Metabolomic discrimination of the edible mushrooms *Kuehneromyces mutabilis* and *Hypholoma capnoides* (Strophariaceae, Agaricales) by NMR spectroscopy

Aino-Liisa Alanne[1*], Jouni Issakainen[2], Kati Pihlaja[2], Johanna Jokioja[1], and Jari Sinkkonen[1]

[1]The Instrument Centre, Department of Chemistry, University of Turku, FI-20014 Turku, Finland; [2]Herbarium, Department of Biology, University of Turku, FI-20014 Turku, Finland

*Corresponding author: Tel: +358 50 966 4721. Fax: +358 29 450 5040. E-mail: alanne.aino@gmail.com

Abstract

Two edible, cultivable mushroom species of the family Strophariaceae; *Kuehneromyces mutabilis* (sheathed woodtuft) and *Hypholoma capnoides* (conifer tuft) were studied using proton nuclear magnetic resonance (^{1}H NMR) metabolomic approach. The variation in the metabolites of the two species and their metabolic behavior regarding caps and stipes and different collection sites were analyzed by multivariate analysis methods. Altogether 169 cap and stipe samples of the mushrooms were investigated. The clearest difference between the species was in the sugar composition, which was more diverse in *H. capnoides*. When mushroom samples collected from different locations were compared, more variance was found in *H. capnoides* whereas *K. mutabilis* appeared more homogeneous as a species. As far as the caps and stipes were concerned, in both species the amount of α-α-trehalose was clearly higher in the stipes and the caps contained a larger proportion of the amino acids and organic acids.

Keywords: *Kuehneromyces mutabilis*, *Hypholoma capnoides*, NMR metabolomics, multivariate analysis, cultivable mushrooms

1. Introduction

The worldwide production of cultivated mushrooms has increased tenfold during last 50 years and mushroom cultivation is a common part of agriculture in several countries [1]. Mushrooms have a high nutritional value since they are low in fat and contain fiber and essential amino acids not produced by the human body [2]. Besides the nutritional value and delicious taste of mushrooms, the popularity of mushroom cultivation arises also from the facts that it does not necessarily require high capital investment and can be rather easily organized. In addition, mushroom cultivation is a recycling process, which enables the utilization of renewable bio-resources, such as lignocellulosic waste, in food production.

Kuehneromyces mutabilis (sheathed woodtuft) and *Hypholoma capnoides* (conifer tuft) from the family Strophariaceae are edible, wood-decaying fungi growing in the wild on broad-leaved and coniferous trees, respectively. They can be cultivated on waste wood and thus lignocellulosic waste is recycled in the process. Despite the fact that these species have been traditionally gathered for food in numerous countries and cultivated in e.g. Germany, little of their chemistry is known so far. Polyacetylenes and total phenolic content of *K. mutabilis* were clarified earlier [3-4]. A couple of studies on nutrients of *H. capnoides* [5-6] and on the uptake of some metals (Cu, Zn, Cd, Pb) by both of the species have been published [7-8]. In addition, we have recently exploited these species in order to clarify whether dried herbarium specimens are utilizable in nuclear magnetic resonance (NMR) metabolomic studies of mushrooms [9].

In general, the chemistry of mushrooms is less studied compared to that of plants. The focus has mostly been on nutritional value [10-11] and metal contents of the mushrooms [12-14] as well as on the identification of toxins [15-16]. NMR studies on mushrooms have focused on the structural identification of extracted compounds such as toxins, polysaccharides and terpenes [17-20]. However, only very few papers on NMR metabolomics of mushrooms with rather small samplings have been published encompassing the species *Agaricus brasiliensis*, *Taiwanofungus camphoratus*, *Ganoderma lucidum, Lentinus edodes*, *Ophiocordyceps sinensis, Tricholoma matsutake* and *Amanita muscaria* [21-25].

NMR is most commonly used for identification of molecular structures of individual compounds but also for analysis of mixtures, quantitative analysis and for the study of chemical equilibrium processes, just to name a few. In metabolomics research, the metabolic profiles of different organisms are screened and compared to study biological phenomena. NMR is highly suitable for metabolomics, since it allows the detection of several metabolites (from dozen even to hundred) simultaneously in a quantitative manner. NMR is accurate and reproducible, which makes the analysis of large amount of samples essential to metabolomics studies comparable and trustworthy. Native serum and plasma, often used as sample matrices in metabolic screening, offer information e.g. on disease pathogenesis as well as metabolic biomarkers. Statistical methods, such as multivariate data analysis are utilized in spectral analysis to enable observing discrimination between studied groups. During the past decades, the number of NMR metabolomics studies has increased due to the development in automation starting from transferring samples to NMR tubes through the measurements to the automated spectral analysis using libraries of known metabolites [26-31]. Besides in medicine, NMR metabolomics has reached a strong position in plant science in order to explore various aspects of plant physiology and biology [32-33].

The aim of the present work was to apply ^{1}H NMR metabolomics method for the study of the metabolic aspects distinguishing two mushroom species *K. mutabilis* and *H. capnoides* from each other. Besides mapping the discriminating metabolites of these species, their behavior considering different parts of mushroom (caps and stipes) and different collection sites was compared. One goal of this study was to widen the perspective of NMR metabolomics of mushrooms with two cultivable, commonly eaten but not much studied species.

2. Materials and methods

2.1 Chemicals

D_2O (99.90% D), CD_3OD (99.80% D) and 3-(trimethylsilyl) propionic acid sodium salt (TSP) (98% D) were purchased from Euriso-top, KH_2PO_4 and K_2HPO_4 from J.T. Baker and NaN_3 (99%) from Sigma Aldrich.

2.2 Sample collection and preparation

The mushroom samples were collected from ten different locations in the area of Turku (within 50 km ratio from Turku), Southwest Finland during July - October 2015. Collection sites (i.e. locations) were defined as separate when they were at least 200 m from each other and the mycologist of the team estimated from e.g. physical obstacles of the terrain, that they could not form a continuous mycelial clone. Usually, the distance between collection sites was several kilometers.

Fruiting bodies (50 *K. mutabilis*, 50 *H. capnoides*) were collected in ten separate locations, 5 locations per species, ten fruiting bodies at each location. The collected mushrooms of each location were chosen by different sizes and ages to get as comprehensive as possible selection of the sample material. Old, damaged or insect-eaten fruiting bodies were not chosen as samples.

The fruiting bodies were divided into the analyzed samples as follows: The caps and the stipes were separated from each other by cutting straight below the gills and the short, visibly dirty portion in the base of each stipe was cut away and omitted from the analysis. All the caps were used separately as one sample each. In case a given stipe was bulky enough for analysis (21 *K. mutabilis*, 39 *H. capnoides*), it was used as a separate sample. However, fruiting bodies of 29 *K. mutabilis* and 11 *H. capnoides* had too small stipe for being analyzed alone. The too small stipes of the same collection site were combined from fruiting bodies of similar developmental phase into larger samples, using maximally four stipes / sample (see Tables S1 and S2 in Supporting Information). One too small *H. capnoides* stipe and three *K. mutabilis* stipes were not measured at all. In addition, due to measurement errors altogether seven samples needed to be left out of the analysis. In total 79 samples of *K. mutabilis* (49 cap, 19 stipes, 11 combined stipes) and 90 samples of *H. capnoides* (49 cap, 36 stipes, 5 combined stipes) were analyzed successfully. One additional voucher sample of each collection site was deposited in the Herbarium of the University of Turku (TUR) and the correct identification of the species was verified by a mycologist.

Immediately after collection (no more than 5 min), the samples were placed in plastic tubes (Low temperature freezer vials, VWR, Leuven, Belgium) and frozen in liquid nitrogen at the collection site. The sample tubes were kept in liquid nitrogen before freeze-drying. After drying, samples were grinded to fine powder and stored in -18 °C until extractions. A solution (v/v 1/1) of CD_3OD and 0.1 M phosphate buffer (KH_2PO_4

and K_2HPO_4) in D_2O (pH 7.0, 2 mM NaN_3, 5 mM TSP) was prepared. Next, 0.5 mL of the solution was added to 50 mg of the mushroom powder, sonicated for 10 min at room temperature, vortexed for 15 min and centrifuged (21130 g, 15 min). The supernatant was removed and the procedure repeated once. The supernatants were combined and 600 µl of the extract was taken for the NMR-measurements.

2.3 NMR analyses and metabolite identification

^1H spectra were measured with a Bruker Avance 500 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) operating at 500.13 MHz. Spectra were recorded at 25 °C with a non-spinning sample in a 5 mm NMR-tube (Norell S500) and locked for CD_3OD. The solvent residual signals of CHD_2OD at 3.31 and water/OH at 4.81 ppm were suppressed by the 1D version of noesyprph using double presaturation during relaxation delay and mixing time (lc1pnf2 pulse programme). 256 scans collecting 64k datapoints were measured. The acquisition time was 3.28 s, the recycle delay 5.0 s, mixing time 0.1 s and receiver gain 181. Metabolite identification was conducted by using ^1H, DQF-COSY and 1D-TOCSY NMR-measurements, Human Metabolome Database (HMDB; http://www.hmdb.ca/), Chenomx NMR Suite 8.1 software (evaluation version, Chenomx Inc., Edmonton, Canada) and the addition of reference compounds to the NMR sample.

2.4 Data processing

Spectral data were processed with the TopSpin 3.5 software (Bruker BioSpin GmbH, Rheinstetten, Germany). The spectra were baseline and phase corrected and referenced to the TSP signal at 0.00 ppm. Amix software (version 3.9.15, Bruker BioSpin GmbH, Rheinstetten, Germany) was used for binning the spectra (from 0.5 to 10 ppm) into widths of 0.005 ppm resulting in a total of 1900 integrated regions per spectrum. The bins of residual methanol and water signals (3.18 - 3.45 and 4.53 - 5.65, respectively) were excluded. Positive intensities was used as the integration mode and the spectral scaling was done to the total intensity.

2.5 Statistical analysis

The data was transferred to SIMCA-P+ 12.0.1 software (Umetrics AB, Umeå, Sweden) to perform the multivariate analyses. Total number of observations and variables were 169 and 1900, respectively. The datasets were Pareto-scaled and principal

component analysis (PCA) was used as the multivariate method. The signals of nine metabolites were clearly separated in the spectra and thus were chosen for the statistical comparison of the relative integrals by IBM SPSS Statistics 23 software (IBM Corp., Armonk, NY). The relative integrals of the metabolite signals were created by combining the buckets of the certain area, divided by the amount of protons in the signal and multiplying them so that the highest average of the integrals became 100. The statistical significance of the metabolite integral differences was examined by using the non-parametric Kruskal-Wallis test (34) and Mann-Whitney U test (35) with Bonferroni correction since the values were not normally distributed. Significance level was considered to be $p < 0.05$.

3. Results and discussion

3.1 Selection of suitable solvent system

NMR samples are usually measured in deuterated solvents due to measurement technical reasons. The variation of pH in NMR samples causes variation in the NMR spectra which affects the results of multivariate analysis. The pH of the mushroom samples in deuterated water (D_2O) varied from about 6 to 7 and required use of a buffer (0.1 M phosphate buffer (KH_2PO_4 and K_2HPO_4) in D_2O, pH 7.0, 2 mM NaN_3, 5 mM TSP). The stability of the samples was not good enough in buffer alone (changes in some metabolites were observed already after 7 hours of storage), and the addition of deuterated methanol (CD_3OD) was noticed to increase the stability remarkably. On the other hand, applying CD_3OD in the solvent system resulted in small changes in the metabolite profiles, but it provided reasonably wide overview on the mushroom metabolites with significantly enhanced sample stability. Moreover, the spectra of the deuterated chloroform ($CDCl_3$) extracts were measured but they were not informative and thus the comparisons were decided to be focused on the more water-soluble metabolites. According to the experiments, taking the stability and the solubility of the metabolites into account, 50% CD_3OD–D_2O-buffer was considered to be the most suitable solvent system.

3.2 ^{1}H NMR spectra, identification and statistical comparison of the metabolites

Table 1 shows 17 metabolites identified from the spectra of mushroom extracts. Visual inspection of the spectra revealed that the signals of the sugar region were most

dominant and that the concentration of α-α-trehalose (later referred as trehalose) was much higher compared to any other compounds (Table 1, Fig. S1, S2 and S3). Signals in the area of about 0.8 to 3 ppm gave clearly stronger intensities than the ones in the region of 6 to 9.5 ppm. Moreover, a singlet at 6.53 ppm arising from fumaric acid and the doublet of doublets signal of malic acid (4.28 ppm) stood out from the spectra due to their high intensities.

The signals that were clearly separated from other signals were chosen for the statistical comparison of the relative integrals. The relative integrals of nine metabolites were compared between the caps and stipes within each mushroom species. Similarly, the constituents of a given fruiting body part (cap or stipe) were compared between the two mushroom species. The results are shown as box and whisker plots in Fig. 1, where the line segments above the boxes indicate the statistical difference ($p < 0.05$) between the groups. The variation in the amounts of metabolites are discussed later to support the multivariate analysis results.

3.3 Multivariate analysis of *Hypholoma capnoides*

The PCA analysis of 49 cap and 41 stipe samples of *H. capnoides* -species collected from five different locations in Southwest Finland is shown in Fig. 2. In the PCA model ($R^2X_{(cum)} = 0.976$, $Q^2_{(cum)} = 0.941$) the first two components explained 67.8% of the total variation and showed rather clear separation of caps and stipes leaving the stipe samples on the positive side of the first component and most of the cap samples on the negative side (Fig. 2A). The corresponding loadings plot (Fig. 2C) revealed the variables causing separation on the first principal component. The signals of trehalose (for chemical shifts, see Table 1) were on the positive side and all the variables in the areas of 0.5 - 3 ppm and 5.5 - 9.5 ppm were on the negative side. Thus, there was more trehalose in the stipes and other compounds, such as amino acids and organic acids, in the caps.

Accordingly, the univariate statistical analysis showed statistically significant difference in the amount of trehalose between the caps and stipes (Fig. 1). However, the relative amount of β-glucose did not differ significantly between the caps and stipes. Malic acid, alanine, aspartic acid, fumaric acid, succinic acid and valine occured significantly less in the stipes.

Similar results were previously found in *Amanita muscaria* where higher concentrations of sugars were found in stipes and the metabolites the signals of which were in the aliphatic region more profuse in caps [22]. Trehalose protects several organisms from heat, cold, desiccation and anoxia [36]. In *Agaricus bisporus* trehalose was shown to be synthesized in the mycelium, translocated to the fruiting body and degraded to glucose. Glucose is further used by the mushroom as a carbon source for growth and mannitol production. [37]

The PCA model also showed grouping of samples collected from different locations (Fig. 2B). The cap samples of collection sites 1, 3 and 5 were overlapping quite in the middle of the two components. However, samples from site 5 were located slightly more on the negative side of the first component. The samples of two collection sites (2 and 4) were clearly discriminated from the others. Both the caps and stipes from site 2 were on the most negative side of the second component, stipes on the positive and caps on the negative side of the first component. The caps from site 4 were situated on the most negative side of the first component and on the positive side of the second component.

The spectra of cap samples from site 4 were visually compared with the ones from other geographical origins (sites 1, 3 and 5; individual samples locating in the middle of the scores plot). Samples from site 4 were noticed to contain fewer sugars (trehalose) and more amino acids such as isoleucine, leucine, valine, threonine, alanine, ornithine and glutamic acid than the other samples. They also had a rather strong singlet of acetic acid at 1.91 ppm, which did not occur in the spectra of other sites. Furthermore, the amount of citric acid was lower.

The production of metabolites in mushrooms is dependent on both growth conditions and genetic features. Differences in temperature, humidity, pH and nutrient availability as well as the stage of development affect the metabolite composition of mushrooms from different geographical origins. [2, 38] The size of a mushroom could indicate its development stage and thus explain differences in metabolites. The sizes of the fruiting bodies were variable since the dry weights (DW) of the caps varied from 78 mg to 1.10 g (Tables S1 and S2). The DWs of the caps from collection sites 1 - 3 were rather similar varying from 100 to 455 mg. The ones from site 4 were slightly smaller (78 - 147 mg) and the ones from site 5 bigger (197 - 1101 mg). Due to high variation

of the cap DWs inside the group, the discrimination of the collection sites could not be explained by sample size.

During the time scale of sample collection (the 1st – 16th of October) the weather conditions varied. As the samples from locations 3 - 5 were collected, the temperature had decreased a few degrees under 0 °C during nights but no clear effect of the low temperature on specific metabolites could be detected.

It is possible that the genetic variation of this species is quite wide and causes the metabolic differences between the samples from different locations. The chemical composition within a species can vary clearly, even if the mushrooms were cultivated and treated under the same conditions [38]. Zhang et al. showed in their NMR metabolomics study that the metabolic characteristics of *O. sinensis* grown in nature (three different locations) were more similar than the ones of cultured mycelia (three strains from different regions) [25]. In this study, where the growth circumstances of the mushrooms were completely natural, in addition to the genetic variation between fungal strains, several other variables such as substrate, accompanying microorganisms, preceding weather and other growth conditions may contribute to the chemical composition of a fruiting body.

3.4 Multivariate analysis of *Kuehneromyces mutabilis*

Fig. 3A shows the distinction between 49 cap and 30 stipe samples of the mushroom species *K. mutabilis* by PCA. Caps and stipes were separated by the first component in the PCA model ($R^2X_{(cum)}$ = 0.964, $Q^2_{(cum)}$ = 0.900) where the first two components accounted for 68.6% of the total variance. Most of the cap samples were on the negative side of the first component, and about one fifth of the total cap sample amount on the positive side, which was dominated by the stipe samples.

The amount of trehalose was clearly higher in stipes than in the caps of *K. mutabilis*, as can be seen in the loadings plot (Fig. 3C) and in Fig. 1 (statistically significant difference). Alanine, aspartic acid, β-glucose and succinic acid were found significantly more in the caps than in the stipes. The loadings plot was rather similar with the one of *H. capnoides* except for fumaric acid, which was seen more in the stipes than in the caps of *K. mutabilis*. However, when analyzed by univariate analysis, the difference was not significant.

Some minor grouping of the *K. mutabilis* samples from different locations could be seen (Fig. 3B). The cap samples from site 2 were located on the positive side of the first component unlike most of the other cap samples. Visual comparison of the cap spectra of location 2 with the cap spectra of other locations revealed that the samples from site 2 contained more trehalose. The samples from site 1 were collected at the end of July, two months earlier compared with the other four collection sites. Based on the PCA model the collection time did not affect much on the metabolites observed (Fig. 3B). The discrimination between samples according to growth locations was not as clear as by *H. capnoides* (Fig. 2B) which could refer to that *K. mutabilis* might be genetically more homogenous species than *H. capnoides*.

3.5 Comparison of the metabolites of *H. capnoides* and *K. mutabilis* by multivariate methods

The complete spectral dataset was analyzed by unsupervised PCA for objective interpretation. The observations were marked by different symbols according to species *H. capnoides* and *K. mutabilis* and by the different parts of the mushroom i.e. caps and stipes. The PCA model ($R^2X_{(cum)} = 0.973$, $Q^2_{(cum)} = 0.937$) showed somewhat clear separation between both the species by the second component and between caps and stipes by the first component (Fig 4A). The two components explained 60.9 % of the total variation. *K. mutabilis* samples were situated on the positive side of the second component while most of the *H. capnoides* samples were on the negative side, except a group from location 4 that clearly separated from the others.

The spectra of the caps of these two species were analyzed in detail to clarify the distinction between metabolites. Typically only the caps of these species are collected for eating because the structure and the taste of the stipes are unpleasant. The PCA analysis ($R^2X_{(cum)} = 0.962$, $Q^2_{(cum)} = 0.887$) for the caps was conducted excluding the dissimilar collection site 4 of *H. capnoides* to better observe the differences between species and to simplify the interpretation of the loadings plot (Fig. 4B and 4C). The first two principal components explained 56.1% of the model and the caps of the species were nicely separated by the first component leaving *K. mutabilis* caps on the positive and *H. capnoides* caps on the negative side.

The most significant difference in the metabolites was the number of different sugars, which was higher in *H. capnoides*. In addition to trehalose, in cap spectra of *H.*

capnoides there were four signals of sugar anomeric protons (4.59, 4.55, 4.53, 4.41 ppm) with coupling constants of 7.9, 7.6, 7.6 and 7.9 Hz, respectively (Fig. S1, Fig. 4C). The sugar with the anomeric proton shift at 4.59 ppm was identified as β-glucose by 1D TOCSY -measurement. In addition, β-xylose (anomeric proton shift at 4.53 ppm) could be identified based on the addition of the reference compound and 1D TOCSY -spectrum. The 1D TOCSY -spectra of the two other anomeric protons (4.55 and 4.41 ppm) resembled very closely the one of β-xylose and were deduced to be derivatives of β-xylose. Nevertheless, the exact structures could not be determined since the concentration of the mushroom extracts was not enough for high quality ^{1}H - ^{13}C - correlation spectra. The multiplet signals at 4.09 - 4.14 and 3.92 - 4.02 ppm at least partially arising from these sugars were only detected in the spectra of *H. capnoides*.

Previously, also mannitol (0.38 ± 0.04 g/100g DW) was detected in *H. capnoides* by high performance liquid chromatography (HPLC) [6]. It is definitely possible, that mannitol was present in small amounts in our *H. capnoides* samples as well. However, since the signals were overlapping extensively with the ones of trehalose, it could not be detected. In *K. mutabilis* caps there was only a very tiny signal of β-glucose (significantly less than in *H. capnoides* caps, Fig. 1). Besides β-glucose and trehalose no other sugars were visible in *K. mutabilis* caps. Despite the number of sugars was higher in the caps of *H. capnoides* the relative amount of trehalose was significantly higher in the caps of *K. mutabilis* (Fig. 1). Likewise, the stipes of *K. mutabilis* contained significantly more trehalose compared to the *H. capnoides* stipes.

The sugar content of the mushrooms can contribute to the fungal metabolism. Glucose, mannitol and trehalose mainly represent the sugar content of the mushrooms [39-40]. Little attention has been paid on the different carbohydrates of mushrooms, however. [41-42] In addition to the previously mentioned sugars and xylose found in *H. capnoides;* ribose, fructose, sucrose and mannose, for example, have been found in different mushrooms [2, 41]. In *Agaricus bisporus* the amount of mannitol was much higher compared to other sugars and it was shown to increase during the mushroom growth, whereas the concentrations of trehalose and fructose remained constant [42].

Another substantial difference between the two species studied by us was observed in the aromatic region of the spectra where the two doublets of tyrosine (chemical shifts in Table 1, Fig. S3) were detected solely in *K. mutabilis*. Furthermore, the relative

amounts of malic acid, fumaric acid and succinic acid were significantly higher in *K. mutabilis* caps, as compared to the caps of *H. capnoides* whereas alanine was found significantly more in the *H. capnoides* caps (Fig. 1). *K. mutabilis* stipes contained more malic acid, fumaric acid and valine than *H. capnoides* stipes, other differences being not statistically significant. The heterogeneity of *H. capnoides* species was also visible in box and whisker plots (Fig. 1) where several outliers were detected and e.g. in the case of trehalose the deviation was high.

4. Conclusions

In this NMR metabolomics study statistically significant differences in the metabolites of *H. capnoides* and *K. mutabilis* were found. Caps and stipes of both species were discriminated clearly in the PCA model. *H. capnoides* showed heterogeneity in relation to growth locations whereas *K. mutabilis* was a more homogeneous species. The usability of the used NMR metabolomics method concerning subsequent mushroom research was demonstrated and the number of samples was high enough to get reliable results on discrimination. In future, the NMR method could be applied to studying variable aspects of mushrooms, e.g. different fruiting body parts (hymenium, flesh etc.), the cooking or preserving methods (fresh, dried, boiled, frozen) and even to comparing different species, families and other taxa.

Aknowledgements

Jukka Vauras (Herbarium, Åbo Akademi University) is kindly thanked for mycological expert help.

Supporting information description

^{1}H NMR spectra of selected mushroom samples and their zoomed figures (**Fig. 1S** - **Fig. 3S**) and tables including the dry weights of the caps and stipes of collected mushroom samples (**Tables 1S and 2S**) are found in the Supporting Information.

References

1. Zhang Y, Geng W, Shen Y, Wang Y, Dai YC. Edible mushroom cultivation for food security and rural development in China: Bio-innovation, technological dissemination and marketing. *Sustain* 2014;6(5):2961–73.

2. Reis FS, Barros L, Martins A, Ferreira ICFR. Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: An inter-species comparative study. *Food Chem Toxicol* 2012;50:191–197.

3. Del Signore A, Romeo F, Giaccio M. Content of phenolic substances in basidiomycetes. *Mycol Res* 1997;101:552–556.

4. Hearn MTW, Jones ERH, Pellatt MG, Thaller V, Turner JL. Natural acetylenes. Part XLII. Novel C7, C8, C9, and C10 polyacetylenes from fungal cultures. *J Chem Soc, Perkin Trans* 1973;1:2785–88.

5. Heleno SA, Barros L, Sousa MJ, Martins A, Ferreira ICFR. Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. *Food Chem* 2010;119:1443–50.

6. Heleno SA, Barros L, Sousa MJ, Martins A, Ferreira ICFR. Study and characterization of selected nutrients in wild mushrooms from Portugal by gas chromatography and high performance liquid chromatography. *Microchem J* 2009;93:195–199.

7. Petkovšek SAS, Pokorny B. Lead and cadmium in mushrooms from the vicinity of two large emission sources in Slovenia. *Sci Total Environ* 2013;443:944–954.

8. Vinichuk MM. Copper, zinc, and cadmium in various fractions of soil and fungi in a Swedish forest. *J Environ Sci Health. A Tox Hazard Subst Environ Eng* 2013;48:980–987.

9. Jafari T, Alanne A-L, Issakainen J, Pihlaja K, Sinkkonen J. Suitability of dried herbarium specimens for NMR metabolomics of mushrooms. A comparison of four species of the genera *Kuehneromyces* and *Hypholoma* (Strophariaceae). Fungal Biol 2018;122:138-146.

10. Manzi P, Aguzzi A, Pizzoferrato L. Nutritional value of mushrooms widely consumed in Italy. *Food Chem* 2001;73:321–325.

11. Mattila P, Könkö K, Eurola M, Pihlava J-M, Astola J, Vahteristo L, et al. Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *J Agric Food Chem* 2001;49:2343–2348.

12. Mendil D, Uluözlü ÖD, Tüzen M, Hasdemir E, Sarı H. Trace metal levels in mushroom samples from Ordu, Turkey. *Food Chem* 2005;91:463–467.

13. Ouzouni PK, Veltsistas PG, Paleologos EK, Riganakos KA. Determination of metal content in wild edible mushroom species from regions of Greece. *J Food Compos Anal* 2007;20:480–486.

14. Zavastina D, Biliutăb G, Dodic G, Macsimb A-M, Lisac G, Ghermana S, et al. Metal content and crude polysaccharide characterization of selected mushrooms growing in Romania. *J Food Compos Anal* 2018;67:149-158.

15. Gonmori K, Fujita H, Yokoyama K, Watanabe K, Suzuki O. Mushroom toxins: a forensic toxicological review. *Forensic Toxicol* 2011;29:85–94.

16. Matsuura M, Kato S, Saikawa Y, Nakata M, Hashimoto K. Identification of

cyclopropylacetyl-(*R*)-carnitine, a unique chemical marker of the fatally toxic mushroom *Russula subnigricans*. Chem Pharm Bull 2016;64:602-608.

17. Ghosh K, Chandra K, Ojha AK, Islam SS. NMR and MALDI-TOF analysis of a water-soluble glucan from an edible mushroom, *Volvariella diplasia*. *Carbohydr Res* 2008;343:2834–2840.

18. Kleinwächter P, Luhmann U, Schlegel B, Heinze S, Härtl A, Kiet TT, et al. New fasciculol-type triterpene compounds from *Hypholoma fasciculare*. *J Basic Microbiol* 1999;39:345–349.

19. Pramanik M, Chakraborty I, Mondal S, Islam SS. Structural analysis of a water-soluble glucan (Fr.I) of an edible mushroom, *Pleurotus sajor-caju*. *Carbohydr Res* 2007;342:2670–2675.

20. Yin X, Feng T, Shang JH, Zhao YL, Wang F, Li ZH, et al. Chemical and toxicological investigations of a previously unknown poisonous European mushroom *Tricholoma terreum*. *Chemistry* 2014;20:7001–7009.

21. Cho IH, Kim YS, Choi HK. Metabolomic discrimination of different grades of pine-mushroom (*Tricholoma matsutake* Sing.) using ^{1}H NMR spectrometry and multivariate data analysis. *J Pharm Biomed Anal* 2007;43:900–904.

22. Deja S, Wieczorek PP, Halama M, Jasicka-Misiak I, Kafarski P, Poliwoda A, et al. Do differences in chemical composition of stem and cap of *Amanita muscaria* fruiting bodies correlate with topsoil type? *PLoS One* 2014;9:1–20.

23. Lo Y-C, Chien S-C, Mishchuk D, Slupsky C, Mau J-L. Quantification of water-soluble metabolites in medicinal mushrooms using proton NMR spectroscopy. *Int J Med Mushrooms* 2016;18(5):413–424.

24. Wen H, Kang S, Song Y, Song Y, Sung SH, Park S. Differentiation of cultivation sources of *Ganoderma lucidum* by NMR-based metabolomics approach. *Phytochem Anal* 2010;21:73–79.

25. Zhang J, Zhong X, Li S, Zhang G, Liu X. Metabolic characterization of natural and cultured *Ophicordyceps sinensis* from different origins by ^{1}H NMR spectroscopy. *J Pharm Biomed Anal* 2015;115:395–401.

26. Cevallos-Cevallos JM, Etxeberria E, Danyluk MD, Rodrick GE. Metabolomic analysis in food science: a review. *Trends Food Sci Technol* 2009;20:557–566.

27. Kortesniemi M, Vuorinen AL, Sinkkonen J, Yang B, Rajala A, Kallio H. NMR metabolomics of ripened and developing oilseed rape (*Brassica napus*) and turnip rape (*Brassica rapa*). *Food Chem* 2015;172:63–70.

28. Kortesniemi M, Sinkkonen J, Yang B, Kallio H. ^{1}H NMR spectroscopy reveals the effect of genotype and growth conditions on composition of sea buckthorn (*Hippophaë rhamnoides* L.) berries. *Food Chem* 2014;147:138–146.

29. Bingol, K. Recent advances in targeted and untargeted metabolomics by NMR and MS/NMR methods. *High-Throughput* 2018, 7, 9.

30. Larive CK, Barding Jr GA, Dinges MM. NMR Spectroscopy for metabolomics and

metabolic profiling. *Anal Chem* 2015, 87, 133-146.

31. Würtz P, Kangas AJ, Soininen P, Lawlor DA, Smith GD, Ala-Korpela M. Quantitative serum nuclear magnetic resonance metabolomics in large-scale epidemiology: a primer on -omic technologies. *Am J Epidemiol* 2017, 186(9), 1084–1096.

32. Hong J, Yang L, Zhang D, Shi J. Plant metabolomics: an indispensable system biology tool for plant science. *Int J Mol Sci* 2016;17:767.

33. Schripsema J. Application of NMR in plant metabolomics: techniques, problems and prospects. *Phytochem Anal* 2010;21:14-21.

34. Kruskal WH, Wallis WA. Use of ranks in one-criterion variance analysis. *J Am Stat Assoc* 1952, 47, 583-621.

35. Mann HB, Whitney, DR. On a test of whether one of two random variables is stochastically larger than the other. *Ann Math Stat* 1947, **18**(1), 50–60.

36. Elbein AD. New insights on trehalose: a multifunctional molecule. *Glycobiology* 2003;13:17R–27.

37. Wannet WJB, Aben EMJ, van der Drift C, van Griensven LJLD, Vogels GD, Op den Camp HJM. Trehalose phosphorylase activity and carbohydrate levels during axenic fruiting in three *Agaricus bisporus* strains. *Curr Microbiol* 1999;39: 205–210.

38. Manzi P, Gambelli L, Marconi S, Vivanti V, Pizzoferrato L. Nutrients in edible mushrooms: an inter-species comparative study. *Food Chem* 1999;65:477–482.

39. Kalač P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. *J Sci Food Agric* 2013;93:209–218.

40. Kalač P. Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chem* 2009;113:9–16.

41. Kim M-Y, Chung I-M, Lee S-J, Ahn J-K, Kim E-H, Kim M-J, et al.. Comparison of free amino acid, carbohydrates concentrations in Korean edible and medicinal mushrooms. *Food Chem* 2009,113:386–393.

42. Wannet WJB, Hermans JHM, van der Drift C, Op den Camp HJM. HPLC Detection of soluble carbohydrates involved in mannitol and trehalose metabolism in the edible mushroom *Agaricus bisporus*. *J Agric Food Chem* 2000;48:287–291.

Table 1. Identified compounds of *Hypholoma capnoides* and *Kuehneromyces mutabilis* and their ^{1}H NMR shifts in 50% CD_3OD - phosphate buffer (D_2O, pH 7) mixture at 25 ^{0}C.

Compound	Position	δ, ppm[1]
Isoleucine	δ-CH_3	0.96 (t, 7.4)
	γ'-CH_3	1.03 (d, 7.0)
Leucine	δ-CH_3, δ'-CH_3	0.98 (t, 6.5)
Valine	γ-CH_3	1.01 (d, 7.0)
	γ'-CH_3	1.05 (d, 7.0)
Threonine	γ-CH_3	1.34 (d, 6.6)
Alanine	β-CH_3	1.49 (d, 7.2)
Ornithine	γ-CH_2	1.71 - 1.88 (m)
	β-CH_2	1.88 - 2.00 (m)
	δ-CH_2	3.04 (t, 6.7)
Acetic acid [a]		1.91 (s)
Succinic acid	H2, H2', H3, H3'	2.41 (s)
Glutamic acid	β-CH_2	2.02 - 2.19 (m)
	γ-CH_2	2.39 (t, 7.6)
Malic acid	H3	2.36 (dd, 10.0, 15.2)
	H3'	2.67 (dd, 3.0, 15.2)
	H2	4.28 (dd, 3.0, 10.0)
Citric acid	H3a, H3'a	2.48 - 2.59 (m)
	H3b, H3'b	2.70 - 2.75 (m)
Aspartic acid	β-CH	2.65 (dd, 9.1, 17.3)
	β'-CH	2.81 (dd, 3.6, 17.3)
β-Xylose	H1	4.53 (d, 7.9)
β-Glucose	H1	4.59 (d, 7.9)
α-α-Trehalose	H1	5.18 (d, 3.9)
	H2	3.59 (dd, 3.9, 9.9)
	H3, H6a	3.83 - 3.88 (m)
	H4	3.41 (dd, 9.4, 9.4)
	H5	3.80 - 3.83 (m)
	H6b	3.73 (dd, 5.3, 12.0)
Fumaric acid	H2, H3	6.53 (s)
Tyrosine[b]	δ-CH, δ'-CH	6.85 (d, 8.5)
	ε-CH, ε'-CH	7.18 (d, 8.5)
Phenylalanine	δ-CH, δ'-CH, ε-CH, ε'-CH, ζ-CH	7.32 - 7.43 (m)

1) Multiplicity of the signals and the coupling constants in hertz shown in the parenthesis after the chemical shift. Meaning of the letters: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet.

a) Found only in *H. capnoides* samples from collection site D

b) Found only in *K. mutabilis* samples

Figures

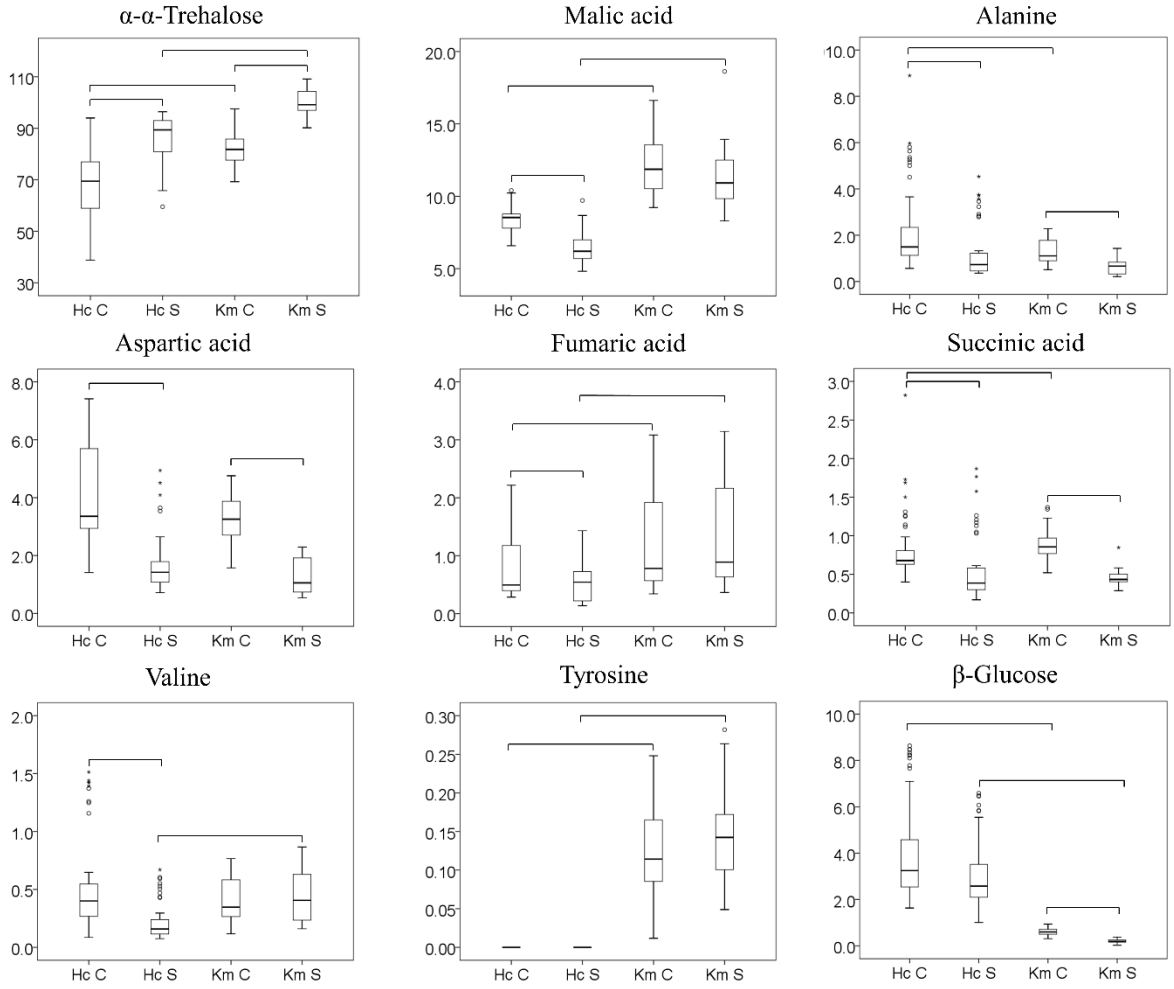

Figure 1. Box and whisker plots of the chosen metabolites of *Kuehneromyces mutabilis* and *Hypholoma capnoides* cap and stipe samples. The line segments above the boxes indicate statistically significant difference in the significance level $p < 0.05$. Y-axis scale is relative to the signal integrals.

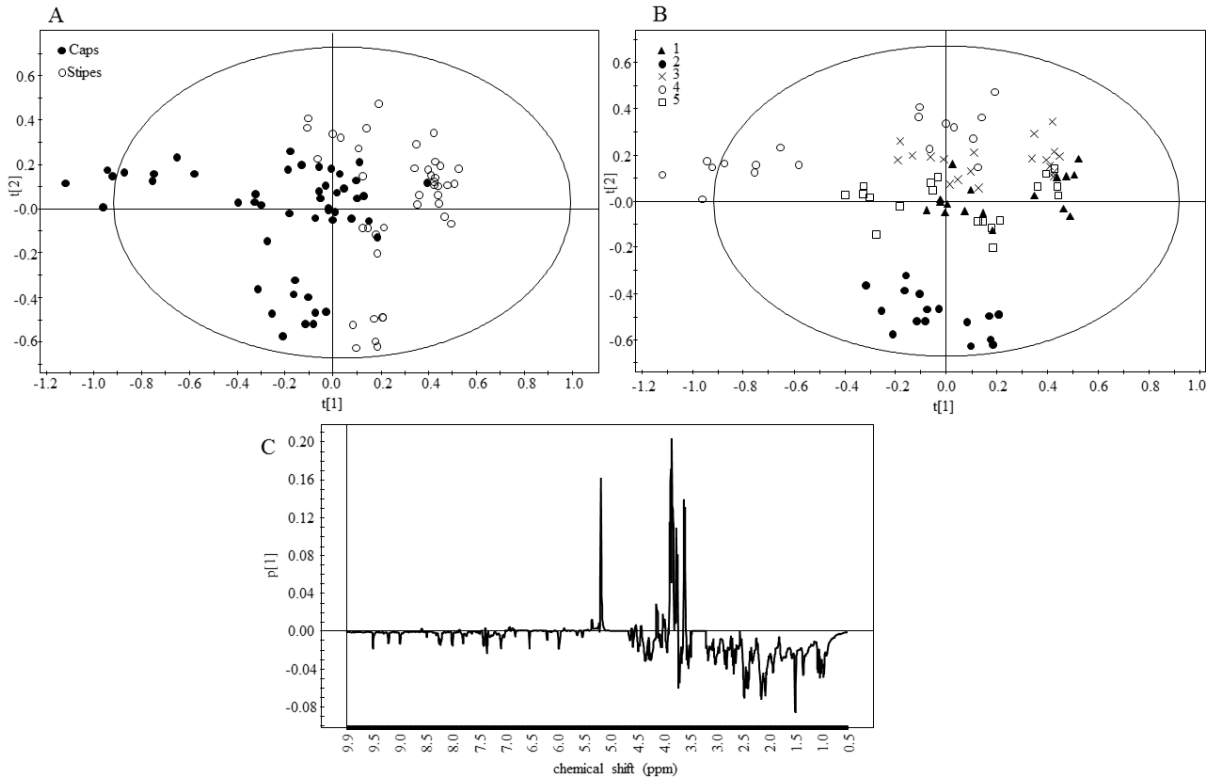

Figure 2. PCA model ($R^2X_{(cum)}$ = 0.976, $Q^2_{(cum)}$ = 0.941) of *Hypholoma capnoides* cap and stipe samples. (A) Scores plot *t*[2] vs. *t*[1], caps and stipes marked by different symbols, (B) Scores plot *t*[2] vs. *t*[1], observations marked according to growth locations 1 - 5, (C) The PCA loadings line plot p[1].

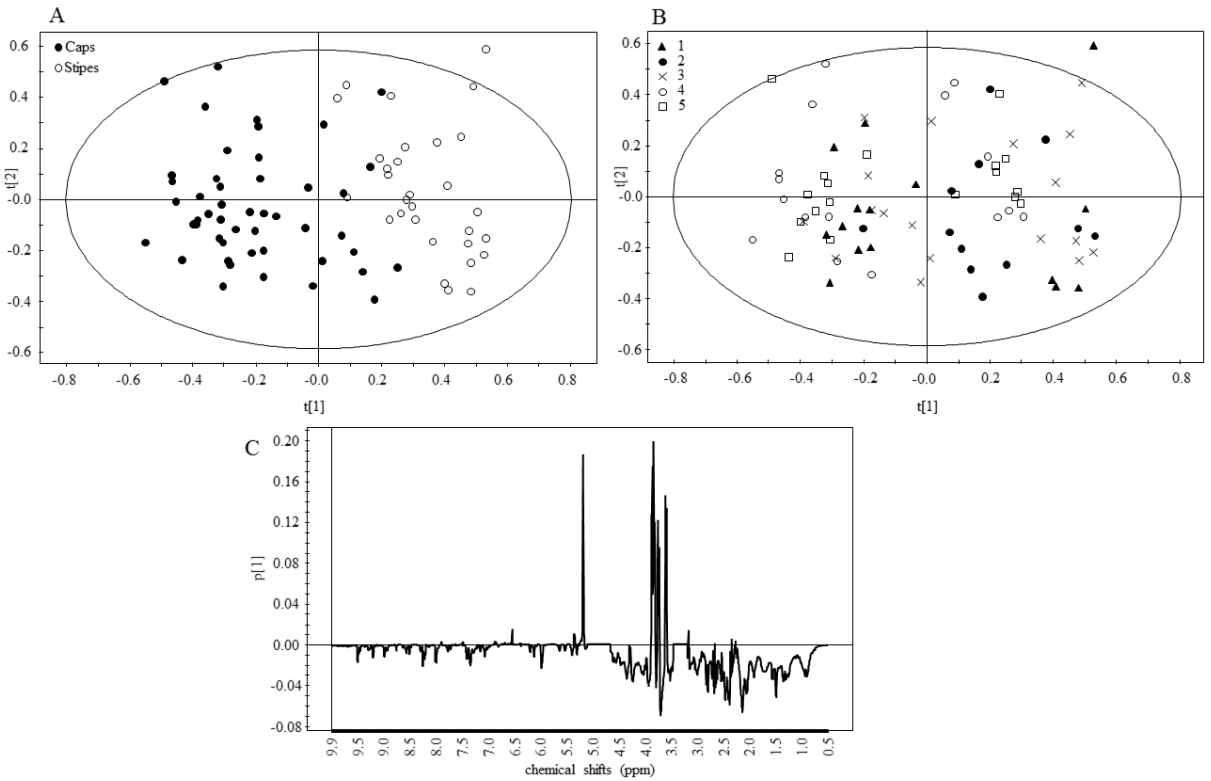

Figure 3. PCA model ($R^2X_{(cum)}$ = 0.964, $Q^2_{(cum)}$ = 0.900) of *Kuehneromyces mutabilis* cap and stipe samples. (A) Scores plot *t*[2] vs. *t*[1], caps and stipes marked by different symbols, (B) Scores plot *t*[2] vs. *t*[1], observations marked according to growth locations 1 - 5, (C) The PCA loadings line plot p[1].

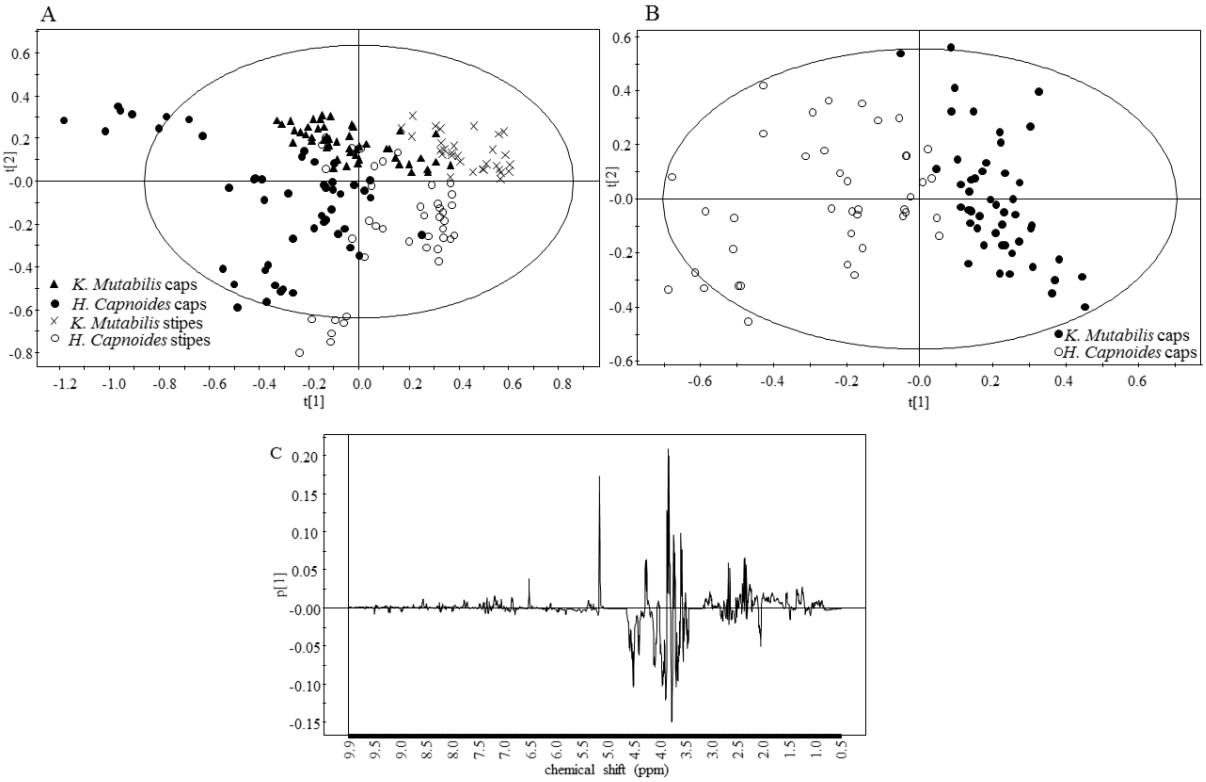

Figure 4. (A) The PCA model ($R^2X_{(cum)}$ = 0.973, $Q^2_{(cum)}$ = 0.937) of *Kuehneromyces mutabilis* and *Hypholoma capnoides* cap and stipe samples, n = 169, scores plot *t*[2] vs. *t*[1], (B) The PCA model ($R^2X_{(cum)}$ = 0.962, $Q^2_{(cum)}$ = 0.887) of *Kuehneromyces mutabilis* and *Hypholoma capnoides* caps, n = 89 (collection site 4 of *H. capnoides* samples excluded), scores plot *t*[2] vs. *t*[1], (C) The PCA loadings line plot of the same model as in B (p[1]).