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Solvent extraction, ultrasound assisted extraction (UAE), and microwave assisted extraction (MAE) were examined for the extraction of astaxanthin from *Haematococcus pluvialis*. In all cases, acetone was found to give the highest astaxanthin recovery compared with other selected solvents, i.e., methanol, ethanol, and acetonitrile. Among the various methods, MAE at 75°C for 5 min resulted in the highest astaxanthin recovery (74 ± 4%).

Keywords accelerated extraction; conventional extraction method; microwave-assisted; ultrasound-assisted

INTRODUCTION

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a highly efficient antioxidant (1) and displays anti-cancer properties. This carotenoid plays vital roles in immune system enhancement and in fighting off tissue damage and cardiovascular diseases (2, 3, 4, and 5). Astaxanthin is naturally produced by various microorganisms and microalgae species, an important example of which is the unicellular microalga *Haematococcus pluvialis*, which produces 0.5–5.0% dry weight of astaxanthin. The thick cell wall of *H. pluvialis* makes astaxanthin extraction difficult. For this reason, various extraction techniques have been proposed, including solvent extraction (6), enzyme-assisted solvent extraction (7), extraction with vegetable oils (8), pressurized fluid extraction (9), and supercritical fluid extraction (10, 11, 12, 13, 14, and 15).

Recently, accelerated techniques, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have gained considerable interest for the extraction of bioactive substances from plants and microorganisms (16,17). These methods offer many advantages, such as short extraction times, reduced solvent usage, and higher extraction yield. In UAE, as ultrasound passes through a solvent, the expansion cycle of the ultrasonic waves creates cavities. These micro-bubbles grow during the expansion cycles and contract during each compression cycle. The increase in pressure and temperature caused by the localized compression leads to the collapse of the bubbles, resulting in enhanced mass transfer and consequent disruption of algal cell walls (18). By contrast, MAE promotes rapid heating and pressure buildup within the sample tissue due to the rotation of the solvent's molecular dipole in the microwave field. As a result, transfer of biological compounds from the cells into the extraction medium is accelerated. Although optimization of MAE for *H. pluvialis* has been carried out and shown to be more effective than conventional extraction methods (19), a detailed comparison of MAE, UAE, and other conventional methods requires additional investigation. This study sought to determine suitable conditions (time, temperature, and solvent type) for UAE and MAE of astaxanthin from *H. pluvialis*. The extraction efficiencies at these conditions were then compared with each other and with those offered by conventional extraction techniques (maceration and Soxhlet extraction).

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MATERIALS AND METHODS

Materials

Red dried samples of feed grade *Haematococcus pluvialis* were purchased from Cyanotech Corporation, Hawaii Ocean, Science and Technology Park, USA (supported by Professor Motonobu Goto, Kumamoto University, Japan). The samples were stored in a dry, tight aluminum pack at 5°C to prevent degradation. Analytical grade solvents (for astaxanthin extraction) i.e., methanol, ethanol, acetone, and acetonitrile and HPLC grade methanol were all purchased from Fisher Scientific. Calibration curves of astaxanthin in various types of solvent were provided from a standard compound of >92% purity purchased from Sigma-Aldrich, USA.

Methods

For each extraction method (maceration, Soxhlet extraction, UAE, and MAE), trials were first carried out

TABLE 1
Experimental variables and ranges studied

Methods	Temperature (°C)	Time (min)
Maceration	30, 45, and 60	0, 5, 15, 30, 45, 60, 75, and 90
Soxhlet	Boiling point of solvent	240
Ultrasonic	30, 45, and 60	0, 5, 15, 30, 45, 60, 75, and 90
Microwave	30, 45, 60, 75, and 78	0, 5, 15, 30, 45, and 60

to investigate the extraction potential of, in turn, methanol, ethanol, acetonitrile, and acetone, solvents chosen on account of their polarity relative to astaxanthin. The most suitable solvent was then used in subsequent determination of the optimal extraction conditions, by varying temperature and reaction time as summarized in Table 1. All experiments were performed in triplicate and the standard errors are presented for all data.

Maceration

For maceration, *Haematococcus pluvialis* powder was extracted in a 125 ml flask at 30–60°C. A sample-to-solvent ratio of 0.1 g algae powder to 10 ml of organic solvent was used, since our preliminary results had shown this ratio to be optimal (data not show). Each extraction was carried out for 0–90 min. The extract mixure was then passed through a 0.45 μm pore size polyethylene terephthalate (PET) syringe filter (Whatman, USA) and the astaxanthin content of the filtrate was determined by High Performance Liquid Chromatography (HPLC).

Soxhlet Extraction

Soxhlet extractions were also carried out to compare possible recovery of astaxanthin in a continuous solvent extraction system. To this end, 0.5 g of the algae sample was placed in a thimble cartridge which was connected to a 500 ml round-bottom flask containing 150 ml of solvent. The flask was placed in a heater pocket (Electro mantle, USA) and extracted for 4 h. The solution was then passed through a 0.45 μm pore size PET syringe filter (Whatman, USA) and the filtrate was subsequently analyzed by HPLC.

UAE

The ultrasonic bath (275DAE, 270 W, Crest Ultrasonics, USA) was a rectangular container (23.5 cm × 13.3 cm × 10.2 cm), featuring two 38.5 kHz transducers annealed at the bottom. The extraction of algae was conducted by adding alga (0.1 g) in a 30 ml amber glass bottle of solvent (10 ml). The bottle was immersed in tap water (2.2 L) and positioned at the center of the ultrasonic bath, 5 cm clear of its base. Extraction runs were performed at various temperatures between 30 and 60°C for 0–90 min (Table 1). After extraction, the solution was filtered through a 0.45 μm pore size PET syringe filter (Whatman, USA) and the filtrate then analyzed by HPLC for the astaxanthin content.

MAE

The extraction was performed on 12 × 100 ml closed polyetheretherketone (PEEK) vessels covered with special TFM® sleeves, a power sensor, a temperature sensor, and a temperature controller of MARS 5™ (1200 W, 2450 MHz), microwave accelerated reaction system from CEM Corp. (Mathews, NC, USA). The extraction was conducted by adding algae (0.1 g) to each of the six vessels containing the extraction solvent (10 ml). The vessels were closed and then placed symmetrically in the microwave field. In all MAE experiments, 60% of 1200 W power output was used and the ramping time was 2 min. The experimental conditions (irradiation time and temperature) are listed in Table 1. After each extraction, the solution was filtered through a 0.45 μm pore size PET syringe filter (Whatman, USA) and the filtrate analyzed by HPLC for astaxanthin content.

Analysis of Astaxanthin

The extracts were analyzed by HPLC (Venisep GES C18 4.6 × 150 mm, 5 μm HPLC column) at 475 nm using a methanol-water (95:5 v/v) mobile phase at a flow rate of 1 ml/min. Astaxanthin concentration of samples was determined by the peak area based on our calibration using an astaxanthin standard. As the basis for the determination of extraction efficiency, the total amount of astaxanthin in *H. pluvialis* was determined from the amounts of astaxanthin repeatedly extracted by MAE in acetone at the condition giving the highest % recovery. The percentage recovery of astaxanthin was defined as the ratio between the amount of astaxanthin extracted and the total amount of astaxanthin in the alga according to Eq. 1.

$$\% \text{ Recovery} = \frac{\text{astaxanthin from the extraction}}{\text{total astaxanthin in the alga}} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Effect of Solvent Types

Acetone gave the highest astaxanthin recovery for all extraction methods (Fig. 1). It should be noted that the ranges of extraction conditions selected for the study were not the same for all extraction methods since each method differs in nature and thus the operation limits. The discussion of the results was therefore given first for each extraction method. The results obtained at the

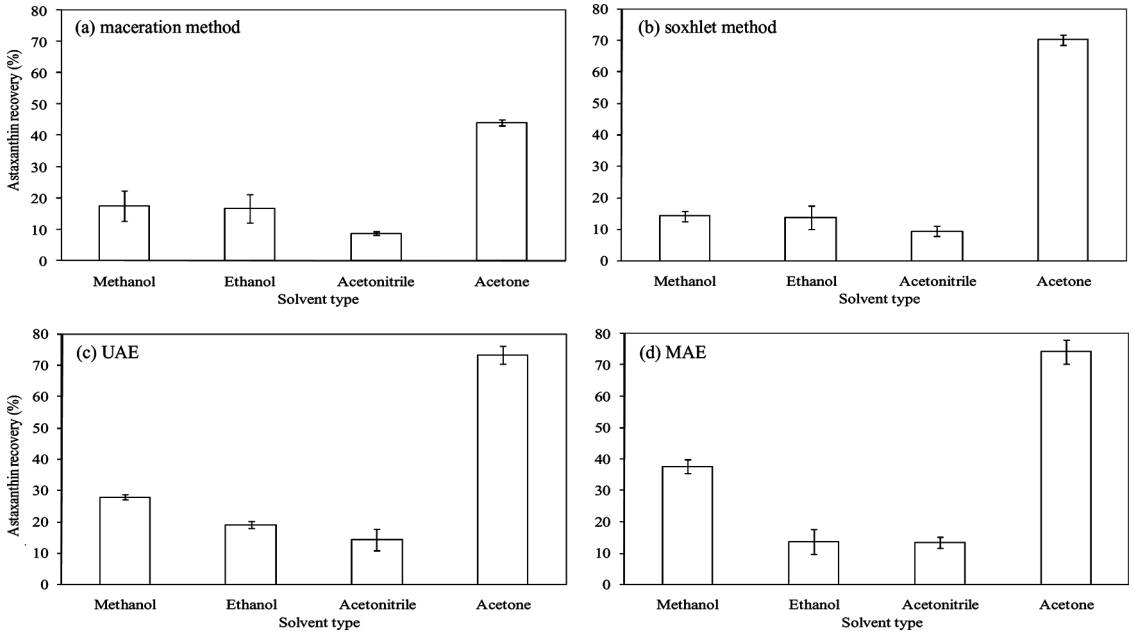

FIG. 1. Effect of solvent type on astaxanthin extraction (a) maceration at 30°C, 30 min, (b) Soxhlet extraction for 4 h, (c) UAE at 45°C, 60 min, 18.40 W, and (d) MAE at 75°C, 5 min, power 720 W.

most suitable condition for each method were then compared.

Maceration

The determination of the suitable solvent for maceration was conducted at 30°C for 30 min. As shown in Fig. 1(a), acetone resulted in the highest astaxanthin recovery, ($44 \pm 1\%$), followed by methanol ($18 \pm 5\%$), ethanol ($17 \pm 5\%$), and acetonitrile ($9 \pm 1\%$). Of all the solvents studied, acetone has the lowest polarity, indicated by the dielectric constant (Table 2), making it the most appropriate solvent to dissolve the fat soluble molecules like astaxanthin. Moreover, the structure of astaxanthin is most similar to that of acetone, in as much as both feature carbonyl groups.

TABLE 2
Properties of solvents used for extraction

Type of solvents	Dielectric constant (ε')	Surface tension (mN/cm)	Vapor pressure (mmHg)	Viscosity (cP)
Methanol	32.6	22.6	127.05	0.6
Ethanol	24.6	23.7	59.02	1.2
Acetonitrile	37.5	19.1	88.47	0.38
Acetone	20.7	23.7	229.52	0.32

Data from (20).
Determined at 20°C.

Soxhlet Extraction

Soxhlet extraction in acetone gave as high as $70 \pm 2\%$ astaxanthin recovery (Fig. 1(b)), compared with only $44 \pm 1\%$ obtained by 30 min maceration at 30°C. The increased extraction efficiency was expected since Soxhlet extraction provides the algae continual contact with the fresh solvent. Nevertheless, none of the other solvents showed similar improvement in the astaxanthin recovery. Since Soxhlet extractions must be done at the solvent boiling temperatures (56.5, 64.7, 78.5, and 81.6°C for acetone, methanol, ethanol, and acetonitrile respectively), possible thermal degradation of the astaxanthin in higher boiling point solvents could account for these unimproved recoveries.

UAE

The results in Fig. 1(c) show the effects of extraction solvents on astaxanthin recovery for UAE carried out at 18.40 watt at 45°C and 60 min. The highest % astaxanthin recovery ($73 \pm 3\%$) was again obtained with acetone. It should be noted that, in UAE, acoustic cavitation is an important phenomenon that is responsible for enhanced extraction recovery other than the solvent polarity. The degree of ultrasonic cavitation depends on various thermodynamic properties of the solvent. In solvents with low vapor pressures, bubble collapses tend to be strong, facilitating the disruption of algal cells and the release of astaxanthin. However, the localized severe high temperatures and pressures could prompt compound degradation and thus have the opposite effect. Less severe bubble

collapses in high vapor pressure solvents like acetone or methanol, by contrast, should therefore lead to minimal product degradation. This could explain the high % astaxanthin recovery obtained by UAE when using acetone. Methanol, which also has relatively high vapor pressure, was also observed to give rather high recovery (compared with ethanol) despite the greater differences in polarities and molecular structures between this solvent and the astaxanthin. These results suggest that, in UAE, acoustic cavitation significantly influences the extraction efficiency of the solvents.

Another solvent property generally known to affect the extraction recovery in UAE is surface tension. Specifically, bubble cavitation occurs more readily in solvents with higher surface tension (20). In this study however, the effect of solvent surface tension was deemed negligible since the surface tensions of the selected solvents did not differ considerably (Table 2).

MAE

To determine the effect of the solvent type on MAE, the experiments were carried out at 75°C for 5 min. The extraction recoveries obtained with MAE followed the same order as the data obtained with maceration, that is, acetone $(74 \pm 4\%) >$ methanol $(38 \pm 2\%) >$ ethanol $(14 \pm 4\%) >$ acetonitrile $(13 \pm 2\%)$. Nevertheless, the degree of enhancement of astaxanthin recovery in methanol and acetone was higher than the other solvents. In general, MAE enhances the extraction capability of solvents because the electromagnetic field causes rapid heating of the solvent, the rate of which depends on a parameter called the dissipation factor ($\tan\delta$), defined as follows.

$$\tan\delta = \frac{\varepsilon''}{\varepsilon'} \qquad (2)$$

where ε' is the dielectric constant or relative permittivity and ε'' is the dielectric loss factor. ε' describes the polarizability of the solvent molecule in an electric field, a measure of the ability of the solvent to store electromagnetic radiation. ε'' is a measure of the efficiency by which the absorbed microwave energy is converted into heat when an electric field is applied. From this definition, the dissipation factor ($\tan\delta$) therefore represents the ability of the solvent to absorb the microwave energy and dissipate that energy into heat. The rate of heating under microwave irradiation is generally expected to be high if both the dielectric constant and dissipation factor of the solvent are high. It is likely, therefore, that the enhanced astaxanthin recoveries observed using acetone and methanol under microwave irradiation were due to the relatively high ε' and $\tan\delta$ values of these solvents (20) (Table 3). By comparison, ethanol and acetronitrile are characterized by low dielectric loss factors, which explain their poorer ability to dissipate the absorbed microwave energy into heat, thus lower the astaxanthin recovery.

TABLE 3
Properties of solvents used for MAE

Type of solvents	ε'^{a} (F/m)	ε''^{b}	$\tan\delta$
Methanol	32.6	15.2	0.5032
Ethanol	24.3	6.1	0.2564
Acetonitrile	37.5	2.3	0.0614
Acetone	20.7	11.5	0.6207

Data from (20).
[a]Determined at 20°C.
[b]At 2450 MHz.

In short, since acetone consistently gave the highest astaxanthin recovery, it was chosen for subsequent optimization studies.

Suitable Extraction Conditions

Maceration

The effects of time and temperature on extraction efficiency (Fig. 2) indicate that for all extraction temperatures the astaxanthin recovery was rapid and essentially complete after 5 min. It is likely that the high initial astaxanthin extraction rate was due to the high concentration gradient of astaxanthin mass transfer between inside and outside the algal cell. Beyond this time, the rate of further astaxanthin extraction appears to drop markedly. The highest astaxanthin recovery ($57 \pm 4\%$) was obtained after 15 min at 45°C, whereas lower recoveries were observed beyond 60 min at 60°C. Higher extraction temperatures should increase astaxanthin solubility and decrease solvent viscosity, thereby increasing the astaxanthin recovery. However, prolonged exposure to high temperatures could also lead to compound degradation, accounting for our observed drop in astaxanthin levels.

UAE

For UAE, all extraction temperatures showed rapid astaxanthin recovery (up to $41 \pm 2\%$) within the first 5 min, and thereafter, between 5 to 30 min, increasing only gradually (Fig. 3). It should be noted that, despite the cavitation effect of UAE, astaxanthin recovery did not increase significantly, compared with maceration, for the first 30 min. This could be due to the fact that ultrasonic cavitation gives rise to localized hot spots that destroy astaxanthin molecules (21,22). After 30 min, however, the extraction recovery increased again until 60 min to around $70 \pm 2\%$. Following this maximum, the astaxanthin level finally dropped as the extraction time approached 90 min. The maximum astaxanthin recovery for UAE was found at the extraction temperature of 45°C and 60 min.

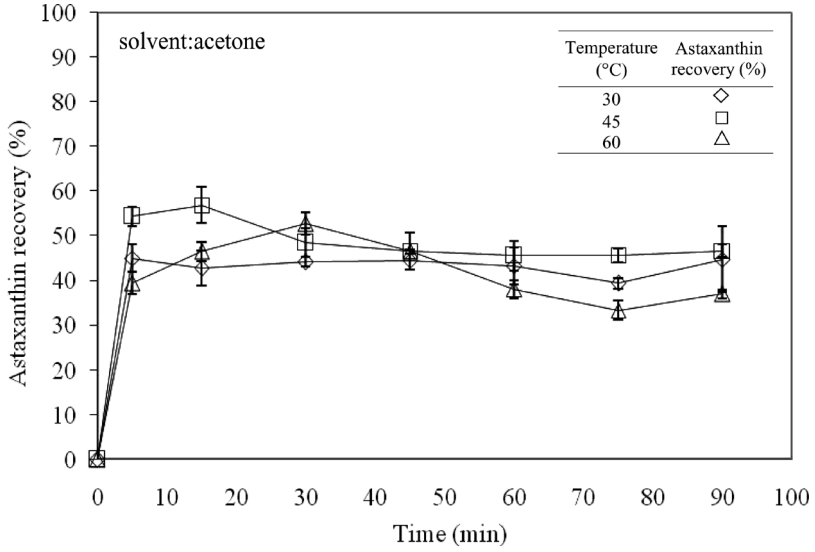

FIG. 2. Effect of time and temperature on astaxanthin recovery by maceration.

MAE

In a closed, microwave-irradiated system, astaxanthin recovery was seen to increase instantly in the first 5 min at all extraction temperatures (Fig. 4). For extraction temperatures of 30, 45, and 60°C, astaxanthin recovery remained relatively constant over time, whereas for extraction temperatures of 70 and 75°C, the highest % astaxanthin recovery was again reached at 5 min, immediately dropping thereafter. The highest recovery ($74 \pm 4\%$) was obtained with MAE at 75°C after 5 min. The increase in temperature to 78°C did not further increase the astaxanthin recovery, possibly due to its structural decomposition of astaxanthin, i.e, via the conversion from (all-E)-astaxanthin to (13Z)-astaxanthin (21).

Comparison of Extraction Methods

The % astaxanthin recovery obtained by different methods at the most suitable conditions are summarized in Table 4. The highest % astaxanthin recovery ($74 \pm 4\%$) was obtained by 5 min MAE at 75°C, followed by UAE at 45°C ($73 \pm 3\%$). The closed system used for MAE allowed high extraction temperature (above the boiling

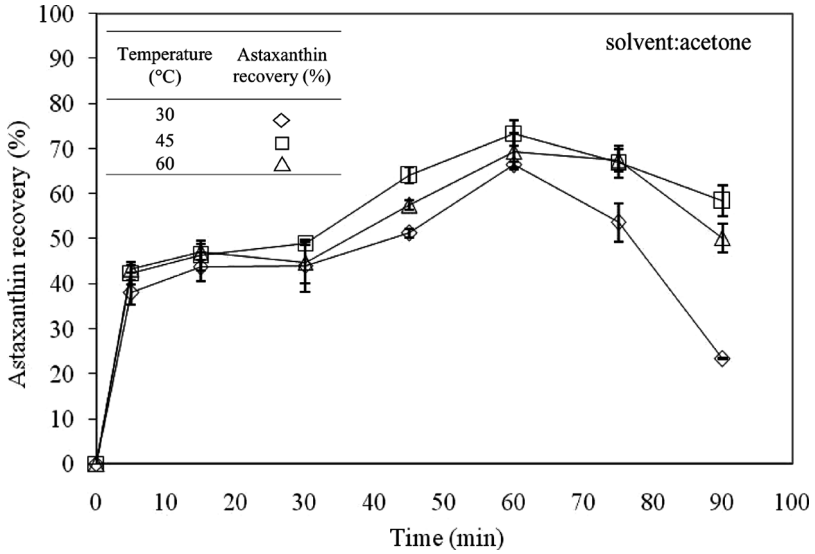

FIG. 3. Effect of time and temperature on astaxanthin recovery by UAE (power 18.40 W).

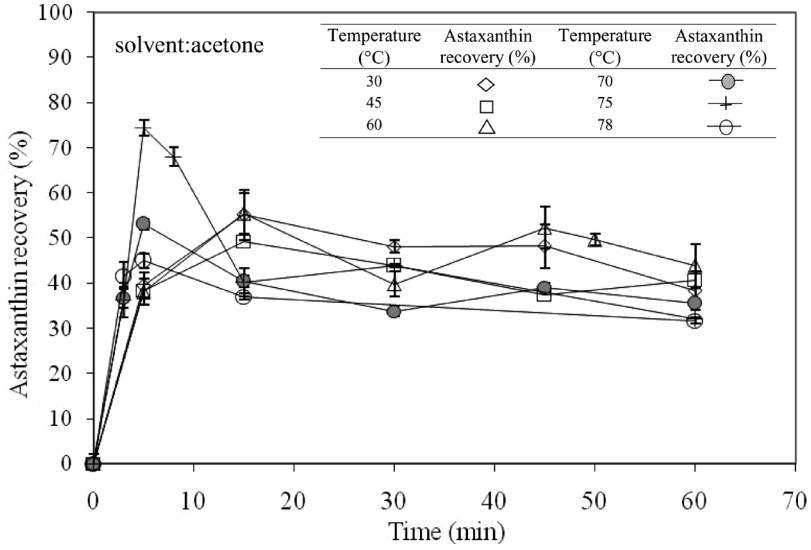

FIG. 4. Effect of time and temperature on astaxanthin recovery by MAE (power 720 W).

point of acetone), and because of rapid heating caused by microwave irradiation, shorter extraction time is required. Brief extraction times are considered favorable since they are expected to minimize compound degradation. Although comparable astaxanthin recovery could be achieved with UAE, it required much longer extraction time (60 min). When compared with the extraction recoveries by other nonconventional methods such as supercritical carbon dioxide extraction (60–83%) (11,13,14), MAE achieves equally impressive recoveries but in a much shorter extraction time. Despite an enhanced recovery, careful considerations must be made when employing MAE and UAE as compound degradation can easily occur. The results on the astaxanthin yield as well as the possible degradation observed in this study nevertheless are in good agreement with the detailed optimization study of MAE reported by Zhao et al. (2009), thus supporting the potential use of MAE for astaxanthin recovery from *H. pluvialis* (19).

TABLE 4
Comparison of astaxanthin recoveries obtained with various extraction methods

Methods	Temperature (°C)	Time (min)	% astaxanthin recovery
Maceration	45	15	57 ± 4
Soxhlet	56.5	240	70 ± 2
UAE	45	60	73 ± 3
MAE	75	5	74 ± 4

CONCLUSIONS

For all extraction methods, acetone was found to give the best astaxanthin recovery. The use of UAE and MAE considerably enhances astaxanthin recovery though there are limits to the conditions in which each of these methods can be employed. MAE at 75°C, using acetone as the extraction solvent, was found to give the highest astaxanthin recovery in a relatively short time. The results of this study suggest therefore that MAE features great potential for the extraction of valuable compounds from *H. pluvialis* microalgae.

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