Journal of Traditional Medicines 18, 39−47, 2001

Equality-evaluation of Wakan-yaku (Japanese-Chinese traditional medicines) with liquid chromatography-mass spectrometry (LC-MS): comparison of constituents of original plants for Chinese traditional medicine "Dan-shen (Tan-jin)"

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(*Accepted January 18, 2001.*)

Abstract

Wakan-yaku (Japanese-Chinese traditional medicines) are natural products and its constituents and/or activities depend on several factors, such as species, timing of collection, producing area, individual, *etc.* Thus, in order to use a homogeneous Wakan-yaku (Japanese-Chinese traditional medicines), it is necessary to use it with an evaluation on the equality of the constituents and/or activities of them, and the effective method for detecting the qualitative and/or quantitative change of the constituents and/or activities is required. However, almost all methods currently in use are based on the comparison of some representative constituents or activities and thus are not suitable for Wakan-yaku (Japanese-Chinese traditional medicines) which usually have many constituents and activities. On the other hand, application of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) has recently been attempted, due to the possibility of measuring many compounds at once. This review deals with the LC-MS method, applicable to several types of compounds, by taking the case of our comparative study on the seventeen *Salvia* plants.

Key words Liquid chromatography-mass spectrometry (LC-MS), equality-evaluation, quality-evaluation, Tan-jin (Dan-shen, 丹参), genus *Salvia*, aldose reductase inhibition.

Abbreviations AR, aldose reductase ; APCI, atomospheric pressure chemical ionization ; ESI, electrospray ionization ; FID, flame ionization detector ; GC, gas chromatography ; GC-MS, gas chromatography-mass spectrometry ; HPLC, high-performance liquid chromatography ; LC-MS, liquid chromatography-mass spectrometry ; MS, mass spectrometry ; PC, principal component ; PCA, principal component analysis ; Shin-kyou-tan-jin (Xin-jiang-dan-shen), 新疆丹参 ; Tan-jin (Dan-shen), 丹参 ; TIC, total ion chromatogram ; UV, ultraviolet.

I. Introduction

Wakan-yaku (Japanese-Chinese traditional medicines) consists of natural products, mainly herbal drugs, and its quality is affected by several factors, such as species, timing of collection, producing area, individual, *etc.* For consistency in result of treatment, use of homogeneous Wakan-yaku is necessary and Japanese and Chinese Pharmacopoeias describe the origin(s), timing of collection, producing area, properties, discrimination methods, quantity measurement methods, *etc.* However, most of them are not directly related to their quality as Wakan-yaku, which should be judged from a viewpoint of effectiveness for patients; *i.e.*, effective for patients is good quality and

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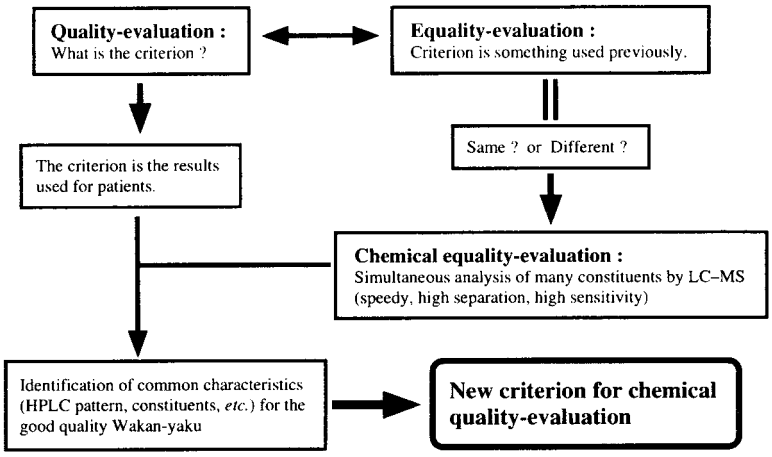

Fig. 1 Quality-evaluation and equality-evaluation. Quality-evaluation of Wakan-yaku is difficult, or impossible, but equality-evaluation is relatively easy. By combining the information of the results used for patients and of chemical analysis of many constituents by LC-MS, common characteristics for the good quality Wakan-yaku would be identified and those characteristics would lead to new criterion for chemical quality-evaluation.

ineffective is bad quality. Therefore, quality-evaluation of Wakan-yaku is very difficult, or impossible, before use on patients. On the other hand, use of homogeneous Wakan-yaku is possible through an evaluation of equality, *i.e.*, equality-evaluation, to clarify whether the Wakan-yaku now in use is the same as the one previously used or not (Fig. 1). This review deals with our comparative study, *i.e.*, equality-evaluation, on the seventeen *Salvia* plants by the use of liquid chromatography-mass spectrometry (LC-MS).

II. LC-MS as a method for equality-evaluation

For quality-evaluation of Wakan-yaku, some representative constituents and/or activities have been analyzed, but the methods now in use have no or only little correlation to the efficacy on patients. On the other hand, those methods would be useful for equality-evaluation, because they could compare the constituents and/or activities of the Wakan-yaku now in use with ones previously used. However, since Wakan-yaku is a complex system having many constituents and many activities, it is required to compare as many constituents and activities as possible, not only some representative ones. Because simultaneous analysis of many constituents would be possible but simultaneous assay of many activities is impracticable, the former should be adequate for the equality-evaluation of Wakan-yaku. Usually, this has been conducted by gas chromatography (GC) with a flame ionization detector (FID) or by high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector.

GC separation has merits that each constituent could be identified through an analysis of fragmentation and that better separation could be attained than other separation methods, while it has also demerits that derivatization to a volatile compound is usually needed, that compounds being unstable against heating or having high molecular weight could not be analyzed, and that isolation of constituents is difficult. On the other hand, HPLC separation has merits that derivatization is needless, that it is applicable to compounds also being unstable against heating or having high molecular weight, and that it is easily applicable to isolation of constituents, while it has demerits that an identification of each constituent is difficult because of less (almost no) fragmentation and that the separation is not as good as GC separation.

Constituents of Wakan-yaku, especially characteristic ones, usually have polar functionality and are non-volatile and/or unstable against heating. The

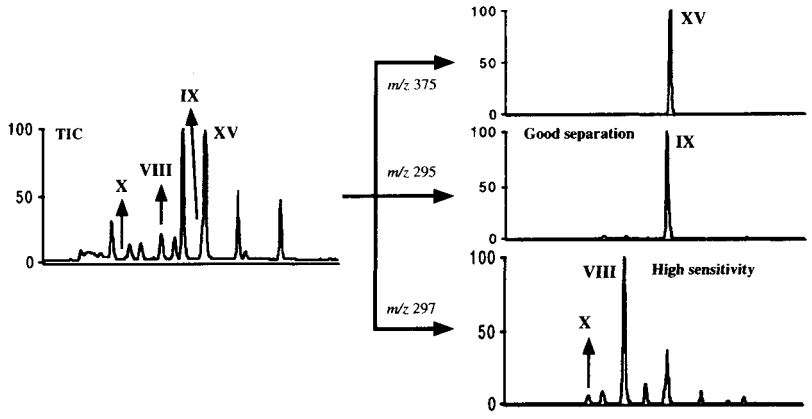

Fig. 2 Effect of separation by mass number. In the mass chromatograms of m/z 375 and m/z 295, two peaks of **XV** and **IX**, overlapped in TIC, were clearly separated. While in the mass chromatogram of m/z 297, the peak of **X**, not detected in TIC, was clearly detected.

HPLC separation, thus, is more adequate for an analysis of constituents of Wakan-yaku. However, the separation of HPLC is not as good as that of GC, and many constituents have no UV absorption; *i.e.*, UV detection only is not enough for the purpose. Thus, the use of mass spectrometry (MS) as a detector, *e.g.*, LC-MS, has recently been attempted for such a purpose, because of the possibility of measuring many compounds, regardless of UV absorption, at once. In addition, detection with MS has an additional merit that better separation and high sensitivity could be obtained through the analysis of each mass chromatogram (Fig. 2) (more detail on MS and LC-MS, see references 1-3). Thus, we use LC-MS for equality-evaluation of Wakan-yaku or crude drugs used in Wakan-yaku.

III. Wakan-yaku "Tan-jin (Dan-shen, 丹参, Radix Salviae miltiorhizae)"

Tan-jin (Dan-shen, 丹参, Radix Salviae miltiorhizae), one of the famous Wakan-yaku, is officially listed in the Chinese Pharmacopoeia[4] and used for treatment of menstrual disorder, menopause, menorrhagia, insomnia, blood circulation diseases, and angina pectoris as well as against inflammation.[5,6] The Chinese Pharmacopoeia prescribes that Dan-shen is prepared from the dry roots and rhizomes of *Salvia miltiorhiza* BUNGE (Lamiaceae).[4] In the People's Republic of China, however, one hundred and ten *Salvia* species are grown, and twelve of them (*S. bowleyana, S. deserta, S. miltiorhiza, 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 used as resources of Dan-shen.[7,8]

In the course of our chemical study on Wakan-yaku, we examined the constituents of roots of *S. miltiorhiza* (Tan-jin, Dan-shen, 丹参)[9,10] and *S. deserta* (Shin-kyou-tan-jin, Xin-jiang-dan-shen, 新疆丹参).[11] *Salvia miltiorhiza* contained "tanshinones" (*e.g.*, tanshinone IIA) as abietane-type diterpenes and a tetramer (magnesium lithospermate B) as the main caffeic acid derivative,[12] while *S. deserta* contained "royleanones" (*e.g.*, horminone) as abietane-type diterpenes and a trimer (salvianolic acid K) as the main caffeic acid derivative. In our assay system of aldose reductase (AR) inhibition, the main active constituents of *S. miltiorhiza* were "tanshinones",[10] while those of *S. deserta* were caffeic acid derivatives.[13] These results aroused our interest in the equality of the plants used as resources of Dan-shen in the People's Republic of China. But there were only few comparative studies on their composition and activities, and thus, we conducted the examination of AR inhibitory activity and LC-MS analysis of seventeen *Salvia* plants (Table I), including *S. miltiorhiza* and *S. deserta*, among which ten species are used as Dan-shen resources in the People's Republic of China.

Table I List of plant name, locality, and AR inhibitory activities of seventeen *Salvia* plants

Sample No.	Plant name	Locality	AR inhibition (IC_{50} in μg/ml)			
			Water ext.	MeOH ext.	EtOAc-soluble	EtOAc-insoluble
1	*S. bowleyana* DUNN	Gaoan, Jiangxi province	364.7	99.3	70.2	90.0
2	*S. bowleyana* DUNN	Kaihua, Zejiang province	365.1	98.0	60.9	86.1
3	*S. bulleyana* DIELS	Dali, Yunnan province	96.9	99.8	93.3	87.1
4	*S. deserta* SCHANG.	Urumuqi, Xinjiang province	84.3	78.5	76.8	7.2
5	*S. flava* FORREST et DIELS	Lijiang, Yunnan province	228.2	98.6	86.3	70.0
6	*S. meiliensis* S. W. SU	Huoshan, Anhui province	298.0	97.2	61.0	92.6
7	*S. miltiorhiza* BUNGE	Chuxian, Anhui province	223.6	93.1	10.8	89.0
8	*S. miltiorhiza* BUNGE (cultivated)	Zhongjiang, Sichuan province	199.8	93.8	11.2	91.3
9	*S. miltiorhiza* BUNGE	Heze, Shandong province	211.5	93.0	9.9	91.9
10	*S. miltiorhiza* BUNGE var. *miltiorhiza* f. *alba* C. Y. WU et H. W. LI (cultivated)	Zhangqiu, Shandong province	197.3	95.2	12.5	88.6
11	*S. paramiltiorhiza* H. W. LI et X. L. HUANG	Shucheng, Anhui province	348.7	96.6	40.1	84.0
12	*S. paramiltiorhiza* f. *purpureo-rubra* H. W. LI	Tongling, Anhui province	346.5	99.1	41.2	87.8
13	*S. przewalskii* MAXIM.	Lijiang, Yunnan province	83.5	33.1	8.6	8.0
14	*S. przewalskii* MAXIM. var. *mandarinorum* STIB.	Saotong, Yunnan province	84.2	27.9	9.3	8.3
15	*S. przewalskii* MAXIM. var. *mandarinorum* STIB.	Dali, Yunnan province	86.2	29.8	7.9	7.2
16	*S. sinica* MIGO f. *purpurea* H. W. LI	Chongyang, Anhui province	213.1	97.5	86.7	90.9
17	*S. trijuga* DIELS	Lijiang, Yunnan province	79.9	98.8	19.5	70.1

IV. Comparative study of seventeen *Salvia* plants

From the plants listed in Table I, we prepared water and methanol (MeOH) extracts, and the latter was separated to ethyl acetate (EtOAc)-soluble and -insoluble parts. On these two extracts and two parts, we conducted the examination of AR inhibitory activity and LC-MS analysis.

1. AR inhibitory activity[13]

As shown in Table I, the MeOH extracts generally inhibited AR more strongly (IC_{50}, 27.9-99.8 μg/ml) than the water extracts. In addition, EtOAc-soluble parts of *S. miltiorhiza* (Nos. 7-9), *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10), *S. przewalskii* (No. 13), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15), and *S. trijuga* (No. 17) and EtOAc-insoluble parts of *S. deserta* (No. 4), *S. przewalskii* (No. 13), and *S. przewalskii* var. *mandarinorum* (Nos. 14, 15) showed strong activity (IC_{50}, 7.2-19.5 μg/ml). Thus, as active constituents, *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10) and *S. trijuga* (No. 17) would contain less-polar compounds (*e.g.*, "tanshinones") as *S. miltiorhiza* (Nos. 7-9), while *S. przewalskii* (No. 13) and *S. przewalskii* var. *mandarinorum* (Nos. 14, 15) contain both the less-polar and polar compounds.

Though the activity of water extracts was weaker than that of MeOH extracts, water extracts of five species [*S. bulleyana* (No. 3), *S. deserta* (No. 4), *S. przewalskii* (No. 13), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15), *S. trijuga* (No. 17)] showed AR inhibitory activity comparable to that of MeOH extracts (Fig. 3). Among the five, three (*S. deserta, S. przewalskii, S. przewalskii* var. *mandarinorum*) were the species in which EtOAc-insoluble part showed stronger AR inhibitory activity than the corresponding EtOAc-soluble part.

This result suggests that, with regard to the AR inhibitory activity, the seventeen plants are not equal and there are at least three types: the first type containing less-polar active compounds, the next type containing polar active compounds, the third type containing both active compounds. However, it is noteworthy in that the activities of the same species [*S. bowleyana* (Nos. 1, 2), *S. miltiorhiza* (Nos. 7-9), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15)] were almost the same and that *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10), *S. paramiltiorhiza* f. *purpureo-rubra* (No. 12), and *S. przewalskii* var. *mandarinorum* (No. 14, 15) showed similar inhibitory activities to their corresponding species [*S. miltiorhiza* (Nos. 7-9), *S. paramiltiorhiza* (No. 11), and *S. przewalskii* (No.

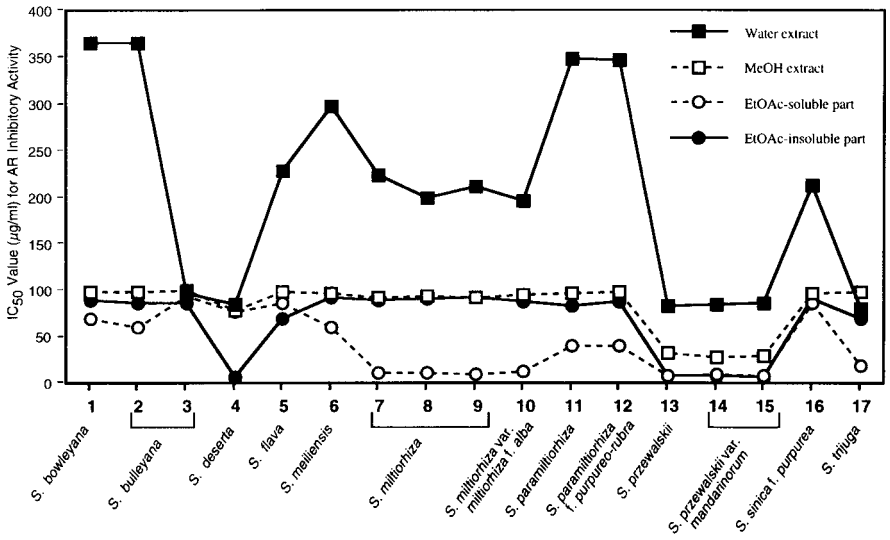

Fig. 3 AR inhibitory activity (IC_{50}, μg/ml) of water and MeOH extracts and EtOAc-soluble and -insoluble parts.

13), respectively].

2. LC-MS analysis of water extracts[13]

The LC-MS analysis of the water extracts was conducted by using the caffeic acid derivatives **I**–**IV** (Fig. 4) as standards, with an electrospray ionization (ESI) method.[2] They showed a slightly overlapped total ion chromatogram (TIC) but were well separated on a mass chromatogram at the respective protonated molecular ion. We thus calculated their amounts from the mass chromatogram (Table II), except for that of **IV** whose ion strength did not show linearity against the amount. The amount of **I** was large (100-260 μg/mg) as usual, but it was small in *S. bulleyana* (No. 3, 15.9 μg/mg), *S. deserta* (No. 4, 0.3 μg/mg), *S. flava* (No. 5, 7.3 μg/mg), *S. przewalskii* (No. 13, 39.0 μg/mg), *S. przewalskii* var. *mandarinorum* (No. 14, 19.0 μg/mg; No. 15, 6.2 μg/mg), and *S. trijuga* (No. 17, 77.5 μg/mg) (Fig. 5). On the other hand, the ratio of **I** against the total amount of **I**–**III** was high (>90%) as usual, but that of *S. deserta* (No. 4), *S. flava* (No. 5), *S. przewalskii* (No. 13), and *S. przewalskii* var. *mandarinorum* (Nos. 14, 15) was low (0.94-80.4%) (Fig. 5). Thus, with regard to the amount of **I**, the seventeen plants are not equal and

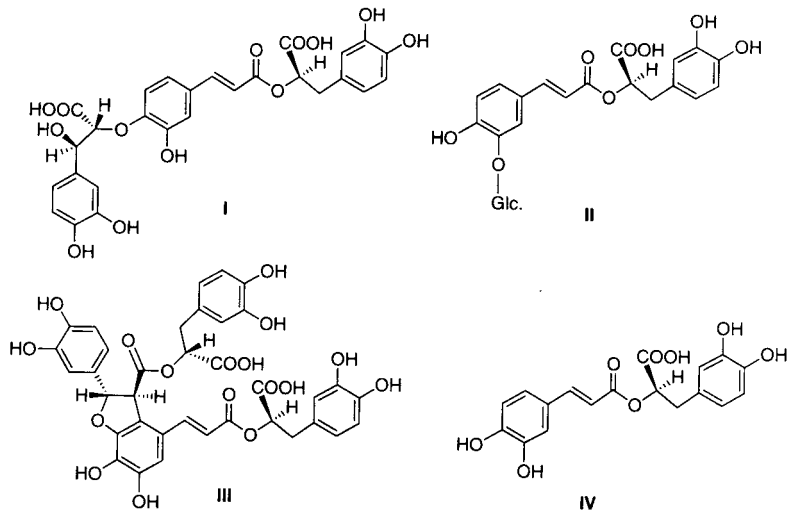

Fig. 4 Structures of caffeic acid derivatives **I**–**IV** used as standards.

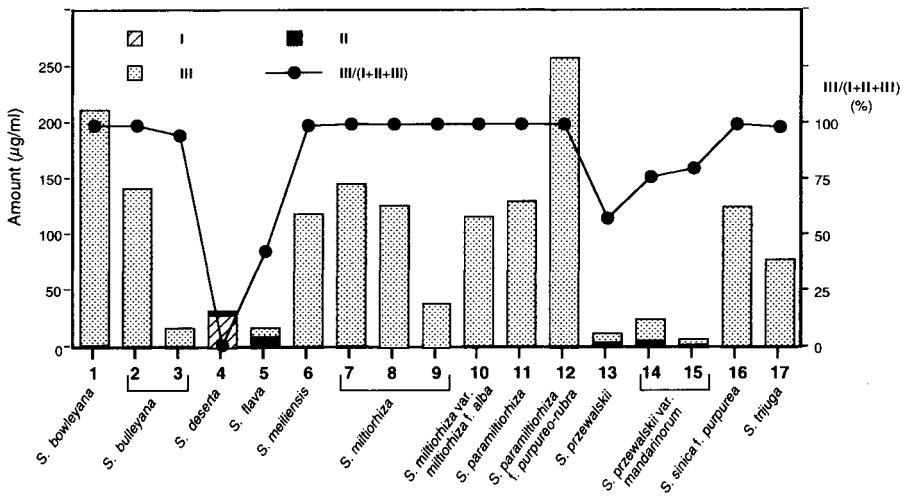

Fig. 5 Amounts (μg/ml) of caffeic acid derivatives **I**−**III** and the ratio (%) of **III** in the total of **I**−**III**.

there are at least three types: the first containing a large amount of **I**, the second containing a small amount but a high ratio of **I**, and a third containing a small amount and a low ratio of **I**. In addition, it should be noted that the plants of the same species [*S. bowleyana* (Nos. 1, 2), *S. miltiorhiza* (Nos. 7-9), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15)] were not the same in their content of **I**−**III**, though they belong to the same group.

3. *LC-MS analysis of MeOH extracts*[14]

The LC-MS analysis of the EtOAc-insoluble parts of MeOH extracts gave the identical result with that of water extracts, but that of the EtOAc-soluble parts gave no satisfactory result under the same conditions. This would be due to the low polarity of diterpenes **V**−**XVII** (Fig. 6), contained in the EtOAc-

Table II Amounts (μg/mg) of compounds **I**−**III** in water extracts by ESI-LC-MS and relative intensity of the $[M+H]^+$ ions of diterpenoids in EtOAc-soluble parts of MeOH extract by APCI-LC-MS

Sample No.	Plant name	Water extract				EtOAc-Soluble part of MeOH extract				
		III	**II**	**I**	**I/(I+II+III)**	**X**	**VII**	**VIII**	**XI**	**IX**
		(7.48)[a]	(8.82)[a]	(9.20)[a]	(%)	(5.69)[a]	(5.96)[a]	(8.08)[a]	(8.97)[a]	(10.84)[a]
1	*S. bowleyana*	1.7	0.40	210.3	99.0	416.9	836.1	2453.9	404.7	2920.4
2	*S. bowleyana*	0.6	0.10	141.1	99.5	3.2	12.3	21.8	31.4	76.7
3	*S. bulleyana*	0.5	0.36	15.9	94.9	0.1	0.2	0.1	0.1	0.0
4	*S. deserta*	2.5	29.28	0.3	0.94	0.0	0.0	0.0	0.1	0.0
5	*S. flava*	9.7	0.03	7.3	42.9	0.1	0.2	0.0	0.0	0.0
6	*S. meiliensis*	0.8	0.18	118.1	99.2	16.0	45.1	90.7	20.9	109.3
7	*S. miltiorhiza*	0.2	0.10	145.4	99.8	468.8	1140.0	2574.3	149.9	3430.6
8	*S. miltiorhiza* (cultivated)	0.1	0.05	127.1	99.9	158.5	195.0	1820.6	51.9	2026.8
9	*S. miltiorhiza*	0.1	0.06	39.0	99.6	182.0	234.0	1923.8	58.1	2049.9
10	*S. miltiorhiza* var. *miltiorhiza* f. *alba* (cultivated)	0.3	0.14	116.5	99.6	104.5	598.6	1999.8	52.1	1496.6
11	*S. paramiltiorhiza*	0.4	0.02	130.0	99.7	40.0	140.7	290.5	44.8	470.9
12	*S. paramiltiorhiza* f. *purpureo-rubra*	0.6	0.04	258.3	99.8	17.3	95.9	756.2	25.6	1744.5
13	*S. przewalskii*	5.0	0.02	6.9	57.9	18.7	37.4	270.2	95.9	2201.3
14	*S. przewalskii* var. *mandarinorum*	5.6	0.19	19.0	76.6	0.1	0.4	0.6	0.2	0.6
15	*S. przewalskii* var. *mandarinorum*	1.5	0.01	6.2	80.4	3.1	4.3	114.4	45.6	64.3
16	*S. sinica* f. *purpurea*	0.4	0.11	125.8	99.6	0.1	0.1	0.0	0.1	0.0
17	*S. trijuga*	1.2	0.04	77.5	98.4	56.1	56.1	1169.0	1630.8	2887.4

[a]Retention time in minutes. The relative intensity of **V** (t_R 4.69 min), **VI** (t_R 6.67 min), **XII** (t_R 16.14 min), **XIII** (t_R 16.97 min), **XIV** (t_R 13.27 min), **XV** (t_R 13.79 min), **XVI** (t_R 11.04 min), and **XVII** (t_R 9.53 min) were very small, and thus they are excluded from this Table.

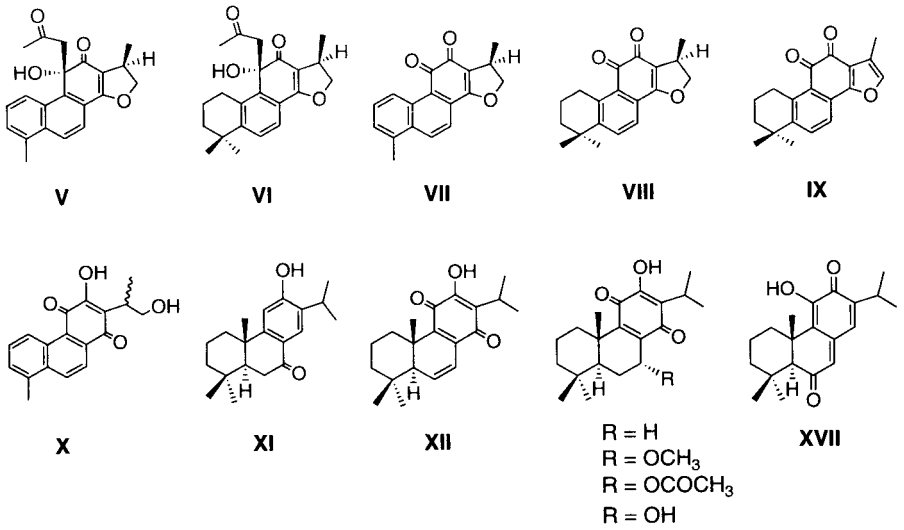

Fig. 6 Structures of diterpenoids **V**−**XVII** used as standards.

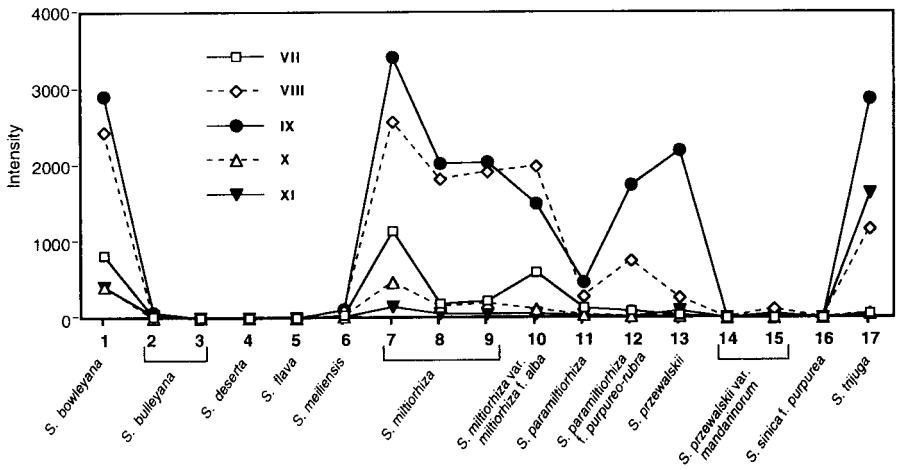

Fig. 7 Intensity of diterpenoids **V**−**XVII** in mass chromatograms of EtOAc-soluble parts monitored by the protonated molecular ion of each compound. The intensities of diterpenoids **V**, **VI**, and **XII**−**XVII** were less than 12 in all measurements, and thus they are excluded.

insoluble parts, because the ESI method is more adequate to an ionization of polar compounds. Indeed, all compounds could be ionized well with an atomospheric pressure chemical ionization (APCI) method, being adequate to an ionization of less polar compounds.[2] In addition, the mass chromatograms monitored by the respective protonated molecular ion of **V**−**XVII** revealed good separation, and the intensities of the protonated molecular ion corresponding to each compound were obtained (Table II).

As can be seen in Fig. 7, the intensities of cryptotanshinone (**VIII**) and tanshinone IIA (**IX**) were high in the EtOAc-soluble parts of *S. bowleyana* (No. 1), *S. miltiorhiza* (Nos. 7-9), *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10), *S. paramiltiorhiza* f. *purpureo-rubra* (No. 12), and *S. trijuga* (No. 17), while they were low in the others. The intensity could not reflect a difference of the quantity between compounds in each extract, because the ionization efficiency of each compound was different. However,

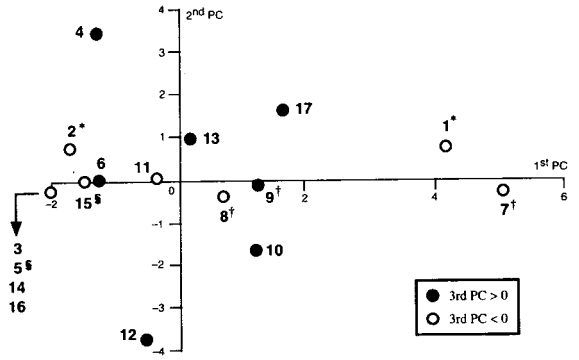

Fig. 8 Karhunen-Loeve plot of 1st-2nd PC. The numbers indicate the sample number in Table I. *, †, §. belong to the same species, respectively.

being measured under carefully controlled conditions, the intensity reflects the difference of quantity of each compound between extracts and is usable as a simple index for comparing the quantity between extracts. Thus, the difference of the intensity indicates the difference of the quantities of each compound between plants; *i.e.*, the seventeen plants are not equal.

This difference was more clearly indicated by applying principlal component analysis (PCA)[15,16] on the intensities. The result indicates that the first three principal components (PCs) could account for 71.6% of variance in the data set. The scores plotted in terms of the 1st and 2nd PCs (Fig. 8) 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), *S. przewalskii* var. *mandarinorum* (No. 14), and *S. sinica* (No. 16) could form another group. However, *S. miltiorhiza* at Chuxian (No. 7) stands out from *S. miltiorhiza* at Zhongjiang (No. 8) and Heze (No. 9); *S. paramiltiorhiza* f. *purpureo-rubra* (No. 12) stands out from *S. paramiltiorhiza* (No. 11); and *S. deserta* (No. 4) stands out from other *Salvia* plants. Thus, the seventeen plants should be not equal in a viewpoint of diterpene constituents, although they belong to the same *Salvia* genus.

V. Conclusion: from chemical equality-evaluation to chemical quality-evaluation

As noted in the preceding section, the seventeen *Salvia* plants were not equal with regards to AR inhibitory activity and chemical constituents. Since these plants were collected without any attention to timing of collection, producing area, individual, *etc.*, such variation might be reasonable, but it might also suggest that without any attention to the equality of the crude drugs, homogeneity of the Wakan-yaku could not be maintained. In addition, equality-evaluation would prevent an accident due to misuse of a similar crude drug, as in the case of Chinese herbs nephropathy, which had been caused by misuse of *Aristolochia fangchi* as *Stephania tetrandra*.[17,18] As a method for such equality-evaluation, the LC-MS analysis of the crude drugs should be a simple, easy, and useful method. In addition, by comparing the LC-MS data of the crude drugs and the efficacy of the Wakan-yaku on patients, characteristics common to the effective crude drugs would be clarified. Once the characteristics (*i.e.*, characteristic constituents) are identified, they will be useful indexes for quality-evaluation.

Acknowledgment

The author is grateful to Prof. Shigetoshi Kadota in Department of Natural Products Chemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, and the other persons concerned with this work, for their sincere support and valuable assistance.

和文抄録

和漢薬は天然物であり，その成分や活性は種，採集時期，産地，個体等，種々の要因に依存する。そのため，均質な和漢薬を用いるには，その成分や活性の同等性を評価した上で用いる必要があり，成分含量や活性の変化を検出する有効な方法が必要とされている。しかし従来法の多くは，少数の代表的成分や活性の比較であり，多成分，多活性を有する和漢薬に適した方法ではなかった。一方，多成分の同時測定が可能なことから，ガスクロマトグラフィ連結質量分析計 (GC-MS) や液体クロマトグラフィ連結質量分析計 (LC-MS) の応用が近年試みられている。本稿では，種々のタイプの化合物に適応容易なLC-MS 法について，我々が行なった漢薬"丹参"の基源植物間の比較を例として述べた。

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