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Analytical Methods

An optimized ultrasound-assisted extraction and simultaneous quantification of 26 characteristic components with four structure types in functional foods from ginkgo seeds

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ABSTRACT

An optimized method of ultrasound-assisted extraction followed by ultra-high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UAE–UHPLC–TQ/MS^2) was proposed for the simultaneous extraction and determination of 26 characteristic components covering four structure types (flavonoids, terpene lactones, ginkgolic acids and phenylpropanols) in ginkgo seeds (GSs). The UAE parameters (ultrasound power, time and solvent-to-material ratio) were optimized using a response surface methodology. This is the first report of the simultaneous analysis of 26 compounds in *Ginkgo biloba* using UHPLC–TQ/MS^2; this analysis afforded good linearity, precision, repeatability and accuracy. UAE–UHPLC–TQ/MS^2 was successfully applied to ginkgo seed samples, and the analysis showed that GSs are rich in terpene lactones and could be selected as a healthy food resource. The results suggest that UAE–UHPLC–TQ/MS^2 might be able to be utilized as a tool for the quality assessment of samples from GSs or other related products using flavonoids, terpene lactones, ginkgolic acids and phenylpropanols as markers.

1. Introduction

As an archaic living fossil, *Ginkgo biloba* has existed on the earth for two hundred million years (Major, 1967). However, only during the last couple of decades has its true value has been recognized. *G. biloba* leaves (GBLs) and extracts (GBEs) have attracted considerable attention mainly because they contain distinct types of compounds that exhibit synergistic action, such as flavonoids and ginkgolides, which have been used in commercial medical products and dietary supplements in many countries (Van Beek & Montoro, 2009). The flavonoids are responsible for free radical scavenging effects, and the terpene lactones can selectively inhibit platelet-activating factor (Stromgaard & Nakanishi, 2004; Van Beek & Montoro, 2009). Commercial *G. biloba* products have usually been standardized based on the contents of terpene lactones and flavonoids (Dubber & Kanfer, 2006). However, ginkgolic acids have been identified as potentially hazardous constituents in GBLs and GBEs (Fukuda et al., 2009). Tests for limiting the ginkgolic acids were presented in a draft monograph in Europe (Van Beek & Montoro, 2009). Up until now, larger studies have concentrated on the three types of characteristic constituents mentioned above in GBLs or GBEs and have overlooked the importance of ginkgo seeds (GSs). GSs have been used in traditional medicine and foodstuff in China as early as 5000 years ago. As a foodstuff, GSs can be added to desserts, glazed fruit, beverages and tipple (Deng et al., 2011). Numerous studies have been published on commonplace nutritional and water-soluble components in GSs, such as protein, carbohydrates, amino acids, vitamins and microelements (Zhou et al., 2013). Characteristic compounds of GSs are also important constituents that have often been ignored in the past. In our previous work, 4 terpene lactones, 7 flavonoids, 2 ginkgolic acids and 1 phenylpropanol were isolated from GSs (Zhou et al., 2012a,b). Hence, exploring the characterisation of compounds of the four structure types mentioned above (flavonoid, terpene lactone, ginkgolic acid and phenylpropanol) in GSs would be very helpful for identifying their potential value as food and medicine.

Several different techniques have been used for analyzing target compounds, including (1) gas chromatography–mass spectrometry

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(GC–MS) (Deng & Zito, 2003), (2) capillary electrophoresis with ultraviolet detection (CE–UV) (Goh, Barlow, & Yong, 2003), (3) liquid chromatography with ultraviolet detection (LC–UV) (Tang et al., 2010), (4) liquid chromatography with evaporative light scattering detection (LC–ELSD) (Pushpinder, Abha, Bikram, & Gopichand, 2009), (5) liquid chromatography with nuclear magnetic resonance (LC–NMR) (Qiu, Friesen, Mcalpine, & Pauli, 2012) and (6) LC–MS (Ding, Chen, Zhou, & Lindsay, 2004). However, most of the present methods, which required multiple and/or time-consuming sample preparations, exhibited low sensitivity and/or bad resolution and only allowed the determination of one or two types of analytes. Yao et al. reported LC–MS for the determination of three types of compounds in GBLs (Yao et al., 2013). However, this validated method did not apply to the simultaneous determination of the four structure types of characteristic compounds and did not report the important matrix effects. For these reasons, in this study, ultra-high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UHPLC–TQ/MS^2) was developed for the simultaneous determination of characteristic compounds of four structure types from GSs.

An effective sample extraction process was also necessary. Recently, various extraction techniques have also been developed for the extraction of target compounds from functional foods, including microwave-assisted extraction (Chan, Yusoff, & Ngoh, 2013), ultrasound-assisted extraction (Şahin, Aybastıer, & Işık, 2013), supercritical fluid extraction (Costa et al., 2012), pressurized liquid extraction (Li et al., 2012), solid-phase microextraction (Pino & Febles, 2013) and hollow fiber liquid-phase microextraction (Hadjmohammadi, Karimiyan, & Sharifi, 2013), among which ultrasound-assisted extraction (UAE) exhibited the best mass transfer, cell disruption, solvent penetration and capillary effect (Da Porto, Porretto, & Decorti, 2013). The principal advantage of UAE was that it was the simplest and most economical technique, and it was easy to scale up to the industrial level (Carrera, Ruiz-Rodríguez, Palma, & Barroso, 2012). Moreover, UAE did not damage the botanical materials, which are sensitive to temperature (Sereshti, Rohanifar, Bakhtiari, & Samadi, 2012). Response surface methodology (RSM), which evaluates multiple parameters and their interactions based on quantitative data (Li et al., 2011), was used to optimize the UAE conditions (Şahin et al., 2013). To the best of our knowledge, there has been no report about the application of RSM to the optimization of UAE conditions for the extraction of four types of compounds in *G. biloba*.

The objectives of the present study were (1) to develop an analytical method for the rapid, accurate and repeatable determination of the target compounds by UHPLC–TQ/MS^2, (2) to optimize UAE by RSM, (3) to apply the hyphenated method of UAE–UHPLC–TQ/MS^2 to investigate the 26 characteristic compounds covering four structure types in GSs and (4) to explore the utilization of GSs as a resource.

2. Experimental

2.1. Materials and samples

The acetonitrile and formic acid were of HPLC grade and were obtained from Merck (Darmstadt, Germany). All other reagents and chemicals were of analytical grade.

The structures of the 26 standards are shown in Fig. 1. Standards of **1**, **3** and **4** were purchased from Sigma–Aldrich (St. Louis, MO, USA). Compounds **5**, **7**, **9**, **11**, **15**–**19** and **21** were isolated from *G. biloba* in our laboratory and were identified by means of extensive chemical and spectroscopic analysis. The purity of each compound was >98%.

Individual standards were prepared by dissolving 1.0 mg of the standard in 25 mL of 70% (v/v) methanol. Working standard mixtures (10–8000 ng mL^{-1}) were prepared by diluting each primary stock solution with 70% methanol. All of the standard solutions were stored at 4 °C until further use.

Ginkgo seed samples (48), consisting of 15 samples from five different parts of the seeds (episperm, mesosperm, endopleura, endosperm and plumule), which are shown in Fig. S1, 11 samples from trees of different ages (8, 10, 15, 20, 25, 30, 40, 50, 100, 300 and 600 years of age) and 22 samples from different habitats in China, were analyzed. Details of each sample are listed in Table 1. The botanical origins of the samples were identified as the seeds of *G. biloba* L. by Dr. Hui Yan (Department of Pharmacognosy, Nanjing University of Chinese Medicine, China). After collection, the samples were air-dried.

2.2. Extraction procedures

2.2.1. Ultrasound-assisted extraction process

The extraction process was performed with an ultrasonic device (KH300SP, 25 kHz, 300 W, Kunshan Ultrasonic Instrument Co. Jiangsu, China) equipped with a digital timer and a temperature controller. A sample of 0.5 g of 40 mesh dry powder was accurately weighed and extracted under several sets of designed UAE conditions. After the ultrasonic extraction, the sample was centrifuged at 13,000 rpm for 10 min to collect the supernatant for analysis.

2.2.2. Experiment design for the optimization of UAE

A three-factor, three-level Box–Behnken design (BBD) was applied to determine the optimal conditions for UAE in this study. The experimental BBD consisted of 17 treatments, including five replicates of the central point (Table 2). The ultrasonic power (W, x_1), extraction time (min, x_2) and S/M ratio (mL g^{-1}, x_3) were chosen as the independent variables. The data from the BBD were analyzed by multiple regression to fit the following quadratic equation:

$$Y = \varphi_0 + \sum_{i=1}^{3}\varphi_i x_i + \sum_{i=1}^{3}\varphi_{ii}x_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{3}\varphi_{ij}x_i x_j \quad (1)$$

where Y, φ_0, φ_i, φ_{ii} and φ_{ij} indicate the predicted response, the intercept term, the linear coefficient, the squared coefficient and the interaction coefficient, respectively.

2.3. UHPLC–TQ/MS^2 analysis conditions

UHPLC was performed using a Waters ACQUITY UPLC system (Waters, Milford, MA, USA). An Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm) maintained at 35 °C was used with an injection volume of 2 μL. Mobile phase A was a 0.1% formic acid/water solution (1/1000, v/v), mobile phase B was a formic acid/acetonitrile solution (1/1000, v/v), and the flow rate was 0.4 mL min^{-1}. The linear gradient conditions were: 0–7 min, 90–65% A; and 7–11 min, 65–40% A.

Mass spectrometric analysis was carried out using a Waters Xevo TQ tandem quadrupole mass spectrometer (Micromass MS Technologies, Manchester, UK). All of the target compounds were detected using an ESI ion source. The parameters in the source were set as follows: capillary voltage = 3.0 kV; source temperature = 150 °C; desolvation temperature = 550 °C; cone gas flow = 50 L h^{-1} and desolvation gas flow = 1000 L h^{-1}. The MS parameters were individually optimized for each target compound and are listed in Table 3.