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Article

A Chemometric Model Applied to Fatty Acid Determination in Blood

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Mudanças no perfil de ácidos graxos têm sido associadas a vários processos patofisiológicos. Dados obtidos por cromatografia gasosa-espectrometria de massas (GC-MS) usando monitoramento seletivo de íons foram empregados para revelarem, após análise por análise de componentes principais (PCA), um conjunto relevante de íons para quantificação e caracterização de ácidos graxos. Este protocolo foi aplicado com sucesso na análise de ácidos graxos presentes em diversas frações lipídicas de sangue, permitindo a quantificação de diversos ácidos graxos e revelando seus números de insaturações. Adicionalmente, a presença de contaminantes, artefatos e co-eluições no cromatograma também foram reveladas sem análises adicionais. Por fim, ácidos graxos presentes em triacilglicerídeos, fosfolipídios e ésteres de colesterol presentes em plasma e membrana de eritrócitos foram determinados com exatidão adequada, repetibilidade e baixos limites de detecção e de quantificação.

Changes in fatty acid profiles have been associated with several pathophysiological processes. Gas chromatography-mass spectrometry (GC-MS) data monitoring of selected ions was used with principal components analysis (PCA), revealing a set of relevant ions for quantification and characterization of fatty acids. This protocol was successfully applied to the analyses of fatty acids in different human blood lipids, allowing the quantification of several fatty acids and revealing their unsaturation numbers. Moreover the presences of contaminants, artifacts and co-eluitions in the chromatogram were also revealed without any additional analyses. Thus, fatty acid constituents of triglycerides, phospholipids and esters of cholesterol present in plasma and erythrocyte membranes were accurately determined, with repeatability, low limits of determination and of quantification.

Keywords: fatty acid methyl esters, principal component analysis, selected ion monitoring

Introduction

Fatty acids (FA) are lipid building blocks that can be saturated, monounsaturated or polyunsaturated, depending on the presence of double bonds.[1,2] Fatty acids present in human blood have typical compositions which have been used to investigate fat intakes and pathological,[3-5] dietary and/or drug influences.[6-9] Consequently, improving analytical tools to access the blood FA profile is valuable in clinical trials and lipid research.

Determinations of FA in biological samples generally involve multiple-step methods: (*i*) lipid extraction procedures, based on Folch or Bligh and Dyer methodologies;[10] (*ii*) separation of individual lipids by preparative thin-layer chromatography or solid phase extraction; (*iii*) derivatization of FA to fatty acid methyl ester (FAME) and (*iv*) FAME analysis by gas chromatography (GC).[1]

GC-FID FAME analysis is robust, displaying high detectivity and reproducibility.[11] However, GC-FID fails in unequivocal identification of the analyte, consequently the hyphenated GC-MS is a better analytical platform. The full scan mode is not recommended in quantitative analyses due low detectivity and selectivity. These limitations are overcome by the selective ion monitoring (SIM) mode that increases detectivity by monitoring a few characteristic ions, at the cost of a significant loss of qualitative information.[12,13] Addressing this issue by choosing a certain ensemble of characteristic ions could lead to a fast and reliable method. Employing principal components analysis

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(PCA)[14] to recognize representative mass fragment ions bearing all desired quanti and qualitative information would serve this purpose. Consequently, PCA was employed to screen mass spectra data of FAME from human blood, selecting ions of *m/z* 74, 79, 81 and 87 for GC-MS/SIM analyses, providing fast and accurate FA quantification and unsaturation numbers.

Experimental

Subjects

Five healthy women were recruited for this study. The blood samples were collected at the Laboratory of Investigation in Metabolism and Diabetes at the Universidade Estadual de Campinas (LIMED-UNICAMP). All volunteers gave formal consent and the study was realized in agreement with the Research Ethics Committee of the Medical Sciences, approved in 15/10/2009, number 836/2009.

Chemical standards

A Supelco 37-component FAME mix was acquired from Sigma-Aldrich (Brazil), nonadecanoic acid (19:0) and oleic (18:1), linoleic and (18:2n6) α-linolenic (18:2n3) methyl esters were purchased from ACROS.

Samples

Blood samples were collected and processed as described by Risé *et al.*[9] The lipids were extracted from 400 µL of sample applying a modified Folch's methodology.[10,15] Phospholipids (PL), triacylglycerides (TG) and cholesteryl esters (CE) from plasma were separated using preparative thin-layer chromatography and the respective FAME were prepared as described by Croset *et al.*[16]

GC-MS analysis

FAME were analyzed by GC-MS (Agilent, 6890 series and Hewlett Packard, 5973 mass selective detector). 1.0 µL of sample was injected in the *splitless* mode at 250 °C. Separations were achieved with a DB-5 column (30 m, 0.25 mm, 0.25 µm, Agilent) with helium as carrier gas at 1.2 mL min^{-1}. The GC oven temperature program started at 50 °C (hold time 1 min), heated to 170 °C at 50 °C min^{-1}, then to 260 °C at 3 °C min^{-1} and finally to 290 °C at 50 °C min^{-1}, the final temperature was maintained for 5 min. The ion source temperature was set at 230 °C. Mass spectra were obtained in full scan mode (*m/z* 40-440) or SIM mode, monitoring four fragment ions (of *m/z* 74, 79, 81 and 87). The spectra were recorded at a rate of five scans per second with ionization energy of 70 eV after a solvent delay of 4.0 min. FAMEs were identified by mass spectra using the software MSD ChemStation, G1701EA. Additionally, unsaturated FAME identifications were confirmed by fractional chain lengths as described by Härtig.[17] FAME standards were used to confirm the identifications and obtain calibration curves.

Multivariate and statistical analysis

Pirouette® (v3.11, Infometrix) was used to process the 96 spectra from 32 different FAME in full scan and SIM mode. In full scan mass spectra the ions between *m/z* 40 and 200 were used as variables after mean data centering. PCA was used as an explorative tool to investigate similarities between FAME and the loadings were examined in order to find ions related to clusters observed for samples in the scores plot. The measures were expressed as mean ± standard deviation (SD) and significant differences between measurements were detected by performing a two-tailed *t*-test with Excel®. The level of statistical significance was set as $p < 0.05$.

Results and Discussions

Selection of ions for FAME analysis by GC-MS/SIM

The PCA of 96 mass spectra obtained by electron ionization in full scan mode for 32 different FAME reduced the data matrix from 161 (ions with *m/z* between 40 and 200) to three PC describing 86.65% of the original data information, for mean-centered data. The scores plots the first two PC (81.45%) revealed five FAME classes, according to the alkyl chain double bond number (Figure 1a). The PCA analyses were conducted only employing mass spectra information.

The loading plots (Figure 1b) were used to investigate which ions (variables) were responsible for FAME class (samples) clusterings in score plot (Figure 1a). The highlighted ions in Figure 1b are the most representative in FAME SIM analyses. Therefore the essential ion selection based on modeling power was conducted by directing the PCA model for the best selectivity, increasing the signal to noise-ratio. Only four ions (nominally *m/z* 74, 79, 81 and 87) were necessary to describe all FAME.

Moreover the ions selection protocols do not require any previous identification of the investigated substances, as the objective is to reach good identification based on statistical parameters (PCA results) which can be confirmed by fragmentation patterns (Figure 2).

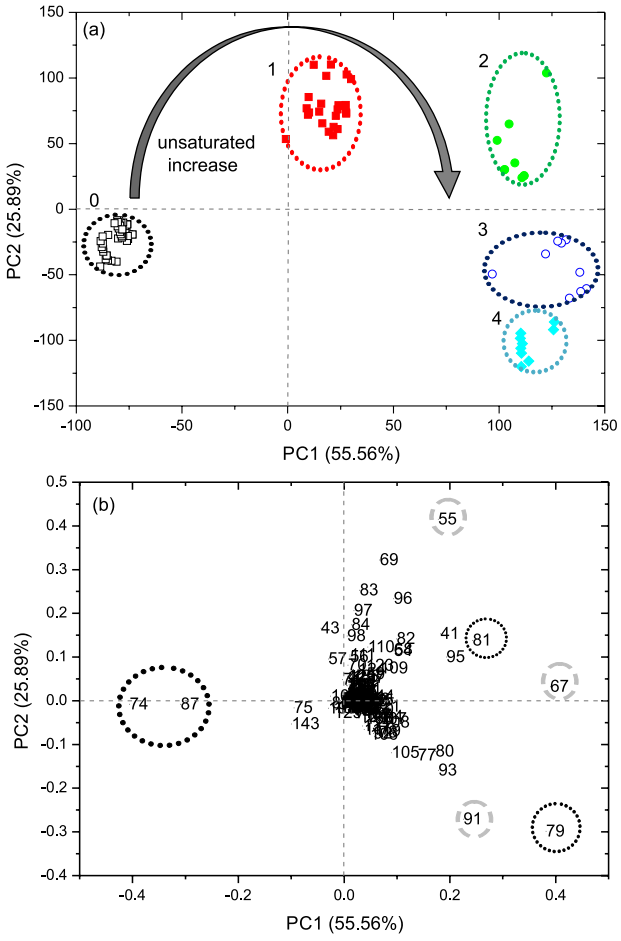

Figure 1. (a) Scores plot reveals the cluster of several FAME into five classes, according to the alkyl chain double bond number and (b) loading plot highlights the most important ions of the mass spectra. Legend: FAME class: 0, saturated (□); 1, one double bond (■); 2, two double bonds (●); 3, three double bonds (○); ≥ 4, four, five or six double bonds (◆).

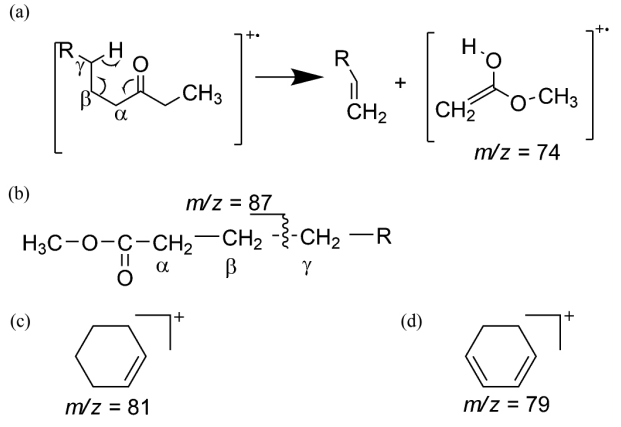

Figure 2. Main fragmentation pathways for the PCA selected ions

These results were confirmed by acquiring a new set of mass spectra in the SIM mode (*m/z* 74, 79, 81 and 87) for all FAME and processing a new PCA analysis (Figure S1 in the Supplementary Information (SI) section).

FAME double bond number by mass spectra in the SIM mode

The relative abundances of the ions of *m/z* 74, 79, 81 and 87 in the SIM spectra did not change significantly for FAME of equal double bond numbers. Additionally, FAME with four, five and six double bonds presented similar abundances for this ensemble of ions and were gathered in a unique class (≥ 4). Table 1 shows the relative abundances of these ions in the mass spectra of five FAME classes as defined by PCA, for the individual FAME spectra (see Table S1 in the SI section).

Table 1. Relative abundance of the ions with *m/z* 74, 79, 81 and 87 in mass spectra obtained by SIM for the FAME class

FAME class[b]	Relative abundance of fragments in MS-SIM, mean ± SD[a] / %			
	m/z 74	*m/z* 79	*m/z* 81	*m/z* 87
0 (n[c] = 13)	100	0.96 ± 0.13	3.40 ± 1.00	72.26 ± 5.49
1 (n = 8)	100	17.06 ± 2.97	50.89 ± 7.27	72.96 ± 1.75
2 (n = 3)	17.98 ± 5.15	42.15 ± 2.03	100	10.93 ± 1.69
3 (n = 3)	11.65 ± 1.16	100	64.51 ± 6.75	19.86 ± 0.94
≥ 4 (n= 3)	13.16 ± 2.61	100	29.20 ± 8.51	5.64 ± 2.77

[a]Mean ± SD of eight injections; [b]FAME class: 0, saturated; 1, one double bond; 2, two double bonds; 3, three double bonds; ≥ 4, four, five or six double bonds; [c]number of FAME in the class.

The relative abundances of the ion ensembles can be used to confirm the FAME identification based on the retention time of FA eluting closely together, which usually causes identification problems, mainly when these occur in such small amounts that full scan spectra acquisitions is not adequate (Figure 3).

Selectivity evaluation

SIM chromatograms using the four selected ions (*m/z* 74, 79, 81 and 87) were useful for the detection of contaminations, artifacts and co-elution, without additionally analyses. The relative abundances of the fragment ions and PCA were was successfully applied to distinguish an alcohol (decanol), an aldehyde (dodecanal) and a hydrocarbon (pentacosane) from FAME in the SIM-chromatogram (Figure 4).

Additionally, the method was also useful to detected FAME co-elutions. For example, the SIM mass spectrum of co-eluting compounds (20:1 plus 20:3n3) was significantly different from that obtained for pure standards, as indicated by the scores plot. The individual relative abundance of the SIM spectra is show in Table S2 in the SI section.

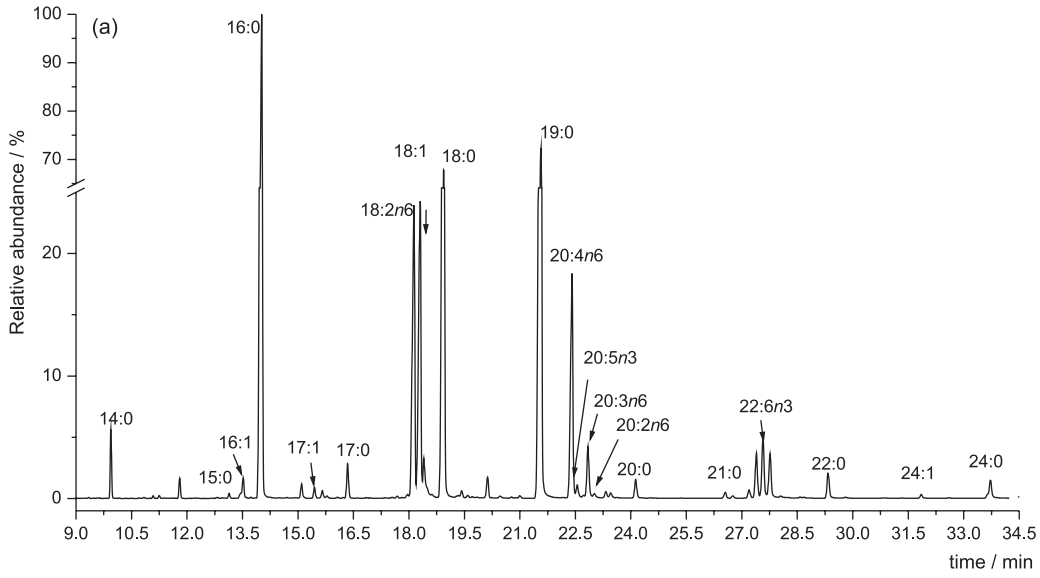

Figure 3. Representative FAME ion chromatogram showing FA present in red blood cell membranes.

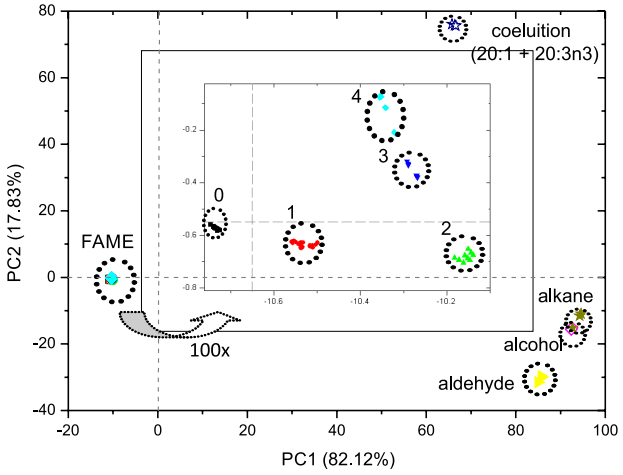

Figure 4. PCA scores plot based on mass spectra (SIM mode for the selected ions) confirm the clustering of FAME into five classes and revel other contaminants or artifacts eventually present in the sample. Legend: FAME class: 0, saturated (■); 1, one double bond (●); 2, two double bonds (▲); 3, three double bonds (▼); ≥ 4, four, five or six double bonds (◆).

After data acquisition the chromatogram peak areas are used in FAME quantification, while the SIM mass spectra are used to confirm the identification and reveal contaminations, artifacts or/and co-elution by PCA analysis.

FAME quantification

Quantitative analysis usually is conducted by single ion monitoring with additional ions used to confirm the identity of a substance.[13,18] Alternatively, we suggest the use of all four ions selected to determine the peak area with a large gain in detectivity, although with a small loss of repeatability, although it is still satisfactory (Table 2).

Calibration curves were obtained in quintuplicate at six different concentrations relative to nonadecanoic acid (19:0; 10.832 μg mL^{-1}) with FAME standards by GC-MS/SIM (*m/z* 74, 79, 81 and 89) analyses. Response factor (RF), correlation coefficient (R), linear range and limits of detection (LOD) and quantification (LOQ) were obtained from calibration curves.

RF is used to allow the FAME quantification with a wide range of chromatographic conditions.[19] Notwithstanding the use of a unique selected ions ensemble, the RF values depend on alkyl chain unsaturation and carbon numbers. RF are inversely proportional to FAME carbon numbers, probably due to a volatility decrease with carbon chain increase.[19]

LOD and LOQ were estimated based on parameters of the analytical curves taking into account the confidence interval of the regression. The limits in this case were defined as the substance minimum detectable concentration which can be quantified with 95% confidence.[20] The calculations were carried out using an available spreadsheet validation.[21]

The values of LOD and LOQ for several FAME are given in Table 2. The LOD reported here are slightly higher than those reported by Dodds *et al.*[12] in the FAME analysis by GC-MS/SIM, however they used a variable ensemble of three selected ions, which are also common to other compounds, such as alkenes and aromatics, decreasing method selectivity and not allowing the determination of the unsaturation number or selectivity check point.

The correlation coefficient (R) values in Table 2 suggest that the calibration curves are linear. Additionally, the linearity test performed by comparing the residuals of

Table 2. Response factor (RF), correlation coefficient (R), linear range, LOD_C, LOQ_C and repeatability response of individual FAME

FAME	RF[a]	R	Linear range / (μg mL^{-1})	LOD_c / (μg mL^{-1})	LOQ_c / (μg mL^{-1})	Mean AR	RSD / %
14:0	0.990	0.9881	13.146-400.0	8.835	13.146	5.31[b]	0.57
16:1	0.6229	0.9881	6.578-200.0	4.425	6.578	0.84[c]	0.94
16:0	1.063	0.9994	4.495-600.0	3.207	4.495	7.93[d]	0.34
17:0	1.148	0.9990	1.922-200.0	1.294	1.922	2.56[c]	1.13
18:3n6	0.520	0.9988	2.063-200.0	1.389	2.063	1.04[c]	0.69
18:2n6	0.531	0.9923	5.268-200.0	3.543	5.268	0.96[c]	1.75
18:1	0.607	0.9839	7.667-200.0	5.156	7.667	0.78[c]	2.31
18:0	0.996	0.9985	4.690-400.0	3.157	4.690	4.94[b]	0.50
20:4n6	0.676	0.969	10.763-200.0	7.235	10.763	0.155[c]	10.30
20:5n3	0.546	0.9648	11.504-200.0	7.737	11.504	0.164[c]	4.56
20:3n6	0.381	0.9948	4.341-200.0	2.920	4.341	0.837[c]	3.06
20:2n6	0.572	0.9891	6.305-200.0	4.240	6.305	1.017[c]	1.71
20:0	1.033	0.9980	5.342-400.0	3.596	5.342	4.836[b]	0.63
21:0	1.007	0.9906	5.845-200.0	3.930	5.845	2.285[c]	1.35
22:6n3	0.407	0.9879	6.629-200.0	4.458	6.629	0.639[c]	9.46
22:2n6	0.642	0.9828	7.936-200.0	5.338	7.936	0.623[c]	3.72
22:1n9	0.645	0.9922	5.309-200.0	3.571	5.309	0.958[c]	6.87
22:0	1.055	0.9969	6.725-400.0	4.537	6.725	4.636[b]	1.43
23:0	1.041	0.9937	4.753-200.0	3.198	4.753	2.135[c]	1.06
24:1	0.673	0.9672	11.086-200.0	7.455	11.086	0.725[c]	2.23
24:0	0.955	0.9933	9.819-400.0	6.607	9.819	4.201[b]	3.09

[a]Response factor relative to 19:0 (10.832 μg·mL^{-1}), used as internal standard; [b]mean of quintuplicate injection for a concentration of 44.44 μg·mL^{-1}; [c]mean of quintuplicate injections for a concentration of 22.2 μg·mL^{-1}; [d]mean of quintuplicate injections for a concentration of 66.67 μg·mL^{-1}.

the linear and quadratic regression using an *F*-test. The calculations were also carried out using a spreadsheet validation.[21]

The mean area ratio (AR) of each compound in relation to the internal standard was calculated based on the analyses of FAME standards. Method variability was computed and expressed as relative standard deviation (RSD). These results are provided in Table 2 and represent the repeatability of the FAME analysis.

Application of the methodology: blood lipid fatty acids composition

FA from red blood cell membrane (RBCM) and plasma PL, TG and EC were methylated and analyzed by GC-MS/SIM (Table 3, Figure S2 in the SI section). The relative abundances of the selected ions in the SIM mass spectra do not change significantly (Table 1 and Table S1 compared to Table S3, SI section), and were used to confirm FAME identification and check the analysis selectivity by PCA.

RBCM and plasma FA profiles are significantly different (Table 3). Sixteen FA were quantified in RBCM, revealing higher levels of arachidonic (20:4n6), palmitic (16:0), stearic (18:0) and oleic acids (18:1), and lower levels of long chain fatty acids (> 20C).

In plasma PL, 19 FA were quantified, including 16:0, lauric (18:2n6), 20:4n6 and 18:0 acids, which are main PL constituents. About 60% of FA present in PL are unsaturated. The FA distribution is important and has a significant influence on metabolic activity; e.g., arachidonic acid is only converted into eicosanoids, after PL phospholipase hydrolysis.[22] Plasma TG alterations are more susceptible to dietary fat intake oscillations preceding the analysis, which is responsible for their larger RSD values than plasma PL and CE. We quantified 15 FA in plasma TG, including 16:0, 18:2n6 and 18:1, which are major components. TG has lower polyunsaturated fatty acid levels compared to other lipids and FA with more than 20 carbon atoms were not detected. Esterification of cholesterol depends on lecithin:cholesterol acyltransferase catalysis to transfer the FA from the lecithin (phosphatidylcholine) *sn*-2 position to free cholesterol.[23] Consequently CE has a prevalence of unsaturated FA at position *sn*-2 of the glycero-PL, almost 75% FA from CE have at least one unsaturation.

Table 3. Esterified Fatty acid compositions of RBCM and from plasma phospholipids, triglycerides and cholesteryl esters

FA	Total fatty acid[a] / % by weight							
	RBCM		Plasma					
			PL fraction		TG fraction		EC fraction	
	Mean ± SD	RDS / %	Mean ± SD	RDS / %	Mean ± SD	RDS / %	Mean ± SD	RDS / %
14:0	0.23^{b,c,d} ± 0.01	4.35	0.30^{c,d} ± 0.06	19.70	1.38 ± 0.32	0.32	0.95 ± 0.31	32.62
15:0	n.d.		0.28^{c} ± 0.08	28.15	0.50^{d} ± 0.11	0.11	0.34 ± 0.03	8.80
16:1	0.20^{b,c,d} ± 0.06	30.01	0.40^{c,d} ± 0.06	15.16	3.15^{d} ± 0.60	0.60	4.85 ± 0.38	7.83
16:0	25.70 ± 2.46	9.57	25.28^{d} ± 1.31	5.18	24.83^{d} ± 1.51	1.51	15.86 ± 0.90	5.67
17:1	n.d.		0.15 ± 0.05	32.84	0.22 ± 0.06	0.06	0.19 ± 0.02	10.76
17:0	0.57^{d} ± 0.07	12.29	0.45^{d} ± 0.13	29.11	0.51^{d} ± 0.17	0.17	0.17 ± 0.03	18.15
18:3n6	n.d.		0.10^{d} ± 0.03	29.55	0.21^{d} ± 0.10	0.10	0.42 ± 0.13	30.70
18:2n6	7.51^{b,c,d} ± 0.95	12.65	18.13^{c,d} ± 0.72	3.97	21.05^{d} ± 1.19	1.19	39.83 ± 3.10	7.78
18:1	13.40^{b,c,d} ± 0.71	5.30	8.77^{c,d} ± 0.88	10.03	34.53^{d} ± 2.38	2.38	20.70 ± 1.31	6.33
18:0	22.49^{b,c,d} ± 2.39	10.63	15.00^{c,d} ± 0.97	6.47	8.81^{d} ± 1.41	1.41	2.34 ± 0.25	10.66
20:4n6	20.77^{b,c,d} ± 2.48	11.94	16.46^{c,d} ± 1.02	6.20	3.71^{d} ± 0.26	0.26	11.86 ± 0.30	2.53
20:5n3	0.28^{b} ± 0.03	10.72	0.74^{c,d} ± 0.14	18.89	0.18^{d} ± 0.12	0.12	0.35 ± 0.09	25.63
20:3n6	1.38^{b,c} ± 0.37	26.82	5.72^{c,d} ± 0.39	6.82	0.47^{d} ± 0.19	0.19	1.47 ± 0.45	30.68
20:2n6	0.25 ± 0.08	32.01	0.26 ± 0.08	30.31	0.29 ± 0.21	0.21	0.40 ± 0.26	64.55
20:0	0.23^{d} ± 0.07	30.45	0.28^{d} ± 0.04	14.07	0.18 ± 0.12	0.12	0.08 ± 0.03	36.31
22:6n3	2.83^{b,d} ± 0.43	15.20		17.06	n.d.		0.19 ± 0.10	53.79
22:0	0.50 ± 0.08	16.01	0.55 ± 0.07	12.77	n.d.		n.d.	
24:1	1.31^{b} ± 0.35	26.73	0.74 ± 0.19	25.64	n.d.		n.d.	
24:0	2.35^{b} ± 0.83	35.33	0.41 ± 0.16	39.40	n.d.		n.d.	
ΣSFA	52.07^{b,c,d} ± 4.77	9.16	42.55^{c,d} ± 2.11	4.96	36.19^{d} ± 1.93	1.93	19.75 ± 0.79	4.00
ΣMUFA	14.91^{b,c,d} ± 0.73	4.89	10.06^{c,d} ± 0.96	9.54	37.90^{d} ± 1.88	1.88	25.74 ± 1.56	6.06
ΣPUFA	33.02^{b,c,d} ± 4.16	12.60	47.39^{c,d} ± 1.27	2.68	25.90^{d} ± 0.97	0.97	54.51 ± 2.61	4.79

[a]Values are mean ± SD (n = 5, analyzed in duplicate); [b]$p < 0.05$ compared with plasma PL; [c]$p < 0.05$ compared with plasma TG; [d]$p < 0.05$ compared with plasma CE; n.d.: not detected.

Conclusion

PCA based on full mass spectra was a useful tool to select important ions for the GC-MS/SIM analysis, requiring no specific mass fragmentation knowledge. The ensemble of the PCA-selected ions allows quantification of several FA present in different lipids. PCA based on MS-SIM mode (*m/z* 74, 79, 81 and 87) was employed to determine double bonds and contaminants in fatty acid analyses. The chemometrics approach to select the best ion ensemble for GC-MS/SIM quali and quantitative analysis is novel to the literature and can be applied to other classes of compounds. We report the first application of this procedure for human blood FA analyses. The results show that fatty acids were accurately determined, with repeatability, low LOD and LOQ.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Supplementary Information

A Chemometric Model Applied to Fatty Acid Determination in Blood

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Table S1. Retention time and relative abundance of the ions with *m/z* 74, 79, 81 and 87 in mass spectra obtained by SIM for individual FAME

FAME	t_R ± SD[b]	Relative abundance of fragments in MS-SIM, mean ± SD[a] / %			
		m/z 74	*m/z* 79	*m/z* 81	*m/z* 87
10:0 [c]	5.563 ± 0.00	100	0.63 ± 0.05	1.45 ± 0.28	54.64 ± 1.17
11:0 [c]	7.377 ± 0.05	100	0.61 ± 0.28	1.80 ± 0.95	58.21 ± 0.71
12:0	8.458 ± 0.08	100	0.77 ± 0.05	1.52 ± 0.22	62.27 ± 1.15
13:0	8.690 ± 0.08	100	0.89 ± 0.07	3.19 ± 3.66	64.44 ± 0.80
14:1	9.949 ± 0.04	100	15.34 ± 0.69	42.06 ± 10.04	72.43 ± 0.75
14:0	10.122 ± 0.04	100	0.91 ± 0.04	2.46 ± 1.24	67.31 ± 1.14
15:1	11.783 ± 0.03	100	14.89 ± 0.69	41.78 ± 13.74	73.16 ± 0.65
15:0	11.993 ± 0.03	100	0.96 ± 0.07	3.52 ± 3.48	68.53 ± 0.57
16:1	13.718 ± 0.03	100	17.48 ± 0.80	50.38 ± 6.2	73.31 ± 0.74
16:0	14.156 ± 0.02	100	0.95 ± 0.02	2.58 ± 0.48	70.71 ± 1.22
17:1	16.068 ± 0.03	100	16.74 ± 0.71	46.54 ± 15.21	73.66 ± 0.82
17:0	16.552 ± 0.02	100	1.02 ± 0.05	5.01 ± 6.10	71.30 ± 0.55
18:3n6	17.908 ± 0.02	11.72 ± 0.19	100	58.20 ± 1.49	18.78 ± 0.80
18:2n6	18.298 ± 0.02	12.80 ± 3.17	43.85 ± 10.91	100	9.27 ± 2.29
18:3n3 [d]	18.573 ± 0.02	10.37 ± 1.48	100	63.85 ± 1.22	20.38 ± 1.12
18:1 [d]	18.573 ± 0.02	100	23.07 ± 1.79	59.81 ± 15.22	75.95 ± 0.71
18:0	18.603 ± 0.02	100	1.04 ± 0.03	3.06 ± 0.49	72.83 ± 0.87
19:0 [e]	20.896 ± 0.02	100	1.02 ± 0.03	3.20 ± 0.38	74.38 ± 0.69
20:4n6	22.525 ± 0.02	16.13 ± 0.60	100	37.17 ± 2.42	8.20 ± 0.26
20:5n3	22.695 ± 0.02	12.13 ± 0.49	100	30.18 ± 13.25	6.04 ± 0.17
20:3n6	22.769 ± 0.02	12.65 ± 0.48	100	71.57 ± 0.76	20.43 ± 0.54
20:2n6	23.071 ± 0.02	18.02 ± 1.00	42.70 ± 1.36	100	10.87 ± 0.29
20:1	23.507 ± 0.13	100	18.06 ± 0.38	60.26 ± 0.83	72.86 ± 1.79
20:0	24.377 ± 0.02	100	1.09 ± 0.02	3.08 ± 0.64	75.21 ± 0.85
21:0	27.014 ± 0.02	100	0.77 ± 0.53	4.00 ± 1.08	76.15 ± 0.45
22:6n3	27.626 ± 0.00	1.22 ± 0.65	100	20.24 ± 0.32	2.69 ± 2.11
22:2n6	28.828 ± 0.00	23.11 ± 1.53	39.89 ± 1.22	100	12.65 ± 1.11
22:1	28.924 ± 0.04	100	17.83 ± 0.37	55.60 ± 13.53	72.78 ± 1.15
22:0	29.614 ± 0.03	100	1.10 ± 0.02	3.55 ± 0.80	77.64 ± 0.90
23:0	32.156 ± 0.04	100	0.79 ± 0.54	5.24 ± 2.99	78.61 ± 0.52
24:1	32.842 ± 0.06	100	13.04 ± 0.39	50.61 ± 5.89	69.57 ± 1.77
24:0	33.756 ± 0.03	100	1.15 ± 0.04	3.80 ± 1.53	80.05 ± 0.93

[a]Mean ± SD of seven injections; [b]chromatogram obtained as described in material and methods; [c]$p < 0.05$ compared with relative abundance of 0 class (satureted); [d]available as individual standard; [e]internal standard; t_R, retention time.

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Table S2. Relative abundance of the ions with *m/z* 74, 79, 81 and 87 in mass spectra obtained by SIM for contaminations, artifacts and co-eluition

	Relative abundance of fragments in MS-SIM, mean ± SD[a] / %			
	m/z 74	*m/z* 79	*m/z* 81	*m/z* 87
Decanol[b]	3.45[e,f,g,h,i] ± 0.19	28.69[e,f,g,h,i] ± 0.35	100[e,f,h,i]	7.37[e,f,h] ± 0.94
Dodecanal	0.33[e,f,g,h,i] ± 0.15	18.83[e,g,h,i] ± 1.00	100[e,f,h,i]	1.53[e,f,g,h] ± 0.21
Pentacosane	0.51[e,f,g,h,i] ± 0.24	32.46[e,f,g,h,i] ± 2.56	100[e,f,h,i]	6.47[e,f,h] ± 0.45
20:1 + 20:3n3 [c]	15.59[e,f,h,I] ± 0.31	100[e,f,g]	38.45[e,f,h,i] ± 0.46	8.02[e,f,h] ± 0.14

[a]Mean ± SD of triplicate analyses; [b]chromatogram obtained as described in material and methods; [c]co-eluted FAME; [e]$p < 0.05$ compared with 0 class; [f]$p < 0.05$ compared with 1 class; [g]$p < 0.05$ compared with 2 class; [h]$p < 0.05$ compared with 3 class; [i]$p < 0.05$ compared with ≥ 4 class.

Table S3. Relative abundance of the ions with *m/z* 74, 79, 81 and 87 in mass spectra obtained by SIM from FA present in blood lipids

FAME	Relative abundance of fragments in MS-SIM, mean ± SD[a] / %			
	m/z 74	*m/z* 79	*m/z* 81	*m/z* 87
14:0	100	0.77 ± 0.64	1.82 ± 0.84	65.76 ± 2.52
15:0	100	0.00	1.43 ± 1.79	73.94 ± 4.34
16:1	100	14.15 ± 2.99	52.53 ± 6.77	72.89 ± 6.34
16:0	100	0.83 ± 0.37	2.13 ± 0.94	74.59 ± 2.14
17:1	100	15.42 ± 7.79	54.58 ± 13.73	73.88 ± 8.48
17:0	100	0.32 ± 0.54	2.40 ± 1.15	70.89 ± 2.17
18:3n6	8.98 ± 4.55	100	55.59 ± 5.70	19.86 ± 1.60
18:2n6	11.32 ± 3.63	44.95 ± 1.18	100	8.95 ± 1.62
18:1	100	23.22 ± 2.61	58.72 ± 8.16	73.68 ± 8.42
18:0	100	0.65 ± 0.44	1.87 ± 1.31	74.67 ± 3.76
19:0 [b]	100	0.97 ± 0.08	2.99 ± 0.27	77.53 ± 2.85
20:4n6	15.02 ± 0.92	100	35.79 ± 2.76	7.69 ± 0.31
20:5n3	12.43 ± 0.68	100	31.46 ± 2.66	7.23 ± 2.12
20:3n6	11.34 ± 0.73	100	71.50 ± 6.41	19.97 ± 1.16
20:2n6	17.34 ± 1.43	44.67 ± 6.17	100	9.35 ± 2.76
20:0	100	1.38 ± 1.61	2.60 ± 1.89	77.51 ± 4.38
22:6n3	1.64 ± 1.36	100.00 ± 0.00	20.82 ± 3.03	1.88 ± 0.92
22:0	100	0.72 ± 0.74	4.05 ± 2.20	77.29 ± 1.66
24:1	100	10.59 ± 4.08	46.51 ± 14.15	65.27 ± 3.93
24:0	100	1.56 ± 3.42	3.36 ± 1.66	79.45 ± 4.54

[a]Values are mean ± SD (n = 8); [b]internal standard.

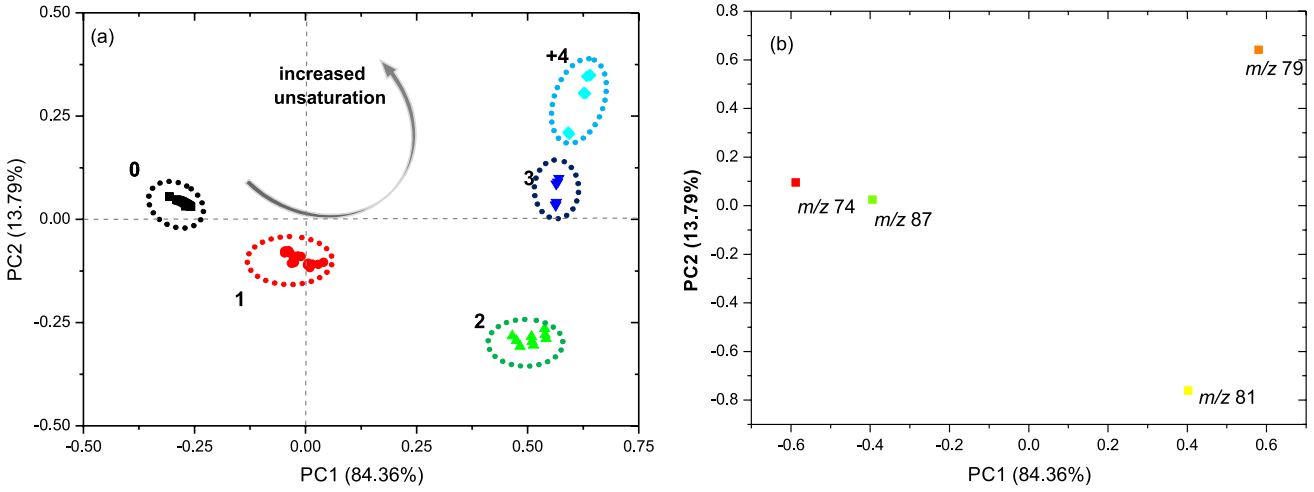

Figure S1. (a) Scores plot from PCA based on mass spectra in SIM mode (*m/z* 74, 79, 81 and 87), confirming the cluster of several FAME in five classes, according to the alkyl chain double bond number and (b) Loading plot highlighting the most important ions of the mass spectra. Legend: FAME class: 0, saturated (■); 1, one double bond (●); 2, two double bonds (▲); 3, three double bonds (▼); ≥ 4, four, five or six double bonds (◆).

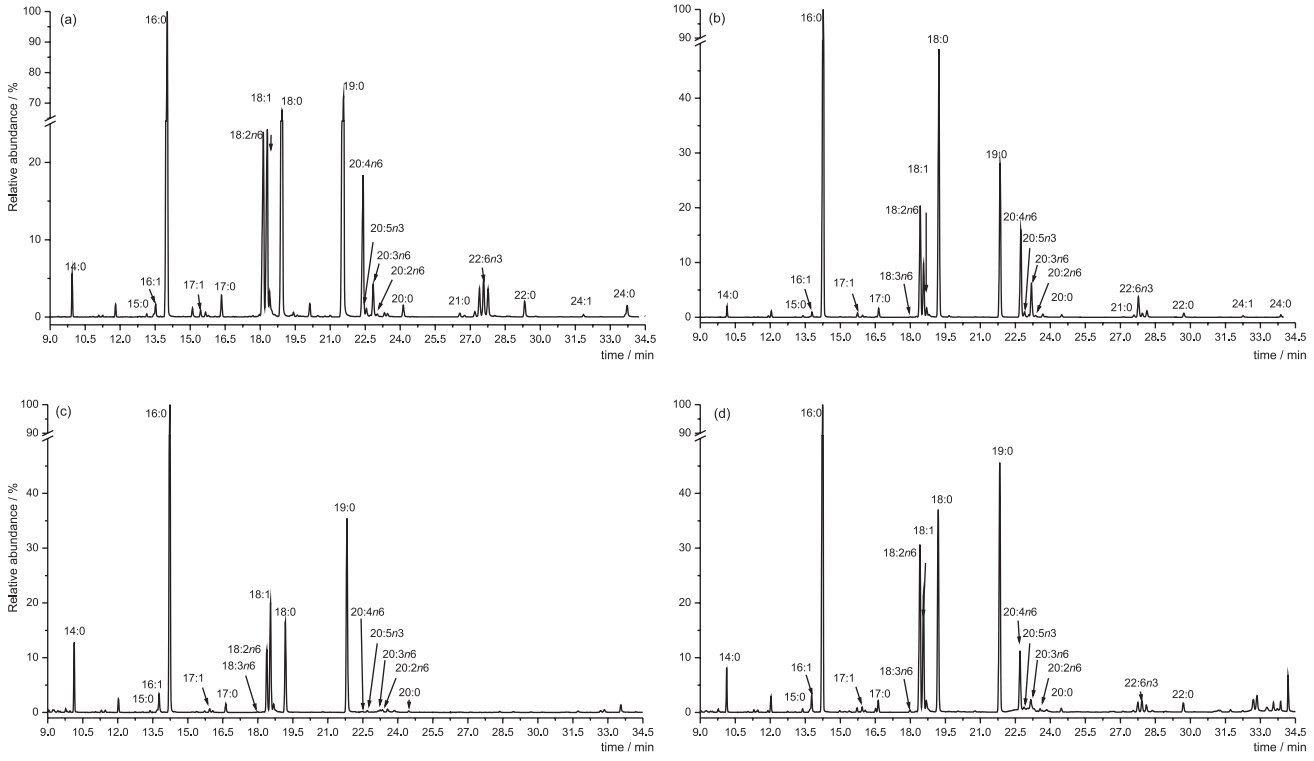

Figure S2. Representative FAME ion chromatogram showing FA present in: (a) RBCM; (b) plasma phospholipids(PL); (c) plasma triglycerides (TG) and (d) plasma cholesteryl esters (CE). Chromatographic conditions: described into CG-MS section.

The unmarked chromatographic peaks were not identified, but also do not match known fatty acid methyl esters due to different retention times and relative abundance of ions with *m/z* 74, 79, 81 and 87. Some fatty acid methyl esters, like 17:1 in RBCM; 21:0 in plasma phospholipids and cholesteryl esters were detected only in few samples and remained below the limit of quantification.