Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 464–470

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Bioactive phenols in algae: The application of pressurized-liquid and solid-phase extraction techniques

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ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 15 March 2009
Accepted 25 March 2009
Available online 5 April 2009

Keywords:
Phenols and aldehydes
Algae
Antioxidants
Benzoic and cinnamic acid derivatives

ABSTRACT

A new extraction technique based on the off-line combination of pressurized-liquid with solid-phase extraction (PLE–SPE) is described. The method was used for the extraction of bioactive phenolic acids (protocatechuic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, chlorogenic, vanillic, caffeic, *p*-coumaric, salicylic acid), cinnamic acid and hydroxybenzaldehydes (*p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, vanillin) from *in vitro* culture of two freshwater algae (*Anabaena doliolum* and *Spongiochloris spongiosa*) and from food products of marine macroalgae *Porphyra tenera* (nori) and *Undaria pinnatifida* (wakame). For the identification and quantification of the compounds the molecular ions $[M-H]^-$ and specific fragments were analyzed by quadrupole mass spectrometry analyzer connected on-line with a reversed-phase HPLC system. Our analysis showed that the freshwater algae and marine algal products contained submicrogram or microgram level of above-mentioned phenols per gram of lyophilized sample. In addition, the total phenol content (Folin–Ciocalteu assay) and antioxidant activity (TEAC assay, Trolox equivalent *antioxidant* capacity assay) of the PLE–SPE extracts were determined and discussed.

1. Introduction

Algae are important sources of various bioactive compounds, including phenols, with different physiological effects (toxic or curative) on human health. Many of them possess antioxidant, antimicrobial and antiviral activities that are important for the protection of algal cells against stress conditions. The discovery of new analytical methods and techniques is important for the study of metabolites in algae and similar organisms with respect to their applications in pharmacology and the food industry [1–4].

Applications of modern extraction and isolation techniques have been described by Herrero et al. [5]. Supercritical-fluid extraction (SFE), pressurized-liquid extraction (PLE) and microwave-assisted or solid-phase extraction (SPE) techniques, or their combination, were frequently used (for comparison of extraction efficiencies of these methods see recent papers [6–8]). In addition, high-speed counter-current chromatography provides an effective liquid/liquid partition technique that has been applied for metabolite isolation. These techniques have been applied to the extraction of amino acids, fatty acids, natural pigments, saccharides, vitamins, toxins, and other metabolites in cyanobacterial, micro- and/or macro-algal species [1].

We report here results of the extraction, identification and antioxidant activity analysis of phenolic compounds from selected freshwater (*Spongiochloris spongiosa*, *Anabaena doliolum*) and marine (*Porphyra tenera*, *Undaria pinnatifida*) algal material. Phenols are an important group of natural products with antioxidant and other biological activities. These compounds play an important role in algal cell defence against abiotic and biotic stress. Several authors have recently published results regarding the total phenol content and antioxidant activity of algae [9,10]. Halogen derivatives of *p*-hydroxybenzoic acid [11] were also identified in algae. Cinnamic acid esters (*n*-butyl 3,5-dimethoxy-4-hydroxycinnamate and isopropyl 3,5-dimethoxy-4-hydroxycinnamate) and methyl 3,4,5-trihydroxybenzoate were studied using ^1H and ^{13}C NMR in brown algae *Spatoglossum variabile* [12]. Some of the first polyphenols found in algae (*Fucus* and *Ascophyllum* spp.) were phlorotannins. They are formed from the oligomeric structures of phloroglucinol (1,3,5-trihydroxybenzene) [13]. Flavonoids or similar polyphenols have not so far been found in algae. However, the compounds with a flavonoid skeleton were analyzed by atmospheric pressure chemical ionization and ESI-mass spectrometry, and other methods in supercritical-fluid extracts of *Spirulina platensis* [14].

Abbreviations: ACN, acetonitrile; TEAC, Trolox equivalent antioxidant capacity; FC, Folin–Ciocalteu; PLE–SPE, combined pressurized-liquid and solid-phase extraction; SFE, supercritical-fluid extraction; HPLC, high-performance liquid chromatography; ESI-MS, electrospray-mass spectrometry; M, molecular ion $[M-H]^-$; TIC, total-ion chromatograms; SIM, single-ion monitoring.

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doi:10.1016/j.jpba.2009.03.027

The aims of this work are: (a) to develop new experimental procedure for the extraction of phenols from algal samples, (b) to perform the identification and quantification of particular phenols (phenolic acids, cinnamic acid and aldehydes) by chromatography and mass spectrometry in selected algal species, and (c) to determine the antioxidant activity and total content of phenolic compounds in methanolic extracts of algae.

2. Materials and methods

2.1. Chemicals

Standards of phenolic compounds were purchased from Sigma–Aldrich (St. Louis, MI, USA) and Fluka (Deisenhofen, Germany), as well as HPLC grade acetonitrile, methanol, acetic acid and NH_4OH. The standards were prepared by dissolution in methanol/2% acetic $acid_{aq}$ 90/10 (%, v/v); in concentration range from 5 ng ml^{-1} to 5 μg ml^{-1}. The solutions were stored in darkness at 4 °C. All solutions were filtered through a 0.45 μm Teflon membrane discs (MetaChem, Torrance, CA, USA) prior to HPLC analysis.

Chemicals for Folin–Ciocalteu and TEAC method [2,2′-azinobis-(3-ethylbenzothiazolin-6-sulfonate) diammonium salts] were purchased from Sigma–Aldrich; gallic acid monohydrate, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), Folin–Ciocalteu (FC) reagent were from Fluka Chemie (Buchs, Switzerland). Other chemicals of p.a. purity were purchased from Pliva-Lachema (Brno, Czech Republic). All reagents and standard solutions were prepared using Milli Q deionised water (Millipore, Bedford, USA).

2.2. Algae cultivation and preparation

Algal and cyanobacterial strains were obtained from the Culture Collection of the Center of Phycology, Institute of Botany, Třeboň, Czech Republic (*S. spongiosa* STARR, strain VISCHER 1942/318), and from the Culture Collection of the Institute of Soil Biology, South Bohemia University, České Budějovice, Czech Republic (*A. doliolum* BHARADWAYA, strain SINGH/CPB 758). Unialgal, but not necessarily axenic, cultures were determined by microscopic observation and re-purified by further plating if necessary, according to the methodology described earlier [15]. Cyanobacterial isolates were placed in a medium of Allen and Arnon [16], algal isolates were cultivated in a Šetlík and Simmer medium [17]. All strains were grown in 450 ml glass tubes through which a mixture of air and 2% carbon dioxide was bubbled at a constant temperature (28 °C) under low continuous illumination with banks of cool-white fluorescent lights of 50 W m^{-2} (Philips, Osram Dulux L, 55W/12-950). After two weeks of cultivation, the strains were harvested by centrifugation (Hettich 320 centrifuge) at 5000 rpm. The biomass of all strains was lyophilized (Lyovac GT3, Leybold-Heralens) and used for extraction. Dried marine algal material (as food products) *P. tenera* and *U. pinnatifida* were purchased from Sunfood and Country Life Ltd. (Czech Republic for both).

2.3. Extraction (PLE–SPE) procedure

2.3.1. PLE

All extractions were carried out using an extractor PSE-one from Applied Separations (USA). The extraction technique is based on a two-step elution. The first step was to eliminate water insoluble compounds and other pigments using the solvent hexane/acetone (1:1 v/v) and then the sample was re-extracted by 80% methanol in water for elution of phenolic compounds. Real samples (0.2 g) were packed into filter paper and placed into a 10 ml stainless steel extraction cell and extracted under controlled conditions. First step: preheating period (5 min), the solvent acetone/hexane (1:1 v/v); temperature 130 °C; pressure 130 bar; three cycles (each 5 min). Second step: the solvent 80% methanol in water (v/v); temperature 130 °C; pressure 130 bar; two cycles (each 10 min). The final PLE extract (80% methanol) was collected in glass vials with PTFE coated rubber caps.

2.3.2. Acid hydrolysis

The PLE extracts were evaporated to dryness in a rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (IKA-Werke) and then the samples were resolved in 10 ml 1 M HCl. The samples were hydrolyzed in a thermomixer (Eppendorf) at 25 °C, 1400 rpm for 30 min and then hydrolyzates were purified using SPE.

2.3.3. SPE

Hydrolyzed PLE-extracts were purified by SPE cartridge Oasis MCX 3cc (60 mg, particle size 38 μm). The cartridge was conditioned with 3 ml of methanol followed by 3 ml deionised water. The samples (10 ml for each) were passed through the cartridges at a flow rate of 50 μl s^{-1}. The cartridge was then rinsed using 2% acetic acid in a 5% methanol solution. The retained analytes were subsequently eluted using aqueous methanol solutions (fractions: 5%, 10%, 15%, 20% methanol, 2 ml for each) with 2% NH_4OH. Prior to the analyses all fractions were collected and evaporated to dryness in a rotary vacuum evaporator (see above), dissolved in 500 μl of mobile phase and injected directly into the HPLC/MS system. Blank extractions were performed to test analyte residues in the system.

2.4. HPLC/electrospray-mass spectrometry

The HPLC chromatographic system HP 1100 (Hewlett Packard, Waldbronn, Germany) equipped with a vacuum degasser, a binary pump, an autosampler, a column thermostat and a diode array detector was used. The system was coupled on-line to an MS detector.

The Zorbax SB-C_{18} chromatographic column (150 mm × 4.6 mm, 3.5 μm, Agilent Technologies, USA) was used. The injection volume was 0.5–10 μl for standard solutions and 25 μl for real sample extracts. The mobile phase consisted of 0.2% acetic $acid_{aq}$ (solvent A) and acetonitrile (solvent B). A linear gradient elution was applied as follows (%, v): from start to 4 min (4% B), from 4 to 6 min (17% B), from 6 to 10 min (30% B), and from 10 to 15 min (4% B). Flow rate was 1.1 ml min^{-1} and the temperature of the column oven was set at 30 °C.

The column effluent was directly introduced into a single quadruple mass-selective HP MSD detector (Hewlett-Packard, Palo Alto, USA) operated in a negative ESI mode. The nebulizer gas pressure was 50 psi, the drying gas was nitrogen at 13 l min^{-1}, the temperature was 350 °C and the capillary voltage was 4 kV. Additional structural information was obtained by detecting diagnostic product ions produced by in-source CID (collision-induced dissociation) of the precursor ion. For the in-source CID, the fragmentor voltage was set to 100 V for most of the analytes. Both aldehyde analytes were stable at 100 V and a higher fragmentor voltage (130 V) was used to produce confirm ions of these compounds.

2.5. Determination of total phenolic compounds in algae extracts

FC assay, based on the reduction of a phosphowolframate–phosphomolybdate complex by phenolics to blue reaction products, was used to determine phenolic compounds [18,19]. Absorbance was measured three times for each sample at 760 nm against blank (water instead of sample) using gallic acid as standard. Five point calibration was linear to concentration 0.2 mM in the reaction mixture.

Table 1
Analyzed compounds and their retention times and quantitative parameters.

No.	Compound	t_R (min)	Molecular ion $[M-H]^-$ (m/z)	Confirm ion (m/z)	R^2	Calibration equation	LOD (ng ml^{-1})	LOQ (ng ml^{-1})
1	Protocatechuic acid	3.4	152.9	109.1	0.999	$y = 64421x - 1239$	0.02	0.07
2	3,4-Dihydroxybenzaldehyde	4.7	137.2	93.1	0.998	$y = 111709x - 12485$	0.01	0.04
3	*p*-Hydroxybenzoic acid	4.9	137.2	93.1	0.998	$y = 36723x - 2524$	0.04	0.12
4	2,3-Dihydroxybenzoic acid	5.0	153.2	109.1	0.999	$y = 64621x + 6598$	0.02	0.07
5	Chlorogenic acid	5.2	353.3	190.9	0.999	$y = 88459x - 6635$	0.02	0.07
6	Vanillic acid	5.8	167.2	122.3	0.998	$y = 25606x - 2038$	0.06	0.18
7	Caffeic acid	6.1	179.2	135.1	0.999	$y = 103210x - 2490$	0.015	0.04
8	*p*-Hydroxybenzaldehyde	6.4	121.2	93.1	0.999	$y = 134103x - 1942$	0.01	0.03
9	Vanillin	7.6	151.2	107.9	0.999	$y = 53977x + 1630$	0.03	0.08
10	*p*-Coumaric acid	7.8	163.2	119.1	0.999	$y = 89486x - 742$	0.017	0.05
11	Salicylic acid	8.9	137.2	93.1	0.999	$y = 99789x - 7539$	0.015	0.05
12	Cinnamic acid	5.4[a]	147.2	103	0.998	$y = 25150x - 3308$	0.06	0.18

[a] Condition (gradient elution profile) for separation of cinnamic acid was modified, see Section 3.1.

2.6. Trolox equivalent antioxidant capacity (TEAC) method

A working solution was diluted to absorbance values between 1.0 and 1.5 AU at 734 nm with phosphate buffer solution (constant initial absorbance values must be used for standard and samples). Standards or studied extracts (from 25 to 50 μl according to reaction intensity) were mixed with the working solution (900 μl) and adjusted to 1000 μl with deionised water. The decrease in absorbance was measured at 734 nm after 20 min. For other details see [20].

2.7. Recovery and reproducibility

Recovery was evaluated with model standard solutions of phenols. The algal samples (1 g, *S. spongiosa*) were spiked with the standards (0.3, 1, and 3 μg) prior to extraction; for other details see [21]. Intra-day HPLC/ESI-MS reproducibility was verified on standard solutions with concentrations close to those expected in real samples. A series of analyses ($n = 6$) was started at 7:00, 9:00 and 11:00 AM and at 1:00, 3:00 and 5:00 PM. The post-time was slightly shortened to allow 6 analyses to be performed during a 2-h period. Similarly, a series of six analyses was measured for six consecutive working days at 9:00 for determination of the inter-day reproducibility.

3. Results

In this paper, we describe results from the analysis of phenolic compounds in PLE–SPE extracts prepared from *in vitro* cultures and the food products of the algae. The selected benzoic and cinnamic acids derivatives, *p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde and vanillin (4-hydroxy-3-methoxybenzaldehyde) were investigated (Table 1). First, we optimized the HPLC/ESI-MS system and PLE–SPE extraction conditions for analysis of these compounds.

3.1. Separation and mass spectrometry of phenols

For analysis of phenols reversed-phase HPLC with electrospray mass spectrometry detector (ESI-MS) was used. Linear gradient elution was carried out with an acetonitrile (ACN) and 0.2% acetic acid aqueous solution. The procedure (Section 2.4) could be used for simultaneous separation of phenols (no. 1-11) whose numbers and retention times (at column temperature 30 °C and mobile phase flow rate 1.1 ml min^{-1}) are shown in Table 1. Only in the case of cinnamic acid (for its separation a higher content of an organic modifier in mobile phase is useful) it was necessary to adjust the linear gradient profile: 0–7 min (20–46% ACN), 7–15 min (46–20% ACN).

The effluent was introduced on-line into an ESI-MS detector operating in negative mode. The compounds were identified by quasi-molecular ions $[M-H]^-$ and specific products of fragmentation, usually $[M-CO_2]^-$ (Table 1). Our optimized method could be used for the detection of sub-nanogram amount of phenols per ml of analyzed sample. The average limit of detection (LOD; 3.S/N) 26 pg ml^{-1} and limit of quantification (LOQ; 10.S/N) 82 pg ml^{-1} of studied compounds were obtained. The best values of LOD (10 pg ml^{-1}) and LOQ (30 pg ml^{-1}) were found for *p*-hydroxybenzaldehyde. The calibration curves of all compounds were linear in concentration range from 0.5 to 30 μg ml^{-1} with correlation coefficients (R^2) in the interval 0.998–0.999. Calibration equations and other qualitative and quantitative parameters are shown in Table 1.

Finally, we tested HPLC/ESI-MS reproducibility ($n = 6$) for all compounds (experimental procedure is described in Section 2.7 and in our previous paper [22]). Excellent average 99.9% reproducibility was found for intra-day and inter-day measurements. Relative standard deviations (R.S.D.) of intra-day and inter-day reproducibility tests varied in the ranges 0.97–3.85% and 0.79–4.31%, respectively.

3.2. PLE–SPE extraction of phenols

For the extraction of phenolic compounds in samples of algae the PLE technique was used. The real samples (original or with addition of phenol standard) were packed into filter paper and placed into a PLE extraction cell. We used the following two-step strategy for phenol and aldehydes extraction. Firstly, the water insoluble compounds and different algal pigments were eluted by cyclic PLE extraction with a mixture of acetone and hexane (1:1 was the optimal v/v ratio). Three extraction cycles were applied (time of one cycle: 5 min). After this, an extraction of more polar compounds with an 80% aqueous methanol solution was performed (see Section 2.3). Two extraction cycles (time of one cycle: 10 min) for this re-extraction were applied. The majority of the analyzed phenols indicated a best extraction recovery at pressure 130 bar and temperature 130 °C; 80% methanol as extraction modifier was used for all experiments. Acquired PLE extracts were hydrolyzed by 1 M hydrochloric acid. For final purification of PLE extracts an SPE cartridge with a sorbent based on ion-exchange/reversed-phase copolymer containing SO_3^- groups was applied. Phenols presented in acid hydrolyzates were retained on the sorbent surface and then eluted using 2% ammonium hydroxyde.

Prepared PLE–SPE extracts were analyzed by the HPLC/ESI-MS procedure described in the previous section. The system of combined PLE and SPE allowed extraction of the compounds with an average 96% extraction recovery. The extraction recoveries (the algal samples (1 g) were spiked with the 0.3 μg of standards) were in the interval from 93% to 98% with average R.S.D. 3.1% for $n = 6$

Table 2
PLE–SPE recovery ($n = 6$). For other details see Section 2.7.

Compound	Recovery [%]	R.S.D. [%]
Protocatechuic acid	96.9	2.6
3,4-Dihydroxybenzaldehyde	97.8	3.4
p-Hydroxybenzoic acid	97.3	1.0
2,3-Dihydroxybenzoic acid	97.6	2.6
Chlorogenic acid	94.3	6.3
Vanillic acid	93.8	4.0
Caffeic acid	93.3	2.8
p-Hydroxybenzaldehyde	97.8	2.8
Vanillin	95.3	5.1
p-Coumaric acid	93.5	1.8
Salicylic acid	98.3	2.6
Cinnamic acid	93.9	3.1

(Table 2). The amount of 0.2 g of lyophilized algal material was sufficient for the analysis.

3.3. Identification and quantification of phenols in algae

The identification and quantification of phenols in algae samples were based on results presented in Section 3.1 and proposed PLE–SPE extraction procedure (Section 3.2). Phenolic content was determined in freshwater cyanobacterium (*A. doliolum*) and microalgae (*S. spongiosa*). In addition, we analyzed phenols in food products from marine macroalgae nori and wakame (prepared from species *P. tenera* and *U. pinnatifida*). In PLE–SPE extracts, low amounts (submicrogram or microgram) of phenols per gram of lyophilized sample were found (Table 3).

The sum of the amounts of phenols identified in the cyanobacterium was 3.6 $\mu g\,g^{-1}$. However, in the case of *S. spongiosa* extracts, a higher amount was confirmed (around 5.1 $\mu g\,g^{-1}$). The extracts prepared from algae nori and wakame contained rather less amounts of phenols than *in vitro* algae culture. The sum of phenol amounts in wakame and nori were 1.05 and 1.9 $\mu g\,g^{-1}$, respectively. High concentrations of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were found in the algal species, by contrast, a very low concentration was observed for cinnamic acid derivatives. In PLE–SPE extracts, all phenols, except for vanillic acid and vanillin, were identified. All phenols were found only in extracts of *S. spongiosa* (Table 3). Vanillic acid was identified in extracts prepared from *A. doliolum* or *S. spongiosa* and vanillin was observed only in extracts of *S. spongiosa*. Total-ion chromatograms (TIC) of single extracts of algae are presented in Fig. 1. For the identification and quantification of phenols single-ion monitoring mode (SIM) was used. The two m/z values ($[M-H]^-$ and specific product of fragmentation) were used for this purpose. MS spectra and SIM chromatograms of fundamental compounds in phenols metabolism (cinnamic, *p*-coumaric and salicylic acid, see below) measured in PLE–SPE extracts of *A. anabaena* are presented in Figs. 2 and 3. The MS spectra (Fig. 2) were obtained in peaks of phenols from the TIC chromatograms in Fig. 1.

3.4. Total phenol content and antioxidant activity of extracts

In subsequent experiments, we focused our interest on the observation of the total content of phenolic compounds (analyzed using Folin–Ciocalteu assay, Section 2.5) and antioxidant activity (analyzed using TEAC method, Section 2.6) in PLE–SPE extracts. The reactivity of phenol standards was studied according to the previously published methodology [23] before real sample measurements. It was confirmed that individual phenols provided different reactivity (quantitative response) for FC and TEAC assay. Enhanced responses were observed especially for phenols with a higher number of hydroxy (–OH) groups per benzene ring. Reactivity could be higher for compounds with hydroxy group in *meta*

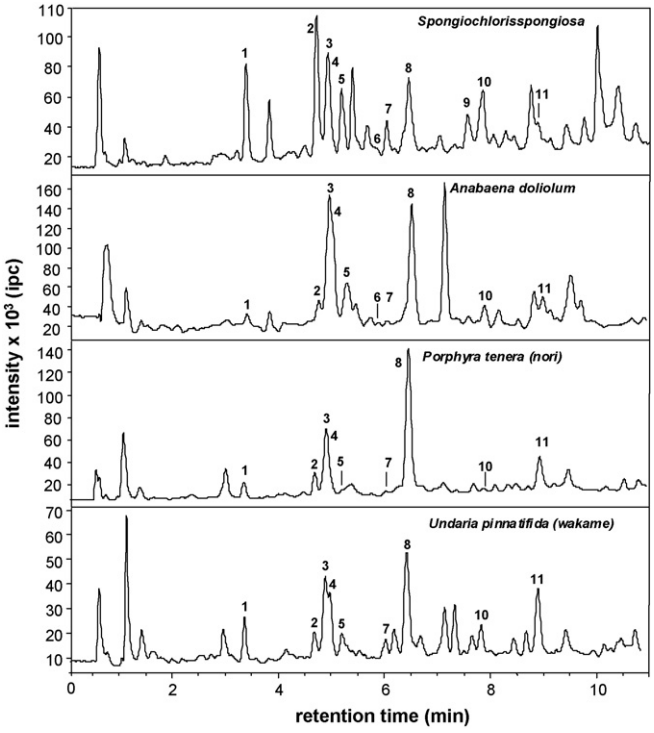

Fig. 1. Chromatograms of selected algae (detected in TIC mode). For peak numbers see Table 1.

and *para* positions as well as cinnamic acid derivatives. The 3,4-dihydroxybenzaldehyde, chlorogenic (five –OH groups) and caffeic acids (two –OH groups) yielded relatively high responses. On the other hand, no reactivity was observed for cinnamic acid (not containing –OH groups) and very low reactivity was measured in the case of salicylic acid with only one –OH group in *ortho* position (Fig. 4a and b).

The results of the content of total phenols and antioxidant activity of PLE–SPE extracts are shown in Fig. 4c. The measured values

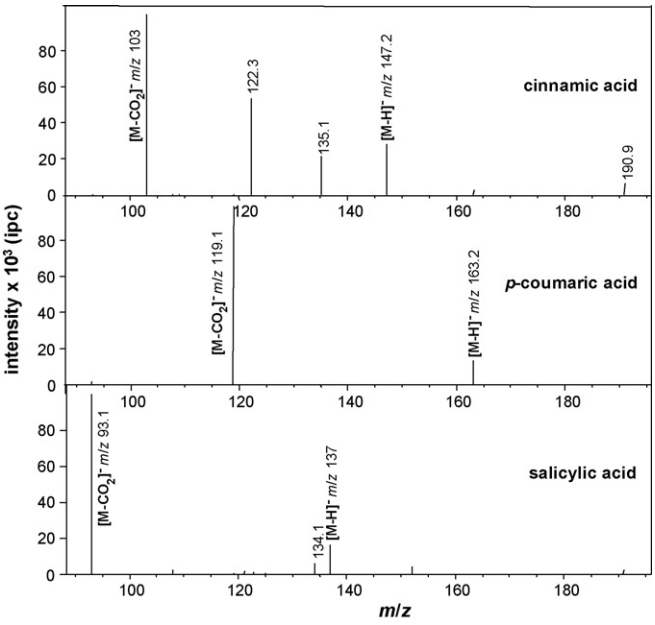

Fig. 2. ESI-MS spectra of cinnamic, *p*-coumaric and salicylic acid in PLE–SPE extract from *A. doliolum*. Fragmentor voltage (ESI negative mode): 100 V for *p*-coumaric acid and 130 V for others; for details see Section 2.4.

Table 3
Content of compounds in studied algae.

Compound	*Spongiochloris spongiosa* (ng g^{-1})	*Anabaena doliolum* (ng g^{-1})	*Porphyra tenera* [nori] (ng g^{-1})	*Undaria pinnatifida* [wakame] (ng g^{-1})
Protocatechuic acid	657 ± 3.6	152 ± 3.6	169 ± 4.7	175 ± 1.8
3,4-Dihydroxybenzaldehyde	583 ± 3.1	109 ± 9.1	132 ± 7.0	60 ± 3.8
p-Hydroxybenzoic acid	788 ± 3.4	760 ± 0.9	690 ± 1.1	211 ± 1.0
2,3-Dihydroxybenzoic acid	210 ± 1.9	780 ± 1.4	149 ± 6.4	98 ± 2.3
Chlorogenic acid	260 ± 5.3	82 ± 6.6	19 ± 11.9	10 ± 11.9
Vanillic acid	73 ± 1.4	92 ± 5.8	-	-
Caffeic acid	145 ± 5.8	17 ± 11.6	8 ± 12.1	16 ± 10.3
p-Hydroxybenzaldehyde	1800 ± 0.9	1460 ± 3.5	85 ± 1.8	155 ± 1.1
Vanillin	248 ± 1.3	-	-	-
p-Coumaric acid	211 ± 1.1	45 ± 4.7	25 ± 6.7	17 ± 8.2
Salicylic acid	108 ± 2.3	44 ± 6.2	530 ± 1.3	226 ± 1.5
Cinnamic acid	73 ± 3.8	61 ± 8.4	104 ± 1.6	86 ± 3.8

Value ± relative standard deviation in %.

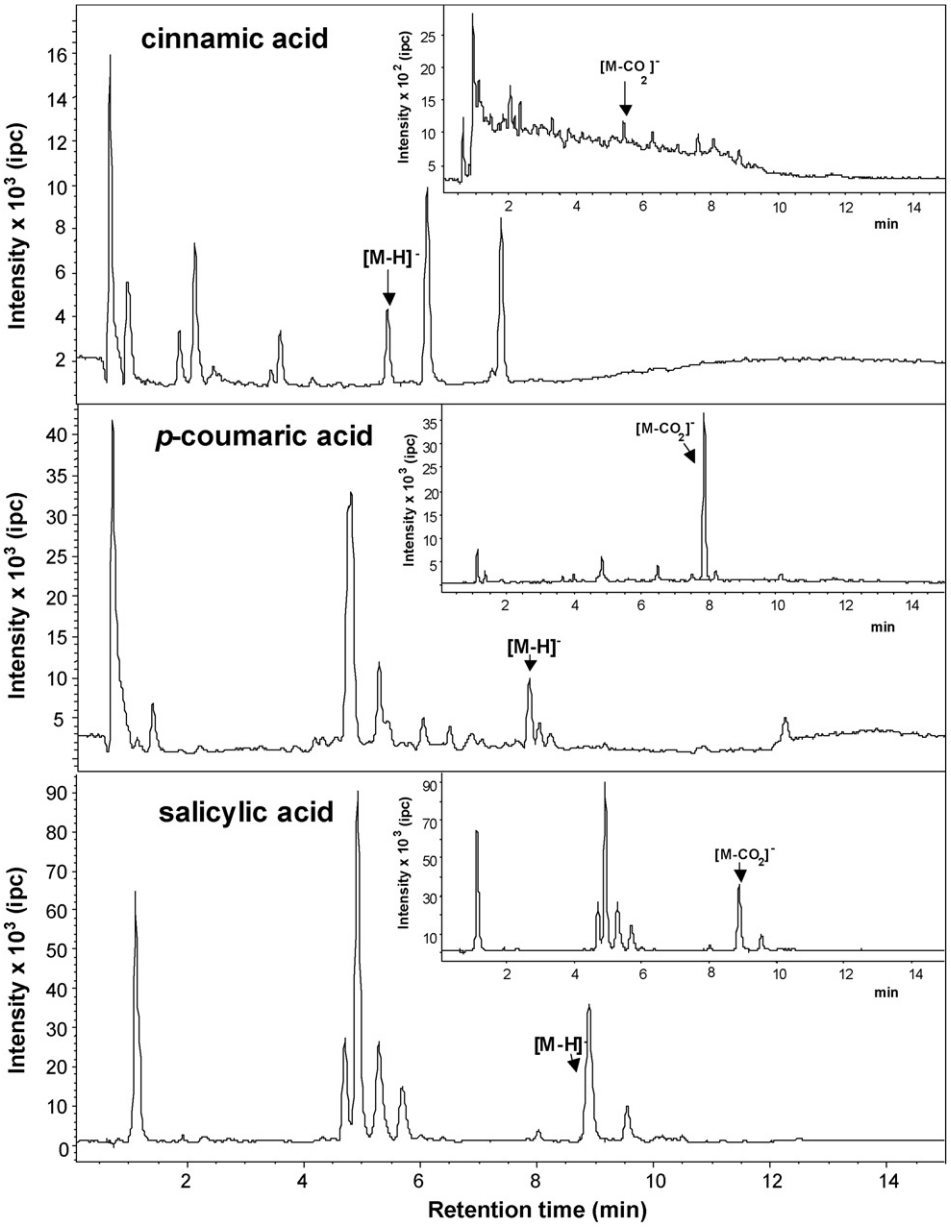

Fig. 3. SIM chromatograms (molecular ions $[M-H]^-$ and fragments $[M-CO_2]^-$) of cinnamic, *p*-coumaric and salicylic acid in PLE–SPE extract from *A. doliolum*. For other details see Fig. 2.

Table 4
Antioxidant activities of PLE and PLE–SPE algal extracts; $n = 3$.

Extracts	*Spongiochloris spongiosa* (μmol g^{-1})	*Anabaena doliolum* (μmol g^{-1})	*Porphyra tenera* [nori] (μmol g^{-1})	*Undaria pinnatifida* [wakame] (μmol g^{-1})
PLE	23.1	27.5	12.5	13.8
PLE–SPE	25.7	26.3	22	19.3

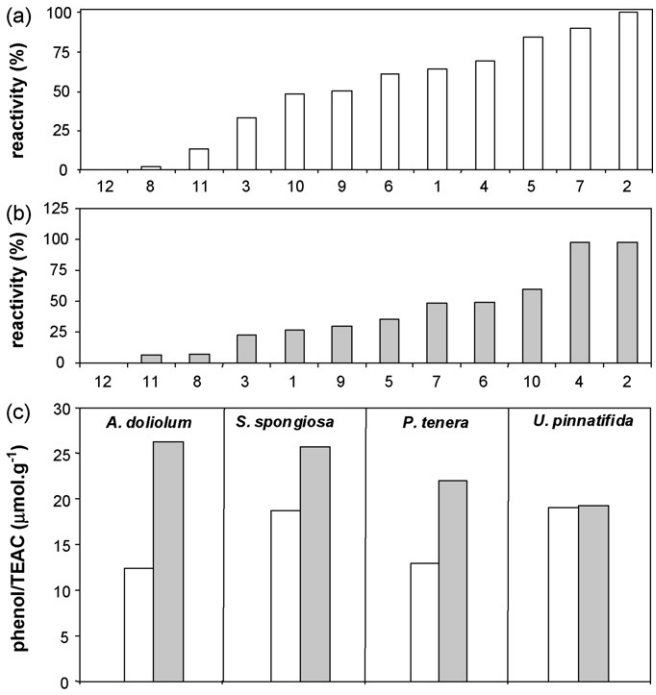

Fig. 4. Comparison of reactivity of compounds using FC (a) and TEAC test (b). Results of total phenol content (FC test) and antioxidant activity (TEAC) measurement in PLE–SPE extracts (c). For number of phenols see Table 1. Values 24.6 mA (for a) and 97.8 mA (for b) corresponding with 100% reactivity; $n = 3$.

are calculated as equivalent of gallic acid and Trolox for FC and TEAC assays, respectively. Concentrations of total phenolic compounds in lyophilized algal samples were in the interval from 10 to 20 μmol g^{-1}.

In the case of TEAC test, antioxidant activity of PLE–SPE extracts varied between 20 and 25 μmol g^{-1}. Significant differences for antioxidant activity of extracts of *in vitro* algae cultures and marine food products were not observed. Furthermore, it was proved that the antioxidant activity of PLE–SPE extracts is similar to the antioxidant activity of PLE extracts (especially for freshwater algae, Table 4) that were not hydrolyzed and purified by SPE procedure; for extracts preparation see Section 2.3. We concluded that the analyzed phenols formed an important part of antioxidant compounds in methanolic PLE and PLE–SPE extracts.

4. Discussion

Different methodologies for natural products (including phenols) analysis in algae and other biological materials were reported by many authors [24–26]. Most of them used the Folin–Ciocalteu assay for total phenols analysis (e.g. in cyanobacterium *Nostoc commune* [27]). There is not yet sufficient information either on individual phenols and hydroxybenzaldehydes in algae or about their physiological function in the human organism.

The phenols studied in this work, especially cinnamic and *p*-coumaric acid, are crucial precursors in the synthesis of different bioactive phenols (usually polyphenols) in photosynthetic organisms. The biosynthesis of cinnamic acid from phenylalanine is controlled by L-phenylalanine ammonia-lyase. This enzyme was found for the first time in cyanobacteria (*Anabaena variabilis* and *Nostoc punctiforme*) and characterized using X-ray crystallography in 2007 [28]. Simple phenols were previously studied using a special solid-phase/supercritical-fluid extractor in *S. spongiosa* and cyanobacterial strains (*S. platensis, A. doliolum*, *Nostoc* sp., and *Cylindrospermum* sp.) [22]. According to previously published results and our experiments presented here, a group of nine phenolic acids (protocatechuic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, vanillic, syringic, caffeic, *p*-coumaric, salicylic, and chlorogenic acid) were found in freshwater cyanobacteria and algae. On the basis of these results, it is probable that similar phenylalanine dependent biosynthesis [29] of simple phenols occurs in cyanobacteria and algae as well as in higher plants. However, the concentrations and spectrum of phenols in algae could be different in comparison to terrestrial plant taxonomic groups. L-Phenylalanine ammonia-lyase distribution and its activity in different cyanobacteria and algae need to be investigated in more detail to confirm this hypothesis. In addition, salicylic acid which participates in regulation of induced plant resistance to pathogens was also identified in PLE–SPE extracts. The biosynthetic pathway of cinnamic acid derivatives, including salicylic acid, from phenylalanine and shikimic acid (well-known in higher plants) is presented in [30]. Our results demonstrate that phenols are *in vivo* synthesized in lower concentration levels in freshwater cyanobacteria than in the case of freshwater algae (see Table 3 and Ref. [22]). The higher amount of phenols in algae as compared to cyanobacteria is probably due to the fact that algae (eukaryotic organisms) are more evolutionary advanced organisms than cyanobacteria (prokaryotic organisms). Algae probably have more developed phenol-based metabolic pathways than cyanobacteria.

Phenolic compounds fall into group of bioactive components of photosynthetic organisms and participate in the antioxidant activity of pharmaceutical and food products. The antioxidant activities of algae extracts were recently studied by several authors by TEAC and other methods [9,10,31,32]. We reported here the antioxidant activity of PLE–SPE extracts containing simple phenols and possibly compounds with similar physicochemical properties. The fraction of pigments and other non-polar compounds was excluded from PLE–SPE extracts (it is described in Section 2.3). Relatively high antioxidant activities were observed since extracts containing only group of selected compounds.

Not only phenolic acids but also derivatives of benzaldehydes (quantitatively: *p*-hydroxybenzaldehyde $\gg$ 3,4-dihydroxybenzaldehyde > vanillin) were identified in PLE–SPE extracts (see Section 3.3 and Table 3). Benzaldehyde and selected volatile organic compounds were previously analyzed by gas chromatography–MS in pentane extracts prepared from cyanobacterium *Oscillatoria perornata* [33]. In addition, *p*-hydroxybenzaldehyde was found in the red alga *Corallina pilulifera* using MS and NMR methods [34]. Bromoaldehydes 3-bromo-4,5-dihydroxybenzaldehyde and 3,5-dibromo-4-hydroxybenzaldehyde have recently been analyzed in the red alga *Polysiphonia urceolata* [35]. Finally, it was confirmed that aldehydes (or their halogen derivatives) are natural antioxidant constituents of cyanobacteria and algae [35], which is in good agreement with our results.

5. Conclusion

Algae represent a source of interesting natural compounds for human nutrition (Japanese people are the main consumers [36]). The application of new extraction strategies and analytical methodologies applicable for the study of these components of algae is one of the main goals in current natural compounds research.

In this paper we have described a new method for quantitative extraction of phenols in algae. The results showed the possibilities of the application of PLE–SPE extraction and HPLC/ESI-MS analysis of phenolic compounds in selected algal species in sub-nanomolar concentrations (corresponding to the sub-nanogram level). Using optimal extraction conditions, the average recovery for studied phenols was 96%. In addition, the antioxidant activity analysis indicated that algae PLE–SPE extracts could be used as a source of antioxidants. We concluded that our proposed extraction procedures can be useful for the rapid extraction of bioactive phenols in various cyanobacteria or algae materials and their food products.

Acknowledgments

This study was supported by grant no. 525/07/0338 from Grant Agency of the Czech Republic. Partial funding was also provided by project MSM6007665808 of the Ministry of Education, Youth and Sports of the Czech Republic. The authors also thank Dr. A. Lukešová for technical support and for her kind providing of some algal strains.

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