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# Determination of Astaxanthin in Trout by High Performance Liquid Chromatography Coupled with Visible Detection

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***ABSTRACT***

**Background**: Astaxanthin is widely and naturally distributed in marine animals including, crustaceans, such as shrimp, crabs, and fish, such as salmon and trout. **Methods**: A total of 20 trout samples were collected from local market and were analyzed for astaxanthin via HPLC-UV. Analyte extraction was done by water, methanol, dichloromethane, and acetonitrile 4.5:85:5:45.5, *v/v/v/v*. **Results**: Despite the optimal conditions, analytical property of the method containing lower limit of quantitation (2.00 ng g-1) and upper limit of quantitation (1000 ng g-1), linearity ($r_2$=0.992), and recovery (103%) were acceptable. The results showed the range of 105 ± 0.09 - 875 ± 0.020 ng g-1. **Conclusion**: The method was successfully used to quantify the target analytes in all trout samples.

**Keywords:** *Astaxanthin; High performance liquid chromatography; Fish; Upper limit of quantification (ULOQ); lower limit of quantification (LLOQ)*

## Introduction

Astaxanthin is a lipid soluble xanthophyll carotenoid produced by various bacterias, yeasts, and microalgaes and found in different microorganisms and marine animals (Ambati *et al.*, 2014). Among these said cases, *Haematococcus pluvialis* is the most important source for biological production of this pigment. It is a red fat-soluble pigment which has more potent biological activity than other carotenoids (Mezzomo and Ferreira, 2016). The United States Food and Drug Administration (USFDA) has agreed the use of astaxanthin as food colorant in animal and fish feed . Astaxanthin is safe, with no side effects when it is consumed with foodstuff. The mild conditions with Bychemical reactions can synthesize astaxanthin (Kumar *et al.*, 2021). Astaxanthin is composed of two terminal rings joined by a polyene chain. This pigment has two asymmetric carbons located at the 3, 3′ positions of the β-ionone ring with hydroxyl group (-OH) on either end of the molecule. Astaxanthin is necessary for Trout reproduction, developing embryos and decreasing the mortality (Yang *et al.*, 2013). Aquatic is the best source for astaxanthin. The antioxidant properties of astaxanthin have been proven and this pigment has many nutritional properties (Lim *et al.*, 2018). Also astaxanthin shows anti-inflammatory feature and enhances human immune system. Trout is filled with protein, omega-3 fatty acids, vitamin D, and astaxanthin (Park *et al.*, 2010). High performance liquid chromatography-ultraviolet detector (HPLC-

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UV) was preferred as powerful technique for determination of astaxanthin due to its high-resolution power, flexibility, sensitivity, and reproducibility (Vervoort *et al.*, 1992). UV detector is a very commonly used detector for HPLC (Rapala *et al.*, 2002). In a similar study conducted on shrimp, astaxanthin content in the procambarus clarkia shell reached 239.96 µg/g. This study aims to determine the reliability and validation of an efficient and reliable method for accurate determination of astaxanthin in trout samples using HPLC-UV (Hu *et al.*, 2019). In this procedure, after validating the reliable method, it was used to identify astaxanthin in trout samples. To this end, the samples obtained from different regions of Iran were analyzed.

## Materials and Methods

*2.1. Chemicals and solutions:* The standard of astaxanthin with purity greater than 97% was obtained from LGC group (Teddington, UK). HPLC grade methanol and acetonitrile were obtained from DUKSAN (Gyeonggi-do, South Korea). Dichloromethane (≥99.8%) was from VWR (Radnor, Pennsylvania, United States of America). Milli–Q water system (Millipore, Billerica, MA, USA) was used for preparation of pure water.

*Instrumentation:* The chromatographic system was a KNAUER HPLC instrument (Knauer, Berlin, Germany) consisting of a Detector S2500 Knauer equipped with a Biotech 2003 degasser (United State), K-1000 Knauer controller Quaternary pump and Rheodyne sample valve fitted with a 20 µL loop (United State). The analytical column was SCIEX AAA C18 column 150 × 4.6 mm, 5 µm (Foster City, USA). The mobile phase was prepared freshly every day by a mixture of water, methanol, dichloromethane, and acetonitrile 4.5:85:5:45.5, *v/v/v/v*). The eluant flow rate was 1 ml/min and wavelength for recording chromatograms was 476 nm.

*Samples:* In this study, 20 trout samples produced in Iran were purchased from local vendors. All sample were stored a -20 °C in the dark prior to analysis.

*Sample preparation:* The studied fish muscle samples were washed with tap water and then with deionized water. After that, the samples were cut to small parts and blended. The homogenized sample was used in analytical procedure.

Firstly, 1.0 g of homogenized fish sample was transferred into a glass test tube and 5.0 ml mobile phase was added to it. The mixture was vortexed for 20 s and then sonicated for 5 min at 320 W and 50 Hz (at 25 °C). Then, the mixture was centrifuged for 15 min at 4000 rpm and all of the supernatant phase was removed and filtered through 0.22 µm PTFE membrane syringe filter. Then, 20 µL of the obtained clear solution was injected to HPLC-UV system.

*Method of validation parameters:* The HPLC-UV method for the determination of astaxanthin was validated for linearity, accuracy, and precision. Calibration curve was prepared by spiking six concentrations (2, 10, 100, 250, 500, and 1000 ng g-1) of astaxanthin in a blank. The linearity was calculated using these six concentrations in triplicate, also linearity requirements were fulfilled when the correlation coefficient was greater than 0.99. The calibration range included concentrations from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ). The LLOQ is defined as the lowest concentration of astaxanthin that can be determined with acceptable precision and accuracy, and the highest amount of astaxanthin that can be quantitatively determined with precision and accuracy is ULOQ. Recovery and precision were evaluated over three consecutive days at three nominal astaxanthin concentrations (80, 200 and 400 µg/l) by spiking an uncontaminated matrix (Safavizadeh *et al.*, 2020).

## Results

*Method validation:* The present method was validated by evaluation of several parameters containing LLOQ and ULOQ, linearity, selectivity, accuracy, intra- and inter-day precisions. The mobile phase was adjusted at 1.0 mL min-1. The calibration range included concentrations from the



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LLOQ to the ULOQ. The LLOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy, and the highest amount of analyte that can be quantitatively determined with precision and accuracy is ULOQ. The results showed that LLOQ and ULOQ for astaxanthin was 2.00 ng g-1 and 1000 ng g-1. The linearity of a calibration graph is evaluated by the calculation of the square of correlation coefficient ($r^2$). The calibration graph was plotted by the analyte concentrations versus analytical signals (peak area) after performing the method. In this study, six different concentrations of astaxanthin (2, 10, 100, 250, 500, and 1000 ng g-1) were spiked to analyte trout samples and after performing the method, the analytical signals (peak area) were plotted versus their initial concentrations. The data showed good linearity with $r^2$=0.992. It was found that the RSDs were in the ranges of 1.5-1.9% and 4.1-5.8% for intra- (n=3) and inter-day (n=3) percision. The results showed that the method is sufficiently repeatable for reliable determination of astaxanthin in fish samples. Chromatograms showed that there was no interfering peak in retention time of astaxanthin. The recovery of the sample was 103% which indicated that the recovery rate of the method was good and the method was feasible.

*Real sample analysis:* The developed method was performed on 20 trout samples. The results showed that all of the trout samples were not free of the astaxanthin, while the astaxanthin was found in samples in the range of 105 ± 0.09 - 875 ± 0.020 ng g-1. In trout samples, astaxanthin was naturally produced because of the fish feeding from microalgaes. However on the trout samples obtained from Iranian fish market.

## Discussion

Fish color is one of the most important characteristics in valuation and sales (Maciel *et al.*, 2013). The color of fish is controlled by the endocrine nervous system, but food sources and pigments also play an important role in determining color. The effect of carotenoid sources from the point of view of pigment is to create a color specific to each species (Lim *et al.*, 2018). Astaxanthin is one of the most important carotenoid sources that has gained special importance in aquaculture and its consumption is expanding. In the present study, differences in different levels of astaxanthin on blood factors related to the health of rainbow trout progenitors, including blood sugar, the ratio of albumin to serum globulin, cholesterol, triglycerides, total plasma protein, globulin, and serum albumin caused significant differences (Ambati *et al.*, 2014, Higuera-Ciapara *et al.*, 2006, Hussein *et al.*, 2006). Carotenoids have been widely used for their various benefits in warm-blooded and aquatic animals, including growth stimulation and immunity, increasing resistance to disease and stress, and creating the right color. Astaxanthin has an effective effect on the color of fish muscles and skin and it is most widely used in aquaculture due to its effect on fish color (Guerin *et al.*, 2003, Naguib, 2000).

For example, in 2010, Yasir and Qin studied the effect of nutrition with different types of carotenoids (astaxanthin, beta-carotene, cantazantine, and zeaxanthin) and its effect on skin color and the amount of pigment scales in fish and concluded that astaxanthin was higher in the red color of the skin (Yasir and Qin, 2010). Sawanboonchun reported in 2008 that increasing the average number of eggs and increasing the probability of fertilization were the benefits of using astaxanthin. Studies show that higher fertility rates can be expected in eggs with high astaxanthin than eggs with low astaxanthin (Sawanboonchun *et al.*, 2008).

## Conclusion

A simple, efficient, and environmentally friendly method based on ultrasonic assisted liquid phase extraction prior to HPLC-UV was developed and validated for determination of astaxanthin in fish samples. The method was used for identification of astaxanthin in trout samples which are sold in Iran markets. This method showed low LLOD and LLOQ, and acceptable recovery. Finally, the method was performed on



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trout samples and this pigment was found in all the samples.

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## Conflict of interest

No conflict of interest.

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