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ANALYSIS OF PHOSPHOLIPIDS IN COW'S MILK BY HIGH-TEMPERATURE INJECTION GAS CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An investigation of the high-temperature injection (350°C) gas chromatographic behaviour of standards of various classes of phospholipids has elucidated certain characteristic fragments ("tracers") in the pyrogram of each class. Under these conditions of pyrolysis of the phospholipids, a natural mixture of such substances (extracted and purified from cows' milk) has provided evidence for the same "tracers" as for the standards. Analogous results were obtained with a more representative specimen of cows' milk of various breeds grown in areas of different altitudes.

The content of fatty acids in phospholipids, tested on each class (separated by means of radial compression high-performance liquid chromatography from polar lipids of milk) appeared to be relatively similar for all the phospholipid classes, and over half the content consisted of unsaturated fatty acids. The major component was Δ^9-octadecenoid (oleic) acid.

INTRODUCTION

The biological significance of phospholipids is that they are among the most important constituents of membranes. They also have a technological relevance because they are used as emulsifiers. They are generally found in complex matrices, associated with lipids of different polarities and in very small amounts.

Despite the existence of a considerable literature on the subject[1,2], the analysis of phospholipids is far from established, in particular the details of the extraction and purification techniques, which influence the final analytic data[3], given the high oxidation sensitivity of the substances in question[4,5]. It is difficult therefore to divide the mixtures into single classes, because they have very similar solubilities and polarities[6].

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In our study, we examined the lipids extracted from a natural matrix (cows' milk). The chromatographic study, carried out on a sample taken from a cow of the Bruno-Alpina breed from the Appennino Modenese, was intended to determine the single classes of phospholipids present and to separate them so that the various types of fatty acids present could be detected.

MATERIALS AND METHODS

Solvents and reagents

Sulphuric acid (96%), nitric acid (65%), glacial acetic acid, anthranilic acid, sodium hydroxide, sodium sulphite heptahydrate, hexamethyldisilazane and trimethylchlorosilane were analytical grade products from Carlo Erba, as were the solvents *n*-hexane, chloroform, methanol, ethanol, acetone and benzene, which were distilled before use when necessary. The substances supplied by Merck (analytical-grade) were: ammonium monohydrate molybdate, ascorbic acid, Kieselgel 60 plates (20 × 20 cm, with a layer thickness of 0.25 mm), pyridine (dried, for analysis), acetonitrile and methanol for HPLC. Silicic acid (325 mesh) was from Bio-Rad, potassium dichromate from J. T. Baker, and boron trifluoride (12% p/p) in methanol from Supelco (Bellefonte, PA, U.S.A.). The phospholipid standards (phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) from *Escherichia coli*, phosphatidylserine (PS) from bovine brains, phosphatidylinositol (PI) from bovine liver, and sphingomyelinn (Sph) from egg yolk) were from Calbiochem-Behring. The water for elution was doubtly distilled in the laboratory from deionized water. The solvents for high-performance liquid chromatography (HPLC) were degassed before use.

Instruments

A semi-industrial instrument (BVF/RB No. 2812/69 type from Brizio Basi, Milan, Italy), was used for the lyophilization. Spectrophotometric measurements were carried out on a Beckman Model ACTA CIII instrument.

The gas chromatographic analysis were performed with a Carlo Erba Model 4160 high-resolution gas chromatography (HRGC) instrument, interfaced with a Spectra-Physics Model 4100 integrating calculator. The HPLC analyses were performed with a Model 440 Waters instrument, fitted with a "Z-module", for radial compression chromatography (RCC).

Glass columns were used for gas chromatographic analyses (25 or 10 m × 0.32 mm I.D.), covered with SE-52 film of 0.10–0.15 μm thickness. For the HPLC analysis a cartridge packed with 10-μm silicas particles was inserted in the "Z-module".

The detector linked to the gas chromatograph was of the flame ionization type, and for HPLC a Waters Model 401 refraction index detector was used.

Analytical procedure

The crude lipids were obtained from a sample of milk, freeze-dried for 15 h, after freezing at −40°C in a layer 2-cm thick, by extraction with chloroform and methanol, according to the method developed by Sprecher[7]. The phospholipid fraction was separated from the lipids by means of liquid chromatography on a silicic acid column, following the Hirsch method[8]. The methanolic eluate was dried in a

stream of nitrogen, and dissolved in chloroform to give a 1% chloroform solution of the phospholipid mixture M. The purity of this mixture, with respect to both neutral lipids and inorganic phosphorus was verified by thin-layer chromatoigraphy (TLC), following the Parson and Patton method[9] (for the first elution only), visualizing phosphate compounds with Bochner's reagent[10], and determining the total phosphorus present with the Morrison method[11].

HRGC chromatograms of the mixture M were compared with chromatograms obtained, under identical experimental conditions, from the standards of single phospholipid classes, in order to identify common peaks and determine the various classes present. The separation into classes was then carried out on the same mixture by means of HPLC and the results were checked using TLC, according to the method developed by Parson and Patton[9].

The use of HRGC is novel in the present context, and the results obtained by HPLC show a considerable improvement on separations made by previous authors[12], thanks to the type of column (RCC) and the solvent mixture used.

The HRGC analysis was performed with both the "on-column system"[13] and the "split system" of injection using the 10-m capillary column. In the first case, the column temperature was programmed from 30°C to 350°C at 10°C/min, with a helium flow-rate of 2 ml/min. In the second case, the injector was maintained at 350°C and the column temperature was raised from 80°C to 350°C at 8°C/min; the splitting ratio was 1:80 and the helium flow-rate was 2 ml/min. In both cases the detector was maintained at 360°C. HRGC analysis of the mixture M by these two systems of injection was performed both after silanization and without etherification of the sample.

For the silanization, to 4 mg of the phospholipid mixture (dried under nitrogen) were added 500 μl of a mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane (5:2:1, v/v). After 15 min the mixture was evaporated to dryness under nitrogen, and 250 μl of benzene were added to the residue. The analysis of the non-silanized mixture was performed on a benzene solution (corresponding to 4 mg of phospholipid residue in 250 μl of solvent).

The test mixture was fractionated into classes at room temperature by means of HPLC, with a mobile phase of acetonitrile–methanol–water (50:45:2, v/v) at a flow-rate of 0.4 ml/min. The peaks were identified either by comparison with the retention times of corresponding standards or by the "enhancing method" through simyltaneous injection of the mixture and single standards of the various classes.

A counter-check on the classes produced by HPLC fractionation was obtained using two-dimensional TLC[9] with chloroform–methanol–water–28% ammonium hydroxide (130:70:8:0.4, v/v) as a solvent for the first elution, and chloroform–acetone–methanol–acetic acid–water (100:40:20:20:10, v/v) for the second. The determination of total phosphorus[11] on the TLC spots corresponding to the single classes made it possible, by means of their respective coefficients (23.84 for PE; 25.00 for PC; 24.85 for Sph; 25.00 for PI; 25.28 for PS), to trace back the percentage of each class present in the mixture.

Each class of phospholipids, as well as the total mixture, was then subjected to transmethylation (methanolysis) with boron trifluoride (12% p/p) in methanol[14], in order to determine the fatty acids present in each of these. This kind of methylation has been shown to be well suited even for the Sph class, which has an amide rather than an ester bond.

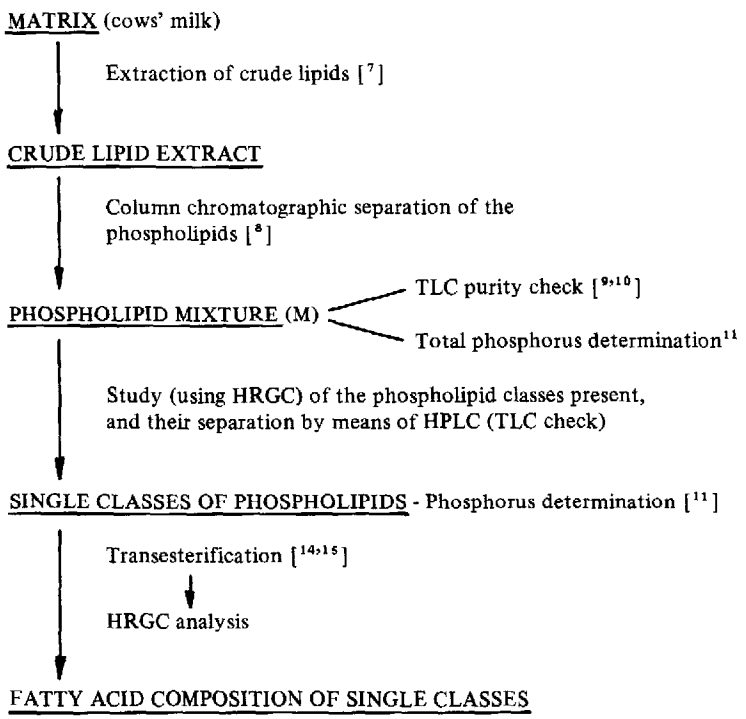

Fig. 1. Scheme of the analytical procedure.

The amount of substance required for each class test was obtained by pooling the eluates of successive injections.

To verify the absence of volatile fatty acids, a vial methylation was also performed, using methanol–zinc–zinc chloride[15].

The HRGC analysis of methyl esters was performed with a 25-m column, described above. The temperature was programmed from 100°C to 300°C at 8°C/min. The sample was injected at room temperature for the "on-column system", the flow-rate of helium was 2.5 ml/min, and the detector temperature was 300°C.

Fig. 1 gives a diagrammatic outline of the analysis procedure.

RESULTS AND DISCUSSION

From a comparative examination of the results of the HRGC analysis, performed with the "split system" injection under identical experimental conditions on both the natural phospholipid mixture (M) and on the standards of each phospholipid class (see the corresponding chromatograms in Fig. 2), the peaks of the mixture that coincide with those "characteristic"' of each given class have been identified. ("Characteristic" means those present in that class only.)

The retention times of the peaks, both of the natural mixture (M) and of the standards of each class, are shown in Table I. The symbols with which each class is identified also mark the characteristic peaks of each one. For the PS, the three peak at retention times 22.53, 27.81 and 28.93 min are common to the classes of PI, the

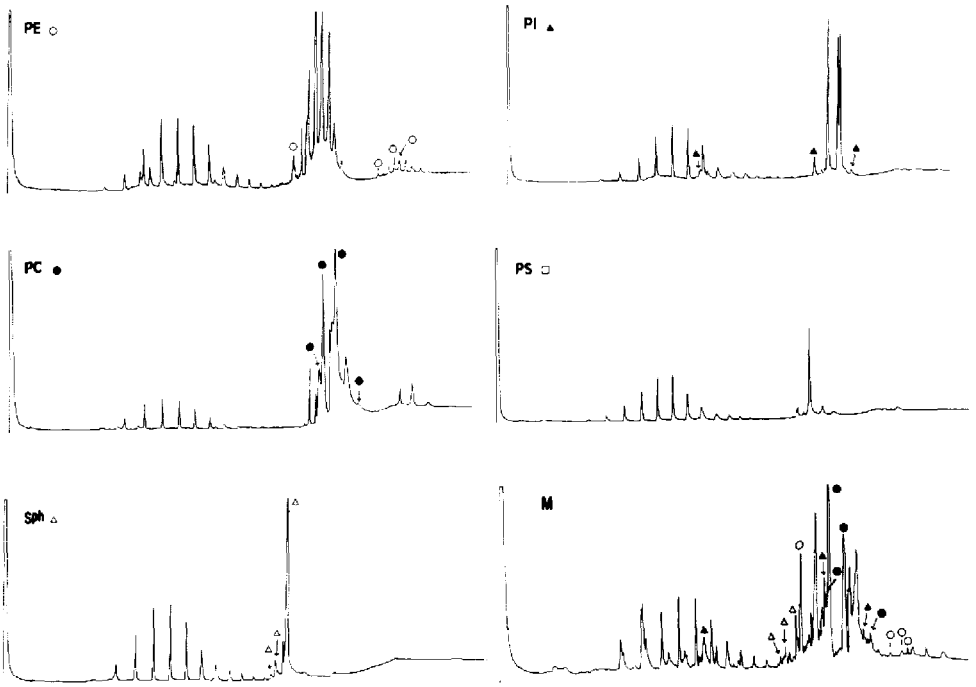

Fig. 2. HRGC chromatograms, under the experimental conditions described, of the phospholipid mixture (M) and of the standards of each phospholipid class. The peaks labelled with corresponding symbols both for the standards and for the mixture are those specific to each class (PE = ○; PC = ●; Sph = △; PI = ▲; PS = □; mixture = M).

first and the third, and to that of PC, the second.

In the natural mixture, 44 peaks were recorded, of which 31 were found in the various standard classes. Table I and Fig. 2 show that all the characteristic peaks of each class are present in the mixture.

The quantitative determination, using phosphorus determination[11], in each class of the natural mixture after TLC fractionation[9], gave the following percentages: 37.2, 33.7, and 32.0 for PE, PC and Sph, and 2.3 and 1.9 for PI and PS, respectively.

Pyrolysis tracings with a completely analogous pattern were also found in samples of milk from various areas (mountains and plains), of cows of different breeds (Frisona, Bruna Alpina, Bianca Modenese) from the province of Modena (Italy).

The HRGC analysis by means of "on column" injection did not provide any valid tracings, irrespective of silanization.

Fig. 3 shows the chromatogram of HPLC separation under the elution conditions described for the phospholipid classes present in the mixture in question. As can be seen, PS and PE classes that were not separated under isocratic conditions[12] are separated here. PS appears to be eluted together with the chloroform residue in the mixture. This, however, does not create problems either of fraction recovery or in the subsequent transmethylation reaction. The time of the analysis (*ca.* 20 min), with a flow-rate of 0.4 ml/min, is considerably shorter than that attained by other

TABLE I

HRGC RETENTION TIMES (MINUTES) OF THE PEAKS PRESENT IN THE PHOSPHOLIPID MIXTURE (M) AND IN THE STANDARDS OF EACH CLASS

The peaks labelled with symbols are characteristic of the respective class.

Natural phospholipid mixture (M)	*PE* ○	*PC* ●	*Sph* △	*PI* ▲	*PS* □
12.20	12.13	12.15	–	–	–
13.45	–	–	13.40	–	13.45
13.85	13.73	13.75	–	–	–
15.31	15.21	15.25	–	–	–
16.75	16.63	16.68	–	–	–
▲ 17.45	–	–	–	▲17.45	–
18.08	18.00	18.05	–	–	–
19.41	19.30	19.33	–	–	–
20.60	20.53	20.60	–	–	–
21.71	21.63	21.70	–	–	–
22.55	–	–	–	22.51	22.53
22.78	22.66	22.71	–	–	–
△ 23.98	–	–	△23.90	–	–
△ 24.35	–	–	△24.28	–	–
△ 25.35	–	–	△25.28	–	–
○ 25.71	○25.65	–	–	–	–
27.01	26.95	26.95	–	–	–
27.55	27.56	27.53	–	–	–
▲ 27.73	–	–	–	▲27.68	–
27.84	–	27.85	–	–	27-81
● 28.18	–	● 28.20	–	–	–
29.00	–	–	–	28.91	28.93
● 29.48	–	● 29.38	–	–	–
29.88	–	29.90	–	29.90	–
▲ 30.98	–	–	–	▲30.98	–
● 31.31	–	● 31.31	–	–	–
○ 33.31	○33.23	–	–	–	–
○ 34.28	○34.18	–	–	–	–
○34.71	○34.70	–	–	–	–
35.21	35.15	35.10	–	–	–
36.31	36.28	36.31	–	–	–

authors[12] (*ca.* 60 min), who did not obtain the separation of PS and PE.

The use of non-polar columns (SE-52 type) for the HRGC analysis of methyl esters of fatty acids present in the various separated classes, for the natural phospholipid mixture under examination, resulted in the chromatogram shown in Fig. 4.

Table II lists the percentage composition of the fatty acids and the relative retention times, for the mixture and for each class. The percentages were derived with an integrating calculator: the peaks due to the unavoidable impurities (given the high sensitivity conditions under which it was necessary to operate), were excluded. These data show that the predominat fatty acid in all classes is Δ^9-octadecenoic acid (oleic, $C_{18:1}$), with percentage ranging from 38.1 (PE) to 31.0 (PS); then comes hexadecanoic acid (palmitic, $C_{16:0}$), with percentages from 23.9 of (PI) to 19.8 (Sph). On the whole, in each class, unsaturated fatty acids predomninate (from 54.1% of PE to 48.7% of

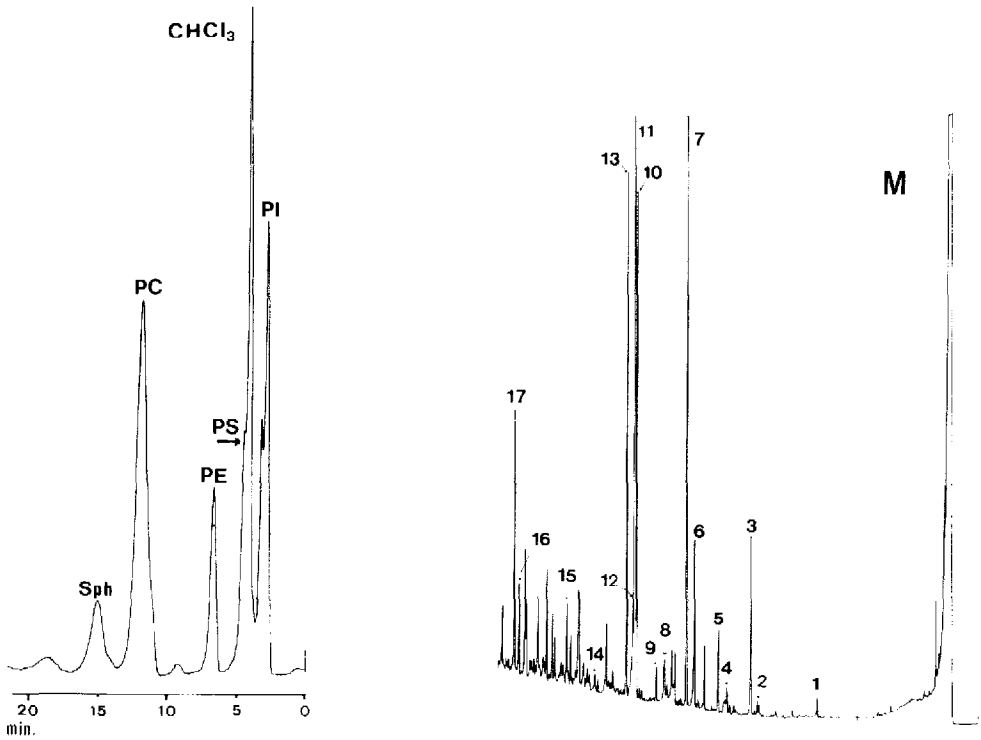

Fig. 3. Chromatogram of the phospholipid classes of the natural mixture (M), separated by means of HPLC.

Fig. 4. HRGC traces (SE-52) of fatty acid methyl esters of the phospholipid mixture (M). Peaks: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{14:0}$; 4 = $C_{15:1}$; 5 = $C_{15:0}$; 6 = $C_{16:1}$; 7 = $C_{16:0}$; 8 = $C_{17:1}$; 9 = $C_{17:0}$; 10 = $C_{18:2}$; 11 = $C_{18:1}$; 12 = $C_{18:1}$ iso; 13 = $C_{18:0}$; 14 = $C_{19:0}$; 15 = $C_{20:0}$; 16 = $C_{22:1}$; 17 = $C_{22:0}$.

PS), which is consistent with the fact that phospholipids are particularly sensitive to oxidation.

CONCLUSION

The high-temperature (350°C) injection gas chromatographic behaviour of the standards of the various phospholipid classes (standards from egg yolk, *Escherichia coli*, bovine brains and bovine liver) has shown the presence of pyrolysis products, each characteristic for its own class (except that of sphingomyelins). The mixture of standards, under the same experimental conditions, has confirmed the possibility of recognizing the classes present by means of the peaks formed by the "tracer" products of each class.

A mixture of polar lipids, extracted and purified from cows' milk, a natural complex system, has provided a trace very similar to that corresponding to a mixture of standards of various phospholipid classes. The same HRGC determination performed on polar lipids from a more significant sample of milk obtained from cows of different breeds bred in both mountain and plain environments, has confirmed the

TABLE II

FATTY ACID COMPOSITION (AND RETENTION TIMES) OF THE NATURAL PHOSPHOLIPID MIXTURE (M) AND OF EACH CLASS (FRACTIONATED FROM THE MIXTURE BY HPLC)

No. of C atoms	*Retention time (min)*	*Fatty acids*					
		Phospholipid mixture (%)	*PI (%)*	*PS (%)*	*PE (%)*	*PC (%)*	*Sph (%)*
$C_{12:0}$	6.68	0.4	0.9	1.0	–	0.4	Trace
$C_{514:0}$	9.39	3.9	8.9	6.2	4.2	4.1	3.2
$C_{15:1}$	10.37	0.6	0.7	0.6	Trace	0.8	–
$C_{15:0}$	10.72	1.9	1.8	2.7	2.2	1.6	2.2
$C_{16:1}$	11.71	4.6	4.2	6.0	5.3	3.6	5.4
$C_{16:0}$	12.02	20.5	23.9	20.7	20.4	22.6	19.8
$C_{17:1}$	12.81	1.8	0.9	1.7	1.4	1.4	1.8
$C_{17:0}$	13.26	0.8	0.9	1.0	1.1	0.8	1.0
$C_{18:2}$	14.09	10.6	7.3	7.9	9.3	9.8	9.7
$C_{18:1}$	14.17	40.1	33.3	31.0	38.1	33.2	34.0
$C_{18:1}$ iso	14.24	1.5	1.8	1.5	Trace	2.4	1.9
$C_{18:0}$	14.48	10.8	9.6	13.5	11.7	12.6	13.4
$C_{19:0}$	15.76	Trace	–	0.4	0.4	1.2	1.0
$C_{20:0}$	16.94	2.5	2.6	5.8	5.0	2.6	6.8
$C_{22:1}$	18.90	Trace	2.3	Trace	Trace	0.7	Trace
$C_{22:0}$	19.17	Trace	2.3	Trace	0.8	2.4	Trace

possibility of a rapid screening to show the various classes present in the phospholipid mixture.

The use of radial compression chromatography, under isocratic conditions, for the separation of phospholipids leads to better separation than that mentioned in the literature[12], as well as considerably shorter elution periods.

The fatty acid content of each phospholipid class [extracted from milk and fractioned using HPLC (RCC)], determined by means of HRGC by elution in a non-polar column (SE-52), has shown in all cases the presence of acids of between fourteen and 22 carbon atoms, with a prevalence of unsaturated acids. The distribution of fatty acids, consistent with that reported in the literature[16], is very similar in each class: the major component is Δ^9-octadecenoic acid (oleic), followed by hexadecanoic acid (palmitic).

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