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Metabolomic discrimination of different grades of pine-mushroom (*Tricholoma matsutake* Sing.) using ^{1}H NMR spectrometry and multivariate data analysis

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Abstract

Metabolomic analysis of raw and cooked pine-mushrooms (*Tricholoma matsutake* Sing.) of different grades was performed using ^{1}H nuclear magnetic resonance (NMR) spectrometry and principal component analysis (PCA). PCA of the ^{1}H NMR spectra of aqueous fractions allowed different grades of raw pine-mushroom to be discriminated by a combination of principal component (PC) 1 and PC 2, which accounted cumulatively for 94.1% of the variation in all variables. The major peaks in the ^{1}H NMR spectra that contributed to discrimination of raw mushrooms were assigned to choline, trehalose, threonine, leucine/isoleucine, succinic acid, alanine, and fumaric acid. The combination of PC 1 (70.8%) and PC 3 (7.5%) allowed different grades of cooked pine-mushroom to be discriminated, and the major peaks in the ^{1}H NMR spectra that contributed to discrimination of cooked mushrooms were assigned to succinic acid, trehalose, and fumaric acid. This metabolomic analysis-based method allows different grades of pine-mushroom to be distinguished without any prepurification.

Keywords: Pine-mushroom (*Tricholoma matsutake* Sing.); ^{1}H NMR; Metabolomic analysis; Principal component analysis

1. Introduction

Mushrooms have been used widely since ancient times not only as a source of foods and to flavor food but also for medicinal and functional purposes. The pine-mushroom (*Tricholoma matsutake* Sing.) is the most valuable species of mushroom throughout the world; this mushroom exhibits several useful biological activities in humans, such as lowering cholesterol concentrations, antioxidant effects, immunomodulation, and antitumor effects [1–4]. Pine-mushrooms that are cultivated in the pine forests of the Republic of Korea are particularly highly valued, mainly due to the unique environment and climate of this region. Pine-mushrooms can be classified according to their appearance, and a standard for the classification of pine-mushrooms was developed by the National Forestry Cooperatives Federation of the Republic of Korea [5].

The term "metabolome" has been used to describe the observable chemical profile or fingerprint of the metabolites in whole tissues [6]. To obtain the most complete metabolomic profile, it is necessary to use a wide spectrum of analytical techniques that are rapid, reproducible, and stable over time; ideally, these methods should be compatible with simple sample preparations. Nuclear magnetic resonance (NMR) is one of the techniques that meet the aforementioned requirements. Although the development of NMR has been driven mainly by the need to obtain qualitative information about general structures, the quantitative aspects of NMR have been recognized since NMR was first developed [7]. Over the last decade, several techniques were devised to use NMR spectrometry as a fingerprinting tool to assess the quality of crude plant materials. Multivariate or pattern recognition techniques such as principal component analysis (PCA) are used to analyze data obtained using NMR. Recently, NMR used in combination with PCA has been used to obtain metabolomic profiles of several types of plant and traditional phytomedicines [8–11]. Determining the grade of pine-mushrooms has depended conventionally on visual inspection, which is unreliable. Some

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studies have been carried out to determine the effects of the origin [12], grade [13,14], and thermal processing [15] of pine-mushrooms on which volatile components are present. Cho et al. [13] evaluated pine-mushrooms according to grade using profiling of volatile metabolites, but the non-volatile components that are associated with different grades of pine-mushroom have not been determined.

In this study, we describe a simple and efficient method that can be used to discriminate among different grades of pine-mushroom and to fingerprint the non-volatile metabolites in these mushrooms; this method comprises ^{1}H NMR spectroscopy and multivariate analysis. In addition, the revelation of the major components contributing the discrimination is performed.

2. Materials and methods

2.1. Pine-mushroom samples

Pine-mushroom samples were collected in Korea, during fall, 2004. Pine-mushrooms could be classified according to their appearance. Pine-mushrooms of the first grade are of the highest quality, and are over 8 cm long with an unopened pileus. Pine-mushrooms of the second grade are generally 6–8 cm long, but their widths are irregular and their pilei are not opened. Pine-mushrooms of the third grade are less than 6 cm long or have one-third opened pilei and pine-mushrooms of the fourth grade have completely opened pilei [5]. Pine-mushrooms of four grades were deposited as a voucher specimen in Herbarium of Chung-Ang University. Raw pine-mushrooms were wrapped in low-density-polyethylene-film and stored at −70 °C until used, when they were thawed at 4 °C in a refrigerator for 3 h and then sliced using a cutter (Shinomura, Sanjŏ, Niigata, Japan). The sliced mushrooms were heat-treated at 190 ± 3 °C for 1 min on both sides in a convection broiler (Toastmaster, Boonville, MO, USA). The cooking time was determined by the time when juice was split from pileus of pine-mushrooms. The raw or cooked mushrooms were placed into a stainless steel container, frozen in liquid nitrogen, and then ground in a blender (Hanil Electric, Seoul, Korea).

2.2. Solvent and chemicals

Chloroform, methanol, and D_2O (99.9%) of first grade were purchased from Sigma–Aldrich (St. Louis, MO, USA), and $CDCl_3$ (99.8%) and NaOD were obtained from Cambridge Isotope Laboratories (Miami, FL, USA) and Cortec (Paris, France), respectively.

2.3. Extraction of pine-mushrooms

One gram of ground material was placed into a centrifuge tube. Five milliliters of a 50% water–methanol mixture and 5 ml of chloroform were added to the mushroom sample in the tube, and then vortexed and sonicated for 1 min, respectively. The materials were then centrifuged at 2000 rpm for 20 min. The extraction was performed twice. The aqueous and organic fractions were transferred separately into a 50 ml round-bottomed flask and dried with a rotary vacuum evaporator. Each experiment was performed in triplicate.

2.4. NMR measurements

KH_2PO_4 was added to D_2O as a buffering agent to make up 0.1 M of final concentration. The pH of the D_2O used for NMR measurements was adjusted to 6.0 using a 1N NaOD solution. 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 for aqueous fractions and, for $CHCl_3$ fractions, to residual solvent at 7.26 ppm. Hexamethyl disiloxane (HMDSO, 0.01%, v/v) and TSP (0.01%, w/v) were used as internal standards for $CDCl_3$ and D_2O, respectively. 2D NMR experiments using ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) were performed using XWIN-NMR software (3.5 version, Bruker, Germany).

2.5. 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 HMDSO for $CHCl_3$ extracts and TSP for aqueous extracts. The spectral region $\delta = 0.52$–10.00 was segmented into regions of 0.04 ppm width giving a total of 237 integrated regions per NMR spectrum. The region from 4.60 to 4.90 was excluded from the analysis because of the residual signal of water in aqueous extracts, whereas that from 7.00 to 7.50 was excluded because of the residual signal of $CHCl_3$ in organic fractions. 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).

3. Results and discussion

3.1. Visual inspection of ^{1}H NMR spectra and assignment of components

Pine-mushrooms are used in a variety of culinary dishes, including stews, soups, and steamed dishes. Even though many different methods are used to cook pine-mushrooms, these mushrooms can be also consumed raw, which preserves the original taste and aroma. Therefore, we analyzed the non-volatile components in raw and cooked pine-mushrooms of different grades.

The spectra of the $CHCl_3$ extracts of the different grades of raw pine-mushrooms were not obviously different. In addition, there were no differences in the spectra of the $CHCl_3$ extracts of the different grades of cooked pine-mushrooms also (data not shown). Different grades of pine-mushrooms

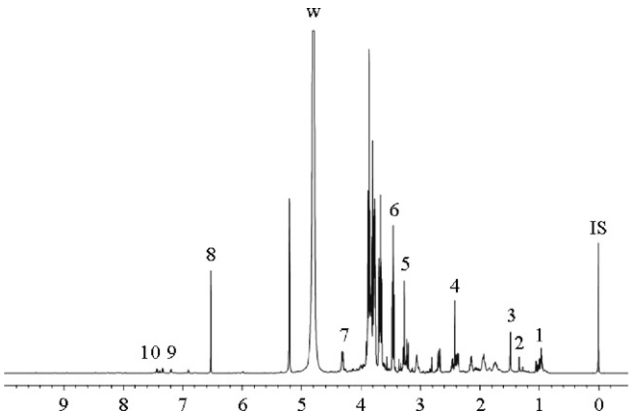

Fig. 1. A representative ^{1}H NMR spectrum of the aqueous fraction of pine-mushroom extract. IS, internal standard; w, residual water; 1, leucine/isoleucine; 2, threonine; 3, alanine; 4, succinic acid; 5, choline; 6, trehalose; 7, fructose; 8, fumaric acid; 9, tyrosine; 10, phenylalanine.

might be developed in different environmental conditions. It is assumed that the composition of water soluble compounds in pine-mushrooms might be changed, while the composition of hydrophobic compounds (compounds in $CHCl_3$ extracts) remained unchained during development into different grades of pine-mushrooms. Therefore, only the aqueous fractions of raw and cooked pine-mushrooms were analyzed further, respectively. Representative ^{1}H NMR spectra of aqueous fractions of pine-mushrooms are presented in Fig. 1. The peaks were identified by comparison with the chemical shifts of standard components and 2D NMR using ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC). In the aliphatic region of the spectra (0–3 ppm), peaks that corresponded to leucine/isoleucine were observed at $\delta = 0.96$ (m), threonine was observed at $\delta = 1.34$ (d, $J = 7.5$ Hz), alanine was at $\delta = 1.46$ (d, $J = 7.3$ Hz), and succinic acid was at $\delta = 2.42$ (s). The midfield region (3–6 ppm) contained choline at $\delta = 3.21$ (s) and trehalose at $\delta = 3.46$ (t, $J = 9.6$ Hz). The peaks in the aromatic region of the spectrum (6–10 ppm) revealed the presence of fumaric acid at $\delta = 6.54$ (s), tyrosine at $\delta = 6.90$ (d, $J = 8.5$ Hz), and phenylalanine at $\delta = 7.42$ (m).

3.2. PCA analysis of raw pine-mushrooms

To ensure the objective interpretation of the results, the samples were analyzed using PCA. 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 [16]. 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).

As shown in Fig. 2, different grades of raw pine-mushroom could be distinguished clearly, and the first two principal components (PC) accounted cumulatively for 94.1% of the total variance. Separation of the different grades of raw pine-mushroom in score plots was achieved by combining PC 1 and PC 2. The combination of PC 1 and PC 2 generated clusters that were separated

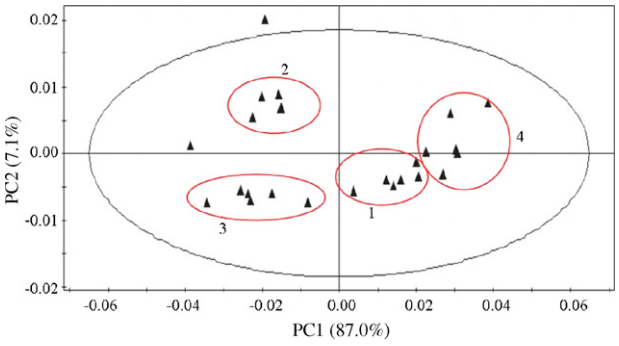

Fig. 2. PCA score plots for aqueous extracts of raw pine-mushrooms of four different grades (1–4) generated using a combination of PC 1 and PC 2.

according to the different grades of mushroom. Second- and third-grade raw pine-mushrooms could be differentiated from first- and fourth-grade mushrooms mainly according to the score of PC 1, while second-grade mushrooms could be differentiated from first- and third-grade mushrooms according to the score of PC 2.

The metabolites that could be used for discrimination were clearly distinguishable in the loading plots for PC 1 and PC 2 (Fig. 3), and the score and loading plots complemented each other. The position of objects in a given dimension in a score plot is influenced by variables that lie in the same dimension in the loading plot. The major components that contributed to the discrimination of different grades of raw pine-mushroom were choline, trehalose, threonine, leucine/isoleucine, succinic acid, alanine, and fumaric acid. Choline, trehalose, threonine, leucine/isoleucine, succinic acid, alanine, and fumaric acid were related to the differentiation by PC 1. The amount of

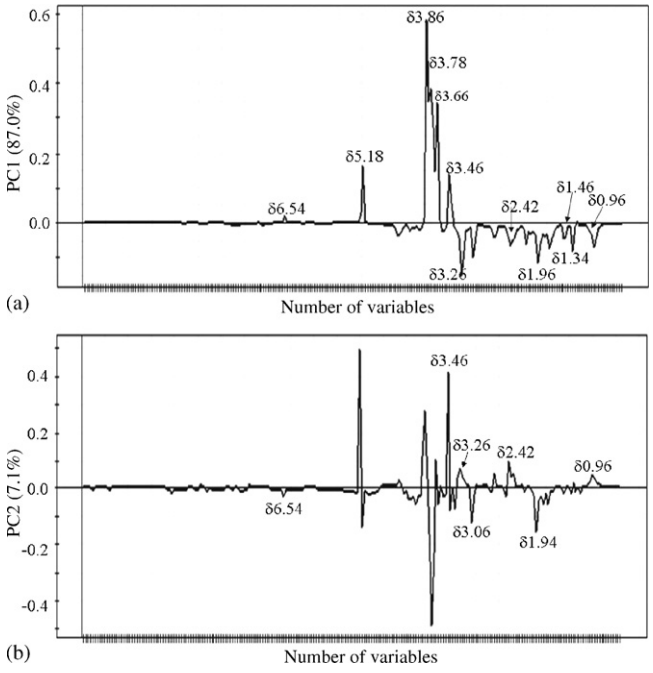

Fig. 3. PCA loading plots for aqueous extracts of raw pine-mushrooms associated with PC 1 (a) and PC 2 (b). Number of variables refers to the chemical shifts binned at intervals of 0.04 ppm from 0.52 to 10.00 ppm.

trehalose and fumaric acid was greatest in fourth-grade pine-mushrooms, whereas the reverse was true for choline, threonine, leucine/isoleucine, succinic acid, and alanine (Fig. 3a). On the other hand, trehalose, choline, succinic acid, leucine/isoleucine, and fumaric acid contributed to the discrimination by PC 2. The second-grade raw pine-mushrooms contained much greater amounts of trehalose, choline, succinic acid, and leucine/isoleucine than the other grades (Fig. 3b). In third-grade pine-mushrooms, threonine and alanine were the dominant components, while fumaric acid was associated with first-grade mushrooms. Based on the aforementioned observations, we concluded that choline, trehalose, threonine, leucine/isoleucine, succinic acid, alanine, fumaric acid are the components that allow for the discrimination of different grades of raw pine-mushroom.

3.3. PCA analysis of cooked pine-mushrooms

The different grades of cooked pine-mushroom could be distinguished using PCA, and PC 1–3 accounted cumulatively for 93.1% of the total variance. The major separation among the different grades of cooked pine-mushroom in score plots was achieved by combining PC 1 with PC 2, PC 1 with PC 3, and PC 2 with PC 3 (Fig. 4). In the score plot generated by combining PC 1 and PC 2, the fourth-grade cooked pine-mushrooms were separated from the other grades, and the first-, second-, and third-grade cooked pine-mushrooms exhibited similar metabolic patterns (Fig. 4a). The third- and fourth-grade mushrooms could be discriminated clearly in the score plots of generated by combining PC 1 with PC 3 (Fig. 4b) and PC 2 with PC 3 (Fig. 4c). The combination of PC 1 and PC 3 generated well-separated clusters according to the different grades of cooked pine-mushroom. However, discrimination between first- and second-grade cooked pine-mushrooms was difficult in each of the three score plots.

It is possible to determine the major non-volatile metabolites that contribute to the discrimination among different grades of cooked pine-mushroom by analyzing the PCA scores (Fig. 4) and loading plots (Fig. 5). Succinic acid, trehalose, and fumaric

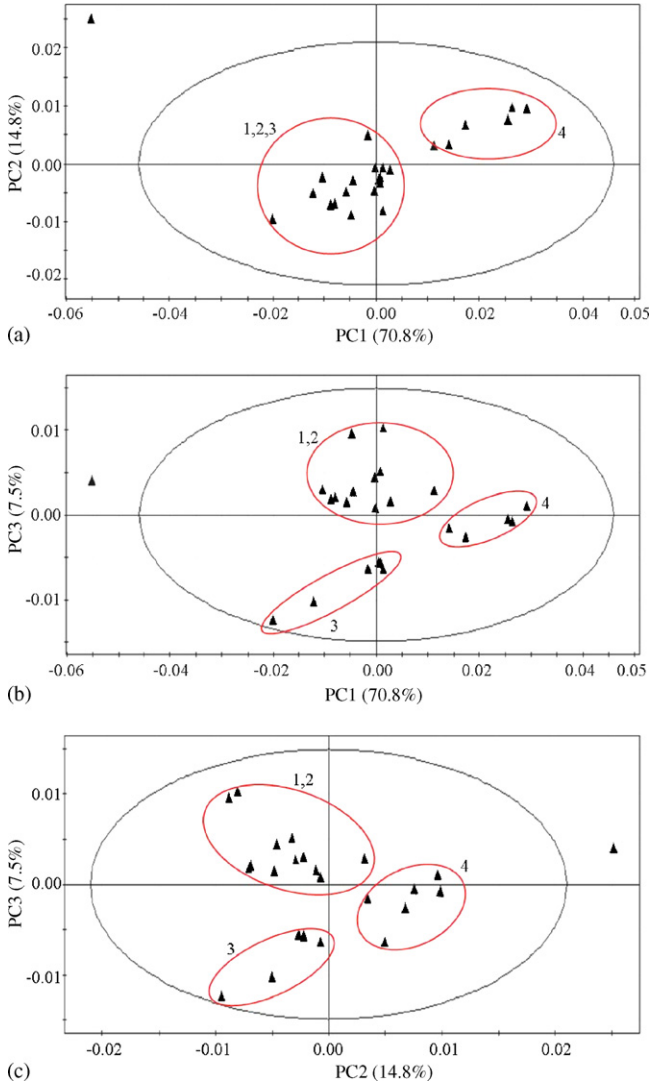

Fig. 4. PCA score plots for aqueous extracts of cooked pine-mushrooms of four different grades (1–4) generated using combinations of PC 1, PC 2, and PC 3.

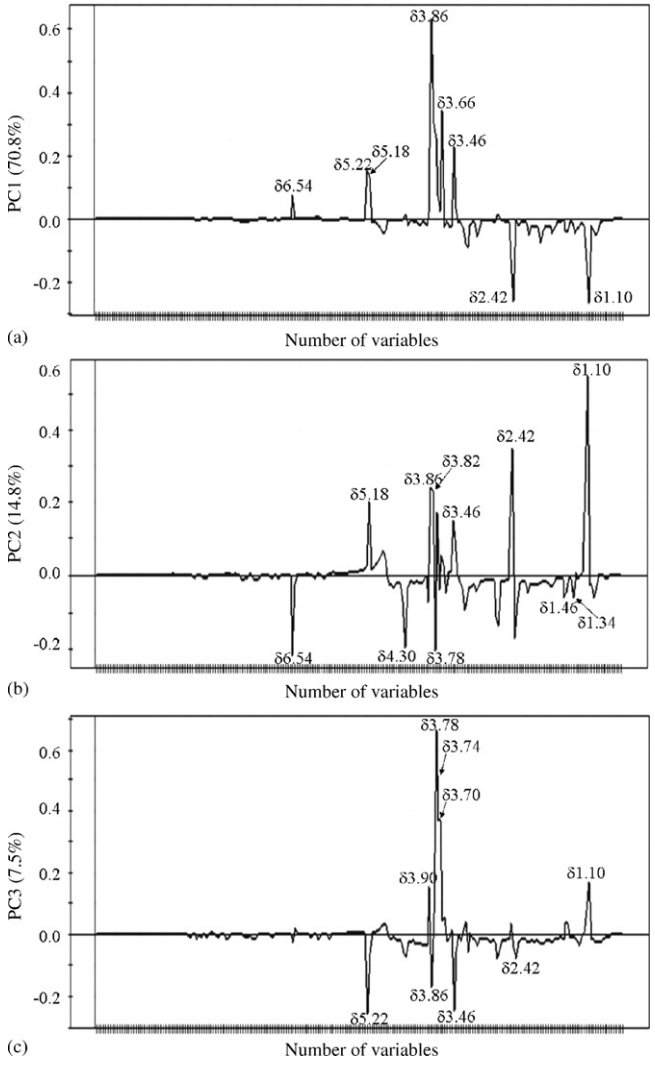

Fig. 5. PCA loading plots for aqueous extracts of cooked pine-mushrooms associated with PC 1 (a), PC 2 (b), and PC 3 (c). Number of variables refers to the chemical shifts binned at interval of 0.04 ppm from 0.52 to 10.00 ppm.

acid were associated with the differentiation by PC 1 (Fig. 5a). The amount of trehalose and fumaric acid was highest in fourth-grade mushrooms (Fig. 4a and b), whereas succinic acid was the main component in first-, second-, and third-grade mushrooms. On the other hand, succinic acid, fumaric acid, fructose, trehalose, alanine, and threonine were related to the discrimination by PC 2 (Fig. 5b). As shown in Fig. 4c, the third- and fourth-grade pine-mushrooms could be discriminated clearly in the score plots generated using PC 2 and PC 3. Succinic acid and trehalose were the dominant components in fourth-grade cooked pine-mushrooms, whereas threonine and alanine were the main components in third-grade mushrooms. The major components associated with separation according to PC 3 were succinic acid and trehalose (Fig. 5c). The first- and second-grade cooked pine-mushrooms contained substantially less succinic acid and trehalose than the other grades (Fig. 4b and c). Based on the aforementioned observations, we concluded that trehalose, succinic acid, and fumaric acid are the components that allow for the differentiation of different grades of cooked pine-mushroom.

4. Conclusions

Except for appearance, there are no objective criteria available to classify and evaluate the quality of pine-mushrooms. In this study, we demonstrated that it is possible to discriminate among different grades of raw and cooked pine-mushroom using PCA of ^{1}H NMR spectra of non-volatile metabolites. Our NMR-based method of metabolic profiling may be a useful tool to elucidate differences among the various grades of pine-mushroom, and might be used to discover an objective criterion that could be used to assess that the quality of different grades of pine-mushroom. In addition, our method could be applied to analyze the non-volatile metabolic profiles of other substances, particularly plants materials that might have medical benefits.

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