100. Metabolism of Carotenoids in Salmonids[1])

Part 2[2])

Distribution and Absolute Configuration of Idoxanthin in Various Organs and Tissues of One Atlantic Salmon (*Salmo salar*, L.) Fed with Astaxanthin

by **Katharina Schiedt**[a])*, **Hans Mayer**[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)

The content of total carotenoids and the ratio astaxanthin/idoxanthin (= 3,3'-dihydroxy-β,β-carotene-4,4'-dione/3,3',4'-trihydroxy-β,β-caroten-4-one) in various organs and tissues of one Atlantic salmon (*Salmo salar*, L.) reared indoors in a tank were analyzed after feeding 'racemic' ((3*R*,3'*R*)/(3*R*,3'*S*; *meso*)/(3*S*,3'*S*) 1:2:1) astaxanthin (90 mg/kg feed) during one year. Configurational analysis of astaxanthin was carried out *via* the (−)-dicamphanate derivative and that of idoxanthin after reaction with (+)-(*S*)-1-(1-naphthyl)ethyl isocyanate. Separation of all eight optical isomers of idoxanthin-tricarbamate derivatives by HPLC is described. In salmon, enzymatic reduction of astaxanthin was found to be stereospecific leading to the (4'*R*)-hydroxy group irrespective of the configuration at C(3'), thus resulting in four different stereoisomers of idoxanthin formed from (3*R*,3'*R*), (3*R*,3'*S*; *meso*)-, and (3*S*,3'*S*)-astaxanthin present in the diet.

Introduction. – In previous papers, the formation and deposition of idoxanthin (= 3,3',4'-trihydroxy-β,β-caroten-4-one) in the flesh of Atlantic salmon after feeding astaxanthin (= 3,3'-dihydroxy-β,β-carotene-4,4'-dione) under particular experimental conditions has been reported [1] [2]. In the present study, the distribution of the two major carotenoids astaxanthin and idoxanthin in the body of one whole fish reared in a tank and fed synthetic 'racemic' astaxanthin is presented. Emphasis was laid on configurational analysis of the two carotenoids.

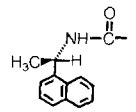
After administration of 'racemic' astaxanthin, a mixture of the three optical isomers (3*R*,3'*R*), (3*R*,3'*S*; *meso*), and (3*S*,3'*S*) in the ratio of 1:2:1, and subsequent reduction of the 4'-carbonyl group, theoretically, eight stereoisomers of idoxanthin may be expected (see *Table 1*).

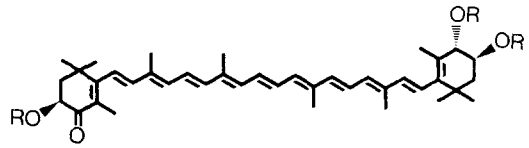
---

[1]) Parts of this series were presented at the 8th Int. Symp. on Carotenoids, Boston, USA, 1987, by *K. Schiedt*.

[2]) Part 1 [1].

Table 1. *Idoxanthin Stereoisomers* (R = H) *and Derivatives* (R = R[1]) *for HPLC Analysis*

R^1 = 

Peak No.	Isomer	Configuration
1		(3*S*,3′*S*,4′*S*)
2		(3*R*,3′*S*,4′*S*)
3		(3*S*,3′*R*,4′*R*)[a])
4		(3*R*,3′*R*,4′*R*)[a])
5		(3*S*,3′*S*,4′*R*)[a])
6		(3*R*,3′*S*,4′*R*)[a])
7		(3*S*,3′*R*,4′*S*)
8		(3*R*,3′*R*,4′*S*)

[a]) Detected in Atlantic salmon.

Results. – *Content and Distribution of Carotenoids in Various Tissues.* The weights of the various tissues analyzed, their content of total carotenoids, the ratio astaxanthin-idoxanthin, and the percent distribution of total carotenoids in the body are presented in *Table 2.*

Stereoisomeric Composition of Astaxanthin and Idoxanthin. The optical isomers of astaxanthin were determined *via* their (−)-dicamphanates and subsequent separation of the diastereoisomers by HPLC [3] [4].

A base-line resolution of the eight optical isomers of idoxanthin was achieved *via* the tricarbamate derivatives (see *Table 1*, R = R[1]), which were prepared by slight modification of the method described by *Rüttimann et al.* [5] in the zeaxanthin series. The procedure had to be adapted to the minute quantities of idoxanthin isolated from the

Table 2. *Distribution of Tissue Weights, Carotenoids, and Ratio Astaxanthin/Idoxanthin in One Salmon Fed with Astaxanthin during One Year* (90 mg/kg feed)

Organ/tissue	Weight [g]	% of body weight	Total carotenoids			% of total carotenoids		
			µg/g tissue	total amount µg	% in the various tissues	astaxanthin	idoxanthin	other carotenoids
Flesh	410	60	4	1640	74	65	31	4 n.i.[b])
Skin/fins	89	13	4	356	16	23	5	60 xanthophylls 9 adonixanthin 3 n.i.[b])
Intestine + pyloric caeca	49	7	1.3	63	2.8	22	78	–
Liver	6.4	0.9	7.5	48	2.2	27	39	34 xanthophylls
Kidneys	3.6	0.5	8	29	1.3	14	68	8 xanthophylls 10 n.i.[b])
Ovaries	2.6	0.4	28	73	3.3	68	32	–
Flesh, skin, and organs	560[a])	82		2209	(100)[a])			

[a]) Estimate without head, heart, and bones.
[b]) Not identified.

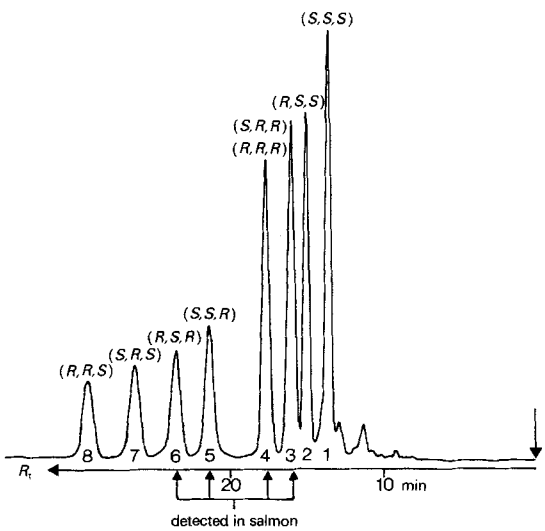


Figure. *HPLC separation of (3ξ,3′ζ,4′ξ)-idoxanthin tricarbamates.* Peaks 1–4: 3′,4′-*trans*-configurated isomers; peaks 5–8: 3′,4′-*cis*-configurated isomers.

biological samples. Assignment of the eight peaks *(Figure)* was possible by comparison with authentic standards prepared from the eight optical isomers of idoxanthins. The latter had been prepared by partial reduction of the three astaxanthin stereoisomers and characterized by ^{1}H-NMR and CD spectroscopy [6].

The stereoisomeric composition of both carotenoids in the various tissues is presented in *Table 3*.

Table 3. *Astaxanthin/Idoxanthin Content and Ratio (%) of Configurational Isomers in Various Tissues of One Salmon Fed with Astaxanthin during One Year* (90 mg/kg feed)

Tissue	Astaxanthin				Idoxanthin				
	µg/g tissue	Ratio of configurational isomers			µg/g tissue	Ratio of configurational isomers			
		(3*R*,3′*R*)	(3*RS*,3′*RS*; meso)	(3*S*,3′*S*)		(3*R*,3′*R*,4′*R*)	(3*R*,3′*S*,4′*R*)	(3*S*,3′*R*,4′*R*)	(3*S*,3′*S*,4′*R*)
Flesh	2.5	27	48	25	1.2	24	32	23	21
Skin/fins	0.92	22	51	27	0.21	17	50	16	17
Intestine + pyloric caeca	0.28	23	49	28	1.0	25	35	21	19
Liver	2.0	37	46	17	2.9	37	27	26	10
Kidneys	1.1	22	53	25	5.4	25	35	25	15
Ovaries	19	22	48	30	9	26	26	29	19

Discussion. – The flesh contained 74% of total carotenoids in the body and the ratio astaxanthin/idoxanthin was *ca.* 2:1. The immature ovaries showed already a high content of carotenoids (28 µg/g) and the same ratio astaxanthin/idoxanthin as in flesh. An accumulation of idoxanthin was found in the intestine and pyloric caeca, where obviously reduction takes place during absorption, and in kidneys. In the skin, a ratio astaxanthin/idoxanthin of 5:1 was found, but the major carotenoids were zeaxanthin, 3′-epilutein, lutein, and β-adonixanthin. Details on metabolites of astaxanthin in the skin of salmon are reported in the following paper of this series [7]. Idoxanthin in skin was present in the free form, while most other carotenols were esterified.

The finding that the isomeric composition of astaxanthin *(Table 3)* in the investigated tissues of salmon was almost unchanged in comparison with that of the synthetic 'racemic' mixture administered is in agreement with earlier results in flesh [8]. Only in liver, a slight decrease of (3*S*,3′*S*)-astaxanthin was noted in favour of the (3*R*,3′*R*)-isomer, which might be explained by preferred metabolization of the (3*S*,3′*S*)-isomer to zeaxanthin or by a favourite deposition of the (3*R*,3′*R*)-isomer.

As to the stereoisomeric composition of idoxanthin *(Table 3)*, it is evident that only four configurational isomers were encountered. Consequently, enzymatic reduction of the 4′-carbonyl was stereospecific and led to the (4′*R*)-hydroxy group. The ratio of (3*R*,3′*R*,4′*R*)- and (3*S*,3′*S*,4′*R*)-idoxanthin was almost identical with that of the respective enantiomers of astaxanthin from which they are derived. The sum of the other two stereoisomers of idoxanthin, (3*R*,3′*S*,4′*R*) and (3*S*,3′*R*,4′*R*), the reduction products of *meso*-astaxanthin, is also in agreement with the 50% previously found in the stereoisomeric mixture of astaxanthin. In conclusion, it may be stated that all three astaxanthin stereoisomers were reduced to the same extent.

So far, a complete configurational analysis of idoxanthin was only carried out by *Matsuno* and *Sakaguchi* [9], who assigned the (3*S*,3′*S*,4′*R*)-configuration to the compound isolated from the Japanese sandfish, *Arctoscopus japonicus*. However, after the detection of the wide distribution of enantiomeric and *meso*-astaxanthin in various aquatic animals [10], other configurational isomers of idoxanthin might be expected as their metabolites after enzymatic reduction.

Experimental Part

Animals and Experimental Conditions. As described in [11] and [1], 75 1.5-year-old salmons (starting weight 62 g) were reared indoors in a tank (1 × 1 × 1.5 m). They were fed with a dry diet containing 90 mg of 'racemic' astaxanthin/kg ((3*R*,3′*R*)/(3*R*,3′*S*)/(3*S*,3′*S*) = 1:2:1) during 1 year. The average weigth of the fish at the end of the pigmenting period was 0.4 kg. One of the larger, well pigmented fish (live weight 684 g) was chosen for the analysis of carotenoids in the various organs and tissues. It was stored deepfrozen (−25°) until analyzed.

Extraction and Analysis of Carotenoids. The fish was thawed, skin and fins were removed from the flesh and the various organs excised. The whole organs, skin, and fins were employed, whereas an aliquot of 280 g of flesh was used. The samples were dehydrated with $MgSO_4$ and extracted with acetone. After evaporation to dryness, all lipid extracts, except that of skin, were partitioned in hexane/dimethyl sulfoxide (DMSO) for removal of colourless lipids, which were discarded. Only in the case of liver, the hexane phase remained yellow and was, therefore, further analyzed. The free xanthophylls were re-extracted from the DMSO phase. Experimental details are described in [3]. The hexane was washed with H_2O and evaporated. TLC and spectrophotometry of the residue revealed a UV (λ_{max} 330 nm in hexane) typical for a mixture of vitamin A_1 and A_2 (esters). According to the VIS, no carotenoids were present in the hexane phase.

UV/VIS Spectrophotometry. The carotenoids of all extracts and chromatographic fractions were determined by VIS spectrophotometry in hexane/EtOH 97:3 using *E* (1%, 1 cm) = 1910 for astaxanthin and idoxanthin and 2300 for xanthophylls (3′-epilutein, zeaxanthin).

Chromatographic Separations and Isolation of Astaxanthin and Idoxanthin. The residues of the reextracted DMSO phase of *liver, kidney,* and *ovaries* (10–50 mg) were submitted to prep. TLC on silica-gel plates (*Merck,* silica gel *60* F_{254}; 20 × 20 cm, 0.25 mm; AcOEt/hexane 2:1). The astaxanthin zone and the two zones of idoxanthin (3′,4′-*cis*- and 3′,4′-*trans*-glycols) were desorbed with Et_2O/EtOH 95:5 and the ratio of the two carotenoids determined spectrophotometrically. Subsequently, both fractions were submitted to HPLC and the all-*trans*-isomers of astaxanthin and that of the 3′,4′-*cis*- and 3′,4′-*trans*-glycols of idoxanthin isolated separately for configurational analysis.

The residue of the reextracted DMSO phase of the *flesh* (760 mg) and that of *intestine* (191 mg) were purified by column chromatography on silica gel (0.063–0.2 mm, *Merck*), starting with Et_2O/hexane 1:9 and continuing with increasing polarity. Astaxanthin was eluted with Et_2O/hexane/MeOH 30:67:3, idoxanthin with Et_2O/MeOH/AcOH 94:3:3. The idoxanthin fraction was washed with H_2O. After evaporation, the carotenoid content was determined spectrophotometrically and the ratio astaxanthin/idoxanthin calculated. The two fractions were repurified by prep. TLC as described above and then submitted to HPLC for the isolation of the respective all-*trans*-isomers.

The lipid extract of the *skin* and *fins* was chromatographed on a column of silica gel in order to obtain a crude separation of the yellow xanthophyll esters from astaxanthin esters, astaxanthin, and idoxanthin. *Fraction 1* (xanthophyll esters) was eluted with Et_2O/hexane 1:9, *Fraction 2* with Et_2O (astaxanthin di- and monoesters, xanthophyll monoesters), and *Fraction 3* with Et_2O/MeOH/AcOH 94:3:3 (free astaxanthin, epilutein, zeaxanthin, and idoxanthin according to TLC). Subsequently, prep. separation of *Fraction 3* was effected on one silica-gel plate in the above mentioned solvent system. The three zones, astaxanthin, xanthophylls, and idoxanthin were desorbed as indicated above and quantified by VIS spectrophotometry. The purified astaxanthin and idoxanthin fractions were submitted to HPLC and their all-*trans*-isomers isolated for configurational analysis. *Fractions 1* and *2* were saponified, neutral parts and 'acids' extracted and analyzed for idocin (= 3′,4′-dihydroxy-β,β-carotene-3,4-dione) and astacene (= all-*trans*-β,β-carotene-3,3′,4,4′-tetrone). In *Fraction 2,* astacene and β-oradecin (= all-*trans*-β,β-carotene-3,4-dione) were identified by TLC on citric acid coated silica gel plates with AcOEt/hexane 1:1, whereas no idocin was found in the ester *Fractions 1* and *2*. The neutral components of *Fraction 1* consisted of 24% of 3′-epilutein, 0.5% of lutein and 74% of zeaxanthin according to HPLC.

Configurational Analysis of Astaxanthin. The material isolated by HPLC (3–50 μg/sample) was transformed to the (−)-dicamphanate [3] and the diastereoisomers separated by HPLC [4].

Configurational Analysis of Idoxanthin. A mixture of 2–10 μg of idoxanthin, 2 drops of Et_3N, 1 drop of $CHCl_3$, ½ drop of (+)-(*S*)-(1-naphthyl)ethyl isocyanate, and 1–2 small crystals of 4-(dimethylamino)pyridine was incubated at r.t. under N_2 overnight, then dissolved in Et_2O and applied to a commercial silica-gel plate (5 × 20 cm) or, preferably, to a 10 × 10-cm plate for high potency TLC using AcOEt/hexane 1:1 as developing system (R_f 0.47 and 0.56 for 3′,4′-*cis*- and 3′,4′-*trans*-glycol derivatives, resp.). The two zones were desorbed with Et_2O and submitted to HPLC.

HPLC Systems and Chromatographic Conditions. Column: stainless steel, length 25 cm, i.d. 4 mm; stationary phase: *Nucleosil 100-5;* detector: *UVIKON 725-* E 0.2 - λ 450 nm.

Isolation of astaxanthin and idoxanthin, 3',4'-*cis*- and 3',4'-*trans*-glycols: mobile phase: hexane/CH_2Cl_2/i-PrOH 88.5:10:1.5 for astaxanthin, hexane/CH_2Cl_2/i-PrOH 83:10:7 for idoxanthin; pressure/flow: 75 bar, 1.2 ml/min.

Separation of idoxanthin tricarbamates: mobile phase: hexane/AcOEt/EtOH/$(i\text{-Pr})_2$EtN 88.9:10:1:0.1; pressure/flow: 48 bar, 1.2 ml/min.

The individual diastereoisomeric derivatives were quantified by peak area.

We should like to thank the following members of *F. Hoffmann-La Roche & Co. AG,* Basle, for their valuable contributions: Drs *G. Englert* and *K. Noack* for NMR and CD spectroscopy, Mr. *K. Holzhauser* for partial synthesis of the idoxanthin isomers and Mr. *S. Bischof* for his excellent experimental work.

REFERENCES

[1] K. Schiedt, T. Storebakken, P. Foss, S. Liaaen-Jensen, submitted to *Comp. Biochem. Physiol.*
[2] P. Foss, T. Storebakken, E. Austreng, S. Liaaen-Jensen, 7th International Symposium on Carotenoids, Munich, 27–31 August, 1984, abstract of contributed paper, p. 69.
[3] K. Schiedt, F. J. Leuenberger, M. Vecchi, *Helv. Chim. Acta* **1981,** *64,* 449.
[4] M. Vecchi, R. K. Müller, *J. High Res. Chromat. Commun.* **1979,** *2,* 195.
[5] A. Rüttimann, K. Schiedt, M. Vecchi, *J. High Res. Chromat. Commun.* **1983,** *6,* 612.
[6] H. Mayer, G. Englert, K. Noack, M. Vecchi, K. Schiedt, in preparation.
[7] K. Schiedt, M. Vecchi, E. Glinz, T. Storebakken, *Helv. Chim. Acta* **1988,** *71,* 887.
[8] T. Storebakken, P. Foss, E. Austreng, S. Liaaen-Jensen, *Aquaculture* **1985,** *44,* 259.
[9] T. Matsuno, S. Sakaguchi, *Bull. Jpn. Soc. Scient. Fish.* **1983,** *49,* 1475.
[10] T. Matsuno, T. Makao, M. Katsuyama, M. Ookubo, K. Katagiri, H. Jimura, *Bull. Jpn. Soc. Scient. Fish.* **1984,** *50,* 1589.
[11] T. Storebakken, P. Foss, K. Schiedt, E. Austreng, S. Liaaen-Jensen, U. Manz, *Aquaculture* **1987,** *65,* 293.