APPLICATION NOTE
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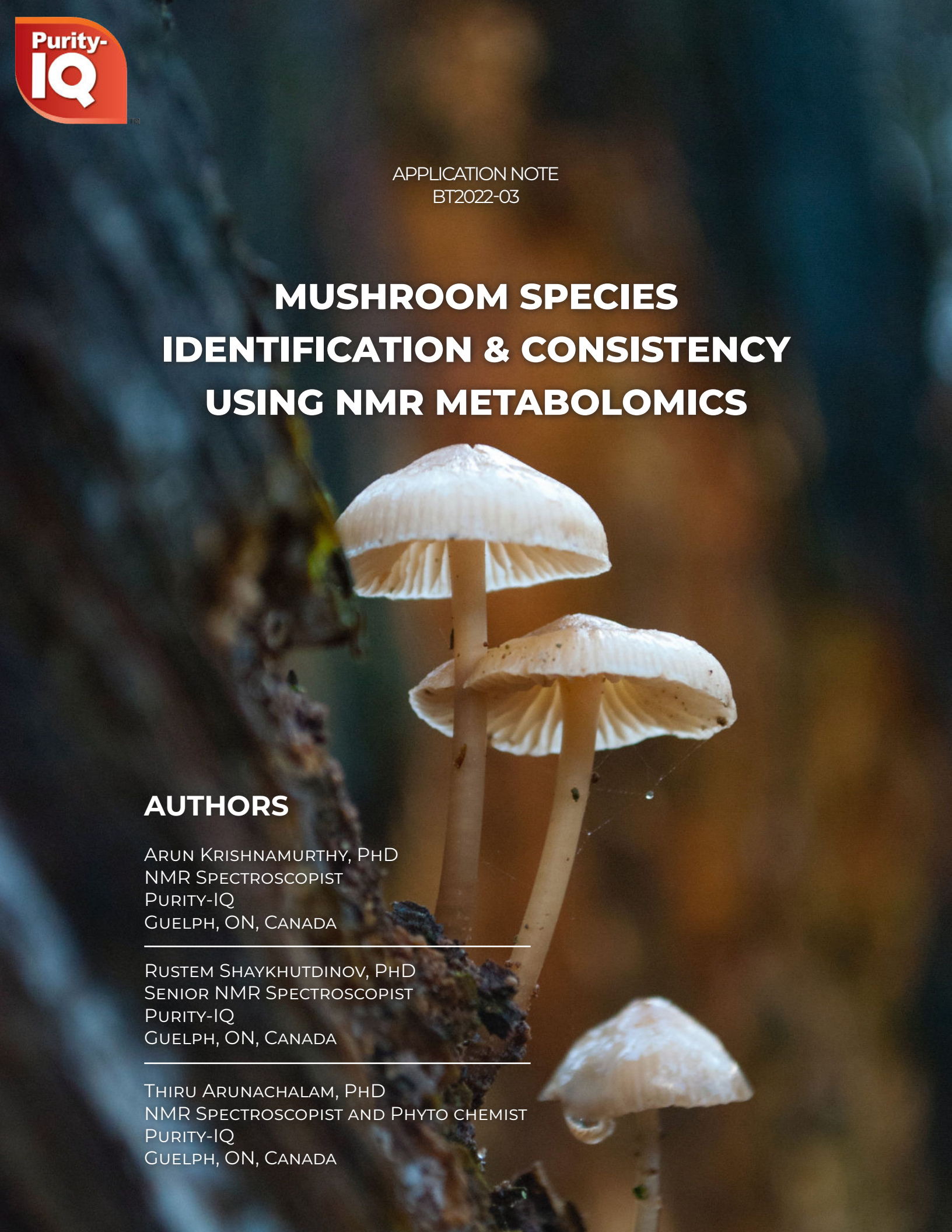
MUSHROOM SPECIES IDENTIFICATION & CONSISTENCY USING NMR METABOLOMICS



AUTHORS

Arun Krishnamurthy, PhD
NMR Spectroscopist
Purity-IQ
Guelph, ON, Canada

Rustem Shaykhutdinov, PhD
Senior NMR Spectroscopist
Purity-IQ
Guelph, ON, Canada

Thiru Arunachalam, PhD
NMR Spectroscopist and Phyto chemist
Purity-IQ
Guelph, ON, Canada

Abstract

Mushrooms have gained immense popularity as dietary supplements due to their favourable health benefits attributable to distinct classes of biomolecules specific to certain species. In this report, we demonstrate the superiority of NMR metabolomics in conjunction with multivariate statistical analysis in the identification of various mushroom species and the evaluation of batch-to-batch product consistency.

Introduction

Mushrooms are a class of fungi cultivated on a large scale across the globe due to their highly sought-after edible attributes. Mushrooms are protein-rich, high in nutritional value, low in calories and packed with nutrients such as essential fatty acids, vitamins, and minerals. They are a rich source of selenium, vitamin D, glutathione, and ergothioneine and help in treating ailments such as hypertension, hypercholesterolemia, atherosclerosis, and cancer. The market analysis report from Grand view research valued the global mushroom market size at US$50.3B in 2021, at an expected compound annual growth rate of 9.7% from 2022 to 2030, with a revenue forecast of US$115.8B in 2030 [1]. In addition to being a prominent component of dietary supplements, dried mushroom powders and mushroom extracts are ubiquitously used in skincare and beauty products, making mushroom species identification pertinent as not all mushroom species confer health benefits. In addition, a huge disparity exists in the pricing of the mushrooms, increasing the risk of adulteration among specialty mushroom products with low-cost species alternatives.

Nuclear Magnetic Resonance (NMR) Spectroscopy-based metabolomic assay of dried mushroom samples and their extracts serves as an elegant approach in the identification and classification of mushroom species as the metabolic makeup of the mushroom fruiting body is specific to each species. NMR has gained in popularity in the botanical sector in recent years as a species identification tool due to its high reproducibility, accuracy, ease of use and simple sample preparation methods. For a detailed explanation of NMR-based metabolomics and the related concepts, readers are encouraged to read the review by Emwas et al. [2]. Several articles highlighting the efficacy and the suitability of NMR spectroscopy to validate herbarium mushroom specimens [3], and to study metabolite diversity [4], development process and anti-cancer effect [5], and medicinal mushrooms [6] have been published. In addition to its species differentiation ability, the metabolite profile of a plant product is also sensitive to product processing and manufacturing conditions, cultivars, growth conditions, and the geography of cultivation, which further amplifies the robustness of NMR across different product formats. Furthermore, the unique nature of the metabolomic profile functions as a fingerprinting technique to test the consistency between samples from different batches (i.e., batch-to-batch consistency) in the manufacturing sector, and to establish the purity and origin of raw materials used in the production of consumer products. In this report, we demonstrate the utility of NMR-based metabolomics in identifying mushroom species and measuring the consistency between different batches of same type.

Materials and Methods

Several dried samples of *Inonotus obliquus* (IO), *Cordyceps militaris* (CM), *Hericium erinaceus* (HE), *Grifola frondosa* (GF), *Pleurotus ostreatus* (PO), *Phellinus linteus* (PL), *Wolfiporia extensa* (WE), *Ganoderma lingzhi* (GL), *Lentinula edodes* (LE), *Tremella fuciformis* (TF), *Trametes versicolor* (TV) mushroom powders and their extracts were sourced commercially. Metabolites from these mushroom products were extracted using different solvents such as water, methanol, chloroform, acetone, ethanol, hexane, and their mixtures. The solvent system that rendered the highest concentration of both polar and non-polar metabolites was adopted for this study. All samples were prepared in triplicates to account for the statistical variations during extraction and NMR spectra acquisition. One-dimensional NMR spectra were collected on a 400 MHz Bruker Avance III spectrometer with tetramethyl silane (TMS, 0 ppm) as an internal chemical shift reference. The collected spectra were processed, baseline and phase corrected, and binned using mrbin package in R for further multivariate statistical analysis, performed on the R platform.

Results and Discussion

The ^{1}H NMR spectra of different mushroom species samples are plotted in Figure 1. Key distinctions can be observed in the spectral profile of different species which spans the whole spectral width. Some of the common metabolites observed in mushroom extracts and their chemical shift ranges [4,5] are:

1-3 ppm: leucine, valine, isoleucine, isobutyrate, 3-methyl-2-oxovalerate, propylene glycol, ethanol, lactate, alanine, arginine, lysine, acetate, proline, methionine, 4-aminobutyrate, glutamate, pyruvate, succinate, glutamine, citrate, 2-oxoisocaproate, malate, aspartate, asparagine, creatinine, malonate, proline, acetoacetate, methylamine, and others

3.1-6 ppm: choline, sn-glycero-3-phosphocholine, myo-inositol, glycerol, glucitol, mannitol, betaine, threonine, xylose, glucose, uracil, guanosine, trehalose, xylitol, and others

6.1-10 ppm: fumarate, tyrosine, histidine, phenylalanine, tryptophan, xanthine, formate, adenosine, trigonelline, phenylacetate, and others.

Observed changes in the peak package of the studied samples is attributed to the presence of metabolites of different classes (sugars, amino acids, carboxylic acids, mono-, di-, and polysaccharides, aldehydes, alcohols, ketones, lipids, fatty acids, molecules with aromatic functional groups, and others) including the above-listed molecules in different concentrations. The comparison of these spectra alone emphasizes the difference in the metabolite chemistry of the studied mushroom species, which serves as a versatile tool for differentiating them.

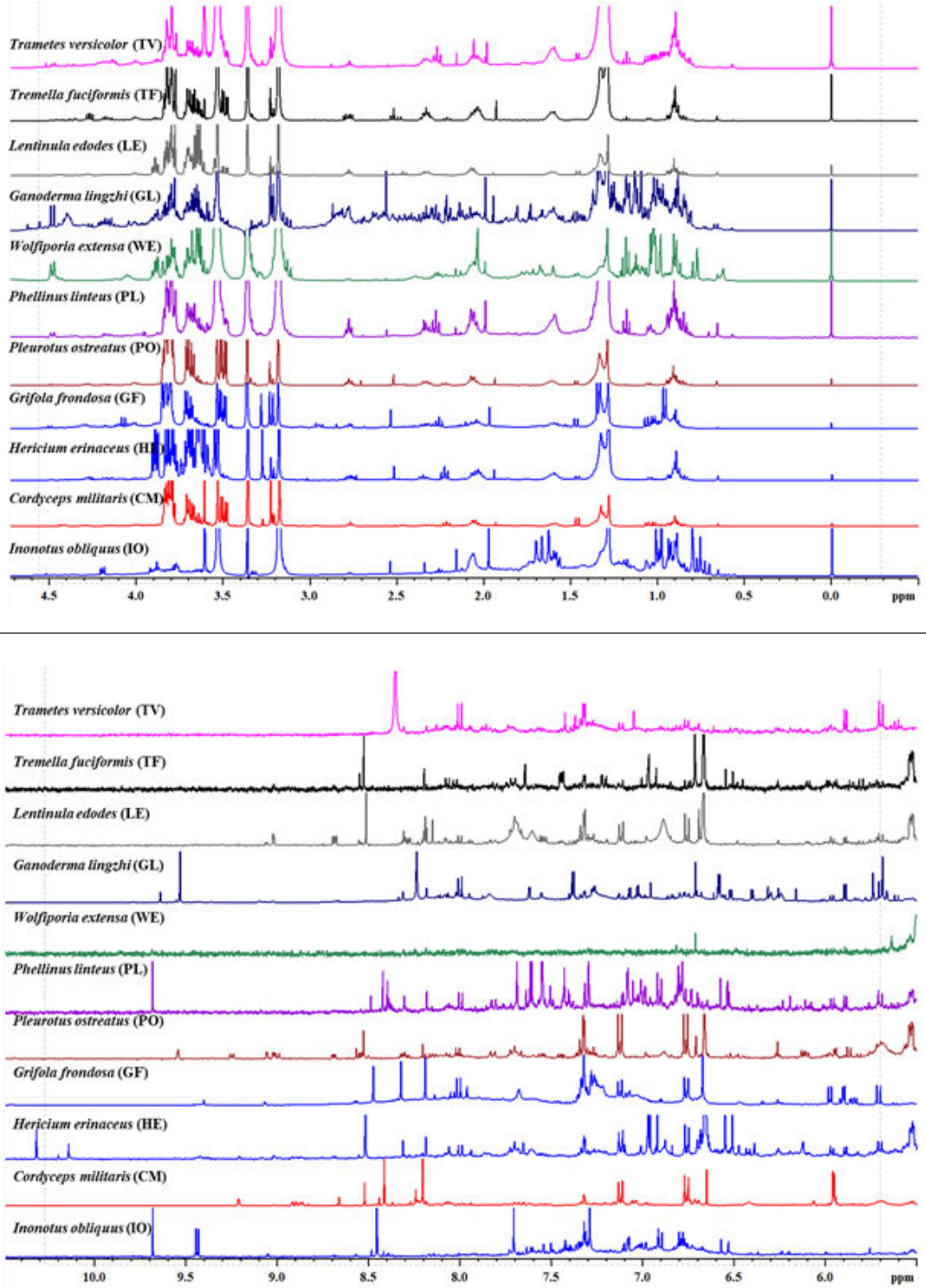


Figure 1. ^{1}H NMR spectra of the mushroom samples, top: 4.7-0 ppm; bottom: 10.5-5.5 ppm.

These spectra were binned and the spectral attributes such as the peak positions and their intensities in each bin were considered for a supervised multivariate statistical analysis, the results of which are plotted in ***Figure 2***. The linear discriminant analysis (LDA) successfully differentiates the sample pool into distinct clusters/ groups wherein the clusters represent different species, therefore attesting to the capability of NMR metabolomics as an analytical tool for species identification. The same approach can be implemented to establish the batch consistencies of the samples, the example of which is demonstrated using the Ganoderma lingzhi (GL) mushroom samples (***Figure 2***). The ordination plot consists of data points from mushroom products of the same type which are clustered within three ellipsoids marking different confidence intervals. The innermost ellipsoid marks ≥98% confidence level between the samples, i.e., a ca. 2% variation in their metabolite makeup. The second and third ellipsoids mark ≥95-<98%, and ≥90-<95% confidence intervals, respectively. Data points representing the samples which land in the grey area outside the three ellipsoids are graded as unrated and are considered to be inconsistent. The majority of the data points representing the GL samples from different batches fall within the innermost ellipsoid, inferring the spectral similarity to be of the highest degree (≥98%). Overall, the NMR metabolomics approach proves to be a versatile tool for mushroom species identification and establishing the consistencies of samples from different batches.

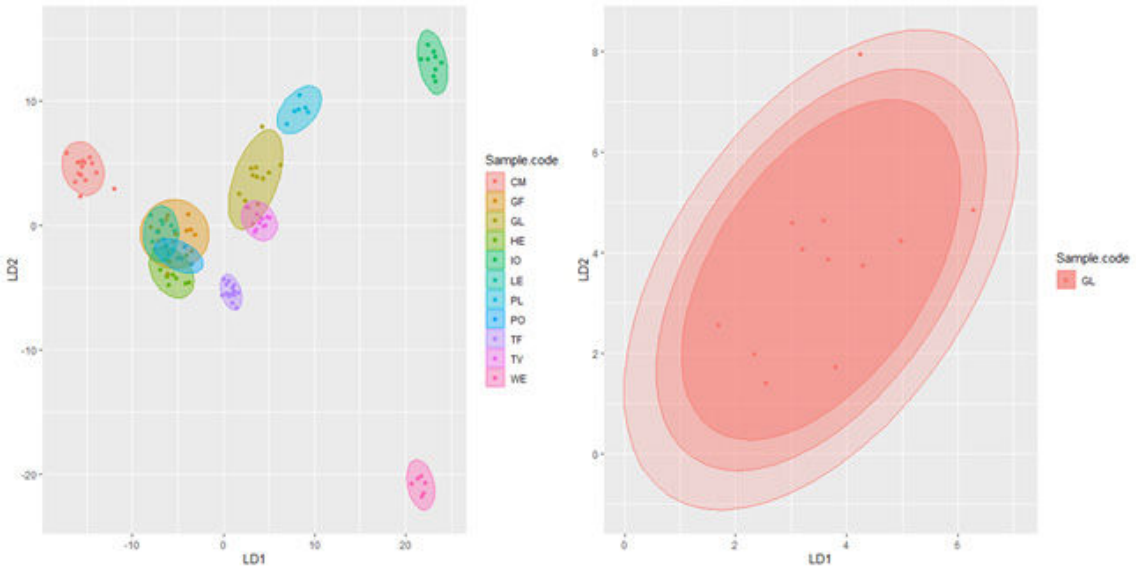


Figure 2. Linear discriminant analysis (LDA) plot (left) showing the clustering of data points representing different mushroom species, and an ordination plot (right) showing different confidence intervals (ellipsoids) and the consistency between *Ganoderma lingzhi* (GL) mushroom samples.

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info@purity-iq.com

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