Authentication of Traditional Chinese Prescriptions Using Comprehensive 2D-LC

The Agilent 1290 Infinity 2D-LC Solution

Suitable for Agilent
1290 Infinity III LC

Application Note

Small Molecule Pharmaceuticals and Generics

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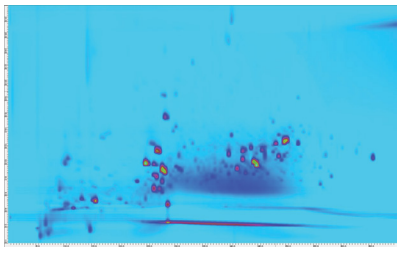
Abstract

The authentication of Chinese herbal medicine (CHM) is a challenging task, a fact that can be attributed to the highly complex nature of plants and preparations used in CHM and the natural variability of the individual plants. Generally, chromatographic fingerprinting is regarded as one of the most effective methods. As an example of the analysis of a traditional Chinese prescription, this Application Note shows the comprehensive 2D-LC analysis of Si-Wu-Tang as well as of the individual herbs contained in Si-Wu-Tang. For each herb, characteristic component peaks are selected, and a template is created. The templates are matched to the peaks detected in Si-Wu-Tang to investigate the possibility to detect characteristic components of the individual herbs in a traditional Chinese prescription. Additionally, changes in template matching following the omission and replacement of one herb from Si-Wu-Tang are examined.



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Introduction

Chinese herbal medicine (CHM), one aspect of traditional Chinese medicine (TCM), uses single plants or preparations of several plants, mainly in form of decoctions prepared by extraction with boiling water[1-3]. Especially in poorer countries, such as parts of Africa and Asia, counterfeiting of drugs is a huge problem[4]. Counterfeit versions of preparations used in CHM might have omission or replacement of one or more herbs in the preparation. Due to the highly complex nature of plants and preparations used in CHM and the natural variability of the individual plants, the authentication of CHM is a challenging task[3]. Generally, one or a few main compounds or pharmaceutically active components are currently used as marker compounds in the analysis of CHM[1,3]. A more complete characterization of CHM can be achieved by chromatographic fingerprinting. The construction of a chromatographic fingerprint and subsequent comparison with the fingerprint of a clinically proven reference product is regarded as an effective method for authentication of CHM[1-3].

One example for a preparation of several plants used in CHM is Si-Wu-Tang. Si-Wu-Tang is composed of the four herbs *Radix Angelicae Sinensis*, *Rhizoma Chuanxiong*, *Radix Paeoniae Alba*, and *Radix Rehmanniae Preparata*, and is widely used for the treatment of female disease[5,6].

According to the literature, *Radix Angelicae Sinensis* contains a range of phthalides, such as Z-ligustilide and senkyunolide A, amongst others, as well as several organic acids such as ferulic acid, phthalic acid, and vanillic acid[7-9]. Several components of *Radix Angelicae Sinensis* can also be found in *Rhizoma Chuanxiong* as it also contains phthalides and organic acids[10,11]. Compounds identified in *Radix Paeoniae* include gallic acid, catechin, albiflorin, paeoniflorin, and paeonol[12], and *Radix Rehmanniae* contains acteoside, isoacteoside, echinacoside, leonuride, catalpol, aucubin, and melittoside[13].

This Application Note shows the analysis of a decoction from Si-Wu-Tang using comprehensive two-dimensional liquid chromatography (comprehensive 2D-LC). Because of its inherent high peak capacity, comprehensive 2D-LC is ideally suited for the analysis of complex samples such as CHM.

The separate analysis of the herbs contained in Si-Wu-Tang and detection by Q-TOF mass spectrometry enables the selection and tentative identification of characteristic components of each herb. Those characteristic components are then detected in Si-Wu-Tang as a means of authentication of traditional Chinese prescriptions. In addition, changes following the omission and replacement of one herb from Si-Wu-Tang are investigated.

Experimental

Equipment

The Agilent 1290 Infinity 2D-LC Solution was comprised of the following modules:

- Two Agilent 1290 Infinity Binary Pumps (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/4-port duo-valve (2D-LC valve head, 1,200 bar (p/n 5067-4214) equipped with two 40-µL loops
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light cartridge cell (G4212-60008)

MS measurements were done using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system with Jet Stream ESI source (G1958-65538).

Software

- Agilent OpenLAB CDS ChemStation Edition, revision C.01.07 [27] with Agilent 1290 Infinity 2D-LC Acquisition Software, revision A.01.02 [24]
- Agilent MassHunter Workstation Software, LC/MS data acquisition for Agilent 6200 series TOF/6500 series Q-TOF, revision B.05.01, qualitative analysis, revision B.06.00.
- GC Image LCxLC-HRMS Edition software, revision 2.5b0 for 2D-LC data analysis from GC Image LLC., Lincoln, NE, USA.

Columns

First dimension

Agilent ZORBAX RRHT SB-Aq
2.1 × 100 mm, 1.8 µm
(p/n 828700-914)

Second dimension

Agilent Poroshell 120 Bonus-RP
3.0 × 50 mm, 2.7 µm
(p/n 699968-301)

Chemicals

All solvents were LC grade. Acetonitrile and methanol were purchased from Merck, Darmstadt, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA). Formic acid was purchased from Agilent (p/n G2453-85060).

Samples

Samples of the traditional Chinese prescription Si-Wu-Tang, which is composed of four herbs, as well as of individual herbs used in CHM, were kindly provided by Patrick Kwik from Congress Pharmacy in Karlsruhe, Germany.

Samples were prepared as decoctions, in the same manner as they are prepared for pharmaceutical use. The samples were weighed and a tenfold amount of water was added. The samples were allowed to soak in the cold water for 60 minutes and were then boiled for 30 minutes. After cooling, aliquots of the decoctions were centrifuged at 10,000 rpm for 15 minutes. Aliquots of the supernatant phases were filtered using a 1-mL plastic syringe with Captiva Premium Syringe Filters Regenerated Cellulose, 15 mm, 0.45 µm (p/n 5190-5109) before injection into the HPLC system.

Thermostatted column compartment

- First dimension column on right side at 30 °C
- Second dimension column on left side at 50 °C

2-position/4-port duo-valve

The 2-position/4-port duo-valve was switched automatically after each second dimension modulation cycle of 30 seconds. The loops were used in a cocurrent manner (filling and elution of the loops in the same flow direction).

Autosampler

Parameter	Value
Injection volume	5 µL
Sample temperature	6 °C
Needle wash	10 seconds in methanol/water (50/50; v/v)

Diode array detector

Before detection, the effluent from the second dimension column was split approximately 4:1 between the DAD and the MS using a T-piece. The connection from the T-piece to the MS was made using a 0.075-mm id capillary (340-mm length) to minimize peak broadening.

Parameter	Value
Wavelength	254 nm/4 nm, Ref. 380 nm/40 nm
Data rate	80 Hz

Comprehensive 2D-LC method

First dimension pump	
Solvent A	Water + 0.1 % formic acid
Solvent B	Methanol + 0.1 % formic acid
Flow rate	0.05 mL/min
Gradient	0 minutes 0 %B; 10 minutes 0 %B; 70 minutes 95 %B; 80 minutes 95 %B
Stop time	80 minutes
Post time	30 minutes
Second dimension pump	
Solvent A	Water + 0.1 % formic acid
Solvent B	Acetonitrile + 0.1 % formic acid
Flow rate	2.5 mL/min
Gradient and gradient modulation	0.00 minutes 2 %B; 30 minutes 2 %B; 52 minutes 22 %B; 70 minutes 40 %B 0.40 minutes 35 %B; 30 minutes 35 %B; 52 minutes 60 %B; 70 minutes 95 %B
^{2}D Gradient stop time	0.40 minutes
Modulation time	0.50 minutes

Mass spectrometer

The Agilent 6530 Accurate-Mass Q-TOF LC/MS system was operated in positive and negative ionization mode with an acquisition rate of 10 spectra/second and the following conditions for the Jet Stream ESI source (G1958-65538).

Parameter	Value
Gas temperature	300 °C
Gas flow	8 L/min
Nebulizer	50 psig
Sheath gas temperature	300 °C
Sheath gas flow	9 L/min
Capillary	3,000 V
Nozzle	500 V

Results and Discussion

Based on the comprehensive 2D-LC analysis of decoctions from plants used in CHM shown in a previous Application Note[14], a method for comprehensive 2D-LC analysis of a decoction from Si-Wu-Tang was developed. Figure 1 shows the resulting separation with UV detection at 254 nm. Especially in the first dimension retention time ranges from 25 to 30 minutes and 40 to 50 minutes, peaks that would coelute in a one-dimensional analysis are separated in the second dimension.

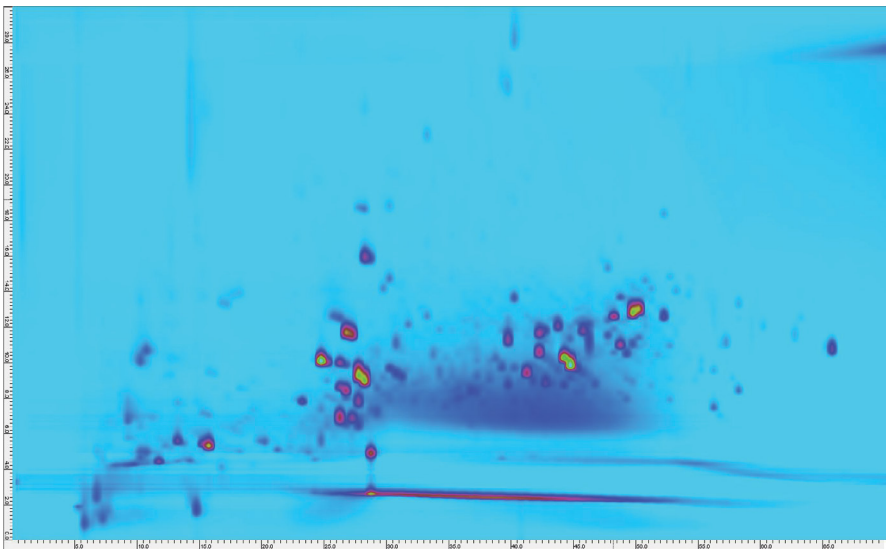

Figure 1. Comprehensive 2D-LC analysis of a decoction from Si-Wu-Tang with UV detection at 254 nm.

An important aspect of the analysis of traditional Chinese prescriptions is their authentication, that is, to ensure that the correct herbs are contained in the prescription. To enable authentication of Si-Wu-Tang, a separate comprehensive 2D-LC analysis of each herb contained in Si-Wu-Tang was performed. Detection of accurate masses by Q-TOF mass spectrometry in positive as well as negative ionization mode in connection with literature data enabled the tentative identification of characteristic components of each herb. In this way, senkyunolide A, Z-ligustilide, and ferulic acid were tentatively identified in *Radix Angelicae Sinensis*, and senkyunolide A, Z-ligustilide, ferulic acid, and caffeic acid could be detected in *Rhizoma Chuanxiong*. *Radix Paeoniae Alba* was shown to contain gallic acid, catechin, and the isomeric compounds albiflorin and paeoniflorin. In *Radix Rehmanniae Preparata*, acteoside or isoacteaoside, as well as echinacoside could be tentatively identified.

In addition to the peaks that were tentatively identified, further high abundant peaks were selected as characteristic components of each herb. Using the LCxLC-HRMS Edition software, a template was constructed from the selected peaks for each herb. Each template was then matched to the peaks detected in Si-Wu-Tang in terms of first and second dimension retention times as well as agreement of the base peak in the respective mass spectra. Figure 2 shows the analysis of *Rhizoma Chuanxiong* with the peaks selected for the templates (A, B) as well as the analysis of Si-Wu-Tang with the matched peaks from *Rhizoma Chuanxiong* (C, D). In positive ionization mode, 11 out of 13 template peaks were matched. In negative ionization mode, the template contained 17 peaks, out of which 10 were matched. This shows the possibility to detect characteristic components of an individual herb in a traditional Chinese prescription.

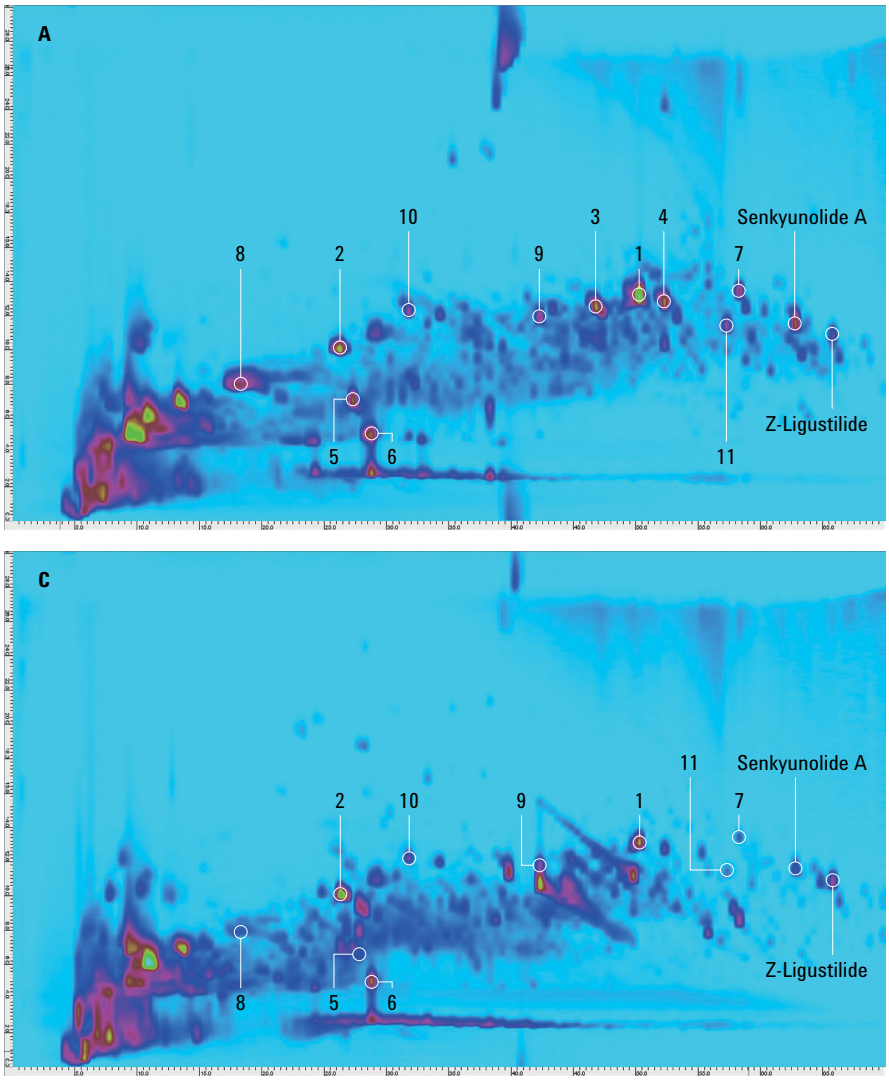

Figure 2. Comprehensive 2D-LC analysis of decoctions from *Rhizoma Chuanxiong* (A, B) and Si-Wu-Tang (C, D) with MS detection in positive (A, C) and negative (B, D) ionization mode. Peaks selected for the templates from *Rhizoma Chuanxiong* and peaks matched in Si-Wu-Tang are marked. (continued on page 5)

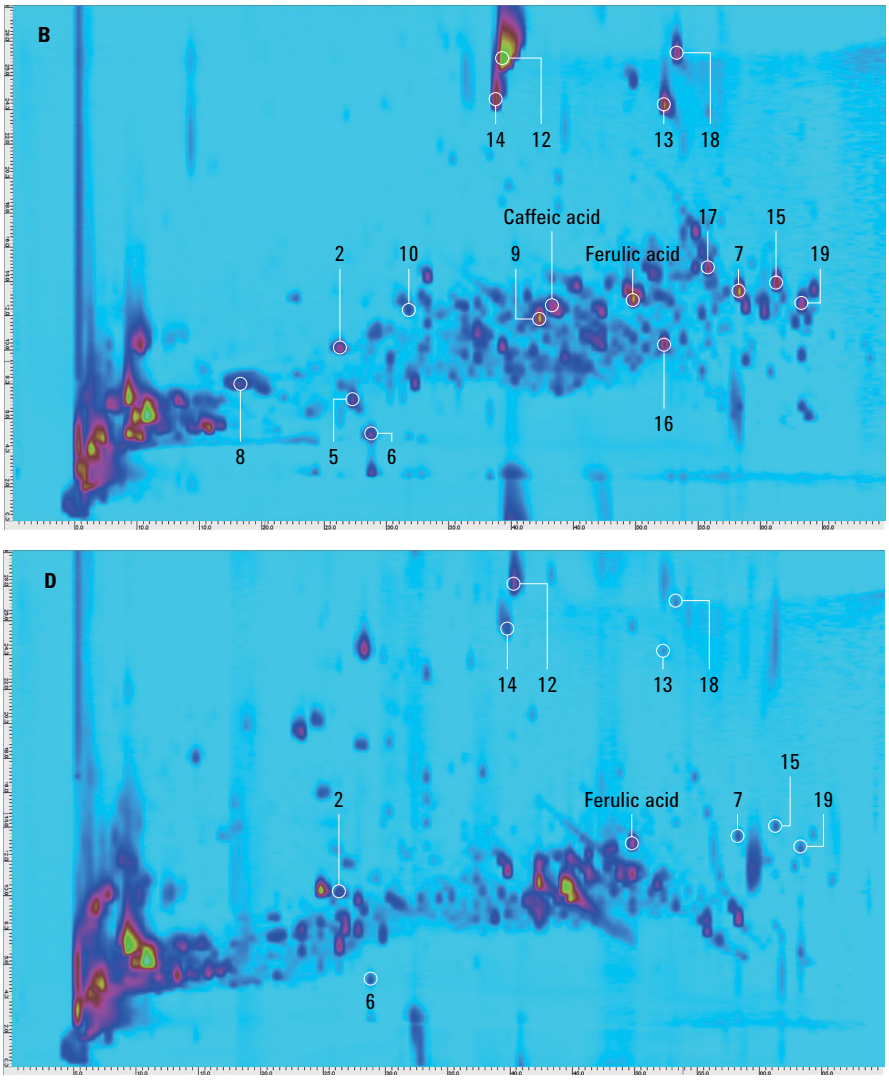

Figure 2. Comprehensive 2D-LC analysis of decoctions from *Rhizoma Chuanxiong* (A, B) and Si-Wu-Tang (C, D) with MS detection in positive (A, C) and negative (B, D) ionization mode. Peaks selected for the templates from *Rhizoma Chuanxiong* and peaks matched in Si-Wu-Tang are marked. (continued from page 4)

Table 1 shows the number of peaks that were selected for the templates of the individual herbs contained in Si-Wu-Tang as well as the number of matched peaks in positive and negative ionization modes. With two exceptions, 75 % or more of the template peaks could be matched to peaks detected in Si-Wu-Tang. Generally, nonmatching of peaks could be explained by the fact that the peaks did not show the same base peak in the mass spectrum, which can occur when partial coelution of peaks is observed in Si-Wu-Tang.

Table 1. Matching of template peaks for each herb contained in Si-Wu-Tang.

	MS positive		MS negative	
Herb	Number of template peaks	Number of matched peaks	Number of template peaks	Number of matched peaks
Radix Angelicae Sinensis	9	7	9	7
Radix Paeoniae Alba	9	5	10	10
Rhizoma Chuanxiong	13	11	17	10
Radix Rehmanniae Preparata	12	9	11	9

Figure 3 shows the analysis of Si-Wu-Tang in positive (A) and negative (B) ionization mode with the peaks matched from the templates of the individual herbs. Several peaks detected in Si-Wu-Tang can be attributed to more than one template peak from the individual herbs, for example, senkyunolide A, Z-ligustilide and ferulic acid from *Radix Angelicae Sinensis* and *Rhizoma Chuanxiong*.

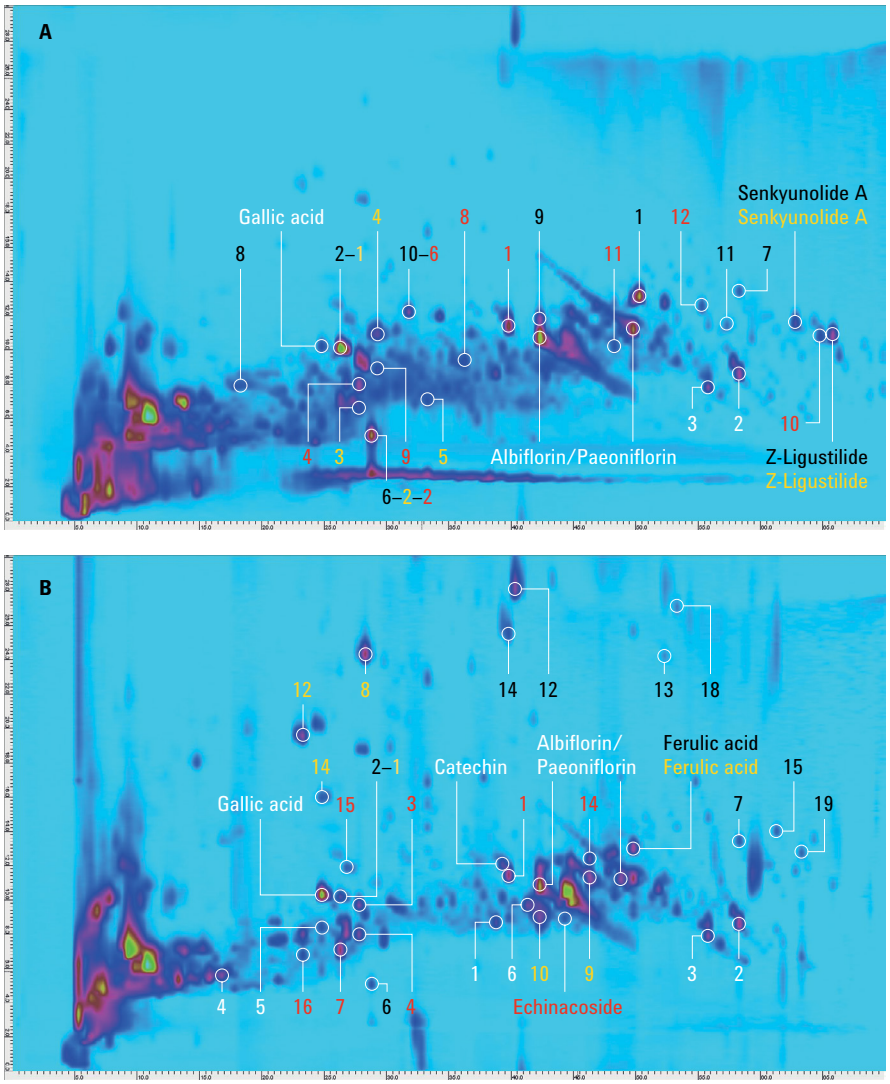

Figure 3. Comprehensive 2D-LC analysis of a decoction from Si-Wu-Tang with MS detection in positive (A) and negative (B) ionization mode. Peaks matched from the templates of the individual herbs are marked: *Radix Angelicae Sinensis* (yellow), *Rhizoma Chuanxiong* (black), *Radix Paeoniae Alba* (white), *Radix Rehmanniae Preparata* (red).

To investigate the possibility of detecting an adulteration of Si-Wu-Tang, an analysis of Si-Wu-Tang without *Rhizoma Chuanxiong* and of Si-Wu-Tang with *Rhizoma Chuanxiong* replaced by the rhizome of another herb was performed. Figure 4 shows the analysis of Si-Wu-Tang without *Rhizoma Chuanxiong* with peaks matched from the template of *Rhizoma Chuanxiong*. In positive ionization mode, six out of 13 template peaks were matched, whereas in negative ionization mode, five out of 17 template peaks were matched. The matching of template peaks from *Rhizoma Chuanxiong* with peaks detected in an adulteration of Si-Wu-Tang that did not contain *Rhizoma Chuanxiong* can be explained by the fact that not all compounds selected as template peaks are unique to *Rhizoma Chuanxiong*. For example, Z-ligustilide and ferulic acid are also contained in *Radix Angelicae Sinensis*. Even though matching of template peaks occurred, the matching of a considerably smaller number of template peaks disclosed that the analyzed sample did not contain *Rhizoma Chuanxiong*. Further, it is illustrated that one or a few marker compounds are not sufficient for the authentication of traditional Chinese prescriptions when those compounds are not uniquely contained in one herb.

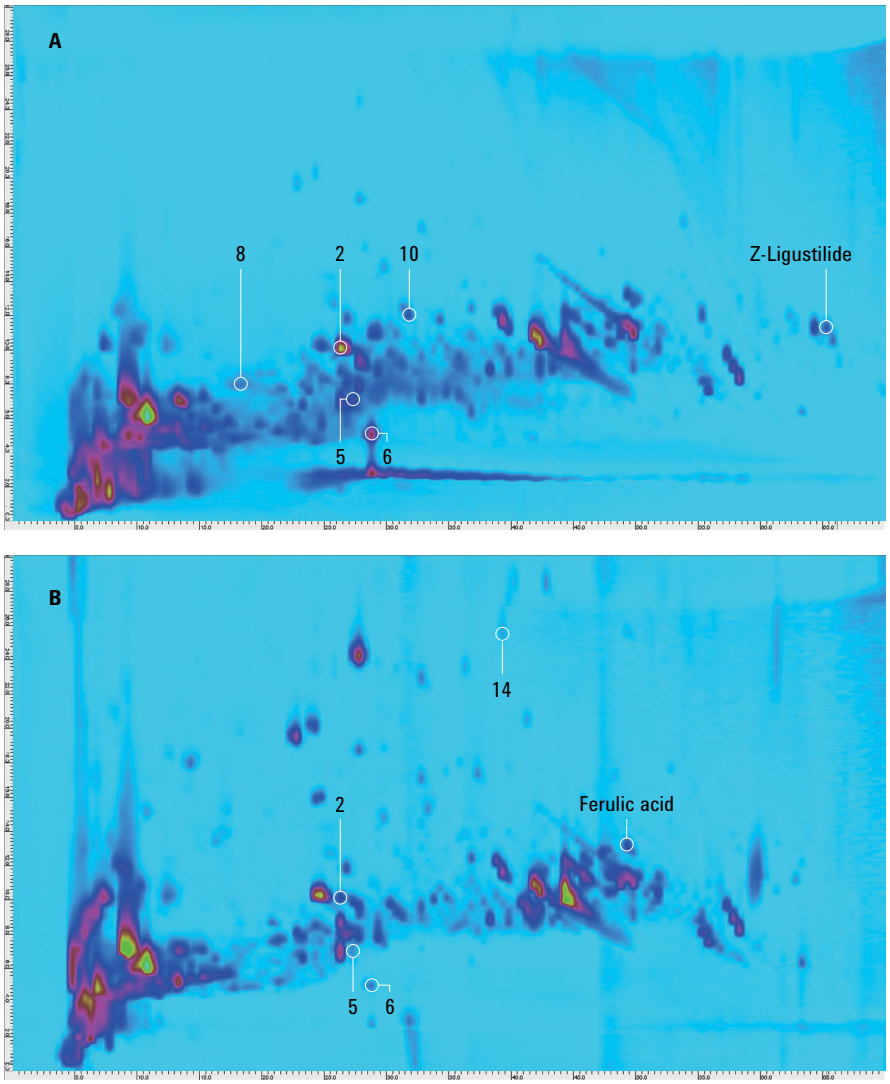

Figure 4. Comprehensive 2D-LC analysis of a decoction from Si-Wu-Tang without *Rhizoma Chuanxiong* with MS detection in positive (A) and negative (B) ionization mode. Peaks matched from the template of *Rhizoma Chuanxiong* are marked.

Figure 5 shows the analysis of Si-Wu-Tang with *Rhizoma Chuanxiong* replaced by the rhizome of another herb and peaks matched from the template of *Rhizoma Chuanxiong*. Compared to Figure 4, additional peaks originating from the replacement herb were detected, but the number of matched peaks did not increase. This shows that the replacement herb did not contain any of the compounds selected for the template of *Rhizoma Chuanxiong*. Table 2 compares the matching of template peaks from *Rhizoma Chuanxiong* in Si-Wu-Tang and the adulterations of Si-Wu-Tang.

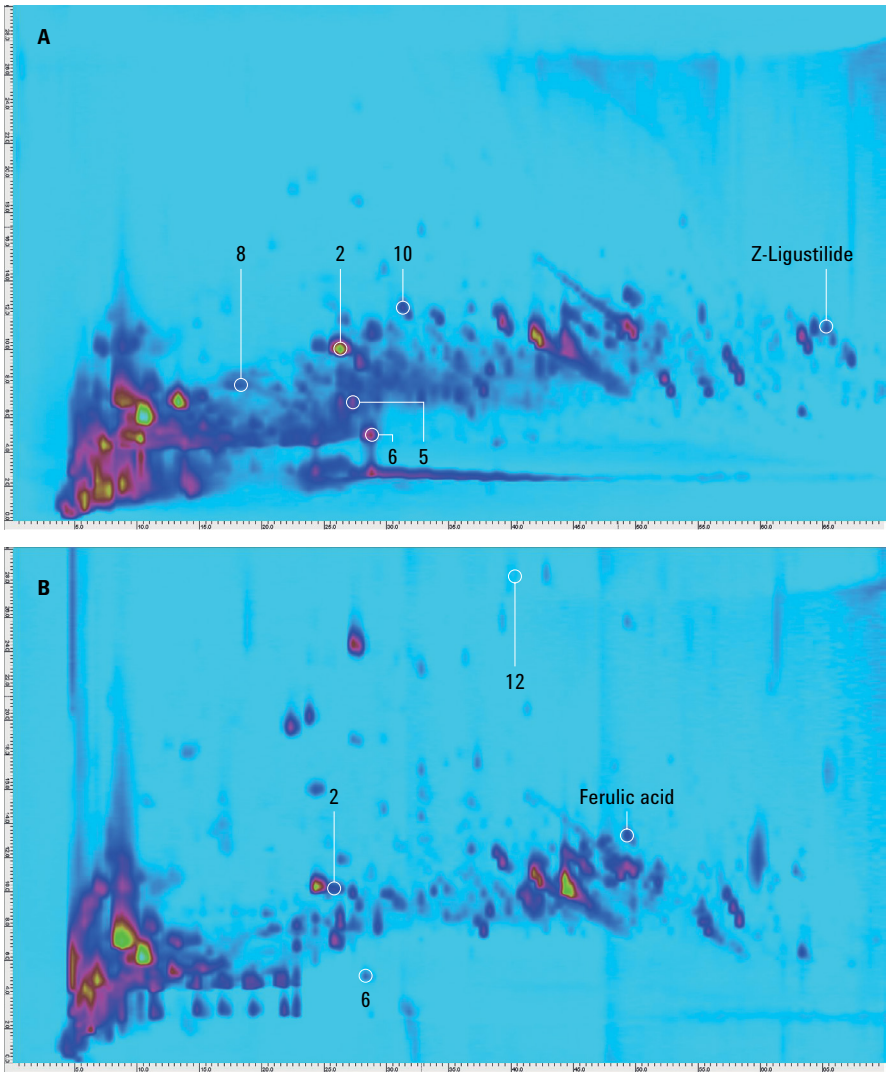

Figure 5. Comprehensive 2D-LC analysis of a decoction from Si-Wu-Tang with *Rhizoma Chuanxiong* replaced by the rhizome of another herb with MS detection in positive (A) and negative (B) ionization mode. Peaks matched from the template of *Rhizoma Chuanxiong* are marked.

Table 2. Matching of template peaks for *Rhizoma Chuanxiong* in Si-Wu-Tang and adulterations of Si-Wu-Tang.

	MS positive		MS negative	
Sample	Number of template peaks	Number of matched peaks	Number of template peaks	Number of matched peaks
Si-Wu-Tang	13	11	17	10
Si-Wu-Tang without *Rhizoma Chuanxiong*	13	6	17	5
Si-Wu-Tang with replacement instead of *Rhizoma Chuanxiong*	13	6	17	4

Conclusion

This Application Note shows the comprehensive 2D-LC analysis of the traditional Chinese prescription Si-Wu-Tang, which is composed of four herbs, as well as the analysis of the individual herbs. For each herb, characteristic component peaks were selected and a template was created. The templates were matched to the peaks detected in Si-Wu-Tang and, with two exceptions, at least 75 % of template peaks could be matched. This shows that it is possible to detect characteristic components of the individual herbs in a traditional Chinese prescription. Additionally, adulteration through omission and replacement of one herb from Si-Wu-Tang could be detected by the matching of a considerably reduced number of template peaks, which provides a means of authentication of Si-Wu-Tang.

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