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Localization of Double Bonds in Wax Esters by High-Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometry Utilizing the Fragmentation of Acetonitrile-Related Adducts

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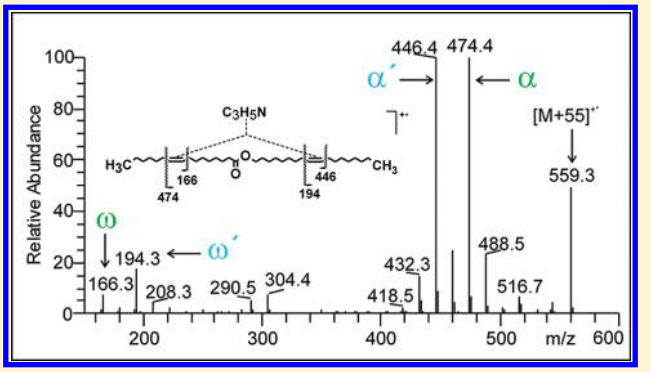
Supporting Information

ABSTRACT: Unsaturated wax esters (WEs) provided molecular adducts with C_3H_5N ($[M + 55]^{+\bullet}$) in APCI sources in the presence of acetonitrile. CID MS/MS of $[M + 55]^{+\bullet}$ yielded fragments allowing the localization of double bond(s) in the hydrocarbon chains of the WEs. These fragments were formed by a cleavage on each side of the double bond. In methylene-interrupted polyunsaturated WEs, diagnostic fragments related to each double bond were detected; the most abundant were those corresponding to the cleavage of the C−C bond next to the first and the last double bond. To differentiate between those fragments differing in their structure or origin, a simple nomenclature based on α and ω ions has been introduced. Fragmentation of the α-type ions (fragments containing an ester bond) provided information on the occurrence of a double bond in the acid or alcohol part of the WEs. While no significant differences between the spectra of the WEs differing by cis/trans isomerism were found, the isomers were separated chromatographically. A data-dependent HPLC/APCI-MS^2 method for the comprehensive characterization of WEs in their complex mixtures has been developed and applied to natural mixtures of WEs isolated from jojoba oil and beeswax. More than 50 WE molecular species were completely identified, including the information on the acid and alcohol chain length and the position of the double bonds.



The presence of double bonds and their number, position, and cis/trans isomerism strongly affect the physicochemical properties of lipids and their functions in biological systems.[1−3] The localization of double bonds in unsaturated long-chain compounds is a classical analytical problem, especially challenging when the analytes are present in complex mixtures and low quantities. The methods for localization of the double bonds based on mass spectrometry have recently been reviewed.[4,5]

Acetonitrile was recognized as a useful gas-phase reagent for chemical ionization (CI) mass spectrometry (MS) in the early 1980s.[6] Moneti et al. implemented CI-MS with acetonitrile on an ion-trap instrument and used it to determine the molecular weight of hydrocarbons based on their $[M + C_2H_2N]^+$ adducts.[7] They subsequently showed that monounsaturated hydrocarbons form $[M + 54]^+$ adducts, providing fragments indicative for the position of a double bond.[8] These adducts were identified as $[M + C_3H_4N]^+$ cations formed by the addition of a reactive species, (1-methylenimino)-1-ethenylium ($H_2C{=}N^+{=}C{=}CH_2$), to the C=C double bond.[9−13] The subsequent cleavage of the former double bond yielded a pair of ions corresponding to two halves of the molecule plus 54 mass units.[8] The same fragmentation of unsaturated hydrocarbons upon CI with acetonitrile was observed by Oldham and Svatoš.[14] However, when the analytes were allowed to react only with $C_3H_4N^+$ using lower cutoff mass settings for the ion trap, a different fragmentation behavior was observed. The $[M + 54]^+$ adducts were fragmented by a cleavage of the C−C bonds in the β-position with respect to the double bond (allylic-type cleavage). Both types of fragmentation enabled the unambiguous assignment of the position of the double bond. CI with acetonitrile has also been used for the localization of double bonds in various functionalized long-chain compounds.[14−19] Functionalized monoenes, with the exception of amines, provided fragments characteristic for the position of a double bond.[14,15] Brenna et al. published several papers on

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acetonitrile CI tandem mass spectrometry for locating double bonds in polyunsaturated fatty acid methyl esters (FAMEs).[16−21] Regardless of the number of double bonds, FAMEs formed $[M + 54]^+$ adducts that yielded diagnostic fragments upon collision-induced dissociation (CID). It was proposed that $C_3H_4N^+$ adds covalently across double bonds and $[M + 54]^+$ represents a mixture of isomeric species.[16] The mechanisms of $[M + 54]^+$ formation and fragmentation were described for FAMEs with various numbers of double bonds.[20] CI with acetonitrile was successfully applied for the localization of double bonds in homoallylic (methylene-interrupted)[17] as well as in nonmethylene-interrupted[19] polyunsaturated FAMEs. It was also shown that cis/trans geometry on double bonds can be established based on the fragment intensities.[18,19,21]

The majority of methods for localizing double bond(s) require electron ionization, which is typically accomplished by GC/MS instruments. This approach is highly successful, but there are principal limitations connected with the need for sufficient volatility and thermal stability of the lipids or their derivatives. Therefore, lipids not amenable to GC have to be hydrolyzed. Lipid hydrolysis is not only an additional step in the analytical workflow but also causes a loss of information on fatty acid binding in original lipids. To avoid the need for lipid hydrolysis, procedures based on atmospheric pressure ionization MS coupled to HPLC are being sought. One successful approach is based on reactions with ozone.[22−24] The gas-phase chemistry of acetonitrile has also been adapted for the ion sources operating at atmospheric pressure. An APCI-MS/MS method for the structure elucidation of triacylglycerols (TGs) containing monoene and diene fatty acyl groups has been developed.[25]

We have recently published an HPLC/MS method for analyzing complex mixtures of wax esters (WEs) using acetonitrile/ethyl acetate gradient and APCI-MS detection.[26] The APCI mass spectra of unsaturated WEs were found to contain $[M + 55]^{+\bullet}$ adducts related to acetonitrile. In this work, we report on their use for the localization of the double bond position(s) in WEs. We describe an HPLC/APCI-MS^n method for a complete characterization of WE molecular species in mixtures, comprising the determination of the molecular weight, identification of the fatty-acid and alcohol-chain length, and localization of the double bond position(s) within the chains.

EXPERIMENTAL SECTION

Chemicals. The WE standards were purchased from Nu-Chek-Prep (Elysian, MN) and Sigma-Aldrich (St. Louis, MO), or synthesized by a procedure published elsewhere.[26] The standard solutions were prepared in chloroform (0.4 μmol/mL) and further diluted in acetonitrile/ethyl acetate (1:1, by vol). Natural mixtures of WEs were isolated from honeybee wax and jojoba oil by a semipreparative TLC[26] and dissolved in ethyl acetate/chloroform (1:1, by vol) at concentrations of 10 mg/mL and 20 mg/mL, respectively.

Instrumentation. The experiments were performed using three different mass spectrometers equipped with an APCI source and coupled to HPLC: An LCQ Fleet ion-trap, an LTQ Orbitrap XL hybrid mass spectrometer (both Thermo Fisher Scientific), and a Q-Tof micro (Waters). The WE standard solutions were directly introduced into the ion sources. The WE mixtures were separated using Nova-Pak C18 columns and an acetonitrile/ethyl acetate gradient. For the settings of the instruments and conditions of the flow injection and chromatography, see Supporting Information.

WE Abbreviations and Nomenclature. In this work, a simple abbreviated nomenclature for WEs has been used.[27] The first part of the abbreviations refers to an alcohol segment of the molecule, whereas the second part indicates the fatty acid. The position of the double bond (or a group of methylene-interrupted double bonds) is indicated as (n-x), where x is the distance from the terminal end of the hydrocarbon chain. If not specified further, cis double-bond geometry is assumed. Thus, for instance, the abbreviation WE 22:0-16:1(n-7) is used for docosanoyl-*cis*-7-hexadecenoate (behenyl palmitoleate). In accordance with previously published work,[16] the diagnostic ions denoted as α are fragments containing an ester moiety, whereas ω ions carry the terminal-carbon end and do not include an ester group. The diagnostic fragments are further specified as follows: The α and ω symbols are used if the fragmented double bond is located in the acid part. The ions are denoted α' and ω' if the fragmented double bond is in the alcohol part. To distinguish between double bonds in polyunsaturated chains, a subscript specifying the position of the fragmented double bond is added. For example, a fragmentation of WE 18:3(n-3)-18:1(n-9) can generate α, ω, α'_{n-3}, ω'_{n-3}, α'_{n-6}, ω'_{n-6}, α'_{n-9}, and ω'_{n-9} (assuming a methylene-interrupted arrangement of the double bonds). Note that α/α' and ω/ω' fragments in this work are structurally different from those in ref 16.

RESULTS AND DISCUSSION

The $[M + 55]^{+\bullet}$ Adducts in the APCI Spectra of WEs. Figure 1a shows the APCI mass spectrum of WE 18:1(n-9)-18:0 dissolved in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source. The elemental composition of m/z 589 ($[M + 55]^{+\bullet}$) was established by orbitrap ($C_{39}H_{75}NO_2^{+\bullet}$; expt mass: 589.5788, calcd mass: 589.5792; error: −0.8 ppm). The mass difference between this ion and $[M + H]^+$ was 54.0341 Da, which corresponded to C_3H_4N (calcd mass: 54.0344; error: −4.7 ppm). Hence, the ion m/z 589 was a molecular adduct $[M + C_3H_5N]^{+\bullet}$. The series of experiments revealed that unsaturated esters form these adducts regardless of the number of double bonds. Interestingly, $[M + 55]^{+\bullet}$ adducts were significantly more abundant in the spectra of esters with double bond(s) located in the alcohol part. Saturated WEs did not form $[M + 55]^{+\bullet}$ adducts. The formation of $[M + 55]^{+\bullet}$ was also noticed in the mobile phases composed of acetonitrile/2-propanol and acetonitrile/acetone. Thus, the origin of these adducts was most likely related to acetonitrile.

The formation of $[M + 55]^{+\bullet}$ was not reported previously. To the best of our knowledge, the only work dealing with acetonitrile-related adducts generated by APCI was focused on TGs.[25] TGs formed several adducts ($[M + 40]^+$ $[M + 54]^+$, $[M + 81]^+$, $[M + 95]^+$), but not $[M + 55]^{+\bullet}$. We repeated the published experiments with 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol (POO) and 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol (POP) and confirmed the formation of the above-mentioned ions. In addition, $[M + 55]^{+\bullet}$ adducts were observed, providing substantially more abundant peaks than $[M + 54]^+$. The $[M + 55]^{+\bullet}$ of POP was observed at m/z 887.7942 ($C_{56}H_{105}O_6N^{+\bullet}$; calcd mass: 887.7936, error: 0.6 ppm). Clearly, the $[M + C_3H_5N]^{+\bullet}$ adducts were generated also for TGs. It is, however, unclear why the previous researchers[25] did not detect them. We speculate that the formation of acetonitrile-related adducts might be related to the

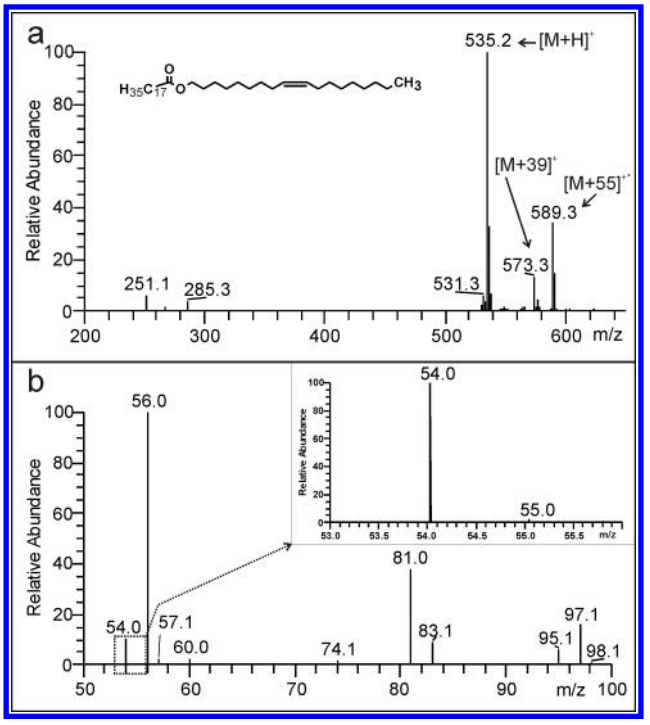

Figure 1. The APCI full-scan mass spectrum of oleyl stearate (WE 18:1(n-9)-18:0) prepared in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source of LCQ Fleet (a) and the APCI full-scan mass spectrum of acetonitrile infused (500 μL/min) into the ion source of LTQ Orbitrap XL (b).

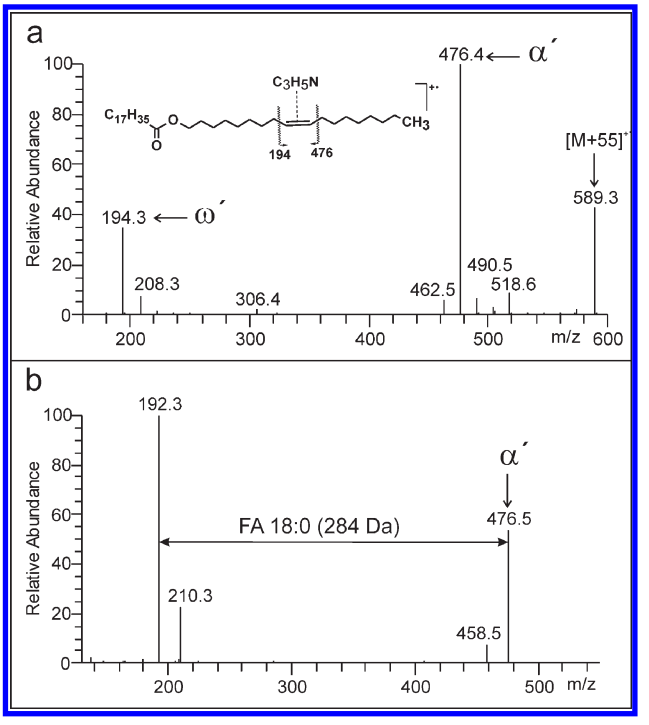

Figure 2. The APCI MS/MS spectrum of the $[M + 55]^{+\bullet}$ adduct of oleyl stearate (WE 18:1(n-9)-18:0) (a) and the MS^3 spectrum of the α′-fragment (m/z 476) (b). The sample was prepared in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source of LCQ Fleet.

construction of the ion source and/or conditions and settings used. To study acetonitrile-related species possibly involved in the $[M + 55]^{+\bullet}$ formation, a high-resolution APCI spectrum of acetonitrile was recorded (Figure 1b). The ions were characterized based on their exact masses and literature data.[9−11,28−30] The elemental composition of m/z 54.0336 was C_3H_4N (calcd mass: 54.0338, error: −3.4 ppm). This cation was likely identical to (1-methylenimino)-1-ethenylium ($H_2C{=}C{=}N^+{=}CH_2$),[9,10] known to be a product of a reaction of CH_2CN^+ with acetonitrile proceeding via the $C_4H_5N_2^+$ (m/z 81) intermediate.[29] A radical cation $C_3H_5N^{+\bullet}$ provided very weak signal m/z 55.0414 (calcd mass: 55.0417, error: −3.6 ppm). An ion with the same composition and linear structure as $CH_3{-}C^+{=}N{-}C^{\bullet}H_2$ is generated by a reaction of $CH_3CN^{+\bullet}$ with acetonitrile.[11] The spectrum base peak m/z 56.0493 appeared to be $C_3H_6N^+$ (calcd mass: 56.0495, error: −3.7 ppm) and was probably the same as $CH_3{-}C^+{=}N{-}CH_3$ (ref 11). Its fragmentation was shown to generate $C_3H_5N^{+\bullet}$ (ref 31). The ions at higher m/z values were probably clusters formed at elevated pressure in the ion source. The structure of m/z 81.0447 ($C_4H_5N_2^+$; calcd mass: 81.0447, error: −0.9 ppm) might be identical to $CH_3{-}C^+{=}N{-}CH_2{-}C{\equiv}N$ (ref 30), formed by a reaction of CH_2CN^+ with CH_3CN (ref 29). The m/z 83.0603 ($C_4H_7N_2^+$; calcd mass: 83.0604, error: −1.0 ppm) was likely formed from protonated and neutral acetonitrile; a cyclic structure is assumed.[30] The ions m/z 95.0603 and 97.0760 were consistent with $C_5H_7N_2^+$ (calcd mass: 95.0604, error: −1.0 ppm) and $C_5H_9N_2^+$ (calcd mass: 97.0760, error: −0.8 ppm), respectively. A structure of $CH_3{-}C^+{=}N{-}C(=CH_2){-}N{=}CH_2$ was suggested[30] for m/z 95.

The investigation of the mechanism leading to $[M + 55]^{+\bullet}$ adducts is beyond the scope of this paper, but we suppose that direct [2 + 2] cycloaddition[20] of the odd-electron species is unlikely. The putative reagent $C_3H_5N^{+\bullet}$ was detected, but its abundance was very low. We speculate that a different mechanism, possibly involving highly abundant $C_3H_6N^+$, is involved.

Fragmentation of the $[M + 55]^{+\bullet}$ Adducts. *Monounsaturated WEs.* Figure 2a shows the CID MS/MS spectrum of the $[M + 55]^{+\bullet}$ adduct of WE 18:1(n-9)-18:0. The most abundant fragments were even-electron species formed by the cleavages of C−C bonds next to the double bond. Thus, the cation m/z 476, denoted as α′, contained an ester moiety and was formed after the elimination of the radical $C_8H_{17}^{\bullet}$. The cation m/z 194, denoted as ω′, was formed by a cleavage of the C−C bond next to the double bond on its opposite side. Both of these ions comprised the acetonitrile-related addition species C_3H_5N, and the exact mass measurement confirmed the expected elemental compositions of $C_{31}H_{58}O_2N$ (expt mass: 476.4462, calcd mass: 476.4462, error: 0.0 ppm) and $C_{13}H_{24}N$ (expt mass: 194.1904, calcd mass: 194.1903, error: 0.6 ppm). These ions were accompanied by several less abundant peaks differing from the α′ and ω′ ions by 14 mass units ($\pm(CH_2)_n$). An ion m/z 306 was a cation formally explained by a loss of the fatty acid (18:0) radical. A CID spectrum of the $[M + 55]^{+\bullet}$ adduct of isomeric WE with a double bond in the acid part, WE 18:0-18:1(n-9) (Figure 3a), closely resembled the previous one with a double bond in the alcohol part. The important difference was the absence of m/z 306. Instead, m/z 318 and m/z 336 were detected. They were even-electron species containing oxygen formed by the elimination of the radical $C_{18}H_{37}^{\bullet}$ from the alcohol part of the WE (m/z 336) and the loss of water (m/z 318). Further confirmation of the location of the double bond in the alcohol/acid part was provided by the CID MS^3 spectra of the α′ (Figure 2b) and α (Figure 3b) fragments. When the double bond was located in the

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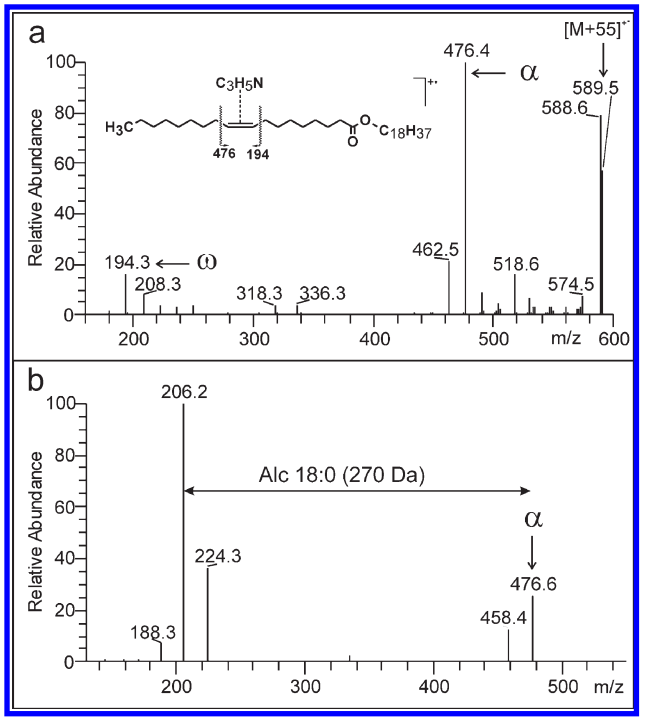

Figure 3. The APCI MS/MS spectrum of the $[M + 55]^{+\bullet}$ adduct of stearyl oleate (WE 18:0-18:1(n-9)) (a) and the MS^3 spectrum of the α-fragment (*m*/*z* 476) (b). The sample was prepared in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source of LCQ Fleet.

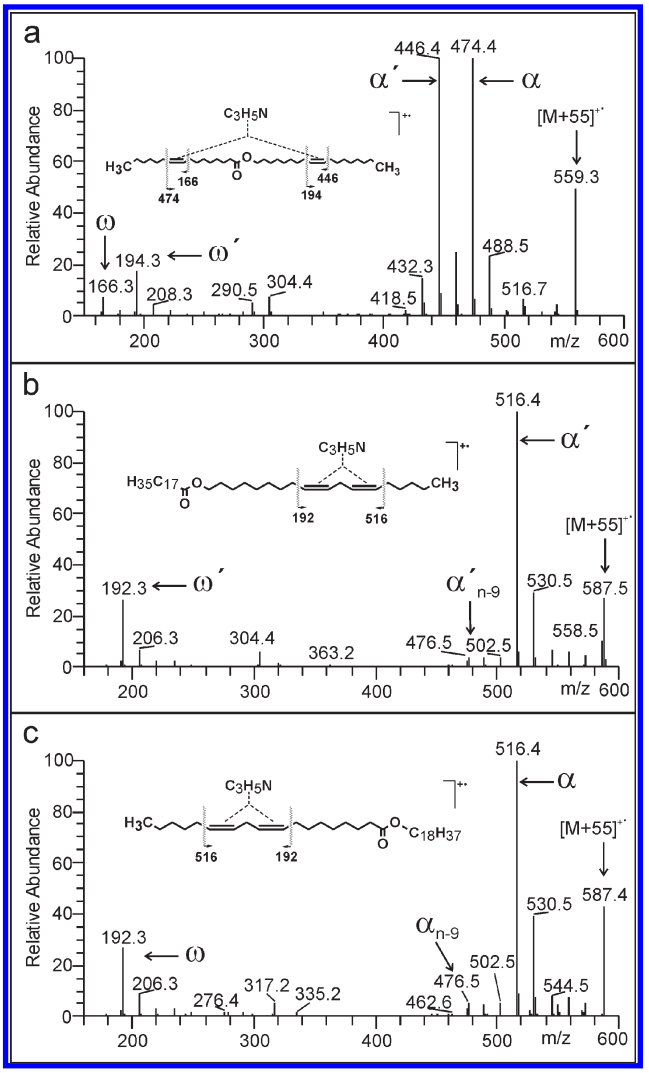

Figure 4. The APCI MS/MS spectrum of the $[M + 55]^{+\bullet}$ adduct of oleyl palmitoleate (18:1(n-9)-16:1(n-7)) (a), linoleyl stearate (WE 18:2(n-6)-18:0) (b), and stearyl linoleate (WE 18:0-18:2(n-6)) (c). The sample was prepared in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source of LCQ Fleet.

alcohol part, the α′ fragment eliminated the neutral fatty acid. When the double bond was located in the acid part, the α fragment eliminated the neutral alcohol. Thus, the fragmentation of $[M + 55]^{+\bullet}$ provided information on the existence of a double bond in the alcohol/acid chain as well as the exact position of the double bond within the chain.

WEs with One Double Bond in Both Parts. The fragmentation of the $[M + 55]^{+\bullet}$ adducts of the WEs with one double bond in the alcohol and one double bond in the acid part was analogous to monounsaturated esters. Because of the presence of two double bonds, two pairs of fragments (α/ω, α′/ω′) formed. Figure 4a depicts the fragmentation spectrum of the $[M + 55]^{+\bullet}$ adducts of WE 18:1(n-9)-16:1(n-7) (*m*/*z* 559). The ions corresponding to the double bond in the acid part were found at *m*/*z* 474 (α) and *m*/*z* 166 (ω); the fragmentations of the adduct formed with a double bond in the alcohol part were at *m*/*z* 446 (α′) and *m*/*z* 194 (ω′). Further cleavage of the α/α′ fragments (MS^3) provided the anticipated ions corresponding to a loss of the unsaturated alcohol or acid (Figure S-1a,b in the Supporting Information).

Methylene-Interrupted Polyunsaturated WEs. The $[M + 55]^{+\bullet}$ adducts of the methylene-interrupted polyunsaturated WEs fragmented mostly via cleavages of the C−C bonds next to the first and last double bond in the polyunsaturated part of esters. Figure 4b and 4c present the MS/MS spectra of the C_3H_5N adducts of isomeric WEs 18:2(n-6)-18:0 and 18:0-18:2(n-6), respectively. The α′/α fragments appeared at *m*/*z* 516, whereas the ω′/ω fragments were detected at *m*/*z* 192. The MS^3 allowed for a clear differentiation between the isomers (Figure S-1c,d in the Supporting Information). Like in the previous cases, the loss of saturated acid or alcohol with 18 carbons reliably indicated double bonds in the alcohol/acid chain. A closer inspection of the MS/MS spectra revealed low-intensity fragments resulting from a cleavage of the C−C bond next to the second double bond, i.e., between the methylene-interrupted double bonds. These fragments were found at *m*/*z* 476 and denoted as α'_{n-9} (WE 18:2(n-6)-18:0) and α_{n-9} (WE 18:0-18:2(n-6)). Note that these fragments can be used for the exact location of all of the double bonds, which is particularly important in the case of the nonmethylene-interrupted arrangement of double bonds. Analogous fragments related to the ω′/ω ions could not be detected because of the low-mass cutoff of the ion trap. WEs with 3- and 4-methylene-interrupted double bonds yielded a similar fragmentation pattern. With the increasing number of methylene-interrupted double bonds, the satellite fragments differing from the α′/α and ω′/ω ions by 14 mass units became more intense. Figure 5a depicts the MS/MS spectrum of the $[M + 55]^{+\bullet}$ adducts of WE 16:0-20:4(n-6), where the α (*m*/*z* 512) and ω (*m*/*z* 272) fragments were still easily distinguishable from the other ions. Like in the previous

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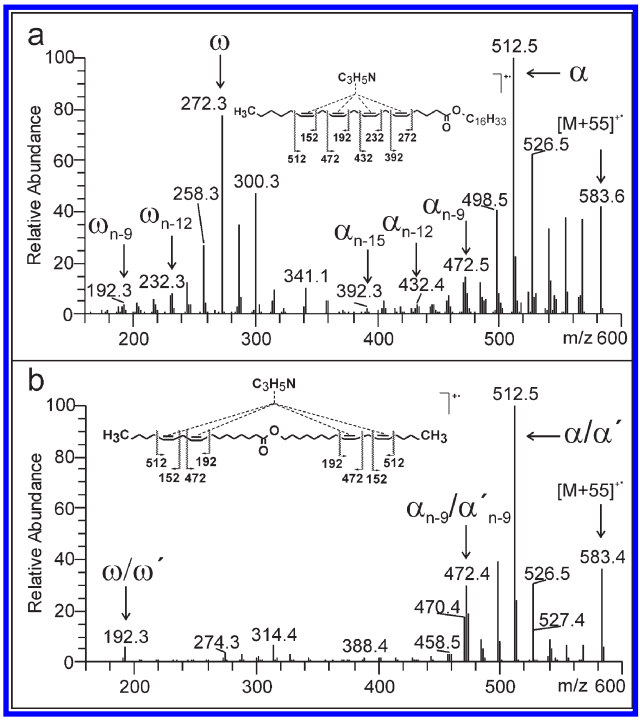

Figure 5. The APCI MS/MS spectra of the $[M + 55]^{+\bullet}$ adducts of palmityl arachidate (WE 16:0-20:4(n-6)) (a) and linoleyl linoleate (WE 18:2(n-6)-18:2(n-6)) (b). The samples were prepared in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source of LCQ Fleet.

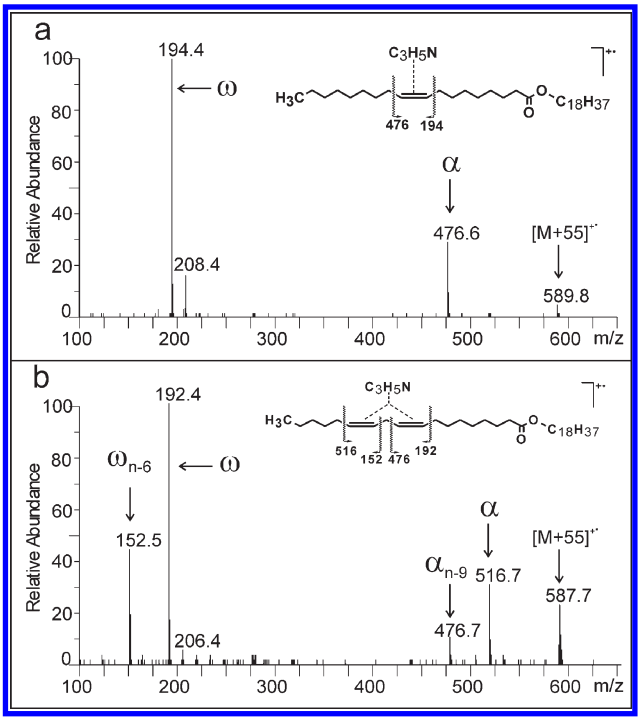

Figure 6. The APCI MS/MS spectra of the $[M + 55]^{+\bullet}$ adducts of stearyl oleate (WE 18:0-18:1(n-9)) (a) and stearyl linoleate (WE 18:0-18:2(n-6)) (b). The samples were prepared in acetonitrile/ethyl acetate (1:1, by vol) and directly infused into the ion source of Q-Tof micro.

case, low-intensity fragments resulting from cleavages between the double bonds were detected as well: α_{n-9} (m/z 472), α_{n-12} (m/z 432), α_{n-15} (m/z 392), ω_{n-12} (m/z 232) and ω_{n-9} (m/z 192). The MS^3 spectrum of the α fragment showed the expected 16:0 loss of alcohol. The MS/MS spectrum of the C_3H_5N adducts of WE 18:2(n-6)-18:2(n-6) is an example of the situation when both chains contain methylene-interrupted double bonds (Figure 5b). The α'/α and ω'/ω fragments as well as the $\alpha'_{n-9}/\alpha_{n-9}$ fragments observed at relatively high intensity were clearly distinguishable, obviously because of the symmetrical structure of the chains providing fragments at the same mass (m/z 472).

Spectra of WEs Recorded by q-tof. The fragmentation of the $[M + 55]^{+\bullet}$ adducts was studied also using a q-tof type instrument equipped with an APCI Z-spray source. The full-scan spectra were very clean, showing only protonated molecules and $[M + 55]^{+\bullet}$ adducts; no other adducts or fragments were detected. The $[M + 55]^{+\bullet}$ adducts were fragmented in a quadrupole collision cell using argon collision gas (Figure 6a). The ω and α ions were quite abundant, and hardly any other fragments were detected. When compared with the ion trap, the q-tof spectra were simpler by far, showing only diagnostic ions (for stearyl oleate, compare Figure 3a). In the case of polyunsaturated WEs, the fragments resulting from a cleavage of the C−C bonds next to all of the double bonds were clearly visible, enabling the unambiguous location of all of the double bonds (Figure 6b; compare with Figure 4c). Importantly, low-mass diagnostic ions could be detected, because the q-tofs have no mass range cutoff. Therefore, q-tofs seem to offer important advantages over ion traps for this particular application.

Unsaturated TGs. The $[M + 54]^+$ and $[M + 55]^{+\bullet}$ adducts of POO were isolated in the ion trap and fragmented by CID. The $[M + 54]^+$ adduct fragmented as published previously[25] (Figure 7a). The α fragment appeared at m/z 814 as a result of the cleavage of the C−C bond next to the methylene group adjacent to the double bond (the C−C bond allylic to the double bond). The most abundant peaks at m/z 656 and m/z 630 corresponded to the elimination of the neutral molecules of palmitic and oleic acid, respectively. Owing to the low mass cutoff of the ion trap, the ω fragment was not present. The CID MSMS spectrum of the $[M + 55]^{+\bullet}$ adduct was markedly different (Figure 7b). The base peak appeared at m/z 800, which was the α fragment resulting from a cleavage of the C−C bond vinylic to the double bond. The fragmentation was analogous to that observed in this work for unsaturated WEs. The intense signals at m/z 657 and m/z 631 corresponded to the elimination of the neutral molecules of the fatty acids, like in the previous case. The spectra recorded using the q-tof instrument (Figure S-2 in the Supporting Information) compared well to the ion trap data. The mass range was extended to lower m/z values, which made it possible to detect ω-type ions. Like in the case of WEs, the q-tof MS/MS spectrum of $[M + 55]^{+\bullet}$ showed highly abundant α and ω ions, allowing for a straightforward localization of double bonds. The diagnostic fragments in the spectrum of $[M + 54]^+$ were substantially less abundant. Identification of the α fragment was difficult because of other peaks with similar intensities in the same region. Thus, TGs formed both types of adducts ($[M + 54]^+$ and $[M + 55]^{+\bullet}$), each of which provided a unique MS/MS spectrum, making it possible to localize the double bond. The use of $[M + 55]^{+\bullet}$ was found to be advantageous because of the higher intensities of the α and ω fragments, thus enabling an easier and more straightforward spectral interpretation.

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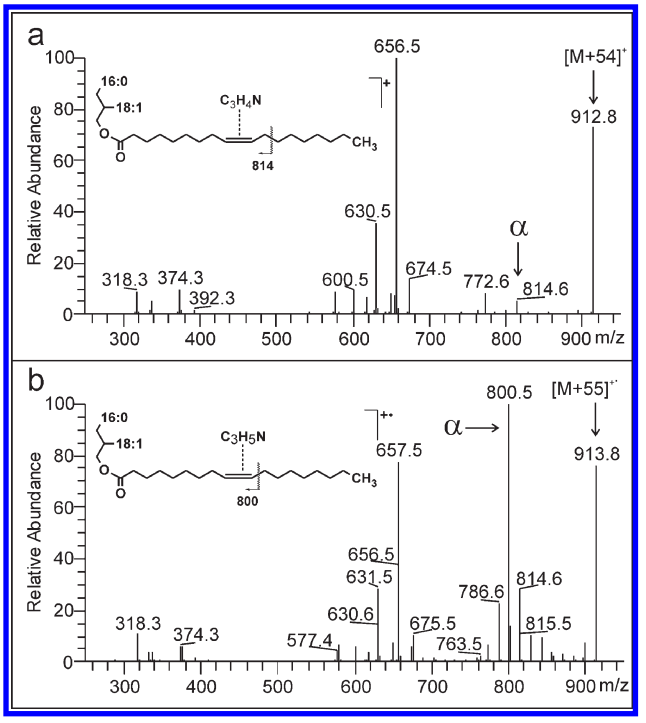

Figure 7. The APCI MS/MS spectra of the C_3H_4N ($[M + 54]^+$) (a) and C_3H_5N ($[M + 55]^{+\bullet}$) (b) adducts of 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol (POO). The samples were prepared in acetonitrile/chloroform (9:1, by vol) at a concentration of 100.0 μg/mL and directly infused into the ion source of LCQ Fleet, with a flow rate of 25 μL/min.

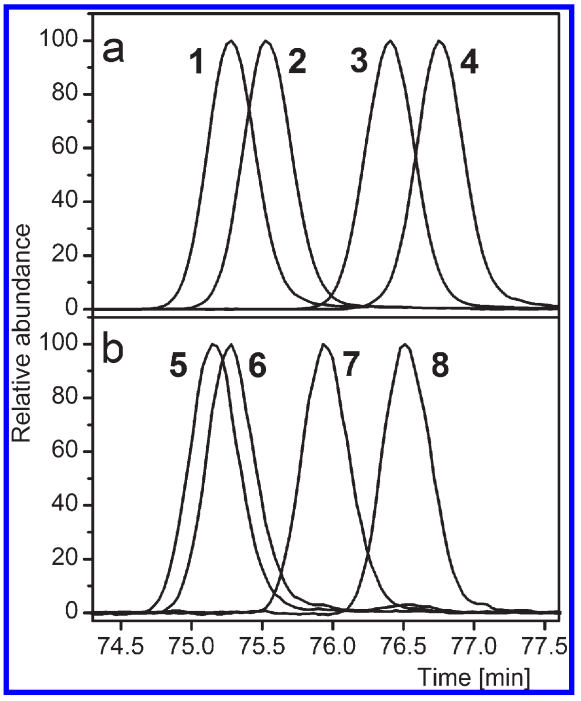

Figure 8. The normalized chromatographic peaks of WE 16:0-18:1(n-7) (1), WE 16:0-18:1(n-9) (2), WE 16:0-18:1(n-12) (3), WE 16:0-18:1(*trans*-(n-9)) (4) (a) and WE 18:1(n-7)-16:0 (5), WE 18:1(n-9)-16:0 (6), WE 18:1(n-12)-16:0 (7), WE 18:1(*trans*-(n-9))-16:0 (8) (b) recorded under optimized conditions using a (15 + 30) cm column at 30 °C.

HPLC of Unsaturated WE Standards. As WEs commonly occur in complex mixtures, a highly efficient HPLC method is required for their comprehensive characterization. The chromatographic behavior of WEs with various chain lengths, numbers of double bonds, and positions of the ester moiety or branching was studied in our previous work.[26] In this report, the study is complemented by data on the chromatographic separation of WEs varying in the position and cis/trans geometry of the double bonds. The WEs were separated in a nonaqueous reversed-phase system under optimized conditions.[26] Figure 8a shows the normalized chromatographic peaks of WEs with a double bond in the acid part (WE 16:0-18:1; *cis*-(n-7), *cis*-(n-9), *cis*-(n-12), and *trans*-(n-9)). In the series of cis isomers, retention increased when the double bond was closer to the center of the molecule (i.e., to the ester bond). Good chromatographic resolution was achieved for isomers with sufficiently different double-bond positions; the (n-7) isomer was separated from the (n-12) one but partially coeluted with the (n-9) isomer. In agreement with the previous studies on other lipids,[32] the *cis*-(n-9) isomer eluted faster than its trans analogue; the cis/trans isomers were baseline separated. An analogous chromatographic behavior was observed for WEs with a double bond in the alcohol part (Figure 8b). In the case of all of the isomers, the retention times were slightly shorter for esters with a double bond in the alcohol part (compare Figure 8a and 8b). The CID spectra of the $[M + 55]^{+\bullet}$ adducts of all of the isomers provided the anticipated α'/α and ω'/ω fragments. There were no significant differences between the spectra of the cis/trans analogues (Figures S-3 and S-4 in the Supporting Information). The mass spectra taken from partially coeluting peaks allowed us to differentiate between the isomers. For instance, HPLC of a 1:1 mixture of WE 16:0-18:1(n-7) and WE 16:0-18:1(n-9) (peaks 1 and 2 in Figure 8) resulted in an almost unresolved wide peak. When the MS/MS spectrum of the $[M + 55]^{+\bullet}$ adducts was taken across the whole peak, two pairs of α and ω fragments appeared. The spectrum enabled the unambiguous identification of both isomers, as only one combination of the ion masses provided a meaningful result. When the spectra were taken in the ascending or descending part of the peak, the α and ω fragments corresponding to the earlier or later eluting isomer predominated, which allowed us not only to identify them but also to determine their elution order. Similar results were obtained using a mixture of WE 18:1(n-7)-16:0 and WE 18:1(n-9)-16:0 (peaks 5 and 6 in Figure 8). The MS/MS spectra from these experiments are included in Figures S-5 and S-6 in the Supporting Information.

Applications. An optimized chromatographic separation system was combined with tandem APCI mass spectrometry to comprehensively characterize the intact WE molecular species in their complex mixtures. The method comprised the separation of WEs in an acetonitrile/ethyl acetate gradient and APCI mass spectrometry with data-dependent scanning. The WEs were identified based on the protonated molecules in full-scan spectra (the total number of carbons and double bonds in WE), MS/MS of $[M + H]^+$ (the number of carbons and double bonds in acid/alcohol parts), and MS/MS of $[M + 55]^{+\bullet}$ (location of double bond(s)). In principle, the MS^3 of $[M + 55]^{+\bullet}$ could also be used to characterize the alcohol and acid part, but the signal intensities were usually too low for an additional MS step. The method applicability was demonstrated on natural mixtures of WEs isolated from jojoba oil and beeswax. In our previous report,[26] we showed that jojoba oil contains at least thirty WE molecular species with 38−48 carbons and 1−4 double bonds. We

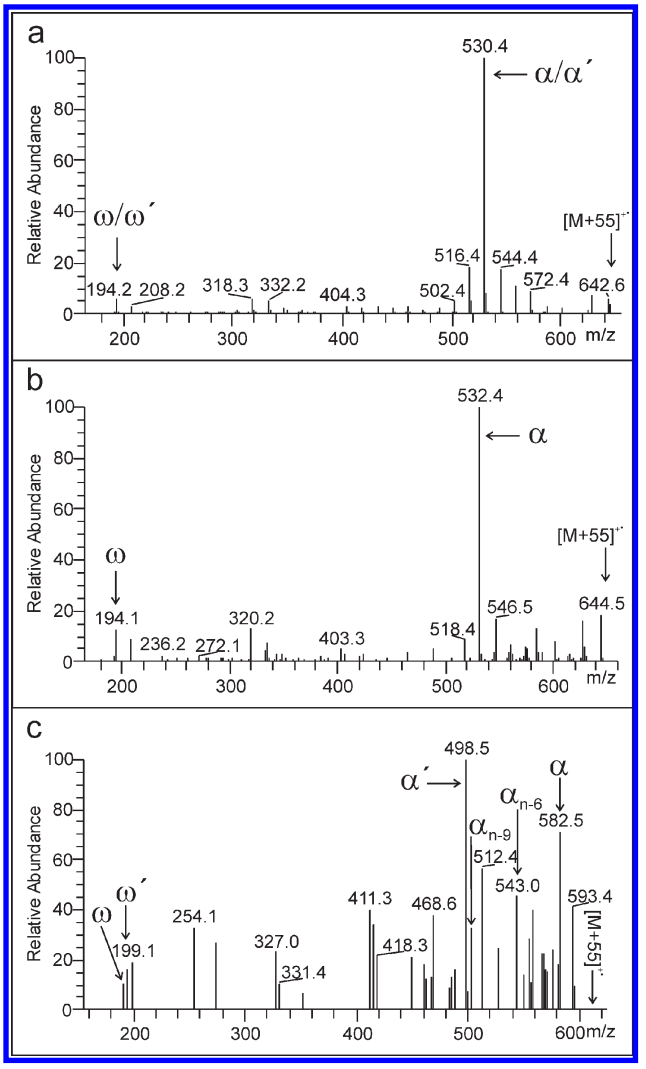

Figure 9. The examples of the APCI MS/MS spectra of the $[M + 55]^{+\bullet}$ adducts of WEs from jojoba oil recorded under optimized chromatographic conditions using a (30 + 15) cm column at 30 °C, with 100 μg of WEs injected. The WEs were identified as follows: a mixture of WE 20:1(n-9)-20:1(n-9) and WE 22:1(n-9)-18:1(n-9), peak t_R = 75.3 min, rel area 20.6% (a), a mixture of WE 20:0−20:1(n-9) and WE 22:0-18:1(n-9), peak t_R = 83.8 min, rel area 0.2% (b), and WE 20:1(n-9)-18:3(n-3), peak t_R = 54.2 min, rel area 0.7% (c).

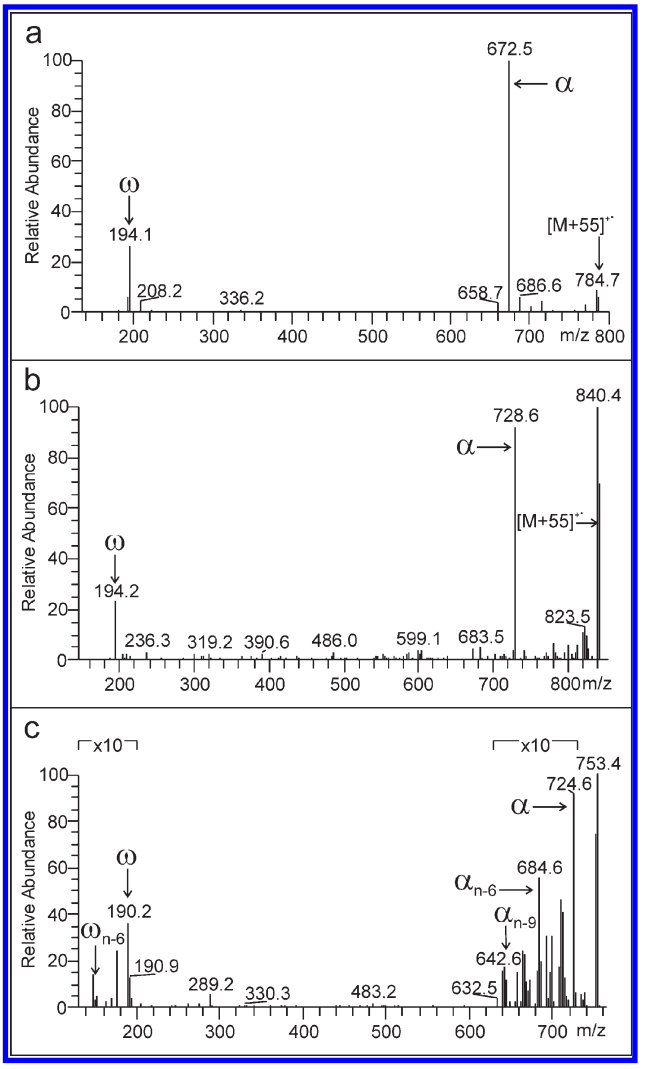

Figure 10. The examples of the APCI MS/MS spectra of the $[M + 55]^{+\bullet}$ adducts of WEs from beeswax recorded under optimized chromatographic conditions using a 30 cm column at 40 °C, with 200 μg of WEs injected. The WEs were identified as follows: a mixture of WE 32:0-18:1(n-9) and WE 30:0-20:1(n-9), peak t_R = 98.7 min, rel area 18.7% (a), a mixture of WE 34:0-20:1(n-9) and WE 32:0-22:1(n-9), peak t_R = 105.9 min, rel area 0.2% (b), and WE 32:0-18:3(n-3), peak t_R = 88.9 min, rel area 2.1% (c).

identified most of them but with no direct evidence for double-bond positions. Other researchers showed that alkyls in jojoba WEs are mostly monounsaturated straight chains with double bonds almost exclusively in the (n-9) position.[33,34] In beeswax, we have detected a similar number of WEs. The molecular species were composed of 40−54 carbons and up to three double bonds.[26] According to the literature, the most abundant unsaturated fatty acid of beeswax WEs is oleic acid.[35] The chromatograms of jojoba and beeswax WEs under optimized HPLC conditions were shown in our previous report[26] along with a detailed commentary on how to interpret the fragmentation spectra of the WE protonated molecules ($[M + H]^+$). Therefore, the following discussion is focused on an interpretation of the MS/MS spectra of $[M + 55]^{+\bullet}$ adducts to obtain the remaining piece of information: the localization of the double bonds.

Jojoba Oil WEs. The complete list of identified WEs, including the α- and ω-type ions used for double-bond localization, is available in Supporting Information (Table S-1). We have confirmed (n-9) double bonds in the alcohol and acid chains of most of the jojoba oil WEs. The localization of the double bonds was straightforward for the most abundant esters. For example, a chromatographic peak at 75.3 min represented two coeluting WEs. On the basis of the MS/MS of $[M + H]^+$ (m/z 589), they were characterized as WE 20:1−20:1 and WE 22:1−18:1. Figure 9a shows the MS/MS spectrum of the $[M + 55]^{+\bullet}$ adduct (m/z 643). The α/α′ (m/z 530) and ω/ω' (m/z 194) ions clearly revealed (n-9) double bonds. As no other diagnostic fragments were detected at a comparable intensity, all the double bonds were considered to be in (n-9) (WE 20:1(n-9)-20:1(n-9), WE 22:1(n-9)-18:1(n-9)). The α- and ω-type diagnostic fragments were easily generated also in the case of less

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abundant peaks. The double bonds were reliably located even when the relative peak area was below 1% and the intensities of $[M + 55]^{+\bullet}$ close to the noise level. An example in Figure 9b depicts the MS/MS spectrum taken from a peak with the relative area of 0.2%. Both the α and ω fragments were easy to recognize, allowing for an unambiguous location of the (n-9) double bond. The fragmentation of very-low-abundance, noise-level precursors provided weak spectra, but the diagnostic fragments were distinguishable in some cases (Figure 9c). Some polyunsaturated WEs were not identified, as the fragments indicating the acid or alcohol chain were difficult to discern in the MS/MS spectra of $[M + H]^+$. However, the fragments indicating the position(s) of double bond were commonly clearly recognizable in the MS/MS spectra of $[M + 55]^{+\bullet}$.

Beeswax WEs. Double bonds in beeswax esters were located mostly in the acid part generating an α and ω pair of diagnostic ions (Table S-2 in Supporting Information). Like in the case of jojoba, the double bonds in monounsaturated fatty acids were identified in the (n-9) positions. The mass spectrum in Figure 10a shows the fragmentation of the $[M + 55]^{+\bullet}$ adduct of a mixture of WE 32:0-18:1 and WE 30:0−20:1 taken from a chromatographic peak. There was a single pair of α and ω ions (m/z 672.5 and m/z 194.2, respectively) indicating double bonds in the (n-9). High-quality spectra were also recorded for some WEs present in trace amounts, e.g., a mixture of WE 34:0-20:1(n-9) and WE 32:0-22:1(n-9), providing $[M + H]^+$ with a relative percent area 0.2% (Figure 10b). Low-abundance WEs containing fatty acid 18:3 provided $[M + 55]^{+\bullet}$ adducts at the noise level. The fragmentation spectra were weak and noisy, but the ions diagnostic for the double-bond positions were still possible to discern. The WE providing the MS/MS spectrum given in Figure 10c was identified based on the α, α_{n-6}, α_{n-9}, ω, and ω_{n-6} fragments as WE 30:0-18:3(n-3). As diagnostic ions in the other WEs with 18:3 indicated the same arrangement of methylene-interrupted double bonds, the fatty acid is the most likely α-linolenoic acid.

CONCLUSIONS

The acetonitrile-related molecular adducts of unsaturated WEs generated in the APCI sources are useful for the location of double-bond position(s) using mass spectrometry. The adducts can be generated and fragmented during a chromatographic run to unambiguously localize the double bonds in WE-molecular species. An HPLC/APCI-MS^2 based on a CID fragmentation of the protonated molecules and acetonitrile-related adducts offers a comprehensive characterization of the complex mixtures of WEs found in nature. Preliminary experiments have indicated that the formation of $[M + 55]^{+\bullet}$ adducts is not restricted only to WEs, which promises the applicability of this method also to other lipids, e.g., FAMEs, TGs, and others ionizable under APCI conditions.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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