Celite mixture until level of solvent reaches top of mixture. Add 50 ml acetonitrile to extract sorbic acid from column, eluting into 50 ml Erlenmeyer flask. Let all acetonitrile elute completely from column (total elution time is ca 3 hr, yielding ca 40 ml eluate). Partially remove acetonitrile by evaporation over steam bath, using nitrogen or filtered air. When ca 15 ml acetonitrile remains in receiving flask, quantitatively transfer residual eluate into 25 ml volumetric flask. Rinse receiving flask several times with acetonitrile, transfer all washings into 25 ml volumetric flask, and dilute extract to volume. Inject 2–8 μl extract for GLC analyses.

Obtain standard curves daily in range 4–12 μg, with 6 μg sorbic acid giving 50% full scale deflection. Plot area of sorbic acid peak against μg sorbic acid.

Results and Discussion

Cheddar cheese manufactured by USDA, Dairy Products Laboratory, Beltsville, Maryland 20705, containing no sorbic acid, and commercially available Cheddar cheese, with and without label claim of sorbic acid added as a preservative, were used in this study.

The dose-response curve was determined for sorbic acid over a weight range of 4–12 μg. The area under the GLC peak was calculated by triangulation and a straight line relationship between peak area and mass was obtained for sorbic acid under these conditions.

Figure 1A shows a typical gas chromatogram of the separation of sorbic and benzoic acids; Fig. 1B is a gas chromatogram of Cheddar cheese with sorbic acid and 1C is Cheddar cheese without sorbic acid. GLC analyses of Cheddar cheese prepared at the USDA Dairy Products Laboratory were similar to analyses of commercial brands having no label claim of added sorbic acid. It is apparent that the retention time of benzoic acid is more than twice that of sorbic acid and it does not interfere with the sorbic acid analyses.

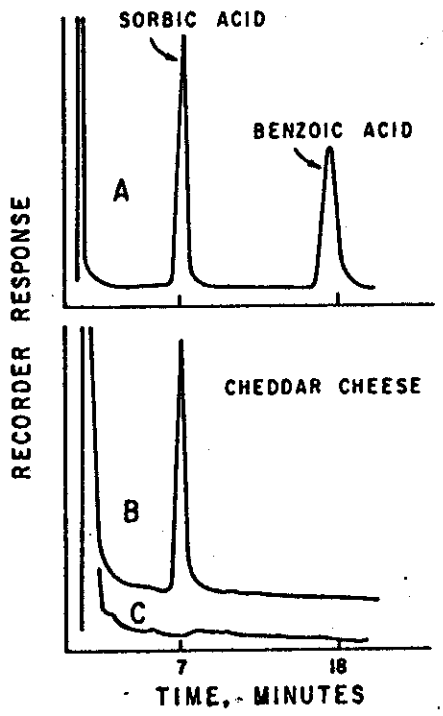

FIG. 1—Gas chromatograms of A, sorbic and benzoic acids; B, Cheddar cheese with sorbic acid; and C, Cheddar cheese without sorbic acid.

The amount of sorbic acid added to Cheddar cheese was equivalent to 0.1 and 0.3%. Twenty g USDA Cheddar cheese was ground thoroughly with Celite 545, and 20 or 60 mg sorbic acid in acetone was added to the homogeneous mass. This mixture was ground thoroughly, packed into the column, and extracted with acetonitrile as described previously. The recoveries fell within a range of 100–112%, based on a total of 20 determinations, with an overall coefficient of variation of 3.35%.

Federal standards for Cheddar cheese, sliced or cut, allow it to contain sorbic acid or its sodium or potassium salt not to exceed 0.3% by weight calculated as sorbic acid (14). Since sorbic acid is preferentially added as a dip for the cut cheese or as a dusting powder to the surface of the cellophane wrapper (15), it would be expected that the distribution of sorbic acid would not be uniform throughout the cheese.

To demonstrate this, a 4 × 14 cm bar of a commercial cheese, having a label claim of added sorbic acid, was sampled at various places. The bar was cut in half and one-half was ground and sampled as representative of the total bar. The other half was cut into slices 1 cm thick, starting from the center of the bar. The first slice was ground and sampled as the center slice. Two samples were obtained from the second slice. One contained the outer 1 cm circumference of the slice and the other a 2 cm square from the center. The values obtained were 0.22, 0.16, 0.26, and 0.095% sorbic acid for total bar, center slice, exterior edge, and center square, respectively. Higher values of sorbic acid were obtained from those sections of the bar having more surface area exposed to the wrapper. Since sorbic acid migrates toward the center from all faces (2), the entire sample must be ground and mixed thoroughly in order to obtain a representative value of sorbic acid. Analyses of several commercially available samples of cheese with a label declaration of sorbic acid showed that sorbic acid values ranged from trace amounts to the legal limit.

The acetonitrile extraction of a cheese-impregnated Celite 545 column provides a fast and efficient method for the isolation of sorbic acid from Cheddar cheese. Subsequent GLC analyses

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of the extract provide a rapid, quantitative procedure which is free from interference by benzoic acid and is applicable for routine analyses of a large number of samples. The method is not applicable to cottage cheese, but it should extract other cheeses with a composition similar to Cheddar cheese.

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Gas Chromatographic Determination of Propionic, Sorbic, and Benzoic Acids in Rye Bread and Margarine

By A. GRAVELAND (Institute for Cereals, Flour and Bread TNO, Wageningen, The Netherlands)

A rapid and accurate method is presented for the GLC determination of propionic and sorbic acids in bakery products and benzoic acid in margarine. The acids are extracted with ether containing orthophosphoric acid, to which valeric acid is added as internal standard. The extract is injected directly into the gas chromatograph. Glass columns (2 m × 2.0 mm id) containing 5% Carbowax 20M-terephthalic acid coated on Chromosorb W (AW-DMCS) are used for analysis. The simplicity and convenience of the method make it suitable for routine determinations.

Low molecular weight carboxylic acids are frequently occurring constituents of food products. They may be inherent to the product, because they are present in the raw materials or formed in the course of the manufacturing process, or they may have been added as a preservative to prevent the development of mold and bacteria.

Because of the use of these acids as preservatives it is desirable to have a rapid method at hand to examine products for their presence. Until recently, routine checking was impossible, as the acids had to be isolated from the materials by steam distillation and recovered as the sodium salts; then they were determined with rather cumbersome methods. The introduction of thin layer chromatographic techniques for the separation and identification of these acids (1, 2) was a step forward. These procedures, however, are also cumbersome and lack the desirable sensitivity.

The newly developed liquid phases for gas chromatography have now made it possible to make a qualitative and quantitative determination of the low molecular weight carboxylic acids within a short time. Complete resolution of all C_1 to C_5 normal and *iso*-acids was attained by Jackson (3), using a sebacic acid column as the liquid phase. The C_2 to C_{11} acids, including some unsaturated and branched isomers, were separated by Nikelly (4) on a column filled with glass microbeads as the solid phase and a mixture of Carbowax and isophthalic acid as the liquid phase. Nieuwenhof and Hup (5) determined free fatty acids (C_2 to C_{18}) by using a Carbowax 20M terephthalic acid column. We found this column to be equally suitable for the estimation of acetic, propionic, and sorbic acids in rye bread and benzoic acid in margarine.

METHOD

Gas Chromatography

A gas chromatograph (Fractovap Model GV Carlo Erba Co.) with a flame ionization detector was used; the carrier gas was nitrogen. Glass columns (U-shaped, 2 m × 2 mm id) were packed with 5% Carbowax 20M-terephthalic acid (Hewlett-Packard) coated on 60–80 mesh Chromosorb W (acid-washed, DMCS-treated). Column packing was prepared by dissolving the liquid phase in ethylene chloride, adding the solution to the solid support, and evaporating the volatile solvent in an evaporator. The material was packed into the column by attaching a vacuum pump to one end and vigorously tapping the tube until no more material was accepted. Column ends were plugged with glass wool. A Honeywell recorder (1 mv span) with an Infortronics automatic digital integrator Model CRS-204 was employed for the recording of the components. Operating conditions: column temperature programmed from 100 to 210°C at 5°/min; injection port 260°C; detector 270°C; carrier gas 65 ml/min.

Reagents

(a) *Acids.*—Analytical grade acetic, propionic, butyric, isobutyric, valeric, isovaleric, sorbic, and benzoic acids (Fluka, Basel, Switzerland).

(b) *Internal standard solution.*—1 mg valeric acid/ml ether.

Procedure

To 10 ml glass tube (12 mm diameter) add 1 g rye bread, 2 ml ether containing 3% (v/v) concentrated orthophosphoric acid, and 1 ml valeric acid stock solution. Homogenize mixture by disintegrating bread with glass rod during 5 min at 0°C (ice-water bath). Inject 5 μl supernatant liquid directly onto GLC column.

To determine benzoic acid in margarine, suspend 1 g margarine in 2 ml ether containing 3% orthophosphoric acid. Add 1 ml valeric acid stock solution. Centrifuge (2800× *g*) and inject 5 μl clear supernatant liquid into gas chromatograph.

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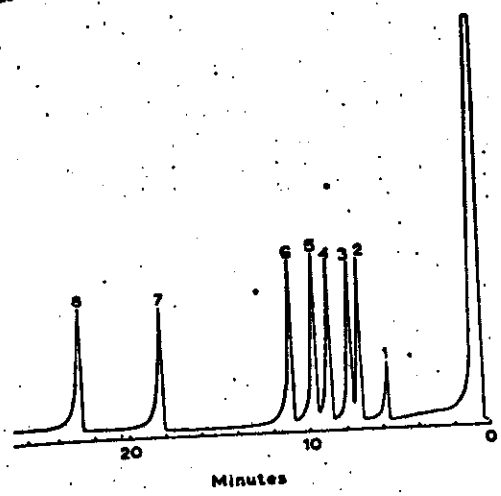

FIG. 1—Gas chromatogram of standard acids: 1, acetic; 2, propionic; 3, isobutyric; 4, butyric; 5, isovaleric; 6, valeric (internal standard); 7, sorbic; 8, benzoic. See text for GLC conditions.

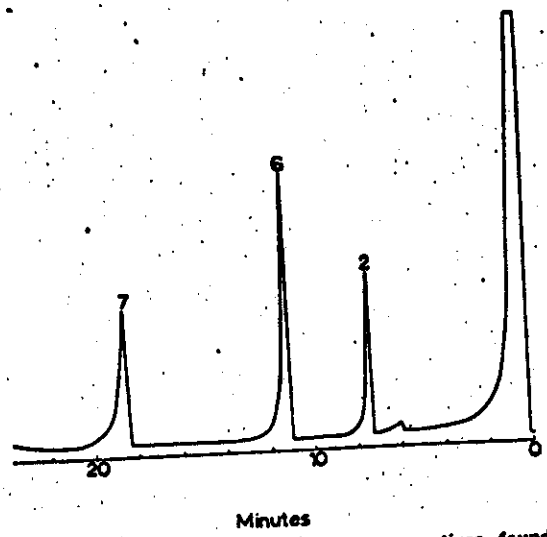

FIG. 2—Gas chromatogram of 2 preservatives found in rye bread: 2, propionic acid; 6, valeric acid (internal standard); 7, sorbic acid.

Measure peak areas with integrator; calculate acids on basis of internal standard and response factors.

Results and Discussion

Figure 1 shows a typical chromatogram of a mixture containing 30 ng of each of the low molecular weight carboxylic acids on a column of Carbowax 20M-terephthalic acid. Both propionic and sorbic acids as well as benzoic acid can be separated on the same column. Figures 2 and 3 show chromatograms of low molecular weight carboxylic acids found in rye bread and margarine, respectively. Valeric acid is the internal standard.

Because an internal standard is used, it is necessary to establish the response factor for the above free fatty acids. The response factors were determined by analyzing solutions containing equal quantities of propionic, sorbic, benzoic, and valeric acids (internal standard). The analysis was repeated 6 times. The average values of the response factor and the standard deviations are summarized in Table 1. It is essential to determine the response factors for each series, because they depend on the column and the flow rate of the carrier gas. Besides, a small change in the temperature programming may affect the response factor.

To be informed about the accuracy and reproducibility of the results, the method was checked by replicate analysis of 5 μl portions of ether extracts of a rye bread sample. The results of the test presented in Table 2 indicate that the method is accurate and precise.

The stability of the packing described above appears to be quite satisfactory. Our column was

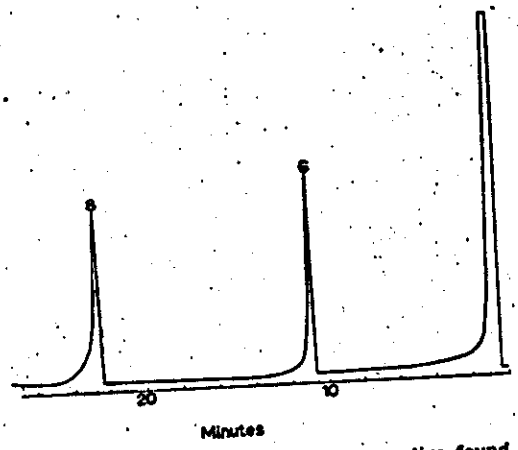

FIG. 3—Gas chromatogram of a preservative found in margarine: 6, valeric acid (internal standard); 8, benzoic acid.

Table 1. Determination of response factors for free fatty acids, calculated with reference to valeric acid

Acid	Av.	Std Dev.
Propionic	1.53	0.018
Sorbic	0.95	0.0094
Benzoic	0.88	0.0082

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Table 2. Determination of propionic and sorbic acids (mg/g) in rye bread and benzoic acid in margarine, 6 replicates

Acid	1	2	3	4	5	6	Av.	Std Dev.
Propionic	0.080	0.078	0.079	0.080	0.081	0.077	0.079	0.0015
Sorbic	0.151	0.143	0.161	0.148	0.152	0.141	0.150	0.0072
Benzoic	0.088	0.086	0.089	0.088	0.087	0.088	0.088	0.0010

operated continuously between 100 and 210°C for more than 4 months without deterioration.

Results obtained by the present method on 4 rye bread samples were compared with those obtained by the colorimetric method of Schmidt (6) for sorbic acid, and by the gas-liquid chromatographic method of James and Martin (7, 8) for propionic acid. The findings are given in Table 3. We obtained higher results for propionic acid with our method than with the method of James and Martin. The steam distillation involved in the latter method may have been incomplete.

The recovery of the free fatty acids was checked by adding the acids in known amounts to the rye bread sample previously analyzed. Recovery of the added free fatty acids varied from 98 to 101%.

Table 3. Comparison of present method and existing methods for determining propionic acid and sorbic acid in rye bread (4 samples analyzed)

Acid	Method	Replicates	Samples, mg/g 1	2	3	4
Propionic	GLC (7, 8)	2	0.16	0.11	0.07	0.07
	GLC	5	0.19	0.12	0.09	0.09
Sorbic	colorim. (6)	3	0.11	0.17	0.12	0.08
	GLC	5	0.10	0.17	0.12	0.09

The lowest amount of preservative detectable in foods is about 5 ng. The present method is simple and comparatively convenient means of obtaining accurate quantitative results. We tested the method only on rye bread and margarine. In all probability, the method may be used to estimate preservatives in other food products.

Acknowledgments

The author wishes to thank N. H. Visser for assistance in experiments and analyses.

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SERIES 1, SCIENCES: NO. 17

THE IDENTIFICATION OF SORBIC ACID BY MEANS OF SELENITES

STUDY ON THE APPLICATION OF THE FARADAY-TYNDALL EFFECT IN ANALYTICAL REACTIONS

BY HECTOR FERNANDEZ AND WALTER JUNG

SUMMARY

Small quantities of sorbic acid may be identified by means of selenites; the reaction is carried out in a hydrochloric medium and at water-bath temperature, observing the precipitation of elemental selenium. The sensitivity of the reaction may be increased considerably by applying the Faraday-Tyndall phenomenon.

This change may be generally applied and was studied in a series of type tests. In all cases a pronounced increase of sensitivity was confirmed, which could be important for resolving certain analytical problems.

In a previous work[1] we mentioned that sorbic acid and its salts can be easily identified by means of the reaction of selenious anhydride or sodium selenite in a strongly hydrochloric medium. At ambient temperature, the reaction is very slow, but relatively rapid at boiling water-bath temperature. At the start the formation of a precipitate is noticed of the red selenium change which settles slowly; following heating, the color changes slowly from red to gray, that is to the stable change of selenium.

For the determination of the sensitivity of this reaction we followed the current technique: A solution of sodium sorbate was prepared by neutralizing an aqueous suspension of 100 mg. of sorbic acid with 0.1 N sodium hydroxide using phenolphthalein as an external indicator; the sodium sorbate solution was taken to 100 ml. in a measuring flask using water as a solvent; therefore, each ml of the solution contains .1 mg. of sorbic acid. By dilution of these main solutions definite concentration solutions were obtained.

The reagent is a solution of sodium selenite at 5% p/v. The reaction is carried out by mixing in test tubes 1 ml. of the dilution of sodium sorbate with 1 ml. of reagent. Under these conditions no reaction takes place. 1 ml. of concentrated hydrochloric acid was then added and heat was applied, after homogenization during 30 minutes by boiling water-bath, noting the chart described previously.

The main solution (1:1000) and its dilution 1:10,000 gave clearly positive reactions, but not greater dilutions; we deduced that the dilutions of high coefficient will be able to produce the separation of elemental selenium, in a colloidal form which escapes direct observation, which was confirmed by applying the Farady-Tyndall phenomenon to the tests under observation[2].

In all cases the corresponding blank tests were made, treating 1 ml. of distilled water with 1 ml. of reagent and 1 ml. of concentrated hydrochloric acid, heating for 30 minutes by boiling water-bath.

The optical device used is of simple construction: in a microprojector a beam of white light is produced which is parallel and which crosses the problem solution.

In the blank tests, the medium is optically clean; instead, in the presence of vestiges of selenium formed in the reduction of the selenite by the action of sorbic acid or other reducers, the Faraday-Tyndall phenomenon is clearly produced. A notable increase of the sensitivity of the reaction is noted. In the dilution $1:10^5$ the result is clearly positive, but weak in the dilution $1:10^6$ and detectable only in comparison with the blank test.

In the tests carried out with high dilutions the tubes should be covered in order to avoid contamination with the air which could produce false positive results.

We have studied the same reaction using as a reagent instead of the selenite, sodium telurite; the reaction was carried out in a sulphuric medium. To 1 ml. of the sodium sorbate solution of known dilution 1 ml. of 10% p/v sodium telurite and 1 ml. of concentrated sulphuric acid is added. Heating by boiling

water-bath for 30 minutes then follows. In positive reactions a grayish solution which settles slowly appears. The reaction is less sensitive than with the selenite as a reagent.

At simple sight a positive reaction is only observed up to the dilution $1:10^3$ and on applying the Faraday-Tyndall test up to the dilution $1:10^4$. Logically, the Faraday-Tyndall phenomenon can be applied by us only to analytical precipitation tests; however, even so it does not lack value in Qualitative Chemical Analysis taking into account the appreciable increase of sensitivity. This fact is evident in the reaction of sodium thiosulphate with different types of acids and of different concentrations. Solutions of sodium thiosulphate (1 ml. to 0.1%, 1% and 10% p/v) were used adding 1 ml. of acid and measuring the time until formation of colloidal sulphur. t: 18°C. In all the cases with the Faraday-Tyndall effect the separation of colloidal sulphur is observed prior to observation at simple sight.

$Na_3S_2O_3$	Tyndall effect	Direct observation
	HCl N	
0.1%	10 min.	20 min.
1.0%	30 sec.	1 min. 27 sec.
10.0%	8 sec.	8 sec.
	HCl 0.1 N	
0.1%	18 min.	30 min.
1.0%	1 min.	2 min. 30 sec.
10.0%	10 sec.	10 sec.
	CH_3COOH N	
0.1%	35 min.	5 h. 30 min.
1.0%	1 min. 55 sec.	5 min.
10.0%	12 sec.	12 sec.
	CH_3COOH 0.1 N	
0.1%	4 h.	-

1.0%	3 min.	10 min.
10.0%	15 sec.	40 sec.

Therefore, the observation time depends on the concentrations of the solutions used and the strength of the acids.

In the last series of observations (CHE_3COOH 0.1 N) the value of the 0.1% sodium thiosulphate at "direct observation" was not recorded since it is excessively great. In the tests with sodium thiosulphate of high concentration (10%), the figures for the first three observation series are practically identical for the "direct observation" and the "Faraday-Tyndall Effect".

With the purpose of simplifying the terminology to express the sensitivity (limit concentration) of qualitative reactions in different dilutions of a primary solution, we believe advisable to substitute the convention denomination by logarithmic terms (PSE), similar to the procedure followed for pH values, that is:

DILUTION	PSE
1 : 100.....	2
1 : 1,000.....	3
1 : 10,000.....	4, etc.

On transcribing this publication we learned that the logarithmic expression of the sensitivity found support from other authors; Burriell-Marti and Ramirez-Munoz(3) apply it especially in the photometry of flame, using the symbol pD. However, we prefer the symbol P_{SE} to avoid confusion with similar terms. We have extended the study to some reactions which are already known in order to learn to scope and value of the phenomenon described.

I. PRECIPITATION OF SILVER HALIDES

We prepared aqueous solutions at 1 per mil p/v of potassium chloride sodium bromide and potassium iodide; using 0.6 ml. of said dilutions. 1 drop of concentrated nitric acid (free of halogenides) and 3 ml. of silver nitrate 10^{-5}N. On applying the Faraday-Tyndall effect it is possible to visualize the precipitation of the corresponding silver halides prior to the observations made at simple sight.

II. PRECIPITATION OF ALKALINE EARTHS SULPHATES

In these cases the results of the previous series are confirmed fully. Primary solutions at 1 % p/v were prepared of calcium chloride, strontium nitrate and barium chloride which were diluted with water as advisable. In each experiment 3 mls. of the solutions of the alkaline earths ions were used in which there were added in each case, 0.6 ml. of 5 N sulphuric acid:

	P_{SE}	Direct observation	Tyndall
Cl_2Ca	2	-	+
$(NO_3)_2Sr$	2	+	+
$(NO_3)_2Sr$	4	-	+
Cl_2Ba	2	+	+
	4	+	+
	6	-	+
	7	-	+

III. HYPOPHOSPHITES AND PHOSPHITES WITH SODIUM SELENITE IN THE PRESENCE OF CONCENTRATED HYDROCHLORIC ACID.

The qualitative reaction was already studied in a previous work ([4]).

Primary solutions were prepared of sodium phosphite and sodium hypophosphite at 1% p/v from which aqueous dilutions of definite concentration were obtained.

One ml. of each dilution was mixed with 1 ml. of sodium selenite at 5% p/v and 1 ml. of concentrated hydrochloric acid; after homogenizing heating was applied for 30 minutes by boiling water-bath.

P_{SE}	Direct observation Hypophosphites	Tyndall
3	+	+
4	-	+
5	-	-

Phosphites

2	+	+
3	-	+
4	-	-

100 mg. of As_2O_3 which was diluted in order to obtain solutions of definite concentration. In each test 1 ml. of the arsenite solution was mixed with 1 ml. of the sodium hyposulphite solution and 1 ml. of concentrated hydrochloric acid, heating for 30 minutes by boiling water-bath.

P_{SE}	Direct observation	Tyndall
3	+	+
4	+	+
5	-	+
6	-	-

Again the result is an increase of at least ten times the sensitivity by applying the Faraday-Tundall effect.

V. REACTION OF 2.1 NITROSONAPHTHANOL WITH COBALT IONS

Also with this reaction, very sensitive and highly specific, we have had a publication on another occasion ([7]).

It was started with a solution of cobalt sulphate 10^{-3}N which was successively diluted up to 10^{-7}N.

The reagent is 2,1 nitrosonaphthol at 1 % p/v in ethyl alcohol. The reaction was carried out on 3 ml. of the solutions of the cobalt salt, adding 5 drops of the reagent 2,1 nitrosonaphthol and alkalizing with 5 drops of NaOH N.

Simultaneously the corresponding blank test was carried out under the same conditions, but in the absence of cobalt salts.

Results

	P_{SE}[1]	Direct observation	Tyndall
Blank		-	-
	8	-	(+)(very weak)
	7	-	- (weak)
	6	-	+
	5	-	+
	4	reddish	+
	3	reddish	+

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University of Buenos Aires
School of Pharmacy and Biochemistry
Dept. of Physicochemistry
General Chemical Orientation.

[4]In this case the term P_{SE} refers to the cologarithmic expression of the normality of the solution of heptahydrated cobalt sulphate solution used.

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dificación roja de selenio que se sedimenta paulatinamente; siguiendo el calentamiento, el color vira lentamente de rojo a gris, es decir a la modificación estable del selenio.

Para la determinación de la sensibilidad de esta reacción seguimos la técnica corriente: Se preparó una solución de sorbato de sodio neutralizando una suspensión acuosa de 100 mg de ácido sórbico con hidróxido de sodio 0,1 N usando la fenolftaleína como indicador externo; la solución de sorbato de sodio se llevó a 100 ml en un matraz aforado empleando agua pura como disolvente; por lo tanto, cada ml de la disolución contiene 1 mg de ácido sórbico. Por dilución de estas soluciones madres se obtuvieron diluciones de concentracción definida.

El reactivo es una solución de selenito de sodio al 5 % p/v. La reacción se efectúa mezclando en tubos de ensayo 1 ml de la dilución de sorbato de sodio con 1 ml de reactivo. En estas condiciones no se produce ninguna reacción. A continuación se agregó 1 ml de ácido clorhídrico concentrado y se calentó, previa homogeneización, durante 30 minutos a baño maría hirviente, observándose el cuadro descripto anteriormente.

La solución madre (1:1000) y su dilución 1:10.000 dieron reacciones netamente positivas, pero no las diluciones mayores; deducimos que las diluciones de cociente elevado podrían producir la separación de selenio elemental, en forma coloidal que se escapa a la observación directa, lo que se confirmó aplicando el fenómeno Farady-Tyndall a los ensayos en observación [2].

En todos los casos se practicaron los correspondientes ensayos en blanco, tratando 1 ml de agua destilada con 1 ml del reactivo y 1 ml de ácido clorhídrico concentrado, calentando durante 30 minutos a baño maría hirviente.

El dispositivo óptico usado es de construcción sencilla; en un microproyector se produce un haz de luz blanca y paralelo que atraviesa la solución problema.

En los ensayos en blanco, el medio es óptimamente limpio; en cambio, en presencia de vestigios de selenio formado en la reducción del selenito por la acción de ácido sórbico u otros reductores, se produce nítidamente el fenómeno Faraday-Tyndall. Se observa un sensible aumento de la sensibilidad de la reacción. En la dilución $1:10^5$ el resultado es netamente positivo, pero débil en la dilución $1:10^6$ y detectable únicamente en comparación con el ensayo en blanco.

En los ensayos realizados con altas diluciones los tubos deben taparse para evitar la contaminación con el aire que podría producir falsos resultados positivos.

Hemos estudiado la misma reacción empleando como reactivo en lugar del selenito, el telurito de sodio; la reacción se realizó en medio sulfúrico. A 1 ml de la solución de sorbato sódico de dilución conocida se agregó 1 ml de telurito de sodio al 10 % p/v y 1 ml de ácido sulfúrico concentrado. A continuación se calienta a baño maría hirviente durante 30 minutos. En las reacciones positivas aparece una coloración grisácea que sedimenta paulatinamente. La reacción es menos sensible que con el selenito como reactivo

A simple vista solamente se observa reacción positiva hasta la dilución $1:10^3$, y aplicando el ensayo Faraday-Tyndall hasta la dilución $1:10^4$. Lógicamente, el fenómeno Faraday-Tyndall podemos aplicarlo solamente en los ensayos analíticos de precipitación; pero aún así no carece de valor en la Química Analítica Cualitativa teniendo en cuenta el apreciable aumento de la sensibilidad. Este hecho se hace evidente en la reacción de tiosulfato de sodio con distintos tipos de ácidos y de diferentes concentraciones. Se usaron soluciones de tiosulfato de sodio (1 ml al 0,1 %, 1 % y 10 % p/v) agregando 1 ml de ácido y midiendo el tiempo hasta formación de azufre coloidal. t:18° C. En todos los casos se observa con el efecto Faraday-Tyndall la separación de azufre coloidal con anterioridad que a la observación a simple vista.

$Na_2S_2O_3$	Efecto Tyndall	Observación directa
	HCl N	
0,1 %	10 min.	20 min.
1,0 %	30 seg.	1 min. 27 seg.
10,0 %	8 seg.	8 seg.
	HCl 0,1 N	
0,1 %	18 min.	30 min.
1,0 %	1 min.	2 min. 30 seg.
10,0 %	10 seg.	10 seg.

$Na^2S_2O_3$	Efecto Tyndall	Observación directa
	CH_3COOH N	
0,1 %	35 min.	5 h. 30 min.
1,0 %	1 min. 55 seg.	5 min.
10,0 %	12 seg.	12 seg.
	CH_3COOH 0,1 N	
0,1 %	4 h.	—
1,0 %	3 min.	10 min.
10,0 %	15 seg.	40 seg.

Por lo tanto, el tiempo de observación depende de las concentraciones de las soluciones empleadas y de la fuerza de los ácidos.

En la última serie de observaciones (CH_3COOH 0,1 N) no se consignó el valor para el tiosulfato de sodio 0,1 % a "observación directa" por ser excesivamente grande. En los ensayos con tiosulfato de sodio de elevada concentración (10 %), los valores de las tres primeras series de observación son prácticamente idénticos para la "observación directa" y el "Efecto Faraday-Tyndall".

Con el objeto de simplificar la terminología para expresar la sensibilidad (concentración límite) de reacciones cualitativas en diferentes diluciones de una solución madre, creemos conveniente sustituir la denominación convencional por términos logarítmicos (P_{SE}), similar al procedimiento seguido para los valores de pH, es decir:

Dilución	P_{SE}
1 : 100	2
1 : 1.000	3
1 : 10.000	4, etc.

Al transcribir esta publicación nos informamos que la expresión logarítmica de la sensibilidad encontró apoyo de otros autores; Burriell-Martí y Ramírez-Muñoz [3] la aplican especialmente en la fotometría de llama, empleando el símbolo pD. Sin embargo preferimos el símbolo P_{SE} para evitar confusiones con términos si-

milares. Hemos extendido el estudio a algunas reacciones ya conocidas con el fin de conocer el alcance y valor del fenómeno descripto.

I. Precipitación de haluros de plata

Preparamos soluciones acuosas al 1 por mil p/v de cloruro de potasio, bromuro de sodio y yoduro de potasio; empleando 0,6 ml de dichas diluciones. Se agregó 1 gota de ácido nítrico concentrado (libre de halogenuros) y 3 ml de nitrato de plata 10^{-5} N. Aplicando el efecto Faraday-Tyndall es posible visualizar la precipitación de los correspondientes haluros de plata con anterioridad a las observaciones realizadas a simple vista.

II. Precipitación de sulfatos alcalinotérreos

En estos casos se confirman ampliamente los resultados de la serie anterior. Se prepararon soluciones madres al 1 % p/v de cloruro de calcio, nitrato de estroncio y cloruro de bario que se diluyeron con agua en forma conveniente. En cada experiencia se emplearon 3 ml de las soluciones de los iones alcalinotérreos en las cuales se agregó en cada caso 0,6 ml de ácido sulfúrico 5 N:

	P_{SE}	Observación directa	Tyndall
Cl_2Ca	2	—	+
$(NO_3)_2Sr$	2	+	+
$(NO_3)_2Sr$	4	—	+
Cl_2Ba	2	+	+
	4	+	+
	6	—	+
	7	—	+

III. Hipofosfitos y fosfitos con selenito de sodio en presencia de ácido clorhídrico concentrado

La reacción cualitativa ya se estudió en un trabajo anterior ([4]).

Se prepararon soluciones madres de fosfito de sodio y hipofosfito de sodio al 1 % p/v de las cuales se obtuvieron diluciones acuosas de concentración definida.

Un ml de cada dilución se mezeló con 1 ml de selenito de sodio al 5 % p/v y 1 ml de ácido clorhídrico concentrado; después de homogeneizar se calentó 30 minutos a baño maría hirviente.

Resultados

P_{SE}	Observación directa	Tyndall
	Hipofosfito	
4	+	+
5	—	+
6	—	+ (débil)
7	—	—
	Fosfito	
3	+	+
4	—	+
5	—	+ (débil)
6	—	—

En las dos series, se nota la superioridad de las observaciones mediante aplicación con el efecto Faraday-Tyndall.

Como ya se describió en otra parte, la reacción es de sensibilidad inferior con el telurito de sodio en medio sulfúrico ([5] y [6]).

A un ml de las soluciones acuosas de fosfito de hipofosfito respectivamente, se agregó 1 ml de solución de telurito de sodio al 10 % p/v y luego 1 ml de ácido sulfúrico concentrado; se calentó las mezclas durante 30 minutos a baño maría hirviente.

P_{SE}	Observación directa	Tyndall
	Hipofosfito	
3	+	+
4	—	+
5	—	—
	Fosfito	
2	+	+
3	—	+
4	—	—

Resultados

P_{SE} [1]	Observación directa	Tyndall
Blanco	—	—
8	—	(+) (muy débil)
7	—	– (débil)
6	—	+
5	—	+
4	rojizo	+
3	rojizo	+

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Universidad de Buenos Aires
Facultad de Farmacia y Bioquímica
Dto. de Fisicoquímica
Orientación Química General

[1] En este caso el término P_{SE} se refiere a la expresión cologarítmica de la normalidad de la solución de sulfato de cobalto heptahidratado utilizado.

Unid. Danish pub., pages 1107-1111

Microbiological method for determination of sorbic acid

By C. Trolle-Lassen

Report from the Laboratory of Danmarks Apotekforeningen, Copenhagen

Sorbic acid can be determined quantitatively in various ways, both physical and chemical. As examples of these it can be stated that Melnick & Luckmann (1954) and Luckmann & Melnick (1955), use a spectrophotometric method in ultraviolet light, Diemair et al (1957) use a colorimetric measurement on a reaction product, and that sorbic acid can be determined by an acidimetric or iodometric titration of the acid group or the ethylene bonds, respectively. These methods, however, are more or less unspecific, linked as they are to certain groups in the molecule.

There is a need for a microbiological method to supplement these mentioned above. Such a method will, for example, on the one hand provide information on the growth-inhibiting properties of a sorbic acid sample, and on the other hand it will permit determination of active sorbic acid in mixtures in which a physical or chemical method would require prior, difficult, perhaps impracticable isolation.

Attempts to work out an agar plate technique yielded unsatisfactory results. On the one hand, the sorbic acid concentrations in the test solutions had to be too high, and on the other hand the zone limits were not sharp. The turbidimetric method described below, did, however, meet our requirements.

As a rule, turbidimetric methods require a thoroughly asceptic technique, which means that these methods are difficult, and relatively costly. In the method discussed here, however, it is possible to restrict the sterilization work because the hydrogen ion concentration of the substrate is so high

that it excludes bacterial growth.

Below the method is presented in its final form, followed by a discussion of the research behind it.

As substrate, malt water was used, in which the pH = 4, established with the aid of citric acid (12.9 g $C_6H_8O_7$, H_2O per liter) and sodium phosphate (13.7 g Na_2HPO_4, 2 H_2O per liter). For production of "diagonal" (crushed) agar, we added 2.5% agar to this substrate.

For test organism we used saccharomyces cerevisiae (2147.2; Alfred Jørgensens Gaeringsfysiologiske Laboratorium), which is kept on diagonal agar. Regrafting takes place at least every 6 months, followed by incubation at about 37° for 1-2 days. The graft culture is produced by dissemination in liquid substrate and incubation at about 37° for 1 - 2 days. The said incubation may optionally be undertaken at room temperature, when the incubation time is extended correspondingly.

For the same solutions (standard and test solutions) we used concentrations of between 50 gamma and 150 gamma of sorbic acid per ml dissolved in the liquid substrate. A suitsble interval of concentrations is 10-20 gamma/ml.

For the research we used preparation glasses containing 10 ml of the sample liquids. These were inoculated at the start with a drop of the inoculation culture, except for a blind sample, which serves as a comparison glass in the measurements. The glasses were incubated for 3 days at 25°, after which the extinction in each individual glass was measured relative to the blind sample. We used a particular colorimeter with a suitable filter (for example, yellow-red).

In a suitable distribution of sample solutions we used 3 identical rows of glasses with 5-7 concentrations in each row, both in the standard and the test research.

The measurements from the standard research were plotted on a coordinate system and the concentrations in the test research, falling within this measurement range were read off, thereby calculating the sorbic acid content of the test substance.

Test organism

As test organism we selected the abovementioned yeast stock, although other yeasts could be used with equally good results. The choice of the yeast for this purpose was made partly because this micro-organism is extremely sensitive to sorbic acid and partly because the growth takes place in a way that easily yields a homogenous cell suspension with a suitable density for the measurements (cf. fig. 1). This also means that the yeast can grow in substrates which, owing to substances in the contents, temperature and hydrogen ion concentration, offer poor growth conditions for bacteria.

Fig. 1 - The growth of micro-organisms at pH 4 in media containing various concentrations of sorbic acid (0-150 gamma/ml). The experiments were carried out for 1, 2, 3 and 4 days at 25° and 37° C. The growth in the tubes containing 10 ml of medium was measured turbidimetrically and expressed as extinction values. (E. 1 cm).

Substrate

As substrate, malt water proved to be very appropriate. When the hydrogen ion concentration was established in the medium, there were various factors to consider. Of essential importance is the fact that the growth inhibiting effect of sorbic acid is linked to the free acid, while the anion is almost without effect in this respect. (Trolle-Lassen, 1958). This means that the pH of the substrate must be established at such an acid reaction that all, or nearly all of the quantity of sorbic acid present in the sample solutions appears in the undissociated form. And for the same reason, the reaction must be precisely identical in all the solutions (cf. fig. 2).

Aside from this it is also important that the growth of the test organism be satisfactory. In illustration of this we undertook growth studies in substrates with various pH values, and the results of these appear in fig. 3. On the basis of these results, and the considerations in the foregoing section, the pH of the substrate was established at 4.

Fig. 2 The theoretical distribution of undissociated sorbic acid, and the corresponding anion in solutions with different pH values. 86% is found as the acid form at pH 4.

Fig. 3. The growth of micro-organisms at different pH values after incubation for 1, 3 and 5 days at 25°C. The growth in the tubes containing 10 ml of medium was measured turbidimetrically and expressed as extinction values. (E. 1 cm).

Research time and temperature

In fig. 1 we see the results of various studies with variations in time and temperature. From this we can see that the dose-response curves at 25° are straighter than at 37°, and since with a study time of 3 days (25°) we obtain a practically linear relationship, this time period was selected for the studies.

Turbidimetry

Measurements of light absorption in the individual glasses were made with a filter colorimeter (Biochem., Hilger). The particular absorption of the substrate is relatively high, and an equalization of this as a possible source of error, should therefore be carried out with the use of filters, if the absorption range covers that of the substrate as much as possible.

Precision of research

To enhance the precision of the method, two studies were conducted in which twice 50 glasses with, respectively, 25 gamma and 125 gamma sorbic acid

per ml were inoculated and incubated. From the measurements in the two studies we calculated the average value ($m_{25\ gamma/ml} = 0.426$ E and $m_{125\ gamma/ml} = 0.204$ E) and the spreads ($s_{25\ gamma/ml} = 0.034$ E and $s_{125\ gamma/ml} = 0.032$ E) for the extinction values. It was thus found that the spreads were independent of the concentrations in the measurement solutions, which means that the method works with greater relative precision at the high concentrations than at the low.

We calculated, with the aid of the slope of the measurement curve $\frac{(0.426 - 0.204)}{(125 - 25)}$ the spread of the extinctions (s = 0.033 E) until the spread of the concentrations was obtained s = 14.7 gamma/ml.

With 3 . 7 = 21 glasses in both standard and test rows) we obtained an average error of $e = \frac{14.7}{\text{square root of } 21}$. square root of 2 = 4.5, and a research uncertainty (p = 0.05) of ± 1.96 . 4.5 = ± 8.8 gamma/ml, corresponding to ± 35% at the concentration of 25 gamma/ml, ± 12% at 75 gamma/ml and ± 7.0% at 125 gamma/ml.

By reducing or increasing the number of glasses in the experiment to, for example, 3 . 5 = 15 glasses or 3 x 10 = 30 glasses, we obtained, for the concentration 75 gamma/ml, an uncertainty of respectively ± 1.96 . 5.4 = ± 10.6 gamma/ml approximately ± 14% and ± 1.96 . 3.8 = ± 7.5 gamma/ml approximately ± 10%.

It is clear from these calculations that the research should be carried out at concentrations which do not lie too far beyond the range of 75-125 gamma/ml.

(For Summary in English, and Bibliography, see page 1111.

Translated by Carl Demrick Associates, Inc./GCT/db

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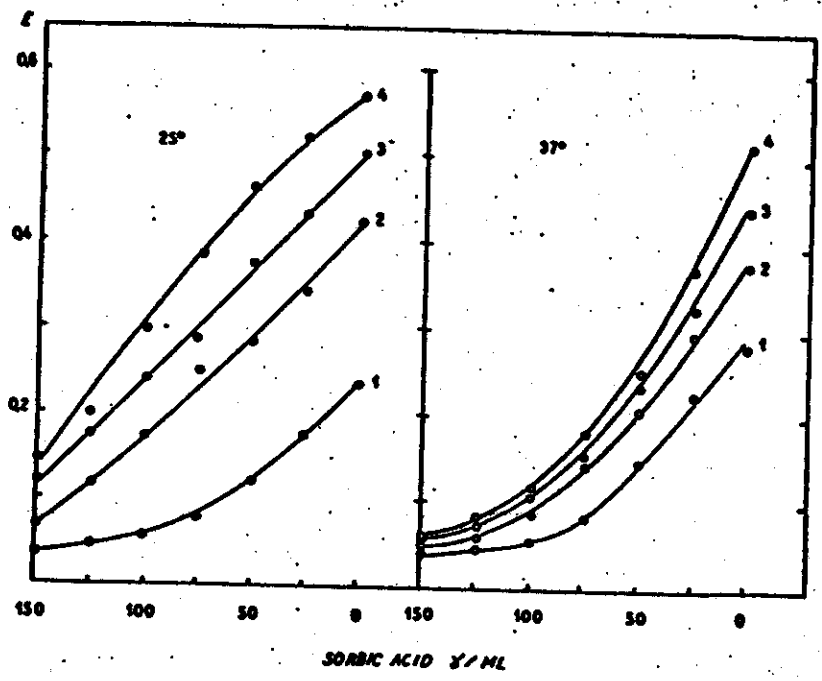


Figure 1.

The growth of microorganisms at pH 4 in media containing various concentrations of sorbic acid (0-150 γ/ml.). The experiments were carried out for 1, 2, 3, and 4 days at 25° C and 37° C. The growth in the tubes containing 10 ml. medium was measured turbidimetrically and expressed as extinction values. (E. 1 cm.).

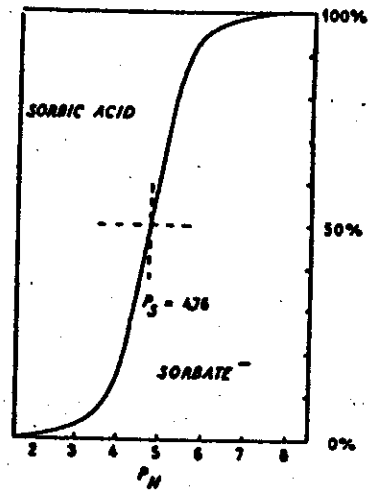
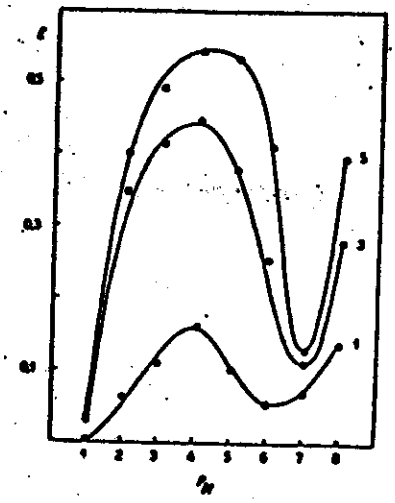


Figure 2.

The theoretical distribution of undissociated sorbic acid and the corresponding anion in solutions with different pH values. 86 per cent is found as the acid form at pH 4.



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The growth of microorganisms at different pH values after incubation for 1, 3, and 5 days at 25° C. The growth in the tubes containing 10 ml. medium was measured turbidimetrically and expressed as extinction values. (E. 1 cm).

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Sorbic Acid
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SUMMARY

From the laboratory of Danmarks Apotekerforening, Copenhagen

The principal basis for the test sample control of the DAK-Laboratory

By Gunnar Hansen

The DAK-Laboratory (The Control Laboratory of the Danish Pharmacies) has commenced, on a test sample basis, an analytical control on galenicals produced or bought by the pharmacies. In the paper there is given an account of some of the considerations underlying the analytical methods chosen. In broad terms is further discussed what information of fundamental nature about drugs and their quality one may expect from an analytical control of this character.

Meddelelse fra Danmarks Apotekerforenings Kontrollaboratorium

Mikrobiologisk metode til bestemmelse af sorbinsyre

Af C. Trolle-Lassen

Sorbinsyre kan bestemmes kvantitativt på adskillige måder, såvel fysiske som kemiske. Som eksempler herpå kan nævnes at *Melnick & Luckmann* (1954) og *Luckmann & Melnick* (1955) anvender en spektrofotometrisk metode i ultraviolet lys, at *Diemair et al.* (1957) bruger en kolorimetrisk måling på et reaktionsprodukt, samt at sorbinsyre kan bestemmes ved en acidimetrisk eller ved en jodometrisk titrering af henholdsvis syregruppen eller ætylenbindingerne. Disse metoder er imidlertid mere eller mindre uspecifikke, bundet som de er til visse grupper i molekylet.

Der savnes en mikrobiologisk metode til supplement af de ovenfor nævnte. En sådan metode vil f. eks. dels kunne give oplysninger om en sorbinsyreprøves væksthæmmende egenskaber, og dels vil den tillade bestemmelse af aktiv sorbinsyre i blandinger, hvor en fysisk eller kemisk metode ville kræve en forudgående besværlig, eventuelt uigennemførlig, isolering.

Forsøg med udarbejdelse af en agarpladeteknik gav utilfredsstillende resultater. Dels krævedes for høje sorbinsyrekoncentrationer i prøveopløsningerne, og dels var zonegrænserne uskarpe. Den nedenfor beskrevne turbidimetriske metodik fungerede derimod efter ønske.

Metode

Almindeligvis kræver de turbidimetriske metoder en gennemført aseptisk teknik, som bevirker, at disse metoder bliver besværlige og temmelig kostbare. Ved den her omtalte metodik er det imidlertid muligt at indskrænke steriliseringsarbejdet, fordi substratets brintionkoncentration er så høj, at den udelukker vækst af bakterier.

Nedenfor er metoden angivet i sin endelige udformning, efterfulgt af en omtale af de forsøg, der ligger til grund herfor.

Som substrat anvendes maltvand, hvori pH = 4 er fastlagt ved hjælp af citronsyre (12,9 g $C_6H_8O_7$, H_2O pr. l) og natriumfosfat (13,7 g Na_2HPO_4, 2 H_2O pr. l). Til fremstilling af skråagar tilsættes dette substrat 2,5 % agar.

Som testorganisme anvendes *saccharomyces cerevisiae* (2147,2; *Alfred Jørgensens Gæringsfysiologiske Laboratorium*), der opbevares på skråagar. Ompodning finder sted mindst hver 6. måned, efterfulgt af inkubering ved ca. 37° i 1-2 døgn. Podekulturen fremstilles ved udsåning i flydende substrat og inkubering ved ca. 37° i 1-2 døgn. De nævnte inkuberinger kan eventuelt foretages ved stuetemperatur, når inkuberingstiden samtidig forlænges.

Til prøveopløsningerne (standard- og testopløsningerne) anvendes koncentrationer i området mellem 50 γ og 150 γ sorbinsyre pr. ml opløst i det flydende substrat. Et passende spring mellem koncentrationerne er 10-20 γ/ml.

Til forsøget anvendes præparatglas indeholdende 10 ml af prøvevæskerne. Disse podes ved starten med en dråbe af podekulturen, når undtages en blindprøve, der tjener som sammenligningsglas ved målingerne. Glassene inkuberes i 3 dage ved 25°, hvorefter ekstinktionen (event. E, 1 cm) i hvert enkelt glas måles overfor blindprøven. Der anvendes et egnet kolorimeter med et passende filter (f. eks. gult-rødt).

I en formålstjenlig fordeling af prøveopløsningerne benyttes 3 ens rækker glas med 5-7 koncentrationer i hver række, både i standard- og i testforsøget.

Målingerne fra standardforsøget indtegnes i et koordinatsystem, og koncentrationerne i testforsøget, der falder indenfor dette måleområde, aflæses, hvorpå teststoffets sorbinsyreindhold beregnes.

Testorganisme

Som testorganisme valgtes den ovenfor nævnte gærstamme, men andre gærarter vil formodentlig kunne bruges med lige så godt resultat. Valget af gær til dette formål skyldes dels, at denne mikroorganisme er yderst følsom overfor sorbinsyre og dels, at væksten foregår på en måde, der let giver en homogen cellesuspension med en for målingerne passende tæthed

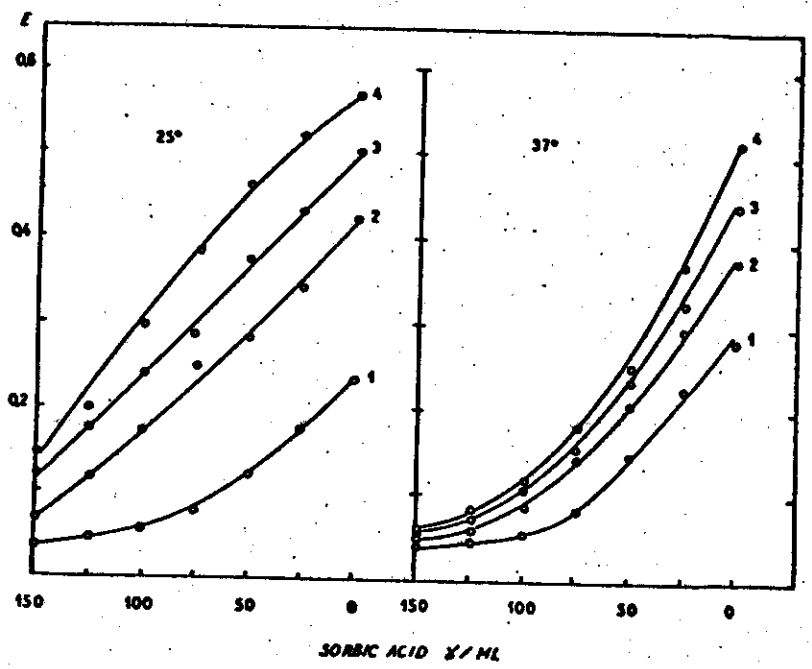


Figure 1.

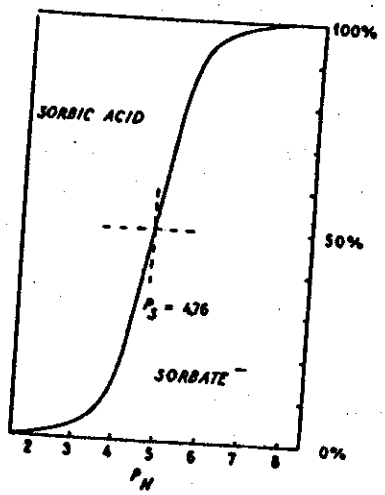
The growth of microorganisms at pH 4 in media containing various concentrations of sorbic acid (0-150 γ/ml.). The experiments were carried out for 1, 2, 3, and 4 days at 25° C and 37° C. The growth in the tubes containing 10 ml. medium was measured turbidimetrically and expressed as extinction values. (E. 1 cm.).

(jvnf. fig. 1). Dertil kommer så, at gær kan vokse i substrater, som med hensyn til indholdsstoffer, temperatur og brintionkoncentration byder på dårlige vækstforhold for bakterier.

Substrat

Som substrat viste maltvand sig at være velegnet. Ved fastlæggelsen af brintionkoncentrationen i mediet var der forskelligt at tage hensyn til. Af essentiel betydning er det, at sorbinsyres væksthæmmende virkning er knyttet til den fri syre, mens anionen nærmest er inaktiv i så henseende (*Trolle-Lassen,* 1958). Dette medfører at substratets pH-værdi må fastlægges ved så sur en reaktion, at hele eller så godt som hele sorbinsyremængden i prøveopløsningerne optræder i den udissocierede form. Endvidere må af samme årsag reaktionen være nøjagtig ens i alle opløsningerne (jvnf. fig. 2).

Herudover er det også af betydning, at testorganismens vækst er tilfredsstillende. Til belysning heraf foretoges vækstforsøg i substrater med forskellige pH-værdier, og resultaterne herfra fremgår af figur 3. På baggrund af disse resultater og overvejelserne i forrige afsnit fastlagdes substratets pH-værdi ved 4.



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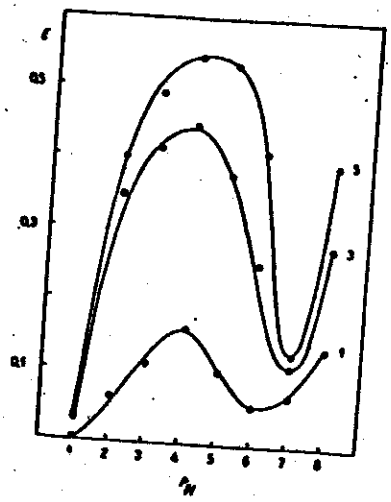

Figure 3.

The growth of microorganisms at different pH values after incubation for 1, 3, and 5 days at 25° C. The growth in the tubes containing 10 ml. medium was measured turbidimetrically and expressed as extinction values. (E. 1 cm).

Forsøgstid og forsøgstemperatur

I figur 1 er resultaterne fra nogle forsøg med variationer i tid og temperatur angivet. Det fremgår heraf, at dosis-virkningskurverne ved 25° er mindre krumme end ved 37°, og da der ved en forsøgstid på 3 dage (25°) opnåedes en så godt som lineær afhængighed valgtes dette tidsrum til forsøgene.

Turbidimetri

Måling af lysabsorptionen i de enkelte glas foretoges med et filterkolorimeter (*Biochem, Hilger*). Substratets egenabsorption er temmelig stor, og en udligning af dette som en eventuel fejlkilde bør derfor ske ved anvendelse af filtre, hvis absorptionsområde så vidt muligt dækker substratets.

Forsøgsnøjagtighed

Til vurdering af metodens nøjagtighed udførtes 2 forsøg hvor 2 gange 50 glas med henholdsvis 25 γ og 125 γ sorbinsyre pr. ml podedes og inkuberedes. Fra målingerne i de to forsøg beregnedes middeltal ($m_{25\gamma/ml}$ = 0,426 E og $m_{125\gamma/ml}$ = 0,204 E) og spredninger ($s_{25\gamma/ml}$ = 0,034 E og $s_{125\gamma/ml}$ = 0,032 E) for ekstinktionsværdierne. Det viste sig herved, at spredningerne var uafhængige af koncentrationerne i måleopløsningerne, hvilket betyder

at metoden arbejder med større relativ nøjagtighed ved de høje koncentrationer end ved de lave.

Omregnes ved hjælp af målekurvens hældning $\left(-\frac{0{,}426-0{,}204}{125-25}\right)$ spredningen på ekstinktionerne ($s = 0{,}033$ E) til spredningen på koncentrationerne fås $s = 14{,}7\,\gamma/ml$.

Med $3 \cdot 7 = 21$ glas i både standard- og testrækken fås en middelfejl på $e = \frac{14{,}7}{\sqrt{21}} \cdot \sqrt{2} = 4{,}5$, og en forsøgsusikkerhed ($p = 0{,}05$) på $\pm 1{,}96 \cdot 4{,}5 = \pm 8{,}8\gamma/ml$, svarende til $\pm$ 35 % ved koncentrationen 25γ/ml, $\pm$ 12 % ved 75γ/ml og $\pm$ 7,0 % ved 125γ/ml.

Ved at nedsætte eller øge antallet af glas i forsøget til f. eks. $3 \cdot 5 = 15$ glas eller $3 \times 10 = 30$ glas, fås for koncentrationen 75 γ/ml en usikkerhed på henholdsvis $\pm 1{,}96 \cdot 5{,}4 = \pm 10{,}6\ \gamma/ml \sim \pm 14$ % og $\pm 1{,}96 \cdot 3{,}8 = \pm 7{,}5\ \gamma/ml \sim \pm 10$ %.

Det fremgår af disse beregninger, at forsøget bør udføres med koncentrationer der ikke ligger for langt udenfor området 75-125 γ/ml.

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SORBIC ACID

Sorbic acid (*trans,trans*-2,4-hexadienoic acid), $CH_3{-}CH{=}CHCH{=}CHCOOH$, is a white crystalline solid. It is a naturally occurring product which was first obtained from the oil of the rowanberry (sorbapple or mountain ash berry) in 1859 by treatment with alkali or mineral acid.

Sorbic acid and its water-soluble potassium salt (collectively referred to as sorbates) are of major commercial significance as preservatives for foods and, to a lesser extent, for drugs and cosmetics. Following the initial recommendations of Gooding (1) in 1945, the sorbates have been adopted in a wide range of consumer products where the inhibition of yeast, mold, and certain bacterial growth is essential to the safe and economical marketing of the products. The use of the sorbates parallels, and in many food categories, dominates the applications historically served by other organic carboxylate acids and salts, such as sodium and calcium propionate, sodium benzoate, and sodium diacetate.

Properties and Reactions

The solubility of sorbic acid in water increases from 0.15% at 20°C to 3.8% at 100°C, hence water is a convenient medium for purification by recrystallization. The comparative solubilities of sorbic acid and potassium sorbate are shown in Table 1.

Table 1. Solubilities of Sorbic Acid and Potassium Sorbate at 20°C, g/100 g soln

Solvent	Sorbic acid	Potassium sorbate
acetic acid, glacial	11.5	
acetone	9.2	0.10
benzene	2.3	< 0.01
carbon tetrachloride	1.3	< 0.01
cyclohexane	0.28	
ethanol, anhydrous	12.9	2.0
20%	0.20	54.6
ethyl ether	5.0	0.1
glycerol	0.31	0.2
methanol	12.9	16
cottonseed oil	1.0	0.01
propylene glycol	5.5	24
sodium chloride, 15%	0.038	15
water	0.15	58.2

The preferred form of the preservative for inclusion in foods normally depends upon the method of addition, ie, the more soluble salt form is selected for spray, dip, or solution applications and the acid for dusting and dry-blending.

Other physical properties of sorbic acid and potassium sorbate are given in Table 2.

Sorbic acid is a doubly unsaturated aliphatic acid. The conjugated unsaturation and carboxylic groups define the nature of its reactivity. As an acid, sorbic acid forms salts readily by reaction with alkalis. Esters, amides, and the acid chloride are formed in the conventional manner (2,3).

The addition reactions of sorbic acid and of its carboxyl derivatives are complex because of the different modes of addition to the unsaturated positions. Partial hydrogenation (reduction) and bromination, for example, can occur at different

Table 2. Physical Properties of Sorbic Acid and Potassium Sorbate

Property	Sorbic acid	Potassium sorbate
boiling point, °C		
760 mm	decomposes	
50 mm	143	
10 mm	119[a]	
melting point, °C	134.5	decomposes over 270°C
density (g/ml), 20°C		1.360
vapor pressure,[a] mm Hg		
20°C	<0.01	
120°C	10	
140°C	43	
flash point,[b] °C	126	
ionization constant at 25°C	1.73×10^{-5}	

[a] Solid–vapor equilibrium. [b] Cleveland open-cup tester, ASTM D-92.

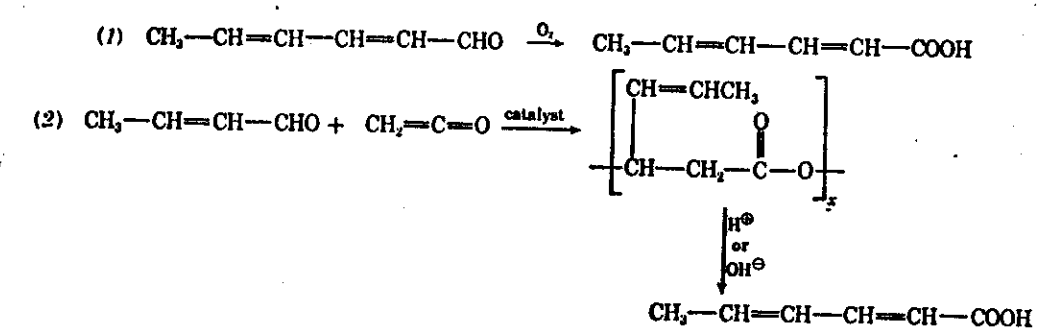
positions in the molecule, depending on the reaction condition and catalysts. Sorbic acid undergoes the Diels-Alder reaction with many dienophiles (styrene, maleic anhydride, acrylates, etc) (4,5). In many cases, the products are complex mixtures owing to the different modes of addition, shifting of double bonds, and other rearrangements and isomerizations.

Sorbic acid polymerizes readily when catalyzed by free radicals. Copolymers with other unsaturated monomers, including butadienes, vinyls, and acrylics, have been studied. No commercial uses of significant volume have been developed for sorbic acid polymers, primarily because of the relatively high cost of the sorbic acid.

Oxidizing agents attack sorbic acid and its salts and esters at the olefinic bonds. Autoxidation results in formation of peroxides, followed by degradation and polymerization. Of the various oxidizing agents, only ozone attacks both double bonds. Peracetic acid gives a 4,5-dihydroxy acid, which yields fumaric acid upon further oxidation.

Manufacture

Sorbic acid is produced commercially by two different processes, the oxidation of 2,4-hexadienal, and the reaction of ketene and crotonaldehyde, through a polymeric ester.

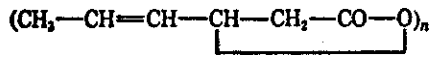


Oxidation of Hexadienal. 2,4-Hexadienal is obtained by the condensation of three molecules of acetaldehyde. This synthesis, and the synthesis by the condensation of acetaldehyde and crotonaldehyde, have been well known for many years, but

the yields were low, and large amounts of the branched isomer (2-vinyl-2-butenal) and higher condensation products were formed and could be separated only with difficulty. The present commercial process results in good yields of 2,4-hexadienal with very little formation of the branched isomer (6,7).

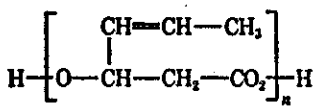
The oxidation of 2,4-hexadienal is effected with air as the oxidizing agent by using specially developed equipment and procedures. Conventional methods of oxidizing aldehydes are not satisfactory in the case of hexadienal because of peroxidation of the ethylenic bonds and polymerization. In the commercial process the oxidation is carried out continuously in a solvent, and in the presence of an oxidation catalyst, under precisely controlled conditions in a manner such that the aldehyde is converted to sorbic acid rapidly and completely.

Ketene–Crotonaldehyde Route. One of the first commercial methods (8) for making sorbic acid was the reaction of ketene and crotonaldehyde in the presence of a Lewis acid catalyst, preferably boron trifluoride (9,10). This reaction yielded a polymeric product, identified as poly(hexeno-β-lactone):



The polymeric lactone was converted to sorbic acid by boiling with an aqueous solution of hydrochloric acid.

A more recent modification of the basic process employs as catalysts carboxylic salts of bivalent transition metals (eg, zinc isovalerate) in the ketene–crotonaldehyde reaction (11). As with acid catalysts, a polymeric intermediate is obtained; using more modern analytical techniques, the polymer has been identified as a polyester of 3-hydroxy-4-hexenoic acid (12):



Many foreign patents have been issued for processes using the ketene–crotonaldehyde route to sorbic acid; most of the syntheses use zinc salts as catalysts.

The polyester obtained from this reaction can be isomerized into sorbic acid using several different types of catalysts and techniques. Patents have been issued on processes using alkali metals (11), alkalis (13), strong inorganic acids (10,14), phosphoric acid (15), or *p*-toluenesulfonic acid (16). A wide variety of yields are obtained, depending upon the catalyst used in the depolymerization–isomerization step as well as in the reaction step.

Purification and Specifications

Sorbic acid and its salts obtained by the previously described processes must be refined to a high degree of purity for use as a fungistatic agent in foods, the principal commercial use for these products. Purification can be achieved by recrystallization of the acid from water or mixed solvents. Another method of purification is by codistillation under vacuum with an organic solvent having the same general distillation range (17). Food-grade sorbate salts are prepared from the purified acid. Sorbic acid and potassium sorbate are now defined by Food Chemicals Specifications (18) having the quality requirements shown in Table 3.

Sorbic acid is routinely assayed in foods using a spectrophotometric method described by Alderton and Lewis (19). In foods where interfering substances absorb in the 260-mμ range, Ciaccio (20) selectively oxidizes the sorbic acid after determination of the optical density and computes the sorbic content by difference. Where a spectrophotometer is not available, the bromine titrimetric method of Spanyar and Sandor (21) may be used or acidimetry (22) when other acids are not present. The thiobarbituric acid (TBA) method of Schmidt (23) is also used in the quantitative determination of sorbic acid. There are also several qualitative methods for detecting sorbates. An excellent review on this entire subject may be found in an article by Schuller and Veen (24).

Table 3. *Food Chemicals Codex* Specifications for Sorbic Acid and Potassium Sorbate

Specification	Sorbic acid	Potassium sorbate
melting point, °C	133–137	
water, wt %, max	0.5	1.0
purity by titration, wt %	99–101	98–102
heavy metals, ppm, max	10	10
ash, %, max	0.2	
arsenic, ppm, max	3	3

Use of Sorbic Acid and Its Salts

The major commercial use of sorbic acid and its alkali salts is as a fungistatic agent in foods. The use of sorbic acid for this application was patented initially in 1945 (1) and has been the subject of additional patents in many different food areas (25–31). Extensive studies related to its effectiveness in various foods, its toxicity, metabolism of fungistatic action, fate in foods, methods of application, and its analytical methodology have been undertaken. An excellent, although dated, bibliography of these studies may be found in an article by Melnick (32) and in a more recent bulletin put out by Chas. Pfizer & Co., Inc. (33).

The effectiveness of sorbic acid as a fungistatic agent is attributed to its α,β-unsaturated character and is undoubtedly enhanced by its fatty acid structure. In normal saturated fatty acid oxidation, essential to fungal metabolism, dehydrogenation occurs at the α,β position, forming an unsaturated fatty acid (β-oxidation pathway). The dehydrogenase enzyme system required for this reaction is inhibited by an excess of unsaturated fatty acids (eg, sorbic acid), the initial product of the β-oxidation pathway. As these products accumulate, they inhibit the dehydrogenase enzyme system, thereby significantly depressing fatty acid metabolism. This stops the growth of the microorganisms, but does not necessarily exert a lethal effect on them. In high mold populations, sorbic acid is not able to inhibit the highly active enzyme system and serves as a metabolite for molds (34), or is degraded to a harmless product (35).

Sorbic acid inhibits the growth of most fungi and a few of the bacteria normally associated with food spoilage. Its activity, as previously stated, is entirely static with respect to fungi and selected bacteria. Its use in a liver infusion media has been reported as a means of selective growth of catalase-negative lactic acid bacteria and *Clostridia* sp, where it inhibited the growth of catalase-positive actinomycetes, bacteria, molds, and yeasts in acidic media (pH 5.0, 5.5) in the absence of phosphate salts (36).

The antimicrobial activity of sorbic acid is enhanced as the pH decreases. Bandelin (37) has shown that as the pH decreases from 9.0 to 3.0 the minimum concentration of sorbic acid required to inhibit the growth of four molds decreases from more than 0.2 to 0.005%. Other workers have also confirmed the interrelationship of low pH and the effectiveness of sorbic acid as a preservative. Beneke and Fabian (38) found 0.05% sorbic acid an effective antimycotic in tomato juice at pH 4.4, and at a 0.075% level in strawberry puree at a pH of about 4.0. Bell and co-workers (39) in examining the activity of sorbic acid for use in pickling applications used over 60 species of fungi, over 30 species of yeast, and 6 species of the lactobacilli. They found 0.1% sorbic acid inactive at pH 7.0, but quite active against the yeasts and fungi at pH 4.5. Below pH 3.5, even the lactic acid bacteria were inhibited.

Food in which sorbates have been demonstrated to have commercially effective activity include baked goods, cheese, confectionery (cake frostings and icings), chocolate coatings, dried fruits, fish products, fruit butters, fruit juices and fresh fruit salads, jellies (artificially sweetened), margarines, nonalcoholic beverages, pastry fillings, pet foods, pickled foods, poultry, prepared salads, salad dressings, sauces, sausage casings, dry sausage, syrups, and wines. They are also effective in some pharmaceutical preparations and in cosmetics. Other nonfood applications in which sorbic acid is used as a preservative include tobacco and silk-screen inks. Sorbic acid is generally more effective than other commercially used chemical food preservatives, such as benzoic and propionic acid, and as a consequence lesser amounts are required. This is a distinct advantage in bland foods where the taste and odor contributions of the preservative to the food are of critical concern. Like benzoic and propionic acids, sorbic acid requires an acidic pH for effectiveness, but functions at higher pH values than either benzoic or propionic acid. The concentration of sorbic acid used in foods varies from about 0.02 to about 0.3% by weight of the food.

The toxicity of sorbic acid is extremely low, about one third that of benzoic acid. Feeding studies have established that it is metabolized as a fatty acid ultimately into carbon dioxide and water in the same manner (ie, β-oxidation) as other unsaturated fatty acids normally found in foods. Comprehensive pharmacological data have established the safety of sorbic acid used in food packaging materials which are in intimate contact with the food. Studies which have shown that sorbic acid can migrate (40) from a packaging material into a foodstuff have been the basis for the utilization of sorbic acid-coated films to protect cheese.

On the basis of expert opinion that sorbic acid is safe for its intended use as a food preservative, the U.S. Food and Drug Administration regards it as GRAS (generally recognized as safe), Food Additives Regulation §121.101. It may be used without limitation in all foods not covered by Federal Standards of Identity, provided the use conforms to good manufacturing practice. Foods subject to Federal Standards of Identity in which the use of sorbic acid has been included in the Standards are shown in Table 4. In Table 5, a listing of uses for sorbates in products not covered by Standards is presented. The starred (*) foods mentioned in the following sections may be found in Table 4 and the daggered (†) foods in Table 5.

Bakery Products. At the present time, sorbic acid is limited to nonyeast-raised bakery products because of its inhibitory effect on yeast growth. The use of a post-baking spray of sorbic acid applied to yeast-raised products, such as English muffins,† has been used as a means of circumventing its effect on yeast. Sorbic acid, which is four times as effective as sodium propionate or sodium benzoate at a 0.2% level, is currently

Table 4. Foods Covered by Standards of Identity Which Include the Use of Sorbates

Foods covered by Standards of Identity	Food Additives Regulation or Food Standards	Sorbic acid or potassium sorbate
Cheese, processed cheese, cheese spreads		
cheddar, curd, colby, granular, swiss, gruyère, brick, muenster, edam, gouda, monterey, monterey jack, high-moisture jack, provolone, caciocavallo, siciliano parmesan, low-moisture mozzarella, low-moisture scamorze, low-moisture part-skim mozzarella, low-moisture part-skim scamorze, romano, asiago fresh, asiago medium, asiago old; and other hard, semisoft, semisoft part-skim, spiced, part-skim spiced, and hard grating cheeses not otherwise specifically defined by names or Standards of Identity	§19.500–19.680	up to 0.3% (either or both)
pasteurized process; pasteurized process with fruits, vegetables or meats; pasteurized process pimento; pasteurized blended; pasteurized blended with fruits, vegetables or meats; pasteurized process cheese food, pasteurized process cheese food with fruits, vegetables or meats	§19.750–19.770	up to 0.2% (either or both)
pasteurized process cheese spread; pasteurized cheese spread; pasteurized process cheese spread with fruits, vegetables, or meats; pasteurized cheese spread with fruits, vegetables, or meats; cold-pack cheese	§19.775–19.785	up to 0.2% (sorbic acid only)
cold-pack cheese food; cold-pack cheese food with fruits, vegetables, or meats	§19.787–19.788	up to 0.2% (sorbic acid only)
Jellies		
fruit jellies, preserves, jams	§29.2, 29.3	no
Jellies, artificially sweetened		
artificially sweetened fruit jellies, artificially sweetened fruit preserves	§29.4, 29.5	up to 0.1% (either or both)
Lemonade, colored lemonade, limeade		
	§27.99, 27.100, 27.131	no limit (potassium sorbate only)
Margarine, liquid margarine		
	§45.1, 45.2 Consumer and Marketing Service, USDA	up to 0.1% (potassium sorbate only)
Orange juice with preservative, concentrated orange juice with preservatives		
	§27.113, 27.115	up to 0.2% (sorbic acid only)

Table 4 (*continued*)

Orange-juice drink, orangeade, orange drink		
	§27.120–27.122	no limit (sorbic acid only)
Sausage, dry		
	Consumer and Marketing Service, USDA	2.5% in aqueous dip (potassium sorbate only)
Soda water, carbonated, plain, sweetened and/or flavored; club sodas, colas, tonics, etc		
	§31.1	no limit

Table 5. Use of Sorbates in Nonstandardized Foods

Product	Reference	Product	Reference
apple cider	(42,50,51)	fruit salads	(42,53)
apple juice	(52,53)	grape juice	(66)
bananas, slices	(53)	icing	(74)
cakes	(55,56)	maraschino cherries	(67)
carrots, pre-peeled	(57)	margarine, imitation diet	(68)
celery	(41,58)	mushrooms, brined in oil	(68)
chappaties	(59)	pancake batter	(68)
cherries	(44)	peach slices	(42)
chicken, smoked	(45)	pet-food patties	(69)
citrus salad	(43)	pickles	(70)
cucumbers	(61)	prunes	(71)
English muffins	(54)	strawberries	(38,44,53)
figs	(62)	tangarine, sherbet base	(72)
fish, smoked and salted	(63–65)	tomato juice	(38)
fruit-pie fillings	(55)	wine	(73)

used to protect cakes†, fruit-pie fillings†, cake frosting and icings, and the toasted fruit-filled tart products. The level of sorbate used varies from 0.075% (acidic fruit fillings) to 0.125% in "neutral" cakes (chocolate, cheese) to as much as 0.3% in devil's food cake.

Dairy Products. One of the principal uses of sorbic acid on foods today is in the protection of packaged cuts and slices of processed cheese. The quantities of sorbate that may be added to various cheeses covered by Standards of Identity are summarized in Table 4. The sorbate is added in one of five general methods: dip, spray, direct, dust, or as a coating on the wrapper film. The quantity and manner of application of sorbate required for protection varies for the different varieties. Dips and sprays use potassium sorbate solutions of 20–40% concentration.

Sorbates, while proven effective in extending the shelf life of cottage (60) and cream cheeses, are not named as ingredients in Federal Standards of Identity for these products and may not be used in interstate commerce. The users should consult state regulations for use of sorbates in intrastate commerce.

Potassium sorbate is the only form permitted by the Federal Standards of Identity for incorporation in margarine*. Used at a concentration of 0.1%, sorbate effectively inhibits the growth of spoilage organisms in salted, unsalted, and low-calorie, imitation margarine. The potassium sorbate may be blended into the fat emulsion, sprayed on the surface of the finished margarine, or incorporated into the wrapper film.

Fruit and Vegetable Products. There is limited use of sorbates with some fermented vegetable products, such as pickles†, where sorbic acid reduces surface yeast scum formation in brining tanks. Hall (41) has reported the use of potassium sorbate to control the discoloration of the butts of prepackaged celery†. After studying fruit products, Robinson et al. (42) found sorbic acid to be an effective preservative with peach slices†, apple cider†, and fruit salad†. Rushing and Senn (43), working with citrus salads†, and Salunke et al. (44), testing strawberries† and cherries†, also found sorbic acid useful as a preservative.

A considerable amount of work has been done in studying the effectiveness of sorbates in fruit juice† preservation. The shelf life of apple juice or cider† has been extended significantly with sorbic acid and it is also used to prevent fermentation in grape juice† stored in holding tanks. Other juices that have been preserved with sorbates include tomato†, orange*, and lemonade*.

Dips or sprays of potassium sorbate solutions are routinely used to preserve such dried fruit products as prunes†, figs†, apricots, and mixed dried fruits.

Various convenience foods which are kept under refrigeration, such as potato salad, cole slaw, macaroni salad, and gelatin (especially with fruit added), demonstrate marked shelf-life improvement with the addition of sorbates. The preservative may be added either in the cream dressing or in the hot water (gelatin).

Beverages, Beverage Syrups, and Syrups. Sorbates are used, often in conjunction with benzoates, to protect and to extend the shelf life of noncarbonated beverages (especially those containing fruit) and in various fountain syrups. Chocolate syrup, which is prepared at relatively high pH levels, requires sorbate protection. Recently, a trend toward the use of sorbates with maple-flavored syrups has been established.

Wines. Sorbate is added to various red and white table wines† in conjunction with sulfur dioxide to effectively inhibit yeast fermentation and increase product life. In practice, as little as 0.05% potassium sorbate plus 10 ppm SO_2 provides the desired protection.

Low-Calorie Foods. Sorbic acid or its potassium salt has been found to be an excellent preservative in low-calorie foods where the normal high sugar content, which acts as a preservative, has been lowered. Removal of fat and substitution of water also increases product susceptibility to microbial attack. Examples of this new generation of foodstuffs include imitation jams, jellies, and preserves*, fruit-flavored syrups, chocolate and maple syrups, soda*, margarine*, salad dressings, and candies.

Meat, Fish, Poultry, and Pet Food. Pet-food patties† of the soft-moist variety generally contain ~25% moisture. To permit long-term storage of these products, about 0.3% sorbate is added to the meat, poultry, or fish emulsion, in conjunction with propylene glycol. This combination provides the antimycotic stability necessary for this product.

Potassium sorbate is permitted, under present Meat and Poultry Regulations, as an additive only in dry sausage*. Here a 2½% potassium sorbate solution is used as a dip for the casing either before or after stuffing.

Sorbates have been used to protect salted and smoked fish†. They are generally applied as 10% aqueous sprays or used at a 5% concentration in a dip solution. There is also a reported use of sorbate for protecting smoked chicken† (45). Users are advised to check appropriate Federal, State, or local regulations prior to use of sorbates in meat, poultry, and fish products.

Miscellaneous. Sorbates have been used for many other products, generally at a concentration between 0.025 and 0.10%. Improvement in keeping quality was found in such diverse products as Chinese egg roll dough, cucumbers, maraschino cherries, pancake batter, etc (see Table 5).

Sorbic acid may be applied to foods in a variety of ways, including direct incorporation, as a spray, as a powder dusting, and via an organic carrier such as ethanol, propylene glycol, or vegetable oil. The potassium salt, or to a very limited extent the sodium salt, of sorbic acid is used principally because of its high water solubility. However, for fungistatic activity, the pH of the foodstuff must be below 6.5. On an equivalent weight basis, the potassium salt has 74% of the activity of sorbic acid. Therefore, higher concentrations are required to obtain the antimycotic activity comparable to that of sorbic acid.

The nonfood uses of sorbic acid which are of significance include cosmetics, tobacco, and latex-based emulsions such as silk-screen inks. In cosmetic formulations, sorbic acid, unlike the *p*-hydroxybenzoate esters, is not inactivated by the commonly used nonionic emulsifier systems, and formulations with excellent physical and biological stability have been prepared (46,47). A comprehensive survey of cosmetic preparations by Lück (48) covers a wide range of applications for sorbic acid and presents convincing bibliographic material which indicates that sorbic acid and its salts can be used without pharmacological and dermatological contraindications for preserving cosmetic emulsions.

Sorbic acid is used domestically to prevent mold growth during the aging of tobacco, and, in Japan, to destroy molds upon which beetles feed, thereby eliminating a tobacco beetle problem (49). Inks which contain significant amounts of latexes in their formulations also are protected from mold attack by sorbic acid.

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S. W. Moline, C. E. Colwell,
and J. E. Simeral
Union Carbide Corporation

SORBITE. See Steel.

SORBITOL, $CH_2OH(CHOH)_3CH_2OH$. See Alcohols, polyhydric.

SORGHUMS. See Wheat and other cereal grains.

SOYBEANS

The soybean, *Glycine max* (L.) Merrill, also known as soya bean, soja bean, Chinese pea, and Manchurian bean, is a native of eastern Asia. It is grown rather extensively in various parts of the world, particularly in the temperate zones. In the Far East, it is an important source of edible protein. In the United States, soybeans have been used primarily as a source of food fat and animal feed. Commercially, no single variety of beans dominates the market, because the soybean is markedly affected by the length of day. Agronomic research has developed a number of varieties well suited to the different parts of the Mississippi Valley where more than 80% of U.S. soybeans are grown. Since most of these varieties were unknown before 1955, further improvement should be expected. Present varieties have an oil content 2–4% higher than the beans grown in 1930. Soybean breeders of the U.S. Department of Agriculture (USDA) and their cooperators in many state experiment stations have been primarily responsible for this change. Leading varieties recommended in 1968 for the major growing areas were the following: Amsoy, Clark 63, Corsoy, Hark, Harosoy 63, and Wayne for the Midwest; Bragg, Hill, and Lee for the South; and Chippewa 64, Hark, Merit, and Portage for Minnesota and Canada (1,2).

This typical legume seed is attached to the plant in short hairy pods containing two to three seeds. Seeds are separated from the pods in the field by combines. Size, shape, and color of the bean varieties are quite different, ranging from small, round beans to large, oblong, rather flat seeds and including yellow, brown, green, and reddish hues, as well as black. As shown in Figure 1 for the Lincoln soybean, formerly grown widely in Illinois, Iowa, and Indiana, the hilum or seed scar, *h*, is linear-elliptical. The chalaza, *c*, is at one end of the hilum where the seed coat joins the body of the ovule. The micropyle, *m*, is the minute opening at the other end of the hilum where the primary root of the germinating seed emerges. The outline of the hypocotyl, *hy*, can be seen beneath the seed coat. The seed consists primarily of hull and embryo, but it has a very rudimen-

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Toxicity Test on Food Additives by Chlorella and Saccharomyces cerevisiae var. Sake (Report No. 2).

Bacterostatic or Bactericidal Action of Food Preservatives and Antimicrobials (1).

(The manuscript was received on June 28, 1967).

By Sakae Yamamoto, Yoko Ito and Shoji Noda (The Research Institute of Shochikubai Wine Brewery Co., Ltd., Hama-cho, Nishinomiya, Hyogo-ken)

(For a brief English synopsis, refer to the original text on p. 408.)

Introduction:

As a toxicity test on the food additives by the disturbance of the life cycle of chlorella and saccharomyces cerevisiae var. Sake, the toxicity of pigments (coloring agents (coloring agents) for food was examined in the previous report (1) and its utilization value as a tentative preliminary test method was recognized. Therefore, a test has been carried out this time on such antimicrobials and preservatives as those which are mentioned in Table 1.

Table 1 on p. 408. Food Preservatives and antimicrobials examined in the current toxicity test.)

1. Benzoic acid
2. Sodium benzoate
3. Salicylic acid
4. Sorbic adid
5. Potassium sorbate
6. Sodium sorbate
7. Dehydroacetic acid (D.H.A.)
8. Sodium dehydroacetate
9. Isobutyl-p-oxybenzoate (Isobutyl-p-OB)
10. Isopropyl-p-OB
11. Ethyl-p-OB
12. Secondary Butyl-p-OB
13. Butyl-p-OB
14. Propyl-p-OB
15. Calcium propionate
16. Sodium propionate
17. Lauryl-trimethyl ammonium-2,4,5-trichlorophenoxide

Antimicrobials

1. 2-(2-Furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2)
2. Chloramine B
3. Chloramine T

Since the purpose of preservatives lies in bacteriostatic action (2), the substances which show a desired inhibitive action on these micro organisms at low concentration and which show low toxicity even at high concentration can be said to have a high degree of safety and a correspondingly high value as preservatives. In addition, since the preservatives show selective toxicity to general bacteria, it is not possible to assume that those which have a high degree of safety against chlorella and yeasts also have a high degree of safety against living cells.

When the results of various animal tests which have thus far been carried out on individual preservatives (3 - 7) and examinations of the mechanism of metabolism in living organisms are taken into consideration, however, those which are believed to have a high degree of safety against living cells were also found to be low in the extent of the inhibition of the life cycle of micro-organisms in the current simple test.

In this concise test, since it was easily possible to make a comparison in inhibitive action amongst a large number of preservatives under the same conditions, a comparison between those acidic preservatives themselves and those of the ester type produced a conclusion that those of the ester type clearly have a greater inhibitive action in the optimal pH range between 4.5 and 7.0 against micro-organisms.

Experimental Method:

Samples: Reagents of the special grade purchased on the market were used. (These included the preservatives and antimicrobials whichhave not been approved as food additives.)

Bacterial stocks: Chlorella vulgaris and saccharomyces cerevisiae var. Sake, brewery testing station No. 6.

Culture solution: As in the previous report, culture medium A (Arnon culture ground) and culture medium B (Arnon culture ground plus one per cent of glucose plus 0.01 per cent of $Mg(OAc)_2$) were used for chlorella and a Henneberg culture medium was used for yeasts.

Table 2. Influence of 0.1 per cent food preservatives on chlorella vulgaris.

Food preservatives	Cell number (per ml) 10^8	f/l	Sample/Std.	Cell diameter (mu)	f/l	Sample Std.
Group 1						
Arnon (first	1.59	1.00		3.30	1.00	
standard(last	5.08	3.21	1.00	3.24	0.98	1.00
Salicylic acid	(9.16		1.08	4.08		1.26
Benzoic acid	(7.92		1.55	4.33		1.33
•Chloramine B	(0.56		0.10			
•Chloramine T last	(0.98		0.19			
Sorbic acid	(0.82		0.16	4.15		1.28
•D.H.A.	(0.78		0.15	4.16		1.42
Group 2						
Arnon (first	1.41	1.00		4.09	1.00	
standard (last	2.43	1.82	1.00	5.47	1.33	1.00
•Na-Benzoate	(1.01		0.42	3.86		0.71
•K-Sorbate	(1.26		0.52	3.82		0.70
•P-O-B-Butyl last(	1.23		0.51			
°P-O-B-Ethyl	(1.28		0.53	3.71		0.67
•P-o-B-Propyl	(1.41		0.56	2.52		0.46
Group 3						
Arnon (first	1.1	1.00		3.91	1.00	
standard(last	39.2	35.6	1.00	3.62	0.93	1.00
n-Butyl-benzoate	(48.0		1.20	4.37		1.21
°n-Propyl-benzoate	(9.2		0.20	3.56		0.99
Propionic acid last	(59.0		1.50	4.57		1.26
P.O.B.	(58.0		1.50	4.44		1.23
Group 4						
Arnon (first	1.54	1.00		4.04	1.00	
standard(last	2.65	1.72	1.00	3.90	0.97	1.00
•Chloramine B	(1.21		0.46	2.91		0.75
°Chloramine T	(1.39		0.52	2.92		0.75
•Na-D.H.A. last	(1.23		0.46	3.05		0.78
°Ca-Propionate	(1.40		0.53	4.67		1.20
↓AF-2	(1.31		0.49	2.81		0.72
Group 5						
Arnon (first	1.43	1.00		3.48	1.00	
standard(last	2.92	2.04	1.00	3.78	1.09	1.00
•Benzoic acid	(1.93		0.66	4.02		1.06
P.O.B.	(10.1		3.46	4.55		1.20
°n-Butyl-benzoate	(1.41		0.48	3.00		0.79
•P-O-B-Butyl	(1.40		0.48	2.54		0.67
•n-Propyl-benzoate last	(1.11		0.38	3.41		0.89
•P-O-B-Propyl	(1.17		0.40	3.00		0.79
Salicylic acid	(1.25		0.43	3.49		0.91
•Butyl salicylate	(1.28	0.[illegible]	0.44	3.24		0.8
•Ethyl salicylate	(1.02		0.35	3.40		0.89

•,o: discolor strong or weakly
P-O-B: p-Hydroxybenzoic acid
f/l: Ratio of cell number or diameter between at first and last periods of culture

Table 3. Influence of 0.1 per cent preservatives on chlorella vulgaris in B medium.

Food Preservatives	Cell number (per ml) 10^8	f/l	Sample/Std.	Cell diameter (mu)	f/l	Sample Std.
Group 1						
Arnon (first	0.77	1.00		3.14	1.00	
standard(last	1.19	1.55	1.00	3.82	1.22	1.00
Salicylic acid	(3.48		2.92	3.78		0.99
Benzoic acid	(2.00		1.69	4.11		1.08
•Chloramine B	last (0.63		0.53			
•Chloramine T	last (0.58		0.48			
Sorbic acid	(1.08		0.91	4.52		1.19
•D.H.A.	(0.71		0.60	3.56		0.93
Group 2						
Arnon (first	0.82	1.00		3.83	1.00	
standard(last	2.92	3.56	1.00	3.73	0.99	1.00
Na-Benzoate	(3.21		1.10	4.63		1.21
K-Sorbate	(1.24		0.42	4.49		1.19
•P-O-B-Butyl	last(0.59		0.21			
•P-o-B-Ethyl	(0.70		0.24	4.27		1.15
•P-O-B-Propyl	(0.80		0.27	3.03		0.81
Group 3						
Arnon (first	1.22	1.00		4.03	1.00	
standard (last	1.60	1.36	1.00	3.57	0.88	1.00
On-Butyl-benzoate	(1.23		0.80	3.51		0.98
On-Propyl-benzoate	last (1.18		0.74	3.66		1.02
Propionic acid	last (1.67		1.04	3.61		1.01
P.O.B.	(1.38		0.89	3.71		1.04
Group 4						
Arnon (first	0.72	1.00		3.53	1.00	
standard(last	3.51	4.88	1.00	3.65	1.03	1.00
•Chloramine B	(1.19		0.34	3.24		0.89
•Chloramine G	(1.84		0.52	3.55		0.97
•Na-D.H.A.	last(1.31		0.37	4.22		1.16
Ca-Propionate	(4.46		1.27	3.73		1.02
AF-2	(1.28		0.36	3.59		0.99

Table 4. Influence of 0.1 per cent preservatives on chlorella vulgaris in B medium.

Food Preservatives	Cell number (per ml) 10^8	f/l	Sample/Std.	Cell diameter (mu)	f/l	Sample Std.
Group 1						
Arnon (first	0.66	1.00		4.00	1.00	
standard(last	1.69	2.56	1.00	5.47	1.37	1.00
•Salicylic acid	(0.60		0.36			
•Benzoic zcid	(0.56		0.33			
Chloramine G	last(0.51		0.30	2.88		0.53
•Sorbic acid	(0.52		0.30	2.88		0.53
•D.H.A.	(0.63		0.35			
Group 2						
Arnon (first	0.54	1.00		3.61	1.00	
standard(last	30.5	5.65	1.00	3.99	1.10	1.00
Salicylic acid	(19.2		0.63	5.42		1.36
Benzoic acid	(12.8		0.42	6.02		1.50
•Chloramine B	last{(0.70		0.22			
•Chloramine T	(0.47		0.02	3.39		0.85
Sorbic acid	(13.1		0.43	5.88		1.48
•D.H.A.	(0.62		0.02			
Group 3						
Arnon (first	0.50	1.00		4.11	1.00	
standard(last	57.8	11.6	1.00	5.15	1.24	1.00
°Na-Benzoate	(1.51		0.26	3.80		0.74
•K-Sorbate	(0.46		0.08	3.90		0.76
•P-O-B-Butyl	last(0.44		0.08	3.54		0.67
•P-O-B-Ethyl	(0.46		0.08	3.95		0.77
•P-O-B-Propyl	(0.41		0.07	3.35		0.65
Group 4						
Arnon (first	1.00	1.00		3.91	1.00	
standard(last	5.90	5.67	1.00	4.57	1.14	1.00
•n-Butyl-benzoate	(0.79		0.13	3.84		0.84
°n-Propyl-benzoate	last{(1.17		0.20	4.68		1.02
Propionic acid	(11.6		1.96	4.71		1.03
P-O-B	(7.40		1.25	4.95		1.08
Group 5						
Arnon (first	1.54	1.00		4.04	1.00	
standard(last	49.1	31.9	1.00	4.89	1.17	1.00
Chloramine B	(1.70		0.35	5.45		1.11
Chloramine T	(2.20		0.45	5.01		1.02
•Na-D.H.A.	last(1.25		0.025	3.73		0.71
Ca-Propionate	(1.50		0.03	4.66		0.95
•AF-2	(1.51		0.03	2.85		0.59
Group 6						
Arnon (first	1.43	1.00		3.48	1.00	
standard(last	45.7	31.9	1.00	4.88	1.29	1.00
Benzoic acid	(1.65		0.03	4.35		0.89
P.O.B.	(24.2		0.53	4.10		0.84
•n-Butyl-benzoate	(1.39		0.03	3.73		0.77
•P-O-B-Butyl	(1.14		0.025	2.85		0.58
•n-Propyl-benzoate	last(1.49		0.03	2.96		0.61
•P-O-B-Propyl	(1.22		0.03	3.10		0.63
•Salicylic acid	(7.90		0.18	5.78		1.18
•Butyl salicylate	(1.28		0.026	3.53		0.72
°Ethyl salicylate	(1.45		0.03	3.08		0.63

Method of operation: A culture solution was charged into a test tube equipped with a Morton cock, which had been dried, heated and sterilized, in such a way that the final concentration of the preservatives would be 0.1 per cent and 0.01 per cent per 10 milliliters. In the case of 0.1 per cent, 10 milligrams of the preservative was added and in the case of 0.01 per cent, one milliliter of the 0.1 per cent solution was added. In all cases, an adjustment was made to pH 6.5 in a five per cent $NaHCO_3$ solution. Only in the case of group 1 in Table 4 in connection with culturing in Medium B for chlorella, however, no operation for the adjustment of pH was carried out. The subsequent operation was exactly the same as in the previous report.

The insolubles were dissolved in boiling water bath and a certain fixed amount of those which were heated and in a dissolved state was poured into the culture medium.

Items examined: Same as in the previous report.

Experimental Results:

In such a manner that the preservatives would be 0.1 per cent and 0.01 per cent, Tables 2 and 3 show the result obtained when it was added to medium A, Tables 4 and 5 show results obtained when it was added to medium B, and Tables 6 and 7 show results which were obtained at the time when it was added to the Henneberg medium. Table 8 summarizes only the ratios of the test groups to the standards of the actually measured values shown in Tables 2 through 7.

In the Table, those marked with a dot show a characteristic of discoloration, the green color of the chlorophylls of these chlorellas being either faded or disclored, the milky white color of the yeast being changed to yellow, faded or discolored.

The cells of both microorganisms which have received this effect were found not to have any power for further life as a result of re-culturing. Therefore, it was considered to be a bactericidal action and those in which there was no discoloration or color fading, with only a reduction in the number of solid cells or stoppage thereof, were considered to show bacteriostatic action in order to differentiate these from the former.

In the case where 0.01 per cent was added in terms of chlorella, the bactericidal action was restricted to only a part, with only the bacteriostatic action being observed. In the case of yeast, there were more cases where a bacteriostatic action was received than those in which the bactericidal action was received even when its amount was 0.1 per cent.

Table 6. Influence of 0.1 per cent preservatives on sachharomyces cerevisiae var. Sake.)

Food preservatives	Cell Number (per ml) 10^8	f/l	Sample /Std	Cell diameter (mu)	f/l	Sample/Std.
Group 1						
Henneberg(first	1.28	1.00		5.37x4.59	1.00	
standard(last	2.99	2.29	1.00	5.30x4.16	0.98x0.92	1.00
Salicylic acid	(2.98		1.00	5.07x4.12		0.96x0.91
Benzoic acid	(2.16		0.72	5.58x4.44		1.05x1.06
•Chbramine B	last(2.82		0.94	4.99x3.93		0.91x0.95
•Chloramine T	last(2.17		0.72	4.81x4.16		0.91x1.00
Sorbic acid	(1.04		0.35	5.81x4.65		1.10x1.11
•D.H.A.	(0.54		0.18	5.33x4.48		1.01x1.08
Group 2						
Henneberg(first	0.21	1.00		5.75x4.44	1.00	
standard(last	(3.37	16.0	1.00	5.61x4.31	0.98x0.97	1.00
°Na-Benzoate	(2.05		0.61	5.66x4.83		1.00 x 1.11
°K-Sorbate	(1.07		0.32	5.92x4.73		1.06x1.09
•P-O-B-Butyl	last(0.27		0.08			
•P-O-B-Ethyl	(0.32		0.09	5.48x4.37		0.99x1.01
•P-O-B-Propyl	(0.25		0.08			
Group 3						
Henneberg(first	0.21	1.00		5.33x4.29	1.00	
standard(last	3.24	15.4	1.00	5.88x4.38	1.01x1.02	1.00
n-Butyl-benzoate	(2.30		0.71	5.87x4.53		1.00x1.03
°n-Propyl-benzoate	(2.96		0.91	5.28x4.14		0.90x0.94
Propionic acid	(1.92		0.59	5.90x4.38		1.00x1.00
P.O.B.	(2.31		0.71	5.94x4.37		0.97x1.00
Group 4						
Henneberg(first	0.11	1.00		5.89x4.54	1.00	
standard(last	3.88	35.3	1.00	5.64x4.44	0.96x0.97	1.00
Chloramine B	(3.46		0.89	5.91x4.50		1.05x1.01
Chloramine T	(2.58		0.65	4.75x4.65		1.02x1.04
•Na-D.H.A.	last(0.22		0.06	5.72x4.45		1.02x1.00
Ca-Propionate	(1.92		0.59	5.89x4.42		1.04x0.99
•AF-2	(0.12		0.03	5.92x4.82		1.05x1.09
Group 5						
Henneberg(first	0.18	1.00		5.59x4.36	1.00	
standard(last	3.20	18.0	1.00	5.53x4.21	1.00x0.96	1.00
Benzoic acid	(1.56		0.48	5.64x4.47		1.02x1.02
P.O.B.	(2.50		0.78	5.17x4.37		0.93x1.03
•n-Butyl-benzoate	(0.25		0.08	5.04x3.39		0.92x0.94
•P-O-B-Butyl	(0.20		0.06	4.92x3.60		0.89x0.85
n-Propyl-benzoate	last(1.42		0.44	5.39x4.32		0.97x1.02
•P-O-B-Propyl	(0.21		0.06	5.28x4.39		0.95x1.04
Salicylic acid	(2.69		0.84	5.07x4.24		0.92x1.00
Butyl saliylate	(0.19		0.06	5.10x3.99		0.92x0.94
•Ethyl salicylate	(0.24		0.07	5.11x3.95		0.92x0.89

•,o: Color change strong or weak

Table 7. Influence of 0.01 per cent food preservatives on saccharomyces cerevisiae var. Sake.

Food preservatives	Cell Number (per ml) 10^8	f/l	Sample/Std	Cell diameter (mu)	f/l	Sample/Std.
Group 1						
Henneberg (first	1.07	1.00		5.07x4.14	1.00	
standard (last	3.72	3.48	1.00	5.48x4.14	1.08x0.99	1.00
Salicylic acid	(3.02		0.96	4.52x4.28		0.82x1.04
Benzoic acid	(2.46		0.66	5.48x4.37		1.00x1.06
Chloramine B	(2.72		0.73	5.49x4.42		1.00x1.07
•Chloramine G	last (3.45		0.93	5.11x3.97		0.93x0.91
Sorbic acid	(1.96		0.53	5.74x4.25		1.29x1.02
•D.H..A.	(2.40		0.64	5.53x4.29		0.82x1.03
Group 2						
Henneberg (first	1.34	1.00		5.19x4.38	1.00	
standard (last	34.9	26.0	1.00	5.55x4.40	1.07x1.01	1.00
Na-Benzoate	(28.6		0.81	5.51x4.39		0.99x1.00
K-Sorbate	(20.3		0.58	5.63x4.59		1.01x1.04
°P-O-B-Butyl	last (18.6		0.53	5.34x4.57		0.96x1.04
P-O-B-Ethyl	(19.8		0.57	5.12x4.48		0.92x1.01
°P-O-B-Propyl	(22.8		0.65	5.69x4.38		1.02x0.99
Group 3						
Henneberg(first	1.41	1.00		5.52x4.75	1.00	
standard (last	3.68	2.61	1.00	5.44x4.15	0.98x0.98	1.00
n-Butyl-benzoate	(3.64		0.99	5.41x4.22		0.99x1.01
n-Propyl-benzoate	(3.26		0.88	5.33x4.23		0.97x1.01
Propionic acid	last (3.20		0.87	5.50x4.20		1.01x1.01
P.O.B.	(3.02		0.82	5.68x4.41		1.05x1.06
Group 4						
Henneberg(first	1.77	1.00		5.55x4.32	1.00	
standard(last	28.3	16.0	1.00	5.65x4.17	1.02x0.96	1.00
Chloramine B	(14.2		0.50	5.55x4.18		0.98x1.00
Chloramine G	(26.3		0.93	5.48x4.04		0.97x0.97
Na-D.H.A.	last (16.6		0.98	5.69x4.22		1.01x1.01
Ca-Propionate	(28.4		1.00	5.64x4.25		1.00x1.01
°AF-2	(3.60		0.13	6.76x4.82		1.20x1.15

A comparison between Group 1 in Table 8 in which no pH adjustment was carried out and the values after the adjustment shows that salicyclic acid, benzoic acid and sorbic acid showed stronger toxicity and bactericidal effect when pH was lower.

The chloramines B and T which are antimicrobials were discolored and showed a bactericidal effect. D. H. A. which is a preservative works as a bactericidal rather.than as a bacteriostatic as in Group 2. After pH adjustment, sorbic acid shows only an effect of inhibiting the multiplication of individual cells and showed a bacteriostatic effect.

In Group 3, sodium benzoate and potassium sorbate were found to have a small-or inhibitive character than p-oxybenzoic acid ester, with discoloration being observed at 0.1 per cent. In all cases, the esters showed a strong anti-microbial effect.

In Group 4, the butyl and propyl ester of benzoic acid showed a strong inhibitive action as compared with free p-oxybenzoic acid and propionic acid.

In chloramines B and T in Group 5, there were found some numerical differences from the same test in Group 2. In connection with culturing in A medium using 0.1 per cent chlorella, a discoloring nature was observed. Therefore, it is believed that its bactericidal effect is strong. D.H.A.-Na and AF-2 also showed a strong bactericidal effect. Propionic acid Ca (calcium propionate) showed no discoloring nature and it was not found to be bactericidal.

Group 6 shows the result of a comparative test on benzoic acid, p-oxybenzoic acid, salicilyic acid and the esters of these. It is clearly shown that the esters have a strongly inhibitive nature.

Table 8. Summary of Cell Number and Cell Diameter Ratios.

Food preservatives	Cell number						Cell diameter					
	A	B	Yeast	A	B	Yeast	A	B	Yeast	A	B	Yeast
	0.1%			0.01%			0.1%			0.01%		
Group 1												
Standard		2.56						1.37				
Salicylic acid		0.36										
Benzoic acid		0.33										
Chloramine T		0.30						1.00				
Sorbic acid		0.30						0.35				
D.H.A.		0.35										
Group 2												
Standard	3.21	5.65	2.29	1.55	2.02	3.48	0.98	1.10	0.98x0.92	1.22	1.51	1.08x0.99
Salicylic acid	1.80	0.63	1.00	2.92	1.22	0.91	1.26	1.36	0.96x0.91	0.99	0.86	0.82x1.04
Benzoic acid	1.55	0.42	0.72	1.69	0.73	0.66	1.33	1.50	1.05x1.06	1.08	0.90	1.00x1.06
Chloramine B	1.10	0.22	0.94	0.53	0.88	0.73			0.91x0.95		1.02	1.00x1.07
Chloramine T	0.19	0.02	0.72	0.48	1.07	0.93		0.85	0.91x1.00		0.88	0.93x0.91
Sorbic acid	0.16	0.43	0.35	0.91	0.47	0.54	1.28	1.48	1.10x1.11	1.19	0.83	1.29x1.02
D.H.A.	0.15	0.02	0.18	0.60	0.07	0.64	1.42		1.01x1.08	0.93	0.88	0.82x1.03
Group 3												
Standard	1.82	11.6	16.0	3.56	31.1	26.0	1.33	1.24	0.98x0.97	0.99	1.48	1.07x1.01
Na-Benzoate	0.42	0.26	0.61	1.10	0.62	0.81	0.71	0.74	1.00x1.11	1.21	0.77	0.99x1.00
K-Sorbate	0.52	0.08	0.32	0.42	0.53	0.58	0.70	0.76	1.06x1.09	1.19	0.91	1.01x1.04
P-O-B-Butyl	0.51	0.08	0.08	0.21	0.03	0.53		0.69			0.80	0.96x1.04
P-O-B-Ethyl	-.53	0.08	0.09	0.24	0.03	0.57	0.67	0.77	0.99x1.01	1.15	1.10	0.92x1.01
P-O-B-Propyl	0.56	0.07	0.08	0.27	0.04	0.65	0.46	0.65		0.81	0.67	1.02x0.99
Group 4												
Standard	35.6	5.67	15.4	1.36	1.14	2.61	0.93	1.14	1.01x1.02	0.88	1.15	0.98x0.98
n-Butyl-benzoate	1.20	0.13	9.71	0.80	1.17	0.99	1.21	0.84	1.00x1.03	0.98	1.36	0.99x1.01
n-Propyl-benzoate	0.20	0.20	0.91	0.74	1.23	0.88	0.99	1.02	0.90x0.94	1.02	0.93	0.97x1.01
Propionic acid	1.50	1.96	0.59	1.04	0.90	0.87	1.26	1.03	1.00x1.00	1.01	1.03	1.01x1.01
P-O-B	1.50	1.25	0.71	0.89	0.99	0.82	1.23	1.08	0.97x1.00	1.04	1.05	1.05x1.06
Group 5												
Standard	1.72	31.9	35.3	4.88	46.0	16.0	0.97	1.17	0.96x0.97	1.03	1.39	1.02x0.96
Chloramine B	0.46	0.35	0.89	0.34	0.97	5.00	0.75	1.11	1.05x1.01	0.89	0.92	0.98x1.00
Chloramine T	0.52	0.45	0.65	0.52	0.97	0.93	0.75	1.02	1.02x1.04	0.97	0.93	0.97x0.97
Na-D.H.A.	0.46	0.02	0.06	0.37	0.04	0.98	0.78	0.71	1.02x1.00	1.16	0.94	1.01x1.01
Ca-Propionate	0.53	0.03	0.59	1.27	0.98	1.00	1.20	0.95	1.04x0.99	1.02	1.02	1.00x1.01
Af-2	0.49	0.03	0.03	0.36	0.04	0.13	0.72	0.59	1.05x1.09	0.99	0.94	1.20x1.15

Table 9. Summary of test results on chlorella and saccharomyces.)

Preservatives and antimicrobials	Influence on Chlorella vulgaris				Influence on Saccaromyces cerevisiae var. Sake	
	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
Group 1						
Salicylic acid			·pH: 4.2 distorted. slightly green.			
Benzoic acid			·pH: 4.4			
Chloramine T			pH: 6.0 green. Cells in divisional term are few.			
Sorbic acid			·pH: 4.4 light green. Number of cell is few.			
D.H A.			·pH: 5.2-5.4 slightly green. Cells are few and small.			
Group 2						
Salicylic acid	Green color is deeper than that of standard. Number of cells is more than that of standard.		Cell are many. Large cells are many	4.2-5.6 mu cells are many. Cells in divisional term are few.		Unchanged.
Benzoic acid	Diameter is larger that of standard.			Small cells are many, too.		Unchanged.

Table 9 continued.

	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
Chloramine B	·Almost white	·Discolor	·Almost discolor. Cells are few.	·White	°Slightly yellow.	
Chloramine T	·Almost white.	·White			°Slightly yellow.	Small cells are many. Germinating cells are few.
Sorbic acid	Scarcely changed.	Green Unchanged	Color is deeper than that of std.	°Light green. Cell is abnormal.		Unchanged.
D.H.A.	·Slightly green.	·White	·White, Light green cells are few.	·Scarvely green	·Slightly yellow.	Number of cells and germinating cells are few.
Group 3						
Na-Benzoate	·Slightly green.	Scarcely changed Cells in divisional term are few.	°Light green, Elliptic cells are few.	Green color ≐. std.	Inhibit Fragrant	Scarcely changed
K-Sorbate	·Slightly green.		·Slightly green	Green ≐.std.	°Scarcely inhibit. Fragrant.	Scarcely changed.
P-O-B-Butyl	·Slightly green Cell is abnormal.	·Discolor.	·Discolor	·Slightly green	·Inhibit. No growth and no multiplication. Infragrant.	Inhibit Fragrant.
P-O-B-Ethyl	°Light green	°Light green Inhibit. -Butyl.	°Discolor. Cell is abnormal.	°Green> -Butyl.	·Diameter and number> -Butyl.	

Table 9 continued.

	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
P-O-B-Propyl	·Slightly green.	·Scarcely green	·Discolor Cell is small	·Scarcely Green	·Cell is abnormal as the case of -Butyl.	Slightly inhibit.
Group 4						
n-Butyl-benzoate	Green. Scarcely	Small cells are many. changed.	Scarcely green. Small.	Small cells are many.	Inhibit.	Scarcely unchanged.
n-Propyl-benzoate	·Discolor. Cell is abnormal.	"	·Discolored cells are many.	Unchanged.	Number of cell is few. Cell form is distorted.	Scarcely unchanged.
Propionic acid	Unchanged.	Scarvely unchanged.	Deep green.	Unchanged.	Scarcely changed. Bubbling is active	Scarcely unchanged.
p-Oxy-benzoate	"	"	Large cells are many.	"	No inhibition.	"
Group 5						
Chloramine B	·Discolor Cell is distorted. Small.	·Almost white. Small cells are many.	Normal. Large cells are many.	≒ standard. Da cells or small cells are many.	≒ standard. Slightly yellow.	Diameter< std.
Chloramine T	·Scarcely green "	·White. Diameter<B	Normal. Diameter<B	≒ std.	"	"
Na-D.H.A.	·" "	·Large and white cell.	·Small cells are many.	·Slightly green.	·Discolor. Cells are few.	
Ca-Propionate	Slightly changed and abnormal.	Normal.	Normal.	·≒std.		Cells are few. Cell is large.

Table 9 continued.

	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
AF-2	•Dye cell to brown. Cell is distorted.	•Dye cell to orange.	•Dye cell to orange Cell is distorted.	°Yellow. Cells are few. No multiplication.	•Dye cell to orange. Cell is distorted.	•Inhibit. Dye cell to orange.
Group 6 Benzoic acid	Green>std. Small cells are few	(pH:5.0)	Inhibited Cells are few. Normal.	9pH: 5.0 -5.2	Normal.	(pH:4.5)
p-Oxy-benzoate	Deep green>std.	(pH: 6.6 -6.8)	Deep green. Normal	(pH:6.8)	Normal.	(pH:4.4 -4.6)
n-Butyl-benzoate	•White Cell is distorted. Small.	(pH: 4.2 -4.4)	•Light green.	(pH:5.6 -5.8)	•No fragrant	(pH: 4.8 -5.0)
p-O.B.-Butyl	•White, small and abnormal	(pH: 4.2 -4.4)	•White small and distorted.	9pH:5.6 -5.8)	•No fragrant.	(pH 5.6: -5.8)
n-Propyl-benzoate	•Discolor Small and abnormal.	(pH: 4.2)	•White, small	(pH: 5.2 -5.4)	Fragrant.	(pH:4.6 -4.8)
P.O.B.-Propyl	•White	(pH:4.2 -4.4)	•White, Abnormal.	(pH:5.2	•No fragrant.	(pH:5.6 -5.8)
Salicylic acid	•Discolor.	(pH: 4.2 -4.4)	Green, Large cells are many. Active multiplication.	(pH: 6.2 -6.4)	Fragrant.	(pH: 4.4 -4.6).
Butyl salicylate	•Distorted.	(pH: 4.2)	•White Cell membrane is broken.	(pH: 5.6)	•No fragrant.	(pH: 5.4 -5.6)
Ethyl salicylate	•White.	(pH: 4.2)	•White.	(pH: 5.6)	. "	(pH: 5.6 -6.0)

Observations:

A summary of the test results obtained in the current study is shown in Table 9. Observations will be made below onthe major items:

Nutrients: Food additives are significant only in the state of being added to foodstuff. However, since the number of foodstuffs is so tremendously large, it would be extremely compoicated to carry out the tests on each individual case. If the foodstuff can be classified according to the main nutrients, tests can be conducted on the basis of combinations between single nutrients and additives. In this manner, it is believed possible to determine the value systematically, both intheory and in practice.

Chlorella culture medium B (11) and the Henneberg culture medium both contain grape sugar (dextrose) and it is believed that the test was conducted in the state of an additive being added to carbohydrate food. Even though the differentiation between culture media A and B of the chlorella method or whether carbohydrates were contained or not contained could not be made clear, it was possible to easily make a comparison between the test group and standards because the dextrose markedly promotes the multiplication as a source of carbon for the chlorella.

pH (12): It is possible to conceive of such a case where that which is acidic and shows a preservative character is neutralized by the qualities of an additive foodstuff, with a result that it no longer serves as a preservative. Accordingly, in order to compare the inhibitive nature of preservatives, the optimal pH in the culture medium to which an additive had been added was adjusted to 6.5 to avoid the effect of different pH values.

In the case of an acidic preservative, when the pH of the culture medium tends to be acidic, there is an increase in the rate of shift toward microorganic cells, with a result that, as is known, there is an increase in the inhibitive effect.

Judging from Groups 1 and 2 in Table 8, the difference in pH and the difference in strength were clearly observed. It can be seen that stipulations of object foods in terms of individual preservatives and antimicrobials are also

important from the standpoint of differentiation amongst the nutrients.

Bacteristatic and bactericidal effects: It is desirable that the preservatives and anti-microbials have bacteriostatic and bactericidal effects depending upon their purposes. In the case of chloramines B and T as well as AF-2, discoloration and color fading are observed, thereby indicating the fact that they have a strongly inhibitive character as antimicrobials. However, there are some preservatives which show a bactericidal effect and it is not possible to draw a clear-cut line of demarcation between the antimicrobials and preservatives.

At 0.1 per cent in this test, there was observed a bactericidal discoloration in the esters of benzoic acid, p-oxybenzoic acid, salicylic acid and D. H.A. and its salts in addition to antimicrobials. After the adjustment of pH, benzoic acid, salicylic acid and sorbic acid show a mildly inhibitive nature and propionic acid shows no bactericidal effect at all as a preservative should be. After the adjustment of pH, p-oxybenzoic acid itself showed no inhibitive nature. However, it is interesting to note that its esters show a strongly inhibitive character. Generally speaking, the inhibitive effect is greater against chlorella than against yeast. This is believed to be due not only to the difference in the life cycle of both of these micro-organisms between synthesis and decomposition but also to the selective toxicity of the preservatives. It is believed that there is need for re-examination of this.

Safety (2): The difference in the toxicity of preservatives stems from the difference in the metabolic mechanism which takes place after they have been absorbed into a living body. It is said that the essence of safety guaranty lies in receiving some kind of metabolism. The reason why propionic acid and sorbic acid do not shown any toxicity in the conventional animal tests lies in the fact that the latter is converted into CO_2 and H_2O in metabolism in a living body and the former enters a TCA cycle via citric acid and receives complete metabolism to be non-toxic. Therefore, it is believed to be a preservative having an extremely high degree of safety both in terms of theory and practice.

In the current test, too, their inhibitive character is extremely weak as

compared with the others and, between the two, the inhibitive nature of sorbic acid is said to be greater. This is believed to be indicative of the fact that it is correct to assume that those which were found in this test to have a small inhibitive character have a high degree of safety. Thus, it is possible to state that this test has a utilization value not only as a test on preservatives and antimicrobials but also as a preliminary test on safety.

Free Acids and Their Esters: As can be observed in Group 6 in Table 8, the esters were found to have a far stronger inhibitive effect and toxicity than the free acids. According to existing literature, the difference in toxicity seems to stem from the difference in the surface of bacteria or the difference in the rate of movement between lipoid and the culture medium or the difference in the metabolic mechanism (8). We could not carry out a comparison amongst the various esters in terms of the strength of toxicity (13).

Conclusion:

Twenty (20) items including chloramines B and T, n-butyl benzoic acid, n-propyl benzoic acid, p-oxy benzoic acid, propionic acid, butyl salicylate and the ester of the same in addition to 12 preservatives and antimicrobials (refer to Table 1) which have been approved as food additives, were treated in the same manner as in the previous report and their effect upon the life cycles of chlorella and sake yeast (saccharomyces cerevisiae var. S ke) was examined. As a result, the following conclusion was obtained:

1. Insofar as chlorella and saccharomyces cerevisiae var. Sake (sake yeast) are concerned, it is found comparatively easily possible to determine the bacteriostatic and bactericidal effects of preservatives and antimicrobials on the basis of a comparison of discoloration, color fading and the number of individual cells before and after culturing.

2. The safety of preservatives is determined by the size of the difference in the concentratinn shown by the bactericidal effect and the concentration showing the bacteriostatic action. If a multiple dilution method is used in this operation, it will become more advantageous as a safety testing method. However, the safety in both micro-organisms and safety in the living cells do

not go side by side. Thus, they are significant only as references.

3. The change in pH has a great effect upon the development of toxicity in the acidic preservatives. In acidity, benzoic acid, salicylic acid and sorbic acid, etc. show a powerfully inhibitive character. When culturing is carried out after pH has been adjusted to 6.5, its effect is markedly mitigated.

4. The esters showed a markedly strong inhibitive and toxic nature as compared with their free acids.

5. As a test on the combination of food additives and nutrients, method B was carried out this time in which dextrose was added relative to chlorella. Since the multiple dilution method was not used, however, no mentionable difference was observed.

Even though the current test was conducted by using chlorella and Sake yeast (saccharomyces cerevisiae var. Sake) as the object, the result obtained was found to be in agreement with the determinations obtained relative to safety in the result of examinations of the metabolic mechanism in living bodies and in the conventional animal tests. Only in the case of salicylic acid, the result obtained was not in agreement with the determination of "not suitable as a food additive" as described in the report of the WHO/FAO Food Additive Joint Committee.

At the end of this report, the authors would like to express their deep gratitude to Dr. Otoharu Ishizaka, Professor, School of Pharmology, Municipal University of Nagoya, who was kind enough to guide them and proof-read the entire manuscript.

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クロレラおよび清酒酵母による食品添加物の毒性試験（第2報）

保存料，殺菌料の[illegible]殺菌作用（1）

（昭和42年6月28日受理）

山本　栄*　伊東洋子*　野田昇治*

Toxicity Test of Food Additives by the Disturbance of Life Cycles of *Chlorella* and *Saccharomyces cerevisiae* var. *Sake* (II).

Bacteriostatic or Bactericidal Action of Food Preservatives and Antimicrobials (1).

Sakae YAMAMOTO, Yoko ITO and Shoji NODA

(The Research Institute of Shochikubai Wine Brewing Co., LTD.: Hama-cho, Nishinomiya, Hyogo)

According to the previous method, twelve sorts of official food preservatives and antimicrobials, chloramine B and T, *n*-butyl and *n*-propyl benzoate, *p*-oxybenzoate, propionic acid, butylsalicylate, and other carboxylic esters were examined. Results of the test were as follows:

1. By the disturbance of life cycles of *Chlorella* and *Saccharomyces*, the bacteriostatic or bactericidal action was inspected easily as a comparative reference to bacteria.
2. This method might be available as a comparative safety test of food preservatives or antimicrobials when used by the multiple dilution culture.
3. In the case of acidic preservatives such as benzoic acid, salicylic acid or sorbic acid, the correlation of pH between additives and medium or food had great influence to the preservative action.
4. Esters of acidic preservatives indicated generally a greater bacteriostatic action than that of the free acids.

In this paper, the effect of preservatives or antimicrobials was tested only by addition of sugar as one of five principal nutrients.

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緒　言

クロレラと清酒酵母の生活環に基づく食品添加物の毒性試験として，[illegible]は食用色素の毒性を検討し，一[illegible]予備試験法としての利用価値を認めたので，今回は全[illegible]

Table 1. Food Preservatives and Antimicrobials Examined by This Toxicity Test

Preservatives:

1. Benzoic acid
2. Sodium benzoate
3. Salicylic acid
4. Sorbic acid
5. Potassium sorbate
6. Sodium sorbate
7. [illegible] acid (D.H.A.)
8. [illegible] hydroacetate
9. Isobutyl-*p*-oxybenzoate (Isobutyl *p*-OB)
10. Isopropyl-*p*-OB
11. Ethyl-*p*-OB
12. *Secondary* Butyl-*p*-OB
13. Butyl-*p*-OB
14. Propyl-*p*-OB
15. Calcium propionate
16. Sodium propionate
17. Lauryl dimethyl ammonium 2,4,5 trichlorophenoxyacetate

Antimicrobials:

1. 2 (2 Furyl) 3 (5-nitro-2-furyl) acrylamide (AF 2)
2. Chloramine B
3. Chloramine T

* 松竹梅酒造株式会社：兵庫県西宮市浜町 13-10

Table 2. Influence of 0.1% Food Preservatives on *Chlorella* [illegible]

Food preservatives		Cell number			Cell diameter		
		[illegible]		Sample/Std.	(μ)	f/i	
Group 1							
Arnon standard	first	1.5[illegible]	1.0[illegible]		3.30	1.00	
	last	5.08	3.21	1.00	3.24	0.98	[illegible]
Salicylic acid	last	9.16		1.08	4.08		[illegible]
Benzoic acid	last	7.92		1.55	4.33		1.33
•Chloramine B	last	0.56		0.10			
•Chloramine T	last	0.98		0.19			
Sorbic acid	last	0.82		0.16	4.15		1.28
•D. H. A	last	0.78		0.15	4.16		1.42
Group 2							
Arnon standard	first	1.41	1.00		4.09	1.00	
	last	2.43	1.82	1.00	5.47	1.33	1.00
•Na-Benzoate	last	1.01		0.42	3.86		0.71
•K-Sorbate	last	1.26		0.52	3.82		0.70
•P-O-B-Butyl	last	1.23		0.51			
•P-O-B-Ethyl	last	1.28		0.53	3.71		0.67
•P-O-B-Propyl	last	1.41		0.56	2.52		0.46
Group 3							
Arnon standard	first	1.1	1.00		3.91	1.00	
	last	39.2	35.6	1.00	3.62	0.93	1.00
n-Butyl-benzoate	last	48.0		1.20	4.37		1.21
•*n*-Propyl-benzoate	last	9.2		0.20	3.56		0.99
Prop[illegible]	last	[illegible]		[illegible]	4.5[illegible]		1.2[illegible]
P. O. P	last	58.0		1.50	4.44		1.23
Group 4							
Arnon standard	first	1.54	1.00		4.04	1.00	
	last	2.65	1.72	1.00	3.90	0.97	1.00
•Chloramine B	last	1.21		0.46	2.91		0.75
•Chloramine T	last	1.39		0.52	2.92		0.75
•Na-D. H. A.	last	1.23		0.46	3.05		0.78
•Ca-Propionate	last	1.40		0.53	4.67		1.20
•AF-2	last	1.31		0.49	2.81		0.72
Group 5							
Arnon standard	first	1.43	1.00		3.48	1.00	
	last	2.92	2.04	1.00	3.78	1.09	1.00
•Benzoic acid	last	1.93		0.66	4.02		1.06
P. O. B.	last	10.1		3.46	4.55		1.20
•*n*-Propyl-benzoate	last	1.41		0.48	3.00		0.79
•P-O-B-Butyl	last	1.40		0.48	2.54		0.6[illegible]
•*n*-Propyl benzoate	last	1.11		0.38	3.41		0.89
•P-O-B-Propyl	last	1.17		0.40	3.00		[illegible]
Salicylic acid	last	1.25		0.43	3.49		0.91
•Butyl salicylate	last	1.28		0.44	3.24		[illegible]
•Ethyl salicylate	last	1.02		0.35	3.40		0.89

•, ∘: discolor strong or weakly
P-O-B: *p*-Hydroxybenzoic acid
f/i: Ratio of cell number or diameter between at first and last periods of culture

[illegible] Table 1 に示す殺菌料，保存料について行なった．

保存料は[illegible]用を目的とするため，これらの微生物に低濃度で再現の阻害性を示し，かつ高濃度においても低い毒性を示す物質は，安全性も高く保存料としての価値が高いということになる．また保存料は，一般細菌に対し選択的毒性を示すため，クロレラや酵母に対し安全性の高いものは，ただちに生体細胞に対して安全性が高いという推定は成立しない．

しかし，従来保存料の個々について行なわれた動物試験[illegible]や，生体内代謝代[illegible]の究明結果と照合したとき，生体細胞に安全性の高いと結論されているものは，今回の簡易試験においても，微生物の生活環阻害度が低い結果となった．

なお，この簡易試験では，同一条件下で多くの保存料について阻害性の比較が容易にできるため，酸性保存料そのものとエステル型のものとの比較を行なった結果，両微生物の至適pH 4.5～7.0程度ではエステル型の[illegible]かに阻害性が高いという結論を得た．

実験方法

試料：市販試薬特級品を用いた（ただし現在保存料，殺菌料として食品添加不許可のものも含む）．

藻，菌種：*Chlorella vulgaris* と *Saccharomyces cerevisiae* var. *Sake*，醸造試験所 No. 6

培養液：前報と同様，クロレラ用にA培地（Arnon 培地）とB培地（Arnon 培地 +1% glucose −0.01% Mg

Table 3. Influence of 0.01% Food Preservatives on *Chlorella vulgaris* in A Medium

Food preservatives		Cell number (per ml) 10^8	f/l	Sample/Std.	Cell diameter (μ)	f/l	Sample/Std.
Group 1							
Arnon standard	first	0.77	1.00		3.14	1.00	
	last	1.19	1.55	1.00	3.82	1.22	1.00
Salicylic acid	last	3.48		2.92	3.78		0.99
Benzoic acid	last	2.00		1.69	4.11		1.08
•Chloramine B	last	0.63		0.53			
•Chloramine T	last	0.58		0.48			
Sorbic acid	last	1.08		0.91	4.52		1.19
•D. H. A.	last	0.71		0.60	3.56		0.93
Group 2							
Arnon standard	first	0.82	1.00		3.83	1.00	
	last	2.92	3.56	1.00	3.73	0.99	1.00
Na-Benzoate	last	3.21		1.10	4.63		1.21
K-Sorbate	last	1.24		0.42	4.49		1.19
•P-O-B-Butyl	last	0.59		0.21			
•P-O-B-Ethyl	last	0.70		0.24	4.27		1.15
•P-O-B-Propyl	last	0.80		0.27	3.03		0.81
Group 3							
Arnon standard	first	1.22	1.00		4.03	1.00	
	last	1.60	1.36	1.00	3.57	0.88	1.00
•n-Butyl-benzoate	last	1.23		0.80	3.51		0.98
•n-Propyl-benzoate	last	1.18		0.74	3.66		1.02
Propionic acid	last	1.67		1.04	3.61		1.01
P. O. B.	last	1.38		0.89	3.71		1.04
Group 4							
Arnon standard	first	0.72	1.00		3.53	1.00	
	last	3.51	4.88	1.00	3.65	1.03	1.00
•Chloramine B	last	1.19		0.34	3.24		0.89
•Chloramine T	last	1.84		0.52	3.55		0.97
•Na-D. H. A.	last	1.31		0.37	4.22		1.16
Ca-Propionate	last	4.46		1.27	3.73		1.02
AF-2	last	1.28		0.36	3.59		0.99

Table 4. Influence of 0.1% Preservatives on *Chlorella vulgaris* in B Medium

Food preservatives		Cell number (per ml) 10^4	f/l	Sample/Std.	Cell diameter (μ)	f/l	Sample/Std.
Group 1							
Arnon standard	first	0.66	1.00		4.00	1.00	
	last	1.69	2.56	1.00	5.47	1.37	1.00
•Salicylic acid	last	0.60		0.36			
•Benzoic acid		0.56		0.33			
Chloramine T		0.51		0.30	5.47		1.00
•Sorbic acid		0.52		0.30	2.88		0.53
•D. H. A.		0.63		0.35			
Group 2							
Arnon standard	first	0.54	1.00		3.61	1.00	
	last	30.5	5.65	1.00	3.99	1.10	1.00
Salicylic acid	last	19.2		0.63	5.42		1.36
Benzoic acid		12.8		0.42	6.02		1.50
•Chloramine B		0.70		0.22			
•Chloramine T		0.47		0.02	3.39		0.85
Sorbic acid		13.1		0.43	5.88		1.48
•D. H. A.		0.62		0.02			
Group 3							
Arnon standard	first	0.50	1.00		4.11	1.00	
	last	57.8	11.6	1.00	5.15	1.24	1.00
•Na-Benzoate	last	1.51		0.26	3.89		0.74
•K-Sorbate		0.40		0.08	3.90		0.76
•P-O-B-Butyl		0.44		0.08	3.54		0.67
•P-O-B-Ethyl		0.46		0.08	3.95		0.77
•P-O-B-Propyl		0.4[illegible]		0.07	3.35		0.65
Group 4							
Arnon standard	first	1.0[illegible]	1.00		3.91	1.00	
	last	5.9[illegible]	5.67	1.00	4.57	1.14	1.00
•n-Butyl-benzoate	last	0.79		0.13	3.84		0.84
•n-Propyl-benzoate		1.17		0.20	4.68		1.02
Propionic acid		11.6		1.96	4.71		1.03
P-O-B		7.40		1.25	4.95		1.08
Group 5							
Arnon standard	first	1.54	1.00		4.04	1.00	
	last	49.1	31.9	1.00	4.89	1.17	1.00
Chloramine B	last	1.[illegible]0		0.35	5.45		1.11
Chloramine T		2.[illegible]		0.45	5.01		1.02
•Na-D. H. A.		1.[illegible]		0.025	3.73		0.71
Ca-Propionate		1.50		0.03	4.66		0.95
•AF-2		1.51		0.03	2.85		0.59
Group 6							
Arnon standard	first	1.43	1.00		3.48	1.00	
	last	45.7	31.9	1.00	4.88	1.29	1.00
Benzoic acid	last	1.65		0.03	4.35		0.89
P. O. B.		24.[illegible]		0.53	4.10		0.84
•n-Butyl benzoate		1.39		0.03	3.73		0.77
•P-O-B-Butyl		1.[illegible]4		0.025	2.85		0.58
•n-Propyl benzoate		1.[illegible]9		0.03	2.96		0.61
•P-O-B-Propyl		1.[illegible]2		0.03	3.10		0.63
•Salicylic acid		7.90		0.18	5.78		1.18
•Butyl salicylate		1.28		0.026	3.53		0.72
•Ethyl salicylate		1.45		0.03	3.08		0.63

Table 5. Influence of 0.01% Food Preservatives on *Chlorella vulgaris* in B Medium

Food preservatives		Cell number (per ml) 10^8	f/l	Sample/Std.	Cell diameter (μ)	f/l	Sample/Std.
Group 1							
Arnon standard	first	9.6	1.00		3.55	1.00	
	last	19.9	2.07	1.00	5.43	1.51	1.00
Salicylic acid	last	24.2		1.22	4.67		0.86
Benzoic acid	last	14.6		0.73	4.90		0.90
Chloramine B	last	7.7		0.88	5.52		1.02
Chloramine T	last	21.5		1.07	4.79		0.88
Sorbic acid	last	9.4		0.47	4.49		0.83
•D. H. A.	last	1.5		0.07	4.78		0.88
Group 2							
Arnon standard	first	0.88	1.00		3.85	1.00	
	last	28.0	31.1	1.00	5.69	1.48	1.00
Na-Benzoate	last	17.4		0.62	4.35		0.77
K-Sorbate	last	14.8		0.53	5.18		0.91
•P-O-B-Butyl	last	0.84		0.03	4.51		0.80
P-O-B-Ethyl	last	0.98		0.03	6.26		1.10
•P-O-B-Propyl	last	1.18		0.04	3.79		0.67
Group 3							
Arnon standard	first	1.22	1.00		4.02	1.00	
	last	1.40	1.14	1.00	4.04	1.15	1.00
n-Butyl-benzoate	last	1.64		1.17	6.30		1.36
n-Propyl-benzoate	last	1.73		1.23	4.37		0.93
Propionic acid	last	1.25		0.90	4.78		1.03
P. O. B.	last	1.38		0.99	4.86		1.05
Group 4							
Arnon standard	first	0.72	1.00		3.52	1.00	
	last	32.2	46.0	1.00	4.92	1.39	1.00
Chloramine B	last	31.0		0.97	4.52		0.92
Chloramine T	last	31.2		0.97	4.59		0.93
•Na-D. H. A.	last	1.42		0.04	4.64		0.94
Ca-Propionate	last	31.7		0.98	5.05		1.02
•AF-2	last	1.43		0.04	4.62		0.94

$(OAc)_2$), 酵母用に Henneberg 培地を用いた.

操作法: 乾熱滅菌済みの Morton 栓付き試験管に, 保存料の最終濃度が 0.1%, 0.01%/10 ml となるよう, 培養液を入れ, 0.1%の場合は, 保存料を 10 mg, 0.01%の場合は, その 0.1%溶液 1 ml を加え, すべて 5% $NaHCO_3$ 溶液で pH 6.5 に調整する. ただし, クロレラの B培地培養の際の Table 4 の Group 1 のみは, pH 調整操作を行なわなかった. 以下の操作は全く前報と同様である. 不溶性のものは, 沸騰水浴中で溶解し, 熱時溶解状態のものの一定量を培地に注加した.

検査項目: 前報と全く同様.

実験結果

保存料を0.1%, 0.01%になるよう, Table 2, Table 3 はクロレラA培地に, Table 4, Table 5 はB培地に, Table 6, Table 7 は酵母の Henneberg 培地に添加し, それぞれ培養した結果を示したものである. Table 8 は Table 2~7 までの実測値の標準に対する試験群の比率だけをまとめたものである. Table 中・印は, 変色性を示し, これらはクロレラのクロロフィルの緑色を退色または脱色し, 酵母の乳白色を黄色化, 退色, 脱色する. この作用をうけた微生物の細胞は, 再度培養の結果, もはや生存力がないので, これを殺菌作用とみなし, また脱色, 退色はみられず個体細胞数の減少または停止作用をみたものを静菌作用とし区別した. クロレラで 0.01%添加の場合は, 殺菌作用はごく一部に限られ, 静菌作用のみがみられ, 酵母では 0.1% でも殺菌的より静菌的

Table 6. Influence of 0.1% Food Preservatives on *Saccharomyces cerevisiae* var. *Sake*

Food preservatives		Cell number (per ml) 10^4	f/l	Sample/Std.	Cell diameter (μ)	f/l	Sample/Std.
Group 1							
Henneberg standard first		1.28	1.00		5.37×4.59	1.00	
Henneberg standard last		2.99	2.29	1.00	5.30×4.16	0.98×0.92	1.00
Salicylic acid	last	2.98		1.00	5.07×4.12		0.96×0.91
Benzoic acid	last	2.16		0.72	5.58×4.44		1.05×1.06
•Chloramine B	last	2.82		0.94	4.99×3.93		0.91×0.95
•Chloramine T	last	2.17		0.72	4.81×4.16		0.91×1.00
Sorbic acid	last	1.04		0.35	5.81×4.65		1.10×1.11
•D. H. A.	last	0.54		0.18	5.33×4.48		1.01×1.08
Group 2							
Henneberg standard first		0.21	1.00		5.75×4.44	1.00	
Henneberg standard last		3.37	16.0	1.00	5.61×4.31	0.98×0.97	1.00
•Na-Benzoate	last	2.05		0.61	5.66×4.83		1.00×1.11
•K-Sorbate	last	1.07		0.32	5.92×4.73		1.06×1.09
•P-O-B-Butyl	last	0.27		0.08			
•P-O-B-Ethyl	last	0.32		0.09	5.48×4.37		0.99×1.01
•P-O-B-Propyl	last	0.25		0.08			
Group 3							
Henneberg standard first		0.21	1.00		5.33×4.29	1.00	
Henneberg standard last		3.24	15.4	1.00	5.88×4.38	1.01×1.02	1.00
n-Butyl-benzoate	last	2.30		0.71	5.87×4.53		1.00×1.03
•*n*-Propyl-benzoate	last	2.96		0.91	5.28×4.14		0.90×0.94
Propionic acid	last	1.92		0.59	5.90×4.38		1.00×1.00
P. O. B.	last	2.31		0.71	5.94×4.37		0.97×1.00
Group 4							
Henneberg standard first		0.11	1.00		5.89×4.54	1.00	
Henneberg standard last		3.88	35.3	1.00	5.64×4.44	0.96×0.97	1.00
Chloramine B	last	3.46		0.89	5.91×4.50		1.05×1.01
Chloramine T	last	2.58		0.65	4.75×4.65		1.02×1.04
•Na-D. H. A.	last	0.22		0.06	5.72×4.45		1.02×1.00
Ca-Propionate	last	1.92		0.59	5.89×4.42		1.04×0.99
•AF-2	last	0.12		0.03	5.92×4.82		1.05×1.09
Group 5							
Henneberg standard first		0.18	1.00		5.59×4.36	1.00	
Henneberg standard last		3.20	18.0	1.00	5.53×4.21	1.00×0.96	1.00
Benzoic acid	last	1.56		0.48	5.64×4.47		1.02×1.02
P. O. B.	last	2.50		0.78	5.17×4.37		0.93×1.03
•*n*-Butyl-benzoate	last	0.25		0.08	5.04×3.39		0.92×0.94
•P-O-B-Butyl	last	0.20		0.06	4.92×3.60		0.89×0.85
n-Propyl benzoate	last	1.4[illegible]		0.44	5.39×4.32		0.9[illegible]×1.02
•P-O-B-Propyl	last	0.21		0.06	5.28×4.39		0.95×1.0[illegible]
Salicylic acid	last	2.69		0.84	5.[illegible]×4.24		0.92×1.0[illegible]
•Butyl salicylate	last	0.19		0.06	5.10×3.99		0.92×0.94
•Ethyl salicylate	last	0.24		0.07	5.11×3.95		0.92×0.89

•, ∘: Color change strong or weak

Table 7. Influence of 0.01% Food Preservatives on *Saccharomyces cerevisiae* var. *Sake*

Food preservatives		Cell number (per ml) 10^8	f/l	Sample/Std.	Cell diameter (μ)	f/l	Sample Std.
Group 1							
Henneberg standard first		1.07	1.00		5.07×4.14	1.00	
Henneberg standard last		3.72	3.48	1.00	5.48×4.14	1.08×0.99	1.00
Salicylic acid	last	3.02		0.96	4.52×4.28		0.82×1.04
Benzoic acid	last	2.46		0.66	5.48×4.37		1.00×1.06
Chloramine B	last	2.72		0.73	5.49×4.42		1.00×1.07
•Chloramine T	last	3.45		0.93	5.11×3.97		0.93×0.91
Sorbic acid	last	1.96		0.53	5.74×4.25		1.29×1.02
•D. H. A.	last	2.40		0.64	5.53×4.29		0.82×1.03
Group 2							
Henneberg standard first		1.34	1.00		5.19×4.38	1.00	
Henneberg standard last		34.9	26.0	1.00	5.55×4.40	1.07×1.01	1.00
Na-Benzoate	last	28.6		0.81	5.51×4.39		0.99×1.00
K-Sorbate	last	20.3		0.58	5.63×4.59		1.01×1.04
•P-O-B-Butyl	last	18.6		0.53	5.34×4.57		0.96×1.04
P-O-B-Ethyl	last	19.8		0.57	5.12×4.48		0.92×1.01
•P-O-B-Propyl	last	22.8		0.65	5.69×4.38		1.02×0.99
Group 3							
Henneberg standard first		1.41	1.00		5.52×4.75	1.00	
Henneberg standard last		3.68	2.61	1.00	5.44×4.15	0.98×0.98	1.00
n-Butyl-benzoate	last	3.64		0.99	5.41×4.22		0.99×1.01
n-Propyl-benzoate	last	3.26		0.88	5.33×4.23		0.97×1.01
Propionic acid	last	3.20		0.87	5.50×4.20		1.01×1.01
P. O. B.	last	3.02		0.82	5.68×4.41		1.05×1.06
Group 4							
Henneberg standard first		1.77	1.00		5.55×4.32	1.00	
Henneberg standard last		28.3	16.0	1.00	5.65×4.17	1.02×0.96	1.00
Chloramine B	last	14.2		0.50	5.55×4.18		0.98×1.00
Chloramine T	last	26.3		0.93	5.48×4.04		0.97×0.97
Na-D. H. A.	last	16.6		0.98	5.69×4.22		1.01×1.01
Ca-Propionate	last	28.4		1.00	5.64×4.25		1.00×1.01
•AF-2	last	3.60		0.13	6.76×4.82		1.20×1.15

作用を受けているものの方が多かった.

pH 調整を行なっていない Table 8 の Group 1 を, 調整後の値と比較すると, サリチル酸, 安息香酸, ソルビン酸は pH が低いほど毒性, 殺菌性の強く現われるのが認められた.

殺菌料のクロラミンBとTは, いずれも脱色し, 殺菌性を示した. D. H. A. は保存料ではあるが, Group 2 のように, 静菌的に殺菌的に[illegible]る. ソルビン酸は pH 調整後は, 個体細胞の増殖抑制のみ現われ, 静菌作用を示した.

Group 3 では, 安息香酸ナトリウム, ソルビン酸カリウムは, *p*-オキシ安息香酸エステルに比べ, 阻害性弱く, 脱色も 0.1% においてみられ, エステル類はいずれも強い殺菌力が認められた.

Group 4 では, 安息香酸のブチル, プロピルエステルが, 遊離の *p*-オキシ安息香酸やプロピオン酸に比べ強い阻害性を示した.

Group 5 [illegible]るクロラミンBとTは, Group 2 の同じ[illegible]比べ数値上の差異はあるが, 0.1% [illegible], 脱色性が認められ[illegible]ら, 殺菌力は[illegible]と判断される. D. H. A. Na, AF-2 [illegible]い殺菌性を[illegible]プロピオン酸-Ca は脱色性はみられず殺菌的ではな[illegible].

Group 6 は, 安息香酸, *p*-オキシ安息香酸, サリチル酸と, これらのエステル類の比較検査の結果を示し, 明らかにエステル類の強い阻害性がみられた.

Table 8. Summary of Cell Number and Cell Diameter Ratios

Food preservatives	Cell number A	Cell number B	Cell number Yeast	Cell number A	Cell number B	Cell number Yeast	Cell diameter A	Cell diameter B	Cell diameter Yeast	Cell diameter A	Cell diameter B	Cell diameter Yeast
	0.1%			0.01%			0.1%			0.01%		
Group 1												
Standard		2.56						1.37				
Salicylic acid		0.36										
Benzoic acid		0.33										
Chloramine T		0.30						1.00				
Sorbic acid		0.30						0.35				
D. H. A.		0.35										
Group 2												
Standard	3.21	5.65	2.29	1.55	2.02	3.48	0.98	1.10	0.98×0.92	1.22	1.51	1.08×0.99
Salicylic acid	1.80	0.63	1.00	2.92	1.22	0.91	1.26	1.36	0.96×0.91	0.99	0.86	0.82×1.04
Benzoic acid	1.55	0.42	0.72	1.69	0.73	0.66	1.33	1.50	1.05×1.06	1.08	0.90	1.00×1.06
Chloramine B	1.10	0.22	0.94	0.53	0.88	0.73			0.91×0.95		1.02	1.00×1.07
Chloramine T	0.19	0.02	0.72	0.48	1.07	0.93		0.85	0.91×1.00		0.88	0.93×0.91
Sorbic acid	0.16	0.43	0.35	0.91	0.47	0.54	1.28	1.48	1.10×1.11	1.19	0.83	1.29×1.02
D. H. A.	0.15	0.02	0.18	0.60	0.07	0.64	1.42		1.01×1.08	0.93	0.88	0.82×1.03
Group 3												
Standard	1.82	11.6	16.0	3.56	31.1	26.0	1.33	1.24	0.98×0.97	0.99	1.48	1.07×1.01
Na-Benzoate	0.42	0.26	0.61	1.10	0.62	0.81	0.71	0.74	1.00×1.11	1.21	0.77	0.99×1.00
K-Sorbate	0.52	0.08	0.32	0.42	0.53	0.58	0.70	0.76	1.06×1.09	1.19	0.91	1.01×1.04
P-O-B-Butyl	0.51	0.08	0.08	0.21	0.03	0.53		0.69			0.80	0.96×1.04
P-O-B-Ethyl	0.53	0.08	0.09	0.24	0.03	0.57	0.67	0.77	0.99×1.01	1.15	1.10	0.92×1.01
P-O-B-Propyl	0.56	0.07	0.08	0.27	0.04	0.65	0.46	0.65		0.81	0.67	1.02×0.99
Group 4												
Standard	35.6	5.67	15.4	1.36	1.14	2.61	0.93	1.14	1.01×1.02	0.88	1.15	0.98 0.98
n-Butyl-benzoate	1.20	0.13	0.71	0.80	1.17	0.99	1.21	0.84	1.00×1.03	0.98	1.36	0.99×1.01
n-Propyl-benzoate	0.20	0.20	0.91	0.74	1.23	0.88	0.99	1.02	0.90×0.94	1.02	0.93	0.97×1.01
Propionic acid	1.50	1.96	0.59	1.04	0.90	0.87	1.26	1.03	1.00×1.00	1.01	1.03	1.01×1.01
P-O-B	1.50	1.25	0.71	0.89	0.99	0.82	1.23	1.08	0.97×1.00	1.04	1.05	1.05×1.06
Group 5												
Standard	1.72	31.9	35.3	4.88	46.0	16.0	0.97	1.17	0.96×0.97	1.03	1.39	1.02×0.96
Chloramine B	0.46	0.35	0.89	0.34	0.97	5.00	0.75	1.11	1.05×1.01	0.89	0.92	0.98×1.00
Chloramine T	0.52	0.45	0.65	0.52	0.97	0.93	0.75	1.02	1.02×1.04	0.97	0.93	0.97×0.97
Na-D. H. A.	0.46	0.02	0.06	0.37	0.04	0.98	0.78	0.71	1.02×1.00	1.16	0.94	1.01×1.01
Ca-Propionate	0.53	0.03	0.59	1.27	0.98	1.00	1.20	0.95	1.04×0.99	1.02	1.02	1.00×1.01
AF-2	0.49	0.03	0.03	0.36	0.04	0.13	0.72	0.59	1.05×1.09	0.99	0.94	1.20×1.15
Group 6												
Standard	2.04	31.9	18.0				1.09	1.29	1.00×0.96			
Benzoic acid	0.66	0.03	0.48				1.06	0.89	1.02×1.02			
P-O-B	3.46	0.53	0.78				1.20	0.84	0.93×1.03			
n-Butyl-benzoate	0.48	0.03	0.08				0.79	0.77	0.92×0.94			
P-O-B-Butyl	0.48	0.02	0.06				0.67	0.58	0.89×0.85			
n-Propyl-benzoate	0.38	0.03	0.41				0.89	0.61	0.97×1.02			
P-O-B-Propyl	0.40	0.03	0.06				0.79	0.63	0.95×1.04			
Salicylic acid	0.43	0.18	0.84				0.91	1.18	0.92×1.00			
Butyl salicylate	0.44	0.06	0.06				0.86	0.72	0.92×0.94			
Ethyl salicylate	0.35	0.07	0.07				0.89	0.63	0.92×0.89			

A, B, Yeast: Sort of Test method

Table 9. Summary of Test Results on *Chlorella* and *Saccharomyces*

Preservatives and antimicrobials	Influence on *Chlorella vulgaris*				Influence on *Saccharomyces cerevisiae* var. *Sake*	
	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
Group 1						
Salicylic acid			•pH: 4.2 distorted. slightly green.			
Benzoic acid			•pH: 4.4			
Chloramine T			pH: 6.0 green. Cells in divisional term are few.			
Sorbic acid			•pH: 4.4 light green. Number of cell is few.			
D. H. A.			•pH: 5.2~5.4 slightly green. Cells are few and small.			
Group 2						
Salicylic acid	Green color is deeper than that of standard. Number of cells is more than that of standard.		Cell are many. Large cells are many.	4.2~5.6 μ cells are many. Cells in divisional term are few		Unchanged.
Benzoic acid	Diameter is larger that of standard.			Small cells ary many, too.		Unchanged.
Chloramine B	•Almost white.	•Discolor.	•Almost discolor. Cells are few.	•White.	•Slightly yellow.	
Chloramine T	•Almost white.	•White.	•		•Slightly yellow.	Small cells are many. Germinating cells are few.
Sorbic acid	Scarcely changed.	Green. Unchanged.	Color is deeper than that of std.	•Light green. Cell is abnormal.		Unchanged.
D. H. A.	•Slightly green.	•White.	•White. Light green cells are few.	•Scarcely green.	•Slightly yellow.	Number of cells and germinating cells are few.
Group 3						
Na-Benzoate	•Slightly green.	Scarcely changed. Cells in divisional term are few.	•Light green. Elliptic cells are few.	Green color ≒ std.	Inhibit. Fragrant.	Scarcely changed

Table 9. Continue

Preservatives and antimicrobials	Influence on *Chlorella vulgaris*				Influence on *Saccharomyces cerevicine* var. *Sake*	
	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
K-Sorbate	•Slightly green.		•Slightly green	Green≒std.	•Scarcely inhibit. Fragrant.	Scarcely changed.
P-O-B-Butyl	•Slightly green. Cell is abnormal.	•Discolor.	•Discolor.	•Slightly green.	•Inhibit. No growth and no multiplication. Infragrant.	Inhibit. Fragrant.
P-O-B-Ethyl	•Light green.	•Light green. Inhibit. -Butyl.	•Discolor. Cell is abnormal.	•Green > -Butyl.	•Diameter and number > -Butyl.	
P-O-B-Propyl	•Slightly green.	•Scarcely green.	•Discolor. Cell is small.	•Scarcely green.	•Cell is abnormal as the case of -Butyl.	Slightly inhibit.
GROUP 4						
n-Butyl-benzoate	Green. Scarcely changed.	Small cells are many.	Scarcely green. Small.	Small cells are many.	Inhibit.	Scarcely unchanged.
n-Propyl-benzoate	•Discolor. Cell is abnormal.	〃	•Discolored cells are many.	Unchanged.	Number of cell is few. Cell form is distorted.	Scarcely unchanged.
Propionic acid	Unchanged.	Scarcely unchanged.	Deep green.	Unchanged.	Scarcely changed. Bubbling is active	Scarcely unchanged.
p-Oxy-benzoate	〃	〃	Large cells are many.	〃	No inhibition.	〃
GROUP 5						
Chloramine B	•Discolor. Cell is distorted. Small.	•Almost white. Small cells are many.	Normal. Large cells are many.	≒standard. Da cells or small cells are many.	≒standard. Slightly yellow.	Diameter < std.
Chloramine T	•Scarcely green. 〃	•White. Diameter < B	Normal. Diameter < B	≒std.	〃	〃
Na-D. H. A.	•〃 〃	•Large and white cell.	•Small cells are many.	•Slightly green.	•Discolor. Cells are few.	
Ca-Propionate	Slightly changed and abnormal.	Normal.	Normal.	≒std.		Cells are few. Cell is large.
AF-2	•Dye cell to brown. Cell is distorted.	•Dye cell to orange.	•Dye cell to orange. Cell is distorted.	•Yellow. Cells are few. No multiplication.	•Dye cell to orange. Cell is distorted.	•Inhibit. Dye cell to orange.
GROUP 6						
Benzoic acid	Green > std. Small cells are few.	(pH: 5.0)	Inhibited cells are few. Normal.	(pH: 5.0 ~5.2)	Normal.	(pH: 4.5)
p-Oxy-benzoate	Deep green > std. Normal.	(pH: 6.6 ~6.8)	Deep green. Normal.	(pH: 6.8)	Normal.	(pH: 4.4 ~4.6)
n-Butyl-benzoate	•White. Cell is distorted. Small.	(pH: 4.2 ~4.4)	•Light green.	(pH: 5.6 ~5.8)	•No fragrant.	(pH 4.8 ~5.0)

Table 9. Continue

Preservatives and antimicrobials	Influence on *Chlorella vulgaris*				Influence on *Saccharomyces cerevisiae* var. *Sake*	
	(A) 0.1%	0.01%	(B) 0.1%	0.01%	0.1%	0.01%
p-O. B.-Butyl	•White, small and abnormal.	(pH: 4.2 ~4.4)	•White, small and distorted.	(pH: 5.6 ~5.8)	•No fragrant.	(pH 5.6: ~5.8)
n-Propyl-benzoate	•Discolor. Small and abnormal.	(pH: 4.2)	•White, small.	(pH: 5.2 ~5.4)	Fragrant.	(pH: 4.6 ~4.8)
P. O. B.-Propyl	•White.	(pH: 4.2 ~4.4)	•White. Abnormal.	(pH: 5.2 ~5.4)	•No fragrant.	(pH: 5.6 ~5.8)
Salicylic acid	•Discolor.	(pH: 4.2 ~4.4)	Green. Large cells are many. Active multiplication.	(pH: 6.2 ~6.4)	Fragrant.	(pH: 4.4 ~4.6)
Butyl salicylate	•Distorted	(pH: 4.2)	•White. Cell membrane is broken.	(pH: 5.6)	•No fragrant.	(pH: 5.4 ~5.6)
Ethyl salicylate	•White.	(pH: 4.2)	•White.	(pH: 5.6)	•〃	(pH: 5.6 ~6.0)

考　察

今回の試験結果を要約すれば，Table 9 に示すごとくで，以下主要項目について考察する．

栄養素： 食品添加物は，本来食品に添加された状態ではじめて意味がある．しかし食品の種類は多種多様で，個々のものについて行なうことはきわめて困難なため，食品を，その主栄養素別に分類し，それら単一栄養素と添加物との組合わせで試験を行なえば，理論上も実際上も系統的にその利用価値が判定できると考えられる．クロレラのB培地[11]と清酒酵母の Henneberg 培地はともにブドウ糖を含み，炭水化物食品に添加物を加えた状態での試験と解釈される．クロレラ法のA，B培地，すなわち炭水化物の含不含の差は明確にはでなかったが，ブドウ糖がクロレラの炭素源として増殖を著しく促進するため，試験群と標準との比較が容易に行なわれた．

pH[12]： 酸性で保存性を十分示すものが，添加食品の性質により中和され保存料としての目的を果さない場合が考えられる．したがって，保存料の阻害性の比較を行なうのに，本実験では添加物を添加した培地の至適 pH を 6.5 に調整し，pH の違いによる影響をさけた．酸性の保存料では，培地の pH が酸性に傾くと，微生物細胞への移行率が増加し，それに伴って阻害効果が増すことが知られている．Table 8 の Group 1 と Group 2 からみて，pH の相違と阻害力の強度の相違が明らかに認められた．対象食品を個々の保存料，殺菌料につき指定する規定は，含有される栄養素の種類別の観点からも重要なことがわかる．

静菌作用と殺菌作用： 保存料と殺菌料は，その目的に応じそれぞれ静菌作用または殺菌作用を示すことが望ましい．クロラミンB，TおよびAF-2においては，脱色，退色がみられ殺菌料として阻害性の強いことを示した．しかし，保存料においても殺菌性を示すものがあり，殺菌料と保存料の限界は判然とは区別しがたい．本実験で0.1%では，殺菌料のほか，安息香酸，*p*-オキシ安息香酸，サリチル酸のエステル類と D. H. A. とその塩にも殺菌性脱色がみられた．安息香酸，サリチル酸，ソルビン酸はpH調整後のものは阻害性が緩和になり，プロピオン酸は全く殺菌性がなく保存料の目的にそっている．pH 調整後，*p*-オキシ安息香酸自体は全く阻害性を認められないが，このエステル類が強い阻害性を示すことは興味深い．また一般的に酵母に対するよりも，クロレラに対する方が阻害作用が強く現われている．これは両微生物の合成と分解との生活環上の相違もあるが，保存料の選択毒性に由来しているものと考えられ，再試験の必要がある．

安全性[3]： 保存料の毒性の相違は，生体内に吸収されてからの代謝機構の違いに起因し，なんらかの代謝をうけることが安全性保証の要点とされている．ソルビン酸やプロピオン酸が従来の動物試験では，なんら毒性を示していない理由として，前者は生体内代謝において CO_2 と H_2O になり，後者はクエン酸を経ず TCA サイクルにはいり完全に代謝をうけ無毒化されるので，理論的にも実際的にも非常に安全度の高い保存料とされている．本試験でも，他のものに比べ阻害性が非常に弱く，両者のうちではソルビン酸の方がやや阻害性が強いと判定された．このことは，本試験で阻害性が少ないものは安全性が高いと考えることの妥当性を示すものと考えられ，本試験が保存，殺菌料においても，安全性の予試験とし

て利用価値を認めることができる.

遊離酸とそのエステル: Table 8 の Group 6 にみられるように，エステル類はその遊離酸よりはるかに強力な阻害性，毒性を示すことがわかったが，報文によればこの毒性の差は一般に菌体表面の，たとえばリポイドと培地間における移行率の差異によるか，または代謝機構の相違からくるものと思われる[9]. 本試験では，各種エステル間の毒性強度の比較はできなかった[13].

結　論

食品添加物として許可された保存，殺菌料12品目 (Table 1) と他にクロラミンB, T, *n*-ブチル安息香酸，*n*-プロピル安息香酸，*p*-オキシ安息香酸，プロピオン酸，サリチル酸ブチル，同エステルの計20品目につき，前報の場合と同様に処理し，クロレラと清酒酵母の生活環に及ぼす影響を検査し，つぎのような結論を得た.

1. クロレラと清酒酵母に関する限り，脱色，退色現象と培養前後の個体細胞数の比較から，保存料と殺菌料について，静菌作用と殺菌作用の判別が比較的容易にできることがわかった.

2. 保存料の安全性は，静菌作用を示す濃度と殺菌作用を示す濃度の差の大小によって決定される. 本操作で倍数希釈法を適用すれば安全性試験法として有利となる. しかし，安全性は，両微生物と生体細胞とで必ずしも並行しないので，資料としてのみ意義をもつものである.

3. pH の変動は酸性保存料の毒性発現に大きく影響し，酸性において安息香酸，サリチル酸，ソルビン酸などは強力な阻害性を示すが，pH を 6.5 に調整して培養を行なうとき，その作用は著しく緩和された.

4. エステル類は，その遊離酸より著しく強い阻害，毒性を示した.

5. 食品添加物と栄養素との組合わせ試験として，今回はクロレラにブドウ糖を添加したB法を行なった. しかし倍数希釈法によらなかったため，特別注目される差異は認めえなかった.

本試験は，クロレラと清酒酵母のみを対象とした試験ではあるが，従来の動物試験や生体内代謝機構の研究結果による安全性の判定とほぼ一致した成績を得た. たゞサリチル酸は WHO/FAO 食品添加物合同委員会報告による食品添加物不適の判定とは一致しなかった.

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DATA ON THE MECHANISM OF THE EFFECT OF SORBIC ACID

Deak, F. Elelmezesi Ipar 23 (8), 251-254

Sorbic acid as a preservative became known following World War II and its use has become widespread, especially in the food insutries. The number of publications dealing with sorbic acid exceeds 1000. Most of the papers are concerned with potential applications of sorbic acid; the products in which and the microorganisms against which it is to be used. There are many publications of the chemical and physical properties and the methods of production, detection and determination of the acid. In addition, as with all preservatives, the toxic and pharmacological properties of sorbic acid were investigated at a very early date. In spite of the many studies, there is relatively little information avail able concerning the basis of the antimicrobial effect of the preservative and the mechanism of its inhibiting action. Data actually presented offer more of contradiction than of agreement. Thus, several possible mechanism of the inhibition of anaerobic metabolism by sorbic acid were described, such as alcohol dehydrogenase (Whitaker, 1959, Martiadoprowito, Whitaker, 1963), enolase (Azukas & al., 1961), or aldolase (Springer, 1964, Springer and Schegk, 1967, Rasmussed, 1967) inhibition as a method of antimicrobial effect. Other authors reported inhibition by sorbic acid of different steps in the aerobic metabolism, i.e. with fumarase (York & Vaughn, 1955), coenzyme A (Palleroni & de Pritz, 1960), cytochrome oxidase (Anderson, 1963; Anderon & Costilow, 1963), catalase (Troller, 1965) and with numerous sulfhydryl type enzymes participating in oxidation (York & Vaughn, 1964) The author began his studies with an investigation of the effect of sorbic acid on the propagation of different yeast fungi. Results were rpoerted in detail elsewhere (Deak, 1996; a), b), (Deak and Novak, 1966, 1968, 1969). (Deak, Tuske & Novak, 1969), three of the most important conclusions are summarized here. It was found that the magnitude of propagation inhibition was substantially greater under anaerobic than under aerobic conditions. The effect is also more lasting in the first case than in the second, where even higher acid concentrations lead to temporary, partial inhibition only. As an example, in Fig. 1, the

results of an investigation with Procandida Albicans are presented, as an example. It is seen that with anaerobic incubation 250 mu g/l potassium sorbate completely inhibited propagation, while with aerobic incubation, four times this amount was still insufficient.

An explanation of this phenomenon was found -- in addition to the different effect of the acid on anaerobic and aerobic metabolism -- in a decrease of the concentration of the inhibitor which occurs in aerobic cultures only. Sorbic acid determinations, performed simultaneously with propagation measurements, showed great depletion, occasionally even the complete disappearance of the inhibitor. The reduction in the sorbic acid content was found not only in live cultures but also in cell suspensions shaken in a phosphate buffer, in addition, in a synthetic nutrient composed of inorganic salts, certain yeast fungi (Procandida albicana, Pc. tropicalis, Candida claussenii, C. pseudotropicalis. C. Utilis, but not C. krusei, C. Beverwilkii, Saccharomyces cerevisiae, S. carlsbergensis) were capable of propagating on sorbic acid as the only source of carbon and energy. Results of such an experiment are shown in Fig. 2. Figures in parenthesis indicate the proportional disappearance of the initial potassium sorbate concentration. It is seen that the magnitude of propagation is proportional to the amount of sorbate consumed and assimilated.

FIGURE 1. Effect of Potassium Sorbate (KSz) of the Propagation of Procandida albicans. Saboraud Glucose Cultures with Aerobic (shaken T tubes) Incubation. ● : control; O 1000 mu g/ml KSz; or (semianaerobic standing tubes), △ : control; ▲ 250 mu g/ml KSz

Key: 1. Hours.

FIGURE 2. Potassium Sorbate Assimilation of Yeast Fungi. Synthetic nutrient with 250 mu g /ml KSz, pH 5.5. Aerobic incubation (shaken T tubes). O : Procandida albicans, _ : Candida claussenii; ● : Candida crusei ▲ : Saccharomynes cerevisiae.

TABLE 1. The Effect of Potassium Sorbatd (KSz) on the Carbohydrate Assimilation of Certain Yeast Fungi Strains

Inhibition of the propagation rate constant by 750 mu g/ml KSz. Aerobic shaken cultures in Sabouraud liquid on (D) Glucose, (S) Saccharose (M) maltose substrates.

Strain	Inhibition, %			Enzyme decomposing the oligosaccharide
	D	S	M	
C. beverwijkii	45	43	43	Invertase, endomaltase
C. claussenii	18	10	14	Endosaccharase
Pc. albicans	23	24	37	Endosaccharase
Pc. tropicalis	31	31	45	Endosaccharase
S. carlsbergensis	32	32	36	Invertase, endomaltase

The differences in the inhibition of the consumption of glucose, saccharose and maltose cannot be explained by the effect of sorbic acid on glycolysis or the enzymes of the respiratory chain, as described in the literature, because the consumption of saccharose or maltose differs from that of glucose in absorption and cleavage only. The cause of the difference in the effect of sorbic acid therefore must be sought in these two steps.

Next, therefore, the effect of sorbic acid endosaccharsase and endomaltase prepared from different strains, was examined. As shown by the results of one investigation in Fig. 3, in extracts frree of cells, the activityóf these enzymes did not vary under the effect of potassium sorbate

FIGURE 3. Effect of Potassium Sorbate (KSz) on the Oligosaccharide Decomposing Enzymes of Procandida albicans. Decomposing activity of extract free of cells on maltose (△, ▲), saccharose (O, ●), measured with the glucoseoxidase of the glucose formed. △ O : control, ▲ , ● : 1000 mu g/ml KSz.

Key: 1. Gluscose, 2. minute

These results lead to the direct investigation of the effect of sorbic acid on the assimilation and transport of the sugars. With S. cerevisiae, which transports sugar in a passive mode, by a so-called transferred or assisted diffusion, no inhibiting effect was found. It was possible, however, to find certain yeast fungi strains (Deak & Kotyk, 1969; Deal & Novak, 1969 b, c) which actively transport sugars by using the energy of metabolism, against a concentration gradient. It is shown that in the case of transport of this type, sorbic acid separates the active part of the transport from the passive, assisted diffusion, thus inhibiting the supply of energy of metabolism, necessary foractive transport. In Fig. 4, the D-xylose assimilation by Pc. albicans is shown. In the presence of sorbic acid the amount of sugar arriving in the cells with all three of the concentrations investigated, attained levels corresponding to the diffusion equilibrium only.

The third important conclusion of the investigations is related to the effect of sorbic acid on the respiration of yeast fungi. According to manometer measurements, under the effect of sorbic acid the endogenic respiration of yeast suspensions exhibited a significant increase. (Fig. 5).

FIGURE 4. D-xylose assimilation by Procandida albicans, Initial sugar concentrations; 1 (□), 5 (△) and 25 (o) mg/ml. Aerobic shaken incubation.

Potassium sorbate concentration at all three sugar levels 50 nM (▲, ■ , ●).

Key: 1. mg sugar/g dry weight, 2. minute

FIGURE 5. Effect of Potassium Sorbate on the Respiration of Pc. albicans. 50 mg wet cells, 2000 g KSz, in 0.03 M phosphate buffer, total volume 2 ml. pH: 3.0 (o), 5.5 (▲). Endogenic values (●) are the averages of oxygen consumption measured at the three pH levels. Addition of KSz at 60th minute (arrow).

Key: 1. hour, 2. minute

However, this increase in respiration is not connected with the dissociation of the inhibitor its oxidative metabolisation, because the reduction in sorbic acid occurred much later than the enhancement of oxygen intake caused by the inhibitor, the pH optimum of the two processes also differed. The enhancement of endogenic respiration by sorbic acid proved to be similar to the separating effect of 2,4-dinitrophenol), it may thus be assumed that sorbic acid inhibits the energy producing system of cells at the mechanism of terminal oxidation.

The disruption of the energy system of cells may be directly responsible for the inhibiting effect of sorbic acid on metabolism and -- as one of the primary symptoms -- transport processes associated with metabolic energy are inhibited. However, in cases where transport is by a passive mode and glycolytic energy recovery takes place under anaerobic conditions, the cause of the inhibition of metabolism and propagation by sorbic acid must be found at different points of attack. Other possible causes are indicated in the literature. It is likely that in the interpretation of the effective mechanism of sorbic acid, the possibility of several attack locations must be considered. This complex problem is far from closed. The author attempted to make a contribution to a solution by his studies summarized in the foregoing.

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Translated by Carl Demrick Associates, Inc./LR/db

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УПАКОВКА ЗАМОРОЖЕННЫХ ПОЛУФАБРИКАТОВ И ГОТОВЫХ БЛЮД

И. Варсани

В венгерских условиях, наиболее приемлемым кажется упаковка в синтетическую пленку допускающую в ку продукта в пакете. Автор, изучил наиболее приемлемые для этой цели пластические материалы и установил, что для замороженного пакета с весом 5[illegible] наиболее пригодна пленка в 0,[illegible] мм, а для пакета с большим весом в 0,08 мм толщины, с большим удельным весом полиэтиленовая пленка.

ÜBER DIE VERPACKUNG VON TIEFGEFRORENEN HALBFERTIGEN UND FERTIGEN SPEISEN

I. Varsányi

Für die einheimische Tiefkühlindustrie [illegible] Verpackung in kochfestem Kunststoffbeutel [illegible] bewährt. Bei der Untersuchung der Kunststoffe [illegible] wurde festgestellt, dass die Polyethylenfolie (mit großer Wichte) mit einer Stärke von 0,04 mm [illegible] Packungsgewichte bis 350 g geeignet ist. Über dieses Packgewicht muss die Stärke der Polyethylenfolie auf [illegible] erhöht werden.

PACKAGING OF HALF-PREPARED AND OF READY-MADE FOODS PRESERVED BY QUICK-FREEZING

I. Varsányi

Under Hungarian conditions for the packaging of quick-frozen foods heat-tolerable plastic bags seem to be the most suitable. Appropriate kinds of plastics were examined and it was found that with a net weight con[illegible] 0,04 mm thick high density polyethylene over this packing weight that of 0,08 mm thickness proved to be useful.

Adatok a szorbinsav [illegible] hatásmechanizmusához*

[illegible] T I B O R

[illegible]mezőgazdasági Egyetem [illegible] Mikrobiológiai [illegible] Budapest

A [illegible] tartósítószer, [illegible] ágban ború [illegible] ismeretes [illegible] szerek [illegible] elterjedt, [illegible] az élelmiszer- [illegible] feldolgozó ágazatai [illegible] szorbinsavval [illegible] közlemények [illegible] mik meg [illegible] az [illegible]. A dolgozat [illegible] tömör [illegible] e a szorbinsav [illegible] termelési lehetőségeivel foglalkozik [illegible] ilyen termékek tartósítása [illegible] mikroorganizmusok [illegible] szelektív [illegible] gátló. Számos [illegible] munkacsoport [illegible] mennyiségben [illegible] az általános fizikai, kémiai tulajdonságaik [illegible] hatásának, kimutatási módszereinek vizsgálata is. Emellett [illegible] minden tartósítószer esetében, a szorbinsav mikrobiológiai, farmakológiai tulajdonságait is [illegible] vizsgálták. A nagyszámú tanulmány [illegible] viszonylag csak nagyon kevés [illegible] arról, hogy mi az alapja e tartósítószer mikrobaellenes hatásának, mi [illegible] gátló hatás a mechanizmusa. Az e tárgyban közölt adatok [illegible] is talán több az ellentmondás, mint a [illegible] egy pl. az anaerob metabolizmus szorbitol [illegible] gátlásának több lehetséges módját [illegible] ták, az alkoholdehidrogenáz (*Whitaker* 1955, *Martoadiprowito* és *Whitaker* 1963), az enoláz (*Azukas, Costilow* és *Sadoff* 1961, *Azukas* 1962) vagy az aldoláz (*Springer* 1954, *Springer* és *Schegk* 1967, *Rasmusen* 1957) gátlása [illegible] magyarázva a mikrobaellenes hatás. Mások viszont az aerob anyagcsere különböző lépéseinek szorbinsavas gátlásáról számol-tak [illegible] a fumár [illegible] (*York* és *V*[illegible] 1955), a koenzim-A-nál [illegible] *eroni* és *de* [illegible] 1960), [illegible] tokrómoxid [illegible] *Anderson* 1[illegible] *Anderson* [illegible] *Costilow* 1[illegible] katalázná[illegible]), az oxidáció[illegible] zt vevő, sz[illegible] -igyá enzim[illegible] *York* [illegible] *Va*[illegible] gátló hatás a [illegible]. Vizsgálataim[illegible] n[illegible] nak különböző [illegible] a [illegible] szaporodásgátló h[illegible] nyo[illegible]. A vizsgálat[illegible] más helyek[illegible] már beszámolt[illegible] 1966, a [illegible] *Novák* 1966, 19[illegible] a), b), *Leák*, [illegible] *Novák* 1969), it[illegible] eredmények hatá[illegible] sabb konklúzió[illegible] [illegible]om össze.

Megállapítot[illegible] hogy a szorbinsav [illegible] mértéke anaerob [illegible] kísérletben [illegible] sem erősebb és [illegible] mint a [illegible] mellett, ahol n[illegible] szorbinsav koncentráció csak részleg[illegible] időlegesen tá[illegible] okoz. Az *E. coli* esetén *Brán* példaké[illegible] a *Proteus vulgaris*-szal végzett vizsgá[illegible] adatok be[illegible] hogy az anaerob inkubá[illegible] 250 μg/ml [illegible] szorbinsavat is teljesen meg[illegible] kényszeríti. Az aerob inkubáció[illegible] ennek [illegible] elegendő.

E jelenség [illegible] — a szorbinsav az aerob, ill. an[illegible] anyagcserére gátló hatásán tú[illegible] — a gátlószer k[illegible] nak lecsökkené[illegible] találhat[illegible] meg [illegible] az aerob tenyészetben [illegible] vé[illegible] rodásméréssel [illegible] vép[illegible] sav-meghatározás[illegible] anyagban [illegible] gomba-faj aerob [illegible] tenyészetében [illegible]

* A Mikrobiológiai Szakosztály Tartósítóipari Mikrobiológiai Enketjén, 1969. május 5-én, elhangzott előadás anyaga.

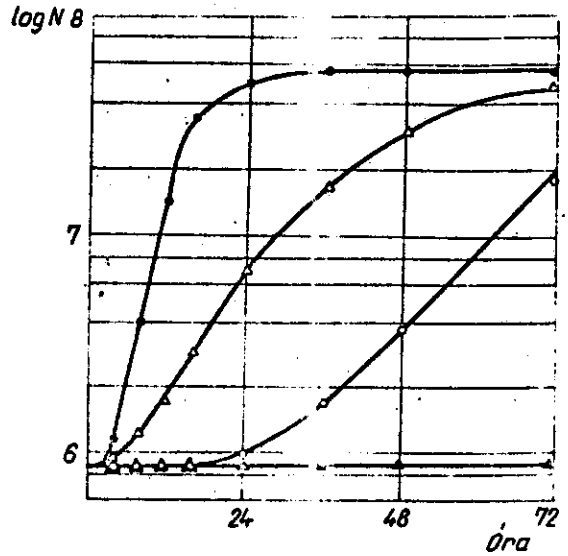
nagymértékű csökkenését, olykor teljes elfogyását mutatták. A szorbinsav-csökkenést nemcsak szaporodó tenyészetekben, hanem foszfátpufferben rázatott sejtszuszpenziók esetében is tapasztaltam, sőt, szervetlen sókból álló szintetikus tápoldatban egyes élesztőgombák (*Procandida albicans, Pc. tropicalis, Candida claussenii, C. pseudotropicalis, C. utilis,* de a *C. krusei, C. beverwijkii, Saccharomyces cerevisiae, S. carlsbergensis* nem) szorbinsavon, mint egyedüli szén- és energiaforráson szaporodni is tudtak. Egy erre vonatkozó kísérlet eredményeit a 2. *ábrán* mutatom be. A zárójelben közölt számok a kezdeti káliumszorbát-koncentráció százalékos elfogyását jelzik. Látható, hogy a szaporodás mértéke arányos a felhasznált, asszimilált szorbát mennyiségével.

Tudnak ehető geről, hogy penészgombák, különösen erős populáció esetén képesek a szorbinsavat lebontani, mások már beszámoltak (*Melnick, Luckmann* és *Gooding* 1954, *Lukas* 1964 a). b), élesztőgombák ilyen képességéről azonban a rendelkezésemre álló irodalomban nem találtam említést. A szorbinsav élesztőgombák általi lebontásának és asszimilációjának bizonyítása magyarázatot ad arra a tartósítóipari gyakorlatban mások (*Alderton* és *Lewis* 1958, *Costilow, Coughlin, Robbins* és *Hsu* 1957, *Bell, Etchells* 1956, *Weaver, Robinson, Hills* 1957) és általam is (*Deák* 1960 a), b), 1964) tapasztalt jelenségre, hogy a tartósítandó termékben a szorbinsav-koncentráció idővel lecsökken, ami a termék romlásához vezethet.

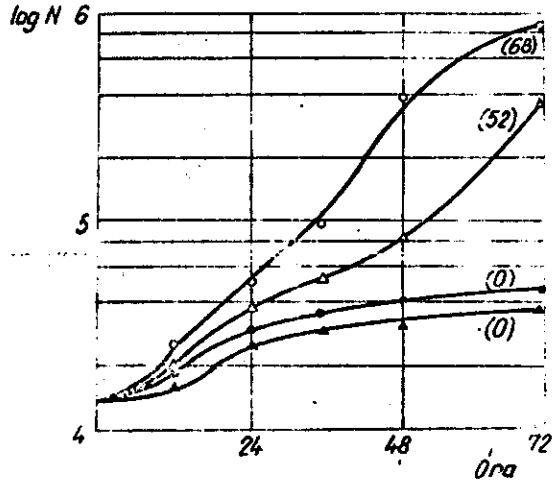
A másik lényeges megállapításhoz a szorbinsavnak a különböző növekedési szubsztrátokon való szaporodásra kifejtett gátló hatása vizsgálata vezetett. Az eredmények szerint az extracellulárisan hasított oligoszaharidokon, ill. a glükózon való szaporodás gátlásának mértéke közel azonosnak bizonyult, míg az intracellulárisan hasított oligoszaharidok esetében a glükóztól eltérő, általában nagyobb mértékű szaporodásgátlást tapasztaltam. A különböző szénhidrátok légzésének és erjesztésének vizsgálata [illegible] erősítette ezeket az eredményeket. Példaként a glükóz, szaharóz és maltóz asszimilációjának szorbinsavas gátlását mutatom be néhány élesztőgomba-fajnál, megjelölve az oligoszaharid bontásában részt vevő enzimek típusát is (*1. táblázat*). Az asszimiláció mértékét az egyes szubsztrátokon való szaporodás sebességével jellemezve a közölt értékek a szaporodási sebességi állandó százalékos gátlását jelentik 750 μg/ml káliumszorbát hatására.

A glükóz, szaharóz és maltóz felhasználásának gátlásában mutatkozó különbségeket nem lehet a szorbinsavnak a glikolízis vagy a légzési lánc enzimeire gyakorolt, az irodalomban eddig leírt gátló hatásával magyarázni, hiszen a szaharóz vagy maltóz felhasználása a glükózétól csak a felvételben és a hasításban tér el. A szorbinsav hatásában mutatkozó különbség oka tehát e két lépésnél kereshető.

A továbbiakban ezért megvizsgáltam a szorbinsav hatását különböző fajokból preparált



1. ábra. A káliumszorbát (KSz) hatása a Procandida albicans szaporodására. Sabouraud-glükóz leves tenyészetek, aerob (rázott T-csövek) inkubálással, ○: kontroll, ○: 1000 μg/ml KSz; vagy anaerob (szemianaerob, álló csövek) inkubálással, △: kontroll, ▲: 250 μg/ml KSz



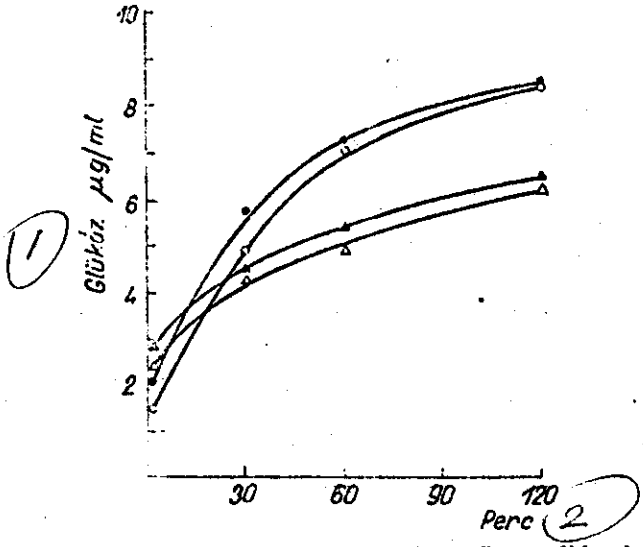
2. ábra. Élesztőgombák káliumszorbát (KSz) asszimilációja. Szintetikus tápoldat 250 μg/ml KSz-tal, pH 5,5. Aerob inkubálás (rázott T-csövek). ○: Procandida albicans, △: Candida claussenii, ○: Candida krusei, ▲: Saccharomyces cerevisiae. A zárójelben közölt számok a KSz %-os csökkenését jelzik 72 órás tenyésztés után

1. táblázat

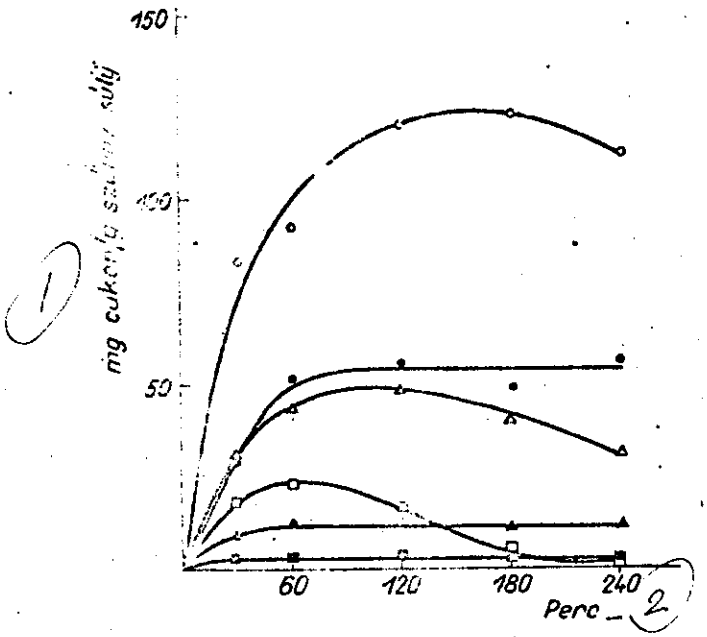
A káliumszorbát (KSz) hatása néhány élesztőgombafaj szénhidrát asszimilációjára

A szaporodási sebességi állandó gátlása 750 μg/ml KSz hatására. Aerob, rázott tenyészetek Sabouraud levesben, glükóz (*D*), szaharóz (*S*), ill. maltóz (*M*) szubsztráttal

Faj	Gátlás %-ban			Oligoszaharid hasító enzim
	D	*S*	*M*	
C. beverwijkii	45	43	43	Invertáz, endomaltáz
C. claussenii	18	10	14	Endoszaharáz, exomaltáz
Pc. albicans	23	24	37	Endoszaharáz, endomaltáz
Pc. tropicalis	31	31	45	Endoszaharáz, endomaltáz
S. carlsbergensis .	32	32	36	Invertáz, endomaltáz



3. ábra. A káliumszorbát (KSz) hatása a Procandida albicans oligoszaharid bontó enzimjeire. Sejtmentes kivonat maltóz (△, ▲), ill. szacharóz (○, ●) bontó aktivitása, a keletkezett glükóz glükózoxidázzal mérve. △, ○: kontroll, ▲, ●: 1000 µg/ml KSz

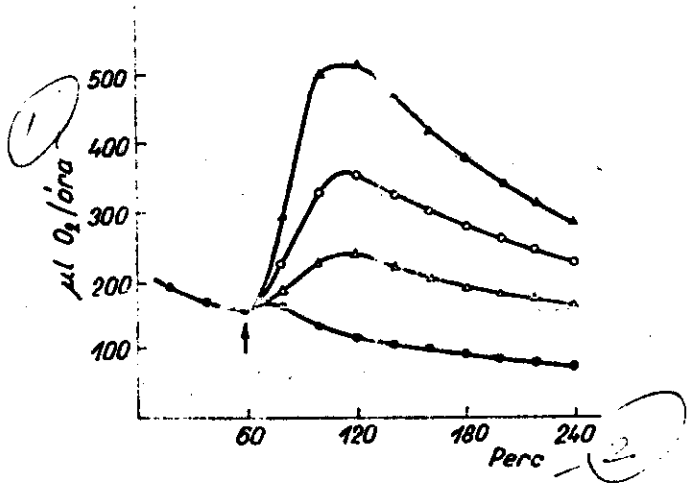


4. ábra. A Procandida albicans D-xilóz felvétele. Aerob, rázott inkubáció 28 °C-on. Kezdő cukorkoncentrációk: 1 (□), 5 (△) és 25 (○) mg/ml. Káliumszorbát koncentráció mindhárom cukorkoncentráció szinten 50 mM (▲, □, ●)

endoszaharázra és endomaltázra. Amint az a 3. *ábrán* bemutatott egyik ilyen vizsgálat eredményéből is látható, sejtmentes kivonatokban ezeknek az enzimeknek az aktivitása nem változott káliumszorbát hatására.

Ezek az eredmények vezettek a szorbinsavnak a cukrok felvételére, transzportjára gyakorolt közvetlen hatása vizsgálatához. A *S. cerevisiae*-nél, amely a cukrokat vagy közvetített vagy elősegített diffúzióval, passzív módon transzportálja, a szorbinsavnak a transzportra gyakorolt gátló hatását nem tudtam kimutatni. Sikerült azonban olyan élesztőgomba-fajokat találnunk (*Deák és Kovár 1968, Deák és Novák* 1969, b), c), amelyek a cukrokat az anyagcsere-energia felhasználásával, koncentráció gradienssel szemben, aktív módon transzportálják. Az ilyen típusú transzportnál kimutattam, hogy a szorbinsav szétkapcsolja a transzport aktív részét a passzív, elősegített diffúziótól, vagyis gátolja az aktív transzporthoz szükséges anyagcsere energia ellátást. A 4. *ábrán* példaként a *Pc. albicans* D-xilóz felvételét mutatom be. Szorbinsav jelenlétében a sejtekbe jutott cukor mennyisége mindhárom vizsgált koncentrációnál csak a diffúziós egyensúlynak megfelelő szintet érte el.

Vizsgálataim harmadik fő tovább megállapítása a szorbinsavnak az élesztőgombák légzésére gyakorolt hatásával kapcsolatos. Manometriás mérések eredményei szerint a szorbinsav hatására az élesztőszuszpenziók endogén légzése jelentős növekedést mutatott (5. *ábra*).



5. ábra. A káliumszorbát (KSz) hatása a Procandida albicans légzésére. 50 mg nedves sejt, 1000 µg KSz, 0,03 M foszfátpufferben, össztérfogat 2 ml, pH: 3,0 (○), 5,5 (△), ill. 8,0 (△). Az endogén értékek (●) a három pH-n mért oxigénfogyasztás átlagai. KSz az 50. percnél (nyíl)

Ez a légzésfokozódás azonban nem volt összefüggésben a gátlószer lebontásával, oxidatív metabolizációjával, mivel a szorbinsav csökkenés csak jóval később következett be, mint a gátlószer által kiváltott oxigénfelvétel-fokozódás és a két folyamat pH optimuma is eltért. Az endogén légzés szorbinsav általi fokozódása hasonlónak bizonyult a 2,4-dinitrofenol szétkapcsoló hatásához, ily módon feltételezhető, hogy a szorbinsav a sejt energiatermelő rendszerét a terminális oxidáció mechanizmusánál gátolja.

A sejtek energiatermelő rendszerének megbontása közvetlenül felelős lehet a szorbinsav anyagcsere gátló hatásáért és ennek egyik elsőként jelentkező következményeként az anyagcsere energiához kötött aktív transzport folyamatok gátlódhatnak. Azokban az esetekben azonban ahol egyrészt a transzport passzív módon történik, másrészt anaerob körülmények között a glikolitikus energianyerés folyik, a szorbinsav hatására ugyancsak tapasztalható anyagcsere, ill. szaporodásgátlás okát más támadás-

pontokon kell keresni. A gátlás más lehetséges pontjaira az irodalom is utal. Valószínű, hogy a szorbinsav hatásmechanizmusának értelmezéséhez több támadáspont lehetőségét kell figyelembe vennünk. E bonyolult probléma még korántsem tekinthető lezártnak. A megoldáshoz igyekeztem azokkal a vizsgálataimmal is hozzájárulni, amelyekről itt összefoglalást adtam.

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ДАННЫЕ К МЕХАНИЗМУ ДЕЙСТВИЯ СОРБИНОВОЙ КИСЛОТЫ

Т. Деак

Статья подводит итоги результатов многолетних серий исследований относящихся к торможению размножения и механизма воздействия на микроорганизмы сорбиновой кислоты. Исследования привели к многочисленным новым установлениям результативности сорбиновой кислоты связанным метаболизмом дрожевых грибков с расщеплением системы энерготворности клеток и прекращением активных транспортных процессов.

ANGABEN ZUM WIRKUNGSMECHANISMUS DER SORBINSÄURE

T. Deák

Es werden die wichtigsten Ergebnisse einer bezüglich des vermehrungshemmenden Wirkungsmechanismus der Sorbinsäure durchgeführten mehrjärigen Versuchsserie zusammengefasst. Es wurden zahlreiche neue Feststellungen hinsichtlich des Metabolismus der Sorbinsäure, der Zerstörung des energieerzeugenden Systems der Zellen und der Hemmung der aktiven Transportprozesse erzielt.

DATA ON THE MECHANISM OF THE EFFECT OF SORBIC ACID

T. Deák

The present publication deals with the antimicrobial effect and with the mechanism of the effect of sorbic acid: summarizes the main results of a series of investigation of several years. Numerous new observations were collected in connection with the metabolism of sorbic acid by yeasts, with the disruption of the energy producing system of cells and with the inhibition of the active transport processes.

From the State Vinicultural Institute Freiburg/Br. (German pp. 304-312)

Headed by Director Dr. B. Götz

Effect of sorbic acid on polyphenol oxidase under inactivating conditions

By R. Enkelmann

A look at what has been over the decades the maximum permissible quantity of SO_2 for wine shows a continual increase (1). In 1890 it was 80 mg/ltr, in 1900, 200 mg/ltr, and at present, 300 mg/ltr. The increase is attributable to the fact that in the course of the years the yeasts have become more and more resistant to SO_2 (2). Besides, there has been a trend in recent times to leave the wine with a certain residual sweetness. By more intensive sulfurization the attempt was made to stabilize the residual sweetness. However, even in the maximum quantity permissible today the SO_2 is unable to kill yeast cells; it simply has an inhibiting effect on the growth and hence is fermentation-retarding. Intensive sulfurization is, however, undesirable because in combined as well as in free form SO_2 impairs the digestability of the wines (3). There have been many attempts, therefore, to limit the use of SO_2 in the preparation of wine. Before fermentation one can save sulfite by using sound vintage material, reducing the standing and pressing time, and adding suitable pure-strain yeasts (4). To prevent after-fermentation, one can apply physical methods or use chemical agents just before filling the bottles. The physical methods include cold sterile filling and pasteurization, but both these methods require large-scale technical facilities and trained personnel. The chemical agents include pyro-carbon dioxide ester and the - for some years - much discussed sorbic acid (5, 6, 7).

Sorbic acid is a diolefinic carbonic acid. It is related to caproic acid, the hydrated compound, which is contained in nutrient fats. Like all other saturated and unsaturated fatty acids, sorbic acid is burned up by the organism; other preservatives, instead, must be excreted by the body after de-

toxication. Sorbic acid, therefore, is a physiologically harmless substance. It has an anti-microbial effect on mould fungi and yeasts and thus offers a possibility of preventing after-fermentation in low-germ wines and reducing the SO_2 requirement to the absolutely necessary quantity.

Apart from binding the acetaldehyde, the main function of SO_2 is the inactivation of the polyphenol oxidase (PPO), which not only is impossible by sorbic acid, but even results in considerable disturbances when using sorbic acid at the beginning of the wine-making process.

Our studies were intended to clarify whether and how the addition of sorbic acid affects the inactivation of the polyphenol oxidase in all three treatment methods.

Execution of experiments and results

For all experiments a crude enzyme was used, which is obtained by acetone precipitation from cider of the Silvaner brand. Descriptions on the preparation are found in Bayer, Born and Reuther (8) and in Poux (9). As the crude enzymes are not soluble in a clear form, only the Warburg method was used to determine the activity. With it the oxygen consumption of a substrate can be observed manometrically directly during the action of the enzyme. The manometric technique is reported in detail by Umbreit, Burris and Stauffer (10) and by Sällman (11).

The effect of the H+ ions on the PPO in the presence of sorbic acid was examined at 25°C in citric acid-phosphate buffers of pH values of 2.5; 3.0; 3.5; 4.0; 4.5. After the desired contact time, the enzyme-buffer mixtures are adjusted with 2n sodium hydroxide solution to pH 5.8 - 6.0. At this pH value the PPO has its reaction optimum. Each batch contains in the reactor 0.4 pyrocatechin units of PPO, corresponding to 15 mg crude enzyme, 3.0 ml citric acid-phosphate buffer, 0.15 ml 0.1 m potassium sorbate solution (300 mg sorbic acid/ltr) and enough water to obtain after neutralization with 2n NaOH and addition of 1 ml 0.1 m pyrocatechin solution a total of 5.0 ml from the side arm before the start of reaction. Agitation is carried out at 200

vibrations per minute, and the oxygen consumption is read every 2 minutes for 20 minutes. From the consumption of the first 10 minutes and the maximum consumption at pH 5.8 the residual activity is calculated. For each pH value a test series is set up with sorbic acid and a parallel series without sorbic acid. As can be seen from fig. 1, the sorbic acid-containing enzyme solutions are on the average 20% more stable to hydrogen ions.

Fig. 1. Inactivation of PPO (in %) by hydrogen ions.

o with addition of 300 mg sorbic acid per liter

x without sorbic acid

The same formulation serves also for the heating tests. A reaction solution with sorbic acid and one without is heated for 30 minutes at 20, 25, 30, 35, 40, 45, 50 and 55°C at pH 3.5. Before the measurement the vessels are chilled in an ice bath and the reaction solution is adjusted with 2n NaOH to pH 5.8 - 6.0. The results are summarized in fig. 2. Sorbic acid-containing enzyme solutions are found to be 5-27% more stable, depending on the temperature.

Fig. 2. Inactivation of PPO (in %) by heat treatment for 30 min at pH 3.5.

o with addition of 300 mg sorbic acid per liter

x without sorbic acid

Fig. 3 shows the curve of the PPO inactivation in a solution free of sorbic acid and one containing sorbic acid by addition of increasing quantities of SO_3. In a pH 3 buffer (with 200 mg sorbic acid per liter) 15 mg of crude enzyme (80 pyrocatechin units per liter) are dissolved and placed in a bath of 25°C. After exactly 5 minutes sulfurization takes place, which is increased from 2 mg SO_2/liter to 42 mg SO_2/liter and to which the enzyme or respectively the enzyme-sorbic acid mixture is exposed for 5 minutes. After the second 5 minutes, the reaction solution is adjusted to pH 5.8 and immedi-

ately the residual activity is measured in the Warburg apparatus. The PPO shows a distinct resistance to sulfurization in sorbic acid-containing solution.

If an enzyme solution is admixed in a pH 3.0 buffer with increasing quantities of sorbic acid and is tested for its residual activity after being left standing for 2.5 hours at 25°C, one finds that at an enzyme concentration of 80 units/ltr 200 mg of sorbic acid per liter are perfectly sufficient for stabilization (fig. 4). Higher sorbic acid concentrations remain without effect.

Fig. 3. Inactivation of PPO (in %) at 25°C in pH 3.0 buffer by sulfurization. After 5 min SO_2 is added, after another 5 min, reading.

o with addition of 200 mg sorbic acid/liter

x without sorbic acid

Fig. 4. The stabilizing effect of sorbic acid on PPO at 25°C in the pH 3.0 buffer. Activity read after 2.5 hours.

g of sorbic acid per liter

Attempt at interpretation of PPO in activation

As our studies on the stability of the PPO to temperature and pH variations show (12), the stability of the PPO is greatly dependent on the hydrogen ion concentration. The PPO loses 90% of its acitvity if it is exposed for two hours at 25°C to a pH of 2.5. At pH 3.5, the activity is largely reduced in the course of 8-10 hours. At a pH of 4.5, instead, the PPO is practically completely stable already.

The PPO consists of two essential constituents, namely colloidal carrier protein and the prosthetic action group, the copper ion. The carrier protein is also called apoenzyme, and the active group coenzyme. It is the presence of the intact enzyme,the so-called holoenzyme, that is the prerequisite of the enzyme effect. The apoenzyme with its specific configuration

of the peptide chain is responsible for the substrate specificity, while the prosthetic group supplies the substance conversion. There are thus two possibilities to be taken into consideration for the mechanism of inactivation: Disturbance or blockage of the function of the prosthetic group, or alterations in the highly compicated configuration of the colloidal apoenzyme.

In view of the relatively slow inactivation by H^+ ions and partial reactivation (6%) of the enzyme by acetone precipitation at low temperature, as well as the improbability of an interaction between the hydrogen ion and the copper ion of the prosthetic group because both are charged in the same sense, one might regard a disturbance of the configuration of the colloidal apoenzyme as very likely for the inactivation of the enzyme by H^+ ions. In the living cell, the swelling of colloidal proteins, which is influenced by the ions contained in the surrounding liquid and hence is dependent on the respective pH, is of great importance. By adsorption of hydrogen ions one might conceive of a loosening and subsequent separation of certain bonds, expecially of the low-energy hydrogen bond. Hydrogen bonds are known to contribute considerably to the fixation of the complicated configuration of the colloidal apoenzyme. Since certainy a large number of such hydrogen bonds exists, it takes a while until enough bonds are dissolved for the substrate-specific effect to be cancelled.

The inactivation of the PPO by sulfurous acid is probably subject to another mechanism. Hydrogen ions inactivate at 25°C and a concentration of 0.005 mole per liter - according to a pH value of 2.5 - 95% of the PPO in two hours. An equally complete inactivation is brought about by SO_2 in a concentration of only 0.0001 mole per liter and a reaction time of only 5 minutes. This comparison leads to a very remarkable result: Despite 50-fold dilution, the SO_2 brings about an at least 24 times faster inactivation. No doubt the reaction between SO_2 and the PPO takes place in a still much shorter time, but this contact time of 5 minutes is inevitable in Warburg

measurements for temperature equalization. Lengthening the contact time of SO2 with the enzyme from 5 min to 10, 30 or 60 min causes no change in the number of inactivated enzyme units. It follows that the contamination of the enzyme by SO_2 must indeed take place quickly. The sulfite ions are attracted by oppositely charged points of the enzyme and bound with immediate inactivation. By increasing the sulfite quantity it is possible, as in a titration, to determine quite accurately the quantity which is just sufficient to eliminate 90% or 95% of the activity. For 80 munits of pyrocatechin per liter about 4.5 to 5.0 mg of SO_2 are needed. A colloidal protein molecule, such as the apoenzyme, is able to adsorb ions from the solution surrounding them. If the inactivation of the PPO is due to a reaction of the sulfite with the carrier protein, one should expect a slow inactivation, as in the reaction with the hydrogen ions. Despite the higher dilution, the reaction takes place rapidly and irreversibly. It is logical to assume, therefore, that an ion reaction is involved and the SO_2 reacts with the relatively few Cu^{++} ions of the enzyme, with the valence change between Cu^{++} and Cu^{+} being blocked (13). Among the inhibitors are predominantly sulfur compounds, such as hydrogen disulfide, cysteine, thiourea, diethyl dithiocarbamate and some compounds which form stable complexes with copper, such as cyanide and salicyl aldoxime.

The inactivation of the PPO by heating is always based on a denaturation of the colloidal protein. The coenzyme, usually a compound from the vitamin B group or a metal ion, is, on the contrary, thermally very stable. The thermal inactivation is also slow, like the inactivation by hydrogen ions. The first signs of a decrease in activity are observable at 35°C. Even at 45° and 50° C the enzyme is still intact for a while. At 45°C there still remains 50% of the activity after 2.5 hours, 25% after 5 hours. This gradual collapse of the protein configuration points to the dissolution of weak bonds, such as hydrogen bridge bonds, which are about 10 times weaker than covalent bonds.

Attempt at interpretation of the stabilization effect of sorbic acid

The presence of sorbic acid in a concentration of 200-250 mg per liter causes under the described three possibilities of inactivation a distinct retardation of the decrease in activity. The inactivation by hydrogen ions undergoes at 25°C and pH 2.5 a deceleration of about 20%. After 30 minutes there remains without sorbic acid 30% of the activity, while in the presence of sorbic acid 50% of the activity remains. A two-hour action of a pH 2.5 buffer results in a 97% inactivation, but in the presence of sorbic acid only a 73% inactivation has occurred. In the pH 3.0 buffer there remains after 5 hours at 25° C 5%, with addition of sorbic acid, 25% of the activity. Also at pH 3.5 and 4.0 this stabilizing effect is evident. Only at pH 4.5, a pH value at which the PPO is stable for a prolonged time anyway, the effect of the sorbic acid becomes small. The possibility of a mobilization of the enzyme by sorbic acid is thus precluded. Hence we can attribute this phenomenon to a stabilization of the apoenzyme.

Due to the -E effect of the CH_3 group of the sorbic acid, the intermediate conjugated system, and the end-positioned carboxyl group which brings about a +E effect, the sorbic acid is present in a highly polar form. It has the possibility of becoming bond-proportional on corresponding other molecules. Due to the two double bonds, the sorbic acid molecule is still relatively rigid, and therefore it can become bond-proportional on both sides on other molecules only if opposite charges are present at exactly defined intervals. With the colloidal protein of the PPO with its specifically folded peptide chain, this condition seems to be fulfilled several times over. Sorbic acid molecules become added to the apoenzyme and cause an additional cross-linkage of the peptide chain. Thereby the configuration of the colloidal apoenzyme is so strengthened that now a higher hydrogen ion concentration is necessary to inactivate the enzyme or, in the case of thermal inactivation, more energy is necessary. For the stabilization of 80 pyrocatechin units of

PPO per liter, 250 mg of sorbic acid, corresponding to 0.0022 mole, are just barely sufficient; smaller quantities cause an incomplete stabilization. Excess sorbic acid remains without effect (fig. 4). Bonding of sorbic acid to the copper ion of the coenzyme seems improbable. 22 times more sorbic acid molecules than SO_2 molecules are necessary to achieve a maximum effect. Also it seems questionable whether such a bond can have a stabilizing effect at all; an inhibiting effect would be more likely. However, such an effect was not observable. Also the result of the thermal treatment (fig. 2) argues against a sole bonding between copper and sorbic acid. The bonding of sorbic acid only to the copper ion would have to result in the coincidence of both curves. With the thermal treatment of an intact enzyme and one contaminated by SO_2 the two curves would have to coincide, but this is very difficult to prove.

The stabilizing effect of the sorbic acid is dependent on the respective prevailing temperature. In the range of 20-25°C it is 7-8%, it rises slowly and reaches the maximum of 27% at 40°C, then decreasing slowly and dropping to 5% at 50° C. To get the same degree of inactivation, the sorbic acid-containing enzyme solution must be exposed for 30 minutes to a 5-7° C higher temperature than the sorbic acid-free solution. The greater energy consumption is easy to explain by assuming an additional cross-linking of the colloidal apoenzyme by adsorption of sorbic acid. A protein molecule which is stabilized by additional cross-linkage needs a higher temperature for the destruction of the specific configuration of its peptide chains.

An enzyme solution is inactivated in a very short time by SO_2. 5 mg of SO_2 per liter are sufficient for a 95% inactivation of 80 pyrocatechin units (fig. 3). But if before the sulfurization ther occurs an addition of 200 mg of sorbic acid per liter, one observes a considerable resistance of the PPO against SO_2. One must reckon with a residual activity of 42% when a sorbic-

acid-containing enzyme solution is admixed with only 5 mg of SO_2 per liter. At 10 mg SO_2 per liter there remains a residual activity of 23%, and at 18 mg SO_2 per liter still 10% residual activity is present. Only with 30 mg SO_2/ liter a 95% inactivation is achieved. An interpretation of this resistance phenomenon is not quite simple. If the copper ion would have an exponential position on the surface of the apoenzyme, then no difference should appear in the behavior of the two enzyme solutions toward the SO_2, provided the contamination of the enzyme by SO_2 - as Schirmacher-Göllner assumes (13) - is a reaction with the copper ion. The SO_2 would react with the copper, regardless of whether sorbic acid has added itself to the apoenzyme. But since there is a considerable deviation in the shape of the two curves, one must assume a central position of the copper ion. By the addition of sorbic acid on the surface of the enzyme there occurs an additional cross-linkage, which causes a strengthening of the configuration of the colloidal apoenzymes, whereby in the end the penetration of the SO_2 is rendered difficult. The resistance of the enzyme in sorbic acid-containing solution is thus very probably attributable to a steric effect. To obtain in a short time the same degree of inactivation, 5 times as much SO_2 is required in the presence of sorbic acid.

Summary

The different methods of inactivating the PPO are compared. Inactivation by H^+ ions and inactivation by heating are reactions which require a certain amount of time. On the other hand, contamination of the PPO by SO_2 occurs very rapidly even in high dilution. Probably the relatively weak hydrogen bonds are dissolved, whereby the substrate-specific configuration of the apoenzyme is abolished. The spontaneous contamination of the PPO by SO_2 points to a reaction with the copper ion of the coenzyme. In the presence of sorbic acid a distinct retardation occurs with all three methods of inactivation. The sorbic acid-containing enzyme solutions are on the average 20% more

stable to hydrogen ions. The stabilization effect upon heating is dependent on the respective temperature; it is especially strong at 40°C and then amounts to 27%. The stabilization of the enzyme is probably due to an adsorption of sorbic acid molecules to the apoenzyme. There occurs an additional cross-linkage of the complicated peptide chain, whereby the configuration of the colloidal apoenzyme is strengthened. An enzyme thus stabilized now requires for its inactivation a higher hydrogen ion concentration or respectively a greater thermal energy. Also the inactivation of the enzyme by SO_2 occurs in sorbic acid-containing solution with a considerable retardation. Steric reasons seem to be the cause. Due to the strengthening of the configuration of the apoenzyme by adsorption of sorbic acid, the penetration of the SO_2 is made difficult.

For the practice it follows from this: Sorbic acid may be used only after inactivation of the PPO.

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Translated by Carl Demrick Associates, Inc./LH/db

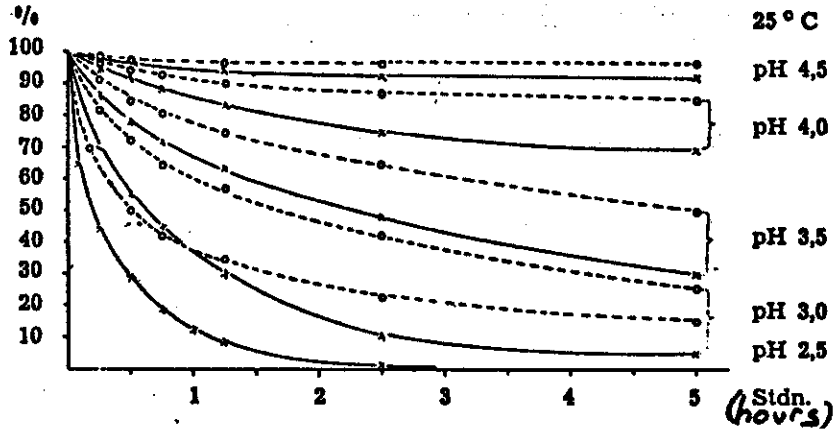

Fig. 1

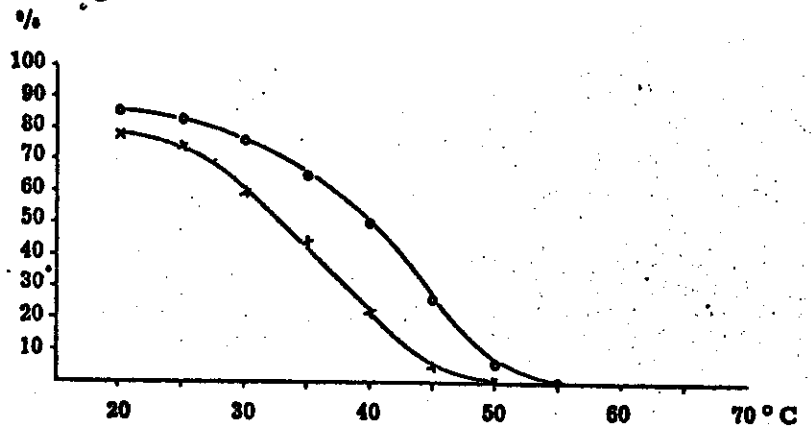

Fig. 2

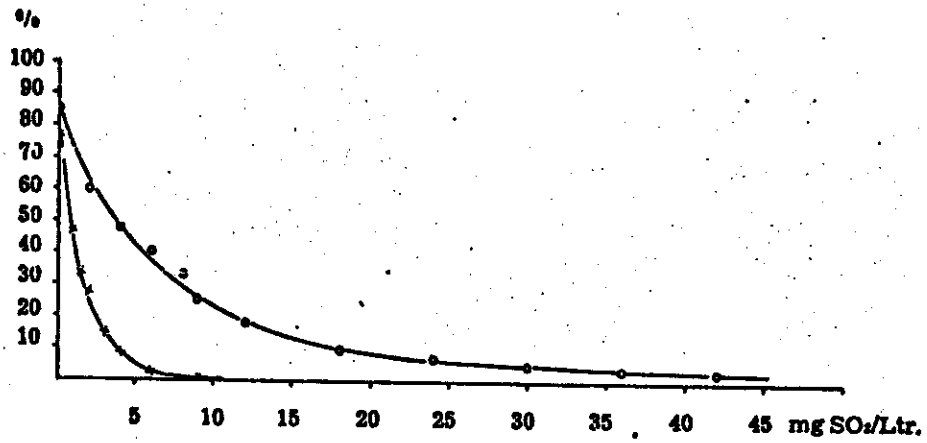

Fig. 3

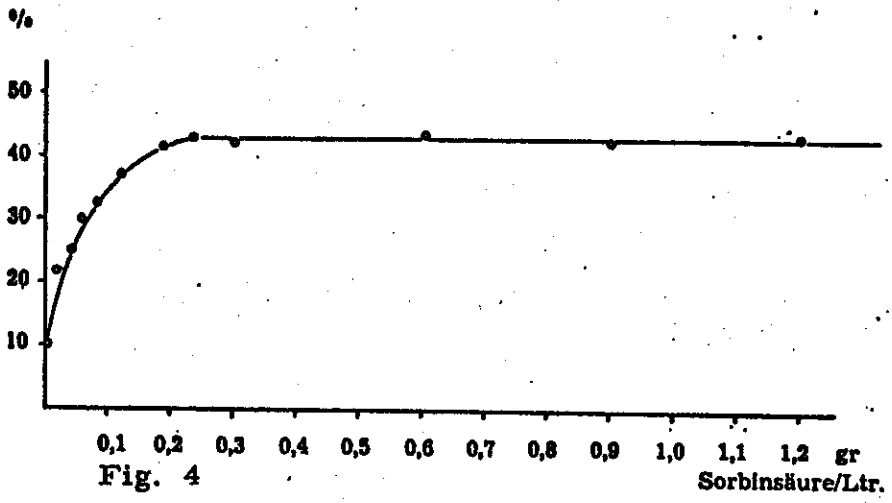

Fig. 4

Aus dem Staatlichen Weinbau-Institut Freiburg/Br.
Leiter: Direktor Dr. B. Götz

Die Wirkung der Sorbinsäure auf die Polyphenoloxidase unter inaktivierenden Bedingungen

Von R. Enkelmann

Verfolgt man über einige Jahrzehnte hinweg die zulässige Höchstmenge an S für Wein, so beobachtet man einen fortwährenden Anstieg (1). 1890 war 80 mg/Ltr. zulässig, 1900 waren es 200 mg/Ltr. und zur Zeit sind es 300 mg/Lt Die Steigerung ist darauf zurückzuführen, daß die Hefen im Laufe der Jah gegen SO_2 immer mehr resistent wurden (2). Hinzu kommt in neuerer Zeit no die Tendenz, dem Wein eine gewisse Restsüße zu belassen. Durch stärke Schwefelung versuchte man, die Restsüße zu stabilisieren. Jedoch vermag d SO_2 selbst in der heute zulässigen Höchstmenge Hefezellen nicht zu töten, s wirkt lediglich hemmend auf das Wachstum und damit gärverzögernd. Ei starke Schwefelung ist aber unerwünscht, weil die SO_2 in gebundener wie i freier Form die Bekömmlichkeit der Weine beeinträchtigt (3). Es hat daher nic an Versuchen gefehlt, den Gebrauch an SO_2 bei der Weinbereitung einzuschrä ken. Vor der Gärung kann man Sulfit einsparen, indem man gesundes Lesegu verwendet, Stand- und Kelterzeit herabsetzt und geeignete Reinzuchthefen zu setzt (4). Zur Verhütung einer Nachgärung kann man kurz vor der Flasche füllung physikalische Verfahren anwenden oder chemische Mittel einsetzen. Z den physikalischen Verfahren zählt die Kalt-Sterilfüllung und die Pasteuris tion; beide Verfahren erfordern aber größere technische Einrichtungen und ge schultes Personal. Zu den chemischen Mitteln gehört der Pyrokohlensäureeste und die seit einigen Jahren viel diskutierte Sorbinsäure (5, 6, 7).

Die Sorbinsäure ist eine Diolefinkarbonsäure. Sie steht der Capronsäure, de aufhydrierten Verbindung nahe, welche in Nahrungsfetten enthalten ist. Wi alle anderen gesättigten und ungesättigten Fettsäuren wird die Sorbinsäu vom Organismus verbrannt; andere Konservierungsmittel müssen dagegen vo Körper nach Entgiftung wieder ausgeschieden werden. Sorbinsäure ist also ei physiologisch-unbedenkliche Substanz. Sie wirkt antimikrobiell gegen Schim melpilze und Hefen und bietet damit eine Möglichkeit, die Nachgärung in kein armen Weinen zu verhindern und den SO_2-Bedarf auf die unbedingt notwendig Menge zu reduzieren.

Abgesehen von der Bindung des Acetaldehyds besteht die Hauptaufgabe de SO_2 in der Inaktivierung der Polyphenoloxidase (PPO), was durch Sorbinsäu nicht nur unmöglich ist, sondern sogar erhebliche Störungen zur Folge hat, wen man Sorbinsäure zu Beginn des Weinbereitungsprozesses einsetzt.

Unsere Untersuchungen sollten klären, ob und ggf. wie sich bei allen dr Behandlungsmethoden die Zugabe von Sorbinsäure auf die Inaktivierung de Polyphenoloxidase auswirkt.

Versuchsdurchführung und Ergebnisse

Für alle Versuche wurde ein Rohenzym verwandt, welches durch Acetonfällung aus Traubenmost der Sorte Silvaner gewonnen wird. Beschreibungen für die Darstellung findet man bei Bayer, Born und Reuther (8) sowie bei Poux (9). Da die Rohenzyme nicht klar löslich sind, wurde für die Aktivitätsbestimmung nur die Warburg-Methode benutzt. Diese gestattet es, den Sauerstoffverbrauch eines Substrates direkt während der Einwirkung des Enzyms manometrisch zu verfolgen. Über die manometrische Technik berichteten ausführlich Umbreit, Burris und Stauffer (10) sowie Süllmann (11).

Der Einfluß der H^+-Ionen auf die PPO in Gegenwart von Sorbinsäure wurde bei 25 ° C in 4 Citronensäure-Phosphat-Puffern mit pH-Werten von 2,5; 3,0; 3,5; 4,0; 4,5 untersucht. Nach Ablauf der gewünschten Einwirkungszeit werden die Enzym-Puffer-Gemische mit 2n Natronlauge auf pH 5,8 - 6-0 eingestellt. Bei diesem pH-Wert hat die PPO ihr Reaktionsoptimum. Jeder Ansatz enthält im Reaktionsgefäß 0,4 Brenzkatechineinheiten PPO, entsprechend 15 mg Rohenzym, 3,0 ml Citronensäure-Phosphat-Puffer, 0,15 ml 0,1 m Kaliumsorbatlösung (300 mg Sorbinsäure/Ltr) und soviel dest. Wasser, daß nach Neutralisation mit 2n NaOH und Zugabe von 1 ml 0,1 m Brenzkatechinlösung aus dem Seitenarm vor Reaktionsbeginn ein Gesamtvolumen von 5,0 ml erhalten wird. Es wird mit 200 Schwingungen in der Minute geschüttelt und über 20 Min. in Abständen von 2 Min der Sauerstoffverbrauch abgelesen. Aus dem Verbrauch der ersten 10 Min und dem Maximalverbrauch bei pH 5,8 wird die Restaktivität berechnet. Für jeden pH-Wert wird eine Versuchsreihe mit Sorbinsäure und eine Parallelreihe ohne Sorbinsäure angesetzt. Wie aus der Abb. 1 ersichtlich ist, sind die sorbinsäurehaltigen Enzymlösungen im Mittel um 20 % stabiler gegen Wasserstoffionen.

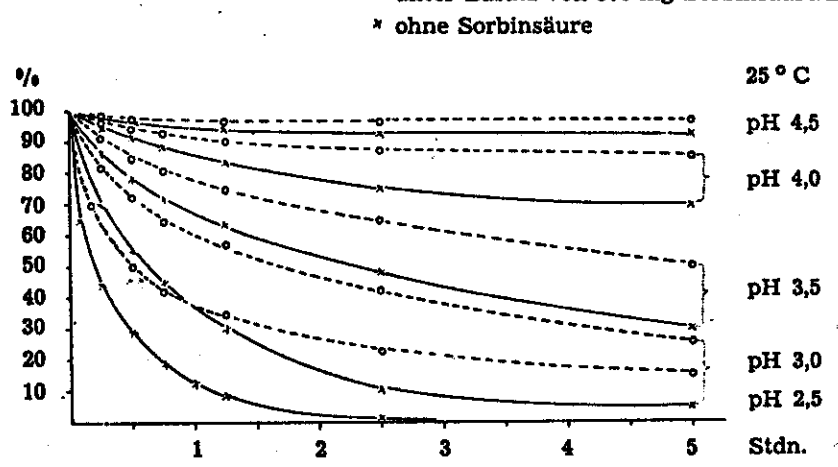

Abb. 1
Inaktivierung der PPO (in %) durch Wasserstoffionen

Der gleiche Reaktionsansatz dient auch für die Erhitzungsversuche. Je eine sorbinsäurehaltige und eine sorbinsäurefreie Reaktionslösung wird bei pH 3,5 30 Min auf 20, 25, 30, 35, 40, 45, 50 und 55 ° C erwärmt. Vor der Messung werden die Gefäße im Eisbad abgekühlt und die Reaktionslösung mit 2n NaOH auf pH 5,8 - 6,0 eingestellt. Die Meßergebnisse sind in Abb. 2 zusammengefaßt. Sorbinsäurehaltige Enzymlösungen sind danach je nach Temperatur 5 - 27 % stabiler.

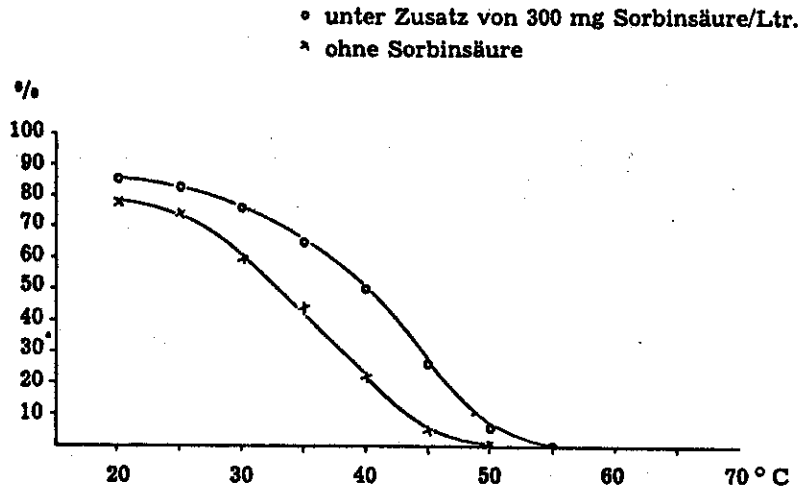

Abb. 2
Inaktivierung der PPO (in %) durch Wärmebehandlung 30 Min bei pH 3,5

Den Verlauf der PPO-Inaktivierung in einer sorbinsäurefreien und einer sorbinsäurehaltigen Lösung durch Zugabe steigender Mengen SO_2 zeigt Abb. 3. In einem pH 3-Puffer (mit 200 mg Sorbinsäure im Liter) werden 15 mg Rohenzym (80 Brenzkatechineinheiten im Ltr.) gelöst und in ein Bad von 25° C gestellt. Nach genau 5 Min erfolgt die Schwefelung, welche von 2 mg SO_2/Ltr. bis auf 42 mg SO_2/Ltr. gesteigert wird und 5 Min auf das Enzym bzw. Enzym-Sorbinsäure-Gemisch zur Einwirkung gelangt. Nach Ablauf der zweiten 5 Min wird die Reaktionslösung auf pH 5,8 eingestellt und sofort im Warburg-Apparat die Restaktivität gemessen. Die PPO zeigt in sorbinsäurehaltiger Lösung gegen Schwefelung eine deutliche Resistenz.

Wird eine Enzymlösung in einem pH 3,0-Puffer mit steigender Menge Sorbinsäure versetzt und nach einer Standzeit von 2,5 Stunden bei 25 ° C auf ihre Restaktivität untersucht, so stellt man fest, daß bei einer Enzymkonzentration von 80 Einheiten/Ltr. 200 mg Sorbinsäure im Liter zur Stabilisierung vollkommen ausreichen (Abb. 4). Höhere Sorbinsäure-Konzentrationen bleiben ohne Wirkung.

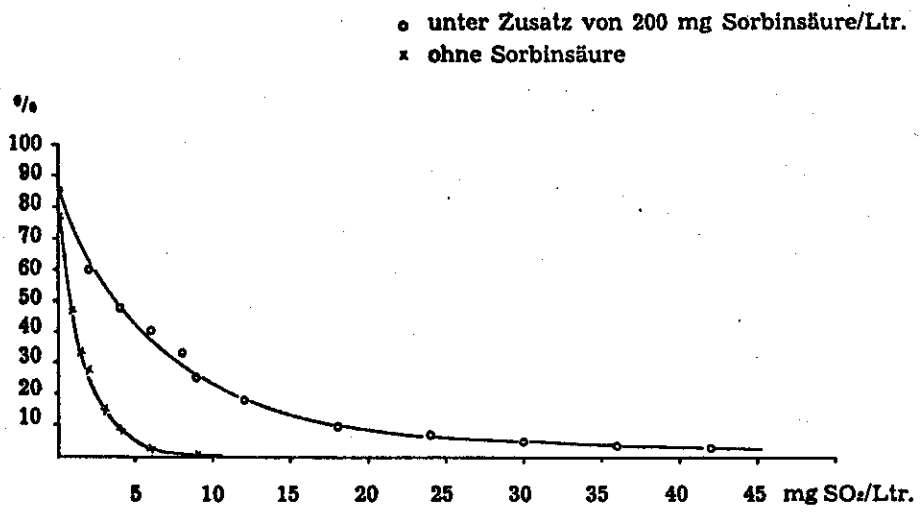

Abb. 3

Inaktivierung der PPO (in %) bei 25 °C im pH 3,0 Puffer durch Schwefelung. Nach 5 Min SO_2-Gabe, nach weiteren 5 Min Messung.

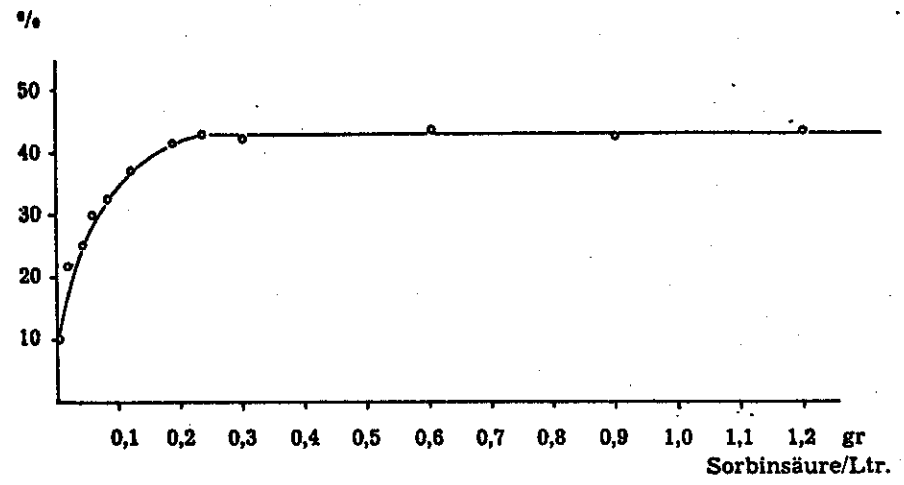

Abb. 4

Der stabilisierende Effekt der Sorbinsäure auf die PPO bei 25 °C im pH 3,0 Puffer. Messung der Aktivität nach 2,5 Stunden.

Versuch einer Deutung der Inaktivierung der PPO.

Wie unsere Untersuchungen über die Stabilität der PPO gegenüber Tempera und pH-Änderungen zeigen (12), ist die Stabilität der PPO stark von der W serstoffionenkonzentration abhängig. Die PPO verliert 90 % ihrer Aktiv wenn sie zwei Stunden lang bei 25 ° C einem pH von 2,5 ausgesetzt wird. pH 3,5 wird die Aktivität im Verlauf von 8 - 10 Stunden weitgehend abgeb Dagegen ist die PPO bei einem pH von 4,5 praktisch schon vollkommen stabi

Die PPO besteht aus zwei wesentlichen Bestandteilen, nämlich aus dem kol den Trägereiweiß und der prosthetischen Wirkgruppe, dem Kupferion. Trägereiweiß nennt man auch Apoferment und die Wirkpruppe Coferment. das Vorliegen des intakten Fermentes, des sog. Holofermentes, bildet die V aussetzung für die Fermentwirkung. Das Apoferment mit seiner spezifisc Konfiguration der Peptidkette ist verantwortlich für die Substratspezifität, prosthetische Gruppe vermittelt den Stoffumsatz. Für den Mechanismus Inaktivierung können somit zwei Möglichkeiten in Betracht gezogen werd Störung bzw. Blockierung der Funktion der prosthetischen Gruppe oder V änderungen an der hochkomplizierten Konfiguration des kolloiden Apoferme tes.

Auf Grund der verhältnismäßig langsamen Inaktivierung durch H^+-Ionen u der teilweisen Reaktivierung (6 %) des Enzyms durch Acetonfällung bei nie riger Temperatur, sowie der Unwahrscheinlichkeit einer Wechselwirkung zw schen dem Wasserstoff- und dem Kupferion der prosthetischen Gruppe weg der Gleichnamigkeit der Ladungen beider Ionen, könnte man für die Inaktivi rung des Fermentes durch H^+-Ionen eine Störung der Konfiguration des kollo den Apofermentes als sehr wahrscheinlich betrachten. In der lebenden Zelle die Quellung kolloider Eiweißkörper, welche durch die in der umgebend Flüssigkeit enthaltenden Ionen beeinflußt wird, also vom jeweiligen pH abhä gig ist, von großer Bedeutung. Durch Adsorption von Wasserstoffionen könn man sich eine Lockerung und spätere Trennung gewisser Bindungen, besonde der energiearmen Wasserstoffbrückenbindung vorstellen. Vermutlich tragen d Wasserstoffbrückenbindungen einen bedeutenden Teil zur Fixierung der kom plizierten Konfiguration des kolloiden Apofermentes bei. Da sicher eine groß Anzahl solcher Wasserstoffbrücken vorliegt, braucht es auch eine gewisse Zei bis soviel Bindungen gelöst sind, daß die substratspezifische Wirkung aufge hoben wird.

Einem anderen Mechanismus unterliegt wahrscheinlich die Inaktivierung de PPO durch schweflige Säure. Wasserstoffionen inaktivieren bei 25 ° C und eine Konzentration von 0,005 Mol pro Liter - entsprechend einem pH-Wert von 2,5 in zwei Stunden 95 % der PPO. Eine ebenso vollständige Inaktivierung bewirk SO_2 in einer Konzentration von nur 0,0001 Mol pro Liter und einer Reaktions zeit von nur 5 Min. Dieser Vergleich bringt ein sehr bemerkenswertes Ergebnis trotz 50facher Verdünnung bewirkt die SO_2 eine mindestens 24 mal schneller Inaktivierung. Sicher läuft die Reaktion zwischen SO_2 und der PPO in noch vie kürzerer Zeit ab, aber diese Verweilzeit von 5 Min ist zum Temperaturausgleic bei Warburgmessungen unumgänglich. Eine Verlängerung der Einwirkungszei der SO_2 auf das Enzym von 5 Min auf 10, 30 oder 60 Min bringt keine Änderung in der Anzahl der inaktivierten Enzymeinheiten mit sich. Daraus folgt, daß die Vergiftung des Enzyms durch SO_2 in der Tat schnell verlaufen muß. Die Sulfit-

ionen werden von entgegengesetzt geladenen Stellen des Enzyms angezogen und unter sofortiger Inaktivierung gebunden. Durch Steigerung der Sulfitmenge läßt sich wie bei einer Titration recht genau diejenige Menge ermitteln, die gerade ausreicht, um 90 % oder 95 % der Aktivität auszuschalten. Für 80 Brenzkatechineinheiten im Liter werden etwa 4,5 bis 5,0 mg SO_2 gebraucht. Ein kolloides Eiweißmolekül, wie das Apoferment, vermag Ionen aus der sie umgebenden Lösung zu adsorbieren. Sollte die Inaktivierung der PPO auf eine Reaktion des Sulfits mit dem Trägereiweiß beruhen, so sollte man wie bei der Reaktion mit den Wasserstoffionen eine langsame Inaktivierung erwarten. Trotz der höheren Verdüngung läuft die Reaktion schnell und irreversibel ab. Die Vermutung liegt daher nahe, daß es sich um eine Ionenreaktion handelt und die SO_2 mit den verhältnismäßig wenigen Cu^{++}-Ionen des Enzyms reagiert, dabei wird der Wertigkeitswechsel zwischen Cu^{++} und Cu^{+} blockiert (13). Zu den Hemmstoffen gehören überwiegend Schwefelverbindungen, wie Schwefelwasserstoff, Cystein, Thioharnstoff, Diäthyldithiocarbamat und einige Verbindungen, die mit Kupfer stabile Komplexe bilden, wie Cyanid und Salicylaldoxim.

Die Inaktivierung der PPO durch Erhitzung beruht immer auf einer Denaturierung des kolloiden Eiweißkörpers. Das Coferment, meist eine Verbindung aus der Vitamin B-Gruppe oder ein Metallion, hingegen ist thermisch sehr stabil. Die thermische Inaktivierung läuft wie die Inaktivierung durch Wasserstoffionen ebenfalls langsam. Die ersten Anzeichen einer Aktivitätsabnahme sind bei 35 ° C zu beobachten. Selbst bei 45 ° und 50 ° C ist das Enzym noch längere Zeit intakt. Bei 45 ° C sind nach 2,5 Stunden noch 50 % und nach 5 Stunden noch 25 % der Aktivität vorhanden. Dieser allmähliche Zusammenbruch der Eiweißkonfiguration deutet auf die Lösung schwacher Bindungen hin, wie Wasserstoffbrückenbindungen, die etwa 10 mal schwächer als kovalente Bindungen sind.

Versuch einer Deutung des Stabilisierungseffektes der Sorbinsäure.

Die Gegenwart von Sorbinsäure in einer Konzentration von 200 - 250 mg pro Liter bewirkt bei den beschriebenen drei Möglichkeiten der Inaktivierung eine deutliche Verzögerung in der Aktivitätsabnahme. Die Inaktivierung durch Wasserstoffionen erfährt bei 25 ° C und pH 2,5 eine Verlangsamung von etwa 20 %. Nach 30 Min sind ohne Sorbinsäure noch 30 % der Aktivität vorhanden, in Gegenwart von Sorbinsäure sind aber noch 50 % der Aktivität erhalten. Eine zweistündige Einwirkung eines pH 2,5 - Puffers hat eine 97prozentige Inaktivierung zur Folge, in Gegenwart von Sorbinsäure ist aber nur eine 73prozentige Inaktivierung eingetreten. Im pH 3,0-Puffer sind bei 25 ° C nach 5 Stunden noch 5 %, unter Zusatz von Sorbinsäure aber noch 25 % der Aktivität vorhanden. Auch bei pH 3,5 und 4,0 tritt dieser Stabilisierungseffekt in Erscheinung. Erst bei pH 4,5, einem pH-Wert, bei dem die PPO ohnehin längere Zeit stabil ist, wird der Effekt der Sorbinsäure klein. Die Möglichkeit eine Mobilisierung des Enzyms durch Sorbinsäure ist damit ausgeschlossen. Somit kann man diese Erscheinung auf eine Stabilisierung des Apofermentes zurückführen.

Auf Grund des — E-Effektes der CH_3-Gruppe der Sorbinsäure, dem dazwischen liegenden konjugierten System und der endständigen Carboxylgruppe, die einen + E-Effekt bewirkt, liegt die Sorbinsäure in einer stark polaren Form vor. Sie hat die Möglichkeit, an entsprechenden anderen Molekülen bindungsanteilig zu werden. Durch die beiden Doppelbindungen ist das Sorbinsäuremolekül noch

verhältnismäßig starr, es kann daher nur dann doppelseitig an anderen Mo
küleu bindungsanteilig werden, wenn entgegengesetzte Ladungen in gen
definierten Abständen vorhanden sind. Bei dem kolloiden Eiweißkörper
PPO mit seiner spezifisch gefalteten Peptidkette scheint diese Bedingung mel
fach erfüllt zu sein. Sorbinsäuremoleküle lagern sich an das Apoferment
und bewirken eine zusätzliche Vernetzung der Peptidkette. Dadurch wird
Konfiguration des kolloiden Apofermentes derart gefestigt, daß nunmehr
Inaktivierung des Fermentes eine höhere Wasserstoffionenkonzentration nö
ist oder im Falle der thermischen Inaktivierung mehr Energie erforderlich wi
Zur Stabilisierung von 80 Brenzkatechineinheiten PPO pro Liter reichen 250
Sorbinsäure, entsprechend 0,0022 Mol, gerade aus; kleinere Mengen bewirk
eine unvollständige Stabilisierung. Überschüssige Sorbinsäure bleibt ohne W
kung (Abb. 4). Eine Bindung von Sorbinsäure an das Kupferion des Coferment
scheint unwahrscheinlich. Es sind 22mal soviel Sorbinsäuremoleküle erforderli
als SO_2 Moleküle, um eine maximale Wirkung zu erzielen. Es scheint auch fra
würdig, ob eine derartige Bindung überhaupt stabilisierend wirken kann; ei
Hemmwirkung wäre dagegen naheliegender. Ein derartiger Effekt konnte ab
nicht beobachtet werden. Gegen eine alleinige Bindung zwischen Kupfer u
Sorbinsäure spricht auch das Ergebnis der Wärmebehandlung (Abb. 2). Die Bi
dung von Sorbinsäure nur an das Kupferion müßte den Zusammenfall beid
Kurven zur Folge haben. Bei der Wärmebehandlung eines intakten und ein
durch SO_2 vergifteten Enzyms aber müßten beide Kurven zusammenfallen, j
doch ist dieses sehr schwer zu beweisen.

Der stabilisierende Effekt der Sorbinsäure ist abhängig von der jeweils her
schenden Temperatur. Im Bereich von 20 - 25 °C beträgt er 7 - 8 %, steige
sich langsam und erreicht bei 40 °C das Maximum von 27 %, nimmt dar
wieder langsam ab und beträgt bei 50 °C nur noch 5 %. Um den gleichen Inak
tivierungsgrad zu bekommen, muß die sorbinsäurehaltige Enzymlösung für
Min einer 5 - 7 °C höheren Temperatur ausgesetzt werden als die sorbi
säurefreie Lösung. Der größere Energieaufwand läßt sich leicht erklären, inde
man eine zusätzliche Vernetzung des kolloiden Apofermentes durch Adsorptio
von Sorbinsäure annimmt. Ein Eiweißmolekül, welches durch eine weite
Vernetzung stabilisiert ist, braucht zur Zerstörung der spezifischen Konfigura
tion seiner Peptidketten eine höhere Temperatur.

Eine Enzymlösung wird durch SO_2 in sehr kurzer Zeit inaktiviert. 5 mg SO
pro Liter sind ausreichend für eine 95prozentige Inaktivierung von 80 Brenz
katechineinheiten (Abb. 3). Erfolgt jedoch vor der Schwefelung ein Zusatz vo
200 mg Sorbinsäure pro Liter, so beobachtet man eine erhebliche Resistenz d
PPO gegen SO_2. Man muß mit einer Restaktivität von 42 % rechnen, wenn ein
sorbinsäurehaltige Enzymlösung mit nur 5 mg SO_2 pro Liter versetzt wird. B
10 mg SO_2 pro Liter verbleiben noch 23 % Restaktivität und bei 18 mg SO_2 i
Liter sind immer noch 10 % Restaktivität vorhanden. Erst mit 30 mg SO_2/Lt
wird eine 95prozentige Inaktivierung erreicht. Eine Deutung dieser Resistenz
erscheinung ist nicht ganz einfach. Würde das Kupferion eine exponierte Lag
auf der Oberfläche des Apofermentes haben, dann müßte im Verhalten d
beiden Enzymlösungen gegenüber der SO_2 kein Unterschied auftreten, voraus
gesetzt, daß die Vergiftung des Enzyms durch SO_2 - wie Schirrmacher-
Göllner annimmt (13) - eine Reaktion mit dem Kupferion ist. Die SO_2 würd

mit dem Kupfer reagieren, gleichgültig ob sich Sorbinsäure am Apoferment angelagert hat oder nicht. Da aber eine erhebliche Abweichung im Verlauf der beiden Kurven besteht, muß man eine zentrale Lage des Kupferions annehmen. Durch die Anlagerung von Sorbinsäure auf der Oberfläche des Fermentes tritt eine zusätzliche Vernetzung ein, die eine Festigung der Konfiguration des kolloiden Apofermentes bewirkt, wodurch schließlich das Eindringen der SO_2 erschwert wird. Die Resistenz des Fermentes in sorbinsäurehaltiger Lösung ist also sehr wahrscheinlich auf einen sterischen Effekt zurückzuführen. Um in kurzer Zeit den gleichen Inaktivierungsgrad zu erreichen, ist in Gegenwart von Sorbinsäure 5 mal soviel SO_2 erforderlich.

Zusammenfassung

Drei verschiedene Methoden zur Inaktivierung der PPO werden miteinander verglichen. Die Inaktivierung durch H^+-Ionen und jene durch Erwärmung sind Reaktionen, die eine gewisse Zeit benötigen. Dagegen erfolgt die Vergiftung der PPO durch SO_2 selbst in hoher Verdünnung sehr schnell. Daraus folgt, daß Wasserstoffionen bzw. Wärmeenergie das Apoferment schädigen. Es werden wahrscheinlich die verhältnismäßig schwachen Wasserstoffbrückenbindungen gelöst, wodurch die substratspezifische Konfiguration des Apofermentes aufgehoben wird. Die spontane Vergiftung der PPO durch SO_2 deutet auf eine Reaktion mit dem Kupferion des Cofermentes hin. In Gegenwart von Sorbinsäure tritt bei allen drei Methoden der Inaktivierung eine deutliche Verzögerung ein. Die sorbinsäurehaltigen Enzymlösungen sind gegen Wasserstoffionen im Mittel um 20 % stabiler. Der Stabilisierungseffekt beim Erhitzen ist abhängig von der jeweiligen Temperatur, er ist besonders stark bei 40 °C und beträgt dann 27 %. Die Stabilisierung des Fermentes beruht wahrscheinlich auf einer Adsorption von Sorbinsäuremolekülen an das Apoferment. Es erfolgt eine zusätzliche Vernetzung der komplizierten Peptidkette, wodurch die Konfiguration des kolloiden Apofermentes gefestigt wird. Ein derart stabilisiertes Ferment benötigt nunmehr zu seiner Inaktivierung eine höhere Wasserstoffionenkonzentration bzw. eine größere thermische Energie. Auch die Inaktivierung des Enzyms durch SO_2 erfolgt in sorbinsäurehaltiger Lösung mit einer erheblichen Verzögerung. Sterische Gründe scheinen hier die Ursache zu sein. Infolge der Verfestigung der Konfiguration des Apofermentes durch Adsorption von Sorbinsäure wird das Eindringen der SO_2 erschwert.

Für die Praxis ergibt sich daraus: Sorbinsäure darf nur nach Inaktivierung der PPO zur Anwendung kommen.

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The Catalytic Action of Blood and Blood Pigment on the Oxidation of Unsaturated Fatty Acids.

By Chiaki Shimizu and Tadanobu Fukhuhara (Department of Fisheries, Nagasaki University).

(The manuscript was received on September 4, 1958).

(For a brief English synopsis, refer to the original text on p. 760.)

Introduction:

H. Suellman (1) reported the fact that unsaturated fatty acids are oxidized by the catalytic action of hemin and A. J. Tappel and Y. T. Lew (2) published a report to the effect that hemoglobin, myoglobin and cytochrome are powerful catalysts for the oxidation of unsaturated fatty acids. F. Haurowitz, P. Schwerin and M. Yensen (3) et al. stated that unsaturated fatty acids are oxidized by the catalytic action of hemin and that the oxidation products destroy hemin.

In the previous report, the authors reported on the oxidation of vitamin A and unsaturated fatty acids by the catalytic action of hemin (4). In the current experiment, the authors examined the oxidation of unsaturated fatty acids by the blood and blood pigment in accordance with the crocin method (5) and the measurement of the ultraviolet absorption curve. As a result, it was confirmed that, in the presence of a fatty acid having the structure of $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$ in the molecule, not only the said fatty acid but also the co-existing unsaturated fatty acids,and unsaturated hydrocarbons are oxidized but that, in the presence of a conjugate unsaturated fatty acid and unsaturated hydrocarbon, etc. individually without the said fatty acid, they are hardly oxidized even in the presence of blood or hemin, etc.

Experimental Method:

1. Oxidation Action of Mackerel Scomber Tapeinocephalus Bleeker Blood upon Unsaturated Fatty Acids.

Blood obtained from raw fish was diluted to a proper concentration after repeating freezing and melting twice. In other words, in a 50 cubic centimeter triangular flask, two cubic centimeters of an M/15 phosphoric acid buffer solution (pH in the range between 5.0 and 8.3), two cubic centimeters of the crocin solution of a proper concentratioh, 0.5 cubic centimeters of an aceton solution of 10 per cent linolic acid methyl ester (iodine value being 174.0) and 0.1 cubic centimmeter of the sample were added and the reaction was carried out at the temperature of 20 degrees centigrade. It is mentioned that, instead of diluted blood, the same diluted blood, emulsified after quietly boiling it for a period of five minutes, 0.1 cubic centimeter of an alkaline solution of medical hemoglobin (manufactured by Wako) (hemoglobin: ca. 50 (gamma)), 0.1 cubic centimeter of an alkali hematin solution using crystal hemin (6) (hemin: five (gamma)), 0.1 cubic centimeter of a hemoglobin solution which was reduced by a $Na_2S_2O_4$ (of a proper concentration) and 0.1 cubic centimeter of a hematin solution (of a proper concentration) were caused to act upon linolic acid methyl ester.

In other words, after the reaction of each of the aforementioned mixture solutions, 10 cubic centimeters of ethanol and 0.2 cubic centimeters of a 20 per cent lead acetate solution were added and centrifugal separation was carried out for a period of 10 minutes (3,000 r.p.m.). Then, the remaining crocin was colorimetrically determined by using a 480 m(mu) filter to check the extent of oxidation. The result of this experiment is shown in Figure 1.

(Figure 1 Effect of pH on the Oxidation of Fatty Acid by Mackerel Blood and Blood Pigment.)

2. Catalytic Action of Mackerel Blood on Two or Three Unsaturated Fatty Acids and Squalene.

A result was obtained in the previous report (4) to the effect that hemin, like lipoxidaze, acted only on some specific unsaturated fatty acids. Therefore, a similar experiment was carried out by using mackerel blood.

Palmitic Acid Methyl Ester: After the esterification of palmitic acid of the reagent first class, it was distilled under reduced pressure and made into a five per cent acetone solution.

Linolic Acid Methyl Ester: Fatty acid was freed from linseed oil and was esterified. Then, it was distilled under reduced pressure and the main fraction (whose iodine value was 174.0) was made into a five per cent acetone solution.

(alpha)-eleostearic acid methyl ester: Fatty acid was freed from tung oil and was esterified. The main fraction which had been distilled under reduced pressure was cooled at a temperature of minus 15 degrees centigrade for a period of more than 50 hours. After filtration at low temperature, the filtration solution (iodine value being 110.7) was made into a five per cent solution.

Sorbic Acid Methyl Ester: Sorbic acid manufactured by Taito was esterified and recrystallized. Then, it was made into a five per cent acetone solution.

Squalene: Liver oils obtained from Yume sharks and Hetsurano sharks were distilled under reduced pressure. Metallic sodium was added to the main fraction and this was redistilled. The main fraction was cleaned with a one N NaOH alcohol solution, the same was cleaned with water and dried. The liquid portion obtained at the temperature of minus 25 degrees centigrade (with the precipitates having been removed therefrom) (the iodine value being 378.1, n_d^{19} 1.4947) was made into a five per cent acetone solution.

In a test tube, 0.1 cubic centimeters of an acetone solution of 2.5 per cent linolic acid methyl ester was added to one cubic centimeter of M/20 phosphoric acid buffer solution (pH 7.0), two cubic centimeters of the crocin aqueous solution of some proper concentration, and 0.2 cubic centimeters of an acetone solution of hemin (hemin: five (gamma)). To each of these, 0.1 per cent cubic centimeter of a five per cent acetone solution of each methyl

ester and squalene of linolic acid, (alpha)-eleostearic acid, sorbic acid and palmitic acid was added. After carrying out the reaction for a certain period of time at the temperature of 35 degrees centigrade, 10 cubic centimeters of ethanol and 0.2 cubic centimeters of a 20 per cent lead acetate solution were added and centrifugal separation was carried out for a period of 10 minutes. Then, the remaining crocin was colorimetrically determined and the extent of the oxidation of the fatty acid was examined. A result shown in Figure 2 was obtained.

(Figure 2 Effect of fatty acid on the decrease of crocin.)

3. Ultraviolet Absorption Curve of the Oxidation Products of Unsaturated Fatty Acids and Squalene.

An experiment was carried out by using the samples which were used in 2. In each test tube, one cubic centimeter each of an eight milligram per cubic centimeter of linolic acid methyl ester, (alpha)-eleostearic acid methyl ester and sorbic acid methyl ester and 35 milligrams per cubic centimeter of squalene was added and, further, one cubic centimeter of an ether solution of linolic acid methyl ester was supplementarily added to these. These were placed in warm bath and ether was evaporated. Then, one cubic centimeter of an N/100 NaOH solution of hemin (hemin 0.2 milligrams) was added and the reaction was carried out at the temperature of 35 degrees centigrade.

As a control test, further, one cubic centimeter of N/100 NaOH was used instead of the hemin solution. After carrying out the reaction for a fixed period of time, one cubic centimeter of N/100 HCl was added and, after these were well mixed, the fatty acid was extracted out by using five cubic centimeters of ether and the light absorption co-efficient was measured.

In the case of the measurement of an absorption curve of squalene, the ether was evaporated after the extraction of ether, one cubic centimeter of

a 2N NaOH alcoholic solution was added, saponification was carried out at the temperature of 50 degrees centigrade, squalene was extracted with ether and the co-efficient of light absorption was measured. The result of this experiment is shown in Figure 3.

(Figure 3 Figure 3a. Absorption spectra of the oxide of methyl ester of linoleic acid in ether, and Figure 3b. Absorption spectra of the oxide of methyl ester of (alpha)-eleostearic acid in ether.)

(Figure 3 Figure 3c. Absorption spectra of the oxide of methyl ester of sorbic acid in ether, and Figure 3d. Absorption spectra of the oxide of squalene in ether.)

Observation of the Result Obtained and Conclusion

The oxidation of unsaturated fatty acids by the catalytic action of mackerel blood which has been properly diluted is the strongest in the neighborhood of pH 7.0 as shown in Figure 1. In other words, the optimal pH is observed as in the case of enzyme action. When the said blood is boiled, however, the optimum pH is not only no longer observed but the capacity of oxidation is reduced to two-thirds of the original capacity. As compared with the acid side, moreover, the oxidation action becomes stronger on the alkali side. Such tendencies were also observed in the case of medicinal hemoglobin, alkali hematin, reduced hemoglobin and reduced hematin.

In other words, the hemin pigment has a greater oxidation action on the alkaline side irrespective of the atomic value in the blood pigment or whether there is globin or not.

From the above result, it is obvious that the oxidation action of the boiled blood is due to the hemin pigment in the blood. In the case of raw blood, there is observed the optimum pH for oxidation in the neighborhood of 7.0 and, in view of the fact that this value is the same as the optimum pH for lipoxidase-like enzyme in the liver, it seems that an enzyme similar to lipoxidase exists in the blood and that the same oxidizes the unsaturated fatty acids

together with blood pigment.

As was stated in the previous report to some extent (4), since the catalytic actions of hemin and lipoxidase-like enzyme upon the oxidation of unsaturated fatty acids are considerably different for different fatty acids used, diluted mackerel blood was caused to act upon the unsaturated fatty acids and aqualene. As a result, it was observed that there is no reduction in crocin at all in the case of (alpha)-eleostearic acid, methyl ester of sorbic acid and aqualene, etc. which have a high degree of unsaturation when they are used individually but that there was observed a reduction in crocin when some linolic acid methyl ester is mixed.

In other words, (alpha)-eleostearic acid and aqualene, etc. are not directly oxidized by the catalytic action of the blood but the oxidation products of linolic acid methyl ester which has been produced by the catalytic action of the blood oxidize the other unsaturated fatty acids and squalene, etc., and, moreover, these oxidation products oxidize the crocin like the oxidation products of linolic acid methyl ester. Exactly like crocin, carocin and vitamin A, etc. are naturally oxidized by these oxidation products. However, since it is possible to think of such oxidation products as will not oxidize crocin, the ultra-violet absorption curves of the products which were oxidized by the catalytic action of hemin were examined.

As is shown in Figure 3, the light-absorption co-efficient at 234 m(mu) increases markedly by the catalytic action of the hemin in the case of linolic acid methyl ester ((delta)^{9,12}). This is a result of the production of a conjugate double bond ((delta)^{10,12} or (delta)^{9,11}) due to isomerization stemming from the oxidation. It is mentioned in this connection that a slight increase in the light-absorption co-efficient at 270 m(mu) is believed to be due to the mixture of hydroperoxide or a small amount of linoleic acid methyl ester which was mixed.

When existing alone, (alpha)-eleostearic acid methyl ester is only absorbed to hemin, even when it exists, without being oxidized thereby. There is observed a slight reduction in Figure 3b; however, the said reduction is almost the same as in the case of (alpha)-eleostearic acid without containing hemin. When linolic acid methyl ester exists, however, there was observed a reducton lin light-absorption co-efficients at 260 m(mu), 270 m(mu) and 280 m(mu), thereby showing the fact that conjugate double bond ((delta)^{9,11,13}) is oxidized. At the same time, the light absorption co-efficient at 234 m(mu) increases as in the case of linolic acid methyl ester. This is believed to be due to the fact that there are some conjugate double bonds remaining inasmuch as oxidation progresses from the double bond at the end.

Such oxidation as the above progresses somewhat even if there is no hemin, provided that there is linolic acid methyl ester. In this case, the light-absorption co-efficient of co-existing linolic acid methyl ester does not have any bearing because the unit is different as shown in Figure 3. When existing alone, sorbic acid methyl ester and aqualene are hardly oxidized but are only adsorbed to hemin when they are alone as in the case of (alpha)-eleostearic acid methyl ester. In the presence of linolic acid, however, the light-absorption co-efficient at 253 m(mu) is reduced in the former and the light-absorption co-efficient at 275 m(mu) is increased in the latter, thereby showing the progress of oxidation.

It is further mentioned that the amount of linolic acid methyl ester is related to the oxidation of (alpha)-eleostearic acid methyl ester, etc. due to the catalytic action of hemin and, when the same is more than the optimum amount or when it is less than the optimum amount, oxidation does not progress satisfactorily. As in the result (3) obtained by F. Haurowitz (3), further, there was observed the destruction of hemin as the oxidation progressed.

From the above experiments, it can be stated that such a fatty acid as linolic acid which contains a molecular structure of $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$ in the molecule or vitamin F is required in the oxidation of an unsaturated fatty acid by the catalytic action of blood. Both blood and blood pigment hardly have any catalytic action in the oxidation of high unsaturated fatty acids and hydrocarbons which do not contain such a molecular structure. When they co-exist with linolic acid, these unsaturated fatty acids and hydrocarbons gradually progress oxidation by the autoxidation of linolic acid even without the catalytic action of the blood.

The authors would like to express their gratitude to Mr. Kenzo Yamakawa of the Fisheries Research Institute for the Eastern Seas who was kind enough to donate squalene in carrying out the current experiments.

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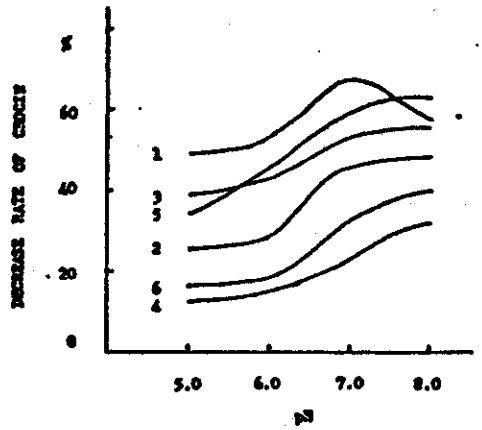

Fig. 1. Effect of pH on the oxidation of fatty acid by the mackerel blood and blood pigment (20°C., 20 min.).
methyl ester of linoleic acid: 50 mg. acetone solution.
1. diluted mackerel blood. 2. boiled blood (100°C., 5 min.). 3. medicinal hemoglobin. 4. reduced medicinal hemoglobin. 5. alkali hematin. 6. reduced alkali hematin.

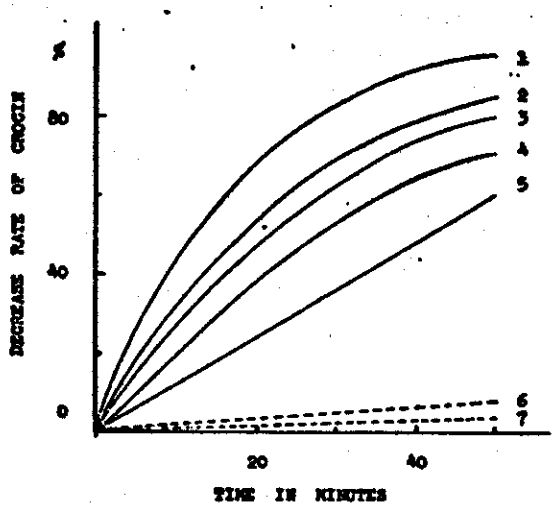

Fig. 2. Effect of fatty acid on the decrease of crocin (35°C., pH 7.0).
hemin: 5γ acetone solution.
fatty acid: mixture of 2.5 mg. methyl ester of linoleic acid and 5 mg. methyl ester of other fatty acids or squalene.
1. linoleic acid. 2. α-eleostearic acid. 3. squalene. 4. sorbic acid. 5. palmitic acid. 6. linoleic acid and none hemin. 7. 7.5 mg. α-eleostearic acid, palmitic acid, sorbic acid or squalene but none linoleic acid.

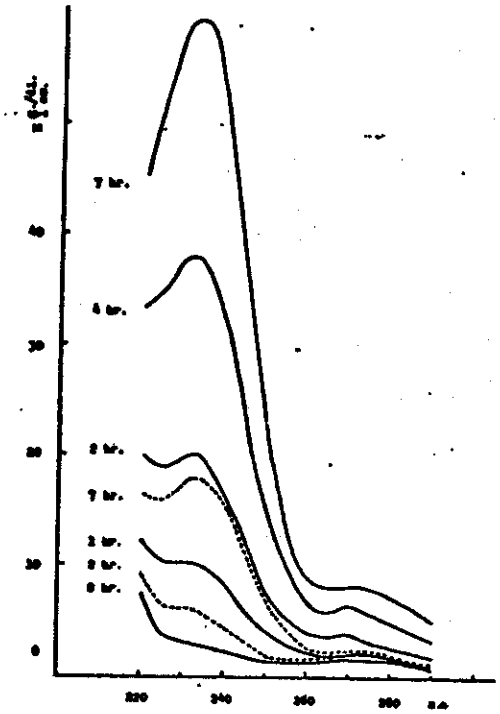

Fig. 3a. Absorption spectra of the oxide of methyl ester of linoleic acid in ether.
reaction temperature: 35°C.
hemin: 0.2 mg. N/100 NaOH solution.
methyl ester of linoleic acid: 16 mg.
dotted lines: none hemin.

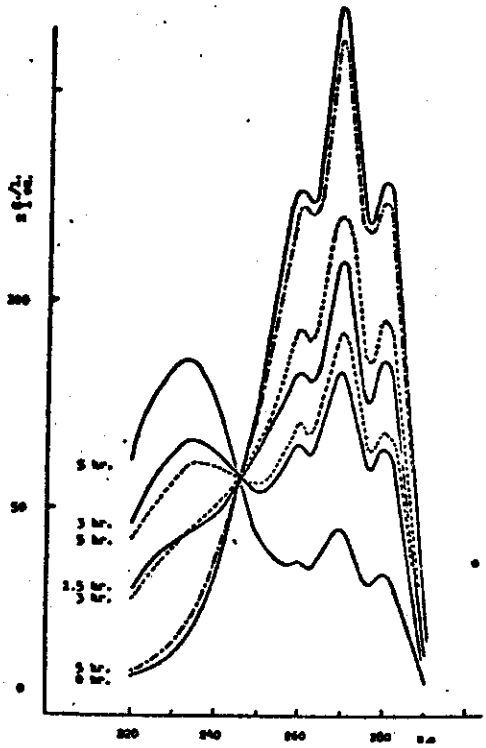

Fig. 3b. Absorption spectra of the oxide of methyl ester of α-eleostearic acid in ether.
solid lines: 8 mg. methyl ester of α-eleostearic acid and methyl ester of linoleic acid.
dotted lines: none hemin.
chain line: none methyl ester of linoleic acid.

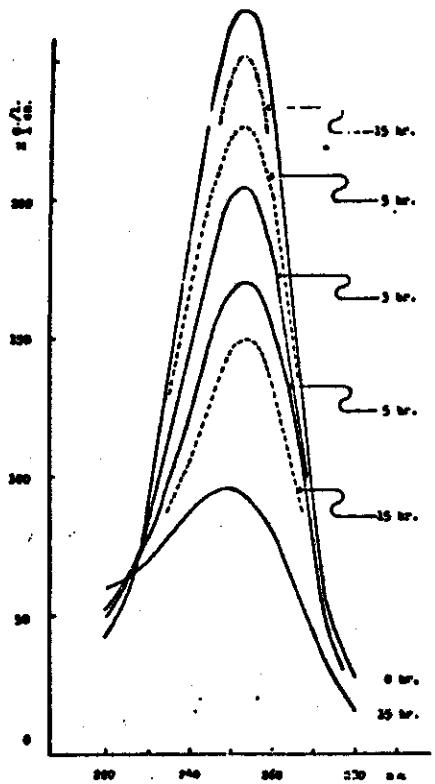

Fig. 3c. Absorption spectra of the oxide of methyl ester of sorbic acid in ether.
solid lines: 8 mg. methyl ester of sorbic acid and methyl ester of linoleic acid.
dotted lines: none hemin.
chain line: none methyl ester of linoleic acid.

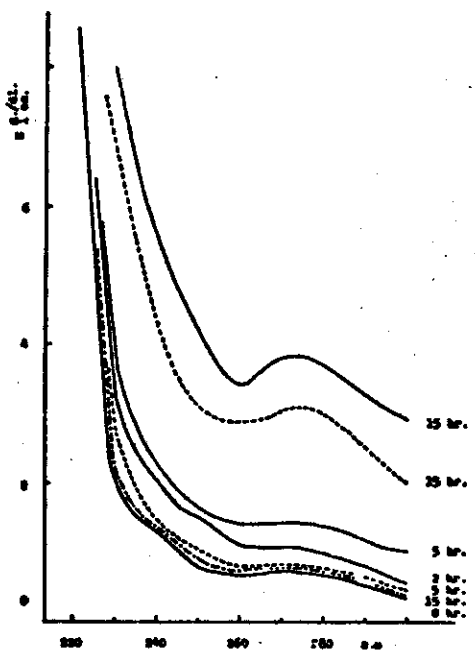

Fig. 3d. Absorption spectra of the oxide of squalene in ether.
solid lines: 35 mg. squalene and 8 mg. methyl ester of linoleic acid.
dotted lines: none hemin.
chain line: none methyl ester of linoleic acid.

Sorbic Acid 414

不飽和脂肪酸の酸化に対する血液ならびに血液色素の触媒作用

清水千秋・福原忠信
(長崎大学水産学部)

The Catalytic Action of Blood and Blood Pigment on the Oxidation of Unsaturated Fatty Acid and Hydrocarbon

Chiaki SHIMIZU and Tadanobu FUKUHARA

The blood of mackerel oxidizes the linoleic acid, and the oxidizing action of the blood is caused by the catalytic action of hemin pigment and lipoxidase-like enzyme (Fig. 1).

As mentioned above hemin oxidizes the linoleic acid (vitamin F), but it does not oxidize sorbic acid, eleostearic acid and squalene. These acids and the hydrocarbon are oxidized by the oxide of linoleic acid. The oxides of sorbic acid, eleostearic acid and squalene oxidize the carotinoid pigment as the oxide of the linoleic acid (Fig. 2, 3).

緒言

H. SUELLMAN[1] はヘミンの触媒作用で不飽和脂肪酸が酸化されることを，A. L. TAPPEL, Y. T. LEW[2] は hemoglobin, myoglobin, cytochrome が不飽和脂肪酸酸化の強力な触媒であることを報告しており，F. HAUROWITZ, P. SCHWERIN, M. YENSON[3] 等は不飽和脂肪酸はヘミンの触媒作用で酸化され，しかもその酸化生成物でヘミンが破壊されると報告している。著者等も前報[4] でヘミンの触媒作用による不飽和脂肪酸ならびにビタミンAの酸化について若干報告したが今回は血液ならびに血液色素による不飽和脂肪酸および不飽和炭化水素の酸化をクロチン法[5] および紫外部吸収曲線の測定でしらべたところ，分子中に $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$ なる構造をもつた脂肪酸が存在するときは該脂肪酸は勿論共存する他の不飽和脂肪酸，不飽和炭化水素も酸化されるが該脂肪酸を含まず，共軛系不飽和脂肪酸，不飽和炭化水素等が単独のときは血液，ヘミン等が共存しても殆んど酸化されないこと等を確かめたのでこれらについて報告する。

実験方法

1. サバ *Scomber tapeinocephalus* BLEEKER 血液の不飽和脂肪酸酸化作用

生魚から採つた血液を二度凍結融解を繰返した後適当濃度に稀釈して試料とした。即ち 50cc 三角フラスコに M/15 燐酸緩衝液 (pH 5.0～8.3) 2cc, 適当濃度のクロチン水溶液 2cc, 10% リノール酸メチルエステル (沃素価: 174.0) のアセトン溶液 0.5cc, 試料 0.1cc を加え 20°C で反応させた。なお稀釈血液の代りに同稀釈血液を5分間静かに煮沸後乳化したもの，薬用 hemoglobin (和光製) アルカリ溶液 0.1cc (hemoglobin: ca. 50γ), 結晶ヘミン[6] を使用した alkali hematin 溶液 0.1cc (hemin: 5γ), $Na_2S_2O_4$ で還元した hemoglobin 溶液 0.1cc (適当濃度) および hematin 溶液 0.1cc (適当濃度) をリノール酸メチルエステルに作用させた。即ち以上の各混合液を20分間反応させた後エタノール 10cc, 20% 酢酸鉛溶液 0.2cc を加え 10分間遠心分離 (3,000 r.p.m.) 後 480mμ フイルターを使用して残存クロチンを比色定量して酸化

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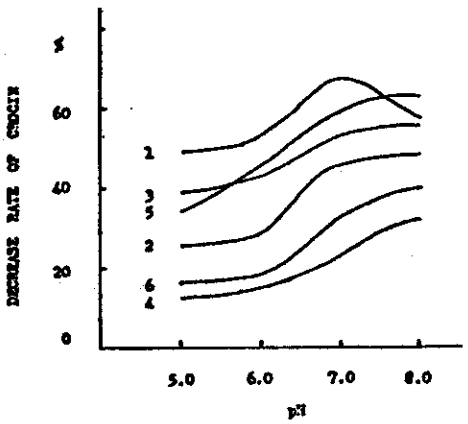

Fig. 1. Effect of pH on the oxidation of fatty acid by the mackerel blood and blood pigment (20°C., 20 min.).
methyl ester of linoleic acid: 50 mg. acetone solution.
1. diluted mackerel blood. 2. boiled blood (100°C., 5 min.). 3. medicinal hemoglobin. 4. reduced medicinal hemoglobin. 5. alkali hematin. 6. reduced alkali hematin.

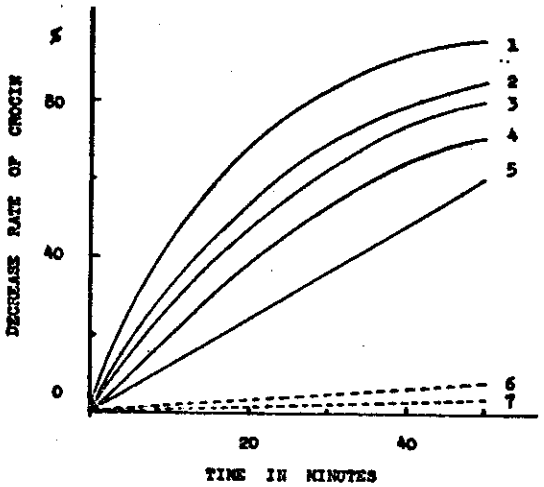

Fig. 2. Effect of fatty acid on the decrease of crocin (35°C., pH 7.0).
hemin: 5γ acetone solution.
fatty acid: mixture of 2.5 mg. methyl ester of linoleic acid and 5 mg. methyl ester of other fatty acids or squalene.
1. linoleic acid. 2. α-eleostearic acid. 3. squalene. 4. sorbic acid. 5. palmitic acid. 6. linoleic acid and none hemin. 7. 7.5 mg. α-eleostearic acid, palmitic acid, sorbic acid or squalene but none linoleic acid.

の程度をしらべた。実験結果は Fig. 1 に示す。

2, 2, 3 の不飽和脂肪酸およびスクアレンに対するサバ血液の触媒作用

前報[4] でヘミンはリポキシダーゼと同じように特定の不飽和脂肪酸にだけ作用するような結果をえたので今回はサバ血液で同様試験を行つた。

パルミチン酸メチルエステル　試薬1級パルミチン酸をエステル化後減圧蒸溜し5% アセトン溶液とした。

リノール酸メチルエステル　局方亜麻仁油から脂肪酸を遊離し，エステル化後減圧蒸溜して主溜部分（沃素価: 174.0）を 5% アセトン溶液とした。

α-エレオステアリン酸メチルエステル　局方桐油から脂肪酸を遊離し，エステル化後減圧蒸溜した主溜部分を −15°C に 50 時間以上冷却し，低温沪過後沪液（沃素価: 110.7）を 5% アセトン溶液とした。

ソルビン酸メチルエステル　台糖製ソルビン酸をエステル化して再結した後 5% アセトン溶液とした。

スクアレン　ユメザメ，ヘラツノザメの肝油を減圧蒸溜した主溜部分に金属ナトリウムを加えて再蒸溜しその主溜部分を 1N NaOH アルコール液で洗滌した後水洗乾燥し −25°C で出来た沈澱物をとり除いた液状部分（沃素価: 378.1, n_D^{19} 1.4947）を 5% アセトン溶液とした。

試験管に M/20 燐酸緩衝液 (pH 7.0) 1cc, 適当濃度のクロチン水溶液 2cc, ヘミンのアセトン溶液 0.2cc (hemin: 5γ) に 2.5% リノール酸メチルエステルのアセトン溶液 0.1cc を加え，各々にリノール酸，α-エレオステアリン酸，ソルビン酸，パルミチン酸の各メチルエステルおよびスクアレンの 5% アセトン溶液 0.1cc を加え 35°C で一定時間反応させた後エタノール 10cc, 20% 酢酸鉛溶液 0.2cc を加えて 10 分間遠心分離後残存クロチンを比色定量して脂肪酸の酸化程度をしらべたところ Fig. 2 に示すような結果をえた。

3. 不飽和脂肪酸およびスクアレンの酸化生成物の紫外部吸収曲線

2で使用した試料を用いて実験を行つた。即ち各々の試験管にリノール酸メチルエステル，α-エレオステアリン酸メチルエステルおよびソルビン酸メチルエステルの各々 8 mg/cc エーテル溶液，スクアレンの 35 mg/cc エーテル溶液を各々 1 cc ずつ加えたものおよびこれらにさらにリノール酸メチルエステルのエーテル溶液 1 cc ずつを追加したものを温湯中に入れてエーテルを蒸発させた後ヘミンの N/100 NaOH 溶液 1 cc (hemin: 0.2 mg) を加え 35°C で反応させた。なお対照試験としてヘミン溶液の代りに N/100 NaOH 1 cc を使用した。これらの混液を一定時間反応させた後 N/100 HCl 1 cc を加えてよく混合した後 5 cc のエーテルで脂肪酸を抽出し吸光係数を測定した。なおスクアレンの吸収曲線測定ではエーテル抽出後エーテルを蒸発させ 2N NaOH アルコール溶液 1 cc を加え 50°C で鹸化後スクアレンをエーテルで抽出して吸光係数を測定した。実験結果は Fig. 3 に示す。

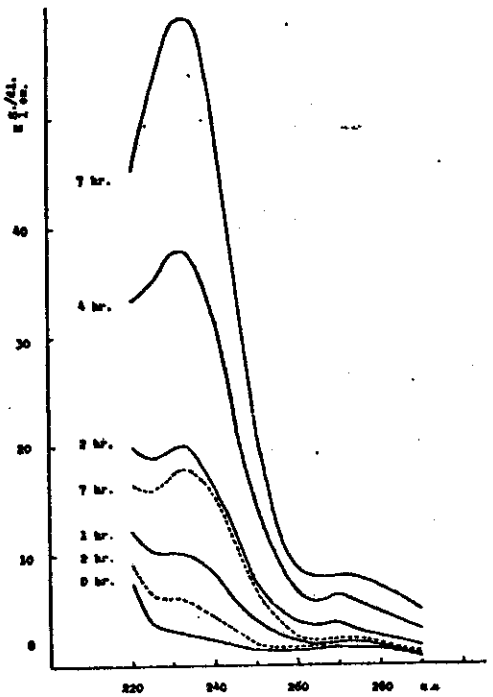

Fig. 3a. Absorption spectra of the oxide of methyl ester of linoleic acid in ether.
reaction temperature: 35°C.
hemin: 0.2 mg. N/100 NaOH solution.
methyl ester of linoleic acid: 16 mg.
dotted lines: none hemin.

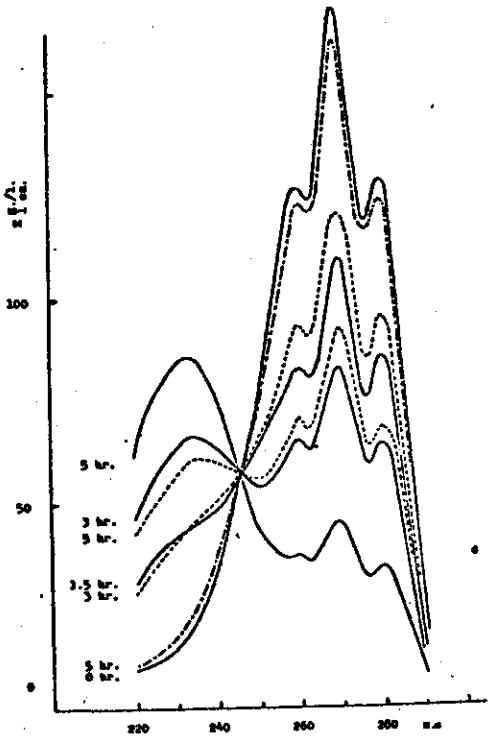

Fig. 3b. Absorption spectra of the oxide of methyl ester of α-eleostearic acid in ether.
solid lines: 8 mg. methyl ester of α-eleostearic acid and methyl ester of linoleic acid.
dotted lines: none hemin.
chain line: none methyl ester of linoleic acid.

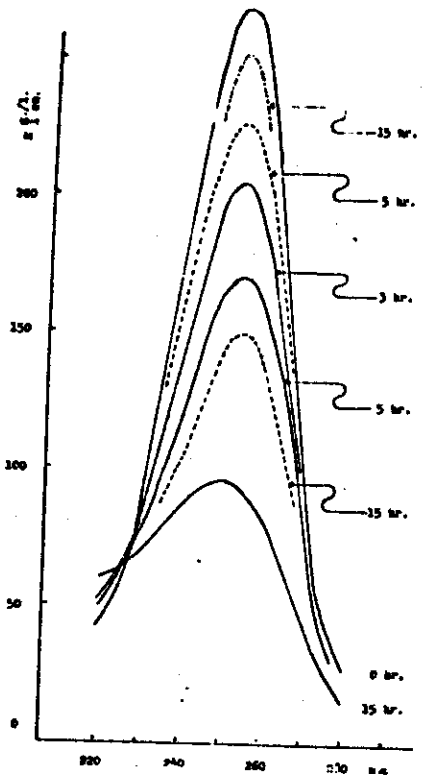

Fig. 3c. Absorption spectra of the oxide of methyl ester of sorbic acid in ether.
solid lines: 8 mg. methyl ester of sorbic acid and methyl ester of linoleic acid.
dotted lines: none hemin.
chain line: none methyl ester of linoleic acid.

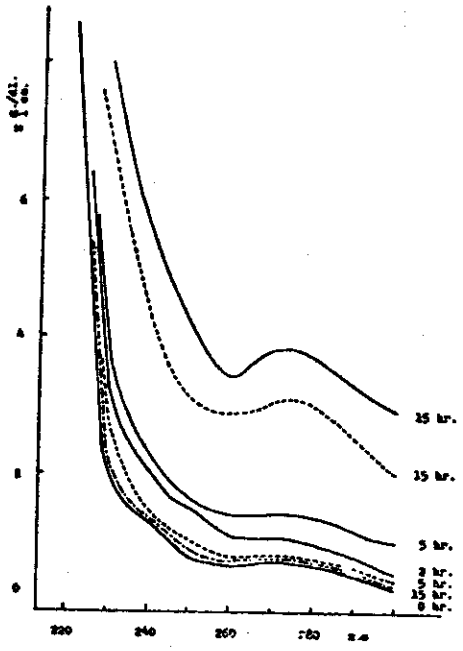

Fig. 3d. Absorption spectra of the oxide of squalene in ether.
solid lines: 35 mg. squalene and 8 mg. methyl ester of linoleic acid.
dotted lines: none hemin.
chain line: none methyl ester of linoleic acid.

結果の考察ならびに結論

Fig. 1 に示すように適当に稀釈したサバ血液の触媒作用による不飽和脂肪酸の酸化は pH 7.0 附近で最も強い。即ち酵素作用のように最適 pH が認められるが該血液を煮沸した場合には最適 pH が認められなくなるのみならず酸化力が 3 分の 2 に減少し，しかも酸化作用は酸側に比較しアルカリ側で強くなる。このような傾向は薬用 hemoglobin, alkali hematin, reduced hemoglobin および reduced hematin の場合にも見られる。即ち血色素中の鉄の原子価，globin の有無にかかわらずヘミン色素はアルカリ側で酸化作用が大きい。以上の結果から煮沸血液の酸化作用が血液中のヘミン色素によることは明白である。生の血液では 7.0 附近に酸化の最適 pH が認められ，しかもこの値は肝臓中のリポキシダーゼ様酵素の最適 pH と同じであることから考えて，多分血液中にもリポキシダーゼ様酵素が存在し，これが血液色素と共に不飽和脂肪酸を酸化するものと考えられる。

前報[4] でも若干のべたが不飽和脂肪酸の酸化に対するヘミンおよびリポキシダーゼ様酵素の触媒作用は作用する脂肪酸によつてかなり異なるので不飽和脂肪酸およびスクアレンに，稀釈したサバ血液を作用させた結果 Fig. 2 に示すように α-エレオステアリン酸，ソルビン酸のメチルエステルおよびスクアレン等不飽和度の高いものも単独の時はクロチンが全然減少しないが，リノール酸メチルエステルが多少混入するとクロチンが減少する。即ち α-エレオステアリン酸，スクアレン等は直接血液の触媒作用では酸化されないが，血

液の触媒作用で出来たリノール酸メチルエステルの酸化生成物が他の不飽和脂肪酸，スクアレン等を酸化しかもこれらの酸化生成物がリノール酸メチルエステルの酸化生成物と同じようにクロチンを酸化するのであり，カロチン，ビタミンA等も当然クロチン同様にこれらの酸化生成物で酸化される。しかしクロチンを酸化しないような酸化生成物も考えられるのでヘミンの触媒作用で酸化した生成物の紫外部吸収曲線をしらべた。

Fig. 3 に示すようにリノール酸メチルエステル ($\Delta^{9,12}$) はヘミンの触媒作用で 234 mμ の吸光係数が著しく増大するが，これは酸化のために異性化して共軛二重結合 ($\Delta^{10,12}$ あるいは $\Delta^{9,11}$) が出来た結果である。なお 270 mμ の吸光係数が多少増加するのは多分 hydroperoxide あるいは僅かに混ざつていたリノレイン酸メチルエステルのためと考えられる。

α-エレオステリアン酸メチルエステルは単独の時はヘミンが存在してもこれに吸着されるだけで殆んど酸化されない。Fig. 3b では僅かに減少しているがこれはヘミンを含まずに α-エレオステアリン酸だけの時と殆んど同じ程度である。しかしリノール酸メチルエステルが存在すると 260 mμ, 270 mμ, 280 mμ の吸光係数が減少して共軛二重結合 ($\Delta^{9,11,13}$) が酸化されることを示す。またリノール酸メチルエステルと同じように 234 mμ の吸光係数が増加するがこれは酸化が端の二重結合から進むためまだ共軛二重結合が残つているためと考えられる。このような酸化はヘミンがなくてもリノール酸メチルエステルが存在すると多少進む。この場合共存しているリノール酸メチルエステルの吸光係数は Fig. 3 から明らかなように単位が異なつているので影響しない。ソルビン酸メチルエステルおよびスクアレンも α-エレオステアリン酸メチルエステルと同じように単独の時はヘミンに吸着される以外殆んど酸化されないが，リノール酸が共存すると前者では 253 mμ の吸光係数が減少し，後者では 275 mμ の吸光係数が増加して酸化の進行を示す。

なおヘミンの触媒作用による α-エレオステアリン酸メチルエステル等の酸化にはリノール酸メチルエステルの量が関係し，これが適量以上或いは以下の時は酸化があまり進行しない。また F. HAUROWITZ[3] 等の結果と同様に酸化が進行するにしたがつてヘミンが破壊されるのを認めた。

これらの実験から血液の触媒作用による不飽和脂肪酸の酸化にはリノール酸のように分子内に $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$ なる分子構造を含んだ脂肪酸即ちビタミンFが必要であり，血液ならびに血色素はこのような分子構造を含まない高度不飽和脂肪酸および炭化水素等の酸化には殆んど触媒作用がない。しかしこれらの不飽和脂肪酸および炭化水素等もリノール酸と共存する時はたとえ血液の触媒作用がなくてもリノール酸の自働酸化によつて次第に酸化が進む。

本実験を行うに当りスクアレンを御恵贈下さつた東海区水研山川健重氏に深謝する。

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Name	Function, Usage	Levels of Use
Sorbate, Calcium	Fungistats	
Sorbate, Potassium		
Sorbate, Sodium	Beverages	0.003 - 0.03%
Sorbic Acid	Baked goods	0.004 - 0.1%
Sorbates	Chocolate sirups	0.05 - 0.2%
	Soda-fountain sirups	0.05 - 0.1%
	Fresh fruit cocktail	0.05 - 0.1%
	Tangerine purée (sherbet base)	0.05 - 0.1%
	Salads (potato, macaroni, cole slaw, gelatin)	0.05 - 0.1%
	Cheesecake	0.05 - 0.1%
	Pie fillings	0.05 - 0.1%
	Cake	0.1%
	Cheeses in consumer-size packages. Up to	0.2%
	Artificially sweetened jellies and preserves. Up to	0.1%
Sulfites and Sulfur Dioxide	Dual-purpose preservatives and antioxidants	
	(See Group 2, Antioxidants)	