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Short communication

Metabolomic differentiation of deer antlers of various origins by ^{1}H NMR spectrometry and principal components analysis

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Abstract

The metabolomic analysis of various types of deer antler was performed by ^{1}H NMR spectrometry and principal components analysis (PCA). The PCA of the ^{1}H NMR spectra of the aqueous fractions allowed a clear discrimination between antler samples according to their origins by the first three principal components (PC1, PC2, and PC3), which cumulatively accounted for 93.5% of the variation in all variables. In particular, the score plots by the combination of PC1 and PC3 allowed an excellent separation of the antler samples. In addition, the major peaks in ^{1}H NMR spectra contributing to the discrimination were assigned to lactate, alanine, acetic acid, choline, glycine, valine, tyrosine, and phenylalanine. This metabolomic-analysis-based method allows various types of deer antler to be efficiently differentiated without any pre-purification steps.
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1. Introduction

Deer antlers are widely used as a traditional oriental medicine in many Asian countries such as Korea and China, with their consumption being associated with anti-stress [1], anti-aging [2], anti-inflammatory [3], and phagocytic [4] effects. Various types of antlers from various countries including Russia, New Zealand, and China are sold commercially for use in traditional medicines in Korea. Determining the origin of deer antlers has conventionally relied upon visual inspection, which is unreliable. Chemical components analysis has also been attempted, with many biochemical components including lipids, peptides, carbohydrates, and inorganic substances reportedly being present [5]. Cho et al. both qualitatively and quantitatively evaluated deer antler using near-infrared spectrometry [6], but the chemical compounds contributing to the discrimination were not determined. Therefore, the chemical classification or discrimination of each species based on classical chromatographic or spectrometric methods is still unclear.

The term 'metabolome' has been used to describe the observable chemical profile or fingerprint of the metabolites present in whole tissues [7]. In metabolites profiling, it is preferable to use a wide spectrum of chemical analysis techniques that are rapid, reproducible, and stable over time while requiring only simple sample preparation. NMR is one of the techniques that potentially meets such demands, which has been widely used as a fingerprinting tool for the interpretation and quality assessment of industrial and natural products, with multivariate or pattern recognition techniques such as the well-known principal components analysis (PCA) having been specifically designed to analyze complex data sets [8]. Accordingly, NMR and PCA methods have been applied to the metabolic profiling of diverse types of products including coffee, juice, wine, beer, and plants [9–16].

In this study, we used a ^{1}H NMR spectrometry method coupled with PCA for the metabolomic differentiation of various types of deer antler, with the aim of elucidating the major metabolites contributing to the discrimination between samples.

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2. Materials and methods

2.1. Deer antler samples

Antler samples (5-year-old *Cervus elaphus*) were collected in China, New Zealand, and Russia during fall, 2004. The top and middle sections of the main beams of the collected antlers were analyzed. The inner parts (i.e. not the velvet) were ground and homogenized in liquid nitrogen using a mortar and pestle, and stored at −80 °C before analysis.

2.2. Solvents and chemicals

First-grade chloroform, methanol, and D_2O (99.9%) were purchased from Sigma (St. Louis, MO, USA), and $CDCl_3$ (99.8%) and NaOD were purchased from Cambridge Isotope Laboratories (Miami, FL, USA) and Cortec (Paris, France), respectively.

2.3. Extraction of antler materials

Three hundred milligrams of ground material was transferred into a centrifuge tube. Five milliliters of a 50% water–methanol mixture and 5 ml of chloroform were added to the antler sample in the tube and vortexed for 30 s and sonicated for 1 min. The materials were then centrifuged at 3000 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. 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, GB = 0, and PC = 1.0. 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 disilane (HMDS, 0.01%, v/v) and TSP (0.01%, w/v) were used as internal standards for $CDCl_3$ and D_2O, respectively.

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 HMDS for $CHCl_3$ extracts and TSP for aqueous extracts. The spectral region $\delta = 0.50$–9.00 was segmented into regions of 0.04 ppm width giving a total of 213 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 assignments of the compounds

Little difference was observed between the spectra of the $CHCl_3$ extracts of the various samples (data not shown), and so only the aqueous fractions were further analyzed. The representative ^{1}H NMR spectra of the aqueous extracts are shown in Fig. 1. The signals were smaller in the aromatic region ($\delta = 6.0$–8.0) than in the aliphatic and sugar regions. The signals of the main aromatic compounds in the extract were assigned as follows: formic acid at $\delta = 8.46$ (s), tyrosine at $\delta = 6.90$ (d, $J = 8.5$ Hz), and phenylalanine at $\delta = 7.42$ (m) (Fig. 1). In addition, 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), HMQC (heteronuclear multiple quantum coherence), and HMBC (heteronuclear multiple bond coherence): lactate at $\delta = 1.34$ (d, $J = 6.6$ Hz); alanine at $\delta = 1.48$ (d, $J = 7.3$ Hz); acetic acid at $\delta = 1.90$ (s); choline at $\delta = 3.22$ (s); glycine at $\delta = 3.56$ (s); and valine at $\delta = 3.58$ (d, $J = 4.3$ Hz). To ensure the objective interpretation of the results, the samples were analyzed using PCA.

3.2. PCA analysis

PCA is an unsupervised clustering method that does not require any knowledge of the data set and acts to reduce the

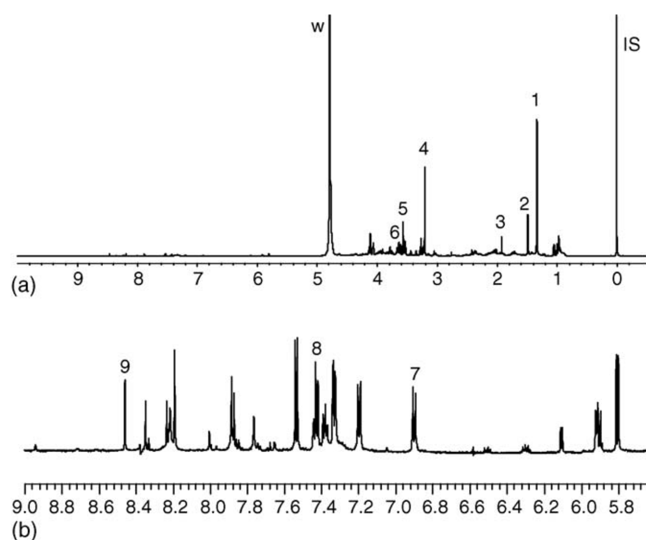

Fig. 1. Representative ^{1}H NMR spectra of the total (a) and aromatic (b) regions of the aqueous fraction of an antler sample. IS: internal standard, w: residual water, 1: lactate, 2: alanine, 3: acetic acid, 4: choline, 5: glycine, 6: valine, 7: tyrosine, 8: phenylalanine, 9: formic acid.

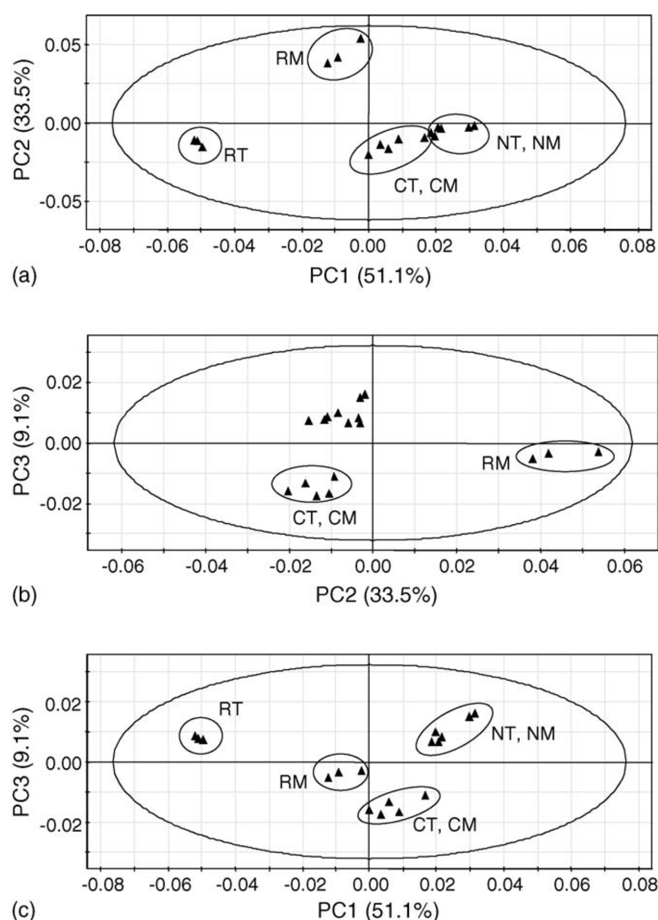

Fig. 2. Score plots of PCA of various antler aqueous extracts by the combination of PC1 and PC2 (a), PC2 and PC3 (b), and PC1 and PC3 (c). The ellipse represents the Hotelling T2 with a 95% confidence in the score plot. RM: middle part of Russian antler, RT: top part of Russian antler, CM: middle part of Chinese antler, CT: top part of Chinese antler, NM: middle part of New Zealand antler, NT: top part of New Zealand antler.

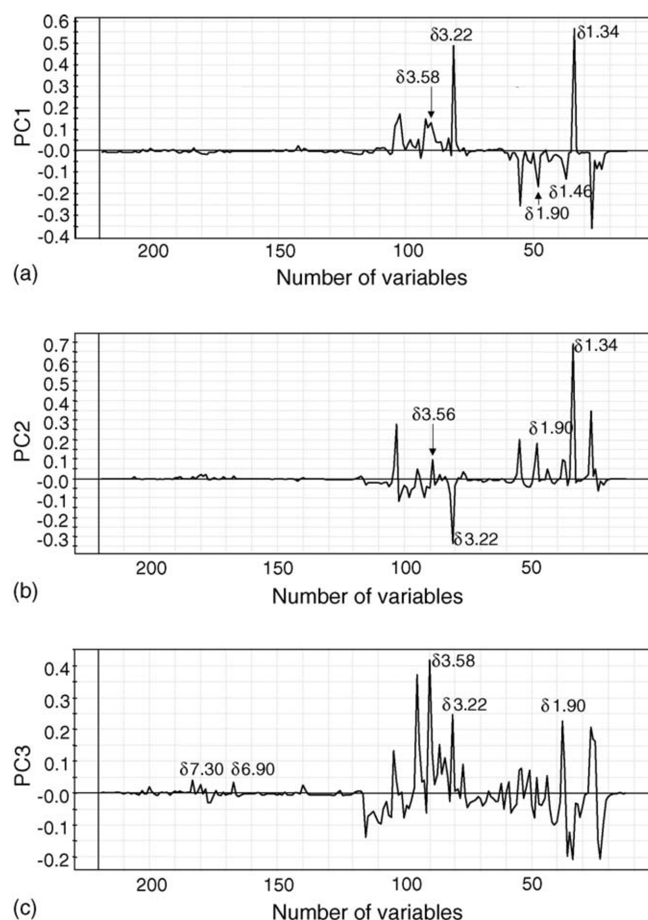

Fig. 3. Loading plots of PCA of various antler aqueous extracts by PC1 (a), PC2 (b), and PC3 (c). Number of variables means the chemical shifts bucketed with interval of 0.04 ppm from 0.50 to 9.00 ppm.

dimensionality of multivariate data while preserving most of the variance therein [17]. The present study applied the covariance method for PCA because it produced a better separation than the correlation method (data not shown). As seen in Fig. 2, the samples of the various antlers could be clearly distinguished, with the first three principal components cumulatively accounting for 93.5% of the variance. The major separation between antler samples in score plots was easily achieved by combining principal component 1 (PC1) with principal component 2 (PC2), principal component 2 (PC2) with principal component 3 (PC3), and PC1 with PC3. In the score plot of the combination of PC1 and PC2, antler samples from Russia were well separated from other samples, and the top and middle parts of Russian antler could also be discriminated. The extracts of antlers from China and New Zealand, however, exhibited similar metabolic patterns (Fig. 2a). The antler samples from China and the middle part of the Russian antlers could be clearly discriminated in the score plots using a combination of PC2 and PC3 (Fig. 2b). Among the principal components, the combination of PC1 and PC3 gave well-separated clusters according to different origins for all antler samples. The antler samples from China, Russia, and New Zealand were well separated from other samples in the score plots of PC1 versus PC3 (Fig. 2c). However, the differentiation between the top and middle parts of the antler samples was difficult in the three combinations of score plots mentioned above (except for the Russian antler samples).

The discriminating metabolites are clearly distinguishable in the loading plots of PC1, PC2, and PC3 (Fig. 3), and the score and loading plots complement each other. The position of objects in a given direction in a score plot is influenced by variables lying in the same direction in the loading plot. The lactate ($\delta = 1.34$) and choline ($\delta = 3.22$) levels were higher in the New Zealand than in the Russian antler samples, whereas the reverse was true for alanine ($\delta = 1.46$). As an example, the intensities of ^{1}H NMR signals for choline ($\delta = 3.22$) were 0.060–0.068 and 0.025–0.027 in the New Zealand and Russian samples, respectively. Choline is essential to the synthesis of acetylcholine (a neurotransmitter) and phosphatidylcholine (a major constituent of cell membranes) [18], and is assumed to be an important component in antlers. The major compounds contributing to the discrimination were lactate, alanine, acetic acid, choline, glycine, valine, tyrosine, and phenylalanine. Lactate, alanine, acetic acid, choline, and valine were mainly related to the discrimination by PC1; lactate, acetic acid, choline, and glycine contributed to the discrimination by PC2; and lactate, choline,

valine, tyrosine, and phenylalanine contributed to the discrimination by PC3. The contributions of tyrosine and phenylalanine peaks were less than the other peaks in aliphatic region, but those compounds showed the higher contribution than the other peaks in aromatic region. Although there were a few peaks in the aromatic region, the peaks contributing to the discrimination were mainly for $\delta = 0.00$–4.50 (except for tyrosine and phenylalanine).

4. Conclusions

This study has proven that it is possible to discriminate various types of deer antler by using PCA of ^{1}H NMR spectra of crude extracts. The per-weight price of deer antler used for oriental medicine varies according to the origin, and hence the technique for discriminating antlers developed in this study can be used for commercial quality-control purposes, especially since it is simple, efficient, and does not require any pre-purification steps. Furthermore, the method is easily applicable to the metabolomic fingerprinting of other medicinal sources such as herbs.

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