

## Rapid Liquid Chromatographic Method to Distinguish Wild Salmon from Aquacultured Salmon Fed Synthetic Astaxanthin

SALEH A. TURUJMAN, WAYNE G. WAMER, RONG RONG WEI, and RICHARD H. ALBERT

U.S. Food and Drug Administration, Office of Cosmetics and Colors, 200 C St, SW, Washington, DC 20204

**Analytical methods are needed to determine the presence of color additives in fish. We report a liquid chromatographic (LC) method developed to identify the synthetic form of the color additive astaxanthin in salmon, based on differences in the relative ratios of the configurational isomers of astaxanthin. The distributions of configurational isomers of astaxanthin in the flesh of wild Atlantic and wild Pacific salmon are similar, but significantly different from that in aquacultured salmon. Astaxanthin is extracted from the flesh of salmon, passed through a silica gel Sep-Pak cartridge, and analyzed directly by LC on a Pirkle covalent L-leucine column. No derivatization of the astaxanthin is required—an important advantage of our approach, which is a modification of our previously described method. This method can be used to distinguish between aquacultured and wild salmon. The method has general applicability and can also be used to identify astaxanthins derived from other sources such as *Phaffia* yeast and *Haematococcus pluvialis* algae.**

The oxycarotenoid astaxanthin is responsible for the distinctive color of salmon flesh (1). Because salmon cannot synthesize astaxanthin de novo, their flesh color is derived entirely from astaxanthin in their diet (2). Wild salmon acquire their pink-to-red color from astaxanthin in their prey. To obtain a flesh color similar to that of wild salmon, aquacultured salmon are fed with fish feed supplemented with color additives (3). Two oxycarotenoids are believed to be widely used as color additives in fish feed to enhance the color of aquacultured salmonids: canthaxanthin,  $\beta,\beta$ -carotene-4,4'-dione (Figure 1), and astaxanthin, 3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione (Figure 2).

Only color additives are listed in the *Code of Federal Regulations* (CFR) may be used legally in the United States to enhance the color of salmon and other animals used as food (4). Astaxanthin recently was listed by the U.S. Food and Drug Administration (FDA) as a color additive in salmonid feed to pigment the flesh of salmonids in the United States (5). Because a

validated analytical method was unavailable to distinguish synthetic astaxanthin in aquacultured salmon from astaxanthin in wild salmon, a method was needed to determine the presence of added synthetic astaxanthin in the fish, as required by the CFR. Canthaxanthin is listed in the CFR as a food color additive (6). A petition has been submitted for its use as a color additive to color the flesh of salmonids (7). Astaxanthin, however, and not canthaxanthin, is normally found in wild salmon (Atlantic salmon, *Salmo salar*, and Pacific salmon, *Oncorhynchus*). Canthaxanthin can be distinguished easily from astaxanthin by thin-layer chromatography (TLC; 8) and liquid chromatography (LC; 9).

All-*trans* astaxanthin is the major geometric isomer in wild salmon flesh (10) and also in the stabilized synthetic astaxanthin beadlet added to the fish feed of aquacultured salmon. All-*trans* astaxanthin has 2 chiral centers, C-3 and C-3', and can exist as 3 configurational isomers: 2 enantiomers (3*R*,3'*R* and 3*S*,3'*S*) and a meso form (3*R*,3'*S*) (Figure 2). Synthetic all-*trans* astaxanthin consists of a racemic mixture of the 2 enantiomers and the meso form.

Studies with rainbow trout (*Oncorhynchus mykiss*; 11) and Atlantic salmon (12) have shown that when synthetic astaxanthin or the individual configurational isomers are added to fish feed, they are deposited in the flesh of the salmon with no change in the configurational isomer distribution. These results indicate the absence of selective absorption or deposition of the different configurational isomers and of epimerization at C-3 and C-3'. Therefore, the ratio of configurational isomers in salmon flesh reflects the configurational isomer distribution in the diet.

Maoka et al. (13) resolved all-*trans* astaxanthin on a covalent D-phenylglycine Pirkle-type column manufactured in Japan; however, the analysis required 70 min. Astaxanthin can also be derivatized with enantiomerically pure chiral reagents, such as camphanic acid chloride, to give diastereomers that can be separated on an achiral stationary phase (14). In our laboratory, other derivatizing reagents, such as 1-naphthoyl chloride, that enhance the affinity of the configurational analyte for the chiral stationary phase without changing the enantiomeric relationship of the configurational astaxanthin isomers to each other, were also used successfully.

Although in some cases the derivatization of synthetic astaxanthin was spontaneous, avoidance of the extra step of making and purifying an astaxanthin derivative was deemed advan-

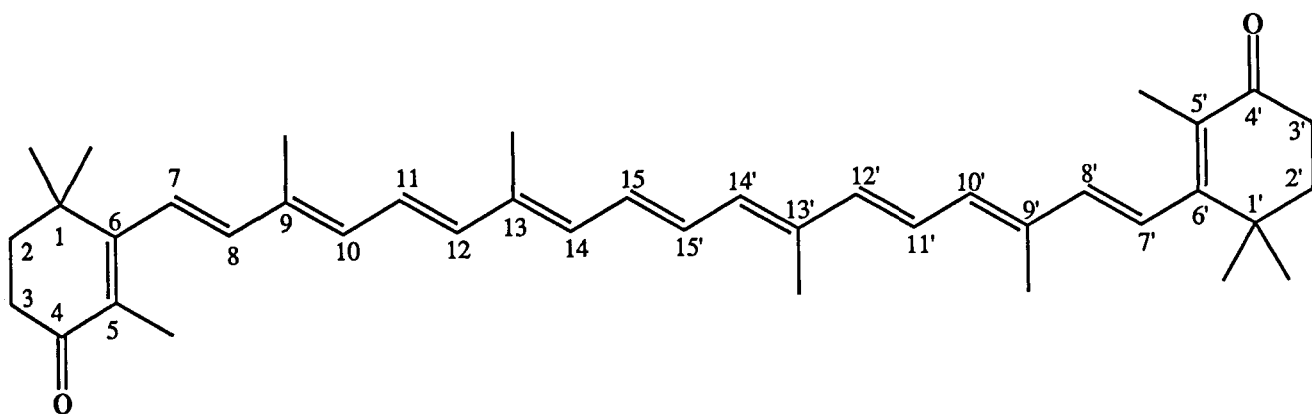


Figure 1. Canthaxanthin.

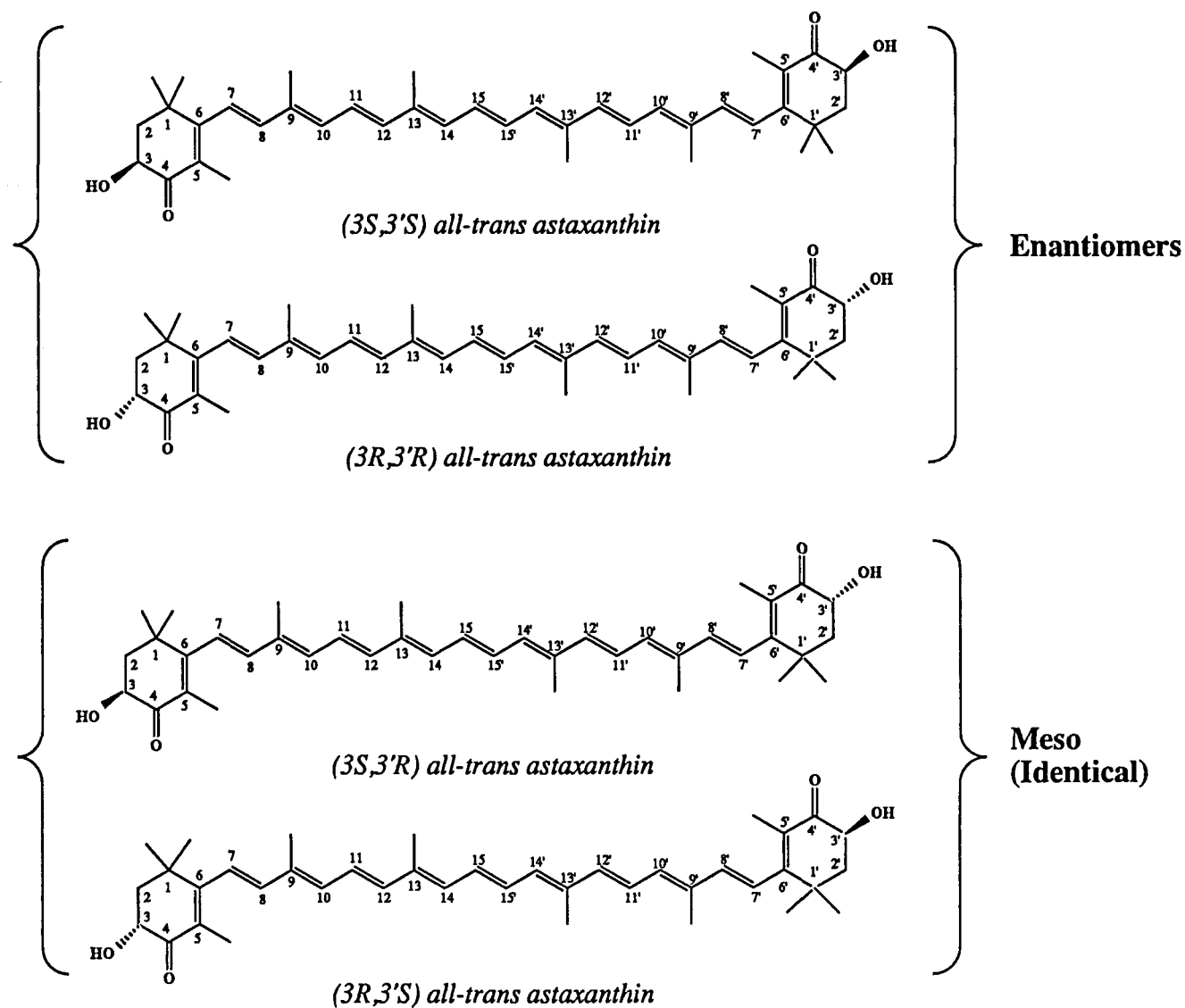
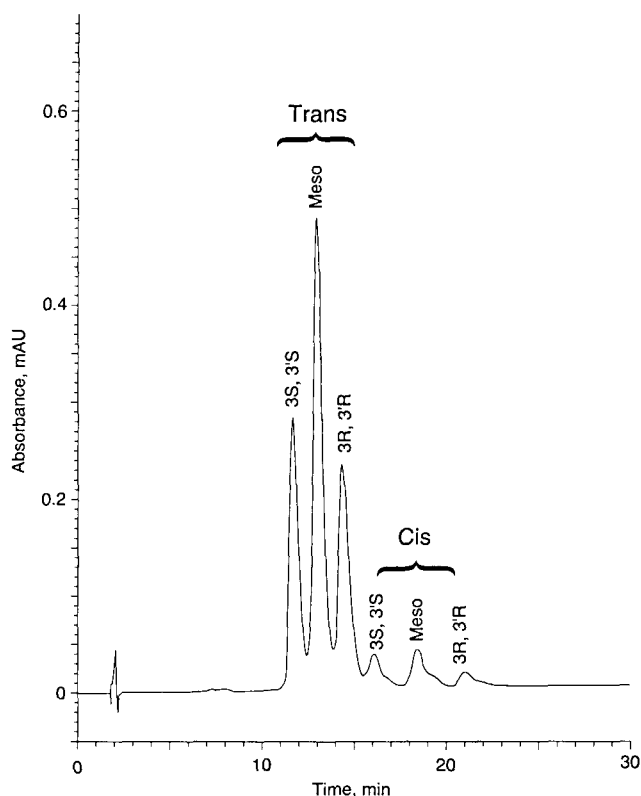


Figure 2. Configurational isomers of all-trans astaxanthin.

tageous. Moreover, when the astaxanthin extracted from the flesh of salmon was used, residual fish oil frequently interfered with and sometimes inhibited the derivatization reaction.

This paper describes an LC method based on direct resolution of the configurational isomers of underivatized astaxanthin (15). We previously described an LC method for efficiently separating and identifying the configurational isomers of synthetic astaxanthin in salmon (16). The present method is faster than the LC method of Lura and Saegrov (17), in which astaxanthin is derivatized before LC analysis. It entirely avoids the derivatization step in which a residual amount of colorless lipids may interfere and, therefore, must be removed before derivatization (10). Previously, we reported that mobile phases of similar polarity allowed direct resolution of the configurational isomers of all-*trans* astaxanthin on a covalent L-leucine Pirkle column in 10–15 min (15). However, when a significant amount of *cis*-astaxanthin was present, analysis time was sometimes as long as 25 min (Figure 3).

With the modified method described here, we can distinguish between synthetic astaxanthin extracted from the flesh of salmon and naturally occurring astaxanthin extracted from the flesh of wild salmon by comparing their chromatographic profiles. During the method development phase of this study, the color extracts from the flesh of salmon and the synthetic astaxanthin standard were chromatographed by using mobile phase A described in the **Experimental** section.



**Figure 3.** Chromatogram of synthetic astaxanthin. LC conditions: Pirkle covalent L-leucine column; mobile phase B; flow rate, 1.5 mL/min; monitoring wavelength, 474 nm.

To devise a regulatory scheme to identify the color additive astaxanthin in salmon flesh and thereby distinguish between aquacultured salmon and wild marine salmon, it is necessary to know the ratio of the configurational isomers of the all-*trans* astaxanthin in each species of salmon from a broad-based set of authentic wild marine Atlantic and Pacific salmon. A range of each of the configurational isomers in astaxanthin extracted from wild salmon was determined by Schiedt et al. (10): 78–85% of the (3*S*,3'*S*) enantiomer, 12–17% of the (3*R*,3'*R*) enantiomer, and 2–6% of the (3*R*,3'*S*) meso form. This pioneering work, however, was based on 4 Atlantic salmon and 1 salmon from each of 3 Pacific species. The data set was too narrow to be generalized to the larger population of wild salmon.

We initiated a study to determine the configurational isomer distribution that could be generalized to wild marine salmon (*Salmo salar* and *Oncorhynchus*). Such a distribution would form the reference standard with which the distribution of configurational isomers of astaxanthin in any salmon could be compared to determine whether the salmon was aquacultured or wild. Thus, a total of 80 specimens consisting of authenticated wild, male and female, Atlantic and Pacific salmon were obtained, as described in the **Experimental** section. These salmon constitute a broad-based set that can be generalized to wild marine salmon. The identification of the species of wild Pacific salmon was reconfirmed in-house by analysis of fish scale patterns (18, 19).

We report here the results of the analysis of at least 6 wild salmon from each of the 6 species.

## Experimental

### Apparatus

(a) *Liquid chromatograph*.—Method development was conducted and initial analysis of salmon extracts was performed by using an HP 1090 Series II/M liquid chromatograph equipped with a DR5 ternary solvent delivery system, helium sparge, autosampler, diode array detector, and workstation (Hewlett-Packard, Inc., Avondale, PA). Analysis of the astaxanthin extracts from marine-caught, authenticated wild salmon was performed by using a Waters solvent delivery system Model 510 equipped with a Waters Model 990 diode array detector, a workstation (Waters Chromatography Division, Millipore Corp., Milford, MA), and a Beckman Model 504 autosampler (Beckman Instruments, Inc., Fullerton, CA). LC conditions: Isocratic conditions and ambient temperature were used for all analyses, and solvents were filtered and sparged with He before use.

(b) *LC columns*.—For initial analyses, including analysis of derivatized astaxanthin, a Pirkle covalent D-phenylglycine column, 5  $\mu$ m particle size, 25 cm  $\times$  4.6 mm (Regis Chemical Co., Morton Grove, IL) was used. For remaining analyses, including the study of the configurational isomer distribution of astaxanthin in authenticated wild salmon, the Pirkle covalent L-leucine column previously described (15) was used.

(c) *Homogenizer*.—Polytron (Brinkmann Instruments, Inc., Westbury, NY).

(d) *Centrifuge*.—Sorvall Instruments Model RC5C fitted with a GSA rotor (DuPont Co., Instruments Div., Newton, CT).

(e) *Spectrophotometer*.—Hitachi 200 (Hitachi Ltd., Tokyo, Japan).

(f) *Freezer*.—Model 8416, ultralow temperature, upright freezer (Forma Scientific, Div. of Mallinckrodt, Inc., Marietta, OH).

(g) *Microscope*.—Nikon Optiphot (Nikon, Inc., Melville, NY).

(h) *Solid-phase extraction cartridge*.—Sep-Pak, silica gel (Waters Chromatography Div., Millipore Corp.).

(i) *Rotary evaporator*.—Büchi Rotavapor R 110 (Brinkmann Instruments, Inc.).

(j) *Molecular sieve*.—Union Carbide Type 4 Å, 1/16 in., 8–12 mesh (Fluka Chemical Corp., Ronkonkoma, NY).

### Reagents

(a) *Solvents*.—Hexane, tetrahydrofuran (THF), methylene chloride, and 2-propanol (all LC grade; Baxter Diagnostics, Inc., Scientific Products Div., McGaw Park, IL); triethylamine ( $\geq 99.5\%$ ; Fluka Chemical Corp.); ethanol (200 proof) and chloroform (stabilized with 1% ethanol; EM Science, Gibbstown, NJ); and pyridine (99+%; Aldrich Chemical Co., Inc., Milwaukee, WI).

(b) *Standards*.—Synthetic astaxanthin (a gift from Hoffmann-La Roche, Inc., Nutley, NJ); 4-*N,N*-dimethylaminopyridine (DMAP; 99+%), 3,5-dinitrobenzoyl chloride (98%), 1-naphthoyl chloride and 2-naphthoyl chloride (Aldrich Chemical Co., Inc.); (1*S*)-(–)-camphanic chloride (98%; Fluka Chemical Corp.); and L-menthoxyacetyl chloride (American Tokyo Kasei, Inc., Portland, OR).

(c) *LC mobile phase A*.—Hexane–THF–ethanol (77 + 22 + 1). The flow rate was 1.5 mL/min, and the monitoring wavelength was 470 nm.

(d) *LC mobile phase B*.—Hexane–THF–2-propanol–triethylamine (77 + 17 + 3 + 3). The flow rate was 1.5 mL/min, and the monitoring wavelength was 474 nm.

### Salmon

Samples for method development were purchased from supermarkets and fish markets. The initial authenticated wild salmon used in method development were obtained through the Office of Seafood, FDA. The authenticated wild salmon used for determination of the configurational isomer distribution of astaxanthin are described below.

#### Determination of Configurational Isomer Distribution of All-trans Astaxanthin in Marine-Caught, Authenticated Wild Salmon

(a) *Marine-caught, authenticated wild salmon*.—A minimum of 12 authenticated wild salmon from each of the 5 species of Pacific salmon—sockeye (red), chum, pink, coho (silver), and chinook (king)—were collected in marine waters under the supervision of the Seattle District, FDA, Washington State, and shipped to Washington, DC, in dry ice. Some salmon were received whole, and others were gutted before shipping. All the Pacific salmon were measured, photographed, and weighed. The Pacific salmon were certified to be wild either

through collection of the fish by FDA inspectors (Seattle District) or by purchase of the salmon directly from a boat whose itinerary at sea had been established. Speciation of the wild Pacific salmon was determined by morphological examination and by the location of the catch. Sex was also determined by morphological examination.

Twelve authenticated wild Atlantic salmon were caught off the coast of Cartwright, Newfoundland, and were filleted before being shipped to Washington, DC. The Atlantic salmon were certified to be wild through collection of the fish by scientists of the Quebec Labrador Foundation, Ipswich, MA.

The wild salmon were stored in a freezer at  $-77^{\circ}\text{C}$ . Each fish was assigned a number that was used throughout the study and in data reporting. Results of the analysis of at least 6 wild salmon from each of the above-mentioned species are reported in this study.

(b) *Preparation of authenticated wild salmon flesh for extraction of astaxanthin*.—Salmon received whole were decapitated and gutted. For all salmon, whether received whole or gutted, the skin was removed from the desired sampling area (see **Results and Discussion**), and a portion of the flesh ( $\geq 10$  g) was excised. The sample of salmon flesh was then cleaned of extraneous material (scales, fat, bones, etc.) and dried by blotting with a paper towel. A 10 g portion was accurately weighed on an analytical balance to 3 significant figures.

(c) *Extraction of astaxanthin from wild salmon flesh for chiral LC analysis*.—The 10 g test portion of wild salmon flesh was transferred to a 150 mL centrifuge tube and homogenized for 2 min with 20 mL hexane to remove a significant amount of the lipid. The homogenate was centrifuged for 5 min at 3000 rpm, and the hexane was decanted. The amount of astaxanthin extracted into hexane was determined by recording the volume and measuring the absorbance of the hexane extract at 474 nm ( $\lambda_{\text{max}}$  of astaxanthin in hexane). Astaxanthin was extracted from the partially delipidified flesh remaining in the centrifuge tube by homogenizing the residue for 1 min with 20 mL acetone. The homogenate was centrifuged for 5 min at 3000 rpm, and the supernatant was decanted. Acetone was added to the homogenate, the homogenate was centrifuged again, and the process was repeated. The acetone extracts were combined, and the acetone was removed with a rotary evaporator. Approximately 4 mL water (extracted by acetone from the salmon flesh) remained. The wet residue was mixed with 20 mL methylene chloride, and the mixture was swirled to dissolve astaxanthin. The water layer was removed with a separatory funnel, and the organic layer was dried over ca 1 g anhydrous sodium sulfate. The amount of astaxanthin extracted into methylene chloride was determined by recording the volume and measuring the absorbance of the methylene chloride extract at 494 nm ( $\lambda_{\text{max}}$  of astaxanthin in methylene chloride).

The astaxanthin was purified by loading the dried methylene chloride extract onto a Waters silica gel Sep-Pak cartridge that had been pretreated with hexane. The cartridge was eluted with 20 mL methylene chloride to remove residual salmon flesh lipids in the extract. Astaxanthin was eluted from the cartridge with chloroform, which was then removed under a stream of nitrogen. The residue was reconstituted in

methylene chloride, and a portion was injected into the liquid chromatograph.

(d) *Precision of LC analysis.*—We determined the precision of the LC analysis of synthetic astaxanthin (Table 1) by using mobile phase B. Six replicate analyses were performed with the synthetic astaxanthin standard.

(e) *LC analysis.*—Each astaxanthin extract from wild salmon was analyzed in duplicate. The average of the 2 analyses is reported in all cases. Synthetic astaxanthin was used as a standard before each run. Analyses were performed with mobile phase B.

(f) *Determination of lipid content of wild salmon flesh.*—The hexane extract of the salmon flesh and the methylene chloride washes from the silica gel Sep-Pak cartridge [see (c) above] were placed in tared 12 × 35 mm (or 1/2 dram) vials. The solvent was evaporated under a gentle stream of nitrogen, and the tube was weighed. This process was repeated until a constant weight was obtained. The amount of lipid in each extract was recorded.

(g) *Identification of Pacific salmon species by microscopic examination of scales.*—AOAC Official Method 979.15 was used (17). From each salmon, a minimum of 4 scales were selected from the area beneath the dorsal fin and above the lateral line. Only well-formed scales with intact areas were used. Each scale was mounted and examined separately, and a separate worksheet was completed for each scale examined. For measurement of scale vertical dimensions, observation of circuli and wave striations, and overall scale morphology, a 2× objective lens was used with a 10× eyepiece. For inspection of reticulations, a 4× objective lens was used with a 10× eyepiece.

A minimum of one representative scale from each fish was photographed for documentation. For photomicrography of scales, a 2× objective lens was used for all except the pink salmon, for which a 4× objective lens was used.

## Results and Discussion

### Method Development

First attempts to resolve the configurational isomers of synthetic all-*trans* astaxanthin on a Pirkle covalent D-phenylglycine column failed to duplicate the results obtained by Maoka et al. (13) under the same LC conditions. We used a commercial column packed with chiral stationary phase from the manufac-

turer of the Sumipax OA-2000 column used by Maoka et al. (13).

*Derivatization of astaxanthin.*—When minor adjustments to the LC conditions failed to duplicate the resolution obtained by Maoka et al. (13), we experimented with various derivatizing reagents in an attempt to obtain optimum conditions for making diastereomers that could be easily resolved on a chiral or an achiral column. These derivatizing reagents included L-menthoxyacetyl chloride, 3,5-dinitrobenzoyl chloride, 1-naphthoyl chloride, 2-naphthoyl chloride, and camphanic acid chloride. Only camphanic acid chloride is described in the literature for this purpose (14). The reaction between synthetic astaxanthin and the benzoyl derivatizing agent proceeded rapidly in anhydrous pyridine with a catalytic amount of dimethylaminopyridine (see **Experimental** section). The derivatization reaction was also performed successfully with 3,5-dinitrobenzoyl chloride, a  $\pi$  acid, which enhanced interaction of the derivatized astaxanthin enantiomer with a Pirkle  $\pi$ -electron donor chiral stationary phase. Similarly, the reaction was performed with 1-naphthoyl chloride and 2-naphthoyl chloride,  $\pi$  bases, which enhanced interaction of the derivatized astaxanthin enantiomer with a Pirkle  $\pi$ -electron acceptor chiral stationary phase.

When derivatization was performed with astaxanthin extracted from salmon flesh, erratic results were obtained. Fortunately, the initial derivatization of astaxanthin extracted from salmon proceeded rapidly without problems. Subsequent reactions were sometimes incomplete or did not proceed at all. Other reactions proceeded very slowly, interspersed with reactions that proceeded very quickly.

When astaxanthin extracted from salmon flesh was derivatized successfully, the derivatized astaxanthin was analyzed by LC. For example, the camphanoyl derivative of astaxanthin extracted from the flesh of salmon purchased from a fish market in Washington, DC, and labeled "Washington State" salmon was analyzed by LC under the conditions used by Maoka et al. (13) without modification. The camphanoyl derivative of synthetic astaxanthin was also analyzed under the same LC conditions. The LC profile of the extracted astaxanthin is different from that of synthetic astaxanthin, as shown by the overlay of the 2 profiles (Figure 4). The astaxanthin extracted from the "Washington State" salmon is therefore not synthetic astaxan-

**Table 1. Precision of LC analysis of synthetic astaxanthin**

Run	<i>trans</i>			<i>cis</i>		
	S,S, %	Meso, %	R,R, %	S,S, %	Meso, %	R,R, %
1	24.6	48.6	22.5	0.47	3.06	0.76
2	24.3	48.6	22.4	0.38	2.43	0.61
3	25.0	48.6	22.6	0.58	2.58	0.74
4	25.0	48.5	22.3	0.47	2.12	0.87
5	24.5	48.7	22.8	0.23	2.23	0.55
6	24.9	48.9	22.7	0.65	2.13	0.62
Av. ± SD	24.7 ± 0.3	48.6 ± 0.15	22.6 ± 0.19	0.46 ± 0.15	2.57 ± 0.66	0.86 ± 0.35

thin. The LC profile of the extracted astaxanthin resembles that of wild salmon, and the configurational isomeric ratio is within the range expected for wild salmon (*see below*).

The LC profile in Figure 4 clearly illustrates that optimization of LC conditions will reduce analysis time significantly. However, the need to ascertain that the last trace of oil was removed before derivatization led us to abandon this approach, because the amount of colorless lipid in salmon flesh was variable. This problem and the need to purify the derivative formed led us to reexamine the possibility of direct chiral LC analysis of underivatized astaxanthin. LC conditions were subsequently found that permitted chiral resolution on a Pirkle covalent L-leucine column (15). With the aging of the L-leucine column, the mobile phase (mobile phase A) was modified (to mobile phase B) to obtain the same chiral resolution of astaxanthin (*see Experimental section*).

Regardless of whether the astaxanthin was first derivatized or analyzed directly by chiral LC, the configurational isomer distribution of all-*trans* astaxanthin, the predominant geometric isomer, had to be established in marine-caught, authenticated wild salmon.

#### Determination of Configurational Isomer Distribution of All-*trans* Astaxanthin in Marine-Caught, Authenticated Wild Salmon

To ascertain that there was little or no variation in the configurational isomer distribution in different parts of salmon muscle, test portions were taken from 3 locations along the lateral line: near the head, at the center below the dorsal fin, and near the tail above the anal fin. At least 6 salmon were sampled from each of the Pacific salmon species for this part of the study. No appreciable variation was found, as shown in Tables 2–6. On the basis of these results, the remaining Pacific

salmon were sampled at one location only, the center of the fish. The Atlantic salmon were received as filets and sampled at the center only (Table 7).

The distributions of the configurational isomers of all-*trans* astaxanthin in 38 marine-caught, authenticated wild salmon (*Oncorhynchus* and *Salmo salar*) are listed in Tables 2–7. No variation was observed between male and female salmon for any species. The ranges of the configurational isomers in Atlantic and Pacific salmon were 47.1–90.0% of the (3*S*,3'*S*) enantiomer, 7.7–45.2% of the (3*R*,3'*R*) enantiomer, and 0–8.6% of the (3*R*,3'*S*) meso form. The range of each configurational isomer is much broader than that found by Schiedt et al. (10), who used a very narrow database and did not include chinook salmon, which has a significantly wider range of each isomer than do the other species. When combined with the other 5 species, chinook salmon appreciably broadens the range, as discussed below. The result, however, supports the basic conclusion of Schiedt et al. (10): The configurational isomer distributions of astaxanthin in wild marine salmon are similar. We have expanded it in this study to include the 6 common species of wild salmon.

The configurational isomer distributions in the 6 species of wild salmon are significantly different from that of synthetic astaxanthin, which consists of 25% of each of the enantiomers and 50% of the meso form. These results show that the distribution of the configurational isomers of astaxanthin in wild salmon flesh indeed provides a basis for distinguishing wild salmon from aquacultured salmon fed synthetic astaxanthin.

The range of each of the configurational isomers of all-*trans* astaxanthin is much wider in chinook (king) salmon than in the 5 other species (Table 6). For example, the range of the (3*S*,3'*S*) enantiomer for chinook is 47.1–80.5%, compared with 65.4–77.0% for sockeye, 77.1–89.4% for coho, 78.5–90.0% for pink,

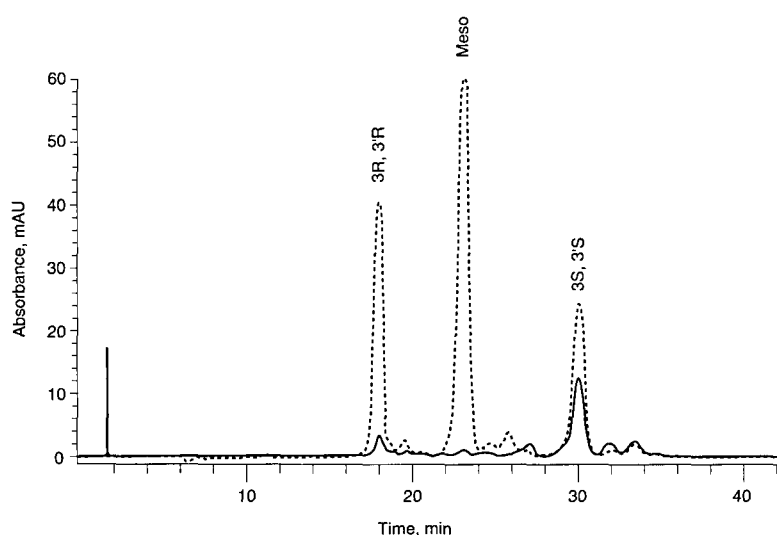


Figure 4. Overlay of LC profiles of the camphanoyl derivative of astaxanthin extracted from wild salmon and synthetic astaxanthin. Solid line = astaxanthin extracted from "Washington State" salmon purchased from the SW pier fish market, Washington, DC; --- = synthetic astaxanthin. LC conditions: Pirkle covalent D-phenylglycine column; mobile phase, hexane–methylene chloride–ethanol (73.3 + 24.4 + 2.4), flow rate, 1.6 mL/min; monitoring wavelength, 490 nm.

**Table 2. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild sockeye salmon**

Salmon						
No.	Sex	Sampling location	S,S, %	Meso, %	R,R, %	Total isomers, ppm
44	F <sup>a</sup>	A <sup>b</sup>	73.8	4.8	21.4	31.1
		B <sup>c</sup>	74.6	4.6	20.8	31.7
		C <sup>d</sup>	74.6	4.6	20.9	32.4
48	F	A	76.8	3.8	19.4	30.0
		B	76.8	3.8	19.4	34.6
		C	77.0	3.9	19.1	39.2
50	F	A	72.8	4.8	22.5	45.7
		B	73.6	4.7	21.7	48.0
		C	73.5	4.7	21.8	59.7
76	F	A	71.1	4.6	24.2	33.7
		B	71.0	4.8	24.4	28.6
		C	71.3	4.6	24.2	37.7
57	M <sup>e</sup>	A	65.4	5.8	28.8	47.8
		B	65.6	5.8	28.6	50.9
		C	65.4	5.8	28.8	58.9
74	M	A	73.2	4.4	22.4	32.4
		B	74.5	4.6	21.2	43.3
		C	73.1	4.5	22.4	42.2
Range			65.4–77.0	3.8–5.8	19.1–28.8	30.0–58.9

<sup>a</sup> F = female.<sup>b</sup> A = sample taken near the head, along the lateral line.<sup>c</sup> B = sample taken at the center below the dorsal fin, along the lateral line.<sup>d</sup> C = sample taken near the tail above the anal fin, along the lateral line.<sup>e</sup> M = male.

77.4–89.8 for chum, and 79.3–82.6 for Atlantic salmon. Chinook salmon (*Oncorhynchus tshawytscha*) are different from the other salmon species because they occur along the Pacific coast of North America in 2 distinct forms known as red-fleshed and white-fleshed chinook (20). The white-fleshed chinook is the only wild Pacific or Atlantic salmon that apparently does not contain deposits of colored dietary carotenoids in the flesh of the sexually maturing adult (20). In a study of intestinal absorption of astaxanthin, investigators concluded that the poor flesh pigmentation was due to rapid metabolism of the absorbed astaxanthin to colorless derivatives rather than to failure of the salmon to absorb astaxanthin (21). Only 2 of 6 chinook salmon samples in our study had very pale flesh (No. 22 and No. 23). The amount of astaxanthin in one sample (No. 22) was too low to determine the configurational isomeric ratios (Table 6). Furthermore, there seemed to be 2 groups of configurational isomeric distributions in the red-fleshed chinook salmon (Table 6). One group resembled the rest of the wild Pacific and Atlantic salmon (samples 23, 52, and 54) with a range of 64.5–80.5% for the (3*S*,3'*S*) enantiomer compared with a range of 65.4–90.0% for the 5 other species. The other group had about equal distribution of the (3*S*,3'*S*) enantiomer (47.1–51.0%) and the (3*R*,3'*R*) enantiomer (40.6–45.2%), with the former slightly

higher than the latter. Inclusion of this second group broadens the range of the entire survey. A much larger database, however, would be required to determine whether those fish with the enantiomers as the 2 major components constitute a distinct subgroup within the red-fleshed chinook salmon.

The results of analyses of the remaining authenticated wild salmon are not expected to appreciably affect the configurational isomeric distribution reported here. Results of all 80 samples will be reported separately.

### Methodology

The LC profile of astaxanthin extracted from the flesh of salmon was examined to determine the configurational isomeric ratio and to compare the LC profile and isomeric ratios with those of synthetic astaxanthin. Aquacultured salmon fed a diet supplemented with synthetic astaxanthin would be easy to identify because the configurational isomeric ratios and the LC profile of the extracted astaxanthin would be identical to those of synthetic astaxanthin. For wild, marine-caught salmon, the ratio of configurational isomers is expected to lie within the range we have established for wild salmon. Furthermore, the LC profile of astaxanthin extracted from wild salmon would be different from the LC profile of synthetic astaxanthin. Exam-

**Table 3. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-trans astaxanthin in wild coho salmon**

Salmon			S,S, %	Meso, %	R,R, %	Total isomers, ppm
No.	Sex	Sampling location				
70	F <sup>a</sup>	A <sup>b</sup>	83.2	3.2	13.6	13.0
		B <sup>c</sup>	81.7	2.8	15.4	12.2
		C <sup>d</sup>	82.8	3.0	14.2	12.7
71	F	A	77.6	3.8	18.6	13.8
		B	77.6	3.7	18.7	14.4
		C	77.1	3.7	19.2	13.0
68	M <sup>e</sup>	A	87.8	2.8	9.5	10.7
		B	89.4	2.1	8.6	9.9
		C	86.8	3.0	10.2	13.8
69	M	A	81.6	3.0	15.4	9.6
		B	82.9	2.8	14.2	9.8
		C	84.4	2.8	12.8	11.7
72	M	A	81.0	3.6	15.6	25.5
		B	79.9	3.2	16.9	16.7
		C	79.6	3.2	17.1	28.0
75	M	A	83.8	2.9	13.4	12.8
		B	84.4	2.8	12.7	10.7
		C	81.6	2.8	15.6	10.6
Range			77.1–89.4	2.1–3.7	8.6–19.2	9.6–28.0

<sup>a</sup> F = female.<sup>b</sup> A = sample taken near the head, along the lateral line.<sup>c</sup> B = sample taken at the center below the dorsal fin, along the lateral line.<sup>d</sup> C = sample taken near the tail above the anal fin, along the lateral line.<sup>e</sup> M = male.

ples are given below for wild salmon and aquacultured salmon fed synthetic astaxanthin.

This method could be also used to determine the presence in aquacultured salmon of astaxanthin derived from other sources such as *Phaffia* yeast and *Haematococcus pluvialis* algae. Astaxanthin in *Phaffia* yeast consists of >98% of the (3R,3'R) enantiomer (22), giving it a very distinctive LC profile that is easy to recognize. Similarly, the astaxanthin extract of salmon fed *Haematococcus pluvialis* algae would consist almost entirely (99%) of the (3S,3'S) enantiomer (23). Its LC profile also would be highly distinctive and easy to characterize.

#### Aquacultured Salmon Fed Synthetic Astaxanthin

We extracted astaxanthin from a Norwegian salmon filet purchased from a local supermarket and analyzed it by LC on a Pirkle covalent L-leucine column eluted with mobile phase A. Synthetic astaxanthin was also analyzed under the same LC conditions. The LC profiles of the astaxanthin peaks were very similar: Each of the 3 configurational isomers eluted at practi-

**Table 4. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-trans astaxanthin in wild pink salmon**

Salmon			S,S, %	Meso, %	R,R, %	Total isomers, ppm
No.	Sex	Sampling location				
18	F <sup>a</sup>	A <sup>b</sup>	84.8	3.4	12.5	6.5
		B <sup>c</sup>	83.0	3.6	13.4	6.2
		C <sup>d</sup>	82.5	3.4	14.2	6.5
55	F	A	83.8	3.3	13.0	5.6
		B	85.4	2.9	11.8	4.9
		C	80.4	3.2	16.4	5.3
63	F	A	84.0	4.1	11.8	5.3
		B	78.5	3.0	18.6	6.1
		C	80.8	3.2	16.0	6.9
14	M <sup>e</sup>	A	87.8	2.9	9.4	7.6
		B	86.4	2.5	11.0	6.2
		C	86.9	2.4	10.6	6.6
59	M	A	80.6	3.6	15.8	7.2
		B	81.3	3.8	14.9	6.9
		C	80.9	4.0	15.1	7.3
65	M	A	85.6	2.3	12.1	3.1
		B	86.6	2.6	10.8	3.4
		C	85.4	2.4	12.2	4.2
77	X <sup>f</sup>	A	87.1	2.0	10.8	3.9
		B	88.4	2.1	9.4	3.9
		C	87.4	2.4	10.2	3.3
78	X	A	88.6	2.2	9.2	4.5
		B	88.2	0.0	11.8	4.2
		C	90.0	1.0	9.0	5.0
Range			78.5–90.0	0–4.1	9.0–18.6	3.1–7.6

<sup>a</sup> F = female.<sup>b</sup> A = sample taken near the head, along the lateral line.<sup>c</sup> B = sample taken at the center below the dorsal fin, along the lateral line.<sup>d</sup> C = sample taken near the tail above the anal fin, along the lateral line.<sup>e</sup> M = male.<sup>f</sup> X = sex unknown.

cally identical retention times, and the ratios of peaks were the same in both profiles (Figure 5). Overlay of peaks eluting at 10.1, 10.8, and 11.5 min showed them to be an almost perfect match. We concluded that the astaxanthin extracted from the Norwegian flesh was synthetic astaxanthin, which must have been added to the fish feed. Consequently, the Norwegian salmon was presumed to be aquacultured and not wild. The retention time and the UV/VIS absorption spectrum of the peak at 3.87 min suggest that it is the diester(s) of astaxanthin.

Similar results were obtained for salmon purchased from a local supermarket and labeled as "imported" from Idaho, as well as for salmon purchased from the delicatessen of an up-



**Table 5. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-trans astaxanthin in wild chum salmon**

Salmon						
No.	Sex	Sampling location	S,S, %	Meso, %	R,R, %	Total isomers, ppm
29	F <sup>a</sup>	A <sup>b</sup>	81.1	3.4	14.8	6.5
		B <sup>c</sup>	80.3	4.2	15.5	7.0
		C <sup>d</sup>	81.5	3.8	14.7	6.9
36	F	A	84.5	1.7	13.8	2.5
		B	84.0	1.7	14.3	1.8
		C	83.7	1.9	14.5	1.1
42	F	A	88.8	2.2	9.0	6.7
		B	89.8	2.4	7.7	6.2
		C	88.4	2.4	9.2	5.7
56	F	A	85.0	3.2	11.8	5.0
		B	85.5	3.4	11.2	4.6
		C	84.8	3.3	11.8	5.4
58	M <sup>e</sup>	A	81.2	3.0	15.8	7.2
		B	80.6	3.6	15.8	7.8
		C	81.2	3.8	15.0	6.8
62	M	A	79.3	3.6	17.4	7.1
		B	77.4	3.6	19.0	5.7
		C	78.2	3.7	18.1	6.6
Range			77.4–89.8	1.7–4.2	7.7–19.0	1.1–7.8

<sup>a</sup> F = female.<sup>b</sup> A = sample taken near the head, along the lateral line.<sup>c</sup> B = sample taken at the center below the dorsal fin, along the lateral line.<sup>d</sup> C = sample taken near the tail above the anal fin, along the lateral line.<sup>e</sup> M = male.

scale department store and advertised as being caught off the icy waters of Canada and Scotland.

#### Wild Salmon

We extracted astaxanthin from a wild pink salmon that had been authenticated by the Office of Seafood, FDA, but was not part of the broad-based set of specimens used for the determination of configurational isomers of all-trans astaxanthin in wild salmon. The extracted astaxanthin was analyzed by LC on a Pirkle covalent L-leucine column with mobile phase A. Synthetic astaxanthin was also analyzed under the same LC conditions. The LC profiles of the astaxanthin peaks were very different (Figure 6). Moreover, the LC profile of the extracted astaxanthin was similar to the LC profile of the marine-caught, authenticated wild salmon, as expected.

#### Concentration of Astaxanthin in Wild Salmon Flesh

We determined the amount of astaxanthin in the flesh of the 38 wild salmon studied, including 2 pale-colored chinook

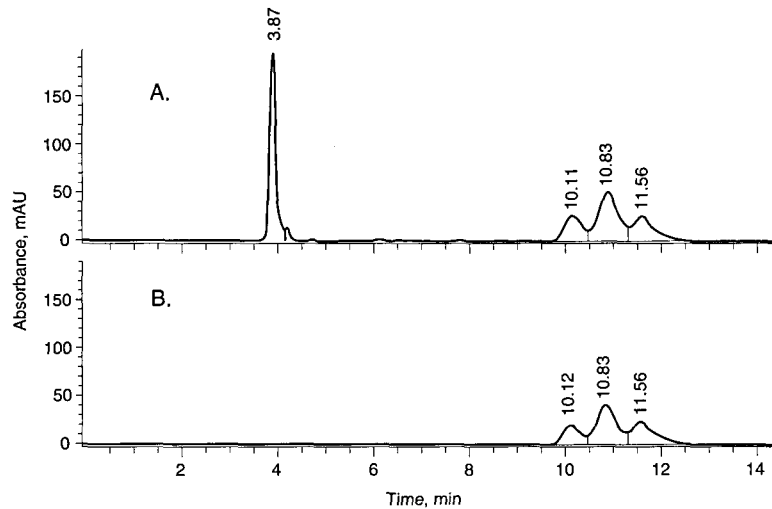
**Table 6. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-trans astaxanthin in wild chinook (king) salmon**

Salmon						
No.	Sex	Sampling location	S,S, %	Meso, %	R,R, %	Total isomers, ppm
22	F <sup>a</sup>	A <sup>b</sup>	ND <sup>c</sup>	ND	ND	0.7
		B <sup>d</sup>	ND	ND	ND	0.8
		C <sup>e</sup>	ND	ND	ND	0.8
23	M <sup>f</sup>	A	65.6	3.6	31.1	0.9
		B	65.1	2.8	32.1	0.9
		C	64.5	2.0	33.5	1.0
17	X <sup>g</sup>	A	50.1	8.6	41.3	12.9
		B	51.0	8.3	40.6	13.1
		C	50.2	8.6	41.2	11.0
19	X	A	48.5	8.1	43.4	11.7
		B	48.3	7.9	43.8	10.4
		C	47.1	7.7	45.2	11.4
52	X	A	79.1	3.5	17.4	18.8
		B	80.5	3.3	16.3	19.6
		C	79.8	3.3	17.0	22.4
54	X	A	71.1	5.3	23.7	7.3
		B	70.8	5.3	23.9	8.3
		C	70.8	5.3	24.0	8.3
Range			47.1–80.5	2.0–8.6	16.3–45.2	0.7–22.4

<sup>a</sup> F = female.<sup>b</sup> A = sample taken near the head, along the lateral line.<sup>c</sup> ND = configurational isomeric ratio not determined (astaxanthin concentration too low).<sup>d</sup> B = sample taken at the center below the dorsal fin, along the lateral line.<sup>e</sup> C = sample taken near the tail above the anal fin, along the lateral line.<sup>f</sup> M = male.<sup>g</sup> X = unknown.**Table 7. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-trans astaxanthin in wild Atlantic salmon**

Salmon					
No.	Sex	S,S, %	Meso, %	R,R, %	Total isomers, ppm
2	F <sup>a</sup>	79.3	4.9	15.9	5.1
6	F	82.6	3.2	14.3	7.2
1	M <sup>b</sup>	81.0	3.7	15.7	5.1
3	M	80.1	4.3	15.6	4.9
4	M	79.3	3.6	17.1	4.5
5	M	80.0	3.4	16.6	4.9
Range		79.3–82.6	3.2–4.9	14.3–17.1	4.9–7.2

<sup>a</sup> F = female.<sup>b</sup> M = male.



**Figure 5.** Comparison of LC profiles of astaxanthin extracted from Norwegian salmon and synthetic astaxanthin. (A) Astaxanthin extracted from Norwegian salmon file, purchased from Safeway. (B) Synthetic astaxanthin. LC conditions: Pirkle covalent L-leucine column; mobile phase A; flow rate, 1.5 mL/min; monitoring wavelength, 470 nm.

salmon. The results were consistent with literature values for each of the species (10). The amounts of astaxanthin were within a defined range for each of the wild salmon species (Tables 2–7). These ranges of astaxanthin content, however, overlapped sufficiently to preclude speciation on the basis of color content alone.

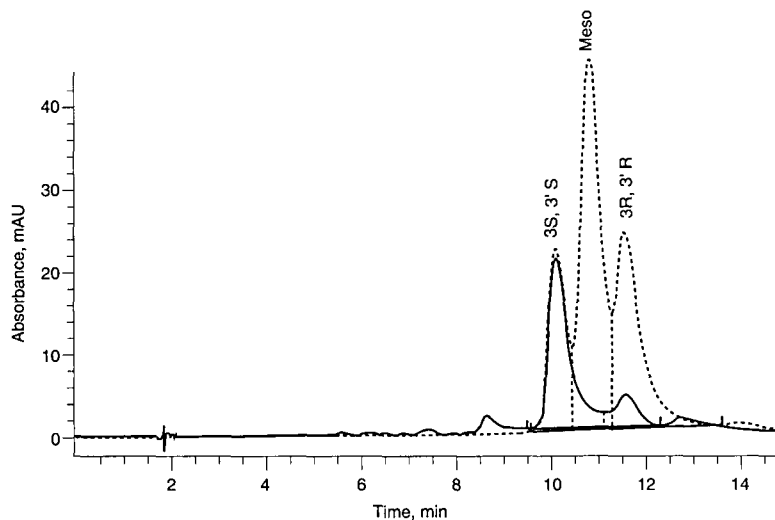
#### Determination of Lipid Content of Wild Salmon Flesh

The amount of lipid in the flesh of wild salmon was also determined (Table 8). Results were consistent with literature values (10). The amount of lipid varied even within the same species. For example, Atlantic salmon No. 7 had twice the amount of lipid found in Atlantic salmon No. 9, whereas chi-

nook salmon No. 21 had 6 times the amount of lipid found in chum salmon No. 35. This variation and the differences in fatty acid profiles of lipids extracted from wild and aquacultured salmon (24) may explain the difficulties encountered in attempts to derivatize astaxanthin extracted from the flesh of salmon (*see Method Development*).

#### Conclusions

A rapid LC method to distinguish between wild salmon and aquacultured salmon fed synthetic astaxanthin has been developed. Validation demonstrated good LC method precision. Preliminary study of the distribution of the configurational isomers



**Figure 6.** Overlay of LC profiles of astaxanthin extracted from wild salmon and synthetic astaxanthin. Solid line = astaxanthin extracted from wild pink salmon obtained from FDA's Office of Seafood; --- = synthetic astaxanthin. LC conditions: Pirkle covalent L-leucine column; mobile phase A; flow rate, 1.5 mL/min; monitoring wavelength, 470 nm.

**Table 8. Lipid content of authenticated wild salmon flesh**

Salmon No.	Total lipid, % <sup>a</sup>	Lipid in hexane, % <sup>b</sup>	Lipid in methylene chloride, % <sup>c</sup>
7	4.61	74.6	25.4
8	4.39	68.4	31.6
9	2.76	50.0	50.0
11	4.66	77.0	23.0
12	4.70	70.9	29.1
13	1.86	30.2	69.8
15	2.85	46.1	53.9
16	1.64	35.8	64.2
20	3.81	62.2	37.8
21	6.68	79.4	20.6
25	3.25	72.5	27.5
26	2.03	37.1	62.9
27	5.84	80.6	19.4
28	5.13	80.7	19.4
30	1.33	16.9	83.1
31	1.23	19.6	80.4
32	1.39	61.0	39.0
33	2.52	45.7	54.3
34	1.50	34.4	65.6
35	1.09	31.3	68.7
39	1.52	20.9	79.1
41	4.67	68.5	31.5
43	4.75	61.3	38.7
45	2.72	51.8	48.2
46	2.52	52.4	47.6
47	2.78	46.5	53.5
49	2.03	32.5	67.5
51	2.51	51.1	48.9
53	3.54	54.5	45.5
61	1.96	43.0	57.0
64	2.70	50.3	49.7
66	1.43	26.2	73.8
67	2.27	38.7	61.3

<sup>a</sup> Total lipid, % =  $\frac{\text{g total lipid}}{\text{g sample}} \times 100$ .

<sup>b</sup> Lipid in hexane, % =  $\frac{\text{g lipid in hexane extract}}{\text{g total lipid}} \times 100$ .

<sup>c</sup> Lipid in methylene chloride, % =  $\frac{\text{g lipid in methylene chloride extract}}{\text{g total lipid}} \times 100$ .

of astaxanthin in wild authenticated salmon confirm the basic tenet of the method: The configurational isomeric ratio falls within a defined range and can be used as a basis for determining whether the salmon is wild. This method also can be used to determine the presence of astaxanthin derived from other sources such as *Phaffia* yeast and *Haematococcus pluvialis* algae.

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