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Determination of metabolites in *Uncaria sinensis* by HPLC and GC–MS after green solvent microwave-assisted extraction

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ABSTRACT

Uncaria sinensis (Oliv.) Havil (Rubiaceae) has been used as an important Traditional Chinese Medicine (TCM) herb for the treatment of fevers and various nervous disorders. The major bioactive secondary metabolites from different classes of chemical compounds, i.e. organic acid, flavonoid and alkaloid, present in this TCM herb, namely catechin, caffeic acid, epicatechin and rhynchophylline, were extracted by microwave-assisted extraction (MAE) method with ultra-pure water as the extraction solvent. The optimal extraction conditions for this green solvent MAE method were found to be 100 °C for 20 min. The recoveries of the compounds were found to be comparable to that of heating under reflux using ultra-pure water for 60 min. The method precision (RSD, $n = 6$) was found to vary from 0.19% to 5.60% for the proposed method on different days for the secondary metabolites. Simultaneously, the key primary metabolites such as sucrose and phenylalanine for the biosynthesis of bioactive secondary metabolites were successfully characterized by GC–MS. Furthermore, an approach using the combination of primary and secondary metabolite profiling based on their chemical fingerprints with Principal Component Analysis (PCA) was successfully developed to evaluate the quality of *U. sinensis* obtained from different sources. This approach was shown to be feasible in discriminating *U. sinensis* from different origins and thus a potential application for the quality control of other medicinal herbs.

1. Introduction

The hooks and stems of dried *Uncaria sinensis* (Oliv.) Havil (Rubiaceae) have been used as important Traditional Chinese Medicine (TCM) herb for the treatment of fevers and various nervous disorders [1]. Various reports have attributed this medicinal herb's pharmacological activities to the different classes of bioactive secondary metabolites such as caffeic acid (CA, an organic acid), rhynchophylline (RH, an alkaloid), and catechin (CT, a flavonoid) and epicatechin (epiCT, another flavonoid) [2–4]. It has been shown that flavonoids such as epicatechin and catechin are the active components that protect the cultured cerebellar granule cells against glutamate-induced neuronal death through the inhibition of Ca^{2+} influx [3]. Other report demonstrated the antidementia effects of phenolic compounds (e.g. caffeic acid) and indole alkaloids (e.g. rhynchophylline and isorhynchophylline) which are present in *U. sinensis* [4]. The molecular structures of these four biomarker compounds are given in Fig. 1. Generally the bioactive marker compounds from herbs are from one class of chemical compounds but *U. sinensis* seems to have three classes of bioactive compounds.

These bioactive secondary metabolites are generally produced in specific organisms and is an expression of its species uniqueness [5]. In contrast, plant primary metabolites such as amino acids and carbohydrates are involved in fundamental plant physiological processes, e.g., growth and development [6]. The analysis of these metabolites can be achieved by metabolomics tools such as extracting, separating, and analyzing a broad spectrum of metabolites from complex matrices [7]. At present, it is impossible to perform the analysis of primary and secondary metabolites present in the complex plant extracts using a single analytical technique.

In the extraction of bioactive compounds from botanicals, conventional extraction methods such as Soxhlet, reflux [8], maceration and decoction [9] are commonly used. These techniques, however, require long extraction time, labour intensive manual procedures and relatively high solvent consumption [8]. With the advancement in extraction technology, modern direct extraction techniques such as supercritical fluids (SFE), pressurized liquids and microwave-assisted extraction (MAE) have been developed to replace these tedious classical techniques [10]. MAE is a relatively new extraction method using non-ionizing microwave energy [11].

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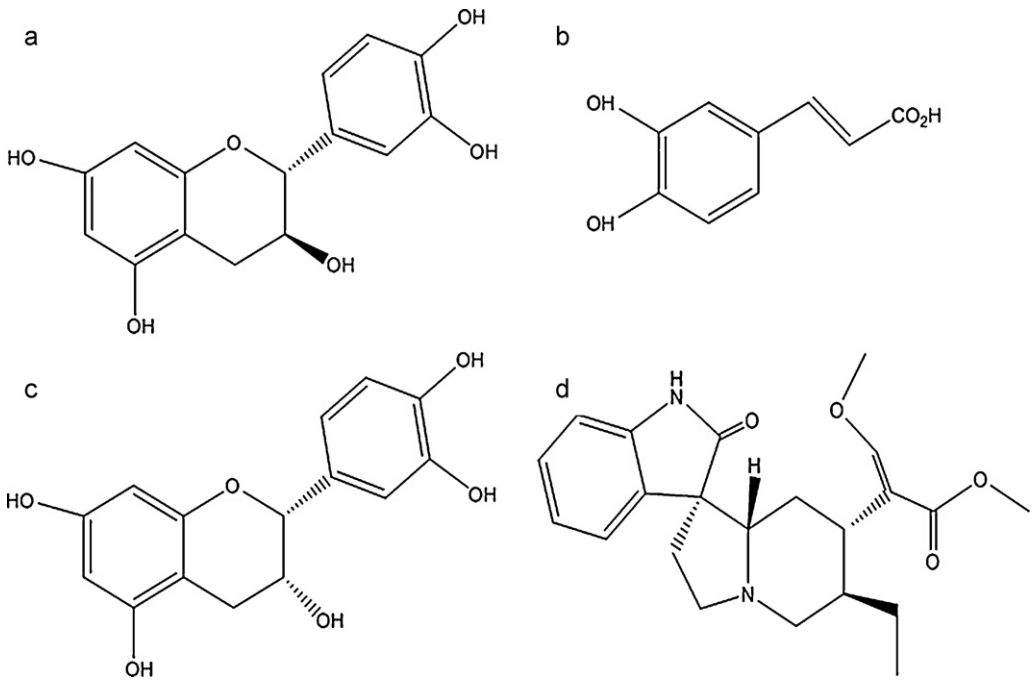

Fig. 1. Molecular structures of biomarker compounds (a) catechin, (b) caffeic acid, (c) epicatechin and (d) rhynchophylline in *U. sinensis*.

The advantages of MAE over the conventional extraction methods include shorter extraction time, low solvent consumption and also being a "green technique" and having with high-throughput capability [11,12]. It is noticed that the primary metabolites are usually directly extracted using an aqueous alcohol mixture [9]. In contrast, water is steadily becoming the choice solvent because it is non-toxic, non-flammable, cheap and also can be recycled without the need for treatment of wastes [13]. At high temperatures, superheated water has a permittivity similar to typical organic solvents which could dissolve a wide range of medium and low polarity analytes [13]. The versatility of MAE can be demonstrated by its applications in extracting alkaloids from *Tussilago farfara* [14], diterpene glycosides from *Stevia rebaudiana* [15], glucosinolates from *Eruca sativa* seeds and soil [16], oleanolic acid and ursolic acid from fruits of *Chaenomeles sinensis* [17], and epicatechin and catechin from green tea [18]. The combination of MAE with ultra high performance liquid chromatography coupled with diode array detection and time-of-flight mass spectrometry (UHPLC-DAD-TOF-MS) method was also developed for the simultaneous determination of 14 phenolic compounds in the root of *Pueraria lobata* (Wild.) Ohwi and *Pueraria thomsonii* Benth [19]. Furthermore, it was reported that MAE had been successfully used to extract some phenolic acids present in *Radix Salviae miltiorrhizae* with water [20]. Earlier reports showed that only one or two classes of bioactive secondary metabolites such as the oxindole alkaloids in this medicinal herb were extracted by the SFE method [21]. Currently, the application of green solvent MAE method to extract various classes of different molecular weights compounds such as CT, epiCT, CA and RH in a single extraction step is rather limited. Chromatographic fingerprinting has been accepted by the World Health Organization as a strategy for the identification and evaluation of the quality of herbal medicines [22,23]. The chromatographic fingerprints based on primary and secondary metabolites profiling are usually analyzed by gas chromatography (GC) and high performance liquid chromatography (HPLC), respectively [24,25]. The combination of secondary metabolite chromatographic fingerprint with chemometric tools such as Principal Component Analysis (PCA) has been used successfully in various earlier reports to characterize, classify and discriminate samples of different origins [23,26–28]. We have earlier reported the successful combination of green solvent MAE method with chromatographic fingerprints based on secondary metabolite profiling and PCA to evaluate the quality of *Stevia* obtained from different Good Agriculture Practice (GAP) cultivation conditions [15]. However, the combination of this approach with plant primary metabolite profiling for the assessment of quality of medicinal herbs is limited.

The aim of the current work is to perform a green solvent MAE method to extract the different classes of active biomarker compounds (CA, RH, CT and epiCT) under optimized conditions for chemical standardization in assessing the quality of different sources of *U. sinensis* in a single extraction run which is seldom reported in the literature. The extraction efficiency of the optimized extraction method is evaluated by comparing with heating under reflux using ultra-pure water as the extraction solvent. Finally, the quality of the medicinal herbs from different sources is evaluated by combining chromatographic fingerprints of primary and secondary metabolites with PCA. The key primary metabolites that can influence the biosynthesis of bioactive secondary metabolites are highlighted in the study. Furthermore, our work demonstrates the feasibility of using the combination of primary and secondary metabolite profiling with PCA to provide an exhaustive and complete assessment of the quality of medicinal herbs as analysis of two classes of metabolites for quality control of herbs is rarely reported. Such an approach can be applicable for other herbs for future applications.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade. Catechin (CT), epicatechin (epiCT), caffeic acid (CA) and rhyncholphylline (RH), all with purity ≥98%, were purchased from Shanghai Standard Biotech Co. Ltd. (Shanghai, China). Ultra-pure water was obtained from Millipore Alpha-Q water system (Millipore, Bedford, MA, USA). Methanol (MeOH), formic acid (FA), *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (purity > 99%) and

other chemical reference standards (proline, serine, tyrosine, lysine, phenylalanine, glycine, leucine, tryptophan, fructose, glucose, galactose, sucrose, lactose, maltose, mannose, cinnamic acid, fumaric acid and citric acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyridine was purchased from Univar (Rotterdam, The Netherlands).

2.2. Preparation of herbal plant samples

Three different batches of *U. sinensis* were purchased from separate Chinese medicinal halls, of which, two were from Kluang, Malaysia and one from Shanghai, China. The hooks and stems were freeze-dried overnight using Alpha 1-2 freeze dryer (Martin Christ, Germany). They were ground using an IKA MF10 microfine grinder (Staufen, Germany) and sieved through an insert of hole size 0.5 mm.

2.3. Preparation of reference standards

2.3.1. For HPLC analysis

Stock solutions of catechin, epicatechin, caffeic acid and rhyncholphylline at 200 mg L^{-1} were prepared in ultra-pure water. Linearity of each standard was established between 2 and 10 mg L^{-1} with correlation coefficient $r^2 \geq 0.999$. To quantify these marker compounds in the botanicals, a three point calibration based on the linearity established was used. The system precisions (RSD, $n = 6$) for each standard was found to be less than 1% on different days. The accuracy of the calibration plot was greater than 99%.

2.3.2. For GC–MS analysis

Stock solutions of all the chemical reference standards were prepared at 200 mg L^{-1} in pure pyridine. Then 50 μL of each standard was incubated in the dark with 60 μL BSTFA for derivatization. The derivatized analytes were finally transferred to amber vials for the GC–MS analysis after a 4 h incubation period.

2.4. Extraction

2.4.1. Organic solvent mixture

Typically 50 mg of the ground plant sample was accurately weighed in an Eppendorf tube. Then 1 mL of aqueous mixture of methanol and water (1:1) was added to the sample and vortexed for 1 min [29]. The sample was centrifuged in Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) for 5 min at 12,000 rpm. The supernatant was collected and evaporated to dryness using a Thermo Fisher Scientific model SPD 2010-230 (MA, USA) speedvac system and then subsequently freeze-dried overnight. The derivatization of the sample was carried out by an addition of 100 μL pure pyridine to reconstitute the dried sample and followed by 60 μL BSTFA before incubated in the dark overnight prior to GC–MS analysis.

2.4.2. Green solvent microwave-assisted extraction

A closed vessel system (under controlled temperature and pressure) was employed using Start E from Milestone (Sorisole, Italy). The extraction efficiency with different (i) extraction temperatures at 40, 60, 80, 100 and 120 °C and (ii) extraction times at 5, 10, 15, 20 and 30 min, was investigated. A microwave power of 600 W was used for the extraction work as described in our earlier works [15,30]. For each set of experiment, an accurately weighed 0.5 g sample was placed in a vessel and 20 mL of ultra-pure water were added. A magnetic stirrer was added in each vessel except for the reference vessel. After the samples were extracted by MAE, the extract was quantitatively transferred into a 50 mL volumetric flask. Each extract was filtered through a 0.45 μm nylon membrane before HPLC analysis.

2.4.3. Heating under reflux method

An accurately weighed 0.5 g plant sample was extracted of 60 mL of ultra-pure water for 60 min by heating under reflux method as optimized in our earlier works [15,30]. After cooling, the extract was collected and rota-evapourated using a Buchi rotary evaporator (Flawil, Switzerland) and quantitatively transferred into a 50 mL volumetric flask. Each extract was filtered with a 0.45 μm nylon membrane before HPLC analysis.

2.5. Analysis

2.5.1. GC–MS analyses of primary metabolites

A Shimadzu GC 17A system (Kyoto, Japan), equipped with an auto-sampler model AOC-20i was used with a HP-5MS capillary column (5%-phenyl)-methylpolysiloxane; 30 m, 0.25 mm i.d., 0.25 μm film thickness (Santa Clara, CA, USA), interfaced to a Shimadzu QP5000 MS system (Kyoto, Japan). The GC inlet temperature was set at 280 °C and MS interface at 300 °C. The oven was programmed to rise from an initial temperature of 100 °C to 300 °C at 10 °C min^{-1}, and held for 2 min with a total run time of 42 min. The inlet was operated in splitless mode, with 0.7 min purge-on time. Helium was the carrier gas (>99.99%), with a constant flow of 1 mL min^{-1}. The MS was performed in scan mode (m/z 50–720) and electron ionization energy was 70 eV.

2.5.2. HPLC analyses of secondary metabolites

An Agilent 1200 series High Performance Liquid Chromatography (HPLC) (Waldhronn, Germany) was employed to quantify the amount of extracted biomarker compounds. The analysis of the extracts was performed with a Waters XTerra C_{18} (150 × 3.9 mm, 5 μm) maintained at 40 °C. For all experiments, 10 μL of standard and sample extracts were injected. Gradient elution was carried out with acidified water (0.1% FA) as mobile phase A (MPA) and acidified MeOH (0.1% FA) as mobile phase B (MPB). The initial condition was set at 10% MPB and ramped to 100% MPB in 25 min before returning to the initial condition for the next 10 min. A flow rate of 1.0 mL min^{-1} was used with a detection wavelength at 280 nm.

2.6. Principal Component Analysis

For the GC–MS analysis, each sample was represented by a total ion chromatogram (TIC). Among the detected peaks, a multidimensional vector was constructed manually to characterize the biochemical patterns. Each vector was normalized to the total sum of vector intensity, thereby partially accounting for concentration due to the different sample size used. Peaks due to column bleed and derivatization reagent were removed. The identification of peaks was based on the use of reference standards and NIST 98 library. The mass spectra obtained were inspected manually and only those molecules with probability matching higher than 90% were considered.

For the HPLC-UV analysis, the peak areas of the chromatographic fingerprints of different wavelengths at 230, 240, 250, 254, 260, 270 and 280 nm were used as input data. A total of 200 peaks for each botanical were computed for their peak areas. The peak area for each chromatogram was normalized to a constant sum. All these results were input into a Simca-P+ Software Package (Umetrics, Umea, Sweden) for subsequent evaluation of the similarities of different chromatograms based on PCA. Typically PCA translates the peak areas obtained from n-dimensional variables space into principal component (PC) where there is a score describing different chromatograms obtained. PCA score plots have been used for the classification of samples from their measured properties. The distribution pattern generated from the data in this plot can be correlated to general characteristics of the samples analyzed.

Table 1
The reproducibility of the retention time and peak area (RSD, $n = 6$) at 6 mg L^{-1} and the linearity equation established between 2 and 10 mg L^{-1} for each biomarker compound.

Active biomarker		Mean retention time		Mean peak area		Equation of calibration curve	R^2	LOD (mg mL^{-1}) LOQ (mg mL^{-1})
		Min	RSD (%)	Mean	RSD (%)			
1	Catechin	4.73	0.01	285	0.50	$y = 2.420x - 0.181$	0.999	1.0 3.5
2	Epicatehin	6.37	0.02	42	0.41	$y = 0.609x - 0.363$	0.999	1.0 3.3
3	Caffeic acid	6.16	0.01	184	0.52	$y = 6.359x + 0.374$	0.999	1.2 3.2
4	Rhynchophylline	8.81	0.06	50	0.50	$y = 0.740x - 0.113$	0.999	1.1 3.3

LOD denotes limit of detection.
LOQ denotes limit of quantification.

3. Results and discussion

3.1. Validation of chromatography methods

The precision and accuracy of the MAE-HPLC method were carried out by analyzing six injections of different plant extracts. The retention times of the peaks were found to be stable on the intra-day and inter-day with variation less than 0.5% (relative standard deviation (RSD), $n = 6$). The RSD values for normalized peak areas for the intra-day ($n = 6$) and inter-day ($n = 6$)) were determined with variation less than 1.5%. For the validation of HPLC method, the response characteristics for the various biomarker compounds are summarized in Table 1. Good linear relationship between peak area and concentration was obtained for each biomarker compound over the tested concentration range 2.0–10.0 mg L^{-1} with correlation coefficient >0.999 for all the biomarkers, as listed in Table 1. The reproducibility of the retention time and peak area for the four biomarker compounds was investigated under optimum HPLC conditions. It was achieved by doing repeated injections ($n = 6$) of a mixture of the standards at a concentration of 6 mg L^{-1}. It was found that a relative standard deviation (RSD) of less than 0.06% could be reproduced for retention time and *ca.* 0.52% for peak area. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise (S/N) of about 3 and 10 respectively. The LOD defined as the concentration resulting in a signal of three times the noise level is shown in Table 1. The lowest concentration of the calibration range was considered to be the limit of quantification (LOQ), which was from 3.2 to 3.5 mg L^{-1} for the biomarker compounds.

The precision and accuracy of the GC–MS method were carried out by analyzing six injections of different plant extracts. The retention time of the peaks was found to be stable on the intra-day and inter-day with variation less than 0.5% (relative standard deviation (RSD), $n = 6$). The RSD values for normalized peak areas for different compounds for the intra-day ($n = 6$) and inter-day ($n = 6$)) were found to vary from 1.06% to 7.45%. The metabolites detected using GC–MS on different plant extracts were summarized in Table 1, with RSD ranging from 1.17% to 20.0% ($n = 6$).

3.2. Optimization of MAE method (closed vessel system)

In developing a MAE method, the parameters most commonly studied were pressure, temperature, extraction time, microwave power, solvent nature and volume, and use of modifiers and additives [31]. In a closed vessel system, temperature is a preferred controlled parameter since pressure is directly dependent on temperature. Temperature could affect the overall extraction efficiency of the MAE method. The elevated temperatures could increase diffusivity of the solvent into the internal parts of the plant matrix and thus enhance the desorption and partition of components from the active sites of the plant matrix into the extraction solvent [31]. In addition, high temperatures significantly alter the solvent properties of water, rendering the attractive forces of water closer to those of non-polar compounds. This enhances the solubility of less polar compounds in water [12].

The effect of temperature from 40 °C to 120 °C on the extraction efficiencies of the 4 biomarker compounds was shown in Fig. 2. All the compounds could be extracted in the studied temperature range. The extraction temperature profile of both epiCT and RH showed that they could be extracted with higher efficiencies with higher temperature (Fig. 2). On the other hand, the recoveries of CT and CA decreased with increasing temperature due to compound degradation or hydrolytic processes (Fig. 2). The low yield of CT at 40 °C and 80 °C compared to 60 °C and the low yield of CA at 80 °C and 120 °C compared to 100 °C was found to be significant based on a two-tailed Student's *t*-test ($p < 0.05$) (Fig. 2).

It was interesting to note that even though CT and epiCT are both flavonoids, their temperature extraction profiles differed greatly. As depicted in Fig. 1, the hydroxyl group on epiCT is less able to interact with the surrounding water molecules as it is sterically hindered by the presence of a di-hydroxybenzyl group on the same plane. Therefore, this makes epiCT less polar compared to CT, which could explain the different temperature extraction profiles and elution times obtained (Figs. 2 and 3). Since the four biomarker compounds had different properties and behaviour at different extraction temperatures, 100 °C was thus selected as a compromised extraction temperature for all the compounds in the subsequent experiments. The effect of extraction time on the recoveries of the four bioactive compounds was shown in Fig. 4. The extraction of CA and RH was almost constant for the different extraction times studied (Fig. 4). The extractions of the flavonoids were observed to increase with longer extraction time before

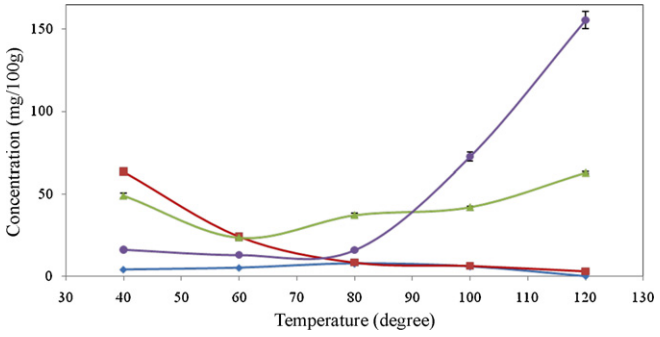

Fig. 2. Effect of different extraction temperatures on the recovery of biomarker compounds from *U. sinensis* by MAE at 20 min ($n = 3$): (a) caffeic acid and rhynchophylline and (b) epicatechin and catechin. The decrease of catechin at 40 °C and 80 °C compared to 60 °C and the decrease of caffeic acid at 80 °C and 120 °C compared to 100 °C was found to be significant based on a two-tailed Student's *t*-test ($p < 0.05$). (♦) Catechin, (■) Caffeic acid, (▲) Epicatechin, and (●) Rhynchophylline.

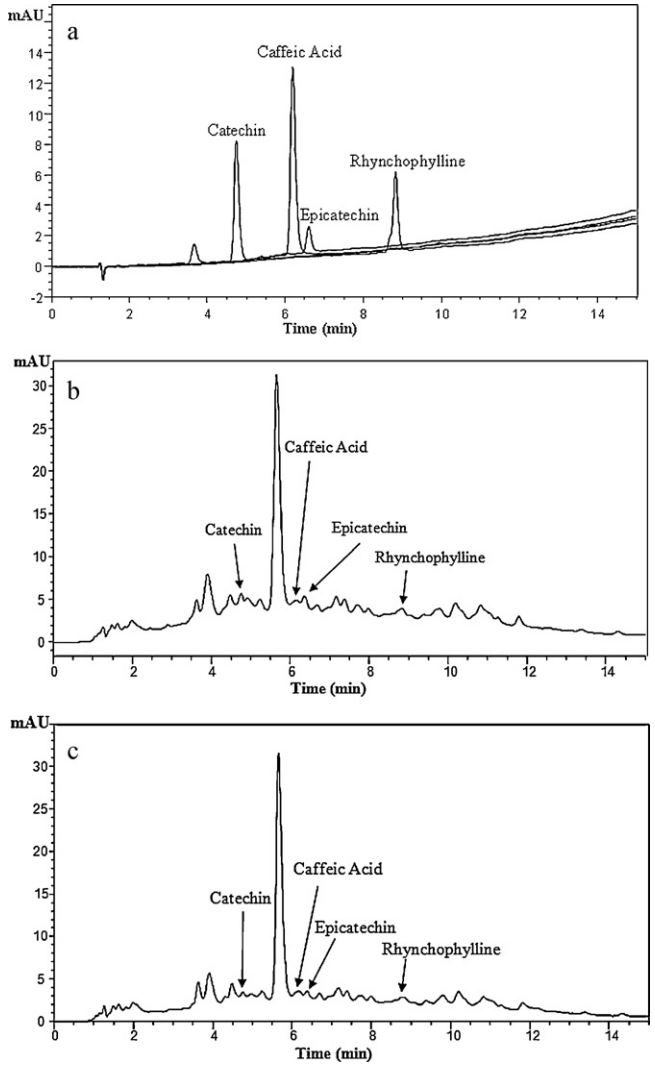

Fig. 3. Chromatograms obtained for (a) the standards at 10 mg L^{-1} and the four biomarker compounds extracted from *U. sinensis* obtained by (b) heating under reflux method for 60 min and (c) MAE at 100 °C for 20 min. HPLC conditions: ultrapure water with 0.1% formic acid as mobile phase A and methanol with 0.1% formic acid as mobile phase B. Initial condition was set at 10% of B, gradient up to 100% B in 25 min before returning to initial condition for 10 min. C_{18} reversed phase column: 150 mm × 3.9 mm, 5 μm. Detection wavelength at 280 nm and temperature of 40 °C at a flow rate of 1.0 mL min^{-1}.

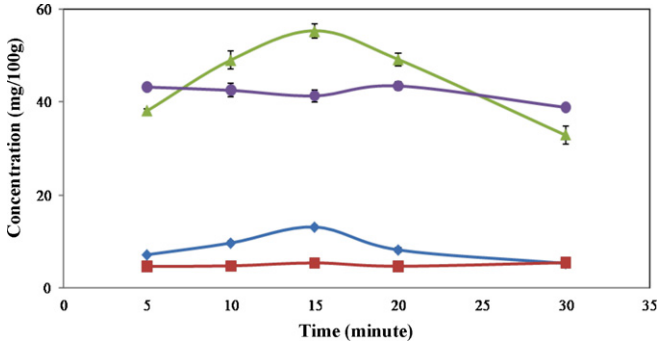

Fig. 4. Effect of extraction time on the recovery of biomarker compounds from *U. sinensis* by MAE at 100 °C ($n = 3$). The difference in the means of catechin at 5, 10, 15, 20 and 30 min and the difference in the means of rhynchophylline at 15, 20 and 30 min are found to be significant based on a two-tailed Student's *t*-test ($p < 0.05$). (♦) Catechin, (■) Caffeic acid, (▲) Epicatechin, and (●) Rhynchophylline.

they started to decrease on prolonged heating due to compound degradation (Fig. 4). There is a significant increase of recovery for epiCT with increasing extraction times up to 15 min compared to the other biomarkers (Fig. 4). All the compounds were typically extracted within 20 min at 100 °C. The differences in the means of the extraction times of CT, RH and CA at 15, 20 and 30 min were found to be significant based on a two-tailed Student's *t*-test ($p < 0.05$) (Fig. 4). Thus, the optimized conditions of MAE for the extraction of the four bioactive secondary metabolites were determined to be at 100 °C for 20 min.

3.3. Comparison of extraction efficiency between MAE and reflux method

From the chromatograms in Fig. 3 and Table 2, the extraction efficiency of MAE was observed to be comparable to heating under reflux. The identities for extracted biomarker compounds were determined by the retention times of their pure standards obtained under the same chromatographic conditions (Fig. 3). The amount of extracted biomarker compounds were computed to a 100 g dry mass of the herb (Table 2). The comparable extraction efficiencies achieved by MAE is due to its extraction mechanism, i.e. the direct interaction of microwave energy with free water molecules present in the sample which resulted in the subsequent rupture of the cell wall and release of intracellular products into the surrounding solvent [29,30,32,33]. The constant stirring improves solvent to sample contact, facilitating the dissolution process and therefore leads to faster desorption rate of analytes from sample matrix to solvent [34]. MAE could also perform multiple extractions using different vessels in a single extraction [31]. The only drawback for MAE is that vessels need to cool down after the extraction process to minimize loss of any volatile solutes [29,34].

3.4. GC–MS analyses of primary metabolites

Metabolomics enables the study of the metabolic composition of an organism or biological system, in which both secondary and primary metabolites are characterized [35]. Among the various analytical techniques, capillary GC–MS possesses high sensitivity, stability and separation efficiency [25]. Thus, it is the most robust and well established methodology for the analysis of primary plant metabolites [7,24].

Three samples of *U. sinensis* (CYY, YSF and SH) from different sources were examined. A typical GC–MS total ion chromatogram (TIC) for primary metabolites detected was shown in Fig. 5. The derivatization conditions for the reference standards and plant samples were determined to be different. Due to the complexity of the metabolites present in the plant samples, a longer incubation time (i.e. an overnight period) for their optimal derivatization was needed. A list of key primary metabolites detected in *U. sinensis* was given in Table 3. The results revealed that the primary metabolites detected were mainly carbohydrates and sugars (Table 3) which concurred with the metabolites found in phloem tissues used in transporting food manufactured in the leaves to other parts of the plant [36]. In addition, it was observed although similar primary metabolites were detected in all samples, their concentrations varied greatly between the different samples (Table 3).

Sucrose, a major source of energy for plant respiration [37], was detected in all extracts (Table 3). The breakdown products of sucrose were fructose and glucose. Glucose was then involved in glycolysis to generate other metabolites such as amino acids and fatty acids (Table 3). The high concentrations of fructose and glucose and the corresponding low concentrations of sucrose in SH suggested that sucrose was extensively broken down to fuel the glycolysis process (Table 3). The detection of higher concentrations of other sugars such as ribitol in SH, compared to CYY and YSF, showed

Table 2
Comparison of amount of biomarker compounds extracted from different batches of *U. sinensis* using MAE and heating under reflux method with ultra-pure water as the extraction fluid.

Extraction method	Concentration of biomarker compounds (mg 100 g^{-1}) ± SD			
	Catechin	Caffeic acid	Epicatechin	Rhynchophylline
MAE[a]	10.5 ± 0.47 (RSD: 4.46%, n = 6)	5.4 ± 0.21 (RSD: 0.19%, n = 6)	62.5 ± 3.50 (RSD: 5.60%, n = 6)	48.8 ± 2.25 (RSD: 4.61%, n = 6)
Reflux[b]	11.9 ± 0.01 (n = 2)	4.6 ± 0.19 (n = 2)	84.8 ± 0.57 (n = 2)	66.0 ± 0.16 (n = 2)
MAE[a] (Repeat)	35.9 ± 0.69 (RSD: 1.92%, n = 6)	16.3 ± 0.44 (RSD: 2.68%, n = 6)	44.0 ± 2.19 (RSD: 4.97%, n = 6)	31.5 ± 0.50 (RSD: 1.59%, n = 6)
Reflux[b] (Repeat)	53.6 ± 0.41 (n = 2)	17.0 ± 0.19 (n = 2)	61.3 ± 1.13 (n = 2)	33.5 ± 0.43 (n = 2)
MAE[a] (Repeat)	55.3 ± 1.71 (RSD: 3.09%, n = 6)	20.0 ± 0.18 (RSD: 0.92%, n = 6)	154.5 ± 1.42 (RSD: 0.92%, n = 6)	47.2 ± 1.25 (RSD: 2.65%, n = 6)
Reflux[b] (Repeat)	68.9 ± 1.03 (n = 2)	22.6 ± 0.68 (n = 2)	195.0 ± 1.06 (n = 2)	62.8 ± 1.37 (n = 2)

[a] Extraction solvent: water, extraction time: 20 min, extraction temperature: 100 °C.
[b] Extraction solvent: water, extraction time: 60 min.

that glucose was highly utilized (Table 3) [38]. On the other hand, the amount of fructose and glucose detected in YSF suggested that the former was preferably used instead (Table 3). For CYY, the rate of sucrose utilization was not as fast as that of SH (Table 3). Therefore, the amount of glucose and fructose were relatively lower in CYY compared to SH. Inherently, these subtle differences demonstrated a differentiation in the metabolic pathways utilized by these plants to produce primary metabolites.

From the PCA score plot shown in Fig. 6a, samples of each source could be clearly discriminated from each other. The same clusters were associated with similar chemical constituents, properties or characteristics. The score plot thus showed a clear batch-to-batch

Table 3
GC–MS analysis of primary metabolites in different sources of *U. sinensis*.

Compounds	Retention time (min)	Relative peak area		
		CYY (n = 6)	YSF (n = 6)	SH (n = 6)
Amino acids				
Phenylalanine*	10.59	0.13 ± 0.01 (RSD: 7.69%)	0.0144 ± 0.0005 (RSD: 3.47%)	0.010 ± 0.001 (RSD: 10.00%)
L-Lysine*	18.70, 19.03	0.79 ± 0.07 (RSD: 8.86%)	0.05 ± 0.0006 (RSD: 1.20%)	0.14 ± 0.01 (RSD: 7.14%)
Sugars				
Sucrose*	31.87, 33.85	2.30 ± 0.04 (RSD: 1.73%)	0.223 ± 0.003 (RSD: 1.34%)	0.15 ± 0.03 (RSD: 20.00%)
D-Fructose*	16.73, 32.29	3.66 ± 0.10 (RSD: 2.73%)	0.341 ± 0.004 (RSD: 1.17%)	17.14 ± 1.21 (RSD: 7.05%)
Ribitol	18.14	0.68 ± 0.008 (RSD: 1.17%)	0.217 ± 0.005 (RSD: 2.30%)	1.08 ± 0.04 (RSD: 3.70%)
Sorbitol*	19.38, 19.79	0.76 ± 0.01 (RSD: 1.31%)	0.111 ± 0.004 (RSD: 3.60%)	0.061 ± 0.008 (RSD: 13.11%)
D-Arabinose*	13.01	0.25 ± 0.01 (RSD: 1.41%)	0.25 ± 0.01 (RSD: 1.27%)	0.27 ± 0.01 (RSD: 2.63%)
Arabitol	14.75	0.92 ± 0.13 (RSD: 14.12%)	1.83 ± 0.09 (RSD: 5.34%)	0.68 ± 0.02 (RSD: 3.48%)
Lactose*	32.01	0.30 ± 0.03 (RSD: 10.00%)	0.72 ± 0.01 (RSD: 1.39%)	0.161 ± 0.0006 (RSD: 0.37%)
Lyxose*	17.21, 18.53	0.78 ± 0.01 (RSD: 1.71%)	0.07 ± 0.01 (RSD: 1.67%)	0.52 ± 0.01 (RSD: 1.71%)
D-Glucose*	17.28, 17.72, 18.33	0.78 ± 0.11 (RSD: 14.10%)	1.78 ± 0.02 (RSD: 1.12%)	1.82 ± 0.11 (RSD: 6.04%)
D-Galactose*	18.60	0.79 ± 0.06 (RSD: 7.59%)	0.061 ± 0.0001 (RSD: 0.16%)	1.29 ± 0.06 (RSD: 4.65%)
Xylitol	19.16	3.65 ± 0.04 (RSD: 1.19%)	0.14 ± 0.01 (RSD: 2.02%)	0.62 ± 0.12 (RSD: 19.4%)
Inositol*	20.37	3.31 ± 0.10 (RSD: 3.08%)	0.27 ± 0.01 (RSD: 5.73%)	0.72 ± 0.01 (RSD: 1.74%)
Others				
Cinnamic acid*	19.41	4.33 ± 0.04 (RSD: 0.92%)	0.38 ± 0.025 (RSD: 6.57%)	0.092 ± 0.006 (RSD: 6.52%)
Glycerol	5.03	3.25 ± 0.15 (RSD: 4.62%)	3.45 ± 0.19 (RSD: 5.53%)	3.44 ± 0.05 (RSD: 1.52%)
Malic acid	9.32	1.03 ± 0.01 (RSD: 0.97%)	1.08 ± 0.04 (RSD: 3.70%)	0.18 ± 0.01 (RSD: 5.55%)
Propanoic acid	6.05	0.21 ± 0.01 (RSD: 4.76%)	0.75 ± 0.01 (RSD: 1.33%)	0.15 ± 0.01 (RSD: 6.67%)

* Denoted those metabolites identified with standards and searches from NIST Mass Spectral Library 2002.

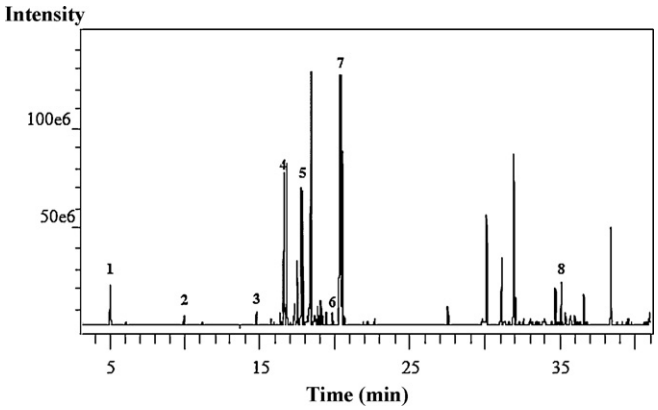

Fig. 5. A representative total ion chromatogram (TIC) from the analysis of derivatized aqueous mixture fraction of *U. sinesis* plant extract using GC–MS. (1) Glycerol; (2) phenylalanine; (3) arabinitol; (4) fructose; (5) glucose; (6) cinnamic acid; (7) inositol; (8) sucrose. Injector: splitless at 280 °C. Carrier: helium. Oven: initial at 100–300 °C at 10 °C/min. Detector: mass selective detector, 300 °C, scan mode: 50–720 *m/z*. Column: HP5, 30 m × 0.25 mm i.d., 0.25 μm.

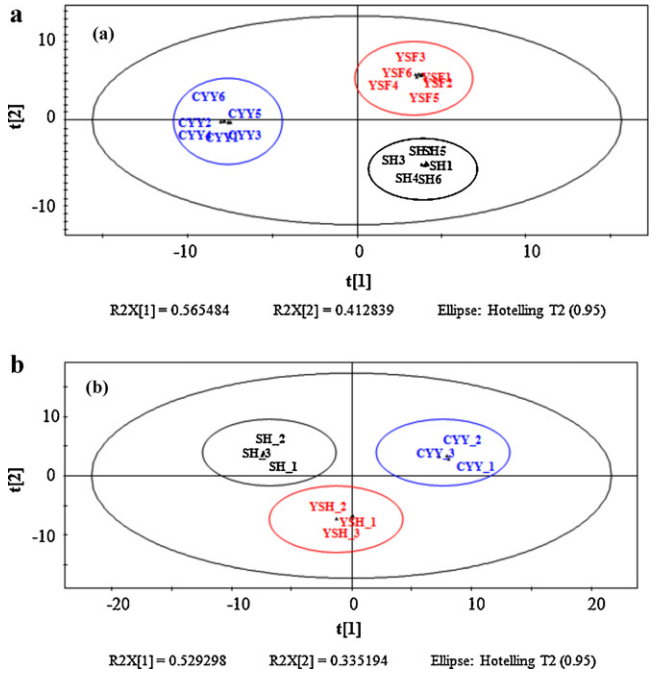

Fig. 6. The PCA score plots obtained using a combination of PC1 and PC2 for (a) primary metabolites and (b) secondary metabolites present in different sources of *U. sinensis* extracts (S1–S3). Abbreviation: S: Source.

differentiation in the amount of primary metabolites present in the samples. The application of chemometric methods for chromatographic fingerprinting offers several benefits; it is not subjective, analysis is consistent, and the results are reproducible [39].

3.5. HPLC analyses of bioactive compounds

The HPLC is still the most popular separation technique with wide applicability and high accuracy for the qualitative and quantitative analyses of TCM herbs [25]. The use of HPLC was previously reported in evaluating the quality of *S. rebaudiana* Bertoni [15], quantifying concentrations of damnacanthal in *Morinda citrifolia* [40] and analysis of anthocyanins in red cabbage [41]. The four bioactive compounds in the MAE extracts were successfully separated and quantified by the HPLC analysis (Fig. 3). Although all the samples were hooks and stem parts of *U. sinensis*, our results revealed that the concentrations of their bioactive secondary metabolites varied for the different sources (Table 4).

Phenylalanine is an important precursor for the biosynthesis of flavonoids. Phenylalanine ammonia-lyase converts the amino acid phenylalanine into cinnamic acid, which is also an important intermediate in the biosynthesis of flavonoids [42,43]. From Table 3, it was observed that the concentration of phenylalanine and cinnamic acid was lowest in SH. The high recoveries of CT and epiCT, both flavonoids, from SH suggested that phenylalanine and cinnamic acid were extensively used for the synthesis of these bioactive compounds (Table 4). Cinnamic acid can also be hydroxylated to give *p*-coumaric acid and caffeic acid [43]. From Table 3, it was noted that CYY has the highest concentration of cinnamic acid. The corresponding low recovery of caffeic acid from CYY suggested that cinnamic acid was not extensively used in its production (Table 4). From the results obtained, SH source should have the highest potency compared to the other two sources (Table 4).

It was interesting to note that certain amino acids, such as tryptophan, which were required for alkaloid biosynthesis, might be in trace amounts and thus not detected (Table 3) [5,44]. This observation proposed that tryptophan was used to synthesize the indole alkaloid rhynchophylline which was present as the major bioactive compound in all the medicinal herbs (Table 4). Other amino acids such as lysine were reported to be the precursor in the biosynthesis of alkaloids [45,46]. The extremely low level of lysine and rhyncophylline detected in YSF extract could support the mechanism that precursor pool size might actually regulate bioactive product accumulation [43]. In addition, the small pool of lysine could be further depleted via a lysine catabolism pathway to produce acetyl-coA and glutamate [47].

From Fig. 6b, batch-to-batch differentiation of the samples was observed in the PCA score plot based on their secondary metabolite profiling obtained.

The PCA score plots for either the primary or secondary metabolites could be used to evaluate the quality of the medicinal herbs (Fig. 6a and b). It was noted that all the sources contain similar metabolites but in different concentrations (Tables 2 and 3). Consequently, this showed that different cultivation conditions arising from different geographical locations, soils and climate change could have a significant impact on the production of plant metabolites [48]. Thus, the combination of the primary and secondary metabolite profiling with PCA could generate a model suitable for an overall assessment of the quality of the medicinal herbs.

Table 4
Amount of biomarker compounds extracted from different sources of *U. sinensis* using MAE method with ultra-pure water as the extraction fluid.

MAE[a] at 100 °C	Concentration of biomarker compounds (mg 100 g^{-1}) ± SD			
	Catechin	Caffeic acid	Epicatechin	Rhynchophylline
CYY	15.8 ± 1.28 (RSD: 8.07%, *n* = 3)	6.4 ± 0.08 (RSD: 1.21%, *n* = 3)	20.2 ± 1.28 (RSD: 6.35%, *n* = 3)	117.6 ± 5.59 (RSD: 4.76%, *n* = 3)
YSF	36.5 ± 1.60 (RSD: 4.38%, *n* = 3)	17.8 ± 0.52 (RSD: 2.91%, *n* = 3)	44.0 ± 1.22 (RSD: 2.78%, *n* = 3)	59.9 ± 2.58 (RSD: 4.31%, *n* = 3)
SH	98.3 ± 1.18 (RSD: 1.20%, *n* = 3)	11.9 ± 0.24 (RSD: 2.02%, *n* = 3)	81.5 ± 1.99 (RSD: 2.44%, *n* = 3)	109.2 ± 1.84 (RSD: 1.69%, *n* = 3)

[a] Optimized extraction conditions: water as the extraction solvent, extraction time: 20 min.

4. Conclusions

The proposed MAE method using ultra-pure water as the extraction solvent, under 100 °C and 20 min extraction time, was shown to be feasible for the extraction of the different classes of active biomarker compounds in *U. sinensis*. These optimized MAE method could extract biomarker compounds from different classes of chemical compounds, namely flavonoid, organic acid and alkaloid. The proposed green solvent MAE method was able to give precise and reproducible results, and showed comparable extraction efficiency to the time consuming conventional method of heating under reflux. Compared with usual boiling and maceration methods commonly used in TCM, MAE was able to reduce the extraction time and eliminate the usage of organic solvents. Therefore, the green solvent MAE method can be proposed to be the standard extraction assay for *U. sinensis* as well as other medicinal herbs in the future. The primary and secondary metabolite profiling combined with PCA analysis could provide a rapid approach in the classification and comparison of the quality of *U. sinensis* obtained from different sources. Additionally, this current work also demonstrates the feasibility of using green solvent MAE with metabolic fingerprints and PCA for the quality assessment of other TCM and medicinal herbs.

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