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Microwave Multi-Stage Countercurrent Extraction of Dihydromyricetin from *Ampelopsis grossedentata*

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Summary

Microwave-assisted extraction (MAE) technique in combination with multi-stage countercurrent extraction (MCE), namely microwave multi-stage countercurrent extraction (MMCE), was evaluated for the extraction of dihydromyricetin (DMY) from *Ampelopsis grossedentata*. Ethanol, methanol and water were used as extract solvents in the MMCE method. Of the three solvents used, water was found to be the best in extracting DMY from *Ampelopsis grossedentata* because it had a good extraction yield and is inexpensive, non-toxic and environmentally friendly. The optimal conditions of MMCE for the extraction of DMY can be determined to be the ratio of the extraction solvent to plant material of 30:1, the extraction time of 5 min, the extraction temperature of 110 °C and the microwave power of 600 W. In addition, the extraction efficiency of the MMCE method was compared with that of the microwave static batch extraction (MSBE) under the optimum extraction conditions. It was found that the MMCE method offered higher extraction efficiency than the MSBE method. Thus, the study suggests that the MMCE method provides an alternative technique in terms of both cost and efficiency.

Key words: microwave-assisted extraction, microwave multi-stage countercurrent extraction, dihydromyricetin, *Ampelopsis grossedentata*, extraction efficiency

Introduction

The development of techniques for the extraction of components from medicinal plants plays an important role in ensuring and providing high-quality natural products to consumers worldwide. Microwave-assisted extraction (MAE) has received an increasing attention as an alternative to solid-liquid extraction for the extraction of secondary metabolites from plants. In contrast with

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the conventional liquid-solid extraction, the use of MAE method results in significant reduction of the extraction time because the microwave heats the solvent rapidly. Furthermore, microwave can keep the temperature gradient to a minimum and accelerate the speed of heat transfer. Additionally, the MAE method allows for a significant reduction in consumptions of solvents and energy and increases extraction efficiency (*1*,*2*). Regarding the extraction, the advantage of microwave heating is the disruption of weak hydrogen bonds promoted by the dipole rotation of the molecules. The migration of dissolved ions increases penetration of the solvent into the matrix and, thus, facilitates the salvation of the target compounds. Microwave acts directly on water molecule within the cells *in situ*, resulting in a rapid increase in the cell temperature. The pressure resulting from the vapor of the cell leads to rupture of the cell membranes and cell walls (*3*,*4*) and generates cavities during MAE operation. This makes the internal material flow out and the solvent penetrates into the cell easily, thereby increasing the extraction efficiency (*5*,*6*).

Multi-stage countercurrent extraction (MCE) as a novel extraction technique combines circulatory dynamic extraction and continuous countercurrent extraction (*7*). The MCE method offers a high extraction yield and is considered to save time and reduce consumption of both energy and solvent. Furthermore, the concentration difference between the solid phase and the liquid phase in different extraction vessels can be maintained as constant as possible during the MCE. Consequently, the use of MCE method can increase extraction efficiency and obtain a rapid and complete extraction of bioactive compounds from herbs (*8*).

Ampelopsis grossedentata, which grows wild south of the Yangtze River region in China, has been used as a Chinese medicinal herb for over 2000 years. Dihydromyricetin (DMY), one of the flavonols (shown in Fig. 1), is a major bioactive component in *Ampelopsis grossedentata* (*9*). DMY has a wide range of therapeutic effects and pharmacological activity, such as antioxidant ability (*10–13*), antiviral and antibacterial properties (*14–16*), high UV-absorbing ability (*17–19*), cell proliferation (*20*) and hepatoprotective properties (*21–23*).

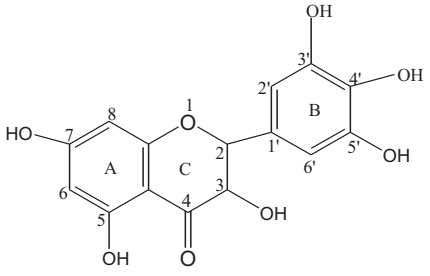

Fig. 1. The chemical structure of DMY

In this study, the MAE technique in combination with the MCE method was used to extract DMY from *Ampelopsis grossedentata*. The extraction conditions of the MMCE technique were evaluated. In addition, the MMCE technique in terms of extraction yield and efficiency was analysed in comparison with the microwave static batch extraction (MSBE) method.

Materials and Methods

Ampelopsis grossedentata

Dried *Ampelopsis grossedentata* leaves produced in the Wuyi mountain region of China were purchased from the Chinese Traditional Medicine Market in Guangzhou. The content of DMY in the dried *Ampelopsis grossedentata* was about 40 % based on the analysis by a TSP-2000A high performance liquid chromatograph (HPLC, Thermoelectric Company, USA).

Reagents

The standard DMY was provided by the Laboratory of Food Chemicals at South China University of Technology. Methanol and double distilled water were of HPLC grade, while other reagents were of analytical grade.

DMY determination

DMY content was determined according to the method of He *et al*. (*24*), with a slight modification. Methanol (20 mL) was added to 1.0 g of the dried *Ampelopsis grossedentata*. Microwave extraction was carried out using the Microwave Accelerated Reaction System (MARS, CEM Corporation, Mattews, NC, USA) (300 W) with closed extraction vessels. The mixture was extracted to stand for 5 min at 60 °C using a magnetic stirrer. The obtained mixture was filtered through filter cloth and the filtrate was then collected. The residue was extracted 6 times. All the filtrates were combined. DMY content was determined by HPLC on a Kromasil 5-μm C_{18} column (250×4.60 mm). The column was eluted by a mobile phase consisting of a binary mixture of methanol and water (methanol/water volume ratio=32:68), spiked with 0.1 % acetic acid as a solvent modifier. The flow rate was maintained at 1.5 mL/min. The eluent was monitored using a UV detection at 291 nm and the DMY content was calculated according to the following formula: $\mathrm{DMY}/\% = [(A+11755)/38121 \times m] \times N \times V \times 10^{-3} \times 100$; where A, N and V were the sample peak area, the sample dilution times, and the total volume (mL) of the extract solution, respectively, while m was the mass (in mg) of *Ampelopsis grossedentata*.

Five-stage MMCE

A five-stage MMCE was carried out according to the method of Wang *et al*. (*7*), with some modifications. Five vessels were used simultaneously in the MARS. This MARS can provide 14 simultaneous extractions in Teflon-lined closed vessels which allow the extraction solvent to be heated above the boiling point of the solvent. The cover for the extraction vessel used as control had a small hole through which a fiber-optic temperature probe was inserted to monitor the internal temperature. The MMCE was divided into the conditioning stage (the first phase) and the extraction stage (the second phase). In the conditioning stage, the *Ampelopsis grossedentata* sample was mixed with water in each vessel and was continuously extracted for a predetermined period of time. The different extraction time used for various vessels was predesigned to produce a systematic extract concentration along the vessel sequence. The concentration gra-

dients for the five-stage MMCE were obtained by extracting the sample in vessels 1–4 for different intervals ($T/5$, $2T/5$, $3T/5$ and $4T/5$ respectively, where T is the total running time of the MMCE). The extraction in vessel 4 was transferred to vessel 5, while the extraction in vessel 1 was exchanged with vessel 3. Meanwhile, fresh solvent and fresh sample were added to vessels 4 and 5, respectively. The MMCE was performed according to a series of five steps. The duration of every step was $T/5$. Each step included four basic operations. For example, the first step consisted of the following operations: (*i*) sample extraction for $T/5$; (*ii*) discharge of the residue from vessel 4 and collection of the extract from vessel 5; (*iii*) transfer of solvent from 1→4, 2→5, 3→1 and 4→2; and (*iv*) addition of fresh sample and fresh water to vessels 4 and 3, respectively. This extraction was followed by the second phase, while the extraction sequence from step 1 to 5 was repeated. The collected extract from two cycles was analyzed by HPLC to determine DMY concentration. The extraction yield (Y) was calculated according to the following formula: $Y/\% = [(C \times V)/(m \times D)] \times 100$, where Y was the DMY extraction yield (%), and C, V and m were the DMY concentration (mg/mL) of the collected extract, the volume (mL) of the collected extract and total mass (mg) of fresh *Ampelopsis grossedentata* from the two cycles, respectively, while D was the DMY content (%) of *Ampelopsis grossedentata*.

Microwave static batch extraction (MSBE)

Five vessels containing the same amount of *Ampelopsis grossedentata* and the same volume of water for the MSBE or MMCE method were simultaneously run through the MARS. Other extraction conditions were the same as for the MMCE method described above.

Determination of grinding degree for Ampelopsis grossedentata

Ampelopsis grossedentata leaves were divided into 6 grades according to the grinding degrees (whole leaves, 20, 40, 80, 180 and >180 mesh) and then used for the analyses of extraction yield. Mesh was the number of aperture per square inch. The extraction conditions used were the temperature of 95 °C, the time of 2 min, the ratio of plant material to solvent of 1:20 and the microwave power of 600 W.

Determination of solvents

The extraction conditions were the time of 5 min, the ratio of solvent to plant material of 20 (by volume per mass) and the microwave power of 600 W at the temperature of 60 °C for methanol, 75 °C for ethanol, or 95 °C for water as a solvent. The sediment yield (Y_s) was calculated according to the following formula: $Y_s/\% = [(C_e - C_s)/C_e] \times 100$, where Y_s was the DMY sediment yield (%) and C_e was the DMY concentration (mg/mL) in the extract, while C_s was the DMY concentration (mg/mL) in the solvent after 24 h at 20 °C.

Orthogonal method

A 4-factor plus 3-level L_9 (3^4) orthogonal experimental design was applied to determine the optimal conditions (*25*). The orthogonal experimental design is an effective method for investigating the effects of the factor on the extraction yield by reducing the experimental numbers. Furthermore, the optimal extraction conditions and the significance of every factor's effect on the yield can be obtained. The factor levels of the orthogonal experiments are shown in Table 1. Four factors (time, temperature, solvent to plant material ratio (M/S) and power) of the five-stage MMCE were considered to evaluate the variables in the design. The experiments were conducted three times and the data were analyzed by analysis of variance (ANOVA). Water was used as solvent in all orthogonal experiments.

Table 1. Factors and levels of the L_9 (3^4) orthogonal experiments for the MMCE

Level	Factor			
	A	B	C	D
	Time / min	t / °C	M/S / g/mL	Power / W
1	5	70	1:10	300
2	10	90	1:20	600
3	20	110	1:30	900

Results and Discussion

Evaluation of grinding degrees for extraction yield of DMY

No obvious increase in the extraction yield was observed when the shiver gradient was <80 mesh (Fig. 2). Considering that it was very difficult to obtain fine granularity of *Ampelopsis grossedentata*, the intact leaves were used in this study.

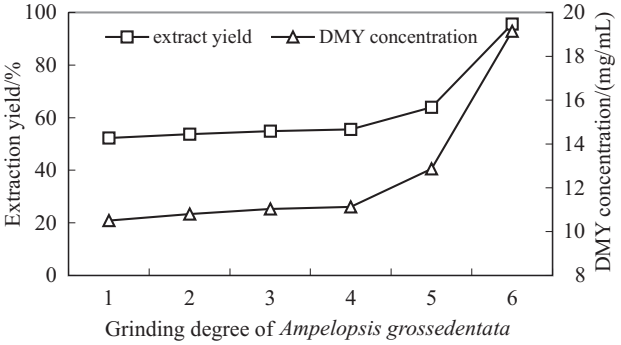

Fig. 2. Effects of grinding degrees on extraction efficiency of DMY from *Ampelopsis grossedentata*. 1–6, whole leaves, and 20, 40, 80, 180 and >180 meshes

Effect of solvents on DMY yield

Appropriate solvents are essential to achieve an optimal extraction. The MAE method depends on the dielectric susceptibility of solvents and microwave energy that causes molecular motion by migration of ions and rotation of dipoles (*26*). To determine an effective solvent for the DMY extraction from *Ampelopsis grosseden-*

tata, methanol, ethanol and water were used in this study. The DMY extraction yield and sediment yield were shown in Table 2. The highest extraction yield with no sediment was noticed when methanol was used as the solvent. It is interesting to notice that water as the solvent extracted DMY well (83.62 % of the sediment yield),

Table 2. Extraction yield and sediment yield of DMY by different solvents*

Solvent	Y/%	Y_s/%
Methanol	60.02±0.82	No sediment
Ethanol	55.44±0.66	No sediment
Water	56.39±0.62	83.62

*Y_s, DMY sediment yield and Y, DMY extraction yield

because water is inexpensive, nontoxic and environmentally friendly. Thus, water can be chosen as the optimal solvent to extract DMY from *Ampelopsis grossedentata* in terms of both extraction cost and safety. Guo *et al*. (*27*) have recently demonstrated that the extraction of puerarin from *Radix puerariae* by MAE method took 1 min when water was used as the extraction solvent, because water not only efficiently absorbed the microwave power but it also readily dissolved the polar active constituents.

DMY content in solid and liquid phases after conditioning process

Fig. 3 showed the DMY content when various extraction conditions were used. However, there was no direct correlation for DMY content with extraction time.

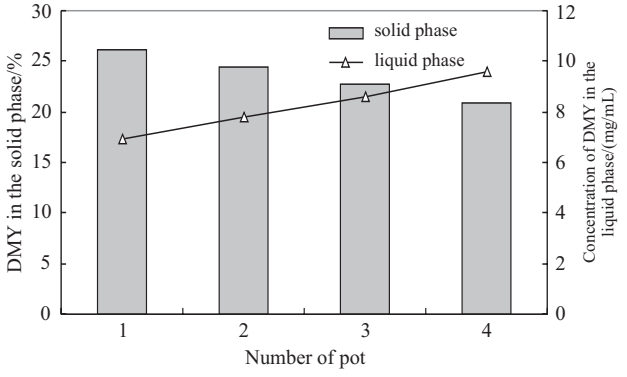

Fig. 3. DMY content in the solid and liquid phases after conditioning process

DMY content after exchange

DMY content in the solid and liquid phases of vessels 1–4 is shown in Fig. 4. There were similar differences in the concentration between the solid phase and the liquid phase in each vessel, especially in vessels 1–3. The result indicated that there was higher DMY concentration in the liquid phase, responding to the higher DMY content in the solid phase.

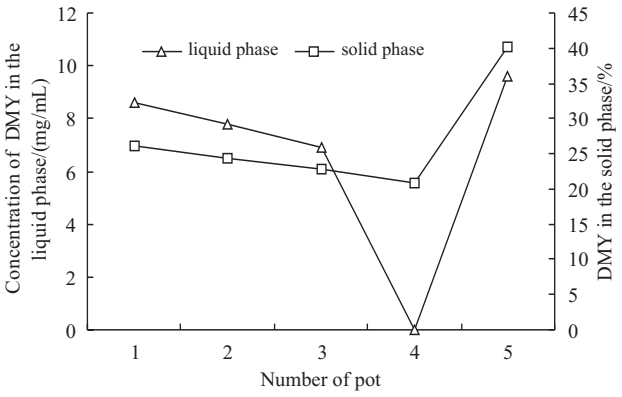

Fig. 4. DMY content in the solid and liquid phases after the exchange of the extract

Determination of extraction conditions for MMCE

The extraction conditions of the MMCE method included temperature, time, microwave power and material/solvent ratio. The extraction efficiency was greatly influenced by the extraction temperature in the five-stage MMCE, as indicated by the high F-values (Tables 3 and 4). The mean values of the extraction yields were 42.2 % at 70 °C and 75.6 % at 110 °C. Ruan *et al*. (*28*) reported that temperature greatly affected DMY solubility in water, while elevated temperature could enhance the extraction efficiency as a result of the increased diffusivity of the solvent into cells and an enhanced DMY desorption from the cells (*29*). However, the extract yield of DMY decreased when the temperature was over 110 °C in this study, possibly due to unstable DMY in hot water. Lin *et al*. (*30*) reported that the ultraviolet spectrum changed when DMY was treated in boiling water and confirmed further that the B-ring of DMY was cracked and then oxidized to quinine. A similar result was observed in notoginseng saponins from *Panax notoginseng* (*31*).

The extraction yield increased notably with increasing ratio of solvent to plant material (Tables 3 and 4) but the sediment yield decreased greatly when the ratio of solvent to plant material was higher than 30 (by volume per mass). In addition, microwave energy might be absorbed and dispersed by a larger volume of solvent, which is disadvantageous to the MMCE method. Thus, the ratio of solvent to plant material of 30 (by volume per mass) was suitable for the five-stage MMCE.

Extraction time affected markedly the extraction efficiency. In this study, as the extraction time increased from 5 to 10 min the extraction yield increased by 3.8 %, but the yield decreased from 68.6 to 52.7 % when the extraction time extended from 10 to 20 min. Similarly, Hao *et al*. (*32*) found that the extraction rate of artemisinin increased with increasing duration of microwave radiation and then decreased, and suggested that overexposure to microwave could cause artemisinin loss. Pan *et al*. (*33*) reported that the percentage of the extraction of tanshinones decreased with increasing extraction time. In addition, the increased dissolution of the polymer matrix from cells probably caused an increase in viscosity (*34*).

Table 3. Arrangements and results of the orthogonal experiments for the MMCE

Number	A	B	C	D	Extraction yield/%				
					Y_1	Y_2	Y_3	$\Sigma=Y_1+Y_2+Y_3$	Mean value±S.D.
1	1	1	1	1	35.8	37.6	37.3	111	36.9±1.0
2	1	2	2	2	71.4	68.7	67.5	208	69.2±2.0
3	1	3	3	3	87.9	88.7	87.7	264	88.1±0.5
4	2	1	2	3	46.4	43.8	45.4	136	45.2±1.3
5	2	2	3	1	83.7	85.3	85.4	254	84.8±0.9
6	2	3	1	2	74.3	76.9	75.7	227	75.6±1.3
7	3	1	3	2	45.4	44.1	43.8	133	44.4±0.8
8	3	2	1	3	50.9	52.6	49.5	153	51.0±1.6
9	3	3	2	1	63.7	64.1	61.5	189	63.1±1.4
I	583	380	491	554				Total=1675	
II	617	615	533	568					
III	475	680	651	553					
I/9	64.8	42.2	54.6	61.6					
II/9	68.6	68.3	59.2	63.1					
III/9	52.8	75.6	72.3	61.4					
R	15.8	33.4	17.7	1.7					

I, the total of level 1; II, the total of level 2; III, the total of level 3; and R, the difference between the maximum and the minimum; A, extraction time; B, extraction temperature; C, material/solvent ratio; D, microwave power

Table 4. Results of the ANOVA analysis

Source	Sum of squares of variance	Degree of freedom	Mean square	F-value	p
A	1222	2	611	128.8	<0.01
B	5535	2	2767	583.4	<0.01
C	1529	2	764.5	161.2	<0.01
D	15.63	2	7.815	1.648	>0.05
Error	85.38	18	4.743		
Total	8387	26			

$F_{0.01(2,18)}=6.01$ and $F_{0.05(2,18)}=3.55$; A, extraction time; B, extraction temperature; C, material/solvent ratio; D, microwave power

No obvious effect of the microwave power on the DMY extraction efficiency was observed (Table 3), as indicated by the low F-value (Table 4). Thus, the power of 600 W was suitable for the five-stage MMCE method in this study.

Based on the above analysis, the optimal extraction conditions were determined to be $A_1B_3C_3D_2$, where A_1 was the first level of extraction time of 5 min (1 min for every step), B_3 was the third level of extraction temperature of 110 °C, and C_3 was the third level of ratio of solvent to plant material of 30 (by volume per mass), while D_2 was the second level of power of 600 W.

Comparison of the MMCE method with the MSBE method

Figs. 5 and 6 represent the variation of DMY content in the solid phase and the liquid phase for the MSBE and MMCE methods. The extraction yield using the MMCE method was 88.5 %, as compared to 63.1 % using the MSBE, while the DMY content in the residue using the MMCE method was 4.6 %, compared to 14.8 % using the MSBE method. Thus, the MMCE method exhibited better DMY extraction efficiency from *Ampelopsis grossedentata* than the MSBE method.

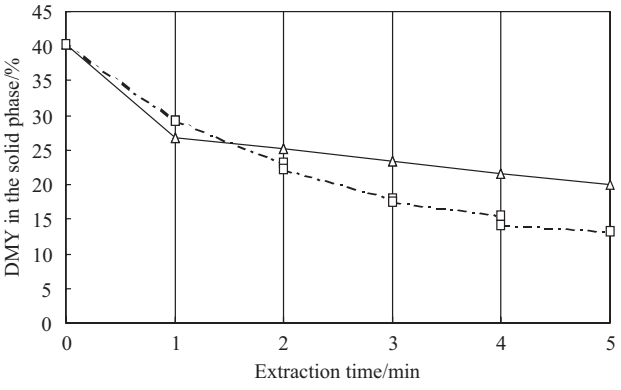

Fig. 5. Percentage of DMY in the solid phase for both MSBE and five-stage MMCE; □ ----five-stage MMCE and △— MSBE

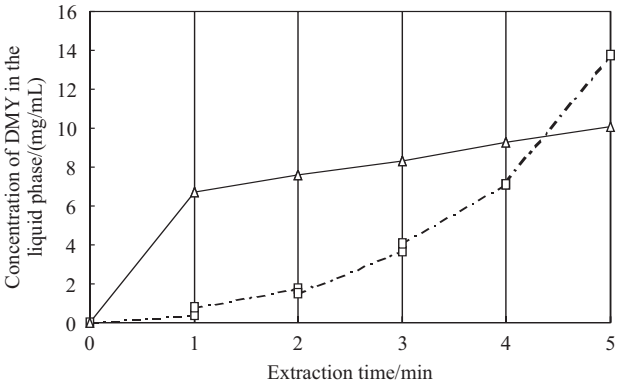

Fig. 6. Concentration of DMY in the liquid phase for both MSBE and five-stage MMCE; □ –––-five-stage MMCE and △— MSBE

Conclusions

The MMCE technique is an original combination of the MAE and MCE methods and it exhibited an obvious difference in the concentration between the extract phase and the residue phase, compared to the MSBE method. The MMCE method was effective in increasing extraction yield. The extraction yield using the MMCE method was higher (20–30 %) than that using the MSBE method under the similar experimental conditions. The MMCE method provided an attractive alternative in both the cost and efficiency of the DMY extraction and it can be expected to be applied in other natural products extracted from plants.

Acknowledgements

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