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QUANTITATIVE ANALYSIS OF FATTY ACID METHYL ESTERS AND DIMETHYL ACETALS ON A POLAR (FREE FATTY ACID PHASE) CAPILLARY COLUMN

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SUMMARY

Separation of fatty acid methyl esters and dimethyl acetals from complex biological samples has been achieved by gas–liquid chromatography on a capillary column coated with free fatty acid phase. Response-correcting factors were determined, showing rather large variations with fatty acid length. Polyunsaturated fatty acid methyl esters were shown to have lower responses than saturated species, whereas dimethyl acetals and equivalent methyl esters were found to give similar responses. Total fatty acid and aldehyde compositions of human and simian erythrocytes were determined and compared, showing a somewhat higher level of linoleate and arachidonate, and a lower level of plasmalogens in simian erythrocytes.

INTRODUCTION

It is now generally acknowledged that each cell, membrane and phospholipid (PL) has a characteristic fatty acid (FA) composition[1–3]. Recent studies involving changes in erythrocyte membrane phosphatidylcholine species mediated by the phospholipid exchange protein[4,5] showed that changes as small as a 4% increase in PL saturated species result in hemolysis. Membrane fatty acid composition also has fluidity-mediated effects on membrane-associated proteins such as enzymes, receptors, and transport-proteins (for a review see ref. 1).

Plasmalogens are the most abundant ether-bond-containing lipids in mammalian tissues[6], but even the most precise studies of fatty acid composition do not always include their quantitation[2,7] even though their influence on membrane fluidity has been reported[8].

The superiority of capillary columns over packed columns in the analysis of fatty acid methyl esters (FAMEs) is now well documented[2,7,9–11]. The phases currently used are SP 2340[7,12] Silar CP-88[2,11], which now supplants the less polar Silar 10 C, and free fatty acid phase (FFAP)[13,14].

In this paper, we report on the simultaneous analysis of FAMEs and dimethyl acetals (DMAs) on a fused-silica capillary column coated with FFAP [poly(ethylene glycol) ester of nitroterephthalic acid], including determination of their response-

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correcting factors. This system was used to compare the overall fatty acid composition of human and simian (*Macaca fascicularis*) erythrocytes.

MATERIALS AND METHODS

Chemicals and samples

Boron trifluoride–methanol (14:100, w/v), butylated hydroxytoluene (BHT; 2,6-di-*tert.*-butyl-*p*-cresol), all fatty acid and aldehyde standards (more than 99% pure) were purchased from Sigma (St. Louis, MO, U.S.A.). PUFA 2 is a standard FAME mixture from Supelco (Bellefonte, PA, U.S.A.). Palmitaldehyde was obtained as a sodium bisulphitic combination from Pfaltz and Bauer (Stamford, CT, U.S.A.). Phosphatidylethanolamine (PE) plasmalogen and SRM-06 standard FAME mixture were Alltech products obtained from Interchim (Montluçon, France). All solvents were of analytical grade. Human blood was provided by the local blood bank. Monkeys were obtained (splenectomized) from SANOFI (Montpellier, France) one year before the experiment, and fed *ad libitum* with water and diet No. 107 from UAR (Epinay sur Orge, France). They weighed *ca.* 4 kg.

Blood samples from both humans and simians were collected on citric acid–dextrose. Red cells were washed three times with RPMI 1640 (GIBCO, Flow Lab., France) and passed through a cellulose powder column (CF-11, Whatman, U.S.A.) to remove white blood cells[15]. Osmotic fragility was determined as previously reported[16]. Lipids were extracted using the Folch method[17], and transmethylated by boron trifluoride–methanol (14:100, w/v) at 100°C for 3 or 90 min in the case of standard FAs (or aldehydes) and in the case of biological samples, respectively[18]. At least 1 ml of reagent was used per milligram of lipid. After cooling and addition of pentane (containing 50 mg BHT/1), 5 *N* sodium hydroxide was added to prevent reversion of DMAs[18].

For identification purpose, FAMEs and DMAs were separated using thin-layer chromatography (TLC) in toluene at 4°C[19].

Analysis

Gas–liquid chromatography (GLC) was performed on a Carlo Erba 2900 apparatus, equipped with a cooled on-column injector and a fused-silica capillary column (25 m × 0.32 mm I.D.) coated with FFAP (Chrompack, The Netherlands). Sample size was 0.5–1 μl in hexane.

Optimum velocity (75.8 cm/s) of the helium carrier gas was determined for octadecanoic FAMEs (using an Alltech SRM-06 reference mixture), according to efficiency and resolution[20]. Complete analysis of FAMEs was performed in 40 min and recorded on a Shimadzu ICR-1-B integrator. Subsequent calculations based on area measurements were done on an Apple IIe computer, using basic programs. Results are given as the mean ± standard error of the mean (S.E.M.).

Identification and quantitation

FAMEs and DMA 16:0 were identified, using reference compounds, according to retention times relative to the methyl ester of the internal standard (I.S.) FA 19:0 (carbon chain length:number of double bonds; unsaturation is designed by omega followed by the number of carbon atoms from the methyl end of the molecule to the

middle of the nearest double bond). An exception was FAME 20:3ω9, which was identified according to the previously reported FA composition of human erythrocyte[19] and the structure–retention relationship discussed elsewhere[21].

DMAs 18:0, 18:ω9 and 18:1ω7 were identified on the following basis: (1) compounds recovered only when sodium hydroxide is added after transmethylation of erythrocyte lipid extracts or PE plasmalogen; (2) analysis of the DMA fraction isolated by TLC in toluene at 4°C[19]; (3) elution from the column before FAME 18:0, like DMA 16:0 and DMA 16:ω6 before FAME 16:0; (4) available data on human erythrocyte FA and aldehyde composition[19,22]; (5) saturation in the case of DMA 18:0 as well as unsaturation in the case of DMAs 18:1ω9 and 18:1ω7 was checked by transmethylation in leaking tubes resulting in a rise in boron trifluoride concentration leading to the disappearance, on the corresponding chromatogram, of peaks due to unsaturated species[18]. Double bond was situated on the molecule considering DMA 18:1ω6 retention, data from the literature[19,22] and elution order of the corresponding FAME.

The response-correcting factor (RCF) for a FAME or DMA (i), relative to I.S. (FAME 19:0) was calculated using the equation given by Patton *et al.*[23]:

$$RCF_i = \frac{A_{\text{I.S.}}\,[i]}{A_i\,[\text{I.S.}]}$$

where A stands for the area of a peak. Concentrations are expressed in terms of the weight of the corresponding FA or aldehyde.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of a standard FAME mixture used for calibration, whose composition approximates that of human erythrocytes (see Fig. 2). FAMEs and DMAs were calibrated using separated standard mixtures because the DMA 18:1ω6 that was used for calibration, but is absent in erythrocyte extracts, migrates too close to FAME 18:0. Hence, DMAs are not shown in Fig. 1.

The RCF values of the most abundant FAMEs and of some DMAs shown in Table I are always at least equal to 1. They are significantly higher for saturated FAMEs shorter than 16:0 and also for 24:0. Monounsaturation causes a moderate increase in the RCF, and only for FAs more than nineteen carbon atoms long. Polyunsaturated FAs (PUFAs) 20 and 22 carbon atoms long show a drastic increase in the RCF. Thus, if these RCF values are not determined and applied, the errors in the FA analyses are considerable, (over 30% for 20:4ω6) only for the PUFAs.

The reproducibility of these RCF values (in S.E.M.) inter- or intra-day is better than 2% except for FAMEs 10:0 and 12:0, for which it is 5%. These rather low variation coefficients for short-chain fatty acids are probably the consequence of using a cooled on-column injector, since split injectors lead to variation coefficients rising from 6% in the case of FAME 15:0, to 15% in the case of 14:0 (ref. 2). The RCF values are checked before each series of analyses, then about every week or month, and did not change over 6 months.

Positional isomers of FAs, such as 20:3ω6/ω3 and 18:1ω9/ω7 show similar responses. Thus the RCF of FAME 20:3ω6/ω3 was extrapolated to FAME 20:3ω9,

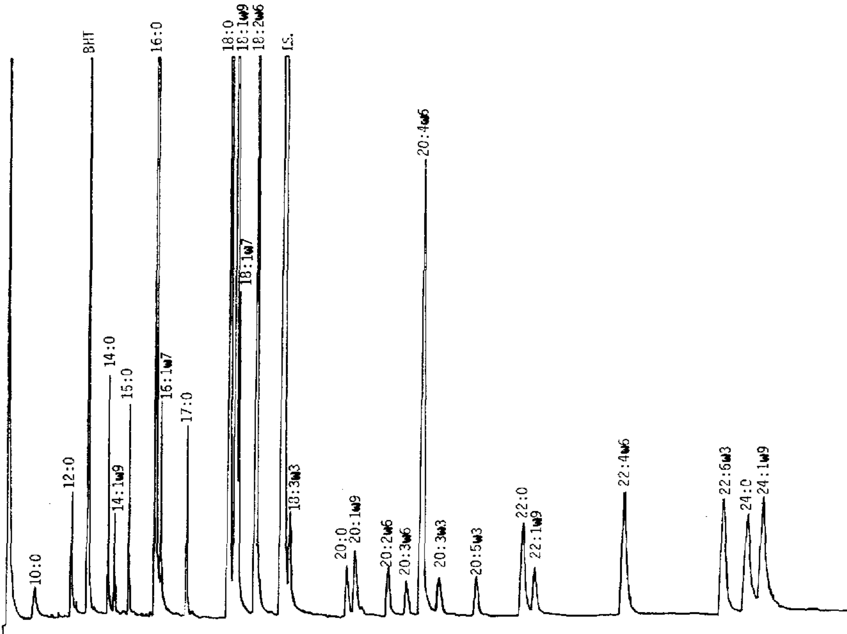

Fig. 1. Gas chromatogram of a standard mixture of fatty acid methyl esters on a 25 m × 0.32 mm I.D. FFAP capillary column; temperature programme, 15°C/min from 60 to 170°C followed by 1°C/min up to a final temperature of 225°C held for 10 min before cooling; injector, cooled on-column; detector temperatures, 255°C; carrier gas, helium at a mean velocity of 75.8 cm/s. I.S. = $C_{19:0}$ methyl ester.

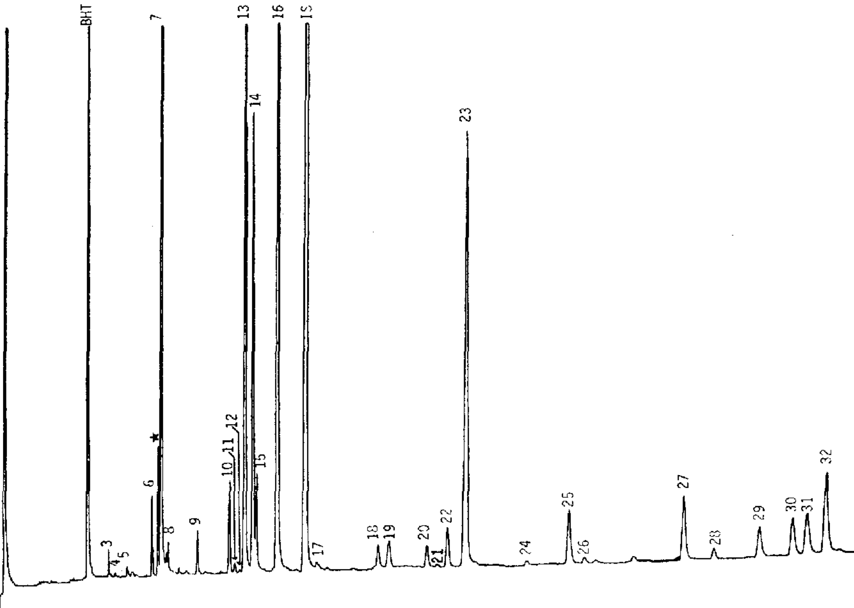

Fig. 2. Gas chromatogram of methylated total fatty acids and aldehydes from simian erythrocytes; for chromatographic conditions, see Fig. 1; compounds corresponding to numbers are listed in Table II. The peak marked with an asterisk is the sum of a BHT artefact (observed after derivatization) plus DMA 16:1 (see text for details).

TABLE I

RESPONSE-CORRECTING FACTORS OF FAMEs AND DMAs

Compounds are listed in elution order; operating conditions are detailed in Fig. 1; response-correcting factors (RCF values) of the fatty acids (F) or aldehydes (D) are given in relation to FA 19:0 (I.S.), and were calculated using the equation given in the text; means of more than five analyses of three standard mixtures.

Fatty acid or aldehyde	*RCF*	*Fatty acid or aldehyde*	*RCF*
F10:0	1.23	F18:3ω3	1.10
F12:0	1.10	F20:0	1.00
F14:0	1.07	F20:1ω9	1.07
F14:1ω9	1.10	F20:2ω6	1.18
F15:0	1.07	F20:3ω6	1.30
D16:0	1.05	F20:4ω6	1.46
F16:0	1.05	F20:3ω3	1.30
F16:1ω7	1.09	F22:0	1.05
F17:0	1.02	F22:1ω9	1.15
D18:1ω6	1.01	F22:4ω6	1.30
F18:0	1.00	F22:6ω3	1.40
F18:1ω9	1.01	F24:0	1.12
F18:1ω7	1.02	F24:1ω9	1.20
F18:2ω6	1.08		

and the RCF of DMA 18:1ω6 was extrapolated to DMA 18:1ω9 and 18:1ω7, compounds that were not commercially available. The RCF of both FAMEs 22:5 was calculated by averaging those of FAME 22:4 and 22:6. Contrary to Dodge and Phillips[19], we did not find any difference between the RCF for DMA (16:0 and 18:1) and the equivalent FAMEs. Consequently, the RCF of FAME 18:0 was extrapolated to DMA 18:0.

Fig. 2 shows a typical gas chromatogram of methylated total FAs and aldehydes from simian erythrocytes.

The column used in this study can separate most naturally occurring FAs and aldehydes, except *cis–trans* isomers (which was verified for oleic/elaidic isomers). However, *trans*-FAs represent no more than 1.8% of total human erythrocytes FAs[7]. Thus FFAP appears to be as suitable as Sil CP-88[2,10] for the FA analysis of biological samples, and more suitable than SP 2340 which is unable, for a similar column design, to separate the biologically significant arachidonic (20:4ω6) and behenic (22:0) methyl esters[7].

Like other authors[7,19], we observed the formation of a BHT derivative during the 90-min long, boron trifluoride-catalysed, methanolysis. This derivative elutes from the column between DMA 16:0 and FAME 16:0 (see Fig. 2). DMA 16:1, probably ω9, which is a very minor component according to TLC in toluene followed by DMA analysis, elutes with the same retention time. Hence the latter compound was not routinely quantified.

From the data presented in Table II, it can be seen that the total FA compositions of human and simian erythrocytes are fairly different. Firstly, simian erythrocytes did not show detectable amounts of the short-chain FAs 10:0 and 12:0.

TABLE II

TOTAL FATTY ACID AND ALDEHYDE COMPOSITION OF HUMAN AND SIMIAN ERYTHROCYTES

Fatty acids (F) and aldehydes (D) are numbered in elution order (see Fig. 2); values (mol per 100 mol of total fatty acid plus aldehyde) are means ± S.E.M. of four separate determinations; tr = traces (less than 0.1% of the total); comparisons were made by Student's *t*-test; M = sum of monounsaturated species; P = sum of polyunsaturated species.

Elution order	*Fatty acid or aldehyde*	*Human erythrocytes*	*Simian erythrocytes*
1	F10:0	0.86 ± 0.12	
2	F12:0	0.57 ± 0.13	
3	F14:0	2.2 ± 0.28	0.39 ± 0.01**
4	F14:1ω9	2.88 ± 0.22	0.17 ± 0.02**
5	F15:0	1.03 ± 0.12	0.26 ± 0.004**
6	D16:0	2.99 ± 0.30	1.25 ± 0.04**
7	F16:0	21.35 ± 0.87	21.96 ± 0.05
8	F16:1ω7	2.91 ± 0.07	1.09 ± 0.14**
9	F17:0	0.62 ± 0.05	0.63 ± 0.005
10	D18:0	2.76 ± 0.22	1.46 ± 0.03**
11	D18:1ω9	0.48 ± 0.04	0.20 ± 0.01**
12	D18:1ω7	trace	trace
13	F18:0	11.42 ± 0.56	13.39 ± 0.18*
14	F18:1ω9	12.71 ± 0.32	7.64 ± 0.25**
15	F18:1ω7	1.23 ± 0.07	1.48 ± 0.04*
16	F18:2ω6	7.09 ± 0.22	17.62 ± 0.35**
17	F18:3ω3	trace	0.40 ± 0.03**
18	F20:0	0.44 ± 0.04	0.68 ± 0.05*
19	F20:1ω9	0.45 ± 0.03	0.91 ± 0.04**
20	F20:2ω6	0.33 ± 0.02	0.76 ± 0.02**
21	F20:3ω9	trace	0.19 ± 0.004**
22	F20:3ω6	1.23 ± 0.09	1.37 ± 0.02
23	F20:4ω6	12.06 ± 0.69	15.95 ± 0.34**
24	F20:5ω3	trace	trace
25	F22:0	1.25 ± 0.02	1.59 ± 0.01**
26	F22:1ω9	trace	0.22 ± 0.01**
27	F22:4ω6	2.65 ± 0.07	2.44 ± 0.04
28	F22:5ω6	0.70 ± 0.05	0.43 ± 0.01**
29	F22:5ω3	1.13 ± 0.05	1.21 ± 0.03
30	F22:6ω3	2.99 ± 0.07	1.72 ± 0.04**
31	F24:0	2.93 ± 0.08	1.63 ± 0.04**
32	F24:1ω9	2.74 ± 0.07	2.90 ± 0.06
Sum for 10^{10} cells			
μmol		6.68 ± 0.19	6.34 ± 0.10
UI		1.28	1.49
$M/(M + P)$		0.454	0.259

* $P < 0.05$.
** $P < 0.01$.

The principal quantitative difference between both species is the greater (*ca.* 2.5-fold) amount of linoleic acid present in simian erythrocytes. This, together with their higher arachidonic acid content (1.3-fold) explains why the unsaturation index (UI) is higher in simian (UI = 1.49) than in human (UI = 1.28) erythrocytes. On the other hand,

monounsaturated species are not so abundant in simian erythrocytes (a quarter of the unsaturated species) as they are in human erythrocytes (about half of the unsaturated species; see Table II). Furthermore, data presented in Table II show that plasmalogens are more abundant in human erythrocytes (more than 2-fold), but have about the same aldehyde composition in both species. The results concerning human erythrocyte FAs and aldehydes composition are in good agreement with those previously reported[19].

Quantitatively, human and simian erythrocytes have similar FA contents (see Table II). When exposed to a buffered sodium chloride solution of gradually decreasing concentration, both erythrocytes exhibited a 50% lysis at 0.3% sodium chloride. Thus, they also show the same osmotic fragility under the conditions used[16].

Total fatty acid analysis, including a quantitation of vinyl-ether-linked aldehydes, provides a precise pattern of the saponifiable lipid content of a cell. Statistically significant differences were obtained between erythrocytes of related species and similar osmotic fragility. Using capillary GLC, detailed analysis as well as total quantitation is obtained in one injection, without the need for other chromatographic procedures[19], or ozonization. FAME analyses do not provide FA location on the glycero-backbone of PLs (as when using the phospholipase C–trimethylsilyl derivatives method[24,25]). Nevertheless, they are not restricted to glycero-PL analyses, their standards are easier available and the lower peak number makes total and precise quantitation easier. In future, both techniques may be used to provide complementary results.

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