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Enhancement of the LC/MS Analysis of Fatty Acids through Derivatization and Stable Isotope Coding

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This paper focuses on the development of an enhanced LC/ESI-MS method for the identification and quantification of fatty acids through derivatization. Fatty acids were derivatized with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide, forming 3-acyloxymethyl-1-methylpyridinium iodide (AMMP). This process attaches a quaternary amine to analytes and enabled ESI-MS in the positive mode of ionization with common LC mobile phases. Moreover, detection sensitivity was generally 2500-fold higher than in the negative mode of ionization used with underivatized fatty acids. The limits of detection were roughly 1.0−4.0 nM (or 10 pg/injection) for standard fatty acids from C10 to C24 and spanned ~2 orders of magnitude in linearity. AMMP derivatives had unique tandem mass spectra characterized by common ions at *m/z* 107.0, 124.0, and 178.0. Individual fatty acids also had unique fingerprint regions that allowed identification of their carbon skeleton number, number of double bonds, and double bond position. The derivatization method also allowed coding of analytes as a means of recognizing derivatives and enhancing quantification. ^{2}H-Coding was achieved through derivatization with deuterated 3-carbinol-1-methyl-d_3-pyridinium iodide. The ^{2}H-coded derivatization reagent, 3-acyloxymethyl-1-methyl-d_3-pyridinium iodide, was used in two ways. One was to differentially label equal fractions of a sample such that after being recombined and analyzed by ESI-MS all fatty acids appeared as doublet clusters of ions separated by roughly 3 amu. This greatly facilitated identification of fatty acids in complex mixtures. Another use of stable isotope coding was in comparative quantification. Control and experimental samples were differentially labeled with nondeuterated and deuterated isotopomers of CPM, respectively. After mixing the two samples, they were analyzed by ESI-MS. The abundance of a fatty acid in an experimental sample relative to the control was established by the isotope ratio of the isotopomeric fatty acids. Absolute quantification was achieved by adding differentially labeled fatty acid standards to experimental samples containing unknown quantities of fatty acids. Utility of the method was examined in the analysis of human serum samples.

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The analysis of fatty acids and metabolites containing a carboxyl group is of major interest in metabolomics because of their prominent role in metabolic pathways[1] and importance in food.[2] Moreover, ~8% of organic compounds of pharmaceutical and biomedical interest possess a carboxyl group.[3] Although it would seem that their analysis by gas chromatography (GC), high-performance liquid chromatography (HPLC), or capillary electrophoresis would be straightforward,[4−6] extracts from biological fluids are of such great complexity and difference in concentration there is a problem with resolving and detecting all the components. One of the ways to deal with this problem is through mass spectrometry (MS). MS has the great advantage of increasing detection selectivity and sensitivity of unresolved components while providing structural information at the same time.[7] Traditionally, GC/MS methods employed derivatization by sialylation or methylation with detection by electron impact ionization,[8−12] but HPLC/MS has become a powerful alternative with the advent of atmospheric pressure ionization (API) and "soft" ionization techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization.[13−15]

A major issue in LC/MS is relative ionization efficiency and the ease with which analytes are ionized. In the case of carboxyl-containing species, ionization is best achieved in the negative ion current mode using a basic mobile phase in which carboxyl groups are ionized.[16−20] Unfortunately, best chromatographic resolution with reversed-phase columns is achieved at acidic pH where the

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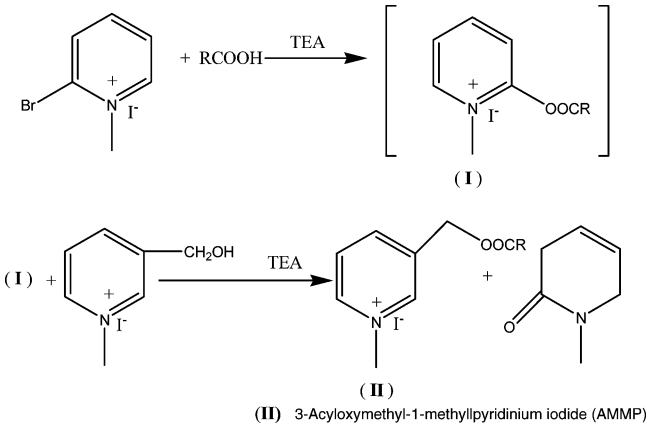
ionization of carboxyl groups is suppressed.[21] This problem could be circumvented by using a postcolumn pH adjustment with ammonia,[18] but would increase the complexity of the analysis and dilute analytes. Another possibility would be to derivatize carboxyl groups with a reagent that causes them to have a positive charge in acidic mobile phases. This strategy has been very successful in the HPLC/ESI-MS analysis of amino acids and peptides[22,23] along with neutral analytes.[24,25]

Quantification in LC/MS is also related to ionization efficiency. Matrix suppression of ionization can greatly reduce sensitivity and complicate quantification. The problem is that ionization efficiency of analytes is variable, being related to the concentration of other analytes in the mixture that suppress their ionization. One way to deal with this problem has been to increase ionization efficiency through the introduction of positive charge into analytes, as discussed above. Another is to add an internal standard that experiences the same suppression of ionization. When it is the objective to quantify large numbers of analytes as in proteomics and metabolomics, large numbers of isotopically coded internal standards of structure identical to analytes are added to a mixture. Isotope coded affinity tagging[26] and global internal standard technology[27] are examples of the large-scale use of internal standards for relative quantification in proteomics. Very similar differential coding methods have been described for metabolomics.[28,29] Functional groups in a control sample are derivatized in vitro with an isotopically coded reagent to target a particular functional group or molecular feature of analytes in a mixture. The experimental sample in contrast is derivatized with an isotopic isoform of the reagent. After the differentially coded samples are mixed, they are analyzed and as a last step the isotope ratio of the isotopomers is determined by mass spectrometry. In this way, differences in concentration between control and experimental samples are easily determined. Although there is substantial similarity between global internal standard coding methods in proteomics and metabolomics, there are differences. The major one is that almost all peptides contain an amino group or carboxyl group that can be isotopically coded and used in relative quantification studies. Metabolites on the other hand have no single, common functional group that can be used for isotope coding. This means that a number of reagents will have to be used to achieve truly global internal standard quantification.

In the analysis of carboxylic acids, stable isotope coded fatty acids are often used as internal standards due to matrix suppression of ionization.[19,30,31] The advantage of this approach is that internal standards have the same chromatographic properties as the analytes but can still be differentiated from analytes on the basis of mass. The disadvantage of this strategy is that a different internal standard has to be synthesized for each analyte. Also, this can only be done with known analytes with known structure. An alternative approach is to attach a stable isotope coded derivatizing agent to analytes.[32] The attractive feature of this strategy is that it allows all analytes in a sample to be coded with one isotopomer of the derivatizing agent while those from another sample can be coded with a second isotopomer.[33] Moreover, both relative and absolute comparisons of concentration between samples are easily achieved with this method.

This paper describes a strategy in which carboxyl-containing analytes are derivatized with a reagent that both increases their ionization efficiency in HPLC/ESI-MS analysis and isotopically codes them for internal standard-based quantification.

Scheme 1. Fatty Acid Derivatization Reactions



(II) 3-Acyloxymethyl-1-methylpyridinium iodide (AMMP)

EXPERIMENTAL SECTION

Materials and Reagents. Fatty acids (FAs), human serum, 2-bromopyridine, 3-carbinolpyridine, triethylamine (TEA), iodomethane, and anhydrous acetonitrile (ACN), were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile, acetone, and formic acid were obtained from Mallinckrodt Baker (Phillipsburg, NJ). Iodomethane-d_3 was a product of Cambridge Isotope Laboratories (Andover, MA). Double-deionized water was produced by a Milli-Q gradient A10 system from Millipore (Bedford, MA).

Synthesis of 2-Bromo-1-methylpyridinium Iodide (BMP), 3-Carbinol-1-methylpyridinium Iodide (CMP), and 3-Carbinol-1-methyl-d_3-pyridinium Iodide (CMP-d_3). Five-fold excess iodomethane-d_0 or -d_3 was added to 2-bromopyridine (10 mmol, 0.97 mL) or 3-carbinolpyridine (10 mmol, 0.96 mL). The solution was stirred at room temperature for 1 h. The crystals were washed with cold acetone and dried in vacuum. ^{1}H NMR properties were as follows: (200 MHz, ACN-d_3) for BMP, δ 9.12 (d, 1H, C(3)H),

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Table 1. Relative Mass Spectral Response of Fatty Acids (%) Affected by Formic Acid (FA) and Acetonitrile (ACN) Concentration in Mobile Phases

		mobile phase					
FA	pK_a[a]	0 mM FA, 50% ACN, pH 4.30	5 mM FA, 50% ACN, pH 2.66	20 mM FA, 50% ACN, pH 2.36	50% ACN, 5 mM FA, pH 2.66	70% ACN, 5 mM FA, pH 2.82	95% ACN, 5 mM FA, pH 4.02
C10:0	7.1−7.3	100	12.3	1.8	100	50.7	7.8
C12:0	∼7.5	100	16.8	3.4	100	59.0	13.1
C14:0	8.1−8.2	100	25.5	8.1	100	58.7	20.0
C16:0	8.6−8.8	100	41.6	25.0	100	48.0	22.6
C18:0	10.5	100	62.8	57.7	100	36.6	22.7
C20:0		100	90.0	80.0	100	55.5	58.3
C22:0		100	150.0	125.0	100	57.1	162.3
C24:0		100	225.8	161.3	100	85.7	375.0

[a] pK_a values for C10:0 to C16:0 were from ref 53, which were determined in water solution. The value for C18:0 was from ref 54, which was determined in water solution.

8.72−8.76 (d, 1H, C(6)H), 8.49−8.53 (d, 1H, C(4)H), 8.12−8.16 (m, 1H, C(5)H), 4.44 (s, 3H, NCH_3). For CMP, δ 8.90 (s, 1H, C(2)H), 8.70−8.76 (d, 1H, C(6)H), 8.51−8.56 (d, 1H, C(4)H), 8.10−8.15 (m, 1H, C(5)H), 4.43 (s, 3H, NCH_3).

Derivatization of Fatty Acids. Ten microliters of standard fatty acid mixture (0.5 μmol/mL each, ∼5 μmol/mL total FA, in acetone) was mixed with 20 μL of BMP (15.0 mg/mL or 50 μmol/mL, in ACN) and 20 μL of CMP (20.0 mg/mL or 200 μmol/mL, in ACN). After mixing, 1.0 μL of TEA was added, and the solution was heated in water at 50 °C for 30 min. The derivatizing reaction is presented in Scheme 1. The resulting solution was diluted 7:3 with DI water (solution/water, v/v) before it was subjected to analysis. The derivatizing reagents and derivatives were stable in ACN at ambient temperature for at least one week.

HPLC/ESI-MS. The HPLC/ESI system consisted of a capillary HPLC system (1100 Series LC, Agilent) and an ESI source of time-of flight (TOF) mass spectrometer (MSD TOF, Agilent). The system was controlled by ChemStation software (Agilent). The instruments were set up at nontemperature control. The separations were performed on a low TFA Vydac analytical column (C4 Mass Spec, 4.6 mm × 250 mm). Typical elution condition were to begin by maintaining 70% mobile phase A (H_2O + 0.05% formic acid) and 30% mobile phase B (95% ACN + 5% H_2O + 0.05% formic acid) for 0−5 min and then increase solvent B from 30 to 40% in 5 min followed by an increase in solvent B from 40 to 100% in 35 min with a 5-min hold at 100% B. The flow rate was set at 0.75 mL/min, and the injection volume was 40.0 μL. The column was preconditioned by running a blank with the above elution profile and then pumping mobile phase A for an additional 15 min. LC/ESI-MS chromatograms were acquired in the positive ion mode with the capillary voltage set at 4000 V and fragmentor at 175 V while the dry temperature was set at 350 °C and dry gas flow was maintained at 13 L/min. The mass acquisition range was from m/z 150 to 1299.

ESI-MS/MS. ESI-MS or ESI-MS/MS analyses were performed on an API QSTAR (Applied Biosystems, Framingham, MA) equipped with an ESI ion source. Free fatty acids or FA derivatives were prepared in various ACN/H_2O ratio solvents containing various concentrations of formic acid and injected into the ESI source by infusion at 10 μL/min. Both single-dimension mass spectra and tandem mass spectra were acquired typically for 2 min in the negative mode for free fatty acids within an m/z range of 150−400 or in the positive mode for fatty acid derivatives within the m/z range of 250−500.

Analysis of Human Serum Fatty Acids. Total fatty acid extraction was carried out by following a protocol suggested by Valianpour et al.[19] with minor modifications. Briefly, 10 mL of ACN/HCl (37%) (4:1, v/v) and 1 mL of serum were added to a glass vial. The vial was capped with a Teflon/silicone disk in a closed screw cap and incubated at 90 °C for 2 h. After cooling to room temperature, 7.5 mL of hexane was added. The vial was vortex-mixed for 20 s and centrifuged at 1500*g* for 1 min. A 1.0-mL aliquot of upper phase was placed in a 15-mL centrifuge tube and flushed with nitrogen to dryness. Meanwhile, 100 μL of the standard fatty acid mixture (even carbon number only from C10:0 to C24:0 along with C16:1, C18:1, and C18:2; 0.2 μM concentration) were processed and derivatized with CMP-d_3 in parallel. These were used as internal standards for quantitative analysis. For qualitative analysis, dried sample was reconstituted with 0.4 mL of acetone. Two 0.1-mL aliquots were derivatized with CMP and CMP-d_3 separately, following the procedure described above, and then mixed at a ratio of 1:1. The mixture was subjected to HPLC/MS analysis after 7:3 dilution with H_2O (solution/H_2O, v/v). The derivatization for quantitative analysis was performed as follows: 20 μL of the above CMP-labeled serum was added into the above dried internal standard vial; the mixture was diluted to 1.0 mL by ACN, further diluted to 70% ACN with water, and then analyzed by HPLC/MS.

RESULTS AND DISCUSSION

Enhancing Electrospray Ionization by Derivatization. It has been reported[21] that increasing mobile-phase concentration of formic acid in reversed-phase chromatography (RPC) from 0 to 10 mM decreased the detection sensitivity of carboxylic acid compounds 5.9-fold in the negative ion current mode of ESI-MS. Similar effects were noted with fatty acids in this work (Table 1). The problem is larger with smaller fatty acids as noted in Table 1. This is an important issue because it is necessary to use acidic mobile phases in RPC to eliminate the impact of residual silanol groups on separations.

It is worth noting that the change in response seen in Table 1 is not proportional to the change in carboxyl group dissociation as a function of pH. For example, it can be calculated that the degree of dissociation of C10:0 in going from zero to 5 mM formic

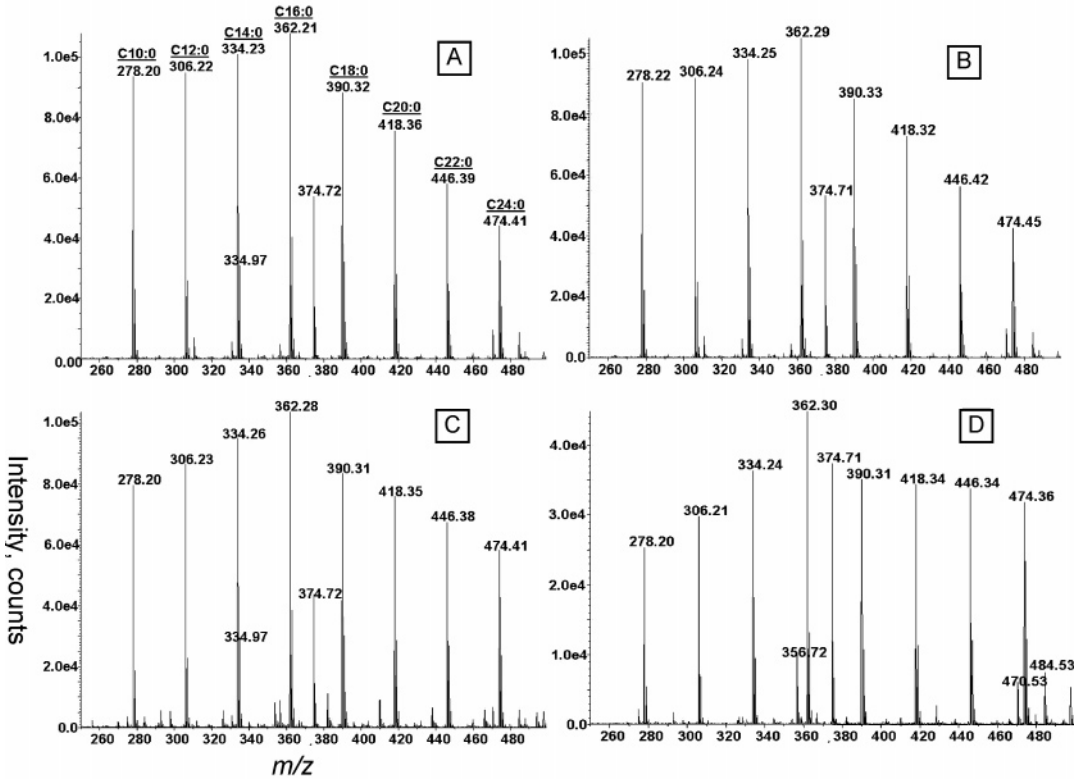

Figure 1. Mass spectra of derivatized fatty acids in various matrixes: (A) 50% ACN + 0 mM formic acid; (B) 50% ACN + 5 mM formic acid; (C) 50% ACN + 20 mM formic acid; (D) 95% ACN + 5 mM formic acid.

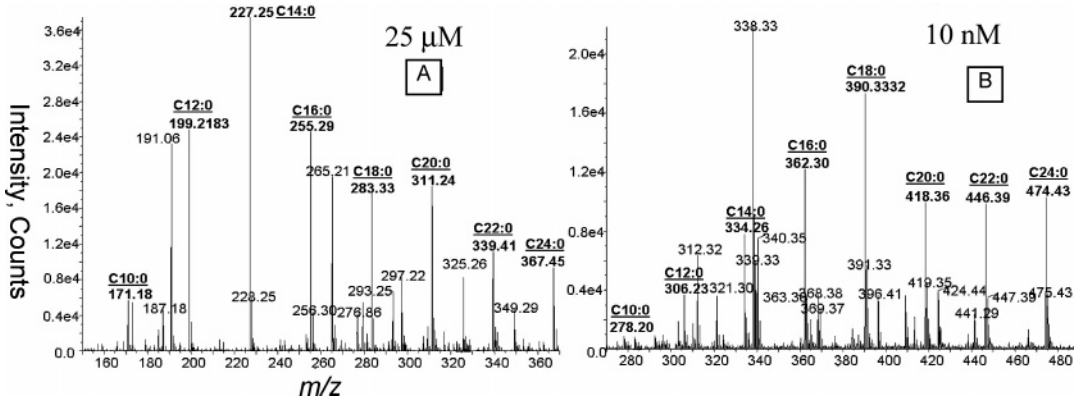

Figure 2. Comparison of detection limits by mass spectrometry for (A) free fatty acids (25 μM, in 80% ACN + 5 mM formic acid) and (B) derivatized fatty acids (10 nM, in 80% ACN + 5 mM formic acid).

acid would be ~43-fold. Going to 20 mM would produce another 2-fold decrease, i.e., an 86-fold change compared to no formic acid. However, as shown in Table 1, the actual changes were 8.1- and 55-fold, respectively. This is below what would be expected, suggesting that formic acid might be making some additional contribution to ionization.

Ionization efficiency in the negative ion current mode of ionization is also impacted by the concentration of organic solvent (Table 1). Again the effect is greater with the smaller C10 than the larger C20 fatty acid in the range of 50−95% acetonitrile. In fact, detection sensitivity increased with larger fatty acids. Clearly, fatty acid dissociation is suppressed in acetonitrile[34−36] as seen in the cases of the C10, C12, and C14 fatty acids. But beyond C20, the impact of carboxyl dissociation on ionization in 95% acetonitrile is not a factor.

The fatty acid mixture was then derivatized with BMP and CMP. Esterification of carboxylic acids with this reaction has been previously reported[37] as proceeding via an intermediate 2-acyloxy-1-methylpyridinum iodide that undergoes nucleophilic attack by the alcohol in the presence of a tertiary amine. The amine sequesters the hydrogen iodide produced in the reaction. The fact that activation of carboxylic acids and esterification takes place in a single step is an obvious advantage that has been exploited in the preparation of fluorescent derivatives of fatty acids for HPLC analysis.[38−40]

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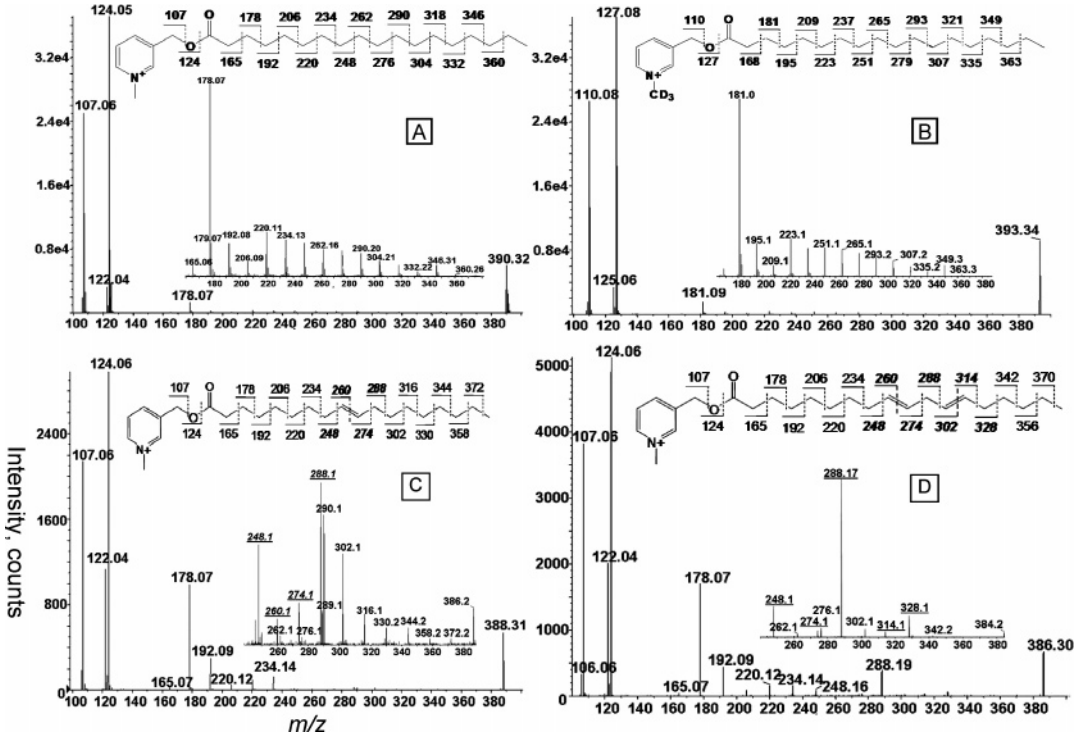

Figure 3. ESI tandem mass spectra showing fragmentation of derivatized C18:0, C18:1, and C18:2 fatty acids. The zoom ratio of the inset spectra are 18:1 in (A), 18:1 in (B), 11:1 in (C), and 7:1 in (D). The boldface and italicized numbers in (C) and (D) indicate the unique masses for unsaturated, derivatized fatty acids.

Quaternization of fatty acids according to Scheme 1 greatly increased the ease with which they were ionized under acidic conditions (Figure 1). No significant change in ESI response in the positive ion mode was observed (Figure 1A−C) with increasing concentrations of formic acid. The fatty acid detection limit before and after quaternization can be seen in Figure 2. Signal intensity with 25 μM free fatty acids was close to that of 10 nM derivatized fatty acids. Detection sensitivity was enhanced roughly 2500-fold by derivatization. Even though acetonitrile still reduced detection sensitivity by 2−3-fold (Figure 1B, D), the detection limits were far below those of free fatty acids. As with free fatty acids, the improvement of detection sensitivity was greater with the larger (>C18) than smaller (<C18) fatty acids.

Tandem Mass Spectrometry. Collision-induced fragment ion spectra of the C18:0, C18:1, and C18:2 fatty acid derivatives were found to correlate with their structure as seen in Figure 3. Ions common to all the fatty acid spectra were at m/z 107, 124, and 178. The ions at m/z 107 and 124 were derived from the *N*-pyridylcarbinol moiety. The first lacks the oxygen, and the second is from a rearranged proton-transfer fragmentation with retention of the oxygen. The fragment ion at m/z 165 is the result of fragmentation with a McLafferty rearrangement.[41] An ion at m/z 178 was also seen in all the spectra that results from fragmentation of the fatty acid ester γ to the C-terminus of the fatty acids. The series of ions at m/z 178, 192, 206, 220, 234, 248, 262, 276, 290, 304, 318, 332, 346, and 360, all differing by 14 amu, indicated this is the spectrum of a saturated fatty acid. Comparing spectra derived from the d_0 and d_3 isotopomers of derivatized C18:0, it is seen that all the ions in the spectrum of the d_3 isotopomer (Figure 3B) are 3 amu higher than those in the d_0 isotopomer (Figure 3A). This confirms that the *N*-pyridylcarbinol group is attached to all the fragments ions in the fatty acid spectra. Panels C and D in Figure 3 show that it is possible to locate the position of double bonds in the C18:1 and C18:2 acids examined. The spectrum of C18:1 (Figure 3C) shows an interruption of the aliphatic series noted above after m/z 248. The difference between m/z 248 and 274 in the spectrum is 26 amu, equivalent to a −CH=CH− group. The prominent ion at m/z 288 is from

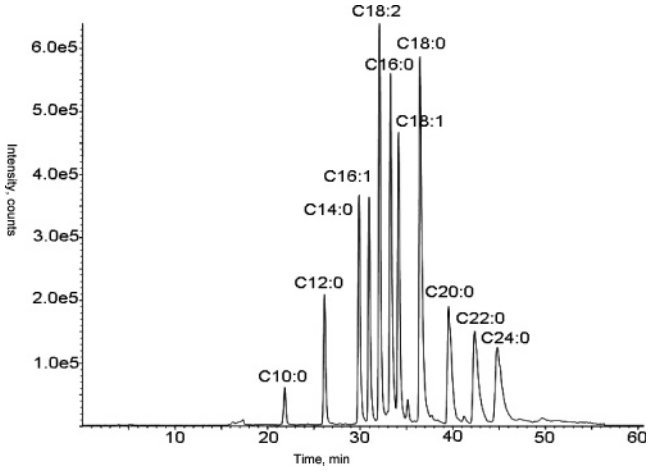

Figure 4. Reconstructed ion chromatogram of CMP derivatives of standard fatty acid mixture. The concentration was 200 nM for each, and the injection volume was 40 μL. Elution protocol is given in the Experimental Section.

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Table 2. Mass, Retention Time, and Detection Sensitivity of Fatty Acid Derivatives

derivative	m/z	t_r (min)	linearity[a]	dynamical range (μM)	detection limit (nM/pg)
C10:0	278.21	22.1	$y = 6064.2x + 336.5$ $r^2 = 0.9959$	0.02−1.0	4.0/27.6
C12:0	306.24	26.4	$y = 34382x + 488.4$ $r^2 = 0.9968$	0.004−0.4	2.0/16.0
C14:0	334.27	30.1	$y = 73297x + 383.3$ $r^2 = 0.9993$	0.004−0.2	2.0/18.3
C16:1	360.28	31.2	$y = 61084x + 1946.1$ $r^2 = 0.9954$	0.004−1.0	2.0/20.3
C18:2	386.30	42.3	$y = 79985x + 1093$ $r^2 = 0.9962$	0.004−0.2	2.0/22.4
C16:0	362.30	33.4	$y = 147025x + 677.6$ $r^2 = 0.9993$	0.004−0.2	2.0/20.5
C18:1	388.31	34.3	$y = 93147x + 550.0$ $r^2 = 0.9946$	0.002−0.2	2.0/22.6
C18:0	390.33	36.5	$y = 97140x + 788.9$ $r^2 = 0.9937$	0.002−0.2	1.0/11.3
C20:0	418.36	39.7	$y = 34445x + 339.3$ $r^2 = 0.9959$	0.002−0.2	1.0/12.5
C22:0	446.39	42.5	$y = 32115x + 629.4$ $r^2 = 0.9926$	0.002−0.2	1.0/13.6
C24:0	474.42	45.0	$y = 38064x + 1188.4$ $r^2 = 0.9966$	0.002−0.2	1.0/14.7

[a] In the linearity equation, y is instrument response and x is concentration (μM).

cleavage allylic to the double bond. In the case of the C18:2 fatty acid (Figure 3D), the dominant ion in the cluster of ions associated with the double bonds is again at m/z 288, allylic to the first double bond. Cleavage allylic to the second double bond at m/z 328 is also apparent in the spectrum. The fragmentation pattern of the fatty acid derivatives in Figure 3 is similar to the extensive description of the fragmentation of picolinyl esters of fatty acids.[42−46]

Absolute and Relative HPLC/ESI-MS Quantitation by ^{2}H-Coded Derivatization. Long-chain fatty acids and their esters are strongly retained on reversed-phase columns. Mobile phases containing up to 95% organic solvent are often required to effect elution. Even with this, the retention is still so strong on C18 columns that investigators often go to C8 columns[18,41,47] or C6 columns.[48] A C4 reversed-phase column was use in this work. Figure 4 shows the reconstructed ion chromatogram of 3-acyloxymethyl-1-methylpyridinium iodide (AMMP) derivatives of a standard mixture of eight saturated and three unsaturated fatty acids. Although difficult to achieve in the past,[41] complete separation of all the physiological significant fatty acids is seen in Figure 4. The retention times and m/z values identifying these fatty acids are summarized in Table 2.

Quantitative aspects of the method were examined in three trials using a sample of 11 fatty acids in the concentration range from 2.0 μM to 1.0 nM that had been derivatized with CMP. Peak height and peak area were derived from extracted ion chromatograms using characteristic ions. Instrument response for individual fatty acids was fitted to a linear regression analysis of concentration. Linearity spanning roughly 2 orders of magnitude for most fatty acids was observed with regression coefficients greater than 0.99. The limit of detection (LOD) for standard AMMP mixtures was determined from mass spectra using a signal-to-noise ratio limit of 3. The LOD varied from 1.0 to 4.0 nM, i.e., 10-pg level/inject (40 μL), for the fatty acids studied here. Detailed data are summarized in Table 2.

Table 3. Linearity and Dynamical Range of Light and Heavy Isotope Labeled Mixtures of Fatty Acid Derivatives

fatty acid	m/z light	m/z heavy	linearity[a]	dynamical ratio range
C10:0	278.21	281.23	$y = 1.003x$; $r^2 = 0.9992$	1:1−1:50
C12:0	306.23	309.26	$y = 0.8902x$; $r^2 = 0.9972$	1:1−1:100
C14:0	334.23	334.26	$y = 1.0934x$; $r^2 = 0.9984$	1:1−1:100
C16:1	360.28	363.30	$y = 1.0745x$; $r^2 = 0.9991$	1:1−1:100
C18:2	386.30	386.32	$y = 0.9185x$; $r^2 = 0.9998$	1:1−1:100
C16:0	362.30	365.32	$y = 1.0247x$; $r^2 = 0.9995$	1:1−1:100
C18:1	388.31	391.33	$y = 1.0745x$; $r^2 = 0.9991$	1:1−1:100
C18:0	390.33	393.35	$y = 0.9774x$; $r^2 = 0.9998$	1:1−1:100
C20:0	418.36	421.38	$y = 1.0179x$; $r^2 = 0.9986$	1:1−1:100
C22:0	446.39	449.41	$y = 0.7967x$; $r^2 = 0.9997$	1:1−1:50
C24:0	474.42	477.44	$y = 0.8727x$; $r^2 = 0.9954$	1:1−1:10

[a] x, theoretic concentration ratio; y, experimental mass spectrometric peak intensity ratio.

In vitro stable isotope coding for comparative quantification has been very useful in proteomics.[33] This strategy has generally been performed in the following manner. Sample and control are derivatized separately with isotope coded or non isotope coded derivatizing reagents, respectively. When these differentially coded samples are then mixed at a certain ratio, e.g., 1:1, and analyzed by ESI-MS, doublet clusters of ions will be seen that are separated by the difference in mass between the isotopically coded derivatizing reagents. The MS peak height (area) ratio of this pair of

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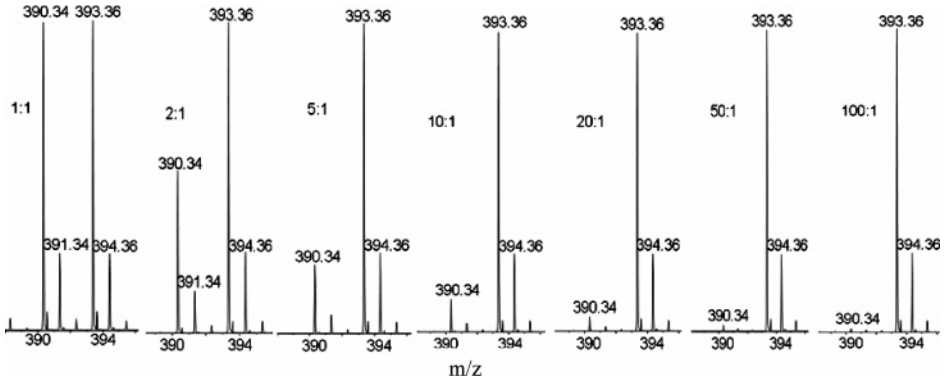

Figure 5. Mass spectra of light (d_0) and heavy (d_3) isotope-labeled CMP derivatives of stearic acid mixed at different ratios.

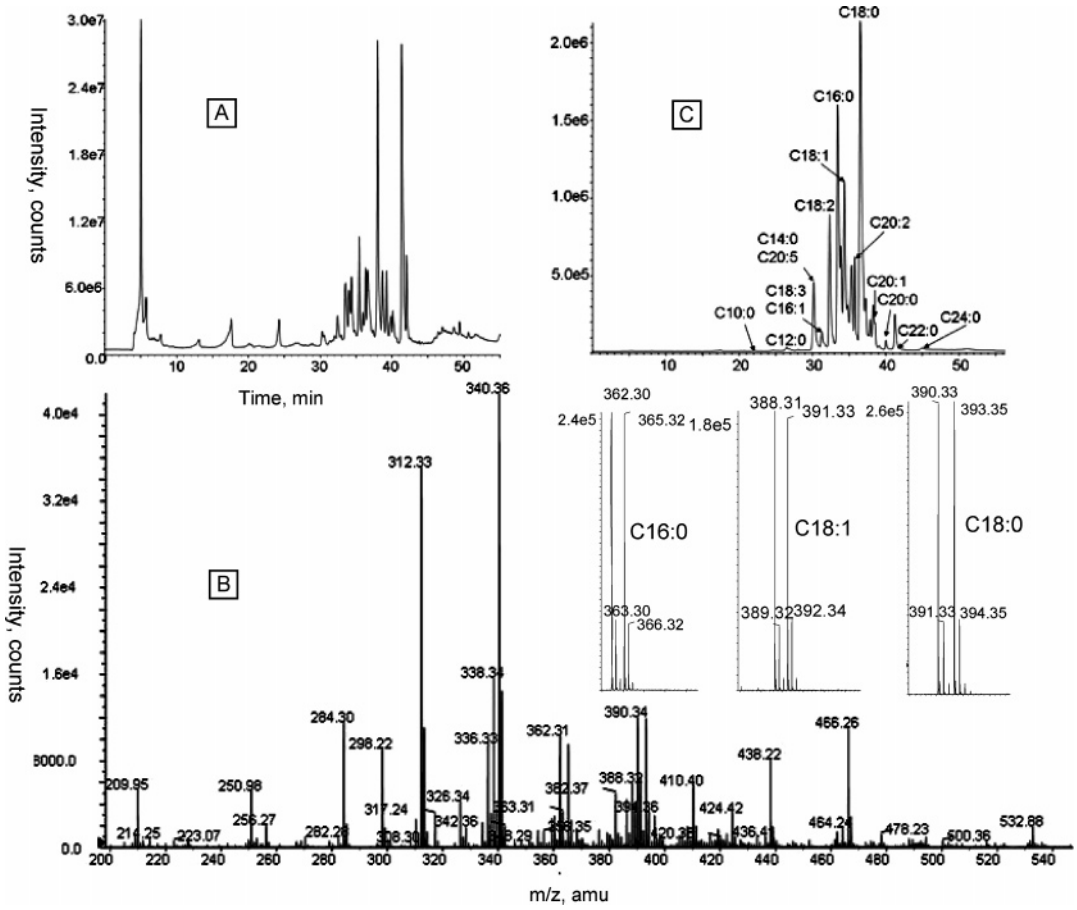

Figure 6. (A) Total ion chromatogram of human serum extract derivatized with light (d_0) and heavy (d_3) isotope-labeled CMP at the isotope ratio of 1:1; (B) accumulated mass spectrum within the elution period from 20 to 55 min in (A) with the insets of extracted spectra of d_0/d_3-labeled C16:0, C18:1, and C18:0; (C) reconstructed ion chromatogram of identified fatty acids from (B).

isotopomers is proportional to their concentration ratio in the sample and control.

A similar strategy will be of value in metabolomics and other fields, where it is the objective to compare the concentration of analytes between samples, especially when the stable isotope coded isoforms of analytes coelute from chromatographic systems. Coelution of coded isoforms is necessary to minimize several problems. One is that ionization of isotopomers may be differentially suppressed if they do not coelute. A second is that the isotope ratio of isotopomers will change across a peak if they are partially resolved.[49] Both of these phenomena can impact linearity and the accuracy of quantification. These issues were examined with the CMP and CMP-d_3 derivatizing agent. The results show that this method exhibits linearity for concentration ratios ranging from 1:10 to 1:100 for the various fatty acids studied with high correlation coefficients (Table 3). Figure 5 illustrates a series of spectra from isotopically derivatized stearic acid. No chromatographic isotope effect was observed with the CMP reagent. Although the presence of deuterium in a coding agent can cause chromatographic isotope effects, placing the deuterium on a quaternary amine as was done here negates the isotope effect.[22]

Applications. The utility of the method was examined in the analysis of fatty acids in human serum. Human serum or plasma contains several physiologically important fatty acids, among

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Table 4. Fatty Acids Found in Human Serum[a]

fatty acid found	*m/z* light labeled	*m/z* heavy labeled	retention time (min)	concentration (μM) determined (mean, % RSD, $n = 4$)	concentration (μM) ref 21 (mean, % RSD, $n = 8$)	concentration (μM) ref 53[b] (mean ± SD, $n = 23$)
C10:0	278.21	281.23	22.1	21.8, 6.8		13.9 ± 17.2
C12:0	306.23	309.26	26.4	32.1, 4.7		37.6 ± 24.7
C14:0	334.27	337.29	30.1	411.4, 4.5		156.8 ± 84.8
C20:5	408.25	413.28	30.8	nq[c]		256.7 ± 73.8
C18:3	384.29	387.31	31.0	nq		88.0 ± 52.6
C16:1	360.28	363.30	31.2	123.7, 5.6		204 ± 62.3
C18:2	386.30	389.32	32.3	1822.5, 6.2		3990.9 ± 646.5
C16:0	362.30	365.32	33.4	2280.0, 5.1	1166, 3.8	2255.4 ± 452.1
C18:1	388.31	391.33	34.3	1485.0, 5.9		1929.9 ± 586.4
C20:2	414.30	417.33	36.2	nq		
C18:0	390.33	393.35	36.5	2445.0, 6.3	1175, 4.1	683.0 ± 204.6
C20:1	416.32	419.34	38.5	nq		
C20:0	418.36	421.38	39.7	72.0, 4.2	74, 4.0	
C22:0	446.39	449.41	42.5	73.1, 6.5	34, 4.3	
C24:0	474.42	477.44	45.0	42.2, 6.9	67, 4.6	

[a] Parallel experiments were performed in quadruplicate from the identical derivatized serum sample. The results were the average of four runs. [b] The results represent the concentration range of total fatty acid in normal serum. The original data were reported in the total concentration of total fatty acid and percentage of individual fatty acid in serum. These values were converted to the present form by the authors. [c] Not quantified.

which six are most noteworthy: myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and linoleic (C18:2).[50] The analysis of fatty acids in human serum/plasma has been the object of numerous HPLC development studies.[39,40,47,48] Because of the complexity of serum, identification of fatty acids directly by HPLC/MS/MS may be time-consuming. We have developed a simple method for this objective by first tagging and sorting fatty acids through derivatization and then performing MS/MS for the confirmation of structure. Serum samples were first derivatized with CMP and CMP-d_3 separately, and the two resulting solutions were then mixed at a 1:1 ratio and analyzed by HPLC/ESI-MS. From the total ion chromatogram (scan range 200.0−550.0) in Figure 6A, the accumulated mass spectrum in Figure 6B was obtained. From this mass spectrum, fatty acids were recognized by looking for the doublet ion clusters with a 3.01−3.04 amu difference in mass and a peak height ratio of 1:1. Doublet cluster recognition was achieved both manually and with an algorithm designed for this purpose in proteomics.[51] Fatty acids were then identified by MS/MS analysis. Figure 6C is the reconstructed ion chromatogram of fatty acids identified by this method. Fifteen fatty acids were identified in the human serum sample.

Absolute quantitation of serum fatty acids was achieved by using isotopically derivatized internal standards. It has been common to use ^{2}H-coded fatty acids as internal standards for GC or HPLC/MS quantitation of serum fatty acids. It is relatively conveniently achieved through the addition of ^{2}H-coded fatty acids into the sample as internal standards. Nevertheless, ^{2}H-coded fatty acids are expensive, but they are commercially available by custom synthesis. The strategy used here was to generate ^{2}H-coded esters of fatty acids as internal standards by derivatizing fatty acids with ^{2}H-coded derivatizing reagents (CMP-d_3). CMP-d_3 is easily and inexpensively prepared by the methylation of 3- carbinolpyridine with iodomethane-d_3. In order to obtain reliable results, the standard fatty acids were processed and derivatized with CMP-d_3 simultaneously with the serum sample and then put into derivatized serum as a set of internal standards to perform HPLC/ESI-MS quantitation analysis. The results are reported in Table 4. Some fatty acids were found in the sample but not quantified because they were not included in our standard mixture of fatty acids. The results were close to those previously reported.[16,52]

CONCLUSIONS

It can be concluded from this work that in vitro derivatization of fatty acids affords the opportunity to incorporate features that can substantially enhance their detection and quantification by HPLC/ESI-MS/MS. One of these features is the introduction of a quaternary amine. Clearly this greatly increases detection sensitivity in the positive ion mode of detection by avoiding suppression of ionization in acidic mobile phases. A second feature of the derivatized fatty acid esters was that they showed unique fragmentation in ESI-MS/MS that made it very easy to identify their position in chromatograms along with the position of double bonds in their carbon skeleton. A third feature is the introduction of isotopic coding that facilitates both qualitative and quantitative analysis. Comparative quantitation and absolute quantitation were realized by ^{2}H-coding of derivatization reagents. The reported methods were validated by the analysis of fatty acids in human serum. Finally, it is concluded that the new technique described here should find wide application in future studies that require the analysis of fatty acids in complex samples.

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