Identification and Analysis of Wax Esters by Mass Spectrometry¹

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ABSTRACT

Several ions in the mass spectra of wax esters were related to the molecular structures. Assigned structures of ions were confirmed by deuterium labeling. A simple, direct method for quantitative analyses of mixtures was developed. The method involved a comparison of sets of three ions, RCO_2H^+ , RCO_2H_2^+ and $[\text{R'-1}]^+$ from all compounds in the mixture. The method was found applicable for mixtures of unsaturated wax esters after reduction with tetradeuterio hydrazine.

INTRODUCTION

The mass spectra of esters of long chain fatty acids and short chain alcohols (1) and of short chain acids and long chain alcohols (2) have been thoroughly studied and reviewed (3,4). Although they occur widely in nature, esters of long chain acids and alcohols have received much less attention and the mass spectrum of only one such wax ester has been published (3) previously. Wax esters are encountered in the lipids of a wide variety of species and they occur in a range from C_{26} to C_{40} and in a variety of isomers. The mass spectra of a series of synthetic wax esters were measured and studied for the purposes of identification and of quantification of individual components in mixtures of such esters. The spectra of some specifically labeled wax esters were recorded to determine the atomic composition of some ions.

Shorthand formulae for wax esters have been used, i.e., hexadecyl octadecanoate is denoted as 16-18, listing the alcohol moiety first. However, in the general formula $\text{RCO}_2\text{R}'$, R and R' are the alkyl moieties of the acyl group and the alcohol group, respectively.

EXPERIMENTAL PROCEDURES

The mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6D instrument at 70 eV and a pressure of approximately 10^{-7} torr. Wax esters, individually or in mixtures, were introduced through the direct inlet system. The spectra were recorded at temperatures that gave constant total ion concentration between 60 C and 250 C, depending on molecular weight. High resolution mass spectra were measured on an AEI MS9 double focussing mass spectrometer.

Mixtures of wax esters were analyzed by temperature-programmed gas chromatography mass spectrometry, using a 0.3 x 90 cm column packed with Gas Chrom P (100-120 mesh) coated with 2% Versamid 900.

Long chain acyl chlorides and alcohols were purchased from The Hormel Institute Lipids Preparation Laboratory. Deuterium-labeled fatty acids and 2,2-dideuteriotetradecanol were prepared as previously described (5). 1,1-Dideuterio-octadecanol was prepared by reduction of methyl octadecanoate with lithium aluminum deuteride (6). Wax esters labeled in the acyl moiety were synthesized by heating a mixture of the labeled fatty acid and an excess of the long chain alcohol at about 1 mm and 80-90 C for 3-4 hr in the presence of *p*-toluenesulfonic acid as catalyst. Other wax esters, nonlabeled or labeled in the alcohol moiety, were synthesized as previously described (7), or were prepared by treating the alcohol with excess acyl chloride for 3-4 hr at 1 mm and 60 C. The esters were purified by preparative thin layer chromatography (TLC) and recrystallized twice from petroleum ether. Yields and melting points are given in Table I.

A synthetic mixture of isomeric unsaturated wax esters, octadecyl octadec-9-enoate, 56.5 mg, and octadec-9-enyl octadecanoate, 64.1 mg, was reduced with tetradeuterio hydrazine as previously described (8,9). Residual unreacted wax esters amounted to about 20%, judging from a peak at m/e 534, and were removed by subjecting a 20 mg portion to ozonolysis (10). The mass spectrum of the product (10.1 mg) which was obtained after preparative TLC, had no peak at the molecular weight of the parent unsaturated esters (m/e 534) but a prominent molecular ion for the

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TABLE I

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Ester	Alcohol-acid	Melting points, C	Yield
Octadecyl 2,2-dideuteriotetradecanoate	18-14(2,2-d ₂)	51.8	84%
Octadecyl 3,3-dideuteriopentadecanoate	18-15(3,3-d2)	55.2-55.6	90%
Octadecyl 4,4-dideuteriohexadecanoate	18-16(4,4-d2)	59.5-60	62%
1,1-Dideuteriooctadecyl octadecanoate	18(1,1-d2)-18	52.5-52.9	95%
2,2-Dideuteriotetradecyl octadecanoate	14(2,2-d2)-18	61.5-61.8	60%
Octadecyl dodecanoate	18-12	43.5-44	59%
Octadecyl decanoate	18-10	37.5	43%
Dodecyl octadecanoate	12-18	44,4-44.8	74%
Decyl octadecanoate	10-18	37.5-38	74%
Octyl octadecanoate	8-18	32	66%
Hexyl octadecanoate	6-18	27.5-28	61%
Butyl octadecanoate	4-18	27	59%
Propyl octadecanoate	3-18	30.5-31	33%

Melting Points and Yields of Wax Esters^a

^aSee also reference 7.

dideuterio- addition product (m/e 536).

RESULTS AND DISCUSSION

Stenhagen et al. (3) found the diagnostically important ions M^+ , $RCO_2H_2^+$, $CO_2R'^+$ and $[R' - 1]^+$. Our deuterium-labeled and nonlabeled wax esters produced ions agreeing with the previously postulated (3) structures. In addition, five more characteristic ions were observed and their structures have been postulated. The elemental compositions of these ions were confirmed by high resolution measurements in the case of octadecyl octadecanoate as follows: ion $[RCO_2H\text{-propyl}]^+$, measured 241.21625, calculated 241.21674; $[R'-1]^+$, 252.28096, 252.28168; RCO⁺, 267.26710, 267.26880; RCO_2H⁺, 284.27212, 284.27173; RCO_2H_2⁺, 285.27845, 285.27954; CO_2R'⁺, 297.27872, 297.27954; CH₂=COH-OR'⁺, 313.30890, 313.31055.

RCO₂H₂⁺

This ion was found to be the base peak in all

TABLE II

Intensities of Characteristic Ions Co	ontaining the .	Alcohol Moiety	in Mass	Spectra of Sa	aturated Wax	Esters,
RCO ₂ R', Expressed as Per Cent of	$RCO_2H_2^+$ to	Indicate Depen	dence of	f Abundance	Upon Chain	Length

Wax e	ester	[R	.'-1]+	cc	D ₂ R'+	СН ₂	=C + O-R'	CH ₂	=C + O-R'
Alcohol	Acid	Mass	Intensity	Mass	Intensity	Mass	Intensity	Mass	Intensity
18	$14(2,2-d_2)$	252	21.9	297	5.0	314	1.3	315	1.2
18	15(3,3-d2)	252	25.4	297	6.0	312	1.2	313	1.5
18	$16(4, 4-d_2)$	252	24.6	297	6.2	313	1.2	314	2.2
$18(1, 1 - d_2)$	18	254	29.8	299	7.1	314	1.5	315	2.7
$14(2,2-d_2)$	18	а	43.9 ^a	243	7.2	258	1.9	259	5.0
18	10	252	17.7	297	4.3	312	0.8	313	0.3
18	12	252	20.5	297	5.0	312	1.0	313	0.6
18	14	252	22.7	297	4.8	312	1.1	313	1.1
18	16	252	26.3	297	6.1	312	2.3	313	1.8
18	18	252	27.7	297	6.6	312	1.4	313	2.7
16	18	224	36.6	269	6.7	284	b	285	b
14	18	196	53.0	241	12.8 ^c	256	1.4	257	5.5
12	18	168	70.5	213	8.5	228	1.5	229	6.4
10	18	140	91.7	185	13.5	200	1.7	201	9.8
8	18	112	113.3	157	8.4	172	2.1	173	10.5
6	18	84	125.6	129	22.6	144	7.3	145	13.7
4	18	56	194.9	101	20.5	116	44.1	117	29.2
3	18	42	đ	87	38.9	102	160.0	103	68.6

 $a[R'-D]^+ + [R'-H]^+$.

^bCoinciding with RCO₂H⁺ and RCO₂H₂⁺.

Coinciding with [RCO₂H-propyl]⁺.

dNot present. The base peak was found at m/e 61, 359%.

Wах	ester	IRCO	2H-propy1] ⁺		RCO ⁺	Ч	tco ₂ H ⁺	RC	02H2 ^{+a}		M+
Alcohol	Acid	Mass	Intensity	Mass	Intensity	Mass	Intensity	Mass	Intensity	Mass	Intensity
18	14(2,2-d ₂)	q	٩	213	11.9	230	28.1	231	100	482	23.1
18	$15(3, 3-d_2)$	199	3.6	227	10.9	244	26.6	245	100	496	21.2
18	$16(4, 4-d_{2})$	213	3.9	241	9.5	258	28.5	259	100	510	27.4
18(1,1-d ₂)	18	241	4.7	267	9.4	284	22.0	285	100	538	35.3
$14(2,2-d_2)$	18	241	5.0	267	11.5	284	19.8	c	100	482	28.7
18	10	129	7.3	155	14.6	172	17.1	173	100	424	12.8
18	12	157	4.2	183	12.4	200	19.1	201	100	452	15.9
18	14	185	5.8	211	11.1	228	20.7	229	100	480	17.4
18	16	213	4.2	239	10.6	256	22.8	257	100	508	27.9
18	18	241	4.7	267	10.2	284	24.4	285	100	536	30.2
16	18	241	5.3	267	10.9	284	24.6	285	100	508	30.6
14	18	241	12.8d	267	12.8	284	26.1	285	100	480	24.9
12	18	241	8.8	267	15.3	284	27.5	285	100	452	30.8
10	18	241	0.6	267	19.5	284	30.1	285	100	424	38.3
80	18	241	11.4	267	25.1	284	32.7	285	100	396	34.2
9	18	241	10.9	267	31.8	284	32.2	285	100	368	36.0
4	18	241	18.5	267	57.5	284	31.2	285	100	340	61.4
e	18	241	29.2	267	111.9	284	29.2	285	100	326	127.7
^a Base peak i ^b m/e 185, 1 ^c RCO ₂ H ₂ ⁺ dCoinciding	n all spectra except 86, 187: 1.37 , 1.81 + RCO ₂ HD ⁺ . with CO ₂ R'+.	in those of 3, , 1.72.	4, 6, 8-18.								

TABLE III

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cases except those in which the alcohol moiety was shorter than 10 carbon atoms (Tables II and III). It is apparently formed directly from the parent ion, M⁺, judging from a corresponding metastable peak. Hence, the cleavage of the O-R' bond and the transfer of two hydrogens from the R' moiety must be a concerted reaction. Using deuterium-labeled n-butyl propionates, Djerassi et al (11) established that in these compounds the double hydrogen transfer involved positions 2 and 3 of the alkoxy group to a higher degree than any other position. The spectra of wax esters labeled in positions 1 or 2 of the alcohol moiety revealed increased $(RCO_2H+1)^+$ ions indicating transfer of some deuterium. The ratio RCO₂H₂+: RCO₂HD+ was found to be 90:10 and 77:23, respectively.

RCO₂H⁺

The ion formally equivalent to the carboxylic acid might be formed by a McLafferty rearrangement. Djerassi et al. (11) found, however, that in the cases of butyl propionate and benzoate the transferred hydrogen originated to some extent from positions other than the 2 position of the alkyloxy group. The spectrum of 2,2-dideuteriotetradecyl octadecanoate showed the ratio of $RCO_2H^+:RCO_2D^+$ to be 85:15. This implies that alkoxy fission with transfer of one hydrogen atom from position 2 (McLafferty rearrangement) is responsible for this ion only to a small degree.

[R'-1]+

The ratio of the ions [R'-H]⁺ to [R'-D]⁺ of 2,2-dideuterioin the spectrum tetradecyl octadecanoate was found to be 70:30, indicating that the main portion of the ion [R'-1]⁺, is not formed in a McLafferty rearrangement with charge retention on the hydrocarbon. This corresponds with the finding of Djerassi et al. (11) for butyl benzoate that only 25% of this ion is produced by a McLafferty rearrangement. At least part of this ion is produced by fragmentation of [CH₂=COH-OR']⁺ since a corresponding metastable peak was always found.

RCO⁺ and CO₂R'⁺

These ions are formed by normal cleavage (12) of the bonds adjacent to the carbonyl group. Absence of $[RCO-1]^+$ and $[CO_2R'-1]^+$ ions in the spectra of deuterium-labeled wax esters indicates that if scrambling takes place between the alkyl moieties, RCO^+ and $CO_2R'^+$ are formed prior to the scrambling. This agrees with reports for diglycerides (13) and triglycerides (5) labeled in the acyl moieties in which

the deuteriums apparently were also confined to their original acyl groups.

[RCO2H-C3H7]+

All unlabeled wax esters exhibited a relatively weak ion 43 mass units lower than the RCO_2H^+ ions. In the cases where positions 3 and 4 of the acyl group were gem. deuterated this interval increased to 45 mass units. When the 2 positions were occupied by deuteriums a cluster corresponding to loss of 43, 44 and 45 was observed. This pattern is analogous to earlier findings for long chain methyl esters (14,15) and triglycerides (5) which apparently expel three methylene groups adjacent to the carbonyl group plus one hydrogen. A similar extrusion must take place with wax esters too. The cluster of three peaks in the case of labeled 2 positions is the result of partial loss of exchange deuterium due to reactions. Deuterium adjacent to the carbonyl group in long chain methyl esters (14,15) and triglycerides (5) is known to be exchangeable with hydrogen atoms in positions 5, 6 and 7 of the acyl chain. In the case of 14-18 this ion coincided with $CO_2 R'^+$ at m/e 241, relative intensity 12.8%. These ions were found at different masses, m/e 241 and 243, respectively, in the spectrum of 14(2,2-d,)-18, and the sum of their relative intensities, 12.2%, is nearly the same as that of the double ion.

$$\begin{array}{ccc} OH_{+} & OH_{2} + & OH_{2} + \\ | & | & | \\ CH_{2}=C=OR', \ CH_{2}=C-O-R' \ and \ CH_{2}=C-OH \end{array}$$

The formation of the first two ions involves

TABLE IV

Quantitative Analyses of Mixtures of Wax Esters

Alcohol-acid	Molecular weight	Gravimetric composition	Found %
18-16	508	48.3	48.9
18-16(4,4-d ₂)	510	51.7	51.1
18-10	424	49.4	52.1
10-18	424	50.6	47.9
18-12	452	41.1	43.4
12-18	452	58.9	56.6
18-14	480	46.4	47.9
14-18	480	53.6	52.2
18-16	508	62.3	62.8
16-18	508	37.7	37.2
14-16	452	34.3	34.7
18-12	452	27.0	27.2
12-18	452	38.7	38.1
16-14	452	21.0	22.0
14-16	452	27.1	26.4
18-12	452	21.3	20.9
12-18	452	30,6	30.8

transfer of one and two hydrogen atoms, respectively, from the acyl moiety. Both ions showed retention of the deuterium atoms when the position adjacent to the carbonyl group was doubly labeled as in 18-14(2,2-d₂), suggesting that fission of the β -bond occurred prior to exchange reactions. Retention of these labile deuterium atoms indicates that a McLafferty rearrangement is involved (5,14,15). Further evidence for such a rearrangement was found in the retention of one deuterium atom in both ions when positions 4 of the acyl moiety were labeled. The origin of the second hydrogen transferred is unknown.

The base peak in the spectrum of propyl stearate was found at m/e 61 rather than the expected m/e 42 ($[R'-1]^+$, see Table II). This ion,, $[CH_2=C(OH_2)-OH]^+$, which involves a triple hydrogen transfer (16,17), is probably formed from $[CH_2=C(OH)-O-R']^+$, m/e 102, since a corresponding strong metastable peak was found at m/e 36.5 (Calculated 36.48). This ion was also found in spectra of other wax esters but to a much smaller extent. The increased relative intensity of this ion in the case of propyl might be due to absence of steric hindrance.

Quantitative Analysis of Mixtures

Since several of the pronounced, characteristic ions of wax esters are readily recognized, a method was sought for quantitative analyses of mixtures. Analyses of mixtures by means of mass spectrometry have been performed primarily on hydrocarbons (18,19) and amino acids (20), but this method should be applicable to lipids as well.

Wax Esters Having the Same Molecular Weight. Ions characteristic of each wax ester component in model mixtures were used singly or in combination as bases of calculations of composition. The most favorable results were obtained when the sums of the heights of the ions RCO_2H^+ , $RCO_2H_2^+$ and $[R'-1]^+$ for each component were compared. The following relationship was derived and empirically verified:

$$\%a = \frac{100 \Sigma_a}{\Sigma_a + \Sigma_b + \dots \Sigma_n}$$

where Σ_a = the sum of the peak heights of ions RCO_2H^+ , RCO_2H_2^+ and $[\text{R}' - 1]^+$ for component a, etc. The results obtained using this method are given in Table IV. Calculations based upon individual ions gave greater deviations from true values. For instance, the observed content of 18-10 (49.4%) in the mixture (18-10, 10-18) was calculated to be 22.9%, 65.6% and 52.3% when ions [R'-1]^+, RCO_2

 H_2^+ and RCO_2H^+ , respectively, were used as the single bases of calculation, whereas 52.1% was calculated (Table IV) using the formula. Differences in running conditions did not significantly influence the results either. The mixture (18-16, 16-18) was repeated one month later and the composition, 62.1% and 37.9%, was found to be consistent with the earlier finding, 62.8% and 37.2% (Table IV). Several more examples are given in Table IV proving the validity of the method. It must, however, be used with caution when one or several of the components of the mixture are present in very small quantities.

Wax esters Having Different Molecular Weights. The method outlined above was not applicable for wax esters having different numbers of carbon atoms. For instance, the four-component mixture (18-18, 18-16, 18-14, 18-12) gave 6.9%, 11.5%, 31.0% and 60.5%, respectively, whereas the gravimetric composition was 22.9%, 19.5%, 29.9% and 28.4%. The deviations are probably due to differences in volatility. Wax esters having different molecular weights are, however, readily separated by chromatographic (GLC) methods (21). Combined gas chromatography-mass spectrometry was applied on a synthetic mixture (18-18, 18-14, 14-18) with known composition. Using the method outlined above, the mass spectrum obtained for the GLC peak representing 18-14 and 14-18 gave the ratio 46.1:53.9 whereas the known composition was 43.9:56.1.

Unsaturated Wax Esters. Unsaturated wax esters can be separated by argentation chromatography into groups having different numbers of double bonds (22). Subsequent deuteration of the double bonds with tetradeuterio hydrazine followed by gas chromatography-mass spectrometry allows quantitative analysis of each group provided residual unreduced esters have been removed either by argentation chromatography (22) or by ozonolysis (10).

A synthetic mixture consisting of 18-18:1 and 18:1-18 was treated accordingly. When the contribution of ${}^{13}C$ and the presence of an [M-1]⁺ ion (2.2% of M⁺) in the spectrum of 18-18 were taken into account, the ratio of d₀, d₁ and d₂ in the wax esters were 3.3:17.8:78.8. The peak heights of the appropriate ions were corrected for isotopic impurities (${}^{13}C$, d₀ and d₁ wax esters) giving m/e 252 29.1 mm, m/e 254 22 mm, m/e 284 27 mm, m/e 285 113.2 mm, m/e 286 23 mm and m/e 287 91mm. Using the formula described above, the content of 18 d₂-18 was thus found to be 53.1% [100(22 + 27 + 113.2/305.3)]. The original mixture consisted of 18-18:1 and 18:1-18 in the ratio 46.8:53.2. Since all the ions used in the calculation are formed by loss or capture of one or two hydrogens, an isotope effect might interfere when certain double bonds in the chain are deuterated.

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