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Quality evaluation of Astragali Radix using a multivariate statistical approach

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ABSTRACT

The quality of 43 Astragali Radix samples collected in China and Mongolia was evaluated using multivariate statistical analysis of data obtained from liquid chromatography-ion trap-time of flight (LC-IT-TOF) mass spectrometry. The samples were classified into four characteristic groups and most of the marker compounds were identified by elemental composition data and the results of MS/MS analysis. The approach provides useful information and gives an overview of the difference between crude drugs originating from different production environments and the genetic nature of the medicinal plants. In addition, the ease with which particular marker compounds could be identified and the effectiveness of the comparison by means of multivariate statistics, such as principal component analysis (PCA), indicates that this method could be utilized for the establishment of standardization and quality control procedures for crude drugs.

1. Introduction

Astragali Radix, one of the most commonly used traditional Chinese crude drugs, is prepared from the roots of *Astragalus membranaceus* (Fisch) Bunge and *Astragalus mongholicus* Bunge, and is prescribed as an immunostimulant, hepatoprotective, antiperspirant, a diuretic or a tonic in Traditional Chinese Medicines (Sinclair, 1998). Both species grow in the northeast, north and northwest of China as well as in Mongolia and Korea (*A. membranaceus*). The natural resources of Astragali Radix are diminishing due to the increasing demand for it as a raw material for health foods and hence the bulk of the commercial supply is mainly taken from farming sources in China (Ma et al., 2000).

Regarding the chemical constituents of Astragali Radix, more than 100 compounds, such as isoflavonoids, triterpene saponins, polysaccharides and amino acids, have been identified so far, and various biological activities of the compounds have been reported (Xu et al., 2006). It is well known that the level of active compounds varies widely depending on the region where the plants were grown and the period when they were harvested (Ma et al., 2002). So far, only a few studies have been carried out on evaluating the quality of Astragali Radix collected in China and Japan, in which the isoflavonoids, such as calycosin, calycosin 7-*O*-glucoside (**1**), formononetin 7-*O*-glucoside, methylnissolin 7-*O*-glucoside and isomucronulatol 7-*O*-glucoside, as well as astragalosides I (**4**), II (**5**), III and IV (**6**) (Fig. 1) have been used as indices of the quality of the Radix (Ma et al., 2000; Anetai et al., 1998).

Currently, the quality of the crude drug is predicated on the analysis of one or more selected compounds. Ideally, these compounds should be responsible for the action and efficiency of the crude drugs. However, crude drugs consist of numerous compounds and their bioactivity can be considered to be due to additives and/or the synergetic effects of several compounds. Therefore, to demonstrate the comprehensive properties of crude drugs, the development of a new evaluation strategy is required. For the differentiation of a large number of similar samples, several approaches using multivariate statistical analysis have proved to be effective (Gonzales et al., 2001; Goodall et al., 1995).

Time of flight mass spectrometry (TOF MS) has a high acquisition speed and provides accurate mass measurements. In addition, the sensitivity under the full scan mode is higher than that of other types of mass measuring instrument. Accurate mass measurement gives the elemental composition of the detected ions, and it is remarkably effective for the identification of unknown compounds (Lacorte and Fernandez-Alba, 2006). Liquid chromatography (LC) mass spectrometry with an ion trap mass analyzer is a useful tool for qualitative analysis due to: its important characteristic, that is,

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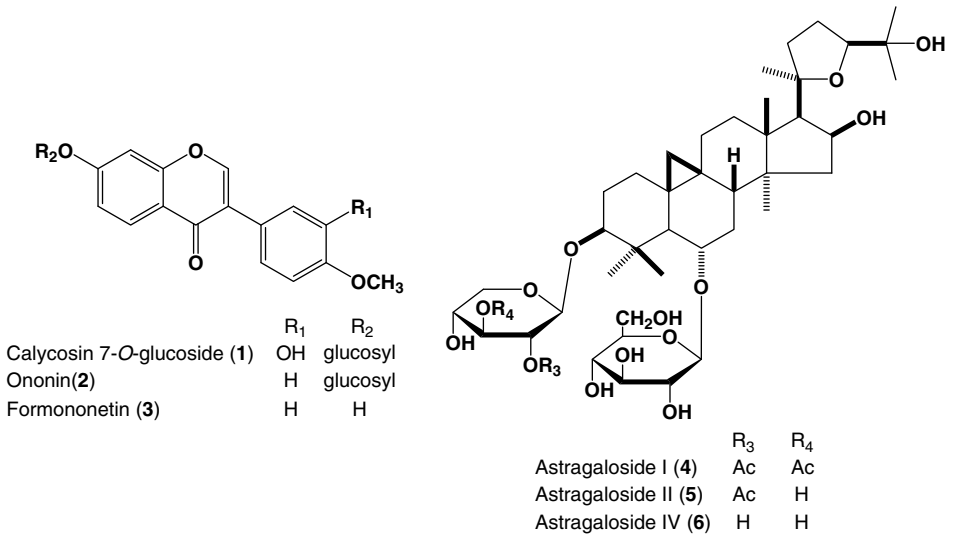

Fig. 1. Structure of the major constituents of Astragali Radix.

the efficient formation of fragmentary ions by collision-induced dissociation, which provides useful structural information, as well as the capability of conducting MS^n analyses that establish the linkage and identity of product ions. The combination of ion trap MS/MS for structural information and TOF MS for the accurate mass measurement and elemental composition of ions represents a powerful analytical approach for the identification of constituents in the crude extract.

In the present study, a multivariate statistical approach of the data obtained from LC-IT-TOF MS analysis was used for evaluating the quality of Astragali Radix grown in China and Mongolia. In addition, the identification of marker compounds for classification and evaluation of the quality of crude extracts was performed by means of MS/MS analysis.

2. Results and discussion

2.1. Mass spectrum of isoflavones and astragalosides

For identification of compounds contained in the crude extract, mass fragmentation with high resolution mass spectrometry data provided variable information. To accumulate knowledge of the fragmentation processes of isoflavones and astragalosides (Fig. 1), which have been reported as the major constituents of Astragali Radix, positive and negative ion mass spectra of **1**, ononin (**2**), formononetin (**3**), **4**–**6**, were measured as shown in Fig. 2. All the glycosides gave $(M+H)^+$ and $(M+Na)^+$ ions in the positive ion spectra and $[(M+CH_3COOH)-H]^-$ ions in the negative ion spectra. Regarding the cleavage of the sugar moiety, the astragalosides provided a series of fragments by deglucosidation, acetylated dexylosidation and a series of dehydrations. On the other hand, isoflavone glucoside gave fragment ions originating from the cleavage of dehydrated glucose from $(M+H)^+$ ions.

2.2. Total ion chromatograms of extracts of Astragali Radix from China and Mongolia

In Fig. 3, total ion chromatograms and mass chromatograms monitored by the protonated molecular ions $(M+H)^+$ of **1**–**6**, as well as the ion corresponding to sapogenin in astragalosides in extracts of Astragali Radix collected in China and Mongolia are shown. Some differences can be seen between the chromatograms of the Chinese and Mongolian samples. Isoflavones and astragalosides **1**–**5** are present in both samples, but the amounts of the compounds are markedly different according to the sample origin. In addition, the presence of different peaks, such as the peak observed at a retention time of 8.20 min in the chromatogram of the Mongolian sample, is apparent. Although there are clear visual differences between the chromatograms of the Chinese and Mongolian samples, for an easier and non-biased interpretation of the results and to reduce the dimensionality of the multivariate data obtained from the LC–MS results, we analyzed the LC–MS chromatographic data using principal component analysis (PCA).

2.3. Principal component analysis

To compare the chromatographic differences among the extracts of Astragali Radix collected from different places, principal component analysis (PCA), an unsupervised method for pattern recognition, was performed.

PCA is an unsupervised method of multivariate data analysis and used for the investigation of metabolomic profile analysis in complex mixtures such as plant extracts. The data analysis procedure requires preprocessing of the raw data to generate a data matrix, the columns of which represent variables and the rows of which contain the samples that are included for analysis. These peak picking and peak alignment processes are important for creating a productive data matrix

The process used in this study was as follows:

(1) First derivation of the total ion chromatogram (TIC)

The solvent peak (retention time 0–4 min) was removed from the original TIC data. To create the original data set, the TIC data was converted to ASCII format. To compensate the baseline drift of the TIC and enhance the information of the shoulder peaks, the first derivation process was applied to the original data set.

(2) Removing data having small loading values

PCA was applied to the original processed data set. The outlier of the samples (outside the 95% line of the Mahalanobis distance) and variables with small loading values (less than 50% of the larg-

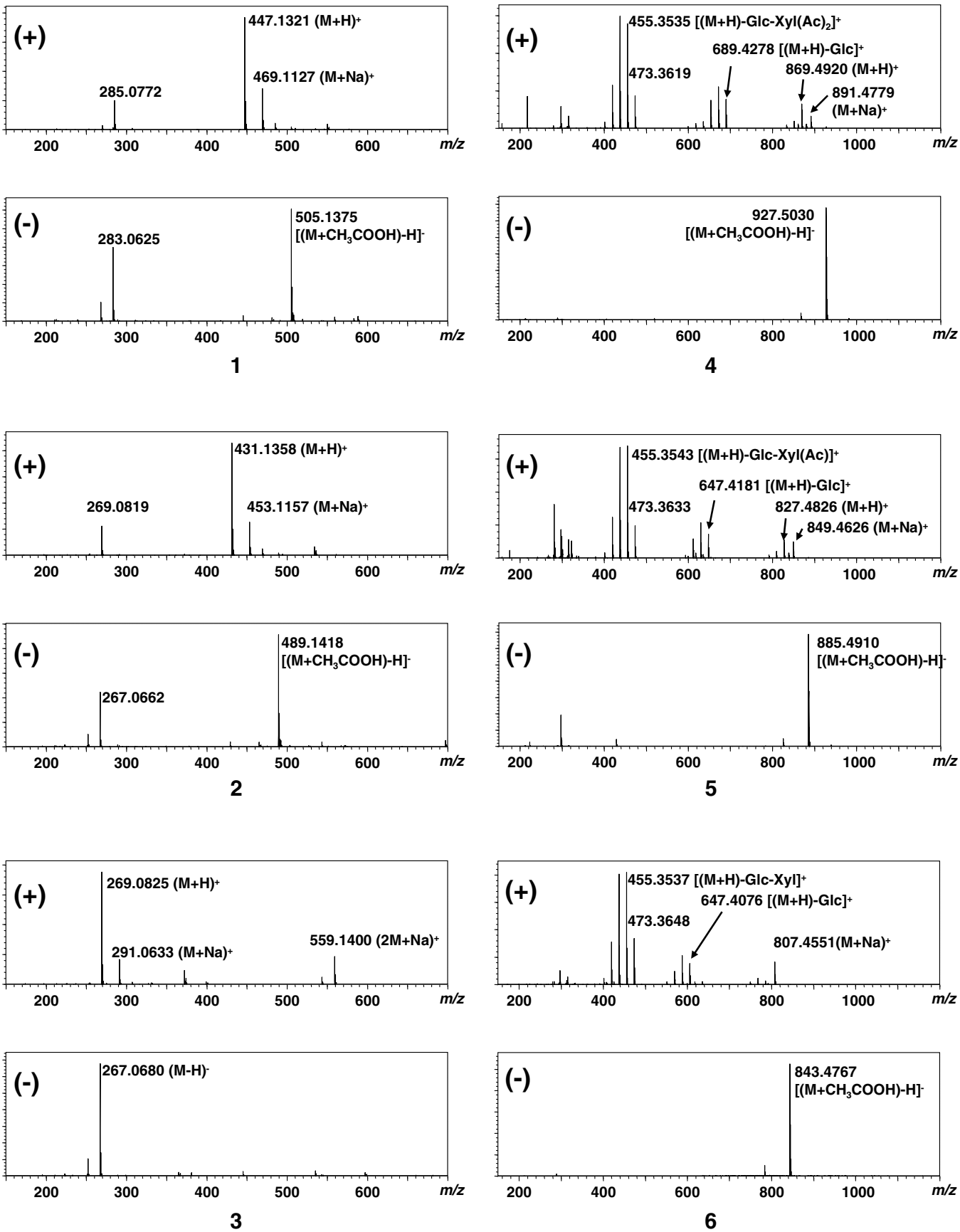

Fig. 2. Positive (+) and negative (−) ion LC-TOF mass spectra of compounds **1**–**6**.

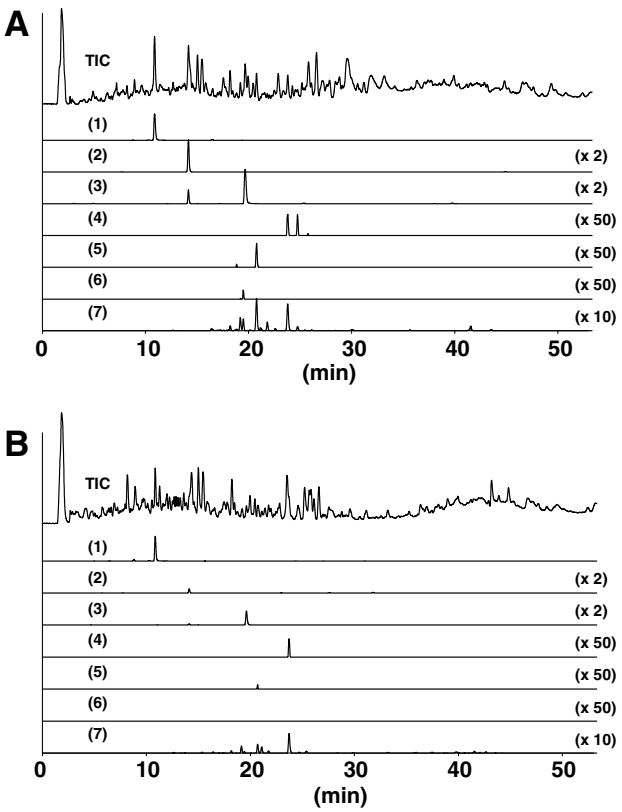

Fig. 3. Total ion chromatograms and mass chromatograms of (A) sample 10 (Chinese Astragali Radix) and (B) 27 (Mongolian Astragali Radix) measured in the positive (+) scan mode. (1), *m/z* 447.1321 ($(M+H)^+$ of **1**); (2), *m/z* 431.1358 ($(M+H)^+$ of **2**); (3), *m/z* 269.0825 ($(M+H)^+$ of **3**); (4), *m/z* 869.4920 ($(M+H)^+$ of **4**); (5), *m/z* 827.4826 ($(M+H)^+$ of **5**); (6), *m/z* 785.4551 ($(M+H)^+$ of **6**); (7), *m/z* 445.3537 ((bis-dehydrated sapogenin+H)$^+$ of the astragalosides). The magnitude factors of the chromatograms are indicated in parentheses.

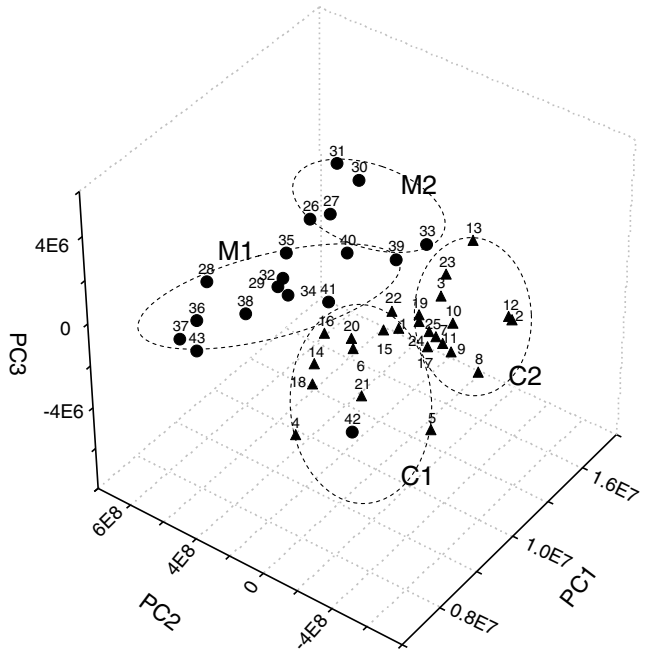

Fig. 4. PCA scores plot of the Chinese and Mongolian Astragali Radix samples. (●) Mongolian Astragali Radix and (▲) Chinese Astragali Radix. The numbers in the scores plot indicate the sample no. in Table 1.

est loading value) were removed from the original data set to create a new analytical data set.

(3) PCA on the new data set

PCA was applied to the analytical data set obtained from preliminary processing.

The original data matrix, ***X***, was decomposed into the product of a score matrix, ***T***, and a loading matrix, ***L*** by PCA. The columns of ***L*** are the principal components (PCs), the new factors which are linear combinations of the original variables. The score matrix ***T*** is the projection of the samples onto the axes defined by the loadings. Each sample had a coordinate on each new axis. The score plots were used to objectively classify samples by their measured properties. The distribution of the samples on this graph establishes a pattern that correlates to the general characteristics of the samples.

In Fig. 4, the PCA scores plot is shown, where each Astragali Radix sample represents a marker. The first three PCs account for 89.6% of the total variance (PC1, 73.2%; PC2, 12.7%; PC3, 3.7%). The scores plot clearly indicates that the TIC patterns of the extracts from Chinese and Mongolian Astragali Radix are different due to their different constituents. Furthermore, it is clear that the respective Chinese and Mongolian groups can be further classified into two sub-groups, which we tentatively named C1 and C2 for the Chinese samples, and M1 and M2 for the Mongolian samples (Fig. 4). In the chemometric analysis, the peaks with large loading values can be considered to be markers that strongly contribute to the classification of the samples by PCA. In the present study, 12 peaks in the TICs were distinguished as marker compounds and these were identified by analysis of the positive ion mass spectra with elemental composition data as well as the MS/MS data, as shown in Table 2. The isobaric compound showing the peaks at retention times of 8.20 min ($(M+H)^+$, *m/z* 285.1928) and 15.50 min ($(M+H)^+$, *m/z* 285.0763) gave different exact masses, and the elemental compositions of the compounds were calculated to be $C_{13}H_{24}N_4O_3$ and $C_{16}H_{12}O_5$, respectively. From reported data (Buckingham, 2007) on the constituents of the *Astragalus* species, we identified the compounds as smyrnovinine and calycosin. This identification was supported by the MS/MS spectroscopic data analysis shown in Fig. 5. In the TIC of the Mongolian samples belonging to the sub-group M1, a characteristic intense peak was observed at a retention time of 37.06 min. The MS/MS analysis indicated that the peak consisted of three compounds having the elemental compositions, $C_{24}H_{47}N_5O_2$, $C_{22}H_{43}N_5O$ and $C_{20}H_{39}N_5$, though the compounds could not be identified.

The concentration of isoflavones and astragalosides, such as **1**, **2**, **3** and **5**, in the Chinese samples were higher than those in the Mongolian samples, whereas smyrnovinine was detected only in the Mongolian samples. In addition relatively large amounts of trihydroxy-octadecadienoic acid and methylnissolin-3-glucoside were detected in the Mongolian samples. The following differences in the constituents of the respective sub-groups were observed: (1) the sub-group C2, consisting of wild Astragali Radix, contained larger amounts of **1**, **2** and **5** than C1 derived from cultivated plants and (2) the sub-group M1 contained larger amounts of three compounds having the elemental compositions, $C_{24}H_{47}N_5O_2$, $C_{22}H_{43}N_5O$ and $C_{20}H_{39}N_5$, than M2.

It is interesting that sample 42 cultivated in Mongolia using seeds originating from China is located near the Chinese sub-group C1, in the PCA scores plot (Fig. 4). On the other hand, sample 43 cultivated in the same place as sample 42 using Mongolian seeds is classified in the Mongolian sub-group M1. These results suggest that the genetic properties of the plants might be more important

Table 1
Materials used in present study

No.	Place of market (production area)	Purchased from	Collected date	TMPW[a] No.
1	Shanxi Province, China	Uchida Wakanyaku Co. Ltd., Tokyo	2002.4	21493
2	Nei Menggu Autonomous Resion, Kökekhota, China (cultivate)	Zhongshanlu Drug Store	2002.9	21551
3	Nei Menggu Autonomous Resion, Chifeng, China (wild)	Chifengrongxiangtang Pharmaceutical Co.	2002.9	21553
4	Nei Menggu Autonomous Resion, Chifeng, China (wild)	ibid.	2002.9	21554
5	Nei Menggu Autonomous Resion, Chifeng, China (cultivate)	ibid.	2002.9	21555
6	Nei Menggu Autonomous Resion, Chifeng, China (cultivate)	ibid.	2002.9	21556
7	Nei Menggu Autonomous Resion, Chifeng, China	ibid.	2002.9	21564
8	Nei Menggu Autonomous Resion, Chifeng, China (cultivate)	ibid.	2002.9	21566
9	Shanxi Province, China (wild)	Huangqizhuang Co.	2002.9	21571
10	Shanxi Province, China (wild)	ibid.	2002.9	21572
11	Shanxi Province, China (wild)	ibid.	2002.9	21573
12	Shanxi Province, China (wild)	ibid.	2002.9	21574
13	Shaanxi Province, China	ibid.	2002.9	21575
14	Gansu Province, China (cultivate)	ibid.	2002.9	21576
15	Nei Menggu Autonomous Resion, China, Guyang (cultivate)	ibid.	2002.9	21577
16	Nei Menggu Autonomous Resion, China, Guyang (cultivate)	ibid.	2002.9	21578
17	Shaanxi Province, China (wild)	ibid.	2002.9	21579
18	Shaanxi Province, China (wild)	ibid.	2002.9	21580
19	Shaanxi Province, China	ibid.	2002.9	21581
20	China (cultivate)	ibid.	2002.9	21582
21	Hebei Province, China	ibid.	2002.9	21583
22	China	Bejing Tongrentang Daren Drug Store	2002.9	21638
23	China	Uchida Wakanyaku Co. Ltd., Tokyo	2003.3	21984
24	China	ibid.	2003.4	21998
25	China	Bejing Tongrentang Drug Store	2003.9	22259
26	Sogo-Tsetserleg, Mongolia		2002.7	M-668
27	Sogo-Tsetserleg, Mongolia		2002.7	M-670
28	Sogo-Tsetserleg, Mongolia		2002.7	M-674
29	Tsetserleg-Songino, Mongolia		2002.7	M-684
30	Tsetserleg-Songino, Mongolia		2002.7	M-685
31	Guulin-Buutsagaan, Mongolia		2002.8	M-880
32	Guulin-Buutsagaan, Mongolia		2002.8	M881
33	Baga Bogdyn Nuruu-Bayanteeg, Mongolia		2002.8	M-947
34	Ulaanbaatar-Terelj, Mongolia		2004.7	M-1086
35	Gachuurt, Mongolia		2004.7	M-1099
36	Ereen-Bayan-Uul, Mongolia		2004.7	M-1197
37	Ereen-Bayan-Uul, Mongolia		2004.7	M-1199
38	Erdenetsagaan, Suhbaatar, Mongolia		2004.7	M-1230
39	Shiliyn Bogd-Dariganga, Mongolia		2004.7	M-1235
40	Shiliyn Bogd-Dariganga, Mongolia		2004.7	M-1241-3
41	Hayrhandulaan, Mongolia		2006.7	M-1324
42	Guulin (cultivate, seeds from China)		2006.8	M-1456
43	Guulin (cultivate, seeds from Mongolia)		2006.8	M-1458

[a] The specimen reference number of the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW).

Table 2
Detected marker compounds for the classification of astragali radix by principal component analysis

No.	Retention time (min)	$(M+H)^+$ or $(M+Na)^+$ ion	Product ions from $(M+H)^+$ or $(M+Na)^+$ ion	Compounds
1	8.20	285.1928 [$C_{13}H_{24}N_4O_3$+H]	241.2027 [$C_{12}H_{25}N_4O$], 199.1811 [$C_{11}H_{23}N_2O$], 173.1394 [$C_7H_{17}N_4O$], 156.1126 [$C_7H_{14}N_3O$]	Smyrnovinine
2	10.88	447.1294 [$C_{22}H_{22}O_{10}$+H]	285.0762 [$C_{16}H_{13}O_5$]	**1**
3	14.17	431.1354 [$C_{22}H_{22}O_9$+H]	269.0812 [$C_{16}H_{13}O_4$]	**2**
4	14.35	351.2159 [$C_{18}H_{32}O_5$+Na]	333.2036 [$C_{18}H_{30}O_4$Na], 315.1967 [$C_{18}H_{28}O_3$Na], 245.1517 [$C_{14}H_{22}O_2$ Na]	Trihydroxy-octadecadienoic acid
5	15.05	463.1611 [$C_{23}H_{26}O_{10}$+H]	301.1076 [$C_{17}H_{17}O_5$]	Methylnissolin 3-glucoside
6	15.50	285.0763 [$C_{16}H_{12}O_5$+H]	270.0524 [$C_{15}H_{10}O_5$], 253.0500 [$C_{16}H_9O_4$], 225.0540 [$C_{14}H_8O_3$]	Calycosin
7	18.23	313.2285 [$C_{18}H_{32}O_4$+H]	295.2280 [$C_{18}H_{31}O_3$], 277.2168 [$C_{18}H_{29}O_2$]	Dihydroxy-octadecadienoic acid
8	19.68	269.0814 [$C_{16}H_{12}O_4$+H]	254.0584 [$C_{15}H_{10}O_4$], 237.0550 [$C_{15}H_9O_3$]	**3**
9	20.73	827.4798 [$C_{43}H_{70}O_{15}$+H] 849.4620 [$C_{43}H_{70}O_{15}$+Na]	Not measured	**5**
10	26.56	295.2278 [$C_{18}H_{30}O_3$+H]	277.2150 [$C_{18}H_{29}O_2$]	Hydroxy-octadecatrienoic acid
11	37.06	438.3807 [$C_{24}H_{47}N_5O_2$+H] 394.3546 [$C_{22}H_{43}N_5O$+H] 350.3283 [$C_{20}H_{39}N_5$+H]	421.3542 [$C_{24}H_{45}N_4O_2$], 239.1492 [$C_{11}H_{19}N_4O_2$] 377.3278 [$C_{22}H_{41}N_4O$], 195.1241 [$C_9H_{15}N_4O$] 333.3015 [$C_{20}H_{37}N_4$], 151.0973 [$C_7H_{11}N_{42}$]	Unidentified
12	43.21	391.2857 [$C_{24}H_{38}O_4$+H]	279.1591 [$C_{16}H_{23}O_4$], 261.1472 [$C_{16}H_{21}O_3$], 167.0344 [$C_8H_7O_4$]	Unidentified

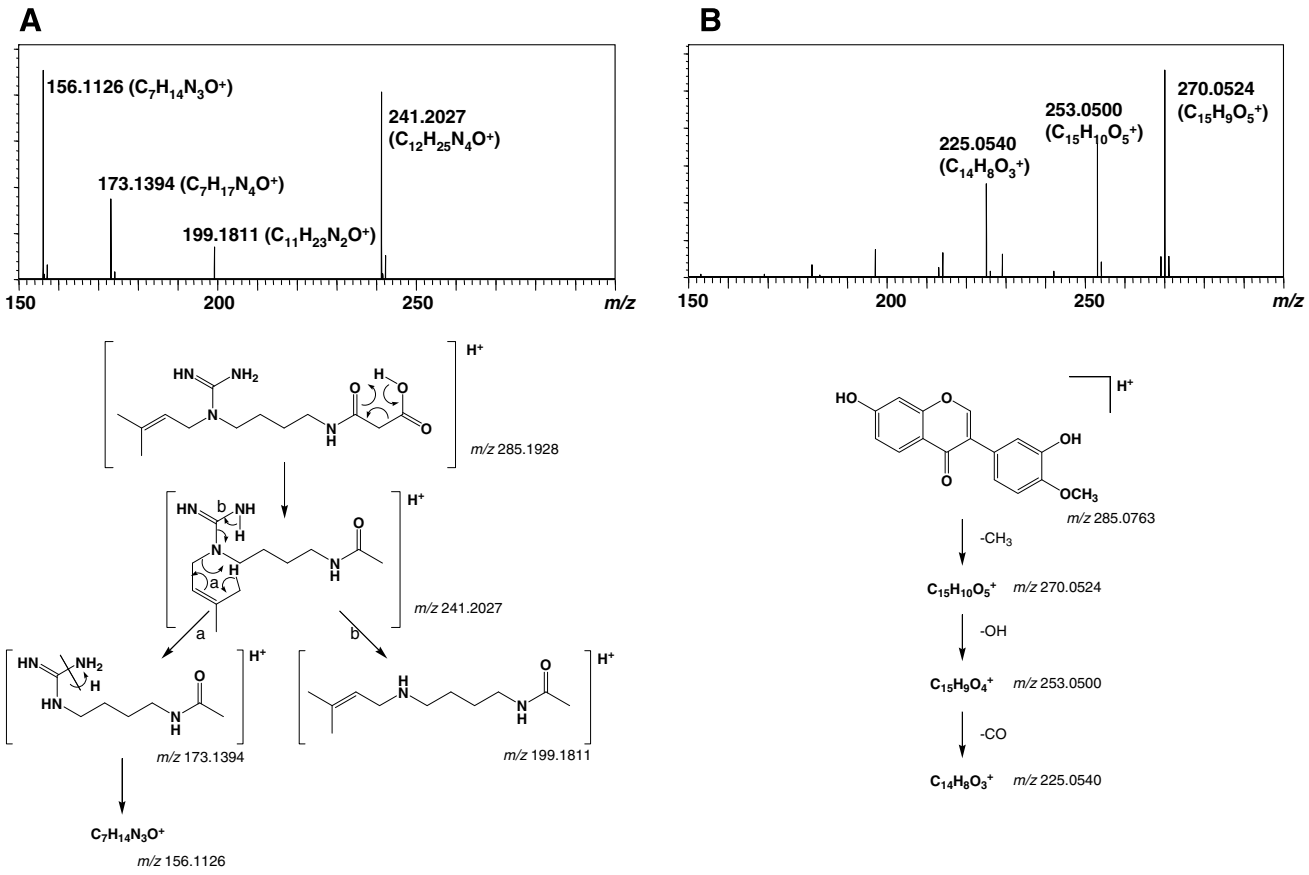

Fig. 5. MS/MS spectra and estimated fragmentations of (A) smyrnovinine and (B) calycosin.

factors in determining the quality of the Astragali Radix than the cultivation and environmental factors, even though only one sample was examined here.

3. Conclusions

In conclusion, we have examined a reliable multivariate statistical approach for the evaluation of Astragali Radix obtained from different places using LC-IT-TOF MS. Forty three Astragali Radix samples collected in China and Mongolia were classified characteristically into four groups and most of the marker compounds, such as smyrnovinine, isoflavones (**1**, **2**, **3**, methylnissolin 7-*O*-glucoside and calycosin), **5** and hydrocarbons, were identified from the elemental composition data and the results of the MS/MS analysis. In addition, it was revealed that Mongolian Astragali Radix contained relatively large amounts of alkaloid compounds compared to the Chinese samples. The approach provides useful information to indicate the differences between crude drugs arising from the genetic nature and the various environmental production factors of the source medicinal plants. In addition, it was shown that the ease with which particular marker compounds could be identified and the effectiveness with which they could be compared using multivariate statistics, such as PCA, could be utilized for the establishment of new standardization and quality control procedures for crude drugs.

4. Experimental

4.1. Materials and analytical sample preparation

Forty three crude drug samples and plant specimens were purchased in markets or collected from the areas where they were grown. All samples and specimens were deposited in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW, Table 1).

The samples were individually pulverized and with the corresponding powders screened through 150 μm sieves. Each fine powder (2 g) was accurately weighed and extracted four times with EtOH–H_2O (50 ml, 7:3) by ultrasonication at room temperature for 30 min. After centrifugation, the organic solvents were combined and evaporated *in vacuo* to give each extract. Each extract was then dissolved in (MeOH 10 ml) and filtered through an 0.2 μm Millipore filter, with 2 μl individually injected into the LC–MS.

4.2. Standard samples and reagents

Calycosin-7-*O*-glcoside (**1**), ononin (**2**), formononetin (**3**), astragalosides I (**4**), II (**5**) and IV (**6**) were isolated from the drug sample (TMPW No. 24238) purchased from Uchida Wakanyaku Co., Ltd., and their structures are shown in Fig. 1. The isolated compounds were identified by comparison of their ^1H and ^{13}C NMR spectra with those reported in the literature (Xiao et al., 2005; Kitagawa et al., 1983). All chemicals were of analytical grade, and the chromatographic solvents were of HPLC grade.

4.3. Analytical instruments

LC–MS analyses were performed with a Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface. The ESI parameters were as follows: source voltage +4.5 kV (positive mode) and −3.5 kV (negative mode), capillary temperature 200 °C, nebulizer gas 1.5 l/min. The mass spectrometer was operated in both positive and negative ion modes scanning from *m/z* 100 to 1500. A Shimadzu VP-ODS 2 column (2.0 mm i.d. × 150 mm) was used and the

column temperature was maintained at 40 °C. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution, (B) CH_3CN under the following gradient conditions: 0–40 min linear gradient from 5% to 100% B, 40–60 min isocratic at 100% B. The flow rate was 0.2 ml/min.

4.4. Data analysis

The chromatograph was recorded at 10 Hz. Before statistical analysis of the data by means of multivariate methods, the mass spectrometric data were converted to ASCII format by Shimadzu LC–MS solution software.

PCA is a multivariate statistical method which allows a large number of sample datasets to be described in terms of a much smaller number of principal components (PCs), each of which represents significant variability in the data but which are uncorrelated with each other. The $I \times J$ data matrix, $\boldsymbol{X}$, can be decomposed into an $I \times K$ scores matrix, $\boldsymbol{T}$, a $K \times J$ loadings matrix, $\boldsymbol{L}$, and a $I \times J$ residual matrix, $\boldsymbol{E}$, according to the following equation:

$$\boldsymbol{X} = \boldsymbol{T} \cdot \boldsymbol{L} + \boldsymbol{E}$$

where I are the number of samples, J the number of variables (chromatographic data) and K the number of principal components. PCA provides a score plot that displays the chemical differences between the chromatograms, i.e. differences or similarities of the samples, and provides a loading plot that correlates how much influence each variable has on the creation of the PCs.

All the statistical analyses were carried out using Piroruet software (GL Science Inc., Tokyo).

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