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Comparison of conventional and fast gas chromatography in human plasma fatty acid determination☆

Isabel Bondia-Pons, Ana I. Castellote, M. Carmen López-Sabater*

Department de Nutrició i Bromatologia, Centre de Referència en Tecnologia dels Aliments (CeRTA), Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n 08028, Barcelona, Spain

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Abstract

A fast gas chromatographic technique for the determination of fatty acids in human plasma was developed. Its validation and comparison to a conventional method are here reported. The fast method significantly reduced the time required for analysis by a factor of 5 (total time of 3.2 min) while maintaining a similar resolution. Reproducibility of qualitative and quantitative data was measured in both applications. The results demonstrated that the applied fast gas chromatography (GC) conditions do not affect the analytical quality of the assays, allowing a short analysis time.

Keyword: Fatty acids

1. Introduction

The determination of fatty acids in human plasma by gas chromatography (GC) has become a useful and routine tool to understand the importance of dietary fat for human health [1].

Since the first GC application on human fatty acids, published by the end of the 1950s [2], numerous and diverse biomedical and nutritional studies have been designed to evaluate the effects of these fat biomarkers on nutritional status and to establish their relationships with some major pathologies such as cardiovascular diseases [3–7].

Plasma fatty acid content varies depending on the quality and quantity of fat in the habitual diet, the cholesterol level, the degree of fat replacement and the time period between measurements, among other factors.

The high sample throughput, that characterises this kind of population study, increases the need for a significant reduction in analysis time. Nowadays this reduction is possible thanks to instrumental developments in narrow-bore fast GC. In practical terms, this implies the use of high inlet pressures, accurate split flow control, fast oven heating rates and fast electronics [8]. This type of approach allows several replicate analyses of a sample in the same time as a single conventional GC separation.

The development of a fast GC method requires careful optimization of the experimental conditions. The fast GC method has already been proved to be successful in the field of essential oils [8,9], PCB mixtures [8,10,11], drugs and pesticides [10,12]. Excellent fast GC separations have also been obtained on fatty acid methyl esters (FAME) in complex matrices such as natural fats [13,14]. But, as far as the authors know, the fast GC determination of fatty acids in biological samples, such as plasma, has not yet been reported.

Until now, research in the field of fatty acids analysis has focussed on the optimization of the sample preparation steps, which basically consist in lipid extraction, fractionation and final derivatization normally into their corresponding fatty acid methyl esters [1,15].

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* Corresponding author. Tel.: +34 93 4024 512; fax: +34 93 4035 931.
E-mail address: mclopez@ub.edu (M.C. López-Sabater).

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The work presented here aims to compare conventional and fast gas chromatography in human plasma fatty acid determination. All other analytical steps, in the current methodology, were previously optimized.

2. Experimental

2.1. Reagents and standards

Boron trifluoride in methanol (20% w/v), *n*-hexane, sodium chloride and anhydrous sodium sulphate were purchased from Merck (Darmstadt, Germany); sodium methylate from Fluka (Buchs, Switzerland) and dry methanol from Panreac (Barcelona, Spain).

Supelco^{TM} 37 Component Fatty Acid Methyl Esters Mix and Menhaden Oil, used for peak identification; and tridecanoic acid ($C_{13:0}$), used as the internal standard (IS), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock standard solutions were prepared by dissolving fatty acid methyl esters standards in *n*-hexane and were stored at 4 °C until usage.

Human plasma used for the validation of the fast GC method was also purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other human plasma samples were kindly obtained from a clinical nutritional study.

2.2. Sample preparation

Plasma samples used for fatty acid analyses were stored at −80 °C, prior to analysis. One hundred microliters plasma samples were saponified in PTFE screw-capped Pyrex tubes containing 20 μg of the IS, by adding 1 ml of sodium methylate (0.5% w/v) and heating to 100 °C for 15 min. After cooling to 25 °C, samples were esterified with 1 ml of boron trifluoride-methanol reagent (also at 100 °C) for 15 min. Once the tubes were cooled, FAME were isolated by adding 500 μL of *n*-hexane. After shaking for 1 min, 1 mL of a saturated sodium chloride solution was added. Finally, the tubes were centrifuged for 8 min at 2200 × *g*. After drying with anhydrous sodium sulphate, the clear *n*-hexane top layer was transferred into an automatic injector vial equipped with a volume adapter of 300 μL.

2.3. Gas chromatography conditions

2.3.1. Conventional GC

Conventional GC analyses were performed on HP-6890 Series GC System (Hewlett Packard, Waldbronn, Germany), equipped with a flame ionization detector (FID) and a HP-6890 Series Injector. Separation of FAME was carried out on a fused-silica capillary column (30 m × 0.25 mm i.d.), coated with SP-2330 non-bonded stationary phase (poly (80% biscyanopropyl–20% cyanopropylphenyl) siloxane, 0.20 μm film thickness) from Supelco (Bellefonte, PA, USA).

Operating conditions were as follows: the split–splitless injector was used in split mode with a split ratio of 1:30. The injection volume of the sample was 1 μL. The injector and detector temperatures were kept at 250 °C and 270 °C respectively. The temperature program was as follows: initial temperature 160 °C, increased at 5 °C/min to 250 °C and held at this temperature for 3 min (total run time: 21 min). Helium was used as the carrier gas, with a linear velocity of 22.5 cm/s (average at 160 °C). Pressure: 100 kPa; detector gas flows: H_2: 30 ml/min; air: 350 ml/min; make-up Gas (N_2): 30 ml/min. Sampling frequency: 20 Hz. Data acquisition and processing were performed with an HP-Chemstation software for GC systems.

2.3.2. Fast GC

Fast GC analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector. Separation of FAME was carried out on a capillary column (10 m × 0.10 mm i.d.), coated with a SGE-BPX70 cross-linked stationary phase (70% cyanopropyl Polysilphenylene-siloxane, 0.20 μm film thickness) from SGE (SGE Europe Ltd., United Kingdom).

Operating conditions were as follows: the split–splitless injector was used in split mode with a split ratio of 1:100. The injection volume of the sample was 1 μL. The injector and detector temperatures were kept at 250 °C and 270 °C respectively. The temperature program was as follows: initial temperature 150 °C, increased at 25 °C/min until 250 °C (total run time: 4 min). Helium was used as the carrier gas, with a linear velocity of 59.4 cm/s (average at 150 °C) Pressure: 560.5 kPa; detector gas flows: H_2: 50 ml/min; air: 400 ml/min; make-up Gas (N_2): 50 ml/min. Sampling frequency: 50 Hz. Data acquisition and processing were performed with a Shimadzu-Chemstation software for GC systems.

2.4. Identification and quantification

The identities of sample methyl ester peaks were determined by comparison of their relative retention times with those of well-known FAME standards. Quantification was accomplished by standard normalisation.

2.5. Validation of fast GC method

Intra-assay and inter-assay precision, limit of detection and limit of quantification were the parameters determined for the validation of the fast GC method.

Intra-assay precision was assessed by the coefficient of variation relative to 10 replicates of the commercial human plasma under the same experimental conditions and carried out by the same operator. The inter-assay precision was also assessed by the relative coefficient of variation, which in this case, was determined by analysing 10 aliquots of plasma by two operators in successive days during 2 months. Triplicate determinations were performed for each aliquot.

The FAME absolute response factors (RF) were determined with a quantitative mixture of $C_{10:0}$–$C_{22:1}$ (10 mg/ml in methylene chloride; Supelco (Bellefonte, PA, USA)). RF for each fatty acid methyl ester was calculated as $RF_i = (W_i \times \sum A)/(A_i \times \sum W)$ were W_i is the amount of the FAME weighed in the mixture and A_i is the measured area.

The detection and quantification limits were obtained following the USP criteria [16]. The magnitude of analytical background response was measured by analysing 10 blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor of 3 (detection limit) or 10 (quantification limit) provided an estimate of the limit of detection and limit of quantification respectively.

2.6. Statistical analysis

All data are mean values ± standard deviation; each sample was analysed in triplicate. Results were processed with the SPSS 11.5 statistical package (SPSS, Chicago, IL). The statistical analysis included the Student's test for differences between groups.

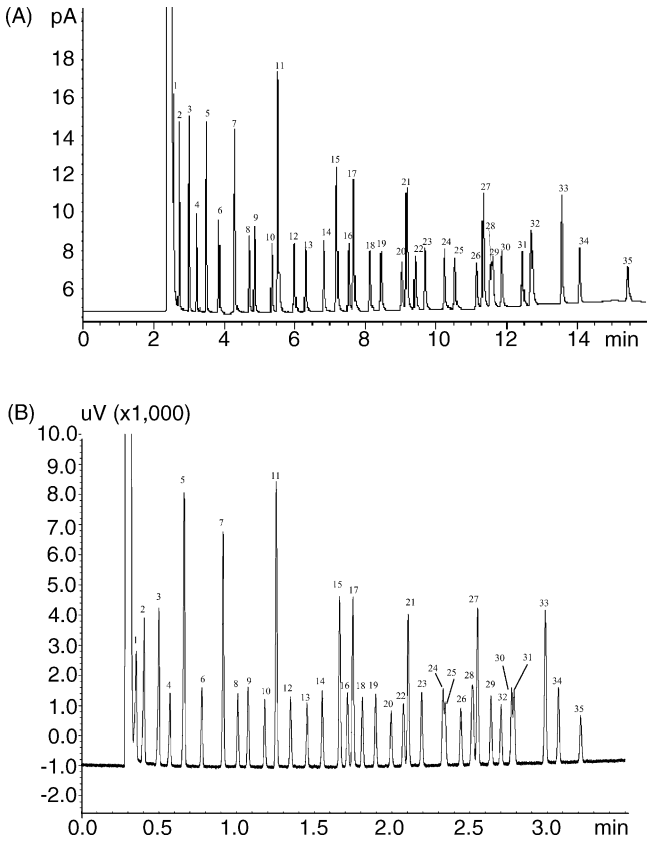

Fig. 1. (A) Conventional GC chromatogram and (B) fast GC chromatogram of a FAME37 mixture. For experimental conditions see Section 2.3. Peak identification: (1) $C_{6:0}$; (2) $C_{8:0}$; (3) $C_{10:0}$; (4) $C_{11:0}$; (5) $C_{12:0}$; (6) $C_{13:0}$; (7) $C_{14:0}$; (8) $C_{14:1}$; (9) $C_{15:0}$; (10) $C_{15:1}$; (11) $C_{16:0}$; (12) $C_{16:1\,n-7}$; (13) $C_{17:0}$; (14) $C_{17:1}$; (15) $C_{18:0}$; (16) $C_{18:1\,(n-9)trans}$; (17) $C_{18:1\,(n-9)cis}$; (18) $C_{18:2\,(n-6)trans}$; (19) $C_{18:2\,(n-6)cis}$; (20) $C_{18:3\,n-6}$; (21) $C_{20:0}$; (22) $C_{18:3\,n-3}$; (23) $C_{20:1\,n-9}$; (24) $C_{21:0}$; (25) $C_{20:2\,n-6}$; (26) $C_{20:3\,n-6}$; (27) $C_{22:0}$; (28) $C_{20:4\,n-6}$; (29) $C_{22:1\,n-9}$; (30) $C_{22:2\,n-6}$; (31) $C_{23:0}$; (32) $C_{20:5\,n-3}$; (33) $C_{24:0}$; (34) $C_{24:1\,n-9}$; (35) $C_{22:6\,n-3}$.

3. Results and discussion

Conventional separation of human plasma fatty acid methyl esters was obtained using a standard column (SP-2330 30 m × 0.25 mm × 0.20 μm). Fast CG analysis was performed with a SGE-BPX70 (10 m × 0.10 mm × 0.20 μm) narrow-bore column.

In order to choose the appropriate operational parameters, the demands of present-day nutritional trials were considered. Nowadays, technological advances enable the study of specific relative to minor fatty acids in lipids, that represent even less than 1% of the total fatty acid profile [1]. This fact, summed to the concept that the greater the stationary phase selectivity, the faster the analysis can be performed, is the reason why a SGE-BPX70 stationary phase (see Section 2) was chosen. It allows geometric and positional FAME isomers separation, and maintains a high thermal stability due to its modified silphenylene siloxane backbone in contrast with other polar columns in use.

According to Klee and Blumberg [17], any fully optimized chromatographic method is a tuned compromise between speed, sample capacity, and resolution. The limited sample capacity is one of the major drawbacks of fast GC techniques [14], which can cause the lack of detection of minor quan-

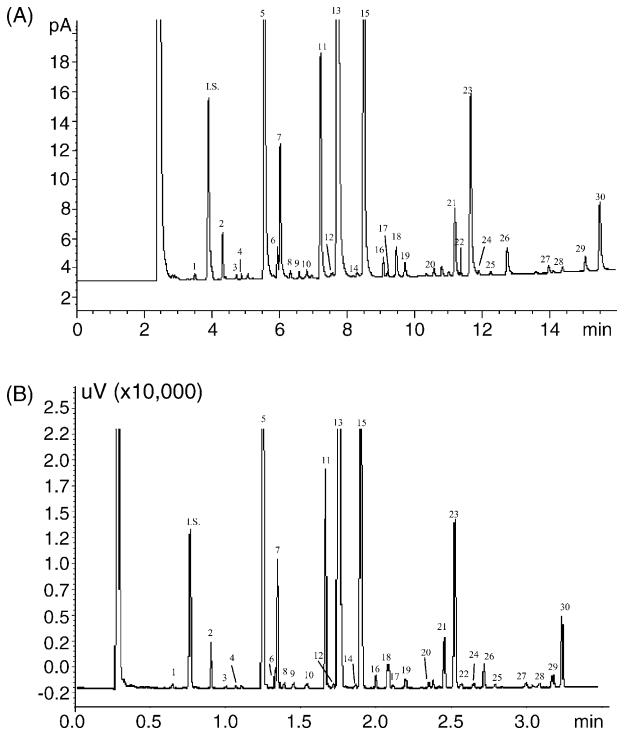

Fig. 2. (A) Conventional GC chromatogram and (B) fast GC chromatogram of a human plasma sample. For experimental conditions see Section 2.3. See Table 1 for peak identification.

Table 1
Peak identification, average retention times (min) and relative standard deviation values relative to conventional and fast GC analyses on plasma FAME

	FAME	Conventional GC		Fast GC	
		t_R	R.S.D. (%)	t_R	R.S.D. (%)
1	$C_{12:0}$	3.487	0.129	0.662	0.040
2	$C_{14:0}$	4.297	0.130	0.914	0.040
3	$C_{14:1}$	4.621	0.124	1.008	0.040
4	$C_{15:0}$	4.813	0.131	1.074	0.040
5	$C_{16:0}$	5.526	0.126	1.256	0.060
6	$C_{16:1\,n-9}$	5.990	0.117	1.348	0.040
7	$C_{16:1\,n-7}$	6.023	0.120	1.353	0.010
8	$C_{16:2\,n-4}$	6.381	0.114	1.507	0.050
9	$C_{16:3\,n-4}$	6.604	0.110	1.609	0.039
10	$C_{16:4\,n-1}$	6.824	0.124	1.636	0.030
11	$C_{18:0}$	7.181	0.111	1.664	0.038
12	$C_{18:1\,(n-9)trans}$	7.555	0.114	1.714	0.030
13	$C_{18:1\,(n-9)cis}$	7.666	0.110	1.749	0.048
14	$C_{18:2\,(n-6)trans}$	8.152	0.106	1.810	0.040
15	$C_{18:2\,(n-6)cis}$	8.452	0.104	1.895	0.030
16	$C_{18:3\,n-6}$	9.033	0.094	1.995	0.039
17	$C_{20:0}$	9.075	0.088	2.104	0.040
18	$C_{18:3\,n-3}$	9.417	0.110	2.074	0.030
19	$C_{20:1\,n-9}$	9.685	0.200	2.191	0.020
20	$C_{20:2\,n-6}$	10.539	0.099	2.343	0.029
21	$C_{20:3\,n-6}$	11.150	0.080	2.444	0.030
22	$C_{22:0}$	11.498	0.081	2.551	0.038
23	$C_{20:4\,n-6}$	11.610	0.099	2.518	0.030
24	$C_{22:1\,n-9}$	11.901	0.100	2.637	0.020
25	$C_{22:2}$	12.001	0.107	2.717	0.065
26	$C_{20:5\,n-3}$	12.698	0.171	2.702	0.029
27	$C_{24:0}$	13.924	0.069	2.987	0.038
28	$C_{22:4\,n-6}$	14.332	0.080	3.100	0.088
29	$C_{22:5\,n-3}$	15.000	0.063	3.157	0.028
30	$C_{22:6\,n-3}$	15.432	0.065	3.215	0.038

n = 10.

Table 2
Intra-assay precision values

	FAME	Conventional GC		Fast GC	
		FAME (%)	R.S.D. (%)	FAME (%)	R.S.D. (%)
1	$C_{12:0}$	0.12	3.76	0.11	3.84
2	$C_{14:0}$	1.57	0.89	1.55	2.36
3	$C_{14:1}$	0.10	3.10	0.11	3.35
4	$C_{15:0}$	0.09	2.29	0.10	3.21
5	$C_{16:0}$	22.10	0.50	22.13	0.61
6	$C_{16:1\,n-9}$	0.41	2.34	0.42	2.31
7	$C_{16:1\,n-7}$	3.25	1.74	3.20	1.33
8	$C_{16:2\,n-4}$	0.12	3.01	0.11	3.35
9	$C_{16:3\,n-4}$	0.12	2.89	0.12	3.31
10	$C_{16:4\,n-1}$	0.44	3.34	0.45	2.85
11	$C_{18:0}$	7.28	0.38	7.30	1.12
12	$C_{18:1\,(n-9)trans}$	0.11	2.63	0.10	2.45
13	$C_{18:1\,(n-9)cis}$	21.30	0.35	21.34	1.02
14	$C_{18:2\,(n-6)trans}$	0.15	4.36	0.13	4.15
15	$C_{18:2\,(n-6)cis}$	29.50	0.38	29.60	0.65
16	$C_{18:3\,n-6}$	0.36	2.02	0.32	2.29
17	$C_{20:0}$	0.19	4.08	0.17	4.19
18	$C_{18:3\,n-3}$	0.50	3.53	0.52	3.22
19	$C_{20:1\,n-9}$	0.26	3.50	0.24	3.92
20	$C_{20:2\,n-6}$	0.19	1.85	0.19	3.98
21	$C_{20:3\,n-6}$	1.75	0.55	1.77	2.23
22	$C_{22:0}$	0.05	4.46	0.05	4.10
23	$C_{20:4\,n-6}$	6.41	0.31	6.38	1.72
24	$C_{22:1\,n-9}$	0.10	3.51	0.10	3.68
25	$C_{22:2}$	0.10	4.16	0.10	4.40
26	$C_{20:5\,n-3}$	0.48	0.65	0.45	1.02
27	$C_{24:0}$	0.18	3.25	0.17	4.21
28	$C_{22:4\,n-6}$	0.15	3.18	0.14	3.95
29	$C_{22:5\,n-3}$	0.31	2.80	0.30	3.37
30	$C_{22:6\,n-3}$	2.31	0.51	2.33	1.28

Average fatty acid methyl ester content in plasma samples and relative standard deviation values. n = 10.

tity peaks. For this reason, the risk of band broadening, due to column overloading, was solved with a highly controlled split flow, by increasing the split ratio to 1:100 respect to the 1:30 ratio for the conventional GC approach.

Fig. 1A–B shows the chromatograms of conventional and fast FAME GC analysis for a commercial mixture of 37 fatty acids. A significant reduction in the analysis time was observed in the rapid application. The fatty acids analysed differ not only in chain length but also in the position of double bonds. The analysis with a conventional GC column took about 16 min, while the fast analysis allowed the separation of the same components in about 3.2 min with a similar resolution. Therefore, the fast GC technique performs the same separation with a significant speed gain of a factor of 5.

Also to be emphasised is the different elution order relative to a series of compounds on both columns. Three peak inversions were observed for the following analyte pairs: $C_{20:0}$ and $C_{18:3\,n-3}$; $C_{22:0}$ and $C_{20:4\,n-6}$ and $C_{22:2}$ and $C_{20:5\,n-3}$.

As it can be observed in Fig. 1B, the fast GC application does neither achieve the total separation of $C_{21:0}$ and $C_{20:2}$ FAME nor of $C_{23:0}$ and $C_{22:2}$ FAME, whereas the same compounds separate with a good resolution in the conventional application. This overlapping is irrelevant in human plasma samples due to their absence in $C_{21:0}$ and $C_{23:0}$.

Once all fatty acid methyl esters were separated and identified, the same analytical conditions were applied to validate the fast GC method with human plasma matrices.

Conventional and fast GC chromatograms relative to FAME contained in the same human plasma sample are shown in Fig. 2A–B. Thirty peaks were identified in both chromatograms. As observed, the human plasma sample presented important differences in the relative abundance of their fatty acids. The five major compounds, in the range of 29.9% to 6.4% relative FAME content, were linoleic ($C_{18:2\,(n-6)cis}$), oleic ($C_{18:1\,(n-9)cis}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and arachidonic acid ($C_{20:4\,n-6}$). Both chromatograms showed a very similar resolution and no overlapping of critical peaks occurred. Peak shapes and symmetry were also satisfactory in both applications.

For routine analyses, qualitative data (retention time values) and quantitative data (relative sample composition) should remain constant. Table 1 reports the qualitative results obtained with both columns, showing the average retention times. It is worth noting the significantly lower relative standard deviation values observed for the fast GC analysis

Table 3
Inter-assay precision values

	FAME	Fast GC	
		FAME (%)	R.S.D. (%)
1	$C_{12:0}$	0.12	4.01
2	$C_{14:0}$	1.60	2.48
3	$C_{14:1}$	0.10	3.71
4	$C_{15:0}$	0.11	3.91
5	$C_{16:0}$	22.34	1.61
6	$C_{16:1\,n-9}$	0.39	3.31
7	$C_{16:1\,n-7}$	3.20	2.33
8	$C_{16:2\,n-4}$	0.13	3.85
9	$C_{16:3\,n-4}$	0.10	3.62
10	$C_{16:4\,n-1}$	0.48	3.02
11	$C_{18:0}$	7.10	1.25
12	$C_{18:1\,(n-9)trans}$	0.10	3.99
13	$C_{18:1\,(n-9)cis}$	21.08	1.75
14	$C_{18:2\,(n-6)trans}$	0.12	4.65
15	$C_{18:2\,(n-6)cis}$	29.92	1.00
16	$C_{18:3\,n-6}$	0.30	2.99
17	$C_{20:0}$	0.14	4.55
18	$C_{18:3\,n-3}$	0.49	3.99
19	$C_{20:1\,n-9}$	0.26	4.02
20	$C_{20:2\,n-6}$	0.18	4.17
21	$C_{20:3\,n-6}$	1.74	2.85
22	$C_{22:0}$	0.07	4.50
23	$C_{20:4\,n-6}$	6.35	1.95
24	$C_{22:1\,n-9}$	0.10	3.98
25	$C_{22:2}$	0.09	4.40
26	$C_{20:5\,n-3}$	0.40	1.62
27	$C_{24:0}$	0.18	4.45
28	$C_{22:4\,n-6}$	0.16	4.48
29	$C_{22:5\,n-3}$	0.40	3.95
30	$C_{22:6\,n-3}$	2.25	1.53

Average fatty acid methyl ester content in plasma samples and relative standard deviation values relative to the fast GC method. $n = 10$.

in comparison to the conventional application. These results agree with the relative standard deviations found by other authors using a similar fast GC approach [9].

The reproducibility of quantitative data in passing from one technique to the other is shown in Table 2. The reported information regards relative average FAME content and relative standard deviation for a human plasma separation. These data show good agreement between fast and conventional results, with no significant differences. The relative standard deviation values, in the range of 0.31% to 4.46% are within the limits of acceptable variability for the analyte concentrations of this kind of samples [18].

Table 3 reports the inter-assay precision for the fast GC method, which has proven to be robust. Some minor peaks (<1% relative quantity) were those with the highest relative standard deviation values, but all values were always lower than 5%.

The values of absolute response factors for a quantitative mixture of $C_{10:0}$–$C_{22:1\,n-9}$ fatty acid methyl esters can be seen in Table 4. As observed, data show good agreement between fast and conventional results, giving all compounds a similar response.

Table 4
Absolute response factors (RF) of the quantitative mixture of FAME for the conventional GC method and the fast GC method

FAME	Conventional GC, RF	Fast GC, RF
$C_{10:0}$	1.11	1.18
$C_{12:0}$	1.05	1.09
$C_{13:0}$	1.02	1.13
$C_{14:0}$	1.00	1.07
$C_{14:1\,(n-9)cis}$	1.00	1.19
$C_{15:0}$	1.06	0.95
$C_{16:0}$	1.12	1.14
$C_{16:1\,(n-9)cis}$	0.93	1.00
$C_{17:0}$	1.28	1.23
$C_{18:0}$	1.13	1.04
$C_{18:1\,(n-9)trans}$	0.96	0.91
$C_{18:1\,(n-9)cis}$	0.91	0.91
$C_{18:2\,(n-6)cis}$	0.93	0.94
$C_{20:0}$	1.18	1.18
$C_{20:1}$	0.90	1.05
$C_{18:3\,n-3}$	0.92	0.96
$C_{22:0}$	1.12	1.10
$C_{22:1\,n-9}$	0.89	1.00

Table 5
Detection (DL) and (QL) quantification limits (QL) for the conventional GC and the fast GC method

FAME	Conventional GC		Fast GC	
	DL (ng)	QL (ng)	DL (ng)	QL (ng)
$C_{10:0}$	4.0	16.0	3.8	12.6
$C_{12:0}$	3.8	15.2	3.6	12.1
$C_{13:0}$	3.6	14.7	3.8	12.6
$C_{14:0}$	3.6	14.4	3.7	12.4
$C_{14:1\,(n-9)cis}$	3.6	14.4	3.7	12.3
$C_{15:0}$	3.8	15.4	4.1	13.8
$C_{16:0}$	4.0	16.3	3.8	12.5
$C_{16:1\,(n-9)cis}$	3.3	13.5	3.4	11.2
$C_{17:0}$	4.6	18.5	5.0	16.8
$C_{18:0}$	4.1	16.4	4.3	14.3
$C_{18:1\,(n-9)trans}$	3.4	13.8	3.8	12.6
$C_{18:1\,(n-9)cis}$	3.3	13.1	3.0	10.0
$C_{18:2\,(n-6)cis}$	3.3	13.4	3.2	10.5
$C_{20:0}$	4.2	17.0	4.8	16.1
$C_{20:1}$	3.2	12.9	3.6	12.0
$C_{18:3\,n-3}$	3.3	13.3	3.2	10.7
$C_{22:0}$	4.0	16.2	4.4	14.6
$C_{22:1\,n-9}$	3.2	12.8	3.5	11.8

Detection and quantification limits values are shown in Table 5. Both methods present limits of detection and quantification in the range of nanograms (3.0–10.0 ng; 3.2–18.5 ng). These values are in concordance with those established for gas chromatography methods [19].

4. Conclusions

The analytical results achieved indicate that the fast GC method developed in the present research allows the separation of a considerable number of fatty acid methyl esters in a short time (3.2 min).

In comparison to traditional chromatography, it has been demonstrated that fast GC conditions do not affect the analytical quality of the assays. Its application to human plasma samples has been shown to be robust and reliable for quick and correct identification in routine analysis.

Furthermore, the new developed method is useful for clinical studies wherein the simultaneous preparation of a large number of samples is always required.

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