J. Microbiol. Biotechnol. (2007), **17**(10), 1682–1687

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Determination of Differences in the Nonvolatile Metabolites of Pine-Mushrooms (*Tricholoma matsutake* Sing.) According to Different Parts and Heating Times Using ^{1}H NMR and Principal Component Analysis

CHO, IN HEE[1], YOUNG-SUK KIM[1], KI WON LEE[2], AND HYUNG-KYOON CHOI[3]*

[1]*Department of Food Science and Technology, Ewha Womans University, Seoul 120-750, Korea*
[2]*Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Korea*
[3]*College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea*

Received: May 15, 2007 Accepted: July 6, 2007

Abstract The differences in the nonvolatile metabolites of pine-mushrooms (*Tricholoma matsutake* Sing.) according to different parts and heating times were analyzed by applying principal component analysis (PCA) to ^{1}H nuclear magnetic resonance (NMR) spectroscopy data. The ^{1}H NMR spectra and PCA enabled the differences of nonvolatile metabolites among mushroom samples to be clearly observed. The two parts of mushrooms could be easily discriminated based on PC 1, and could be separated according to different heat-treated times based on PC 3. The major peaks in the ^{1}H NMR spectra that contributed to differences among mushroom samples were assigned to trehalose, succinic acid, choline, leucine/isoleucine, and alanine. The content of trehalose was higher in the pileus than in the stipe of all mushroom samples, whereas succinic acid, choline, and leucine/isoleucine were the main components in the stipe. Heating resulted in significant losses of alanine and leucine/isoleucine, whereas succinic acid, choline, and trehalose were the most abundant components in mushrooms heat-treated for 3 min and 5 min, respectively.

Keywords: Pine-mushroom (*Tricholoma matsutake* Sing.), ^{1}H nuclear magnetic resonance, principal component analysis, different parts, heat treatment, metabolomic approach

Multivariate metabolite profiling has, for some time now, provided a potential means for studying global changes in metabolite concentrations related to varying chemical, physiological, genetic, or pathological status [1, 14, 16, 23, 30]. In general, metabolomic approaches are based on spectroscopic or spectrometric data of complex samples, mainly as obtained from ^{1}H nuclear magnetic resonance (NMR) spectroscopy and liquid or gas chromatography with mass spectrometry [4, 12, 13, 15]. In particular, since NMR spectroscopy is a rapid, reproducible, and stable method that requires simple sample preparation, it has often been applied to the interpretation and quality assessment of complex materials. NMR spectra are numerically complex, as they contain potentially thousands of different metabolites [9]. This complexity can be untangled by the application of multivariate statistical techniques that are used to reduce the dimension of the NMR data so as to reveal underlying patterns [25]. Recently, NMR spectroscopy and principal component analysis (PCA) have been combined to characterize and classify food or medicinal sources according to factors such as their origin, quality, variety, and so on [3, 6–8, 24].

Pine-mushroom (*Tricholoma matsutake* Sing.) is one of the most valuable species of mushroom worldwide. These mushrooms can be consumed raw or used in a variety of cooked culinary dishes, including stews, soups, and steamed dishes. The composition profiles of mushrooms vary with species and varieties [19, 26], and can also be influenced by the cultivating conditions [20, 21]. In particular, some researchers have found that the various portions of mushroom exhibited significantly different compositions [17, 22]. Noël-Suberville *et al.* [22] reported that the contents of certain fatty acids and aromatic compounds were higher in the gills rather than in the pileus or stipe. In addition, since raw mushrooms contain numerous reactive components, any processing (*e.g.*, drying, canning, γ-irradiation, or other thermal treatments) normally results in significant changes in the compositions of diverse components [5, 10, 28]. On the other hand, we demonstrated that it is possible to discriminate among different grades of pine-mushrooms using PCA of ^{1}H NMR spectra of nonvolatile metabolites [7]. However, there are no reports on the effects of different parts or heating times on the nonvolatile metabolites of pine-mushrooms.

*Corresponding author
Phone: 82-2-820-5605; Fax: 82-2-816-7338;
E-mail: hykychoi@cau.ac.kr

In this study, a metabolomic approach applying multivariate statistical techniques to complex instrumental data sets was used to understand and compare differences in the nonvolatile metabolite profiles in the pileus and stipe of pine-mushrooms heat-treated for different times.

MATERIALS AND METHODS

Chemicals

Chloroform, methanol, and D_2O (99.9%) of first grade were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), and NaOD were purchased from Cortec (Paris, France), respectively.

Sample Materials

We used first-grade pine-mushrooms that were cultivated in Inje-eup, Gangwon-do, Korea in 2005. Fresh pine-mushrooms were wrapped in low-density polyethylene film before being stored at −70°C until used. Frozen mushrooms were thawed at 4°C for 3 h, divided into pileus and stipe, and then sliced using a cutter (Shinomura, Sanjŏ, Niigata, Japan). The sliced mushrooms were heat-treated at 190±3°C for 1 min, 3 min, or 5 min on both sides in a convection broiler (Toastmaster, Boonville, MO, U.S.A.). The raw or heat-treated mushrooms were placed into a stainless steel container, frozen in liquid nitrogen, and then ground in a blender (Hanil Electric, Seoul, Korea).

Extraction

A 1 kg sample (from 40 to 50 individual mushrooms) of the pileus and stipe of first-grade pine-mushroom with and without heat treatment were ground in a blender as mentioned above, respectively, to obtain the representative data of each sample. Among 1 kg of each sample, one gram of ground material was placed into a centrifuge tube. Five ml 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 2,000 rpm for 20 min. The extraction was performed twice. The aqueous fractions was transferred separately into a 50-ml round-bottomed flask and dried with a rotary vacuum evaporator. Because the spectra of the $CHCl_3$ extracts of pine-mushrooms had not been obviously different in our previous study [7], in this study, only the aqueous fractions of mushrooms were analyzed. Each experiment was performed in triplicate.

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 1 N NaOD solution. All spectra were obtained by an 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. TSP (0.01%, w/v) was used as the internal standard for D_2O. 2D NMR experiments using ^1H-^1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) were performed using XWIN-NMR software (version 3.5, Bruker, Germany).

Data Analysis

The ^1H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Biospin, Bruker). Spectral intensities were scaled to TSP for aqueous 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 4.60 to 4.90 was excluded from the analysis because of the residual signal of water in aqueous extracts. The remaining regions were normalized to the whole spectrum for PCA. All spectral data were mean centered with no scaling, and then analyzed by PCA based on the covariance matrix. PCA was performed with SIMCA-P software (Umetrics, Umeå, Sweden).

RESULTS AND DISCUSSION

^1H NMR Spectra and Assignment of Nonvolatile Metabolites

Fig. 1 and Fig. 2 show the representative ^1H NMR spectra from aqueous fractions according to different parts and heating times of pine-mushrooms, respectively. Differences were observed between the spectra of the mushroom samples. The peaks were identified by comparison with the chemical shifts of standard components and 2D NMR using ^1H-^1H 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 δ=0.96 (m), alanine was at δ=1.46 (d, J=7.3 Hz), and succinic acid was at δ=2.42 (s). The midfield region (3−6 ppm) contained choline at δ=3.21 (s) and trehalose at δ=3.46 and 5.18 (t, J=9.6 Hz). The peaks in the aromatic region of the spectrum (6−10 ppm) revealed the presence of fumaric acid at δ=6.54 (s), tyrosine at δ=6.90 (d, J=8.5 Hz), and phenylalanine at δ=7.42 (m). These assignments were in accord with our previous ones [7]. Although changes may be noted in all regions between different mushrooms, for easier

and nonbiased interpretation of the results, and to reduce the dimensionality of the multivariate data obtained with the NMR data sets, we analyzed the samples using PCA.

Comparison of Nonvolatile Metabolites in Different Parts of Pine-Mushrooms According to Different Heat-treated Times

PCA is an unsupervised clustering method that does not require any knowledge of the data set and which reduces the dimensionality of multivariate data while preserving most of the variance therein [11]. The data for PCA can be scaled in different ways, such as using covariance and correlation methods. An advantage of the covariance matrix is that the loadings preserve the scale of the original data, whereas the correlation method is better for discriminating weak and strong signals simultaneously [29]. In this study, the covariance method produced better separation results. The PCs can be displayed graphically as a score plot, which is useful for observing any groupings in the data sets. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings, whose numerical values indicate how much the variable has in common with that component [18].

PCA models were constructed using all the samples in the study, which were designated based on the sample type (P, pileus body; S, stipe) and whether they were raw (R) or heat-treated for 1 min (H1), 3 min (H3), or 5 min (H5).

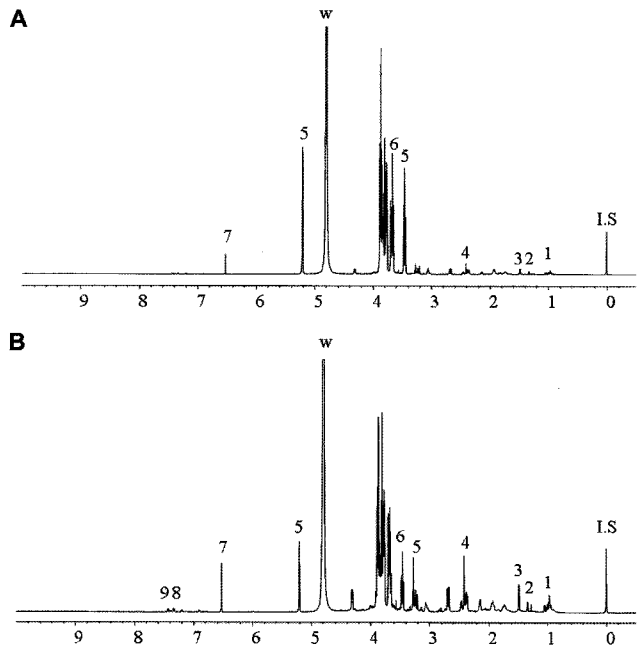

Fig. 1. Representative ^{1}H NMR spectra from the aqueous fraction in the pileus (**A**) and stipe (**B**) of pine-mushrooms.
IS, internal standard; w, residual water; 1, leucine/isoleucine; 2, threonine; 3, alanine; 4, succinic acid; 5, choline; 6, trehalose; 7, fumaric acid; 8, tyrosine; and 9, phenylalanine.

The various samples (*i.e.*, R-P, R-S, H1-P, H1-S, H3-P, H3-S, H5-P, and H5-S) could be distinguished using PCA, with PCs 1–3 cumulatively accounting for 98.5% of the total variance. The best separation among mushroom samples in score plots was achieved by combining PC 1 with PC 2, PC 1 with PC 3, and PC 2 with PC 3 (Fig. 3). In the score plot generated by combining PC 1 and PC 2, the pileus and stipe could be separated from mushroom

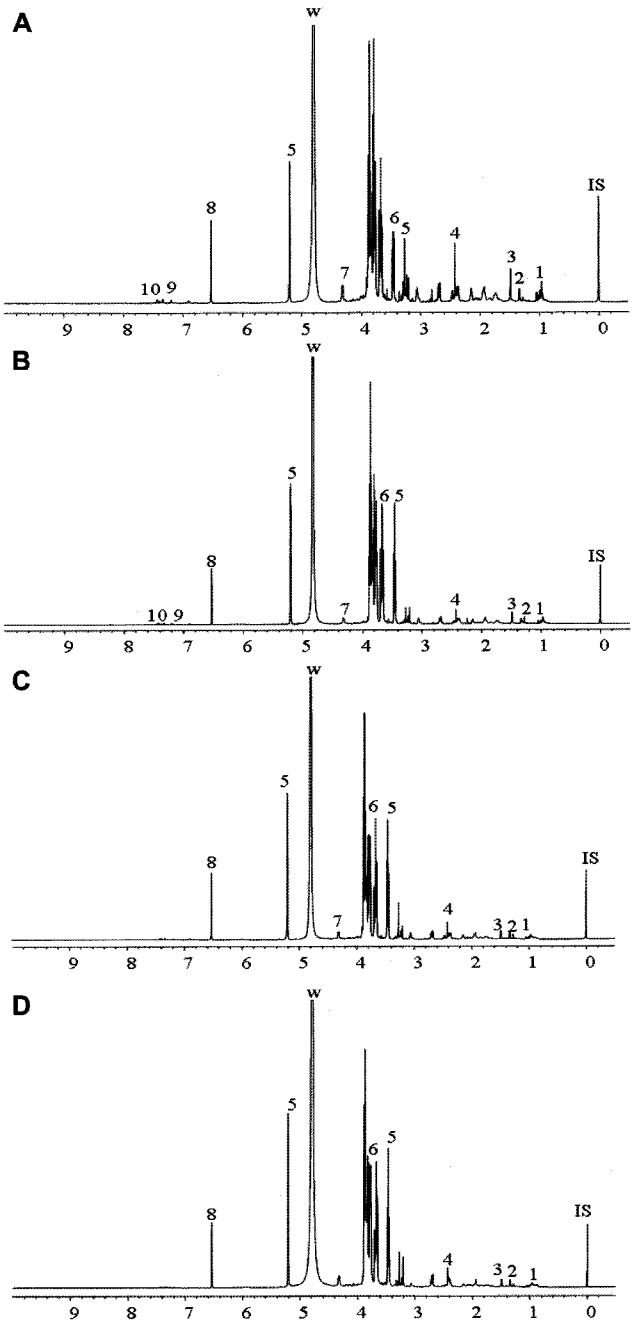

Fig. 2. Representative ^{1}H NMR spectra from the aqueous fraction of raw (**A**) and heat-treated pine-mushrooms for different heating times, such as 1 min (**B**), 3 min (**C**), and 5 min (**D**).

samples, with the two different parts exhibiting a similar metabolite pattern (Fig. 3A). All of the mushroom samples could be discriminated clearly in the score plots generated by combining PC 1 and PC 3 (Fig. 3B). On the other hand, discriminating the stipe of pine-mushrooms heat-treated for 1 min (H1-S) and 3 min (H3-S) was difficult in each of the three score plots.

We found that it was possible to determine the major nonvolatile metabolites that contribute to the differences among mushroom samples according to different parts and heating times by analyzing the PCA scores (Fig. 3) and loading plots (Fig. 4). Trehalose, succinic acid, choline, and leucine/isoleucine were associated with the separation based on PC 1 (Fig. 4A). In all of the raw and heat-treated mushrooms, the amount of trehalose was higher in the pileus (R-P, H1-P, H3-P, and H5-P) than in the stipe, whereas succinic acid, choline, and leucine/isoleucine were the main components in the stipe (R-S, H1-S, H3-S, and H5-S). Beecher *et al.* [2] reported that trehalose was the second abundant component in all tissues (lower stipe, upper stipe, gills, inner cap, and peel) of *Agaricus bisporus*. Trehalose concentration was greatest in the lower stipe, followed by the inner cap, upper stipe, peels, and gills [2]. However, in this study, the amount of trehalose was higher in the pileus than in the stipe. On the other hand, trehalose, succinic acid, alanine, and leucine/isoleucine were related to the discrimination based on PC 2 (Fig. 4B). However, mushroom samples could still not be discriminated clearly in the score plots generated by combining PC 2 and PC 3 (Fig. 3C). In addition, the major components associated with separation according to PC 3 were trehalose, succinic acid, alanine, choline, and leucine/isoleucine (Fig. 4C). The raw mushrooms and those heat-treated for 1 min (R-P, R-S, and H1-P) contained substantially more alanine and leucine/isoleucine and less succinic acid, choline, and trehalose than mushrooms heat-treated for a longer time (H1-S, H3-P, H3-S, H5-P, and H5-S).

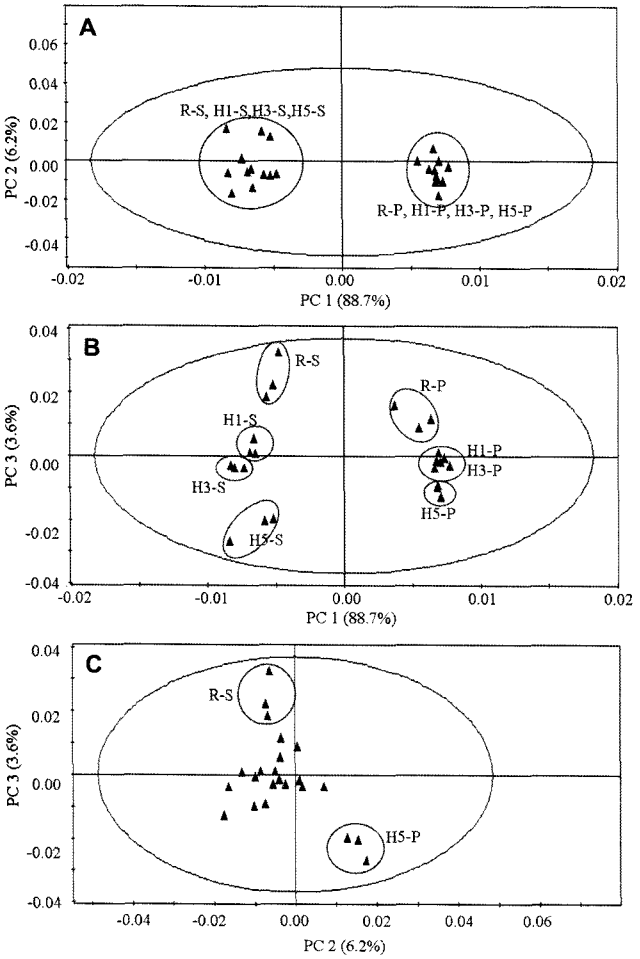

Fig. 3. PCA score plots for aqueous extracts of raw and heat-treated pileus and stipe of pine-mushrooms, generated using combinations of PC 1, PC 2, and PC 3.

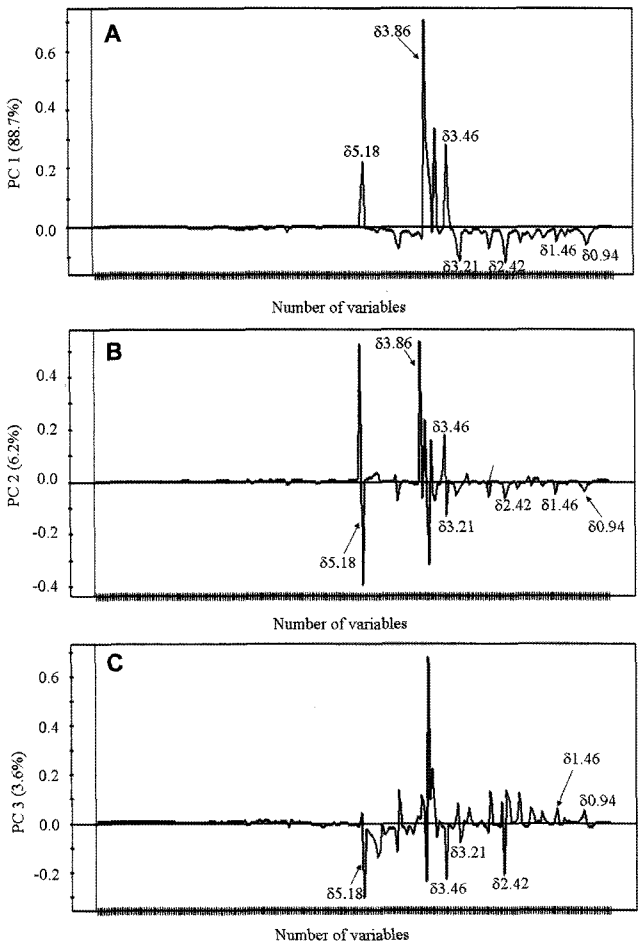

Fig. 4. PCA loading plots for aqueous extracts of raw and heat-treated pileus and stipe of pine-mushrooms associated with PC 1 (**A**), PC 2 (**B**), and PC 3 (**C**).
Number of variables refers to the chemical shifts binned at the interval of 0.04 ppm, from 0.52 to 10.00 ppm.

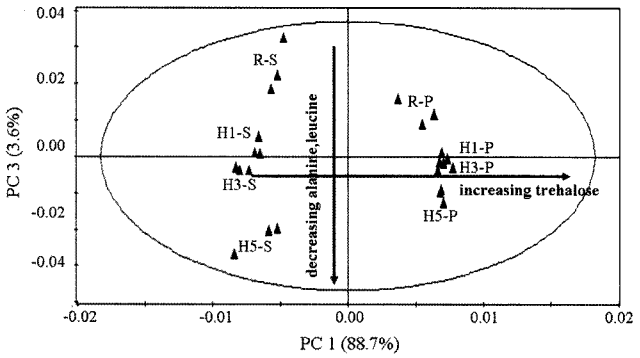

Fig. 5. Summary of the scores plot of PC 1 and PC 3, after examination of the associated loading plots, indicating the metabolites responsible for the greatest variance.

Based on the aforementioned observations, we concluded that trehalose, succinic acid, choline, leucine/isoleucine, and alanine are the components that allow for the differentiation of pine-mushrooms according to different heat-treatments and parts. In general, the pileus and stipe of pine-mushrooms exhibited similar metabolite patterns. However, as shown in Fig. 5, the content of trehalose was higher in the pileus than in the stipe of all mushroom samples, whereas succinic acid, choline, and leucine/isoleucine were the main components in the stipe. On the other hand, heating resulted in decreases in the contents of alanine and leucine/isoleucine in the mushroom samples. This may be attributable to the occurrence of Maillard or nonenzymatic browning reactions, which involve condensation between amino groups of amino acids in the protein (or in peptide linkages or even in free amino acids) with reducing sugars [2]. This study shows that it is possible to investigate the differences in the nonvolatile metabolites according to different parts and heating times of mushroom samples using multivariate analysis of their metabolite fingerprints in ^{1}H NMR spectra of crude mushroom extracts.

Acknowledgments

This study was funded in part by the Korea Science and Engineering Foundation (R01-2004-000-10276-0) and the Korea Research Foundation (KRF-2005-908-C00064).

References

1. Asenjo, J. A., P. R. I. Rapaport, J. Aracena, E. Goles, and B. A. Andrews. 2007. A discrete mathematical model applied to genetic regulation and metabolic networks. *J. Microbiol. Biotechnol.* **17:** 496–510.
2. Beecher, T. M., N. Magan, and K. S. Burton. 2001. Water potentials and soluble carbohydrate concentrations in tissues of freshly harvested and stored mushrooms (*Agaricus bisporus*). *Postharvest Biol. Technol.* **22:** 121–131.
3. Brescia, M. A., V. Caldarola, A. De Giglio, D. Benedetti, F. P. Fanizzi, and A. Sacco. 2002. Characterization of the geographical origin of Italian red wines based on traditional and nuclear magnetic resonance spectrometric determinations. *Anal. Chim. Acta* **458:** 177–186.
4. Campo, G. D., J. I. Santos, N. Iturriza, I. Berregi, and A. Munduate. 2006. Use of the ^{1}H nuclear magnetic spectra signals from polyphenols and acids for chemometric characterization of cider apple juices. *J. Agric. Food Chem.* **54:** 3095–3100.
5. Card, A. and C. Avisse. 1997. Comparative study of the aroma of raw and cooked mushrooms (*Agaricus bisporus* L.). *Ann. Technol. Agric.* **27:** 287–293.
6. Charlton, A. J., W. H. H. Farrington, and P. Brereton. 2002. Application of ^{1}H NMR and multivariate statistics for screening complex mixtures: Quality control and authenticity of instant coffee. *J. Agric. Food Chem.* **50:** 3098–3103.
7. Cho, I. H., Y.-S. Kim, and H.-K. Choi. 2007. Metabolomic discrimination of different grades of pine-mushroom (*Tricholoma matsutake* Sing.) using ^{1}H NMR and multivariate analysis. *J. Pharm. Biomed. Anal.* **43:** 900–904.
8. Choi, H.-K., K.-H. Kim, K. H. Kim, Y.-S. Kim, M.-W. Lee, and W. K. Whang. 2006. Metabolomic differentiation of deer antlers of various origins by ^{1}H nuclear magnetic resonance spectrometry and principal components analysis. *J. Pharm. Biomed. Anal.* **41:** 1047–1050.
9. Cloarec, O., M. E. Dumas, J. Trygg, A. Craig, R. H. Barton, J. C. Lindon, J. K. Nicholson, and E. Holmes. 2005. Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in ^{1}H NMR spectroscopic metabonomic studies. *Anal. Chem.* **77:** 517–526.
10. Dijkstra, F. Y. 1976. Studies on mushroom flavours. 3. Some flavour compounds in fresh, canned and dried edible mushrooms. *Z. Lebensm. Unters. Forsch.* **160:** 401–405.
11. Eriksson, L., E. Johansson, N. Kettaneh-Wold, and S. Wold. 2001. *Multi- and Megavariate Data Analysis*. Umetrics Academy, Umeå, Swden.
12. Fragaki, G., A. Spyros, G. Siragakis, E. Salivaras, and P. Dais. 2005. Detection of extra virgin olive oil adulteration with lampante olive oil and refined olive oil using nuclear magnetic resonance spectroscopy and multivariate statistical analysis. *J. Agric. Food Chem.* **53:** 2810–2816.
13. Jonsson, P., A. I. Johansson, J. Gullberg, J. Trygg, A. J. Okay as, B. Grung, S. Marklund, M. Sjöström, H. Antti, and T. Moritz. 2005. High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. *Anal. Chem.* **77:** 5635–5642.
14. Jung, H. J. and H. J. Kwon. 2006. Chemical genomics with natural products. *J. Microbiol. Biotechnol.* **16:** 651–660.
15. Karlshøj, K. and T. O. Larsen. 2005. Differentiation of species from the *Penicillium requeforti* group by volatile metabolite profiling. *J. Agric. Food Chem.* **53:** 708–715.
16. Lee, K. W., H.-S. Joo, Y.-H. Yang, E. J. Song, and B.-G. Kim. 2006. Proteomics for streptomyces: "Indusrial

proteomics" for antibiotics. *J. Microbiol. Biotechnol.* **16:** 331–348.
17. Maga, J. 1981. Influence of maturity, storage and heating on the flavor of mushroom (*Agaricus bisporus*) caps and stems. *J. Food Process. Preserv.* **5:** 95–101.
18. Massart, D. L., B. G. M. Vandeginste, S. N. Deming, Y. Michotte, and L. Kauffman. 1988. *Chemometrics: A Textbook.* Elsevier, New York.
19. Mau, J.-L., C.-C. Chyau, J.-Y. Li, and Y.-H. Tseng. 1997. Flavor compounds in straw mushrooms *Volvariella volvacea* harvested at different stages of maturity. *J. Agric. Food Chem.* **45:** 4726–4729.
20. Mau, J.-L., R. B. Beelman, and G. R. Ziegler. 1992. 1-Octen-3-ol in the cultivated mushroom, *Agaricus bisporus*. *J. Food Sci.* **57:** 704–706.
21. Mau, J.-L., R. B. Beelman, and G. R. Ziegler. 1993. Factors affecting 1-octen-3-ol in mushrooms at harvest and during postharvest storage. *J. Food Sci.* **58:** 331–334.
22. Noël-Suberville, C., C. Cruz, J. Guinberteau, and M. Montury. 1996. Correlation between fatty acid content and aromatic compound release in fresh Blewit (*Lepista nuda*). *J. Agric. Food Chem.* **44:** 1180–1183.
23. Oh, M.-K., M.-J. Cha, S.-G. Lee, L. Rohlin, and J. C. Liao. 2006. Dynamic gene expression profiling of *Escherichia coli* in carbon source transition from glucose to acetate. *J. Microbiol. Biotechnol.* **16:** 543–549.
24. Pereira, G. E., J.-P. Gaudillere, C. van Leeuwen, G. Hilbert, M. Maucourt, C. Deborde, A. Moing, and D. Rolin. 2006. ^{1}H NMR metabolite fingerprints of grape berry: Comparison of vintage and soil effects in Bordeaux grapevine growing area. *Anal. Chim. Acta* **563:** 346–352.
25. Ramadan, Z., D. J. M. Grigorov, and S. Kochhar. 2006. Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. *Talanta* **68:** 1683–1691.
26. Rapior, S., S. Gavalié, C. Andary, Y. Pélissier, C. Marion, and J.-M. Bessière. 1996. Investigation of some volatile components of seven fresh wild mushrooms (*Basidiomycetes*). *J. Essent. Oil Res.* **8:** 199–201.
27. Sumner, L. W., P. Mendes, and R. A. Dixon. 2003. Plant metabolomics: Large-scale phytochemistry in the functional genomics era. *Phytochemistry* **62:** 817–836.
28. Thomas, A. F. 1973. An analysis of the flavor of the dried mushroom, *Boletus edulis*. *J. Agric. Food Chem.* **21:** 955–958.
29. Ward, J., C. Harris, J. Lewis, and H. Beale. 2003. Assessment of ^{1}H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopis thaliana*. *Phytochemistry* **62:** 949–957.
30. Yu, J. W., W. H. Chung, T.-K. Sohn, Y.-H. Park, and H. I. Kim. 2006. Development of a meta-information system for microbial resources. *J. Microbiol. Biotechnol.* **16:** 178–183.