

Electrospray mass spectrometry of human hair wax esters

Mark Fitzgerald and Robert C. Murphy¹

Department of Pharmacology, University of Colorado at Denver and Health Sciences Center, Aurora, CO 80045-0511

Abstract Wax esters extracted from human hair have been examined by capillary GC-MS and by nano electrospray ionization (ESI) mass spectrometry using a tandem quadrupole mass spectrometer. Initially, the wax esters were examined by capillary GC-MS using conventional means, thus revealing an incomplete chromatographic resolution of the complex array of >200 wax esters ranging from 28 to 40 carbons in length, including saturated/straight-chained, unsaturated/straight-chained, saturated/branched, and unsaturated/branched molecular species. ESI of wax esters produced ammonium adduct ions $[M+NH_4]^+$, and collisional activation of these ions formed abundant $[RCO_2H_2]^+$ product ions. Wax esters containing a double bond in the fatty acyl or fatty alcohol portion of the molecule revealed identical behavior, suggesting little influence of the double bond on the ionization process or subsequent decomposition. The wax ester mixture was analyzed by ESI and tandem mass spectrometry using multiple reaction monitoring and neutral loss scanning. The neutral loss experiment [loss of NH_3 and $CH_2=CH-(CH_2)_nCH_3$] was particularly effective at rapidly surveying the complex biological mixture, identifying >160 different wax esters that range from 24 to 42 total carbons.—Fitzgerald, M., and R.C. Murphy. *Electrospray mass spectrometry of human hair wax esters*. *J. Lipid Res.* 2007. 48: 1231–1246.

Supplementary key words electrospray ionization • tandem mass spectrometry • gas chromatography-mass spectrometry • human hair lipids

Naturally occurring wax esters, consisting of a fatty acid esterified to a fatty alcohol, are structurally diverse and carry out a variety of biological functions in plants, marine creatures, insects, and mammals. The mostly saturated wax esters, which coat the aerial surfaces of plants, provide protection against ultraviolet light, some pathogens, and desiccation attributable to water loss (1). Wax esters may also provide buoyancy, insulation, or a source of energy in some marine species, such as fish, pelagic invertebrates (particularly krill and other crustaceans), and whales (2).

In the insect realm, coatings of wax esters restrict water movement across the cuticle, protecting against desiccation (3), whereas mammalian wax esters are secreted by meibomian glands as a constituent of the protective coating of the eyes or secreted from sebaceous glands (4, 5).

The most commonly used techniques for the study of complex biological mixtures of wax esters have used separation by gas chromatography and capillary gas chromatography combined with analyses by mass spectrometry and electron ionization (EI) (6). GC-MS has been used to study these mixtures either as the intact esters or after breaking down the wax esters into their constituent fatty acids and alcohols, followed by derivatization to increase volatility and make the products more amenable to study by GC-MS. Human sebaceous wax esters in vernix caseosa (7–9), on the surface of the adult skin (10), and in follicular casts (11) have been examined using GC-MS, and in each case the isolated wax esters were found to be composed of a complex mixture of mostly straight-chained/saturated or monoene fatty acids and fatty alcohols. Using a different approach, three diunsaturated wax esters that are the major constituents in jojoba oil were analyzed by atmospheric pressure chemical ionization. These species were quantified by measuring the abundance of protonated fatty acid product ions obtained by collisional activation for the $[M+H]^+$ ions using multiple reaction monitoring (MRM) experiments (12). Only one study has been published concerning the use of electrospray ionization (ESI) to characterize wax esters, perhaps because of the apparent insensitivity of this technique for neutral lipids (13). In that study, ESI was used to define the molecular weight of an aromatic wax ester isolated from *Rhodococcus* bacteria by measuring its sodium adduct $[M+Na]^+$ ion.

In the study presented here, the value of using ESI mass spectrometry to characterize a naturally occurring mixture of wax esters was compared with the data obtained by GC-MS analysis of the same sample. A complex mixture of wax esters was obtained from human hair and analyzed both

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¹To whom correspondence should be addressed.
e-mail: robert.murphy@uchsc.edu

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as intact wax esters and as individual components after hydrolysis and derivatization to trimethylsilylated fatty alcohols and fatty acid methyl esters (FAMES). This biological wax ester mixture was found to contain >200 individual molecular species by GC-MS, although examination of the intact esters did not reveal the alkyl branch or double bond positions. The same complex mixture was also examined by nano electrospray using a triple quadrupole/linear ion trap mass spectrometer. Individual wax ester molecular ions, as the ammonium adducts $[M+NH_4]^+$, were collisionally activated and then studied by MRM as well as neutral loss scanning. The identification of individual molecular species was then compared with those molecular species identified by traditional GC-MS methods. Considerable information about the complexity of the mixture as well as individual molecular species was obtained using both approaches, although the ESI approach was considerably easier and faster.

EXPERIMENTAL PROCEDURES

Materials

All synthetic wax esters were purchased from Nu-Chek Prep, Inc. (Elysian, MN). Methyl 13-methylpentadecanoate and methyl 14-methylpentadecanoate were obtained from Matreya LLC (Pleasant Gap, PA), whereas methyl palmitate, palmitic acid, and hexadecanol were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were purchased from Fischer Scientific (Fair Lawn, NJ) and were of the highest purity available. The shampoo was Alberto V05 (Melrose Park, IL) for normal hair. Thin-layer chromatography was performed using activated, prewashed Silica Gel G TLC plates (Alltech, Deerfield, IL), and bands of interest were identified by comparisons of Rf values with standards.

Sample preparation

The hair was collected from a 61 year old Caucasian male. The fibers were cut distal to the root end (>1 cm) and stored in vacuum bags at -20°C until needed. A portion of the hair sample (0.5 g) was washed with water (20 ml) and shampoo (1 ml) for 10 min, then rinsed with water for an additional 30 min (11). The hair was then dried under nitrogen (10 min) and sonicated in dichloromethane (20 ml, 40 min) (14), and the extract was filtered and dried down to give 1.0 mg of total lipid extract.

The total lipid extract (1.0 mg) was purified by TLC [hexane-diethyl ether (9:1)] to yield 300 μg of wax esters. A portion of this wax ester mixture was set aside for analysis by GC-MS and electrospray tandem mass spectrometry. The purified wax esters (200 μg) were transesterified (excess 3 N methanolic acid, 60°C , 30 min) to give equimolar amounts of FAMES and fatty alcohols. These were separated by TLC [hexane-diethyl ether-acetic acid (80:19:1)], and the FAMES were stored for analysis by GC-MS. The fatty alcohols (25 μg) were trimethylsilylated [25 μl of bis(trimethylsilyl)trifluoroacetamide/ CH_3CN (1/1)] by heating at 60°C for 30 min. Methyl 13-methylpentadecanoate and methyl 14-methylpentadecanoate were reduced to fatty alcohols using previously published procedures (15, 16) and then trimethylsilylated as described above.

For analysis by GC-MS, all lipids (trimethylsilylated alcohols, FAMES, and wax esters) were made up to 100 ng/ μl dichloromethane, whereas the concentrations used for ESI were 50 ng/ μl isopropyl alcohol-methanol-5 mM ammonium acetate-dichloromethane (4:3:2:1, v/v/v/v) as a solvent compatible with ESI.

Gas chromatography-mass spectrometry

The GC-MS experiments were carried out using a 30 m ($30\text{ m} \times 0.2\text{ mm}$ inner diameter $\times 0.25\text{ }\mu\text{m}$ film thickness) ZB-1 polydimethylsiloxane capillary gas chromatograph column (Phenomenex, Torrance, CA) attached to a ThermoFinnigan (San Jose, CA) Trace DSQ mass spectrometer. The injector temperature was

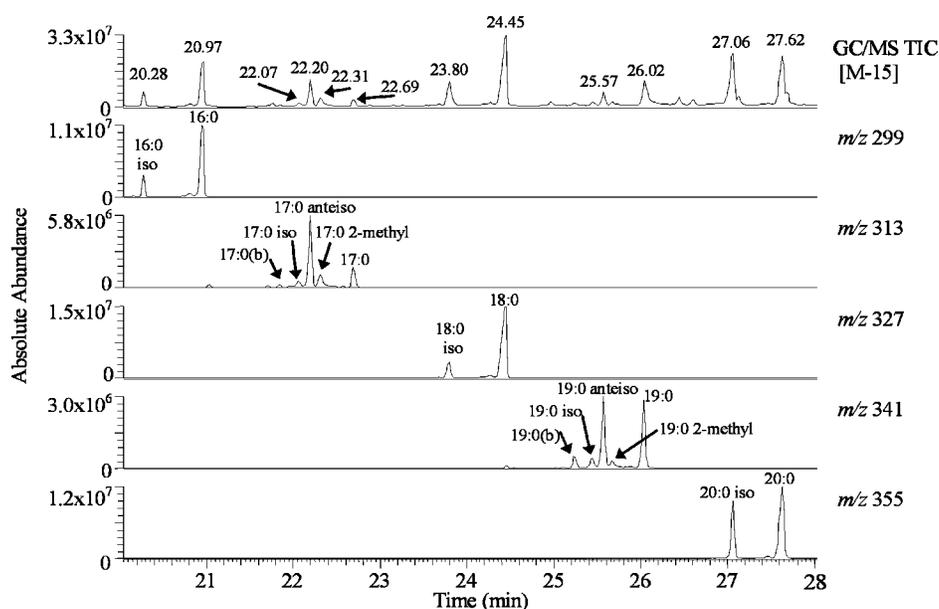


Fig. 1. Gas chromatographic separation and mass spectrometric detection as total ion current (TIC) of the silylated fatty alcohols derived from the human hair wax esters. Labels on the TIC trace refer to time (min), whereas those on the mass chromatograms of the specific ions indicated depict fatty acyl total carbon chain lengths. For experimental conditions, see Experimental Procedures.

TABLE 1. GC-MS data for the wax ester-derived silylated fatty alcohols

| Fatty Alcohols (Silylated) | Retention Time | [M-15] ⁺ | Relative Peak Area |
|----------------------------|----------------|---------------------|--------------------|
| | <i>min</i> | | <i>%</i> |
| 14:0 | 17.23 | 271 | 5 |
| 15:0 | 19.11 | 285 | 6 |
| 16:0 iso | 20.28 | 299 | 11 |
| 16:0 | 20.97 | 299 | 54 |
| 17:0 (branched) | 21.84 | 313 | 1 |
| 17:0 iso | 22.07 | 313 | 2 |
| 17:0 anteiso | 22.20 | 313 | 23 |
| 17:0 2-methyl | 22.31 | 313 | 4 |
| 17:0 | 22.69 | 313 | 6 |
| 18:0 iso | 23.80 | 327 | 17 |
| 18:1 | 23.85 | 325 | <0.5 |
| 18:0 | 24.45 | 327 | 100 |
| 19:09 (branched) | 25.23 | 341 | 2 |
| 19:0 iso | 25.45 | 341 | 2 |
| 19:0 anteiso | 25.57 | 341 | 12 |
| 19:0 2-methyl | 25.66 | 341 | 1 |
| 19:0 | 26.02 | 341 | 14 |
| 20:0 iso | 27.06 | 355 | 36 |
| 20:1 | 27.13 | 353 | 14 |
| 20:0 | 27.62 | 355 | 63 |
| 21:0 (branched) | 28.39 | 369 | 1 |
| 21:0 iso | 28.57 | 369 | 1 |
| 21:0 anteiso | 28.69 | 369 | 16 |
| 21:0 | 29.13 | 369 | 8 |
| 22:0 iso | 30.07 | 383 | 10 |
| 22:1 | 30.16 | 381 | <0.5 |
| 22:0 | 30.62 | 383 | 30 |

maintained at 230°C, and the transfer line was kept at 290°C. The mass spectrometric experiments were performed in positive EI mode (70 eV) with a source temperature of 200°C. Helium was used as the carrier gas, with a constant flow rate of 0.8 ml/min. The trimethylsilylated fatty alcohols were injected into the gas chromatograph, which was initially set to 60°C, then temperature programmed at 5°C/min to 230°C. FAMES were chro-

matographed with a temperature program from 60°C to 300°C at 5°C/min, and the intact wax esters were chromatographed from 150°C to 320°C at 4°C/min.

ESI/tandem mass spectrometry

Samples were infused using a NanoMate 100 (Advion Bio-Sciences, Ithaca, NY) into a Sciex API 4000 Q TRAP hybrid triple quadrupole/linear ion-trap mass spectrometer (Applied Biosystems, Toronto, Canada). Nano electrospray was initiated using the NanoMate by applying a 1.39 kV spray voltage and a 0.50 p.s.i. nitrogen head pressure to the sample in the pipette tip, combined with an 80 V declustering potential and a 10 V entrance potential. All experiments were carried out in positive ion mode, and collisional activation of [M+NH₄]⁺ ions was carried out with a 20 V collision energy and a 6 V collision cell exit potential using nitrogen as the collision gas.

RESULTS

Wax esters were obtained by extraction of washed human hair using dichloromethane, then separated from other extracted lipids by normal-phase TLC. This same wax ester mixture was analyzed either by GC-MS or ESI/tandem mass spectrometry without further purification. An aliquot of the wax ester mixture was hydrolyzed to the constituent fatty acids and fatty alcohols, which were derivatized as the FAMES or fatty alcohol trimethylsilyl ethers before GC-MS analysis.

Fatty alcohol analysis

Capillary gas chromatographic and mass spectral analysis of the trimethylsilylated fatty alcohols revealed a complex mixture partially represented in Fig. 1 by the total

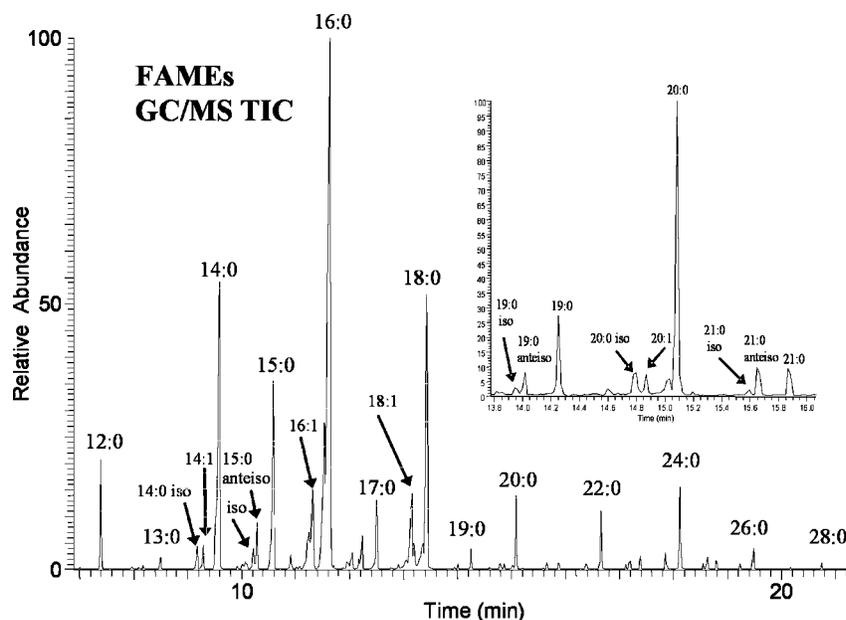


Fig. 2. GC-MS analysis and total ion current chromatogram (TIC) of the fatty acid methyl esters (FAMES) derived from human hair wax esters. Peak labels depict fatty acyl total carbon chain lengths. For experimental conditions, see Experimental Procedures.

ion current. EI of fatty alcohol-trimethylsilyl ethers is known to yield abundant M-15 ions, which can be used to determine molecular weight (17). Mass chromatograms for the [M-15]⁺ ions for common saturated fatty alcohols containing 16, 17, 18, 19, and 20 carbon atoms in their acyl chain at *m/z* 299, 313, 327, 341, and 355, respectively, revealed multiple components (Fig. 1). As expected, separated GC peaks from molecules of the same molecular weight (i.e., isobaric [M-15]⁺) were consistent with isomeric fatty alcohols esterified in these wax esters. Structural assignments for the separated peaks were made by comparison of the observed retention times for authentic standards, when available, and by plotting the retention times of saturated, straight-chained as well as branched, chained fatty alcohol trimethylsilyl ethers against carbon number to identify homologs in a structural series (18). The even carbon numbered chain fatty alcohols showed a simple distribution pattern consisting of a straight-chained alcohol and a single iso branched isomer that was typically present in lesser abundance. The odd carbon numbered alcohols displayed a more complex distribution consisting of major peaks for the straight-chained and anteiso species and a less abundant isomer corresponding to an iso branched alcohol. There were also 2-methyl branched fatty alcohols observed (Fig. 1, *m/z* 313 and *m/z* 341 traces), as shown by the characteristic ion at *m/z* 117 [TMSOCHCH₃]⁺ in the EI mass spectra of these species.

The full gas chromatogram (data not shown) displayed prominent peaks attributable to trimethylsilylated fatty alcohols ranging from 14 to 22 carbons in length. The even carbon numbered 18:0, 20:0, and 16:0 alcohols were the most abundant (in decreasing order) in this wax ester mixture, but branched isomers were also surprisingly major constituents. Odd carbon numbered alcohols, both branched and straight-chained, were generally minor. Only a few alcohols contained a double bond, and these were identified as 18:1, 20:1, and 22:1, but these were present in very low abundance and did not appear on the total ion trace (Fig. 1). The double bond positions in the alkyl chain could not be determined for these fatty alcohols. The full list of alcohol moieties detected in this wax ester mixture is presented in **Table 1**.

Fatty acid (methyl ester) analysis

The fatty acid components that made up the wax esters were transesterified and analyzed as FAMES by capillary GC-MS. FAMES were identified by their EI mass spectra, and the assignment of iso or anteiso branching was based upon characteristic fragmentations (19), comparisons of retention times with authentic standards, and correlation of the corresponding carbon numbers in the FAME against retention times (20). The gas chromatographic separation of the wax ester-derived FAMES (**Fig. 2**) revealed a complex mixture, with the most abundant components corresponding to saturated fatty acids. The only unsaturated fatty acids were 14:1, 16:1, 18:1, and 20:1. The complete list of species identified is presented in **Table 2**. FAMES with acyl chains ranging from 12 to 28 carbons were identified, with the even chained 16:0, 14:0, and 18:0

TABLE 2. Gas chromatographic retention times and observed molecular ions for the wax ester-derived fatty acid methyl esters

| Fatty Acid Methyl Ester | Retention Time | Molecular Ion M ⁺ | | Relative Peak Area |
|-------------------------|----------------|------------------------------|--|--------------------|
| | | <i>min</i> | | % |
| 12:0 iso | 6.98 | 214 | | <0.5 |
| 12:0 | 7.38 | 214 | | 8 |
| 13:0 (branched) | 7.96 | 228 | | <0.5 |
| 13:0 iso | 8.08 | 228 | | <0.5 |
| 13:0 anteiso | 8.16 | 228 | | <0.5 |
| 13:0 | 8.48 | 228 | | 1 |
| 14:0 iso | 9.17 | 242 | | 3 |
| 14:1 | 9.28 | 240 | | 2 |
| 14:0 | 9.57 | 242 | | 33 |
| 15:0 iso | 10.21 | 256 | | 2 |
| 15:0 anteiso | 10.30 | 256 | | 4 |
| 15:0 | 10.59 | 256 | | 21 |
| 16:1 | 11.33 | 268 | | 9 |
| 16:0 | 11.66 | 270 | | 100 |
| 17:0 iso | 12.17 | 284 | | 2 |
| 17:0 anteiso | 12.24 | 284 | | 4 |
| 17:0 | 12.49 | 284 | | 9 |
| 18:2 | 12.91 | 294 | | 1 |
| 18:1 | 13.16 | 296 | | 9 |
| 18:0 | 13.40 | 298 | | 32 |
| 19:0 iso | 13.94 | 312 | | <0.5 |
| 19:0 anteiso | 14.02 | 312 | | <0.5 |
| 19:0 | 14.25 | 312 | | 1 |
| 20:0 iso | 14.78 | 326 | | <0.5 |
| 20:1 | 14.84 | 324 | | <0.5 |
| 20:0 | 15.09 | 326 | | 5 |
| 21:0 iso | 15.58 | 340 | | <0.5 |
| 21:0 anteiso | 15.65 | 340 | | <0.5 |
| 21:0 | 15.87 | 340 | | 1 |
| 22:0 iso | 16.36 | 354 | | <0.5 |
| 22:0 | 16.65 | 354 | | 4 |
| 23:0 iso | 17.12 | 368 | | <0.5 |
| 23:0 anteiso | 17.17 | 368 | | 1 |
| 23:0 | 17.37 | 368 | | 1 |
| 24:0 iso | 17.84 | 382 | | 1 |
| 24:0 | 18.12 | 382 | | 6 |
| 25:0 iso | 18.57 | 396 | | <0.5 |
| 25:0 anteiso | 18.62 | 396 | | 1 |
| 25:0 | 18.81 | 396 | | 1 |
| 26:0 iso | 19.22 | 410 | | <0.5 |
| 26:0 | 19.48 | 410 | | 2 |
| 27:0 | 20.17 | 424 | | <0.5 |
| 28:0 | 20.75 | 438 | | <0.5 |

most abundant. Odd chain fatty acids were also observed, including 15:0, 17:0, and 13:0 in decreasing abundance. The only polyunsaturated fatty acid detected was 18:2; however, because it was of such low abundance, it was difficult to observe on the total ion plot (Fig. 2). The double bond position in the unsaturated fatty acids was not determined, and EI mass spectra alone could not be used to locate the double bond position (21).

GC-MS wax ester molecular species analysis

The intact wax esters were separated by capillary gas chromatography, but this was constrained by their volatility and stability at the temperature limits of the gas chromatographic column (**Fig. 3A**). A very complex series of components could be detected, ranging from 28:0 to 40:0 wax esters (total number of carbon atoms in both the fatty alcohol and fatty acid portion of the molecule:total number of double bonds). It was possible to identify several specific components based upon gas chromato-

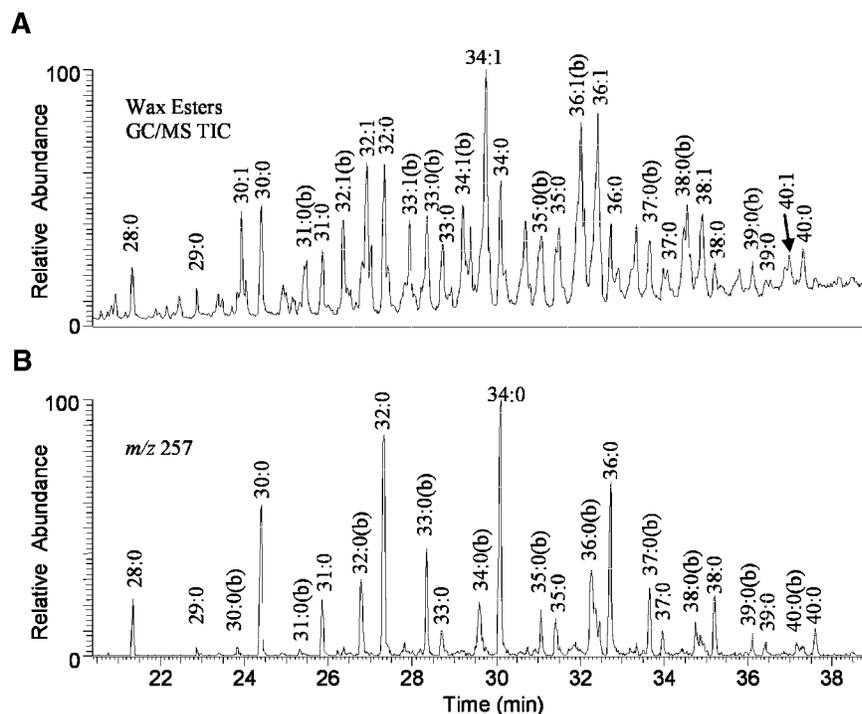


Fig. 3. A: Gas chromatographic separation total ion current (TIC) of the intact human hair wax esters. B: Mass chromatogram of m/z 257 $[\text{C}_{15}\text{H}_{31}\text{CO}_2\text{H}_2]^+$ used to identify straight-chained and branched (b) 16:0 fatty acids formed from the intact wax esters under electron ionization (EI) conditions. For experimental conditions, see Experimental Procedures. All labels depict the total number of carbons in both the fatty acyl and fatty alcohol portions of the molecules.

graphic retention times as well as upon the EI mass spectrometric data, such as the observed molecular ion $[\text{M}]^+$ and characteristic ions derived from the decomposition of the molecular ion. Specifically, the saturated wax esters were known to yield diagnostic ions corresponding to the fatty acids $[\text{RCO}_2\text{H}_2]^+$ and $[\text{RCO}]^+$ (22). The fatty alcohol portion of the wax ester could be identified by the characteristic $[\text{OCOR}]^+$ and $[\text{CH}_2=\text{CH}(\text{CH}_2)_n\text{CH}_3]^+$ ion products (22). The mechanisms involved with the formation of these ions have been described (23). Interestingly, when a double bond was located in the alcohol portion of the wax ester, the most abundant ion observed in the EI mass spectrum corresponded to $[\text{CH}_2=\text{CH}(\text{CH}_2)_n\text{CH}_3]^+$.

Using these characteristic fragment ions in the EI mass spectrum, it was possible to interrogate the GC-MS data for the appearance of wax esters containing specific structural moieties. For example, extracting the ion at m/z 257 from the GC-MS data set, which corresponded to the protonated carboxylic acid $[\text{C}_{15}\text{H}_{31}\text{CO}_2\text{H}_2]^+$, wax esters that contained palmitic acid were revealed (Fig. 3B). Interestingly, the chromatographic peak widths for the components that contained palmitate were somewhat less than those observed for the total ion current, indicating that under each total ion current chromatographic peak there were likely multiple components either coeluting or eluting quite close to the major components (see below). Branched chain fatty alkyl containing wax ester species could be separated from those containing straight-chain

fatty alkyl esters; for example, for the 16:0 fatty acid containing wax esters, two peaks were observed corresponding to molecular ions for 34:0 at 29.2 and 30.1 min. The assignment of the retention time for the *n*-octadecylhexadecanoate (34:0) was found to be 30.1 min; therefore, the component eluting earlier was in agreement with the gas chromatographic behavior of a component containing either a branched chain fatty acyl substituent or a branched fatty alcohol moiety, or both.

Assignment of the wax ester components could be made upon closer examination of the EI mass spectra of each gas chromatographic peak. The mass spectrum of synthetic 16:0/16:0 wax ester (Fig. 4A) revealed the characteristic ions described above and was dominated by protonated palmitate at m/z 257. There was considerable similarity between this mass spectrum and that obtained at retention time 27.3 min from the wax ester mixture (Fig. 4B). Both mass spectra displayed a base peak at m/z 257 attributable to $[\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H}_2]^+$ and peaks at m/z 239 $[\text{CH}_3(\text{CH}_2)_{14}\text{CO}]^+$, m/z 224 $[\text{CH}_2=\text{CH}(\text{CH}_2)_{13}\text{CH}_3]^+$, and m/z 269 $[\text{OCO}(\text{CH}_2)_{15}\text{CH}_3]^+$, which were consistent with the wax ester 16:0/16:0. However, the mass spectrum of the hair extract also contained ions at m/z 297 $[\text{OCO}(\text{CH}_2)_{17}\text{CH}_3]^+$, m/z 285 $[\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H}_2]^+$, m/z 211 $[\text{CH}_3(\text{CH}_2)_{12}\text{CO}]^+$, and m/z 229 $[\text{CH}_3(\text{CH}_2)_{12}\text{CO}_2\text{H}_2]^+$, consistent with a mixture also containing 14:0/18:0 and 18:0/14:0 wax esters (fatty acid/fatty alcohol) that were not resolved by the gas chromatographic column. Nearly all of

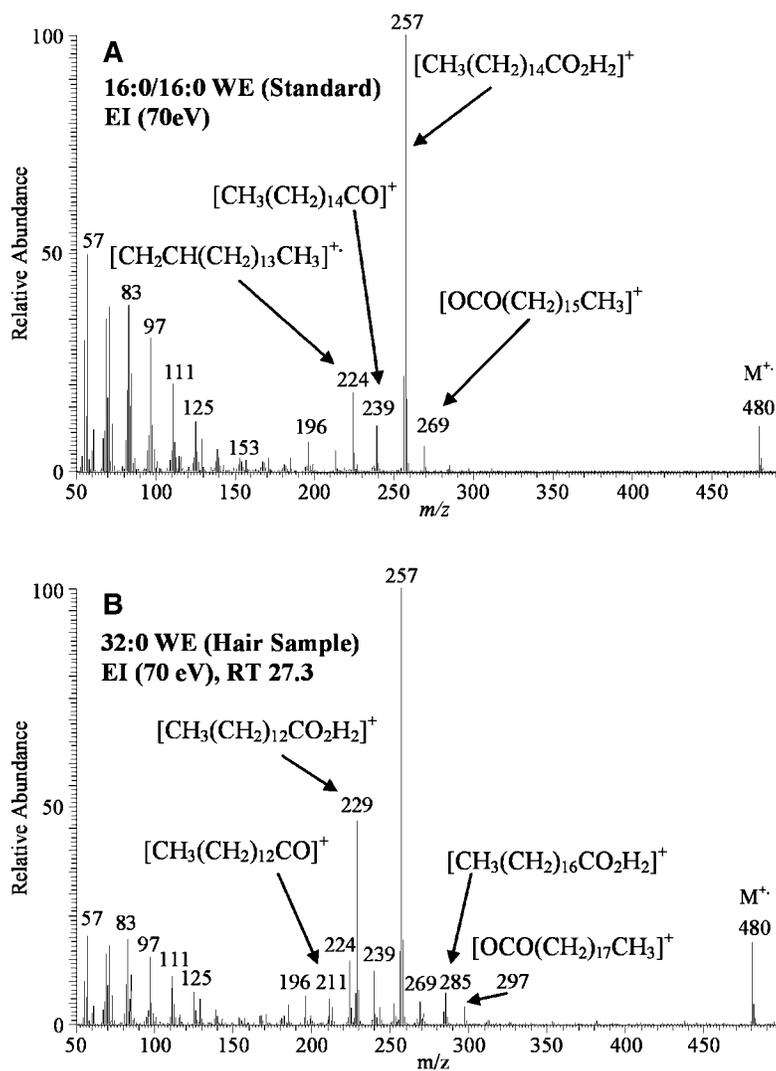


Fig. 4. A: Positive ion EI (70 eV) mass spectrum of synthetic 16:0/16:0 wax ester (WE). B: Positive ion EI mass spectrum of the 32:0 wax esters from the hair sample that chromatographed at 27.3 min. For experimental conditions, see Experimental Procedures.

the gas chromatographic peaks separated in the wax ester extract contained isomeric mixtures of molecular species. For example, the mass spectrum (data not shown) of 30:0 wax esters was observed to contain the molecular species 14:0/16:0, 16:0/14:0, 15:0/15:0, 12:0/18:0, 18:0/12:0, and 13:0/17:0. Similarly, the saturated, branched chained wax esters were also found to be mixtures of fatty acid and fatty alcohol moieties of varying carbon chain length; however, the mass spectral data did not allow for the definitive assignments of the positions of methyl branching. The list of molecular species identified is given in **Table 3**.

The presence of a single double bond within the wax ester, either fatty acid or fatty alcohol sites, substantially changed its fragmentation behavior (**Fig. 5A**). The EI mass spectrum of 16:1/16:0 displayed a characteristic molecular ion at m/z 478 M^+ and fragment ions at m/z 255 $[C_{15}H_{29}CO_2H_2]^+$ and m/z 236 $[C_{15}H_{28}CO]^+$. These abundant ions could be seen in the mass spectrum recorded at 26.9 min and tentatively identified by the molecular ion as corresponding to 32:1 wax esters. However, this mass spectrum also displayed a number of other abundant ions, including at m/z 264, which would likely be attributable to

$[C_{17}H_{32}CO]^+$. Therefore, this mass spectrum was likely a mixture of at least 16:1/16:0 and 18:1/14:0 wax esters. Furthermore, minor wax ester components for which diagnostic ions were difficult to discern were also present. Inspection of other gas chromatographic components containing straight-chained wax ester monoenes from the hair extract revealed similar or more complex mixtures.

Analysis of the intact wax esters by capillary GC-MS revealed >200 molecular species ranging from 28 to 40 total carbon atoms. The most abundant molecular species appeared to be saturated and straight-chained fatty alcohol and fatty acid wax esters and wax esters containing a single double bond in the fatty acid with an esterified, saturated fatty alcohol. The wax esters eluted from the capillary gas chromatographic column as isobaric components that differed in fatty acid and fatty alcohol chain length but separated from the branched chain series, which also likely differed in the position of a methyl substituent. The even carbon numbered wax esters predominantly contained even carbon numbered fatty acids and fatty alcohols rather than two odd carbon numbered moieties.

TABLE 3. Observed molecular ions from GC-MS analysis and molecular species designations for intact human hair wax esters

| Wax Esters (Generic Name) | M ⁺ | Wax Esters Identified in Hair Sample |
|---------------------------|----------------|--|
| 28:0 | 424 | 14:0/14:0, 16:0/12:0, 12:0/16:0, 13:0/15:0, 15:0/13:0 |
| 29:0 | 438 | 14:0/15:0, 15:0/14:0, 13:0/16:0, 16:0/13:0, 17:0/12:0 |
| 30:0 (branched) | 452 | 14:0/16:0, 16:0/14:0, 12:0/18:0, 15:0/15:0 |
| 30:1 | 450 | 18:1/12:0, 16:1/14:0, 16:0/14:1 |
| 30:0 | 452 | 15:0/15:0, 14:0/16:0, 16:0/14:0, 12:0/18:0, 18:0/12:0, 13:0/17:0 |
| 31:0 | 466 | 15:0/16:0, 16:0/15:0, 17:0/14:0, 14:0/17:0 |
| 32:1 (branched) | 478 | 16:1/16:0(b), 14:1/18:0(b), 16:0(b)/16:1, 18:0(b)/14:1 |
| 32:0 (branched) | 480 | 14:0/18:0, 18:0/14:0, 16:0/16:0 |
| 32:1 | 478 | 16:1/16:0, 18:1/14:0 |
| 32:0 | 480 | 16:0/16:0, 14:0/18:0, 18:0/14:0 |
| 33:1 (branched) | 492 | 16:1/17:0(b), 17:0(b)/16:1, 15:0(b)/18:1, 19:0(b)/14:1, 18:0(b)/15:1 |
| 33:0 (branched) | 494 | 15:0/18:0, 16:0/17:0, 17:0/16:0, 18:0/15:0, 19:0/14:0, 14:0/19:0, 20:0/13:0 |
| 33:0 | 494 | 15:0/18:0, 18:0/15:0, 16:0/17:0, 17:0/16:0, 14:0/19:0, 13:0/20:0 |
| 34:1 (branched) | 506 | 16:1/18:0(b), 15:1/19:0(b), 18:1/16:0(b), 14:1/20:0(b), 12:0(b)/22:1, 14:0(b)/20:1, 12:1/22:0(b), 22:0(b)/12:1, 18:0(b)/16:1, 14:0(b)/20:1 |
| 34:0 (branched) | 508 | 18:0/16:0, 16:0/18:0, 20:0/14:0, 14:0/20:0, 12:0/22:0, 10:0/24:0 |
| 34:1 | 506 | 16:1/18:0, 18:1/16:0, 14:1/20:0, 20:1/14:0, 22:0/12:1, 20:0/14:1, 14:0/20:1, 18:0/16:1, 16:0/18:1 |
| 34:0 | 508 | 20:0/14:0, 16:0/18:0, 18:0/16:0, 17:0/17:0, 22:0/12:0, 14:0/20:0, 12:0/22:0, 24:0/10:0 |
| 35:0 (branched) | 522 | 17:0/18:0, 18:0/17:0, 19:0/16:0, 15:0/20:0, 14:0/21:0, 11:0/24:0, 13:0/22:0, 16:0/19:0, |
| 35:0 | 522 | 16:0/19:0, 17:0/18:0, 18:0/17:0, 19:0/16:0, 20:0/15:0, 14:0/21:0, 15:0/20:0, 22:0/13:0 |
| 36:1 (branched) | 534 | 16:1/20:0(b), 18:1/18:0(b), 16:0(b)/20:1, 22:0(b)/14:1, 18:0(b)/18:1, 16:0(b)/20:1 |
| 36:0 (branched) | 536 | 14:0/22:0, 22:0/14:0, 16:0/20:0, 20:0/16:0, 18:0/18:0, 17:0/19:0 |
| 36:1 | 534 | 19:1/17:0, 18:1/18:0, 16:1/20:0, 15:1/21:0, 14:1/22:0, 16:0/20:1, 14:0/22:1 |
| 36:0 | 536 | 14:0/22:0, 22:0/14:0, 16:0/20:0, 20:0/16:0, 18:0/18:0, 13:0/23:0, 15:0/21:0, 17:0/19:0 |
| 37:0 (branched) | 550 | 19:0/18:0, 20:0/17:0, 21:0/16:0, 22:0/15:0, 14:0/23:0, 18:0/19:0, 17:0/20:0, 16:0/21:0, 15:0/22:0 |
| 37:0 | 550 | 17:0/20:0, 20:0/17:0, 21:0/16:0, 15:0/22:0, 23:0/14:0, 18:0/19:0, 16:0/21:0, 14:0/23:0 |
| 38:0 (branched) | 564 | 14:0/24:0, 24:0/14:0, 20:0/18:0, 22:0/16:0, 18:0/20:0, 16:0/22:0 |
| 38:1 | 562 | 16:1/22:0, 18:1/20:0, 22:1/16:0, 24:1/14:0, 16:0/22:1, 18:0/20:1, 22:0/16:1, 20:0/18:1, 14:0/24:1 |
| 38:0 | 564 | 14:0/24:0, 24:0/14:0, 16:0/22:0, 22:0/16:0, 18:0/20:0, 20:0/18:0, 15:0/23:0, 17:0/21:0, 19:0/19:0 |
| 39:0 (branched) | 578 | 17:0/22:0, 16:0/23:0, 15:0/24:0, 14:0/25:0 |
| 39:0 | 578 | 17:0/22:0, 16:0/23:0, 15:0/24:0, 14:0/25:0, 19:0/20:0, 21:0/18:0 |
| 40:0 (branched) | 592 | 16:0/24:0, 18:0/22:0, 24:0/16:0, 14:0/26:0 |
| 40:1 | 590 | 22:0/18:1, 24:0/16:1, 26:0/14:1, 20:1/20:0, 16:0/24:1, 14:1/26:0, 16:1/24:0, 18:1/22:0 |
| 40:0 | 592 | 16:0/24:0, 18:0/22:0, 20:0/20:0, 17:0/23:0, 22:0/18:0, 24:0/16:0, 26:0/14:0 |

The generic designations 32:0, 32:1, and 32:1(b) denote wax esters that have 32 carbons and are straight-chained, 32 carbons and are straight-chained monoenes, and 32 carbons and are branched monoenes, respectively. An example of the nomenclature when there is additional structural specificity for the fatty acid and fatty alcohol moieties is 18:1/16:0(b). This depicts a wax ester consisting of a fatty acid moiety that is an 18 carbon, straight-chained monoene attached by an ester linkage to a saturated, branched fatty alcohol possessing 16 carbons.

ESI analysis of wax ester molecular species

Synthetic 16:0/16:0 wax ester (palmityl palmitate) produced an abundant $[M+NH_4]^+$ ammonium adduct ion (m/z 498) under ESI conditions. Upon collisional activation (Fig. 6A), the ammonium adduct predominantly formed the protonated wax ester molecular ion $[M+H]^+$ (m/z 481) and abundant fragment ions at m/z 257 $[CH_3(CH_2)_{14}CO_2H_2]^+$ and m/z 225 $[(CH_2)_{15}CH_3]^+$. There was also an ion of substantially less abundance at m/z 275, which was likely attributable to an ammoniated carboxylic acid group, but this was an odd electron species (radical cation). This general behavior of saturated wax esters was observed with four other synthetic products, supporting the rather simple decomposition behavior of those lipids (data not shown).

The collision-induced dissociation of the 32:0 ammonium adduct wax ester ions (m/z 498) from the extract of human hair yielded several ions that could be readily assigned as protonated carboxylic acids (Fig. 6B). For example, there was evidence for a series of saturated fatty acids based upon m/z 229 $[C_{13}H_{27}CO_2H_2]^+$ (14:0), m/z 243 $[C_{14}H_{29}CO_2H_2]^+$ (15:0), m/z 257 $[C_{15}H_{31}CO_2H_2]^+$ (16:0), and m/z 285 $[C_{17}H_{35}CO_2H_2]^+$ (18:0). MS³ analysis of the most abundant protonated carboxylic acid ion at

m/z 257 yielded an identical product ion mass spectrum to that obtained from the same MS³ experiment with synthetic 16:0/16:0 wax esters (data not shown). The observed product ions were consistent with a mixture of wax esters at this single molecular weight that included the molecular species 14:0/18:0, 15:0/17:0, 16:0/16:0, and 18:0/14:0 (fatty acids/fatty alcohols), all of which were identified by GC-MS with the exception of 15:0/17:0.

Monoene wax esters also yielded ammonium adduct ions under electrospray conditions, and the collision-induced mass spectra of synthetic 16:1/16:0 (palmitoyl palmitoleate) and 14:0/16:1 (palmitoleyl myristate) are included in Fig. 7. These species underwent collisional activation and decomposition in a manner similar to that observed for the saturated analogs. The mass spectrum after collisional activation of the 16:1/16:0 ammonium adduct ion (Fig. 7A) yielded the expected product ion characteristic of the fatty acyl moiety of the molecule, namely m/z 255 $[C_{15}H_{29}CO_2H_2]^+$, and the most abundant ion corresponding to the simple loss of ammonia and the formation of $[M+H]^+$ at m/z 479. An ion was also observed at m/z 237, which most likely corresponded to the ketene-like structure $[C_{15}H_{29}CO]^+$. The ion at m/z 219 was not examined further. The scale was magnified between

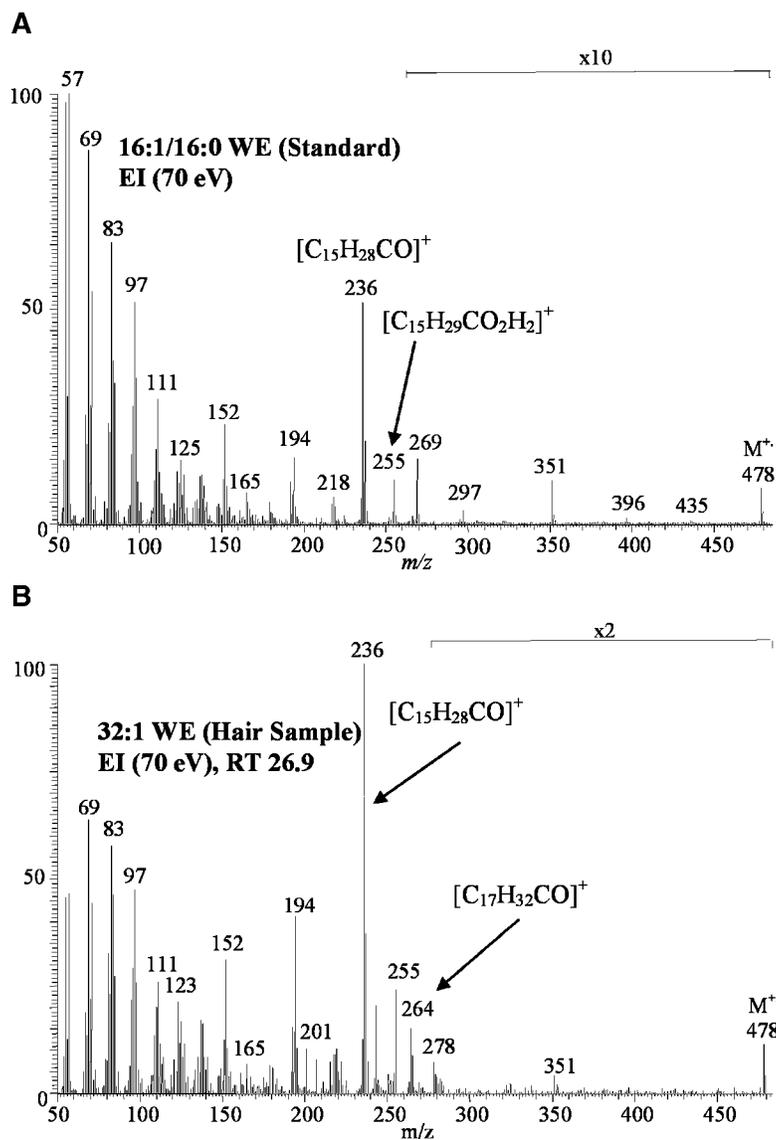


Fig. 5. A: Positive ion EI mass spectrum of synthetic 16:1/16:0 wax ester (WE). B: Positive ion EI mass spectrum of the 32:1 wax esters from the hair sample that chromatographed at 26.9 min. For experimental conditions, see Experimental Procedures.

m/z 50 and 215 by 20-fold, revealing a hydrocarbon series that likely corresponded to the collision-induced formation of hydrocarbon ions from both the fatty alcohol and the fatty acyl portions of the molecule.

When the double bond was present in the fatty alcohol portion of the wax ester, the product ion spectrum (Fig. 7B) displayed a prominent ion corresponding to the fatty acid portion of the molecule (i.e., m/z 229 $[C_{13}H_{27}CO_2H_2]^+$). This was in contrast to the EI behavior of monoene wax esters, in which a double bond in the fatty alcohol moiety significantly altered the EI mass spectrum (see above). In the case of EI, this was most likely attributable to the ionization and charge site at a double bond, whereas with ESI the charge site likely was confined to the ester moiety. The only obvious difference in the products obtained after collisional activation of the wax esters containing an unsaturation of the fatty alcohol group was more abundant ions at low mass between m/z 69 and 181, which likely correspond to an olefin series of ions.

The collision-induced decomposition of the 32:1 follicular wax ester at m/z 496 (Fig. 7C) yielded the expected neutral loss of NH_3 (m/z 479) as well as a series of protonated fatty acid product ions, including m/z 283 $[C_{17}H_{33}CO_2H_2]^+$, m/z 255 $[C_{15}H_{29}CO_2H_2]^+$, and m/z 227 $[C_{13}H_{25}CO_2H_2]^+$. Ions of lesser abundance corresponding to C_{15} and C_{17} protonated fatty acid monoenes were present at m/z 241 and 269. As observed for wax esters having a double bond in the fatty acyl portion, a series of corresponding ketene ions were seen at m/z 265, 237, and 209 that confirmed the identification of the protonated carboxylic acid ions described above. This collision-induced mass spectrum of the wax ester extract revealed a mixture of 18:1/14:0, 17:1/15:0, 16:1/16:0, 15:1/17:0, and 14:1/18:0 wax esters. This was a somewhat more complex mixture than that revealed by GC-MS; however, this latter analysis did reveal the likely presence of 20:0/12:1, which was not detected in the ESI experiment. There was little evidence for the occurrence of unsaturated fatty alcohols in this

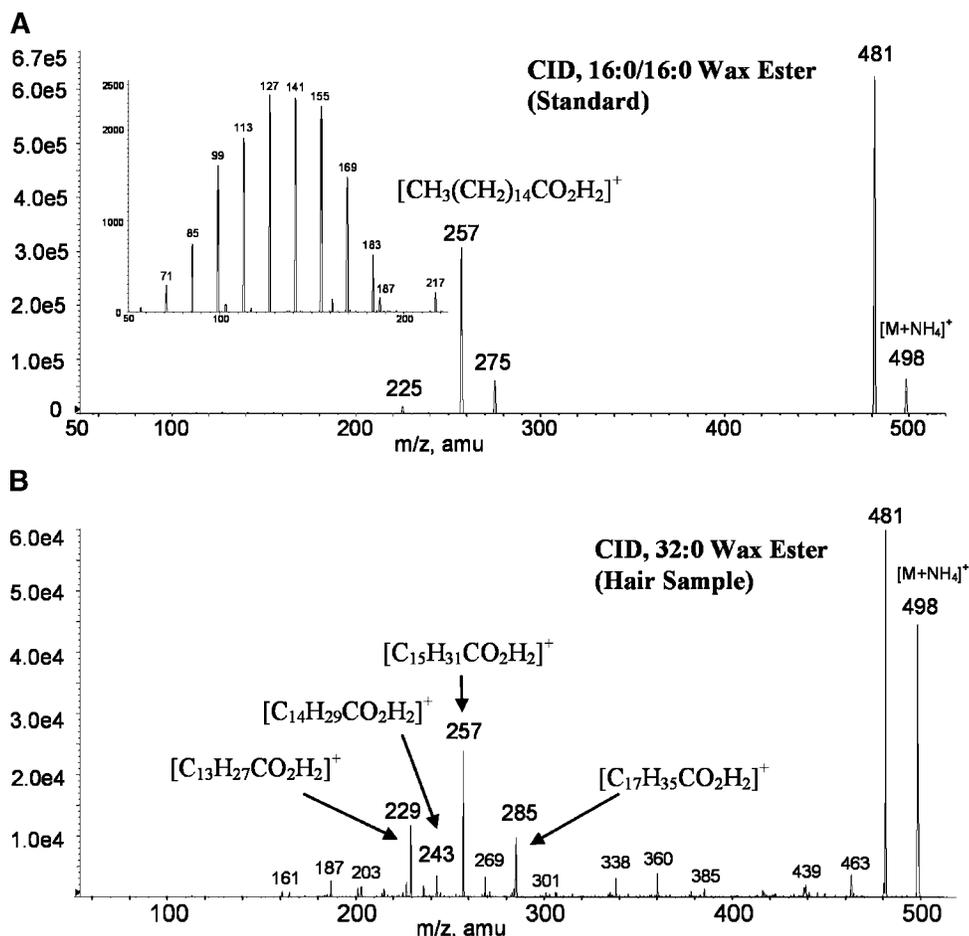


Fig. 6. A: Positive ion collision-induced dissociation (CID) mass spectrum of the 16:0/16:0 (synthetic) ammonium ion adduct formed under electrospray ionization (ESI) conditions. B: Positive ion collision-induced dissociation mass spectrum of the mixture of 32:0 wax ester ammonium adducts (m/z 498) formed from the hair extract under ESI conditions.

extract, specifically those having a $[\text{M}+\text{NH}_4]^+$ at m/z 496 and a saturated $[\text{RCO}_2\text{H}_2]^+$ mass-to-charge ratio.

Mixture analysis by tandem mass spectrometry

The rather simple behavior of wax ester ammonium adduct ions in response to collisional activation, with the formation of an abundant ion corresponding to the protonated fatty acyl portion of the wax ester, suggested an alternative approach to assess the occurrence of specific components in a complex mixture that could be carried out without prior separation of molecular species by chromatographic means. This alternative approach involved the unique capabilities of the tandem quadrupole mass spectrometer to carry out rapid MRM experiments and neutral loss scans. As exemplified by the complex mixture in the human hair wax ester extract, a large number of fatty acid and fatty alcohol moieties were present, but most were saturated and a few monounsaturated moieties were present. Thus, one could construct a list of expected molecular weights that would correspond to the combinatorial mixture of these fatty acids and fatty alcohols as well as specific $[\text{RCO}_2\text{H}_2]^+$ product ions that would define

both the saturated and unsaturated fatty acyl and fatty alkyl portions of the wax ester. This list could then be used to generate a series of MRM experiments that could examine the mixture for specific wax esters displaying these properties (Fig. 8).

MRM experiments for the transitions of the saturated wax esters 32:0 (m/z 498) to the protonated saturated fatty acids $[\text{C}_n\text{H}_{2n+1}\text{CO}_2\text{H}_2]^+$ ($n = 3-23$) (Fig. 8A) and unsaturated wax esters 32:1 (m/z 496) that underwent transitions to yield $[\text{C}_n\text{H}_{2n-1}\text{CO}_2\text{H}_2]^+$ ($n = 3-23$) revealed signals for some of the protonated fatty acid cations. Specifically, for the saturated species, the components containing C_{11} to C_{19} fatty acyl groups were significantly above the signal-to-noise limit, with the signals corresponding to the even carbon number fatty acids being most abundant. The C_4 to C_{10} and C_{20} to C_{24} fatty acyl moieties, if indeed they were present, did not produce ions of sufficient intensity to be detected by this protocol. These results suggested that the mixture of 32:0 follicular wax esters was composed of at least nine different isomers of varying fatty acyl and fatty alkyl chain lengths, compared with the three identified by EI GC-MS or the

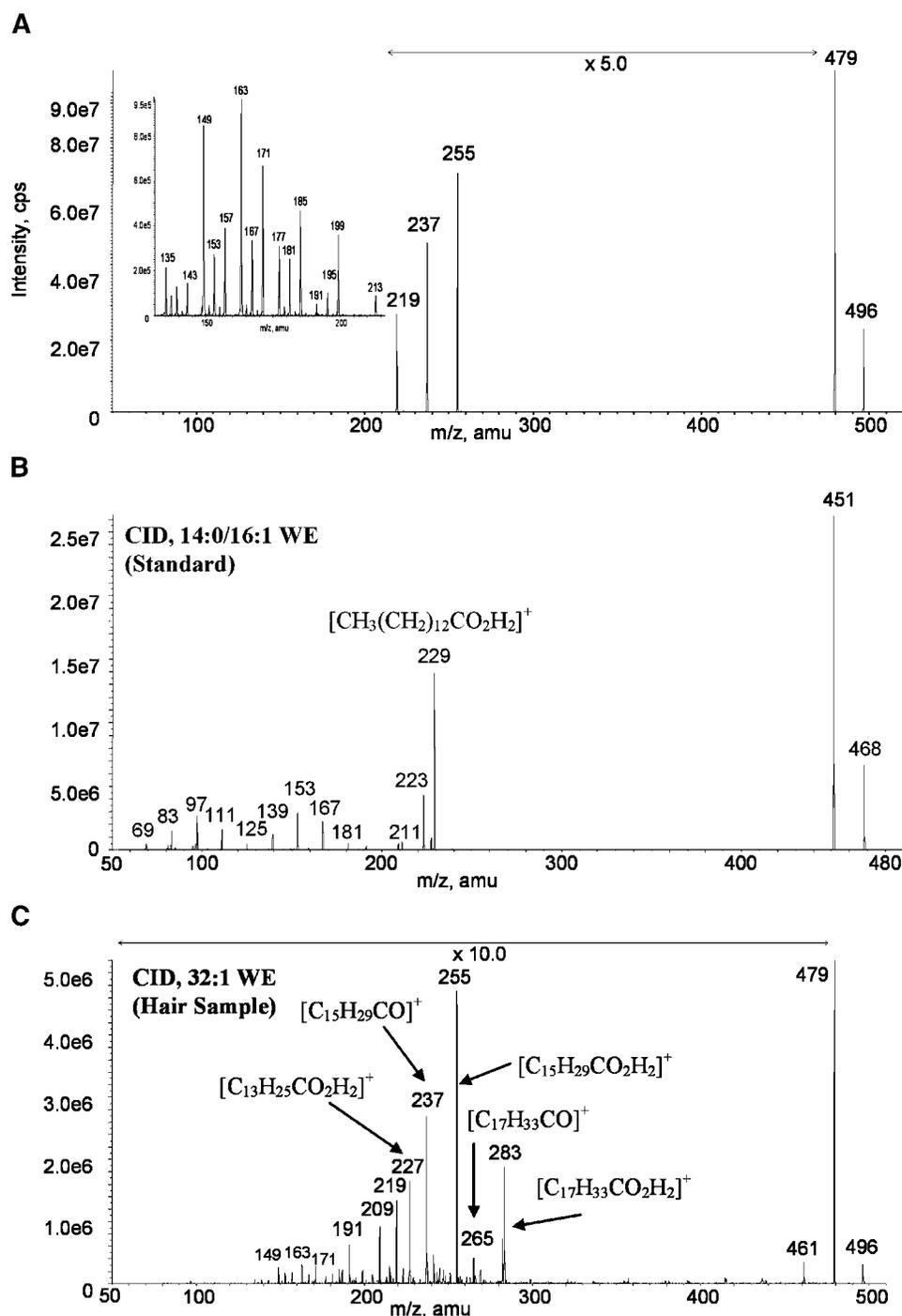


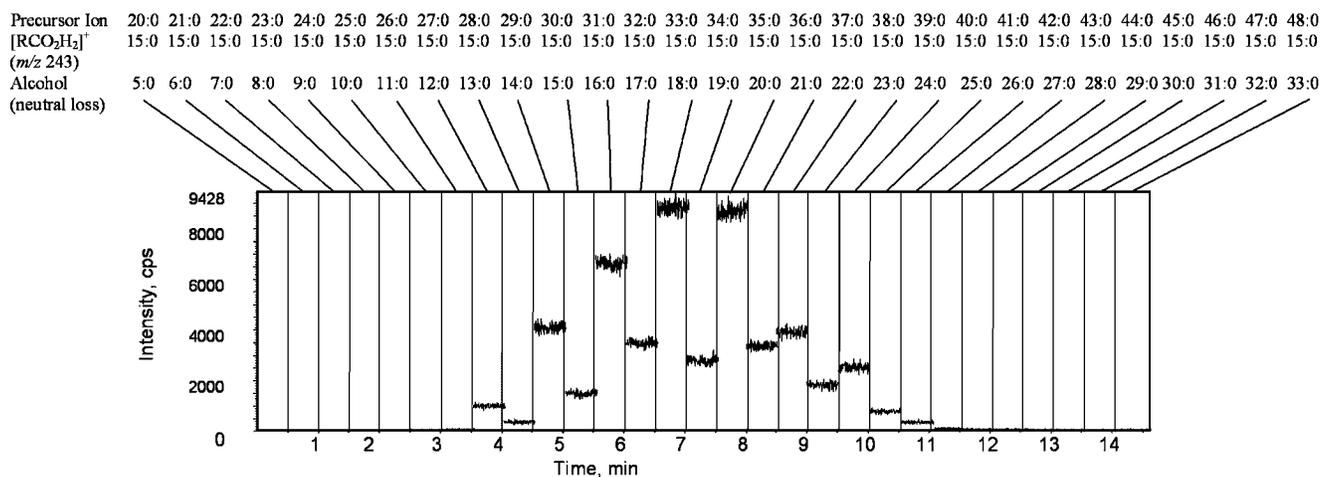
Fig. 7. A: Positive ion collision-induced dissociation (CID) mass spectrum of the 16:1/16:0 (synthetic) ammonium adduct formed under ESI conditions. B: Positive ion collision-induced dissociation mass spectrum of the 14:0/16:1 (synthetic) ammonium adduct formed under ESI conditions. C: Positive ion collision-induced dissociation mass spectrum of the mixture of 32:1 wax ester (WE) ammonium adducts (m/z 496) formed from the hair extract under ESI conditions.

four identified by electrospray product ion analysis (MS/MS experiments). The MRM experiment probing the mixture for 32:1 wax esters revealed only abundant ions corresponding to C_{16} , C_{14} , and C_{18} (in decreasing order) monoene fatty acids and some indication for C_{15} and C_{17} fatty acyl wax ester molecular species. This proved to be a sensitive and specific technique to probe the complex

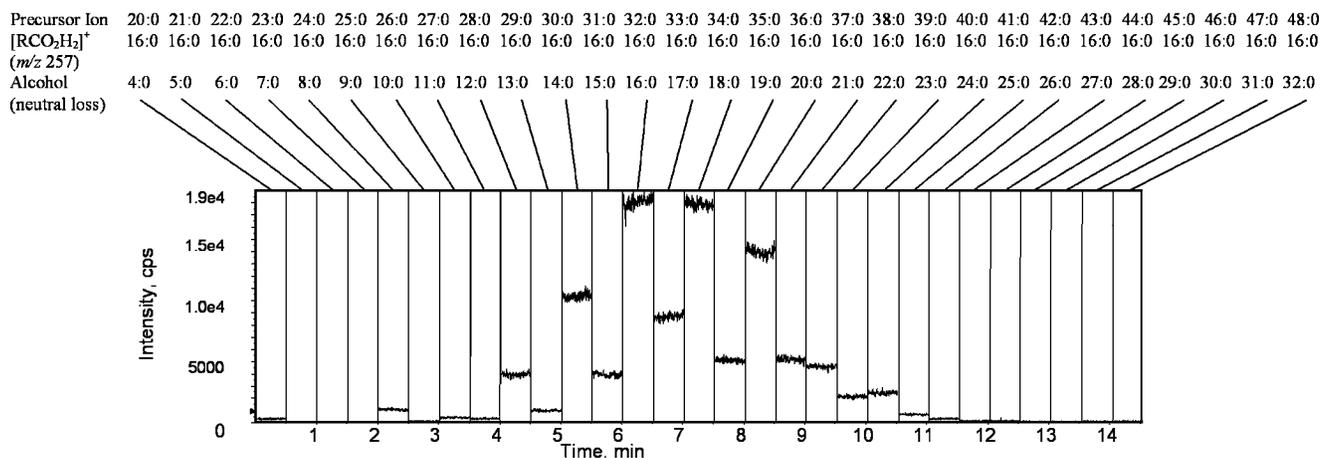
mixture without prior separation and to reveal relative abundances of unique molecular species. It did not, however, reveal the existence or positions of methyl branched fatty acyl or fatty alkyl components of the wax esters.

A variation of the MRM survey of wax esters described above examined all $[\text{M}+\text{NH}_4]^+$ ions that yielded a single diagnostic $[\text{RCO}_2\text{H}_2]^+$ ion. The transitions for saturated

A



B



C

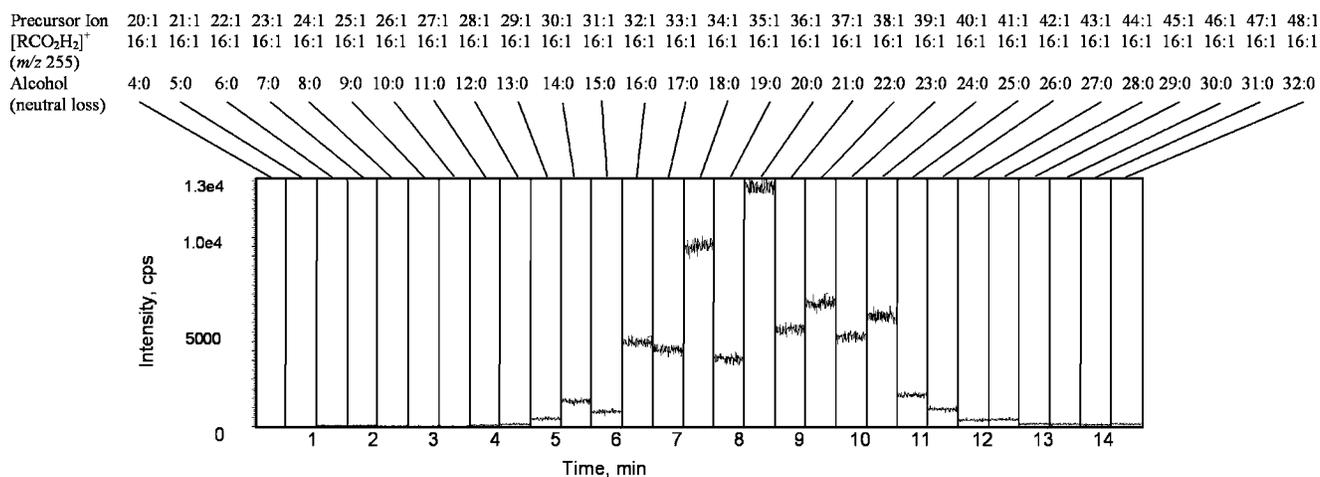


Fig. 9. A: MRM experiment measuring the ion transitions from human hair 20:0 to 48:0 ammonium adduct wax esters to the same protonated fatty acid $[\text{C}_{14}\text{H}_{29}\text{CO}_2\text{H}_2]^+$ corresponding to 15:0 at *m/z* 243. B: MRM experiment measuring the transitions from human hair 20:0 to 48:0 ammonium adduct wax esters to the protonated fatty acid $[\text{C}_{15}\text{H}_{31}\text{CO}_2\text{H}_2]^+$ corresponding to 16:0 at *m/z* 257. C: MRM experiment measuring the transitions from human hair 20:1 to 48:1 ammonium adduct wax esters to the protonated fatty acid $[\text{C}_{15}\text{H}_{29}\text{CO}_2\text{H}_2]^+$ corresponding to 16:1 at *m/z* 255.

containing wax esters (Fig. 9B), the even chained wax esters were consistent with the GC-MS results (Table 4). Most prominent were 16:0/14:0, 16:0/16:0, 16:0/18:0, and 16:0/20:0; however, odd and even species possessing the 16:0 fatty acyl group were identified in wax esters ranging from 25 to 42 carbon atoms in total length. Even the monoene wax esters containing the 16:1 esterified fatty acyl group (Fig. 9C) yielded a population of molecular species with 16:1/18:0 and 16:1/20:0 most abundant.

An alternative to the MRM approach was to perform a neutral loss experiment in which the mass of NH_3 plus the mass corresponding to the fatty alcohol chain minus one hydrogen atom (Table 5) could be used to detect those specific molecular species containing unique esterified alcohol components. This neutral loss approach would yield an abundant ion when a specific $[\text{M}+\text{NH}_4]^+$ ion was being scanned in the first stage of the tandem quadrupole mass spectrometer and then collisionally activated to a unique $[\text{RCO}_2\text{H}_2]^+$ product ion that was detected by the third mass spectrometer when the offset corresponded to the neutral loss of ammonia plus the fatty alcohol group as an olefin. This neutral loss scan was used to probe the

complex mixture of hair wax esters, testing for the presence of a range of fatty alcohols from C_{10} (loss of NH_3 and $\text{C}_{10}\text{H}_{20}$) to C_{26} (loss of NH_3 and $\text{C}_{26}\text{H}_{52}$). Specific examples of the neutral loss scan for C_{16} , C_{17} , and C_{18} revealed the abundance of all wax ester species that underwent this neutral loss behavior (Fig. 10A, B, C respectively). The neutral loss scan for 241 u (Fig. 10A) revealed the presence of molecular species with an esterified 16:0 fatty alcohol ranging from 26 to 37 carbons in length. The 16:0/16:0 and 16:1/16:0 wax esters were the most abundant, along with major species corresponding to 14:0/16:0, 15:0/16:0, and 14:1/16:0 as additional major components. Several prominent anomalous peaks were present in the full spectrum (data not shown), including m/z 404, 408, and 532. These components did not appear to be wax esters and were most likely attributable to the presence of other contaminating lipids detected by this process. The spectrum of the neutral loss for 17:0 fatty alcohol wax esters (Fig. 10B) displayed a range of components from C_{28} to C_{34} total carbon length and was dominated by the odd chained species 16:1/17:0, 16:0/17:0, and 14:0/17:0.

TABLE 4. Wax ester ammonium adducts (electrospray ionization) detected by neutral loss scanning and assignment of molecular ion species

| Neutral Alcohol | Loss Scanned | Precursor Ion (m/z)/Wax Ester/Abundance (%) |
|-----------------|--------------|--|
| | | <i>amu</i> |
| C12 | 185 | 358, 12:0/12:0 (5%); 372, 13:0/12:0 (3%); 386, 14:0/12:0 (5%); 400, 15:0/12:0 (18%); 414, 16:0/12:0 (76%); 428, 17:0/12:0 (62%); 440, 18:1/12:0 (33%); 442, 18:0/12:0 (100%); 454, 19:1/12:0 (11%); 456, 19:0/12:0 (19%); 468, 20:1/12:0 (6%); 470, 20:0/12:0 (10%) |
| C13 | 199 | 400, 12:0/13:0 (13%); 414, 13:0/13:0 (91%); 428, 14:0/13:0 (51%); 442, 15:0/13:0 (48%); 454, 16:1/13:0 (100%); 456, 16:0/13:0 (88%) |
| C14 | 213 | 414, 12:0/14:0 (40%); 428, 13:0/14:0 (58%); 440, 14:1/14:0 (35%); 442, 14:0/14:0 (86%); 456, 15:0/14:0 (72%); 468, 16:1/14:0 (92%); 470, 16:0/14:0 (100%); 482, 17:1/14:0 (64%); 484, 17:0/14:0 (28%); 496, 18:1/14:0 (25%); 498, 18:0/14:0 (14%) |
| C15 | 227 | 412, 11:1/15:0 (17%); 414, 11:0/15:0 (32%); 428, 12:0/15:0 (23%); 442, 13:0/15:0 (48%); 454, 14:1/15:0 (26%); 456, 14:0/15:0 (85%); 468, 15:1/15:0 (25%); 470, 15:0/15:0 (80%); 482, 16:1/15:0 (100%); 484, 16:0/15:0 (92%); 496, 17:1/15:0 (39%); 498, 17:0/15:0 (24%); 510, 18:1/15:0 (20%); 512, 18:0/15:0 (15%) |
| C16 | 241 | 414, 10:0/16:0 (5%); 426, 11:1/16:0 (3%); 428, 11:0/16:0 (5%); 442, 12:0/16:0 (16%); 456, 13:0/16:0 (24%); 468, 14:1/16:0 (32%); 470, 14:0/16:0 (79%); 482, 15:1/16:0 (15%); 484, 15:0/16:0 (55%); 496, 16:1/16:0 (100%); 498, 16:0/16:0 (91%); 510, 17:1/16:0 (15%); 512, 17:0/16:0 (16%); 524, 18:1/16:0 (16%); 526, 18:0/16:0 (15%); 554, 20:0/16:0 (24%); 568, 21:0/16:0 (3%) |
| C17 | 255 | 440, 11:1/17:0 (28%); 442, 11:0/17:0 (9%); 456, 12:0/17:0 (14%); 470, 13:0/17:0 (30%); 482, 14:1/17:0 (24%); 484, 14:0/17:0 (56%); 496, 15:1/17:0 (25%); 498, 15:0/17:0 (45%); 510, 16:1/17:0 (100%); 512, 16:0/17:0 (80%); 524, 17:1/17:0 (19%); 526, 17:0/17:0 (13%) |
| C18 | 269 | 414, 8:0/18:0 (2%); 428, 9:0/18:0 (16%); 454, 11:1/18:0 (2%); 456, 11:0/18:0 (2%); 468, 12:1/18:0 (1%); 470, 12:0/18:0 (13%); 484, 13:0/18:0 (19%); 496, 14:1/18:0 (29%); 498, 14:0/18:0 (47%); 510, 15:1/18:0 (13%); 512, 15:0/18:0 (46%); 524, 16:1/18:0 (100%); 526, 16:0/18:0 (58%); 538, 17:1/18:0 (13%); 540, 17:0/18:0 (13%); 552, 18:1/18:0 (14%); 554, 18:0/18:0 (6%) |
| C19 | 283 | 468, 11:1/19:0 (53%); 470, 11:0/19:0 (35%); 484, 12:0/19:0 (17%); 498, 13:0/19:0 (40%); 510, 14:1/19:0 (35%); 512, 14:0/19:0 (67%); 524, 15:1/19:0 (24%); 526, 15:0/19:0 (71%); 538, 16:1/19:0 (100%); 540, 16:0/19:0 (95%) |
| C20 | 297 | 496, 12:1/20:0 (3%); 498, 12:0/20:0 (9%); 512, 13:0/20:0 (33%); 524, 14:1/20:0 (35%); 526, 14:0/20:0 (44%); 538, 15:1/20:0 (11%); 540, 15:0/20:0 (42%); 552, 16:1/20:0 (100%); 554, 16:0/20:0 (49%); 566, 17:1/20:0 (13%); 568, 17:0/20:0 (18%); 580, 18:1/20:0 (19%); 582, 18:0/20:0 (2%) |
| C21 | 311 | 468, 9:1/21:0 (18%); 482, 10:1/21:0 (8%); 496, 11:1/21:0 (35%); 498, 11:0/21:0 (10%); 510, 12:1/21:0 (11%); 512, 12:0/21:0 (18%); 524, 13:1/21:0 (18%); 526, 13:0/21:0 (33%); 538, 14:1/21:0 (45%); 540, 14:0/21:0 (84%); 552, 15:1/21:0 (23%); 554, 15:0/21:0 (73%); 566, 16:1/21:0 (100%); 568, 16:0/21:0 (82%); 580, 17:1/21:0 (31%); 582, 17:0/21:0 (9%); 594, 18:1/21:0 (22%) |
| C22 | 325 | 482, 9:1/22:0 (3%); 496, 10:1/22:0 (14%); 510, 11:1/22:0 (7%); 512, 11:0/22:0 (7%); 524, 12:1/22:0 (13%); 526, 12:0/22:0 (6%); 540, 13:0/22:0 (31%); 552, 14:1/22:0 (40%); 554, 14:0/22:0 (27%); 566, 15:1/22:0 (16%); 568, 15:0/22:0 (49%); 580, 16:1/22:0 (100%); 582, 16:0/22:0 (31%); 594, 17:1/22:0 (23%); 596, 17:0/22:0 (10%); 608, 18:1/22:0 (14%); 610, 18:0/22:0 (6%); 624, 19:0/22:0 (6%) |
| C23 | 339 | N/A |
| C24 | 353 | 482, 8:1/24:0 (14%); 496, 9:1/24:0 (19%); 510, 10:1/24:0 (17%); 524, 11:1/24:0 (34%); 538, 12:1/24:0 (31%); 540, 12:0/24:0 (11%); 552, 13:1/24:0 (26%); 554, 13:0/24:0 (17%); 568, 14:0/24:0 (30%); 580, 15:1/24:0 (42%); 582, 15:0/24:0 (35%); 596, 16:0/24:0 (55%); 608, 17:1/24:0 (100%); 610, 17:0/24:0 (56%); 622, 18:1/24:0 (49%); 624, 18:0/24:0 (28%) |

TABLE 5. Fatty alcohol chain length and corresponding neutral loss mass from wax esters containing esterified saturated fatty alcohols

| Fatty Alcohol Total Carbon Chain Length | Neutral Mass Loss | Molecular Losses from Precursor Ions ^a |
|---|-------------------|---|
| 10 | 157 | NH ₃ and CH ₂ =CH(CH ₂) ₇ CH ₃ |
| 11 | 171 | NH ₃ and CH ₂ =CH(CH ₂) ₈ CH ₃ |
| 12 | 185 | NH ₃ and CH ₂ =CH(CH ₂) ₉ CH ₃ |
| 13 | 199 | NH ₃ and CH ₂ =CH(CH ₂) ₁₀ CH ₃ |
| 14 | 213 | NH ₃ and CH ₂ =CH(CH ₂) ₁₁ CH ₃ |
| 15 | 227 | NH ₃ and CH ₂ =CH(CH ₂) ₁₂ CH ₃ |
| 16 | 241 | NH ₃ and CH ₂ =CH(CH ₂) ₁₃ CH ₃ |
| 17 | 255 | NH ₃ and CH ₂ =CH(CH ₂) ₁₄ CH ₃ |
| 18 | 269 | NH ₃ and CH ₂ =CH(CH ₂) ₁₅ CH ₃ |
| 19 | 283 | NH ₃ and CH ₂ =CH(CH ₂) ₁₆ CH ₃ |
| 20 | 297 | NH ₃ and CH ₂ =CH(CH ₂) ₁₇ CH ₃ |
| 21 | 311 | NH ₃ and CH ₂ =CH(CH ₂) ₁₈ CH ₃ |
| 22 | 325 | NH ₃ and CH ₂ =CH(CH ₂) ₁₉ CH ₃ |
| 23 | 339 | NH ₃ and CH ₂ =CH(CH ₂) ₂₀ CH ₃ |
| 24 | 353 | NH ₃ and CH ₂ =CH(CH ₂) ₂₁ CH ₃ |
| 25 | 367 | NH ₃ and CH ₂ =CH(CH ₂) ₂₂ CH ₃ |
| 26 | 381 | NH ₃ and CH ₂ =CH(CH ₂) ₂₃ CH ₃ |

^aThe double bond indicated in these structures corresponds to an olefin product ion formed in the neutral loss experiment.

Additional wax esters were clearly present but were close to the signal-to-noise limit of this experiment. The neutral loss mass spectrum for 18:0 fatty alcohols in this wax ester mixture (Fig. 10C) revealed a similar mass range of wax esters as that observed for the 16:0 fatty alcohol-containing components. However, this mass spectrum was dominated by the higher mass wax ester components.

DISCUSSION

Wax esters are an interesting class of lipids in that they have an extremely hydrophobic character and serve very specialized roles in both the plant and animal kingdoms. In spite of this, very few advances have been made in the analysis of this class of biomolecules. It is known that only a few tissues or cells in animals have the enzymatic activity capable of producing these molecules, and only recently have those enzymes responsible for catalyzing the esterification of fatty acids with fatty alcohols been cloned and expressed (24). The chemical properties of wax esters render them relatively easy to isolate and extract as well as chromatographically separate from other classes of lipid substances. Soon after EI mass spectrometry was applied to the analysis of wax esters isolated from biological matrices, it was observed that these simple molecules were present in a complex mixture of individual molecular species (25).

These complex mixtures of wax ester molecular species have been separated using gas chromatography, but GC peaks consisted of multiple isobaric components that were not separated chromatographically. This rendered peaks broader than expected because of closely eluting species (i.e., 18:0/16:0 and 16:0/18:0). A second approach to the complex mixture was to degrade these molecules and use the powerful tools of gas chromatography and mass spectrometry to more fully characterize the resulting fatty

acids and fatty alcohols, including structural information such as positions of double bonds, locations of methyl substituents, or other characteristics difficult to analyze within the intact wax ester. Nonetheless, degradation of these molecules resulted in the loss of specific molecular species information that is known in some cases to have a profound effect on the chemical and physical properties of the wax esters. For example, introduction of a single double bond can decrease the melting temperature as much as 30°C when the double bond is present either in the alcohol or the fatty acyl portion of the wax ester (26). On the other hand, analysis of the intact wax ester by capillary GC-MS was also limited in that a great deal of structural information related to individual molecular species could not be obtained, such as the positions of double bonds and/or methyl branching in either the fatty alcohol or the fatty acyl portion of the molecule. However, it was possible from the mass spectrometric data to recognize characteristic fragment ions that depicted a different carbon chain length within the fatty acyl or fatty alcohol portion of the molecule as a result of the quite different fragmentation pathways and abundant characteristic ions produced. More than 200 different wax esters could be identified at this level by EI GC-MS, but this required combining mass spectrometric and chromatographic data in a somewhat time-consuming process.

ESI has been found to generate molecular ion species corresponding to the attachment of alkali metals or ammonium ions, most likely through association with the ester group. Collision-induced decomposition of synthetic wax esters as the ESI generated ammonium adduct ion [M+NH₄]⁺ resulted in the formation of an abundant and very characteristic [RCO₂H₂]⁺ ion for all wax esters studied, including wax esters containing double bonds in the fatty acyl as well as the fatty alcohol portion of the molecule. The number of different product ions formed after collisional activation of the [M+NH₄]⁺ was remarkably simpler than the EI-induced fragmentation of wax esters. In part, this was most likely attributable to a single site of adduct ion formation during ESI as opposed to possibly multiple sites of ionization for EI (e.g., different regions of the wax ester could become ionized when double bonds were present, and in these cases the charge site could be localized at the ester carbonyl or localized initially at the double bond).

Collision-induced decomposition of [M+NH₄]⁺ ions from wax esters provided information regarding the carbon chain length of the fatty acid portion of the molecule and whether or not double bonds were present in that moiety. From the known molecular weight of the wax ester, as defined by the precursor [M+NH₄]⁺, information about the alcohol portion of the molecule as well as the presence or absence of double bonds in this portion of the molecule was readily deduced. Very little variation in the behavior was found in a series of synthetic wax esters examined by tandem mass spectrometry after ESI.

Because wax esters behave in a very similar manner after collisional activation of the [M+NH₄]⁺ ion, it was possible to design a series of tandem mass spectrometric experi-

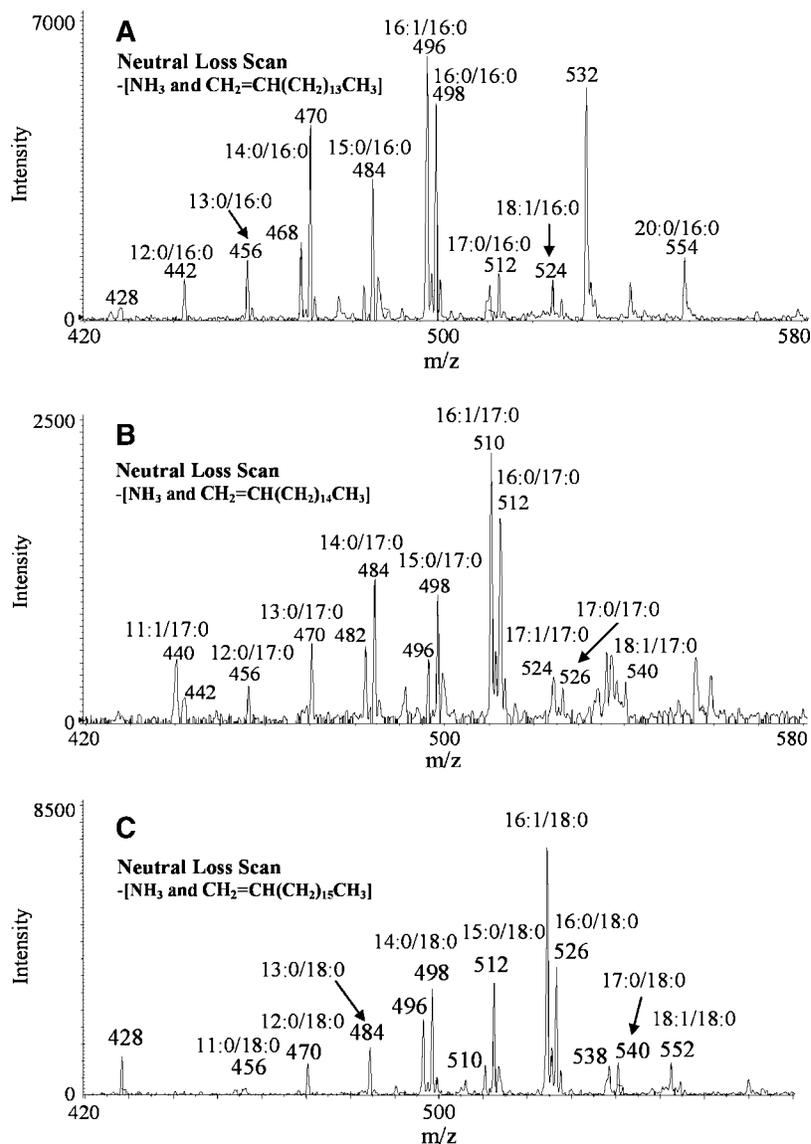


Fig. 10. A: Mass spectrum produced by scanning the neutral loss of NH_3 and $\text{CH}_2=\text{CH}(\text{CH}_2)_{13}\text{CH}_3$, which displayed precursor human hair wax ester ammonium adducts possessing the 16:0 fatty alcohol moiety. B: Mass spectrum produced by scanning the neutral loss of NH_3 and $\text{CH}_2=\text{CH}(\text{CH}_2)_{14}\text{CH}_3$, which displayed precursor human hair wax ester ammonium adducts possessing the 17:0 fatty alcohol moiety. C: Mass spectrum produced by scanning the neutral loss of NH_3 and $\text{CH}_2=\text{CH}(\text{CH}_2)_{15}\text{CH}_3$, which displayed precursor human hair wax ester ammonium adducts possessing the 18:0 fatty alcohol moiety.

ments to capitalize on this property. MRM experiments could be designed that would define the presence of a chosen molecular species possessing a specific esterified fatty acid and fatty alcohol in a complex mixture without prior separation. In such MRM experiments, one merely predicted the mass-to-charge ratio for each $[\text{M}+\text{NH}_4]^+$ ion for the molecular species of interest as well as the expected $[\text{RCO}_2\text{H}_2]^+$ product ion. A rapid scanning tandem quadrupole mass spectrometer allows several tens of such MRM experiments to be carried out in a reasonable length of time. In a similar vein, neutral losses that involve the loss of a fatty alcohol portion of the molecule as an olefin can be examined by scanning the quadrupole for all precursor ions that undergo this decomposition. In

this way, all of the $[\text{M}+\text{NH}_4]^+$ ions that contain a specific fatty alcohol would yield a unique $[\text{RCO}_2\text{H}_2]^+$ ion.

It was found using this method that the complex mixture of wax esters isolated from human hair could be very rapidly analyzed by ESI and neutral loss scanning. Furthermore, the GC-MS data obtained from the degraded hair wax ester could be used to appropriately choose the neutral loss masses. Using this method, it was possible to rapidly identify both saturated and monoene wax esters possessing known fatty alcohol constituents from 12 to 24 carbon atoms in length and thereby identify wax esters at 24–42 carbon atoms. In total, the neutral loss scan approach detected >160 wax ester molecular species in the human hair sample without prior chromatographic sepa-

ration, including many that were not identified using GC-MS. The ESI/tandem mass spectrometry approach was particularly effective at detecting monoenes present in the fatty acid portion of the molecule. It was also surprisingly more sensitive at detecting molecular species that appeared at lower molecular weights. One of the distinct advantages of using ESI/tandem mass spectrometry was the removal of the requirement that the wax ester be volatile. This could be especially critical for those wax esters of higher molecular weight or those containing polyunsaturated fatty alkyl groups, which might undergo thermal decomposition during the high temperatures used in the gas chromatographic separation.

In summary, ESI/tandem mass spectrometry can be used to analyze wax ester molecular species present in complex mixtures isolated from biological extracts in a facile manner. It is possible to use these techniques without chromatographic separation and identify a large number of individual molecular species in a complex mixture at a level of structural identification comparable to that obtained by EI GC-MS of the intact wax esters. A particularly powerful technique is neutral loss scanning of the complex mixture of $[M+NH_4]^+$ ions for the loss of NH_3 and the olefin corresponding to the alcohol portion of the molecule. This approach afforded a rapid means to assess changes in molecular species, particularly changes in saturation, which are known to change the physical properties of wax esters.^{11r}

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