



Determination of Three Carotenoids in Microalgae by Matrix Solid-Phase Dispersion Extraction and High-Performance Liquid Chromatography

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Abstract

A HPLC method based on matrix solid-phase dispersion (MSPD) was developed for simultaneous determination of fucoxanthin, lutein and astaxanthin in different microalgae. First, the powder of microalgae was blended with C8 adsorbent. Then the SPE column made from the microalgal matrix/sorbent (1:2) was eluted with 4 mL methanol (containing 0.1% BHT). The eluent was analyzed by HPLC. A C8 column and acetonitrile (ACN)/H₂O as the mobile phase were used. The coefficient (R^2) of each calibration curve was higher than 0.998. Limits of detection (LODs) and limits of quantification (LOQs) for each carotenoid were 0.30 $\mu\text{g g}^{-1}$ and 1.00 $\mu\text{g g}^{-1}$, respectively. The recovery at three concentration levels ranged from 69.1 to 106.5%, and the RSDs were lower than 5%. As a new sample preparation method for microalgal analysis, the MSPD procedure was optimized, validated and compared with conventional methods including ultrasonic and ice-bath extraction. The MSPD–HPLC method was accurate and reproducible. It was suitable for the quantitative determination of carotenoids in microalgae.

Keywords Matrix solid-phase dispersion · High-performance liquid chromatography · Microalgae · Carotenoid

Abbreviations

BHT Butylated hydroxytoluene
MTBE Methyl tert-butyl ether
PTFE Polytetrafluoroethylene

Introduction

Carotenoids are known as important antioxidants for human health and have been used as food additives. In addition, several trials have reported that carotenoids can protect people from diseases such as lung cancer, amyotrophic lateral sclerosis and several other degenerative diseases [1]. For example, lutein is frequently used as a food additive because of its ability to prevent or ameliorate cardiovascular diseases and some types of cancer [2]. Astaxanthin is vitamin A precursor and a more efficient antioxidant than β -carotene and vitamin E [3]. Among the various sources of carotenoids, microalgae have recently attracted a wide interest [1]. Some microalgae can produce large amounts of specific carotenoids, including β -carotene in *Dunaliella salina*, astaxanthin in *Haematococcus pluvialis*, and lutein in *Scenedesmus almeriensis* and *Muriellopsis* sp. [4].

Microalgae have special advantages as a natural source of different secondary metabolites [1]. It is easy to cultivate and can be used to purify and take up nutrients from wastewater. Besides, the more important characteristics of microalgae are that directed induction is feasible in its culture to accumulate target compounds. Method development of analytical

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separation is critical to the extraction process of carotenoids from microalgae, particularly in procedures of microalgal screening and directed induction. When constructing an analytical method, the pretreatment played an important role. The pretreatment method should not only avoid the matrix effect but also consider solvent consumption and throughput. Solvent extraction was the most frequently used method to extract carotenoids from microalgae. To decrease the degradation of the carotenoids, ultrasonic and ice-bath extraction were frequently used [5, 6]. However, these methods were time consuming and used large amount of organic solvent. Some new methods such as supercritical fluid extraction, microwave-assisted or enzyme-assisted extraction have been used to improve yields [7, 8]. Although these methods have advantages, they were dependent on special instruments and the cost was high.

Matrix solid-phase dispersion (MSPD) was regarded as a sample preparation technique allowing for a quick and efficient isolation of the analyte from the plant matrix [9]. MSPD comprised sample homogenization, cellular disruption, fractionation and purification in a single process [10]. This method has been successfully applied to detect macroconstituent and illegal additive with satisfactory results [11–17]. The aim of this study was to develop a reliable MSPD–HPLC method for the simultaneous determination of fucoxanthin, lutein and astaxanthin in different microalgae. In this method, the effects of several parameters such as solid-phase support, elution solvents, volume, etc. were investigated to optimize the MSPD conditions. The MSPD–HPLC method was also validated with reproducibility, linearity and consistent recovery.

Materials and Methods

Chemicals and Materials

The solid-phase materials used for MSPD were silica-based C8 and C18 (60 μm) from Acchrom (Beijing, China). The reference standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Their structures are shown in Fig. 1. Acetonitrile (ACN) and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q purification system (Billerica, MA, USA). Dichloromethane, acetone, ethanol, isopropanol, methyl tert-butyl ether (MTBE) and tetrahydrofuran (THF) were from Kaimei (Dalian, China). Butylated hydroxytoluene (BHT) were obtained from J&K Scientific (Beijing, China). The powder of two *Isochrysis zhangjiangensis* (named – 1 and – 2) from different culture conditions, *Nannochloropsis oculata* and *Platyonas subcordiformis* were provided by professor Song Xue in another group of the institute; the microalgae were cultivated in their laboratory.

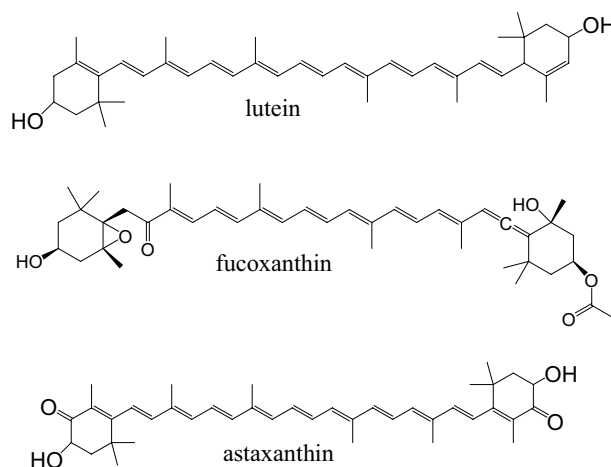


Fig. 1 The structures of three reference standards

Apparatus

The chromatographic evaluation was performed in a Chromaster HPLC system equipped with a 5110 HPLC pump, a 5430 diode array detector (DAD), a 5210 autosampler and a 5310 column oven (HITACHI, Tokyo, Japan). Also, an Alliance 2695 system equipped with a Waters e2695 HPLC pump and a Waters 2998 photodiode array detector (PDA) (Waters, Milford, MA, USA) was also applied. Chromatographic data were all recorded on a computer with Empower workstation software.

Chromatographic Conditions

The columns used were Unitary C8 and C30 (150 mm \times 4.6 mm, I.D., 5 μm) from Acchrom (Beijing, China). Flow rate was 1.0 mL min^{-1} and the column temperature was 30 $^{\circ}\text{C}$. The mobile phase consisted of ACN (A) and water (B). Gradient program was adopted as follows: linear from 60 to 100% A (0–60 min) and held for 20 min. The wavelength was 450 nm.

Sample Preparation

Preparation of Standards

About 10 mg of lutein and astaxanthin was accurately weighed and dissolved in a 5-mL volumetric flask with dichloromethane (containing 0.1% BHT) to yield a stock solution of about 2 mg mL^{-1} , respectively. Also, about 10 mg of fucoxanthin was accurately weighed and dissolved in acetone (containing 0.1% BHT) to yield a stock solution of about 2 mg mL^{-1} . 1 mL of each stock solution was mixed

in a 5 mL volumetric flask with acetone (containing 0.1% BHT) to yield a working solution of about 0.4 mg mL⁻¹. This solution was serially diluted with methanol to give calibration standards at 40, 20, 10, 5, 2, 0.5, 0.2 and 0.1 µg/mL.

Sample Preparation Using MSPD Extraction

About 0.1 g of each dried microalgae was weighed and blended with C18 or C8 adsorbent at a ratio of 1:1, 1:2, 1:3 and 1:4 by grinding with a pestle in an agate mortar to produce a homogenous packing material. The mixture was then transferred to a 6-mL solid-phase extraction (SPE) cartridge with a PTFE gasket on the bottom. The blend was compressed in the barrel using the syringe plunger with another PTFE gasket on the top. The column was eluted first with 2 mL of methanol/H₂O (3:7, v:v) to wash out ionic and polar matrix components. After this clean-up procedure, the target compounds were eluted with different organic solvents (containing 0.1% BHT) to optimize the elution procedure. The volume of the elution solvent was also optimized to separate target compounds from strongly retained components. The eluent was concentrated to dry by vacuum centrifuge and redissolved with 1 mL of methanol for HPLC analysis. To verify if all target carotenoids were eluted completely, a stronger elution solvent, tetrahydrofuran (THF) was used to clean up the column.

Reproducibility was assessed by evaluating the peak areas and retention times of fucoxanthin, lutein and astaxanthin analyzed by HPLC. The target compounds in different microalgae after MSPD procedure and HPLC analysis were repeated three times. The recovery was assessed by measuring the recovery of 10 µL, 25 µL and 50 µL of 400 µg mL⁻¹ mix stock solution after it was added to the agate mortar and ground together with 0.1 g of *Nannochloropsis oculata* and adsorbent in the same way at optimized MSPD conditions.

Sample Preparation Using Ultrasonic Extraction

Approximately 0.1 g of *Isochrysis zhangjiangensis-1* and *Nannochloropsis oculata* powder with two copies was accurately weighed in a centrifugation tube, respectively. 10 mL of methanol or acetone was added, respectively. The mixture was sonicated for 10 min. The extract was collected by centrifugation at 4000 rpm for 5 min. The process was repeated until both the precipitate and supernatant became colorless [5]. The pooled supernatant was rotary evaporated to dryness and redissolved in 1 mL of methanol for HPLC analysis.

Sample Preparation Using Ice-Bath Extraction

Approximately 0.1 g of *Isochrysis zhangjiangensis-1* and *Nannochloropsis oculata* powder with two copies was

accurately weighed in a centrifugation tube, respectively. And 10 mL of methanol or acetone was added, respectively. The mixture was placed in an ice bath and vortexed for 10 min [5]. The extract was separated from the powder by centrifugation at 4000 rpm for 5 min. The procedure was repeated until both the precipitate and supernatant became colorless. The pooled supernatant was rotary evaporated to dryness and redissolved in 5 mL of methanol for HPLC analysis.

Results and Discussion

Development of HPLC Method

Carotenoids were usually analyzed by RPLC–UV method [18–22]. And C30 column was always used to increase shape selectivity for separating carotenoid isomers [23]. Here, C30 and C8 columns were used to compare the separation of fucoxanthin, lutein and astaxanthin. As shown in Fig. 2, the resolution between lutein and astaxanthin on C8 column was larger than that on C30 column. Subsequently, C8 column was used for the separation of microalgal extract. Considering the retention and practicability of ACN, methanol, acetone and MTBE, the most commonly used ACN/H₂O was selected as the mobile phase. The chromatogram from *Nannochloropsis oculata* extract was more complex, it was used to develop HPLC method. Other microalgal extracts used the same HPLC method. The optimized separation chromatogram of *Nannochloropsis oculata* is shown in Fig. 3. To make the target component baseline separating in the actual complex microalgal extract to avoid matrix effect, a little longer analysis time was optimized. The retention times for the target compounds, fucoxanthin, lutein and astaxanthin

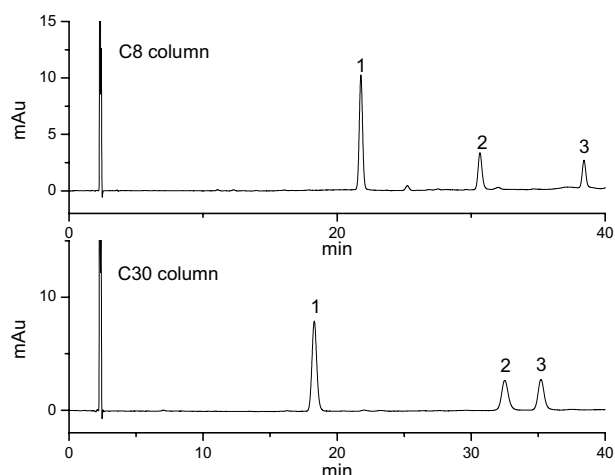


Fig. 2 The chromatogram of three reference standards on C8 and C30 column. 1: Fucoxanthin, 2: astaxanthin, 3: lutein

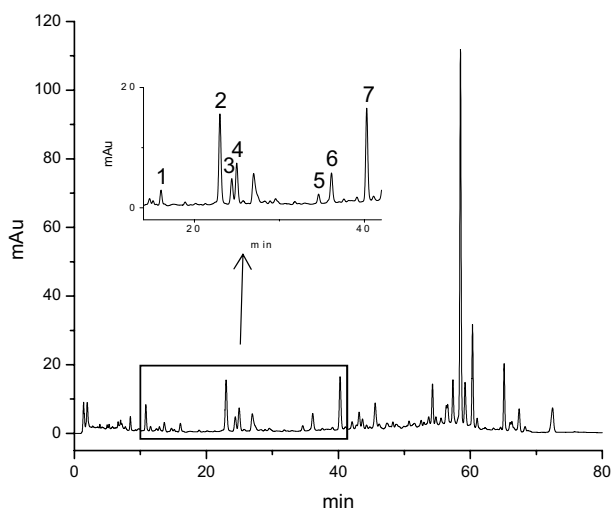


Fig. 3 The chromatogram of *Nannochloropsis oculata* extract by C8 column. Peaks 1–7 were selected to optimize the MSPD method. The HPLC conditions were the same as mentioned in “[Chromatographic conditions](#)”

were shorter than 45 min. Weaker polarity compounds after 45 min were not concerned and they can be removed most by MSPD method. Therefore, seven large peaks with retention time between 10 and 45 min were selected to express the optimization progress of MSPD.

Optimization of MSPD Method

Column Packing

In MSPD method, the solid or semi-solid samples were blended with a suitable adsorbent (e.g., C18) to form homogenous packing materials. The packing materials were then packed into SPE cartridge and eluted with a different solvent. First, it was washed with water or low concentration of organic solvent to remove matrix

Fig. 4 Influence of the adsorbent (C8 and C18) on MSPD procedure. Peak area of peaks 1–7 in Fig. 3 was the marker; the ratio between matrix and different adsorbent was 1:4 and 7 mL acetone was used for elution in the MSPD procedure; the HPLC conditions were the same as mentioned in “[Chromatographic conditions](#)”

interferences like ionic compounds. Then the target compounds were eluted with suitable organic solvent. The extraction, clean-up and enrichment of the target compounds were completed in a single progress [10, 11]. For carotenoids, silica-based C8 and C18 adsorbents were tried. In this MSPD procedure, the normal ratio of 1:4 for sample to adsorbent was used. The *Nannochloropsis oculata* powder sample and adsorbent (C8 or C18) were ground in an agate mortar, respectively. The homogenous sample (green color) was packed into SPE column and compressed with a syringe plunger. After it was washed with 2 mL methanol/H₂O (3:7/v:v) to remove ionic and strong polarity components, the target carotenoids were eluted with acetone (which was the usual solvent for carotenoids extraction). The target components can be completely eluted by 7 mL of acetone and the SPE column became nearly colorless [5]. As shown in Fig. 4, when C8 adsorbent was used, the peak area of seven peaks marked in Fig. 3 in the elution was a little larger than that with C18 adsorbent. The retention of carotenoids on C18 adsorbent was much stronger. The recovery of carotenoids from C8 adsorbent was much better than that from C18 adsorbent. Therefore, C8 adsorbent was chosen in this MSPD method.

Elution Solvents

Elution solvent was another important factor in the MSPD method. The regularly used extraction solvents (acetone, methanol, ethanol, isopropanol and MTBE) for carotenoids were tried in the current work. BHT (0.1%, v:v) was added in each solvent as antioxidant to protect the carotenoids from oxidation during the sample preparation [24, 25]. Fixing the ratio between matrix and C8 adsorbent at 1:4 and the solvent volume of 7 mL, the peak area of peak 1 to peak 7 in different elution solvents is shown in Fig. 5. It can be seen that the peak area with methanol elution was larger than other elutes for most peaks.

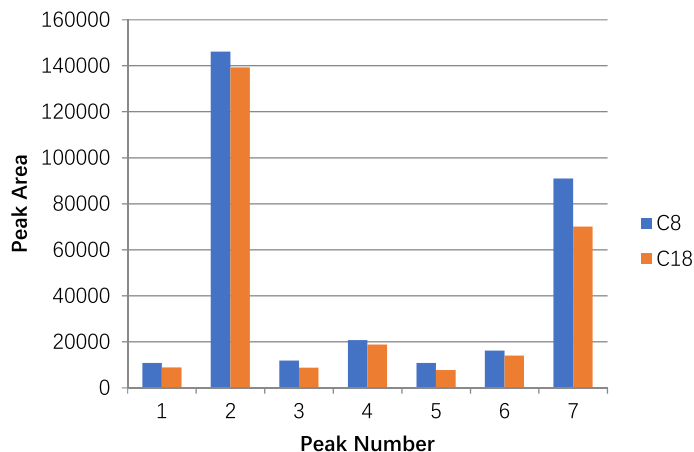
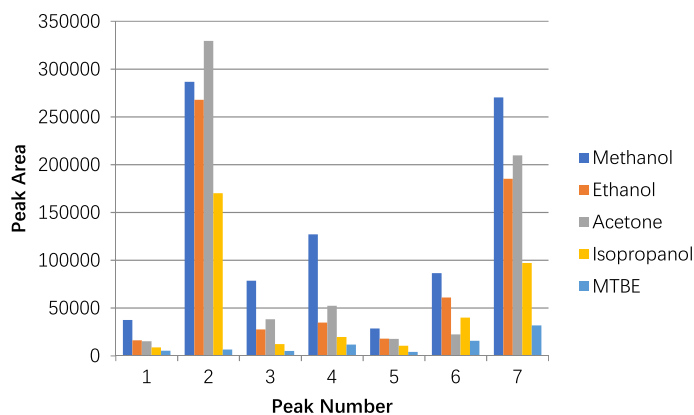


Fig. 5 Influence of the elution solvent (methanol, ethanol, acetone, isopropanol and MTBE) on MSPD procedure. Peak area of peaks 1–7 in Fig. 3 was the marker; the ratio between matrix and C8 adsorbent was 1:4 and 7 mL solvent volume was used for elution in the MSPD procedure; the HPLC conditions were the same as mentioned in “[Chromatographic conditions](#)”



The Ratio Between Matrix and Adsorbent

Another critical parameter in the MSPD is the ratio between matrix and adsorbent. Ratios of 1:1, 1:2, 1:3 and 1:4 (*Nannochloropsis oculata* powder : C8) were evaluated. The sample was 0.1 g and then 0.1 g, 0.2 g, 0.3 g and 0.4 g C8 adsorbent were mixed, respectively. The solvent volume of 7 mL methanol was used in the MSPD procedure. The results are presented in Fig. 6. It showed that when sample to adsorbent mass ratio was smaller than 1:2 (1:3 and 1:4), the extraction efficiency for the seven peaks in the microalga sample was almost the same, which means that the peak areas for seven peaks in the methanol elution with different ratios between matrix and C8 adsorbent were almost the same. Thus, the ratio of 1:2 between sample and adsorbent was chosen in this MSPD method.

Elution Solvent Volume

To evaluate the elution volume of methanol, each elution fraction with 1 mL of methanol was collected from the packed SPE column, respectively. Using the optimized ratio between matrix and C8 adsorbent at 1:2, the fractions were collected until the SPE column was almost colorless. Then

each 1 mL of methanol fraction was adjusted to a constant volume and analyzed by HPLC. The result showed that the fourth fraction contained few target compounds. There were no target compounds in later fractions.

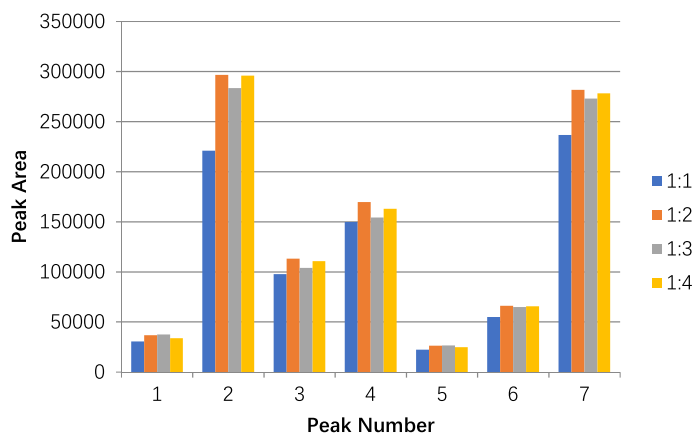
Summarizing the above results, the optimized conditions of MSPD method for carotenoids were C8 adsorbent, 1:2 ratio of sample to adsorbent, and elution with 4 mL of methanol. When the SPE column was re-eluted with THF, it can be seen that no more target compounds could be eluted (data not shown). It indicated that the MSPD conditions were reliable.

Validation of the HPLC–MSPD Method

Reproducibility of HPLC Method

The reproducibility of HPLC method was assessed by comparing the peak area and retention time of the three reference standards (lutein, fucoxanthin and astaxanthin) in seven replicate tests. The reproducibility of the peak areas, retention times of fucoxanthin, astaxanthin and lutein were very good. The relative standard deviation (RSD) values for retention times were less than 0.70%, and the RSD values for peak

Fig. 6 Influence of the ratio between matrix and C8 adsorbent (1:1, 1:2, 1:3 and 1:4) on MSPD procedure. Peak area of peaks 1–7 in Fig. 3 was the marker; The volume of 7 mL methanol was used for elution on the MSPD procedure; the HPLC conditions were the same as mentioned in “[Chromatographic conditions](#)”



areas were less than 1.41%. Therefore, it can be concluded that the reproducibility of HPLC method was satisfactory.

Linearity and Sensitivity

The calibration curves for three reference standards are listed in Table 1. Their determination coefficients were all higher than 0.998. The LODs ($S/N=3$) for fucoxanthin, lutein and astaxanthin were all $0.30 \mu\text{g g}^{-1}$ and the LOQs ($S/N=10$) could get $1.00 \mu\text{g g}^{-1}$ for each analyte.

Recovery

Recovery and precision of MSPD method were measured using *Nannochloropsis oculata* samples spiked at three concentration levels for three carotenoids (each level $n=6$). The results are shown in Table 2. The recoveries ranged from 69.1 to 106.5% for each concentration. The same experiment was repeated for 3 days to obtain the inter-day precision. The RSDs were 2.11% to 4.76% for three target compounds. The recovery results with *Isochrysis zhangjiangensis-1* matrix and solvent blank were also satisfied.

Comparison of MSPD Extraction with Ultrasonic and Ice-Bath Methods

The MSPD extraction method was compared with two conventional extraction methods, ultrasonic and ice-bath extraction methods. The chromatograms of *Nannochloropsis oculata* extracts by three methods are shown in Fig. 7. It can be seen that the elution profile was the same. However, the peak area of the target compounds was different. The extraction efficiency of the MSPD method was higher than ultrasonic and ice-bath extraction methods. In addition, the solvent consumption of MSPD method was only 4 mL in comparison to 30 mL of the other two extraction methods. The corresponding quantitative results for lutein and the solvent consumption of the three methods are shown in Table 3.

The MSPD method integrated the disruption, extraction and clean-up process. When it was applied to the microalgal samples, 4 mL of methanol can elute the target compounds—fucoxanthin, lutein and astaxanthin. Most weak polar compounds were retained on the MSPD column. The target carotenoid samples were purified. On the other hand, for the ultrasonic and ice-bath extraction methods, the target

compounds and weak polar components were extracted together. When the sample was analyzed by HPLC, the column repeatability and lifetime may be affected by these less polar compounds.

Application of Different Microalgal Samples

The applicability of the MSPD method was assessed through the analysis of different microalgal samples. The chromatograms for *Isochrysis zhangjiangensis-1*, *Isochrysis zhangjiangensis-2*, *Nannochloropsis oculata* and *Platyonas subcordiformis* extracts are shown in Fig. 8. *Isochrysis zhangjiangensis* contained more fucoxanthin, it was 1.13 mg/g ($n=3$, $\text{RSD}=8.78\%$) and $31.9 \mu\text{g/g}$ ($n=3$, $\text{RSD}=13.3\%$) for *Isochrysis zhangjiangensis-1* and *Isochrysis zhangjiangensis-2*, respectively. Lutein was detected in *Nannochloropsis oculata* and *Platyonas subcordiformis* only. The level was $28.5 \mu\text{g/g}$ ($n=3$, $\text{RSD}=0.54\%$) in *Nannochloropsis oculata* and $153.1 \mu\text{g/g}$ ($n=3$, $\text{RSD}=4.77\%$) in *Platyonas subcordiformis*. As reported in reference, astaxanthin and lutein in different microalgae ranged from 0 to 2.26 and 0 to 4.49 mg/g [1], respectively. Fucoxanthin in different diatom ranged from 2.24 to 21.67 mg/g [4]. Different microalgae varied greatly. Then the high-throughput quantification method was very important for screening microalgae, especially in the culture for the production of carotenoids. This MSPD method can meet the demand.

Conclusions

The carotenoids in different microalgae were extracted effectively by MSPD method and then determined by HPLC. The MSPD–HPLC method exhibited acceptable reproducibility, recovery, extraction efficiency and lower solvent consumption relative to conventional extraction techniques such as ultrasonic and ice-bath extraction. The method described here is suitable for the routine analysis of carotenoids in the progress of inducing a culture of microalgae.

Table 1 The calibration curves and linearity of three standards

Name	Linear equation	Linear range (ng)	R^2
Fucoxanthin	$y=20780x - 5055.7$	0.1–40	0.9994
Astaxanthin	$y=82102x - 14627$	0.1–20	0.9985
Lutein	$y=66116x - 32480$	0.1–40	0.9982

Table 2 The recovery results of MSPD method

Name	Day 1			Day 2			Day 3			Inter-day precision (%)
	Spiked (µg)	Found (µg)	Recovery (%)	Spiked (µg)	Found (µg)	Recovery (%)	Spiked (µg)	Found (µg)	Recovery (%)	
Fucoxanthin	4.03	2.91	72.2	4.03	2.82	70.0	4.03	2.82	69.9	2.11
	4.03	2.94	73.0	4.03	2.81	69.7	4.03	2.92	72.4	
	10.07	7.84	77.9	10.07	7.78	77.3	10.07	7.88	78.3	2.13
	10.07	8.03	79.7	10.07	7.78	77.3	10.07	7.52	74.7	
	20.14	18.64	92.6	20.14	17.76	88.2	20.14	19.07	94.7	2.98
	20.14	17.73	88.0	20.14	17.97	89.2	20.14	18.55	92.1	
Astaxanthin	40.11	3.06	74.5	4.11	2.92	71.0	4.11	2.84	69.1	2.96
	40.11	3.03	73.7	4.11	2.88	70.1	4.11	2.98	72.5	
	100.07	8.24	81.8	10.07	8.06	80.0	10.07	8.16	81.0	3.08
	100.07	8.49	84.3	10.07	8.09	80.3	10.07	7.73	76.8	
	200.14	19.91	98.9	20.14	18.96	94.1	20.14	20.42	101.4	2.99
	200.14	19.27	95.7	20.14	18.95	94.1	20.14	19.74	98.0	
Lutein	3.99	4.19	105.0	3.99	3.94	98.7	3.99	4.06	101.8	3.40
	3.99	4.32	108.3	3.99	3.98	99.7	3.99	4.13	103.5	
	10.07	9.89	98.2	10.07	9.51	94.4	10.07	9.51	94.4	4.76
	10.07	10.20	101.3	10.07	9.44	93.7	10.07	8.85	87.9	
	20.14	22.56	112.0	20.14	20.47	101.6	20.14	21.44	106.5	3.50
	20.14	21.23	105.4	20.14	20.84	103.5	20.14	20.77	103.1	

Fig. 7 The chromatograms of *Nannochloropsis oculata* extract from different preparation methods. **a** MSPD method; **b** ultrasonic extraction by methanol; **c** ice-bath extraction by methanol. The HPLC conditions were the same as mentioned in “Chromatographic conditions”

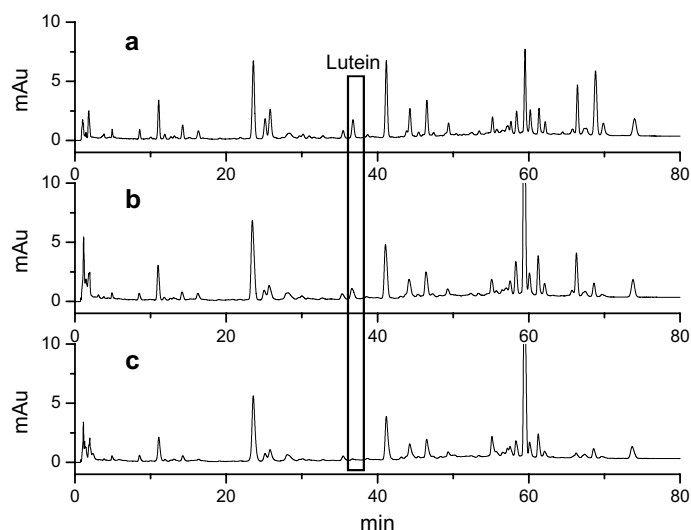
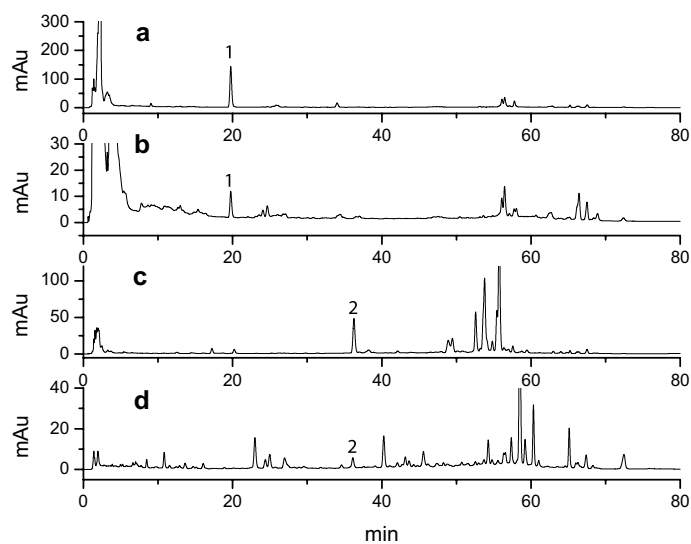


Table 3 The quantitative results of three extraction methods

Name	Method	Content ($\mu\text{g/g}$)	Solvent consumption (mL)
Lutein in <i>Nannochloropsis oculata</i>	MSPD	115.9	4
	Ultrasonic (methanol)	112.3	30
	Ultrasonic (acetone)	22.0	30
	Ice bath (methanol)	5.40	30
	Ice bath (acetone)	—	30

Fig. 8 The chromatograms of different microalgal extract from MSPD method. **a** *Isochrysis zhangjiangensis-1*; **b** *Isochrysis zhangjiangensis-2*; **c** *Platyonas subcordiformis*; **d** *Nannochloropsis oculata*. 1 Fucoxanthin; 2 lutein. The HPLC conditions were the same as mentioned in “Chromatographic conditions”



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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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