



## Analytical Methods

# Simultaneous extraction, identification and quantification of phenolic compounds in *Eclipta prostrata* using microwave-assisted extraction combined with HPLC–DAD–ESI–MS/MS

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## ABSTRACT

A simple and rapid method was developed using microwave-assisted extraction (MAE) combined with HPLC–DAD–ESI–MS/MS for the simultaneous extraction, identification, and quantification of phenolic compounds in *Eclipta prostrata*, a common herb and vegetable in China. The optimized parameters of MAE were: employing 50% ethanol as solvent, microwave power 400 W, temperature 70 °C, ratio of liquid/solid 30 mL/g and extraction time 2 min. Compared to conventional extraction methods, the optimized MAE can avoid the degradation of the phenolic compounds and simultaneously obtained the highest yields of all components faster with less consumption of solvent and energy. Six phenolic acids, six flavonoid glycosides and one coumarin were firstly identified. The phenolic compounds were quantified by HPLC–DAD with good linearity, precision, and accuracy. The extract obtained by MAE showed significant antioxidant activity. The proposed method provides a valuable and green analytical methodology for the investigation of phenolic components in natural plants.

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## 1. Introduction

Phenolic compounds are broadly distributed in plants and have various biological activities in the prevention and treatment of many diseases such as cardiovascular and cerebrovascular diseases, liver disease, inflammation, cancer, and AIDS (Mink et al., 2007; Terao, Kawai, & Murcita, 2008). The edible natural products that contain phenolic compounds would help to promote health benefits (Yahia, 2010). There is therefore strong current interest in discovering active polyphenols in edible natural products with medicinal properties. Although different approaches toward the analysis of polyphenols have been attempted, the simultaneous extraction and determination of different groups of polyphenols by a single analysis remain difficult. Satisfactory extraction and separation are the key methods for the qualitative and quantitative analysis. Conventional extraction methods such as heat reflux extraction and maceration are time consuming, hazardous to health, and expensive because of the required use of high volumes of organic solvents. In addition, the extraction methods may influence the antioxidant capacity of the sample (Michiels, Kevers, Pincemail, Defraigne, & Dommes, 2012).

In general, phenolic compounds are chemically unstable. Therefore, developing an efficient extraction method is essential for the accurate identification and quantification of phenolic components that exist in plants. Microwave-assisted extraction (MAE) has been used in life science and environmental science (Plotka et al., 2013; Xiao, Yuan, & Li, 2013). MAE may be more suitable for the extraction of phenolic compounds as compared to traditional extraction methods (Karabegović, Stojičević, Veličković, Nikolić, & Lazić, 2013; Lou et al., 2012). Methanol (or ethanol) and water are the conventional solvents for extraction. A comprehensive investigation of the effect of mixed solvent systems of methanol/water and ethanol/water on MAE may improve the efficiency of MAE and economize the use of energy, organic solvents and time.

*Eclipta prostrata* Linn (Mo Han Lian in Chinese, abbreviated as MHL) is an annual herb in China and many other countries in the world. The overground part of the herb is used as a traditional medicine and is also used as a vegetable. It is used for the treatment of hemorrhages, hepatic disease, renal injuries, hair loss, tooth mobility, and viper bites in traditional Chinese medication (National Commission of Chinese Pharmacopoeia, 2010). Modern pharmacological research has demonstrated that MHL exhibits various bioactivities including anti-tumor, anti-snake venom, anti-inflammatory, anti-oxidation, anti-HIV-1 integrase, reduction

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of blood lipids, and prevention of CCL<sub>4</sub>-induced liver damage (Lee, Ha, & Yang, 2008; Santhosh, Govindasamy, & Sukumar, 2006; Tewtrakul, Subhadhirasakul, Cheenpracha, & Karalai, 2007). Our preliminary study indicated that phenolic compounds are abundant in MHL. These metabolites may be the important bioactive components in MHL.

Several phenolic compounds such as luteoloside and wedelolactone have been isolated from *Herba Ecliptae* (Wu, Hou, Zhang, & Han, 2008; Zhang & Guo, 2001). To the best of our knowledge, no comprehensive information on the study of phenolic compounds in *Herba Ecliptae* is available. In our preliminary investigations, dicaffeoylquinic acids and wedelolactone were found to be chemically unstable if exposed to high temperature for a long time. In addition, isomers and similar adsorptions of some flavonoid glycosides and phenolic acids made the separation difficult in column chromatography. The objective of this study was to develop a green analytical method for simultaneous extraction, identification, and quantification of the main phenolic components in MHL using MAE and HPLC–DAD–ESI/MS. In order to obtain good extraction and avoid the degradation of the phenolic compounds, the key parameters of MAE including solvent type, microwave power, temperature, solvent to material ratio and extraction time were optimized. For good separation and determination, the analytical methods of HPLC–DAD–ESI/MS were optimized and validated. The extraction efficiency of heat reflux extraction and ultrasonic assisted extraction using different solvents was compared with that of MAE. Furthermore, the antioxidant activity of the extracts was also evaluated.

## 2. Material and methods

### 2.1. Plant materials, chemicals, and reagents

The overground part of *E. prostrata* L. (collected from Taian city in Shandong province of China) was fragmented into small segments. All segments were dried in a drying room with active ventilation at room temperature (about 25 °C) until a constant weight was obtained. Standard substances including chlorogenic acid (CGA), caffeic acid (CFA), quercetin 7-*O*-glucoside (QTG), luteolin 7-*O*-glucoside (LLG), and wedelolactone (WL) were obtained from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Other substances including 3,4-dicaffeoylquinic acid (3,4-DCQA), 3,5-dicaffeoylquinic acid (3,5-DCQA) and 4,5-dicaffeoylquinic acid (4,5-DCQA) were obtained from Tauto Biotech company (Shanghai, China). The purity of the reference standards was determined to be more than 98% by HPLC. Methanol for LC–MS analysis was purchased from Merck (Darmstadt, Germany). Formic acid, acetonitrile (HPLC grade), and absolute ethanol (analytical grade) were purchased from Tianjin Kemiou Chemical Reagent Company (Tianjin, China). Double-distilled water was prepared in our laboratory.

### 2.2. Extraction methods

#### 2.2.1. MAE

MAE was carried out using MAS-I Microwave Extraction Testing Equipment (Shanghai SINEO Microwave Chemic Technology Co., Ltd., China). The slices of MHL were crushed and sieved using a standard sieve (80–100 mesh). The powder (1.000 g) was put into a clean and dry 100-mL flask and soaked for 5 min with an accurately measured extraction solvent and the extraction was carried out according to the experimental design using different MAE conditions. After extraction, the weight loss was complemented with the solvent. The solvent type (10–100% (v/v) aqueous methanol and 10–100% (v/v) aqueous ethanol), microwave power (400,

600, 800, and 1000 W), temperature (50, 60, 70, and 80 °C), solvent to material ratio (10, 20, 30, and 40 mL/g) and extraction time (0.5, 1, 2, 3, 5, and 10 min) were optimized using extraction yield as response.

#### 2.2.2. Ultrasonic assisted extraction

Ultrasonic assisted extraction (UAE) was conducted in an ultrasonic bath (Shumei® KQ-5200DE ultrasonic instrument, Kunshan, China). The frequency and maximum power of the instrument were 40 kHz and 250 W, respectively. In brief, 1.000 g sample powder and 30 mL solvent were accurately added into a clean and dry 100-mL flask and sonicated in a water bath for 30 min. The ultrasonic power was 250 W. After extraction, the weight loss was complemented with the solvent and the extraction efficiency of UAE with different solvents was evaluated.

#### 2.2.3. Heat reflux extraction

Heat reflux extraction (HRE) was conducted in a water bath. The sample powder (1.000 g) and 40 mL solvent were accurately added into a clean and dry 100-mL glass flask and extracted for 60 min at the boiling point of the solvent. After extraction, the weight loss was complemented with the solvent. The extraction efficiency of HRE with different solvents was evaluated.

### 2.3. Qualitative and quantitative analysis

The qualitative analysis was carried out using LC–DAD–ESI/MS. The instrument consisted of a Thermo HPLC system coupled with photodiode array detection and LCQ Deca XP MAX IT-MS (Thermo Fisher Scientific Inc., USA). The HPLC system was equipped with a micro vacuum degasser, a high pressure quadrupole pump, an autosampler, and Xcalibur Data Software. An Xterra® MS-C18 column (50 mm × 2.1 mm, 2.1 μm, Waters, USA) was used for LC–MS analysis. The mobile phase consisted of methanol (A) and 0.1% (v/v) aqueous formic acid (B) using the following gradient elution program for separation: 0–30 min, 15–50% (A); 30–35 min, 50–100% (A). The column temperature was maintained at 30 °C. The flow rate was 0.2 mL/min and the injection volume was 5 μL. UV spectra were obtained from 200 to 400 nm. Mass spectrometric analyses were performed on a LCQ™ Deca XP MAX IT-MS equipped with an electrospray ionization (ESI) source. Negative ion mode was found to be selective and sensitive for the ionization of the flavonoid glycosides and phenolic acids and full scan ESI mass spectra were measured between *m/z* 100–800 in the negative ion mode. Nitrogen was used as the nebulizer gas. The instrumental parameters for the mass spectrometric analysis were as follows: sheath flow rate, 30 arbitrary units; sweep flow rate, 10 arbitrary units; capillary voltage, 3500 V; drying gas (N<sub>2</sub>) temperature, 350 °C; collision induced dissociation (CID) spectra were obtained using helium as the collision gas. The collision energy ranged from 10% to 60%.

The components could not be separated on baseline using the MS-C18 column, and therefore it was not suitable for the quantitative analysis. Therefore, a longer chromatographic column was used for the quantitative analysis. A Waters HPLC system equipped with Waters 600 pump, photodiode array detection, Empower™ 2 Chromatography Data Software (Waters Technologies, USA), and an Agilent ZORBAX SB-C18 column (250 mm × 4.6 mm, 5 μm) were used for the quantitative analysis. The mobile phase consisted of acetonitrile (A) and 0.05% (v/v) aqueous solution of phosphoric acid (B) was suitable to separate the compounds in a shorter time. The gradient elution program was as follows: 0–30 min, 10–25% A; 30–35 min, 25–30% A; 35–45 min, 30–50% A, and then increased to 100% A at 50 min. After 5 min, the system was returned to the initial conditions and allowed to equilibrate for 5 min prior to the subsequent injection. The flow rate was

1 mL/min and the injection volume was 20  $\mu$ L; UV spectra were scanned from 210 to 400 nm. Individual stock standard solutions of CGA, CFA, QTC, LLG, 3,4-DCQA, 3,5-DCQA, 4,5-DCQA and WL were prepared in methanol with the concentration of 0.62, 0.31, 0.53, 0.665, 1.20, 1.78, 0.88, and 1.30 mg/mL, respectively. All solutions were stored at 4 °C. The mixed working solutions were prepared by diluting the stock solutions with methanol before use. The standard working solutions and sample solutions were filtered through a 0.22  $\mu$ m membrane before injection. Quantitative determination was performed using external standards by means of a six-point calibration curve.

#### 2.4. Method validation

Standard stock solutions containing all reference compounds were prepared and diluted to the appropriate concentrations for the construction of calibration curves. Six concentrations of the eight analyte solutions were analyzed in triplicate and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The coefficient of determination was used to evaluate the linearity of the calibration curves. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte were evaluated at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. The intra-day precision was determined by analyzing three concentrations of the eight analytes five times during one day. For the inter-day precision, the same solution was examined in triplicate on three consecutive days. The relative standard deviation (RSD) was taken as a measure of precision. The recovery was used to evaluate the accuracy of the method. Three concentration levels of the standard solutions were added to 0.5 g of the MHL samples. The samples were then extracted and analyzed using the developed MAE and HPLC–DAD. Three replicates were performed for each test. The recovery was calculated using the following formula: recovery (%) = (amount found – original amount)/amount spiked  $\times$  100%.

#### 2.5. Determination of antioxidant capacity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was used to evaluate the antioxidant activity of the phenolic compounds (Brand-Williams, Cuvelier, & Berset, 1995). The test sample solutions (2 mL) at different concentrations were mixed with 2 mL DPPH (0.06 mg/mL) and allowed to stand for 30 min in the dark at room temperature. The UV absorbance of the reaction mixtures was measured at 517 nm using a UV–Visible spectrophotometer (UV 2450 Shimadzu, Japan). The experiment was performed in triplicate and the average absorption was noted for each concentration. The DPPH-scavenging activity was calculated by the following formula: scavenging activity =  $100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$ ,  $A_{\text{control}}$  = absorbance of control,  $A_{\text{sample}}$  = absorbance of sample.  $IC_{50}$ , the concentration of sample needed to scavenge 50% of DPPH radical, was obtained by plotting the DPPH-scavenging percentage of each sample against the sample concentration (Kumarasamy et al., 2007).

#### 2.6. Statistical analysis

All experiments were performed in triplicate and data were expressed as means  $\pm$  standard deviation. A one-way analysis of variance (ANOVA) was performed to calculate significant differences in treatment means, and multiple comparisons of means were done by the LSD (least significance difference) test. The difference of the yields between the same concentration of methanol and ethanol was determined by Student's *t*-test. A probability value of <0.05 was considered significant. A correlation analysis (Pearson's correlation coefficient) was performed to evaluate the

relationship between the content of phenolic compounds and the DPPH-scavenging activity. All analyses were carried out using SPSS 17.0 statistical analysis software (SPSS Inc., IL, USA).

### 3. Results and discussion

#### 3.1. Identification of phenolic compounds

##### 3.1.1. Identification of phenolic acids

Multifarious phenolic compounds in the MHL extract were efficiently separated using the Xterra<sup>®</sup> MS-C18 column (50 mm  $\times$  2.1 mm, 2.1  $\mu$ m). The HPLC chromatograms at different wavelengths are presented in Supplementary data Fig. S1. Under the optimal LC–ESI/MS conditions, most of the compounds detected had intensive signals corresponding to the pseudomolecular ion  $[M-H]^-$  and formation of  $[M+HCOOH-H]^-$ . The MS/MS spectra of the compounds provided considerably more structural information. Definitive identification was accomplished using UV, mass spectrometry, comparison to known standard compounds, and literature data. The results are shown in Table 1.

Compound 1, in the ESI/MS with negative ion mode, produced the  $[M+HCOOH-H]^-$  and  $[M-H]^-$  ions at  $m/z$  399 and 353, respectively. According to the UV spectra, the  $\lambda_{\text{max}}$  were present at 326 and 240 nm with a shoulder band at 290 nm. Furthermore, the deprotonated molecular ions  $[M-H]^-$  yielded the fragments  $[\text{quinic acid}-H]^-$  191 and  $[\text{caffeic acid}-H]^-$  179, which represented the diagnostic fragmentation of caffeoylquinic acids. The fragmentation behavior with a base peak at  $m/z$  191 was in accord with literature data (Clifford, Knight, & Kuhnert, 2005; Fujioka & Shibamoto, 2008). Compound 1 was definitively identified as 5-*O*-caffeoylquinic acid (chlorogenic acid, 5-CQA) by comparison to the authentic standard. The fragment  $[353-CO_2-H]^-$  at  $m/z$  309.8 was due to the loss of  $CO_2$ . The ESI/MS and UV spectra of compound 2 were similar to those of compound 1. Considering the retention time, compound 2 was tentatively identified as 4-*O*-caffeoylquinic acid (Luo et al., 2013). Compound 3 gave the  $[M+HCOOH-H]^-$  and  $[M-H]^-$  ions at  $m/z$  225 and 179 with  $\lambda_{\text{max}}$  (UV) at 325 and 240 nm and a shoulder band at around 290 nm. By comparison to the authentic standard, compound 3 was identified as caffeic acid.

Compounds 8, 9, and 11 showed similar UV spectra with  $\lambda_{\text{max}}$  at 326 and 245 nm and a shoulder at 295 nm, typical characteristic of caffeic acid derivatives. The three compounds had the same  $[M-H]^-$  ion at  $m/z$  515,  $[M+HCOOH-H]^-$  ion at  $m/z$  561, and a fragment at  $m/z$  353. The  $[M-H]^-$  ion at  $m/z$  515 is indicative of dicaffeoylquinic acid isomers, which have been previously reported (Clifford et al., 2005). The ion at  $m/z$  353 corresponds to the loss of one dehydrated molecule of caffeic acid  $[M-\text{caffeic acid}]^-$ . ESI–MS/MS spectra of the dicaffeoylquinic acids (DCQAs) provided more structural information in the negative ion detection mode. The MS/MS spectra data of the three DCQAs are displayed in Table 1. Based on the fragmentation ions at  $m/z$  353, 191 and 179 obtained due to the loss of 1 caffeoyl, 2 caffeoyl, and 1 caffeoyl plus one quinic acid moiety in the ESI–MS/MS, authentic standards, and literature data (Deng & Yang, 2013; Luo et al., 2013), compounds 8, 9, and 11 were definitively identified as 3,4-DCQA, 3,5-DCQA, and 4,5-DCQA, respectively.

The ESI–MS/MS spectra showed the major difference between the three isomeric compounds (Fig. 1). The relative intensity of the MS/MS ion  $[CQA-H_2O-H]^-$  335 was different. 3,4-DCQA was more intense (12% of base peak) at  $m/z$  335, but 3,5-DCQA was barely detectable (2% of base peak) and 4,5-DCQA was only detectable to the extent of 1%. Secondly, the MS/MS ion at  $m/z$  299 was detected because of the fragmentation of quinic acid for 3,5-DCQA and 4,5-DCQA, but not for 3,4-DCQA. Furthermore, the

**Table 1**

The components identified by LC–DAD and mass spectra in MHL.

Peak no.	Rt (min)	UV (nm) ( $\lambda_{\max}$ )	ESI–MS ions $m/z$ (%)	MS <sup>2</sup> ions $m/z$ (%)	Identified compounds
1	3.66	326, 240, 290 (sh)	[M–H] <sup>–</sup> : 353.3 (55) [M–H+HCOOH] <sup>–</sup> : 399.3 (100)	MS <sup>2</sup> [353]: 353.6 (7); 191.3 (100), 179.0 (8), 309.8 (4)	5-O-caffeoylquinic acid <sup>a</sup>
2	4.37	326, 240, 295 (sh)	[M–H] <sup>–</sup> : 353.3 (49) [M–H+HCOOH] <sup>–</sup> : 399.3 (100)	MS <sup>2</sup> [353]: 353.6 (6); 191.3 (100), 179.0 (10)	4-O-caffeoylquinic acid <sup>b</sup>
3	7.21	325, 240, 290 (sh)	[M–H] <sup>–</sup> : 179.0 (53) [M–H+HCOOH] <sup>–</sup> : 225.0 (100)	MS <sup>2</sup> [179]: 179.2 (100); 135.3 (18)	Caffeic acid <sup>a</sup>
4	14.08	355, 260	[M–H] <sup>–</sup> : 479.0 (36) [M–H+HCOOH] <sup>–</sup> : 525.0 (100)	MS <sup>2</sup> [479]: 317.0 (100)	Myricetin-glucoside <sup>b</sup>
5	16.75	345, 280, 255	[M–H] <sup>–</sup> : 463.0 (30) [M–H+HCOOH] <sup>–</sup> : 509.0 (100)	MS <sup>2</sup> [463]: 317.2 (100)	Myricetin-rhamnoside <sup>b</sup>
6	17.37	369, 256	[M–H] <sup>–</sup> : 463.0 (55) [M–H+HCOOH] <sup>–</sup> : 509.0 (100)	MS <sup>2</sup> [463]: 301.4 (100)	Quercetin-7-O-glucoside <sup>a</sup>
7	19.75	349, 255	[M–H] <sup>–</sup> : 447.0 (60) [M–H+HCOOH] <sup>–</sup> : 493.0 (100)	MS <sup>2</sup> [447]: 285.3 (100)	Luteolide <sup>a</sup>
8	20.31	326, 245, 290 (sh)	[M–H] <sup>–</sup> : 515.0 (100) [M–H+HCOOH] <sup>–</sup> : 561.0 (20)	MS <sup>2</sup> [515]: 353.0 (100), 335.4 (12), 191.3 (5), 179.3 (10), 173.0 (20), 255.3 (5)	3,4-Dicaffeoylquinic acid <sup>a</sup>
9	20.86	326, 245, 290 (sh)	[M–H] <sup>–</sup> : 515.0 (30) [M–H+HCOOH] <sup>–</sup> : 561.0 (100)	MS <sup>2</sup> [515]: 353.0 (100), 335.4 (2), 299.2 (1), 191.3 (3), 179.0 (2)	3,5-Dicaffeoylquinic acid <sup>a</sup>
10	22.62	350, 250	[M–H] <sup>–</sup> : 447.0 (50) [M–H+HCOOH] <sup>–</sup> : 493.0 (100)	MS <sup>2</sup> [447]: 286.0 (100)	Luteolin-glucoside <sup>b</sup>
11	24.48	327, 245, 290 (sh)	[M–H] <sup>–</sup> : 515.0 (100) [M–H+HCOOH] <sup>–</sup> : 561.0 (10)	MS <sup>2</sup> [515]: 353.0 (100), 335 (1), 299.0 (12), 255.0 (12), 203.4 (15), 173.3 (10), 179.3 (6), 191.3 (1), MS <sup>2</sup> [433]: 301.0 (100)	4,5-Dicaffeoylquinic acid <sup>a</sup>
12	27.10	350, 255	[M–H] <sup>–</sup> : 433.0 (70) [M–H+HCOOH] <sup>–</sup> : 479.0 (40)		Quercetin-arabinoside <sup>b</sup>
13	30.60	352, 250	[M–H] <sup>–</sup> : 313.0 (100) [M–H+HCOOH] <sup>–</sup> : 359.0 (30)	MS <sup>2</sup> [313]: 298.4 (10), 269.3 (30), 313.0 (100)	Wedelolactone <sup>a</sup>

<sup>a</sup> Identified by UV spectra, MS/MS, authentic standards and previous literatures.<sup>b</sup> Identified by UV spectra, MS/MS and previous literatures.

MS/MS ion at  $m/z$  173 in the spectra of 3,4-DCQA and 4,5-DCQA is indicative of acylation at the 4-position. In addition, the MS/MS ion at  $m/z$  255 was detected because of the fragmentation of quinic acid in 3,4-DCQA and 4,5-DCQA (Clifford et al., 2005).

### 3.1.2. Identification of flavonoid glycosides and wedelolactone

Compound 4, UV  $\lambda_{\max}$  at 355 and 260 nm with  $m/z$  479 [M–H]<sup>–</sup>,  $m/z$  525 [M–H+HCOOH]<sup>–</sup>, and fragmentation at  $m/z$  317 [myricetin–H]<sup>–</sup>, was tentatively identified as myricetin-glucoside (Rattmann et al., 2012). Compound 6, UV  $\lambda_{\max}$  at 369 and 256 nm with  $m/z$  463 [M–H]<sup>–</sup>,  $m/z$  509 [M–H+HCOOH]<sup>–</sup> and fragmentation at  $m/z$  301 [quercetin–H]<sup>–</sup>, was definitively identified as quercetin-7-O-glucoside by comparison with authentic standards and literature data (Tewtrakul et al., 2007). Compound 7, UV  $\lambda_{\max}$  at 349 and 255 nm with  $m/z$  447 [M–H]<sup>–</sup>,  $m/z$  493 [M–H+HCOOH]<sup>–</sup> and fragmentation at  $m/z$  285 [luteolin–H]<sup>–</sup>, was definitively identified as luteolin 7-O-glucoside by comparison with authentic standards and published data (Lee, Ha, Yang, Sung, & Kim, 2009; Liu, Zhao, Zhong, & Jiang, 2012). Compound 13, UV  $\lambda_{\max}$  at 352 and 250 nm with  $m/z$  313 [M–H]<sup>–</sup> and  $m/z$  359 [M–H+HCOOH]<sup>–</sup>, was definitively identified as WL by comparison with authentic standards and published data (Tewtrakul et al., 2007). The fragmentation ions at  $m/z$  298 and 269 corresponded to the fragments of [M–CH<sub>3</sub>–H]<sup>–</sup> and [M–CO<sub>2</sub>–H]<sup>–</sup>, respectively. Compounds 5, 10, and 12 were tentatively identified as myricetin-rhamnoside (Ricardo & Quezia, 2013), luteolin-glucoside (Fattahi et al., 2013), and quercetin-arabinoside (Rattmann et al., 2012), respectively, according to the UV spectra and MS/MS (Table 1).

## 3.2. Optimization of MAE

### 3.2.1. The effect of mixed solvents on MAE

A correct choice of solvent is fundamental to obtain a green extraction process. In order to find an optimal solvent for MAE, mixtures of methanol–water and ethanol–water were investigated in detail. One gram of powder was extracted with 30 mL different

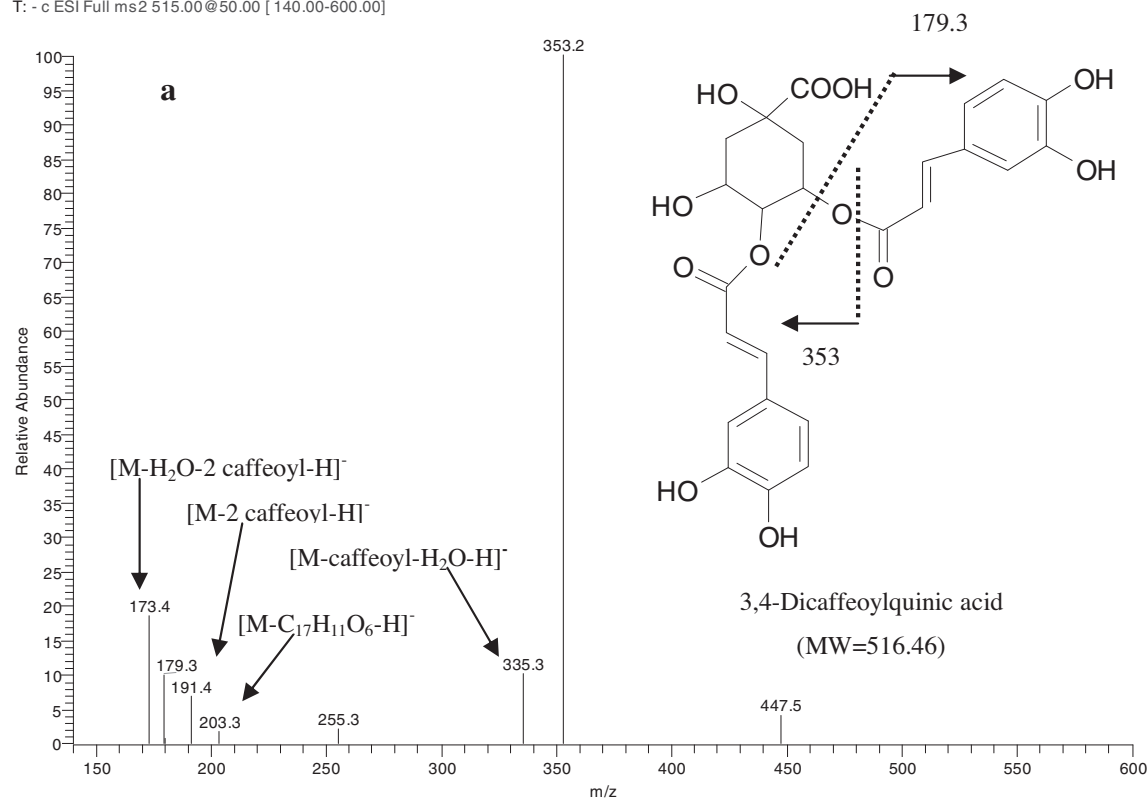
mixed solvent for 5 min. The microwave power was 400 W and the temperature was 60 °C. The results are shown in Fig. 2. The yields of CGA, CFA, LLG, and 3,5-DCQA increased when the solvent changed from 10% to 50% aqueous methanol (or ethanol). However, for 3,4-DCQA and 4,5-DCQA, the highest yield was obtained with 40% and 50% ethanol. For CFA, QTG, 3,5-DCQA, and WL, the highest yield was obtained using 50–70% ethanol. The yields of all components decreased when the solvent changed from 70% to 100% methanol (or ethanol). For QTG, 3,4-DCQA, and WL, the yields with the ethanol–water mixtures were higher than those with the methanol–water mixtures when the ratios were between 10% and 50%. The yields of all the components with the methanol–water mixtures were significantly higher ( $p < 0.05$ ) than those with the ethanol–water mixtures when the ratio changed from 90% to 100%. In MAE, the dissipation factor of the mixed solvent, the solubility of the components in the solvent system and the polarity of the compound all influence the extraction efficiency (Eskilsson & Björklund, 2010; Fang, Wang, Wang, Zhang, & Wang, 2013). The yield of WL was very low when the solvent ratio was between 10% and 40%, which indicated that solubility was an important factor for this low polarity component when the solvent ratio was low. The variation of the yields of 3,4-DCQA and 4,5-DCQA showed a different trend compared with that of 3,5-DCQA, which indicated that the structure of the three dicaffeoylquinic acids would also be important factor for MAE in addition to the solvent and the polarity of the components. The highest yields of the eight components could be simultaneously obtained when the solvent was 50% aqueous ethanol. Therefore, 50% (v/v) aqueous ethanol was chosen as the optimal solvent.

### 3.2.2. Effect of microwave power on MAE

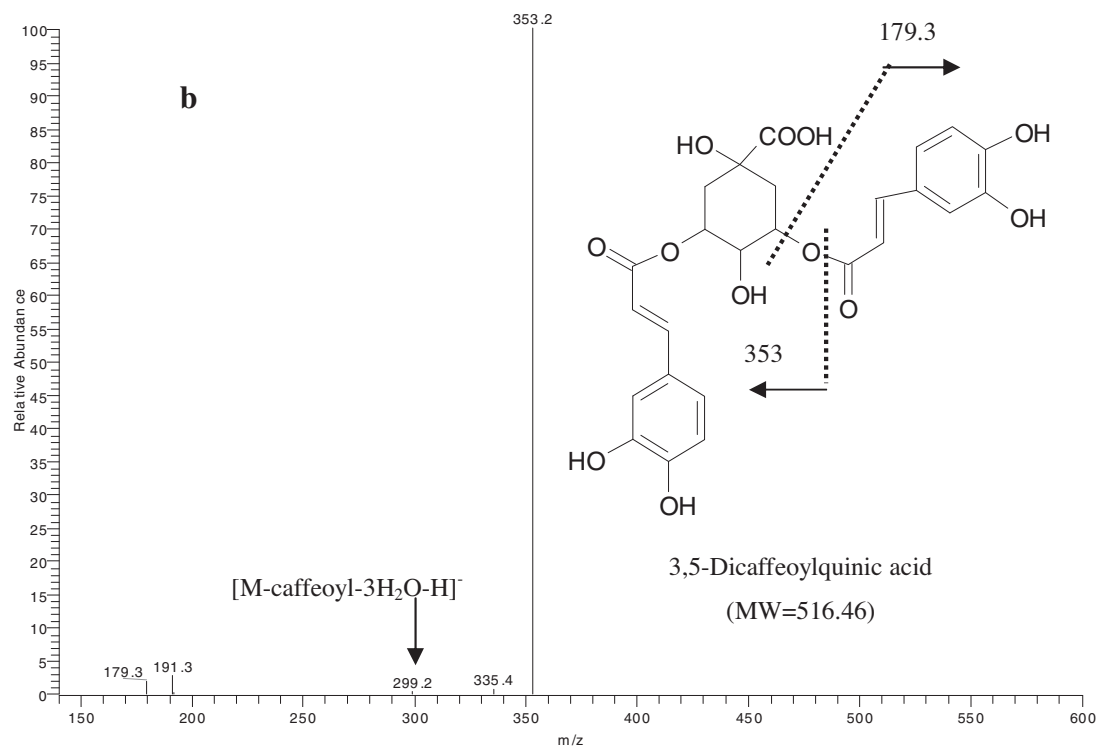
One gram powder was extracted with 30 mL 50% ethanol for 5 min at 60 °C. The extraction yields obtained using different microwave powers were evaluated (Fig. 3a). The yields of CGA, CFA, QTG, 3,4-DCQA and WL showed insignificant differences ( $p > 0.05$ ) when the microwave power was changed from 400 to



data13 #540 RT: 20.53 AV: 1 NL: 1.78E5  
T: - c ESI Full ms2 515.00@50.00 [ 140.00-600.00]



data13 #558 RT: 21.09 AV: 1 NL: 2.63E5  
T: - c ESI Full ms2 515.00@50.00 [ 140.00-600.00]



**Fig. 1.** MS/MS spectra and proposed fragmentation mechanism of the  $[M-H]^-$  ions of 3,4-DCQA (a), 3,5-DCQA (b) and 4,5-DCQA (c).

data11 #737 RT: 24.70 AV: 1 NL: 5.73E5  
T: - c ESI Full ms 2 515.00 @50.00 [ 140.00-600.00]

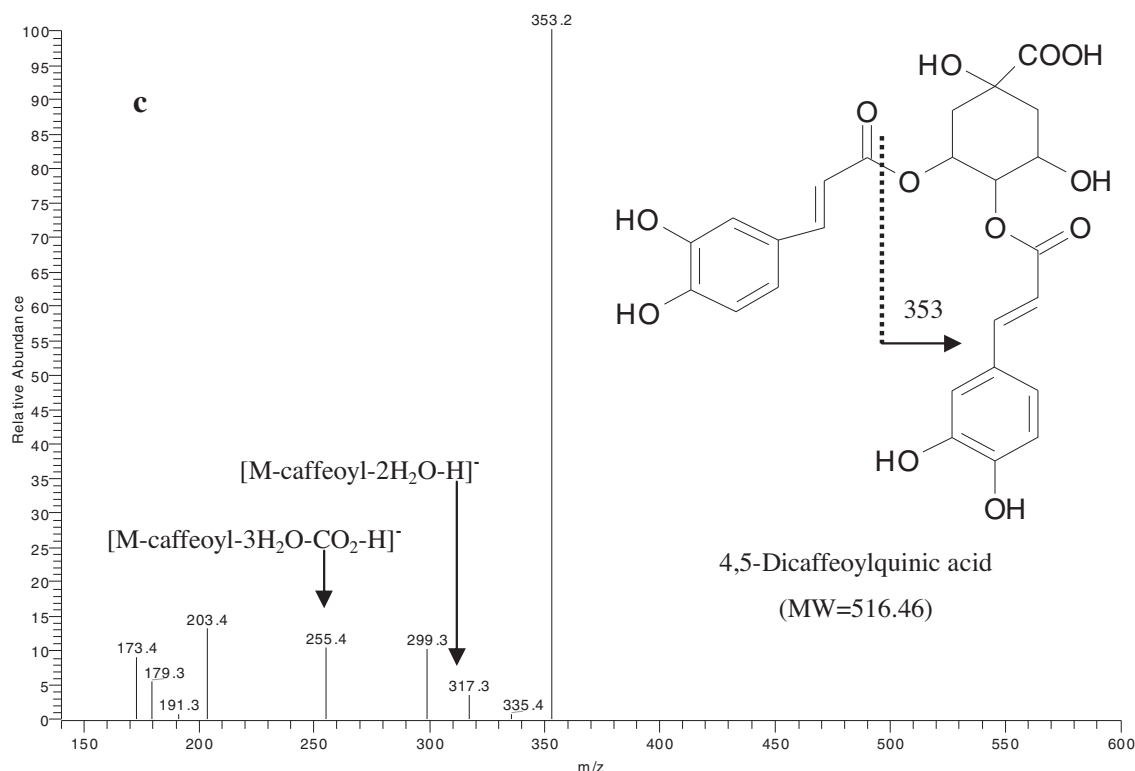


Fig. 1 (continued)

800 W. The yield of 3,5-DCQA with 400 W was significantly higher ( $p < 0.05$ ) than those of 600 and 800 W. The yield of 4,5-DCQA with 800 W was significantly higher ( $p < 0.05$ ) than those of 400 and 600 W. Therefore, in order to save energy and avoid degradation, 400 W was utilized for the extraction.

### 3.2.3. Effect of temperature on MAE

The extraction yields at different temperatures were evaluated under the conditions obtained from Section 3.2.2 (Fig. 3b). The extraction yields of all the components increased with the temperature increasing from 50 to 70 °C. The yields of CGA, LLG, 3,4-DCQA, 3,5-DCQA, 4,5-DCQA and WL at 70 °C were significantly higher ( $p < 0.05$ ) than those at 60 °C, respectively. The yields of CGA, LLG, 3,5-DCQA and WL decreased significantly ( $p < 0.05$ ) when the temperature changed from 70 to 80 °C, which maybe due to the degradation in high temperature. The highest yields of all the components were obtained when the temperature was 70 °C. Therefore 70 °C was chosen as the optimal temperature for the extraction.

### 3.2.4. Effect of solvent to material ratio on MAE

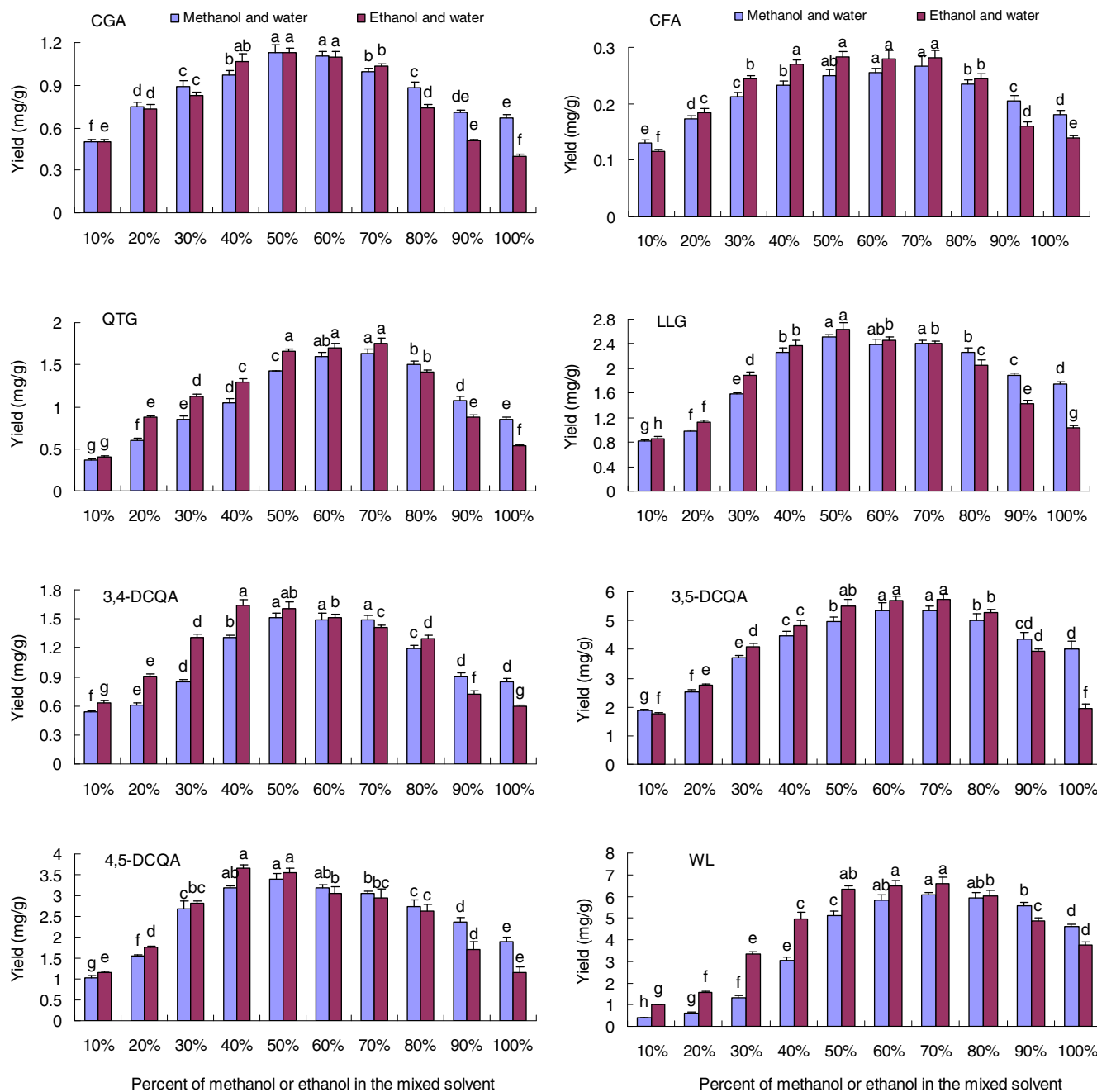
The extraction yields using different solvent to material ratios were measured under the conditions obtained from Section 3.2.3 (Fig. 3c). The yields of all the components increased when the solvent to material ratio changed from 10 to 30 mL/g. The yields of CGA, CFA, QTG, LLG, 3,4-DCQA and 3,5-DCQA decreased when the solvent to material ratio was 40 mL/g. The yields showed not significant differences ( $p > 0.05$ ) between 30 and 40 mL/g solvent to material ratio for 4,5-DCQA and WL. Therefore, 30 mL/g was the most suitable solvent-to-material ratio for all eight components.

### 3.2.5. Effect of extraction time on MAE

The extraction yields using different time period were compared under the conditions obtained from Section 3.2.4 (Fig. 3d). The yields of all the components increased significantly with the increase of extraction time (0.5–2 min). But the yields of 3,5-DCQA and WL decreased when the time was higher than 5 min, which indicated that the two compounds degraded because of extended heating. The yields of 4,5-DCQA and CGA increased after 10 min and this might be due to the transformation of the other components. The yields of CGA, QGT, 3,5-DCQA and WL at 3 min were higher than those at 2 min. However, the yields showed not significant difference between ( $p > 0.05$ ) 2 min and 3 min. When the extraction time was 2 min, the yields of all the components reach the maximum, and therefore 2 min was the optimal extraction time for all eight components.

### 3.3. Method validation

The components were separated on the baseline using the ZORBAX SB-C18 column (250 mm × 4.6 mm, 5 μm) for quantitative analysis. Fig. 4 shows the chromatogram of the standards and one MHL sample obtained by the optimized MAE. All calibration curves exhibited an excellent coefficient of determination ( $r^2 \geq 0.9993$ ) within the range of tested concentrations (Supplementary data Table S1). The measured LOD and LOQ were in the range of 0.035–0.212 μg/mL and 0.130–0.760 μg/mL, respectively (Supplementary data Table S1). The method precision was checked by intra-day and inter-day variability. From the results obtained, the present method was found to have acceptable precision for the intra-day RSD values (0.26–0.43%) and the inter-day RSD values (1.66–5.19%) (Supplementary data Table S2). The



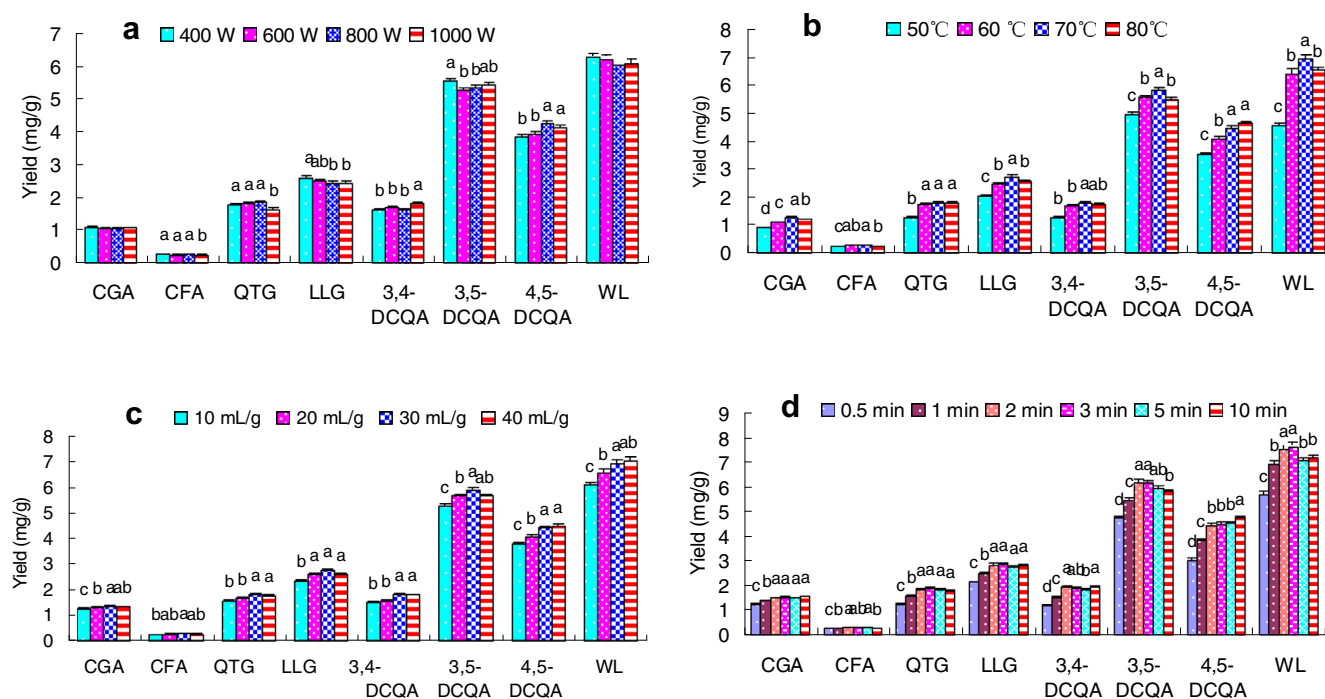
**Fig. 2.** Effect of the mixed ratio of methanol/water and ethanol/water on the extraction efficiency of different components in MAE. Temperature, 60 °C; microwave power, 400 W; solvent to material ratio, 30 mL/g; extraction time, 5 min. The abbreviations of every component were showed on the top left corner of every figure. Different letters within each bar of the same color mean a statistical difference at  $p < 0.05$ .

recovery was used to evaluate the accuracy of the method. The recoveries of the analytes were between 95.1% and 103.0% with RSDs between 2.59% and 5.38% (Supplementary data Table S3). All of the measured precision and accuracy values were found to be acceptable for the quantification of the components in MHL.

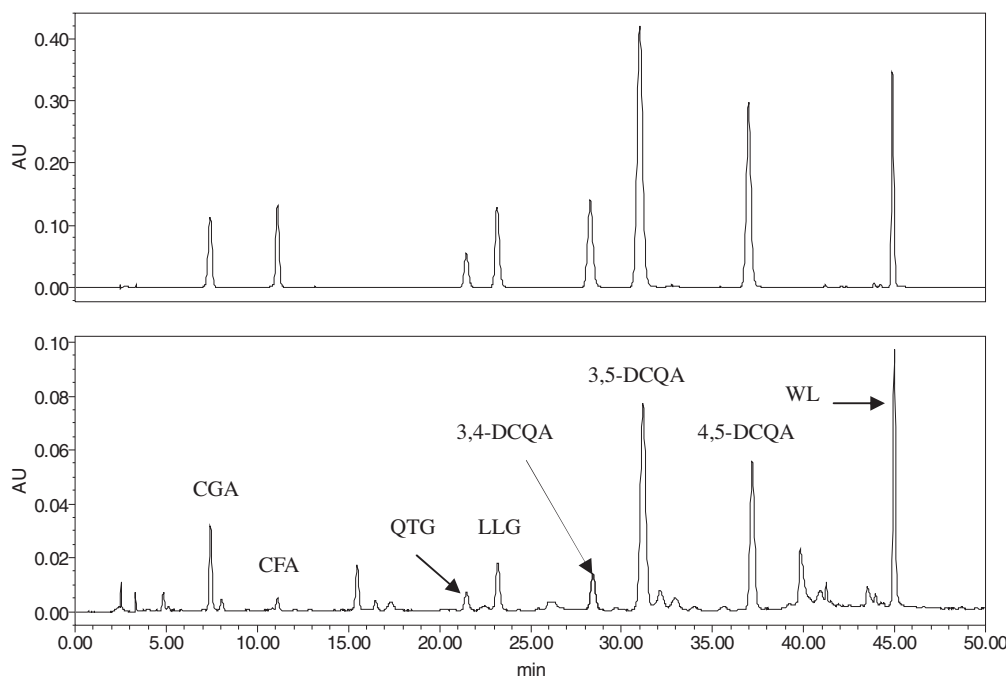
### 3.4. Comparison with UAE and HRE

UAE and HRE were also applied for the extraction of the phenolic compounds from MHL. The extraction yields of UAE and HRE with different mixed solvents (10–100% methanol and 10–100% ethanol) were compared (Supplementary data Fig. S2). The results indicated that for UAE, 60% methanol was the most suitable for the

extraction of five phenolic acids, but for QTG, LLG, and WL 70–80% ethanol was most suitable. For HRE, 70% ethanol was suitable for the extraction except for 3,4-DCQA which could be obtained in a higher yield using 30% ethanol. The highest yield of the components obtained by UAE and HRE using different solvents are shown in Table 2. However, the extraction yields of the three dicaffeoylquinic acids by HRE were significantly different from those obtained by MAE and UAE (Fig. 2 and Supplementary data Fig. S2). The yield of 3,5-DCQA by HRE was less than those obtained by MAE and UAE, but the maximum yields of 3,4-DCQA and 4,5-DCQA with HRE were higher than those obtained by MAE and UAE. The component of 3,5-DCQA was obviously degraded and mainly transformed into 3,4-DCQA when the solvent was



**Fig. 3.** Effect of microwave power (a), temperature (b), solvent to material ratio (c) and extraction time (d) on the extraction efficiency of MAE. Different letters within each bar of the same compound mean a statistical difference at  $p < 0.05$ .



**Fig. 4.** The HPLC chromatogram (330 nm) of the standards (upper) and one sample of MHL (under).

10–40% methanol (or ethanol). However, when the solvent was 50–90% methanol (or ethanol), 3,5-DCQA was isomerized into 4,5-DCQA. Therefore, HRE was not suitable for the analysis of phenolic compounds. Although the phenolic acids were not degraded with UAE, the eight components could not be simultaneously extracted with high yields. However, the highest yields of all the analytes could be simultaneously obtained by MAE in a shorter amount of time and using less solvent. Furthermore, the phenolic compounds could remain stable with MAE, which allowed for the

determination of the content in the sample. Therefore, the optimized MAE was the most suitable method for the simultaneous determination of the phenolic compounds.

### 3.5. Evaluation of antioxidant capacity

The DPPH radical-scavenging assay is a simple and sensitive method used to evaluate the antioxidant activity of chemical substances. The antioxidant capacity of different extract of MHL is



**Table 2**

The content of components and antioxidant capacity of different extract of MHL.

Extraction method and solvents	Contents of the analytes (mg/g) (n = 3)								Total	DPPH IC <sub>50</sub> (μg/mL)
	CGA	CFA	LLG	QGT	3,4-DCQA	3,5-DCQA	4,5-DCQA	WL		
MAE – 50% ethanol	1.50 ± 0.05a	0.31 ± 0.01ab	1.75 ± 0.04a	2.91 ± 0.07a	1.98 ± 0.06b	6.20 ± 0.16a	4.34 ± 0.09b	7.53 ± 0.12a	26.51	7.15
UAE – 60% methanol	1.30 ± 0.05b	0.29 ± 0.01b	1.50 ± 0.05b	2.55 ± 0.11c	1.41 ± 0.06c	5.40 ± 0.15b	2.96 ± 0.10c	7.18 ± 0.16b	22.58	8.03
UAE – 70% ethanol	1.13 ± 0.04c	0.24 ± 0.01c	1.72 ± 0.05a	2.72 ± 0.08b	1.19 ± 0.03d	5.11 ± 0.12b	2.57 ± 0.10d	7.26 ± 0.11b	21.93	8.69
HRE – 30% ethanol	1.17 ± 0.04c	0.25 ± 0.01c	0.52 ± 0.02d	0.96 ± 0.05d	2.71 ± 0.10a	0.99 ± 0.04d	2.72 ± 0.13c	2.59 ± 0.09d	11.90	30.60
HRE – 70% ethanol	1.55 ± 0.07a	0.34 ± 0.02a	1.16 ± 0.07c	2.48 ± 0.09c	1.14 ± 0.03d	1.56 ± 0.08c	7.50 ± 0.33a	6.38 ± 0.15c	22.12	12.57

IC<sub>50</sub> is the concentration (calculated according to the total content) of sample needed to scavenge 50% of DPPH radical.Data are expressed as means ± standard deviation of triplicate samples. Different letters in the same column indicate significant differences ( $p < 0.05$ ).

shown in Table 2. The results indicated that the extract obtained by MAE using 50% ethanol as solvent showed the strongest radical scavenging activities with IC<sub>50</sub> values of 7.15 μg/mL. The antioxidant activity of the extracts obtained by UAE was slightly lower than that of MAE. The content of 3,4-DCQA was higher and that of 3,5-DCQA was very low in the extract obtained by HRE using 30% ethanol as solvent. The radical-scavenging activity of this extract was minimal compared to those of other four extract. The total content of the eight compounds showed significant correlation with the DPPH radical-scavenging activity ( $|r|=0.96$ ,  $p < 0.01$ ), which indicated that the phenolic compounds would be the major antioxidant in MHL.

#### 4. Conclusion

In the present study, an improved method utilizing MAE and HPLC–DAD–ESI/MS was developed for the simultaneous extraction, identification, and quantification of the phenolic compounds in *E. prostrata* L. The developed method was proved to be rapid and efficient for the simultaneous analysis of caffeoylquinic acids, flavonoid glycosides, and coumarins. The most suitable solvent system for the MAE was elucidated by comparing different mixed ratio (10–100%) of methanol/water and ethanol/water solvent systems. Compared to UAE and HRE, the optimized MAE avoids the degradation of dicaffeoylquinic acids and wedelolactone and simultaneously obtains the highest yields of all components in a shorter amount of time and using less ethanol and energy. The negative ion mode of ESI was selective and sensitive for the ionization of these phenolic compounds. The components were simultaneously determined by MAE and HPLC–DAD with good separation, linearity, precision, and accuracy. Two isomers, 3,4-DCQA and 4,5-DCQA, were more stable than 3,5-DCQA. The degradation of 3,5-DCQA was different in different solvent systems. The extract obtained by MAE showed the strongest antioxidant activity. *E. prostrata* may be used as a natural antioxidant in food and medicine. The proposed method would provide a valuable and green methodology for the investigation of phenolic components in natural food and plants.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.05.037>.

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