Fatty Acid Analyses of Known Mixtures of Purified Methyl Esters[1]

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ACCURATE quantitative analysis of the fatty acid composition of fats and oils has been dependent upon a preliminary separation of the acids, usually by fractional distillation of their methyl or ethyl esters at reduced pressures, into a series of fractions intended to contain not more than two saturated and not more than two homologous groups of unsaturated acids. A useful, if not in many cases essential, first step in the analysis of a mixture of fatty acids has been the segregation, so far as possible, of the saturated from the unsaturated acids. In the majority of reported analyses use has been made of the relative insolubility in alcohol of the lead salts of saturated acids to accomplish this end; and in some cases the same result has been attained simply by careful fractional crystallization of the mixed acids themselves. If proper preliminary separations have been accomplished so that the final fractions collected for physical and chemical analysis meet the conditions mentioned above, and if in the process stearic acid and higher molecular weight saturated acids have been removed quantitatively from the predominately unsaturated acid group, then it is possible to calculate the composition of each individual fraction from analytical data such as the saponification number or equivalent, the iodine number, and the thiocyanogen number by a series of simultaneous equations. From the analysis of each fraction the composition of the original entire fatty acid mixture can be readily ascertained.

In a discussion of this usual procedure, involving lead-salt separation of the mixed acids prepared from a fat and the fractional distillation of their methyl esters, Hilditch (1) estimated that the analysis of resulting fractions was reliable to about one unit per cent. This accuracy, although not so satisfactory as would frequently be desired, has been acceptable in the characterization of many naturally occurring fats and products produced or derived from them (2).

On several occasions repeated analyses of the same fat have yielded results in agreement with the precision indicated by Hilditch. There has not been published, however, an analysis of known mixtures of highly purified acids by this technique. Crowther and Hynd (3) did analyze a known mixture of the methyl esters of oleic acid and saturated acids from C_4 to C_{18}. Their calculated analysis agrees incredibly well with the known composition of the mixture. It is clear, however, that with the procedures used at that time the calculations would have been invalid if more than one unsaturated component had been present.

The very realization that it has been desirable to resolve a group of mixed acids into predominantly saturated and predominantly unsaturated groups has not infrequently limited the use of the "ester distillation" procedure. Time and materials are consumed in effecting the resolution of the mixed acids and the eventual analysis of the two separate groups. Doubtless for these reasons there has been an occasional offering of quantitative data based on analyses of ester fractions resulting from the fractional distillation of esters prepared (a) directly from a complex group of saturated and unsaturated acids or (b) by simple methanolysis of the fat itself. Analyses of the resulting fractions by determinations of the mean molecular weight, the iodine value, and the thiocyanogen value may be grossly inaccurate if, for example, there is present in a single fraction a mixture of palmitic, palmitoleic, stearic, oleic, and linoleic acids. At best, the procedure can be used reliably only when accompanied by satisfactory qualitative identification.

Although it has been pointed out elsewhere (2) that direct methanolysis of a fat is not a satisfactory procedure to use in quantitative fatty acid analytical work, the reasons seem to need reemphasis. Alcoholysis is a convenient and quick means of preparing esters from fats. The reaction does not go to completion, however, and there is no satisfactory means of removing the unchanged glycerides from the ester mixture. It is usually not feasible to distill all the methyl (or ethyl) esters produced. Consequently the "residue" from any such analytical distillation must inevitably contain a mixture of methyl (or ethyl) esters of high molecular weight acids plus the unreacted glycerides. The quantitative analysis of such a mixture for its methyl (or ethyl) ester content would be difficult indeed; and the amount of the uncalculated esters throws in error other calculations on the distilled fractions.

There has been a desire in this laboratory for some time to accomplish a simplification of the expensive, time-consuming procedures in fatty acid analyses without reducing the accuracy and precision of the results from those possible with the customary procedures. Toward that end Mattil and Longenecker (4) studied the use of the refractometer in acid and ester analyses and found that, with only small samples, considerable information could be derived from refractive index measurements. In many instances the physical measurement has replaced the more cumbersome but not more accurate chemical determination of the mean molecular weight.

The development of the technique for quantitative determination of highly unsaturated fatty acids by spectral analysis after their isomerization at high temperatures (5) has led to a further simplification of the procedure for fatty acid analysis in which no preliminary separation of acids into predominantly saturated and predominantly unsaturated groups is necessary. This simplified procedure has been in use in this laboratory for more than two years with very satisfactory results. It is described in detail as applied in the analysis of known mixtures of highly purified methyl esters.

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Materials and Methods

Preparation of Methyl Esters.[3] The esters used in these analyses consisted of a series of highly purified methyl esters. Saturated esters of lauric, myristic, palmitic, and stearic acids were prepared from fatty acids with essentially theoretical saponification equivalents and zero iodine value. Methyl oleate was prepared by the crystallization procedure of Brown and Shinowara (6). Methyl linoleate and methyl linolenate were prepared by debromination of tetrabromostearic acid (m.p. 115) and hexabromostearic acid (m.p. 185) in methanol (7). In Table I are presented some analytical constants of the methyl esters used in compounding the mixtures.

TABLE I

Criteria of Purity of Methyl Esters Used

Methyl ester	Iodine value (Wijs)		Saponification equivalent		Refractive index ($n_D^{40.0°}$)
	Theory	Found	Theory	Found	
Laurate	0.0	0.0	214.2	214.1	1.42363
Myristate	0.0	0.0	242.2	242.4	1.42882
Palmitate	0.0	0.0	270.3	270.5	1.43310
Stearate	0.0	0.0	298.3	298.4	1.43608
Oleate	85.7	84.7	296.3	296.4	1.44380
Linoleate	172.5	173.2	294.3	294.5	1.45312
Linolenate	260.6	260.0	292.2	292.4	1.46320

Distillation of the mixtures. The purified methyl esters were mixed in definite proportions by weight and fractionally distilled at reduced pressure using an electrically heated column of about 12 theoretical plates (8), packed with glass helices and equipped with a total condensation, partial take off distilling head (9) which permitted only a minimum admixture of successive fractions. The course of the distillations was followed by noting the change in refractive indices of the fractions as they were received.

TABLE II

Analytical Data for Mixture 1

Fraction	Weight	Iodine value	Saponification equivalent	Methyl linoleate	Methyl linolenate	Refractive index 40.0°C.
	gm.	*(Wijs)*		*Pct.*	*Pct.*	
1	2.286	0.2	226.5	0.0	0.0	1.42398
2	1.902	0.1	229.6	0.0	0.0	1.42684
3	2.783	0.0	239.7	0.0	0.0	1.42821
4	3.251	0.0	250.4	0.0	0.0	1.43002
5	4.579	3.3	268.7	0.46	0.43	1.43288
6	2.082	19.3	278.0	2.96	1.82	1.43458
7	2.785	62.4	282.8	10.81	7.76	1.44050
8	4.109	93.9	290.2	15.45	11.15	1.44438
9	19.294	100.1	295.2	14.05	13.60	1.44563
Res.	2.079	101.8	296.5	12.61	14.23	1.45310

TABLE III

Analytical Data for Mixture 2

Fraction	Weight	Iodine value	Saponification equivalent	Methyl linoleate	Methyl linolenate	Refractive index 40.0°C.
	gm.	*(Wijs)*		*Pct.*	*Pct.*	
1	1.088	0.2	214.6	0.0	0.0	1.42402
2	2.096	0.0	219.5	0.0	0.0	1.42410
3	3.170	0.0	239.0	0.0	0.0	1.42822
4	1.706	0.0	249.6	0.0	0.0	1.42970
5	3.279	0.0	268.0	0.0	0.0	1.43235
6	3.448	1.0	272.4	0.16	0.24	1.43291
7	2.883	12.1	274.6	1.81	2.84	1.43480
8	2.964	100.9	291.1	9.79	16.26	1.44505
9	1.943	114.4	295.0	10.79	19.30	1.44690
10	16.173	88.9	297.0	6.02	16.80	1.44382
Res.	1.153	54.0	295.9	2.80	9.79	.....

Analyses of Fractions. Analytical data for the various fractions collected from two separate ester mixtures are indicated in Tables II and III. The percentages of methyl linoleate and methyl linolenate in each fraction were determined by measurement with a Beckman quartz spectrophotometer of the spectral absorption of the soaps resulting from alkali isomerization of the esters. The method used was essentially that developed by Mitchell, Kraybill, and Zscheile (5). Alkaline glycol used in the isomerization was prepared by dissolving in redistilled ethylene glycol 10 gm. of reagent grade potassium hydroxide per 100 ml. of glycol. Solution was heated before use to 190°C., cooled to room temperature and made up to 100 ml. with ethylene glycol Approximately 0.1 gram of fat to be analyzed was weighed into the bottom of a standard-taper, glass-stoppered, pyrex test tube. Two ml. of the alkaline glycol was added by pipette to the samples and to a blank, and the loosely stoppered test tubes were placed in a constant temperature oil bath at 185°C. At three successive one-minute intervals the tubes were removed and shaken to mix thoroughly the fat and reagent. After exactly 30 minutes total heating time the tubes were removed from the oil bath and placed immediately in cool water. The isomerized soaps and excess reagent were transferred with triple distilled water to volumetric flasks and further diluted to optical densities suitable for measurement in the Beckman spectrophotometer. The peak optical densities of the soap solutions were then measured at 234 mμ and 270 mμ against the blank diluted to the same concentration. $E^{1\%}_{1\ cm.}$ values were calculated by the relationship, $E^{1\%}_{1\ cm.} = \frac{d}{cl.}$ where d is the optical density, c is the concentration of fat in the dilution measured, and l is the length of the absorption cell in centimeters.

TABLE IV

Compositions of Methyl Ester Mixtures

Methyl ester	Mixture 1		Mixture 2	
	Actual weight	Found weight	Actual weight	Found weight
	Pct.	*Pct.*	*Pct.*	*Pct.*
Laurate	6.1	6.4	8.1	8.4
Myristate	14.3	15.1	12.2	11.4
Palmitate	21.0	20.6	24.3	24.8
Stearate	11.5	11.8	18.4	18.0
Oleate	29.8	29.2	24.1	24.0
Linoleate	9.2	8.8	3.8	3.9
Linolenate	8.1	8.1	9.1	9.5

For calculation of the percentage of methyl linolenate the observed $E^{1\%}_{1\ cm.}$ at 270 mμ was compared with that obtained when a highly purified methyl linolenate was isomerized. The methyl linoleate present was obtained by comparison of the observed $E^{1\%}_{1\ cm.}$ at 234 mμ after correcting for absorption due to triene material, with the corresponding value obtained for isomerized purified methyl linoleate.

The composition of each fraction was then calculated from the analytical data given in Tables II and III, using the equations described previously (10). The component esters in the fractions having essentially zero iodine values were calculated directly from their saponification equivalents. The composition of the more complex fractions, those containing methyl palmitate, stearate, oleate, linoleate, and linolenate, was facilitated by calculation first of the methyl linoleate and linolenate from the spectral analyses. The

[3]The authors are indebted to Drs. T. R. Wood and F. L. Jackson for their kindness in furnishing some of the materials used in this study.

iodine value was then corrected for these components to give the amount of methyl oleate. The proportions of the saturated esters were computed from the saponification equivalents after correction for the known amounts of the unsaturated esters.

In Table IV are compared the original composition of the two mixtures and the composition calculated from a summary of the analyses of all the fractions.

Discussion

THE results of these analyses indicate that an accuracy of somewhat less than one unit per cent of the methyl ester in question can be obtained by using the above procedure. In fact, the calculated analyses and the actual analyses of the *individual components* differ on the average by less than 4% of the amount present.

It is recognized that hydrogenated fats may have complicating factors which might tend to decrease the accuracy somewhat but a series of duplicate analyses of hydrogenated shortenings in this laboratory have been found to agree well within the one unit per cent range. Natural fats such as milk fats (9, 11) and guinea pig body fats (12) have been analyzed using the above method and it has been our experience that the complex highly unsaturated C_{20} esters require further study by bromination or crystallization techniques to characterize completely their individual components. However, this is also true of the other procedures for fatty acid analyses. It should be remembered, as Hilditch has pointed out, that the methyl ester distillation technique of fat analysis is insufficient in itself for the identification with certainty of very small amounts of any specific component. The spectrophotometric analyses will indicate quantitatively diene, triene, and tetraene material and this information together with iodine values and saponification equivalents will afford a very close approximation to the composition of most fractions resulting from a methyl ester distillation.

Summary

Mixtures of known composition of purified methyl esters of lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acids have been prepared. Fractional distillation under reduced pressure followed by spectrophotometric determination of methyl linoleate and methyl linolenate in each of the fractions and determination of iodine values and saponification equivalents allowed calculation of the compositions which agreed well with the compositions of the original mixtures.

REFERENCES

1. Hilditch, T. P., Biochem. J., *28*, 779 (1934).
2. Hilditch, T. P., The Chemical Constitution of the Natural Fats, John Wiley and Sons, N. Y. (1941).
3. Crowther, C., and Hynd, A., Biochem. J., *11*, 139 (1917).
4. Mattil, K. F., and Longenecker, H. E., Oil and Soap *11*, 16 (1944).
5. Mitchell, J. H., Jr., Kraybill, H. R., and Zscheile, F. P., Ind. Eng. Chem., Anal. Ed., *15*, 1 (1943).
6. Brown, J. B., and Shinowara, G. Y., J. Am. Chem. Soc., *59*, 6 (1937).
7. Rollett, A., Z. physiol. Chem., *62*, 410 (1909).
8. Longenecker, H. E., J. Soc. Chem. Ind., *56*, 199T (1937).
9. Baldwin, A. Richard, and Longenecker, H. E., J. Biol. Chem., *154*, 255 (1944).
10. Longenecker, H. E., Oil and Soap, *17*, 53 (1940).
11. Baldwin, A. Richard, and Longenecker, H. E., J. Biol. Chem., *155*, 407 (1944).
12. Baldwin, A. Richard, Longenecker, H. E., and King, C. G., Arch. Biochem., *5*, 137 (1944).

Report of the Bleaching Methods Committee 1944-45

THIS Committee's work during the 1943-44 season was concentrated on studying the bleaching response of refined soybean oil against various natural and activated clays as the oils were aged under normal storage conditions. A rather marked change, a deterioration of the bleach in the case of the more active natural clays and activated clay led to a further study designed to establish whether the change was primarily in the oil, or in the bleaching material, or both. As reported in a supplement to last year's report, Oil & Soap, *22*, 22 (1945), this change was shown to reside in the refined oils, and early work during the current season furnished evidence that the deterioration was associated with the fairly rapid development of peroxide in stored refined oil samples.

Since the 4% activated clay bleach test was proposed specifically for the grading of the refined bleach color of crude soybean oils, the attention of the Committee was then directed to learning if the bleaching response on stored crude oil (freshly refined just before making each bleach test), also deteriorated and at what rate.

The results were brought together at a meeting of the Committee in Chicago in October, 1944. Bleach response-aging data were presented covering seven crude expeller oils studied in five different laboratories. The results are shown in Table I and indicate that the deterioration of refined bleach color in stored crude is negligible or non-existent. One member determined the change in bleaching response on storing a quantity of refined oil derived from the same crude, at the same time that the crude itself was being held and periodically tested. Peroxide values were run on both crude and refined. The results rather strikingly show the apparent connection between formation of peroxide in the refined oil and partial inactivation of the activated clay. (See Sander's data, Table I and Fig. 1; also see Table III). In this particular case the bleaching response of the refined oil against official A.O.C.S. Fuller's Earth also deteriorated whereas in most cases that remains substantially constant. Attention is called to the constancy of the 4% activated clay bleach in the case of the crude oil.

At the meeting in Chicago one member pointed out that all of these results were obtained on only one type of crude soy i.e., expeller. Arrangements were then made to get similar data on extracted oil, degummed and non-degummed, and on hydraulic oil. The results on these have just been completed and are given in Table II. Again the bleaching response remained uniform well within the normal irregularities of reading colors. At least one of the laboratories ran peroxide numbers on the oil and found them to remain very low as compared with the rapid increase noted in the case of holding refined oils. We con-