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Capillary gas chromatography of underivatized fatty acids with a free fatty acid phase column and a programmed temperature vaporizer injector

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Abstract

Underivatized fatty acids occurring in lipids of a number of biological specimens (blood plasma, tissues, food) were studied using a capillary column with a chemically bonded free fatty acid phase and a programmed temperature vaporizer. Non-linear calibration dependences were obtained for almost all the acids at lower concentrations because of losses in the column, non-linearity increasing with increasing carbon number and degree of unsaturation. Sample introduction at temperatures below the solvent boiling point eliminates losses in the injector. The lower recovery of long-chain polyunsaturated acids is caused by interactions occurring in the column. Some critical pairs remain unresolved in the underivatized form (18:1*n*9–18:1*n*7, 20:4*n*6–20:3*n*3), whereas the corresponding methyl esters exhibit baseline resolution.

1. Introduction

Although the first gas chromatographic paper concerned the analysis of free fatty acids (FFA) [1], the determination of these compounds, especially long-chain highly unsaturated acids, has still not been completely satisfactorily solved. The main problem lies in the relatively high boiling points of the analytes, which also represent the temperatures of thermal decomposition, hence these compounds do not exist in the form of vapours. Separation of individual FFA with respect to the carbon number and degree of unsaturation requires highly polar stationary phases with thermal stability up to about 280°C because of the high elution temperatures of long-chain unsaturated homologues. The recently developed chemically-bonded free fatty acid phase (FFAP) capillary column has permitted good qualitative resolution of underivatized fatty acids with up to twenty carbon atoms (docosanoic acid, 20:0) and three double bonds (linolenic acid, 18:3*n*6) [2]. However, quantification has only been checked for lower saturated homologues [3]. As lipid analyses often present problems regarding quantification [4,5], it seemed useful to check it also for underivatized fatty acids. The losses of the individual components are strongly influenced by the injection technique [6], chromatographic process [7] and the structure of the molecule [8].

The aim of this study was to investigate the

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possibility of determining the spectra of fatty acids in biological samples without previous methylation, using an FFAP capillary column and a programmed temperature vaporizer (PTV) injector.

2. Experimental

2.1. Reagents

Analytical-reagent grade solvents were supplied by Merck (Darmstadt, Germany) and Lachema (Brno, Czech Republic) and were distilled prior to use, if necessary. Standards of individual fatty acids (14:0, 16:0, 16:1, 18:0, 18:1*n*9, 18:2*n*6, 18:3*n*6, 18:3*n*3, 20:0, 20:1*n*9, 20:3*n*6, 20:3*n*3, 20:4*n*6, 22:6*n*3) were obtained from Sigma (St. Louis, MO, USA), and their stock standard solutions in methanol (10 mg/ml) were stored at −20°C. Boron trifluoride–methanol (12%, w/w) was purchased from Supelco (Gland, Switzerland). Silica gel 60 $HF_{254+366}$ for thin-layer chromatography (Merck) was used for the preparation of TLC plates.

2.2. Sample preparation

Total lipid was extracted as described by Folch *et al.* [9]. Polar lipids were separated by TLC with chloroform–methanol–water (60:30:5, v/v/v). The phosphatidylcholine fraction was scraped off and phophatidylcholine was isolated by a dry column technique with mixture of chloroform–methanol–acetic acid–water (13:5:1:0.2, v/v). The dried samples were saponified with 0.5 *M* potassium hydroxide in methanol for 7 min at 100°C. Fatty acids were extracted from the acidified mixture into two portions of hexane. After hexane evaporation, the samples were dissolved in a small amount of methanol and injected into the gas chromatograph. In parallel with GC analysis of the free acids, aliquots of the samples were methylated using BF_3–methanol for 3 min at 100°C and the methyl esters were extracted twice with hexane, concentrated under a stream of nitrogen and again analysed by GC. Working standard solutions for calibration dependences were prepared by diluting the stock standard solution to obtain concentrations of 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 ng/μl.

2.3. Apparatus and operating conditions

Gas chromatography was performed with Model 438A and 438S gas chromatographs (Chrompack, Middelburg, Netherlands) equipped with a capillary split–splitless injector (Chrompack), programmed-temperature vaporizer (Gerstel, Mulheim, Germany) and flame ionization detectors. The chromatographs were interfaced with an IBM PC/2 Model 30 computer and Epson LQ 550 printer. Chrompack integration software was used for data acquisition and handling.

Analyses of FFA were performed on a fused-silica capillary column (25 m × 0.32 mm I.D.) coated with chemically bonded FFAP (layer thickness 0.3 μm) (Chrompack). The oven temperature programme was isothermal at 80°C for 1 min, followed by a temperature gradient to 200°C at 30°C/min and a second gradient to 265°C at 5°C/min. The PTV injector was used in the split mode with the programme isothermal at 60°C for 1 min, followed by a temperature gradient to 300°C at 2°C/s and a hold at 300°C for 2 min. The detector temperature was 300°C. The carrier gas (hydrogen) was maintained at a head pressure of 100 kPa.

Analyses of fatty acid methyl esters (FAME) were performed on a fused-silica capillary column (25 m × 0.25 mm I.D.) coated with chemically bonded CP-WAX 52 (layer thickness 0.25 μm) (Chrompack). The oven temperature was programmed from 160 to 220°C at 2°C/min and then kept isothermal for 10 min. The injector and detector temperatures were 250 and 270°C, respectively. The carrier gas (hydrogen) was maintained at a head pressure of 80 kPa with a splitting ratio of 1:20.

2.4. Evaluation of the results

The molar correction factors were calculated according to the equation

$$f_{MR} = \frac{m_i}{m_s} \cdot \frac{A_s}{A_i} \cdot \frac{M_s}{M_i} \qquad (1)$$

where m, A and M are the analyte mass, peak area and molecular mass, respectively, subscript i refers to the test compounds and s to the internal standard (palmitic acid). Statistical calculations were carried out using Scientific Calculator Windows 3.1 Graphs and the calibration dependences were processed using the MicroCal Origin 2.4 program.

The detection limits were calculated according to the equation [10]

$$c_{\text{lim.det.}} = \frac{k s_{xy}}{S} \quad (2)$$

where $k = 4$ for a significance level of $\alpha = 0.01$, s_{xy} is the standard deviation estimate for the regression straight line and S is the slope of the calibration dependence.

3. Results and discussion

3.1. *Effect of injector temperature and carrier gas flow-rate*

It is known that for intact lipids [6,7] and highly unsaturated FAME [11], cold on-column or cold split injection techniques yield the highest recovery and lowest variability of results. A similar effect was also observed for FFA. The recovery of the acids decreases with increasing initial temperature of the injector in the split mode. Virtually identical results were also obtained with dilute analyte solutions and the injector in the splitless mode. The variability of the detector response is nearly one order of magnitude higher for hot split injection than with the cold split technique, as indicated in Table 1.

The carrier gas flow-rate is another factor that substantially influences the recovery of intact lipids [4,7] and FFA. The recovery is inversely proportional to the flow-rate because the retention times become shorter with increasing flow-rate and thus sample losses during the chromatographic process are suppressed. However, a compromise must be found for the carrier gas flow-rate in analyses of biological samples because of the poor resolution of critical FFA pairs at higher flow-rates.

Table 1
Dependence of the detector response on the injector temperature

Parameter	FFA			
	20:4*n*6		22:6*n*3	
Injector temperature (°C)	240	30	240	30
x[a]	0.25	0.28	0.24	0.27
R.S.D. (%)	8.8	1.2	9.8	1.3

[a] Mean A_i/A_s ($n = 5$).

3.2. *Effect of injected amount and molecular structure of the analytes*

The concentration, molecular mass and number of double bonds significantly affect the recovery of the individual components of lipid samples. The losses occurring during the injection and chromatographic process are generally inversely proportional to the concentration and directly proportional to the molecular mass and degree of unsaturation [7,8]. Our main interest was to check these deviations from the regular chromatographic behaviour with the FFAP column. Calibration dependences for monoenoic and polyenoic fatty acids were measured over the concentration range 20–1000 ng/μl and the parameters of the linear calibration plots are

Table 2
Parameters of the calibration data

FFA	*A*	*B*	*r*	*N*
16:1*n*7	0.0026	1.00	0.9998	8
18:1*n*9	4.92×10^{-5}	0.95	0.9998	8
20:1*n*9	−0.0031	0.81	0.9999	8
22:1*n*9	−0.0047	0.80	0.9998	8
24:1*n*9	−0.0072	0.76	0.9998	8
18:2*n*6	−0.0045	0.97	0.9998	6
18:3*n*6	−0.0037	0.95	0.9996	6
18:3*n*3	−0.0062	0.94	0.9996	6
20:3*n*3	−0.0060	0.80	0.9998	6
20:4*n*6	−0.0167	0.78	0.9992	6
22:6*n*3	−0.0100	0.67	0.9994	6

A and *B* = intercept and slope of the regression calibration dependence, respectively; *r* = correlation coefficient; *N* = number of measurements.

given in Table 2. All the fatty acids exhibit linear calibration plots, the slope of which decreases with increasing carbon number and degree of unsaturation. However, at low concentrations (below 100 ng/μl) the molar correction factor f_{MR} differs from unity and its dependence on the amount injected is characteristic for each component. Fig. 1 depicts the dependence of the relative molar response (RMR) on the amount

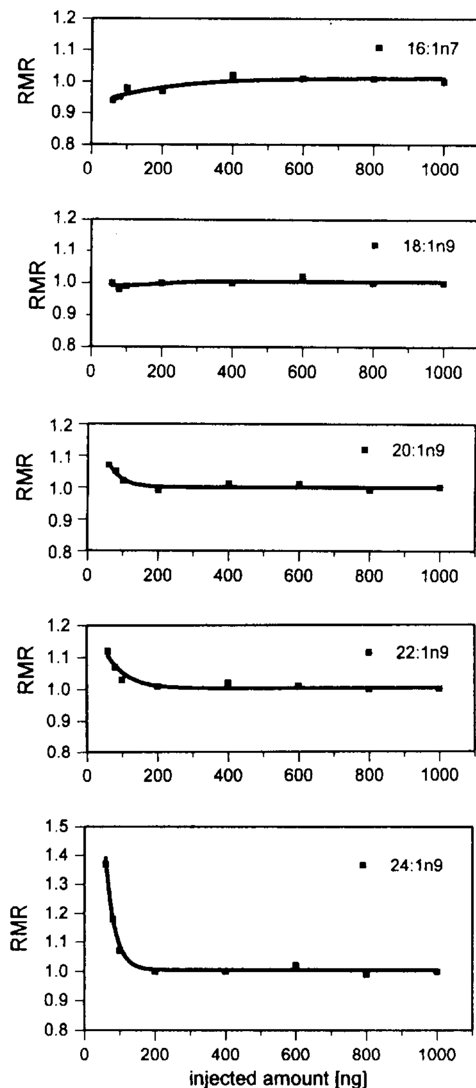

Fig. 1. Dependence of the relative molar response on the amount injected for monoenoic acids. Internal standard, palmitic acid (1000 ng).

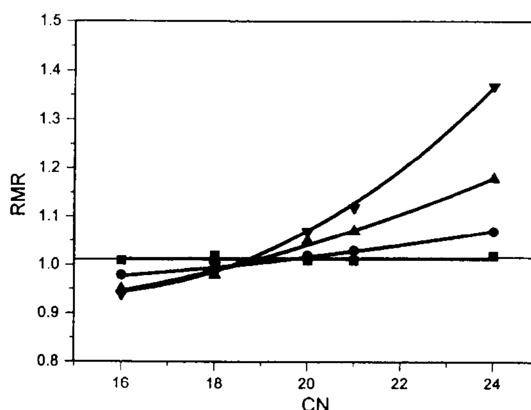

Fig. 2. Dependence of the relative molar response on the carbon number for different amounts of monoenoic acids. ■ = 600; ● = 100; ▲ = 80; ▼ = 60 ng. Internal standard, palmitic acid (1000 ng).

injected for individual monoenoic fatty acids. Similar dependences were also obtained for polyenoic acids. The higher the carbon number and degree of unsaturation, the more curved is this dependence in the low concentration range. The different behaviour of palmitoleic acid is probably caused by the very similar retention times for this compound and palmitic acid used as the internal standard, although they are baseline resolved.

Fig. 2 shows the dependence of the molar correction factor on the carbon number for various concentrations of individual monoenoic acids. At higher concentrations, the molar cor-

Table 3
Molar correction factors for saturated fatty acids

Concentration (ng/μl)	f_{MR}				
	14:0	18:0	20:0	22:0	24:0
1000	1.00	1.00	1.00	1.00	1.00
800	1.02	1.03	1.04	1.05	1.02
600	1.12	1.14	1.18	1.14	1.28
400	1.22	1.26	1.26	1.23	1.53
200	1.24	1.44	1.56	1.81	2.21
100	1.52	1.32	1.61	1.99	2.24
80	1.27	1.29	1.71	2.07	2.71
60	1.26	1.34	1.67	2.10	–
40	1.22	1.36	1.71	2.08	–

Table 4
Detection limits for individual fatty acids

FFA	Limit of detection in the sample analyzed (ng)
16:1*n*7	0.3
18:1*n*9	0.4
20:1*n*9	0.5
22:1*n*9	0.6
24:1*n*9	0.7
18:2*n*6	0.5
18:3*n*6	0.5
18:3*n*3	0.4
20:3*n*3	0.7
20:4*n*6	1.2
22:6*n*3	1.6

rection factor is independent of the carbon number, whereas for concentrations below 100 ng/μl the molar correction factor increases with increasing carbon number.

Intact lipids containing saturated fatty acids exhibit lower losses than their unsaturated homologues during the chromatographic process. Minimal losses were also expected with saturated FFA because of their higher stability; however, the losses were unexpectedly high and the reproducibility was poor. This effect is probably caused by the limited solubility of underivatized fatty acids. Their higher homologues are soluble in organic solvents only at increased temperatures and hence a poor solubility in the stationary phase can also be expected. Table 3 gives the molar correction factors measured over the whole calibration range.

3.3. Determination of fatty acids in biological samples

The above results demonstrate a limited possibility of determining the wide range of underivatized fatty acids in biological samples. Highly unsaturated fatty acids, which are often present in trace amounts, have poorer detection limits (Table 4). A comparison of three techniques for the determination of fatty acids in a plasma phosphatidylcholine sample showed the highest losses of polyunsaturated acids in underivatized form with cold split injection. Determination of the corresponding FAME by both hot and cold split injection techniques yield higher results for these acids. The results are summarized in Table 5. Chromatograms of both FFA and FAME with the FFAP column are given in Fig. 3.

Another limitation is an insufficient resolution of some critical pairs. In underivatized form, fatty acids 18:1*n*9–18:1*n*7 and 20:4*n*6–20:3*n*3 are not resolved, whereas in the form of FAME they

Table 5
Determination of fatty acids in blood plasma phosphatidylcholine in the form of FFA and FAME

FFA	A		B		C	
	M (%)[a]	R.S.D. (%)	*M* (%)[a]	R.S.D. (%)	*M* (%)[a]	R.S.D. (%)
14:0	1.40	2.9	1.28	4.3	1.95	3.4
16:0	35.25	1.0	34.65	2.1	37.59	1.2
16:1	2.87	2.5	2.26	3.1	2.24	2.9
18:0	15.75	1.9	15.94	1.6	15.59	1.2
18:1	18.0	1.5	17.77	2.2	18.52	1.7
18:2*n*6	18.07	3.7	18.14	2.5	16.99	3.1
20:3*n*6	2.03	1.6	2.03	4.9	1.88	5.1
20:4*n*6	5.54	3.9	5.55	3.7	4.44	3.5
22:6*n*3	1.67	4.4	2.07	4.8	1.40	4.1

A = Determination of FAME; split injection, 250°C, CP-WAX 52 column. B = Determination of FAME; PTV injector, 60°C, FFAP column. C = Determination of FFA; PTV injector, 60°C, FFAP column.
[a] Molar per cent, mean of five measurements.

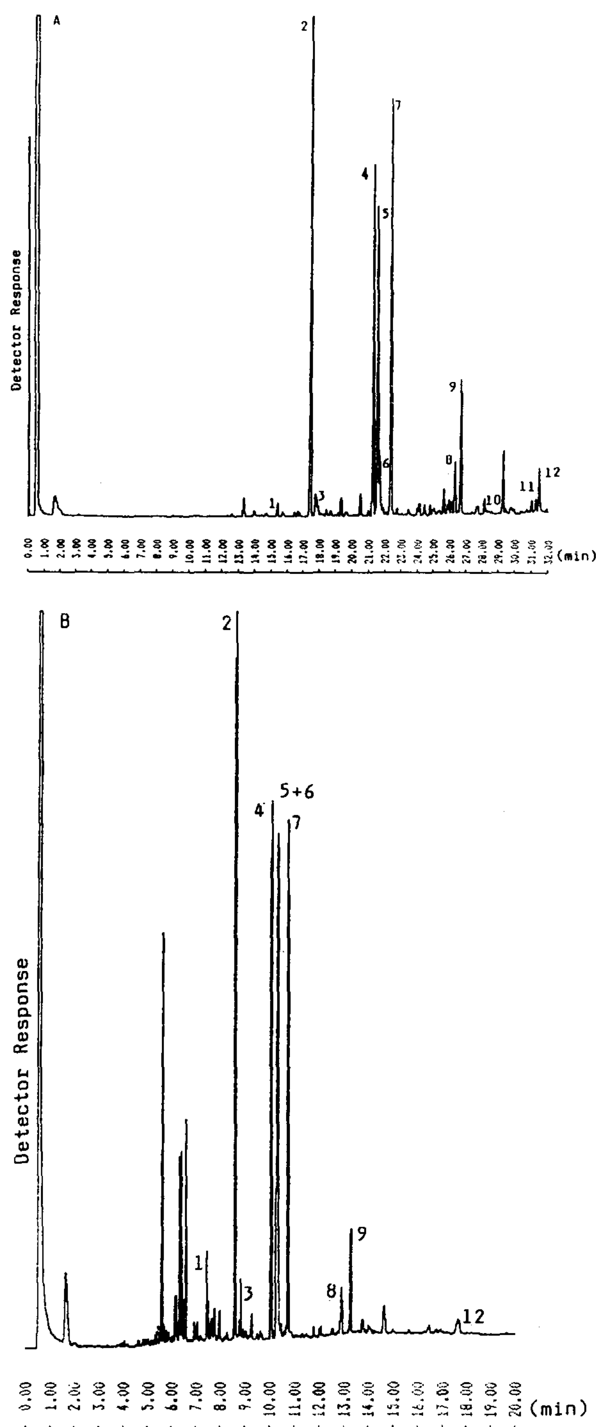

Fig. 3. Chromatograms of (A) FAME and (B) FFA from human plasma phosphatidylcholine on the FFAP column. Fatty acid designation: 1 = 14:0; 2 = 16:0; 3 = 16:1; 4 = 18:0; 5 = 18:1*n*9; 6 = 18:1*n*7; 7 = 18:2*n*6; 8 = 20:3*n*6; 9 = 20:4*n*6: 10 = 20:5*n*3; 11 = 22:5*n*3; 12 = 22:6*n*3. Analytical conditions are given in the text. The peaks preceding peak 1 in chromatogram (B) are unidentified sample matrix components that disappeared on formation of FAME.

are resolved satisfactorily. The first critical pair, oleic and vaccenic acid, is present in many biological samples (blood cells and plasma, most animal and plant tissues) and the second, arachidonic and dihomoalphalinolenic acid, is encountered more rarely (*e.g.*, in fish tissues). Saturated fatty acids in biological samples are primarily represented by palmitic and stearic acid, which can be determined without serious complications.

4. Conclusions

The described method for the determination of underivatized acids is simple and rapid. On the other hand, not all critical acid pairs can be adequately separated and it must be expected that calibration plots would be non-linear at lower analyte concentrations.

5. References

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