

Double Off-line Two-dimensional Liquid Chromatography for Separation and Identification of Compounds in *Salvia Miltiorrhiza* (Danshen)

Ji-xia Wang^a, Xiu-li Zhang^{*a}, Fan Yang^a, Hong-li Jin^a, Li-ying Shi^{a,b}, Wei-jia Zhou^a, Yan-fang Liu^a and Xin-miao Liang^a

^aKey Lab of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China

^bInstitute of Materia Medica, Dalian University, Dalian, China

*Corresponding authors: Xiuli Zhang, Tel.: +86 411 84379521; fax: +86 411 84379539, E-mail addresses: zhangxiuli@dicp.ac.cn

ABSTRACT

Background: Danshen is an important traditional Chinese medicine (TCM) used for the treatment of cardiovascular and cerebrovascular diseases. Separation and analysis of its components have been widely investigated. However, the systematical two dimensional liquid chromatography (2D-LC) methods have not been developed to comprehensively separate and characterize its components.

Objective: In this work, double off-line 2D-LC methods were aimed to develop for the systematical separation of compounds from Danshen.

Methods: Using solid phase extraction (SPE), the Danshen extract was divided into a medium-polar fraction (Sample I) and a weak-polar fraction (Sample II) according to their polarities. Based on reversed-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) modes, a 2D-HILIC × RPLC system and a 2D-RPLC × RPLC system were designed for the separation of Sample I and Sample II, respectively. According to reversed-phase and HILIC columns selectivities characterized in our previous reports, ZIC-HILIC and XTerra C18 were employed to build the 2D-HILIC × RPLC system and Click TE-CD and XTerra C18 for the 2D-RPLC × RPLC system, respectively.

Results: The 2D-HILIC × RPLC and 2D-RPLC × RPLC systems exhibited excellent orthogonality for the separation of Sample I and Sample II, respectively. Their orthogonalities were 88.42% and 63.24%. Based on these double 2D-LC systems combined with mass spectrometry, at least 200 compounds were found and 33 compounds of them were identified, including 16 phenolic acids and 17 diterpenoid quinines.

Conclusion: These results suggest that these two off-line 2D-LC methods are effective for the separation and characterization of components in Danshen.

Key words: two-dimensional liquid chromatography, separation, identification, Danshen

INTRODUCTION

Traditional Chinese medicines (TCMs) are now receiving considerable attention for drug discovery given their wide variety of biological activities. Analysis and purification of compounds from TCMs is a critical step for biochemical, pharmaceutical and clinical research^[1–3]. High-performance liquid chromatography (HPLC) is the most widely used separation technique for analysis and purification of compounds from TCMs^[4–6]. However, one-dimensional liquid chromatography fails to provide sufficient resolving power for the separation of targeted compounds from TCMs, which usually contain hundreds of thousands of compounds with great differences in category, polarity and concentration. Therefore, two-dimensional liquid chromatography (2D-LC), introduced by Frei and Erni in 1978, was developed to improve peak capacity and reduce sample complexity to an acceptable level^[7]. A 2D-LC method can be developed based on the same or different chromatography modes, including reversed-phase, ion exchange, size exclusion or hydrophilic interaction chromatography. Among these modes, reversed-phase liquid chromatography (RPLC) and hydrophilic

interaction liquid chromatography (HILIC) are the most widely used in sample separation.

2D-RPLC × RPLC is one of the most prospective separation systems due to its robustness, the outstanding peak capacity in each dimension and the compatibility of mobile phase in each dimension with mass spectrometry (MS)^[8–10]. The resolving power of 2D-RPLC × RPLC systems depends on the different selectivity of the two stationary phases in both dimensions. Due to traditional C18 columns with high similarities, some new stationary phases have been synthesized and used in constructing 2D-RPLC × RPLC systems with good orthogonality. Chen et al. designed a comprehensive 2D-RPLC × RPLC system using CN and ODS columns for the separation of components in *Rhizoma chuanxiong*^[11]. Based on these two columns, a 2D-RPLC × RPLC system was established for analysis of components in *Swertia franchetiana Smith*^[12]. In light of differences in selectivity between C18 and phenyl columns, they were used to design a 2D-RPLC × RPLC system for the evaluation of retention behaviors of polycyclic aromatic hydrocarbons^[13]. A novel click oligo(ethylene glycol) (Click OEG) stationary phase and a C18 column were employed to develop a excellent

orthogonal system to separate *LignumDalbergiae Odoriferae*^[14]. Furthermore, this 2D-RPLC × RPLC system was used to purify compounds from this herb^[15]. Although 2D-RPLC × RPLC systems have been widely used in the separation of TCMs, their orthogonality has a certain limitation for the similar retention mechanisms.

HILIC is an effective technique for the separation of polar compounds^[16]. For its retention mechanisms different from RPLC, HILIC provides complementary selectivity to RPLC, which is useful for constructing highly orthogonal 2D-LC systems. Based on XTerra C18 and Click β-CD, a 2D-RPLC × HILIC system was developed for the separation of polar and medium-polar components in TCMs^[8]. This 2D-RPLC × HILIC system was also used for the isolation of flavonoids from licorice extract^[17]. Guo et al. used a C18 column and an XAmide column to develop a 2D-RPLC × HILIC system for the separation of saponins from leaves of *Panax notoginseng*^[18]. These 2D-LC methods provide a powerful means for the analysis of TCMs.

Danshen, the dried root of *Salvia miltiorrhiza*, is a traditional Chinese medicine (TCM) widely used in China for the treatment of cardiovascular and cerebrovascular diseases^[19,20]. The Danshen Dripping Pill is currently in phase III clinical trial with a great hope to be the first Food and Drug Administration (FDA) approved TCM. Danshen extract contains two main types of ingredients, including water-soluble phenolic acids and lipophilic diterpenoid quinines. Until now, more than 100 compounds have been isolated^[21]. High-speed counter-current chromatography^[22–24], capillary zone electrophoresis^[25,26] and HPLC^[27–31] with UV detector or mass spectrometry have been used to separate and identify compounds from Danshen. Zhu et al. has identified forty constituents from *Radix Salvia miltiorrhizae* using RPLC combined with diode-array detection, electrospray ionization time-of-flight mass spectrometry and electrospray ionization quadrupole ion trap mass spectrometry^[27]. Using normal phase HPLC, tanshinone I, tanshinone IIA and cryptotanshinone were successfully separated from *Salvia miltiorrhiza* Bunge^[28]. Although the analysis of components from Danshen has been widely investigated^[20], the proper 2D-LC methods have not been developed for its systematical separation.

In this work, the Danshen extract was divided into the medium-polar fraction and the weak-polar fraction using solid phase extraction (SPE). Subsequently, a 2D-RPLC × HILIC system and a 2D-RPLC × RPLC system were developed for the separation and identification of components in the medium-polar fraction and the weak-polar fraction, respectively. And by combination with MS, compounds in these fractions were identified or tentatively characterized.

EXPERIMENTAL

1. Reagents and chemicals

Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Formic acid (FA) was from Acros (Fair Lawn, NJ, USA). Water (H₂O) was prepared by a Milli-Q water purification

system (Billerica, MA, USA). XTerra C18, Atlantis T3 and Atlantis HILIC Silica were supplied by Waters (Milford, MA, USA). XAqua C18, XAqua CN, XAmide and Unitary NH₂ were from Acchrom (Beijing, China). Click OEG, Click TE-CD and Click TE-Cys were homemade^[32–34]. TSKgel Amide-80 and ZIC-HILIC were purchased from Tosoh Biosciences (Shanghai, China) and Merck SeQuant (Darmstadt, Germany), respectively. Except as noted, all of these columns were 150 mm × 4.6 mm with 5 μm particle size.

2. Sample preparation

Danshen, the dried root of *Salvia miltiorrhiza*, was collected in Henan province (China) and authenticated by Institute of Medication, Xiyuan hospital of China Academy of Traditional Chinese Medicine. The procedures of extraction were as follows: the dried root was ground into powder and sieved through a No. 40 mesh. 1 g of powder sample was extracted for 30 min by sonication using 25 mL of MeOH-H₂O (70/30, v/v). After centrifugation for 15 min at 5000 rpm, 5 mL of the supernatant was filtered through a 0.45 μm cellulose membrane and evaporated to 3 mL under a gentle stream of nitrogen. The obtained solution contained about MeOH-H₂O (50/50, v/v).

An XAqua C18 cartridge (200 mg, Acchrom, Beijing, China) was first activated with 1 mL of MeOH and equilibrated with 1 mL of MeOH-H₂O (50/50, v/v). 600 μL of the obtained solution was loaded onto this cartridge. It was eluted with 1 mL of ACN-H₂O containing 0.5% FA (50/50, v/v) for Sample I and eluted with 1 mL of ACN-H₂O containing 0.5% FA (90/10, v/v) for Sample II. This step was repeated thrice and the respective fractions were combined. These fractions were evaporated to dryness under a gentle stream of nitrogen. The Sample I and Sample II were dissolved in 800 μL of MeOH-H₂O (50/50, v/v) and 700 μL of MeOH-H₂O (65/35, v/v), respectively. These solutions were stored at approximate 4 °C before use.

3. Chromatographic conditions

The samples were analyzed by HPLC system (Waters, MA, USA) equipped with an Alliance 2695 quaternary pump and an ultraviolet detector (UV) at 280 nm. The mobile phases consisted of 0.5% FA in H₂O (A) and ACN (B) at a constant flow rate of 1.0 mL/min. The column temperature was set at 30 °C. The total sample was separated on the XAqua C18 using the following gradients: (1) 0–15 min, 10%–30% B; (2) 15–20 min, 30%–60% B; (3) 20–30 min, 60%–95% B; (4) 30–35 min, 95%–95% B.

3.1. Chromatographic conditions for Sample I

The Sample I was separated on different columns. After optimization, the suitable gradient conditions were obtained. The gradients performed on the XTerra C18 were from 5% B to 25% B within 20 min and then increased linearly to 45% B within another 10 min. The gradients on TSKgel Amide-80 were as follows: (1) 0–20 min, 95%–90% B; (2) 20–30 min, 90%–60% B. The gradients on XAmide started from 90% B to 60% B within 30 min. The eluted conditions on ZIC-HILIC

were as follows: (1) 0–20 min, 95%–83% B; (2) 20–30 min, 83%–50% B. The gradients on Click TE-Cys, Atlantis HILIC Silica and Unitary NH₂ were: 0 ~ 15 ~ 30 min, 95% ~ 85% ~ 10% B; 0 ~ 20 ~ 30 min, 95% ~ 95% ~ 60% B; 0 ~ 30 min, 95% ~ 10% B; respectively.

3.2. Chromatographic conditions for Sample II

To effectively separate Sample II, different columns were tested. The gradients performed on XAqua C18 were as follows: (1) 0–20 min, 50%–75% B; (2) 20–30 min, 75%–90% B. The gradients on Atlantis T3 were the same as those on XAqua C18. The gradients on XTerra C18 were from 40% B to 65% B within 20 min and then linearly increased to 80% B within another 10 min. The gradients on Click TE-CD were: 0 ~ 25 ~ 30 min, 25% ~ 30% ~ 45% B. The gradients on Click OEG started from 25% B to 55% B within 30 min. The gradients performed on XAqua CN were as follows: (1) 0–20 min, 20–35% B; (2) 20–30 min, 35–70% B.

3.3. Two-dimensional liquid chromatography for separation of Sample I and II

The separation conditions of Sample I were as follows: ZIC-HILIC was used as the first dimensional column. A volume of 50 µL of Sample I was injected into the first dimension. The linear gradient of the mobile phase was as follows: 0 ~ 20 ~ 30 min, 95% ~ 83% ~ 50% B. Fractions were collected manually from 3 min to 18 min at 1-min interval and denoted orderly as Fraction SI-F1 to Fraction SI-F16. Each fraction was concentrated to dryness and redissolved in 100 µL of MeOH-H₂O (50/50, v/v). 10 µL of each fraction was injected directly into the second dimension. XTerra C18 (150 mm × 2.1 mm, I.D. 5 µm) was used as the second dimensional column with the flow rate at 0.2 mL/min. The linear gradient elution on this column was: 0 ~ 20 ~ 30 min, 8% ~ 28% ~ 48% B.

The separation conditions of Sample II were as follows: Click TE-CD was used as the first dimensional column. A volume of 50 µL of Sample II was injected into the first dimension. The linear gradient of the mobile phase was as follows: 0 ~ 20 ~ 25 min, 25% ~ 29% ~ 55% B. Fractions were collected manually from 6 min to 21 min at 1-min interval and denoted orderly as Fraction SII-F1 to Fraction SII-F16. Each fraction was concentrated to dryness and redissolved in 100 µL of MeOH-H₂O (50/50, v/v). 10 µL of each fraction was injected directly into the second dimension. XTerra C18 (150 mm × 2.1 mm, I.D. 5 µm) was used as the second dimensional column with the flow rate at 0.2 mL/min. The linear gradient elution on this column was: 0 ~ 20 ~ 30 min, 45% ~ 70% ~ 85% B.

4. LC-MS analysis

Compounds of fractions in Section 3.3 (EXPERIMENTAL) were characterized using an Agilent 1290 Infinity LC instrument coupled to Agilent 6540 series Q-TOF-MS (Agilent Technologies Inc., USA), which equipped with electrospray ionization (ESI) source. Fractions of Sample I and Sample II were separated using the same chromatographic conditions as

in Section 3.3 (EXPERIMENTAL). Compounds in fractions of Sample I were detected in the negative ion mode. While compounds in fractions of Sample II were detected in the positive ion mode. The mass spectrometer conditions were as follows: nebulizer gas pressure (35 psi), drying gas flow rate (8 L/min), gas temperature (350 °C), capillary voltage (3500 V) and collision energy (25 eV). The MS scan ranged from 100 to 1000 m/z and the MS/MS scan ranged from 50 to 1000 m/z.

5. Data analysis

The orthogonality of any two chromatographic systems was calculated according to the literature^[8, 35]. Retention times in the second dimension are normalized according to Eq. (1),

$$t_R^{i(norm)} = \frac{t_R^i - t_R^{min}}{t_R^{max} - t_R^{min}} \quad (1)$$

where t_R^{max} and t_R^{min} represent the retention times of the most and least retained solute in the data set, respectively. The retention times are converted to the normalized $t_R^{i(norm)}$ ranging from 0 to 1. For the x -axis, the grid number is the number of fractions in the first dimension and the grid number of the y -axis is calculated according to Eq. (2),

$$Grid_y = \frac{Num_p}{Frac_x} \quad (2)$$

where Num_p is the number of peaks detected by the two dimensional chromatography and $Frac_x$ is the grid number of the x -axis. The coverage of data points can describe the orthogonality calculated by Eq. (3),

$$0\% = \frac{\Sigma bins - \Sigma bins(blank)}{0.63P_{max}} \times 100 \quad (3)$$

where $\Sigma bins$ is the number of bins containing data points in the two dimensional plot and P_{max} is the total peak capacity obtained as a sum of all bins. $\Sigma bins(blank)$ is the number of bins containing data points in the two dimensional plot when the fractions are analyzed using identical conditions for the first and second dimensions, namely a non-orthogonal system, in which the data points would be lined up along the diagonal and the surface coverage should be 10%. The data was calculated using Microsoft excel 2010 and Origin 8.0.

RESULTS AND DISCUSSION

1. Design of 2D-LC separation systems

1.1. Pretreatment of Danshen extracts

Given that the chemical compositions of Chinese herbs are very complex, it is necessary to reduce sample complexity by pretreatment procedures. The Danshen extracts was first separated on an XCharge C18 column (Fig. 1A). According to the retention of compounds on this column under this gradient conditions, the extracts mainly contained medium-polar components and weak-polar components. After SPE pretreatment, the extracts can be divided into two sections:

the medium-polar components (Sample I) eluted with ACN-H₂O containing 0.5% FA (50/50, v/v) and the weak-polar components (Sample II) eluted with ACN-H₂O containing 0.5% FA (90/10, v/v) (Fig. 1B and Fig. 1C). Because the medium-polar components have good retention under RPLC and HILIC modes and these two modes have different separation selectivity for their different retention mechanisms. Herein, a 2D-HILIC × RPLC system was designed to separate Sample I. While the weak-polar components could not be retained by HILIC columns but had good retention on RPLC columns. Therefore, a 2D-RPLC × RPLC system was designed to separate Sample II. Thus, double off-line 2D-LC systems were designed for the systematical separation of Danshen.

1.2. Optimization of separation conditions for Sample II

12 kinds of reversed-phase columns have been characterized using linear solvation energy relationships (LSERs) combined with fundamental retention equations in our previous

work^[36]. According to these column selectivities, representative columns were selected, including XTerra C18, Atlantis T3, XAqua C18, XAqua CN, Click OEG and Click TE-CD. The separation conditions of Sample II on these columns were optimized. Sample II was separated on these columns under optimal chromatographic conditions and their chromatographs are shown in Fig. 2. As expected, the elution orders of the components of Sample II on XTerra C18 (Fig. 2A), Atlantis T3 (Fig. 2B) and XAqua C18 (Fig. 2C) are similar, meaning that these columns are near-equivalent. Among these three columns, XTerra C18 could provide sharpest peaks and was selected. Based on the elution orders of these components on XAqua CN, Click OEG and Click TE-CD (Fig. 2D–F), Click TE-CD can provide highest difference in pattern from XTerra C18. Additionally, samples are well separated in the second dimension, which is useful for the improvement of the resolving power of 2D-LC^[37]. Therefore, Click TE-CD and XTerra C18 were used in the first and second dimension respectively to develop a 2D-RPLC × RPLC system for the separation of Sample II.

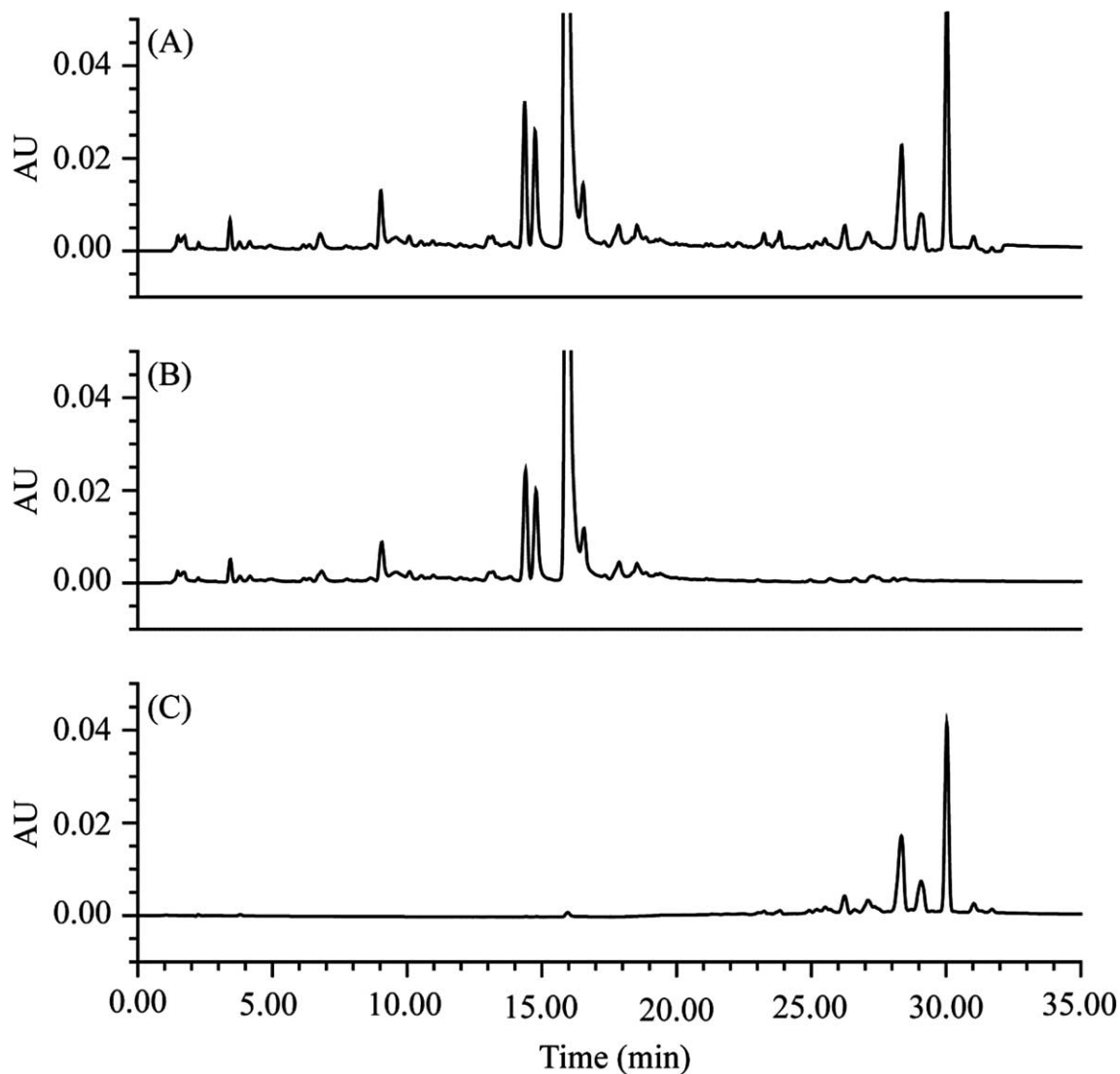


Fig. 1 The chromatograms of samples on XCharge C18 column. (A) the total extracts of Danshen; (B) Sample I (the medium-polar components); (C) Sample II (the weak-polar components).

1.3. Optimization of separation conditions for Sample I

In the process of optimization of separation conditions for Sample II, XTerra C18 could provide better separation power and was tested for the separation of Sample I (Fig. 3A). It can be seen that Sample I is well separated on XTerra C18. Therefore, this column was selected and used for constructing a orthogonal system for the separation of Sample I. According to selectivities of HILIC columns evaluated using our hydrophilic-subtraction model^[38], representative columns were selected, including TSKgel Amide-80, XAmide, ZIC-HILIC, Click TE-Cys, Atlantis HILIC Silica and Unitary NH₂. Separation of Sample I on these HILIC columns under the optimal experimental conditions is showed in Fig. 3. The elution orders of components of Sample I on TSKgel Amide-80, XAmide, ZIC-HILIC and Click TE-Cys are similar

(Fig. 3B-E). Among these columns, the sharp peaks of components are obtained on ZIC-HILIC (Fig. 3D). It can be explained as follows: Click TE-Cys is synthesized by immobilizing cysteine onto the silica surface and it possesses positive charges on the stationary phase surface under the acid conditions^[34], which will interact with the partially ionized phenolic acids, resulting in peak tailing (Fig. 3E). The neutral TSKgel Amide-80 and XAmide cannot provide symmetry peaks of the main components for molecular interactions between them (Fig. 3B and C). While ZIC-HILIC is prepared by grafting sulfobetaine to the silica surface with negatively charged sulfonate groups as a distal moiety and positively charged quaternary ammonium in the proximal location to the silica surface^[39]. The negative charges of this stationary phase surface lead to a certain ionic repulsion to phenolic acids, which is useful for obtaining good peaks

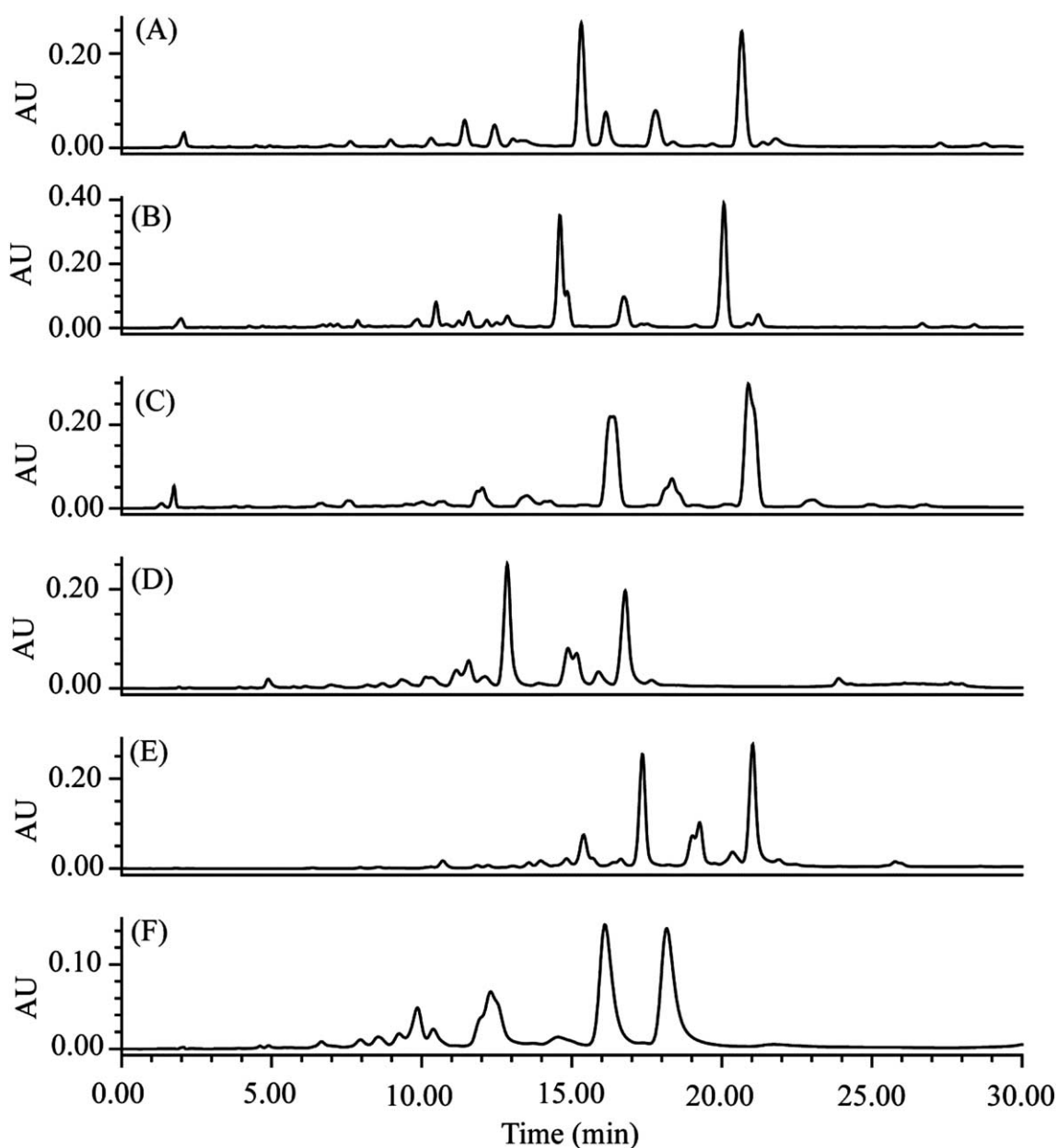


Fig. 2 The chromatograms of Sample II on XTerra C18 (A), Atlantis T3 (B), XAqua C18 (C), XAqua CN (D), Click OEG (E) and Click TE-CD (F).

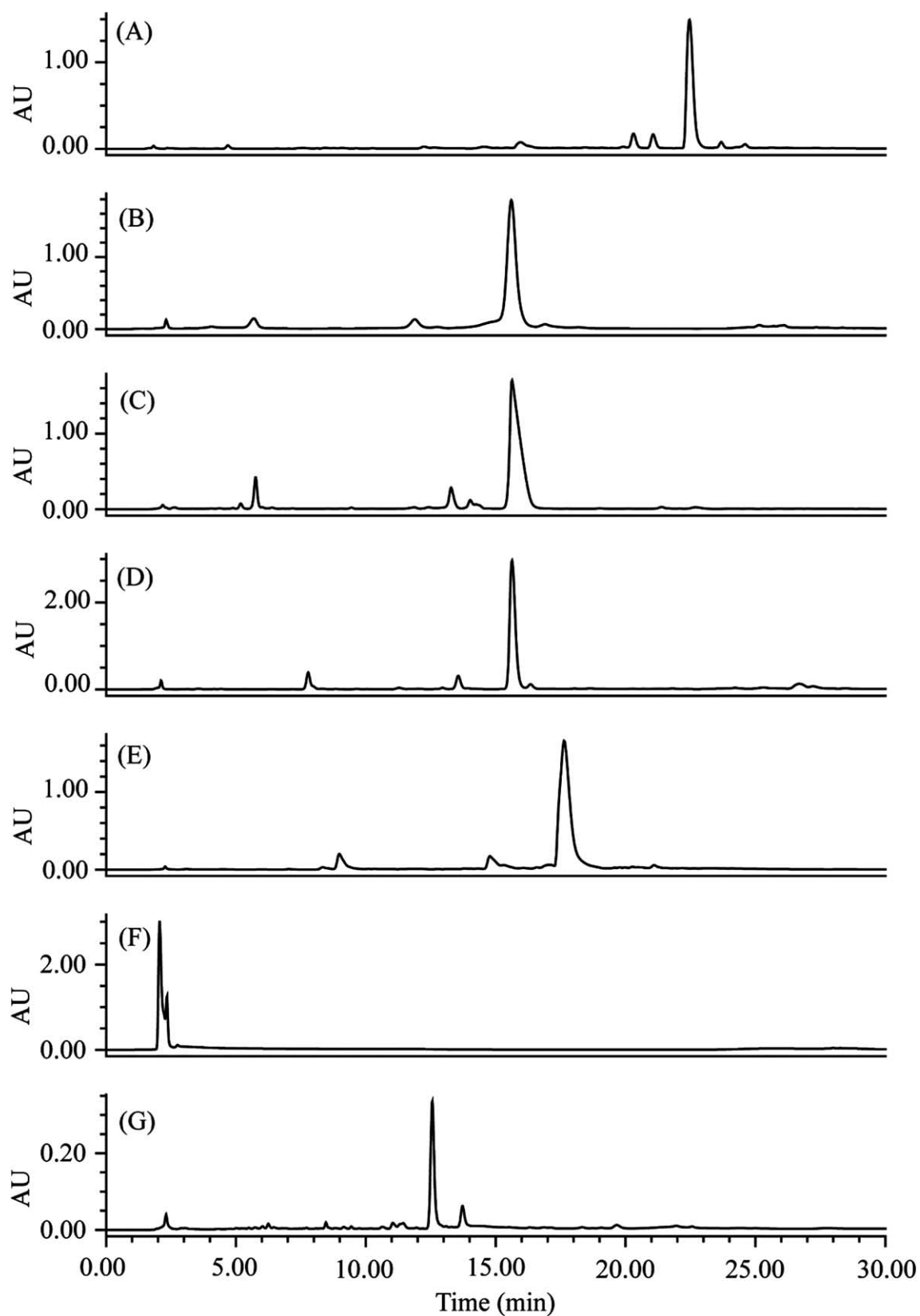


Fig. 3 The chromatograms of Sample I on XTerra C18 (A), TSKgel Amide-80 (B), XAmide (C), ZIC-HILIC (D), Click TE-Cys (E), Atlantis HILIC Silica (F) and Unitary NH₂ (G).

(Fig. 3D). This similar phenomenon has also been reported in the separation of basic compounds^[40]. Due to the relatively weak hydrophilicity of Atlantis HILIC Silica, Sample I was eluted at dead time even under the condition of 95% ACN (Fig. 3F). When Sample I was separated on Unitary NH₂, the peak high decreased greatly for death absorption of phenolic acids (Fig. 3G). In summary, ZIC-HILIC was the optimal one for the separation of Sample I. Therefore, ZIC-HILIC and XTerra C18 were used in the first and second dimension respectively to develop a 2D-HILIC × RPLC system for the separation of Sample I.

2. 2D-HILIC × RPLC system for separation of sample I

Using the 2D-HILIC × RPLC system developed in Section 1.3 (RESULTS AND DISCUSSION), ZIC-HILIC and XTerra C18 were employed as the first and second dimension columns respectively to separate Sample I. Of note, XTerra C18 (150 mm × 2.1 mm, I.D. 5 μm) was used in the second dimension to enhance the detected concentration of components. During the separation of Sample I, 366 peaks were detected in SI-F1 ~ SI-F16 fractions. According to Eq. (2), the normalized data points were distributed in space of 16 × 23 bins (368, which was approximate to 366) and bins containing data points were 257 (Fig. 4A). $\Sigma bins(blank)$ was calculated by the non-orthogonal system that ZIC-HILIC was used in both dimensions to analyze these 16 fractions. As presented in Fig. 4B, $\Sigma bins(blank)$ was 52. According to Eq. (3), the degree of orthogonality of ZIC-HILIC and XTerra C18 in the separation of Sample I was 88.42%. The corresponding three dimensional chromatogram is displayed in Fig. 5. The results demonstrated that this 2D-HILIC × RPLC system was highly orthogonal in the separation of medium-polar components in Danshen.

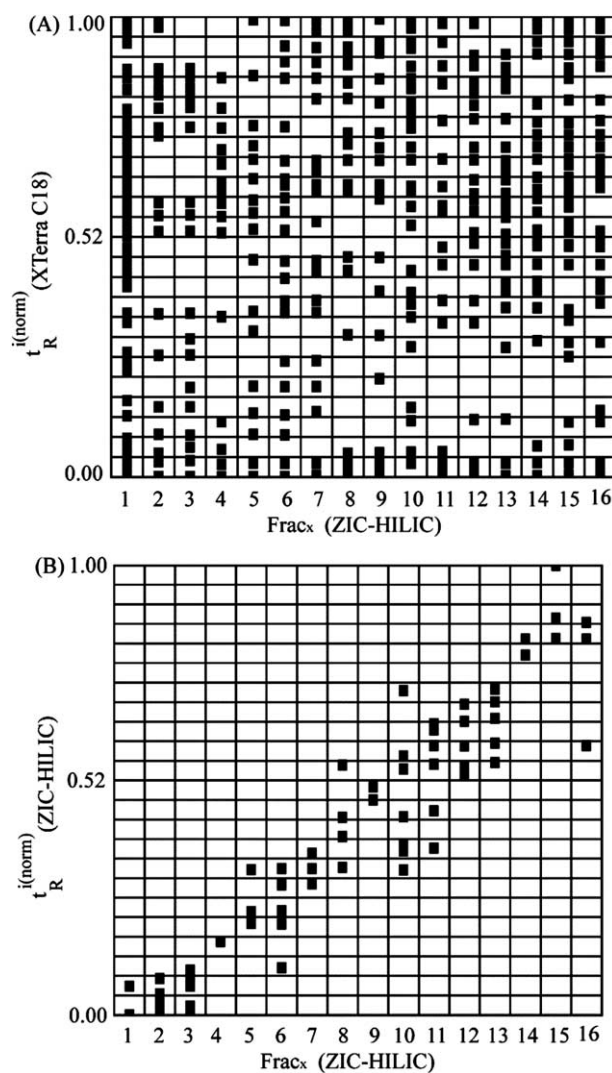


Fig. 4 Normalized plots of this 2D-HILIC × RPLC system for the separation of Sample I: (A) ZIC-HILIC × XTerra C18 system; (B) ZIC-HILIC × ZIC-HILIC system.

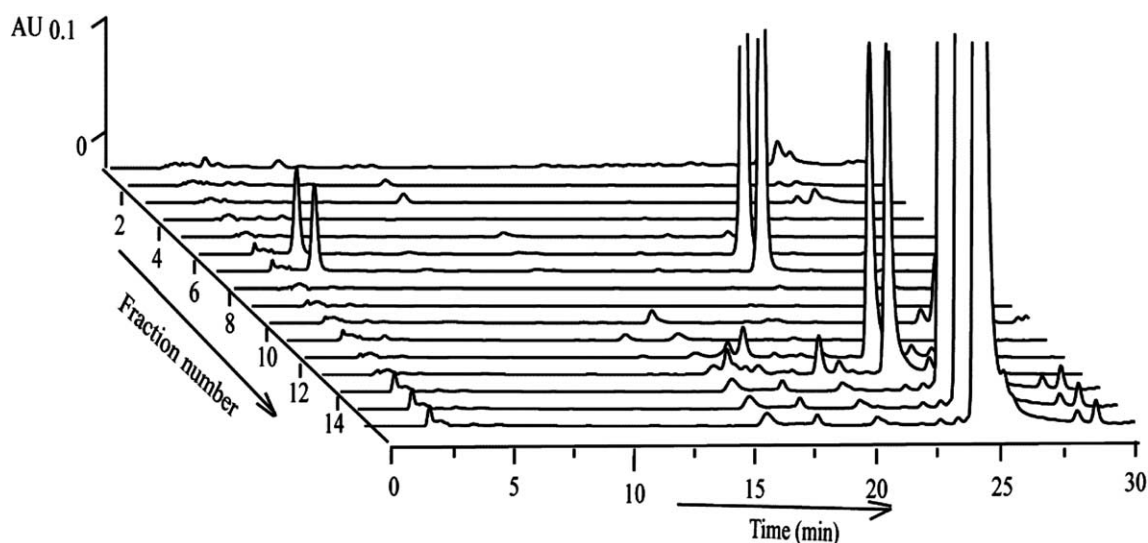


Fig. 5 Three dimensional chromatogram of SI-F1 ~ SI-F16 fractions analyzed on XTerra C18.

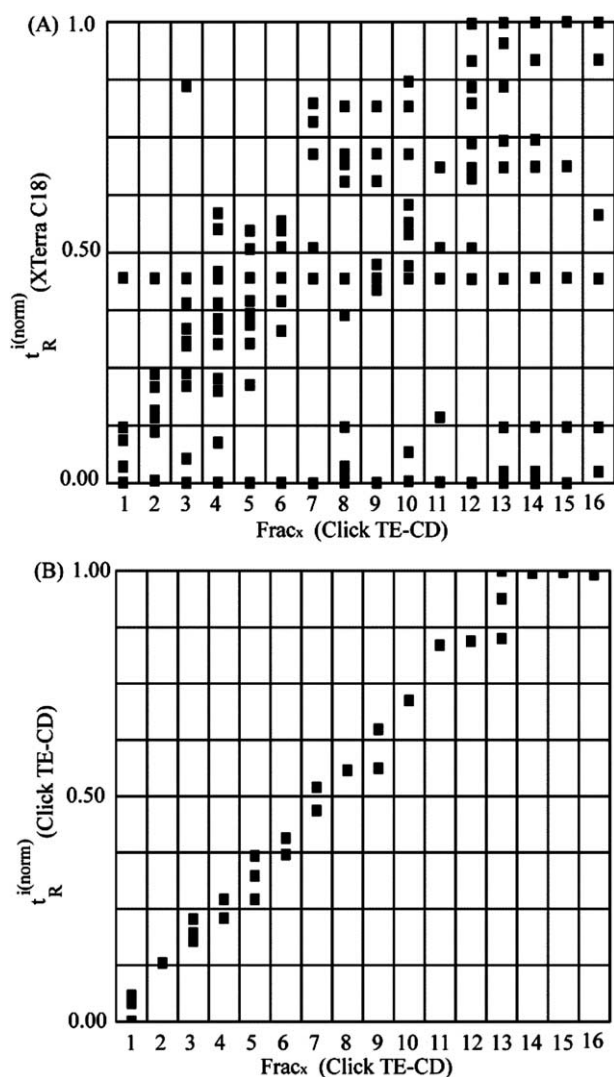


Fig. 6 Normalized plots of this 2D-HILIC \times RPLC system for the separation of Sample II: (A) Click TE-CD \times XTerra C18 system; (B) Click TE-CD \times Click TE-CD system.

3. 2D-RPLC \times RPLC system for separation of sample II

Using the 2D-RPLC \times RPLC system developed in Section 1.2 (RESULTS AND DISCUSSION), Click TE-CD and XTerra C18 (150 mm \times 2.1 mm, I.D. 5 μ m) were employed as the first and second dimension columns respectively to separate Sample II. During the separation of Sample II, 129 peaks were detected in SII-F1 ~ SII-F16 fractions. According to Eq. (2), the normalized data points were distributed in space of 16 \times 8 bins (128, which was approximate to 129) and bins containing data points were 71 (Fig. 6A). $\Sigma bins(blank)$ was calculated by the non-orthogonal system that Click TE-CD was used in both dimensions to analyze these 16 fractions. As presented in Fig. 6B, $\Sigma bins(blank)$ was 20. According to Eq. (3), the degree of orthogonality of Click TE-CD and XTerra C18 in the separation of Sample II was 63.24%. The corresponding three dimensional chromatogram is displayed in Fig. 7. The results demonstrated that this 2D-RPLC \times RPLC system was highly orthogonal in the separation of weak-polar components in Danshen.

4. Characterization of components in sample I and sample II

Fractions of Sample I and Sample II were analyzed using high resolution ESI-Q-TOF-MS/MS. At least 200 compounds were found. Generally, water-soluble phenolic acids and lipophilic diterpenoid quinines in Danshen extract are believed to be active ingredients. In these fractions, a total of 33 compounds including 16 phenolic acids and 17 diterpenoid quinines were identified in Table 1. Among them, 11 compounds were identified unambiguously by comparing their retention times and mass spectra with standard compounds, including protocatechuic aldehyde (1), caffeic acid (2), danshensu (4), rosmarinic acid (5), salvianolic acid C (6), salvianolic acid A (11), lithospermic acid (13), salvianolic acid B (16), dihydrotanshinone (23), cryptotanshinone (32) and

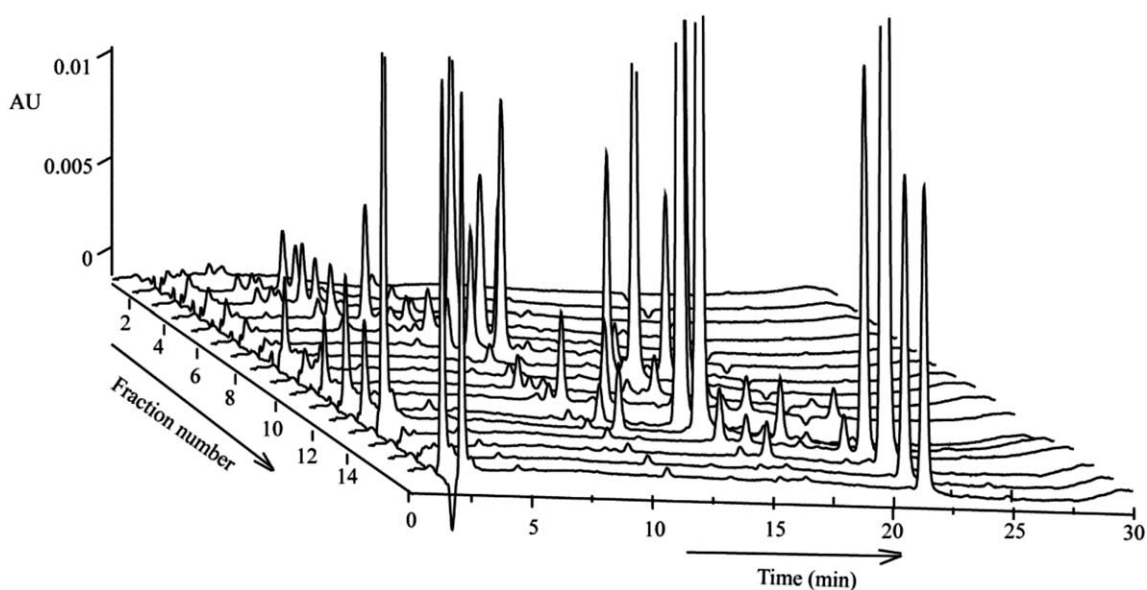


Fig. 7 Three dimensional chromatogram of SII-F1 ~ SII-F16 fractions analyzed on XTerra C18.

Table 1. Compounds identified using MS/MS Danshen.

No.	Fraction	RT (min)	m/z	Measured ion	Error (ppm)	MS/MS	Identification
1	SI-F1	6.39	137.0249	[C7H5O3] ⁻	-3.38	108(100), 136(96), 92(55), 53(62)	Protocatechuic aldehyde
2	SI-F2	8.14	179.0371	[C9H7O4] ⁻	0.24	135(100), 134(43), 107(7), 89(12), 79(5)	Caffeic acid
3	SI-F4	4.66	153.0197	[C7H5O4] ⁻	-2.1	109(100)	Protocatechuic acid
4	SI-F6	3.82	197.0453	[C9H9O5] ⁻	1.12	135(90), 123(82), 109(10), 73(100)	Danshensu
5	SI-F6	17.47	359.0781	[C18H15O8] ⁻	-2.35	179(22), 161(100), 135(16), 73(31)	Rosmarinic acid
6	SI-F7	25.08	491.0986	[C26H19O10] ⁻	-0.51	293(100), 197(12), 135(7), 109(12)	Salvianolic acid C
7	SI-F8	20.84	551.1193	[C28H23O12] ⁻	0.27	327(11), 309(98), 197(100)	9''-Methyl lithospermate
8	SI-F8	21.86	551.121	[C28H23O12] ⁻	-2.63	353(100), 321(12), 309(24), 197(22), 135(23)	αMethyl salvianolate H/I
9	SI-F9	24.28	731.1647	[C37H31O17] ⁻	-3.95	533(100), 339(5), 321(7)	9''-Methyl salvianolate B
10	SI-F10	21.93	731.164	[C37H31O16] ⁻	-3.08	551(7), 533(100), 353(29), 335(45), 309(6)	4-Methoxyl-salvianolic acid B
11	SI-F10	21.41	493.1147	[C26H21O10] ⁻	-1.41	295(93), 185(100), 109(70)	Salvianolic acid A
12	SI-F12	12.85	539.1208	[C27H23O12] ⁻	-2.36	359(18), 341(18), 315(58), 297(23), 197(84), 179(91), 161(81), 135(100)	6-[(1E)-3-[1-carboxy-2-(3,4-dihydroxyphenyl)ethoxy]-3-oxo-1-propenyl]-3-(3,4-dihydroxyphenyl)-8-hydroxy-7-oxo-bicyclo[2.2.2]oct-5-ene-2-carboxylic acid
13	SI-F12	18.1	537.1061	[C27H21O12] ⁻	-4.08	295(100), 197(15), 185(76), 109(15)	Lithospermic acid
14	SI-F13	15.51	537.1035	[C27H21O12] ⁻	0.62	339(100), 295(73), 185(17), 109(18)	Salvianolic acid H/I
15	SI-F13	16.38	537.1046	[C27H21O12] ⁻	-1.46	537(84), 493(100), 295(5)	Salvianolic acid J
16	SI-F14	19.59	717.1479	[C36H29O16] ⁻	-2.47	519(100), 339(19), 321(78), 295(8)	Salvianolic acid B
17	SII-F2	4.87	295.0965	[C18H15O4] ⁺	-1.61	277(46), 251(28), 249(87), 221(100), 219(31), 178(28)	3α-Hydroxymethylenetanshinquinone
18	SII-F2	5.12	297.1119	[C18H17O4] ⁺	0.94	261(97), 233(100), 205(45), 234(16), 190(15)	Danshenxinkun A
19	SII-F3	10.62	311.1278	[C19H19O5] ⁺	-0.13	265(42), 237(30), 225(27), 197(18), 185(100)	1-Ketoisocryptotanshinone
20	SII-F3	11.09	339.1226	[C20H19O5] ⁺	0.37	261(86), 233(100), 205(38)	Methyl tanshinonate
21	SII-F4	7.29	297.1122	[C18H17O4] ⁺	-0.2	261(99), 233(100), 237(20), 234(25), 209(24), 206(16), 205(71), 190(20)	Tanshinone VI
22	SII-F5	10.51	281.1173	[C18H17O3] ⁺	-0.39	263(21), 235(100), 220(28), 207(27), 192(37)	Trijuganone B
23	SII-F5	9.56	279.1025	[C18H15O3] ⁺	-3.44	233(100), 205(68), 169(34)	Dihydrotanshinone
24	SII-F6	11.35	293.1167	[C19H17O3] ⁺	1.76	293(50), 278(41), 275(51), 247(100), 219(26), 204(33)	1, 2-Didehydrotanshinone IIA
25	SII-F6	8.72	293.1166	[C18H13O4] ⁺	1.94	293(38), 278(47), 279(20), 275(32), 251(17), 247(100), 219(38), 207(27), 192(37)	Monohydroxytanshinone I
26	SII-F7	15.68	279.102	[C18H15O3] ⁺	-1.44	261(30), 233(100), 205(89), 191(7)	Methylenetanshinquinone
27	SII-F8	13.88	277.0859	[C18H13O3] ⁺	-0.07	249(42), 168(100)	Tanshinone I
28	SII-F9	9.92	297.1482	[C19H21O3] ⁺	1.14	269(100), 270(23), 227(18), 251(10), 209(9), 199(29), 171(70)	Cryptotanshinone isomer
29	SII-F9	9.06	311.1278	[C19H19O4] ⁺	-0.12	293(60), 275(51), 266(9), 247(100), 219(18)	Tanshinone IIB
30	SII-F10	11.37	295.133	[C19H19O3] ⁺	-0.54	295(100), 280(46), 262(20), 277(44), 249(40), 225(29)	1, 2-Didehydrocryptotanshinone
31	SII-F10	4.65	313.1435	[C19H21O4] ⁺	-0.15	269(100), 267(40), 253(58), 171(87)	17-Hydroxycryptotanshinon
32	SII-F11	13.33	297.1484	[C19H21O4] ⁺	0.29	297(100), 279(47), 251(64)	Cryptotanshinone
33	SII-F13	18.56	295.133	[C19H19O3] ⁺	-0.34	277(27), 262(37), 234(35), 206(21), 191(100)	Tanshinone IIA

tanshinone IIA (33). And other compounds were identified tentatively based on MS/MS fragmentation rules reported in reference^[27, 41, 42]. As shown in Table 1, retention times of compounds 8 and 10 were 21.86 min and 21.93 min in the second dimension, but they were fractioned into Fraction SI-F8 and Fraction SI-F10 in the first dimension, respectively. The same phenomenon can be found in compounds 24 and 30. Therefore, compounds with very close retention times in one-dimensional LC system were identified obviously by such off-line 2D-LC separation systems. Compared to the published work using a one-dimensional LC system^[27, 41, 42], these off-line 2D-LC separation systems provided great potential in separating more compounds, especially in isomer compounds or compounds with close retention times in one-dimensional LC system.

Additionally, the unidentified components were listed in Table S1, which were difficult to be identified based on the current information and needed to be characterized via further work.

CONCLUSION

Double off-line 2D-LC methods were successfully developed for the systematical separation of compounds from Danshen. The Danshen extract was first divided into the medium-polar fraction (Sample I) and the weak-polar fraction (Sample II) using solid phase extraction (SPE). Subsequently, ZIC-HILIC and XTerra C18 were used to construct the 2D-HILIC×RPLC system for the separation of Sample I and Click TE-CD and XTerra C18 were used to establish the 2D-RPLC×RPLC system for the separation of Sample II. These two off-line 2D-LC systems exhibited excellent orthogonality, reaching 88.42% and 63.24%, respectively. By identification using MS, 33 compounds were identified, including 16 phenolic acids and 17 diterpenoid quinines. These double off-line 2D-LC methods exhibited excellent performance in the separation and characterization of components in Danshen.

ACKNOWLEDGEMENTS

This work was funded by Project of National Science Foundation of China (81473436, 81274077 and 81403100).

REFERENCES

- Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products* 2007,70(3):461–477.
- Newman DJ, Cragg GM. Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010. *Journal of Natural Products* 2012,75(3):311–335.
- Harvey AL. Natural products in drug discovery. *Drug Discovery Today* 2008,13(19–20):894–901.
- Liang XM, Jin Y, Wang YP, Jin GW, Fu Q, Xiao YS. Qualitative and quantitative analysis in quality control of traditional Chinese medicines. *Journal of Chromatography A* 2009, 1216(11):2033–2044.
- Yang M, Sun JH, Lu ZQ, Chen GT, Guan SH, Liu X, Jiang BH, Ye M, Guo DA. Phytochemical analysis of traditional Chinese medicine using liquid chromatography coupled with mass spectrometry. *Journal of Chromatography A* 2009,1216(11):2045–2062.
- Zhao HY, Jiang JG. Application of Chromatography Technology in the Separation of Active Components from Nature Derived Drugs. *Mini-Reviews in Medicinal Chemistry* 2010, 10(13):1223–1234.
- Erni F, Frei RW. Two-dimensional column liquid chromatographic technique for resolution of complex mixtures. *Journal of Chromatography A* 1978,149(0):561–569.
- Liu YM, Xue XY, Guo ZM, Xu Q, Zhang FF, Liang XM. Novel two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography, an excellent orthogonal system for practical analysis. *Journal of Chromatography A* 2008, 1208(1–2):133–140.
- Sticher O. Natural product isolation. *Natural Product Reports* 2008,25(3):517–554.
- Stephanowitz H, Lange S, Lang D, Freund C, Krause E. Improved Two-Dimensional Reversed Phase-Reversed Phase LC-MS/MS Approach for Identification of Peptide-Protein Interactions. *Journal of Proteome Research* 2012,11(2):1175–1183.
- Chen XG, Kong L, Su XY, Fu HJ, Ni JY, Zhao RH, Zou HF. Separation and identification of compounds in Rhizoma chuanxiong by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry. *Journal of Chromatography A* 1040(2):169–178.
- Liu YM, Xu Q, Xue XY, Zhang FF, Liang XM. Two-dimensional LC-MS analysis of components in Swertia franchetiana Smith. *Journal of Separation Science* 2008, 31(6–7):935–944.
- Kayillo S, Dennis GR, Shalliker RA. An assessment of the retention behaviour of polycyclic aromatic hydrocarbons on reversed phase stationary phases: Selectivity and retention on C18 and phenyl-type surfaces. *Journal of Chromatography A* 2006,1126(1–2):283–297.
- Liu YM, Guo ZM, Jin Y, Xue XY, Xu Q, Zhang FF, Liang XM. “Click oligo(ethylene glycol)”: An excellent orthogonal stationary phase to C18 for two-dimensional reversed-phase/reversed-phase liquid chromatography. *Journal of Chromatography A* 2008,1206(2):153–159.
- Feng JT, Xiao YS, Guo ZM, Yu DH, Jin Y, Liang XM. Purification of compounds from Lignum Dalbergia Odorifera using two-dimensional preparative chromatography with Click oligo (ethylene glycol) and C18 column. *Journal of Separation Science* 2011,34(3):299–307.
- Alpert AJ. HHydrophilic interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *Journal of Chromatography* 1990,499:177–196.
- Zhang H, Guo ZM, Li W, Feng JT, Xiao YS, Zhang FF, Xue XY, Liang XM. Purification of flavonoids and triterpene saponins from the licorice extract using preparative HPLC under RP and HILIC mode. *Journal of Separation Science* 2009,32(4):526–535.
- Guo XJ, Zhang XL, Feng JT, Guo ZM, Xiao YS, Liang XM. Purification of saponins from leaves of Panax notoginseng using preparative two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography. *Analytical and Bioanalytical Chemistry* 2013,405(10):3413–3421.
- Zhou LM, Zuo Z, Chow MSS. Danshen: An overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *Journal of Clinical Pharmacology* 2005,45(12):1345–1359.
- Li YG, Song L, Liu M, Zhi BiH, Wang ZT. Advancement in analysis of Salviae miltiorrhizae Radix et Rhizoma (Danshen). *Journal of Chromatography A* 2009,1216(11):1941–1953.
- Chen XP, Guo JJ, Bao JL, Lu JJ, Wang YT. The Anticancer Properties of Salvia Miltiorrhiza Bunge (Danshen): A Systematic Review. *Medicinal Research Reviews* 2014,34(4):768–794.
- Tian GL, Zhang YB, Zhang TY, Yang FQ, Ito Y. Separation of tanshinones from Salvia miltiorrhiza Bunge by high-speed counter-current chromatography using stepwise elution. *Journal of Chromatography A* 2000,904(1):107–111.
- Gu M, Wang X, Su Z, Fan O. One-step separation and purification of 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde from Salvia miltiorrhiza Bunge by high-speed counter-current chromatography. *Journal of Chromatography A* 2007,1140(1–2): 107–111.
- Meng J, Yang Z, Liang J, Zhou H, Wu S. Comprehensive multi-channel multi-dimensional counter-current chromatography for separation of tanshinones from Salvia miltiorrhiza Bunge. *Journal of Chromatography A* 2014,1323:73–81.

25. Che AJ, Zhang JY, Li CH, Chen XF, Hu ZD, Chen XG. Separation and determination of active components in Radix Salviae miltiorrhizae and its medicinal preparations by nonaqueous capillary electrophoresis. *Journal of Separation Science* 2004,27(7-8):569–575.
26. Qiao C, Zhao L, Jiang S, Song P. Separation and determination of water soluble active components in Salvia miltiorrhiza Bunge and its pharmaceutical preparations by capillary zone electrophoresis with diode array detection. *Journal of Liquid Chromatography & Related Technologies* 2007,30(19):2819–2833.
27. Zhu Z, Zhang H, Zhao L, Dong X, Li X, Chai Y, Zhang G. Rapid separation and identification of phenolic and diterpenoid constituents from Radix Salvia miltiorrhizae by high-performance liquid chromatography diode-array detection, electrospray ionization time-of-flight mass spectrometry and electrospray ionization quadrupole ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry* 2007,21(12):1855–1865.
28. Wan X, Wang Y, Row KH. Separation of Tanshinone I, Tanshinone IIA, and Cryptotanshinone from Salvia miltiorrhiza Bunge by Normal Phase HPLC. *Journal of Liquid Chromatography & Related Technologies* 2009,32(4):544–552.
29. Guo YX, Zhou LL, Li T, Wang LH. Preparative separation of lithospermic acid B from Salvia miltiorrhiza by polyamide resin and preparative high-performance liquid chromatography. *Journal of Chromatography A* 2011,1218(29):4606–4611.
30. Chen XF, Lou ZY, Zhang H, Tan GG, Liu ZR, Li WH, Zhu ZY, Chai YF. Identification of multiple components in Guanxinling injection using hydrophilic interaction liquid chromatography/time-of-flight mass spectrometry and reversed-phase liquid chromatography/time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 2011,25(11):1661–1674.
31. Song JZ, Qiao C-F, Li SL, Zhou Y, Hsieh MT, Xu HX. Rapid optimization of dual-mode gradient high performance liquid chromatographic separation of Radix et Rhizoma Salviae Miltiorrhizae by response surface methodology. *Journal of Chromatography A* 2009,1216(42):7007–7012.
32. Guo ZM, Jin Y, Liang T, Liu YF, Xu Q, Liang XM, Lei AW. Synthesis, chromatographic evaluation and hydrophilic interaction/reversed-phase mixed-mode behavior of a “Click beta-cyclodextrin” stationary phase. *Journal of Chromatography A* 2009,1216(2):257–263.
33. Guo ZM, Liu YF, Xu JY, Xu Q, Xue XY, Zhang FF, Ke YX, Liang XM, Lei AW. Novel reversed-phase high-performance liquid chromatography stationary phase with oligo(ethylene glycol) “click” to silica. *Journal of Chromatography A* 2008,1191(1–2):78–82.
34. Shen AJ, Guo ZM, Cai XM, Xue XY, Liang XM. Preparation and chromatographic evaluation of a cysteine-bonded zwitterionic hydrophilic interaction liquid chromatography stationary phase. *Journal of Chromatography A* 2012,1228:175–182.
35. Gilar M, Olivova P, Daly AE, Gebler JC. Orthogonality of separation in two-dimensional liquid chromatography. *Analytical Chemistry* 2005,77(19):6426–6434.
36. Wang J, Wang C, Guo Z, Dong X, Xiao Y, Xue X, Zhang X, Liang X. A novel method for characterization and comparison of reversed-phase column selectivity. *Journal of Chromatography A* 2014,1361:153–161.
37. Liang Z, Li KY, Wang XL, Ke YX, Jin Y, Liang XM. Combination of off-line two-dimensional hydrophilic interaction liquid chromatography for polar fraction and two-dimensional hydrophilic interaction liquid chromatography x reversed-phase liquid chromatography for medium-polar fraction in a traditional Chinese medicine. *Journal of Chromatography A* 2012,1224:61–69.
38. Wang J, Guo Z, Shen A, Yu L, Xiao Y, Xue X, Zhang X, Liang X. Hydrophilic-subtraction model for the characterization and comparison of hydrophilic interaction liquid chromatography columns. *Journal of Chromatography A* 2015,1398:29–46.
39. Jiang W, Irgum K. Covalently bonded polymeric zwitterionic stationary phase for simultaneous separation of inorganic cations and anions. *Analytical Chemistry* 1999,71(2):333–344.
40. Wang CR, Guo ZM, Long Z, Zhang XL, Liang XM. Overloading study of basic compounds with a positively charged C18 column in liquid chromatography. *Journal of Chromatography A* 2013,1281:60–66.
41. Liu AH, Guo H, Ye M, Lin YH, Sun HH, Xu M, Guo DA. Detection, characterization and identification of phenolic acids in Danshen using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. *Journal of Chromatography A* 2007,1161(1–2):170–182.
42. Yang M, Liu AH, Guan SH, Sun JH, Xu M, Guo D. Characterization of tanshinones in the roots of Salvia miltiorrhiza (Dan-shen) by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2006,20(8):1266–1280.

Summary

This supporting information file includes additional information and results as described in the article.

Table S1 The components were found and unidentified in Danshen.

No.	Fraction	RT (min)	m/z	No.	Fraction	RT (min)	m/z	No.	Fraction	RT (min)	m/z	No.	Fraction	RT (min)	m/z
1	SI-F1	12.766	317.1060	26	SI-F4	3.91	231.0339	51	SI-F7	17.461	719.1655	76	SI-F11	2.51	111.0084
2	SI-F1	17.103	315.1280	27	SI-F4	5.313	181.0509	52	SI-F8	4.386	299.0785	77	SI-F11	2.832	277.0038
3	SI-F1	20.416	347.1870	28	SI-F4	5.351	228.9820	53	SI-F8	4.774	311.0788	78	SI-F11	3.512	439.0556
4	SI-F1	21.502	501.0983	29	SI-F4	9.333	499.1963	54	SI-F8	10.378	523.2444	79	SI-F11	4.186	343.1042
5	SI-F1	21.622	373.0880	30	SI-F4	9.517	325.0568	55	SI-F8	15.28	723.5070	80	SI-F11	10.18	375.0743
6	SI-F1	24.38	359.0592	31	SI-F4	10.038	259.0649	56	SI-F8	16.569	187.0981	81	SI-F11	10.186	151.0402
7	SI-F1	25.224	373.0390	32	SI-F4	10.456	371.1000	57	SI-F8	19.11	835.4691	82	SI-F11	16.094	495.0957
8	SI-F1	25.284	299.1316	33	SI-F4	11.597	385.1162	58	SI-F8	19.112	819.4461	83	SI-F11	21.619	701.1557
9	SI-F1	25.378	297.1168	34	SI-F4	13.585	403.1631	59	SI-F8	23.828	715.1697	84	SI-F11	26.914	209.0850
10	SI-F1	25.827	341.1042	35	SI-F4	15.254	581.1909	60	SI-F8	25.013	671.3301	85	SI-F12	18.114	295.0625
11	SI-F1	26.249	379.1228	36	SI-F4	15.256	535.1850	61	SI-F8	25.798	711.3279	86	SI-F14	19.666	519.0984
12	SI-F1	26.368	329.1398	37	SI-F4	15.685	567.2116	62	SI-F8	29.672	193.0877	87	SI-F15	3.822	197.0454
13	SI-F1	27.665	365.1806	38	SI-F4	19.096	845.4943	63	SI-F9	6.627	507.1754	88	SI-F15	15.575	715.1318
14	SI-F1	27.703	375.1860	39	SI-F4	20.132	405.0666	64	SI-F9	16.152	535.1488	89	SI-F15	18.626	731.1294
15	SI-F1	28.121	247.0982	40	SI-F4	20.556	343.0830	65	SI-F9	20.372	521.1132	90	SI-F15	23.537	745.1426
16	SI-F1	28.827	309.0770	41	SI-F4	22.037	493.2446	66	SI-F9	20.693	477.1216	91	SII-F1	2.836	327.1234
17	SI-F4	2.462	147.0300	42	SI-F4	27.891	331.1927	67	SI-F9	23.897	685.1587	92	SII-F1	3.953	327.1227
18	SI-F4	2.513	273.0551	43	SI-F5	3.595	229.0354	68	SI-F9	25.428	727.3954	93	SII-F1	5.732	387.1680
19	SI-F4	3.592	515.0004	44	SI-F5	9.341	509.2254	69	SI-F9	26.429	705.1769	94	SII-F1	5.737	409.1526
20	SI-F4	3.593	481.0631	45	SI-F5	9.531	193.0498	70	SI-F10	2.516	391.0333	95	SII-F1	5.792	285.1435
21	SI-F4	3.594	141.0559	46	SI-F5	15.28	571.1620	71	SI-F10	2.518	191.0199	96	SII-F1	5.803	329.1354
22	SI-F4	3.594	113.0608	47	SI-F5	17.338	577.1935	72	SI-F10	2.567	233.0132	97	SII-F1	6.364	267.0989
23	SI-F4	3.595	185.0460	48	SI-F5	17.493	161.0240	73	SI-F10	11.658	269.0826	98	SII-F1	7.094	393.2863
24	SI-F4	3.598	213.0231	49	SI-F5	19.305	665.2483	74	SI-F10	11.674	439.0356	99	SII-F1	7.998	415.2112
25	SI-F4	3.601	443.0680	50	SI-F5	26.766	209.0856	75	SI-F11	2.509	206.9966	100	SII-F1	17.242	149.0232
101	SII-F1	27.551	331.2845	126	SII-F3	8.68	309.1116	151	SII-F5	9.758	609.1884	176	SII-F9	9.053	643.2304
102	SII-F1	27.558	353.2656	127	SII-F3	8.688	449.2174	152	SII-F5	10.502	583.2093	177	SII-F9	9.069	333.1095
103	SII-F2	2.828	283.1332	128	SII-F3	9.116	335.1252	153	SII-F5	10.509	281.1171	178	SII-F9	9.894	319.1301
104	SII-F2	3.862	345.1326	129	SII-F3	10.571	303.0988	154	SII-F6	9.557	579.2227	179	SII-F9	27.231	585.5326
105	SII-F2	5.12	319.0939	130	SII-F3	10.694	307.0962	155	SII-F6	12.113	269.1172	180	SII-F10	3.516	353.1359
106	SII-F2	5.703	527.2761	131	SII-F3	13.702	519.2922	156	SII-F6	12.117	559.2093	181	SII-F10	3.52	683.2826
107	SII-F2	5.739	723.3079	132	SII-F4	5.731	105.0569	157	SII-F6	12.121	291.0988	182	SII-F10	5.388	647.2614
108	SII-F2	5.752	315.1446	133	SII-F4	5.747	721.2912	158	SII-F6	17.222	579.2924	183	SII-F10	10.898	299.2005
109	SII-F2	5.869	719.2815	134	SII-F4	6.182	293.1167	159	SII-F7	2.813	647.1889	184	SII-F10	10.9	619.3757
110	SII-F2	5.918	451.2337	135	SII-F4	7.109	773.4934	160	SII-F7	5.74	795.3311	185	SII-F10	11.372	611.2396
111	SII-F2	5.927	333.1098	136	SII-F4	7.287	319.0941	161	SII-F7	11.373	487.3420	186	SII-F10	11.384	317.1147
112	SII-F2	6.416	303.0630	137	SII-F4	7.323	659.2249	162	SII-F7	13.823	671.2615	187	SII-F10	16.208	293.1172
113	SII-F2	7.114	327.1226	138	SII-F4	7.329	363.1199	163	SII-F7	13.841	623.2038	188	SII-F10	16.214	607.2091

Table S1 (Continued)

No.	Fraction	RT (min)	m/z	No.	Fraction	RT (min)	m/z	No.	Fraction	RT (min)	m/z	No.	Fraction	RT (min)	m/z
114	SII-F2	7.668	297.1124	139	SII-F4	7.377	341.1380	164	SII-F7	13.876	575.1463	189	SII-F10	16.219	315.0992
115	SII-F2	8.004	555.3171	140	SII-F4	8.038	777.3247	165	SII-F7	13.881	299.0678	190	SII-F10	16.481	595.3030
116	SII-F2	9.462	577.2983	141	SII-F4	8.309	627.2929	166	SII-F7	15.689	301.0837	191	SII-F10	16.481	287.1641
117	SII-F2	17.628	301.1407	142	SII-F4	8.311	325.1408	167	SII-F8	2.82	313.1075	192	SII-F11	2.832	711.2411
118	SII-F3	3.225	365.1000	143	SII-F4	8.312	285.1486	168	SII-F8	8.02	437.1937	193	SII-F11	9.53	675.2935
119	SII-F3	3.232	325.1072	144	SII-F4	9.099	313.1434	169	SII-F8	10.137	297.1488	194	SII-F11	9.535	327.1591
120	SII-F3	4.269	355.1174	145	SII-F4	9.739	639.1979	170	SII-F8	12.262	441.3364	195	SII-F11	13.332	615.2716
121	SII-F3	5.092	307.0963	146	SII-F4	11.083	361.1044	171	SII-F8	15.608	579.1779	196	SII-F11	13.34	319.1306
122	SII-F3	5.53	305.1280	147	SII-F4	11.084	699.2200	172	SII-F8	15.66	261.0910	197	SII-F12	10.418	315.1589
123	SII-F3	6.339	643.2295	148	SII-F4	11.088	479.2290	173	SII-F8	17.474	293.1539	198	SII-F12	18.552	611.2406
124	SII-F3	7.382	703.2515	149	SII-F5	3.592	318.3003	174	SII-F9	6.424	451.3211	199	SII-F13	18.563	317.1149
125	SII-F3	7.405	481.2445	150	SII-F5	9.559	301.0837	175	SII-F9	7.066	471.3473	200	SII-F14	13.304	437.2546