

Discrimination of Producing Areas of *Astragalus membranaceus* Using Electronic Nose and UHPLC-PDA Combined with Chemometrics

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Abstract

Wei W., Li J., Huang L. (2017): Discrimination of producing areas of *Astragalus membranaceus* using electronic nose and UHPLC-PDA combined with chemometrics. *Czech J. Food Sci.*, 35: 40–47.

Electronic nose (E-nose) and ultra-high-performance liquid chromatography (UHPLC) were simultaneously developed for quality evaluation and diversity research of *A. mongholicus*. Instrumental data were subjected to principal component analysis (PCA) and discriminant factor analysis (DFA) to rapidly distinguish *A. mongholicus* derived from different main producing regions in China. E-nose results showed that *A. mongholicus* that originated from main producing areas (Gansu, Shanxi, and Inner Mongolia in China) could be accurately classified into three groups through PCA and DFA. The response values of samples from Inner Mongolia and Shanxi were similar, and better than those of the samples from Gansu. Identification by the E-nose was verified through UHPLC analysis, and the content of the main ingredient was consistent with the response value of *A. mongholicus*. In conclusion, E-nose and UHPLC combined with chemometrics can be used as reliable techniques to distinguish the geographic origins of *A. mongholicus* effectively, and evaluate the quality of traditional Chinese herbs.

Keywords: E-nose; *Astragalus mongholicus*; astragaloside IV; campanulin; UHPLC

Astragalus membranaceus var. *mongholicus* (Bunge) P.K.Hsiao, known as Meng Gu Huang Qi in Chinese, is a medicinal plant that has been widely used in China for thousands of years (LUI *et al.* 2015). Currently, *A. mongholicus* is extensively utilised worldwide for its numerous medicinal properties, such as immune stimulant, tonic, antioxidant, hepatoprotectant, diuretic, antidiabetic, anticancer, and expectorant (CHO & LEUNG 2007; AGYEMANG *et al.* 2013; HONG *et al.* 2013; SHAHZAD *et al.* 2016). Daodi herbs prove that herbs grown in some regions possess better quality and efficacy than those grown in other regions under certain natural, ecological, and environmental conditions. Such regions can be classified as top-geoherb or

non-top geoherb. It is well known that top-geoherbs (daodi herbs) are featured with proverbial superior qualities and popularly used in traditional Chinese medicinal clinical practice compared with non-top geoherbs. Daodi research included that evaluated quality difference, explored the reason for quality difference, etc. *A. mongholicus* is a herb with multiple geographic origins. The provinces of Gansu, Shanxi, and Inner Mongolia are the major producing areas in China, of which Inner Mongolia and Shanxi are traditionally regarded as top-geoherb regions (FU *et al.* 2014). However, quality variations have existed in different herb-producing areas (YAO *et al.* 2012), and the rapid, effective, and non-destructive

Supported by the National Natural Science Foundation of China, Grants No. 81274013 and No. 81473315, and CAMS Innovation Fund for Medical Sciences, Grant CIFMS No. 2016-I2M-3-015.

Wenlong Wei and Jinhua Li contributed equally to this work.

doi: 10.17221/126/2016-CJFS

method should be established for quality evaluation of daodi herbs.

In general, the aroma of *A. mongholicus* is an important index to evaluate its quality (Qi *et al.* 2006; ZHENG *et al.* 2015). Samples derived from various regions possess different odour intensity, and the better quality samples are usually processed more aromatic than those of low quality. Therefore, the detection of a difference in odours could be a feasible way to evaluate the geographic origins and quality of *A. mongholicus* (Li *et al.* 2013; Russo *et al.* 2014). Compared to those traditional methods, the main advantage of an electronic nose (E-nose) is that the data normalisation can perform the odour assessment on a continuous basis with the characteristics of being non-invasive, rapid, precise, and requiring no pre-treatment. In this study, an E-nose was employed to analyse the response value of *A. mongholicus* samples obtained from top-geoherb and non-top geoherb regions. Meanwhile, the contents of campanulin, calycosin, kaempferol, formononetin, ononin, quercetin, and astragalus polysaccharide (APS) were determined to confirm the quality variation of the *A. mongholicus* samples.

MATERIAL AND METHODS

Experimental material. Astragaloside IV, D(+)-glucose, formononetin, kaempferol, and quercetin were purchased from the Chinese Control of Pharmaceutical and Biological Products (SFDA, China). Ononin, calycosin, and campanulin were purchased from the Institute of Beijing Heng Yuan Qi Tian (China). Three samples of *A. mongholicus* (GS1, GS2, GS3) were collected from Gansu province, three samples (NM1, NM2, NM3) were collected from Inner Mongolia

province, and three samples (SX1, SX2, SX3) were collected from Shanxi province in China. All herbal samples were authenticated by Professor Linfang Huang, and the voucher specimens were deposited in the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, China. The details of the samples are listed in Table 1.

E-nose. A FOX-4000 E-nose (Alpha MOS, France), which consists of a sampling apparatus, an array of sensors, an HS-100 autosampler, air generator equipment, and pattern recognition software (SOFTV 9.1) for data recording and analysis was used to analyse the odours of *A. mongholicus*. The sensor array was composed of 18 metal oxide semiconductors, namely, LY2/LG, LY2/G, LY2/AA, LY2/GH, LY2/gCTL, LY2/gCT, T30/1, P10/1, P10/2, P40/1, T70/2, PA/2, P30/1, P40/2, P30/2, T40/2, T40/1, and TA/2. These chemical sensors are resistive; each sensor has a specific sensitivity and selectivity characteristic. Their characteristics depend on the materials used in the manufacturing of the sensors. Alpha-Fox automatically collects data (autosampling, data acquisition, and data processing) through a complete software package (BLEIBAUM *et al.* 2002). A schematic of the E-nose system is shown in Figure 1, and the sensors and their main applications are listed in Table 2.

The typical sensing signals of the 18 sensors for *A. mongholicus* are shown in Figure 2. When a volatile gas reaches the measurement chamber, each curve represents a sensor conductivity induced by an electrovalve action. The E-nose sensor response of the samples and odour intensity were calculated using the formula:

$$R = (R_0 - R_t)/R_0$$

where: R – E-nose sensor response; R_t – value of the sensors; R_0 – value of the sensors at time = 0 seconds

Table 1. Sample information of *A. mongholicus* used for E-nose analysis

No.	Origin	Harvest time
GS1	Xiadiema village, Qingshui township, Min county, Dingxi city, Gansu province	2014–10–21
GS2	Chelu village, Meichuan township, Min county, Dingxi city, Gansu province	2014–10–23
GS3	Chelu village, Meichuan township, Min county, Dingxi city, Gansu province	2014–10–23
NM1	Puranxin village, Kebuer township, Wulanchabu city, inner Mongolia province	2014–10–27
NM2	Nanmen village, Guyang county, Baotou city, inner Mongolia province	2014–10–27
NM3	Shanggou township, Wuchuan county, Huhehaote city, inner Mongolia province	2014–10–27
SX1	Heishi village, Guaner township, Hunyuan county, Datong city, Shanxi province	2014–10–29
SX2	Mazhuang village, Guaner township, Hunyuan county, Datong city, Shanxi province	2014–10–29
SX3	Hunyuan county, Datong city, Shanxi province	2014–10–30

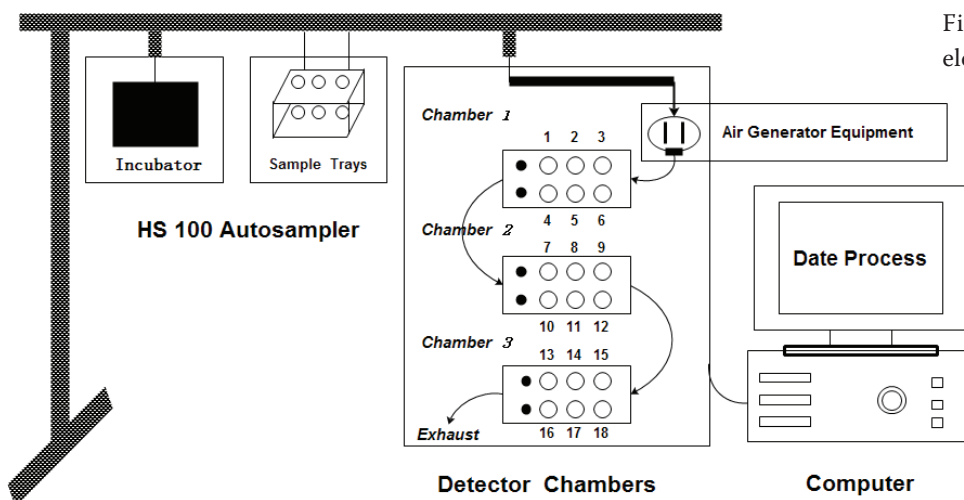


Figure 1. Schematic of the electronic nose system

In the initial period, the intensity of each sensor was low. Subsequently, the intensity increased continuously, and finally stabilised after a few seconds or minutes. The horizontal axis is the timeline, with a total of 120 seconds; the vertical axis is the intensity of the response, and each curve represents a sensor response to the changes within 120 seconds. The maximum response values of the sensor were extracted and analysed individually. The response values representing different curves were explored and response values with low significance were discarded.

E-nose measurements. The *A. mongholicus* samples (0.50 g) were placed in 10 ml headspace vials. The headspace vials were then sealed and loaded into the autosampler tray. The time of the headspace was set to 600 s, and temperature was set to 55°C. Afterward, 1000 µl of purified air was injected into the testing chamber through a syringe at a flow rate of 150 ml/min and injection rate of 1000 µl/s. The acquisition time of parameters was 120 s, and a delay of 600 s was used to allow the sensors to return to their baseline value before the next injection. This amount of time is sufficient for

Table 2. The FOX4000 response characteristics of eighteen sensors

Matrix chamber	No.	Sensor	Sensitivity property	Reference materials
Sensor chamber 1	1	LY2/LG	oxidising gas	chlorine, fluorine, oxynitride
	2	LY2/G	toxic gas	ammonia, amines, carbon oxide
	3	LY2/AA	organic compounds	ethyl alcohol
	4	LY2/GH	toxic gas	ammonia, amines
	5	LY2/gCTL	toxic gas	hydrogen sulfide
	6	LY2/gCT	inflammable gas	propane, butane
Sensor chamber 2	7	T30/1	organic compounds	organic compounds
	8	P10/1	combustible gas	hydrocarbon
	9	P10/2	inflammable gas	methane
	10	P40/1	oxidising gas	fluorine
	11	T70/2	aromatic compounds	methylbenzene, xylene
	12	PA/2	organic compounds and toxic gas	ammonia, amines, ethyl alcohol
Sensor chamber 3	13	P30/1	combustible gas and organic compounds	hydrocarbon, product of combustion
	14	P40/2	oxidising gas	chlorine
	15	P30/2	organic compounds	ethyl alcohol, product of combustion
	16	T40/2	oxidising gas	chlorine
	17	T40/1	oxidising gas	fluorine
	18	TA/2	organic compounds	ethyl alcohol

Source: Electronic nose brochure and YE (2013) as the source for this table

doi: 10.17221/126/2016-CJFS

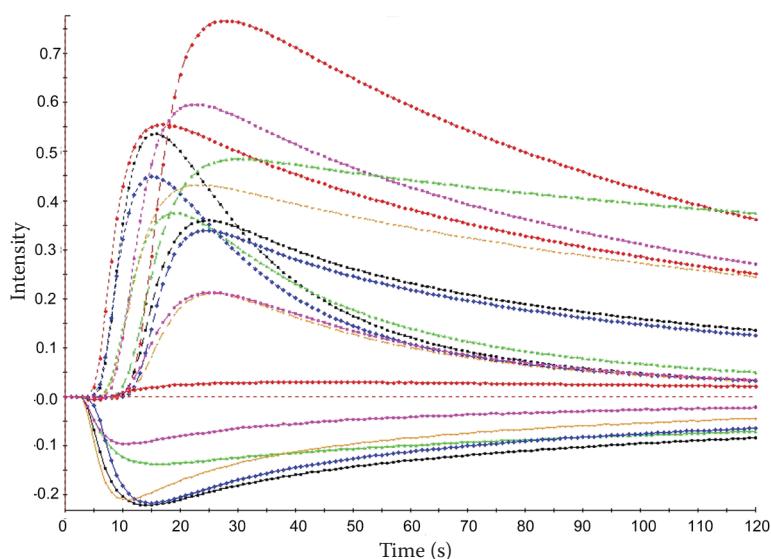


Figure 2. Typical sensing signals of 18 sensors during the measurement of *A. mongholicus*

the sensors to obtain stable values. Each sample was measured three times repeatedly and independently. The response values of the 18 sensors corresponding to each sample were recorded, and response curves were generated. After the measurement, the clean phase was activated to clean the test chamber and allow the sensors to return to their baseline values; this phase lasted for 400 seconds. In the previous work, sample volume, incubation temperature, time of sampling, injection time, and temperature were optimised to generate reproducible responses. Each sample was measured in triplicate, and the average of the final results was used for a subsequent statistical analysis (Cosio *et al.* 2007).

Chemical analysis. To verify the discrimination performed with E-nose, a Waters Acquity™ UHPLC-PDA detector (Waters, USA) was used to determine the main markers. An evaporative light scattering detector (ELSD) was mainly used for detection of non-ultraviolet-absorbing compounds. An AB 135-S electronic analytical balance (Mettler, Switzerland), classic UVF pure water meter (ELGA, UK), and KQ-400KDE ultrasonic cleaner (Kunshan, China) were applied to pre-process the samples. Six flavonoids including campanulin, calycosin, kaempferol, formononetin, ononin, and quercetin were determined by UHPLC-PDA. *A. mongholicus* samples were crushed, and the powder was poured into a No. 4 screen ($250 \pm 9.9 \mu\text{m}$). The screen was shaken, and 2 g of the powder was accurately weighed to dissolve with 25 ml of a methanol solution. The solution was placed in an ultrasonic extractor and sonicated for 40 minutes. Then the solution was filtered with a $0.22 \mu\text{m}$ filter membrane for a subsequent analysis (Lv *et al.* 2015).

A Waters Acquity™ UHPLC column for BEH C18 ($100 \text{ mm} \times 2.1 \text{ mm}$, $1.7 \mu\text{m}$), water (containing 0.1% formic acid) as mobile phase A, and acetonitrile-isopropyl alcohol (7 : 3) (containing 0.1% formic acid) as mobile phase B were used. The gradient elution procedure was as follows: 0~1 min, 10~20% B; 1~6 min, 20~25% B; 6~8 min, 25~30% B; 8~10 min, 30~40% B; 10~12 min, 40~100% B; 12~13 min, 100~10% B; 13~14 min, 10% B. The PDA parameter was set between 190–400 nm, detection wavelength was set at 254 nm. The injection volume was $2 \mu\text{l}$, and the column temperature was 30°C . The sample was injected into the channel at a flow rate of 0.25 ml/minute.

Astragaloside IV was determined through UHPLC-ELSD. *A. mongholicus* samples were crushed and filtered with No. 4 screens, and 2 g of the powder was accurately weighed to dissolve with 25 ml of a methanol solution. The solution was placed in an ultrasonic extractor and sonicated for 60 minutes. Samples of the supernatant were removed with a glass volumetric pipette and diluted to 25 ml in a glass volumetric flask with methyl alcohol. Then the solution was filtered with a $0.22 \mu\text{m}$ filter membrane for subsequent analysis. Separation was performed on a Waters Acquity™ UHPLC column for BEH C18 ($100 \text{ mm} \times 2.1 \text{ mm}$, $1.7 \mu\text{m}$) in an isocratic elution mode, using acetonitrile-water (8 : 2) as the mobile phase at a flow rate of 0.25 ml/minutes. The injection volume was $2 \mu\text{l}$, and the column temperature was maintained at 30°C . The ELSD parameters were temperature of 45°C and N_2 pressure of 25 Psi.

APS was determined using the phenol-sulfuric acid method. *A. mongholicus* samples were crushed and filtered with No. 4 screens. The powders were ac-

curately weighed (1 g) to dissolve with a petroleum ether solution and refluxed at 70°C for 1 hour. Then the extract was refluxed with 75% ethanol (500 ml) three times for 1 h each time. The combined filtrates were concentrated and ethanol was added at a concentration of 80%. Crude polysaccharides (0.05 g) were obtained through stewing and drying, dissolved in water, and diluted with distilled water in a volumetric flask (500 ml). The prepared sample solution (1 ml) was added to another volumetric flask (2 ml) and diluted with ultrapure water. The absorbance values were determined at 490 nm wavelength.

Statistical processing. Principal component analysis (PCA) and discriminant function analysis (DFA) provided a clear visualisation of differences between the different regions and allowed these regions to be classified independently of their response values and contents. In each dataset, the digital odour print of the samples was acquired, and each odour print was obtained by recording the percentage ratio, $\Delta R/R_0$, versus time (s). The class assignment was done by finding the model that best fits the unknown specimen at a specified statistical significance (BICCIATO *et al.* 2003; TAN *et al.* 2015). DFA is an algorithm used to build models and identify the category of unknown samples. This algorithm can narrow down the difference between similar sets of data as much as possible, and expands the difference between different groups as much as possible to establish a

data recognition model via mathematical transformation. The difference between DFA and PCA is that DFA reorganises collected information according to known information to make the results consistent with the known information as much as possible (HAN *et al.* 2010). Experimental data were analysed by IBM SPSS Statistics 20.0. PCA was used to extract the integrated indexes of the odour response for discrimination analysis with the content.

RESULTS AND DISCUSSION

Radar charts and fingerprint response values of *A. mongholicus* samples from different producing areas. The radar chart and response value map for nine samples of *A. mongholicus* from the three main producing areas are shown in Figures 3 and 4, respectively. The arrays reveal highly effective operations for the discrimination of the samples. The samples possessed consistency and differences in their response value. Samples from the same province are consistent, and samples from different provinces are different (Figure 3). Province as observation, the response trajectories of *A. mongholicus* samples from Inner Mongolia and Shanxi are similar. The response values also document that the samples from Inner Mongolia are consistent with those from Shanxi, and exhibit an obvious difference in response values when

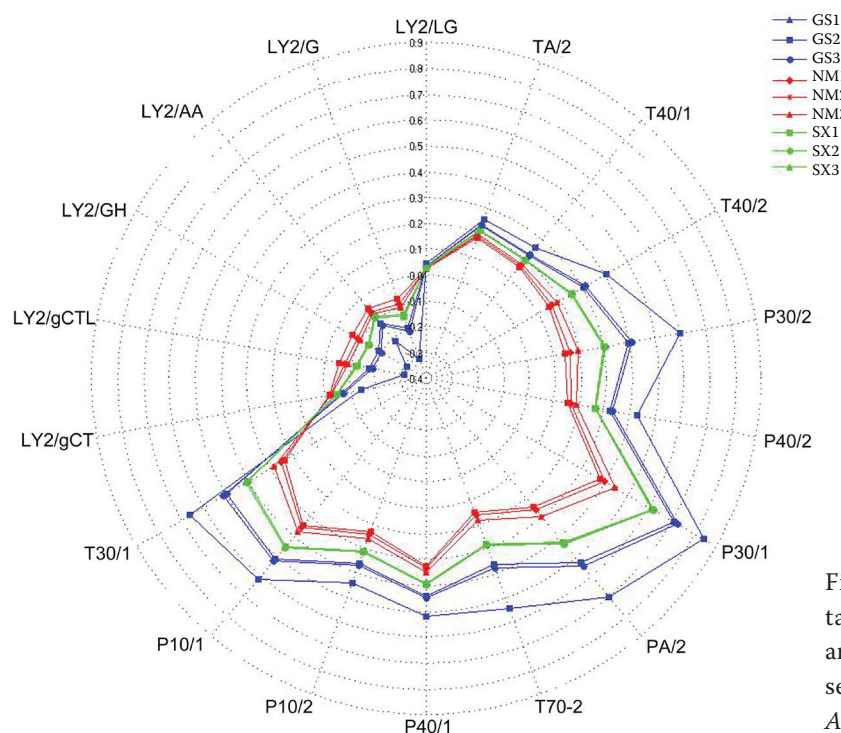


Figure 3. Radar plots of samples obtained from different main producing areas; blue, green, and red lines represent Gansu, Shanxi, and Inner Mongolia *A. mongholicus*, respectively.

doi: 10.17221/126/2016-CJFS

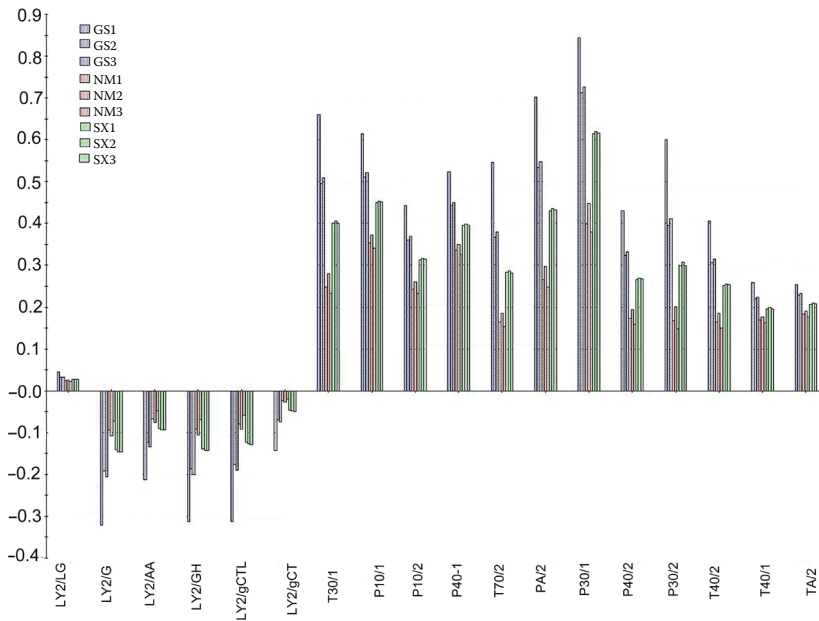


Figure 4. Response values of 18 sensors of *A. mongholicus* obtained from different main producing areas

compared with samples from Gansu. While the two groups showed a significant discrepancy. The odour intensity of *A. mongholicus* samples from the three different producing areas differs (Figure 4). There existed a distinct discrepancy in the sensor response between the various producing areas.

Classification of producing areas using PCA and DFA. The *A. mongholicus* samples were classified into three parts according to the producing area by PCA (Figure 5). The maximum response values of the sensors were extracted and analysed individually, and the samples from different producing areas were clearly distinguished with the total variance of 94%. The first principal component (PC1) and the second principal component (PC2) were used as the axes to analyse the *A. mongholicus* samples. The PC1 value, which is the most important factor in the discrimi-

nation of clusters, was 98.71%. The second principal component was 1.24%. Each point represented one sample, and the distance between two points represented the degree of differences in characteristics between the samples. The result expounded that the samples from the same region were divided into uniform parts, and the samples from different producing areas were completely separated. It is indicated that the E-nose can be used to distinguish *A. mongholicus* from different regions effectively.

DFA is a statistical analysis that projects a categorical dependent variable by one or more continuous or binary independent variables. The DFA of *A. mongholicus* samples is shown in Figure 6. The *A. mongholicus* samples were grouped into three regions, and the contribution rates of DF1 and DF2 were 89.81 and 2.10%, respectively. The result documented that the

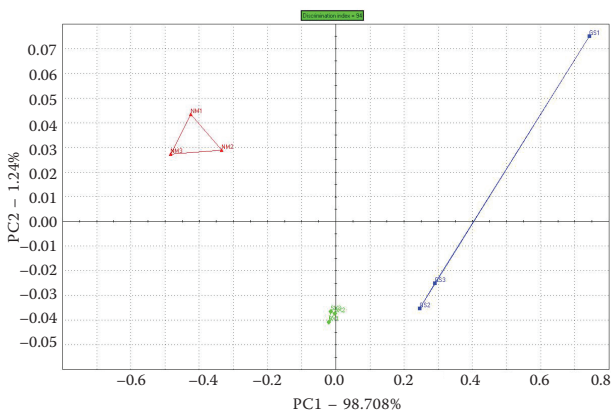


Figure 5. PCA recognition pattern analysis performed with the E-nose data of *A. mongholicus* samples obtained from main producing areas

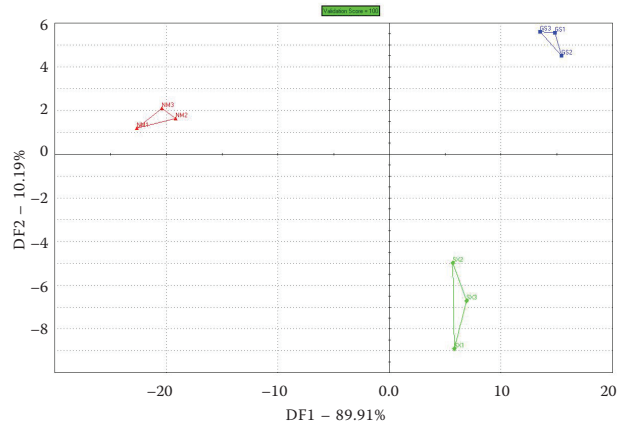


Figure 6. DFA recognition pattern analysis of *A. mongholicus* samples obtained from main producing areas

Table 3. The content of chemical constituents in *A. mongholicus* ($\mu\text{g/g}$, $n = 3$)

No.	AstragalosideIV	Campanulin	Ononin	Quercetin	Calycosin	Kaempferol	Formononetin	APS (%)
GS1	1132	1266.4	833.5	841.3	804.7	516.3	704.2	14.531
GS2	1139	1089.9	819.1	799.6	797.6	517.1	719.6	15.02
GS3	1126	1078.1	823.5	856.2	800.8	510.4	709.3	15.281
NM1	1198	1537.2	963.2	1100.9	437.2	628.6	488.1	19.390
NM2	1189	1488.6	971.9	1049.8	457.6	634.1	473.5	18.903
NM3	1153	1476.2	967.2	1053.2	432.7	653.7	470.3	17.909
SX1	1199	1603.9	1065.1	978.5	407.2	711.9	361.1	20.899
SX2	1173	1579.2	1102.5	969.2	413.1	703.5	353.4	21.020
SX3	1186	1533.1	1037.6	973.1	411.9	701.2	355.1	22.061

E-nose analysis can be applied to the classification of *A. mongholicus* samples from main producing areas, which is consistent with the result of PCA.

Content of the chemical components of *A. mongholicus*. Flavonoids and polysaccharides were the main effective compounds in *A. mongholicus*. Studies have shown that astragaloside IV and campanulin are active compounds with good pharmacological effects and they are indicators in Chinese Pharmacopoeia (ZHANG *et al.* 2011). Therefore, the contents of astragaloside IV, campanulin, calycosin, kaempferol, formononetin, ononin, quercetin, and APS were determined for the quality evaluation of *A. mongholicus*, and the methodology has been established by our team in a previous study (FU *et al.* 2014). The contents of chemical constituents in *A. mongholicus* are listed in Table 3.

Based on the contents of the eight chemical components of *A. mongholicus*, PCA was performed as shown in Figure 7. It was shown that the results of chemical analysis are consistent with those of the

E-nose analysis. The samples from Mongolia and Shanxi are gathered in the right part of the figure, and those from Gansu are gathered in the left part. With regard to the contents of the seven compounds and APS of the *A. mongholicus* samples, the results showed that the quality of samples from Inner Mongolia and Shanxi is similar, and better than that of the Gansu samples. The quality of samples from Inner Mongolia is the best. *A. mongholicus* from disparate producing areas exhibited quality discrepancy, which is consistent with the E-nose analysis. Quality is closely related to pharmacodynamic activities and could reflect the efficacy of *A. mongholicus*. Therefore, quality evaluation and daodi research on *A. mongholicus* are necessary.

CONCLUSION

A method involving innovative E-nose and UHPLC combined with chemometrics was developed to evaluate the quality and geotherbism of *A. mongholicus* originated from top-geotherb and non-top geotherb regions. The samples from different producing areas exhibited a quality difference and were successfully divided into three parts based on the detection of odour which is difficult for the human olfactory system to recognise. Furthermore, the content of seven constituents was determined by UHPLC, and the results are consistent with those of odour detection by the E-nose. Although UHPLC-UV or ELSD is a mature technology, its sensitivity efficiency and accuracy still need to be improved for the quality evaluation of *A. mongholicus*. In this study, the E-nose performed well in distinguishing and qualifying the samples of *A. mongholicus*. With regard to measurement complexity, detection cost, and result accuracy,

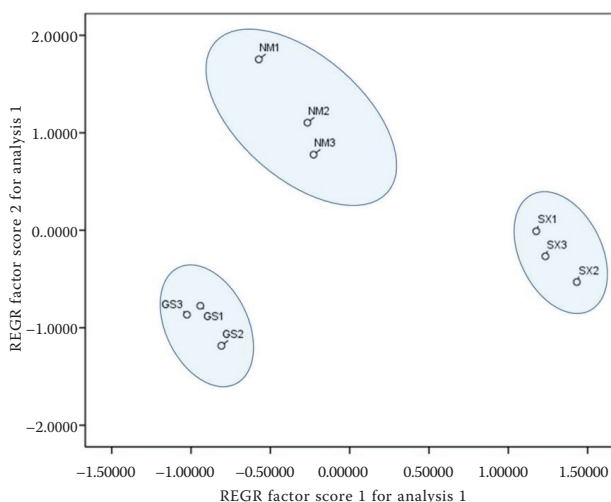


Figure 7. Principal component analysis of *A. mongholicus* samples based on the content of chemical constituents

doi: 10.17221/126/2016-CJFS

the E-nose is a potentially viable method for distinguishing *A. mongholicus* from different main producing areas. This method also presents other advantages including fast response, easy operation, and non-destructiveness. The results imply that Chinese medicinal herbs grown in different producing areas present a distinct quality difference, and further studies should focus on variation and diversity for quality evaluation and daodi research.

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Received: 2016–04–06

Accepted after corrections: 2017–01–19