Optimization of Dynamic Microwave-Assisted Extraction of Dihydromyricetin from *Ampelopsis grossedentata* using Response Surface Methodology

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Summary: Dihydromyricetin is a natural flavonoid and principal component of the Chinese herbal tea, *Ampelopsis grossedentata*, with numerous health-promoting bioactivities. Response surface methodology (RSM) was employed to optimize the extraction of dihydromyricetin from *Ampelopsis grossedentata* leaves using dynamic microwave assisted multi-stage countercurrent extraction. The influence of temperature, pH, time and solvent/material ratio on the yield of extraction was investigated. It was found that the extraction data were sufficiently fitted into a second-order polynomial model ($R^2 = 0.9992$). The extraction parameters of temperature, pH and solvent/material ratio, the quadrics of time, temperature and pH, and the interaction between each two of the four extraction parameters had a significant effect on the yield. The optimal conditions for extracting dihydromyricetin were predicted to be: temperature, 96.8 °C; time, 8.8 min; solvent/material ratio, 26.4/1 and pH, 5.3. Under those conditions, the predicted yield was 92.3% of dihydromyricetin in the leaves.

Keywords: Dihydromyricetin, Ampelopsis grossedentata, Dynamic microwave-assisted extraction, Response surface methodology

Introduction

Ampelopsis grossedentata (Hand.-Mazz) W. T. Wang is a vine plant which is found abundantly in southern China, especially in regions south of the Yangtze River [1]. As a Chinese herbal medicine, dry leaves of the plant are used for a number of purposes including treatment of inflammation, detoxification and for improving blood circulation [2]. Leaves of the plant are also brewed for tea, known as rattan tea, which is widely consumed in southern China, and is believed to provide many health-promoting functions [3, 4]. In recent years, many of the perceived pharmaceutical and general health-promoting properties of *Ampelopsis grossedentata* have been confirmed by modern scientific investigations, including clinical studies [5-7].

The principal bioactive component of *Ampelopsis grossedentata* is 3,5,7,3',4',5'-hexahydroxyl-2,3 dihydroflavonol, commonly known as dihydromyricetin (DMY) and ampelopsin [8]. As a natural aglycone flavonoid with six hydroxyl groups, especially the pyrogallol group (the three adjacent hydroxyl groups at 3'-, 4'- and 5'- positions), dihydromyricetin is a strong antioxidant with comparable antioxidant capacity to quercetin [9]. Recent studies have shown that it has many other health-promoting properties including anti-inflammatory [10], antimicrobial [11, 12], cholesterol-lowering [13], hepta-protective [7] and anti-cancer activities [14-16]. Therefore, this compound is regarded as a bioactive ingredient with a strong potential of application in a wide range of functional food and pharmaceutical products. *Ampelopsis grossedentata* is extremely rich in dihydromyricetin, with young leaves containing up to 20% (dry matter) of this compound. This makes the plant a valuable source for large scale production of dihydromyricetin.

Microwave-assisted extraction is a relatively new extraction technique which has been shown to significantly improve extraction efficiency over traditional batch extraction procedures. The technique has been used to extract bioactive components from a wide variety of plant materials with much improved yield compared with batch extraction [17-19]. Another technique that is attracting growing attention in recent years is the multi-stage countercurrent extraction process (MCE). The key principle of the MCE process is that the extracts in different stages of extraction are frequently exchanged, whereby establishing a constant and substantial concentration differential between the extraction medium and the matrix [20].

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This constant concentration differential allows the efficiency of extraction to be greatly improved. Furthermore, because the extraction medium is recycled at different stages of extraction, the technology also requires much less extraction medium [20]. We have previously reported the development of a novel extraction technique which combines microwave assisted extraction with multi-stage countercurrent extraction processes (MAMCE) [21]. Compared with conventional microwave-assisted extraction, the MAMCE process was found to be highly efficient for the extraction of dihydromyricetin from *Ampelopsis grossedentata*. The aim of the study was to systematically examine the influence of temperature, pH, solvent/material ratio and time on extraction efficiency and to optimize the conditions of MAMCE for the extraction of dihydromyricetin from *Ampelopsis grossedentata.*

Experimental

*Plant sample and reagents***:** Dry leaves of *Ampelopsis grossedentata* were purchased from a local market of Chinese traditional medicine in Guangzhou, China. The leaves contained approximately 40g dihydromyricetin/100 g dry matter as determined by HPLC (described below). Standard dihydromyricetin (purity > 99.4%) was kindly provided by the Laboratory of Food Chemicals, South China University of Technology. Methanol and de-ionized water were of HPLC grade; all other reagents were of analytical grade unless otherwise stated.

Microwave assisted multi-stage countercurrent extraction (MAMCE) Microwave assisted MCE was carried out using the Microwave Accelerated Reaction System (MARS, CEM Corporation, Mattews, NC, USA). Five concurrent extraction pots and a control pot, all 100 ml in volume, were placed symmetrically in the pot holders of the system to ensure that each pot will receive exactly the same microwave treatment. The lid for the control pot had a small hole through which a fibre-optic temperature probe was fitted to monitor and control the internal temperature.

Details of the MAMCE procedure was described elsewhere [21]. Briefly, the extraction was carried out in two phases: the conditioning phase and the extraction phase. The purpose of the conditioning phase was to establish a pre-designed initial concentration gradient between the sample matrix and extraction solvent in all the extraction pots. The extraction phase consisted of five extraction stages, each with four basic operations: sample extraction, discharge of sample residue from (and adding fresh sample to) one of the pots, collection of extract from one of the pots and transfer of extracts. The microwave power used was 600 W throughout the extraction process in all experiments. Extracts from two extraction cycles were pooled and analyzed by HPLC to determine the concentration of dihydromyricetin.

Optimization of extraction conditions using response surface methodology Four extraction parameters, namely extraction time, temperature, material/solvent ratio and pH, were investigated to determine their influence on the extraction yield of DMY from the leaves of *Ampelopsis grossedentata* using the response surface methodology (RSM). The experiments were designed according to the central composite design (CCD) using the software Design Expert 7 (Stat-Ease, MN) with the experimental conditions shown in Table-1. Individual experiments were carried out in random order. A second-order polynomial equation was used to express the extraction yield as a function of the independent variables as follows:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4 \quad (1)$$

where Y represents the extraction yield expressed as the proportion (%) of extracted DMY in the total amount of DMY in *Ampelopsis grossedentata* leaves and aij are co-efficients of the equation.

Table-1: Experimental conditions and results for the extraction of DMY.

Experiment No.	Time (min) (A)	Temperature (°C) (B)	Solvent /material ratio (ml/g) (C)	pH (D)	Yield (%)
1	10	100	20	7	86
2	5	70	10	3	41
3	5	100	30	11	62
4	5	100	10	11	55
5	15	100	30	3	91
6	10	85	20	7	78
7	10	85	20	7	78
8	15	85	20	7	75
9	10	85	20	7	78
10	10	70	20	7	44
11	5	70	30	3	53
12	10	85	30	7	85
13	10	85	10	7	70
14	10	85	20	7	78
15	15	100	10	3	66
16	10	85	20	11	61
17	10	85	20	7	78
18	15	70	30	11	39
19	5	85	20	7	74
20	15	70	10	11	35
21	10	85	20	3	87

Determination of dihydromyricetin The dihydromyricetin content in *Ampelopsis grossedentata* and the extracts was determined according to the method of He et al. [22], with slight modifications. For the measurement of dihydromyricetin content in the leaves, 1.0 g of dry *Ampelopsis grossedentata* leaves was mixed with 20 ml methanol in a MARS apparatus (CEM Corporation, Mattews, NC, USA). The mixture was extracted at 60 °C for 5 min with a microwave power of 600W, filtered with sintered disc filter funnel and the filtrate collected. The extraction was repeated on the residue 6 times and the filtrates were combined. Dihydromyricetin in the filtrate or in the extracts, obtained as described above, was determined by HPLC using a TSP-2000A HPLC system (Thermo Electron Corporation, MA, USA) with a Kromasil 5µm C18 column (250 mm x 4.6 mm, i.d.). Samples were filtered through a 0.45 µ membrane filter and were injected without further treatment. Elution was performed isocratically with a mixture of methanol and water at a 32/68 (v/v) ratio, spiked with 0.1% acetic acid (w/v) as a solvent modifier. The flow rate was maintained at 1.5 ml/min and peaks were detected by a UV detector at 291 nm. Concentration of dihydromyricetin was obtained by referring to a standard curve constructed from the standard compound analyzed the same way. Extraction yield (extraction rate) was calculated according to the following formula:

$$Y\,(\%) = ((C \times V)/(M \times D)) \times 100\%$$

where Y was extraction yield (%); C and V were the dihydromyricetin concentration (mg/mL) and volume of the collected extract, respectively; M was the total mass (mg) of *Ampelopsis grossedentata* samples used and D was the concentration of dihydromyricetin (% dry matter) in the sample.

Results and Discussion

Model fitting

A number of extraction experiments were conducted according to the RSM design, and Table-1 presents the extraction conditions and yields of dihydromyricetin in all the experiments. The experimental data were applied to a quadratic polynomial model of the software, which resulted in the following equation.

$$Y(\%)=-537.20125+6.31319\times A+12.29278\times B+0.21160\times C+1.26247\times D-0.073333\times A\times B+0.025000\times A\times C+0.38750\times A\times D+0.013333\times B\times C-0.033333\times B\times D-0.081250\times C\times D-0.15962\times A^2-0.059958\times B^2-9.90446E\text{-}003\times C^2-0.28065\times D^2$$

where Y is extraction yield (%), A is extraction time (min), B is extraction temperature, C is solvent/material ratio and D is the pH.

Table-2 presents the results of ANOVA for the model. As can be seen, the F-value and P-value of the model were 567.12 and <0.0001, respectively, demonstrating that the model was highly significant. All the linear parameters, with the exception of extraction time, quadric parameters (except solvent/ material ratio) and the interactive parameters are significant model terms.

Based on the model, the optimal extraction conditions predicted were temperature, 96.8 °C; time, 8.8 min; solvent/material ratio, 26.4/1 and pH 5.3. Under those conditions, the predicted extraction yield for DMY was 92.3%.

Table-2: ANONA results of the fitted quadratic model for extraction yield of DMY.

Term	Sum of squares	Degree of freedom	Mean square	F value	Prob>F	Significance
Model	**5796.29**	**14**	**414.02**	**567.12**	**<0.0001**	**Yes**
A	**0.50**	**1**	**0.50**	**0.68**	**0.4396**	**No**
B	**882.00**	**1**	**882.00**	**1208.15**	**<0.0001**	**Yes**
C	**396.90**	**1**	**396.90**	**543.67**	**<0.0001**	**Yes**
D	**338.00**	**1**	**338.00**	**462.99**	**<0.0001**	**Yes**
AB	**48.40**	**1**	**48.40**	**66.30**	**0.0020**	**Yes**
AC	**12.50**	**1**	**12.50**	**17.12**	**0.0061**	**Yes**
AD	**96.10**	**1**	**96.10**	**131.64**	**<0.0001**	**Yes**
BC	**32.00**	**1**	**32.00**	**43.83**	**0.0006**	**Yes**
BD	**6.40**	**1**	**6.40**	**8.77**	**0.0253**	**Yes**
CD	**84.50**	**1**	**84.50**	**115.75**	**<0.0001**	**Yes**
A^2	**40.65**	**1**	**42.62**	**55.68**	**0.0003**	**Yes**
B^2	**464.60**	**1**	**464.60**	**636.40**	**<0.0001**	**Yes**
C^2	**2.50**	**1**	**2.50**	**3.43**	**0.1135**	**No**
D^2	**51.48**	**1**	**51.48**	**70.51**	**0.0002**	**Yes**
R^2	**0.9992**					
Signal/noise ratio	**78.380**					

Surface response and interactions between extraction parameters

Extraction time (A) and temperature (B) Fig. 1 presents the influence of extraction time and temperature, as well as their interactions on the yield of DMY. The influence of extraction time was markedly smaller than that of the temperature. When temperature was relatively low, the yield increased only slightly with increases in extraction time. However, when temperature was raised to about 80 °C and higher, the yield began to decline with increasing time, indicating that thermal decomposition of DMY may have occurred. The effect of temperature on extraction yield was highly significant. When temperature was low, the yield was kept at a low level even with prolonged extraction. With rising temperature, the yield increased rapidly although some thermal degradation of DMY occurred at higher temperatures. Thus, optimal extraction conditions were found in high temperature and short extraction time regions.

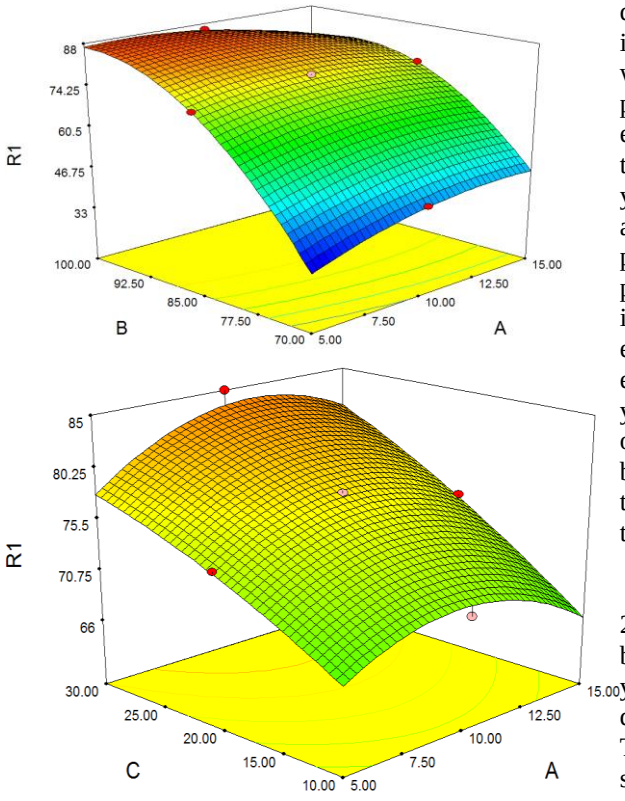

Fig. 1: Interactive influence of extraction time, temperature and solvent/material ratio on the extraction yield of dihydromyricetin.

A, Extraction time (min); B, Temperature (˚C); C, Liquid-material ratio (ml:g); R1, Extraction yield (%).

Extraction time and solvent/material ratio Fig. 1 also shows the interactive influence of extraction time and solvent/material ratio on the extraction yield of DMY. When extraction time is shorter than 10 min, an increase in extraction time led to a rise in extraction yield. However, when extraction time was increased further, the yield of DMY declined, indicating that thermal decomposition probably occurred with prolonged exposure to high temperatures. This pattern was rather prominent when the solvent/solid ratio is low and it became less so with increasing solvent/material ratio. In general, higher yields were obtained at high solvent/material ratios where the influence of extraction time was less significant. This was probably a reflection of the good solubility of DMY at relatively high temperatures used for the experiments.

Extraction time and pH Fig. 2 shows the interactive influence of extraction time and pH on the yield of DMY. Within the range studied, pH had a decisive influence on the extraction yield, which increased rapidly with lowering pH. Highest yield was obtained at pH 3 while lowest yield occurred at pH 11. At pH 3, highest yield was obtained for short extraction times and, interestingly, longer extraction times resulted in small decreases in the extraction yield, indicating possible decomposition or structural alteration to DMY when exposed to low pH for prolonged periods. Under high pH conditions, prolonging extraction time only led to marginal increases in the yield. Li *et al* [21] showed that when extraction of DMY was conducted at pH 3 for extended period of time, the extract showed a slight yellowish colour. The solution of pure DMY, on the other hand, is colourless. The yellow colour was believed to be due to either impurity extracted from the sample or decomposition products. The results in the present study appeared to agree with that finding.

Temperature and solvent/material ratio Fig. 2 also illustrates the influence of the interactions between temperature and solvent/material ratio on the yield of DMY. It is clear that temperature had a more decisive influence on the extraction yield. Temperature also affected the influence of solvent/material ratio on DMY yield. At low temperatures around 70 °C, increasing the solvent/material ratio from 10 to 30 only caused incremental rises in DMY yield. At high temperatures around 100 °C, however, increases solvent/material ratios caused drastic increases in the yield.

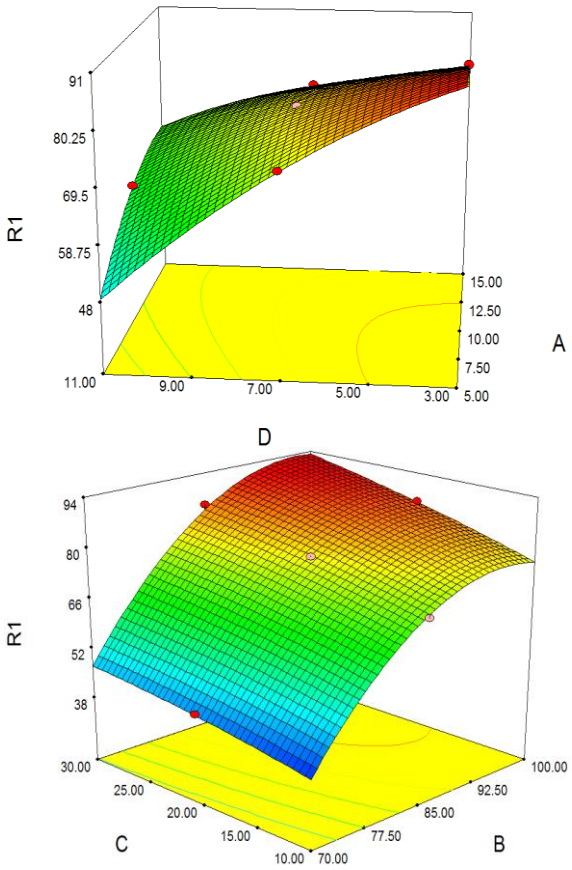

Fig. 2: Interactive influence of extraction time, pH, temperature and solvent/material ratio on the extraction yield of dihydromyricetin.

A, Extraction time (min); B, Temperature (˚C); C, Liquid-material ratio (ml:g); R1, Extraction yield (%), D, pH.

Temperature and pH Fig. 3 shows the interactive influence of temperature and pH on the extraction yield of DMY. Both temperature and pH strongly affected the yield but the influence of temperature was more decisive than pH. When temperature was low, the extraction rate was rather low regardless of the pH. When temperature was high, lowering pH resulted in substantial increases in the yield.

Solvent/material ratio and pH Fig. 3 also illustrates the influence of interactions between pH and solvent/material ratio on the extraction yield of DMY. Of the two parameters, the influence of pH was much more decisive than solvent/material ratio. At high pH values around 11, the yield was low regardless of the solvent/material ratio. At low pH values around 3 however, the yield was much higher and it increased significantly with increasing solvent/material ratio.

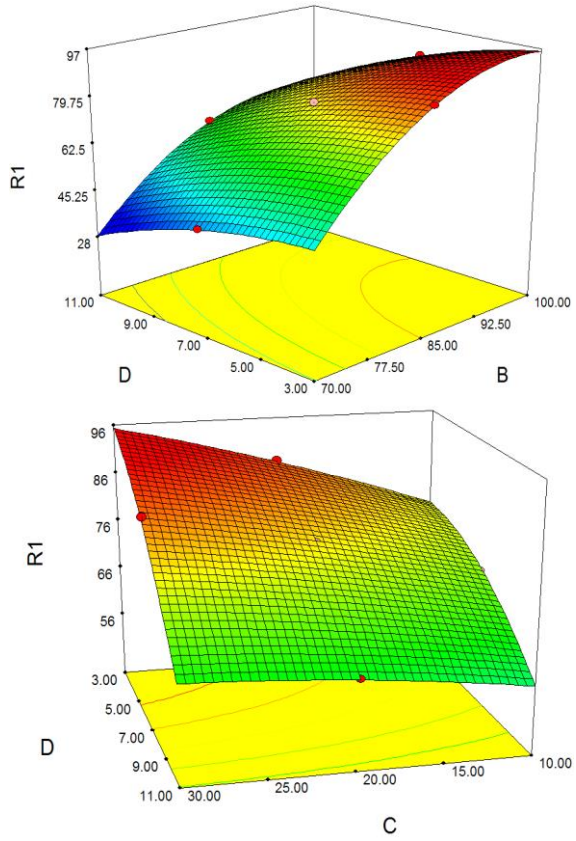

Fig. 3: Interactive influence of temperature, pH and solvent/material ratio on the extraction yield of dihydromyricetin.

B, Temperature (˚C); C, Liquid-material ratio (ml:g); D, pH; R1, Extraction yield (%).

There are only a few reports in the literature on the extraction of DMY from *Ampelopsis grossedentata* and other plants. These studies have explored the use of several extraction techniques including countercurrent, reflux, microwave and ultrasound assisted techniques [22, 23]. Li *et al* [22] showed that both reflux and microwave-assisted extraction were very efficient in extracting DMY from *Ampelopsis grossedentata* leaves and achieved an extraction yield of slightly over 95%. However, they used a 70% ethanol-water solution as the extraction medium, and thus the result cannot be directly compared with ours as hot water alone was used as the extraction medium in the present study. We have developed a microwave assisted multi-stage countercurrent extraction (MAMCE) technique and found it to be efficient in extracting DMY from *Ampelopsis grossedentata* leaves [21, 23]. Using this technique with orthogonal method of experimental design, a yield of 83.6% DMY in the leaf sample was obtained. However, none of the studies attempted to

optimize the extraction by response surface methodology (RSM). Using MAMCE coupled with RSM, an improved yield of 92.3% (w/w) was achieved in the present study.

Conclusion

In the present study a second-order polynomial model was developed to describe the influence of extraction conditions on the yield of DMY from *Ampelopsis grossedentata* using RSM. The model was shown to be capable of predicting the extraction yield under different extraction conditions within the ranges studied. Extraction temperature, pH and solvent to material ratio, the quadrics of time, temperature and pH, as well as the interactive terms of the four extraction parameters significantly influenced the yield of DMY. The optimal conditions for extracting dihydromyricetin as predicted by the model are: temperature, 96.8 °C; time, 8.8 min; solvent/material ratio, 26.4/1 and pH 5.3. Under these conditions, the predicted extraction yield was 92.3% (w/w) of the original dihydromyricetin in the *Ampelopsis grossedentata* leaves.

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