The Lipides of Green Peas[1,2]

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THE LIPIDES of green peas *(Pisum sativum)* have not been studied to any great extent during the past four decades, and their composition has remained largely unknown. The crude lipide content of peas is about 2%, quite low in comparison to that of oil-bearing seeds (22). The importance of pea lipides has been emphasized by the recent observation that off-flavor development during storage of frozen raw peas occurs in the lipide fraction (15) and that the enzymes lipoxidase and lipase, acting upon lipide substrates, are largely responsible for production of off-flavors during frozen storage (16, 25).

The present study was undertaken to determine the quantitative composition of pea lipides in order to further the study of the role that this important class of compounds plays in the development of off-flavors and other deteriorative changes in the quality of raw and processed vegetables.

Experimental

Thomas Laxton peas, grown on Experiment Station plots were harvested, processed, and lyophilized as described previously (15, 25).

Analytical procedures were as follows: nitrogen by the micro-Kjeldahl method, using selenium as catalyst; long-chain base nitrogen by the method of McKibbin and Taylor (18); phosphorus by the method of Harris and Popat (13); glycerol by the method of Blix (3); sugars by the anthrone procedure, using sucrose standards (19); fatty acid content and saponification equivalent by the method employed by Scholfield and Dutton (20); saponification number and acid value as outlined by Maera (17); soluble and insoluble acids and iodine number (macro) by A.O.A.C. procedures (2); acid number and iodine number also by micro-modifications of A.O.A.C. procedures (15).

The several lipide fractions were partitioned by countercurrent distribution (CCD) between n-heptane and 95% methanol in a seven-transfer system with single withdrawal, employing 125 ml. separatory funnels as receptacles (7). The stationary phase was n-heptane saturated with 95% methanol; the mobile phase was 95% methanol saturated with n-heptane. The samples were dissolved and transferred to tube 0 with 50 ml. of the stationary phase. Then 50 ml. of mobile phase were added, and the tubes were inverted 25 times. After collection of the fractions 5.0-ml. aliquots were removed by pipette for determinations of weight distribution and sugar content. The end tubes (0 and 8) were analyzed for nitrogen and phosphorus content.

Hydrolysis of lipides for chromatographic studies was accomplished by heating 50-mg. samples with 0.5 ml. of mineral acid in sealed tubes under the following conditions: for liberation of sugars, 0.5 N H_2SO_4 for 12 hrs. at 100°C.; for liberation of glycerol and inositol, 6 N H_2SO_4 for 6 hrs. at 150°C.; for liberation of ninhydrin-positive materials, 6 N HCl for 30 min. at 171°C. The contents of the tubes were filtered,[3] neutralized to pH 4.0 by stirring with Dowex 2 resin (8% cross-linked) in the bicarbonate phase, again filtered and lyophilized. The dried materials were dissolved in 50 λ distilled water and spotted onto Whatman No. 1 paper strips; the strips were developed by ascending chromatography.

The following solvent systems and spray reagents were used in the chromatographic studies: reducing sugars, phenol-water 4:1, sprayed with aniline acid phthalate, heated 5 min. at 100°C. (23); sucrose and oligosaccharides, n-propanol-ethyl acetate-water 7:1:2 (1), sprayed with 1% alcoholic α-naphthol-phosphoric acid 10:1, heated 5 min. at 85°C. (4); glycerol and inositol, n-butanol-acetic acid-water 4:1:5 (BAW), sprayed with 2% $NaIO_4$-1% $KMnO_4$ in 2% Na_2CO_3 4:1; amino acids, BAW or n-propanol-acetic acid-water 3:1:1 (PAW), sprayed with 0.2% ninhydrin in 50% aqueous pyridine, heated 5 min. at 100°C.; choline, BAW, sprayed with 0.4% alcoholic bromcresol green (23); phosphates, BAW or PAW, sprayed with Hanes-Isherwood reagent (11), dried and developed for several hours in direct sunlight at room temperature.

Preparation of Crude Pea Lipides

Lyophilized raw peas were ground to pass 40-mesh in a laboratory Wiley mill and extracted in lots of 1 kg. with 5 liters of chloroform-methanol 2:1 in 12-liter flasks on a shaking machine for 3 hrs. at room temperature (25–30°C.). The extract was decanted and filtered with suction. The pea meal was then re-extracted with an additional 5 liters of solvent mixture. The solvent from the second extraction was filtered and used for the extraction of a fresh batch of pea meal. Fresh solvent mixture was then used for the second extraction of each succeeding batch of pea meal. The solvent ratio was 10:1. The solvent was removed by evaporation in a stream of nitrogen under reduced pressure at temperatures not exceeding 40°C. The yield of crude pea lipides was 809 g. from 13.4 kg. of lyophilized peas (6.0%). The crude lipide was stored under nitrogen at −17.8°C. (Fraction I).

In order to determine the completeness of lipide extraction the extracted pea meal was analyzed for unsaponifiable matter and fatty acid content (20). Whereas the above method of extraction did not completely remove all of the lipides, it was better suited for handling large quantities of pea meal and did remove 94% of the total pea lipides. Furthermore the crude pea lipides contained considerably smaller amounts of non-lipide contaminants than extracts prepared in the Waring blendor by the procedure of Folch (8), by which more than 99% of the total lipides were extracted. About 25% of the dry weight of peas consists of sucrose and stachyose (21), and these sugars are not readily removed from lipides. These non-lipide impurities, mainly sugars and nitrogenous compounds, were extracted from the peas be-

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[2] Approved by the director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1070.

[3] In this study all materials were filtered through fritted Pyrex glass funnels of medium porosity.

TABLE I

Composition of Pea Lipides

Fraction	Percentage of total lipide	Nitrogen	Phosphorus	Molar N/P	Long-chain base nitrogen	Sugars
		%	%		%	%
Crude pea lipides (I)	100	1.42	1.65	1.91	0.069	11.0
Acetone-soluble (II)	51					
More acetone-soluble (III)	39	0.83	0.67	2.74	.....	3.8
Less acetone-soluble (IV)	12	0.93	0.74	2.80	.....	4.9
n-Heptane-soluble (V)	28.8	0.21	0.12	3.88	0.092	0.9
95% methanol-soluble (VI)	22.2	1.85	1.24	3.30	0.180	7.7
Acetone-insoluble (VII)	49					
Chloroform-methanol-insoluble (IX)	9.4	1.97	0.05	.....	0.002	81.2
Chloroform-methanol-soluble (VIII)	39.6	2.41	3.20	1.66	0.116	4.3
Glacial acetic-insoluble (XI)	4.1	0.28	4.50	.....	0.077	2.2
Glacial acetic-soluble (X)	35.5	2.15	2.10	2.26	0.095	5.9
95% ethanol-soluble (XII)	19.8	3.08	2.05	3.22	0.136	.....
95% ethanol-insoluble (XIII)	15.7	1.23	2.85	0.96	0.136	.....

cause of the semi-polar nature of the chloroform-methanol used as solvent. The use of such a solvent mixture is justifiable on the grounds that certain important minor constituents, such as the calcium-magnesium salt of phosphatidyl inositol, might not have been as readily extractable and thus have been overlooked had a strictly non-polar solvent system been employed.

The scheme for solvent fractionation of crude pea lipides into several major fractions, along with certain analytical data, is shown in Figure 1 and Table I.

Solvent Fractionation of Crude Pea Lipides

Preparation of Acetone-Insoluble Lipides (Fraction VII). A sample of 798.7 g. of crude pea lipide was stirred with 1,500 ml. of acetone and allowed to settle. The supernatant liquid was decanted and filtered. The insoluble residue was treated as above with 10 additional portions of acetone. The total volume of acetone extracts was 13 liters. Acetone was removed from the insoluble matter in a vacuum desiccator by means of a water pump.

The acetone-insoluble lipides were then dissolved in 1,450 ml. of chloroform-methanol, 2:1, allowed to stand over-night at room temperature, and then clarified by low-speed centrifugation. Clarification of the supernatant liquid was completed by filtration. The insoluble residue was washed five times at the centrifuge with chloroform-methanol solvent mixture and dried *in vacuo* over P_2O_5. The washings were combined with the original solution, and the solvent was removed on a rotary evaporator, followed by vacuum desiccation over P_2O_5. The yield of acetone-insoluble pea lipides (Fraction VIII) was 316.1 g. This material in the form of a crumbly, light brown solid was stored under acetone at —17.8°C. The lecithin content of pea fat has been reported as 30.46% and 50.25% (14), based on the phosphorus content of the lipides extracted with alcohol-ether.

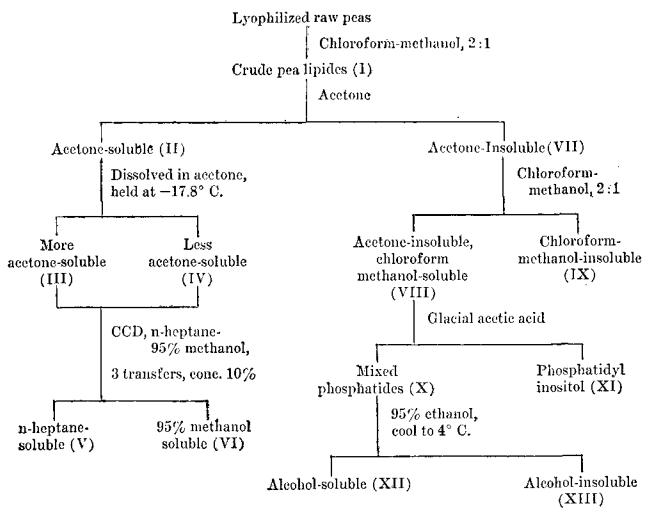

FIG. 1. Solvent fractionation of pea lipides.

Chloroform-Methanol-Insoluble Material (Fraction IX). The residue of material insoluble in chloroform-methanol, 74.9 g., was readily soluble in water and was found to be non-lipidic; it contained only 1.25% fatty acids. The very high sugar content was not caused by starch, no color was obtained with iodine solution, but rather by sucrose, which was detected chromatographically. A bright cherry red color, similar to that produced by fructose, was obtained with Bial's reagent. The material gave strongly positive Molisch and Selivanoff tests, slightly positive xanthoproteic and Millon tests, and a negative biuret test. After acid hydrolysis, fructose, glucose, and several ninhydrin-positive materials were detected chromatographically. That this amount of non-lipide material was obtained from pea lipides is a dramatic demonstration of the solubilizing power of lipides.

Acetone-Soluble Lipides (Fraction II). The solvent was removed from the acetone extract of crude pea lipides by evaporation to a small volume under reduced pressure in a stream of nitrogen, and the process was completed in a rotary evaporator, followed by vacuum desiccation over P_2O_5.

The acetone-soluble, pea lipides were dissolved in 500 ml. of acetone and held for two days at —17.8°C. The precipitate was filtered with suction at —17.8°C., and the acetone was removed in a vacuum desiccator with a water pump. This material was taken up in a minimum amount of benzene and lyophilized to a semi-fluid viscous mass. The acetone was removed from the filtrate on a rotary evaporator.

Countercurrent Distribution Studies

A two-gram portion of the "less acetone-soluble" lipide (Fraction IV) was used at a concentration of 4% with respect to each solvent (Figure 2). The majority of the material was concentrated in the two end-tubes. The more heptane-soluble portion (tube 0) gave negative Molisch and Selivanoff tests, indicating the apparent absence of carbohydrate material, was devoid of phosphorus, and contained but 0.11% nitrogen. The anthrone determination on this fraction was 0.62%. This material was presumably mainly triglyceride in character. The more methanol-soluble portion (tube 8) gave positive Molisch and Selivanoff tests and contained 9.45% sugar by anthrone, 2.38% nitrogen, and 1.36% phosphorus (molar N/P ratio = 3.88). Sucrose constitutes the major portion of

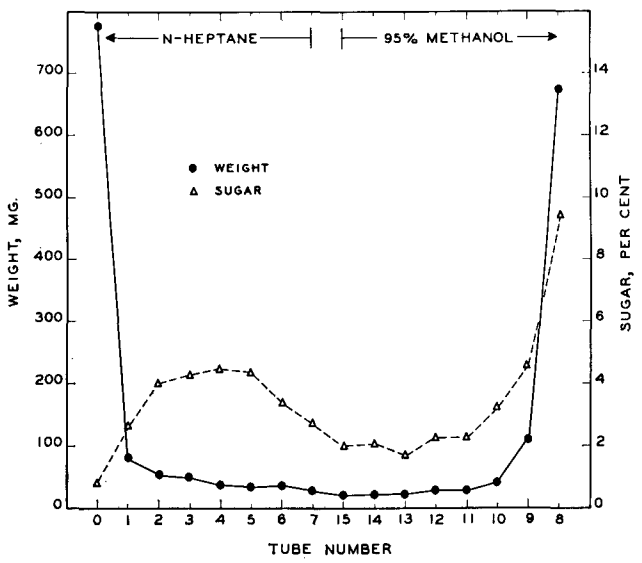

FIG. 2. Countercurrent distribution of "less acetone-soluble" pea lipides (Fraction IV).

the sugar of tube 8. The small anthrone-positive peak in the heptane fractions is of some interest inasmuch as it is not caused by sucrose, any common hexose, pentose, or fructose-containing oligosaccharide, as determined chromatographically. An unknown material, which gave a yellow color with aniline acid phthalate and with an Rf of 0.92, was noted in both tubes 4 and 8.

A similar pattern of distribution was obtained with the "more acetone-soluble" pea lipides (Fraction III). Analytical data for nitrogen, phosphorus, and sugar further indicated that these two acetone-soluble fractions were very similar in composition. They were therefore pooled prior to further fractionation.

When the acetone-insoluble pea lipides (Fraction VIII) were subjected to CCD in the above system, again the majority of the material was concentrated in the two end-tubes, but with this material more than 60% of the weight was found in tube 8 and about 20% in tube 0. The more heptane-soluble material gave negative Molisch and Selivanoff tests, and the anthrone on this fraction was 0.49%. The nitrogen content was low, 0.36%, but the phosphorus content was surprisingly large, 4.59%, when compared to similar fractions obtained from the acetone-soluble lipides. This finding of material of high phosphorus content in the non-polar phase was similar to that observed in a 400 transfer CCD of a complex inositol phosphatide from corn by Carter and Galanos (6), who deemed it to be due to phytin, which is present in peas to the extent of 0.3% (9). The more alcohol-soluble portion (tube 8) gave positive Molisch and Selivanoff tests, contained 3.37% nitrogen, 1.93% phosphorus (molar N/P = 3.86), and 6.24% sugar by anthrone. Sucrose was detected chromatographically in this fraction.

The fact that 84% of the total weight was contained in the four end tubes (0, 1, 8, 9) with very little material in the intervening tubes and the nearly complete elimination of nitrogen and phosphorus containing materials and sugars from the heptane fraction was taken as evidence that CCD had effected a reasonably good separation of two major classes of components in the acetone-soluble pea lipides.

For large-scale separation it was necessary to increase the solute concentration and decrease the number of transfers. When the solute concentration was increased to 10% and the number of transfers decreased to three (with no withdrawal), the end tubes were found to contain 82.3% of the total weight. Therefore the method was deemed suitable for large-scale work.

A sample of 345 g. of acetone-soluble pea lipides (Fractions III and IV pooled) was partitioned under the above conditions between n-heptane and 95% methanol in four-liter, separatory funnels. No difficulties with emulsions were encountered. After separation of the phases all of the heptane fractions were combined (Fraction V), the solvent was removed by distillation under reduced pressure in a stream of nitrogen at 40°C., and the dark-colored oil was dried *in vacuo* over Drierite. The methanol fractions (Fraction VI) were pooled and treated in a similar manner. These lipides were then stored under nitrogen at —17.8°C.

Some of the properties of crude pea lipides and the three major subfractions (Fractions V, VI and VIII) obtained in this study are given in Table II. The heptane fraction very closely resembles the "fixed pea oil" obtained by Grimme (10).

Benzene-Acetic Acid Fractionation and Acid Hydrolysis

During the purification of lipides the removal of undesirable, non-lipide contaminants often can be facilitated by subjecting lipides to a distribution between benzene and aqueous acetic acid (5). Accordingly portions of the three major fractions of pea lipides were dissolved in glacial acetic acid, water was added to adjust the acetic acid level to 50%, and the aqueous mixtures were extracted in separatory funnels with benzene. The benzene and aqueous acetic acid layers and any interfacial material were separated by centrifugation, and the solvents were removed: benzene by lyophilization and acetic acid and water by distillation under reduced pressure at moderate temperatures. The final traces of acetic acid were removed by evacuation over moistened KOH; and the materials were dried *in vacuo* over P_2O_5. The distribution of weight and some of the properties of the various fractions are summarized in Table III. Samples of these materials were hydrolyzed with mineral acids in order that their composition might be studied with paper chromatography. Sucrose was determined chromatographically on unhydrolyzed materials.

Heptane Fraction (Fraction V). The heptane fraction of acetone-soluble pea lipides was completely soluble in glacial acetic acid and may be assumed to consist primarily of mixed triglycerides. The fatty acids obtained by saponification of the benzene-soluble material had a mean molecular weight of 307, which is indicative of the presence of component fatty acids containing more than 18 carbon atoms. Maera (17) states that the mixed fatty acids of *Leguminosae* seed fats usually contain about 5% of arachidic acid along with behenic and lignoceric acids. Jacobson (14) prepared a pea oil containing 7.44% of solid fatty acids, arachidic and palmitic,

TABLE II

Properties of Pea Lipides

Fraction	Saponification value	Acid value	Ester value	Soluble acids	Insoluble acids (Hehner No.)	Iodine No. (Hanus)	Saponification equivalent	Glycerol
				%				%
Crude lipide (I)	168.7	30.8	137.9	5.9	60.3	71.6	333.3	.....
Acetone-insoluble (VIII)	205.2	38.9	166.3	10.2	48.5	50.1	273.4	.....
Methanol-soluble (VI)	170.3	49.4	120.9	10.3	54.3	67.7	329.4	14.50
Heptane-soluble (V)	183.9	16.5	167.4	1.9	91.9	104.3	305.1	.....
Fixed oil of peas[a]	194.5	6.2	178.3	.....	92.3	106.0	306.1	9.74

[a] From Grimme (10)

and 80.46% of liquid fatty acids, reported as oleic acid.

The low content of nitrogen, phosphorus, and sugar probably represents contaminating material carried over because of the well-known solubilizing action of triglycerides rather than actual constituents of the lipide.

The acid hydrolysates of the benzene-soluble portion of the heptane fraction contained three ninhydrin-positive materials but no sugars. Sucrose was detected in the aqueous acetic acid portion prior to acid hydrolysis; hydrolyzed material contained five ninhydrin-positive materials.

Methanol Fraction (Fraction VI). The methanol fraction of acetone-soluble pea lipides was completely soluble in glacial acetic acid. The molar ratio of N/P of 1.13 in the benzene-soluble portion suggests that it probably is a mixture of triglycerides and phosphatides, which contain one nitrogen per phosphate radical. Upon acid hydrolysis this material liberated glycerol, choline, a trace of fructose, and a mixture of nine ninhydrin-positive materials.

The aqueous, acetic acid-soluble portion of the methanol fraction was a mixture, consisting largely of sugars and other non-lipide materials. Judging from the relatively low phosphorus and fatty acid content there was very little phosphatide in this material. After acid hydrolysis this fraction was found to contain glucose, galactose, fructose, ribose, glycerol, inositol, choline, and nine ninhydrin-positive spots on paper chromatograms.

Acetone-Insoluble Fraction (Fraction VIII). A considerable portion of the acetone-insoluble fraction of pea lipides was insoluble in glacial acetic acid. This material (Fraction XI), when purified by extraction with benzene from an aqueous, acetic acid suspension, was found to consist of very nearly pure phosphatidyl inositol in the form of the calcium-magnesium salt and was obtainable as a white fluffy powder in yields up to 11.4% of the acetone-insoluble pea lipides (24).

The bulk of the acetone-insoluble fraction after removal of phosphatidyl inositol was soluble in benzene. This material (Fraction X) was a mixture of phosphatides, with molar N/P ratio of 1.11, which still contained various impurities mainly in the form of sugars. After acid hydrolysis of the benzene-soluble portion, paper chromatograms revealed the presence of galactose, glycerol, glycerophosphate, inositol, inositol phosphate, choline, ethanolamine, and eight other ninhydrin-positive materials. The foregoing indicate the presence of lecithin, phosphatidyl ethanolamine, and inositol lipides in the phosphatides of peas.

The aqueous, acetic acid-soluble portion of the acetone-insoluble fraction was a mixture consisting largely of sugars and other non-lipide materials and containing only 4.6% of fatty acids. Sucrose was detected chromatographically prior to hydrolysis, and glucose and fructose were found after mild acid hydrolysis. Inositol, glycerophosphate, glycerol, choline, and nine ninhydrin-positive spots were observed in the acid hydrolysate of this fraction.

The insoluble material formed at the interface between the benzene and aqueous acetic acid, although too small in quantity for extensive investigation, was of some interest. On hydrolysis it was found to contain galactose, glycerol, inositol, several materials of acidic character (yellow to bromcresol green), ethanolamine, serine, and eight other ninhydrin-positive materials.

Separation of Lecithin and Other Phosphatides. The lecithin content of pea lipides, calculated from the phosphorus content of alcohol-ether extracts of peas, is 0.5% in unripe peas and varies from 0.57% up to 2.34% in ripe peas (12). Lecithin, phosphatidyl choline, is readily soluble in alcohol, and the alcohol-insoluble portion consists primarily of a mixture of phosphatidyl ethanolamine (cephalin), phosphatidyl serine, and other complex phospholipides. Accordingly acetone-insoluble pea lipide, from which phosphatidyl inositol had been removed by treatment with glacial acetic acid (Fraction X), was freed of

TABLE III

Distribution of Pea Lipides Between Benzene and 50% Aqueous Acetic Acid

Fraction	Subfraction	Percentage of total wt. of fraction	N	P	Long-chain base N	Sugar	Molisch test	Sucrose	Glycerol
			%	%	%	%			%
Heptane-soluble (V)	Benzene	99.5	0.11	0.23	0.055	1.7	Neg.	Neg.	9.08
Heptane-soluble (V)	Acetic acid	0.5	.....	.....	.....	.....	++	+	.....
Methanol-soluble (VI)	Benzene	77.0	0.73	1.43	0.194	6.2	Neg.	Neg.	9.38
Methanol-soluble (VI)	Acetic acid	23.0	3.30	0.41	0.035	29.5	++++	+	.....
Acetone-insoluble (VIII)	Insoluble in glacial acetic acid	7.7	0.28	4.50	0.077	1.7	Neg.	.....	11.06
Acetone-insoluble (VIII)	Benzene	62.8	1.38	2.75	0.095	4.9	Neg.	Neg.	12.03
Acetone-insoluble (VIII)	Acetic acid	24.9	1.60	1.20	0	18.1	++++	+	.....
Acetone-insoluble (VIII)	Interfacial material	3.6	.....	.....	.....	.....	+	.....	.....

acetic acid by lyophilization and evacuation over KOH and P_2O_5 as described above, stirred with 10 volumes of 95% ethanol, and gently heated to 45–50°C. to aid in softening the hard mass. A mixture of flocculent and gummy materials remained undissolved. After being held over-night at 4°C., the mixture was filtered at 4°C. and the precipitate was washed with small portions of cold ethanol. Following removal of alcohol, the two fractions were dried *in vacuo* over P_2O_5.

Sphingolipides. The recent discovery of a long-chain base, phytosphingosine, in corn phosphatides (5) was cause for the examination of pea lipides to determine whether sphingolipides are also present in peas. Accordingly the long-chain base nitrogen content of the various pea lipide fractions was determined. These data, included in Tables I and III, indicate that, whereas there is some type of long-chain base present in pea lipides, peas do not constitute a very good source for the preparation of sphingolipides. The nature of the long-chain base present in pea lipides was not determined in the present study.

Summary

Crude pea lipides were prepared by extracting lyophilized raw peas with chloroform-methanol, 2:1, and found to comprise 6% of the dry weight of the peas. The composition of the various fractions of pea lipides was studied by measuring the nitrogen, phosphorus, glycerol, fatty acid, and sugar contents, also by means of paper chromatography following acid hydrolysis. The crude lipides were fractionated with acetone, and the acetone-soluble portion was subjected to countercurrent distribution between n-heptane and 95% methanol. The heptane fraction was found to consist nearly entirely of mixed triglycerides; the methanol fraction was a mixture of triglycerides, phosphatides, sugars, and nitrogenous materials. The acetone-insoluble fraction contained 10% of phosphatidyl inositol and nearly equal amounts of alcohol-soluble and alcohol-insoluble phosphatides.

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Stability of *d*-*a*-Tocopherol Alone, in Solvents, and in Methyl Esters of Fatty Acids[1]

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THE PREVENTION of oxidative spoilage in fats and fatty foods by adding small quantities of antioxidants has been widely studied during the past 20 years. Tocopherol, the most important naturally-occurring fat stabilizer, apparently interrupts the oxidation reaction as a free radical acceptor (7). Since the rate and extent of destruction of tocopherol during this stabilizing process vary with natural fats or fats of mixed composition (4, 8), the present experiments were undertaken to study the behavior of tocopherol in simple systems.

Materials and Methods

The materials used were *d*-*a*-tocopherol, "stable" solvents (methyl silicone,[2] polymer of trifluorovinyl chloride,[3] and dibutyl phthalate), and pure fatty acid methyl esters[4] (stearate, oleate, linoleate, and linolenate). The tocopherol was added to the solvents and/or esters in various concentrations, and these mixtures and pure tocopherol were stored at several temperatures in the dark. Periodic weighings indicated that the materials did not volatilize appreciably during the experiments. The residual tocopherol was determined at intervals by the dipyridyl method, after preliminary removal of interfering substances with sulfuric acid (6). Tests showed that this method could be used to determine tocopherol in silicone, dibutyl phthalate, and polymer of trifluorovinyl chloride as well as in fatty acid esters. The oxidation of solvents and esters was followed by determinations of peroxide oxygen (3).

Results

The stability of tocopherol in the three "stable" solvents was studied at temperatures from 60 to 120°C. (Table I). Actually silicone was the only completely stable solvent; the polymer of trifluoro-

[1] N.R.C. No. 4429 Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
[2] General Electric LTNV40.
[3] Fluorolube HO, from Hooker Electrochemical Company.
[4] From Hormel Institute, Austin, Minn.