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Development and Validation of a High-Performance Liquid Chromatography Method for the Determination of Astaxanthin in Fish Samples; Application of the Method in Identification of Fake Salmon Marketed in Iran

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Astaxanthin is a carotenoid produced by different microalgae, bacteria, and yeasts in fish samples. Some fishes like salmon contain astaxanthin in their muscles and it can be a proper indicator for the identification of fishes. In this work, a simple and validated ultrasonic-assisted liquid-liquid extraction coupled to high-performance liquid chromatography-ultraviolet detector was developed for the determination of astaxanthin in fish samples in order to identify the trout from colored salmon sold in Iranian fish markets. Under the optimum conditions, analytical features of the method include a limit of detection (1.46 ng g^{-1}) and quantification (5.46 ng g^{-1}), linearity (r^2 = 0.995), precision (RSD ≤ 5.2%), accuracy (RSD ≤ 8%), and recovery (90%) were acceptable. The method was successfully used to determine the target analyte in several fish samples including thirty salmon produced in Iran, five salmon produced in Norway, and five trout samples. The results showed that trout samples were free of astaxanthin, while salmon samples produced in Iran have astaxanthin in the range of 179 ± 9-782 ± 40 ng g^{-1}. The content of astaxanthin in Norwegian salmons is much higher than in salmon samples produced in Iran.

Keywords: Astaxanthin, High performance liquid chromatography, Fish, Salmon, Liquid-liquid extraction, Trout

INTRODUCTION

Astaxanthin is a lipid-soluble xanthophyll carotenoid produced by a number of bacteria, yeasts, and microalgae [1]. Among these sources, *Haematococcus Pluvialis* is the most important source for the biological production of astaxanthin [2]. This pigment can be synthesized by chemical reactions under mild conditions [3]. The results showed that astaxanthin has useful effects on treating Alzheimer's and Parkinson's, liver diseases, and stroke. It is also used for metabolic syndrome, a group of conditions that increase the risk of stroke, heart disease, and diabetes [4,5]. Astaxanthin shows antioxidation and anti-inflammatory properties and enhances the immune functions of the human body [6]. Based on these advantages astaxanthin was used in the pharmaceutical and food industries as a drug to prevent oxidizing tissue damage and in healthy food preparations, respectively [7,8]. Astaxanthin has an

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attractive red color and can bind non-specifically. Therefore, adding astaxanthin to aquaculture feed can improve the color of the skin and flesh of the fish produced [9]. In addition, astaxanthin has important effects on fish growth and reproduction; such as encouraging roe fertilization, increasing the growth rate of developing embryos, decreasing the mortality rate, increasing the maturation rate and decreasing the age that the fish can do. It proliferates and increases disease resistance [10-12]. The use of foods containing astaxanthin is advised by the nutritionist. Salmon fish is known as the best source of astaxanthin. Salmon is a general name for several species of ray-finned fish Salmonidae group. Astaxanthin can be considered useful food for the human health due to possessing high contents of protein, omega-3 fatty acids, and vitamin D [13]. Unfortunately, the studies showed that trout fed by astaxanthin is very similar to salmon and it can be sold in the markets as salmon fish. This is a big fraud and it should be controlled by the Regulatory Commissions. The development of a simple, easy, valid, and accurate method for the analysis of astaxanthin in fish samples is an essential request by the communities. The spectrophotometric methods can be used for total carotenoids determination [14], but the accurate determination of astaxanthin cannot be performed.

High-performance liquid chromatography-ultraviolet detector (HPLC-UV) was preferred as a powerful technique for the determination of astaxanthin due to its high-resolution power, flexibility, sensitivity, and reproducibility. Recently Wang *et al.* extracted astaxanthin from shrimp shells and studied the effects of different treatments on its content. In this report, astaxanthin was extracted in different conditions and its concentration was determined by HPLC [15]. In this work, ethanol in water solution (10%, *v/v*) was used under sonication for 2.5 h. It is obvious that the proposed extraction method is time-consuming and boring. However, relatively high recoveries (nearly 54.3-84.6%) were obtained by this method. In another report, astaxanthin was determined in a green microalga. In this procedure, total pigments in the samples were extracted using a methanol/dichloromethane mixture (3:1, *v/v*) on an HPLC-UV system [16]. Up to now, different extraction methods including liquid-liquid extraction [17], solid-phase extraction [18], dispersive solid-phase extraction [19], hollow-fiber liquid phase extraction [20], dispersive liquid-liquid extraction [21] and single drop microextraction were reported as sample preparation methods for the analysis of different compounds.

The goal of this study was the development and validation of an easy and reliable extraction method for the extraction and determination of astaxanthin in fish samples using HPLC-UV. In this work, ultrasonic-assisted liquid-phase extraction was used for extraction of the analyte from the fish sample. Sonication irradiations were used to penetrate the extraction solvent into the sample and accelerate the extraction efficiency. The effect of various factors on the efficiency of this step like extraction solvent composition, extraction solvent volume, sonication time, and frequency was studied by a “one-factor-at-one-time” strategy. Then, the optimized method was validated completely and performed on different trout and salmon samples for the identification of fake salmon marketed in Iran. Short extraction time, easiness, cheapness, and high efficiency are the most important advantages of the method.

MATERIALS AND METHODS

Chemicals and Solutions

The standard of astaxanthin with purity greater than 98% was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol, water, and acetonitrile were obtained from DUKSAN (Gyeonggi-do, South Korea). Dichloromethane (≥99.8%) was bought from Merck (Darmstadt, Germany). A stock solution of the analyte was prepared in methanol at a concentration of 100 mg l^{-1} and it was used for the preparation of working solutions using its dilution with deionized water.

Instrumentation

A Knauer (Berlin, Germany) HPLC system equipped with a ultraviolet detector (S2500), a Biotech 2003 degasser (MPLS, USA), a K-1000 Knauer controller Quaternary pump, and a Rheodyne sample valve fitted with a 20 µl loop was used for separation and determination of astaxanthin. The analytical column was the SCIEX AAA C_{18} column (150 × 4.6 mm, 5 µm) (Foster, USA). The mobile phase was prepared by mixing water: methanol: dichloromethane: acetonitrile at a ratio of 5.0:85:5.0:5.0 (v/v/v/v). The eluent

flow rate was 1 ml min^{-1} and the detection wavelength was 475 nm. A Hettich centrifuge, model D-7200 (Germany), was used for accelerating phase separation. A Labsonic LB S2 (Falc, Treviglio, Italy) was used for sonication.

Real Samples

In this study, thirty salmon and five trout samples, produced in Iran, were purchased from local vendors in Tabriz (East Azarbaijan Province, Iran) and Tehran (Tehran Province, Iran). One trout sample was bought and used in the validation step as a blank sample. Also, five frozen Norwegian salmons were purchased for comparing the obtained results with other salmons produced in Iran. All samples were stored at -20 °C in a dark place prior to their analysis. The studied fish muscle samples were washed with tap water and deionized water, respectively. After that the samples were cut into small parts and blended. The homogenized sample was used in the analytical procedure. To prepare the spiked samples, 50 g of each fish sample was spiked with the analyte and the mixture was left to complete penetration of the analyte for at least 15 min prior to performing the proposed extraction method.

Ultrasonic-assisted Liquid-liquid Extraction

A 1.0 g of homogenized trout fish or real samples spiked with astaxanthin at a concentration of 500 ng g^{-1} or fish sample was transferred into a glass test tube and then 5.0 ml of mobile phase was added into the tube. After manually shaking the mixture, the tube was transferred into a sonication bath adjusted at 40 °C and it was sonicated for 6 min at 40 kHz. After that, 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 into the HPLC-UV system.

RESULTS AND DISCUSSION

The main objective of the present study is the development of a validated HPLC-UV method for extraction and quantification with high efficacy, short analysis time, and best chromatographic conditions from fish samples. Effective extraction and chromatographic parameters were studied and the method was validated and used on different fish samples.

Optimization of HPLC-UV Conditions

Optimization of mobile phase composition. Optimization of the mobile phase composition was done by using various combinations of water, methanol, and acetonitrile and the obtained chromatograms for the analyte were compared considering retention time, tailing factor, peak width at half-height, peak width at 0.05 of height, and the number of theoretical plates. The obtained results are shown in Table 1. Based on these results, the highest number of theoretical plates and lowest retention time and

Table 1. Chromatograms Characteristics at Different Compositions of Mobile Phase

Mobile phase composition (Water:methanol:acetonitrile, v/v/v)	Retention time (min)	$W_{1/2}$[a]	N[b]	$W_{0.05}$[c]	T[d]
10:0.0:90	-	-	-	-	-
10:90:0.0	13.7	0.62	2693	1.11	2.84
5.0:95:0.0	16.3	0.74	2678	1.30	3.17
5.0:90:5.0	12.9	0.51	3543	0.93	1.02
10:85:5.0	15.3	0.61	3473	1.06	1.29
5.0:85:10	13.2	0.56	3068	1.02	1.36
7.5:85:7.5	13.6	0.72	1967	1.32	1.73
10:80:10	17.9	0.82	2636	1.51	1.22
15:75:10	18.2	0.76	3170	1.42	1.24
10:75:15	19.5	0.93	2434	1.73	1.32

[a]Peak width at half-height. [b]Number of theoretical plates. [c]Peak width at 0.05 of maximum height. [d]Tailing factor.

tailing factor were obtained at the water: methanol: acetonitrile was 5.0:90:5.0 (v/v/v). It should be considered that in all tests a constant analysis time was considered. However, in these conditions retention time for the analyte and tailing factor were not satisfactory. Therefore, dichloromethane was added to the mobile phase and a quaternary composition was used. Different percents of dichloromethane (1-5%) were added into the mobile phase and the methanol amount was decreased, simultaneously. The obtained chromatograms (Fig. 1) showed that the most suitable mobile phase was obtained to be composed of water/methanol/dichloromethane/acetonitrile of 5.0:85:5.0:5.0 (v/v/v/v).

Optimization of Mobile Phase Flow Rate and Column Temperature

Optimization of the mobile phase flow rate was investigated in the range of 0.5-1.2 ml min^{-1} (at 0.1 ml min^{-1} interval) while the other conditions were kept constant. The results showed that broad peaks at high retention times were obtained at flow rates ≤1.0 ml min^{-1}. It is noted that at flow rates of higher than 1.0 ml min^{-1} there was no significant improvement in the peak shape except its retention time. Therefore, 1.0 ml min^{-1} was selected for the next steps.

The effect of column temperature was studied in the range of 20-40 °C. The obtained results showed that there was no important improvement in the analyte retention time while the column pressure was decreased. It can be attributed to the decrease in the mobile phase viscosity. Subsequently, the column temperature was adjusted at 40 °C.

Selection of Monitoring Wavelength

The method sensitivity is strongly related to the detection wavelength. To obtain high sensitivity it was studied in the range 470-480 nm (at 5 nm intervals). The outcomes showed that the best efficiency was obtained at 475 nm and it was selected for the next steps.

Optimization of Effective Parameters on Sample Preparation Step

The effect of extraction solvent volume has a significant role on the method performance and it affects the extraction efficiency by altering the volume ratio of sample solution to extraction solvent. In this method, the extractant volume was assayed by adding the different volumes of mobile phase into 1.0 g of spiked trout sample at a concentration of 500 ng g^{-1} within the range of 1-8 ml. The obtained data in Fig. 2a showed that the extraction efficiency of the method increased up to 5.0 ml and then remain constant. Since at higher volumes (>5 ml) dilution of the analyte in the extractant phase decreases its analytical signal and therefore, 5.0 ml was preferred to use in the following steps.

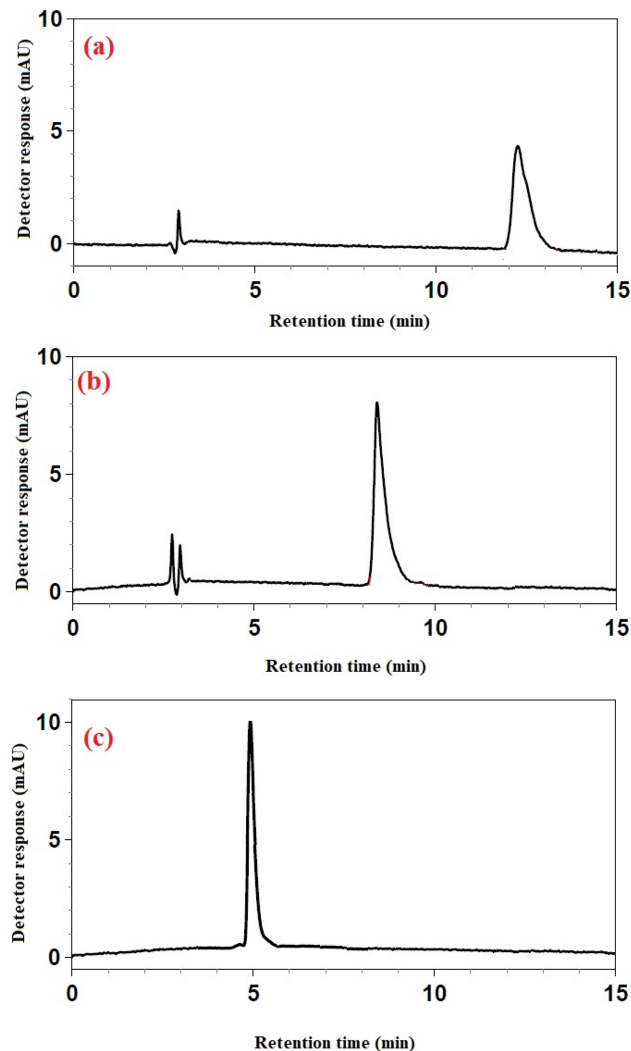

Fig. 1. Effect of mobile phase composition on astaxanthin peak. Direct injection of standard solution prepared in mobile phase at a concentration of 500 mg l^{-1} containing (a) 0%, (b) 2.5%, and (c) 5.0% dichloromethane.

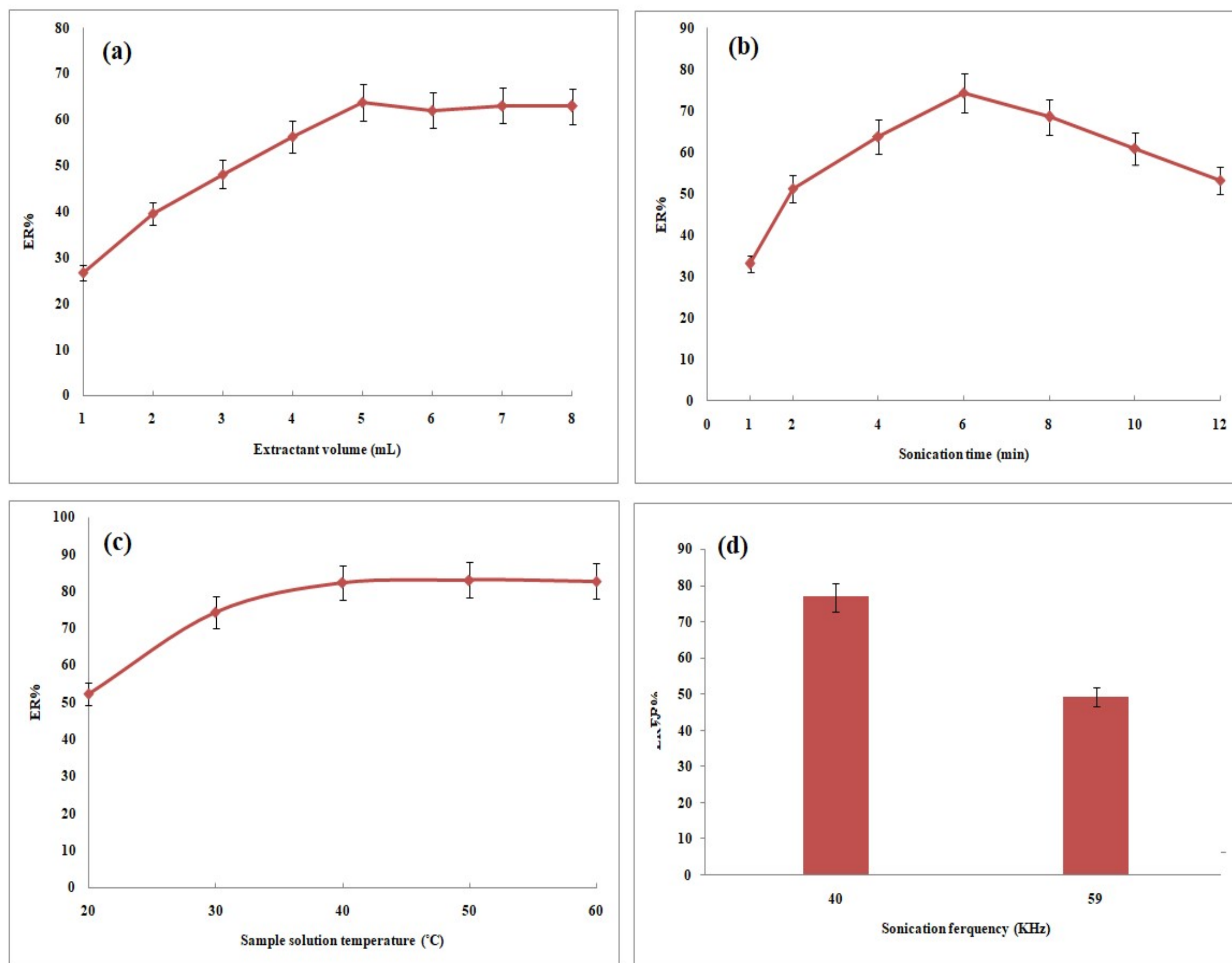

Fig. 2. Optimization of effective parameters in extraction method.
(a) Optimization of extraction solvent volume. Extraction conditions: sample, 1.0 g of trout sample spiked with astaxanthin at a concentration of 500 ng g^{-1}; sonication time, 4 min; sample solution mixture, 30 °C; sonication frequency, 40 kHz. The error bars indicate the minimum and maximum of three determinations.
(b) Optimization of sonication time. Extraction conditions: sample, 1.0 g of trout sample spiked with astaxanthin at a concentration of 500 ng g^{-1}; extraction solvent composition (volume), water/methanol/dichloromethane/acetonitrile of 5.0:85:5.0:5.0 v/v/v/v (0.5 ml); sample solution mixture, 30 °C; sonication frequency, 40 kHz. The error bars indicate the minimum and maximum of three determinations.
(c) Optimization of sample solution temperature. Extraction conditions: sample, 1.0 g of trout sample spiked with astaxanthin at a concentration of 500 ng g^{-1}; extraction solvent composition (volume), water/methanol/dichloromethane/ acetonitrile of 5.0:85:5.0:5.0 v/v/v/v (0.5 ml); sonication time, 6 min; sonication frequency, 40 kHz. The error bars indicate the minimum and maximum of three determinations.
(d) Optimization of sonication frequency. Extraction conditions: sample, 1.0 g of trout sample spiked with astaxanthin at a concentration of 500 ng g^{-1}; extraction solvent composition (volume), water/methanol/dichloromethane/acetonitrile of 5.0:85:5.0:5.0 v/v/v/v (0.5 ml); sonication time, 6 min; sample solution temperature, 40 °C. The error bars indicate the minimum and maximum of three determinations.

The time of sonication has a key effect on the method performance by increasing the contact area of the sample with extraction solvent. On the other hand, the analyte destruction can occur at higher sonication times. Evaluation of sonication time effect on the method efficiency was studied in the range of 1.0-12 min and the results (Fig. 2b) showed that ER% increased till 6 min and then decreased. This alteration can be related to the effect of sonication on the migration rate of the analyte from sample to extraction solvent up to 6 min and decomposition of the analyte at higher times. Subsequently, 6 min was selected for the next steps.

The temperature of the sonication bath may increase the extraction solvent penetration into the sample matrix and the efficiency of the method. Also, the sample solution temperature can enhance the analyte migration rate into the extraction solvent. Investigation of the sample solution temperature was performed by performing different tests in the range of 20-60 °C. Based on the obtained %ER at different temperatures, 40 °C was selected for the next experiments.

Sonication is a crucial step for increasing the method efficiency. Sonication produces small bubbles in the sample solution which are nucleated and grown at sufficient frequencies. The bubbles collapse forms many local sections with high temperatures and pressures. These areas can act as an efficient media for fast extraction of the analyte. In this method, the effect of sonication effect was investigated at two frequencies and the obtained data were shown in Fig. 2d. According to the results, the method efficiency at 40 kHz is higher than 59 kHz and it was chosen for the next experiments.

Method Validation

The present method was validated considering several parameters including limit of detection (LOD), limit of quantification (LOQ), linearity (LR), selectivity, accuracy, intra‐ and inter‐day precisions, and ER using the International Council Harmonization protocol [23].

Limit of Detection and Limit of Quantification

The lowest concentration of an analyte that can be consistently determined by an analytical approach is considered as LOD. Typically, LOD is measured to be in the range where the peak height (S) of an analyte to noise (N) ratio is equal to 3 (S/N = 3). The LOQ is the lowest concentration level that can be quantitatively measured with acceptable accuracy and precision and is calculated based on S/N = 10. The results (Table II) showed that LOD and LOQ for astaxanthin were 1.46 and 5.46 ng g^{-1}, respectively.

Linearity

The linearity of the calibration graph is evaluated by the calculation of coefficient of determination (r^2). In this method, the calibration graph was plotted by analyzing analyte-free trout fish samples spiked at seven concentrations of astaxanthin including 25, 50, 100, 250, 500, 1000, and 5000 ng g^{-1} using the developed method. After performing the developed method, the analytical signals were plotted *versus* the concentration and calibration curve equation and the obtained $r^2 = 0.995$ confirmed good linearity.

Repeatability

Repeatability of a method is defined as the nearness of the results obtained from independent analysis with the same method. In extraction methods, usually, repeatability was considered as the method precision and it is expressed as relative standard deviation (RSD) for replicate analysis. In this method, the method precision was evaluated by analyzing spiked blank trout samples spiked with astaxanthin at four concentrations consisting of 25, 100, 250, and 400 ng g^{-1}. It was found that the RSDs were in the ranges of 1.1-2.4% and 3.3-5.2% for intra- (n = 5) and inter-day (n = 3) precisions, respectively. The results (Table 2) showed that the method is sufficiently repeatable for the reliable determination of astaxanthin in fish samples.

Accuracy

The closeness of the mean experimental results obtained by an analytical method to the accurate concentration is expressed as the accuracy. The best approach to evaluate the accuracy of an analytical method is performing the method on Certificated Reference Materials (CRMs). In this case, there is no CRM for astaxanthin in fish samples and the standard addition method was used for accuracy assessment. In this method, four blank trout samples were spiked with

Table 2. Figures of Merit of the Developed Method for Astaxanthin

Analyte	Calibration curve equation	LR[a]	r[b]	LOD[c]	LOQ[d]	ER[e]	RSD[f]							
							Intra-day percision (n = 5) at a concentration of (ng g^{-1})				Inter-day percision (n = 3) at a concentration of (ng g^{-1})			
							25	100	250	400	25	100	250	400
Astaxanthin	Y = 21017 x + 1936	5.46-5000	0.995	1.46	5.46	90 ± 2	1.9	2.1	2.4	1.1	3.3	5.2	3.9	4.6

[a]Linear range (ng g^{-1}). [b]Correlation coefficient. [c]Limit of detection (S/N = 3) (ng g^{-1}). [d]Limit of quantification (S/N = 10) (ng g^{-1}). [e]Extraction recovery ± standard deviation (n = 3). [f]Relative standard deviation.

Table 3. Accuracy Assessment of the Developed Method in Determination of the Analyte

Analyte	Added (ng/g)	Sample #1				Sample #2				Sample #3				Sample #4			
		Found (ng/g)	SD (ng/g)[a]	Recovery (%)	RSD (%)[b]	Found (ng/g)	SD (ng/g)	Recovery (%)	RSD (%)	Found (ng/g)	SD (ng/g^{-1})	Recovery (%)	RSD (%)	Found (ng/g)	SD (ng/g)	Recovery (%)	RSD (%)
Astaxanthin	25	21.6	0.89	86	4.1	20.9	1.1	83	5.2	21.3	0.74	85	3.4	21.2	1.2	84	5.6
	200	182.3	3.6	91	1.8	181.4	3.2	90	1.7	172.6	4.1	86	2.3	179.2	3.4	89	1.8
	400	368.2	5.2	92	1.4	371.2	7.2	92	1.9	370.3	9.9	92	2.6	363.4	12.4	90	3.4

[a]Standard deviation. [b]Relative standard deviation.

astaxanthin at three concentrations including 25, 200, and 400 ng g^{-1}, and the method was performed on them. The obtained results are shown in Table 3. The data showed that RSDs were less than 5.6% for astaxanthin.

Selectivity

The selectivity of the developed method was investigated by analyzing different fish samples (3 salmon and 3 trout samples) obtained from different sources and the chromatograms showed that there was no interfering peak in the retention time of astaxanthin and the developed method is selective for the selected analyte in fish samples.

ER

ER is defined as the percentage of the total amount of analyte (n_0) that is transferred into the extractant (n_{ex}). The following equation is used for ER calculation.

$$ER\% = \frac{n_{ex}}{n_0} \times 100 = \frac{C_{ex} \times V_{ex}}{M \times C_0} \times 100 \quad (2)$$

where V_{ex} and M represent the volume of the extractant and the weight of the fish sample, respectively. The recovery of the sample was 90.2% and the RSD was 2.3%, which indicated that ER of the method is good.

Real Sample Analysis

The developed method was performed on different fish samples including 30 wild salmons marketed in Iran, five trout, and five Norwegian salmons and the content of astaxanthin was determined in them using the standard addition method. The results showed that all of the trout samples were free of the astaxanthin while the astaxanthin was found in Norwegian salmons in the range of 1300 ± 72 - 2000 ± 269 mg kg^{-1}. In Norwegian salmon samples, astaxanthin was naturally produced because the fish feed from microalgae. However, on the salmon samples obtained from Iranian fish markets, the astaxanthin content was in the range of 179 ± 9 - 782 ± 40 ng g^{-1} which is much lower than Norwegian salmon samples. It can be attributed to the addition of astaxanthin to the ration of the trout and coloring their muscles. The concentration of astaxanthin in salmon

Table 4. The Concentration of Astaxanthin (ng g^{-1}) in Salmon Fishes Marketed Iranian Fish Markets. All Determinations were Done in Triplicates

Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Sample #6	Sample #7	Sample #8	Sample #9	Sample #10
276 ± 14	179 ± 9	643 ± 33	193 ± 10	782 ± 40	186 ± 9	218 ± 11	184 ± 9.5	640 ± 33	299 ± 15
Sample #11	Sample #12	Sample #13	Sample #14	Sample #15	Sample #16	Sample #17	Sample #18	Sample #19	Sample #20
188 ± 10	224 ± 12	430 ± 23	240 ± 12	321 ± 16	224 ± 11	229 ± 12	344 ± 17	425 ± 22	445 ± 23
Sample #21	Sample #22	Sample# 23	Sample #24	Sample #25	Sample #26	Sample #27	Sample #28	Sample #29	Sample #30
351 ± 18	345 ± 18	265 ± 13	265 ± 13	332 ± 17	374 ± 20	424 ± 20	236 ± 12	260 ± 13	451 ± 24

Table 5. Results of Assays to Check the Samples Matrix Effects. The Analyte Content of the Samples was Subtracted and all Determinations were Done in Triplicates

Analyte	Added (ng g^{-1})	Found (ng g^{-1}) (Mean relative recoveries ± SD)		
		Sample #1[a]	Sample #2[b]	Sample #3[c]
Astaxanthin	0	1410 ± 62	224 ± 11	ND[d]
	100	97 ± 4	98 ± 5	96 ± 4
		(97 ± 4)	(98 ± 5)	(96 ± 4)
	200	186 ± 24	192 ± 9	196 ± 8
		(93 ± 6)	(96 ±9)	(98 ± 8)
	400	402 ± 22	406 ± 14	417 ± 13
		(100 ± 5)	(101 ± 3)	(104 ± 3)

[a]Norwegian salmon. [b]Iranian salmon. [c]Trout sample. [d]Not detected.

samples obtained from Iranian fish markets was mentioned in Table 4. To evaluate the matrix effect standard-addition method was performed on different samples spiked at three concentrations including 100, 200, and 400 ng g^{-1} and the method was performed on them (three times for each concentration). The obtained concentrations and mean relative recoveries were shown in Table 5. The results showed that the matrices of the samples had no significant effect on the efficiency of the method. Figure 3 shows typical HPLC-UV chromatograms of direct injection of standard solution at 10 mg l^{-1} and unspiked trout and salmon samples after performing the developed method. Sensitive and selective extraction and determination of astaxanthin was performed by using the present green extraction method. The present method can be also easily applied to different samples for the determination target analyte.

Comparison of the Method with other Methods

Table 6 shows RSD, LR, extraction time, extraction solvent volume, and LOD values of the introduced method and other previously published methods for the determination of the analyte. The presented method LODs are lower than those of the compared procedures. The method precision is good and the RSD% values in the present work are less than the RSD values of the other approaches. The method extraction time is shorter than other methods except for the method developed by López-Cervantes and co-workers [25]. According to the results, the proposed method is a rapid, sensitive, and repeatable technique that can be used for the

extraction/preconcentration and determination of the target analyte in fish samples.

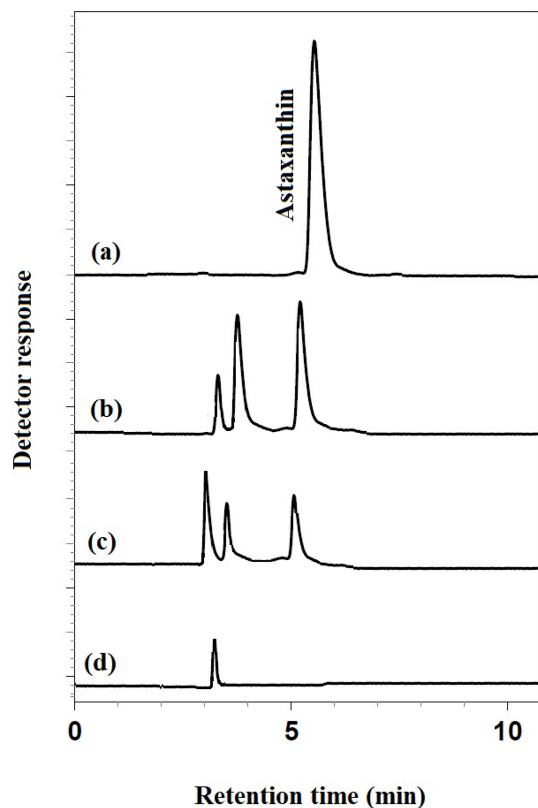

Fig. 3. Typical HPLC-UV chromatograms (a) standard solution at 10 mg l^{-1} astaxanthin (direct injection), (b) un-spiked Norwegian fish after performing the method, (c) un-spiked salmon fish produced in Iran after performing the method, and (d) un-spiked trout after performing the method.

CONCLUSIONS

A simple, efficient, and validated method based on ultrasonic-assisted liquid-phase extraction coupled to HPLC-UV was developed for extraction and determination of astaxanthin in fish samples. The method was used for the identification of fake salmons sold in Iran. The proposed method showed low LOD and LOQ, and acceptable recovery. Finally, the method was performed on different samples and unfortunately, it was found that most of the sold salmons in the markets of Iran are colored trout (astaxanthin was added to the fish ration).

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Table 6. Comparison of the Method with other Procedures in Determination of the Analyte

Method	Sample	LOD (ng g^{-1})[a]	LR (ng g^{-1})[b]	RSD (%)[c]	Extraction solvent volume (ml)	Extraction time (min)	Ref.
LLE-HPLC-UV[d]	Feed	100	-	≤ 4.2	50	~ 40	[23]
LLE-HPLC-UV	Eggs	440	440-10000	≤ 1	15	~ 15	[24]
SPE-HPLC-UV[e]	Shrimp waste	-	520-2600	≤ 2.2	5	~ 2	[25]
LLE-HPLC-UV	Fish	1.4	5.4-5000	≤ 2.4	5	~ 21	Present work

[a]Limit of detection. [b]Linear range. [c]Relative standard deviation. [d]Liquid-liquid extraction-high performance liquid chromatography-ultraviolet detector. [e]Solid phase extraction-high performance liquid chromatography-ultraviolet detector.

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