



HAL
open science

Determination of astaxanthin stereoisomers and colour attributes in flesh of rainbow trout (*Oncorhynchus mykiss*) as a tool to distinguish dietary pigmentation source

Vittorio Maria Moretti, Tiziana Mentasti, Federica Bellagamba, Umberto Luzzana, Fabio Caprino, Giovanni Turchini, Ivan Giani, Franco Valfrè

► To cite this version:

Vittorio Maria Moretti, Tiziana Mentasti, Federica Bellagamba, Umberto Luzzana, Fabio Caprino, et al.. Determination of astaxanthin stereoisomers and colour attributes in flesh of rainbow trout (*Oncorhynchus mykiss*) as a tool to distinguish dietary pigmentation source. *Food Additives and Contaminants*, 2006, 23 (11), pp.1056-1063. 10.1080/02652030600838399 . hal-00577494

HAL Id: hal-00577494

<https://hal.science/hal-00577494>

Submitted on 17 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Determination of astaxanthin stereoisomers and colour attributes in flesh of rainbow trout (*Oncorhynchus mykiss*) as a tool to distinguish dietary pigmentation source

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2005-384.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	01-Jun-2006
Complete List of Authors:	Moretti, Vittorio; University of Milan, VSA Mentasti, Tiziana; University of Milan, VSA Bellagamba, Federica; University of Milan, VSA Luzzana, Umberto; ASA - Naturalleva Caprino, Fabio; University of Milan, VSA Turchini, Giovanni; School of Ecology and Environment, Deakin University Giani, Ivan; University of Milan, VSA Valfrè, Franco; University of Milan, VSA
Methods/Techniques:	Chromatography - HPLC, Traceability
Additives/Contaminants:	Additives general, Colours, Feeding, Nutrition
Food Types:	Animal, Animal feed, Feeding, Fish

SCHOLARONE™
Manuscripts

1
2
3
4 **Determination of astaxanthin stereoisomers and colour attributes in flesh of**
5 **rainbow trout (*Oncorhynchus mykiss*) as a tool to distinguish the dietary**
6 **pigmentation source**
7
8
9
10
11
12
13
14

15 Vittorio Maria Moretti^{1*}, Tiziana Mentasti¹, Federica Bellagamba¹, Umberto Luzzana², Fabio
16
17 Caprino¹, Giovanni Mario Turchini³, Ivan Giani¹ and Franco Valfrè¹
18
19
20
21

22 ¹ *Department of Veterinary Science and Technology for Food Safety, University of Milan.*
23 *Via Trentacoste 2, 20134 - Milan, Italy*
24

25 ² *A.S.A. S.p.a. – Naturalleva*
26 *Viale del Lavoro, 45, 37036 – San Martino Buon Albergo (VR), Italy*
27
28

29 ³ *School of Ecology & Environment, Deakin University*
30 *PO Box 423, Warrnambool, Victoria 3280, Australia*
31
32
33
34
35
36
37
38

39 * To whom correspondence should be addressed:
40

41 Phone: 0039-02-50315760

42 Fax: 0039-02-50315746

43 e-mail: vittorio.moretti@unimi.it
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

The presence of carotenoids in animal tissue reflects their sources along the food chain. Astaxanthin, the main carotenoid used for salmonid pigmentation, is usually included in the feed as a synthetic product. However, other dietary sources of astaxanthin such as shrimp or krill wastes, algae meal or yeasts are also available on the market. Astaxanthin possesses two identical asymmetric atoms at C-3 and C-3' making possible three optical isomers with all-*trans* configuration of the chain: 3*S*,3'*S* ; 3*R*,3'*S* ; 3*R*,3'*R*. The distribution of the isomers in natural astaxanthin differs from that of the synthetic product. This latter is a racemic mixture, with a typical ratio of 1:2:1 (3*S*,3'*S* : 3*R*,3'*S* : 3*R*,3'*R*), while astaxanthin from natural sources has a variable distribution of the isomers deriving from the different biological organism that synthesised it. The high performance liquid chromatographic (HPLC) analysis of all-*trans* isomers of astaxanthin was performed in different pigment sources, such as red yeast *Phaffia rhodozyma*, alga meal *Haematococcus pluvialis*, krill meal and oil, and shrimp meal. With the aim to investigate astaxanthin isomer ratios in flesh of fish fed different carotenoid sources, three groups of rainbow trout were fed for 60 days diets containing astaxanthin from synthetic source, *Haematococcus pluvialis* algae meal and *Phaffia rhodozyma* red yeast. Moreover, the distribution of optical isomers of astaxanthin in trout purchased on the Italian market was investigated. A characteristic distribution of astaxanthin stereoisomers was detected for each pigment sources and such distribution was reproduced in the flesh of trout fed with that source. Colour values measured in different sites of fillet of rainbow trout fed with different pigment sources showed no significant differences. Similarly, different sources of pigment (natural or synthetic) produced colour values of fresh fillet with no relevant or significant differences. The coefficient of distance computed amongst the feed ingredient and the trout fillet astaxanthin stereoisomers was a useful tool to identify the origin of the pigment used on farm.

Keywords:-**Introduction**

Carotenoids are widely distributed in nature and present in many plants, algae, micro-organisms and animals. Although carotenoids are largely diffused in the plant and animal kingdoms, they are synthesized *de novo* only in higher plants and protists (Goodwin, 1992). Consequently, the traceability of carotenoids in animal reflects the presence of their sources along with the food chain. Animal carotenoids mainly result from metabolic transformation (oxidation and/or reduction) of those present in the food chain. Aquatic animals constitute a considerable rich source of carotenoids, which are in many cases responsible of organoleptic characteristics considered by the consumer (bright colours in lobster, shrimp, salmon, fish eggs) (Shahidi et al. 1998, Matsumo 2001). Moreover, among the large number of pigments present in living organisms (i.e. chlorophylls, anthocyanins and porphyrins), carotenoids play an important role in protecting cells against photosensitised oxidation (Palozza and Krinsky 1992).

In farmed salmonid, astaxanthin is mainly used for pigmentation and it is usually included in the feed as a synthetic product. However, other natural dietary sources of astaxanthin such as shrimp or krill wastes, algae meal or yeasts are also available on the market. All-*trans* astaxanthin may exist as three stereoisomers: two enantiomers ($3R,3'R$ and $3S,3'S$) and a *meso* form ($3R,3'S$; $3S,3'R$). The distribution of the all-*trans* stereoisomers in natural astaxanthin differs from that of the synthetic product. This latter is a racemic mixture of three isomers, with a typical ratio of 1:2:1 ($3S,3'S$: $3R,3'S$: $3R,3'R$), while astaxanthin from natural sources has a

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

variable distribution of the isomers deriving from the different biological organism that synthesised it.

Crustaceans belong to the class of marine animals that can oxidise carotenoids at the 3,3' and 4,4' position. Particularly, shrimp (*Penaeus spp.*) are able to convert by oxidative transformation dietary carotenoids such as β -carotene, zeaxanthin and canthaxanthin into astaxanthin and they are also able to absorb astaxanthin (Matsumo 1992, Schiedt et al. 1993, Sachindra et al. 2005). Similarly, in krill (*Euphausia spp.*) astaxanthin and its esters represent the major carotenoid component. A metabolic transformation to astaxanthin stereoisomers from other carotenoid sources is hypothesized as well in zooplanktonic crustaceans (Takaichi et al. 2003).

The red yeast *Phaffia rhodozyma* produces and accumulates carotenoids in lipid droplets into the cytoplasmatic membranes. Andrewes et al., (1976) reported that free astaxanthin was the most abundant carotenoid in *Phaffia* with 83-87% of the total pigment mixture. Other carotenoids such as β -carotene (2-2.5% of total carotenoids), echinenone (2-4% of total carotenoids), 3-hydroxyechinenone (3-4.5% of total carotenoids) and phoenicoxanthin (5-7% of total carotenoids) were also isolated red yeast culture. The predominant optical isomer of *Phaffia rhodozyma* unesterified astaxanthin is the 3*R*,3'*R* form.

The *Haematococcus pluvialis* microalgae mainly contains astaxanthin monoesters linked to short chain fatty acids. The astaxanthin pool of encysted *Haematococcus* is approximately 70% monoesters, 25% diesters and 5% free (Lorenz and Cysewski 2000). All of free astaxanthin and its mono and di- esters in *Haematococcus pluvialis* have predominantly the 3*S*,3'*S* form. Since commercially grown *Haematococcus pluvialis* can accumulate >30 g astaxanthin of dry matter,

1
2
3
4 the alga meal represents a good source of natural astaxanthin that found an increasing use in
5
6 nutrition of farmed fish (Olaizola and Huntly 2003).
7
8
9

10
11 The distribution of astaxanthin stereoisomers has been investigated in different tissues of
12
13 crustaceans (Renstrøm et al 1981, Coral-Hinostroza and Bjerkeng 2002), trout (*Oncorhynchus*
14
15 *mykiss*) (Storebakken and No 1992, Bjerkeng et al. 1997, Østerlie et al. 1999,) and salmon
16
17 (*Oncorhynchus spp* and *Salmo salar*) (Arai et al. 1987, Turujman et al. 1997). Astaxanthin
18
19 deposition in white muscle of salmonid reflects the optical isomers ratio of dietary astaxanthin
20
21 (Foss et al. 1984, Storebakken and No, 1992, Storebakken et al. 2004). On the contrary, the
22
23 distribution of astaxanthin stereoisomers in skin and posterior kidney of rainbow trout has been
24
25 observed to be different from that of dietary source, indicating some selectivity in the tissue
26
27 metabolism of the optical isomers of astaxanthin. In particular Østerlie et al. (1999) found in
28
29 rainbow trout fed synthetic astaxanthin an higher percentage of 3S,3'S isomer and a lower
30
31 percentage of 3R,3'R isomer in kidney and skin, if compared to the percentage of optical
32
33 isomers in the diet, suggesting a degree of epimerization of (3S,3'S)-astaxanthin in these tissues.
34
35
36
37
38
39
40
41

42 High performance liquid chromatographic (HPLC) analysis of all-*trans* isomers of astaxanthin
43
44 has been described in recent years by several authors (Vecchi and Müller 1979, Maoka et al.,
45
46 1985, Turujman 1993, Turujman et al. 1997, Østerlie et al. 1999). The separation of optical
47
48 isomers could be achieved on an achiral column after derivatization with a suitable derivatizing
49
50 reagent in order to obtain the diastereoisomers, that could be easily resolved on an achiral phase
51
52 column. The derivatizing reagent more used for this purpose in literature is camphenic acid
53
54 chloride (Vecchi and Müller 1979, Bowen et al. 2002). Another approach is to perform direct
55
56 HPLC analysis of underivatized astaxanthin on a chiral column. This latter method permits to
57
58
59
60

1
2
3
4 avoid various problems encountered during the derivatization step and to reduce the analysis
5
6 time to less than 15 minutes (Turujman 1993, Turujman et al. 1997).
7
8
9

10
11 This study was designed in order to investigate the ratios of optical isomers of astaxanthin from
12 different synthetic and natural sources used for rainbow trout pigmentation. Different
13 ingredients such as yeasts of the genus *Phaffia* (*Phaffia rhodozyma*), *Haematococcus pluvialis*
14 algae meal, Antarctic krill meal, krill oil and shrimp meal were analysed. Afterwards, with the
15 aim to investigate astaxanthin isomer ratios in flesh of fish fed different carotenoid sources,
16 three groups of rainbow trout were fed for 60 days diets containing 60 mg kg⁻¹ of astaxanthin
17 from synthetic source, *Haematococcus pluvialis* algae meal and *Phaffia rhodozyma* yeast.
18 Moreover, in order to investigate the reliability of the optical isomers distribution to distinguish
19 the type of pigmentation of commercially farmed trout available on the Italian market, nine
20 specimens of pigmented rainbow trout farmed in different aquaculture plants of Northern Italy
21 were also purchased in local retailers and analysed.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39

40 **Materials and methods**

41 *Chemicals and reagents*

42
43
44
45 Standard astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) was a gift from Hoffmann-La-
46 Roche Inc. (Basel, Switzerland).
47
48
49

50
51 Hexane, tetrahydrofuran (THF), methylene chloride, 2-propanol, acetonitrile, acetone,
52 petroleum ether were LC grade and from Merck (Darmstadt, Germany); dimethyl sulfoxide
53 (DMSO), triethylamine, sodium sulphate decahydrate and sodium sulphate anhydrous supplied
54 by Sigma-Aldrich (St. Louis, MO, USA). Cholesterol esterase from *Pseudomonas fluorescens*
55
56
57
58
59
60

1
2
3
4 used for hydrolysis of carotenoids esters was purchased from Sigma (St. Louis, MO, USA). Sep-
5
6 Pak silica gel cartridges were from Waters (Milford, MA, USA).
7
8
9

10 11 *Feed ingredients*

12
13 Unesterified astaxanthin (Carophyll[®] pink) and canthaxanthin (Carophyll[®] red) were from
14
15 Hoffmann-La-Roche Inc. (Basel, Switzerland), microalgae meal from *Haematococcus pluvialis*
16
17 (Naturrose[®]) purchased from Cyanotech Corporation (Kailua-Kona, HI, USA) and red yeast
18
19 meal (Astaxin[®]) (*Phaffia rhodozyma*) was from Igene Biotechnology Inc. (Columbia, MD,
20
21 USA). Antarctic krill meal, krill oil and shrimp meal were supplied by Tepual SA (Santiago,
22
23 Chile).
24
25
26
27
28
29
30

31 *Fish and experimental conditions*

32
33 Three outdoor raceways (L=50 X W=4 X H=1.5) were stocked each with 1900 kg rainbow trout
34
35 (average weight 350 g) and were reared under normal production scale for 60 days. During the
36
37 trial, oxygen levels were always over 80% saturation and water temperature was 11±1 °C. Three
38
39 pigmented diets were used in this study. Diet 1 contained Carophyll Pink (CP), diet 2 contained
40
41 *Haematococcus pluvialis* algae meal (HP) and diet 3 contained *Phaffia rhodozyma* red yeast
42
43 (PR). The level of inclusion in all three diets was 60 mg kg⁻¹ astaxanthin (free or esterified). The
44
45 three groups of fish were fed with commercial extruded feed (41% protein, 26% fat) coated with
46
47 different synthetic source. The feed ratio was set at 1% of the biomass per day. No mortalities
48
49 occurred during the experiment. At the end of the trial, ten fish per group were taken and
50
51 transported to the laboratory for colour measurements and astaxanthin analysis. Parallely, nine
52
53 specimens of pigmented rainbow trout from unknown origin were purchased from local retailers
54
55 and analysed.
56
57
58
59
60

1
2
3
4
5
6
7 *Astaxanthin extraction from feed ingredients*
8

9 Carotenoids from algae and yeast pigments were extracted by suspending 25 mg of sample in 5
10 mL of dimethyl sulfoxide (DMSO) and incubating in a thermostatic shaking bath at 50°C for 30
11 minutes. At the end, the sample was centrifuged at 4000 rpm for 10 minutes and the supernatant
12 collected. The obtained pellet was homogenized for 2 min using an UltraTurrax (Janke &
13 Kunkel, IKA, Staufen, Germany) in 5 mL of acetone, then centrifuged at 4000 rpm for 15
14 minutes. Acetone extraction was repeated until the organic phase resulted colourless. Similarly,
15 acetone extraction was used with Antarctic krill meal, krill oil, shrimp meal and with synthetic
16 astaxanthin source. To avoid oxidation and isomerisation of astaxanthin, all manipulations were
17 performed in diffuse light and conditioned room (20°C), under acid-free conditions and using
18 pure peroxide-free solvents (Liaaen-Jensen 1971).
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

35 *Enzymatic hydrolysis of Haematococcus pluvialis extract*
36

37 Three mL of *Haematococcus* acetone extract was added to 2 mL of Tris HCl (0.05 M, pH 7.00)
38 were added and put in a thermostatic bath at 37°C for 2 minutes. Then, 100 µL of cholesterol
39 esterase solution (50 unit/mL) were added and incubated in a water bath at 37°C for 30 minutes
40 with gently shaking. After the incubation 1.0 g of sodium sulphate decahydrate and 2 mL of
41 petroleum ether were added and mixed vigorously by vortex. The upper petroleum ether phase
42 was collected in a clean tube and the procedure repeated two times to make the organic phase
43 clear. Petroleum ether phase containing astaxanthin was added with sodium sulphate anhydrous
44 mixing by vortex for 30 seconds and the ethereal phase transferred in a clean tube, then dried
45 under a stream of nitrogen and reconstituted in 200 µL of hexane.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Colour measurements in fillet

The fish were decapitated, gutted, filleted and the right fillet used for colour analysis. Colour measurements of the flesh samples were performed in the following sites: posterior to the head (neck), at the center below the dorsal fin (center) and anterior to the caudal fin (tail). Colour was measured using a Minolta Chroma Meter II Reflectance instrument (Minolta Camera Co. Ltd., Osaka, Japan). Results were recorded as a^* , b^* , L^* values where a^* describes redness, b^* yellowness and L^* lightness. Chroma values (brightness, C^*) were calculated according to the following equation: $C^* = \sqrt{a^{*2} + b^{*2}}$. Two measurements were taken on each site and the measuring head was rotated 90° between each measurement. The left fillet was packed under vacuum and stored at -20°C until astaxanthin analysis, which was performed within a few days.

Astaxanthin extraction from fish fillets

Astaxanthin was extracted from flesh according to the method of Turujman et al. (1997). Briefly, 10 g of tissue were sampled at the centre of the fillet and homogenized using an Ultra Turrax for 2 min with 10x2 mL of hexane, to remove most of lipids. The homogenate was centrifuged at 4000 rpm for 10 minutes and hexane phase was discharged. Astaxanthin was extracted from the pellet resulted after hexane extraction with 10x2 mL of acetone, following the procedure mentioned above. The acetone phase was evaporated with a rotary evaporator (Rotavapor R 110, Büchi, Switzerland) and the aqueous residue (approximately 4 mL) reconstituted in 20 mL of methylene chloride and vigorously swirled to dissolve astaxanthin. The residual water was removed with a separatory funnel and the organic phase was dried over approximately 1 g of anhydrous sodium sulfate. The methylene chloride phase containing astaxanthin was transferred in a clean tube. The amount of astaxanthin extracted was determined

1
2
3
4 recording the volume and measuring the absorbance of the methylene chloride extract a 474 nm
5
6 using a Jasco model V-530 spectrophotometer (Ishikawa-cho, Japan).
7
8
9

10 11 *HPLC analysis of astaxanthin*

12
13 The astaxanthin was purified by loading the methylene chloride phase onto a silica gel Sep-Pak
14 cartridge (Waters, Milford, MA, USA) pretreated with hexane. Astaxanthin was eluted from the
15 cartridge with 5 mL of chloroform and the eluate dried under a stream of nitrogen. Dry residue
16
17 was dissolved in 200 μ L of hexane and 20 μ L were injected into the HPLC system.
18
19
20
21

22
23 The HPLC system consisted of a Jasco model 980-PU pump (Ishikawa-cho, Japan), a Jasco
24 model LG-980-02 gradient unit, a Jasco model AS-950 autosampler and a Waters model 996
25
26 Photodiode Array Detector (Waters, Milford, MA, USA). Chromatograms were recorded and
27
28 integrated on a Millennium 32 Chromatography Software (Waters, Milford, MA, USA).
29
30

31
32 Analyses were performed with a Pirkle L-leucine chiral column (Regis Technologies, Morton
33
34 Grove, IL, USA). Mobile phase was hexane-tetrahydrofuran-triethylamine-acetonitrile
35
36 (77:19:2:2 v/v) at a flow rate of 1.5 mL min⁻¹. Isocratic condition and ambient temperature were
37
38 used, and the monitoring wavelength was 474 nm. In these conditions the retention times of the
39
40 3*S*,3'*S*, meso and 3*R*,3'*R* forms were approximately 10.5, 11.5 and 12.5 minutes, respectively.
41
42
43 Each astaxanthin extract was analyzed in duplicate.
44
45
46
47
48

49 *Statistical analysis*

50
51 Data are reported as mean value \pm standard error of the mean. Homogeneity of variance was
52
53 confirmed and comparison between means was by one-way ANOVA. Significance was
54
55 accepted at probabilities of 0.05 or less.
56
57
58
59
60

For comparison of whole astaxanthin stereoisomers profile of trout fillet and the pigment ingredients of the diet, the coefficient of distance D (McIntire et al. 1969) between “reference” and “test” samples (pigment source and trout fillet, respectively) was computed.

This coefficient was originally developed to estimate the similitude of aquatic animals on the base of their fatty acids composition. Therefore, the smaller the value of the coefficient of distance D the higher the probability that trout have been fed with a diet containing that specific pigment source.

For comparison of the whole astaxanthin stereoisomers profile of “reference” (feed ingredient) and “test” (trout fillet), the coefficient of distance D (McIntire *et al.*, 1969) was computed using the following equation:

$$D_{jh} = \left[\sum_{i=1}^n (P_{ij} - P_{ih})^2 \right]^{1/2}$$

where, D_{jh} is the degree of difference (coefficient of distance) between samples j (“reference” diet) and h (“test” trout fillet), P_{ij} and P_{ih} are percentage of astaxanthin stereoisomers i in sample j and h , for each i stereoisomers. All the statistical analysis were performed by SPSS 12.0 (SPSS Inc. Chicago, Illinois).

Results and Discussion

In Figure 1 the chromatograms of a standard solution ($32 \mu\text{g mL}^{-1}$) of synthetic astaxanthin and of the extracts obtained from different sources are presented. The three peaks corresponding to $3S,3'S$ isomer, meso form and $3R,3'R$ isomer were eluted at 10.5, 11.5 and 12.6 minutes, respectively.

1
2
3
4 Percentage distribution of stereoisomers was calculated from chromatograms of different
5 pigment extracts by means of relative peak area measurements. The results are presented in
6
7 Figure 2. A characteristic distribution of astaxanthin optical isomers was detected for each
8
9 pigment sources and such distribution was reproduced in trout fed with that source.
10
11 *Haematococcus pluvialis* algae meal contained 87.6% of the 3*S*,3'*S* enantiomer, 10.1% of the
12 meso form and 2.3% of the 3*R*,3'*R* enantiomer, while the red yeast *Phaffia rhodozyma* contained
13
14 1.5% of the meso form and 98.5% of the 3*R*,3'*R* enantiomer. The values of optical isomers in
15
16 krill meal and krill oil were similar. In fact, 27.4% of 3*S*,3'*S* enantiomer, 21.8% of the meso
17
18 form and 50.8% of 3*R*,3'*R* enantiomer were found in the krill meal whilst 35.8% of 3*S*,3'*S*
19
20 enantiomer, 13.6% of the meso form and 50.6% of 3*R*,3'*R* enantiomer were found in krill oil.
21
22 On the other hand, shrimps meal contained 65.4% of 3*S*,3'*S* enantiomer, 27.9% of the meso
23
24 form and 6.7% of 3*R*,3'*R* enantiomer. These results were consistent with literature values. In
25
26 particular, Foss et al. (1987) analysed the content of astaxanthin stereoisomers in several species
27
28 of crustaceans and demonstrated that in *Euphausia superba* (krill) the 3*R*,3'*R* isomer
29
30 predominated, whereas in *Thysanoessa inermis* and in *Calanun finmarchicus*, the 3*S*,3'*S* isomer
31
32 was dominant. These latter two small crustaceans are important ingredient of diets of wild
33
34 salmonids, and their isomeric composition has been demonstrated similar to the isomeric
35
36 composition encountered in wild salmon, in which the S-form predominated (Turujman et al.
37
38 1997). Astaxanthin isomer ratios in the flesh of fish fed different carotenoid sources is reported
39
40 in Table 1. Dietary treatment influenced significantly the distribution of the optical isomers in
41
42 the fillets, and the average values of the optical isomers in the flesh reflected the isomeric ratio
43
44 of the dietary astaxanthin source. The astaxanthin isomers distribution in microalgae and in the
45
46 corresponding trout flesh were similar and ranged from 80.0% to 87.6%, from 11.7% to 10.1%
47
48 and from 2.4% to 8.3% for the 3*S*,3'*S* isomer, meso form and 3*R*,3'*R* isomer, respectively. In the
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4 red yeast and in the corresponding trout flesh the *3R,3'R* isomer predominated, ranging from
5
6 98.5% to 74.9%, while the meso form and the *3S,3'S* isomer ranged from 1.5% to 10.7% and
7
8 from 0% to 14.4%, respectively. In the flesh of trout fed synthetic astaxanthin, the averages of
9
10 the distribution of optical isomer were 29.6%, 48.4% and 22.0% for the *3S,3'S* isomer, meso
11
12 form and *3R,3'R* isomer, respectively. Also in this case the distribution resembles that of the diet
13
14 and these results were in agreement with those of Østerlie et al. (1999), who found in rainbow
15
16 trout fed syntetic astaxanthin a distribution of isomers in muscle of 28.5%, 49.0% and 22.5% for
17
18 *3S,3'S*, meso and *3R,3'R* isomers, respectively.
19
20
21
22

23 Colour values measured in different sites of fillet of rainbow trout fed with different pigment
24
25 sources are presented in table 2. No significant differences were registered in different parts of
26
27 the flesh. Similarly, different sources of pigment (natural or synthetic), used with the three
28
29 different diets, produced colour values of fresh fillet (table 3) with no significant differences,
30
31 although there was a tendency for the CP diet to give an higher value of redness (a^*) and
32
33 brightness (C^*) when compared to HP diet. This confirms the better pigmenting efficacy of free
34
35 astaxanthin in comparison with esterified astaxanthin from microalgae. (White et al. 2003).
36
37
38
39

40 Among the specimens purchased on the market (table 4), two samples showed the algal
41
42 astaxhantin isomeric profile (trout 3 and 4), and three (trout 2, 5, 7) were found to contain a
43
44 different pigment source that on the basis of its retention time and UV/VIS absorption spectra
45
46 was reasonably recognized as canthaxanthin. Three fish samples showed to contain only
47
48 synthetic astaxanthin (trout 1, 8, 9) and one (trout 6) both synthetic carotenoids: astaxanthin and
49
50 canthaxantin.
51
52

53 The coefficient of distance (table 5) computed amongst the feed ingredient and the trout fillet
54
55 astaxanthin stereoisomers is a simple geometrical tool which can be useful to tentatively identify
56
57 the origin of the pigment used on farm.
58
59
60

1
2
3
4 The smallest D values registered in trout fed the Carophyll pink and trout fed microalgae were
5
6 5.4 and 9.8 when computed using the synthetic astaxanthin and the microalgae as “reference”,
7
8 respectively. The other D values, computed using the astaxanthin stereoisomers composition of
9
10 the other analysed ingredients, were considerably higher. In trout fed the read yeast, D value
11
12 (29.1) was similar to those calculated for krill meal (29.5) and krill oil (32.5).
13
14

15
16 This information suggests that the coefficient of distance can be used as a general tool to explain
17
18 pigment source only amongst some sources and it is particularly useful to distinguish amongst
19
20 synthetic and natural sources whilst is not sensitive enough to distinguish amongst different
21
22 natural astaxanthin sources, or amongst mixtures of synthetic and natural sources.
23
24

25
26 Using the coefficient of distance to estimate the potential origin of the astaxanthin found in the
27
28 market purchased trout, it is possible to point out that samples trout 1, trout 8 and trout 9 were
29
30 with high probability fed with a diet containing a synthetic source of astaxanthin.
31
32

33
34 At the best of our knowledge, this is the first time the coefficient of distance was used as a tool
35
36 for the identification of the astaxanthin origin in salmonids and further information, particularly
37
38 as regard the inter- and intra- population variability and the dose-response effects are requested.
39
40
41

42 **References**

43
44
45 Andrewes, A.G., Phaff, H.J., and Starr, M.P., 1976, Carotenoids of *Phaffia rhodozyma* a
46
47 red pigmented fermenting yeast. *Phytochemistry*, 15, 1003-1007.
48
49

50
51
52 Arai, S., Mori, T., Miki, W., Yamaguchi, K., Konosu, S., Satake, M., and Fujita, T., 1987,
53
54 Pigmentation of juvenile coho salmon with carotenoid oil extracted from Antarctic krill.
55
56 *Aquaculture*, 66, 255-264.
57
58
59
60

1
2
3
4 Bjerkg, B., Følling, M., Lagocki, S.; Storebakken, T., Olli, J.J., Alsted, N., 1997,
5 Bioavailability of all-E-astaxanthin and Z-isomers of astaxanthin in rainbow trout
6 (*Oncorhynchus mykiss*). *Aquaculture*, 157, 63-82.
7
8

9
10
11 Bowen, J., Soutar, C., Serwata, R.D., Lagocki, S., White, D.A., Davies, S.J., and Young, A.J.,
12 2002, Utilization of (3S,3 ϕ S)-astaxanthin acyl esters in pigmentation of rainbow trout
13 (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, 8, 59-68.
14
15

16
17
18 Coral-Hinostroza, G.N., and Bjerkg, B., 2002, Astaxanthin from the red crab langostilla
19 (*Pleuroncodes planipes*): optical R/S isomers and fatty acid moieties of astaxanthin esters.
20 *Comparative Biochemistry and Physiology Part B*, 133, 437-444.
21
22

23
24
25 Foss, P., Storebakken, T., Schiedt, K., Liaaen-Jensen, S., Austreng, E., and Streiff, K., 1984,
26 Carotenoids in diets for salmonids. I. Pigmentation of rainbow trout with the individual optical
27 isomers of astaxanthin in comparison with canthaxanthin. *Aquaculture*, 41,213–226.
28
29

30
31
32 Foss, P., Renstrøm, B., Liaaen-Jensen, S., 1987, Natural occurrence of enantiomeric and meso
33 astaxanthin: 7. Crustaceans including zooplankton. *Comparative Biochemistry and Physiology*
34 *B*, 86, 313-314.
35
36

37
38
39 Goodwin, T.W., 1992, Distribution of carotenoids. *Methods in Enzymology, Carotenoids, Part*
40 *A*, edited by L. Packer (Academic Press, Inc), vol. 213, pp. 167-172.
41
42

43
44
45 Liaaen-Jensen S., 1971, III. Isolations, reactions. *Carotenoids*, edited by O. Isler (Basel:
46 Birkhäuser Verlag), pp. 61-188.
47
48

49
50
51 Lorenz, R.T., and Cysewsky, G.R., 2000, Commercial potential for *Heamatococcus microalgae*
52 as natural source of astaxanthin. *Trends in Biotechnology*, 18, 160-167
53
54

55
56 Maoka, T., Komori, T., and Matsuno,T., 1985, Direct diastereomeric resolution of carotenoids:
57 I. 3-hydroxy-4-ocy β -end group. *Journal of Chromatography*, 318, 122-124.
58
59
60

1
2
3
4 Matsuno, T., 1992, Structure and characterization of carotenoids from various habitats and
5 natural sources. Methods in Enzymology, Carotenoids, Part A, edited by L. Packer (Academic
6 Press, Inc), vol. 213, pp. 22-31.
7
8
9

10
11 Matsumo, T., 2001, Aquatic animal carotenoids. Fisheries Science, 67, 771-783.
12
13

14
15 McIntire, C.D., Tinsley, I.J. and Lowry, R.R., 1969, Fatty acid in lotic periphyton: another
16 measure of community structure. Journal of Phycology, 5, 26-32.
17
18

19
20 Olaizola, M., and Huntley, M.E., 2003, Recent advances in commercial production of astaxanthin
21 from microalgae. Biomaterials and Bioprocessing, edited by Fingerman M., Nagabhushanam R.
22 (Science Publishers).
23
24

25
26
27 Østerlie, M., Bjerkgeng, B., and Liaaen-Jensen, S., 1999, Accumulation of astaxanthin all-E, 9Z
28 and 13Z geometrical isomers and 3 and 3' RS optical isomers in rainbow trout (*Oncorhynchus*
29 *mykiss*) is selective. Journal of Nutrition, 129, 391-398.
30
31
32

33
34 Palozza, P., and Krinsky, N.I., 1992, Antioxidant effects of carotenoids in vivo and in vitro: an
35 overview. Methods in Enzymology, Carotenoids, Part A, edited by L. Packer (Academic Press,
36 Inc), vol. 231, pp. 403-420.
37
38
39

40
41 Renstrøm, B., Borch, G., and Liaaen-Jensen, S., 1981, Natural occurrence of enantiomeric and
42 meso-astaxanthin 4. Ex shrimps (*Pandalus borealis*). Comparative Biochemistry and
43 Physiology Part B, 69, 621-624.
44
45
46

47
48 Sachindra, N.M., Bhaskar, N., and Mahendrakar, N.S., 2005, Carotenoids in different body
49 components of Indian shrimps. Journal of the Science of Food and Agriculture, 85, 167-172.
50
51
52

53
54 Schiedt, K., Bischof, S., and Glinz, E., 1993, Metabolism of carotenoids and *in vivo*
55 racemization of (3S,3'S)-astaxanthin in the crustacean *Penaeus*. Methods in Enzymology,
56 Carotenoids, Part B, edited by L. Packer (Academic Press, Inc), vol. 214, pp. 148-152.
57
58
59
60

1
2
3
4 Shahidi, F., Metusalach, and Brown, J., A., Carotenoid pigments in seafoods and aquaculture,
5 1998, *Critical Reviews in Food Science*, 38, 1-67.
6
7

8
9
10 Storebakken, T., Foss, P., Scheidt, K., Austreng, E., Jensen, S.-L. and, Manz, U., 1987,
11 Carotenoids in diets for salmonids IV. Pigmentation of Atlantic salmon with astaxanthin,
12 astaxanthin dipalmitate and canthaxanthin. *Aquaculture*, 65, 279–292.
13
14

15
16
17 Storebakken, T and No, H.K., 1992, Pigmentation of rainbow trout. *Aquaculture*, 100, 209–229.
18

19
20 Storebakken, T., Sørensen, M., Bjerkgeng, B., and Hiu, S., 2004, Utilization of astaxanthin from
21 yeast, *Xanthophyllomyces dendrorhous*, in rainbow trout, *Oncorhynchus mykiss*: effect of
22 enzymatic cell wall disruption and feed extrusion temperature. *Aquaculture* 236, 391-403.
23
24

25
26
27 Takaichi, S, Matsui, K., Nakamura, M., Muramatsu, M., and Hanada, S., 2003, Fatty acids of
28 astaxanthin esters in krill determined by mild mass spectrometry. *Comparative Biochemistry*
29 *and Physiology Part B*, 136, 317-322.
30
31

32
33
34 Turujman, S.A., 1993, Rapid direct resolution of the stereoisomers of all-*trans* astaxanthin on a
35 Pirkle covalent L-leucine column. *Journal of Chromatography*, 631, 197-199.
36
37

38
39
40 Turujman, S.A., Wamer, W.G., Wie, R.R., and Albert, R.H., 1997, Rapid liquid chromatographic
41 method to distinguish wild salmon from aquacultures salmon fed synthetic astaxanthin. *Journal*
42 *of AOAC International*, 80, 622-632.
43
44

45
46
47 Vecchi, M., and Müller, R.K., 1979, Separation of (3*S*,3'*S*)-, (3*R*,3'*R*)- and (3*S*,3'*R*)-astaxanthin
48 via (-)-camphanic acid esters. *Journal of High Resolution Chromatography*, 2, 195-196.
49
50

51
52
53 White, D.A., Moody, A.J., Serwata, R.D., Bowen, J., Soutar, C., Young, A.J., and Davies, S.J.,
54 2003, The degree of carotenoid esterification influences the absorption of astaxanthin in rainbow
55 trout, *Oncorhynchus mykiss* (Walbaum), *Aquaculture Nutrition*, 9, 247-251.
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review Only

Figure 1: Chromatograms of stereoisomers composition of all-*trans* astaxanthin of: (a) a standard solution of synthetic astaxanthin ($32 \mu\text{g mL}^{-1}$); (b) an *Haematococcus pluvialis* extract; (c) a *Phaffia rhodozyma* extract. HPLC conditions: Pirkle L-leucine chiral column; mobile phase hexane-tetrahydrofuran-triethylamine-acetonitrile (77:19:2:2 v/v); flow rate of 1mL min^{-1} ; monitoring wavelength 474 nm.

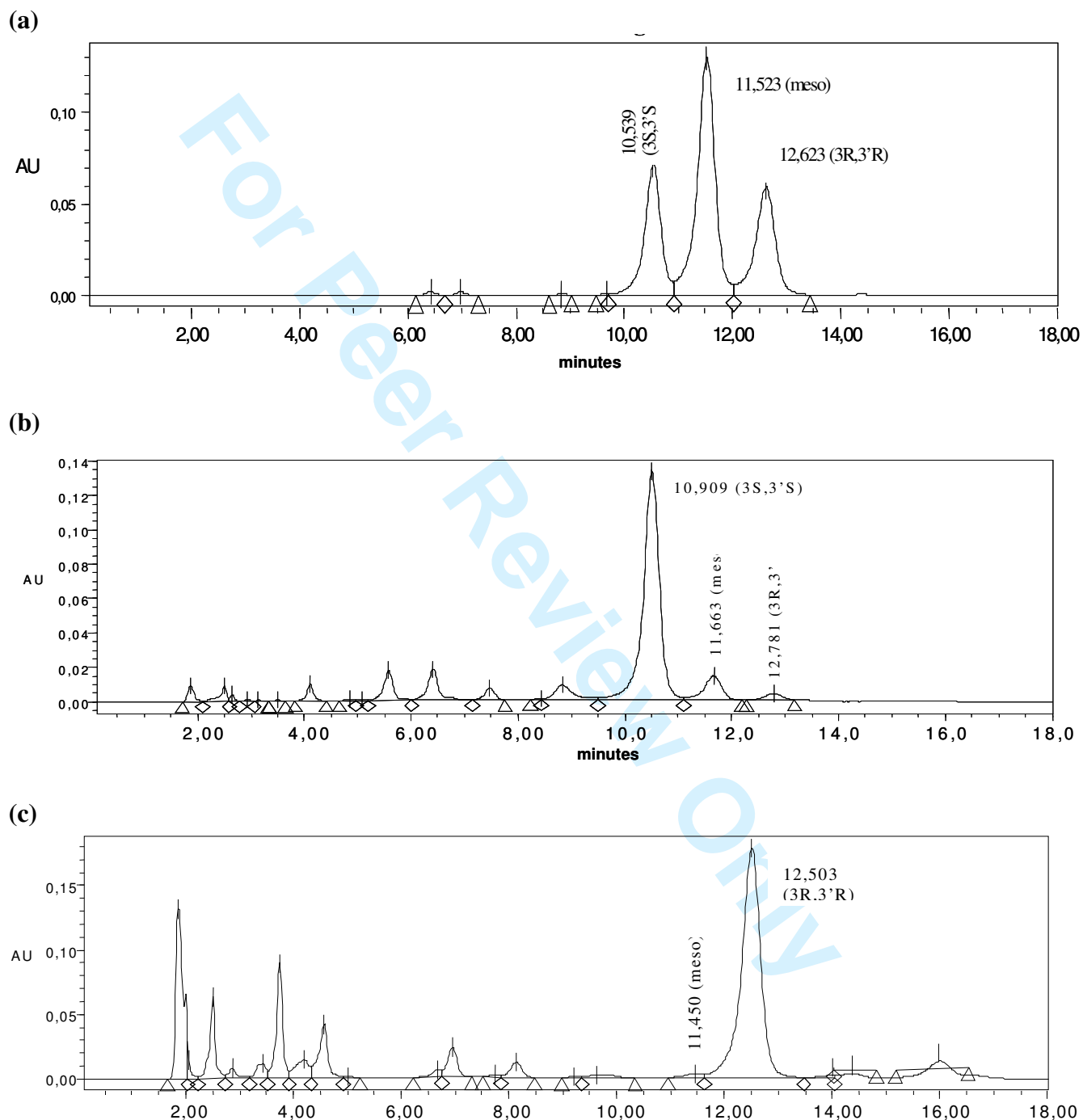
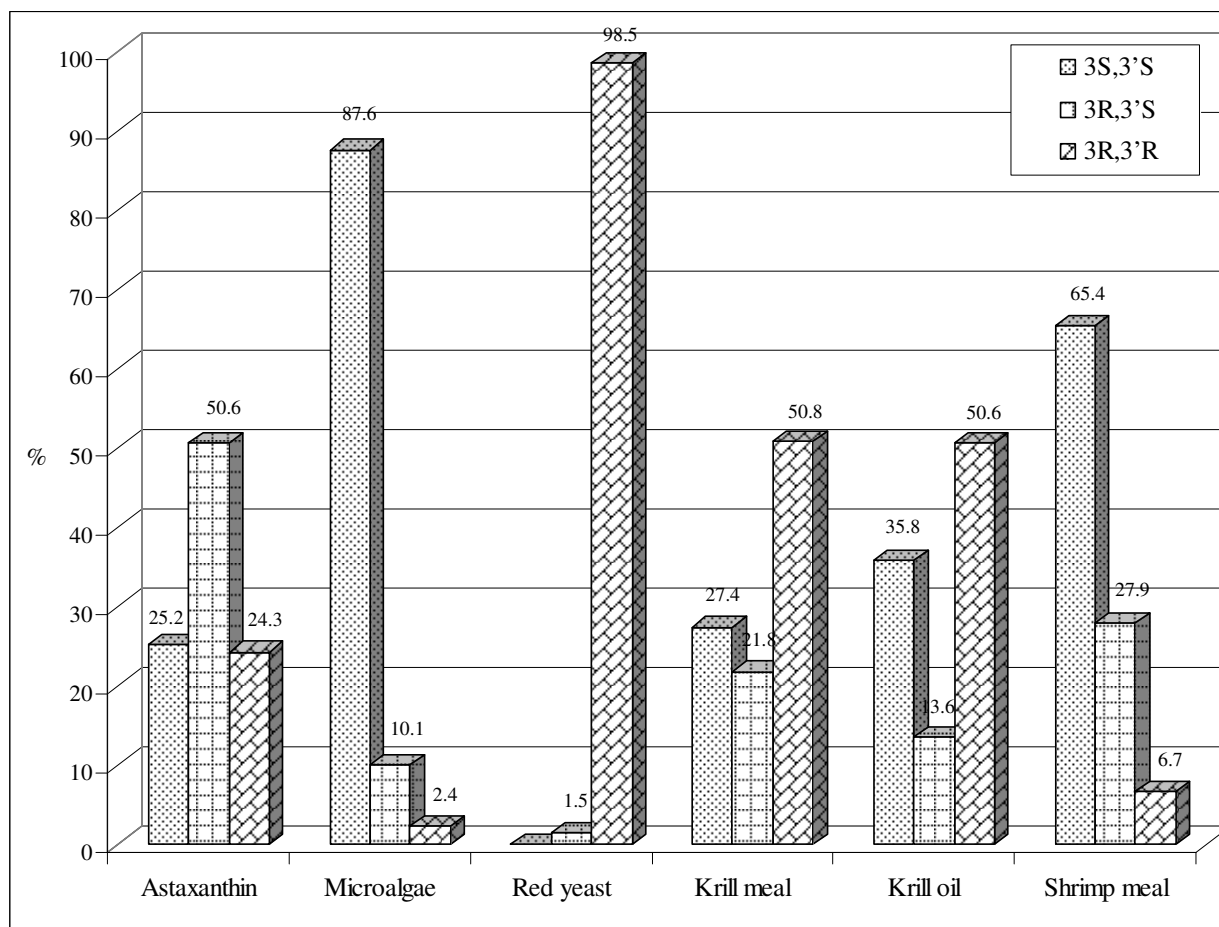


Figure 2. Percentage distribution of astaxanthin stereoisomers in pigment sources



View Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Percentage distribution of astaxanthin stereoisomers in fillet of rainbow trout fed with different pigment sources (means±s.e.)

Samples (n=10)	3S,3'S enantiomer (%)	3R,3'S meso (%)	3R,3'R enantiomer (%)
Carophyll® pink fed trout	29.6±3.62	48.4±4.26	22.0±7.50
Microalgae (<i>Haematococcus pluvialis</i>) fed trout	80.0±2.01	11.7±1.14	8.3±1.03
Red yeast (<i>Phaffia rhodozyma</i>) fed trout	14.4±2.33	10.7±2.28	74.9±4.52

For Peer Review Only

Table 2. Colour values measured in three different part of fillet of rainbow trout fed with different pigment sources (means±s.d., n=10)

Color value	Neck	Center	Tail	<i>P</i> ¹
a*	14.8±0.74	14.5±0.66	15.8±0.33	0.28
b*	18.9±1.13	17.3±1.17	19.3±0.87	0.40
L*	44.8±1.31	42.3±1.42	43.8±1.60	0.48
C*	24.1±1.31	22.6±1.28	25.0±0.86	0.37

¹ Significance accepted at probabilities of 0.05 or less

Table 3. Colour values measured in fillet of rainbow trout fed with different pigment sources (means \pm s.d., n=10)

Color value	Synthetic astaxanthin	<i>Phaffia rhodozyma</i>	<i>Haematococcus pluvialis</i>	P ¹
a*	15.3 \pm 0.58	15.7 \pm 0.56	14.2 \pm 0.64	0.19
b*	18.7 \pm 1.06	18.6 \pm 1.04	18.2 \pm 1.20	0.95
L*	42.9 \pm 1.40	43.4 \pm 1.50	44.7 \pm 1.50	0.64
C*	24.1 \pm 1.18	24.4 \pm 1.14	23.1 \pm 1.28	0.74

¹ Significance accepted at probabilities of 0.05 or less

Table 4. Percentage distribution of astaxanthin stereoisomers in flesh of rainbow trout purchased on the market.

Samples	3S,3'S enantiomer (%)	3R,3'S meso (%)	3R,3'R enantiomer (%)	Presence of canthaxanthin
Trout 1	30.7	48.0	21.3	no
Trout 2	nd	nd	nd	yes
Trout 3	71.2	19.9	8.9	no
Trout 4	68.4	21.8	9.8	no
Trout 5	nd	nd	nd	yes
Trout 6	40.5	26.9	32.6	yes
Trout 7	nd	nd	nd	yes
Trout 8	28.2	49.1	22.7	no
Trout 9	29.8	48.3	21.9	no

nd = not detected

Table 5. The coefficient of distance (D value) of the astaxanthin stereoisomers amongst known and unknown trout samples and the analysed astaxanthin sources

	Synthetic astaxanthin	Microalage (<i>H.pluvialis</i>)	Red yeast (<i>X. dendrorhous</i>)	Antartic Krill meal	Krill oil	Shrimps meal
Trout fed Carophyll® pink	5.4	72.2	94.5	39.3	45.5	44.0
Trout fed microalage (<i>H.pluvialis</i>)	69.1	9.8	121.0	68.4	61.2	21.9
Trout fed red yeast (<i>X. dendrorhous</i>)	65.3	103.0	29.1	29.5	32.5	86.9
Trout 1	6.8	70.9	95.2	39.6	45.5	42.7
Trout 3	57.4	20.2	115.9	60.6	55.1	<i>10.1</i>
Trout 4	53.9	23.7	113.8	58.0	52.9	7.5
Trout 6	29.4	58.4	81.4	23.0	22.9	35.9
Trout 8	3.7	73.9	93.8	39.2	45.8	45.7
Trout 9	5.7	72.0	94.6	39.3	45.4	43.8

In bold font, the actual coefficient of distance amongst pigment source and trout fillet of the known samples; in italics the possible coefficient of distance amongst pigment source and trout fillet of the unknown samples.