SUMMARY

1. The melting points of binary mixtures of oleic, linoleic, and linolenic acids have been reported.

2. The oleic-linoleic acid system has eutectics for the α and β forms of oleic acid of 75.2 and 76.3 mole per cent linoleic acid, at —10.0° and —9.8°, respectively.

3. Linoleic and linolenic acid mixtures show only melting points intermediate between the pure acids.

4. The oleic-linolenic acid system has eutectics for the α and β forms of oleic acid of 82.7 and 85.5 mole per cent linolenic acid, at —15.7° and —15.1°, respectively.

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A Convenient Method for the Rapid Estimation of Carotene in Butterfat

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Experimental

MOST colorimetric methods for the quantitative determination of carotene in biological material are based upon a comparison of the natural yellow color of this pigment in fat-solvents with that of known concentrations of carotene or other suitably prepared standards. These methods usually require saponification of the material and subsequent extraction with solvents which separate carotene from xanthophyll and associated carotenoid pigments of a similar color. This separation of pigments is especially important whenever the results of carotene determinations are to be interpreted in terms of vitamin A activity. Of the carotenoid pigments found in plant material, only carotene and cryptoxanthin are known to have value as a source of vitamin A.

In the case of butterfat it appears possible to make a reasonably accurate estimate of its carotene content from a determination of total yellow color. Palmer (7) first showed carotene to be the principal yellow pigment in butterfat. Baumann and Steenbock (2) concluded from spectrophotometric studies that about 95 per cent of the natural pigment in butterfat was carotene. Their work was substantially confirmed by Gillam (3) who found 94 per cent of the color in the unsaponifiable matter of butterfat to be due to carotene, the remaining 6 per cent being due to xanthophyll. Gillam has suggested that for routine work a spectrophotometer be used to determine the carotene content of butterfat. He suggests that the light absorption of the unsaponifiable matter be determined at 455-460 millimicrons and that the absorption due to carotene be taken as 94 per cent of this value.

Shrewsbury and Kraybill (8) found that the determination of carotene in butterfat by direct color comparison with potassium dichromate standards, as in the Willstätter and Stoll method used by Palmer (7), gave results which were much too high. Their high results were attributed to the greater color intensity of carotene in butterfat than in the usual solvents (4).

Barnett (1) proposed a colorimetric method which made allowance for the increased color of carotene in oil. This investigator determined the carotene of butterfat with the aid of a spectrophotometer, the results being taken as the true carotene content of the sample. In these determinations cocoanut oil was used to dilute the butterfat and also as a blank. The carotene content of the same samples was then determined colorimetrically. In the colorimetric determinations 0.2 per cent solution of potassium dichromate was used as a color standard and the concentration of carotene calculated from Palmer's chart (7). By dividing the concentrations obtained by the spectrophotometric method by those obtained by the colorimetric method an average correction factor of 0.28 was secured to be applied to the colorimetric determinations. This factor when applied to the colorimetric determinations corrected for the increased color intensity of the carotene in fat. Only a narrow range of carotene concentrations was covered in this work.

Baumann and Steenbock (2) determined the carotene content of butterfat spectrophotometrically, carotene dissolved in purified cottonseed oil being used for a standard. Shrewsbury and Kraybill (8) and Leuschen *et al.* (6) diluted butterfat with petroleum solvents previous to spectrophotometric determinations of carotene. Treichler, Grimes, and Fraps (9) deemed it advisable for routine work to devise a spectrophotometric method whereby the carotene content of butterfat could be determined without previous dilution with other oils or solvents and without the need of a blank to correct for the absorption of light by the fat itself. These investigators have prepared a table of conversion factors which when applied to spectrophotometric density readings at 470 and 480 millimicrons express the carotene content of butterfat in parts per million.

Hand and Sharp (5) devised a photoelectric colorimeter fitted with suitable glass filters for the rapid determination of carotene in milk fat. This instrument in the hands of a capable operator should prove very satisfactory.

The fact that equipment for spectrophotometric methods of analysis is not available in many laboratories often makes it necessary to rely upon the less direct methods for the determination of carotene. A method which is applicable to butterfat and which has been found to fit in well with the procedures ordinarily employed in determining butterfat constants, i.e., melting point, iodine number, etc., is herein described. The method is based upon the direct comparison of the color of butterfat samples contained in cylindrical bottles of uniform diameter with that of solutions of potassium dichromate of known concentration. Although not designed to replace the more accurate methods of analysis, the proposed procedure should be of practical value in grading butterfat samples for carotene content.

Experimental

The butterfat was obtained from butter produced by Jersey cows receiving graded levels of prairie hay as the sole source of vitamin A in the ration. Regulation of the carotene intake of the cows during complete lactation periods made it convenient to secure a large number of butterfat samples of varying carotene content. Further, it has made possible a study of normal variations in carotene content of both the blood plasma and butterfat of the Jersey breed during successive lactation periods. The results of these latter studies are to be published in due time.

The butter was representative of that produced by individual cows during each month of lactation. It was melted at 60° C. and the fat separated by filtration into cylindrical glass bottles fitted with plastic screw tops. During this procedure, exposure to light was minimized and the filtered fat samples were stored in an electric refrigerator until used.

The selection of uniform sample bottles in this work is important since they serve not only as storage containers holding an ample amount of fat for chemical determinations but also as receptacles in matching the color of the fat with that of potassium dichromate standards. They take the place of test tubes of uniform bore commonly employed in colorimetric work. These bottles which have a capacity of 40 ml., are 110 mm. in height and have an inside diameter of 22 mm. They can be purchased from most supply houses at a small cost and need only be selected with respect to uniformity of diameter and color of glass. As a rule, only a few bottles need be discarded as unsuitable.

Solutions of potassium dichromate of progressively increasing concentration were prepared from a 0.2 per cent stock solution, 1, 2, 3, ..., 20 ml. of the stock solution being diluted with the proper amount of redistilled water to make the total volume of each 25 ml. These dilutions were made in standard sample bottles described above and each bottle labeled according to the number of ml. of 0.2 per cent dichromate used in making the dilution. A comparator block placed in front of a daylight lamp was used to facilitate comparison of the color of the butterfat samples with that of the potassium dichromate standards. The block held six bottles by means of vertical holes bored 70 mm. deep and 30 mm. in diameter. Smaller holes bored perpendicular to these completely through the block allowed light to pass through the solutions. The fat sample was warmed to 60° C. and placed in the block with a series of five dichromate solutions that closely approached the sample in color. The position of the fat sample was then shifted between bottles of the dichromate solutions until a match in color was obtained. The sample was then numbered to correspond with the label on the matching dichromate solution.

The actual carotene content of the butterfat was determined with a Bausch and Lomb Spectrophotometer according to the procedure outlined by Treichler, *et al.* (9). Color density readings were made at 470 and 480 millimicrons.

Results

Table 1 summarizes the important data obtained in making the color comparisons and carotene determinations. The several concentrations of potassium dichromate solution used as color standards with which the color of the butterfat was compared are given in the second column of this table. The minimum, maximum and average carotene content (spectrophotometric) of butterfat samples that matched the color of each standard is given in the last four columns.

The figures in Table 1 are in substantial agreement with the accepted fact that an increase in the carotene content of butterfat is associated with an increase in depth of color. Due to the green tinge of very dilute dichromate solutions there was difficulty in matching the color of many of the samples of low carotene content. Possible errors in obtaining a correct match are very likely reflected in the spread of the minimum and maximum carotene values shown in Table 1. Although more refined methods of colorimetry which are available would probably overcome this difficulty, they would detract from the simplicity of the procedure. Further, it was found that the order of results was not changed when the color comparisons were made with test tubes of considerable smaller diameter than that of the bottles specified in the method. It appears therefore that with some experience the method may be adapted to even smaller samples of butterfat than those available in the present study.

When the average values were plotted against color, the latter being expressed in terms of potassium dichromate concentration, the plotted points tended to fall on a straight line connecting the point of lowest concentration with that of the highest. It is possible therefore with the aid of the figures in Table 1 to estimate with a fair degree of accuracy the carotene content of butterfat samples which fall between these two extremes, 0.23 and 8.07 micrograms per gram. The formula for a straight line $y = ax + b$ was used in obtaining the average values given in the last column of Table 1. In this formula, rearranged to give $x = \frac{y - b}{a}$, x is the micrograms of carotene per gram of fat, y is the concentration of matching dilute dichromate solution in per cent, and b and a are factors, 0.012 and 0.018 respectively. Where y is more conveniently expressed in terms of ml. of stock dichromate used in making up the dilute standard, the factors b and a become 1.45 and 2.30 respectively. The observed and calculated average values are in fair agreement. It is recognized that the figures obtained for the actual carotene content of the butter fat samples are based upon spectrophotometric methods involving calculations that may introduce some error. The method and calculations are discussed in detail by Treichler, *et al.* (9). In view of the instability of carotene and its alteration in physical properties with change of solvent as pointed by Hand and Sharpe (5) and others it appeared more desirable to base results upon spectrophotometric determinations made on actual butterfat samples than upon solutions of carotene in oil or other solvents.

Summary

A simple method is described for determining the carotene content of butterfat under conditions where extreme accuracy is not required. A direct comparison is made of the color of the melted fat with that of known concentrations of potassium dichromate solution. Since there is no loss of fat from dilution or in transferring from one container to another, the method is adaptable to small samples that later can be used for the determination of the usual fat-constants.

The dichromate solutions are prepared by dilution of measured amounts of a 0.2 per cent stock solution to a volume of 25 ml. These dilute solutions and the fat samples are contained in cylindrical sample bottles of uniform diameters and their color matched with the aid of a comparator block placed before a "daylight" lamp.

TABLE I.

MINIMUM, MAXIMUM AND AVERAGE CAROTENE CONTENT OF BUTTERFAT SAMPLES WHICH MATCHED IN COLOR VARIOUS CONCENTRATIONS OF POTASSIUM DICHROMATE SOLUTION.

Stock dichromate solution (0.2 per cent) in dilute standard	Strength of dilute dichromate standard	Number of samples	Carotene content micrograms per gram of fat			
			Minimum*	Maximum*	Average	Calculated average**
ml.	per cent					
2	0.016	13	0.10	0.40	0.23	0.23
3	.024	25	0.40	1.15	0.60	0.67
4	.032	16	1.01	1.70	1.26	1.11
5	.040	23	1.18	2.00	1.55	1.54
6	.048	5	1.05	2.24	1.64	1.97
7	.056	8	1.81	3.58	2.42	2.41
8	.064	7	2.21	3.74	3.03	2.85
9	.072	6	2.20	3.98	3.07	3.28
10	.080	8	2.20	5.15	4.16	3.72
11	.088	2	4.17	4.41	4.29	4.15
12	.096	4	3.98	4.74	4.18	4.58
13	.....	....	.....	.....	.....	5.02
14	.....	....	.....	.....	.....	5.46
15	.120	5	4.90	7.15	5.86	5.89
16	.....	....	.....	.....	.....	6.33
17	.136	10	5.56	8.84	6.62	6.76
18	.....	....	.....	.....	.....	7.20
19	.152	1	.....	.....	7.50	7.63
20	.160	5	6.93	10.31	8.25	8.07

* Spectrophotometric determinations.

** The figures in this column were obtained with the formula $y = ax + b$ (see text).

The carotene content of the fat in micrograms per gram can be obtained from the figures given in Table 1 of the text or calculated with the formula $x = \frac{y - b}{a}$ in which x is the micrograms of carotene per gram of fat, y is the concentration of the matching dichromate solution in per cent, and b and a are factors, 0.012 and 0.018 respectively.

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The Mechanism of the Autoxidation of Fats*

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THE autoxidation of fats and oils exhibits some of the typical characteristics of an autocatalytic reaction: a latent or induction period of variable length during which the amount of oxygen absorbed is small, followed by a period of rapidly accelerating oxygen absorption. Not all fats show a sharply defined termination of the induction period; the reasons for this varying behavior are inherent in the source and nature of the fat.

The changes occurring during the induction period are very different from those that follow it. They are less obvious, but from a practical point of view they are more important because once the induction period is past the damage is done. Arbitrarily the end of the induction period usually coincides with or immediately precedes the first appearance of the products of organoleptic rancidity. For their detection and estimation various tests have been devised and standardized and these tests have been employed in making almost countless observations on the deterioration of fats (1).

The first event is probably the addition of oxygen at the vulnerable double bonds of unsaturated fat acids, with the formation of fat acid oxides. For such oxides several formulas have been proposed, ethylene oxide, peroxide, moloxide, dative or oxonium peroxide, ozonide,—all of which have been assumed to occur among the primary products of oxidation. Attempts have recently been made by variations in the titration methods, to distinguish between different kinds of fat peroxides (2). Some of the fat peroxides are prooxygenic and their removal by a properly chosen adsorbent may make for greater stability of the fat; others may be quite inactive; some are highly unstable, others very stable. Some are volatile, others nonvolatile.

The fact that some of these early oxidation products are chemically more active than others may be explained by differences in the configuration of the oxygen linkage to the fat but differences between the original fat molecules may also be recognized.

In their survey of autoxidation Moureu and Dufraisse (3) recall the conception of Arrhenius that in a fluid made up of a definite chemical species, all of the molecules are not in the same state; a small proportion of them may be described as activated. At a given moment only a small portion of the entire number of molecules can enter into reaction. The velocity of a reaction is regulated precisely by the proportion of active molecules, and by the speed with which they are formed. It is possible to calculate the additional energy which must be acquired by molecules in the mean state to become activated for a given reaction. This minimum additional energy requirement Arrhenius called the critical increment of energy.

Many factors may facilitate the acquisition of a sufficient critical increment of energy by fat molecules in the average state. The agencies may be mechanical, such as collision of the molecules with the walls of the containing vessel; physical, such as light, especially ultraviolet, and heat, or they may be chemical, such as metals like copper and iron and their salts, and the fat peroxides themselves. The actual state of an activated molecule may be visualized in various ways, such as the possession of a higher potential, or of molecular valence electrons (4). Not only does it possess a sufficient amount of energy to take part in a reaction, but when it does so, a highly reactive product molecule is formed, whose excess energy is transferred to another average reagent molecule, enabling

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