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Gas chromatographic analysis of fatty acid methyl esters: avoiding discrimination by programmed temperature vaporizing injection

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ABSTRACT

The programmed temperature vaporizing injection technique was used in its three operation modes (split injection, splitless injection and solvent elimination injection) for injection of fatty acid methyl esters into a gas chromatographic system. The relative response factors of standard fatty acid methyl esters and their coefficients of variation were determined. Using programmed temperature vaporizing injection, discrimination between high- and low-boiling-point fatty acid methyl esters can be avoided and also high precision can be achieved by injecting small amounts of fatty acid methyl esters. High precision was also demonstrated by the injection of small amounts of fatty acid methyl esters from rat red blood cell membrane phosphatidylcholine and phosphatidylethanolamine. Contrary to the programmed temperature vaporizing injection technique, sample discrimination occurred when classical split injection with the usual injection conditions was used for injection of standard fatty acid methyl esters.

INTRODUCTION

In high-resolution gas chromatography (GC) sample injection is the most critical step for achieving high accuracy and precision [1–4]. For injection of samples into a GC system the classical split and splitless injection techniques, the on-column injection technique and the programmed temperature vaporizing (PTV) injection technique are used. The classical split injection, introducing the sample into the hot injection chamber, is the most widely used techique for injection of fatty acid methyl esters (FAMEs) [5–10]. However, controversy exists about the accuracy and precision of results obtained by GC analysis of FAMEs with classical split injection. While many studies have shown that this technique may cause discrimination between acids with low and high boiling points [5,10] because of the high temperature of the injection chamber, others [6,9] have shown that using the same technique it is possible to obtain excellent results. The problems of the classical split injection resulting from the high temperature of the injection chamber can be avoided by cold injection of the sample using the on-column or PTV injection technique. Some authors have shown that, using cold injection techniques, excellent accuracy and precision of GC determination of hydrocarbons [11–18] can be achieved. In spite of this, cold injection has been applied by only a few authors for the determination of FAMEs [19,20].

In the present work standard FAMEs were injected using PTV injection in its three operation modes, namely cold split and splitless injection and solvent elimination injection for injection of standard FAMEs into a GC system. The accuracy and precision of the results were checked by measuring the relative response factors of standard FAMEs and their coefficients of variation. Moreover, as an example for practical application, FAMEs of rat red blood cell phospholipid classes were injected. Besides the PTV technique classical split injection technique was also used for injection of standard

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FAMEs in order to compare the suitability of both injection techniques for injection of FAMEs.

EXPERIMENTAL

Reagents, samples and chromatographic equipment

Fatty acid standards were procured from Sigma (Taufkirchen, Germany). The purity of all fatty acid standards was at least 99%. A standard solution was prepared which contained $C_{8:0}$–$C_{24:1}$ fatty acids in concentrations of 10–100 mg/l [in dichloroethane, butylated hydroxytoluene (BHT) as an antioxidant; the composition is shown in Table I]. Preparation of standard FAMEs was carried out with methanolic boron trifluoride according to Morrison and Smith [21].

Blood samples were taken from male Sprague–Dawley rats. GC analysis of FAMEs was performed on a Sichromat 2 GC system (Siemens, Karlsruhe, Germany) equipped with a 50 m × 0.25 mm I.D. CP-Sil 88 wall-coated (film thickness 0.25 μm) fused-silica column (Chrompack, Middelburg, Netherlands) and a flame ionization detector (300°C). For injection either a PTV injection system or a classical split injection system was used. Hydrogen was used as the carrier gas. Peak areas were measured using a Merck-Hitachi (Darmstadt, Germany) D-2500 integrator. Separation of rat red blood cell phospholipid classes was performed on a Merck-Hitachi high-performance liquid chromatographic (HPLC) system consisting of a gradient pump (L-6200), a diode array (L-3000), an integrator (D-2000), a Merck Si-60 (5 μm) column and a fraction collector (Gilson, Model 201, Villiers-le-Bel, France).

TABLE I

COMPOSITION OF THE FATTY ACID STANDARD SOLUTION USED IN THE PRESENT EXPERIMENTS

FAME	Concentration (mg per 100 ml)
8:0	10.65
10:0	7.98
11:0	8.06
12.0	6.57
14:0	10.94
14:1	6.50
16:0	6.78
16:1	12.56
17:0	6.63
18:0	11.24
18:1	6.41
18:2	10.37
18:3	6.68
20:0	10.26
20:1	5.89
20:2	1.59
20:3	2.71
20:4	7.27
21:0	7.59
22:0	9.57
22:1	7.79
22:2	2.54
22:3	4.76
22:6	8.48
24:0	9.30
24:1	2.60

Preparation of FAMEs from rat red blood cell membrane phospholipid classes

Red blood cell membranes were prepared according to ref. 22. Phospholipids were extracted from red blood cell membranes with isopropanol [23]. Extracted phospholipid classes were separated using normal-phase HPLC according to ref. 24 and collected with a fraction collector. Transesterification of separated phospholipid classes was carried out with sodium methoxide [25].

Chromatographic conditions

Classical split injection. A 2.0-μl aliquot of standard FAME solution was manually fast-injected with a 2-μl Hamilton syringe into the injection chamber (temperature 300°C). The carrier gas flow-rate was 2.0 ml/min; the split ratio was 1:25. The injection insert consisted of a plain tube packed with silanized glass wool. The oven temperature program was: 120°C held for 3 min, 30°C/min to 160°C, 15°C/min to 200°C, 200°C held for 1.5 min, 10°C/min to 225°C, 225°C held for 15 min. The relative response factors (RRFs) of FAMEs were calculated in relation to either one ($C_{17:0}$) or three ($C_{11:0}$, $C_{17:0}$, $C_{21:0}$) internal standards.

PTV injection. The PTV injection program was: 25°C held for 1 min after injection, 800°C/min to 300°C, 300°C held for 10 min, then the PTV injector was cooled. The oven temperature program was: 50°C held for 1 min, 30°C/min to 160°C, 15°C/min to 200°C, 200°C held for 1.5 min, 10°C/min to

225°C, 225°C held for 15 min. Note that the oven temperature was initially low in order to avoid peak splitting. A typical chromatogram is shown in Fig. 1.

Injection of standard FAMEs. For cold splitless injection the split vent was closed for the first 5 min to allow total entrance of the vaporized sample into the column and was then opened to allow purging of the vaporizer. A 2.0-μl sample of the standard FAME solution (diluted 1:36) was injected.

For cold split injection the split vent was opened during the whole analysis. Split ratios were 1:25, 1:6 and 1:2. A 2.0-μl sample of the standard FAME solution (undiluted for a split ratio of 1:25, 1:6 diluted for a split ratio of 1:6, and 1:36 diluted for a split ratio of 1:2) was injected.

For solvent elimination injection a 2.0-μl aliquot of FAME standard solution (1:36 diluted) was injected. The split vent (split ratio 1:100) was opened for the first 6 s after injection in order to allow elimination of the solvent by passage through the split exit and was then closed in order to allow complete entrance of the sample vapors from the injector to the column after heating the vaporizer.

Injection of FAMEs from red blood cell membrane

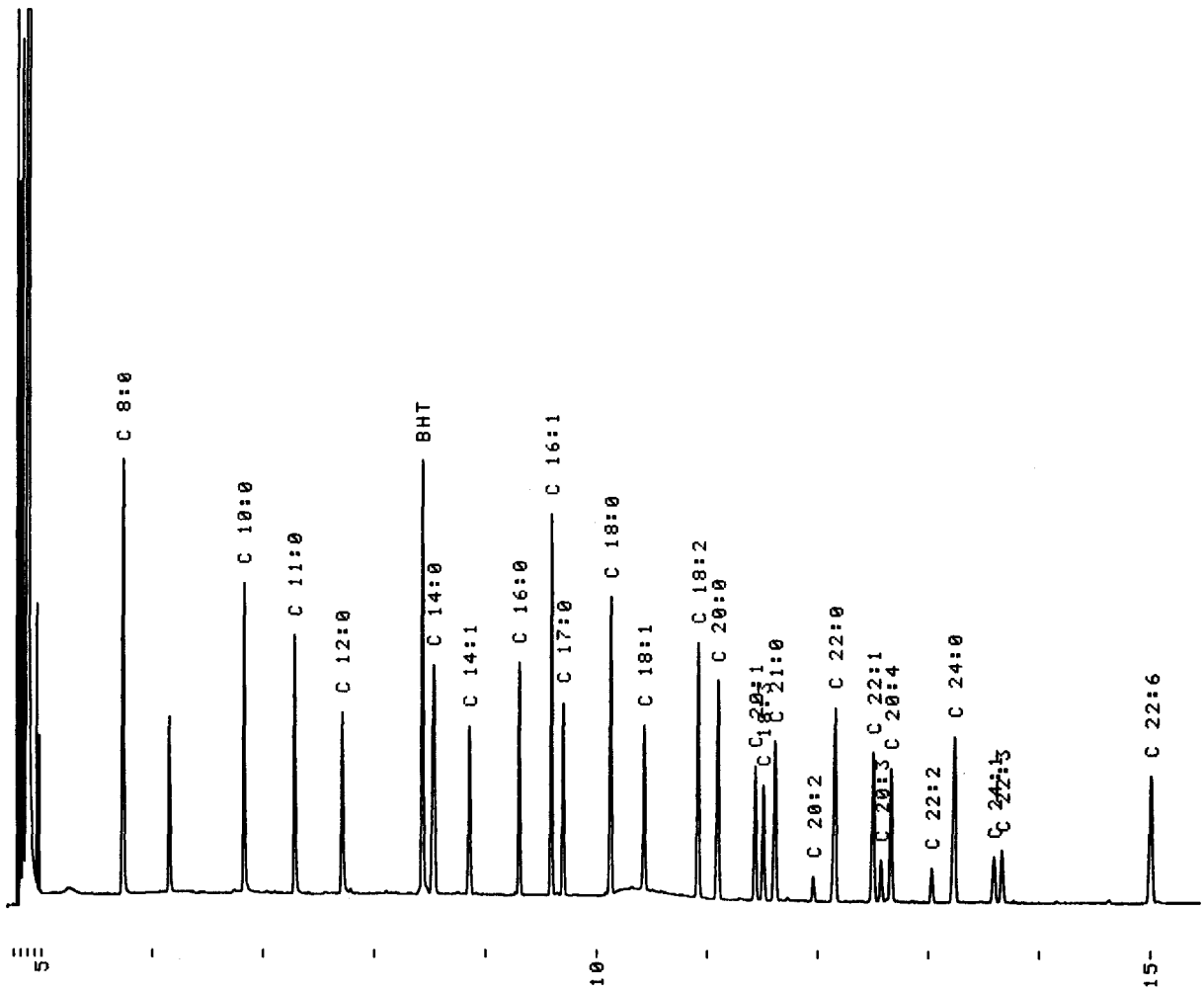

Fig. 1. Separation of standard FAMEs using PTV split injection. Split ratio was 1:25; the amounts of FAMEs injected into the injection chamber were between 32 and 252 ng. Time scale in min.

phospholipid classes. A 2.0-μl sample of the FAME extract was injected using the cold split injection technique with a split ratio of 1:2. Other chromatographic conditions were as described above.

RESULTS AND DISCUSSION

The aim of the present study was to check the accuracy and precision of the GC analysis of FAMEs with either PTV or classical split injection. For the investigation of accuracy, response factors of standard FAMEs were determined. Ackman and Sipos [26] proposed theoretical response factors for all FAMEs depending on the weight percentage in the molecule of "active" carbon atoms, including all carbon atoms except that of the carbonyl group. Bannon and co-workers [27–30] and Albertyn *et al.* [31] have shown in experiments that these theoretical response factors are highly accurate for all FAMEs. Therefore the occurrence of sample discrimination can be recognized by the deviation of determined response factors from the theoretical values.

The classical split injection technique is the most widely used technique for injection of FAMEs. In the present study the classical split injection technique was used for injection of standard FAMEs with injection conditions similar to those used by many authors [5–8,10,32,33] with the exception of a higher injector temperature (300°C), which has been reported to be useful in order to avoid discrimination between acids with high- and low-boiling points [9]. The results of the present study (Table II) show that determination of FAMEs using the classical split injection and the conditions chosen gives results with low accuracy and precision. Determined RRFs of FAMEs deviated from theoretical RRFs with an increase in their chain length. This means that there occurred discrimination of FAMEs which was highest for high-boiling-point FAMEs. The high coefficients of variation show that the precision of the results was also low when $C_{17:0}$ was used as the internal standard. The use of additional internal standards for calculation reduced the coefficients of variation somewhat, but the precision achieved by this method was also unacceptable. Similar results were obtained by other authors using the classical split injection technique with similar conditions. Their results also showed differences between determined and theoretical RRFs of FAMEs [32,33], and coefficients of variation were similar to those obtained in the present study [5,10]. However, besides these studies demonstrating that the classical split injection technique causes problems due to discrimination effects, a few authors [6,9] have shown that if all injection parameters (injector temperature, split vent flow-rate, volume injected, speed of injection, injector insert design, etc.) are optimized

TABLE II

RRFs OF STANDARD FAMEs RELATED TO EITHER ONE ($C_{17:0}$) OR THREE ($C_{11:0}$, $C_{17:0}$, $C_{21:0}$) INTERNAL STANDARDS DETERMINED USING CLASSICAL SPLIT INJECTION TECHNIQUE

Coefficients of variation (%) are stated in parentheses.

FAME[a]	RRF (n = 5)	
	$C_{17:0}$[b]	$C_{11:0}$, $C_{17:0}$, $C_{21:0}$[c]
8:0	0.91 (24.0)	1.35 (16.5)
10:0	0.71 (13.9)	1.06 (3.0)
11:0	0.67 (13.8)	1.00
12:0	0.72 (12.3)	1.06 (2.9)
14:0	0.76 (9.3)	1.13 (5.6)
14:1	0.78 (9.7)	1.16 (5.2)
16:0	0.86 (3.7)	0.86 (3.7)
16:1	0.88 (4.6)	0.88 (4.6)
17:0	1.00	1.00
18:0	1.12 (5.0)	1.12 (5.0)
18:0	1.08 (3.2)	1.08 (3.2)
18:2	1.12 (3.8)	1.12 (3.8)
18:3	1.28 (3.9)	1.28 (3.9)
20:0	1.48 (15.3)	0.88 (8.0)
20:1	1.33 (13.9)	0.79 (8.9)
20:2	1.58 (15.3)	0.92 (2.3)
20:3	1.65 (14.4)	0.99 (9.6)
20:4	1.56 (11.5)	0.93 (10.8)
21:0	1.71 (24.0)	1.00
22:0	1.72 (26.3)	1.00 (5.3)
22:1	1.85 (25.1)	1.07 (4.8)
22:2	1.86 (22.0)	1.08 (5.7)
22:3	2.20 (25.5)	1.28 (4.5)
22:6	3.58 (22.6)	2.09 (3.6)
24:0	2.08 (35.3)	1.19 (12.8)
24:1	2.14 (33.1)	1.23 (10.1)

[a] Amounts of FAMEs injected into the injection chamber were between 32 and 252 ng.

[b] $C_{17:0}$ was used as internal standard for calculation of all FAMEs.

[c] $C_{11:0}$ was used as internal standard for calculation of C_8 to C_{14}, $C_{17:0}$ for calculation of C_{16} to C_{18} and $C_{21:0}$ for calculation of C_{20} to C_{24}.

discrimination of FAMEs can be totally eliminated and thus high accuracy and precision in GC determination of FAMEs with classical split injection can be achieved. This means that it is possible to achieve results with high accuracy and precision using classical split injection. However, it seems that achieving high accuracy and precision is not easy and the injection conditions used by many authors, including those of our classical split injection technique, might not allow it.

The PTV injection technique was introduced by Vogt and co-workers [34–36] in order to inject very large sample volumes using the solvent elimination mode. However, other authors [14–18] have shown that PTV injection is also able, like the on-column injection, to avoid sample discrimination during injection, a problem often demonstrated with classical split injection because of the high temperature of the injection chamber. In spite of the excellent results of GC determination of hydrocarbons [14–

TABLE III

RRFs (RELATING TO $C_{17:0}$) OF FAMEs DETERMINED BY PTV INJECTION IN DIFFERENT OPERATION MODES IN COMPARISON WITH THEORETICAL RRF

Coefficients of variation (%) of RRFs are stated in parentheses.

FAME	Theoretical RRF[a]	RRF determined				
		Split injection			Splitless injection (n = 5) (0.9–7 ng)	Solvent elimination injection (n = 6) (0.9–7 ng)
		Split ratio				
		1:2 (n = 5) (0.9–7 ng)[b]	1:6 (n = 5) (5.4–42 ng)	1:25 (n = 8) (32–252 ng)		
8:0	1.18		0.98 (4.51)	0.78 (8.95)		
10:0	1.12	0.74 (3.57)	0.83 (2.99)	0.69 (8.67)	2.19 (14.22)	
11:0	1.09	0.72 (4.12)	0.92 (4.59)	0.73 (6.58)	2.40 (7.20)	
12:0	1.07	0.81 (11.33)	0.92 (6.01)	0.94 (4.39)	1.12 (13.48)	
14:0	1.04	0.90 (3.21)	0.98 (1.15)	1.00 (2.26)	0.99 (9.19)	1.12 (14.78)
14:1	1.03	0.98 (5.11)	1.00 (1.86)	1.02 (2.42)	1.03 (2.35)	1.29 (16.33)
16:0	1.02	0.98 (2.27)	1.01 (1.08)	0.99 (0.83)	1.01 (1.06)	0.98 (2.30)
16:1	1.01	1.00 (1.67)	1.00 (0.96)	1.00 (0.53)	0.97 (0.40)	1.00 (2.40)
17:0	1.00	1.00	1.00	1.00	1.00	1.00
18:0	1.00	1.00 (1.95)	1.01 (1.31)	1.00 (1.07)	0.95 (5.68)	1.02 (0.74)
18:1	0.99	1.02 (2.39)	1.01 (2.80)	1.01 (0.52)	1.00 (1.23)	1.02 (0.24)
18:2	0.99	1.01 (1.38)	1.01 (1.17)	1.02 (0.19)	1.01 (0.47)	1.02 (0.56)
18:3	0.98	1.04 (1.74)	1.02 (2.30)	1.03 (0.44)	1.02 (1.36)	1.02 (0.57)
20:0	0.98	1.01 (2.36)	1.02 (1.36)	1.00 (0.41)	0.97 (2.48)	1.01 (3.07)
20:1	0.98	0.97 (2.07)	0.99 (1.18)	1.01 (0.43)	1.02 (0.68)	1.02 (0.44)
20:2	0.97	1.02 (1.19)	1.04 (3.87)	1.02 (0.69)	1.03 (1.02)	1.03 (1.07)
20:3	0.97	1.03 (4.80)	1.03 (3.51)	1.02 (1.23)	1.04 (5.22)	1.04 (2.82)
20:4	0.96	1.06 (5.55)	1.04 (4.20)	1.05 (0.96)	1.03 (0.66)	1.05 (0.94)
21:0	0.98	1.00 (1.95)	0.98 (1.38)	1.04 (0.66)	0.99 (0.60)	1.01 (3.43)
22:0	0.97	0.95 (1.49)	0.95 (1.61)	1.01 (0.53)	0.99 (2.03)	0.98 (0.83)
22:1	0.97	0.96 (0.83)	0.97 (1.45)	1.04 (0.53)	1.02 (1.63)	0.99 (0.40)
22:2	0.96	1.01 (4.39)	1.01 (2.39)	1.02 (2.75)	1.08 (2.02)	1.03 (3.65)
22:3	0.96	1.03 (3.14)	1.02 (1.31)	1.03 (0.95)	1.06 (9.27)	1.05 (0.84)
22:6	0.94	1.06 (1.76)	1.03 (1.43)	1.06 (0.91)	1.06 (0.94)	1.07 (1.21)
24:0	0.96	1.02 (2.25)	1.00 (1.70)	0.98 (0.58)	1.01 (2.88)	1.01 (1.47)
24:1	0.96	1.02 (3.43)	0.99 (2.35)	1.00 (0.82)	1.01 (3.60)	1.02 (0.65)

[a] As proposed by Ackman and SIPOS [26].

[b] FAME amounts injected into the injector chamber.

18] achieved by injection with PTV, this injection technique is very seldom used for injection of FAMEs.

Table III shows that determined RRFs of $C_{12:0}$ and higher deviated only slightly from their theoretical RRFs when PTV injection was used. Moreover, RRFs of FAMEs were similar for all PTV injection techniques used. This indicates that discrimination of high-boiling-point FAMEs during injection did not occur. The precision of the results was good even for small amounts of FAMEs. The RRFs of polyunsaturated FAMEs were somewhat higher than those of saturated FAMEs. The reason for this might be that the purity of available polyunsaturated FAME standards is lower because of possible autoxidation of these FAMEs during storage, methylation or injection. The RRFs of the low-boiling-point FAMEs deviated more from their theoretical RRFs. The coefficients of variation were also higher for these FAMEs. When the solvent elimination technique was used the recovery of the low-boiling-point FAMEs was far below 100% (below 10% for $C_{8:0}$ and $C_{10:0}$, 18% for $C_{11:0}$, 23% for $C_{12:0}$, 88 and 92% for $C_{14:0}$ and $C_{14:1}$ and between 99 and 101% for the higher boiling acids) because they are lost at the same time as the solvent through the split exit.

Figs. 2 and 3 show the separation of FAMEs from rat red blood cell membrane phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The amount of FAMEs injected into the injector chamber was for PC in the range from 0.26 ng ($C_{20:0}$) to 52.16 ng ($C_{16:0}$) and for PE in the range from 0.08 ($C_{16:1}$) to 8.14 ng ($C_{20:4}$). The amount of internal standard injected ($C_{17:0}$) was in both cases 11.8 ng.

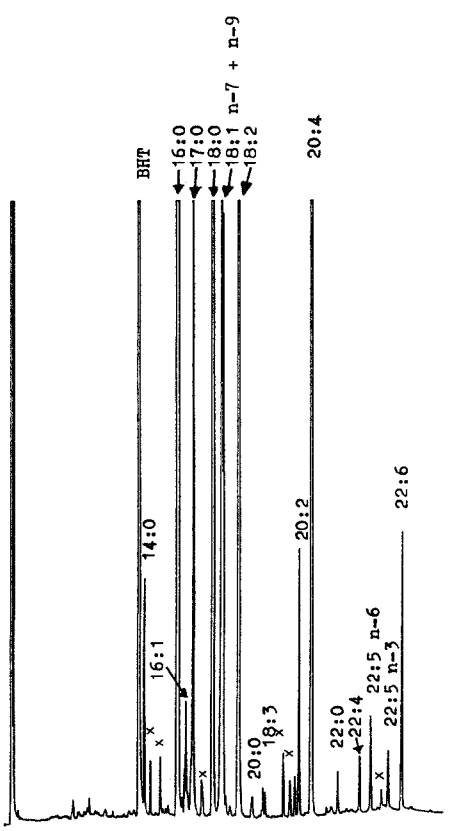

Fig. 2. Separation of FAMEs from rat red cell blood cell phosphatidylcholine ($C_{17:0}$ = internal standard, x = non-identified peaks).

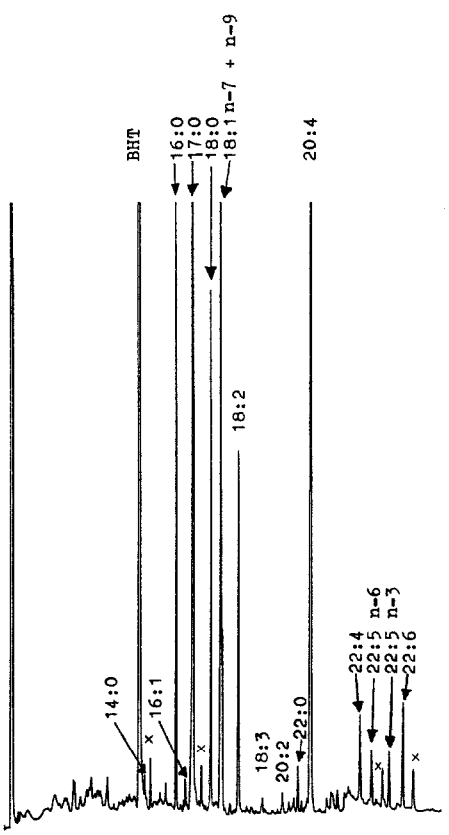

Fig. 3. Separation of FAMEs from rat red cell blood cell phosphatidylethanolamine ($C_{17:0}$ = internal standard, x = non-identified peaks).

TABLE IV

PRECISION OF GAS CHROMATOGRAPHIC DETERMINATION OF FAMEs FROM RAT RED BLOOD CELL MEMBRANE PC AND PE

C.V. = coefficient of variation. Each FAME extract was injected three times.

FAME	PC		PE	
	Amount (ng)[a]	C.V. (%)	Amount (ng)	C.V. (%)
14:0	1.08	2.83	–	
16:0	52.16	0.13	2.52	3.45
16:1	0.80	3.56	0.32	5.91
18:0	24.68	0.46	2.02	2.12
18:1	16.60	0.65	4.61	2.82
18:2	16.58	0.38	1.84	2.09
20:0	0.26	4.34	–	
20:2	2.24	1.98	0.14	4.56
20:4	20.16	0.76	8.14	0.58
22:0	0.36	1.64	0.24	3.89
22:4	0.58	1.67	0.36	3.87
22:5 n-3	0.56	1.95	0.30	4.64
22:5 n-6	1.06	2.08	0.32	4.34
22:6	2.96	0.88	0.54	3.32

[a] Amounts of FAMEs injected into the injector chamber.

Although the injected amounts of FAMEs were small, precise results were achieved. The coefficients of variation when the same FAME extract was injected three times were between 0.13 and 4.34% for FAMEs from PC and between 0.58 and 5.91% for FAMEs from PE (Table IV).

These results show that although the determined RRFs of low-boiling-point FAMEs differed from their theoretical RRFs, PTV might be useful for injection of FAMEs from most lipids, because most lipids contain few or no fatty acids with ten or fewer carbon atoms. Thus it seems that with PTV accurate and precise results can be achieved more easily than with classical split injection. Moreover, injection of FAMEs with PTV in most cases might be a good alternative to on-column injection, which is considered to be the optimal technique for injection of FAMEs with high accuracy and precision [37]. Compared with on-column injection, PTV injection offers the advantage that it can be used in various operation modes. Using the split injection mode, samples containing very different amounts can be determined without the risk of exceeding the capacity of the capillary column; using the splitless injection mode compounds of very diluted samples can be determined. PTV solvent elimination injection is useful for injection of large sample volumes. However, the solvent elimination technique is restricted to determination of fatty acids containing sixteen or more carbon atoms.

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