



An on-line analytical approach for detecting haptens in Shuang-huang-lian powder injection



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ABSTRACT

Shuang-huang-lian powder injection (SHLPI) is a traditional Chinese medicine injection (TCMI) frequently used in the clinical treatment of faucitis, bronchitis, and other viral and bacterial infections of upper respiratory tract. However, its allergenic reactions, being the main adverse effects (AEs) of SHLPI, have been a serious problem of its clinical safety. This problem has not been solved due to short of methods for detecting haptens in complex TCMI. In this study, an on-line high-performance liquid chromatography-diode-array detector-mass spectrometry combined with bovine serum albumin-fluorescence detector (HPLC-DAD-MS-BSA-FLD) system was established for the first time, validated and applied for identification of haptens in SHLPI. Fourteen of 35 identified compounds showed BSA binding activity, and they were six flavonoids, six caffeoylquinic acids (CQAs), and two phenylethanoid glycosides. The structure-activity relationships of 10 active components were studied, and their ability of sensitization together with that of two CQAs were further verified by ELISA assay. It was found that 10 compounds had sensitization, and flavonoids showed stronger sensitizability than CQAs while the diCQAs were slightly stronger than caffeoylquinic acids. The system was validated using 3-CQA as a positive control, and was proved to have good reproducibility, stability, precision (RSD < 0.1%) and linearity ($R^2 > 0.9993$). This online system is fast, sensitive and efficient for screening haptens in traditional Chinese medicine injection (TCMI), provides a new approach to reveal the chemical basis of haptens in TCMI.

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1. Introduction

Traditional Chinese medicine injections (TCMIs) as a new formation of TCM is widely applied in China. However, the associated allergic reactions caused by haptenic components have become a big concern, and consequently impeded further applications of TCMI [1]. Haptens are small molecules that can induce allergic reactions only after binding to proteins but cannot elicit immune responses alone [2]. Among these TCMI, shuang-huang-lian powder injection (SHLPI) has a bad fame because of its allergic reactions (side effects) [3,4]. SHLPI is composed of the extract of three herbs: *Radix Scutellariae*, *Flos Lonicerae* and *Fructus Forsythiae* [5], and is an important TCMI in Chinese Pharmacopoeia. Currently little

is known about its haptens, although various types of chemical constituents including phenolic acids, flavonoids, phenylethanoid glycosides and iridoid glycosides have been reported previously [6–8].

Immunoglobulin E (IgE) mediated anaphylactic reactions are known as allergic reactions, leading to histamine release, acute allergic reactions and inflammatory responses [9]. IgE mediated drug hypersensitivity is a major type of hapten induced allergy [10]. Because of this, chemical haptens in compound drugs have become a serious public concern all over the world [11]. Hapten-induced allergic symptoms vary from slight abdominal discomforts, skin disorders and acute respiratory reactions to life-threatening anaphylactic shock reactions [12,13]. Rapid identification and verification of haptens in drugs have become an urgent issue [14,15].

Some methods have been developed to identify haptens, including commonly used technologies such as gold immunity chromatographic assay, immunosensor [16], immuno chromatographic test [17], immuno-chip [18] and enzyme linked immunosorbent assay (ELISA). Take ELISA for example, this technique uses specific binding of antigen-antibody in the immune response for qualita-

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tive and quantitative analysis. The ELISA method has been used as a diagnostic tool in medicine and plant pathology, as well as a quality-control assay in industry. It also has been widely used in clinical medicine, biology and analytical chemistry. ELISA has the advantages of high sensitivity, strong specificity and good accuracy. However, the ELISA method can be only used to test pure compounds, and it is time-consuming, complicated. In addition, the purification process of compounds may lose minor active compounds. Therefore, the ELISA method is more suitable for verification than for screening purpose. Recently, Han et al. have reported a two-dimensional analytical method for identifying allergenic components in Shuang-huang-lian injection [19], however only identified one active compound indicating the inefficiency of this technique. Thus, developing sensitive and rapid analytical methods for high-throughput screening of potential allergens are of great necessity.

Being one of the main transport proteins and the most abundant protein in human blood, HSA is a key protein in the allergic reactions triggered by small molecules. Bovine serum albumin (BSA) is composed of 583 amino acids. With only 2 amino acid residues different from human serum albumin (HSA), the structure of BSA is extremely similar to HSA. Moreover, BSA can be easily obtained without arousing ethic problems, making it a commonly used protein in the screening of haptens in TCMI. BSA has three homologous domains (I–III), and its two sub-domains IIA and IIIA are the primary binding sites of compounds/haptens [20], highlighting potential applications of BSA as a screening probe for haptens. In addition, BSA has two tryptophan residues which give intrinsic fluorescence under excitation wavelength [21]. However, after binding to compounds, the dynamic or static fluorescence of BSA can be quenched and gave a decreased fluorescent signal [22]. Based on this mechanism, several hapten compounds [23,24] such as 3-caffeoylquinic acid (3-CQA) have been successfully identified in previous studies, indicating that BSA can be used to detect potential hapten compounds.

In this study, we established an efficient and sensitive method using high-performance liquid chromatography-diode-array detector-electrospray ionization-ion-trap-time-of-flight-mass spectrometry combined with bovine serum albumin-fluorescence detector (HPLC-DAD-ESI-IT-TOF-MS-BSA-FLD) system for the first time, and had successfully identified potential haptens in SHLPI. These potential haptens were further validated by ELISA, and the results suggested that our developed technique is a promising high-throughput screening approach for haptens in TCMI.

2. Experimental

2.1. Materials and reagents

SHLPI (Lot. No. 1306505) was purchased from the Second Chinese Medicine Factory, Harbin Pharmaceutical Group Co., Ltd (Heilongjiang, China). Standard compounds of baicalin (**32**), 3-CQA (**8**), aschrysin-7-O-glucuronide (**33**), oroxylin A-7-O-glucuronide (**34**), scutellarin (**22**), forsythoside A (**24**), 4,5-dicaffeoylquinic acid (4,5-diCQA) (**29**) and baicalin (**31**) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 5-CQA (**6**), 4-CQA (**10**), acteoside (**25**), 3,4-diCQA (**26**), 1,3-diCQA (**27**) and baicalein (**35**) were obtained from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China). The purity of each compound was above 98.0%.

HPLC grade acetonitrile and methanol (purity > 99.9%) were purchased from Fisher Scientific Products (Fair Lawn, NJ, USA). Formic acid (purity > 99.0%) was purchased from Dikma Technologies Inc. (Beijing, China). Deionized water was purified by Milli-Q water

purification system (Millipore, Bedford, MA, USA) with a 0.22 μm membrane.

Freund's reagent, 0.15 mol·L⁻¹ phosphate buffer solution (pH 7.4) and HRP-SPA were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, USA). Tween-20 and Na₂HPO₃ were purchased from Tianjin Kemiou chemical reagent Co., Ltd (Tianjin, China). Na₂CO₃ and NaHCO₃ were purchased from Tianjin Tianli chemical reagent Co., Ltd (Tianjin, China). OPD was purchased from Shanghai Kefeng industrial Co., Ltd (Shanghai, China). The purity of each chemical reagents was above 99.0%.

2.2. Sample preparation

The stock solutions of individual standard compounds was prepared by accurately weighting the required amounts into the volumetric flask and dissolved in 50% methanol-water, the stock solution was further diluted to obtain appropriate concentrations as working solutions. 10 mg of SHLPI sample was accurately weighed and dissolved in 10 mL 50% methanol-water (*v/v*) solution. All solutions were filtered through a 0.22 μm membrane, and stored at 4 °C prior to use.

BSA was purchased from Scien B&C (BR grade, Beijing, China). Tris (Lot. No. 527K073) were purchased from Solarbio (BR grade, Beijing, China). Stock solution of BSA (1.0 $\times 10^{-6}$ mol·L⁻¹) was prepared by dissolving BSA in 0.05 mol·L⁻¹ Tris-HCl buffer (pH 7.4) and then stored at 4 °C. The 0.05 mol·L⁻¹ Tris-HCl buffer (0.20 mol·L⁻¹, pH 7.4) was employed to increase the solubility of BSA and maintain the pH of reaction system.

2.3. Instruments and design of the on-line HPLC-DAD-IT-TOF-MS-BSA-FLD detection

Instruments and materials used for ELISA experiment were as follows: electronic balance (Sartorius Industrial Weighing Equipment Co., Ltd., Beijing, China), vortex mixer, HH-S6 water bath (Beijing Kewei Yongxing instrument Co., Ltd, Beijing, China), centrifuge, 96-well plates, rabbit IgE ELISA kit (Shanghai Guangrui biotech Co., Ltd, Shanghai, China).

The on-line screening system (Fig. 1) is composed of two major sections: HPLC-DAD-MS section and BSA-FLD section. The first section was composed of two LC-20AD pumps, a CTO-20A column oven, a DGU-20A3 degasser, an SIL-20AC autosampler, an SPD-M20AD detector and an ESI-IT-TOF mass spectrometer (Shimadzu LC-MS-IT-TOF, Kyoto, Japan). The BSA-FLD section was composed of an LC-20AD pump, a reaction coil, a CTO-20A column oven and an RF-20AXS fluorescence detector (Shimadzu, Kyoto, Japan).

Sample solution was injected by autosampler and separated by the HPLC column, the fingerprint was recorded by DAD detector. Then eluents was divided into two streams by an adjustable T-piece, one of which (0.5 mL·min⁻¹) was introduced into the MS detector to obtain the MS data for structural identification. The other stream (0.5 mL·min⁻¹) flowed into the reaction coil. Once an active compound reacted with BSA which was pumped at a flow rate 0.5 mL·min⁻¹, the decrease of the fluorescence signal can be detected by FLD. After the complete fingerprint and decreased fluorescence signal were recorded by DAD and FLD, respectively, a figure containing liquid chromatogram and fluorescence spectra was obtained. All data were processed by Shimadzu LCMS solution software (ver.3.8).

2.4. Conditions of the on-line HPLC-DAD-IT-TOF-MS-BSA-FLD system

2.4.1. HPLC conditions

The chromatographic analysis was performed on a Shimadzu HPLC system (Kyoto, Japan). The separation was carried out on

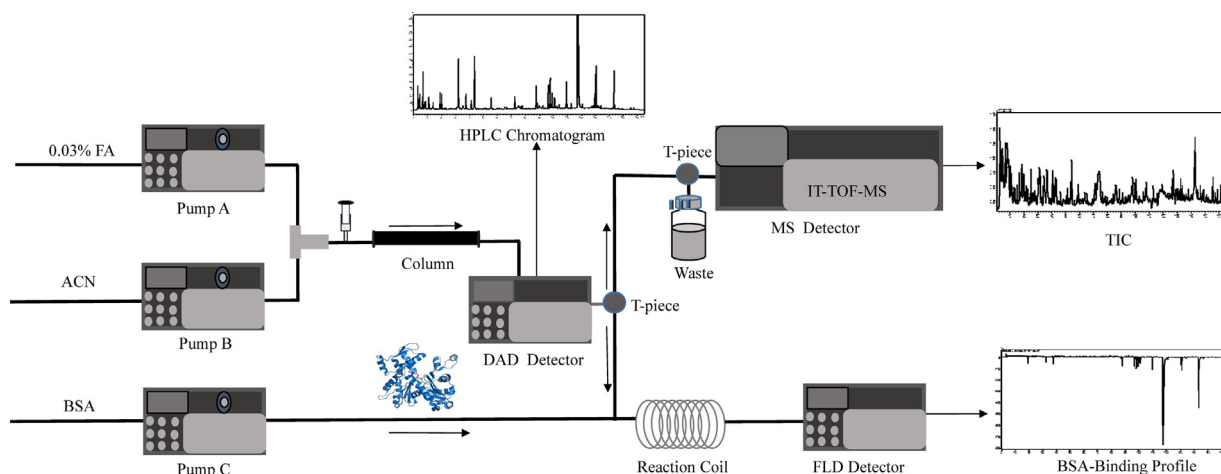


Fig 1. The on-line HPLC-DAD-ESI-IT-TOF-MS-BSA-FLD system for screening of BSA-binding compounds.

a Kromasil column (250 mm × 4.6 mm, 5 μm, 100 Å) (Kromasil, Bohus, Sweden) maintained at a column temperature of 40 °C. The mobile phase was composed of water containing 0.03% formic acid (A) and acetonitrile (B) at a flow rate of 1.0 mL·min⁻¹ with the following gradient: 3–5% B, 0–15 min; 5–10% B, 15–60 min; 10–15% B, 60–90 min; 15–22% B, 90–120 min; 22–40% B, 120–150 min; 40–70% B, 150–165 min. To comprehensively reflect the total components in SHLPI, the UV spectra were recorded in the range of 200–400 nm. In this range of wavelength, the UV signal of all components were recorded at their maximum absorption wavelength.

2.4.2. ESI-IT-TOF-MS conditions

The optimized MS conditions were as follows: negative ion mode was used with a scan range of m/z 100–800. The curved desolvation line (CDL) temperature and block heater temperature were both set at 200 °C. The capillary voltage, CDL voltage and detector voltage were fixed at 4.5 kV, 10 kV and 1.75 kV, respectively. The flow rate of nitrogen as nebulizer gas was 1.5 L·min⁻¹. The collision energy was adjusted to 70% in the HPLC–MS analysis. Data were acquired and processed by LC/MS solution software (version 3, Shimadzu, Kyoto, Japan).

2.4.3. Conditions of FLD system for BSA-binding activity detection

Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) equipped with 1.0 cm quartz cells. The excitation and emission spectra were set at a scan range of 250–340 nm and 300–500 nm, respectively. The slit widths of excitation and emission slits were both set at 5 nm. The fluorescence of buffer solution was subtracted for fluorescence background correction.

The BSA solution (4.0×10^{-7} mol·L⁻¹) maintained at 0 °C was pumped into the reaction coil (2.0 m, I.D. 0.25 mm) at a constant flow of 0.5 mL·min⁻¹. The reaction temperature was set at 27 °C. The excitation and emission wavelengths of FLD were set at 292 nm and 347 nm, respectively.

2.5. Enzyme-linked immunosorbent assay

2.5.1. Preparation of un-sensitized serum

All animal experiments were performed in accordance with the animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Shaanxi University of Chinese Medicine (Xi'an, China). The un-sensitized serum was prepared from 10 mL blood sample drawn from the ear center arteries of New Zealand rabbits [25].

2.5.2. Preparation of immune holoantigen

Accurately weighed SHLPI and 12 compounds were dissolved in saline to produce 4 mg/mL test solutions, respectively. 0.5 mL of the test solution was mixed with 0.5 mL un-sensitized serum, then the mixed solution was incubated 2 h at 37 °C. An equal volume of Freund's adjuvant was then added into the above solution, mixed well by vortex to produce immune holoantigen [26].

2.5.3. Preparation of specific antibody

The immune holoantigen for first immunization was injected subcutaneously and multi-pointly on both sides of the back along the rabbit spine with 0.2 mL per point, 10 points in total. Ten days after the first immunization, the immune holoantigen was used to strengthen immunization at the same dose as the first immunization. A strengthened immunization was carried out once every week. From the fourth immunization, blood sample drawn from the rabbit ear artery was used for titer of the antibody on the 7th day after the immunization. The non-anticoagulant whole blood was taken from the common carotid artery, incubated at 37 °C for 2 h, and stored overnight at 4 °C. The whole blood was centrifuged for 15 min at 3000 r/min, then the upper antiserum was collected and stored at –20 °C after its titer reached the desired value.

2.5.4. Determination of antiserum titer by ELISA

After diluting the antigen with 0.05 M sodium carbonate buffer (pH 9.6) to a proper concentration, the 96-well plate was coated with diluted antigen at 110 μL per well. The plate was stored in a 4 °C humid chamber overnight. After washing the 96-well Plate 3 × 4 min with PBST, 130 μL of 2% BSA was added to each well, and the plate was sealed and stored overnight. The sealing fluid was abandoned and the 96-well plate was washed with PBST, then 100 μL antiserum solutions diluted with 0.15 M PBS (pH 7.4) in a ratio of 1: 1, 1: 2, 1: 4, 1: 8, 1: 16, 1: 32, 1: 64 and 1: 128 were added into the 96-well plate, respectively. The plate was incubated at 37 °C for 2 h, washed with PBST, added 100 μL HRP-SPA, incubated for 1 h at 37 °C, and washed with PBST. 100 μL of substrate solution was added, mixed, and colored for 15 min at room temperature protected from light. The reaction was stopped by adding 50 μL of H₂SO₄, and the absorbance value (OD value) was recorded at wavelengths of 450 nm and 630 nm using a microplate reader.

The wells containing un-sensitized serum and without serum were used as negative and blank controls, respectively. Each sample was tested 5 times. OD values two times higher than or equal to the OD value of the negative control wells were considered positive. The data were expressed as “mean ± standard deviation”, and SPSS 20.0 was used for statistics analysis.

2.5.5. Determination of antibody specificity by indirect competitive ELISA (ciELISA)

2.5.5.1. Determination of optimal concentration of coated antigen.

The immune holoantigen was immunized according to method 2.2, and diluted with physiological saline to the concentrations of 4.00, 2.00, 1.00, 0.50 and 0.25 mg/mL. 110 μ L of diluted sample was used to coat the ELISA plate, which was washed with PBST for 3×4 min. 130 μ L of 2% BSA was added to each well, and the plate was blocked overnight. The sealing fluid was abandoned, and the 96-well plate was washed. 100 μ L of antiserum diluted in a ratio of 1:64 was added into the plate and incubated at 37 °C for 2 h. After the plate was washed three times, 100 μ L of enzyme-labeled antibody HRP-SPA, which was diluted with PBS in a ratio of 1:200, was added. Then the plate was incubated at 37 °C for 1 h. After the plate was washed, 100 μ L of substrate solution was added, mixed, and colored for 15 min at room temperature protected from light. The reaction was stopped by adding 50 μ L of H_2SO_4 , and the OD values were recorded at wavelengths of 450 nm and 630 nm using a microplate reader. The OD value of the blank control was less than 0.1, and the optimal coating concentration is the standard antigen dilution with an OD value of about 1.0.

2.5.5.2. Method of ciELISA assay. The ELISA plate was coated with the coating antigen at optimal concentration. After washing the plate, compound stock solution was diluted with 50 μ L of physiological saline to a series concentrations of 0.25, 0.50, 1.00, 2.00, 4.00 mg/mL, and mixed with antisera (1:1), and added to the plate, respectively. Then operations were done according to method 2.5.5.1. The OD value of each well was measured by a microplate reader at 450 nm and 630 nm. Wells containing diluent were used as blank controls, and wells without sample solution were used as controls. The inhibitory activity was calculated using the following equation.

$$\text{Inhibition ratio}(\%) = \frac{\text{OD}_0 - \text{OD}}{(\text{OD}_0 - \text{OD}_{\min})} \times 100\%$$

Where OD is the absorbance of the test solution, OD_0 is the absorbance of control well, $\text{OD}_{\min}$ is the absorbance of the blank control well.

The inhibitory rates were plotted on the ordinate, and the logarithm of the concentrations of the 12 small molecular haptens and SHLPI were plotted on the abscissa to give the suppression curves. The regression equation was established, and the correlation coefficients (R^2), the half-inhibition concentration of sample solution (IC_{50}) and the limit of detection (IC_{10}) were calculated by regression analysis.

2.6. Statistical analysis

The values in this study are reported as the means $\pm$ SD (or SE). The significances of the differences among treatments were estimated by unpaired, two-tailed Student's *t*-test with $p < 0.05$ considered as being statistically significant. All statistical analyses were performed using SPSS 20.0.

3. Results and discussion

3.1. Optimization of the on-line BSA-binding analysis system

3.1.1. Determination of the detection wavelength of BSA

As shown in Fig. 2A, the maximum excitation wavelength was 292 nm and maximum emission wavelength was 347 nm. Under such wavelengths, different amounts of 3-CQA were added to BSA solution (1.0×10^{-4} mol·L $^{-1}$). The fluorescence intensity of BSA decreases gradually as the concentration of 3-CQA increases from 25 to 200 μ g/mL (Fig. 2B). The results suggested that 3-CQA used as

a positive control can bind to BSA, and gradually saturate the binding sites of BSA.

3.1.2. Optimization of the on-line hapten-BSA binding system

Factors, including the concentration of BSA, the pH value, the concentration of NaCl, the temperature of reaction coil, and organic solvents in mobile phase, may affect the fluorescence of BSA-binding activities. Thus, these variables were investigated, each experiment was carried out in triplicate with RSDs < 4.8%.

3.1.2.1. Effects of the BSA concentrations. Firstly, various concentrations of BSA (1.000×10^{-8} , 1.000×10^{-7} , 2.000×10^{-7} , 4.000×10^{-7} , 6.000×10^{-7} , 8.000×10^{-7} and 1.000×10^{-6} mol·L $^{-1}$) were evaluated. As shown in Fig. 2C, the fluorescence intensity was gradually enhanced with increasing concentration of BSA. However, viscosity of BSA induced an abnormal pump pressure at concentrations higher than 4.000×10^{-7} mol·L $^{-1}$. Thus, BSA at a concentration of 4.000×10^{-7} mol·L $^{-1}$ was chosen because of favorable fluorescence intensity and smooth baseline for the detection.

3.1.2.2. Effects of the pH values. Various pH values affect the structural behavior of the BSA, which consequently influence the fluorescence intensity [27,28]. As shown in Fig. 2D (pH 5.3–8.7), when pH is higher than 8.0 or lower than 6.1, the fluorescence intensity drops dramatically. Thus, pH 7.4 producing highest fluorescence was chosen to screen active compounds.

3.1.2.3. Effects of the NaCl concentrations. Appropriate ionic environment is favorable for protein stability [29]. The influence of NaCl concentration was assessed by various concentrations (0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 mol·L $^{-1}$). As shown in Fig. 2E, concentration at 0.15 mol·L $^{-1}$ yields a strong fluorescence intensity and was therefore chosen to adjust the ionic strength of BSA.

3.1.2.4. Effects of the reaction temperatures. Moreover, temperatures of the reaction coil can also affect the fluorescence intensity [30]. According to Fig. 2F, the fluorescence intensity is only slightly affected by the reaction temperatures, although lower temperature does favor fluorescence intensity to some extent. Finally, room temperature (27 °C) was selected as the optimal reaction temperature.

3.1.2.5. Effects of the organic solvents. Methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were commonly used organic reagents in HPLC. According to Fig. 2G–I, the fluorescence intensity was significantly reduced by increased concentrations of MeOH, ACN and FA. 100% MeOH inhibited the 39% of the highest fluorescence while 70% ACN inhibited 23.8% fluorescence. Formic acid is critical for the fluorescence intensity (Fig. 2I) as 0.1% formic acid inhibited more than 35% of the highest fluorescence. Thus, the ratio of organic phase and FA should be carefully controlled, on the condition that the HPLC peaks were well separated.

3.2. Validation of the on-line method

The interaction mechanism of 3-CQA and BSA was studied previously [31]. In this study, 3-CQA was applied as a positive control to verify the established system. The HPLC chromatogram and activity profile of 3-CQA are shown in Fig. 3. The method was validated in terms of precision, stability, reproducibility, linearity, limit of detection (LOD) and limit of quantification (LOQ).

3.2.1. Precision

The precision of the on-line system was evaluated by consecutive injections ($n=6$) of 3-CQA solution. The relative standard

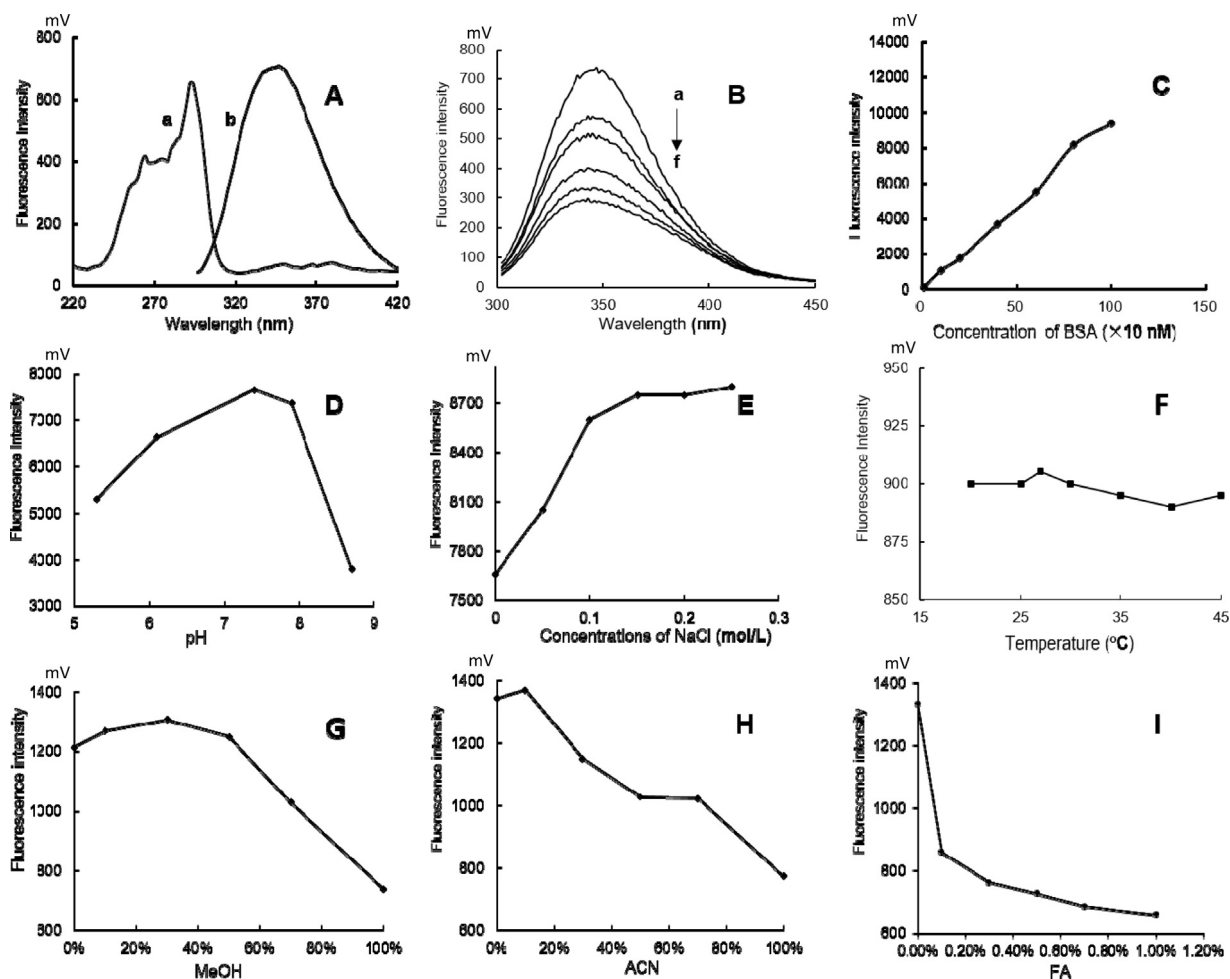


Fig. 2. Determination of excitation wavelengths at 292 nm (a) and emission at 347 nm (b) (A); the fluorescence spectra of BSA (a) and 3-caffeoylquinic acid-bound BSA complex (b–f, concentration of 3-CQA: 25, 50, 100, 150, 200 µg/mL) (B); optimization of reaction conditions: concentration of BSA (C), pH value (D), concentration of NaCl (E), reaction temperature (F), and different solvents of mobile phase (G, H and I) ($n=3$, the RSDs of all data are less than 2%).

deviations (RSDs) of HPLC peak areas and BSA-binding peak areas were 0.83% and 0.40%, respectively.

3.2.2. Stability

The stability of the on-line system was performed by injecting the same 3-CQA solution at 0 h, 3 h, 6 h, 9 h, 12 h, 15 h ($n=6$). The RSDs of HPLC peak area and BSA-binding peak areas were 0.71% and 0.37%, respectively.

3.2.3. Reproducibility

Reproducibility was evaluated by injecting the same amount of six independently prepared samples. The RSDs of HPLC peak area and BSA-binding peak areas were 0.88% and 0.39%, respectively. All RSD values were less than 1.00%, suggesting that the established on-line system was reliable for the analysis of active compounds.

3.2.4. Linearity, LOD and LOQ

The relationship between 3-CQA and fluorescence intensity was established by plotting the HPLC peak area (y_1) and active peak area (y_2) versus the corresponding injection amount (x , nmol). The regression equations were as follows: $y_1 = 1.65E+06x - 2.48E+05$, $R^2 = 0.9999$; $y_2 = -6.79E+05x + 8.05E+05$, $R^2 = 0.9935$. The result suggested good linearity of on-line system.

The LOD and LOQ were determined to be 0.780 nmol and 1.17 nmol when signal-to-noise (S/N) was 3 and 10 as criteria,

respectively. These data indicated that the established method was sensitive enough for on-line activity analysis.

3.3. Identification of major compounds in SHLPI

The optimized conditions of the on-line system were applied to identify and screen potential hapten components in SHLPI. The HPLC chromatogram, the total ion chromatogram (TIC) and the BSA-binding activity profile are shown in Fig. 4.

Thirty-five compounds were identified or characterized by their MS behavior (Table 1) from SHLPI, including 10 chlorogenic acids, 10 phenylethanoid glycosides, eight iridoid glycosides, six flavonoids and one lignan. Structures of these compounds are shown in Fig. 5.

3.3.1. Identification of 10 chlorogenic acids

Peak 2 provided two fragments at m/z 123.0481 and 108.0249 corresponding to the elimination of a CO_2 molecule and the subsequent loss of a methyl group. It was tentatively identified as vanillic acid [2,6]. Peak 3 was glycosylated form of peak 2. In its MS^2 , aglycone ion (m/z 167.0393) was achieved after the loss of glucosyl group (162 Da) [32]. Therefore, it was tentatively identified as vanillic acid-*O*-glucopyranoside [33]. Peak 5 was assigned as protocatechuic acid with an additional carboxyl group, which gave the $[M-H-CO_2]^-$ ion at m/z 109.0341 in MS^2 [34].

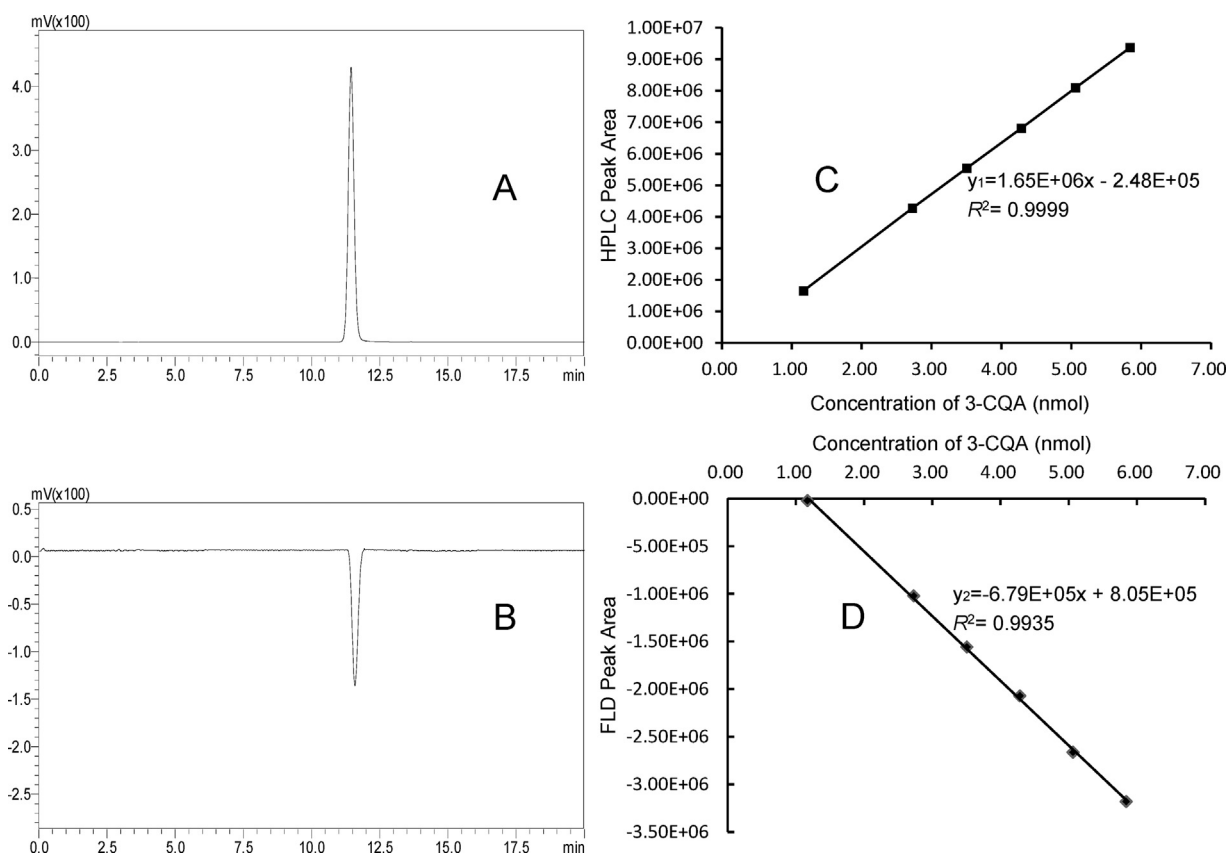


Fig. 3. HPLC chromatogram (A), BSA-binding activity profile (B) and the calibration curves of HPLC (C) and active profile (D) for 3-caffeoylquinic acid.

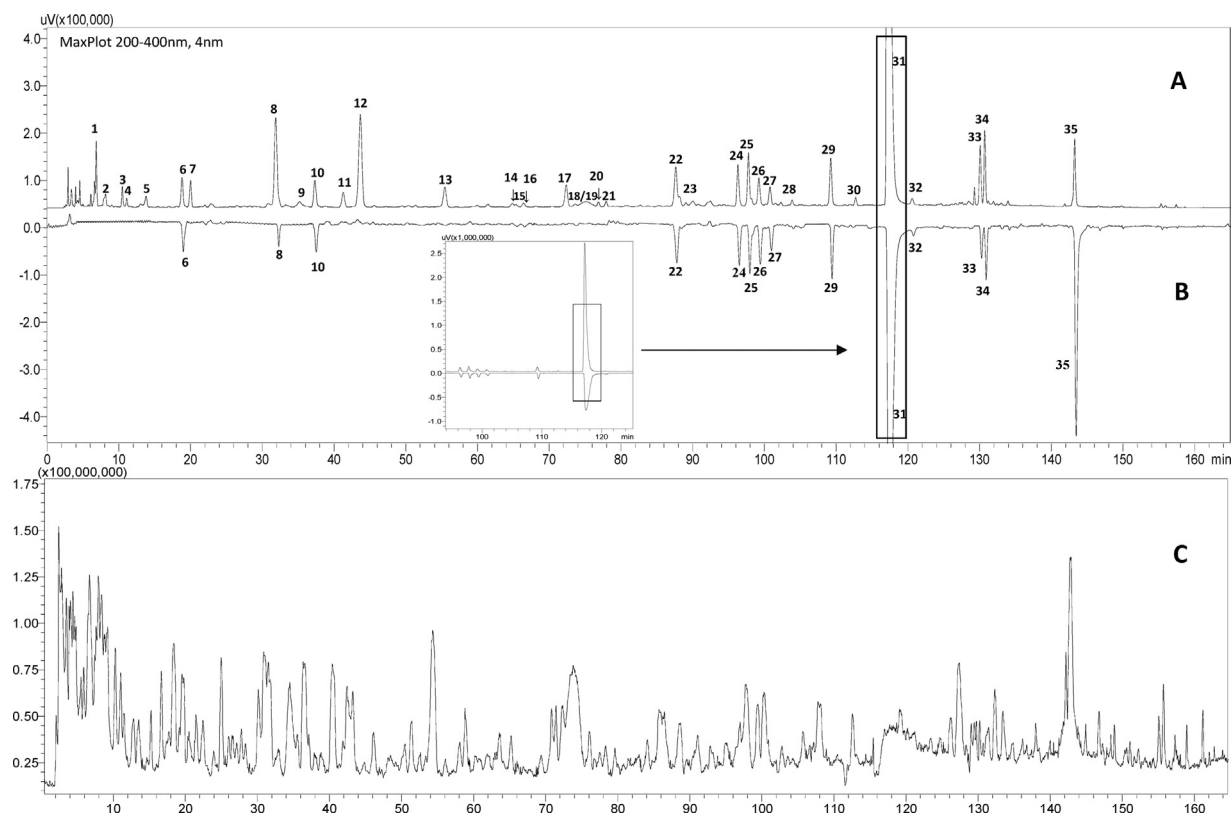


Fig. 4. HPLC fingerprint (A), the BSA-binding activity profile (B) and the total ion chromatogram (C) of Shuang-huang-lian powder injection.

Table 1
Identification of 35 major components in SHLPI by HPLC-DAD-ESI-IT-TOF-MS in negative-ion mode.

No.	t_R (min)	UV_{max}	[M-H] ⁻ (error in ppm)	Formula	(-) ESI-MS ⁿ		Identification
					MS ⁿ (Precursor)	Fragments ions (Intensity)	
1	6.367	231, 287	315.1064 (5.08)	C ₁₄ H ₂₀ O ₈	MS ² [315]:	153.0586 [M-H-Glc] ⁻ (37.19%) 135.0491 [M-H-Glc-H ₂ O] ⁻ (100%) 179.0570, 161.0516, 143.0400 [Glc] ⁻	Hydroxytyrosol -1-glucopyranoside
2	7.533	215, 291	167.0372 (13.17)	C ₈ H ₈ O ₄	MS ² [167]:	123.0481 [M-H-CO ₂] ⁻ 108.0249 [M-H-CO ₂ -CH ₃] ⁻	Vanillic acid
3	10.475	215, 289	329.0893 (4.56)	C ₁₄ H ₁₈ O ₉	MS ² [329]:	167.0393 [M-H-Glc] ⁻	Vanillic acid-O- glucopyranoside
4	10.592	225, 283	477.1634 (4.19)	C ₂₀ H ₃₀ O ₁₃	MS ³ [167]:	123.0513 [M-H-Glc-CO ₂] ⁻	Forsythoside D isomer
					MS ² [477]:	459.1479 [M-H-H ₂ O] ⁻	
					MS ³ [459]: MS ⁴ [193]:	253.0826 [^{0.2} X] ⁻ , 223.0600 [^{0.3} X] ⁻ 193.0495 [^{0.4} X] ⁻ 151.0418 [dehydrophenethanol] ⁻ 123.0514 [dehydrophenethanol-CO] ⁻	
5	13.697	217, 256, 292	153.0228 (19.61)	C ₇ H ₆ O ₄	MS ² [153]:	109.0341 [M-H-CO ₂] ⁻	Protocatechuic acid
6 ^a	18.433	217, 233, 324	353.0894 (4.53)	C ₁₆ H ₁₈ O ₉	MS ² [353]:	191.0635 [M-H-caffeoyl] ⁻ (100%)	5-CQA
					MS ³ [191]:	179.0376 [CA-H] ⁻ 173.0488 [M-H-caffeoyl-H ₂ O] ⁻ 127.0443 [M-H-caffeoyl-2H ₂ O-CO] ⁻	
7 ^a	19.33	234	375.1295 (1.07)	C ₁₆ H ₂₄ O ₁₀	MS ² [375]:	213.0764 [M-H-Glc] ⁻	Loganic acid
					MS ³ [213]:	169.0859 [M-H-Glc-CO ₂] ⁻ 151.0799 [M-H-Glc-CO ₂ -H ₂ O] ⁻ 125.0646 [^{1.4} F] ⁻ , 87.0085 [3-oxopropanic acid-H] ⁻	
8 ^a	31.03	218, 233, 326	353.0880 (0.57)	C ₁₆ H ₁₈ O ₉	MS ² [353]:	191.0655 [M-H-caffeoyl] ⁻ (100%)	3-CQA
					MS ³ [191]:	179.0368 [CA-H] ⁻ 173.0480 [M-H-caffeoyl-H ₂ O] ⁻ 127.0428 [M-H-caffeoyl-2H ₂ O-CO] ⁻	
9	35.412	234, 276, 324	367.1041 (3.27)	C ₁₇ H ₂₀ O ₉	MS ² [367]:	193.0529 [FA-H] ⁻ (100%)	3-FQA
					MS ³ [193]:	191.0542 [M-H-feruloyl] ⁻ 173.0453 [M-H-feruloyl-H ₂ O] ⁻ 134.0448 [FA-H-CH ₃ -CO ₂] ⁻	
10 ^a	36.737	218, 233, 326	353.0892 (3.97)	C ₁₆ H ₁₈ O ₉	MS ² [353]:	191.0591 [M-H-caffeoyl] ⁻	4-CQA
					MS ³ [191]:	179.0375 [CA-H] ⁻ 173.0515 [M-H-caffeoyl-H ₂ O] ⁻ (100%) 93.0377 [M-H-caffeoyl-3H ₂ O-CO ₂] ⁻	
11	40.788	233	389.1099 (3.85)	C ₁₆ H ₂₂ O ₁₁	MS ² [389]:	345.1178 [M-H-CO ₂] ⁻ 209.0458 [M-H-Glc-H ₂ O] ⁻ 183.0682 [M-H-Glc-H ₂ O-C ₂ H ₂] ⁻ 165.0577 [M-H-Glc-H ₂ O-CO ₂] ⁻ 121.0280 [M-H-Glc-H ₂ O-2CO ₂] ⁻	Secologanoside
12 ^b	42.435	246	419.1177 (-3.10)	C ₁₆ H ₂₂ O ₁₀	MS ² [419]:	373.1161 [M-H] ⁻	Swertiamarin
					MS ³ [373]:	193.0519 [M-H-Glc-H ₂ O] ⁻ 167.0724 [M-H-Glc-H ₂ O-C ₂ H ₂] ⁻ 149.0642 [M-H-Glc-H ₂ O-C ₂ H ₂ -H ₂ O] ⁻ 123.0408 [M-H-Glc-H ₂ O-C ₂ H ₂ -CO ₂] ⁻	
13 ^b	54.12	246	403.1264 (4.47)	C ₁₆ H ₂₂ O ₉	MS ² [403]:	357.1192 [M-H] ⁻	Sweroside
					MS ³ [357]: MS ⁴ [195]:	195.0668 [M-H-Glc] ⁻ 151.0769 [M-H-Glc-CO ₂] ⁻ 125.0286 [M-H-Glc-CO ₂ -C ₂ H ₂] ⁻	
14	63.553	233, 287, 325	639.1918 (-1.10)	C ₂₉ H ₃₆ O ₁₆	MS ² [639]:	621.1844 [M-H-H ₂ O] ⁻	R-suspensaside
					MS ³ [621]: MS ⁴ [469]:	487.1294 [M-H-H ₂ O-134] ⁻ 469.1342 [M-H-H ₂ O-134-H ₂ O] ⁻ 459.1484 [M-H-H ₂ O-caffeoyl] ⁻ 441.1384 [M-H-H ₂ O-caffeoyl-H ₂ O] ⁻ 245.0457, 233.0429, 203.0357 179.0253 [CA-H] ⁻ , 161.0243 [CA-H ₂ O] ⁻	
15	64.111	233, 288, 325	639.1960 (4.54)	C ₂₉ H ₃₆ O ₁₆	MS ² [639]:	621.1829 [M-H-H ₂ O] ⁻	Isocampneoside II
					MS ³ [621]: MS ⁴ [469]	487.1449 [M-H-H ₂ O-134] ⁻ 469.1334 [M-H-H ₂ O-134-H ₂ O] ⁻ 459.1479 [M-H-H ₂ O-caffeoyl] ⁻ 441.1388 [M-H-H ₂ O-caffeoyl-H ₂ O] ⁻ 245.0457, 233.0429, 203.0370 179.0365 [CA-H] ⁻ , 161.0271 [CA-H ₂ O] ⁻	

Table 1 (Continued)

No.	t_R (min)	UV_{max}	[M-H] [−] (error in ppm)	Formula	(-) ESI-MS ^a		Identification
					MS ^b (Precursor)	Fragments ions (Intensity)	
16	65.131	232, 280, 327	639.1933 (0.31)	C ₂₉ H ₃₆ O ₁₆	MS ² [639]: MS ³ [621]: MS ⁴ [469]:	621.1846 [M-H-H ₂ O] [−] 487.1413 [M-H-H ₂ O-134] [−] 469.1317 [M-H-H ₂ O-134-H ₂ O] [−] 459.1485 [M-H-H ₂ O-caffeoyl] [−] 441.1381 [M-H-H ₂ O-caffeoyl-H ₂ O] [−] 245.0476, 233.0487, 203.0362 179.0360 [CA-H] [−] , 161.0270 [CA-H ₂ O] [−]	S-suspensaside
17	71.097	234	403.1240 (−1.49)	C ₁₇ H ₂₄ O ₁₁	MS ² [403]: MS ³ [371]:	371.0959 [M-H-CH ₃ OH] [−] 223.0607 [M-H-Glc-H ₂ O] [−] 197.0834 [M-H-Glc-H ₂ O-C ₂ H ₂] [−] 179.0595 [M-H-Glc-H ₂ O-CO ₂] [−] 209.0498 [M-H-CH ₃ OH-Glc] [−] 181.0568 [M-H-CH ₃ OH-Glc-CO] [−] 165.0588 [M-H-CH ₃ OH-Glc-CO ₂] [−]	Secologanoside-7-methyl ester
18 ^b	74.007	237	433.1357 (1.15)	C ₁₇ H ₂₄ O ₁₀	MS ² [433]:	387.1259 [M-H] [−] 225.0771 [M-H-Glc] [−] 207.0654 [M-H-Glc-H ₂ O] [−] 193.0535 [M-H-Glc-CH ₃ OH] [−] 181.0563 [M-H-Glc-H ₂ O-C ₂ H ₂] [−] 149.0454 [M-H-Glc-CH ₃ OH-CO ₂] [−]	Vogeloside
19 ^b	74.25	234	433.1356 (0.92)	C ₁₇ H ₂₄ O ₁₀	MS ² [433]:	387.1256 [M-H] [−] 225.0770 [M-H-Glc] [−] 207.0654 [M-H-Glc-H ₂ O] [−] 193.0535 [M-H-Glc-CH ₃ OH] [−] 181.0563 [M-H-Glc-H ₂ O-C ₂ H ₂] [−] 149.0498 [M-H-Glc-CH ₃ OH-CO ₂] [−]	7- <i>epi</i> -Vogeloside
20	75.678	216, 281, 334	639.1933 (−2.97)	C ₂₉ H ₃₆ O ₁₆	MS ² [639]: MS ³ [621]: MS ⁴ [459]:	621.1808 [M-H-H ₂ O] [−] 487.1427 [M-H-H ₂ O-134] [−] 469.1299 [M-H-H ₂ O-134-H ₂ O] [−] 459.1468 [M-H-H ₂ O-caffeoyl] [−] 441.1343 [M-H-H ₂ O-caffeoyl-H ₂ O] [−] 151.0445 [dehydrophenethanol] [−]	R-campneoside II
21	76.912	230, 285, 328	639.0618 (−0.78)	C ₂₉ H ₃₆ O ₁₆	MS ² [639]: MS ³ [621]: MS ⁴ [469]:	621.1840 [M-H-H ₂ O] [−] 487.1429 [M-H-H ₂ O-134] [−] 469.1348 [M-H-H ₂ O-134-H ₂ O] [−] 459.1482 [M-H-H ₂ O-caffeoyl] [−] 441.1374 [M-H-H ₂ O-caffeoyl-H ₂ O] [−] 151.0430 [dehydrophenethanol] [−]	S-campneoside II
22 ^a	85.37	281, 333	461.0737 (3.69)	C ₂₁ H ₁₈ O ₁₂	MS ² [461]: MS ³ [285]:	285.0395 [M-H-GlcA] [−] 267.0334 [M-H-GlcA-H ₂ O] [−] 239.0295 [M-H-GlcA-H ₂ O-CO] [−] 223.0446 [M-H-GlcA-H ₂ O-CO ₂] [−] 167.0431 [^{1,3} A] [−]	Scutellarin
23	86.083	218, 289, 327	623.1969 (−1.12)	C ₂₉ H ₃₆ O ₁₅	MS ² [623]: MS ³ [461]: MS ⁴ [315]:	461.1674 [M-H-caffeoyl] [−] 443.1553 [M-H-caffeoyl-H ₂ O] [−] 315.1059 [M-H-caffeoyl-Rha] [−] 205.0723 [M-H-caffeoyl-Rha-110] [−] 135.0501 [M-H-caffeoyl-Rha-Glc-H ₂ O] [−]	Forsythoside H
24 ^a	94.933	219, 275, 327	623.1995 (2.25)	C ₂₉ H ₃₆ O ₁₅	MS ² [623]: MS ³ [461]: MS ⁴ [315]:	461.1738 [M-H-caffeoyl] [−] 443.1557 [M-H-caffeoyl-H ₂ O] [−] 315.1075 [M-H-caffeoyl-Rha] [−] 205.0721 [M-H-caffeoyl-Rha-110] [−] 135.0482 [M-H-caffeoyl-Rha-Glc-H ₂ O] [−]	Forsythoside A
25 ^a	96.675	219, 292, 329	623.1973 (−1.28)	C ₂₉ H ₃₆ O ₁₅	MS ² [623]: MS ³ [461]: MS ⁴ [315]:	461.1680 [M-H-caffeoyl] [−] 443.1554 [M-H-caffeoyl-H ₂ O] [−] 315.1054 [M-H-caffeoyl-Rha] [−] 205.0719 [M-H-caffeoyl-Rha-110] [−] 135.0482 [M-H-caffeoyl-Rha-Glc-H ₂ O] [−]	Acteoside
26 ^a	98.147	234, 290, 327	515.1205 (1.94)	C ₂₅ H ₂₄ O ₁₂	MS ² [515]: MS ³ [353]: MS ⁴ [179]:	353.0954 [M-H-caffeoyl] [−] 191.0584 [M-H-2caffeoyl] [−] 179.0362 [CA-H] [−] 173.0490 [M-H-2caffeoyl-H ₂ O] [−] (100%) 135.0484 [CA-H-CO ₂] [−]	3,4-DiCQA
27	99.325	234, 290, 327	515.1186 (−1.75)	C ₂₅ H ₂₄ O ₁₂	MS ² [515]: MS ³ [353]: MS ⁴ [179]:	353.0956 [M-H-caffeoyl] [−] 191.0593 [M-H-2caffeoyl] [−] (100%) 179.0360 [CA-H] [−] 173.0482 [M-H-2caffeoyl-H ₂ O] [−] 135.0484 [CA-H-CO ₂] [−]	1,3-DiCQA

Table 1 (Continued)

No.	t_R (min)	UV_{max}	[M-H] [−] (error in ppm)	Formula	(-) ESI-MS ^a		Identification
					MS ⁿ (Precursor)	Fragments ions (Intensity)	
28	105.624	231, 281	519.1844 (−4.24)	C ₂₆ H ₃₂ O ₁₁	MS ² [519]:	357.1304 [M-H-Glc] [−]	Epipinoresinol-4'-O-glucopyranoside
					MS ³ [357]:	342.1095 [M-H-Glc-CH ₃] [−] 327.0852 [M-H-Glc-2CH ₃] [−] 151.0412 [M-H-Glc-206] [−] 136.0206 [M-H-Glc-206-H ₂ O] [−]	
29 ^a	108.352	220, 290, 329	515.1202 (1.36)	C ₂₅ H ₂₄ O ₁₂	MS ² [515]: MS ³ [353]: MS ⁴ [179]:	353.0951 [M-H-caffeoyl] [−] 191.0575 [M-H-2caffeoyl] [−] 179.0360 [CA-H] [−] 173.0484 [M-H-2caffeoyl-H ₂ O] [−] (100%) 135.0465 [CA-H-CO ₂] [−]	4,5-DiCQA
30	111.619	234	757.2554 (−0.13)	C ₃₄ H ₄₆ O ₁₉	MS ² [757]: MS ³ [595]: MS ⁴ [525]:	693.2009 [M-H-2CH ₃ OH] [−] 595.2004 [M-H-Glc] [−] 397.1317 [M-H-2Glc] [−] 525.1583 [M-H-Glc-C ₄ H ₆ O] [−] 493.1684 [M-H-Glc-3-oxoMAQ] [−] 467.1483 [M-H-Glc-3-oxoMAQ-C ₂ H ₂] [−] 345.1071 [M-H-Glc-C ₄ H ₆ O-Glc] [−]	Aldosecologanin
31 ^a	116.913	215, 276, 316	445.0771 (−1.12)	C ₂₁ H ₁₈ O ₁₁	MS ² [445]: MS ³ [269]:	269.0455 [M-H-GlcA] [−] 251.0340 [M-H-GlcA-H ₂ O] [−] 241.0488 [M-H-GlcA-CO] [−] 223.0413 [M-H-GlcA-H ₂ O-CO] [−] 195.0451 [M-H-GlcA-H ₂ O-2CO] [−] 179.0535 [^{1,2} A] [−] , 167.0502 [^{1,3} A] [−]	Baicalin
32 ^a	118.888	218, 276, 317	431.1003 (4.41)	C ₂₁ H ₂₀ O ₁₀	MS ² [431]: MS ³ [269]:	269.0435 [M-H-Glc] [−] 251.0374 [M-H-Glc-H ₂ O] [−] 241.0506 [M-H-Glc-CO] [−] 223.0397 [M-H-Glc-H ₂ O-CO] [−] 195.0460 [M-H-Glc-H ₂ O-2CO] [−] 179.0540 [^{1,2} A] [−] , 167.0553 [^{1,3} A] [−]	Baikalin
33	129.368	204, 267, 305	429.0833 (2.56)	C ₂₁ H ₁₈ O ₁₀	MS ² [429]: MS ³ [253]:	253.0502 [M-H-GlcA] [−] 209.0608 [M-H-GlcA-CO ₂] [−] 185.0640 [M-H-GlcA-C ₃ O ₂] [−] 165.0757 [^{1,2} A] [−] , 151.0099 [^{1,3} A] [−]	Chrysin-7-O-glucuronide
34	130.168	213, 272, 312	459.0927 (1.09)	C ₂₂ H ₂₀ O ₁₁	MS ² [459]: MS ³ [283]: MS ⁴ [268]:	283.0621 [M-H-GlcA] [−] 268.0364 [M-H-GlcA-CH ₃] [−] 240.0432 [M-H-GlcA-CH ₃ -CO] [−] 239.0353 [M-H-GlcA-CH ₃ -HCO] [−] 211.0389 [M-H-GlcA-CH ₃ -HCO-CO] [−] 167.0500 [^{1,3} A] [−]	OroxylinA-7-O-glucuronide
35 ^a	143.42	216, 275, 323	269.0449 (−0.37)	C ₁₅ H ₁₀ O ₅	MS ² [269]:	251.0382 [M-H-H ₂ O] [−] , 241.0528 [M-H-CO] [−] 223.0437 [M-H-H ₂ O-CO] [−] 195.0469 [M-H-H ₂ O-2CO] [−] 179.0526 [^{1,2} A] [−] , 167.0530 [^{1,3} A] [−]	Baicalein

Abbreviations: t_R : retention time; CQA: caffeoylquinic acid; FQA: feruloylquinic acid; DiCQA: dicaffeoylquinic acid; CA: Caffeic acid; Glc: glucose; GlcA: glucuronic acid; Rha: rhamnose.

^a Compounds confirmed by comparison with reference standards.

^b m/z values of [M+HCOO][−] ion.

Peak **9** showed the deprotonated molecular ion at m/z 367.1041 which yielded diagnostic fragments at m/z 193.0529 [FA-H][−], 191.0542 [M-H-feruloyl][−] together with a base peak at m/z 173.0453 [M-H-feruloyl-H₂O][−]. It was tentatively identified as 3-O-feruloylquinic acid (3-FQA) [35].

Peaks **6**, **8** and **10** were authenticated as mono-caffeoylquinic acid (CQA) after comparison with standard compounds. They gave the same [M-H][−] ion at m/z 353, and were distinguished by the diagnostic ions at m/z 179 [CA-H][−], 191 [M-H-caffeoyl][−] and 173 [M-H-caffeoyl-H₂O][−] [36]. The base peaks at m/z 173 for peak **10** and m/z 191 for **6** and **8** were found in their MS². After careful comparison, peaks **6**, **8** and **10** were clearly identified as 5-CQA, 3-CQA and 4-CQA, respectively [37–39]. Peaks **26**, **27** and **29** displayed [M-H][−] ions at m/z 515, which afforded a common ion at m/z 353 in MS² due to the elimination of a caffeoyl moiety, and further

produced the diagnostic fragment ions at m/z 179, 191 and 173 in MS³ and MS⁴. After comparison of standard compounds, peaks **26**, **27** and **29** were identified as 3,4-diCQA, 1,3-diCQA and 4,5-diCQA, respectively [35].

3.3.2. Identification of 10 phenylethanoid glycosides

Peak **1** showed [M-H][−] ion at m/z 315.1064 (C₁₄H₂₀O₈). In MS² and MS³, ions at m/z 153.0541 and 135.0481 were observed due to the loss of a glucosyl or glucose residue. It was tentatively characterized as hydroxytyrosol-1-glucopyranoside [6].

Peak **4** gave [M-H][−] ion at m/z 477.1634 (C₂₀H₃₀O₁₃). An [M-H-H₂O][−] ion at m/z 459.1479 in MS² with a further fragment at m/z 151.0418 [dehydrophenethanol][−] in MS⁴ suggested the existence of a hydroxyl group on the β -position. Thus, peak **4** was tentatively identified as forsythoside D isomer. Peaks **23**, **24** and **25** gave [M-

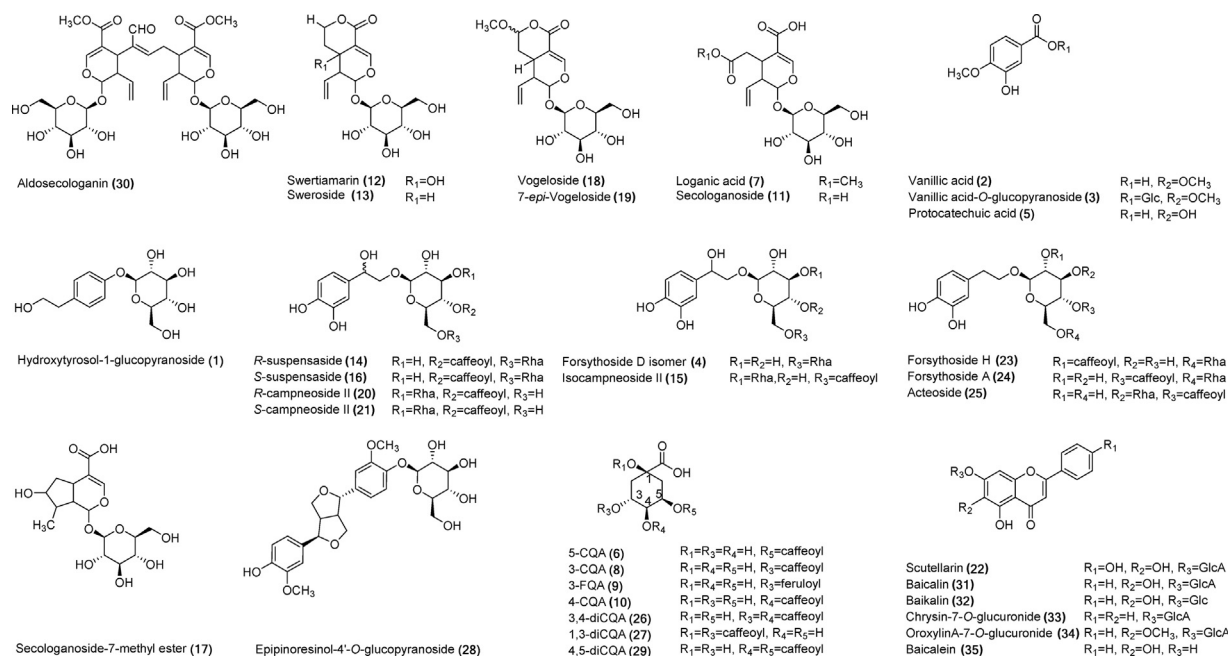


Fig. 5. Structures of 35 major compounds identified in SHLPI.

H][−] ions at m/z 623 (C₂₉H₃₆O₁₅). Ions at m/z 461 in MS², m/z 315 and m/z 135 in MS⁴ were observed after successive elimination of a caffeoyl residue, a rhamnosyl residue and a glucose molecule [40]. Comparing their retention times, UV spectra and MS behaviors with their reference compounds, peak 23 was tentatively identified as forsythoside H, and peaks 24 and 25 were unequivocally identified as forsythoside A and acteoside [41].

Peaks 14, 15, 16, 20 and 21 exhibited [M-H][−] ions at m/z 639 (C₂₉H₃₆O₁₆). Ion at m/z 621 in MS² after losing a H₂O, and ions at m/z 487 ([M-H-H₂O-134][−]) in MS³, 469 ([M-H-H₂O-caffeoyl][−]) and 441 ([M-H-2H₂O-caffeoyl][−]) in MS⁴ were also discovered. They were tentatively characterized as *R*-suspensaside, isocampneoside II, *S*-suspensaside, *R*-campneoside II and *S*-campneoside II, respectively [42,43].

3.3.3. Identification of eight iridoid glycosides

Peak 7 displayed [M-H][−] ion at m/z 375.1295. MS² fragmentation gave [M-H-Glc][−] ion at m/z 213.0764, while MS³ fragmentation showed ion at m/z 169.0859 [M-H-Glc-CO₂][−] suggesting the presence of a carboxyl group. Therefore, peak 7 was identified as loganic acid after comparing with standard compound.

Peak 11 gave [M-H][−] ion at m/z 389.1099 (C₁₆H₂₂O₁₁) as a base peak. In MS², ion at m/z 345.1178 [M-H-CO₂][−] was obtained, suggesting the presence of a carboxyl group at C4 position. In addition, ions at m/z 165.0577 and 121.0280 by sequential eliminations of CO₂ from ion at m/z 209.0458 were produced, indicating the presence of two carboxyl groups. Thus, it was characterized as secologanoside [6].

Peak 17 yielded [M-H][−] ion at m/z 403.1240. Its MS² spectrum provided two typical fragments at m/z 371.0959 ([M-H-CH₃OH][−]) and 179.0595 ([M-H-Glc-H₂O-CO₂][−]), validating the presences of a methyl ester group and a carboxyl group. Peak 17 was characterized as secologanoside-7-methyl ester.

Peaks 12, 13, 18 and 19 contain a lactone group at their C4 positions, due to the observation of fragments after loss of CO₂. Peak 13 was identified as sweroside by comparison with standard reference. It formed its deprotonated molecular ion at m/z 357.1192. Its MS³ and MS⁴ spectra presented a series of ions after successive losses of glucosyl moieties, CO₂ and C₂H₂. Peak 12 gave [M-H][−]

ions at m/z 373.1161, 16 Da more than that of peak 13. Their ions at m/z 149.0642 [M-H-Glc-H₂O-C₂H₂-H₂O][−] were noteworthy since it supports the presence of a hydroxyl group in their structures [44]. Peak 12 was identified as swertiamarin after comparing with standard compound. Peaks 18 and 19 were a pair of isomers, both of which gave [M-H][−] ions at m/z 387 (C₁₇H₂₄O₁₀), 30 Da more than that of peak 13. The appearance of ion at 193.0535 ([M-H-Glc-CH₃OH][−]) indicated the presence of a methoxyl group. Thus peaks 18 and 19 were tentatively identified as vogeloside or 7-*epi*-vogeloside [45].

Peak 30 was iridoid glycoside dimer with its [M-H][−] ion at m/z 757.2554 (C₃₄H₄₆O₁₉). Its characteristic ions at m/z 693.2009 ([M-H-2CH₃OH][−]), 595.2004 ([M-H-Glc][−]), 525.1583 ([M-H-Glc-C₄H₆O][−]), 397.1317 ([M-H-2Glucose][−]) were observed. Peak 30 was thus tentatively identified as aldosecologanin [46].

3.3.4. Identification of six flavonoids

Peak 22 showed [M-H][−] ion at m/z 461.0737 and exhibited its aglycone ion at m/z 285.0395 by elimination of a glucuronic residue in MS² [47]. After comparing the standard compound, peak 22 was identified as scutellarin [32].

Peak 35 produced [M-H][−] ion at m/z 269.0449 (C₁₅H₁₀O₅). And it further yielded ^{1,3}A[−] ion at m/z 167.0530 in MS², suggesting the presences of three OH groups on A-ring. Referring to molecular formula, fragmentation pattern and reference substance, peaks 35 was identified as baicalein. Peaks 31 and 32 showed [M-H][−] ions at m/z 445 (C₂₁H₁₈O₁₁) and 431 (C₂₁H₂₀O₁₀), respectively. They gave the same aglycone ions (baicalein) in MS³ after losing a glucuronic residue (176 Da) and glucosyl group (162 Da), respectively [48]. Thus, peak 31 was identified as baicalin, and peak 32 was characterized as baikalin.

Peak 33 displayed [M-H][−] ion at m/z 429.0833 (C₂₁H₁₈O₁₀) in MS and [M-H-GlcA][−] at m/z 253.0502 in MS². MS³ spectrum gave ^{1,3}A[−] ion at m/z 151.0099 due to cross-ring cleavage of C-ring and an additional ion at m/z 185.0640 ([M-H-GlcA-C₃O₂][−]), indicating that C5 and C7 positions on A-ring were hydroxylated. Peak 33 was therefore identified as chrysin-7-O-glucuronide [49].

Peak 34 gave [M-H][−] ion at m/z 459.0927 (C₂₂H₂₀O₁₁). Ions at m/z 283.0621 in MS² and m/z 268.0364 in MS³ in accordance with

Table 2
Activity contribution of individual active compound in SHLPI sample (n = 3).

No.	Compound	Chromatogram contribution (%)	Activity contribution* (%)
1	5-CQA (6)	1.92	2.08
2	3-CQA (8)	7.03	1.05
3	4-CQA (10)	2.19	2.00
4	Scutellarin (22)	3.04	2.89
5	Forsythoside A (24)	2.52	2.50
6	Acteoside (25)	3.40	3.45
7	3,4-DiCQA (26)	1.71	2.55
8	1,3-DiCQA (27)	1.32	1.57
9	4,5-DiCQA (29)	2.99	3.09
10	Baicalin (31)	63.22	58.60
11	Baicalin (32)	0.29	0.47
12	Chrysin-7-O-glucuronide (33)	1.01	1.70
13	OroxylinA-7-O-glucuronide (34)	1.83	2.57
14	Baicalein (35)	2.02	15.48

The RSDs of all data are less than 3%.

*The activity contribution of each compounds were calculated based on their negative peak areas.

the losses of a glucuronosyl residue and a successive loss of methyl group. Peak **34** was thus tentatively identified as oroxylin A-7-O-glucuronide [50].

3.3.5. Identification of one lignan

Peak **28** gave $[M-H]^-$ ions at m/z 519.01844 with a molecular formula of $C_{26}H_{32}O_{11}$. The ion at m/z 357.1304 in MS^2 formed by loss of a glucosyl moiety, the $[M-H-Glc-CH_3]^-$ ion at m/z 342.1095 and the $[M-H-Glc-2CH_3]^-$ ion at m/z 327.0852 indicated the presence of two methoxyl groups on their structures [51]. The presences of ions at m/z 151.0412, 136.0206 were produced from the cleavage of the tetrahydrofuran ring. Hence, peak **28** was proposed as (+)-epipinoresinol-4'-O-glucopyranoside [52].

3.4. BSA-binding activity of active compounds in SHLPI

Among 35 compounds, 14 active compounds belonging to three types of structures showed significant BSA-binding activity. They are six chlorogenic acids, six flavonoids and two phenylethanoid glycosides.

3.4.1. Activity contribution of individual active compound

Firstly, the amounts of these active compounds were preliminarily evaluated by their HPLC peak areas. The total peak area of 35 identified compounds was considered as 100%. As shown in Table 2, positive peak areas of 14 active compounds (**6**, **8**, **10**, **22**, **24**, **25**, **26**, **27**, **29**, **31**, **32**, **33**, **34** and **35**) account for about 95% of the total peak areas (%), suggesting that they are main constituents in SHLPI.

To evaluate the activity contribution of each compound, the following equation was used to calculate the activity contribution of an individual compound to the overall BSA-binding activity of the sample (CTB: contribution to binding):

$$CTB(\%) = (A_i/A_t) \times 100\%$$

Where A_i represents the negative peak area of an individual compound in the BSA binding profile, A_t represents the sum of negative peak areas of all active compounds in SHLPI. CTB is used as a preliminary indicator to evaluate the overall impact of an active compound.

As shown in Table 2, flavonoids were the major activity contributors responsible for more than 80% of the total BSA-binding ability, of which baicalein (**35**) alone contributed 58.60% to the overall activity. The CTB of chlorogenic acids and phenylethanoid glycosides were 12.33% and 5.95%, respectively. There were four compounds (**25**, **29**, **31** and **35**) with CTBs greater than 3.0% in

Table 3
The HPLC calibration curves and BSA-binding activity calibration curves of 10 constituents in SHLPI.

No.	Compound	Calibration curves of compounds	R^2	Calibration curves of BSA-compounds	R^2	Linear range (nmol)	Compound amount in SHLPI (nmol) ^a	BSA-binding activity (10^6 nmol ⁻¹)
1	Scutellarin (22)	$y = 1.4908E + 06x + 7.5163E + 04$	0.9999	$y = -6.8267E + 05x + 2.6348E + 04$	0.9999	0.660–2.860	1.10	0.68
2	Baicalin (31)	$y = 1.7226E + 06x + 4.9855E + 05$	0.9993	$y = -1.0071E + 06x - 3.0392E + 06$	0.9993	7.188–31.148	11.98	1.00
3	Baicalein (35)	$y = 1.0843E + 06x + 5.9953E + 04$	0.9996	$y = -4.8468E + 06x + 7.3319E + 06$	0.9996	2.646–11.466	4.41	4.84
4	Forsythoside A (24)	$y = 5.5758E + 06x + 5.9820E + 04$	0.9997	$y = -1.4753E + 06x + 9.4747E + 04$	0.9997	0.726–3.146	2.66	1.47
5	Acteoside (25)	$y = 3.1176E + 06x - 2.0799E + 04$	0.9997	$y = -8.6278E + 05x + 1.2702E + 05$	0.9997	0.428–1.712	2.19	0.86
6	5-CQA (6)	$y = 5.3166E + 06x - 1.5696E + 05$	0.9998	$y = -1.7230E + 06x + 6.2860E + 04$	0.9998	0.331–3.310	3.31	1.72
7	3-CQA (8)	$y = 3.2126E + 06x - 2.8299E + 05$	0.9998	$y = -1.1129E + 06x + 4.2519E + 05$	0.9998	0.780–3.900	3.90	1.11
8	4-CQA (10)	$y = 2.4980E + 06x - 3.1075E + 05$	0.9999	$y = -8.3279E + 05x + 1.2402E + 06$	0.9999	2.280–6.270	2.850	0.83
9	3,4-diCQA (26)	$y = 6.4066E + 06x - 6.0955E + 05$	0.9999	$y = -3.5818E + 06x + 1.8844E + 06$	0.9999	1.752–4.818	1.21	3.58
10	4,5-diCQA (29)	$y = 5.9543E + 06x - 5.2231E + 05$	0.9995	$y = -2.8044E + 06x + 8.7879E + 05$	0.9995	1.569–6.916	1.07	2.80

^aThis represents the amounts in 10 μ L injection volumes of SHLPI (1 mg/mL).

Table 4The titer results of rabbit antiserum by ELISA (average $\pm$ SD, $n = 5$).

Group (peak No.)	OD values (titer) of antiserum at different dilution ratios					OD values before sensitization
	1:8	1:16	1:32	1:64	1:128	
SHLPI	1.070 $\pm$ 0.01	0.996 $\pm$ 0.01	0.866 $\pm$ 0.05	0.722 $\pm$ 0.01	0.579 $\pm$ 0.03	0.117 $\pm$ 0.01
Scutellarin (22)	2.523 $\pm$ 0.36	2.248 $\pm$ 0.05	2.133 $\pm$ 0.03	2.152 $\pm$ 0.15	1.812 $\pm$ 0.02	0.103 $\pm$ 0.03
Baicalin (31)	2.104 $\pm$ 0.09	2.016 $\pm$ 0.06	1.965 $\pm$ 0.05	1.540 $\pm$ 0.04	1.265 $\pm$ 0.10	0.134 $\pm$ 0.01
Baicalein (35)	2.009 $\pm$ 0.03	2.022 $\pm$ 0.01	1.730 $\pm$ 0.08	1.492 $\pm$ 0.17	1.222 $\pm$ 0.04	0.161 $\pm$ 0.01
Forsythoside A (24)	0.534 $\pm$ 0.01	0.496 $\pm$ 0.03	0.485 $\pm$ 0.02	0.466 $\pm$ 0.04	0.458 $\pm$ 0.04	0.133 $\pm$ 0.02
5-CQA (6)	0.408 $\pm$ 0.02	0.340 $\pm$ 0.01	0.284 $\pm$ 0.01	0.233 $\pm$ 0.01*	0.183 $\pm$ 0.01*	0.119 $\pm$ 0.01
3-CQA (8)	0.521 $\pm$ 0.01	0.457 $\pm$ 0.01	0.387 $\pm$ 0.01	0.330 $\pm$ 0.01	0.274 $\pm$ 0.02	0.116 $\pm$ 0.02
4-CQA (10)	0.637 $\pm$ 0.01	0.491 $\pm$ 0.01	0.322 $\pm$ 0.01	0.247 $\pm$ 0.01*	0.178 $\pm$ 0.01*	0.160 $\pm$ 0.01
3,4-diCQA (26)	0.298 $\pm$ 0.01	0.272 $\pm$ 0.01	0.258 $\pm$ 0.02	0.259 $\pm$ 0.01	0.245 $\pm$ 0.01	0.094 $\pm$ 0.02
1,3-diCQA (27)	0.711 $\pm$ 0.05	0.706 $\pm$ 0.01	0.579 $\pm$ 0.04	0.513 $\pm$ 0.04	0.443 $\pm$ 0.02	0.102 $\pm$ 0.01
4,5-diCQA (29)	0.725 $\pm$ 0.01	0.639 $\pm$ 0.01	0.570 $\pm$ 0.01	0.535 $\pm$ 0.02	0.525 $\pm$ 0.01	0.136 $\pm$ 0.01
1,5-diCQA**	0.556 $\pm$ 0.06	0.472 $\pm$ 0.04	0.383 $\pm$ 0.03	0.374 $\pm$ 0.01	0.374 $\pm$ 0.01	0.102 $\pm$ 0.02
3,5-diCQA**	0.455 $\pm$ 0.01	0.470 $\pm$ 0.03	0.441 $\pm$ 0.02	0.453 $\pm$ 0.01	0.436 $\pm$ 0.01	0.119 $\pm$ 0.01
normal saline	0.279 $\pm$ 0.01*	0.183 $\pm$ 0.01*	0.137 $\pm$ 0.01*	0.138 $\pm$ 0.01*	0.121 $\pm$ 0.01*	0.149 $\pm$ 0.01

*OD value (titer) is lower than 2 times of that of un-sensitized serum, **Standard compound which is not component of SHLPI.

Table 5

Determination of serum specificity of rabbit anti-SHLPI by ciELISA.

Group (Peak No.)	Regression equation	R ²	IC ₅₀	LOD (IC ₁₀)
SHLPI	I = 0.561lgC + 0.430	0.946	1.335 mg/mL	0.258 mg/mL
Scutellarin (22)	I = 0.163lgC + 0.527	0.954	0.684 mg/mL	2.442 μ g/mL
Baicalin (31)	I = 0.135lgC + 0.270	0.872	0.506 mg/mL	0.055 μ g/mL
Baicalein (35)	I = 0.140lgC + 0.404	0.949	0.485 mg/mL	6.838 mg/mL
Forsythoside A (24)	I = 0.249lgC + 0.505	0.905	0.954 mg/mL	0.024 mg/mL
5-CQA (6)	I = 3.179lgC + 0.843	0.914	>10.0 mg/L	1.933 mg/mL
3-CQA (8)	I = 0.639lgC + 0.350	0.934	1.716 mg/mL	0.406 mg/mL
4-CQA (10)	I = 2.546lgC + 0.638	0.891	>10.0 mg/L	1.640 mg/mL
3,4-diCQA (26)	I = 0.395lgC + 0.457	0.978	0.657 mg/mL	0.340 mg/mL
1,3-diCQA (27)	I = 0.183lgC + 0.327	0.903	0.873 mg/mL	0.057 mg/mL
4,5-diCQA (29)	I = 0.417lgC + 0.296	0.902	1.086 mg/mL	0.339 mg/mL
1,5-diCQA*	I = 0.137lgC + 0.291	0.822	0.349 mg/mL	0.040 mg/mL
3,5-diCQA*	I = 0.287lgC + 0.355	0.938	1.188 mg/mL	0.129 mg/mL

Abbreviations: SHLPI = Shuanghuanglian powder injection; CQA = caffeoylquinic acid; diCQA = dicaffeoylquinic acid; R² = correlation coefficient; IC₅₀ = half maximal inhibitory concentration; LOD = limit of detection. *Standard compound which is not component of SHLPI.

the sample. Although accounting for a small percentage (2.02%) of total HPLC peak area, compound **35** (baicalein) was one of the main contributor to the overall BSA-binding activity. Therefore, the BSA-binding activity of active compounds might be closely related to their structure.

3.4.2. Structure-activity relationships of active compounds

3.4.2.1. Calibration curves of 10 active compounds in SHLPI. Calibration curves of 10 active compounds including 5-CQA (**6**), 3-CQA (**8**), 4-CQA (**10**), 3,4-diCQA (**26**), 4,5-diCQA (**29**), acteoside (**25**), forsythoside A (**24**), scutellarin (**22**), baicalin (**31**) and baicalein (**35**) were studied to calculate the exact amount of each compound. The calibration curves of these compounds were constructed by plotting the HPLC peak area vs. the amount of injected standard compound (x , nmol). As shown in Table 3, their correlation coefficients ($R^2 > 0.9993$) indicate that all calibrations have good linearity within the test ranges. The amounts of 10 active compounds in 10 μ L SHLPI sample (1 mg/mL) were calculated and shown in Table 3.

3.4.2.2. Evaluation method of BSA-binding activity. To evaluate the binding ability of individual compound bind to BSA, the activities of these ten active constituents including five chlorogenic acids (**6**, **8**, **10**, **26** and **29**), three flavonoids (**22**, **31** and **35**) and two phenylethanoid glycosides (**24** and **25**) were calculated to investigate their structure-activity relationships.

The calibration curves of BSA-binding activity (Table 3) were constructed by plotting the negative peak area vs. the corresponding injection amount (x , nmol). Good linearity of each calibration

curve was observed. The regression equations could be used to assess the BSA-binding ability of individual active compound since the slopes of the calibration curves represent negative peak areas ($10^6 \cdot \text{nmol}^{-1}$) generated by a certain amount (nmol) of active compound. The BSA-binding activity of 5-CQA (**6**), 3-CQA (**8**), 4-CQA (**10**), 3,4-diCQA (**26**), 4,5-diCQA (**29**), acteoside (**25**), forsythoside A (**24**), scutellarin (**22**), baicalin (**31**) and baicalein (**35**) were 1.72, 1.11, 0.83, 3.58, 2.80, 0.86, 1.47, 0.68, 1.00, $4.84 \times 10^6 \cdot \text{nmol}^{-1}$, respectively.

3.4.2.3. Comparison of BSA-binding activity. The BSA-binding ability of each type of compounds ranked in the following order: baicalein > baicalin > scutellarin; 3,4-diCQA > 4,5-diCQA > 5-CQA > 3-CQA > 4-CQA; forsythoside A > acteoside.

The flavonoids were close to the tryptophan residue when a flavonoid-BSA complex formed [31]. From Table 3, the activity of flavonoid (**35**) is stronger than that of flavonoid glycosides (**22** and **31**). For baicalin (**31**) and its aglycone baicalein (**35**), the steric hindrance of **31** may weaken the BSA-binding affinity due to its C7 glycosylation. In addition, the activity of scutellarin (**22**) with an additional C4'-OH is smaller than that of baicalin (**31**) which may due to the increased polarity and size of scutellarin causing possible reduced capacity to penetrate into the tryptophan rich hydrophobic regions of BSA.

Chlorogenic acids, with aromatic ring and adjacent hydroxyl groups exhibited strong BSA-binding ability. The dicaffeoylquinic acids (3,4-diCQA and 4,5-diCQA) showed stronger ability than that of caffeoylquinic acids (3-CQA, 4-CQA and 5-CQA). The diCQAs with more aromatic rings would affect more of the microenvironment

near tryptophan residue of BSA, thus were able to obtain more extensive interaction with BSA than that of mono-CQAs. Alternatively, diCQAs with more adjacent hydroxyl groups may result in more hydrogen bonds between compound and BSA.

There are glycosyl and cinnamoyl groups in phenylethanoid glycosides. The BSA-binding ability of forsythoside A (**24**) is stronger than acteoside (**25**). Compound **24** has a rhamnoside on the C6 position of the central glycosyl while the rhamnoside of compound **25** is linked to C3 position, indicating that glycosyls on C6 position may result in stronger activity due to the more flexible three-dimensional structures than that on C3.

3.5. Validation of BSA-binding active compounds as haptens by ELISA

Based on the BSA-binding activity of phenylethanoid glycosides and the structure–activity relationships study, we chose nine active compounds (**6**, **8**, **10**, **22**, **24**, **26**, **29**, **31** and **35**) and three caffeoylquinic acids (1,3-diCQA, 1,5-diCQA and 3,5-diCQA) to carry out the ELISA assay.

The ELISA results (Table 4) showed that titers of the antiserum were all positive when the serum concentrations were diluted from 1:8 to 1:128, indicating that ectogenic antibodies were then generated in rabbit serum. Among these compounds, SHLIP and 10 compounds (baicalein, 3,5-diCQA, 3-CQA, 1,5-diCQA, baicalin, 3,4-diCQA, 4,5-diCQA, forsythiaside A, 1,3-diCQA and scutellarin) showed higher OD values than that of before sensitization, suggesting their potential sensitization effects. In contrast, 5-CQA (**6**) and 4-CQA (**10**) had lower OD values than that of before sensitization. Moreover, the ELISA results (Table 4) also showed that, according to their OD values, the flavone (**35**) and flavonoids (**22** and **31**) have stronger sensitization than caffeoylquinic acids and forsythiaside A (**24**). The strong sensitization of flavones and flavonoids was consistent with the ciELISA results (Table 5), which showed that the flavones and flavonoids also had high specificity as haptens. This information may be utilized to screen other haptens for future studies.

To verify the specificity of antibodies, we analyzed the 12 compounds using ciELISA assay. As shown in Table 5, the antisera inhibition rate of sample solutions increases as its concentration goes up, indicating that the antibodies were specific and were generated due to the stimulation of the haptens in serum. The results indicated that 10 compounds have potential sensitization activity with specific immune binding, while the ciELISA assay showed that 5-CQA and 4-CQA were unable to stimulate the production of antibodies since their IC₅₀ values greatly exceeded the concentrations applied.

4. Conclusion

In this study, an on-line HPLC-DAD-MS-BSA-FLD system was established by combining biochemical detection with HPLC–MS for identifying haptens in TCMIs for the first time. Applying this system, 35 constituents were identified in SHLPI, 14 (six caffeoylquinic acids, six flavonoids and two phenylethanoid glycosides) of which showed differential BSA-binding activity. The subsequent ELISA results showed that 10 compounds were identified as haptens in 12 selected BSA-binding activity compounds. Flavonoids shown stronger allergenic activity than caffeoylquinic acids and phenylethanoid compounds, suggesting that flavonoids are the main sensitizers in SHLPI. The established system is rapid, stable and reliable for isolation, identification and screening of haptens in SHLPI sample, and thus could be a promising approach to study the chemical basis of allergic side effects for other TCMIs.

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