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Electron Ionization Mass Spectrometry-based Metabolomics Studies of *Sophora flavescens* can Identify the Geographical Origin of Root Samples

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An electron ionization mass spectrometry (EI-MS)-based metabolomic approach was applied to *Sophora flavescens* to identify the geographical origin of each sample. The score plot from principal component analysis using the EI-MS data showed that Japanese *S. flavescens* samples tended to cluster away from Chinese *S. flavescens* samples. Statistical techniques showed that ions arising from kurarinol and kushenol H, which we previously identified as marker molecules for Japanese *S. flavescens,* were characteristic of Japanese *S. flavescens*. Therefore, metabolomics based on EI-MS data is a valuable tool for confirming the geographical origins of *S. flavescens* samples. The results suggest that EI-MS-based metabolomics is suitable for the quality control of traditional medicines containing many components.

Keywords: EI-MS based metabolomics, *Sophora flavescens*, Kurarinol and Kushenol H.

The oriental natural drug called "kujin" in Japanese (SOPHORAE RADIX) comprises the dry roots of *Sophora flavescens* Aiton (Leguminosae) grown in Japan, China and Korea. *Kujin* is used as a Chinese traditional medicine to treat gastric disorders, and for its antifebrile, anodyne, and anthelmintic activities [1]. Alkaloids, pterocarpans, and flavonoids have been isolated from *kujin* [2]. In past, Japanese and Chinese *S. flavescens* were given different scientific names each other. Japanese plant was given *S. angustifolia*, and Chinese plant was given *S. flavescens*. Therefore, the constituents of *S. flavescens* might be changed according to growing area.

Metabolic profiling techniques are used to find possible correlations between the metabolic profile of a compound and its biological activity [3, 4]. Loading plot analysis is a useful strategy for the identification of biological compounds. We previously used NMR-based metabolomics techniques and loading plot analysis to demonstrate that the components of *S. flavescens* differ with their location of growth. For example, kurarinol and kushenol H are characteristic compounds in Japanese *kujin* [5].

Slight differences in the components of several traditional medicines grown in different areas can be detected using metabolic profiling techniques. The biological and pharmaceutical effects of natural drugs depend on their components, and differences in the components affect the activities of the traditional medicine; consequently, controlling the components of natural drugs is of paramount importance for both the supplier and the consumer. Although several components are specified as markers for the quality control of natural drugs, using two or three marker compounds is insufficient to guarantee the quality of the drug. Furthermore, these markers are not always biologically active compounds contributing to the efficacy of the drug. Metabolomics can assess all analytical targets and therefore metabolic profiling is suitable for the quality management of multi-component products such as traditional medicines.

Mass spectrometry has gained importance in metabolomics analyses due to its unparalleled sensitivity and specificity, high resolution, and wide dynamic range. Electron ionization mass spectrometry (EI-MS) is a commonly used analytical instrument, and its widespread availability makes it suitable for the quality control of natural drugs by manufacturers.

We here evaluated differences in the components of *S. flavescens* roots grown in different geographical locations using metabolic analysis coupled with EI-MS (Table 1). In this case to utilize an advantage of mtabolomics, we did not particularly focus on matrine alkaloids which are maker compounds for quality control of *S. flavescens*.

Sample extracts were prepared as previously described [5]. Briefly, twenty batches of *S. flavescens* roots (eleven grown in China, eight in Japan and one Korea) were individually extracted with methanol under reflux. The extract was partitioned with diethyl ether/water, and the diethyl ether layer was evaporated *in vacuo*. DMSO was added to provide a residue concentration of 5 mg/ml and the samples were analyzed by EI-MS.

Table 1: Twenty batches of roots of *S. flavescens*.

No.	Sample name	Growing area	Harvest time (Month. Year)	Latitude	Longitude
1	Hangzhou-1	China	10.2010	30.25	120.17
2	Hangzhou-2	China	3.2011	30.25	120.17
3	Hangzhou-3	China	3.2008	30.25	120.17
4	Hangzhou-4	China	9.2009	30.25	120.17
5	Beijing-1	China	10.2010	39.91	116.39
6	Beijing-2	China	10.2010	39.91	116.39
7	Beijing-3	China	3.2010	39.91	116.39
8	Shanghai-1	China	10.2010	31.17	121.48
9	Shanghai-2	China	10.2010	31.17	121.48
10	Shanghai-3	China	3.2011	31.17	121.48
11	*Kujin*	China	-----	38.03	114.52
12	Yamanashi	Japan	3.2008	35.65	138.57
13	Kagoshima	Japan	10.2010	31.60	130.57
14	Tokushima	Japan	10.2010	34.07	134.55
15	Shiga	Japan	10.2010	35.02	135.85
16	Kanagawa	Japan	3.2011	35.43	139.63
17	Chiba	Japan	7.2011	35.60	140.12
18	Saitama-Ogose	Japan	7.2012	35.95	139.28
19	Saitama-Moroyama	Japan	7.2012	35.95	139.28
20	Seoul	Korea	8.2011	37.57	126.98

A microsyringe was used to load 1 μL of each extract solution onto glass targets, then the glass targets were loaded into a direct-insertion (DI) sample probe (MS-DIP25, JEOL, Tokyo, Japan) and the EI-MS were measured. Each sample was measured independently three times. Integration analysis between approximately *m/z* 50-500 of the total ion chromatogram was used to classify the geographical origin of each sample.

The observed ions were statistically analyzed by *t*-tests; those providing a *p*-value below 0.05 were considered to be characteristic of Japanese, Chinese and Korean *S. flavescens*. Many intense, characteristic ions were detected between *m/z* 440-500 and these were used for principal component analysis (PCA). The PCA score plot of the EI-MS data for *S. flavescens* is shown in Figure 1.

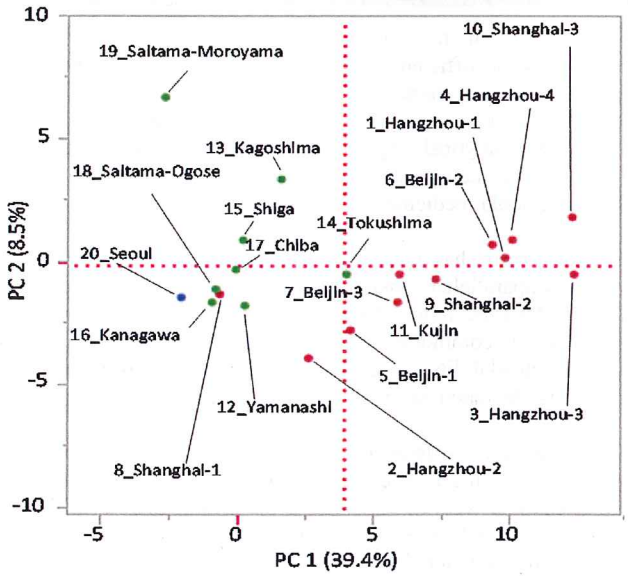

Figure 1: PCA score plot based on EI-MS data of *S. flavescens*.

A score plot represents the distribution of samples in multivariate space. The PCA score plots showed that Japanese *S. flavescens* localized in a different area of the plot from Chinese *S. flavescens*, whereas Korean S. *flavescens* localized near Japanese S. *flavescens* samples. The features contributing to the separation of the two groups were identified by calculating the *F*-value of each ion; the *F*-value of each ion with an *F*-value above 10 is shown in Figure 2.

White bars indicate ions derived from Japanese *S. flavescens* and black bars indicate ions derived from Chinese *S. flavescens*. The *m/z* 440 and *m/z* 454 ions belonging to Japanese *S. flavescens* were chosen as the primary and secondary ions, respectively; whereas the *m/z* 471 and 481 ions were attributed to being characteristic of Chinese *S. flavescens*. We then considered the relationship between the components and the geographical origin of the samples and found that the representative latitude and longitude of harvest (Table 1) correlated with the components' of *S. flavescens*. In particular, the signals at *m/z* 499 and *m/z* 454 correlated significantly with latitude and longitude, respectively.

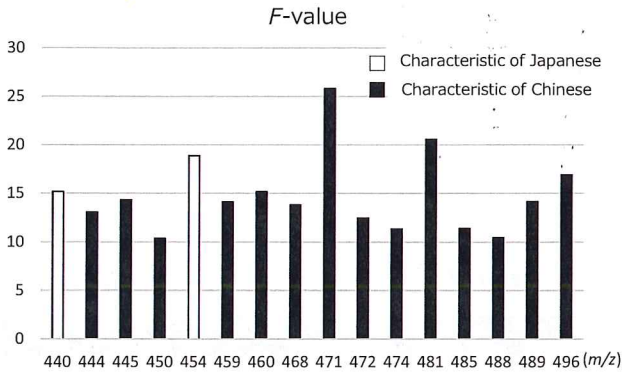

Figure 2: *F*-value of each ion observed on EI-MS of *S. flavescens (F≥10)*.

Our previous metabolomics-based research on *S. flavescens* using ^{1}H NMR assigned kurarinol and kushenol H as characteristic compounds of Japanese *S. flavescens* [5]. We here measured the EI-MS of kurarinol and kushenol H to confirm the *m/z* 440 and *m/z* 454 ions. EI-MS of kurarinol provided *m/z* 440 as the highest molecular ion and kushenol H provided *m/z* 454 as the highest molecular ion. These results indicated that *m/z* 440 and *m/z* 454, identified as characteristic ions based on EI-MS metabolomics profiling, might be attributed to two compounds characteristic of Japanese *S. flavescens*, kurarinol and kushenol H, respectively (Figure 3). Furthermore, *m/z* 454 was also correlated with longitude.

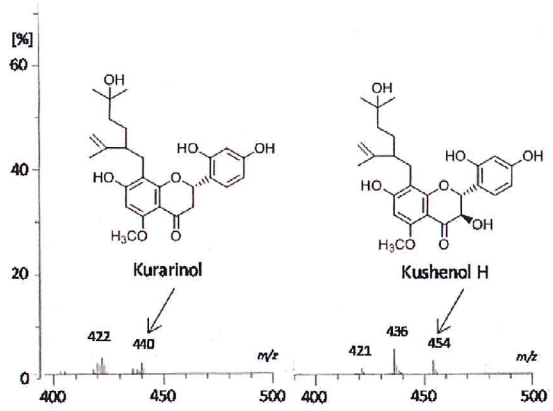

Figure 3: EI-MS of kurarinol and kushenol H isolated from *S. flavescens*.

Ions generally fragment during EI-MS, making it difficult to assign particular signals to specific compounds in multi-component systems such as traditional medicines. However, the particular signals assigned here from EI-MS analyses may be useful in the quality control of traditional medicines, and the observation that particular signals correlate with the latitude and longitude of harvest is useful for distinguishing the origins of medical plants growing in the same country.

EI-MS analysis provides less structural information compared to NMR analysis, but EI-MS methodologies are widely used and there is a substantial database. Most importantly, EI-MS analysis is highly sensitive, allowing detection of low concentration components that would be undetectable by NMR. We therefore believe that metabolomics profiling using EI-MS is useful for the quality control of traditional medicines.

Experimental

Sample collection: Nineteen batches of *S. flavescens* roots were collected from different regions (Table 1). All voucher specimens identified by Professor Yoshiaki Shirataki were deposited in the laboratory of Pharmacognosy & Natural Medicines, Faculty of Pharmaceutical Science, Josai University, Japan. Sample No. 11 (SOPHORAE RADIX, *Kujin*) was purchased from UCHIDA WAKANYAKU Ltd. (Tokyo, Japan).

Preparation of* S. flavescens *root extracts for EI-MS analysis: Each of the 20 batches of dried *S. flavescens* roots was extracted in methanol for 3 h under reflux. The methanolic extract was evaporated *in vacuo* and the residue dissolved in water. The aqueous soluble fraction was partitioned with diethyl ether. After evaporation of the diethyl ether, DMSO was added to provide a residue concentration of 5 mg/ml. The samples were analyzed using electron ionization-mass spectroscopy (EI-MS).

EI-MS measurements: EI-MS measurements were made using a double-focusing mass spectrometer (JMS GC-mate II; JEOL, Tokyo, Japan) fitted with a heated direct-insertion sample probe. The MS detector parameters were: interface temperature, 320 °C; ion-source temperature, 280 °C; ionization mode, EI; ionization voltage, 70 eV; scan speed, 0.3 sec/scan; inter delay, 0.2 sec; scan range, *m/z* 50-500; accelerating voltage, 2500 V; ionization current (emission), 0.3 mA.

EI-MS data reduction procedures, statistical analysis, and pattern recognition analysis: The resulting EI-MS data sets were imported into JMP10 software (SAS Institute Inc., Cary, NC) for multivariate statistical analysis and univariate analysis. The range *m/z*. 50-500 ion from each sample was used for statistical analysis. The *m/z* of each peak was represented as an integral value, rounding down the decimal place. Variables were standardized with a mean of 0 and standard deviation of 1. Principal component analysis (PCA), an unsupervised pattern recognition method, was conducted to confirm any relationship between the analyzed variables from *S. flavescens*.

All statistical analyses were carried out using JMP10 to identify the feature contributing to group separation. Independent *t*-test and analysis of variance (ANOVA) analyses were used to determine if there were significant differences in the EI-MS marker ion levels between Japanese and Chinese *S. flavescens*. Significance was defined at $p<0.05$

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