Available online at www.sciencedirect.com
ScienceDirect
Journal of Ethnopharmacology 111 (2007) 265–270
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Fingerprinting quality control of Qianghuo by high-performance liquid chromatography-photodiode array detection

Fuquan Jiang [a,b], Yanduo Tao [a,*], Yun Shao [a]

[a] *Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining 810001, China*
[b] *Graduate University of Chinese Academy of Sciences, Beijing 100049, China*

Received 9 March 2006; received in revised form 17 August 2006; accepted 19 November 2006
Available online 1 December 2006

Abstract

A novel, simple and accurate fingerprint method was developed using high-performance liquid chromatography-photodiode array detection (HPLC-DAD) for the quality control of Qianghuo, a Tibetan folk and Chinese herbal medicine used as a diaphoretic, an antifebrile and an anodyne. For the first time, the feasibility and advantages of employing chromatographic fingerprint were investigated and demonstrated for the evaluation of Qianghuo by systematically comparing chromatograms of aqueous extracts with the professional analytical software recommended by State Food and Drug Administration (SFDA). Our results revealed that the chromatographic fingerprint combing similarity evaluation could efficiently identify and distinguish raw herbs of Qianghuo from different sources and different species. The effects on *Notopterygium forbesii* Boiss (Apiaceae) chromatographic fingerprints resulted from collecting locations, harvesting time were also examined.

Keywords: Quality control; Chromatographic fingerprint; *Notopterygium forbesii*; *Notopterygium incisum*; HPLC-DAD

1. Introduction

It is well known that the therapeutic effect of the herbal medicine is based on the synergic effect of its mass constituents, which is different from that of western medicines (Van Beek, 2003; Xie, 2001). However, a few marker or pharmacologically active constituents are generally employed to assess the quality and authenticity of the complex herbal medicine or preparations in traditional standards. Unfortunately, those markers and/or pharmacologically active constituents are hardly demonstrated to stand for the complex herbal medicine or preparation. It is absolutely necessary to develop new analytic methods for quality control of herbal medicine (Gu et al., 2004).

Recently, chromatographic fingerprint technique, as a more meaningful formulation for controlling the quality of herbal samples or their products, has been attracting more and more people's attention because the fingerprint technique emphasizes on the systemic characterization of compositions of samples and focus on identifying and assessing the stability of the plants. Fingerprint analysis has been introduced and accepted by WHO as a strategy for the assessment of herbal medicines (WHO, 1991). And it is also required by the Drug Administration Bureau of China to standardize injections made from traditional Chinese medicines and their raw materials. Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice (Hasler and Sticher, 1992; WHO, 2002; Calixto, 2000; FDA, 2000).

The roots and rhizomes of *Notopterygium forbesii* Boiss (Apiaceae) and *Notopterygium incium* Ting ex H.T (Apiaceae), two of the most popular traditional Tibetan folk herbal medicine of medicinal herbs, are included in Chinese Pharmacopoeia under the same name Qianghuo in Chinese. This herbal has been used as diaphoretic, antifebrile and anodyne (Pharmacopoeia of PR China, 2000; Yang, 1991; Gu et al., 1990). Volatile oil (including α-Pinene and β-Pinene), coumarins (including isoimperaorin, imperaorin, notopterol, etc.) and organic acid (ferulic acid, linoleic acid, etc.) are major active constituents of Qianghuo (Xin and Ling, 1988; Li and Gao, 2004). Though the two medicinal plants were widely applied under the same name in clinic prescriptions, no comparative study on the chemical profile of NF and NI has been reported. Most reported methods (Okuyama et al., 1993; Ji and Xu, 1997) for the

* Corresponding author. Tel.: +86 971 6117264; fax: +86 971 6117264.
E-mail address: yanduotao1963@yahoo.com.cn (Y. Tao).

0378-8741/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2006.11.024

analysis of Qianghuo were based on its organic reagent extracts and volatile compounds though water extracts were commonly used in traditional medicines. In the present investigation, we used high-performance liquid chromatography-photodiode array detection (HPLC-DAD) to develop a simple, rapid, and valid chromatographic fingerprint method for qualitative analysis of Qianghuo and qualitative distinguishing *Notopterygium forbesii* Boiss (NF) and *Notopterygium incium* Ting ex H.T (NI). Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A) was employed to synchronize the chromatographic peaks, to calculate the correlation coefficients between entire chromatographic profiles and to do quantitative standard fingerprint/chromatogram for a group of chromatograms. Besides, the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak related to the reference peak were calculated for quantitative expression of the chemical properties in the chromatographic pattern of herbs.

2. Materials and methods

2.1. Instrumentation and reagents

An Agilent/HP 1100 series HPLC-DAD system consisting of a vacuum degasser, thermo stated column compartment and DAD (Agilent, Palo Alto, CA, USA) was used to obtain chromatograms and UV spectra; an ultrasonic cleaner was used for extraction. Reverse osmosis water (18M, simplicity 185, Millipore, France) was used for all the solutions and dilutions. Acetonitrile was HPLC grade. Standards (Fig. 1) of ferulic acid, imperatorin and isoimperatorin were purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China).

2.2. Plant materials

Eighteen raw herbs of Qianghuo from Qinghai, Gansu and Sichuan provinces of China, which are main bases of Qianghuo, were investigated and collected (Table 1). These herbal samples were authenticated by Professor Jin-Tang Pan (Northwest Plateau Institute of Biology, Chinese Academy of Sciences). Voucher specimens were stored at the Herbarium Center of Northwest Plateau Institute of Biology.

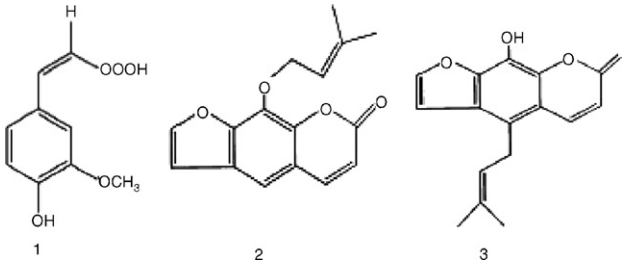

Fig. 1. Chemical structures of ferulic acid (1), imperatorin (2) and isoimperatorin (3).

Table 1
A summary of tested samples

Sample no.	Sample species	Origins	Harvesting time (2004)
01	*Notopterygium forbesii* Boiss	Yongchang, Gansu, China	May
02	*Notopterygium forbesii* Boiss	Ganzi, Sichuan, China	May
03	*Notopterygium forbesii* Boiss	Xinghai, Qinghai, China	May
04	*Notopterygium forbesii* Boiss	Huangyuan, Qinghai, China	May
05	*Notopterygium forbesii* Boiss	Xunhua, Qinghai, China	May
06	*Notopterygium forbesii* Boiss	Datong, Qinghai, China	May
07	*Notopterygium forbesii* Boiss	Guinan, Qinghai, China	May
08	*Notopterygium forbesii* Boiss	Huzhu, Qinghai, China	May
09	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	April
10	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	May
11	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	June
12	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	July
13	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	August
14	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	September
15	*Notopterygium forbesii* Boiss	Ping'an, Qinghai, China	October
16	*Notopterygium incisum* Ting ex H.T.	Ganzi, Sichuan, China	May
17	*Notopterygium incisum* Ting ex H.T.	Yongchang, Gansu, China	May
18	*Notopterygium incisum* Ting ex H.T.	Ping'an, Qinghai, China	May

2.3. Sample preparation

A 2.0 g powder of dried materials was extracted with 25 ml distilled water in an ultrasonic water bath for 15 min. This extraction was repeated two times. The aqueous extracted solution was mixed and filtered through Whatman No.1 filter paper. The resulting clear solution was precipitated by ethanol (final concentration 50% (v/v)) at 4 °C for 12 h and filtered again. The continual filtered was diluted to 100 ml volumetric flask by water and filtered through a 0.45 μm filter membrane prior to HPLC analysis.

2.4. HPLC analysis

The column was a reversed-phase column (C_{18}, 5 μm, 250 mm × 4.6 mm i.d., Thermo, USA). The mobile phase consisted of 0.1% H_3PO_4 in water (A) and acetonitrile (B) using a gradient program of 10–30% (B) in 0–12 min, 30–100% (B) in 12–35 min. The flow rate was 1.0 ml/min and column

temperature was maintained at 30 °C. DAD detector was set at 310 nm for obtaining chromatograms. UV spectra were acquired from 200 to 400 nm. The loading volume was 10 μl.

2.5. Data analysis

Data analysis was performed by a professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), which was recommended by SFDA, used for evaluating similarities of different chromatograms by calculating the correlative coefficient and/or cosine value of vectorial angel.

3. Results

3.1. Reproducibility and repeatability of analytical method

Method reproducibility and repeatability were evaluated by the analysis of five injections of sample solution and five replicates of solid sample, respectively. Precision of retention times and peak areas of compounds 2–5 and 7–10 for replicated injection were found in the range of 0.01–0.09 and 0.32–2.15% of R.S.D. ($n = 5$). The R.S.D. of peak area of all characteristic peaks in sample replicates were estimated to be 1.28–2.86% ($n = 5$). All results indicated that the conditions for the fingerprint analysis were satisfactory.

Table 2
The similarities of 15 chromatograms of *Notopterygium forbesii* Boiss

No.	Similarities[a]	No.	Similarities[a]	No.	Similarities[a]
01	0.881	06	0.990	11	0.985
02	0.925	07	0.995	12	0.980
03	0.970	08	0.982	13	0.983
04	0.974	09	0.994	14	0.994
05	0.981	10	0.992	15	0.996

[a] The reference fingerprint was developed with the median of all chromatograms.

3.2. HPLC fingerprints of NF

Altogether 15 samples of NF were analyzed with the developed procedure. These samples were collected from a variety of source and conditions. These included different season, different geographical location and different soil types. The results indicated that their chromatographic patterns were generally consistent although the absorption intensity of some peaks was different (Fig. 2a and Table 2). The correlation coefficient of

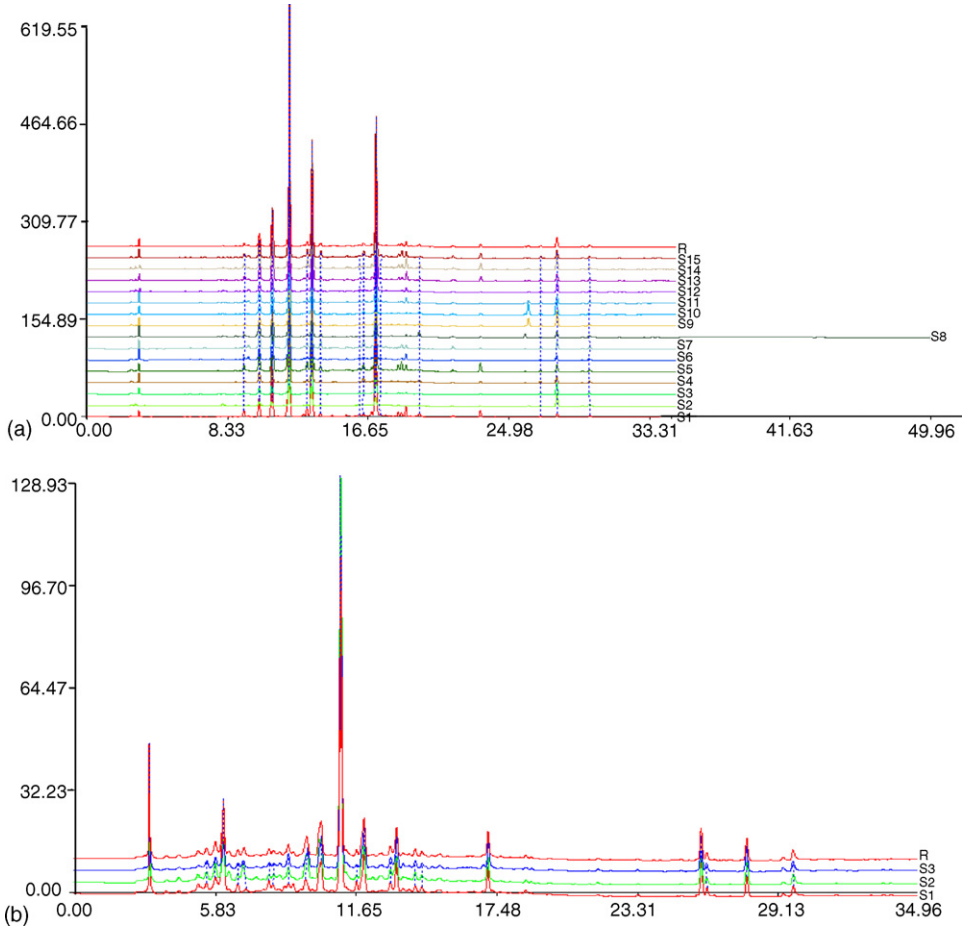

Fig. 2. HPLC chromatograms of (a) (*R* is the simulative median chromatograms of *Notopterygium forbesii* Boiss) 15 *Notopterygium forbesii* Boiss samples; (b) (*R* is the simulative median chromatograms of *Notopterygium incisum* Ting ex H.T) 3 *Notopterygium incisum* Ting ex H.T. samples.

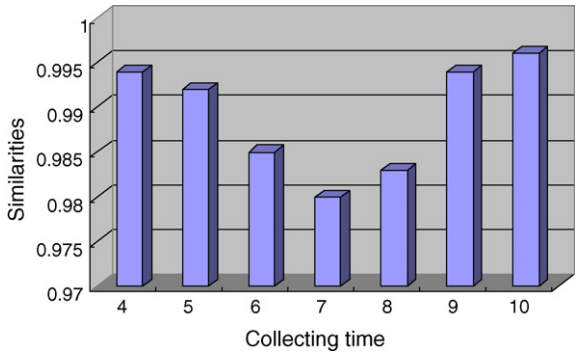

Fig. 3. The similarities of *Notopterygium forbesii Boiss* in different time.

each chromatogram to their simulative median chromatogram was 0.976 ± 0.10.

The HPLC chromatograms of NF were further quantitatively expressed in terms of RRT and RPA. Peak 4 (ferulic acid) was assigned as the reference peak as it was the highest peak in the chromatogram. Besides, peaks 1–3 and 5–10 were also chosen as characteristic peaks, which were used to distinguish NF from NI (Fig. 4).

3.3. The effects on NF of collecting time

Seven raw herbs of NF were collected every month from April to October, at the same location, Ping'an, Qinghai, China. The collecting details and similarities of the chromatograms were shown in Fig. 3. The results clearly show that the similarities were distributed symmetrically. The regular changes of secondary metabolites provided us guidelines for harvesting the herbal. From Fig. 3, the best herbs of NF were harvested in April and October.

3.4. The effects on NF of various locations

According to Sections 2.3 and 2.4, 15 samples were extracted and the extractions were analyzed by HPLC system. The 15 chromatograms obtained were compared with the software presented in Section 2.5. Based on the similarity values of all herbal chromatograms (Table 2), it is interesting to note that all the samples fell into three groups: group A, group B, group C, were 0.881, 0.925, and 0.970–0.996, which were consistent with their collecting provinces (Tables 1 and 2). The secondary metabolites of NF would vary greatly in different locations. Moreover, the effects brought from collecting locations were more visible than those from harvesting time.

Table 3
Similarity comparison of the chromatographic pattern of these herbal samples

Sample	*Notopterygium forbesii* Boiss	*Notopterygium incisum* Ting ex H.T.
Notopterygium forbesii Boiss	0.976 ± 0.10[a] (*n* = 15)	0.413[b]
Notopterygium incisum Ting ex H.T.		0.977 ± 0.05[a] (*n* = 3)

[a] The correlation coefficient of each chromatogram to themselves simulative median chromatogram, mean ± S.D.
[b] The correlation coefficient between simulative median chromatograms.

3.5. Distinguishing between NF and NI

The chromatograms of the three NI samples were found resembling to each other (Fig. 2b). The correlation coefficient of each chromatogram to their simulative median chromatogram was 0.977 ± 0.05 (Table 3). However, the chromatogram of NI showed drastic differences from that of NF (Figs. 2a and b and 4) and the correlation coefficient of the simulative median chromatogram of NI to that of NF was 0.413.

Comparing the chemical components in the NF and NI chromatograms, peak 4 (ferulic acid) was the highest peak in the NF whilst peak 3 was the highest in the NI chromatogram. Besides, the RPA of (Table 4; Fig. 4) peaks 2, 3, 8, 9 and 10 in NI were 2, 26, 11, 6 and 12 times higher than that in the NF. As is shown in Fig. 4, there are 8 common peaks in NF and NI chromatograms. However, peak 1 and peak 6 were not found in NI. Comparison of these peaks was made on the basis of their ultraviolet absorption spectra and retention times.

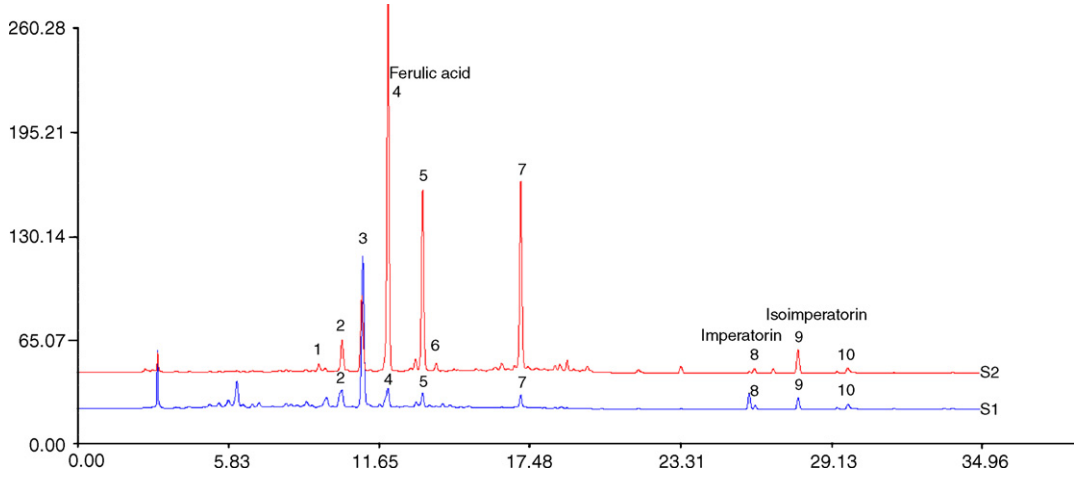

Fig. 4. Simulative median chromatograms of (S_1) *Notopterygium incisum* Ting ex H.T. samples; (S_2) *Notopterygium forbesii* Boiss samples.

Table 4
The relative retention time (RRT) and relative peak area (RPA) of characteristic peaks in simulative median chromatograms of *Notopterygium forbesii* Boiss and *Notopterygium incisum* Ting ex H.T.

Peak no.	*Notopterygium forbesii* Boiss[a]		*Notopterygium incisum* Ting ex H.T.[a]	
	RRT	RPA	RRT	RPA
2	0.852	0.098	0.845	0.225
3	0.916	0.233	0.919	6.138
4	1.000	1.000	1.000	1.000
5	1.116	0.510	1.112	0.597
7	1.428	0.523	1.428	0.510
8	2.182	0.013	2.184	0.142
9	2.322	0.071	2.323	0.429
10	2.483	0.019	2.483	0.239

[a] The simulative median chromatograms of *Notopterygium forbesii* Boiss and *Notopterygium incisum* Ting ex H.T.

4. Discussion

The obtained fingerprints are usually complex chromatograms, and their application relies on a comparison of profiles. Hence, fingerprint development has a stringent demand on resolution and peak capacity in the separation process. However, it is really difficult to separate all active components of herbal medicines in a single chromatographic run. Therefore, the development and optimization of methodology to create fingerprints seems to be very important. To develop a fingerprint for Qianghuo, an optimized strategy for HPLC conditions was performed. Both systems with methanol had longer duration of analysis than those with acetonitrile. The acetonitrile–water system had the same analytical time as the acetonitrile–0.1% H_3PO_4 solution, but the former had poor resolution. So the acetonitrile (10–30% in 0–12 min, 30–100% in 12–35 min)–0.1% H_3PO_4 solution (90–70% in 0–12min, 70–0% in 12–35 min) was chosen for its well baseline resolution and suitable duration for analysis.

Selection of detection wavelength was one of the key factors contributing to a reliable and reproducible HPLC fingerprint of Qianghuo. In order to obtain a sufficiently large number of detectable peaks on the HPLC chromatogram, the spectra of all main peaks were investigated and 310 nm was selected as detection wavelength.

Ferulic acid, imperatorin and isoimperatorin are commonly found in Qianghuo and other related umbelliferae herbs. In the fingerprint ferulic acid indicated a high and stable content, therefore it was chosen as the reference substance. All common peaks' relative retention time and relative peak area were obtained with this substance.

Herbal medicine may consist of hundreds of phytochemicals, and their contents vary depending on climate, regions of cultivation and seasons of harvest. Moreover, these ingredients have significant concentration differences. The same is true in Qianghuo. Our study demonstrated that many peaks area, such as peak 4 (ferulic acid), peak 8 (imperatorin) and peak 9 (isoimperatorin) were distinctly influenced by regions and harvesting time (Figs. 2a and b and 3). Along with more and more extensive application of Qianghuo, it is absolutely and urgent to develop a novel quality standard to validly control its quality. Comparing the quantification of a few markers or pharmacologically active constituents, the chromatographic fingerprint has more predominance in showing the authenticity of herb. So we used high-performance liquid chromatography-photodiode array detection (HPLC-DAD) to develop a simple, rapid, and valid chromatographic fingerprint method for qualitative analysis of Qianghuo.

The rootstock and root of NI are used in the important traditional medicine "Qianghuo". The rootstock and root of NF are used in some districts instead of NI for the important traditional medicine "Qianghuo". In market, the price of NI was higher than NF, sometimes NF was masqueraded as NI to sell. Apart from macroscopic and microscopic authentication, chemical identification of herbal medicine is an important and useful mean as it directly associates with the medicinal functions of herbal medicine. In this study, for the first time a simple and rapid chromatographic fingerprint method was developed for distinguishing NF from NI.

5. Conclusion

In the present work, a novel, valid and rapid chromatographic method and a new fingerprint analysis method were developed and applied. Fifteen samples of NF were identified. According to their similarities, those herbs were assorted to three groups. The taxonomy based on similarities had a fair consistency with simulative median chromatographic profiles (Fig. 2a).

The most relevant factor on secondary metabolites of NF was the collecting location and then was the harvesting time. So in order to get the consistent raw materials of NF, the collecting location should be fixed and then the harvesting time.

The HPLC method developed in this study and characteristic peaks 1–10 can be used for the rapid identification and evaluation of Qianghuo and their aqueous supplements, and differentiation of NF from NI conveniently.

Acknowledgements

The project was supported by the Leading Innovation Research Program of Chinese Academy of Sciences (No. CXLY-2002-9). The authors would like to acknowledge Professor Mei Lijuan, Mr. Zhao Xiaohui, Mr. Zhou Jianbo, Miss Wen huaixiu for their help with the HPLC experiments and collecting some herbal samples.

References

Calixto, J.B., 2000. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines. Brazilian Journal of Medical and Biological Research 33, 179–189.

FDA, 2000. FDA Guidance for Industry Botanical Drug Products (Draft Guidance). US Food and Drug Administration, Rockville, pp. 18–22.

Gu, M., Ouyang, F., Su, Z.G., 2004. Comparison of high-speed counter-current chromatography and high-performance liquid chromatography on fingerprinting of Chinese traditional medicine. Journal of Chromatography A 1022, 139–144.

Gu, Z.M., Zhang, D.X., Yang, X.W., Hattori, M., Namba, T., 1990. Isolation of two coumarin glycosides from *Notopterygium forbesii* and evaluation of a Chinese crude drug, Qian-Huo, the underground parts of *N. incisum* and *N. forbesii*, by high performance liquid chromatography. Chemical and Pharmaceutical Bulletin 38, 2498–2502.

Hasler, A., Sticher, O., 1992. Identification and determination of the flavonoids from Ginkgo biloba by high performance liquid chromatography. Journal of Chromatography A 605, 41–48.

Ji, L., Xu, Z.L., 1997. Analysis on volatile oil chemical components of Qianghuo. Natural Product Research and Development 9, 4–8.

Li, Y.X., Gao, C.H., 2004. Advances in chemical components and pharmacology of Qianghuo. Journal of Liaoning College of TCM 6, 22–23.

Okuyama, E., Nishimura, S., Ohmori, S., Ozaki, Y., Satake, M., Yamazaki, M., 1993. Analgesic component of *Notopterygium incisum* Ting. Chemical and Pharmaceutical Bulletin 41, 926.

The State Pharmacopoeia Commission of Republic of China, 2000. Pharmacopoeia of the People's Republic of China, Vol. 1. Chemical industry press, Beijing, China, pp. 144–145.

Van Beek, T.A., 2003. Chemical analysis of Ginkgo biloba leaves and extracts. Journal of Chromatography A 967, 21–55.

WHO, 1991. Guidelines for the Assessment of Herbal Medicines. World Health Organization, Munich.

WHO, 2002. WHO Traditional Medicine Strategy 2002–2205. World Health Organization, Geneva.

Xie, P.S., 2001. A feasible strategy for applying chromatography fingerprint to assess quality of Chinese herbal medicine. In: International Symposium on Quality of TCM with Chromatograpic Fingerprint, Guangzhou, p. 8.

Xin, L.J., Ling, L.Q., 1988. Studies on the chemical constituents of *Notopterygium forbesii* Boiss. Acta Botan. Sin. 30, 562–564.

Yang, Y.C (Ed.), 1991. Tibetan Medicines. Qinghai People Press, Qinghai.