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Assay of free ferulic acid and total ferulic acid for quality assessment of *Angelica sinensis*

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Abstract

Activity of Chinese Danggui (DG), the processed root of *Angelica sinensis* (Oliv.) Diels, is linked to the ferulic acid content but the stability of ferulic acid during extraction for medicinal use is not known. The stabilities of ferulic acid and coniferyl ferulate were evaluated in the extracts of DG using a variety of extraction solvents. These included various combinations and proportions of methanol, water, formic acid, 1 M aqueous hydrochloric acid and 2% sodium hydrogen carbonate ($NaHCO_3$) in water. Coniferyl ferulate was found liable to hydrolyze into ferulic acid in neutral, strongly acidic and basic solvents, where heat and water could facilitate this hydrolysis. However, the hydrolysis was relatively resisted in weakly organic acid. Based on the stability evaluation, two new terms, namely: free ferulic acid and total ferulic acid, were suggested and defined. Free ferulic acid refers to the natural content of ferulic acid in herbs. Total ferulic acid means the sum of free ferulic acid plus the amount of related hydrolyzed components. Meanwhile, the high-performance liquid chromatographic (HPLC) method was developed to assay free ferulic acid and total ferulic acid in DG using methanol–formic acid (95:5) and methanol–2% $NaHCO_3$ in water (95:5) as extraction solvents, respectively. Ten DG samples were investigated on their contents of free and total ferulic acid. The results indicated that the amount variety of free ferulic acid was larger than that of their counterparts, and the ratio of total ferulic acid to free ferulic acid was 4.07 ± 2.73 (mean $\pm$ SD, $n = 10$). The chemical assay of DG using total ferulic acid content would be a better choice to assess the herbal quality and was recommended.

Keywords: Ferulic acid; Coniferyl ferulate; *Angelica sinensis*; HPLC; Pharmaceutical analysis

1. Introduction

Chinese Danggui (Radix *Angelicae Sinensis*, DG) is the processed root of *A. sinensis* (Oliv.) Diels, which is one of the widely used traditional Chinese medicinal (TCM) materials to enrich blood, activate blood circulation, regulate menstruation, relieve pain and relax bowels, etc. There are over eighty composite formulae of TCM containing DG [1]. Furthermore, this herb is commonly used as a female tonic, dietary supplements and one of the cosmetic ingredients sold in China, Europe, USA and/or other countries [2–5]. Its medicinal value has been demonstrated by numerous clinical trials, pre-clinical studies and traditional or modern experiences [6–11].

Ferulic acid was isolated from DG and was also found in other plants [12–14]. Pharmacological studies showed that ferulic acid and/or sodium ferulate had been found to inhibit platelet aggregation, increase coronary blood flow, relax or stimulate smooth muscle, possess anti-arrhythmic affects, anti-oxidate, immunostimulate, anti-inflammatory effects, etc. [2,6,15–19]. Some of these bioactivities were related to the medicinal functions of DG. Therefore, ferulic acid was widely used as one of the marker compounds to assess the quality of DG and its products [20–26]. However, the reported content of ferulic acid in DG varies within the range of 0.211–1.43 mg/g, and which were quantified by a variety of methods (Table 1) [20–36]. Apart from the variation in natural abundance among the samples, the

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Table 1
The variation in contents of ferulic acid for *Angelica sinensis* (Oliv.) Diels analyzed by the different methods in literatures

Extraction solvent	Extraction method	Analytical method	Content (mg/g)	Reference
Methanol	Reflux	TLC	1.08	27
Methanol	Reflux	HPLC	0.418–1.20	28
Methanol	Sonication	HPLC	0.423–1.03	20, 21
Methanol	Sonication	CE	0.211–0.226	22
Methanol–formic acid (95:5)	Immersion	HPLC	0.425	29
Methanol–formic acid (95:5)	Immersion	TLC	0.529	30
Methanol–formic acid (95:5)	Soxhlet	TLC	0.673–1.17	23
Methanol–formic acid (95:5)	Sonication	HPLC	0.233–0.479	31
Aqueous methanol	Sonication	HPLC	0.35–1.43	24
70% methanol	Reflux	HPLC	0.915–1.37	32
Ethanol	Reflux	HPLC	0.468	33
70% Ethanol	Sonication	CE	0.486–1.02	34
50% Ethanol	Reflux	HPLC	0.582–0.606	35, 36
Diethyl ether–methanol (20:1)	Reflux	HPLC	0.271	25
Water	Reflux	CE	0.415	26

nature of extraction solvents and methods were likely to be a critical cause. In the reported literatures, DG sample was commonly extracted using a variety of solvents, namely: methanol, methanol–formic acid (95:5), ethanol, diethyl ether–methanol (20:1) and/or water under reflux, sonication, immersion or soxhlet extraction (Table 1) [20–36]. However, coniferyl ferulate, the ester of ferulic acid, was also found in DG sample [37–39]. Kobayashi et al. reported that coniferyl ferulate was liable to hydrolyze into ferulic acid and coniferyl alcohol even if the pulverized sample of *Cnidium officinale* Makino was heated in water for 1 h (Fig. 1) [40]. According to this reported result, coniferyl ferulate is likely to be hydrolyzed in a variable extent in different extraction solvents and therefore resulting in a variety of level of ferulic acid determined in herbs. It is worth noting that TCM prescription is usually prepared and then decocted in boiling water. In this regard, coniferyl ferulate in TCM prescription materials are being easily converted into ferulic acid during extraction with boiling water. Therefore, ferulic acid remained as the major chemical constituent in this aqueous extract. If this is true, ferulic acid should be the principal functional compound instead of coniferyl ferulate in DG material according to the TCM practice. Another concern is about the reported levels of ferulic acid in literatures which might not represent the natural content in herbs or the actual amount for medicinal functions. Therefore, it is of top importance to examine the stability of ferulic acid in the different extraction conditions and to develop an accurate method for ferulic acid in DG sample for its quality assessment and evaluation of the therapeutic effect of DG.

This paper focuses mainly on studying the stability and relationship of ferulic acid with coniferyl ferulate, and developing a quantitative analysis method for the assay of ferulic acid in DG sample. Firstly, ferulic acid and coniferyl ferulate were identified in the HPLC chromatograms of DG extracts based on the on-line HPLC-atmospheric pressure chemical ionization (APCI)-MS and UV techniques. Then, the stabilities of ferulic acid and coniferyl ferulate were examined in extracts of DG samples by comparing their amounts using a variety of solvent/solvent combination and extraction methods. These solvents include methanol, methanol–formic acid, methanol–formic acid–water, methanol–hydrochloric acid (HCl) in water, methanol–water, water and methanol–sodium hydrogen carbonate ($NaHCO_3$) in water (Table 2). The results showed that coniferyl ferulate was liable to hydrolyze into ferulic acid in neutral, strongly acidic or basic media including methanol, methanol–water, water, methanol–1 M HCl in water and methanol–2% of $NaHCO_3$ in water resulting in a variety amount of ferulic acid being determined when the DG samples were extracted by different methods. However, a relatively stable amount of ferulic acid could be obtained in herb extracted with methanol–formic acid or methanol–2% $NaHCO_3$ in water.

Based on the observation, two new terms, ‘free ferulic acid’ and ‘total ferulic acid’, were suggested and defined. Free ferulic acid means the freely available ferulic acid and represents the natural content of ferulic acid in herb. Total ferulic acid is the sum of free plus the ferulic acid obtained from hydrolysis of conjugated ferulate, which represents the amount of ferulic acid in medicinal function. Through a

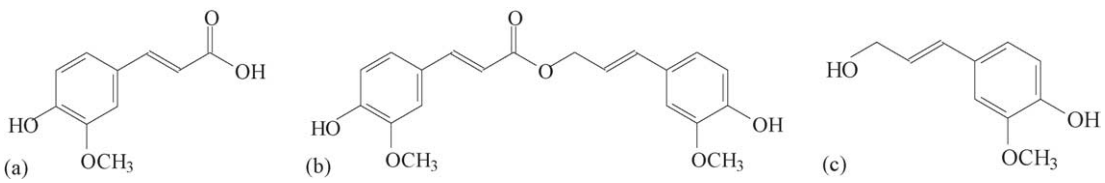

Fig. 1. Chemical structure of (a) ferulic acid, (b) coniferyl ferulate and (c) coniferyl alcohol.

Table 2
Comparison of ferulic acid and coniferyl ferulate in the sample of *Angelica sinensis* (Oliv.) Diels extracted with different solvents and methods

Number	Solvent	Method	Temperature	Time (min)	Ferulic acid[a]	Coniferyl ferulate[a]	Ratio[b]
1	Methanol	Sonication	Ambient	100	1097.7	691.5	1.59
2	Methanol	Sonication	50 °C	100	1429.2	315.1	4.54
3	Methanol	Reflux	Boiling	60	1445.5	52.7	27.44
4	Methanol	Reflux	Boiling	120	1463.7	46.8	31.31
5	Methanol	Reflux	Boiling	180	1657.1	4.7	351.08
6	Methanol–formic acid (99:1)	Sonication	Ambient	100	656.0	1200.5	0.55
7	Methanol–formic acid (95:5)	Sonication	Ambient	100	665.2	1205.6	0.55
8	Methanol–formic acid (90:10)	Sonication	Ambient	100	681.2	1188.3	0.57
9	Methanol–formic acid–water (90:5:5)	Sonication	Ambient	100	707.7	1227.1	0.58
10	Methanol–formic acid (99:1)	Sonication	50 °C	100	693.3	1163.4	0.60
11	Methanol–formic acid (95:5)	Sonication	50 °C	100	720.7	1164.1	0.62
12	Methanol–formic acid (90:10)	Sonication	50 °C	100	756.6	1155.3	0.65
13	Methanol–formic acid–water (90:5:5)	Sonication	50 °C	100	751.2	1136.5	0.66
14	Methanol–1 M HCl (95:5)	Sonication	Ambient	100	1281.4	481.8	2.66
15	Methanol–1 M HCl (90:10)	Sonication	Ambient	100	1396.4	510.5	2.74
16	Methanol–1 M HCl (95:5)	Sonication	50 °C	100	1501.7	81.8	18.36
17	Methanol–water (95:5)	Sonication	Ambient	100	1239.5	545.5	2.27
18	Methanol–water (90:10)	Sonication	Ambient	100	1289.3	519.6	2.48
19	Methanol–water (85:15)	Sonication	Ambient	100	1329.9	508.1	2.62
20	Methanol–water (80:20)	Sonication	Ambient	100	1560.3	365.9	4.26
21	Methanol–water (70:30)	Sonication	Ambient	100	1700.8	136.8	12.43
22	Methanol–water (95:5)	Sonication	50 °C	100	1634.0	120.2	13.59
23	Methanol–water (95:5)	Reflux	Boiling	60	1676.1	22.1	75.94
24	Methanol–water (95:5)	Reflux	Boiling	120	1698.8	2.3	745.07
25	Water	Sonication	Ambient	100	573.2	[c]	N/A
26	Water	Sonication	50 °C	100	1081.8	[c]	N/A
27	Water	Reflux	Boiling	120	1134.6	[c]	N/A
28	Methanol–2% $NaHCO_3$ in water (99:1)	Sonication	Ambient	100	1648.3	25.0	65.93
29	Methanol–2% $NaHCO_3$ in water (97:3)	Sonication	Ambient	100	1629.9	[c]	N/A
30	Methanol–2% $NaHCO_3$ in water (95:5)	Sonication	Ambient	100	1704.8	[c]	N/A
31	Methanol–2% $NaHCO_3$ in water (93:7)	Sonication	Ambient	100	1673.5	[c]	N/A
32	Methanol–2% $NaHCO_3$ in water (90:10)	Sonication	Ambient	100	1677.4	[c]	N/A

N/A: not applicable.
[a] Specific peak area. The value is the ratio of peak area to sample weight, mAU s/g.
[b] Ratio of the peak area of ferulic acid to coniferyl ferulate.
[c] Not detected.

series of method validation, two new HPLC methods were developed to quantitatively analyze free ferulic acid and total ferulic acid in DG samples, respectively. Altogether 10 DG samples including four whole roots, two root heads, two rootlets and two slices were investigated on their contents of free ferulic acid and total ferulic acid (Table 3). The results indicated that the variety of the content of free ferulic acid in herbs were generally larger than that of total ferulic acid, and the average amount of total ferulic acid was more than four times of that of free ferulic acid. Total ferulic acid should be a better chemical marker for assessment of herbal quality.

Table 3
Contents of free ferulic acid and total ferulic acid in the samples of *Angelica sinensis* (Oliv.) Diels

Sample (voucher number)	Sampling part	Source	Free ferulic acid[a]	Total ferulic acid[a]	Ratio[b]
1 (020118-09)	Whole root	Minxian, Gansu, China	0.124 ± 0.002	1.21 ± 0.007	9.73
2 (020407-01)	Whole root	Dangchang, Gansu, China	0.377 ± 0.004	0.899 ± 0.002	2.38
3 (030328-02)	Whole root	Pingwu, Sichuan, China	0.358 ± 0.001	1.26 ± 0.036	3.53
4 (020812-01)	Whole root	Diqing, Yunnan, China	0.172 ± 0.004	0.956 ± 0.009	5.56
5 (020407-16)	Root head	Minxian, Gansu, China	0.184 ± 0.004	0.588 ± 0.007	3.20
6 (020118-11)	Root head	Weiyuan, Gansu, China	0.100 ± 0.001	0.767 ± 0.010	7.67
7 (020407-17)	Rootlet	Minxian, Gansu, China	0.354 ± 0.001	1.09 ± 0.007	3.08
8 (020407-03)	Rootlet	Dangchang, Gansu, China	0.384 ± 0.009	0.942 ± 0.008	2.45
9 (020407-18)	Root slice	Minxian, Gansu, China	0.529 ± 0.006	0.811 ± 0.003	1.53
10 (020407-08)	Root slice	Weiyuan, Gansu, China	0.296 ± 0.004	0.477 ± 0.001	1.61

[a] The value is mean ± SD ($n = 4$), mg/g. The value is expressed in three significant figures.
[b] The value is the ratio of the content of total ferulic acid to that of free ferulic acid in herbs.

2. Experimental

2.1. Instrumentation

An Agilent/HP 1100 series HPLC-DAD system consisting of a vacuum degasser, binary pump, autosampler, thermostated column compartment and diode array detection (DAD) (Agilent, Palo Alto, CA, USA) was used for acquiring chromatogram, UV spectra and 3D-plots of retention time–absorbance–wavelength. An Applied Biosystems/PE-SCIEX API 365 LC–MS–MS system with atmospheric pressure chemical ionization source (Applied Biosystems, Foster City, CA, USA) was used for mass spectrometric measurements. A Branson 5210E-MTH ultrasonic processor (Branso ultrasonics corporation, CT, USA) was used for sample extraction. An Alltima C_{18} column (5 μm, 250 mm × 4.6 mm) with a suitable guard column (C_{18}, 5 μm, 7.5 mm × 4.6 mm) was used for chromatographic analysis. The mobile phase consisted of 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% **B** in 0–18 min, 19–100% **B** in 18–60 min and 100% **B** in 60–75 min. The flow rate was 1.0 mL/min and column temperature was maintained at 30 °C. DAD detector was set at 320 nm for acquiring the chromatogram. UV spectra were acquired from 200 to 400 nm. The APCI–MS spectra were acquired in both the positive and negative ion mode.

2.2. Solvents and chemicals

Analytical grade of methanol (Labscan, Bangkok, Thailand), formic acid, sodium hydrogen carbonate (Unichem, Warsaw, Poland) and hydrochloric acid (Farco, Beijing, China) were used for preparation of standard and/or sample solutions. HPLC grade acetonitrile (Labscan, Bangkok, Thailand) and analytical grade of glacial acetic acid (Unichem, Warsaw, Poland) was used for preparation of mobile phase. Deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Ferulic acid was obtained from the Institute for the Control of Pharmaceutical and Biological Products of the People's Republic of China (Beijing, China).

2.3. Plant materials

Herb samples of *A. sinensis* (Oliv.) Diels were collected from various cultivation areas in China. Sample voucher number, source and sampling part were summarized in Table 3. Voucher specimens were preserved at the School of Chinese Medicine, Hong Kong Baptist University (Hong Kong, China). Samples 1 and 6 were collected in November 2001 and dried in shade. Sample 1 was harvested in Minxian, Gansu, China whilst sample 6 was purchased in one of the markets in Weiyuan, Gansu, China. Samples 2, 5, 7–10 were collected and supplied by Gansu Shengtai Traditional Chinese Medicine Development Limited (Gansu, China) in April 2002. Sample 4 was a commercial product from Diqing Pharmaceutical Company (Yunnan, China), and was provided by Professor Hao Zhang (West China School of Pharmacy, Sichuan University, Chengdu, China) in June 2002. Sample 3 was harvested in November 2002 and dried in the shade by Professor Liang Li (Institute of Mianyang Traditional Chinese Medicine, Sichuan, China).

2.4. Preparation of standard solution for calibration and linearity studies

For assay of free ferulic acid, the stock solution of ferulic acid was prepared at a concentration of 100 mg/L in methanol–formic acid (95:5). Calibration standard solutions were prepared in the concentration range of 1–15 mg/L with methanol–formic acid (95:5). For assay of total ferulic acid, the stock solution of ferulic acid was prepared at a concentration of 500 mg/L in methanol–2% $NaHCO_3$ in water (95:5). Calibration standard solutions were prepared in the concentration range of 5–45 mg/L with methanol–2% $NaHCO_3$ in water (95:5). An aliquot of 10 μL solution for each calibration standard solution was injected for HPLC analysis. The calibration curve was constructed by plotting the peak areas of the analyte against the concentration of ferulic acid.

2.5. Sample preparation

Representative samples were cut into smaller pieces and further grounded into powder, and passed through a 20-mesh (0.9 mm) sieve. The grounded powders were stored at about 4 °C before use.

For assay of free ferulic acid, 0.5 g of sample powder was accurately weighed and transferred into a 60 mL amber vial. Twenty-five milliliters of methanol–formic acid (95:5) was added and sonicated for 100 min. The extract was normalized to 25 mL by adding additional extraction solvent. The extract was filtered through a 0.2 μm membrane filter. An aliquot of 10 μL solution was injected for HPLC analysis. Sample duplicates were prepared.

For assay of total ferulic acid, the sample preparation was made under similar treatment as the assay of free ferulic acid except replacing the extraction solvent with methanol–2% $NaHCO_3$ in water (95:5).

3. Results and discussion

3.1. Identification of ferulic acid and coniferyl ferulate

Ferulic acid was identified in the HPLC chromatogram of DG extracts by spiking authentic standard and compared their UV and APCI–MS spectra. Owing to the unavailability of authentic compound, the identification of coniferyl ferulate in the chromatogram was based on the on-line HPLC–APCI–MS and UV spectra [41].

3.2. Stability of ferulic acid and coniferyl ferulate

Both ferulic acid and its ester, coniferyl ferulate, are coexisting in DG [37–39]. Kobayashi et al. reported that coniferyl ferulate was readily hydrolyzed into ferulic acid and coniferyl alcohol that would be easily decomposed [40]. In literature, a variety of extraction solvents and conditions had been employed in the determination of ferulic acid in DG samples. The content of ferulic acid was found to be within the range of 0.211–1.43 mg/g (Table 1) [20–36]. It was suggested that the significant variation in contents of ferulic acid was probably attributed in a certain extent to the difference in sample preparation and extraction procedures. In this study, the stabilities and levels of ferulic acid and coniferyl ferulate in the different extraction conditions were examined. Aqueous methanolic solvents in different pH values had been employed for extraction by sonication or reflux (Table 2). Although coniferyl ferulate standard was not available, specific peak area (peak area/sample weight, mAU s/g) was used for the assay purpose in this study.

For the evaluation of optimal extraction time, a comparative study of different sonication time, namely, 20, 40, 60, 80, 100, 120 and 150 min, was conducted by sonication at ambient temperature using the ultrasonic processor. Results showed that after extraction for 40 min, the amounts of ferulic acid were not obviously changed further in these extracts. Considering the possible difference in the nature of herbal samples, a duration of 100 min was chosen as the optimal extraction time for sonication extraction whilst 60 min was selected as the optimal extraction time under refluxed condition to ensure the quantitative extraction of ferulic acid.

The stabilities and amounts of ferulic acid and coniferyl ferulate with different extraction conditions are described as follows:

3.2.1. Sample extraction with methanol

Methanol was the most commonly used extraction solvent in the assay of ferulic acid in herbs in literatures. DG samples were usually extracted with methanol by sonication for 20–30 min or under reflux for 3 h (Table 1) [20–22,27–28]. In this study, the amounts of ferulic acid and coniferyl ferulate were compared in the methanolic extracts of DG by sonication for 100 min at ambient temperature, 50 °C and reflux for 60, 120 and 180 min, respectively (Table 2, numbers 1–5). In Fig. 2, the remarkable differences in levels were observed in the chromatographic patterns. The results showed that their extracts were obviously different in relative levels. The differences among the two extremities in specific peak areas were 1.5 times for ferulic acid and 147.1 times for coniferyl ferulate. The ratios of peak area of ferulic acid to coniferyl ferulate were also ranging from 1.59 to 351.1. It indicated that coniferyl ferulate was unstable in methanol at elevated temperatures. In general, the higher the extraction temperature was, the more coniferyl ferulate were hydrolyzed and hence the larger peak area of ferulic acid was being observed. This chemical transformation of

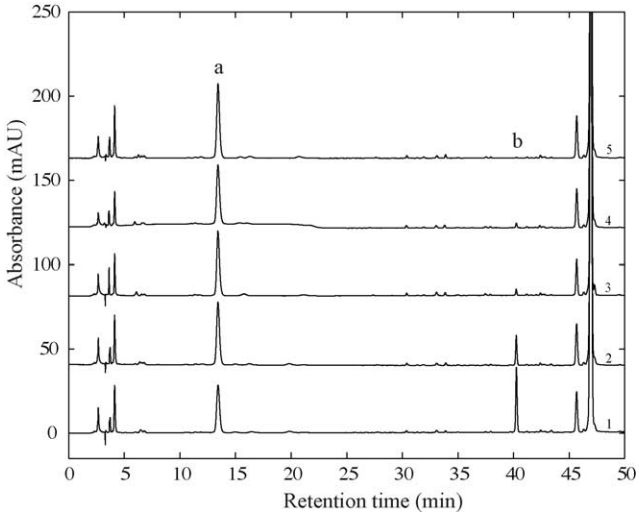

Fig. 2. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with methanol by sonication for 100 min at (1) ambient temperature and (2) 50 °C, reflux for (3) 60 min, (4) 120 min and (5) 180 min, respectively. (a) Ferulic acid; (b) coniferyl ferulate (analytical column: Alltima C_{18}, 5 μm, 250 mm × 4.6 mm; guard column: C_{18}, 5 μm, 7.5 mm × 4.6 mm; injected sample volume: 10 μL; mobile phase: 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% **B** in 0–18 min, 19–100% **B** in 18–60 min and 100% **B** in 60–75 min; flow rate: 1.0 mL/min; temperature: 30 °C; measured at UV 320 nm.).

coniferyl ferulate to ferulic acid could be visualized in the 3D-plots of retention time–absorbance–wavelength (Fig. 3).

The stabilities of ferulic acid and coniferyl ferulate in methanolic extract during storage were further evaluated. DG sample was extracted with methanol by sonication for 100 min at ambient temperature. The amounts of ferulic acid and coniferyl ferulate were determined in the sample over the storage period of 0.7–127 h after sonication. By comparing peak areas of ferulic acid and coniferyl ferulate, the peak area of ferulic acid was increased by 60.5% at 127 h with the RSD of 19.0% ($n = 11$) while that of coniferyl ferulate was decreased by 58.1% with the RSD of 26.1% ($n = 11$) (Fig. 4A). It indicated that coniferyl ferulate was relatively unstable and readily decomposed into ferulic acid whilst the amount of ferulic acid was almost quantitatively increased in the methanolic extracts during storage.

3.2.2. Sample extraction with methanol–formic acid

Methanol–formic acid (95:5) was another widely used solvent for the assay of feruilic acid in DG sample in some recent publications (Table 1) [23,29–31]. The amounts of ferulic acid and coniferyl ferulate in DG extracts were evaluated with methanol–formic acid (99:1, 95:5 and 90:10) and methanol–formic acid–water (90:5:5) by sonication for 100 min at ambient temperature and 50 °C, respectively (Table 2, numbers 6–13). The results showed that the chromatographic pattern of DG samples were similar (Fig. 5). The RSD of the specific peak area were 5.3% ($n = 8$) for ferulic acid and 2.6% ($n = 8$) for coniferyl ferulate in these extracts. The ratio of the peak area of ferulic acid to

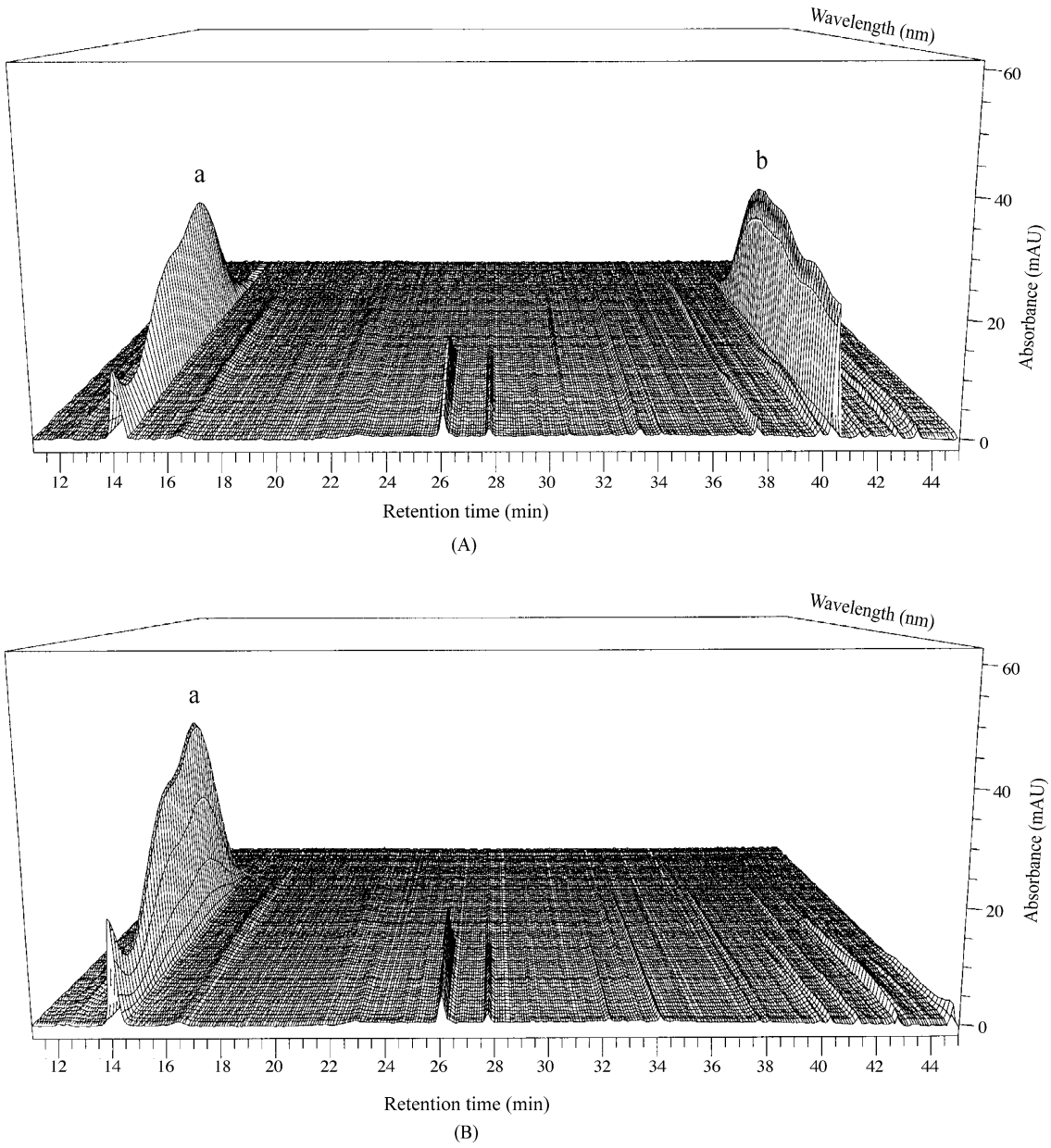

Fig. 3. 3D-plots of retention time-absorbance-wavelength of the root of *Angelica sinensis* (Oliv.) Diels extracted with methanol by (A) sonication for 100 min at ambient temperature and (B) reflux for 3 h, respectively. (a) Ferulic acid; (b) coniferyl ferulate (analytical column: Alltima C_{18}, 5 μm, 250 mm × 4.6 mm; guard column: C_{18}, 5 μm, 7.5 mm × 4.6 mm; injected sample volume: 10 μL; mobile phase: 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% **B** in 0–18 min, 19–100% **B** in 18–60 min and 100% **B** in 60–75 min; flow rate: 1.0 mL/min; temperature: 30 °C; wavelength: 250–400 nm).

coniferyl ferulate was relatively constant at 0.597 ± 0.044 (mean ± SD, $n = 8$). It indicates that the extent of hydrolysis of coniferyl ferulate can be minimized in methanol–formic acid. Although elevated extraction temperature can facilitate the hydrolysis of coniferyl ferulate, the hydrolysis is not significant in methanol–formic acid. The absolute deviation from mean (ADM) of specific peak area in the extracts of methanol–formic acid by sonication for 100 min at ambient temperature and 50 °C were 3.76% ($n = 3$) for ferulic acid and 2.14% ($n = 3$) for coniferyl ferulate (Table 2, numbers 6–8 and 10–12). However, the corresponding ADM of ferulic acid were 13.1% in methanol and 13.7% in methanol–water (95:5) (Table 2, numbers 1–2), and that of coniferyl ferulate were 37.4% in methanol and 63.9% in methanol–water (95:5) (Table 2, numbers 17 and 22). It indicated that formic acid could minimize the hydrolysis of coniferyl ferulate even in variable temperatures.

To examine the influence of the water content in methanol–formic acid to the stabilities of ferulic acid and coniferyl ferulate, the amounts of ferulic acid and coniferyl ferulate in the extracts of methanol–formic acid (95:5) and methanol–formic acid–water (90:5:5) by sonication for 100 min at ambient temperature and 50 °C were determined. The results showed that the ADM of specific peak

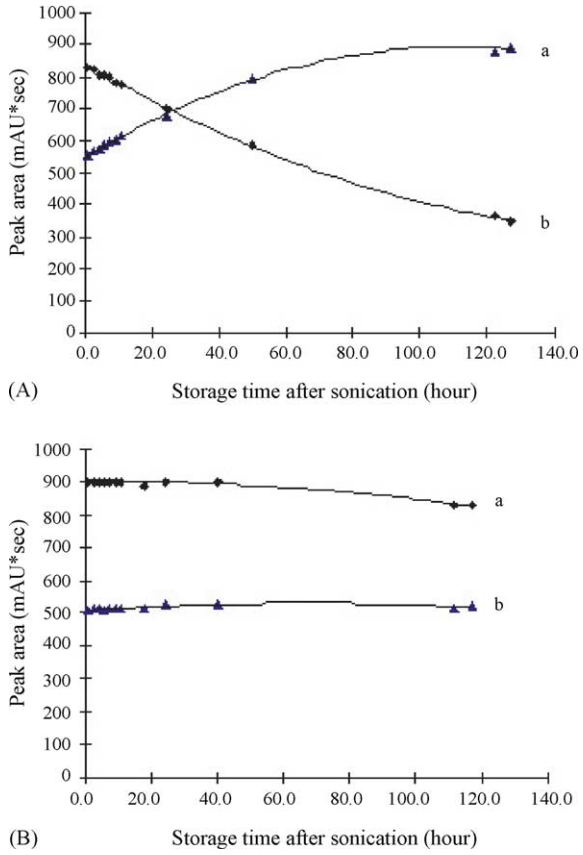

Fig. 4. Peak areas of (a) ferulic acid and (b) coniferyl ferulate in the sample solutions of (A) methanol and (B) methanol–formic acid (95:5), respectively after storage for different time, which were extracted by sonication for 100 min at ambient temperature.

area in the extracts with methanol–formic acid (95:5) and methanol–formic acid–water (90:5:5) were 2.56% ($n = 2$) for ferulic acid and 0.13% ($n = 2$) for coniferyl ferulate (Table 2, numbers 7, 9, 11 and 13). It indicated the effect of the water content on the hydrolysis was not significant in this weak organic acid.

Furthermore, the stabilities of ferulic acid and coniferyl ferulate in the extract of methanol–formic acid (95:5) by sonication for 100 min at ambient temperature during storage were evaluated. The extract was analyzed in a storage period of 0.6–117 h after sonication. By comparing the chromatographic peak areas, the peak area of ferulic acid was slightly increased with RSD of 1.18% ($n = 12$) while that of coniferyl ferulate acid was slightly decreased with RSD of 3.07% ($n = 12$) (Fig. 4B). It indicated that ferulic acid and coniferyl ferulate were relatively stable for up to five days in this medium. It is suggested that the weakly acidic solution suppresses coniferyl ferulate hydrolyzing into ferulic acid and maintains a relative balance between the chemical constituents. In this regard, the amount of ferulic acid extracted with methanol–formic acid (95:5) should be considered as the naturally occurring amount existing in the herbs.

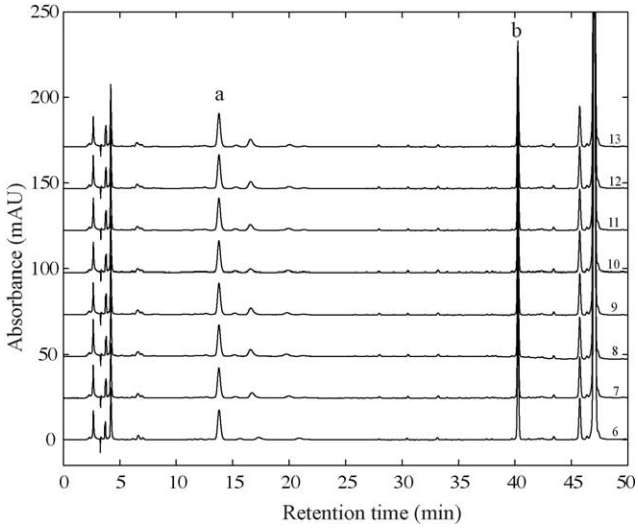

Fig. 5. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with methanol–formic acid at ratio of (6) 99:1, (7) 95:5, (8) 90:10 and (9) methanol–formic acid–water (90:5:5) by sonication for 100 min at ambient temperature, and with methanol–formic acid at ratio of (10) 99:1, (11) 95:5, (12) 90:10 and (13) methanol–formic acid–water (90:5:5) by sonication for 100 min at 50 °C. (a) Ferulic acid; (b) coniferyl ferulate (analytical column: Alltima C_{18}, 5 μm, 250 mm × 4.6 mm; guard column: C_{18}, 5 μm, 7.5 mm × 4.6 mm; injected sample volume: 10 μL; mobile phase: 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% **B** in 0–18 min, 19–100% **B** in 18–60 min and 100% **B** in 60–75 min; flow rate: 1.0 mL/min; temperature: 30 °C; measured at UV 320 nm).

3.2.3. Sample extraction with methanol–hydrochloric acid

In order to examine the effect of strong acid on the stability of coniferyl ferulate, methanol–1 M HCl in water (95:5 and 90:10) as extraction solvent for DG sample was examined by sonication for 100 min at ambient temperuature and 50 °C, respectively (Table 2, numbers 14–16). The specific peak areas of ferulic acid in the extracts with methanol–1 M HCl in water were about two times larger than that in the extracts with methanol–formic acid whilst the specific peak areas of coniferyl ferulate in the extracts with methanol–1 M HCl were only about one-third of that in the extracts with methanol–formic acid. The ratio of the peak area of ferulic acid to coniferyl ferulate in the extracts with methanol–1 M HCl in water was about 13 times larger than that in the extracts with methanol–formic acid (Table 2, numbers 6–16). It indicated that coniferyl ferulate was partially hydrolyzed in methanol–1 M HCl in water. At an elevated temperature (50 °C), most of coniferyl ferulate was hydrolyzed in the extract of DG with methanol–1 M HCl in water (95:5). The results revealed that coniferyl ferulate was liable to hydrolyze into ferulic acid in a strongly acidic medium.

3.2.4. Sample extraction with methanol–water

Aqueous methanol and 70% methanol were used as extraction solvents for the assay of ferulic acid in DG sample in literatures (Table 1) [24,32]. The effect of the

water content in the extraction medium to the stabilities of ferulic acid and coniferyl ferulate were examined by addition of water into methanol. DG samples were extracted with six different ratio of methanol to water by sonication for 100 min at ambient temperature (Table 2, numbers 1 and 17–21). The results showed that the specific peak area of ferulic acid increased by 54.9% whilst the specific peak area of coniferyl ferulate decreased by 80.2% for extraction medium with 30% water. It indicated that water facilitated the hydrolysis of coniferyl ferulate in methanol.

The effect of different extraction methods to the stability of ferulic acid and coniferyl ferulate was also examined in the extracts of methanol–water (95:5). Samples were sonicated for 100 min at ambient temperature, 50 °C and being refluxed for 1 and 2 h (Table 2, numbers 17, 22–24). It was found that the hydrolysis of coniferyl ferulate was more significant at elevated temperatures.

3.2.5. Sample extraction with water

Since TCM decoctions were always prepared in water, water was also used as one of the extraction solvents for the assay of ferulic acid in DG sample by capillary electrophoresis (CE) in literatures (Table 1) [26]. The stabilities of ferulic acid and coniferyl ferulate in water extracts were examined in this study. DG samples were extracted with water by sonication for 100 min at ambient temperature, 50 °C and reflux for 2 h (Table 2, numbers 25–27). The specific peak area of ferulic acid was different. The higher the extraction temperature was, the more ferulic acid was determined. However, coniferyl ferulate was not observed in these extracts, which indicated that ferulic acid remained the principal bioactive contributing to the therapeutic effect of DG in TCM practices. Further research works are required to clarify their pharmacological effects.

3.2.6. Sample extraction with weakly basic solvent

All of the above results showed that coniferyl ferulate was easily hydrolyzed into ferulic acid. It was suggested that coniferyl ferulate could be easily hydrolyzed in weakly basic solvent. Methanol–2% $NaHCO_3$ in water at different ratios was used as extraction solvents by sonication for 100 min at ambient temperature (Table 2, numbers 28–32; Fig. 6). The results showed that coniferyl ferulate was not observed in these extracts except in the extract of methanol–2% $NaHCO_3$ in water (99:1) (Fig. 6, number 28). A large peak of ferulic acid at specific peak area of 1666.8 ± 28.8 (mean $\pm$ SD, $n = 5$) was determined in these extracts. The results demonstrated that coniferyl ferulate was readily hydrolyzed into ferulic acid in a weakly basic solvent and hence a large amount of ferulic acid could be determined.

3.3. Definition of free ferulic acid and total ferulic acid

Literatures and the above results show that both ferulic acid and coniferyl ferulate exist in DG. Coniferyl ferulate is readily hydrolyzed into ferulic acid in neutral, basic and strongly acidic media. Elevated temperature and water content can also facilitate this hydrolysis. However, coniferyl ferulate is relatively stable in a weakly organic acid. As ferulic acid is a weak acid, it is suggested that the amount of ferulic acid extracted with a weak organic acid is freely available in herbs and is defined as 'free ferulic acid'. This may represent the natural amount of ferulic acid existing in herbs. The sum of free ferulic acid and the ferulic acid from hydrolysis of conjugated ferulate, such as coniferyl ferulate, is defined as 'total ferulic acid'. Total ferulic acid is likely to be one of the indicators of medicinal value in herbs.

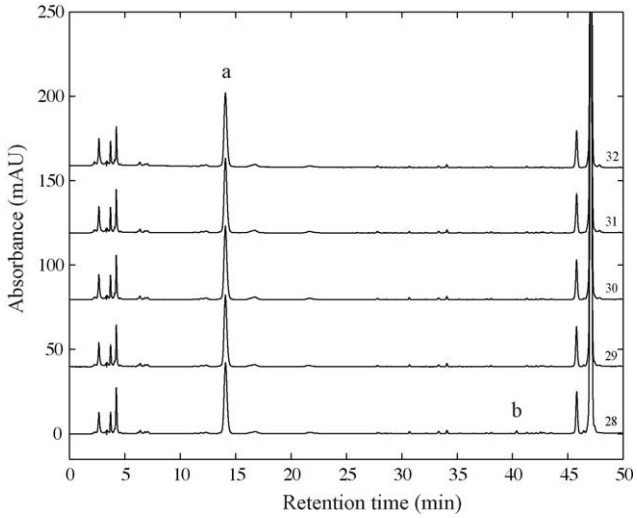

Fig. 6. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with methanol–2% of $NaHCO_3$ in water at ratio of (28) 99:1, (29) 97:3, (30) 95:5, (31) 93:7 and (32) 90:10 by sonication for 100 min at ambient temperature. (a) Ferulic acid; (b) coniferyl ferulate (analytical column: Alltima C_{18}, 5 μm, 250 mm × 4.6 mm; guard column: C_{18}, 5 μm, 7.5 mm × 4.6 mm; injected sample volume: 10 μL; mobile phase: 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% **B** in 0–18 min, 19–100% **B** in 18–60 min and 100% **B** in 60–75 min; flow rate: 1.0 mL/min; temperature: 30 °C; measured at UV 320 nm).

3.4. Quantitative analysis

Although coniferyl ferulate may be a bioactive compound activating the blood circulation and removing blood stasis [42], it is unstable and is readily hydrolyzed into ferulic acid in neutral, basic or strongly acidic solvents. As a general practice, TCM materials are usually decocted in boiling water resulting in the conversion of coniferyl ferulate into ferulic acid. Therefore, it would be appropriate to assume that ferulic acid was the functional component contributing to the therapeutic effect of DG in TCM practices. In this study, both free ferulic acid and total ferulic acid were determined in ten DG samples.

3.4.1. Selection of extraction solvent and measurement wavelength

For the assay of free ferulic acid in DG sample, methanol–formic acid (95:5) was chosen as the extraction

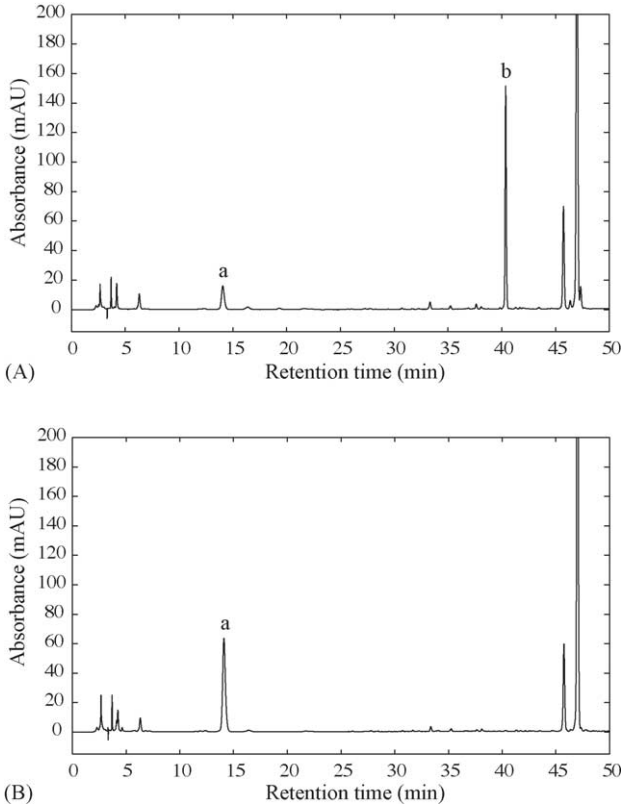

Fig. 7. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with (A) methanol–formic acid (95:5) and (B) methanol–2% of $NaHCO_3$ in water (95:5) by sonication for 100 min at ambient temperature. (a) Ferulic acid; (b) coniferyl ferulate (analytical column: Alltima C_{18}, 5 μm, 250 mm × 4.6 mm; guard column: C_{18}, 5 μm, 7.5 mm × 4.6 mm; injected sample volume: 10 μL; mobile phase: 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% **B** in 0–18 min, 19–100% **B** in 18–60 min and 100% **B** in 60–75 min; flow rate: 1.0 mL/min; temperature: 30 °C; measured at UV 320 nm).

solvent based on the stability study. Although coniferyl ferulate could be fully hydrolyzed to ferulic acid in DG sample powder with methanol by reflux of more than 3 h, or with methanol–2% $NaHCO_3$ in water (95:5) by sonication at ambient temperature for 60 min, methanol–2% $NaHCO_3$ in water (95:5) was recommended as extraction solvent for the assay of total ferulic acid in DG sample because of its ease of handling in terms of operational procedures. The chromatograms for the assay of free ferulic acid and total ferulic acid were shown in Fig. 7.

The stability of ferulic acid in storage was evaluated by dissolving ferulic acid in methanol–formic acid (95:5) and methanol–2% $NaHCO_3$ in water (95:5). Two DG samples were also extracted according to the above procedures. The amounts of ferulic acid in these solutions were determined after storage for 0, 5, 10, and 24 h, 2, 6 and 19 days, respectively. The RSD of ferulic acid content in methanol–formic acid (95:5), methanol–2% $NaHCO_3$ in water (95:5), free ferulic acid in extract of methanol–formic acid (95:5) and total ferulic acid in the extract of methanol–2% $NaHCO_3$ in water (95:5) were found to be 2.85, 1.49, 3.54 and 1.59% ($n = 7$), respectively. It indicated that ferulic acid was relatively stable in methanol–formic acid (95:5) and methanol–2% $NaHCO_3$ in water (95:5). The stability finding of free ferulic acid was generally agreed with the observation made in Section 3.2.2.

The corresponding maximum absorption of ferulic acid and coniferyl ferulate were located at UV 323 and 318 nm, respectively by HPLC–DAD analysis. The peak area of ferulic acid measured at 320 nm was estimated as 99.1 ± 0.39% (mean ± RSD, $n = 5$) to that of 323 nm whilst the peak area of coniferyl ferulate measured at 320 nm was calculated as 100.0 ± 0.12% ($n = 5$) to that of 318 nm. It indicated that the loss in sensitivity was not significant for ferulic acid and coniferyl ferulate measured at 320 nm with respect to their maximum UV absorptions. In order to detect ferulic acid and coniferyl ferulate simultaneously, UV at 320 nm was chosen as measuring wavelength in this study. In fact, UV 320 nm was also chosen as measuring wavelength for the assay of ferulic acid in literatures [21,35].

3.4.2. Linearity and calibration graphs

Ferulic acid was quantified in DG samples using external standard calibration method with reference marker. As two solvent systems were used for extracting free ferulic acid and total ferulic acid, two sets of calibration curve for the assay of free ferulic acid and total ferulic acid were prepared by dissolving standards of ferulic acid in methanol–formic acid (95:5) and methanol–2% $NaHCO_3$ in water (95:5), respectively in order to match the matrix for chromatography. Both of them showed linearity over the selected concentration ranges from 1.00–15.01 mg/L in methanol–formic acid (95:5) and 5.16–46.42 mg/L in methanol–2% $NaHCO_3$ in water (95:5). The linear regression equations of the calibration curves were calculated to be $y_{free} = 55.4792x - 2.5757$ with correlation coefficient $R^2 = 0.9996$ ($n = 7$) and $y_{total} = 55.8846x - 9.9054$ with $R^2 = 0.9992$ ($n = 6$). The method limits of detection, for the assay of free ferulic acid and total ferulic acid in DG sample were approximately 121.2 and 148.6 μg/mL in sample solution (corresponding to 6.00 and 7.34 μg/g ferulic acid in the herbs) at a signal-to-noise ratio of 3, respectively. Results also indicated that there was no solubility problem or UV absorbance difference for ferulic acid standard in methanol–formic acid (95:5) and methanol–2% $NaHCO_3$ (95:5) in water. A plot of the concentration range from 1.00 to 46.62 mg/L in the two solvents versus peak areas also gave linearity $y = 55.828x - 6.5268$ with $R^2 = 0.9995$ ($n = 13$).

3.4.3. Method validation

Method reproducibility and repeatability were evaluated by seven replicated analysis of standard solution and solid sample, respectively. Precisions of free ferulic acid and total ferulic acid for seven replicated injections of standard solution were found to be 0.37 and 0.92% RSD ($n = 7$), respectively. The RSD of the content of free ferulic acid and total ferulic acid in solid samples replicated were estimated to be 1.53 and 0.69% ($n = 7$), respectively.

The recovery and bias of ferulic acid were determined by spiking the sample with different concentration levels, namely 50, 100 and 150% of ferulic acid in the samples. For the assay of free ferulic acid, the recoveries were estimated to be $93.15 \pm 1.30\%$, $95.66 \pm 3.69\%$ and $101.50 \pm 1.19\%$ (Mean $\pm$ RSD, $n = 3$), respectively. The overall recovery was $96.18 \pm 4.20\%$ ($n = 9$). Similarly, the recoveries for assay of total ferulic acid were calculated as $97.17 \pm 1.92\%$, $98.25 \pm 1.06\%$ and $98.23 \pm 4.12\%$ ($n = 3$), respectively. The overall recovery was $97.88 \pm 2.03\%$ ($n = 9$).

3.4.4. Quantification of herb samples

The contents of free ferulic acid and total ferulic acid in 10 DG samples including four whole root samples, two root head samples, two rootlet samples and two root slice samples were determined (Table 3). The results showed that the RSD of the amounts for free ferulic acid and total ferulic acid were estimated as 47.9 and 27.9% ($n = 10$), respectively. It indicated that the variation of the content of free ferulic acid was larger than that of total ferulic acid, which implied that the amount of total ferulic acid was more consistent than that of free ferulic acid. Furthermore, the ratios of total ferulic acid to free ferulic acid were obviously different with an average of 4.07 ± 2.73 (mean $\pm$ SD, $n = 10$). The difference of the ratio of total ferulic acid to free ferulic acid content was 6.4 times between the two extreme samples. This variation of total ferulic acid to free ferulic acid was likely to be due to the different processed method, water content in herb, humidity in storage condition, etc. All these indicated that total ferulic acid was a better marker for the quality assessment of DG samples.

4. Conclusions

Ferulic acid is a bioactive compound existing in the root of *A. sinensis* (Oliv.) Diels and other plant samples. This compound is used as one of the marker compounds for quality assessment of some herbs. However, ferulic acid commonly exists with its esters and conjugated ferulated, and the latter is easily hydrolyzed into the former in different solvents and conditions which results in the variable amount of ferulic acid determined. In order to obtain a more representative amount of ferulic acid in the herbs, two ways for the assay of ferulic acid are recommended: namely, to prevent other compounds from being hydrolyzed into ferulic acid or to drive the hydrolysis to completeness. Methanol–formic acid (95:5) and methanol–2% $NaHCO_3$ in water can be used as extraction solvents, respectively for the purpose.

Two new terms of 'free ferulic acid' and 'total ferulic acid' are introduced in this study. Free ferulic acid indicates the natural content of ferulic acid in herbs, which maybe considered as a characteristic of herbal species. Total ferulic acid indicates the sum of free ferulic acid and those hydrolyzed from conjugates. The relatively unstable property of coniferyl ferulate and its absence in the water extract of DG implies that ferulic acid rather than coniferyl ferulate is the functional compound contributing to the therapeutic effect of DG in TCM decoction. However, further research works are required to clarify their pharmacological effects. The results on the amounts of free ferulic acid and total ferulic acid in ten DG samples showed that the chemical assay of herbs using total ferulic acid content would be a better choice for the quality assessment of the herb.

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