Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Identification of *Rhodiola* species by using RP-HPLC*

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Received Oct. 25, 2004; revision accepted Jan. 3, 2005

Abstract: An approach was established using RP-HPLC (reversed-phase high-performance liquid chromatography) to identify ten species of *Rhodiola*, *R. coccinea* A. Bor, *R. junggarica* C.Y. Yang et N.R. Cui spn., *R. heterodonta* A. Bor, *R. linearifolia* A. Bor, *R. pamiro alaiucm* A. Bor, *R. kaschgarica* A. Bor, *R. litwinowii* A. Bor, *R. gelida* schrenk, *R. rosea* L. and *R. quadrifide* Fisch et Mey collected from the Tianshan Mountains areas of China. Chromatograms of alcohol-soluble proteins, generated from these ten *Rhodiola* spp. were compared. Each chromatogram of alcohol-soluble proteins came from a single seed of one wild species only. The results showed that when using a Waters Delta Pak. C18, 5 μm particle size reversed phase column (150 mm×3.9 mm), a linear gradient of 22%−55% solvent B with a flow rate of 1 ml/min and a run time of 67 min, the chromatography gave optimum separation of *Rhodiola* alcohol-soluble proteins. Chromatogram of each species was different and could be used to identify those species. Cluster analysis of genetic similarity coefficients of 37% to 60% showed a medium degree of genetic diversity among the species in these eco-areas. Cluster analysis showed that the ten species of *Rhodiola* can be divided into four clusters and yielded the general and unique biochemical markers of these species. RP-HPLC was shown to be a rapid, repeatable and reliable method for *Rhodiola* species identification and analysis of genetic diversity.

Key words: *Rhodiola*, Genetic diversity, Species identification, RP-HPLC
doi:10.1631/jzus.2005.B0477 **Document code:** A **CLC number:** Q94

INTRODUCTION

The roots of *Rhodiola* species, one of the important traditional Chinese herbal medicines, have anti-viral (Sun *et al*., 1993), anti-tumor (Wang *et al*., 1992), anti-ageing (Yi *et al*., 1992), anti-oxygen deficiency, anti-radiation (Luo *et al*., 1996) effects and other pharmacological properties (Xu *et al*., 2000). Ten *Rhodiola* species distribute in the Tianshan Mountains areas of China. Biologically active compounds content in the roots has large variation and the value of utilization is different among the species. *Rhodiola* grows at higher altitude mountain areas where the ecological environment is vulnerable to unfavorable factors. Wild *Rhodiola* is a rare genetic resource due to limitation of ecological adaptation, some of which is depleted (Zu *et al*., 1998). In order to make full use of valuable *Rhodiola* resources, it is necessary to build up an efficient way to identify the species of *Rhodiola.*

Alcohol soluble seed storage proteins are genetically stable molecules, which can be analyzed by electrophoresis or HPLC to identify species and cultivars. Electrophoresis studies greatly enhanced knowledge of cereal proteins. Brink *et al*.(1989) used isoelectic focussing of zeins and an immunoassay system to evaluate the genetic purity of maize. Dinelli and Bonetti (1992) used capillary electrophoresis identifying *Phaseolus vulgaris* L. cultivars.

* Project (No. 30470330) supported by the National Natural Science Foundation of China

RP-HPLC has been used to characterize species and individual inbred lines or varieties of many cultivated species including wheat (Batey, 1984), barley (Marchylo and Kruger, 1984), oats (Lookhart and Pomeranz, 1984), maize (Smith and Smith, 1992). RP-HPLC has also been used for identifying cultivated genotypes and for revealing varietal mixtures and hybrid pedigrees. In this paper, we report the identification of wild species of *Rhodiola* by using RP-HPLC protein markers and compare the genetic diversity of various species.

MATERIALS AND METHODS

Plant materials

Ten species of *Rhodiola* were collected from the Tianshan Mountains of China in Aug./Sept. 1997 to 2000. The habitat, altitude and coordinate of *Rhodiola* spp. are summarized in Table 1. The *Rhodiola* spp. add. collected by us were identified by Prof. Wang Bing and Prof. Yang Chang-you, Department of Forestry, Xinjiang Agriculture University. Three to five populations per species, ten to fifteen mature plants per population and five seeds per plant were taken in the experiment for analyzing 250−300 seed samples per species. The seeds of each plant were sorted into fractions by size and infertile particles were removed by using sieves. Seed with size of 0.8 to 1.0 mm were used in the experiment.

Extraction of protein

Single seed was ground with a small pestle in 0.5 ml tube, then extracted for 2 h with 50 μl of 70% ethanol. The samples were ground for a further 10 min, and then centrifuged at 12000×g for 10 min. The aqueous phase was filtered through a 0.45 μm pore size filter before injection into an RP-HPLC column.

Chemicals and reagents

Unless noted otherwise, all chemicals were of analytical grade. Acetonitrile and methanol (spectra analyzed grade) were purchased from TEDIA (USA), Trifluoroacetic acid (spectra analyzed grade) from British Drug Houses (London, UK). Water was purified by using a Millipore Milli-Q purification system.

Apparatus and mobile phases

RP-HPLC was performed with a Shimadzu Associates Model LC 6A equipped with VP5.0 multi-solvent delivery system and SCL-6A system controller (Shimadzu, Japan). Ten ml of samples were injected through a Rheodyne 7000 injection valve connected to an LKB 2157 auto sampler (LKB, Bromma, Sweden). Eluted proteins were monitored at 210 nm by using Shimadzu LC-10A TVP photodiode and array detector (PDA). A Shimadzu CTO-6A Column oven was used to maintain constant column temperature at 50 °C.

Computation

Data acquisition was by means of a Shimadzu CR-3A Chromatopack connecting to Intel Personal Computer with Nelson Analytical software. Peak areas of protein chromatograms were determined through a manual interactive integration process on the monitor screen and by aligning the cursor at positions corresponding to the beginning and the end of an eluting peak. The area under this curve was calculated in the same time intervals for each chromato-

Table 1 Location of *Rhodiola* spp. collection sites in Tianshan Mountains of China

Species	Habitat, altitude and coordinate
Rhodiola coccinea A. Bor	Hejin County, along the snowline/3280 m/43°18′/93°34′
Rhodiola junggarica C.Y. Yang et N.R. Cui spn.	Wnquan County, meadow, near the snowline with more stones/2700 m/45°31′/79°37′
Rhodiola heterodonta A. Bor	NiYa County, above the snowline, along sandy roadside/3400 m/37°35′/85°15′
Rhodiola linearifolia A. Bor	Eawati County, meadow edge, along small sandy creek/3000 m/37°31′/80°22′
Rhodiola pamiro alaiucm A. Bor	Hotian County, along the snowline, more sandy and stone/3200 m/37°18′/79°38′
Rhodiola kaschgarica A. Bor	Wush County, meadow edge, along small creek/2700 m/39°37′/76°02′
Rhodiola litwinowii A. Bor	Ahqi County, meadow edge, along sandy riverbed/2900 m/40°51′/78°32′
Rhodiola gelida schrenk	Barkol County, meadow along roadeide/2700 m/41°12′/79°07′
Rhodiola rosea L.	Heavenly Lake of Tianshan Mountains, meadow edge along forest/2100 m/43°9′/87°
Rhodiola quadrifide Fisch et Mey	Bogada Peak of Tianshan Mountains, along sandy riverbed/3400 m/43°17′/86°12′

graphy to ensure a reproducible calculation of peak areas. Statistical analysis of data was done by using Minitab statistical package (supplied by Minitab Inc. Pennsylvanian, USA). Retention time in the text was expressed as mean±*SE*.

Data evaluation

The peaks of the elution of chromatogram were converted into "0" or "1", where "1" and "0" indicates the presence and absence of a peak, respectively. Genetic similarities (GS) were estimated for all comparisons of samples according to $GS=2n_{xy}/(n_x+n_y)$ in which n_x and n_y are the total number of peaks in the chromatogram of the samples x and y, respectively, and n_{xy} is the number of peaks shared by the two samples. The genetic similarity coefficients were applied to cluster samples based on their degree of genetic similarity, by using the UPGMA (unweighted pair group method using arithmetic averages) option of the SPSS (Version 11.0) software. After this process, RP-HPLC chromatograms of all *Rhodiola* samples were made and then each chromatogram with different retention times of peaks from each sample were compared.

RESULTS

Optimum condition for separation of alcohol-soluble proteins

Chromatographic conditions were optimized by using water and acetonitrile containing 0.1% (v/v) trifluoroacetic acid as solvents A and B, respectively. Use of a Waters Delta Pak. C18, 5 μm particle size reversed phase column (150 mm×3.9 mm), linear gradient of 22%−55% solvent B with flow rate of 1 ml/min and a run time of 67 min, was found to give optimum separation of *Rhodiola* alcohol-soluble proteins. Under these conditions, more than 50 different constituents could be identified in ethanol extracts from a single seed. At the end of each gradient program, solvent B was increased to a final concentration of 60% over a period of 4 min before returning to initial conditions. After this, the extract was treated by periodic washing with a 0−100% acetonitrile gradient over 15 min to remove any strongly absorbed proteins that may accumulate on the column from previous elution.

Reproducibility of column retention time for the methods

In order to test the reproducibility of column retention times for different extracts of the same species, ethanol extracts of *R. coccinea* A. Bor and *R. quadrifide* Fisch et Mey were analyzed on different days. In this case, samples of *R. rosea* L were also analyzed on the same day. When tested on the same day, the coefficient of variation for five chosen peaks was found to vary little−average value 1.59% (Table 2). When tested on different days, the reproducibility of retention times for five chosen peaks was variable with average coefficients of variation of 3.56% and 4.13% for two different species (Table 3). Comparison of elution profiles indicated that this level of reproducibility was very acceptable for identification of the species.

Table 2 Reproducibility of retention time (within one day) for reversed phase separation of alcohol-soluble proteins in *R. rosea*

Peak number	Mean retention time±*SD* (min)	Coefficient of variation (%)
a	2.627±0.091	3.46
b	3.430±0.098	2.86
c	14.403±0.125	0.09
d	17.425±0.183	1.05
e	36.482±0.185	0.51
Average (*n*=5)		1.59

Table 3 Reproducibility of retention time (next day) for reversed phase separation of *R. coccinea* A. Bor and *R. quadrifide* Fisch et Mey alcohol-soluble proteins

Protein	Peak number	Mean retention time±*SD* (min)	Coefficient of variation (%)
R. coccinea A. Bar	a	2.627±0.159	6.05
	b	3.430±0.218	6.36
	c	16.278±0.325	2.00
	d	17.425±0.355	2.04
	e	34.382±0.455	1.32
	Average (*n*=5)		3.56
R. quadrifide Fisch et Mey	a	2.627±0.161	6.13
	b	3.430±0.245	7.14
	e	14.403±0.415	2.88
	d	16.278±0.475	2.92
	e	32.402±0.505	1.56
	Average (*n*=5)		4.13

Effect of identification for the species of *Rhodiola*

The RP-HPLC alcohol-soluble proteins profiles for the ten species of *Rhodiola* are shown in Fig.1. Repeated analyses of the alcohol-soluble proteins extracts under the same conditions yielded almost identical elution patterns, characteristic of each species.

There were 109 peaks, among which peak areas beyond 50 chosen for analysis of alcohol-soluble proteins in the seed of ten of *Rhodiola* spp.

The elution profiles in Fig.1a and Fig.1j presented the same number (52) of peaks from *R. coccinea* A. Bor and *R. quadrifide* Fisch et Mey. Figs.1b, 1d, 1g, 1h and 1i show the elution profiles with 54, 55, 49, 44 and 50 peaks from *R. junggarica* C.Y. Yang et N.R. Cui spn., *R. linearifolia* A. Bor, *R. litwinowii* A. Bor, *R. gelida* schrenk and *R. rosea* L., respectively. In Figs.1c, 1e and 1f, there are the same number (51) of peaks from *R. heterodonta* A. Bor, *R. pamiro alaiucm* A. Bor and *R. kaschgarica* A. Bor.

Cluster analysis

RP-HPLC data from 10 *Rhodiola* species were sorted into four clusters at GSC (genetic similarity coefficients)=0.52 level, designated I to IV (Tables 4 and 5). Cluster I including two species, *R. coccinea* A. Bor and *R. junggarica* C.Y. Yang et N.R. Cui spn.; Cluster II was composed of five species, *R. heterodonta* A. Bor, *R. kaschgarica* A. Bor, *R. litwinowii* A. Bor, *R. gelida* schrenk, *R. rosea* L.; two species including *R. linearifolia* A. Bor and *R. pamiro alaiucm* A. Bor were grouped in Cluster III; only one species *R. quadrifide* Fisch et Mey was putin Cluster IV.

Tables 4 and 5 reveal the relationships among the 10 species of *Rhodiola* L. in Tianshan Mountains areas. The greatest GSC (0.63) was found between *R. linearifolia* A. Bor and *R. pamiro alaiucm* A. Bor in Cluster III. The lowest GSC (0.40) was observed in different clusters, such as, *R. coccinea* A. Bor vs *R. heterodonta* A. Bor.

DISCUSSION AND CONCLUSION

The separation of gliadins by using RP-HPLC was successfully used for wheat (Bietz and Cobb, 1985; Ram *et al*., 1995), maize (Bietz, 1983), rye (Kubiezek *et al*., 1993), rice (Huebner *et al*., 1990). The results obtained in this study indicated that the separation technique can be used identifying wild species; each chromatogram of *Rhodiola* is distinct. The peaks eluting at 0.273 min, 0.795 min, 1.138 min, 1.178 min, 2.657 min, 3.428 min, 26.525 min, 28.677 min, 40.757 min and 47.352 min are characteristic of elution proteins from 10 species of *Rhodiola*, and can be used as general biochemical markers of these species.

The peaks eluting at 10.335 min and 32.917 min represent alcohol-soluble proteins specially for *R. litwinowii* A. Bor; the peak eluting at 20.022 min represents protein specially for *R. linearifolia* A. Bor; the peak eluting at 23.032 min represents protein specially for *R. junggarica* C.Y. Yang et N.R. Cui spn.; the peak eluting at 25.025 min represents protein specially for *R. pamiro alaiucm* A. Bor; the peaks eluting at 29.168 min and 49.682 min represent proteins specially for *R. quadrifide* Fisch et Mey; the peak eluting at 36.328 min represents protein specially for *R. gelida* schrenk; These peaks are the unique biochemical markers for the species, respectively.

In addition to the high sensitivity and resolution of the method (HPLC theoretical plates=10^5 vs 10^3 for SDS-PAGE (sodium dodecyl sulfate-polyacryl amide gel)), it is worth mentioning that the rapidity of analysis (ca. 67 min) and the possibility of monitoring the separations allow for possible modifications in real-time. Automated sample handling is another advantage of modern chromatographic systems. But, the disadvantages of HPLC system are: (1) only one sample can be tested at one time, while SDS-PAGE allows for fifteen or more samples to be analyzed simultaneously; (2) it is very difficult to determine the molecular weight and the isoelectric point of the analyzed proteins; (3) it is difficult to predict their elution order. In light of these considerations, RP-HPLC and SDS-PAGE need not be seen as competing methods, but as complements to each other. SDS-PAGE could be used to screen analyses because of its rapidity and simplicity, whereas RP-HPLC could be used to differentiate varieties not well characterized or indistinguishable with the use of electrophoretic banding patterns.

According to the result of cluster analysis, the ten species of *Rhodiola*, which distribute in Tianshan Mountains areas, can be divided into four clusters. The species of *R. linearifolia* A. Bor and *R. pamiro*

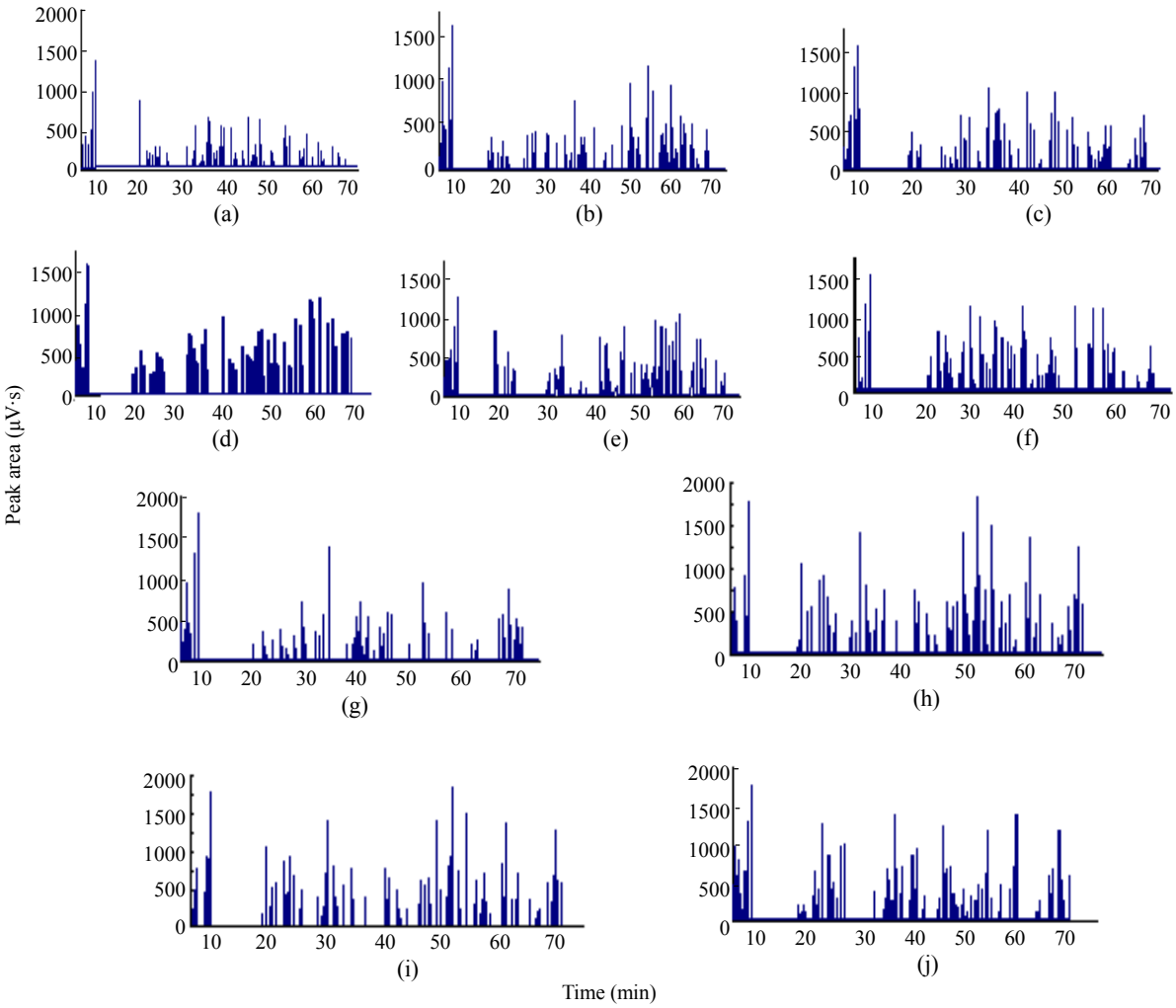

Fig.1 RP-HPLC of alcohol-soluble proteins from 10 *Rhodiola* species (a) *R. coccinea* A. Bor; (b) *R. junggarica* C.Y. Yang et N.R. Cui spn.; (c) *R. heterodonta* A. Bor; (d) *R. linearifolia* A. Bor; (e) *R. pamiro alaiucm* A. Bor; (f) *R. kaschgarica* A. Bor; (g) *R. litwinowii* A. Bor; (h) *R. gelida* schrenk; (i) *R. rosea* L., (j) *R. quadrifide* Fisch et Mey

Table 4 Cluster analysis based on genetic similarity coefficients of *Rhodiola* samples in UPGMA

Stage	Cluster combined		Coefficients (%)	Stage cluster first appears		Next stage
	Cluster I	Cluster II		Cluster I	Cluster II	
1	d	e	37.000	0	0	7
2	c	h	39.000	0	0	5
3	f	g	44.000	0	0	6
4	a	b	44.000	0	0	9
5	c	i	45.000	b	0	6
6	c	f	48.000	e	c	8
7	d	j	52.000	a	0	8
8	c	d	57.000	f	g	9
9	a	c	60.000	d	h	0

Table 5 the results of cluster analysis

Case	a	b	c	d	e	f	g	h	i	j
4 Clusters	I	I	II	III	III	II	II	II	II	IV

Letter indicates the same species as in Fig.1

alaiucm A. Bor in Cluster III have more homogeneity and their genetic relationship was much more closer. The species of *R. quadrifide* Fisch et Mey in Cluster IV shows less homogeneity among the inter-species. Thus, further studies should be made in order to elucidate the causative reason for these phenomena. In summary, this method is simple, rapid, reproducible and accurate. Data from RP-HPLC can be readily compared and analyzed by computer.

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