tion space is at a premium, use of mild aeration should be considered.

Rapid freezing of the culture supernatant with storage at a low temperature affords a simple procedure for prolonged storage of small quantities of enzyme. However, since the concentration, dialysis, and lyophilization procedure will reduce several liters of material to a few grams, it has obvious advantages for any extensive study.

It is reasonable to expect that further study on the interrelationships of pH, aeration, and supply of nutrients during the entire growth cycle would bring about additional increases in yield of these lipases. However, with the media and conditions of growth described here, coupled with proper recovery and storage procedures, stable preparations can be routinely prepared which are suitable for the study of the structure of triglycerides.

REFERENCES

1. Alford, J. A., and L. E. Elliott, Food Research *25*, 296–303 (1960).
2. Alford, J. A., and D. A. Pierce, J. Food Sci. *26*, 518–524 (1961).
3. Alford, J. A., and D. A. Pierce, J. Bacteriol. *86*, 24–29 (1963).
4. Alford, J. A., D. A. Pierce and F. G. Suggs, J. Lipid Res. *5*, 390–394 (1964).
5. Jensen, R. G., J. Sampugna, J. G. Quinn, D. L. Carpenter, T. A. Marks and J. A. Alford, JAOCS *42*, 1029–1032 (1965).
6. Jensen, R. G., J. Sampugna, J. G. Quinn, D. L. Carpenter and J. A. Alford, J. Dairy Sci. *48*:767 (1965).
7. Kohn, J., Nature *183*, 1055 (1959).

The Unsolved Problems of Triglyceride Analysis[1]

M. H. COLEMAN, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England

Abstract

The modern techniques of countercurrent distribution between solvents, GLC and TLC (especially on silver nitrate impregnated silica gel), and enzymic hydrolysis have greatly simplified and speeded up the analysis of triglyceride mixtures, such as are found in natural fats. The application of these methods has provided data for the formulation of a number of new theoretical descriptions of the fatty acid distributions found in fats.

But it is clear that neither the experimental techniques, nor the theories of glyceride structure arrived at with their aid, are entirely satisfactory. On the one hand analytical methods are inadequate for such fats as the nut oils; and in no case can the accuracy of the analysis be regarded as complete. On the other hand present theories have yet to accommodate minor components, enantiomorphic forms, and recent evidence concerning the biosynthesis of natural triglycerides.

Attention is drawn to some of the outstanding problems still facing those concerned with the analysis of triglyceride mixtures; and an attempt it made to indicate the direction in which solutions to these problems may be sought.

Introduction

NATURAL FATS ARE COMPOSED of triglycerides, the esters of a variety of fatty acids with the trihydric alcohol, glycerol. The consequence of the trihydric character of glycerol is that triglycerides may be simple (containing one fatty acid species) or mixed (containing two or three fatty acid residues per molecule). The presence of two primary and one secondary hydroxyl group in glycerol gives rise to positional isomers of the same fatty acid composition: two in the case of diacid triglycerides, and three in the case of triacid triglycerides. If the two primary hydroxyls are esterified with different fatty acids, the central glycerol C atom becomes asymmetric; and such triglycerides can exist in two enantiomorphic forms. For the complete analysis of a mixture of mixed triglycerides of the kind encountered in natural fats, it is therefore necessary to know: (a) the overall fatty acid composition; (b) the distribution of these acids between the various triglycerides present; (c) the distribution of the acids between the primary and secondary hydroxyls, within each mixed triglyceride molecule; (d) the distribution between the two primary hydroxyls of each asymmetric, mixed, triglyceride molecule.

The methods for making each of these determinations will be considered separately, as far as present techniques allow.

Experimental Methods Available

(a) To Determine Overall Fatty Acid Composition

It is not proposed to consider here the identification of unusual fatty acids by chemical means, although much remains to be done. This work of Korn (1) in identifying and analysing the fatty acids of *Euglena gracilis* may be cited as an example of the kind of careful work required in a comprehensive analysis.

There are a number of methods available for fatty acid analysis (see Ref. 2), but for most purposes the speed and high resolution of gas-liquid chromatography (GLC) renders it superior to other methods. It is very versatile, and is probably the easiest method to operate for quantitative analyses: and in biological work the small size of the sample required is a further advantage. A comprehensive review (3) of the application of GLC to fatty acid analysis, renders detailed consideration of the technique unnecessary here.

Sources of error or difficulty with this method are now well documented. Thus it is necessary to make sure that samples for analysis contain only fatty acids: a preliminary purification by thin-layer chromatography (TLC) may be advisable. Samples are usually run as methyl esters, and care should be taken to ensure that methylation is complete (4) and to avoid artifacts (1,2,5). It may be desirable to purify the methyl esters (6). For identification of the emergent peaks it is unwise to rely on retention volume alone; Fontell et al. (7) have advocated the use of two stationary phases, or two column temperatures, to be sure of identification. Certain classes of fatty acids are subject to alteration during the process of GLC (8,9).

[1] Presented at the AOCS meeting in Houston, Texas, 1965.

Since all methods of triglyceride analysis depend ultimately on fatty acid analysis, the question of quantitation in GLC is very important. In general the error appears to be within ±5% of the total of any one component; and this may be reduced to ±2.5% with extra care. This may be regarded as reasonably satisfactory, and doubtless will be improved further. It must be remembered that errors in fatty acid analysis may be multiplied three-fold when used to calculate triglyceride composition.

(b) To Determine Distribution Between the Triglycerides

For this purpose it is necessary to resolve the mixture of triglycerides into its components, unless some assumption is made about the way in which the fatty acids are distributed; e.g., the theories of Kartha (10), VanderWal (11) or Gunstone (12).

In the past this resolution was attempted by oxidation (13) or fractional crystallisation (14); and more recently counter-current distribution between solvents (15). It is clear that resolution was never complete by any of these methods.

Two methods which have been gaining in importance recently are the separation by degree of unsaturation on silica gel impregnated with $AgNO_3$ using column (16) or TLC (17); and by molecular weight using GLC (18). Neither of these methods alone, or in combination, can effect the complete resolution of a triglyceride mixture. Only a limited number of positional isomers can be separated by $AgNO_3$ chromatography, and numbers of triglycerides are known with different fatty acid compositions, but with the same molecular weight, and containing the same number of double bonds (see Table I).

Another technique in which the separation depends both upon the molecular weight and the degree of unsaturation is reversed phase chromatography. Kaufmann and Wessels (19) have used TLC on paraffin-impregnated kieselguhr; Vereshchagin (20) has employed paper chromatography in a similar way. A combination of TLC on $AgNO_3$-silica gel, followed by reversed phase separation can effect a complete separation of the triglycerides listed in Table I, into their individual groups.

(c) To Determine Distribution Between Primary and Secondary Positions

Undoubtedly the simplest technique for determining the distribution of the commoner fatty acids between the primary and secondary positions of a triglyceride, is to subject it to hydrolysis with pancreatic lipase (21,22). This enzyme catalyses the hydrolysis of fatty acid residues from the primary positions of the triglyceride; so the 2-monoglyceride produced contains the acid originally present at the 2-position of the triglyceride. The other acid, or acids, known to be present from the overall fatty acid analysis must therefore have been present in the 1- and 3-positions. The positional specificity of pancreatic lipase has been demonstrated by the use of synthetic mixed triglycerides (21,22); and although no completely pure monoglyceride appears to have been reported in such experiments, it seems likely that this is due to impurities in the triglycerides, or to the conditions under which the hydrolysis is conducted, rather than a lack of complete positional specificity of the enzyme. The partial glycerides produced are liable to undergo acyl migration, so that acids of the 2-positions by migrating to the 1- or 3-positions may be hydrolysed, and appear in the free fatty acids. On the other hand the equilibra lie in favour of 1,3-diglycerides or 1-monoglycerides, and hydrolysis should be faster than acyl migration; hence there is little likelihood of acids of the 1- and 3-positions being found in the monoglyceride fraction. It is important to remember that under physiological conditions of pancreatic hydrolysis, Hofmann (23) has found no 1-monoglycerides in human intestinal contents.

TABLE I

A Classification of the 40 possible Triglycerides Prepared from the 4 Acids Listed [a]

No. of acyl C atoms	No. of double bonds						
	0	1	2	3	4	5	6
48	PPP						
50	PPS PSP	PPO POP	PPL PLP				
52	PSS SPS	POS PSO SPO	POO OPO PLS PSL SPL	POL PLO LPO	PLL LPL		
54	SSS	SSO SOS	SOO OSO SSL SLS	SOL SLO LSO OOO	SLL LSL OOL OLO	LLO LOL	LLL

P—Palmitic S—Stearic O—Oleic L—Linoleic

[a] Enantiomorphic forms are not distinguished here.

It is clearly important to achieve the maximum yield of monoglyceride in the shortest convenient time; i.e., the conditions for the optimal activity of the enzyme should be employed. These include: (a) a pH of 8–9 maintained throughout the hydrolysis (24); (b) high ionic strength (21); (c) presence of bile salts (21); (d) presence of Ca^{++} ions to remove inhibiting free fatty acids (25); (e) temperature around 40C; (f) good dispersion of the insoluble substrate (26).

Unless the amount of triglyceride used is small compared with the amount of buffer, the release of the fatty acids will cause a marked drop in pH; and pancreatic lipase activity falls sharply below pH 8 (24). This may be avoided by the continuous addition of alkali, which also serves as a check on the extent of the hydrolysis, and the point of maximum monoglyceride yield; since at this point the rate of hydrolysis tails off. Bile salts serve not merely to emulsify the substrate, but also activate the enzyme (27). If Ca^{++} ions are omitted the reaction rapidly ceases (25), due to inhibition of the enzyme by the free fatty acids (24). The use of phosphate buffers precludes the use of Ca^{++} ions.

When conditions (a) to (e) are satisfied the reaction rate depends upon condition (f) and the enzyme concentration. This last is best adjusted to give the required degree of hydrolysis (ca. 65%) in the shortest convenient time. It is also necessary to make sure the enzyme preparation is free of extractable lipid, and nonspecific esterase activity.

When, as is usually the case, a mixture of triglycerides is subjected to hydrolysis, there is the additional complication that different acids may be hydrolysed at different rates from the primary positions (28,29). This, together with the early appearance of glycerol (30) may lead to difficulties if the composition of the free fatty acids is used as a measure of the composition of acids occupying the 1- and 3-positions.

The anomalous behaviour of triglycerides containing short-chain acids is now well established (28,29); and it seems doubtful that pancreatic hydrolysis can give a completely quantitative picture of the fatty acid distribution of fats such as butterfat, which contain appreciable quantities of these acids (especially butyric). There are other examples of anomalous be-

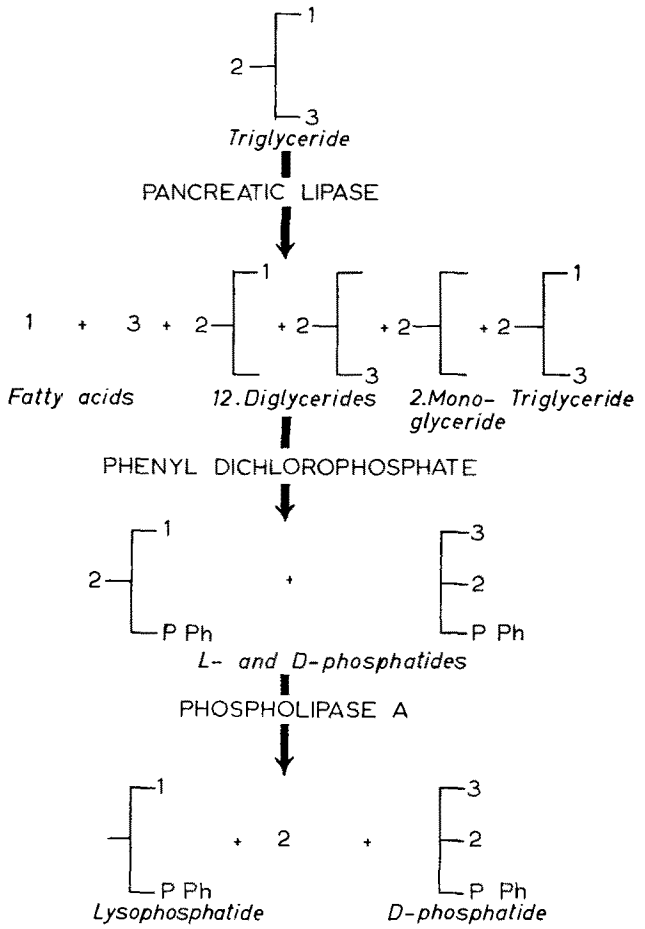

FIG. 1. Differentiation of 1 and 3 positions (Brockerhoff).

haviour during pancreatic hydrolysis, where the fats contain unusual fatty acids (31,32).

Where the triglyceride composition is sufficiently simple, the configuration of positional isomers may be determined by infrared spectroscopy, in the way which Chapman et al. (33) have used for cocoa butter. It seems doubtful if mass spectrometry, which can distinguish individual positional isomers from each other, could be applied to mixtures such as occur in natural fats.

(d) To Determine the Distribution Between the Primary Positions

The recent work of Brockerhoff (34,35) has introduced a method in which the fatty acid composition of the 1-positions may be determined independently of those of the 3-positions. The method involves removing one fatty acid residue from the triglyceride with pancreatic lipase, converting the diglyceride to a phosphatidyl phenol with phenyl dichlorophosphate, and subjecting this to hydrolysis with a phospholipase A of snake venom. Since this hydrolysis is stereospecific, only the L-isomer is attacked. The fatty acids found in this lyso-phosphatidyl phenol are derived from the original 1-positions of the triglycerides. The composition of the acids of the 2-positions is known from that of the 2-monoglycerides produced in the original hydrolysis; and the composition of the 1-, 2- and 3-positions is known from the overall fatty acid analysis of the original fat. The composition of the 3-positions may then be calculated by difference (Fig. 1).

A difficulty of this scheme is that all fatty acids are not hydrolysed from the primary positions at exactly the same rate. This may render the diglycerides unrepresentative, which in turn affects the compositions of the phosphatidyl and lyso-phosphatidyl phenols, and hence the calculated compositions of the acids of the 1- and 3-positions. Brockerhoff (35) has added hexane to the hydrolysis, to reduce this effect, but this is not entirely effective. The problem might be solved by preparing the diglyceride without Ca^{++} ions, and with a high enzyme concentration, in the hydrolysis; the monoglyceride being prepared separately under the usual conditions.

Another approach (36) which merely establishes qualitatively the existence of any difference in the composition of the acids of the 1- and 3-positions, is suitable for vegetable fats containing appreciable amounts of asymmetric monosaturated triglycerides. After hydrolysis with pancreatic lipase to yield as much diglyceride as possible, the products are re-esterified with acetic acid (37), and subjected to a Von Rudloff oxidation (38). It is to be expected that the product should contain an optically active component, if the original asymmetric monosaturated triglyceride existed exclusively, or largely, in one enantiomorphic configuration. In this method, the more rapid removal of oleic acid from say, 1-stearodiolein, is actually an advantage, since the diglyceride will contain more of the mixed 1-stearo 2-oleo diglyceride, which gives rise to the optically active product (Fig. 2).

Another method has been used by Morris (39). The largely asymmetrical disaturated (lard) or monosaturated (malabar tallow) fraction of the fat is isolated by TLC on $AgNO_3$-silica gel. The fraction is subjected to pancreatic lipolysis, and the 1, 2-diglycerides isolated by TLC on silica gel. These are converted to the corresponding trimethylsilyl ethers (40), and then are fractionated by TLC on $AgNO_3$-silica gel. In each case the mixed acid ethers are examined for optical activity.

Two other methods have been introduced by Schlenk (41), employing X-ray diffraction, or the piezoelectric effect. A difficulty here is the necessity for ioslating individual triglycerides, which at present can only be achieved for a few fats of comparatively simple triglyceride composition.

Little asymmetry has been detected in such vegetable fats as cocoa butter (41), corn oil (34) or malabar tallow (36,39,42). Brockerhoff (43) has reported asymmetry in mammalian depot fats (lard, human depot fat) but not in fats of birds and crustaceans. Morris (39) has detected asymmetry in lard.

Scope and Limitations of Present Methods

We may now consider the mixtures of triglycerides which are encountered in natural fats and how far the methods outlined have been successful in elucidating their compositions. It is easily shown (44) that the number of distinct triglycerides, N, which can be prepared from n different fatty acids is given by the expression:

$$N = (n^2 + n^3) \div 2$$

(In this formula, only positional isomers are distinguished, enantiomorphic forms are not.) If we consider only the major component acids, a large number of natural fats contain at least four, including palmitic, stearic, oleic and linoleic; which means that 40 different triglycerides may be present in these. These are listed in Table I, where it will be seen they

fall into 4 groups by C number, and 7 groups by degree of unsaturation.

It is possible to separate such a mixture in both ways, by unsaturation with column chromatography (16) or TLC (45) on $AgNO_3$-silica gel; and by acyl carbon number with GLC of the oxidised (46) or hydrogenated (47) mixture. But the important point to note is that the methods are not (at present) commutative. Fractions from $AgNO_3$-silica gel chromatography may be subjected to GLC, (or enzymic hydrolysis etc.); but it is not possible to examine further, fractions obtained by GLC. The consequence of this restriction is that it is not possible to isolate and estimate all of the 40 triglycerides listed in Table I, individually. Tripalmitin may be estimated by GLC alone, and tristearin by GLC after oxidation. Trilinolein may be obtained by $AgNO_3$-silica gel chromatography; and triglycerides containing 4 or 5 double bonds may be estimated by a combination of these two techniques with enzymic hydrolysis. But there remain 29 triglycerides which cannot be estimated individually, with these methods.

Various simplifications of the problem have been made to meet this experimental difficulty. Thus Youngs (48) originally used a combination of column chromatography (after oxidation) with lipase hydrolysis to estimate the proportions of the six glyceride classes, trisaturated, symmetrical and unsymmetrical di- and monosaturated, and triunsaturated. Youngs and Subbaram (46) then extended the scope of this analysis by combining it with the GLC of an oxidised sample of the fat. This permits the differentiation of the two saturated acids considered, palmitic and stearic. The analysis was further extended by these two authors (49) by combining the previous techniques with a separation by degree of unsaturation (16) on a column of $AgNO_3$-impregnated silica gel. As lipolysis was not applied to the individual fractions, no estimate of the proportions of isomeric triglycerides was possible. In another publication (50) these authors have examined 14 animal and vegetable fats, by their technique of GLC of an oxidised sample; and compared the results with those calculated from lipolysis data, using VanderWal's theory. In this instance, the unsaturated acids were considered together.

Barrett et al. (45) have simplified the problem by considering the saturated acids together. Six natural fats were examined by TLC on $AgNO_3$-impregnated silica gel, and by lipase hydrolysis. It was possible to distinguish 10 triglyceride groups in terms of unsaturation; but the isomeric monosaturated dioleins, triolein and monosaturated oleolinolein, and triglycerides containing 4 or more double bonds were not isolated individually. Of the positional isomers, only the 1- and 2-oleo disaturated classes were distinguished, because lipolysis was applied only to the whole fat, not to the fractions isolated by TLC.

Jurriens and Kroesen (47) have also used TLC on $AgNO_3$-impregnated silica gel; but in this case part of each fraction so obtained was subjected to lipase hydrolysis, whilst the remainder was hydrogenated and analysed by GLC. In this instance also, it was not possible to determine the distribution of all the saturated acids, between and within the triglycerides present; and the assumption was made that these were statistically distributed amongst the 1- and 3-positions, subject to the restriction that each group separated by TLC contained triglycerides with the same number of double bonds. For the more unsaturated

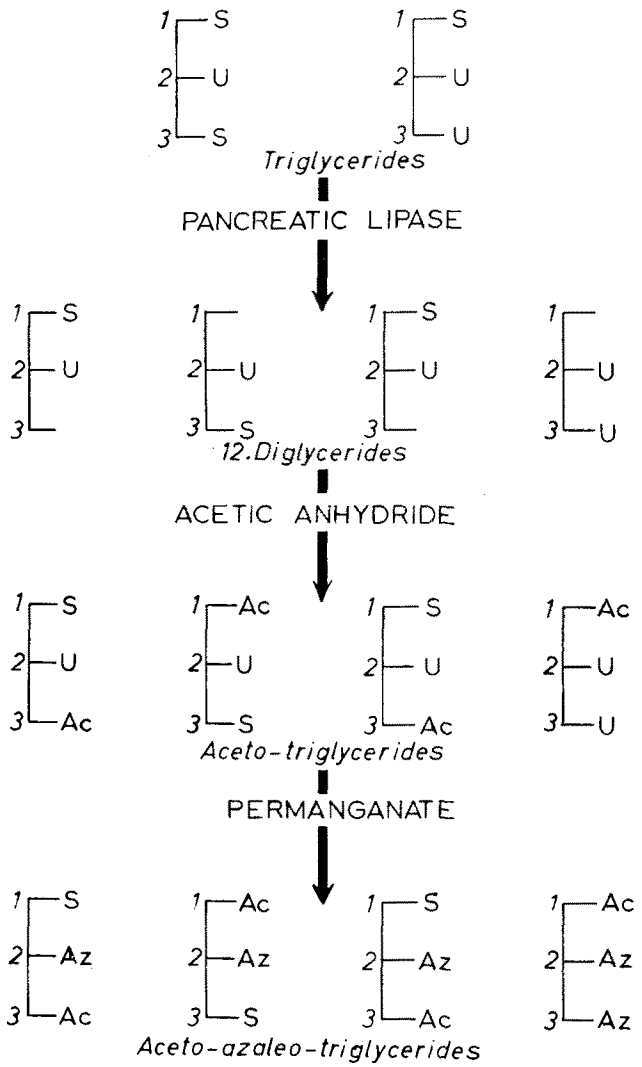

FIG. 2. Differentiation of 1 and 3 positions (Coleman).

vegetable oils examined, there was the experimental difficulty of examining the very small amounts of triglycerides containing more than 6 double bonds; and the theoretical difficulty resulting from the introduction of a fifth acid, linolenic, which is discussed below.

If the separations by GLC and $AgNO_3$-impregnated silica gel chromatography were commutative, complete resolution of the 40 triglycerides of Table I would be possible, when these techniques are combined with enzymic hydrolysis. After an initial separation by C number into 4 groups, the triglycerides could be further separated into the 16 groups of Table I. Further resolution within each group is then possible by lipolysis; and may be illustrated for the 5 triglycerides with an acyl carbon number of 52, each containing 2 double bonds. The proportions of POO, PLS and PSL will be given directly by the molar proportions of oleic, linoleic and stearic acids in the monoglycerides produced by lipolysis. The remaining stearic content of the 1- and 3-positions unaccounted for by PLS will give the amount of SPL; and OPO can be calculated by difference. Similar calculations permit the estimation of the remainder of the 40 possible triglycerides.

An alternative to gas-chromatography for the separation by molecular weight is reversed-phase TLC. To eliminate the effects of unsaturation on this separation, Kaufmann and Wessels (19) have first separated sunflower oil by unsaturation, on $AgNO_3$-silica

TABLE II

Lipolysis Data and Calculated Glyceride Composition (VanderWal's theory) of Sow-Milk Fat

Fatty acid	Fatty acid composition (M %)		Triglyceride composition (M %)		
	Triglyc.	Monoglyc.	Glyc. class	Sow milk fat	Lard[a]
8	1.0	0.8			
10	0.3	—	SSS	10.1	4.9
12	0.5	0.5			
14	4.0	6.9	SSU	30.2	27.0
14:1	0.5	0.2			
16	36.5	54.3	SUS	5.9	1.9
16:1	13.0	15.8			
?17:1	0.3	t	SUU	17.8	11.2
18	4.4	0.5			
18:1	27.2	12.4	USU	22.7	38.9
18:2	10.8	8.6			
20	1.0	—	UUU	13.3	16.1
20:1	0.5	—			

[a] Data from JAOCS *38*, 685 (1961).

gel plates. The fractions from this separation were then further resolved by molecular weight, using reversed-phase TLC. The examination of each fraction by lipolysis would also permit all the triglycerides to be estimated individually.

When we consider the wider group of fats which include linolenic in addition to the four acids so far considered, a theoretical difficulty arises in connection with triglyceride analysis. The number of possible triglycerides is 75; and they fall into 22 groups of differing acyl carbon number and degrees of unsaturation. It will be found that even if such a mixture can be first separated by acyl carbon number, then by $AgNO_3$-silica gel chromatography, and finally by enzymic hydrolysis, there remain two groups (acyl carbon number 54 containing 4 or 5 double bonds) each containing 7 triglycerides, of which only one in each group can be estimated.

Our inability to estimate the triglycerides of a fat containing these 5 fatty acids means that fats, such as the fish oils, with an even wider range of the polyunsaturated fatty acids, are even less amenable to our present methods of analysis.

When we turn to another group of fats, the nut oils, which include such commercially important fats as coconut and palm kernel oils, the situation is even less satisfactory. The wide range in molecular weight of the fatty acids present, and their largely saturated character, offer very limited opportunities for separating their mixed triglycerides. The 6 normal, even-numbered saturated fatty acids from caprylic to stearic can give rise to 126 different triglycerides, ranging from acyl carbon numbers of 24 to 54. Kuksis and McCarthy (51) using GLC have reported 14 components in coconut oil, with acyl C numbers of 28 to 54. If such fractions could be subjected to enzymic hydrolysis, those with 48 or more carbon atoms in the acyl residues could be estimated individually: but this would still leave over 100 possible triglycerides unresolved.

Another important group of fats which is not susceptible to present methods of analysis, includes many of the milk fats. It is true that sow-milk fat for example, contains very little short-chain acid, and is therefore suitable for examination by pancreatic hydrolysis. Table II gives hydrolysis data for this fat (36), in which the short-chain acids (ca. 2%) have been neglected. The triglyceride composition calculated from VanderWal's theory, resembles that of lard: this is to be expected from the preponderance of palmitic acid in the monoglycerides (52). The presence of appreciable amounts of short-chain acids in most milk fats makes it difficult to assign quantitative significance to the results obtained by lipolysis. Jensen et al. (53) has suggested that the native lipase of milk exhibits the same positional specificity as pancreatic lipase, but does not differentiate between long and short-chain acids. For this group of fats therefore, this enzyme may be more suitable for use in hydrolysis studies.

TABLE III

Triglyceride Composition of Human Depot Fat

Glyceride	Calculated for 1 random, 2 random 3 random FA distribution	Calculated for 13 random, 2 random FA distribution (VanderWal)
SSS	5.1	5.3
SSU	12.5	12.1
SUS	15.9	16.5
SUU	39.1	37.7
USU	6.7	6.9
UUU	20.7	21.5

Data from Brockerhoff (35).

A consideration of the methods for triglyceride analysis currently available, clearly gives no justification for complacency. But there is one aspect of the situation we have not yet considered. The foregoing discussion has been confined to the problems of experimental analysis, without reference to any theory of fatty acid distribution in natural fats. If the theory of VanderWal (11), (which represents a more empirical approach than that of Gunstone's theory (12) for the vegetable oils), is valid, then this provides a very considerable short-cut for triglyceride analysis of many of the commoner natural fats. Evidence for the validity of VanderWal's theory is provided by the agreement between results calculated from lipolysis data with its aid, and those obtained experimentally by other methods. Comparisons with oxidation, fractional crystallisation and countercurrent distribution have been made elsewhere (2,11,52); more recently, comparisons have been made with TLC (17,19), GLC of oxidised samples (50), and a combination of TLC, GLC and lipolysis (47). In general the agreement is very good, although small departures have been noted for palm oil (47,50) and human depot fat (50); but whereas one investigation reports differences for lard (47) the other does not (50). Similarly, differences for cocoa butter are noted in one (50) but not in the other (47). One fat which has been reported as not conforming to VanderWal's theory is that of the bitter gourd seed (32), and there is some doubt if the fat from *Cuphea llavia* seed (54) conforms to this theory. But both these fats contain more than 50% of an acid other than those specified by Savary and Desnuelle (55) as being hydrolysed at much the same rate; the anomalous results may therefore arise from this source. It may be noted in this connection that the analysis of castor oil (56) gave results in agreement with VanderWal's theory, even though it contains about 90% of hydroxy-acids. The independence of the monoglyceride composition of the extent of hydrolysis is also consistent with (30), but not conclusive evidence in favour of (57) VanderWal's theory.

If VanderWal's theory is true, the analysis of a fat suitable for pancreatic hydrolysis (55) is a relatively simple matter. But it must be stressed that fats containing short-chain or unusual acids cannot be analysed quantitatively in this way. From the appropriate lipolysis data, complete information on the distribution of fatty acids between the triglycerides, and the primary and secondary positions within each molecule, may be calculated using the theory. But the theory assumes that each primary position has the same fatty acid composition, i.e., the asymmetric

mixed triglycerides cannot exist in one enantiomorphic form.

The close agreement between the proportions of various triglycerides calculated from the theory and those determined experimentally for a number of fats suggests that these triglycerides do not exist exclusively in one enantiomorphic configuration. But Brockerhoff's data for human depot fat (35) give the fatty acid compositions of the 1- and 3-positions separately. If it is assumed that the fatty acids of each position are randomly distributed with respect to those of the other two positions, it is possible to calculate the triglyceride composition of the original fat. A triglyceride composition may also be calculated in the usual way (52) from VanderWal's theory (assuming an average composition in the 1- and 3-positions). Table III gives these results in terms of the six main classes, and it will be seen that there is very little difference between them. This indicates that although the triglyceride compositions calculated for vegetable oils (2,17,47), and fats (2,11,17,47), and some animal fats (2,11,47,52), may be fairly accurate, they give no indication of the existence of particular enantiomorphs. But this leaves all those fats such as the nut oils, milk fats, fish oils and fats containing unusual fatty acids, for further investigation by other methods.

Future Investigations

It is now clear which problems seem likely to be resolved in the near future, and which require a radically different approach from those already investigated. But it is convenient to mention here two general topics which have not so far received consideration.

Firstly there is the question of whether a sample of any particular fat can be regarded as more than a rather arbitrary collection of triglycerides. This question arises from a number of observations that the fatty acid composition in one oilseed may vary quite markedly from one part of the seed to another (58–60). This suggests that the triglyceride composition may vary throughout the seed, and the composition of the extracted fat may have no particular significance. But against this must be set the fact that repeated analyses of the same kind of fat agree in assigning a fairly characteristic triglyceride spectrum to it; and that different kinds of fat with similar fatty acid compositions often differ very markedly in triglyceride composition (Table IV). The investigations of Litchfield and Reiser (61) have shown that even though the fatty acid composition may vary dramatically in different parts of a seed, the triglyceride composition calculated from data for the whole seed scarcely differs from that calculated from the sum of the component parts.

Secondly, little has so far been said of the minor component acids of natural fats. Now it is true that they may complicate the analysis of triglyceride mixtures, but those containing more than 18 carbon atoms are probably easily detected with GLC, taking the usual precautions for the measurement of small peaks (62). Pancreatic lipolysis shows that many of these minor components are confined to the primary positions of triglycerides (63). As far as present knowledge goes, most of these minor components appear to be without biological significance; and for this reason they do not seem to warrant particular consideration, until the problems associated with the analysis of the triglycerides of major component acids have been resolved.

TABLE IV

Difference in Monoglyceride Compositions for Two Fats with Similar Overall Fatty Acid Compositions

Fatty acid	Fatty acid composition (M. %)			
	Triglycerides		Monoglycerides	
	Pig	Pheasant	Pig	Pheasant
14	1.7	0.9	4.1	t
16	24.8	25.2	60.1	8.9
16:1	3.9	8.6	7.9	5.9
17	t	—	2.2	—
17:1	t	—	1.7	—
18	14.3	5.4	4.4	2.8
18:1	43.6	46.7	15.2	62.6
18:2	11.7	10.9	4.4	16.2
18:3	—	2.3	—	3.6

If all possible triglycerides of a mixture containing palmitic, stearic, oleic and linoleic are to be estimated individually, it will be necessary to effect an initial separation by molecular weight, before further separation by $AgNO_3$-silica gel chromatography and examination by enzymic hydrolysis, is to be applied. This might be achieved by preparative GLC, but liquid/liquid column chromatography appears a more attractive prospect; especially since several methods of detection have been described (64–66), and at least one detector is available commercially. Alternatively the fat may first be fractionated by degree of unsaturation with $AgNO_3$-silica gel TLC, then by molecular weight by reversed phase chromatography, and finally the fractions examined individually by enzymic hydrolysis.

If a fifth acid, linolenic, is added to the other four, there remains the difficulty of estimating the triglycerides containing 54 acyl C atoms, and 4 or 5 double bonds. If the separation of triglycerides pairs such as SLL and LSL, or LOO and OLO can be achieved on $AgNO_3$ impregnated silica gel, this problem can be overcome.

The prospects for the complete resolution of triglyceride mixtures containing appreciable amounts of short-chain fatty acids remain unpromising. Again GLC provides one method of separation by molecular weight, but liquid/liquid chromatography either on columns or by TLC, would be more convenient; particularly for providing samples of the fractions for further investigations. Alternatively displacement chromatography may offer some advantages. But methods for the subsequent investigation of the fractions are more difficult to envisage, at the present time.

One possibility for triglyceride analysis is the separation by GLC both by molecular weight and degree of unsaturation, on a suitable stationary phase. But apart from the difficulty of devising such a material capable of withstanding the high temperatures needed for such relatively nonvolatile samples, the difficulty arises of collecting samples unchanged, for further investigation. Liquid/liquid chromatography would probably be more suitable in this instance, also.

There remain the fats such as the fish oils, with a wide range of polyunsaturated acids, which will clearly require radically different methods from those now in prospect, if they are to be analysed exhaustively.

REFERENCES

1. Korn, E. D., J. Lipid Res. *5*, 352 (1964).
2. Coleman, M. H., in "Advances in Lipid Research," ed. R. Paoletti and D. Kritchevsky, 1963, Vol. 1, p. 1.
3. James, A. T., in "Methods of Biochemical Analysis," ed. D. Glick, 1960, Vol. 8, p. 1.
4. Schlenk, H., and J. L. Gellerman, Anal. Chem. *32* 1412 (1960).
5. Johnston, P. V., and B. I. Roots, J. Lipid Res. *5*, 477 (1964).
6. Stoffel, W., F. Chu and E. H. Ahrens, Anal. Chem. *31*, 307 (1959).
7. Fontell, K., R. T. Holman and G. Lambertsen, J. Lipid Res., *1*, 391 (1960).

8. Morris, L. J., R. T. Holman and K. Fontell, JAOCS *37*, 323 (1960).
9. Morris, L. J., R. T. Holman and K. Fontell, J. Lipid Res. *1*, 412 (1960).
10. Kartha, A.R.S., JAOCS *30*, 326 (1953).
11. VanderWal, R. J., *Ibid. 37*, 18 (1960).
12. Gunstone, F. D., Chem. Ind. (London) —1214 (1962).
13. Hilditch, T. P., and C. H. Lea, J. Chem. Soc. —3106 (1927).
14. Hilditch, T. P., and P. N. Williams, "The Chemical Constitution of Natural Fats," 4th Ed. Chapman & Hall London, 1964.
15. Dutton, H. J., and J. A. Cannon, JAOCS *33*, 46 (1956).
16. De Vries, B., Chem. Ind. (London) —1049 (1962).
17. Barrett, C. B., M. S. J. Dallas and F. B. Padley, *Ibid.* —1050 (1962).
18. Kuksis, A., and H. J. McCarthy, Can. J. Biochem. Physiol. *40*, 679 (1962).
19. Kaufmann, H. P., and H. Wessels, Fette Seifen Anstrichmittel *66*, 81 (1964).
20. Vereshchagin, A. G., Biokhimiya *27*, 866 (1962).
21. Mattson, F. H., and L. W. Beck, J. Biol. Chem. *214*, 115 (1955).
22. Savary, P., and P. Desnuelle, Compt. Rend. *240*, 2571 (1955).
23. Hofmann, A., J. Lipid Res. *3*, 391 (1962).
24. Schonheyder, F., and K. Volqvartz, Acta Physiol. Scand. *10*, 62 (1945).
25. Desnuelle, P., and M. J. Constantin, Res. Commun. 2e Congr. Int. Biochem., Paris, 1952, p. 153.
26. Sager, C. A., Biochem. Z. *321*, 44 (1950).
27. Fritz, P. J., and P. Melius, Can. J. Biochem. Physiol. *41*, 719 (1963).
28. Entressangles, B., L. Pasero, P. Savary, P. Desnuelle and L. Sarda, Bull. Soc. Chim. Biol. *43*, 583 (1961).
29. Clement, G., J. Clement and J. Bezard, Biochem. Biophys. Res. Comm. *8*, 238 (1962).
30. Coleman, M. H., JAOCS *40*, 568 (1963).
31. Bottino, N. R., G. Vanderburg, and R. Reiser, in preparation.
32. Subbaram, M. R., M. M. Chakrabarty, C. G. Youngs and B. M. Craig, *Ibid. 41*, 691 (1964).
33. Chapman, D., A. Crossley and A. C. Davis, J. Chem. Soc. —1502 (1957).
34. Brockerhoff, H., J. Lipid Res. *6*, 10 (1965).
35. Brockerhoff, H., Arch. Biochem. Biophys., in press.
36. Coleman, M. H., unpublished.
37. Mattson, F. H., and R. A. Volpenhein, J. Lipid Res. *5*, 374 (1964).
38. Von Rudloff, E., Can. J. Chem. *34*, 1413 (1956).
39. Morris, L. J., in preparation.
40. Sweeley, C. C., R. Bentley, M. Makita and W. W. Wells, J. Am. Chem. Soc. *85* 2497 (1963).
41. Schlenk, W., "Festschrift Carl Wurster zum 60 Geburstag," (1960), p. 105.
42. Brockerhoff, H., personal communication to the author.
43. Brockerhoff, H., R. J. Hoyle and N. Wolmark, Federation Proc. *24*, 662, 1965.
44. Kaufmann, H. P., and H. Wessels, Fette Seifen Anstrichmittel *66*, 13 (1964).
45. Barrett, C. B., M. S. J. Dallas and F. B. Padley, JAOCS *40*, 580 (1963).
46. Youngs, C. G., and M. R. Subbaram, *Ibid. 41*, 218 (1964).
47. Jurriens, G., and A. C. J. Kroesen, *Ibid. 42*, 9 (1965).
48. Youngs, C. G., *Ibid. 38*, 62 (1961).
49. Subbaram, M. R., and C. G. Youngs, *Ibid. 41*, 445 (1964).
50. Subbaram, M. R., and C. G. Youngs, *Ibid. 41*, 595 (1964).
51. Kuksis, A., and H. J. McCarthy, Can. J. Biochem. Physiol. *40*, 679 (1962).
52. Coleman, M. H., JAOCS *38*, 685 (1961).
53. Gander, G. W., R. G. Jensen and J. Sampugna, J. Dairy Sci. *44*, 1980 (1961).
54. Litchfield, C., Margaret Farquhar and R. Reiser, JAOCS *41*, 588 (1964).
55. Savary, P., and P. Desnuelle, Arch. Sci. Biol. *39*, 689 (1955).
56. Achaya, K. T., B. M. Craig and C. G. Youngs, JAOCS *41*, 783 (1964).
57. Coleman, M. H., *Ibid. 41*, 247 (1964).
58. Kartha, A. R. S., J. Sci. Food Agric. *14*, 515 (1963).
59. Galloppini, C., and G. Lotti, Chim. Ind. (Milan) *45*, 812 (1963).
60. Harlow, R. D., C. Litchfield, H. C. Fu and R. Reiser, JAOCS *42*, 747–750 (1965).
61. Litchfield, C., and R. Reiser, *Ibid. 42*, 757–762 (1965).
62. James, A. T., op. cit. (3), p. 41.
63. Coleman, M. H., JAOCS, in press.
64. Claxton, G., J. Chromatog. *2*, 136 (1959).
65. Haahti, E., T. Nikkari and K. Juva, Acta Chem. Scand. *17*, 538 (1963).
66. James, A. T., J. R. Ravenhill and R. P. W. Scott. Chem. Ind. (London), (1964), p. 746.

The Triglycerides of Sable Fish (*Anaplopoma fimbria*). II. Fatty Acid Distribution in Triglyceride Fractions as Determined with Pancreatic Lipase[1]

AMI DOLEV and H. S. OLCOTT,
Department of Nutritional Sciences, Institute of Marine Resources, University of California, Berkeley, California

Abstract

Sable fish muscle lipids were fractionated on a silicic acid column with mixtures of chloroform and methanol as eluting solvents. Three main peaks containing only triglycerides were isolated; 11 additional peaks contained phosphorous. Each of the 3 triglyceride peaks was separately fractionated into 300 fractions on silica gel columns impregnated with silver nitrate. Mixtures of petroleum ether and ethyl ether were the eluting solvents. About 25 distinct fractions were isolated from each column. The fractions were characterized for fatty acid content by gas chromatography of the methyl esters. The results showed that the fractionation did not depend upon the presence of single fatty acids but upon total unsaturation. Fatty acid distribution within each fraction was determined with the use of hog pancreatic lipase, followed by thin-layer chromatography and gas chromatography.

Introduction

PREVIOUS ATTEMPTS to characterize triglycerides (1) have depended upon the use of total carbon number with gas-liquid chromatography (2,3), according to partition number on rubber columns (4,5) and liquid-liquid column partition chromatography (6). The development of adsorption chromatography on silicic acid impregnated with silver nitrate (7,8) for fractionation of the triglycerides was a significant break-through. This combined with the use of pancreatic lipase to determine the positional distribution of the fatty acids (9), were the methods used in the present study. While it was in progress, several other papers based on the same combination of methods were published (10,11).

In this study, we describe the nature of the triglycerides of a marine oil that contains 21 different fatty acids.

Experimental

The methods used for extraction of the lipids, and the fractionation of the different triglycerides by column chromatography, were those described previously (12).

Separation of Neutral Lipids and Phospholipid Fractionation

Neutral lipids were separated from the rest of the lipid components by column chromatography on silicic acid gel column using a modified method of that described by Shuster et al. (13). The silica gel (Mallinckrodt, 100 mesh) was washed three times with chloroform and two identical columns were packed wet (4.0 cm I.D., 55 cm long, packed silica gel height 46 cm). Chloroform was run continuously through the columns for 24 hr before application of the lipid. A sample containing 11.30 g of sable fish muscle lipids mixed with 15 ml chloroform was applied to one of

[1] Presented at the AOCS Meeting in Houston, Texas, 1965.