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Short communication

Comparison of two methods for the determination of fatty acid profiles in plasma and erythrocytes

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Abstract

We have validated and compared two methods for the determination of fatty acid profiles in biological samples by capillary gas chromatography. Method I consisted of a previous lipid extraction and esterification of fatty acids using boron trifluoride–methanol. Method II was a direct method that combined extraction and esterification of freeze-dried samples in a single step, using acetyl chloride as the reagent. The two methods were applied to the analysis of plasma and erythrocyte samples. Both methods gave similar results in plasma, whereas in erythrocytes, the direct method gave significantly higher contents of total fatty acids. Precision and recovery rates were determined and the results were satisfactory. Detection and quantification limits showed that both methods had excellent sensitivity. It was concluded that the direct method has substantial advantages over the conventional method, such as higher values in erythrocytes, rapidity and less possibility of contamination or fatty acid losses. Therefore, it is preferable for the analysis of biological samples such as plasma and erythrocytes. © 1997 Elsevier Science B.V.

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1. Introduction

Fatty acid analysis in biological samples has acquired relevance during the last decades due to evidence of the influence of dietary lipids on the development of some human diseases, such as heart disease and cancer [1–3]. Both official [4] and common methods to determine fatty acids consist of many steps, i.e. lipid extraction with organic solvents, derivatization and quantification by capillary gas chromatography [5]. Derivatization involves the conversion of fatty acids into volatile compounds, giving rise to fatty acid methyl esters. This step can be carried out by acid catalysis using hydrochloric

acid [6,7], sulfuric acid [8] or boron trifluoride [9,10], all in methanol, by alkaline catalysis using sodium methoxide in anhydrous methanol [11] or by a combination of the two procedures [12]. Diazo-methane has also been used as a methylation reagent [13]. However, many difficulties arise when conventional methods are applied to biological samples, since there is a high risk of contamination and recovery losses in multistep procedures. In addition, these methods are impractical when the number of samples being analyzed is large and the availability of samples is limited.

To overcome these problems, some authors have recently developed methods that combine extraction and derivatization in a single step. Direct (i.e., without previous lipid extraction) methylation of

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fatty acids has recently been carried out by several investigators using acetyl chloride [14], hydrochloric acid in methanol [13] or boron trifluoride in methanol [15]. Here, as part of a study in which a large number of biological samples were to be analysed, we compared the conventional method for the determination of fatty acids (method I) with a modification of the direct method proposed by Lepage and Roy [14] (method II). The two methods were validated for routine analysis and were applied to the determination of fatty acid patterns in human plasma and erythrocyte samples.

2. Experimental

2.1. Reagents and standard solutions

The solvents used, such as chloroform, methanol and toluene (all of ACS grade) were purchased from Probus (Badalona, Spain), and *n* and $C_{22:6w-3}$. The following parameters were determined: linearity, precision, recovery rates and limits of detection and quantification. In order to check the linearity of response of the fatty acid methyl esters, calibration curves were calculated by adding five increasing amounts of fatty acid methyl ester standards to the same amount of I.S. ($C_{17:0}$, 40 μg). Standard amounts ranged from 3.06 to 122.4 μg for $C_{16:0}$, from 2.9 to 116 μg for $C_{18:1w-}$ and $C_{18:2w-6}$, from 7.5 to 40 μg for $C_{20:4w-6}$ and from 2.5 to 25 μg for $C_{22:6w-3}$. Each concentration level was determined in triplicate. The inter-assay precision was assessed by the coefficient of variation, which was determined by analysing ten aliquots of both pooled plasma and erythrocytes by the two methods. The standard addition method was used to evaluate the recovery rates of both methods. Five different standard amounts (the same ranges as for linearity tests) were added to a known volume of plasma or erythrocytes. Then, samples were analysed by the two procedures described and fatty acid methyl ester solutions were injected into the column. Triplicates determinations were performed for each standard amount. Recovery rates were calculated by comparison of the values obtained with the amounts of standards added.

For the theoretical limits of detection and quantification, we determined the analytical background

response for each fatty acid by injecting ten blank samples into the gas chromatograph and calculating the standard deviation of this response [18].

2.2. Results and discussion

Fatty acid analysis of a large number of biological samples is part of many clinical trials [1]. In this study, we have compared the conventional method for the analysis of fatty acids, which includes a lipid extraction step, with a direct method that combines extraction and derivatization in a single step. The two methods were applied to the analysis of plasma and erythrocyte samples. To our knowledge, the use of the direct method proposed by Lepage and Roy [14] for the analysis of erythrocyte samples has not been reported before. Despite the fact that haemoglobin breakdown products were present in the solution to be injected into the gas chromatograph, we did not observe any alteration in the resolution of the column after processing over 80 samples. The direct method presented here included some modifications to the method reported by Lepage and Roy [14]. First, toluene was used instead of benzene, which is more toxic. Secondly, the samples were freeze-dried to eliminate water interference before analysis, mainly because erythrocytes were resuspended to a blood cell to plasma ratio, by volume, of about 45%. Freeze drying also prevented a violent reaction occurring between acetyl chloride and water. In Method II, strict adherence to the protocol is needed to achieve good accuracy. Additionally, in the neutralization step, potassium carbonate must be added very slowly to eliminate acid traces and, therefore, to avoid column damage. With these preventive measures, many samples can be analysed without column damage. Fig. 1 shows a typical gas chromatographic profile of fatty acid methyl esters from plasma. The resolution was satisfactory and totals of nineteen and twenty fatty acids were identified in plasma and erythrocytes, respectively. The possibility of contamination or of interfering peaks was eliminated by injecting a reagent blank processed by the two methods. However, it must be pointed out that since more material is involved, more care is needed using Method I, to avoid contamination. The peaks with retention times >52 min, which appeared in the chromatograms, were

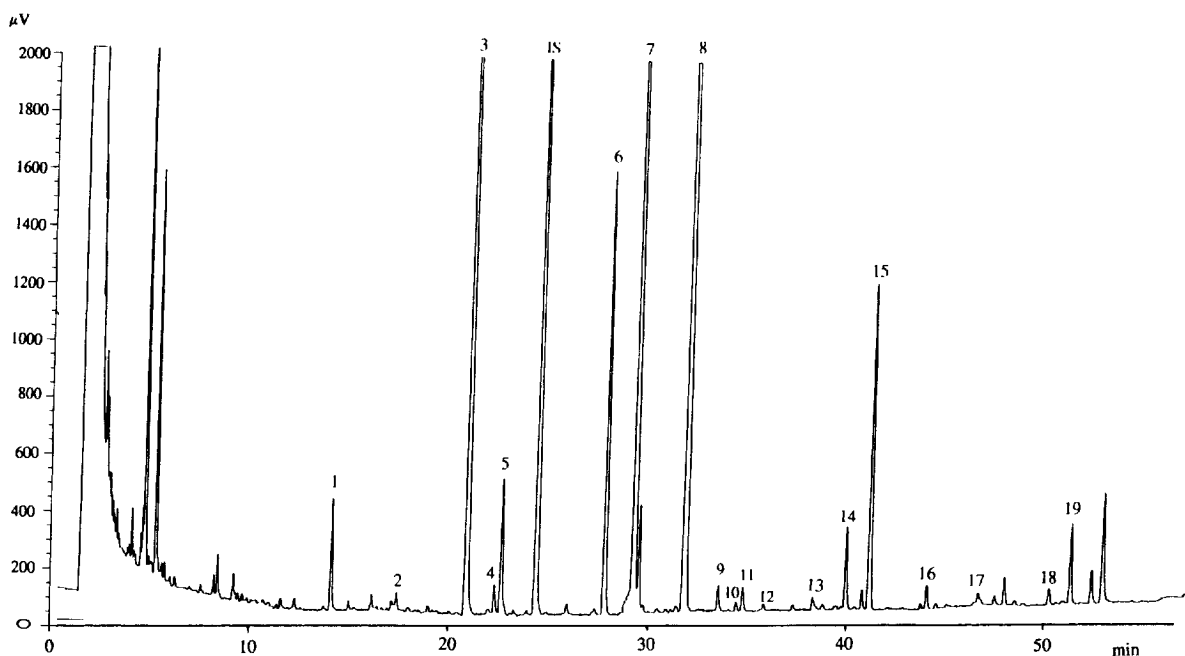


Fig. 1. Typical chromatogram of a fatty acid profile of a plasma sample that was processed using Method II. Peaks: 1=C_{14:0}; 2=C_{15:0}; 3=C_{16:0}; 4=C_{16:1w-9}; 5=C_{16:1w-7}; 6=C_{18:0}; 7=C_{18:1w-9/w-7}; 8=C_{18:2w-6}; 9=C_{18:3w-}; 10=C_{20:0}; 11=C_{18:3w-3}; 12=C_{20:1w-9}; 13=C_{20:2w-6}; 14=C_{20:3w-6}; 15=C_{20:4w-}; 16=C_{20:5w-3}; 17=C_{22:4w-6}; 18=C_{22:5w-} and 19=C_{22:6w-3}.

identified by other authors as cholesterol ester degradation products [19].

The fatty acid composition of plasma and erythrocytes, as determined by Methods I and II, is presented in Table 1. Both methods gave comparable results in plasma and no significant differences were observed between them. These results contrast with those of other authors, who reported higher fatty acid yields in plasma [14] and lipoproteins [15] using a direct method. Similarity of the values obtained by the two methods in our study may be the result of the addition of the I.S. before lipid extraction of the samples, which allowed us to quantify fatty acids in Method I without the risk of losses being incurred during the procedure. In erythrocytes, the total fatty acid content was higher when Method II was used. The content of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids were also significantly higher using Method II ($p < 0.05$, Student's *t*-test). The higher yields obtained using Method II in erythrocytes can be attributed to the greater aggressiveness of reagents, which allows complete fatty acid methylation despite the complex-

ity of the sample. The addition of butylhydroxytoluene (BHT) at a concentration of 1 mg/200 μ l sample did not result in higher fatty acid contents. Other authors have also reported similar results in the presence and absence of an antioxidant [15].

The two methods compared in this work were also validated. Validation was performed with five fatty acids of different chain lengths and degrees of unsaturation: C_{16:0}, C_{18:1w-9}, C_{18:2w-6}, C_{20:4w-} and C_{22:6w-3}. The detector response showed good linearity for all fatty acids in the range of concentrations studied. A plot of the ratio of fatty acid peak area to that of the I.S. versus the amount of fatty acid gave the calibration curves and their correlation coefficient (r^2), which was > 0.99 in all cases. The inter-assay precision of the two methods was evaluated and is implied by data shown in Table 1. Relative standard deviations obtained in plasma using Methods I and II ranged from 4.22 to 5.92% and from 3.42 to 5.15%, respectively. In erythrocytes, coefficients of variation varied from 3.89 to 7.49% and from 3.56 to 5.28%, respectively. These values were within the limits of acceptable variability for the analyte concentrations

Table 1
Fatty acid content ($\mu\text{g/ml}$) in pooled plasma and erythrocytes determined by Methods I and II

	Plasma		Erythrocytes	
	Method I	Method II	Method I	Method II
C _{14:0}	39.00±2.07	43.97±2.46	3.20±0.28	5.42±0.44
C _{15:0}	7.72±0.77	7.01±0.16	1.34±0.11	1.49±0.12
C _{16:0}	588.09±24.82	656.88±29.88	167.55±7.67	208.90±11.04
C _{16:1w-9}	14.88±1.42	15.04±1.28	n.d.	n.d.
C _{16:1w-7}	64.34±3.00	65.78±2.00	3.77±0.43	4.02±0.31
C _{18:0}	188.23±7.88	200.98±8.37	130.20±5.80	181.67±8.33
C _{18:1w-9/w-7}	533.97±29.32	544.07±19.61	132.11±6.70	147.70±7.50
C _{18:2w-6}	935.79±45.13	942.78±32.39	102.98±6.26	103.66±4.20
C _{18:3w-6}	12.68±0.44	14.07±0.70	n.d.	n.d.
C _{20:0}	tr	5.41±0.44	2.90±0.20	4.87±0.47
C _{18:3w-3}	12.68±0.67	12.91±0.82	n.d.	n.d.
C _{20:1w-9}	3.91±0.34	3.34±0.30	3.85±0.35	3.28±0.25
C _{20:2w-6}	7.85±0.27	9.08±0.90	2.34±0.30	2.84±0.27
C _{20:3w-6}	44.87±1.74	44.83±2.62	12.86±0.50	15.92±0.73
C _{22:0}	n.d.	n.d.	0.64±0.08	1.82±0.14
C _{20:4w-6}	170.54±10.10	179.05±6.12	104.86±4.07	140.15±5.43
C _{20:5w-3}	12.97±0.74	12.75±0.68	4.74±0.55	5.31±0.45
C _{22:4w-6}	4.87±0.39	4.44±0.44	24.64±1.72	24.80±0.27
C _{24:1w-9}	n.d.	n.d.	4.11±0.24	4.32±0.29
C _{22:5w-6}	n.d.	n.d.	4.78±0.50	7.80±0.60
C _{22:5w-3}	10.58±0.64	11.59±0.85	12.81±0.74	17.48±0.75
C _{22:6w-3}	68.50±3.30	65.71±3.28	45.38±3.40	55.06±1.96
Total	2799.39±103.81	2859.27±117.90	746.68±37.80	929.76±34.13 ^a
SFA	889.44±36.99	924.50±36.85	313.64±17.90	420.09±19.40 ^a
MUFA	617.98±36.14	629.87±23.95	138.66±7.35	159.41±7.36 ^a
PUFA	1300.12±47.92	1306.74±51.90	294.37±17.05	350.26±6.94 ^a

^aData are presented as means±SD, $n=10$.

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n.d., not detected; tr, traces.

^aSignificantly different from Method I, $p<0.05$ (Student's t test).

of these samples [20]. The mean fatty acid recovery rates determined by the two methods are presented in Table 2. Since no significant differences were observed with these methods between the five levels of

addition (analysis of variance, Anova), global values are given in Table 2. The average recoveries obtained with Methods I and II were satisfactory in all cases. Detection and quantification limits were de-

Table 2
Average recovery rates in plasma and erythrocytes using Methods I and II

	Plasma		Erythrocytes	
	Method I	Method II	Method I	Method II
C _{16:0}	97.80±5.42	99.08±6.52	98.55±3.07	94.96±6.61
C _{18:1w-9}	98.97±8.59	98.96±4.52	101.61±3.66	101.24±6.04
C _{18:2w-6}	95.04±8.83	95.92±3.22	97.08±2.77	98.21±5.06
C _{20:4w-6}	99.23±6.38	97.24±9.76	91.47±2.69	103.89±4.39
C _{22:6w-3}	101.37±6.25	96.10±9.92	100.03±7.20	107.32±9.31

Values are expressed as the means±SD. Data are means of five levels of concentration and each level was performed in triplicate. The following amounts of standards were added: C_{16:0}, 3.06, 15.3, 30.6, 91.8 and 122.4 μg ; C_{18:1w-9}, 2.9, 14.5, 29, 87 and 116 μg ; C_{18:2w-6}, 2.9, 14.5, 29, 87 and 116 μg ; C_{20:4w-6}, 7.5, 11.25, 15, 25 and 40; C_{22:6w-3}, 2.5, 5, 7.5, 15 and 25 μg .

Table 3
Limits of detection and quantification of Methods I and II

Fatty acid	Limit of detection ^a		Limit of quantification ^b	
	Method I	Method II	Method I	Method II
C _{16:0}	3.19	3.12	3.55	3.43
C _{18:1w-9}	1.25	1.17	1.75	1.54
C _{18:2w-6}	1.69	1.63	2.09	1.95
C _{20:4w-6}	0.23	0.13	0.62	0.33
C _{20:5w-3}	0.25	0.21	0.56	0.48

The results are expressed in μg .

^aSignal to noise ratio=3.

^bSignal to noise ratio=10.

terminated by analysis of ten blank samples by both methods and the results are shown in Table 3. It was found that both methods gave comparable low values for all of the fatty acids studied.

In conclusion, the direct method for fatty acid analysis of biological samples, consisting of trans-methylation of freeze-dried samples, has substantial advantages over the conventional method. The direct method is reliable, accurate and rapid, involves a minimum consumption of reagents and material and gives complete information about the fatty acid profile. Moreover, it gave higher contents of fatty acids in erythrocytes, whereas in plasma, the contents of fatty acids were comparable to those obtained using the conventional method. Therefore, the direct method is suitable for the screening of blood samples from selected populations.

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