Histochemistry. XII.[1)]
Morphological, histological and chemical evaluation of roots of *Bupleurum falcatum* L. cultivated at eighteen producing districts in Japan

Tadato TANI,* Tadahisa KATSUKI and Shigeru ARICHI

The Research Institute of Oriental Medicine, Kinki University

(Received November 20, 1987. Accepted February 1, 1988.)

Abstract

Bupleurum falcatum roots, the seeds of which had been harvested in Shizuoka Prefecture and cultivated at 18 producing fields in seven prefectures, were examined from morphological, histological and chemical viewpoints. It was proved that saikosaponin d concentration varied markedly among the 18 samples and that saikosaponin a concentration showing a smaller variation correlated negatively with main root diameter. The regional characteristics of the roots were clarified by factor analysis using 12 variables. Saikosaponin a concentration was found to be estimable by multiple regression analysis, revealing that light and thin roots tended to contain more saikosaponin a than weighty and thick ones.

Key words Chinese crude drug, *Bupleurum falcatum* L., saikosaponin, herbal drug evaluation, HPLC, plasma emission spectrometer, multivariate analysis

Abbreviations HPLC, high-performance liquid chromatography ; ICP, inductively-coupled plasma emission spectrometer

Introduction

A Chinese crude drug Bupleuri Radix, Saiko in Japanese, which has recently attracted pharmacognostical, pharmacological and clinical interest, is defined as the roots of *Bupleurum falcatum* L. (Mishima-saiko in Japanese) and its varieties (Umbelliferae) by the XIth Japanese Pharmacopoeia. As about 90% or much more of the Radix currently used in Japanese Kampo prescriptions is imported from China, cultivation of *B. falcatum* growing naturally in Japan has been attempted to stabilize the procurement of crude drug resources in some places in Japan.

Recently, one-year-old roots of *B. falcatum* cultivated at eighteen producing districts in seven prefectures in Japan were obtained. Although the seeds harvested at the same producing field in Shizuoka Prefecture were sowed in this test cultivation, the 18 batches of *B. falcatum* roots had varied morphological characteristics such as weight and diameter of main roots.

B. falcatum roots cultivated in Japan have been examined as to their concentrations and composition of saikosaponins[2)] and elements[3)] from the histological and chemical viewpoints. In the course of continuing investigations on Japanese *B. falcatum* roots, we further examined the relationship between saikosaponin concentrations and morphological and geographical characteristics of the roots obtained from 18 producing fields by means of multivariate analysis, which seems to be a convenient method for characterizing crude drugs possessing various morphological and chemical variables, as reported previously.[1,3,4)]

Materials and Methods

Materials : One-year-old roots of *B. falcatum* L., the seeds of which had been collected at

*〒589 大阪府大阪狭山市大野東377-2
近畿大学東洋医学研究所 谿 忠人
377-2 Ohno-higashi, Osaka-sayama, Osaka 589, Japan

Gotemba in Shizuoka Prefecture in October 1981, were sowed and cultivated in the following 18 producing districts : A, B, Iwate Prefecture (Daitoh) ; C, D, Ibaraki Pref. (Yasato) ; E, F, G, Gifu Pref. (Minokamo) ; H, Hyogo Pref. (Ono) ; I, Hyogo Pref. (Yasutomi) ; J, K, L, Nara Pref. (Totsukawa) ; M, N, O, Kochi Pref. (Mihara) ; P, Q, Kumamoto Pref. (Johnan) ; and R, Kumamoto Pref. (Minamata).

Cultivation and harvesting procedures are generally standardized as follows : the seeds were sowed in the soil treated with vegetable manure, oil cake, artificial manure and superphosphate of lime at the end of March or the beginning of April 1982, and additional composts were given in June, July and September. The flower-stalk was trimmed to about 40 cm from the ground surface between August and September. Roots were harvested from February to March 1983, and washed, root hairs removed and dried naturally for two or three weeks.

Morphological and Histological Characteristics : The two morphological characteristics of the roots, the whole root weight (g) and the maximum diameter of the main root (mm), were measured using 10 whole roots sampled randomly from each batch, in the manner previously reported.[1]

Thin cross-sections of the roots from each batch were prepared using a freezing microtome (MA-101, Komatsu Electronic) and examined histologically using a light microscope (Vanox, Olympus) with a microphotographic camera (PM -10, Olympus). The xylem ratio (%) : (xylem diameter)×100/(main root diameter) was calculated via microscopic examination.

Chemical Characteristics : Ten whole roots randomly sampled from each batch were pulverized using a coffee mill. About 500 mg, exactly weighed, of each powder was extracted with hot water (80 ml) for 2 hr. After cooling, the water-soluble portion was made up to 100 ml with water and then measured as to pH value, the fourth variable, using a pH meter (F-8L, Horiba).

Five elements, magnesium (Mg), phosphorus (P), potassium (K), calcium (Ca) and iron (Fe) in the ash prepared from the powder with a plasma asher (ASH-302, Hitachi) were determined by an inductively-coupled plasma emission spectrometer (ICP, Hitachi 300) by the same procedures as reported previously.[3]

Five hundred mg of the powder, exactly weighed, was extracted twice with hot methanol (80 ml each time). The methanol extract yield (%) obtained from the powder was determined after evaporation of methanol under reduced pressure.

Bioactive saikosaponins a and d in the methanol extract were determined quantitatively via high - performance liquid chromatography (HPLC). The pre-treatment of the methanol extract with a SEP-PAK C_{18} cartridge (Waters), the HPLC system (LC-3A, Shimadzu) equipped with an ultra-violet spectrophotometric detector (SPD-6A, Shimadzu) and the computing integrator (C-R1A, Shimadzu), stationary phase (NOVA-PAK C_{18}, Waters), mobile phase (methanol-water) and other determination conditions were the same as those in our previous report.[2] The authentic saikosaponins a and d used for the identification and calibration of chromatograms were kindly supplied by the Shionogi Research Laboratory. The concentrations (%) of two saikosaponins were based upon the whole root weight.

Statistical Analysis : Factor loadings of five factors (I-V as shown in Table II) were rotated by the normal varimax method. Each factor score calculated using 12 factor loadings was figured out using radar charts (Fig. 1) for comparison of 18 samples.

Correlation coefficients (r) in Table III and Fig. 3 were statistically evaluated in accordance with the equation $t_{0.05,df16}=r\sqrt{(1-r^2)/(n-2)}$, where t represents the t-value (1.746) of the critical rate (5%) and degree of freedom (16) obtained from a statistical t-table and n is the sample number (18). Thus, correlation coefficients with an absolute value of more than 0.40 are found to be statistically meaningful.

The regression equations (Fig. 3) between saikosaponins a and d concentrations (y : %) and main root diameter (x : mm) are obtained by a least squares method.

The multiple regression equations estimating

saikosaponin a concentration, a dependent variable, were calculated using 10 independent variables.

These statistical calculations were performed on a personal computer (PC - 9801 Vm 2, NEC) with multivariate analysis software packages programmed by Nippon Maikon Gakuin and Kyoritsu Press.

Results and Discussion

The relationship between morphological characteristics and saikosaponin concentration of *B. falcatum* roots cultivated in Nara Prefecture has already been examined histochemically.[1,2] In the present study, characteristics of one-year-old *B. falcatum* roots cultivated at 18 producing fields in seven prefectures using the same seeds harvested in Shizuoka Prefecture were statistically examined by multivariate analysis.

The means and standard deviation (S.D.) of 12 variables determined for 180 roots of 18 samples are summarized in Table I. Morphologically, whole root weight and main root diameter exhibited about two or three - fold variance. Among 12 variables, the largest coefficient of variation (C.V.) was found in saikosaponin d concentration (C.V.=73.3%), which exhibited about 11-fold variance by the sample (sample A, 0.05%, and sample K, 0.56%) and about five-fold variance by prefecture (Kochi Pref., 0.09% and Nara Pref., 0.43%), whereas saikosaponin a concentration (0.15−0.28%) showed a smaller variation (C.V.=15.2%).

The present samples are found to have similar saikosaponin concentrations to those reported.[5] Concentration of saikosaponins (a, 0.25 ± 0.01% and d, 0.27 ± 0.08%) in the roots from Ibaraki Prefecture, the area in which medicinal plants have been extensively cultivated, is also similar to that of commercial Radix from the same prefecture.[6]

To compare regional characteristics of the samples by producing district, factor analysis was performed using 12 variables for 18 batches of *B. falcatum* roots to obtain five factors (I - V) with an eigen value greater than 1.00 (Table II). High communalities (0.50−0.98), which means the sum of squared factor loadings of each variable, are obtained in this factor analysis.

Factor I, with the largest eigen value (2.92), has high factor loadings of saikosaponin a concentration (−0.805) together with whole root weight, main root diameter and Ca concentration. Factor II, with an eigen value of 2.02, contains high factor loadings of Mg and P concentrations. Factor loading of xylem ratio (−0.553) as an index of root flexibility constitutes Factor III, to-

Table I Twelve variables for *B. falcatum* roots cultivated at 18 producing fields in Japan.

Variables	Mean±S.D.	C.V. (%)	Minimum value	Maximum value
[1] Whole root weight (%)	1.65±0.65	39.3	0.88	2.80
[2] Main root diameter (mm)	5.83±1.20	20.6	4.40	8.53
[3] Xylem ratio (%)	61.8±4.75	7.7	52.3	68.6
[4] pH of aqueous decoction	5.49±0.12	2.1	5.20	5.72
[5] Mg (mg/g)	2.27±0.66	27.1	1.34	3.24
[6] P (mg/g)	4.38±0.72	16.5	2.94	5.44
[7] Ca (mg/g)	3.67±1.52	41.3	1.96	7.44
[8] K (mg/g)	6.78±1.57	23.2	5.11	10.44
[9] Fe (mg/g)	0.05±0.04	68.8	0.03	0.17
[10] MeOH extraction yield (%)	24.5±5.31	21.7	13.0	32.0
[11] Saikosaponin a (%)	0.21±0.03	15.2	0.15	0.28
[12] Saikosaponin d (%)	0.20±0.15	73.3	0.05	0.56

Values represent the mean±S.D. from measurement of 18 samples.

Table II Factor loadings of five factors for *B. falcatum* roots.

Variables\factors	I	II	III	IV	V	Communality
[1] Whole root weight (%)	0.84	−0.04	−0.00	−0.06	−0.09	0.71
[2] Main root diameter (mm)	0.93	0.19	0.02	−0.18	0.21	0.98
[3] Xylem ratio (%)	0.07	0.34	−0.55	−0.52	0.10	0.71
[4] pH of aqueous decoction	−0.05	−0.19	−0.74	0.14	0.30	0.69
[5] Mg (mg/g)	−0.03	−0.83	0.32	−0.07	0.36	0.93
[6] P (mg/g)	−0.08	−0.91	−0.16	0.13	−0.10	0.88
[7] Ca (mg/g)	0.76	0.27	0.07	0.08	0.25	0.72
[8] K (mg/g)	0.16	0.41	0.10	−0.53	0.12	0.50
[9] Fe (mg/g)	−0.04	−0.09	0.91	−0.07	0.17	0.87
[10] MeOH extraction yield (%)	−0.23	0.09	−0.12	0.84	0.09	0.81
[11] Saikosaponin a (%)	−0.81	0.23	0.13	0.25	0.20	0.82
[12] Saikosaponin d (%)	0.09	−0.05	−0.08	−0.01	0.80	0.66
Eigen value	2.92	2.02	1.86	1.40	1.08	
Contribution ratio (%)	24.3	16.9	15.5	11.7	9.0	
Cumulative contribution ratio (%)	24.3	41.2	56.7	68.3	77.4	

I ~ V : Factors calculated using 12 factor loadings rotated by normal varimax method.

gether with pH value of H_2O decoction and Fe concentration. Factor IV has high factor loadings of MeOH extraction yield and K concentration. Factor loading of saikosaponin d concentration (0.804) is an essential part of Factor V. The morphological, histological and chemical variance of the 18 batches was clarified via factor analysis using these five factors, with a cumulative contribution ratio of 77.4%.

Five factor scores of 18 samples, calculated using 12 factor loadings, are shown in seven pentagonal radar charts for every cultivation prefecture (Fig. 1). There are individual differences by producing district and by prefecture. Samples cultivated in Ibaraki, Nara and Kumamoto Prefectures show their own characteristics by regional group. The roots from Ibaraki Prefecture, with a smaller score for Factor I and larger score for Factor V, tend to contain more saikosaponins a and d ($0.51 \pm 0.09\%$) than do roots ($0.35 \pm 0.06\%$) from Kumamoto Prefecture, whose climate is warmer. This is in agreement with the finding that *B. falcatum* roots cultivated in lower temperature areas in a test field are apt to contain more saikosaponins than are roots in higher temperature areas in the same field.[7]

There are regional differences in roots even from the same prefecture, as in those from Iwate, Gifu and Hyogo. Detailed comparison is made between sample H with small score Factor I and large score Factor III, and sample I with large score Factor I and small score Factor III, both cultivated in Hyogo Prefecture (Fig. 2). According to considerations of the absolute values of factor loadings constituting each Factor, a large score for Factors I and III corresponds to weighty, thick roots with small xylem ratio. As noted in Fig. 2, sample H is lighter and thinner than sample I ($p < 0.05$), as was supposed. Sample H, with its small xylem ratio and consequently wide phloem and outer parenchyma, in which saikosaponins are concentrated tissue-specifically,[2] shows a higher concentration of saikosaponins ($p < 0.05$) than does sample I, with its large xylem ratio. These morphological, histological and chemical differences between the two samples are in agreement with those of previous results[1] in that the thin and flexible *B. falcatum* roots cultivated in Nara Prefecture are apt to contain more bioactive saikosaponins than the large and thick roots with large xylem ratio.

As it is clear from the correlation matrices among the 12 variables (Table III), saikosaponin a concentration is closely related not only to whole root weight ($r = -0.59$) and main root diameter ($r = -0.72$), but also to Ca concentration ($r = -0.52$) and MeOH extraction yield ($r = 0.42$).

As shown in Fig. 3, the regression equation

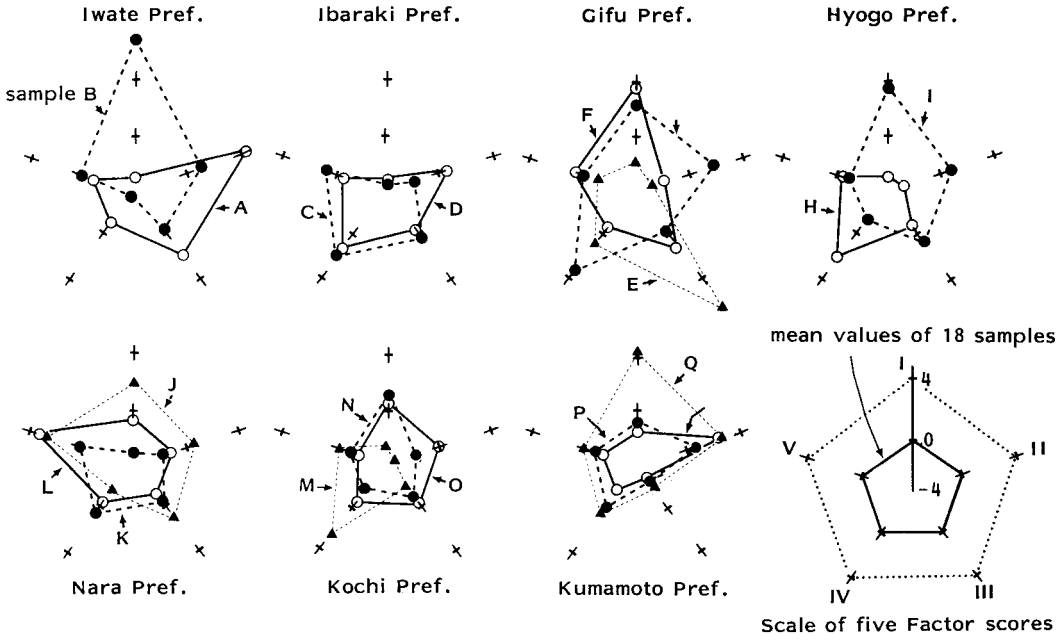
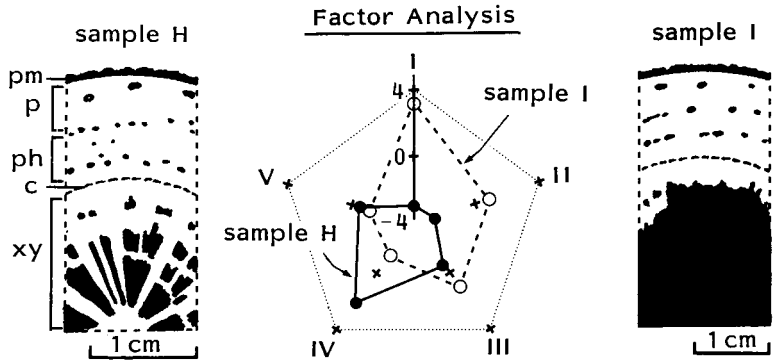


Fig. 1 Comparison of 18 *B. falcatum* roots cultivated at seven prefectures and examined by factor analysis.

Each pentagon contains five factor scores, calculated using 12 factor loadings, as shown in Table II.



1.05±0.44	whole root weight (g)	1.56±0.79*
4.83±0.79	main root diameter (mm)	7.02±1.84*
56.5±1.79	xylem ratio (%)	63.9±2.59*
0.21±0.004	saikosaponin a (%)	0.16±0.018*
0.16±0.001	saikosaponin d (%)	0.11±0.004*

Fig. 2 Comparison of two *B. falcatum* roots cultivated in Hyogo Prefecture.

Samples H and I are illustrated diagrams of transverse sections. Factor analysis is shown in pentagonal radar chart with five factor (I-V) scores examined by factor analysis (Table II). *$p<0.05$ (sample H vs. sample I) evaluated by Student's t-test. Abbreviations : c : cambium ; p : parenchyma ; ph : phloem ; pm : periderm ; xy : xylem.

Table III Correlation matrix of 12 variables determined for *B. falcatum* roots.

Variable\variable	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
[1] Whole root weight	1											
[2] Main root diameter	0.77	1										
[3] Xylem ratio	−0.02	0.18	1									
[4] pH of aqueous decoction	−0.01	−0.04	0.22	1								
[5] Mg	−0.08	−0.09	−0.41	−0.04	1							
[6] P	−0.05	−0.32	−0.24	0.22	0.68	1						
[7] Ca	0.49	0.79	0.20	−0.13	−0.11	−0.28	1					
[8] K	0.12	0.39	0.34	−0.22	−0.21	−0.45	0.24	1				
[9] Fe	−0.05	−0.10	−0.47	−0.66	0.42	−0.10	0.06	0.04	1			
[10] MeOH extraction yield	−0.39	−0.38	−0.33	0.17	−0.13	0.12	−0.04	−0.43	−0.17	1		
[11] Saikosaponin a	−0.59	−0.72	−0.21	0.03	−0.09	−0.16	−0.52	−0.10	0.14	0.42	1	
[12] Saikosaponin d	0.05	0.25	0.14	0.31	0.28	−0.03	0.21	0.06	0.10	0.04	0.08	1

Correlation coefficients (r) with absolute value above 0.40 are statistically meaningful, as shown in Materials and Methods.

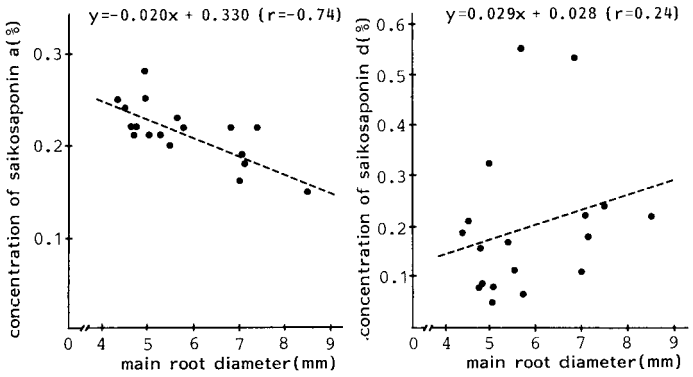


Fig. 3 Relationship between saikosaponins a and d concentrations and main root diameters of *B. falcatum* roots.

between saikosaponin a concentration (y : %) and main root diameter (x : mm) is $y=-0.020x+0.330$, whose correlation coefficient ($r=-0.74$) is statistically meaningful. The samples prepared to have thin and flexible roots must contain a higher saikosaponin a concentration. The main root diameter could be used as a parameter for evaluating *B. falcatum* roots on the basis of saikosaponin a concentration. Main root diameter variability is related to whole root weight ($r=0.77$) and Ca concentration ($r=0.79$). As the Ca concentration of roots is affected by soil and manure in cultivated fields, it is necessary to investigate soil and manure conditions in conjunction with saikosaponin concentration.

No linear relation between saikosaponin d concentration and main root diameter was observed : $y=0.029x+0.028$ ($r=0.24$).

Furthermore, the saikosaponin a concentration in 18 *B. falcatum* roots was estimated by multiple regression analysis using 10 characteristics as independent variables of (1) to (10), as shown in Table I. Thus, the multiple regression equation estimating saikosaponin a concentration is $y=-0.019x_2+0.325$ for the first step analysis, in which the partial regression coefficient of the main root diameter (x_2) is −0.019 (partial F value 17.40). The coefficient of determination adjusted from the degree of freedom of the equation is 0.491, which means that about 49% of the variance of saikosaponin a concentrations of the 18 samples could be estimated using only the main root diameter (x_2). The second step equation is calculated as $y=-0.023x_2-0.019x_6+0.430$, whose partial regression coefficients are −0.023 (32.31) for x_2 and −0.019 (8.29) for P concentration (x_6).

As shown in Fig. 4, the estimated concentrations of saikosaponin a are in good accord with

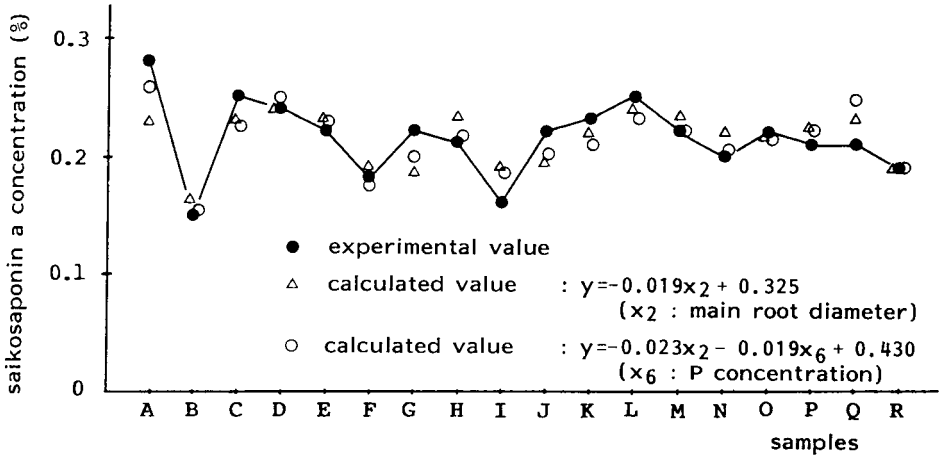


Fig. 4 Comparison of experimental and calculated values of saikosaponin a concentration of *B. falcatum* roots examined by multiple regression analysis.

the experimental values of the 18 samples. Although the saikosaponin d concentrations of 18 samples were also estimated by multiple regression analysis, they are difficult to be estimated, in view of the extreme variation (C.V.= 73.3%) among the 18 samples, and in view of their having no relationship with other variables examined in this study.

All the evidence mentioned above indicates that morphological, histological and chemical characteristics were widely dispersed in 18 batches of *B. falcatum* roots, whose seeds had been harvested in the same field in Shizuoka Prefecture. The variation might be due to the inequality of inheritance factors of the starting plant materials besides environmental conditions. Further biotechnological studies are required to produce the Radix being uniform in quality.

As herbal crude drugs are evaluated mainly by their morphological characteristics, it was necessary to investigate the relationship between conventional crude drug evaluation and analytical methods. In the present study, morphological and chemical analysis demonstrated that the concentration of saikosaponin a correlates negatively to the main roots diameter. This is in good accord with the previous results using roots cultivated in Nara Prefecture[1,2] and with the comparative study using light, thin and flexible products prepared from Ibaraki Prefecture and heavy, thick and hard roots collected from China.[6]

Acknowledgements

The authors wish to thank Dr. H. Ishii of Shionogi Research Laboratory and Mr. S. Fukuda of Nippon Tokushu Nosanbutsu Kyokai for supplying authentic saikosaponins and the cultivated *B. falcatum* roots. They are also indebted to Prof. Y. Okazaki of the Faculty of Pharmaceutical Sciences, Kinki University for his helpful discussions in ICP analysis.

和文抄録

静岡県で採取した種子を用いて，7県の18カ所で栽培したミシマサイコの根を形態学的，組織学的，および化学的な見地から検討を行った。Saikosaponin d 濃度は18検体間で著しく変動し，変動の少ない saikosaponin a 濃度は，主根部の直径と負の相関のあることが明らかとなった。各根の地域的な特徴は12変数を用いた因子分析を行うことにより明らかとした。Saikosaponin a 濃度は重回帰分析によって予測することができ，軽くて細い根は重くて太い根より saikosaponin a 含量が高い傾向のあることが明らかとなった。

References

1) Part XI : Tani, T., Katsuki, T., Kubo, M., Okazaki, Y. and Arichi, S. : Histochemistry. XI. Histological and chemical characteristics of bolting and non-bolting roots of cultivated *Bupleurum falcatum* L. *Chem. Pharm. Bull.* **35**, 4530-4536, 1987

2) Tani, T., Katsuki, T., Kubo, M. and Arichi, S. : Histochemistry. IX. Distribution of saikosaponins in *Bupleurum falcatum* root. *J. Chromatogr.* **360**, 407 - 416, 1986

3) Tani, T., Katsuki, T., Okazaki, Y. and Arichi, S. : Histochemistry. X. Distribution of aluminum, phosphorus and other elements in *Bupleurum falcatum* root cultivated in Japan. *Chem. Pharm. Bull.* **35**, 3323-3328, 1987

4) Tani, T., Katsuki, T., Kubo, M. and Arichi, S. : Numerical discrimination of Persicae Semen and Armeniacae Semen. *Shoyakugaku Zasshi* **40**, 281-288, 1986

5) Shimizu, K., Amagaya, S. and Ogihara, Y. : Separation and quantitative analysis of saikosaponins by high-performance liquid chromatography. *J. Chromatogr.* **268**, 85-91, 1983

6) Lin, M., Tani, T., Katsuki, T. and Arichi, S. : Analysis of Chinese Bupleuri Radix (1). Proc. XVth Symp. for Crude Drug Analysis (Kobe), pp. 1-8, 1986

7) Shimokawa, Y., Ushio, N., Uno, N. and Ohashi, H. : Cultivation and breeding of *Bupleurum falcatum* L. (1). Effect of temperature on growth, development and saikosaponin content of one-year-old plants. *Shoyakugaku Zasshi* **34**, 209-214, 1980