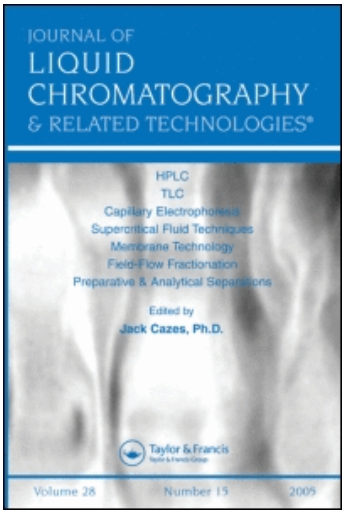

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Li, Yulin , Wang, Lingyun , Wang, Xiaoyan , Suo, Yourui , Chen, Guichen , Hu, Fengzu , Du, Yuzhi and You, Jinmao(2007) 'Separation and Determination of the Major Active Components in Tibetan Folk Medicinal Species *Swertia franchetiana* by HPLC with DAD', Journal of Liquid Chromatography & Related Technologies, 30: 11, 1687 — 1696

To link to this Article: DOI: 10.1080/10826070701224986

URL: http://dx.doi.org/10.1080/10826070701224986

Journal of Liquid Chromatography & Related Technologies®, 30: 1687–1696, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701224986

Separation and Determination of the Major Active Components in Tibetan Folk Medicinal Species *Swertia franchetiana* by HPLC with DAD

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Abstract: A sensitive and specific reversed-phase high performance liquid chromatography (RP-HPLC) method with diode array detection (DAD) was established for the quantitative determination of the nine active components, namely, swertiamarin (SWM, 1), mangiferin (MA, 2), gentipicroside (GE, 3), sweroside (SWO, 4), isoorientin (IS, 5), swertisin (SWS, 6), swertianolin (SWN, 7), 7-O-[α-L-rhamnopyranosyl-1 $\rightarrow$ 2)-β-D-xylopyranosyl]-1,8-dihydroxy-3-methoxyxanthone (RX, 8), and bellidifolin (BE, 9) used as the external standard, in Tibetan folk medicinal species *Swertia franchetiana*. Based on the baseline chromatographic separation of most components from the methanolic extract of *Swertia franchetiana* on a reversed-phase Eclipse XDB-C8 column with water-acetonitrile-formic acid as mobile phase, the nine components were identified by comparison with standard samples and qualified by using the external standard method with DAD at 254 nm. The correlation coefficients of all the calibration curves were found to be higher than 0.9980.

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The relative standard deviations (RSDs) of the peak areas and retention times for the nine standards were less than 2.07% and 2.86%, respectively.

Keywords: Swertiamarin, Gentipicroside, Sweroside, Swertianolin, Bellidifolin, *Swertia franchetiana*

INTRODUCTION

Swertia franchetiana, belonging to the family of *Swertia* in gentianaceae, is a traditional Tibetan folk medicine called "Zang yin chen" and is used to treat diseases of the liver and gall. There are about 170 species of this genus recorded in the world and about 79 species are found in China.[1] Currently, *Swertia* has been proven effective in the treatment of hepatitis, cholecystitis, and gastroenteritis.[2,3] Various chemical and pharmacological studies on *S. franchetiana* had demonstrated that the major bioactive constituents were iridoid glycosides, xanthones, xanthone glycosides, flavone glycosides, and triterpenoids.[4–10] Most of them were SWM, SWO, GE, SWN, SWS, MA, RX, IS, and BE. Recent pharmacological studies showed that SWM, SWO, and GE had antibacterial, free radical scavenging activities and general toxicity;[11,12] SWN and BE had anti-AChE activity effects, and BE showed similar activity to galanthamine;[13] IS showed significant hypoglycemic and antihyperlipidemic effects.[14] Therefore, the quality control of *S. franchetiana* was focused mainly on the determination of these major active components.

Traditionally, chromatography and related techniques represent the primary techniques that are used to evaluate the quality of herbal materials.[15] Among the different analytical methods of chromatography, HPLC is the most powerful. In the past several years, many HPLC analyses of major active components in *S. franchetiana* had been performed.[16–21] However, most of these HPLC methods were insufficient to qualify the major active components, since the resolutions were limited to less than seven components with an external standard method, which made the reproducibility and extraction yield become critical for the quantification. Tian et al. developed a HPLC/MS method for simultaneous determination of five and six glycosides in the extract of *S. franchetiana.*[22,23]

We describe here, the development of a direct and sensitive method for simultaneous determination of nine components in *S. franchetiana* within 60 min. The chemical structures of the nine components are shown in Figure 1.

EXPERIMENTAL

Instrumentation

Experiments were performed using a Agilent 1100 Series liquid chromatograph consisting of a quaternary pump (model G1311A), an autosampler

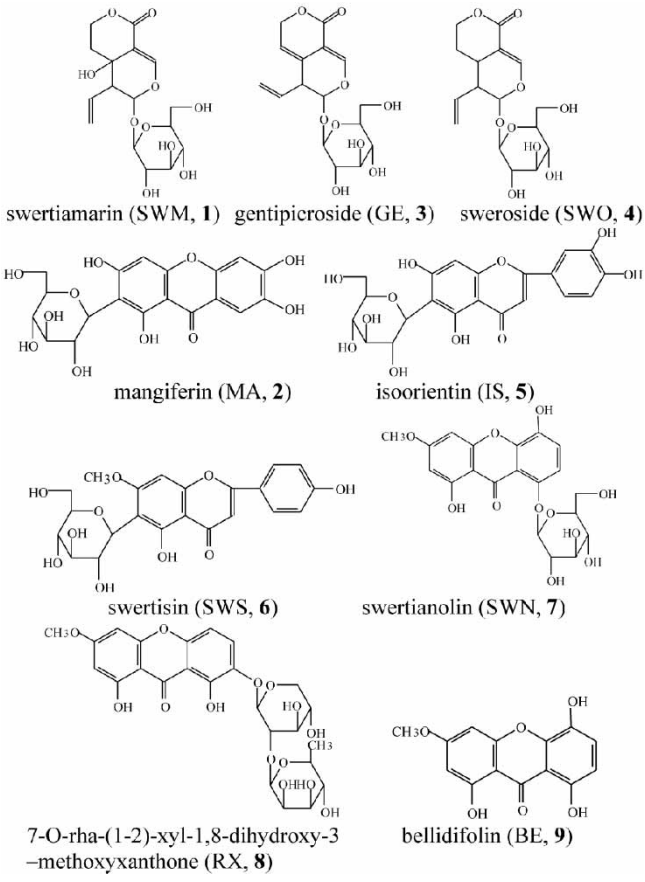

Figure 1. The chemical structures of the standards.

(model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (model G1315A). The HPLC system was controlled by HP Chemstation software.

Chemicals and Materials

SWM, MA, GE, and SWO standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). IS, SWS, SWN, RX, and BE standards (purity $\geq$ 98.0%) were isolated from the extract of *Lomatogonium rotatum* belonging to the gentianaceae, and their structures were elucidated by NMR data.[24] The chemical structures of all standards are shown in Figure 1. HPLC grade acetonitrile was

purchased from Merck Co. (Germany). Methanol and formic acid were of analytical grade from Shanghai Chemical Reagent Co. Ultra-pure water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

Chromatographic Conditions

Chromatographic analysis was carried out by a reversed-phase Eclipse XDB-C8 column (150 × 4.6 mm 5 μM). The binary gradient employed was solvent A (CH_3CN-H_2O-HCOOH, 10 : 90 : 0.1, v/v/v) and B (CH_3CN-H_2O, 90:10, v/v) according to Table 1. The mobile phase was filtered through a 0.2 μm nylon membrane filter (Alltech, Deerfiled, IL). The samples were detected with DAD at 240, 254, and 265 nm.

Preparation of the Standard Mixture Solution and Sample Solution

A standard mixture solution of the nine standards was prepared by dissolving weighted quantities and mixing into methanol, at concentration of 0.94 mg/mL for SWM, 0.48 mg/mL for MA, 0.20 mg/mL for GE, 0.14 mg/mL for SWO, 0.52 mg/mL for IS, 0.78 mg/mL for SWS, 0.62 mg/mL for SWN, 0.53 mg/mL for RX, and 0.60 mg/mL for BE. All solutions were filtered through 0.45 μm membrane filters (Millipore), and directly injected. When not in use, all the solutions were stored at −4°C in the refrigerator. The whole plant of *S. franchetiana* was collected from Qinghai-Tibet Plateau in September, 2004. The air dried sample was powdered with a pulverizer and a 1.529 g powder sample was extracted in an ultrasound bath with 10 mL methanol for 1 h. The extracting process was repeated. The extraction solutions were combined in a 25 mL volumetric flask and diluted to volume with methanol. A volume of 2 mL of the solution

Table 1. The mobile phase gradient program

Time (min)	A (%)	B (%)
0	100	0
14	90	10
15	85	15
25	75	25
40	65	35
48	60	40
50	50	50
65	35	65

(A) 5% CH_3CN + 0.1% HCOOH; (B) 90% CH_3CN.

was filtered through the membrane filter (Millipore) into a HPLC sample vial before analysis.

RESULTS AND DISCUSSION

HPLC Separation Optimization

The selection of the HPLC condition was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short analysis time. In this experiment, two mobile phase systems, including methanol-water and acetonitrile-water in combination with formic acid were tested. Eventually, it was found that an acetonitrile-water system containing 0.1% formic acid gave the best separation of SWM, SWO, GE, SWN, SWS, MA, RX, IS, and BE. Figure 2 and Figure 3 demonstrated the separation obtained for the nine standards and all components from the extracted plant samples. They all showed that good separation could be achieved within 60 min using the conditions described.

The presence of acid in the mobile phase could improve peak trailing of compounds and change the pH value of the mobile phase, having a significant effect on the resolution of compounds. Therefore, on the basis of the acetonitrile-water system, adding formic acid to the mobile phase was considered at this point. It is necessary to choose a proper volume percent formic acid that gives enough differences in the values of the capacity factor for a good resolution in the RP-HPLC. In this experiment, the concentration of formic acid in the mobile phase was chosen at 0.1%. The higher concentration acid added in the mobile phase may produce better sample separation, which in turn shortens the HPLC column lifetime. For the above reason, 0.1% of formic acid was chosen.

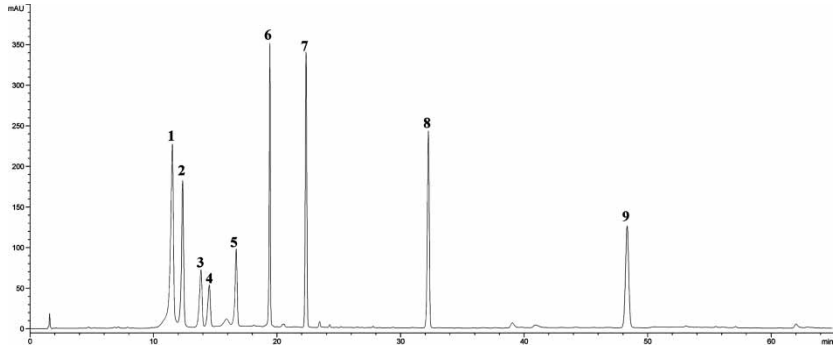

Figure 2. HPLC Chromatograms of the standards, DAD detection at 254 nm. Peak **1** for SWM, **2** for MA, **3** for GE, **4** for SWO, **5** for IS, **6** for SWS, **7** for SWN, **8** for RX, and **9** for BE.

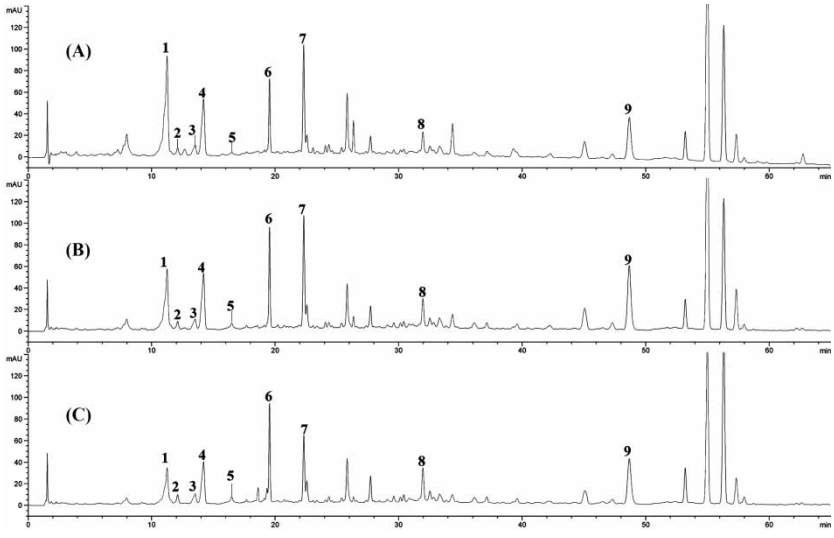

Figure 3. HPLC Chromatograms of the extract of *S. franchetiana*, DAD detection at 240 nm (A), 254 nm (B) and 265 nm (C). Peak **1** for SWM, **2** for MA, **3** for GE, **4** for SWO, **5** for IS, **6** for SWS, **7** for SWN, **8** for RX, and **9** for BE.

Selection of the Detection Wavelength and Identification of the Components

The choice of a proper detection mode is crucial to ensure that all the components are detected. With DAD, this problem can be overcome by using a multiple wavelengths scanning program, which is capable of monitoring several wavelengths simultaneously. It provides assurance that all the UV absorbing components are detected, if presented in sufficient quantity. DAD is used to record spectrochromatograms of compounds simultaneously. So DAD was used to optimize determination of the wavelength in this work.

The UV-vis spectrums of SWM, MA, GE, SWO, IS, SWS, SWN, RX, and BE standards dissolved in the mobile phase were obtained by DAD. From the spectrograms of SMW, MA, GE, SWO, IS, SWS, SWN, RX, and BE, we can see their stronger absorbing peaks as 240, 258, 276, 245, 270, 270, 252, 260, and 254 nm, respectively. Under the optimized HPLC conditions mentioned in experimental section, good separation of the standards and extracted *S. franchetiana* were obtained in 60 min with the DAD detector recorded at 240, 254, and 265 nm, as shown in Figure 3. By comparison, the stronger absorbance occurred at the wavelength of 254 nm. Therefore, 254 nm was chosen in the following study.

Identification of the nine components in the extract of *S. franchetiana* was made by comparison of retention times and the UV-vis spectra with the opposite nine standards, under the same chromatographic conditions.

Table 2. Linear regression equations, correlation coefficients, detection limits and repeatability for peak area and retention time (n = 5)

No	Components	Standard curves	Correlation	Detection limits (mg/ml)	Retention time RSD (%)	Peak area RSD (%)
1	SWM	$y = 3311.9\ x - 75.2$	0.9989	0.0047	2.07	2.58
2	MA	$y = 3655.2\ x + 87.8$	0.9986	0.0030	1.50	2.82
3	GE	$y = 4899.2\ x + 45.4$	0.9999	0.0032	1.72	2.05
4	SWO	$y = 3073.2\ x - 10.7$	0.9996	0.0031	1.61	2.86
5	IS	$y = 1278.9\ x - 3.1$	0.9998	0.0062	1.08	1.81
6	SWS	$y = 1230.7\ x - 12.2$	0.9986	0.0026	0.46	2.10
7	SWN	$y = 7283.7\ x - 11.2$	0.9996	0.0021	0.39	1.01
8	RX	$y = 5495.6\ x + 2.7$	0.9999	0.0025	0.12	1.67
9	BE	$y = 4660.8\ x - 9.0$	0.9999	0.0054	0.11	1.22

Linearity, Detection Limits, and Reproducibility

A series of the standard mixture solutions of these nine compounds were tested to determine the linearity between the standard mixture concentration and peak areas. Under the chromatographic conditions used in this study, all nine calibration curves exhibited good linear regressions. The results of analysis on calibration curves and detection limits are presented in Table 2. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3 (S/N = 3) and between 0.0061 and 0.0026 mg/mL for the nine compounds.

A standard mixture solution was analyzed five times to determine the repeatability for the peak areas and retention time under the optimum conditions in this experiment. The relative standard deviations (RSDs) of the peak areas and retention times were less than 2.07% and 2.86%, respectively (see Table 2).

Separation and Determination of Sample

The methanol extracted solution of *S. franchetiana* was injected directly and separated under the optimum condition mentioned earlier. Baseline separation of the nine components from each other and the unknown chemicals was achieved within 60 min. The typical chromatogram in the extract of *S. franchetiana* is shown in Figure 3. The peaks of the nine components were identified by spiking the standards. As can be seen in this study, the established method was suitable for the determination of these components from the extracted medicinal plant, with satisfactory results. The calculated contents of the nine components are given in Table 3.

The recovery experiments of the nine components were performed by adding known amounts of the standard solutions to the powder of *S. franchetiana*, which were treated according to the procedure described in Preparation of the Standard Mixture Solution and Sample Solution, and determined at

Table 3. Contents of the nine components in the extract of *S. franchetiana* (n = 5)

No.	Components	Contents (mg/g)	RSD (%)
1	SWM	1.84	1.31
2	MA	0.06	2.86
3	GE	0.34	2.53
4	SWO	2.19	1.54
5	IS	0.77	2.15
6	SWS	9.69	1.36
7	SWN	3.70	1.28
8	RX	0.65	1.42
9	BE	5.33	1.12

the same optimal conditions. The recoveries for the nine components were between 97.02 and 101.86%.

CONCLUSION

The method reported here was the first HPLC-DAD assay with which the nine major components could be quantitated simultaneously in the extracted medicinal plant *S. franchetiana*. This method was accurate, sensitive, and reliable, providing a useful quantitative method for the analyses of *S. franchetiana*. Furthermore, it suggested that it was valuable to deeply explore the phytopharmaceuticals of *S. franchetiana*.

ACKNOWLEDGMENT

Support for this research was provided by the Chinese Academy of Sciences (the Talent Cultivation Plan "Hope of the West China" and the Knowledge Innovation Program).

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Received November 15, 2006
Accepted December 12, 2006
Manuscript 6989