

# Pressurized fluid extraction of carotenoids from *Haematococcus pluvialis* and *Dunaliella salina* and kavalactones from *Piper methysticum*

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## Abstract

Pressurized fluid extraction (PFE) was examined as an alternative technology for the extraction of carotenoids in the green algae *Haematococcus pluvialis* and *Dunaliella salina* and kavalactones in *Piper methysticum*. The extraction process was optimized by varying the key extraction factors of solvent, sample-solvent ratio, temperature, and time. The selectivity and efficiency of extraction parameters were determined with high performance liquid chromatography (LC) and LC–mass spectrometry (LC–MS). Results showed that PFE utilization of conventional solvents under controlled temperature and pressure in an oxygen and light-free environment could result in the use of less solvent in a shorter period of time. PFE showed higher or equal extraction efficiencies as compared with traditional solvent extractions while maintaining the integrity of chemical components. PFE showed high potential for extraction of natural products and nutraceuticals, particularly labile and light sensitive chemicals.

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**Keywords:** *Haematococcus pluvialis*; *Dunaliella salina*; *Piper methysticum*; Pressurized fluid extraction; Carotenoids; Kavalactones

## 1. Introduction

Natural product extraction forms the basis for a growing nutraceutical industry. Although traditional solvent extraction techniques are widely applied throughout the industry, these techniques utilize large quantities of organic solvents, are labor-intensive, and can expose the extracts to excessive heat, light and oxygen, promoting the potential isomerization and oxidation of labile compounds. Pressurized fluid extraction (PFE) utilizes conventional solvents at controlled temperatures and pressures and has been well established for environmental analysis [1,2]. Since PFE uses less solvent in a shorter period of time, is automated, and involves retaining the sample in an oxygen and light-free environment, it has the potential to be a powerful tool in the nutraceutical industry. Recent applications have demonstrated the advantage of PFE for the extraction of natural products from plant materials [3–9]. Other biological applications include the extraction of fatty acids from plant and animal matrices [10] and the determination of zearalenone mycotoxin in corn

[11]. However, PFE has not been widely applied as a routine tool in natural product extraction. Therefore, it was our interest to apply PFE parameters to the efficient extraction of two economically valuable nutraceuticals, carotenoids and kavalactones, selected as model chemicals in this study.

The carotenoids are powerful antioxidants and bright colorants, and thus are used as human diet supplements and fish food pigmentors, respectively. The green alga *Haematococcus pluvialis* biosynthesizes the carotenoid astaxanthin in high concentrations (1.5–3.0%) compared to other microbial sources [12]. Environmental stresses can cause *H. pluvialis* to accumulate large quantities of astaxanthin and other carotenoids in the cytoplasm enclosed by a thick cyst-like cell wall (aplanospore) [13]. This protective barrier can hinder carotenoid extraction and bioavailability [14,15]. *Dunaliella salina* is a halophilic alga that produces  $\beta$ -carotenes, chlorophylls, and the xanthophylls lutein, zeaxanthin, and neoxanthin [16]. *D. salina* cells lack a rigid cell wall structure [17].

There are *cis* and *trans* isomeric forms of astaxanthin that are often esterified to fatty acids in *H. pluvialis*. *Trans*-astaxanthin is readily isomerized with heat and light to 9-*cis* and 13-*cis* isomers [18] that are more susceptible to oxidation than the *trans* isomer [19].  $\beta$ -Carotene and

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lutein are also oxygen-sensitive and are subject to isomerization under heat and light. To quantify carotenoids in the crude extract, carotenoid esters have been hydrolyzed using a non-base-dependent hydrolysis reaction [20] to minimize side reactions [21,22]. Methods for the separation of carotenoid isomers have been well-established using C<sub>30</sub> reverse phase chromatography [23,24]. Use of this stationary phase in combination with diode array (DAD) and mass spectrometry (MS) detection allows for an efficient means of monitoring the possible formation of the less desirable isomers that may arise as a result of an inferior extraction process.

Kavalactones are primarily present in the roots and stump of the kava plant, *Piper methysticum*, and have psychoactive, anxiety relieving, and sleep inducing effects [25]. The lipophilic kavalactones are  $\alpha$ -pyrones with methoxy and aromatic styryl or phenylethyl substituents at the 4- and 6-positions, respectively [26]. Six of the known kavalactones have been identified as the major, pharmaceutically relevant components [27]. Accurate analysis of the chemical constituents of kava products has recently been of concern due to putative hepatotoxicity of prepared kava products [28]. High performance liquid chromatography analysis combined with UV detection (LC–UV) is used as a standard method throughout the kava industry for verification of kavalactone content and quality assurance [29].

This study was designed to demonstrate the application potential of PFE in the extraction of the carotenoids from the green algae *H. pluvialis* and *D. salina* and kavalactones from *P. methysticum*. The extraction process was optimized by varying the key extraction factors of solvent, sample-solvent ratio, temperature, and time. LC–UV and LC–mass spectrometry (LC–MS) were used to analyze these sensitive compounds, to determine the selectivity and efficiency of extraction parameters, and to verify the integrity of the chemical constituents throughout the extraction process.

## 2. Experimental

### 2.1. Solvents, reagents and chemical standards

Acetone, acetonitrile, ethanol, methanol, and methylene chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA); isopropyl alcohol from Baxter Healthcare Corporation (Muskegon, MI, USA); and phosphoric acid from J.T. Baker (Phillipsburg, NJ, USA). The water was prepared by a double distillation and Millipore deionization process. All solvents were of HPLC grade and were filtered through a 0.45  $\mu$ m membrane filter prior to use. Astaxanthin,  $\beta$ -carotene, and lutein standards, cholesterol esterase and TRIZMA buffer were obtained through Sigma Corporation (St. Louis, MO, USA). Kavalactones were isolated from the kavaroot extracts and identified by their UV absorbance, rel-

ative LC retention times, and gas chromatographic (GC)–MS spectra as compared to those in the literature [26,30,31]. The authentic kavalactone, kavain, was used for individual kavalactone quantification using conversion factors from the Institute for Nutraceutical Advancement (INA) method [29].

### 2.2. Samples

Freshly homogenized *H. pluvialis* cysts were obtained from Cyanotech Corporation (Kona, HI, USA), and *D. salina* from Drs. Roger Babcock and JoAnn Radway at the University of Hawaii. Algae samples were lyophilized, nitrogen packed, and stored at  $-20^{\circ}\text{C}$ . Dried kava root samples (Hawaiian variety, Mo'i) were provided by Dr. H.C. Bittenbender at the University of Hawaii and purchased from the local market. The lateral root samples were pulverized to pass a 60 mesh particle sieve and homogenized before extraction.

### 2.3. Extraction

PFE was carried out with a Dionex ASE 200 extractor (Salt Lake City, UT, USA). The 11 ml stainless steel extraction cells were filled in consecutive layers with a cellulose filter (20  $\mu$ m pore size) on the bottom, a 2–3 cm<sup>3</sup> layer of inert Ottawa sand, the homogenized sample, and Ottawa sand again layered on top to fill the extraction cells. Extraction cells and collection vials were loaded onto the automated carousel. The heating time of the cells was 5 min. Extracts were collected into glass vials by flushing the system with 6.6 ml of fresh solvent after extraction. Finally, the system was purged for 1 min with nitrogen. It is known that extraction pressure does not significantly affect extraction efficiency [32,33]. The default pressures of 1500 and 2000 psi (for the carotenoids and kavalactones, respectively) were retained for all analyses.

Traditional extraction of carotenoids from *H. pluvialis* and *D. salina* followed a simple sonication and centrifugation procedure [34] designed for limited exposure of the sample to heat and light. Samples were sonicated in 5 ml of organic solvent and then centrifuged at 10,000  $\times g$  for 5 min. The supernatant containing pigments was retained and the pellet was again sonicated in fresh solvent and subsequently centrifuged. This process was repeated for up to six cycles rendering the cell pellet colorless, where upon the supernatants were combined.

PFE kava extracts were concentrated with a rotary evaporator to dryness. The residues were re-dissolved in aqueous methanol (70% v/v) to a final volume of 25 ml. For all carotenoid extracts in organic solvents other than acetone, the solvent was removed under a gentle stream of nitrogen and the dried extracts were re-dissolved in acetone to a final volume of 50 ml. All extracts were filtered through a GHP Acrodisc filter (0.45  $\mu$ m  $\times$  25 mm) and were stored at  $-20^{\circ}\text{C}$  until analysis.

## 2.4. Astaxanthin hydrolysis

Astaxanthin esters in crude extracts were hydrolyzed with cholesterol esterase in accordance with the procedure of Jacobs et al. [20] as modified by Cyanotech Corporation [35]. Basic hydrolysis of the astaxanthin esters in crude extracts was carried out according to the procedure of Yuan and Chen [36].

## 2.5. Liquid chromatography and mass spectrometry

Carotenoids in 20  $\mu$ l aliquots were separated on a Waters C<sub>30</sub> carotenoid column (4.6 mm  $\times$  12.5 cm; 5  $\mu$ m) held at 22 °C. The LC instruments were an Agilent 1100 (Palo Alto, CA, USA) and Dionex BioLC workstation each equipped with a binary pump, thermostated column compartment, and UV diode array absorbance detector. The solvent system was optimized as an isocratic mixture of 84% acetone and 16% water for the first 21 min, followed by a 4 min linear gradient to 97% acetone and 3% water for the remainder of the 50 min run, modified from Dachtler et al. [24]. The flow rate was 1 ml/min.

Mass spectra were obtained with an Agilent single quadrupole LC–MSD with an APCI interface using a modified method [24]. They were collected in a positive ion mode in a mass range of  $m/z$  200–800. The drying gas was nitrogen at a flow rate of 4.0 l/min at 300 °C, creating a nebulizing pressure of 15 psi. The capillary voltage was 5 kV resulting in an 8  $\mu$ A corona current.

LC–UV chromatographic peaks were measured at a wavelength of 480 nm for primary carotenoid detection. Total absorbance was recorded from 250 to 700 nm. The major carotenoid peaks were identified by comparison of retention times and spectra against authentic standards (astaxanthin,  $\beta$ -carotene, and lutein). The minor carotenoid components and carotenoid isomers were determined by comparison of UV–Vis and MS spectra with published data [24,37–39]. Ratios of *trans*–*cis* carotenoid isomers were estimated using absorbance values. A slight shift of the maximal absorbance and presence of a characteristic shoulder “*cis*” peak (~360 nm) allowed for rough differentiation of the *trans* from the *cis*-9, 13 astaxanthin isomers [40]. Retention times distinguished esterified from unesterified astaxanthin pigments. All concentrations were measured from standard calibration curves prepared regularly for authentic astaxanthin,  $\beta$ -carotene, and lutein standards.

Kavalactones were analyzed with a Dionex BioLC using the validated INA method [29]. Extracts in 5  $\mu$ l aliquots were separated on a YMC J'sphere ODS-H80 basic reverse phase column (25 cm  $\times$  4.6 mm; 4  $\mu$ m). The isocratic mobile phase was made up of 0.1% phosphoric acid (60.9%), acetonitrile (20.6%), and isopropyl alcohol (18.5%) at a flow rate of 0.6 ml/min. Column temperature was held constant at 40 °C. Total absorbance was recorded from 200 to 400 nm with quantification of kavalactones at 220 nm.

## 3. Results and discussion

### 3.1. Separation and determination of carotenoids and kavalactones

Fig. 1 shows the LC–UV and LC–MS chromatograms of the crude algal extracts (A<sub>1</sub>, A<sub>2</sub>, C<sub>1</sub> and C<sub>1</sub>). Carotenoid separation using the C<sub>30</sub> stationary phase allowed for enhanced selectivity between the major carotenoids. The elution order of the pigments was relative to the polarity of carotenoid analytes. In *H. pluvialis* the relatively polar astaxanthin and lutein are eluted first followed by the non-polar astaxanthin monesters,  $\beta$ -carotene, and astaxanthin diesters. Among the total pigments in *H. pluvialis* were *trans*-astaxanthin (1%), lutein (2%), astaxanthin monoesters (71%) and diesters (19%), and other minor carotenoid components and chlorophylls (7%). These results agreed with those found by Renstrom et al. [41] in *H. pluvialis* aplanospores. Total astaxanthin in the extracts was determined following enzymatic hydrolysis (Fig. 1B<sub>1</sub> and B<sub>2</sub>) that proved to be fast, specific and complete under mild conditions as compared with basic hydrolysis. This method also avoided co-elution and chlorophyll interference, and allowed for the monitoring of any occurrence of *cis*-astaxanthin isomers.

In the chromatographic separation of the *D. salina*, the major pigments elute in an order of lutein, chlorophyll *b*, chlorophyll *a*, and  $\beta$ -carotene making up 32, 18, 13 and 13%, respectively, of pigments in the crude extract. The LC–UV chromatogram of kavalactones in the crude kava extract is shown in Fig. 1D, where the major kavalactones methysticin, dihydromethysticin (DHM), kavain, dihydrokavain (DHK), yangonin, and desmethoxyyangonin (DMY), were well resolved.

### 3.2. Optimization of PFE of kavalactones from *P. methysticum*

In general, kavalactones are more stable than the oxygen-sensitive and thermolabile carotenoids. The kava samples consisted of ground lateral root powder, in contrast to *H. pluvialis* having a thick cell wall impermeable to common organic solvents and requiring cyst disruption prior to extraction. Kava was, therefore, selected for initial PFE optimization. The six major kavalactones monitored to test PFE parameters were DMY, DHK, DHM, kavain, methysticin, and yangonin. Methanol was used as the extracting solvent as it was seen in initial screening to have sufficient extraction capabilities and is used as the extracting solvent in nutraceutical analyses [29]. The extraction time and number of cycles were optimized for complete extraction, varying from a one 1 min cycle to three 6 min cycles at 60 °C and 2000 psi using 0.750 g of the same homogenized kava sample. Initial results (not shown) indicated little difference in extracted kavalactones with relation to extraction cycle time and number of cycles.

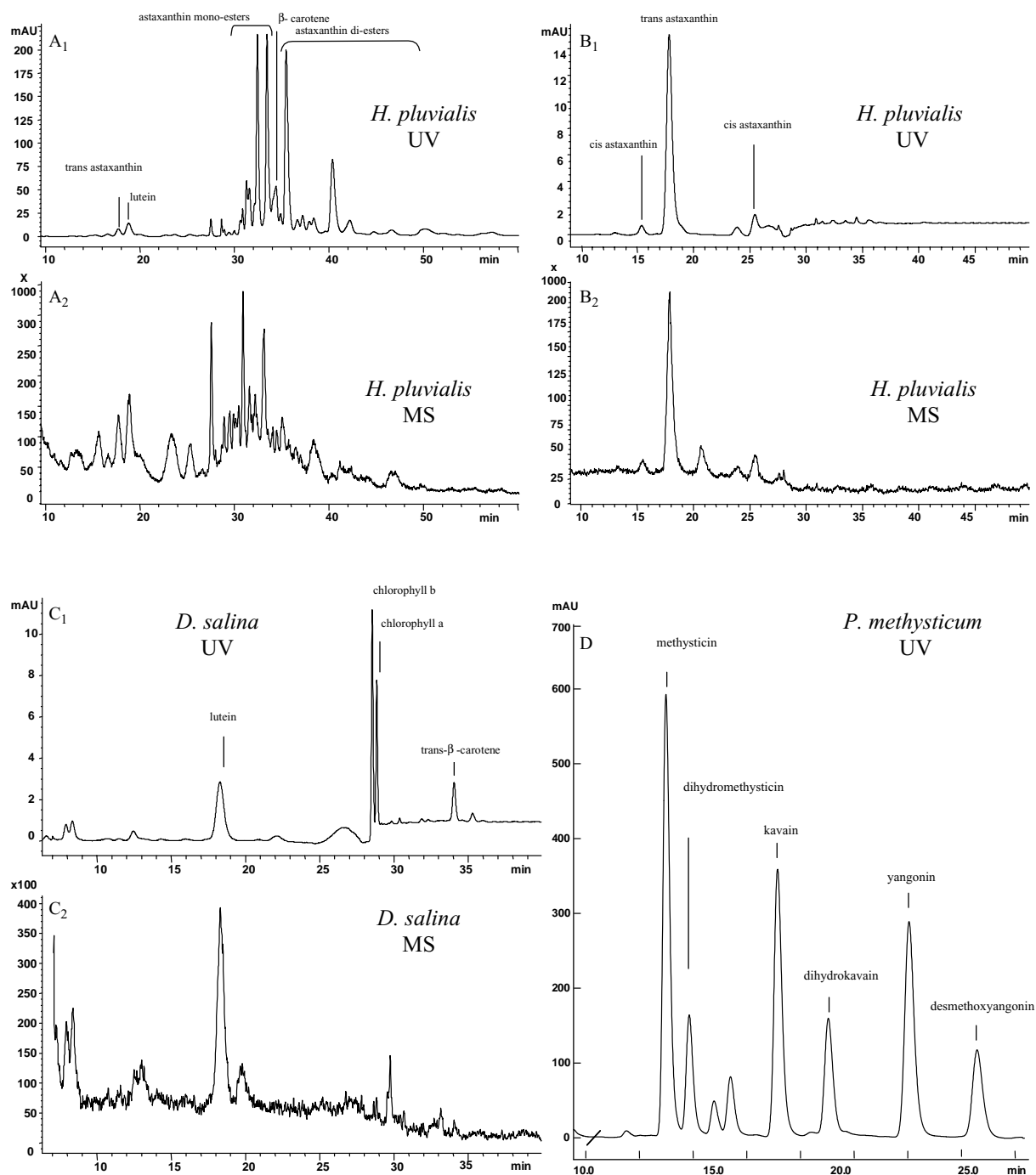


Fig. 1. UV and MS chromatograms of crude extracts of *H. pluvialis* (A<sub>1</sub> and A<sub>2</sub>), enzymatically hydrolyzed extracts of *H. pluvialis* (B<sub>1</sub> and B<sub>2</sub>) crude extracts of *D. salina* (C<sub>1</sub> and C<sub>2</sub>), and crude extract of *P. methysticum* (D).

Experiments were conducted to determine the effects of sample-solvent ratios on extraction efficiency. Quantitative PFE extractions of the six kavalactones were obtained with 15 ml methanol for each of the 0.5–5 g of kava samples (Fig. 2). As the sample size increased from 2 to 5 g, the extraction efficiency of the six kavalactones decreased. The solid line in Fig. 2 is a theoretical line of extraction yield proportional to the sample mass, where the y-axis is the product

yield normalized to reflect an ideal slope of 1 (designated as the  $k$  value). The experimental values below the theoretical line indicate incomplete extraction (Fig. 2) or possible chemical alterations such as isomerization occurring during the extraction. In Fig. 2, the highlighted data point represents the chemical alteration of yangonin extracted from a 3 g kava sample and stored for 5 months prior to analysis. The  $k$  value ratio of yangonin ( $k = 0.5$ ) as compared with

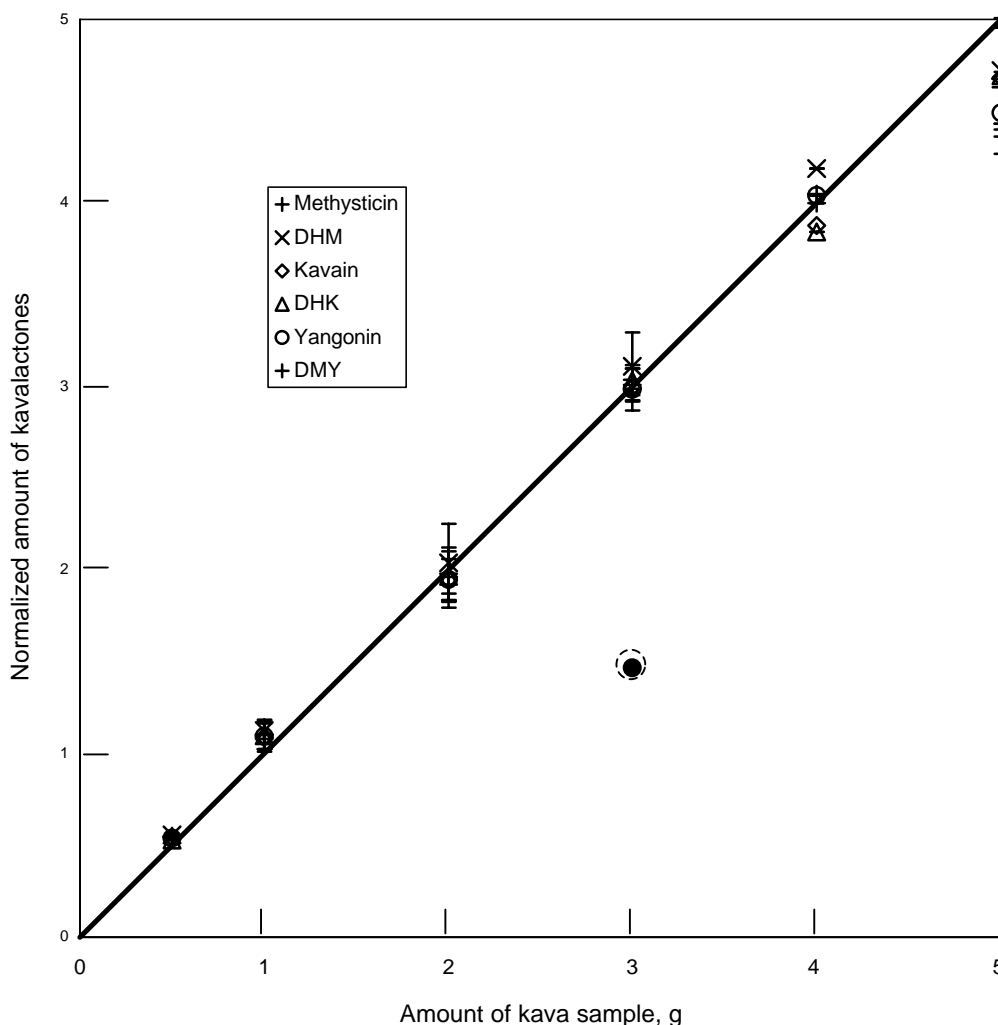


Fig. 2. Extraction efficiency of six kavalactones as a function of sample size. The amount of solvent used for each sample (0.5–5 g) was 15 ml of methanol. The line is a theoretical correlation between the sample size and the amount of kavalactones extracted, where the slope, designated as a  $k$  value indicates extraction efficiency. The data points below the line indicate an incomplete extraction of the six kavalactones (5 g sample size) and isomerization of yangonin (dotted circle) after long-term storage.

the rest of the kavalactones in this extraction sample is quite below the  $k$  value (i.e.,  $k < 1$ ) due to what is believed to be *cis* isomerization of yangonin from exposure to light and subsequent long-term storage [42]. If the experimental values were above the theoretical line (i.e.,  $k > 1$ ), this would indicate co-eluting interference or chemical alteration. The valuable use of  $k$  values in extraction optimization is demonstrated in Fig. 2 as a means of reducing solvent consumption while maximizing extraction efficiency.

### 3.3. Optimization of PFE of carotenoids from *H. pluvialis*

The extraction efficiency of five solvent systems was investigated at the following conditions: 1500 psi, 40 °C, 2–5 min extraction cycles with 1 g of the *H. pluvialis* cysts (Table 1). These five solvents were found to be commonly used in traditional carotenoid extractions and were initially seen as most effective in a preliminary screen. Acetone gave the highest extraction efficiency for the un-esterified

*trans*-astaxanthin (0.2 mg/g), lutein (0.9 mg/g), and total pigments (47.7 mAU of relative absorbance), except for the total hydrolyzed astaxanthin (9.5 mg/g). A mixture of methylene chloride and methanol (1:3, v/v) allowed for a greater recovery of total astaxanthin (10.9 mg/g). *Trans*-to-*cis* isomerization was not observed under any of these extraction conditions. This increase in extraction efficiency of the total astaxanthin with the methylene chloride and methanol mixture may be due to the higher solubility of astaxanthin in methylene chloride (~30 g/l) as compared to acetone (0.2 g/l) [18].

Temperature affects viscosity and solubility of solvents, but it also may promote isomerization and decomposition of labile target chemicals. Table 2 shows the results obtained when extraction temperature was increased from 20 to 100 °C. A slight decrease in the amount of extracted lutein and total pigment was observed as the extraction temperature increased, a possible consequence of carotenoid degradation at higher temperatures.

Table 1  
Selection of solvents for PFE of carotenoid compounds from *H. pluvialis*

Solvents	Concentration of target analytes <sup>a</sup>			
	Unesterified astaxanthin (mg/g)	Total astaxanthin (mg/g)	Lutein (mg/g)	Total pigment <sup>b</sup>
Acetone	0.2	9.5	0.9	47.7
Ethanol	0.1	8.4	0.7	36.7
Acetone:ethanol (7:3, v/v)	0.1	9.9	0.7	43.6
Acetone:methanol (7:3, v/v)	0.1	10.3	0.7	41.6
Methylene chloride:methanol (1:3, v/v)	0.1	10.9	0.7	47.1

<sup>a</sup> Average values of two replicates; 1 g sample size; extraction conditions: 1500 psi, 40 °C, two 5 min extraction cycles.

<sup>b</sup> Normalized absorbance values measured at 480 nm.

Table 2  
Effect of temperature on PFE efficiency of carotenoid compounds from *H. pluvialis*

Temperature (°C)	Concentration of target analytes <sup>a</sup>		
	Total astaxanthin (mg/g)	Lutein (mg/g)	Total pigment <sup>b</sup>
20	11.4 ± 0.4	1.1 ± 0.1	57.7 ± 2.7
40	10.0 ± 1.0	1.0 ± 0.1	52.1 ± 5.3
60	9.5 ± 0.7	0.9 ± 0.1	50.0 ± 6.2
80	11.0 ± 0.3	0.8 ± 0.1	49.3 ± 7.1
100	10.7 ± 1.6	0.7 ± 0.1	42.4 ± 6.9

<sup>a</sup> Average values of three replicates; 25 mg sample size; extraction conditions: acetone as extraction solvent, 1500 psi, three 5 min extraction cycles.

<sup>b</sup> Normalized absorbance values measured at 480 nm.

### 3.4. Comparison of recoveries between PFE and traditional extraction

Efficiency of PFE was compared with that of traditional extraction for two sample-solvent ratios for the extraction of

lyophilized *H. pluvialis* and *D. salina* cells (Table 3). The total volume of solvent used in PFE and traditional extraction was approximately 15 and 30 ml, respectively. PFE extracted higher or equal amount of astaxanthin, β-carotene, lutein, and total pigment from *H. pluvialis* and *D. salina* as compared with the traditional method (Table 3). As noted in the kava extraction (Fig. 2), sample sizes (i.e., sample-solvent ratio) affect extraction efficiency. However, the extraction efficiency for the carotenoids varied little in a range from 25 mg to 1 g of sample under the extraction conditions (Table 3).

It should be noted that all traditional extraction steps were carefully conducted in order to avoid isomerization and degradation of carotenoids. All steps were carried out in the dark at low temperature, where applicable, such as centrifugation at 4 °C. In another preliminary study, significant isomerization and degradation of the carotenoids was observed in extracts using an AOAC procedure [43] involving 2–4 h of sonication and up to 100 ml of solvent. Therefore, the procedure of Yuan and Chen [34] was used for comparison with PFE.

Table 3  
PFE and traditional extraction of carotenoid compounds from *H. pluvialis* and *D. salina*

Solvent	Target analyte	Concentration of pigment <sup>a</sup>			
		PFE <sup>b</sup> (1 g)	Traditional <sup>c</sup> (1 g)	PFE <sup>b</sup> (25 g)	Traditional <sup>c</sup> (25 g)
<i>H. pluvialis</i>					
Acetone	Astaxanthin (mg/g)	12.6 ± 2.5	12.7 ± 1.0	13.4 ± 2.1	14.3 ± 2.5
	Lutein (mg/g)	0.7 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.0
	Total pigment <sup>d</sup>	62.7 ± 5.6	54.9 ± 6.4	42.6 ± 3.7	37.8 ± 3.1
Methylene chloride:methanol (1:3, v/v)	Astaxanthin (mg/g)	10.2 ± 2.0	9.5 ± 2.1	13.0 ± 0.1	11.6 ± 2.6
	Lutein (mg/g)	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
	Total pigment <sup>d</sup>	40.5 ± 3.7	36.6 ± 2.2	38.0 ± 5.7	39.5 ± 2.3
<i>D. salina</i>					
Acetone	β-Carotene (mg/g)	1.6 ± 0.1	1.4 ± 0.1	2.7 ± 0.3	2.3 ± 0.5
	Lutein (mg/g)	3.8 ± 0.7	4.5 ± 0.3	4.8 ± 1.0	4.3 ± 0.8
	Total pigment <sup>d</sup>	50.3 ± 7.4	59.0 ± 4.7	61.7 ± 8.0	54.2 ± 7.1

<sup>a</sup> Average values of three replicates ± standard deviations.

<sup>b</sup> PFE conditions: 1500 psi, two (for 1 g) to four (for 25 mg) 5 min extraction cycles.

<sup>c</sup> Traditional extraction conditions: five to six 5 min sonication and centrifugation cycles.

<sup>d</sup> Normalized absorbance values measured at 480 nm.



#### 4. Conclusion

Carotenoids in the algae *H. pluvialis* and *D. salina* and kavalactones in *P. methysticum* were selected as model systems to demonstrate the application potential of PFE in natural product extractions. The results of this study showed that PFE had comparable extraction efficiency to traditional extraction techniques. However, PFE required approximately half the amount of extracting solvent as traditional extraction, and about 20 min per sample as compared with 90 min or longer required with traditional techniques. PFE showed an advantage for the extraction of oxygen- and light-sensitive carotenoids and kavalactones. The PFE conditions can be readily optimized with simple *k* value determinations. Such a simple and quick optimization of PFE conditions allows for complete extraction by using a minimum amount of solvent in a short period of time, with minimum chemical alteration of labile molecules such as carotenoids. One would expect that PFE might be a powerful tool for extraction of a wide range of chemicals (from non-polar to polar) for metabolomic studies.

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