of experimental error and to have the plausible explanation of contamination of the standards with traces of linoleic and saturated acids (28). In any case, such a revision would result in only an insignificant modification of the computations. However, the deviation of linoleic acid is real and unquestionably significant in the analysis of the semi-drying oils. It therefore appears desirable that in reporting thiocyanogen values, the exact experimental conditions should be indicated, and where the 0.2 N reagent is used the specified conditions the modified calculations proposed above should be used, while with the 0.1 N reagent, either the 3 hour reaction period and the theoretical equations, or the following equations based on an average value of 93 for the 24 hour period may be tentatively recommended for closer approximation to the true composition of mixed free fatty acids:

$$\% \text{ Linoleic Acid} = 1.135 \text{ (I. V. — T. V.)}$$
$$\% \text{ Oleic Acid} = 2.287 \text{ T. V. — } 1.174 \text{ I. V.}$$
$$\% \text{ Solid Acids} = 100 - \% \text{ (L. A. + O. A.)}$$


### Summary

1. Only one form of linoleic acid, identical with the octadecadienoic acid regenerated from either *alpha* or *beta* tetrabromostearic acids, was found in a series of seed fats of widely different compositions.

2. The tetrabromide number was shown to be of questionable significance in that the precipitation of *alpha* tetrabromostearic acid is markedly affected by the component fatty acids of the oil.

3. Although the iodine numbers (Wijs) of pure linoleic and linolenic acids agreed closely with the theoretical, their thiocyanogen values determined according to the officially recommended methods were found to be 96.3 and 171 respectively, these values further varying with the conditions of the determination. The substitution of the empirical values for the theoretical constants in the accepted equations for the calculation of the concentration of unsaturated fatty acids was therefore suggested.


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# The Use of Fatty Acid Ester Distillation Methods in Fat Analysis*


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THE first serious efforts to elaborate the composition of natural fats by distillation methods were made towards the end of the nineteenth century. They followed the laborious attempts of Heintz, Bömer, Klimont, Amberger and others to determine fat compositions by repeated crystallizations. As early as 1877 Krafft (1) attempted the distillation of castor oil but succeeded only in cracking the oil. In later studies between 1880 and 1903, Krafft and his students (2) used high vacuum techniques for the distillation of fatty acids and the derived aldehydes and esters. In the same year, 1903, both Krafft (2) and Kreis and Hafner (3) resorted to fractional distillation in order to obtain relatively pure palmitic and stearic acids for glyceride synthesis.

Haller and Youssoufian (4) described the fractional distillation of methyl esters of coconut oil fatty acids in 1906, but made no claim for quantitative results. These workers obtained methyl esters by methanolysis of the fat as described by Haller (5). Hilditch (6) has since pointed out that direct alcoholysis is an unfortunate method to use for the preparation of fatty acid esters in quantitative fat analysis because it offers no opportunity for an initial separation of the mixed fatty acids into saturated and unsaturated groups. The alkali required for the reaction inevitably causes some soap formation although soaps may be removed from the esters. Unchanged glycerides, however, cannot be removed by any known method.

Bull (7) contributed materially to the problem of fat analysis by ester distillation in 1906. His careful fractionation of the methyl esters of cod liver oil fatty acids was carried out with a heated column provided with total reflux.

Fractional distillation of coconut oil itself in high vacuum was undertaken in 1909 by Caldwell and Hurtley (8) with some degree of success. A similar attempt was described later by Bömer (9).

In 1911, both Smedley (10), in England, and Holland (11), in this country, recorded their observations on the composition of butter fat based on methyl ester fractionations. Smedley obtained evidence for the existence of lower members of the oleic acid series.

The percentage composition of fatty acids in coconut and palm kernel oils was published in 1913 and 1914 by Elsdon (12). The results were calculated from analytical data obtained for esters separated by fractional distillation.


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Crowther and Hynd (13) in 1917 converted the mixed acids from butter fat into methyl esters for repeated fractional distillations. Unsaturated material was associated with even the lowest boiling fractions. Similar results on butter fat were reported by Holland and his associates (14) who desired to use methyl ester distillations as a practical analytical method to measure the effect of dairy cattle feed on the composition of the resulting butter fat. In 1924, Channon, Drummond and Golding (15) also submitted the methyl esters of butter fatty acids to the fractional distillation procedure as a step in their analytical examination of butter fat. They were lead to the conclusion, however, that "as an exact quantitative method, it is of little value."

It was at this time, when the ester distillation method was receiving its most serious criticism, that it also received rather suddenly its greatest impetus. Armstrong, Hilditch, Allan and Moore at Crossfield's laboratories in England published in 1925 the results of several detailed examinations of natural fats (16, 17, 18). These papers established the fact that even the most complex mixtures of fatty acids obtained from natural fats could be resolved to yield significant information regarding composition.

Under the guidance of Hilditch, the Liverpool school soon developed an extensive program for the study of fatty acid compositions and glyceride structures of natural fats. The Merseyside oil and soap industry undoubtedly gave this group of investigators a stimulus for research. The research group, in return, has supplied much reliable data on the composition of commercially and biologically important fats, and this represents an invaluable contribution to general progress in the technology and physiology of lipids. New incentive for lipid research in its broad applications was given other investigators in many parts of the world. Consequently, the present situation is, as Hilditch has remarked (19), ". . . . . it may now be claimed that our knowledge of the subject is fully abreast of that of other important groups of natural products."

In this country, Brown and Beal (20) first published their studies on the character of the unsaturated fatty acids of fish oils in 1923 and were able to use ester distillations effectively in making separations. With others (21), Brown continued to use the method for purification and analytical purposes.

Two factors unquestionably facilitated research with the ester distillation methods. First, the introduction into the distillation system of vacuum pumps capable of continuously evacuating laboratory distillation apparatus to 1 mm. or less of mercury made the process of fractional distillation of high boiling acids or their esters much less difficult than with the older high vacuum technique. Second, the fractionation equipment available for ester distillations was quite simple until the last decade. However, the rapid concurrent development of theoretical knowledge of the fundamental principles involved in fractional distillations has brought about the construction of entirely new types of distillation equipment. This equipment was gradually designed to satisfy so far as possible all theoretical requirements for maximum separation of mixtures.

Analysis of a fat for its constituents by the ester distillation method requires a maximum resolution of mixed esters into samples containing not more than two adjacent groups of homologous series, e.g. $C_{16}$ and $C_{18}$, not $C_{14}$, $C_{16}$, and $C_{18}$. In 1930, several reports emphasized the necessity of utilizing every means possible to effect such separations. Jantzen and Tiedcke (22) described a packed distillation column which they built for the separation of saturated acids of high molecular weight. The column itself was protected from temperature fluctuations by the use of an evacuated jacket and an electrical heating unit. Other workers (23,24,25) have reported excellent results with this column for the separation of ester mixtures. Somewhat similar columns were discussed by Evans and his associates (26,27) and Bush and Schwartz (28).

Several other refinements in fractional distillation practice were introduced with a few years to give the operator better control over the conditions of a distillation. A still head designed for a variable up to a total reflux was used in a fractionating column by Whitmore and Lux (29). A similar still head, capable of being removed and fitted to other columns of varying length and diameter by employing interchangeable ground glass connections, was described by Wagner and Simons (30). The effect of packing materials on distillation efficiency (as measured by the production of a low height equivalent to the theoretical plate, H. E. T. P., (31) ) has been demonstrated by Fenske and associates (32). Single turn glass helices (33) were shown to be very efficient. They are particularly desirable for fatty acid ester distillations. Several excellent methods are available for their preparation (34,35).

Since 1934, we have used such a column in which these refinements are combined. Its usefulness in the separation of fatty acid ester mixtures has been demonstrated repeatedly for both the qualitative and quantitative examination of natural fats (36,37,38,39,62). The main features of the column are (a) the use of single turn glass helices for the packing, (b) electrical heating of the column which may be regulated to control equilibrium conditions between the liquid and vapor phases, (c) direct measurement of boiling points, (d) total condensation of the vapor, and (e) adjustment of the distillation rate so as to maintain a high reflux ratio (ratio of the vapor condensate returned to the column to that collected as distillate).

The quantity of material used for distillation with this column has ranged from 1 to 500 grams. Twenty grams or more of a fatty acid mixture may be analyzed to within 1 per cent of the total acids, but it has not been possible to attain the same degree of success in separating smaller amounts of material. It is believed, however, that these difficulties are not due to an incapacity of the column to separate small amounts but rather to drainage problems during distillate collections. Klenk (40) suggested the use of a capillary tube in an apparatus of his own design, partially to avoid this difficulty. The capillary tube has been found to be of considerable service in microfractions using both Klenk's column and a modification of the packed column mentioned above. This modification consisted simply of the use of a capillary tube and stopcock in the stillhead, replacing the 7 mm. tube previously used. The holdup of this column (40 x 1.2 cm.) was consistently less than 50 mg.

Other workers have reported descriptions of microfractionation equipment. The column of Cooper and Fasce (41) was improved by Wesson (42). Schoen-

heimer and Rittenberg (43) constructed a unit consisting of three concentric tubes through which the vapors were deflected before collection. This equipment proved quite satisfactory for palmitatestearic mixtures. Klenk (24) claimed a high efficiency for a similar apparatus designed independently as a modification of the one he had described earlier (40).

Klem (44) found a small Podbielniak apparatus (45) quite satisfactory for the separation of a mixture of palmitic, stearic, oleic and elaidic methyl esters. The Stedman column (46,47) has been used by Whitekamp and Brunstrum (48) to effect good separations of various ester mixtures. For the distillation of highly unsaturated acids of fish oils, Farmer and van den Heuvel (49) have suggested the use of molecular distillation equipment to reduce the opportunity for polymerization at the very reactive double bonds.

Details of the methods used for the quantitative analysis of fats by the ester distillation procedure have been published several times (18,19,50,51). Hilditch (6) has correctly pointed out that the order of accuracy obtainable depends almost entirely on the degree to which it is possible to obtain ester fractions with "no more than two adjacent homologous saturated, and two adjacent homologous unsaturated members." Perhaps this ideal condition is rarely attainable. It is, nevertheless, true that it may be approached very positively, as Hilditch suggests, first, by taking advantage of available means for making preliminary separations of saturated from unsaturated acids as complete as possible, and second, by using efficient fractionating equipment for both the primary ester distillations and refractionations. It is possible to limit the occurrence of more than two adjacent members of an homologous series with the strictest observance of these two suggestions.

Even with efficient fractionation equipment, ester fractions containing both saturated and unsaturated esters are commonly obtained in the course of fat analysis. With a knowledge of the qualitative composition of these mixtures the quantitative composition may be quite readily calculated by using simultaneous equations for (1) weight of ester fractions, (2) equivalent weights, and (3) iodine values so long as the mixture contains not more than two saturated and two unsaturated components.

Seven types of such ester mixtures found in distilled fractions may be calculated. They are (I) two saturated, (II) two unsaturated, (III) one saturated and one unsaturated, (IV) two saturated and one unsaturated, (V) one saturated and two unsaturated, (VI) one saturated and three unsaturated, and (VII) two saturated and two unsaturated. Components of these mixtures usually may be calculated from fractionation data including weight, saponification equivalent, and iodine value of the ester fractions. The following equations are used:

$$(1)\quad s + u + u' = w$$

$$(2)\quad \frac{s}{S} + \frac{u}{U} + \frac{u'}{U'} = \frac{w}{W}$$

$$(3)\quad u.I_u + u'.I_{u'} = w.I_w$$

where s, u and u' represent the weights of saturated and two unsaturated esters in an ester fraction of weight w, and S, U, U' and W are the corresponding equivalent weights, and $I_u$, $I_{u'}$ and $I_w$ are the corresponding iodine values.

The simplest mixtures obtainable are ester fractions which include only saturated (or unsaturated) esters of two groups in the homologous series (e.g., $C_{14}$ and $C_{16}$ or $C_{16}$ and $C_{18}$). The two saturated components (Type I) may be evaluated directly from their saponification equivalents as indicated for a methyl palmitate — stearate mixture (from equation 2 above):

$$\frac{\text{wt. Me palmitate}}{270.3} + \frac{\text{wt. Me stearate}}{298.3} = \frac{\text{wt. of ester fraction}}{\text{sap. equiv. (fraction)}}.$$

Two unsaturated esters (Type II) are best calculated from iodine values (equation 3) rather than equivalent weights since a difference of only 0.1 in the latter corresponds to a change of 4-5 units in the iodine value.

Type III is also a simple case. The amount of only one unsaturated component is obtained directly from the iodine values of the ester fraction and theoretical iodine values of the unsaturated component (equation 3). For example, the amount of methyl oleate in a mixture with methyl palmitate is given by:

$$\text{wt. Me oleate} = \text{wt. ester fraction} \times \frac{\text{I.V. (ester fraction)}}{\text{I.V. (Me oleate)}}.$$

The amount of the single saturated component is obtained by difference.

When there are two saturated esters present with only one unsaturate (type IV) the procedure is identical in obtaining the weights of unsaturated and total saturated components. The equivalent weight of the saturated esters must be calculated from equation (2), however, before the individual components are estimated:

$$\text{Sap. equiv. (sat'd esters)} = \frac{\text{wt. of sat'd. esters}}{\frac{w}{W} - \frac{u}{U}}$$

In type V, with two unsaturated esters of the same carbon content, it is necessary to establish the ratio of the occurrence of the unsaturated esters. This is determined from the iodine value — equivalent weight curves plotted for both the ester fractions and the theoretical values of the unsaturated esters. The mean unsaturation deduced for a group of esters, e. g., oleate and linoleate, may then be used to obtain the weight of total unsaturated esters (exactly as for Me oleate in type III above). Equation 2 is then employed for the weights of the individual unsaturated components, and equation 1 for the saturated component. In this connection it is important to note no appreciable separation of methyl oleate from methyl linoleate has yet been accomplished.

The case of one saturated and three unsaturated components (Type VI) may be represented by palmitate, palmitoleate, oleate and linoleate esters. Here again, the ratio of occurrence of the esters in the same group (oleate and linoleate) must first be ascertained. The individual components are then calculated by using the data thus obtained in equations 1, 2, and 3 simultaneously.

When two saturated and two unsaturated esters of adjacent groups (type VII) are found in a single ester fraction information in addition to the weights, saponification equivalents, and iodine values of the ester frac-

tion is required before calculations can be made. It will be observed that the problem may be reduced to that indicated in type V if the equivalent weight of the mixture of saturated esters is known. This value may be obtained after the oxidation and removal of the unsaturated esters by the method of Armstrong and Hilditch (17) or by the modification of Steger and van Loon (52) using the precautions given by Harper, Hilditch and Tereleski (51). The results of such oxidations usually show that the equivalent weight of the saturated esters is very close to the equivalent weight of the whole ester fraction. This is especially true when an efficient column has been used for the ester distillations.

In this way, the approximate composition of each ester fraction obtained during a distillation may be determined. It is then a relatively simple procedure to total the compositions for both the saturated and unsaturated groups of esters (corresponding to the same groups of acids separated originally), convert these values for esters to values for acids, and then, using the data obtained from the original separation of the mixed acids, construct the fatty acid composition of the original fat.

Spadola and Ellis (53) included a fourth working equation based on thiocyanogen values in their calculations of ester fractions ultimately obtained from rat body fat. There is justification for the use of the thiocyanogen number (in addition to the iodine number) in the analysis of fats containing linoleic acid. In this connection it is important to mention the recent findings of Kass (54) who has proposed corrected formulas for the percentage of linoleic, oleic and saturated acids in a given sample as follows:

% linoleic acid = 1.180 (I. No. — CNS. No.)
% oleic acid = 2.377 CNS. — 1.265 I. No.
% saturated acids = 100 — (% linoleic + % oleic acids).

Reference has been made briefly to the usefulness of efficient distillation equipment for the qualitative determination and separation of fatty acids present in natural fats. As recently as 1925, Grün (55) pointed out the paucity of our knowledge regarding the fatty acids of fats and waxes. The explanation lies, of course in the fact that fatty acids are difficult to separate in pure state, completely free of isomers and homologs. Since 1925, our knowledge of the extent to which particular fatty acids occur in natural fats has been increased almost entirely as the result of diligent application of distillation methods.

Quantitative examinations of natural fats have so sharpened the ester distillation tool that it is now recognized as a logical means of approach to the study of such widely differing problems as the fundamental biochemistry of fat metabolism to industrial trouble shooting. Whereas previously many investigators relied solely on the simpler analytical constants such as iodine value, saponification value, etc., there is now a steadily growing feeling, based on experience, that these values by their very nature are insufficient to indicate fatty acid compositions. Several cases (out of many which could be cited) are given to illustrate the utility of the procedure.

In industries using fats and oils, a knowledge of the fatty acid composition is frequently indispensable. With such information it is indeed unlikely that technologists shall have to bear again the chagrin of their forerunners when hydrogenated fats were being introduced in 1908-1910. Whale oil, hardened to the same melting point and iodine value as a natural tallow, did not produce by any means the same soap as tallow as was expected. The difference in the nature of the soaps may be attributed, as is well known, to the fact that whale oil contains mainly $C_{14}$, $C_{16}$, $C_{18}$, and $C_{20}$ acids while tallow consists mainly of $C_{16}$ and $C_{18}$ acids. The quantitative differences in fatty acid composition of partially hydrogenated whale oil and a beef tallow with approximately the same iodine value and equivalent weight are striking.

| | Saponification equivalent | Iodine value |
|---|---|---|
| Beef tallow (Hilditch and Longenecker (37) | 286.4 | 44.7 |
| Partially-hydrogenated whale oil (Hilditch and Tereleski (56) ) | 286.5 | 48.8 |

| | Partially-hydrogenated whale oil | | Beef tallow | |
|---|---|---|---|---|
| Acids | Saturated acids | Unsaturated acids | Saturated acids | Unsaturated acids |
| | % mol. | % mol. | % mol. | % mol. |
| C14 | 11.1 | 2.2 | 3.9 | 0.5 |
| C16 | 25.6 | 8.2 | 26.5 | 2.6 |
| C18 | 10.4 | 25.9 | 23.1 | 42.2 |
| C20 | 2.6 | 9.5 | 0.7 | 0.5 |
| C22 | 0.3 | 4.2 | — | — |

Another important contribution has been the detailed study of the process of selective hydrogenation of the more highly unsaturated fatty acids. Hilditch and Jones (57) made a very thorough quantitative investigation of the course of hydrogenation of olive and cottonseed oils confirming earlier qualitative indications that linoleic acid was hydrogenated almost completely to the oleic acid stage before appreciable amounts of stearic acid were found. The Liverpool group have contributed other similar studies on more complicated glycerides with results that indicate a stepwise reduction of polyethenoid acids rather than direct formation of the saturated derivatives (56,58, 59).

In the biological field, increasing use is being made of the ester distillation tool. Lovern (60) has been particularly active in demonstrating the fatty acid compositions of various fishes. The experiments of Ellis and his coworkers (53, 61) showed beyond any question the quantitative effects of dietary fat upon body fat. Our discovery (39) of large amounts (to 15%) of $\Delta^{9,10}$ — hexadecenoic acid in rat body fat which was synthesized from carbohydrate or protein was made easier by recourse to efficient ester distillations for both the qualitative and quantitative analysis of the fat. Smith and Dastur (62) contributed to the study of milk fats the finding that after several days' fasting the fatty acids of lower molecular weight, $C_4$ to $C_{14}$, were decreased about 75%, the deficiency being made up by an increase in oleic acid. Interestingly enough, this parallels our results on the ultilization during inanition of rat depot fat containing high amounts of fatty acids of low molecular weight (37).

The knowledge of fatty acid compositions forms the basis for several other significant developments. (a) The exceedingly difficult determination of glyceride structures has been remarkably clarified by Hilditch and his associates by a combination of ester distillations with either acetone-permangenate oxidations or crystallizations of relatively simple fats. These studies have elaborated the main principle of "even distribution" of fatty acids among the glycerides. (b) A new classification of natural fats in a completely rational manner is now possible. Hilditch (19) attempted the

first such classification on the basis of fatty acid compositions in 1935 and extended the effort in 1937 (50). Vegetable reserve fats were divided into fruit coat fats consisting almost entirely of varying proportions of palmitic, oleic and linoleic acids, for the most part "evenly distributed" as glycerides and seed fats with a considerable variety of fatty acids distributed to form mixed glycerides. Broad similarities in composition were observed when due regard for botanical family was considered. (c) Hilditch and Lovern (63) have pointed out some interesting relationships between progressive differences in plant or animal fat compositions and the place of the plant or animal in the scale of evolutionary development. In general, there appears to be a marked simplification in fatty acid composition as one proceeds in the direction of more highly developed plants or animals.

Ester distillation methods have proven an invaluable aid to the extension of our knowledge of lipid components and the structural patterns in natural lipids. It is to be expected that increasing use of the methods will continue to prove of assistance in varied fields of lipid research.

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