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Targeted quantitative analysis of fatty acids in atherosclerotic plaques by high sensitivity liquid chromatography/tandem mass spectrometry

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Abstract

The quantitative analysis of fatty acid composition in atherosclerotic plaques provides a way to monitor the underlying etiology of atherosclerosis. Previously, the method of choice for analyzing fatty acids in biological samples was gas chromatography/mass spectrometry (GC/MS); however, recent developments in electrospray ionization (ESI)/liquid chromatography (LC)/tandem mass spectrometry have made it a superior alternative. Previous research has largely focused on global analyses of intact lipids rather than more targeted analysis of the fatty acids themselves. We have now developed a targeted, stable isotope dilution LC–electrospray ionization/multiple reaction monitoring/MS method for the quantitative analysis of 10 fatty acids (myristic, palmitic, stearic, oleic, linoleic, α-linolenic, γ-linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acids) using their trimethylaminoethyl ester (TMAE) derivatives to improve sensitivity. The method was validated, had a detection limit in the fmol range, and was used in the analysis of fatty acids in atherosclerotic plaques from carotid arteries.

Keywords: Trimethylaminoethyl ester; Fatty acids; Stable isotope dilution; Liquid chromatography/mass spectrometry; Electrospray ionization; Atherosclerosis

1. Introduction

Fatty acids play a role in the pathology of multiple diseases [1], e.g., asthma [2], cystic fibrosis [3], type II diabetes [4], depression and epilepsy [5–7], inflammatory bowel diseases [8,9], atherosclerosis [10,11], and breast and ovarian cancers [12,13]. Thus, researchers began to develop methodology to monitor lipid composition in biological samples [14]. The earliest attempts used gas chromatography (GC) to separate the fatty acids [15,16]. These attempts were followed by the coupling of GC and mass spectrometry (MS) to provide structural data, such as the position of double bonds and heteroatoms [17–19]. GC/MS analysis became the method of choice, and GC capillary columns were used in combination with positive chemical ionization/MS to analyze the methyl ester derivatives of isomeric fatty acids [20,21]. However, protonated methyl ester derivatives do not readily fragment, preventing collision-induced dissociation (CID) and MS/MS studies that would result in increased specificity.

High performance liquid chromatography (HPLC) systems have also been used for the global analysis of lipids. GC/MS and LC–UV analysis of fatty acids are roughly equivalent in terms of ease of use and sensitivity. However, LC–UV methods using phenacyl derivatives to improve chromatographic separation improved sensitivity 20–50-fold over GC/hydrogen flame detector methods [22,23]. Miwa et al. have reported LC–UV analysis of more than 20 fatty acids including geometrical isomers as their 2-nitrophenyl hydrazine derivatives with limits of detection from 500 fmol to 1 pmol [24]. Sensitivity is further increased in LC–MS studies by using atmospheric pressure chemical ionization (APCI), especially for the analysis of unsaturated fatty acids. Overall, LC–MS methodology is much more sensitive than LC–UV methods, and LC–MS/MS methods with selected ion monitoring (SIM) are even more sensitive [25].

In 2000, Rezanka [26] showed that LC–APCI/MS is as reliable as GC/MS for the analysis of methyl esters, and

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this methodology allowed for the analysis of thermally unstable polyunsaturated fatty acids. LC–APCI/MS/MS analysis of pentafluorobenzyl (PFB) derivatives of fatty acids provided intense negative ions that readily underwent CID to give useful product ions that can be used for high sensitivity applications [27]. This technique has proven to be particularly useful for analyzing the PFB derivatives of unsaturated fatty acids and their chiral metabolites [28,29]. This methodology, however, is not applicable to the analysis of saturated fatty acids and cannot be used to monitor subtle quantitative changes that occur in fatty acid composition in tissues. On the other hand, Johnson et al. found that trimethlaminoethyl (TMAE) derivatives (quaternary ammonium salts) of long chain fatty acids were readily ionized under positive ESI conditions. They also readily underwent CID to give analytically useful product ions [30,31].

In order to monitor saturated and unsaturated fatty acids in atherosclerotic plaques, we developed a targeted stable isotope dilution LC–ESI/MRM/MS method using TMAE derivatives. We found that TMAE derivatives of fatty acids have excellent positive ESI characteristics for a wide range of saturated, unsaturated, and polyunsaturated fatty acids, in addition to their excellent CID and chromatographic properties. This method allows for detection of 10 fatty acids (Fig. 1) in a single run. All analyses were performed on atherosclerotic plaques from endarterectomized patients. The method was validated and had a detection limit in the fmol range.

2. Materials and methods

2.1. Chemicals and materials

Oleic acid (OA, C18:1), linoleic acid (LA, C18:2), alpha linolenic acid (αLNA, C18:3), gamma linolenic acid (γLNA, C18:3), arachidonic acid (AA, C20:4), eicosapentaenoic acid (EPA, C20:5), docosahexaenoic acid (DHA, C22:6), linoleic-d_4 acid ([$^2H_4$]-LA), and AA-d_8 acid ([$^2H_8$]-AA) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Myristic acid (MA, C14:0), palmitic acid (PA, C16:0), stearic acid (SA, C18:0), [$^2H_{27}$]-MA, [$^{13}C_1$]-PA, [$^2H_3$]-SA, oxalyl chloride solution in dichloromethane, *N*,*N*-dimethylethanolamine, diethyl ether, hydrochloric acid, potassium hydroxide, and sodium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). [$^{13}C_{18}$]-αLNA and [$^2H_4$]-LA were purchased from Spectra gases (Columbia, MD, USA). Sulfuric acid and sodium chromate were purchased from Alfa Aesar (Ward Hill, MA, USA). Methyl iodide was purchased from Chem Service (West Chester, PA, USA). HPLC-grade ammonium acetate, HPLC-grade water, acetonitrile, ethanol, methanol, chloroform, dichloromethane, and hexane were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Gases were supplied by BOC Gases (Lebanon, NJ, USA). Atherosclerotic plaques were obtained from endarterectomized patients in the SS. Annunziata Hospital in Chieti, Italy. The Varian Pursuit diphenyl 3 μm column (150 mm × 20 mm i.d.) was obtained from Varian (Palo Alto, CA, USA).

2.2. Removal of fatty acids from glassware

To avoid fatty acid contamination of the glass tubes used to perform the lipid extraction, hydrolysis, and derivatization, the glass tubes were cleaned with a sulphochromic mixture, an indiscriminative oxidizing agent obtained by the addition of aqueous sodium chromate (1 g/ml) to concentrated sulfuric acid (100 ml). The glass tubes and pipettes needed for the extraction were soaked overnight in the sulphochromic mixture, then they were repeatedly rinsed with distilled water and dried in an oven.

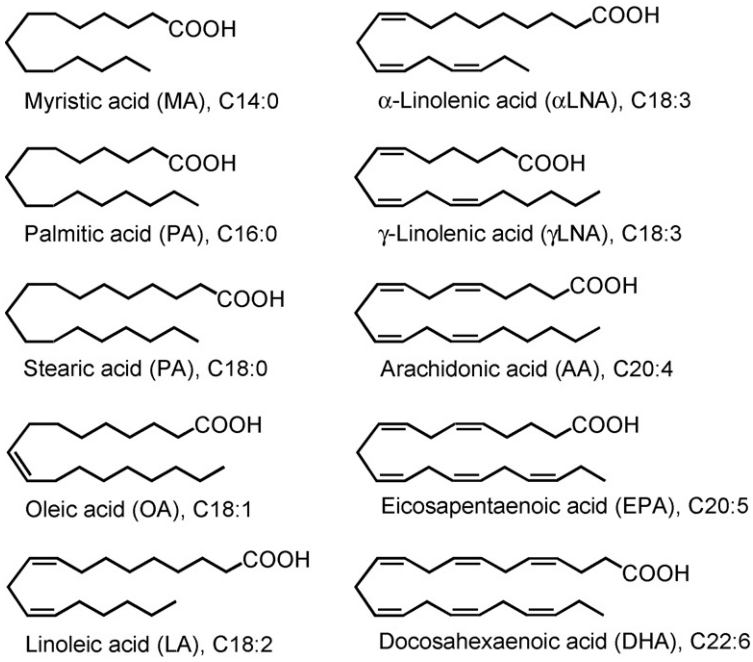

Fig. 1. Structures of fatty acids analyzed in the current study. The 14 in C14:0 is the number of carbons; the 0 after the colon is the number of *cis* double bonds in the carbon chain.

2.3. Tissue sample preparation and extraction

Both the fatty acid standards and tissue samples underwent the same steps to obtain TMAE-derivatives for LC–MS/MS analysis. An internal standard mixture was prepared containing [$^{13}C_1$]-PA, [$^{2}H_3$]-SA, [$^{13}C_{18}$]-OA, and [$^{2}H_4$]-LA at 2 μg/μl and [$^{2}H_{27}$]-MA, [$^{13}C_{18}$]-αLNA, and [$^{2}H_8$]-AA at 0.1 μg/μl. The Folch method was modified to extract the fatty acids from the plaques [32]. Weighed fractions of homogenized and lyophilized tissue samples from endarterectomized patients (1.0–5.0 mg) were placed in separate tubes, and the internal standard mixture (10 μl/sample) was added. Seven standards were prepared containing PA, SA, OA, LA in the range of 0.2–20 μg and MA, AA, EPA, DHA, αLNA, and γLNA in the range of 10–1000 ng, and the internal standard mixture (10 μl/standard) was added. Then a chloroform/methanol solution (5 ml, 2:1, v/v) was added to each tube, and the tubes were shaken for 15 min in a high speed shaker and centrifuged at 5000 rpm for 10 min. The supernatants were transferred to new tubes and washed with 1 ml of 0.9% sodium chloride solution. After vortex mixing and centrifugation to separate the two phases, the upper phases from each tube were removed. The steps for washing with 0.9% NaCl solution and separation were repeated and combined lower phases were evaporated to dryness under the nitrogen.

2.4. Fatty acid hydrolysis

The lipid esters were solubilized with a methanol/chloroform solution (850 μl, 8:1, v/v). Hydrolysis was performed under basic conditions (150 μl, 40% KOH in water) in a nitrogen atmosphere at 60 °C for 30 min. At the end of the incubation, 700 μl of 50 mM phosphate buffer (pH 7.4) was added and the pH of reaction mixture was adjusted with 300 μl of 2.5 M hydrochloric acid to pH 2–3. Free fatty acids were extracted with diethyl ether/hexane (2 ml, 1:1, v/v) twice. The organic layer was then evaporated to dryness under nitrogen.

2.5. Fatty acid derivatization

The free fatty acids were analyzed as TMAE esters iodide in positive ionization mode. The reaction was performed using a modification of the method of Johnson [30]. The dried residue from the hydrolysis was treated with 200 μl of oxalyl chloride (2 M in dichloromethane) at 65 °C for 5 min and evaporated to dryness under nitrogen. Dimethylaminoethanol (60 μl) was added to the residue, and the samples were left at room temperature for 5 min followed by evaporation to dryness under nitrogen. A methyl iodide/methanol mixture (150 μl, 1:1, v/v) was added to obtain the positively charged iodide trimethylaminoethyl ester. After 2 min at room temperature, the reaction mixture was evaporated, and the residue was dissolved in ethanol (100 μl). The TMAE derivatives were further diluted at a ratio 1:1000 and analyzed by LC–ESI/MRM/MS. The solution is stable for at least 1 week at room temperature [30].

2.6. LC

Reversed-phase LC–MS analysis was performed using a Hitachi L-2130 pump equipped with Hitachi Autosampler L-2200 (Hitachi, San Jose, CA, USA). The separation employed a Varian Pursuit Diphenyl 3 μm column (150 mm × 2 mm i.d., 3 μm). Solvent A was 5 mM ammonium acetate in water, solvent B was 5 mM ammonium acetate in acetonitrile. The gradient elution was as follows: 40% B at 0 min, 40% B at 3 min, 60% B at 13 min, 80% B at 15 min, 80% B at 20 min, 40% B at 25 min, 40% B at 35 min. The flow rate was 0.5 ml/min. The separation was performed at ambient temperature. After diluting the samples 1:1000 to reach pg/μl concentration, they were maintained at 4 °C in the autosampler tray with injections of 10 μl/sample.

2.7. MS

Quantitative analysis of fatty acids was conducted using a Finnigan TSQ Quantum Ultra AM mass spectrometer (Thermo Electron Corporation, San Jose, CA) equipped with an ESI source in the positive ionization mode. Operating conditions were as follows: spray voltage was 4.5 kV, and the heated capillary temperature was 250 °C. Nitrogen was used for the sheath gas and auxiliary gas, set at 45 psi and 10 (in arbitrary units), respectively. CID was performed using argon as the collision gas at 1.5 mTorr in the second (rf-only) quadrupole. An additional dc offset voltage was applied to the region of the second multipole ion guide (Q_0) at 10 V to impart enough translational kinetic energy to the ions so that solvent adduct ions dissociate to form sample ions. For MRM analysis, unit resolution was maintained for both precursor and product ions. The MRM transitions monitored are reported in Table 1.

Table 1
Parent and product ions of the fatty acids with applied collision energy to conduct LC–LC–MRM/MS analysis

Fatty acid	$[M]^+$ (Da)	$[M-59]^+$ (Da)	Collision energy (eV)
MA-TMAE	314	255	25
PA-TMAE	342	283	25
SA-TMAE	370	311	25
OA-TMAE	368	309	23
LA-TMAE	366	307	20
αLNA and γLNA-TMAE	364	305	18
γLNA-TMAE	364	243	18
AA-TMAE	390	331	18
EPA-TMAE	388	329	17
DHA-TMAE	414	355	19
[$^{2}H_{27}$]-MA-TMAE	341	282	25
[$^{13}C1$]-PA-TMAE	343	284	25
[$^{2}H_3$]-SA-TMAE	373	314	25
[$^{13}C1_8$]-OA-TMAE	386	327	23
[$^{2}H_4$]-LA-TMAE	370	311	20
[$^{13}C1_8$]-αLNA-TMAE	382	323	18
[$^{2}H_8$]-AA-TMAE	398	339	18

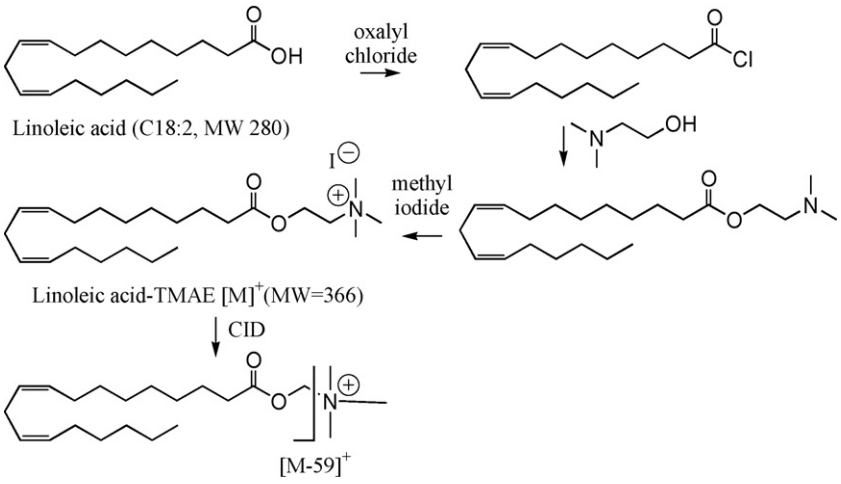

Fig. 2. TMAE iodide derivatization of LA and its fragmentation on MS/MS analysis.

3. Results

3.1. MS analysis of standard fatty acids

The ESI–MS analysis and MS/MS spectra of TMAE fatty acids showed identical fragmentation patterns for all the acids, from the $[M]^+$ ion (fatty acid plus the 89 Da from the trimethylaminoethyl group) to the corresponding $[M-59]^+$ ion (derived from the neutral loss of the trimethylamine group, Fig. 2 lower). For γLNA acid we used the $[M-121]^+$ ion to distinguish it from αLNA acid, which has the same retention time in the LC chromatogram (Fig. 3). The transition from *m/z* 370 to *m/z* 311 is typical of both $[^2H_4]$-LA and SA; however, they were readily separated by LC with retention times of 10.76 and 13.36 min,

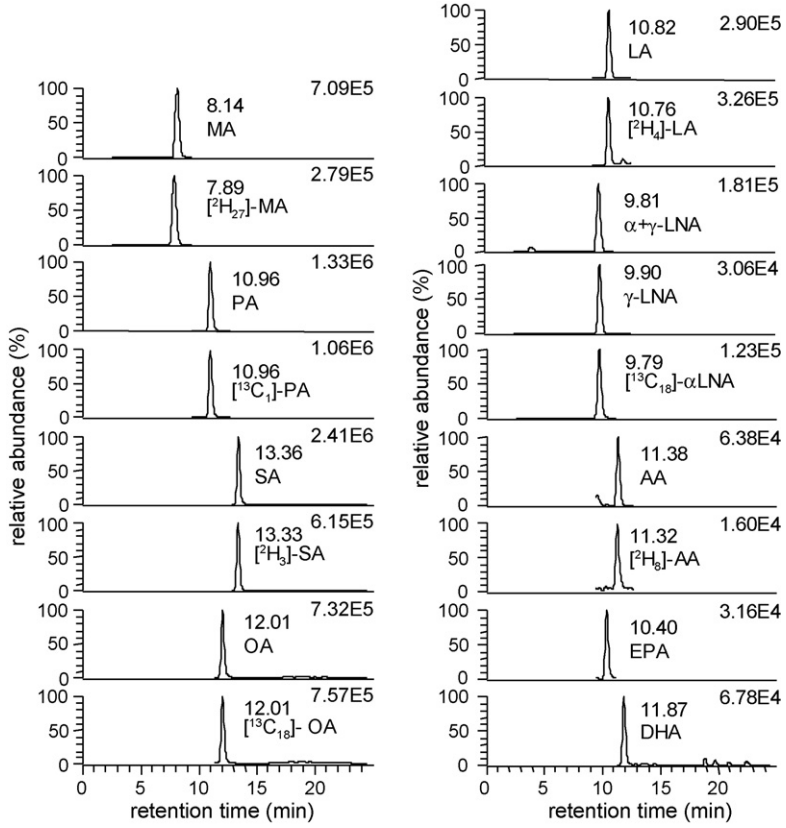

Fig. 3. LC–ESI/MRM/MS analysis of a mixture of authentic fatty acid standards together with corresponding internal standards as TMAE iodide derivatives. MRM chromatograms are shown for: (Left panel) MA-TMAE (*m/z* 314 → 255), $[^2H_{27}]$-MA-TMAE (*m/z* 341 → 282), PA-TMAE (*m/z* 342 → 283), $[^{13}C_1]$-PA-TMAE (*m/z* 343 → 284), SA-TMAE (*m/z* 370 → 311), $[^2H_3]$-SA-TMAE (*m/z* 373 → 314), OA-TMAE (*m/z* 368 → 309), $[^{13}C_{18}]$-OA-TMAE (*m/z* 386 → 327), (Right panel) LA-TMAE (*m/z* 366 → 307), $[^2H_4]$-LA-TMAE (*m/z* 370 → 311), αLNA- and γLNA-TMAE (*m/z* 364 → 305), γLNA-TMAE (*m/z* 364 → 243), $[^{13}C_{18}]$-αLNA-TMAE (*m/z* 382 → 323), AA-TMAE (*m/z* 390 → 331), $[^2H_8]$-AA-TMAE (*m/z* 398 → 339), EPA-TMAE (*m/z* 388 → 329), DHA-TMAE (*m/z* 414 → 355).

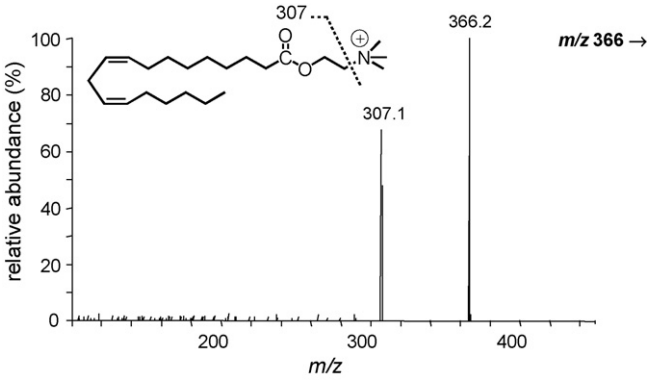

Fig. 4. Typical product ion spectra from CID of $[M]^+$ ion for LA-TMAE.

respectively (Fig. 3). Specific parent ions $[M]^+$ and product ions $[M - C_3H_9N]^+$ were selected for each fatty acid-TMAE ester derivatives in order to perform LC/MRM/MS analysis (Table 1), which provides high sensitivity and selectivity of the analysis. All the samples were diluted by 1:1000 to minimize the signal/noise ratio. Therefore, pg/μl concentrations of sample were present on column. A typical MS/MS fragmentation pattern of TMAE-FA for LA is shown in Fig. 4.

3.2. Validation assays using standards

3.2.1. Linearity

To determine the linearity of response, the fatty acid standards were extracted, hydrolyzed, derivatized, and analyzed using our LC–ESI/MRM/MS method. Calibration curves were constructed for each fatty acid in a range related to its expected in vivo concentrations: 1–100 ng/μl for MA, αLNA, γLNA, AA, EPA, and DHA; 20–2000 ng/μl for PA, SA, OA, and LA. Typical regression lines were: MA, $y = 1.8155x + 7.3827$ ($r^2 = 0.9992$); PA, $y = 0.039x + 0.1628$ ($r^2 = 0.9993$); SA, $y = 0.0034x + 0.1041$ ($r^2 = 0.9969$); OA, $y = 0.0046x + 0.0002$ ($r^2 = 0.9961$); LA, $y = 0.0042x + 0.1520$ ($r^2 = 0.9923$); αLNA, $y = 1.5892x + 0.6769$ ($r^2 = 0.9981$); γLNA, $y = 0.1173x + 0.0314$ ($r^2 = 0.9975$); AA, $y = 0.1001x - 0.3165$ ($r^2 = 0.9873$); EPA, $y = 0.0475x + 0.0847$ ($r^2 = 0.9959$); DHA, $y = 0.2016x + 0.3304$ ($r^2 = 0.9961$) (Fig. 5).

3.2.2. Intra- and inter-day precision and accuracy

Assay precision and accuracy were determined by analyzing quality control (QC) samples in triplicate on three separate days at three different concentrations: lower quality control (LQC, 20 ng/μl of PA, SA, OA, LA, and 1 ng/μl of MA, AA, EPA, DHA, LNAs), middle quality control (MQC, 200 ng/μl of PA, SA, OA, LA, and 10 ng/μl of MA, AA, EPA, DHA, LNAs), and higher quality control (HQC, 2000 ng/μl of PA, SA, OA, LA, and 100 ng/μl of MA, AA, EPA, DHA, LNAs). The coefficients of variation (CV) were measured with consideration of the intra- and inter-day standard deviation percentages for each fatty acid at each concentration (Table 2). The accuracy for triplicate QC samples on three separate days was between 85 and 115% (data not shown).

Table 2
Intra- and inter-day precision assay on standards

	Intra-day precision (%)			Inter-day precision (%)		
	LQC	MQC	HQC	LQC	MQC	HQC
MA	13.3	9.35	3.23	11.6	12.4	10.7
PA	7.97	6.89	4.50	10.5	13.9	12.1
SA	11.5	6.86	3.94	13.8	8.88	13.2
OA	7.48	11.7	2.35	3.96	7.91	13.7
LA	7.27	12.0	4.59	1.25	10.7	9.58
αLNA	13.0	6.89	3.84	11.2	11.5	8.61
γLNA	13.0	9.91	9.94	9.41	13.3	7.20
AA	13.4	10.0	0.87	6.41	13.2	13.7
EPA	6.90	9.13	10.9	8.57	5.30	10.0
DHA	1.23	8.08	7.69	7.93	7.02	12.4

Table 3
LOD and LOQ values found with standard mixtures

	LOD (pg)	LOD (fmol)	LOQ[a] (pg)	LOQ (fmol)
MA	2.0	8.8	4.0	17.6
PA	2.0	7.8	40.0	156
SA	5.0	17.6	40.0	141
OA	2.0	7.1	40.0	142
LA	5.0	17.9	40.0	143
αLNA	5.0	18.0	7.5	27.0
γLNA	5.0	18.0	7.5	27.0
AA	10.0	32.9	20.0	65.8
EPA	10.0	33.1	20.0	66.2
DHA	10.0	30.5	15.0	45.7

[a] LOQ was calculated according to FDA definition (CV < 20%) from analysis of three standard solutions.

3.2.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD values with a signal to noise ratio (S:N) > 5:1 were 2 pg for MA, PA, and OA; 5 pg for SA, LA, and LNAs; and 10 pg for AA, EPA, and DHA on column (Table 3). The LOQ values were 4 pg for MA; 40 pg for PA, SA, OA, and LA; 7.5 pg for LNAs; 20 pg for AA and EPA; and 15 pg for DHA. They were calculated from dilutions of the standard mixture that showed a CV < 20% in triplicate analysis. Different LOD/LOQ for various fatty acids was due to the different sensitivity of their molecular cations: a better sensitivity of molecular cation was observed in shorter chain fatty acids.

3.2.4. Carryover between injections

The potential for carryover of analytes from consecutive injections was measured by injection of the blank after the highest point of the calibration curve. No significant peaks appeared in the blank sample for any of the fatty acids analyzed.

3.3. Validation in plaque tissue samples

3.3.1. Linearity

A typical chromatogram for fatty acids extracted from a plaque is shown in Fig. 6. To determine the linearity of response and evaluate any matrix effects, calibration samples were prepared in the presence of the same amount of plaque. The samples were extracted, hydrolyzed, derivatized,

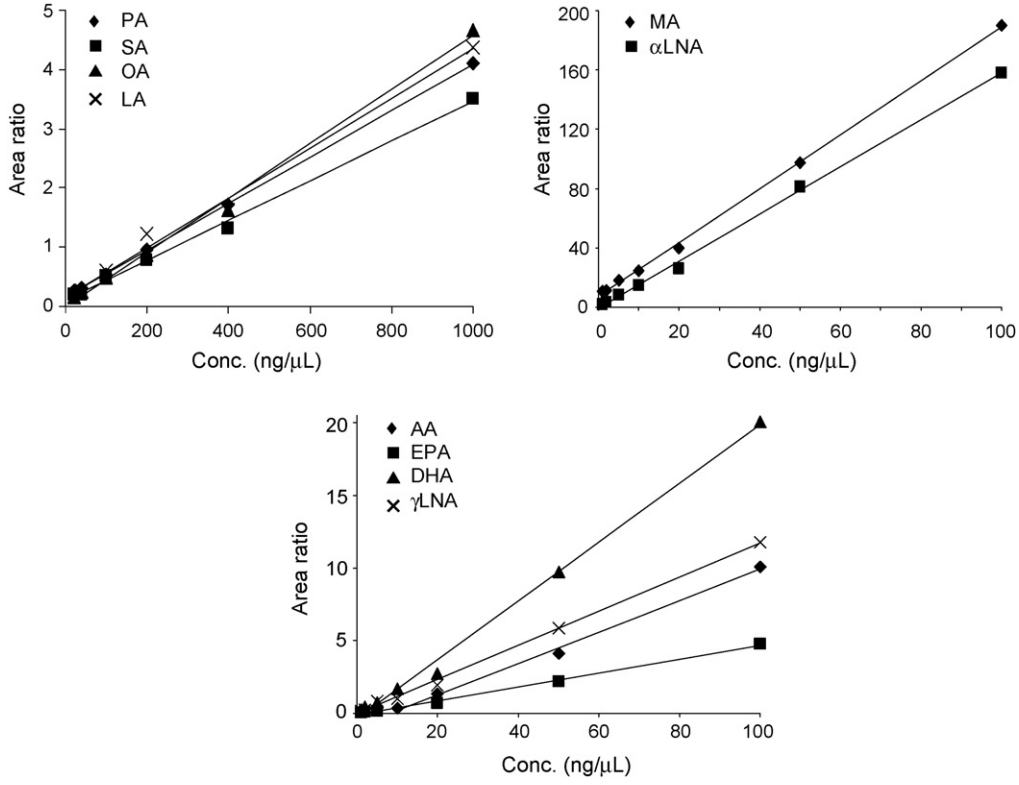

Fig. 5. Linearity of response from increasing amount of fatty acid standards.

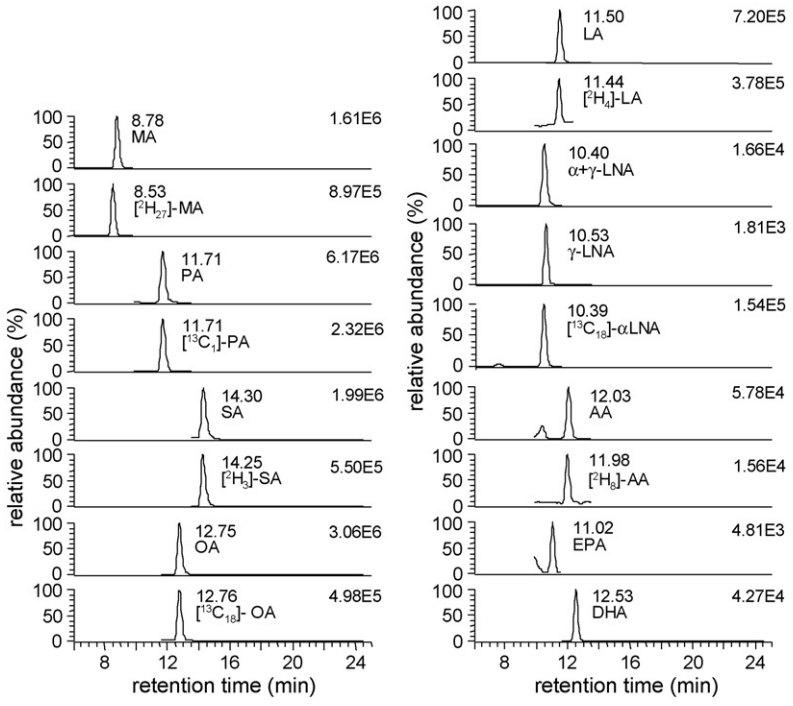

Fig. 6. Typical chromatogram from LC–ESI/MRM/MS analysis of a plaque extract (plaque weight 6.1 mg). MRM chromatograms are shown for: (Left panel) MA-TMAE (*m/z* 314 → 255), [$^2H_{27}$]-MA-TMAE (*m/z* 341 → 282), PA-TMAE (*m/z* 342 → 283), [$^{13}C_1$]-PA-TMAE (*m/z* 343 → 284), SA-TMAE (*m/z* 370 → 311), [$^2H_3$]-SA-TMAE (*m/z* 373 → 314), OA-TMAE (*m/z* 368 → 309), [$^{13}C_{18}$]-OA-TMAE (*m/z* 386 → 327), (Right panel) LA-TMAE (*m/z* 366 → 307), [$^2H_4$]-LA-TMAE (*m/z* 370 → 311), αLNA- and γLNA-TMAE (*m/z* 364 → 305), γLNA-TMAE (*m/z* 364 → 243), [$^{13}C_{18}$]-αLNA-TMAE (*m/z* 382 → 323), AA-TMAE (*m/z* 390 → 331), [$^2H_8$]-AA-TMAE (*m/z* 398 → 339), EPA-TMAE (*m/z* 388 → 329), DHA-TMAE (*m/z* 414 → 355).

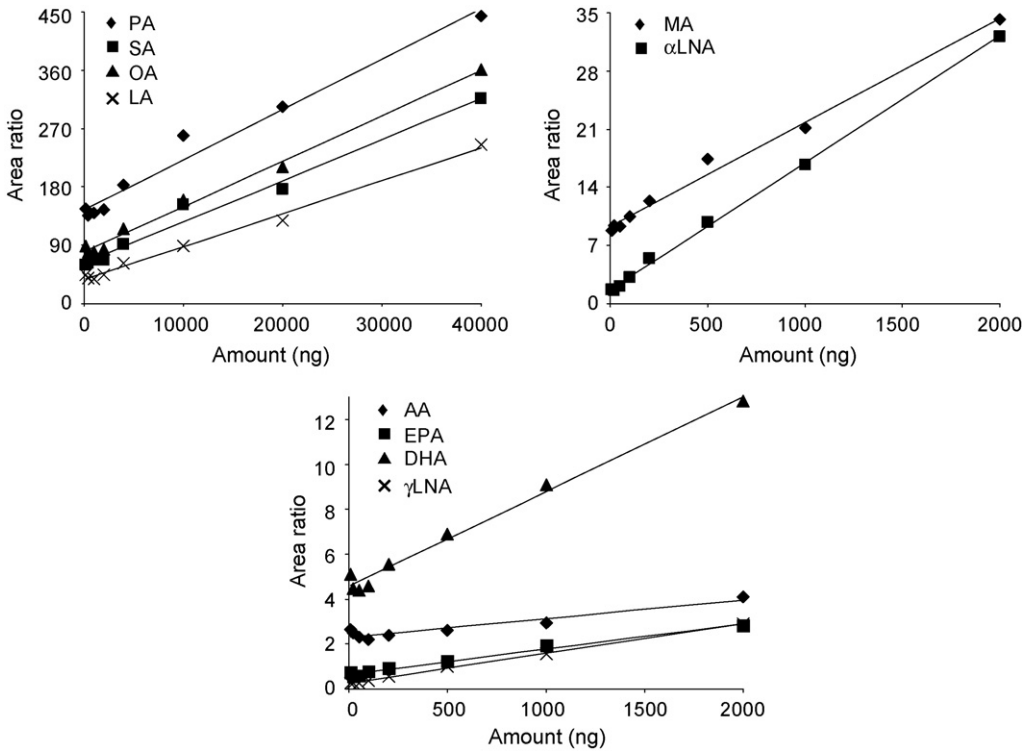

Fig. 7. Linearity of response from fractions containing the same amount of plaque (1 mg) spiked with increasing amounts of fatty acid standards.

and analyzed using our LC–ESI/MRM/MS method. Calibration curves were constructed for each fatty acid. Typical regression lines were MA, $y = 0.0125x + 9.2823$ ($r^2 = 0.9910$); PA, $y = 0.0078x + 144.16$ ($r^2 = 0.9752$); SA, $y = 0.0064x + 62.589$ ($r^2 = 0.9835$); OA, $y = 0.007x + 79.751$ ($r^2 = 0.9932$); LA, $y = 0.0051x + 36.921$ ($r^2 = 0.9940$); αLNA, $y = 0.0152x + 1.6831$ ($r^2 = 0.9985$); γLNA, $y = 0.0013x + 0.2595$ ($r^2 = 0.9974$); AA, $y = 0.0008x + 2.3077$ ($r^2 = 0.9022$); EPA, $y = 0.0011x + 0.6566$ ($r^2 = 0.9861$); DHA, $y = 0.0042x + 4.5657$ ($r^2 = 0.9879$). The higher intercepts in each calibration curves (Fig. 7) compared with those obtained without matrix (Fig. 5) represent the endogenous concentrations of fatty acids in plaque. However, the linearity of the results was not significantly affected by matrix effects from the plaque; all correlation coefficients were >0.97, except for AA with a correlation coefficient of 0.9022 (Fig. 7). Unknown interference from plaque was co-eluted with AA, which caused a suppression of ionization and affected the linearity of AA response.

3.3.2. Precision of plaque analysis

The precision of this method was evaluated in two different assays. In the first method, samples with an increasing amount of plaque were prepared to take into account any matrix effects, and the fatty acid content of each was related to its unit of weight (mg of plaque). The CV's were all within ±15% range (Table 4). In the second method, five different fractions (∼1 mg each) of the same plaque were analyzed in an intra-day assay (Table 5). The CVs were lower in this second method suggesting that plaque matrix can influence the results, even though they remain within acceptable limits. The results obtained from an inter-day assay over 3 days were not significantly different.

4. Discussion

LC–MS has been used previously for the analysis of intact lipids [33] and the quantitative analysis of PUFAs and their chiral hydroxylated products [28,29]. However, these methods are not suitable for the precise and specific quantitative analysis of saturated fatty acids that are present as esterified lipids. A previous report by Nichols et al. [25] showed that phenacyl esters of fatty acids can be analyzed using LC–APCI/MS giving LOD values lower than those obtained using GC/MS on methyl esters. Also, Han and Gross have recently proposed so-called "shotgun lipidomics," a methodology that bypasses chromatographic separation, using the ESI ionization source to separate the main lipid classes present in an infused solution (intra-source separation). Through a coordinated series of sequential 2D mass spectra, over 20 lipid classes are identified and quantified to obtain a cellular lipidome [34–36]. This methodology is evolving, but it currently requires subsequent MS analyses in order to analyze all the lipids present in a tissue.

In the present study we have optimized an alternative stable isotope dilution LC–ESI/MRM/MS method for saturated and unsaturated fatty acid analysis. Using TMAE derivatives of fatty acids (Fig. 2 upper), the sensitivity of the analysis was greatly improved over phenacyl esters, especially for saturated fatty acids. MS/MS fragmentation of the TMAE moiety gave the cation of the ethyl ester of the fatty acid, which allowed for good selectivity between the different acids (Fig. 2 lower). Also, it was possible to distinguish between the isomers of αLNA and γLNA using different product ions. *Cis*- and *trans*-isomers such as oleic acid and elaidic acid, were separated from each other as a consequence of different strengths of interaction with a sta-

Table 4
Precision calculated using increasing amounts of plaque

Plaque amount (mg)	6.1 (ng/mg)	11 (ng/mg)	15 (ng/mg)	21.8 (ng/mg)	Mean (ng/mg)	CV (%)
MA	121.4	115.9	109.0	99.7	111.5	8.4
PA	153.7	158.6	149.9	144.9	151.8	3.8
SA	933.0	1127.4	1068	1033	1040	7.8
OA	3811	3281	3186	3020	3324	10.3
LA	4588	3449	3399	3740	3794	14.5
αLNA	73.1	68.0	59.5	55.7	64.1	12.4
LNA	110.0	100.6	137.2	107.3	113.8	14.2
AA	921.9	824.7	834.2	874.1	863.7	5.1
EPA	160.8	155.6	126.4	126.5	142.3	13.0
DHA	196.9	205.4	213.9	230.2	211.6	6.7

Table 5
Intra-day assay to measure the precision of response in plaque samples

Fatty acid	CV (%)
MA	5.45
PA	3.52
SA	5.67
OA	3.70
LA	3.29
αLNA	8.21
γLNA	0.33
AA	7.75
EPA	0.08
DHA	5.70

tionary phase (data not shown). Reversed-phase LC was able to readily separate 10 fatty acids after extraction from atherosclerotic plaque tissue. Our method was then used to evaluate the involvement of fatty acids in atherosclerotic plaque stability [10,11].

Acute ischemic complications of atherosclerosis, such as stroke, myocardial infarction, and clinically transitory ischemic attacks, remain the primary cause of morbidity and mortality in Western countries [37]. Prognostic evaluation of atherosclerotic plaques, initially based upon a qualitative approach linked to the severity of arterial stenosis, has progressed to a quantitative approach to plaque composition. Also, during the development of atherosclerosis, the lipid fraction of the plaque undergoes a progressive change in fatty acid composition [38,39,40].

Our analysis was performed directly on atherosclerotic plaque tissue. This kind of tissue, which contains primarily carotid arteries, is particularly hard to analyze because it is so heterogeneous. The fatty acid content is very variable depending on the patient and the progression of pathology (e.g., calcific plaques have a lower content of every fatty acid) [38,39,40]. In most of the plaques, the predominant fatty acids are LA, OA, PA, and SA (ranging from 1000 to 3800 ng/mg); followed by AA, DHA, EPA, γLNA, MA, αLNA (ranging from 800 ng/mg of the AA to 60 ng/mg of αLNA) (Table 4).

The validated method we have developed provides an excellent way to analyze saturated and unsaturated fatty acids and can be used for analysis of many different tissues and cell systems. Moreover, the LC–MS method can readily be modified to analyze additional fatty acids, such as arachidic acid (C20:0, ACA), behenic acid (C22:0, BA), lignoceric acid (C24:0, LCA), cerotic acid (C26:0, CA) (data not shown). These data illustrate that TMAE derivatization of fatty acids coupled with analysis by stable isotope dilution LC–ESI/MRM/MS provides an alternative to GC/MS for the sensitive and specific analysis of saturated and unsaturated fatty acids. Our method separates and quantifies fatty acids present in human tissues with a high degree of sensitivity (LOD in fmol range) and reproducibility (CV < 15% for all the acids); this compares favorably with other traditional GC and HPLC methods.

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References

[1] A.P. Simopoulos, Am. J. Clin. Nutr. 70 (1999) 560S.
[2] H.M. Schachter, J. Reisman, K. Tran, B. Dales, K. Kourad, D. Barnes, M. Sampson, A. Morrison, I. Gaboury, J. Blackman, Evid. Rep. Technol. Assess. (Summ.) (2004) 1.
[3] S.D. Freedman, P.G. Blanco, M.M. Zaman, J.C. Shea, M. Ollero, I.K. Hopper, D.A. Weed, A. Gelrud, M.M. Regan, M. Laposata, J.G. Alvarez, B.P. O'Sullivan, N. Engl. J. Med. 350 (2004) 560.
[4] C. Phillips, D. Owens, P. Collins, G.H. Tomkin, Atherosclerosis 181 (2005) 109.
[5] M. Peet, C. Stokes, Drugs 65 (2005) 1051.
[6] L.M. Bodnar, K.L. Wisner, Biol. Psychiatry 58 (2005) 679.
[7] A.W. Yuen, J.W. Sander, D. Fluegel, P.N. Patsalos, G.S. Bell, T. Johnson, M.J. Koepp, Epilepsy Behav. 7 (2005) 253.
[8] P. Socha, J. Ryzko, B. Koletzko, D. Celinska-Cedro, M. Woynarowski, P. Czubkowski, J. Socha, Scand. J. Gastroenterol. 40 (2005) 573.
[9] Y.Z. Almallah, S. Richardson, T. O'Hanrahan, N.A. Mowat, P.W. Brunt, T.S. Sinclair, S. Ewen, S.D. Heys, O. Eremin, Am. J. Gastroenterol. 93 (1998) 804.
[10] T. Yamada, J.P. Strong, T. Ishii, T. Ueno, M. Koyama, H. Wagayama, A. Shimizu, T. Sakai, G.T. Malcom, M.A. Guzman, Atherosclerosis 153 (2000) 469.
[11] F. Thies, J.M. Garry, P. Yaqoob, K. Rerkasem, J. Williams, C.P. Shearman, P.J. Gallagher, P.C. Calder, R.F. Grimble, Lancet 361 (2003) 477.
[12] P.D. Schley, H.B. Jijon, L.E. Robinson, C.J. Field, Breast Cancer Res. Treat. 92 (2005) 187.
[13] A. Sharma, J. Belna, J. Logan, J. Espat, J.A. Hurteau, Gynecol. Oncol. 99 (2005) 58.
[14] I. Brondz, Anal. Chim. Acta 465 (2002) 1.
[15] B. Hallgren, A. Svanborg, Scand. J. Clin. Lab. Invest. 14 (1962) 179.

[16] W. Schrade, E. Boehle, R. Biegler, V. Meder, R. Teicke, Klin. Wochenschr. 38 (1960) 126.
[17] R.C. Murphy, J. Fiedler, J. Hevko, Chem. Rev. 101 (2001) 479.
[18] W.J. Griffiths, Mass Spectrom. Rev. 22 (2003) 81.
[19] J.A. Hankin, R.C. Murphy, Int. J. Mass Spectrom. 200 (2000) 201.
[20] G. Dobson, W.W. Christie, Eur. J. Lipid Sci. Technol. 104 (2002) 36.
[21] T. Seppänen-Laakso, I. Laakso, R. Hiltunen, Anal. Chim. Acta 465 (2002) 39.
[22] R.F. Borch, Anal. Chem. 47 (1975) 2437.
[23] H. Chen, R.E. Anderson, J. Chromatogr. 578 (1992) 124.
[24] H. Miwa, M. Yamamoto, T. Asano, J. Chromatogr. 568 (1991) 25.
[25] D.S. Nichols, N.W. Davies, J. Microbiol. Methods 50 (2002) 103.
[26] T. Rezanka, J. High. Resolut. Chromatogr. 23 (2000) 338.
[27] G. Singh, A. Gutierrez, K. Xu, I.A. Blair, Anal. Chem. 72 (2000) 3007.
[28] S.H. Lee, M.V. Williams, R.N. DuBois, I.A. Blair, Rapid Commun. Mass Spectrom. 17 (2003) 2168.
[29] S.H. Lee, M.V. Williams, I.A. Blair, Prostaglandins Other Lipid Mediat. 77 (2005) 141.
[30] D.W. Johnson, Rapid Commun. Mass Spectrom. 14 (2000) 2019.
[31] D.W. Johnson, M.U. Trinh, T. Oe, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 798 (2003) 159.
[32] J. Folch, M. Lees, G.H. Sloane Stanley, J. Biol. Chem. 226 (1957) 497.
[33] A. Mehta, A.M. Oeser, M.G. Carlson, J. Chromatogr. B Biomed. Sci. Appl. 719 (1998) 9.
[34] X. Han, K. Yang, H. Cheng, K.N. Fikes, R.W. Gross, J. Lipid Res. 46 (2005) 1548.
[35] X. Han, R.W. Gross, Mass Spectrom. Rev. 24 (2005) 367.
[36] X. Han, R.W. Gross, Expert Rev. Proteomics 2 (2005) 253.
[37] A.D. Callow, Vascul. Pharmacol. 45 (2006) 302.
[38] H.C. Stary, A.B. Chander, R.E. Dinsmore, Circulation 92 (1995) 1355.
[39] R. Virmani, F.D. Kolodgie, A.P. Burke, A. Farb, S.M. Schwartz, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1262.
[40] E. Stachowska, B. Dolegowska, D. Chlubek, T. Wesolowska, K. Ciechanowski, P. Gutowski, H. Szumilowicz, R. Turowski, Eur. J. Nutr. 43 (2004) 313.