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Journal of Chromatography B, 770 (2002) 91–99

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Effect of column and software on gas chromatographic determination of fatty acids

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Abstract

Four capillary columns (A: CP-WAX 52 CB 25 m×0.25 mm; B: CP WAX 52 CB 30 m×0.25 mm; C: CP-WAX 58 CB 25 m×0.25 mm, Chrompack; D: OMEGAWAX™ 320 30 m×0.32 mm, Supelco) and two integration software (Mosaic v.5.10, Chrompack and CSW v.1.7, Data Apex®) were compared for analysis of fatty acids. Column A was mounted stepwise in two different instruments. Fatty acids of blood plasma phosphatidylcholine and standard mixture of saturated fatty acids were analysed as methyl esters under identical chromatographic conditions. Both integrating software did not differ significantly in most results; differences were observed only for minor components: 16:1n9 (0.10±0.020 vs. 0.17±0.005 *M*%, $P<0.0001$, column A1; 0.09±0.011 vs. 0.16±0.007 *M*%, $P<0.0001$, column A2; 0.09±0.010 vs. 0.17±0.003 *M*%, $P<0.0001$, column C; 0.09±0.008 vs. 0.19±0.003 *M*%, $P<0.0001$, column D), 20:0 (0.10±0.001 vs. 0.06±0.005 *M*%, $P<0.05$, column C) and 20:2n6 (0.43±0.030 vs. 0.91±0.016 *M*%, $P<0.0001$, column A2). Increased values for 16:1n9 and 20:2n6 integrated by MOSAIC are caused by cointegration of two poorly resolved peaks: fatty acid and impurity from sample matrix. Lower values for 20:0 are caused by incomplete integration of minor peak. Differences between columns were observed mostly for minor fatty acids. The results indicate that CSW is more suitable software for integration of complicated chromatograms. Linear calibration dependences measured with standard mixture of saturated fatty acids (carbon number 10–24) were observed in wide range of concentrations (three orders). Slope close to unity and minimal value of intercept confirmed theoretical relations when analyses are run under optimal conditions. Use of one column is advisable in small intervention or experimental metabolic studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Fatty acids

1. Introduction

Large and long-lasting clinical studies are often prone to difficulties resulting from changes of methodology. Thus, continuity of results within longer period of time (years) is important especially in studies searching for metabolic changes under different pathophysiological conditions.

Gas chromatographic (GC) analysis of fatty acids (FA) has old history dating back to the time when James and Martin [1] published first paper on gas chromatography dealing with underivatized FA and 3 years later with their methyl esters (ME) [2], which remained up today the most convenient form. Technical progress in the last 50 years resulted in the development of sophisticated instrumental techniques, minimising sample losses during chromatographic process and errors due to measurement of peak area.

Practical reasons led to wide use of hot split-splitless injection technique and flame ionisation

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PII: S1570-0232(01)00630-4

detection (FID). Optimisation of operating conditions of FID increases effective linear range with good quantitative results [3]. Temperature programming enables to reach similar peak shapes for all components, which would be problematic under isothermal conditions [4]. Correct adjustment of the system empowers us to use theoretical response factors [5], confirmed also by other authors [6,7], rather than apply the response correction factors [3].

Capillary columns with chemically bonded stationary phases of middle polarity are predominantly used for analyses of FA in biological samples (columns with highly polar phases are used when the separation of *cis–trans*-geometrical isomers is desirable). Elution order reflects at first the number of carbon atoms (CN) and then the degree of unsaturation (FAME with CN=X and four or five double bonds is eluted ahead of saturated FAME with CN=X+2).

Quantitative analysis in GC–FID is based on the relationship between the mass of ''effective carbons'' (carbon atoms of methylene units, for FAME all carbon atoms except that of carboxyl group) and the detector response. Quantitation of individual FA is thus based on the comparison of their peak areas, A_i, and the peak area of suitable internal standard, A_{is} (usually saturated FA with odd CN, which is originally not present in the biological sample). For those purposes were used margaric acid (17:0) [6–9], nonadecanoic acid (19:0) [10–12], in case of samples with high levels of polyunsaturated FA (PUFA) also docosatrienoic [13,14], tricosanoic (23:0) and nervonic acids [15]. Relative standard deviation is

Table 1
Fatty acid content in plasma phospholipid: sample 1

Column software	A1		A2		C		D	
	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC
14:0	0.42±0.02	0.43±0.02	0.40±0.01^{b}	0.41±0.01	0.43±0.01^{b}	0.43±0.01	0.46±0.01	0.46±0.01
16:0	26.11±0.42	26.00±0.43	25.68±0.30	25.53±0.30	26.52±0.29	26.34±0.32	26.31±0.25	26.16±0.31
16:1n9	0.17±0.01^{ccc}	0.17±0.01	0.12±0.01	0.18±0.01*	0.11±0.02^{ccc}	0.18±0.04***	0.13±0.02	0.20±0.01****
16:1n7	0.59±0.01	0.60±0.02	0.57±0.01	0.58±0.01	0.57±0.01	0.58±0.01	0.59±0.01	0.60±0.01
18:0	14.05±0.15	13.99±0.14	14.04±0.13	13.98±0.16	14.19±0.06	14.11±0.07	13.80±0.06	13.72±0.13
18:1n9	11.55±0.07	11.62±0.03	11.48±0.12	11.83±0.07	11.34±0.09	11.70±0.07	11.38±0.10	11.64±0.06
18:1n7	1.88±0.05^{c}	1.93±0.01	1.93±0.04	1.95±0.02	2.02±0.04^{c}	2.04±0.03	1.92±0.01	1.94±0.02
18:2n6	26.26±0.11	26.27±0.08	26.75±0.12	26.71±0.16	26.45±0.11	26.33±0.10	26.33±0.08	26.39±0.10
18:3n6	0.10±0.01	0.13±0.00	0.11±0.00^{d}	0.11±0.01	0.10±0.00	0.11±0.01	0.08±0.01^{d}	0.09±0.01
18:3n3	0.47±0.01	0.48±0.01	0.46±0.01	0.46±0.01	0.47±0.01	0.43±0.01	0.46±0.01	0.46±0.03
20:0	0.07±0.01^{aaaa}	0.07±0.01	0.07±0.00^{bbbb}	0.07±0.00	0.12±0.01$^{bbbb}_{aaaa}$	0.10±0.05	0.09±0.02	0.09±0.00
20:1n9	0.18±0.01	0.18±0.01	0.17±0.01	0.17±0.01	0.18±0.01	0.25±0.12	0.16±0.03	0.16±0.01
20:2n6	0.58±0.02^{aaa}	0.59±0.01	0.56±0.01^{bb}	0.55±0.02	0.54±0.01	0.53±0.01	0.38±0.01$^{aaa}_{bb}$	0.52±0.02
20:3n6	2.41±0.05	2.41±0.05	2.44±0.05	2.42±0.05	2.41±0.03	2.39±0.03	2.40±0.03	2.39±0.03
20:4n6	9.71±0.17	9.67±0.16	9.80±0.20	9.73±0.19	9.51±0.11	9.43±0.10	9.65±0.09	9.66±0.11
20:5n3	0.97±0.02	0.98±0.02	0.98±0.02	0.96±0.03	0.92±0.02	0.91±0.03	0.97±0.01	0.95±0.03
22:4n6	0.35±0.01	0.36±0.01	0.34±0.01	0.34±0.01	0.39±0.02	0.40±0.05	0.47±0.18	0.49±0.04
22:5n6	0.22±0.01	0.23±0.01	0.22±0.01	0.21±0.02	0.20±0.01	0.21±0.01	0.23±0.01	0.23±0.02
22:5n3	1.04±0.04	1.04±0.04	1.02±0.02	0.99±0.03	0.95±0.03	0.95±0.03	1.02±0.02	1.02±0.02
22:6n3	2.89±0.10	2.91±0.10	2.85±0.05	2.82±0.05	2.58±0.10	2.59±0.09	2.87±0.06	2.85±0.06
Σsatur	40.64±0.34	40.49±0.35	40.19±0.32	39.99±0.35	41.26±0.35	40.98±0.37	40.65±0.28	40.43±0.41
Σmono	14.36±0.11	14.50±0.05	14.28±0.14	14.71±0.09	14.22±0.08	14.75±0.13	14.19±0.09	14.54±0.08
Σn6	39.62±0.21	39.64±0.17	40.22±0.27	40.07±0.29	39.60±0.27	39.40±0.22	39.85±0.25	39.76±0.26
Σn3	5.37±0.16	5.37±0.23	5.31±0.09	5.27±0.10	4.92±0.13	4.88±0.12	5.31±0.08	5.23±0.07

Fatty acid content was measured using four different chromatographic systems (see text) and two types of integration software. Each sample was measured 10 times, typical injected volume was 1–2 μl. Conditions: injector, splitter 250 °C; detector, FID 270 °C; temperature programme, 150–240 °C 2°/min, carrier gas hydrogen, head pressure 70 kPa.

The data (molar percentages) are in the average±SD format. Symbols and abbreviations used: *Σ*, the sum; *significant difference (Student's *t*-test) from CSW v.1.7 integration in the same column, data bearing the same letters indicate difference (GLM with post hoc comparison analysis) both using CSW v.1.7 software. Number of symbols indicates *P*: <0.05, <0.01, <0.001, and <0.0001

proportional to the difference of CN of the determined component and I.S.; compensation is achieved by the use of several I.S. [7,16]. Results of FA composition are mostly expressed as molar percentage of individual components; only few papers deal with absolute concentrations related to the volume of plasma or separated lipoproteins [12,13].

The aim of our study was to compare four capillary columns installed in different GC instruments as well as two integration systems for the analysis of FA in biological matrix.

2. Experimental

Standards of FA were purchased from Sigma–Aldrich (Prague, Czech Republic), 1,2-dinonanoyl-*sn*-glycero-3-phosphocholine ($I.S._{PL}$) from Larodane (Malmö, Sweden). Stock solutions of FAME with CN10, 12, 14, 16, 18, 20, 22, 24 and CN19 (I.S.) as well as of $I.S._{PL}$ were prepared in concentration 10 mg/ml. Nine calibration samples were prepared from the equimolar mixture and solution of I.S. covering c_i:c_{is} ratio from 10 to 0.01. Calibration samples were analysed undiluted (D0), diluted 1:10 (D1) and 1:20 (D2).

Total lipid was extracted from 0.5 ml of plasma containing 8 μl of $I.S._{PL}$ solution by the method of Folch et al. [17] using dichloromethane instead of chloroform [18]. Phospholipids were separated by TLC with the mobile phase heptane–diethyl ether–acetic acid (80:20:1, v/v/v) and transmethylated to FAME with 1 *M* sodium methoxide in dry methanol under nitrogen atmosphere in darkness without previous separation from the layer material (60 min at ambient temperature). The reaction mixture was

Table 2
Fatty acid content in plasma phospholipid: sample 2

Column software	A1		A2		C		D	
	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC
14:0	0.22±0.01	0.23±0.01	0.21±0.01	0.22±0.01	0.23±0.01	0.24±0.00	0.25±0.01	0.25±0.01
16:0	26.99±0.90	26.66±0.86	26.64±0.59	26.49±0.59	28.01±0.75	27.62±0.74	27.61±0.60	27.44±0.64
16:1n9	0.12±0.01^{bbbb}	0.18±0.00	0.11±0.02	0.17±0.01****	0.12±0.01^{bbbb}	0.18±0.04****	0.14±0.01	0.21±0.01****
16:1n7	0.61±0.01	0.62±0.01	0.58±0.02	0.60±0.01	0.63±0.06	0.63±0.02	0.64±0.02	0.65±0.02
18:0	14.29±0.40	14.15±0.41	13.92±0.13	13.86±0.11	14.50±0.43	14.33±0.46	13.70±0.08	13.62±0.09
18:1n9	9.49±0.19	10.13±0.14***	9.88±0.18	10.27±0.08	9.78±0.21	10.23±0.15	9.81±0.09	10.16±0.10
18:1n7	2.22±0.10^{aaaa}	2.28±0.03	2.33±0.02	2.32±0.02	2.42±0.05^{aaaa}	2.44±0.04	2.29±0.03	2.31±0.01
18:2n6	20.72±0.28	20.63±0.28	21.28±0.17	21.23±0.19	21.00±0.49	20.83±0.32	21.24±0.15	21.13±0.16
18:3n6	0.12±0.01	0.14±0.01	0.12±0.00	0.11±0.01	0.12±0.01	0.13±0.01	0.10±0.01	0.10±0.01
18:3n3	0.29±0.01	0.30±0.01	0.29±0.00	0.29±0.00	0.30±0.01	0.28±0.01	0.30±0.01	0.24±0.01
20:0	0.06±0.00^{aaaa}	0.07±0.01	0.06±0.00^{bbbb}	0.06±0.01	0.12±0.01$^{bbbb}_{aaaa,cc}$	0.08±0.01**	0.08±0.01^{cc}	0.08±0.01
20:1n9	0.16±0.01	0.17±0.01	0.16±0.01	0.15±0.01	0.16±0.01	0.18±0.02	0.15±0.01	0.14±0.00
20:2n6	0.65±0.03^{aaaa}	0.69±0.03	0.61±0.02^{bbbb}	0.60±0.02	0.58±0.10^{cccc}	0.62±0.01	0.31±0.01$^{bbbb}_{aaaa,cccc}$	0.55±0.03****
20:3n6	3.85±0.11	3.83±0.11	3.87±0.09	3.85±0.10	3.38±1.18	3.73±0.08	3.77±0.07	3.75±0.08
20:4n6	12.75±0.32	12.59±0.33	12.82±0.27	12.74±0.26	12.19±0.38	12.07±0.30	12.55±0.25	12.46±0.23
20:5n3	1.17±0.04	1.20±0.03	1.18±0.04	1.17±0.03	1.10±0.05	1.11±0.06	1.18±0.03	1.11±0.04
22:4n6	0.39±0.02	0.39±0.03	0.37±0.02	0.36±0.02	0.41±0.04	0.40±0.04	0.36±0.02	0.34±0.01
22:5n6	0.31±0.02	0.32±0.04	0.30±0.02	0.30±0.02	0.27±0.01	0.28±0.01	0.28±0.01	0.27±0.02
22:5n3	1.17±0.07	1.17±0.09	1.13±0.05	1.11±0.05	1.03±0.06	1.03±0.05	1.12±0.04	1.11±0.04
22:6n3	4.33±0.21^{aaaa}	4.27±0.23	4.13±0.20^{bbb}	4.08±0.20	3.65±0.23$^{bbbb}_{aaaa}$	3.60±0.22	4.12±0.14^{ccc}	4.08±0.13
Σsatur	41.57±1.27	41.11±1.24	40.84±0.64	40.64±0.63	42.85±1.19	42.26±1.20	41.64±0.61	41.40±0.67
Σmono	12.67±0.29	13.37±0.19*	13.06±0.16	13.51±0.11	13.11±0.27	13.66±0.18	13.03±0.10	13.46±0.10
Σn6	38.79±0.72	38.59±0.76	39.37±0.41	39.19±0.40	37.96±1.00	38.06±0.73	38.60±0.44	38.60±0.45
Σn3	6.97±0.32^{aaaa}	6.93±0.34	6.72±0.20^{b}	6.54±0.19	6.08±0.33$^{aaaa}_{b,c}$	6.02±0.33	6.73±0.29^{c}	6.65±0.27

Fatty acid content was measured using four different chromatographic systems (see text) and two types of integration software. Each sample was measured 10 times, typical injected volume was 1–2 μl. Conditions: injector, splitter 250 °C; detector, FID 270 °C; temperature programme, 150–240 °C 2°/min, carrier gas hydrogen, head pressure 70 kPa.
For explanation see Table 1.

neutralized with 1 *M* acetic acid, ME were extracted twice into hexane and passed through the column (5×20 mm) of anhydrous sodium sulphate. The combined extracts were dried under nitrogen, dissolved in an appropriate volume of isooctane and stored at −20 °C until analysed.

Gas chromatography was performed with Chrompack Model 438A, 438S, 9000 and 9001 gas chromatographs (Chrompack, Middelburg, The Netherlands). All chromatographs were equipped with a capillary split-splitless injector and FID.

Analyses of FAME were performed on the fused-silica capillary columns coated with chemically bonded stationary phases: (A) CP-WAX 52 CB, 25 m×0.25 mm I.D., (B) CP-WAX 52 CB 30 m×0.25 mm I.D., (C) CP-WAX 58 CB 25 m×0.32 mm I.D., (Chrompack), (D) Omegawax 320, 30 m×0.32 mm I.D. (Supelco, Bellefonte, PA). The oven temperature was programmed from 80 to 160 °C at 10°/min, to 240 °C at 2°/min, then isothermal 15 min. The injector and detector temperatures were 250 and 270 °C, respectively. Hydrogen carrier gas was maintained at a head pressure of 80 kPa, split ratio 1:20.

Integration software CSW v.1.7 (Data Apex, Prague, Czech Republic) and MOSAIC v.5.10 (Chrompack) were used for data acquisition and handling.

Statistical analyses were performed with the statistical software Statistica for Windows, v.4.0 (StatSoft, Tulsa, USA).

3. Results and discussion

Composition of FA in PL of five different plasma samples is shown in Tables 1–5. Column A was

Table 3
Fatty acid content in plasma phospholipid: sample 3

Column software	A1		A2		C		D	
	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC
14:0	0.34±0.02[aaaa]	0.34±0.02	0.32±0.01[bbbb]	0.33±0.01	0.33±0.01[cccc]	0.34±0.01	0.38±0.01[bbbb] [aaaa,cccc]	0.38±0.01
16:0	27.00±1.09	26.84±1.09	26.48±0.34	26.41±0.39	27.05±0.54	26.87±0.58	27.74±0.21	27.47±0.23
16:1n9	0.22±0.01[bbbb] [cccc]	0.30±0.01	0.21±0.02	0.29±0.01****	0.23±0.02[bbbb]	0.29±0.01****	0.22±0.02[cccc]	0.34±0.01****
16:1n7	1.62±0.06	1.61±0.06	1.64±0.02	1.47±0.52	1.60±0.04	1.62±0.04	1.75±0.02	1.73±0.02
18:0	14.97±0.65	14.91±0.62	14.72±0.07	14.69±0.07	15.13±0.64	15.05±0.66	14.42±0.09	14.35±0.07
18:1n9	16.19±0.50	16.19±0.44	16.54±0.08	16.76±0.09	16.15±0.35	16.26±0.34	16.43±0.10	16.46±0.04
18:1n7	2.90±0.09	2.92±0.07	3.02±0.02	3.04±0.02	3.01±0.12	3.01±0.06	2.99±0.02	2.98±0.01
18:2n6	16.56±0.52	16.69±0.50	16.94±0.07	16.98±0.11	16.68±0.36	16.67±0.34	16.87±0.07	16.75±0.08
18:3n6	0.21±0.01	0.23±0.02	0.21±0.00	0.21±0.00	0.20±0.01	0.21±0.01	0.19±0.01	0.20±0.01
18:3n3	0.32±0.01	0.33±0.02	0.32±0.00	0.32±0.01	0.32±0.01	0.32±0.02	0.31±0.01	0.27±0.03
20:0	0.07±0.00[aaaa]	0.07±0.01	0.06±0.01[bbbb]	0.06±0.01	0.12±0.01[bbbb] [aaaa]	0.07±0.01****	0.09±0.00	0.09±0.01
20:1n9	0.22±0.01	0.22±0.01	0.21±0.01	0.20±0.01	0.22±0.01	0.23±0.01	0.20±0.01	0.20±0.01
20:2n6	1.84±0.07[aaaa]	1.88±0.07	1.86±0.03[bbbb]	1.86±0.04	1.80±0.04[cccc]	1.80±0.04	1.34±0.23[bbbb] [aaaa,cccc]	1.77±0.04****
20:3n6	3.87±0.14	3.88±0.13	3.96±0.05	3.95±0.05	3.89±0.09	3.90±0.08	3.85±0.04	3.84±0.03
20:4n6	8.38±0.28	8.34±0.28	8.44±0.11	8.42±0.11	8.26±0.19	8.28±0.18	8.23±0.06	8.19±0.05
20:5n3	0.99±0.04	1.02±0.04	0.97±0.02	0.96±0.04	0.95±0.03	0.96±0.04	0.96±0.09	0.96±0.02
22:4n6	0.44±0.02	0.44±0.02	0.43±0.01	0.43±0.01	0.51±0.03	0.51±0.03	0.38±0.11	0.42±0.00
22:5n6	0.43±0.02	0.43±0.02	0.42±0.01	0.41±0.02	0.40±0.01	0.40±0.01	0.36±0.03	0.39±0.01
22:5n3	0.91±0.04	0.90±0.06	0.89±0.02	0.87±0.02	0.85±0.03	0.86±0.04	0.91±0.10	0.87±0.01
22:6n3	2.47±0.11	2.45±0.12	2.36±0.05	2.33±0.05	2.30±0.08	2.36±0.09	2.37±0.03	2.34±0.03
Σsatur	42.37±1.66	42.16±1.63	41.58±0.31	41.51±0.43	42.62±1.16	42.33±1.21	42.63±0.25	42.29±0.19
Σmono	21.22±0.62	21.24±0.54	21.62±0.07	21.76±0.42	21.21±0.38	21.41±0.44	21.59±0.10	21.71±0.03
Σn6	31.72±0.94	31.89±0.92	32.27±0.22	32.26±0.25	31.75±0.70	31.76±0.66	31.23±0.21	31.55±0.16
Σn3	4.69±0.18	4.70±0.22	4.55±0.15	4.45±0.05	4.42±0.14	4.50±0.14	4.53±0.09	4.48±0.10

Fatty acid content was measured using four different chromatographic systems (see text) and two types of integration software. Each sample was measured 10 times, typical injected volume was 1–2 μl. Conditions: injector, splitter 250 °C; detector, FID 270 °C; temperature programme, 150–240 °C 2°/min, carrier gas hydrogen, head pressure 70 kPa.
For explanation see Table 1.

Table 4
Fatty acid content in plasma phospholipid: sample 4

Column software	A1		A2		C		D	
	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC
14:0	0.24±0.01	0.25±0.01	0.22±0.01	0.23±0.01	0.23±0.01	0.24±0.00	0.26±0.01	0.26±0.01
16:0	29.19±0.35	29.10±0.36	28.79±0.35	28.71±0.34	28.93±0.14	28.81±0.14	29.64±0.26	29.45±0.31
16:1n9	0.14±0.02	0.16±0.01	0.11±0.01	0.17±0.00***	0.11±0.01	0.17±0.00***	0.13±0.02	0.20±0.01****
16:1n7	0.80±0.01	0.81±0.01	0.79±0.01	0.80±0.01	0.78±0.01	0.80±0.01	0.82±0.01	0.82±0.01
18:0	13.04±0.08	12.99±0.08	13.11±0.05	13.07±0.04	13.21±0.05	13.17±0.05	12.94±0.07	12.87±0.05
18:1n9	10.09±0.05	10.06±0.05	10.09±0.04	10.20±0.02	10.06±0.07	10.15±0.07	9.98±0.06	10.07±0.03
18:1n7	1.78±0.01	1.78±0.01	1.79±0.01	1.80±0.01	1.80±0.02	1.84±0.01	1.77±0.02	1.79±0.02
18:2n6	19.91±0.10	20.02±0.12	20.23±0.04	20.24±0.07	20.10±0.13	20.12±0.12	20.09±0.05	20.03±0.05
18:3n6	0.13±0.00	0.14±0.01	0.13±0.00	0.12±0.01	0.12±0.01	0.13±0.00	0.11±0.00	0.12±0.01
18:3n3	0.21±0.00	0.21±0.01	0.20±0.00	0.20±0.01	0.21±0.01	0.20±0.01	0.20±0.01	0.20±0.01
20:0	0.04±0.00[aaaa]	0.05±0.00	0.04±0.00[bbbb]	0.05±0.00	0.09±0.00[bbbb] [aaaa]	0.05±0.00****	0.07±0.00	0.07±0.01
20:1n9	0.17±0.00	0.18±0.01	0.17±0.00	0.17±0.00	0.18±0.00	0.19±0.00	0.17±0.01	0.17±0.01
20:2n6	0.72±0.01[aaaa]	0.75±0.02	0.71±0.02[bbbb]	0.71±0.02	0.71±0.01[cccc]	0.71±0.01	0.44±0.01[bbbb] [aaaa,cccc]	0.68±0.02****
20:3n6	3.98±0.05	3.98±0.05	4.04±0.05	4.03±0.04	4.04±0.02	4.04±0.07	3.97±0.04	3.95±0.04
20:4n6	11.87±0.12	11.84±0.12	11.99±0.13	11.95±0.13	11.89±0.04	11.89±0.05	11.81±0.11	11.74±0.09
20:5n3	1.52±0.02	1.53±0.02	1.51±0.03	1.53±0.02	1.50±0.02	1.49±0.03	1.53±0.01	1.53±0.02
22:4n6	0.36±0.01	0.36±0.01	0.36±0.01	0.35±0.01	0.39±0.01	0.39±0.01	0.36±0.09	0.35±0.00
22:5n6	0.20±0.00	0.20±0.01	0.20±0.01	0.19±0.01	0.19±0.01	0.18±0.01	0.16±0.01	0.19±0.00
22:5n3	1.03±0.02	1.03±0.02	1.02±0.02	1.00±0.02	1.00±0.02	1.00±0.02	1.02±0.02	1.00±0.03
22:6n3	4.59±0.09	4.55±0.09	4.49±0.07	4.46±0.07	4.45±0.07	4.42±0.06	4.54±0.08	4.51±0.07
Σsatur	42.51±0.35	42.39±0.36	42.17±0.31	42.06±0.31	42.47±0.17	42.28±0.16	42.91±0.21	42.66±0.27
Σmono	12.98±0.08	12.99±0.06	12.95±0.04	13.15±0.02	12.94±0.11	13.15±0.09	12.86±0.07	13.06±0.05
Σn6	37.17±0.22	37.29±0.23	37.66±0.21	37.60±0.21	37.44±0.12	37.46±0.12	36.95±0.16	37.05±0.16
Σn3	7.35±0.13	7.33±0.13	7.28±0.09	7.24±0.11	7.16±0.09	7.11±0.10	7.22±0.12	7.19±0.11

Fatty acid content was measured using four different chromatographic systems (see text) and two types of integration software. Each sample was measured 10 times, typical injected volume was 1–2 μl. Conditions: injector, splitter 250 °C; detector, FID 270 °C; temperature programme, 150–240 °C 2°/min, carrier gas hydrogen, head pressure 70 kPa.
For explanation see Table 1.

stepwise installed in two instruments, CP 438A (A1) and CP 9000 (A2). All analyses were evaluated by both integration systems. The most consistent findings in integration are significant differences in the content of Δ7 hexadecenoic acid, 16:1n9, reached with all three columns, and in the content of Δ12 eicosadienoic acid, 20:2n6, observed only with column D. These differences are caused by an incomplete resolution of the relevant acids and impurities from sample matrix, which were not distinguished using the MOSAIC software. Total chromatogram of plasma PL analysis is shown in Fig. 1, the detail of both integrations for columns A1, A2, and C in Fig. 2. The CSW integration software is more flexible to distinguish unidentified component eluted between 16:1n9 and 16:1n7 acids.

Most important differences between columns were observed for column D, which differed from the other columns used in case of FA 14:0, 16:1n9, 20:0 and 20:2n6. An unidentified impurity with changing elution time was observed, which disturbed integration of 22:4n6 and 22:5n6. Results in Tables 1–5 for these acids are based on peak height measurement with CSW and manual correction for MOSAIC. Column D was thus disqualified from calibration measurements, as determination of these acids in plasma triacylglycerols and cholesteryl esters, where their content is much lower, would be impossible. On the other hand, resolution of unidentified impurity and FA 20:2n6 was possible only using column D as documented by different results reached with CSW and MOSAIC integration software.

Determination of this acid with columns A and C leads to its overestimation, as both compounds are not separated at all (Tables 1–5). Correction using peak height measurement (correction factors 0.739,

Table 5
Fatty acid content in plasma phospholipid: sample 5

Column	A1		A2		C		D	
software	CSW 1.7	MOSAIC	CSW 1.7	CSW 1.7	CSW 1.7	MOSAIC	CSW 1.7	MOSAIC
14:0	0.24±0.01	0.25±0.01	0.22±0.01	0.23±0.01	0.24±0.01	0.25±0.01	0.27±0.01	0.27±0.01
16:0	27.89±0.49	27.87±0.47	27.62±0.56	27.55±0.55	28.02±0.32	27.87±0.31	28.79±0.22	28.57±0.27
16:1n9	0.10±0.02	0.17±0.01****	0.09±0.01	0.16±0.01****	0.09±0.01	0.17±0.00****	0.09±0.01	0.19±0.00****
16:1n7	0.89±0.02	0.90±0.03	0.89±0.02	0.89±0.02	0.89±0.02	0.90±0.02	0.91±0.01	0.93±0.01
18:0	14.97±0.23	14.97±0.22	15.04±0.05	15.01±0.04	15.15±0.16	15.07±0.15	14.85±0.06	14.74±0.04
18:1n9	11.38±0.05	11.37±0.05	11.44±0.05	11.52±0.05	11.37±0.09	11.47±0.09	11.33±0.04	11.36±0.02
18:1n7	1.74±0.02	1.74±0.01	1.76±0.01	1.77±0.01	1.77±0.02	1.80±0.02	1.75±0.01	1.75±0.01
18:2n6	16.87±0.08	16.90±0.10	17.17±0.08	17.20±0.10	17.05±0.09	17.03±0.10	17.13±0.04	17.03±0.03
18:3n6	0.18±0.00	0.17±0.01	0.17±0.00	0.17±0.01	0.15±0.01	0.17±0.00	0.16±0.01	0.16±0.00
18:3n3	0.24±0.00	0.24±0.00	0.24±0.00	0.24±0.01	0.24±0.01	0.23±0.01	0.23±0.01	0.23±0.04
20:0	0.05±0.01[aaaa]	0.06±0.01	0.05±0.01[bbbb]	0.05±0.01	0.10±0.00[bbbb] [aaaa]	0.06±0.01*	0.07±0.00	0.07±0.01
20:1n9	0.18±0.01	0.17±0.01	0.18±0.01	0.18±0.01	0.18±0.00	0.19±0.02	0.17±0.01	0.17±0.01
20:2n6	0.97±0.02[aaaa]	0.96±0.02	0.95±0.02[bbbb]	0.93±0.02	0.95±0.01[cccc]	0.96±0.01	0.43±0.03[bbbb] [aaaa,cccc]	0.91±0.02
20:3n6	5.09±0.08	5.09±0.08	5.12±0.11	5.11±0.11	5.09±0.0 5	5.08±0.04	5.00±0.05	4.96±0.05
20:4n6	11.98±0.16	11.96±0.17	12.06±0.25	12.02±0.25	11.83±0.11	11.82±0.12	11.79±0.10	11.72±0.09
20:5n3	1.40±0.02	1.41±0.02	1.37±0.04	1.39±0.04	1.36±0.03	1.39±0.04	1.38±0.03	1.39±0.03
22:4n6	0.47±0.01	0.46±0.01	0.45±0.02	0.46±0.02	0.49±0.01	0.49±0.01	0.45±0.02	0.44±0.00
22:5n6	0.36±0.01	0.37±0.01	0.36±0.02	0.35±0.03	0.34±0.01	0.33±0.04	0.40±0.12	0.34±0.00
22:5n3	1.08±0.03	1.06±0.03	1.04±0.05	1.03±0.05	1.02±0.03	1.04±0.02	1.06±0.02	1.06±0.03
22:6n3	3.92±0.13	3.89±0.13	3.75±0.19	3.73±0.18	3.68±0.12	3.67±0.13	3.73±0.06	3.72±0.07
Σsatur	43.16±0.37	43.14±0.34	42.95±0.57	42.85±0.56	43.51±0.34	43.25±0.32	43.98±0.19	43.66±0.25
Σmono	14.29±0.08	14.35±0.08	14.35±0.07	14.52±0.08	14.29±0.11	14.54±0.13	14.25±0.04	14.39±0.03
Σn6	35.91±0.23	35.91±0.22	36.29±0.36	36.24±0.36	35.90±0.19	35.88±0.21	35.36±0.11	35.56±0.13
Σn3	6.64±0.17	6.60±0.17	6.40±0.07	6.39±0.14	6.30±0.18	6.33±0.18	6.41±0.27	6.38±0.27

Fatty acid content was measured using four different chromatographic systems (see text) and two types of integration software. Each sample was measured 10 times, typical injected volume was 1–2 μl. Conditions: injector, splitter 250 °C; detector, FID 270 °C; temperature programme, 150–240 °C 2°/min, carrier gas hydrogen, head pressure 70 kPa.
For explanation see Table 1.

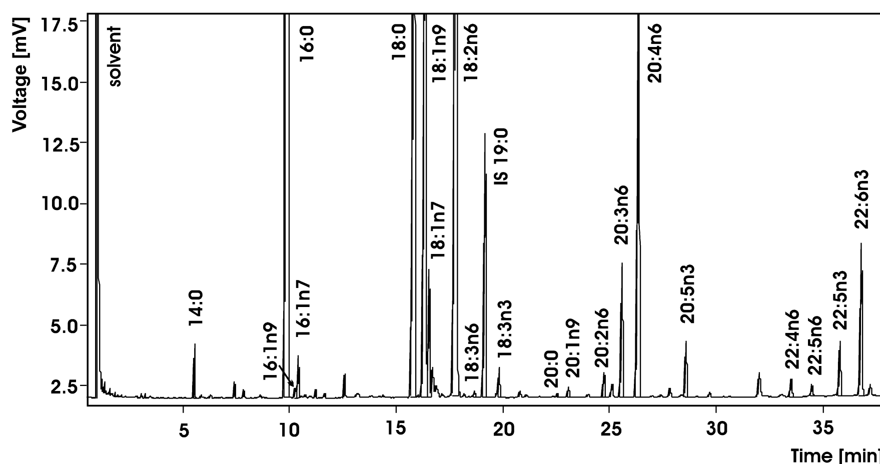

Fig. 1. Chromatographic analysis of plasma phospholipid sample. I.S., internal standard; solvent, isooctane; injected volume of the sample, 1 μl.

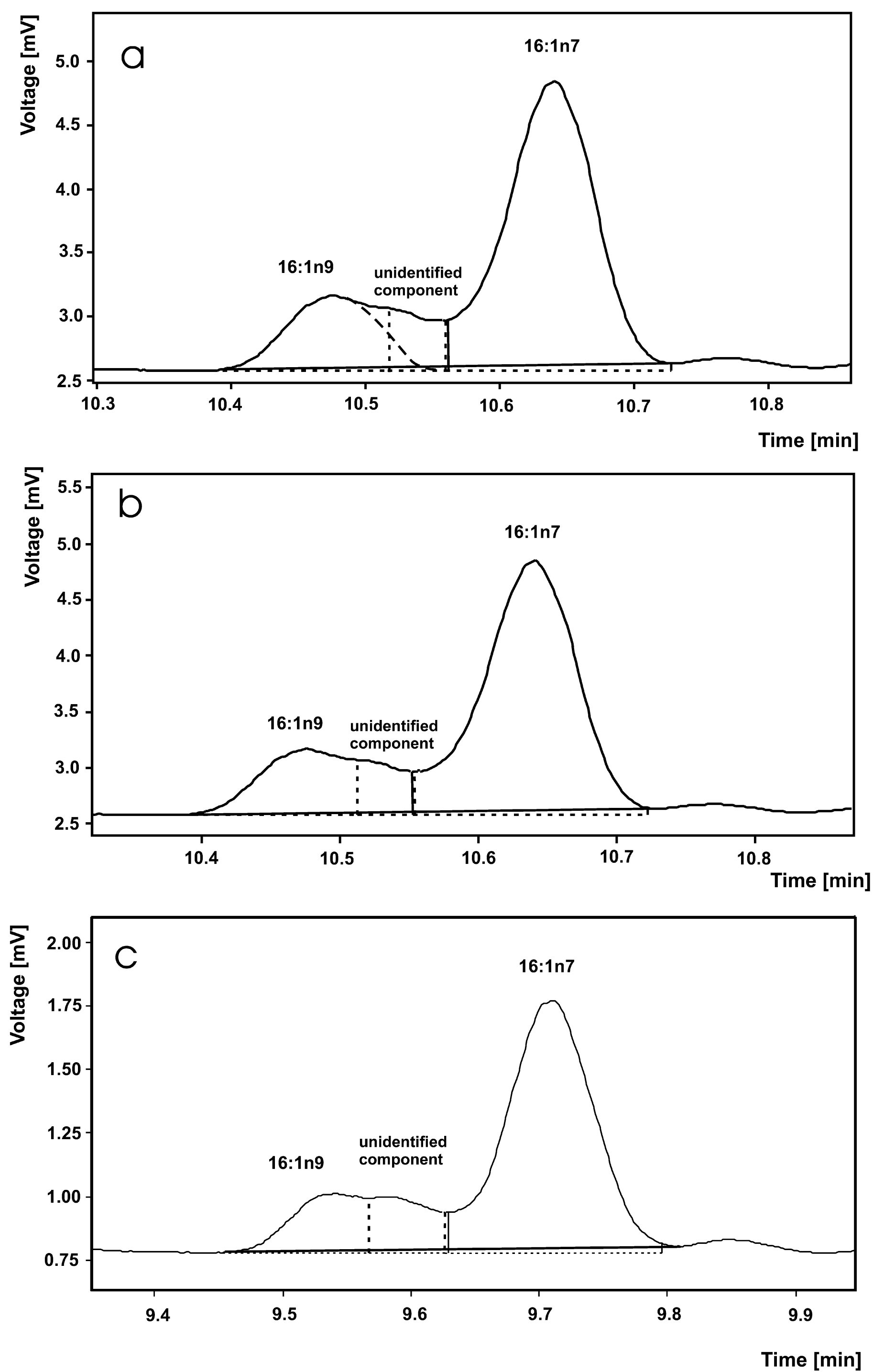

Fig. 2. (a) Detail of plasma phospholipid sample integration for column A1. Dashed lines, CSW software; solid lines, MOSAIC software. Theoretically resolved peak of 16:1n9 is indicated. (b) Detail of plasma phospholipid sample integration for column A2. Dashed lines, CSW software; solid lines, MOSAIC software. (c) Detail of plasma phospholipid sample integration for column C. Dashed lines, CSW software; solid lines, MOSAIC software.

Table 6
Calibration dependencies—undiluted standards ($n=7$)

	Column A	R^2	Column B	R^2	Column C	R^2
Dilution 0						
10:0	$y=0.988x+0.018$	0.9976	$y=0.973x-0.038$	0.9974	$y=1.003x-0.006$	0.9990
12:0	$y=1.006x-0.007$	0.9988	$y=0.978x-0.054$	0.9973	$y=1.028x-0.029$	0.9990
14:0	$y=1.013x-0.008$	0.9988	$y=0.983x-0.030$	0.9975	$y=1.037x-0.021$	0.9989
16:0	$y=1.024x+0.012$	0.9987	$y=1.001x+0.014$	0.9979	$y=1.028x-0.002$	0.9992
18:0	$y=1.026x-0.007$	0.9984	$y=1.021x+0.001$	0.9983	$y=1.034x-0.006$	0.9987
20:0	$y=1.035x-0.002$	0.9980	$y=1.044x-0.013$	0.9987	$y=1.026x-0.006$	0.9984
22:0	$y=1.033x-0.007$	0.9978	$y=1.052x-0.060$	0.9989	$y=1.013x-0.025$	0.9981
24:0	$y=1.036x-0.027$	0.9977	$y=1.038x-0.120$	0.9984	$y=1.006x-0.061$	0.9978

x, concentration ratio $c_i/c_{I.S.}$; y, peak area ratio $A_i/A_{I.S.}$; I.S., 19:0.

0.705, 0.861, 0.705, 0.746 for samples 1, 2, 3, 4, 5, respectively) led to values similar to those reached with column D.

Column C differed from the columns A1 and A2 in case of FA 20:0 (in all samples), 16:1n9 (in samples 1,2, and 3), 18:1n7 (in samples 1 and 2) and 22:6n3 (in sample 2). Column C is slightly more polar than column A with identical elution order of all components; however, slightly different resolution of closely eluted components as well as some unexpected impurities of sample matrix can influence the results. Determination of vaccenic acid (18:1n7) is influenced by incomplete resolution from preceding excess of oleic acid (18:1n9), which is in phospholipids 5–6-fold, the difference between the retention times being approximately 0.17–0.18 min, similarly as in cholesteryl esters. The influence could be higher in triacylglycerols, where the excess of oleic acid is 10–12-fold, and the difference between the retention times being 0.12–0.13 min. Another critical pair represent palmitoleic (16:1n7) and Δ7 hexadecenoic acids. The loss of separation ability for above-mentioned critical pairs, which cannot be improved by the change of analytical conditions, signals the end of column life.

Results reached with column A installed in two different instruments did not differ significantly, which confirms negligible contribution of optimally conditioned chromatographic system to overall procedure error.

Statistical evaluation of the calibration dependences (peak area ratio versus concentration ratio) for columns A, B and C measured with undiluted standard solutions is given in Table 6, that for columns B and C with diluted solutions is shown in Table 7. The results confirm linear dependence for the whole range of concentrations (three orders) for all three dilutions. Slopes of all dependences are close to unity and intercepts on the y-axis have only minimal value. The correlation coefficients confirm straight lines passing through origin. Very close results document that all instruments worked under optimal conditions.

Observed differences in content of minor FA are negligible in clinical studies where large groups of patients are compared; however, in small intervention studies (e.g., repeated measures design for clamp studies) or experimental studies with well-defined

Table 7
Calibration dependencies—diluted standards ($n=7$)

	Column B	R^2	Column C	R^2
Dilution 1				
10:0	$y=1.000x-0.075$	0.9991	$y=0.966x-0.054$	0.9970
12:0	$y=1.023x-0.096$	0.9989	$y=0.988x-0.080$	0.9982
14:0	$y=1.021x-0.081$	0.9988	$y=0.995x-0.076$	0.9986
16:0	$y=1.025x-0.032$	0.9987	$y=0.997x-0.045$	0.9986
18:0	$y=1.024x-0.021$	0.9986	$y=0.989x-0.051$	0.9989
20:0	$y=1.030x+0.003$	0.9985	$y=0.994x-0.039$	0.9990
22:0	$y=1.031x+0.004$	0.9985	$y=0.981x-0.037$	0.9991
24:0	$y=1.025x-0.016$	0.9984	$y=0.991x-0.073$	0.9990
Dilution 2				
10:0	$y=0.993x+0.028$	0.9987	$y=1.014x+0.031$	0.9980
12:0	$y=0.992x-0.007$	0.9987	$y=1.022x-0.039$	0.9979
14:0	$y=0.988x-0.010$	0.9984	$y=1.009x-0.050$	0.9984
16:0	$y=0.991x+0.014$	0.9982	$y=1.005x-0.030$	0.9987
18:0	$y=0.992x-0.011$	0.9977	$y=1.005x-0.029$	0.9987
20:0	$y=0.999x-0.015$	0.9972	$y=1.008x-0.005$	0.9987
22:0	$y=1.003x-0.037$	0.9977	$y=1.002x+0.028$	0.9982
24:0	$y=1.004x-0.052$	0.9976	$y=1.014x-0.021$	0.9988

x, concentration ratio $c_i/c_{I.S.}$; y, peak area ratio $A_i/A_{I.S.}$; I.S., 19:0.

animal strains or cell lines such differences could play an important role in statistical significance. Thus, use of one column installed in one chromatographic system is for those purposes necessary. Another improvement of the precision of results could be reached by running duplicate analyses.

The use of one I.S. eluted approximately in the middle of analysis seems to be sufficient for the determination of FA in biological samples which do not contain extremely volatile components. Comparison of enzymatic determination of individual lipid classes and chromatographic one based on FA determination with heptadecanoic acid as I.S. showed good general agreement between both methods [12]. The precision is maintained within wide range of concentrations, which enables the addition of I.S. with only approximate knowledge about the concentration of the relevant lipid class.

4. Conclusions

(1) Both integrating software did not differ significantly in most results; differences were observed only for minor components. The results indicate that CSW is more suitable software for integration of complicated chromatograms.
(2) Differences between columns were observed mostly for minor FA and episodically for vaccenic acid.
(3) Linear calibration dependences measured with standard mixture of saturated fatty acids confirmed theoretical relations when analyses are run under optimised conditions.
(4) Use of one column installed in one instrument is advisable in small intervention or experimental metabolic studies.

Acknowledgements

The study was supported by the research grant of International Grant Agency of Charles University Prague No. 72/1999 and by the research project J 13/98 1111 0000 2-1 from Ministry of Education, Czech Republic.

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