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♣Compositional Analysis of Natural Wax Ester Mixtures by Tandem Mass Spectrometry

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ABSTRACT

Tandem mass spectrometry is particularly suited for the analysis of complex, natural wax ester mixtures R_1-CO_2-R_2. Reduction of the mixture with deuterium provides species that are separable (through mass spectrometry) based on the number of original double bonds. Chemical ionization with isobutane produces high yields of protonated molecular ions and very little further fragmentation. These ions are separated by the first mass filter and then dissociated through collisions with argon. The positively charged dissociation products are almost exclusively the protonated acid ions $(R_1\text{-}CO_2H_2)^+$ that can then be separated by the second mass filter before detection and quantitation. The technique overcomes many of the obstacles previously faced during wax ester analysis. Results from this method are compared with those obtained by previous work, and the isomer composition of a new wax ester oil, orange roughy oil, is given.

INTRODUCTION

The analysis of naturally occurring wax ester mixtures for the relative abundances of isomers within each chain length is a somewhat formidable task. Since Aasen et al. showed that electron impact (EI) mass spectra could be used to quantitate saturated wax ester mixtures (1), mass spectrometry has become the method of choice of many workers (2-4). Complications encountered in these analyses include the difficulties associated with gas chromatographic separation of these relatively high molecular weight compounds and the problem of quantitatively introducing them into the mass spectrometer. Further, EI gave a great deal of nonspecific fragmentation with a relatively small percentage of the total ion current attributed to diagnostically important ions.

Recently, we showed that chemical ionization (CI) with isobutane gave spectra with intense protonated molecular ions and very little further fragmentation (5). Although this feature was not beneficial for structural information, it appeared to be particularly advantageous for mass spectrometry/mass spectrometry (MS/MS), because a high yield of ions representing the molecular species could be formed. When subsequent experiments showed that these ions could be dissociated to yield essentially one daughter ion per acyl radical (the protonated acid), a method was needed to identify and quantitate unsaturated isomers and analogs. Tris(triphenylphosphine)chlororhodium(I) catalyzes the reduction of double bonds with very little exchange between substrate and reagent (6), which results in saturates that include two atoms of deuterium per original double bond. Therefore, the protonated molecular ion and associated protonated acid ion from unsaturated compounds have m/z values 2 units greater per double bond and, although they behave chemically like their fully protonated counterparts, they are easily distinguished by mass spectrometry. Thus conditions were available to conduct the analysis in a single MS/MS experiment, because the entire wax ester mixture could be reduced with deuterium and the protonated molecular ions separated by the first mass filter. Following dissociation, the ions arising from the component acids could be analyzed in the second mass filter. In this paper we describe methods used to conduct such analyses and the results obtained from four natural wax ester mixtures.

EXPERIMENTAL

Purified wax ester standards were prepared from appropriate alcohols and acyl chlorides (2). Saponification (and recovery of unsaponifiables) was carried out essentially as prescribed by the AOCS (Method Ca 6b-53); the combined aqueous layers were then acidified and the free acids were

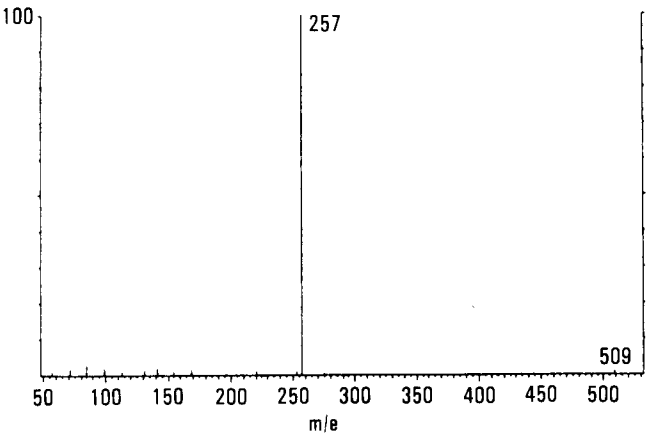

FIG. 1. Daughters of the protonated molecular ion from stearyl palmitate (18:0-16:0) produced by collision-induced dissociation with Ar; collision energy = −15 V.

TABLE I

Comparison of Isomer Compositions Obtained by Different Methods

Chain length: unsaturation	Alkoxy-acyl groups	Isomer composition (%)	
		Present work MS/MS	Sample (method, ref.)
			Sperm whale oil (EI/MS, 2)
	12-20	tr	4
	13-19	–	4
	14-18	8	6
	15-17	tr	1
32:0	16-16	64	63
	17-15	4	2
	18-14	20	19
	19-13	tr	1
	20-12	4	2
	14:0-20:1	4	4
	14:1-20:0	tr	–
	16:0-18:1	61	47
34:1	16:1-18:0	1	15
	18:0-16:1	6	9
	18:1-16:0	25	23
	20:0-14:1	tr	–
	20:1-14:0	2	2
	14:0-22:1	–	4
	14:1-22:0	2	6
	16:0-20:1	67	46
36:1	16:1-20:0	1	6
	18:0-18:1	16	17
	18:1-18:0	8	11
	20:0-16:1	1	5
	20:1-16:0	4	4
	22:0-14:1	tr	1
	22:1-14:0	1	–
	14:1-22:1	–	2
36:2	16:1-20:1	9	9
	18:1-18:1	86	87
	20:1-16:1	4	3
			Jojoba oil (HPLC-GC, 9)
	16:1-24:1	tr	1
	18:1-22:1	1	4
40:2	20:1-20:1	89	82
	22:1-18:1	10	12
	24:1-16:1	tr	1
	18:1-24:1	tr	2
42:2	20:1-22:1	25	21
	22:1-20:1	74	75
	24:1-18:1	1	2
			Spermaceti (HPLC-GC, 10)
	14:0-16:0	9	12
30:0	16:0-14:0	84	79
	18:0-12:0	7	9
32:0	14:0-18:0	1	3
	16:0-16:0	81	82
	18:0-14:0	16	15

recovered by extraction with diethyl ether. Free acids were methylated with diazomethane and alcohols were acetylated in pyridine/acetic anhydride (1:2). Analysis of the wax esters and their hydrolysis products by gas chromatography has been described previously (2).

The sample (5 mg) and internal standard, palmityl $[^2H]_6$-stearate (0.5 mg), were dissolved in ca. 2 mL of $CHCl_3$ in a 30-mL test tube equipped with a side-arm to which a small balloon was attached. Ca. 20 mg of tris-(triphenylphosphine)chlororhodium(I) catalyst (6) was added and the flask was stoppered. A three-way stopcock with one arm through the stopper, one arm to vent and one arm connected to a deuterium cylinder facilitated saturation of the atmosphere with deuterium. Flushing was accomplished by repeatedly inflating the balloon with deuterium and then exhausting through the vent. After 8-10 cycles, the system was assumed to be saturated. The reduction medium was vigorously stirred (with the balloon inflated) for 7-8 hr; by this time, some insolubles had formed. The solution volume was reduced to a minimum

TABLE II

Isomer Composition of the Major Wax Esters of Orange Roughy Oil

Chain length and percent[a]	Monoenoid alkoxy-acyl	(%)	Dienoid alkoxy-acyl	(%)
	18:1-14:0	4	18:2-14:0	9
32	18:0-14:1	6	18:1-14:1	14
Monoenoid = 6.1	16:1-16:0	1	16:1-16:1	65
Dienoid = 0.3	16:0-16:1	55	14:1-18:1	11
GC = 4.9	14:0-18:1	35		
	20:1-14:0	3	20:1-14:1	8
	20:0-14:1	tr	18:1-16:1	50
34	18:1-16:0	1	16:1-18:1	41
Monoenoid = 19	18:0-16:1	5	14:1-20:1	tr
Dienoid = 3.0	16:0-18:1	87		
GC = 18	14:0-20:1	3		
	22:1-14:0	2	22:1-14:1	1
	22:0-14:1	tr	20:1-16:1	23
36	20:1-16:0	28	18:1-18:1	72
Monoenoid = 15	20:0-16:1	1	16:1-20:1	4
Dienoid = 11	18:0-18:1	33		
GC = 22	16:0-20:1	33		
	14:0-22:1	2		
	24:1-14:0	2	24:1-14:1	tr
	22:1-16:0	5	22:1-16:1	9
38	22:0-16:1	2	20:1-18:1	74
Monoenoid = 3.4	20:0-18:1	17	18:1-20:1	15
Dienoid = 16	18:0-20:1	31	16:1-22:1	1
GC = 25	16:0-22:1	43		
40	24:1-16:0	4	24:1-16:1	2
Monoenoid = 1.0	24:0-16:1	2	22:1-18:1	58
Dienoid = 15	22:0-18:1	25	20:1-20:1	31
GC = 19	20:0-20:1	19	18:1-22:1	8
	18:0-22:1	50		
42	24:0-18:1	26	24:1-18:1	26
Monoenoid = 0.2	22:0-20:1	35	22:1-20:1	45
Dienoid = 8.0	20:0-22:1	38	20:1-22:1	28
GC = 9.1				
44	24:0-20:1	33	24:1-20:1	32
Monoenoid = 0.1	22:0-22:1	66	22:1-22:1	67
Dienoid = 2.6				
GC = 2.9				

[a]Total number of carbon atoms in alkoxyl and acyl moieties. Monoenoid and dienoid figures are calculated from MS/MS data; GC is proportion found by GC of intact oil.

(creating more insoluble matter) and this entire mass, together with the stirring bar, was extracted in a Butt-type apparatus with diethyl ether for 2 hr. Finally, the waxes were purified from this extract by TLC (0.25 mm layers of silica gel, hexane/ether 80:20 development solvent).

The Finnigan triple quadrupole model 4535/TSQ™ MS/MS/DS (mass spectrometer/mass spectrometer/data system) was operated in the CI mode with isobutane as the reagent gas at a source pressure of 0.25 torr and at a source temperature of 140 C. Samples (1-5 μg) were introduced through the direct insertion probe that was heated from ambient to 330 C over ca. 2 min. The collision gas was argon at ~1 m torr and the collision energy was −15 V.

RESULTS

The types of instruments and procedures available for MS/MS have been described in recent reviews (7,8). Our experiments were designed to acquire daughter spectra exemplified by Figure 1, the daughters of the protonated molecular ion (m/z 509) from stearyl palmitate (shorthand notation 18:0-16:0 [1]). Only one ion of consequence (m/z 257) representing the protonated acid group CH_3-$(CH_2)_{14}CO_2H_2^+$ is produced. Because the original molecular weight is known, this intense ion provides all of the information necessary to discern the original wax ester structure. However, increased fragmentation would be promoted by unsaturation or by methane as a reagent gas (5). In addition, fragmentation is influenced by the location(s) of double bond(s) in unsaturated compounds (5). These complications in the spectra would make this technique virtually unusable for natural products. We therefore needed a way to generate "saturated" waxes that still retained enough diagnostic information to ascertain their original degree of unsaturation. This kind of compound could be produced by reduction with deuterium if no exchange between substrate and reagent occurred. Tris-(triphenylphosphine)chlororhodium(I) has been shown to saturate double bonds effectively with a minimum of exchange (6) and proved to be an ideal catalyst to produce these "saturated" waxes. For example, the isomers 16:1-18:0, 16:0-18:1, 18:1-16:0 and 18:0-16:1 all have a protonated molecular ion at m/z 511 after reduction with deuterium, but their intense daughter ions are found at m/z 285(18:0), 287(18:1), 257(16:0) and 259(16:1), respectively.

To determine the extent of exchange during reduction, ion intensities from the reduction product of 16:1-18:1 were compared. The isotope at m/z 514 was 41% as large as the protonated molecular ion at m/z 513, which is very close to the 38% value calculated from natural isotopic abundance. Moreover, the intensity found for the isotope of the protonated acid ion (m/z 288) was 25% as large as that of m/z 287, compared to the theoretical value of 20%. These data indicate that the extent of exchange is minimal (6) and should not contribute serious errors to the analysis.

Data Acquisition/Reduction

The data system performs experiments through a program of procedural commands and is in complete control of the mass filters during data acquisition. Thus the first quadrapole (Q_1) was set so that only ions of a particular mass (protonated molecular ion) would be passed to the collision chamber Q_2. Here, these ions were dissociated by collisions with Ar (7) to form the daughters that were separated by the second mass filter Q_3. Because only one ion per isomer needed to be measured, it was unnecessary to take full scans with Q_3. Instead, this mass filter was sequentially set to pass to the detector only those daughter ions of interest. By conducting the experiments in this way, more precise measurements could be made at expected m/z values during the 1-1.5 min that ions were available for analysis than would have been possible if Q_3 had been scanned normally.

Because each natural wax ester mixture has a unique composition, the masses examined were different for each sample. GC analysis of intact and hydrolyzed samples provides the information necessary to select which parent and daughter ions need to be analyzed. A large number of combinations may be possible, but the time available for analysis is limited to the time that ions are being formed in the source. In addition to analysis time, time must be allotted to change and stabilize the mass filters at new m/z values before data can be taken. We found that examining each of 14 protonated molecular ions for 7 possible daughters provided a reliable set of data points during the time that ions were being produced. Attempting to take more data by increasing the number of parent or daughter ions scrutinized and increasing the rate of data acquisition decreased the accuracy of the measurements. Therefore, a simple mixture like spermaceti could be analyzed in one

TABLE III

Percentages of Major Wax Ester Components Obtained by Different Methods

Chain length: unsaturation	Sperm whale oil			Jojoba oil		Orange roughy oil	
	MS/MS	EI/MS (2)	Hydrolysis (13)	MS/MS	Hydrolysis (9)	MS/MS	Hydrolysis
Fatty acids							
12:0	4.1	4.3	2.0	–	–	–	–
14:0	7.0	5.1	6.2	–	–	1.5	1.4
14:1	7.6	8.6	4.8	–	–	0.7	0.4
16:0	6.7	5.5	11	–	–	4.6	1.5
16:1	20	20	17	–	–	11	13
18:0	1.5	3.1	1.6	–	–	–	–
18:1	25	28	27	12	11	56	45
20:1	16	11	11	73	71	18	21
22:1	6.3	6.0	4.6	14	14	7.4	9.0
24:1	–	–	–	1	1	3.1	1.3
Alcohols							
14:0	4.7	3.9	3.3	–	–	3.4	2.1
16:0	27	21	28	–	–	26	17
16:1	7.7	12	6	–	–	2.1	2.3
18:0	–	4.6	4.4	–	–	7.8	5.6
18:1	41	45	43	2	1.1	13	15
20:0	–	–	–	–	–	–	–
20:1	4.2	3.9	3.8	52	44	26	27
22:1	–	–	–	39	45	16	21
24:1	–	–	–	7	9	3.4	4.6

run, whereas a complex sample such as sperm whale oil required 5 experiments to gather data from the full range of possible combinations.

Reduction of the data can also be accomplished through procedural commands. Provisions are made to construct a smooth curve representing the ion current for each ion of interest. If sufficient current has been recorded to produce a "peak," its area can be integrated and stored. Reduced data, then, consisted of a series of areas for the daughter ions from each protonated molecular ion.

Calibration and Quantitation

To calibrate the technique, the responses of a range of standard wax esters were compared to the response of the internal standard, palmityl $^2H_6$-stearate [16:0-($^2H_6$-18:0)]. Mixtures of known compositions comprising 14:0, 16:0, 18:0, 20:0 and 22:0 alcohols were esterified to 14:0, 16:0 and 18:0 acyl groups. A converse group of standards with 14:0, 16:0, 18:0, 20:0 and 22:0 acyl groups esterified to 14:0, 16:0 and 18:0 alcohols was also prepared. Three different concentrations of each standard mixture were combined with an aliquot of the internal standard and analyzed. The area:area ratio of each protonated acid ion:internal standard acid ion ($^2H_6$-18:0) was compared to the weight:weight ratios of each parent wax:internal standard parent wax in order to determine a calibration curve. It was immediately obvious that a third set of variables, the chain lengths of the parent and daughter species, was also very important. Multiple regression curves with area:area/weight:weight and the chain lengths as coordinates gave parabolic functions of the form shown that reasonably fit the data (standard error = 5.40; R = 0.970).

$$\frac{\text{wt X}}{\text{wt IS}} \div \frac{\text{area X}}{\text{area IS}} = a - b\,(Ac) - c\,(Al) + d\,(Ac \cdot Al)$$

where X = isomer examined, IS = internal standard, Ac = chain length of acyl radical, and Al = chain length of alkoxy radical.

MS/MS of the unknowns provided the chain lengths of the radicals plus the areas of the protonated acid ions from the unknowns and the internal standard. The functions could then be solved for weight of unknowns. Now that the weight of each parent species was known, an overall wax ester composition and independent alkoxy and acyl compositions could be easily calculated.

Table I illustrates a comparison of the results obtained by MS/MS with those from previous work. Extensive tabulation would be necessary to compare complete information for all chain lengths, so only representative data are given. A complete table of the MS/MS results is available on request. Excellent agreement is evident for almost all of the isomer compositions. One group of components which are not in good agreement are those containing a 16:1 alkoxy group in sperm whale oil. As was pointed out previously (2), the EI spectrum of 16:0-18:1 has a moderately intense ion that coincides with the diagnostic ion for a 16:1 alkoxy moiety, so the discrepancy seen in the results for 34:1 is probably due (at least in part) to errors inherent in the EI technique.

In Table II, the isomer composition of orange roughy oil is given. This oil has recently been proposed as a replacement for sperm whale and jojoba oils (11) and indeed appears to have a composition somewhere in between these two in terms of chain lengths and degrees of unsaturation. The figures in the far left column give a comparison between composition calculated solely on the basis of MS/MS data and those obtained by GC of the intact oil. Agreement here is again quite good considering the dissimilarity between the two techniques. This orange roughy oil has a slightly different composition than that published by Buisson et al. (11) in that a larger proportion of shorter chain length compounds is present.

DISCUSSION

Physical separation of intact wax esters by chain length, followed by hydrolysis and analysis of the acids and alcohols, is only feasible for relatively simple mixtures such as jojoba oil or spermaceti. Even then, the process is tedious. Mass spectrometry in the EI mode or in the CI mode with methane as the reagent gas is a far more commodious

undertaking but also has some shortcomings. These include the formation of large numbers of nondiagnostic ions (some of which interfere with the measurement of critical ions), calibration of a system to determine quantitatively compounds with differing degrees of unsaturation and the GC separation and transfer of wax esters to a mass spectrometer. Although capillary columns greatly enhance the GC separation of analogs by degree of unsaturation, they still are not particularly efficient when large numbers of positional isomers (such as are found in sperm whal oil) are present (12). Additional precautions must be taken to minimize adsorptive losses and degradation on the way to the mass spectrometer (4). These obstacles are not encountered in MS/MS.

A critical test of the results obtained by any MS method is to calculate, from these results, the alcohol and acid composition of the overall wax ester mixture. The calculated composition can then be compared to that found by hydrolysis which we consider the most accurate. These types of comparisons are made in Table III. Apparently, either MS technique gives reasonable answers, but the specificity, simplicity and sensitivity of the MS/MS procedure make it very attractive. As instruments become more widespread and less expensive (7), MS/MS analysis of wax ester mixtures should become routine.

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Effect of Water Quality on Degumming and Stability of Soybean Oil

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ABSTRACT

Solvent-extracted crude soybean oil was degummed with deionized distilled water containing various amounts of $CaCO_3$-$MgCO_3$, $FeCl_2$, and NaCl. The total phosphorus content remaining in the degummed oil was determined and the peroxide value of the degummed oil held at 98-101 C was measured daily for 10 days. The results were compared statistically with those from oil degummed with deionized distilled water as a control. It was found that 250 mg/L of $CaCO_3$-$MgCO_3$ significantly reduced the efficiency of the degumming process. $FeCl_2$ at concentrations of 150 and 250 μg/L and NaCl at 300 mg/L resulted in the removal of more phosphorus than the control at the 5% level of significance. Generally, the stability of the degummed oils decreased as the salt concentrations increased. The rate of oxidation was greater for oils degummed in the presence of $FeCl_2$ than of NaCl and $CaCO_3$-$MgCO_3$ under the same conditions.

INTRODUCTION

Degumming is a process by which 1-3% water with or without degumming agents such as phosphoric acid is mixed with crude oil at 300-500 rpm for 30-60 min at 60-70 C to render fat-soluble impurities insoluble by hydration – these are then removed by centrifugation (1). Carr (2) reported that degumming removed 80-95% of the phosphorus present in soybean oil. Most of the hydratable phosphatides can be eliminated by degumming, but the nonohydratable phosphatides (Ca and Mg phosphatides) remain in the degummed oil. Letan and Yaron (3) reported that the presence of Ca and Mg in crude and degummed oil prevented complete elimination of phosphatides by hydration.

There is no available information on $FeCl_2$ or NaCl solutions as degumming agents for degumming vegetable oils. However, it has been reported that phosphatides have the ability to form combinations with salts of various heavy metals. Tompsett (4) indicated that Fe^{+3} but not Fe^{+2} salts formed complexes with phosphatides. According to Thurman (5), a 10% solution of NaCl aided the breaking of emulsions and he recommended that NaCl solutions be used for washing phosphatides or precipitates because of its solvent action.

The oxidation of lipids and oils and the decomposition of hydroperoxides are catalyzed by heavy metals, particularly those possessing two or more valency states (Fe^{+2} and Fe^{+3}) with a suitable oxidation-reduction between them (6). According to List et al. (7), $FeCl_2$ at concentrations of 0.1, 0.5 and 1.5 ppm significantly lowered the flavor stability of soybean oil. Alkali and alkali earth metals possess catalytic acitivity in oil oxidation due to induced hydroperoxide decomposition of free radicals (8).

There is an increased interest in the use of vegetable oil as a replacement for or extender of diesel fuel, and an interest in processing the oil on the farm. The water used for degumming can be a factor in the quality of the final

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