differed by 4.91, 13.79 and 11.36%, respectively. Tocopherol contents were much more variable, especially margarines 3 and 6. Sterols were more consistent, and varied less than 18% (relative). From these limited data, it appears that the compositions of single samples of margarine are representative in general of the lipids in specific brands, and that linoleate and the tocopherols will be most variable.

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Analysis of Lipids by High Performance Liquid Chromatography—Chemical Ionization Mass Spectrometry

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ABSTRACT

The apparatus and techniques for the analysis of lipids by high performance liquid chromatography in conjunction with computer controlled chemical ionization-mass spectrometry are presented. The interfaces between the liquid chromatograph and the mass spectrometer and between the computer and the mass spectrometer are described. The identification of lipid classes separated by adsorption chromatography and molecular species by reverse phase chromatography also is presented. The techniques are applied to reference compounds and to examples of plant and animal lipids.

INTRODUCTION

The inherent difficulties of combining high performance liquid chromatography (HPLC) with conventional mass spectrometry have been elaborated by McFadden and coworkers (1,2). Present methods generally are limited to the narrow band of compounds that are too unstable for analysis by gas liquid chromatography (GLC), but are sufficiently stable or volatile to be introduced directly into the mass spectrometer. Kuksis and his colleagues (3-5) have successfully applied HPLC with chemical ionization mass spectrometry (CIMS) to the molecular species of triglycerides by a Direct Liquid Inlet (DLI) technique in which the species are simultaneously separated and analyzed.

Jungalwala et al. (6) reported the use of the Finnegan moving belt system for the analysis of spingolipid bases after hydrolysis and derivatization. More recently (7), these investigators applied this system to the analysis of phospholipids which they found could be separated by HPLC by the use of a solvent system containing ammonium hydroxide. The use of ammonium hydroxide in solvent systems for the separation of phospholipids was first used by Rouser et al. (8) in the gravity flow chromatography of lipid classes. We reported the use of NH_4OH-methanol in a gradient solvent system in the HPLC of plant and animal glycolipids and phospholipids about 1973 (9,10). More recently, we reported similar systems for the separation of lipofusion substances, neutral and polar lipid classes (11) and in our method for the quantitative analysis of the lipid classes (12).

Patton et al. (13) and Smith and Jungalwala (14) developed methods for the separation of phospholipids by HPLC using conventional UV detectors, but methods based on the use of photometric detectors are very difficult to employ for quantitative analysis as illustrated in the work of Perrin and Naudet et al. (15).

In previous work (16-18), we developed a simple interface for the purpose of combining HPLC with CIMS for the analysis of lipids based on their continuous conversion to volatile products by reduction with hydrogen prior to their introduction into the mass spectrometer. The operation of the interface was demonstrated on the lipid classes and related compounds by CIMS with methane as the reagent gas (16,17). Hydrogen was introduced into the reactor in this system to convert the acyl groups of the lipids to hydrocarbons. However, some of the double bonds in the acyl groups were hydrogenated, voiding identification of the fatty acid constituents. Hence we turned to the use of an inert gas in place of hydrogen and developed a rapid method (ca. 30 seconds) for the quantitative analysis of the 19 most common fatty acids found in plant and animal tissues directly by CIMS (19). With an inert carrier gas in our system, the acyl groups of the lipid classes are split from the backbone structures and identified as the $[RCOOH + 1]^+$ and $[RCO]^+$ ions. Since the products of the backbone structures of the lipid classes are produced by thermal degradation, their formation does not appear to be affected by the change to an inert gas. The application of this technique to the lipid classes and molecular species of triglycerides is described here.

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EXPERIMENTAL PROCEDURES

Materials and Methods

The solvents employed in HPLC must be rigorously purified. Methylene chloride was shaken with concentrated sulfuric acid in a separatory funnel several times until both upper and lower phases were clear. This solvent was then washed successively with dilute sodium bicarbonate and distilled in an all-glass still. Methanol was distilled in an all-glass still over potassium hydroxide pellets and zinc dust. Reagent grade acetonitrile, obtained from Fisher Scientific Company, Fairlawn, New Jersey, was treated with phosphorus pentoxide and fractionally distilled through a 2-meter Hyper-Cal Padbielniak column at a reflux ratio of 20:1. The above procedures removed nonvolatile contaminants and insured uniformity of the solvents from batch to batch.

Fractionation of the lipid classes using a column of silicic acid treated with ammonium hydroxide was carried out with an Instrument Specialties Company, Model 384 liquid chromatograph using a 40-min linear gradient solvent system starting with a 1:1 mixture of chloroform and methylene chloride and finishing with methanol containing 6% NH_4OH as previously described (16). The neutral lipids are not separated with this solvent system but eluted in a large band at the solvent front. The major phospholipids, however, are separated by the above gradient and were studied in the present work.

Pure dipalmitoyl, dipalmitoleoyl and dilinoleoyl phosphatidylcholines were prepared according to the procedures described by Brockerhoff and Yurkowski (20) and Robles and Van Den Berg (21) and were purified by thin layer chromatography.

Soybean oil, which provided a model for the analysis of triglyceride species, was a commercial sample in which the triglyceride fraction was purified as previously described (22).

HPLC of the molecular species of triglycerides was performed with the same liquid chromatograph, using a combination of two Zorbax reversed-phase columns obtained from E.I. Dupont de Nemours and Company and a gradient solvent system of methylene chloride in acetonitrile (22,23).

A schematic of the interface for combining these techniques in the present work is shown in Figure 1. The main difference in the present interface and that of previous models (16,17) is the modification of the transfer line between the reactor and the mass spectrometer to reduce dead volume and provide a more uniformly heated channel.

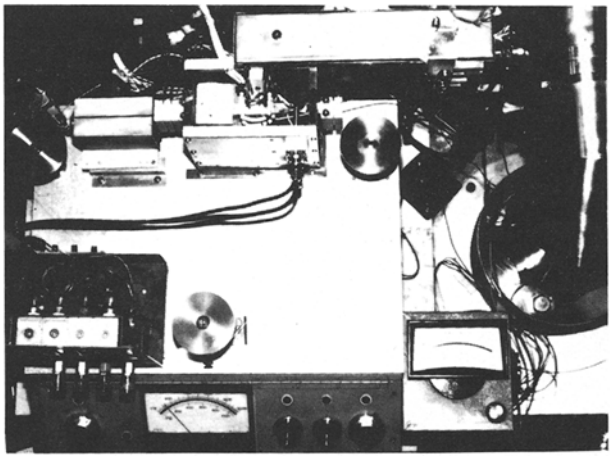

FIG. 2. Picture showing relation of HPLC-CIMS interface to the HPLC column and mass spectrometer.

A picture of the interface is shown in Figure 2 in order to put it in perspective with the liquid chromatographic column and the mass spectrometer. Details of construction of the interface blocks are illustrated in Figures 3A and 3B. Figure 3A shows the sandwich construction of the MACOR (Corning Glass, Ithaca, New York) blocks with H_2 being introduced into the stack of the flame detector via a tee connection, thereby eliminating reduction of the sample taken for mass spectrometry. Figure 3B shows the arrangement of the gas inlets into the reactor and to the mass spectrometer as well as the flame detector compartment. The two compartments of the reactor block are made by decreasing the width of the channel between them and by introducing nitrogen to block the volatile sample intended for mass spectral analysis from entering the flame section. The flame section is used for the final cleaning of the belt before it enters the outside atmosphere. It may be used as a flame ionization detector if only a small portion of the sample is removed for introduction into the mass spectrometer. Because of its high insulating properties, the use of MACOR enables different temperatures to be applied to the mass spectrometer and flame sections. Typically the reactor is operated at 250 C, while the flame section is operated at 500 C. Nitrogen is introduced into the front and rear parts of the reactor channel at 100 ml/min and 30 ml/min, respectively, while the helium flow is 60 ml/min. Helium is used in the flame section to prevent hydrogen absorption by the

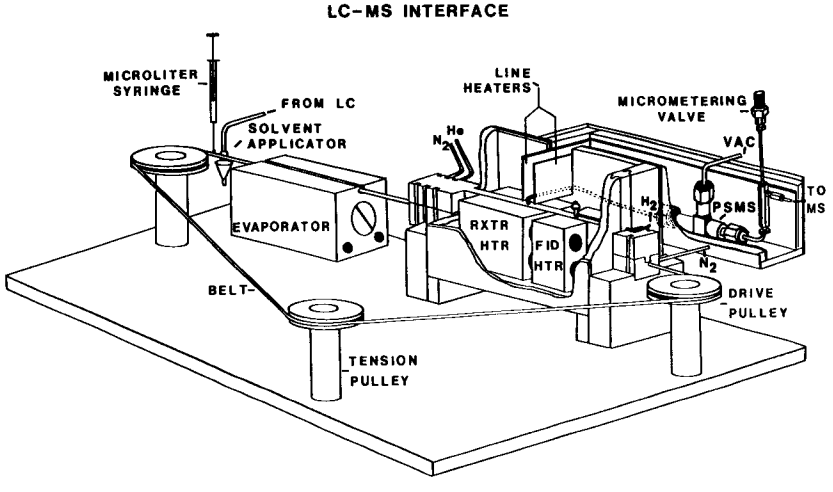

FIG. 1. Schematic of HPLC-CIMS interface system showing methods of introduction of sample onto the belt to its introduction into the mass spectrometer. RXTR HTR, reactor heater; FID HTR, flame ionization heater; VAC, vacuum; PSMS, porous silver membrane separator; MS, mass spectrometer; LC, liquid chromatograph.

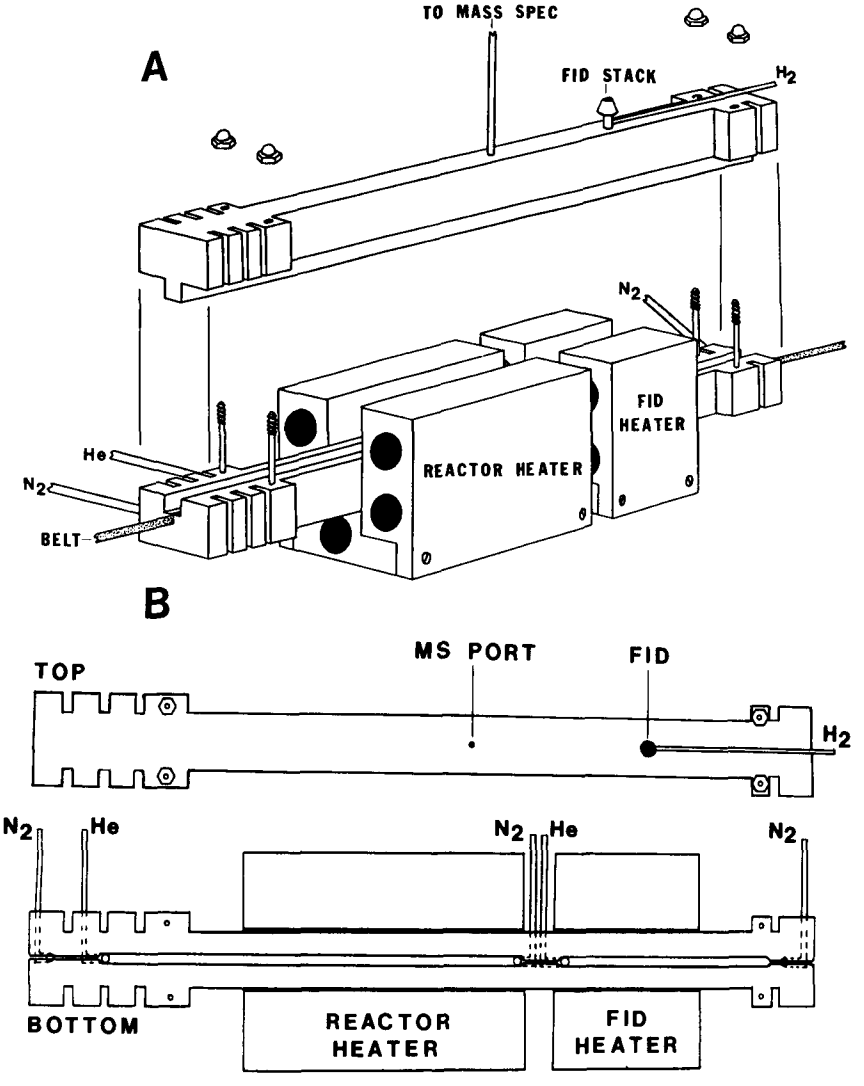

FIG. 3. A. Schematic of the reactor-FID unit showing the sandwich type construction and the tee used to introduce hydrogen into the flame. B. Schematic showing a top view of the top and bottom sections ot the reactor-FID unit. Gas inlets are shown in the bottom section and the hydrogen on the top section. Outlet ports to the mass spectrometer and hydrogen flame as shown in the top section.

belt, which would be evidenced by hydrogenation of double bonds, especially in polyunsaturated fatty acids. The helium flow rate at this point is 20 ml/min, and the nitrogen blocking the end is 20 ml/min. The flow rate of hydrogen into the tee is ca. 30 ml/min.

Simultaneous mass spectral and FID analyses can be made, but only the HPLC/CIMS technique is reported here. Special line heaters are used to maintain an even temperature in the connecting tube between the interface and the mass spectrometer. A porous silver membrane separator (24) is used to remove carrier gas and light volatiles produced in the reactor. An auxiliary vacuum pump is used as the main vacuum for the operation of the separator; the vacuum from the source of the mass spectrometer draws a sample for an analysis. The vacuums from both sources are regulated in coordination with each other by valves to give the highest concentration of sample in the amount of gas that can be accommodated by the mass spectrometer.

The eluent is applied continuously to the transport system, which is a specially designed stainless steel belt (25). The solvent is evaporated in the aluminum block at 180 C in a stream of nitrogen. The solvent vapors are then passed through a dry ice trap to collect the solvent. The sample which remains on the belt is transported into the reactor, where it is evaporated if it is volatile or converted to volatile compounds if it is involatile, as in the case of most lipids. Traces of remaining solvent and light volatiles produced in the reactor from the sample are removed by the separator as the volatilized compounds from the sample are drawn into the mass spectrometer for analysis.

The mass spectrometer is interfaced with a PDP 11/34 minicomputer which controls the analysis as illustrated in Figures 4 and 5.

Figure 4 shows a block diagram of the hardware used to control the mass spectrometer, collect and store the data. The mass spectrometer, which is a Biospect Model 7501 (Scientific Research Instruments, Inc., Baltimore, Maryland), is controlled through a 16-bit high resolution digital to analog (D/A) converter (Datel Systems Inc., DAC-169 series). This converter accurately regulates the quadrupole controller. Sample entering the source of the mass spectrometer from the interface system is analyzed, and the signal from the electron multipler tube is amplified by a high speed current amplifier (Keithly Model 428). The current then passes to the 12-bit high speed analog to digital (A/D) converter, sending digitized data to the computer for processing and storage. The raw or processed data can be retrieved by the video graphics terminal (DEC VT-55) and

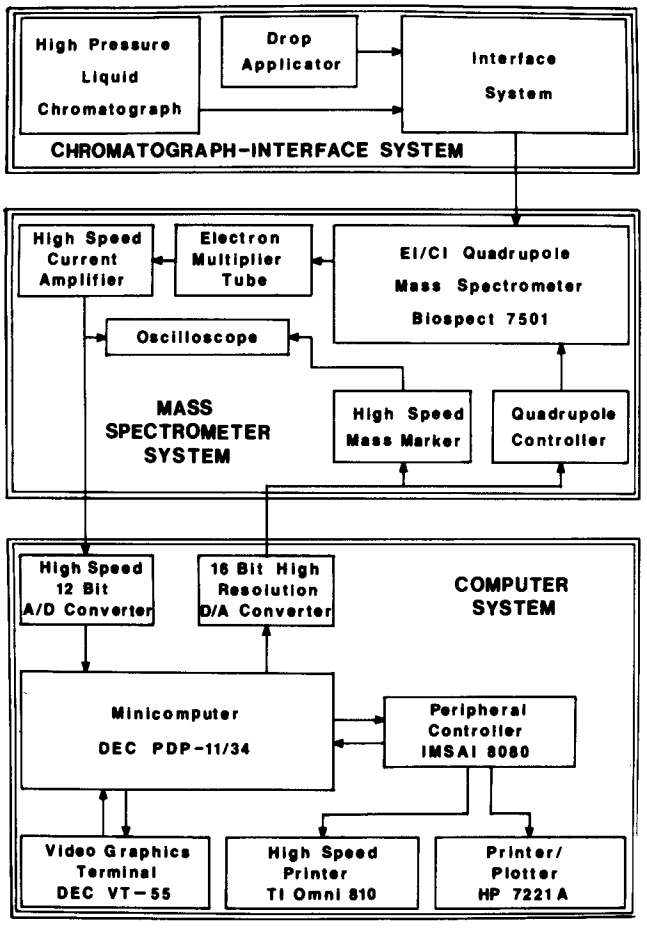

FIG. 4. Block diagram of the LC-IF-MS and computer hardware. IF, interface; EI, electron impact; CI, chemical ionization; A/D, analog to digital; D/A, digital to analog; DEC, Digital Equipment Corporation; TI, Texas Instruments Inc.; HP, Hewlett Packard; IMSAI, IMSAI Manufacturing Corporation.

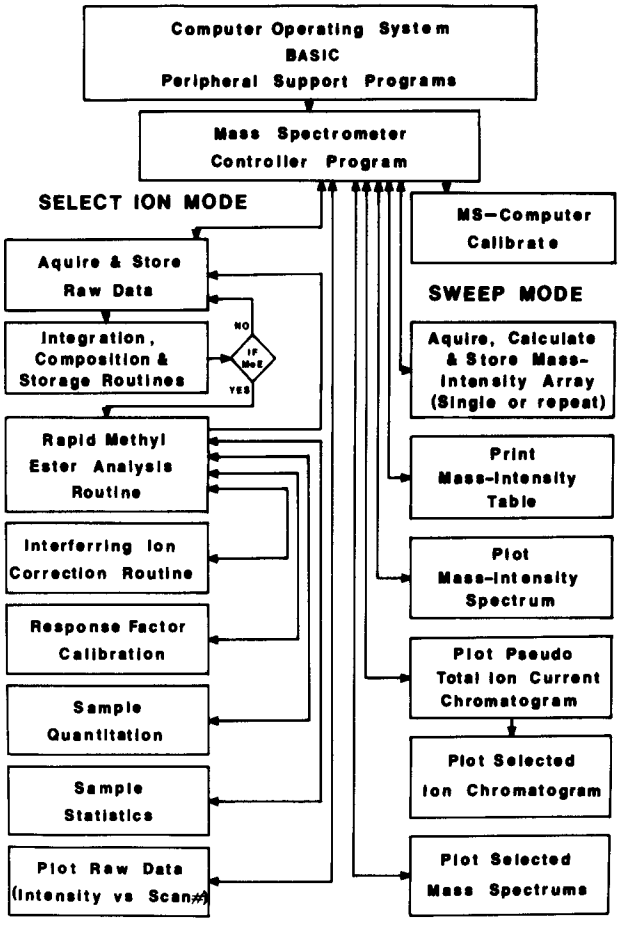

FIG. 5. Block diagram of the LC-CIMS system software.

subsequently sent to the printer/plotter (Hewlett Packard 7221 A) or the high speed printer (Texas Instrument Omni 810) through a peripheral controller (IMSAI 8080 microcomputer).

A diagram outlining the software used to control the mass spectrometer, collect, process and output data is shown in Figure 5.

With the exception of the computer operating system and BASIC, all other software was developed in this laboratory. After calling up the mass spectrometer controller program, the first operation was to calibrate the D/A converter in the computer to the mass ions being displayed.

There are then two modes of data collection, a Select Ion Mode, which we have used in previous work (19), and a Sweep Mode. In this work, the Sweep Mode with repetitive sweeps was used to obtain a pseudo-total ion current chromatogram and subsequent mass-intensity tables or spectograms.

RESULTS

The total ion current chromatogram for the analysis of normal rat kidney phospholipids is shown in Figure 6. Each of the runs shown on the horizontal axis consists of an average of 36 separate sweeps taken over 10-second intervals between the range of 230 to 330 amus. These were connected together by the computer to give the chromatogram shown in Figure 6. The peaks were identified from their retention times compared to those of reference compounds. The mass spectrum of the samples of pure phosphatidylcho-

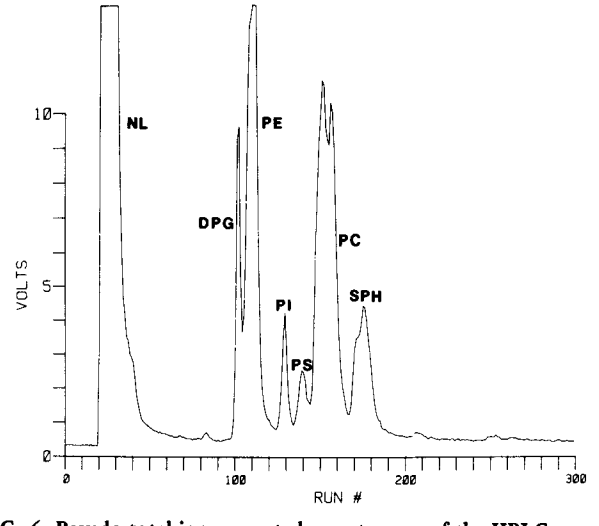

FIG. 6. Pseudo-total ion current chromatogram of the HPLC separation of normal rat kidney total lipid extract. Column 1–2.1 mm X 450 mm packed with 8 μ NH_4OH treated Spherisorb silicic acid. Solvent system: 40 min linear gradient starting with methylene chloride/chloroform (1:1) and ending with 6% NH_4OH in methanol at a flow rate of 0.5 ml/min. CIMS: 1.0 torr methane; reactor, 345 C; transfer lines, 315 C; source, 245 C. NL, neutral lipids; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine, and SPH, sphingomyelin fractions.

line (PC) (Fig. 7) shows that the acyl groups split from the backbone structure yield $[RCOOH+1]^+$ and $[RCO]^+$ ions, as illustrated in Figure 8. The strong ion with an amu of 72 in these spectra (Fig. 7) arises from the choline moiety

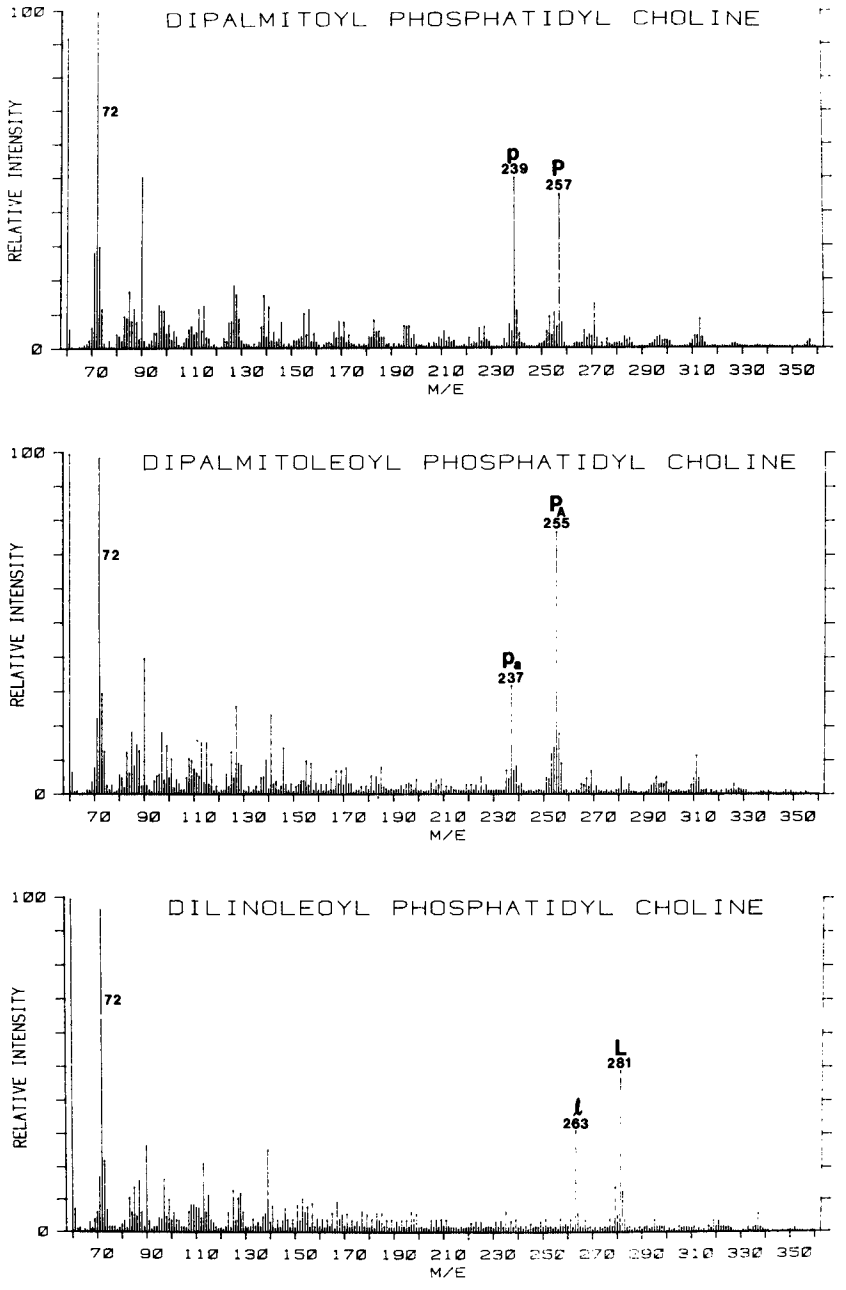

FIG. 7. Mass spectrograms of synthetic PC standards. CIMS: 0.75 torr isobutane; reactor, 325 C; transfer line, 315 C; source, 245 C; P, $[RCOOH+1]^+$ ion and p, $[RCO]^+$ ion of palmitic acid; P_A, $[RCOOH+]^+$ ion and P_a, $[RCO]^+$ ion of palmitoleic acid; and L, $[RCOOH+]^+$ ion and ℓ, $[RCO]^+$ ion of linoleic acid.

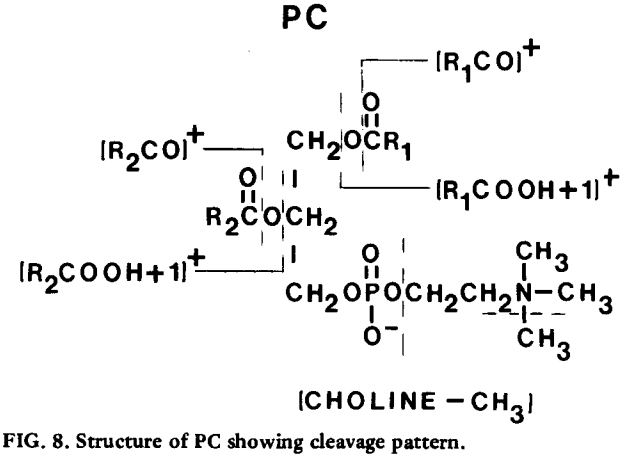

FIG. 8. Structure of PC showing cleavage pattern.

(Fig. 8), and might be used for the detection and identification of PC.

The mass spectra of the PC and phosphatidylethanolamine (PE) fractions selected from runs near the top of the peaks for these compounds, run #113 for PE and #157 for PC, are shown in Figure 9. These analyses indicate that the primary fatty acids of these compounds consist of palmitic, oleic, linoleic and arachidonic acids.

Preliminary studies on the quantitative analysis of the fatty acid composition of these fractions determined on the basis of the proportionality of the sum of both ions corrected for mass discrimination (19) are shown in Table I. The agreement between the GLC and HPLC-MS indicates that the acyl groups are converted essentially quantitatively to the $[RCOOH+1]^+$ and $[RCO]^+$ ions. However, it should be noted that the plasmogens cannot be separated by HPLC. Thus, the calculated fatty acid composition by both HPLC-

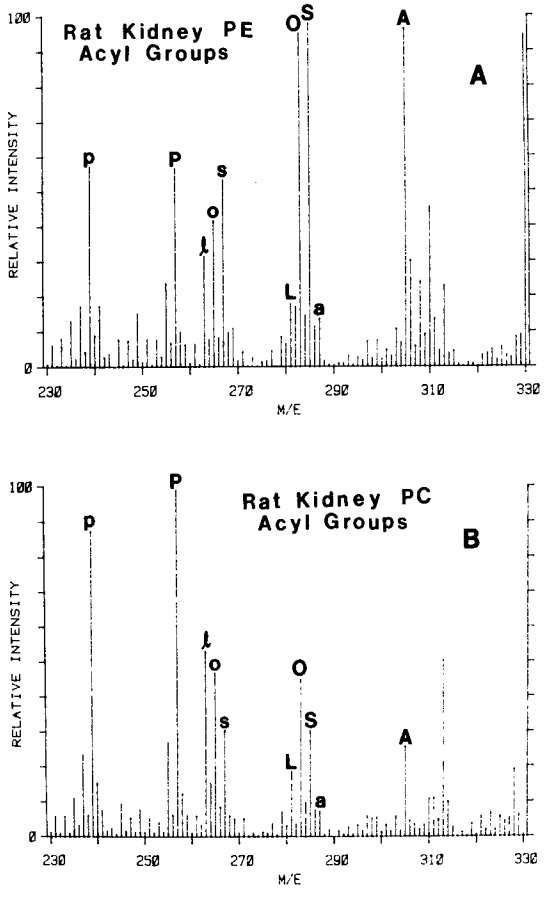

FIG. 9. Mass spectrograms of normal rat kidney PE and PC lipid class fractions taken at run numbers 113 and 157, respectively. Nomenclature used is the same as in Figure 7. P, p, palmitic acid ions; S, s, stearic acid ions; O, o, oleic acid ions; L, ℓ, linoleic acid ions; and A, a, arachidonic acid ions (large letters = $[RCOOH+1]^+$, small letters = $[RCO]^+$).

CIMS and GLC represent that of the total PC and PE fractions.

Triglyceride species analysis also can be determined by HPLC-CIMS, as illustrated by the chromatogram of soybean oil triglycerides in Figure 10. This oil has been analyzed by several investigators using conventional photometric detectors (15,26,27), as well as by Marai et al. (4) by HPLC-CIMS. Hence, it serves as an excellent model to demonstrate the application of our method to the triglyceride species analysis of natural oils. This analysis agrees well with that which we reported with a flame ionization detector (22) and is similar to those generally reported in the literature. In the analyses shown in Figure 10, the mass spectrometer is used as a universal detector within a range of 230-290 amus. This range covers the ions given by the acyl groups of the common triglycerides of vegetable oils and permits a species identification on the basis of the $[RCOOH+1]^+$ and $[RCO]^+$ ions produced from the fatty acid constituents as indicated in Figure 8 and for pure triglycerides (not shown).

It is also possible to obtain the direct identification of poorly separated minor species by ion subtraction as shown for the LLS peak in soybean oil in Figure 11. In this analysis, the minor component is identified by a subtraction technique in which the peaks on either side of the minor component, i.e. LOO and LOP, are identified (Figs. 11A and 11B). The primary ions of the minor peak are identified from a run at or near its top, as shown in Figure 11C. Contamination of the minor peak with species containing oleic acid is readily recognized, and the true fatty acid spectrum of the minor component is determined by the subtraction

TABLE I

Fatty Acid Analyses of Rat Kidney Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) Fractions

	PE		PC	
FFA species	MS	GLC	MS	GLC
16:0	14.2	10.7	34.4	32.7
18:0	25.1	28.7	14.8	16.2
18:1	15.5	11.8	15.1	13.2
18:2	10.5	8.7	20.9	19.2
20:4	34.7	38.0	14.8	15.5

GLC = gas liquid chromatography; MS = HPLC-CIMS.

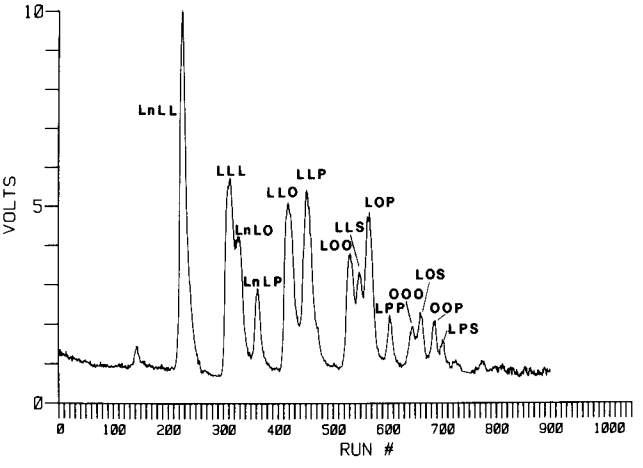

FIG. 10. Pseudo-total ion current chromatogram of the reverse phase HPLC separation of soybean oil triglyceride. Columns, two DuPont 4.6 mm × 250 mm packed with Zorbax 5μ ODS connected together. Solvent system: 40 min linear gradient of methylene chloride/acetonitrile going from 36-51% followed by a 40-min linear gradient of the same solvents going from 51-57% by volume at a flow rate of 0.4 ml/min. CIMS: 1.0 torr methane; reactor, 280 C; transfer line, 280 C; source, 265 C. Triglycerides are identified by the fatty acids present in the spectrum and do not indicate stereospecificity. P = palmitic acid; S = stearic acid; O = oleic acid; L = linoleic acid and Ln = linolenic acid.

of the species containing oleic acid, mainly LOO in this case (Fig. 11D).

DISCUSSION

The work presented here demonstrates that our technique provides a simple system for coupling HPLC with CIMS for determination of a profile of the lipid classes and molecular species of triglycerides. The acyl groups appear to be split out primarily as free acids from the backbone structure of both triglycerides and phospholipids in the reactor, giving $[RCOOH+1]^+$ and $[RCO]^+$ ions on chemical ionization in the mass spectrometer. Identification of the fatty acid constituents of the parent compounds is made on the basis of these ions.

No attempt was made to separate the plasmalogens of kidney PC or PE, the latter of which is estimated to be a total of ca. 10% (28). Only traces of the PC plasmogen appear to be present in kidney (28). Nevertheless, the acyl group of these compounds also gives $[RCOOH+1]^+$ and $[RCO]^+$ ions and thus, our analysis of these compounds represents that of the total fractions. Ether groups also are degraded (16), but no effort was made to detect them in this study.

The main use of HPLC-CIMS is for the detection and identification of peaks, particularly minor components and

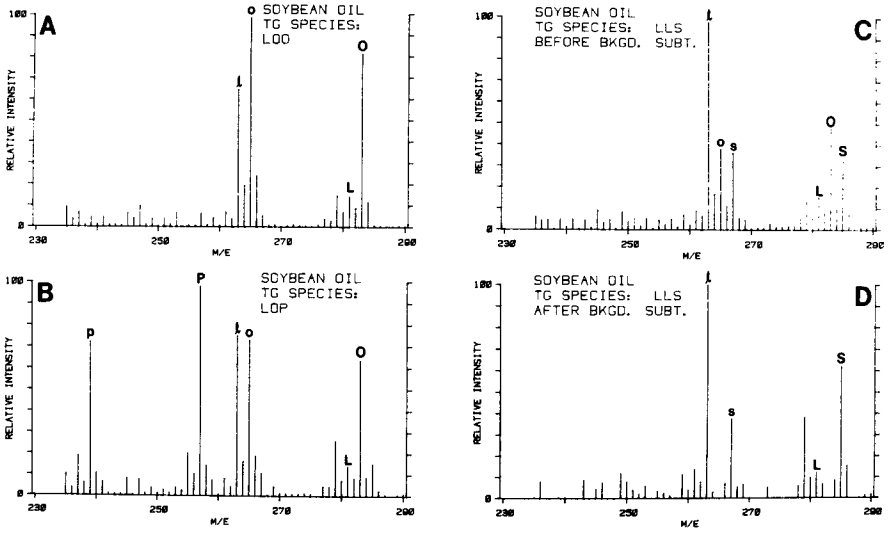

FIG. 11. Mass spectrogram taken in the LOO, LDP and LLS species = A, B and C, respectively. D) mass spectrogram of the LLS species after the contribution of LOO species is subtracted. The nomenclature is the same as in Figures 7 and 9.

those not well resolved. Identification of minor constituents was demonstrated here with the detection of LLS in soybean oil by an ion subtraction technique commonly used in GLC-MS (29). A different technique is employed in the identification of the molecular species by the DLI method because the major ions are given by diacylglycerides. These ions are not unequivocal in that some different combinations of fatty acids have the same molecular weight. Thus, reference must be made eventually to the fatty acid composition. With closely separated peaks and minor constituents, identification by the DLI method is made by the process of elimination upon multiple ion monitoring, which is time consuming and involved (3). Similar problems exist with the analysis of the phospholipids by the Finnegan Moving Belt system, in that a number of peaks are produced when intact compounds are introduced directly into the mass spectrometer.

Our technique does not permit a simultaneous determination of the lipid classes and their molecular species as attempted by Jungalwala et al. (7). The triglycerides and individual phospholipids must be analyzed separately inasmuch as completely different chromatographic systems are used for their separations, and components of the latter, in turn, must be isolated for a follow-up isomer analysis. It must be considered further that these compounds are far too complex for complete resolution by even the most sophisticated HPLC methods at their present state of development. For example, using a combination of physical and enzymatic techniques, we found that the PC fraction of milk serum contained over 100 different molecular species (30). A partial analysis of the triglycerides of milk serum gave over 150 molecular species (31). At this time, the application of HPLC-CIMS to the analysis of the lipid classes and triglyceride species is at an interim stage, with its greatest utility being for identification purposes; quantitative analysis is accomplished readily with a flame ionization detector (22) insofar as these compounds can be separated.

ACKNOWLEDGMENTS

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