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Accelerated solvent extraction of metabolites from marine fungi

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The accelerated solvent extraction method was used to extract metabolites from marine fungi and the process parameters were optimized. The influences of the times of extraction, temperature, pressure and extraction time duration on the extraction yield were discussed using single-factor experiment. The optimum process parameters were then obtained using orthogonal experiment. The optimal conditions for accelerated solvent extraction of metabolites from marine fungi were obtained: extracted for 2 times with methanol and ethyl acetate (80:20, V/V), extraction pressure of 1.2 × 10^7 Pa, extraction temperature of 105°C, and static extraction time of 15 min. Under the optimum conditions, the extraction yield was much higher for accelerated solvent extraction method when compared with soaking method, whereas, the accelerated solvent extraction method took only 1/3 of time for soaking method and saved over 80% of extraction solvent. The results showed that accelerated solvent extraction method was suitable for extracting metabolites from marine fungi in batch.

Key words: Accelerated solvent extraction, high-performance, liquid chromatography, marine fungi, solid fermentation.

INTRODUCTION

The research on the metabolites of marine microorganisms began in the 1960s (Burkholder et al., 1966; Wang et al., 2010), and many extraction methods have been developed since then. The statistics showed that the most common method for extraction was soaking extraction (Li et al., 2010, 2011; Zhang et al., 2008; Feng and Ma, 2010; Yen et al., 2003), and different extractants were choose due to the physicochemical properties. For example, 3,3''-DHT and 3HT were extracted by ethyl acetate (Yen et al., 2003) and three bioactive metabolites were obtained by extracted with petroleum ether, ethyl acetate and n-butanol (Zhang et al., 2008); but soaking extraction method was not only time, but also, highly solvent-consuming. When it comes to processing a large number of microorganisms to build a library comprising lots of fractions and compounds, it is necessary to develop a method which takes the advantages of shorter operation time, higher yield and lower solvent consumption.

Accelerated Solvent Extraction (ASE) (Richter et al., 1996) was first developed in 1996 and then validated on an automated extraction system. ASE method uses organic solvents to extract samples under high pressure and at high temperature, which is usually higher than the solvents' boiling point. The ASE method provides many advantages: rapid, low solvent consumption, simple

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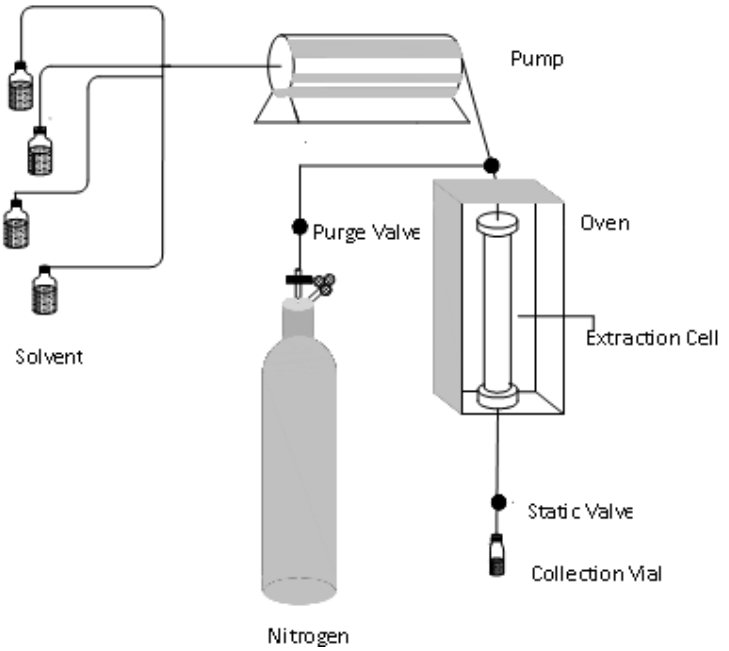

Figure 1. Schematic diagram of accelerated solvent extraction device.

operation, highly automated, high yield and environmentally friendly (Richter et al., 1996; Bjorklund et al., 2000; Holt et al., 2012; Sun et al., 2012). It has been shown to be equivalent to standard environmental protection agency (EPA) extraction methodology in terms of recovery and precision, and it was proposed as a standard method and appeared as method 3545 in update III of the U.S. EPA SW-846 methods (USEPA SW-846). ASE method is widely used in extracting trace amounts of ingredients from foods and medicine (Hubert et al., 2000; Conte et al., 1997; Adou et al., 2002; Suchan et al., 2004; Chuang et al., 2001; Chen et al. 2007). So far, few reports have been published on the application of ASE in extracting metabolites from marine fungi. The study described in this paper aimed to optimize extraction conditions of ASE for processing a marine fungus with single-factor experiments and orthogonal test.

The qualitative and quantitative analysis was performed by high performance liquid chromatography (HPLC) using both diode array detector (DAD) and evaporative light scattering detector (ELSD). The extraction yields were calculated to evaluate the performance of different extracting methods.

MATERIALS AND METHODS

Materials and reagents

The solid fermentation of cladosporium sphaerospermum MCCC 3A00023, which was obtained from the Marine Culture Collection of China (MCCC, Third Institute of Oceanography, State Oceanic Administration, Xiamen, Fujian, China) was used as raw materials. Methanol (HPLC Grade) was obtained from Spectrum Chemical Mfg. Corp (California, United States). Methanol (AR, analytical reagent) and ehyl acetate (AR) were obtained from Shantou Dahao Fine Chemicals Co., Ltd (Guangdong, China). The ASE instrument (SP-100QSE) was obtained from Spectrum Shanghai (Shanghai, China). HPLC (Waters 2695, alliance system) equipment was from Waters Corporation (America), and ELSD was from Unimicro Technologies, Inc (Shanghai, China).

Accelerated solvent extraction (ASE) technology

Figure 1 is a schematic diagram of accelerated solvent extraction device. The general working process is as follows: Firstly, ferment substances were loaded into the extraction cell and the cell was then tightened. After the extraction, temperature, pressure, static extraction time and extraction cycles were set; the extractant was introduced into the cell automatically. Then, the extraction cell was heated and pressurized to the set points. The static period for extraction started after that. After the static extraction period, fresh gas purged the lines and the cell, and the extract was collected in the collection vial.

Evaluation methodology

The qualitative analysis was performed by HPLC using both DAD and ELSD. The separation was performed on a Hypersil BDS C18 (150 × 4.6 mm i.d., 5 µm particle size) column (Dalian Elite Analytical Instruments Co. Ltd.) with a guard column. The column was maintained at 30°C with a column heating block and the absorption of the eluate was monitored at 254 nm with a Waters 2996 diode array detector. All solvents used for chromatography were of HPLC grade. Solvent A was water and solvent B was methanol. Both solvents were degassed by sonication and further degassed using the on-line degassing device during chromategraphy. The flow rate was 0.8 ml/min and the injection volume was 20 µl. The elution started with a linear gradient from 5 to 100% B over a period of 35 min. The latter percentage of solvent B was then maintained for 20 min. The total separation time was 55 min. The column was equilibrated for about 10 min with the initial eluting

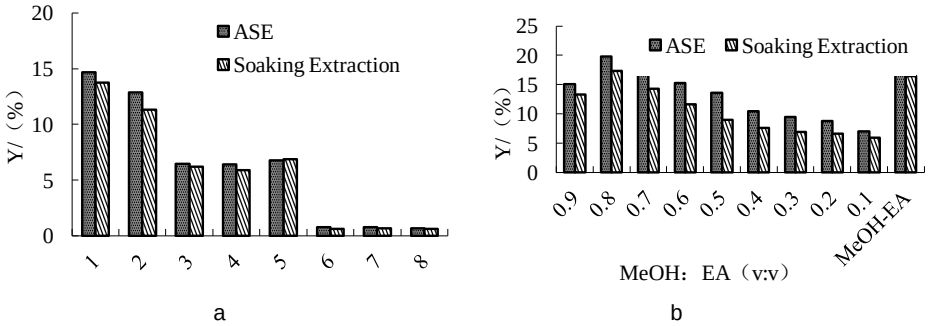

Figure 2. Effect of different extractants for ASE method and soaking extraction method. The abscissa represents the types of the extractants. A: 1 methanol 2 ethanol 3 ethyl acetate 4 chloroform 5 n-butanol 6 petroleum ether 7 n-hexane 8 cyclohexane. B: MeOH→EA shows that the sample was extracted with methanol and ethyl acetate in order.

solvent mixture (95% A and 5% B) before the next run was started. The gas velocity for ELSD was 2.5 ml/min and the atomization temperature was set at 30°C.

Extraction yields were used for quantitative analysis. The extraction yields were calculated using the following formula:

$$\text{Extraction yield (Y/\%)} = \frac{\text{Weight of the extract}}{\text{Weight of the sample}} \times 100$$

Extraction by ASE method

Firstly, the required parameters for ASE were set up. Then 10 g of solid fermentation were loaded into the extraction cell. Different extractants, including methanol, ethanol, chloroform, n-butyl alcohol, ethyl acetate, petroleum ether, hexamethylene and n-hexane were applied to extract the sample. After the static extraction period, the extracts were collected and evaporated to dryness using a rotary evaporator at 40°C. The extraction yield was then calculated based on the dry weight of the extracts.

Soaking extraction

Ten grams of solid fermentation were mixed with the extractant. The mixture was then shearing stirred at high speed, after which homogenization was conducted. Extracts were collected after centrifugation and filtration. The effects of various factors, including the extractant applied, times of extraction (1, 2 and 3 times), extraction duration time (5, 10, 15, 20 and 25 min) and ratio of sample to extractant (1:4, 1:6, 1:8, 1:10 and 1:12) were studied. The optimum conditions of soaking extraction were as follows: a mixture of methanol and ethyl acetate (80:20, V/V) as the extractant, extraction for two times 20 min each and a 1:8 ratio of sample to extractant.

RESULTS AND DISCUSSION

Selection of extractants

The objective of extractant selection is to ensure high extractability, low cost and non or low toxicity (Zhang, 2011; Park et al., 2012; Chebrolu et al., 2010; Gong, 2008). In this study, the performance of ASE method and soaking extraction method were compared and the results are shown in Figure 2A. When methanol or ethanol was used as the extractant, the extraction yields were the highest and the extracts obtained covered a wide polarity range (Figures S1 and S2). The extraction yields were much lower when petroleum ether, n-hexane or cyclohexane was used as the extractant and the extracts obtained were of relatively weak polarity (Figures S3, S4 and S5). Ethyl acetate, chloroform and n-butanol were all capable of extracting the most diverse extracts (Figures S6, S7 and S8); however, n-butanol was difficult to recycle and chloroform was highly toxic. Taking all these into account, methanol and ethyl acetate were chosen as extractants.

Two research protocols were then employed and compared. One was to extract the sample with methanol and ethyl acetate in order, and the other was to extract with a mixture of the two. The results were shown in Figure 2B. Different proportions of methanol and ethyl acetate were used and the yield was the highest when methanol and ethyl acetate was mixed with a ratio of 80:20. Taking the HPLC results into consideration, the following conclusions could be drawn: the optimal mixing ratio of methanol and ethyl acetate is 80:20.

Single-factor experiments

Effect of extraction times

The effects of extraction times were investigated, and the results were displayed in Table 1 and Figure 3. Equal amount of extractant was used for each round of extraction but the corresponding yield was getting smaller. The yield for the 1st extraction accounted for more than 70% of the total extraction yields and the sum of the extraction yields for the 1st and 2nd extraction was greater than 95% of the total extraction yields. The times of extraction is considered to be enough when almost the entire target components were extracted (Li et al., 2010). Considering the extraction efficiency and solvent consumption (Zhao

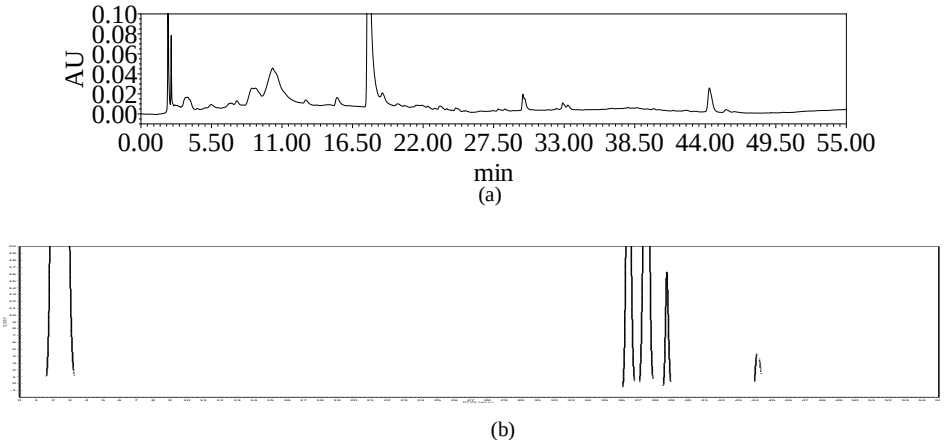

Figure S1. Chromatograms of methanol extracts. (a) UV detection, and (b) evaporative light scattering detection.

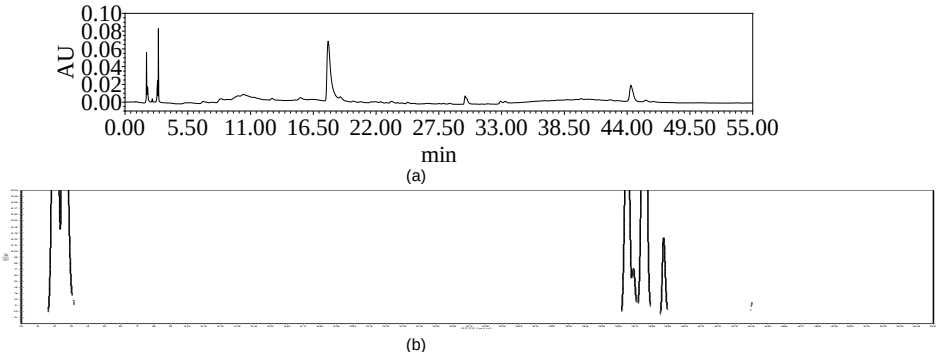

Figure S2. Chromatograms of ethanol extracts. (a) UV detection, and (b) evaporative light scattering detection.

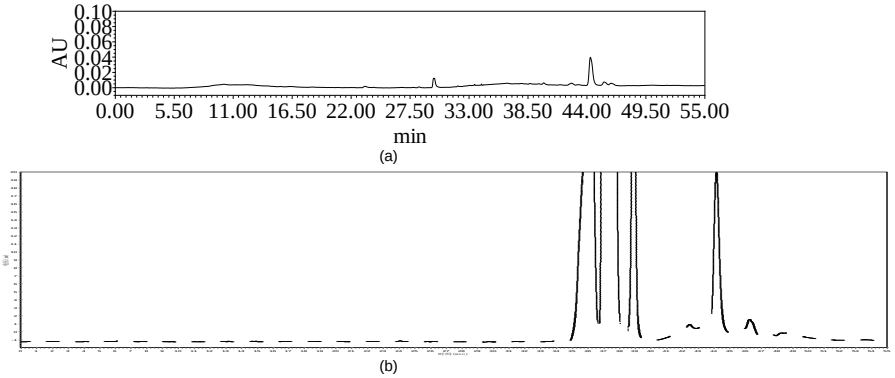

Figure S3. Chromatograms of petroleum ether extracts. (a) UV detection, and (b) evaporative light scattering detection.

et al., 2012), two times of extraction was acceptable (Li et al., 2012). All the following experiments were carried out with two times of extraction.

Effect of temperature on extraction

To evaluate whether the temperature influences the

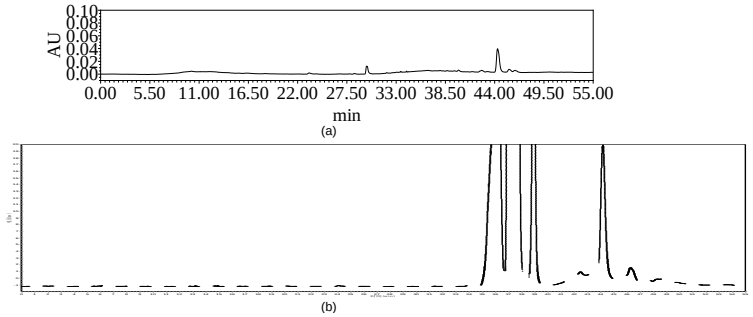

Figure S3. Chromatograms of petroleum ether extracts. (a) UV detection, and (b) evaporative light scattering detection.

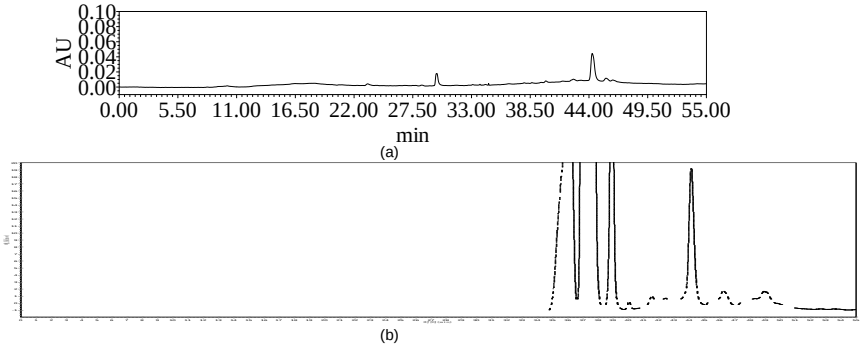

Figure S4. Chromatograms of n-hexane extracts. (a) UV detection (b) evaporative light scattering detection.

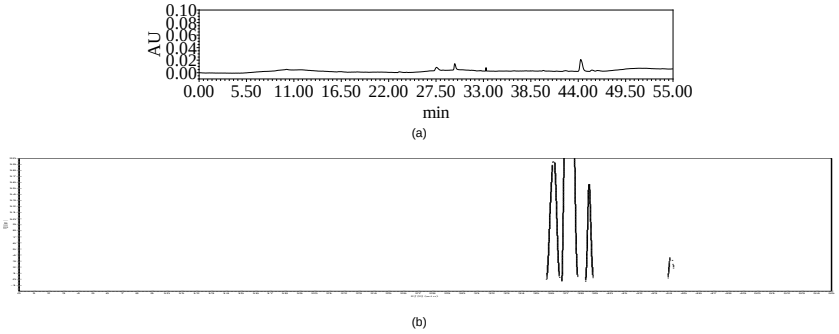

Figure S5. Chromatograms of cyclohexane extracts. (a) UV detection, and (b) evaporative light scattering detection.

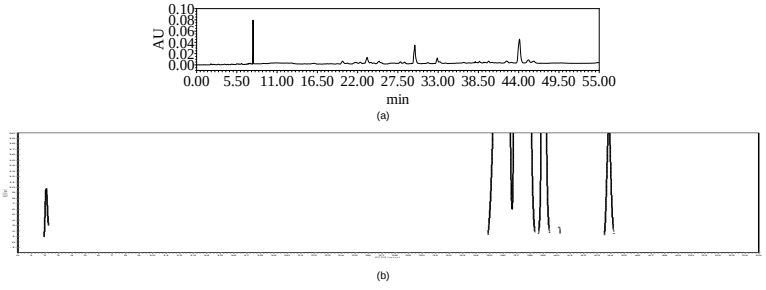

Figure S6. Chromatograms of ethyl acetate extracts. (a) UV detection, and (b) evaporative light scattering detection.

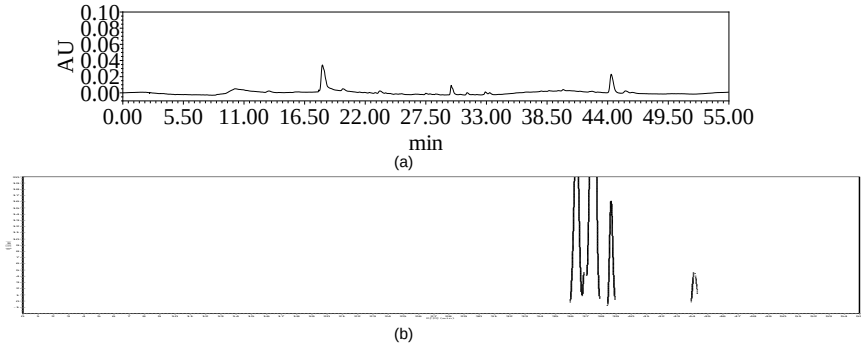

Figure S7. Chromatograms of chloroform extracts. (a) UV detection (b) evaporative light scattering detection.

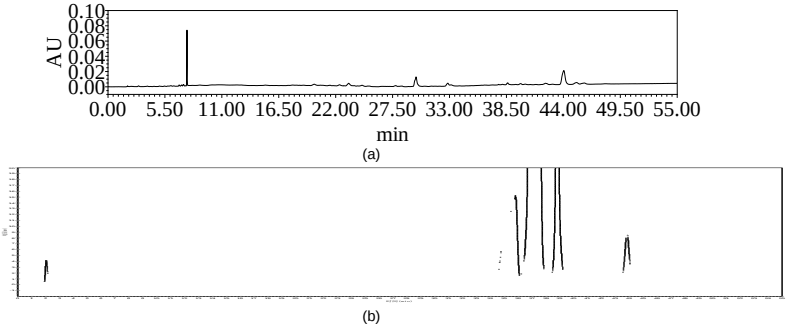

Figure S8. Chromatograms of n-butanol extracts. (a) UV detection, and (b) evaporative light scattering detection.

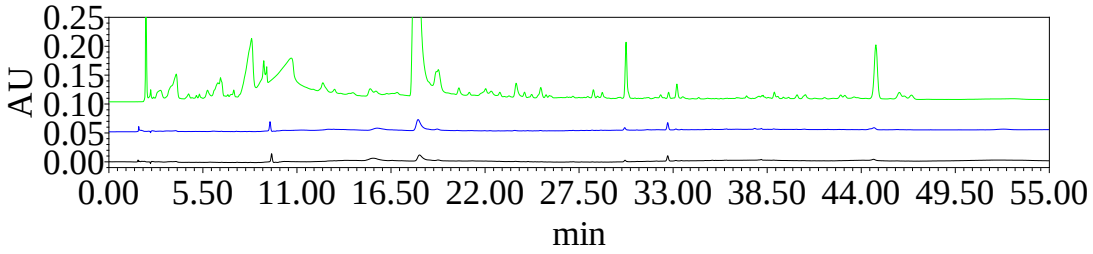

Figure 3. HPLC chromatograms of methanol extracts (from above: 1st, 2nd and 3rd).

extraction process, different extraction temperatures were tested and the results were shown in Figures 4 and 5. The extraction temperature has an effect on the extraction yield (Xue et al., 2012). A suitable temperature can increase diffusion rate, solubility of analytes and the mass-transfer kinetics, while at the same time it decreases the viscosity and surface tension of the solvent (Jeong-Heui et al., 2012; Chen et al., 2011; Liao et al., 2012). It was shown in Figure 4 that as the extraction temperature was increased from 60 to 130°C, with the highest increase from 60 to 75°C. Further elevation of temperature resulted in smaller rise probably due to decomposition of target components (Jeong-Heui et al., 2012; Quénéa et al., 2012).

As shown in Figure 5, extraction at 130°C lead to a different patternin HPLC chromatogram as compared to lower extraction temperatures (marked in the round circle), which we believed to be a sign that some thermally unstable substances changed under this temperature. Therefore, the optimal temperature was 120°C.

Effect of pressure on extraction

Similar to the temperature, an appropriate pressure also can increase the diffusion and density of the extractant to increase extraction efficiency and extractability (Jeong-Heui et al., 2012). Therefore, we investigated a set of pressures from 0.8×10^7 to 1.2×10^7 Pa. In Figure 6, the

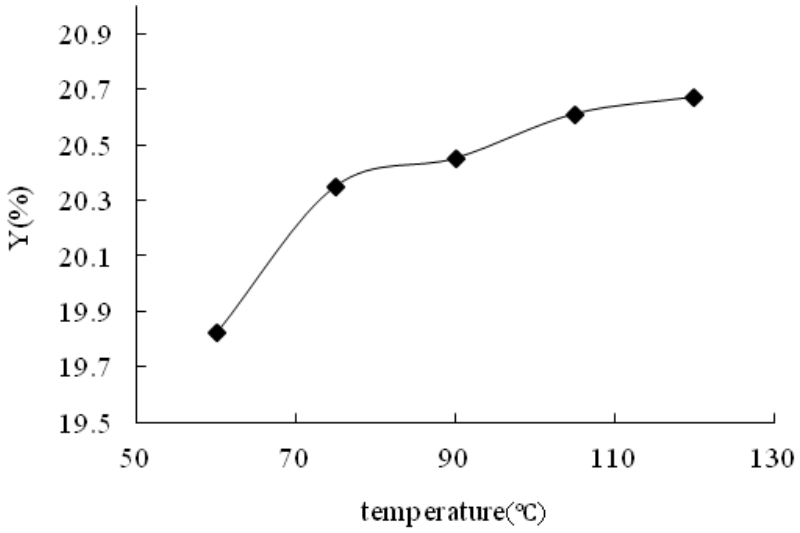

Figure 4. Effect of temperature on the extraction yield

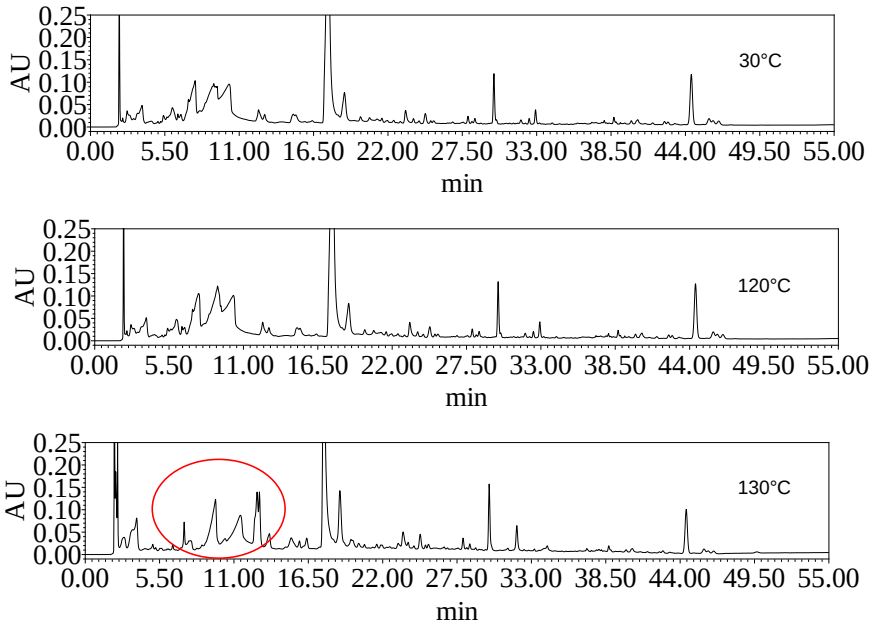

Figure 5. ELSD spectra at various extraction temperatures.

results indicated that the extraction yield increased as the pressure increased except for 0.9 × 107 Pa. However, one should take into consideration that the solvent may leak at higher pressure. So 1.2 × 10^7 Pa was selected as the extraction pressure.

Effect of static extraction time on extraction

The static extraction time cannot be neglected because it influences the dosage of extraction solvent and extraction efficiency (Liao et al., 2012). The length of static extraction time was set between 5 and 25 min (5, 10, 15, 20 and 25 min). The effect of static extraction time on the extraction yield was presented in Figure 7. The extraction yield increased as the static extraction time rose from 5 to 15 min, and then slightly decreased at longer static extraction time. Hence, 15 min was applied for further experiments.

Orthogonal experiment

To investigate the relationships between different variables (temperature, pressure and static extraction time), the 3-level 3-factor orthogonal experiments were employyed (Table 2); while the extraction times was set as

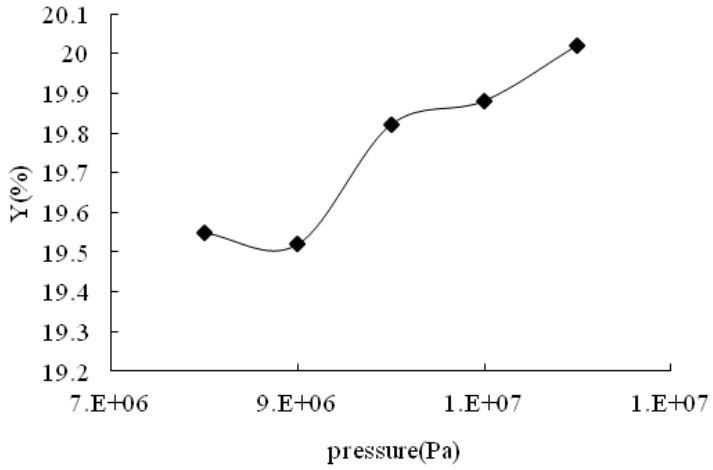

Figure 6. Effect of pressure on the extraction yield.

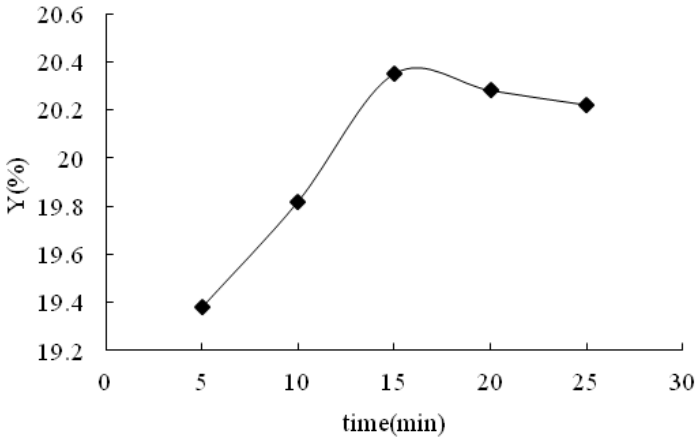

Figure 7. Effect of duration on the extracting yield.

Table 2. Factors and levels of the orthogonal test.

level	A	B	C
	Pressure(Pa)	Temperature(°C)	Extraction time(min)
1	1.2×10^7	120	20
2	1.1×10^7	105	15
3	1.0×10^7	90	10

2. The orthogonal experimental results were shown in Table 3. The influences of various factors on ASE were determined by range analysis and it showed that temperature, pressure and static extraction time had different effects on extraction yield. Static extraction time was the major influence, the second most important factor was pressure, and temperature had minimal impact. Therefore, the optimal conditions were as followed: extraction pressure of 1.2×10^7 Pa, extraction temperature of 105°C; the static extraction time of 15 min.

Comparison between ASE method and soaking extraction method

Soaking extraction and ASE were compared under their optimal conditions, and the results indicated that the extraction yield of ASE method was higher than that of soaking extraction (Table 4). Further advantages are that

Table 3. Results of the orthogonal test.

Number	A	B	C	Y (%)
	Pressure/Pa	Temperature(°C)	Extraction time(min)	(Extraction yield)
1	1.2×10^7	120	20	22.62
2	1.2×10^7	105	15	25.41
3	1.2×10^7	90	10	22.49
4	1.1×10^7	120	15	25.41
5	1.1×10^7	105	10	21.42
6	1.1×10^7	90	20	21.32
7	1.0×10^7	120	10	20.67
8	1.0×10^7	105	20	21.97
9	1.0×10^7	90	15	24.11
Average 1	23.51	22.9	21.97	
Average 2	22.72	22.93	24.98	
Average 3	22.25	22.64	21.53	
R	1.26	0.29	3.45	

Table 4. Effect of different extraction methods on the extracting yields.

Extraction method	Extraction yield (Y/%)
ASE	25.41
Soaking extraction	18.03

Table 5. Comparison between soaking method and ASE method.

	Soaking extraction	ASE
Static extraction time	50 min/time	15 min/time
Ratio of sample/solution	1:8	1:1.5
Operation	complex	simple

the ASE method was highly automated, as well as simpler, more rapid, less solvent consuming and thus environmentally friendlier than the soaking extraction method (Table 5).

Conclusions

The process parameters of ASE were optimized using single-factor experiments and orthogonal experiment. The optimum conditions were to extract for 2 times with a mixture of methanol and ethyl acetate (80:20, V/V) at a pressure of 1.2×10^7 Pa, extraction temperature of 105°C, and static extraction time of 15 min. The ASE method provides many advantages as compared to other extraction methods, and is especially suitable for extracting metabolites from marine fungi in batch.

ACKNOWLEDGEMENTS

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