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## <sup>1</sup>H NMR-based metabolomic study of *Cornus officinalis* from different geographical origin

Youngae Jung<sup>1</sup>, Young-Sang Jung<sup>1</sup>, Geum-Sook Hwang<sup>1,2,\*</sup>

<sup>1</sup>Seoul Center, Korea Basic Science Institute, Seoul 136-713, Republic of Korea,

<sup>2</sup>Graduate School of Analytical Science and Technology,  
Chungnam University, Daejeon 305-764, Republic of Korea

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**Abstract:** *Cornus officinalis* (Cornaceae) is primarily grown in Asian countries. The pericarp of *C. officinalis* (Corni Fructus) is a well-known traditional medicine with tonic, analgesic, and diuretic properties. We analyzed methanolic extracts of Corni Fructus (grown in Korea and China) by <sup>1</sup>H NMR spectroscopy. Metabolite profiling was performed to characterize the metabolic difference between different Corni Fructus origins (Korea or China). Principal components analysis revealed significant separation between Corni Fructus from different origins. The metabolites responsible for differences were identified using loading plots, coefficients plots, and variable influence on projection followed by *t*-tests. As a result, 16 metabolites were identified and quantified; tyrosine, acetate, sucrose, and malate differed the most between origins. These data suggest that NMR-based metabolomics can be used to identify differences between Corni Fructus samples obtained from different regions.

**Keywords:** Metabolomics; <sup>1</sup>H NMR; Multivariate analysis; *Cornus officinalis*; Geographical origin

### INTRODUCTION

*Cornus officinalis* typically grows as a large, spreading, multistemmed, deciduous shrub 38–64 cm tall in Korea, China, and Japan. *C. officinalis* belongs to the Cornaceae family. The pericarp of *C. officinalis* (Corni Fructus) is a well-known traditional medicine with tonic, analgesic, and diuretic

\* To whom correspondence should be addressed. E-mail : gshwang@kbsi.re.kr

properties<sup>1</sup>. Corni Fructus has been reported to possess cognitive-enhancing, antidiabetic, anti-oxidative, and advanced glycation end products (AGE)-mediated renal injury protective effects<sup>2,3</sup>, as well as therapeutic effects on diabetes<sup>4</sup>, cancer<sup>5</sup>, and shock<sup>6</sup>. Moreover, *C. officinalis* seeds have antihyperglycemic effects<sup>1</sup>. The effects of *C. officinalis* have been well studied, but a metabolomics approach had not been applied to characterize the *C. officinalis* metabolite profile.

Metabolomics is important in plant sciences and natural products chemistry<sup>7-12</sup>. Among the several techniques used for metabolomics, nuclear magnetic resonance (NMR) spectroscopy has many advantages for plant metabolomics. For example, sample preparation is simple; one can simultaneously detect diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids) as well as primary metabolites (sugars, organic acids, amino acids); signals are proportional to their concentration, which allows a direct comparison of metabolite concentrations; and it can be used for structure elucidation<sup>13</sup>. After data collection, multivariate analysis is required because metabolomic analysis generates a large number of variables and multivariate data sets that are difficult to analyze.

In this study, one-dimensional proton (<sup>1</sup>H) NMR spectroscopy was used to investigate the difference between *C. officinalis* from two different regions (Korea and China). We performed principal components analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), which clearly distinguished *C. officinalis* from two different regions based on the metabolite profiles. The metabolites that primarily contributed to origin discrimination were identified based on loading plots, coefficient plots, and variable influence on projection (VIP), followed by *t*-tests to evaluate the

statistical significance of metabolite concentration differences between the regions.

## EXPERIMENTAL

### Plant material

We obtained 40 *C. officinalis* pericarp (Corni Fructus) samples from Korea (20) and China (20). All samples used in this study were dried after Corni Fructus seeds were removed. Samples were ground into powder using liquid nitrogen. All powder samples were stored at  $-80^{\circ}\text{C}$  until used for NMR analysis.

### Extraction and NMR analysis

Methanol (500  $\mu\text{L}$ ,  $d_4$ ), 400  $\mu\text{L}$  of 0.2 M (pH 7) sodium phosphate buffer, and 100  $\mu\text{L}$  of 0.5 mM DSS in  $\text{D}_2\text{O}$  were added to 100 mg of dried powder, vortexed for 1 min, and sonicated for 20 min at  $40^{\circ}\text{C}$ . The mixture was adjusted to pH 7 using 0.1 M HCl and 0.1 M NaOH, and centrifuged at  $16609 \times g$  for 10 min. The supernatants (600  $\mu\text{L}$ ) were transferred to 5-mm NMR tubes.  $\text{D}_2\text{O}$  and DSS provided a field frequency lock and a chemical shift reference ( $^1\text{H}$ ,  $\delta 0.00$ ), respectively.

$^1\text{H}$  NMR spectra were acquired on a VNMRs 600-MHz NMR using a triple resonance HCN salt-tolerant cold probe (Agilent Technologies Inc., Santa Clara, CA). For the polar fraction, a NOESYPRESAT pulse sequence was applied to suppress the residual water signal. For each sample, 32 transients were collected into 67,568 data points using a spectral width of 8445.9 Hz with a

relaxation delay of 2.0 s, an acquisition time of 4.00 s, and a mixing time of 100 ms. A 0.5-Hz line-broadening function was applied to all spectra prior to Fourier transformation. Assignments of NMR signals were based on the Chenomx NMR Suite 7.1 (Chenomx Inc., Edmonton, AB, Canada) database, spiking experiments, and two-dimensional (2D) NMR analyses.

### **NMR data analysis**

All NMR spectra were phased and baseline-corrected using the Chenomx NMR suite version 7.1. Regions corresponding to water, methanol, and DSS (4.79–4.97 ppm, 3.28–3.33 ppm, 0.0–0.7 ppm, respectively) were excluded, and the remaining spectral regions were divided into 0.005 ppm bins. The spectra were then normalized to the total spectral area and converted to ASCII format. The ASCII format files were imported into MATLAB (R2006a; Mathworks, Inc., Natick, MA), and all spectra were aligned using the correlation optimized warping method<sup>14</sup>.

The NMR data sets were imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for chemometric analyses. All imported data were mean-centered for multivariate analysis. PCA was used to examine intrinsic variation in the data set and obtain an overview of variation among groups. The quality of the models was determined by  $R^2$  and  $Q^2$  values.  $R^2$  is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit.  $Q^2$  is defined as the proportion of variance in the data predictable by the model and indicates predictability<sup>15</sup>.

Metabolites from NMR analysis were identified using a Chenomx Profiler, a module of Chenomx NMR Suite version 7.1. All standard NMR spectra used for metabolite identification are commercially available (Chenomx Inc.). The metabolites were also quantified using the Chenomx NMR suite 7.1 library software, which uses the concentration of a known reference signal (in this case, DSS) to determine the concentration of individual compounds.

A PLS-DA was applied to the relative concentration data of assigned metabolites. To identify which metabolites contributed most to clustering or trends in the data, coefficient plots and the VIP parameters were examined.

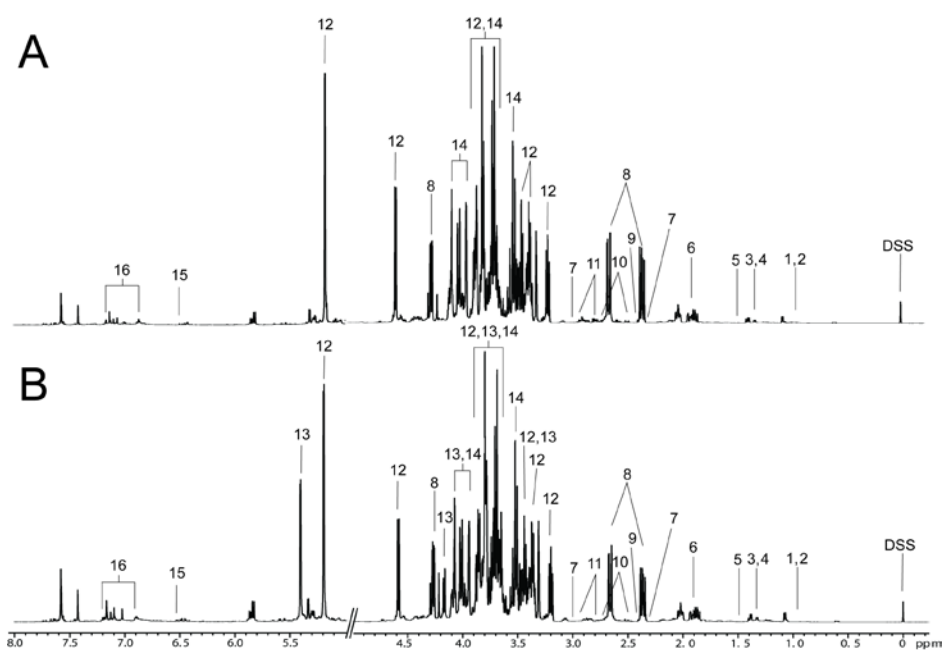
A two-tailed *t*-test, the Mann–Whitney test (non-Gaussian distribution), and the Wilcoxon signed-rank test (non-Gaussian distribution) were performed using GraphPad PRISM version 5.0 (GraphPad Software, Inc., La Jolla, CA) to evaluate significance differences in metabolite levels between samples from different origins.

## RESULTS AND DISCUSSION

### <sup>1</sup>H NMR spectroscopy and multivariate statistical analysis

Representative <sup>1</sup>H NMR spectra of Korean and Chinese *Cornus Fructus* samples are shown in Figure 1. The vertical scale of the aromatic region is tripled to improve visibility since signals in the aromatic region ( $\delta$ 6.0–8.0) were smaller than those in the aliphatic and sugar regions. No clear

differences were observed in the overall spectroscopic fingerprints between Corni Fructus samples obtained from the two origins. However, close inspection revealed that the chemical composition of metabolites in the aliphatic ( $\delta$ 1.0–3.0) and aromatic region ( $\delta$ 6.0–8.0) were different between Corni Fructus samples from different countries. A clear difference was observed between the sucrose peaks of the sugar region ( $\delta$ 3.0–6.0) between the two groups.



**Figure 1.** Representative <sup>1</sup>H NMR spectra of Corni Fructus extracts obtained from Korea (A) and China (B). The vertical scale of the aromatic region is tripled to improve visibility. Peaks: 1, leucine; 2, valine; 3, lactate; 4, threonine; 5, alanine; 6, acetate; 7, 4-aminobutyrate; 8, malate; 9, succinate; 10, citrate; 11, asparagine; 12, glucose; 13, sucrose; 14, fructose; 15, fumarate; 16, tyrosine.

In aqueous extracts, the majority of metabolites were amino acids, organic acids, and sugars. Sixteen metabolites were identified in the <sup>1</sup>H NMR spectra of Corni Fructus extracts (Table 1). These metabolites were assigned based on comparison with the chemical shifts of standard compounds using Chenomx NMR Suite 7.1 software. Assignment of the ambiguous metabolites was determined through spiking experiments and 2D NMR analyses.

**Table 1.** Identification and quantification of metabolites from <sup>1</sup>H NMR spectra of Corni Fructus samples grown in Korea and China

metabolite <sup>a</sup>	chemical shift (multiplicity) <sup>b</sup>	Concentration, mean±S.E.M. (μM)		<i>p</i> value <sup>c</sup>	VIP rank
		Korea	China		
4-aminobutyrate	1.89(m), 2.28(t), 3.00(t)	115.9±12.16	129.1±15.23	0.5005	15
acetate	1.90(s)	1762±115.7	700.9±51.25	<0.0001†	2
alanine	1.47(d), 3.77(q)	62.37±14.35	11.24±2.283	<0.0001†	11
asparagine	2.85(dd), 2.94(dd), 3.99(dd)	3427±695.8	887.1±150.8	<0.0001†	9
citrate	2.51(d), 2.69(d)	914.3±72.49	534.3±42.93	0.0001†	7
fructose	3.50–3.57(m), 3.63–3.73(m), 3.77–3.88(m), 3.93–4.10(m)	78007±1582	64184±2215	<0.0001	6
fumarate	6.53(s)	24.65±3.739	39.96±1.896	0.0036†	13
glucose	3.20(dd), 3.33–3.90(m), 4.59(d), 5.19(d)	67748±2216	57088±1860	0.0007	12
lactate	1.31(d), 4.03(q)	110.5±15.09	123.6±4.255	0.0009†	14
leucine	0.94(d), 0.96(d), 1.74(m), 3.73(m)	52.35±4.956	16.91±1.273	<0.0001†	4

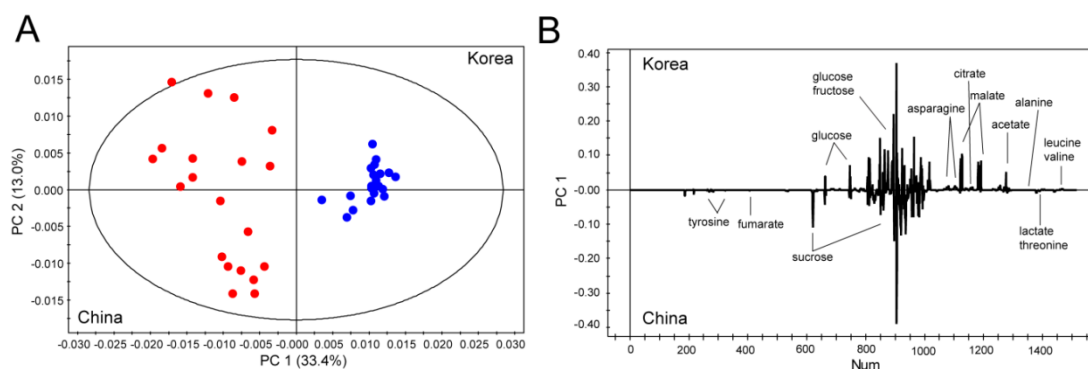
malate	2.36(dd), 2.66(dd), 4.27(dd)	69291±3216	37236±1248	<0.0001†	3
succinate	2.40(s)	123.4±11.08	97.63±16.18	0.1975	16
sucrose	3.43(t), 3.51(dd), 3.65(s), 3.75(t), 3.76–3.87(m), 4.02(t), 4.17(d), 5.40(d)	0	9160±1747	0.0002‡	5
threonine	1.32(d), 3.58(d), 4.22(m)	320.7±24.07	448.4±19.76	0.0002	10
tyrosine	3.06(dd), 3.20(dd), 3.95(dd), 6.90(d), 7.19(d)	119.2±12.24	347.4±17.98	<0.0001	1
valine	1.00(d), 1.04(d), 2.28(m), 3.62(d)	43.71±7.716	11.89±1.232	<0.0001†	8

<sup>a</sup>Identification and quantification of metabolites was performed using Chenomx. <sup>b</sup>Letters in parentheses denote the peak multiplicities: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; q, quartet; and m, multiplet. <sup>c</sup>*P* values are the results of *t*-tests; †, Mann–Whitney *U* test; ‡, Wilcoxon signed-rank test

PCA is an unsupervised clustering method requiring no *a priori* knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving most of the variance<sup>16</sup>. A PCA score plot was used to determine whether the Corni Fructus metabolic fingerprints were sufficiently unique to identify metabolic markers specific for the different origins. The PCA score plot derived from the NMR spectra of Corni Fructus extracts from the two countries are given in Figure 2A. The plot showed clear separation according to the first component (PC 1). Chinese samples were more widely distributed than Korean samples, possibly because Chinese samples were collected from a broader region.  $R_x^2$  represents the goodness of fit to the PCA model, and  $Q^2$  reveals the predictability of the PCA model. The PCA model revealed  $R_x^2$  and  $Q^2$  values of 0.762 and 0.419, respectively. The



loading plots allow for identification of the spectral regions with the greatest influence on separation and clustering of the samples, and allows for deduction of the compounds responsible for this clustering (markers). Observations lying in the same direction in a score plot are influenced by variables with the same behavior in the loading plot (Figure 2B). The upper and lower sections of the loading plot show metabolites that had higher and lower concentrations, respectively, in Korean Corni Fructus samples. Acetate, alanine, asparagine, citrate, fructose, glucose, leucine, malate, and valine were more abundant in Korean Corni Fructus samples, while fumarate, lactate, sucrose, threonine, and tyrosine were more abundant in Chinese samples. These metabolites were responsible for the observed separation between Korean and Chinese Corni Fructus samples.

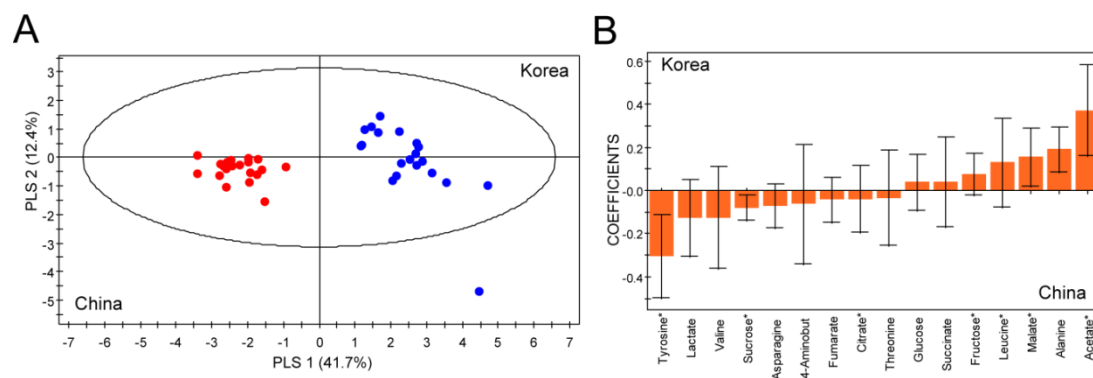


**Figure 2.** PCA score plot (A) and complementary loading plot of the first component (B) derived from <sup>1</sup>H NMR spectra of Corni Fructus extracts obtained from Korea (blue circles) and China (red circles):  $R_x^2 = 0.711$  and  $Q^2 = 0.419$ . The ellipse represents the 95% confidence interval for Hotelling's  $T^2$ .

### Targeted metabolite profiling

A targeted profile of metabolites was created to expand our understanding of the Corni Fructus metabolic pattern. Metabolic concentrations were determined using the Chenomx NMR Suite 7.1 library, which compares the integral of a known reference signal (DSS) with signals derived from a library of compounds containing chemical shifts and peak multiplicities for all resonances of a compound. Individual metabolite levels in the Corni Fructus extracts are shown in Table 1.

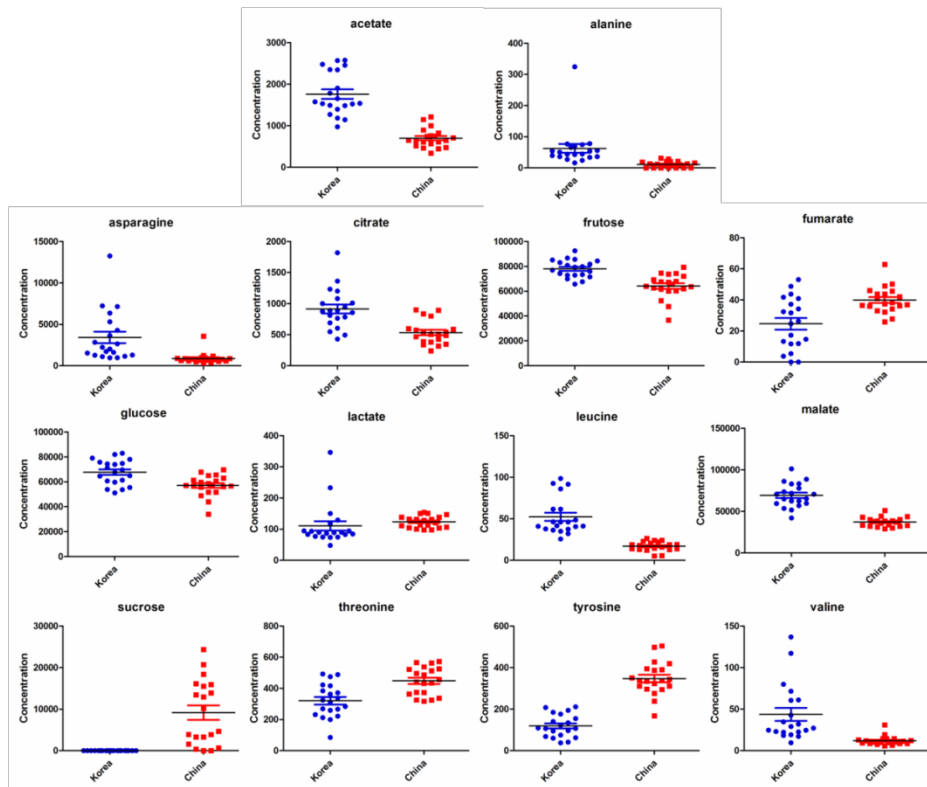
To minimize the effects of unknown materials and noise on the multivariate analysis, PLS-DA was conducted with the metabolic profiling data. The score plot showed clear separation according to the first component (PLS 1) (Figure 3A):  $R_X^2 = 0.711$ ,  $R_Y^2 = 0.954$ , and  $Q^2 = 0.904$ . One outlier, caused by unusually high levels of alanine, asparagine, and citrate, was identified in the Korean samples. We applied a combination of statistical approaches to further evaluate differences in metabolite concentrations between the groups. We selected biomarkers using *t*-tests, as well as jackknifing of coefficients and VIP values<sup>17,18</sup>.



**Figure 3.** PLS-DA score plot (A) and coefficients plot with jackknifed confidence intervals (B) derived from metabolite concentrations obtained by targeted profiling of *Corni Fructus* extracts from Korea (blue circles) and China (red circles). The asterisk (\*) indicates which metabolites contribute significantly to the PLS-models (VIP of >1).

PLS regression coefficients of the variables showed the extent that each X-variable contributed to Y. Precision was derived from the 95% confidence interval using jackknifing. Results are considered statistically significant if the error bars in the figures do not cross the 0 line<sup>19</sup>. A PLS-DA regression coefficient plot was generated to identify metabolites responsible for differentiation of the score plot (Figure 3B), which showed that acetate, alanine, and malate were positively correlated, while sucrose and tyrosine were negatively correlated. In particular, since acetate and tyrosine had the highest correlations (over |0.02|), these metabolites are the most important in differentiating between the two groups. Among the metabolites shown in the PLS-DA coefficient plot, seven metabolites (tyrosine, acetate, malate, leucine, fructose, sucrose, and citrate) showed high VIP values of >1

(Figure 3B). In addition, 14 metabolites (excluding 4-aminobutyrate and succinate) had statistically significant differences of  $p < 0.05$  (Table 1 and Figure 4). 4-Aminobutyrate and succinate had the lowest VIP values (Table 1).



**Figure 4.** Quantification of the identified metabolites with significant differences ( $p < 0.05$ ) between extracts of Corni Fructus grown in different regions. Error bars indicate the mean  $\pm$  standard error. Each point represents a single Corni Fructus sample.

In summary, a complementary approach using both non-targeted and targeted metabolite profiling was applied to distinguish between Corni Fructus samples obtained from Korea and China, and to identify the metabolites responsible for clustering. Through various statistical approaches (i.e., *t*-tests, jackknifing of coefficients, and VIP values), we found that acetate, malate, sucrose, and tyrosine are responsible for discrimination between the Corni Fructus samples. Our data suggest that <sup>1</sup>H NMR-based metabolomics fingerprinting combined with multivariate statistical analysis is a useful tool to determine the origin of Corni Fructus extracts.

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