Metabolic Discrimination of Safflower Petals of Various Origins Using ^{1}H NMR Spectroscopy and Multivariate Statistical Analysis

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The metabolic discrimination of safflowers from various geographical origins was performed using ^{1}H nuclear magnetic resonance (NMR) spectroscopy followed by principal components analysis. With a combination of these techniques, safflower samples from different origins could be discriminated using the first two principal components (PC) of the ^{1}H NMR spectra of the 50% methanol fractions. PC1 and PC2 accounted cumulatively for 91.3% of the variation in all variables. The major peaks in the ^{1}H NMR spectra that contributed to the discrimination were assigned to fatty acid (terminal CH_3), lactic acid, acetic acid, choline derivatives, glycine, and safflower yellow derivatives. In this study, we suggest that various types of safflower can be discriminated using PCA and ^{1}H NMR spectra.

Key Words : Safflowers, Metabolic discrimination, ^{1}H NMR, Principal components analysis

Introduction

Petals of safflowers (*Carthamus tinctorius* L.) are used as a traditional medicine in Korea and China. Composition of safflower petals is associated with thrombolytic, anti-inflammatory, and antioxidative effects.[1-3] At present, petals of safflowers from various countries including China, Japan and Korea are sold commercially for use in traditional medicines in Korea. The yellow and red pigments present within safflower petals have been reported to be carthamin, safflower yellow B, and safflomin A.[4,5] Analyses of the chemical components other than pigments have revealed the presence of organic acids, phytosterols, free sugars, fatty acids, minerals, and polyphenolic compounds.[6,7] Recently, intraspecific variation among Chinese safflowers was analyzed by examing the expression of AFLP (amplified fragment length polymorphic) markers.[8] However, discriminating among petals of safflowers from different origins based on chemical composition has not been achieved to date. The term 'metabolome' has been used to describe the observable chemical profile or fingerprint of the metabolites present within whole tissues.[9] To produce a metabolic profile, it is preferable to use a broad spectrum of chemical analytical techniques that are rapid, reproducible, and stable over time; ideally, these techniques should not require complex methods of sample preparation. Nuclear magnetic resonance (NMR) is a technique that meets the aforementioned criteria. Although the development of NMR has been driven mainly towards the gathering of qualitative information that is related to the general elucidation of structure, the quantitative aspects of NMR have been recognized since NMR was first developed.[10] Moreover, several techniques have been developed in the past decade to use NMR spectroscopy as a fingerprinting tool to assess the quality of crude plant materials. Multivariate or pattern recognition techniques such as principal components analysis (PCA) are important tools that are used to analyze data obtained using NMR. Recently, NMR used in combination with PCA was applied to generate metabolomic profiles for several types of plants and traditional phytomedicines.[11-16] In this study, we used ^{1}H NMR spectroscopy and PCA to discriminate among various types of safflower petal using metabolomic profiling and thereby elucidated the major compounds that contribute to discrimination.

Experiments

Safflower petal samples. A 200 g of safflower petal samples (2-year-old *Carthamus tinctorius* L.) were collected in China (Yunnan), Korea (Sanchung-gun), and Japan (Yamagata), respectively, during late summer in 2005. The petals of the safflowers were analyzed in the present study, and samples of the petals of safflowers from each of the aforementioned regions were deposited as voucher specimens at the herbarium at Chung-Ang University. To avoid the individual variability of safflower samples, a 100 g of safflower petal samples, respectively, were ground and homogenized in liquid nitrogen using a mortar and pestle before being stored at 80 °C until analyzed.

Solvent and chemicals. First-grade methanol and D_2O (99.9%) were purchased from Sigma (St. Louis, MO, USA), and methanol *d*4 (99.8%) and NaOD were purchased from Cambridge Isotope Laboratories (Miami, FL, USA) and Cortec (Paris, France), respectively. Standard safflower yellow compound was purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan).

Extraction and NMR measurements. For metabolomic comparison of safflower samples with different origins, 50 mg of material was transformed to a 2 mL microtube. Then, 1.5 mL of a mixture of KH_2PO_4 buffer (90 mM, pH 6.0) in D_2O containing 0.05% trimethyl silane propionic acid sodium salt (TSP) and methanol *d*4 (1:1) was added to the safflower samples. The mixture was vortexed for 30 s and sonicated for 1 min. The materials were then centrifuged at 13,000 rpm

for 20 min. The supernatant was taken for NMR analysis. Each experiment was performed in six times replication.

All spectra were obtained by a NMR spectrometer (Avance 600 FT-NMR, Bruker, Germany) operating at a proton NMR frequency of 600.13 MHz. For each sample, 128 scans were recorded with the following parameters: 0.155 Hz/point, pulse width of 4.0 μs (30°), and relaxation delay of 1.0 s. Free induction decays were Fourier transformed with LB = 0.3 Hz. The spectra were referenced to trimethyl silane propionic acid sodium salt (TSP) at 0.00 ppm. TSP (0.01%, w/v) was used as internal standards for 50% methanol *d*4. 2D NMR experiments using ^{1}H-^{1}H correlation spectroscopy (COSY), and heteronuclear single quantum coherence (HSQC) were performed using XWIN-NMR software (Bruker, Germany).

Data analysis. The ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Biospin, Bruker). Spectral intensities were scaled to TSP for 50% methanol extracts. The spectral region δ = 0.52-10.00 was segmented into regions of 0.04 ppm width giving a total of 237 integrated regions per NMR spectrum. The region from 3.30 to 3.40 and 4.70 to 5.00 were excluded from the analysis because of the residual signal of methanol and water in aqueous extracts, respectively. The remaining regions were normalized to the whole spectrum for PCA. All spectral data were mean centered with no scaling, then analyzed by PCA based on the covariance matrix. PCA was performed with SIMCA-P software (Umetrics, Umeå, Sweden). The statistical significances of the mean values of lactic acid, choline derivatives, and safflower yellow derivatives were tested by Tukey's multiple t-test of one-way ANOVA using SPSS 12.0 software (SPSS Inc., Chicago, USA).

Results and Discussion

The representative ^{1}H NMR spectra of the 50% methanol extracts are presented in Figure 1. The signals within the aromatic (δ = 6.0–9.0) and aliphatic (δ = 0.5-3.6) regions were smaller than those within the sugar regions (δ = 3.6–6.0). The signals associated with the main aromatic compounds within the extracts were assigned to safflower yellow derivatives (C-glycosylquinochalcone) at δ = 6.90 (*d*, *J* = 8.4 Hz) and δ = 7.54 (*d*, *J* = 7.2 Hz), δ = 7.49 (*d*, *J* = 15.6 Hz) (Fig. 1) by comparison with those of standard safflower yellow compound from TCI (Tokyo Kasei Kogyo Co., Ltd). The following signals were assigned based on comparisons with the chemical shifts of standard compounds and 2D-NMR using ^{1}H-^{1}H COSY (correlation spectroscopy) and HSQC (heteronuclear single quantum coherence): fatty acids (terminal CH_3) at δ = 0.94 (*t*, *J* = 6.0 Hz); valine δ = 1.09 (*d*, *J* = 7.2 Hz); lactic acid at δ = 1.30 (*d*, *J* = 6.8 Hz); alanine at δ = 1.48 (*d*, *J* = 7.2 Hz); acetic acid δ = 2.02 (*s*), choline derivatives at δ = 3.26 (*s*), glycine at δ = 3.54 (*s*), β-glucose δ = 4.58 (*d*, *J* = 7.2 Hz); α-glucose δ = 5.18 (*d*, *J* = 4.2 Hz); and fumaric acid at δ = 6.54 (*s*).

To ensure the objective interpretation of the results, the samples were analyzed using PCA. As shown in Figure 2, samples from different types of safflowers could be distinguished clearly, and the first two principal components (PCs) accounted cumulatively for 91.3% of the total variance. In the score plot of the combination of PC1 and PC2 (Figure 2), samples of safflower petals from Korea were separated from samples from China and Japan. Samples from Korea were separated from those of China and Japan mainly by PC1 (which explained the 75.2% of the total variance), which meant that the samples from China and Japan exhibited a closer metabolomic profile compared to

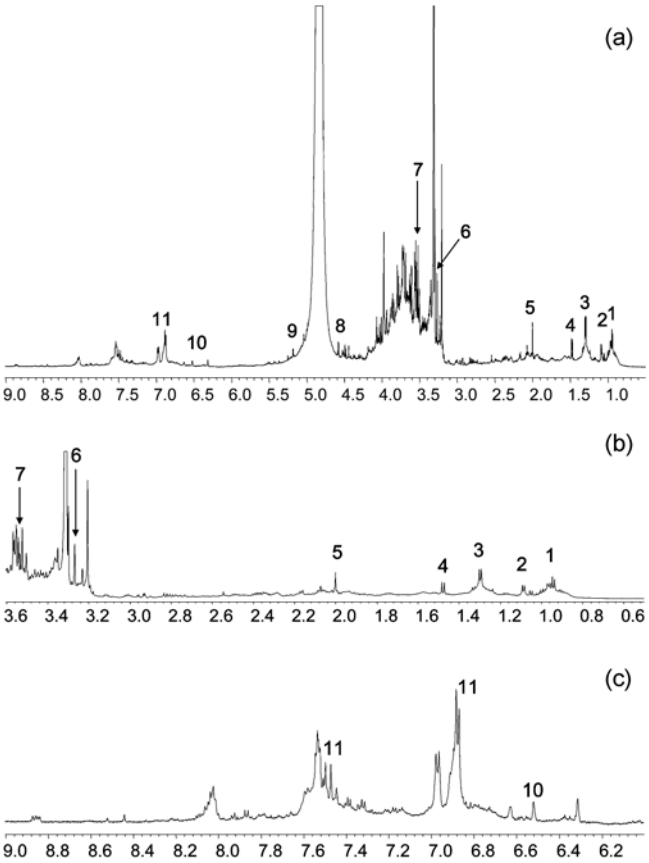

Figure 1. Representative ^{1}H NMR spectra of the total (a), aliphatic (b), and aromatic (c) regions of the 50% methanol fraction of a safflower sample. IS: internal standard, w: residual water, 1: fatty acid (terminal CH_3), 2: valine, 3: lactic acid, 4: alanine, 5: acetic acid, 6: choline derivative, 7: glycine, 8: β-glucose, 9: α-glucose, 10: fumaric acid, 11: safflower yellow derivatives.

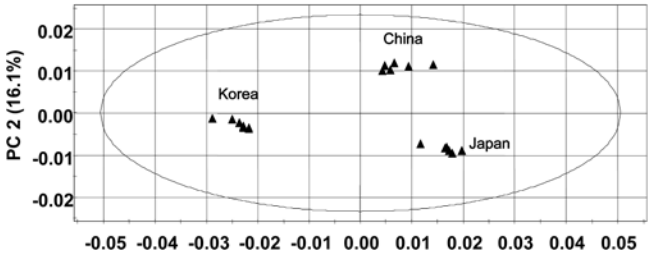

Figure 2. PCA-derived score plots for the 50% methanol extracts of various safflower petal samples generated by combining PC1 and PC2. The ellipses represent the Hotelling's T2 with 95% confidence.

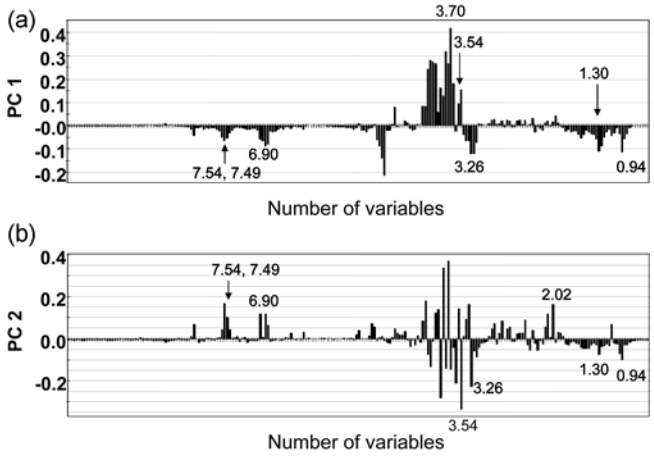

Figure 3. PCA-derived loading plots for the 50% methanol extracts for PC1 (a) and PC2 (b). The number of variables refers to the chemical shifts in 0.04-ppm bins for the range 0.52 to 10.00 ppm.

the samples from Korea. Separation between samples from China and Japan was achieved by PC2 (which explained the 16.1% of the total variance).

The compounds that contributed to discrimination were clearly discriminable in the loading plots of PC1 and PC2 (Figure 3), and the score and loading plots complemented each other. The position of an object in a given direction within a score plot is influenced by variables that lie in the same direction within the loading plot. The major compounds that contributed to discrimination by PC 1 were fatty acid (terminal CH_3), lactic acid, choline derivatives, glycine, and safflower yellow derivatives. Fatty acid (terminal CH_3), lactic acid, acetic acid, choline derivatives, glycine, and safflower yellow derivatives contributed to the discrimination by PC2. The levels of safflower yellow derivatives (δ = 6.90) levels were higher in the samples from China and Korea than in the samples from Japan. In addition, the results of our PCA analysis also suggested that lactic acid (δ = 1.34) and choline derivatives (δ = 3.26) levels were higher in Korean samples than the other samples, and those were clearly summarized in Table 1.

PCA is an unsupervised clustering method that does not require any knowledge of the data set and reduces the dimensionality of multivariate data while preserving most of the variance therein.[17] We used the covariance method of PCA in the present study, because this method produced a better separation than the correlation method (data not shown). The Safflower petal samples were collected from a multitude of fields at different days and 100 g of petals were pooled together for further analysis to obtain the representative data of each sample and to avoid the intra region variability. Therefore it can be claimed that the results in this research are clearly ensure that there are regional differences in each metabolome of safflower samples from different region not just arbitrary fluctuations of each samples. In sugar region, there were major peaks compared to the aliphatic (0.5-3.6 ppm) or aromatic regions. The sugar region (3.6-6.0 ppm) mainly contributed to the discrimination between samples of Korea, China and Japan, although each peaks could not be assigned. On the whole, the sugar levels in samples of China and Japan were higher than those of samples from Korea. In addition, majority of compounds assigned, such as fatty acids, lactic acid, acetic acid, choline derivatives, glycine, and safflower yellow derivatives were dominated in Korean samples. It was assumed that the characteristic compositions of safflower petals were influenced by the light, temperature, watering, and soil conditions of the growing sites of safflower plants.

Safflower yellow derivatives are water-soluble yellow pigments that are present within safflower petals; these pigments include safflomin A, safflomin C, carthamin precursor, safflower yellow B,[4,5] tinctromine,[18] and cartomin.[19] The aforementioned pigments have a unique C-glycosylquinochalcone structure that has not been detected in other products that occur naturally. It was assumed that the characteristic peaks as shown in Figure 1(c) were derived from unique C-glycosylquinochalcone structure, though there are many kinds of water soluble safflower yellow derivatives including various tautomers. Therefore, relative comparison of contents of safflower yellow derivatives was possible as shown in Table 1.

This study has demonstrated that petals of safflowers can be discriminated according to their geographic origin by using PCA to analyze the ^{1}H NMR spectra of nonvolatile metabolites. Although some minor metabolites were not included in this approach, this study illustrates the potential of using NMR to obtain metabolic profiles of safflower petals. In addition, the method used in the present study can be used for nonvolatile metabolic fingerprinting of other plants that are used as medicines. In future, we will investigate the bioactivities associate with different metabolic profiles of safflower petals.

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Table 1. The mean values and standard deviations of lactic acid, choline derivatives and safflower yellow derivatives for each petal samples from China, Korea and Japan. *The mean differences are statistically significant ($p < 0.05$).

Compounds	Samples		
	China	Korea	Japan
lactic acid	0.0043 ± 0.0002	*0.0065 ± 0.0002	0.0043 ± 0.0001
choline derivatives	0.0112 ± 0.0005	*0.0159 ± 0.0004	0.0112 ± 0.0001
safflower yellow derivatives	0.0077 ± 0.0002	0.0090 ± 0.0004	*0.0046 ± 0.0001

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