Simultaneous Qualitative and Quantitative Analyses of the Major Constituents in the Rhizome of *Ligusticum Chuanxiong* Using HPLC-DAD-MS

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An HPLC-DAD-MS method was developed for the qualitative and quantitative analysis of the major constituents in *Chuanxiong* (the dried rhizome of *Ligusticum chuanxiong* Hort). Twenty compounds including phenolic constituents, alkylphthalides and phthalide dimers were identified using online ESI-MS and comparisons with literature data and standard compounds, and six of them were quantified by HPLC-DAD simultaneously. A comprehensive validation of the method including sensitivity, linearity, repeatability and recovery was conducted. The linear regressions were acquired with $R^2>0.99$ and limit of detection (LOD, S/N=3) values were between 1.5 and 2.5 ng. The repeatability was evaluated by intra- and inter-day assays, and relative standard deviation (RSD) values were reported within 1.87%. The recovery studies for the quantified compounds were observed in the range of 96.36—102.37% with RSD values less than 2.63%. These phenolic constituents and alkylphthalides, the major constituents in *Chuanxiong*, are generally regarded as the index for the quality assessment of this herb. The overall procedure is accurate and reproducible, which is considered suitable for the qualitative and quantitative analyses of a large number of *Chuanxiong* samples.

Key words *Ligusticum Chuanxiong*; phenolic constituent; alkylphthalide; HPLC-DAD-MS; quality assessment

Chuanxiong (Tousenkyu in Japanese and Chuang-xiong in Chinese), the dried rhizome of *Ligusticum chuanxiong* Hort (Umbelliferae), is one of the major clinically used cardiovascular-protective botanic medicines in China.[1)] Having a reputation for facilitating blood circulation and dispersing blood stasis, this herb is commonly prescribed for the treatment of angina pectoris, cardiac arrhythmias, hypertension and stroke.[2—4)]

Currently, the quality assessment standard of *Chuanxiong* is based on the content of the marker compounds ferulic acid or/and ligustilide, the bioactive compounds identified in *Chuanxiong* herb.[5—8)] However, due to multiple constituents that might be involved in the therapeutic functions, the content of a single or a few marker compounds cannot accurately reflect the quality of herbal products.[9,10)] To ensure the quality, efficacy and safety (QES) of *Chuanxiong* herb and *Chuanxiong*-containing preparations, the demand for a quality assessment standard that is based on the chemical identification of its major constituents is necessary.

In this paper, an HPLC-DAD-MS method with a comprehensive validation protocol for the qualitative and quantitative analyses of the major constituents in *Chuanxiong* is presented.

Experimental

Materials and Reagents Samples of *Chuanxiong* herb were collected in a number of cultivation bases in mainland China. The respective sources of the plant materials are listed in Table 1. The identities of these herbs were confirmed based on morphologic appearance, microscopic and physiochemical analyses according to the Chinese Pharmacopoeia.[1)] Voucher specimens were deposited in the Herbarium Centre, Hong Kong Baptist University. Representative samples were cut into smaller pieces, further ground into powder, and passed through a 20-mesh (0.9-mm) sieve. The ground powders were stored at about 4 °C before use.

Acetonitrile, methanol and acetic acid used in HPLC analysis were HPLC grade purchased from Lab-scan (Bangkok, Thailand). Deionized water was generated from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Ferulic acid was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The standard compounds of senkyunolide I, senkyunolide H, senkyunolide A, *Z*-ligustilide and levistolide A were isolated from *Rhizoma Chuanxiong* in our laboratory with silica gel column chromatography, preparative TLC and semi-preparative HPLC. The purity of each isolated compound was determined by HPLC, and their identities were confirmed by the comparison of their respective NMR and MS spectra with the published data.[11—13)] The details of separation and structural elucidation will be reported in another paper.

HPLC System and Conditions An Agilent 1100 series HPLC-DAD system comprising a vacuum degasser, binary pump, autosampler, thermostated column compartment and DAD (Agilent, Palo Alto, CA, U.S.A.) was used for acquiring chromatograms and UV spectra.

For the determination of the major constituents in *Chuanxiong*, the UV detector was also set at 280 nm. An Alltima C_{18} column (5 μm, 4.6 mm×250 mm) with a compatible guard column (C_{18}, 5 μm, 4.6 mm×7.5 mm) was used. The mobile phase consisted of 0.5% acetic acid in water (A) and acetonitrile (B) using a gradient program of 15—20% (B) at 0—10 min, 20—53% at 10—40 min, and 53—100% at 40—60 min. The mobile phase flow rate was 1 ml/min, and the column temperature was set at 30 °C.

HPLC-MS System and Conditions An Applied Biosystems/PE-SCIEX API 365 LC-MS system with an electrospray ionization source (Applied Biosystems, Foster City, CA, U.S.A.) was used for mass spectrometric measurements. The HPLC conditions for HPLC-MS analysis were the same as those used for HPLC-DAD analysis.

The mass spectrometer conditions were first optimized using flow injection analysis of the standards without the HPLC column. For identification of the major constituents in *Chuanxiong*, the conditions of MS analysis were as follows: drying gas air flow, 8 l/min; gas temperature, 300 °C; full scan range, 50—500 u of *m*/*z*; orifice voltage, 30 V; focusing voltage, 175 V; and electrospray voltage, 5500 V.

Standard Solution Preparation For assay of the six analytes in *Chuanxiong* herb, stock solutions of the standard compounds were prepared at a concentration of 1000 mg/l in methanol. Calibration standard solutions were prepared in concentration range of 1—100 mg/l with methanol. An

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Table 1. Contents of Six Constituents in *Chuanxiong*

Materials	Source and year of harvest	Contents of six constituents (mg/g)[a)]					
		Ferulic acid	Senkyunolide I	Senkyunolide H	Senkyunolide A	*Z*-Ligustilide	Levistolide A
CX-1	Sichuan, China (2003)	0.8046±0.0044	1.3319±0.0063	0.3662±0.0021	7.3133±0.0581	8.3490±0.0264	0.0733±0.0008
CX-2	Chongqing, China (2003)	0.3335±0.0021	1.8298±0.0085	0.3384±0.0018	3.3518±0.0269	10.1028±0.0273	0.0565±0.0006
CX-3	Yunnan, China (2002)	0.7146±0.0040	1.7258±0.0083	0.3274±0.0020	4.5067±0.0358	6.5820±0.0205	0.0763±0.0007
CX-4	Fujian, China (2002)	0.4927±0.0026	1.9191±0.0089	0.3748±0.0024	5.4229±0.0432	7.4284±0.0232	0.0910±0.0009
CX-5	Shanxi, China (2003)	0.5161±0.0027	2.4184±0.0112	0.4634±0.0027	2.4507±0.0201	4.2001±0.0133	0.0928±0.0009
CX-6	Sichuan, China (2002)	0.3255±0.0019	1.3032±0.0063	0.2494±0.0015	2.5507±0.0206	5.3297±0.0169	0.0756±0.0008
CX-7	Cangxi, Sichuan, China (2002)	0.3996±0.0022	1.6016±0.0076	0.3659±0.0021	3.5217±0.0284	6.3153±0.0199	0.0649±0.0007
CX-8	Chongzhou, Sichuan, China (2003)	0.4417±0.0027	2.4068±0.0113	0.5220±0.0028	5.1089±0.0409	6.4442±0.0205	0.0468±0.0005
CX-9	Chongqing, Sichuan, China (2001)	0.4520±0.0028	2.2430±0.0105	0.5488±0.0031	2.0528±0.0166	5.2047±0.0168	0.0684±0.0007
CX-10	Dujiangyan, Sichuan, China (2003)	0.8505±0.0053	1.8911±0.0088	0.4324±0.0025	4.6823±0.0376	5.3164±0.0172	0.0812±0.0008
CX-11	Market I, Sichuan, China (2003)	0.9387±0.0057	1.3429±0.0065	0.2456±0.0015	3.9658±0.0319	8.2579±0.0257	0.0604±0.0007
CX-12	Market II, Sichuan, China (2003)	0.2187±0.0017	1.6430±0.0077	0.3354±0.0019	7.0951±0.0569	10.8929±0.0333	0.0558±0.0006
CX-13	Market III, Sichuan, China (2003)	0.7075±0.0045	1.9796±0.0093	0.3901±0.0022	5.9672±0.0479	9.0095±0.0277	0.0655±0.0007
CX-14	Pengzhou, Sichuan, China (2003)	0.4110±0.0046	2.0106±0.0094	0.4378±0.0025	3.0876±0.0249	8.5213±0.0266	0.0517±0.0006
CX-15	Yunnan, China (2003)	0.5898±0.0036	1.3545±0.0064	0.2803±0.0017	5.3273±0.0428	12.6107±0.0383	0.0735±0.0008
CX-16	Shanxi, China (2002)	0.2492±0.0017	1.6411±0.0078	0.3127±0.0019	3.4316±0.0277	9.6578±0.0294	0.0567±0.0006
CX-17	Chongqing, China (2002)	0.6556±0.0041	1.6019±0.0076	0.2978±0.0018	3.1562±0.0256	8.3462±0.0259	0.0637±0.0007
CX-18	Fujian, China (2003)	0.4904±0.0031	1.0286±0.0051	0.2189±0.0014	4.3263±0.0347	10.9112±0.0329	0.0523±0.0006
CX-19	Dujiangyan, Sichuan, China (2002)	0.3258±0.0022	1.5632±0.0074	0.3326±0.0019	6.2147±0.0499	8.4607±0.0263	0.0769±0.0008
CX-20	Cangxi, Sichuan, China (2003)	0.7856±0.0048	1.6837±0.0078	0.3167±0.0019	4.3582±0.0349	10.5474±0.0317	0.0854±0.0009

CX-1 to CX-20 were the rhizome of *Ligusticum chuanxiong* Hort. *a*) The value is mean±S.D. (n=3).

aliquot of 10 μl of 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 the standard compound.

Sample Solution Preparation A Branson 5210E-MTH ultrasonic processor (Branson Ultrasonics Corporation, CT, U.S.A.) was used for sample extraction. Accurately weighed *Chuanxiong* powder of 0.25 g was extracted with 8 ml of methanol by means of sonication at room temperature for 30 min. The operations were repeated three times. The pooled extracts were transferred into a 25-ml volumetric flask and made up to the full volume with methanol. An aliquot of 10 μl of solution was injected for HPLC analysis. Sample duplicates were prepared as shown above for analysis.

Validation of the Present Method and Quantification of Major Constituents in *Chuanxiong* Freshly prepared calibration curves were established for each standard compound. Repeatability was evaluated in the intra- and inter-day (n=3) assays. Recovery of all the quantified constituents was determined by samples at different concentration levels using a mixture of standards with 50, 100 and 200% of the quantified levels of constituents in the samples. Twenty batches of *Chuanxiong* acquired in various regions were compared quantitatively by the present method.

Results and Discussion

Optimization of Preparation of Sample and Stock Solutions For sample extraction, sonication was chosen as the extraction method due to its confirmed efficacy and ease of handling. The choice of extraction solvent was compared between methanol and acetonitrile. Persistent turbidity was observed in samples extracted with acetonitrile, while the methanolic counterparts remained clear throughout; in addition, most of the standard compounds are volatile oils under ambient conditions and dissolve completely in methanol. Therefore methanol was selected as the solvent for preparation of sample solution and stock solution.

Choice of Detection Wavelength for HPLC-DAD Analysis By comparing the HPLC chromatograms of *Chuanxiong* acquired at different wavelengths in the range of 210—500 nm and the corresponding UV absorption maximum for each standard compound, it was found that 280 nm well represents the profile of the major constituents. The UV absorption maximum for each identified compound is listed in Table 2. A representative HPLC chromatogram is shown in Fig. 1.

HPLC-MS Identification of Major Constituents in *Chuanxiong* The combined HPLC-diode array detection and mass spectral techniques can provide online UV and MS information for each peak in a chromatogram, which has been shown to be a powerful approach for the rapid identification of the constituents in botanical extracts and herbal products.[14—16] In most cases, direct identification of the peaks is possible based on a comparison with those published data standard compounds.[17—19]

In the present paper, the mass spectral conditions were optimized in both positive- and negative-ion modes, and the positive-ion mode was found to be more sensitive (Fig. 2). Most of constituents exhibited quasi-molecular ions $[M+H]^+$ and $[M+Na]^+$ in this mode.

Based on comparisons with standard compounds, six peaks were unambiguously identified as ferulic acid (**1**), senkyunolide I (**3**), senkyunolide H (**4**), senkyunolide A (**7**), *Z*-ligustilide (**12**) and levistolide A (**17**). Fourteen other peaks were tentatively identified as senkyunolide J (**2**), senkyunolide F (**5**), coniferyl ferulate (**6**), butylphthalide (**8**), cnidilide (**9**), *E*-ligustilide (**10**), *E*-butylidenephthalide (**11**), *Z*-butylidenephthalide (**13**), angelicide (**14**), riligustilide (**15**), tokinolide B (**16**), senkyunolide P (**18**), *Z*-ligustilide dimmer E-232 (**19**) and *Z*,*Z*′-3,3′,8,8′-diligustilide (**20**) by comparing their *m*/*z* values and UV spectra with the data reported in the literature.[20—22] The results are listed in Table 2, and the structures of the identified compounds are shown in Fig. 3.

From the assay results, the major constituents in *Chuanxiong* were phenolic constituents, alkylphthalides and phthalide dimmers. For the unknown peaks, further chemical characterization is in process and the structures will be elucidated in a future report.

Validation of the Method and Quantification of Major Constituents in *Chuanxiong* A comprehensive validation of the present method was conducted, and the results are

Table 2. The Mass Spectral and Spectrometric Data of the Identified Compounds in *Chuanxiong*

Peak No.	Compound	$[M+H]^+$ (*m*/*z*)	$[M+Na]^+$ (*m*/*z*)	Other positive ion (*m*/*z*)	λ_{max} (nm)
1	Ferulic acid	195	—	—	294sh, 324
2	Senkyunolide J	227	249	191, 209	213, 274
3	Senkyunolide I	225	247	189, 207	278
4	Senkyunolide H	225	247	189, 207	278
5	Senkyunolide F	—	229	161, 189	294sh, 324
6	Coniferyl ferulate	—	379	—	270, 298sh, 318
7	Senkyunolide A	193	215	175	280
8	Butylphthalide	191	213	145, 173	230, 276
9	Cnidilide	195	217	177	—
10	*E*-Ligustilide	191	213	173	290sh, 328
11	*E*-Butylidenephthalide	189	211	153, 171	262, 312
12	*Z*-Ligustilide	191	213	173	282, 328
13	*Z*-Butylidenephthalide	189	211	153, 171	262, 312
14	Angelicide	381	403	191	283
15	Riligustilide	381	403	213, 191	284
16	Tokinolide B	381	403	213, 191	280
17	Levistolide A	381	403	191	230, 276
18	Senkyunolide P	383	405	215, 191	226, 282
19	*Z*-ligustilide dimmer E-232	381	403	191	278
20	*Z*,*Z*′-3,3′,8,8′-Diligustilide	381	403	213, 191	282

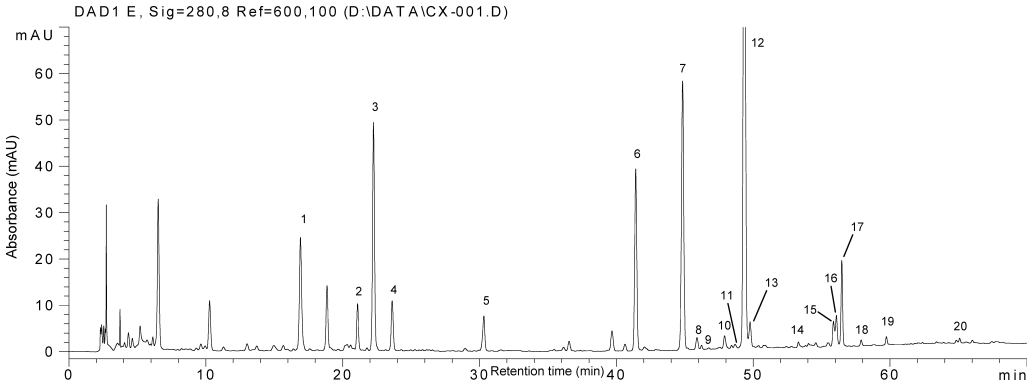

Fig. 1. Typical HPLC Chromatogram of *Chuanxiong* Samples at 280 nm

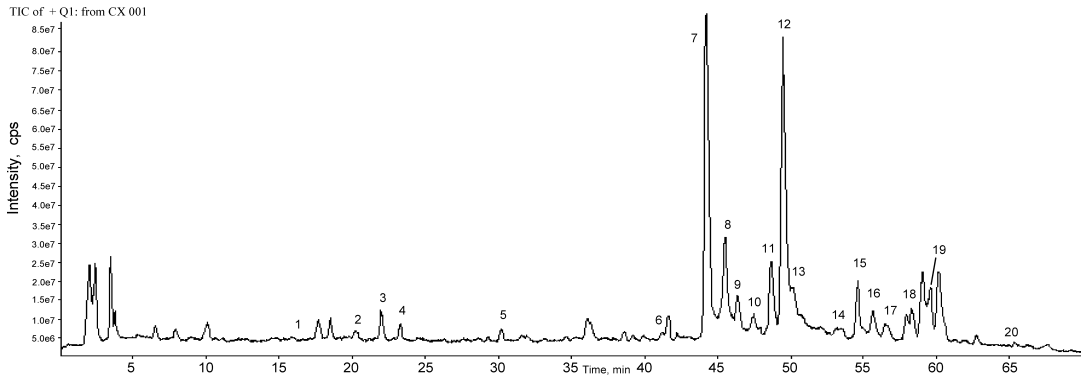

Fig. 2. The TIC Chromatogram of *Chuanxiong* Sample in Positive-Ion Mode

listed in Tables 3 to 5. For all of the quantified constituents, a good linearity with R^2>0.99 was achieved. The RSD values were within the range of 0.21—1.09% for intra-day assays and 0.63—1.87% for inter-day assays. Based on visual evaluation with a signal-to-noise ratio of about 3 : 1, the LOD values of the quantified constituents were found within 1.5 and 2.5 ng. The average recovery rates of ferulic acid, senkyunolide I, senkyunolide H, senkyunolide A, *Z*-ligustilide and levistolide A were 101.17% (RSD 1.59%), 98.69% (RSD 2.20%), 98.67% (RSD 2.29%), 98.72% (RSD 2.63%), 100.98% (RSD 1.77%) and 98.06% (RSD 1.48%), respectively. Twenty batches of *Chuanxiong* samples acquired from various regions were evaluated by the present method, and the results are listed in Table 1. The contents of the quantified constituents in *Chuanxiong* varied as a result of differences in cultivation conditions and year of harvest. However, the overall analytical procedure was accurate and reproducible, which is considered suitable for the qualitative and

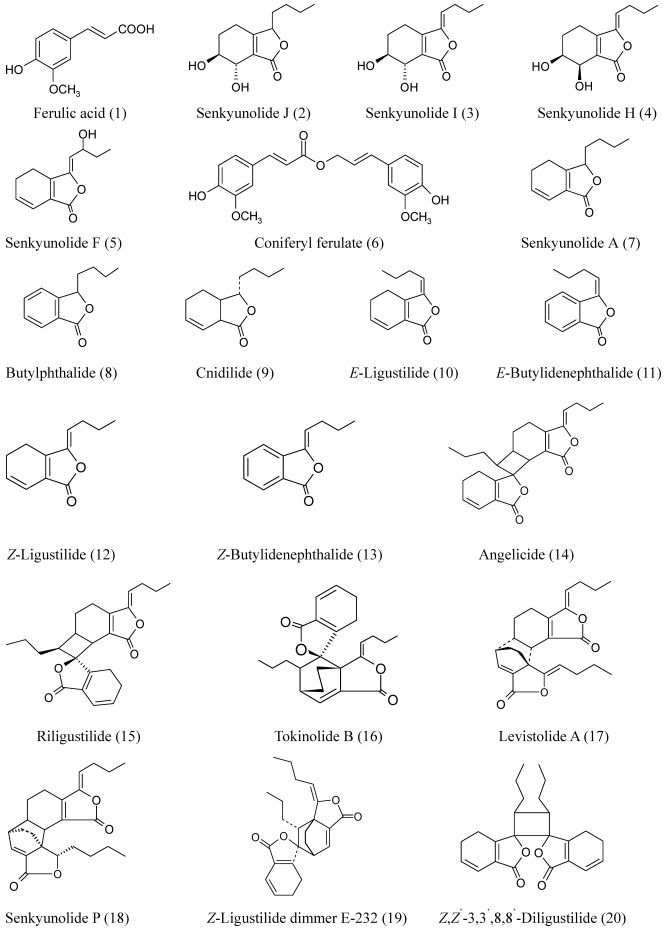

Fig. 3. Chemical Structures of the Constituents Identified in *Chuanxiong* Herb

quantitative analyses of a large number of *Chuanxiong* samples.

Conclusion

The present paper describes a method for the identification and quantification of major components in the medicinal plant *Ligusticum Chuanxiong*. Twenty compounds of different types, including phenolic constituents, alkylphthalides and phthalide dimers, were identified. Six of those compounds were quantified simultaneously, and the developed method exhibited good sensitivity, repeatability and accuracy. Phenolic constituents and alkylphthalides, the major constituents in *Chuanxiong*, are generally regarded as the index for quality assessment of this herb.

On the other hand, tetramethylpyrazine (TMP, chuanxiongize, ligustrazine) was previously isolated from *Chuanxiong* herb and chemically synthesized in 1977,[23,24] and various preparations using synthesized TMP have been clinically used for the treatment of cerebro- and cardiovascular diseases.[25—27] TMP is considered to be one of the main bioactive components and is used as a chemical marker for the quality assessment of *Chuanxiong* herb and *Chuanxiong*-

Table 3. Linearity Calibration Curve Factors and LOD of Six Constituents in *Chuanxiong*

Peak No.	Compound	Slope (A)	Intercept (B)	R^2	LOD (ng)
1	Ferulic acid	13.332	+6.372	0.9999	2.3
3	Senkyunolide I	7.336	+2.826	0.9998	2.2
4	Senkyunolide H	6.857	+3.143	0.9997	2.3
7	Senkyunolide A	4.885	+2.372	0.9998	2.5
12	*Z*-Ligustilide	22.787	+7.003	0.9999	1.8
17	Levistolide A	26.682	+4.254	0.9998	1.5

Table 4. Repeatability of the Method

Peak No.	First day		Third day		Fifth day		Inter-days RSD (%)
	Calculated content (mg/g)[a]	RSD (%)	Calculated content (mg/g)	RSD (%)	Calculated content (mg/g)	RSD (%)	
1	0.8046±0.0044	0.55	0.8052±0.0032	0.40	0.8157±0.0035	0.43	0.77
3	1.3319±0.0063	0.47	1.3540±0.0059	0.44	1.3364±0.0061	0.46	0.87
4	0.3662±0.0021	0.57	0.3684±0.0026	0.71	0.3792±0.0030	0.79	1.87
7	7.3133±0.0581	0.79	7.3296±0.0476	0.65	7.2273±0.0356	0.49	0.75
12	8.3490±0.0264	0.32	8.4423±0.0232	0.27	8.4391±0.0178	0.21	0.63
17	0.0733±0.0008	1.09	0.0746±0.0006	0.80	0.0737±0.0006	0.81	0.90

a) The value is mean±S.D. (n=3).

Table 5. Recovery of Six Constituents in *Chuanxiong*

Spike level (%)	Recovery of six constituents (%)[a]					
	Ferulic acid	Senkyunolide I	Senkyunolide H	Senkyunolide A	*Z*-Ligustilide	Levistolide A
50	99.34±0.80	96.55±1.12	96.39±0.98	101.51±0.73	101.43±0.83	96.95±0.96
100	101.80±1.26	98.63±1.16	98.70±1.01	98.30±1.07	99.01±1.60	99.70±1.16
200	102.37±0.82	100.89±0.95	100.91±0.37	96.36±0.67	102.49±0.63	97.52±0.73
Mean	101.17±1.59	98.69±2.20	98.67±2.29	98.72±2.63	100.98±1.77	98.06±1.48

a) The value is mean±RSD (n=3).

containing preparations.[28—30] However, in the present study, TMP was not detected in any of the *Chuanxiong* samples (detection limit of 8×10^{-8} g/g, by LC-MS-MS, S/N=3). This result suggests that TMP is present very low levels in *Chuanxiong* herb. Therefore TMP is not a suitable chemical marker for the quality assessment of *Chuanxiong* herb and *Chuanxiong*-derived herbal products.

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