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Development of an on-line analytical platform to screen haptens in shuxuening injection based on high-performance liquid chromatography coupled with ion-trap multistage mass spectrometry and human serum albumin fluorescence detection

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ABSTRACT

Shuxuening injection (SXNI), one of the traditional Chinese medicine injections (TCMI), is widely used for the treatment of cardiovascular diseases in the clinic. However, its allergic reactions have impeded the clinical applications of SXNI, such adverse reactions have not been well understood due to the lack of methods for detecting haptens. In this study, a high-performance liquid chromatography–diode-array detector–multi-stage mass spectrometry–human serum albumin–fluorescence detector (HPLC–DAD–MSⁿ–HSA–FLD) system was established to identify and screen haptens for the first time. Flavones, flavonols and their glycosides in SXNI showed strong HSA binding ability in different degrees. Fifteen of these compounds were used to study the association of HSA binding ability and sensitizability using isothermal titration calorimetry (ITC) and fluorescence techniques, furthermore, RBL-2H3 cell experiments were conducted to verify the results. It was found that ginkgolides showed no sensitizability, while flavones and flavonol aglycones showed stronger sensitizability than their glycosides. The system was proven to be precise, stable and reproducible, which lays a foundation for screening haptens in SXNI and relevant samples.

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1. Introduction

Shuxuening injection (SXNI) is a sterile aqueous solution made of Ginkgo biloba leaf [1]. It is famous for its good clinical efficacy as a treatment of ischemic cerebrovascular disease, coronary heart disease, cerebral thrombosis and cerebral vasospasm [2,3]. However, its adverse reactions caused by its haptens have aroused great concern for its expansion on clinical applications. Previous studies have revealed that its major constituents include flavonoid glycosides and ginkgolides [4,5], however, whether these constituents are the haptens causing allergic reactions is still uncertain.

Major adverse reactions of SXNI include anaphylaxis and anaphylactoid reaction. Anaphylaxis occurs when the same substance stimulated the immunized organism again, while anaphylactoid reaction is similar to anaphylaxis except that it occurs when the antigen enters the body for the first time [6]. In anaphylaxis, the hapten can be a small molecule, which after binding to its carrier proteins, forms a hapten–protein complex (complete antigen) to induce allergic reactions [7]. Several known carrier proteins include keyhole limpet hemocyanin, concholepas concholepas hemocyanin, bovine serum albumin, ovalbumin and human serum albumin (HSA) [8–11]. Among them, HSA is the most relevant carrier responsible for human sensitivity. HSA is the most abundant protein accounting for about 60% of total plasma protein in human circulation system. It is composed of 585 amino acid residues, among which tryptophan, lysine and phenylalanine residues confer endogenous fluorescence to HSA [12]. When a compound binds to HSA, its fluorescence decreases [13]. Based on this mechanism, potential HSA binders can be detected by fluorescence detection for

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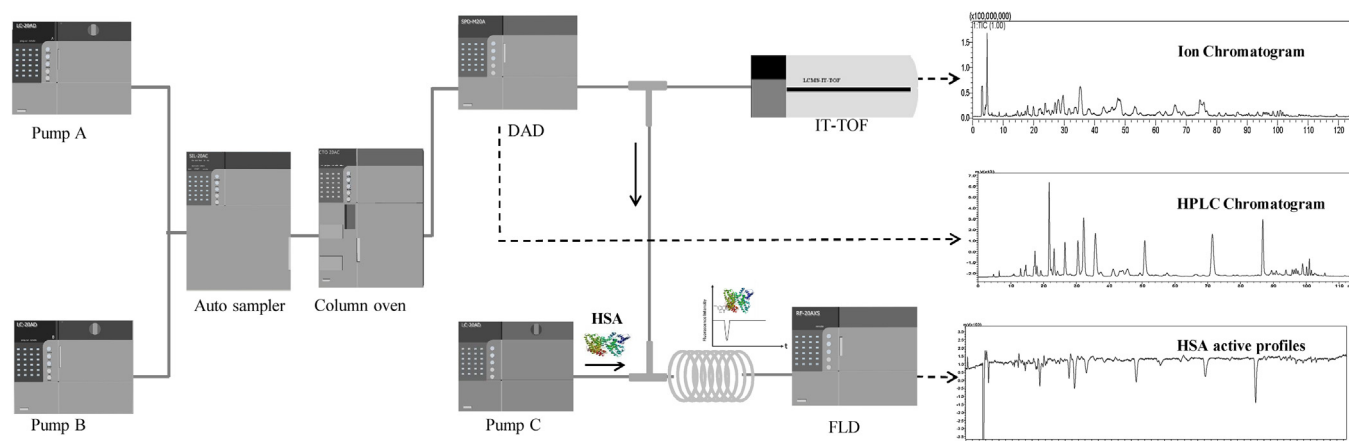


Fig. 1. Schemes of on-line HPLC-DAD-MSⁿ-HSA-FLD system. DAD: diode array detector, IT: ion-trap, TOF: time-of-flight, MS: mass spectrometer, HSA: human serum albumin, FLD: fluorescence detector, HPLC: high-performance liquid chromatography.

further evaluation of their hapten properties. Isothermal titration calorimetry (ITC) was also applied to explore the interaction mode between HSA and flavonoids in Shuxuening injection [14,15].

RBL-2H3 cell is one type of basophilic leukemia cells isolated from basophils, which is commonly used in studies of high-affinity IgE receptors (also known as FcεRI receptors). FcεRI receptor is able to distinguish substances leading to anaphylaxis and anaphylactoid reaction. In anaphylaxis, the allergic model can be established by co-cultured with IgE for cross-linking activation of cell surface receptor [16]. In sensitized state, RBL-2H3 cells will release allergic medium when co-cultured with allergens. Meanwhile, co-culturing compound directly with RBL-2H3 cells can determine whether compound causes class allergic reaction. Histamine and tryptase content then can be detected to determine the degree of allergic reactions and class allergic reactions [16,17].

In this study, a method using high-performance liquid chromatography–diode-array detector–multi-stage mass spectrometry–human serum albumin–fluorescence detector (HPLC–DAD–MSⁿ–HSA–FLD) system was established for the first time. HSA binders were identified in SXNI, and their hapten properties were then validated on RBL-2H3 cells. This system can serve as a high-throughput screening approach for screening and validating haptens from complex samples.

2. Experimental

2.1. Materials and reagents

SXNI (Lot. No.: 20150809) was obtained from Shi Yao Yin Hu Group Co., Ltd (Shijiazhuang, China). Quercetin (**42**) was obtained from Sinopharm Group Chemical Reagent Co., Ltd. Quercetin-3-O-rutinoside (**8**), quercetin-3-O-glucoside (**11**), apigenin-7-O-glucoside (**25**), luteolin-7-O-glucoside (**30**), myricetin (**51**) and luteolin (**52**) were purchased from China Food and Drug Administration Research Institute (Beijing, China). Myricetin-3-O-glucoside (**4**), kaempferol-3-O-rutinoside (**17**), isorhamnetin-3-O-rutinoside (**19**) and apigenin (**56**) were obtained from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China). Kaempferol-3-O-glucoside (**21**), isorhamnetin-3-O-glucoside (**24b**), kaempferol (**57**) and isorhamnetin (**58**) were purchased from Beijing Si Mian Ti Technology Co., Ltd (Beijing, China). The purity of each compound was determined to be > 98% by HPLC. HSA (lyophilized powder) were purchased from Sigma-Aldrich. RBL-2H3 cells and IgE ELISA-kit were obtained from Beijing Beiluo Biotechnology Co., Ltd. (Beijing, China). Minimum essential medium (MEM) and penicillin-streptomycin were purchased from GE Healthcare Life Sciences

Hyclone laboratories (Beijing, China). NaOH was purchased from Beijing Chemical Works Co., Ltd (Beijing, China).

HPLC grade methanol and acetonitrile were purchased from DUKSAN Co., Ltd (Duksan, Korea). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. On-line detection solutions and sample solutions preparation

2.2.1. On-line detection solutions

SXNI was filtered using a 0.22 μm membrane. All solutions of standard compounds were dissolved in MeOH with a concentration of 0.1 mM and stored at 4 °C prior to use. HSA (1.66×10^{-7} M) was dissolved in 0.05 M PBS buffer (pH 7.40) and stored at 4 °C prior to use.

2.2.2. Sample preparation for ITC experiment

The stock solution of individual standard was dissolved in 0.05 M PBS buffer (pH 7.40) containing 0.05% DMSO (v/v) with concentrations of 0.25 mM and 0.1 mM for ITC experiment. HSA solutions (0.25 mM and 1.0 mM) were prepared in 0.05 M PBS buffer (pH 7.40) containing 0.05% DMSO (v/v).

2.3. Methods

2.3.1. Design and conditions of on-line HPLC-DAD-MSⁿ-HSA-FLD system

The on-line HPLC-DAD-MSⁿ-HSA-FLD system (Fig. 1) was composed of two major sections: HPLC-DAD-MSⁿ section and HSA-FLD section. HPLC-DAD-MSⁿ section was composed of DGU-20A3 degasser, two LC-20AD pumps, an SIL-20AC injector, a CTO-20AC column oven, an SPD-M20A detector and an ESI-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). The HSA-FLD section was composed of an LC-20AD pump, a reaction coil, a CTO-20AC column oven and a RF-20Axs fluorescence detector. The flow rate of HPLC-DAD part was 0.6 mL/min, which was divided into two parts. One of which was pumped into the MS detector to obtain the MS data for structural analysis, the other stream (0.5 mL/min) flowed into HSA-FLD section for on-line screening analysis.

The separation part was carried on an Inertsil column (250 mm × 4.6 mm, 3 μm) maintained at 35 °C. The mobile phase was composed of water containing 0.05% formic acid (A) and acetonitrile (B) at a flow rate of 0.6 mL/min with the following gradient: 0–20 min, 18.5–20% B; 20–30 min, 20–18.5% B; 30–45 min, 18.5–20.5% B; 20.45–60 min, 5–21% B; 60–90 min, 21–29% B; 90–115 min, 29–38% B; 115–140 min, 38–100% B. The UV spectrum was recorded at 200–400 nm.

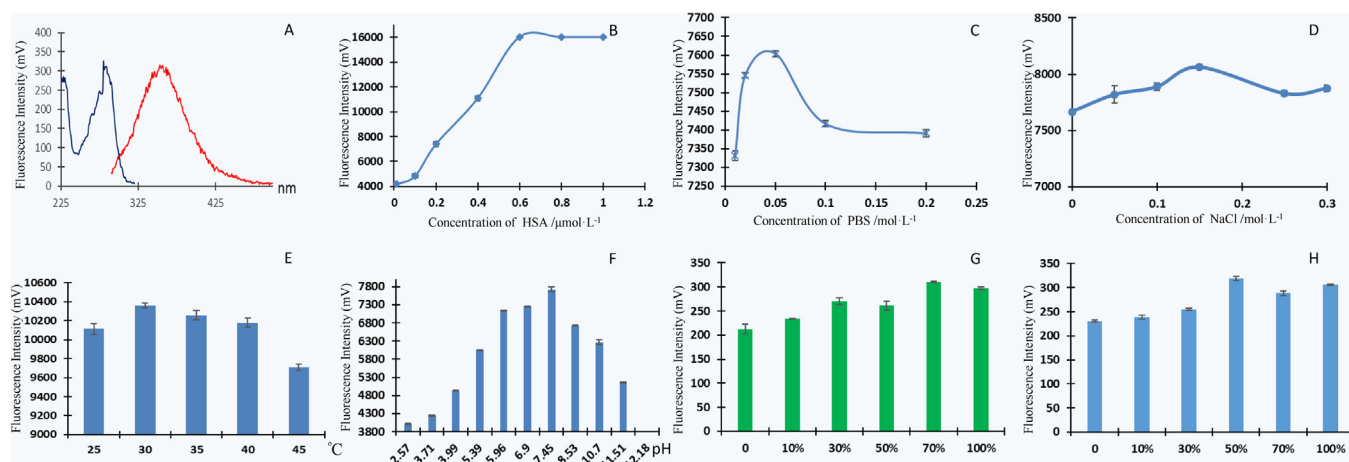


Fig. 2. Fluorescence spectra of HSA and rutin (A). Effects of the concentration of HSA (B), the concentration of PBS (C), the concentration of NaCl (D), the column temperature (E), the pH value (F), the concentrations of ACN (G) and MeOH (H) on the fluorescence intensity of the on-line system.

The optimized MS conditions were as follows: data was collected in negative ion mode with a scan range of m/z 100–800. The curved desolvation line (CDL) temperature was set at 200°C . The detector voltage, capillary voltage and CDL voltage were fixed at 1.75 kV, 4.5 kV, 10 kV, respectively. The collision energy was adjusted to 70% in the analysis. The flow rates of nitrogen and nebulizer gas were 1.5 L/min. All data were acquired and analyzed by LC/MS solution software (Shimadzu, Kyoto, Japan).

The optimized HSA-binding activity conditions were as follows: the excitation and emission wavelengths of FLD were set at 285 nm and 350 nm, respectively. The HSA solution (1.66×10^{-7} M) was pumped at a flow rate of 0.5 mL/min. The reaction temperature was set at 35°C consistent with the liquid phase analysis.

2.3.2. Isothermal titration calorimetry (ITC) measurements

ITC measurement was carried on a Nano-ITC machine (MicroCal ITC 200, Malvern Instruments Ltd.) at 25°C . To achieve complete equilibration, HSA solutions ($40 \mu\text{L}$, 0.25 mM) were titrated into $300 \mu\text{L}$ of flavonols (0.1 mM) and flavones (0.1 mM), respectively, with a stirring rate of 500 rpm. Another HSA solution (1.00 mM) was titrated into $300 \mu\text{L}$ flavonoid glycosides (0.25 mM). The reference cell contained $300 \mu\text{L}$ water. All the protein solution, flavonols, flavones and their glycosides solutions were prepared using PBS (0.05 mM , pH 7.4) containing 5% DMSO (v/v). In this titration experiment, the drop was set at $1.8 \mu\text{L}$, and the time between two injections was set at 110 s.

2.3.3. RBL-2H3 cell assays

2.3.3.1. Anaphylactoid test using RBL-2H3 cells. RBL-2H3 cells were cultured in MEM medium (containing 15% fetal bovine serum and 1% penicillin/streptomycin) under a humidified atmosphere of 5% CO_2 at 37°C , then seeded with a cell density of 2×10^5 cells/well in 96-well plates. After 24 h incubation, the culture solution was removed. The cells were washed with $2 \times 150 \mu\text{L}$ balanced salt solution, and then added another $150 \mu\text{L}$ balanced salt solution. According to the previous methods [18,19], all the samples [myricetin-3-*O*-glucoside (**4**), quercetin-3-*O*-rutinoside (**8**), quercetin-3-*O*-glucoside (**11**), kaempferol-3-*O*-rutinoside (**17**), isorhamnetin-3-*O*-rutinoside (**19**), kaempferol-3-*O*-glucoside (**21**), isorhamnetin-3-*O*-glucoside (**24b**), apigenin-7-*O*-glucoside (**25**), luteolin-7-*O*-glucoside (**30**), quercetin (**42**), apigenin (**56**), kaempferol (**57**), isorhamnetin (**58**), myricetin (**S1**) and luteolin (**S2**)] were prepared using 0.9% NaCl solution containing 5% DMSO. $100 \mu\text{L}$ of each compound (0.1 mM) was added to a 96-well plate and incubated for one hour, followed by addition

of 0.05 mL NaOH (0.4 M) and $10 \mu\text{L}$ *o*-phthalaldehyde. After incubation for 15 min, $20 \mu\text{L}$ HCl (0.1 M) was added to quench the reaction. The OD value at 450 nm was then determined by measuring the content of histamine.

2.3.3.2. Anaphylaxis test using RBL-2H3 cells. All animal assays were performed in accordance with the animal use guidelines. The protocol was in line with the Institutional Animal Care and Use Committee (IACUC) of Peking University Health Science Center. According to published methods [18,19], 0.5 mL mix-solution (10 mg/mL human serum albumin and 0.25 mL SXNI) mixed with an equal volume of complete Freund's adjuvant was injected into the back of the rat. On the 7th day and 14th day after the injection, allergic symptoms including shortness of breath of rats were observed, the rats were then injected with the same amount of mix-solution mixed with an equal volume of incomplete Freund's adjuvant, respectively. On the 14th day, blood was withdrawn from the heart, centrifuged at 1500 g for 10 min. Serum was isolated and transferred into another sterile tube and stored under 4°C . Contents of total serum IgE was detected with ELISA method following the manufacturer's instructions.

The anaphylaxis test of RBL-2H3 cells were prepared in the same way as the anaphylactoid test. RBL-2H3 cells were cultured in MEM medium supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin under a humidified atmosphere of 5% CO_2 at 37°C . Cells were seeded in 96-well plates (2×10^5 cells/well). After 24 h, the supernatant was removed by aspiration, the cells were washed with $2 \times 150 \mu\text{L}$ balanced salt solution, and added another $150 \mu\text{L}$ balanced salt solution. All 15 compounds (see above) were prepared in 0.9% NaCl solution containing 5% DMSO. $100 \mu\text{L}$ of each compound (0.1 mM in 0.9% NaCl solution) containing 0.5 mM HSA was added to cells followed by a 1 h incubation. The cells were then added 0.05 mL NaOH (0.4 M) and $10 \mu\text{L}$ *o*-phthalaldehyde. After 15 min incubation, $20 \mu\text{L}$ HCl (0.1 M) was added to quench the reaction. The OD value at 450 nm was then determined by measuring the content of histamine.

3. Results and discussion

3.1. Optimization of HSA-binding analysis system

3.1.1. Determination of the detection wavelengths of HSA

Fluorescence spectra of off-line detection were performed on a Cary Eclipse fluorescence spectrophotometer (Agilent, Australia). The excitation and emission spectra were set in the scanning ranges

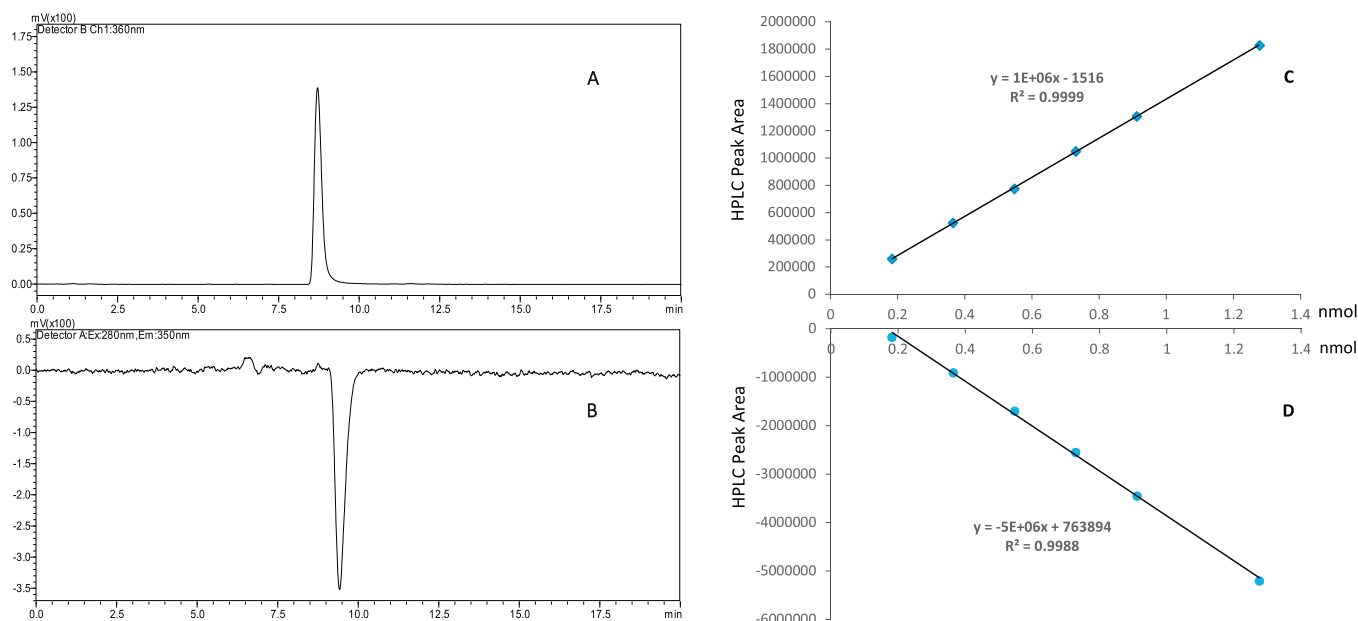


Fig. 3. The on-line detection of rutin: the HPLC chromatogram (A), HSA-rutin activity profile (B), the linearity relationships of HSA-rutin: between the amount and peak areas (C for HPLC and D for active profile).

of 225–325 nm and 300–600 nm, respectively. The maximum excitation wavelength was set at 285 nm and the maximum emission wavelength was set at 350 nm as shown in Fig. 2A.

3.1.2. Optimization of the on-line HSA binding system

Various conditions including HSA concentration, the pH value, the concentration of NaCl, the reaction temperature, and the mobile phase, may affect the fluorescence of HSA-binding activity. Thus, these conditions were investigated for appropriate detection conditions.

3.1.2.1. Effects of the HSA concentrations. Firstly, various concentrations of HSA (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} M) were evaluated (Fig. 2B). Fluorescence intensity increased with the increase of HSA concentrations. However, when the concentration of HSA was higher than 8.000×10^{-7} M, the fluorescence intensity of HSA reached the upper detection limit of the fluorescence detector. The concentrations of HSA within the range of 1.000×10^{-7} M and 8.000×10^{-7} M were thus acceptable.

3.1.2.2. Effects of the PBS concentrations and NaCl concentrations. The concentrations of PBS and NaCl had an effect on the fluorescence intensity of protein [20]. The influence of PBS concentration was evaluated by a series of concentrations (0.01 M, 0.02 M, 0.05 M, 0.1 M, 0.2 M) shown in Fig. 2C. The concentration of PBS was finally set at 0.05 M, which displayed stronger fluorescence intensity than any other concentrations. The influence of NaCl concentrations (0.00, 0.05, 0.10, 0.15, 0.25, 0.30 M) was investigated as well (Fig. 2D). The concentration of NaCl was finally set at 0.15 M, which gave the strongest intensity.

3.1.2.3. Effects of the reaction temperatures. The reaction temperature also influences the fluorescence intensity [20,21]. According to Fig. 2E, the reaction temperature had little effect on fluorescence intensity of HSA. 35 °C was used to be consistent with the temperature in liquid chromatography.

3.1.2.4. Effects of the pH values. The pH values can also affect the fluorescence intensity of the HSA. As shown in Fig. 2F (pH 2.57–12.18),

when pH value was between 5.96 and 8.53, the fluorescence intensity was relatively high (pH 7.45 gave the highest fluorescence intensity). Thus, the physiological pH 7.4 was chosen in this study.

3.1.2.5. Effects of the organic solvents. Acetonitrile (ACN) and methanol (MeOH) were commonly used as mobile phase reagents in liquid chromatography analysis. Thus, their effects on the fluorescence intensity of HSA were also investigated. According to Fig. 2G–H, ACN and MeOH did not significantly affect the fluorescence intensity of HSA. Therefore, the appropriate proportion of organic solvents was selected based on the LC analysis program. In addition, the maximum emission wavelength spectrum of HSA was not obviously changed by concentrations of ACN (Fig. S1).

3.2. Validation of HSA-binding analysis system

In a previous study [22], the interaction mechanism between HSA and rutin has been studied, and rutin was used as a positive control to verify the on-line system in our study. The liquid chromatogram and activity profile of HSA-rutin were shown in Fig. 3. To avoid the occurrence of false positives, we conducted a blank control experiment, and the results showed that there was no negative peak in the fluorescence spectrum of HSA (Fig. S2).

3.2.1. Precision, stability and reproducibility

The precision was evaluated by six consecutive injections ($n=6$) of rutin solution (0.18 mM). The relative standard deviations (RSDs) of liquid chromatogram peak areas and HSA-binding peak areas were 0.89% and 0.70%, respectively. The stability experiment of this system was evaluated by injecting the same rutin solution at 0, 2, 4, 8, 16 and 24 h. The RSDs of liquid chromatogram peak area and HSA-binding peak areas were 0.90% and 2.70%, respectively. Reproducibility of the system was evaluated by analyzing the equal amount of six independently samples. The RSDs of liquid chromatogram peak areas and HSA-binding peak areas were 1.88% and 1.55%, respectively. All the results indicated that this on-line system was suitable for the analysis of HSA-binding active compounds.

3.2.2. LOD, LOQ and linearity

The LOD and LOQ were determined to be 0.81 nmol and 1.32 nmol based on the calculation when the signal to noise (S/N) ratios was 3 and 10, respectively. The linearity relationship between HSA and rutin amount (x , nmol) was determined by plotting the HPLC peak area (y_1) and active peak area (y_2). The regression equations were as follows: $y_1 = 1E+06x - 1516$, $R^2 = 0.9999$; $y_2 = -5E+06x + 763894$, $R^2 = 0.9988$. These results suggested that this system was sufficiently sensitive and had good linearity for on-line analysis.

3.3. Identification of major constituents in shuxuening injection

In the present work, 63 compounds (vitamin C, five ginkgolides, and 57 flavones, flavonols and their glycosides) were identified or characterized in SXNI. The established system was applied to screen potential hapten components in SXNI. The HPLC fingerprint and activity profile of SXNI were shown in Fig. 4. The list of identified compounds and their structures were shown in Table S1 and Fig. 5, respectively.

3.3.1. Identification of flavones, flavonols and their glycosides

Based on the previous report [23], the main compounds in SXNI were flavonoid glycosides such as quercetin (42), kaempferol (57) and isorhamnetin glycosides. They all showed the typical UV spectra of flavonol compounds in the ranges of 250–280 and 310–360 nm. The results also showed characteristic fragments of aglycones. In addition, according to previous study [20], comparing the abundance of the radical aglycone ion $[Y_0-H]^+$ with that of aglycone ion $[Y_0]^-$, the glycosylation sites of flavonoids could be identified. In the flavonoid-*O*-monoglycosides, the relative abundance of $[Y_0-H]^+$ was higher than that of $[Y_0]^-$ presented in flavonoids-3-*O*-glycosides. In the contrary, the relative abundance of $[Y_0]^-$ was higher than $[Y_0-H]^+$ in flavonoids-7-*O*-glycosides. Among flavonoid-*O*-diglycosides, flavonoid-3-*O*-diglycosides with a 1→2 linkage produced stronger $[Y_0-H]^+$ ions than $[Y_0]^-$ with the 1→6 linkage. In this study, 57 flavonoids were identified by comparing their UVmax and MS data with standard compounds and reported data.

3.3.1.1. Identification of flavonoid-*O*-monoglycosides. All mass spectra were obtained in the negative ion mode. Flavonoids are cleaved in a similar manner, and here are some examples. The MS² spectrum of flavonoid-*O*-monoglycosides gave the $[Y_0]^-$ after losing a sugar moiety (glucose or rhamnose). Compounds 11 and 16 both produced $[M-H]^-$ ion at m/z 463 ($C_{21}H_{20}O_{12}$). In MS², they both gave aglycone ion $[Y_0]^-$ at m/z 301 and the radical aglycone ion $[Y_0-H]^+$ at m/z 300 by losing a glucosyl group. In the MS³, they all gave the characteristic ion fragments $[(Y_0-H)-(H+CO)]^-$ m/z 271, $[(Y_0-H)-(CO+H+CO)]^-$ m/z 255, 179 ($[^{1,2}A]^-$) and 151 ($[^{1,3}A]^-$), indicating that the A ring had two OH groups and the C ring had one OH group. Therefore, we inferred that the aglycone was quercetin. At the same time, the abundance of $[Y_0]^-$ at m/z 301 was weaker than that of $[Y_0-H]^+$ at m/z 300 in compound 11. Based on literature report [24], compound 11 could be flavonoid-3-*O*-glycoside. Different from compound 11, the abundance of $[Y_0]^-$ at m/z 301 was higher than $[Y_0-H]^+$ at m/z 300 in compound 16. Based on standard compounds and literature [25], compounds 11 and 16 were determined to be quercetin-3-*O*-glucoside (11) and quercetin-7-*O*-glucoside (16), respectively. Compounds 21 and 23 both exhibited $[M-H]^-$ at m/z 447 ($C_{21}H_{20}O_{11}$). In MS², they both gained the aglycone ion $[Y_0]^-$ at m/z 285 and the radical aglycone ion $[Y_0-H]^+$ at m/z 284 by losing a glucosyl group. In the MS³, they gained the fragments at m/z 255, 179 ($[^{1,2}A]^-$) and 151 ($[^{1,3}A]^-$), indicating that the A ring had two OH groups and the C ring had one OH group. Compared with standard compound, the

aglycone was inferred as kaempferol. Compounds 21 and 23 were identified as kaempferol-3-*O*-glucoside (21) and kaempferol-7-*O*-glucoside (23), respectively. Compounds 24b and 27 both exhibited $[M-H]^-$ at m/z 477 ($C_{22}H_{22}O_{12}$). Their molecular weight had a 15 Da (CH_3) difference from that of quercetin. In MS³, they displayed $[A-H-CH_3]^-$ ion at m/z 300, and $[^{1,3}A]^-$ ion at m/z 151 originating from RDA cleavage. After comparison with standard compound, their aglycone was determined as isorhamnetin (58). Different from compound 24b, the abundance of $[Y_0]^-$ at m/z 315 was higher than $[Y_0-H]^+$ at m/z 314 in compound 27. According to the standard compound and reference [23,26], compounds 24b and 27 were identified as isorhamnetin-3-*O*-glucoside (24b) and isorhamnetin-7-*O*-glucoside (27), respectively.

Compound 4 exhibited $[M-H]^-$ at m/z 479 ($C_{21}H_{20}O_{13}$). It gave the $[Y_0]^-$ at m/z 317 by losing a glucosyl group and a weaker signal of its $[Y_0-H]^+$, indicating the presence of flavonoid-3-*O*-glycosides. In the MS³, compound 4 gave the characteristic ion fragments $[(Y_0-H)-(H+CO)]^-$ at m/z 271, $[(Y_0-H)-(CO+H+CO)]^-$ at m/z 255, 179 ($[^{1,2}A]^-$) and 151 ($[^{1,3}A]^-$), indicating that the A ring had two OH groups and the C ring had one OH group. The aglycon of this compound differs from the aglycone of quercetin by a molecular weight of 16 Da, and has the same cleavage mode as quercetin, thus its B ring has one more OH group than that of quercetin. According to the standard compound and reference [23], compound 4 was identified as myricetin-3-*O*-glucoside (4).

3.3.1.2. Identification of flavonoid-*O*-diglycosides. Flavonoid aglycones of SXNI are mainly quercetin (42), apigenin (56), kaempferol (57), isorhamnetin (58), syringetin and luteolin (52) as described previously [23]. Compounds 8, 17, 19 and 20 all gained the $[Y_0]^-$ with the loss of 308 Da. Their MS² fragmentation gave ions after losing 146 and then 162 Da, suggesting that the rhamnosyl group was on the outside of the glucosyl group. All the relative abundance of $[Y_0]^-$ was higher than that of $[Y_0-H]^+$. According to the reference [27], the interglycosidic connection was identified as 1→6 linkage. Meanwhile, compounds 15, 24a and 28a all gained the $[Y_0]^-$ by the loss of 308 Da after losing 146 Da. In contrast, the relative abundance of $[Y_0]^-$ was weaker than that of $[Y_0-H]^+$, implying that their interglycosidic connection was 1→2 linkage. Compounds 8 and 15 both gained $[Y_0]^-$ at m/z 301. In their MS³ spectra, they both obtained the characteristic ion fragments $[(Y_0-H)-(H+CO)]^-$ at m/z 271, $[(Y_0-H)-(CO+H+CO)]^-$ at m/z 255, 179 ($[^{1,2}A]^-$) and 151 ($[^{1,3}A]^-$), indicating that their aglycone ion was quercetin. Furthermore, according to the reference and standard MS spectra [26], compounds 8 and 15 were assigned as quercetin-3-*O*-rutinoside (8) and quercetin-3-*O*-2''-glucosylrhamnoside (15), respectively. Both 17 and 28a displayed $[Y_0]^-$ at m/z 285 which showed identical fragmentation pattern as kaempferol (57). According to the reference and standard MS spectra [28], they were identified as kaempferol-3-*O*-rutinoside (17) and kaempferol-3-*O*-2''-glucosylrhamnoside (28a), respectively. Compounds 20 and 24a both gave $[Y_0]^-$ at m/z 345 with the similar fragmentation pattern with that of syringetin. Based on the reference [23], they were identified as syringetin-3-*O*-rutinoside (20) and syringetin-3-*O*-2''-glucosylrhamnoside (24a), respectively. Compound 19 obtained $[Y_0]^-$ at m/z 316 with the similar fragmentation pattern as isorhamnetin (58), according to the standard MS spectra, compound 19 was positively assigned as isorhamnetin-3-*O*-rutinoside (19).

3.3.1.3. Identification of ginkgolides. The other kind of major ingredients in SXNI was ginkgolides. Compound 14 gave the $[M-H]^-$ at m/z 439.1234 ($C_{20}H_{24}O_{11}$). In MS², it obtained the predominant peak at m/z 383 with losing a dimethylvinyl group. In MS³, it gave the fragments at m/z 365, 321, 303 and 259 with the losses of H_2O , H_2O+CO_2 , $2H_2O+CO_2$ and $2(H_2O+CO_2)$, indicating that compound 14 contained ester structure and more than two hydrox-

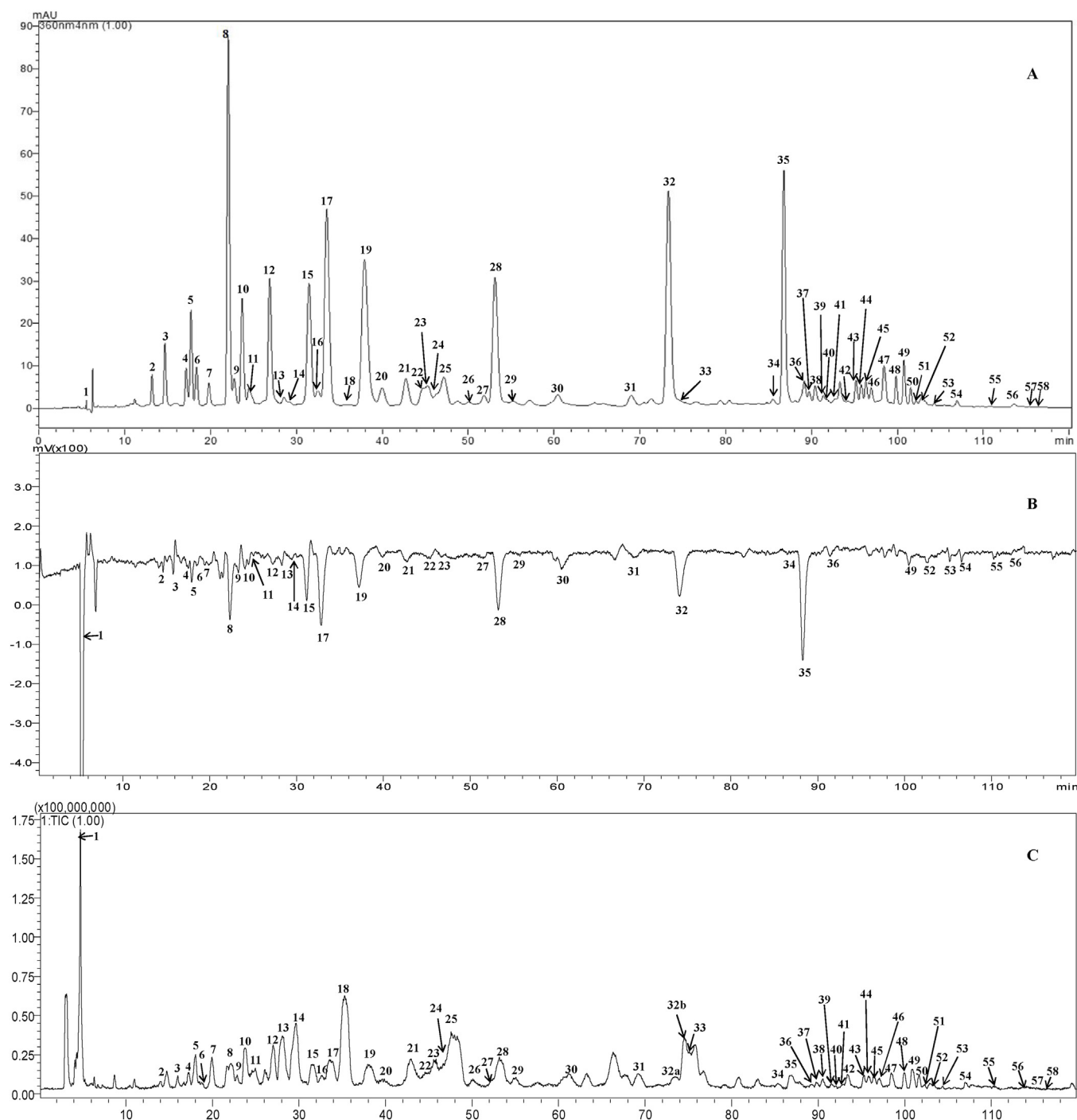


Fig. 4. The HPLC chromatogram (A), activity profile (B) and total ion chromatogram (C) of Shuxuening injection.

yls. According to the standard substance and references [29,30], compound **14** was tentatively identified as ginkgolide C (**14**). Compound **18** obtained $[M-H]^-$ at m/z 325.0923 ($C_{15}H_{18}O_8$). In MS^2 spectrum, it obtained ions at m/z 251 after losing C_4H_8 . After comparing with reference spectra [29], compound **18** was identified as bilobalide (**18**). Compound **13a** and **33** gave the $[M+COOH]^-$ at m/z 423 ($C_{20}H_{24}O_9$). They both obtained ion at m/z 367 after losing C_4H_8 . In MS^3 spectra, they gave the fragments at m/z 349 and 305 with the loss of H_2O and H_2O+CO_2 , indicating that compounds **13a** and **33** contained hydroxyl and ester structures. Comparing with reference mass spectra [29,31], compounds **13a** and **33** were temporarily assigned as ginkgolide J (**13a**) and B (**33**), respectively.

3.4. HSA-binding activity of active compounds in SXNI

Flavonoids in SXNI had a different degree of binding ability to HSA. As shown in Fig. 4, the peak areas of the fluorescence peaks are positively correlated with the peak areas in chromatogram, suggesting a dose-dependent manner of the active compounds. To further study the binding ability of compounds and HSA, we selected compounds with various aglycons based on the structures of flavonoids identified in SXNI. As a result, 15 compounds [myricetin-3-*O*-glucoside (**4**), quercetin-3-*O*-rutinoside (**8**), quercetin-3-*O*-glucoside (**11**), kaempferol-3-*O*-rutinoside (**17**), isorhamnetin-3-*O*-rutinoside (**19**), kaempferol-

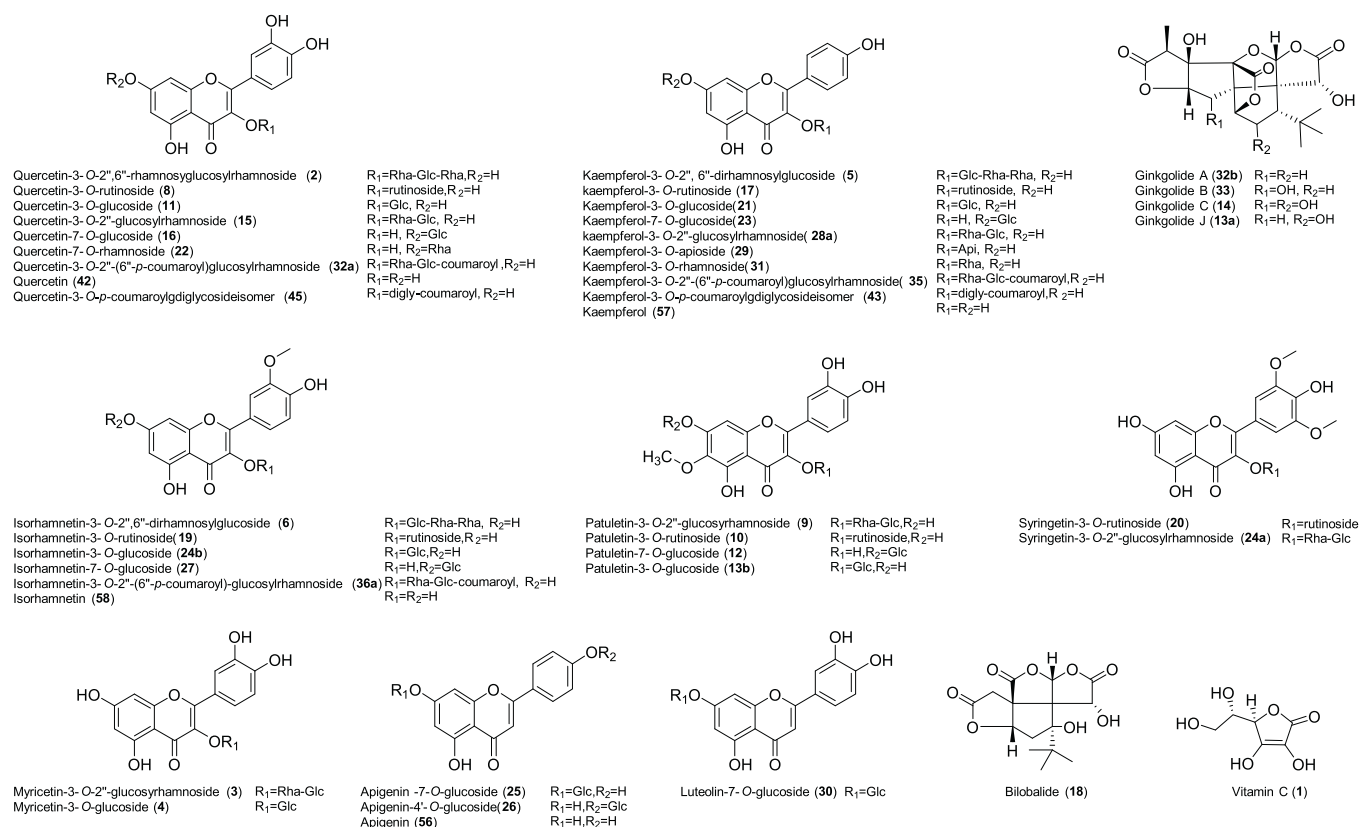


Fig. 5. Structures of the identified compounds of Shuxuening injection.

3-O-glucoside (21), isorhamnetin-3-O-glucoside (24b), apigenin-7-O-glucoside (25), luteolin-7-O-glucoside (30), quercetin (42), apigenin (56), kaempferol (57), isorhamnetin (58), myricetin (S1) and luteolin (S2)] with different aglycones at the same concentration were used to compare their absolute HSA-binding activities.

3.4.1. Binding activities relationships of 15 compounds and HSA

3.4.1.1. Binding activities relationships of 15 active compounds and HSA by on-line system. Fifteen compounds were dissolved in MeOH with the same concentration (0.1 mM) to compare the HSA-binding ability. To evaluate HSA-binding ability of each compound, the following evaluation was used:

$$NP = X_1 / Y_1 \times 100\%$$

Where X_1 represents the peak area of the compound in has-binding profile. Y_1 represents the chromatographic peak area of the compound. NP represents the ratio of the negative peak area over the positive peak area. The bigger the NP, the stronger the HSA-binding ability. The results of these compounds were shown in Table 1, which showed that the order of activity for tested compounds was as follows: apigenin (56) > luteolin (S2) > kaempferol (57) > myricetin (S1) > quercetin (42) > isorhamnetin (58).

All flavone and flavonol aglycones exhibited stronger HSA-binding ability than their glycosides. The order of the binding ability of flavone and flavonol glycosides was consistent with that of aglycones. For example, apigenin-7-O-glucoside (25) with one more glucoside group than apigenin (56) showed weaker HSA-binding affinity. This may indicate that the presence of a glucoside group impairs the HSA binding capacity due to an increased polarity and molecular size for apigenin (56), resulting in reduced affinity required for penetration into the HSA hydrophobic region. Apigenin (56) and luteolin (S2) had no substitution at C3 position, while the other C3 substituted compounds [quercetin (42), kaempferol (57), isorhamnetin (58) and myricetin (S1)] showed weaker HSA-

Table 1

Structure-activity relationships of 15 active compounds by on-line system and binding affinity among the compounds and HSA from SXNI.

Peak No.	Compound	NP	RSD%	K _D
4	Myricetin-3-O-glucoside	3.51	2.01	7.01×10^{-5} M
8	Quercetin-3-O-rutinoside	3.43	2.01	3.41×10^{-5} M
11	Quercetin-3-O-glucoside	3.76	0.48	6.89×10^{-5} M
17	Kaempferol-3-O-rutinoside	4.82	1.33	2.14×10^{-5} M
19	Isorhamnetin-3-O-rutinoside	2.32	1.06	4.10×10^{-5} M
21	Kaempferol-3-O-glucoside	6.03	0.95	5.12×10^{-5} M
24b	Isorhamnetin-3-O-glucoside	3.08	2.91	3.58×10^{-5} M
25	Apigenin-7-O-glucoside	20.42	1.22	1.05×10^{-5} M
30	Luteolin-7-O-glucoside	18.3	2.05	4.28×10^{-5} M
42	Quercetin	9.57	1.04	7.87×10^{-6} M
56	Apigenin	55	0.06	1.16×10^{-6} M
57	Kaempferol	11.55	1.61	1.85×10^{-6} M
58	Isorhamnetin	6.42	0.82	2.35×10^{-6} M
S1*	Myricetin	10.77	2.01	7.59×10^{-6} M
S2*	Luteolin	34	2.45	1.69×10^{-6} M

NP represents the ratio of the peak area value of the negative peak to the positive peak. *Myricetin and luteolin were not identified in Shuxuening injection, and are used as standard substance 1 (S1) and standard substance 2 (S2).

binding activities. Therefore, we speculated that the presence of C3 substituents was not beneficial for the binding between compounds and HSA. In addition, among the C3 substituted compounds [quercetin (42), kaempferol (57), isorhamnetin (58) and myricetin (S1)], kaempferol (57) showed stronger HSA-binding activities than others, and it was the only one that had no substitution on the C3' position. Furthermore, apigenin (56) showed the highest NP value than all other compounds, the only difference between apigenin (56) and luteolin (S2) was that apigenin (56) had no C3' substitution group on the B ring. Therefore, we speculated that the presence of C3'-substituents on the B ring was also not conducive to the HSA-binding activity.

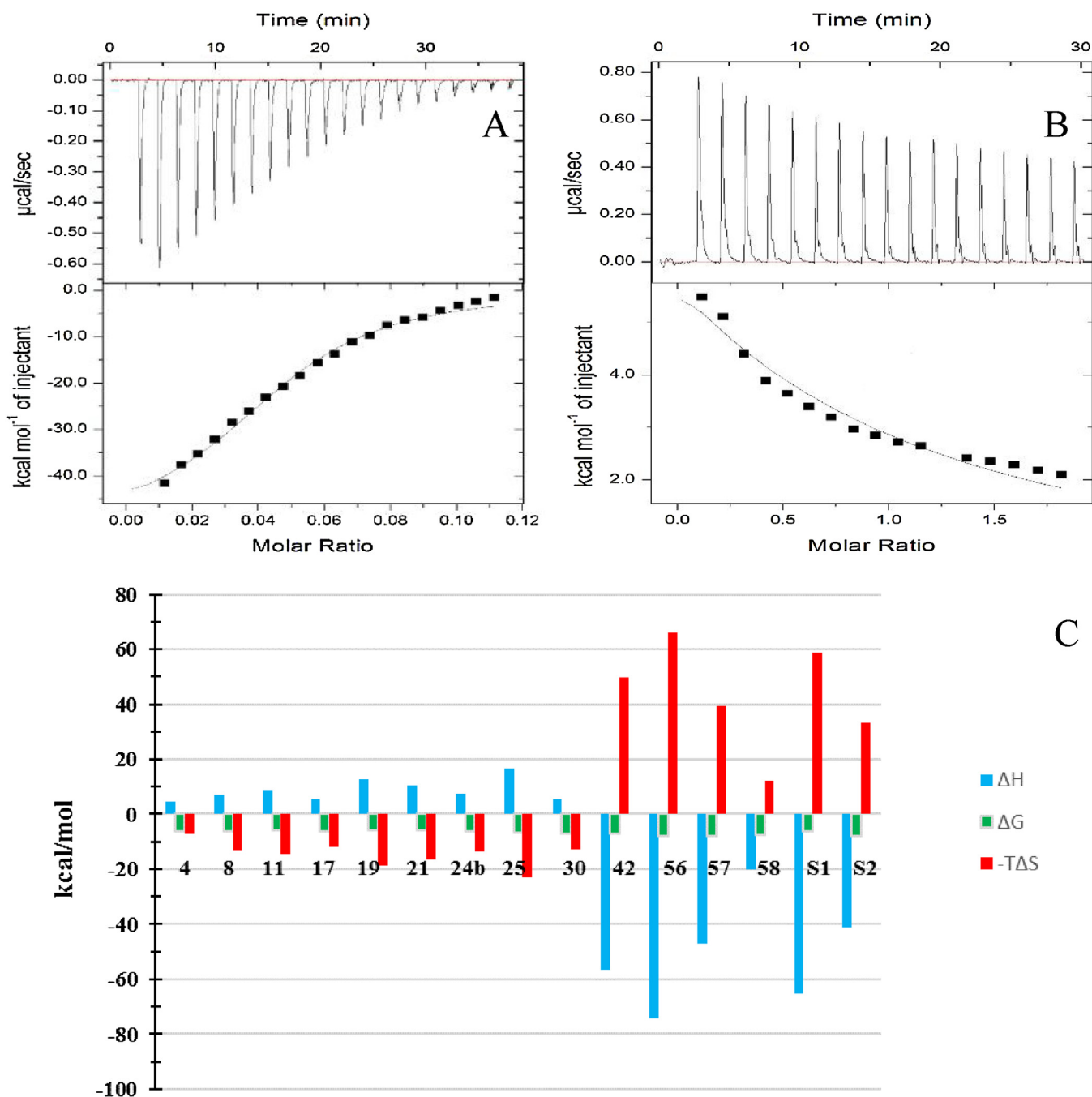


Fig. 6. Isothermal titration calorimetry for HSA interaction with kaempferol (A) and kaempferol-3-O-glucoside (B) at 285 K, pH 7.4. Column chart contained ΔH , ΔG and $-T\Delta S$ in flavonoids and their glycosides (C). (4. myricetin-3-O-glucoside, 8. quercetin-3-O-rutinoside, 11. quercetin-3-O-glucoside, 17. kaempferol-3-O-rutinoside, 19. isorhamnetin-3-O-rutinoside, 21. kaempferol-3-O-glucoside, 24. isorhamnetin-3-O-glucoside, 25. apigenin-7-O-glucoside, 30. luteolin-7-O-glucoside, 42. quercetin, 56. apigenin, 57. kaempferol, 58. isorhamnetin, S1. myricetin, S2. luteolin. Myricetin and luteolin were not identified in Shuxuening injection and were used as standard compounds S1 and S2.).

3.4.1.2. Binding activities relationships of 15 compounds and HSA by ITC. The ITC experiment could be used to investigate the binding affinity, enthalpy changes (ΔH), entropy change (ΔS) and free energy change (ΔG) of the interactions between HSA and ligands [14,32]. Negative free energy change ($\Delta G < 0$) reflected that the interaction of all 15 compounds with HSA was spontaneous [33]. According to the literature reports [11,33], the positive values of ΔH and ΔS indicated that the main force was hydrophobic effect, the negative values of ΔH and ΔS indicated that the predominant interaction force was van der Waals interaction and hydrogen bonding.

The results showed that the interaction between flavonoid aglycones and HSA was van der Waals interaction and hydrogen bonding, while the interaction between flavonoid glycosides and HSA was hydrophobic effect. As an example, the ΔH , ΔS and ΔG of kaempferol (57) and its glycosides after binding to HSA were shown in Fig. 6.

Furthermore, both the online system and the ITC experiments were used to study the binding ability of these 15 compounds to HSA. As shown in Table 1, the results showed good consistency of results from the online system and the ITC experiments. Aglycone compounds have stronger binding ability to HSA than their

Table 2

The result of anaphylactoid reaction test and anaphylaxis reaction test of RBL-2H3 cell assay.

Peak No.	Compound	The OD value of anaphylactoid reaction test (n = 3)	The OD value of anaphylaxis reaction test (n = 3)
4	Myricetin-3-O-glucoside	0.58 ± 0.021	0.43 ± 0.029
8	Quercetin-3-O-rutinoside	0.42 ± 0.031	0.39 ± 0.020
11	Quercetin-3-O-glucoside	0.48 ± 0.098	0.48 ± 0.098
14	Ginkgolide C	0.30 ± 0.016	–
17	Kaempferol-3-O-rutinoside	0.47 ± 0.020	0.36 ± 0.014
18	Bilobalide	0.32 ± 0.047	–
19	Isorhamnetin-3-O-rutinoside	0.36 ± 0.020	0.31 ± 0.060
21	Kaempferol-3-O-glucoside	0.51 ± 0.100	0.48 ± 0.010
24b	Isorhamnetin-3-O-glucoside	0.41 ± 0.039	0.41 ± 0.073
25	Apigenin-7-O-glucoside	0.77 ± 0.012	0.80 ± 0.038
30	Luteolin-7-O-glucoside	0.72 ± 0.026	0.68 ± 0.022
32b	Ginkgolide A	0.33 ± 0.041	0.28 ± 0.032
33	Ginkgolide B	0.29 ± 0.046	–
42	Quercetin	0.71 ± 0.083	0.87 ± 0.011
56	Apigenin	0.93 ± 0.113	1.08 ± 0.014
57	Kaempferol	0.87 ± 0.011	0.84 ± 0.030
58	Isorhamnetin	0.52 ± 0.080	0.77 ± 0.013
S1*	Myricetin	0.70 ± 0.131	0.85 ± 0.011
S2*	Luteolin	0.85 ± 0.033	0.97 ± 0.015
–	Blank group	0.26 ± 0.043	0.28 ± 0.032

OD is the abbreviation of optical density, which indicates the optical density absorbed by the detected substance. *Myricetin and luteolin were not identified in Shuxuening injection, and are used as standard substance 1 (S1) and standard substance 2 (S2).

respective glycosides, and flavonoids showed stronger binding ability than flavonols. Both apigenin (56) and luteolin (S2) exhibited strong HSA binding ability than other compounds. In addition, it was also verified that the presence of a C3 substitution and a C3' substitution is disadvantageous for the HSA binding.

Meanwhile, the predominant forces between aglycones and HSA are van der Waals or hydrogen bond interactions. The main force between flavonoid glycosides and HSA is hydrophobic interaction, which is weaker than van der Waals or hydrogen bonding. Furthermore, according to literature reports [32,34,35], HSA has three homologous domains (I, II and III), and each domain has two subdomains (A and B). Studies [34–36] have shown that some drug molecules interacted with HSA in the hydrophobic cavities (domains IIA and IIIA). Meanwhile, the binding sites of baicalin, baicalin aglycon and flavonoid derivatives in HSA are all located in IIA [36,37], indicating that different binding abilities of flavonoids are structure-dependent. In a word, the HSA binding ability of flavonoid aglycones is stronger than that of flavonoid glycosides. The molecular volume of flavonoid glycosides is larger than that of flavonoid aglycones, which is not conducive to HSA binding.

3.5. Validation of 15 compounds as haptens in RBL-2H3 cell assay

3.5.1. Anaphylactoid reaction test of RBL-2H3 cell assay

Fifteen flavonoids (flavonols aglycone, flavones aglycone and their glycosides) and four Ginkgolides were selected for anaphylactoid reaction test in RBL-2H3 cells. Results (Table 2) showed that ginkgolides had almost no sensitization and no HSA binding capacity. In the anaphylactoid reaction test, the OD values of aglycone and their glycosides were evaluated by student's *t*-test. The results showed that the *p* value was less than 0.05, indicating a significant difference in sensitivity between aglycone and glycosides. Aglycone was more likely to induce histamine release from RBL-2H3 cells. At the same time, aglycones had stronger HSA binding capacity than glycosides, and apigenin (56) showed stronger sensitization than other compounds in allergic experiments.

3.5.2. Anaphylaxis reaction test of RBL-2H3 cell assay

The IgE standard curve was as follows: $Y = 0.0203X + 0.3903$, $R^2 = 0.9985$. *Y* stands for OD value and *X* represents IgE concentration. Compared with the normal control group, the level of IgE increased in the experimental group. In the anaphylaxis reaction

(Table 2), the OD values of aglycone and their glycosides were evaluated by student's *t*-test. The results showed that the *p* value was also less than 0.05, indicating a significant difference between aglycone and the glycosides. For anaphylaxis reaction test, aglycone was also more likely to induce histamine release from RBL-2H3 cells than their glycosides.

By comparing the results of anaphylaxis reaction test and anaphylactoid reaction test, it was found that aglycones were more likely to cause RBL-2H3 cells to release histamine than their glycosides. In addition, HSA-binding activity of compounds in SXNI is positively correlated with their sensitization activity.

4. Conclusion

In this study, an on-line HPLC–DAD–MSⁿ–HSA–FLD system was established to screen haptens in SXNI for the first time. Using this system, 63 ingredients were identified in SXNI, flavonoids in which showed different HSA-binding activity. In fluorescence detection and ITC experiments, flavonoid aglycones had stronger HSA binding activity than their glycosides. The aglycones also displayed strong sensitization in RBL-2H3 cell allergic test and class-allergic test. In summary, the workflow of this study including the established on-line system is of great importance for rapid screening of potential haptens in complex samples.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.chroma.2019.04.011>.

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Supporting information

Development of an on-line analytical platform to screen haptens in Shuxuening injection based on high-performance liquid chromatography coupled with ion-trap multistage mass spectrometry and human serum albumin fluorescence detection

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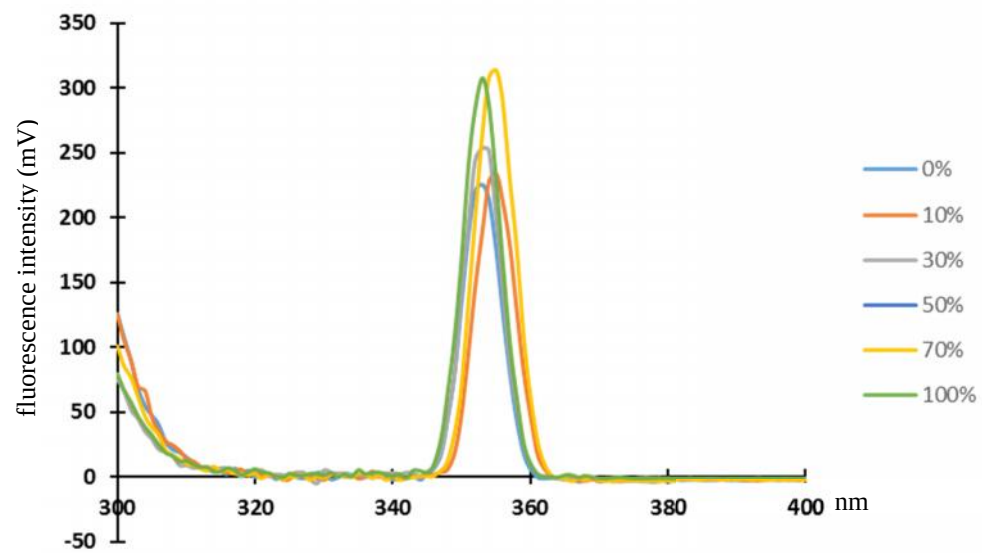


Fig.S1. Emission spectrum of HSA in different ratios of acetonitrile.

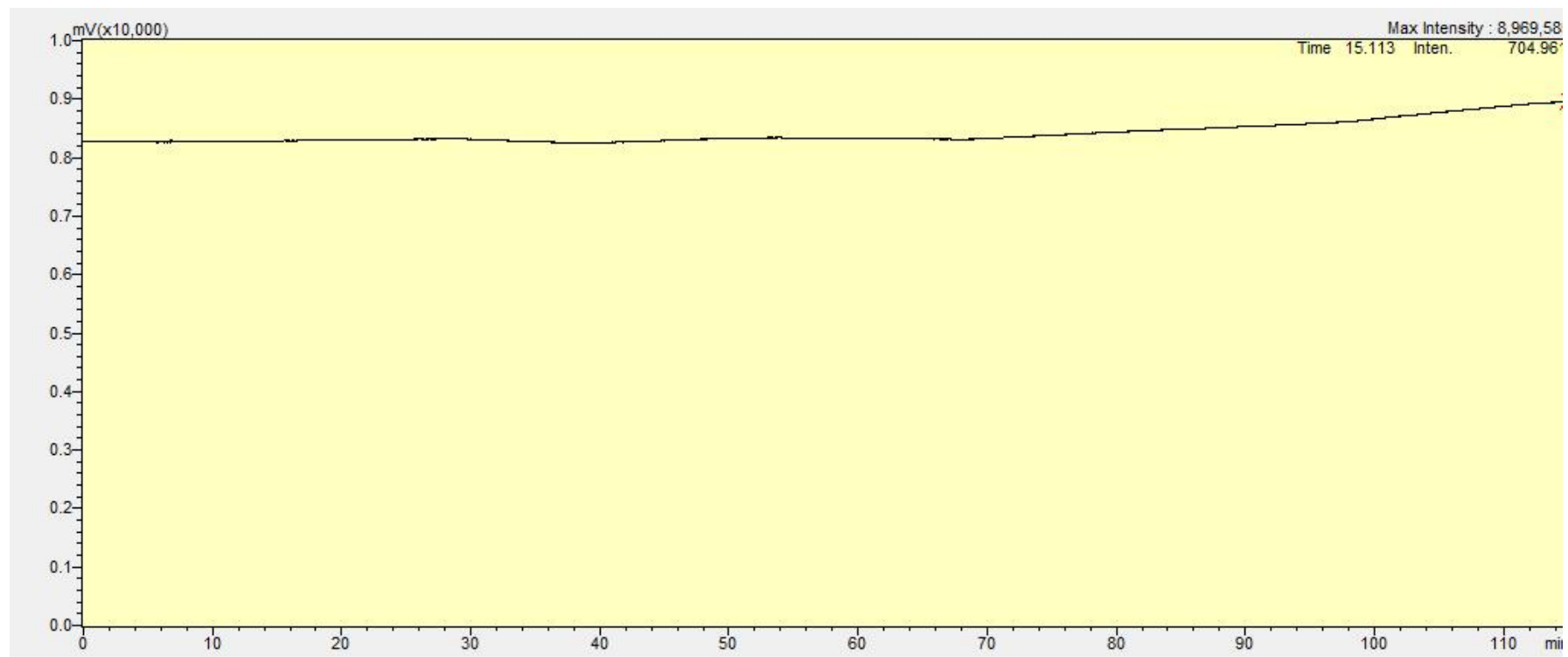


Fig.S2. Blank control fluorescence spectrum of HSA binding activity study.

Table S1 The UV and MSⁿ data for identification of 63 compounds in SXNI.

Peak No.	t _R (min)	UV(nm)	Formula	[M-H] ⁺ / [M-H] ⁻	ESI-MS ⁿ	Identification
1	4.473	224,242	C ₆ H ₈ O ₆	175.0246(+1.71)	MS ² (175.0246):131.014(100),115.0103	Vitamin C
2	12.853	255,349	C ₃₃ H ₄₀ O ₂₀	755.2005(-4.63)	MS ² (755.2005):300.0241(100),609.1422,489.1005 447.0175,301.0294 MS ³ (300.0241):271.0231(100),179.0044,151.0004	Quercetin-3-O-2",6"-rhamnosyglucosylrhamnoside
3	15.047	258,356	C ₂₇ H ₃₀ O ₁₇	625.1387(-2.88)	MS ² (625.1387):316.0203(100),317.0270,287.0230,271.0230 MS ³ (316.0203):271.0217(100),179.0036,151.0116	Myricetin-3-O-2"-glucosyrhamnoside
4	16.815	265,327	C ₂₁ H ₂₀ O ₁₃	479.0832(+1.25)	MS ² (479.0832):316.0222(100),317.0298,287.0175 271.0279 MS ³ (316.0221):271.0224(100),150.9980	Myricetin-3-O-glucoside
5	17.845	266,346	C ₃₃ H ₄₀ O ₁₉	739.2073(-2.44)	MS ² (739.2073):575.1353(100),593.1456,473.1045 447.1015,285.0361 MS ³ (575.1353):257.0456(100),285.0389 MS ⁴ (285.0389):151.0079(100)	Kaempferol-3-O-2",6"-dirhamnosylglucoside
6	18.330	254,353	C ₃₄ H ₄₂ O ₂₀	769.2195(+0.26)	MS ² (769.2195):314.0405(100),623.1510,503.1236315.0478,300.0280,271.0255 MS ³ (314.0406):299.0176(100),300.0248,271.0233151.0115 MS ³ (300.0248):271.0239,243.0288,151.0062	Isorhamnetin-3-O-2",6"-dirhamnosylglucoside
7	19.670	231,316	C ₁₄ H ₂₀ O ₇	299.1118(-6.02)	MS ² (299.1118):193.1124(100),175.1154	Unknow
8	22.350	256,354	C ₂₇ H ₃₀ O ₁₆	609.1439(-3.61)	MS ² (609.1439):301.0336(100),463.0896,343.0428	Quercetin-3-O-rutinoside

					300.0245,271.0262	
					MS ³ (300.0284):151.0061(100),271.0221,255.0289	
					179.0000	
9	22.723	264,346	C ₂₈ H ₃₂ O ₁₇	639.1569(+0.31)	MS ² (639.1569):316.0186(100),331.0424,317.0208,287.0151,287.0174,271.0220	Patuletin-3- <i>O</i> -2"-glucosyrhamnoside
					MS ³ (331.0424):316.0198(100)	
					MS ⁴ (317.0240):271.0231(100),178.9988,151.0046	
10	23.760	267,346	C ₂₈ H ₃₂ O ₁₇	639.1555(-1.88)	MS ² (639.1555):331.0432(100),477.0951,330.0333,315.0124,287.0174,271.0220	Patuletin-3- <i>O</i> -rutinoside
					MS ³ (330.0333):316.0197(100),178.9984,151.0066	
11	24.583	256,355	C ₂₁ H ₂₀ O ₁₂	463.0885(0.65)	MS ² (463.0885):300.0242(100),343.0407,301.0320,343.0387,	Quercetin-3- <i>O</i> -glucoside
					MS ³ (301.0320):151.0068(100),255.0289,178.9999	
12	26.890	255,354	C ₂₂ H ₂₂ O ₁₃	493.1001(2.64)	MS ² (493.1001):331.0463(100),330.0384,316.0217	Patuletin-7- <i>O</i> -glucoside
					315.0142,271.0226	
					MS ³ (331.0462):315.0125(100),287.0261,271.0261	
					179.0030,151.0065	
13a	27.995	nd	C ₂₀ H ₂₄ O ₁₀	423.1289(-2.36)	MS ² (423.1289):349.0895(100),323.1140,	Ginkgolide J
13b	28.698	268,326	C ₂₂ H ₂₂ O ₁₃	493.0963(-3.85)	MS ² (493.0963):315.0103(100),331.0434,330.0350	Patuletin-3- <i>O</i> -glucoside
					316.0164	
					MS ³ (315.0103):287.0181(100),271.0222,151.0246	
14	28.997	nd	C ₂₀ H ₂₄ O ₁₁	439.1234(-2.50)	MS ² (439.1234):383.1302(100),365.1249,321.1325	Ginkgolide C
					303.1225,277.1461,259.1312	

					MS ³ (383.1302):365.1249(100)	
					MS ³ (383.1347):259.1307(100)	
15	32.140	255,350	C ₂₇ H ₃₀ O ₁₆	609.1432(-4.76)	MS ² (609.1432):300.0218(100),505.0919,447.0919 429.0772,301.0297 MS ³ (300.0218):271.0235(100),255.0305,179.0007 151.0118	Quercetin-3-O-2"-glucosylrhannoside
16	32.438	269,346	C ₂₁ H ₂₀ O ₁₂	463.0883(+0.22)	MS ² (463.0883):301.0340(100),300.0315(5.2) MS ³ (300.0340):151.0086(100),255.0326	Quercetin-7-O-glucoside
17	33.438	265,347	C ₂₇ H ₃₀ O ₁₅	593.1481(-5.23)	MS ² (593.1481):285.0380(100),447.0915,284.0306 MS ³ (285.0380):257.0460(100),229.0514,163.0070	Kaempferol-3-O-rutinoside
18	35.605	nd	C ₁₅ H ₁₈ O ₈	325.0923(3.38)	MS ² (325.0923):251.0953(100) MS ³ (325.0923):163.1230(100)	Bilobalide
19	37.673	254,354	C ₂₈ H ₃₂ O ₁₆	623.1595(-2.73)	MS ² (623.1595):315.0486(100),316.0505,301.0326,300.0240,271.0211,255.0279 MS ³ (315.0486):300.0263(100) MS ⁴ (300.0263):271.0217(100),255.0289,151.0060	Isorhamnetin-3-O-rutinoside
20	40.512	254,359	C ₂₉ H ₃₄ O ₁₇	653.1725(+1.07)	MS ² (653.1725):345.0593(100),344.0511,330.0350,315.0122 MS ³ (345.0593):330.0357(100),315.0132 MS ⁴ (315.0132):287.0184(100),271.0222,151.0129	Syringetin-3-O-rutinoside
21	42.728	265,345	C ₂₁ H ₂₀ O ₁₁	447.0921(-2.68)	MS ² (447.1006):284.0301(100),327.0493,285.0371 MS ³ (285.0371):255.0299(100),163.0068,151.0071	Kaempferol-3-O-glucoside

22	44.732	266,372	C ₂₁ H ₂₀ O ₁₁	447.0910(-3.80)	MS ² (447.0910):301.0136(100),300.0244,271.0222 MS ³ (300.0244):151.0067(100),255.0271,178.9992	Quercetin-7-O-rhamnoside
23	45.275	256,348	C ₂₁ H ₂₀ O ₁₁	447.0929(+0.40)	MS ² (447.0929):285.0373(100),284.0308, 271.0326 MS ³ (284.0308):151.0151(100),	Kaempferol-7-O-glucoside
24a	46.410	254,358	C ₂₉ H ₃₄ O ₁₇	653.1697(-3.98)	MS ² (653.1671):345.0592(100),507.1111,330.0340,315.0121 MS ³ (345.0592):330.0345(100),315.0111,271.0230 163.0081 MS ³ (330.0345):315.0128(100),271.0233,151.0128	Syringetin-3-O-2"-glucosylrhamnoside
24b	46.990	265,344	C ₂₂ H ₂₂ O ₁₂	477.1041(+0.42)	MS ² (477.1041):314.0389(100),357.0572,300.0236285.0371,243.0257 MS ³ (314.0389):285.0396(100),271.0246,243.0296 MS ⁴ (271.0246):227.0345(100)	Isorhamnetin-3-O-glucoside
25	47.560	266,327	C ₂₁ H ₂₀ O ₁₀	431.0987(+0.70)	MS ² (431.0987):269.0432(100),268.0356, MS ³ (269.0432):225.0647(100),149.0264	Apigenin-7-O-glucoside
26	50.172	270,319	C ₂₁ H ₂₀ O ₁₀	431.0958(-5.78)	MS ² (431.0958):269.0458(100),268.0383 MS ³ (269.1152):225.0542(100),149.0236	Apigenin-4'-O-glucoside
27	51.478	264,340	C ₂₂ H ₂₂ O ₁₂	477.1007(-6.49)	MS ² (477.1007):299.0173(100),357.0504,315.0461 314.0396,300.0255 MS ³ (299.0173):243.0224(100),271.0255	Isorhamnetin-7-O-glucoside
28a	52.937	264,343	C ₂₇ H ₃₀ O ₁₅	593.1484(-4.72)	MS ² (593.1484):284.0295(100),431.0945,413.0842285.0364 MS ³ (284.0295):255.0279(100),227.0364,151.0063	Kaempferol-3-O-2"-glucosylrhamnoside

28b	53.908	265.342	C ₁₅ H ₁₂ O ₆	287.0561(0.0)	MS ² (287.0561):259.0588(100),243.0702 MS ³ (259.0587):173.0621(100),215.0651	Katuranin
29	55.490	264,354	C ₂₀ H ₁₈ O ₁₀	417.0805(-5.27)	MS ² (417.0805):284.0291(100),285.0372,255.0285 MS ³ (284.0291):255.0366(100),227.0395	Kaempferol-3-O-apioside
30	60.473	267,339	C ₂₁ H ₂₀ O ₁₁	447.0922(-2.46)	MS ² (447.0922):285.0371(100) MS ³ (285.0371):175.0421(100), 151.0240	Luteolin-7-O-glucoside
31	68.823	263,323	C ₂₁ H ₂₀ O ₁₀	431.0973(-2.55)	MS ² (431.0973):285.0378(100),255.0274 MS ³ (285.0378):255.0291(100),227.0316,163.0042	Kaempferol-3-O-rhamnoside
32a	73.028	267,317	C ₃₆ H ₃₆ O ₁₈	755.1827(-0.26)	MS ² (755.1827):609.1408(100),429.0824,301.0335 300.0225,271.0230 MS ³ (609.1416):300.0232(100),271.0227,255.0279 MS ⁴ (300.0232):271.0225(100),255.0282,151.0041	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside
32b	74.173	nd	C ₂₀ H ₂₄ O ₉	453.1393(-1.99) [M+COOH] ⁻	MS ² (453.1393):351.1435(100),407.1316,333.1387 319.1491 MS ³ (351.1435):333.1277(100),289.1366,245.1456,	Ginkgolide A
33	74.747	Nd	C ₂₀ H ₂₄ O ₁₀	423.1287(-2.36)	MS ² (423.1287):367.1375(100),349.1265,305.1266 287.1267 MS ³ (367.1387):349.1265(100),261.1483	Ginkgolide B
34	85.770	257,319	C ₃₆ H ₃₆ O ₁₈	755.1824(-0.66)	MS ² (755.1824):300.0282(100),609.1481,301.0334 271.0257,255.0274	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer

					MS ³ (300.0282):271.0261(100),255.0338,151.0103	
					117.5418	
35	86.448	267,315	C ₃₆ H ₃₆ O ₁₇	739.1867(-1.76)	MS ² (739.1867):593.1471(100),431.0972,413.0881	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside
					285.0397	
					MS ³ (593.1471):284.0300(100),285.0362,255.0273	
					MS ⁴ (284.0316):255.0306(100),151.0047	
36a	88.938	264,316	C ₃₇ H ₃₇ O ₁₈	769.1990(+0.65)	MS ² (769.1990):623.1548(100),461.0998,315.0472	Isorhamnetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside
					MS ³ (623.1548):315.0480(100),299.0181,271.0187	
					MS ⁴ (315.0480):299.0176(100),300.0252,271.0233	
36b	89.143	257,315	C ₃₆ H ₃₆ O ₁₈	755.1811(-2.38)	MS ² (755.1811):609.1404(100),447.0924,301.0307,300.0231	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (609.1404):300.0235(100),447.0904,301.0306,271.0215	
					255.0315	
					MS ⁴ (300.0234):271.0219(100),255.0277,178.9972,151.0033	
37	90.065	254,347	C ₃₆ H ₃₆ O ₁₈	755.1796(-4.37)	MS ² (755.1796):300.0226(100),609.1426,301.0324	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (609.1426):300.0215(100),447.0916,429.0795,255.0254	
					MS ⁴ (300.0215):271.0217(100),178.9923,151.0031	
38	90.652	257,349	C ₃₆ H ₃₆ O ₁₈	755.1816(-1.72)	MS ² (755.1816):609.1381(100),447.0882,429.0796	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (609.1381):300.0218(100),301.0306,271.0207,	
					MS ⁴ (300.0218):271.0239(100),255.0308,179.0055,151.0054	
39	91.553	255,347	C ₃₆ H ₃₆ O ₁₈	755.1801(-3.71)	MS ² (755.1801):609.1375(100),447.0872,429.0781	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer

					MS ³ (609.1373):300.0243(100),301.0318,271.0217	
					MS ⁴ (300.0243):271.0207(100),255.0318,178.9955,151.0057	
40	91.777	254,357	C ₃₆ H ₃₆ O ₁₈	755.1807(-2.91)	MS ² (755.1807):609.1401(100),447.0939,429.0786	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (609.1401):300.0231(100),301.0310,271.0219	
					MS ⁴ (301.0310):271.0207(100),255.0257,179.0044,151.0013	
41	92.670	254,356	C ₃₆ H ₃₆ O ₁₈	755.1830(+0.13)	MS ² (755.1830):609.1415(100),447.1030,429.0812	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (609.1415):300.0235(100),301.0359,271.0219	
					MS ⁴ (300.0235):271.0188(100),255.0347	
42	94.137	260,350	C ₁₅ H ₁₀ O ₇	301.0344(-3.32)	MS ² (301.0344):271.0246(100),178.9980,151.0071	Quercetin
43	95.237	265,345	C ₃₄ H ₃₆ O ₁₉	747.1816(+5.09)	MS ² (747.1816):593.1475(100),413.0856,255.0285	Kaempferol-3-O-p-coumaroylgdiglycoside isomer
					MS ³ (593.1475):284.0302(100),285.0372,255.0292	
					MS ⁴ (284.0302):255.0309(100),227.0323,151.0013	
44	95.852	263,348	C ₃₄ H ₃₆ O ₁₉	747.1790(+1.61)	MS ² (747.1790):593.1490(100)	Kaempferol-3-O-p-coumaroylgdiglycoside isomer
					MS ³ (593.1490):284.0298(100),413.0856,255.0262	
					MS ⁴ (284.0298):255.0312(100),227.0323,163.0020	
45	96.550	264,349	C ₃₄ H ₃₆ O ₁₉	747.1826(+6.42)	MS ² (747.1826):609.1446(100),593.1505,413.0853	Quercetin-3-O-p-coumaroylgdiglycoside
					MS ³ (609.1446):300.0229(100),301.0284,271.0205	
					MS ⁴ (301.0283):271.0219(100),255.300,178.9917,151.0071	
46	96.783	265,349	C ₃₄ H ₃₆ O ₁₉	747.1801(+3.08)	MS ² (747.1790):593.1484(100),413.0871,255.0274,	Kaempferol-3-O-p-coumaroylgdiglycoside isomer
					MS ³ (593.1484):284.0310(100),285.0374,255.0280	

					MS ⁴ (284.0298):255.0293(100),227.0328,163.0202,151.0059	
47	97.788	254,347	C ₃₆ H ₃₆ O ₁₈	755.1795(-4.38)	MS ² (755.1795):609.1387(100),300.0252 MS ³ (609.1387):300.0244(100),447.0916,429.0795,301.0370 MS ⁴ (301.0370):271.0247(100),255.0297,179.0044	Quercetin-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
48	98.497	264,344	C ₂₂ H ₂₂ O ₁₂	739.1840(-5.41)	MS ² (739.1840):593.1461(100),413.0831 MS ³ (593.1461):285.0211(100),284.0291,255.0266 MS ⁴ (284.0297):255.0296(100),227.0322,151.0146	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
49	99.960	264,344	C ₂₂ H ₂₂ O ₁₂	739.1844(-4.87)	MS ² (739.1844):593.1477(100),413.0862 MS ³ (593.1477):285.0211(100),284.0298,255.0266 MS ⁴ (284.0298):255.0296(100),227.0377,151.0044	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
50	100.890	265,344	C ₂₂ H ₂₂ O ₁₂	739.1864(-2.16)	MS ² (739.1864):593.1484(100),413.0877 MS ³ (593.1484):285.0367(100),284.0302,255.0287 MS ⁴ (284.0302):255.0274(100),227.0361,151.0066	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
51	101.692	264,345	C ₂₂ H ₂₂ O ₁₂	739.1838(-5.68)	MS ² (739.1838):593.1459(100),413.0863 MS ³ (593.1459):285.0378(100),284.0312,255.0290 MS ⁴ (284.0312):255.0276(100),227.0352,151.0050	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
52	102.093	265,349	C ₂₂ H ₂₂ O ₁₂	739.1816(-8.65)	MS ² (739.1816):593.1483(100),413.0883 MS ³ (593.1483):285.0361(100),284.0235,255.0331 MS ⁴ (284.0235):255.0325(100),227.0252	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
53	102.772	263,347	C ₂₂ H ₂₂ O ₁₂	739.1882(+0.27)	MS ² (739.1882):593.1429(100),413.0857	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer

					MS ³ (593.1429):285.0297(100),284.0306,255.0266	
					MS ⁴ (284.0297):255.0296(100),227.0322,151.0146	
54	104.262	265,347	C ₂₂ H ₂₂ O ₁₂	739.1873(-0.94)	MS ² (739.1873):593.1462(100),413.0831	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (593.1462):285.0374(100),284.0306,255.0277	
					MS ⁴ (285.0374):255.0280(100),227.0329,151.0129	
55	107.073	266,343	C ₂₂ H ₂₂ O ₁₂	739.1843(-5.01)	MS ² (739.1873):593.1443(100),413.0861	Kaempferol-3-O-2''-(6''-p-coumaroyl)glucosylrhamnoside isomer
					MS ³ (593.1462):284.0304(100),284.0368,255.0291	
					MS ⁴ (284.0304):255.0289(100),227.0387,151.0059	
56	110.842	260,354	C ₁₅ H ₁₀ O ₅	269.0445(-3.72)	MS ² (269.0445):201.0516(100),225.0532,149.0236	Apigenin
57	115.138	254,354	C ₁₅ H ₁₀ O ₆	285.0400(-1.40)	MS ² (285.0400):255.0222(100),151.0362	Kaempferol
58	116.723	251,365	C ₁₆ H ₁₂ O ₇	315.0494(-4.76)	MS ² (315.0494):300.0237(100)314.0396,300.0255	Isorhamnetin
					MS ³ (300.0237):271.0298(100),151.0052	

*nd: not detected. t_R represents the retention time of the compound in LC/MS analysis.