(*11*). However, the fractions contained a sugar moiety. Fractions 27 to 35 were possibly a mixture of lysolecithin and a sugar lysolipid–e.g., lysosulfolipid. Yagi and Benson (*23*) have shown that the diglyceride sulfolipid of Benson *et al.* (*2*) can be converted enzymatically to a lysosulfolipid.

The free fatty acids, which amounted to 7% of the total lipid extract, contained relatively more palmitic and linolenic acids and less stearic and oleic acids than Garton (*10*) found using fresh pasture grasses. The high percentage of linolenic acid in the free fatty acids is to be expected since the total lipids have a similar fatty acid distribution (Table III).

The process of dehydrating alfalfa is such that oxidation and degradation of the lipids could well occur. Possibly the high content of C_8 and C_{10} acids, free fatty acids, and the unsaponifiable fraction are at least in part a reflection of changes occurring during the dehydration process. Studies now underway will compare the lipids of fresh alfalfa with those found in the dehydrated meal.

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MILK FAT ANALYSIS

The Protection of Milk Fat Tocopherols during Saponification with Ascorbic Acid

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Difficulties encountered in attempting to determine the total tocopherols on the unsaponifiable matter of milk fat were overcome through the use of ascorbic acid in the saponification mixture. An analytical procedure utilizing this step in the chemical determination of tocopherols, that enables one to determine concurrently and with high degree of precision vitamins A and E and carotenoids on the unsaponifiable matter of fat, permitting the analysis of six samples in one day, was evolved. Information on the protective influence which ascorbic acid extends to tocopherols in alkaline medium is of value in connection with studies of organoleptic and nutritional properties of lipid-containing food products processed with ionizing energy, and of metabolic processes involving vitamin E.

Moore and Tosic (*7*) reported, on the basis of recovery tests, that the loss in *d,α*-tocopheryl acetate during saponification may be completely prevented by a suitable procedure for adding pyrogallol before saponification. They remarked that the need for saponification in the determination of tocopherols in lipids results from the fact that the Emmerie and Engel (*3*) reaction is not given by tocopheryl acetate, and thus vitamin E present in esterified form in natural sources might escape estimation if saponification is omitted.

Quaife and Harris (*11*), in their paper on molecular distillation as a step in the chemical estimation of tocopherols, indicated that saponification, which would appear to be a proper procedure for concentrating tocopherols from lipids, has not proved satisfactory for fats of low tocopherol content, even if conducted in a closed system according to directions given by Chipault *et al.* (*1*) with the use of pyrogallol as an antioxidant.

More recently, Handwerk and Bird (*4*) evolved a procedure for the determination of unoxidized tocopherols in milk fat based on the Moore and Tosic saponification step to protect vitamin E

and to destroy reducible tocoquinones, chromatography of benzene solutions of unsaponifiable matter to remove carotenoids and similar substances that interfere with Emmerie and Engel's assay, and color development by the procedure of Devlin and Mattill (*2*). Difficulties were encountered by them in obtaining adequate recoveries of *d*,α-tocopherol added to fat as the solution in benzene, and they claimed that recoveries ranging from 90 to 94% were obtained when this vitamin was added directly to fat.

In connection with these studies, it should be noted that pyrogallol is an artifact that plays no role in human nutrition. On the contrary, ascorbic acid, an essential to human nutrition, is also known to be involved in either retardation or promotion of harmful-to-flavor reactions in oxidation-sensitive, lipid-containing food products, especially milk, depending upon its concentration and the availability of occluded oxygen in these foods (*6*). It seemed pertinent, therefore, to learn if the loss in tocopherols that occurs during the saponification can be avoided by a suitable addition of ascorbic acid before saponification. There was reason to believe that this step would permit the use of simpler techniques for the neutralization of substances in the solutions of unsaponifiable matter that interfere with the Emmerie and Engel assay, namely, the Parker and McFarlane (*8*) acid–alkali treatment, and Quaife and Biehler (*9*) hydrogenation technique. Moreover, it would determine concurrently vitamins A and E and carotenoids on the unsaponifiable matter of milk fat. After numerous trials, the following procedure, based to some extent on the related work of earlier investigators, was developed.

Table I. The Protection of Pure *d*,α-Tocopherol during Saponification with l-Ascorbic Acid

Additions before Saponification			Tocopherol Content of Unsaponifiable Matter, μg.		Recovery of Tocopherols, %	
Ascorbic acid, mg.	Tocopherol, μg.	Saponification Time, Min.	H[a]	AAT[b]	H[a]	AAT[b]
...	265	10	52	29	19	10
300	265	10	...	269	..	101
...	271	60	...	32	..	11
300	271	60	...	266	..	98
...	530	10	110	62	20	11
300	530	10	...	527	..	99
...	542	60	...	63	..	11
300	542	60	...	544	..	100
...	795	10	166	89	20	11
300	795	10	...	792	..	99
...	813	60	...	98	..	12
300	813	60	...	780	..	95
...	1060	10	225	117	21	11
300	1060	10	...	1016	..	95
...	1084	60	...	109	..	10
300	1084	60	...	1032	..	95

[a] H = hydrogenated. [b] AAT = acid–alkali-treated solutions of unsaponifiable matter.

Experimental

Reagents. Alcoholic l-ascorbic acid solution, 3%. Three grams of ascorbic acid are dissolved in enough 94% methanol to make 100 ml.

Absolute ethanol (*14*). Commercial absolute ethanol is purified by refluxing 4 liters of ethanol with 2.1 grams of potassium permanganate and 4.2 grams of potassium hydroxide pellets for 1 hour, then distilled from an all-glass apparatus and the first 100-ml. portion effluxed is discarded.

Petroleum naphtha (*15*). Petroleum naphtha (Skellysolve B) is purified by shaking 1 liter of Skellysolve B with 120 ml. of 6% aqueous potassium hydroxide and 60 ml. of 13% aqueous silver nitrate for 1 hour and allowing it to settle. Separated supernatant liquid is dried overnight over lumpy calcium chloride, and distilled from an all-glass apparatus. The first 100-ml. portion effluxed is discarded.

Alcoholic ferric chloride and α,α′-dipyridyl solutions (*12*). One-tenth gram of ferric chloride hexahydrate is dissolved in enough absolute ethanol to make 50 ml.; 0.25 gram of dipyridyl is dissolved in enough absolute ethanol to make 50 ml.

Sulfuric acid solution (*8*). This is prepared by mixing 85 ml. of 96.3% sulfuric acid (Baker Chemical Co.) with 15 ml. of glass-redistilled water.

Potassium hydroxide solution, 2% (*8*). Two grams of potassium hydroxide pellets are dissolved in enough glass-redistilled water to make 100 ml.

Sample Preparation. Milk fat is prepared by churning cream separated from milk pasteurized at approximately 61 ° C. for 30 minutes immediately after its withdrawal from the mammary gland, melting and centrifuging the butter, and filtering the supernatant liquid.

Procedure. Ten grams of milk fat are accurately weighed into a 500-ml. borosilicate glass boiling flask with round bottom, long neck, and standard taper joint. Then 10 ml. of 3% ascorbic acid, 10 ml. of absolute methanol, and 5 ml. of saturated aqueous potassium hydroxide are added in the order given. The flask, connected by a ground joint to a Liebig condenser, is placed in a circular opening on the top of a boiling water bath, so that the level of the opening is slightly below the level of the liquid in the flask.

The mixture is refluxed for 10 minutes after condensate begins to drip from the tip of the condenser. It is then diluted with 40 to 50 ml. of glass-redistilled water, cooled to room temperature, and transferred to a 500-ml. Squibb separatory funnel; 40 to 50 ml. of redistilled water are used to rinse the flask. The unsaponifiable matter is extracted from the mixture by shaking for 30 seconds, once with 100 ml. and two or three times with 50-ml. portions of Mallinckrodt's anhydrous ether, depending on the carotenoid content of the sample. The glass stopcock of a separatory funnel must be lightly coated with silicone grease (Dow Corning) so that it turns easily. The inside walls of the funnel must be entirely free from grease. The contamination of ether extract with grease causes haziness in 1:1 Skellysolve B–absolute ethanol solution of unsaponifiable matter that undergoes acid–alkali treatment (*8*) just prior to assay for total tocopherols. The combined ether extracts are washed successively with 100-ml. portions of redistilled water until the washings are free of alkali, using phenolphthalein as an indicator. The first 100 ml. of water are added slowly, however, and the mixture is allowed to settle undisturbed to avoid formation of the relatively stable emulsion. The extract is dried over anhydrous granular sodium sulfate, and then transferred to a 200-ml. volumetric flask. The sulfate is eluted 3 or 4 times with 10-ml. portions of dried ether, and the combined extracts and washings are diluted to volume with ether.

Tocopherol Analysis. A suitable volume of ether extract [from 50 to 100 ml., depending on its color intensity (*6*)] is transferred to a boiling flask and taken to dryness under reduced pressure on the warm water bath, making sure that the level of water is below the level of ether in the flask. Just as the ether is completely removed, the stopcock in the connecting tube is closed and the flask cooled to room temperature and detached from the vacuum device. Then the vacuum is released and the dry residue, which should not be oily in appearance, is transferred to a 25-ml. volumetric flask and diluted to volume with either Skellysolve B, or 1:1 Skellysolve B–absolute ethanol mixture, depending on the method employed for the

Table II. The Protection of Pure d,γ-Tocopherol during Saponification with 1-Ascorbic Acid

Additions before Saponification		Saponification Time, Min.	Tocopherol Content of Unsaponifiable Matter, μg.		Recovery of Tocopherols, %	
Ascorbic acid, mg.	Tocopherol, μg.		H[a]	AAT[b]	H[a]	AAT[b]
...	177	10	50	52	28	29
300	177	10	...	174	..	98
...	192	60	...	68	..	35
300	192	60	...	187	..	97
...	354	10	115	93	32	26
300	354	10	...	347	..	98
...	384	60	...	100	..	26
300	384	60	...	381	..	99
...	531	10	151	154	28	29
300	531	10	...	517	..	97
...	576	60	...	161	..	27
300	576	60	...	565	..	98
...	708	10	172	168	24	23
300	708	10	...	704	..	99
...	768	60	...	181	..	23
300	768	60	...	750	..	97

[a,b] See footnotes, Table I.

Table III. The Protection of Tocopherols of the Milk Fat (Natural, or Natural Plus Added d,α-Tocopherol) during Saponification with 1-Ascorbic Acid

Additions before Saponification			Quantities, μg./100 Grams, Fat Determined on Unsaponifiable Matter of 10-Gram Sample				Recovery of Tocopherols, %	
Ascorbic acid, mg.	Tocopherol, μg.	Saponified, Min.	Vitamin A	Carotenoids	Tocopherols H[a]	Tocopherols AAT[b]	H[a]	AAT[b]
...	...	10	413	497	2744	2,171	92.4	73.0
300	...	10	418	499	2898	2,960	97.6	99.6
...	244	10	421	486	4877	3,300	90.1	60.9
300	244	10	415	490	5471	5,361	101.1	99.0
...	...	30	414	493	2682	2,143	90.3	72.1
300	...	30	422	487	2946	2,899	99.2	97.6
...	...	60	425	488	2698	2,210	90.9	74.4
300	...	60	428	492	2918	2,951	98.3	99.3
...	244	60	420	481	4764	3,249	88.1	60.0
300	244	60	425	490	5406	5,350	99.9	98.8
Av. of all samples		10–60	420 ± 4	490 ± 5	...	...	...	...
Av.	...	10–60	...	...	2708 ± 26	2,174 ± 27	91.2	73.1
300	...	10–60	...	...	2920 ± 19	2,936 ± 26	98.4	98.8
...	244	10–60	...	...	4820 ± 56	3,274 ± 25	89.1	60.4
300	244	10–60	...	...	5438 ± 32	5,355 ± 5	100.5	98.9
...	...	10	5256	4351	...	10,240	...	74.2
300	...	10	5364	4311	...	13,782	...	100.0
...	958	10	5283	4322	...	14,785	...	63.2
300	958	10	5310	4364	...	23,033	...	98.5
Av.		10	5303 ± 40	4377 ± 21	...	...	...	...

[a,b] See footnotes, Table I.

neutralization of substances which interfere with the Emmerie and Engel reaction. The residue may also be dissolved directly in 20 ml. of either solvent.

If the interfering substances are to be removed by the method of Parker and McFarlane (*8*), the Skellysolve B solution of unsaponifiable matter is treated as follows. Twenty milliliters of the solution are transferred to a 50-ml. conical centrifuge tube and 4 ml. of sulfuric acid solution are added. The tube is inverted several times and centrifuged. The supernatant liquid is siphoned to a second conical tube, 2 ml. of 2% potassium hydroxide are added, and the tube is vigorously shaken once or twice and centrifuged. Four milliliters of supernatant liquid are transferred to a spectrophotometer tube which contains exactly 4 ml. of absolute ethanol and the solution is assayed for total tocopherols by the procedure of Emmerie and Engel (*3*), according to the following directions given by Quaife and Harris (*10*), using calibration data for pure d,α-tocopherol. The tube with 8 ml. of unknown plus 1 ml. of dipyridyl solution is placed in a spectrophotometer with a 520 mμ filter, and the galvanometer is set at 100. The tube is removed, 1 ml. of ferric chloride solution is added, and the tube is shaken for at least 5 seconds. It is reinserted and the reading taken 50 seconds after addition of the last drop of ferric chloride. Blank and unknown are read identically. The color due to tocopherol reaction of unknown is L_{520} of unknown minus L_{520} of the blank.

On the other hand, the unsaponifiable matter is dissolved in 1 to 1 Skellysolve B–absolute ethanol mixture if the interfering substances are to be neutralized via the hydrogenation technique of Quaife and Biehler (*9*), using their apparatus and procedure, just prior to the spectrophotometric assay.

Vitamin A and Carotenoid Analysis. From 100 to 150 ml. of ether solution of unsaponifiable matter are transferred to a boiling flask, and the solution is taken to dryness under reduced pressure on a warm water bath. The residue is transferred to a 10-ml. volumetric flask and diluted to volume with chloroform. It is then assayed for vitamin A by the antimony trichloride method (*5*), and for carotenoids by reading its fraction in a spectrophotometer according to directions given by the U. S. Technical Committee on Vitamin A researches (*13*), using calibration data for vitamin A alcohol and crystalline beta carotene solutions in chloroform.

Results and Discussions. The protection of tocopherols during saponification with ascorbic acid has been checked for quantitative recoveries of pure d,α- and d,γ-tocopherols (Distillation Products Inc., Rochester, N. Y.) alone, used in amounts varying from 265 to 1084 and from 177 to 768 μg., respectively, and by recovery experiments using d,α-tocopherol added to 10 grams of milk fat at the levels of 244 and 958 μg. These were added to empty boiling flasks as the solutions in Skellysolve B, and taken to dryness under reduced pressure on a warm water bath prior to the addition of milk fat and saponification of the sample. Control assays were made of the original Skellysolve B solutions of tocopherols, diluted 1 to 1 with absolute ethanol, and the samples were held under vacuum at −15° to −20° C. until saponified. Then 10 ml. of 3% ascorbic acid (or 10 ml. of 94% methanol for the controls), 10 ml. of absolute methanol, and 5 ml. of saturated potassium hydroxide were added to samples in the order given, except that an extra 10 ml. of absolute methanol were added to some of the flasks to offset the lack of 10 grams of fat. Thereafter, the samples were saponified from 10 to 60 minutes, and extraction and assaying of unsaponifiable matter solutions for tocopherols were carried out as described earlier. In addition, two 10-gram samples of fat were processed in like manner, but without added tocopherols.

As a rule, saponification with ascorbic acid is accompanied by the development of an orange-red color in the mixture. The coloring substances are not removed from the mixture by ether, and are readily decolorized on standing or upon

neutralization of separated alkaline soap solutions with concentrated sulfuric acid.

The results listed in Tables I, II, and III show conclusively that ascorbic acid protects tocopherols during saponification. The recovery figures reveal also that pure tocopherols are much more readily decomposed in alkaline medium by heat than the natural tocopherols of the milk fat. Moreover, the data in Tables I and III show that the recovery of pure α-tocopherol, and especially that of natural tocopherols of the milk fat, were appreciably improved when solutions of unsaponifiable matter underwent hydrogenation (*9*) treatment, instead of acid–alkali (*8*) treatment, just prior to assay, presumably because of reduction of some of the tocoquinones formed. Under the same experimental conditions, the recovery of pure γ-tocopherol was affected but slightly.

The recovery data in the upper part of Table III were computed *vs.* corresponding distillation data. According to distillation analysis (*11*), the fat contained 2967 and 2970 μg. of natural tocopherols per 100-gram samples when the solutions of distillates underwent hydrogenation and acid–alkali treatment, respectively, just prior to assay. However, the recovery data in the lower part of the same table (colostrum fat) were computed *vs.* saponification data of the author.

The data in Table III reveal also that no significant change occurred in the vitamins A and E and the carotenoid values of the fresh milk fat, determined after saponification six times as long as specified in the outlined procedure.

On the basis of these results, it would be logical to assume that ascorbic acid might also extend its protective influence to lipid-containing food products that are exposed to ionizing energy, and that this vitamin plays an important role in the metabolic processes involving vitamin E.

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