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# Non-targeted and targeted analysis of wild toxic and edible mushrooms using gas chromatography–ion trap mass spectrometry

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## ABSTRACT

Mushrooms are known all over the world both due to the remarkable gastronomic value of some species and for severe intoxications mediated by other species that are frequently difficult to distinguish from the edible ones, by the common user. Therefore, it is important to develop strategies to discover molecules that can identify mushroom species. In the present work, two GC–MS methodologies were applied in the chemical characterization of 22 mushroom species (12 edible, 3 toxic and 7 potentially toxic) – a multi-target procedure to simultaneously determine amino acids (AA), fatty acids (FA) and sterols by previous derivatization procedure with MSTFA, and a Head Space-Solid Phase Microextraction method to determine volatiles. For both methods, two approaches to data analysis were used: (I) targeted analysis, to identify and quantify AA, FA sterols and volatiles; (II) untargeted analysis, including Principal Component Analysis and Partial Least Square Discriminant Analysis, in order to identify metabolites/metabolite pattern with potential species identification and/or differentiation. Multi-target experiment allowed the identification and quantification of twenty one primary metabolites (9 AA, 11 FA and 1 sterol). Furthermore, through untargeted data analysis, it was possible to identify a 5-carbon sugar alcohol structure molecule, which was tentatively identified as xylitol or adonitol, with potential to be a species-marker of the edible *Suillus bovinus* mushrooms. Volatile profiling studies resulted in the identification of the main volatiles in mushrooms. Untargeted analysis allowed the identification of 6 molecules that can be species- or genus-specific: one secondary metabolite specific to the edible species *Lycoperdon perlatum*, an ester of hexanoic acid, tentatively identified as allyl or vinyl caproate; and five other secondary metabolites, whose identification was not achieved, which were only detected in *Lactarius aurantiacus* specimens (edibility/toxicity unknown).

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## 1. Introduction

In recent years there has been an increased interest in wild edible mushrooms. Their high popularity in many countries around the world, is mainly due to their gastronomic value, owned by unique organoleptic properties, such as aroma and flavor [1,2]. Additionally, mushrooms' nutritional value and pharmacological potentialities have increased their importance and consumption [3]. Indeed, mushrooms are rich in proteins, amino acids, vitamins

and minerals and have low caloric and fat contents [4–6], and some compounds recently isolated from mushrooms have shown promising immunomodulatory, antitumoral, antioxidant, cardiovascular, antiviral, antibacterial, antiparasitic, antihypercholesterolemic, hepatoprotective and antidiabetic properties [7]. In many countries, including Portugal, mushrooms picking in forests and grasslands is a common family activity [8]. Although mushrooms have been mainly collected by pickers to own consumption, they recently gained importance in the rural economy due to their commercialization, especially of wild edible ectomycorrhizal species [8].

However, some mushroom species contain toxins and their consumption could result in severe, sometimes lethal, intoxications. The consumption of toxic mushrooms is, usually, due to

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misidentification of such specimens with an edible species, since some edible and toxic mushrooms are morphologically very similar [9–11]. Despite the very small number of toxic or poisonous species in the nature, the high risk of misidentification with toxic species, create a potential barrier to mushroom marketing in many places. The proper identification of a species is the only safe way to ensure edibility.

The classical method for identifying mushroom species involves macroscopic and microscopic examination of tissues, spores and sporing structures. This method is not accurate due to gaps and inconsistencies in morphology-based identification. Moreover, climatic conditions and other environmental factors can change the macroscopic characteristics of the species, making it more difficult to identify.

Once the unequivocal identification of a mushroom species through this process is very difficult [12,13], it is essential to find other features for correctly identifying the species – mainly those which can have clinical value, i.e., properties which can be rapidly identified in an emergency/hospital context. Thus, the study of the chemical composition of mushroom species constitutes an important strategy to identify chemotaxonomical markers [14].

The use of chemical composition [14–20] and/or DNA analysis [21–25] of species to its identification and classification has already been applied in the study of mushrooms. Although a large number of mushrooms' metabolites have been identified and characterized, few studies regarded the chemotaxonomic importance/utility of these molecules. Even though, different groups of molecules have been analyzed with chemotaxonomical purposes (e.g. amino acids [20] and fatty acids [19]), DNA and secondary metabolites (e.g. pigments [16,17] and volatiles [18]) are the most used in this context [15].

In the present work, two GC–MS methodologies were applied to the chemical characterization of 22 mushroom species (12 edible, 3 toxic and 7 potentially toxic). The first one was a multi-target procedure to simultaneously determine amino acids (AA), fatty acids (FA) and sterols by using a derivatization process; the second one was a HS-SPME/GC–MS method to study the volatile profiles of the species. In both methodologies, data analysis was conducted in two separate ways: (i) targeted analysis to identify and quantify expected metabolites, such as AA, FA and sterols and to identify the most common volatiles present in mushrooms; and (ii) non-targeted analysis through multivariate analysis, namely Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA), metabolites/metabolite pattern with chemotaxonomical value.

## 2. Material and methods

### 2.1. Samples

Five to seven specimens of each of 22 different wild mushroom species were collected in Trás-os-Montes and Douro Litoral regions of Portugal (Table 1). After harvest, species were taxonomically identified by using morphological features and appropriate keys and monographs including Courtecuisse [26] and Courtecuisse and Duhem [27]. Following identification, three different carpophores of each species were cleaned and fragmented into small pieces. 5 g of each species were put into cap-sealed 20 mL vials and frozen at  $-20\text{ }^{\circ}\text{C}$  until HS-SPME–GC–MS analysis. The remaining of each mushroom was lyophilized and frozen. Lyophilized samples were powdered and screened through a  $910\text{ }\mu\text{m}$  fine sieve before being stored in hermetically sealed bags until extraction (multi-target experiment). The species harvested in 2010 were stored frozen, hence volatile analysis was not performed on those species (Table 1). Representative voucher

specimens were deposited at the herbarium of School of Agriculture of the Polytechnic Institute of Bragança (Portugal).

### 2.2. Standards

Reference compounds, with the highest purity, used in multi-target experiment were purchased from two suppliers: alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, *trans*-4-hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, norvaline, arachidonic acid, capric acid, docosahexaenoic acid (DHA), 5,8,11,14,17-eicosapentaenoic acid (EPA), gondoic acid, lauric acid, margaric acid, linoleic acid, linolenic acid, myristic acid, oleic acid, palmitic acid, pelargonic acid, pentadecyclic acid and stearic acid, as well as methyl linoleate, cholesterol, cholestanol, ergosterol, fucosterol,  $\beta$ -sitosterol, desmosterol and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were obtained from Sigma (St. Louis, MO, USA); absolute ethanol was obtained from Carlo Erba Reagents (Milan, Italy).

For volatile profiling, the reference compounds were obtained from Sigma (St. Louis, MO, USA) – *trans*-2-octenal, *trans*-2-octen-1-ol and linalool; and from SAFC (Steinheim, Germany) – 1-octen-3-ol, 3-octanol and 3-octanone.

### 2.3. Preparation of standard solutions

For multi-target experiments, stock solutions of amino acids, fatty acids, sterols and internal standards (IS) – norvaline ( $0.30\text{ mg mL}^{-1}$ ), methyl linoleate ( $10.00\text{ mg mL}^{-1}$ ) and desmosterol ( $2.00\text{ mg mL}^{-1}$ ), were prepared individually in absolute ethanol and kept at  $-20\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  until analysis.

The stock solutions of volatile standards ( $0.25\text{ mg mL}^{-1}$ ) were prepared individually in deionized water and kept at  $4\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  until analysis.

### 2.4. Multi-target experiment

The multi-target experiment methodology was performed according to Pereira et al. [28], with some minor modifications.

#### 2.4.1. Amino acids, fatty acids and sterols metabolites extraction

$100.00 \pm 2.00\text{ mg}$  of lyophilized sample were transferred to a glass vial and the internal standards were added:  $80\text{ }\mu\text{L}$  of norvaline ( $0.30\text{ mg mL}^{-1}$ ),  $20\text{ }\mu\text{L}$  of methyl linoleate ( $10.00\text{ mg mL}^{-1}$ ), and  $80\text{ }\mu\text{L}$  of desmosterol ( $2.00\text{ mg mL}^{-1}$ ). The volume was then completed to  $2.00\text{ mL}$  with absolute ethanol. Samples were vortexed for 1 min and then filtered through a  $0.45\text{ }\mu\text{m}$  membrane (Millipore). A  $50\text{ }\mu\text{L}$  aliquot of extract was transferred to a glass vial, the solvent evaporated under nitrogen stream, and  $50\text{ }\mu\text{L}$  of the derivatization reagent MSTFA added to the dried residue. The vial was capped, vortexed and heated for 20 min in a dry block heater maintained at  $40\text{ }^{\circ}\text{C}$ . All analyses were performed in triplicate.

#### 2.4.2. Gas chromatography–ion trap–mass spectrometry analysis

GC–MS analysis was performed with a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 4000 ion trap mass selective detector and a Saturn GC/MS workstation software version 6.8. A VF-5 ms ( $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$ ) column (VARIAN) was used. A CombiPAL automatic autosampler (Varian, Palo Alto, CA) was used for all experiments. The injector port was heated to  $250\text{ }^{\circ}\text{C}$ . Injections were performed in split mode, with a ratio of 1/40. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of  $1\text{ mL min}^{-1}$ . The oven temperature was set at  $100\text{ }^{\circ}\text{C}$  for

**Table 1**  
Characterization of the mushroom species analyzed.

Code	Species	Edibility	Origin	Habitat	Date of collection	Analysis methodology
AA	<i>Agrocybe aegerita</i> (V. Brig.) Singer	Edible	Porto	<i>Platanus x acerifolia</i>	Nov. 2011	MultiT+Vol
AC	<i>Amanita caesarea</i> (Scop.) Pers.	Edible	Bragança	<i>Castanea sativa</i>	Sept. 2011	MultiT+Vol
AM	<i>Amanita muscaria</i> (L.) Lam.	Toxic	Bragança	<i>Castanea sativa</i>	Dec. 2011	MultiT+Vol
AS	<i>Agaricus sylvicola</i> (Vittad.) Perck	Edible	Bragança	Meadow	Nov. 2011	MultiT+Vol
AV	<i>Amanita vaginata</i> (Bull.) Lam.	Toxic <sup>a,c,d</sup>	Bragança	<i>Castanea sativa</i>	Oct. 2010	MultiT
BE	<i>Boletus edulis</i> Bull.	Edible	Bragança	<i>Castanea sativa</i>	Sept. 2011	MultiT+Vol
CB	<i>Collybia butyracea</i> (Bull.) P. Kumm.	Edible	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Nov. 2011	MultiT+Vol
CD	<i>Clitocybe dealbata</i> (Sowerby) P. Kumm.	Toxic	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Nov. 2011	MultiT+Vol
HS	<i>Hebeloma sinapizans</i> (Paulet) Gillet	Toxic <sup>a,c,e</sup>	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Nov. 2011	MultiT+Vol
LA	<i>Lactarius aurantiacus</i> (Pers.) Gray	Toxic <sup>b</sup>	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Dec. 2011	MultiT+Vol
LC	<i>Lactarius controversus</i> (Pers.) Pers.	Toxic <sup>a,c</sup>	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Oct. 2010	MultiT
LP	<i>Lycoperdon perlatum</i> Pers.	Edible	Bragança	<i>Castanea sativa</i>	Nov. 2011	MultiT+Vol
MP	<i>Macrolepiota procera</i> (Scop.) Singer	Edible	Bragança	<i>Castanea sativa</i>	Nov. 2011	MultiT+Vol
MR	<i>Mycena rosea</i> Gramberg	Toxic <sup>a,f</sup>	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Nov. 2011	MultiT+Vol
RC	<i>Russula cyanoxantha</i> (Schaeff.) Fr.	Edible	Bragança	<i>Castanea sativa</i>	Oct. 2010	MultiT
RD	<i>Russula delicata</i> Fr.	Edible	Bragança	<i>Castanea sativa</i>	Oct. 2010	MultiT
RL	<i>Rhizopogon luteolus</i> Fr.	Toxic <sup>b</sup>	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Dec. 2011	MultiT+Vol
SB	<i>Suillus bovinus</i> (Pers.) Roussel	Edible	Bragança	<i>Pinus pinaster</i>	Dec. 2011	MultiT+Vol
SI	<i>Sarcodon imbricatus</i> (L.) P. Karst.	Edible	Bragança	<i>Pinus pinaster</i>	Nov. 2011	MultiT+Vol
TA	<i>Tricholoma acerbum</i> (Bull.) Vent.	Toxic <sup>a,c</sup>	Bragança	<i>Castanea sativa</i>	Nov. 2011	MultiT+Vol
TE	<i>Tricholoma equestre</i> (L.) P. Kumm.	Toxic	Bragança	<i>Pinus pinaster</i>	Dec. 2011	MultiT+Vol
TP	<i>Tricholoma portentosum</i> (L.) P. Kumm.	Edible	Bragança	<i>Castanea sativa</i> + <i>Pinus pinaster</i>	Dec. 2011	MultiT+Vol

MultiT – Multi-target experiment ; Vol – Volatile profiling experiment.

<sup>a</sup> Suspected to be toxic – Considered toxic for comparison purposes.

<sup>b</sup> Edibility/Toxicity unknown – Considered toxic for comparison purposes.

<sup>c</sup> It is suspected that causes gastrointestinal disorders.

<sup>d</sup> It is suspected that causes hemolytic disorders.

<sup>e</sup> May have cytotoxic cucurbitacins.

<sup>f</sup> It is suspected to contain the toxin muscarine.

1 min then increasing 20 °C min<sup>-1</sup> to 250 °C held for 2 min, 10 °C min<sup>-1</sup> to 300 °C and held for 10 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first 2.5 min to avoid solvent overloading. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50, and 180 °C, respectively. The scanned mass ranged from 50 to 600 *m/z* with a scan rate of 6 scan/s. The emission current was 50 μA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25.000 μs, with an ionization storage level of 35 *m/z*. The injection volume was 2 μL and the analysis was performed in Full Scan mode.

The identification of AA, FA and sterols was achieved by comparison of their retention times and mass spectra with those from pure standards analyzed under the same conditions, and from NIST05 MS Library Database, respectively. For quantification and comparison purposes, each sample was injected in triplicate and the amount of metabolites present in samples was achieved from the calibration curves of the respective full scan TMS standards. The linoleic and oleic acids were quantified using the diagnostic ions *m/z* 262, 337 and 352 and *m/z* 264, 339 and 354, respectively.

## 2.5. Volatile profiling experiment

### 2.5.1. SPME fibers

Several commercial fibers can be used to extract volatile compounds. According to previous experiments [2] the fiber chosen was coated with divinylbenzene/polydimethylsiloxane (DVB/PDMS), 65 μm.

### 2.5.2. HS-SPME extraction and gas chromatography–ion trap-mass spectrometry analysis

Previous to HS-SPME extraction, mushroom samples were unfrozen for 10 min at room temperature.

HS-SPME extraction was performed with CombiPAL automatic autosampler (Varian, Palo Alto, CA). In pre-incubation, samples were stirred at 500 rpm for 5 min at 40 °C. Then the fiber was exposed to headspace for 45 min (250 rpm, 40 °C). Afterwards, the fiber was pulled into the needle sheath, the SPME device was removed from the vial and inserted into the injection port of GC system for thermal desorption. After 4 min, the fiber was removed and conditioned for 10 min at 250 °C in the same chromatograph.

The same GC–MS was used with different operational conditions. The injector port was heated to 220 °C. The injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 ml min<sup>-1</sup>. The oven temperature was set at 40 °C for 1 min, then increased at 5 °C min<sup>-1</sup> to 250 °C held for 20 min. All mass spectra were acquired in EI mode. Ionization was maintained off during the first minute. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50, and 180 °C, respectively. The mass ranged from 50 to 600 *m/z* with a scan rate of 6 scan/s. The emission current was 50 μA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25.000 μs, with an ionization storage level of 35 *m/z*. All analyses were performed in Full Scan mode.

Volatile compounds were identified in samples by comparing the retention times/Kovats indices of the chromatographic peaks with those of standards, when available, analyzed in the same condition, and also by comparison of MS fragmentation patterns with the mass spectra present in the NIST 05 MS Library Database, when standards are not available.

## 2.6. Data analysis

### 2.6.1. Multi-target experiment

Two different approaches were used to analyze multi-target experiment chromatographic data: targeted and non-targeted approaches.

In targeted approach, three classes of compounds were studied – AA, FA and sterols. Quantification of such metabolites was performed by using calibration curves according to the method previously reported by Pereira et al. [28]. The evaluation of statistical significance was performed by ANOVA, followed by Tukey's HSD test. The level of significance was set at  $p < 0.05$  (95% statistical confidence level). The PCA of quantified metabolites was also performed. This analysis was carried out using SPSS software, version 20.0 (IBM corporation, NY, U.S.A.).

In non-targeted approach, the ASCII file of chromatographic data obtained from each sample was extracted and a matrix created containing all the chromatogram data. The matrix was imported to Excel for manual chromatogram alignment. The main objective of this procedure is to correct small differences in retention times. Then, chromatograms were normalized by the IS desmosterol, through division of the intensity of each scan (expressed as kcount) by the intensity of the higher desmosterol scan, i.e., the intensity of each data point (scan) was converted to a ratio of "scan intensity"/"intensity of desmosterol high intense scan". After normalization, data were submitted to multivariate analysis with PCA and PLS-DA using The Unscrambler X version 10 (Camo Software, Oslo, Norway). PCA shows similarities between samples projected on a plane and makes it possible to determine which variables determine these similarities and in what way. PLS validation was performed by cross-validation method.

The TIC (total ion current) chromatogram alignment and processing was used as screening procedure in order to extract more information from the data set using a non-target approach, by collapsing MZ dimension some subtle will be lost. This first step is done to supervise and validate the metabolic differences between species. Next step would use more sophisticated signal processing techniques each allows us to presence the 3D structure of the GCMS data from each sample.

## 2.6.2. Volatile profiling experiment

A non-targeted approach was used to compare the volatile profiles of mushroom samples. The ASCII file of spectra data obtained from each sample was extracted and a matrix was created containing all chromatograms. The matrix was imported to Excel for manual spectral alignment. Then chromatograms were normalized through division of intensity of each scan (expressed as kcount) by the sum of intensities of all scans, i.e., the intensity of each data point (scan) was converted to a ratio of "scan intensity"/"sum of scan intensities". After normalization, data were submitted to multivariate analysis with PCA and PLS-DA using the same software described in Section 2.6.1.

## 3. Results and discussion

### 3.1. Multi-target experiment

The previous established multi-target method for the identification and quantification of AA, FA and sterols [28] was successfully applied to all 22 mushroom species. Despite AA, FA and sterols compositions were already studied for several mushroom species [1,4,29–31], no reference has been found in literature in which these compounds were studied through a single multi-target methodology. Furthermore, to our best knowledge this is the first time that these compounds were studied in some toxic species.

#### 3.1.1. Targeted approach: AA, FA and sterols profiling

The applied extraction and derivatization procedure allowed the quantification of 21 compounds in the mushroom samples – 9 AA, 11 FA and 1 sterol (Tables 2–4).

It was possible to identify 13 AA, and among them 9 were quantified (alanine, isoleucine, glycine, leucine, phenylalanine, proline,

**Table 2**  
Quantification of amino acids in mushroom samples (mg/100 g fw).

Mushroom species	Amino acids <sup>1</sup> [Retention time]									
	Ala [3,21 min]	Gly [3,34 min]	Val [3,97 min]	Leu [4,35 min]	Ile [4,50 min]	Pro [4,58 min]	Ser [4,92 min]	Thr [5,09 min]	Phe [6,69 min]	Total <sup>2</sup>
AA	121 (16) <sup>bcd</sup>	nd <sup>a</sup>	15.1 (0.2) <sup>efgh</sup>	4.26 (0.42) <sup>a</sup>	16.5 (2.2) <sup>ef</sup>	3.63 (0.72) <sup>ab</sup>	2.82 (1.30) <sup>ab</sup>	2.63 (0.48) <sup>abc</sup>	4.02 (0.10) <sup>a</sup>	170 (16) <sup>bcd</sup>
AC	169 (1) <sup>def</sup>	13.3 (3.0) <sup>bc</sup>	12.1 (1.7) <sup>bcd</sup>	34.1 (8.5) <sup>d</sup>	17.1 (1.3) <sup>f</sup>	9.41 (1.62) <sup>abc</sup>	12.8 (1.8) <sup>ab</sup>	9.80 (1.59) <sup>bcd</sup>	37.9 (11.0) <sup>ghi</sup>	316 (17) <sup>ef</sup>
AM	118 (12) <sup>bcd</sup>	19.6 (9.6) <sup>c</sup>	7.79 (1.85) <sup>abc</sup>	28.1 (6.4) <sup>d</sup>	5.80 (0.92) <sup>abc</sup>	3.35 (1.27) <sup>ab</sup>	5.15 (0.23) <sup>ab</sup>	4.95 (1.24) <sup>abcd</sup>	34.0 (10.1) <sup>fgh</sup>	227 (22) <sup>cde</sup>
AS	347 (7) <sup>g</sup>	1.75 (1.45) <sup>a</sup>	22.2 (4.3) <sup>hij</sup>	23.2 (2.9) <sup>bcd</sup>	35.0 (4.9) <sup>h</sup>	20.2 (14.3) <sup>c</sup>	21.2 (8.8) <sup>ab</sup>	11.1 (0.6) <sup>cde</sup>	33.3 (4.1) <sup>fgh</sup>	515 (34) <sup>h</sup>
AV	124 (1) <sup>bcd</sup>	2.20 (1.03) <sup>a</sup>	19.2 (2.2) <sup>ghi</sup>	34.3 (2.5) <sup>d</sup>	20.1 (2.0) <sup>fg</sup>	2.55 (0.64) <sup>ab</sup>	3.36 (1.22) <sup>ab</sup>	3.82 (0.91) <sup>abcd</sup>	26.1 (5.5) <sup>defg</sup>	236 (12) <sup>de</sup>
BE	503 (4) <sup>h</sup>	56.3 (6.9) <sup>e</sup>	25.7 (5.0) <sup>ij</sup>	55.2 (14.5) <sup>e</sup>	25.5 (6.2) <sup>g</sup>	12.2 (4.9) <sup>bc</sup>	53.7 (27.84) <sup>d</sup>	30.1 (12.5) <sup>f</sup>	45.3 (6.7) <sup>hij</sup>	807 (78) <sup>j</sup>
CB	209 (13) <sup>f</sup>	1.09 (0.28) <sup>a</sup>	16.2 (1.9) <sup>efghi</sup>	8.35 (0.87) <sup>a</sup>	20.0 (2.2) <sup>fg</sup>	5.61 (0.69) <sup>ab</sup>	23.1 (2.6) <sup>ab</sup>	12.6 (0.4) <sup>de</sup>	16.0 (1.6) <sup>abcde</sup>	312 (21) <sup>ef</sup>
CD	153 (32) <sup>def</sup>	nd <sup>a</sup>	2.42 (0.20) <sup>ab</sup>	3.10 (0.38) <sup>a</sup>	4.66 (0.32) <sup>ab</sup>	2.65 (0.43) <sup>ab</sup>	14.9 (3.8) <sup>ab</sup>	8.13 (1.55) <sup>abcd</sup>	11.2 (0.4) <sup>abc</sup>	200 (37) <sup>bcd</sup>
HS	117 (27) <sup>bcd</sup>	2.36 (0.67) <sup>a</sup>	3.78 (0.52) <sup>abc</sup>	4.55 (0.33) <sup>a</sup>	6.43 (0.80) <sup>abcd</sup>	2.94 (0.17) <sup>ab</sup>	7.10 (1.40) <sup>ab</sup>	2.71 (0.08) <sup>abc</sup>	19.8 (0.93) <sup>bcd</sup>	167 (30) <sup>bcd</sup>
LA	283 (13) <sup>g</sup>	nd <sup>a</sup>	4.95 (1.13) <sup>abcd</sup>	3.53 (0.06) <sup>a</sup>	3.09 (0.23) <sup>ab</sup>	3.74 (0.19) <sup>ab</sup>	24.4 (4.4) <sup>bc</sup>	7.09 (0.56) <sup>abcd</sup>	9.89 (2.04) <sup>abc</sup>	340 (11) <sup>fg</sup>
LC	181 (2) <sup>def</sup>	35.3 (2.5) <sup>d</sup>	53.6 (13.1) <sup>k</sup>	63.1 (6.3) <sup>e</sup>	35.6 (4.8) <sup>h</sup>	11.0 (6.6) <sup>abc</sup>	19.9 (4.8) <sup>ab</sup>	nd <sup>a</sup>	61.3 (5.7) <sup>k</sup>	461 (52) <sup>gh</sup>
LP	178 (15) <sup>def</sup>	4.58 (1.84) <sup>a</sup>	8.77 (1.63) <sup>ab</sup>	6.57 (1.26) <sup>a</sup>	15.0 (1.5) <sup>ef</sup>	12.6 (1.0) <sup>bc</sup>	48.1 (6.2) <sup>cd</sup>	18.4 (0.8) <sup>e</sup>	24.4 (5.0) <sup>cdefg</sup>	316 (33) <sup>ef</sup>
MP	698 (64) <sup>j</sup>	19.0 (5.8) <sup>c</sup>	17.3 (2.4) <sup>fghi</sup>	9.37 (1.68) <sup>ab</sup>	13.5 (3.3) <sup>cdef</sup>	11.7 (2.5) <sup>bc</sup>	119 (17) <sup>e</sup>	43.6 (5.7) <sup>g</sup>	11.9 (1.7) <sup>abcd</sup>	943 (80) <sup>k</sup>
MR	76.5 (8.8) <sup>ab</sup>	2.16 (1.34) <sup>a</sup>	3.90 (0.43) <sup>abc</sup>	7.32 (0.59) <sup>a</sup>	6.55 (0.12) <sup>abcd</sup>	3.35 (0.47) <sup>ab</sup>	6.48 (0.46) <sup>ab</sup>	5.63 (0.45) <sup>abcd</sup>	13.8 (1.6) <sup>abcd</sup>	126 (10) <sup>abc</sup>
RC	184 (9) <sup>ef</sup>	5.57 (1.12) <sup>ab</sup>	32.1 (2.0) <sup>j</sup>	51.9 (4.2) <sup>e</sup>	37.8 (3.4) <sup>h</sup>	6.63 (2.05) <sup>ab</sup>	5.83 (3.21) <sup>ab</sup>	11.5 (3.0) <sup>cde</sup>	49.7 (4.8) <sup>ijk</sup>	385 (9) <sup>fg</sup>
RD	90.0 (8) <sup>bc</sup>	2.80 (2.08) <sup>a</sup>	6.28 (0.27) <sup>abcd</sup>	25.6 (5.4) <sup>cd</sup>	9.12 (0.25) <sup>bcd</sup>	4.45 (1.47) <sup>ab</sup>	12.9 (1.1) <sup>ab</sup>	7.73 (0.65) <sup>abcd</sup>	28.9 (3.9) <sup>efg</sup>	185 (7) <sup>bcd</sup>
RL	15.9 (2.9) <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	0.991 (0.065) <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	2.75 (0.23) <sup>ab</sup>	1.39 (0.21) <sup>ab</sup>	5.84 (0.26) <sup>ab</sup>	26.9 (3.6) <sup>a</sup>
SB	580 (29) <sup>i</sup>	2.86 (0.10) <sup>a</sup>	13.7 (2.6) <sup>cdefgh</sup>	5.80 (1.19) <sup>a</sup>	3.53 (2.62) <sup>ab</sup>	nd <sup>a</sup>	nd <sup>a</sup>	5.53 (0.74) <sup>abcd</sup>	53.7 (8.9) <sup>jk</sup>	665 (31) <sup>i</sup>
SI	114 (6) <sup>bcd</sup>	nd <sup>a</sup>	0.968 (0.356) <sup>a</sup>	2.66 (0.28) <sup>a</sup>	4.68 (0.85) <sup>ab</sup>	2.28 (0.11) <sup>ab</sup>	13.8 (4.4) <sup>ab</sup>	3.08 (0.89) <sup>abcd</sup>	10.6 (0.1) <sup>abc</sup>	152 (11) <sup>bcd</sup>
TA	85.8 (7.8) <sup>bc</sup>	nd <sup>a</sup>	3.21 (0.24) <sup>abc</sup>	12.9 (1.6) <sup>abc</sup>	6.59 (0.30) <sup>abcd</sup>	2.42 (0.05) <sup>ab</sup>	nd <sup>a</sup>	8.69 (0.72) <sup>abcd</sup>	6.60 (1.11) <sup>ab</sup>	126 (9) <sup>abc</sup>
TE	290 (13) <sup>g</sup>	1.82 (0.01) <sup>a</sup>	8.10 (0.12) <sup>abcd</sup>	9.01 (0.46) <sup>a</sup>	13.9 (0.9) <sup>def</sup>	6.32 (1.32) <sup>ab</sup>	15.1 (1.6) <sup>ab</sup>	5.80 (0.76) <sup>abcd</sup>	12.4 (0.2) <sup>abcd</sup>	363 (9) <sup>fg</sup>
TP	85.9 (19.6) <sup>bc</sup>	nd <sup>a</sup>	1.06 (0.12) <sup>a</sup>	13.5 (1.3) <sup>abc</sup>	1.91 (0.73) <sup>ab</sup>	2.19 (0.18) <sup>ab</sup>	nd	2.48 (0.32) <sup>abc</sup>	7.28 (0.54) <sup>ab</sup>	114 (20) <sup>ab</sup>
<b>Average total</b>	215	11.4	13.26	18.5	14.4	6.47	21.7	9.85	23.8	325 <sup>3</sup>

<sup>1</sup> Results are expressed as mean (standard deviation) of three determinations.

<sup>2</sup> Mean of total AA of three determinations (data not shown).

<sup>3</sup> Mean of totals (values in the same column). nd – not detected; Ala – alanine; Gly – glycine; Val – valine; Leu – leucine; Ile – isoleucine; Pro – proline; Ser – serine; Thr – threonine; Phe – phenylalanine. Values not sharing the same superscript letter (a–k) within the vertical column are different according to the Tukey test ( $p < 0.05$ )



**Table 3**  
Quantification of fatty acids in mushroom samples (mg/100 g fw).

Mushroom species	Fatty acids <sup>1</sup> [Retention time]											
	Pel (C9:0)	Cap (C10:0)	Lau (C12:0)	Myr (C14:0)	Pen (C15:0)	Pal (C16:0)	Mar (C17:0)	Lin (C18:2, n-6)	Ole(C18:1, n-9)	Ste (C18:0)	Ara (C20:0)	Total <sup>2</sup>
	[5,00 min]	[5,64 min]	[6,81 min]	[7,89 min]	[8,39 min]	[8,91 min]	[9,49 min]	[9,95 min]	[10,01 min]	[10,14 min]	[11,80 min]	
AA	nd <sup>a</sup>	nd <sup>a</sup>	6.57 (1.83) <sup>abcd</sup>	55.0 (0.9) <sup>b</sup>	nd <sup>a</sup>	94.5 (9.8) <sup>bc</sup>	1.95 (0.41) <sup>ab</sup>	6.42 (0.26) <sup>abc</sup>	24.4 (4.6) <sup>ab</sup>	14.3 (7.6) <sup>abc</sup>	nd <sup>a</sup>	203 (11) <sup>bcde</sup>
AC	4.48 (0.56) <sup>b</sup>	0.517 (0.108) <sup>ab</sup>	30.7 (12.5) <sup>c</sup>	93.2 (5.6) <sup>c</sup>	nd <sup>a</sup>	238 (56) <sup>ef</sup>	3.52 (2.68) <sup>bc</sup>	5.42 (0.12) <sup>a</sup>	41.2 (4.2) <sup>ab</sup>	132 (23) <sup>i</sup>	2.73 (1.36) <sup>b</sup>	552 (81) <sup>g</sup>
AM	nd <sup>a</sup>	0.498 (0.008) <sup>ab</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	178 (14) <sup>de</sup>	nd <sup>a</sup>	5.82 (0.37) <sup>ab</sup>	1172 (51) <sup>e</sup>	131 (6) <sup>i</sup>	11.5 (0.7) <sup>d</sup>	1499 (36) <sup>j</sup>
AS	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	7.22 (0.08) <sup>cd</sup>	93.5 (8.1) <sup>bc</sup>	2.65 (0.28) <sup>bc</sup>	12.1 (2.6) <sup>bcde</sup>	nd <sup>a</sup>	22.5 (3.4) <sup>abcd</sup>	nd <sup>a</sup>	135 (12) <sup>abcd</sup>
AV	4.12 (0.14) <sup>b</sup>	0.609 (0.167) <sup>b</sup>	14.5 (1.1) <sup>d</sup>	nd <sup>a</sup>	nd <sup>a</sup>	191 (2) <sup>de</sup>	1.64 (0.12) <sup>ab</sup>	5.88 (0.17) <sup>ab</sup>	nd <sup>a</sup>	67.8 (2.5) <sup>fg</sup>	nd <sup>a</sup>	286 (4) <sup>ef</sup>
BE	4.52 (0.37) <sup>b</sup>	0.480 (0.012) <sup>ab</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	28.3 (0.8) <sup>a</sup>	1.74 (0.21) <sup>ab</sup>	5.03 (0.09) <sup>a</sup>	nd <sup>a</sup>	4.86 (0.43) <sup>a</sup>	nd <sup>a</sup>	44.9 (0.7) <sup>a</sup>
CB	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	60.8 (9.6) <sup>ab</sup>	2.10 (0.04) <sup>bc</sup>	10.8 (0.1) <sup>abcd</sup>	26.9 (1.8) <sup>ab</sup>	39.5 (3.2) <sup>cde</sup>	nd <sup>a</sup>	140 (11) <sup>abcd</sup>
CD	nd <sup>a</sup>	nd <sup>a</sup>	5.12 (0.01) <sup>ab</sup>	nd <sup>a</sup>	nd <sup>a</sup>	48.2 (14.5) <sup>ab</sup>	1.78 (0.25) <sup>ab</sup>	6.39 (0.11) <sup>abc</sup>	19.2 (6.7) <sup>ab</sup>	17.7 (7.3) <sup>abcd</sup>	nd <sup>a</sup>	98.7 (26.8) <sup>abc</sup>
HS	5.62 (0.46) <sup>b</sup>	4.18 (0.78) <sup>cd</sup>	5.35 (0.06) <sup>abc</sup>	nd <sup>a</sup>	nd <sup>a</sup>	76.3 (7.7) <sup>ab</sup>	1.78 (0.17) <sup>ab</sup>	21.4 (7.4) <sup>e</sup>	85.6 (21.2) <sup>b</sup>	33.3 (7.0) <sup>bcd</sup>	nd <sup>a</sup>	234 (48) <sup>cde</sup>
LA	nd <sup>a</sup>	nd <sup>a</sup>	6.40 (1.64) <sup>abcd</sup>	nd <sup>a</sup>	nd <sup>a</sup>	50.7 (0.4) <sup>ab</sup>	nd <sup>a</sup>	8.04 (0.29) <sup>abcd</sup>	50.9 (5.0) <sup>ab</sup>	64.0 (2.9) <sup>efg</sup>	nd <sup>a</sup>	180 (5) <sup>abcde</sup>
LC	nd <sup>a</sup>	0.689 (0.141) <sup>ab</sup>	29.6 (1.6) <sup>d</sup>	nd <sup>a</sup>	nd <sup>a</sup>	286 (28) <sup>f</sup>	nd <sup>a</sup>	8.44 (1.52) <sup>abcd</sup>	270 (32) <sup>cd</sup>	457 (15) <sup>k</sup>	12.1 (1.2) <sup>d</sup>	1064 (57) <sup>i</sup>
LP	nd <sup>a</sup>	nd <sup>a</sup>	5.28 (0.34) <sup>abc</sup>	nd <sup>a</sup>	nd <sup>a</sup>	49.5 (8.8) <sup>ab</sup>	2.42 (0.39) <sup>bc</sup>	7.19 (0.03) <sup>abc</sup>	10.1 (3.2) <sup>ab</sup>	14.7 (8.5) <sup>abc</sup>	nd <sup>a</sup>	89.2 (20.6) <sup>ab</sup>
MP	4.55 (0.23) <sup>b</sup>	nd <sup>a</sup>	6.04 (0.79) <sup>abcd</sup>	nd <sup>a</sup>	9.99 (4.95) <sup>d</sup>	140 (29) <sup>cd</sup>	2.86 (0.70) <sup>bc</sup>	13.7 (3.5) <sup>d</sup>	44.5 (5.0) <sup>ab</sup>	29.1 (7.3) <sup>abcd</sup>	nd <sup>a</sup>	251 (51) <sup>def</sup>
MR	7.68 (2.05) <sup>c</sup>	4.37 (0.22) <sup>d</sup>	5.67 (0.67) <sup>abc</sup>	nd <sup>a</sup>	2.54 (0.23) <sup>ab</sup>	107 (25) <sup>bc</sup>	2.01 (0.88) <sup>ab</sup>	9.54 (3.73) <sup>abcd</sup>	53.6 (6.9) <sup>ab</sup>	60.8 (9.8) <sup>efg</sup>	nd <sup>a</sup>	253 (40) <sup>def</sup>
RC	4.09 (0.08) <sup>b</sup>	0.551 (0.018) <sup>ab</sup>	13.9 (0.4) <sup>cd</sup>	nd <sup>a</sup>	nd <sup>a</sup>	220 (16) <sup>e</sup>	nd <sup>a</sup>	5.79 (0.37) <sup>ab</sup>	345 (11) <sup>d</sup>	95.7 (3.6) <sup>h</sup>	nd <sup>a</sup>	685 (5) <sup>gh</sup>
RD	4.16 (0.21) <sup>b</sup>	0.564 (0.033) <sup>ab</sup>	11.5 (0.3) <sup>bcd</sup>	nd <sup>a</sup>	nd <sup>a</sup>	188 (46) <sup>de</sup>	1.87 (0.03) <sup>ab</sup>	5.22 (0.12) <sup>a</sup>	335 (101) <sup>d</sup>	200 (17) <sup>j</sup>	nd	747 (146) <sup>h</sup>
RL	5.37 (1.25) <sup>b</sup>	3.73 (0.16) <sup>c</sup>	5.14 (0.22) <sup>ab</sup>	nd <sup>a</sup>	5.22 (0.46) <sup>bc</sup>	51.2 (3.4) <sup>ab</sup>	1.57 (0.10) <sup>ab</sup>	7.16 (1.31) <sup>abc</sup>	25.7 (10.0) <sup>ab</sup>	11.6 (1.5) <sup>ab</sup>	5.28 (0.28) <sup>c</sup>	122 (17) <sup>abcd</sup>
SB	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	44.7 (10.1) <sup>ab</sup>	1.81 (0.10) <sup>ab</sup>	5.56 (0.01) <sup>a</sup>	29.0 (7.2) <sup>ab</sup>	17.0 (4.9) <sup>abcd</sup>	nd <sup>a</sup>	98.1 (20.7) <sup>abc</sup>
SI	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	nd <sup>a</sup>	68.6 (6.3) <sup>ab</sup>	1.85 (0.06) <sup>ab</sup>	6.54 (0.30) <sup>abc</sup>	32.8 (7.7) <sup>ab</sup>	22.7 (0.8) <sup>abcd</sup>	nd <sup>a</sup>	132 (11) <sup>abcd</sup>
TA	nd <sup>a</sup>	nd <sup>a</sup>	5.14 (0.19) <sup>ab</sup>	nd <sup>a</sup>	nd <sup>a</sup>	104 (9) <sup>bc</sup>	4.05 (0.30) <sup>c</sup>	5.58 (0.38) <sup>a</sup>	190 (2) <sup>c</sup>	70.9 (4.8) <sup>gh</sup>	nd <sup>a</sup>	380 (13) <sup>f</sup>
TE	nd <sup>a</sup>	nd <sup>a</sup>	5.32 (0.07) <sup>abc</sup>	nd <sup>a</sup>	26.6 (1.1) <sup>e</sup>	72.4 (5.9) <sup>ab</sup>	2.41 (0.21) <sup>bc</sup>	12.8 (0.9) <sup>cde</sup>	84.2 (9.3) <sup>b</sup>	43.1 (1.6) <sup>def</sup>	nd <sup>a</sup>	247 (11) <sup>def</sup>
TP	nd <sup>a</sup>	nd <sup>a</sup>	5.50 (0.31) <sup>abc</sup>	nd <sup>a</sup>	nd <sup>a</sup>	65.9 (4.6) <sup>ab</sup>	1.62 (0.09) <sup>ab</sup>	5.51 (0.07) <sup>a</sup>	39.1 (1.9) <sup>ab</sup>	26.1 (1.2) <sup>abcd</sup>	nd <sup>a</sup>	144 (6) <sup>abcd</sup>
<b>Average total</b>	4.95	1.62	10.1	74.1	10.3	112	1.82	8.20	152	71.7	7.92	345 <sup>3</sup>

<sup>1</sup> Results are expressed as mean (standard deviation) of three determinations.

<sup>2</sup> Mean of total FA of three determinations (data not shown).

<sup>3</sup> Mean of Totals (values in the same column) nd – not detected; Pel – pelargic ac.; Cap – capric ac.; Lau – lauric ac.; Myr – myristic ac.; Pen – pentadecylic ac.; Pal – palmitic ac.; Mar – margaric ac.; Lin – linoleic ac.; Ole – oleic ac.; Ste – stearic ac.; Ara – arachidonic ac. Values not sharing the same superscript letter (a–m) within the vertical column are different according to the Tukey test ( $p < 0.05$ )

serine, threonine and valine) and 4 other were identified but not quantified (glutamic acid, methionine, tyrosine and tryptophan). As presented in Table 2, substantial differences were found in the levels of AA and total AA in mushroom species. The AA present in highest amounts was alanine the content of which varied between 15.9 and 698.4 mg/100 g of fresh weight (fw); and was highest in *Boletus edulis* Bull. [BE] (689.4 mg/100 g), *Macrolepiota procera* (Scop.) Singer [MP] and *Suillus bovinus* (Pers.) Roussel [SB] species the highest amounts (698.4, 579.9 and 503.1 mg/100 g fw, respectively). A significant high level ( $p < 0.05$ ) of glycine was observed in BE (56.3 mg/100 g fw) and *Lactarius controversus* (Pers.) Pers. [LC] species (35.3 mg/100 g fw). The highest amounts of valine, leucine and isoleucine were found in LC and *Russula cyanoxantha* (Schaeff.) Fr. [RC] species. A significant high level of leucine was also observed in BE species. *Agaricus sylvicola* (Vittad.) Perck [AS], *Amanita caesarea*

(Scop.) Pers. [AC], BE, LC, *Lycoperdon perlatum* Pers. [LP] and MP species showed the highest levels of proline. The highest levels of serine and threonine were found in MP species (118.9 and 43.6 mg/100 g fw, respectively), and they were significantly different from the amounts in all the other species. Threonine was also present in high levels in BE species. LC, RC and SB presented the highest levels of phenylalanine.

MP, BE and SB were the species with highest free AA contents of 944.1, 806.9 and 665.1 mg/100 g fw, respectively; whereas *Rhizopogon luteolus* Fr. [RL], *Tricholoma acerbum* (Bull.) Vent. [TA] and *Tricholoma portentosum* (L.) P. Kumm. [TP] were the species with the lowest amounts. Results obtained are in agreement with previous reports that showed that BE species present a high AA content [4,32,33], being characterized by high amounts of alanine and glycine.

FA are among the most widely studied compounds in mushrooms with several reports available in literature [1,34–36]. In the present work, 11 FA were identified (pelargonic, capric, lauric, myristic, pentadecylic, palmitic, margaric, linoleic, oleic, stearic and arachidonic acids) – Table 3. The FA contents varied considerably, oleic and palmitic acids being those who presented higher amounts – 151.6 and 111.6 mg/100 g fw, respectively. Pelargonic and capric acid were present at higher amounts in *Mycena rosea* Gramberg [MR] species (7.7 and 4.4 mg/100 g fw, respectively). The highest levels of lauric and myristic acids were found in AC species. Besides AC, myristic acid was only detected in specimens belonging to *Agrocybe aegerita* (V. Brig.) Singer [AA] species. Similarly, pentadecylic acid was only found in few species; *Tricholoma equestre* (L.) P. Kumm. [TE] showed the highest amount (26.6 mg/100 g fw), which was significantly different from the levels in the other four species. The highest amounts of palmitic acid were found in LC and AC species – 285.6 and 237.6 mg/100 g fw,

respectively. AC, AS, *Collybia butyracea* (Bull.) P. Kumm. [CB], LP, MP, TA and TE presented a higher level of margaric acid than the other species. The levels of linoleic acid varied from 5.0 and 21.4 mg/100 g fw, presenting HS species the highest amount. Oleic acid was not detected in AS, *Amanita vaginata* (Bull.) Lam. [AV] and BE species; however, a significant level of 1172.4 mg/100 g fw were found in *Amanita muscaria* (L.) Lam. [AM] species. The highest contents of stearic acid were found in LC and *Russula delica* Fr. [RD] species (457.4 and 200.0 mg/100 g fw, respectively). As also observed for myristic and pentadecylic acids, only few species presented arachidonic acid; a maximum of 11.5 and 12.1 mg/100 g fw were found in AM and LC species, respectively. In terms of total FA, mushroom species presented a content varying between 44.9 mg/100 g fw (BE species) and 1500.3 mg/100 g fw (AM species). These results are in agreement with other studies reported in literature, where oleic and palmitic acids were reported as the main FA in mushrooms [1,33,37]. The high FA content in AM species, mainly due to the large quantity of oleic acid, was also previously described in literature reports. [19,38]

**Table 4**  
Quantification of sterols in mushroom samples (mg/100 g fw).

Mushroom species	Sterols <sup>1</sup> [Retention time]
	Ergosterol [18,58 min]
AA	50.1 (6.4) <sup>cd</sup>
AC	137 (7) <sup>jk</sup>
AM	78.6 (6.0) <sup>def</sup>
AS	145 (15) <sup>jk</sup>
AV	240 (12) <sup>m</sup>
BE	123 (6) <sup>hij</sup>
CB	81.8 (6.6) <sup>efg</sup>
CD	95.8 (8.2) <sup>figh</sup>
HS	107 (7) <sup>ghi</sup>
LA	86.6 (1.0) <sup>efg</sup>
LC	58.6 (11.4) <sup>cde</sup>
LP	79.4 (14.8) <sup>efg</sup>
MP	191 (21) <sup>l</sup>
MR	32.8 (2.6) <sup>bc</sup>
RC	nd <sup>a</sup>
RD	16.9 (0.7) <sup>ab</sup>
RL	92.9 (3.5) <sup>fg</sup>
SB	123 (10) <sup>hij</sup>
SI	160 (34) <sup>kl</sup>
TA	87.7 (4.8) <sup>fg</sup>
TE	22.1 (0.7) <sup>ab</sup>
TP	125 (4) <sup>ij</sup>
<b>Average total</b>	<b>102</b>

nd – not detected.

Values not sharing the same superscript letter (a–m) are different according to the Tukey test ( $p < 0.05$ ).

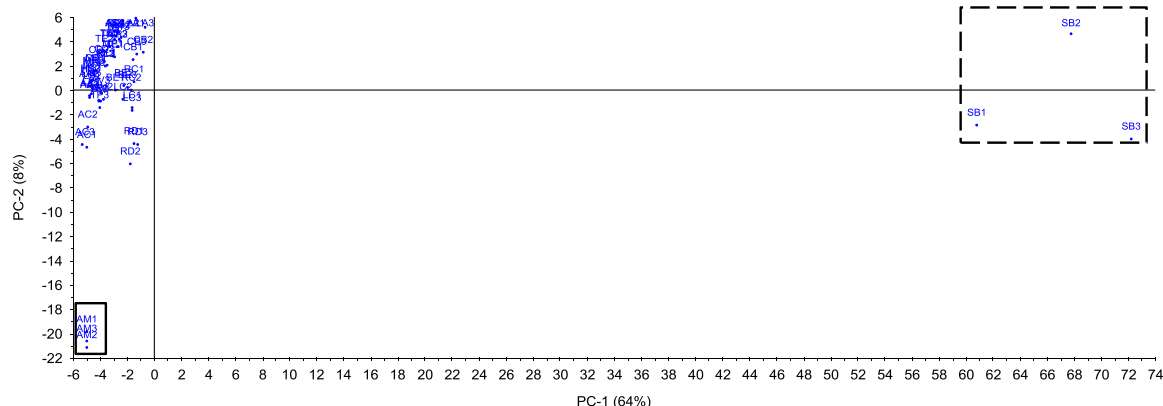
<sup>1</sup> Results are expressed as mean (standard deviation) of three determinations

Ergosterol (ergosta-5,7,22-trien-3 $\beta$ -ol) is the main sterol in several mushrooms [39]. In the present study, ergosterol, the vitamin D2 precursor, was identified in all samples, except in RC, and was present in high amounts in AV species (240 mg/100 g fw) – Table 4. Other sterol-like compounds were also present in almost all species and by comparison with literature database one of them was tentatively identified as fungisterol, as it showed intense ions at  $m/z$  255 and 472 [39].

Despite the fact that in certain species the levels of some AA and FA differ significantly from all other specimens (e.g. alanine in MP and oleic acid in AM), the use of those compounds as biomarkers is not recommended, as they are primary metabolites that exist in almost all species and their amounts are dependent on different variables (environmental conditions, growth stage, etc.) [19]. Nevertheless, they are important molecules in biosynthetic process and their higher expression in specific species can be due to their role in synthetic pathways that can be specific for such species or the respective genera.

### 3.1.2. Non-targeted approach: multivariate analysis

In addition to the three compound classes referred, this multi-target procedure also result in the extraction and derivatization of several other metabolites, such as sugar-like compounds, since the derivatization reagent used (MSTFA) reacts with other metabolites from different classes [28]. Thus, it seems important to analyze those results through a non-targeted approach, in order to explore if there are relevant differences among metabolites composition which were not detected in targeted analysis.



**Fig. 1.** Scores plot resulting from principal component analysis of multi-target experiment data of the several mushrooms samples. AM and SB species, which are apart from the main cluster, are flagged with boxes (— and — —, respectively).

After chromatograms pre-processing (peak alignment and normalization), the resulting data were submitted to two different statistical tools: PCA and PLS-DA.

PCA analysis was used to verify if there is discriminating metabolites among mushroom species. In samples scores plot presented in Fig. 1, principal component 1 (PC1) and principal component 2 (PC2) have a discriminant power of 64 and 8%, respectively, explaining more than 70% of variance. Sample distribution in the PC1 vs. PC2 space (Fig. 1), shows a cluster that includes almost all species. SB and AM species were out from cluster, and showed distinct projections in PC1 and PC2, respectively. The recognition of chromatographic peaks responsible for distribution among axes can be done analyzing the loading plot of the singular value decomposition analysis of those components (Fig. 2).

Chromatographic peaks labeled in Fig. 2 were found in almost all species and were tentatively identified through NIST05 MS Library Database (Table 5). Thus, (PC1-1) and (PC1-2) presented 5- and 6-carbon sugar alcohol structures, respectively. According to NIST05 MS Library Database, (PC1-1) and (PC1-2) were identified as being

one of two isomers: xylitol or adonitol and sorbitol or mannitol, respectively. Indeed, xylitol and sorbitol were previously identified in a mushroom species from northern Thailand [40] and mannitol is the main representative of monosaccharide derivatives [37] in several mushroom species [3,40–42]. PC2 loadings tentatively identified, (PC2-1) and (PC2-2), were shown to be monosaccharides with 6-carbon skeletons. However, it was neither possible to understand if those compounds correspond to the open-chain or the cyclic structures nor to identify the kind of isomer (galacto-, gluco-, manno-, etc.). As this part of the study aims to understand the applicability and potential of this metabolomic approach on data analysis to mushrooms identification/discrimination, a detailed identification of unknown compounds was not performed.

The ratio “metabolite peak area/desmoterol peak area” (quantification ratios) were obtained for the referred metabolites and ANOVA of that data revealed statistical differences among species. Indeed, in agreement with scores plot (Fig. 1), the higher amounts of (PC1-1) and (PC1-2) sugar alcohols in SB and of (PC2-1) and (PC2-2) monosaccharides in AM were statistically different from the values in all other species. The bar charts presented on Fig. 3

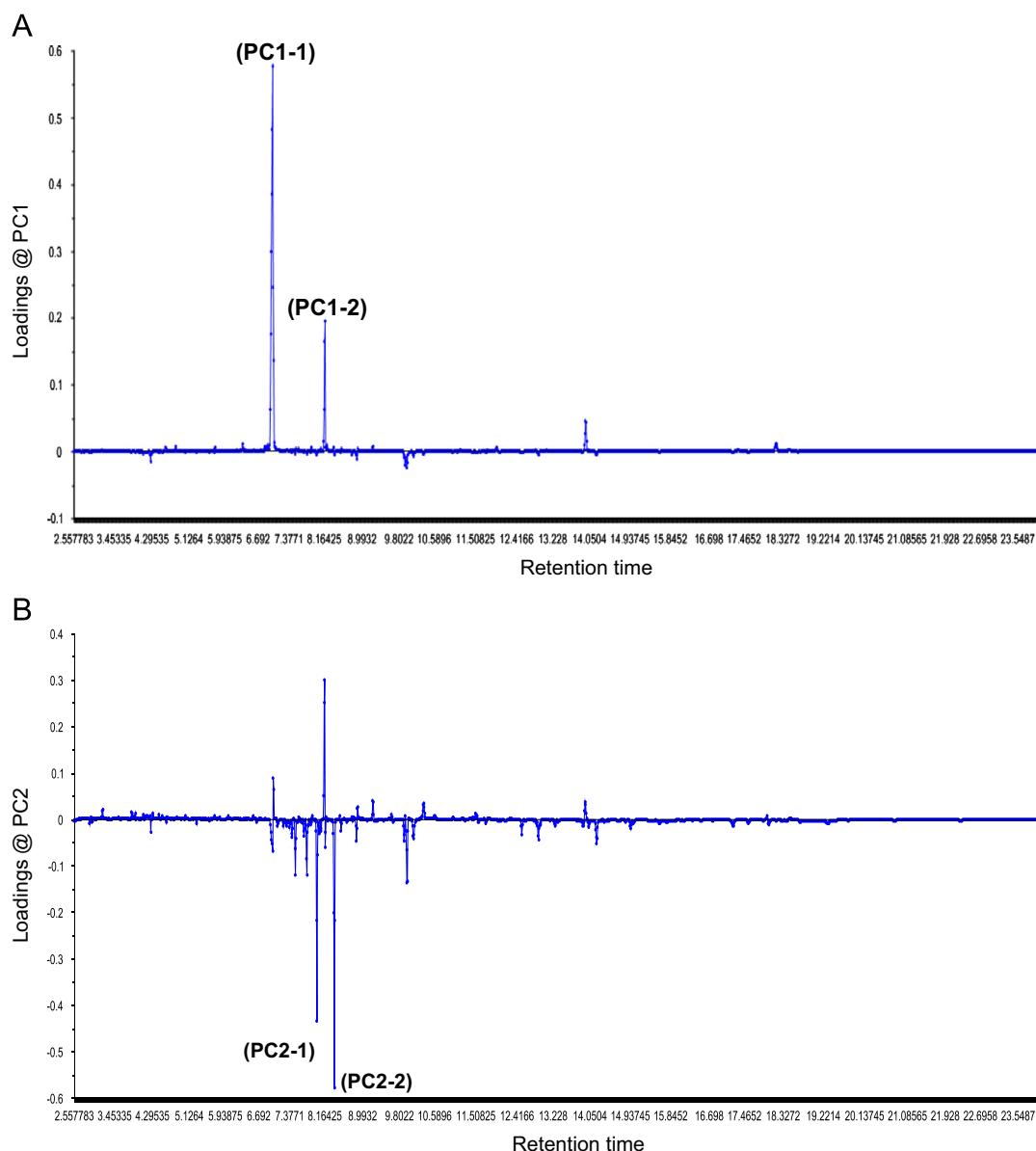
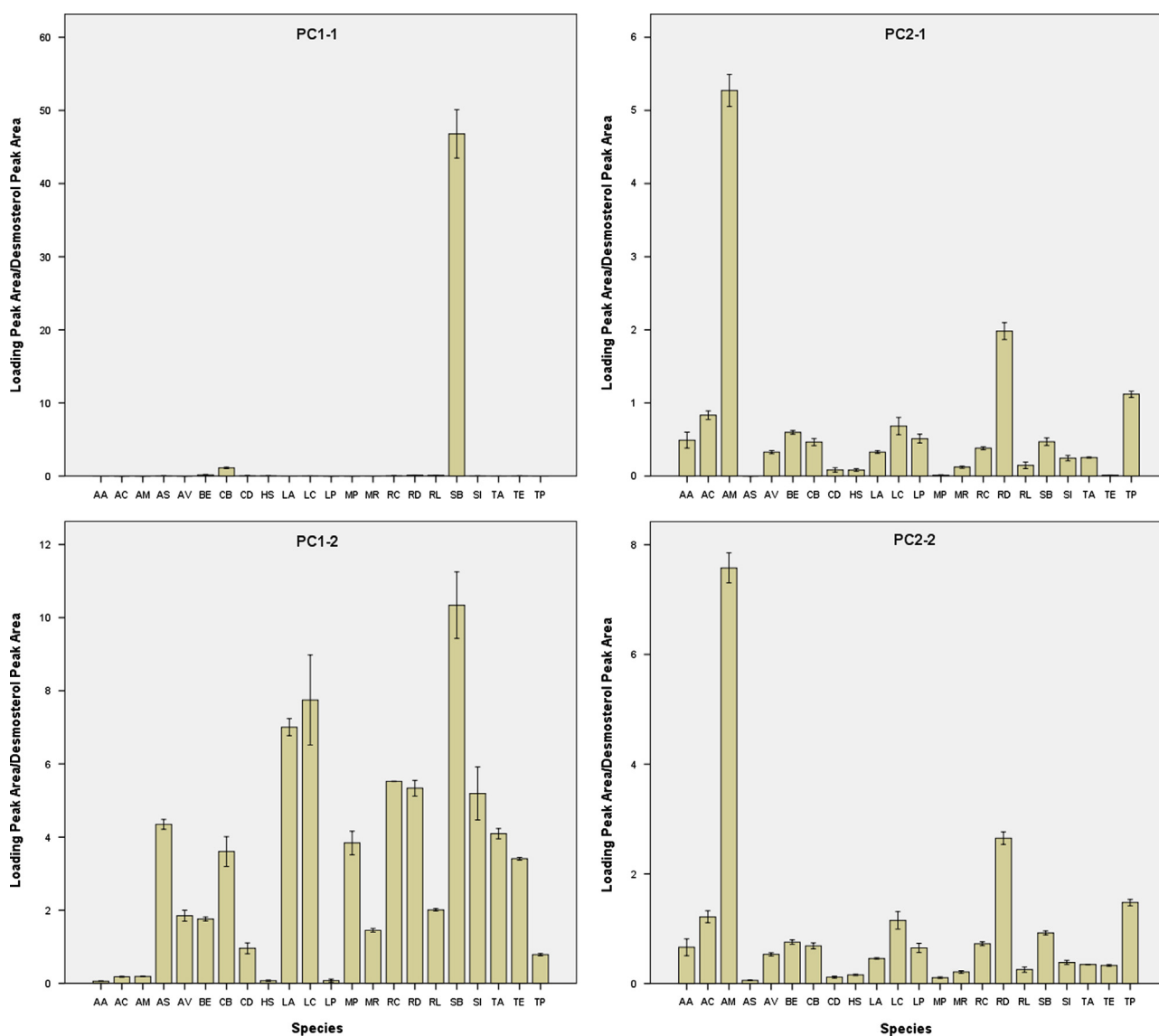


Fig. 2. Loadings plots corresponding to PC1 (A) and PC2 (B) of multi-target experiment. The annotations correspond to peaks selected for further analysis and identification.

**Table 5**

Tentative identification of the main loadings of PC1 and PC2 resulting from the principal component analysis of multi-target experiment data.

Label	Retention time (min)	Presence in mushrooms	Ions ( <i>m/z</i> )	Tentative identification	
				Structure	Compound [KI] <sup>a</sup>
PC1-1	7,02	All species, except AC, AM and AV	73 (1); 217 (0.70); 129 (0.36); 147 (0.35); 243 (0.27); 103 (0.23); 117 (0.16); 205 (0.16); 319 (0.10)	5-Carbon sugar alcohol	Xylitol [1739] or adonitol [1756]
PC1-2	8,18	All species	73 (1); 147 (0.52); 217 (0.39); 205 (0.32); 319 (0.25); 117 (0.18); 157 (0.13); 103 (0.12)	6-Carbon sugar alcohol	Sorbitol [1981] or mannitol [1975]
PC2-1	8,00	All species, except AS	73 (1); 204 (0.82); 191 (0.38); 147 (0.29); 217 (0.17); 205 (0.15); 129 (0.11); 133 (0.09)	6-Carbon monosaccharide	Unknown
PC2-2	8,38	All species	73 (1); 204 (0.67); 191 (0.37); 117 (0.30); 147 (0.26); 75 (0.19); 217 (0.18); 205 (0.17); 129 (0.16)	6-Carbon monosaccharide	Unknown

<sup>a</sup> Kovats Index presented by NIST05 MS Library Database for a VF-5MS column, or similar.**Fig. 3.** Main loadings of PC1 and PC2 distribution among mushroom species. Values represent mean  $\pm$  SE of three replicates.

show the quantification ratios of the analyzed PC1 and PC2 loadings in each mushroom species. Thus, based on those charts it is possible to infer that (PC1-1) metabolite is the most promising compound to be a chemotaxonomical marker since it is the one that shows the highest difference among species. All the others, despite showing statistical significant differences among species,

presented smaller variations which indicates a lower discriminating power. It is important to notice that metabolic synthesis is dependent on growth stage and abiotic conditions [19], and thus the differences showed by (PC1-2), (PC2-1) and (PC2-2) could be attenuated if mushrooms were in different maturation stages and had grown under different abiotic conditions. The same postulate



cannot be applied to (PC1-1) as this compound was present in much higher concentration in one of the species. However, it is obvious that several other studies are required to confirm the discrimination skills of this metabolite.

A supervised statistical method (PLS-DA) was applied to data analysis in order to understand if there were metabolite similarities within the edible and toxic mushrooms that allow discriminating those two groups, i.e. common metabolites within the edible species that differentiate them from the toxic species and the opposite. Results did not show clustering (data not shown) which reveals that there were not enough common features in edible species able to distinguish them from the toxic ones, i.e. the metabolite patterns of edible and toxic species are not discriminant among them.

### 3.2. Volatile profiling experiment

#### 3.2.1. Targeted approach: main volatiles qualitative analysis

Mushrooms are known for their diversified composition on volatiles, which can include volatile carboxylic acids, alcohols, aldehydes, ketones, esters, phenols, lactones and terpenic compounds [2,43]. Among the main volatiles responsible for mushroom flavor – aliphatic 8-carbon molecules [2,42–44], 5 were selected to be evaluated in samples, namely 1-octen-3-ol, 3-octanone, 3-octanol, *trans*-2-octenal, *trans*-2-octen-1-ol. In addition, the presence of linalool (terpene alcohol) in mushrooms samples was also determined.

The results obtained are in agreement with previous reports, since 1-octen-3-ol, 3-octanone and 3-octanol were the most common volatiles in the studied mushroom species (Table 6). Among these, 3-octanone was present in all mushroom specimens, with exception to AS and BE, although 3-octanone was once reported in BE species [43]. However, in a much more recent study, 3-octanone was not detected in a BE specimen collected in Bragança (Trás-os-Montes) [2], the same place where the samples for the present study were harvested. That difference is, probably, due to the fact that those specimens grew under different environmental conditions, which, as previously referred, influence the secondary metabolites synthesis. It was also shown by [52] that the main eight-carbon volatiles can vary according to the mushroom preparation. Enzymes responsible for the formation of these C8-compounds are located in cells, and therefore

the method of disruption of cells influence the quantity of metabolites produced. 1-Octen-3-ol, also known as “mushrooms alcohol”, has been reported as the main volatile responsible for the flavor of most mushrooms [2,37,45]. Indeed it was present in 18 of the 22 mushroom specimens studied and was the main volatile in five of those species – AC, BE, HS, RL, SB and SI. 3-Octanol was also present in almost all mushrooms, although it was not detected in 4 species – AS, BE, TE and TP. Despite it was present in several specimens it was the main volatile compound in AA and CB species. As in the case of 3-octanone, 3-octanol was also previously reported in BE [43] but was not detected in the samples of this species analyzed in the present study. Once again, the differences in environmental conditions can be the reason for such differences.

The other volatile compounds, *trans*-2-octenal, *trans*-2-octen-1-ol and linalool, were identified in different species but they were less distributed among the species when compared to other molecules analyzed. Nevertheless, linalool was found to be the main volatile compound in TA species.

#### 3.2.2. Non-targeted approach: multivariate analysis

The 8-carbon molecules are present in several species, as previously referred, and therefore are not the most suitable metabolites to distinguish species. Thus, to achieve that goal it is important to study other volatiles, namely those present in low amounts, as they can be more species-/genus-specific. Since the chromatograms of volatile profiles showed the presence of other compounds beyond those with an 8-carbon skeleton (Supplementary data 1) a non-targeted analysis of the data obtained was performed in order to verify the existence of molecules which can have some chemotaxonomical value.

As in the multi-target experiment, data resulting from chromatograms pre-processing (peak alignment and normalization), were submitted to a non-targeted analysis through two different statistical tools: PCA and PLS-DA.

PCA resulted in the samples score plot presented in Fig. 4, in which the principal component 1 (PC1) and principal component 2 (PC2) presented a discriminant power of 28 and 19%, respectively, explaining almost 50% of variance. In Fig. 4 it is possible to observe that there are 3 species more isolated (flagged with boxes), specifically BE and SB, which are ahead in PC1, and LP, which is detached in PC2. That apartness is due to the high amounts of 1-octen-3-ol and 3-octanone in BE/SB and LP species, respectively, once the peaks of those compounds represent the main loadings in PC1 and PC2 (Fig. 7), respectively.

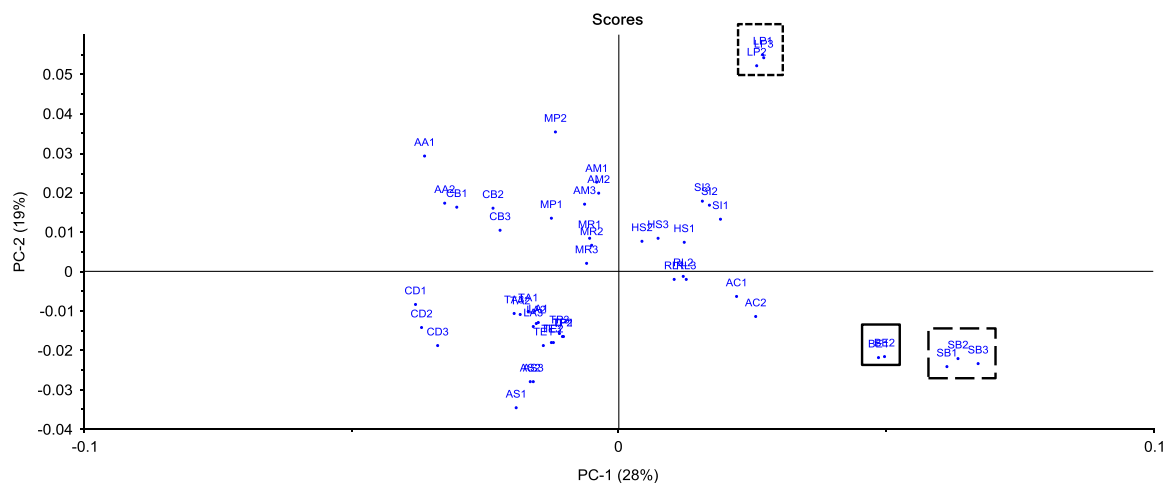
Besides 1-octen-3-ol and 3-octanone, there is one, PC1/1 (tentatively identified as a sesquiterpene) and two other volatiles marked in PC2/1 and PC2/2 loadings plot (Fig. 5) (an ester of hexanoic acid and a sesquiterpene alcohol), respectively. Those were the molecules selected to be studied in order to understand their potential for species discrimination. They were tentatively identified through NIST05 MS Library Database (Table 7) and semi-quantified (Fig. 6), i.e. a ratio “peak area”/“sum of scan intensities” was calculated in the species in which they exist.

According to NIST05 MS Library Database, (PC1/1) and (PC2/2) correspond to a sesquiterpene and a sesquiterpene alcohol, respectively. (PC1/1) was identified as being one of two compounds:  $\delta$ -guaiane or sativene. Despite the absence of reports indicating the existence of  $\delta$ -guaiane in mushrooms, its isomers  $\alpha$ -guaiane and  $\beta$ -guaiane were already described in mushrooms [43] and other fungal species [46], respectively. Thus, it is possible that some mushrooms, share a common ancestral biosynthetic pathway with those species, as a result of evolution specialized in the synthesis of that specific isomer. On the other hand, sativene was already described in mushroom species, such as *Fomitopsis pinicola* (Swartz ex Fr.) Karst. [47] and *Coprinus cinereus* (Schaeff.) Gray [48]. Concerning (PC2/2), it was

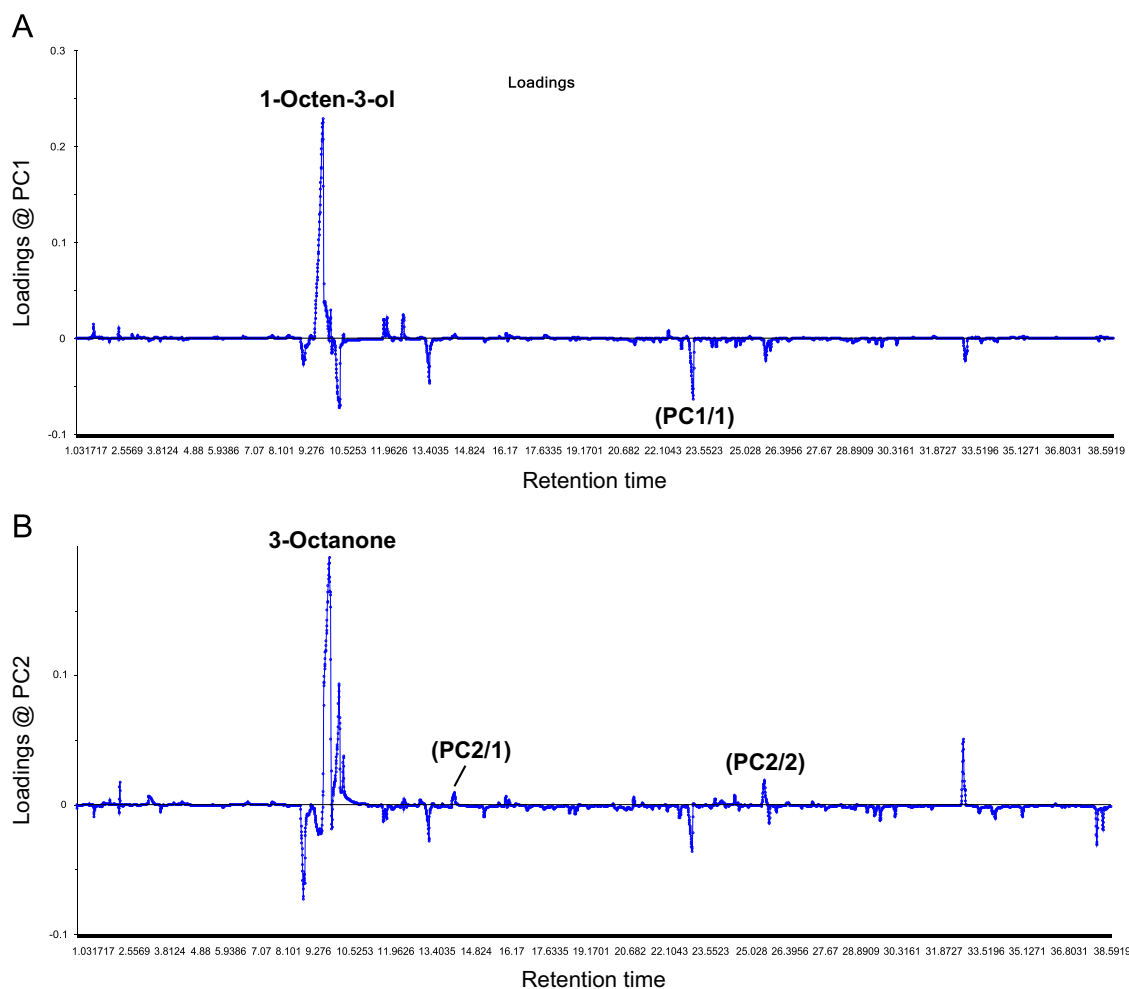
**Table 6**  
Volatile composition of mushroom species.

Mushroom species	Compounds					
	1-Octen-3-ol	3-Octanone	3-Octanol	<i>trans</i> -2-Octenal	<i>trans</i> -2-Octen-1-ol	Linalool
AA	–	+	+	–	+	–
AC	+	+	+	–	–	–
AM	+	+	+	–	–	–
AS	–	–	–	–	–	–
BE	+	–	–	+	+	–
CB	+	+	+	+	–	–
CD	–	+	+	–	–	+
HS	+	+	+	+	–	+
LA	+	+	+	–	–	–
LP	–	+	+	+	–	–
MP	+	+	+	+	–	–
MR	+	+	+	–	–	+
RL	+	+	+	–	–	–
SB	+	+	+	–	+	–
SI	+	+	+	+	+	+
TA	+	+	+	+	+	+
TE	+	+	–	–	–	+
TP	+	+	–	+	–	–

+ Compound was identified in species; – Compound was not identified in species.



**Fig. 4.** Scores plot resulting from PCA of mushrooms' volatile composition data. The species more isolated, i.e. BE, LP and SB, are flagged with boxes (—, --- and - - -, respectively).



**Fig. 5.** Loadings plots corresponding to PC1 (A) and PC2 (B) resulting from PCA of volatile profiling experiment data. The peaks marked with a (PC1/1), (PC2/1) and (PC2/2) labels correspond to those selected to further analysis and identification.

identified as *trans*-nerolidol, a sesquiterpene alcohol previously reported in mushrooms [2,44,49,50]. (PC2/1) was identified as an ester of hexanoic acid (C6:0). Despite some esters of hexanoic acid being already described in mushroom species, namely methyl [51] and ethyl hexanoate [2], the compounds identified as being the more

probable by NIST05 MS Library Database, i.e. allyl or vinyl caproate, were not hitherto found in mushrooms.

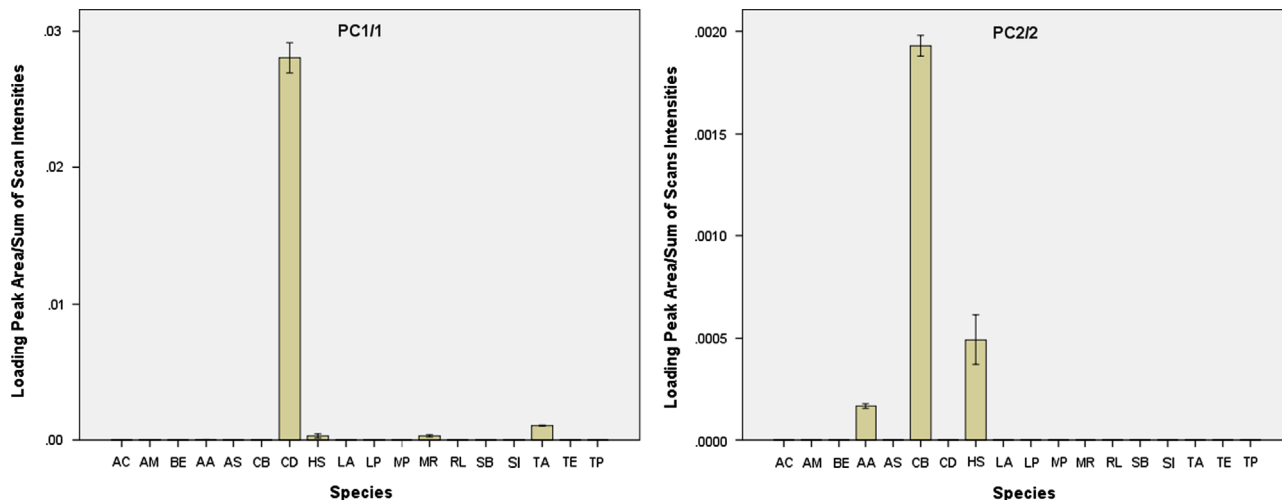
Although the unequivocal identification of (PC2/1) was not possible, such compound seems to be a promising metabolite for species identification/discrimination, since it was only present in LP samples.

**Table 7**  
Tentative identification of the main loadings of PC1 and PC2 resulting from the principal component analysis of volatile profiling experiment data.

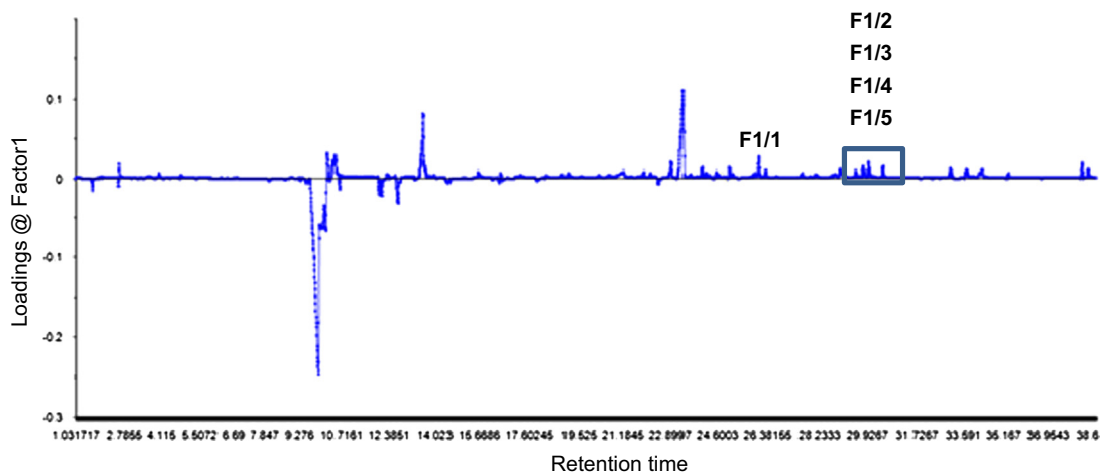
Label	Retention time (min)	Presence in mushrooms	Ions ( <i>m/z</i> )	Tentative identification	
				Structure	Compound [KI] <sup>a</sup>
PC1/1	23,37	AA, AM, CD, HS, LP, MR, SI and TA	108 <sup>b</sup> (1); 81 (0.63); 79 (0.37); 107 (0.32); 109 (0.31)	Sesquiterpene	δ-Guaiene [1505] or -sativene [1405]
PC2/1	14,55	LP	43 (1); 39 (0,38); 99 (0.38); 41 (0,37); 55 (0,28); 71 (0.21)	Ester of hexanoic acid	Allyl caproate [1080] or vinyl caproate [974]
PC2/2	25,94	AA, CB and HS	69 (1); 107 (0,96); 41 (0.88); 81 (0.79); 121 (0.49); 161 <sup>b</sup> (0.44)	Sesquiterpene alcohol	<i>trans</i> -Nerolidol [1568]

<sup>a</sup> Kovats Index presented by NIST05 MS Library Database for a VF-5MS column, or similar.

<sup>b</sup> Ions used in semi-quantification.



**Fig. 6.** Main loadings of PC1 and PC2 distribution among mushroom species subjected to volatile profiling. Values represent mean  $\pm$  SE of three replicates.



**Fig. 7.** Loadings plots corresponding to Factor 1 resulting from PLS-DA of volatile profiling experiment data. The peaks labeled correspond to those selected to further analysis and identification.

This is in agreement with the score plot (Fig. 4), in which LP is the species more detached in PC2. The other loadings studied were present in few species (Table 7 and Fig. 6) and among those an ANOVA revealed that there were statistical significant differences among those species. (PC1/1) showed better discrimination potential than (PC2/2) – Fig. 6, since it presented a higher difference between the two most concentrated species (i.e. *Clytocybe dealbata* (Sowerby) P. Kumm. [CD] and TA, respectively) – approximated ratio of 27:1.

The results of PLS-DA did not show clustering of edible neither toxic species (data not shown), i.e. there were not sufficient

similarities among edible or toxic species to group them. Nevertheless, some of the loadings, F1/1, F1/2, F1/3, F1/4 and F1/5, resulting from PLS-DA were studied (Fig. 7). The semi-quantification of compounds corresponding to these loadings revealed that they are only present in the toxic *Lactarius aurantiacus* (Pers.) Gray [LA] species and thus they can be particular for the *Lactarius* Pers. genus or even more specific, existing only in the referred species. However, the identification of such compounds was not possible, since the identification's probability presented by NIST 05 MS Library Database for the suggested compounds

were not significant. On the other hand, the comparison of mass spectra of the different compounds revealed that the fragment ions at  $m/z$  105, 199, 214, 215 and 217 are common to all the molecules and several other fragments are present in almost all of them ( $m/z$  189 and 232). These similarities indicate that those compounds can probably share a common structure and the same biosynthetic pathway. Thus, it is possible that this pathway was a result from evolution and it is specific to certain mushrooms taxa, being an important tool for chemotaxonomy of such taxonomic groups. However, several other studies are required to confirm such hypothesis.

#### 4. Conclusion

In this work, it was possible to confirm the applicability of the multi-target GC-ITMS method to the identification of AA, FA and ergosterol in mushrooms, a matrix that was analyzed for the first time with this methodology. The analysis of the resulting data also allowed concluding that this methodology results in the extraction and derivatization of other molecules, such as sugar-like compounds, and that the extracted metabolites are not the most suitable for species discrimination.

On the other hand, the study of mushrooms' volatile profiles by HS-SPME/GC-ITMS allowed the identification of the main volatile compounds in macrofungi, i.e. 8-carbon skeleton compounds.

Finally, the main conclusion of this work is that the use of non-targeted data analysis approaches is an important strategy to identify species-/genus-specific metabolites, once in the present investigation it was possible to identify 7 candidates to chemotaxonomical markers: one primary metabolite, with a 5-carbon sugar alcohol structure, present in higher amount in SB samples, the compound being tentatively identified as xylitol or adonitol; one secondary metabolite, specific to LP species – an ester of hexanoic acid, tentatively identified as allyl or vinyl caproate; and five other secondary metabolites, whose identification was not possible, which were only detected in LA specimens. On the other hand, PLS-DA showed that the volatile composition did not allow a clear separation between edible and toxic mushrooms, i.e. there were not detected specific volatiles that enable predicting if a mushroom is toxic or edible.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.talanta.2013.09.038>.

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