www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18: 1053-1061 (2018) DOI: 10.4194/1303-2712-v18_9_05

RESEARCH PAPER

Quantification of Astaxanthin and Canthaxanthin in Muscle Tissues of Rainbow Trout *Oncorhynchus mykiss* and Brook Trout *Salvelinus fontinalis*

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Received 22 July 2016
Accepted 20 October 2017

Abstract

This study reports the quantification of two main pigments in Salmonidae- astaxanthin and canthaxanthin, in different muscle tissues (skeletal and cardiac) of rainbow trout *Oncorhynchus mykiss* W. and brook trout *Salvelinus fontinalis* M. The fish were fed with conventional no pigment supplemented feed. The xanthophyll quantities in flesh and heart were determined by high performance liquid chromatography and photodiode array detection after single-laboratory validation of the method. The target analysis of surrounding water showed concentrations under detection limit. This study demonstrates the ability of rainbow trout and brook trout to absorb two main xanthophylls, from environment containing them in concentrations under the detection limit. The total xanthophyll content in the cardio muscle is larger than those in the skeletal muscle in both fish species and higher in the skeletal muscle of *Salvelinus fontinalis* M. than those of *Oncorhynchus mykiss*.

Keywords: Salmonidae, astaxanthin, canthaxanthin, muscle tissue.

Introduction

Carotenoids are the most widespread and important pigment class in living organisms. They are produced by plants and many photosynthetic microorganisms and are brightly colored (yellow to red). As for the animals including fish, the carotenoids are not essential in nutritional sense, but they are useful for their health (reviewed by Maoka, 2011). Besides the coloration properties, the carotenoids have important biological functions. Carotenoids have excellent antioxidative activities for quenching the Singlet oxygen molecule ($^1O_2$) and inhibiting lipid peroxidation (Krinsky, 1994, Skibsted, 2012). Lysozyme and complement proteins in teleost fish are responsible for the cell protection from diverse pathogens (Whyte, 2007; Saurabh & Sahoo, 2008) and both of them are influensed by carotenoid availability in birds and fish (Amar, Kiron, Satoh, & Watanabe, 2001, 2004; McGraw, & Klasing, 2006; Cucco, Guasco, Malacarne, & Ottonelli, 2007; Lin, Nieves-Puigdollers, Brown, McGraw, & Clotfelter, 2010).

However, the salmonids are not able to synthetize carotenoids *de novo*, but these species are able to absorb carotenoids from their diet and the pigments, that are accumulated in largest quantities, are astaxanthin (AX) and canthaxanthin (CX). Studies show that feeding diets supplemented with synthetic or native AX reduce serum lipid peroxide content (Nakano, Tosa, & Takeuchi, 1995), reduce susceptibility of liver to lipid oxidation (Nakano, Miura, Wazawa, Sato, & Takeuchi, 1999), normalize liver function and reduce serum lipids and lipid peroxides in rainbow trout fed with oxidized oil (Nakano, Kanmuri, Sato, Takeuchi, 1999). Dietary deficiency of astaxanthin increases the hepatocytic recovery of desaturated and elongated products of polyunsaturated fat acids (Bell, McEvoy, Tocher, & Sargent, 2000). This activity may explain the positive effects of astaxanthin on growth and disease resistance of atlantic salmon, e.g. (Christiansen, Glette, Lie, Torrissen, & Waagbø, 1995, Bjerkeng, 2000).

Salmonids absorb and deposit AX and CX in the muscles during the grow-out period and mobilize them predominantly to the gonads and also to the skin during the sexual maturation (Garner, Neff, & Bernards, 2010; Rajasingh, 2006). A combination of both pigments in the diet gives a higher total carotenoid deposition in the flesh than either one alone. Deposition increases along with the increasing fish weight. Foss, Storebakken, Austreng, and Liaaen-Jensen (1987) and Torrissen (1989) demonstrated that

astaxanthin is deposited in the flesh of rainbow trout more effectively than canthaxanthin due to preferred absorption in the digestive tract as well as deposition in the flesh. Foss *et al*. (1987) proposed that reasons for this are differences in digestibility, better ability of muscle actomyosin to bind astaxanthin, and a higher metabolic turnover of canthaxanthin.

In 1994 Meyers reviewed the factors affecting carotenoid absorption in fish. Among them are pigment source, form and concentration, diet composition, especially fat content, fish size, physiological state and stage of sexual maturation, and genetic background. Astaxanthin absorption also depends on the concentration used and whether it is provided in its free form or as a diester. The effects of abiotic factors, i.e., temperature and salinity, have also been examined in studies involving both immature and mature salmonids.

The fish muscle tissues have relatively low astaxanthin concentrations but are responsible for the largest body pool of this carotenoid (Bjerkeng, 2000). The specific pink flesh color provided mostly by astaxanthin has always been associated with salmonids and has differentiated the salmonids from other fish species. When it comes to making purchasing decisions about salmon, consumers state that color is very important: the redder colour, the better flavor and higher quality. From biochemical perspective it is important also to establish the basic concentration levels of both main carotenoids- AX and CX and their distribution in different muscle tissues- skeletal and cardio of farmed salmonids fed with specialized feed without extra added pigments and to prove the ability of this species to absorb xanthophylls from environment containing them in very low concentrations.

Materials and Methods

Fish, Feeding, Hydro Chemical Parameters and Sample Preparation

In this study as biological material were used tissue from skeletal muscle and heart of rainbow trout *Oncorhynchus mykiss* and brook trout *Salvelinus fontinalis* M breed in a Bulgarian fish farm near to Tundzha River and Middle Forest Mountain under ambient conditions suitable for the normal growth of these species. The qualitative and quantitative requirements to the water in Bulgarian trout farms are according to Regulation 44/ 20.04.2006 of the Bulgarian ministry of agriculture and food. The supply water for the farm facilities comes from Tundzha River and also contains rain- and ground-water and has temperature from 4.2°C to 19.8°C and contains dissolved oxygen from 9.1 $mg.l^{-1}$ to 10.8 $mg.l^{-1}$ depending on the season ant daytime.

To achieve the trial aims, the fish was fed with specialized slowly sinking extruded feed without extra added pigments delivered from certified European producer. The content of the used feed according manufacturer's specifications is: 42% raw proteins, 20% raw fats, 1,5% raw fiber, 1,3% phosphorus, 10000 I.U. Vit A, 1500 I.U. Vit D_3 and 200 mg Vit E. The digestible energy is 19,8 MJ. The raw materials used for the feed production are: fish meal, animal proteins, fish oil, sunflower concentrate, colza oil, wheat, wheat meal, powdered hemoglobin and colza expeller. This feed was picked because of the following advantages: extruded and slowly sinking; suitable for growing up fish; high content of fish meal, animal proteins and fish oil of great quality; high digestibility; low water pollution and no content of GMOs, according Regulation (EC) 1829/2003. The diet plan is in accordance with the manufacturer's recommendations, which are consistent with the size of the fish and the water temperature.

The fish species of rainbow trout *Oncorhynchus mykiss* were 36 months old with body mass of 823 ± 51 g. The brown trout *Salvelinus fontinalis* M. were 24 months old with body mass of 261 ± 13 g. The biological material was sampled in the period June - October 2015, immediately frozen and stored for a maximum 14 days at -12°C prior to the analyses. Xanthophyll extraction was carried out according to the method developed by Schweigert (2009).

Xanthophyll Quantification

A number of authors analyze the content of carotenoids in biological samples, both plant and animal origin (reviewed by Rodriguez-Amaya, 2001; Regal, Amorim-Carrilho, Cepeda, & Fente, 2014). The analysis starts always with an extraction followed often by separation, identification and quantification by liquid chromatography. In this study for quantification of AX and CX in muscle tissues after adaption was applied HPLC method for determination of xanthophyll in fish eggs, developed by Tzanova, Argirova, and Atanasov (2016).

The reference standards astaxanthin (min 97%, HPLC) from *Haematococcus pluvailis* and canthaxanthin OEKANAL® (min 97%, HPLC), were purchased from Sigma-Aldrich (St. Louis, MO and Seelze, Germany, respectively). All solvents – methanol and chloroform CHROMASOLV® HPLC grade, ethanol p.a., n-hexane p.a., i-propanol p.a. were also delivered from Sigma-Aldrich. Deionized water ($\sigma \leq 0.4$ $\mu S.cm^{-1}$) was used thoroughly.

Analytical HPLC was performed with a C-18 column Hypersil Gold (5µm; 150 mm x 4.6 mm) on a Thermo system composed of a Surveyor LC Pump Plus, Surveyor Autosampler Plus, and Surveyor photodiode array detector PDA Plus. The mobile phase was a mixture of methanol and water (97:3, v/v) was filtered through a 0.45 μm membrane and degased before use. Under isocratic conditions, the analysis was carried out at a flow rate of 1.0 $ml.min^{-1}$ at room temperature for 6 min run time. Chromatograms were recorded at 474 nm with a

photodiode-array detection system.

Stock solutions of AX and CX were prepared by dissolving 2.5 mg of each pigment (weighed to the nearest ± 0.1 mg) in 25 ml chloroform (HPLC grade) and stored in dark at –12°C. The standard solutions used for the calibration were obtained by diluting the stock solutions in methanol yielding a final concentration of 0.10; 1.00; 2.00; 4.0 and 10.00 mg.l^{-1}) of both, AX and CX. Figure 1A illustrates typical chromatogram of standard solution.

The biological material (skeletal muscle and hearts) was thawed at room temperature, 1.5 – 2.0 g were weighed to the nearest ± 0.0001 g, and homogenized with mechanical tissue homogenizer in 2 cm^3 1N urea and 5 cm^3 of a mixture of ethanol-i-propanol-n-hexane (1:2:6 v/v/v) for 3 min in ice bath. The homogenized samples were centrifuged for 2 min at 4°C at relative centrifugal force of 604 g. The samples were vortexed for 30 s with the solvent mix and centrifuged at the same conditions. This procedure was repeated. The extraction step is crucial for the analysis of these compounds because they are light- and oxygen-sensitive. The extraction procedure was carried out as quickly as possible in a dark room to minimize xanthophyll exposure to air and sunlight and to avoid compromising the experimental results.

The solvents were removed from the collected supernatants by rotary evaporator at 40°C and the dry residue was dissolved in 2 cm^3 of methanol. The methanol extracts were stored overnight at -12°C prior to the HPLC-analysis. A small quantity of each extract was transferred into a capped vial and placed in the HPLC system autosampler.

Daily in a period of two weeks 2 water samples, each 2 dm^3, were collected from the ponds for growing fish immediately before and 30 min after feed throwing. 1 dm^3 of each was filtered through a 0.45 μm membrane and the filter was washed up with deionized water. The extraction procedure described above was followed.

Results

Single-Laboratory Validation (SLV) of the Method

The single-laboratory validation is a demonstration that the method is applicable by establishment of the method characteristics. Typical performance characteristics of analytical methods are: calibration, linearity, operating range, precision/repeatibility, accuracy/recovery, limit of quantification and detection (Thomson, Ellison, & Wood, 2002).

In the present study, linearity was studied in the

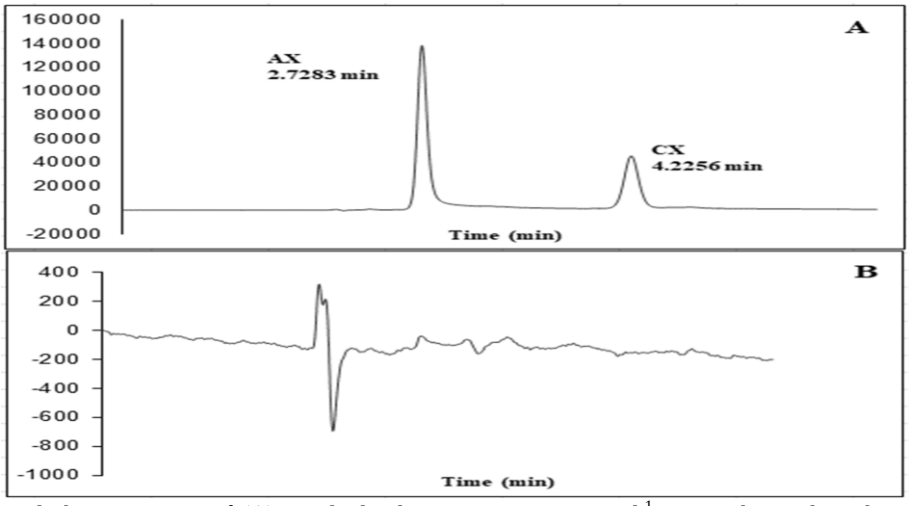

Figure 1. Typical chromatograms of (A) standard solution containing 1 mg.l^{-1} astaxanthin and canthaxanthin and (B) blank solution.

Table 1. Linearity and operating range. Results of assessment of the linearity of the HPLC method for quantification of astaxanthin and canthaxanthin

Astaxanthin				Canthaxanthin			
Calibration level	Concentration, mg.l^{-1}	Mean Peak Area	RSD*, %	Calibration level	Concentration, mg.l^{-1}	Mean Peak Area	RSD*, %
1	0.10	14215	0.15	1	0.10	9528	1.70
2	0.92	161382	1.92	2	1.00	127870	0.63
3	1.84	307216	0.13	3	1.68	243281	0.73
4	3.68	672822	0.08	4	3.36	470831	0.11
5	9.20	1746481	0.17	5	8.40	1172594	0.22
Correlation coefficients: r^2 = 0.9995				Correlation coefficients: r^2 = 0.9997			
Equation for regression line: y = 191450x – 22263 (n = 3)				Equation for regression line:y = 140167x – 2786.1 (n = 3)			

* RSD – Relative Standard Deviation

concentration range 0.10-10.00 mg.l^{-1}. Each of the calibration standards was run in triplicate (Table 1). Relative standard deviation (RSD), intercept and slope were calculated (Figures 2A and 2B).

Accuracy/recovery was determined by analyzing a sample of known concentration (reference materials) and comparing the measured value to the true value: four different solutions were prepared with known added amounts of AX and CX and injected in triplicate. Percent recoveries of response factor (area/concentration) were calculated. The results of accuracy studies are shown in Table 2. The recovery of AX and CX in the sample solutions are in the range 86.2- 100.3% and 87.9- 104.5%, respectively.

The repeatability of the method was verified on the basis of relative standard deviation (RSD) of one solution at 100% of expected concentration level injected 10 times during the same day and under the same experimental conditions (Table 3).

The detection and quantification limits of the method were determined by comparing the peak areas of blank solutions and calculated by the formulas: LOD= C_{av} + 3*STD and LOQ = C_{av} + 10*STD, where C_{av} is the average concentration of the corresponding pigment, and STD is the standard deviation of the measurements. LOD and LOQ were 0.019 mg.l^{-1} and 0.034 mg.l^{-1} for AX. Similar values were found for CX, 0.016 mg.l^{-1} and 0.021 mg.l^{-1}, respectively. Figure 1B illustrates typical chromatogram of blank solution.

AX and CX Content in Muscle Tissue of Salmonids

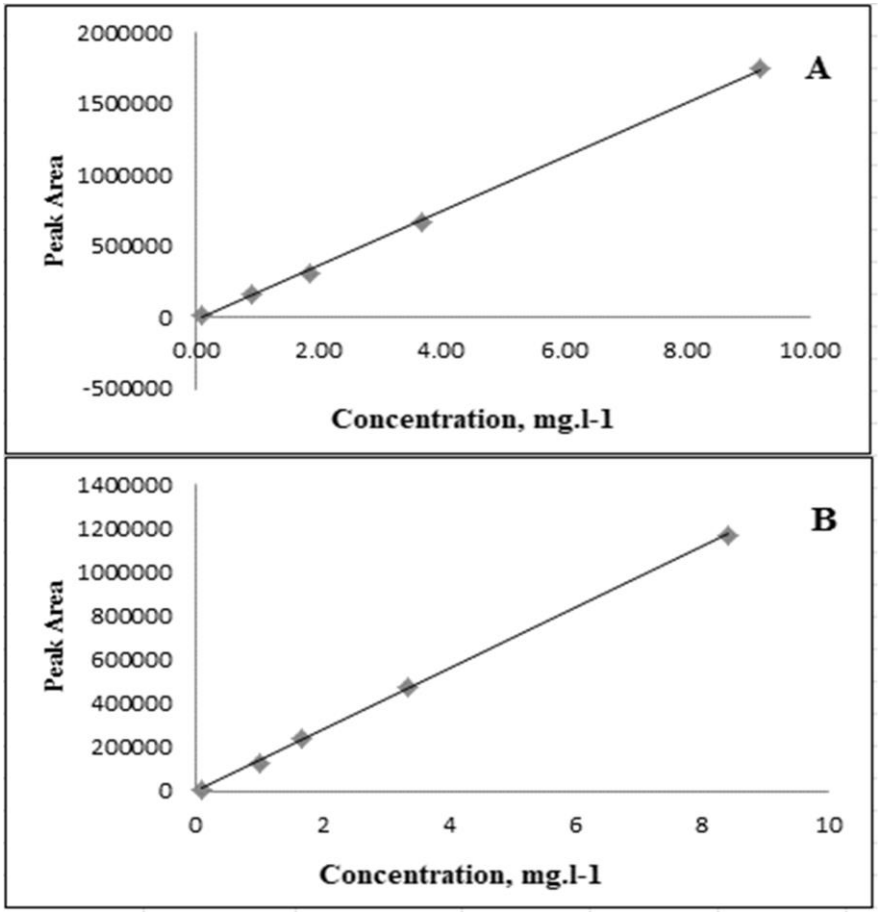

Figure 2. Linearity plot of (A) astaxanthin, and (B) canthaxanthin (n = 3).

Table 2. Accuracy/Recovering of astaxanthin and canthaxanthin from samples with known concentration

Astaxanthin			Canthaxanthin		
Concentration level	Recovery%	RSD*, %	Concentration level	Recovery%	RSD*, %
1	86.2	0.15	1	87.9	1.70
3	93.5	0.13	3	104.5	0.73
5	100.3	0.17	5	99.8	0.22

* RSD – Relative Standard Deviation of area response factor

Table 3. Precision/repeatability of the assay for astaxanthin and canthaxanthin evaluated on the basis of 10 replicate injections of one and the same solution at 100% of expected concentration level (1 $mg.l^{-1}$)

Astaxanthin			Canthaxanthin		
Injection No	Retention Time	Peak Area	Sample No	Retention Time	Peak Area
1	2.72	160288	1	4.22	127645
2	2.73	161014	2	4.22	127656
3	2.74	160145	3	4.21	128230
4	2.72	161556	4	4.23	128015
5	2.74	161984	5	4.21	127651
6	2.71	161875	6	4.23	128192
7	2.72	162049	7	4.21	127521
8	2.72	161922	8	4.23	127121
9	2.71	162349	9	4.24	128012
10	2.72	161535	10	4.21	128112
Mean	2.72	161472	Mean	4.22	127816
RSD%	0.39	0.47	RSD%	0.26	0.28

* RSD – relative standard deviation

The chromatographic method described in this study, was applied to measure the content of AX and CX in muscle tissue of rainbow trout *Oncorhynchus mykiss* and brook trout *Salvelinus fontinalis* M. bred in a Bulgarian fish farm. The number of the analyzed samples is 11 of each muscle tissue of rainbow trout *Oncorhynchus mykiss* and of brook trout *Salvelinus fontinalis* M., respectively. Figures 3A, 3B, 4A and 4B illustrate typical chromatograms of sample solutions.

The results are expressed in mg/kg calculated by the formula: Xanthophylls, mg/kg = Xanthophylls, $mgl^{-1}*V*M^{-1}$, where: V is the volume of the methanol solution in cm^3 and M is the mass of the sample in g. The average contents of AX and CX of rainbow trout species were 0.198 ± 0.018 $mg.kg^{-1}$ and 0.037 ± 0.005 $mg.kg^{-1}$ in skeletal muscle and 0.248 ± 0.032 $mg.kg^{-1}$ and 0.045 ± 0.010 $mg.kg^{-1}$ and heart, respectively. The quantities of AX and CX in the brook trout samples were 0.233 ± 0.012 $mg.kg^{-1}$ and 0.054 ± 0.006 $mg.kg^{-1}$ in skeletal muscle and 0.314 ± 0.052 $mg.kg^{-1}$ and 0.065 ± 0.012 $mg.kg^{-1}$ and heart, respectively.

Water Analysis

No xanthophyll peak was detected in тhe chromatograms of water samples (Figure 5). So, the xanthophyll content in water samples is lower than quantification limits of the method- 0.034 $mg.l^{-1}$ and 0.021 $mg.l^{-1}$, respectively for astaxanthin and canthaxanthin.

Discussion

Considering the use of AX and CX as food additives in order to improve the organoleptic and dietary-preventive qualities of the salmonid products, the analysis of the stored pigments is required. This study reports the single-laboratory validation of a rapid method for quantification of astaxanthin and canthaxanthin in muscle tissue of rainbow trout *Oncorhynchus mykiss* and brook trout *Salvelinus fontinalis* M. An advantage of the proposed method is the perfect combination of selective extraction of the xanthophylls and analysis of the extract by high performance liquid chromatography and photodiode array detection. The method validation was carried out in terms of linearity, accuracy/recovery, precision/repeatability and limits of detection and quantification. The linearity range was determined according to International Conference on Harmonization (ICH) guidelines. ICH specified a minimum of five concentration levels, along with certain minimum specified ranges. The regression coefficient (r^2) > 0.998 is generally considered as evidence of acceptable fit of the data to the regression line. The correlation coefficients (r^2) obtained for the regression lines (0.9995 for AX and 0.9997 for CX) demonstrated the excellent relationship between peak area and concentration in the range of 0.10 $mg.l^{-1}$ to 10.00 $mg.l^{-1}$. Accuracy can be determined by analyzing a sample of known concentration (reference materials) and comparing the measured value to the true value. The ICH (1997) recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels covering the operating range (e.g., three concentrations, three replicates each). Recovery of AX and CX in the sample solutions are in the range 86.2- 100.3% and 87.9- 104.5%, respectively and it is evident that the method is accurate enough within the desired recovery range. Precision is the other parameter obtained by this single-laboratory validation and it is expressed by the measure of the degree of repeatability of an analytical method under normal operation, and it is normally expressed as the percent relative standard deviation for a statistically significant number of samples. In this study precision of the method was evaluated through the repeatability

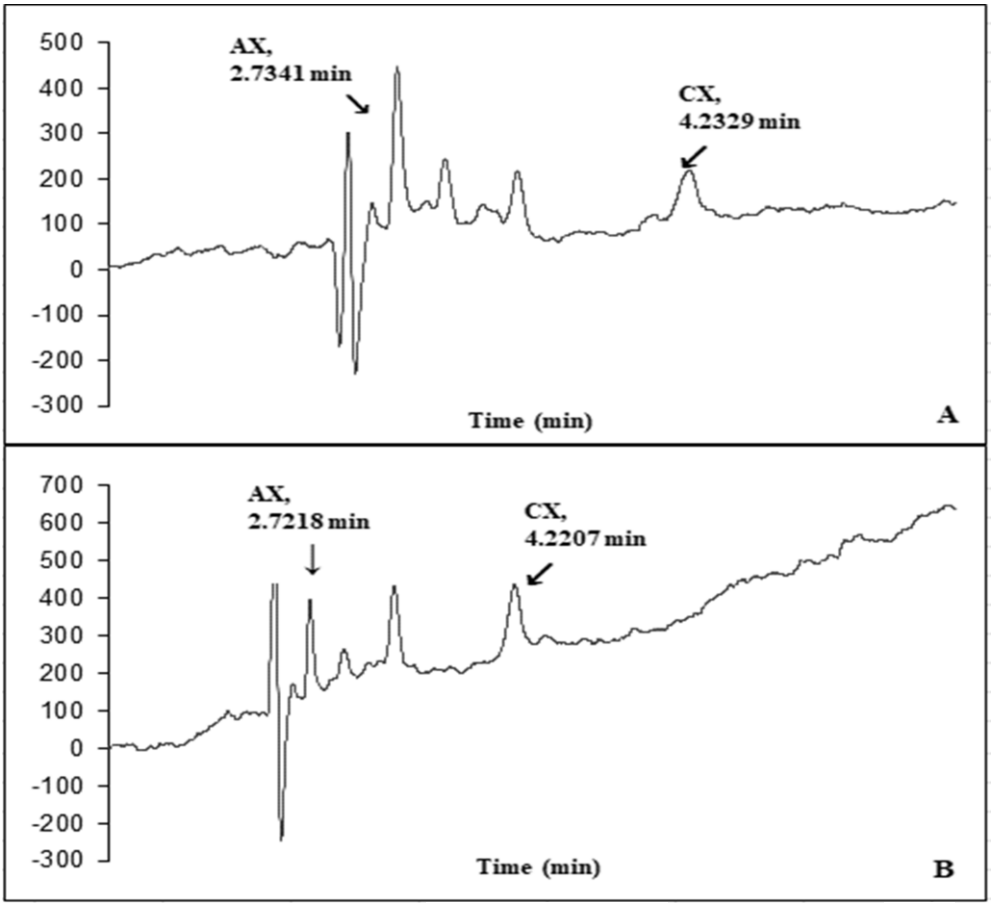

Figure 3. Typical chromatograms of sample solutions of *Oncorhynchus mykiss*. (A) – skeletal muscle*;* (B) – heart.

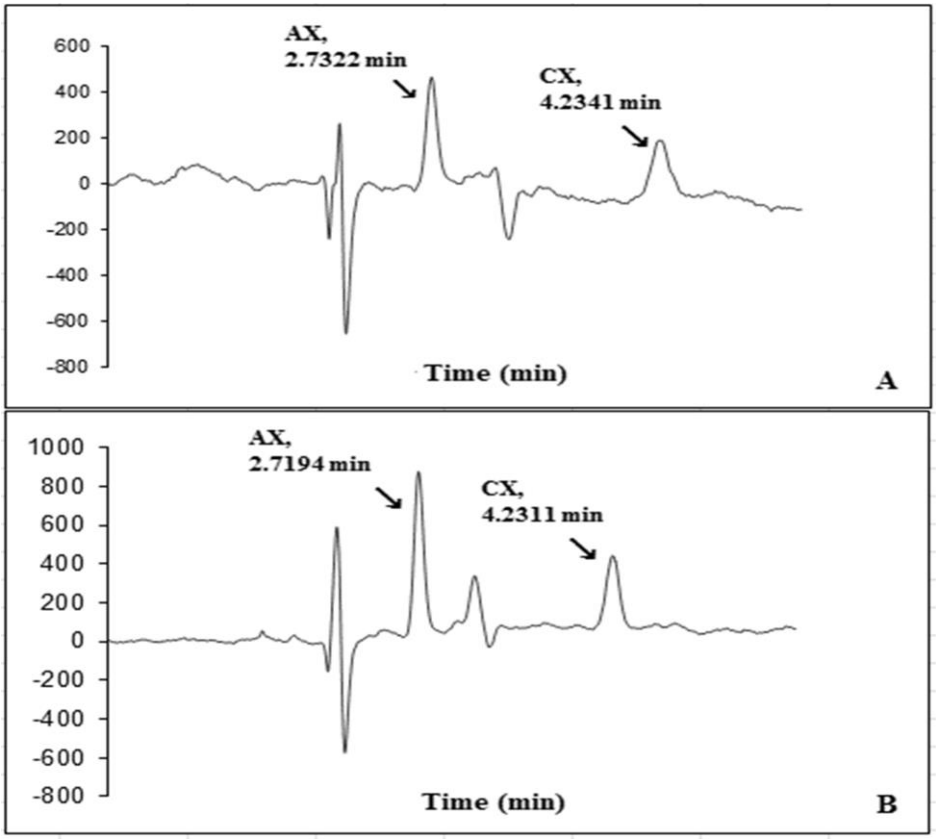

Figure 4. Typical chromatograms of sample solutions of *Salvelinus fontinalis* M. (A) – skeletal muscle*;* (B) – heart.

(intra-assay precision). ICH has a requirement to the precision of an assay method and the criteria is RSD 1%. The repeatability study shows that the RSD criteria are satisfied, the RSD are less than 1%. The Limits of Detection and Quantification 0.034 $mg.l^{-1}$ and 0.021 $mg.l^{-1}$, respectively for AX and CX show the high sensibility of the applied method. The values of the validated parameters are suitable for routine determination of these two carotenoids in fish muscle tissue samples.

Figure 6 shows compared results of quantification of astaxanthin and canthaxanthin in the different muscle tissues of rainbow trout *Oncorhynchus mykiss* and of brook trout *Salvelinus fontinalis* M. The obtained results document astaxanthin accumulations in salmonid in different muscle tissues are larger than those of canthaxanthin. It confirms the statement of Foss *et al.* (1987) and Torrissen (1989) - astaxanthin is deposited in the flesh of rainbow trout *Oncorhynchus mykiss* more effectively than canthaxanthin. Foss *et al.* (1987) explained this by the better ability of muscle actomyosin to bind astaxanthin, and the faster metabolic turnover of canthaxanthin. The present study demonstrates the same results also for brook trout *Salvelinus fontinalis* M.

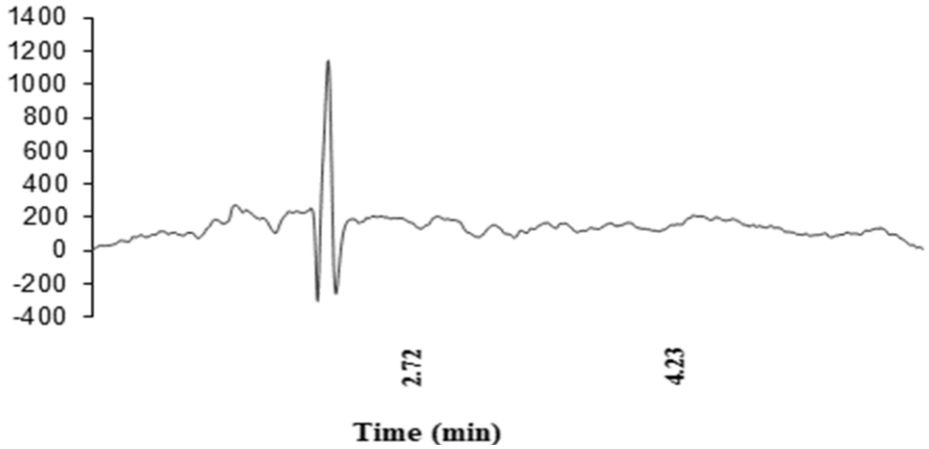

Figure 5. Typical chromatograms of water sample from ponds for growing fish.

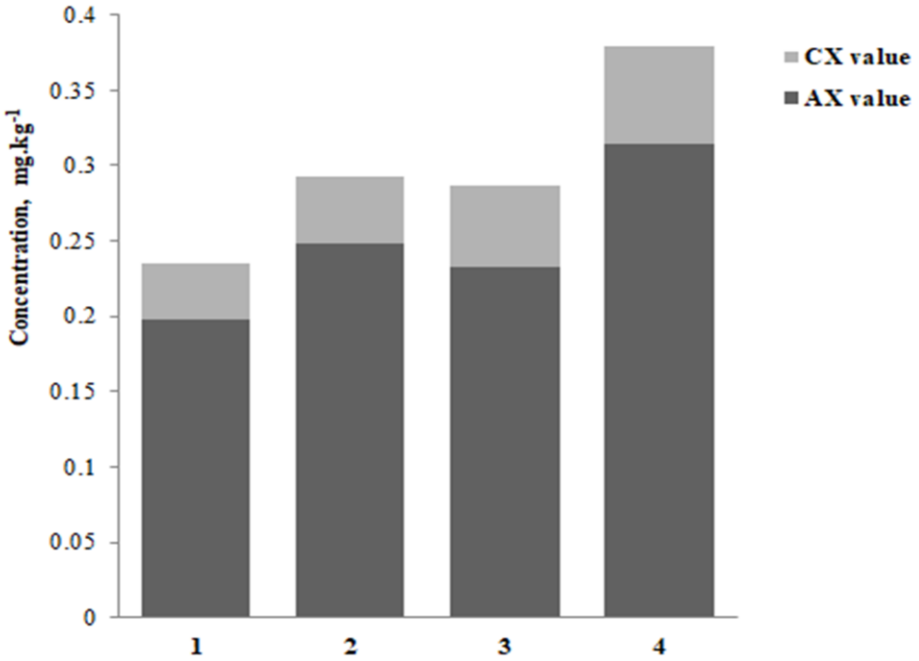

Figure 6. Chart of distribution of astaxanthin and canthaxanthin in muscle tissue of *Oncorhynchus mykiss* and *Salvelinus fontinalis M*: 1- Skeletal muscle of *Oncorhynchus mykiss;* 2- Heart of *Oncorhynchus mykiss*; 3- Skeletal muscle of *Salvelinus fontinalis* M; 4- Heart of *Salvelinus fontinalis* M.

Total xanthophyll level in the muscle tissue of brook trout *Salvelinus fontinalis M* is higher than those of rainbow trout *Oncorhynchus mykiss*. This can explain the darker colored brown flesh of brook trout *Salvelinus fontinalis* M. Total xanthophyll content in the cardio muscle of salmonid is larger than that in the skeletal muscle. This corresponds to the greater cell concentration per volume of cardiac muscle tissue.

Fish can supply xanthophylls only with the feed. The feed used in this experiment didn't contain extra added pigments. The analysis of the environment water shows also absence of astaxanthin and canthaxanthin. This fish species are able to absorb and deposit AX and CX in their organism under the described abiotic factors regardless of very low contentration levels, less than 0.034 $mg.l^{-1}$ and 0.021 $mg.l^{-1}$, for AX and CX respectively.

Acknowledgements

Author thanks to the fish farm "HAPPY FISH" Ltd, Bulgaria, for providing biological material for this study and especially to Prof. Vasil Atanasov, DSc for technical support and evaluation of experimental results.

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