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Quantification of Water-Soluble Metabolites in Medicinal Mushrooms Using Proton NMR Spectroscopy

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ABSTRACT: The water-soluble metabolites in 5 mushrooms were identified and quantified using proton nuclear magnetic resonance (NMR) spectroscopy and software for targeted metabolite detection and quantification. In total, 35 compounds were found in *Agaricus brasiliensis*, 25 in *Taiwanofungus camphoratus*, 23 in *Ganoderma lucidum* (Taiwan) and *Lentinus edodes*, and 16 in *G. lucidum* (China). Total amounts of all identified metabolites in *A. brasiliensis*, *T. camphoratus*, *G. lucidum*, *G. lucidum* (China), and *L. edodes* were 149,950.51, 12,834.18, 9,549.09, 2,788.41, and 111,726.51 mg/kg dry weight, respectively. These metabolites were categorized into 4 groups: free amino acids and derivatives, carbohydrates, carboxylic acids, and nucleosides. Carbohydrates were the most abundant metabolites among all 4 groups, with mannitol having the highest concentration among all analyzed metabolites (848–94,104 mg/kg dry weight). Principal components analysis (PCA) showed obvious distinction among the metabolites of the 5 different kinds of mushrooms analyzed in this study. Thus PCA could provide an optional analytical way of identifying and recognizing the compositions of flavor products. Furthermore, the results of this study demonstrate that NMR-based metabolomics is a powerful tool for differentiating between various medicinal mushrooms.

KEY WORDS: medicinal mushrooms, NMR, principal component analysis, water-soluble metabolite

ABBREVIATIONS: DW, dry weight; **GABA**, 4-aminobutyric acid (γ-aminobutyric acid); **HPLC**, high-performance liquid chromatography; **NMR**, nuclear magnetic resonance; **PCA**, principal component analysis

I. INTRODUCTION

Mushrooms have been consumed as food and food flavorings because of their unique and subtle flavors. Mushroom flavor derives primarily from nonvolatile components such as amino acids and nucleotides.[1] Mushrooms have been reported to be low in calories and fat but rich in proteins, polysaccharides, and vitamins, and they exhibit abilities to lower the level of cholesterol in humans.[2,3] Moreover, some mushroom species have also shown certain medicinal effects and are widely used to alleviate the symptoms of certain diseases such as diabetes, hypercholesterolemia, and cancer.[4] Among the mushrooms typically used to treat disease are species such as *Ganoderma lucidum*, *Agaricus brasiliensis*, *Taiwanofungus camphoratus*, and *Lentinus edodes*.

The lingzhi or reishi medicinal mushroom, *G. lucidum* (Curtis: Fr.) P. Karst. (Ganodermataceae, Agaricomycetes), has long been used in traditional Chinese medicine as an anti-inflammatory, antitumor, antiviral, antibacterial, and antiparasitic agent, as well as in blood pressure regulation, cardiovascular disorders, kidney toxicity, hepatotoxicity, and chronic bronchitis. It has also been suggested for use as a protective nerve tonic.[5]

A. brasiliensis S. Wasser et al. (=*A. blazei* Murrill s. Henem., Agaricaceae, Agaricomycetes) is

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reportedly used as a healthy food for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis.[6] It has also been traditionally used in food because of its unique flavor. Research *in vitro* and *in vivo* showed that *A. brasiliensis* has the ability to stimulate the body's immune system and show positive clinical results in colorectal and gynecological cancers.[7,8]

T. camphoratus (M. Zang & G.H. Su) Sheng H. Wu et al. (=*Antrodia cinnamomea*, Polyporales, Basidiomycetes) is a traditional medicinal mushroom in Taiwan and is found only in the high mountainous regions of Taiwan, with *Cinnamomum kanehirai* as its single host. This fungus grows inside the hollow trunk of the tree, where it is dark and humid. The fruiting body is difficult to culture successfully, even in a well-controlled environment. Such unique growth conditions not only enable this medicinal mushroom to generate important compounds that are difficult to replicate in the laboratory, but they also make it one of the most expensive medicinal fungi in the world. Extracts from this fungus have been found to exhibit various anticancer activities, and its active components, such as triterpenoids and polysaccharides, have shown potent cytotoxicity against cancer cells.[9] In addition, *T. camphoratus* has been used as a remedy for drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer.[10]

The shiitake mushroom, *L. edodes* (Berk.) Singer (=*Lentinula edodes,* Marasmiaceae, Agaricomycetes), is a traditional delicacy in Asia and can be ground to powder form and used as a flavoring to enhance the taste of foods and increase a product's appeal.[11]

Nuclear magnetic resonance (NMR)–based metabolomics is a modern and effective analytical technique used to identify and quantify components in different biological fluids and in extracts from tissues, including plants and mushrooms. NMR was discovered in 1938 by Isidor Rabi. Since then, NMR spectroscopy has become a widely used analytical technique.[12] One important application of NMR-based metabolomics is in identifying biomarkers of disease in human body fluids such as urine and serum.[13–15] This complicated task can be effectively accomplished by combining NMR spectroscopy with metabolite identification and quantification in NMR spectra using a technique called "targeted profiling."[16]

The Chenomx NMR Suite is a commercial metabolomics software package that uses targeted profiling and contains standard spectral libraries/databases that include hundreds of metabolites.[16] Targeted profiling is based on a combination of mathematical modeling and a database of NMR spectral signatures of individual metabolites.[17] NMR spectroscopy is an efficient technology for metabolomics because it is not destructive, does not rely on separating mixtures before analysis, and can concurrently identify hundreds of metabolites in samples.[16]

Once metabolites in biological samples are identified and quantified, the information can be analyzed further using different methods of multivariate analysis, including principal component analysis (PCA). PCA is an unsupervised statistical analysis technique that is used to find sets of correlated variables that can describe differences between data sets.[17] In this study a combination of ^{1}H NMR spectroscopy and PCA was used to identify the metabolites that differentiate *A. brasiliensis*, *L. edodes*, *T. camphoratus*, and *G. lucidum* from 2 different locations (one grown in Taiwan and other obtained in China).

II. MATERIALS AND METHODS

A. Samples and Preparation

Four different species of mushrooms, including *A. brasiliensis*, *T. camphoratus*, *G. lucidum*, and *G. lucidum* (China), were obtained from the Biotechnology Center, Grape King Inc., Chungli City, Taiwan; *L. edodes* was purchased from Q-Yo Bio-Technology Farm, Pusin, Chunghua, Taiwan. All 5 mushrooms were obtained in the dried form and randomly divided into 3 samples (~50 g each), ground to powder, and stored in desiccators before use. The moisture content of the freeze-dried samples was measured using an MF-50 Infrared Moisture Analyzer (A&D Co., Tokyo, Japan). Each sample powder (1 g) was mixed with 10 mL

ultrapure (type I) water obtained from a Synergy UV system (Millipore, Billerica, MA); they were preheated to 80°C, vortexed for 15 minutes, and centrifuged using a model 5810 centrifuge (Eppendorf, Hauppauge, NY) at 2000 × *g* for 15 minutes. The collected supernatant was lyophilized using a FreeZone 4.5-L Benchtop Freeze Dry System (Labconco, Kansas City, MO), sealed, and stored at −80°C until further analysis.

B. NMR Spectroscopy

Each lyophilized extract was redissolved in 1 mL of ultrapure water, vortexed, and transferred into a 0.5-mL Amicon Ultra centrifugal filter with a 3-K pore size (Millipore), then centrifuged at 14,000 × *g* for 30 minutes at 4°C using an Eppendorf 5415R microcentrifuge. The volume of the filtrate was adjusted with ultrapure water to a total volume of 585 µL, and 65 µL of an internal standard solution (Chenomx Inc., Edmonton, Alberta, Canada) containing 4.8566 mmol/L 3-(trimethylsilyl)-1-propanesulphonic acid-d_6 (DSS-d_6) and 0.2% NaN_3 in 98% D_2O was added. Small amounts of 1 N HCl or NaOH (certified grade; Thermo Fisher Scientific, Pittsburgh, PA) were added to each sample to adjust the pH value to 6.8. Each sample (650 µL) was transferred into a 5-mm NMR tube (Bruker, Billerica, MA), and stored at 4°C until NMR data acquisition (within 24 hours of sample preparation). All other chemicals used were of analytical grade.

Data were acquired using the nuclear Overhauser effect spectroscopy PR1D pulse sequence on an Avance 600 MHz NMR spectrometer (Bruker) equipped with a SampleJet, as previously described.[18] Spectra were manually phased and baseline corrected using a Chenomx NMR Suite 7.6 Processor (Chenomx Inc.). Metabolites were detected and quantified using a Chenomx NMR Suite 7.6 Profiler (Chenomx Inc.). This software uses a known concentration of the internal standard solution (DSS-d_6) to determine the concentrations of individual compounds.[16] Metabolite concentrations were quantified (milligrams per deciliter), exported from the Chenomx program, and recalculated as milligrams per kilogram dry weight (DW).

C. Statistical Analysis

The metabolite concentrations were expressed as the mean ± standard error of 3 triplicate extraction/analyses and subjected to an analysis of variance for a completely random design using a SAS version 9.4 (SAS Institute, Inc., Cary, NC). To determine the significance of differences between means, Duncan multiple range tests at the level of $\alpha = 0.05$ were used. PCA was carried out using Xlstat 2014 (Addinsoft, Brooklyn, NY), in which the computations were performed using a Pearson correlation matrix. PCA was elucidated to show the main differences in variation between each sample.[19] Results from the PCA score and loading plots were used to elucidate correlations between different kinds of mushrooms and the amounts of water-soluble metabolites.

III. RESULTS AND DISCUSSION

A. Free Amino Acids and Derivatives

Figure 1 shows a representative NMR spectrum from each variety of mushroom. In total, 35 metabolites were measured, quantified, and categorized into 4 groups: free amino acids and derivatives, carbohydrates, carboxylic acids, and nucleosides. Of the identified amino acids and derivatives, 13 were hydrophilic and 8 were hydrophobic (Table 1). Eight of 9 essential amino acids were found in *A. brasiliensis* and *L. edodes* (no tryptophan was found). Tryptophan and threonine were not detected in *G. lucidum*, whereas tryptophan, lysine, and methionine were not present in *T. camphoratus*. Only threonine and methionine were found in *G. lucidum* (China). These essential amino acids are used in the human body to make proteins and act as a source of carbon and nitrogen to synthesize other biochemical compounds. The total amounts of hydrophilic and hydrophobic amino acids was highest in *A. brasiliensis* and lowest in *G. lucidum* (China). Surprisingly, 2 *G. lucidum* samples differed in the profiles of free amino acids: *G. lucidum* from Taiwan contained more types of amino acids and in higher concentrations (Table 1).

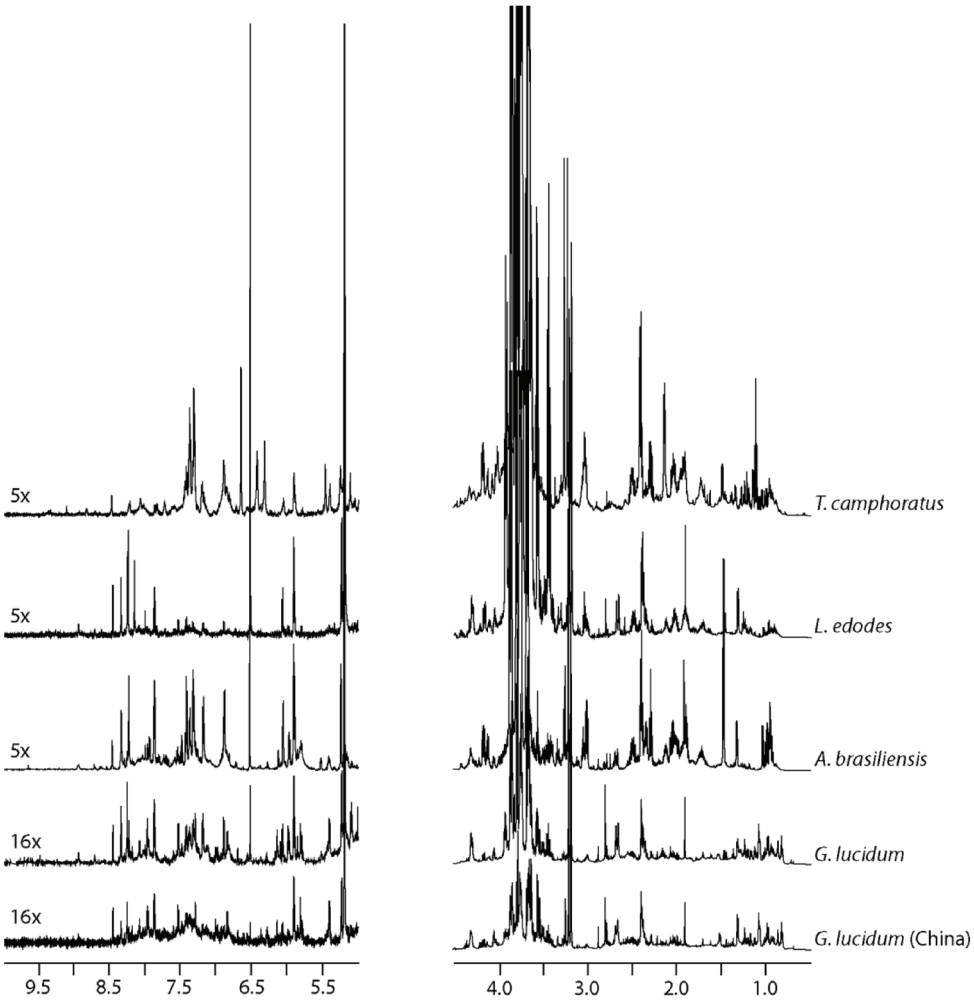

FIG. 1: Nuclear magnetic resonance (NMR) spectra of the 5 varieties of mushrooms in this study. The aliphatic region (right) and the aromatic region (left) of the NMR spectra are shown. The spectra on the left were scaled as indicated relative to the aliphatic region.

Pyroglutamate (2-oxo-pyrrolidone carboxylic acid) was the most abundant free amino acid derivative in all mushrooms analyzed in this study. A previous study in animals indicated that this compound improves learning and memory in old rats.[20] 4-Aminobutyric acid (γ-aminobutyric acid [GABA]), had the highest concentrations in *A. brasiliensis*. GABA is a secondary metabolite that can be found in most mushroom fruiting bodies and mycelia.[21] In this study it was found in all mushrooms except for *G. lucidum*. GABA acts as an inhibitory neurotransmitter to block nerve impulses and functions to inhibit neural excitatory activity in the human body, thereby alleviating stress induced by mental tasks, stimulating relaxation, and diminishing anxiety.[22] However, low amounts of GABA have been related to the seizure activity, and a GABA deficiency could lead to symptoms characteristic of Huntington disease.[23,24]

Alanine was the hydrophobic amino acid found in the largest amount in *A. brasiliensis*. In the human body, alanine is one of the major substrates for the alanine-glucose cycle, a pathway that helps to remove lactate from muscles and regenerate glucose from lactate in the liver.[25]

TABLE 1: Free Amino Acid and Derivative Contents of Medicinal Mushrooms

	Content* (mg/kg Dry Weight)				
	Agaricus brasiliensis	***Taiwanofungus camphoratus***	***Ganoderma lucidum***	***Ganoderma lucidum* (China)**	***Lentinus edodes***
Hydrophilic					
GABA	3784.27 ± 78.68A	1157.89 ± 30.91B	—	20.88 ± 0.44D	188.14 ± 3.08C
L-Arginine	497.59 ± 6.37B	579.95 ± 4.34A	—	18.96 ± 0.58C	596.72 ± 20.02A
L-Asparagine	402.44 ± 13.13A	247.10 ± 7.33B	—	—	-
L-Aspartate	781.70 ± 41.63A	60.65 ± 2.31B	—	—	-
Betaine	636.40 ± 15.00B	1686.91 ± 9.80A	—	45.18 ± 0.36C	-
L-Glutamine	1367.24 ± 47.07A	390.43 ± 15.71B	60.16 ± 1.36C	—	-
L-Histidine	18.28 ± 0.15B	63.04 ± 3.21A	—	—	-
L-Lysine*	1336.43 ± 40.68A	—	16.66 ± 0.77B	—	23.39 ± 0.34B
L-Ornithine	2403.42 ± 91.38A	235.29 ± 4.62C	—	—	931.84 ± 41.35B
L-Pyroglutamate	10049.50 ± 179.37A	3884.59 ± 25.58B	476.84 ± 7.29D	415.93 ± 4.64D	2459.01 ± 69.57C
L-Serine	961.03 ± 13.41A	119.99 ± 3.43B	—	—	—
L-Threonine*	1234.20 ± 37.66A	157.05 ± 5.09B	—	113.38 ± 3.36B	15.18 ± 0.59C
L-Tyrosine	743.12 ± 18.55A	82.62 ± 2.80B	82.78 ± 4.11B	—	100.99 ± 3.08B
Total	24215.62 ± 295.70	8665.50 ± 38.51	636.44 ± 5.30	614.33 ± 9.21	4315.26 ± 131.40
Hydrophobic					
L-Alanine	5146.74 ± 280.62A	283.03 ± 9.24B	69.82 ± 3.11B	—	189.10 ± 9.27B
Glycine	821.39 ± 20.51A	252.80 ± 9.48B	76.01 ± 0.64C	—	108.67 ± 3.45C
L-Isoleucine*	993.60 ± 47.22A	150.84 ± 6.78B	18.80 ± 0.89C	—	43.96 ± 1.84C
L-Leucine*	1673.34 ± 48.07A	154.98 ± 3.44B	54.37 ± 0.56C	—	4.38 ± 0.17C
L-Methionine*	50.16 ± 2.15A	—	7.55 ± 0.14C	13.61 ± 0.54B	10.77 ± 0.41C
L-Phenylalanine*	1045.99 ± 32.55A	304.30 ± 4.90B	57.28 ± 2.26C	—	98.86 ± 0.31C
L-Proline	3914.47 ± 86.24A	147.52 ± 3.86B	43.40 ± 0.38B	—	—
L-Valine*	1528.03 ± 74.30A	243.31 ± 2.29B	44.50 ± 2.13C	9.03 ± 0.31C	105.01 ± 3.28C
Total	15173.69 ± 499.87	1536.78 ± 18.54	371.73 ± 6.37	22.64 ± 0.77	560.75 ± 15.93

Each value is expressed as mean ± standard error (n = 3). Means with different letters within a row differ significantly ($P < 0.05$).
*Essential amino acid.

B. Carbohydrates

Three sugars (glucose, *myo*-inositol, and trehalose) and 2 sugar alcohols (arabinitol and mannitol) were identified in extracts of medicinal mushrooms analyzed in this study (Table 2). The total carbohydrate content was the highest in *A. brasiliensis* and *L. edodes* (>100 g/kg DW) and the lowest (>2 g/kg DW) in *T. camphoratus*, *G. lucidum*, and *G. lucidum* (China). Mannitol was the most significant compound found in all mushroom samples, ranging from 848 to 94,104 mg/kg. Trehalose was the second most

TABLE 2: Carbohydrate Contents of Medicinal Mushrooms

	Content (mg/kg Dry Weight)				
	Agaricus brasiliensis	***Taiwanofungus camphoratus***	***Ganoderma lucidum***	***Ganoderma lucidum (China)***	***Lentinus edodes***
Arabinitol	1521.08 ± 65.51B	—	1628.55 ± 59.65B	373.25 ± 4.67B	23550.94 ± 1275.27A
Glucose	4030.60 ± 101.09A	—	554.96 ± 14.79C	—	2650.48 ± 88.79B
Mannitol	94104.34 ± 4038.22A	2311.05 ± 44.45C	4266.18 ± 156.71C	848.44 ± 22.53C	49383.01 ± 2756.33B
myo-Inositol	572.60 ± 7.79A	—	225.92 ± 9.98B	246.48 ± 8.21B	—
Trehalose	5627.07 ± 290.03B	—	1490.42 ± 26.22C	550.94 ± 22.34C	29933.91 ± 1461.33A
Total	105855.70 ± 4283.57	2311.05 ± 44.45	8766.03 ± 228.23	2019.12 ± 41.97	105518.30 ± 5540.66

Each value is expressed as mean ± standard error (n = 3). Means with different letters within a row differ significantly ($P < 0.05$).

TABLE 3: Carboxylic Acid Contents of Medicinal Mushrooms

	Content (mg/kg Dry Weight)				
	Agaricus brasiliensis	***Taiwanofungus camphoratus***	***Ganoderma lucidum***	***Ganoderma lucidum* (China)**	***Lentinus edodes***
Citrate	171.58 ± 7.00A	—	—	—	—
Formate	16.84 ± 0.32B	—	5.29 ± 0.12C	—	94.09 ± 0.81A
Malate	2499.81 ± 38.46A	—	—	—	—
Nicotinate	14.88 ± 0.32A	—	—	—	—
Succinate	426.37 ± 3.94A	216.27 ± 4.92B	40.99 ± 1.93D	23.05 ± 0.24E	54.24 ± 2.07C
Total	3129.48 ± 40.44	216.27 ± 4.92	46.28 ± 2.05	23.05 ± 0.24	148.32 ± 2.76

Each value is expressed as mean ± standard error (n = 3). Means with different letters within a row differ significantly ($P < 0.05$).

abundant carbohydrate found in all mushrooms except *T. camphoratus*. These 2 carbohydrates are used as energy reserves in the mushrooms.[26] While mannitol was the major respiratory substrate in the harvested mushrooms, it is also the major taste-active component among mushroom sugars.[27,28]

In addition to mannitol and trehalose, other sugars and sugar alcohols, such as arabinitol, glucose, and *myo*-inositol, were also identified; these could contribute to a sweet taste and used as sweeteners in the food industry because of their low calorie content.[1,29] Interestingly, mannitol was shown to inhibit an angiotensin I–converting enzyme resulting in an antihypertensive effect in animals.[30] In both *in vitro* and *in vivo* studies of Parkinson disease, mannitol was shown to decrease the action of α-synuclein, which is a key factor found in patients with Parkinson disease.[31]

C. Carboxylic Acids

Five carboxylic acids were found in the analyzed medicinal mushroom samples, including citrate, formate, malate, nicotinate, and succinate (Table 3). *A. brasiliensis* had the largest amount of carboxylic acids (>3g/kg DW), whereas *G. lucidum* (China) had the smallest amount (<0.02 g/kg DW). Citrate is the main substance responsible for removing excess

TABLE 4: Nucleoside Contents of Medicinal Mushrooms

	Content (mg/kg Dry Weight)				
	Agaricus brasiliensis	***Taiwanofungus camphoratus***	***Ganoderma lucidum***	***Ganoderma lucidum* (China)**	***Lentinus edodes***
Adenosine	139.96 ± 1.81B	6.60 ± 0.37E	103.61 ± 5.55C	24.22 ± 0.22D	463.52 ± 10.92A
Guanosine	57.23 ± 1.86B	11.17 ± 0.38C	13.97 ± 0.62C	7.23 ± 0.14C	171.58 ± 7.59A
Inosine	32.70 ± 0.97B	15.23 ± 0.24C	64.24 ± 1.17A	9.61 ± 0.49D	—
Uridine	1385.97 ± 57.42A	70.81 ± 3.71C	153.03 ± 4.35C	66.58 ± 2.59C	564.18 ± 15.20B
Total	1576.02 ± 21.87	104.59 ± 2.70	328.61 ± 6.05	109.27 ± 1.89	1183.84 ± 23.99

Each value is expressed as mean ± standard error ($n = 3$). Means with different letters within a row differ significantly ($P < 0.05$).

calcium in the human body. Malate plays many functional roles in animals and plants, such as energy generation, photosynthesis, fatty acid oxidation, nitrogen fixation, amino acid biosynthesis, ion balance, uptake of phosphorus and iron, and aluminum tolerance.[32–35] Nicotinate, also known as nicotinic acid or vitamin B_3, is the anionic form of niacin. The common forms of niacin include nicotinic acid and its amide nicotinamide. In living organisms, NAD+ and NADP+ are important coenzymes for redox reactions and are also responsible for delivering electrons from one reaction to another, in which nicotinate and nicotinamide act as the precursors for generation of these 2 coenzymes.[36]

Formate, malate, and succinate are compounds involved in the citric acid cycle.[37] The citric acid cycle, also called the tricarboxylic acid cycle or the Krebs cycle, is where adenosine triphosphate is produced to provide energy, and it is also a pathway for metabolizing glucose into energy. These results suggest that after harvest, carboxylic acids remain in larger amounts in *A. brasiliensis* than in other mushrooms.

D. Nucleosides

Nucleosides were the least abundant of all analyzed metabolites (Table 4). Of the measured nucleosides, uridine was the most abundant in the 5 analyzed mushroom types, whereas adenosine was the second most abundant in 4 of the mushrooms (not in *T. camphoratus*). Uridine has been shown to participate in the synthesis of membrane constituents and glycosylation.[38] Adenosine has been proposed to protect against myocardial ischemia and lung ischemia-reperfusion injuries by inhibiting proinflammatory interleukin-6 production and increasing anti-inflammatory interleukin-10.[39,40]

In summary, 35 metabolites were identified and quantified in *A. brasiliensis*, 25 in *T. camphoratus*, 23 in *G. lucidum* and *L. edodes*, and 16 in *G. lucidum* (China). Total metabolites in *A. brasiliensis*, *T. camphoratus*, *G. lucidum*, *G. lucidum* (China), and *L. edodes* were 149,950, 12,834, 9,549, 2,788, and 111,726 mg/kg DW, respectively. Detected differences in metabolite concentrations between the 5 types of analyzed mushrooms may explain the differences in their flavor, since many of these metabolites impart unique tastes.

Several nonvolatile components, including 18 free amino acids, 8 sugars and sugar alcohols, and 6 nucleotides, in *G. lucidum* and *L. edodes* were previously analyzed using high-performance liquid chromatography (HPLC).[41,42] Comparison with the current study suggests that more compounds can be identified and quantified using NMR than HPLC. However, not all compounds in the mushrooms were identified and quantified, and the addition of more standards to the database could aid in the identification and quantification of many more metabolites. In terms of quantification, the main difference between the 2 analytical methods is that the quantification by NMR spectroscopy was based on a database containing a pure, authentic compound and thus excludes/

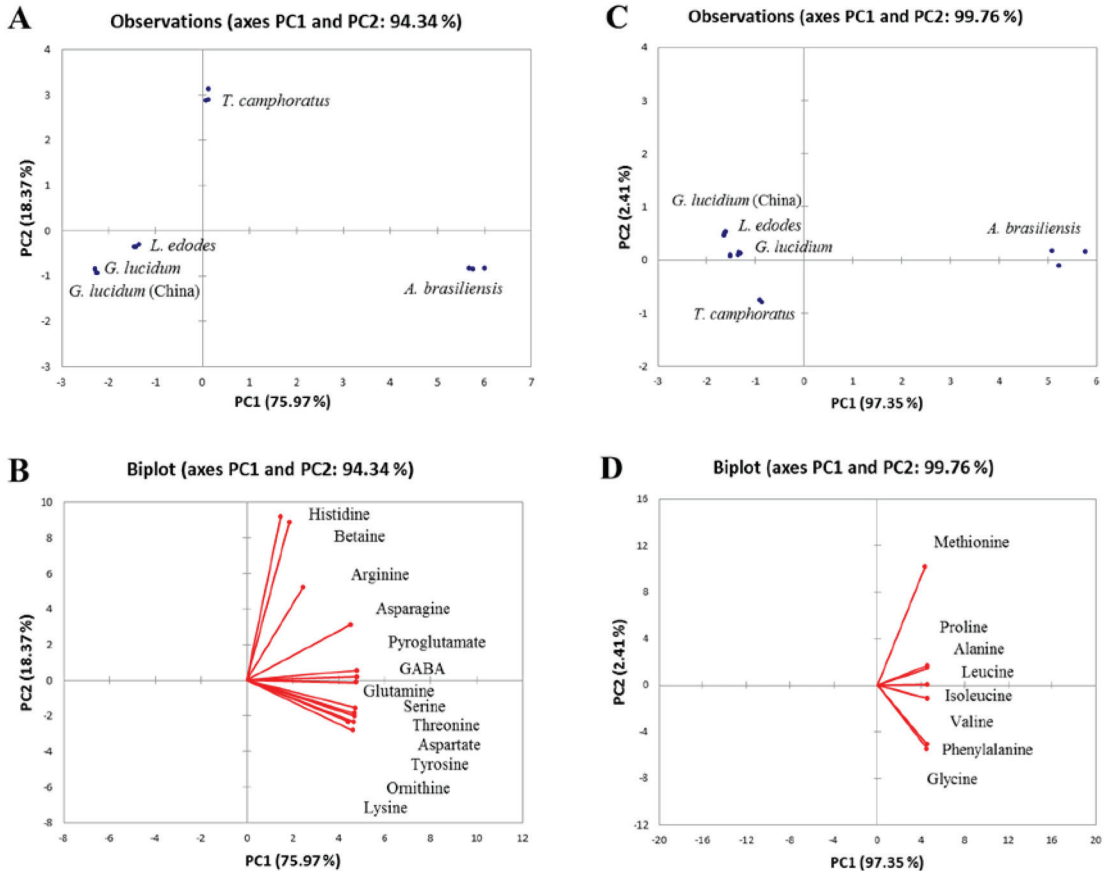

FIG. 2: Principal components (PC) analysis of medicinal mushrooms using free amino acids and derivatives as variables. Graphs show the score (A) and loading plots (B) for hydrophilic amino acids and derivatives, and the score (C) and loading plots (D) for hydrophobic amino acids.

eliminates possible errors that occur during the preparation of standards required for HPLC analysis. Furthermore, considering the cost and time efficiency of the experiment, NMR quantification technology could provide an alternative to such research.

E. PCA Scrutinization of NMR Analysis Data Sets

PCA was used to visualize differences in the 5 mushroom varieties. Using only hydrophilic amino acids and derivatives as variables in discrimination, the first 2 components were responsible for 94.34% of the variance (75.97% for principal component [PC] 1 and 18.37% for PC2), and as shown in Fig. 2A and B. Higher values for PC1 were associated with higher concentrations of these hydrophilic amino acids. *T. camphoratus* was characterized by more betaine and histidine than other varieties; *A. brasiliensis* was characterized by more L-glutamine and L-serine. *G. lucidum*, *G. lucidum* (China), and *L. edodes* had lower concentrations of hydrophilic amino acids and derivatives, along with PC2,. Using hydrophobic amino acids as variables, PCA revealed that the first 2 components were responsible for 99.76% of the variance (97.35% for PC1 and 2.41% for PC2); this is illustrated in Fig. 2C and D. Higher PC1 values were associated with higher concentrations of hydrophobic amino acids. *A. brasiliensis* was characterized by higher levels of L-leucine and L-isoleucine. Separation along PC2 was correlated with *G. lucidum*, *G. lucidum* (China), *L. edodes*, and *T. camphoratus*.

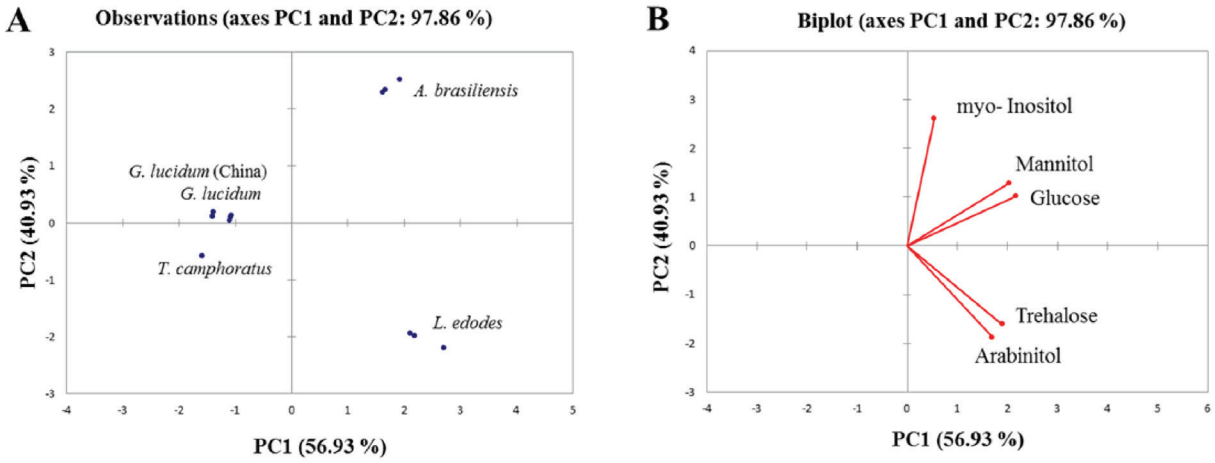

FIG. 3: Principal components (PC) analysis of medicinal mushrooms using carbohydrates as variables. Graphs show the score (A) and loading plots (B).

Using measured carbohydrate compounds as variables in PCA, the first 2 components were responsible for 97.86% of the variance in the mushroom samples (56.93% for PC1 and 40.93% for PC2), as shown in Fig. 3A and B. The higher the value, the greater the contents of mannitol and myo-inositol, along with PC1. *A. brasiliensis* has more mannitol (94,104 mg/kg DW) and myo-inositol than other mushroom varieties. *L. edodes* was associated with a larger amount of arabinitol and trehalose. Along with PC2, separation between *G. lucidum*, *G. lucidum* (China), and *T. camphoratus* was observed. The 2 specimens of *G. lucidum* were indistinguishable because their carbohydrate content was similar (Fig. 3A).

Using measured carboxylic acids as variables in PCA, the first 2 components were responsible for 96.95% of the variance (77.11% for PC1 and 19.84% for PC2), as shown in Fig. 4A and B. *A. brasiliensis* was associated with higher concentrations of citrate, malate, and nicotinate, whereas formate was higher in *L. edodes*. In this study *A. brasiliensis* was the only variety of mushroom that contained citrate, malate, and nicotinate. Formate was detected in *A.*

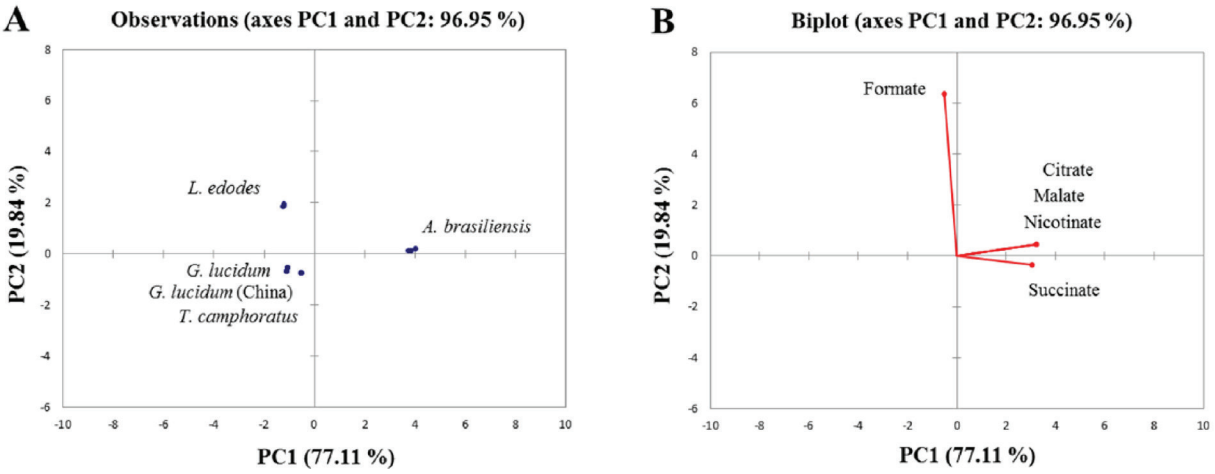

FIG. 4: Principal components (PC) analysis of medicinal mushrooms using carboxylic acids as variables. Graphs show the score (A) and loading plots (B).

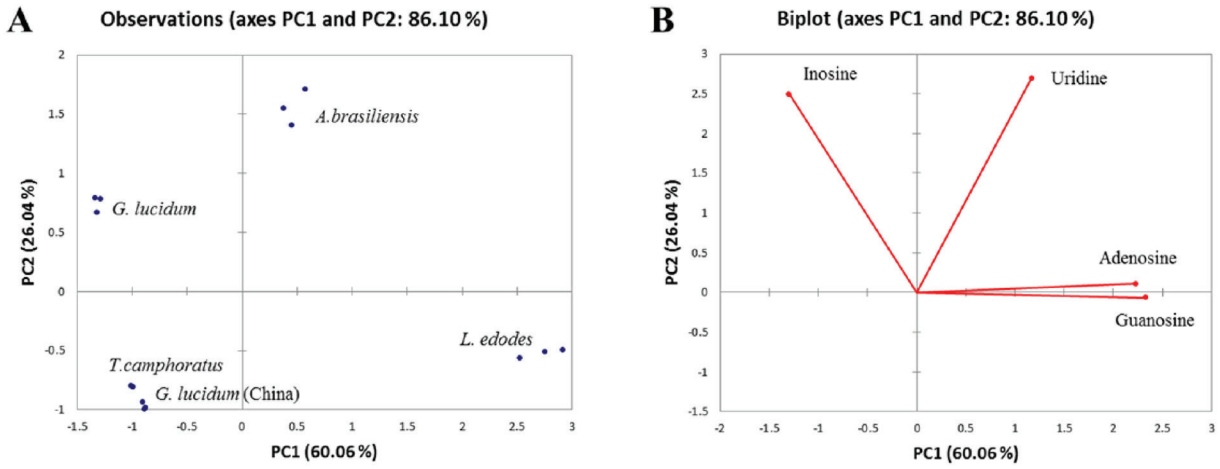

FIG. 5: Principal components (PC) analysis of medicinal mushrooms using nucleosides as variables. Graphs show the score (A) and loading plots (B).

brasiliensis, *G. lucidum*, and *L. edodes* (5.29–94.09 mg/kg DW), but not in *G. lucidum* (China) or *T. camphoratus*. These results are in agreement with previous studies where formate was reported to contribute to the flavor of *L. edodes*.[43]

In PCA using nucleosides as variables, the first 2 components were responsible for 86.10% of the variance (60.06% for PC1 and 26.04% for PC2), as shown in Fig. 5A and B. Higher concentrations of uridine were observed in *A. brasiliensis*, whereas higher concentrations of guanosine were observed in *L. edodes*. Both *G. lucidum* (China) and *T. camphoratus* were similar in terms of nucleoside content.

In summary, the results from PCA demonstrated good distinction between the 5 different mushrooms based on the differences in water-soluble metabolites. Moreover, the results also indicated that combining NMR-based metabolomics with targeted profiling and PCA provided a technique that may be helpful for the differentiation and identification of different species of mushrooms present in formulated food or flavoring.

IV. CONCLUSIONS

In this study NMR-based metabolomics was used to detect and quantify water-soluble metabolites in 5 different mushrooms. In total, 35 metabolites were analyzed. Among 4 groups of identified metabolites, carbohydrates were the highest in concentration. Mannitol was the most significant compound found in the analyzed mushroom samples. The culinary mushrooms *A. brasiliensis* and *L. edodes* contained higher concentrations of water-soluble metabolites than the medicinal mushrooms *G. lucidum*, *G. lucidum* (China), and *T. camphoratus*. In addition, *G. lucidum* (China) contained the smallest number of identified metabolites and in the lowest amounts.

PCA distinguished between the 5 different mushrooms analyzed in this study. In conclusion, NMR-based metabolomics was able to differentiate between various mushroom species based on the water-soluble metabolite content.

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