Exploitation of the Selectivity of Various Chromatographic Techniques for the Study of the Triglyceride Structure of Natural Fats[1]

M. J. McCARTHY and A. KUKSIS, Department of Biochemistry, Queen's University, Kingston, Ontario, Canada

Abstract

The separation of naturally occurring triglyceride mixtures on the basis of mol wt, polarity, and iodine number by the methods of gas-liquid, liquid-liquid, and thin-layer chromatography, respectively, rarely results in fractions representing pure triglycerides. Furthermore, most of the fractions obtained are too complex for detailed structural analyses. An integrated system using all three of these techniques for a sequential fractionation and isolation of triglyceride groups of progressively decreasing complexity, however, while not yielding single glycerides, can provide practical information for the assignment of structures. Since the possibility of the same components overlapping in all three systems is very small, a prudent application of these techniques affords triglyceride groups that can be analyzed meaningfully by enzymatic methods, and, in many cases, can lead to a complete assessment of the triglyceride structure of the mixture.

For an examination of the scope of this integrated analytical system, selected naturally occurring triglyceride mixtures were used as models. The success of the system rests upon accurate quantitative determination of the fatty acids and triglycerides by gas chromatography.

Introduction

A LARGE PART of the present knowledge of glyceride structure is derived from an extensive series of investigations on many different fats and oils carried out over a period of years by Hilditch and coworkers (1). These investigations led to the deduction of the rule of even distribution governing the composition of natural fats and a disproof of the older claim that natural fats contained mostly simple triglycerides with three identical fatty acid chains (2). Since the demonstration of Hilditch that mixed glycerides predominate in nature, long and tedious discussions have taken place to determine whether the chain distribution was really "even," "random" or "partly random."

A partial solution to this problem has been provided by the discovery of the specificity of pancreatic lipase. Thus, by hydrolyzing the triglyceride mixtures with lipase and comparing the composition of the free and the monoglyceride fatty acids, it has been possible to demonstrate that the chain distribution is never random (3,4). It is obvious, however, that enzymatic positional analysis is not an ideal manner of determining glyceride structure, when performed on a complex mixture of triglycerides. Before such studies become meaningful, an effective preliminary segregation of the fat is obligatory. A combination of the oxidative degradation techniques with adsorption chromatography followed by an enzymatic examination of the isolated fractions (5) has yielded superior data, but has also provided new stimuli for statistical speculation. The inability of this system to separate the saturated triglycerides and to distinguish between the mono- and poly-unsaturated fatty acid residues of the unsaturated triglycerides has prevented a complete determination of the structure of any triglyceride mixtures.

Successful fractionation of natural triglycerides by gas chromatography (6) has allowed a new approach to the determination of triglyceride structure. Though the present method of gas chromatography does not allow the isolation of any of the triglyceride peaks, the qualitative and quantitative data obtained by examination of the detector response has permitted a demonstration of the existence of specific non-random distributions for all natural fats investigated (7). The failure to distinguish between different triglycerides of the same mol wt or carbon number, has prevented this method from attaining the ultimate separation and identification of individual triglycerides. A combination of the oxidative techniques of triglyceride degradation with high temp gas chromatography of the oxidation products, while giving both qualitative and quantitative information about the structures of numerous triglycerides, has similarly failed to provide a complete system of analysis on account of not differentiating between the various delta-9-ethylenic fatty acid residues (8).

Other methods for the systematic segregation of natural triglyceride mixtures depend on unsaturation and polarity (a combination of unsaturation and mol wt effects), and produce other groups of inseparable or overlapping triglycerides, none of which are simple enough for an effective enzymatic positional analysis (9,10,11). The recognition that no single technique available presently is capable of such fractionation of natural triglyceride mixtures that would be sufficiently refined for exact positional analysis, has prompted us to look for suitable combinations of these methods in the hope that a practical system could be worked out on this basis.

To utilize effectively the individual chromatographic systems or their combinations for the study of triglyceride structure, it is instructive to review the principles involved in bringing about these separations. The separations obtained on gas chromatography would appear to be based entirely upon differences in mol wt or carbon numbers. They do not distinguish between unsaturated and saturated glycerides having the same carbon numbers. Problems may arise on account of branched or oxygenated fatty acid chains which have migration rates different from those of the normal chain paraffinic acids. The occurrence of these materials in natural products, however, is restricted and need not complicate a general scheme of analysis. When the separations are monitored with a hydrogen

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TABLE I

Methods of Triglyceride Analysis

Chromatographic Method	Basis of Separation	Designation [a]
Gas-liquid	Mol wt or carbon number	C (Carbon number)
$AgNO_3$-thin layer	Unsaturation or iodine number	I [b] (Iodine number)
Partition	Polarity (unsaturation + mol wt)	P (Polarity number)

[a] C = Number of fatty acid carbons in glyceride molecule.
I = (number of double bonds) × 2.
P = C − I.
[b] The iodine number refers to the number of iodine atoms added to the triglyceride molecule when completely iodinated. The polarity number is discussed in the text.

flame ionization detector, the recorded areas can readily be interpreted in terms of wt composition (14).

The triglyceride separations obtained on adsorption chromatography on silver-nitrate impregnated silica plates appear equally simple and are broadly based upon the iodine values. The saturated triglycerides are obtained together in one group. Although certain isomeric unsaturated glycerides may also be resolved, there is no mol wt or carbon number effect. That is, triolein overlaps with tripalmitolein, for example. This system, however, permits the separation of preparative quantities of triglycerides and is amenable to various techniques of qualitative and quantitative analyses (10).

The partition chromatographic techniques of triglyceride separation are the most complex, and are dependent upon both the mol wt and the iodine number or unsaturation. The contributions of the two effects can be readily separated in terms of the number of methylene units and the number of active hydrogens. This joint effect is usually referred to as polarity. As in the case of the fatty acid esters of simple alcohols and sterols, the polarity number of triglycerides can be calculated from the knowledge of fatty acid composition and expressed in terms of effective chain length (15). As a result, the nature of the segregation obtainable on this basis can be predicted for any natural triglyceride mixture. Although these theoretical polarity relationships are perfectly reproduced in practice only when dealing with fatty acid methyl esters, the adherence to the rule is sufficiently close with triglycerides to permit its effective experimental exploitation in partition systems with a limited number of theoretical plates.

It may be seen that all three of these techniques are completely independent and any one of them may serve for the further analysis of the separation products of the other. It remains to be established now, whether a systematic application of these methods can yield information permitting the assignment of absolute triglyceride structures following enzymatic positional analysis of the final fractionation products. This can be decided by selecting representative natural fat samples of known fatty acid composition and considering the separations obtainable with the above systems for all the theoretically possible triglycerides.

It is the purpose of this presentation to show by a critical examination of certain natural triglyceride mixtures that a combination of partition, adsorption and gas chromatographic analyses can yield all the information necessary for the identification and quantitation of the individual triglycerides of many natural fats.

Experimental

The samples of corn oil, lard and butterfat referred to in the theoretical analyses and the discussion have been previously described (6,8), as have been the gas

TABLE II

Triglyceride Composition of a Model Vegetable Oil [a]

Glyceride type	Component fatty acids	Segregation numbers [b] P	I	C
36	18:3, 18:3, 18:3	36	18	54
38	18:2, 18:3, 18:3	38	16	54
40a	16, 18:3, 18:3	40	12	52
40b	18:1, 18:3, 18:3	40	14	54
40c	18:2, 18:2, 18:3	40	14	54
42a	16, 18:2, 18:3	42	10	52
42b	18:0, 18:3, 18:3	42	12	54
42c	18:1, 18:2, 18:3	42	12	54
42d	18:2, 18:2, 18:2	42	12	54
44a	16, 16, 18:3	44	6	50
44b	16, 18:1, 18:3	44	8	52
44c	16, 18:2, 18:2	44	8	52
44d	18, 18:2, 18:3	44	10	54
44e	18:1, 18:1, 18:3	44	10	54
44f	18:1, 18:2, 18:2	44	10	54
46a	16, 16, 18:2	46	4	50
46b	16, 18, 18:3	46	6	52
46c	16, 18:1, 18:2	46	6	52
46d	18, 18:1, 18:3	46	8	54
46e	18, 18:2, 18:2	46	8	54
46f	18:1, 18:1, 18:2	46	8	54
48a	16, 16, 16	48	0	48
48b	16, 16, 18:1	48	2	50
48c	16, 18, 18:2	48	4	52
48d	16, 18:1, 18:1	48	4	52
48e	18, 18, 18:3	48	6	54
48f	18, 18:1, 18:2	48	6	54
48g	18:1, 18:1, 18:1	48	6	54
50a	16, 16, 18	50	0	50
50b	16, 18, 18:1	50	2	52
50c	18, 18, 18:2	50	4	54
50d	18, 18:1, 18:1	50	4	54
52a	16, 18, 18	52	0	52
52b	18, 18, 18:1	52	2	54
54	18, 18, 18	54	0	54

[a] The model vegetable oil was assumed to consist of unspecified quantities of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids.
[b] As explained in Table I.

(6), adsorption (9,10) and partition (11,12) chromatographic, and oxidative (5) techniques. The linseed oil was obtained from a local drug store. The method of the enzymatic positional analysis has been reviewed by Desnuelle (13). The systems for grouping triglycerides on the basis of their carbon, iodine and polarity numbers rest upon the gas chromatographic studies of Kuksis and McCarthy (6), the work of De Vries (9) and Barrett et al. (10) on silver nitrate impregnated silica plates, and the liquid-liquid partitions of Dutton and Scholfield (11) and Hirsch (12), respectively. The validity of the classification has been verified experimentally in our laboratory by demonstrating that the predicted number of triglyceride groups is actually obtained with each of these three methods when lard, corn and linseed oils, and synthetic triglyceride mixtures are analyzed. The above concepts are summarized in Table I.

Results and Discussion

The vegetable oils are among the simplest of natural fats as they contain just five major fatty acids (16:0, 18:0, 18:1,18:2 and 18:3) representing only two different chain lengths. Although the actual concn of the individual acids may vary greatly depending upon the source of the oil, rarely are other acids encountered at significant levels—greater than 1%. (When an acid is present at the 1% level and is associated with two other acids in the triglyceride, there would be 3% of the triglyceride containing that acid. Theoretically, this glyceride could be concd in one of the fractions to a highly significant extent.) Table II lists all the combinations of these major acids as triglycerides, except for positional isomers. The glycerides have been classified into ten groups according to polar-

TABLE III

General Procedure for Complete Triglyceride Analysis [a]

1. Determine fatty acid composition of sample.
2. Compute all glyceride combinations and classify according to C or P.
3. Isolate P's by partition chromatography and weigh.
4. Quantitate C's for each P and the total sample by gas chromatography.
5. Obtain I's for each P by adsorption chromatography on thin layers.
6. Determine total fatty acid composition for each I.
7. Ascertain intraglyceride fatty acid distribution in each I by lipase hydrolyses.
8. Inspect (6) for types of glyceride present; solve algebraically if necessary. Assign structures from (2) and (7).
9. Complete the assignment of structures and quantitation on the basis of (2), (3), and (4).

[a] See Tables I and II for definition of symbols and method of classification.

ity numbers, and each group has been further subdivided according to the iodine and carbon numbers. As a result of such classification, it is possible to construct a flow sheet for an integrated system of triglyceride analysis. This is outlined in Table III in the imperative form. By following the scheme a quantitative measure for each of these triglyceride types may be obtained.

The solutions for most of the triglyceride types listed in Table II are simple, as there is either only one glyceride type per group or each type possesses unique fatty acids when more than one type is present in a group. In the more complicated cases the individual triglyceride type concn may be found algebraically. To illustrate the applicability of the integrated system, the group of seven glycerides with polarity number 48 (Table II) has been chosen as an example. Gas chromatographic analysis of this group will yield the four peaks indicated in the C column. A determination of the areas of these peaks will give a measure of the contribution of glycerides 48a and 48b, as well as the relative ratio of the 48c, 48d doublet and the remaining 48e, 48f, 48g triplet. One could then analyze the entire 48 group by TLC and isolate the bands corresponding to glycerides with I numbers of 4 and 6, and determine their fatty acid compositions. From an examination of the possibilities existing in I group 4, it is apparent that stearic and linoleic acids are unique to glyceride 48c and oleic acid is unique to glyceride 48d. The relative proportions of each of these types can therefore be readily determined from the fatty acid composition. A similar analysis of I group 6 shows that linoleic acid is unique to 48e, and linoleic acid is unique to 48f. Glyceride 48g is either determined by subtracting an amount of oleic acid equivalent to 48f from the total oleic acid and dividing the result by three, or by subtracting the values of 48e and 48f from C peak 54 obtained by gas chromatography. By following a similar sequence with the remaining polarity groups, it can be shown that each of the 35 glycerides listed can be measured. Calculations from simple permutation and combination formulae, however, show that there should be n^3 or 125 different glycerides in the case of the model oil. Since there are, at present, no known chemical means of distinguishing between the one and three positions of a triglyceride molecule, one would expect to find only $(n^3 + n^2)/2$ or 75 chemically distinguishable triglycerides.

The analytical treatment to this point has not included positional isomers, which account for the discrepancy in the estimated triglyceride numbers. In order to identify these isomers all the steps in Table III have to be used. The technique of studying triglyceride structure with pancreatic lipase, as usually employed (13), requires fairly large sample sizes. Suitable microtechniques, however, have recently been developed (16) to permit analysis of the quantity of triglyceride available from preparative scale TLC. Projection of the scheme in this manner permits measurements of 63 of the 75 chemically different triglycerides present. Since six irresolvable doublets occur, it is possible to determine by this means a total of 69 glyceride types (63 singles + 6 doublets) from a theoretical number of 75.

TABLE IV

Positional Isomers of Triglycerides of Polarity Number 50 from Table II

Glyceride type	Component fatty acids [b]	Segregation numbers [a]		
		P	I	C
$50a_1$	16, 16, 18	50	0	50
$50a_2$	16, 18, 16	50	0	50
$50b_1$	16, 18, 18:1	50	2	52
$50b_2$	16, 18:1, 18	50	2	52
$50b_3$	18:1, 16, 18	50	2	52
$50c_1$	18, 18, 18:2	50	4	54
$50c_2$	18, 18:2, 18	50	4	54
$50d_1$	18, 18:1, 18:1	50	4	54
$50d_2$	18:1, 18, 18:1	50	4	54

[a] As explained in Table I.
[b] The component fatty acids in this table have been listed in a definite order from left to right to correspond to the alpha, beta, and alpha' positions of the glyceride molecule.

The mechanics involved in the analysis are illustrated by solving for all the triglyceride types of polarity number 50 (Table II). Table IV lists all the possible positional isomers for this polarity group. Pancreatic lipase hydrolysis of I group O followed by isolation and determination of the monoglyceride fatty acids, yields directly the ratio of glyceride $50a_1$ to glyceride $50a_2$, since all of the palmitic acid would have to have come from the $50a_1$ and all the stearic acid from the $50a_2$ triglyceride. Triglycerides $50b_1$, $50b_2$ and $50b_3$ are given by the ratios of stearic, oleic and palmitic acids, respectively, following a similar series of manipulations with I group 2. The problem with the remaining four glycerides is slightly more complex. The monoglycerides from this group would yield a mixture of three fatty acids; stearic acid being common to $50c_1$ and $50d_2$. The linoleic and oleic acid concn would, however, yield direct estimates of $50c_2$ and $50d_1$, respectively. Since the concn of 50c and 50d (Table II) would have already been determined, subtraction of the values of $50c_2$ and $50d_1$ from these would yield the values for $50c_1$ and $50d_2$. Systematic application of this type of analysis to the 66 other chemically distinguishable triglycerides gives values for the remaining individual glycerides or their doublets.

Theoretically, the triglyceride groups obtained by whichever was the final resolving technique could be submitted to enzymatic positional analysis and the final structure assignment and quantitation made. Practically, however, at the moment at least, not all of these techniques lend equally readily to the isolation of triglyceride groups. Thus, the gas chromatographic method, while giving exact quantitative data, has as yet not been successfully employed for preparative purposes. A close examination of Table II shows that, in fact, there is no need for an actual isolation of all the glyceride groups at every step of separation. It is sufficient, for example, to perform quantitative isolation of the peaks only during partition chromatography or liquid-liquid extraction, as was illustrated in Table III. A subsequent TLC fractionation of these triglyceride groups can then be seen to provide the detailed separation necessary for an enzymatic positional analysis based on fatty acid proportions alone. There is no need to quantitate the tri-

TABLE V
Irresolvable Group of Triglycerides Containing Saturated Acids

Glyceride type	Component fatty acids	Segregation numbers [a]		
		P	I	C
24a	4, 4, 16	24	0	24
24b	4, 6, 14	24	0	24
24c	4, 8, 12	24	0	24
24d	4, 10, 10	24	0	24
24e	6, 6, 12	24	0	24
24f	6, 8, 10	24	0	24
24g	8, 8, 8	24	0	24

[a] As explained in Table I.

glyceride bands themselves on the thin-layer plates, as this information can be obtained from gas chromatography of the triglyceride groups obtained by liquid-liquid partition. In other words, the I's and C's overlap for a given P. This can be clearly seen from an inspection of the triglyceride groupings in Table II. There are several obvious alternatives to the proposed order of examination (Table III), and the actual sequence followed may depend on the type of fat to be examined, the available equipment, as well as the skills and personal preference of the investigator.

This solution is applicable to such vegetable oils as corn, cottonseed, linseed, safflower and soya oils, for example, all of which contains the same major acids, and, hence, theoretically the same glycerides as those considered for the model oil. Furthermore, a survey of the fatty acid composition of various natural fats shows, that in addition to the seed oils, this type of analysis is also applicable to practically all animal body fats, as these too contain only a limited number of different fatty acids of significant concentration. It may be noted again at this point, that in all cases checked, the number of peaks or triglyceride groups predicted on these bases of separation has corresponded exactly to the number of peaks determined experimentally by gas chromatography, the number of bands found in the thin-layer plates, and the number of peaks isolated and weighed by partition chromatography or liquid-liquid extraction. This experimental verification of the predicted number of glyceride groups places the proposed classification and the suggested scheme of examination on a sound experimental footing. A reliable analysis of these fats depends, therefore, only upon an exact quantitation of the fatty acids and triglycerides by gas chromatography, both of which can be meaningfully assessed when using the hydrogen flame ionization detector.

The system fails to identify every triglyceride, however, in those fats that contain significant amounts of five or more different saturated fatty acids. Among such fats are coconut oil, palm kernel oils and the various milk fats. Although exact solutions still can be obtained for many triglyceride groups, there are others that can not be resolved. Table V illustrates the composition of one such saturated triglyceride group from butterfat. Only the glyceride types 24a and 24b of this group may be quantitatively measured since they contain unique fatty acids. Sixty five per cent of the possible trisaturated glycerides of butter occur in such irresolvable combinations. About 30% of butterfat triglycerides are of the completely saturated type (14). Table VI indicates the theoretical composition of another irresolvable butterfat triglyceride group containing saturated and unsaturated fatty acids. This group of glycerides is the only such case encountered in total butter fat. If means could be found to deduce just one of the triglyceride structures by some independent emthod, the rest of them would immediately fall in line resulting in a complete

TABLE VI
Irresolvable Group of Triglycerides Containing Saturated and Unsaturated Acids

Glyceride type	Component fatty acids	Segregation numbers [a]		
		P	I	C
46a	14, 18, 18:2	46	4	50
46b	14, 18:1, 18:1	46	4	50
46c	16, 16, 18:2	46	4	50
46d	16, 16:1, 18:1	46	4	50
46e	16:1, 16:1, 18	46	4	50

[a] As explained in Table I.

solution of all the structures. In spite of these shortcomings, application of the techniques in the manner described above would allow identification and quantitation of 285 of the 368 constitutionally different triglycerides that are possible when the 12 major acids of butterfat are considered. It is thus feasible to assess essentially all of the theoretically possible constitutionally different triglycerides of the common vegetable oils and ca. 77% of those occurring in butterfat, which is the most complex of all known natural fats.

In order to obtain exact structures for the remaining triglycerides experimentally, it will be necessary to refine the analytical methods. Thus, there may be a possibility for designing partition systems that are effective enough (have a sufficient number of theoretical plates) to break up the P's obtained during the fractionation by polarity number. Experimental basis for this may be found in the somewhat unequal contributions made to the polarity number by the chain length of the fatty acids and their contents of active hydrogens (15). Similarly, there is reason to anticipate that gas chromatographic systems will be developed for the separation of unsaturated and saturated triglycerides as well as different unsaturated glycerides of the same carbon number.

The proposed system offers for the first time an integrated experimental system for the complete determination of the triglyceride structures of several natural fats. This system eliminates completely any need for statistical speculation at any of the analytical stages. The separations and quantitative estimations are based upon essentially nondestructive physical techniques the experimental validity of which has been firmly established. Furthermore, it provides numerous cross-checks at most of the steps of separation and quantitation. It must be emphasized that of the actually or potentially available methods, only integrated systems of this type are likely to yield definite triglyceride structures.

REFERENCES

1. Hilditch, T. P., "The Chemical Constitution of Natural Fats", John Wiley & Sons, New York, 1941.
2. Longenecker, H. E., Chem. Revs. *29*, 201 (1941).
3. Savary, P., J. Flanzy and P. Desnuelle, Biochim. Biophys. Acta *24*, 414 (1957).
4. Mattson, F. H., and E. S. Lutton, J. Biol. Chem. *233*, 868 (1958).
5. Youngs, C. G., JAOCS *38*, 62 (1961).
6. Kuksis, A., and M. J. McCarthy, Can. J. Biochem. Physiol. *40*, 679 (1962).
7. Kuksis, A., M. J. McCarthy and J. M. R. Beveridge, JAOCS *40*, 530–535 (1963).
8. McCarthy, M. J., and A. Kuksis, Proc. Can. Fed. Biol. Soc. *6*, 42 (1963).
9. DeVries, B., JAOCS *41*, 403–406 (1964).
10. Barrett, C. B., M. S. Dallas and F. B. Padley, *Ibid. 40*, 580–584 (1963).
11. Dutton, H. J., and C. R. Scholfield, *in* Progress in the Chemistry of Fats and Other Lipids, R. T. Holman, W. O. Lundberg, and T. Malkin, editors, Vol. 6, page 314, London and New York: Pergamon Press, 1963.
12. Hirsch, J., *in* Digestion, Absorption Intestinale et Transport des Glycerides chez les Animaux Superieurs, edited by the Centre National de la Recherche Scientifique, Paris, 1961, p. 11.
13. Desnuelle, P., Advances in Enzymology *23*, 129 (1961).
14. Kuksis, A., M. J. McCarthy, and J. M. R. Beveridge, JAOCS *41*, 201–205 (1964).
15. Kuksis, A., and J. M. R. Beveridge, Can. J. Biochem. Physiol. *38*, 95 (1960).
16. Luddy, F. E., R. A. Barford, S. F. Herb, P. Magidman and R. W. Riemenschneider, JAOCS in press.

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