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Short communication

A rapid GC–MS method for quantification of positional and geometric isomers of fatty acid methyl esters

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ABSTRACT

So far the most frequently used method for fatty acid (FA) analysis is GC coupled to flame ionization detector (FID). However, GC–FID does not allow profiling of FA synthesis and metabolism using stable isotopes. Here we present a rapid and sensitive GC–MS method for determination of fatty acid methyl esters (FAMEs). Fatty acid methylation was carried out by transesterification with acetyl-chloride and methanol. FAME separation applies a short and polar cyano-column resulting in an analysis time of 17.2 min. Separation was achieved for positional and geometrical (*cis*/*trans*) isomers with chain lengths between C8 and C28. Partial overlap of FAMEs (e.g. for C20:2 (*n*−6) and C21:0) could be resolved using selected ion monitoring (SIM). The precisions for human plasma samples were better than 10% coefficient of variation (CV) except for very low abundant FAs and LODs were in the low femtomol range on column. The developed GC–MS method also allows quantification of conjugated FAs such as conjugated linoleic acid (CLA) isomers because lowering the derivatization temperature from 95 °C to room temperature prevented cis to trans double bond isomerization. Finally, profiling of fatty acid synthesis and metabolism was exemplified with stable isotope labeling of macrophages using fatty acid precursors or deuterated fatty acids. In summary, we present a fast and robust GC–MS method for fatty acid profiling of positional and geometrical isomers including CLAs as well as very long chain fatty acids (VLCFAs). The method is suitable for both clinical studies and basic research including application of stable isotope compounds.

1. Introduction

Several studies have shown that *n*−3 poly-unsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) protect against coronary heart disease [1]. In contrast to *n*−6 fatty acids, *n*−3 fatty acids are precursors of signaling molecules with mainly anti-inflammatory effects [2]. Besides *n*−3 fatty acids, conjugated linoleic acid (CLA) has been reported to have various beneficial effects for human health, such as anti-atherogenic and anti-diabetic effects. CLA refers to a group of positional isomers of octadecadienoic acid with conjugated double bonds [3,4].

Thus, the rapid and precise determination of fatty acid profiles within nutritional, epidemiological and clinical studies is becoming of increasing interest for basic research and human health. Numerous different methods have been employed for total fatty acid analyses in biological and cellular samples. Most methods determining fatty acids in biological samples use gas chromatography coupled to a flame ionization detector (GC–FID). A powerful alternative to GC–FID for fatty acid analysis is gas chromatography coupled to mass spectrometry (GC–MS) combining the separation power of GC with structural information obtained by MS detection [5,6]. Additionally, GC–MS in contrast to GC–FID is suitable for metabolic studies with stable isotopes.

GC–MS methods for the analysis of fatty acids described so far suffer from analysis times longer than 30 min [7,8] or do not show separation of geometric and positional isomers [9,10]. Here we present a novel, fast GC–MS method based on a short, small diameter polar column characterized by a run time of 17.2 min and separation of geometrical and positional isomers of fatty acids.

2. Experimental

2.1. Reagents and standards

Acetyl-chloride, methanol and n-hexane were purchased from Merck. As standard a commercial fatty acid methyl ester (FAME)

Abbreviations: CLA, conjugated linoleic acid; CV, coefficient of variation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl esters; FID, flame ionization detector; PUFA, poly-unsaturated fatty acids; SIM, selected ion monitoring; VLCFA, very long chain fatty acids.

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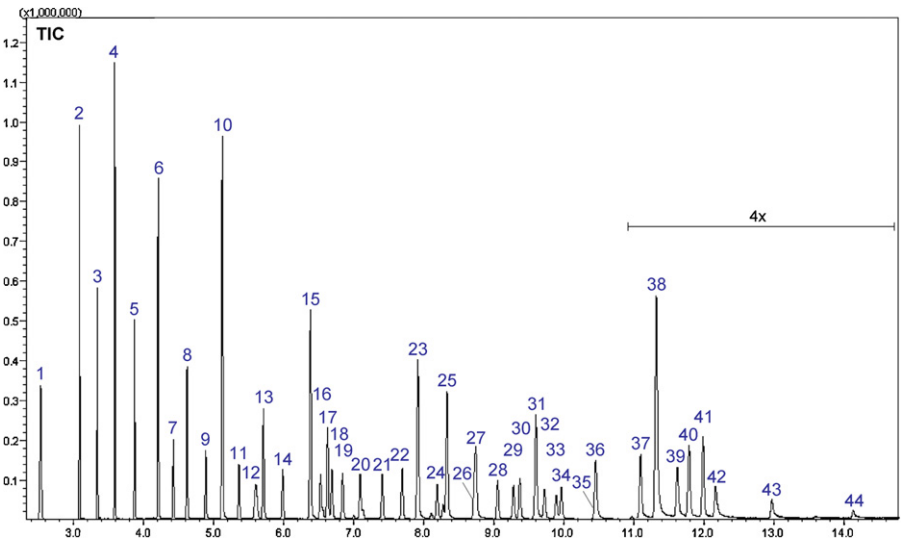

Fig. 1. Chromatogram of a FAME standard mixture. The peaks are assigned in Table 1.

standard mixture (FAME Mix 37, Supelco) spiked with FAMEs of phytanic acid, C18:1 (*cis*-11; *n*−7), C20:4 (*cis*-8,*cis*-11,*cis*-14,*cis*-17; *n*−3), C22:4 (*cis*-7,*cis*-10,*cis*-13,*cis*-16; *n*−6), C25:0, C26:0 and C28:0 (Larodan, Sweden) was used. The internal standards C13:0 and 19-methyleicosanoate (C21:0 *iso*) and the conjugated linoleic acid isomers C18:2 (*trans*-9,*trans*-11), C18:2 (*cis*-9,*trans*-11) and C18:2 (*trans*-10,*cis*-12) were obtained from Larodan (purity of CLAs 89–94%). Stock standard solutions were prepared in n-hexane and stored at −20 °C until usage. Internal standards were dissolved in methanol. The stable isotope labeled 2-^{13}C acetate was obtained from Cambridge Isotope Laboratories, D_3-C16:0 was purchased from Sigma.

Human EDTA-containing plasma was prepared from blood samples freshly drawn from healthy human volunteers; approval was obtained from the University of Regensburg ethics committee. All samples were stored at −80 °C prior to analysis.

2.2. Sample preparation

FAMEs were prepared as described previously [11]. Briefly, 10 μl plasma or cell homogenate corresponding to 50 μg protein were methylated in PTFE screw capped Pyrex tubes. 1 μg each of C13:0 and C21:0 *iso* were added as internal standards in 50 μl methanol. Derivatization was performed with 2 ml of methanolic acetyl-chloride (10%) and 500 μl n-hexane at 95 °C for 1 h under vigorous shaking in a water bath. For CLA analysis derivatization was carried out at RT over night. After cooling down to RT, 5 ml 6% potassium carbonate solution was added. 100 μl of the n-hexane top layer was transferred into a 500 μl auto-sampler vial and crimped.

2.3. Gas chromatography coupled to mass spectrometry

FAMEs were separated by a highly polar BPX70 column (10 m length, 0.10 mm diameter, 0.20 μm film thickness, SGE) coated with 70% cyanopropyl polysilphenyl-siloxane using a GC-2010 coupled to a GCMS-QP2010 detector from Shimadzu. The injection volume was 1 μl and a programmed temperature vaporizer (PTV) was used in the spilt mode 1:20 for 3 s, switched for 1.3 min to the splitless mode and a split ratio of 1:100 until end of the run. Injection temperature was 72 °C; after 3 s it was increased with 240 °C/min to 250 °C and hold for 15 min. The liner was packed with CarboFrit™ (Restek).

The temperature program was as follows: the initial oven temperature 50 °C was hold for 0.75 min, then programmed to increase with 40 °C/min to 155 °C, with 6 °C/min to 210 °C, to reach finally with 15 °C/min 250 °C and hold for 2 min. Helium was used as carrier gas with a constant linear velocity of 50 cm/s. The detector temperature was kept at 250 °C.

Characterization and identification of FAMEs was performed in the scan mode. Quantification was done by selected ion monitoring (SIM) mode of the most intense fragments (saturated: *m*/*z* 74, mono-unsaturated: *m*/*z* 55, di-unsaturated: *m*/*z* 67, poly-unsaturated: *m*/*z* 79). Data acquisition and processing were performed with the GC–MS Solution Software (Shimadzu). Quantification was based on an external calibration with C21:0 *iso* as internal standard. C13:0 added in a constant ratio to C21:0 *iso* was used as a quality control.

2.4. Stable isotope labeling of cells

To study fatty acid synthesis, human primary macrophages were incubated with 10 mM 2-^{13}C acetate for 24 h. For palmitate desaturation and elongation studies cells were incubated with 50 μM D_3-palmitate for 24 h. Cells were harvested in PBS and homogenized by sonication. Enrichment of 2-^{13}C-acetate in palmitate was analyzed by mass isotopomer distribution analysis using SIM of molecular ions (*m*/*z* 270–278) [12,13]. Desaturation and elongation of D_3-C16:0 was determined by SIM of specific molecular or fragment ions of the metabolites (D_3-C16:0: *m*/*z* 273, D_3-C16:1: *m*/*z* 242, D_3-C18:0: *m*/*z* 301).

3. Results and discussion

3.1. Gas chromatographic separation

Aim of this study was to develop an easy and fast method to quantify fatty acids in biological materials. Fatty acid methyl esters (FAMEs) were prepared by transesterification in a one-step reaction with acetyl chloride and methanol based on the protocol by Lepage and Roy [11]. To analyze biological samples with low concentration we decided to use a programmed temperature vaporizer (PTV) as sample inlet. Beside a gain of sensitivity, the use of a PTV showed superior peak shapes compared to conventional split/split-less operation (data not shown). In biological samples it is mandatory to separate positional and geometric isomers of fatty

Table 1
Positional and geometrical FA isomers, retention times (RT), fragment ions used for quantification and nominal masses of the FAMEs. Intra- and inter-day precisions, measured in human plasma samples ($n = 6$) at low FA levels (level 1) or high FA levels (level 2). The number refers to the chromatogram shown in Fig. 1.

Nr.	Fatty acid	cis/trans	RT	*m/z*	Mass	Intra-day precision human plasma ($n = 6$)				Inter-day precision human plasma ($n = 6$)			
						Level 1		Level 2		Level 1		Level 2	
						mmol/l	CV	mmol/l	CV	mmol/l	CV	mmol/l	CV
1	FA 8:0		2.54	74	158								
2	FA 10:0		3.09	74	186	0.06	2.0	0.02	4.6	0.07	5.7	0.02	10.2
3	FA 11:0		3.34	74	200								
4	FA 12:0		3.60	74	214	0.01	5.4	0.05	3.4	0.01	17.7	0.05	5.5
5	FA 13:0		3.88	74	228								
6	FA 14:0		4.23	74	242	0.09	1.8	0.41	3.7	0.10	6.6	0.40	4.4
7	FA 14:1	c9	4.44	55	240								
8	FA 15:0		4.64	74	256	0.02	2.4	0.06	3.4	0.03	5.4	0.06	4.7
–	FA 15:0 (4-Me)		4.84	101	312								
9	FA 15:1	c10	4.91	55	254								
10	FA 16:0		5.15	74	270	2.18	1.6	10.67	3.8	2.35	6.3	10.43	6.2
11	FA 16:1 (*n*−7)	c9	5.39	55	268	0.16	4.0	1.30	4.3	0.17	6.0	1.30	4.6
12	FA 16:0 (4-Me)		5.62	101	326								
13	FA 17:0		5.74	74	284	0.03	4.2	0.07	3.4	0.03	5.8	0.07	5.5
14	FA 17:1	c10	6.01	55	282								
15	FA 18:0		6.41	74	298	0.65	2.5	2.43	3.4	0.69	5.9	2.39	5.8
16	FA 18:1 (*n*−9)	t9	6.55	55	296								
17	FA 18:1 (*n*−9)	c9	6.65	55	296	1.89	2.7	10.05	4.7	2.04	7.3	9.93	5.8
18	FA 18:1 (*n*−7)	c11	6.72	55	296	0.12	4.2	0.87	4.8	0.13	7.4	0.89	3.8
19	FA 18:2 (*n*−6)	t9,t12	6.87	67	294								
20	FA 18:2 (*n*−6)	c9,c12	7.12	67	294	1.88	2.1	7.76	4.9	2.02	7.7	7.68	6.1
21	FA 18:3 (*n*−6)	c6,c9,c12	7.43	79	292	0.02	2.4	0.06	2.8	0.02	7.9	0.06	4.2
22	FA 18:3 (*n*−3)	c9,c12,c15	7.71	79	292	0.06	2.5	0.28	4.0	0.07	7.6	0.27	5.5
23	FA 20:0		7.94	74	326	0.01	7.1	0.05	7.5	0.01	6.8	0.05	9.4
24	FA 20:1 (*n*−9)	c11	8.21	55	324	0.01	3.5	0.09	6.7	0.01	14.6	0.09	8.1
25	FA 21:0 (iso)		8.35	74	340								
26	FA 20:2 (*n*−6)	11c,14c	8.74	67	322								
27	FA 21:0		8.78	74	340								
28	FA 20:3 (*n*−6)	c8,c11,c14	9.08	79	320	0.07	3.9	0.38	4.2	0.08	8.5	0.38	6.6
29	FA 20:4 (*n*−6)	c5,c8,c11,c14	9.30	79	318	0.32	2.3	1.24	4.0	0.36	9.1	1.22	5.7
30	FA 20:3 (*n*−3)	c11,c14,c17	9.40	79	320								
31	FA 22:0		9.62	74	354	0.02	6.1	0.08	3.6	0.03	8.8	0.08	7.5
32	FA 20:4 (*n*−3)	c8,c11,c14,c17	9.76	79	318								
33	FA 22:1 (*n*−9)	c13	9.92	55	352								
34	FA 20:5 (*n*−3)	c5,c8,c11,c14,c17	9.98	79	316	0.04	7.4	0.07	4.8	0.05	11.0	0.07	4.6
35	FA 22:2 (*n*−6)	c13,c16	10.47	67	350								
36	FA 23:0		10.50	74	368								
37	FA 22:4 (*n*−6)	c7,c10,c13,c16	11.13	79	346	0.01	6.3	0.05	4.8	0.01	8.1	0.05	5.8
38	FA 24:0		11.35	74	382	0.01	9.6	0.04	9.4	0.02	10.9	0.05	6.5
39	FA 24:1 (*n*−9)	c15	11.64	55	380	0.05	4.7	0.23	8.1	0.05	8.4	0.23	7.1
40	FA 22:5 (*n*−3)	c7,c10,c13,c16,c19	11.82	79	344	0.02	7.5	0.09	5.2	0.02	10.7	0.08	6.7
41	FA 22:6 (*n*−3)	c4,c7,c10,c13,c16,c19	12.01	79	342	0.08	5.3	0.29	5.5	0.09	9.6	0.29	5.9
42	FA 25:0		12.21	74	396								
43	FA 26:0		13.00	74	410								
44	FA 28:0		14.16	74	438								

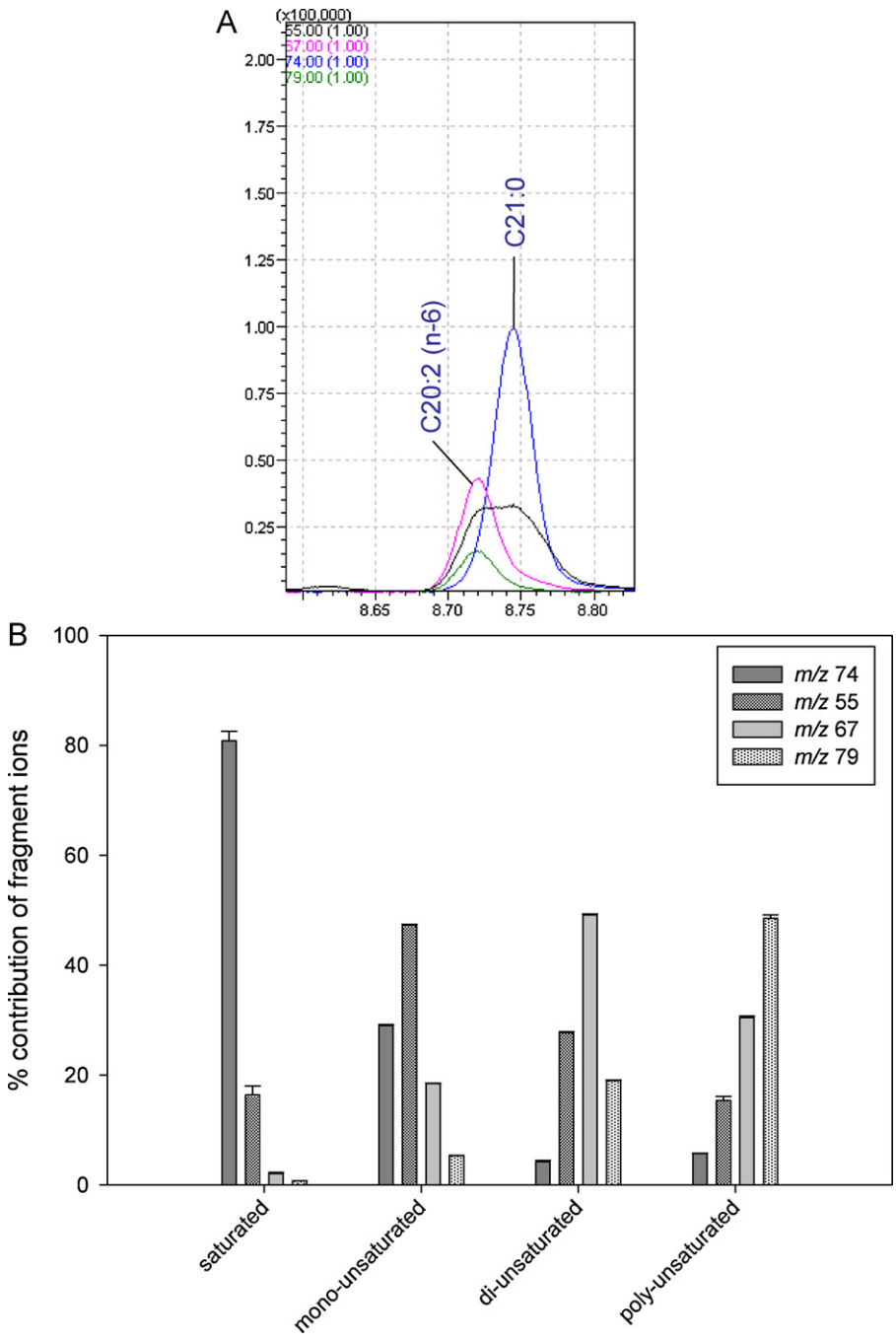

Fig. 2. (A) Differentiation of overlapping peaks using selected ion monitoring (SIM). C20:2 (*n*−6) was quantified with *m*/*z* 67, whereas C21:0 was quantified with *m*/*z* 74 (peaks 26, 27 from Fig. 1). (B) Percent contribution of the fragment ions *m*/*z* 74, *m*/*z* 55, *m*/*z* 67 and *m*/*z* 79 to saturated (C16:0), mono-unsaturated (C18:1 (*n*−9)), di-unsaturated (C18:2 (*n*−6)) and poly-unsaturated FAs (C20:4 (*n*−6)). Mean and standard deviation (*n* = 6).

acids to avoid mis-interpretation [2]. Since polar columns are well suited for isomer separation we chose a column coated with 70% cyanopropyl polysilphenyl-siloxane for this method. In contrast to frequently applied WAX columns, the selected cyano column shows a high temperature stability allowing analysis of very long chain FAs (up to C28:0) and low column bleeding facilitating a MS coupling. In order to achieve a rapid analysis a short (10 m) column with a small internal diameter (0.10 mm) was selected.

We developed a three step temperature program to achieve a clear separation of geometric and positional isomers. Fig. 1 shows a typical GC chromatogram of a FAME standard mix containing fatty acids with chain lengths between C8 and C28. Retention times increase with the number of double bonds and show the following characteristics concerning position and geometry: *n*−6 < *n*−3; trans- < cis-double bonds. Most FAMEs were clearly separated except an overlap of C20:2 (*n*−6) with C21:0 (Fig. 1, peak 26/27), and C22:2 (*n*−6) with C23:0 (Fig. 1, peak 35/36). However, selected ion monitoring (SIM) helped to separate these species (Fig. 2A) because FAME fragmentation results in characteristic pattern with the main ions *m*/*z* 74 for saturated FAs, *m*/*z* 55 for mono-unsaturated FAs, *m*/*z* 67 for di-unsaturated FAs and *m*/*z* 79 for PUFAs (Fig. 2B). Thus, contribution of *m*/*z* 74 to di-unsaturated and the contribution of *m*/*z* 67 to saturated FAs is less than 9%. Moreover, the overlap of di-unsaturated C20 and C22 with the odd-chain C21:0 and C23:0 is not of high relevance for biological samples since these saturated species are usually not present in

Table 2
Percent of detected CLA isomers after derivatization for 1 h at 95 °C or over night at RT.

Experimental conditions	95 °C, 1 h						Room temperature, over night					
Supplied CLA isomer	c9,t11		t10,c12		t9,t11		c9,t11		t10,c12		t9,t11	
	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
c9,t11	14.88	0.32	8.72	0.10	76.40	0.24	92.69	0.17	0.78	0.13	6.54	0.09
t10,c12	8.12	0.21	18.41	0.67	73.47	0.87	1.69	0.04	91.96	0.24	6.36	0.21
t9,t11	8.41	0.09	3.81	0.29	87.79	0.22	0.49	0.06	0.14	0.07	99.36	0.04

substantial quantities. To show the broad applicability of this method we analyzed human plasma (Suppl. Fig. 1A and B) and primary human monocytes derived macrophages (Suppl. Fig. 2).

In summary, we could achieve FAME separation within 17.2 min which is to the best of our knowledge currently the fastest GC–MS method described for quantification of FAMEs in biological samples (Fig. 1).

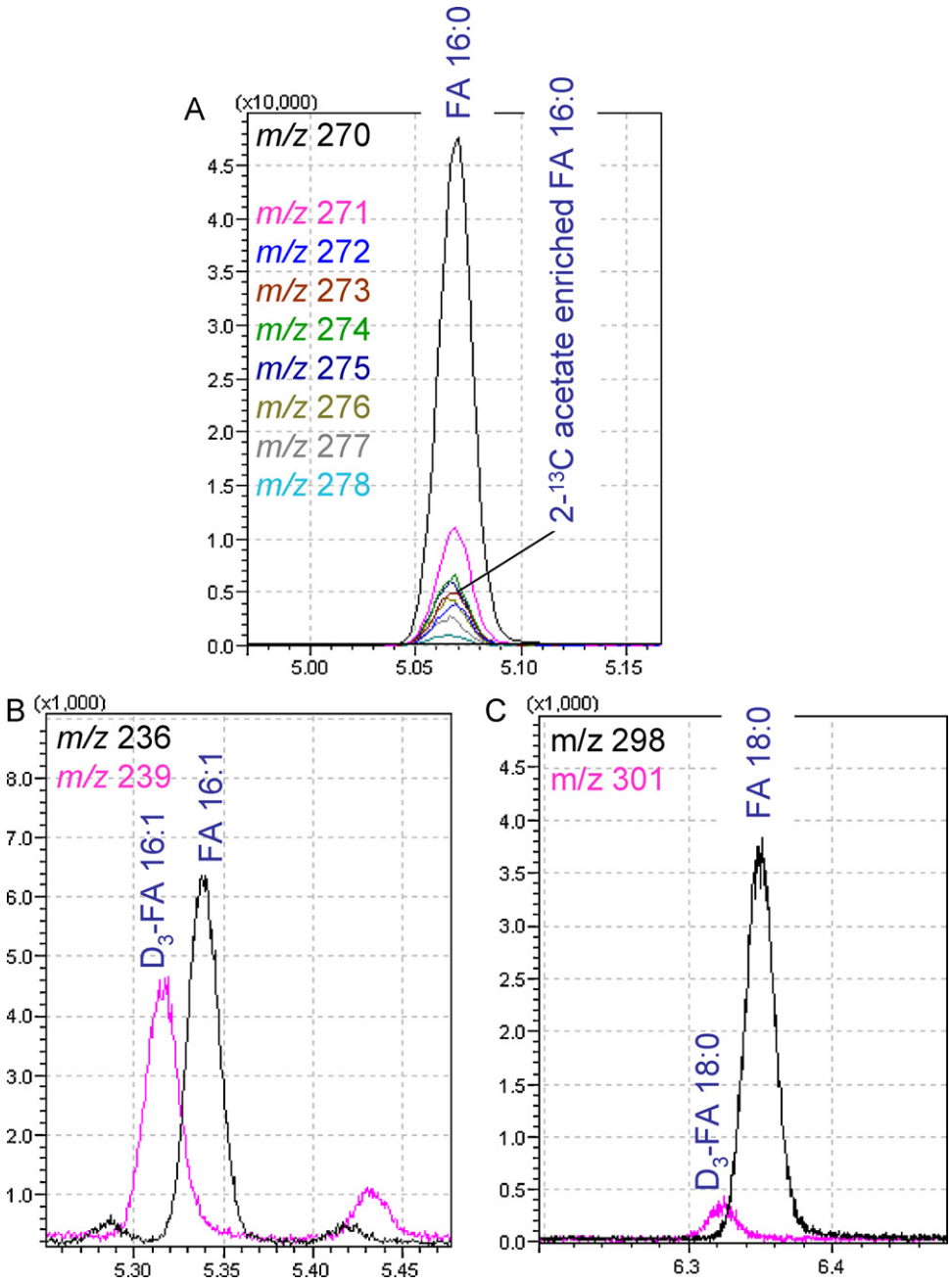

Fig. 3. (A) Analysis of isotope enrichment in palmitate in primary human macrophages incubated for 24 h with 2-^{13}C-acetate to profile FA synthesis. (B) Analysis of FA desaturation using SIM of specific ions (D_3-C16:0: *m/z* 273; D_3-C16:1: *m/z* 242) in primary human macrophages incubated with D_3-palmitate for 24 h. (C) Analysis of FA elongation using SIM of specific ions (D_3-C18:0: *m/z* 301) in primary human macrophages incubated with D_3-palmitate for 24 h.

3.2. Internal standard selection and quantification

To quantify we performed SIM as described above and selected two internal standards C13:0 and C21:0 *iso* (methyl 19-methyleicosanoate). The internal standards were selected instead of the commonly used n-heptadecanoic acid C17:0 (low levels were found in plasma) based on the following two criteria: These fatty acids do not naturally occur in biological samples and their retention times do not interfere with other FAMEs. Moreover, two internal standards allow a quality check of each sample since their ratio is expected to be constant. So C21:0 *iso* was used for quantification since its retention time is in the middle of the run and C13:0 was applied as quality control. Furthermore, ions not used for quantification were used as reference ions to check peak purity. The external calibration consists of four levels with concentrations up to 12 μg/ml with a constant concentration of C21:0 *iso* (2 μg/ml). Calibration lines were linear in this range and showed regression line coefficients $R^2 > 0.995$.

3.3. Method validation

The method reproducibility was examined by determining the intra-day and inter-day precisions in two plasma samples with low and high lipid contents ($n = 6$). 24 different FAMEs were detected in these plasma samples. The major FAs were C16:1, C18:0, C18:1 ($n-7$), C18:2 ($n-6$) and C20:4 ($n-6$) (Table 1). We observed a concentration dependency of the coefficients of variation (CVs). FAs with concentration above 0.02 mmol/L showed overall CVs below 10% whereas lower concentrated species revealed in part CVs over 10%.

The limit of detection (LOD) was determined from diluted standard samples as a signal to noise ratio of 3. LOD varied between 1.9 and 16 fmol on column and increased with FA chain length and degree of unsaturation. The linear range was determined for FAME 16:0 (Suppl. Fig. 3). A linear response was found up to 100 nmol/ml (equal to 100 pmol on column and a dynamic range of ~4 orders of magnitude).

3.4. Determination of conjugated linoleic acid (CLA) isomers

As our laboratory has a major interest in cellular effects of CLA [3,14], we optimized our method to determine individual CLA isomers in cellular samples. CLAs are known to isomerize (cis to trans), particularly under high temperatures [15,16]. Hence, the most critical factors are incubation temperature and time during the sample preparation procedure. Table 2 shows a typical result for the CLA isomers C18:2 (*trans*-9,*trans*-11); C18:2 (*cis*-9,*trans*-11) and C18:2 (*trans*-10,*cis*-12) when sample preparation was performed at 95 °C for 1 h (isomer purity before derivatization: 89–94%). Bondia-Pons et al. showed that CLA isomerization can be avoided by changing from acid-catalyzed methylation to alkali-catalyzed methylation using sodium methylate and boron trifluoride-methanol [17]. Instead of changing the methylation reagent, we preferred to optimize the incubation time and temperature from 1 h at 95 °C to over night at RT, which successfully prevented CLA isomerization (Table 2). This alternative derivatization method is suitable for all fatty acids including CLA analysis. The only disadvantage is the longer time for sample preparation (over night vs. 1 h incubation time). The CLA isomers eluted in the order C18:2 *cis*-9,*trans*-11 (RT 7.90), C18:2 *trans*-10,*cis*-12 (RT 8.07), and C18:2 *trans*-9,*trans*-11(RT 8.28). The peak for C18:2 (*trans*-9,*trans*-11) overlapped with the internal standard *iso* C21:0, but may be differentiated by SIM mode as described above (see Suppl. Fig. 4 for an example).

3.5. Metabolic studies with stable isotopes

A major advantage of GC–MS compared to GC–FID is its potential to differentiate stable isotope labeled compounds from naturally occurring. Thus, the presented method facilitates together with the use of stable isotope labeled substrates studies of fatty acid synthesis and metabolism. To show an example for fatty acid synthesis monitoring, we supplied cells with stable isotope labeled 2-^{13}C-acetate and analyzed palmitate isotopomer composition by SIM of its molecular ion (m/z 270) and the isotopes $m+1$ to $m+8$ (m/z 271–278). Whereas $m+1$ (m/z 271) and $m+2$ (m/z 272) could also be observed in unlabeled samples, the isotopes $m+3$ to $m+8$ were only detected after acetate labeling (Fig. 3A). Calculation of isotope enrichment by mass isotopomer distribution analysis has been extensively described by Lee et al. [12,13]. Another application of stable isotopes labeling is shown with the introduction of stable isotope labeled palmitate (D_3-C16:0) to monitor cellular desaturation (Fig. 3B) and elongation (Fig. 3C) activity. Desaturation and elongation indices can be calculated by D_3-C16:1/D_3-C16:0 and D_3-C16:0/D_3-C18:0, respectively. Applications for cellular metabolic profiling studies using stable isotope substrates have been published previously [18,19].

4. Conclusion

The developed GC–MS method provides a rapid separation of positional and geometrical isomers of fatty acid methyl esters (17.2 min). Its application to human plasma has been shown to be robust (several thousand injections with one column) and reliable for routine analysis. In contrast to other methods, we used a short and highly polar column which allows analysis of complex mixtures of fatty acids including VLCFAs and CLA isomers. As a major advantage compared to GC–FID, GC–MS may be applied to study fatty acid metabolism by the use of stable isotope labeled substrates. This method has been applied successfully already in various studies [14,18–20].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.04.015.

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