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THE DETERMINATION OF TOCOPHEROLS IN MILK FAT

by

Reginald Leo Handwerk

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
the Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Food Technology (Dairy Chemistry)

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I. INTRODUCTION

Tocopherols are among the important natural antioxidants. The data reported in the literature on this subject would seem to indicate that the tocopherols, and possibly alpha-tocopherol, may be the only naturally occurring antioxidants in milk fat. Further study may indicate the occurrence of tocopherols other than alpha-tocopherol, since delta-tocopherol has been reported in hog fat (Chipault, Lundberg and Burr (7)), gamma- and delta-tocopherols in human tissues (Quaife and Dju (62)) and in eggs (Harris, Quaife and Swanson (23)). Moreover, since the tocopherols are indigenous in plant tissues and not synthesized in the animal body, the type of tocopherols in milk fat may be dependent upon the feed ingested.

If the types of tocopherols in milk fat vary, considerable differences in oxidative stability of different milk fat samples may be expected since it has been shown (Griewahn and Daubert (22) and others) that the antioxidative characteristics increase from alpha, to beta, to gamma, to delta, when the oxidative stability is measured by the Swift stability test. The greater oxidative stability of summer butter may result not only from an increase in total tocopherol content but also from an increase of gamma and delta forms.

Antioxidants have been considered to retard (if not inhibit) the autoxidative reaction in fats; it has likewise been con-

sidered that destruction of antioxidants may ensue (Golumbic (20) and Swift, Mann and Fisher (82)) as the fat becomes oxidized. Although the exact mechanisms of the antioxidant's reaction is not known, it has been postulated that the antioxidant serves as a hydrogen donor, blocking the autoxidative free radical peroxide, in the early stages of the autoxidation reaction.

There is a possibility that the semiquinone of the antioxidant may exist in an oxidizing fat since Michaelis _et al_. (50, 51, 52, 53, 73) have established the fact (by potentiometric, magnetometric and colorimetric means) that the semiquinone free radicals can exist. Semiquinones have been obtained in alkaline solution from duroquinone, phenanthrenequinone-3-sulfonic acid and benzil, and in acid solution from aromatic amines.

Whether or not the semiquinones do form, it has been generally considered that the antioxidant is oxidized concurrently with the fat. If this is correct, the quantity of non-oxidized antioxidant present in the fat at any one time should be a function of the degree of oxidation of the fat. This study was initiated to determine, among other things, the procedure by which the residual unoxidized tocopherol could be estimated.

In order that the quantity of oxidizing materials in the fat could be estimated, the Chapman and McFarlane (5) and the

Hills and Thiel (26) methods were studied. These methods are based upon the oxidation of ferrous to ferric iron and measurement of ferric iron colorimetrically (as the thiocyanate complex). The Lea (43) method was employed for comparative purposes. As a result of these comparisons and the published work of Lea (44) and Chapman and McKay (6), the Hills and Thiel method was selected.

The method in general use for determination of tocopherols is that of Emmerie and Engel (13), although other methods have been reported and used to a limited extent. This method is based on the reduction of ferric to ferrous iron, and the subsequent formation of a color complex with alpha,alpha'-dipyridyl. The method is sensitive. It has the disadvantage that it is not specific for tocopherols. It was considered, at the initiation of this investigation, that in addition to vitamin A, beta-carotene and other pigments, peroxides and tocopherol oxidation products might interfere. In addition, a simple method was desired. Therefore, methods involving both saponified and non-saponified fat were investigated.

In general the objectives of the study were to determine the extent to which materials in oxidizing and in normal milk fat interfered with the Emmerie and Engel procedure, to devise means of eliminating the interference of these substances without destruction of the unoxidized tocopherol present and as a result

of the first two objectives to develop a method for the determination of the residual non-oxidized tocopherol in milk fat at any time during the oxidation of the fat.

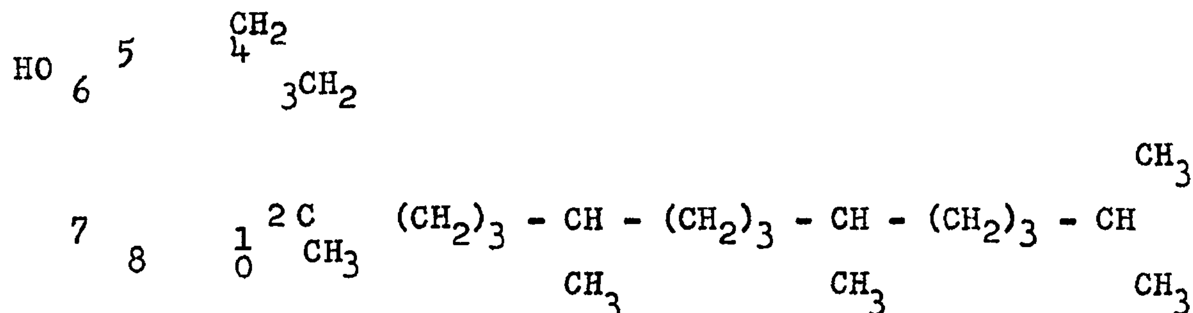
II. LITERATURE REVIEW

A. Preliminary Considerations

The tocopherols are viscous oils which have not been obtained in the crystalline state. Certain esters such as the allophanates, p-nitrophenylurethanes, 3,5-dinitrobenzoates (Rosenberg (70)), and the palmitate, acid succinate, and azobenzene-4-carboxylate (Baxter et al. (2)) have been crystallized. This group of vitamins occurs predominantly in plant materials, particularly in the vegetable oils. Fish oils and animal fats are not good sources (Rosenberg (70), Hickman and Harris (25), Harris, Quaife, and Swanson (23)). Four forms have been reported for naturally occurring tocopherols. These are named alpha-, beta-, gamma- and delta- in the order of their discovery.

Tocopherol structure has been established by Karrer et al. (31) and others. The tocopherols have been synthesized by Karrer et al. (31, 32, 33) and Smith et al. (76). Synthetic dl-alpha-tocopherol acetate is the standard reference substance for vitamin E in an assay. One mg. of the acetate is considered to be 1 I.U. (48). Karrer et al. (31) proposed the term tocol for the general class of tocopherols without substituents in the benzene nucleus. The single members are indicated by following the Geneva-nomenclature principle for chromans.

The tocol nucleus may be represented thus:



alpha-tocopherol is 5,7,8-trimethyltocol

beta-tocopherol is 5,8-dimethyltocol

gamma-tocopherol is 7,8-dimethyltocol

delta-tocopherol is 8-methyltocol

5,7-Dimethyltocol has been synthesized and studied by Kofler (41), but has not been found in nature. Similarly the 5-methyl or 7-methyl isomers of delta-tocopherol have not been found in nature. Water soluble forms of the disodium salts of the phosphate esters are available.

B. Physical Properties of the Tocopherols

1. Color

Pale yellow, viscous oils

2. Empirical Formulae

alpha-Tocopherol - $C_{29}H_{50}O_2$, (70)

beta- and gamma-Tocopherol - $C_{28}H_{48}O_2$, (70)

delta-Tocopherol - $C_{27}H_{46}O_2$, (81)

3. Temperatures for Molecular Distillation of Tocopherols from Natural Materials at Pressures of 1 Micron or Less

215-220° C., Quaife and Harris (64).

140° C., Kjolhede (36).

95° C., Glavind, Heslet and Prange (19).

4. Absorption Maxima and Extinction Coefficients of the Tocopherols and Some of Their Esters and Derivatives (in ethanol)

Compound	Reference	Absorption maximum	$E^{1\%}_{1\ cm.}$
alpha-Tocopherol (nat.)	Baxter *et al*. (2)	292 mu*	73.7
beta-Tocopherol (nat.)	Baxter *et al*. (2)	297 mu	87.6
gamma-Tocopherol (nat.)	Baxter *et al*. (2)	298 mu	92.8
delta-Tocopherol (nat.)	Stern *et al*. (81)	298 mu	91.2
alpha-Toc. *o*-quinone	Baxter *et al*. (2)	480 mu	21.1
gamma-Toc. *o*-quinone	Baxter *et al*. (2)	480 mu	21.1
beta-Toc. *o*-quinone	Baxter *et al*. (2)	465 mu	21.1
delta-Toc. *o*-quinone	Stern *et al*. (81)	373 mu	59.7
delta-Toc. *p*-quinone	Stern *et al*. (81)	435 mu	15.2 (impure preparation)
Tocopheryl *p*-quinone	Stern *et al*. (81)	260 mu	220.
Most tocopherol esters	Baxter *et al*. (2)	286 mu	60% of free phenol

*mu is employed to designate millimicrons throughout this thesis.

Compound	Reference	Absorption maximum	$E^{1\%}_{1\ cm.}$
Coupled product of gamma- or delta-tocopherol with diazotized o-dianisidine	Weisler *et al.* (85)	395 mu and 510 mu[1]	Depend upon alkalinity
Nitroso tocopherols	Quaife (60)	410 mu[2]	
beta			-42.2
gamma			-57.7
delta			-58.4

[1]Solvent, water [2]Solvent, Skellysolve H.

Infrared absorption spectra have been reported by Rosenkrantz (71) and by Stern *et al.* (81) who described assigned bands due to the O-H bond of phenolic hydroxyl and aliphatic carbon chains near 3.0 μ, to c = c at 6.3 μ and to phenolic C-O absorption at 8.0 μ; in addition to unassigned bands near 8.6 μ and 10.9 μ as characteristics of the tocopherol structure.

5. Solubility

Soluble in most organic solvents and fats (70).

6. Optical Activity

Specific rotation of the tocopherols (Stern *et al.* (81))

	$[\alpha]^{25}_{546.1}$ In benzene	$[\alpha]^{25}_{546.1}$ In alcohol
alpha-Tocopherol	-3.0	+0.32
beta-Tocopherol	--	+2.9
gamma-Tocopherol	-2.4	+2.2
delta-Tocopherol	+1.1	+3.4

The direction of rotation changes with the solvents with alpha- and gamma- but not with delta-tocopherol.

C. Chemical Properties of Tocopherols

Stable to heat to 200° C. in the absence of air (Rosenberg (70))

Stable to H_2SO_4 and to HCl up to 100° C. (Rosenberg (70))

Slowly destroyed by alkali (Rosenberg (70))

Oxidizable with ferric chloride, auric chloride, and ceric sulfate to the p-quinone (Karrer, et al. (33), Baxter et al. (2) and Stern et al. (81)) and by nitric acid and silver nitrate to the o-quinone (Furter and Meyer (18) and Karrer et al. (33)).

Upon pyrolysis duroquinone is formed from alpha-tocopherol, trimethylhydroquinone from the beta- and gamma- forms (Rosenberg (70)), and dimethylhydroquinone from the delta- form (Stern et al. (81)).

Tocopherols are not soluble in concentrated aqueous alkali and are part of the ether extract from a saponification mixture (16).

Their activity as antioxidants increases from alpha-, to beta-, to gamma-, to delta-tocopherol (22, 30, 81). The antioxidant properties of the tocopherols are a function of the free phenolic hydroxyl while the acetate esters are biologically

more active than the free forms (25).

The tocopherols are quite stable to visible light but are readily destroyed by ultraviolet light (70).

D. The Physiological Function of Tocopherols

The physiological function of the tocopherols is comparatively obscure. According to Rosenberg (71) and Hickman and Harris (25) they have been reported to be necessary in the prevention of gestation resorption and testicular degeneration in rats and have been named the antisterility vitamin; they have been shown necessary (in animal studies) for proper vitamin A utilization, in prevention of muscular dystrophy, creatinurea, cardiac failure, degeneration of the epithelium of the convoluted tubules of the kidney and proper function of thyroid and pituitary glands. Tocopherol has been reported to prevent abortions in cattle and barrenness in sows. Eggs from tocopherol-deficient hens cannot be hatched successfully due to the development of a lethal ring in the blastoderm from cell proliferation in the mesoderm. Chicks develop an alimentary exudative diathesis, followed by increased capillary permeability and muscular dystrophy when on a tocopherol-deficient diet. Tocopherol deficiency in humans has not been definitely shown, but many cases have been reported in which its clinical

use has been beneficial. Recent research has indicated that tocopherol acts to decrease metabolic rate, inhibit diphosphopyridine nucleotidase, and in general, control tissue metabolism. The tocopherols are non-toxic substances and E-hypervitaminosis is unknown.

E. Methods of Analysis of Tocopherols

1. General Aspects

a. Principles of various methods

Methods for the determination of the tocopherols are varied as to the principle of their action and in their application. A critical review of the available methods was given by Kofler (41) and Baxter (1). A major portion of the work on methods for the tocopherol determination has been done with tocopherol concentrates containing one per cent or more of tocopherol and on materials low in lipid content such as blood and tissue.

Six general methods have been reported: 1. Titrimetric oxidimetric methods (Karrer *et al.* (31), Schulek and Roxsa (72), Smith, Kolthoff, and Spillane (77, 78, 79) and others). 2. Colorimetric oxidimetric methods employing ferrous:dipyridyl (Emmerie and Engel (13)) and ferrous ferricyanide (Meunier and Vinet (49)). 3. Colorimetric measurement of oxidation products of tocopherols with strong oxidizing agents (Furter and Meyer (18)).

4. Spectrophotometric measurement of colored derivatives formed by coupling of diazotized reagents (Weisler, Robeson and Baxter (85)) and by production of nitroso derivatives (Quaife (60)). 5. Measurement of fluorescence of phenazine derivatives formed with the o-quinoid oxidation products of the tocopherol, (Kofler (38, 39, 40)). 6. Direct spectrophotometric measurement at 290 to 298 mu, (Kofler (41)).

Special methods involving one or more of the techniques listed above are many.

b. Concentration of the tocopherol-containing fraction and the removal of the interfering substances may be accomplished by one of the following methods:

(1) Saponification as a means of removing the glycerides and concentrating the unsaponifiables is a method generally used in fat-soluble vitamin work. Its application to the determination of vitamin E has been studied extensively by many and the results reported vary from statements by Quaife and Harris (64) that it cannot be used, to statements (Emmerie and Engel (16)) that it is necessary to saponify in order to get reproduceable results. Studies with the pure tocopherols, however, show that they are unstable in the presence of alkali and any method employing saponification must necessarily provide some means to protect the materials in the saponification mixture from oxidation.

(2) Another general means of concentration of constituents present in small amount is the chromatographic method. It has general application and is used in most of the methods that will be mentioned for the removal of interfering substances.

(3) The third general method of concentrating or "purifying" a constituent of a mixture is by distillation. Molecular distillation has been studied by many [(Kjolhede (36), Glavind, Heslet, and Prange (19), Quaife and Harris (64)], and has been shown to permit only a crude separation. The only advantage this method offers over saponification is that no destruction with alkali results. Some of the disadvantages of the method were given by Glavind, Heslet and Prange (19); they included simultaneous distillation of vitamin A, lack of complete recovery, distillation of low molecular weight glycerides, etc.

(4) Another general separation or "purification" method is found in extraction, or more specifically, partition between solvents. Extraction of the tocopherols from petroleum ether by alcohol or from aqueous mixtures by the use of petroleum ether has been used by many as a means of accomplishing a partial separation, however this again is a crude means of "purification".

c. The effect of peroxidic materials upon the results obtained

The results obtained by a given assay procedure apparently depend upon the means of removal of interfering substances. Kjolhede (36), Kofler (41), Quaife and Dju (62), Quaife, Scrimshaw, and Lowry (66), Kaunitz and Beaver (34) and others have reported effects due to oxidized fats upon the results of tocopherol determinations. It was desired to measure the concentration of peroxidic materials in the samples studied by the most sensitive means available. The ferric thiocyanate color complex was reported as highly sensitive by Young, Vogt, and Nieuland (88) in a colorimetric adaptation of the titrametric method of Yule and Wilson (89) for the determination of peroxides in gasoline. Later Chapman and McFarlane (5) and Lips, Chapman, and McFarlane (46) adapted this technique for use in studying keeping quality of milk powders. Hills and Thiel (26) modified the Chapman and McFarlane adaptation by changing the solvent from acetone to a mixture of benzene:methanol and by using separate solutions of ferrous chloride and ammonium thiocyanate rather than the pre-prepared mixture of ferrous ammonium sulfate and ammonium thiocyanate used by Chapman and McFarlane (5). In a critical study of the Chapman and McFarlane and the Hills and Thiel modifications, Lea (44) and Chapman and McKay (6) found that the Hills and Thiel method was preferable, due to the lesser

effect of oxygen upon the values obtained. Bird *et al.* (4) have shown that the peroxide values obtained by the Chapman and McFarlane method are higher by some variable amount than those obtained by the Hills and Thiel method, and that analysis of solutions of hydrogen peroxide gave erratic results by the former but quite reproduceable results by the latter method. On the basis of the above and its much greater simplicity, the Hills and Thiel method was chosen in the present studies for use in measuring the peroxide content of the samples studied.

2. Tocopherol Methods Employing Mild Oxidation

a. Oxidant, ferric iron

(1) The method of Emmerie and Engel (13) and the many modifications of this method employ solutions of ferric iron and alpha,alpha'-dipyridyl in alcohol or acetic acid as a color development reagent. The tocopherol reduces ferric iron (2 equivalents of Fe^{++} per mol. of tocopherol) to form tocopheryl-*p*-quinone. The ferrous iron forms a color complex with alpha, alpha'-dipyridyl, which has a maximum absorption at 520 mu and a very high molar extinction coefficient.

Any substances which will reduce ferric iron or oxidize ferrous iron, under the conditions of the experiment involved, will cause interference in this method. Furthermore, any substance (not in the blank) which absorbs at 520 mu will also interfere. Generally it has been found necessary to remove

or destroy vitamin A, carotene, chloesterol, peroxides, protein, and some unknown factors in certain products. The means of removal or destruction vary considerably among methods.

(a) Parker and McFarlane (55) shake the petroleum ether solution of the fat or extract with 85 per cent H_2SO_4 to destroy or render water soluble all of the unsaturated systems and quinones, then extract with mild alkali and water.

(b) Quaife and Biehler (61) described a method of mild hydrogenation in which the pigments and other highly unsaturated molecules are decolorized and rendered inert to oxidation by iron. This method has had quite general application to many materials (23, 56, 59, 62, 64, 65, 68).

(c) Emmerie and Engel (14), Kjolhede (36), Devlin and Mattill (9) and others used various adsorbents to remove interfering substances from solutions to be assayed for tocopherol. The substances generally removed by this technique are the same as those removed by H_2SO_4.

(d) Wall and Kelly (84) and others, separate tocopherol and carotene from fat, chlorophyll, xanthophyll and other lipoids by chromatography in which the tocopherol is first adsorbed on the column to separate it from fat and other lipoids not absorbed, then it is eluted by a solvent which elutes only tocopherol and carotene. They destroy carotene by the H_2SO_4 wash as in (a) above.

(2) The method of Meunier and Vinet (49) is similar to that of Emmerie and Engel. Ferric iron is reduced by tocopherols; in this case the reduced iron is measured by a colorimetric method using the ferro-ferri-cyanide complex. Interfering substances are the same as indicated above and are removable by the same techniques as in the Emmerie and Engel procedure.

b. Oxidant, ceric sulfate

The ceric sulfate titration, worked out by Schulek and Roxsa (72) is the only titrimetric method which has been developed for the determination of the tocopherols in the presence of the interfering substances normally encountered. Karrer *et al.* (31) and Smith, Kolthoff and Spillane (77, 78, 79) used pure compounds, and therefore, the applicability of their methods to natural products is not known. Schulek and Roxsa make use of the reversible oxidation of the tocopherols for the determination, oxidizing with ceric sulfate to destroy constituents such as carotene, and converting the tocopherols to tocopheryl-*p*-quinones. These were then reduced to hydroquinones with $SnCl_2$. A second titration with an oxidizing agent determined the tocopherols (and any other reversibly oxidizable substances). This procedure, unless chromatography or other means of removal of quinones is employed, will determine total tocopherol in addition to tocopheryl-quinones.

3. Methods Employing Vigorous Oxidation

a. Kofler (38, 39, 40) oxidized the tocopherols with nitric acid to tocopheryl-o-quinones and condensed the latter with o-phenylenediamine to produce a highly fluorescing phenazine derivative. He compared the fluorescence of this derivative with a standard prepared in the same way with alpha-tocopherol. The phenazine derivative was freed from interfering substances by adsorbing on slightly activated Al_2O_3 (from petroleum ether solution); it was then eluted with benzene. If tocopherol and tocopheryl-quinones were to be determined separately, a petroleum ether solution of the material was chromatographed on Floridin XS, then eluted with benzene; and the eluate was used for assay. (The tocopheryl-quinones remain adsorbed on Floridin XS while tocopherols are eluted; see study of the effect of tocopherol oxidation products p. 86). Lieck and Willstaedt (45) in employing Kofler's method, used a photoelectric fluorometer and an artifical fluorescence standard of fluorescein. They used Al_2O_3 (Merck) which was not specially activated for chromatographic purification of the phenazine derivative.

b. Furter and Meyer (18) used the red color of the oxidized forms of tocopherol (quinoid form) as a measure of tocopherol in butanol-methanol solutions. Quackenbush, Gottlieb and Steenbock (57) report a sensitivity limit of 50 mcg. of tocopherol when using a 7:2:1 mix of n-butanol:chloroform:conc.

nitric acid. They report interference by many substances. Wall and Kelly (84) used the Furter and Meyer (18) and the Emmerie and Engel (13) methods on plant extracts. They employed Hyflo-Supercel plus activated MgO adsorbents to remove chlorophyll and xanthophyll and subsequently destroyed the carotene and tocopheryl-quinones by the treatment of Parker and McFarlane (55). Carotene and tocopherol were eluted from the adsorbent by 80 ml. of 5 per cent acetone in Skellysolve B and 20 ml. of 10 per cent acetone in Skellysolve B. The Furter-Meyer method can be criticized for its low sensitivity as compared to the Kofler or Emmerie and Engel methods.

A modification of the Furter-Meyer reaction which makes use of the oxidizing properties of the tocopheryl-o-quinone formed by HNO_3 oxidation has been presented by Chipault, Lundberg, and Burr (7). Here again all of the objections to an oxidimetric method apply. The final reaction must be carried out in the absence of any substances which have oxidizing or reducing properties since the intensity of the blue color formed by the oxidation of leuco methylene blue is measured. A very elaborate procedure for exclusion of air is followed throughout the procedure.

4. Methods Attempting to Determine Mixtures of (alpha-, beta-, gamma-, and delta-) Tocopherols

a. On the basis of reaction rates with the Emmerie and Engel reagent:

Hove and Hove (28) determined the reaction rates of the tocopherols with the Emmerie and Engel reagent at two different temperatures. They made standard curves with mixtures of alpha-, beta-, and gamma-tocopherol in known ratios. The reaction rates are such for synthetic alpha-, beta-, and gamma-tocopherols in Skellysolve B with the modified Emmerie and Engel reagent of Devlin and Mattill (9) (acetic acid) that they produce equal color intensities during a 15 minute reaction at 35° C. However, at 15° C., alpha-tocopherol produces the same color intensity as at 35° while the beta- and gamma- forms show only about one half the intensity. This difference is the basis of a method of determining alpha-tocopherol in the presence of beta- and gamma-tocopherols. (delta-Tocopherol was not known when this method was reported.)

Baxter (1) reported that the Devlin and Mattill (9) acetic acid reagent was unsatisfactory for the determination of total tocopherols and reported the use of the alcohol reagent of Emmerie and Engel (13) (0.1 g. alpha-,alpha'-dipyridyl in 50 ml. alcohol; 0.1 g. $FeCl_3$ in 50 ml. alcohol). They used a two minute reaction time for total tocopherols. Later Stern and Baxter (80) reported that the time should be increased to 2.5 minutes, when delta-tocopherol is present, because of the slower reactivity of delta-tocopherol.

b. By differential oxidation in various solvents

Fisher (17) presented a modified Furter-Meyer (18) method for the determination of gamma-tocopherol in the presence of alpha-tocopherol by oxidation with nitric acid in acetic acid:chloroform solution. Alpha-tocopherol is not oxidized under these conditions.

Kofler (41) in 1947 studied many of the methods available for the determination of mixtures of tocopherols and reported modifications which make possible the determination of any one of the four types in the presence of the others. He further altered Fisher's (17) modification of the Furter-Meyer (18) technique by employing chloroform as a solvent for oxidation of one sample and alcohol as the solvent for oxidation of another. Alpha-tocopherol is oxidized in alcohol solution with the others. It is not oxidized in chloroform solution while the other three are. Herein lies the basis for a separate determination. He reported studies of the chromatography of mixtures, the direct spectrophotometric assay, a fluorometric method, Emmerie and Engel method, and coupling methods (c. below).

c. By the formation of derivatives

The use of diazotized *o*-dianisidine as a reagent has been reported by Weisler, Robeson and Baxter (85); this is a modification of a discovery of Scudi and Buhs (75) and of

Quaife (58) who originally employed diazotized p-nitroaniline for the assay of mixtures of tocopherols. Tocopherols which are unsubstituted in a position ortho to the phenolic group couple with diazotized anilines while alpha-tocopherol, having a fully substituted ring, will not couple. Quaife (58) reports beta-tocopherol will not couple and proposes an explanation in the Mills-Nixon effect. The percentage of gamma- and delta-tocopherols can be determined by the difference in color of the two compounds in Na_2CO_3 and in KOH solution. beta-Tocopherol if present is determined as alpha- by the Emmerie and Engel method.

The absorption curves of the diazo derivatives have maxima at 395 and 510 mu in Na_2CO_3 and KOH solutions but less interference due to absorption by the fat is experienced at 510 mu.

The nitroso derivatives of beta-, gamma- and delta-tocopherols were prepared by Quaife (60) who separated the derivatives from interferants by chromatography on a $ZnCO_3$ -Celite column and then read the L value on an Evelyn or D value on a Beckman Spectrophotometer at 410 mu. The solvent was Skellysolve H.

5. Miscellaneous Methods and Modifications

a. Application of correction factors

Kaunitz and Beaver (34) (35) applied correction factors for carotene, vitamin A and fat in their application of the

Emmerie and Engel procedure. Their correction equalled 4.8 mcg. of tocopherol per mcg. of carotene determined by the absorption of carotene at 400 mu. The vitamin A correction used was 0.24 x I.U. of vitamin A = mcg. of alpha-tocopherol when vitamin A was determined by an independent means. The inhibition of color formation due to the presence of oil was determined by running tocopherol recoveries on each oil sample and correcting for the reduction in tocopherol assay. A micro method employing this general technique has been reported by Quaife, Scrimshaw, and Lowry (66).

b. Modified vitamin K method

The method of Scudi and Buhs (74) (75) (a modification of their vitamin K method) employs hydrogenation in butanol over Raney's nickel to reduce quinones originally present to hydroquinones. The hydroquinones are then reacted with 2,6-dichloroindophenol and the reduction of the blue color of the dye is measured. A second sample is treated with auric chloride reagent to oxidize all tocopherol to the quinoid form which is then reduced by hydrogenation and the total tocopherol determined. The difference between the first and second readings is considered equivalent to the unoxidized tocopherol in the sample. The first reading gives the amount of oxidized tocopherol. Vitamins K interfere but extrapolation of a reading vs. time curve to zero time gives the reading resulting from

vitamin K. The specificity of the method is increased if the hydrogenated solution is treated with Claisen's alkali (strong alc. KOH) to render the o- and p-hydroquinones water soluble, and is then extracted with petroleum ether to remove cryptophenolic tocopherols. After the tocopherols are separated, water is added to the alkaline solution to hydrolyse the potassium salts of the phenols and hydroquinones present (together with any other phenols which will reduce 2,6-dichloroindophenol).

c. Bioassay methods (reviewed by Mason and Harris (48))

Many workers accept bioassay methods as being the standard. It is not considered necessary in the case where the pure substances are available for standards, to rely upon so tedious and uncertain a method which in itself must be calibrated by means of the pure substances. Where a specific biological action is to be studied with a substance which is not completely known a bioassay may be preferable.

d. Hydroquinone as a basic standard

Rawlings (67) used hydroquinone as a basic standard for the Emmerie-Engel method.

e. Antioxidant properties as a measure of tocopherol content

The antioxidant properties of the tocopherols have been used as a means of assay by Hove and Hove (29), by

Riemschneider *et al.* (69), and by others. The length of the induction period of a specific fat previously standardized, to which the unknown has been added, is measured by an accelerated oxidation method; the increase in induction period is used as a measure of the amount of tocopherol present.

The literature contains considerable data obtained on isolated samples of various foodstuffs by various methods. Recently, however, an effort has been made by Harris, Quaife and Swanson (23) and others to bring the available data together and to gather the additional data necessary to classify foods according to their vitamin E potency. The study of the tocopherol content of blood, organs, and tissues has generally been limited to an estimation of total tocopherols, however here as well as in foodstuffs, there is a need for more data and reliable methods for the determination of the individual tocopherols.

III. EXPERIMENTAL

A. Preparation of Samples

1. From Butter

Butter was melted in 4 ounce screw-capped sample jars in a 45° C. water bath. The melted butter was centrifuged for 30 minutes (in the jars) at 1500 r.p.m. in a Number 2 International Centrifuge, and the fat layer was then transferred into other 4 ounce sample jars and recentrifuged. The sera were composited and centrifuged to recover all the fat possible. The fat was then composited and run through a 6 inch hot water jacketed filter (Whatman #12 fluted filter paper). If the filtrate was not clear and free from water droplets, the filtration was repeated.

2. From Cream

Four to five hundred grams of cream were churned in E.Z. seal quart fruit jars in a reciprocating shaker which held six jars (lying with the long axis in the direction of shaking). When butter granules about the size of walnuts were obtained, the buttermilk was drained and the butter was washed and transferred to four ounce screw-capped sample jars. After holding overnight at below 40° F., the subsequent procedure was the same as 1. above.

B. Preparation of Standards, Reagents, Adsorbents, and Solvents

1. Standards

a. Tocopherol solutions

Pure natural d-alpha-tocopherol (Distillation Products Inc.) (ca. 100 mg.) was weighed in a tared, thin-walled flattened capillary about 1 mm. in diameter and 50 mm. long. The tube was dropped into a 100 ml. volumetric amber flask and crushed with a glass rod, the rod was rinsed with solvent and solvent was added to the 100 ml. mark. This solution contained approximately 1000 mcg.* per ml. It was then diluted to yield solutions containing 100 and 10 mcg. per ml. The solvent used depended upon the subsequent use of the standard.

b. Ferric iron solutions

An accurately weighed sample (ca. 0.5 g.) of electrolytically precipitated iron was dissolved in 1.0 N hydrochloric acid (ca. 79 ml.). This solution was oxidized by dropwise addition of 10 ml. (C.P.) 30 per cent hydrogen peroxide, and boiling off the excess. It was necessary that this be carried out in a long-necked Florence flask to prevent volatilization of the iron. The volume was then made to 500 ml. with water and HCl such that the concentration of HCl was 0.1 N. The concentration of iron at this point was ca. 0.02 N. These standard iron

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*mcg. is used as the abbreviation for micrograms throughout the thesis.

solutions were analyzed iodimetrically (Kolthoff and Sandell (42 p. 635)). One gram of solid, reagent grade, iodate-free potassium iodide was added to a 50 ml. aliquot of the solution and the iodine liberated in 5 minutes was titrated to a starch end point (2 ml. of 1 per cent starch) with 0.05 N sodium thiosulfate.

c. Hydrogen peroxide solutions

A solution of 30 per cent hydrogen peroxide (C.P.) was diluted 1:100 with 7:3 purified benzene (p 38, e.): purified methanol (p 37, d.) solvent; 25 ml. aliquots of this solution were titrated with sodium thiosulfate after the addition of 50 ml. of water, 10 ml. of 1:10 sulfuric acid, 2 g. of solid potassium iodide and 3 drops of 3 per cent neutral ammonium molybdate (Kolthoff and Sandell (42 p 630)). The mixture was allowed to stand three minutes before it was titrated; 2 ml. of 1 per cent starch indicator were employed.

d. Sodium thiosulfate solutions

Solutions of the normalities required were prepared according to Willard and Furman (87 p 267) and were standardized against potassium iodate.

e. Ceric sulfate (1.0 N)

These solutions were prepared from reagent $Ce(HSO_4)_4$ (G. F. Smith Chemical Co.) by solution in 1 N sulfuric acid. They were standardized according to Willard and Furman (87 p 256) against sodium oxalate.

f. Tocopheryl-o-quinone (chroman 5,6-quinone) according to Swift, Mann and Fisher (82)

One gram of dl-alpha-tocopherol (Merck) was dissolved in 900 ml. of U.S.P. absolute alcohol, 100 ml. of concentrated nitric acid were added, and the mixture was heated at 70° C. until the spectral absorption at 470 mu indicated that the concentration of the red product had reached a maximum (about one hour). The reaction mixture was then poured into 2.5 l. of ice water; the reaction product was extracted with a 1:1 mixture of redistilled Skellysolve B and reagent ethyl ether. The extract was washed then with three portions of water and dried with anhydrous sodium sulfate. The solvent was evaporated on a steam bath and the residue dissolved in 100 ml. of redistilled Skellysolve B. This solution was run through a 2 x 20 cm. column consisting of a 2:1 mixture of silicic acid (Davco): Hyflo Supercel (Johns Manville). The silicic acid (8 mesh) was ground in a mortar to pass 40 mesh before it was used. The chromatogram was developed with 200 ml. of Skellysolve B and the column eluted with a 1:1 mixture (by volume) of reagent methyl alcohol and C.P. acetone to remove the red band. The solvents were evaporated and the chromatography repeated. Finally the red oil was taken up in 100 ml. of purified absolute alcohol (p 37).

g. Tocopheryl-p-quinone

0.1 gram of dl-alpha-tocopherol (Merck), dissolved in 10 ml. of C.P. benzene, was titrated with 0.0123 N $Ce(HSO_4)_4$ in 1.0 N sulfuric acid as suggested by Schulek and Roxsa (80). The ceric sulfate was added dropwise to the vigorously stirred solution to which 0.3 ml. of diphenylamine indicator had been added. The titration was continued until the blue "oxidation color" persisted for one minute. After titration, 20 ml. of benzene were added and the solution washed with three 10 ml. portions of 1.0 N sulfuric acid, then with a 25 ml. portion of 5 per cent Na_2CO_3 and finally with water. The benzene solution was dried over anhydrous sodium sulfate; the benzene was then evaporated in vacuo. The yellow oily residue was taken up in 50 ml. of purified benzene. Analysis of the benzene solution for tocopherol with the Devlin and Mattill (17) reagent indicated that less than 1 per cent of the compound was tocopherol.

2. Reagents

a. Diphenylamine sulfonic acid indicator

Prepared as directed by Willard and Diehl (94 p 246) from barium diphenylamine sulfonate (G. F. Smith Chemical Co.).

b. Devlin and Mattill (9) tocopherol reagent (acetic acid)

A 1.0 g. portion of reagent grade $FeCl_3 \cdot 6H_2O$ and 2.5 g. of alpha,alpha'-dipyridyl (Eastman) were weighed into

separate 100 ml. beakers, dissolved in reagent glacial acetic acid (ca. 10 ml.), mixed and the volume made to 1 l. with glacial acetic acid. This reagent is a modification of that of Emmerie and Engel.

c. Quaife and Biehler (61) tocopherol reagents (alcohol)

A 0.25 g. portion of $FeCl_3 \cdot 6H_2O$ was dissolved in 100 ml. of purified absolute ethanol; 0.50 g. of alpha,alpha'-dipyridyl was dissolved in 100 ml. of purified absolute ethanol. This reagent is essentially that of Emmerie and Engel.

d. Hills and Thiel peroxide reagents (26)

Ammonium thiocyanate: A 30 per cent aqueous solution of iron free reagent ammonium thiocyanate was prepared and kept in a dark bottle fitted with a dropping pipette through the stopper.

Ferrous chloride solution: A 0.5 g. sample of reagent ferrous sulfate was dissolved in 80 ml. of 0.1 N HCl and was mixed with 0.4 g. of reagent barium chloride (hydrated) in 50 ml. of water. The precipitated barium sulfate was allowed to settle, the supernatant was decanted and was kept in a tightly stoppered brown bottle.

e. Palladinized calcium carbonate was prepared according to the directions of Quaife and Harris (63):

> Suspend 50 g. of well washed calcium carbonate which has been precipitated while hot, in about 200 cc. of water and mix the suspension by shaking and gentle warming with a solution of 1.0 g. of palladium

> chloride. When the liquid over the calcium carbonate is completely decolorized, pour off, wash the solid a few times with distilled water, and then filter at the pump. Wash the material on the funnel until the filtrate is free of chloride ions, dry in a vacuum desiccator and store in a well stoppered reagent bottle. The adsorbed palladium hydroxide is reduced during the determination.

Baker's purified, precipitated calcium carbonate was found to be suitable.

f. <u>Cobaltous chloride solution</u>

A 9.52 g. portion of $CoCl_2.6\ H_2O$ was dissolved in 100 ml. of solution containing 2 ml. of concentrated hydrochloric acid.

g. <u>Methanolic potassium hydroxide</u>

Two hundred g. of reagent grade potassium hydroxide were dissolved and the volume made to 1 l. with reagent grade absolute methanol.

h. <u>Five per cent pyrogallol</u>

Five g. of acid, pyrogallic, (Eastman) were dissolved in reagent grade absolute methanol and were made to 100 ml. of solution.

i. <u>Two per cent Calgon</u>

Twenty g. of Calgon were dissolved in water and made to 1 l. of solution.

3. Absorbents

a. Floridin columns prepared by the Kjolhede method (36)

A 1.5 to 2 g. portion of Floridin (Floridin XXS, The Floridin Co., Warren, Pennsylvania) and about 0.25 g. of $SnCl_2$ were suspended in 5 ml. of concentrated HCl, heated to boiling, and poured into an adsorption tube. The liquid was sucked down under vacuum (30 mm. Hg); two 5 ml. portions of absolute alcohol and five 5 ml. portions of benzene were added and the liquid sucked down to the surface with each addition, without letting the surface become dry.

b. Floridin columns by the "modified" Kjolhede method

Floridin XXS earth was prepared in the following manner: 500 g. of Floridin XXS were placed in a beaker; 2 l. of 4.5 per cent stannous chloride in conc. C.P. HCl were added and the mixture was boiled for 1 hour. The liquid was removed by suction on a fritted filter and washed on the filter several times with water. This washed adsorbent was then transferred to tall cylinders and washed by a slow stream of distilled water introduced into the bottom of the cylinder by means of a glass tube. The rate of flow was such that there was agitation with a minimum of overflow. Washing was continued for 24 to 48 hours until there was no detectable chloride in a sample of wash water. The pH of the wash water checked that of the distilled water used. When washing was complete, the excess water was removed by suction on a fritted filter; the

adsorbent was air-dried and washed with C.P. or reagent benzene on the filter by suction until the washings gave a 100 per cent transmittance reading against pure benzene as blank in the Coleman spectrophotometer at 400 mu. (First washings were, at times, slightly turbid and colored brown or yellow.) This benzene-treated earth was again dried in a vacuum desiccator overnight, then stored in a shallow dish over a saturated solution of $Ca(NO_3)_2$ (280 g. of $Ca(NO_3)_2 \cdot 4H_2O$/100 ml. of water). Moisture equilibrium was reached in about two weeks. Constancy of weight of a sample placed in a weighing bottle, indicating saturation with moisture, was checked if it was desired to use the adsorbent as soon as possible after preparation.

c. Columns of Kjolhede Floridin and alumina

The Kjolhede Floridin was prepared as described in a. above, with the exception that after preparation in a separate column it was placed above the alumina in a second column. The alumina was prepared according to Kofler (41). One kg. of 80-100 mesh Fisher's Chromatographic Alumina was placed in a 4 liter beaker; 500 ml. of conc. HCl and 50 g. of reagent $ZnCl_2$ in 500 ml. of water were added. This mixture was stirred occasionally for three days and was then washed by decantation a few times. The solid was transferred into a large funnel (about 2 liter capacity) held upright on a ring stand and was further washed by running distilled water into the bottom at a

rate which just kept the stem of the funnel clear of solids. This flow of water was continued until the overflow was clear and 10 ml. of the effluent when tested with 0.1 per cent methyl red indicator, just turned 1 drop of methyl red to a yellow color. When washing was complete (six to eight hours), the wet solid was transferred to a 1 l. fritted Pyrex glass filter; the water was removed; and the alumina was dried by three 100 ml. portions of C.P. acetone and by air drawn through the alumina. When dry the alumina was placed in a vacuum desiccator (no desiccant) and evacuated 4 or 5 times over a period of 2 days to remove acetone. Just prior to use, the alumina was heated for 1 hour in a muffle furnace at 600° C. in Vycor or porcelain crucibles (about 10 g. placed in each crucible). After heating, the crucibles were covered and the adsorbent was transferred, directly while hot, into a 150 x 4 cm. column with a plug of fine glass wool in the base through which a rapid stream of nitrogen was flowing upward. The hot adsorbent was poured slowly down the column against the stream of nitrogen so that it was partially cooled by the time it collected over the glass plug in the base. The nitrogen was allowed to flow until the adsorbent was at room temperature. Airco 99.9 per cent nitrogen was used for this purpose after it had been passed through a tube of Dririte. When the adsorbent was at room temperature, enough for an 8 cm. colum was placed in

a beaker with purified benzene (one of the columns was used as a measure) to make a slurry and this was transferred to the column and washed with two 5 ml. portions of benzene. The 4 cm. of Kjolhede Floridin was then placed on top of the alumina and the tube was washed with two 5 ml. portions of benzene. The columns were then ready for use.

In the experiments in which redistilled Skelly B was used instead of benzene the preparations were the same. The Floridin preparation was the same here as in a. above except that the adsorbent was washed on the column with Skelly B until there were no more "schlieren" in the drops coming from the bottom indicating complete removal of benzene. The Floridin thus prepared was transferred, using Skelly B, to the top of the alumina columns.

When development was complete on the columns and prior to use of elution solvents, the Kjolhede prepared Floridin was carefully removed by use of suction. A glass tube of about 4 mm. bore was connected to a suction flask half filled with water and the water pump turned on. Thrusting the tube gently into the surface of the adsorbent, the layers were removed slowly until only the alumina remained. One precaution was necessary, the tube could not be thrust below the surface or air would be drawn into the bottom of the column by capillary action. The last traces of Floridin were carefully removed by

wrapping a piece of tissue around the tube connected to the vacuum, and carefully wiping the inside of the adsorption tube. The columns were then eluted and the eluent was collected for assay.

4. Solvent Purification

a. Peroxide free ether

Anhydrous, reagent-grade ethyl ether was distilled from 10 g. of KOH pellets and 2 g. of crystalline reagent grade $KMnO_4$ per liter (the first and last 200-300 ml. of a 4 liter batch were discarded) and was stored in a dark bottle at 20° C.

b. Ethanol

U.S.P. absolute alcohol was distilled (after one hour refluxing) with 20 g. of KOH and 5 g. of aluminum per liter.

c. Cyclohexane

Cyclohexane was prepared by shaking 3 liters with five 120 ml. portions (or more if the fifth sulfuric acid portion darkened) of C.P. conc. sulfuric acid per l., then 5 times with 100 ml. water. It was dried over 400 g. of anhydrous sodium sulfate and distilled; the first and the last 50 ml. of distillate were discarded. The cyclohexane distilled between 79.2° and 80° C.

d. Methanol

Methanol was purified by the method of Hills and Thiel (26) by refluxing reagent grade absolute methyl alcohol 4 hours

over 5 g. of magnesium ribbon per liter and distilling.

e. Benzene

Reagent, thiophene-free benzene was refluxed 1 hour over 20 g. KOH + 5 g. aluminum per l. and distilled, discarding the first 50 ml. of a 4 liter batch of distillate.

f. Purified isooctane

According to Graff, O'Connor, and Skau (21) 100 g. of silica gel (Davco 659528-2000) were placed in a column 120 cm. long x 4.0 cm. in diameter and 4 l. of isooctane, (Eastman 99.5+ per cent pure 2,2,4-trimethylpentane) were passed through. The level of the liquid was maintained above the top of the adsorbent by adjusting the flow rate from a large separatory funnel suspended above the column.

C. Apparatus

The following apparatus was employed:

A size 2 International Centrifuge.

A Cenco Number 11604 International Clinical Centrifuge.

Three different model 11, Coleman Universal Spectrophotometers and one set of 23 matched Pyrex cuvettes, calibrated with each spectrophotometer using cobaltous chloride solution (p 32 f.).

Two different Model DU, Beckman Spectrophotometers and matched Beckman Silica cuvettes were employed.

A home-made constant temperature box employing a dark-room fan for circulating air and electric lamps for heat, and regulated by a Fenwall air thermoswitch to operate at 40° C. ± 0.5° C.

A commercial electric household refrigerator operating at 35° F.

A commercial ice cream holding cabinet held at -10 to -15° F.

A Sargent laboratory air oven regulated at 100° C.

A 10 g. butter moisture scale with a sensitivity of 25 mg.

A standard Ainsworth chainomatic analytical balance.

A Roller-Smith micro torsion balance with 125 mg. capacity.

All glass stills fitted with standard taper joints for refluxing and distilling solvents.

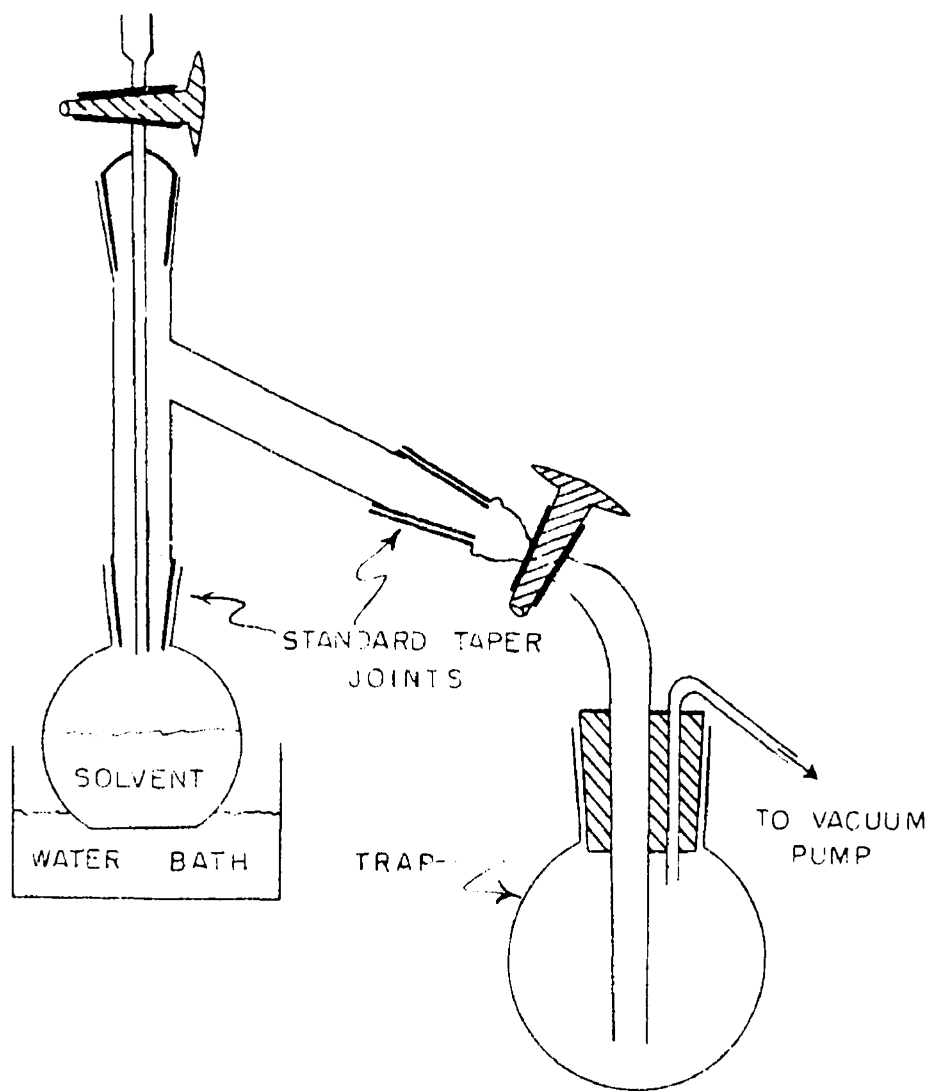

Fig. 1. Vacuum evaporation apparatus for removal of volatile solvents.

A micro hydrogenation apparatus from Vacuum Equipment Division, Distillation Products Inc. as indicated by Quaife and Biehler (61).

A vacuum evaporation apparatus for removal of volatile solvents at temperatures below their boiling points at atmospheric pressure. See Figure 1.

D. Methods

1. Ferrimetric Determination of Peroxide (Hills and Thiel (26))

Weighed fat samples (ca. 1 g.) were dissolved in a 7:3 mixture of purified benzene (p 38 e.) and purified methanol (p 37 d.), and were made to volume (50 ml. if the peroxide content was ca. 3.0 m.e. per kg.). An aliquot (1 to 9 ml. to give a reading between 20 and 90 per cent transmittance) was pipetted into a 10 ml. graduated amber cylinder, the volume was made to 9.90 ml. with the benzene:methanol solvent, one drop of 30 per cent ammonium thiocyanate (p 31 d.) was added, and the cylinder was stoppered and shaken to dissolve the reagent. One drop of the ferrous chloride reagent (p 31 e.) was added and the cylinder again stoppered and shaken until the solution was homogenous.

A (reagent) blank was prepared containing solvent (9.90 ml.) and reagents; a fat blank was prepared containing an aliquot of fat identical with the sample, solvent to make the volume 9.95 ml., and one drop of 30 per cent ammonium thiocyanate.

All cylinders were tightly stoppered and placed in a water bath at 60° C. for three minutes, cooled to room temperature in a water bath and poured into separate matched cuvettes for color intensity measurement.

The sample, the reagent blank and the fat blank were read against benzene:methanol set at 100 per cent transmittance with the wavelength setting on the Coleman Spectrophotometer at 510 mu. The cuvettes were matched with cobaltous chloride solution at 510 mu (p 32 f.). The results were calculated as follows:

I. m.e. perox./kg. =

$$\frac{(\text{2-log T, samp.}) - (\text{2-log T, reagt. blank} + \text{2-log T, fat blank})}{\text{regression constant x g. fat in soln. read}}$$

If the m.e. of peroxide were calculated separately for the sample and blanks from the established regression, the peroxide content of the sample was calculated by equation II:

II. m.e. perox./kg. =

$$\frac{(\text{m.e. perox., samp.}) - (\text{m.e. perox., reagt. blk} + \text{m.e. perox. fat blank})}{\text{g. fat in soln. read}}$$

a. <u>Determination of standard regressions</u>

(1) From ferric chloride. The standard ferric chloride solution (p 27 b.) was diluted with benzene:methanol to obtain a series of solutions (minimum 7) that covered the

range 0.02 to 0.40 microequivalents of iron per ml. of solution. Aliquots (less than 9 ml.) were pipetted from each solution into a 10 ml., glass-stoppered graduated amber cylinder, and the volume made to 9.95 ml. with benzene:methanol solvent; one drop of ammonium thiocyanate was added (but no ferrous chloride) and the analysis completed as with the sample.

(2) From hydrogen peroxide. Standard hydrogen peroxide solutions (p 28 c.) were diluted with benzene:methanol to obtain a series of solutions which cover the range 0.02 to 0.40 micro equivalents of hydrogen peroxide per ml. of solution and these solutions aliquoted as in (1) above. The analysis of these solutions proceeded as for the fat (a. above).

The standard regressions obtained for the Hills and Thiel method were:

III. $$\text{Micro eq. of iron} = \frac{2.007 - \log T}{1.743}$$

IV. $$\text{Micro eq. of hydrogen peroxide} = \frac{2.060 - \log T}{1.248}$$

The two curves had different slopes. The color development per equivalent was less with hydrogen peroxide than with ferric chloride. The ratio of the values by the two methods is:

$$\frac{H_2O_2}{FeCl_3} = \frac{1.743}{1.248} = \frac{1.43}{1}$$

b. <u>Order of addition of reagents</u>

The ammonium thiocyanate solution was added prior to the ferrous chloride in the analytical procedure as directed by

Bird <u>et al</u>. (1). The reagent blanks obtained generally read 91 to 94 per cent transmittance, against the solvent at 100 per cent. There was usually little change until the reagent was one week old.

The maximum absorption at 510 mu was established by plotting T against wavelength of light employed. The maximum is broad; the slope of the curve is nearly zero from 500 to 520 mu.

2. <u>The iodometric determination of peroxide</u>

The Lea (43) method was used. One gram of the melted fat was weighed on an analytical balance into a 250 ml. iodine flask, 50 ml. of 3:2 reagent glacial acetic acid: reagent chloroform mixture was added to dissolve the fat, then 1 ml. of a saturated solution of potassium iodide was added. The flask was stoppered, shaken and placed in the dark exactly one minute, then 100 ml. of water were added and the liberated iodine was titrated with 0.002 N $Na_2S_2O_3$ (p 28 d.) to a starch (2 ml. of 1 per cent solution) end point. The ml. of 0.002 N $Na_2S_2O_3$ is equal to the millimols of peroxide per kilogram of fat. The blank consisting of all the reagents used was titrated in the same manner as the sample and a correction made.

3. <u>Preparation of Standard Regression Equations for the Determination of Tocopherol</u>

The Devlin and Mattill glacial acetic acid reagent (p 30 b.) was used as the color development reagent.

Suitable (purified benzene) dilutions of standard tocopherol solutions (p 27 a.) were made and aliquots of these dilutions were taken, such that quantities of tocopherol of from about 2 to 400 mcg. were obtained in the final test solutions. An aliquot (10 ml. or less containing from 10 to 100 mcg./ml.) was placed in a 25 ml. glass stoppered mixing cylinder. The volume was made to 10 ml. with benzene, 2 ml. of color development reagent were added, and the volume was made to 25 ml. with alcohol. The tube contents were mixed by inverting several times, the mixture allowed to stand ten minutes, then transferred to cuvettes and read against a blank treated in the same manner with no tocopherol added. The regression equation was calculated from the log of the percentage transmittance and the concentration values, and from this regression equation, a table was prepared by calculating the mcg. of tocopherol represented by any percentage transmission reading from 100 to 20 per cent in 0.2 per cent intervals.

The mcg. of tocopherol in the sample were read from this table directly. The mcg. of tocopherol per sample were divided by sample weight to give the tocopherol value in mcg. per g. of fat.

The matched set of 23 tubes was used for this determination. These tubes were checked against one tube as a standard using the solution of $CoCl_2.6H_2O$ in water (p 32 f.).

4. Analysis of Milk Fat Samples for Tocopherol by the Kjolhede and Modified Kjolhede Methods

The modified adsorbent consisted of a slightly packed column of prepared Floridin (p 33 b.) (about 0.9 x 8.0 cm.) which had been previously wetted with about 20 ml. of benzene. Columns were made of 9 mm. glass tubing about 20 cm. long, fused to a piece of 18 mm. tubing about 6 cm. long to serve as a reservoir. A constriction was made about 2.0 cm. from the bottom of the tube to hold a pad of glass wool in place. The glass wool served to retain the adsorbent in the column.

The Kjolhede adsorbent was prepared in the column (p 33 a.) and was used immediately. The two procedures were identical excepting for the adsorbent used. Four g. of fat were weighed into a small Erlenmeyer flask and 10 ml. of benzene were added. The dissolved fat was poured upon the wetted column. When the top meniscus of the fat:solvent mixture just reached the top of the adsorbent, a 5 ml. portion of benzene was added; this was allowed to percolate through until the top meniscus was again at the top of the adsorbent when a second 5 ml. portion of benzene was added. This was repeated until 25 ml. of benzene were added. Percolate and washings were collected in a 125 ml. extraction flask with 24/40 fitting. This flask was attached to a vacuum evaporative apparatus (Figure 1) and the solvent evaporated. A water bath maintained at about 45° C.

was used. The residue was transferred to another 125 ml. extraction flask with about 30 ml. of redistilled Skellysolve B, and the evaporation was repeated. When this solvent was removed, 2 ml. of benzene were introduced into the extraction flask through the stopcock in the top of the evaporation apparatus. The residue in the extraction flask was transferred to a 25 ml. glass stoppered amber graduated mixing cylinder, with a volume of benzene such that the benzene: alcohol ratio stayed constant. To maintain a constant benzene:alcohol ratio, the following calculations were made:

V. ml. of benzene $= \frac{9}{22} \times 25 -$ (ml. of fat + ml. of reagent)

VI. ml. of alcohol $= \frac{13}{22} \times 25 -$ (ml. of fat + ml. of reagent)

Two ml. of the Devlin and Mattill reagent were always used. The total volume was 25 ml.

The order of addition was: Benzene solution of fat, benzene, reagent, and alcohol. The contents of the cylinders were mixed and the tube contents transferred to cuvettes for reading against a blank (set at 100 per cent transmittance) containing reagents and solvents but no fat at 520 mu on the Coleman Model 11 Spectrophotometer. Readings were generally taken 1 minute after the addition of the reagent unless changes in reading with time were being studied.

Note: For alpha-tocopherol there was no appreciable change in the readings of the samples with time.

The spectrophotometer readings were converted to micrograms of tocopherol by reference to the table prepared from the standard curve (p 44 3.).

5. Analysis of Milk Fat Samples for Tocopherol by the Quaife and Biehler (61) Method (as modified by Quaife (59))

Readings in the range between 50 per cent and 90 per cent transmittance were obtained using a sample weight of 1 g. of fat in 15 ml. of 1:1 ethanol (p 37 b.):cyclohexane (p 37 c.) solution. About 0.1 to 0.2 g. of palladinized calcium carbonate (p 31 e.) catalyst were used and the solution was hydrogenated at 20 lbs. pressure for 3 minutes in 50 ml. centrifuge tubes. The hydrogenation was carried out in the right hand tube of the Quaife and Biehler apparatus (61), the left hand tube being filled with solvent to minimize evaporation and cooling in the tube containing fat and catalyst. When hydrogenation was complete, the valve on the apparatus was opened very slightly while the low pressure valve on the regulator was closed. This allowed the pressure to drop, and as soon as the pressure was down to about two pounds on the apparatus gauge, the right hand tube was removed. This cleared fat and solvent from the dispersion tube. The contents of the tube were then transferred to a 15 ml. conical glass stoppered centrifuge tube and immediately placed in a clinical type centrifuge and centrifuged 5 minutes at 2000 r.p.m. A maximum of 4 replicates were run in

this manner; then, immediately 8 ml. aliquots were taken from the supernatant liquid and placed in amber glass stoppered mixing cylinders. A reagent blank was prepared with 8 ml. of solvent only. One ml. of alpha,alpha'-dipyridyl solution (p 31 c.) was added to each tube containing the 8 ml. aliquots of solution, including the reagent blank. Another blank containing only solvent was set at 100 per cent transmittance at 520 mu. One ml. of the $FeCl_3$ (p 31 c.) solution was pipetted rapidly (less than 5 seconds) into the tube, the tube was rapidly inverted twice, the contents transferred to a cuvette and readings taken at exactly 15 seconds and at 10 minutes after addition of last drop of $FeCl_3$. The reagent blank was read in the same manner. All were measured against the solvent set at 100 per cent transmittance. A fat blank of 8 ml. of the hydrogenated solution diluted with 2 ml. of absolute alcohol was also measured against the solvent at 520 mu to correct for absorption due to the fat. The micrograms of tocopherol represented by the per cent transmittance of the blanks was subtracted from the mcg. of tocopherol represented by the per cent transmittance of the sample. This value is the mcg. found in the final test.

$$\text{mcg. of tocopherol/g. fat} = \text{mcg. in final test} \times \frac{15}{8} \times \frac{1}{\text{sample wt. in g.}}$$

The data for the standard regression equation was obtained in the same manner as that for the Kjolhede method (p 43 c.), except the reagents employed were those described for this method

and quantities of tocopherol required in the final 10 ml. volume were about 10/25 of those required for the Kjolhede method. The standard tocopherol solution (p 27 a.) is best made up in cyclohexane solvent.

6. <u>Saponification of Milk Fat and Extraction of Tocopherol from the Saponification Mixture for Tocopherol Assay</u>

Ten g. samples were weighed into 150 ml. extraction flasks with standard taper necks, 5 ml. of 5 per cent pyrogallol (p 32 h.) and 20 ml. of 3.5 N KOH (p 32 g.) were added. The flasks were connected to a reflux condenser fitted with a tube for introducing nitrogen into the extraction flask. A cup with water at 70° C. was placed under the flask and a small flame was placed under the cup to maintain the water temperature at 70° C. When the first drop of condensate fell from the condenser tip, the time was noted and the saponification was allowed to proceed for 10 minutes. The flask was then removed and 40 ml. of water were added immediately, followed by 20 ml. of C.P. absolute methanol. The flask was cooled in water. After cooling, the contents of the flask were transferred to a 250 ml. amber, Squibb-type separatory funnel, using 60 ml. of water and 80 ml. of peroxide-free ether (p 37 a.). The funnel was shaken vigorously 10 times and the layers allowed to separate, the aqueous layer was run off, and the ether layer was poured from the top into a separate flask. The aqueous layer was returned to the funnel

and the extraction repeated twice with 50 ml. of peroxide-free ether. After the last extraction, the combined ether extracts were returned (quantitatively) to the funnel and the extract was washed with 50 ml. of 1 per cent KOH, 50 ml. of 2 per cent Calgon (p 32 i.) and two 50 ml. portions of water. The washed extract was dried by addition of Na_2SO_4 and by passing through a filter paper (18.5 cm. folded Whatman, No. 12). The ether was removed by evaporation in vacuo. When all of the ether was removed, 2 ml. of benzene were introduced through the stopcock of the vacuum evaporation apparatus and the flask filled with nitrogen. The residue was transferred with benzene and made to 25 ml. in a volumetric flask. Aliquots were taken for assay.

7. Kjolhede Floridin:Alumina Chromatographic Method for Tocopherol Determination

The columns were prepared as previously directed (p 34 c.). Solutions of fat in benzene (20 ml. of a solution containing 5 g. of fat or the non-saponifiables from 5 g. of fat) were poured onto the top of the column. Benzene was added in 10 ml. portions until 50 ml. of percolate were collected. The Floridin was removed from the alumina as described in "Preparation of absorbents". Absolute alcohol was poured onto the column in 5 ml. portions until 25 ml. of eluate was collected. The solvent capacity of the 8 cm. column was found to be 5 ml.; thus the eluate contained 5 ml. of benzene and 20 ml. of alcohol.

For assay a 15 ml. aliquot was used, 5 ml. of benzene and 2 ml. of the Devlin and Mattill reagent (p 30 b.) were added, and the volume was made to 25 ml. with benzene in a glass stoppered, graduated amber mixing cylinder. The contents of the cylinder were mixed, a portion of the contents transferred to a cuvette and the percentage transmittance was read at 520 mu 1 minute and 5 minutes after addition of the Devlin and Mattill reagent. A blank of solvents plus reagent in the same proportions as employed with the sample, was prepared and set at 100 per cent transmittance. The mcg. of tocopherol contained in the sample were determined from the tocopherol standard curve prepared with the Devlin and Mattill reagent in the same manner as in the Kjolhede procedure.

E. Results

1. Study of Saponification Methods

a. Preliminary studies with the saponification technique recommended by Emmerie and Engel (10) (11) indicated that saponification was not complete. For this reason the concentration of alkali necessary to obtain complete saponification of the glyceride was determined. In the same study, the effect of the addition of pyrogallol to the saponification mixture was determined. The saponifications were conducted in 150 ml. amber extraction flasks fitted with standard taper

24/40 ground joints attached to reflux condensers. The alkali solutions were added to the weighed fat samples, after the addition of pyrogallol (if pyrogallol was used). The saponification and extraction procedure described (p 50 6.) was followed except for variations noted in the tables.

b. Study of alkali and pyrogallol concentration

The data in Table 1 indicate that in order to have complete saponification it was necessary to employ an alkali stronger than 3.0N; 3.5 N methanolic KOH was selected to assure complete saponification. They likewise indicate that 5 ml. of 5 per cent pyrogallol will prevent destruction of tocopherol during saponification.

Another study was conducted to compare the results obtained with whole fat with those obtained with the non-saponifiable fraction, and to determine the effect of the addition of 5 ml. of 5 per cent pyrogallol when the saponified samples were passed through the Kjolhede and the modified Kjolhede adsorbents.

In all cases, a sample of the fat that was used for saponification was weighed and assayed as such. The data from these experiments appear in Table 2. Statistical analysis of these data shows that at the 10 per cent level there is a significant difference between values obtained by the Kjolhede procedure and those obtained by the modified Kjolhede procedure when pyrogallol was used in the saponification mixture. There

Table 1

Effect of Concentration of Alkali upon Volume of Non-Saponifiable Fraction and Effect of Pyrogallol and Concentration of Alkali upon the Tocopherol Value

Description of fat	Sample number	N of alkali[1]	Ml. of 5% pyrogallol	Tocopherol[2] in mcg./g. of fat	Ml. of non-sap. from 10 g. fat
1.5 month old milk fat	1	2.0	0	0	5.0
	2	2.0	1	0	5.0
	3	2.0	2	0	5.0
	4	2.0	5	0	5.0
8 month old milk fat	1	2.0	2	56.9	2.0
	2	2.0	7	55.0	3.0
Iowa fat 3 mo. at -20° C.	1	2.0	0	52.8	4.0
	2	3.0	5	50.4	1.0
	3	4.0	5	--	1.0
	4	5.0	5	--	1.0
Same fat stored 2 weeks in 40° F. box	1	3.5	0	8.6	trace[3]
	2	3.5	2	39.0	trace
	3	3.5	5	35.5	trace
	4	3.5	7	40.5	trace
Note: The ether used for this group was not peroxide free.					
Same fat one day later	1	3.5	5	70.0	trace
	2	3.5	5	67.4	trace
	3	3.5	0	51.9	trace
	4	3.5	0	43.0	trace
	5	3.5	5	62.5	trace
	6	3.5	5	63.4	trace
	7	3.5	0	33.2	trace
	8	3.5	0	34.6	trace
	9	3.5	5	77.5	trace
	10	3.5	5	57.9	trace
	11	3.5	0	36.0	trace
	12	3.5	0	29.9	trace
Same fat stored 8 days at 45° C.	1	3.5	5	50.5	trace
	2	3.5	5	50.5	trace
	3	3.5	0	17.7	trace
	4	3.5	0	17.0	trace

1. 20 ml. of alkali of the conc. shown was used.
2. Tocopherol values with the Devlin and Mattill reagent after Kjolhede treatment.
3. A few waxy drops.

is no significant statistical difference at the 10 per cent level between the values by these procedures when the samples were not treated with pyrogallol. There is a marked difference in the tocopherol values between the pyrogallol treated and non-treated samples, with both the modified Kjolhede and the Kjolhede methods (without pyrogallol the values are about 20 mcg./g. lower). Not enough samples of the whole fat were run to check the differences between this and saponified fat by statistical methods, but there appear to be slightly higher values as a result of the saponification treatment (with added pyrogallol).

Table 2

Tocopherol Values of a Sample of Fat Analyzed Before and After Saponification, in the Absence and Presence of Pyrogallol, by the Kjolhede and Modified Kjolhede Methods

Sample number	Tocopherol values (mcg./g. of fat)					
	Saponified fat				Whole fat	
	With pyrogallol		Without pyrogallol		No pyrogallol	
	Kjolhede	Modified	Kjolhede	Modified	Kjolhede	Modified
1	70.0	62.3	51.8	41.0	56.6	49.0
2	67.4	57.9	43.0	33.6		55.4
3	62.5	65.0	33.2	37.7	51.2	49.1
4	63.4	53.8	34.6	22.7		
5	77.5	56.5	36.0	37.9	63.2	56.6
6	57.9	63.2	29.9	50.4		
Average	66.5	59.8	38.1	36.2	57.0	52.6
std. error of mean	6.8	4.3				

c. Comparison of 3.5 N methanolic KOH procedure and the vitamin A saponification procedure

For the more rigorous saponification method of the vitamin A determination procedure (83) (5 ml. of a solution containing 150 g. of KOH/165 ml. of solution) about 4.5 g. of KOH are used per 10 g. of fat, while the 20 ml. 3.5 N KOH used here results in 3.94 g. per 10 g. of fat. The concentration in the saponification mixture is 180 g./liter in the first case, 158 g./liter in the latter case. It is of interest that Chipault, Lundberg, and Burr (7) recommend 17 ml. of 3.5 N KOH for 8 or 9 g. of fat. It must be remembered, however, that 5 ml. of 5 per cent pyrogallol added to the saponified mixture reduces the concentration of alkali from 70 to 64.1 millequivalents per sample as shown by calculating the m.e. of alkali needed to react with the pyrogallol.

$$\frac{5 \times 5}{100} = .25 \text{ g. pyrogallol/sample}$$

$$\frac{.25}{126.11} = .00198 \text{ mols.} = 1.98 \text{ m.mols.} = 5.94 \text{ m.e. pyrogallol}$$

20 ml. 3.5 N KOH = 70 m.e. of alkali

Comparison of the saponification method used for the vitamin A procedure (U. S. Technical Committee on Vitamin A Researchs (83)) with that (p 50) using 3.5 N methanolic KOH indicated no significant difference in the tocopherol values obtained with either the Kjolhede or the modified Kjolhede methods (Table 3).

The peroxide was not significant in the non-saponifiables obtained from the vitamin A saponification. (It will be shown later (Table 21) that this is also true of the procedure using 3.5 N methanolic KOH.)

d. Study of saponification procedure in air and in nitrogen

A study was made to determine the effect of a nitrogen atmosphere in the saponification flasks during saponification.

Table 3

Comparison of the Saponification Procedure of the Vitamin A Method with that Employing 3.5 N KOH in Methanol

Sample number	Sapon. procedure	Pyrogallol added	Tocopherol value (mcg./g. of fat) Kjolhede	Tocopherol value (mcg./g. of fat) Modified	Perox. value (m.e./kg.) Kjolhede	Perox. value (m.e./kg.) Modified	Perox. value (m.e./kg.) Untreated
1	Vit. A[1]	+	46.4-46.0	37.8-39.2	0.00	0.00	0.012
2	Vit. A	+	45.8-46.7	42.7-42.7	0.00	0.00	0.007
3	Vit. A	0	30.6-31.2	25.8-26.3	0.00	0.00	0.012
4	Vit. A	0	31.0-32.2	25.8-26.4	0.00	0.00	0.009
5	Not sapon	0	55.4-56.6	46.0-45.8	0.00	0.00	3.19
6	3.5 N KOH	+	46.7-45.8	41.1-42.7	0.00	0.989	0.010
7	3.5 N KOH	+	44.9-46.0	40.3-41.2	--	--	0.015
8	3.5 N KOH	0	32.4-32.8	26.2-26.8	--	--	--
9	3.5 N KOH	0	29.6-30.2	24.2-24.8	--	--	--
10	Not sapon	0	57.3-55.8	39.1-37.7	--	--	3.02

1. Procedure recommended by the U. S. Technical Committee on Vitamin A Researches (83)

Eight 10 g. samples were saponified and extracted by the procedure described (p 50), with the exception that one half of the samples were saponified under N_2 and the other half in air. Pyrogallol was used with one half of the samples and was not used with the other half. All samples were assayed by both the Kjolhede and the modified Kjolhede methods.

The data (Table 4) indicate that there was no difference as a result of the nitrogen treatment.

It may be concluded that the saponification procedure adopted for use compares well with the vitamin A procedure, that in the presence of pyrogallol, nitrogen has no beneficial effect, that peroxide is destroyed by the vitamin A method, and that the Kjolhede method gives higher values than the modified method when the non-saponifiable fraction is employed.

2. Substances Thought to Interfere with the Tocopherol Determination

a. Peroxides were believed to interfere with the Emmerie and Engel color development reaction.

(1) Removal of peroxides by miscellaneous methods. Preliminary studies of several methods which were reported in the literature or postulated as means of removal of peroxides from milk fat were conducted in an attempt to develop a means of peroxide destruction or removal without interfering with the subsequent tocopherol determination.

Table 4

Effect of Nitrogen Atmosphere During Saponification on Tocopherol Values (mcg./g. of fat) Compared with Values Obtained on Whole Untreated Fat

Sapon. Treatment			Adsorbent Treatment			
			Tocopherol (mcg./g. of fat)			
Sample number	Pyro-gallol added	Nitro-gen at-mosphere	Kjol-hede	Modi-fied	Untreated corrected[1]	Untreated uncorrected[2]
1	+	0	39.4	37.7	41.1	58.7
2	+	+	42.5	37.7	40.7	58.3
3	0	+	32.2	--	--	--
4	0	0	25.7	22.3	22.9	40.5
5	+	0	44.0	39.4	41.5	59.1
6	+	+	44.0	37.7	39.5	57.1
7	0	+	24.4	25.7	22.4	41.0
8	0	0	26.6	26.2	25.9	43.5

1. The corrected values were obtained by the method described by Quaife et al. (66) in which the correction due to carotene is obtained by use of the density readings of the solutions used for assay at 460 mu and 520 mu. The corrected values check the Kjolhede method rather well.

Quaife, et al. report that the correction factor involving a dilution of 57.4/69.6 is equal to $0.29 \times D_{460}$. With no dilution involved this would be $0.352 \times D_{460}$. The D_{460} of the yellow solutions above diluted to the same concentration as the final assay equals 0.1201. The D_{520} of this solution equals 0.0410. 0.0410/0.1201 = 0.342. This is believed to be good confirmation of the Quaife et al. value. Calculating the correction due to carotene for our solutions by the method of Quaife: $(0.1201 \times 0.352)/.001460^{*} \times 1/1.6^{x} = 18.2$ mcg. per g.

Calculating the correction due to carotene using D_{520}: $.0410/.00146^{*} \times 1/1.6^{x} = 17.55$ mcg. per g. These corrections check within experimental error.

*The regression constant.
^{x}The weight of fat in assay.

2. No corrections for yellow color of untreated fat.

(a) The Parker and McFarlane method (55) for removal of pigments, vitamin A and other interfering materials was used with milk fat. Preliminary work indicated that peroxides were partially removed and pigments completely removed by the 85 per cent sulfuric acid treatment of Skelly B solutions of milk fat. The method was unsatisfactory for milk fat because troublesome emulsions were formed which caused the results to be erratic.

(b) Potassium iodide, which is known to react with peroxide in acid solution was used in an attempt to produce peroxide free solutions which could be assayed for tocopherol. Peroxides were titrated by the Lea method (p 44); the chloroform layer of the titration mixture was removed and the chloroform evaporated in vacuum. The tocopherol value of the residue was determined with the Devlin and Mattill reagent (p 30 b). Recoveries of alpha-tocopherol added to oxidized fat were low, however, and the method was abandoned since it was indicated that some side reaction had occurred.

(c) Stannous chloride was used in alcoholic solution in an attempt to destroy peroxide.

Two ml. of a freshly prepared solution of stannous chloride in alcohol, (ca. 0.25 g. $SnCl_2$/ml.) were added to 4.25 g. of fat and allowed to stand 5 minutes with occasional shaking (about once/minute). At the end of 5 minutes,

30 ml. of $CHCl_3$ were added and the alcohol removed by washing 3 times with water. The $CHCl_3$ layer became white and translucent. Ten ml. of acetic acid and 1 ml. of a saturated solution of KI were added and the mixture allowed to stand in the dark 10 minutes. At the end of this time the mixture was titrated with 0.002 N thiosulfate to a starch end point. A control was run to which no $SnCl_2$ had been added.

	ml. thio
Control	38.7
Sample	0.0

This experiment indicated apparent removal of peroxide but the translucence of the solution indicated that $SnCl_2$ had not been completely removed. Kjolhede reported that $SnCl_2$ was insoluble in petroleum ether. The fat was treated, therefore, with alcoholic $SnCl_2$, was extracted from the alcohol and $SnCl_2$ mixture with Skelly F., the Skelly was evaporated and the peroxides and tocopherols were determined on the residue (sample I). It was considered that it might be possible to destroy excess $SnCl_2$ by reaction with $HgCl_2$. Another sample (sample II) was treated with 5 ml. of concentrated HCl plus 5 ml. of 10 per cent $HgCl_2$, a third sample (III) was treated with $HgCl_2$ only, and a fourth (IV) with HCl only. The control (V) used was treated with Skelly F only. The HCl was used in an attempt to prevent formation of colloidal $Sn(OH)_2$

from hydrolysis of the $SnCl_2$ in the neutral solution. All samples were run through fritted glass filters to assure removal of precipitates. The samples to which concentrated HCl had been added had a distinct odor of butyric acid. Sample II was the only one for which reasonable values were obtained, i.e., the peroxide was reduced and a higher value was obtained for the apparent tocopherol content.

Table 5

Tocopherol and Peroxide Values Obtained with a Single Fat When Subjected to Various Treatments with $SnCl_2$ and $HgCl_2$

	Treatment	mcg. Tocoph./ g. fat[1]	m.e. Peroxide/ kg. fat
I	Skelly only + $SnCl_2$	excessively high	negative
II	Skelly + $SnCl_2$ + $HgCl_2$ + HCl	58.4	0.0536
III	Skelly + $SnCl_2$ + $HgCl_2$	cloudy	0.360
IV	Skelly + $SnCl_2$ + HCl	cloudy	cloudy
V	Skelly only	34.6	1.34
Blank		0.0	0.0

1. Determined by method (p 46).

An experiment based upon the results obtained above was run to check the recoveries of added toco-

pherol to fat treated in a manner similar to Sample II. The treatment differed in that 5 ml. of ca. 1.2 N HCl were used instead of the 5 ml. of concentrated HCl employed with sample II above. Dry filter paper was used in place of fritted glass filters in order to remove the excess moisture, which had a tendency to cause clouding of the assay mixtures. Table 6 contains the results of this experiment.

Table 6

Recoveries of Alpha-Tocopherol Added to Milk Fat, Which was Subsequently Treated with $SnCl_2$ to Remove Peroxide and with $HgCl_2$ to Remove $SnCl_2$

Sample number	g. Fat	mcg. Tocoph. added	mcg. Tocopherol/ g. fat found[1]	% Recov. of added tocoph.
1	4.25	0.0	9.0	
2	4.25	0.0	13.1	
3	4.25	0.0	12.2	
4	2.12	341.0	10.4	-0.3
5	2.12	341.0	7.1	-1.3

1. Determined by modified Kjolhede method (p 46) (omitting adsorbent treatment).

No added tocopherol was recovered. This negative recovery may result in part from excess $HgCl_2$ for when $FeCl_2$ was added to the tocopherol reagent in an attempt to obtain color development in the presence of $HgCl_2$, it was found that there was only very slight color development. If the $FeCl_2$ was added

first there was good development of color but upon addition of $HgCl_2$ the color receded slowly. The data are given below:

Description of contents and order of addition of constituents of assay mixture	Transmittance (10 min.), solvent set at 100% T at 520 mu
Tocopherol reagent alone	92.0
Tocopherol reagent + $FeCl_2$	20.0
Tocopherol reagent + $FeCl_2$ + $HgCl_2$	26.0
Tocopherol reagent + $HgCl_2$ + $FeCl_2$	90.0

This experiment showed that $HgCl_2$ could not be used for removal of $SnCl_2$, if any $HgCl_2$ was left in the assay mixture. This technique was abandoned.

(d) Heating with carbon disulfide was attempted since it was believed possible that this solvent would act as a reducing agent toward peroxide and thereby prevent interference in the tocopherol assay. The apparent peroxide content of the fat was increased by the carbon disulfide treatment as determined by the Hills and Thiel method (p 41). This treatment was not considered satisfactory.

(e) Sodium bisulfite was used according to Knight and Swern (37) who reported stirring an ether solution of fat with an aqueous solution of bisulfite as a successful means of removal of the interference due to peroxide in the

determination of carbonyl oxygen, iodine value, and saponification value. Some samples were reduced in peroxide value from over 5.0 to values of 0.3 m.e./kg. Other samples contained considerable peroxide after treatment. The tocopherol value was apparently increased by this treatment. This technique was not considered satisfactory.

(2) Chromatographic methods for removal of peroxide.

(a) The use of solid adsorbents for the removal of substances which interfere in the tocopherol determination has been reported in several papers. Kjolhede (36) reported good recoveries; he employed Floridin earth, especially treated with $SnCl_2$ and HCl just prior to use. In a preliminary study, the percolates obtained from Kjolhede's Floridin XXS were peroxide free when a solution of fat which initially contained 0.35 m.e./kg. were employed. The tocopherol assay value of the fat before treatment was 3 mcg./g.; after treatment it was 41 mcg./g. The alcohol eluate from these columns was tested for peroxide and found to contain more peroxide per g. than the original fat.

(b) Floridin XXS preparations. Emmerie and Engel (16) stated that tocopherol recovery was improved when their HCl treated Floridin, water-washed free of chloride, was stored in a desiccator over a salt solution which maintained 18 per cent absolute humidity. They likewise stated

that they had satisfactorily prepared and stored Kjolhede's adsorbent in large batches covered with benzene. It was postulated that if the Kjolhede adsorbent could be stored and if the optimal conditions for storage of the HCl treated, washed Floridin were in an atmosphere of 18 per cent moisture, it might be possible to treat the adsorbent with HCl <u>and</u> $SnCl_2$, wash the adsorbent free of chloride ion and dry and store until used in a desiccator maintained at 18 per cent absolute humidity. This <u>modified</u> Kjolhede adsorbent was prepared (p 33 b.) to determine whether or not accurate results could be obtained, because preliminary work with the Kjolhede adsorbent and the Emmerie and Engel adsorbent had given erratic results for tocopherol.

The modified Kjolhede and Kjolhede preparations of Floridin were studied as means of removal of peroxide.

The initial experiment showed that peroxides were adsorbed by the Kjolhede earth when the sample was dissolved in benzene and that the peroxides could be eluted from the adsorbent by alcohol. The tocopherol values found were much higher when the solutions of the samples were run through a column of this earth than when they were not treated at all. Modified Kjolhede earth was prepared as indicated above except that large batches were made, the earth was water-washed until chloride free, and the earth was held for several months in a

desiccator over a saturated solution of $Ca(NO_3)_2$ before ordinary use. When the samples were chromatographed through columns of the modified earth the tocopherol values of the samples were lower and the peroxide values higher than when the original Kjolhede earth was employed.

Another experiment was conducted in which the fat was run through the adsorbent in purified Skelly B solution. After the fat solution and subsequently five 5 ml. portions of Skelly B had been passed through the column, 10 ml. of benzene were run through the column and then 10 ml. of alcohol. The Skelly B percolate and the benzene and alcohol eluates were collected separately and each was analyzed for peroxides and tocopherols. The data from this experiment are shown in Table 7.

These data indicate that the peroxide is definitely removed by Kjolhede Floridin, in fact there are some negative values for peroxides, indicating that a reducing agent may be removed from the column in some cases. The tocopherol values for the fat are higher than when the modified Kjolhede Floridin is used; the recovery of added tocopherol is low, however, in the Skelly percolates from Kjolhede Floridin. Subsequent benzene and alcohol eluations, however, account for this loss. If 40 mcg./g. is assumed as the correct value for the tocopherol content of the fat, the percentage of the

Table 7

Recoveries of alpha-Tocopherol and Peroxide Values of Fat Solutions and of Eluates, When Fat Solutions Were Passed Through Kjolhede and Modified Kjolhede Floridin Columns

Fraction	Adsorbent used (Floridin)	mcg. alpha-Tocoph. added/ g. fat	Tocoph. found as % of added	mcg. of Tocoph./ g. fat[1] found	m.e. Peroxide/ kg. fat
Skelly[2]	mod. Kjol.	55.6	100.4	79.7	1.37
Benzene[3]			5.5	3.05	1.33
Alcohol[4]			7.5	4.16	0.471
Skelly[2]	mod. Kjol.	55.6	90.5	74.2	1.20
Benzene[3]			7.5	4.16	1.35
Alcohol[4]			3.8	2.14	0.665
Skelly[2]	mod. Kjol.	0.0		Cloudy	0.936
Benzene[3]				1.85	1.30
Alcohol[4]				2.40	0.363
Skelly[2]	mod. Kjol.	0.0		23.9	0.968
Benzene[3]				3.47	1.23
Alcohol[4]				3.47	0.475
Skelly[2]	Kjolhede	55.6	53.0	79.3	
Benzene[3]			38.1	21.2	neg.
Alcohol[4]			5.7	3.20	0.033
Skelly[2]	Kjolhede	55.6	71.6	79.6	neg.
Benzene[3]			9.2	5.11	neg.
Alcohol[4]			8.9	4.91	0.033
Skelly[2]	Kjolhede	0.0		39.8	0.
Benzene[3]				7.16	neg.
Alcohol[4]				2.56	0.033

1. Determined by method (p 46).
2. Milk fat in Skelly B after passage through column.
3. Benzene eluate of column.
4. Absolute alcohol eluate of column.

total naturally occurring tocopherol indicated after treatment by the modified earth will be 78 per cent and 65 per cent respectively for the two samples. This recovery is about the same as those for added alpha-tocopherol calculated for the adsorbent prepared by Kjolhede's method. A study of the effect of peroxide on the tocopherol value will be presented in Table 15.

This experiment also shows results contrary to the initial experiment which indicated that peroxide could be eluted from the Kjolhede-prepared columns with alcohol. No peroxide was recovered from the columns prepared by Kjolhede's method in this experiment. It may be that the 10 ml. of alcohol used as final eluant was insufficient to remove all the peroxides present in the column.

The effect of using Skelly B or benzene as fat solvents was checked. Equal weights of the same fat were dissolved in these solvents. The percolates were analyzed for tocopherol content. The data are presented in Table 8.

The higher values obtained when the fat was percolated through as a benzene solution agree with the previous experiment in which there was considerable tocopherol in the benzene eluate following the Skelly percolate.

An attempt was made to determine the nature of materials adsorbed by the Kjolhede and modified Kjolhede Floridin preparations.

Table 8

The Effect of Solvent (Skelly B or Benzene) on the Tocopherol Values of Fats Treated with Floridin Prepared According to Kjolhede

g. Fat added to column	mcg. Tocoph./g. fat Skelly percolate	mcg. Tocoph./g. fat benzene percolate
4.25	52.5	62.4
4.25	50.4	58.8
4.25	51.0	58.8
4.25	50.8	57.7
4.25	51.0	55.4

1. Determined by method (p 46).

Infrared absorption spectra studies were made on alcohol eluates from Kjolhede Floridin columns through which oxidized fat had been passed. On several occasions after passage of the fat through the Kjolhede Floridin columns, the purple band at the top seemed to be somewhat graded in color from blue at the top to red at the bottom, a phenomena which suggested two constituents might be present. Development with large quantities of benzene did not produce two distinct bands, but the red portion was moved down the column somewhat. Development of the chromatogram with 1 per cent and 2 per cent alcohol in benzene produced eluates high in peroxide (e.g. 84.4 m.e./kg. of eluted material) as determined by the Hills and Thiel method.

Henick (24) made infrared absorption studied of these eluates. Absorption at 3.1 microns, which is typical of hydroperoxide absorption, in addition to the usual spectrum of milk fat was encountered. This indicates that peroxides are adsorbed by the Kjolhede Floridin and that they are not completely destroyed.

In one determination, it was possible to calculate the results upon the basis of the original fat used; 30 per cent of the peroxide was eluted from the column. This may or may not mean that the remainder of the peroxide was destroyed.

Ultra-violet absorption spectra were run on the eluates from Kjolhede and modified Kjolhede Floridin columns. An experiment was set up as follows: A benzene solution of oxidized fat (peroxide 4.42 m.e./kg. fat) was percolated through:

1. Kjolhede Columns
2. Modified Kjolhede columns
3. (a) Kjolhede Columns, then (b) Modified Columns
4. (a) Modified Columns, then (b) Kjolhede Columns

By this procedure, it was believed that the action of each adsorbent might be determined. The assay of these percolates showed that the tocopherol values for 1, 3, and 4 are essentially the same, while the values for 2 are lower by an average of 10 mcg. per gram. The data are presented in Table 9. The peroxide values for all percolates were in the range of 0.0 to 0.05 m.e./kg. of fat excepting those in the modified column percolates prior to passage over Kjolhede columns. From this it may be concluded that either the differences are due wholly to

peroxide, or to the reduction of tocopherol oxidation products by the Kjolhede columns and passage of both the oxidation products and the reduced form of these products by the modified columns.

An attempt was made to determine whether the materials adsorbed by the Kjolhede adsorbent were peroxides or oxidation products of tocopherol. The Kjolhede and the modified Kjolhede Floridin preparations through which whole fat had been passed (4. b. and 3. b. described on p 71) were eluted with 50 ml. of alcohol each, the alcohol evaporated, and the residue of 4. b. taken up in methanol. There was no noticeable residue from 3. b. It was assumed, therefore, that nothing was adsorbed by 3. b. after 3. a. The tocopherol and peroxide assay values calculated on the basis of the weight of non-volatile materials in the eluate from 4. b. were in the range of 10,000 mcg./g. and 7.0 m.e./kg. respectively. The quantities of peroxide and tocopherol were quite uncertain because of the very small quantities of material available for assay. The absorption spectrum of this material in methanol is given in Figure 2. The $E^{1\%}_{1\ cm.}$ for this material at 231 mu is 11.2. This is the region of maxima for conjugated diene. A very slight irregularity in the curve between 265 and 270 mu may indicate a trace of conjugated dienone. It is believed unlikely that the concentration of tocopherol is actually that shown by the assay estimate since no maximum occurs

Table 9

Peroxide and Tocopherol Content[1] of Percolates of Fat Solutions in Benzene Through Kjolhede and Modified Kjolhede Floridin Columns and Through Combinations of These Columns

First treatment	Aliquot 4.0 g. fat	mcg. Tocoph./ g. fat	Per-oxide m.e./kg. fat	Sec-ond treat-ment	mcg. Tocoph./ g. fat	Per-oxide m.e./kg. fat
Kjolhede	1	48.8	0.034			
	2	49.0	Neg.	modi-	54.8	.0418
	3	46.8	0.0517	fied	51.4	.0513
	4	51.2	0.0641			
Modified	5	39.7	1.60			
	6	39.7	1.46	Kjol-	53.5	.0502
	7	39.3	1.52	hede	52.4	.0418
	8	41.3	1.55			
Whole un-treated fat		28.2	4.42			

1. Determined by method (p 46).

Note: The values after <u>both</u> treatments are a little higher than they are by either method <u>before</u> the second treatment; but, this is a small difference upon which one should not place too much emphasis.

in the region of 290-295 mu. The trace of dienone indicated may actually be tocoquinone or chroman p-quinone ("semi" quinone).

The nature of the materials adsorbed by the Kjolhede and modified Kjolhede adsorbents when used for the chromatography of non-saponifiable fractions of a deteriorated fat (containing 4.4 m.e. of peroxide per kg. of fat) was also studied. Columns of both adsorbents over which non-saponifiables

had been passed were eluted with alcohol, the alcohol evaporated and the residues taken up in benzene. The benzene solutions were then passed over the opposite type of column from that over which they had originally been passed, (this double treatment was necessary to eliminate carotene) the benzene was evaporated and the residue was taken up in the solvent used for study of the spectrum; unfortunately, the alcohol eluate from the Kjolhede column was insoluble in purified methanol so it was dissolved in isooctane (p 38 f.) for the study of the spectrum; the spectrum of the eluate from the modified Kjolhede column was studied in methanol solution.

The spectra obtained are plotted on Figure 2; they are markedly similar except that they show little adsorption in the low visible and high ultraviolet but at 270 mu the adsorption begins to increase. These curves indicate that no conjugated diene, or diene peroxide (lack of maximum at 230 mu) is removed by the adsorbent from the non-saponifiable fraction of this fat which substantiates the assumption that the saponification procedure removes peroxide.

These experiments indicate that peroxide is destroyed during saponification, that in whole fat it passes through the modified columns to a variable degree, and that it is reduced to 0.06 m.e./kg. or less by the Kjolhede columns.

The presumptive indication on the basis of the trace of dienone found is that the tocopherol oxidation products (from whole fat) were held to some extent by the Kjolhede column from benzene solution and could be eluted by alcohol.

The effect of the Floridin treatment on the materials adsorbed was studied; it was considered that the "activation state" of the adsorbent might be responsible for the greater retention of peroxidic materials in the case of the Kjolhede adsorbent. To check this, portions of a sample of modified Kjolhede Floridin were used after having been treated in the following manner:

Table 10

Treatment of Modified Kjolhede Floridin Designed to Produce Different States of Adsorbent Activity in the Removal of Interfering Substances

Treatment number	Treatment of adsorbents
1	Benzene wash (the normal modified earth)
2	Alcohol, then benzene wash
3	Conc. HCl, then alcohol, then benzene wash[1]
4	Conc. HCl + $SnCl_2$, then alcohol, then benzene wash (the normal Kjolhede earth)

1. The HCl percolate was very yellow in color. A test with thiocyanate showed a high concentration of ferric iron.

Aliquots of a solution of milk fat in benzene were run through these columns; and the percolates assayed as usual for peroxide and tocopherol. The data appear in Table 11. The table

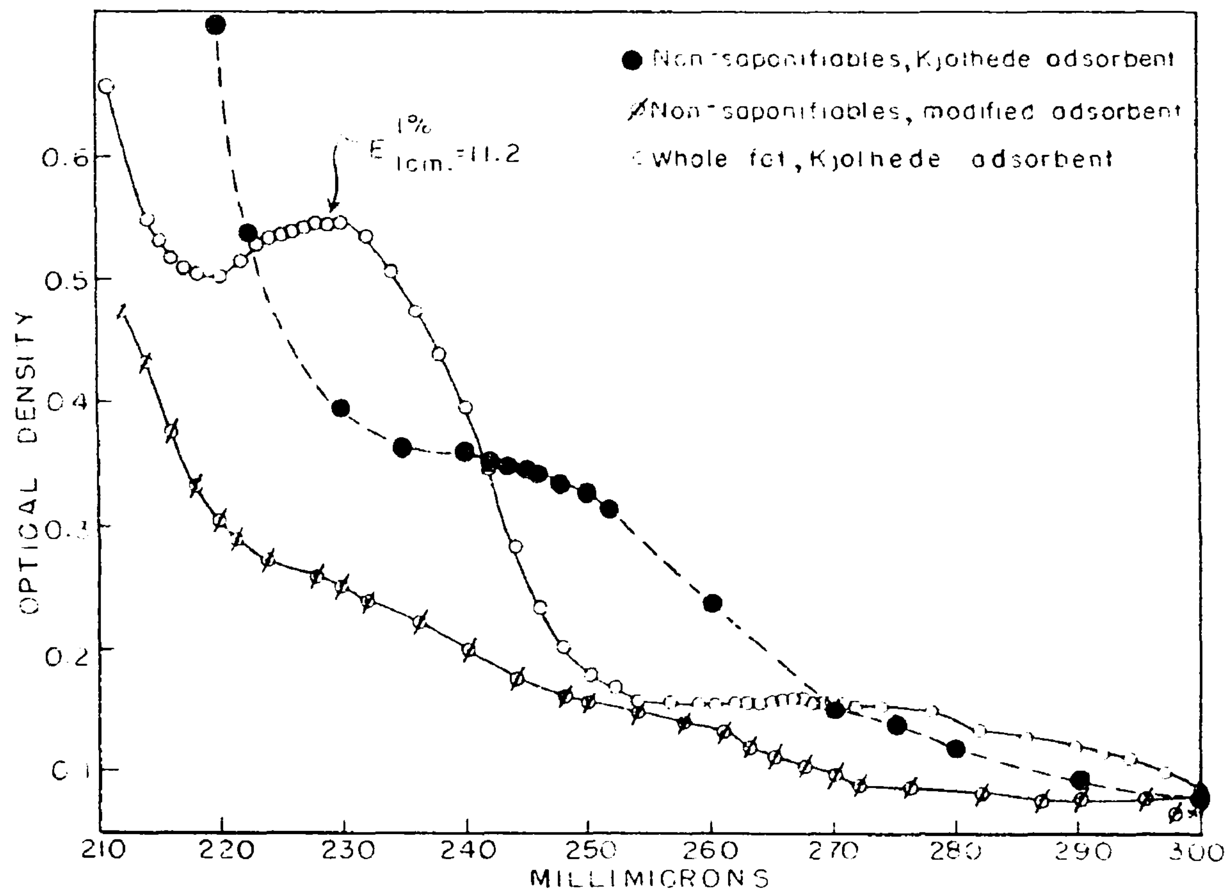

Figure 2. Absorption spectra of the alcohol eluates of materials adsorbed from benzene solutions of whole fat by Kjolhede Floridin, and from benzene solutions of the non-saponifiable fractions of fat by Kjolhede Floridin and modified Kjolhede Floridin.

shows that percolates from 3 and 4 contained more tocopherol and less peroxide than 1 and 2. The alcohol eluates from 3 and 4 also contained more tocopherol and less peroxide.

Another experiment was conducted with a relatively good fat, in the same manner as the above to check these activation effects with a fat of high quality. Duplicate samples were run. Adsorbent treatment 5 consisted of adsorbent prepared by treatment 4 placed above absorbent by treatment 1 in the column. The data in Table 12 indicate that the tocopherol content varied little regardless of treatment of this good fat, but the peroxide was somewhat higher in 1 and 2. Analysis of the eluates indicates very little peroxide or tocopherol in these fractions.

An experiment with three different fats ranging in peroxide value from 0.47 to 6.3 was carried out to verify the results of the last two experiments concerning the activity of HCl treated Floridin as compared with the Kjolhede Floridin. The procedures were the same as in the last two experiments except that only Floridin prepared by treatments 3 and 4 was used. Table 13 shows that, for low peroxide content fats, the benzene percolates and alcohol eluates are essentially peroxide free. With deteriorated fat the percolates and eluates from both adsorbents are high in peroxide. Tocopherol values agree rather well between methods.

Table 11

Tocopherol[1] and Peroxide Content of Benzene Percolates of Oxidized Fat Passed Through Different Adsorbents and Absolute Alcohol Eluates from These Adsorbents After Passage of the Fat Through the Columns

Adsorbent treatment number[2]	Benzene percolate		Alcohol eluate	
	mcg. Tocoph./ g. fat	m.e. Perox./ kg. fat	mcg. Tocoph./[3] g. fat	m.e. Perox./ kg. fat
1	34.2	0.625	2.3	.248
2	28.7	0.291	2.0	.372
3	37.7	0.000	5.9	.094
4	37.7	0.025	7.0	.042
Untreated fat	38.2	3.28		

1. Determined by method (p 46).
2. Descriptions of treatments Table 10.
3. Corrected for carotene by subtracting absorption due to color of the eluate measured against solvent according to the method described (p 58).

These runs indicate that, as far as the peroxide values of the eluate are concerned, the HCl treated earth gives the same values as the $SnCl_2$ + HCl treated earth. In the case of the poorer fat (Table 11) the lower values obtained by adsorbents 1 and 2 are probably due to not washing with the HCl prior to use, rather than not washing with $SnCl_2$ in HCl. In the case of the "good" fat (Table 12) results check rather well regardless of treatment. It may also be of some significance that the values obtained on the whole, untreated yellow fat are

Table 12

Tocopherol[1] and Peroxide Content of Benzene Percolates of High Quality Fat Passed Through Different Adsorbents[2] and Peroxide Values of Absolute Alcohol Eluates Subsequent to the Passage of the Fat Through the Columns

Adsorbent[2] treatment number	Benzene percolate		Alcohol eluate	
	mcg. Tocoph./ g. fat	m.e. Perox./ kg. fat	mcg. Tocoph./ g. fat[3]	m.e. Perox./ kg. fat
1	56.2	.168	-1.14	0.046
	54.6	.148	+1.14	0.072
2	51.2	.0969	+4.57	0.086
	51.3	.1255	+1.12	0.072
3	55.6	.0154	+5.14	0.080
	56.3	.0826	+1.42	0.059
4	53.0	.0473	-0.28	0.040
	54.6	.0154	-1.33	0.040
5	54.4	.00	+0.27	0.062
	52.4	.120	+0.22	0.060
Untreated fat	56.1	.0646		

1. Determined by method (p 46).
2. Described in Table 10.
3. Corrected for carotene color by subtracting absorption due to solution of the eluate measured against solvent according to the method described (p 58).

the same when corrected for carotene, as those obtained by Kjolhede method.

These observations and the experiment with the varying peroxide values (Table 15) seem to indicate that peroxide, as it is found in milk fat does not necessarily interfere in the tocopherol determination when the spectrophotometer

Table 13

Tocopherol[1] and Peroxide Content of Benzene Percolates of Different Fat Samples Passed Through Different Adsorbents[2] and Peroxide Values of Absolute Alcohol Eluates from These Adsorbents

	Treatment of Adsorbent						
	4 (Kjolhede adsorbent)			3 (HCl washed. mod. ads.)			No Adsorbent
	Benzene percolate		Alcohol Eluate:	Benzene percolate		Alcohol Eluate:[3]	
Sample description	mcg. Tocoph./g. of fat	m.e. Perox./kg. of fat	m.e. Perox./ kg. of fat	mcg. Tocoph./g. of fat	m.e. Perox./kg. of fat	m.e. Perox./ kg. of fat	m.e. Perox./kg. of fat
August, Iowa butter 3 mos at 45° C.	0.0 0.0	5.93 5.67	4.90 5.15	0.0 0.0	7.01 6.50	4.56 4.94	6.26 --
May, Iowa butter 1 mo at -20° C.	21.2 19.2	0.105 0.054	Neg. Neg.	15.8 19.9	0.028 0.005	0.002 Neg.	0.487 0.470
May, Nebr. butter 1 mo at -20° C.	27.1 28.3	0.050 0.016	Neg. Neg.	26.2 26.2	0.005 0.074	Neg. 0.000	0.648 0.599

1. Determined by method (p 46).
2. Treatments described in Table 10.
3. Blanks containing only the eluate plus NH_4SCN reagent were quite highly colored presumably due to ferric iron. The apparent peroxide in this blank was 0.523 m.e./kg. of fat.

readings are taken one minute after addition of reagent. The adsorption of some conjugated diene compounds, by the Kjolhede column and non-adsorption of these by the modified Kjolhede column indicates the important difference between these adsorbents in addition to the reducing power imparted to the latter by $SnCl_2$ treatment. It indicates that the Kjolhede Floridin is a more highly active adsorbent.

The necessity for the presence of $SnCl_2$ on the Floridin columns was claimed by Kjolhede (36) and confirmed by Emmerie and Engel (14) and by Kofler (41) in order to prevent destruction of tocopherol. Although it was found here that the adsorptive power of the columns were not increased by the use of $SnCl_2$ as evidenced by the peroxide and tocopherol content of the percolates, it was considered better to continue the use of $SnCl_2$ as a precaution against oxidative destruction particularly by ferric iron (which was encountered in the HCl and in subsequent alcohol washings of the modified Kjolhede adsorbent, (Tables 10 and 13)).

An assay of alcohol eluates, from the adsorbent preparation of Emmerie and Engel, by the Hills and Thiel method for peroxide showed that there was a considerable contamination of the eluates with ferric iron as evidenced by the formation of the ferric thiocyanate color with ammonium thiocyanate prior to addition of any iron. This blank

(footnote 3, Table 13) which contained 0.523 m.e. of Fe/kg. of fat represents a contamination of iron many times larger than the tocopherol content. The lower values observed (Tables 2, 11, 13) when the modified adsorbent treatment was used might be attributable to oxidation of the tocopherol on the columns by ferric iron.

Effect of stannous chloride on the result obtained when Kjolhede Floridin was employed.

The removal of $SnCl_2$ by the alcohol wash subsequent to HCl treatment was checked as follows: One column was washed with the specified 5 ml. of purified absolute alcohol, a second with two 5 ml. portions. Each column was then washed with five 5 ml. portions of purified benzene, the benzene eluates were checked with the Devlin and Mattill iron:dipyridyl reagent. The results presented in Table 14 indicate that 10 ml. of alcohol are necessary to completely remove the $SnCl_2$.

The extent of elution of stannous chloride from the Kjolhede Floridin by solutions of fat in benzene was also checked. The quantity of tocopherols in the benzene percolate as obtained from the column was compared with tocopherol quantities found when the benzene percolate was evaporated, the residue taken up in redistilled Skelly B, transferred to another flask, the Skelly evaporated and the residue taken up in benzene as recommended by Kjolhede (36). (Kjolhede indicated

Table 14

Check of Completeness of Removal of Stannous Chloride from the Kjolhede Floridin Columns by Absolute Ethanol and Benzene

No. 5 ml. benzene rinses after abs. ethanol rinse	mcg. Apparent tocopherol in each benzene rinse after alcohol rinse
	One 5 ml. ethanol pre-rinse
1	18.2
2	11.3
3	6.2
4	2.7
5	5.8
	Two 5 ml. ethanol pre-rinses
1	3.5
2	0.0
3	0.0
4	0.0
5	0.0

1. Determined by method (p 46).

stannous chloride was removed by this latter procedure.) There was no significant difference between these two techniques excepting that closer checks were obtained in the former case, presumably because of the fewer manipulations and transfers involved. Therefore it is indicated that it is not a matter of the benzene (or fat in benzene) eluting some of the stannous chloride which causes the apparently higher assay values when benzene is used for the percolation solvent.

(c) Interference of peroxides in the tocopherol determination.

It was postulated that the effect of peroxides upon the tocopherol determination could be checked by determining tocopherol values of fat samples with varying peroxide content, plotting the tocopherol value against the peroxide value and obtaining the true tocopherol content by extrapolating the curve to zero peroxide. The modified Kjolhede Floridin had been shown to remove only part of the peroxide in a fat sample. It was considered that if columns of different lengths were employed with aliquots of a solution of fat known to contain peroxides, eluates with the same tocopherol and varying peroxide content could be obtained. This was done and the tocopherol and peroxide were determined in the first 25 ml., and in the next 10 ml. through the column; the peroxide content of the fat (untreated) was likewise determined. The solvent was benzene. The data are presented in Table 15.

By plotting mols. of tocopherol vs. mols of peroxide a scatter diagram was obtained, indicating the determined tocopherol value of the assay mixture is not a specific function of the peroxide value of the fat (within the range studied).

In a study of the interaction of peroxides with the glacial acetic acid (Devlin and Mattill) reagent, it

Table 15

Effect of Varying Peroxide Content on the Measured Tocopherol Value[1] of Aliquots of the Same Fat Solution (peroxides removed increment-wise by chromatography)

Approx. length of column cm.	g. of Adsorbent	Tocoph. mcg./g. of fat[2] 1st 25 ml.	Next 10 ml.	Tocoph. mols./g. fat in 1st 25 ml.[2]	Perox. (m.e./kg. of fat) 1st 25 ml.	Next 10 ml.
20	7.05	31.8	3.25	.0735	.414	.059
17	5.65	30.4	1.71	.0704	.960	--
16	5.10	32.5	2.16	.0753	.903	.0395
14	4.20	34.8	1.03	.0806	1.08	--
12	3.80	37.0	2.16	.0856	.739	0.00
10	3.20	34.2	3.08	.0791	1.14	--
8	2.70	34.2	1.65	.0791	1.31	.0316
6	2.25	34.8	2.30	.0806	1.69	--
4	1.60	30.4	2.16	.0704	1.83	.0395
2	1.00	30.8	1.78	.0714	2.03	--
Untreated					5.19	

1. Method (p 46).
2. Tocopherol and peroxide values calculated to the basis of fat added.

was found that the percentage transmittance readings obtained had a tendency to increase with whole fat which contained peroxide (Figure 3). Solutions of tocopherol in absolute ethanol were mixed with solutions of methyl oleate containing 60 m.e. of peroxide/kg. and assayed by the Emmerie and Engel procedure to obtain a graphic picture of the reactions occurring among the three components: tocopherol, methyl oleate peroxide and ferrous dipyridyl. These curves follow those obtained with the oxidized fat (Figure 3).

It has not been possible to demonstrate conclusively but it is indicated that there is no appreciable interaction between tocopherol and peroxide in absolute ethanol, but that the action is between the ferrous iron, or ferrous iron-dipyridyl complex, and the peroxide. If this is the case, removal of peroxides at any point prior to the addition of the iron-dipyridyl reagent should prevent their interference. (Daubert (8) has shown that there is no decrease in absorption of tocopherol at 292 mu when it is treated in alcoholic solution with methyl hydroperoxido-oleate. He concluded that this peroxide did not destroy tocopherol.)

The lower two curves in Figure 3 demonstrate the action of peroxidic materials upon the ferrous: dipyridyl complex. The color recedes rapidly with time after addition of methyl oleate, or hydrogen peroxides. The zero-slope lines at 31 and 40 percentage transmittance obtained with tocopherol plus reagent show that the ferrous:dipyridyl color is stable in the absence of peroxidic materials.

b. <u>The effects of the oxidation products of tocopherol</u> It was not possible to account for the difference between the Kjolhede and the modified Kjolhede adsorbent on the basis of peroxide content of the fat. It was decided, therefore, to determine whether the two possible oxidation products of toco-

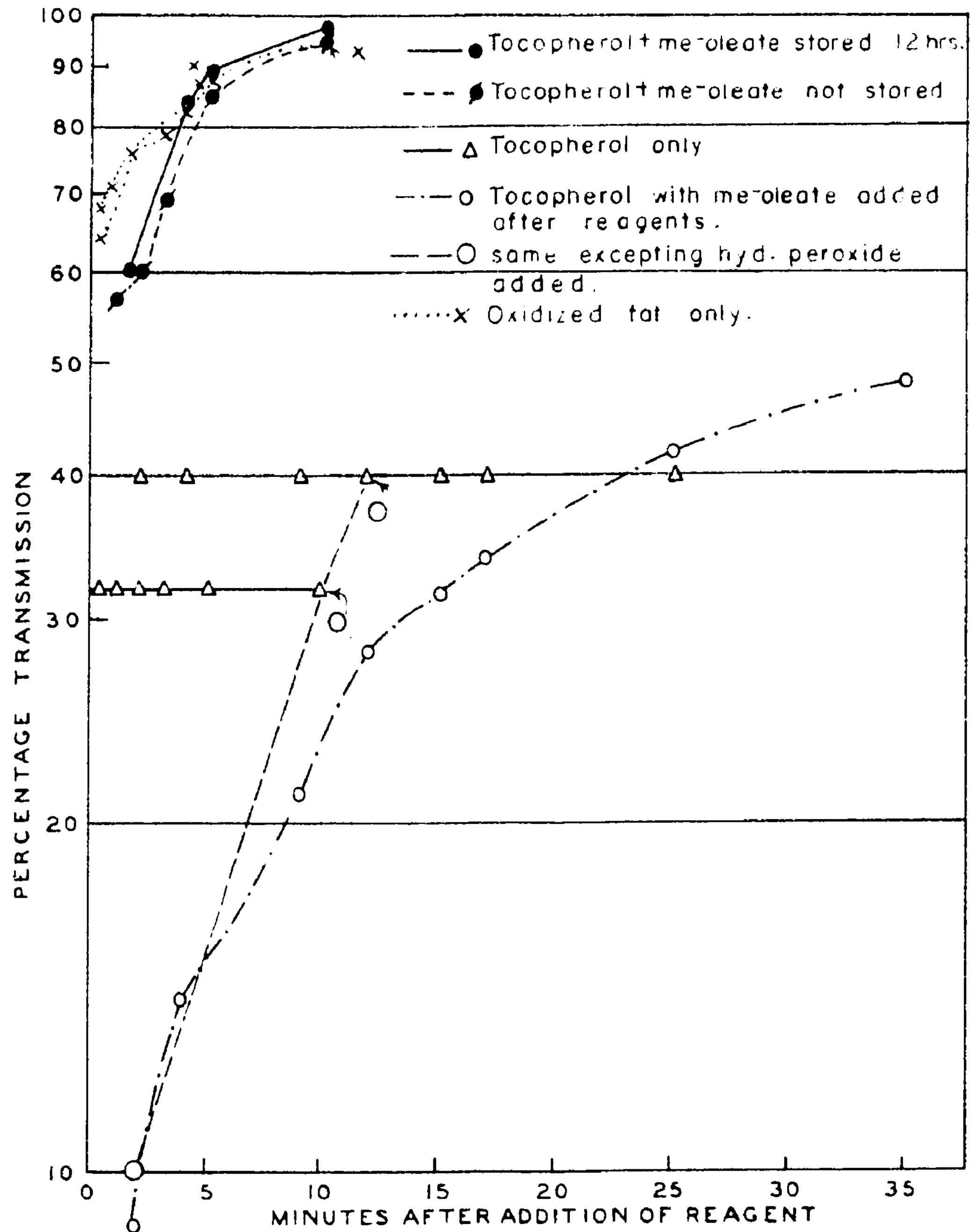

Figure 3. The effect of peroxidic materials, added to solutions of tocopherol prior to and subsequent to the addition of the Devlin and Mattill reagent, upon the stability of the ferrous: dipyridyl color complex.

pherol, the tocopheryl-o- and p-quinones* were responsible for the higher values by the former method.

This was believed necessary because it was demonstrated by a storage study to be reported (paragraph 2, p 145 and Table 41) that the tocopherol assay values on samples treated by the Kjolhede technique did not decrease significantly with storage even after the fat was beyond the acceptable stage by sensory test, while the tocopherol assay values of samples treated with the modified Kjolhede adsorbent decreased about 30 per cent in 20 days. Although peroxide has not been entirely eliminated as the cause for the decrease in the assay values in the latter method, it was indicated (Table 15) that with the method of assay used the tocopherol value is not a specific function of the peroxide content. Therefore, there must have been some other substance which was responsible for the higher values obtained by the Kjolhede method than by the modified Kjolhede method. Since quinoid compounds are easily reduceable it seems highly probably that if quinoid forms are the product of the autoxidation reaction they might be the interfering substances. Two quinoid forms have been demonstrated as oxidation products of tocopherol: the o-quinone and the p-quinone. The former

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*Tocopheryl-o-quinone and tocopheryl-p-quinone will be subsequently referred to as, the o-quinone and the p-quinone, or as the quinoid forms.

have been isolated from oxidizing vegetable oil by Swift, Mann and Fisher (82) and Golumbic (20). Whether or not the latter is produced in autoxidation is not definitely known. A series of experiments were designed to determine the effects, if any, which these quinoid forms have upon the Emmerie and Engel method, especially if non-saponified fat solutions were treated according to Kjolhede prior to assay.

It was shown in these experiments (Table 16) that there was from 0.0 to 11.0 per cent conversion of the o-quinone to reducing substance which gave the Emmerie-Engel test. The percentage conversion calculated was based upon the quantity of tocopherol originally used for preparation of the quinone. The quantities of this derivative used were in the range 13.4 to 134 mcg. per g. The percentage error of the determination due to this quinone varied from -3.3 to 11.2 per cent in one experiment.

In the case of fats which have deteriorated to the extent of destruction of 1/3 of the tocopherol, the value then found would be about 42 mcg./g. and the per cent error of the determination due to this source would be:

$62.5 - 42 = 20$ mcg. of oxidized form.

$20 \times 6.3/100 = 1.26$ mcg. error in assay.

$1.26/42 \times 100 = 3$ per cent error. This is within the limits of error of the determination.

Another experiment indicated 5 per cent error due to the addition of 24.4 mcg. of o-quinone to a fat prior to percolation through the modified adsorbent.

Table 16

The Effect of o-quinone on the Tocopherol Value When Kjolhede Floridin was Employed

Sample	mcg. of o-Quinone added/ g. fat	mcg. Tocoph. found/ g. fat[1]	Difference, control minus sample	% Conversion of o-quinone added	% Error (basis 62.5 mcg.)
1	13.4	60.5	+2	--	-3.3
2	26.8	65.5	-3	11.0	4.7
3	53.5	64.5	-2	3.7	3.3
4	66.9	66.0	-3.5	5.2	5.6
5	134.0	69.5	-7.0	5.2	11.2
6(Control)	0.0	62.5	--	--	--
				Av. 6.3%	

1. Method (p 46).

Some problems were encountered in the preparation of the p-quinoid derivative. Reaction of tocopherol solutions in alcohol with ferric chloride as suggested by Karrer (31) did not produce residues, after removal of iron and alcohol, which showed no reaction with the Emmerie and Engel reagent. Addition of hydrogen peroxide to a solution of tocopherol in alcohol plus a small amount of ferrous iron and alpha,alpha'-dipyridyl caused complete oxidation of the tocopherol as shown by the virtual absence of material which would then react

with the reagent, but the rust red color of this product suggested contamination with products other than the p-quinone.

When this oxidized material was added to solutions of fat and assayed by the Kjolhede method and by the modified Kjolhede method there was an increase in assay value by the first, but not by the second method over the values obtained for the fat to which none was added.

Table 17

Degree of Interference of Large Amounts of p-Quinone Preparation in the Tocopherol Determination when Kjolhede Floridin was Employed[1]

Fat (g.)	Treatment	Quinone added	mcg. of Tocopherol found/g. fat	mcg. Increase due to addition of p-quinone
1.0	Kjolhede	+	128	65.2
1.0	Mod. K.	+	54.6	-0.4
1.0	Kjolhede	0	62.8	--
1.0	Mod. K.	0	55.0	--
0.0	None	+	--	15.1

1. Method (p 46).

There can be no definite check on the quantity of tocopherol actually represented by the aliquots added because of the uncertainty of the yields obtained. For this reason, an attempt was made to oxidize some tocopherol by the ceric sulfate titration method of Schulek and Rozsa (72) (p 30 g.).

There was assurance that all of the tocopherol in the solution was oxidized by this method. When assayed for tocopherol by the Emmerie and Engel method these samples showed no reducing material. But when aliquots of this solution containing 2600 mcg. of p-quinone were added to fat and run through the Kjolhede columns and assayed, a conversion of 923.5 mcg. of this quinone resulted, i.e., a conversion of 35.5 per cent.

Another experiment was conducted in which quantities of p-quinone more nearly in the range one might expect in an oxidized fat were employed. The data from this experiment are presented below.

If for example it were assumed that the assay value of the Kjolhede treated sample, to which no tocopherol quinone had been added, is the correct value, it is found that by adding only 17.8 mcg., an error of 13 per cent is obtained. There is no error in the modified method when the p-quinoid form is added.

It has been shown by these experiments that, if the o- and p-quinoid derivatives of tocopherol are added to fats and the fats are assayed by the Kjolhede procedure, the o-quinoid form shows a slight increase in the assay value while the p-quinoid form shows a very decided increase in the assay value. This latter form may be the interfering substance which causes the differences between the various modifications of the Emmerie and Engel method.

Table 18

Degree of Interference of Quantities of p-Quinone (that approximate those that might be encountered in oxidizing fats) in the Tocopherol Determination on a Good Fat when Kjolhede and Modified Kjolhede Floridin are Employed[1]

Treatment	mcg. of p-Quinone added/g. of fat	mcg. Tocoph./ g. of fat[1]	Difference (sample minus control)	% Conversion of p-quinone added	% Error (basis 65.4 mcg./ g.)
Kjolhede	71.2	103.2	37.8	53.0	57.9
Kjolhede	35.6	93.6	28.2	79.3	43.2
Kjolhede	17.8	74.0	8.6	48.4	13.2
Kjolhede	0.0(control)	65.4	--	--	--
Ave. Kjol.				60.5	
Mod. Kjol.	35.6	57.8	0.0	0.0	0.0
Mod. Kjol.	0.0(control)	57.8	--	--	--

1. Method (p 46).

For verification, the experiment just described was repeated with a fat which was somewhat oxidized at 45° C. storage, to determine whether or not the products of oxidation in the fat changed the results.

Quantities of p-quinone were added to samples of milk fat stored in the 45° C. oven for three months. The tocopherol value of this fat when placed in the oven was 56.0 mcg./g. of fat. The heated fat was no longer acceptable. Table 19 describes the experiment.

This experiment indicates that there was a very large error resulting from the addition of p-quinone to the oxidized fat.

Table 19

Degree of Interference of Quantities of p-Quinone (that approximate those that might be encountered in oxidizing fats) in the Tocopherol Determination[1] on an Oxidized Fat when Kjolhede and Modified Kjolhede Floridin is Employed

Treatment	mcg. of p-Quinone added/g. of fat	mcg. Tocoph. per g. of fat	Difference (sample minus control)	% Conversion (of p-quinone added)	% Error (basis 24.5 mcg. tocoph./g. fat
Kjolhede	0 (control)	24.5	--	--	--
Kjolhede	44.4	38.0	13.5	32.2	55.1
Kjolhede	88.8	51.4	26.9	30.3	109.8
Ave.Kjol.	--	--	--	31.2	--
Mod.Kjol.	0 (control)	7.6	--	--	--
Mod.Kjol.	44.4	5.5	-2.1	Neg.	Neg.
Mod.Kjol.	88.8	4.2	-3.4	Neg.	Neg.

1. Method (p 46).

If the deteriorated tocopherol in an oxidized fat is determined to the extent of 30 per cent by Kjolhede Floridin and not at all by the modified Kjolhede Floridin, the use of the following equation may be in order:

$$\text{VII.}\quad \text{mcg. tocoph./g. orig. milk fat} = \text{mcg. tocoph./g. mod. Kjol., Time T} + \left[\frac{\text{mcg. tocoph./g. Kjol., Time T} - \text{mcg. tocoph./g. mod. Kjol., Time T}}{0.3}\right]$$

The calculation for the above fat would be:

$$7.6 + \frac{24.5 - 7.6}{0.3} = 7.6 + 56.3 = 63.9 \text{ mcg. tocoph./g. contained in orig. milk fat}$$

The fact that the value 63.9 mcg./g. is higher than the assay value of the cold storage sample may mean that some of the tocopherol had already oxidized before the fat was placed in cold storage or had oxidized during storage. On the other hand, if the average percentage conversion of p-quinone, in good and in oxidized fat, is used as the correct figure (average "% conversion" in Tables 18 and 19 equals 48.8%) the equation that would permit an approximate determination of the original tocopherol would be:

$$\text{VIII.}\quad \text{mcg. tocoph./g. orig. milk fat} = \text{mcg. tocoph./g. mod. Kjol., time T} + \frac{\left[\text{mcg. tocoph./g. Kjol., time T} - \text{mcg. tocoph./g. mod. Kjol., time T}\right]}{0.5}$$

and the calculation would be:

$$7.6 + \frac{24.5 - 7.6}{0.5} = 7.6 + 34.6 = 41.4 \text{ mcg. tocoph./g. contained in orig. fat}$$

This calculated value appears to be too low for this fat.

It was considered possible that the tendency for values obtained by the Kjolhede method to decrease to a lesser extent than those obtained by the modified Kjolhede method (as the length of time the fat was stored increased) might possibly be reduced to some mathematical expression (as given above) which could be used to determine to what extent the tocopherol of a fat had been destroyed. Preliminary data, that had been ob-

tained with oxidizing fats indicated that the difference between the two methods was not constant but increased with time. On the assumption that the difference between the two methods resulted from the partial (30 per cent) conversion of the p-quinone to the hydroquinone by the Kjolhede Floridin and not by the modified Kjolhede Floridin, equation VII was used to calculate the original tocopherol values of a fat in two storage studies. These values are presented in Table 20, Column I. These calculated values for original tocopherol content of the fat increase with storage time. It was considered that the factor 0.3 was too small, therefore equation VIII was also employed. The calculated original tocopherol values by this latter equation are relatively constant (Table 20, Column II). By plotting the calculated values, four curves are obtained; the curves for Column II having essentially zero slope beyond ca. 5 days (See Figure 4). This characteristic allows the use of the mean to represent all values and thus provides a rather good method of estimating the original tocopherol content of a given sample in the event that data are available such as are presented in Table 20. The standard error of the mean for Column II equals 6.11 mcg./g. fat for the first storage study and 5.89 for the second; the means are 61.6 and 58.7 for the first and second storage studies respectively. The original tocopherol content of this fat determined by the Kjolhede

Table 20

Calculated "Original" Tocopherol Content of a Fat From the Observed Tocopherol Assay Values of the Stored Sample

Days stored at 45° C.		mcg. Tocopherol/g. of fat Kjolhede	Modified K.	Calculated original Column number (I)[1]	(II)[2]
0	First	51.0	47.0	60.3	55.0
6	storage	59.3	58.0	62.3	60.6
11	study	50.7	39.0	78.0	62.4
13	"	56.4	41.3	91.6	71.5
14	"	56.6	46.7	79.7	66.5
19	"	43.9	35.2	64.2	52.6
20	"	49.9	35.0	84.7	64.8
21	"	49.1	37.6	75.9	60.4
24	"	46.2	29.2	85.8	63.2
25	"	46.0	33.8	80.4	58.2
Averages				76.3	61.6
Standard error of the mean				7.75	6.11
0	Second	53.9	52.2	57.9	55.6
2	storage	56.5	55.1	59.8	57.6
8	study	50.5	48.5	55.1	52.5
16		54.3	49.1	66.4	59.5
22		51.5	43.8	69.5	59.2
28		55.7	42.8	86.0	68.6
35		48.9	40.0	69.7	57.8
Averages				66.3	58.7
Standard error of the mean				7.58	5.89

1. Calculated by formula VII.
2. Calculated by formula VIII.

method on whole fat was 56.0 mcg./g., which value lies within the limits of the standard errors of the means of the calculated original tocopherol content. It is not possible to use the mean to represent the values in Column I because the line does not have zero slope and the standard error has no significance. These comparisons indicate that the first assumption was

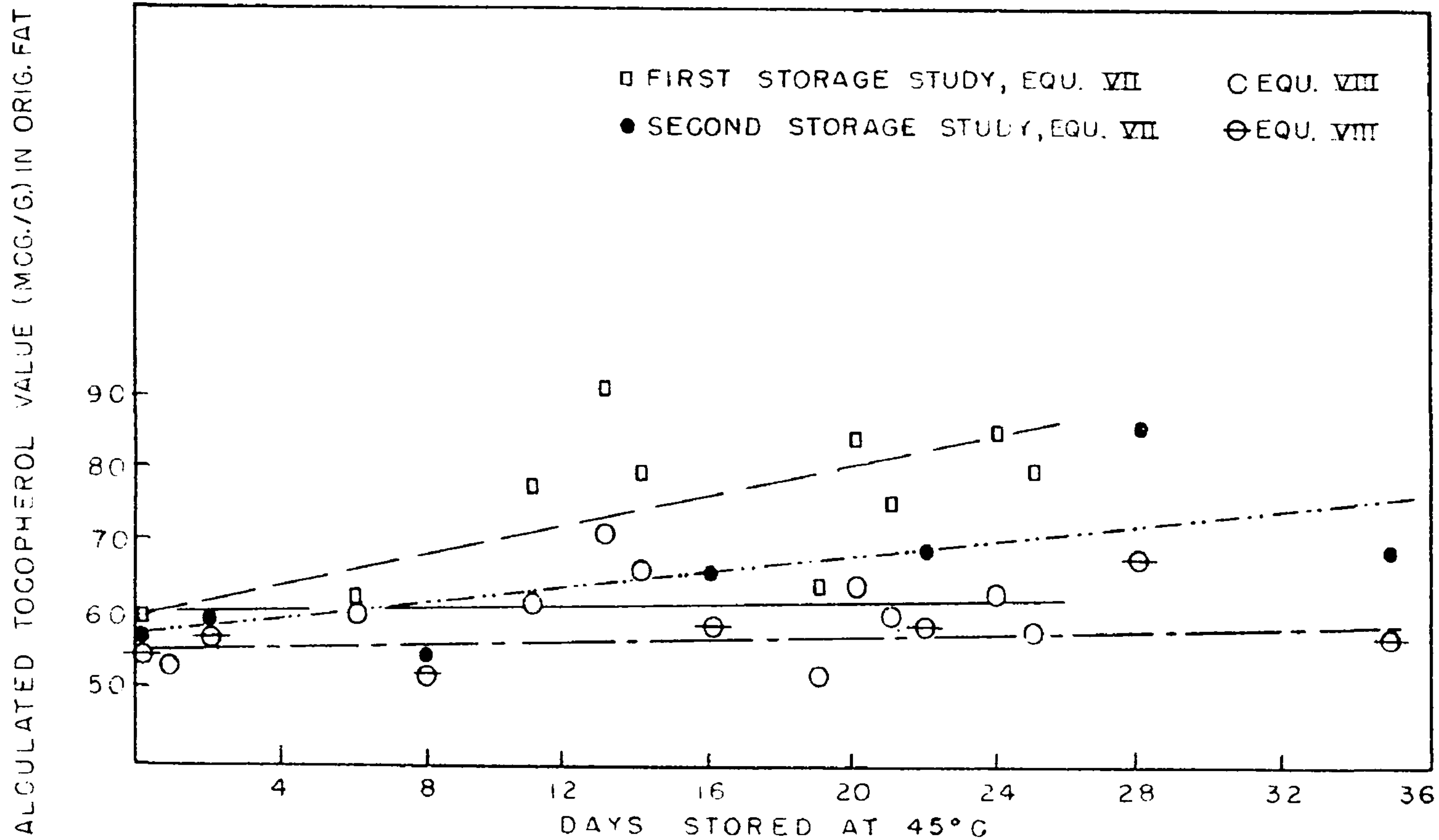

Figure 4. Calculated tocopherol content of the original fat from the tocopherol contents determined for this fat at different storage intervals (45° C.) by the Kjolhede and modified Kjolhede methods.

erroneous (30 per cent conversion of p-quinone) and that the discrepancy between the two methods is better explained by the second expression (50 per cent conversion). It is not intended that these calculations be used to routinely determine the "original" tocopherol content of any given fat sample; it is indicated however that some substance which is reduced by the Kjolhede adsorbent is determined as tocopherol with the Devlin and Mattill reagent.

c. Effect of saponification upon the quinoid forms

This experiment, similar to that with the whole fat (Table 16), was conducted with saponified samples; the tocopherols were determined in the non-saponifiable fraction. The o-quinone was added to fat prior to saponification or in the case of whole fat (used as a control), prior to chromatography. The concentration of added o-quinone was 24.2 mcg. per g. of fat. As indicated in Table 21 saponification was conducted either with or without added pyrogallol. The solutions of the samples obtained by all treatments were divided; one part was run through the Kjolhede adsorbent, the other through the modified Kjolhede adsorbent. Aliquots of these percolates were passed through the second of the two adsorbents. Other aliquots were assayed for tocopherol together with the percolates from the second chromatography. The tocopherol and peroxide values

Table 21

Degree of Interference, Resulting from Addition of o-Quinone to Fat Prior to Saponification, in the Tocopherol Determination by Kjolhede and Modified Kjolhede Methods

Sample	24.2 mcg. o-Quinone added/g. fat	5 ml. of 5% Pyrogallol added	mcg. Tocoph./g. fat				m.e. Perox./kg. fat		
			Thru modified K	Thru Kjolhede	Thru Mod.K then Kjol.	Thru Kjol. then mod.K	Mod.K percolate	Kjol. percolate	Unchromatographed fraction
1 (Sapon)	+	+	40.9	47.4	49.6	35.5	--	.022	.068
2 (Sapon)	0	+	38.8	48.6	48.0	39.4	--	.017	.000
3 (Sapon)	+	0	21.4	32.6	18.7	17.0	--	.000	.00
4 (Sapon)	0	0	21.6	23.4	12.4	15.4	.118	.033	.02
5 Whole fat	+	0	55.4	67.2	60.6	53.6	.128	.082	.43
6 Whole fat	0	0	50.3	53.0	49.9	48.0	--	.049	.45

of the percolates with and without added o-quinone are presented to show the effect of this constituent.

Little significance may be placed upon the values obtained with the non-saponifiable fractions from saponifications not protected with pyrogallol, since there was so much variation between determinations (not only among these data but likewise with other runs).

Peroxide values of the non-saponified fractions (corrected for carotene color) were low (0.00 to 0.068 m.e./kg.). The peroxide value of untreated fat was appreciable (0.45 m.e./kg.). The highest peroxide value of the Kjolhede percolates was 0.08 but those of the modified Kjolhede method were higher (0.118 to 0.128 m.e./kg.). The effect of 24.2 mcg. of added o-quinone is apparent (14 mcg. increase) only in the case of the whole fat which is run through the Kjolhede earth. The sample run through the modified followed by the Kjolhede Floridin shows about 10.7 mcg. increase over the sample to which no o-quinone was added. The sample run through Kjolhede and then modified Kjolhede Floridin showed a much smaller increase (only 5.6 mcg.). The Kjolhede adsorbent apparently reduces o-quinone to hydroquinone to some extent. Saponification appears to destroy the o-quinone since there was no increase in assay value of the non-saponifiable fraction of the samples containing

o-quinone over that for the non-saponifiable fraction of the fat containing no added o-quinone.

The effect of saponification upon the p-quinone was also studied. Five samples were assayed in duplicate simultaneously. To the samples which were saponified, 40 mcg. of tocopherol (oxidized to the p-quinone by $Ce(HSO_4)_4$ titration) were added per gram. To the samples not saponified 66 mcg. per gram were added. The data from the experiment are presented in Table 22. The samples were saponified with 20 ml. of 3.5 N KOH in methanol. When pyrogallol was added, 5 ml. of 5 per cent pyrogallol in methanol was used. The data indicate that there was no increase in assay value as a result of the addition of the p-quinone to the samples which were saponified but as has been shown before, there was an increase when solutions of the whole fat were percolated through the Kjolhede earth though not when they were passed through the modified earth.

It is indicated (Tables 16 to 22) that the Kjolhede earth apparently reduced some of the tocopherol oxidation products and removed or destroyed peroxide from the whole fat, the modified earth either passed these tocopherol oxidation productions through unchanged or retained them on the column. Saponification either with or without pyrogallol added destroyed the quinones and peroxides.

Table 22

Effect of Saponification and of Kjolhede and Modified Kjolhede Floridin on the Degree of Interference, of Added p-Quinone, in the Tocopherol Determination

Sample	5 ml. of 5% Pyrogallol added	mcg. p-Quinone added/g.	Tocopherol mcg./g. of fat Kjol.	Ave.	Mod.Kjol.	Ave.
1 (Sapon)	+	+40	54.0	53.2	52.8	52.7
2 (Sapon)	+	+40	51.5		52.5	
3 (Sapon)	+	0	49.2	47.9	47.4	48.9
4 (Sapon)	+	0	46.6		50.5	
5 (Sapon)	0	+40	34.0	34.0	28.9	29.0
6 (Sapon)	0	+40	--		--	
7 (Sapon)	0	0	33.0	34.5	35.2	36.2
8 (Sapon)	0	0	36.0		37.2	
9 Whole fat	0	0	51.3		53.0	51.6
10 Whole fat	0	+66	68.2		50.3	

3. Studies with a Double Column Method Employing Kjolhede's Floridin and Alumina for Separation of Tocopherols from Glycerides and Interfering Materials

a. Basis of the method

The analysis technique employed activated alumina placed below Kjolhede Floridin in a column. The experiment was based upon the knowledge that the Kjolhede Floridin

produced a percolate in benzene solution which gave virtually a zero test for peroxide (Tables 9, 11, 12, 13, 21) and a tocopherol recovery equivalent to 100 per cent added alpha-tocopherol (subsequently shown in Table 42). It was believed that if percolates from a Kjolhede Floridin column were run through a column of alumina, activated according to Kofler (41), the tocopherols could be separated from peroxides, carotene and vitamin A by the Floridin and from the bulk of the glyceride by the alumina since the tocopherol would be adsorbed by the alumina, while the glyceride would not. Peroxide values were run by the Hills and Thiel (26) method.

b. Comparison of benzene with Skellysolve B as solvents for the chromatographic separation of tocopherols from interfering components of milk fat by the combined Kjolhede Floridin:alumina columns

The first experiment was conducted with 2.5 g. of fat per column dissolved in 5 ml. of the solvent. The alumina used was freshly activated and was cooled in a P_2O_5 desiccator; it was not treated with nitrogen. The second experiment was conducted with 2.5 and with 5 g. of fat per column with the concentration of fat in the solvent being the same. The solutions of fat in Skelly B or benzene were poured on the columns and allowed to percolate through. The same solvent was added in 5 ml. portions to rinse the column until 25 ml. of percolate

were collected. The second solvent used--as noted in Table 23--was then added in 5 ml. portions until 25 ml. of the elute were collected with this solvent. (In the first experiment two or more portions of each solvent were collected.) Aliquots of the percolates and eluates were analyzed, by the method used for the Kjolhede percolates (p 47). Air dry, non-activated alumina, freshly activated air cooled alumina, and nitrogen cooled alumina were used. These data are presented in Tables 23 and 24.

The peroxide value of the untreated fat when analyzed from Skellysolve solution was 0.356 m.e./kg. and from benzene solution was 0.424 m.e./kg. These experiments show that it is necessary to activate the adsorbent just prior to use. There was no advantage of using Skelly B as a solvent for the fat with the air cooled alumina; no comparisons were made with the nitrogen cooled alumina. Higher tocopherol values were obtained with the larger fat samples. Somewhat lower peroxide values and higher tocopherol values were obtained on the eluates from columns which were prepared by cooling the adsorbent with nitrogen. It is also considered to be theoretically correct to saturate the hot adsorbent with some inert gas in order to prevent surface reactions with oxygen. The stream of nitrogen used was dried by passage through a Dririte tube to prevent deactivation of the adsorbent by water vapor. It was not possible,

however, to prevent peroxide formation by the nitrogen treatment of the adsorbent, possibly because residual oxygen was not removed from the nitrogen.

Table 23

Tocopherol Values (mcg./g. of fat) of Skelly B Percolate and of Benzene and Absolute Alcohol Eluates of Fat (Iowa, August butter) Solutions Percolated Through the Kjolhede Floridin: Alumina Columns when the Alumina was Cooled in a P_2O_5 Desiccator after Activation

Initial solvent	g. fat per column	Tocopherol values of[1] fraction on basis of mcg./g. of fat in sample[2]							
		Percolate		Eluates					
		skelly		benzene				alcohol	
		1st	2nd	1st	2nd	3rd	4th	1st	2nd
		25 ml.	25 ml.	25 ml.	25 ml.	25 ml.	10 ml.	25 ml.	10 ml.
Skelly B	2.5	0.0	0.0	0.0	0.0	0.0	0.0	39.2	33.2
Benzene	2.5	--	--	7.33	9.18	--	--	22.8	--

1. Determined by method (p 46).
2. No assay was made where a dash appears on the table.

c. <u>Determination of the fate of the glyceride</u>

The eluates and percolates from Kjolhede Floridin: alumina columns (p 34) were evaporated to dryness on the steam bath and the weight of the non-volatile residue was determined in order that the fate of the bulk of the glyceride might be ascertained. These results show that the major portion of the glyceride was in the first 25 ml. of percolate and washings with Skelly or benzene (Table 25).

Table 24

Tocopherol and Peroxide Values of Skelly B, Benzene and Absolute Alcohol Fractions of the Chromatogram of Fat (Iowa, August butter) Solutions Percolated Through Kjolhede Floridin:Alumina Columns, in which the Alumina was Variously Treated

			Tocopherol value of fraction on basis of mcg./g. of fat in sample[2]			
Adsorbent treatment	Initial solvent	g. Fat per column	Skelly 1st 25 ml.	Benzene 1st 25 ml.	Alcohol 1st 25 ml.	m.e. Perox. per kg. fat alcohol eluate
Double column: air dry, not activated alumina	Skelly	2.5	58.8	--	0.0	Neg.
	Skelly	5.0	--	--	4.0	--
	benzene	2.5	--	50.6	3.6	Neg.
	benzene	5.0	--	--	2.3	--
Activated alumina, cooled in air on column	Skelly	2.5	0.0	--	25.3	1.98
	Skelly	5.0	0.0	--	33.3	.88
	benzene	2.5	--	0.0	27.5	.832
	benzene	5.0	--	0.0	37.9	.909
N_2 Treated activated alumina	benzene	5.0	--	--	50.3	.411
	benzene	5.0	--	--	45.5	.476
	benzene	5.0	--	--	39.5	.234
Kjolhede Floridin only	benzene	5.0	--	48.1	--	--

1. Determined by method (p 46).
2. No assay was made where a dash appears in the table.

Table 25

Weights of Non-Volatile Materials Found in Chromatographic Fractions when Solutions of Iowa August Fat were Percolated Through Columns of Kjolhede Floridin:Alumina[1]

g. of Fat added to column	Solvent fraction	g. of Non-volatile in fraction	g. of Fat added to column	Solvent fraction	g. of non-volatile in fraction
2.5	1. 25 ml. Skelly	2.17	2.5	1. 25 ml. Skelly	2.50
	2. 25 ml. Skelly	.052			
	3. 25 ml. benzene	.120	2.5	1. 25 ml. Skelly	2.03
	4. 25 ml. benzene	.022			
	5. 10 ml. benzene	--	5.0	1. 25 ml. Skelly	4.50
	6. 25 ml. alcohol	.075		2. 25 ml. Skelly	0.10
	7. 10 ml. alcohol	--		Total	4.60
	Total	2.439			
			5.0	1. 25 ml. Skelly	4.25
2.5	1. 25 ml. Skelly	2.055		2. 25 ml. Skelly	.095
	2. 25 ml. Skelly	0.142		Total	4.345
	3. 25 ml. benzene	0.208			
	4. 25 ml. alcohol	0.078	2.5	1. 25 ml. benzene	2.50
	Total	2.483			
			5.0	1. 25 ml. benzene	4.50
2.5	1. 25 ml. benzene	2.20		2. 25 ml. benzene	.075
	2. 5 ml. benzene	0.10		Total	4.575
	3. 5 ml. benzene	0.01			
	4. 5 ml. alcohol	0.01	5.0	1. 25 ml. benzene	4.55
	5. 5 ml. alcohol	0.01		2. 25 ml. benzene	.05
	Total	2.33		Total	4.60

1. Prepared according to method (p 34).

It was shown, Table 25, that when the columns were washed with benzene subsequent to the Skelly wash, there was more non-volatile material in the benzene wash than in the prior Skelly wash. This indicated that there was some material elutable with benzene not elutable with Skelly, but this material was not associated with tocopherol as shown by the fact that benzene percolates from Kjolhede Floridin:activated alumina columns were tocopherol free. Generally less than 100 per cent of the fat introduced to the column was recovered because the Kjolhede Floridin part of the column apparently held the pigment fraction and possibly some fraction of the glyceride.

d. Analysis of the non-saponifiable fractions of fats

Another experiment was conducted on the non-saponifiable fraction from a sample of the same fat used above. The saponification was conducted in the presence of pyrogallol with 20 ml. of 3.5 N KOH in methanol per 10 g. of fat. Ten ml. aliquots of benzene solution of the non-saponifiable fractions equivalent to 4 g. of fat were passed through the Kjolhede:alumina column and over the Kjolhede column alone. Fifteen ml. of benzene were added to develop the chromatogram, the Kjolhede Floridin was removed from the double columns and the alumina portion was eluted with 25 ml. of alcohol. Preparation of the columns was according to the method previously described (p 34).

Table 26

Peroxide and Tocopherol Values[1] of Benzene Percolates and Alcohol Eluates from Kjolhede Floridin and Kjolhede Floridin:Alumina Chromatograms of the Non-Saponifiable Fractions[2] from Samples of August, Iowa, Fat

	Tocopherol (mcg./g. of fat)			Peroxide (m.e./kg. of fat)		
	Kjol. Floridin	Kjol. Floridin: alumina		Kjol. Floridin	Kjol. Floridin: alumina	
Sample number	Benz. perc.	Benz. perc.	Alc. eluate	Benz. perc.	Benz. perc.	Alc. eluate
1	49.1	4.71	39.5	0.00	0.050	0.00
2	39.1	0.00	39.5		0.053	0.00
3	44.2	0.00	41.5		0.030	0.00
4	49.1	0.00	41.5		0.00	0.047
Composite of four samples equiv. to 8 g. fat	--	--	46.0	--	--	--
Average of first 4 samples	45.4		40.5			

1. Method (p 46).
2. Method (p 50).

The composite sample was run to determine whether the capacity of the column for tocopherol was being exceeded by the 4 g. samples being used. The results (Table 26) show that the tocopherol value was higher on the per gram basis when 8 g., rather than 4 g., of fat were employed indicating that the capacity of the column was not exceeded by 4 g. This same result is shown

in Table 24. The essentially zero peroxide values indicate that it must have been the action of alumina on glyceride which produced peroxide since in the absence of glyceride no peroxide was found. The average Kjolhede Floridin assay value was 5 mcg./g. higher than the assay of the alcohol eluate from alumina.

e. Analysis of whole fat

The flowing chromatograms from Kjolhede Floridin: alumina columns were studied. Fifty g. of fat were weighed into a 200 ml. volumetric flask, the fat was dissolved in benzene and made to 200 ml. volume. Columns were prepared in the usual manner (p 34); 20 ml. of the solution of fat were added to columns followed by benzene in 5 ml. portions to a total volume of 50 ml. The top Floridin layer was removed by suction. Elution was accomplished with five 10 ml. portions of 10 per cent absolute ethanol in benzene. The fractions were collected separately and each was analyzed for tocopherol. The reagent proportions were 11 ml. of 1:9 ethanol:benzene eluate, 12 ml. of absolute ethanol and 2 ml. of Devlin and Mattill glacial acetic acid reagent (total volume 25 ml.). Peroxide values were determined on the eluates from one column in order to determine in which fractions the peroxides were to be found. (The solvent capacity of the alumina column was 8 ml.)

Benzene:absolute ethanol (90:10) was used because it was believed that some alcohol-insoluble material might crystallize in the column, retard flow and possibly prevent elution. The analytical data for these eluates are presented in Table 27. One aliquot was analyzed by the usual Kjolhede method as a control.

It is probable that 4 or more separate analyses would not check more closely than the data of Table 27; however, the tendency appears to be to obtain lower values by the double column method than by the Kjolhede method. The higher value by the latter method may result not from tocopherol, but from a more highly adsorbable material which elutes from the alumina rather slowly. This analysis of the flowing chromatogram (Table 27) showed that there were still materials which reacted with the Emmerie and Engel reagent being removed from the columns in the 2nd 10 ml. portion of alcohol, while the bulk of the tocopherol was removed in the 1st 10 ml. of 10 per cent ethanol in benzene. The possibility exists that these materials were tocopherols other than alpha-.

The major portion of the tocopherol was in all cases eluted with 30 ml. of the 10 per cent ethanol in benzene and 100 per cent ethanol in only two cases eluted more than the previous 10 ml. of 10 per cent ethanol in benzene. Comparison of these results with those of the non-saponifiable fraction

Table 27

Tocopherol and Peroxide Content of Fractions of the Chromatograms of Whole Fat from Kjolhede Floridin:Alumina Columns[1]

Sample	Benzene 50 ml.	10% Ethanol in benzene (ml.) 1st 10	2nd 10	3rd 10	4th 10	5th 10	Ethanol (ml.) 1st 10	2nd 10	Total
	Fraction (tocopherol values in mcg./g. of fat)								
1	0.0	23.8[2]		5.8[2]		2.5	2.67[2]		34.8
2	0.0	33.5		3.7		0.0	0.0		37.2
3	0.0	24.1		2.99	1.37	--	3.86		32.2
4	0.0	18.35	14.01	6.0	2.7	2.5	1.9	--	45.5
5	0.0	21.1		6.0	0.3	--	3.8		31.2
								Av.	36.2
Kjolhede	46.4								46.4
Perox. m.e./kg. of fat									
Sample 5		0.260		.0379	.0172	--	.0206		

1. Prepared by method (p 34).
2. The composites of the two fractions underscored were analyzed.

leads one to believe that the replicability and the absence of peroxide in the percolates from the non-saponifiable fraction would indicate that it is better to saponify fat that is to be treated by the double column method.

f. Recoveries of added tocopherols

(1) Analysis of whole fat plus added alpha- and gamma-tocopherols. Another experiment was run in the same manner on the same solution of fat with alpha-tocopherol added to one sample and gamma-tocopherol added to another. The third sample was run as a control. The solutions of alpha- and gamma-tocopherol were assayed without treatment to establish the concentration of the pure tocopherol. The results of this experiment are given in Table 28.

It is obvious from these results that the recoveries of gamma-tocopherol were low by this method; this may result from interference by peroxides. A pure solution containing 100 mcg. of Merck synthetic gamma-tocopherol in 20 ml. of benzene was run through an alumina column; 150 ml. of benzene: alcohol (90:10) and 50 ml. of alcohol failed to elute all of the tocopherol as evidenced by a strong Emmerie and Engel test on the eluates. These data indicated that the highly activated alumina used adsorbed the gamma-tocopherol, very strongly in the absence of fat. Columns over which pure solutions of alpha- or gamma-tocopherol had been run take on

Table 28

Analysis for Tocopherol[1] of Whole Fat Plus Added Alpha- and Gamma-Tocopherols Treated with Kjolhede Floridin:Alumina Columns

g. of fat	Tocopherol added	Tocoph. value of fract. (mcg./g. fat added): Percolate, Benzene 50 ml.	Eluates: 10% Ethanol in benz. 25 ml.	Eluates: 10% Ethanol in benz. 10 ml.	Eluates: 10% Ethanol in benz. 10 ml.	Eluates: Ethanol 10 ml.	Total	Total % recovery
5	0	0.0	35.0	5.6	2.5	3.5	46.6	
5	42.2 mcg. of alpha/g.	0.0	57.4	9.6	4.3	4.9	76.2	
5	112.0 mcg. of gamma/g.	0.0	73.0	6.9	3.4	4.0	87.3	
Diff. due to added alpha			12.4	4.0	1.8	1.4	29.6	70.2
Diff. due to added gamma			38.0	1.3	0.9	0.4	40.7	36.4

1. Method (p 51).

a yellow color which was only partly elutable with alcohol. This yellow color was identical in appearance and behavior with the yellow band appearing on the columns over which fat was passed. Since low recoveries were obtained with these columns, because peroxide was formed on the column, and since pure tocopherol solutions turned yellow on the columns, it was indicated that tocoquinone was formed. It was probably the p-quinone since the ortho- form is not yellow, but red.

(2) Reaction rates of gamma-tocopherol with the Devlin and Mattill reagent. For solutions containing gamma-tocopherol it was found that the percentage transmittance continued to decrease with time after addition of the reagent up to 15 minutes. The values reported in Table 28 were calculated, however, from the one minute reading. A solution containing gamma-tocopherol with no fat present gave readings, with the Devlin and Mattill reagent, which decreased to fifteen minutes:

Time after addition of reagent	Per cent transmittance
1 min.	50.0
2 min.	33.0
5 min.	22.0
10 min.	17.0
15 min.	15.2
20 min.	15.2

However, in the presence of peroxide there was an opposing reaction which apparently took place with the result that the

minimum per cent transmittance value was reached at some time sooner than fifteen minutes; following this the values might increase or remain constant. The per cent transmittance values for a solution containing gamma-tocopherol and fat eluted from a column by 10 per cent ethanol in benzene reach a minimum in five minutes; then increase as shown below:

Time after addition of reagent	Per cent transmission
1 min.	74.0
5 min.	61.2
10 min.	61.6
20 min.	62.0

It is believed that the value most nearly representing the true concentration was the 5 minute reading in this case, although the 10 minute reading was little different. Further information on the reaction rate of gamma-tocopherol was obtained in the following experiment and is presented in Table 29. See also p 118, 3 and 4.

g. Relative adsorption:elution characteristics of alpha- and gamma-tocopherols

Another experiment was conducted for the purpose of studying the relative adsorption characteristics of alpha- and gamma-tocopherol on the Floridin + alumina column that had been used. For this experiment 50 g. of a sample of the same fat that was used in the last experiment were weighed into a 200 ml. volumetric flask, and the volume made to 200 ml.

with benzene. Twenty ml. aliquots of this solution were used. Solutions of alpha- and gamma-tocopherol were added to some of the aliquots prior to running them over the columns. The data from this experiment are presented in Table 29. These data indicate that:

1. Both gamma- and alpha-tocopherols were adsorbed by the alumina used.
2. Both tocopherols were eluted with 10 per cent alcohol in benzene, alpha- to the extent of about 84 per cent and gamma- to the extent of about 90 per cent (calculated on the basis of the 10 minute reading using the alpha-tocopherol standard curve).
3. The increase in tocopherol assay value with time after addition of reagent was essentially zero for fat alone, for fat plus alpha-tocopherol and for alpha-tocopherol alone, but for fat plus gamma-tocopherol and for gamma-tocopherol alone the increase in tocopherol assay value with time was appreciable.
4. This apparent increase in tocopherol content was 30 per cent of the value calculated from the 10 minute reading. These factors indicate that the tocopherol contained in this fat was not gamma-

tocopherol, but a substance with the same adsorption:elution characteristics as alpha-tocopherol.

h. Effect of the tocopherol oxidation products

The results of the last experiment, in which the chromatography of gamma- and alpha-tocopherol was studied, definitely indicated that it would be possible to show the presence or absence of gamma-tocopherol, but possible interference of quinone was suggested. Therefore a series of solutions containing the o- and p-quinones (p 29), alpha-tocopherol and gamma-tocopherol were run through the Kjolhede + Floridin: alumina columns, and the flowing chromatograms analyzed. Table 30 presents the results calculated from the alpha-tocopherol standard regression equation (p 44).

This experiment was designed to find out whether the chromatograms of solutions of the quinones from the Kjolhede Floridin:alumina system were the same as that of the alpha- or of the gamma-tocopherol used in the last experiment. It was found in this experiment that about 5 per cent of the p-quinone reacted as tocopherol after passage over both Kjolhede Floridin and alumina. Untreated p-quinone was active to the extent of 1.5 per cent as tocopherol; untreated o-quinone was not active as tocopherol. There was no apparent tocopherol in the percolate from alpha- or gamma-tocopherol

Table 29

A Study of the Adsorption:Elution Characteristics of
Benzene Solution and Eluted with Alcohol; with

Fraction	50 ml. Benz. perc.		1st 10 ml. 10% Alc.[1] eluate			2nd 10 ml. 10% Alc. eluate			3rd 10 ml. 10% Alc. eluate			10 Al
Sample	1 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min	1 min
5 g. Fat	0.0	0.0	15.4	-	15.7	13.3	13.3	-	3.7	3.7	-	7.1
5 g. Fat	0.0	0.0	19.1	-	19.7	11.3	11.3	-	2.8	3.2	-	5.7
5 g. Fat	-	-	18.8	-	19.7	9.7	9.7	-	3.1	3.1	-	4.9
5 g. Fat plus 42.2 mcg. alpha-tocoph. per g.	0.0	0.0	36.4	-	36.5	21.2	21.2	-	3.7	4.1	-	9.1
" "	0.0	0.0	27.5	-	28.0	25.9	26.0	-	3.7	3.7	-	8.5
" "	-	-	48.4	-	48.4	15.0	15.2	-	3.7	3.7	-	8.9
5 g. Fat plus 56.0 mcg. gamma-tocoph. per g.	0.0	0.0	25.7	35.6	35.8	28.5	39.6	41.5	3.7	-	4.9	6.3
" "	0.0	0.0	43.7	54.5	62.1	14.8	21.4	22.2	7.0	9.1	9.7	1.9
" "	-	-	33.6	47.1	51.0	17.9	26.6	27.4	4.9	-	9.7	2.0
No fat 280 mcg. gamma-tocoph. only	0.0	0.0	11.9	31.2	31.4	9.7	20.4	22.0	2.4	-	6.3	1.9
No fat 211 mcg. alpha-tocoph. only	0.0	0.0	15.1	15.3	15.3	19.7	-	19.8	6.3	6.3	-	1.1

Tocopherol assay values (mcg. per gram) of the fracti

1. 10% Alc. = absolute ethanol 10% by volume in benzene.
2. Calculated from the alpha-tocopherol regression equation p. 44 analysis by the met
3. Calculated from the alpha-tocopherol regression equation using 10 min. % T. readin

NOTE - In the cases where the 5 minute reading was not significantly different from t calculating totals the 5 minute reading was used in lieu of the 10 minute reading.

Table 29

acteristics of alpha- and gamma-Tocopherols Percolated in
Alcohol; with the Kjolhede Floridin:Alumina Columns

of the fractions[2]								Difference 10 min-1 min totals	Per cent recov. of added tocoph.	Per cent of total eluted w/10% alc.
l. 10% uate	10 ml. 50% Alc. eluate			10 ml. 100% Alc. eluate		Totals				
10 min	1 min	5 min	10 min	1 min	5 min	1 min	10 min			
-	7.1	7.1	-	3.0	9.9	42.5	41.7	-0.8	-	78.0
-	5.7	5.7	-	3.0	2.0	41.9	41.9	-0.0	-	79.2
-	4.9	4.9	-	3.7	3.0	40.2	40.4	-0.2	-	78.6
-	9.1	9.1	-	3.2	-	73.6	74.1	-0.5	77.4	83.3
-	8.5	8.5	-	3.0	2.5	68.6	68.7	-0.1	65.3	83.2
-	8.9	8.9	-	3.3	-	79.3	79.5	-0.2	91.5	84.6
4.9	6.3	9.7	9.7	3.7	3.0	67.0	94.9	+27.9	95.8[3]	86.6
9.7	1.9	2.5	2.5	5.2	3.9	72.6	100.4	+27.8	105.6[3]	93.7
9.7	2.0	-	1.9	3.7	3.0	62.1	93.0	+30.9	92.4	94.7
6.3	1.9	-	1.9	3.1	2.7	29.0	64.3	+35.3	115.0[3]	92.8
-	1.1	-	1.1	1.9	1.1	44.1	43.6	-0.5	103.4	93.2

sis by the method p. 51.
n. % T. reading p. 44.
fferent from the 1 minute reading there was no 10 minute reading made and in
ading.

solutions or from an o-quinone solution. The alcohol eluate of p-quinone from alumina contained about 2 per cent of substances that reacted as tocopherol calculated on the basis of 400 mcg. of added p-quinone, but an alcohol eluate of o-quinone did not contain tocopherol. The alpha- and gamma-tocopherols were eluted to the extent of 82 per cent and 100.5 per cent (gamma-tocopherol calculated from 10 minute reading). This assay showed about 1.5 per cent of the p-quinone was active as tocopherol.

We may conclude from this experiment that the differences in the characteristics of the reactions of alpha- and gamma-tocopherols are not due to the contamination of one or the other with quinone derivatives, because neither o- nor p-quinone reacted to an appreciable extent with the tocopherol reagent.

4. Study of the mild hydrogenation technique for removal of interfering materials

The Quaife and Biehler (61) method employs mild hydrogenation to decolorize the plant pigments and possibly inactivates other interfering substances. Quaife (59) modified the Quaife and Biehler method by using cyclohexane:ethanol as the hydrogenation solvent. This latter modification (p 48) was studied as a possible means of assay. The assay values obtained, compared with values by other methods, the recovery of added

Table 30

Analysis of o- and p-Quinones and Tocopherols Treated with the Kjolhede Floridin:Alumina Columns in the Same Manner as Fat Solutions are Treated

Sample	Tocopherol content in micrograms[1]								% Recovered as tocopherol			
	25 ml. Benz. percolate			25% ml. 10% Alc. eluate[2]			10 ml. 50% Alc. eluate[3]		Benz. percolate		10% Alc. eluate	
	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	1 min	10 min	1 min	10 min
400 mcg. of p-Quin.	13.9	19.0	19.0	5.34	8.35	8.90	7.05	7.05	3.5	4.8	1.3	2.2
400 mcg. of o-Quin.	0.0	0.0	0.0	0.0	1.71	1.71	7.00	7.00	0.0	0.0	0.0	0.4
560 mcg. of gamma-Tocoph.	0.0	0.0	0.0	287.0	47.2	557.5	2.33	2.33	0.0	0.0	51.2	101.0
211 mcg. of alpha-Tocoph.	0.00	0.0	0.0	173.3		175.0	13.70	13.70	0.0	0.0	82.1	83.0
	Untreated benz. solution											
400 mcg. p-Quin.	2.40	4.79	6.22						0.6	1.5		

1. Calculated from alpha-tocopherol standard regression (p 44) Method (p 51).
2. 10% alc. = 10% solution of absolute ethanol by volume in benzene.
3. 50% alc. = 50% solution of absolute ethanol by volume in benzene.

alpha-tocopherol, the interference of vitamin A and carotene, and the effect of the oxidation products of tocopherol were all determined. Iodine values (by the Wij's method) of samples of hydrogenated milk fat were found to be zero.

a. Comparison of the Quaife and Harris and the Devlin and Mattill Color development reagents

The reagents (Quaife and Harris (63)) used in the Quaife and Biehler (61) procedure for color development in the hydrogenated solutions are different from the reagent used throughout the rest of this study. The Quaife and Harris reagents consist of an absolute alcohol solution of ferric chloride and an absolute alcohol solution of alpha,alpha'-dipyridyl. The Devlin and Mattill (9) reagent contains both of these constituents in acetic acid solutions. Calculation of the concentrations of ferric chloride and alpha,alpha'-dipyridyl which are present in the assay solution read in the spectrophotometer shows that the concentration of ferric chloride is 0.05 per cent in the Quaife and Biehler assay as compared with 0.04 per cent in the Devlin and Mattill assay. The color is developed in the Quaife modification (59) in an 8 ml. aliquot of a solution of the hydrogenated fat in 1:1 cyclohexane:ethanol solution to which are added 1 ml. of each of the two alcohol solutions of the reagents. In the Devlin and Mattill method, color development is in a 10:13:2

Table 31

Comparison of Tocopherol Assay Values by the Quaife and Biehler and the Devlin and Mattill Reagents on Variously Treated Fat Samples

Description of solution assayed	Tocoph. added mcg./g. of fat	Tocoph. found (mcg./g. of fat) Quaife and Biehler reagent	Tocoph. found (mcg./g. of fat) Devlin and Mattill reagent	% Recov. of added tocoph. Quaife and Biehler reagent	% Recov. of added tocoph. Devlin and Mattill reagent
Hydrogenated fat solution		49.4	47.7		
		74.2	52.7		
		56.0	56.1		
		63.0	61.7		
Hydrogenated fat solution		73.4	68.8		
		69.7	68.8		
		69.7			
Non-saponifiables[1] not decolorized	16.24	57.4	61.6	113	117
	32.48	78.2	80.5	121	114
	48.74	93.8	101.1	112	120
	64.98	107.8	111.1	106	106
Non-saponifiables through Kjolhede Floridin	32.48	90.3	91.2	113	114
	48.74	109.1	105.7	114	106
	64.98	123.1	127.7	107	106
	0.0	54.6	54.0	---	---
	0.0	52.5	54.0	---	---
Whole fat through Kjolhede Floridin	20.31	74.4	81.8	97.5	104
	60.93	121.0	124.3	109	104
	81.23	144.2	145.8	110	105
	0.00	54.6	60.8		
Whole fat untreated[1]	0.00	46.5	47.3		
Tocopherol solutions only	162.5		179.6		110.6
	162.5		179.6		110.6
	162.5		175.6		108.1
	162.5		178.5		109.9
			Ave. 178.3		Ave. 109.8
	81.23	97.3		119.6	
	81.23	90.4		111.2	
	81.23	91.6		112.6	
	81.23	91.6		112.6	
		Ave. 92.7		Ave. 114	

1. Corrected for blank color by the method of Quaife, Scrimshaw and Lowry (66).

benzene:ethanol:acetic acid solution. These two color development reagents were compared to determine whether or not differences, that might exist between the two methods, were the result of the type of solvent mixture employed in preparing the color development reagents. Table 31 presents a comparison of the results obtained with the two reagents. Whole fat, whole fat percolated through Kjolhede adsorbent, non-saponifiable fractions, non-saponifiable fractions percolated through Kjolhede adsorbent, and hydrogenated fat solutions were employed. These experiments show that there was good agreement between the assay values of a given solution containing tocopherol by the two reagents used. Generally, results by Quaife and Biehler reagent were somewhat more erratic and possibly slightly higher on the average, but not significantly so. The differences found between the Quaife and Biehler method and the modified Kjolhede method do not result from the type of reagent.

b. Recovery of alpha-Tocopherol by the Method

Table 32 illustrates the results of some recovery experiments with the Quaife and Biehler procedure. Aliquots of a solution of alpha-tocopherol in cyclohexane-ethanol were added to solutions of fat in the same solvent prior to hydrogenation. The recoveries of alpha-tocopherol added to milk fat were erratic with this method. The recoveries of alpha-

tocopherol from solutions containing no fat were good, however.

c. Comparison of the Quaife and Biehler and the Modified Kjolhede Methods

Since the recoveries of added tocopherol were somewhat erratic in the presence of fat and the tocopherol values for the fat itself were high compared to the values usually obtained by the modified Kjolhede method, these methods were compared on a sample of whole fat and the non-saponifiable fractions of this fat. When the non-saponifiable fraction was used, an aliquot of the benzene solution was evaporated; the non-volatile residue was dissolved in cyclohexane:ethanol and was hydrogenated as usual. This study (Table 33) showed that the hydrogenation technique always yielded higher values for whole fat, regardless of the age or source of the samples used, but that with the non-saponifiable fraction the two methods give essentially the same value. The fact that assay of the non-saponifiables by the two methods gave essentially the same results, indicates that there is some constituent in milk fat which is removed by saponification but which reacts as tocopherol when hydrogenated. The quinones were studied as regards the effect of hydrogenation upon their reaction as tocopherol with the Quaife and Biehler reagents (p 132, sec. e).

Table 32

Recovery of alpha-Tocopherol Added to Fats Prior to Hydrogenation and Assay by the Quaife and Biehler Method

Tocopherol value (mcg./g. of fat) Individuals	Tocopherol value (mcg./g. of fat) Average	Tocopherol added	(mcg./g. fat) Toco-coph. value with added tocoph.	% Re-covery	Description of fat	m.e. Perox./kg. of orig. fat
90.6		78.5	198.5	133.5	Mississippi	
84.4		78.5	190.6	123.3	fat stored	
100.0	93.7	162.6	240.5	90.3	under ni-	2.53
100.0		162.6	245.2	93.2	trogen 4	
90.6		157.0	324.8	148.2	years	
No fat		83.7	86.0	102.8	"	
No fat		83.7	83.0	99.2	"	
94.4	94.4	157.9	245.2	95.6	"	
94.4		157.9	222.8	81.4	"	
53.1	51.9	109.4	183.5	120.0	Fresh August	
50.6		109.5	166.6	104.5	fat, Iowa	0.00
No fat		109.4	108.7	99.1		
No fat		109.4	118.0	107.8		

Table 33

Tocopherol Values of Whole Fat and of Non-Saponifiable Fractions of this Fat by Quaife and Biehler and the Modified Kjolhede Method

No.	Description of fat Source and storage	m.e. Perox./ kg. of orig. fat	Tocopherol values (mcg./g. of fat) Quaife and Biehler	Modified Kjolhede
I	Specially prepared Krafteen stored room temperature 3 weeks. Household refrigerator 8 months. (whole fat)	1.1	52.5	39.0
			56.3	40.1
			58.2	41.8
			60.0	39.6
			71.3	41.5
			--	39.6
II	August, Iowa fat 1 week old in household refrigerator (whole fat)		73.4	25.7
			73.1	15.4
			91.9	28.1
			95.7	17.7
			41.3	18.3
			108.8	24.5
III	June, Iowa fat 3 years old stored under N_2 at -10 to -15° F. (whole fat)	1.5	47.0	Less than 4
			39.0	"
			47.0	"
			47.0	"
			45.0	"
			47.0	"
			51.0	"
IV	Composite sample from 3 cows production date 7-29-48 Iowa fat. Two weeks in refrigerator (whole fat)	0.120	103.1	35.6
			93.8	35.6
			91.9	35.8
			93.8	35.8
				35.6
Va	Same sample 3 mons. in refrig. (whole fat)	0.232	81.8	27.5
			63.0	25.1
VIa	Oct. Iowa fat 1 week in refrig. (whole fat)	0.00	75.4	48.3
			--	47.6

Table 33 (cont'd)

No.	Description of fat Source and storage	m.e. Perox./ kg. of orig. fat	Tocopherol values (mcg./g. of fat) Quaife and Biehler	Modified Kjolhede
	Assay of non-saponifiables			
Vb	Non-saponifiable fraction from samp. #V	--	28.3 26.8	17.6 17.6
VIb	Non-saponifiable fraction from sample #VI	--	24.0 21.2[1] 22.3[1]	30.1 31.2 22.5 23.5

1. Run through modified Kjolhede earth prior to hydrogenation.

d. The Effectiveness of the Method for the Removal of Interference by Vitamin A and Carotene

The Quaife and Biehler method was designed primarily to remove vitamin A, carotenoids and other plant pigments. These experiments were designed to determine if these pigments, when present in the concentrations normally found in milk fat, would be removed by hydrogenation. This was considered necessary since difficulties were encountered in obtaining completely decolorized solutions of fat by hydrogenation. The results of this study are presented in Table 34. The carotene sample used was G.B.I. pure crystalline 90 per cent beta- and 10 per cent alpha-carotene stored one year at -10 to -15° F.

The natural vitamin A ester used was a distilled natural vitamin A ester (DPI Rochester, N. Y.) containing 5.4 per cent vitamin A. The ester used was pure crystalline vitamin A acetate (D.P.I.); pure crystalline vitamin A alcohol was obtained from G.B.I.

These experiments show that vitamin A ester or alcohol do not interfere in concentrations as high as 20 times that expected in a natural milk fat (20 x 4.5 to 10.6), even when not hydrogenated. Carotene in concentrations up to that expected in natural milk (1.5 to 10.7 mcg./g.) interferes to the extent of approximately 1 mcg. increase in apparent tocopherol per mcg. of carotene. The actual interference of these substances is small when the 15 second reading is used to calculate the assay value. (Carotene may be corrected for according to Quaife, Scrimshaw and Lowry (66), Table 31). It is believed that hydrogenation, for the purpose of removal of these pigments, is not necessary. However, when a time interval longer than 15 seconds was used between addition of the ferric iron solution and reading the percentage transmittance a considerable error was introduced by both vitamin A and carotene. It is believed that hydrogenation was effective as a means of preventing this error, since the decrease in per cent transmittance with time (after addition of ferric iron) of hydrogenated solutions was generally the same as the blank. It has

Table 34

Effect of Hydrogenation on the Degree of Interference of Carotene and Vitamin A When Hydrogenated Solutions are Assayed for Tocopherol

mcg. of Toco-pherol[1] added	mcg. of vit. A added as:	mcg. of Carotene[3] added	Apparent Tocoph. from standard curve (mcg.)		% Recovery of tocopherol added	
			Hydro.	Un-treated	Hydro.	Un-treated
	Acetate[2]					
0.0	0.0	1.09	0.0	0.0	--	--
0.0	0.0	5.45	0.0	0.0	--	--
0.0	0.0	8.72	0.0	10.1	--	--
0.0	122.0	0.0	--	0.0	--	--
83.5	0.0	0.0	82.0	56.5	99.4	68.9
83.5	0.0	0.0	95.0	--	115.0	--
83.5	244.0	0.0	89.0	51.0	107.8	62.3
83.5	244.0	0.0	94.0	--	113.8	--
83.5	0.0	218.0	90.0	232.0	109.0	270.0
83.5	0.0	218.0	86.0	--	104.2	--
83.5	244.0	218.0	92.0	232.0	111.4	270.0
83.5	244.0	218.0	95.0	--	115.0	--
	Alcohol[4]					
0.0	547	0.0		4.0		
0.0	547	0.0		1.0		
93.0	0.0	0.0		85.0		91.5
93.0	0.0	0.0		82.0		88.2
93.0				88.0		94.6
93.0	547	0.0		91.0		98.0
0.0	1449	0.0	2.0			
219	1449	0.0	214		97.7	
219	0.0	0.0	214		97.7	
	Distilled nat. ester[5]					
0.0	1.97	0.0	0.0	0.0		
0.0	3.94	0.0	0.0	0.0		
0.0	5.91	0.0	0.0	0.0		

1. D.P.I., pure natural d,alpha-tocopherol.
2. D.P.I., pure crystalline vitamin A acetate.
3. G.B.I., pure crystalline 90 per cent beta- and 10 per cent alpha-carotene.
4. G.B.I., pure crystalline vitamin A Alcohol.
5. D.P.I., distilled natural ester containing 5.4 per cent vitamin A.

been found that incomplete decolorization of fat samples was sometimes the result of incomplete desiccation of the catalyst.

e. The Effect of Hydrogenation upon the Oxidation Products of Tocopherol was considered of interest since it was thought probable that the tocopherol quinones would be present in the oxidized fats and that, if they were reduced by hydrogenation, they might interfere.

o- and p-Quinones were prepared (p 29-30) and their effect studied when hydrogenated alone and when added to solutions of fat, prior to hydrogenation. The percentage increase in tocopherol value (mcg./g. of fat) resulting from the addition of the quinoid forms was calculated in the following manner:

IX. $$\text{\% increase resulting from added quinone} = \frac{\text{TV}^{1}\text{ of fat with added quinone} - \text{TV of fat alone}}{\text{TV of fat alone}} \times 100$$

1. TV is the mcg. of tocopherol/g. of fat as determined by the assay.

Table 35 contains these data. The p-quinone was reduced to a considerable extent and caused 103.8 per cent increase in the tocopherol value while the o-quinone was apparently not reduced in this experiment.

The effect of hydrogenation time and length of standing subsequent to hydrogenation was also studied with solutions of o-quinone. Table 36 illustrates the erratic results obtained.

Table 36 shows that the highest recovery of the quinone as tocopherol was obtained with seven minutes hydrogenation and with the five minute standing after hydrogenation; hydrogenation longer than seven minutes apparently had no additional effect. The length of standing after hydrogenation does not correlate with the recovery values. The erratic results obtained illustrate one of the possible reasons for erratic results with the Quaife and Biehler method, (assuming the presence of o-quinone in oxidized fat). There was apparently some variable among the experimental conditions other than hydrogenation time and time of standing subsequent to hydrogenation which caused these erratic results.

The effect of time of standing after hydrogenation upon the p-quinone was also studied; the same type of experiment was employed. Ten minute hydrogenation was employed.

The effect of hydrogenation upon the p-quinone is clear cut and definite and the length of standing after hydrogenation is inversely related to the recovery.

These experiments indicate that the Quaife and Biehler method for the assay of tocopherols in oxidizing fat is not of value for the study of oxidizing milk fat since recoveries

Table 35

Tocopherol Assay Values[1] (mcg./g. of fat) of Hydrogenated Solutions of the Quinones (3 minute hydrogenation in the Quaife and Biehler apparatus)

Tocopherol assay value (mcg./g. of fat)					% Increase in tocoph. due to added quinone	
p-Quin.[2] only (120 mcg.)	o-Quin.[3] only (109 mcg.)	Fat only	Fat + 120 mcg. p-quin.	Fat + 109 mcg. o-quin.	p-Quinone	o-Quinone
74.1	7.16	59.4 56.9	127.5 109.5	50.1 62.5	103.8	-3.0

1. Quaife and Biehler method (p 48).
2. Prepared by method (p 32).
3. Prepared by method (p 32).

Table 36

Tocopherol Assay Values[1] of Hydrogenated Solutions of o-Quinone

mcg. of o-Quinone added	Hydrog. time (min.)	Standing time after hydrog. (min.)	mcg. Found as tocopherol[1]	% Recovery of added o-quinone
120.0	3	10	17.1	14.2
120.0	5	10	15.1	12.6
120.0	10	10	38.0	31.7
120.0	0	--	Neg.	Neg.
120.0	0	--	8.1	6.7
33.3	0	--	0.0	0.0
33.3	0	--	0.0	0.0
33.3	0	--	0.0	0.0
333.3	10	50	150.0	31.5
333.3	10	5	159.9	48.0
333.3	10	15	56.3	16.9
333.3	10	17	78.6	23.6
333.3	7	5	232.0	69.7
333.3	5	5	9.24	2.77

1. Quaife and Biehler method (p 48).

Table 37

Assay[1] of Hydrogenated Solutions of p-Quinones

mcg. of p-quinone	Standing time after hyd.	mcg. Found as tocoph.	% Recovery of added p-quinone
333	5	157.5	47.3
333	10	134.4	40.7
333	15	161.3	48.4
333	60	68.9	20.7
333	120	44.5	13.4

1. Quaife and Biehler method (p 48).

of added alpha-tocopherol were erratic and the o- and p-quinoid derivatives of tocopherol interfere to a variable degree. The removal of vitamin A is unnecessary when present in concentrations normally found in milk fat. If a correction for carotene color is made (Tables 4, 31), and the percentage transmittance readings are made 15 seconds after the addition of the reagent, there is no apparent interference of carotene.

Recent publications by the proponents of the hydrogenation method (Harris, Quaife and Swanson (31), Quaife and Dju (70) and Quaife and Harris (71)) state that at least six steps are necessary for assay of tocopherols in foods: 1. vacuum dehydration from the frozen state; 2. grinding and homogenization; 3. extraction with solvent; 4. molecular distillation; 5. hydrogenation of the molecular distillate; and 6. colori-

metric assay of tocopherols. Bird (3) found that low temperature crystallization of the hydrogenated glyceride from cyclohexane:ethanol solvent aided in the removal of catalyst and in prevention of fat precipitation during the assay. These additional refinements were not studied.

5. Study of the Effect of gamma-Tocopherol on the Kjolhede and the Kjolhede:Alumina Methods

It has been reported by Hove and Hove (28) that the tendency for the per cent transmittance of the Emmerie and Engel assay solutions to decrease with time is a characteristic of gamma-tocopherol. It was reported by Stern and Baxter (80), that mixtures containing alpha-, beta-, gamma- and delta-tocopherol may not be assayed with the glacial acetic acid reagent of Devlin and Mattill because the intensity of color produced by individual tocopherols was not a linear function of concentration. (They studied a system of fat in Skellysolve plus reagent in glacial acetic acid.) They also reported that gamma-tocopherol was not completely oxidized in 40 minutes and delta-tocopherol required a still longer period. With these observations as a background it was decided to use the Kjolhede and the Kjolhede:alumina procedures (p 46 and p 51) for the analysis of solutions containing alpha- and gamma-tocopherols and solutions of fats with added tocopherols. Unfortunately it was not possible to procure a sample of pure delta-tocopherol.

The methods were modified only in that spectrophotometer readings were taken one minute and five minutes after the addition of the Devlin and Mattill reagent and the tocopherol values were calculated for both readings. It was believed that this technique might make it possible to determine, with a moderate degree of certainty, whether or not the sample analyzed contained gamma- (or delta-) tocopherol. As a preliminary to the recovery experiments reaction rates and assay values of the tocopherols with the glacial acetic acid reagent were necessary.

An experiment was conducted in the manner described for the preparation of standard regression equations using solutions of pure synthetic alpha-tocopherol (Merck) and pure natural gamma-tocopherol (DPI). Dilutions were made from weighed amounts of the pure compounds such that solutions which contained about 50 mcg. per ml. were obtained (p 27). Aliquots of these solutions were assayed in the manner described below. In order to check the reaction rates of these tocopherols and the assay values of the above solutions as determined with the alpha-tocopherol standard curve, the Devlin and Mattill reagent was used in the assay procedures described (p 46 and p 51) and the per cent transmittance was read at 1 minute and 5 minute intervals. The results of this experiment are presented in Table 38.

Table 38

Assay of Pure alpha- and gamma-Tocopherol Solutions with the Devlin and Mattill Reagent Using 1 Minute and 5 Minute Reaction Times

ml. of Solution Used	mcg. of Tocopherol per ml. added	mcg. of Tocopherol per ml. found from alpha-tocopherol standard curve		Assay value as % of tocopherol actually added	
		1 min.	5 min.	1 min.	5 min.
1	57.57 mcg. of alpha-tocopherol	59.0	58.8	102.4	102.0
3		63.3	62.3	109.8	108.0
5		61.1	61.4	105.5	106.7
1		61.6	61.5	107.1	106.9
3		62.9	64.9	109.4	112.4
5		62.5	63.9	108.6	111.2
Averages		61.7	62.1	107.1	107.9
1	50.97 mcg. of gamma-tocopherol	18.1	65.9	35.5	129.4
2		16.5	66.5	32.4	130.5
3		16.9	65.8	33.2	129.2
4		15.7	66.0	30.8	129.6
5		20.3	64.6	39.8	126.8
1		20.7	62.6	40.6	122.9
1		22.1	65.6	43.4	134.3
3		23.4	62.9	45.9	123.5
5		17.8	60.1	34.9	118.0
Averages		19.1	64.4	37.5	126.8

The data of Table 38 indicate a recovery of 107.1 per cent for alpha-tocopherol. The standard curves employed were prepared with alpha-tocopherol obtained from Distillation Products, Inc., Rochester, New York. The solutions above were prepared from a sample obtained from Merck and Co., Rahway, New Jersey. For the assays of fats it is assumed that the standard reference curve presents an accurate value. In the

recovery experiments with fat samples to be presented subsequently, the tocopherol solutions were assayed and the mcg. of added tocopherol were based on these assays, i.e., it was considered that the solutions contained 1.071 times the quantity of alpha-tocopherol actually weighed.

gamma-Tocopherol was recovered to the extent of 126.8 per cent. At the one minute reaction interval all of the alpha-tocopherol that would react, had reacted (Table 38), while but 29.5 per cent of the gamma-tocopherol had reacted. At the 5 minute interval, however, the apparent tocopherol content was 126.8 per cent of that added. If it is assumed that 100 per cent of the gamma-tocopherol reacted at the 5 minute interval, 70.5 per cent of the total added gamma-tocopherol reacted between the 1 and the 5 minute readings. On this basis, the actual weight of alpha-, gamma- and total-tocopherols can be estimated by the following formulae:

X. mcg. gamma-t[1], calc. =

$$\frac{\text{mcg. t., 5 min.} - \text{mcg. t., 1 min.}}{0.705} \times \frac{100}{126.8}$$

XI. mcg. alpha-t., calc. =

$$\text{mcg. t., 1 min.} - \frac{0.295}{0.705}\left[\text{mcg. t., 5 min.} - \text{mcg. t., 1 min.}\right]$$

XII. mcg. total-t. = mcg. alpha-t., calc. + mcg. gamma-t., calc.

1. t = tocopherol

Although there is no assurance that alpha- and gamma-tocopherols are the only tocopherols present in milk fat, it is considered that if they were, equations X and XI afford a means of estimating the relative amounts of the two in milk fat samples.

Recoveries of added alpha- and gamma-tocopherols from 4 milk fat samples obtained from commercial butters were calculated. The solutions, the assays for which were presented in Table 38 were employed as tocopherol sources. The apparent concentrations resulting from the assays of the solutions were employed as actual concentrations.

When the samples were saponified the aliquot of the tocopherol solution (in alcohol) was placed in the saponification flask, the fat was weighed into the flask, the pyrogallol was added and the three were mixed as well as possible. This mixture was then saponified. When the samples were not saponified the tocopherol solution and the fat were placed in the graduated mixing cylinders directly.

The data are presented in Tables 39a and 39b, and are summarized in Table 39. The total- and gamma-tocopherol content of the samples calculated by formulae X to XII are presented in Tables 39c and 39d.

The recoveries of both tocopherols by the Kjolhede method were excellent from whole fat but were somewhat lower

on the average and quite erratic from saponified fat (Tables 39 a, b and c). Recoveries of alpha-tocopherol were low by the Kjolhede + alumina method; while recoveries of gamma-tocopherol were 80.6 and 75.8 per cent for whole fat and non-saponifiables respectively. The recoveries calculated from values obtained for formula X are given in the summary table for comparison. Table 39c indicates that, although from 80 to 98 per cent of added gamma-tocopherol is assayed by the Kjolhede method, there is only a very small amount of gamma-tocopherol found in the samples of whole fat. Calculations from the assay values of the non-saponifiable fractions Table 39d give similar results. Correspondingly lower values are calculated for the Kjolhede + alumina method. The calculated gamma-tocopherol contents of 15 different fats will be presented in Table 44.

By comparison of the percentage recoveries of added gamma-tocopherol shown in Tables 39a and 39b with those shown in 39c and 39d it may be seen that the apparent recoveries of gamma-tocopherol are higher in the former set of tables. This apparent discrepancy is a result of the comparatively greater "recovery" of pure gamma-tocopherol when the alpha-tocopherol standard curve is used for conversion of transmittance readings to tocopherol values. It is considered that the gamma-tocopherol values and their corresponding percentage recoveries shown in

Table 39

Summary of Results of Recovery Studies

Method	Whole fat or non-sap. fraction	Average percentage recovery of added: alpha-Tocopherol	gamma-Tocopherol
Kjolhede	Whole fat	100.3	95.7
same	non-sap.	88.7	93.8
Kjolhede +	Whole fat	56.1	80.6
alumina	non-sap.	69.1	75.8
	<u>Values calculated from the formula X</u>:		
Kjolhede	Whole fat		88.4
Kjolhede	non-sap.		76.3
Kjolhede +	Whole fat		45.6
alumina	non-sap.		45.7

Table 39a

Recovery of alpha- and gamma-Tocopherols Added to Fats Assayed by the Kjolhede and Kjolhede:Alumina Methods

Sample description whole fat (added alpha-tocopherol 62.1 mcg./g. of fat) (added gamma-tocopherol 64.4 mcg./g. of fat	Method of assay							
	Kjolhede method				Kjolhede:alumina method			
	mcg. Tocoph./g. fat found		% Recovery[1]		mcg. Tocoph./g. fat found		% Recovery[1]	
	1 min	5 min	1 min	5 min	1 min	5 min	1 min	5 min
Washington fat, only	25.1	25.2			15.4	14.9		
Fat only	21.5	23.8			15.7	15.7		
Fat + alpha-tocoph.	86.6	88.8	101.9	103.5	46.9	47.3	50.5	51.5
Fat + alpha-tocoph.	88.8	90.8	105.4	106.7	47.3	47.6	51.2	52.0
Fat + gamma-tocoph.	36.1	92.5	19.8	105.5	43.3	75.4	43.1	93.3
Fat + gamma-tocoph.	31.2	88.8	12.3	99.8	48.8	79.9	51.7	100.3
Louisiana fat, only	54.9	56.0			21.6	24.2		
Fat only	54.5	56.5			30.7	32.1		
Fat + alpha-tocoph.	116.8	118.0	100.0	99.5	51.5	54.6	40.9	42.5
Fat + alpha-tocoph.	115.9	118.0	98.5	99.5	48.6	50.1	36.2	35.2
Fat + gamma-tocoph.	66.3	118.0	18.0	95.8	51.5	84.7	39.4	87.7
Fat + gamma-tocoph.	66.3	120.8	18.0	100.2	50.1	85.2	37.2	88.5
Indiana fat, only	32.2	33.7			25.6	23.1		
Fat only	29.7	30.6			29.7	29.5		
Fat + alpha-tocoph.	95.0	95.5	103.1	102.0	75.1	64.4	76.4	62.4
Fat + alpha-tocoph.	92.6	95.0	99.2	101.4	78.2	71.6	81.4	72.9
Fat + gamma-tocoph.	37.6	91.1	10.3	91.5	51.5	64.7	37.1	59.6
Fat + gamma-tocoph.	46.2	88.7	23.6	87.8	54.5	69.3	41.7	66.8
Minnesota fat, only	31.0	34.2			25.4	25.4		
Fat only	28.0	29.8			25.4	24.8		
Fat + alpha-tocoph.	89.4	90.0	96.6	93.8	71.5	68.4	74.2	69.7
Fat + alpha-tocoph.	89.4	90.6	96.6	94.1	65.9	64.1	65.2	62.8
Fat + gamma-tocoph.	41.5	91.7	18.6	92.7	48.6	73.9	36.0	75.8
Fat + gamma-tocoph.	48.0	92.5	28.7	93.9	48.6	73.5	36.0	75.2

1. Based on average of duplicates of whole fat only.

Table 39b

Recovery of alpha- and gamma-Tocopherols Added to Fats Which were then Saponified and Assayed by the Kjolhede and Kjolhede:Alumina Methods

Sample description saponified fat (added alpha-tocopherol 62.1 mcg./g. of fat) (added gamma-tocopherol 64.4 mcg./g. of fat	Method of assay							
	Kjolhede method				Kjolhede:alumina method			
	mcg. Tocoph./g. fat found		% Recovery[1]		mcg. Tocoph./g. fat found		% Recovery[1]	
	1 min	5 min	1 min	5 min	1 min	5 min	1 min	5 min
Washington fat, only	21.5	21.5			25.4	24.5		
Fat only	24.6	24.3			24.6	24.2		
Fat + alpha-tocoph.	65.7	68.4	68.8	73.2	66.0	66.4	66.0	67.8
Fat + alpha-tocoph.	83.1	86.0	96.7	101.6	76.7	76.6	83.3	84.2
Fat + gamma-tocoph.	48.6	88.2	39.8	101.3	45.8	73.2	32.3	75.9
Fat + gamma-tocoph.	31.2	83.0	12.7	93.3	53.0	75.6	43.5	79.7
Louisiana fat, only	50.9	53.4			39.2	40.5		
Fat only	36.4	37.3			35.8	38.0		
Fat + alpha-tocoph.	77.3	83.0	54.3	60.5	64.5	68.2	43.5	46.6
Fat + alpha-tocoph.	104.5	107.1	98.1	99.4	69.6	68.5	51.7	47.1
Fat + gamma-tocoph.	56.6	103.1	20.2	89.6	51.5	84.7	21.7	70.6
Fat + gamma-tocoph.	58.3	103.7	22.8	90.4	51.5	82.9	21.7	67.8
Indiana fat, only	25.0	27.5			27.4	29.7		
Fat only	23.7	27.5			28.4	30.9		
Fat + alpha-tocoph.	72.2	73.7	77.1	74.4	72.4	73.5	71.7	69.6
Fat + alpha-tocoph.	89.3	90.6	104.7	101.6	85.2	87.5	92.3	92.1
Fat + gamma-tocoph.	41.4	88.2	26.6	94.3	53.2	75.1	39.3	69.6
Fat + gamma-tocoph.	46.2	91.1	34.0	98.8	53.0	82.9	39.0	81.7
Minnesota fat, only	22.0	24.1			25.4	27.6		
Fat only	23.7	25.8			25.7	27.1		
Fat + alpha-tocoph.	85.8	89.3	101.4	103.7	73.5	68.8	77.3	66.7
Fat + alpha-tocoph.	82.9	84.0	96.6	95.2	76.0	76.3	81.3	78.8
Fat + gamma-tocoph.	46.0	82.9	36.0	90.1	57.8	80.2	50.3	82.1
Fat + gamma-tocoph.	46.0	85.3	36.0	93.8	57.8	80.2	50.3	82.1

1. Based on average of duplicates of saponified fat only.

Table 39c

Calculated Concentration of gamma-Tocopherol and Total-Tocopherols in Four Samples of Whole Fat with Added alpha- and gamma-Tocopherols Using Formulae X, XI, XIII

Sample description whole fat (added alpha-tocopherol 62.1 mcg./g. of fat) (added gamma-tocopherol 64.4 mcg./g. of fat	Calculation of gamma- and total-tocoph. by Formulae X, XI, XII: Difference 5 min - 1[1] min value		Tot. gamma-tocoph. mcg./g. fat formula X		Tot. alpha + tot. gamma-tocoph. mcg./g. formulae XI & XII		% Recovery of gamma-tocoph. from formula X	
	Kjolhede	Kjolhede: alumina	Kjolhede	Kjolhede: alumina	Kjolhede	Kjolhede: alumina	Kjolhede	Kjolhede: alumina
<u>Washington fat</u>								
Fat only	1.2	0.0	1.34	0.0	24.2	15.4		
Fat + alpha-tocoph.	2.1	0.3	2.35	0.34	89.0	47.3		
Fat + gamma-tocoph.	57.0	32.1	63.8	35.9	73.6	68.5	98.8	55.5
<u>Louisiana fat</u>								
Fat only	1.6	1.6	1.8	1.8	55.8	27.3		
Fat + alpha-tocoph.	1.7	2.3	1.9	2.6	117.5	51.6		
Fat + gamma-tocoph.	53.1	34.2	59.5	38.3	103.5	74.7	92.1	59.2
<u>Indiana fat</u>								
Fat only	1.3	-1.4	1.5	Neg.	31.8			
Fat + alpha-tocoph.	1.5	-8.7	1.68	Neg.	94.8			
Fat + gamma-tocoph.	47.5	14.0	53.2	15.7	75.0	62.9	82.3	29.3
<u>Minnesota fat</u>								
Fat only	2.5	-0.3	2.8	-.3	31.2			
Fat + alpha-tocoph.	0.9	-2.4	1.01	-2.7	90.0			
Fat + gamma-tocoph.	46.3	25.1	51.8	28.1	77.2	66.2	80.2	43.5

1. Values from Table 39a.

Table 39d

Calculated Concentration of gamma-Tocopherol and Total Tocopherols in Four Samples of Saponified Fat with alpha- and gamma-Tocopherols Added Prior to Saponification. Calculations According to Formulae X, XI, XII

Sample description saponified fat (added alpha-tocopherol 62.1 mcg./g. of fat) (added gamma-tocopherol 64.4 mcg./g. of fat	Calculation of gamma- and total-tocoph. by formulae X, XI, XII: Difference 5 min - 1[1] min value		Tot. gamma-tocoph. mcg./g. fat formula X		Tot. alpha + tot. gamma-tocoph. mcg./g. formulae XI & XII		% Recovery of calculated gamma-tocoph.	
	Kjolhede	Kjolhede: alumina	Kjolhede	Kjolhede: alumina	Kjolhede	Kjolhede: alumina	Kjolhede	Kjolhede: alumina
Washington fat								
Non-sap only	0.0	0.0	0.0	0.0	23.0	25.0		
Non-sap + alpha-tocoph.	2.7	0.2	3.1	0.22	76.3	71.5		
Non-sap + gamma-tocoph.	46.0	25.0	51.5	28.0	72.1	66.9	79.7	43.4
Louisiana fat								
Non-sap only	1.7	1.8	1.9	2.0	44.8	38.8		
Non-sap + alpha-tocoph.	5.1	1.3	5.7	1.4	94.5	67.9		
Non-sap + gamma-tocoph.	46.0	32.3	51.6	36.2	89.8	74.1	79.8	56.0
Indiana fat								
Non-sap only	3.2	2.4	3.6	2.7	26.5	29.6		
Non-sap + alpha-tocoph.	1.5	1.7	1.7	1.9	81.8	80.0		
Non-sap + gamma-tocoph.	45.9	25.9	51.4	29.0	75.9	71.2	79.5	44.9
Minnesota fat								
Non-sap only	2.1	1.8	2.35	2.0	24.4	26.9		
Non-sap + alpha-tocoph.	2.3	-2.2	2.6	-2.5	86.0	--		
Non-sap + gamma-tocoph.	38.1	22.4	42.7	25.1	72.7	73.5	66.1	38.8

1. Values from Table 39b.

39c and 39d are more nearly correct since they are obtained from calculations with the formulae (X to XII) designed to correct for the differences in reaction characteristics of alpha- and gamma-tocopherols. "Total tocopherol values" (Tables 39c and 39d) based on formula XII, do not differ significantly from the tocopherol values of Tables 39a and 39b excepting when gamma-tocopherol was added. This again indicates the insignificance of the amounts of tocopherols which react as gamma-tocopherol in the fat samples analyzed.

6. Storage Studies for Intercomparison of Methods

It was considered that a comparison of methods and likewise an estimate of the degree of deterioration to expect in a fat, could be obtained by storage in air at 45° C. Three 300 ml. Erlenmeyer flasks, containing 200 g. of fat each, were placed in an air oven controlled at 45° C. The contents of all flasks were mixed before use by pouring part of the contents of one flask into the second, stirring, pouring part of the contents of this second flask into the third, stirring, pouring part of the contents of the third flask into the first, etc., for three complete cycles. A sample (30 g.) was weighed from one of the flasks into a 100 ml. volumetric flask; the volume was made to 100 ml. with benzene and the contents mixed. Three 9 cm. columns were prepared by Kjolhede's method and three by the modified Kjolhede method. A 15 ml. aliquot of the fat

solution was run through each column and the columns were washed with benzene. The percolates were made to 50 ml. volume; 10 ml. aliquots were assayed for tocopherol. The peroxide was determined on suitable dilutions of the original fat solution and on the chromatographed solutions by the method of Hills and Thiel (26).

The study of the tocopherol and peroxide assay values of July, Iowa fat from 45° C. storage was conducted over a period of 25 days. The data from this study (Figure 5) indicate that the peroxide increases gradually during ca. 10 days. A break occurs in the curve between the 10th and 14th days; the curve continues to rise beyond the 14th day at a relatively constant rate until the end of the study. The tocopherol values when plotted against time in days give a zig-zag curve by both the Kjolhede and modified Kjolhede methods, with about equivalent initial and four day values. Beyond four days the values by the modified method decrease at a greater rate than by the Kjolhede method. It is possible that the zig-zag tendency of this curve was due to sampling error. In other words, the mixing procedure adopted may have caused variation. The detailed data appear in Table 40.

A second study was conducted with the same fat to determine the effect of saponification (with added pyrogallol) upon the assay values of the fat stored at 45° C. as compared with the

assay values of the same samples not saponified. Kjolhede treatment was compared with the modified Kjolhede treatment. To assure homogeneity of the stored sample, 330 g. of fat were placed on a 600 ml. beaker in the oven and prior to each sampling the contents were vigorously stirred with a glass rod and then poured back and forth five times between two beakers, with the fat finally in the beaker in which it was not stored. Samples were then withdrawn for assay. The data are presented in Table 41 and Figure 5.

It will be observed (Figure 5) that the assay values by the Kjolhede and modified Kjolhede treatments for both the saponified and the whole fat samples are nearly the same at the initial points and at two days storage. The curves for the whole fat begin to diverge after the second day; the values by both methods drop to the sixth day. Beyond the sixth day the values by the Kjolhede method rise slightly, while those by the modified Kjolhede method have a downward trend. The data obtained by the Kjolhede method are much more erratic in trend than those by the modified Kjolhede method. The greatest divergence between the methods occurs beyond the 16th day, the point at which the milk fat lost its "good butter" flavor and aroma (as indicated by tasting the liquid fat). It is interesting to note that the sixteenth day was the point beyond which peroxides were formed at a greater rate than they were

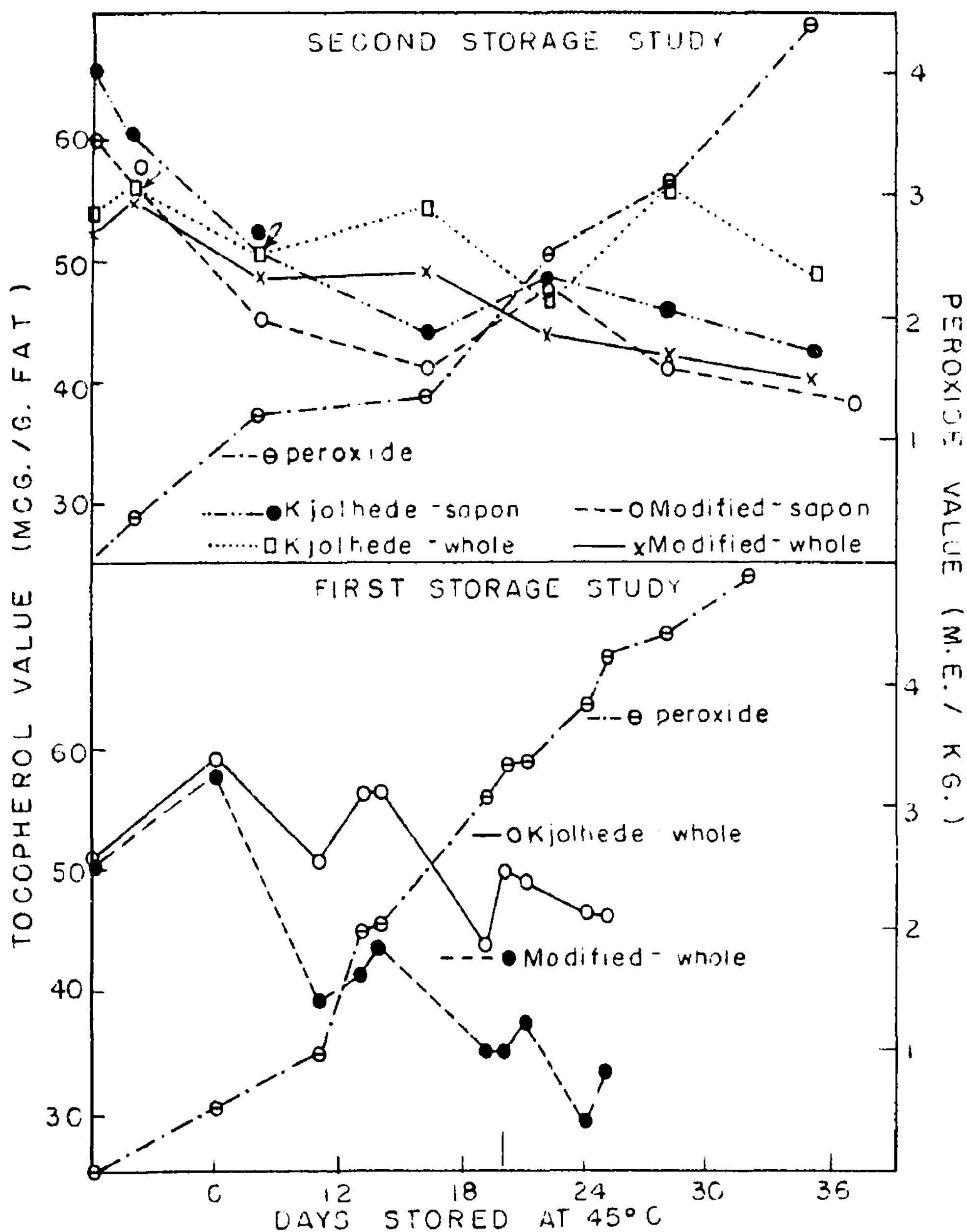

Figure 5. Tocopherol (by the Kjolhede and modified Kjolhede methods) and peroxide contents of milk fat samples stored for various lengths of time at 45° C.

prior to that date. It is possible that there is significance in the break exhibited by the curve of the data by the modified Kjolhede method, or that the divergence of the curves by the two methods is the significant thing.

Values for the saponified samples, which were protected by pyrogallol, have a downward trend by both methods; the values by the Kjolhede method are always higher than those by the modified Kjolhede method. The curves do not diverge to the extent that was found with the whole fat, possibly showing that the substances responsible for lower values by the modified earth, or for higher values by the Kjolhede earth, were removed in the saponification. The results of the study of the effect of saponification upon the oxidation products of tocopherol (p 96-102) indicated that the quinoid oxidation products did interfere in the tocopherol determination by the Kjolhede method if the fat was not saponified, while with the modified Kjolhede method there was no interference with either whole or saponified fat. This agreement between values by the modified Kjolhede method used on whole fat with the Kjolhede and modified Kjolhede methods on saponified fat substantiates these prior indications.

There seems to be no obvious correlation between the peroxide and the tocopherol values unless the increase in slope at 16 days may have some relation to the decrease in slope of

Table 40

First Storage Study

Comparison of Tocopherol values (Kjolhede and modified Kjolhede methods) and Peroxide Values (Hills and Thiel method) of Whole Fat Samples Stored for Different Intervals (up to 25 days) at 45° C.

Storage time days	Perox. value of whole fat (m.e./kg.)	Tocopherol value of whole fat (mcg./g.)				Perox. value of Percolates (m.e./kg.)	
		Kjolhede	Ave.	Mod. Kjol.	Ave.	Kjolhede	Mod. Kjol.
0	.012	52.0 50.0 51.0	51.0	57.0 47.0 47.0	50.3	0.00 0.00 0.00	-- 0.023 0.030
6	.573	60.1 57.5 60.3	59.3	60.1 57.5 56.3	58.0		
11	1.12 0.878	48.9 48.7 54.6	50.7	37.6 38.6 40.9	39.1		
13	2.01	55.3 57.6	56.4	43.2 39.4	41.3	0.038	0.342
14	2.08	56.6 56.6	56.6	47.0 45.4	46.2		
19	1.91 3.10	45.1 42.7	43.9	30.6 37.7 37.2	35.2	0.005	.878
20	3.60 3.22	46.8 54.4 48.6	49.9	35.2 36.4 33.5	35.0	0.147	
21	3.56 3.30	52.1 47.0 48.1	49.1	38.3 36.3 38.3	37.6	Neg.	1.46
24	3.94 3.86	46.6 46.6 46.0	46.2	26.8 27.3 33.4	29.2	0.03	3.56
25	3.98 4.44	46.0	46.0	33.8	33.8	0.085	1.41

the modified Kjolhede curve at this time. In the first storage study this increase in slope occurred at eleven days, this was the first sample assayed in the series which showed a divergence of the Kjolhede and modified Kjolhede curves.

The samples were likewise saponified in the absence of pyrogallol. The values varied in a highly erratic manner indicating the possibility of variations in the action of alkali upon the labile tocopherols.

The rate of oxidation was greater in the first storage study than in the second one, probably because of the larger surface area exposed. In the first storage study it appears that the tocopherol values decreased with time by both Kjolhede and modified methods. In the second study the Kjolhede values do not appear to decrease but those by the modified Kjolhede method do. Analysis of saponified (with pyrogallol added) samples shows a rapid decrease during the first eight days after which the values appear to parallel the values by the modified Kjolhede method for whole fat.

Recovery studies were made with pure alpha-tocopherol solutions (Table 42) added to two samples of oxiding fat from the first storage study. The Kjolhede and the modified Kjolhede methods of analysis were employed. The recoveries of alpha-tocopherol by the Kjolhede method were essentially 100 per cent but by the modified Kjolhede method, the recoveries

Table 41

Second Storage Study

Comparison of Tocopherol Values (Kjolhede and the modified Kjolhede methods) and Peroxide Values (Hills and Thiel method) of Whole Fat and Saponified Fat Samples Stored for Different Intervals (up to 37 days) at 45° C.

Storage time at 45° C.	Whole fat			Saponified fat (pyrogallol added)	
	m.e. Perox./ kg. of fat	mcg. Tocoph./g. of fat		mcg. Tocoph./g. of fat	
		Kjolhede	Modified Kjolhede	Kjolhede	Modified Kjolhede
0 Days	0.085	51.3	53.0	54.0	52.8
				51.5	52.5
				70.0	59.3
		58.2	57.6	70.0	65.3
		55.1	53.2	64.8	58.4
		54.6	51.4	70.0	57.4
		49.8	46.8	65.0	65.0
				60.0	65.0
				65.0	56.5
				61.8	51.1
2 Days	0.437	58.5	55.3	61.0	55.9
		54.8	54.6	57.8	54.1
				61.0	58.0
				60.5	57.5
8 Days	1.23	49.7	49.7	51.6	43.8
	1.27	51.2	48.2	50.1	41.9
				51.5	48.6
				50.2	46.7
16 Days	1.44	53.4	48.2	46.8	38.2
	1.37	55.2	50.0	45.8	38.6
				43.4	43.8
				41.1	43.6
22 Days	2.71	51.5	45.7	49.1	48.3
	2.66	42.0	42.0	48.3	47.8
	2.30			57.9[2]	56.6[2]
	2.58			58.2	54.1

Table 41 (cont'd)

Storage time at 45° C.	Whole fat			Saponified fat (pyrogallol added)	
		mcg. Tocoph./g. of fat		mcg. Tocoph./g. of fat	
	m.e. Perox./ kg. of fat	Kjolhede	Modified Kjolhede	Kjolhede	Modified Kjolhede
28 Days	3.19	55.4	46.0	46.4[3]	38.7[3]
	3.02	55.4	45.8	46.0	39.2
		57.3	39.1	45.8	42.7[3]
		55.8	37.7	46.7	42.7
				46.7	41.1
				45.8	42.7
				44.9	40.3
				46.0	41.2
35 Days	4.42	48.8	39.7		
	4.46	49.0	39.7		
		46.8	39.3		
		51.2	41.3		
37 Days				39.4[4]	37.7[4]
				42.5	37.7
				44.0[4]	39.4[4]
				44.0	37.7

1. Replicate assays were made on same saponification.
2. Abnormal colored band on column when chromatographed, the same color as obtained on whole fat.
3. These samples saponified by vitamin A method.
4. No nitrogen in saponification flasks.

Table 42

Percentage Recoveries of alpha-Tocopherol Added to Oxidized Fat Samples Before Analysis by the Kjolhede and Modified Kjolhede Methods

Treatment Used	Storage	mcg. Tocoph. added/g. fat	mcg. Tocoph. found/g. fat	% Recovery of added alpha-tocoph.
	11 days			
Kjolhede	(PV 2.01)	63.2	125.7	111.4
"	"	63.2	117.0	97.6
"	"	0.0	55.3	--
Modified	"	0.0	43.2	--
"	"	63.2	102.5	93.8
"	"	63.2	103.0	94.6
	25 days (PV 4.21)			
Kjolhede	"	46.35	91.4	98.0
"	"	46.35	95.2	106.9
"	"	0.0	46.0	--
Modified	"	0.0	33.8	--
"	"	46.35	75.8	90.6
"	"	46.35	71.2	80.8

were 94.2 per cent with the fat with 2.01 m.e. peroxide/kg. and 85.7 per cent with the fat with 4.21 m.e. peroxide/kg. Other recovery studies appear in Tables 39a and b.

7. Comparison of the Kjolhede and Kjolhede:Alumina Methods with Saponified and Non-Saponified Fats from Commercial Butters

The tocopherol content (of whole fat and non-saponifiable fractions), as calculated from the alpha-tocopherol standard curve using 1 minute and 5 minute percentage transmittance readings was determined by the Kjolhede and the Kjolhede: alumina methods. Analyses were run in triplicate for 15 samples of milk fat rendered from butter obtained in retail prints. The peroxide content and color index were also determined on these samples. The peroxides were determined in the usual way on a benzene solution of the whole fat. The color index was determined by measuring the per cent transmittance of benzene solutions of the fat against the solvent set at 100 per cent transmittance, at 510 mu. This index is defined as:

$$\text{Color index} = \frac{2 - \log \%\ \text{Trans.}}{\text{g. fat/ml. soln}}$$

The samples, furnished by Mr. W. W. Griese of the A and P Company, Chicago, were selected to represent as nearly as possible all of the major butter production areas of the country. The first thirteen of these samples were from butter manufactured

in the first two weeks of May. The Louisiana and the Washington samples were manufactured in April. A description of these samples is given in Table 43. Two, one pound prints of each sample were obtained. Each print was cut into quarters longitudinally and two non-adjacent quarters were used as the sample. The fat was rendered by the usual procedure and was stored at -30° C. until used. No pronounced weed, feed or grass flavors were noted. The samples were variable in color and it is presumed that color was added to at least some of the samples. The average tocopherol values, color indices and peroxide values are given in Table 44. From the average tocopherol values by the Kjolhede method on whole fat, the concentration of gamma-tocopherol was calculated by use of formula X derived on p 139 for this purpose.

When the tocopherol value is compared with the color index (Table 44) it appears there is a positive correlation which would indicate that the tocopherol values of the fats increase with increasing color. Since these were samples of commercial butter, color may have been added, so that this correlation may be coincidental.

It may be concluded that the analysis technique employing alumina and the Kjolhede adsorbent is not preferable to the Kjolhede method because of low recoveries of added tocopherol and the somewhat erratic results obtained (Tables 39a, 39b).

Table 43

Source and Description of Butter Samples (analyses in Table 44)

Sample	Source of butter	Score	Flavor criticisms and remarks: Observers 1	2	3
1	N. Wisconsin	92	OK	OK	---
2	Illinois	92	OK	OK	---
3	Oklahoma	89	Inedible, old cream	Inedible, old cream	Old cream
4	Indiana	92	Sl. old cream	Sl. stale	None
5	Missouri	90	Sl. old cream	Neut. sl. stale sl. cheese	Old cream
6	Michigan	92	OK	OK	---
7	S. Iowa	90	Off, old cream neut.	Off, old cream neut.	---
9	Wisconsin	90	Old cream	Heated OK	Swiss cheese whey cream
10	N. E. Iowa	92	OK	OK	---
11	Minnesota	92	Disguised weed or feed high starter	Disguised weed or feed high starter	---
12	South Dakota	90	Sl. neut. flavor	Off. neut.	Wheat pasture
13	Kansas	90	Very bad, old cream, utensil	Very salty, cooked sl. cheesy	Wheat pasture
14	Nebraska	90	Sl. old cream, pasty	Old cream	Wheat pasture
15	Louisiana		---	---	---
16	Washington		---	---	---

Table 44

Average Values from Analyses of Milk Fat Samples from Varying Sources for Tocopherols by the Kjolhede and Kjolhede:Alumina Methods

Sample	Spl. no.[1]	Perox. m.e./kg. of fat	Color index[2]	Average tocopherol values (mcg./g. of fat) Whole Kjol.	Whole Kjol: alum.	Sapon Kjol.	Sapon Kjol: alum.	gamma-Tocopherol calc. formula X
N. Wisconsin	1	0.48	0.63	20.6	14.8	17.8	12.1	4.6
Illinois	2	0.42	1.10	39.0	19.8	36.5	23.8	3.8
Oklahoma	3	0.717	1.39	49.1	24.7	42.4	34.4	2.8
Indiana	4	0.412	--	30.9	23.9	24.3	29.5	2.0
Missouri	5	0.738	1.33	32.9	25.4	26.2	30.0	1.4
Michigan	6	0.522	0.64	21.0	10.5	18.6	11.6	5.4
S. Iowa	7	0.441	0.81	20.9	21.5	19.9	24.1	3.6
So. Wisconsin	9	0.615	0.84	14.1	17.7	12.5	13.0	3.0
N. E. Iowa	10	0.320	0.51	14.4	23.9	9.8	25.0	0.3
Minnesota	11	0.465	0.98	31.2	20.7	19.9	23.0	1.8
South Dakota	12	0.956	0.89	22.1	21.4	19.7	12.2	3.3
Kansas	13	1.280	0.84	36.6	21.1	31.3	27.3	5.4
Nebraska	14	0.450	0.62	24.7	26.8	21.1	19.4	2.4
Washington	15	0.308	1.066	30.2	12.9	24.6	18.6	0.9
Louisiana	16	0.820	1.57	48.7	23.2	46.6	32.8	2.2

1. Samples described (Table 43).
2. Color index $= \frac{2 - \log \% T \text{ (in benzene)}}{\text{g. fat/ml. Soln.}}$

It may be that the lack of reproducibility results from destruction by the alumina adsorbent.

An analysis of variance for the randomized block design was computed for the data (Table 45). (Average values are given in Table 44). The experimental errors of the eight factors studied were not homogeneous, as indicated by the analysis of variance, consequently a different error term was computed for each factor and the interactions.

The factors analyzed were:

(S), effect of saponification

(A), effect of alumina treatment after Kjolhede Floridin treatment

(T), effect of time after addition of reagent (1 min vs. 5 min)

(R), effect of the samples themselves

The factors analyzed are given in Table 45. The effect of saponification was found to be non-significant but the interaction SA was highly significant. The effect of time after addition of color development reagent was highly significant, as were the differences between the samples themselves. The interaction RT has a low mean square value which indicates that the differences as a result of time after addition of the reagent are not a function of the sample.

Table 45

Analysis of Variance of Data Obtained from Analyses of 15 Samples of Fat from Commercial Butter[1]

Source of variation	Degrees of freedom		Mean square		F
Samples (R)	14		1361.29		15.91***
Saponification (S)	1		.75		N.S.
Adsorption (A)	1		4191.58		10.49**
Time (T)	1		436.04		33.16***
SA	1		1108.10		11.48**
ST	1		6.35		N.S.
AT	1		3.56		N.S.
SAT	1		1.41		N.S.
Experimental error	98		85.55		
RS		14		68.83	
RA		14		399.69	
RT		14		13.15	
RSA		14		96.52	
RST		14		8.78	
RAT		14		8.75	
RSAT		14		3.13	
Determinations	240		7.02		

1. Average values for triplicate determinations with the 1 minute reading are given in Table 44.

* Significant at P = 0.05
** Significant at P = 0.01
*** Significant at P = 0.001

The fact that the averages for columns I and II of Table 46 agree, indicates the reason for a non-significant change in tocopherol values as a result of saponification, and column I - II shows quantatively why these averages are equal: the <u>magnitude</u> of the differences are the same but the <u>signs</u> are opposite with the two adsorbents used, i.e., the assay values

Table 46

Means of Tocopherol Values by Eight Treatments

	Whole fat I		Sapon. fat II		Differences I - II		Average $\frac{I + II}{2}$	
	1 min.	5 min.	1 min.	5 min.	1 min.	5 min.	1 min.	5 min.
A. Kjolhede	29.1	31.7	25.7	27.9	+3.4	3.8	27.4	29.8
B. Kjolhede: alumina	18.9	21.3	22.7	24.3	-3.8	-3.0	20.8	22.8
$\frac{A + B}{2}$	24.0	26.5	24.2	26.1	-0.4	+0.8	24.1	26.3
Differences due to time	2.5		1.9		+0.4		2.2	

of the saponified fats are higher than those of the whole fats when analyzed by the Kjolhede Floridin:alumina method. By the Kjolhede method the non-saponified values are the higher. It is indicated from these results that the Kjolhede Floridin: alumina columns destroy more tocopherol when glyceride is present (presumably as a result of the formation of peroxide) than when the glyceride is removed by saponification.

8. Modifications of the Emmerie and Engel Method Recommended for the Determination of Total Tocopherols in Milk Fat

On the basis of a cursory examination of the facts, it might be concluded that the best method would be one which analyzed the whole fat pretreated with Kjolhede Floridin, since this method was the one which gave good recoveries of added tocopherol, removed the effect of peroxide, carotene, and vitamin A and had relatively good reproducibility between samples of the same fat. This method is not, however, recommended for tocopherol analyses of samples of oxidized fat because the Kjolhede Floridin reduces the tocopheryl-p-quinones, which are indicated to be constituents of oxidized fats.

The saponification method employing 3.5 N KOH in methanol (with added pyrogallol) was effective in removal of the quinones and is therefore recommended for oxidized fats. Recoveries of alpha- and gamma-tocopherol were quite variable when fats plus tocopherols were saponified and assayed, however, the

variability of the replicates within samples was no greater by this method than with the whole fat (Table 41), which indicates that there is some fault in the technique for recovery studies.

The modified Kjolhede adsorbent was not effective in the removal of peroxide (Tables 5 and 40) and apparently caused destruction of tocopherol as indicated by the recovery studies (Table 42). It did not cause interference by reducing the o- and p-quinones.

On the basis of the results of storage studies (Figure 5) it was shown that (with the exception of values for fresh fat) the tocopherol values determined in whole fat and in saponified fat by the modified Kjolhede method tended to parallel those by the Kjolhede method on saponified fat, as the fat progressively oxidized. In the case of the fresh fat the analytical values for the non-saponifiable fraction were higher than those for the whole fat by either the modified Kjolhede or the Kjolhede treatments. This would indicate that the low recoveries obtained (when tocopherols were added to fat) were not representative of the conditions in the fat alone and that the discrepancies between the results by the Kjolhede and modified Kjolhede methods with whole fats (shown in Table 20) probably resulted largely from reduction of the tocopheryl-quinoid forms.

It is recommended that any further recovery studies be conducted with a slightly modified technique from that employed here. A solution of tocopherol in methanol was added directly to the fat and mixed as thoroughly as possible, 5 ml. of 5 per cent pyrogallol were added and the solutions were again mixed. The methanolic potassium hydroxide was then added and the extraction flask was connected to the reflux condenser to effect the saponification. Incomplete mixing of the tocopherol with the fat (and the pyrogallol) prior to addition of the alkali may be the cause of the variability in tocopherol recoveries. Recovery studies in which the alkali is added to the pyrogallol just prior to its addition to the fat might overcome this difficulty. Some means of providing a more completely homogeneous mixture of the fat and added tocopherol might also prove beneficial. This might be accomplished by addition of the tocopherol to the fat in Skellysolve solution and evaporation of the Skellysolve under vacuum prior to addition of the alkaline pyrogallol solution.

IV. SUMMARY AND CONCLUSIONS

The Emmerie and Engel method was selected for study as a means of determining tocopherols in milk fat. This method is based on the ability of tocopherols to reduce ferric iron in the presence of alpha, alpha'-dipyridyl to form the highly colored ferrous:dipyridyl complex, the intensity of which is measured spectrophotometrically.

Removal of Interfering Materials

It is necessary, prior to the development of the ferrous: dipyridyl color to remove interfering substances. Emmerie and Engel employed Floridin treated with hydrochloric acid for this purpose. A number of modifications of this adsorbent have been proposed. Those which have been employed in this study are the Kjolhede Floridin, which is treated with stannous chloride as well as hydrochloric acid and a modified Kjolhede Floridin, from which it is considered excess stannous chloride was completely removed. The latter adsorbent was used because it was considered that stannous chloride, since it is a reducing agent, might interfere in the color development.

A third chromatographic procedure involved the use of Kjolhede Floridin plus activated alumina. It was considered that the first of these would remove most of the interfering

substances while the latter would separate the tocopherols from the glycerides.

The Quaife and Biehler mild hydrogenation procedure, which was originally designed to prevent interference of carotene and vitamin A by catalytic hydrogenation, was the fourth procedure employed.

A combination of saponification and chromatography was likewise studied.

The materials which were considered to be possible interferents in the Emmerie and Engel method are: Vitamin A, carotene, peroxides, and oxidation products of tocopherols.

Preliminary studies carried out with various treatments for the removal of peroxides were not successful. A sulfuric acid wash treatment; treatments with potassium iodide, stannous chloride and sodium bisulfite; and heating with carbon disulfide were found to be unsatisfactory.

The conclusions drawn regarding the effectiveness of the methods for the removal of interfering substances are indicated below:

The Kjolhede Floridin satisfactorily removed vitamin A, carotene and peroxides. The adsorbent, however, reduced a significant portion of added tocopheryl-p-quinone; the reduced quinone was assayed as tocopherol.

The modified Kjolhede Floridin removed vitamin A and carotene quantitatively. It did not reduce any of the *p*-quinone that might be present. It was unsatisfactory in that it did not quantitatively remove peroxides.

The combination of Kjolhede Floridin and alumina was satisfactory for the removal of carotene and vitamin A. When solutions of the whole fat were employed, the alumina was found to oxidize a portion of the glycerides as evidenced by positive peroxide tests in the percolate and washings. Moreover, the adsorbed tocopherol was either not quantitatively eluted from the alumina or a portion of it was oxidized on the column.

When hydrogenation was complete, the mild hydrogenation procedure removed vitamin A and carotene. The tocopherylquinones were reduced by the procedure and varying quantities of the reduced products were assayed as tocopherols.

Saponification procedures were effective in proportion to the degree of completeness of the saponification of the glycerides. The original procedure recommended by Emmerie and Engel employed 2 ml. of 2.0 N KOH per g. of fat. This alkali concentration did not completely saponify the fat. Adequate saponification was obtained with 2 ml. of 3.5 N KOH per g. of fat. It was necessary to conduct the saponification in the presence of pyrogallol to prevent tocopherol destruction.

When saponification was complete and when pyrogallol was employed the peroxides and tocopheryl-quinones were completely destroyed; the carotene and vitamin A could be satisfactorily removed from the non-saponifiable fraction with Kjolhede Floridin.

It has generally been considered that peroxides did interfere to a considerable extent in the Emmerie and Engel color development reaction. The effect of the peroxides was checked. The data obtained indicate no interaction between peroxides and tocopherols during the preparation and chromatographing of the samples, when the Devlin and Mattill reagent was employed with a 1 minute reaction interval after addition of the color development reagent. The length of time the peroxide is in contact with the ferrous-dipyridyl complex is important; the greater the length of time the lower the assayed tocopherol content.

It can be concluded, therefore, that under the conditions of these experiments, peroxides are unimportant up to the time of the development of the colored complex. After its development, peroxides cause a reduction of the tocopherol assay as the length of time between color development and measurement of its intensity increases (see Figure 3).

<u>Recovery of Tocopherols Added to Fat Samples</u>

Recoveries of alpha- and gamma-tocopherols added to fats

were generally found to be near 100 per cent with the Kjolhede method employing whole fat. With the Quaife and Biehler method, the recoveries of alpha-tocopherol were quite erratic but averaged about 100 per cent. Some tocopherol was apparently destroyed by saponification and recoveries of alpha-tocopherol from the non-saponifiable fractions of fats were quite erratic with the average being 88.7 per cent recovery. It must be pointed out, however, that these recovery studies are not considered representative of the effect of saponification upon naturally occurring tocopherols. In the second storage study (Figure 5) the tocopherol values obtained with saponified fat were higher than those obtained for the whole fat. Furthermore, the assay values of relatively fresh fats (commercial butter) were as reproduceable on the non-saponifiable fractions as they were on the whole fats. These results indicate that there is some fault in the recovery-study technique employed.

Recoveries of gamma-tocopherol which was added to fats prior to saponification were somewhat higher and less erratic than the recoveries of alpha-tocopherol.

With the Kjolhede Floridin and alumina columns there was considerable loss of tocopherols both with the whole fat and the saponified fat.

Tocopherols Other Than alpha-Tocopherol Found in Milk Fat

It was desired to determine whether or not tocopherols other than alpha-tocopherol were present in milk fat. Equations X to XII were derived from data obtained with pure solutions of gamma- and alpha-tocopherols to test a series of commercial butter samples for the presence of materials which react in the same manner as gamma- (and delta-) tocopherol. These equations are based on the difference in rate of color formation of the two compounds. It is concluded from the analysis of variance of these data (Table 45), that if substances which react as gamma-tocopherol were present, their variation among samples must have been within the limits of error of the method.

Interference of Tocopherol Oxidation Products

It is concluded that the differences observed between the tocopherol values by the Kjolhede and modified Kjolhede methods result from reduction of tocopheryl-*p*-quinone to products which react as tocopherol when the Kjolhede but not when the modified Kjolhede method is used. This is presumed to be due to reduction of the quinone by the action of residual stannous chloride on the Kjolhede adsorbent; the modified adsorbent was washed chloride free. Reactivity to the extent of 50 per cent of the added quinone was indicated. Equations VII and VIII, based on these indications, were tested in an attempt

to determine whether the differences in results obtained by the two methods resulted from the reduction of the quinone. The "calculated original tocopherol" content of a fat in two storage studies (formula VIII) indicated that it was possible to estimate the original tocopherol content of the sample, thereby lending support to the assumptions on which equation VIII was based.

Comparison of the Quaife and Biehler Method with the Modified Kjolhede Method

The tocopherol values of several fats as determined by the Quaife and Biehler method and the modified Kjolhede method were compared. The former method gave higher results on whole fat but not on the non-saponifiable fractions. It was shown that the differences between these methods were not a result of the reagents used for the assay. The Quaife and Biehler hydrogenation technique reduced the tocopheryl-quinones which were assayed to a variable extent as tocopherol.

The Recommended Method

The modification of the Emmerie and Engel method which is recommended for the determination of tocopherol in oxidized milk fat is that of Kjolhede. It is recommended that the non-saponifiable fraction obtained by the saponification procedure in which pyrogallol is used to minimize oxidation of tocopherols be employed. Saponification is believed necessary to

destroy quinoid oxidation products of tocopherols (and possible other oxidation products). For the determination of tocopherols in milk fat of known high quality, it is not necessary to saponify for the removal of oxidized tocopherol; the whole fat may be assayed directly after treatment with Kjolhede Floridin. With fats of unknown quality it would be preferable to assay both whole fat and non-saponifiables.

The Kjolhede Floridin adsorbent is indicated as preferable on the basis of recovery studies and the ability to remove peroxidic materials. The presence of stannous chloride on the Kjolhede columns is indicated but interference as a result of stannous chloride is not indicated, excepting as it effects reduction of the quinoid forms.

Results of Analysis of Commercial Fat Samples

The average tocopherol value of the fats from the commercial butter samples is 29.1 mcg./g. of fat when Kjolhede Floridin was employed with solutions of whole fat. The values range from 14.1 to 49.1 mcg./g. of fat. When saponification was employed in conjunction with Kjolhede Floridin the values average 25.7 mcg./g. of fat with a range from 9.8 to 46.6 mcg./g. of fat. These lower values are explainable on the basis of partial oxidation of the tocopherols prior to assay in which case the quinoid substances would be determined as tocopherol in the whole fat but not when the non-saponifiable fraction is assayed.

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