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Short communication

Qualitative and quantitative analysis of *Fructus Corni* using ultrasound assisted microwave extraction and high performance liquid chromatography coupled with diode array UV detection and time-of-flight mass spectrometry

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ABSTRACT

The first successful combination of ultrasound assisted microwave extraction (UAME) with liquid chromatography analysis is described for the quality evaluation of *Fructus Corni*, a commonly used traditional Chinese medicine (TCM). Due to their multifarious biological activities, seven representative bioactive constituents (two phenolics and five iridoids) were chosen as targets for the quality assessment. The chromatographic separation was performed on a C18 Aq column with gradient elution using methanol and aqueous solution containing 0.2% acetic acid. The quantitative method developed was validated and successfully applied to determine the seven markers in 12 batches of *Fructus Corni* extract from various habitats. Significant variations were demonstrated in the contents of seven compounds. Further 13 components were tentatively identified by online TOF mass analysis.

1. Introduction

Fructus Corni (Shan Zhu Yu in Chinese), the fruits of *Cornus officinalis* Sieb. et Zucc., is commonly used in Chinese prescriptions as a tonic agent and has been reported to possess actions such as invigorating stomach, reducing blood glucose, immunological regulation and antiarrhythmia [1–4]. In general, the therapeutic effect of traditional Chinese medicine (TCM) depends on the cumulative effects of a number of bioactive constituents in a selected treatment [5]. However, at present only one bioactive component, loganin is determined through officially conducted quality control of *Fructus Corni* [6]. Thus, an integral quality control approach based on the multiple constituents of *Fructus Corni* is urgently needed to ensure the efficacy and safety of this herbal medicine. This is the objective of the present study.

To date, in terms of authentication and evaluation of *Fructus Corni* extract (FCE), various analytical techniques have been employed based on thin layer chromatography (TLC) [7,8], capillary electrophoresis (CE) [9,10], high-performance liquid chromatography (HPLC) with different detections [11,17]. In FCE, gallic acid, morroniside, sweroside, loganin, and cornuside were generally considered as the bioactive components, and their determination was well documented in an earlier report by Du et al. [12]. In recent years, protocatechuic acid and 7-O-methylmorroniside were reported as other two important bioactive constituents for their predominant pharmacology effect [13,14], and both should also be investigated in the quality control of FCE. To the best of our knowledge, the determination of protocatechuic acid and 7-O-methylmorroniside has not been reported yet. In addition, although HPLC with different detections is among the most widely practiced method, reports on the mass spectrometric analysis of the components in *Fructus Corni* extract (FCE) have been scarce.

Effective extraction is one of the key steps in the investigation and utilization of target compounds from botanical materials. Traditional extraction methods such as Soxhlet, ultrasonication and heat reflux extraction is usually associated with longer extraction time and larger amounts of solvent with relatively lower efficiency. As an alternative, ultrasonic and microwave assisted extraction (UMAE), an extraction technique utilizing microwave dielectric heating and sono-chemistry, could drastically improve the speed and efficiency of extraction, which has been more and more widely used for sample preparation of TCM [15,16].

In this article, UAME coupled with HPLC-DAD/UV-TOF/MS system was developed for simultaneous determination of seven bioactive constituents, namely gallic acid, protocatechuic acid, morroniside, sweroside, loganin, 7-O-methylmorroniside and cor-

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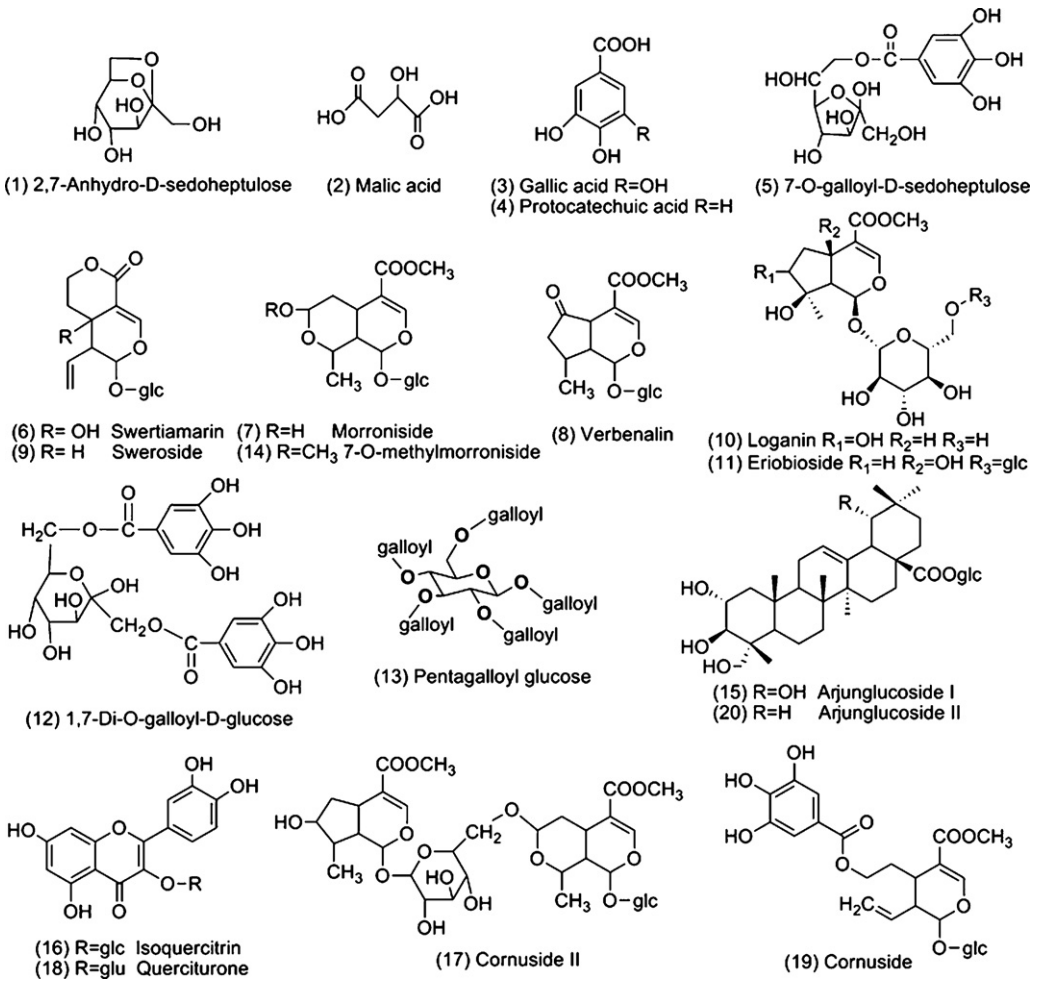

Fig. 1. Chemical structures of the compounds analyzed in FCE. The denotations from 1 to 20 are corresponding to the peak numbers in the chromatogram as listed in Fig. 2.

nuside in FCE (see Fig. 1). Besides, to depict the overall chemical profile of all herbal ingredients, a detailed study on chromatographic characteristics was also carried out by TF/MS. The results demonstrated that this developed method had potential perspective for comprehensive quality evaluation of *Fructus Corni*.

2. Experimental

2.1. Chemicals, reagents and materials

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany), and ultra-pure water was prepared by Milli-Q System (Millipore, Bedford, MA, USA). Acetic acid used in the mobile phase was of analytical grade, purchased from the Beijing Chemical Corporation (Beijing, China). Other reagents used were of analytical grade.

Chemical standards of gallic acid and loganin were purchased from Sigma (St. Louis, Missouri, USA). Protocatechuic acid, morroniside, sweroside, 7-O-methylmorroniside and cornuside were obtained from Xinyi pharmaceutical Co., Ltd. (Shanghai, China). Their structures were elucidated by spectroscopic methods (UV, IR, MS, ^{1}H NMR and ^{13}C NMR), and their purities were determined to be over 98% by normalization of the peak areas detected by HPLC-DAD/UV. Twelve commercial material samples of *Fructus Corni* were collected from different provinces in China and corresponding reference herb materials samples were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NCIPB, Beijing, China). All the voucher specimens, which were authenticated by Prof. Lian-Na Sun, are kept in our department for future reference. The air-dried samples were pulverized and the powder was screened through 40-mesh sieves.

2.2. Sample preparation

Sample preparation was performed by UAME in a simultaneous ultrasonic and microwave extracting apparatus (CW-2000, Shanghai Xintuo Microwave Instrument Co. Ltd., China). An aliquot (0.5 g) of sample was transferred to the extraction vessel and extracted under different UAME conditions according to the experimental design. The four factors of ethanol concentration, solvent to material ratio, temperature and irradiation time were optimized with central composite design (CCD). After that, the extract was clarified by centrifugation at 4000 rpm for 10 min to separate the fine particulates. The supernatant was then collected and transferred into 50 ml volumetric flask which was made up to its volume with water and filtered through a 0.22 μm nylon membrane filter prior to injection into the HPLC system.

2.3. Calculation of extraction yield

The extraction yield (EY) was chosen as the evaluating indicator for UAME extraction efficiency. It was defined as the geometric mean of the contents of 7 target compounds to provide the

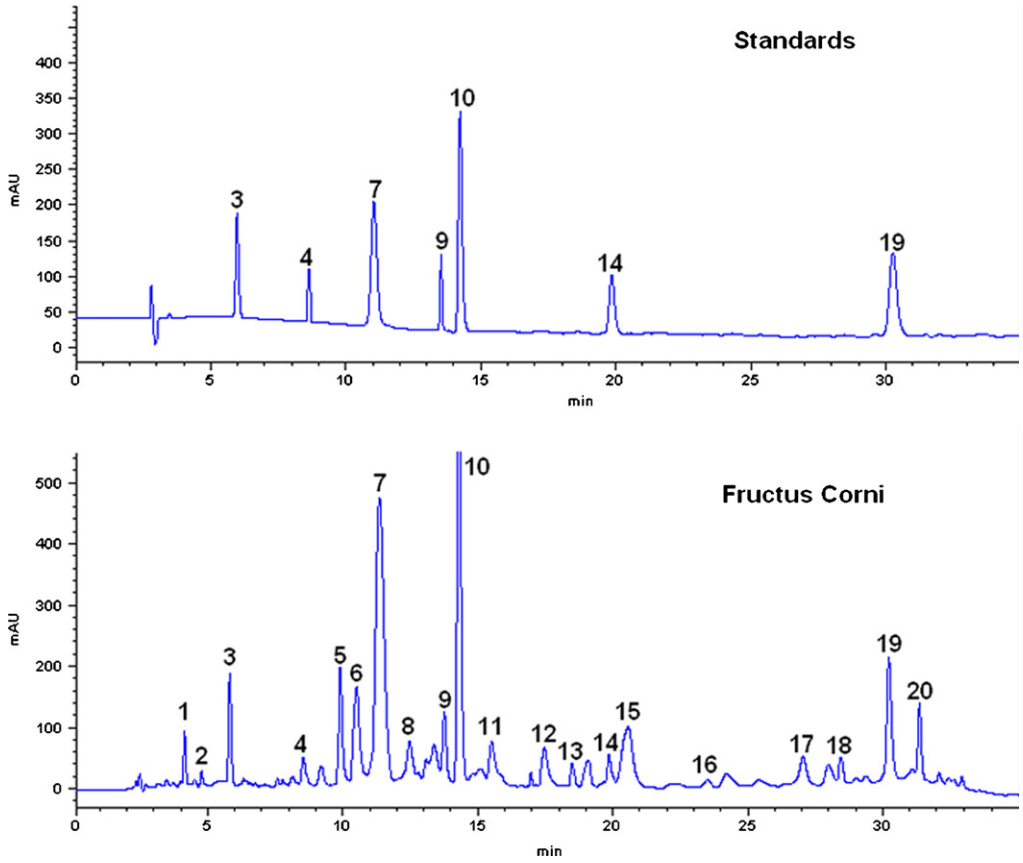

Fig. 2. Representative HPLC chromatograms of mixed standards and FCE. The peaks marked with 3, 4, 7, 9, 10, 14 and 19 are gallic acid, protocatechuic acid, morroniside, sweroside, loganin, 7-O-methylmorroniside and cornuside, respectively.

comprehensive optimum extraction condition.

$$EY = lg^{-1}\left[\frac{\sum lgC}{7}\right] \quad (1)$$

The C was the concentration of each analyte determined by the developed HPLC-DAD/UV quantitative method, whose unite of measure is mg/g.

2.4. HPLC-DAD/UV-TOF/MS system

2.4.1. HPLC chromatography

The qualitative and quantitative analyses were performed on an Agilent 1200 series HPLC system (Agilent, Waldbronn, Germany) consisting of quaternary pump, on-line degasser, well-plate autosampler, thermostatic column compartment and diode-array detector (DAD) interfaced with a 6210 TOF mass spectrometer. The chromatographic separation of samples was carried out at 35 °C on an Agilent Zorbax SB-Aq column (250 mm × 4.6 mm I.D., 5 μm). The mobile phase consisted of 0.2% (v/v) acetic acid (A) and methanol (B) with gradient elution: 0–4 min, isocratic 1% B; 4–18 min, 1–12% B; 18–22 min, 12–20% B; 22–35 min, 20–35% B, 35–40 min, 30–95% B, and the re-equilibration time of gradient elution was 10 min. Flow rate was set at 1.2 ml/min, and by solvent splitting, 0.2 ml/min portion of the column effluent was delivered into the ion source of mass spectrometry. The sample injection volume was 2 μl. For establishment of the fingerprint of FCE, the UV detector was set at 240 nm with full spectral scanning from 190 nm to 400 nm. For the determination of the major constituents, the UV detector was set at 270 nm for gallic acid and protocatechuic acid, and 240 nm for the five iridoids.

2.4.2. Mass spectrometry

The HPLC system was directly connected to the 6210 time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) via an electrospray ionization (ESI) interface. The parameters of ion source were as follows: acquisition mode, positive mode; capillary voltage, 4000 V; drying gas (N_2) temperature, 350 °C; drying gas flowing rate, 10 l min^{-1}; nebulizer gas (N_2) pressure, 35 psig; fragmentor voltage, 150 V; skimmer voltage, 60 V. A second orthogonal sprayer was used to introduce a reference solution as a continuous calibration. The mass axis was automatic recalibrated averaging 11 scans by using the reference solution provided by the manufacturer: m/z 121.050873 and 922.009798 in the positive ion mode. The mass spectra in full-scan mode were recorded between m/z 100 and 1000.

2.5. Data processing

The "Design Expert" software (version 7.1.6, Stat-Ease, Inc., Minneapolis, USA) was used for optimizing UAME experimental procedures. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

3. Results and discussion

3.1. Optimization of UAME

In order to reveal the interaction of different extraction factors and the linear relationship between response and variables, central composite design (CCD) was employed in the procedure of UAME optimization. The parameters including ethanol concentration, solvent to material ratio, temperature and irradiation time were

Table 1
Linear regression data, limit of detection (LOD), limit of quantification (LOQ) of the investigated compounds.

Analyte	Linear regression data			LOD (μg/ml)	LOQ (μg/ml)
	Regression equation	Test range (μg/ml)	r		
Gallic acid	$y = 50.82x + 1.97$	1.077–107.7	0.9999	0.08	0.15
Protocatechuic acid	$y = 47.83x - 2.76$	1.956–97.80	0.9997	0.06	0.18
Morroniside	$y = 63.51x + 1.87$	7.740–774.0	0.9999	0.24	0.75
Sweroside	$y = 97.68x - 0.19$	1.006–100.6	0.9998	0.02	0.09
Loganin	$y = 54.25x - 10.21$	3.936–393.6	0.9999	0.11	0.32
7-O-methylmorroniside	$y = 28.17x - 4.78$	1.024–51.20	0.9998	0.03	0.13
Cornuside	$y = 10.17x + 24.53$	1.012–101.2	0.9999	0.04	0.12

selected as CCD factors. The experiments were arranged according to central composite design table: concentration (50%, 70%, 90%), ratio (20 ml g^{-1}, 30 ml g^{-1}, 40 ml g^{-1}), temperature (50 °C, 60 °C, 70 °C) and time (2 min, 6 min, 8 min), and the total peak area of all components was taken as criteria for optimization. The significance of each coefficient was determined by Student's *t*-test and *P*-values. The best condition of UAME was proposed as: concentration 70%, ratio 24 ml g^{-1}; temperature, 61 °C, time 6.5 min.

3.2. HPLC-DAD/UV-TOF/MS analysis of FCE

In our present study, chemical standards of the seven targets are available. In an effort to reveal the overall profile of unknown compounds in FCE (Fig. 2), HPLC-DAD/UV-TOF/MS experiment was carried out. Being a high resolution mass spectrum, TOF/MS could perform accurate mass measurement, which gives elemental composition of parent and fragment ions. Furthermore, the in-source collision induced dissociation (CID) technique was applied in our experiment to acquire sufficient structure information from TOF/MS. The structure-relevant fragmentation ions can be used as a very useful 'a priori' screening standard for rapidly locating the exact candidates containing such a substructure and/or substituent group. The most probable structure could then be determined from these candidates by fragmentation comparisons. For example, most o-glycosides typically generated a characteristic base peak at $[M-162]^+$ by facile loss of an anhydroglucose residue (162 Da) caused by the cleavage of the glycosidic bond. Peaks 5, 12 and 13 were identified as galloyl glucoses, which showed similar fragmentation pattern $[M\text{-galloyl}]^+$ in the product ion spectra. 13 components in FCE were tentatively assigned by comparing the HPLC/DAD and MS data with those in the literature [17] (Table S1).

3.3. Validation of the chromatographic method

3.3.1. Calibration curves, limits of detection and quantification

Stock solutions containing 7 reference compounds were prepared and diluted to appropriate concentrations for construction of calibration curves. Each concentration of the mixed standard solution was injected in triplicates, and then the calibration curves were constructed by plotting the peak area versus the concentration of each analyte. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The results are summarized in Table 1.

3.3.2. Precision

The quality control samples at low, medium and high were analyzed in a set of five on a single assay day to determine intra-day precision, and analyzed in triplicate on each of three consecutive

Table 2
Intra- and inter-day precisions of seven targets standard solutions at three different concentration levels.

Fortified concentration of target compounds (μg/ml)		Intra-day ($n = 5$)[a]		Inter-day ($n = 3$)[b]	
		Mean ± SD (μg/ml)	RSD (%)[c]	Mean ± SD (μg/ml)	RSD (%)
Gallic acid	4.308	4.331 ± 0.014	0.32	4.302 ± 0.031	0.72
	10.77	10.68 ± 0.131	1.23	10.62 ± 0.208	1.96
	53.85	55.25 ± 1.033	1.87	56.14 ± 0.409	0.73
Protocatechuic acid	3.912	3.971 ± 0.042	1.06	3.983 ± 0.038	0.95
	9.780	9.543 ± 0.154	1.61	9.476 ± 0.068	0.72
	48.90	45.26 ± 0.434	0.96	49.81 ± 0.344	0.69
Morroniside	30.96	30.69 ± 0.248	0.81	31.88 ± 0.293	0.92
	77.40	77.03 ± 0.671	0.87	75.42 ± 0.551	0.73
	387.0	385.6 ± 3.519	0.91	389.8 ± 1.676	0.43
Sweroside	15.74	15.71 ± 0.209	1.33	15.76 ± 0.137	0.87
	39.36	39.12 ± 0.336	0.86	39.31 ± 0.377	0.96
	196.8	194.3 ± 1.205	0.62	194.7 ± 0.487	0.25
Loganin	4.024	4.045 ± 0.078	1.93	4.019 ± 0.071	1.77
	10.06	10.69 ± 0.101	0.94	10.16 ± 0.137	1.35
	50.30	52.13 ± 0.271	0.52	52.78 ± 0.538	1.02
7-O-methylmorroniside	2.048	2.069 ± 0.013	0.63	2.054 ± 0.025	1.22
	5.120	5.192 ± 0.066	1.27	5.170 ± 0.061	1.18
	25.6	24.95 ± 0.202	0.81	26.82 ± 0.255	0.95
Cornuside	4.048	4.032 ± 0.074	1.84	4.087 ± 0.046	1.13
	10.12	10.76 ± 0.063	0.59	10.14 ± 0.153	1.51
	50.60	48.98 ± 0.749	1.53	53.64 ± 0.499	0.93

[a] Intra-day precision on the same day for five times.
[b] Inter-day precision on three different days.
[c] RSD (%) = (SD/Mean) × 100.

Table 3
Recovery experiment of the analytical method for seven components.

Analyte	Original (mg)	Spiked (mg)	Found (mg)[a]	Recovery (%)[b]	RSD%
Gallic acid	1.217	0.6324	1.866	102.7	2.01
		1.173	2.379	99.1	1.67
		1.914	3.079	97.3	0.93
Protocatechuic acid	0.673	0.352	1.017	97.8	1.79
		0.698	1.360	98.4	2.25
		1.006	1.676	99.7	1.92
Morroniside	5.891	2.403	8.275	99.2	0.88
		5.742	11.79	102.7	1.64
		7.063	13.04	101.2	0.92
Sweroside	1.061	0.645	1.697	98.6	1.98
		1.193	2.253	99.9	0.85
		1.943	3.051	102.4	0.73
Loganin	3.173	1.945	5.175	101.8	0.89
		3.892	7.038	99.3	2.11
		4.991	8.204	100.8	1.45
7-O-methylmorroniside	0.424	0.209	0.6347	100.8	1.44
		0.457	0.8709	97.8	1.86
		0.632	1.069	102.1	2.24
Cornuside	1.465	0.693	2.405	96.6	2.03
		1.412	2.854	98.4	1.96
		2.208	3.724	102.3	2.28

[a] Average of three determinations.
[b] Recovery (%) = 100% × (found amount − original amount)/spiked amount.

days for inter-day variation. RSDs of the intra-day and inter-day measurements were all less than 2% for the seven components. The findings of intra- and inter-day precision of the seven targets standard solutions at three concentrations (low, medium and high) are shown in Table 2.

3.3.3. Repeatability and stability

To test the repeatability of our assay, six independently prepared FCE (Linan, Zhejiang, batch no. 20100225) in parallel were prepared and analyzed. Variations were expressed as RSD. The contents of seven compounds were 0.8737, 0.2304, 14.29, 0.6278, 8.196, 0.1903 and 5.714 mg/g, and the RSDs were 0.98%, 1.36%, 1.57%, 1.64%, 0.87%, 1.27% and 1.64%, respectively. Thus repeatability was very good. For stability test, the same sample solution was analyzed every 24 h over 6 days at the room temperature. The RSD of contents of the seven bioactive constituents in the same sample (Linan, Zhejiang, batch no. 20091101) ranged between 0.89% and 1.79%, which indicated that the sample was stable over 6 days under the experimental conditions.

3.3.4. Recovery

In order to evaluate the accuracy of this method, recovery was performed by adding standard solutions at low, medium and high levels (50%, 100% and 150%) to 0.2 g FCE (Linan, Zhejiang, batch no. 20100225) with known content of seven components (the same as repeatability). The samples ($n = 9$) were then extracted according to the procedure described above and analyzed. The recovery of each component was calculated as the percentage of the net amount of each compound obtained after extraction from that had been added prior to the extraction. Table 3 presents the recovery results and they were within satisfactory ranges.

3.4. Quantitative analysis of the investigated compounds in FCE

The developed UAME and HPLC method were subsequently applied to the simultaneous quantification of the 7 investigated compounds. The quantitative analysis data are listed in Table 4. Among the analytes in FCE, morroniside (7) and loganin (10) were determined as the main components whose contents vary from 4.90 to 18.25 mg/g and from 1.14 to 10.26 mg/g, followed by cornuside (19), gallic acid (3) and sweroside (9) at

Table 4
The contents (mg/g) of the 7 targets in 12 FCE samples collected from different habitats.

No.	Batch number	Sample source	**3**[a]	**4**	**7**	**9**	**10**	**14**	**19**	Total
A	20100310	Song county, Henan	0.26[b]	0.23	10.03	0.72	6.97	0.14	7.98	26.33
B	20100405	Xixia, Henan	0.62	0.56	9.49	0.53	4.85	tr[c]	6.16	22.21
C	20080128	Xixia, Henan	1.37	tr	12.14	0.37	9.16	tr	6.37	29.41
D	20091107	Foping, Shananxi	0.37	0.48	15.61	1.20	8.32	tr	8.93	34.91
E	20100321	Yang county, Shananxi	0.66	0.32	9.71	0.96	6.74	tr	4.45	22.84
F	20091116	Chunan, Zhejiang	1.48	0.41	6.89	0.47	8.17	0.19	6.05	23.66
G	20100225	Linan, Zhejiang	1.66	0.36	18.25	0.60	10.26	0.24	8.84	40.21
H	20090901	Shitai, Anhui	0.42	tr	16.83	0.68	6.41	tr	5.79	30.13
I	20090926	Danfeng, Shananxi	1.40	0.38	4.90	0.61	1.14	0.49	2.88	11.80
J	20081017	Anxian, Sichuan	0.90	tr	5.81	0.92	2.10	0.23	3.76	13.72
K	20100415	She county, Anhui	0.34	0.41	5.18	0.99	4.05	0.33	4.18	15.48
L	20090601	Tonglu, Zhejiang	1.83	tr	8.17	0.84	6.71	tr	6.28	23.83

[a] The sum of 7 compounds including Gallic acid (3), Protocatechuic acid (4), morroniside (7), sweroside (9), loganin (10), 7-O-methylmorroniside (14), cornuside (19).
[b] Data calculated as average of three replicates (RSD < 5%).
[c] "tr" Below the linear range of calibration.

a concentration of about 2.88–8.93 mg/g, 0.26–1.66 mg/g and 0.37–1.20 mg/g, respectively. The targets with the lowest content (<0.56 mg/g and <0.49 mg/g) are protocatechuic acid (4) and 7-O-methylmorroniside (14), and they could not be detected in some samples. The contents of the seven bioactive constituents in raw herbs collected from different origins varied dramatically in a range of 11.80–40.21 mg g^{-1}, and the sequence of the total contents of the seven bioactive constituents in different samples was G>D>H>C>A>L>F>E>B>K>J>I. The sample G from Linan of Zhejiang province contained a significantly higher amount of the seven bioactive constituents as compared to the others. These findings are in accordance with the traditional opinion that Linan of Zhejiang Province is the best cultivated regions of *Fructus Corni*. Apart from this, the samples from the same habitat, such as B and C, are partly far apart in total contents of the 7 targets. These phenomena could be caused by variations in the year of cultivation, harvest time, climate and environment. Therefore, in the collection of *Fructus Corni*, not only tough restrictions on region of origin, but also the harvest time and year of cultivation should be indispensably fixed.

4. Conclusions

A UAME coupled with HPLC-DAD/UV-TOF/MS system developed in this work is well suited for simultaneous determination of 7 marker compounds in FCE. Additionally, 13 unknown chromatographic peaks were tentatively identified by accurate mass measurement of TOF-MS. In summary, the developed method is generally applicable and easily expandable to include more quality evaluation of complex herbal matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.02.007.

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