SUPPLEMENTARY MATERIAL

Integrated extraction and purification of total bioactive flavonoids from *Toona sinensis* leaves

Yuping Shen, Minhui Xu, Yufei Chen, Haiyan Wang, Yiying Zhou, Yeting Zhu, Huan Yang*, Jiangnan Yu*

School of Pharmacy, Jiangsu University, Zhenjiang 212013, China

**Corresponding authors*: School of Pharmacy, Jiangsu University, Zhenjiang 212013, Jiangsu Province, PRC. Tel.: +86 (511) 85038170; Fax: +86 (511) 88795939.

E-mail addresses: yanghuan1980@ujs.edu.cn (Huan Yang); yjn@ujs.edu.cn (Jiangnan Yu).

1. Experimental

1.1. Materials and chemicals

TSL was harvested on campus and authenticated to be the leaves of *Toona sinensis* Roemer by Professor Jun Chen, Jiangsu University, China. And, a voucher specimen (No. SP20180401) was deposited in Pharmacognosy Research Facility, School of Pharmacy, Jiangsu University, China. They were dried in a ventilate place under the shade to remove most of water prior to further lyophilization, and the fine powder was collected to be used for the experiments after smash and sieve. NKA-9, D101, HPD400, HPD100, AB-8, S-8 and X-5 macroporous resins were purchased from Zhengzhou Qinshi Keji Co., Ltd., China. Rutin reference material with a purity of 98.2% was purchased from Chengdu Pufei De Biotech Co., Ltd., China. HPLC-grade acetonitrile was purchased from J&K scientific Ltd., Shanghai, China. HPLC-grade formic acid was obtained from Aladdin Industrial Corporation, Shanghai, China. All the other reagents including acetic acid, MeOH and EtOH from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, were of analytical grade, and water was prepared by a Milli-Q water-purification system from Millipore, Bedford, MA,

USA.

1.2. Conventional method

1.2.1 Reflux extraction

The dried TSL powder (9.5 g) was refluxed with 95 mL of 60% EtOH for 2.0 hrs thrice, and suction filtration was performed. The solutions were pooled and then concentrated to about 50 mL under reduced pressure below 50℃ by a rotary evaporator.

1.2.2 Macroporous resin purification

The extract obtained above was purified using a glass column (100 mm × 15 mm) packed with 20 mL of pretreated HPD100 macroporous resin. After a complete loading of the sample, the column was washed with 3.0 BV of water and eluted with 5.0 BV of 70% EtOH successively at a flow rate of 2.0 BV/h. All of the eluent was collected and subject to UV spectrophotometer.

1.3. Determination of total flavonoids

The content of total flavonoids was determined by the colorimetric method (Liu et al. 2011; Pan et al. 2012) with some modifications. Briefly, rutin was used as the reference standard, and a series of standard solution ranging from 3.750×10^{-2} mg/mL to 1.200 mg/mL were prepared in 60% EtOH. 1 mL of individual standard solution and 1 mL of 5% $NaNO_2$ (*w/v*) were swirled for 6 mins, and then 1 mL of 10% $AlCl_3$ (*w/v*) was added and reacted for another 6 mins, followed by the addition of 10 mL of 1 *M* NaOH. After standing for 15 mins, the absorbance (Abs) of the resulting solution was measured at 510 nm by UV-7504 spectrophotometer. The Abs (y) *versus* the concentration of rutin (x) was then plotted for the calibration curve by ordinary linear regression using Microsoft Office Excel 2003. The content of the total flavonoids in all samples was then determined, the Abs was measured, and the concentration of total flavonoids was calculated according to the calibration curve $y = 1.1653x - 0.0013$ ($r^2 = 0.9998$).

1.4. Selection of macroporous resins

1.4.1 Selection of macroporous resins by static adsorption and desorption

25.0 g of dried TSL powder was extracted twice by reflux extraction in 250 mL water for 2 hrs, and the solutions were pooled and scaled up in a 500 mL volumetric flask for subsequent use. Seven macroporous resins, including NKA-9, D101, HPD400, HPD100, AB-8, S-8 and X-5 were investigated for recovery of total flavonoids from TSL extracts by static adsorption at first. Adsorption experiments were carried out in 100 mL flasks, which were maintained at 25℃ for 24 hrs with continuous vibration at 125 rpm. Solid/liquid ratio was fixed at 1:50, namely 1 g of adsorbent (dry weight) for 50 mL of the above TSL extract. Then, desorption experiments were performed in same flasks, and 50 mL of 70% EtOH was added to desorb the total flavonoids from the adsorbents. The adsorption/desorption capacity and desorption ratio of each resin were calculated based on the following formula (1), (2), and (3), respectively:

$$G_e/(mg/g) = \frac{(C_0 - C_e)\,V_0}{M} \qquad (1)$$

$$E_e/\% = \frac{(C_0 - C_e)}{C_0} \times 100\% \qquad (2)$$

$$D_d/\% = \frac{C_d \times V_d}{(C_0 - C_e) \times V_0} \times 100\% \qquad (3)$$

where Γ_e was the adsorption capacity at adsorption equilibrium (mg/g dry resin); E_e was the adsorption ratio (%); D_d was the desorption ratio (%); C_0, C_e and C_d in mg/mL are the initial concentration, the equilibrium concentration of total flavonoids in the extracts, and the concentration of total flavonoids in desorption solution, respectively; V_0 is the volume (mL) of the extract loaded; V_d represents the volume of desorption solution (mL); and M stands for the dry weight (g) of the macroporous resins packed.

1.4.2 Selection of macroporous resins by dynamic adsorption

Two non-polar macroporous resins, namely HPD100 and D101 were further tested for the adsorption capacity of total flavonoids from TSL extracts by dynamic adsorption at room temperature. The extract (10.0 BV) was loaded on glass column (100 mm × 15 mm) packed with 15 mL of HPD100 or D101 at the speed of 2.0 BV/h individually. And, the eluted fractions of 1.0 BV were collected and analyzed by UV spectrophotometry.

1.4.3 Selection of macroporous resins by dynamic desorption

Dynamic desorption tests were carried out on glass columns (100 mm × 15 mm) packed with 15 mL of HPD100 or D101 respectively. 3.0 BV of extracts were loaded at flow rate of 2.0 BV/h, and the column was firstly washed with 3.0 BV of water and eluted with 5.0 BV of 20%, 30%, 40%, 60%, 80% and 95% EtOH successively at same speed. All of the eluted fractions were collected and analyzed by UV spectrophotometry.

1.5. Integrated extraction-adsorption process

1.5.1 The experimental set-up for integrated extraction-adsorption process

The experimental set-up for integrated extraction-adsorption process consisted of two sections, namely pressurized liquid extraction and macroporous resin enrichment (as presented in Fig. S1). For each experimental run, the TSL powder was mixed with fine sand thoroughly, and then they were transferred into the stainless-steel column (200 mm × 15 mm) kept in a thermostat. The extraction solvent (10% EtOH) was pumped by a binary HPLC pump through the column. Then the fluent were directly loaded onto a glass column (200 mm × 25 mm) packed with certain amount of macroporous resin for subsequent adsorption. After the completeness of the integrated extraction-adsorption process, the column was washed with 1.0 BV of 10% EtOH and flushed with 70% EtOH successively, using a peristaltic pump. The eluted fractions were collected and analyzed by UV spectrophotometry. Recovery and purity of the total flavonoids was calculated according to the following formula:

$$\text{Recovery (\%)} = \frac{C_f \times V_e}{C_0 \times V_0} \times 100\% \quad (4)$$

$$\text{Purity (\%)} = \frac{C_f \times V_p}{m_p} \times 100\% \quad (5)$$

where C_f and C_0 represents the concentration (mg/mL) of total flavonoids in the collected fractions and the concentration (mg/mL) of total flavonoids extracted by conventional method; V_e, V_0 and V_p represents the volume (mL) of the collected fractions, the volume of extract obtained by conventional method, and the volume of the fractions tested for recovery and purity; m_p stands for the mass (g) of the dry TSL extracts obtained by conventional method and tested for recovery and purity.

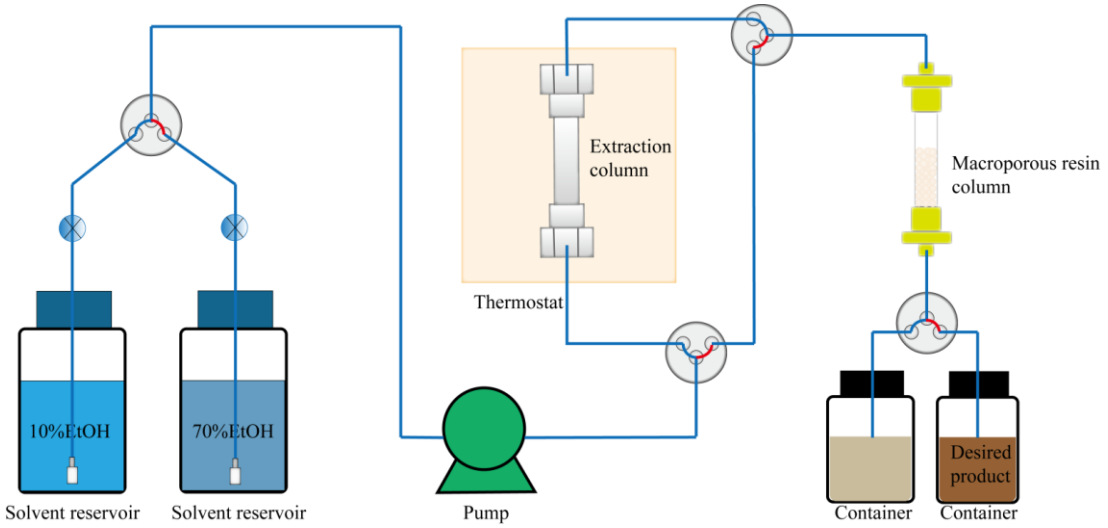

Fig. S1. Schematic diagram of experimental set-up for integrated extraction-adsorption process

1.5.2 Optimization of pressurized liquid extraction conditions

Extraction temperature

To determine the optimal temperature for the extraction of total flavonoids, the TSL powder (7.5 g) blend with sand was loaded into the stainless steel column maintained at 50, 60, 70, 80, 90 or 100℃, with extraction solvent (10% EtOH, liquid/solid ratio 30:1 mL/g) pumped at the flow rate of 1.0 mL/min through the column. The extracts were analyzed by UV spectrophotometry.

Liquid/solid ratio

To determine the optimal liquid/solid ratio for extraction, the TSL powder (7.5 g) blend with sand was loaded into the stainless-steel column maintained at 90℃, with extraction solvent (10% EtOH) pumped at 1.0 mL/min. The eluted solution of each 7.5 mL was collected (300 mL in total) and analyzed by UV spectrophotometry.

Amount of TSL powder

To determine the optimal amount, TSL powder (7.5, 8.5, 9.0, 9.5, 10.0 g) blend with sand was loaded into the stainless-steel column maintained at 90℃, with extraction solvent (10% EtOH) pumped at 1.0 mL/min. And, the liquid/solid ratio was 30:1 mL/g. All the eluted solution was collected and then analyzed by UV spectrophotometry.

1.5.3. Optimization of integrated extraction-adsorption conditions

Effect of amount of macroporous resin on adsorption

To obtain the optimal amount of macroporous resin for the process, 9.5 g of TSL powder blend with sand was loaded into the stainless-steel column maintained at 90℃, with extraction solvent (10% EtOH) pumped at the flow rate of 1.0 mL/min. And, the liquid/solid ratio was 30:1 mL/g. The eluted solution was directly driven into the glass column for subsequent adsorption, with resin/solid ratio at 2-fold, 4-fold, 6-fold and 8-fold of packed macroporous resin respectively. Then the column was washed with 1.0 BV of 10% EtOH and eluted with 5.0 BV of 70% EtOH while the flow rate was 1.0 mL/min. All the eluent was collected and analyzed by UV spectrophotometry.

Effect of flow rate of extraction solvent on extraction-adsorption

To determine the optimal flow rate of extraction solvent for the process, 9.5 g of TSL powder blend with sand was loaded into the stainless-steel column maintained at 90℃, with extraction solvent (10% EtOH) pumped at the flow rate of 1.0 mL/min. The resin/solid ratio was 6:1 mL/g, and liquid/solid ratio was 30:1 mL/g. To integrate the extraction and purification, the eluted solution was directly driven into the glass column for subsequent adsorption. The flow rate was fixed at 0.8 mL/min, 1.0

mL/min, or 1.2 mL/min, for each experiment. Then the column was washed with 1.0 BV of 10% EtOH and eluted with 5.0 BV of 70% EtOH while the flow rate was 1.0 mL/min. All the eluent was collected and analyzed by UV spectrophotometry.

Effect of elution volume on desorption

To determine the optimal elution volume for the process, 9.5 g TSL powder blend with sand was loaded into the stainless-steel column maintained at 90℃, with extraction solvent (10% EtOH) pumped continuously. The resin/solid ratio was 6:1 mL/g, and liquid/solid ratio was 30:1 mL/g. The eluent was directly driven into the glass column for adsorption, and the flow rate of the extraction solvent was fixed at 1.0 mL/min throughout the integrated process. Then the column was washed with 1.0 BV of 10% EtOH and eluted with 5.0 BV of 70% EtOH. The eluent of each 30 mL (0.5 BV) was collected for 300 mL totally and analyzed by UV spectrophotometry.

Effect of flow rate of elution solvent on desorption

To determine the optimal flow rate of elution solvent for the process, 9.5 g of TSL powder blend with sand was loaded into the stainless-steel column maintained at 90℃, with extraction solvent (10% EtOH) pumped at the flow rate of 1.0 mL/min. The resin/solid ratio was 6:1 mL/g, and liquid/solid ratio was 30:1 mL/g. The eluent was directly driven into the glass column for adsorption. After the integrated process was finished, the column was washed with 1.0 BV of 10% EtOH and then eluted with 2.0 BV of 70% EtOH. And the flow rate was fixed at 1.0 mL/min, 1.5 mL/min, or 2.0 mL/min, for each experiment. All the eluent was collected and analyzed by UV spectrophotometry.

1.6. Identification of flavonoids in the purified products

The flavonoids existing in the purified products were analyzed using a UPLC-ESI-MS system (Shimadzu LCMS-8050, Japan). The chromatographic separation was performed on a C_{18} column (2.1 mm × 150 mm, 1.7 μm, Waters, Ireland) according to previous study (Shen et al. 2018). The column temperature was

kept at 35℃. The mobile phase consisted of 0.1% formic acid in ultrapure water (A) and acetonitrile (B). The gradient elution was started from 18%B and increased to 30%B during 10 mins, then maintained for 7 mins. The injection amount was 2 μL and the flow rate was set at 0.35 mL/min. The mass detector was equipped with an ESI source and operated in both positive and negative ionization modes. The conditions of TOF/MS were: capillary voltage, 4.0 *kV*; drying gas (N_2) temperature, 350℃; drying gas (N_2) flow rate, 10.0 L/min; skimmer, 65 *V*; nebulizer pressure, 30 psi; scan range, 100~1000 *m/z*. The acquisition and analysis of data were controlled by Mass Hunter B.04.00 software.

1.7. Statistical analysis

All experiments were performed in duplicated unless otherwise indicated. Data are expressed as Mean ± SD.

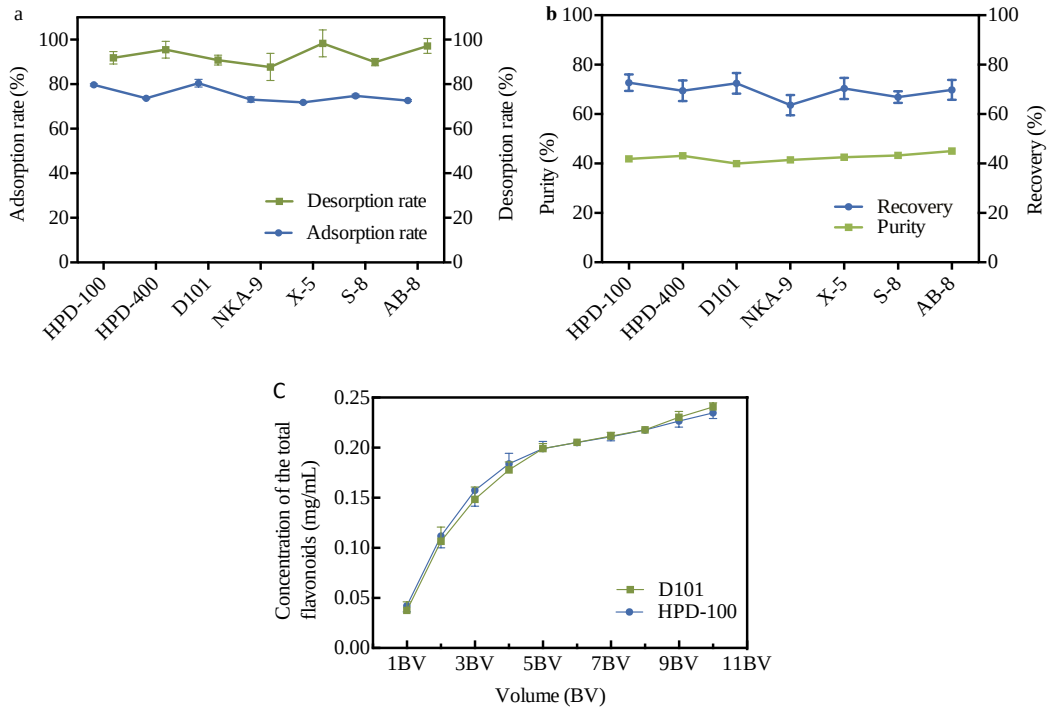

Fig. S2. Adsorption, desorption capacities (A) and recovery and purity (B) of total flavonoids on different resins, and dynamic breakthrough curve of total flavonoids on column packed with D101 and HPD100 resins (C)

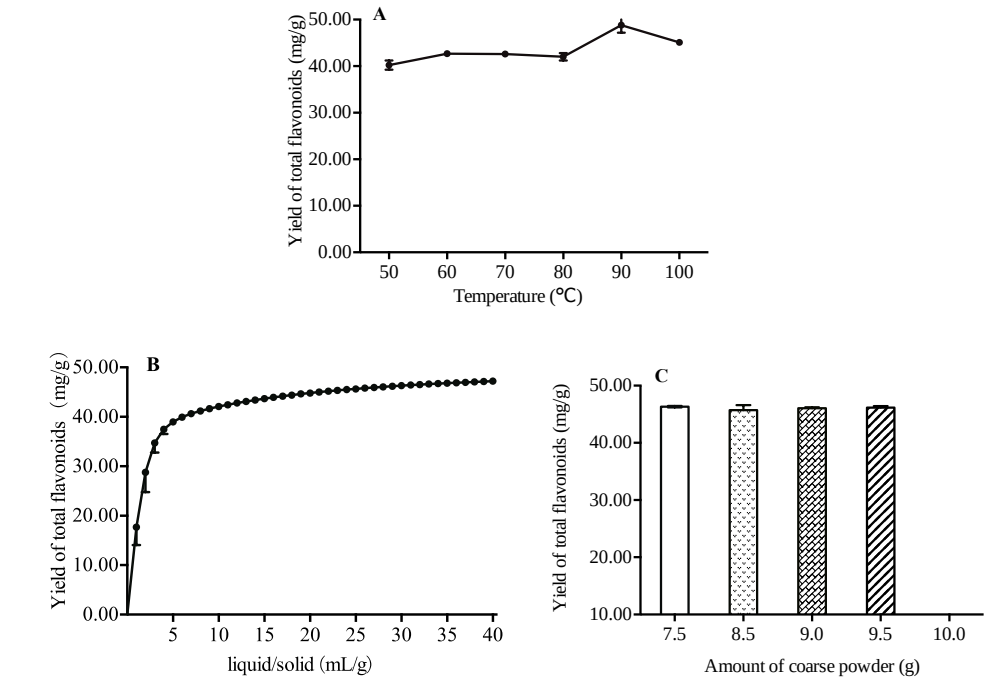

Fig. S3. Results of extraction temperature (A), extraction volume (B) and amount of the TSL powder on the extraction of total flavonoids

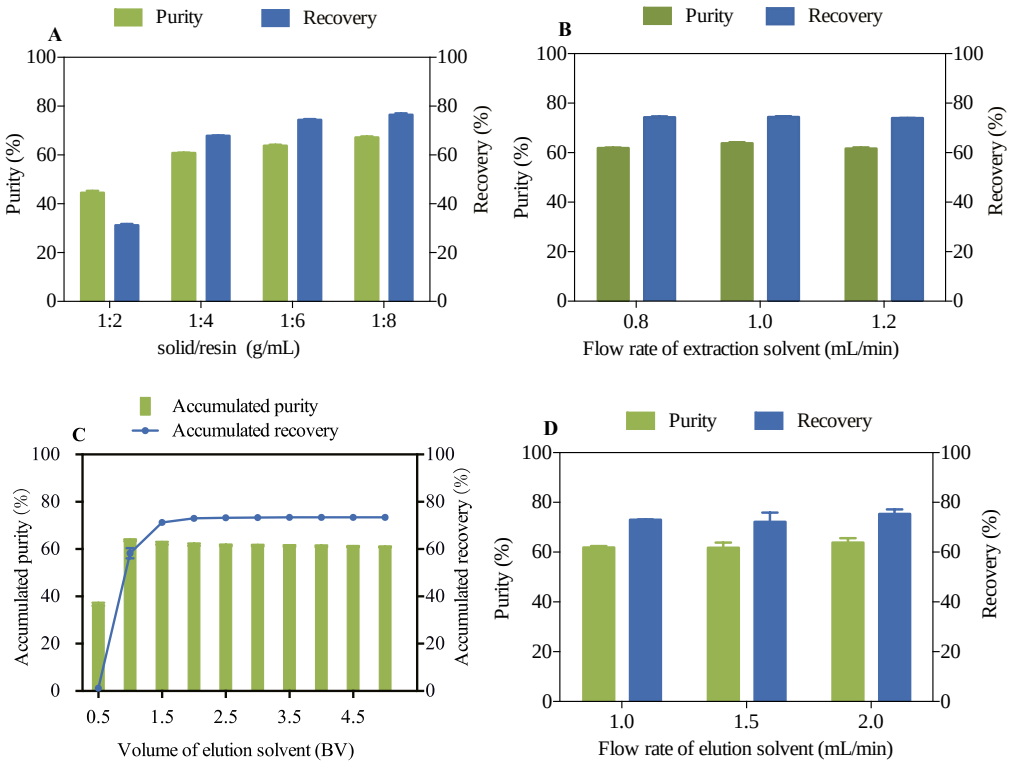

Fig. S4. Results of experiments for integrated extraction-adsorption procedure. (A) Effect of solid/resin; (B) effect of flow rate of elution solvent; (C) effect of volume of elution solvent; (D) effect of flow rate of elution solvent

Table S1. Results of gradient elution of total flavonoids on column packed with D101 (a) and HPD100 (b) resins

(a)

Concentration of EtOH (%)	Mass of dried residue (mg)	Mass of the total flavonoids (mg)	Content of the total flavonoids (%)	Recovery of the total flavonoids (%)
0	40.19	0.93	2.32	6.55
20	10.50	2.73	25.99	19.13
30	4.96	2.11	42.56	14.80
40	4.52	2.15	47.65	15.10
60	2.09	0.25	11.79	1.73
80	0.66	0.06	9.07	0.42
95	0.81	0.02	2.11	0.12

(b)

Concentration of EtOH (%)	Mass of dried residue (mg)	Mass of the total flavonoids (mg)	Content of the total flavonoids (%)	Recovery of the total flavonoids (%)
0	39.56	0.91	2.29	6.36
20	10.75	2.96	27.52	20.74
30	4.16	2.27	54.58	15.92
40	3.70	1.90	51.28	13.31
60	1.64	0.22	13.36	1.54
80	0.88	0.07	7.68	0.47
95	0.76	0.08	10.43	0.56

Table S2. Comparison between conventional method and newly proposed method

Methods	Recovery of total flavonoids (%)	Purity of total Flavonoids (%)	EtOH (mL)	Time (hrs)	Procedure
Conventional method	61.63	54.91	241	14.50	Separated
Integrated process	71.05	66.60	125	7.93	Integrated

Variation	+15.28%	+21.29%	-48.13%	-45.31%	N.A.

Table S3. UPLC-ESI-MS analysis of total bioactive flavonoids purified from TSL

Code	t_R (min)	Precursor (*m/z*)	Molecular Formula	Identification of flavonoids
I	8.19	[M-H] 609	$C_{27}H_{30}O_{16}$	Rutin
II	8.25	[M-H] 463	$C_{21}H_{20}O_{12}$	Myricitrin
III	8.46	[M-H] 463	$C_{21}H_{20}O_{12}$	Quercetin-3-*O*-*β*-*D*-galactoside
IV	8.51	[M-H] 463	$C_{21}H_{20}O_{12}$	Quercetin-3-*O*-*β*-*D*-glucoside
V	8.90	[M-H] 433	$C_{20}H_{18}O_{11}$	Quercetin-3-*O*-*α*-*L*-arabinoside
VI	9.00	[M-H] 447	$C_{21}H_{20}O_{11}$	Astragalin
VII	9.18	[M-H] 447	$C_{21}H_{20}O_{11}$	Quercetin-3-*O*-*α*-*L*-rhamnoside
VIII	9.95	[M-H] 431	$C_{21}H_{20}O_{10}$	Kaempferol-3-*O*-*α*-*L*-rhamnoside

References

Liu J, Lin S, Wang Z, Wang C, Wang E, Zhang Y, Liu J. Supercritical fluid extraction of flavonoids from Maydis stigma and its nitrite-scavenging ability. Food Bioprod Process. 2011; 89: 333-339.

Pan G, Yu G, Zhu C, Qiao J. Optimization of ultrasound-assisted extraction (UAE) of flavonoids compounds (FC) from hawthorn seed (HS). Ultrason Sonochem. 2012; 19: 486-490.

Shen Y, Xu M, Deng P, Gu Q, Yin H, Xia G, Jia X, Yang H, Tam J. Establishment of a rapid method to quantify eight flavonol glycosides for quality assessment of Red Toon using UPLC. Acta Chromatogr. 2018; 30: 31-37.