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STRATEGY OF GLYCEROLIPID SEPARATION AND QUANTITATION BY COMPLEMENTARY ANALYTICAL TECHNIQUES

PLENARY LECTURE

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SUMMARY

Although improved systems for chromatographic resolution continue to be developed there is good reason to believe that no single method will be capable of complete separation of all lipid mixtures including the geometric, positional and stereochemical isomers in each molecular species. Furthermore, the chromatographic systems giving the highest resolution usually yield the least complete recoveries of components and require separate procedures of quantitation. It is therefore necessary to develop appropriate strategies that yield the required resolution as a result of consecutive application of complementary analytical techniques. At the present time, the original combination of thin-layer and gas—liquid chromatography has been joined by the combination of thin-layer and liquid, and liquid and gas—liquid chromatography with both liquid and gas—liquid chromatography being frequently coupled to mass spectrometry with computerized data processing. Internal standardization with hydrogen flame ionization provides a simple quantitative detection for gas chromatography, while mass spectrometry serves a similar purpose in liquid chromatography, although a much more extensive calibration may be required for quantitation. Special advantages for both separation and quantitation of most neutral lipid mixtures are derived from enzymic and chemical modification of the samples prior to chromatography. With imaginative work-up of samples, superior qualitative and quantitative results can frequently be obtained by appropriate combination of chromatographic techniques of limited resolving power.

INTRODUCTION

The glyceryl esters of fatty acids possess the most complex structure of any natural products of comparable molecular weight. Their derivatives are widely distributed in nature and frequently account for the bulk of the mass of cell membranes and lipoproteins. The biochemical and metabolic requirements of the structural complexity, however, remain unknown. Since the individual molecular species of the glycerolipids exhibit marked physico-

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chemical differences in their bulk and monolayer properties, it is widely believed that the glycerolipids serve some physico-chemical function in the cell. Much effort is therefore being expended in identifying and quantitating the molecular make-up of the lipid phase of various cellular components in the hope that this information will lead to elucidation of the mechanism of their physiological action and the recognition of the need for the extreme diversity in their structure.

A complete identification of glycerolipids requires resolution of molecular species differing not only in molecular weight, degree of unsaturation and the overall polarity of the molecules as obtained by conventional methods of chromatography, but also a differentiation among double bonds of different positional distribution and geometric configuration, as well as a determination of the positional and stereoisomeric placement of the fatty acids on the glycerol molecules [1]. No single method of analysis exists that would be capable of resolving all of these molecules and there is good reason to believe that such methods will not be developed in the foreseeable future. It is therefore necessary to derive and utilize appropriate combinations of analytical techniques, which allow the separation and quantitation of a maximum number of components. In recent years the power of these separations has been further increased by combining the chromatographic techniques with mass spectrometry and computerized data processing, and by preceding the separations with appropriate chemical and enzymic transformations. Although no universally applicable routines have emerged, the advantages demonstrated for the separation and quantitation of model mixtures have suggested potential practical applications for work with specific glycerolipids and their mixtures of natural origin. In the following we have reviewed some of the more recent developments in the utilization of the combined techniques of analysis in the separation and quantitation of glycerolipids of model and natural mixtures with special reference to the utilization of the combined liquid chromatographic—mass spectrometric—computer (LC—MS—COM) system.

MATERIALS AND METHODS

The various experimental materials employed in the present illustrations were available in the laboratory from previous studies referred to in the text. The gas—liquid chromatographic (GLC) analyses were performed on a Hewlett-Packard Model 5880 capillary gas chromatograph, the high-performance liquid chromatography (HPLC) on a Hewlett-Packard Model 1084B liquid chromatograph and the mass spectrometry (MS) was done on a Hewlett-Packard 5985B quadrupole mass spectrometer equipped with an LC—MS interface based on the Baldwin—McLafferty split chemical ionization (CI) approach. The stereospecific analyses of triacylglycerols were performed as described by Myher and Kuksis [2]. The final products of the chemical and enzymic transformations were purified by conventional thin-layer chromatography (TLC) but in some instances the individual glycerolipid classes were further resolved by $AgNO_3$-TLC. The operating conditions for the polar capillary GLC of diacylglycerols were as described by Myher and Kuksis [3], while

the non-polar capillary GLC conditions for the examination of the double enzyme digestion products of glycerophospholipids were as given by Kuksis et al. [4]. The GC—MS of the diacylglycerols was performed as reported by Myher et al. [5]. The HPLC conditions of triacylglycerol resolution were modeled on the work of El Hamdy and Perkins [6] and of Herslof and Pelura [7] with the acetone—acetonitrile and acetonitrile—propionitrile systems, respectively. For the LC—MS application a gradient of 20—90% propionitrile in acetonitrile proved the most effective. The general conditions of operation and the performance of the Hewlett-Packard LC—MS interface have been described by Kenyon et al. [8]. In the present study, the MS scans were limited to masses above 200, because the lower mass range was contaminated with ions from impurities in solvents. The greatest offender was propionitrile, especially at the higher concentrations of gradient. To remove the contaminant ions (e.g. *m/z* 252, 391) a scan was made of propionitrile at 90% concentration, where no glycerolipids were being eluted, and the intensities of these ions were subtracted from all scans containing the ions belonging to the glycerolipids. There was no loss or distortion of the glycerolipid ions as verified by detailed examination of the mass spectra before and after the subtraction.

RESULTS AND DISCUSSION

The combination of complementary analytical techniques has been recognized as a useful strategy in lipid separation and quantitation in the past [9, 10] and in specific instances superior results have been obtained [11—13]. The need for strategy in glycerolipid analyses arises from the extreme complexity of natural glycerolipid mixtures, the limited resolving power of individual analytical techniques, the small amounts of sample usually available for analysis, a requirement for a realistic sample turn-around time, and a desire to minimize the cost of the analysis.

The more effective combinations of complementary analytical techniques include a systematic preliminary work-up of the sample by chemical and enzymatic means, which can also be applied to any subfractions of the sample derived following a chromatographic separation. The chemical work-up usually facilitates the separation of lipid classes, while the enzymic hydrolysis leads to a segregation of positional and stereochemical isomers of the glycerolipids. Combinations of complementary chromatographic techniques comprise the well established TLC—GLC routines and the more recently developed couplings of TLC—LC and LC—GLC. In recent years extensive use has been made of the combination of various MS methods with chromatography, of which the most valuable have been the GC—MS and LC—MS. Although these techniques possess an enormous analytical power, especially when combined with computerized data processing, there remain applications which cannot be adequately handled by unidimensional MS. There is a need for MS—MS combinations [14], which must be projected for future application in glycerolipid analysis, although the cost of the initial introduction of the system may be high.

Chemical modification

A number of chemical transformations are generally performed on the total lipid extract as well as on any subfractions of it derived by chromatography, which are not considered as specific combinations of complementary analytical techniques. These include saponification, transmethylation of fatty acids and silylation of various free hydroxyl and carboxyl groups prior to chromatography. However, Grignard degradation of triacylglycerols [15] must be recognized as a special analytical maneuver, which leads to the random release of diacylglycerols necessary for subsequent stereospecific analyses of triacylglycerols via both phospholipase A_2 and phospholipase C. Likewise, synthesis of both phosphatidylphenols [16] and phosphatidylcholines [2] as intermediates in the stereospecific analysis of the triacylglycerols via both phospholipase A_2 and phospholipase C must be considered as complementary steps in the total analytical scheme of determination of stereochemical structure of both diacylglycerols and triacylglycerols.

Specific enzymic hydrolyses

In many instances it has proved informative to subject the glycerolipid sample to a specific enzymic hydrolysis prior to chromatographic analysis. The advantages of using phospholipase C to release the diacylglycerol moieties from the glycerophospholipids are well recognized [17]. It should be noted, however, that phospholipase C is stereospecific and that it attacks the *sn*-3-phosphatides much more readily than the *sn*-1-phosphatides [18]. It can be used to distinguish between the enantiomeric glycerophosphatide types in a mixture and can serve to provide quantitative estimates of the relative proportions of the enantiomers by subsequent isolation and quantitation of the degraded and undegraded phosphatides [2]. A similar resolution of the enantiomeric glycerophospholipids can be obtained by means by phospholipase A_2, except that in the latter instance the identity of the molecular species is lost because of the destruction of the diacylglycerol moiety of the *sn*-3-phosphatide [19]. We have taken advantage of the stereospecificity of phospholipase C in devising a method of stereospecific analysis of triacylglycerols via an intermediary formation of *rac*-1,2-diacylglycerols and *rac*-1,2-diacylglycerophosphorylcholines [2].

Fig. 1 outlines the chemical and enzymic transformations in the generation of the *sn*-1,2- and *sn*-2,3-diacylglycerols. These transformations

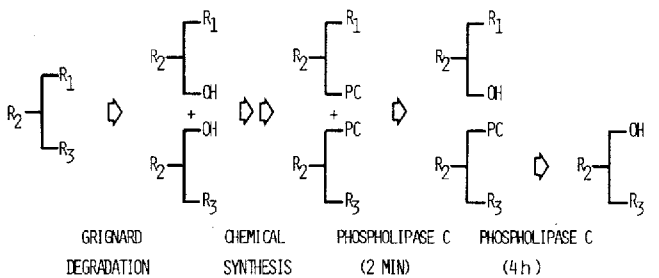

Fig. 1. Resolution of *sn*-1,2- and *sn*-2,3-diacylglycerol moieties of triacylglycerols by complementary chemical and enzymic transformations [2].

allow a direct examination of the pairing of the fatty chains in the various diacylglycerol moieties that are randomly generated from the triacylglycerols by Grignard degradation. Although this knowledge does not yet permit a direct reconstitution of the original triacylglycerol structure, it does allow the testing of the validity of the various random hypotheses about the structure of natural triacylglycerols on the basis of the predicted association of the fatty acids in these positions [20].

In the past phospholipase A_2 has been used to specifically release the fatty acids from the *sn*-2-position of *sn*-3-phosphatides and Brockerhoff [16] had utilized this reaction as the basis for his stereospecific method of analysis of natural triacylglycerols via the intermediate formation of *rac*-phosphatidylphenols. This enzyme destroys the *sn*-1,2-diacylglycerol moiety of the triacylglycerol molecules and traps the *sn*-2,3-diacylglycerol moiety in an enzymically inaccessible form.

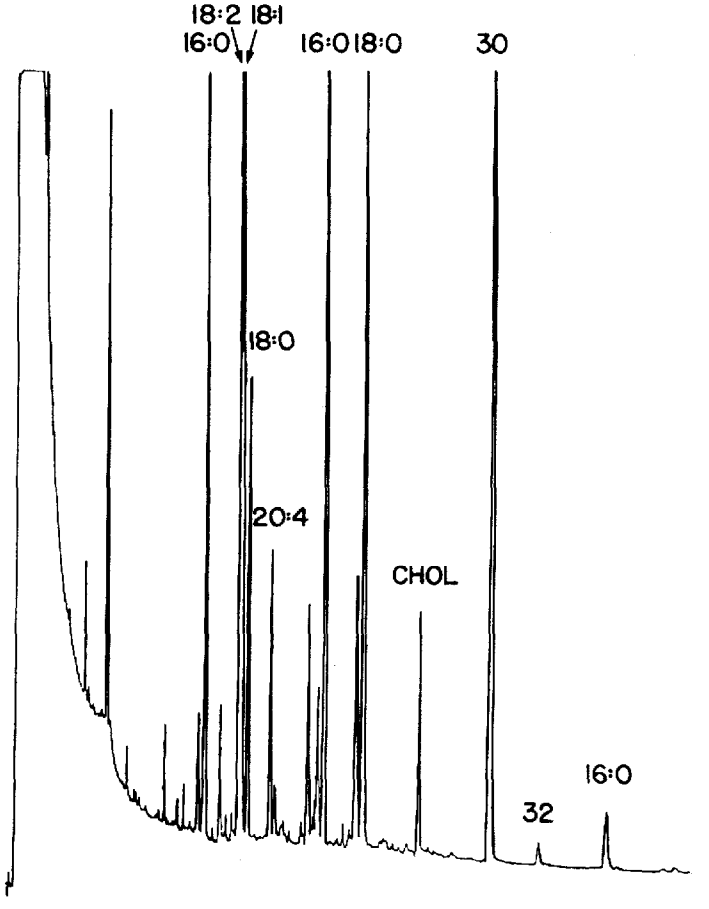

Fig. 2. Analysis of egg yolk phosphatidylcholine (PC) by consecutive digestion with phospholipase A_2 and phospholipase C, and capillary GLC [4]. First series of peaks (Peaks 16:0, 18:2, 18:1 and 20:4), fatty acids released from *sn*-2-position of PC; second series of peaks (Peaks 16:0 and 18:0), fatty acids retained in the *sn*-1-position as monoacylglycerols; CHOL, cholesterol; Peaks 30 and 32, triacylglycerols with 30 and 32 acyl carbons.

We have recently combined the action of phospholipase A_2 and of phospholipase C in a double enzyme digestion routine for the analysis of the structure of phosphatidylcholines in natural mixtures [4]. An initial digestion with phospholipase A_2 converts the diacylglycerophospholipids into the corresponding lysophospholipids with a release of the fatty acids from the *sn*-2-position. A subsequent digestion of the reaction mixture with phospholipase C converts the lysophosphatides into the corresponding monoacylglycerols. A trimethylsilylation of a total lipid extract of the final reaction mixture transforms the fatty acids into the trimethylsilyl (TMS) esters to represent the composition of the *sn*-2-position and the monoacylglycerols into the di-TMS ethers to represent the *sn*-1-position of the diacylglycerol moiety of the glycerophospholipid. All of these components can be completely recovered by means of GLC on both packed and capillary columns containing non-polar liquid phases or polar liquid phases as shown in Fig. 2.

We have also attempted to adopt the double enzyme digestion for an improved determination of plasma total lipid profiles. In the past we had depended upon phospholipase C to convert the choline phosphatides and sphingomyelins into the corresponding mono- and diacylglycerols and free ceramides, which could then be resolved from the free cholesterol, cholesteryl esters and triacylglycerols by high-temperature GLC [21]. In this routine, however, the diacylglycerols and ceramides largely overlap when run as the TMS or *tert.*-butyldimethylsilyl (t-BDMS) derivatives. A preliminary digestion with phospholipase A_2 converts the phosphatidylcholine into the lysophos-

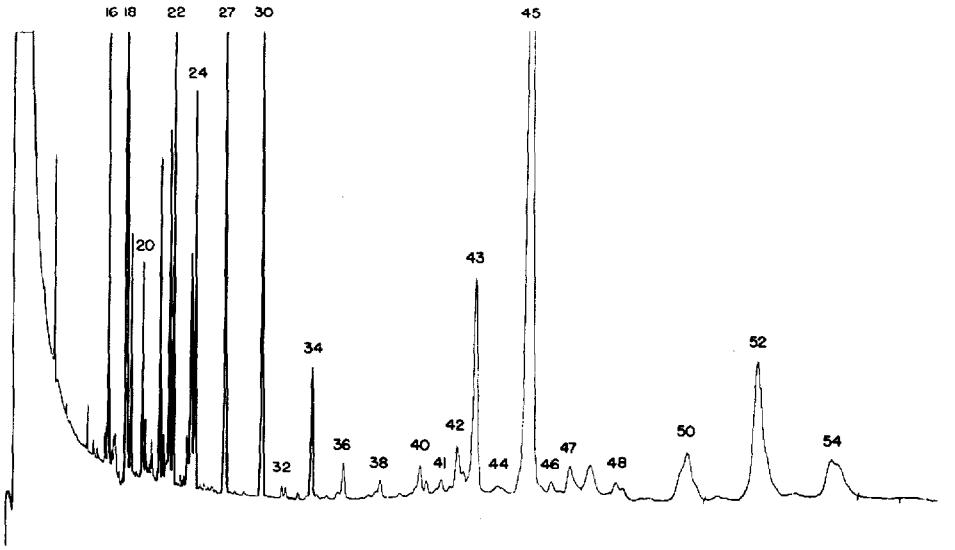

Fig. 3. Analysis of plasma total lipids by consecutive digestion with phospholipase A_2 and phospholipase C, and capillary GLC [4]. First series of peaks (Peaks 16, 18 and 20), fatty acids released from the *sn*-2-position of PC; second series of peaks (Peaks 20, 22 and 24), monoacylglycerols retaining the fatty acids in the *sn*-1-position of phosphatidylcholine; Peak 27, cholesterol; Peak 30, tridecanoylglycerol; Peaks 32, 34, 38, 40 and 42, ceramides with a total of 32—42 carbons; Peaks 41, 43, 45 and 47, cholesteryl esters of fatty acids with 14—20 acyl carbons; Peaks 44, 46, 48, 50, 52 and 54, triacylglycerols with a total number of 44—54 acyl carbons.

phatidylcholine, which can then be further converted along with the sphingomyelins into the corresponding monoacylglycerols and ceramides, which are now completely resolved as the TMS derivatives, as shown in Fig. 3. The liberated fatty acids overlap with the small amounts of plasma free fatty acids and the released monoacylglycerols overlap with the traces of plasma free monoacylglycerols, which do not significantly influence the estimates of plasma total phosphatidylcholines. The ceramides provide a direct estimate of the plasma total sphingomyelins, as well as of their relative composition or carbon number distribution. The free sterols, steryl esters and triacylglycerols remain unaffected during these transformations and are eluted with their characteristic retention times. Theoretically the remaining overlaps could be avoided by combining a double enzyme digestion with a double chemical derivatization [4]. In this procedure the plasma free fatty acids, lysophosphatidylcholines, monoacylglycerol free sterols and sphingomyelins are first converted into the corresponding t-BDMS derivatives. The reaction mixture is then subjected to hydrolysis with phospholipase A_2, which converts the diacylglycerophosphatidylcholines into the lysophosphatidylcholines and free fatty acids. A subsequent digestion with phospholipase C could then be used to remove the phosphorylcholine moieties from the lysophosphatidylcholines and the t-BDMS ethers of sphingomyelins, and the reaction products resolved by GLC following a further derivatization with the TMS reagent.

TLC–GLC

TLC is the most widely applied method of separation of natural glycerolipids and glycerophospholipids. Improved separation of glycerolipid classes has recently been realized by means of high-performance thin-layer chromatography (HPTLC) [22, 23], while improved quantitation of the resolved fractions has been achieved by the use of the Iatroscan system [24]. A combination of the TLC resolution with a subsequent GLC examination of the recovered components, however, can provide further information about the fatty acid composition of the glycerolipid classes. If the transmethylation and GLC analysis are performed in the presence of an internal standard, excellent quantitative measurements of the lipid classes may also be obtained along with the qualitative composition [25]. The TLC method is especially well suited for purification and isolation of the small amounts of material necessary for capillary GLC of the fatty acid methyl esters [26]. Furthermore, TLC may be performed with modified adsorbents, of which the most popular are those containing silver nitrate, which allows the separation of saturated and unsaturated fatty acid esters, as well as of certain geometric isomers. Although the latter method is amenable to work with intact glycerophospholipids [27, 28], more efficient resolutions of the saturated and unsaturated species are obtained following a removal of the polar head groups by phospholipase C [17, 29]. Although most of the present knowledge of the molecular species structure of glycerophospholipids has been obtained by the latter method [30], it is not entirely satisfactory, as the exact combinations of the fatty acids must be calculated from the molecular proportions of the major fatty acids assuming some type of random distribution. Much more certain are the combinations of the fatty acids derived from a high temperature GLC of the

diacylglycerols of uniform double bond content [12, 31]. Since free diacylglycerols tend to isomerize on the $AgNO_3$-TLC plates and yield separate peaks for the *sn*-1,2- and *sn*-1,3-isomers, it is necessary to block the free hydroxyl groups with acetyl [31] or t-BDMS [5] groups. Unlike the TMS ethers, the t-BDMS ethers are stable to moisture and can be purified by TLC prior to GLC. The blocking of the free hydroxyl groups also permits the resolution of the alkyl acyl, alkenyl acyl and diacyl species of the diradylglycerols, that may be present in some of the original glycerophospholipid classes [11].

Combinations of $AgNO_3$-TLC and GLC are also suitable for the investigation of the structure of natural triacylglycerols. High-temperature GLC on both packed [32] and capillary [33] columns allows a carbon number or molecular weight resolution of triacylglycerols of uniform number of double bonds. Transmethylation of the various argentation fractions yields the fatty acid composition as well as a quantitative proportionation of the individual subfractions. The method has been extensively utilized in a preliminary characterization of natural triacylglycerols [34, 35]. A more complete determination of the triacylglycerol structure requires partial degradation of the acylglycerol molecules prior to further resolution. Hammond [36] has outlined a theoretical approach to a complete determination of the molecular species of triacylglycerols by means of combined argentation TLC—preparative GLC and stereospecific analysis via phospholipase A_2. This method, however, is too complicated for practical execution and no natural fats have been analyzed in this manner as yet.

GC—MS

Another popular combination of complementary analytical systems is GC—MS. MS in the form of single ion monitoring provides a specific and sensitive detection for the effluents of the GLC columns, while the total ion current output compares favourably to the output of a hydrogen flame ionization detector. The MS identification of the molecular species within a complex mixture of diacylglycerols is in general limited to determination of carbon and unsaturation number. The technique also requires time-consuming data processing [37] and extensive calibration [38, 39].

When working with deuterium-labelled molecular species of the glycerolipids, it is necessary to perform the GC—MS assays following a preliminary $AgNO_3$-TLC separation. However, recent advances in the development of polar capillary columns may improve the GC—MS application to the natural diacylglycerol moieties of the glycerophospholipids or of triacylglycerols. Preliminary studies have shown that the capillary columns coated with SP-2330 (Silar 9C) produce very little bleed at column temperatures which yield essentially complete resolution of the molecular species of natural diacylglycerols (250°C). Fig. 4 shows that the resolution of diacylglycerols on these columns is sufficiently complete and reproducible and that the need for MS examination of the column effluents is largely eliminated [3]. However, unknowns and deuterium-labelled species must still be identified and quantitated by MS.

Likewise, GC—MS is effective in the characterization of natural triacyl-

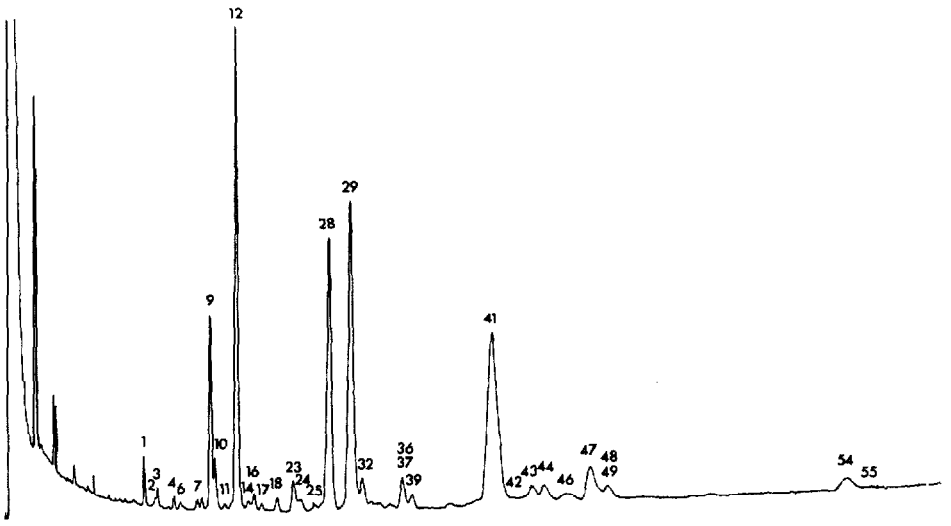

Fig. 4. Capillary GLC of TMS ethers of *sn*-1,2-diacylglycerols of rat liver phosphatidylcholines. The major peaks are: Peak 9, 16:0 18:1w9; Peak 12, 16:0 18:2w6; Peak 28, 18:0 18:2w6; Peak 29, 16:0 20:4w6; Peak 41, 18:0 20:4w6; and Peak 54, 18:0 22:6w3.

glycerols only following $AgNO_3$-TLC. The triacylglycerol fractions of uniform degree of unsaturation can then be admitted to the mass spectrometer as uniform carbon numbers, the fatty acid and diacylglycerol content and composition of which can then be readily recognized in the ammonia ionization mode [40].

Like capillary GLC, GC—MS has been greatly advanced by the development of the flexible quartz capillary column [41], which practically eliminates costly breakages and permits simple exchange of columns as the experimental needs may dictate. The flexible quartz capillaries are readily interfaced with MS instruments without the need of ineffective gas separators. At the present time, however, the flexible quartz capillaries are not available in a complete selection of liquid phases.

LC—TLC

In recent years LC in the form of HPLC has undergone a spectacular development and now provides a favourable alternative choice for the resolution of glycerolipids. While normal-phase HPLC yields essentially the same separations as TLC, reversed-phase HPLC provides separations that were previously possible only by a combination of $AgNO_3$-TLC and GLC. Thus, reversed-phase HPLC resolves natural triacylglycerols on the basis of both molecular weight and degree of unsaturation or polarity [42]. In fact, the reversed-phase HPLC columns are capable of a nearly complete resolution of all molecular species of the neutral acylglycerols, including many critical pairs and triplets [6, 7]. It is also possible to resolve all the major molecular species of the common glycerophospholipids by means of reversed-phase HPLC on C_{18} columns using phosphate buffers and methanol or choline chloride and methanol as the eluting solvents [43, 44].

Since the solutes resolved by HPLC are readily collected, it is possible to

subject them to further chromatographic resolution as such or following a chemical or enzymic transformation. In those instances where the critical pairs of the acylglycerols overlap during the reversed-phase HPLC, a subsequent $AgNO_3$-TLC resolution of the collected peaks may yield evidence of additional components. Bezard and Ouedraogo [45] have noted that certain natural triacylglycerol mixtures are more effectively resolved by HPLC following a preliminary segregation by argentation TLC. Furthermore, since the solutes of interest in the HPLC separation are frequently detected by UV absorption, the collected material may contain components which do not absorb in the UV. It is therefore prudent to subject the collected fractions to TLC to ensure their purity and to resolve any overlapping solutes.

LC—GLC

One of the major shortcomings of the HPLC method of analysis is the absence of a universal detector for lipids. Although the presence of unsaturated fatty acids and their esters may be detected by short-wavelength UV light (190—210 nm), the absorptivity is relatively low and variable, and quantitative analysis is impractical because of the need for extensive calibration. It has therefore proved practical to use the UV absorption only as a guide for peak collection and to perform the quantitative determinations of the solutes by GLC. In addition, complex mixtures of lipids may not be reliably identified by the retention times of standards, hence the peaks must be collected and identified by GLC as the methyl esters of the component fatty acids, which then also serve for their quantitation. Although laborious, the combination of HPLC and GLC provides the most effective means of resolving, identifying and quantitating the molecular species of intact natural glycerophospholipids [44]. Furthermore, in combination with capillary GLC it is possible to identify and quantitate the molecular species differing in the positional distribution and geometric configuration of the double bonds of the component fatty acids which remain unresolved by HPLC. There is no record as yet of the behaviour of the plasmalogens during reversed-phase HPLC of the glycerophospholipids. In a few instances the molecular species of glycerophospholipids have been first dephosphorylated and the released diacylglycerols converted into UV-absorbing derivatives prior to HPLC [46]. This maneuver greatly improves the sensitivity of detection and the accuracy of quantitation of the molecular species, but does not alter the extent of resolution or of the peak identification, which still requires assistance from GLC or MS.

Likewise, only small gains are made in the resolution of the glycerolipid species by converting the original glycerophospholipids into UV-absorbing derivatives. Proper identification of the peaks requires their collection and GLC examination. Consecutive application of reversed-phase HPLC and GLC has also proven to be effective in the resolution and identification of the molecular species of triacylglycerols [6, 7, 45]. Thus, by collecting the effluent from the HPLC columns and examining it by GLC for the fatty acid composition it has been possible to demonstrate that mixtures of acetone—acetonitrile (63.5:36.5) [6] and acetonitrile—propionitrile (50:50 or 30:70) [7] yield excellent resolution of certain critical pairs of triacylglycerols. Since positional isomers and enantiomers are not resolved by any of the HPLC systems, a

complete identification of the triacylglycerol species requires collection of peaks and stereospecific analysis as indicated above.

HPLC—MS

A combination of HPLC with MS is especially useful in glycerolipid work because of the low chromogenicity of the saturated fatty acid esters in the short-wavelength range of UV (190—210 nm), and the general low sensitivity of refractometric assay of all lipids. The mass spectrometer serves as a more sensitive detector and as a further analyzer of the components. The simplest technique for coupling HPLC to MS is the direct introduction of a fraction of the column effluent into the mass spectrometer [47]. The system employed in the present studies includes a provision for extra cryogenic pumping within the mass spectrometer in which the solvent vapour is trapped via cooling with liquid nitrogen. In this system the quantity of solvent introduced into the mass spectrometer is only about 1/100th of the effluent from the liquid chromatograph (1.5 ml/min). The mass spectrometer is operated in the chemical ionization mode, with the HPLC effluent serving as the reagent gas. The less extensive fragmentation in the chemical ionization mode provides increased sensitivity of detection, which is most important in view of the unfavourable effluent split.

Since the natural triacylglycerols are readily resolved by HPLC but not easily detected or identified by the UV and refractometry detection systems, we selected triacylglycerols for the combined LC—MS examination. The acetone—acetonitrile and acetonitrile—propionitrile solvent systems, which have thus far been shown to give the best resolution of triacylglycerols, also appeared to be excellent reagents for chemical ionization. They yielded readily

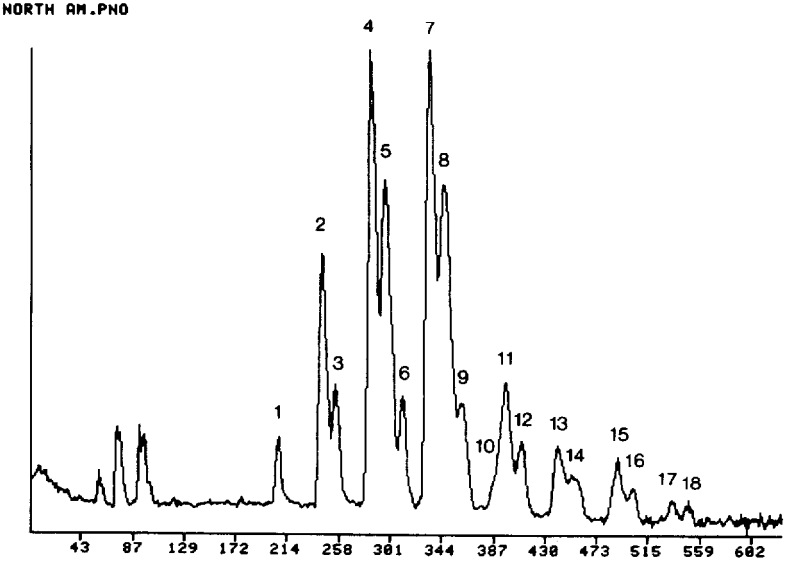

Fig. 5. LC—MS of peanut oil triacylglycerols. The major peaks are: Peak 2, 18:1 18:2 18:2; Peak 4, 18:1 18:1 18:2; Peak 5, 16:0 18:1 18:2; Peak 7, 18:1 18:1 18:1; and Peak 11, 18:1 18:1 18:0. LC—MS conditions: 30—90% propionitrile in acetonitrile in 60 min. Flow-rate, 1.5 ml/min.

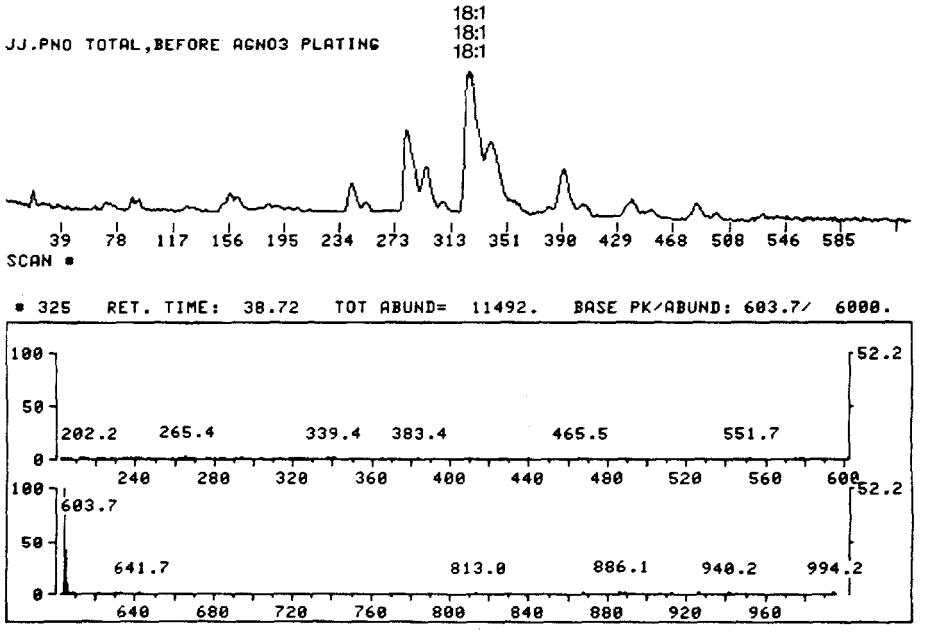

Fig. 6. Chemical ionization spectrum of Peak 7 in Fig. 5. Upper panel, total ion current profile; lower panel, mass spectrum of trioleoylglycerol. LC—MS conditions as in Fig. 5.

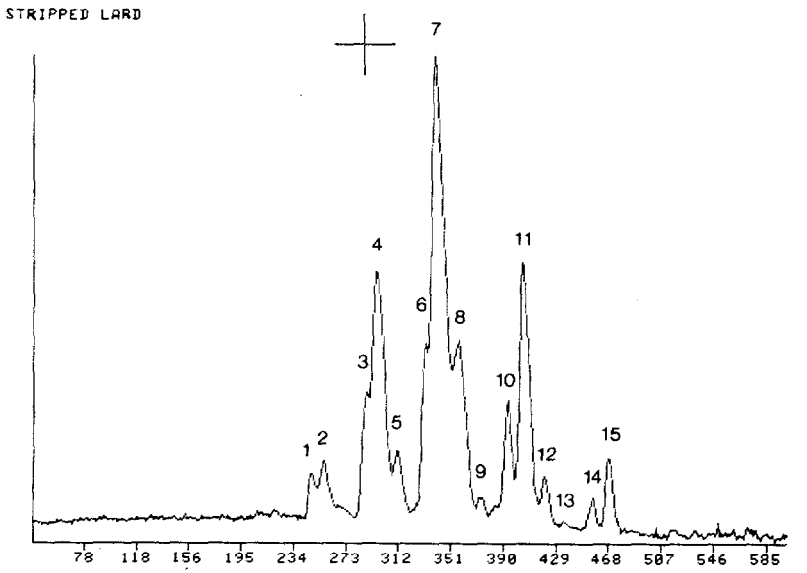

Fig. 7. LC—MS of stripped lard. The major peaks are: Peak 4, 16:0 18:1 18:2; Peak 6, 18:1 18:1 18:1; Peak 7, 16:0 18:1 18:1; Peak 8, 16:0 16:0 18:1; Peak 10, 18:0 18:1 18:1, Peak 11, 16:0 18:0 18:1; Peak 12, 16:0 16:0 18:0; Peak 14, 18:0 18:0 18:1; and Peak 15, 16:0 18:0 18:0. LC—MS conditions as in Fig. 5.

detectable protonated molecular ions as well as characteristic mass ions for the component diacylglycerols. We observed that a 20—90% gradient of propionitrile in acetonitrile provided sharper peaks and more complete recoveries of the saturated species than any of the isocratic solvent systems. Further-

more, this gradient system appears to be universally applicable to diacylglycerol and triacylglycerol mixtures. Fig. 5 shows the total ion current profile obtained for peanut oil triacylglycerols. The numbered peaks represent either individual triacylglycerols or small groups thereof. The three unnumbered peaks in the front represent small amounts of contaminating diacylglycerols. Fig. 6 shows the mass spectrum of one of the major peaks (Peak 7) identified as essentially pure trioleoylglycerol. It yields a small ion for the protonated parent molecule (*m/z* 886) and a single ion for dioleoylglycerol (*m/z* 603). The presence of significant amounts of trioleoylglycerol in the peanut oil was confirmed by LC—MS of the triene band isolated by $AgNO_3$-TLC.

The LC—MS method is equally well suited for analyses of animal fats. Fig. 7 shows the elution pattern obtained for a sample of stripped lard using the propionitrile gradient in acetonitrile. The numbered peaks represent individual triacylglycerols or simple mixtures of them. Peak 6 is made up of pure trioleoylglycerol as indicated by the single prominent diacylglycerol ion at *m/z* 603 representing dioleoylglycerol. Fig. 8 shows that a much smaller peak, Peak 12 in the original pattern, can be identified as largely 16:0 16:0 18:0 triacylglycerol. The presence of this species is indicated by the formation of nearly equal intensities of diacylglycerols corresponding to 16:0 16:0 (*m/z* 551) and 16:0 18:0 (*m/z* 579) ions, although theoretically a ratio of 2:1 would have been anticipated.

Fig. 9 shows the total ion current profile of human plasma triacylglycerols as obtained by LC—MS in the acetonitrile—propionitrile gradient. The numbered peaks correspond to individual triacylglycerols or small groups thereof. Fig. 10 shows that Peak 13 is made up essentially of pure 16:0 18:1 18:0 triacylglycerol as indicated by the ions *m/z* 579 (16:0 18:0), *m/z* 577 (16:0 18:1) and *m/z* 605 (18:1 18:0), in roughly 1:1:1 proportion.

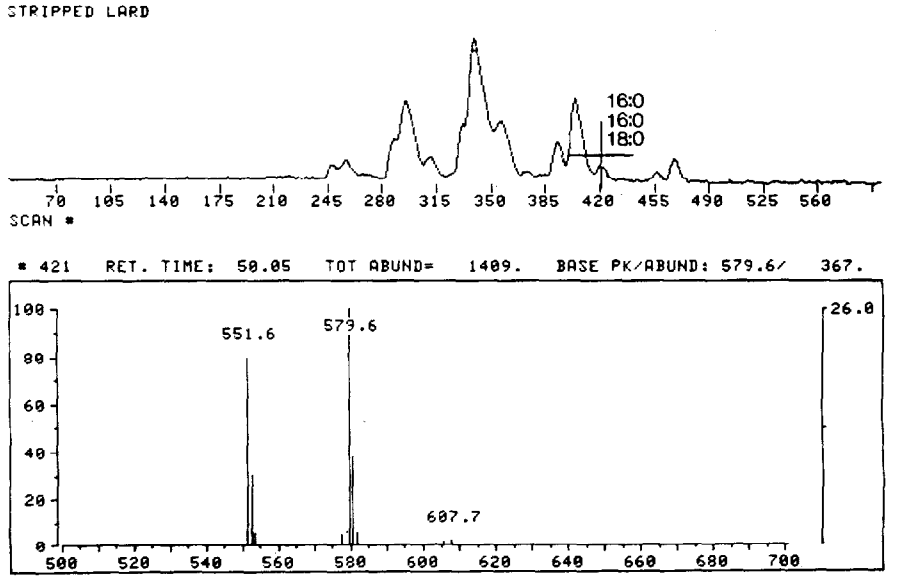

Fig. 8. Chemical ionization spectrum of Peak 12 in Fig. 7. Upper panel, total ion current profile; lower panel, mass spectrum of dipalmitoylmonostearoylglycerol. LC—MS conditions as in Fig. 5.

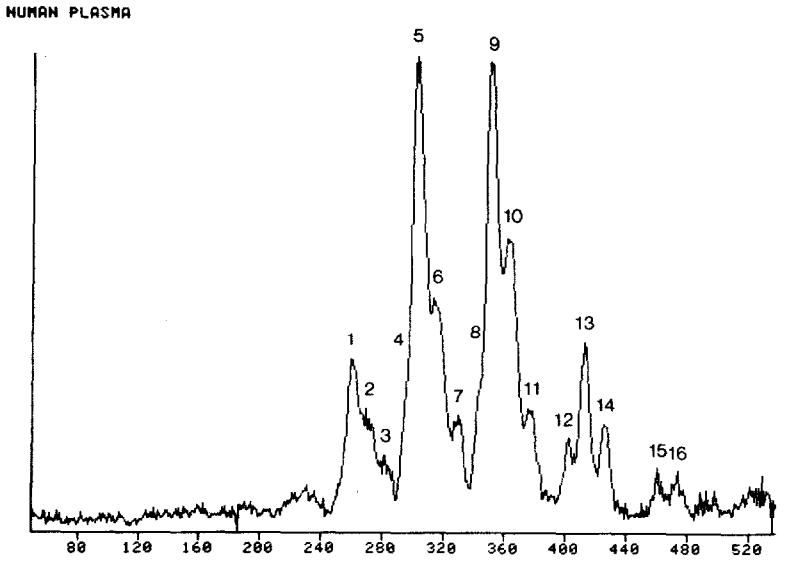

Fig. 9. LC—MS of human plasma triacylglycerols. The major peaks are: Peak 1, 16:0 18:2 18:2; Peak 5, 16:0 18:1 18:2; Peak 6, 16:0 16:0 18:2; Peak 9, 16:0 18:1 18:1; and Peak 10, 16:0 16:0 18:1. LC—MS conditions as in Fig. 5.

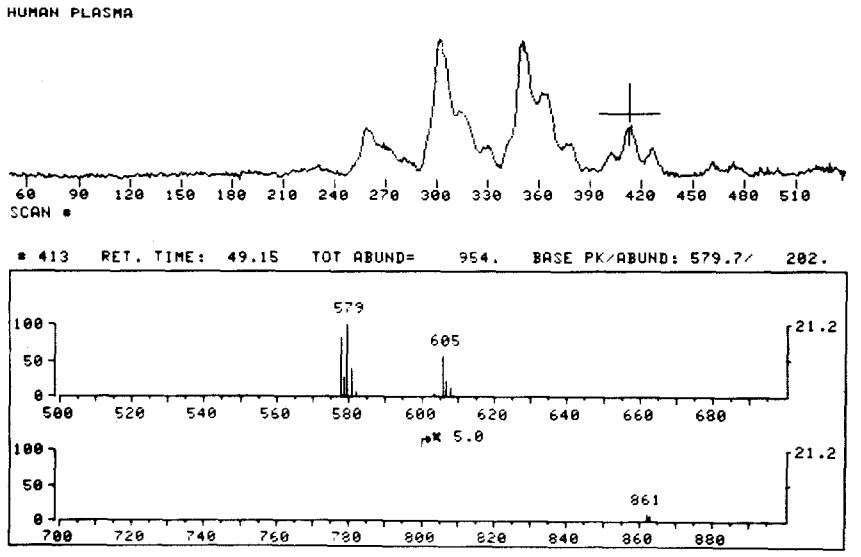

Fig. 10. Chemical ionization spectrum of Peak 13 in Fig. 9. Upper panel, total ion current profile; lower panel, mass spectrum of palmitoyloleoylstearoylglycerol. LC—MS conditions as in Fig. 5.

We have attempted to apply HPLC to the determination of plasma total lipid profile, but these efforts thus far have not been as successful as those obtained by high-temperature GLC. Fig. 11 shows the LC—MS profile of human plasma lipids after treatment with phospholipase C. In the acetonitrile—propionitrile gradient there was an excellent resolution of the diacylglycerols

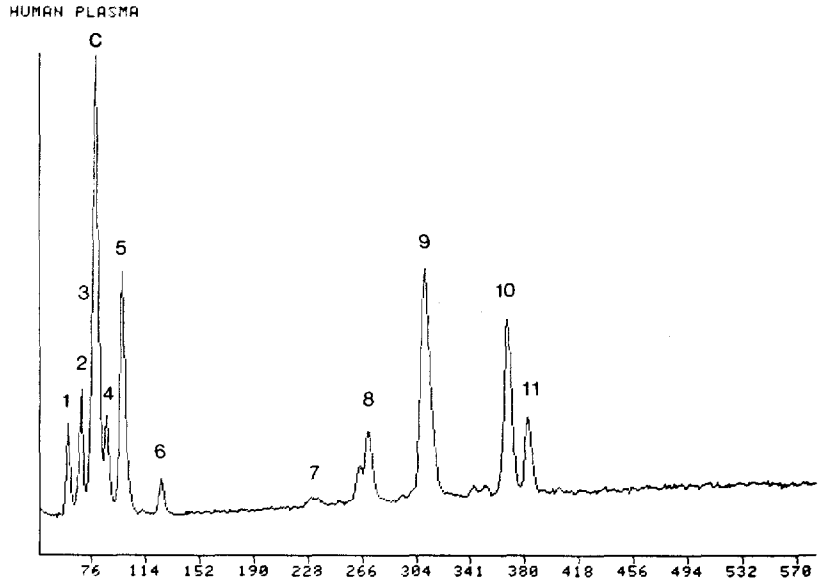

Fig. 11. LC—MS of human plasma lipids after dephosphorylation with phospholipase C. The major peaks are: Peak 1, 16:0 22:6; Peak 2, 16:0 20:4; Peak 3, 18:0 22:6 + 16:0 18:2; Peak 4, 18:0 20:4; Peak 5, 16:0 18:1 + 18:0 18:2; Peak 6, 18:0 18:1; Peak 8, cholesteryl arachidonate; Peak 9, cholesteryl linoleate; Peak 10, cholesteryl oleate; Peak 11, cholesteryl palmitate; and C, cholesterol. LC—MS conditions as in Fig. 5.

released from the plasma phosphatidylcholines by the phospholipase C digestion, and of the cholesteryl esters. Thus, Peaks 1—6 represent the diacylglycerols 16:0 22:6, 16:0 20:4, 18:0 22:6 + 16:0 18:2, 18:0 20:4, 16:0 18:1 + 18:0 18:2, and 18:0 18:1 as the major components. When run in the free form, the diacylglycerols 16:0 18:2 and 18:0 22:6 overlap partially with free cholesterol. In the acetate form, the cholesterol peak overlaps with that of 18:0 20:4 diacylglycerol species. There was also an overlap using the t-BDMS ethers of diacylglycerols and of free cholesterol. Peaks 8—11 represent the cholesteryl esters of arachidonic, linoleic, oleic and palmitic acids, respectively. The cholesteryl esters overlap with the triacylglycerols, which are seen as minor peaks interspersed among the major cholesteryl ester peaks. An overlap of triacylglycerol and cholesteryl ester peaks during HPLC has been previously noted [48, 49]. The triacylglycerol peaks can be best seen by subtracting the ions due to the cholesteryl esters from the total ion current profile. Fig. 12 shows that such a differential plot readily identifies the triacylglycerols as minor Peaks 1—13. Although this plasma sample is different from that analyzed above, it is seen that the same triacylglycerol pattern can be recognized. The removal of the ions due to cholesterol results also in the removal of the free cholesterol peak from the diacylglycerol profile.

Another complex mixture of triacylglycerols which we have subjected to LC—MS—COM analysis is provided by bovine milk fat. Fig. 13 shows the LC—MS profiles of the most volatile 2.5% of butterfat, and of the whole butterfat triacylglycerols. There is an excellent correspondence between the

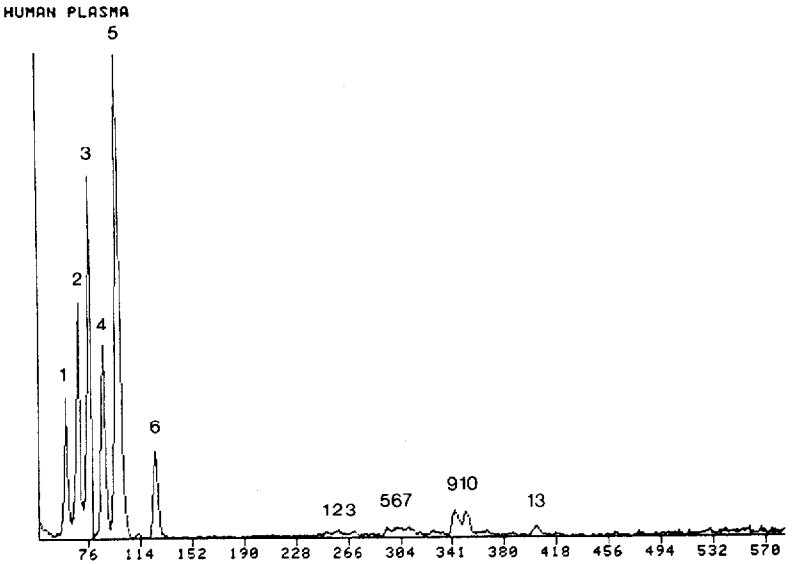

Fig. 12. Differential mass spectrum of plasma lipids as obtained by subtracting the ions for free cholesterol and cholesteryl esters from the total ion current profile in Fig. 11. First set of peaks (Peaks 1—6), diacylglycerols; second set of peaks (Peaks 1—13), triacylglycerols. LC—MS conditions as in Fig. 5.

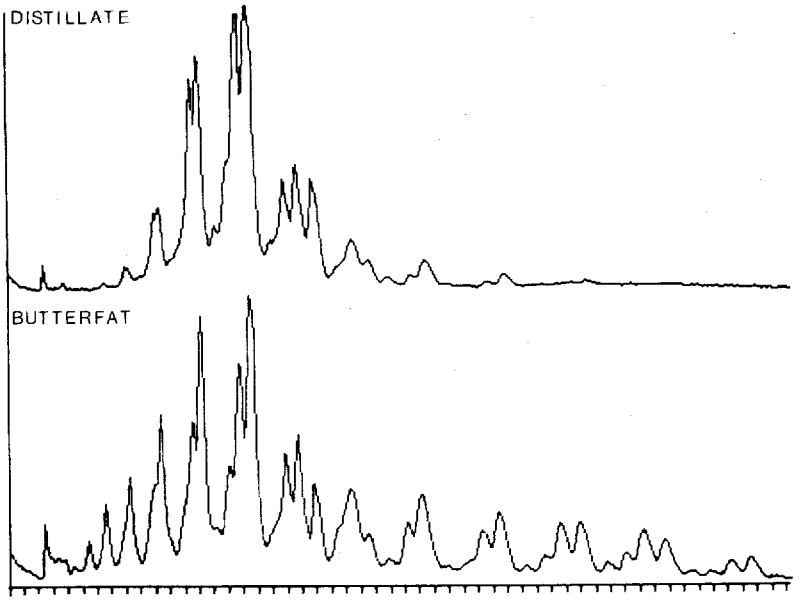

Fig. 13. LC—MS of the most volatile 2.5% distillate and of total bovine milk fat triacylglycerols. LC—MS conditions as in Fig. 5.

distillate peaks and the peaks in the total fat, although the actual sources of the triacylglycerols are not the same. Fig. 14 shows that the second half of the first major peak in the whole fat corresponds to 16:0 14:0 4:0, as the main component. We have identified all the peaks in the distillate and in the whole

butterfat and have verified that the corresponding HPLC peaks possess comparable compositions of molecular species. The composition of the peaks, however, is much more complex than the simple HPLC elution profiles would indicate. Fig. 15 shows the LC—MS—COM plot of the composition of the butterfat, by displaying the mass distribution for the ions of the component diacylglycerols and for some triacylglycerols corresponding to each triacylglycerol peak in the HPLC profile. Although the pattern is too complex to make any quantitative distinction, the qualitative distribution provides a good indication of the complexity of the species. The mass ranges have been given for each peak in a forward and backward direction so as not to obscure any of the masses present.

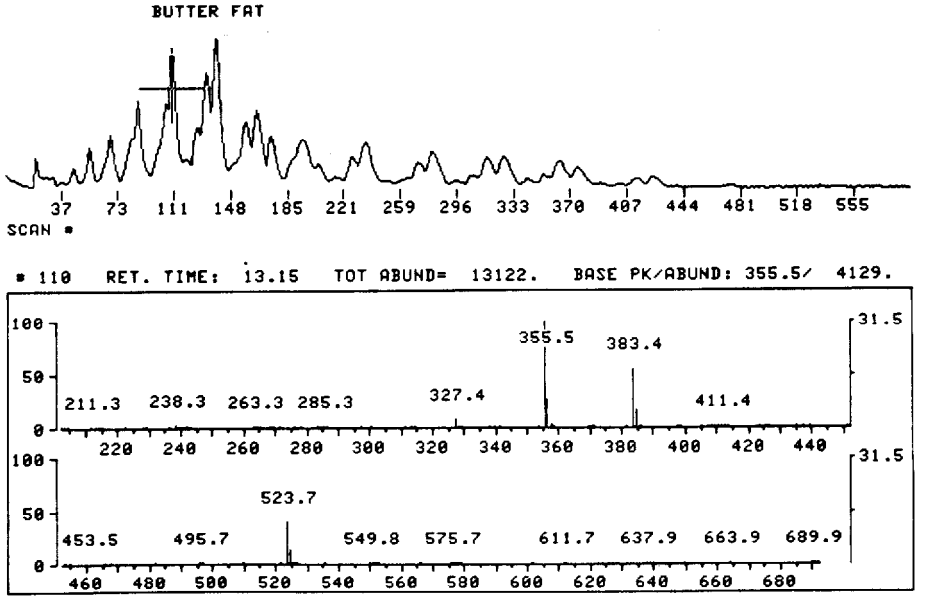

Fig. 14. Chemical ionization spectrum of one of the major peaks in the total bovine milk fat shown in Fig. 13. Upper panel, total ion current profile; lower panel, mass spectrum of palmitoylmyristoylbutyroylglycerol. LC—MS conditions as in Fig. 5.

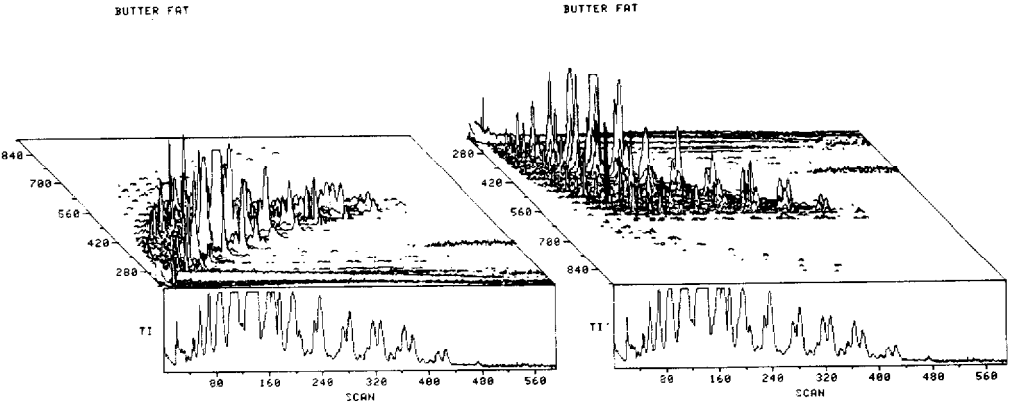

Fig. 15. Three-dimensional computer plot of the LC—MS data obtained for total bovine milk fat triacylglycerols in Fig. 13.

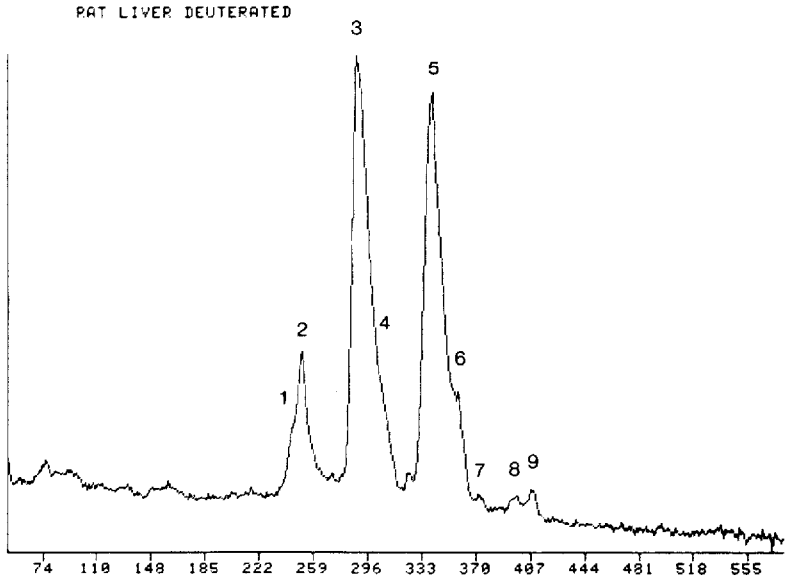

Fig. 16. LC—MS of deuterated rat liver triacylglycerols. The major peaks are: Peak 2, 16:0 18:2 18:2; Peak 3, 16:0 18:1 18:2; Peak 5, 16:0 18:1 18:1; and Peak 6, 16:0 16:0 18:1. LC—MS conditions as given in Fig. 5.

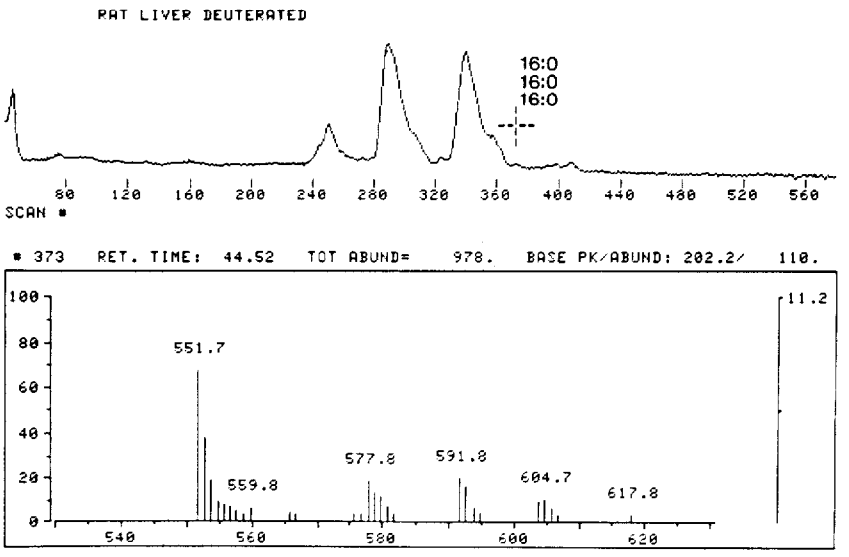

Fig. 17. Chemical ionization spectrum of Peak 7 in Fig. 16. Upper panel, total ion current profile; lower panel, mass spectrum of deuterated tripalmitoylglycerol. Note the presence of deuterium in the ions *m/z* 552—599. LC—MS conditions as in Fig. 5.

Like GC—MS, LC—MS is apparently well suited for the measurement of the stable isotope content and distribution among the various molecular species of triacylglycerols. Fig. 16 shows the total ion current profile of rat liver triacylglycerols obtained from an animal subjected to an overnight infusion of perdeuterated ethanol. Again the numbered peaks represent individual

triacylglycerol species or small groups thereof. Fig. 17 shows that a minor peak can be identified as tripalmitoylglycerol, with smaller amounts of 16:0 18:1 16:0 and a 17:0-containing species trailing from the preceding peak. Clearly the palmitic acid residues contained deuterium as indicated by the elevated intensities at *m/z* 552—559.

Fig. 18 shows the mass spectrum of one of the major triacylglycerol peaks. It can be identified as largely 16:0 18:1 18:2, as indicated by the corresponding ions for the diacylglycerols. Again, the palmitic acid component contains deuterium, but the presence of deuterium in any of the fatty chains in the 18:1 18:2 diacylglycerol moiety is less certain, as the increase in the mass could have arisen from a presence of deuterium in the glycerol moiety of the molecule. Although some of the ambiguity of the distribution of the deuterium among the triacylglycerol species can be eliminated by a preliminary $AgNO_3$-TLC, a complete clarification of the problem is not possible. For this purpose an MS—MS analysis of the peaks would be desirable [14]. In such a system, selected ions of the parent molecule or of any derived fragments could be admitted to another mass spectrometer and the composition of these ions determined by a further fragmentation. In such a case no doubt would remain about the true origin of any of the fragments in the mass spectrum of the total mixture.

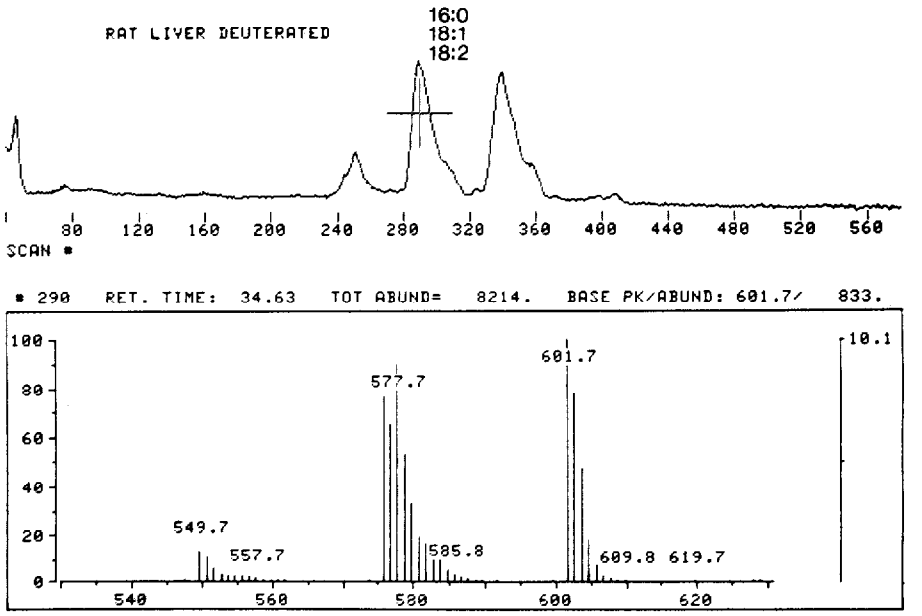

Fig. 18. Chemical ionization spectrum of Peak 3 in Fig. 16. Upper panel, total ion current profile; lower panel, mass spectrum of deuterated palmitoyloleoyllinoleoylglycerol. LC—MS conditions as in Fig. 5.

Quantitation

The most effective quantitation of neutral glycerolipids is obtained by means of hydrogen flame ionization detection, which gives a response directly proportional to the carbon content of the fatty esters over a wide range of

concentrations [50]. This method has been extensively employed in the quantitation of fatty acids and their glycerol and long-chain alcohol esters using both non-polar and polar, packed and capillary GLC columns. It can be applied to the determination of the relative and absolute amounts of the lipid components in the original sample or in any fraction thereof derived by the combined application of complementary analytical techniques, in which GLC provides the final step. An endless belt technique of hydrogen flame ionization detection of solutes in HPLC effluent has also been described and can give highly reproducible results under appropriate conditions of analysis [51]. The Iatroscan system is designed to provide a flame ionization response for the components resolved in the TLC or Chromarod systems, and for some homologous series a close relationship has been found between the mass concentration of the solutes and the peak areas recorded by the flame ionization detector [24]. However, many other mixtures undergo a highly variable decomposition on the absorbent surfaces and yield flame ionization responses that require extensive calibration for quantitative work [52]. A comparable measurement of the mass of the eluted components in a chromatographic system is provided by the total ion current of the mass spectrometer, but this response varies considerably with the molecular weight, unsaturation and chemical stability of the solutes and requires extensive calibration of individual molecular species [5].

The intact glycerophospholipids are best quantitated by their phosphorus content following a perchloric acid digestion to inorganic phosphorus, if necessary, in the presence of the silica gel from the TLC plate [53]. Colorimetric means of quantitation are also available for the fatty acid, glycerol and nitrogenous base moieties of the glycerolipids [54]. The latter methods utilize the Beer—Lambert principles of colorimetric analysis and do not require special discussion here. Of interest is, however, the post-column utilization of some of these methods for the detection and quantitation of the solutes emerging in the effluent of the HPLC columns [55].

In connection with the HPLC separation and detection of lipids must be mentioned the possibility of their detection in the short-wavelength UV range (205—210 nm), which is largely due to the double bonds in the fatty acid molecules [56]. This method of detection and quantitation of the lipid esters requires extensive calibration with the corresponding molecular species before the recorded peak areas may be effectively utilized for calculation of the masses of the solutes. In contrast, the UV-absorbing derivatives of fatty acids and diacylglycerols (e.g. esters of UV-absorbing alcohols and acid) can be quantitated directly on the basis of the molar extinction coefficient of the reagent, which is not significantly affected by the nature of the molecular species of the fatty chains [46, 56]. Extensive calibration is also not necessary for quantitative measurements of lipids in the HPLC column effluents by means of refractometry [42].

Relative composition

A simple GLC elution pattern provides the relative peak area composition of the fatty ester mixture [57]. The individual areas of all components are determined, and from the mean the relative peak areas are established.

$$\%X = 100\ A_x/(A_1 + A_2 + x \ldots + A_n)$$

where A_x is the area of a given peak X and A_1 to A_n the sum of all the peak areas. High-quality packed columns or short capillary columns, which allow complete recoveries of all components, when combined with hydrogen flame ionization detection generally yield area responses, which agree closely with the weight concentration of the fatty esters in the mixture and the correction factor is 1. It is important to verify that the estimates of the relative composition hold over the entire range of concentrations encountered in the samples, not just for the concentrations found in a commercial sample that happens to be used for calibration of the system. This can be tested by means of appropriate dilution of the various components of the standard mixture by adding known amounts of pure components, as well as by injecting different concentrations of the total sample in the presence of an internal standard of a fixed concentration. For fatty esters comprising different homologous series and for analytical systems not giving complete recoveries of all components, it is important to correct the peak area for differences in the detector response. This can be done by means of correction factors (F), which yield corrected individual and total peak areas,

$$\text{Corr. Area} = A_1F_1 + A_2F_2 + \ldots + A_nF_n$$

where A_1 to A_n are the peak areas and F_1 to F_n are the corresponding response correction factors. The correction factors are determined experimentally by reference to a standard known to be completely recovered ($F = 1$), as follows

$$F_x = (A_r/A_x)\ (W_x/W_r)$$

where A_x and A_r are the areas of the solute and reference peaks, respectively. W_x and W_r are the concentrations of the solute and the reference compounds.

It should be noted that in these corrections no distinction is being made between differences in the detector response due to loss or decomposition of the solute on the column and an inherent difference in the total ion yield in the hydrogen flame ionization detector. The weight response can be easily converted into a relative molar response by dividing it out by the molecular weight of each fatty ester and normalizing the result.

Absolute composition

In addition to the relative composition of the sample, it is frequently necessary to ascertain the total amount of the fatty ester in the sample or in a subfraction of it. This can be accomplished by means of either the internal or external standard method. In the internal standard method the concentration of the fatty esters in the mixture is derived by adding to the unknown sample a known amount of an internal standard, which is completely recovered and which does not overlap with any of the solutes in the chromatogram [57]. The concentration of any one of the unknowns can be obtained by relating

its area to the area of the internal standard, and the total weight of the sample to the sum of all the areas.

$$C_x = (C_s/A_s)\, A_x F_x$$

where C_s and A_s are the concentrations and area of the standard and A_x and F_x are the peak area and response factors of the measured compounds. If the volume containing the unknowns is also known, the results can be expressed as weight units per 100 volume units, as an example. For analytical systems, which possess a limited linear response to increasing or decreasing solute concentration, the response relative to the internal standard is plotted against solute concentration over a range of solute concentration with a constant addition of internal standard, and the concentration of the unknown is obtained from the calibration curve. It is recommended, however, that an attempt is first made to adjust the analytical system in such a way that the analyses can be completed within the linear range of the response, as the errors outside this range can be variable (adjust sample size or column length).

External standards are used when it is not possible to use internal standards, when peaks are poorly resolved or when there are too many peaks. A calibration curve is prepared using a range of the external standard concentration by injecting accurately controlled volumes in the column and plotting the response against the concentration of the standard. As the injection volume remains constant, absolute amount of the standard is directly proportional to the concentration. Injection of an equal volume of sample containing the unknown can then be used to obtain its concentration from the graph. Reproducible volumes may be injected with a conventional Hamilton syringe by first drawing up 1 μl of the solvent followed by 1 μl of the standard solution. Emptying the entire contents of the syringe into the injector results in a quantitative delivery of 1 μl of standard solution. It is usually impractical to prepare precise calibration curves by injecting increasing volumes from a constant standard concentration. The external standard technique has been extensively employed in the calibration of the total ion current and of single ion response in the mass spectrometer, where a representative internal standard cannot be used because of the need for closely reproducing both molecular weight and chemical properties of the unknown. In GC—MS and in LC—MS isotope-labelled internal standards may be employed to provide an exact match of the recoveries and ionization intensities for the measured and reference species.

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