Journal of Chromatography, 600 (1992) 257–266
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 084

Optimized method for the determination of 1,2-diacyl-*sn*-glycero-3-phosphocholine and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine molecular species by enzymatic hydrolysis and gas chromatography

N. Urban Olsson* and Peter Kaufmann

Karlshamns LipidTeknik AB, P.O. Box 152 00, S-104 65 Stockholm (Sweden)

(First received November 20th, 1991; revised manuscript received February 6th, 1992)

ABSTRACT

A method was developed for the analysis of molecular species of 1,2-diacyl-*sn*-3-glycerophosphocholine and 1,2-diacyl-*sn*-3-glycerol-phosphoethanolamine from natural sources. The method involves enzymatic hydrolysis of the *sn*-3 position of the glycerophospholipids, acetylation of the newly liberated hydroxyl group and high-temperature gas chromatography of the 1,2-diacyl-3-acetyl-*sn*-glycerols. Both the enzymatic hydrolysis and the gas chromatographic separation were optimized with chemometric methods.

INTRODUCTION

Glycerophospholipis, *i.e.* phospholipids (PLs), are found as major constituents in cell membranes in both the plant and animal kingdoms. They play an important role in biological systems, not only as barriers in cells but also as the site of many reactions that are vital to life [1]. The conformation of the phospholipid molecules, with a polar head group and two fatty acyl chains, provides them with powerful emulsifying capabilities. This fact has made phospholipids interesting commercial products. Some phospholipid mixtures, called lecithins [2], are frequently used as emulsifying agents in the food, cosmetic and pharmaceutical industries. The main sources of vegetable lecithin are soybean, rapeseed and sunflower seed. The predominant polar lipid classes of lecithins are usually 1,2-diacyl-*sn*-3-glycerophosphocholine (PC) and 1,2-diacyl-*sn*-3-glycerophosphoethanolamine (PE) [2].

The fatty acyl composition of glycerophospholipid species has important physicochemical and bioavailability implications. Therefore, the analysis of molecular species with the glycerol backbone intact provides valuable structural and metabolic information.

The analysis of highly polar lipid molecules presents problems in chromatographic systems such as high-performance liquid chromatography, (HPLC) and gas–liquid chromatography (GLC) owing to adsorption and thermal instability. It is therefore desirable to reduce the polarity of the molecules, either by entirely removing the head group or by utilizing some suitable derivative [3–7]. Monoacetyldiacylglycerol (MADG) derivatives of diacylglycerols (of phospholipid origin) have been successfully used in high-temperature GLC analysis [4]. There exist two general methods for the conversion of phospholipids to MADGs: (1) acetolysis of phospholipids with acetic acid anhydride and acetic acid at approximately 150°C [4] and (2) enzymatic hydrolysis to remove the phosphoryl moiety, followed by acetylation at 70°C [4].

Diacylglycerols (DGs) isomerize under the influence of acidic, basic or polar solvents [4,8]. Thus, the DG residues of phospholipids have to be deriv-

0021-9673/92/$05.00

atized promptly into stable MADGs in order to avoid intramolecular acyl migration. Some reports have indicated that this type of isomerization may be promoted by the acetolysis procedure [9]. However, it has been shown that intermolecular acyl migration does not occur [8]. The removal of the phosphoryl group at the *sn*-3 position with a specific enzyme and the subsequent acetylation can in this respect be considered a much less violent procedure.

Complete removal of the polar head group in glycerophospholipids can be accomplished by enzymatic hydrolysis with phospholipase C [7,10–12], a well known but time-consuming reaction. As long as the fatty acyl chains are of sufficient length to possess a hydrophobic region which can bind to the enzyme [13], PC is a suitable substrate for phospholipase C (from *Bacillus cereus*). Soybean PC fulfils this requirement since the fatty acids mainly contain sixteen to eighteen carbon chains. The phospholipase C from *Bacillus cereus* has also been used for the hydrolysis of PE and 1,2-diacyl-*sn*-3-glycerophosphoinositol (PI) [4,7].

The aim of this investigation was therefore to optimize the performance of two well known and existing methods, enzymatic hydrolysis and high-temperature gas chromatography (GC) of MADGs, for the analysis of PC and PE in natural lipid materials.

EXPERIMENTAL

Study design

This study consisted of two separate steps: (1) enzymatic hydrolysis of PLs to 1,2-diacyl-*sn*-glycerols and (2) GLC analysis of the hydrolysis products after their conversion to 1,2-diacyl-3-acetyl-*sn*-glycerols, *i.e.* MADGs. Steps 1 and 2 were separately optimized by the combined use of factorial design and response surface modelling [14,15].

Experimental design

In order to obtain optimal conditions for the enzymatic hydrolysis and subsequent GLC analysis, factorial design was used [14,15]. The design and experimental domains are shown in tables I and II. For the enzymatic hydrolysis (Table I) the effects of the amount of enzyme, amount of substrate (phospholipid) and reaction temperature were explored in a reduced two-level design (2^{3-1}) [14,15]. Two additional points of measurement (experiments) were collected, one for calculating interactions and the centrepoint of the design to check for non-linearities. This was done in order to produce optimal conditions for the complete transformation of PC and PE into their diacylglycerol residues in as short a time as possible. Five minutes were considered a practical goal for this reaction.

A full factorial, with centrepoint, using the temperature programming rate, linear velocity of carrier gas and sample concentrations as design variables (Table II), was used to optimize the GLC analysis. This was done using a mixture of 1,2-diacyl-3-acetyl-*sn*-glycerol standards in equal amounts. In order to ensure stable GLC analysis conditions, a new design was laid out using the conditions from the best run from the previous design as the centrepoint (see Fig. 2). Through response surface modelling (see below), the influence of sample concentration was found to be negligible within

TABLE I

ENZYMATIC VARIABLES AND RATIOS OF UNREACTED PHOSPHOLIPID TO REACTION PRODUCT

	Design			Enzyme concentration (Units)[a]	Substrate concentration (mg/ml)	Temperature (°C)	Phospholipid/ diglyceride (%)
	A	B	C				
1	−	−	+	1	2	37	0.431
2	+	−	−	5	2	22	0.0
3	−	+	−	1	10	22	0.375
4	+	+	+	5	10	37	0.246
5	Centre point			2.5	5	30	0.756
6				2.5	5	22	0.542

[a] One Unit will liberate 1.0 μmol of organic phosphorus from PC per minute at pH 7.3 at 37°C (manufacturer's definition).

TABLE II

CHROMATOGRAPHIC VARIABLES AND CRS VALUES

Run[a]	Design			Temperature rate (°C/min)	Linear velocity[b] (cm/s)	Sample concentration (mg/ml)	CRS value
	A	B	C				
1	−	−	−	2	21.4	0.05	3.42
2	+	−	−	6	24.4	0.05	4.48
3	−	+	−	2	47.6	0.05	3.39
4	+	+	−	6	47.6	0.05	3.22
5	−	−	+	2	21.7	0.25	4.62
6	+	−	+	6	24.4	0.25	4.24
7	−	+	+	2	48.6	0.25	4.97
8	+	+	+	6	47.6	0.25	3.18

[a] Executed in random order.
[b] Actual values.

the explored interval. Therefore only the temperature programming rate and linear velocity of carrier gas were used as design factors in this set of four runs.

Evaluation of experimental data

The ratio of unreacted phospholipid to liberated diacylglycerol (PL/DG) was used as a response (dependent variable) for the enzymatic hydrolysis. In order to obtain a quantitative and objective response from the chromatograms a multivariate ranking function was used, the chromatographic resolution statistic (CRS) [16]

$$\mathrm{CRS} = \{\sum[(R_i - R_{\mathrm{opt}})^2/R_i(R_i - R_{\mathrm{min}})^2] + \\ + \sum (R_i)^2/a\bar{R})^2\}\ (T_t/n)$$

where R_i = resolution element for the ith peak pair, R_{min} = chosen value of minimum resolution, a = total number of resolution elements, T_t = retention time of final peak, n = total number of peaks, $\bar{R}^2$ = square of mean resolution. The optimal chromatographic conditions are found at the minimum of the CRS function. The optimal peak resolution value (R_{opt}) was set to 1.5 in this study.

The above-described experimental responses were fitted to polynomial functions in the experimental variables (Tables I and II). Linear models were found to be sufficient to describe the data. These models were used to generate response surface plots, which were used graphically to locate optimal conditions within the explored domains. All calculations were done in Statgraphics (STSC; Rockville, MD, USA).

Materials

The solvents used in this study were all of pro analysi quality and were purchased from Merck (Darmstadt, Germany). Phospholipase C (No. P-7147), from *B. cereus*, was purchased from Sigma (St. Louis, MO, USA) and suspended in a phosphate buffer (pH 7), composed of 50 cm^3 of 0.1 mol/dm^3 potassium dihydrogenphosphate and 29.1 cm^3 of 0.1 mol/dm^3 sodium hydroxide. The stock enzyme suspension was diluted to 25 U/ml of buffer and stored at 5°C until used. Natural PC and PE from soybean lecithin were obtained through Lipid Teknik (Stockholm, Sweden). All 1,2-diacylglycerol standards were purchased from Larodan (Malmö, Sweden). Phospholipid standards [1,2-di-14:0 PC, 1,2-di-16:0 PC, 1,2-di-18:0 PC, 1,2-di-18:2 PC, 1,2-di-20:0 PC (Larodan) and 1-16:0, 2-18:1 PC (Sigma)] were used to generate stereospecifically intact MADGs, with the optimized method described herein, for a standard GLC run.

Enzymatic hydrolysis

The experimental domain for the enzymatic hydrolysis was chosen on the basis of previous experience in our laboratory. According to standard practice in experimental design (Table I), the experiments were executed in random order, utilizing the following general procedure. A 1-ml volume of substrate suspension (in all experiments PC and PE

were suspended in the above-described phosphate buffer, and it was found to be important to minimize the size of aggregates, especially when preparing the PE suspension) (concentration according to design) was transferred to a test tube equipped with a PTFE-lined screw cap. The test tube was placed in a ultrasonic water bath and held at a temperature given in the design for 5 min for temperature equilibration. Either 40 or 200 μl of the stock enzyme suspension were added to the test tube. While maintaining control of the temperature, the ultrasonic function was activated for 5 min. The hydrolysis was discontinued by the addition of 2 ml of diethyl ether–isooctane (20:5, v/v), and heating the sealed tube in a thermoblock at 140°C for 5 min. After the subsequent addition of 3 ml of water, the test tube was shaken vigorously for 30 s and centrifuged at 3000 *g* for 5 min. The organic layer was recovered and the aqueous phase was extracted twice in the same manner with 1 ml of iso-octane. The three organic phases were combined and washed once with 1 ml of deionized water.

Evaluation of reaction performance was done by thin-layer chromatography (TLC) and scanning densitometry. The organic phase containing the diacylglycerols was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 200 μl of chloroform–methanol (1:1, v/v), of which 1 μl was drawn and subsequently spotted on a HPTLC plate (see Fig. 1). The remaining diacylglycerols were again evaporated to dryness under nitrogen and immediatly derivatized into 1,2-diacyl-3-acetyl-*sn*-glycerols.

Thin-layer-chromatography

TLC evaluation was conducted according to an optimized method [17] on prewashed silica HPTLC plates (Merck). Sample application was conducted with a PS 01 TLC spotter (Desaga, Heidelberg, Germany); the solvent system was chloroform–methanol–1-butanol–ethyl acetate–ammonia (25%)–Ca^{2+} (aq., 0.25%, w/v) (80.2:44.1:4.9:5:5:6, v/v). Densitometric evaluation of the HPTLC plates was performed with the Desaga CD 60 scanning densitometer, utilizing the spot optimization programme.

Derivatization

In order to preserve structural integrity and to render the hydrolysis products amenable to analysis by GC, the hydrolysis products, *i.e.* the 1,2-diacyl-*sn*-glycerols, were acetylated by a modified method [4]. The diacylglycerols were dissolved in 2 ml of a freshly prepared solution consisting of pyridine–acetic acid anhydride (1:5, v/v), and sealed in a test tube at 70°C for 30 min. The reaction was stopped by the addition of 2 ml of isooctane and 3 ml of deionized water, after which the test tube was shaken vigorously for 30 s and centrifuged at 3000 *g* for 5 min. The organic layer, containing the acetylated diacylglycerols, was recovered and the water phase was extracted once again with 1 ml of isooctane. The combined organic phases were evaporated in a stream of nitrogen. The residue was dissolved in isooctane (2.0 ml) and injected into the gas chromatograph.

Gas–liquid chromatography

GLC of the acetylated diacylglycerols was conducted on a moderately polar fused-silica capillary column, TAP (65% phenyl–35% methyl polysiloxane), 25 m × 0.25 mm I.D. (phase thickness: 0.10

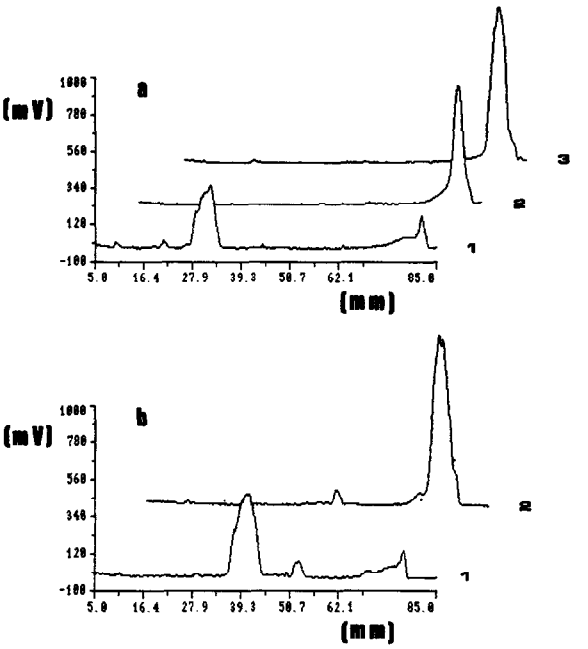

Fig. 1. (a) Densitograms of (1) unreacted PC, (2) reaction product and (3) dioleoylglycerol. (b) Densitograms of (1) unreacted PE and (2) reaction product.

μm), purchased from Chrompack (Middelburg, Netherlands). This column was installed in a Varian 3500 gas chromatograph, equipped with a temperature-programmable on-column injector and a flame ionization detector (FID) (Walnut Creek, CA, USA). A retention gap was installed between the injector and the analytical column. The detector signals were recorded by the PC Integration Pack (Kontron, Milan, Italy). The standard mixture of MADGs was composed of Ac-di-14:0, Ac-di-15:0, Ac-di-18:0, Ac-di-18:1, Ac-di-18:2 and Ac-di-18:3 in equal amounts, dissolved in isooctane. The profile of the oven temperature programme was as follows: from 100°C (hold 1 min) to 335°C at 50°C/min, then from 335°C (hold 1 min) to 360°C at 2 (or 6) °C/min, and hold at the final temperature for 5 min. The temperature programme of the injector was 100°C (hold 1 min) to 360°C at 200°C/min and hold at the final temperature for 2 min. The detector temperature was held at 360°C throughout this investigation.

The fatty acid composition (as methyl esters) of

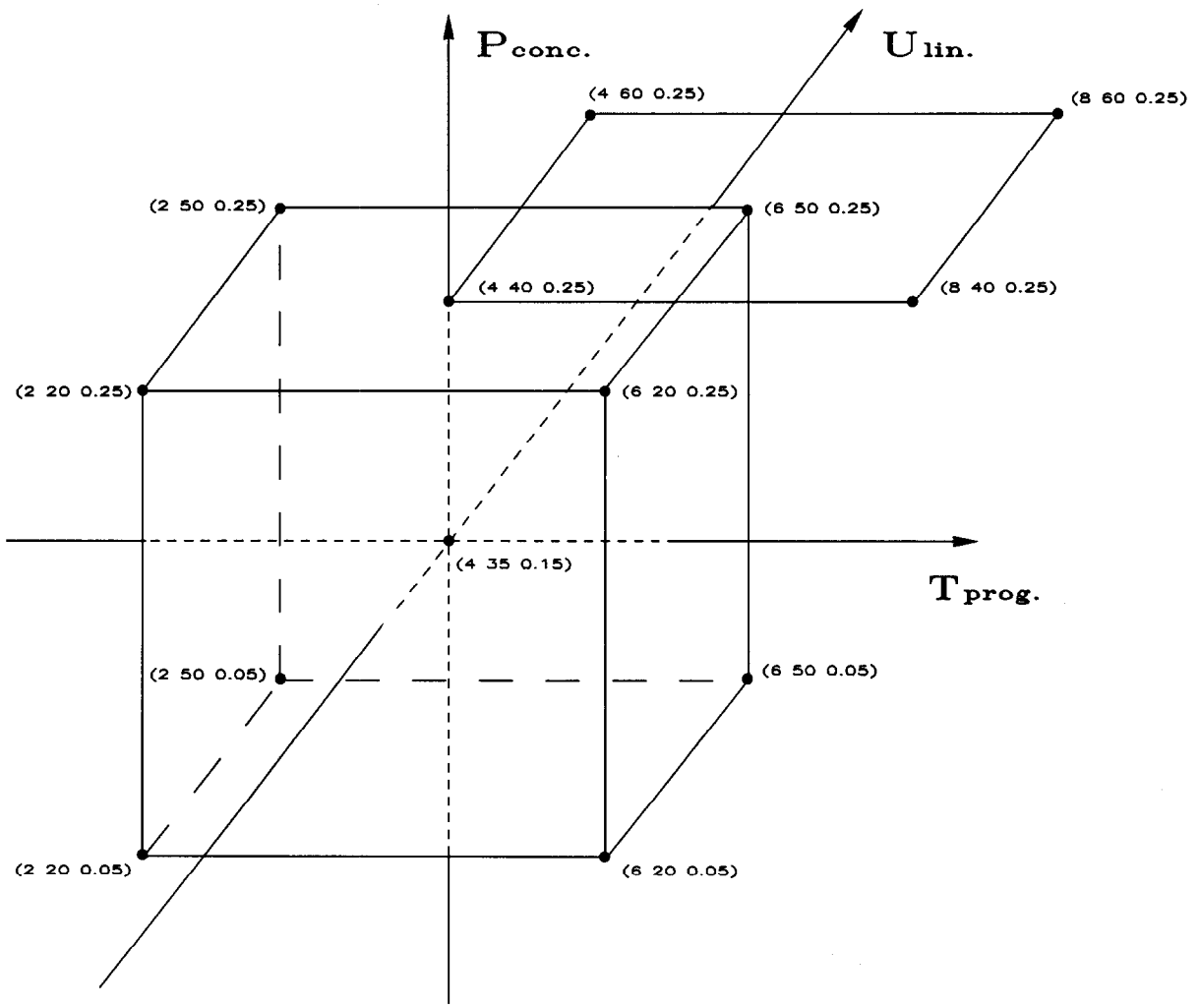

Fig. 2. Two-level, three-factor factorial design (2^3) with centre point, for the gas chromatographic separation of MADGs. Optimal conditions: Temperature programme rate, 6°C/min; linear velocity, 50 cm/s; sample concentration, 0.25 mg/ml. New design with the found optimal conditions as centre point.

PC and PE was determined according to an optimized method which has been described elsewhere [18]. However, a DB-WAX (30 m × 0.25 mm I.D.) column, purchased from J&W (Folsom, CA, USA), was used in this study. Information about the fatty acid composition is essential for the interpretation of peaks representing molecular species in the high-temperature GLC analysis.

RESULTS AND DISCUSSION

The optimum conditions for the enzymatic hydrolysis of PC were located in the experimental domain (Table I) by response surface modelling. Evaluation of reaction performance was done by TLC in combination with scanning densitometry (Fig. 1a). Optimum conditions obtained for PC were also

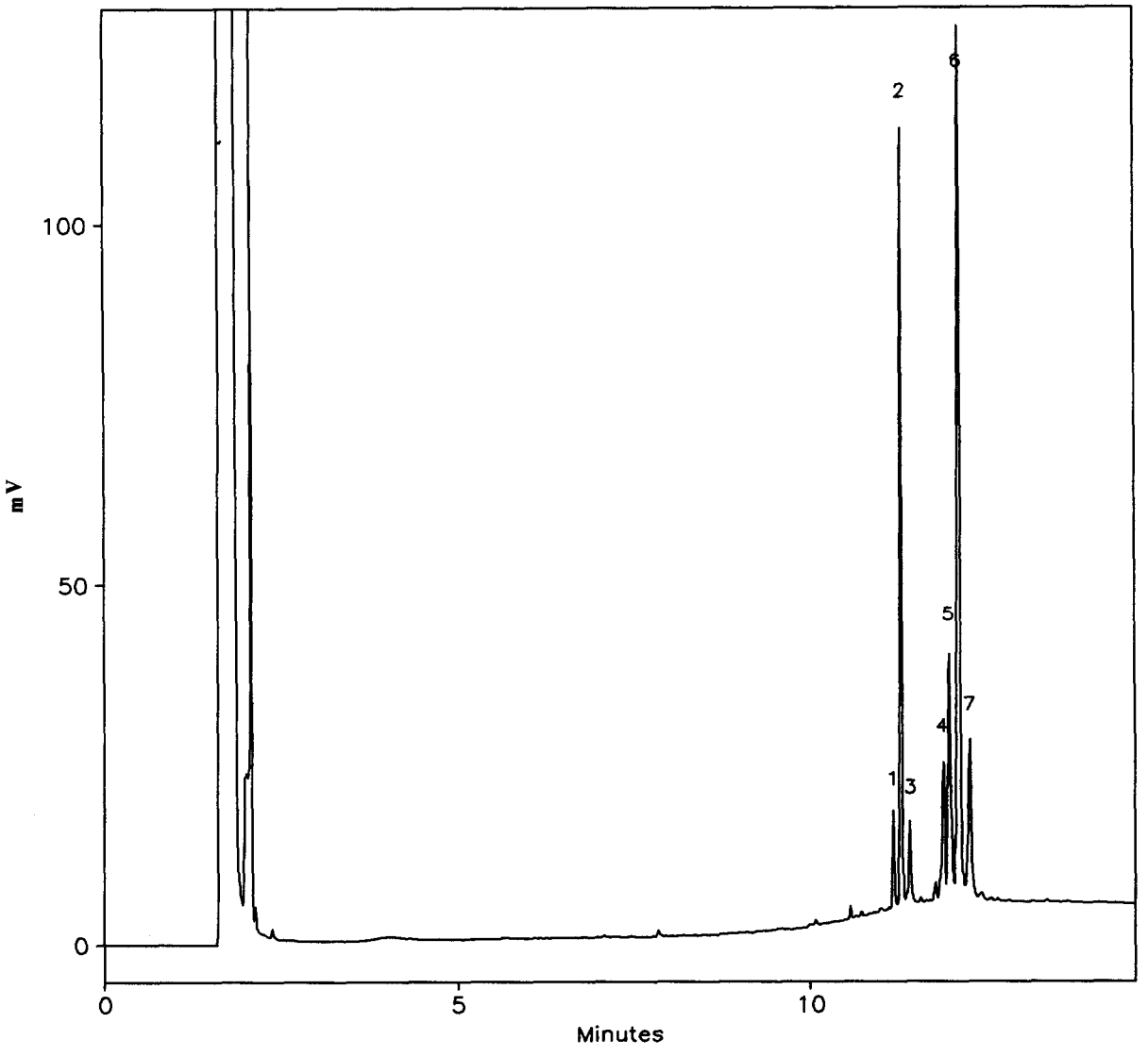

Fig. 3. Gas chromatogram of MADGs of PC origin. Optimized separation for conditions see text. Peaks: 3 = 16:0,18:2; 6 = 18:2,18:2; 7 = 18:2,18:3.

found to be suitable for the hydrolysis of PE, as illustrated in Fig. 1b. The response surfaces (not shown) of the data show a minimum (the optimum) at 22°C and 5 U in the experimental domain investigated. The response surfaces of reaction temperature *vs.* substrate and enzyme concentration *vs.* substrate concentration (not shown) all confirm that the optimal conditions for the enzymatic hydrolysis are at 22°C, 2 mg/ml and 5 U. The hydrolysis of both PC and PE went to 100% completion, with no side-products, after 5 min at ambient temperature.

The response surfaces (not shown) of the GLC experimental data showed that optimal conditions for the GLC analysis were 6°C/min temperature programming, a linear velocity of carrier gas of 50 cm/s (actual value: 47.6 cm/s) and a sample concentration of 0.25 mg/ml.

In order to validate the method, a new design was laid out with optimum conditions as the centre

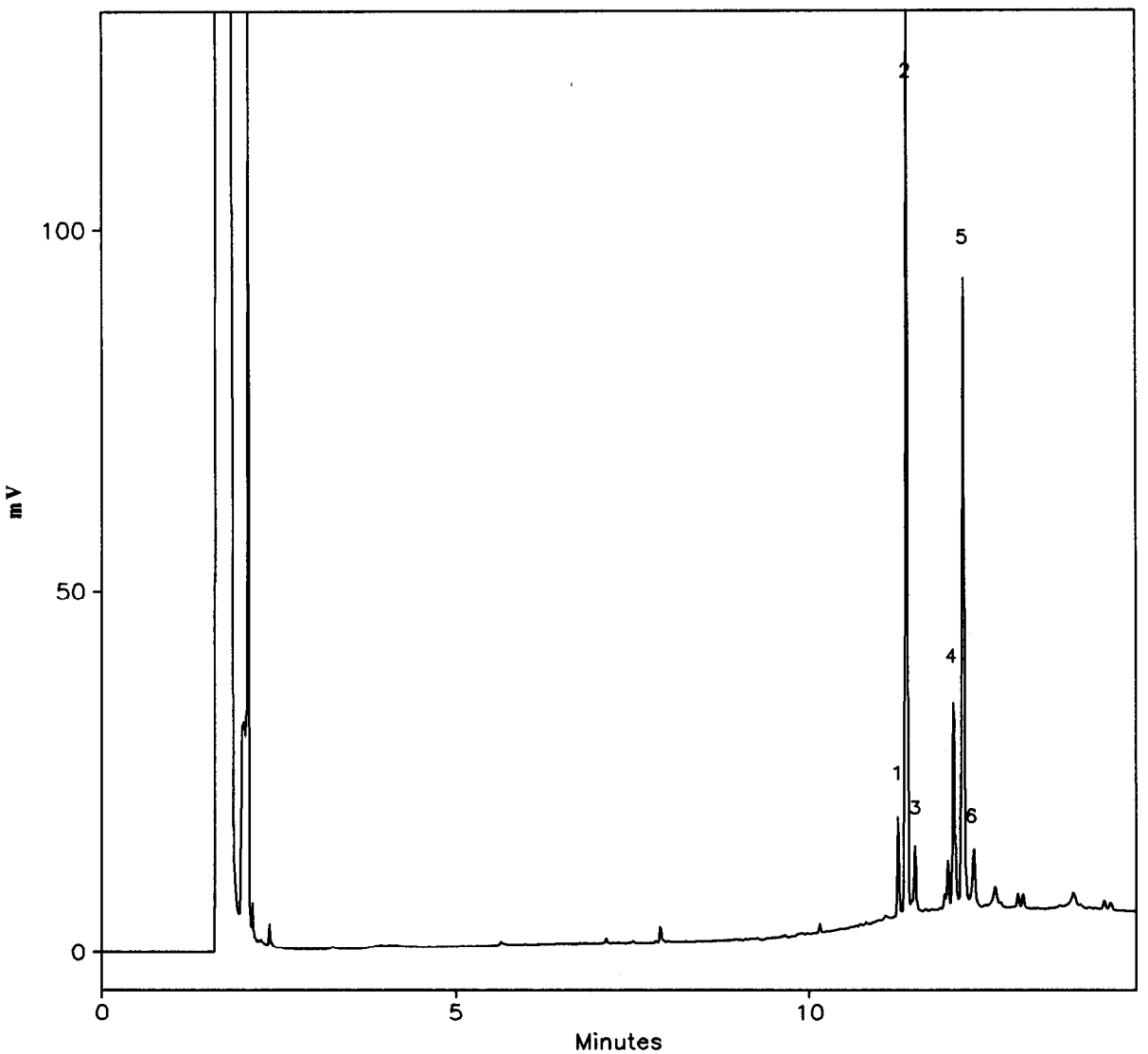

Fig. 4. Gas chromatogram of MADGs of PE origin. Optimized separation. For conditions see text. Peaks: 3 = 16:0,18:2; 5 = 18:2,18:2; 6 = 18:2,18:3.

TABLE III

FATTY ACID COMPOSITION (AS METHYL ESTERS) OF PC AND PE

Fatty acid	Composition (mean ± S.D.) (weight-%)	
	PC	PE
14:0	0.1 ± 0.00	Trace[a]
15:0	Trace	Trace
16:0	14.8 ± 0.14	18.8 ± 0.05
17:0	Trace	Trace
18:0	3.2 ± 0.01	0.8 ± 0.01
20:0	Trace	Trace
22:0	Trace	
24:0	Trace	
16:1(*n*−7)	0.1 ± 0.01	0.1 ± 0.03
17:1[b]	Trace	Trace
18:1(*n*−9)	7.5 ± 0.04	6.2 ± 0.11
18:1(*n*−7)	1.6 ± 0.04	0.6 ± 0.11
20:1(*n*−9)	Trace	
20:1(*n*−11)	Trace	
18:2(*n*−6)	62.6 ± 0.42	64.8 ± 0.30
18:3(*n*−3)	7.6 ± 0.06	7.8 ± 0.04
Unidentified[c]	2.5 ± 0.30	0.9 ± 0.13
Total	100.0	100.0

[a] <0.10%.
[b] Position not determined.
[c] Including minors.

point (Fig. 2). By perturbing the system in such a manner, the stability of the method, measured as the variation in the CRS value, could be deduced. This also served to confirm that the optimal conditions had in fact been located. Naturally, different optimal chromatographic conditions might result by increasing the optimal peak resolution term (R_{opt}) in the CRS function. The resolution obtained for molecular species of natural mixtures of PC and PE can be seen in Figs. 3 and 4.

The overall fatty acid compositions of PC and PE are given in Table III. Since there are fewer fatty acids in PE than there are in PC (4 *vs.* 6; >1.0%), the number of molecular species (as MADGs) should be lower in PE. This is illustrated in Figs. 3 and 4. The fatty acid composition of the underivatized material determines the number of peaks in MADG analysis. Consequently, minor peaks in Figs. 3 and 4 represent unidentified molecular species containing fatty acids (>1.0%) and species comprised of a combination of one of these acids and one of the four or six (PE or PC) major acids. The chromatogram of the system blank (without substrate) shows no peaks at all, and chromatograms of individual stereospecifically intact DG residues, transformed into their respective MADGs, show no extraneous peaks other then those expected from their initally somewhat poor purity level.

TABLE IV

METHOD REPRODUCIBILITY; PC FROM SOYBEAN

Peak No.	GLC separation[a]		Overall[b]		
	Area (%)	S.D. (%)	Area (%)	S.D. (%)	R.S.D. (%)[c]
1	3.2	0.03	3.1	0.18	5.8
2	24.6	0.22	24.9	0.66	2.6
3	3.1	0.09	2.7	0.43	15.9
4	7.0	0.36	7.0	0.37	5.3
5	11.1	0.55	11.2	0.55	4.9
6	37.8	0.24	37.7	0.34	0.9
7	8.3	0.14	8.3	0.17	2.1
Minors[d]	4.9	0.40	5.1	0.48	
Total	100.0		100.0		

[a] Average of six gas chromatographic runs of the same batch of MADGs.
[b] Average of six gas chromatographic runs of six individually prepared batches of MADGs, *i.e.* including the enzymatic hydrolysis step.
[c] Relative standard deviation. Overall method reproducibility = 5.4%.
[d] < 1.0%.

Further, the presence of only six peaks in the chromatogram of the stereospecifically intact standard mixture of MADGs confirms that interacyl migration does not occur with the described derivatization procedure (Fig. 5).

As a measure of the reproducibility of the method, six batches of 1,2-diacyl-*sn*-glycerol (of soybean PC origin) were prepared and subsequently acetylated and submitted to the optimized GLC analysis. The standard deviation of the overall method can be seen in table IV. These figures are not dramatically higher than those obtained with the GLC separation alone. The overall mean relative standard deviation (peaks 1–7) of the method was 5.4%.

Our goal in this work was to develop an optimized method for the GLC analysis of molecular species of naturally occurring phospholipids. To this end, experimental design and response surface modelling were used. The two main steps in this work, the hydrolysis of PLs and the GLC analysis,

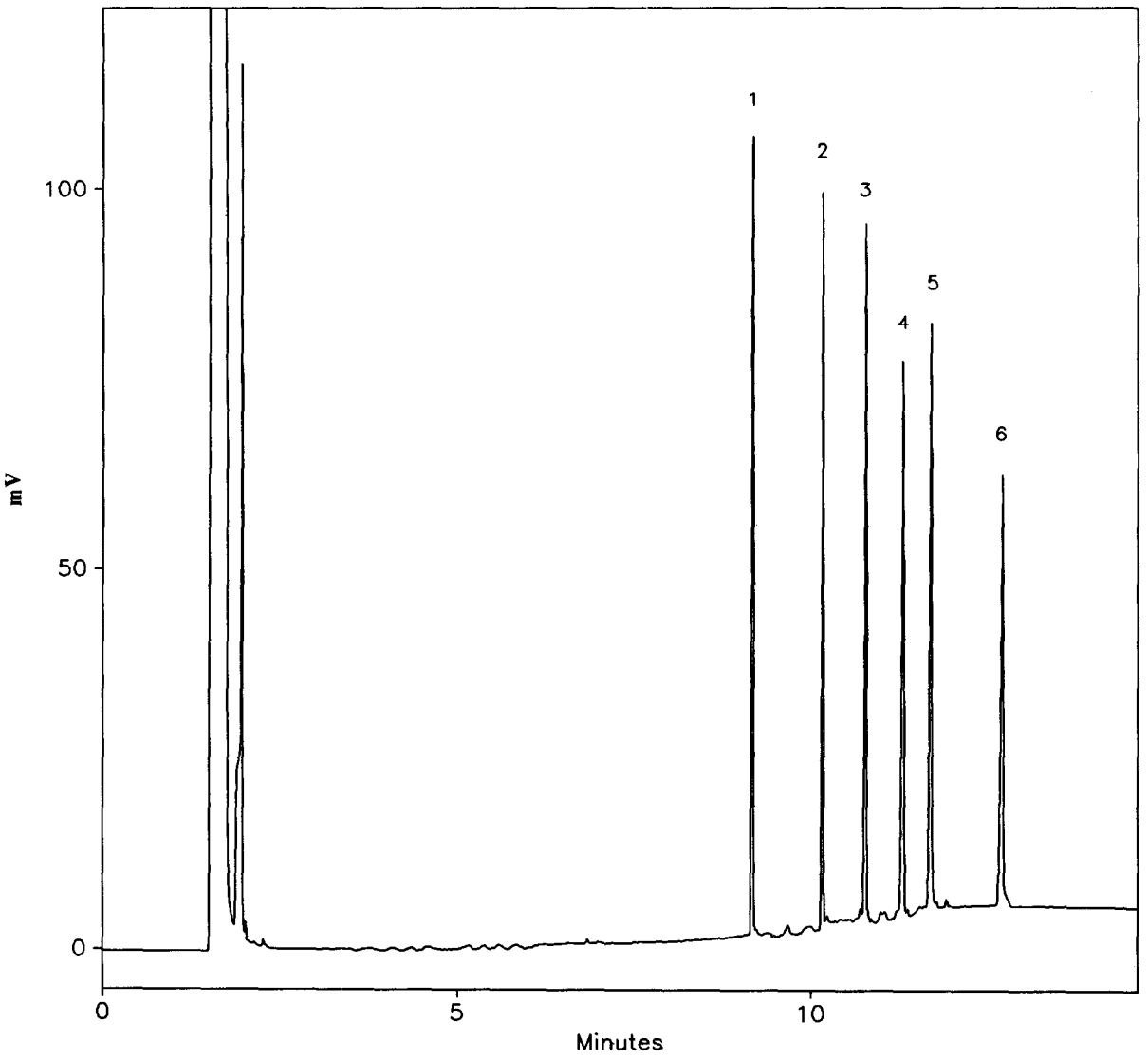

Fig. 5. Gas chromatogram of stereospecifically intact MADGs of PC standards. Optimized separation. For conditions see text. Peaks: 1 = Ac-1,2-di-14:0; 2 = Ac-1,2-di-16:0; 3 = Ac-1-16:0,2-18:1; 4 = Ac-1,2-di-18:0; 5 = Ac-1,2-di-18:2; 6 = Ac-1,2-di-20:0.

were optimized in terms of reaction performance and chromatographic separation, respectively.

ACKNOWLEDGEMENTS

We would like to thank Magnus Bergqvist for skillful technical assistance. The authors are also grateful for financial support given by the Karlshamns Research Council (Karlshamns AB, Karlshamn, Sweden).

REFERENCES

1 R. N. Robertson, *The Lively Membranes*, Cambridge University Press, Cambridge, 1983.
2 B. F. Szuhaj (Editor), *Lecithins: Sources, Manufacture and Uses*, American Oil Chemists' Society, Champaign, IL, 1989.
3 C. S. Ramesha, W. C. Pickett and D. V. Krishna Murthy, *J. Chromatogr.*, 491 (1989) 37–48.
4 W. W. Christie, *GC of Lipids*, The Oily Press, Ayr, 1989, pp. 188–215.
5 J. J. Myher, A. Kuksis, S. Pind and E. R. M. Kay, *Lipids*, 23 (1988) 398–404.
6 J. J. Myer and A. Kuksis, *Biochim. Biophys. Acta*, 795 (1984) 85–90.
7 A. Kuksis and J. J. Myer, R. F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, 1990, pp. 187–219.
8 S. Bloomer, *Triglyceride Interesterification by Lipases*, Licentiate Thesis, University of Lund, 1990, pp. 54–56.
9 K. Hasegawa and T. Suzuki, *Lipids*, 10 (1975) 667–672.
10 A. Kuksis, A. O. Stachnyk and B. Holub, *J. Lipid. Res.*, 10 (1969) 660–667.
11 S. J. Gaskell and G. J. W. Brooks, *J. Chromatogr.*, 142 (1977) 469–480.
12 H. Takamura, H. Kasai, H. Arita and M. Kito, *J. Lipid Res.*, 31 (1990) 709–717.
13 E. Hough, L. Kr. Hansen, S. Hansen, A. I. Hordvik, K. Jynge and C. Little, in V. K. S. Shukla and G. Hølmer (Editors), *Proceedings of the 15th Scandinavian Symposium on Lipids, Rebild Bakker, June 1989, Lipidforum*, (1989) 323–335.
14 G. E. P. Box, W. G. Hunter and J. S. Hunter, *Statistics for Experimenters*. Wiley, New York, 1978.
15 R. Carlson, *Design and Optimization in Organic Synthesis*, Elsevier, Amsterdam, 1991.
16 T. D. Schlabach and J. L. Excoffier, *J. Chromatogr.*, 439 (1988) 173–184.
17 U. Olsson, P. Kaufmann and B. G. Herslöf, *J. Planar Chromatogr.*, 3 (1990) 55–60.
18 U. Olsson, P. Kaufmann and B. G. Herslöf, *J. Chromatogr.*, 505 (1990) 385–394.