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Facile Determination of Double Bond Position in Unsaturated Fatty Acids and Esters by Low Temperature Plasma Ionization Mass Spectrometry

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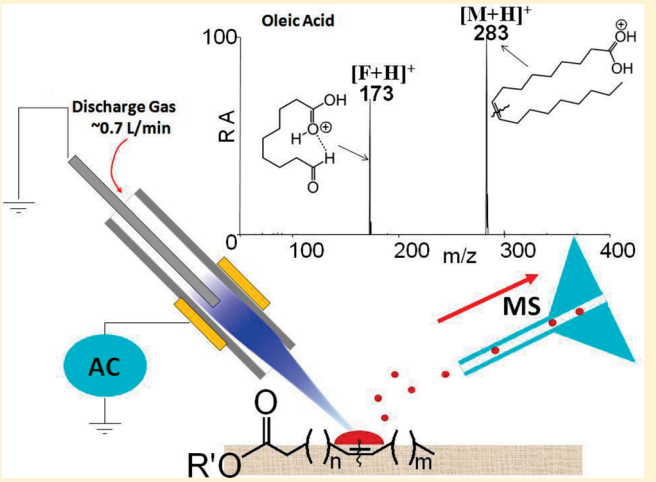
ABSTRACT: Unsaturated fatty acids and esters can be oxidized in situ during ionization using a low temperature plasma (LTP) probe. The discharge generates ozone from air that reacts with and cleaves olefins. The molecular ions of the resulting acid/ester oxidation products are present in the full scan mass spectra and are confirmed by exact mass measurements. The fragmentation information can be used to assign double bond positions. We have successfully applied this strategy to a range of mono-/polyunsaturated fatty acids and fatty acid methyl/ethyl esters to assign their double bond locations. The procedure allows rapid and direct identification of double bond positions in situ at atmospheric pressure without sample preparation prior to mass spectrometric analysis. Microbial fatty acid ethyl ester (FAEE) mixtures from complex bacterial samples were directly analyzed by this method. Structural confirmation of their diagnostic ions by using exact mass measurements and tandem mass spectrometry confirms double bond positions in unsaturated bacterial FAEEs.



Fatty acids play significant biological roles as structural components of cell lipid membranes in all organisms and serve as precursors of oxygenated signaling molecules in living cells.[1,2] Structurally, fatty acids differ in their chain lengths, degree of unsaturation, and double bond location. Locations of double bonds in unsaturated fatty acids have important consequences for the fluid nature of lipid membranes, and the appropriate isomer is critical to biological function.[1,2] Mass spectrometry has been a major tool in the study of the structures of fatty acids and their derivatives for over 50 years and has substantial advantages over alternative methods.[3−5] However, the identification of double bond positions by mass spectrometry has remained a challenge because double bond migration may occur during electron ionization[6,7] or chemical ionization.[8]

Previous attempts to identify double bond positions in unsaturated fatty acids and their esters have mainly been based on charge-remote fragmentation (CRF)[9−12] and double bond modification by chemical reactions.[13−19] CRF, first described by Gross and co-workers, is characterized by decreased cleavage of vinylic carbon−carbon bonds. It is typically implemented using high-energy collision-induced dissociation (CID) to yield tandem mass spectra, which allow double bond localization. In subsequent studies, the activation and ionization methods used were extended to include low energy multiple collisional activation and electrospray ionization. The precursor ion in these experiments can be the $[M - H]^-$ ion,[9] $[M - H + 2X]^+$ ($X = Li^+$, Na^+, K^+, Rb^+, or Cs^+),[10] or $[M + Y - H]^+$ ion ($Y = Mg^{2+}$, Ca^{2+}, Sr^{2+}, or Ba^{2+}).[12] The alternative approach, double bond modification, including off-line chemical derivatization[13−16,19] and covalent adduct formation during chemical ionization (CI),[17,18] utilizes chemical derivatization to "fix" double bonds. Examples include dimethyl disulfide adduct formation,[13,14] Diels−Alder (MTAD: 4-methyl-1,2,4-triazoline-3,5-dione) cycloaddition,[15] deuteration,[16] and acetonitrile CI,[17,18] followed by CID to locate their positions. Recently, Blanksby and co-workers introduced online ozonolysis as a method to cleave[20−22] double bonds present in unsaturated phospholipids, applying this chemistry either during ionization or during dissociation. Structurally diagnostic fragments occur in mass spectra recorded in the course of ozone electrospray ionization (OzESI-MS)[20,21] and also in ozone-induced dissociation (OzID),[22] and allow assignment of double bond positions. In OzESI-MS, ozonolysis occurs in the electrospray ionization source of a commercial mass spectrometer, which simplifies the otherwise complicated sample handling needed prior to mass spectrometry analysis. A limitation of the ionization method is not directly applicable to in situ analysis of

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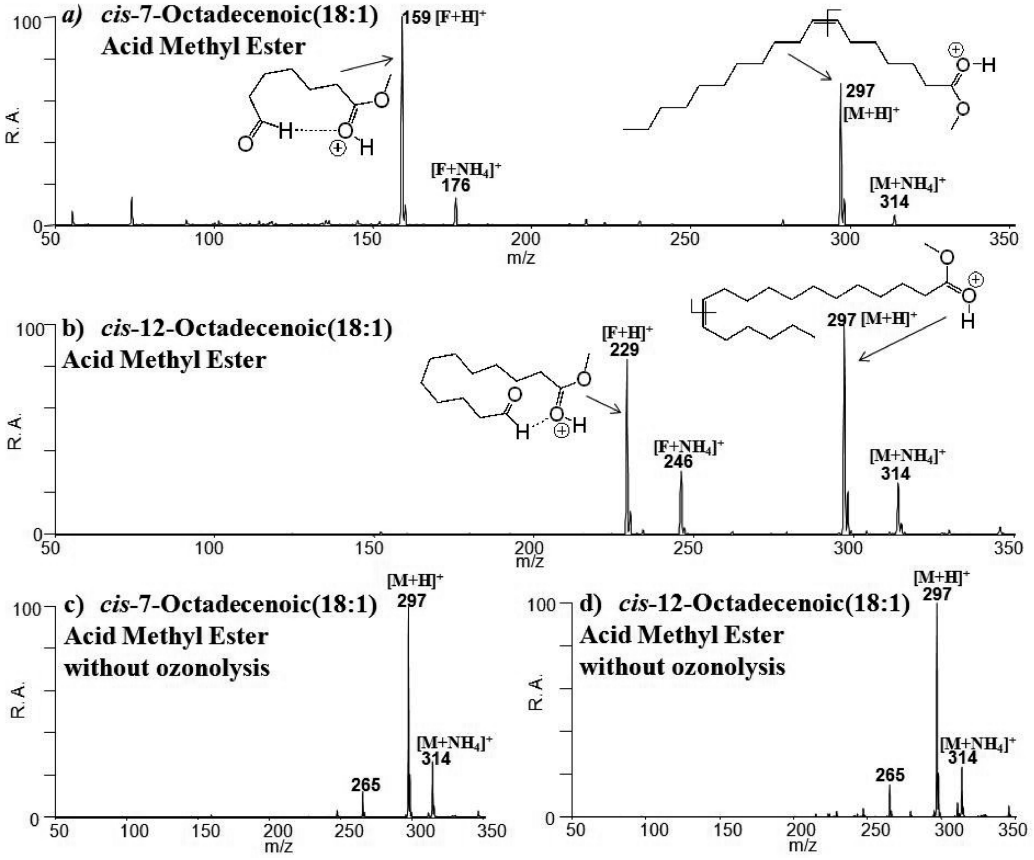

Figure 1. Positive ion mode LTP mass spectra of methyl esters of two isomeric monounsaturated fatty acids (18:1 FA). (a) *cis*-7-Octadecenoic (18:1) acid methyl ester using normal LTP probe, (b) *cis*-12-octadecenoic (18:1) acid methyl ester using normal LTP probe, (c) *cis*-7-octadecenoic (18:1) acid methyl ester using LTP probe with shield to avoid oxidation, and (d) *cis*-12-octadecenoic (18:1) acid methyl ester using LTP probe with shield to avoid oxidation. M designates the fatty acid methyl ester, and F stands for the ester-containing aldehyde oxidation product. None of the samples were heated.

complex samples. In the alternative method, OzID, the analyte can be ionized by any of a number of ionization methods, and the ions are exposed to ozone vapor within an ion trap mass spectrometer.

Direct analysis under ambient conditions is highly desirable for on-site analysis. One appropriate method, desorption electrospray ionization (DESI),[23] allows ambient analysis by directing a flow of charged solvent droplets generated by electrospray ionization onto the surface of the sample being analyzed. Secondary droplets are splashed from the surface and enter the mass spectrometer. Alternatives to DESI based on exposure of the sample to low powered plasmas, including low temperature plasma (LTP) ionization,[24] plasma-assisted desorption ionization (PADI),[25] dielectric barrier discharge ionization (DBDI),[26] and flowing atmospheric-pressure afterglow (FAPA),[27,28] have certain advantages associated with the fact that a spray solvent is not needed.[24,29] Discharge-induced oxidation in ambient air was previously reported in our laboratory[30] in the course of using DESI analysis of saturated hydrocarbons. In the present study, we show that in situ ambient ionization by LTP can be used to determine double bond positions in unsaturated fatty acids and their esters by chemical modification of the double bond. The method uses air as the oxidizing agent, so avoiding the need for special reagents and solvents. This approach was applied to complex bacterial samples to study fatty acid ethyl esters (FAEEs). Under the LTP ionization conditions, every double bond in a molecule can be oxidized and cleaved. The molecular ions of the acid/ester containing oxidation product as well as ions due to intact unsaturated molecule are present in the mass spectrum and were verified by LTQ-Orbitrap exact mass measurements and by tandem mass spectrometry. Together they can be used to assign the double bond position, thus providing a facile method to directly analyze unsaturated fatty acids and their esters in complex biological samples.

EXPERIMENTAL SECTION

Low Temperature Plasma Probe. As described in the literature,[24] the LTP probe consists of a glass tube (o.d. 6.35 mm and i.d. 3.75 mm, which serves as the dielectric barrier) with an internal grounded electrode (stainless steel; diameter, 1.57 mm) centered axially and an outer electrode of copper tape surrounding the outside of the glass tube. An alternating voltage of 2.5−5 kV at a frequency of 2.5 kHz is applied to the outer electrode with the center electrode grounded to generate the dielectric barrier discharge. The discharge ac voltage is provided by a custom-built power supply with total power consumption below 3 W. Helium is used as the discharge gas at an optimized flow rate of 0.7−0.9 L/min. It is fed through the glass tube to facilitate the discharge to direct the plasma onto the sample surface and to transport analyte ions to the mass spectrometer. When it was desirable to avoid ozonolysis, the LTP probe was

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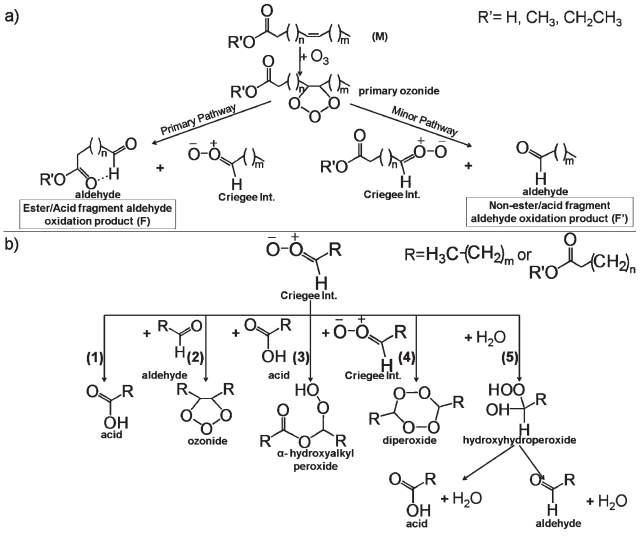
Scheme 1. Reaction Mechanisms for Ozonolysis of Double Bonds[33] in Unsaturated Noncyclic Fatty Acids or Methyl/Ethyl Esters (M)[a]



[a] (a) Formation pathways of aldehydes and Criegee intermediates (Criegee int.). (b) Formation pathways of acids, aldehydes, ozonides, α-hydroxyalkyl peroxides, and diperoxides in the presence and absence of water.

covered by a tubular plastic shield and a stream of helium, which completely eliminated atmospheric oxygen and the olefin fragmentation products.

Mass Spectrometry. A linear ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA) was used to record full scan mass spectra in the normal scan rate using these parameters: capillary temperature, 150 °C; max injection time, 100 ms; 3 microscans/spectrum. For the positive ion mode: capillary voltage, +15 V; tube lens voltage, +65 V. For the negative ion mode: capillary voltage, −15 V; tube lens voltage, −65 V. To confirm oxidation product ions, a Thermo Scientific Hybrid LTQ-Orbitrap mass spectrometer (Bremen, Germany) was used to measure the exact mass of each peak in the full scan mode, and each chemical formula was assigned using the XCalibur 2.0 software. Caffeine (m/z 195.0876) was used as the lock mass for accurate mass measurements in the positive ion mode, and stearic acid (m/z 283.2632) was used in the negative ion mode. The Orbitrap resolution was set to 60 000 (fwhm definition). Other parameters used are the same as those used in the LTQ as listed above. To further verify oxidation product ions, collision-induced dissociation (CID) experiments were performed using an isolation window of 1.5−2.0 mass/charge units, Mathieu q_z value of 0.25, and 20−25% (manufacturer's unit) collision energy. Unless otherwise indicated, the mass spectra shown here using the LTQ were background subtracted and represent the average of 20−40 scans.

Sample Preparation. All standard fatty acids and their methyl or ethyl esters used in this study were purchased from Cayman Chemical (Ann Arbor, MI). Samples were prepared by spotting a 1 μL ethanol solution containing 1 μg of each standard fatty acid or 100 ng of each standard fatty acid methyl/ethyl ester onto a glass microscope slide and allowing it to dry. All of the fatty acid esters were analyzed directly without heating; the fatty acids required mild heating to 60−80 °C for analysis.

The bacterial sample, *Salmonella enterica* Typhimurium INSP24 (SARA1),[31] was supplied by Dr. John H. Callahan and Dr. Rebecca Bell of the Food and Drug Administration (FDA). After being harvested from LB (Luria−Bertani) agar, bacteria were suspended in 70% ethanol and then stored at −80 °C. Cell concentrations were about 10^9 cfu/mL. Aliquots (1.5 μL) of the suspended cells were evenly spread onto a glass microscope slide for LTP analysis. No other sample preparation was performed.

RESULTS AND DISCUSSION

Monounsaturated Fatty Acid Esters. A low temperature plasma was generated using the LTP probe, and a stable low current (microamps) discharge was maintained through the dielectric barrier at atmospheric pressure. Ozone was produced by the dielectric barrier discharge[32] and recognizable by its odor. At the interface of the plasma and the sample, oxygen in the ambient air is converted to ozone, leading to ozonolysis of double bonds.[33]

Evidence of ozonolysis with oxidation of the double bonds is provided in Figure 1, which shows the LTP mass spectra of methyl esters of two isomeric monounsaturated fatty acids (18:1 FA) recorded in the positive ion mode (the fatty acid methyl ester ions were not observed in negative ion mode LTP mass spectra). Scheme 1 shows the proposed ozonolysis reaction mechanism[33] of double bonds in noncyclic fatty acids or esters (M). The ester/acid-containing aldehyde oxidation product (F) is relatively stable due to its intramolecular hydrogen bond (structure shown in Scheme 1a), which makes its formation the primary fragmentation pathway. As shown in Figure 1, the intense peaks at m/z 159 for *cis*-7-octadecenoic (18:1) acid methyl ester (Figure 1a), and at m/z 229 for *cis*-12-octadecenoic (18:1) acid methyl ester (Figure 1b) correspond to protonated forms of the aldehyde oxidation products retaining the ester chain. These product ions can be used to unequivocally assign double bond locations in monounsaturated fatty acid esters, at least in the cases of simple methyl and ethyl esters. Other oxidation fragments, particularly the nonester containing aldehyde/acid oxidation products (cf., Scheme 1b), are not observed in positive ion mode LTP mass spectra. To further validate the ozonolysis process, spectra (Figure 1c and d) were also recorded using a tubular plastic shield over the LTP probe to avoid air oxidation. No double bond oxidation products were present, only protonated intact esters, which confirm the molecular weight but give no information on double bond position. Minor ammonium (+18) adducts of both the intact esters and the ester-containing aldehyde oxidation products are observed and were also validated by exact mass measurements using the LTQ-Orbitrap (data not shown).

Monounsaturated Fatty Acids. The LTP method of ionization has adequate detection sensitivity for volatile and semivolatile molecules, which makes it appropriate for the analysis of unsaturated fatty acid esters for rapid double bond localization as discussed above. In the case of less volatile unsaturated fatty acids, the glass surface on which the sample was deposited was mildly heated to facilitate desorption of analytes. Figure 2a and b shows positive ion mode LTP mass spectra of two monounsaturated fatty acids, *cis*-9-hexadecenoic (16:1) acid and *cis*-9-octadecenoic (18:1) acid, recorded using the LTQ. As was the case for esters (Figure 1), only aldehyde oxidation fragments (m/z 173) containing carboxylic acid groups were observed on the spectra, so indicating double bond positions in the parent

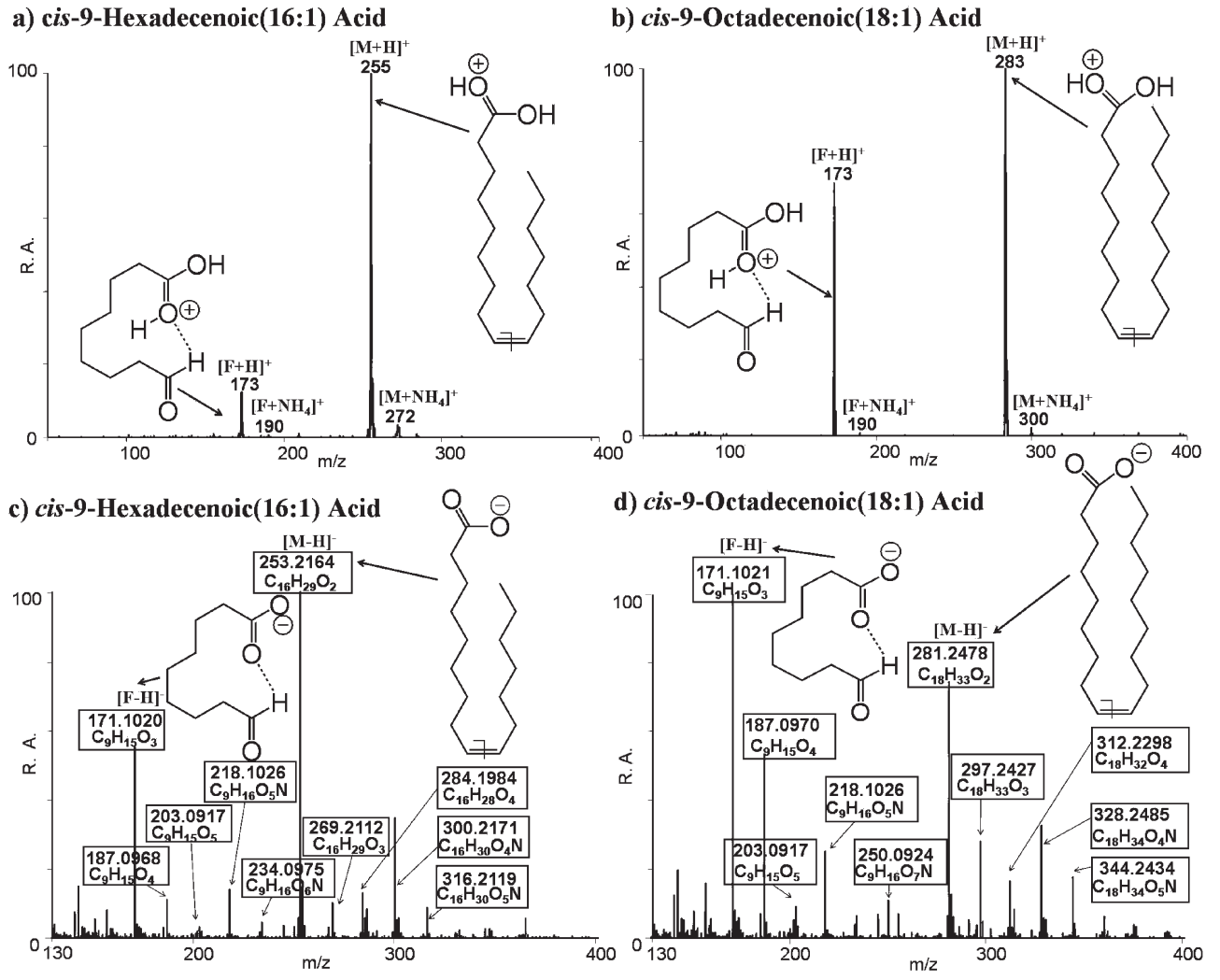


Figure 2. LTP mass spectra of two monounsaturated fatty acids. (a) LTQ mass spectrum of *cis*-9-hexadecenoic (16:1) acid in the positive ion mode. (b) LTQ mass spectrum of *cis*-9-octadecenoic (18:1) acid in the positive ion mode. (c) High-resolution Orbitrap mass spectrum of *cis*-9-hexadecenoic (16:1) acid in the negative ion mode. (d) High-resolution Orbitrap mass spectrum of *cis*-9-octadecenoic (18:1) acid in the negative ion mode. M designates the fatty acid, and F stands for the acid-containing aldehyde oxidation product. The samples were mildly heated to 60−80 °C.

monounsaturated fatty acids. A minor difference between the ester and the fatty acid LTP mass spectra is that the ammonium adduct ions (m/z 190, m/z 272, and m/z 300) are less abundant in the acid spectra, which may be due to the heating as well as the reduced cation affinity.

Figure 2c and d shows negative ion mode LTP mass spectra of *cis*-9-hexadecenoic (16:1) acid and *cis*-9-octadecenoic (18:1) acid recorded at high resolution using the LTQ-Orbitrap. All chemical formulas shown are based on the exact mass measurement and calculated using XCalibur 2.0 software. High abundance peaks corresponding to the acid containing aldehyde oxidation product ($[F - H]^-$) and the unsaturated parent molecule ($[M - H]^-$) can be used to assign the double bond position unambiguously. As compared to the positive ion mode spectra (Figure 2a and b), more plasma associated adducts of F and M are present. A series of +O, $+O_2$, $+HNO_2$, and $+HNO_3$ adducts of F and M were observed in both monounsaturated fatty acid negative ion mode LTP mass spectra.

Polyunsaturated Fatty Acids and Esters. When more than one double bond is present in unsaturated fatty acids and their esters, oxidative fragmentation occurs at each double bond, as illustrated in Figure 3a and b for the γ-linolenic acid (all-*cis*-6,9,12-18:3 FA) methyl ester and the pinolenic acid (all-*cis*-5,9,12-18:3 FA) ethyl ester LTP mass spectra recorded in the positive ion mode. For each of the polyunsaturated samples, in addition to the protonated precursors (γ-linolenic acid methyl ester at m/z 297 and pinolenic acid ethyl ester at m/z 307), three ester group containing aldehyde oxidation products, corresponding to reactions occurring separately at each of the three double bonds, are present as evidenced by peaks m/z 145 ($[F_6 + H]^+$), 185 ($[F_9 + H]^+$), and 225 ($[F_{12} + H]^+$) for γ-linolenic acid methyl ester and peaks m/z 145 ($[F_5 + H]^+$), 199 ($[F_9 + H]^+$), and 239 ($[F_{12} + H]^+$) for pinolenic acid ethyl ester. These ions allow clear assignment of three double bond positions provided one knows the stoichiometry of the original ester. These three oxidation products for each sample, $[F_5 + H]^+/[F_6 + H]^+$, $[F_9 + H]^+$, and $[F_{12} + H]^+$, show decreasing intensities with increasing chain length, likely due to further double bond oxidation of the larger unsaturated oxidation products, F_9 and F_{12} (structures shown in Figure 3a and b). Ammonia adducts (+17) are observed as well as

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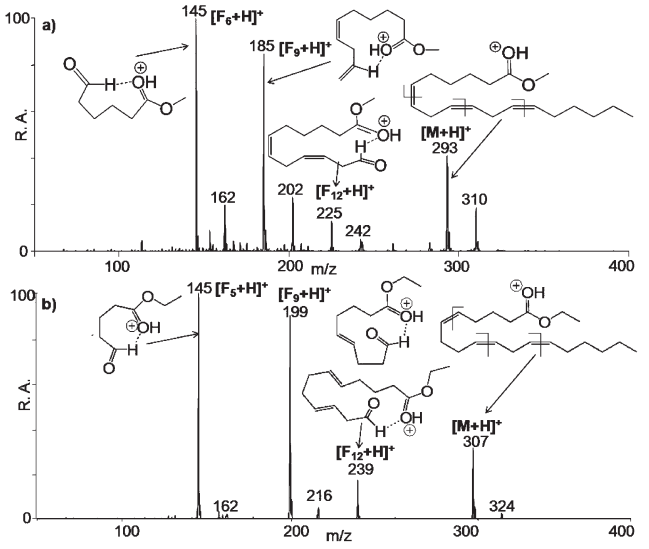

Figure 3. (a) Positive ion mode LTP mass spectrum of γ-linolenic acid (all-*cis*-6,9,12-18:3 FA) methyl ester. (b) Positive ion mode LTP mass spectrum of pinolenic acid (all-*cis*-5,9,12-18:3 FA) ethyl ester. Neither of the samples was heated. M designates the fatty acid methyl/ethyl esters. F_5, F_6, F_9, and F_{12} are ester-containing aldehyde oxidation products corresponding to separate reactions at the n-5, n-6, n-9, and n-12 double bonds.

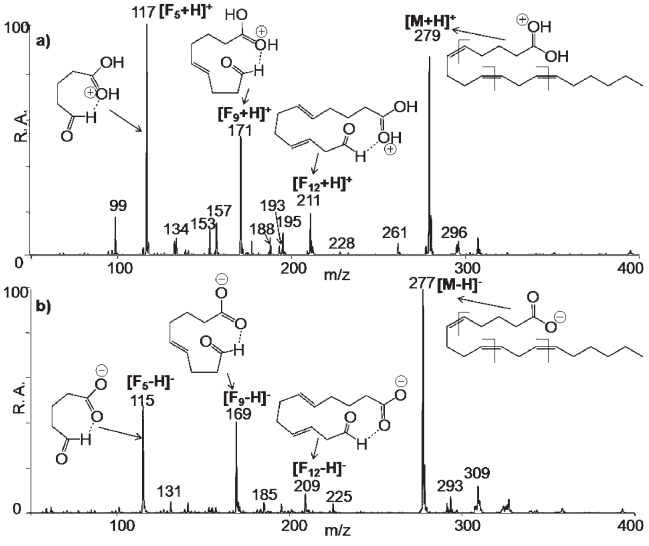

Figure 4. (a) Positive ion mode LTP mass spectrum of pinolenic acid (all-*cis*-5,9,12-18:3 FA). The sample was mildly heated to 60−80 °C. (b) Negative ion mode LTP mass spectra of pinolenic acid (all-*cis*-5,9,12-18:3 FA). The samples were mildly heated to 60−80 °C. M designates the fatty acid. F_5, F_9, and F_{12} are acid-containing aldehyde oxidation products corresponding to separate reactions of n-5, n-9, and n-12 position double bonds.

ions at m/z 310, 162, 202, and 242 corresponding to $[M + H]^+$, $[F_6 + H]^+$, $[F_9 + H]^+$, and $[F_{12} + H]^+$ for γ-linolenic acid methyl ester (Figure 3a) and at m/z 324, 162, and 216 corresponding to $[M + H]^+$, $[F_5 + H]^+$, and $[F_9 + H]^+$ for pinolenic acid ethyl ester (Figure 3b).

With mild heating to 60−80 °C during the analysis of polyunsaturated fatty acids (as Figure 4), the major peaks present

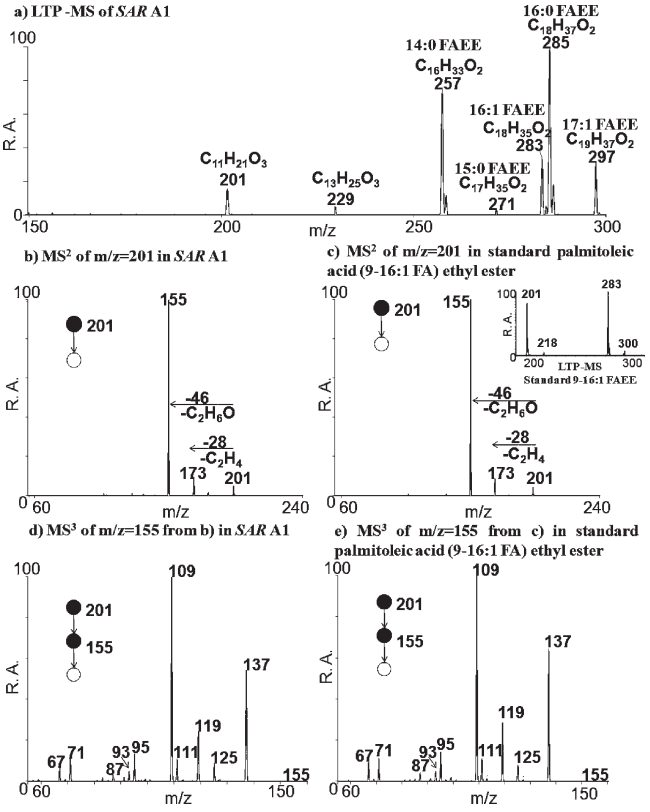

Figure 5. (a) LTP full scan mass spectrum of SARA1 in the mass range between 250 and 300. (b) LTP MS/MS product ion scan of m/z = 201 in SARA1. (c) LTP MS/MS product ion scan of m/z = 201 recorded using standard compound palmitoleic acid (9-16:1 FA) ethyl ester and its LTP full scan mass spectrum shown in the inset. (d) LTP MS/MS/MS of m/z = 155 from MS/MS of m/z = 201 in SARA1. (e) LTP MS/MS/MS of m/z = 155 from MS/MS of m/z = 201 in standard compound palmitoleic acid (9-16:1 FA) ethyl ester. All spectra are in the positive ion mode. The samples were not heated.

in mass spectra correspond to the intact fatty acid molecules and their acid fragment aldehyde oxidation fragments (F), which can be used for double bond position assignments. In addition, many minor peaks in the spectra give more information about other oxidation fragments (nonacid fragment aldehyde/acid oxidation products in Scheme 1b). Figure 4a shows the positive ion mode LTP mass spectrum of pinolenic acid (all-*cis*-5,9,12-18:3 FA). Peaks at m/z 117 ($[F_5 + H]^+$), 171 ($[F_9 + H]^+$), and 211 ($[F_{12} + H]^+$) are protonated forms of the acid fragment aldehyde oxidation products corresponding to reactions occurring separately at the n-5, n-9, and n-12 position double bonds. Besides ammonia adducts (+17) of these ions and protonated pinolenic acid (m/z 134, 188, 228, and 296), a series of dehydration peaks at m/z 99, 153, 193, and 261 is present. Because of the greater stability of F_5 as compared to the other unsaturated fragments F_9 and F_{12}, given the operation of the reaction mechanism illustrated in Scheme 1, the most abundant noncarboxylic acid fragment oxidation product should be an aldehyde (F'_5) formed at the n-5 double bond. This ion is observed as m/z 195 ($[F_5' + H]^+$) in Figure 4a. The n-9 double bond nonacid fragment acid oxidation product is also observed as m/z 157. These minor peaks offer some information about other oxidation products, but due to their low abundances especially after background subtraction, their information content is quite limited.

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Table 1. Exact Mass Measurements of Characteristic Peaks in SARA1 by LTP-LTQ-Orbitrap

experimental m/z (Da)	theoretical m/z (Da)	m/z error (Δ ppm)	molecular formula	structure identified
201.1483	201.1485	−1.1738	$C_{11}H_{21}O_3$	ester fragment aldehyde oxidation product of n-9 double bond
229.1797	229.1798	−0.5166	$C_{13}H_{25}O_3$	ester fragment aldehyde oxidation product of n-11 double bond
257.2477	257.2475	0.5573	$C_{16}H_{33}O_2$	C 14:0 FAEE
271.2633	271.2632	0.6611	$C_{17}H_{35}O_2$	C 15:0 FAEE
283.2634	283.2632	0.7408	$C_{18}H_{35}O_2$	C 16:1 FAEE
285.2790	285.2788	0.7547	$C_{18}H_{37}O_2$	C 16:0 FAEE
297.2790	297.2788	0.7243	$C_{19}H_{37}O_2$	C 17:1 FAEE

Figure 4b shows the negative ion mode LTP mass spectrum of pinolenic acid (all-*cis*-5,9,12-18:3 FA). Three acid fragment aldehyde oxidation product ions (m/z 115 $[F_5 - H]^+$, 169 $[F_9 - H]^+$, and 209 $[F_{12}-H]^+$) corresponding to n-5, n-8, and n-12 position double bonds are clearly present as is the pinolenic acid molecule ion (m/z 277 $[M - H]^-$). These ions provide valuable structural information for the determination of double bond positions in polyunsaturated fatty acids. Adduct ions +O (m/z 131 $[F_5 - H + O]^+$, 185 $[F_9 - H + O]^+$, 225 $[F_{12} - H + O]^+$, and 293 $[M - H + O]^+$) and $+O_2$ adduct ions (as m/z 309 $[M - H + O_2]^+$) were also observed in this polyunsaturated fatty acid negative ion mode LTP mass spectrum.

Microbial Fatty Acid Ethyl Ester Mixtures. There has been some exploration of LTP for direct analysis of compounds in complex biological samples.[24] Because of its sensitivity when applied to volatile or semivolatile molecules, we applied this strategy to directly analyze unsaturated fatty acid esters in bacteria. A bacterial sample, SARA1 in 70% ethanol, was deposited on a glass slide and directly examined by LTP-MS. As shown by the mass spectrum (Figure 5a), there are five FAEEs detected in the m/z range of 250−300. All of the chemical formulas shown in Figure 5a were calculated using XCalibur 2.0 software based on the exact masses measured using the Orbitrap (as Table 1). Nonoxidative ethanol metabolism may be responsible for the production of the FAEEs[34] when fresh harvested bacteria cells are suspended in 70% ethanol. SARA1 as a gram-negative bacterium has thin cell walls comprised of about 20% peptidoglycan with a lipopolysaccharide layer, which may be dissolved away by 70% ethanol, thus exposing the lipid bilayer of the cell to release FAEEs.

To verify these identifications, MS/MS and MS/MS/MS spectra of the oxidation fragment ions generated in bacterial samples were compared to those from standard compounds. Figure 5b−e shows almost identical MS/MS and MS/MS/MS product ion spectra of m/z 201 in bacteria SARA1 (4b and 4d) and in standard palmitoleic acid (9-16:1 FA) ethyl ester (4c and 4e), which demonstrates that the m/z 201 peak in SARA1 is the ester fragment aldehyde oxidation product of the n-9 double bond. Similarly, the observation of the protonated ester-containing aldehyde oxidation product at m/z 229 ($C_{13}H_{25}O_3$) indicates the presence of unsaturated FAEE(s) with the double bond at n-11 position. Among the five FAEEs identified in Figure 5a, there are two monounsaturated FAEEs that occur at m/z 283 (16:1 FAEE) and at m/z 297 (17:1 FAEE), and therefore each of them has two possible positions for the double bond: n-9 or n-11. Palmitoleic acid (16:1 FA) is a common unsaturated fatty acid in biological systems. The heptadecenoic acid (17:1 FA) with an n-11 double bond is an essential ω-6 fatty acid, which has important biological effects.[35]

CONCLUSION

In situ oxidation and ionization of a range of unsaturated fatty acids and fatty acid methyl/ethyl esters has been achieved using LTP. In-source LTP ozonolysis results in the formation of ester/acid fragment aldehyde oxidization products. The full scan mass spectra show exclusively the intact molecular ions, which occur without oxidation products when air is excluded by sheathing the LTP probe. These ions and the oxidation products were confirmed by exact mass measurements. Together the data facilitate the determination of the nature of fatty acid/esters and their double bond position(s). LTP-MS was applied to directly analyze microbial FAEE mixtures in bacterial samples and their diagnostic ions verified by exact mass measurements and tandem mass spectrometry, allowing the recognition of the presence of unsaturated FAEEs with double bonds at the n-9 or n-11 positions.

With its simplicity and advantages as an ambient direct detection method, LTP-MS provides an approach to analyze double bond positions that might find applications in a broad range of problems requiring structural elucidation of unsaturated molecules without explicit chemical derivatization or prior sample treatment. This method is comparable to the OzESI-MS and OzID methods introduced by Blanksby, but it allows in situ analysis and does not require instrumental modifications. On the other hand, the quality of data obtainable for more complex analytes is not as good as that obtained from the earlier methods.

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