Liquid chromatography-mass spectrometry analysis of diterpenoid constituents of seventeen *Salvia* plants

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Abstract

The MeOH extracts of the seventeen *Salvia* plants, including ten species used as resources of the Chinese crude drug, Dan-shen (丹参, Radix Salviae miltiorhizae, Tan-jin in Japanese), were comparatively examined by the liquid chromatography-mass spectrometry (LC-MS) method using thirteen diterpenoids as standards. The principle component analysis (PCA) on the relative intensity of the protonated molecular ion of standard diterpenoids in LC-MS showed the presence of several groups in genus *Salvia* with regard to the diterpenoids, which means that the ten species used as Dan-shen resources were not the same. Their use as a Dan-shen resource, thus, should be based on their activity and/or active constituents.

Key words *Salvia*, liquid chromatography-mass spectrometry (LC-MS), principle component analysis, chemotype, Dan-shen, abietane-type diterpenoid.

Abbreviations APCI, atmospheric pressure chemical ionization ; LC-MS, liquid chromatography-mass spectrometry ; PC, principle component ; PCA, principle component analysis ; Tan-jin (Dan-shen), 丹参 ; TIC, total ion chromatogram ; TFA, trifluoroacetic acid.

Introduction

Dan-shen (丹参, Radix Salviae miltiorhizae, Tan-jin in Japanese) is one of the famous Chinese crude drugs officially listed in the Chinese Pharmacopoeia[1] and is used for treatment of menstrual disorder, menorrhalgia, insomnia, blood circulation diseases, and angina pectoris as well as inflammation.[2,3] The Chinese Pharmacopoeia prescribes that Dan-shen is prepared from the dry roots and rhizomes of *Salvia miltiorhiza* BUNGE (Lamiaceae), but eleven species of genus *Salvia* other than *S. miltiorhiza* (*S. bowleyana, S. deserta, S. miltiorhiza* var. *miltiorhiza* f. *alba, S. paramiltiorhiza, S. paramiltiorhiza* f. *purpureo-rubra, S. przewalskii, S. przewalskii* var. *mandarinorum, S. sinica, S. sinica* f. *purpurea, S. trijuga, S. yunnanensis*) are also used as resources of Dan-shen.[4,5] In the comparative study on their constituents and/or activities, however, few studies have been made.[4]

We previously reported the constituents of *S. miltiorhiza*[6-8] and *S. deserta*,[9] aldose reductase inhibitory activity of seventeen *Salvia* plants including ten species used as Dan-shen resources (Table I) and liquid chromatography-mass spectrometry (LC-MS) analysis of water extracts of the seventeen *Salvia* plants.[10] In this paper, we wish to report the LC-MS analysis of MeOH extracts of the seventeen *Salvia* plants.

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Table I List of plant name, locality, and voucher sample number used in this study.

Sample No.	Plant Name	Locality	Voucher Sample No.
1	*S. bowleyana* DUNN	Gaoan, Jiangxi province	CPU 646
2	*S. bowleyana* DUNN	Kaihua, Zejiang province	CPU 647
3	*S. bulleyana* DIELS	Dali, Yunnan province	CPU 692
4	*S. deserta* SCHANG.	Urumuqi, Xinjiang province	TMPW 15403
5	*S. flava* FORREST et DIELS	Lijiang, Yunnan province	CPU 690
6	*S. meiliensis* S. W. SU	Huoshan, Anhui province	CPU 701
7	*S. miltiorhiza* BUNGE	Chuxian, Anhui province	CPU 698
8	*S. miltiorhiza* BUNGE (Cultivated)	Zhongjiang, Sichuan province	CPU 653
9	*S. miltiorhiza* BUNGE	Heze, Shandong province	TMPW 15481
10	*S. miltiorhiza* BUNGE var. *miltiorhiza* f. *alba* C. Y. WU et H. W. LI (Cultivated)	Zhangqiu, Shandong province	CPU 648
11	*S. paramiltiorhiza* H. W. Li et X. L. HUANG	Shucheng, Anhui province	CPU 649
12	*S. paramiltiorhiza* f. *purpureo-rubra* H. W. LI	Tongling, Anhui province	CPU 686
13	*S. przewalskii* MAXIM.	Lijiang, Yunnan province	CPU 654
14	*S. przewalskii* MAXIM. var. *mandarinorum* STIB.	Saotong, Yunnan province	CPU 651
15	*S. przewalskii* MAXIM. var. *mandarinorum* STIB.	Dali, Yunnan province	CPU 694
16	*S. sinica* MIGO f. *purpurea* H. W. LI	Chongyang, Anhui province	CPU 695
17	*S. trijuga* DIELS	Lijiang, Yunnan province	CPU 700

Materials and Methods

Plant materials : The plant name, locality, and voucher sample number of the plants used in this study are listed in Table I. Among them, fifteen plants (No. 1-3, 5-8, 10-17) were samples which had been identified by Dr. Z.-N. Gong of Department of Medicinal Botany, China Pharmaceutical University and were preserved in the Department. The other two samples, *S. deserta* (sample No. 4) and *S. miltiorhiza* (sample No. 9), were supplied by Alps Pharmaceutical Industry Co., Ltd., Furukawa, Japan and identified by Dr. X.-H. Ma of Xinjiang Medical College, China, and the voucher samples are preserved in the Museum of Materia and Medica, Analytical Research Center for Ethnomedicines, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University.

Chemicals : Standard samples of danshenol A (**1**), danshenol B (**2**), dihydrotanshinone I (**3**), cryptotanshinone (**4**), tanshinone IIA (**5**), danshexinkun A (**6**), and sugiol (**7**) were isolated from *S. miltiorhiza*,[6,7] while those of 6,7-dehydroroyleanone (**8**), royleanone (**9**), 7-*O*-methylhorminone (**10**), taxodione (**11**), 7-*O*-acetylhorminone (**12**), and horminone (**13**) were from *S. deserta*[9] (Fig. 1). All other chemicals and reagents used were the HPLC grade.

Preparation of extracts and fractions : Roots of each plant were chopped into small pieces and extracted with water (15 ml/g, 80°C, 3 h, ×3) and then MeOH (10 ml/g, reflux, 3 h, ×3) to give water and MeOH extracts, respectively. A part of the MeOH extract was suspended in water (5 ml/mg) and extracted with AcOEt (5 ml/mg×3). The AcOEt extract was evaporated to give an AcOEt-soluble fraction, while the water layer was liophylized to yield an AcOEt-insoluble fraction.

LC-MS analysis : A solution of AcOEt-soluble or AcOEt-insoluble fraction in MeOH (10 mg/ml) was filtered using Millipore SJLG 250 filter (0.2 μm) (Bedford, MA, USA), and 10 μl of the filtrate was directly subjected to LC-MS analysis. LC-MS analysis was performed on Finnigan-Mat LCQ system (San Jose, CA, USA) equipped with a Shimadzu LC10A HPLC system (Kyoto, Japan). LC-MS was operated under atmospheric pressure chemical ionization (APCI) mode and the conditions were as follows; vaporizer temperature, 450°C ; capillary temperature, 180°C ; ion injection time, 100 msec ; column, Waters SYMMETRY C_{18} (150 mm×4.6 mm i.d.) (Milford, MA, USA) ; column temperature, 40°C ; mobile phase,

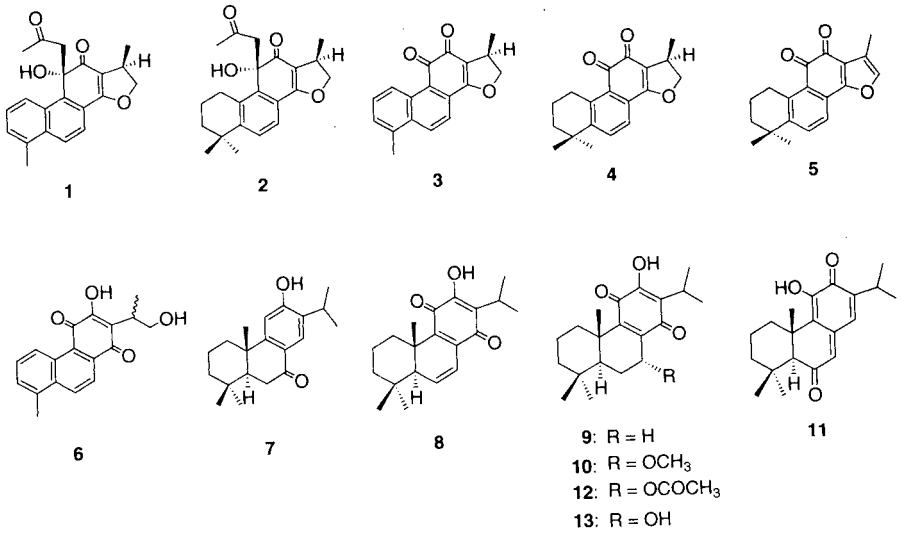

Fig. 1 Structures of standard samples **1**-**13**

linear gradient from 0.1 % trifluoroacetic acid (TFA)-MeOH (20 : 80, v/v) to MeOH for 20 min ; flow rate, 0.8 ml/min ; UV detector, 254 nm.

Principle component analysis (PCA) : The relative intensities of **1**-**13** in the AcOEt-soluble fraction (Table II) were obtained from the intensity of the $[M+H]^+$ ion of **1**-**13** in each AcOEt-soluble fraction by scaling with the intensity ratio of **5**. PCA was done on the relative intensities listed in Table II with a Windows 95™ software, Pirouette™ (Infomatrix Co., Woodinville, WA, USA).

Results and Discussion

HPLC and LC-MS analysis of standard samples

First, we measured the LC-MS spectra of the AcOEt-insoluble fractions under the same conditions as used previously[10] and detected caffeic acid derivatives identical with those of water extracts. Then, in order to measure the LC-MS spectra of the AcOEt-soluble fractions, we examined the HPLC conditions with the thirteen diterpenoids obtained from *S. miltiorhiza* (**1**-**7**) or *S. deserta* (**8**-**13**) (Fig. 1) as the standard samples. The total ion chromatogram (TIC) did not show clear separation of the authentic **1**-**13** (Fig. 2a,h), but in the mass chromatograms monitored by respective $[M+H]^+$ ion of **1**-**13** (Fig. 2b-g,j-n), all compounds could be well separated by the HPLC

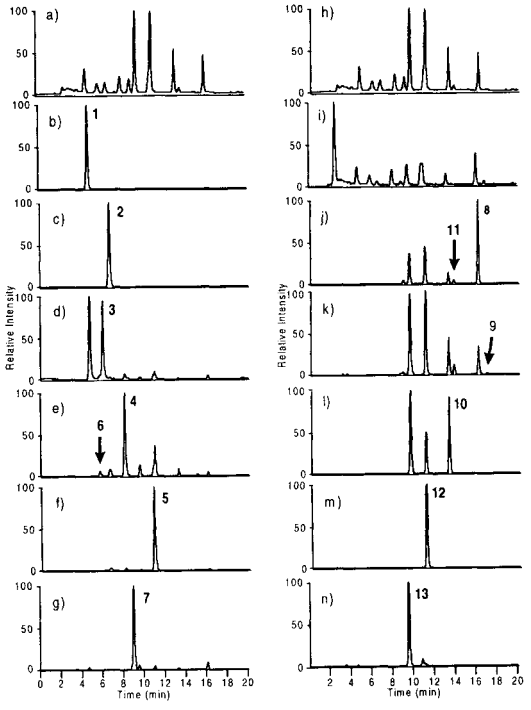

Fig. 2 LC-MS chromatograms of diterpenoids mixture. a), h) Total ion chromatogram (TIC). b-g) Mass chromatograms monitored at the $(M+H)^+$ ion of **1** (m/z 337), **2** (m/z 355), **3** (m/z 279), **4** and **6** (m/z 297), **5** (m/z, 295), and **7** (m/z 301). i) UV-detected chromatogram. j-n) Mass chromatograms monitored at the $(M+H)^+$ ion of **8** and **11** (m/z 315), **9** (m/z 317), **10** (m/z 347), **12** (m/z 375), and **13** (m/z 333).

Table II Relative intensity of the $[M+H]^+$ ions of diterpenoids in the extracts.

Sample No.	Compound (t_R in min)											
	1 (4.69)	**6** (5.69)	**3** (5.96)	**2** (6.67)	**4** (8.08)	**7** (8.97)	**13** (9.53)	**5** (10.84)	**12** (11.04)	**10** (13.27)	**11** (13.79)	**8** (16.14)
1	0.0	416.9	836.1	0.0	2453.9	404.7	1.2	2920.4	0.0	0.6	1.2	0.6
2	0.0	3.2	12.3	0.0	21.8	31.4	0.1	76.7	0.0	0.6	0.1	0.1
3	0.0	0.1	0.2	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0	0.0	0.1	0.3	0.0	0.2	1.0	0.0	1.1
5	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	0.0	16.0	45.1	0.4	90.7	20.9	0.4	109.3	0.0	0.2	0.1	0.5
7	0.0	468.8	1140.0	0.0	2574.3	149.9	1.6	3430.6	0.0	0.0	1.1	1.1
8	0.0	158.5	195.0	0.0	1820.6	51.9	0.6	2026.8	0.0	0.0	0.0	0.6
9	0.0	182.0	234.0	0.0	1923.8	58.1	0.0	2049.9	0.0	0.0	0.0	1.1
10	1.7	104.5	598.6	0.0	1999.8	52.1	0.4	1496.6	0.0	0.0	0.4	0.4
11	0.0	40.0	140.7	0.0	290.5	44.8	0.6	470.9	0.0	0.0	0.6	0.6
12	2.4	17.3	95.9	1.8	756.2	25.6	0.0	1744.5	0.0	0.0	0.0	0.6
13	0.0	18.7	37.4	0.0	270.2	95.9	0.6	2201.3	0.0	0.6	0.6	0.6
14	0.0	0.1	0.4	0.0	0.6	0.2	0.7	0.6	0.0	0.0	0.0	0.0
15	0.0	3.1	4.3	0.0	114.4	45.6	11.4	64.3	0.0	0.0	0.3	0.3
16	0.0	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
17	0.0	56.1	56.1	0.0	1169.0	1630.8	0.0	2887.4	0.0	0.6	0.6	0.6

The relative intensity of **9** (t_R 16.97 min) were all zero.

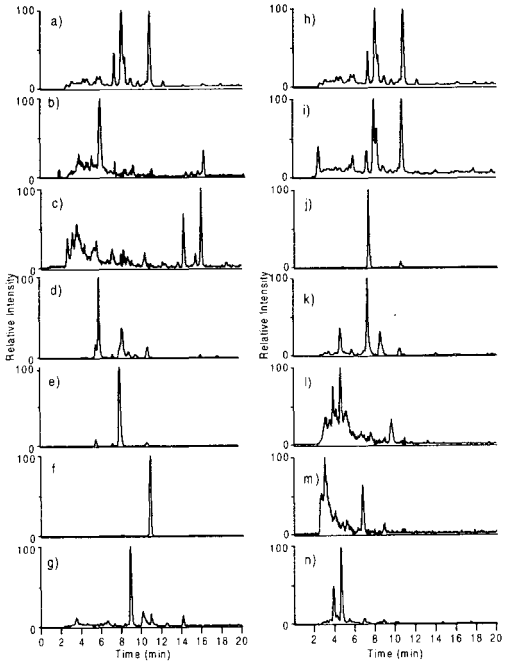

Fig. 3 LC-MS chromatograms of AcOEt-soluble fraction of MeOH extract of roots of *Salvia miltiorhiza* (Sample No. 9)
a), h) Total ion chromatogram (TIC). b-g) Mass chromatograms monitored at the $(M+H)^+$ ion of **1** (*m/z* 337), **2** (*m/z* 355), **3** (*m/z* 279), **4** and **6** (*m/z* 297), **5** (*m/z*, 295), and **7** (*m/z* 301). i) UV-detected chromatogram. j-n) Mass chromatograms monitored at the $(M+H)^+$ ion of **8** and **11** (*m/z* 315), **9** (*m/z* 317), **10** (*m/z* 347), **12** (*m/z* 375), and **13** (*m/z* 333).

conditions (see Materials and Methods). In addition, APCI-MS spectra of the peaks clearly revealed the respective $[M+H]^+$ ion of **1-13**.

LC-MS analysis of AcOEt-soluble fractions of seventeen Salvia plants

The AcOEt-soluble fractions of *Salvia miltiorhiza* and *S. deserta* were first examined under the above conditions. In the case of *S. miltiorhiza*, the peaks of seven abietane-type diterpenoids, **1-7**, were mainly detected (Fig. 3), while in the case of *S. deserta*, the peaks of six abietane-type diterpenoids, **8-13**, were detected (Fig. 4). These indicated that the LC-MS conditions could be applicable to the extracts. Thus, we measured the LC-MS spectra of other fifteen *Salvia* plants, and from the mass chromatograms monitored by respective $[M+H]^+$ ion of diterpenoids **1-13**, relative intensities of the diterpenoids in the extracts have been calculated (Table II).

Principle component analysis (PCA)

In order to compare the contents of the diterpenoids **1-13**, we used a PCA method[11] on the relative intensities of **1-8** and **10-13** (Table II), because that of **9** was 0.0 in all AcOEt-soluble fractions. The results indicated that the first three principle components (PCs) could account for 71.6 % of variance in the data

Table III First three principle components of sixteen *Salvia* plants.

Sample No.	Plant Name	1st PC	2nd PC	3rd PC
1	*S. bowleyana*	4.25	0.72	−0.45
2	*S. bowleyana*	−1.65	0.75	−0.17
3	*S. bulleyana*	−1.96	−0.28	−0.85
4	*S. deserta*	−1.30	3.48	3.03
5	*S. flava*	−1.96	−0.28	−0.85
6	*S. meiliensis*	−1.30	0.01	0.27
7	*S. miltiorhiza*	5.11	−0.32	−0.71
8	*S. miltiorhiza* (Cultivated)	0.72	−0.41	−0.33
9	*S. miltiorhiza*	1.29	−0.22	0.20
10	*S. miltiorhiza* var. *miltiorhiza* f. *alba* (Cultivated)	1.24	−1.72	0.28
11	*S. paramiltiorhiza*	−0.33	0.05	−0.59
12	*S. paramiltiorhiza* f. *purpureo-rubra*	−0.51	−3.77	3.21
13	*S. przewalskii*	0.15	0.99	0.23
14	*S. przewalskii* var. *mandarinorum*	−1.97	−0.28	−0.93
15	*S. przewalskii* var. *mandarinorum*	−1.43	−0.06	−2.03
16	*S. sinica* f. *purpurea*	−1.96	−0.28	−0.85
17	*S. trijuga*	1.61	1.64	0.53

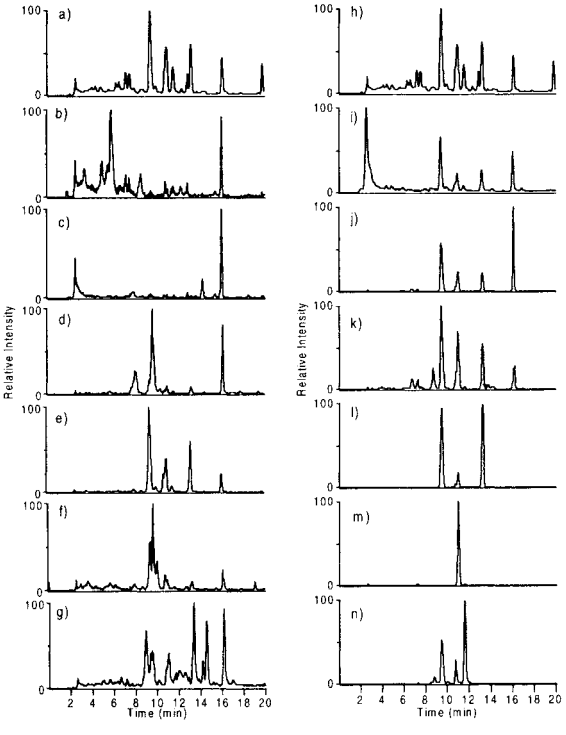

Fig. 4 LC-MS chromatograms of AcOEt-soluble fraction of MeOH extract of roots of *Salvia deserta* (Sample No. 4)
a), h) Total ion chromatogram (TIC). b-g) Mass chromatograms monitored at the $(M+H)^+$ ion of **1** (*m/z* 337), **2** (*m/z* 355), **3** (*m/z* 279), **4** and **6** (*m/z* 297), 5 (*m/z* 295), and **7** (*m/z* 301). i) UV-detected chromatogram. j-n) Mass chromatograms monitored at the $(M+H)^+$ ion of 8 and **11** (*m/z* 315), **9** (*m/z* 317), **10** (*m/z* 347), **12** (*m/z* 375), and **13** (*m/z* 333).

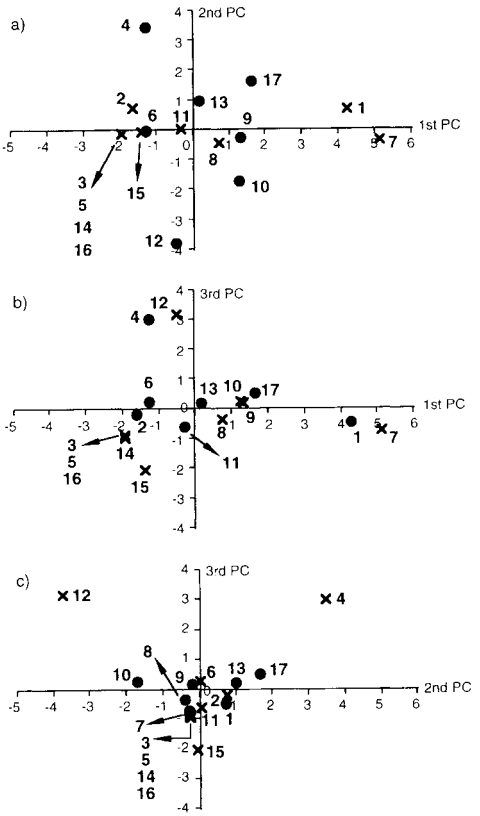

Fig. 5 Plot of scores of first three principle components of seventeen *Salvia* plants.
a)-c) Projection onto the 1st-2nd, 1st-3rd, and 2nd-3rd PC planes, respectively. The number indicates the sample number in Table I. The mark (●) and (×) indicate the sample having positive (●) and negative (×) sign, respectively, with regard to the third axis.

set. The eigenvectors for the first three PCs are listed in Table III, and dihydrotanshinone I (**3**), cryptotanshinone (**4**), tanshinone IIA (**5**), danshexinkun A (**6**), and taxodione (**11**) contributed to the first PC ; danshenol A (**1**), danshenol B (**2**), 7-*O*-methylhorminone (**10**), and 7-*O*-acetylhorminone (**12**) contributed to the second PC ; and **1**, **2**, 6,7-dehydroroyleanone (**8**), **10**, **12**, and horminone (**13**) to the third PC.

The scores plotted in terms of these PCs (Fig. 5) suggested that *S. miltiorhiza* (No. 8, 9) and *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10) could form a group and *S. bulleyana* (No. 3), *S. flava* (No. 5), and *S. przewalskii* var. *mandarinorum* (No. 14, 15) could form another group, which are almost coincident with morphological classification. However, *S. miltiorhiza* at Chuxian (No. 7) stands out from *S. miltiorhiza* at Zhongjiang (No. 8) and Heze (No. 9) for its high content of **3** and **6** and presence of **11**, and *S. paramiltiorhiza* f. *purpureo-rubra* (No. 12) stands out from *S. paramiltiorhiza* (No. 11) for the presence of **1** and **2**. Moreover, *S. deserta* (No. 4) stands out from other *Salvia* plants because of high content of **10** and low content of abietane-type diterpenoids in both plants. Though more comprehensive sampling would be needed, these results would suggest that there are some groups in genus *Salvia* in regard to chemical constituents and that some plants belonging to the same species may not have the same constituents.

Conclusions

In this study, we examined the LC-MS of the AcOEt-soluble fractions of MeOH extracts of seventeen *Salvia* plants, including ten species of Dan-shen resources. The PCA on the relative intensity of diterpenoids in LC-MS indicated that there are some groups in genus *salvia* in regard to chemical constituents. Though more comprehensive sampling would be needed in order to determine whether the frequency of these groups fitted with taxonomic or geographical groupings, this result, together with the previous one,[10] would indicate that the ten species used as Dan-shen resources were not the same with regard to chemical constituents. Thus, their use as a Dan-shen resource should be based on their activity and/or active constituents.

Acknowledgment

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和文抄録

漢薬“丹参”の基原植物として用いられている10種を含む17種のサルビア属植物のメタノールエキスについて，ジテルペン13種を指標としてLC-MSによる比較検討を行なった。マスクロマトから得られる各指標成分のプロトン付加分子イオンに対して主成分分析を適用した結果，サルビア属植物に複数のグループの存在が認められ，丹参の基原植物として用いられる10種はジテルペンに関して同等ではなかった。したがって，それらを丹参の基原植物として用いる場合，その作用や活性成分に関する考慮を払うべきである。

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