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Preparation of phytosterols and phytol from edible marine algae by microwave-assisted extraction and high-speed counter-current chromatography

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ABSTRACT

A rapid method of microwave-assisted extraction coupled with high-speed counter current chromatography and UV detector was established for the preparation of phytosterols from edible marine algae. Extraction conditions, namely microwave power, liquid/solid ratio, irradiation time and extraction temperature were optimized using orthogonal array design. The microwave-assisted extraction of *Undaria pinnatifida* and *Sargassum fusiforme* were separated and purified with a non-aqueous two-phase solvent system composed of n-hexane–acetonitrile–methanol (5:5:3, v/v/v). The isolation was done in less than 220 min, and a total of 13.0 mg fucosterol, 1.5 mg 24-methylenecholesterol and 10.7 mg phytol were obtained from 15.0 g *U. pinnatifida*; 4.6 mg fucosterol, 0.3 mg 24-methylenecholesterol and 3.5 mg phytol were obtained from 15.0 g *S. fusiforme*. The purities of all products were over 97% determined by high performance liquid chromatography. The results demonstrate that microwave-assisted extraction coupled with high-speed counter-current chromatography is a feasible, economical and efficient technique for rapid extraction, separation and purification of effective compounds from natural products.

1. Introduction

Natural products play an important role as one of the major sources of new drugs in the years to come because of their "drug-like" properties. Among them, marine organisms such as edible marine macroalgae or seaweeds are rich in bioactive compounds that could potentially be exploited as functional ingredients for both human and animal health diets, pharmaceuticals and cosmetics [1]. These marine algae are classified as red (Rhodophyta), brown (Phaeophyta) or green algae (Chlorophyta) depending on their nutrient and chemical composition. Seaweeds contain appreciable amounts of phytosterols [2], which form an important group among the steroids and may have particular biological activities including anti-diabetic activities [3], anti-cancer properties [4–6], anti-inflammatory [7] and anti-oxidative effects [8]. However, an accurate determination of the biological activity of individual phytosterols is currently difficult because of the fact that pure phytosterols are expensive and barely available [9]. Fucosterol and 24-methylenecholesterol are characteristic sterols of brown algae such as *Sargassum fusiforme* (Harv.) Setch and *Undaria pinnatifida* that affords several health benefits to humans [10].

Since their structures are closely related, to evaluate phytosterols mixed with a diversity of other non-saponifiable components in food lipids of complex sample matrices is a formidable task and requires reliable analytical techniques for the extraction, isolation, purification, detection and quantitative data analyses [11]. Traditional extraction of phytosterols followed a simple solvent soaking procedure, which is not only laborious and time-consuming but also hazardous [12,13]. Alternatively, the plant samples can be extracted with microwave irradiation to obtain total lipid extracts from which sterols can be enriched and isolated. As a widely used sample preparation technique, microwave-assisted extraction (MAE) shows higher or equal extraction efficiencies as compared with traditional solvent extractions while allowing great reduction in time and solvent consumption, and has attracted growing interest in the extraction of terpenes [14], alkaloids [15] and glycoside [16] in natural products.

Moreover, conventional separation and purification methods are needed a multi-step protocol based on column chromatography, thin-layer chromatography and preparative high performance liquid chromatography [17], these methods have obvious inadequacy in analytical precision. High-speed counter-current chromatography (HSCCC) is a continuous liquid–liquid partition chromatography based on partitioning of compounds between two immiscible liquid phases without support matrix, which has great merits of eliminating the irreversible adsorption or sample denaturation, and has been successfully applied to the isolation of various natural products in the last decade [18,19]. Zhou et al. [20] used HSCCC for the enrichment of β-sitosterol in a phytosterol standard, but no pure standard was obtained. More recently, a HSCCC method for the fractionation and analysis of a commercial

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crude β-sitosterol standard (~60% purity) was developed. n-Hexane/methanol/aqueous silver nitrate solution (34/24/1, v/v/v) solvent system was used and pure β-sitosterol and sitostanol (~99%) were obtained by performing HSCCC fractionation step twice [21]. However, the fractions was only collected according to the elution time and all fractions should be evaporated to dryness, accurately weighed and redissolved in hexane before analysis with GC/MS. A more rapid and efficient method for the extraction, separation and purification of phytosterols from algae and other natural products was needed.

In the present study, a rapid and efficient method based on MAE coupled with HSCCC and UV detector was established. Fucosterol, 24-methylenecholesterol and phytol with high purity were separated and purified from *Undaria pinnatifida* and *Sargassum fusiforme*. The purities of all products were over 97% determined by HPLC and their structures were further identified by GC–MS and ^{1}H NMR.

2. Experimental

2.1. Apparatus

MAE experiments were performed on a MAS-II microwave oven (2450 MHz, Sineo, Shanghai, China) consisting of continuous microwave non-pulsed power supply, advanced IR temperature sensor and uniform temperature throughout the cavity.

A *J*-type instrument (GS10A; Beijing UE Biotech., China) equipped with a 110 m multilayer coil (1.6 mm I.D.) with a total capacity of 240 mL was utilized for high-speed counter current chromatography. The β values of this preparative column varied from 0.8 at the head terminal to 0.5 at the tail terminal ($\beta = r/R$, where r is the spool radius and R is the rotor radius). The HSCCC system was equipped with a Model NS-1007 constant-flow pump (UE Biotech., Beijing, China), a UV6000 detector (ChuangXinTongHeng Science & Technology Co., Beijing, China). HW-2000 chromatography data software (Qianpu, Shanghai, China) was employed to carry out data acquisition.

HPLC analysis was performed on a LC-2010 C system (Shimadzu, Kyoto, Japan) with built-in UV–vis detector and with column oven enabling control of temperature. Chromatographic software Class-VP 6.13 was used for data collection and processing. A GC/MS-QP2010 gas chromatography mass spectrometer (Shimadzu, Kyoto, Japan) was used to carry out GC/MS analysis. The NMR data were obtained on a Mercury Plus 300 spectrometer (Varian Inc., USA).

2.2. Reagents and materials

All solvents used were of analytical grade (Guangzhou Chemical Reagent Factory, Guangdong, China) and distilled water was used. Chromatographic grade methanol and acetonitrile used for HPLC analysis were purchased from Merck (Darmstadt, Germany) and Lab-scan (Bangkok, Thailand), respectively. *U. pinnatifida* and *S. fusiforme* were purchased from seafood market in Guangzhou (Guangdong, China). Seaweeds were all dried at 45 °C for 24 h and ground into powder before extraction.

2.3. Sample preparation

The sample preparation for phytosterols analysis usually starts with saponification, which liberates sterols from their esters, and continues by extracting the unsaponifiables. Based on the literature [22], the samples were saponified by ethanolic solution of potassium hydroxide and the alkali concentration was firstly optimized. In this study, a direct sterol extraction was carried out in 1.5 mol/L ethanolic KOH by MAE. 2.0 g of homogenized algae sample was weighed and transferred to the extraction vessel. According to a pre-designed experimental trial, the respective volumes of the extracting solvents were added into the vessels and extracted under different conditions. After extraction, sample extracts were further clarified by centrifugation at 3500 rpm for 5 min to separate out the fine particles. The supernatant was then re-extracted three times with n-hexane. Finally, the organic phase was evaporated to dryness and reconstituted with ethanol prior to HPLC analysis.

Under the optimum conditions, a suitable amount of algae sample was saponified during microwave-assisted extraction to prepare a crude extract for the HSCCC separation procedure. The combined re-extracted n-hexane extracts were concentrated under reduced pressure at 45 °C and stored at −4 °C for HSCCC use.

2.4. Preparing of two-phase solvent system and sample solution

The two-phase solvent system was selected according to the partition coefficient (K) of each target component. The K-values were determined by HPLC as follows: about 1.0 g of original *U. pinnatifida* sample was put into a test tube, in which 3 mL of up phase and 3 mL of lower phase of the selected two-phase solvents were added. The test tube was shaken violently to get equilibrium. After the two-phases separated completely, 1 mL of each layer was taken and analyzed by HPLC. The K-values of each target components were calculated by peak area obtained from the upper phase to that obtained from the lower phase.

The selected two-phase solvent system composed of hexane–acetonitrile–methanol (5:5:3, v/v/v) was thoroughly equilibrated in a separation funnel at room temperature, it was separated and degassed by ultrasonic bath for 30 min before use.

The sample solution was prepared by dissolving crude extract in 5 mL of each phase of selected solvent system for preparative HSCCC separation. Sample loading of HSCCC was optimized by using different amount of crude extract.

2.5. HSCCC separation procedure

The HSCCC system was operated in head-to-tail mode using the upper phase as stationary phase. The revolution speed of the multilayer-coiled column was set at 800 rpm and the flow rate of mobile phase was 2.0 mL/min. The dissolved samples were injected after the hydrodynamic equilibrium was established. Chromatograms were recorded at 210 nm and the fractions were collected according to the chromatograms. After separation, the solvent in the coil was ejected with nitrogen gas to determine the retention of stationary phase (S_f).

2.6. HPLC analysis and identification of HSCCC fractions

The HPLC analysis was performed using a Kromasil C_{18} (250 mm × 4.6 mm, I.D. 5 μm). The mobile phase composed of methanol: acetonitrile (30:70, v/v) was eluted at a flow rate of 1.0 mL/min. The effluent was monitored by a UV detector at 210 nm. HPLC chromatograms obtained from MAE extracts and a standard solution are shown in Fig. 1. Fucosterol, 24-methylenecholesterol and phytol were successfully extracted from spiked *U. pinnatifida* with recoveries ranging from 91 to 97%. Linear relations between peak areas and concentrations (1.00–200 mg/L each compound) were established with correlation coefficient, i.e., $R = 0.999$ for phytol and 24-methylenecholesterol, and 0.997 for fucosterol. The relative standard deviations were 1.5% for phytol, 2.3% for 24-methylenecholesterol, and 3.8% for fucosterol in triplicates, respectively.

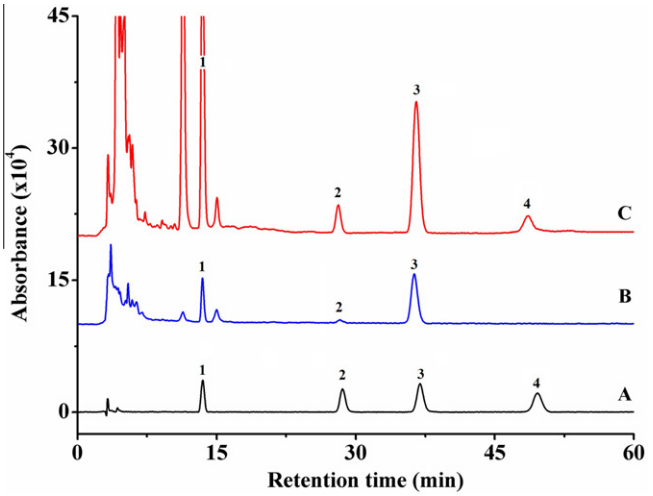

Fig. 1. HPLC chromatograms of the standards (100 mg/L) (A), MAE extract of *S. fusiforme* (B) and *U. pinnatifida* extract (C). Peaks: phytol (1), 24-methylenecholesterol (2), fucosterol (3), and *β*-sitosterol (4).

The structures of collected fractions were elucidated by comparison of their ^{1}H NMR spectra with literature values and were confirmed by GC–MS. The NMR data were obtained on a Mercury Plus 300 spectrometer at 300 MHz with $CDCl_3$ as solvent.

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on a Shimadzu GCMS-QP2010 gas chromatograph mass spectrometer (Shimadzu, Tokyo, Japan) equipped with commercial mass spectral libraries. The instrument was equipped with a split/splitless injector (280 °C) operating in the electron ionization mode at 70 eV and monitored on the full-scan range (*m*/*z* 50–500). Data acquisition and processing, and instrumental control were performed by GC–MS solution software. Analytes were separated with an Agilent J&W DB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm). The column temperature gradient was programmed from 100 °C (hold for 2 min) to 150 °C at 15 °C/min and then, to 315 °C at 10 °C/min (hold for 6 min). The injector operating conditions were as follows: injection volume 1 μL, in the split mode (ratio 10:1). Carrier gas: He delivered at constant pressure: 119.2 kPa; linear velocity: 50 cm/s. Interface temperature 250 °C.

3. Results and discussion

3.1. Optimization of MAE conditions

The concentration of extraction solvent was firstly optimized. Algae samples were extracted with different concentrations of ethanolic KOH solution (0.5, 1.0, 1.5 and 2.0 mol/L). Best extraction yield was obtained when the concentration was 1.5 mol/L. Moreover, microwave irradiation power and irradiation time are two important parameters that effect extraction efficiency (based on the sample/solvent ratio) in the MAE, these significant parameters were optimized with orthogonal array design (OAD) method. Microwave power (factor A), solvent/solid ratio (factor B), irradiation time (factor C) and extraction temperature (factor D) were optimized with a L_9 (3^4) matrix and three levels are defined for each of the factors. Superiority and inferiority levels of the factors are estimated by comparing the average extraction yield of each level (K_i) list in Table 1. ANOVA (analysis of variance) had been employed for the determination of significant variables. The results showed that factors B and C have similar slightly significant (i.e. $p < 0.1$) impact on the extraction yield of these sterols while factors A and D are greatly significant (i.e. $p < 0.01$), which means stronger microwave irradiation and higher extraction temperature are beneficial for extraction. However, when applied 30 min irradiation, the extraction yield of phytol was decreased, which illustrates that phytol could be degradated by microwave irradiation. Moreover, the extraction yield of phytol was decreased with liquid/solid ratio. Taking all these facts into consideration, the optimum MAE conditions was selected as follows: 1.5 mol/L of ethanolic KOH extraction solvent, 500 W microwave power, 20:1 mL/g of liquid/solid

Table 1
The results of orthogonal test L_9 (3^4).

Experiments	A (microwave power/W)	B (liquid/solid ratio)	C (irradiation time/min)	D (extraction temperature/°C)	Extraction yield (μg/g, *n* = 3)		
					Ph	Mc	Fu
1	A_1 (300)	B_1 (10:1)	C_1 (10)	D_1 (30)	437	27	570
2	A_1 (300)	B_2 (20:1)	C_2 (20)	D_2 (50)	708	67	957
3	A_1 (300)	B_3 (30:1)	C_3 (30)	D_3 (70)	530	96	1148
4	A_2 (400)	B_1 (10:1)	C_2 (20)	D_3 (70)	806	87	938
5	A_2 (400)	B_2 (20:1)	C_3 (30)	D_1 (30)	568	63	860
6	A_2 (400)	B_3 (30:1)	C_1 (10)	D_2 (50)	606	75	867
7	A_3 (500)	B_1 (10:1)	C_3 (30)	D_2 (50)	851	99	1094
8	A_3 (500)	B_2 (20:1)	C_1 (10)	D_3 (70)	788	116	1034
9	A_3 (500)	B_3 (30:1)	C_2 (20)	D_1 (30)	593	92	843
Ph							
K_1[a]	558	698	610	533			
K_2[a]	660	688	703	722			
K_3[a]	744	576	650	708			
Mc							
K_1	63	71	73	61			
K_2	75	82	82	80			
K_3	102	88	86	100			
Fu							
K_1	892	867	824	758			
K_2	889	950	913	973			
K_3	990	953	1034	1040			

Note: Each combination was carried out in triplicate so that the error estimation can be done by experiment repetition. Ph, phytol; Mc, 24-methylenecholesterol; Fu, fucosterol.

[a] Average extraction yield of each level, μg/g. $K_i^A = \Sigma$ (extraction yield at A_i)/3.

Table 2
Partition coefficients (*K*) for target components in different solvent systems.

Solvent systems	*K*-values		
	Phytol	24-Methylenecholesterol	Fucosterol
Hexane–ethyl acetate–butanol–methanol–water (7:0.6:1:5:0.6)	2.50	3.13	3.72
Heptane–methanol (1:1)	0.92	1.39	1.38
Hexane–acetonitrile–methylene dichloride (5:5:2)	1.32	1.35	1.51
Hexane–acetonitrile–methanol (5:5:0.5)	1.67	1.68	2.68
Hexane–acetonitrile–methanol (5:5:1)	1.58	1.62	2.54
Hexane–acetonitrile–methanol (5:5:2)	1.43	1.55	2.22
Hexane–acetonitrile–methanol (5:5:3)	1.31	1.44	2.05
Hexane–acetonitrile–methanol (5:5:4)	1.20	1.32	1.95

ratio, 70 °C of extraction temperature, and 20 min of irradiation time. The extraction yields of fucosterol, 24-methylenecholesterol and phytol from *U. pinnatifida* under the optimum condition were 1.21 mg/g, 0.16 mg/g and 0.75 mg/g, respectively.

To evaluate the extraction efficiency of MAE, *U. pinnatifida* was also extracted using solvent extraction method under the similar conditions, that is, 2.0 g sample was extracted with 40 mL 1.5 mol/L of ethanolic KOH at 25 °C for 30 min without microwave irradiation. The extraction yields were 0.35 mg/g, 0.07 mg/g and 0.27 mg/g, respectively. Compared with traditional solvent extraction, MAE gives much higher extraction yields and costs shorter extraction time, indicating that MAE is an efficient sample preparation technique and has good potential on the extraction of phytosterols from the algae.

3.2. Optimization of HSCCC conditions

The selection of the two-phase solvent system for the target compounds is the most important step in HSCCC procedure. According to the gold rules in selecting optimum conditions introduced by Ito [23] and some literatures on HSCCC separation of steroids [21,24,25], several solvent systems were selected and assessed for *K* values. The *K*-values of fucosterol, 24-methylenecholesterol and phytol in different solvent systems were determined by HPLC as described in Section 2.4, the results are listed in Table 2. Of all the results, solvent systems composed of hexane–acetonitrile–methylene dichloride and hexane–acetonitrile–methanol had the suitable *K* value. However, the working wavelength used for UV detection of sterols was 210 nm. In general, a solvent with a UV cutoff higher than the system's operating wavelength generates such a high background absorbance that the target signal could be completely masked, as it occurred in previously published reports [21,24]. Hexane–acetonitrile–methylene dichloride with high UV cutoff wavelength seems to be impracticable for the separation and purification of sterols. And then, the non-aqueous solvent system n-hexane–acetonitrile–methanol was further investigated in order to achieve good resolution and acceptable elution time. Table 2 also gives the *K* values of target compounds in hexane–acetonitrile–methanol solvent system with different volume ratio. Although both of the 5:5:3 (v/v/v) and 5:5:4 (v/v/v) solvent systems have acceptable *K* values, the 5:5:4 (v/v/v/) solvent system produces upper phase much less than lower phase after equilibrium and it is excluded from further consideration for solvent waste issues. Solvent system of hexane–acetonitrile–methanol (5:5:3, v/v/v) was finally selected for the HSCCC separation.

The sample loading is another significant parameter in HSCCC. Large sample loading can increase the separation efficiency in a single HSCCC run, but it is restricted by the volume of multi-layer coil column and the stability of selected solvent system. Different amounts of original sample were extracted with MAE and sample loading was optimized based on peak resolution and product purities. When the sample loading increased from 5.0 to 15.0 g crude sample, the purity of obtained target fractions were similar despite of resolution reduction. Moreover, sample loading larger than 15.0 g not only leaded to excessive loss of stationary phase retention but also caused a large decrease in product purities. So, the maximum sample loading is 15.0 g original sample and three fractions with high purity were obtained. The HSCCC chromatograms of MAE samples extracted from *U. pinnatifida* and *S. fusiforme* are shown in Fig. 2A and B, respectively.

3.3. Purity determination and identification of the collected fractions

Under the optimum MAE and HSCCC conditions, phytosterols and phytol were separated and purified from *U. pinnatifida* and *S. fusiforme* and the results are listed in Table 3. Their purities were

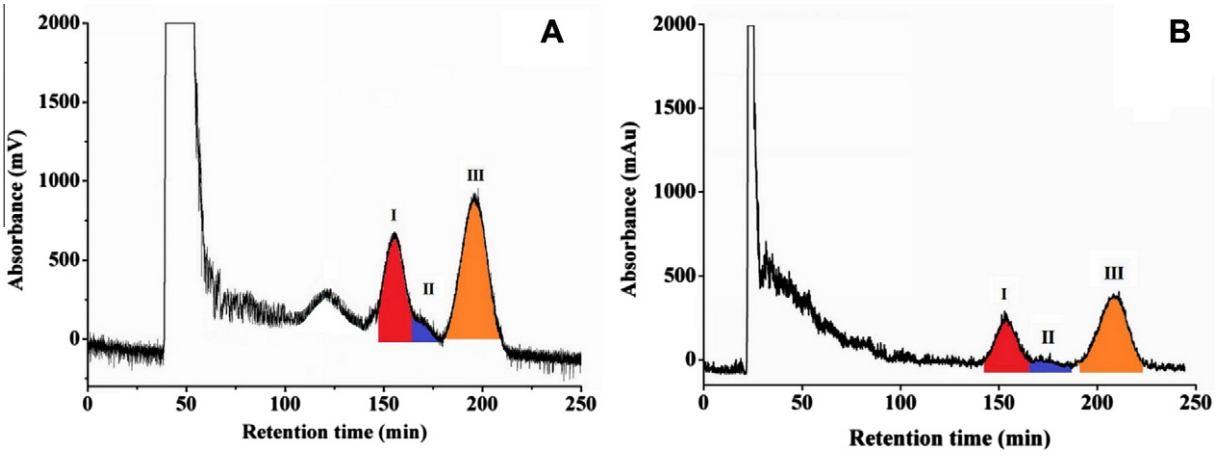

Fig. 2. HSCCC chromatograms of the MAE extract from 15.0 g *U. pinnatifida* (A) and *S. fusiforme* (B) original samples.

Table 3
The separation and purification results of MAE-HSCCC.

Samples	Sample loading (g)	Fucosterol		Phytol		24-Methylenecholesterol	
		Amount (mg)	Purity (%)	Amount (mg)	Purity (%)	Amount (mg)	Purity (%)
U. pinnatifida	15.0	13.0	98.2	10.7	97.3	1.5	97.0
S. fusiforme	15.0	4.6	97.6	3.5	97.4	0.3	97.4

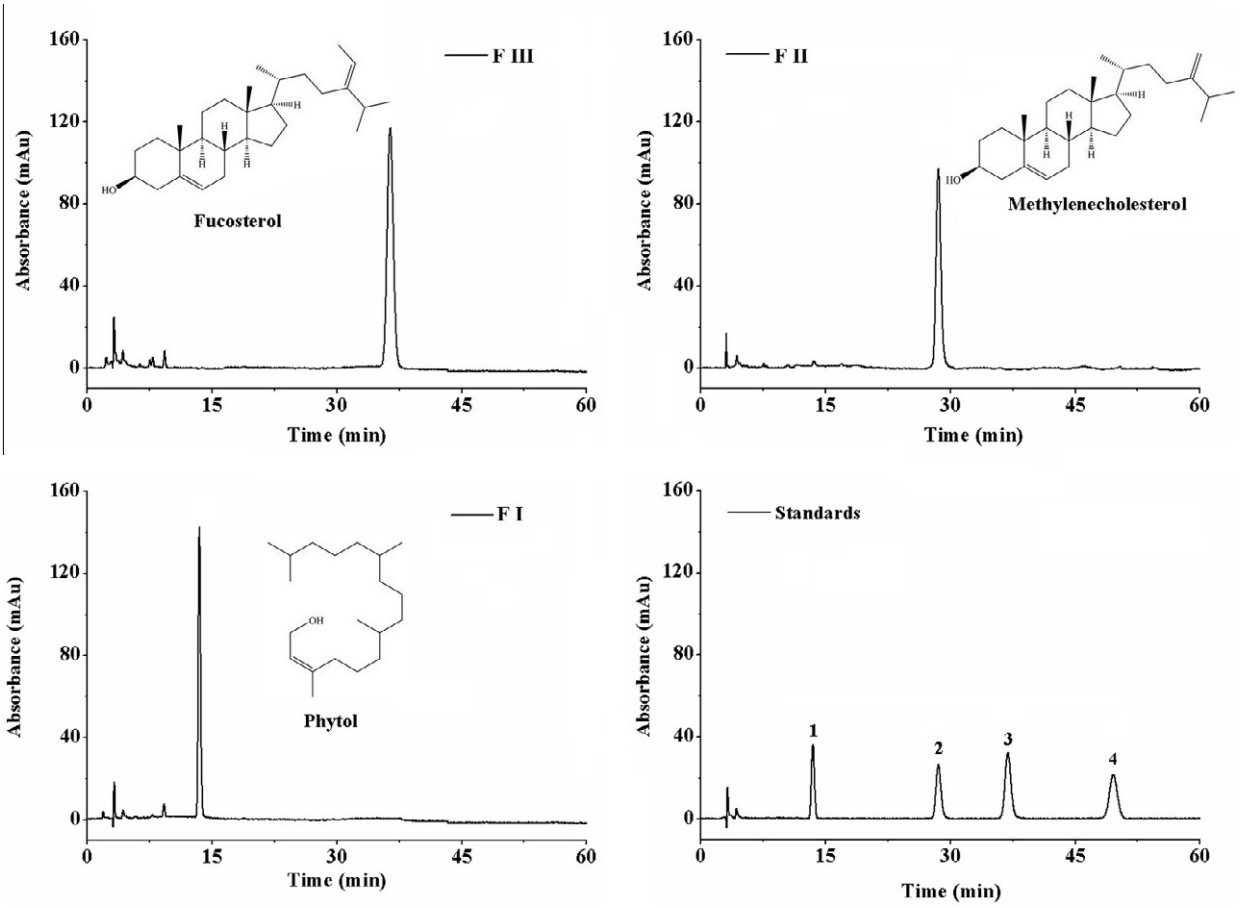

Fig. 3. The HPLC chromatograms of the standards and obtained fractions from *U. pinnatifida*. Peaks: phytol (1), 24-methylenecholesterol (2), fucosterol (3) and *β*-sitosterol (4).

all over 97% as determined and their HPLC chromatograms were shown in Fig. 3. Fucosterol, 24-methylenecholesterol and phytol were obtained from *U. pinnatifida* and *S. fusiforme* in a single HSCCC step within 220 min.

The chemical structure of each peak fractions was further identified by GC–MS and ^{1}H NMR as follows:

Fraction **I**: MS (70 eV, *m*/*z*): 296 $[M]^+$; ^{1}H NMR (300 Hz, CDCl3)δ (ppm): 5.41 (1H, t, H-2), 4.16 (2H, d, H-l), 2.00 (2H, t, H-4), 1.68 (3H, s, H-20), 1.64–1.02 (19H, m), 0.88–0.85 (12H, m). The data were in agreement with previous report [26], fraction **I** was identified as phytol.

Fraction **II**: MS (70 eV, *m*/*z*): 398 $[M]^+$; ^{1}H NMR (300 Hz, CDCl3)δ (ppm): 0.68 (s, 3H, 18-CH_3), 0.95 (d, 3H, 21-CH_3), 1.01 (s, 3H, 19-CH_3), 1.03 (dd, 6H, 26-CH_3 and 27-CH_3), 3.54 (tt, 1H, 3-CH), 4.66 (s, 1H, 28-CH), 4.71 (s, 1H, 28-CH), 5.35 (dd, 1H, 6-CH). Compared with the data given in literature [27], fraction **II** was identified as 24-methylenecholesterol.

Fraction **III**: MS (70 eV, *m*/*z*): 412 $[M]^+$; ^{1}H NMR (300 Hz, CDCl3)δ (ppm): 5.36 (1 H, br d, H-6), 5.19 (1 H, q, H-28), 3.53 (1 H, m, H-3), 1.58 (3 H, d, H-29), 1.02 (3 H, s, H-19), 1.01 (3 H, br s, H-21), 1.00 (3H, d, H-27), 0.98 (3 H, d, H-26), 0.70 (3 H, s, H-18). After comparison of the data with literature [28], fraction **III** was identified as fucosterol.

4. Conclusion

Two edible marine algae, namely *U. pinnatifida* and *S. fusiforme*, were extracted, separated and purified by MAE coupled with HSCCC. Based on the optimized conditions of MAE and HSCCC, fucosterol, 24-methylenecholesterol and phytol with purity over 97% were prepared in a single HSCCC step within 200 min. The results showed that the combination of MAE and HSCCC could be used as a powerful tool for the quickly and efficiently separation and purification of bioactive compounds from natural products.

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References

[1] O. Sticher, Natural product isolation, Nat. Prod. Rep. 25 (2008) 517–554.
[2] N. Bouzidi, Y. Daghbouche, M. El Hattab, Z. Aliche, G. Culioli, L. Piovetti, S. Garrigues, M. de la Guardia, Determination of total sterols in brown algae by Fourier transform infrared spectroscopy, Anal. Chim. Acta 616 (2008) 185–189.
[3] G. García-Llatas, M.T. Rodríguez-Estrada, Current and new insights on phytosterol oxides in plant sterol-enriched food, Chem. Phys. Lipids 164 (2011) 607–624.
[4] A.B. Awad, R. Roy, C.S. Fink, β-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells, Oncol. Rep. 10 (2003) 497–500.
[5] P.G. Bradford, A.B. Awad, Phytosterols as anticancer compounds, Mol. Nutr. Food Res. 51 (2007) 161–170.
[6] A.V. Rao, S.A. Janezic, The role of dietary phytosterols in colon carcinogenesis, Nutr. Cancer 18 (1992) 43–52.
[7] T. Akihisa, Y. Nakamura, M. Tagata, H. Tokuda, K. Yasukawa, E. Uchiyama, T. Suzuki, Y. Kimura, Anti-inflammatory and anti-tumor-promoting effects of triterpene acids and sterols from the fungus *ganoderma lucidum*, Chem. Biodiversity 4 (2007) 224–231.
[8] S. Lee, Y.S. Lee, S.H. Jung, S.S. Kang, K.H. Shin, Anti-oxidant activities of fucosterol from the marine algae *Pelvetia siliquosa*, Arch. Pharm. Res. 26 (2003) 719–722.
[9] P. Fernandes, J.M.S. Cabral, Phytosterols: applications and recovery methods, Bioresour. Technol. 98 (2007) 2335–2350.
[10] S. Gupta, N. Abu-Ghannam, Bioactive potential and possible health effects of edible brown seaweeds, Trends Food Sci. Technol. 22 (2011) 315–326.
[11] S.L. Abidi, Chromatographic analysis of plant sterols in foods and vegetable oils, J. Chromatogr. A 935 (2001) 173–201.
[12] P. Kumari, C.R.K. Reddy, B. Jha, Comparative evaluation and selection of a method for lipid and fatty acid extraction from macroalgae, Anal. Biochem. 415 (2011) 134–144.
[13] J. Roche, M. Alignan, A. Bouniols, M. Cerny, Z. Mouloungui, O. Merah, Sterol concentration and distribution in sunflower seeds (*Helianthus annuus* L.) during seed development, Food Chem. 119 (2010) 1451–1456.
[14] S. Chemat, H. Aït-Amar, A. Lagha, D.C. Esveld, Microwave-assisted extraction kinetics of terpenes from caraway seeds, Chem. Eng. Process. 44 (2005) 1320–1326.
[15] F. Du, X. Xiao, P. Xu, G. Li, Ionic liquid-based microwave-assisted extraction and HPLC analysis of dehydrocavidine in *corydalis saxicola* Bunting, Acta Chromatographica 22 (2010) 459–471.
[16] T. Zhou, X. Xiao, G. Li, Z. Cai, Study of polyethylene glycol as a green solvent in the microwave-assisted extraction of flavone and coumarin compounds from medicinal plants, J. Chromatogr. A 1218 (2011) 3608–3615.
[17] L. Dinan, J. Harmatha, R. Lafont, Chromatographic procedures for the isolation of plant steroids, J. Chromatogr. A 935 (2001) 105–123.
[18] A. Marston, K. Hostettmann, Developments in the application of counter-current chromatography to plant analysis, J. Chromatogr. A 1112 (2006) 181–194.
[19] I.A. Sutherland, D. Fisher, Role of counter-current chromatography in the modernisation of Chinese herbal medicines, J. Chromatogr. A 1216 (2009) 740–753.
[20] Y. Zhou, F. Chen, Z. Li, Preparative separation of β-sitosterol by high speed countercurrent chromatography, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 1693–4588.
[21] M. Schröder, W. Vetter, High-speed counter-current chromatographic separation of phytosterols, Anal. Bioanal. Chem. 400 (2011) 3615–3623.
[22] B. Cañabate-Díaz, A. Segura Carretero, A. Fernández-Gutiérrez, A. Belmonte Vega, A. Garrido Frenich, J.L. Martínez Vidal, J. Duran Martos, Separation and determination of sterols in olive oil by HPLC-MS, Food Chem. 102 (2007) 593–598.
[23] Y. Ito, Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography, J. Chromatogr. A 1065 (2005) 145–168.
[24] L. Huang, Y. Cao, H. Xu, G. Chen, Separation and purification of ergosterol and stigmasterol in *Anoectochilus roxburghii* (wall) Lindl by high-speed counter-current chromatography, J. Sep. Sci. 34 (2011) 385–392.
[25] S. Yao, R. Liu, X. Huang, L. Kong, Preparative isolation and purification of chemical constituents from the root of *Adenophora tetraphlla* by high-speed counter-current chromatography with evaporative light scattering detection, J. Chromatogr. A 1139 (2007) 254–262.
[26] K.H. Kim, K.H. Lee, S.U. Choi, Y.H. Kim, K.R. Lee, Terpene and phenolic constituents of *Lactuca indica* L, Arch. Pharm. Res. 31 (2008) 983–988.
[27] W. Lu, C. Zhang, L. Zeng, J. Su, Synthesis of polyhydroxysterols (V): efficient and stereospecific synthesis of 24-methylene-cholest-5-ene-3β,7α-diol and its C-7 epimer, Steroids 69 (2004) 803–808.
[28] S. Lee, Y.S. Lee, S.H. Jung, H.S. Lee, S.S. Kang, K.H. Shin, Determination of fucosterol in the marine algae *pelvetia siliquosa* by gas chromatography, Nat. Prod. Sci. 10 (2004) 29–31.