THE USE OF µELUTION PLATE AS A PART OF HYPHENATED EXTRACTION METHOD FOLLOWED BY RRLC-MS-MS ANALYSIS FOR THE ISOLATION AND DETERMINATION OF PHENOLIC COMPOUNDS IN SEA ALGAE

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ABSTRACT

Development and aplication of µElution plate for the extraction of phenolic compounds from sea algae and their analysis using RRLC/MS/MS is described. The extraction and identification of phenolic compounds is presented from five different sea algae samples, two brown algae *(Cystoseira abies* + *Cystoseira abies* dried with nitrogen , *Undaria pinnatifida*) and two red algae (*Sargassum muticum*, *Chondrus crispus*) via solid phase extraction (SPE) using Oasis µElution plate. Selected groups of benzoic acid derivatives (*p*-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acid), hydroxybenzaldehydes (4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde) and cinnamic acid derivatives (*o*-coumaric, *p*-coumaric, caffeic, ferulic, sinapic and chlorogenic acid) were investigated.

Recoveries in range 96–100% were obtained, with LOQs 0.01–2.1 pg/inj and LODs 0.03–7.1 pg/inj, i.e. in the range of low µm. The µSPE enabled to avoid the evaporation step and pre-concentrate the analytes directly. The applied method allowed a simultaneous determination of phenols in less than 5 minutes. Thus, the analysis of different plant species containing trace amounts of polar phenols became possible.

Phenolic compounds contained in algae were extracted using Oasis µElution plate for its increased sensitivity and pre-concentration effect – extract volume is only 500 µl. Robotic mechanism of the HPLC instrument is able to use the µElution plate trapping part directly and take samples from it.

Key words: µElution plate, hyphenated techniques, sea algae, phenolic compounds

Acknowledgments: The work has been supported by grants No. 525/07/0338 and 525/08/P540 from Czech Science Foundation and by the grant of the IGA AF Mendelu in Brno no. TP 1/2010. Merichel Plaza thanks CSIC for her I3P fellowship.

INTRODUCTION

In the last years, the development of a systematic, high-throughput analysis that optimizes sample preparation and chromatography to minimize matrix effects in bioanalytical LC/MS/MS assays is increasingly to the fore of interest. The recent introduction of the Waters Oasis µElution SPE plate and Rapid Resolution Liquid Chromatography-Tandem Mass Spectrometry Analysis has become widely spread technique and new trend in separation sciences. The advantages of Oasis µElution plate for the rapid isolation of analytes from complex matrices are that it can perform for clean-up and pre-concentration with very small sample volumes. Produced extracts can be directly injected, eliminating the time-consuming evaporation and reconstitution steps. Reversed-phase and polymeric mixed-mode SPE sorbents (both reversed phase and ion exchange retention mechanisms) produce cleaner extracts and reduce matrix effects without losing the searched analytes. One of the aims of the methodology was to advance various solid phase extractions and selected the one with the best reproducible and recovery results in quantifying a high range of phenolic compounds from different sea algae. Comparisons were made among several sorbents, including mixed-mode sorbents and SampliQ sorbents. First, in hyphenated series of experiments the procedures of samples preparation on Ika Ultra-Turrax® Tube Drive, the extraction techniques pressurized-liquid extraction (PLE) and ultrasound-assisted extraction are described. Used techniques were the off-line combinated with Oasis µElution plate.

Phenolic compounds are one of the most widely occurring groups of bioactive compounds and algae can be their a very interesting natural source. Phenols are classified as secondary metabolites derivates of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants [9]. These compounds are considered to have important physiological and morphological roles in plants; they also play an important role in growth and reproduction, providing protection against pathogens and predators [2]. They can provide physiological benefits, such as anti-artherogenic [6], anti-inflammatory [7], antimicrobial [11], antioxidant [8], anticarcinogenic [7] effects, among others [3, 10]; for instance, they have been associated with the health benefits derived from consuming high levels of fruits and vegetables, which have been attributed to their antioxidant activity [1]. Nowadays, there has been a growing interest in research, development and commercialization of algae as functional food ingredients. These materials are preferred by consumers to have a natural origin (i.e. non-synthetic origin) being commonly extracted from natural sources, such as macroalgae and microalgae, plants and other organisms. Algae contain a secondary metabolism products that induse physiological effects in mammals including human. Many of them posses antioxidant, antimicrobial and antiviral activities. In fact, several researchers have reported the relationship between phenolic content and the antioxidant capacity of micro- and macro-algae [4, 5,

8]. The discovery of new extraction techniques and analytical methods is important for the study of metabolites in algae and similar organisms with respect to their applications in the pharmacology and the food industry. The goal of this work was to report results of methodology available to concentrate analytes using Oasis µElution plate, identification and quantitative LC/MS/MS determination of phenolic compounds from marine algal material.

MATERIALS AND METHODS

Chemicals, standards and solvents

Standards of phenolic compounds were purchased from Sigma-Aldrich (St. Louis, MI, USA) and Fluka (Deisenhofen, Germany). Hydrochloric acid and ammonia were purchased from Penta. Acetic acid was purchased from Fluka and ascorbic acid from Sigma-Aldrich. HPLC grade acetonitrile, methanol and other organic solvents were obtained from Sigma–Aldrich (St. Louis, MI, USA). The standard of phenolic compounds was prepared by dissolution in acetonitrile (5%, v/v) with 0.2% acetic acid aq (95%, v/v). The solutions were stored in darkness at 4 ◦C. All reagents and standard solution were prepared using MilliQ deionised water (Millipore, Bedford, USA).

Real samples

Sea algae samples (Sargassum muticum, Undaria pinnatifida, Chondrus crispus and Cystoseira abies) were obtained from the Laboratory of Foodomics, Institute of Industrial Fermentations, Madrid, Spain. All real samples were obtained in lyofilized and homogenized form in more than sufficient quantity.

Extraction techniques

Ika Ultra-Turrax® Tube Drive

50 mg of algae with 20 mg ascorbic acid and 5 ml aqueous methanol (80%, v/v) was extracted on Ika Ultra-Turrax® Tube Drive, filtered and concentrated to 1 ml on rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (IKA-Werke). Extracts were used for series of alcalic and acid hydrolysis. The produced extracts were purified using Oasis µElution plate.

The same algae samples were sonicated 1 min before extraction on Ika Ultra-Turrax® Tube Drive, though two series of samples were gained. One of them without and one with usage of ultrasound.

Pressurized liquid extraction (PLE)

This technique has received different names, such as pressurized liquid extraction (PLE), accelerated solvent extraction (ASE), and pressurized solvent extraction (PSE).

Pressurized liquid extraction (PLE) is a registered technique that combines elevated temperature and pressures with liquid solvents to achieve efficient and fast extraction. This procedure can be used for the analytes from the solid and semi-solid samples matrix.

The instrument, an extractor PSE-one from Applied Separations (USA) was used for pressurized solvent extraction of phenolic compounds in algae. The extraction technique was based on a two-step elution with aqueous methanol (80 %,v/v). First 50 mg of sample of alga with 20 mg ascorbic acid and 300 µL aqueous methanol (90%, v/v) was sonicated 15 min and than packed into a filter paper and placed into a 10 ml stainless steel extraction cell. The extraction was conducted under the following condition: pre-heating period: 5 min; the solvent 80 % methanol in water (v/v); extraction volume: 10 ml; temperature: 130 °C; pressure: 130 bar; static time: 10 min; 1 min using pressurized nitrogen; static cycle: 2. Both the PLE extracts were collected in glass vials with PTFE coated rubber caps.

Extracts were concentrated to 1 ml and used for series of alcalic and acid hydrolysis.

Ultrasound-assisted extraction

50 mg of sample of algae with 20 mg ascorbic acid were sonicated 1, 2, 3, 4, 5, 8 and 10 min with 1 ml aqueous methanol (80%, v/v) at room temperature using a Bandelin Sonopuls - Ultrasonic homogenisers (Bandelin GmbH & Co. KG, Germany). The obtained extracts were refilled to 5 ml, filtered and concentrated to 1 ml. Extracts were used for series of alcalic and acid hydrolysis. The best results were recognized with 8 min of sonication.

Stationary methanol samples, passive leaching extraction

50 mg of sample of algae with 20 mg ascorbic acid and 5 ml aqueous methanol (80%, v/v) was extracted for 10 min at 5°C. The obtained extracts were filtered and concentrated to 1 ml. Extracts were used for series of alcalic and acid hydrolysis.

The same algae samples were sonicated 1 min before 10 min extraction at 5°C. The obtained extracts were filtered and concentrated to 1 ml. Extracts were used for series of alcalic and acid hydrolysis.

Alcalic and acid hydrolysis

This method was modified according to method used by Ayaz (2007). Samples concentrated under vacuum were acidified by 6 M HCl to pH 2 and refilled with MilliQ water onto 4,5 ml. The aqueous phase was divided into three aliquots, one of which was hydrolysed with 2 M NaOH for 4h under a nitrogen atmosphere at room temperature. After acidification to pH 2 (6 M HCl), phenolic acids released from the soluble esters were extracted at µElution plate. To the second aliquot, 6 M HCl was added and the medium was hydrolysed under a nitrogen atmosphere for 1h in a boiling water bath. Phenolic acids released from soluble glycosides were also separated at µElution plate. The third aliquot was extracted at µElution plate and free phenolic acids were obtained.The solid residue obtained after filtration was dissolved in 2 M NaOH for 4h and, after acidification to pH 2

(6 M HCl), phenolic acids released from methanol-insoluble ester-bound acids were extracted at µElution plate.

Oasis µElution plate

Discovery Oasis µElution plate with different cartridges were used for the efficient extraction of phenolic compounds from the sea algae. The used cartridges were filled with a macroporous copolymers with reversed-phase capability. The specification of five different sorbents in SPE cartridges is presented in **Chyba! Nenalezen zdroj odkazů.**. The interaction between phenolic compounds and *N*-vinylpyrrolidone-divinylbenzene copolymer is based on reversed-phase and mixed mode exchanges mechanisms. For optimization the isolation of the substances the elution study was performed. The elution study allowed to find the best conditions in individual washing and elution steps for the maximum separation selectivity of analytes. Selection of pH was assayed because retention of an analyte depends on the acidic/basic character of a substance and organic solvent concentration. Both parameters were combined to optimize the method. The elution procedure was applied as follows: The sorbents were conditioned with 50 µl 100% methanol and equilibrated with 50 µl MilliQ water than standard solution (500 µl) diluted with 2 M HCl was applied. Interferences at MCX, WAX and HLB were washed with aqueous methanol (%, v/v) with the increasing methanol content (5 – 100% methanol) containing 2% acetic acid. The analytes of interest were eluted with aqueous methanol (%, v/v) with the increasing methanol content (5 – 100% methanol) containing 2% ammonium hydroxide. For MAX and WCX plates wasch and elution solutions were swapped. One of the results of the elution studies for the phenolic compounds are presented in Fig. 1 and Fig. 2. All fractions were collected in the collection plates and directly injected into the RRLC-MS/MS system. Blank extractions were performed to test analyte residues in the system. Developed, optimized, and validated extraction method using Oasis µElution plate was applied for real samples.

Tab. 1 Different sorbents in Oasis Elute Plate

Commercial name	Sorbent type	Particle size/sorbent weight	Supplier
HLB	N-vinylpyrrolidone-divinylbenzene copolymer	30 µm/2 mg	Waters
MCX	Mixed mode Cation-eXchange and reversed-phase (N-vinylpyrrolidone-divinylbenzene copolymer)	30 µm/2 mg	Waters
MAX	Mixed-mode Anion-eXchange and reversed-phase sorbent	30 µm/2 mg	Waters
WCX	Mixed-mode Weak Cation-eXchange and reversed-phase sorbent.	30 µm/2 mg	Waters
WAX	Mixed-mode Weak Anion-eXchange and reversed-phase sorbent	30 µm/2 mg	Waters

Fast chromatography and MS/MS

Instrumentation

As a chromatography analytical tool was used Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) consisted of on-line degasser, binary pumps, high performance SL autosampler, thermostated column compartment, photodiode array UV-VIS detector. The ChemStation software was utilized for chromatography development. The system was coupled on-line to MS detector Agilent Technologies 6460 Triple quad LC/MS. MRM transitions were monitored for the compounds of interest. For identification and quantification of compounds quasi-molecular ions $[M–H]^{-}$ and specific fragments were analysed by quadrupole mass spectrometry analyzer. Final optimized conditions were reached on Zorbax SB-C18 column (2.1 x 50mm, 1.8μm). Phenols were separated at flow rate of 0.8 ml/min with linear gradient of mobile phase acetonitrile (%, v/v) with 0.2% acetic acid aq (%, v/v) set according to the following profile: 0 min 8% ACN, 0.79 min 8% ACN, 1.19 min 20% ACN, 1.99 min 20% ACN, 3.0 min 25% ACN, 3.5 min 8% ACN. Capillary voltage was 4 kV. Fragmentation (V) and collision energy (eV) are as follows: 80, 100, 120 V and 10, 20 eV.

Chromatography:

Column: Zorbax SB-C18, 2.1x50 mm, 1.8 μm

Temperature: 26°C

Mobile Phase: acetonitrile/0.2 % acetic acid (v/v)

Flow: 0,8 ml/min

Gradient: 0 min 8% ACN, 0.79 min 8% ACN, 1.19 min 20% ACN, 1.99 min 20% ACN, 3.0 min 25% ACN, 3.5 min 8% ACN

Stop time: 4.5 min

Post time: 3 min

λ of detection: 254, 210, 280 nm

Mass spectrometry:

Source parameters:

Gas temperature: 350°C

Gas flow: 12 l/min

Nebulizer gas pressure: 50 psi

Sheat gas temperature: 350 °C

Sheat gas flow: 12 l/min

Capillary voltage: 4 kV

RESULTS AND DISCUSSION

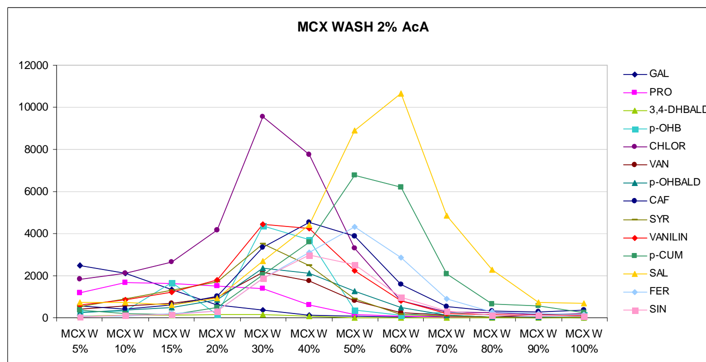


Fig. 1. Elution study of phenolic compounds with MCX μElution plate - % of methanol with 2% acetic acid

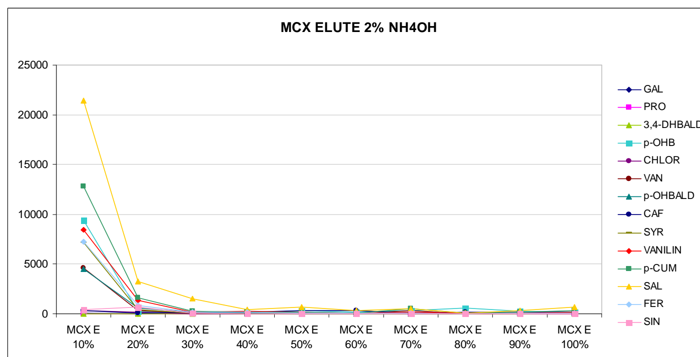


Fig. 2. Elution study of phenolic compounds with MCX μElution plate - % of methanol with 2% amonnium hydroxide

Table 2. Phenolic acids contents ($ng.g^{-1}$ DW) in fractions of sample alga – Cystoseira abies

	GAL	PRO	3,4DHBALD	POH	CHLOR	VAN	POHALD	CAF	SYR	VANIL	PCUM	SAL	FER	SIN
1 F	210.85	399.63	254.53	2278.85	367.13	2402.90	1035.53	25.70	145.51	1958.57	21.88	291.02	31.71	0.00
2 F	721.12	2053.64	361.24	2670.16	18.60	2343.80	1284.45	361.56	221.66	2241.42	77.50	1623.68	108.83	9.47
3 F	496.99	1048.57	202.08	4836.46	198.36	3103.48	1111.37	345.30	202.94	1699.24	52.54	479.68	64.61	0.00
4 F	0.00	23.37	0.00	136.97	60.74	743.80	128.60	0.00	42.39	630.40	4.99	129.83	23.05	0.00

Table 3. Phenolic acids contents (ng.g^{-1} DW) in fractions of sample alga – Chondrus crispus

	GAL	PRO	3,4DHBALD	POH	CHLOR	VAN	POHALD	CAF	SYR	VANIL	PCUM	SAL	FER	SIN
1 F	58.26	596.98	288.81	1797.10	249.38	2770.22	2652.67	38.72	154.52	2843.03	75.42	511.27	59.04	2.82
2 F	56.39	689.66	289.00	1630.93	107.33	1788.90	1943.22	216.40	163.17	2588.49	46.54	596.84	58.41	0.00
3 F	259.58	30151.8	528.31	2304.47	44.92	2821.58	2818.29	366.04	215.59	3926.99	88.23	1813.02	122.91	0.00
4 F	0.00	20.50	0.00	134.21	44.81	843.02	128.86	0.00	42.25	641.63	4.19	99.12	18.48	0.00

Table 4. Phenolic acids contents (ng.g^{-1} DW) in fractions of sample alga – Sargassum muticum

	GAL	PRO	3,4DHBALD	POH	CHLOR	VAN	POHALD	CAF	SYR	VANIL	PCUM	SAL	FER	SIN
1 F	678.98	665.81	197.89	20186.22	290.41	1929.63	1528.66	9.75	116.33	1947.26	33.22	436.07	48.38	0.00
2 F	5640.71	4044.99	490.49	21752.56	261.82	2545.98	1927.06	279.64	189.07	2072.19	141.07	1617.69	123.48	0.00
3 F	3255.60	2021.79	468.87	37505.36	55.88	2047.99	1248.41	454.10	140.79	1829.56	39.34	611.91	27.45	0.00
4 F	0.00	62.25	0.90	345.06	40.48	759.98	185.01	0.00	30.67	1325.73	20.10	89.28	22.14	0.00

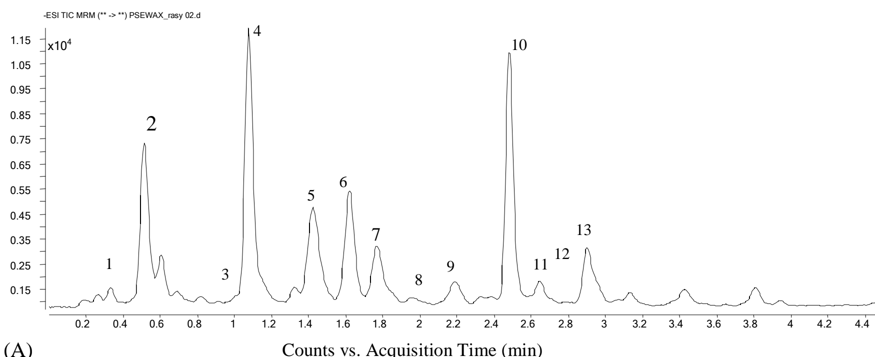
Table 5. Phenolic acids contents (ng.g^{-1} DW) in fractions of sample alga – Cystoseira abies N_2

	GAL	PRO	3,4DHBALD	POH	CHLOR	VAN	POHALD	CAF	SYR	VANIL	PCUM	SAL	FER	SIN
1 F	171.91	309.63	118.27	860.14	328.06	1404.41	980.00	3.62	118.27	1600.97	34.93	308.21	11.20	0.00
2 F	2981.74	3511.61	498.60	1706.86	198.22	2372.83	1888.50	608.65	221.99	2513.91	174.85	2278.46	86.70	0.00
3 F	1711.73	1207.89	304.34	1924.90	129.55	1858.69	979.43	344.83	161.08	1625.10	81.78	683.20	11.00	0.00
4 F	0.00	51.98	32.04	99.81	13.96	664.95	140.85	0.00	16.80	1336.95	21.67	225.02	6.69	0.00

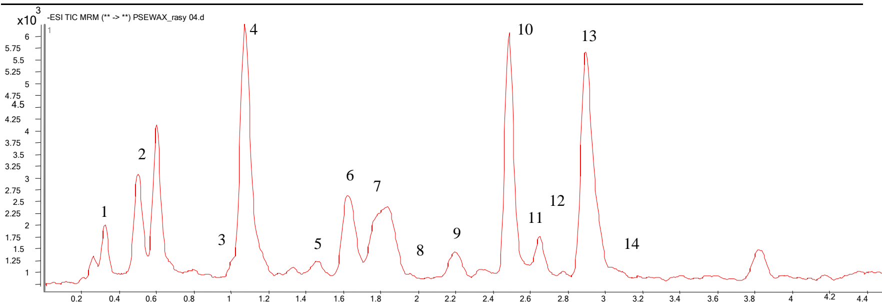
Table 6. Phenolic acids contents (ng.g^{-1} DW) in fractions of sample alga – Undaria pinnatifida

	GAL	PRO	3,4DHBALD	POH	CHLOR	VAN	POHALD	CAF	SYR	VANIL	PCUM	SAL	FER	SIN
1 F	137.01	400.74	181.66	720.54	1170.06	1252.48	1397.01	12.17	104.32	1772.19	34.32	3020.44	21.77	0.00
2 F	1692.01	1659.93	491.92	1327.02	221.71	1569.41	1320.05	403.65	181.40	1774.98	97.08	960.13	16.44	0.00
3 F	3425.86	3613.12	393.73	1076.42	44.05	1277.66	1421.29	684.21	159.95	2069.67	137.69	818.89	94.90	0.00
4 F	0.00	74.11	67.71	273.33	96.76	280.21	67.84	0.00	16.44	947.13	12.95	148.83	1.79	0.00

1 F - Fractions free acids
2 F - Fractions soluble esters
3 F - Fractions glycosides
4 F - Fractions insolubles esters
Used method: *UAPLEμSPE – cartridge WAX*



(A) Counts vs. Acquisition Time (min)



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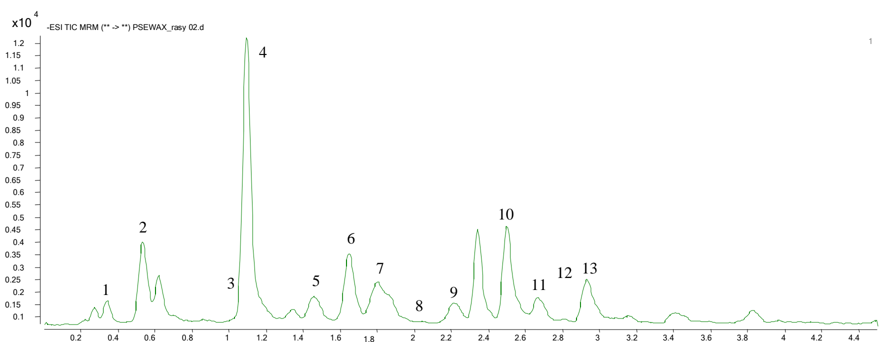
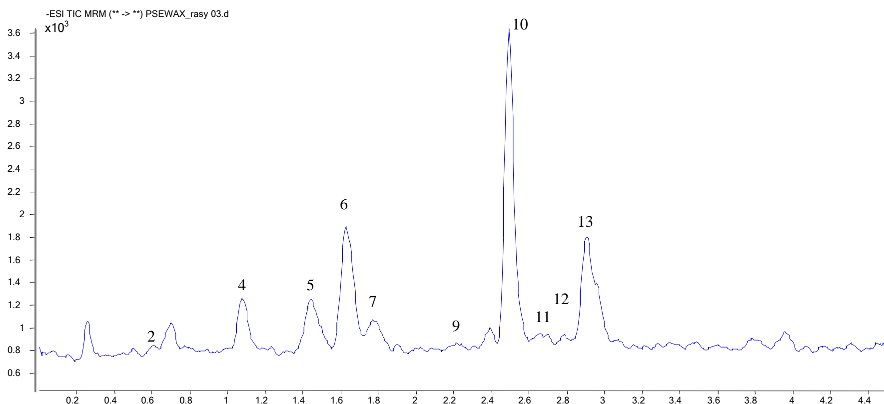


(C) Counts vs. Acquisition Time (min)



(D) Counts vs. Acquisition Time (min)

Fig. 3. Full-scan total ion chromatograms (TIC) for particulars fractions of sea alga sample – Cystoseira abies. (A) 1. Fraction free acids, (B) 2. Fraction soluble esters, (C) 3. Fraction glycosides, (D) 4. Fraction insolubles esters. Peak identification: 1. Gallic acid, 2. Protocatechuic acid, 3. 3,4-Dihydroxybenzaldehyde, 4. p-Hydroxybenzoic acid, 5. Chlorogenic acid, 6. Vanilic acid, 7. p-hydroxybenzaldehyde, 8. Caffeic acid, 9. Syringic acid, 10. Vanilin, 11. p-Coumaric acid, 12. Ferulic acid, 13. Salicylic acid, 14. Sinapic acid.

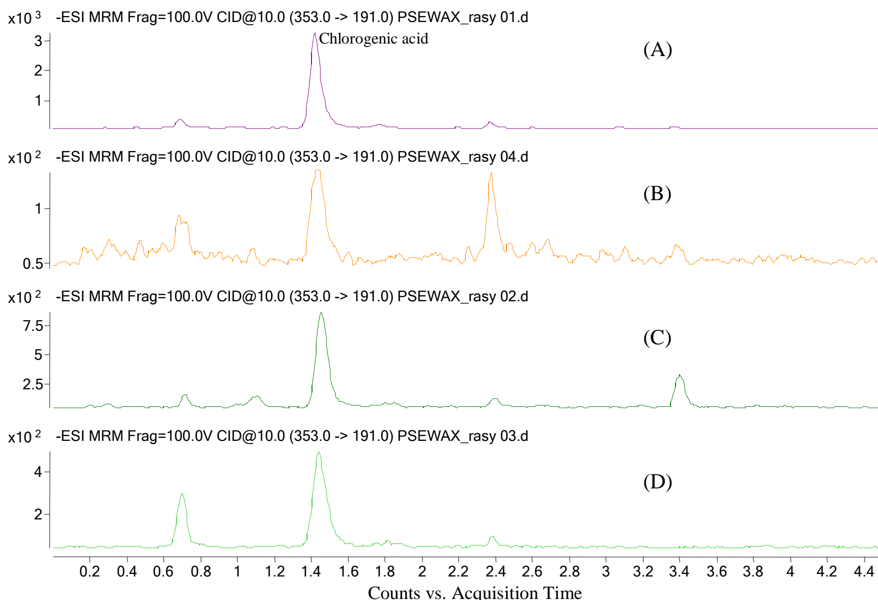


Fig. 4. MRM chromatograms of sea alga sample - Cystoseira abies for chlorogenic acid in particulars fractions: (A) 1. Fraction free acids, (B) 2. Fraction soluble esters, (C) 3. Fraction glycosides, (D) 4. Fraction insolubles esters.

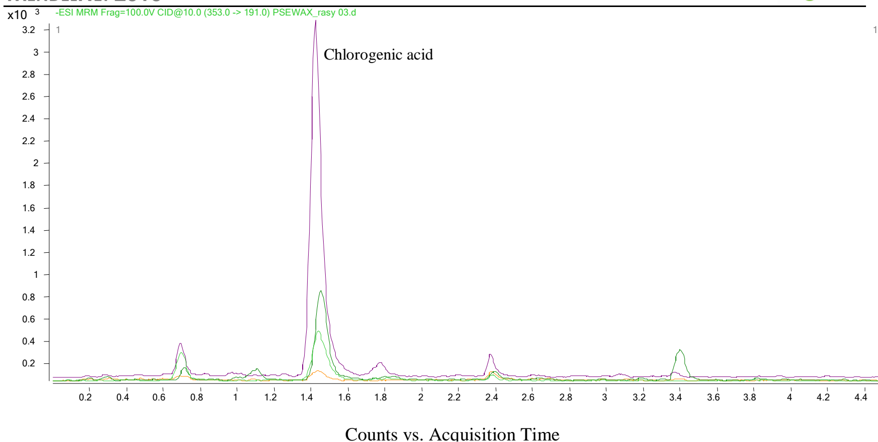


Fig. 5. Comparison of MRM chromatograms of sea alga sample - Cystoseira abies for chlorogenic acid in particulars fractions: (A) 1. Fraction free acids, (B) 2. Fraction soluble esters, (C) 3. Fraction glycosides, (D) 4. Fraction insolubles esters.

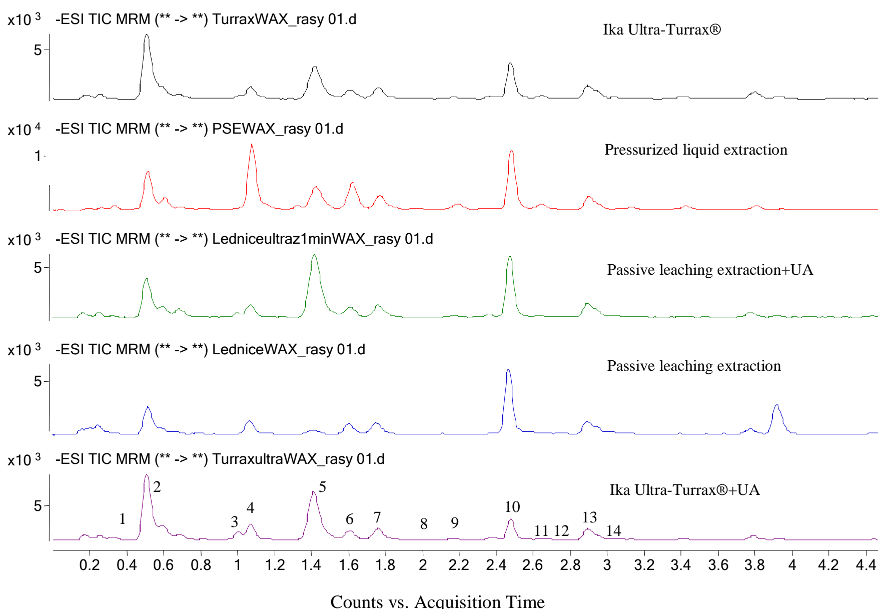


Fig. 6. Comparison of full-scan total ion chromatograms (TIC) of phenolic compunds in different used methods - Ika Ultra-Turrax® Tube Drive, Ika Ultra-Turrax® Tube Drive+Ultrasound-

assisted extraction, Passive leaching extraction, Passive leaching extraction+Ultrasound-assisted extraction, Pressurized liquid extraction (UAPLEμSPE) (cartridge WAX) for Cystoseira abies.

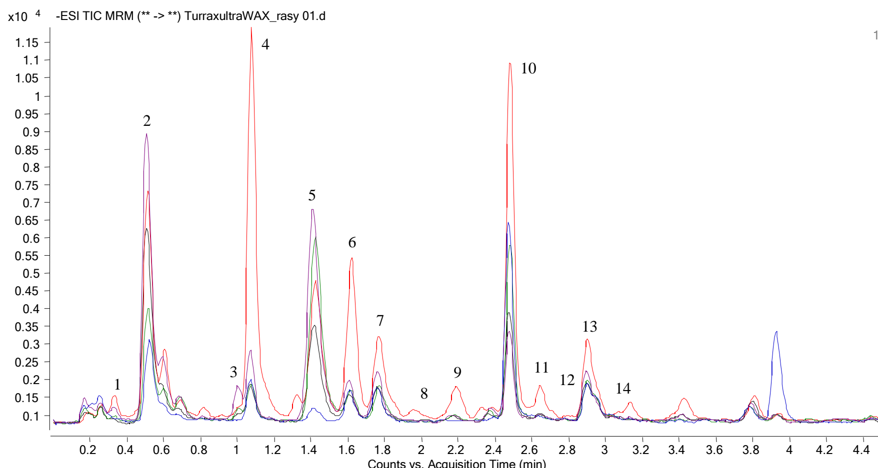


Fig. 7. Comparison of full-scan TIC of phenolic compunds in different used methods - Ika Ultra-Turrax® Tube Drive, Ika Ultra-Turrax® Tube Drive+Ultrasound-assisted extraction, Passive leaching extraction, Passive leaching extraction+Ultrasound-assisted extraction, Pressurized liquid extraction (UAPLEμSPE) (cartridge WAX) for Cystoseira abies.

Table 7. Parameters of MRM transition (negative ESI mode).

Name of phenolic compound	MRM transition (m/z)	Product ions	Fragmentation [V]	Collision energy [eV]
chlorogenic acid	353→191	$[M-H]^-$→$[M-H-caf]^-$	100	10
syringic acid	197→182	$[M-H]^-$→$[M-H-CH_3]^-$	100	10
ferulic acid	193→134	$[M-H]^-$→$[M-H-CO_2^-CH_3]$	100	10
caffeic acid	179→135	$[M-H]^-$→$[M-H-CO_2]^-$	100	10
gallic acid	169→125	$[M-H]^-$→$[M-H-CO_2]^-$	100	10
vanilic acid	167→152	$[M-H]^-$→$[M-H-CH_3]^-$	80	10
p-coumaric acid	163→119	$[M-H]^-$→$[M-H-CO_2]^-$	100	10
protocatechuic acid	153→109	$[M-H]^-$→$[M-H-CO_2]^-$	100	10
vanillin	151→136	$[M-H]^-$→$[M-H-CH_3]^-$	100	10
3,4-dihydroxybenzaldehyde	137→108	$[M-H]^-$→$[M-H-COH]^-$	120	20
salicylic acid	137→93	$[M-H]^-$→$[M-H-CO_2]^-$	100	10
p-hydroxybenzoic acid	137→93	$[M-H]^-$→$[M-H-CO_2]^-$	100	10
p-hydroxybenzaldehyde	121→92	$[M-H]^-$→$[M-H-COH]^-$	120	20

Tab. 8: Analyzed compounds and their retention times and quantitative parameters.

R. T. (min)	Compound	LOD[a] (pg/inj)	LOQ[b] (pg/inj)
0.34	gallic acid	2,10	7,01
0.62	protocatechuic acid	0,01	0,03
1.03	3,4-dihydroxybenzaldehyde	0,04	0,14
1.09	*p*-hydroxybenzoic acid	0,10	0,33
1.42	chlorogenic acid	0,03	0,08
1.63	vanillic acid	0,05	0,15
1.79	*p*-hydroxybenzaldehyde	0,05	0,16
1.85	caffeic acid	0,24	0,80
2.19	syringic acid	0,03	0,10
2.50	vanillin	0,12	0,41
2.66	*p*-coumaric acid	0,09	0,30
3.04	ferulic acid	0,11	0,35
3.12	salicylic acid	0,06	0,19
3.13	synapic acid	0.98	2.94

CONCLUSION

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 several methods for quantitative extraction of phenols in algae. The results showed the possibilities of the application of PLE–µElute plate with ultrasound-assisted extraction and tandem mass spectrometry for 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%. The hyphenated methods PLE–µElute plate with ultrasound-assisted extraction enabled to avoid the evaporation step and pre-concentrate the analytes directly. The applied method allowed a simultaneous determination of phenols in less than 5 minutes. We concluded that our proposed extraction procedures can be useful for the rapid extraction of bioactive phenols in various algae materials and their food products.

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