101. Metabolism of Carotenoids in Salmonids

Part 3[1])

Metabolites of Astaxanthin and Canthaxanthin in the Skin of Atlantic Salmon (*Salmo salar*, L.)

by **Katharina Schiedt**[a])*, **Max Vecchi**[b]), **Ernst Glinz**[b]), and **Trond Storebakken**[c])

[a]) Department of Vitamin and Nutrition Research and

[b]) Central Research Units of *F. Hoffmann-La Roche & Co. AG*, CH-4002 Basel

[c]) The Agricultural Research Council of Norway, Institute of Aquaculture Research, N-1432 As-NLH

(30.III.88)

Diets supplemented with astaxanthin and canthaxanthin, respectively, and a control diet without carotenoid additions, were fed to 1½-year-old Atlantic salmon (*Salmo salar*, L.) for one year. The integuments were investigated as to their quantitative and qualitative carotenoid composition. Astaxanthin and canthaxanthin deposited in the skin amounted to 20 and 14% of the total carotenoids only. Seventy % must be considered as metabolites of astaxanthin and canthaxanthin and 10% as basic xanthophylls also present in the control groups. Astaxanthin apparently underwent the following metabolic pathway: astaxanthin→idoxanthin→adonixanthin→zeaxanthin→zeaxanthin 5,6-epoxides. Reduction of the 4′-carbonyl group was stereospecific leading to the (4′*R*)-idoxanthin. Canthaxanthin was obviously converted to β,β-carotene *via* 4′-hydroxyechinenone, echinenone, and 4-hydroxy-β,β-carotene.

1. Introduction. – In a previous paper, the pigmenting efficacy of astaxanthin (= 3,3′-dihydroxy-β,β-carotene-4,4′-dione), astaxanthin dipalmitate, and canthaxanthin (= β,β-carotene-4,4′-dione) in Atlantic salmon (*Salmo salar*, L.) reared indoors in tanks has been reported by *Storebakken et al.* [2]. In that study, total carotenoids were determined in flesh and skin after administration of 0, 30, 60, and 90 mg of carotenoids per kg feed. An increased formation and deposition of idoxanthin (= 3,3′,4′-trihydroxy-β,β-caroten-4-one) was observed in the flesh and organs of the fish fed astaxanthin [1] [3] [4], whereas astaxanthin was found exclusively in the flesh under normal farming conditions [4] [5].

The purpose of the present study was to analyze the carotenoid composition in the skin, where metabolites are usually found. Particular emphasis was laid on the configurational analysis of astaxanthin, idoxanthin, and zeaxanthin (= β,β-carotene-3,3′-diol). It is known that only a minor portion of the dietary carotenoids is deposited unchanged in the skin of salmon. The major part are metabolized carotenoids [6].

2. Results. – 2.1. *Carotenoid Content and Composition in the Skin of Atlantic Salmon Reared in Tanks.* The content of total carotenoids and the percent composition in the skin of the various experimental groups, after a period of one year, are compiled in *Table 1*. The total lipids amounted to *ca.* 10% of fresh weight in all groups.

[1]) Part 1: [4]; Part 2: [1].

Table 1. *Carotenoid Composition of Salmon Skin after Astaxanthin and Canthaxanthin Administration, Compared with Control groups; % of Total Carotenoids.* Duration, 1 year; dosage $\bar{x}$ 60 mg/kg feed.

	Control group (start) $N = 9$	Control group (end) $N = 9$	Astaxanthin group (end) $N = 9$	Canthaxanthin group (end) $N = 9$
Carotenoid content in skin	8.7 µg/g	1.9 µg/g	5.5 µg/g	5.8 µg/g
β,β-Carotene	2.5%	3.2%	0.6%	33%
Cryptoxanthin	2.5	4.5	–	–
3′-Epilutein	81	67	9.1	4.7
Lutein	5	3	0.3	0.2
Zeaxanthin	6	15	10.3	0.5
Astaxanthin	–	–	19.3	–
β-Adonixanthin[a])	–	–	5.4	–
Idoxanthin	–	–	12.3	–
Canthaxanthin	1.3[b])	–	–	14
Echinenone	–	–	–	15
4′-Hydroxy-β,β-caroten-4-one	1.3[b])	–	–	7.7
β,β-Caroten-4-ol	–	–	–	4.9
β,β-Carotene-4,4′-diol	–	–	–	1.3
Epoxides				
5,6-Dihydro-β,β-carotene 5,6-epoxide[c])	–	–	–	0.6
5,6-Dihydrocryptoxanthin 5,6-epoxide[c])	–	–	1.3	–
5,6-Dihydrozeaxanthin 5,6-epoxide[d])	–	–	6.7	–
5,6,5′,6′-Tetrahydrozeaxanthin 5,6:5′,6′-diepoxide	–	–	4.1	–
Unidentified polar carotenoids				
Trihydroxy and epoxide derivatives	1	7	31	2

[a]) Identified as β-doradecin. [b]) Originating from pre-experimental feed. [c]) Identified as 5,8-epoxides. [d]) Partly identified as 5,8-epoxide.

2.2. *Astaxanthin Group. Absolute Configuration of Astaxanthin, Idoxanthin, and Zeaxanthin.* Regarding the stereoisomeric composition of astaxanthin, idoxanthin, and zeaxanthin *(Table 2)*, the following results were obtained: The maintenance of the 1:2:1 ratio of (3*S*,3′*S*)-, (3*R*,3′*S*; *meso*)-, and (3*R*,3′*R*)-astaxanthin in skin is in agreement with previous findings in the flesh of Atlantic salmon [5] and of rainbow trout [6] [7] confirming again that epimerization at C(3), C(3′) does not occur.

Four optical isomers of idoxanthin, the intermediate between astaxanthin and adonixanthin (see *Scheme 1*), were identified in the skin. All four isomers exhibited the (4′*R*)-configuration confirming the stereospecific reduction found previously also in other tissues and organs of salmon [1].

All three optical isomers (3*S*′,3′*S*)-, (3*R*,3′*R*)-, and *meso*-zeaxanthin were detected in all samples, but their ratios varied considerably.

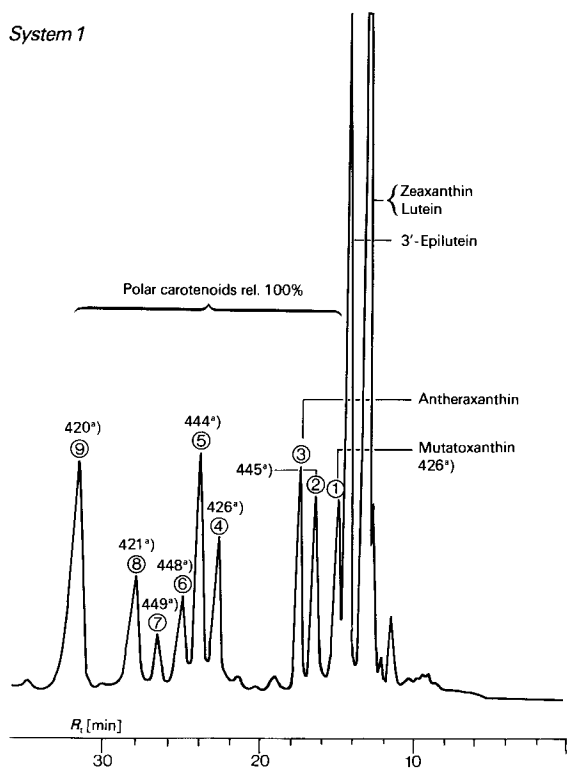
Epoxides. Most of the 5,6-epoxides originally present in the skin of the salmon fed with astaxanthin *(Table 1)* were identified as 5,8-epoxides after furanoid rearrangement by chromatography on silica gel. Chiroptical analyses were not carried out.

Mutatochrome (= 5,8-Epoxy-5,8-dihydro-β,β-carotene). The minute amount of mutatochrome separated from the β,β-carotene fraction was identified according to R_f value and VIS spectrum (λ_{max} 426 nm, hexane) only (see *Exper. Part*).

Table 2. *Configurational Analysis of Zeaxanthin, Astaxanthin, and Idoxanthin in Skin of Salmon*

	Control group (start)	Control group (end)	Astaxanthin group (end)	Canthaxanthin group (end)
Zeaxanthin[a])				
(3*R*,3′*R*)	84	75	9.7	77
(3*R*,3′*S*; *meso*)	4	4	39.4	7
(3*S*,3′*S*)	12	21	50.9	16
Astaxanthin[b])		–		–
(3*S*,3′*S*)			25	
(3*R*,3′*S*; *meso*)			51	
(3*R*,3′*R*)			24	
Idoxanthin[c])	–	–	–	
(3*S*,3′*S*,4′*R*) } 3′,4′-*cis*			24.4	
(3*R*,3′*S*,4′*R*) } 3′,4′-*cis*			57 { 40	
(3*S*,3′*R*,4′*R*) } 3′,4′-*trans*			57 { 17	
(3*R*,3′*R*,4′*R*) } 3′,4′-*trans*			18.3	

[a]) HPLC area [%] of corresponding dicarbamates.
[b]) HPLC area [%] of corresponding dicamphanates.
[c]) HPLC area [%] of corresponding tricarbamates.



[a]) λ_{max} in eluent.

Fig. 1. *HPLC separation of dihydroxy carotenoids and epoxides*

Dihydrozeaxanthin 5,6- and 5,8-Epoxides. From the HPLC (*Fig. 1*, representing a mixture of dihydroxy and more polar skin carotenoids, it may be seen that only *Peaks 1* and *3*, accounting for *ca.* 20 area-%, could be ascribed to antheraxanthin and mutatoxanthin of known configuration. The natural reference substances (isolated from *Viola tricolor*) were (3*S*,5*R*,6*S*,3′*R*)-antheraxanthin and the corresponding (8*R*)-mutatoxanthin previously assigned by ^{1}H-NMR and CD spectroscopy [8]. The *Peaks 4, 8,* and *9* may also be ascribed to diastereoisomeric 5,8-epoxides according to unpublished results on HPLC separations of synthetic and semi-synthetic mixtures of various diastereoisomeric 5,8-epoxides. But, because of lack of material, these unidentified peaks could not be investigated in detail. *Peaks 2, 5, 6,* and *7*, exhibiting VIS spectra with λ_{max} 445–449 nm and more or less fine structure, were also not identified. R_t and VIS of *Peak 5* were consistent with that of violaxanthin (5,6:5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-diol), indicating that these unknown compounds may have more O-functions.

2.3. *Canthaxanthin Group. 4′-Hydroxyechinenone (= 4′-Hydroxy-β,β-caroten-4-one).* The canthaxanthin metabolite 4′-hydroxyechinenone was identified by HPLC and VIS spectrophotometry in comparison with the synthetic racemic reference substance (see *Exper. Part*).

Isozeaxanthin (= β,β-Carotene-4,4′-diol). This further reduction product of canthaxanthin was identified by VIS and HPLC *(Fig. 2)* in comparison with the synthetic racemic reference substance.

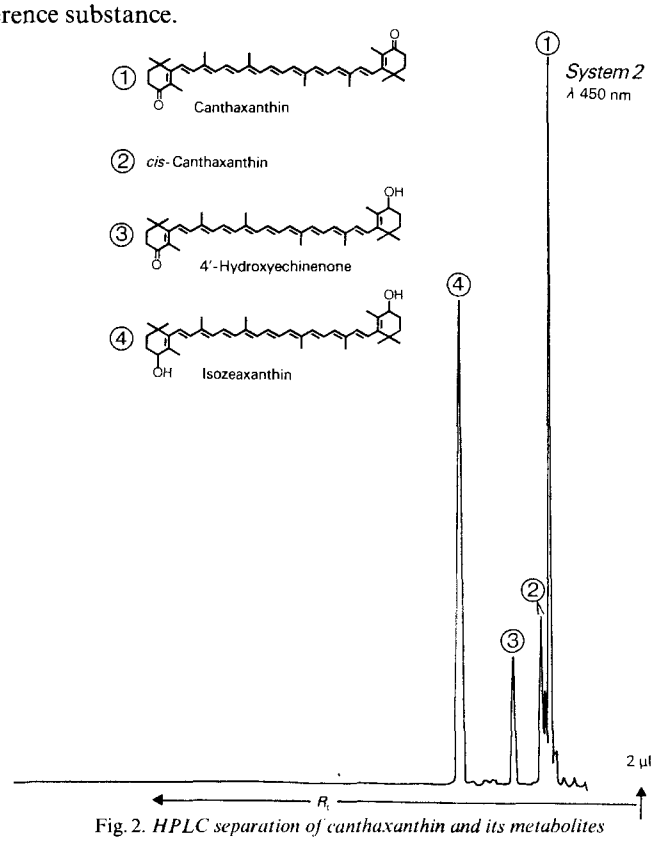


Fig. 2. *HPLC separation of canthaxanthin and its metabolites*

β,β-Caroten-4-ol, for which no reference substance was available, was identified by spectroscopic data (MS, ^{1}H-NMR, VIS, see *Exper. Part*).

No chiroptical analyses were carried out for the canthaxanthin metabolites.

3. Discussion. – The relatively high initial concentration of carotenoids (8.7 μg/g; *Table 1*) and the decrease in the control group to 1.9 μg/g after one year may indicate that the carotenoids in fingerlings are accumulated in the skin, where they are either utilized or redistributed into other target tissues. The relative-percent composition, however, remained the same throughout the study.

The skin of the groups fed with astaxanthin and canthaxanthin showed a higher carotenoid content (5.5 and 5.8 μg/g) than the control group, but differed considerably in their composition, although only a minor portion of astaxanthin (19%) and canthaxanthin (14%), respectively, was present *(Table 1)*. Approximately 70% of the carotenoids in the skin of the pigmented salmon must be attributed to metabolites.

It appears that adonixanthin (= 3,3′-dihydroxy-β,β-caroten-4-one), idoxanthin, zeaxanthin, and 5,6-dihydrozeaxanthin 5,6-epoxide were the main metabolites of astaxanthin, while canthaxanthin was converted to β,β-carotene with the intermediates 4′-hydroxy echinenone, echinenone, and β,β-caroten-4-ol. These results are in agreement with the preliminary findings in Atlantic salmon of the same trial after six months of pigmentation [6] and with those in rainbow trout [6] [9], with the exception of the intermediates idoxanthin and 4′-hydroxyechinenone which were not detected in rainbow trout.

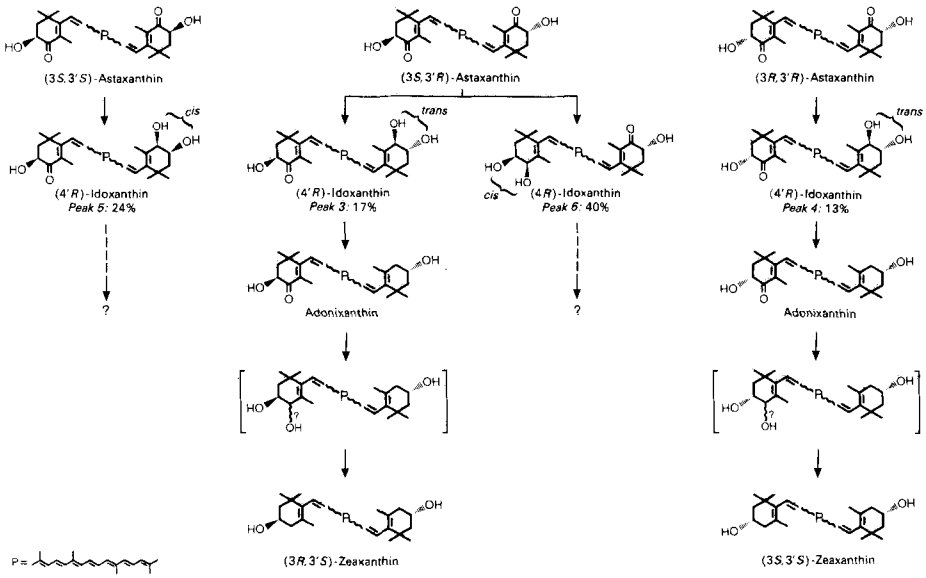
In the control group and in the canthaxanthin group, an almost constant ratio of (3*R*,3′*R*)-, (3*R*,3′*S*;*meso*)-, and (3*S*,3′*S*)-zeaxanthin of 80:5:15 was found *(Table 2)*, whereas a shift of the isomeric composition in favour of the (3*S*,3′*S*)- and (3*R*,3′*S*;*meso*)-zeaxanthin was observed in the astaxanthin group. The latter two compounds may be interpreted as metabolities of (3*R*,3′*R*)- and (3*R*,3′*S*,*meso*)-astaxanthin, confirming earlier findings in rainbow trout and in Atlantic salmon, where the occurrence of (3*R*,3′*S*;*meso*)- and (3*S*,3′*S*)-zeaxanthin was reported for the first time [6]. Further isolation of all three stereoisomers of zeaxanthin, which may also be considered as metabolites of astaxanthin, has been reported by *Maoka et al.* [10] in various other species of aquatic animals.

The conversion of 'racemic' astaxanthin to zeaxanthin merits some consideration from the enzymatic point of view *(Scheme 1)*. When the surplus of (3*R*,3′*S*;*meso*)- and (3*S*,3′*S*)-zeaxanthin is expressed in absolute quantitative figures *(Table 3)*, it must be concluded that these two stereoisomers were formed almost exclusively, while (3*S*,3′*S*)-astaxanthin was not degraded reductively to (3*R*,3′*R*)-zeaxanthin. A tentative metabolic pathway of the three optical astaxanthin isomers is depicted in *Scheme 1*.

Table 3. *Content of the Three Optical Isomers of Zeaxanthin in Skin of the Control and the Astaxanthin Group*

		Control (end)	Astaxanthin (end)
Total carotenoids	μg/g tissue	1.9	5.5
Zeaxanthin	μg/g tissue	0.28	0.56
(3*R*,3′*R*)	μg/g tissue	0.21	0.05
(3*R*,3′*S*; *meso*)	μg/g tissue	0.01	0.22
(3*S*,3′*S*)	μg/g tissue	0.06	0.29

Scheme 1. *Suggested Metabolic Pathway of Astaxanthin to Zeaxanthin in Atlantic Salmon*[a])



[a]) Peak numbers of the idoxanthin isomers refer to HPLC *Figure* of the preceding paper [1].

Chiral analysis of idoxanthin revealed that the configuration of astaxanthin did not influence the enzymatic reduction of the 4'-oxo group; however, the reduction itself was stereospecific leading to the (4'*R*)-hydroxy group irrespective of the configuration at C(3').

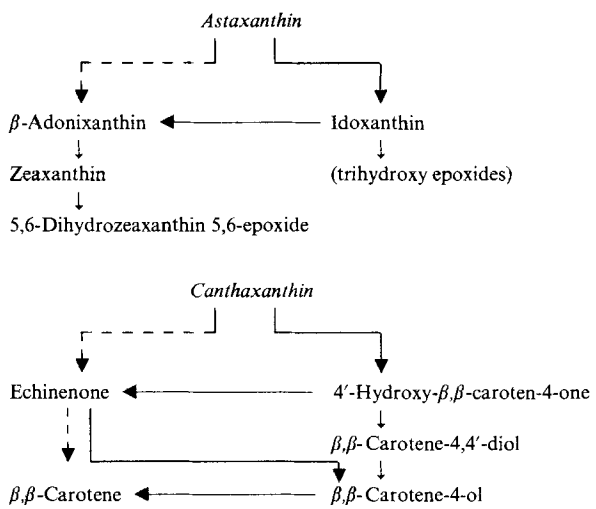
Interpretation of the further transformation of idoxanthin is more difficult. It seems that 3',4'-*trans*-idoxanthin is preferably, if not exclusively, attacked by the enzyme, thus leading to adonixanthin. Unfortunately, the chirality of adonixanthin was not investigated in this study. If the 4-carbonyl group of adonixanthin was further reduced by the same enzyme yielding also a (4*R*)-hydroxy group, then the 3,4-*trans* configuration of the trihydroxy carotenoid, which was not identified, could only originate from (3*R*,3'*R*)-astaxanthin. There was in fact no (3*R*,3'*R*)-zeaxanthin formed, but the considerable increase of (3*R*,3'*S*;*meso*)-zeaxanthin indicates that reduction of the 3-hydroxy-4-oxo end group of adonixanthin is not subject to the same enzymatic reaction as astaxanthin. The surplus of 3',4'-*cis*-idoxanthin isomers in the biological samples supports the theory that they do not follow the usual pathway to zeaxanthin, but are degraded to polar unidentified polyhydroxy carotenoids and epoxides (*Table 1* and *Fig. 1*) as already observed in the skin of sockey salmon (*Oncorhynchus nerka*) by *Evelyn* [11]. Referring to the epoxides, only two of the nine peaks present in *Fig. 1* were assigned: one to (3*S*,5*R*,6*S*,3'*R*)-antheraxanthin and one to (8*R*)-mutatoxanthin according to natural reference samples. However, after administration of 'racemic' astaxanthin, which was obviously reduced to zeaxanthin resulting mainly in the (3*S*,3'*S*)- and (3*R*,3'*S*;*meso*)-isomers, also other diastereoisomers of zeaxanthin, 5,6- and 5,8-epoxides, might be expected. However, not enough material was left for further identification.

We have obtained similar results in earlier trials in rainbow trout fed with 'racemic' tritiated astaxanthin: (3*S*,3′*S*)-zeaxanthin was formed preferably, followed by (3*R*,3′*S*; *meso*)- and only traces of (3*R*,3′*R*)-zeaxanthin (unpublished results). On the other hand, rainbow trout converted tritiated (3*S*,3′*S*)-astaxanthin to adonixanthin and to (3*R*,3′*R*)-zeaxanthin [6] [9]. Thus, either the individual optical isomers are attacked by different enzymes or they are competitive inhibitors for single enzyme systems. Idoxanthin was not detected in rainbow trout which might be due to an immediate elimination of the 4′-hydroxy group.

No chiral analyses were carried out for the canthaxanthin metabolites 4′-hydroxyechinenone and β,β-carotene-4,4′-diol. In hens, the enzymatic reduction of canthaxanthin was also stereospecific leading to (4′*S*)-hydroxyechinenone and (4*S*,4′*S*)-β,β-carotene-4,4′-diol [12]. These two compounds had the same configuration at C(4) as the idoxanthin from salmon. It is evident that the polar epoxides found in the astaxanthin group were missing after canthaxanthin administration (*Table 2*).

In summary, the metabolic pathways of astaxanthin and canthaxanthin in the integuments of Atlantic salmon, as described earlier [6], could be confirmed and extended by the more advanced chiral analysis.

Scheme 2



Experimental Part

1. *Animal Material and Feeding.* In tanks, 1½-year old salmon with an initial weight of 62 g were reared and pigmented with astaxanthin and canthaxanthin. Each carotenoid was admixed to the basic dry diet as H_2O-dispersible beadlets in dosages of 0, 30, 60, and 90 mg/kg. Detailed experimental design and feed composition have been published by *Storebakken et al.* [2]. After 56 weeks of pigmentation, the fish had reached an average weight of 406 g. For the present study, three fish of each experimental group were available. The skin was removed, minced, and stored in liq. N_2 until analyzed. The skin samples (25–30 g/animal) of the three dosages of each carotenoid group were pooled, because no dose-dependent differences in the carotenoid concentrations had been found at the end of the pigmenting period [2]. Qual. and quant. analysis of skin carotenoids of the control group was carried out at the beginning and at the end of the experiment.

2. *Extraction and Analysis of Total Carotenoids.* Extraction and spectrophotometric determination of the total of carotenoids in the skin and flesh of the various experimental groups have been published previously [2]. The pooled skin samples ($N = 9$) weighed *ca.* 300 g, yielding 30 g of lipid extract with a content of 1–2 mg of carotenoids.

3. *Chromatographic Separations and Isolation of Individual Carotenoids. TLC:* Silica gel *60* plates (F_{254}, 0.25 mm; *Merck*). AcOEt/hexane 1:1 for β,β-caroten-4-ol (R_f 0.64), canthaxanthin (R_f 0.5), 4′-hydroxy-β,β-caroten-4-on (R_f 0.4), and β,β-carotene-4,4′-diol (R_f 0.3). AcOEt/hexane 3:97 for β,β-carotene (R_f 0.5), and 5,8-epoxy-5,8-dihydro-β,β-carotene (R_f 0.1).

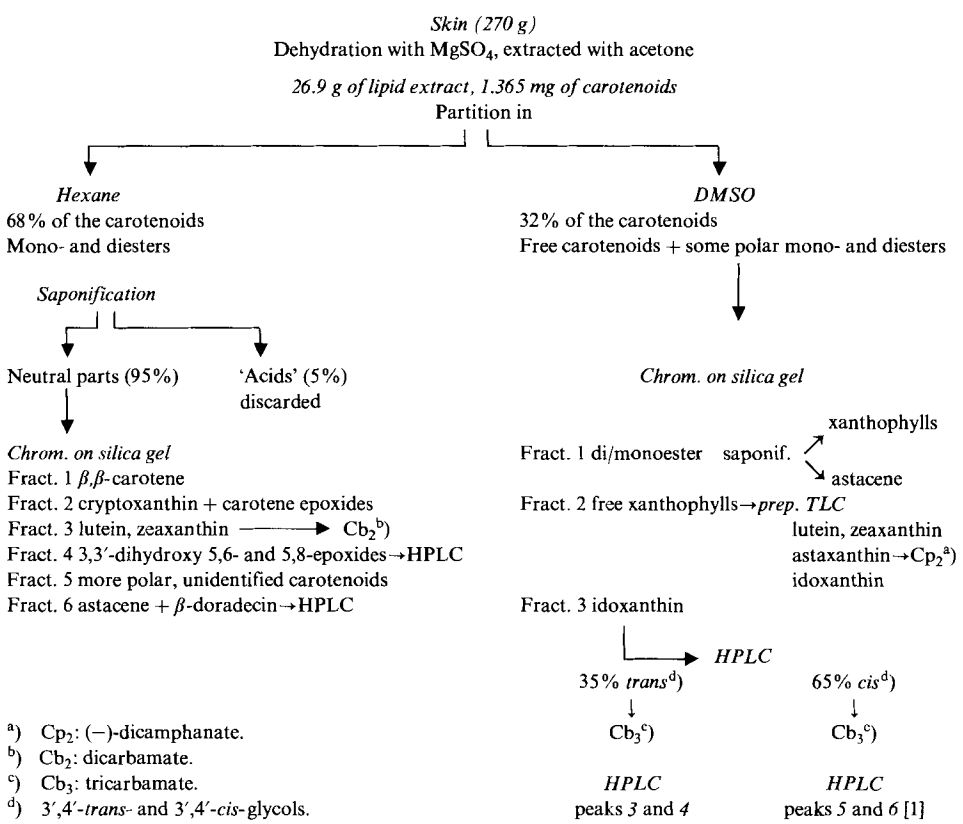
HPLC: System 1: Separation of carotenoid-diols and their 5,6- and 5,8-epoxides *(Fig. 1)*; stainless steel column, length 50 cm, inner diameter 3.1 mm; *Spherisorb S5-CN;* hexane/AcO(i-Pr)/i-PrOH/$(i\text{-Pr})_2$EtN 84.4:13:2.5:0.1; pressure/flow, 86 bar, 0.7 ml/min; detector, λ 450 nm.

System 2: Separation of canthaxanthin and possible metabolites *(Fig. 2)*. See *System 1*, differing from it: *Spherisorb S5-W* (silica); hexane/CH_2Cl_2/i-PrOH/$(i\text{-Pr})_2$EtN 90.9:6.5:2.5:0.1; pressure/flow, 80 bar, 1 ml/min.

4. *Alkaline Saponification.* The lipid extracts of the *negative control* and the *canthaxanthin* group were saponified under mild conditions: 20 ml of a 5% soln. of KOH in EtOH/H_2O 9:1 was added per 1 g of lipid residue dissolved in 10 ml of Et_2O, and the mixture was incubated at r.t. for 2 h under N_2. The non-saponifiable fraction was extracted with hexane/Et_2O 1:1, washed, and evaporated to dryness.

5. *Chromatography on Silica Gel* (0.06–0.2 mm, *Merck*). The carotenoid content of all fractions was determined by VIS spectrophotometry [1]. Separation of carotenes, monohydroxy and ketocarotenoids, dihydroxy-,

Scheme 3. *Isolation of Carotenoids from the Skin of Salmon Fed with Astaxanthin*



a) Cp_2: (−)-dicamphanate.
b) Cb_2: dicarbamate.
c) Cb_3: tricarbamate.
d) 3′,4′-*trans*- and 3′,4′-*cis*-glycols.

trihydroxycarotenoids, and polar epoxide-like compounds was achieved by elution with solvents of increasing polarity, namely Et_2O/hexane and EtOH/Et_2O mixtures. The single fractions were further purified and separated by TLC on commercial silica-gel plates and finally submitted to HPLC (*System 2, Fig. 2*). The individual peaks were quantified by their relative area-%, the carotenoids isolated and identified according to their retention time and UV/VIS spectra in comparison with the authentic synthetic compounds. Moreover, *β,β*-caroten-4-ol, for which no reference substance was available, was identified by MS and ^{1}H-NMR spectroscopy.

For the isolation of the carotenoids from the skin of the *astaxanthin*-pigmented salmon, the procedure had to be modified. The free astaxanthin, idoxanthin, and xanthophylls were separated from the esters by partition in hexane/dimethyl sulfoxide (DMSO). The esters were present in the hexane phase and the free carotenols in the DMSO phase. Re-extraction from DMSO was carried out as described earlier [13]. The ester fraction was saponified and the unsaponifiable matter separated by column chromatography on silica gel. Zeaxanthin was eluted with Et_2O, subsequently isolated from the xanthophylls by HPLC and the dicarbamate derivative prepared for configurational analysis. A more polar fraction containing the epoxides and other higher oxidized carotenoids was analyzed by HPLC (*System 1, Fig. 1*). The spectra of all peaks were recorded. Natural antheraxanthin (= (3*S*,5*R*,6*S*,3′*R*)-5,6-epoxy-5,6-didehydro-*β,β*-carotene-3,3′-diol) and (8*R*)-mutatoxanthin [8] were used as reference substances.

The whole procedure of extraction, saponification, chromatography, and identification is summarized in *Scheme 3*.

6. *Configurational Analysis of Carotenoids Zeaxanthin, Lutein, and 3′-Epilutein.* After saponification of the esters and chromatography, the dihydroxycarotenoids were collected in one fraction which always contained a certain amount of *cis*-isomers. The all-*trans*-fraction of each carotenoid was isolated by HPLC and transformed to dicarbamates, whereby the method [14] was modified and adapted to the 2–10-μg scale. The previous isolation of the all-*trans*-fraction of lutein and zeaxanthin facilitates quantification of the diastereoisomeric derivatives by avoiding coincidence of geometrical *cis*-isomers with different configurational isomers.

Preparation of the dicarbamates of 2–10 μg lutein or zeaxanthin with (+)-(*S*)-1-(1-naphthyl)ethyl isocyanate, see [1]. R_f of zeaxanthin dicarbamate 0.64, zeaxanthin = 0.17. HPLC, see [14]. The diastereoisomeric derivatives were assigned according to the chromatographic data of the authentic synthetic reference substances.

Astaxanthin. The all-*trans*-astaxanthin fraction was isolated by HPLC and transformed to the (–)-dicamphanate. The diastereoisomers were separated by HPLC and the single peaks assigned according to the synthetic reference substances [15].

Idoxanthin. The idoxanthin fractions were purified by TLC, reacted with (+)-(*S*)-1-(1-naphthyl)ethyl isocyanate, and the diastereoisomeric carbamates separated and quantified as described in [1].

β,β-Caroten-4-ol. Microgram amounts of *β,β*-caroten-4-ol isolated by HPLC were subjected to spectroscopic investigation. VIS (hexane): 426 (sh), *449*, 477. Because of impurities, interpretation of the ^{1}H-NMR (400 MHz, $CDCl_3$, 100% D) was restricted to the relevant shift of H–C(4) at 4.01 ppm [16]. MS: 552 (14, M^+, $C_{40}H_{56}O$), 534 (80, $M-$ 18), 442 (20, $M-$ 18– 92), 119 (95), 69 (100).

We should like to thank Dr. *G. Englert* and Mr. *W. Meister* for NMR and mass spectroscopy, Dr. *H. Mayer* for stimulating discussions and advice, and Mr. *S. Bischof* for his diligence in experimental work. The cooperation and continuous support of Prof. *S. Liaaen-Jensen*, Laboratory of Organic Chemistry, University of Trondheim, Norway, is also gratefully acknowleged.

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