44. Natural Occurrence of Enantiomeric and *meso*-Astaxanthin 5. Ex Wild Salmon (*Salmo salar* and *Oncorhynchus*)

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Summary

The configurational isomers of astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) from the flesh of salmon (*Salmo salar* and *Oncorhynchus*) caught at different places in Europe and Canada were isolated and analyzed as (−)-camphanic acid diesters by means of HPLC. The biological variation in the composition of the configurational isomers in seven fish was surprisingly similar: 78 to 85% of (3*S*, 3'*S*)-astaxanthin, 12 to 17% (3*R*, 3'*R*)-astaxanthin and 2 to 6% *meso*-astaxanthin.

In recent years, configurational studies of naturally occurring astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione, *Scheme*, **1**) have been carried out [1–4]. The method described by *Vecchi & Müller* [5] of separating enantiomeric and *meso*-astaxanthin in the form of their di-(−)-camphanates (*Scheme 1*, **3**) by HPLC. renders qualitative and quantitative configurational analyses possible. The co-occurrence of the optically inactive *meso*-astaxanthin with (3*S*, 3'*S*) and (3*R*, 3'*R*)-astaxanthin in lobster eggs was reported for the first time by *Rønneberg et al.* [4]. While astaxanthin from the green algae *Haematococcus pluvialis* has (3*S*, 3'*S*)-configuration [6], that from shrimps *Pandalus borealis* is an almost racemic mixture of all three configurational isomers [7].

Our presentation deals with investigations on salmon of various origins (*Salmo salar* and *Oncorhynchus*). Astaxanthin is the major pigment in salmon flesh together with small amounts of the acetylenic 7,8-didehydroastaxanthin (5–6%), 7,8,7',8'-tetradehydroastaxanthin (1–2%), and minor quantities of less oxidized carotenoids such as idoxanthin (3,3',4-trihydroxy-β,β-carotene-4'-one) and possibly crustaxanthin (3,3',4,4'-tetrahydroxy-β,β-carotene). Enantiomeric and *meso*-astaxanthin were determined according to *Vecchi et al.* [5]. The preparation of di-(−)-camphanates has been adapted to biological samples. Details of the separation of *cis-trans*-isomers as their diacetates (*Scheme*, **2**) by HPLC. are described.

The ratio of *cis*-isomers of the polyene chain in the astaxanthin mixture was determined before and after esterification with camphanoyl chloride. It could be shown that the increase of the *cis*-content by esterification is negligible (0–4%). The naturally occurring *cis*-isomers amounted to 9–23%. It was proven that neither

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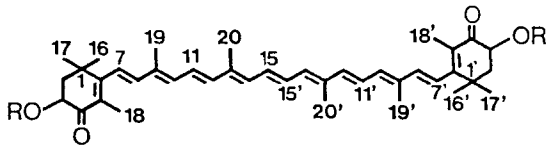
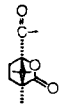
Scheme



1 R = H

2 R = $COCH_3$

3 R =  = (–)-camphanic acid

racemization nor isomerization takes place during the isolation procedure by adding (3*S*, 3′*S*)-astaxanthin to unpigmented flesh of cultured salmon and subsequent analogous isolation and HPLC. analysis.

Experimental Part

General remarks. All operations were carried out under an inert atmosphere and subdued light at RT. The solvents were evaporated under reduced pressure at a bath temperature not exceeding 35°. The astaxanthin content was analyzed by VIS. spectrophotometry using $E^{1\%}_{1cm} = 2100$. Analytical grade solvents were used.

Fish material. Wild salmon caught at different places in Europe and Canada were mailed deep-frozen and kept at –25° until analyzed.

Extraction. The fish were partly thawed and the skin removed from the flesh, which was minced in a meat mincer, mixed with the 1.5–2-fold quantity of anhydrous $MgSO_4$ and allowed to stand for about 1 h. Cooling with dry ice prevents warming up of the mixture by exothermic reaction. The dry powder is then mixed with acetone in a *Waring* blender and exhaustively extracted until the acetone filtrate is colourless. The extracts are drawn through a glass filter and the acetone evaporated. The weight of the residue yields the total lipid extract.

Precipitation of sterols. Sterols were precipitated in acetone at –20°, filtered off and the filtrate evaporated.

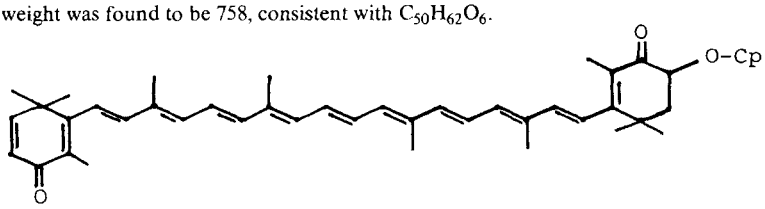
Partition in hexane/dimethyl sulfoxide (DMSO). The remaining lipids were dissolved in hexane yielding a 3% solution and partitioned with the same quantity of DMSO. Free astaxanthin is completely extracted with DMSO., while 90–95% of the lipids can be removed with hexane. Astaxanthin is re-extracted with hexane/ether 1:1 (*v:v*) after dilution of the DMSO. with water, ethanol and saturated NaCl-solution. The ether extract is then washed with water for complete removal of the DMSO, dried (Na_2SO_4) and the solvent evaporated.

Chromatography on silica gel 60 (Merck *0.063–0.2 mm).* Using hexane and increasing amounts of ether, astaxanthin was eluted with 50% ether in hexane. This fraction is generally pure enough for the esterification with camphanoyl chloride. If not, or for smaller quantities of carotenoid extract, preparative TLC. on pre-coated plates (*Merck*, silicagel 60 F 254) can be used instead of column chromatography (ethyl acetate/hexane 2:1 (*v:v*)). The astaxanthin fraction is scraped off and desorbed with 10% methanol in ether. After evaporation of the solvent, the astaxanthin concentrate is dried (P_2O_5). This preparation is used for CD., HPLC. and for the preparation of the derivatives.

Preparation of astaxanthin di-(–)-camphanate. Esterification of pure crystalline astaxanthin takes place spontaneously with a mol-equiv. or slight excess of (–)-camphanoyl chloride at 0°. For biological samples, the conditions had to be adapted, because the concentration of carotenoid relative to uncoloured lipids is generally small (2–10%). These lipids interfere with the esterification.

A solution of 0.5–1.5 mg astaxanthin/20 mg lipids in 0.5 ml dry pyridine was allowed to react with 50 mg (−)-camphanoyl chloride for 10 min. The reaction mixture was adapted to sample size. Lower limit: 5 µg astaxanthin in 0.1 ml pyridine. The relations 100 mg (−)-camphanoyl chloride per ml pyridine should be maintained. The end of the reaction can be checked by TLC. on silica gel plates (ethyl acetate/hexane 2:1).

After longer reaction time or at higher temperature, two less polar artefacts may be formed besides the di-(−)-camphanate. One of the two artefacts was isolated and analyzed by mass spectroscopy. The molecular weight was found to be 758, consistent with $C_{50}H_{62}O_6$.



The less polar artefact might be dehydrated at both ends before esterification takes place. The above solvent system for TLC. does not separate the diastereoisomers which can, however, be separated either by column chromatography or by TLC. on silica gel with toluene/ether/isopropanol 89:9:2 [8].

Preparation of diacetates. The method described by *Kienzle et al.* [9] used for the acetylation of crystalline astaxanthin, has been modified for biological samples. Purified concentrates, containing 0.5–1.5 mg astaxanthin/20 mg lipids, dissolved in 0.2 ml dry pyridine were allowed to react under N_2 with 0.2 ml acetic anhydride at RT., overnight. Solvents were evaporated at 0.02 Torr.

Addition of (3S,3'S)-astaxanthin to unpigmented salmon flesh and subsequent isolation for configurational analysis. Unpigmented salmon flesh was provided by *Unilever,* Scotland. Crystalline (3*S*,3'*S*)-astaxanthin was transferred into a water dispersible gelatine preparation (100 mg astaxanthin, 20 mg EMQ, 20 mg ascorbyl palmitate, 1000 mg gelatine, 400 mg sugar, 400 mg dextrine, dissolved in 4 ml water by ultrasonification, dried and ground). Addition to salmon flesh was made in biological concentration (10 ppm). The astaxanthin-gelatine preparation was dispersed in water and mixed with the meat in a *Waring* blender. Extraction and isolation were carried out in analogy to the wild salmon samples. Results are compiled in *Table 5.*

HPLC. methods. – *Equipment and chromatographic conditions.* All analytical work was performed with a HPLC. unit consisting of an *Altex* 100 delivery solvent system, septum injection port *(Perkin Elmer)* and UV.-VIS. detector LCD-725 *(Kontron)* or *Variscan.* The columns (500 mm length × 3.2 mm ID) were home made.

Although *Spherisorb* 5 CN is a chemically bonded phase, the chromatographic systems containing the base *N*-ethyldiisopropylamine had to be equilibrated for at least 6 h to reach constant retention times. *Figure 1* demonstrates the changes in peak shape and resolution during this equilibration phase. It is concluded that the amine is adsorbed by the stationary phase until a steady state is reached.

All solvents were of analytical grade and used without further purification.

Separation of cis-trans *isomers of underivatized astaxanthin samples.* Although more than 70 chromatographic system were tried, satisfactory separation of the various *cis-trans* isomers of

Table 1. *Chromatographic systems employed.*

Chromatographic system	Mobile phase	Stationary phase
A	hexane/CH_2Cl_2/*N*-ethyldiisopropylamine (74:25:1)	*Spherisorb* S 5 CN (Phase separation)
B	*n*-hexane/ethyl acetate/acetonitrile (88:10:2)	*Lichrosorb* SI 60 (5 µm) *(Merck)*
C	hexane/2-propanol/*N*-ethyldiisopropylamine (89.75:10:0.25)	*Spherisorb* S 5 CN (Phase separation)

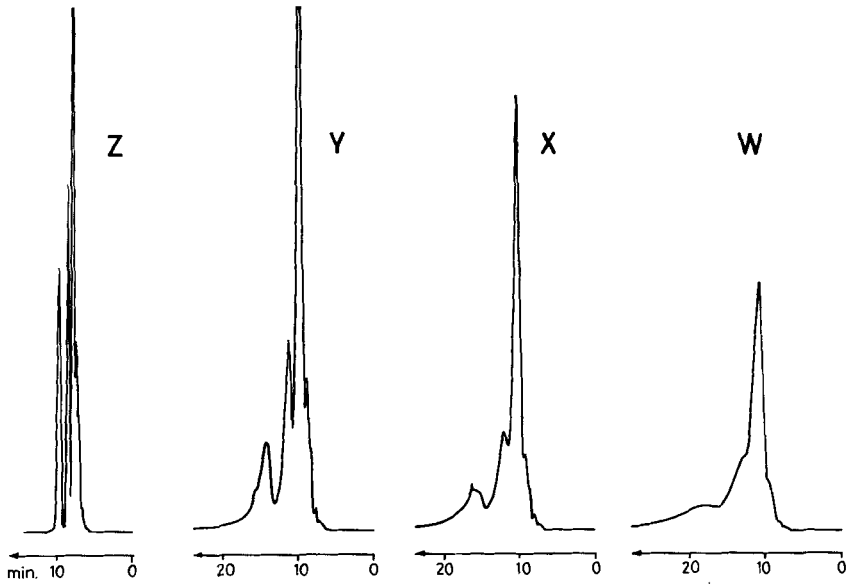


Fig. 1. *Chromatography of a mixture of* cis/trans *isomers of astaxanthin with System A* after different equilibration times (W 1 h, X 2 h, Y 3 h, and Z 4 h).

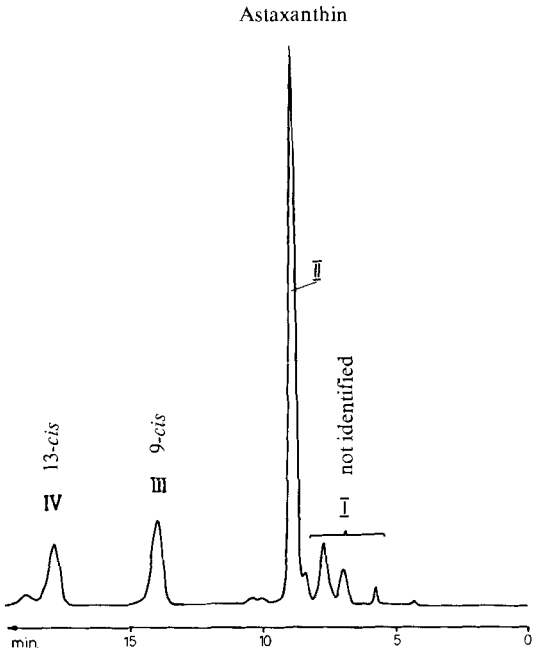


Fig. 2. *Chromatography of a mixture of* cis/trans *isomers of astaxanthin with system A* (I not identified isomers, II all-*trans*-astaxanthin, III 9-*cis*-astaxanthin and IV 13-*cis*-astaxanthin).

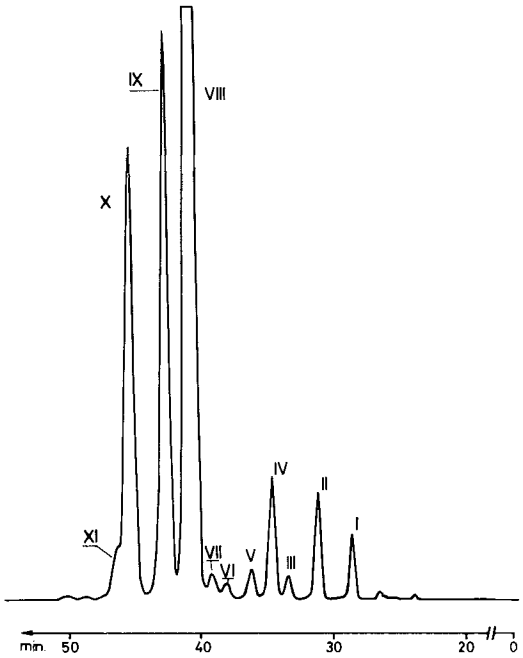


Fig. 3. *Chromatography of a mixture of* cis/trans *isomers of astaxanthin diacetates with system B.*

underivatized astaxanthin was not achieved. The best results were obtained with **system A,** but *(Fig. 2)* only 9-*cis*, 13-*cis* and all-*trans* isomers are well separated. However, separation of astaxanthin, 7,8-didehydroastaxanthin, 7,8,7′,8′-tetradehydroastaxanthin and adonirubin can be achieved with the same system.

Separation of cis-trans *isomers of astaxanthin diacetates.* After thermal or light isomerization of racemic astaxanthin diacetate, a high resolution of 11 different *cis-trans* isomers was obtained with **system B** *(Fig. 3)*. Identification of the single peaks by ^{1}H-NMR. has been described [10]. System B is well-suited for the separation of *cis-trans* isomers of astaxanthin diacetate, but more polar substances are not detected because their retention times are too long.

System C results in an only partial resolution of the *cis*-isomers *(Fig. 4)*. Peak identification was achieved by re-injecting and separating them on system B *(Table 2)*. System C has the advantage that more polar oxidation products such as 3,3′-dihydroxy-2,3-didehydro-β,β-carotene-4,4′-dione and 3,3′-dihydroxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione can be analyzed simultaneously *(Fig. 5)*.

Separation of enantiomeric and meso-*astaxanthin as di-(−)-camphanates.* Separation of the configurational isomers as well as their *cis-trans* isomers has been described [5]. Possible metabolites of

Table 2

Peak	Isomer	Peak	Isomer
I	9,9′-di-*cis* 9,13′-di-*cis*	III	all-*trans*
II	9,13-di-*cis* 9,15-di-*cis* 13,13′-di-*cis*	IV	9-*cis* 13-*cis* 15-*cis* 13,15-di-*cis*

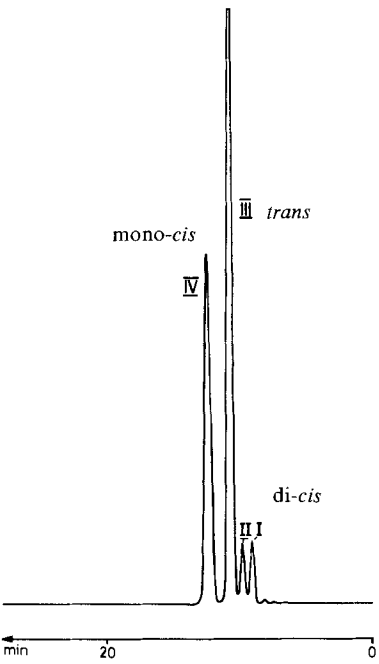


Fig. 4. *Chromatography of a mixture of* cis/trans *isomers of astaxanthin diacetate with system C.* For peak identification see *Table 2.*

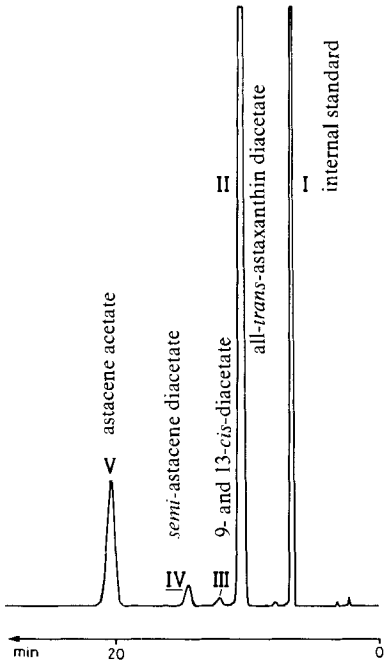


Figure 5.

astaxanthin or by-products of biological samples, such as adonirubin and 3-hydroxyechinenone can be separated under the same experimental conditions and their configuration determined simultaneously [8].

Results and discussion. – Astaxanthin, occurring naturally in salmon of different origin, consists of a mixture of configurational isomers. The total astaxanthin concentration in flesh varied little in *Salmo salar* (3.1 to 8.1 ppm), whereas *Oncorhynchus* showed a wider variation (3.4 to 34 ppm). The composition of the three configurational isomers in seven fish of different provenance was surprisingly similar, namely 12 to 17% (3*R*,3′*R*)-, 78 to 85% (3*S*,3′*S*)- and 2 to 6% *meso*-astaxanthin.

The finding of only 2 to 6% *meso*-astaxanthin must be considered assured by the highly sophisticated analytical work. No racemization takes place during the isolation procedure *(Table 5).* The seeming increase of *meso*-astaxanthin during the formulation process (gelatine preparation) must be explained by the formation of *semi*-astacene (oxidation of one ring system). Under the experimental conditions described above its di-(−)-camphanate has the same retention time as that of *meso-trans*-astaxanthin.

The *cis-trans* isomers in the polyene chain of the configurational isomers of astaxanthin found in seven fish must be considered natural with an average content of *ca.* 20% *cis* and 80% *trans* (see *Table 3* for *Salmo salar* and *Table 4* for *Oncorhynchus*).

From the first-hand HPLC. results of *cis*-(3*S*, 3′*S*)-astaxanthin, the value of *trans*-(3*S*, 3′*S*)-7, 8-didehydroastaxanthin had to be subtracted because its di-(−)-camphanate has the same retention time as that of *cis*-(3*S*, 3′*S*)-astaxanthin.

Table 3. *Wild Salmon*, Salmo salar

Analyses in flesh		Wild Salmon 1 Scotland		Wild Salmon 2 Scotland, Moray Firth		Wild Salmon 3 Ireland		Wild Salmon 4 Norway	
			cis/trans %		*cis/trans* %		*cis/trans* %		*cis/trans* %
Lipid content %		6.8		2.5		5.6		4.5	
Astaxanthin content ppm		6.8		3.1		3.8		8.1	
Underivatized astaxanthin sample		area %		area %		area %		area %	
7,8,7′,8′-Tetradehydro-astaxanthin		2.60		–		–		0.46	
7,8-Didehydroastaxanthin		6.50[a])		6		–		3.71	
Astaxanthin	*cis*	14.61	16.07	–		–		8.27	8.86
Astaxanthin	*trans*	76.29	83.93	–		–		85.06	91.14
Not identified		0						2.5	
di-(−)-Camphanates		rel. area %		rel. area %		rel. area %		rel. area %	
(3*R*,3′*R*)-Astaxanthin	*cis*	2.40 } 11.9	20.2	3.04 } 13.8	22.1	3.31 } 15.01	22.0	2.02 } 17.53	11.5
(3*R*,3′*R*)-Astaxanthin	*trans*	9.51 }	79.8	10.72 }	77.9	11.70 }	78.0	15.51 }	88.5
meso-Astaxanthin	*cis*	0.88 } 4.33	20.3	1.04 } 5.7	18.4	1.15 } 5.02	22.9	0.52 } 4.26	12.2
meso-Astaxanthin	*trans*	3.45 }	79.7	4.62 }	81.6	3.87 }	77.1	3.74 }	87.8
(3*S*,3′*S*)-Astaxanthin	*cis*	(23.18)[b])		(23.93)[b])				(12.04)[b])	
Corrected for[b])	*cis*	18.03 } 83.8	21.5	19.80 } 80.6	24.6	18.32 } 79.98	22.9	8.33 } 78.22	12.3
(3*S*,3′*S*)-Astaxanthin	*trans*	65.71 }	78.5	60.79 }	75.98	61.66 }	79.98	59.49 }	87.7

[a]) Isolated and identified by MS. and ^{1}H-NMR.
[b]) Increased by di-(−)-camphanate of (3*S*,3′*S*)-7,8-didehydroastaxanthin, which has the same retention time as that of (3*S*,3′*S*)-*cis*-astaxanthin.

Table 4. *Wild Salmon*, Oncorhynchus

Analyses in flesh		O. Keta		O. Nerka		O. Kisutch	
			cis/trans %		*cis/trans* %		*cis/trans* %
Lipid content %		1.27		4.7		3.0	
Astaxanthin content ppm		3.4		34.3		20.9	
Underivatized astaxanthin sample		area %		area %		area %	
7,8,7′,8′-Tetradehydroastaxanthin		2.35		1.42		1.62	
7,8-Didehydroastaxanthin		5.95		5.24		5.92	
Astaxanthin	*cis*	20.60	23.45	11.22	12.95	15.59	16.35
Astaxanthin	*trans*	67.23	76.55	79.54	87.05	74.67	83.64
Not identified		3.87		1.98		3.20	
di-(−)-Camphanates		rel. area %		rel. area %		rel. area %	
(3*R*,3′*R*)-Astaxanthin	*cis*	3.32 } 15.56	21.3	1.57 }	12.4	1.85 } 12.25	15.1
(3*R*,3′*R*)-Astaxanthin	*trans*	12.24 }	78.7	11.14 } 12.71	87.6	10.40 }	84.9
meso-Astaxanthin	*cis*	0.51 } 1.93	26.4	0.62 } 2.01	30.8	0.89 } 3.17	28.1
meso-Astaxanthin	*trans*	1.42 }	73.6	1.39 }	69.2	2.28 }	71.9
(3*S*,3′*S*)-Astaxanthin	*cis*	(19.95)[b])		13.47)[b])		(14.77)[b])	
Corrected for[b])	*cis*	14.24 } 82.51	17.3	8.79 } 85.28	10.3	10.44 } 84.57	12.3
(3*S*,3′*S*)-Astaxanthin	*trans*	68.24 }	82.7	76.49 }	89.7	74.13 }	87.7

[b]) See Table 3.

Table 5. *Addition of (3S,3'S)-astaxanthin to unpigmented salmon flesh*

		Crystalline (3*S*,3'*S*)-astaxanthin			Gelatine preparation (5% astaxanthin)			Astaxanthin after addition and extraction from flesh		
		rel. area %		*cis/trans* %	rel. area %		*cis/trans* %	rel. area %		*cis/trans* %
Free astaxanthin										
	di-*cis*				3.95			4.77		
	9-*cis*	2.15		2.7	5.27		29	5.89		30
	13-*cis*	0.52			19.75			19.21		
	trans	97.32		97.3	71.02		71	70.04		70
Di-(–)-Camphanates										
meso-	*cis*	0.15	2.66	7	0.88	4.23	21	0.89	4.11	22
meso-	*trans*	2.51		93	3.35[a]		79	3.22[a]		78
3*S*,3'*S*-	*cis*	6.38	97.49	7	26.41	95.76	28	28.43	95.88	30
3*S*,3'*S*-	*trans*	91.11		93	69.35		72	67.45		70

[a]) Increased by *semi*-astacene.

The results suggest that salmon either have a high selection of feed, or that a C_3-isomerase is present as proposed by *Rønneberg et al.* for lobster eggs [4]. However, feeding experiments with racemic astaxanthin in salmon [12] showed that the astaxanthin extracted from flesh was still optically inactive, which makes the first hypothesis more likely.

Although a more or less unique deposition of free astaxanthin is found in salmon flesh, a wide spectrum of carotenoids is found in integuments [11], where metabolism obviously takes place. It is still unknown, why *e.g.* xanthophylls are deposited in skin as esters of higher fatty acids, while only free astaxanthin is found in flesh.

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