Characterization of Wax Esters in the Roe Oil of Amber Fish, *Seriola aureovittata*

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ABSTRACT: The wax esters of the roe oil of the amber fish, *Seriola aureovittata,* have been resolved by high-performance liquid chromatography (HPLC) in the silver-ion mode. Each of the fractions collected was transmethylated, and the fatty acids and alcohols were identified by gas chromatography/mass spectrometry (GC/MS) as the picolinyl esters and nicotinates, respectively. Their compositions were determined by GC. The fatty acid composition is complex, and the main components are C18:1n-9 (35.5 mol%), C22:6n-3 (20.3 mol%), and C16:1n-7 (10.7 mol%), while fatty alcohols are limited to saturated (C16:0, 60.3 mol%; C18:0, 15.3 mol%; C14:0, 5.1 mol%) and monoenoic alcohols (C18:1n-9, 6.5 mol%; C16:1n-7, 4.5 mol%) with traces (<0.1 mol%) of polyunsaturated fatty alcohols such as C20:3n-3, C20:4n-6, C20:5n-3, and C22:5n-3. Silver-ion HPLC exhibited excellent resolution in which fractions were resolved on the basis of the number and configuration of double bonds as well as the distribution pattern between the acid and alcohol moieties of the molecules with a given number of double bonds. The main wax ester fraction are those of monoenoic acid-saturated alcohol species, hexaenoic acid/saturated alcohol species, and pentaenoic acid/saturated alcohol species. Appreciable specificity was observed in the esterification of fatty acids with alcohols, and surprisingly, no saturated acid–monoenoic alcohol species were detected.
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KEY WORDS: Fatty alcohol, mass spectrometry, nicotinates, picolinyl esters, *Seriola aureovittata,* silver-ion HPLC, wax ester.

Reversed-phase high-performance liquid chromatography (RP-HPLC) with an octadecylsilanyl packing material has been widely used for the structural analysis of triacylglycerols (1), retinoyl fatty acyl esters (2–4), and steryl fatty acyl esters (5,6). Miwa *et al.* (7,8) have also separated the molecular species of jojoba wax esters with RP-HPLC in combination with gas chromatography/mass spectrometry (GC/MS). However, because the elution profile of components is determined by partition number, defined as the effective number of carbon atoms in all the acyl and alkyl moieties less twice the number of double bonds in a molecule, the elution order of components is not easy to interpret, especially with highly unsaturated samples.

Silver-ion chromatography has been used for the separation of molecular species of wax esters by the degree of unsaturation, in conjunction with thin-layer chromatography (TLC) (9–12) or column chromatography (13). Recently, a method, involving HPLC with a cation exchange column loaded with silver ions, has been developed and applied to fractionate fatty acids esters (14–18) and triacylglycerols from seed oils (19–22) and fish oils (23,24) with high resolution. This method is simple, rapid and gives clean fractions without contamination by silver ions.

In this work, silver-ion HPLC has been used to resolve the wax esters from the oil of the roe of the amber fish, *Seriola aureovittata,* for the first time, according to degree of unsaturation of the molecular species. It is known that large amounts of highly unsaturated wax esters are present in the roe (25). Amber fish is regarded as a delicacy in the Far East and is reared by aquaculture on a commercial scale.

EXPERIMENTAL PROCEDURES

Sample and reagents. Two ovaries filled with mature roe of amber fish were collected at a fish market in Pusan, Korea. The oil was extracted from the lyophilized roe with chloroform/methanol (2:1, vol/vol). Wax esters were isolated from the oil by silica gel column chromatography by elution with hexane/diethyl ether (97:3, vol/vol). All solvents and reagents were of Analar (BDH Ltd., Poole, United Kingdom) or HPLC grade and were supplied by FSA Scientific (Loughborough, United Kingdom).

HPLC. The HPLC instrument used in this study was comprised of a Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics Ltd., St. Albans, United Kingdom), a Cunow Model DDL 21 light-scattering detector (Cunow SA, Cergy St. Christophe, France), and a Spectra-Physics SP 4290 integrator. A manual stream-splitter was set up between the column and the detector. The silver-ion column was prepared by loading a column of Nucleosil™ 5SA (250 × 4.6 i.d.; HPLC Technology, Macclesfield, United Kingdom) with silver ions as described previously (26). For analysis of the wax

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esters by silver-ion HPLC, a gradient system of three different solvents was used: (A) 1,2-dichloroethane/dichloromethane (1:1, vol/vol); (B) acetone; (C) acetone/acetonitrile (2:1, vol/vol). The column was eluted with (A) isocratically over 3 min, then with a linear gradient of 100% (A) to 70% (A)-30% (B) over 25 min, and to 90% (B)-10% (C) over 50 min. The elution continued for 10 min more with the final solvent system. The column was kept at ambient temperature, and the flow-rate of the mobile phase was 1.0 mL/min. The sample (1–1.3 mg) in dichloromethane (10–25 mL) was injected onto the column. An internal standard of methyl nonadecanoate in hexane (1.2 mg/mL; 10 mL aliquot) and 100 mL of 0.02% butylhydroxytoluene in hexane were added to each of the fractions.

Transesterification of wax esters and isolation of fatty acid methyl esters (FAME) and fatty alcohols (FAL). A portion (0.5–1 mg) of the total and each of the wax ester fractions collected from the HPLC runs were dissolved in toluene (0.5 mL) for transesterification with 2% sulfuric acid/methanol (2 mL) at 60°C for 36 h (27).

The FAME and the FAL fractions were separated on a Bond Elut™ NH_2 solid-phase extraction column (Analytichem International, Harbour, CA). The transesterified mixture in hexane/diethyl ether (1:1, vol/vol) was dried under a stream of nitrogen. It was dissolved in hexane and loaded onto the column, which was prewashed with 3 mL of hexane. The column was eluted with 10 mL of hexane/methyl-*tert*-butyl ether (M*t*BE) (98:2, vol/vol) to recover the methyl esters, followed with 5 mL of hexane/M*t*BE (90:10, vol/vol). Finally, the column was washed with 10 mL of hexane/M*t*BE (75:25, vol/vol) to elute the FAL. Each of the fractions collected was evaporated under nitrogen and was checked by GC.

GC analysis. GC analyses of FAME and FAL were performed on a Carlo Erba Model 4130 capillary gas chromatograph (Erba Science, Swindon, United Kingdom), equipped with a split/splitless injector and a fused-silica capillary column (25 m × 0.22 mm i.d., film thickness 0.2 mm) coated with Carbowax 20M (Chrompack UK Ltd., London, United Kingdom). Hydrogen was used as the carrier gas. The column temperature was held at 175°C for 3 min, then programmed to 205°C at a rate of 4°C/min (3°C/min for FAL), and kept at the final temperature for 25 min.

Preparation of fatty acid picolinyl esters. Picolinyl esters were prepared essentially according to the method of Balazy and Nies (28). The picolinyl esters were purified from a small column of Florisil™ (3 cm) by elution with hexane/acetone (8:2, vol/vol; 8 mL); the excess solvent was evaporated in a stream of nitrogen before GC analysis.

Preparation of FAL nicotinates. Nicotinates were prepared by reaction of the FAL with nicotinyl chloride in the presence of pyridine (29). The FAL nicotinates were purified by a small Florisil™ column with hexane/acetone (9:1, vol/vol; 7 mL) and were concentrated under nitrogen.

GC/MS. The GC/MS instrument, operating condition and data acquisition/reduction were essentially the same as described previously (14), i.e., a Hewlett-Packard GC/mass spectrometer (Model 5890A) fitted with a 5970 Series mass selective detector (Hewlett-Packard Ltd., Wokingham, Berks, United Kingdom). A fused-silica capillary column (25 m × 0.2 mm i.d.), coated with a cross-linked (5% phenyl methyl) silicone (Hewlett-Packard Ltd.), was connected directly to the ion source. The column temperature was programmed from 60 to 220°C at 50°C/min and then to 270°C at 1°C/min. The mass spectrometer was operated at an ionization energy of 70 eV, and the carrier gas was helium.

RESULTS AND DISCUSSION

In this work, the molecular species profile of wax esters from the roe oil of the amber fish was determined. Wax esters are present only in the roe oil (41.8 wt% of total oil), but were not encountered in the lipids from the other tissues (muscle and intestines) of the specimen from which the roe had been taken. A portion of the intact wax esters was transesterified, and FAME and FAL fractions were cleanly separated from the reaction products on a Bond Elut™ NH_2 solid-phase extraction column.

The structures of FAL were determined by GC/MS of the picolinyl and nicotinate derivatives, respectively, which is a powerful technique for identification of unknown components in a complicated mixture from marine organisms (14,18). These derivatives induce radical cleavages of the chain without double-bond migration when electrons impact on the molecule in a mass spectrometer (30–32). The picolinyl ester of polyunsaturated fatty acids give complex spectra, but a gap of 40 a.m.u. between major peaks usually provides a clue to interpret the structure as shown in Figure 1 (30). In the spectrum of 8,11,14,17-eicosatetraenoic acid (C20:4n-3), the molecular ion is at m/z = 395, and typical ions to locate the position of the double bonds are m/z = 220, 246, 286, 326, 340, and 366 (gaps of 26, 40, 40, 14, and 26 amu).

Similarly, in the fragmentation pattern of 7,10,13,16,19-docosapentaenoic alcohol (C22:5n-3) (Fig. 2), ion peak m/z = 421 represents the molecular ion, and ions diagnostic for location of the double bonds are at m/z = 206, 246, 272, 286, 312, 326, 352, and 392. The gap of 40 amu between m/z = 352 and 392 is diagnostic for the terminal double bond of the alkenyl chain.

The fatty acid composition is in the range expected for fish oils (33). The fatty acid profile (Table 1) is characterized by high levels of monoenoic acids (52.5 mol%), such as C18:1n-9 (35.5 mol%) and C16:1n-7 (10.7 mol%), and polyunsaturated acids (40.3 mol%), mainly composed of C22:6n-3 (20.3 mol%) and C20:5n-3 (8.5 mol%). In particular, the proportion of C22:6n-3 acid in the wax esters of amber fish roe is much higher than has been reported for marine wax esters (34–36) and gourmi wax esters (37). On the other hand, the FAL components are strictly limited (9,34,38), i.e., C16:0, C18:0, and C14:0 alcohols occupy over 86 mol% of the total, followed by monoenoic alcohols (13.1 mol%), such as C18:1n-9 and C16:1n-7, with a small amount of branched and dienoic alcohols. Polyunsaturated FAL of C20:3n-3,

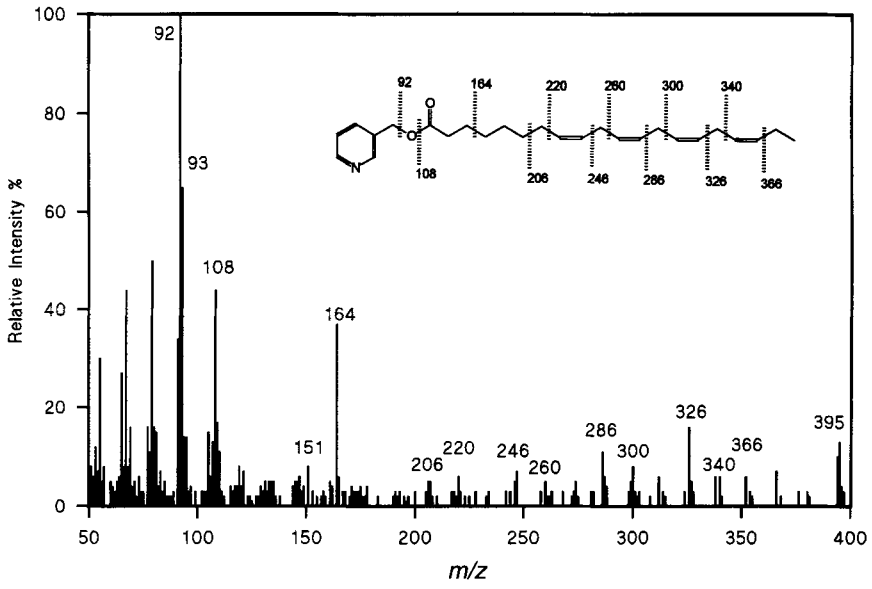

FIG. 1. The mass spectrum of the picolinyl ester of 8,11,14,17-eicosatetraenoic acid.

C20:4n-6, C20:5n-3, and C22:5n-3 (less than 0.1 mol% for each component) were identified by GC/MS for the first time, although these FAL have been identified in the wax esters of marine organisms (39,40) by their relative retention times and of the roe of fresh-water gourmis (37) by a degradative method.

The distribution of alcohol chainlengths is highly different from that of acid chainlengths, in spite of a close resemblance of double-bond position between the corresponding fatty acids and alcohols. This may suggest that the alcohols of wax esters arise from the acids by biological reduction of their carboxyl groups, in which saturated and monoenoic acids have preferential selectivities, as suggested previously (31,34).

When a silver-ion column was used for analysis of the wax esters with the most suitable elution scheme to achieve maximum resolution, the sample was resolved into 16 fractions, which could be categorized as eight groups according to degree of unsaturation: saturated, monoene, diene, triene, tetraene, pentaene, hexaene, and heptaene groups in the order as represented in Figure 3. The resolution in the chromatogram is excellent, even though some fractions—usually positional isomers of species with a given number of double bonds in the polyunsaturated region—overlapped slightly. Each fraction was collected and transesterified for determination of the compositions of fatty acids and alcohols. The data are presented in Table 1. The abbreviations S, M, D, T, Te, P,

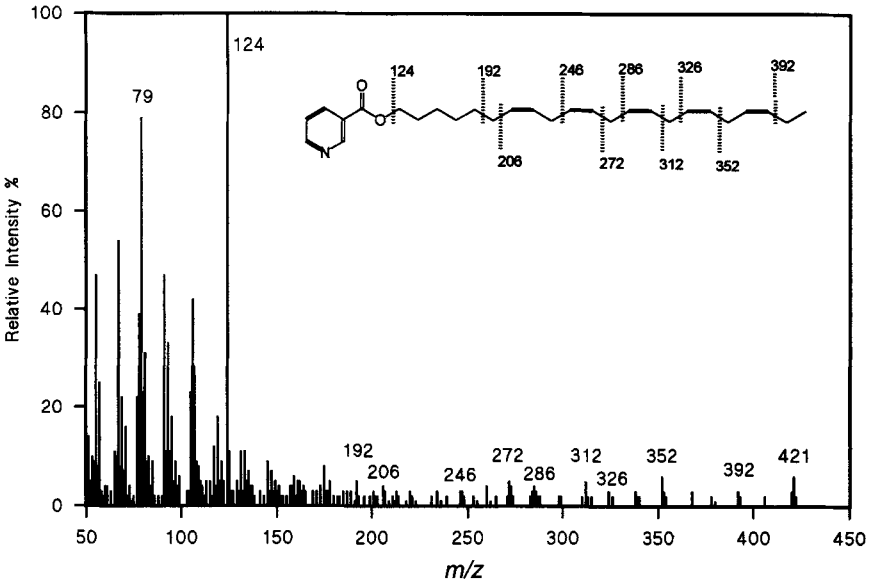

FIG. 2. The mass spectrum of the nicotinyl ester of 7,10,13,16,19-docosapentaenoic alcohol.

TABLE 1
Fatty Acid and Alcohol Compositions (mol% of the total), and Proportions of Wax Ester Fractions Obtained by Silver-Ion High-Performance Liquid Chromatography, from the Roe Oil of Amber Fish, *Seriola aureovittata*[a]

	Total																	
	ac	al	Fr. 1		Fr. 2		Fr. 3		Fr. 4	Fr. 5		Fr. 6		Fr. 7	Fr. 8		Fr. 9	
14:0	1.0	5.1	12.9	3.8	15.9	5.0	0.5	4.1						5.7				3.1
15:0	0.3	1.5	1.7	1.5	2.8	2.2		1.6						1.9				1.4
16:0	4.3	60.3	64.6	66.7	29.7	65.4	1.5	71.7	10.4	11.7	46.5	15.8	26.9	68.2			15.2	64.9
16:1n-7	10.7	4.5			29.2		16.7								3.6	16.5		
16:2n-4	0.4													10.0	6.4			
16:3n-3	0.2																4.3	
15-Me16:0		0.8		1.7		4.6		0.9			9.0			4.1				4.4
14-Me16:0		0.4						0.3			4.5			1.7				
17:0	0.1	1.7	2.4	2.1		1.7		2.1										1.7
17:1n-8	1.4	0.4					2.4		2.3	3.2	5.4	2.6	2.7					
18:0	1.1	15.3	18.4	22.8		19.6		18.5			8.6			17.8			3.5	23.2
18:1n-9	35.5	6.5			17.0		72.3		74.4	76.6	23.3	74.1	56.8		23.6	46.7		
18:1n-7	3.2	0.9					5.6		8.9	6.2	1.1	6.3	8.0			6.5		
18:2n-6	2.4	0.1												75.8	57.0	8.6		
18:2n-4	0.3	0.1												9.8	5.0	10.2		
18:3n-6	0.3																12.1	
18:3n-3	1.2																49.0	
18:4n-3	1.0																	
19:0		0.3																
20:0		1.0		1.3		1.5		0.8						0.6				1.3
20:1n-9	0.5	0.5					0.8		2.6	0.9	1.6	0.7	3.1			3.2		
20:1n-7	0.1	0.1					0.2		1.3	1.2		0.5	0.9					
20:2n-9	trace	0.3														5.6		
20:2n-6	0.1	trace												4.4	4.4	2.7		
20:3n-6	0.2																9.5	
20:3n-3	0.1	trace															6.4	
20:4n-6	2.7	trace																
20:4n-3	0.6																	
20:5n-3	8.7	trace																
21:5n-3	0.3																	
22:1n-11		0.2											1.6					
22:4n-6	0.2																	
22:5n-6	0.5																	
22:5n-3	2.2	trace																
22:6n-3	20.3																	
Amount (mol%)			5.3		0.8		37.7		2.7	1.7		5.8		2.2	0.4		1.9	

(continued on next page)

and H stand for saturated, monoene, diene, triene, tetraene, pentaene, and hexaene residues, respectively, of fatty acids or alcohols, and short-hand formulae for wax ester species have been used, i.e., dienoic acid/saturated alcohol is denoted as D/S, listing the acid moieties first. To check on the recoveries, the fatty acid composition of the whole was reconstituted from the relative proportions in each of the fractions. The composition obtained in this way is in good agreement with that of the intact wax esters.

The saturated group [Fraction (Fr.) 1] has species in which the C16:0 acid and C16:0 alcohol predominate, followed by C18:0 acid and C18:0 alcohol. The main difference in distribution of chainlength in this fraction is a greater abundance of shorter fatty acids and a higher proportion of longer-chain FAL.

Between the saturated and the monoene fraction, a small fraction (two peaks) (Fr. 2) contained fatty acids with the same proportion of saturated-to-monoenoic fatty acids and exclusively saturated alcohols, indicating that the components might be a equimolar mixture of sterol esters with saturated fatty acids, and monoene wax esters of *trans*-monoenoic acids and saturated alcohols. In this work, the species in both peaks could not be applied to a further analysis of their acid and sterol moieties because there were insufficient amounts. In this sample, sterol esters were found in other fractions that eluted later (perhaps 5% in total) and interfered with resolutions of wax ester species; preliminary separation of these two groups is advisable but is technically difficult.

Fraction 3 contains M/S species (the highest proportion of the total) which consist only of monoenoic acids (C18:1n-9, C16:1n-7) and saturated alcohols (C16:0 and C18:0). The molecular species of C18:1n-9/C16:0 is the most abundant in this sample, as reported in the wax esters of gourmi roe (37). No species of saturated acid/monoenoic alcohol (S/M species) are present.

TABLE 1 *(continued)*

	Fr. 10	Fr. 11	Fr. 12	Fr. 13	Fr. 14	Fr. 15	Fr. 16	Recombinant composition[b]
14:0	2.8	4.2	1.2	2.9 11.7	3.5	12.5		1.0
15:0	1.3	1.3	1.1	1.9	1.4	2.1		0.1
16:0	61.4	61.7	63.6	29.0 59.4	64.8	11.2		5.5
16:1n-7	0.8	2.5	0.5	2.3	1.1	24.1	25.8	8.0
16:2n-4								0.3
16:3n-3								0.1
15-Me16:0	1.5	0.7	1.1	1.1	0.9	3.7		
14-Me16:0	2.0	0.3	0.3		0.3	3.0		
17:0		1.8	2.2	1.6	2.0	4.5		0.1
17:1n-8		0.2			0.1	1.6	2.9	1.3
18:0	26.2	17.5	26.8	1.5 20.0	19.2	13.9		1.1
18:1n-9	2.4	6.7	1.2	1.3	4.2	6.0	62.4	35.6
18:1n-7	0.7	1.0			0.6	1.2	8.9	2.9
18:2n-6								2.0
18:2n-4								0.2
18:3n-6								0.2
18:3n-3	4.5							1.0
18:4n-3		30.5						1.0
19:0		0.4	0.4	0.3	0.3			
20:0	0.9	1.1	1.6	0.4	1.0			
20:1n-9		0.5			0.3	7.6		0.5
20:1n-7		0.1			0.1	2.3		0.2
20:2n-9								
20:2n-6								0.1
20:3n-6								0.2
20:3n-3								0.1
20:4n-6	95.5	18.3						2.9
20:4n-3		23.4	1.3					0.8
20:5n-3		3.4	96.9	20.4	5.8			9.4
21:5n-3				5.4	0.2			0.3
22:1n-11					0.2	6.3		
22:4n-6		6.0						0.2
22:5n-6		18.4	1.8	0.9	0.1			0.8
22:5n-3				39.9	1.2	2.7		2.5
22:6n-3					92.7	97.3	100	21.7
Amount (mol%)	2.4	3.2	7.3	5.4	19.0	2.3	1.9	

[a]Abbreviations: Fr., fraction; ac, acid; al, alcohol; trace <0.1 mol%.
[b]Calculated by summation of the fractions and based on the fatty acid components only. Note that some fractions may contain a small amount of sterol esters and Fr. 13 contains vitamin A palmitate.

The components in the minor Fr. 4 are assumed to be sterol monoenoic fatty acid esters because no alcohol residues from this fraction were detected by GC.

Three minor peaks (Fr. 5) that eluted between Fr. 4 and Fr. 6 are regarded as a mixture of diene wax esters (M/M species) bearing *trans*-double bond(s) in the molecules of fatty acid and/or alcohol.

Fr. 6 contains wax ester species composed of monoenoic acids and monoenoic alcohols (M/M species) and has a number of shoulder peaks differing in the position of double bonds and the combined chainlength of the species. It was not possible to separate each of the peaks in its pure form without contamination with neighboring components, but the first main peak is considered to include mainly C18:1n-9/C18:1n-9 species; the second is probably a mixture of C18:1n-9/C16:1n-7 and C16:1n-7/C18:1n-9. The shoulder peak, eluted just before the first main peak, is regarded as C20:1n-9/C22:1n-11 species, and the last one will correspond to C16:1n-7/C16:1n-7. Fr. 7 also has diene ester species in a combination of dienoic acids such as C18:2n-6, C16:2n-4, C18:2n-4, and C20:2n-6, with saturated alcohols, C16:0 and C18:0. Thus, with silver-ion HPLC, an excellent separation of dienoic wax ester species from each other was achieved, a feat that could not be accomplished by RP-HPLC (1,7,8).

The elution pattern of a dienoic species is easily understood from the fact that the retention time of an eluant depends not only on the strength of the interaction between the silver ions and the *pi*-electrons of double bonds, but also on the distance between the double bonds in a molecule from the carbonyl moiety (19,38).

Similar features can be observed in the triene fraction. Fraction 8 is a small peak in which D/M species coeluted with

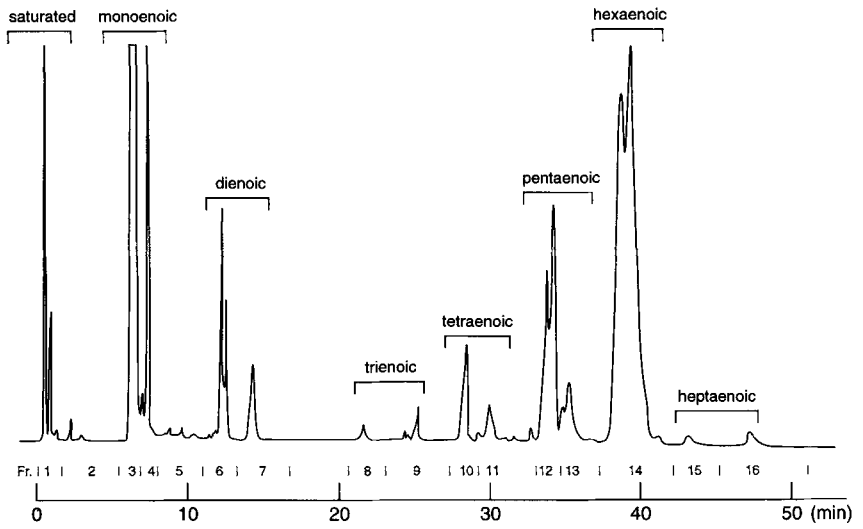

FIG. 3. Fractionation of the wax esters from the roe oil of *Seriola aureovittata* by high-performance liquid chromatography with a silver-ion column and evaporative light-scattering detection. The chromatographic conditions are described in the Experimental Procedures section. Fraction (Fr.) numbers are the same as in Table 1.

M/D species. A resolution of the critical pairs (D/M vs. M/D) was not achieved, but it is of interest that dienoic alcohols in the sample have been esterified with monoenoic acids in a nonrandom fashion.

Fr. 9 is also a triene fraction, consisting of trienoic acids and saturated alcohols (T/S species) and divided into three peaks. The two small peaks, eluted first, are believed to be positional isomers n-6 of T/S species, and the remaining peak is composed of n-3 trienoic acids and saturated alcohols.

The tetraene group was divided into two subfractions. The first one (Fr. 10) is chiefly occupied by Te(n-6)/S species in a combination of C20:4n-6 acid with saturated alcohols, accompanied by a small amount of Tn-3/M esters which are comprised of C18:3n-3 acid and monoenoic alcohols C18:1n-9, C16:1n-7, and C18:1n-7. The other (Fr. 11) is broad, and it was impossible to collect individual small peaks as was intended. Presumably, the first fraction is a mixture of Te(n-6)/S and T(n-3)/M species, and the main one contains P(n-6)/S species.

Two pentaene wax ester peaks emerged. The first (Fr. 12) contains C20:5n-3 predominantly, with traces of C20:4n-3 and C22:5n-6 in the acid composition, and saturated alcohols with small amounts of monoenoic alcohols. So, this fraction is composed mainly of C20:5n-3 acid/saturated alcohols [P(n-3)/S species]. The second peak (Fr. 13) contains mainly C22:5n-3, C20:5n-3, and C21:5n-3 acids, and saturated alcohols. The molecular species with C20:5n-3 acid are well resolved from those containing C22:5n-3 and C21:5n-3 acids, even though the latter two components are not completely separated (it is the position of the first double bond that governs the separation, not that of the terminal one). This separation order has been observed in the analysis of methyl esters of fatty acid derivatives from marine oils (18). This fraction probably contains some vitamin A palmitate as a contaminant.

The hexaene fraction (Fr. 14) has H(n-3)/S species, mainly composed of C22:6n-3 acid/saturated alcohols, with small portions of P(n-3)/M species, such as C20:5n-3/M, C21:5n-3/M and C22:5n-3/M.

Fr. 15 seems to be H(n-3)/M species, in which C22:6n-3 acid is esterified with *trans*-monoenoic alcohols, and the last fraction (Fr. 16) contains H(n-3)/M species of exclusively C22:6n-3 acid and monoenoic alcohols.

The methodology used in this work is extremely efficient, provided detailed information to enable complete structural elucidation of many different fatty acids and fatty alcohols in the marine wax esters, and gave good resolution of the sample into its molecular species. Every component of the fatty acids and alcohols occurring in the wax esters at a level of more than 0.05 mol% was identified. Some of the FAL do not appear to have been found previously in nature.

Silver-ion HPLC can be utilized to resolve complicated marine wax esters into many molecular species according to degree of unsaturation. The chromatogram is highly reproducible, even though some critical pairs that differed just in the position of the double bonds slightly overlapped with one another. The fractions were clean, but some of them were inevitably contaminated with sterol esters and conceivably glyceryl ether diesters, leading to a few difficulties in interpretation.

Taking account of the complicated constituents of the marine wax esters and several variables involved in the order of elution from HPLC, it is difficult to isolate single components by this method alone. We recommend combining silver-ion HPLC with another approach, i.e., RP-HPLC and/or MS. For example, when silver-ion HPLC is utilized with RP-HPLC in a complementary fashion, molecules fractionated from silver-ion HPLC by degree of unsaturation can be separated subsequently into species according to total chainlength.

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