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Component Determination of Total Saponins in Bupleurum Root by High-performance Liquid Chromatography

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Considering not only free saponins (saikosaponin a and saikosaponin d), but also their acylsaponins, the determination for the total amount of saponins in Bupleurum Root was studied. This paper especially describes the optimization of the suitable analytical conditions. A diluted sodium hydroxide test solution (TS) was added to the extract of the sample to saponify acylsaponis. The mixture was neutralized and treated using an octadecylsilanized silica gel (ODS) cartridge column to furnish a sample solution, and analyzed with a HPLC technique using an ODS column. The total amount of saponins, which was defined as the sum amount of saikosaponins a and saikosaponin d, was from 0.33 to 1.49% (average 0.78%). The amount of saikosaponin a increased 1.5 times and that of saikosaponin d did 1.6 times as the result of saponification. The method mentioned above is assumed to be time saving because plural samples can be handled in parallel.

Key words: Bupleurum Root, saikosaponin, component determination, total saponins, acylsaponin, HPLC, cartridge column

In Japanese Pharmacopoeia, 14*th* edition (JP14), "Bupleurum Root" [1] is prescribed in various Kampo medicines and consumption is considerably large. But determination of the main components, saikosaponins, has not been referred in any official codex in any country. It has been difficult to spread the component determination of the saponins because of the low intensity of UV-light absorption, poor separation of analogous saponins by chromatographic analysis, insufficient supply of standards and so on. Recently, the determination of saponins in Ginseng by HPLC was introduced in USP-NF *Official Monograph*[2] and EP.[3] Similarly, many academic reports have also been published on the analysis of saponins in Bupleurum Root by HPLC,[4, 5,6,7,8,9,10] capillary electrophoresis[11] and enzyme-linked immunosorbent assay.[12] Judging from those publications and reports, the HPLC method is the most popular, and Preparation of the sample solution is generally classified into two types: 1) Direct extraction with solvent (generally containing ammonia); [4,5,6,7,8,9,10] 2) Treatment of the extract solution in acidic condition in order to transform the monoene saponins to the diene (cf., Chemical structures in Fig. 1).[8,9] Though the latter preparation is superior to the former one in the measurement because of the high intensity of UV-light absorption, it has the risk of causing glycoside hydrolysis and a secondary reaction in the frame structure.[9] On the other hand, there is a report that informs some part of saikosaponins a and d exists in a plant as acetyl or malonyl esters.[7] If the acylsaponins are sufficient, the content should also be evaluated. There are very few reports focused on the determination of the total amount of saponins.[7] We have tried in recent years to saponify acylsaponins using ammonia water.[13] The method, unfortunately, has a weak point in that ammonia, which causes a large interference peak, must be evaporated to dryness while taking care to not bump the sample. Thus, in this study, we tried to optimize the method, including saponification, extraction condition, HPLC condition, *etc.*, to determine the contents of total saponins.

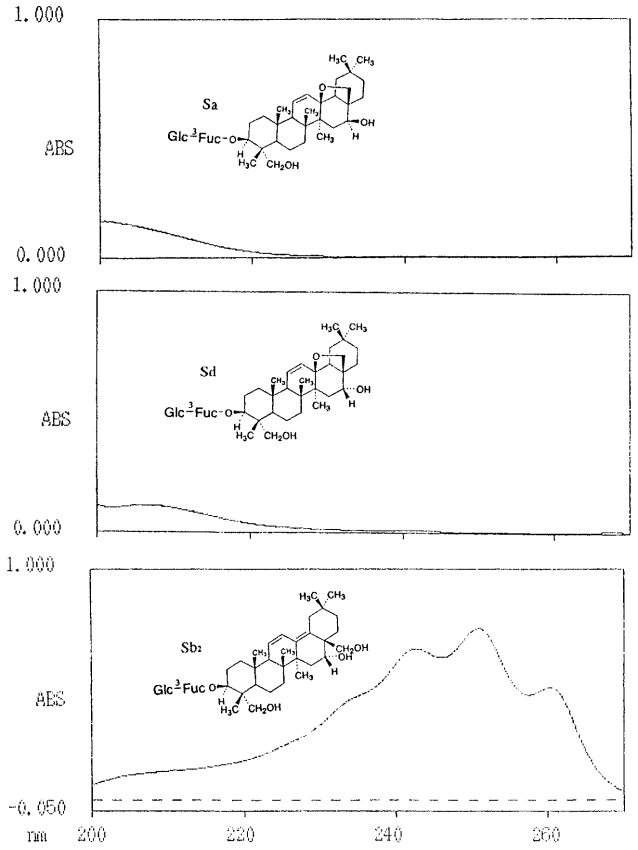

Fig. 1 UV spectra of saikosaponins

Sa: saikosaponin a; Sd: saikosaponin d; Sb_2: saikosaponin b_2

Experimental

Crude drugs All the samples, produced in China and Japan as shown in Table 1, were provided from the Japan Medicinal Plant Federation. The samples were pulverized with a blender till the size became less than 300 μm with the aid of a sieve (No. 50). Loss of drying was examined according to JP (105℃, 5h). Sample P was used for the reference sample to establish the method of determination.

Reagents Reference standards of saikosaponin a (Sa), saikosaponin d (Sd) saikosaponin b_2 (Sb_2) and saikosaponin c (Sc) were of crude-drug-standard grade (labeled purity more than 98%; no other peak but the object peak was observed by HPLC.), purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan) and dried in a desiccator (phosphorus (V) oxide, 40℃) at a pressure not exceeding 2.0 kPa for 5 h before use. Sep-Pack Plus C18 Short-Body Cartridges (Waters, i.d. about 10 mm, solid phase amount about 0.36 g) were used as the ODS cartridge column. The conditioning was performed successively by eluting 10 ml of methanol and 10 ml of water before use. Inertsil ODS-80A (GL Sciences Inc., Tokyo, Japan, 5 μm, 4.6 x 150 mm) and DISMIC-13HP (ADVANTEC, Japan, pore size 45 μm) were used as the HPLC column and membrane filter, respectively. Other reagents were of special grade.

Table 1 Crude drug samples

Sample	Production place	LOD (%)
A	China, Hupeh Province (湖北省)	8.06
B	China, Hupeh Province (湖北省)	7.97
C	China, Hupeh Province (湖北省)	7.41
D	China, Hupeh Province (湖北省)	7.64
E	China, Hupeh Province (湖北省)	7.48
F	China, Hupeh Province (湖北省)	7.70
G	China, Hopeh Province (河北省)	7.86
H	China, Hopeh Province (河北省)	7.77
I	China, Hopeh Province (河北省)	7.38
J	China, Hopeh Province (河北省)	7.23
K	China, Hopeh Province (河北省)	7.62
L	China, Hopeh Province (河北省)	6.81
M	China, Hopeh Province (河北省)	7.25
N	China, Hopeh Province (河北省)	6.65
O	China, Szechwan Province (四川省)	7.82
P	Japan	8.21
Q	Japan	7.81
R	Japan	8.00

LOD: Loss of drying O to R: Labeled "Mishimasaiko"

Preparation of standard solutions and test solutions

Two types of standard solutions were prepared as follows: Standard Sol.I, Sa and Sd (0.0100 g for each) were dissolved in methanol and the volume measured to exactly 50 ml (concentration: 200 μg/ml for each saponin); Standard Sol.II, Standard Sol.I (5 ml) was diluted with methanol and the volume measured to exactly 20 ml (concentration: 50 μg/ml for each saponin).

Dilute sodium hydroxide TS: Sodium hydroxide (4.3 g) was dissolved in freshly boiled water and the volume increased to 1,000 ml with water.

Neutral buffer solution: To 100 ml of 0.2 mol/l potassium dihydrogenphosphate, 69.4 ml of 0.2 mol/l sodium hydroxide was added.

The mobile phase of HPLC was a mixture of water and acetonitrile (3:2), which was degassed by 10-min supersonic irradiation before use. The component solvents used were of HPLC grade.

Apparatus and operating conditions Purification of the extract solution was proceeded by the system assembled with a reservoir, an ODS cartridge column and a manifold. Then, the outer tube of a 50-ml injector was utilized as the reservoir, which was attached onto

the cartridge column. A Sep-Pack Vacuum Manifold (Waters, USA) was used as the manifold.

The HPLC system consisted of the following apparatus: pump; LC-10ATvp, detector; SPD-10Avp, degasser; DGV-12A, autosampler; SIL-10ADvp, column oven; CTO-10ASvp and a recorder, C-R7A plus (all Shimadzu made). The detector wavelength was set at 206 nm for Sa and Sd and 254 nm for Sb_2. The column temperature was 50℃ and the injection volume was 20 μl. The flow rate was adjusted so that the retention time of Sa was about 8 min.

A Shimadzu uv-2400PC was used as the UV photometer.

UV spectorophotometry of standards To a quantity of each standard, Sa, Sd and Sb_2, methanol was added to a concentration of 20 μg/ml.

Drawing of calibration curve Standard Sol.I was diluted stepwise with methanol from 10 to 100 μg/ml and each of the solutions was submitted to HPLC.

Optimizing of extaction solvent Low-concentrated methanol solvents (0, 10, 30%), high-concentrated methanol solvents (80, 90, 100%) and a 50% methanol solvent were prepared. Each solvent (15 ml) was added to 1.0 g each of the powdered sample, shaken for 10 min and centrifuged at 3,000 r.p.m. for 3 min. The same procedure was repeated again. The supernatant liquids were combined and increased to exactly 50 ml with methanol (for determination of free saponins). Successively, 1 ml of this solution and 0.5 ml of dilute sodium hydroxide TS were mixed and warmed in a water bath of 50℃ for 1h (for determination of total saponins). Each of the solutions was submitted to HPLC and the relative peak areas of Sa and Sd were compared.

Optimizing of extraction condition The powdered sample (1.0 g) was shaken with 20 ml of 90% methanol for 10 min and centrifuged at 3,000 r.p.m. for 3 min. To the supernatant liquid, 90% methanol was added and increased to exactly 20 ml. The residue was further extracted in the same manner twice. Each of the extracted solutions was submitted to HPLC and the relative peak areas of Sa and Sd were compared.

Optimizing of saponification From the extract (in Fig. 2), five 5 ml-portions were collected. To each portion 2.5 ml of dilute sodium hydroxide TS was added and the mixture was warmed at 50℃ for different periods (0, 10, 30, 60 and 120 min). To each saponified mixture, 7.5 ml of neutral buffer solution was added and treated with a Sep-Pack cartridge. Each purified fraction was submitted to HPLC and the peak areas of Sa and Sd were compared.

Certification of stability To 1 ml of Standard Sol.II, dilute sodium hydroxide TS was added, and the mixture was kept at room temperature (20 to 30℃). The reacted solutions were sampled every fixed period and submitted to HPLC.

Cleanup by cartridge column To 5 ml of Standard Sol.II, 2.5 ml of dilute sodium hydroxide TS was added, warmed at 50℃ for 1 h and then 7.5 ml of Neutral buffer solution was added. This neutralized solution was poured into a reservoir and the first eluate was collected. Successively, the following 10-ml methanol solutions (30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) were eluted and the eluates were fractionated. Those 9 fractions were submitted to HPLC and the elution movement of Sa and Sd was examined. Separately, the extract solution from the powdered crude drug sample was treated in the same manner.

Preparation of sample solution for component Determination One gram of the powdered sample, accurately weighed, was put in a glass-stoppered centrifuge tube (Fig. 2). Twenty milliliters of 90% methanol was put in it. The mixture was shaken for 15 min, centrifuged at 3,000 r.p.m. for 3 min and then the supernatant liquid was separated. The same procedure was repeated twice with the residue using 15 ml portions of 90% methanol. To all the extracts combined, 90% methanol was added to make exactly 50 ml. To 5 ml of this solution 2.5 ml of dilute sodium hydroxide TS was added and kept in a water bath of 50℃ for 1 h. To this solution, 7.5 ml of neutral buffer solution was added. The mixture was transferred into a reservoir, an ODS cartridge column was attached and the elution was adjusted at the speed of about 2 ml/min under reduced pressure till the reservoir became almost empty. After washing the column with 10 ml of 35% methanol, 100% methanol was passed till the eluate volume became exactly 10 ml. The eluate was used as the sample solution for HPLC.

Measurement of Sb_2 The 90% methanol extract of the sample (extract in the above "Preparation of sample solution for component determination") was submitted to HPLC in the same way, but the detection wavelength was adjusted at 254 nm.

Others All the solutions except the standard solutions and the extracts (in Fig. 2), applied to HPLC, were treated using the cartridge column. The extracts were passed through the membrane filter.

The peak response was evaluated by peak area

measuring method.

The unit % expresses volume % in liquid and weight % in solid.

Results and Discussion

Wavelength of HPLC detector The UV spectra of Sa and Sd were almost the same and showed no absorption over the wavelength of 240 nm. Therefore, the wavelength 206 nm (based on $\pi \rightarrow \pi^*$ transmission of alkene structure) was selected for their detection (Fig. 1), whereas, that of Sb_2 showed a wide and strong absorption curve with a few maxima, and then the wavelength 254 nm was considered to be possible for the detection of HPLC.

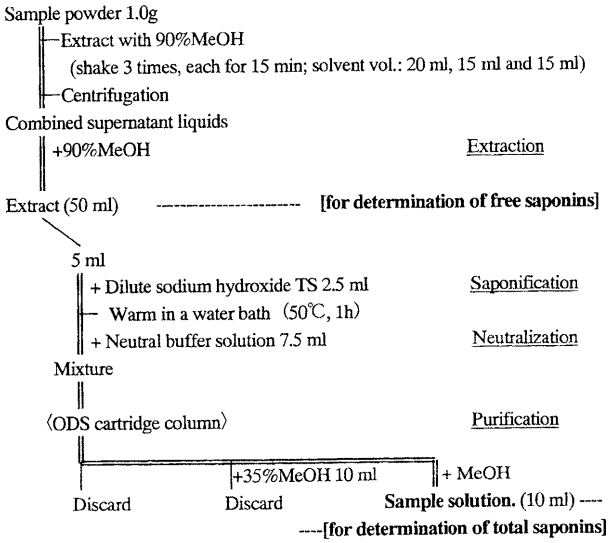

Fig. 2 Flow chart for preparation of sample solution

Calibration curve When the peak responses to the concentrations were plotted, the calibration curves of Sa and Sd almost overlapped and each curve exhibited good linearity (Fig. 3).

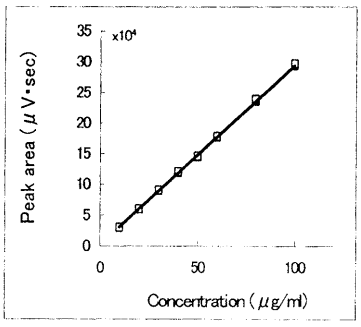

Fig. 3 Calibration curves for Standard Sol.I

■ Sa: $y/10^4 = 0.2928x + 0.1026$ $R^2 = 0.9997$

□ Sd: $y/10^4 = 0.2963x + 0.0675$ $R^2 = 0.9997$

As unexpected peaks sometime appear near the objective peaks in the HPLC analysis of natural products, the choice of internal standard method was abandoned and the absolute calibration method was adopted for this component determination.

Extraction solvent The saponification curves reached constant values at 60 min (Fig.4).

When Dilute sodium hydroxide TS was added to each of the extracted solutions, the following phenomena were observed: In the case of the extract solution prepared with 100% methanol, a faint white turbidity was produced. In the case of low-concentrated methanol, a precipitate-like starch was produced after standing for a while.

From the above phenomena, 90% methanol was chosen from the remaining solvents (80% and 90%) for the extracting solvent.

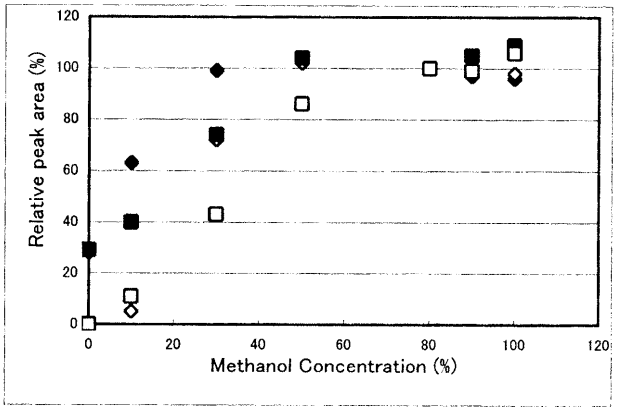

Fig. 4 Selection of extraction solvent

Extractions were performed using different percents of methanol-water mixtures and then the solutions were used for the determination of free saponins. Separately, each extract was treated for saponification with dilute sodium hydroxide TS and then the mixture was used for the determination of total saponins (ref. Fig. 2). Ordinate: relative peak area of HPLC The peak area obtained from the extraction with 80% MeOH = 100)

◆: Before saponification/Sa; ◇: Before saponification/Sd;

■ : After saponification/Sa; □: After saponification/Sd

Extraction condition

It was founded that the extraction of free saponins would proceed successfully if the extractions were repeated two times (Table 2).

More robust conditions were used in this experiment, as follows. The extraction was repeated three times with 90% methanol, shaking with 20 ml, 15 ml and 15 ml, each for 15 min, in order. When the powdered sample was utilized, that satisfactory

repeatability was achieved: the relative standard deviations were 0.459% for Sa and 0.340% for Sd (n=4).

Table 2 Proceeding of extraction

Extraction time	1st	2nd	3rd
Sa	100	11.3	0
Sd	96.7	10.0	0

Extraction was performed by shaking 1.0g of the powdered sample in 20 ml of 90% methanol for 10 min following by centrifuging, and the residue was extracted further in the same manner twice. Ordinate: relative peak area of HPLC (Sa of the 1st extraction = 100).

Saponification As plural acylsaponins exist in Bupleurum Root,[7] the saponification condition should be established using isolated acylsaponins. However, this was abandoned because the acquisition was difficult. As the second best policy, a suitable condition such as if either Sa or Sd occupied the maximum peak area was investigated (Fig. 5). Consequently, saponification was established to be the following: Add 2.5 ml of dilute sodium hydroxide TS to 5 ml of the sample solution and warm the mixture in a water bath at 50℃ for 60 min.

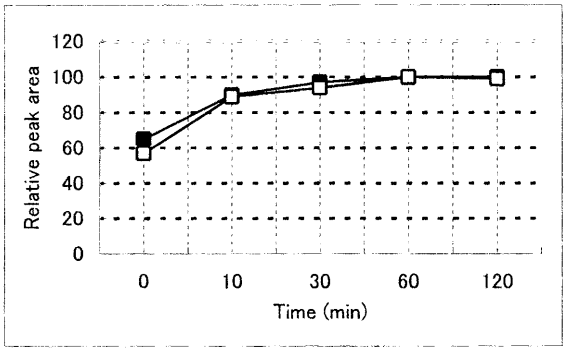

Fig. 5 Saponification proceeding

Saponification was performed at 50℃ for 0 to 120 min, and the mixture was submitted to HPLC. The peak area at the warming for 60 min=100 ■: Sa; □: Sd

After establishing component determination (ref. "Preparation of sample solution"), the peak areas from sample P were compared between the sample solutions before and after saponification (P_4 and P_3 in Fig. 6). Those of Sa and Sd increased 1.5 and 1.6 times, repectively. This example showed that the content of acylsaponins cannot be neglected and saponification is necessary for determination.

Stability of saikosaponins The stability of saikosaponins in the alkaline solution was examined (Table 3). It was speculated that those saponins were stable for at least 3 d under alkaline conditions. The saponins in Standard Sol.II did not show any change of the peak areas until 1 mo when kept in a refrigerator. However, Sa and Sd easily change to the diene form.[9] Then, it was convinced that the secondary reaction was hardly produced if the solution was not acidified.

Table 3 Stability of saponins in alkaline solution

Time	0 h	2 h	8 h	3 d
Sa	100	99	100	101
Sd	100	100	99	100

A mixture of Standard Sol.II (1 ml) and dilute sodium hydroxide TS (0.5mL) was allowed to stand at room temperature. Unit: relative peak area of HPLC (The peak area obtained at 0 h = 100)

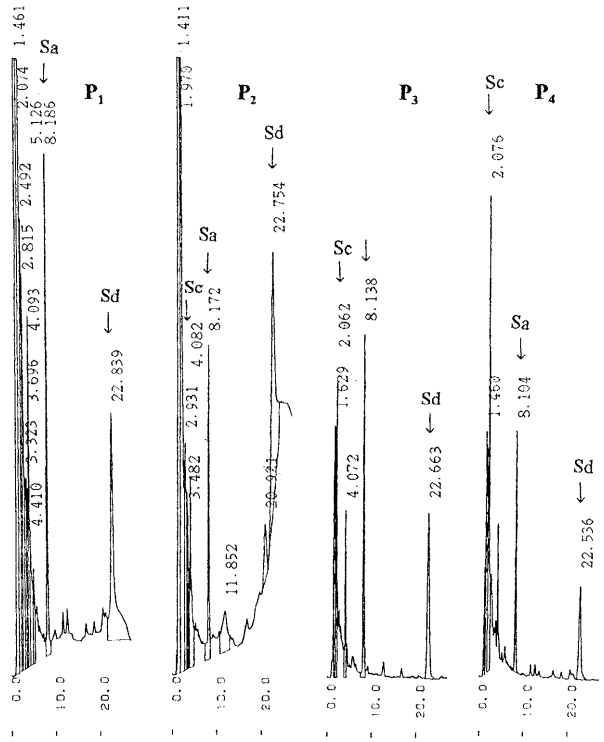

Fig.6 Chromatogram of Bupleurum Root

Sample P was treated. P_1: the extract in Fig. 2 (no dilution); P_2: the mixture of the extract (5 ml) and dilute sodium hydroxide TS (2.5 ml) was warmed at 50℃ for 1 h and then water (7.5 ml) was added; P_3: the sample solution in Fig. 2; P_4: P_1 was passed through a cartridge column. Full scale of the recorder: 32 mV

Cleanup by cartridge column In the case of the standard solution, the elution of saponins began with the fractionation of 70% methanol. In the case of the extract solution, the elution began by the fractionation of 50% methanol. In any case, Sa eluted faster than Sd. The separation efficiency varied a little with the sample loaded. Then robustness was considered and the cartridge column operation was established as the

following: First the neutralized solution was loaded and then the cartridge was washed with 10 ml of 35% methanol, and the saponins were eluted with 100% methanol, and a 10 ml volume of it was collected.

The efficacy, before and after cleanup, was proven by the changes from P_2 to P_3 as well as from P_1 to P_4 in Fig. 6.

The recovery test by the cartridge column operation (after saponification and neutralization) was studied.

When a 5 ml portion of Standard Sol.II was applied, the relative standard deviations were Sa 0.63% and Sd 0.60 % (n=6).

When a 5 ml portion of the extract (in Fig. 2) was applied, the relative standard deviations were Sa 0.53% and Sd 0.57 % (n=5).

When Standard Sol.II (1.25 ml) was added to 5 ml of the extraction solution, the recoveries of the saponins were Sa 101.1% and Sd 98.8 % (average, n=2).

From these results, it was confirmed that optimization with a cartridge column was achieved.

Determination of samples The total amount of saponins was calculated on the dry basis, which was obtained from "Loss of drying" in Table 1. The calculation followed the next equation.

Total amount (%) of saikosaponins =
Amount (%) of Sa + Amount (%) of Sd

Amount (%) of Sa = $C_{Sa}/W \times A_{Ta}/A_{Sa} \times 1/100$

Amount (%) of Sd = $C_{Sd}/W \times A_{Td}/A_{Sd} \times 1/100$

W: Sample weight (g), calculated on the dry basis

C_{Sa}: Sa concentration (μg/ml) in Standard Sol.II

C_{Sd}: Sd concentration (μg/ml) in Standard Sol.II

A_{Ta}: Sa peak area from sample solution

A_{Sa} : Sa peak area from standard solution

A_{Td}: Peak area of Sd from sample solution

A_{Sd}: Peak area of standard solution

When 18 samples were tested, the amount of Sa was 0.19 to 0.72% and that of Sd was 0.16 to 0.77%. The total amount of saikosaponins was calculated to be 0.33 to 1.49%. Samples from China may be special, and the amount varied from province to province. Samples labeled "Mishimasaiko" had a character intermediate between those from Hupeh and Hopeh in content (Fig.7).

The reproducibility of the determination was verified by repeating it (Table 3).

The unity of peaks Sa and Sd from sample P was ascertained by measuring the tailing factor (Sa:0.98; Sd:1.03) and by changing into the other column (YMC-Pak ODS-A, YMC Co. Ltd., 5μm, 4.6x150 mm).

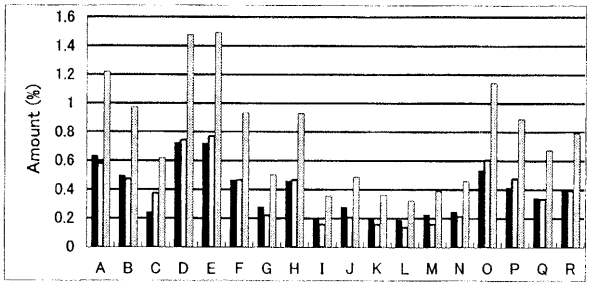

Fig.7 Amount of saikosaponins after saponification

■ Sa; □ Sd; ▯ Sa + Sd Average (A,G,P: n=3; others: n=2, The difference between the values was within 0.04%.)

Table 3 Deviation of component determination

Sample		1st	2nd	3rd	Av (%)	RSD (%)
A	Sa	0.621	0.627	0.650	0.620	2.7
	Sd	0.564	0.575	0.611	0.830	3.6
G	Sa	0.291	0.261	0.290	0.280	6.1
	Sd	0.211	0.214	0.231	0.218	10.78
P	Sa	0.410	0.418	0.416	0.415	0.42
	Sd	0.473	0.476	0.482	0.477	0.96

RSD: Relative standard deviation

Sc is the third major saponin in Bupleurum Root, but the content is generally less than one-third times as much as that of Sa or Sd.[8] The HPLC peak comes out at about 2min, on the beginning slow slope. It is very difficult to determine the content exactly without specific clean-up or other devised means. We cannot propose innovative method at present. In the market, the saponins in Bupleurum Root are often evaluated by the sum content of Sa and Sd, and we followed the same process.

As the HPLC peak of Sb_2 comes out just after that of Sa, if detected at the wavelength of 206 nm, the influence of Sb_2 must be discussed. When the residual content of Sb_2 was measured, it was less than 0.019%. Then it was found that Sb_2 was negligible in the component determination of Sa and Sd.

The determination established is time saving because six or more samples can be handled in parallel to prepare the sample solution.

Table 4 System reproducibility of Standard Sol.II

		Sa	Sd
1st	Peak area	150016	151526
2nd		147706	149110
3rd		147510	150112
4th		146675	150414
5th		148982	150842
6th		148982	150842
Average		147948.8	150430.3
RSD (%)		0.88	0.53

System suitability The detection limit is suggested to be 0.005% from the noise-level calculation (S/N 3) of the chromatogram, and the data on analytical validation were collected and are shown in Table 4 and 5. The results were satisfactory for system suitability.

Table 5 System capacity of standards

	Sa	Sd
Elution time (min)	8.1	22.6
Theoretical plate number	4550	8201
Symmetry factor	1.32	1.43

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