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HISTOCHEMISTRY

IX*. DISTRIBUTION OF SAIKOSAPONINS IN *BUPLEURUM FALCATUM* ROOT

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SUMMARY

The root of *Bupleurum falcatum* cultivated in Japan was examined by histological and chemical means in order to clarify the distribution of saikosaponins in the various kinds of tissues and parts of the root. By means of qualitative thin-layer chromatographic–densitometric and quantitative high-performance liquid chromatographic analyses, saikosaponins, bioactive principles in the root, were found to be abundant in the outer phloem layer, especially the pericycle and its neighbouring parenchyma cells in the root. It was also found that the highest level of saikosaponins was detected in thinner root hairs and that the level decreased towards the thicker root head.

INTRODUCTION

In order to evaluate the medical utility of a crude drug, it is important to establish its original species and the chemical and biological nature of its constituents. Histological analysis is one of the methods for determining the source plant of a crude drug. In order to perform qualitative and quantitative analyses of bioactive components in a crude drug, various chromatographic methods must be applied.

A combination of analysis from both a histological and a chemical viewpoint has been adapted for determining the distribution of bioactive components in plant organs and tissues. This histochemical analysis[1] provides some fundamental data not only for the evaluation of quality but also for the improvement of the development and processing methods for crude drugs. During the course of these investigations, we further examined the distribution of six saikosaponins in the root of *Bupleurum falcatum* L. cultivated in Osaka and Nara Prefectures by a combination of histolog-

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ical, qualitative thin-layer chromatographic–densitometric (TLC–DM) and quantitative high-performance liquid chromatographic (HPLC) analyses.

The dried root of *B. falcatum* is known as Bupleuri Radix, or Chai-hu in Chinese and Saiko in Japanese, and has been used in the traditional Chinese system of medicine. It has been substantiated by many chemical and pharmacological investigations[2,3] of Bupleuri Radix that saikosaponins are one of the bioactive components of the drug.

EXPERIMENTAL

Materials

The roots of 2-year-old *Bupleurum falcatum* L. plants cultivated in Nara Prefecture (harvested in February 1985) and in Osaka at the herbary of the Faculty of Pharmaceutical Sciences of Kinki University (October 1983) were subjected to the experiments.

Histological analysis

Cross-sectioned slices of each fresh root were prepared using a freezing microtome (MA-101; Komatsu Electronic) in the usual manner, and then subjected to microscopic observation. Surface observation of each slice was carried out with a microscope (A-RLB; Olympus Optical) and a three-dimensional diagram was prepared (Fig. 1).

Relatively thick sections obtained from the fresh root were divided concentrically with tweezers and a sharp knife from the outermost cork layer into the five tissues ph(1), ph(2), ph(3), xy(1) and xy(2), as shown in Fig. 3.

The thickness of the phloem layer and xylem layer of each of the slices obtained from six parts (Fig. 5) was measured in order to obtain the phloem/xylem ratio (phloem layer thickness/xylem layer thickness). Five measurements on different samples were made in order to calculate the mean and standard deviation (S.D.) of the ratio.

Qualitative analysis of saikosaponins

A fresh cross-section of a main root was sprayed with saponin-detecting reagent[4], a 2% (w/v) ethanol solution of silicotungstic acid, and heated by blowing hot air onto it to deveop the colour (Fig. 2).

A mucilaginous exudate obtained from the secretory canals in the cortical layer of the fresh root was dissolved in methanol and applied to a pre-coated silica gel plate ($60F_{254}$, Merck), which was then developed with chloroform–methanol–water (30:10:1). The plate was sprayed with a 4% ethanol solution of dimethylaminobenzaldehyde and heated to develop the colour.

TLC-DM profile analysis of the constituents of five tissues (50 mg each) divided from five fresh main roots was conducted as follows. Each test sample was extracted twice with methanol (100 ml each) under reflux. The residue obtained by evaporation of the combined methanol solution under reduced pressure was dissolved in 1 ml of methanol and centrifuged at 3000 rpm. The supernatant was then chromatographed on the TLC plate using the same conditions as those described above. After the dimethylaminobenzaldehyde treatment, the plate was scanned with a dual-

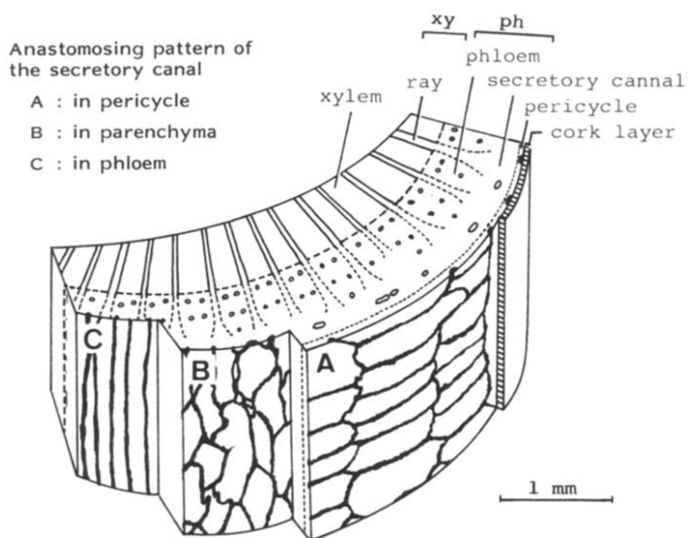


Fig. 1. Histological characteristics of ***Bupleurum falcatum*** root. xy, denotes the inner tissues from cambium: medullary ray, pith, etc. ph, denotes the outer tissues from cambium: phloem, cortical parenchyma, secretory canal, pericycle, cork layer, etc.

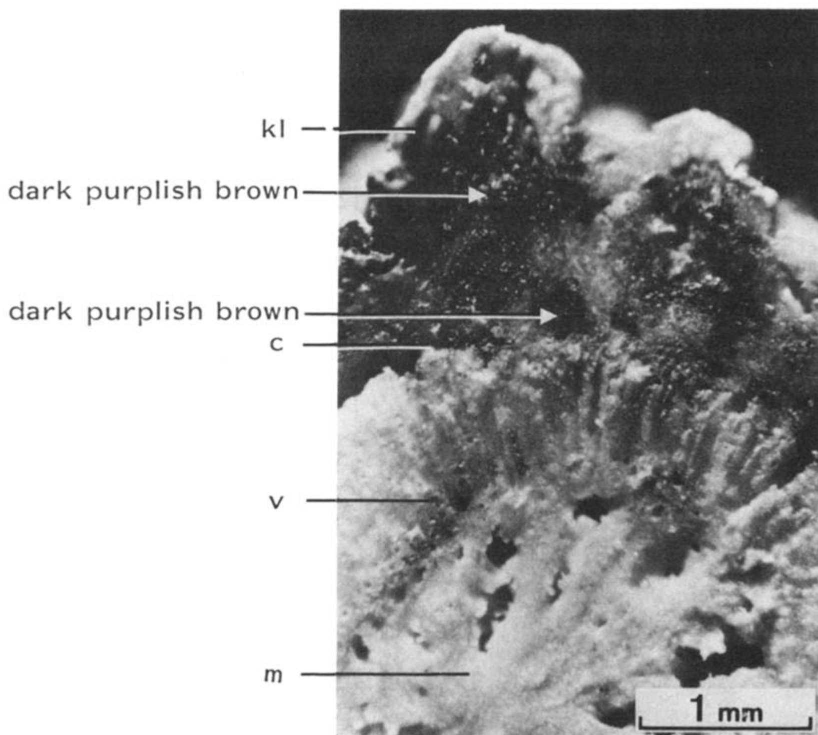
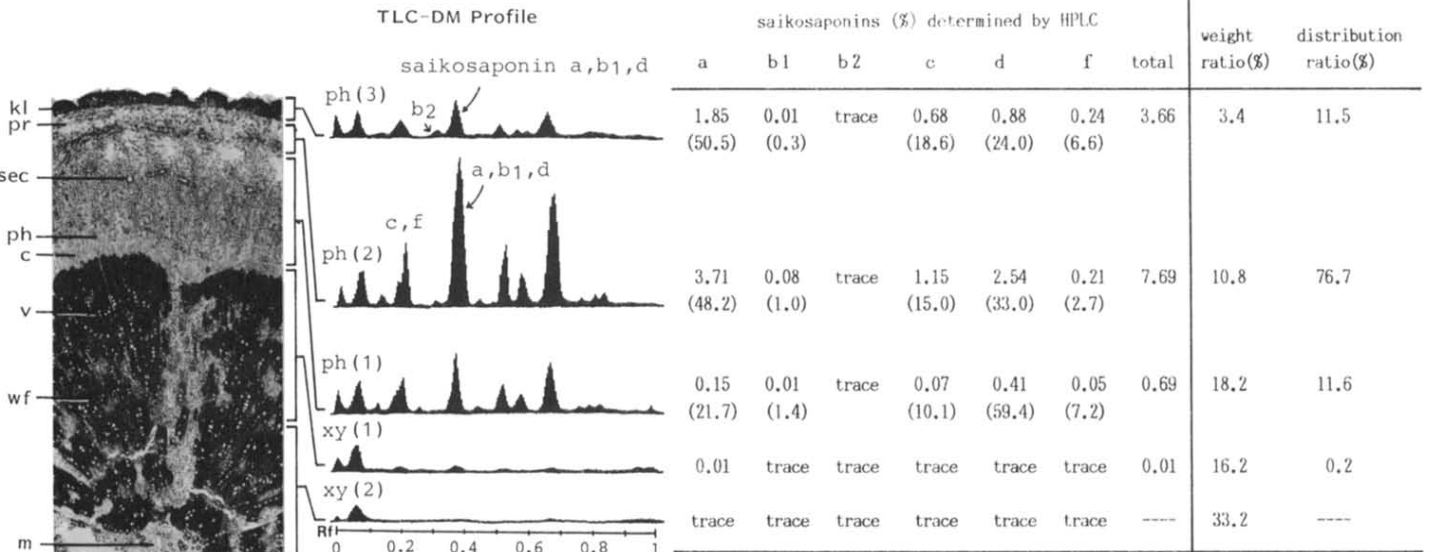


Fig. 2. Colour developed in a cross-section of a Fresh main root of *Bupleurum falcatum* by saponin-detecting reagent. kl, cork layer, c, cambium; v, vessel; m, pith.



saikosaponins (%) determined by HPLC								
a	b1	b2	c	d	f	total	weight ratio(%)	distribution ratio(%)
1.85 (50.5)	0.01 (0.3)	trace	0.68 (18.6)	0.88 (24.0)	0.24 (6.6)	3.66	3.4	11.5
3.71 (48.2)	0.08 (1.0)	trace	1.15 (15.0)	2.54 (33.0)	0.21 (2.7)	7.69	10.8	76.7
0.15 (21.7)	0.01 (1.4)	trace	0.07 (10.1)	0.41 (59.4)	0.05 (7.2)	0.69	18.2	11.6
0.01	trace	trace	trace	trace	trace	0.01	16.2	0.2
trace	trace	trace	trace	trace	trace	----	33.2	----

Fig. 3. Distribution of saikosaponins in different tissues of the main root of *Bupleurum falcatum* cultivated in Osaka. kl, cork layer; pr, pericycle; sec, secretory canal; ph, phloem; c, cambium; v, vessel; wf, wood fibre; m, pith. Each value was obtained from combined tissues separated from five fresh roots. The values in parentheses are percentages of the total value.

wavelength TLC scanner (CS-910; Shimadzu) (zig-zag scan; reflection; 1.25 × 1.25 mm slit; λ_S = 520 nm; λ_R = 700 nm).

Quantitative analysis of saikosaponins

The dried and powdered five tissues (50 mg each) obtained by the procedure described above were extracted twice with refluxing methanol (100 ml each) and the combined solution was concentrated under reduced pressure to yield a residue, which was treated with 10 ml of water. The suspension was treated with a C_{18} cartridge (Sep-Pak; Waters Assoc.) and then the methanol eluate from the cartridge was evaporated under reduced pressure to give a residue, which was dissolved in methanol (1.0 ml) to obtain the sample solution. An aliquot (10 μl) of the sample solution was subjected to HPLC for the quantitative determination of the six saikosaponins (a, b_1, b_2, c, d and f).

Six parts (50 mg each) separated from a root were also treated in the same manner. Five measurements were undertaken for each part obtained from five plants in order to calculate the mean and S.D. (Fig. 5).

The HPLC system was an LC-3A liquid chromatograph (Shimadzu) equipped with an SPD-2A UV detector (Shimadzu). A Nova-Pak C_{18} column, 15 cm × 4.6 mm I.D. (Waters Assoc.) with an eluent consisting of methanol–water (67:33) at a flow-rate of 1 ml/min was used. The peaks were checked by the monitored wavelength at 204 nm (saikosaponin a, c, d and f) and at 254 nm (saikosaponin b_1 and b_2) and identified by co-chromatography with standard solutions of six authentic saikosaponins, which were kindly supplied by Shionogi Research Laboratory[5,6].

The standard regression lines of each saponin (y = amount injected in μg, x = peak area) were obtained with a suitable prepared solution for each saikosaponin (Fig. 4). Peak areas were measured using a C-RlA computing integrator (Shimadzu).

RESULTS

Histological characteristics of B. falcatum root cultivated in Osaka Prefecture

The *B. falcatum* root consists of two types of tissues separated by cambium (c): an outer phloem layer (ph) including a cork layer (kl), pericycle (pr), phloem (ph) and many secretory canals (sc) having various anastomozing patterns in every tissue (Fig. 1), and inner tissues (xy) consisting of xylem, medullary rays and pith.

When an ethanol solution of silicotungstic acid, the saponin-detecting reagent[4], was sprayed over the cross-section of a fresh root of *B. falcatum*, xylem was not coloured but the outer phloem layer, especially the parenchyma around the pericycle and secretory canals, was coloured a dark purplish brown (Fig. 2).

Saikosaponins in different tissues of B. falcatum root cultivated in Osaka Prefecture

According to qualitative TLC-DM profile analysis, it was found that saikosaponins did not have a ubiquitous distribution in the five tissues but were found predominantly in the phloem layers, especially the outer phloem layer possessing many secretory canals [ph(2) in Fig. 3]. A mucilaginous exudate within the secretory canals contained only a small amount of saikosaponins compared with the parenchyma cells located around the pericycles.

The qualitative distribution of saikosaponins checked by the TLC-DM method

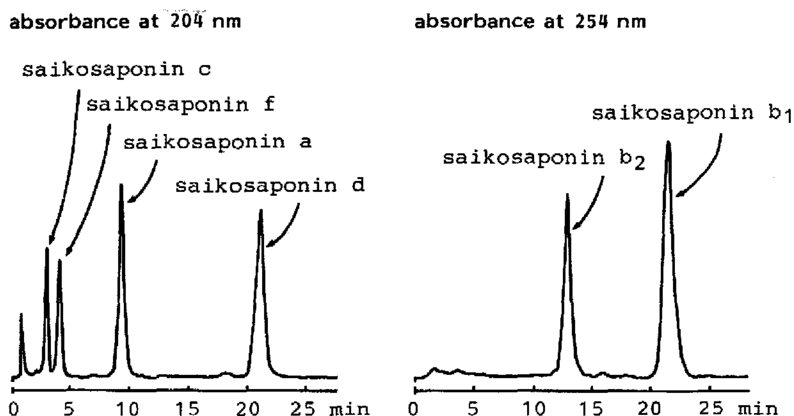


Fig. 4. Chromatograms of standard saikosaponins.

agreed with the quantitative results obtained by HPLC.

The HPLC method described in this paper is adaptable for the routine and reliable analysis of saikosaponins in crude methanol extracts. Retention times of saikosaponins a, b_1, b_2, c, d, and f were 9.21, 22.31, 13.28, 2.95, 20.99 and 3.85 min, respectively (Fig. 4). The concentrations of each saikosaponin in the samples were determined by comparison with each regression line of concentration (y, μg) for each saponin *versus* peak area (x): y(saikosaponin a) = $1.863 \cdot 10^{-4}x + 2.366$ ($r = 0.99$), y(saikosaponin b_1) = $6.070 \cdot 10^{-5}x + 0.054$ ($r = 0.99$), y(saikosaponin b_2) = $7.478 \cdot 10^{-5}x - 0.011$ ($r = 0.99$), y(saikosaponin c) = $1.878 \cdot 10^{-4}x + 0.610$ ($r = 0.99$), y(saikosaponin d) = $1.505 \cdot 10^{-4}x + 0.252$ ($r = 0.99$) and y(saikosaponin f) = $2.548 \cdot 10^{-4}x + 0.519$ ($r = 0.99$).

The relative proportions of each of the tissues based on dry weight were ph(1) 18.2%, ph(2) 10.8%, ph(3) 3.4%, xy(1) 16.2% and xy(2) 33.2%. If the total content of the root is taken as 100%, ph(3), ph(2), ph(1), xy(1) and xy(2) contained 11.5%, 76.7% 11.6%, 0.2% and 0% of saikosaponins, respectively.

The composition of saikosaponins was dependent on the tissues; thus in the outer phloem layer [ph(2) and (3)] there was a relatively higher concentration of saikosaponin a, which decreased towards the inner phloem layer ph(1) having the highest saikosaponin d content.

Similar distributions and compositions of saikosaponins were found for other specimens of *B. falcatum* cultivated in Nara.

Saikosaponins in different parts of B. falcatum root cultivated in Nara Prefecture

The concentrations and compositions of saikosaponins in six root regions as shown in Fig. 5 were quantitated by HPLC. The major and second major components for the six regions were saikosaponins a (0.43 ± 0.08% in whole root) and d (0.28 ± 0.05%). All parts contained only a small concentration (about 0.01%) of saikosaponins b_1 and b_2, which are converted secondarily[6] from genuine saikosaponins a and d during the determination procedure.

The saikosaponins showed an accumulated concentration in root hairs (5.58 ± 1.71%), which possess the widest phloem layer, but this decreased toward the

thicker roots (0.54 ± 0.09%) having a smaller phloem/xylem ratio. For example, the main root head showed a twelve-fold lower concentration of saikosaponin a 0.25 ± 0.04%) than the root hairs (3.08 ± 0.95%). The highest concentration of saikosaponin d was also found in thinner root hairs (1.46 ± 0.42%) and the level declined steadily towards the top of the thicker main root (0.14 ± 0.02%).

Saikosaponin concentrations varied in proportion to the phloem/xylem ratio: the correlation coefficients (r) between the ratio and each saponin were 0.86 for saikosaponin a, 0.87 for c, 0.88 for d, and 0.88 for f. They were negatively correlated with the diameter of the root: r values between the diameter and each saponin were −0.77 for saikosaponin a, −0.80 for c, −0.78 for d and −0.85 for f.

DISCUSSION

In order to evaluate the quality of a crude drug, we have been investigating the distribution of the bioactive components in plants organs and tissues by a combination of histological and chromatographic analyses[1]. In connection with our histochemical investigations on medicinal plants, the distribution of saikosaponins in the biennial roots of *B. falcatum* cultivated in Osaka and Nara Prefectures was analysed by means of qualitative TLC-DM and quantitative HPLC.

Prior to chromatographic analysis, an ethanol solution of silicotungstic acid was sprayed directly over the cross-section of a fresh root to investigate qualitatively the distribution of triterpene glycosides in the tissues. Thus, the phloem layer (ph) was coloured a dark purplish brown, which means that saikosaponins were localized mainly in the phloem layer and not in the woody central cylinder. This method has been applied in our previous histochemical investigation to determine the occurrence of ginsenosides in ginseng[4].

In order to analyse chromatographically the distribution of saikosaponins in each root tissue, the cross-section of the main root of *B. falcatum* was concentrically divided into five tissues: ph(1), ph(2), ph(3), xy(1) and (2), as shown in Fig. 3. A methanol extract of each tissue was subjected to profile TLC-DM analysis. It was found that saikosaponins are not homogeneously distributed throughout the tissues of the main root, but exist especially in parenchyma cells around the secretory canals and pericycle in the outer phloem layer [ph(2)].

This is in agreement with the experimental fact[4] that a higher concentration of ginsenosides was found in the outer periderm of the ginseng root. However, although ginsenosides were detected in mucilaginous exudate within secretory canals, only a small amount of saikosaponins was detected in the mucilage of *B. falcatum* root.

Further quantitative HPLC experiments were carried out in order to verify the results performed by qualitative TLC-DM analysis. Various attempts have been made to analyse saikosaponins by HPLC[7–9]. We have also reported a quantitative HPLC method for saikosaponins in Bupleuri Radix[10] and Kampo prescriptions containing Bupleuri Radix[11].

In this work, a pre-treatment using a Sep-Pak cartridge and an improved column–solvent system was used to quantitate saikosaponin a, b_1, b_2, c, d and f. Fig. 4 shows that the HPLC method described provides a useful means of determining saikosaponins. From the results of this HPLC analysis of methanol extracts of each

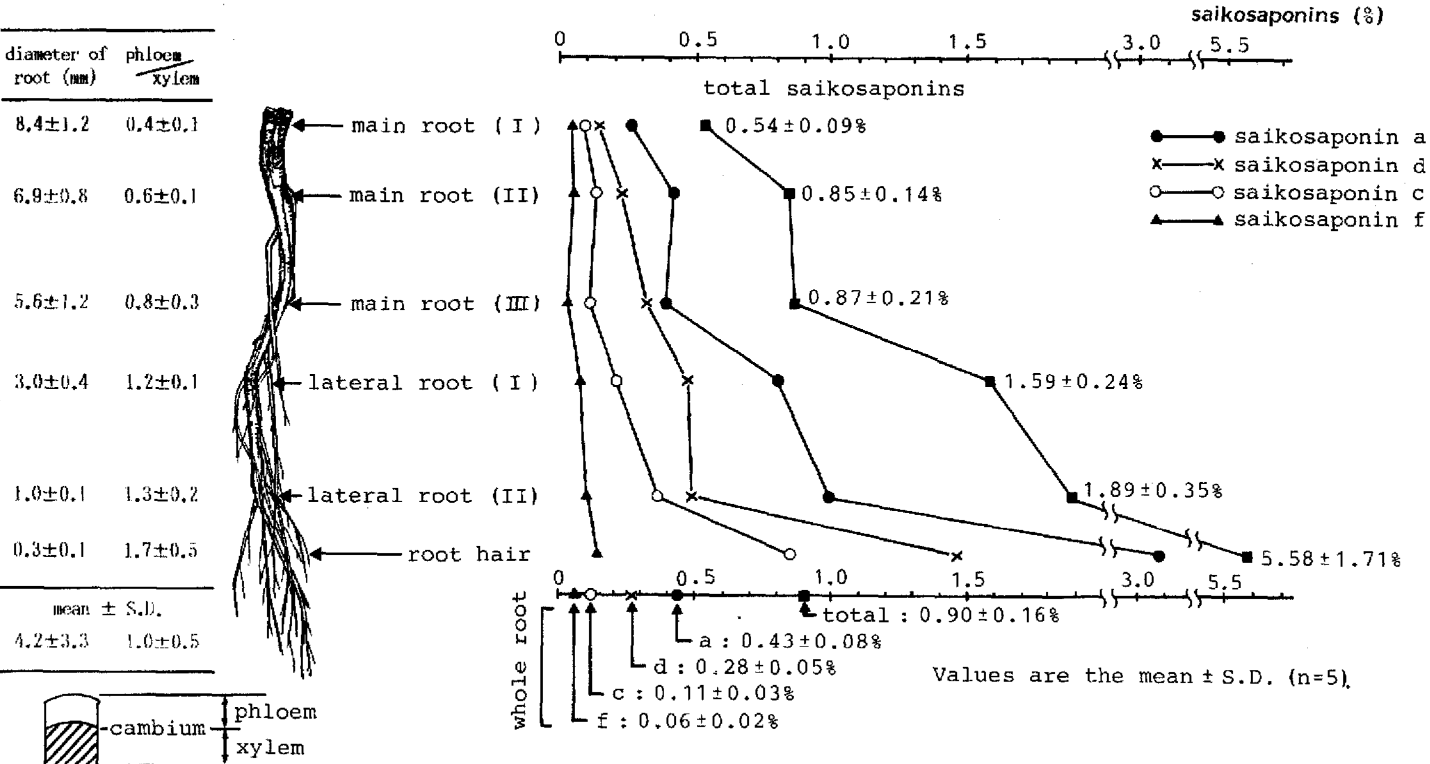


Fig. 5. Concentration of saikosaponins in different parts of *Bupleurum falcatum* root.

kind of tissue divided from the main root of *B. falcatum* as shown in Fig. 3 and the weight ratio of each kind of tissue in the main root, it was confirmed that most of the total amount of saikosaponins in *B. falcatum* root is derived from the phloem layers [ph(1), (2) and (3)]. A tissue-specific accumulation of bioactive constituents of medicinal plants is also found in ginseng[4,12], soybeans[13,14] and Scutellariae Radix[15].

The composition of each saikosaponin varied among the kinds of tissues examined. The main component of the outer phloem was saikosaponin a, whereas that of the inner phloem was saikosaponin d. Similar results were also obtained for another five *B. falcatum* specimens cultivated in Osaka and Nara.

Furthermore, the HPLC method was applied to trace where saikosaponins were located in six different parts of the root: three main roots, two lateral roots and root hairs, as shown in Fig. 5. The highest level of saikosaponins was detected in thinner root hairs containing a wide cortical layer, and that the level declined consistently towards root tops having a larger weight proportion of xylem, which contains much less of the saikosaponins. The regression equation $y = -0.45x + 3.75$ ($r = -0.77$) was established between the total saikosaponin concentration (y, μg) and the root diameter (x, mm). The total saikosaponin concentration (y) was also positively correlated to the phloem/xylem ratio (x): $y = 3.35x - 0.46$, $r = 0.86$.

These results are substantiated by the fact that the relative proportion of the phloem layer, in which saikosaponins are concentrated, to the xylem layer was 0.4 ± 0.1 in the root top and 1.7 ± 0.5 in root hairs. The phloem/xylem ratio of the root could be used as a parameter for the selection of roots with a higher saikosaponin content. The above results are similar to those for the distribution of ginsenosides in ginseng[4,12].

Although the composition of saikosaponins varied in accordance with the kinds of tissues divided from the main root, saikosaponin a was found to be the major ingredient throughout all root parts.

The results presented in this paper confirm that saikosaponins are concentrated in the phloem layer, especially the marginal tissues of the *B. falcatum* root, and that the upper thick root shows about a ten-fold lower concentration than the thinner root hairs. These experimental results are in accordance with those for conventional method for evaluating the quality of Bupleuri Radix, *i.e.*, that a more flexible one containing a smaller hard xylem is superior. Furthermore, it is thought that it would be useful if the cultivating methods of *B. falcatum* were adapted to produce roots with a higher weight proportion of thinner roots in order to obtain a much higher saikosaponin content.

This study will be extended to trace exactly where and how saikosaponins are distributed during the stages of root maturation.

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