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Simultaneous quantification of 12 bioactive components of *Ligusticum chuanxiong* Hort. by high-performance liquid chromatography

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Abstract

A sensitive and specific HPLC-UV method has been developed, for the first time, to simultaneously quantify 12 bioactive ingredients in *Ligusticum chuanxiong* Hort. (Rhizoma Chuanxiong). This assay was fully validated in respect to precision, accuracy and sensitivity. This method was successfully applied to quantify twelve ingredients in six different Chuanxiong samples. The results demonstrated significant variations in the total content and quantity of each of the main bioactive compounds in different herbs, indicating that quality control of bioactive ingredients in Chuanxiong is critical to ensure its clinical benefits. This assay can be readily utilized as quality control method for Chuanxiong.
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Keywords: *Ligusticum chuanxiong* Hort.; Rhizoma Chuanxiong; HPLC quantification; Quality control

1. Introduction

Rhizoma Chuanxiong, derived from the rhizome of *Ligusticum chuanxiong* Hort. (*Umbelliferae*), is a well-known traditional Chinese medicinal (TCM) herb with haemodynamic and analgesic effects [1]. In the TCM practice, this herb is commonly prescribed for the treatment of migraine and various cardiovascular diseases, such as angina pectoris and ischemic stroke [2,3].

From Rhizoma Chuanxiong, more than 30 compounds, which belong to various different structural types, have been isolated, and among them, several components belonging to three types, namely phenolic acid, alkaloid, and phthalide, have been found to be pharmacologically active. For instance, the phenolic acid-type compound: ferulic acid and coniferylferulate; the alkaloid-type compound: tetramethylpyrazine (TMP); and the phthalide-type compound: *Z*-ligustilide, senkyunolide A, 3-butylidenephthalide and levistolide A, have been reported to be the biologically active components contributing to the therapeutic effects of this medicinal herb [4–8].

As a naturally occurring medicinal herb, the type and quantity of the bioactive ingredients in Rhizoma Chuanxiong vary with the growth environments and post-harvesting process [9]. Furthermore, coniferylferulate and several phthalides are thermolabile [10–13], which may also influence the quality of this herb and consequently its therapeutic outcomes if the post-harvesting process alters. Therefore, a reliable quality control method is needed for the qualitative and quantitative determination of the main bioactive components of Rhizoma Chuanxiong.

To date, various analytical methods have been reported for the analysis of the active ingredients of Rhizoma Chuanxiong, including GC–MS [14,15], HPCE-UV [16], HPLC–MS [15,17], and HPLC-UV [15,17–19]. However, the elevated temperatures used in all reported GC methods may not be appropriate for quantification of the thermolabile compounds such as *Z*-ligustilide, coniferylferulate, senkyunolide A and 3-butylidenephthalide [10–13]. On the other hand, all the published HPCE and HPLC methods were unable to simultaneously determine all three types of the active ingredi-

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ents present in Rhizoma Chuanxiong. Therefore, the present work aimed to develop an analytical method to simultaneously determine all reported main active compounds. A simple HPLC-UV assay using an internal standard method has been developed, for the first time, to simultaneously determine and quantify twelve ingredients, which belong to the three active types and are the reported bioactive components present in Rhizoma Chuanxiong. The chemical structures of these 12 compounds are illustrated in Fig. 1. The developed method has been subsequently applied to analyze six different Rhizoma Chuanxiong samples for the quantification of these twelve components.

2. Experimental

2.1. Chemicals and standards

HPLC grade methanol (E. Merck, Darmstadt, Germany) was used for the HPLC analysis. Analytical-reagent grade

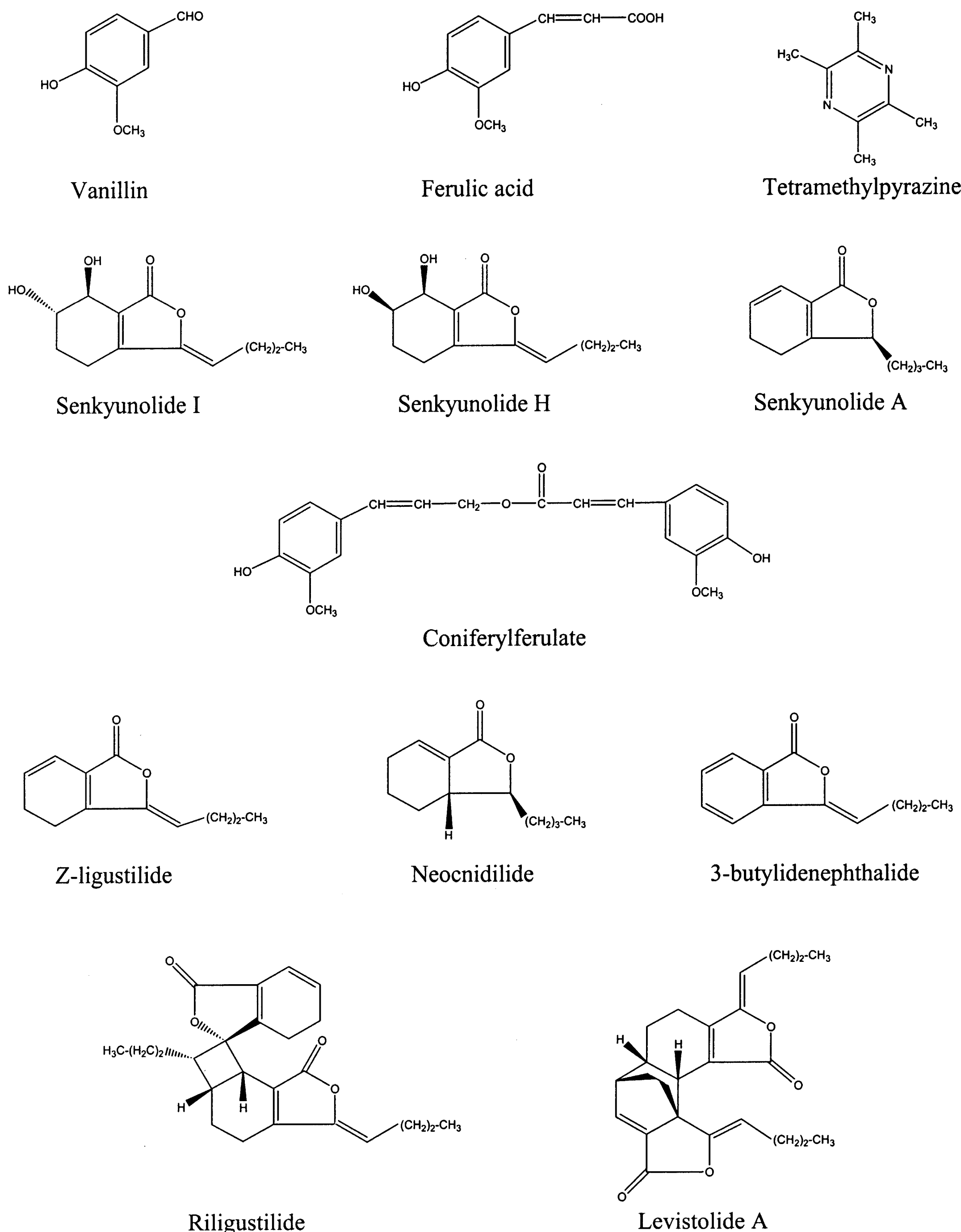

Fig. 1. Chemical structures of 12 bioactive components present in Rhizoma Chuanxiong.

ethanol was used for the extraction of Rhizoma Chuanxiong. Vanillin from Fluka Chemie (Buchs, Switzerland), ferulic acid and TMP from Acros Organics (Geel, Belgium), *Z*-ligustilide from ChromaDex (Santa Ana, CA, USA), sedanolide and α-naphthaflavone (utilized as internal standard) from Sigma (St. Louis, MO, USA), and 3-butylidenephthalide from Aldrich Chemical Company (St. Louis, MO, USA) were purchased. Senkyunolide I, senkyunolide H, senkyunolide A, coniferylferulate, riligustilide and levistolide A were isolated from Rhizoma Chuanxiong previously by our laboratories. Their identities have been confirmed by ^{1}H NMR and HPLC–MS with individual purity not less than 95% [20].

2.2. Plant materials

Rhizoma Chuanxiong samples were purchased from local herb stores in Provinces of Sichuan (CX-R-1, 2, 3, 4), Jiangxi (CX-R-5) and Yunnan (CX-R-6) in Mainland China. Rhizoma Chuanxiong sample (CX-T) used for the preparation of herbal blank and the assessment of reproducibility and accuracy of the developed assay was collected in Dujiangyan County, Sichuan Province, Mainland China. All these herbs were cultivated and harvested in the corresponding local areas in China. The voucher specimens of these samples were deposited at the Department of Pharmacology, the Chinese University of Hong Kong, and their identities were authenticated according to the pharmacognostic standard documented in China Pharmacopoeia [1].

2.3. Apparatus and chromatographic conditions

An Agilent 1100 HPLC system was employed with a Waters Symmetry C_{18} column (5 μm, 150 mm × 4.6 mm) coupled with a guard column (Waters Spherisorb S5 ODS2, 10 mm × 4.6 mm). UV absorption was measured with a spectrum ranging from 190 to 400 nm. The mobile phase consisted of A (0.25% aqueous acetic acid, v/v) and B (methanol). Gradient elution was as follows: 32% B at 0–3 min, increased linearly to 85% B at 36 min and to 100% B at 43 min, then maintained at 100% B for 9 min. The flow rate was 0.7 ml/min.

2.4. Calibration curves

A 95% ethanol stock solution containing all 12 authentic compounds was prepared and diluted to appropriate concentrations to construct calibration curves. Each calibration curve contained six different concentrations and was performed in triplicate. α-Naphthaflavone was used as an internal standard with the concentration of 160 μg/ml for all analytes. The herbal blank was prepared by extracting the raw herbal material (CX-T) thoroughly with 95% ethanol in Soxhlet apparatus till all the compounds of interest were undetectable by HPLC. Calibration curves were constructed by spiking authentic analytes and internal standard into the herbal blank (500 mg) prior to extraction. The extraction was performed as described in Section 2.7. An aliquot (10 μl) of each extract was subjected to HPLC analysis. Four wavelengths at 254, 274, 284 and 294 nm, respectively, were chosen as the monitoring wavelength for different analytes. Calibration curves were constructed by plotting the concentration of each analyte as a function of peak area ratio of spiked analyte to internal standard monitored at the selected wavelength.

2.5. Accuracy and precision

The accuracy and precision of the developed analytical method were determined, including both intra- and inter-day variation. Known quantities of 12 ingredients and the internal standard were added into herbal blank prior to the extraction. The resultant samples were then extracted and analyzed as described in Section 2.7. Quantities for all analytes were calculated from their corresponding calibration curves. Each sample was analyzed in triplicate to determine the intra-day variability. The inter-day reproducibility was determined by analyzing the samples on three separate days. The relative standard deviation (R.S.D.) was taken as a measure of precision and the percentage difference between amounts determined and spiked was considered as a measure of accuracy.

In addition, to further evaluate the reproducibility of the developed assay, Rhizoma Chuanxiong sample (CX-T) was treated and analyzed in triplicate within the same day and also on three separated days by using the procedures described in Section 2.7. The quantity of each of the ingredients present in this herbal sample was determined from the corresponding calibration curves. Moreover, the accuracy of the measurement was further assessed by spiking one known quantity of each of the 12 authentic samples into the Rhizoma Chuanxiong sample (CX-T) followed by addition of the internal standard. The resultant samples were then extracted and analyzed as described in Section 2.7. Each sample was analyzed in triplicate. The total concentration of each of the analytes was determined from the corresponding calibration curve, and accuracy of the measurement for each analyte was calculated by the following equation:

$$\text{accuracy}\,(\%) = \left\{\frac{-[C_{\text{spiked}} - (C_{\text{total}} - C_{\text{original}})]}{C_{\text{spiked}}}\right\} \times 100$$

where C_{total} is the determined total concentration, C_{original} is the concentration in the original herb measured in the above described experiment, and C_{spiked} is the spiked concentration.

2.6. Limits of detection

Aliquots of analytes were added to the herbal blank to provide adequate concentrations for all 12 analytes, ranging from 2 to 100 ng/ml, in the final extract. The resultant samples were extracted and analyzed in the same manner as described in Section 2.7. The detection limit of each analyte was determined when the signal-to-noise ratio of the testing peak was greater than five.

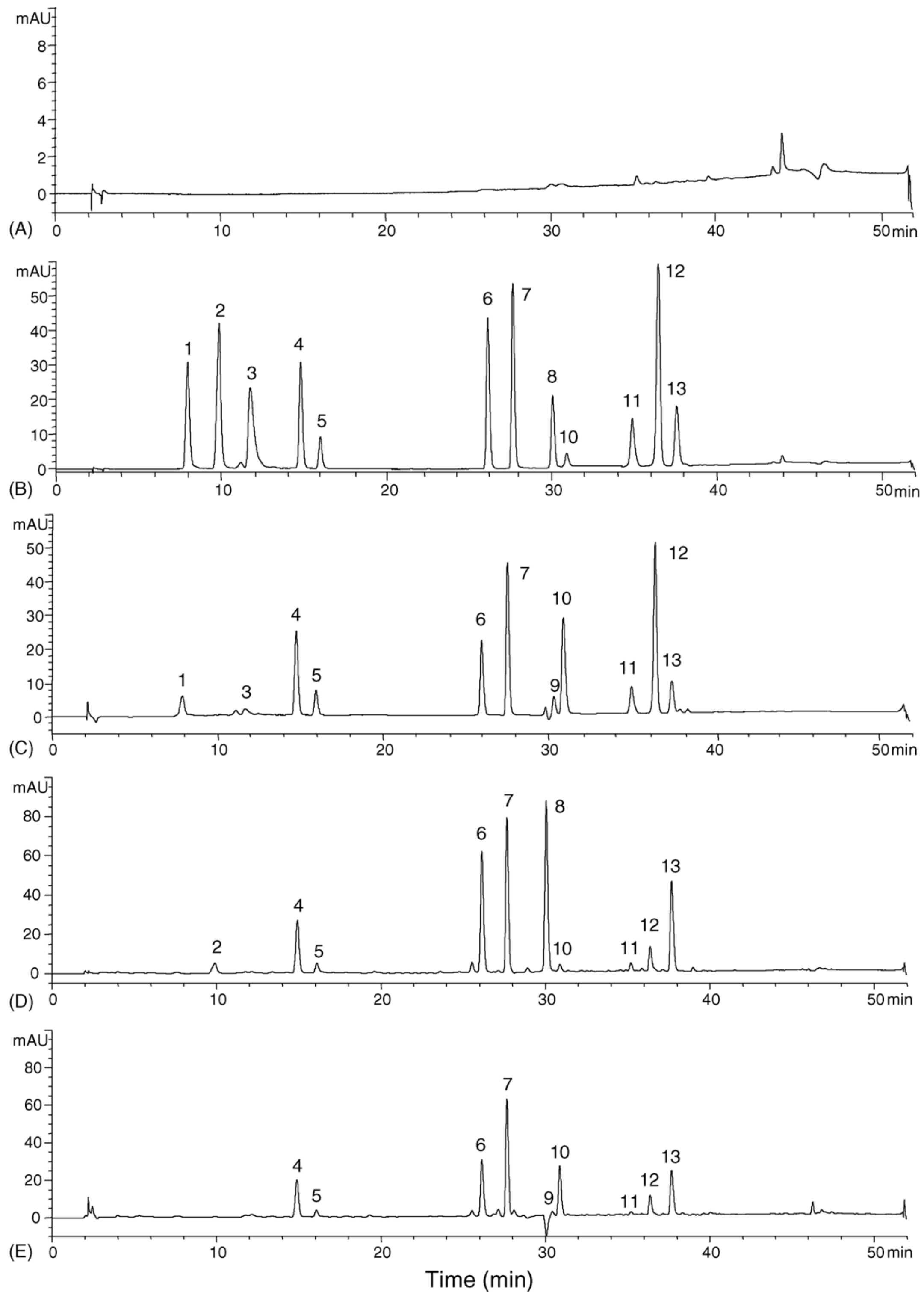

Fig. 2. Representative HPLC chromatograms of the ethanol extracts of herbal blank detected at: 294 nm (A); herbal blank spiked with analytes and internal standard detected at: 294 nm (B) and 254 nm (C); and CX-R-4 detected at: 294 nm (D) and 254 nm (E). Peaks: (1)—vanillin; (2)—ferulic acid; (3)—tetramethylpyrazine; (4)—senkyunolide I; (5)—senkyunolide H; (6)—senkyunolide A; (7)—coniferylferulate; (8)—*Z*-ligustilide; (9)—sedanolide (neocnidilide); (10)—3-butylidenephthalide; (11)—riligustilide; (12)—levistolide A; (13)—α-naphthaflavone (internal standard).

Table 1
Calibration curves for 12 ingredients

Compound	Monitor wavelength (nm)	Retention time (min)	$y=ax+b$		r^2	Concentration range (μg/ml)	Detection limit (ng/ml)
			Slope (a)	Interpret (b)			
Vanillin	294	7.9	0.06261	−0.003084	0.9993	1–45	50
Ferulic acid	294	9.9	0.03943	−0.006506	0.9994	4–90	40
Tetramethylpyrazine	294	11.7	0.05418	−0.003422	0.9988	1–45	100
Senkyunolide I	274	14.6	0.05783	−0.004227	0.9994	3–180	20
Senkyunolide H	274	15.2	0.04556	−0.0008477	0.9995	1–70	10
Senkyunolide A	284	26.1	0.01888	−0.02573	0.9991	24–1220	20
Coniferylferulate	294	27.7	0.02326	−0.03440	0.9987	34–1720	25
Z-ligustilide	284	30.1	0.02093	−0.01400	0.9990	20–1050	10
Sedanolide	254	30.4	0.007065	−0.006063	0.9995	15–710	10
3-butylidenephthalide	254	30.8	0.08646	−0.006835	0.9980	2–88	40
Riligustilide	284	35.2	0.06010	0.008237	0.9991	0.1–9	5
Levistolide A	274	36.4	0.04041	0.005424	0.9993	0.6–62	30

2.7. Quantification of 12 ingredients in Rhizoma Chuanxiong herbal samples

Six Rhizoma Chuanxiong samples, obtained from different herbal stores, were ground. The powder of each herbal sample was spiked with the internal standard and then extracted by 3 ml of 95% ethanol with rotations for 2 h. After centrifugation, the residues were further extracted with 3 ml of fresh 95% ethanol for 2 h. The filtered supernatants were combined. An aliquot (10 μl) of the filtrate was directly subjected to HPLC analysis. Each sample was determined in triplicate. The content of each analyte was calculated from the corresponding calibration curve.

3. Results and discussion

As shown in Fig. 2, all 12 analytes were eluted with baseline separation under the optimized chromatographic condition. Since the 12 ingredients have their maximum UV absorptions (λ_{max}) at different wavelengths, in order to obtain the highest sensitivity to analyze each analyte, four different wavelengths at 254, 274, 284 and 294 nm, respectively, were chosen to detect and quantify all 12 analytes simultaneously. The wavelengths for monitoring different analytes are listed in Table 1. A further advantage of using multiple wavelength monitoring is being able to simultaneously detect neocnidilide at 254 nm, while as shown in Fig. 2, this compound cannot be detected at other wavelengths. This may explain that in a previous study of Rhizoma Chuanxiong by Naito et al. [19], neocnidilide was not determined simultaneously with other ingredients by using a single monitoring wavelength at 270 nm.

Under the present condition, all analytes were detected with relatively high sensitivities. The detection limits calculated as per dried herbal sample were 1.09 μg/g for tetramethylpyrazine, 0.55 μg/g for vanillin, 0.44 μg/g for ferulic acid and 3-butylidenephthalide, 0.34 μg/g for levistolide A, 0.27 μg/g for coniferylferulate, 0.22 μg/g for senkyunolide I and senkyunolide A, 0.11 μg/g for senkyunolide H, *Z*-ligustilide and sedanolide, and 0.05 μg/g for riligustilide, respectively.

As shown in Table 1, the calibration curves for all 12 analytes showed good linearity ($r^2 > 0.998$) in the concentration ranges tested. Furthermore, the validation studies provided good precision, with overall intra- and inter-day variations of less than 7 and 15%, respectively, and accuracy higher than 80% (Table 2). Furthermore, when the 12 analytes were spiked into the real herbal sample (not the herbal blank), a good accuracy of higher than 85% was also obtained for each of all the analytes tested (Table 3). In the case of analysis of neocnidilide, since it was reported as the only diastereomer of sedanolide identified in Rhizoma Chuanxiong [20], but its authentic compound was unavailable, the synthetic sedanolide was utilized for the construction of the calibration curve for neocnidilide.

The developed HPLC-UV analytical method was subsequently applied to simultaneously determine the twelve ingredients in six Rhizoma Chuanxiong herbal samples obtained from different sources. All compounds found in herbal samples were qualitatively determined by comparison of both their UV spectrum and the retention time with those of the authentic compounds. Representative chromatograms, monitored at 294 and 254 nm, are shown in Fig. 2. The quantity of each compound present in the six Chuanxiong samples was determined and the results are summarized in Table 4. Furthermore, as shown in Fig. 3, an excellent reproducibility was obtained for each of the ingredients determined.

The results indicated that, except for TMP and vanillin, all compounds tested were detected in all six Rhizoma Chuanxiong herbal samples. In all six samples analyzed, *Z*-ligustilide was found to be the most abundant component, and coniferylferulate and senkyunolide A were determined as major ingredients. These three major bioactive ingredients account for about 86–95% of the total components determined in the six different Chuanxiong herbal samples. As shown in Fig. 4A, the contents of these three major ingredients vary significantly in different herbs. Furthermore, marked differences in the content of each individual minor bioactive ingredient present in the six different herbal samples are also

Table 2
Intra- and inter-day variability for the assay of 12 ingredients

Compound/concentration spiked (μg/ml)	Intra-day ($n = 3$)			Inter-day ($n = 3$)		
	Detected (μg/ml)	R.S.D. (%)[a]	Accuracy (%)[b]	Detected (μg/ml)	R.S.D. (%)	Accuracy (%)
Vanillin						
2.25	2.45 ± 0.13	5.2	91.1	2.55 ± 0.01	0.4	86.7
11.23	10.94 ± 0.32	2.9	97.4	11.25 ± 0.09	0.8	99.8
22.46	22.40 ± 0.24	1.1	99.7	22.46 ± 0.39	1.7	100.0
Ferulic acid						
9.08	9.35 ± 0.38	4.1	97.0	9.24 ± 0.33	3.6	98.2
22.69	23.01 ± 0.47	2.0	98.6	23.41 ± 0.52	2.2	96.8
90.75	90.09 ± 0.95	1.1	99.3	90.65 ± 2.32	2.5	99.9
Tetramethylpyrazine						
2.29	2.63 ± 0.18	6.9	85.2	2.76 ± 0.01	0.3	79.5
11.46	11.58 ± 0.22	1.9	99.0	11.44 ± 0.37	3.2	99.8
22.92	22.16 ± 0.48	2.2	96.7	22.09 ± 0.40	1.8	96.4
Senkyunolide I						
18.28	17.34 ± 0.29	1.7	94.9	17.63 ± 0.19	1.1	96.4
91.40	92.70 ± 3.01	3.3	98.6	90.77 ± 0.64	0.7	99.3
182.78	182.45 ± 2.22	1.2	99.8	185.45 ± 1.30	0.7	98.5
Senkyunolide H						
6.88	6.58 ± 0.14	2.1	95.6	6.68 ± 0.06	1.0	97.1
34.43	34.95 ± 1.04	3.0	98.5	34.42 ± 0.33	1.0	100.0
68.86	68.72 ± 0.75	1.1	99.8	70.13 ± 0.93	1.3	98.2
Senkyunolide A						
60.96	66.87 ± 2.40	3.6	90.3	68.59 ± 1.20	1.7	87.5
304.79	295.05 ± 5.02	1.7	96.8	294.87 ± 4.68	1.6	96.7
609.59	585.83 ± 5.93	1.0	96.1	586.27 ± 5.71	1.0	96.2
Coniferylferulate						
86.17	95.07 ± 4.75	1.6	89.7	94.57 ± 4.57	4.8	90.3
430.83	445.21 ± 14.13	3.1	96.7	441.02 ± 12.04	2.7	97.6
861.67	895.08 ± 14.04	1.6	96.1	896.45 ± 15.43	1.7	96.0
Z-Ligustilide						
52.48	55.92 ± 2.46	4.4	93.4	58.70 ± 1.56	2.7	88.1
262.41	255.42 ± 3.65	1.4	97.3	254.63 ± 2.71	1.1	97.0
524.83	507.45 ± 4.07	0.8	96.7	513.41 ± 5.60	1.1	97.8
Sedanolide						
35.57	36.68 ± 2.46	6.7	96.9	36.74 ± 2.04	5.5	96.7
177.83	173.33 ± 5.19	3.0	97.5	170.76 ± 3.44	2.0	96.0
355.67	351.60 ± 3.48	1.0	98.9	350.85 ± 1.44	0.4	98.6
3-Butylidenephthalide						
4.4	5.26 ± 0.28	5.3	80.5	4.76 ± 0.69	14.6	91.8
22	20.97 ± 0.38	1.8	95.3	20.86 ± 0.30	1.4	94.8
44	41.95 ± 0.83	2.0	95.3	42.31 ± 1.13	2.7	96.2
Riligustilde						
0.09	0.09 ± 0.002	0.7	99.7	0.085 ± 0.005	2.2	96.4
0.35	0.35 ± 0.01	2.9	99.8	0.35 ± 0.01	2.2	99.1
2.20	2.19 ± 0.04	1.8	99.5	2.14 ± 0.05	2.3	97.1
Levistolide A						
0.62	0.59 ± 0.03	5.1	95.6	0.59 ± 0.02	3.4	95.2
2.46	2.47 ± 0.05	2.0	99.6	2.48 ± 0.06	2.3	99.2
15.40	15.35 ± 0.34	3.7	99.7	15.14 ± 0.18	1.2	98.3

[a] R.S.D. (%) = (S.D./mean) × 100.
[b] Accuracy (%) = [1 − (mean concentration measured − concentration spiked)/concentration spiked] × 100.

Table 3
Assessment of testing accuracies for the 12 ingredients spiked into Rhizoma Chuanxiong sample (CX-T)

Compound	Concentration		Accuracy (%)
	Spiked (μg/ml)	Detected (μg/ml)	
Vanillin	10.0	9.8 ± 0.5	98.0
Ferulic acid	25.0	22.9 ± 1.1	91.6
Tetramethylpyrazine	10.0	9.5 ± 0.5	95.0
Senkyunolide I	21.8	21.2 ± 0.2	97.2
Senkyunolide H	18.5	18.9 ± 0.3	97.7
Senkyunolide A	57.0	51.1 ± 3.1	89.6
Coniferylferulate	72.0	67.5 ± 0.9	93.8
Z-ligustilide	50.0	46.2 ± 1.5	92.4
Sedanolide	120.0	115.3 ± 3.7	96.1
3-butylidenephthalide	47.0	42.9 ± 1.9	91.3
Riligustilde	10.0	10.0 ± 0.0	99.9
Levistolide A	10.0	9.9 ± 0.0	99.0

Table 4
Contents of twelve bioactive ingredients in six Rhizoma Chuanxiong herbal samples

Compound	Content (mg/g of the dried material)					
	CX-R-1	CX-R-2	CX-R-3	CX-R-4	CX-R-5	CX-R-6
Vanillin	nd	nd	nd	nd	nd	nd
Ferulic acid	0.24 ± 0.01	0.22 ± 0.01	0.17 ± 0.004	0.24 ± 0.01	0.25 ± 0.02	0.16 ± 0.01
Tetramethylpyrazine	nd	nd	nd	nd	nd	nd
Senkyunolide I	0.45 ± 0.01	0.99 ± 0.01	0.75 ± 0.01	0.87 ± 0.01	0.93 ± 0.01	0.14 ± 0.01
Senkyunolide H	0.12 ± 0.003	0.30 ± 0.002	0.23 ± 0.002	0.26 ± 0.003	0.26 ± 0.002	0.04 ± 0.003
Senkyunolide A	2.71 ± 0.07	8.99 ± 0.22	5.29 ± 0.06	7.58 ± 0.04	10.55 ± 0.12	3.51 ± 0.09
Coniferylferulate	4.88 ± 010	7.18 ± 0.15	3.14 ± 0.04	6.52 ± 0.08	7.64 ± 0.08	9.08 ± 0.18
Z-ligustilide	9.84 ± 0.26	13.07 ± 0.32	7.57 ± 0.07	11.25 ± 0.04	13.65 ± 0.11	20.74 ± 0.21
Neocnidilide	0.56 ± 0.07	0.55 ± 0.07	0.44 ± 0.01	0.47 ± 0.02	0.47 ± 0.01	0.44 ± 0.02
3-butylidenephthalide	0.31 ± 0.001	0.47 ± 0.002	0.45 ± 0.01	0.43 ± 0.01	0.40 ± 0.01	0.14 ± 0.004
Riligustilide	0.13 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
Levistolide A	0.44 ± 0.01	0.68 ± 0.02	0.32 ± 0.01	0.57 ± 0.01	0.52 ± 0.01	0.57 ± 0.01
Total	19.67 ± 0.29	32.58 ± 0.43	18.52 ± 0.10	28.32 ± 0.11	34.77 ± 0.19	34.93 ± 0.29

Data are represented as mean ± S.D. ($n = 3$); nd: not detectable.

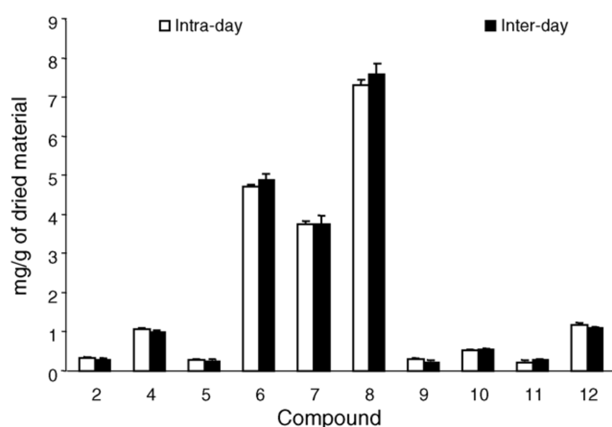

Fig. 3. The reproducibility assessment of 10 ingredients determined in Rhizoma Chuanxiong sample (CX-T). Compound: (2)—ferulic acid; (4)—senkyunolide I; (5)—senkyunolide H; (6)—senkyunolide A; (7)—coniferylferulate; (8)—*Z*-ligustilide; (9)—sedanolide (neocnidilide); (10)—3-butylidenephthalide; (11)—riligustilide; (12)—levistolide A.

been observed (Table 4). With a consideration of the total content of all ingredients determined (Fig. 4B), samples CX-R-1 (19.67 ± 0.29 mg/g) and CX-R-3 (18.52 ± 0.10 mg/g) had very similar contents, which were significantly lower than all the other samples, and three samples containing higher quantities also have similar total contents (CX-R-2: 32.58 ± 0.43 mg/g, CX-R-5: 34.77 ± 0.19 mg/g, CX-R-6: 34.93 ± 0.29 mg/g). However, the quantity ratio of these three major components and the content of each of the three major ingredients present in different herbs are significantly different (Fig. 4A). For example, senkyunolide A, coniferylferulate and *Z*-ligustilide account for 30, 22 and 39% of the total content in sample CX-R-5, and 10, 26 and 59% of the total content in sample CX-R-6, respectively. Therefore, the determination of each of the major bioactive components is more important and meaningful than a measurement of the total content of all bioactive ingredients only. Our results demonstrated significant variations in chemical profiles, in particular the profiles of the major bioactive ingredients, in this TCM herb cultivated in different locations. Therefore, in order to ensure the consistency of therapeutic benefits, it is necessary to quantify each of the major bioactive components in Rhi-

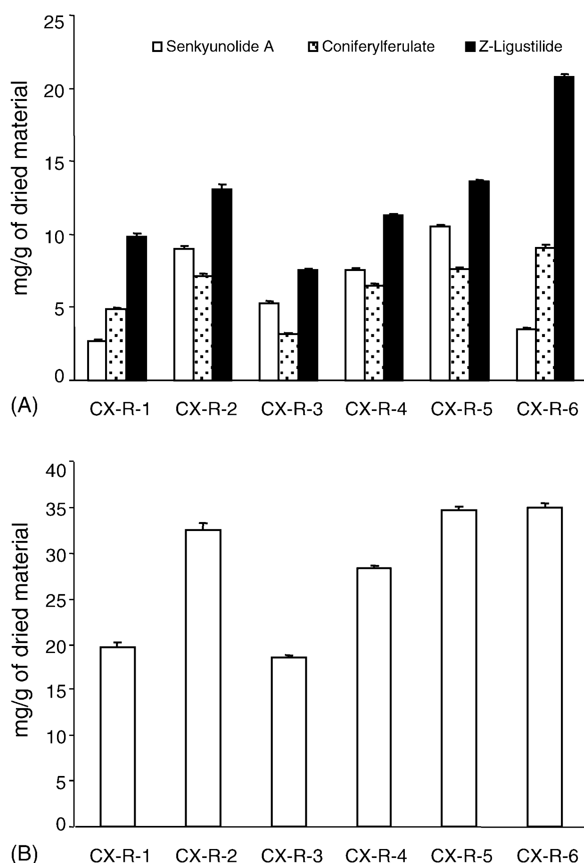

Fig. 4. Contents of three major bioactive ingredients (A) and total contents of all components (B) determined in six Rhizoma Chuanxiong samples.

zoma Chuanxiong prior to its use as herbal prescriptions or as the plant source for the manufacture of natural product based pharmaceutical preparations.

It is interesting to note that TMP was not determined in all six Rhizoma Chuanxiong samples tested and Rhizoma Chuanxiong sample CX-T in the present study, although the detection limit of 1.09 μg TMP/g of the dried herb was considered to be adequate. Since its first isolation from the rhizome of *L. chuanxiong* Hort. in 1977, TMP has been generally considered as one of the main components contributing to the therapeutic effects of Rhizoma Chuanxiong [8,21,22]. In some reports, TMP was employed as a chemical marker for the quality control of Rhizoma Chuanxiong [23,24], or Rhizoma Chuanxiong-containing pharmaceutical preparations [25]. However, in these studies [23–25], the identification of TMP present in Rhizoma Chuanxiong samples was simply conducted by an apparent comparison of the retention time of the chromatographic peak in the tested samples with that of authentic TMP only. Obviously such confirmation of the presence of TMP is insufficient and thus the subsequent quantification of TMP maybe unreliable. In fact, it is worth mentioning that a very much lower content of TMP (0.1 μg/g of the dried herb) in the raw herbal material was reported when it was firstly identified from the rhizome of *L. chuanxiong* Hort. [26]. Moreover, recently Li et al. also reported that TMP was undetectable in various Rhizoma Chuanxiong herbal samples tested [17]. Their results are in a good agreement with our present and previous studies [20]. In addition, the synthetic TMP rather than the one isolated from Rhizoma Chuanxiong was utilized in all the reported pharmacological and clinical studies on TMP [27,28]. Therefore, based on all available information, we believe that TMP is not the main component of Rhizoma Chuanxiong. Further investigation is warranted for the confirmation of whether or not TMP is the major bioactive component contributing to the pharmacological activities of Rhizoma Chuanxiong. However, based on the currently available evidence, we would suggest that TMP is not a suitable chemical marker for the quality control of the major bioactive ingredient for Rhizoma Chuanxiong herbs and herbal products.

4. Conclusions

This is the first report on the development of a simple, sensitive and specific HPLC-UV method to simultaneously quantify all main bioactive ingredients in Rhizoma Chuanxiong. The results demonstrate that the developed method was accurate and reproducible. This method has also been applied successfully to simultaneously quantify twelve constituents in six Rhizoma Chuanxiong samples. The results demonstrate that this method could be readily utilized as a suitable quality control method for quantification of all major bioactive ingredients in Rhizoma Chuanxiong.

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