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Separation and determination of astaxanthin from microalgal and yeast samples by molecularly imprinted microspheres

Jia-Ping Lai [a,b], Yue Jiang [c], Xi-Wen He [b], Jun-Chao Huang [a], Feng Chen [a,d,*]

[a] Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong, PR China
[b] Department of Chemistry, Nankai University, Tianjin 300071, PR China
[c] Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, PR China
[d] South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, PR China

Abstract

In this work, molecularly imprinted microspheres (MIMs) were synthesized by aqueous microsuspension polymerization using astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) as imprinting molecule. The MIMs obtained were subsequently packed into the stainless steel column and the chromatographic characterization of the column was investigated. The effects of pH and composition of the mobile phase on the retention factor (k') were investigated in detail. The mixture of methanol and dichloromethane (DCM) (8:2, v/v) was used as mobile phase A while the mixture of methanol and water (5:5, v/v) as mobile phase B. The separation of astaxanthin and zeaxanthin (3,3′-dihydroxyl-β-carotene) was obtained when the concentration of mobile phase B was higher than 30% (v/v) due to their strong lipophilicity. The method developed was successfully applied to separate astaxanthin in the saponified samples of the microalga *Haematococcus pluvialis* and the yeast *Phaffia rhodozyma*. The recovery of adding 40 mg astaxanthin to 1.0 g microalgal sample was 95.5% with an R.S.D. ($n = 5$) of 5.3%. The results of determination of astaxanthin in the microalga and the yeast were 3.7% (R.S.D. = 1.5%, $n = 9$) and 0.041% (R.S.D. = 7.3%, $n = 9$), respectively.
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Keywords: Molecularly imprinted microspheres; Astaxanthin

1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a carotenoid pigment found in certain marine animals and plants such as fish, shrimps, and algae [1]. It has an attractive pink color and is therefore widely used as food colorant. The Food and Drug Administration of the United States has permitted it for use in the aquacultural industry [2]. Due to its special structure, astaxanthin is a more powerful scavenger of singlet oxygen and peroxyl radicals than β-carotene, cantaxanthin, and zeaxanthin (3,3′-dihydroxyl-β-carotene); its antioxidant activity is much stronger than all other carotenoids [3]. Furthermore, astaxanthin may exert antitumor activities through the enhancement of immune responses [4].

There have been some reports on the separation and determination of astaxanthin and the other carotenoids. These methods include high performance liquid chromatography (HPLC) [5,6], on-line HPLC–UV-APCI (atmospheric pressure chemical ionization) [7], HPLC–TLS (thermal lens spectrometry) and HPLC with Pirkle covalent L-leucine chiral stationary phase [8]. Molecularly imprinted polymers (MIPs) are cross-linked polymers with specific binding sites for a template molecule. These binding sites are tailor-made in situ by the copolymerization of cross-linker and functional monomer in the presence of the template molecule. After removal of the template from the polymers, the recognition sites, in terms of size, shape and functionality, are complementary to the template molecule. MIPs possess advantages of physical robustness, high strength, resistance to elevated temperatures and pressures, and inertness towards acids, base, metal ions and organic solvents compared to enzymes. MIPs have been extensively used in biosensors [9–11], mimic enzyme catalysis [12,13], solid-phase extraction (SPE) [14–16] and as HPLC stationary phase [17–23]. The latter two applications could, sooner or later, be routinely employed for real-life analytical problems [14,24]. In comparison with C18 column, an outstanding advantage of

* Corresponding author. Tel.: +852-2299-0309; fax: +852-2299-0311.
E-mail address: sfchen@hkusua.hku.hk (F. Chen).

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MIPs as HPLC stationary phase is that they can be utilized in strong acidic or basic media [19]. Furthermore, MIPs as HPLC stationary phase are stable after multi-repeated uses [19,22].

In the present paper, the molecularly imprinted microspheres (MIMs) (one form of MIPs) were synthesized by aqueous microsuspension polymerization using astaxanthin as the template molecule. The MIMs were packed into a stainless steel column, which were subsequently used for the separation of astaxanthin, zeaxanthin, Vitamin E, and Vitamin A in HPLC model. The influence of the composition of the mobile phase on chromatographic retention was investigated. The method was successfully employed to determine factual samples such as the microalga *Haematococcus pluvialis* and the yeast *Phaffia rhodozyma*.

2. Experimental

2.1. Chemicals and materials

Astaxanthin, zeaxanthin, Vitamin E, and Vitamin A (Fig. 1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) were purchased from Aldrich Chemical Co., Inc. (Milwaukee WI, USA). 2,2′-Azobis (2-isobutyronitrile) (AIBN) was supplied by the Special Chemical Reagent Factory of Nankai University (Tianjin, China). Methanol, dichloromethane (DCM) and acetonitrile for HPLC were purchased from BDH Laboratory Supplies (Poole, England). All solutions were prepared using Milli-Q water (Millipore, Molsheim, France). The microalga *H. pluvialis* and the yeast *P. rhodozyma* were obtained from our laboratory [25,26].

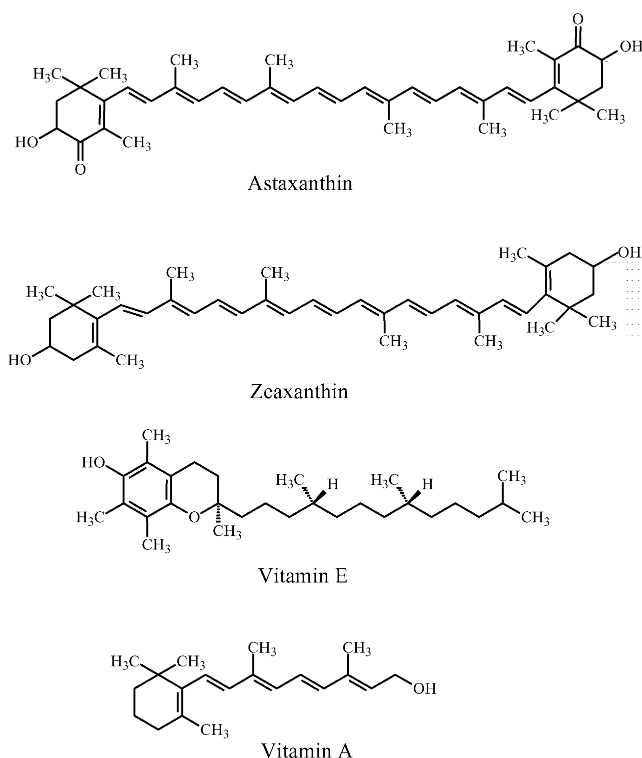

Fig. 1. The chemical structures of astaxanthin and its analogues.

2.2. Preparation of MIMs

Poly(vinyl alcohol) 500 (6.0 g) was dissolved in 150 ml of water while stirring at 90–95 °C and cooled to room temperature. Then the aqueous solution was transferred into a 250 ml three-neck flask. Astaxanthin as template (0.5 mmol) and MAA as functional monomer (8 mmol) were successively dissolved in 15 ml of DCM (porogen) in a glass flask. The organic mixture was placed into a freezer at 0 °C and maintained for 30 min. Then 50 mmol EDMA (cross-linker) and 150 mg AIBN (initiator) were added to this mixture and sonicated to dissolve. The total mixture was added to the water phase in the three-neck flask while stirring at 400 rpm under a gentle stream (at 0.8 l min^{-1}) of nitrogen (99.99%). The temperature was then elevated to 60 °C for polymerization. The process was maintained for 24 h. The microspheres were washed with doubly distilled water, methanol, and organic mixture of acetic acid, dichloromethane, and methanol (1:2:7, v/v), successively. Non-imprinted microshperes were prepared in the same way without the addition of the template molecule.

2.3. Pretreatment of microalgal and yeast samples

The pretreatment of microalgal and yeast samples was carried out according to our previous study [27] with slight modification. The microalgal or yeast sample (0.1 g) in a mortar was ground with a pestle and 10 ml of the extraction solvent, a mixture of dichloromethane and methanol (2:8, v/v), was then added. After grinding the microalgal or yeast cells for 3 min, the mixture of the ground microalgal or yeast cells and the extraction solvent was then separated by centrifugation at 12,000 × *g* for 5 min, and the supernatant containing the pigments was collected. The extraction procedure was repeated at least three times until the microalgal or yeast cell debris was almost colorless. The combined supernatant was centrifuged again at 12,000 × *g* for 15 min. 1.5 ml (for microalgal sample) or 0.4 ml (for yeast sample) of 0.5 mol l^{-1} NaOH solution was added to the extracts obtained under a nitrogen atmosphere. The total mixture was then kept for 12 h in darkness under nitrogen for complete saponification of astaxanthin esters. The saponified pigment extract solution was directly analyzed by the MIMs.

2.4. Chromatographic method

The microspheres obtained (8–20 μm) [23] were packed into a stainless steel column (250 mm × 4.6 mm i.d.) by slurry method using a Haskel DSTV-150 pump at 600 kg cm^{-2} (pressure) and a mixture of methanol and 2-propanol (5:5,

v/v) as slurry medium. HPLC system consisted of two Waters 510 pump systems, a Waters temperature control module and a Waters 996 photodiode array detector (Milford, MA, USA). Column oven temperature was controlled at 25 °C. Detection was performed at 291 nm. The column was rinsed on-line with a mixture of acetic acid, DCM, and methanol (1:2:7, v/v) until a stable baseline was obtained. Mobile phase A was the mixture of methanol and dichloromethane (8:2, v/v), mobile phase B was the mixture of methanol and water (5:5, v/v), mobile phase C was the mixture of acetonitrile and water (5:5, v/v), and mobile phase D was the acetate buffer solution at different pH values. The total flow-rate was maintained at 1.0 ml min^{-1}. The volume of injection was 20 μl. The retention factor (k') was calculated according to standard chromatographic procedure as $k' = (t - t_0)/t_0$, where t and t_0 are retention time and void time, respectively. The void time was determined by the elution time of a solvent peak observed. To serve as a control, an additional column was packed with non-imprinted microspheres and conditioned using an identical procedure.

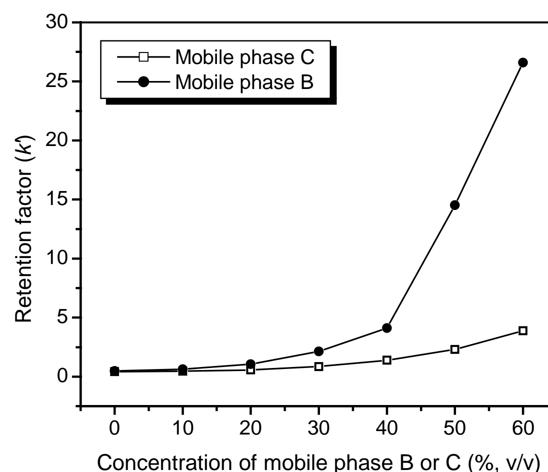

Fig. 3. The influence of mobile phase B or C on the retention factor for astaxanthin (mobile phase A: methanol/dichloromethane = 8/2 (v/v); mobile phase B: methanol/water = 5/5 (v/v); mobile phase C: acetonitrile/water = 5/5 (v/v) and total flow-rate, 1.0 ml min^{-1}; load: 80 μg).

3. Results and discussion

3.1. The effect of pH of mobile phase on retention

According to our previous study [23], the influence of pH of mobile phase on the retention factor for the template molecule was notable. In the present work, thus, pH buffers (i.e., pH values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) were prepared by acetate solution ($C = 0.05$ mol l^{-1}) whose pH was adjusted with HCl or NaOH. Then the buffer with different pH values was used as mobile phase D. The effect of pH on the retention factor was investigated. As can be seen from Fig. 2, the effect of pH on the retention factor was, however, unremarkable. The retention factor was just slightly increased with increasing pH value of the buffer. The results indicated that the retention behavior of astaxanthin on the MIMs was subtly affected by the pH of the mobile phase within the range investigated. This was probably due to that (1) there are no acidity or basicity functional group and (2) there is a long carbon chain in astaxanthin, which presents strong lipophilicity. In the present work, therefore, instead of buffer, water was chosen to adjust the polar properties of the mobile phase.

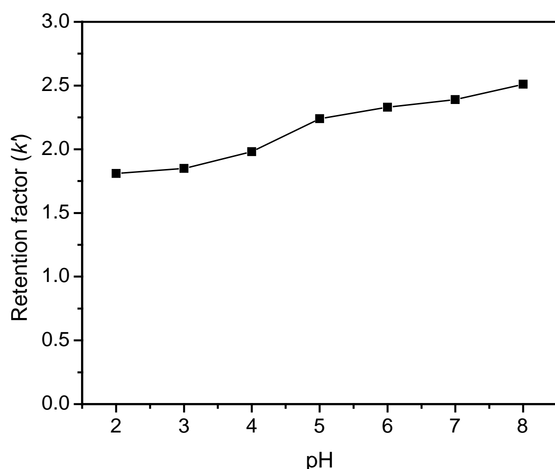

Fig. 2. The effect of pH on the retention factor for astaxanthin (mobile phase A: methanol/dichloromethane = 8/2 (v/v) and flow-rate, 0.8 ml min^{-1}; mobile phase D: acetate buffer solution and flow-rate, 0.2 ml min^{-1}; load: 80 μg).

3.2. The influence of composition of mobile phase on retention

The retention behavior of astaxanthin on MIMs was determined by both the imprinting cavities with recognition sites and the physico-chemical properties of the eluent. Fig. 3 shows that the influences of mobile phase C on the retention factors of astaxanthin appreciably depended on the composition of mobile phase C. In the range of 0–60% of mobile phase C, the retention factor of astaxanthin was slightly increased with increasing concentrations of acetonitrile/water. But the retention factor apparently increased with increasing ratios of mobile phase B when mobile phase B was used. The retention factor was abruptly increased when the concentration of mobile phase B was above 40%. This was probably ascribable to that astaxanthin is insoluble in polar solvents such as methanol but can be dissolved at room temperature in dichloromethane, chloroform, acetone, DMF, acetonitrile, etc. [28]. Either in mobile phase B or C, the retention time was increased with increasing ratio of mobile phase B or C. This phenomenon was perhaps ascribed to the strong lipophilicity of astaxanthin because the hydrophobic interaction between astaxanthin and MIMs played an important role in the retention process when the mobile phase became highly polar. In principle, a high value of retention factor

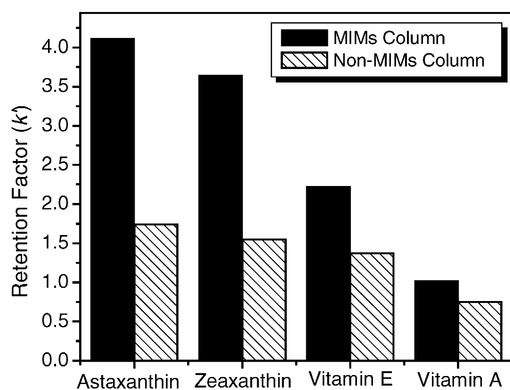

Fig. 4. The retention factors for substrates on MIMs and the control column (mobile phase A: methanol/dichloromethane = 8/2 (v/v) and flow-rate, 0.6 ml min^{-1}; mobile phase B: methanol/water = 5/5 (v/v) and flow-rate, 0.4 ml min^{-1}; load: Vitamin A (10 μg), Vitamin E (100 μg), zeaxanthin (80 μg), and astaxanthin (80 μg)).

favors chromatographic separation, but too long a retention time may widen the peak. So in this study, the concentration of mobile phase B between 30 and 40% (v/v) was adopted.

3.3. The selectivity of MIMs

In order to evaluate the imprinting effect, the blank polymer was synthesized and manipulated in the same condition as for MIMs. Fig. 4 shows the retention factors of the various substrates on the MIMs and the non-imprinted column. It indicated that the MIMs exhibited evident selectivity to astaxanthin. The retention factors for astaxanthin on the MIMs and the non-imprinted column were 4.11 and 1.74, respectively. The special affinity to astaxanthin might be due to both the imprinting process and the lipophilicity of astaxanthin. On the one hand, recognition sites with two points of interaction were created upon removal of the template. When the template molecule rebound with the imprints, a cyclic hydrogen bonding interaction between the functional groups of astaxanthin and methacrylic acid residues of the MIMs as shown in Fig. 5 was probably formed. On the other hand, the astaxanthin has the strong lipophilicity as it contains a long carbon chain and two benzene cycles in its molecule. Thus, the lipophilic interactions between long carbon chain of astaxanthin and the lipophilic cavities of imprints may play a more important role in prolonging the retention time under a polar mobile phase of methanol and water. As shown in Fig. 4, the value of the retention factor for zeaxanthin on the MIMs was close to that for astaxanthin. This was also ascribed to that zeaxanthin has the same long carbon chain as astaxanthin (Fig. 1). In order to further testify this, the ratio of mobile phase B was changed to investigate the influences of concentration of mobile phase B on retention factors for all compounds (Fig. 6). As shown in Fig. 6, the k' values for astaxanthin and zeaxanthin are much greater than that for the other substrates at all concentrations of mobile phase C investigated. This further verifies the above conclusion. Imprinting factors calculated according to Mayes et al. [29]

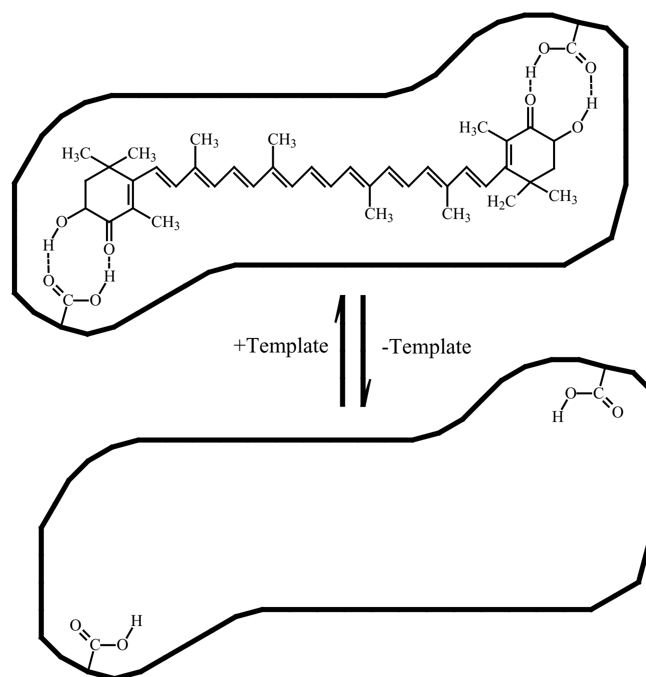

Fig. 5. Scheme of the molecular imprinting process.

show the imprints recognize astaxanthin slightly better than zeaxanthin and much better than Vitamin E and Vitamin A. This also indicates that the MIMs of astaxanthin exhibited specific affinity to the template.

3.4. Separation of astaxanthin from real sample

The aim of this study was to develop a method for the separation of astaxanthin from the biological samples, namely, the microalga *H. pluvialis* and the yeast *P. rhodozyma*. The

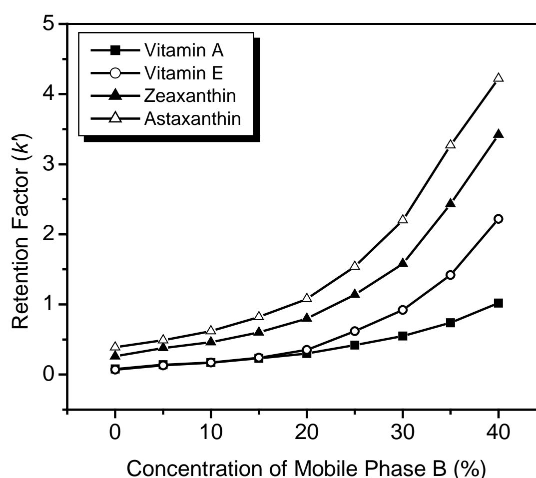

Fig. 6. The influence of concentration of mobile phase B on the retention factor for astaxanthin and its analogues (mobile phase A: methanol/dichloromethane = 8/2 (v/v); mobile phase B: methanol/water = 5/5 (v/v) and total flow-rate, 1.0 ml min^{-1}; load: Vitamin A (10 μg), Vitamin E (100 μg), zeaxanthin (80 μg), and astaxanthin (80 μg)).

Table 1
The gradient elution program

Time (min)	Flow-rate (ml min^{-1})	A (%, v/v)	B (%, v/v)
0.00	1.0	60.0	40.0
13.00	1.0	60.0	40.0
13.05	1.0	80.0	20.0
20.00	1.0	80.0	20.0
20.05	1.0	60.0	40.0
25.00	1.0	60.0	40.0

interferences of analogues such as Vitamin A, Vitamin E, and zeaxanthin (Fig. 1) were investigated. The results indicated that increasing the concentration of mobile phase B facilitated the baseline separation, especially between astaxanthin and zeaxanthin. However, if the concentration of mobile phase B was too high, it would broaden the chromatographic peak, which was unfavorable to chromatographic separation. So in this study, a gradient elution program was employed (Table 1). The chromatogram of separating astaxanthin and its analogues by MIMs stationary phase is shown in Fig. 7a. Baseline separation was observed while it was not obtained when non-imprinted stationary phase was used (Fig. 7b). All retention times for analytes on the non-imprinted stationary phase were much shorter than on the MIMs stationary phase (Fig. 7b). The column efficiency of the MIMs stationary phase could be increased by gravitational settling of the microspheres [30]. The gradient elution program was used for the separation of astaxanthin from the saponified microalgal samples.

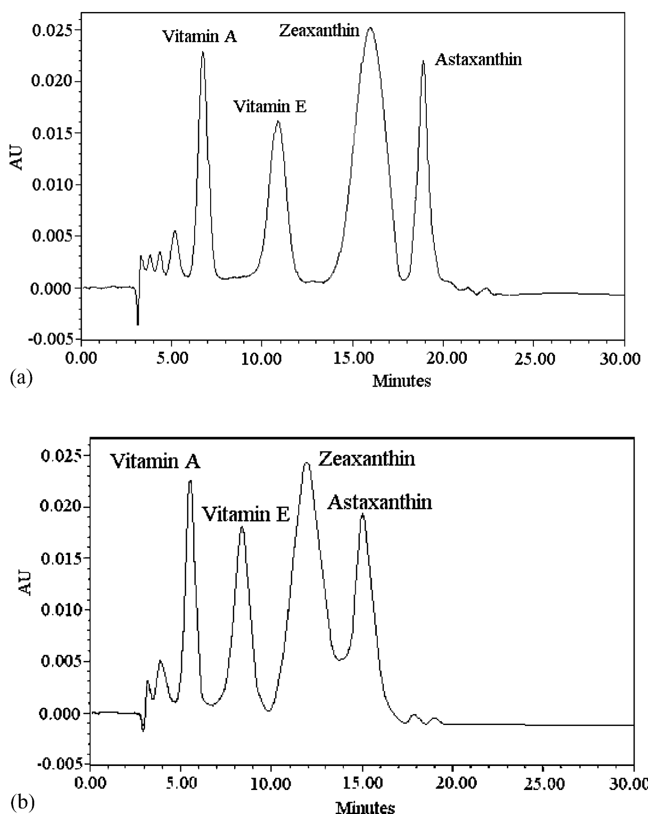

Fig. 7. The representative chromatogram of MIMs for separation of astaxanthin and its analogues on the MIMs and non-imprinted polymer stationary phase (chromatographic conditions: gradient elution; total flow-rate, 1.0 ml min^{-1}; detection wavelength, 291 nm; load: Vitamin A (10 μg), Vitamin E (100 μg), zeaxanthin (80 μg), and astaxanthin (80 μg)).

Table 2
The results of astaxanthin determination of *H. pluvialis* and *P. rhodozyma*

Samples	Means of astaxanthin concentrations (%, w/w)	R.S.D.(%, $n = 9$)
H. pluvialis	3.738 ± 0.056	1.5
P. rhodozyma	0.041 ± 0.003	7.3

3.5. Quantitative analysis

A series of astaxanthin standard calibration solutions in the range of 0.2–2.0 mg ml^{-1} were prepared and investigated under the above conditions. A good linearity in terms of peak area response to the astaxanthin concentration was observed. The equation of the standard calibration line was $A = 1.9394 \times 10^6 C - 126059.5$ with $R^2 = 0.9984$, where A is the peak area and C is the concentration of standard solution. In order to confirm the accuracy of this method for real samples, standard astaxanthin (40 mg) was added to the microalgal sample (1.0 g), and the sample was then treated and determined according to the above optimal conditions. The recovery of astaxanthin was 95.52% (R.S.D. = 5.32%, $n = 5$). The results of astaxanthin determination in the microalga and the yeast are shown in Table 2. The means ($n = 9$) of the astaxanthin concentrations (by weight) from the microalga and the yeast were 3.738 and 0.041%, respectively, and the R.S.D. ($n = 9$) for the microalga and the yeast were 1.5 and 7.3%, respectively. The results coincided with those of the chromatographic method [27].

4. Conclusions

A novel method for the separation of astaxanthin and its analogues by molecularly imprinted microspheres was developed. The MIMs exhibited specific affinity to astaxanthin in a fixed composition of the mobile phase. The retention factor of astaxanthin was affected by both the property of the MIMs and the polarity of the mobile phase due to its lipophilicity. The method developed was successfully applied to separate astaxanthin in the saponified samples of the microalga *H. pluvialis* and the yeast *P. rhodozyma*. The MIMs as solid-phase extraction material might find application in the separation of astaxanthin from other biological sources.

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