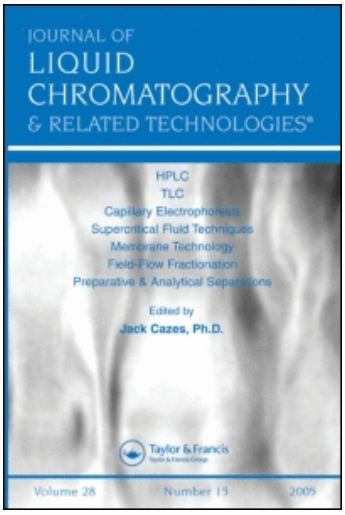

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Lipid Classes by HPLC with the Evaporative Light Scattering Detector

Andrezj Stolyhwo[a]; Michel Martin[b]; Georges Guiochon[c]
[a] Institute of Organic Chemistry Politechnica Gdanska, Gdansk, Poland [b] Laboratoire de Chemie Analytique Physique Ecole Polytechnique, France [c] Department of Chemistry, Georgetown University, Washington, D. C.

To cite this Article Stolyhwo, Andrezj , Martin, Michel and Guiochon, Georges(1987) 'Analysis of Lipid Classes by HPLC with the Evaporative Light Scattering Detector', Journal of Liquid Chromatography & Related Technologies, 10: 6, 1237 — 1253

To link to this Article: DOI: 10.1080/01483918708066765

URL: http://dx.doi.org/10.1080/01483918708066765

JOURNAL OF LIQUID CHROMATOGRAPHY, 1237-1253 (1987)

ANALYSIS OF LIPID CLASSES BY HPLC WITH THE EVAPORATIVE LIGHT SCATTERING DETECTOR

Andrezj Stolyhwo[1], Michel Martin[2], and Georges Guiochon[3]

[1]*Institute of Organic Chemistry*
Politechnica Gdanska
Gdansk, Poland
[2]*Laboratoire de Chemie Analytique Physique*
Ecole Polytechnique
91128 Palaiseau Cedex, France
[3]*Georgetown University*
Department of Chemistry
Washington, D. C. 20057

SUMMARY

The evaporative light scattering detector enables the detection and quantitation of all relatively non-volatile lipids. The mixtures of polar and non-polar lipids were separated in one run, in 20 to 25 minutes on Silica Si-100 columns, using consecutive gradients of pentane to diethylether, to chloroform, to methanol containing a large concentration of ammonia.

The flexibility of the method is illustrated by the change in elution patterns following the treatment of the packing material by ammonia. For example, the elution order of phosphatidyl inositol and phosphatidyl choline is reversed and the separation of the former compound from phosphatidyl serine, which is generally difficult, is now accomplished readily.

The weak dependence of the detector sensitivity on the nature of the analytes permits an easy quantitation, as illustrated by the results of the analyses of lipid classes in blood serum, amniotic fluid, beef brain and other natural samples.

The method is particularly useful for the analysis of lecithin and sphingomyelin in the amniotic fluid. The ratio of the concentration of these two compounds is an indicator of lung maturity and could permit an early diagnosis of the respiratory stress syndrome of neonates.

INTRODUCTION

The separation and analysis of lipid classes is a problem of great importance for the physiologist, the clinical chemist and the physician. Lipids are generally divided into non-polar lipids, such as squalane, squalene, carotenoids, fatty acid methyl esters, triglycerides, i.e. fatty acid esters of glycerol, and sterols, i.e. cholesterol and related compounds (lanosterol, sitosterol, brassicasterol, etc.), and polar lipids (1). The main classes of polar lipids are cerebrosides, sphingolipids, phospholipids, etc. Phospholipids are fatty acid diesters of glycerol, the third alcohol function of glycerol being esterified by phosphoric acid. The phosphoric acid, in turn, can be bonded to a variety of compounds, such as choline, serine, inositol, ethanolamine, etc.

Because of the presence of long hydrocarbon chains and of the weakly polar ester group in these molecules, lipids have a number of common properties. They are important or essential components of cell membranes. They are coextracted by organic solvents, being very soluble in chloroform, acetone and benzene. Most of them are difficult to detect by standard liquid chromatography detectors, since they carry only the weak ester chromophore (except those with a polyunsaturated fatty acid chain).

This last property is illustrated by the data in Table I, which gives the extinction coefficients at 203 nm of several phosphatidylcholines,

T A B L E I

Extinction Coefficients of Synthetic

Phosphatidyl Cholines

Fatty Acid Chain	Extinction Coefficient (Mole-1 x cm-1)*
Di C18:0	200
Di C18:1	4,300
Di C18:2	12,900
Di C18:3	18,900
Di C20:4	32,400

* at 203 nm, cf Ref. 11.

carrying different fatty acid chains, saturated, unsaturated or polyunsaturated. This Table illustrates several aspects of the serious difficulties faced by the analyst who wants to determine the composition of a mixture of lipids using an UV absorption detector.

First, a rather low wavelength should be chosen if a detectable signal is to be observed. Most lipids do not absorb light at wavelengths above ca 230 nm. Exceptions are some sterols and the esters containing conjugated, polyunsaturated fatty acid chains. The sensitivity of most UV detectors is poor in this extreme range of wavelengths, in part because lamps do not emit a very intense light beam in this part of the spectrum, in part because all solvents absorb to some extent at these wavelengths (see Table II), which reduces the sensitivity of the

T A B L E I I

Spectral Properties of Some Solvents*

Solvent	Wavelength below which Transmission is smaller than:	
	20%	80%
n - Hexane	200	225
2 - Propanol	210	230
Acetonitrile	200**	220
Methanol	210	235

*Merck Catalog, 1984.

** 60% transmission

detector, raising to unacceptably high values the detection limits. Traces of oxygen may also interfere with the response around 205 nm.

Second, the response varies considerably with the nature of the fatty acid chain(s) contained in the molecule. The response is very large for the eicosatetraenoique, 160 times smaller for the stearic acic chain (cf Table I). This enormous variation of the response factor for compounds belonging to the same lipid class makes impossible any direct quantitation in lipid class analysis, using the UV detector.

Finally and most importantly, it is not possible to carry out detection of lipids with a UV detector operating at such low wavelengths, because

the analysis of lipids requires the use of solvents which are not transparent in the wavelength range below 210 nm where all lipids absorb light, more or less. Only water, methanol, propanol, hexane, acetonitrile are somewhat transparent in that range (cf Table II). Chloroform, acetone, benzene all absorb strongly. This precludes the use of these solvents, even as additives or moderators in the mobile phase. On the other hand, the weakly polar nature of most lipids makes necessary the use of solvents which have a large polarizability, hence absorb light up to rather large wavelengths. The wide range of polarity of the various classes of lipids requires for class analysis the use of gradient elution, involving several solvents. This is clearly impossible, given the spectral properties of most of these solvents.

Another non selective detector is required. Unfortunately, the refraction index detector cannot be used either. The detection limits of the refraction index detector are barely satisfactory for most applications, but the essential drawback is the impossibility to use this detector in gradient elution. A detector which gives no response at all for the solvent is required for the detection of compounds separated in gradient elution (2).

The quantitative class analysis of lipid samples becomes easy to perform if one uses the evaporative light scattering detector (2-10). We describe here the methodology and some preliminary results.

EXPERIMENTAL

The principle of the detector consists in the nebulization of the column effluent in a gas stream where the solvent is vaporized, leaving the non volatile components of the sample as a cloud of particles (2-5). These particles are detected by measuring the amount of light scattered when they cross a light beam. The detector is usually non linear, but the response factor depends only very little on the nature of the compound.

The main parts of the detector are a gas stream, a nebulizer, a light beam and a light collector (2,7). Figure 1 shows a schematic diagram of

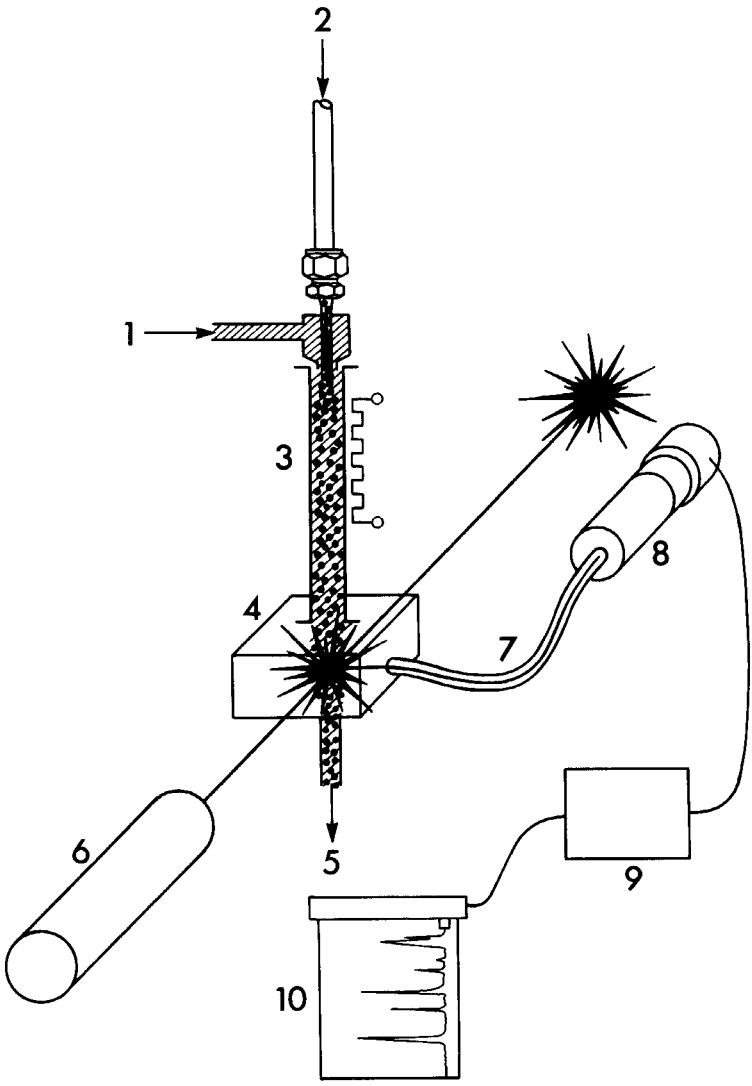

Figure 1. Schematic of the Evaporative Light Scattering Detector. 1, Carrier Gas Stream (constant temperature and flow rate). 2, LC Column Effluent. 3, Drift Tube. 4, Light Scattering Cell. 5, To Water Ejector. 6, He-Ne Laser (632.8 nm). 7, Optical Fiber. 8, Photomultiplier. 9, Electrometer. 10, Recorder.

the detector. A detailed description of the detector and of each of its component has been previously published (9). Some variations, corresponding to previous experiments, have also been discussed (2,7,8).

A stream of carbon dioxide of constant, but adjustable, flow rate is generated through a flow rate controller and heated at a temperature somewhat below the boiling point of the solvent used (a vapor pressure around 0.5 atm seems quite satisfactory).

This gas stream feeds a concentric nebulizer. The column effluent enters through the center of the nebulizer. The gas flow rate is adjusted so that the gas velocity in the annular space around the nebulizer tip is slightly above sound speed (5,6). A stream of droplets with a rather wide size distribution is generated. They are carried by the gas stream through a heated tube where the solvent vaporizes and leaves dry droplets or particles, made of the non volatile fraction of the effluent. Thus the detector cannot be used with buffers, nor with solvents with a high non volatile amount of residue.

The gas stream carries the dry particles through the beam of a He-Ne laser, to a water ejector through which the solvent stream (ca 0.3 mL/min) and the sample are disposed of safely. A laser light beam is used for the sake of simplicity, a laser being the most convenient way to generate an intense, narrow light beam. It does not seem, however, that the coherence properties of the beam give any advantage in the present instance (9).

The light scattered by the particles is collected on a photomultiplier and the current recorded. The amount of light collected is a fonction of the wavelength of the light beam, of the particle diameter and of the angle of collection (6). It is related to the concentration of the solute in the column effluent, but is not proportional (2,5-9). The response is exponential, the exponent being a fonction of the average particle size, i.e., of the characteristics of the nebulizer.

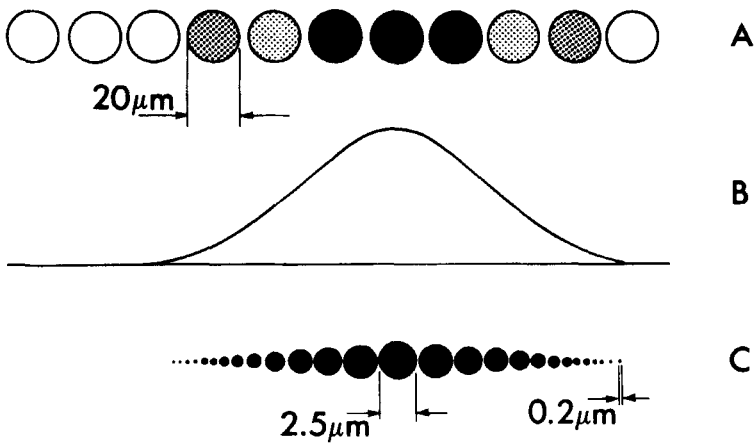

Figure 2. Principle of the Evaporative Light Scattering Detector. A, Stream of droplets generated by the nebulizer (constant average diameter, ca 20 um). B, Elution Profile of the studied compound. C, Stream of Particles entering the light scattering cell (average diameter increasing from ca 0.2 um, to about 2.5 um, and then decreasing back to ca 0.2 um.

Since the particles generated by the nebulizer have a constant average diameter during the elution of the chromatogram, the average dimension of the particles carried in the light beam is equal to the average size of the droplets generated by the nebulizer, multiplied by the cubic root of the concentration of the eluted compound in the eluent (2,5). The average size of the particles of non-volatile solute flowing through the detector changes during the elution of the band (cf Figure 2). If this concentration is 1 ppm, the particles of non-volatile content are 100 times smaller than the solvent droplets. Thus, the particles of solute to be detected have an average size in the 0.05 to 2 um range, i.e., of the same order of magnitude than the wavelength of the scattered light. Under such conditions, the relationship between the amount of light scattered and the droplet diameter is not simple, however, and this is why the detector response is exponential (5-7). In fact precise results require a calibration of the response, but the result of this calibration is independent of the nature of the studied compound within a given class (9).

Experiments were carried out using a 25 cm long, 4.5 mm i.d. column, packed with SI 100, average particle size 10 um, in the gradient elution mode. For the separation of polar lipids, the mobile phase is initially chloroform, followed by a gradient of a mixture of (i) methanol (92%), (ii) a 28% solution of ammonia in water (7%) and (iii) chloroform (1%). The gradient starts at injection and is completed in 20 minutes. The sample size used was 100 ug, diluted in 5 uL of chloroform. When the separation of non-polar lipids is also required, the injection is made with a mixture of diethyl ether and chloroform or acetonitrile and chloroform as mobile phase, and a gradient to pure chloroform is first run, as already explained for the analysis of triglycerides (9).

RESULTS and DISCUSSION

Figure 3 shows the result of a calibration achieved by measuring the area of the peaks obtained in response to the injection of increasingly large amounts of various compounds. Two different, parallel straight lines are obtained, separating the lipids into two groups having a markedly different response.

Thus, the response factor is the same for fatty acid methyl esters, for di and triglycerides and for cholesterol. On the other hand, it is about four times larger for the polar lipids, phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol), for sphingomyeline and for cardiolipin. Within each of these two classes, quantitative analysis can be made directly from the determination of peak areas.

The difference in behavior between the two series of compounds is probably related to the fact that those belonging to the first series condense as liquid particles, whereas those of the second series may condense as solids (the melting point of dimyristoyl phosphatidyl choline is 172C). A similar result was already observed for methyl stearate (7). Solid flakes scatter light more efficiently than liquid droplets: it is more difficult to see the road when driving in a snow

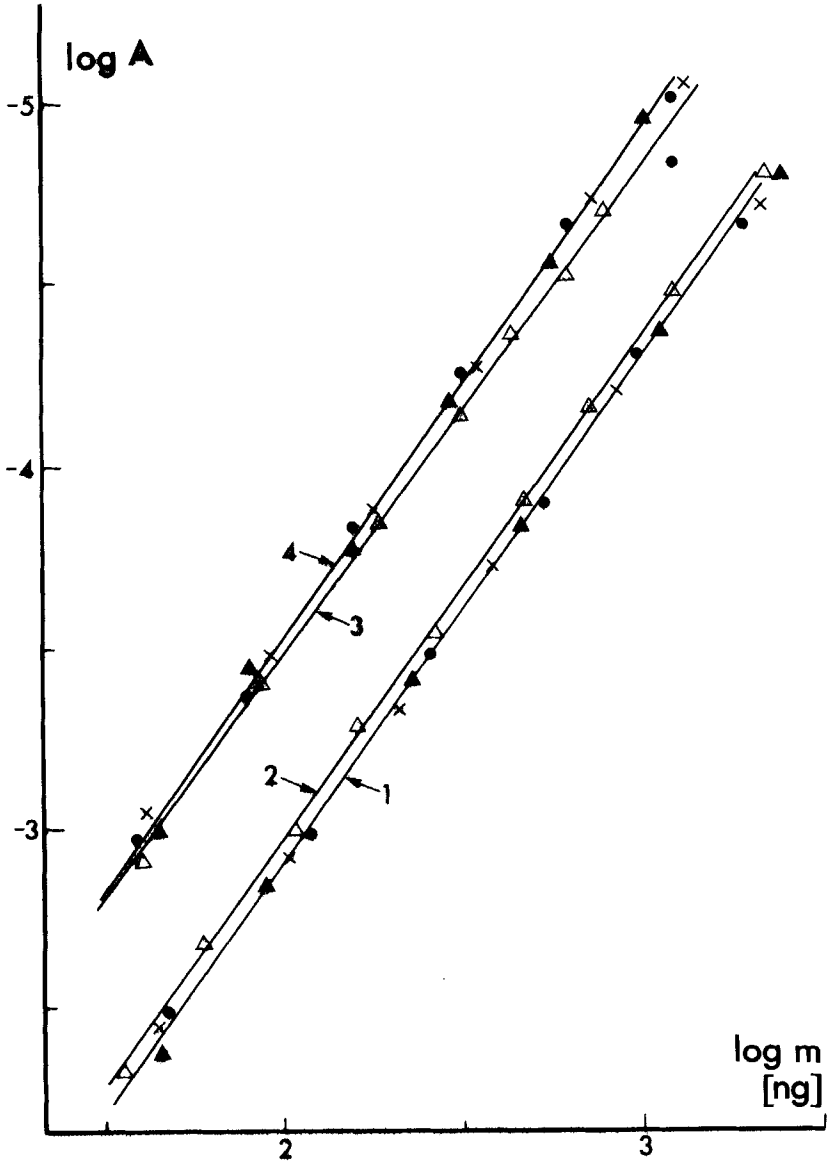

Figure 3. Calibration curves. Variation of the peak area (electronic integrator counts) versus the sample size. Logarithmic coordinates. 1, oleic acid (x), trioleine (●), tripalmitine (▲). 2, cholesterol (▲). 3, cardiolipins (▲). 4, phosphatidylcholine (▲), phosphatidylethanolamine (●), phosphatidylinositol (x).

Experimental conditions, as described in the Experimental Section.

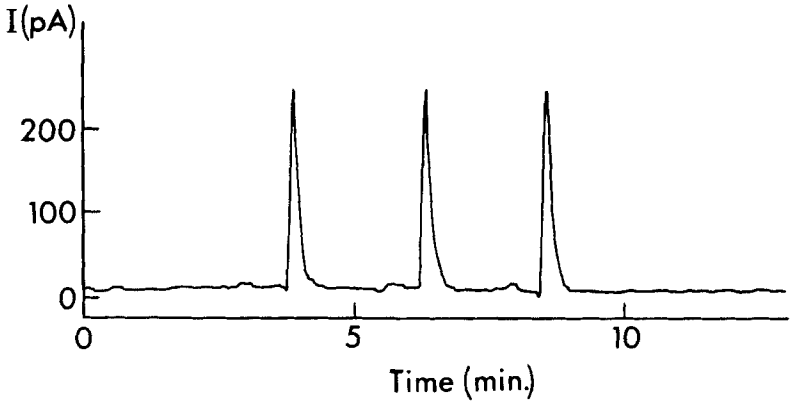

Figure 4. Detection limits of the Evaporative Light Scattering Detector. Repeated injection of 39 ng of cardiolipin. The peak signal corresponds to a current of 245 pA. The noise is 10.5 pA. The detection limit is approximatively 3 ng (detection limits at twice the noise). This corresponds to a concentration of 4.5 ppm in the column effluent and a mass flow rate of 15 ng/sec.

storm than during a rain storm. Although the residence time of the droplets in the drift tube where the solvent vaporizes is a few milliseconds (10 at most, see ref. 9), some compounds have time to crystallize. Most sugars do not (9).

Except for this phenomenon, quantitative analysis with the evaporative light scattering detector would not require positive identification of the analyte and one calibration curve only is needed.

Figure 4 shows the signal obtained for three successive injections of a dilute sample of cardiolipin. The sample size is 39 ng and the signal delivered by the photomultiplier is 245 pA. The noise level is 10 pA. Accordingly, the detection limit (amount corresponding to twice the noise level) is 3 ng for this particular compound. The corresponding concentration at band maximum was 4.5 ppm in the mobile phase, and the mass flow rate 15 ng/sec. Recent experiments have shown that the detection limit may be decreased by a factor 10 to 30 by using a pulsed laser instead of the continuous gas laser used in the present work (10).

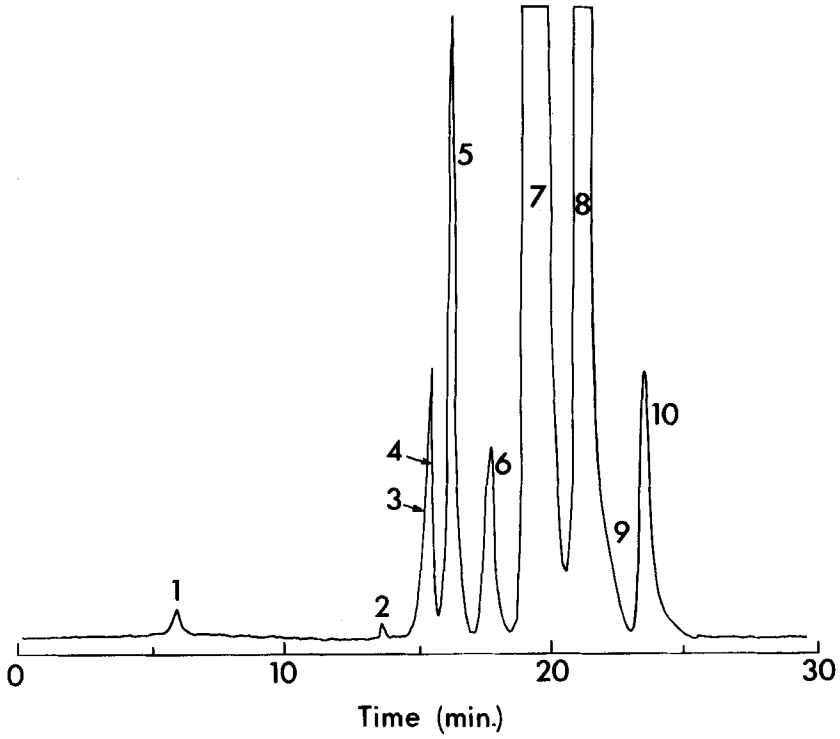

Figure 5. Analysis of a standard mixture of blood serum lipids (Supelco, Bellefonte, PA). Column length: 25 cm, i.d.: 4.6 mm. Stationary phase: Lichrosorb Si 100, 10 um. Mobile phase: 20 min gradient from pure chloroform to a mixture of 92% methanol, 7% of a 28% ammonia solution in water and 1% chloroform. Sample size: 100 ug in 5 uL chloroform.

Compounds are identified by their number. See Table III.

Figures 5 to 7 shows chromatograms obtained for various samples of lipids. Figure 5 shows the separation of a mixture of synthetic standard lipids, among those found in blood serum. The separation of most of them has been easily achieved. No attempt has been made, however, at optimizing the characteristics of the gradient used. The stability of the base line during the gradient is excellent, as has already been observed with this detector (2,7-9).

Figures 6 and 7 shows the analysis of two technical mixtures of polar lipids, known as lecithines. They are obtained, the first one from rape

TABLE III

Analysis of a Standard Mixture

Compound		%
#*	Name	
1	Cardiolipin	1
2	Phosphatidyl Acid	1
3	Phosphatidyl Glycerol	2
4	DiPhosphatidyl Diglycerol	1
5	Phosphatidyl Ethanolamine	6
6	Phosphatidyl Inositol	4
7	Phosphatidyl Choline	53
8	Sphingomyeline	24
9	Phosphatidyl Serine	1
10	Lysophosphatidylcholine	8
	Total	101

* On the chromatogram Figure 5.

seed oil, the second one fron soja beans. Numerous peaks are resolved. They have not been identified yet. The first group of peaks, at the very beginning of the chromatogram contains residual triglycerides and other neutral lipids. the other peaks are those of phospholipids and possibly other polar lipids. Most of the peaks on these chromatograms exhibit some tailing, especially the last eluted ones, which correspond to the

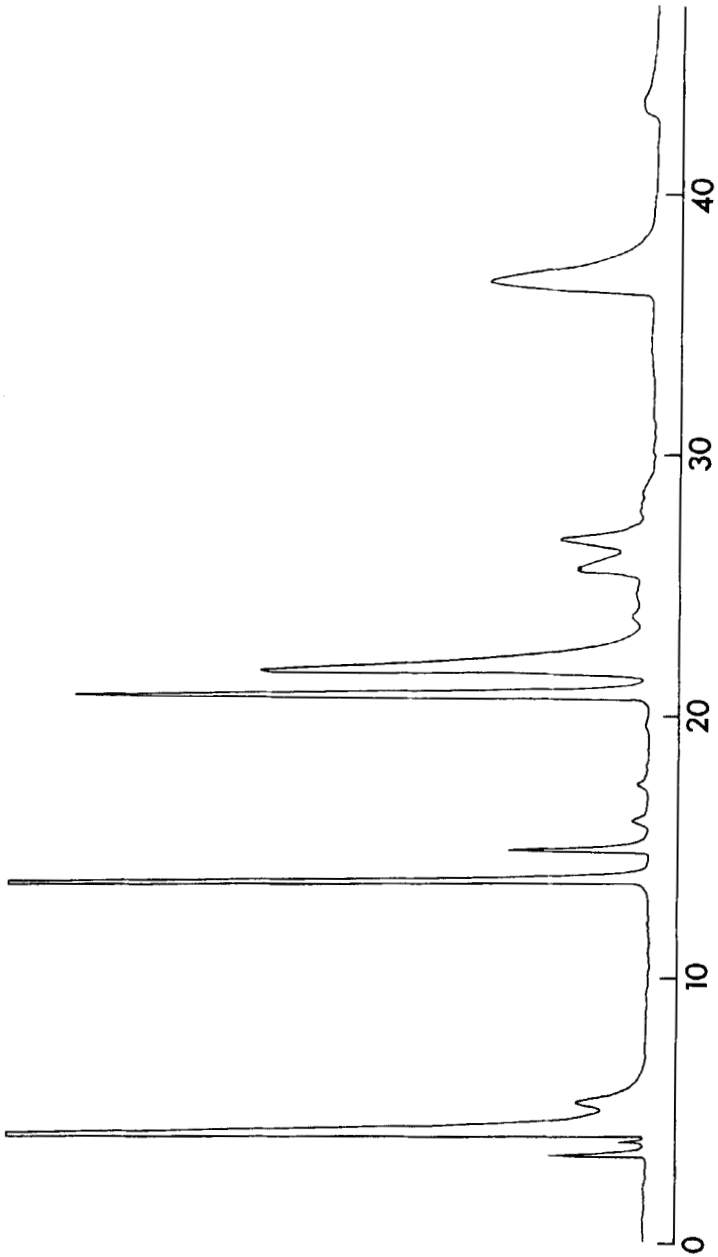

Figure 6. Analysis of technical rape seed oil lecithine (industrial mixture of phospholipids). Same experimental conditions as for Figure 5.

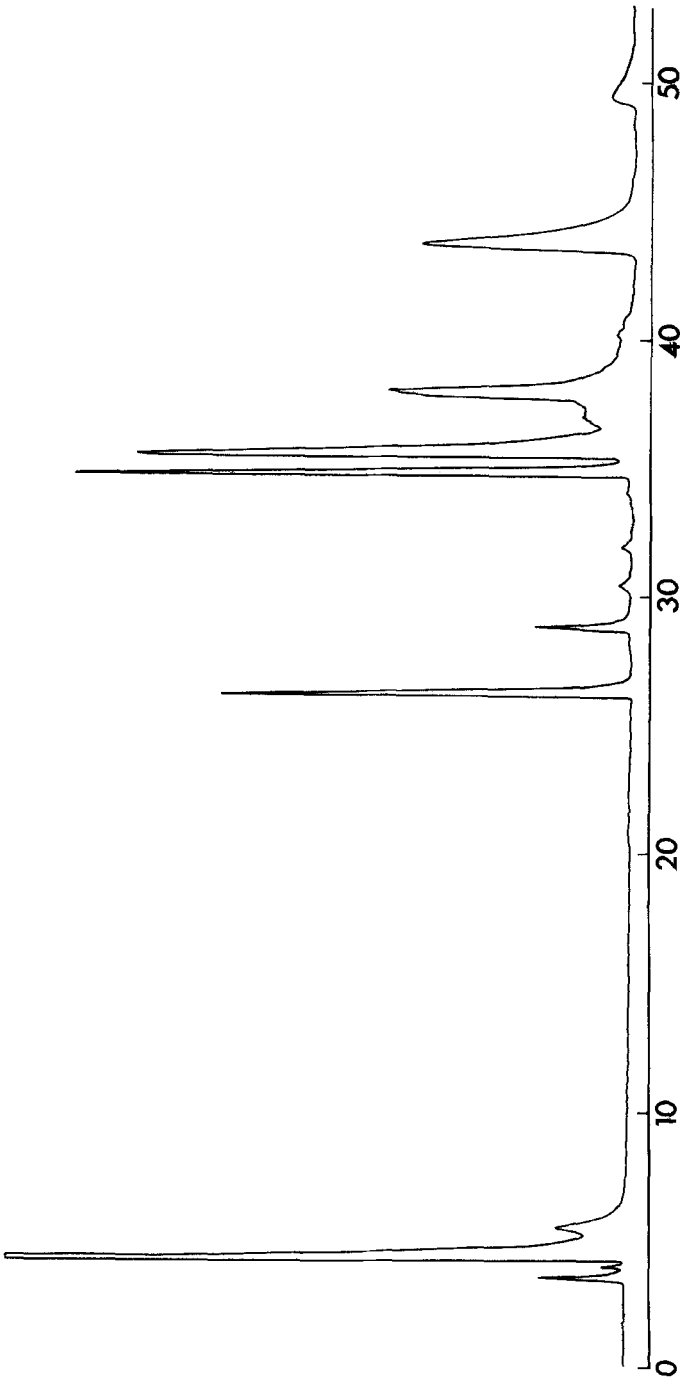

Figure 7. Analysis of Soya bean lecithine (technical product). Same analytical conditions as for Figure 5.

most polar compounds. This may be due to insufficient endcapping of the silica, after chemically bonding the C18 groups, or to the fact that the gradient characteristics have not been optimized.

Polar lipids can be separated by reversed phase liquid chromatography, using gradient elution. The evaporative light scattering detector permits a sensitive detection of the various components of the mixtures of natural origin in which they are encountered and a precise quantitation of these compounds. The determination of the ratio of lecithine to sphingomyeline in amniotic fluid, which will be reported elsewhere, is a case in point (12). This is an indicator of lung maturity in the fetus and can be used to diagnose respiratory distress syndrome in neonates prior to actual birth (13).

Acknowledgments

This project was supported in part by grant DE-FG05-86ER13487 of the Department of Energy.

Literature Cited

(1) A.L. Lehninger, "Principles of Biochemistry", Worth, New York, NY, 1982.

(2) A. Stolyhwo, H. Colin and G. Guiochon, Journal of Chromatography, 265, 1 (1983).

(3) R. Macrae and J. Dick, Journal of Chromatography, 210, 138 (1981).

(4) J.M. Charlesworth, Analytical Chemistry, 50, 1414 (1978).

(5) T.H. Mourey and L.E. Oppenheimer, Analytical Chemistry, 56, 2427 (1984).

(6) T.H. Mourey and L.E. Oppenheimer, Journal of Chromatography, 323, 297 (1985).

(7) A. Stolyhwo, H. Colin, M. Martin and G. Guiochon, Journal of Chromatography, 280, 253 (1984).

(8) J.L. Perrin, A. Prevot, A. Stolyhwo and G. Guiochon, Revue Francaise des Corps Gras, 12, 495 (1984).

(9) A. Stolyhwo, H. Colin and G. Guiochon, Analytical Chemistry, 57, 1342 (1985).

(10) A. Ogonowski and A. Stolyhwo, Journal of Chromatography, to be published.

(11) F. Jungalvala, Biochemistry Journal, 155, 55 (1976).

(12) A. Stolyhwo, in preparation.

(13) L. Gluck, M.V. Kulovich, R.C. Borer Jr. and W.N. Keidel, American Journal of Obstetrical Gynecology, 120, 142 (1974).