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Distinguishing *Glycyrrhiza* species using NMR-based Metabolomics

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Glycyrrhiza species are widely used as natural medicines and as well as food additives. Quality control during methods development is therefore critically important. In this study, we conducted NMR-based metabolomics analyses to distinguish *Glycyrrhiza* species (*G. glabra*, *G. uralensis*, and *G. inflata*). Principle component analysis (PCA) of ^{1}H-NMR spectra of extracts of *Glycyrrhiza* species enabled categorization of the three species. We conducted solid-phase extraction of methanol extracts of *Glycyrrhiza* roots prior to NMR measurements. Most studies of *Glycyrrhiza* species have used mass spectra to distinguish species. By conducting solid-phase extraction prior to NMR analysis, we could distinguish species clearly by PCA score plots. These results indicate that solid-phase extraction enables clear discrimination of *Glycyrrhiza* species in NMR metabolomics analyses. Furthermore, saponin fractions prepared by solid-phase extraction from water extracts were also discriminated according to the species using NMR metabolomics. Saponins such as glycyrrhizin are important constituents of *Glycyrrhiza* species. These results thus indicate that there are species-specific differences in root saponins in *Glycyrrhiza*.

Keywords: *Glycyrrhiza* species, NMR metabolomics.

Licorice, the dried root of *Glycyrrhiza* species, is one of the most popular natural medicines worldwide. Licorice is also widely used as a flavoring and sweetening agent. The pharmacologic effects of licorice, including anti-inflammatory, antitussive, and antispasmodic activity, have been well described [1, 2]. In China and Europe, the roots and rhizomes of *G. uralensis*, *G. glabra*, and *G inflata* are used as licorice without discrimination, whereas only the former two species are used in the United States and Japan [3, 4]. The major bioactive secondary metabolites of licorice include saponins, flavonoid glycosides, and various free phenolic compounds [5, 6].

Quality control in licorice production centers on one component, glycyrrhizin, which is an oleanane-type saponin and the most important constituent. However, the biological activities of licorice described above are attributed to a variety of compounds contained in the extract. Therefore, focusing on one specific compound in quality control is not ideal.

Metabolite fingerprinting approaches aided by nuclear magnetic resonance (NMR) spectroscopy provide valuable metabolite signatures for complex plant extracts. The advantage of ^{1}H-NMR spectroscopy over other metabolomics techniques is that the signal intensity depends only on the molar concentration of each compound in the solution, enabling direct comparisons of the concentrations of all compounds present in the sample.

In a previous report, we demonstrated that an NMR-based metabolomics approach enabled classification of *Sophora flavescens* (Leguminosae) into two groups based on cultivation location, Japan or China. Furthermore, loading plot analyses of principal component analysis (PCA) results identified kurarinol and kushenol H as flavonoid compounds characteristic of Japanese *S. flavescens* [7].

In this study, an NMR metabolomics approach was employed to assess the metabolic differences among the most widespread *Glycyrrhiza* species, namely, *G. glabra*, *G. uralensis*, and *G. inflata*. Although metabolic profiles of *Glycrrhiza* species have already been reported [8-10], these studies were primarily conducted using mass spectrometry such as LC-ESI-MS, LC-TOF-MS and GC-MS. Tada *et al*. have also reported MS and NMR aided multivariate analysis were useful to identify original plant of natural food additive products derived from *Glycrrhiza* species [11]. By contrast, in the present study, we used an ^{1}H-NMR–based metabolomics approach to distinguish *Glycyrrhiza* species. In addition we also applied solid phase extraction by ODS cartridge column before ^{1}H-NMR measurements. The research which focused on licorice root to distinguish their spices using NMR metabolomics technique is extremely limited.

Roots and rhizomes of *G. glabra*, *G. inflata*, and *G. uralensis* were extracted with MeOH under reflux to prepare corresponding extracts. These methanol extracts were evaporated and loaded onto an ODS column and eluted with H_2O → 30% aqueous MeOH → 50% aqueous MeOH → 80% aqueous MeOH →MeOH. The 50% aqueous MeOH fraction was dissolved in dimethyl sulfoxide (DMSO)-d_6 at a concentration of 10 mg/mL for ^{1}H-NMR measurement. ^{1}H-NMR spectral data were processed using the ALICE2 program for Metabolome version 5.0 (JEOL). This program enables integration of NMR spectroscopy and multivariate pattern-recognition methods, such as PCA, into a single interface. ^{1}H-NMR spectral measurements of two batch per each species were conducted and totally six acquired ^{1}H-NMR spectra were subjected to PCA. All 342 variables in the bucketed regions (see Experimental Procedures) were equally accounted for in the data sets. PCA models were depicted as score plots and consisted of two synthetic variables: principal component (PC) 1 (the greatest data

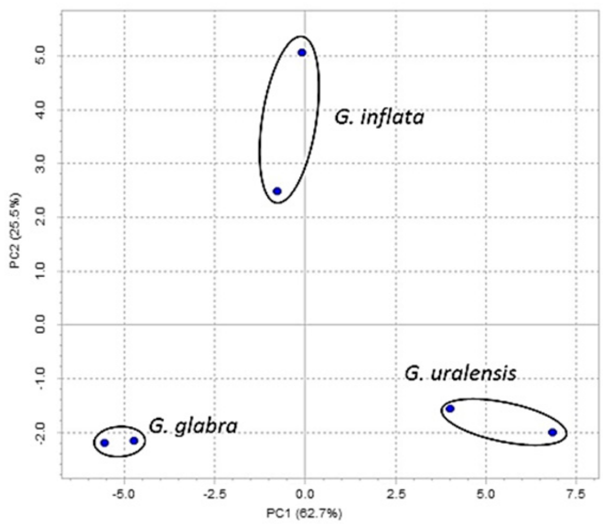

Figure 1: PCA score plot of PC1-PC2, in which 342 variables were equally accounted for in the data sets. PCA score plot of different *Glycyrrhiza* species derived from [1]H-NMR spectral data of 50% aqueous MeOH elute prepared by *Glycyrrhiza* MeOH extract. MeOH extract of *Glycyrrhiza* species was loaded onto ODS cartridge column and eluted with various aqueous methanol. Among of them, 50% aqueous MeOH elute was dried *in vacuo* and resulting residue was dissolved in DMSO-d_6 for [1]H-NMR measurement. [1]H-NMR spectra were procced by ALICE2 for Metabolome to give PCA score plot.

variance) and PC2 (the second greatest data variance, orthogonal with PC1). The PCA score plot of PC1 and PC2 clearly showed three independent groups (Figure 1). The PC1, PC2, PC3 scores and the residue value were 62.7%, 25.5%, 8.4%, and 3.5%, respectively. The values of PC1 and PC2 were used to distinguish the *Glycyrrhiza* species. The score sum of PC1 and PC2 was 88.2%, which was sufficient to identify the *Glycyrrhiza* species. Figure 2 shows a loading plot of the most relevant variables enabling differentiation of the three species at δ 7.02, δ 5.30, and δ 6.74.

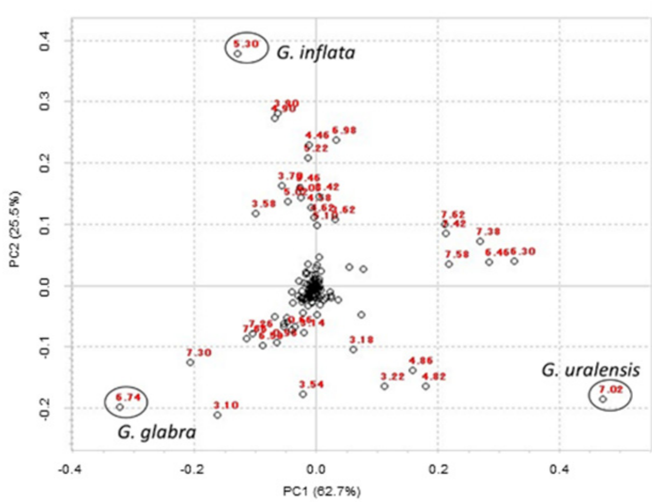

Figure 2: Loading plot derived from the [1]H-NMR spectra of the 50% aqueous methanol elution of a methanol extract of licorice.

In previous LC-MS–aided metabolomics on *Glycyrrhiza* species, glabridin, licochalcone A, and glycycoumarin were identified as characteristic constituents of *G. glabra*, *G. inflata*, and *G. uralensis*, respectively [5, 12]. Thus, we investigated whether these previously reported results would be supported by NMR-based metabolic profiling. Comparisons of [1]H-NMR spectra were conducted using

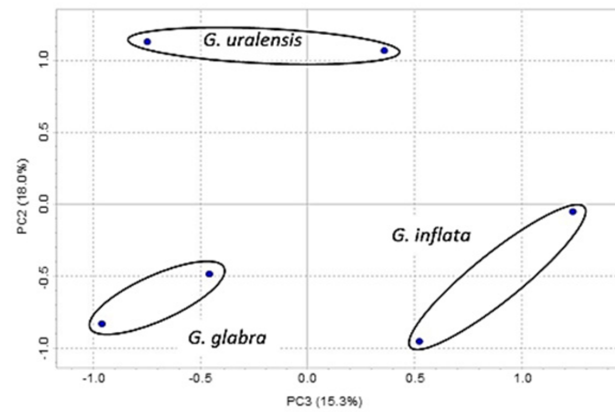

Figure 3: PCA score plot of PC2-PC3, in which 342 variables were equally accounted for in the data sets. PCA score plot of different *Glycyrrhiza* species derived from [1]H-NMR spectral data of 80% aqueous MeOH elute prepared by *Glycyrrhiza* water extract. Water extract of *Glycyrrhiza* species was loaded onto ODS cartridge column and eluted with various aqueous methanol. Among of them, 80% aqueous MeOH elute was dried in vacuo and resulting residue was dissolved in DMSO-d_6 for [1]H NMR measurement. [1]H-NMR spectra were procced by ALICE2 for Metabolome to give PCA score plot.

authentic compounds (glabridin, licochalcone A, and glycycoumarin). Our PCA score plot results did not distinguish the *Glycyrrhiza* species based on these specific compounds. The signal at δ 7.02, which was characteristic of *G. uralensis* in the loading plot of PCA data, could not be assigned to any proton signals of glycycoumarin. The signals at δ 5.30 (characteristic of *G. inflata*) and δ 6.74 (characteristic for *G. glabra*) also could not be assigned to licochalcone A or glabridin, respectively. These results indicate that the characteristic signals derive from other compounds not yet identified as specific to the respective *Glycyrrhiza* species.

We then examined licorice saponin, specifically, the well-known saponin glycyrrhizin. Glycyrrhizin is also used as a marker quality control component in preparation of *Glycyrrhiza* root and rhizome products according to the Japanese Pharmacopoeia [4]. Roots and rhizomes of *G. glabra*, *G. inflata*, and *G. uralensis* were extracted with boiling water to give corresponding extracts. These water extracts were freeze dried, and then the resulting powder was dissolved in water and loaded onto an ODS column eluted with H_2O → 50% aqueous MeOH → 80% aqueous MeOH → MeOH. The 80% aqueous MeOH fraction was dissolved in DMSO-d_6 at a concentration of 10 mg/mL for [1]H-NMR measurement. Our previous investigation revealed that the 80% methanol fraction of the water extract of licorice contains various saponins. We confirmed the presence of saponins in the 80% methanol fraction by HPLC analysis. The [1]H-NMR spectra of the saponin fractions were processed using ALICE2 for Metabolome to give PCA score plots in same manner described above. Although the PCA score plot for PC1 and PC2 did not enable classification according to species, the PCA score plot for PC2 and PC3 showed these saponin extracts of *Glycrrhiza* species located apart from each other according to the species, as shown in Figure 3. The PC2 and PC3 scores were 18.0% and 15.3%, respectively.

In this study, we attempted to distinguish *Glycrrhiza* species using NMR-based metabolomics analysis. Solid-phase extraction of methanol extracts of licorice before NMR measurements enabled discrimination of the species via PCA score plots of the NMR data. Furthermore, NMR-based metabolomics analysis of the saponin fractions prepared from water extracts of licorice enabled us to recognize difference between species. As the saponin glycyrrhizin is a representative component of licorice, these results are extremely interesting. We plan to elucidate the characteristic saponin compounds of these species based on loading plot analysis of PCA data.

Experimental

Sample collection: Roots and rhizomes of *Glycrrhiza* species were purchased from the Ningxia and *Guangzhou markets,* China, in 2015, and identified by Prof. Chen B of the South China Botanical Garden. Furthermore, HPLC analysis were also conducted to confirm their species. The characteristic compounds for each species (glabridin; *G. glabra*, licochalcone A; *G. inflata*, glycycoumarin: *G. uralensis*) were detected.

Preparation of licorice extracts for NMR analysis: Each of 6 batches of dried roots of *Glycrrhiza* species was extracted with methanol for 3 h under reflux. The methanol extracts (150 mg) were loaded onto an ODS cartridge column (5 g, 20 cc, Waters) and separated by eluting with H_2O→ 30% aqueous MeOH → 50% aqueous MeOH → 80% aqueous MeOH → MeOH (each 20 mL). Fractions containing saponin were prepared by water extraction followed by loading onto an ODS column eluted with H_2O → 50% aqueous MeOH → 80% aqueous MeOH → MeOH. The saponin was contained in the 80% aqueous MeOH fraction.

Apparatus and chromatographic conditions: A Waters Alliance HPLC system comprised of a PDA detector coupled with an analytical workstation (Empower 3) was used. A PEGASIL ODS column (Senshu Pak, 5 μm, 150 × 4.6 mm) was used. The sample injection volume was 10 μL, the detection wavelength 254 nm, the flow rate 1.0 mL min^{-1}, and the column temperature was maintained at 40°C. Gradient elution was achieved with solvent A (0.1% formic acid in water, *v/v*) and solvent B (acetonitrile containing 0.1% formic acid, *v/v*). The gradient program was: 5% solvent B for 3 min, 5 to 100% over 15 min, then 100% B for 15 min.

NMR spectroscopy: ^{1}H-NMR spectra were recorded at room temperature on a 400-MHz Agilent 400MR-vnmrs 400 spectrometer (Agilent). Each spectrum consisted of 65,536 complex data points and a spectral width of 6,410.3 Hz, obtained by 16 scans with a repetition time of 5.0 s and a relaxation delay of 1.50 s per scan. The detection pulse flip angle was set at 45°.

NMR data reduction procedures and pattern recognition analysis: Each NMR spectrum was divided into 342 regions, each 0.04 ppm wide, over the range 0.00 to 14.00 ppm. Each segment of the spectral regions (bucket) was integrated. Any integrated regions from 2.36 to 2.52 ppm and 3.24 to 3.40 ppm that contained solvent and water signals were eliminated from the data table, such that the total data were reduced to 342 regions. The remaining integral values for each spectrum were normalized over 100 total summed integrals to compensate for any differences in concentration between licorice extracts. Spectral processing was performed using ALICE2 for Metabolome software, version 5.0 (JEOL).

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