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Intra-specific genetic relationship analyses of *Elaeagnus angustifolia* based on RP-HPLC biochemical markers*

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Abstract: *Elaeagnus angustifolia* Linn. has various ecological, medicinal and economical uses. An approach was established using RP-HPLC (reversed-phase high-performance liquid chromatography) to classify and analyse the intra-specific genetic relationships of seventeen populations of *E. angustifolia*, collected from the Xinjiang areas of China. Chromatograms of alcohol-soluble proteins produced by seventeen populations of *E. angustifolia*, were compared. Each chromatogram of alcohol-soluble proteins came from a single seed of one wild plant 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 25%~60% solvent B with flow rate of 1 ml/min and run time of 67 min, the chromatography yielded optimum separation of *E. angustifolia* alcohol-soluble proteins. Representative peaks in each population were chosen according to peak area and occurrence in every seed. The converted data on the elution peaks of each population were different and could be used to represent those populations. *GSC* (genetic similarity coefficients) of 41% to 62% showed a medium degree of genetic diversity among the populations in these eco-areas. Cluster analysis showed that the seventeen populations of *E. angustifolia* could be divided into six clusters at the *GSC*=0.535 level and indicated the general and unique biochemical markers of these clusters. We suggest that *E. angustifolia* distribution in these eco-areas could be classified into six variable species. RP-HPLC was shown to be a rapid, repeatable and reliable method for *E. angustifolia* classification and identification and for analysis of genetic diversity.

Key words: *E. angustifolia*, Intra-specific genetic relationship, Genetic diversity, Biochemical marker
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INTRODUCTION

Elaeagnus angustifolia Linn. is an Eurasian tree that has become naturalized and has invaded zones along watercourses in many arid and semiarid regions of the world. These habitats are characterized by vertical environmental gradients, and hence trees have developed much plasticity to adapt to the wide range of site conditions (Klich, 2000). *E. angustifolia* plays a very important role in maintaining ecosystem function in the hyper arid areas of Xinjiang, China, because of its tolerance to severe drought, high salinity and alkalinity in soils (Zhang and Zhao, 1996). *E. angustifolia* also has various medicinal uses. The ripe fruits of *E. angustifolia* have been used to treat amoebic dysentery (Perry, 1980). There is general belief that leaves and fruits of the plant have antipyretic effect (Zargari, 1990). In folk medicine, oleaster fruit or flower preparations are used for treating nausea, vomiting, jaundice, asthma, and flatulence (Mirhydar, 1998). An infusion of the fruit has been used in Iranian traditional medicine as an analgesic agent for alleviating pain in rheumatoid arthritis patients. The flower is also traditionally used for treating tetanus (Hosseinzadeh *et al*., 2003). The content of biologically active compounds in the leaves and fruits and the tolerance to drought, salinity and alka-

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linity stress have large variations and the value of utilization is consequently different among the populations. In order to make full use of valuable *E. angustifolia* resources, it is necessary to build up an efficient way to classify the populations of *E. angustifolia*.

Alcohol soluble seed storage proteins are genetically stable molecules, which can be analyzed by electrophoresis or high performance liquid chromatography (HPLC) to classify or identify species and cultivars. Electrophoresis studies greatly enhanced the knowledge of cereal proteins. Brink *et al*.(1989) used isoelectric focusing of zeins and an immunoassay system to evaluate the genetic purity of maize. Dinelli and Bonetti (1992) used capillary electrophoresis as an identification tool for *Phaseolus vulgaris* L. cultivars.

Reversed phrase-HPLC (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, 1985), maize (Smith and Smith, 1992). Wang *et al*.(2005) used RP-HPLC biochemical markers for the identification of *Rhodiola* species. In this paper, we report an approach to establish RP-HPLC analysis of protein biochemical markers of *E. angustifolia* and analysis of the intra-specific genetic relationships of various populations.

MATERIALS AND METHODS

Plant materials

From 2000 to 2003, several extensive trips for collecting *E. angustifolia* were conducted in the Xinjiang region of China. The seeds used in this study were collected from each of seventeen populations studied. These populations were chosen to represent the overall distribution of *E. angustifolia* in Xinjiang. For the sampling localities (Table 1), Population 1 is the most eastern population in the entire distribution region of the plants, while in contrast, Population 17 is one of the westernmost populations of the plants. The populations were separated geographically by at least 80 km, and ranged in area 3500~5000 m^2. In each of the seventeen populations, a representative sample of 15 plants was chosen and five seeds per plant were taken for analysis. The distance between adjacent samples was at least 50 m to increase the possibility of detecting potential variation within each population. Samples were taken from the edges, as well as the interior of populations. Individuals were chosen at random. The seeds were separated from pericarp, dried in the shade and ground.

Extraction of protein

Single seeds were ground with a small pestle in a

Table 1 Location of *E. angustifolia* collection sites in Xinjiang of China

Population	Location	Habitat (m)	Latitude	Longitude
1	Kashitashi	650	37°02′	78°52′
2	Qiaqia	753	36°15′	79°32′
3	Mgaiti	652	39°18′	77°02′
4	Zepu	765	38°40′	77°15′
5	Aktao	565	39°36′	75°47′
6	Mlitamu	535	38°31′	76°35′
7	Ahaqi	560	40°16′	77°02′
8	Wshi	465	40°38′	78°29′
9	Hbukesai	875	46°32′	85°47′
10	Hbahe	865	48°07′	86°17′
11	Alahake	917	47°41′	87°32′
12	Brjin	905	47°03′	86°51′
13	Beitun	865	46°17′	87°44′
14	Jinhe	887	45°36′	90°09′
15	Wrhe	875	45°02′	86°39′
16	Mnasi	905	43°54′	85°27′
17	Srtuomu	917	44°10′	90°32′

1.5 ml tube, then extracted for 2 h with 500 μl of 70% (v/v) 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 the RP-HPLC column.

Chemicals and reagents

Unless noted otherwise, all chemicals were of analytical grade. Acetonitrile and methanol (spectra analysis grade) were purchased from TEDIA (USA). Trifluoroacetic acid (spectra analysis grade) was purchased from British Drug Houses (London, UK). Water was purified 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 microlitres 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 using Shimadzu LC-10A TVP photodiode and ray 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 Chromatopac connected to an Intel Personal Computer with Nelson Analytical software. Peak areas of the 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 chromatogram to ensure a reproducible calculation of peak areas. The data were analyzed using the Minitab statistical package (supplied by Minitab Inc. Pennsylvanian, USA). Retention times in the text are expressed as mean±*SD*.

Data evaluation

The chromatogram peaks were converted into a "1" and "0" matrix, to indicate the presence or absence of a peak, respectively. Seventy-five single seeds were analyzed to represent each population. The peaks in each population were chosen according to peak area and occurrence in every seed. Genetic similarities (GS) were estimated for all comparisons of samples according to Nei (1972) as $GS=2n_{xy}/(n_x+n_y)$ in which n_x and n_y are the total numbers of peaks in the chromatograms of the samples x and y, respectively, and n_{xy} is the number of peaks shared by the two samples. To examine the genetic relationships between populations, a dendrogram was constructed by an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) option of the SPSS (version 11.0) software. After this process, RP-HPLC chromatograms of all *E. angustifolia* samples were made and then data with different peak retention times from each population were compared.

RESULTS

Optimum conditions 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. When using a Waters Delta Pak. C18, 5 μm particle size reversed phase column (150 mm×3.9 mm), a linear gradient of 25%~60% Solvent B with a flow rate of 1 ml/min and a run time of 67 min was found to give optimum separation of *E. angustifolia* alcohol-soluble proteins. Under these conditions, more than 75 different constituents could be identified in ethanol extracts from single seeds. At the end of each gradient program, Solvent B was increased to a final concentration of 60% over a period of 10 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 seed, ethanol extracts of Population 1 (1-12-5) and Population 2 (2-12-4) were analyzed on different days. In this case, samples of Population 3 (3-10-4) 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 1.97 (Table 2, Fig.1a). When tested on different days, the reproducibility of retention times for five chosen peaks was variable with average coefficients of variation of 3.09 and 3.14 for the two different plants (Table 3, Fig.1b, Fig.1c). Comparison of elution profiles indicated that this level of reproducibility was very acceptable for identification and classification of the populations.

Classification for the populations of *E. angustifolia*

Repeated analyses of the alcohol-soluble proteins extract under the same conditions yielded almost identical elution patterns characteristic for each population. There were 121 peaks, among which peak areas above 50 μV·s were chosen for analysis of alcohol-soluble protein in the seed of seventeen populations of *E. angustifolia*. The converted data on the elution peaks of the chromatograms can be seen in

Table 2 Reproducibility of retention time (within one day) for reversed phase separation of alcohol-soluble proteins of Population 3 (3-10-4)

Peak number	Mean retention time±*SD* (min)	Coefficient of variation (%)
a	2.88±0.10	3.47
b	3.87±0.10	2.58
c	7.66±0.12	1.57
d	13.47±0.18	1.34
e	16.15±0.15	0.93
Average (*n*=5)		1.97

Table 3 Reproducibility of retention time (next day) for reversed phase separation of alcohol-soluble proteins of Population 1 (1-12-5) and Population 2 (2-12-4)

Protein	Peak number	Mean retention time±*SD* (min)	Coefficient of variation (%)
Population 1 (1-12-5)	a	2.20±0.10	4.55
	b	3.94±0.15	3.81
	c	7.72±0.25	3.23
	d	20.03±0.30	1.50
	e	41.96±0.35	2.38
	Average (*n*=5)		3.09
Population 2 (2-12-4)	a	3.94±0.20	5.08
	b	7.72±0.35	4.53
	c	10.22±0.35	3.42
	d	21.58±0.35	1.62
	e	37.70±0.40	1.06
	Average (*n*=5)		3.14

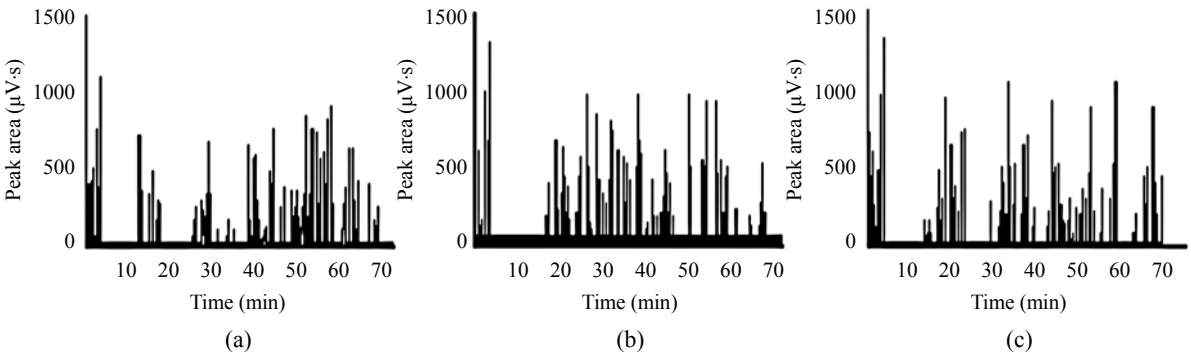

Fig.1 RP-HPLC of alcohol-soluble proteins from different population of *E. angustifolia* (a) Sample No. 3-10-4; (b) Sample No. 1-12-5; (c) Sample No. 2-12-4

Table 4. The maximum number of peaks in an elution profile was 79 from Population 12, while the minimum was 41 from Populations 4 and 13.

Cluster analysis

RP-HPLC data from seventeen populations of *E. angustifolia* were sorted into six clusters at *GSC* (genetic similarity coefficients)=0.535 level, designated I to VI (Fig.2). Clusters I, II, III and IV each included two populations of plants, Populations 1 and 3, Populations 2 and 5, Populations 4 and 6, Populations 7 and 8, respectively. Cluster V was composed of six populations of plants (Populations 9, 10, 11, 12, 13 and 14) and three populations (Populations 15, 16 and 17) were grouped in Cluster VI.

Fig.2 shows the relationships between the seventeen populations of *E. angustifolia* in Xinjiang Province, China. The greatest *GSC* (0.62) was found between Populations 9 and 11 in Cluster V. The lowest *GSC* (0.41) was observed in different clusters, for example Populations 1 vs 4.

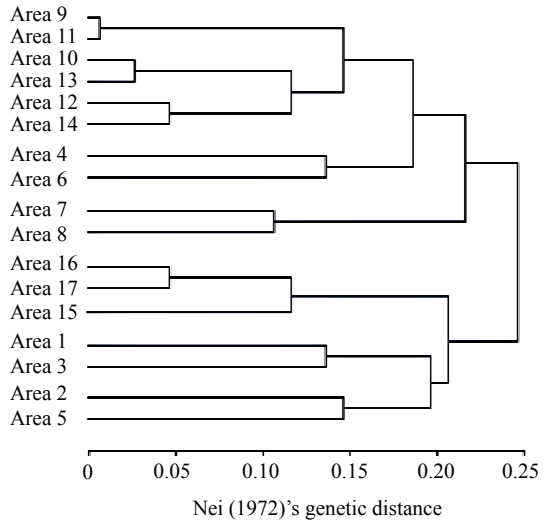

Fig.2 Dendrogram based on the converted data on alcohol-soluble protein peaks in HPLC chromatogram

DISCUSSION AND CONCLUSION

The separation of alcohol soluble proteins using RP-HPLC has been successfully used in wheat (Bietz and Cobb, 1985; Ram *et al*., 1995), maize (Bietz, 1983), rye (Kubiczek *et al*., 1993), and rice (Huebner *et al*., 1990). The results obtained in this study indicated that the separation technique could also be used to analyze the intra-specific genetic relationship of various populations of wild species. The converted data on the elution peaks of different population were distinct (Table 4). The peaks eluting at 2.657, 3.428, 24.383, 34.325, 40.757, 45.822, 46.708, 49.682, 54.657, 64.236 min were characteristic of elution proteins from 17 populations of *E. angustifolia*, and could be used as general biochemical markers of *E. angustifolia*.

The peaks eluting at 10.535, 38.254 and 59.252 min represent alcohol-soluble proteins specific to Cluster I. Similarly, the peaks eluting at 5.732, 6.233 and 25.025 min represent Cluster II; the peaks eluting at 29.168 and 48.248 min represent Cluster III; the peaks eluting at 39.865 min represent Cluster IV; and the peaks eluting at 4.728, 5.394 and 23.657 min represent Cluster V. These peaks could thus be considered as unique biochemical markers for the respective clusters.

According to the result of cluster analysis, the seventeen populations of *E. angustifolia*, which represent the overall distribution in Xinjiang, were grouped into two geographic regions: those located in the south (Populations 1, 2, 3, 4, 5, 6, 7, 8) and the north (Populations 9, 10, 11, 12, 13, 14, 15, 16, 17) of the province. The populations of *E. angustifolia* in south region can be divided into four clusters; the populations in north region are two clusters. The authors think that one cluster of *E. angustifolia* can be named a variable species. There are six variable species of *E. angustifolia* in the Xinjiang eco-area. This result agreed with that of other authors according to morphological data (FLORA of China, 1983).

In addition to the high sensitivity and resolution of the method (HPLC theoretical plates=105 vs 103 for an electrophoresis method such as SDS-PAGE), it is worth mentioning the rapidity of HPLC analysis (ca. 67 min) and the possibility that monitoring the separations allows for possible modifications in real-time. Automated sample handling is another advantage of modern chromatographic systems. Data from RP-HPLC can be readily compared and analyzed by computer. However, there are disadvantages in comparison to electrophoresis, e.g. (1) Only one sample can be tested at one time, while SDS-PAGE can allow for fifteen or more samples to be analyzed simultaneously; (2) It is very difficult to determine the

Table 4 Converted data on elution time on alcohol-soluble protein peaks in seventeen populations of *Elaeagnus*

Retention time (min)	Converted data of protein in seventeen populations																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
0.273	1	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	1
0.795	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	1	1
1.138	1	0	0	1	1	1	1	1	1	0	0	1	1	0	1	0	1
1.778	0	0	0	1	1	1	1	1	0	0	1	0	1	0	0	0	1
2.657	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3.428	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4.107	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
4.728	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
5.394	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
5.732	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6.233	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6.789	1	0	0	0	0	1	0	0	0	1	1	1	0	0	1	0	0
7.472	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
8.222	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
8.732	0	1	0	0	1	0	0	1	0	0	1	0	0	0	1	1	0
9.155	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9.674	1	1	0	1	1	1	1	0	0	0	0	1	0	0	1	1	0
10.535	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11.427	1	0	0	1	1	1	0	1	0	0	0	0	0	0	1	0	0
12.225	1	1	0	1	0	0	1	1	0	0	0	0	0	1	1	0	0
12.874	1	1	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0
13.243	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	0	0
13.838	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0
14.285	0	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	1
14.937	0	0	0	1	0	0	0	0	0	1	0	0	1	1	1	1	1
15.678	1	0	1	1	0	0	0	1	0	0	1	0	0	1	1	1	0
16.278	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
16.903	1	0	1	0	1	0	0	0	1	1	0	0	1	0	1	0	1
17.812	1	0	1	1	1	1	0	1	0	0	0	0	0	0	1	1	1
18.325	1	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	1
18.855	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
19.563	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1
20.022	0	1	0	1	0	0	0	0	0	0	1	1	1	1	0	1	0
20.834	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1
21.558	0	1	0	1	1	0	0	0	1	0	0	0	0	1	0	1	0
22.547	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23.032	0	1	0	1	0	0	0	0	1	0	0	0	0	1	1	0	1
23.657	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
24.383	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25.025	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
25.883	0	0	0	1	0	0	1	0	1	1	0	0	1	1	1	1	1
26.525	0	1	0	0	0	0	1	1	1	0	0	1	1	0	1	0	1
27.068	1	0	1	1	0	0	1	1	0	0	1	0	0	1	0	0	0
27.767	1	1	0	1	0	0	0	1	1	1	0	0	0	0	1	0	0
28.122	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0	1	0
28.677	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0
29.168	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
29.752	0	0	1	1	1	0	1	0	1	0	0	1	1	0	0	1	0
30.118	1	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1	0
30.876	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
31.097	1	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0
31.878	1	0	1	0	1	1	1	1	0	0	0	0	0	0	1	1	1
32.281	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	1
32.917	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1
33.258	0	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	1
33.825	1	0	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0
34.325	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
34.773	0	1	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1
35.162	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	1	1
35.718	0	1	1	0	0	1	0	1	1	1	1	0	0	0	1	1	1
36.328	1	1	0	0	1	0	1	0	1	0	0	1	0	1	1	1	0
36.842	1	1	1	0	1	0	0	0	1	0	0	1	0	1	1	1	0
37.245	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	1	0
37.813	1	1	1	0	0	0	1	1	1	0	1	1	1	1	1	1	1
38.254	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38.925	1	0	1	0	0	0	0	0	1	0	1	1	0	1	1	1	1
39.227	0	0	0	0	1	0	0	1	1	1	0	1	0	1	1	1	1
39.865	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
40.115	0	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1
40.757	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
41.248	1	1	1	0	1	0	1	1	1	0	0	0	0	0	1	0	1
41.838	1	1	1	0	1	1	0	0	1	0	1	0	0	0	1	1	0
42.362	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1	0
42.785	0	0	0	1	0	0	1	0	1	0	1	0	0	1	1	1	1
43.198	1	1	1	0	1	0	0	0	1	0	0	0	0	0	1	1	1
43.772	0	0	0	1	0	0	0	0	1	0	1	1	1	1	0	1	1
44.012	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1
44.503	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1
45.172	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	1	1
45.822	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
46.125	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	1	1
46.708	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
47.352	0	1	1	0	0	1	1	1	0	0	0	0	0	0	1	1	1
47.825	0	1	1	0	1	0	0	0	0	1	0	1	1	0	1	0	1
48.248	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
48.778	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0	1	1
49.133	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1
49.682	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
50.047	0	1	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1
50.895	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1	1	1
51.156	1	0	1	0	0	1	1	1	0	1	0	1	0	0	1	1	1
51.662	0	1	1	0	1	0	0	0	0	0	0	1	0	0	1	1	1
52.592	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53.103	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1
53.785	0	1	0	1	0	1	1	0	1	1	1	1	1	0	1	1	1
54.113	0	0	0	1	0	1	1	0	1	0	1	0	1	1	0	1	1
54.657	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
55.178	1	0	1	1	0	1	0	0	1	1	1	0	1	1	1	0	0
55.821	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	1	1
56.005	0	1	1	0	0	1	1	0	0	1	0	0	1	0	0	0	1
56.210	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1
57.097	0	1	1	0	0	0	1	0	1	1	1	0	0	0	1	1	0
57.622	1	0	1	0	0	1	1	1	1	0	1	0	1	0	1	0	0
58.305	1	1	1	0	1	1	0	0	0	0	0	1	0	0	1	1	0
58.788	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1
59.235	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1
59.767	0	0	1	1	0	1	0	0	0	1	0	1	0	0	0	1	0
60.328	1	1	1	0	1	1	0	0	1	0	1	1	0	0	1	1	1
60.805	1	0	1	1	0	0	0	0	1	0	1	1	0	1	1	0	0
61.137	1	0	1	0	0	1	0	0	1	0	1	1	1	1	1	0	1
61.853	1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1	0
62.028	1	1	0	0	0	0	0	1	1	1	1	1	0	0	1	0	1
62.868	1	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1
63.362	1	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0
63.870	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0
64.236	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
64.766	0	1	1	0	0	0	0	0	1	0	1	1	0	1	1	0	0
65.173	1	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1
65.667	1	1	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1
66.219	1	1	0	0	1	0	1	1	0	0	0	0	0	0	1	1	1
66.692	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0

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 methods such as SDS-PAGE should not be seen as competing methods, but as complementary 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 or samples not well characterized or indistinguishable by use of electrophoretic banding patterns. In summary, this method is simple, rapid, reproducible and accurate.

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