Development of a High Performance Liquid Chromatographic Method for Systematic Quantitative Analysis of Chemical Constituents in Rhubarb

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HPLC methods for the systematic determination of 30 compounds in Rhei Rhizoma (rhubarb) were developed. Using a combination of mobile phase gradient conditions and UV detection at 280 nm, all 30 compounds were separated satisfactorily with low detection limits (0.05—2 μg/ml). The developed methods provided a reliable calibration curve for each compound. By adopting these methods, the determination of 30 compounds in three kinds of rhubarb samples, derived from *Rheum tanguticum*, *R. palmatum* and *R. officinale*, was achieved. The constituent pattern of each rhubarb was clearly characterized through the quantitative composition of 30 major constituents of rhubarb.

Key words *Rheum*; HPLC; RG-tannin; rhatannin; quantitative comparison

Rhei Rhizoma (rhubarb), called *Daio* in Japanese, is one of the important herbal drugs. To date, qualitative analysis of its typical laxative components, anthraquinone derivatives including sennoside A, has been used for the qualitative evaluation of rhubarb by means of several analytical methods.[1)] However, such evaluation is incomplete because rhubarb has been used for the treatment of "Oketsu" (various syndromes caused by the obstruction of blood circulation such as dysmenorrhoea, hypermenorrhea, hematemesis, lower abdominal pain, *etc.*), jaundice, diarrhea and food poisoning, in addition to constipation. Although various individual pharmacological effects related to the above treatments, such as purgative activity,[2,3)] anti-bacterial and anti-fungal activities,[4)] anti-tumor activity,[5)] anti-inflammatory and analgesic activities,[6,7)] improvement of renal disorders,[8—11)] improvement of nitrogen metabolism,[12,13)] psychotropic activity,[14)] anti-allergic effects,[15,16)] anti-cholera toxin activity,[17,18)] promoting blood circulation and removing blood stasis,[19)] and the various involved active compounds have been reported, comprehensive chemical study of the composition of bioactive constituents of rhubarb has been rare.

Kashiwada *et al.*[20)] set up a HPLC method to analyze almost all the phenolic compounds simultaneously, and reported that the majority of the compounds could be separated by a 0.05 M H_3PO_4 solution–acetonitrile gradient elution system. However, several compounds, *i.e.* procyanidin B-1 3-*O*-gallate and 1,2,6-tri-*O*-galloyl-β-D-glucose; resveratrol 4′-*O*-β-D-glucopyranoside, 2-*O*-cinnamoylglucose and 1-*O*-galloyl-2-*O*-*p*-coumaroyl-β-D-glucose; sennoside B, resveratrol 4′-*O*-β-D-(6″-*O*-galloyl)-glucopyranoside and resveratrol 4′-*O*-β-D-(2″-*O*-galloyl)-glucopyranoside; isolindleyin 6″-*O*-gallate, procyanidin B-5 3,3′-di-*O*-gallate and 1-*O*-galloyl-2-*O*-cinnamoyl-β-D-glucose, showed overlapping peaks. In addition, polymeric procyanidins, *i.e.* RG-tannin and rhatannin, showed serious band broadening and tailing. Therefore, it is still necessary to develop a HPLC method which can be used for the effective separation and quantitative determination of the active components, which would then be used to evaluate the quality of rhubarb samples.

In this study, we developed new HPLC methods to analyze 30 compounds (**1**—**30**, Fig. 1) in rhubarb, quantitatively, and the contents of the active components were compared in rhubarb samples of different botanical origins.

Experimental

Materials Three Rhei Rhizoma samples derived from the following species were quantitatively examined: rhizomes of *R. tanguticum* (Huangnan County, Qinghai Prov., TMPW no. 20065), *R. palmatum* (Jiulong County, Sichuan Prov., TMPW no. 20216) and *R. officinale* (Wanyuan County, Sichuan Prov., TMPW no. 20267). The botanical origins of each sample were correctly identified by the molecular biological methods previously reported.[21,22)] The rhubarb sample (Qinghai Prov., TMPW no. 19929) used for the isolation of standard compounds was purchased from Uchida Wakanyaku Co., Ltd. (Japan). Voucher specimens have been deposited in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW).

Chemicals and Reagents Sephadex LH-20 (Amersham Biosciences, Sweden) and reversed phase gel MCI CHP-20P (70—150 mesh, Mitsubishi Chemical Co., Japan) were used for column chromatography. All chemicals were of analytical grade, and chromatographic solvents were of HPLC grade.

Standard Compounds Anthraquinones (**1**—**5**), anthraquinone glucosides (**6**—**10**), dianthrones (**11**, **12**), phenylbutanones (**13**, **14**), stilbenes (**15**, **16**), flavan-3-ols (**17**, **18**), procyanidins (**19**, **20**), galloylglucoses (**21**—**24**), acylglucoses (**25**—**27**) and gallic acid (**28**) were isolated from the rhubarb sample (TMPW no. 19929), and are listed in Fig. 1.

The isolated compounds were identified by comparison of their NMR and mass spectral data with those reported in the literature.[23—35)]

Purification of RG-Tannin (29) and Rhatannin (30) Rhubarb powder (1 kg) was extracted with 8 l of acetone–water (4 : 1, v/v) three times at room temperature. Combined extracts were evaporated *in vacuo* and lyophilized to get the final extract (345 g). One hundred grams of the extract was subjected to chromatography on a Sephadex LH-20. The column was eluted successively with methanol–water mixtures (in 10% increments of methanol concentration from 0 to 100%). After the column was eluted with 100% methanol, procyanidins were eluted with 60% of acetone (1 l). The aqueous acetone fraction was concentrated *in vacuo* and lyophilized to give crude procyanidins (9.35 g). Fifty milliliters of ethyl acetate was added to crude procyanidins (9.35 g), and the mixture was sonicated for 15 min and centrifuged. The precipitation was washed with ethyl acetate and dried to give polymeric procyanidins (5.85 g).

One gram of polymeric procyanidins was subjected to reversed phase gel column chromatography. The column was eluted with methanol–water mix-

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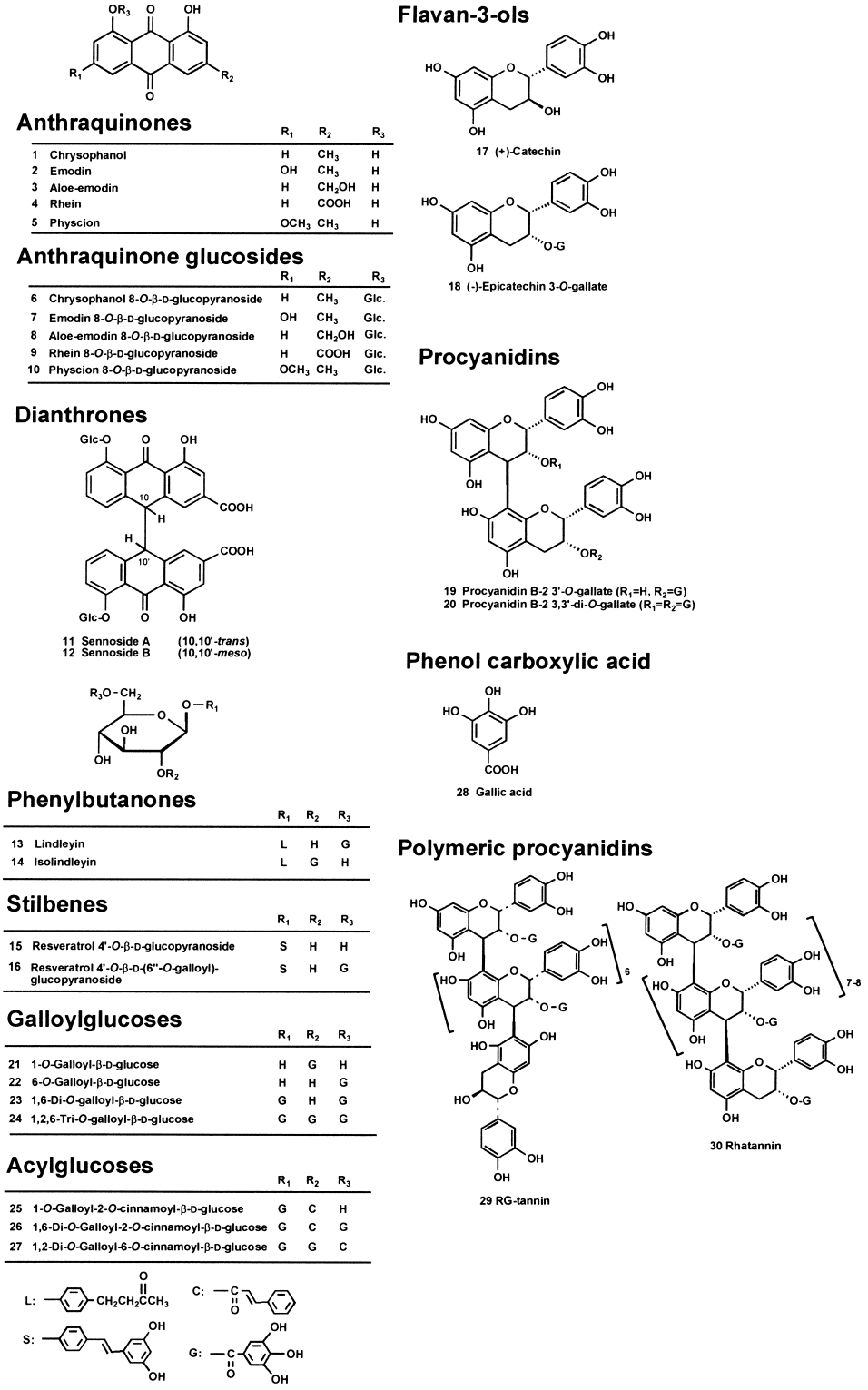

Fig. 1. Structures of 30 Compounds Used for Quantitative Determination

tures (in 10% increments of methanol from 10 to 100%). The twenty to thirty % methanol fraction and 60—70% methanol fraction gave RG-tannin (**29**) and rhatannin (**30**), respectively. The compounds were purified by repeated reversed phase gel column chromatography.

The isolated compounds were identified by comparison of the results of tannase hydrolysis and thiolitic degradation with those reported in the literature.[14,30]

Instrumentation and Analytical Conditions The JASCO HPLC system (Jasco Co. Ltd., Japan) is composed of a PU-1580 intelligent pump, a DG-1580-53 3 line-degasser, a LG-1580-02 ternary gradient unit, a CO-1565 intelligent column oven, an AS-2057 puls intelligent sampler, and an MD-1510 diode array detector. An Inertsil ODS column (5 μm particle size, 4.6 mm i.d.×250 mm, GL Science Inc.) was used throughout all chromatographic experiments. The column temperature was set at 45 °C, and eluted compounds were detected by monitoring the UV absorbance at 280 nm. The chromatographic data were collected and processed using Borwin-PDA Application and Borwin Chromatography Software (Version 1.5, Jasco Co. Ltd., Japan). The mobile phase systems used in this study were 0.05 M H_3PO_4 solution–acetonitrile or 0.05 M H_3PO_4 solution–acetonitrile–methanol, and the compositions of the eluents are given in Tables 1 (system

Table 1. Mobile Phase Gradient System A

Time (min)	0.05 M H_3PO_4	Acetonitrile
0	92	8
50	73	27
60	20	80
70	20	80
80	92	8

Table 2. Mobile Phase Gradient System B

Time (min)	0.05 M H_3PO_4	Acetonitrile	Methanol
0	92	8	0
30	77	10	13
50	77	15.3	7.7
60	20	80	0
70	20	80	0
80	92	8	0

Table 3. Mobile Phase Gradient System C

Time (min)	0.05 M H_3PO_4	Acetonitrile	Methanol
0	77	10	13
10	77	10	13
40	66.8	26.6	6.8
50	20	70	10
60	77	10	13

Table 4. Mobile Phase Gradient System D

Time (min)	0.05 M H_3PO_4	Acetonitrile
0	92	8
25	75	25
35	75	25
50	85	15
75	55	45
85	55	45
90	92	8

A), 2 (system B), 3 (system C) and 4 (system D), respectively. The mobile phase was filtered and degassed before use. The flow rates of the mobile phase were 0.8 ml/min in system A or 0.75 ml/min in systems B, C and D.

Preparation of Standard Solution and Samples Stock solutions of each standard compound were prepared independently by dissolving the appropriate amount of the compound in methanol in order to obtain a final concentration of 1 mg/ml. To draw calibration curves, a series of standard solutions were prepared from the stock solution, then filtrated through a filter (0.2 μm, Advantec, Toyo Roshi Co. Ltd., Japan). Typical calibration curves containing 1, 5 and 10 μg of analytes were prepared, plotting area against injection amount.

Two hundred milligrams of pulverized rhubarb sample was extracted with 80% of acetone (10 ml) for 2 h at room temperature after sonication for 15 min. This extraction procedure was repeated three times. The organic solvents were combined and evaporated *in vacuo* to give a methanol extract. The extract was dissolved in 10 ml of methanol–water (9 : 1, v/v). After filtration through a filter (0.2 μm, Advantec), 20 μl of the filtrate was injected into the HPLC system for analysis.

For quantitation of **29** and **30**, 5 ml of the analytical sample described above was evaporated *in vacuo*. The residue was suspended in 2 ml of methanol, then 8 ml of ethyl acetate–dioxane–acetone (85 : 10 : 5, by vol.) was added to the solution. After centrifugation, the precipitation was dissolved in 5 ml of methanol–water (9 : 1, v/v). The solution was filtered through a filter (0.2 μm, Advantec), and 20 ml of the filtrate was injected into the HPLC system.

Results and Disussion

The HPLC chromatograms of the extracts of rhubarb samples from different origins analyzed by mobile phase gradient system A are shown in Fig. 2. Twenty four compounds, except 4 compounds, could be separated clearly and identified by direct comparisons of their retention time values and UV spectra with those of authentic compounds, as well as co-chromatography.

The calibration curve of 24 standard compounds was investigated between the peak area (y) and the quantity of each compound (x, μg) using a prepared standard working solution. Triplicate injections were performed to obtain the absorption plots ranging from 0.1 to 1 μg for each of 24 compounds. The calibration equations and detection limits of the compounds are shown in Table 5. The results of regression analysis revealed that the calibration curve of each compound had a correlation coefficient very close to one.

On the other hand, aloe-emodin 8-*O*-β-D-glucopyranoside (**8**) and lindleyin (**13**); and rhein 8-*O*-β-D-glucopyranoside (**9**) and sennoside B (**12**), provided overlapping peaks at the retention times of 30 and 34 min, respectively.

In order to separate the two compounds in both cases, gradient systems using three solvents were examined. Figure 3 shows the HPLC chromatogram of standard **9** and **12** analyzed using mobile phase gradient system B. The compounds were separated clearly to give peaks at retention times of 52 min (**9**) and retention time 55 min (**12**), respectively. Both compounds could be detected without interference form the other components in the extracts of rhubarb samples. The HPLC chromatogram of a standard mixture of **8** and **13** analyzed by gradient system C is shown in Fig. 4. Under this analytical condition, both compounds were clearly separated, and quantitation of the compounds in rhubarb samples could be carried out with high accuracy. The calibration equation and detection limit of the compounds by these analytical conditions are shown in Table 5.

Rhubarb is one of the most important tannin-containing crude drugs, due to its high tannin content. It has been reported that **29** has psychotropic and anti-cholera toxin activities,[17,18] and **30** possesses a blood urea nitrogen-decreasing activity.[13] Thus, much attention has been paid to detailed quantitative information regarding the polymeric procyanidins in rhubarb. However, there are very few reports on HPLC analysis of polymeric procyanidins, partially due to the difficulties in isolating pure polymers and to the lack of efficient analytical methods. In Fig. 5, the HPLC chromatogram of a standard mixture of **29** and **30** analyzed by mobile phase gradient system D is shown. **29** and **30** provided peaks at retention times of 20—40 min and 60—70 min, respectively, and the results of regression analysis revealed that the calibration curves of each compound were sufficient. Figure 6 shows HPLC chromatograms of the extracts from rhubarb samples derived from different origins. Though the low molecular weight compounds could not be removed by the pretreatments used in this study, the subtraction of the peak areas of the low molecular weight compounds from the total peak area provided sufficient quantitative results.

By adopting the established methods, quantitative determinations of the 30 compounds in rhubarb samples from different origins were conducted. The results showed that the 30

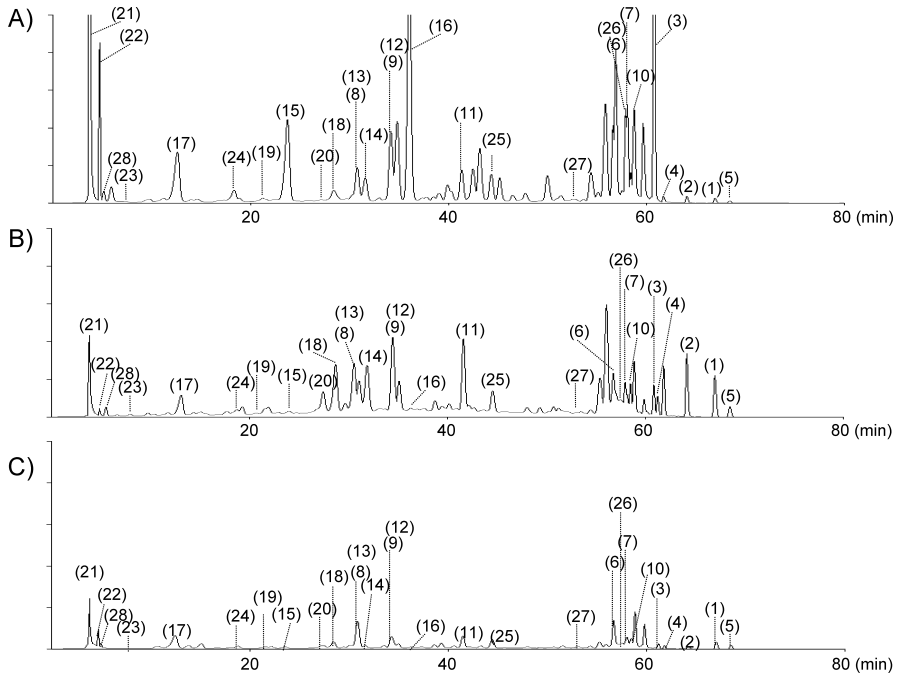

Fig. 2. HPLC Chromatograms of the Extracts of Rhubarb Samples from Different Origins Analyzed by Mobile Phase Gradient System A

A) *Rheum tanguticum* (Huangnan Co., Qinghai Prov., TMPW no. 20065). B) *R. palmatum* (Jiulong Co., Sichuan Prov., TMPW no. 20216). C) *R. officinale* (Wanyuan Co., Sichuan Prov., TMPW no. 20267).

Table 5. Calibration Equation, Correlation Coefficient and Detection Limits of 30 Standard Compounds

Compound	Calibration equation	Detection limit
Chrysophanol (**1**)	$y=2273798x-412567$	0.1
Emodin (**2**)	$y=2406185x+2206886$	0.1
Aloe-emodin (**3**)	$y=1576651x+182696$	0.1
Rhein (**4**)	$y=2098673x+606206$	0.1
Physcion (**5**)	$y=1036602x-150124$	0.1
Chrysophanol 8-*O*-*β*-D-glucopyranoside (**6**)	$y=863581x+34318$	0.1
Emodin 8-*O*-*β*-D-glucopyranoside (**7**)	$y=863581x+34318$	0.1
Aloe-emodin 8-*O*-*β*-D-glucopyranoside (**8**)	$y=2148712x-64298$	0.1
Rhein 8-*O*-*β*-D-glucopyranoside (**9**)	$y=1382746x+259744$	0.1
Physcion 8-*O*-*β*-D-glucopyranoside (**10**)	$y=2622119x+2972489$	0.1
Sennoside A (**11**)	$y=1266518x-40188$	0.1
Sennoside B (**12**)	$y=1157999x+251696$	0.1
Lindleyin (**13**)	$y=1302118x-45554$	0.1
Isolindleyin (**14**)	$y=1619650x-38932$	0.1
Resveratrol 4′-*O*-*β*-D-glucopyranoside (**15**)	$y=2397244x-374828$	0.1
Resveratrol 4′-*O*-*β*-D-(6″-*O*-galloyl)-glucopyranoside (**16**)	$y=2755826x+742738$	0.1
(+)-Catechin (**17**)	$y=700215x+212649$	2.0
(−)-Epicatechin 3-*O*-gallate (**18**)	$y=2292660x-312875$	2.0
Procyanidin B-2 3′-*O*-gallate (**19**)	$y=1645992x+186226$	2.0
Procyanidin B-2 3,3′-di-*O*-gallate (**20**)	$y=1174328x-98248$	2.0
1-*O*-Galloyl-*β*-D-glucose (**21**)	$y=1164902x+703482$	0.1
6-*O*-Galloyl-*β*-D-glucose (**22**)	$y=1450736x+425931$	0.1
1,6-Di-*O*-galloyl-*β*-D-glucose (**23**)	$y=1645992x+186226$	0.1
1,2,6-Tri-*O*-galloyl-*β*-D-glucose (**24**)	$y=2659381x-162988$	0.1
2-*O*-Cinnamoyl-1-*O*-galloyl-*β*-D-glucopyranoside (**25**)	$y=4073608x+874129$	0.1
1,2-Di-*O*-galloyl-6-*O*-cinnamoyl-*β*-D-glucose (**26**)	$y=3062758x-135881$	0.1
1,6-Di-*O*-galloyl-2-*O*-cinnamoyl-*β*-D-glucose (**27**)	$y=2227086x+2363732$	0.1
Gallic acid (**28**)	$y=2718682x-64032$	0.05
RG-tannin (**29**)	$y=1167851x+82772$	2.0
Rhatannin (**30**)	$y=1085600x+2922697$	2.0

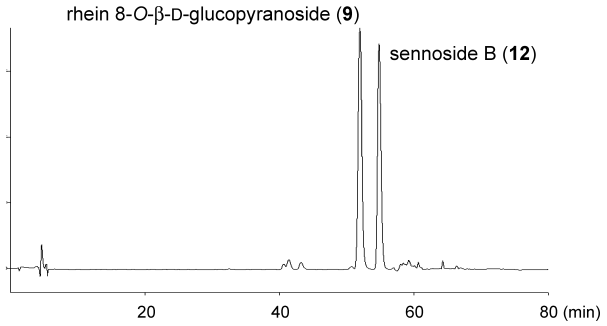

Fig. 3. HPLC Chromatogram of Sennoside B (**12**) and Rhein 8-*O*-*β*-D-Glucopyranoside (**9**) Analyzed by Mobile Phase Gradient System B

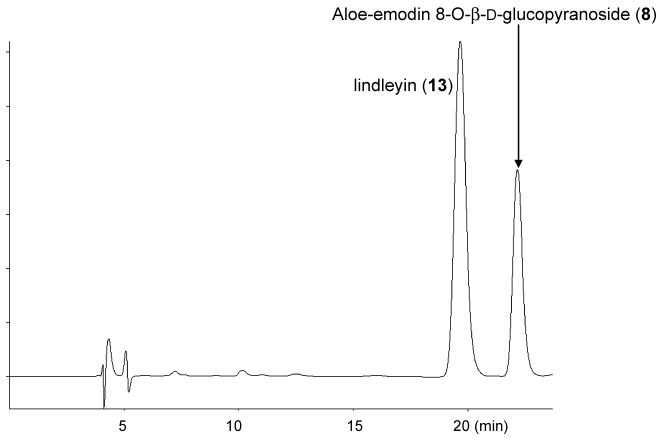

Fig. 4. HPLC Chromatogram of Lindleyin (**13**) and Aloe-Emodin 8-*O*-*β*-D-Glucopyranoside (**8**) Analyzed by Mobile Phase Gradient System C

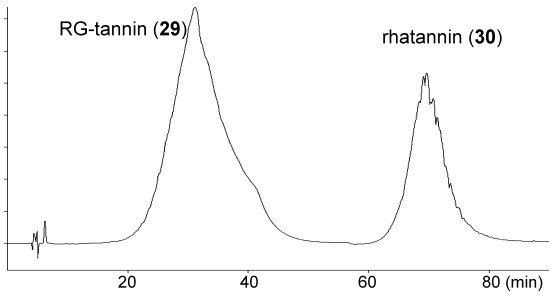

Fig. 5. HPLC Chromatogram of RG-Tannin (**29**) and Rhatannin (**30**) Analyzed by Mobile Phase Gradient System D

compounds were varied considerably in content and composition among the three rhubarb samples (Table 6). The rhubarb sample derived from *R. tanguticum* showed a high anthraquinone content, especially **3**, as well as substantial glucosides, **16** of stilbenes, **17** of flavan-3-ols, **21** of galloylglucoses and **29** of polymeric procyanidins. On the other hand, higher amounts of the following: **11** of dianthrones, **13** of phenylbutanones, **20** of procyanidins and **29** of polymeric procyanidins, were observed in the rhubarb sample from *R. palmatum*. The amount of the initial extract of the rhubarb sample from *R. officinale* was small and, therefore, it gave relatively small concentrations of the 30 compounds. The concentration of **11** in the sample derived from *R. officinale* was smaller than the limit indicated in the Japanese Pharmacopoeia. However, it was shown to have high concentration ratios of anthraquinone glucosides, flavan-3-ols and galloyl-

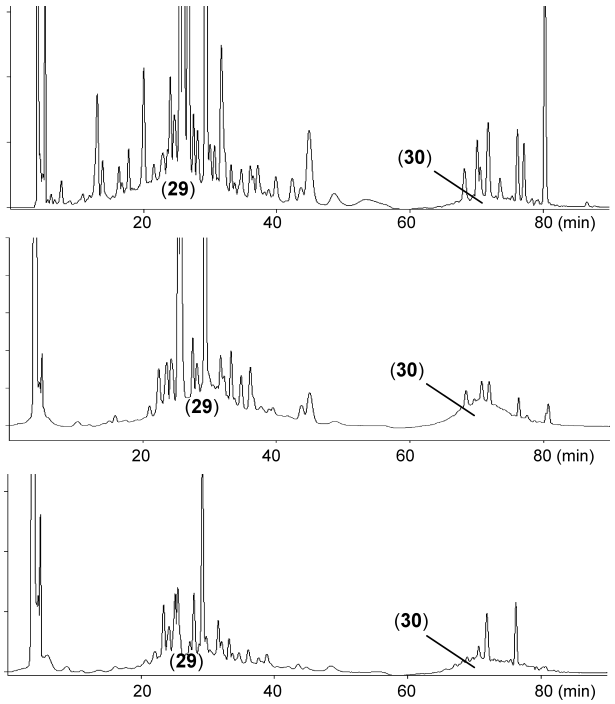

Fig. 6. HPLC Chromatograms of the Extracts of Rhubarb Samples from Different Origins Analyzed by Mobile Phase Gradient System D

A) *Rheum tanguticum* (Huangnan Co., Qinghai Prov., TMPW no. 20065). B) *R. palmatum* (Jiulong Co., Sichuan Prov., TMPW no. 20216). C) *R. officinale* (Wanyuan Co., Sichuan Prov., TMPW no. 20267).

glucoses, relative to the other compounds.

Kashiwada *et al.*[20] have reported the chemical constituents' patterns in rhubarbs produced in Qinghai and Sichuan Provinces. The rhubarb produced in Qinghai Province contained relatively large amounts of phenylbutanones, stilbenes and polymeric procyanidins. On the other hand, the rhubarb produced in Sichuan Province, which contained comparatively large amounts of anthraquinones and their glucosides, could be divided into two groups, types I and II, based on the levels of phenylbutanones, stilbenes and procyanidins. The contents of polymeric procyanidins are high in type I, but low in type II.

The rhubarbs used in this study were mostly obtained commercially, thus their botanical origins were unidentified. Therefore, the constituent patterns only provided information to evaluate the quality of the rhubarb samples. It was unclear whether the constituent patterns obtained from these rhubarb samples were affected by botanical source or producing area. In the present study, we analyzed the genetically identified rhubarb samples by newly developed HPLC conditions, together with Kashiwada's condition. This clarified that the quantitation of phenolic compounds in rhubarb sample provides extremely helpful information in estimating the origin of rhubarb, as well as data to evaluate the quality of rhubarb drugs.

It has been reported that the lower molecular weight procyanidins are usually present in plant tissue in relatively low concentrations compared to that of larger oligomers or polymers.[36,37] Higher concentrations of polymeric procyanidins than procyanidins in all rhubarb samples were confirmed in this study.

In conclusion, we developed a new HPLC method for the

Table 6. Contents (mg/g) of 30 Chemical Components in Rhubarb Samples (Mean, $n=3$)

Compounds	Samples		
Botanical origin	*Rheum tanguticum*	*R. palmatum*	*R. officinale*
Producing area	Huangnan Co., Qinghai Prov.	Jiulong Co., Sichuan Prov.	Wanyuan Co., Sichuan Prov.
TMPW no.	20065	20216	20267
Extract content	451.33	315.00	174.50
1. Anthraquinones			
Chrysophanol (**1**)	0.76	3.56	0.87
Emodin (**2**)	0.01	0.54	n.d.
Aloe-emodin (**3**)	20.91	1.12	n.d.
Rhein (**4**)	0.01	3.70	0.01
Physcion (**5**)	0.67	2.59	0.89
Subtotal	22.36	11.51	1.77
2. Anthraquinone glucosides			
Chrysophanol 8-*O*-*β*-D-glucopyranoside (**6**)	6.17	9.42	3.92
Emodin 8-*O*-*β*-D-glucopyranoside (**7**)	9.90	4.32	2.22
Aloe-emodin 8-*O*-*β*-D-glucopyranoside (**8**)	7.03	1.83	3.15
Rhein 8-*O*-*β*-D-glucopyranoside (**9**)	9.34	9.26	0.95
Physcion 8-*O*-*β*-D-glucopyranoside (**10**)	2.97	0.34	0.05
Subtotal	35.41	25.17	10.29
3. Dianthrones			
Sennoside A (**11**)	6.69	12.16	1.78
Sennoside B (**12**)	3.69	4.48	n.d.
Subtotal	10.38	16.64	1.78
4. Phenylbutanones			
Lindleyin (**13**)	2.46	6.39	4.61
Isolindleyin (**14**)	3.91	4.49	0.46
Subtotal	6.37	10.88	5.07
5. Stilbenes			
Resveratrol 4′-*O*-*β*-D-glucopyranoside (**15**)	9.97	4.11	0.81
Resveratrol 4′-*O*-*β*-D-(6″-*O*-galloyl)-glucopyranoside (**16**)	21.01	1.35	0.01
Subtotal	30.98	5.46	0.82
6. Flavan-3-ols			
(+)-Catechin (**17**)	19.40	5.77	9.27
(−)-Epicatechin 3-*O*-gallate (**18**)	2.37	4.66	1.18
Subtotal	21.77	10.43	10.45
7. Procyanidins			
Procyanidin B-2 3′-gallate (**19**)	1.36	1.22	0.81
Procyanidin B-2 3,3′-di-gallate (**20**)	0.86	7.49	0.85
Subtotal	2.22	8.71	1.66
8. Galloylglucoses			
1-*O*-Galloyl-*β*-glucose (**21**)	21.36	6.01	3.99
6-*O*-Galloylglucose (**22**)	4.91	2.25	0.95
1,6-Di-*O*-Galloyl-*β*-D-glucose (**23**)	0.71	0.03	1.39
1,2,6-Tri-*O*-Galloyl-*β*-D-glucose (**24**)	0.75	0.54	0.49
Subtotal	27.73	8.83	6.82
9. Acylglucoses			
1-*O*-Galloyl-2-*O*-cinnamoyl-*β*-D-glucose (**25**)	2.34	0.71	0.06
1,6-Di-*O*-Galloyl-2-*O*-cinnamoyl-*β*-D-glucose (**26**)	1.61	0.99	0.04
1,2-Di-*O*-Galloyl-6-*O*-cinnamoyl-*β*-D-glucose (**27**)	0.40	0.09	0.13
Subtotal	4.35	1.79	0.23
10. Gallic acid (**28**)	0.73	1.91	0.69
11. Polymeric procyanidins			
RG-tannin (**29**)	31.48	34.85	17.36
Rhatannin (**30**)	0.05	8.45	0.04
Subtotal	31.53	43.30	17.40

systematic quantification of 30 compounds which are major bioactive constituents in rhubarb, by using a combination of mobile phase gradient conditions and UV detection at 280 nm. Using this method, three rhubarb samples from different origins were analyzed to indicate the possibility of characterizing rhubarb samples by chemical constituent pattern. In order to demonstrate the characteristic chemical pattern of each rhubarb, we have subsequently conducted a comparative study on the 30 compounds of 25 rhubarb samples collected from various markets in Qinghai, Sichuan, Gansu and Yunnan Provinces of China, and from Japan. Additionally, we have reported on genetic polymorphisms of genus *Rheum* in the chloroplast *mat*K gene.[21] At the same time, the key nucleotide markers for identifying 3 official species of rhubarb such as *R. palmatum*, *R. tanguticum* and *R. officinale*, and those for distinguishing the 3 intraspecies groups of *R. palmatum* were determined, by which most rhubarb samples were identified correctly.[22] In our future paper, the chemical constituent pattern of rhubarb samples from different sources and the relationship between that and

genetic variation will be reported.

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