Supplementary paper

Title: Extraction, identification and content determination of lignans in *Schisandra chinensis*

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Abstract: Objective： To determine the content of *Schisandra chinensis* lignans (SCL) by rapid resolution liquid chromatography-quadrupole time-of-flight mass/mass spectrometry (RPLC Q-TOF-MS/MS). **Method:** The extraction of SCL was used by a macroporous adsorption resin method. The identification of SCL was used by RRLC-Q--TOF MS/MS method and the contents were also determined. **Results：** The total content of 9 SCL was obtained as about 12.2 mg/g.

1 Materials and Methods

1.1 Materials and chemicals

Schisandra chinensis was provided by the Ji'an *Schisandra* Planting Base of Jilin Province, and identified as the mature dry fruit of *Schisandra* by Professor Han Dong at the Department of Pharmacognosy, College of Pharmacy, Beihua University. Ether was purchased from Shenyang Dongxing Reagent Factory (Liaoning, China). Analytical grade methanol was purchased from Tianjin Yongda Chemical Reagent Co., Ltd. (Tianjin, China). Ethanol (95%) was purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China). Macroporous adsorption resin (HPD100) was purchased from Shanghai Ziyi Reagent Factory (Shanghai, China). Schisandrol A, Schisantherin A, Schisandrol B, Schisandrin B, Schisandrin C, Deoxyschisandrin, Schisanhenol, Gomisin J and Schisantherin B were purchased from Chengdu Preferred Biotechnology Co., Ltd. (Sichuan, China). The purity of above reagents was over 98%. HPLC-grade methanol, acetonitrile and ethyl acetate were purchased from TEDIA (Cincinnati, OH, USA). Deionized water was prepared by a Milli-Q Water Millipore Purification System (Millipore Corp., Bedford, MA, USA).

1.2 Extraction of *Schisandra chinensis* lignans

Dried *Schisandra chinensis* was crushed and sieved by a 40-mesh sieve. Then 150 g of *Schisandra chinensis* powder were added into 1200 mL of 95% ethanol and reflux-extracted twice in succession, 1 h each time. The two filtrates was merged, and the merged filtrate was filtered by vacuum filtration and then the filtrate was dried by rotary evaporation. The dried sample was

dissolved in an appropriate amount of distilled water to adjust the concentration of ethanol to 70%. The extract was left standing for 24 h, then filtered and dried by rotary evaporation, and an appropriate amount of distilled water was added to the extract to prepare the extract solution at a constant volume of 0.1 g/mL. The column was filled with macroporous resin, in which the ratio of column height to diameter was 1:6, and 300 cm^3 of the macroporous resin and 1000 mL of the extract were added. When the extract reached below the macroporous resin, 730 mL distilled water was added to remove impurities, and then 1500 mL 95% ethanol was added to elute the extract. The ethanol eluent was collected for the rotary evaporation and freeze-drying. Finally, 5 g *Schisandra chinensis* lignans（SCL）powder was obtained, with an extraction rate of 3.33%.

1.3 Preparation of standard solutions

3 mg of Schisandrol A, Schisandrol B, Schisantherin A, Deoxyschisandrin and Schisandrin B, and 2 mg of Gomisin J, Schisanhenol, Schisandrin C and Schisantherin B, each, were accurately weighed and dissolved in an appropriate amount of methanol in a 10 mL volumetric bottle for the preparation of the constant volume solutions, to obtain the reference stock solutions, and the stock solutions were kept at 4 ˚C as the mixed standards. 50 mg SCL powder was dissolved in an appropriate amount of methanol in a 10 mL volumetric bottle for the preparation of the constant volume solution, to obtain the sample stock solution at a concentration of 5 mg/mL. The sample stock solution was kept at 4 ˚C as the sample to be tested.

1.4 Conditions of HPLC-Q-TOF-MS/MS analysis

An 1200 rapid resolution liquid chromatography system (Agilent, Santa Clara, CA, USA) was used for liquid chromatography separation. The column was Agilent SB-C18 (100 mm×3.0 mm, 1.8 μm, 600 bar), and the column temperature was 30 ˚C. MeOH and H_2O were used as the mobile phases A and B, respectively. The gradient elution procedures were as follows: 0-15 minutes (60%-100% B) and 15-25 minutes (100% B). The flow rate was 0.3 mL/min, the injection volume was 10 μL and the HPLC system was connected with a Q-TOF-mass spectrometry.

6520 Q-TOF-MS (Agilent, Santa Clara, CA, USA) was selected for mass spectrometry. The scanning range was set to *m/z* 100-1500 under positive ion mode. The conditions of the ESI source were as follows: the flow rate of dry gas (N_2) was 9 L/min, the dry gas temperature was set at 350 ˚C, the sprayer was set at 40 psig, the capillary voltage was 3500 V, the collision cracking voltage was 150 V and the cone voltage was 65 V. The mass axis was calibrated with the calibration fluid

before the analysis of each sample, and the document was saved for the determination when the calibration deviation was less than 1 ppm. Q-TOF was equipped with dual spray port ESI sources, and the second spray port could continuously introduce reference ions to ensure the accuracy. The data were analyzed by Agilent software Masshunter (version B.03.01).

1.5 Establishment of standard curve

The mixed standards were diluted by different multiples to obtain 9 standard stock solutions with concentration gradients ranging from 0.002 to 0.300 mg / mL. Precisely measure 1.5 mL of each standard stock solution, inject it into the sample, and take 10 μL into the tandem mass spectrometer. Using the peak area y as the ordinate and the mass concentration x (mg / mL) as the abscissa, linear regression was performed to obtain the regression equation and correlation coefficient of each component. The results show that the linear relationship of each component is good within the experimental range (Table 1).

Tab. 1 Linear regression data of 9 lignan standards

Name	Regression equation	Correlation coefficient
Schisandrol A	y=53849501x+163432	0.9998
Schisandrol B	y=66971090x+195118	0.9997
Schisantherin A	y=89237976x+340162	0.9993
Schisanhenol	y=43020568x+893155	0.9913
Deoxyschisandrin	y=45796227x+543819	0.9952
Schisandrin B	y=54670620x+52880	0.9985
Schisandrin C	y=39651318x+318588	0.9972
Schisantherin B	y=59284406x+126660	0.9981
Gomisin J	y=19755700x+61197	0.9979

1.6 Sample determination

An aliquot of 10 μL of SCL sample was injected into LC/MS. The relate peak area and retention time was obtained by Agilent software Masshunter, the content of 9 lignans in the sample were calculated according to the regression equation, three times of each sample. The results are shown in Figure 1 and Table 2.

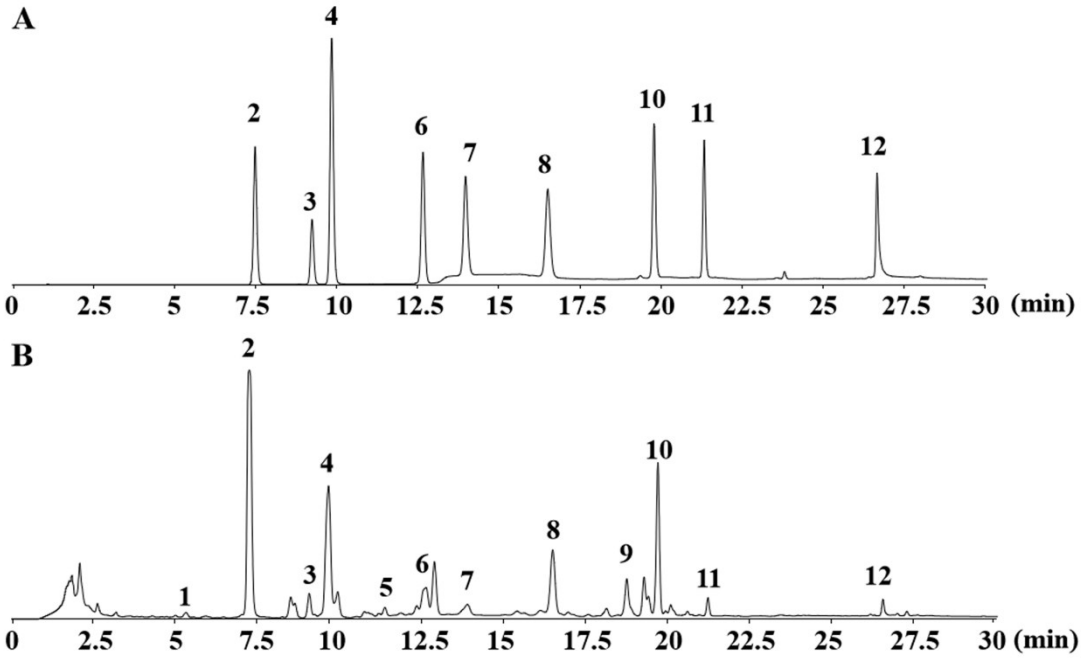

Fig.1 Base peak intensity (BPI) chromatograms of SCL standards (A) and sample (B)

Note: 1: Gomisin G; 2: Schisandrol A; 3: Gomisin J; 4: Schisandrol B; 5: Angeloygomisin H; 6: Schisantherin A; 7: Schisanhenol; 8: Deoxyschisandrin; 9: Schisantherin D; 10: Schisandrin B; 11: Schisandrin C; 12: Schisantherin B.

Table 2. Determination of nine SCL content by RRLC-Q--TOF MS/MS method

(n=3, mean± s, mg/g)

Name	Schisandrol A	Schisandrol B	Schisantherin A	Schisanhenol	Deoxyschisandrin
Content	5.30 ± 0.08	1.77 ± 0.07	0.40 ± 0.07	0.22 ± 0.05	1.17 ± 0.10
Name	Schisandrin B	Schisandrin C	Schisantherin B	Gomisin J	Total
Content	1.67 ± 0.07	0.37 ± 0.04	0.36 ± 0.03	1.94 ± 0.03	12.2 ± 0.13

1.7 Component analysis of SCL

As shown in Fig.1 (Base peak intensity chromatogram of SCL), the Schisandrol A, Gomisin J, Schisandrol B, Schisantherin A, Schisanhenol, Deoxyschisandrin, Schisandrin B, Schisandrin C and Schisantherin B in the samples were identified by comparing the retention time, precise molecular weight and characterization of fragmented ions with those of the SCL standards, and the Gomisin G, Angeloygomisin H and Schisantherin D were identified by comparing the precise molecular mass and tandem mass spectrometry characteristics with those in the network database.

The $[M+H]^+$ ion (*m/z* 537.2111) with a retention time of 5.75 min is taken as an example to illustrate the identification process of SCL. In the positive ion mode, the element composition corresponding to *m/z* 537 was $C_{30}H_{32}O_9$, so it was speculated that it might be Gomisin G by referring to the network database PubChem and related literatures, and based on its chemical structure and the tandem spectrogram information, *m/z* 415 might be generated by *m/z* 537 losing one molecule of benzoic acid at C-6 position ($-C_6H_5COOH$), *m/z* 371 by *m/z* 415 losing one molecule of $-C_2H_4O$ at C-7 position, *m/z* 299 by *m/z* 415 losing one molecule of CH_2O at C-14 position and fragment ions by broken biphenyl ring losing $-C_5H_{10}O$; m/z 371 ion lost the methyl group at C-14 position to obtain m/z 356 ion or lost the methoxy group at C-14 position to obtain *m/z* 340 ion; the biphenyl hexacyclic ring of *m/z* 340 ion lost one molecule of $-C_2H_4$ to obtain *m/z* 312 ion or lost one molecule of methyl group at C-1 position to obtain *m/z* 325 ion; *m/z* 312 ion lost one molecule of CH_2O at C-1 position to obtain *m/z* 282 ion; the methylenedioxy at C-2 and C-3 positions of *m/z* 325 broke to lose one molecule of $C_2H_2O_2$ to obtain *m/z* 267; *m/z* 255 was obtained by *m/z* 325 losing one molecule of CO at the C-1 position and the broken six-membered ring to lose one molecule of methyl group and one molecule C_2H_3, indicating that the substance should be Gomisin G (Fig.2).

Based on the above methods, 12 SCL were identified in the extracts of Schisandra chinensis. Their chemical structures and MS/MS diagrams are shown in Fig.3, and the related information on the mass spectrometry conditions is shown in Table 3.

SCL have a common mother nucleus, i.e. biphenylcycl ooctadiene lignans, with the similar chromatographic behavior and mass spectrometry information, and the similar fragmented ions under the same conditions of mass spectrometry, such as fragmented ions of *m/z* 285 in Schisandrol A, Schisandrol B, Deoxyschisandrin, Schisandrin B and Schisandrin C, those of *m/z* 340 in Schisantherin A, Schisantherin B, Schisantherin D, Schisanhenol, Gomisin G and Gomisin J, and those of *m/z* 315 in Schisandrol A, Schisandrol B, Schisandrin C and Schisanhenol.

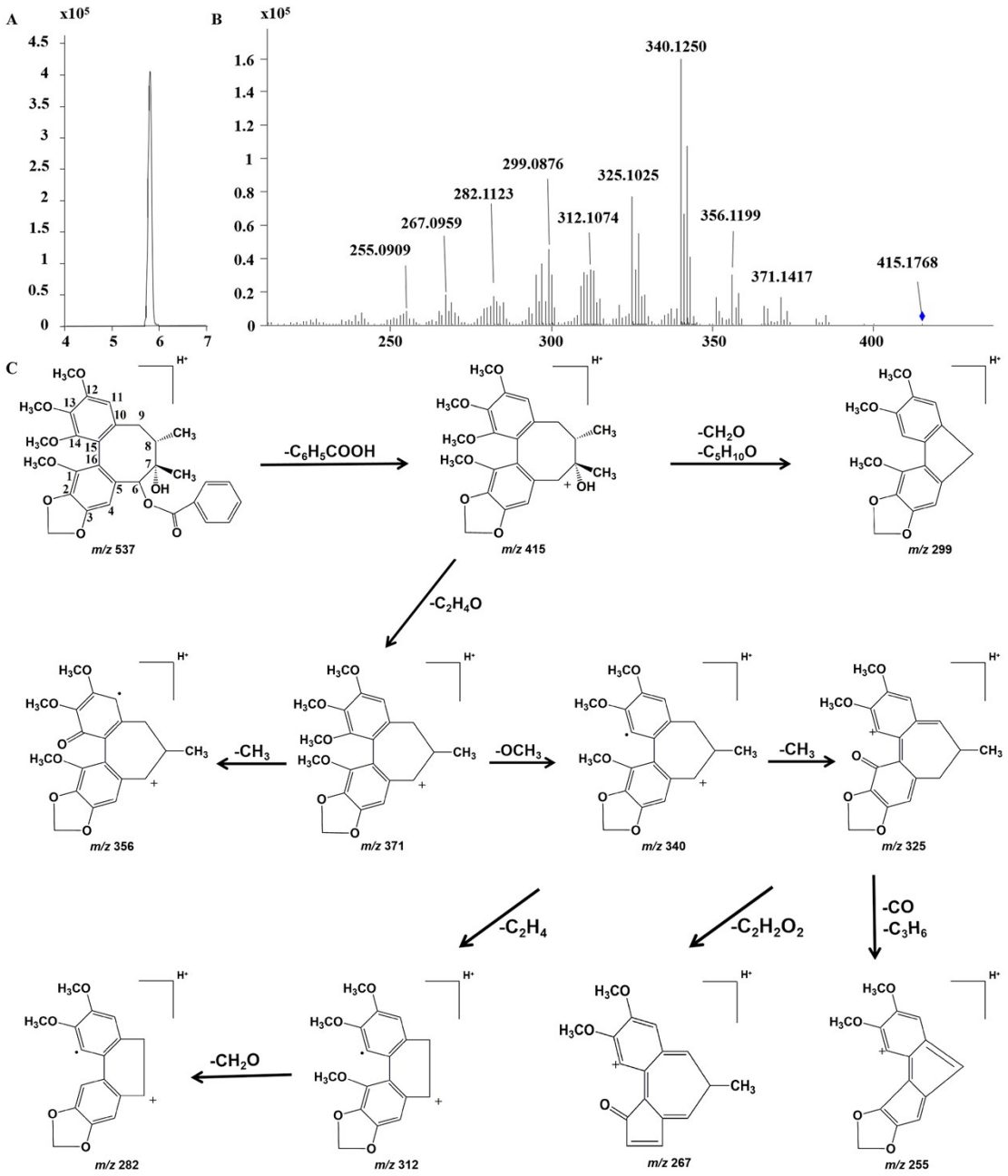

Fig.2. Extracted ion chromatograms (A), MS/MS spectrum (B) in positive ion mode and fragmentation pathways (C) of Gomisin G.

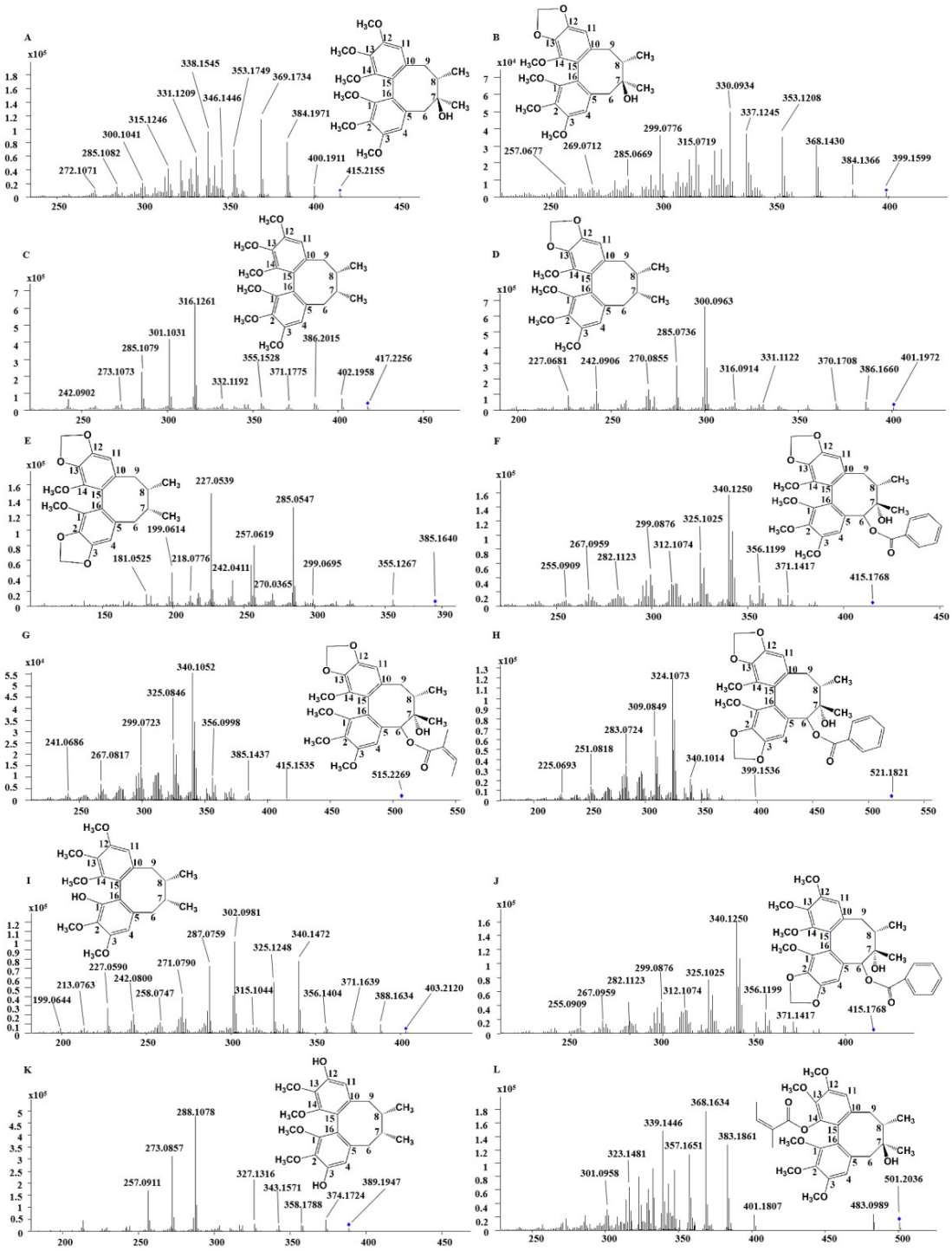

Fig.3. Chemical Structural and MS/MS spectrum of 12 lignans from Schisandra chinensis

Note: (A): Schisandrol A; (B): Schisandrol B; (C): Deoxyschisandrin; (D): Schisandrin B; (E): Schisandrin C; (F): Schisantherin A; (G): Schisantherin B; (H): Schisantherin D; (I): Schisanhenol; (J): Gomisin G; (K): Gomisin J; (L): Angeloygomisin H.

Table 3. Summary information of 12 lignans from Schisandra chinensis

Rt (min)	Mass (Da)	Error (ppm)	Fragment	Formula	Compound
5.75	536	-1.6	415, 371, 356, 340, 325, 312, 299, 282, 267, 255	$C_{30}H_{32}O_9$	Gomisin G
7.31	432	-2.3	415, 400, 384, 369, 353, 346, 338, 331, 315, 300, 285, 272	$C_{24}H_{32}O_7$	Schisandrol A
9.06	388	1.0	389, 374, 358, 343, 327, 288, 273, 257	$C_{22}H_{28}O_6$	Gomisin J
9.69	416	0.8	399, 384, 368, 353, 337, 330, 315, 299, 285, 269, 257	$C_{23}H_{28}O_7$	Schisandrol B
11.69	500	-0.6	501, 483, 401, 383, 368, 357, 339, 323, 301	$C_{28}H_{36}O_8$	Angeloygomisin H
12.50	536	3.5	415, 371, 356, 340, 325, 312, 299, 282, 267, 255	$C_{30}H_{32}O_9$	Schisantherin A
13.88	402	-2.9	403, 388, 371, 356, 340, 325, 315, 302, 287, 271, 258, 242, 227, 213, 199	$C_{23}H_{30}O_6$	Schisanhenol
16.38	416	1.4	417, 402, 386, 371, 355, 332, 316, 301, 285, 273, 242	$C_{24}H_{32}O_6$	Deoxyschisandrin
19.06	520	0.7	521, 399, 340, 324, 309, 283, 251, 225	$C_{29}H_{28}O_9$	Schisantherin D
19.69	400	-1.1	401, 386, 370, 331, 316, 300, 285, 270, 242, 227	$C_{23}H_{28}O_6$	Schisandrin B
21.25	384	2.8	385, 355, 299, 285, 270, 257, 242, 227, 218, 199, 181	$C_{22}H_{24}O_6$	Schisandrin C
26.63	514	1.2	515, 415, 385, 356, 340, 325, 299, 267, 241	$C_{28}H_{34}O_9$	Schisantherin B