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Selective extraction of astaxanthin from crustaceans by use of supercritical carbon dioxide

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Abstract

An on-line supercritical fluid extraction (SFE) system coupled to a continuous flow manifold including a UV detector was used as a screening system to extract astaxanthin from crayfish, which was found to be the major carotenoid present in the samples. This compound constitutes the principal additive used to dye salmon flesh. The flow manifold was used to confirm the presence of astaxanthin in the crustacean samples. Also, an HPLC/UV–vis method was used to ascertain that this compound was the major carotenoid extracted under the optimum SFE conditions employed. The influence of SFE operating variables such as pressure, temperature, equilibration time, extraction time, trap temperature, and volume of CO_2 modifier was examined in order to maximize the efficiency of analyte extraction. The use of supercritical CO_2 enables the expeditious, selective, quantitative extraction of astaxanthin from crustaceans.

Keywords: SFE; Screening; Astaxanthin; Crustaceans

1. Introduction

"Carotenoid" is a generic name used to designate the most common groups of naturally occurring pigments found in the animal and plant kingdoms. These lipid-soluble pigments comprise well over 700 compounds that account for beautiful red, orange, and yellow colors. Most carotenoids are polyunsaturated hydrocarbons containing 40 carbon atoms and two terminal rings systems. Also, carotenoids are highly conjugated polyprenoid nutrients essential in the human diet by virtue of their antioxidant [1] and anti-cancer properties [2]. They can be obtained from a variety of sources including fruits, vegetables, and sea foods [3]. Carotenoids that are composed entirely of carbon and hydrogen are known as carotenes, whereas those that also contain oxygen are termed xanthophylls. Astaxanthin is one conjugated keto-carotenoid and hence a xanthophyll.

Carotenoids are the major pigments in fish, which, however, cannot synthesize these compounds by themselves. Thus, the addition of carotenoids to aquaculture feed provides the color associated with the bright vibrant colors of ornamental fish. Astaxanthin amounts accounting for over 90% of the total carotenoid content have been found in the flesh of wild salmons (salmon and trout) [4,5]. This xanthophyll is 10 times stronger than β-carotene, and up to 500 times stronger than Vitamin E, as an antioxidant [6,7]. It occurs in wild salmon and is used in aqua-feeds to impart this natural, pink–red color to farmed salmon fillets. Salmons cannot synthesize astaxanthin endogenously; therefore, it must be supplemented in fish diet. The astaxanthin absorbed is then transported in the bloodstream to the muscles and skin, where it accumulates [8]. All these facts make the determination of astaxanthin in crustaceans very interesting.

Official and conventional methods based on solvent extraction of carotenoids from natural matrices are time-consuming as they involve a multiple extraction steps and require large amounts of organic solvents, which are often

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expensive and potentially hazardous [9,10]. The problems associated with traditional solvent extraction techniques have aroused growing interest in developing simpler, faster, more efficient methods for the extraction of carotenoids from foods and natural products [11–13]. In recent years, SFE has proved one of the most appealing techniques for solid sample treatment. In fact, supercritical fluids diffuse more readily into matrices than do ordinary liquids, thereby improving the extraction yields of analytes from complex matrices. The SFE technique is a desirable alternative to the solvent extraction of some classes of natural substances from foods. SFE is highly expeditious and efficient; also, it avoids the need for concentration steps and simplifies analytical procedures as a result. One advantage of supercritical CO_2 relative to traditional organic solvents is that it can be used at a moderate temperature; this allows carotenoid losses through heat-induced degradation to be reduced. In addition, because it avoids the use of organic solvents, the extracted compounds can be employed as nutritional additives and in pharmacological applications. The SFE technique has previously been assessed as an alternative to the extraction of carotenoids from complex natural products [14–21].

Only two SFE methods for the selective extraction of astaxanthin from crustaceans appear to have been reported to date [22,23]. The aim of this work was to develop a method for the same purpose, but using the SFE technique in conjunction with a screening system to expeditiously confirm the presence or absence of the astaxanthin.

2. Experimental procedures

2.1. Apparatus

2.1.1. Supercritical fluid extraction–UV detection system

All SFE tests were conducted on a Hewlett-Packard 7680A supercritical fluid extractor equipped with a Hewlett-Packard 1050 isocratic modifier pump and furnished with a 7 ml extraction vessel, an automated variable restrictor and a solid-phase trap packed with Porapack Q, stainless steel (SS) or octadecylsilica (ODS) material. The extractor was controlled via the software HP 7068T, which was run under Microsoft™ Windows 3.1 on an IBM compatible PC. An on-line coupled SFE–continuous flow manifold including a Hewlett-Packard 8453A diode array spectrophotometer controlled via a Hewlett-Packard Vectra 500 computer was used to determine the total carotenoid contents in the samples. The continuous flow system (CFS) allowed the SF-extracted analytes to be transferred to the UV detector.

2.1.2. High performance liquid chromatography (HPLC)

Supercritical fluid extracts were analyzed on an HPLC system consisting of a Knauer 64 HPLC pump, a Rheodyne 7725 high-pressure manual injector valve with a 20 μl injection loop, and a Hewlett-Packard 1040A photodiode array detector. Data was acquired and controlled using Agilent ChemStation software, which was run under Microsoft™ Windows NT on an IBM compatible PC.

2.2. Reagents

Astaxanthin and diatomaceous earth (acid washed and containing ca. 95% SiO_2) were purchased from Sigma and used as received. All solvents and reagents were HPLC grade. SFE/SFC grade CO_2 from Air Products was used as extraction fluid. Butylated hydroxy anisole (BHA), supplied by Sigma, was used to avoid oxidation of astaxanthin in its stock solutions.

2.3. Sample preparation

The crayfish studied was supplied by Ecodryer S.A. (Seville, Spain). Crayfish waste was obtained from a processing plant (Seafood Sevilla) in the marshes of the river Guadalquivir in the province of Seville (southern Spain). Samples were stored in a dryer at room temperature, ground and passed through a no. k 84 sieve of 0.50 mm mesh prior to analysis. No other treatment was applied prior to their supercritical fluid extraction.

Astaxanthin was also extracted manually from crustaceans, shaking an amount of 0.3 g of sample with 5 ml of acetone. The extracts thus obtained were filtered and placed in a 25 ml flask. The process was repeated three times and the final sample diluted to 25 ml with acetone prior to injection of appropriate aliquots into the HPLC system.

2.4. Supercritical fluid extraction

Each extraction thimble was loaded with 0.1 g of ground crustacean sample and 0.6 g of diatomaceous earth in all cases in order to reduce the void volume. Thimbles were placed in the extraction chamber, which was kept at 60 °C throughout. Supercritical CO_2 was aspirated through a dip tube, pressurized to 200 bar (corresponding to a 0.73 g ml^{-1} density at 60 °C) and mixed on-line with 15% (v/v) ethanol. Samples were subjected to dynamic extraction for 15 min. The leached analytes were driven to an ODS trap through a variable restrictor; this avoided plugging to a great extent and ensured a constant flow rate during extraction. In a subsequent step, the trap was depressurized and flushed with a liquid solvent (1.5 ml of acetone) that was pumped through it at a flow rate of 1.5 ml min^{-1} by means of a syringe pump. The trap was kept at 80 and 30 °C during the extraction and flushing steps, respectively.

2.5. Photometric screening

Total carotenoid extracts, which consisted largely of astaxanthin, were analyzed by direct measurement at 450 nm for screening purposes. Calibration solutions containing 0.1–15 μg ml^{-1} astaxanthin were prepared from a

100 μg ml^{-1} stock solution in acetone containing 1% BHA. The resulting dilute solutions were used to construct a calibration plot.

2.6. HPLC method

The presence of astaxanthin in the carotenoid extracts provided by the SFE system was confirmed by using a slightly modified version of a previously reported method [24]. Carotenoid extracts in acetone were passed through a filter of 0.45 μm pore size and directly injected (in 20 μl aliquots) into the HPLC system for separation on a reversed phase Spherisorb® ODS analytical column (25 cm × 4.6 mm i.d., 5 μm particle size) from Waters (Barcelona, Spain). A 67.5:22.5:9.5:0.5 methanol:dichloromethane:acetonitrile:water mixture was used as mobile phase, at a flow rate of 1 ml min^{-1}. The effluent from the column was monitored spectrophotometrically at 450 nm.

3. Results and discussion

SFE variables were optimized in order to maximize the recovery of astaxanthin from real crustacean samples. The carotenoid SF extracts in acetone were screened for the analyte using a UV–vis spectrophotometer. Those samples testing positive for astaxanthin were subsequently subjected to the HPLC method in order to confirm whether it was the main carotenoid extracted.

The amount of carotenoids extracted was determined from absorbance measurements. Carotenoids absorb maximally at 470 nm. However, the linear range obtained was wider at 450 nm than at 470 nm, so the former wavelength was chosen to construct the calibration curve, which was obtained by using astaxanthin standards containing 0.1–15 μg ml^{-1} concentrations of the analyte. The figures of merit of the proposed screening method are given in Table 1.

3.1. Optimization of SFE variables

Tests were conducted with a view to assessing the effects of various factors on the SFE of astaxanthin. The variables optimized were the CO_2 pressure and density, extraction temperature (in the extraction chamber and trap), elution temperature (in the trap), equilibration and extraction time, extraction and elution flow rate, modifier (ethanol) volume, trap type, and amounts of sample and diatomaceous earth. Their optimum values are shown in Table 2.

Table 1
Figures of merit of the proposed screening method

	Measured at 450 nm
Intercept (a)	−0.004 ± 0.018
Slope (b)	0.199 ± 0.002
Regression coefficient (r)	0.9994
Standard deviation of residual ($S_{y/x}$)	0.044
Curve fitting level (R^2) (%)	99.88
R.S.D. (%) ($n = 10$)	15.3
LOD (μg ml^{-1})	0.022
LOQ (μg ml^{-1})	0.074

Table 2
Optimization of SFE variables

Variable	Range studied	Optimum value
Amount of sample (g)	0.05–0.15	0.1
Amount of diatomaceous earth (g)	0–0.6	0.6
Equilibration time (min)	0–5	0
Extraction time (min)	15–25	15
Pressure (bar)	200–350	200
Density (g ml^{-1})	0.73–0.93	0.73
Extraction temperature		
Extraction T in chamber (°C)	40–60	60
Extraction T in trap (°C)	80–85	80
Elution T in trap (°C)	20–40	30
Extraction flow rate (ml min^{-1})	1–3	2
Elution flow rate (ml min^{-1})	0.5–2	1.5
Modifier (ethanol) content (%)	0–20	15
Trap	ODS, PorapackQ, SS ODS	

3.1.1. Sample weight and cell dead volume

Because the extraction chamber volume (7 ml) was much greater than the sample size (<0.5 ml), an inert solid (diatomaceous earth) was added to the vessel in order to fill in as much void volume as possible. The cell dead volume was thus reduced and no additional extraction time was required to flush the SC extract. Diatomaceous earth was placed at the extraction chamber edge of the CO_2 inlet. In this way, variable amounts of diatomaceous earth from 0 to 6 g were used and the amount of carotenoids extracted was found to markedly increase with the addition of this material in amounts up to 0.6 g.

Although variable amounts of crustaceans were studied, the specification of the SFE equipment advised against the use of large amounts of sample to avoid contamination problems in various parts. Hence, the amounts of sample used were restricted to the range 0.05–0.15 g—the latter was the highest concentration that allowed the SFE equipment to be kept in good condition—and 0.1 g was found to be the minimum required to obtain an acceptable signal from the screening system.

3.1.2. Equilibration and extraction times

The effects of the equilibration and extraction times on analyte extractability were examined on constancy of all other operating variables. Three different extraction times (15, 20, and 25 min) were tested. The latter two failed to increase the efficiency of astaxanthin extraction relative to the former, so 15 min was adopted for further work. Also, an equilibration time of 0–5 min prior to extraction for 15 min resulted in no improvement in recovery. No equilibration time was therefore used in subsequent tests.

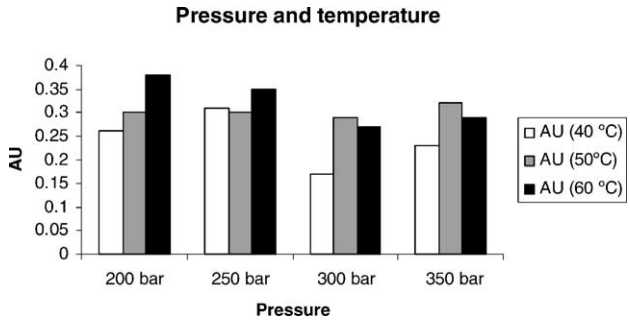

Fig. 1. Influence of the CO_2 pressure (200–300 bar) and temperature (40–60 °C) on the efficiency of extraction of astaxanthin from crustacean samples.

3.1.3. Extraction chamber pressure and temperature

Analyte solubility depends on a complex balance between the supercritical fluid density and solute vapor pressure, both of which are dictated by the temperature and pressure of the supercritical fluid. Rising the temperature decreases the fluid density, but can increase the solute vapor pressure. On the other hand, rising the pressure increases the fluid density and can thus have a two-fold effect, namely: an increase in the solvating power of the supercritical fluid, which facilitates quantitative recovery and a reduced interaction between the fluid and the matrix resulting from the decrease in diffusion coefficient with increasing density [25]. For these reasons, the influence of the extraction pressure and temperature was studied simultaneously. As can be seen in Fig. 1, the temperature ranged from 40 to 60 °C (no higher levels were studied in order to avoid degradation of the analyte); also, the pressure ranged from 200 to 350 bar. The density was defined at a fixed pressure and temperature. As can be seen from Fig. 1, the best recoveries of carotenoids from crustacean samples were achieved by using 200 bar at 60 °C (corresponding to an SF density of 0.73 g ml^{-1}, which was the lowest tested).

3.1.4. Trap temperature

The effect of the trap temperature during the extraction step was studied at 80 and 85 °C. Temperatures below 80 °C were avoided in order to prevent condensation of the organic modifier (ethanol) in the trap, and so were levels above 85 °C in order to avoid thermal decomposition of the analyte. Because analyte recoveries were 25% higher at 80 than at 85 °C, the former temperature was adopted as optimal.

The elution temperature should be lower than the boiling point of acetone (the solvent used to elute the analytes from the trap). Temperatures over the range 20–40 °C were studied and 30 °C chosen as the optimum value for the trap during the elution step.

3.1.5. Flow rate

The effect of the CO_2 flow rate on the extraction yield was examined and the best extraction recoveries were found to be provided by a flow rate of 2 ml min^{-1}. Also, a flow rate of 1.5 ml min^{-1} for the rinsing solvent was found to provide the best analyte recoveries among those tested (1–2 ml min^{-1}).

3.1.6. Modifier concentration

One shortcoming of supercritical CO_2 is that it often fails to quantitatively extract polar analytes from solid matrices owing to its low solvating power and inadequate interaction with such matrices [26]. The addition of an organic modifier can substantially improve the extraction efficiency of CO_2 by raising the solubility of the analytes, reducing their interaction with the sample matrix or altering it in some way; this can significantly facilitate removal of the analytes from the matrix [27–30]. In this work, ethanol was tested as modifier for supercritical CO_2. The ethanol content range studied was chosen in accordance with the polarity of the analyte and reported SFE data for carotenoids [31]. The amounts of astaxanthin extracted by using pure CO_2 and various ethanol–CO_2 mixtures are shown in Fig. 2. As can be seen, the addition of ethanol was indispensable in order to ensure quantitative extraction. A 15:85 ethanol:CO_2 mixture was found to provide the highest recoveries of astaxanthin.

3.1.7. Trapping variables

The trapping/collection efficiency of three different types of trap (viz. Porapack Q, Stainless Steel and ODS) was comparatively assessed. Extractions were performed by using CO_2 at 204 bar, 60 °C (0.73 g ml^{-1}) and 2 ml min^{-1} for 15 min. The trap temperature was 80 °C during extraction and 30 °C during elution. The flushing flow rate was 1.5 ml min^{-1}. ODS, a polar material, proved the most effective choice of trap packing material on account of the also polar nature of the analyte.

3.2. Astaxanthin extraction and confirmation

A previously reported HPLC method [24] was used to confirm whether astaxanthin was the main carotenoid extracted from the crustacean samples using the proposed SFE

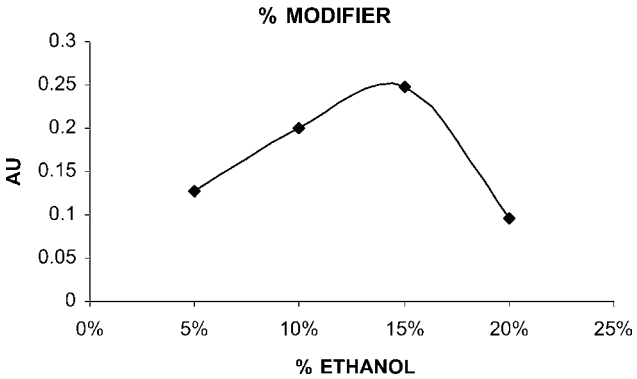

Fig. 2. Optimization of the proportion of ethanol used as CO_2 modifier. *Conditions:* chamber temperature, 40 °C; CO_2 density, 0.88 g ml^{-1}; pressure, 250 bar; equilibration time, 0 min; extraction time, 15 min; trap temperature, 80 °C; and trap packing, ODS.

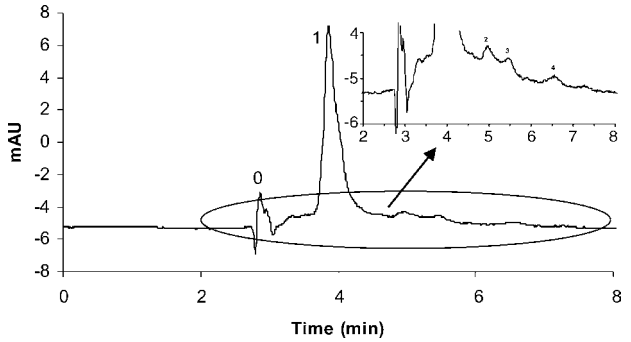

Fig. 3. Chromatogram for an SFE extract. *Conditions:* column, C_{18} reversed phase Spherisorb® (250 mm × 4.6 mm i.d., 5 μm particle size); mobile phase, 67.5:22.5:9.5:0.5 (v/v) methanol:dichloromethane:acetonitrile:water; flow rate, 1.0 ml min^{-1}; detection wavelength, 450 nm. Peak identification: 1, astaxanthin and 2–4, unidentified carotenoids.

method. As can be seen from Fig. 3, such was indeed the case: peak 1 (astaxanthin) was much stronger than peaks 2–4, which corresponded to other extracted carotenoids. Therefore, a simple screening system suffices to confirm the presence or absence of astaxanthin in extracts from crustacean samples.

The SFE of astaxanthin from crustacean samples proved more selective than its extraction by hand. Table 3 shows the results of extracting the same sample four times with both methods and analyzing the extracts using HPLC. As can be seen from the rightmost column, astaxanthin accounted for 98% of all carotenoids extracted with the SFE method versus only 84% with the manual method. Also, the repeatability, as R.S.D., of the SFE method (6%) was higher than that of the manual method (21%). Therefore, although the manual method is required if carotenoids other than astaxanthin are to be extracted as well, the SFE method is more selective and precise for the extraction of astaxanthin as target analyte from crustacean samples.

Table 3
Comparison of manual and supercritical fluid extraction methods for the determination of astaxanthin in crustacean samples by HPLC

Extraction method	A_a [a]	A_t [b]	Percentage astaxanthin [c]
Manual			
	135.4	161.8	83.68
	131.7	154.6	85.19
	95.1	113.3	83.93
	163.9	197	83.20
Average ± standard deviation	131 ± 28		84 ± 1
R.S.D.	21%		
Supercritical fluid extraction			
	89.6	91.8	97.60
	92.8	94.2	98.51
	90.5	90.5	100.00
	102	104.4	97.70
Average ± standard deviation	94 ± 6		98 ± 1
R.S.D.	6%		

Analyses carried out by using 0.1 g of crustacean sample with the SFE method and 0.3 g of crustacean sample with the manual method.

[a] A_a Astaxanthin peak area.

[b] A_t total area. All data were obtained using the HPLC method.

[c] Percentage astaxanthin referred to the other carotenoids extracted from the crustacean sample.

4. Conclusions

A clean, expeditious highly selective automated SFE method for the isolation of carotenoids from crustaceans is proposed that reduces solvent waste and handling times, and provides quite clean extracts in a single step. Because increasing the solvent polarity increases the extraction rate and efficiency, a polar modifier was added to the non-polar supercritical CO_2; to this end, ethanol was preferred to methanol, which is toxic. Also, ODS proved the best trap packing material for collection and elution of extracted analytes.

The proposed method is more expeditious and simple than its manual extraction counterpart, which it also surpasses in efficiency and precision. Moreover, the SFE method avoids the use of large amounts of toxic solvents and is more environmentally benign than the classical method for astaxanthin extraction. Finally, the analyte can be extracted at lower temperatures, which avoids the potential degradation of thermolabile compounds.

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