Targeted metabolites analysis in wild *Boletus* species

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ABSTRACT

In European countries, the edible mushrooms from the *Boletus* genus are the most frequently harvested of all the forest species gathered in the wild. Their popularity is mainly due to their sensory qualities, in particular aroma, taste and texture. In the present work, a targeted metabolites analysis was performed in six wild *Boletus* species. The analysis of primary metabolites revealed proteins, carbohydrates, fatty acids, mainly linoleic acid, sugars, mainly mannitol and trehalose, and vitamins (tocopherols and ascorbic acid). Secondary metabolites, such as phenolic acids, were also identified and quantified, and correlated to *Boletus* antioxidant properties including free radical scavenging activity, reducing power and lipid peroxidation inhibition. As a source of these important metabolites, the edible *Boletus* spp. could be directly used in the human diet as health foods, taking advantage on the synergistic and/or additive effects of all the antioxidants present, while inedible species could represent a source of extractable phenolic compounds to be used as additives in the food industry or as components in pharmaceutical and cosmetic formulations.

Keywords: Wild mushrooms; *Boletus* sp.; Primary/Secondary Metabolites; Bioactivity

1. Introduction

Under natural conditions and in culture, growing fungi take nutrients from their surroundings, that they can easily use as energy sources to produce materials such as proteins and lipids, essential for continued growth and biomass production (primary metabolism). Primary metabolites are formed during the active growth of the fungus and some of them have commercial importance. Large scale cultures are grown industrially with the specific purpose of obtaining large quantities of these fungal products including vitamins (food supplements). Primary metabolites and intermediate compounds that have accumulated in the fungus are further converted to different products (secondary metabolites) which are not normally produced during active growth and are not essential for vegetative proliferation. Secondary metabolites include a wide diversity of molecules and are produced when the fungus is not actively growing; their formation may accompany differentiation and sporulation in the fungus (Isaac, 1997).

Many of the metabolites present in mushrooms (either primary or secondary) have antioxidant activity and may therefore impart a competitive advantage, acting as weapons for survival (Barros, Dueñas, Ferreira, Baptista, & Santos-Buelga, 2009; Heleno, Barros, Sousa, Martins, & Ferreira, 2009; Heleno, Barros, Sousa, Martins, & Ferreira, 2010).

Amino acids are building blocks for the synthesis of proteins, including antioxidant enzymes. Some amino acids and small peptides directly scavenge oxygen free radicals. Thus, a dietary deficiency of protein not only impairs the synthesis of antioxidant enzymes but also reduces tissue concentrations of antioxidants, thereby resulting in a compromised antioxidant status. The Polyunsaturated Fatty Acids, ω-6 PUFAs, in contrast to ω-3 PUFAs inhibit free radical production and decrease plasma

triacylglycerol concentration, exerting beneficial effect on cardiovascular function (Fang, Yang, & Wu, 2002). Some vitamins directly scavenge reactive oxygen species (ROS) and upregulate the activities of antioxidant enzymes. Among them, vitamin E has been recognized as one of the most important antioxidants. Vitamin E inhibits ROS-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA in membrane phospholipids and membrane degeneration, plasma very low density lipoprotein, cellular proteins or DNA from oxidative damage (Fang et al., 2002). Phenolic acids are secondary metabolites that are commonly found in plant-derived foods (Mattila & Hellstrom, 2007) and mushrooms (Barros et al., 2009). As polyphenols, phenolic acids are powerful antioxidants and have been reported to possess antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions (Mattila & Hellstrom, 2007).

Mushrooms represent a rich source of all these biologically active compounds. *Boletus* is a genus of mushrooms, comprising over 100 species. Of all the forest species gathered in the wild, the edible mushrooms from the *Boletus* genus are the most frequently harvested in European countries, including Portugal. Their popularity is mainly due to their sensory qualities, in particular aroma, taste and texture (Jaworska & Bernas, 2009).

Among the many species of fungus belonging to the *Boletus* family, *Boletus edulis* Bull: Fr. is undoubtedly regarded as having the finest flavour. *B. edulis* related species involves a dozen or so varieties, such as *B. aereus* Bull. and *Boletus reticulatus* Schaeff., and may be classified by their natural habitat, the trees they are associated with forming mycorrhizas and finally the morphology of their fruiting body (Jaworska & Bernas, 2009). *B. edulis*, king bolete, is a popular edible mushroom in Europe (in

Portugal is among the most appreciated), North America, and Asia. Fresh and dried king bolete may be marketed in oriental restaurants and oriental, gourmet, and health food stores. The flavor of this dried king bolete including odour and taste is marvellous-nutty, earthy, and meaty all at once (Tsai, Tsai, & Mau, 2007; Tsai, Tsai, & Mau, 2008). The non edible *Boletus* spp. may also be interesting sources of drugs such as bolesatine, a toxic glycoprotein purified from *Boletus satanas* (Ennamany, Lavergne, Reboud, Dirheimer, & Creppy, 1995). This lectin exerts a potent mitogenic activity on human peripheral blood lymphocytes, and induced the release of interleukin-1α, interleukin-2 and tumour necrosis factor-α from mononuclear cell cultures (Wang, Ng, & Ooi, 1998). Herein, a targeted metabolites (primary and secondary) analysis was performed in six different wild mycorrhizal *Boletus* species (edible: *B. aereus*, *B. edulis*, *B. reticulatus*; not-edible: *B. purpureus*, *B. satanas*; *B. rhodoxanthus*) collected in mixed stands, *Quercus pyrenaica* and *Castanea sativa* habitats from Portugal (Table 1).

2. Material and methods

2.1. Mushroom species

Boletus aereus Bull., *Boletus edulis* Bull., *Boletus reticulatus* Schaeff., *Boletus purpureus* Fr. & Hök, *Boletus satanas* Lenz and *Boletus rhodoxanthus* (Krombh.) Kallenb were collected in Bragança (Northeast Portugal), in autumn 2009. Information about the wild *Boletus* species collected is provided in Table 1. Taxonomic identification of sporocarps was made according to several authors (Moser, 1983; Alessio, 1985; Bon, 1988; Courtecuisse & Duhem, 2005), and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. All the samples were lyophilised (Ly-8-FM-ULE, Snijders,

Holland), reduced to a fine dried powder (20 mesh) and kept at -20 ºC until further analysis (~60 days).

2.2. Standards and Reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, ascorbic acid, tocopherols (α-, β-, γ-, δ-tocopherols), sugars (arabinose, mannitol, raffinose, trehalose) and phenolic standards (gallic, protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Primary metabolites

2.3.1. Macronutrients. The edible samples were analysed for chemical composition (moisture, protein, fat, carbohydrates and ash) using the AOAC procedures (1995). Protein content (N × 4.38) of the samples was estimated by the macro-Kjeldahl method; fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15ºC. Carbohydrates were calculated by difference: Carbohydrates = 100 – (g protein + g fat + g ash). Reducing sugars were determined by the DNS (dinitrosalicylic acid)

131 method. Energy was calculated according to the following equation: Energy (kcal) = 4 ×
132 (g protein +g carbohydrate) + 9 × (g lipid).
133
134 *2.3.2. Fatty Acids.* Fatty acids were determined by gas chromatography with flame
135 ionization detection (GC-FID) as described previously by the authors (Heleno et al.,
136 2009). The equipment was a DANI model GC 1000 instrument equipped with a
137 split/splitless injector, a FID (at 260 ºC) and a Macherey-Nagel column (30 m × 0.32
138 mm ID × 0.25 µm d_f). The oven temperature program was as follows: the initial
139 temperature of the column was 50 ºC, held for 2 min, then a 30ºC/min ramp to 125 ºC,
140 5ºC/min ramp to 160 ºC, 20ºC/min ramp to 180 ºC, 3ºC/min ramp to 200 ºC, 20ºC/min
141 ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0
142 ml/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC.
143 Fatty acid identification was made by comparing the relative retention times of FAME
144 peaks from samples with standards. The results were recorded and processed using
145 CSW DataApex 1.7 software and expressed in relative percentage of each fatty acid.
146
147 *2.3.3. Sugars.* Free sugars were determined by high performance liquid chromatography
148 coupled to a refraction index detector (HPLC-RI) as previously described by the authors
149 (Heleno et al., 2009), using raffinose as internal standard (IS). The equipment consisted
150 of an integrated system with a pump (Knauer, Smartline system 1000), degasser system
151 (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer
152 Smartline 2300). The chromatographic separation was achieved with a Eurospher 100-5
153 NH_2 column (4.6 × 250 mm, 5 µm, Knauer) operating at 30ºC (7971 R Grace oven).
154 The mobile phase was acetonitrile:deionized water, 70:30 (v/v) at a flow rate of 1

ml/min. Sugar identification was made by comparing the relative retention times of sample peaks with standards. Data were analysed using Clarity 2.4 Software (DataApex). Quantification was made by internal normalization of the chromatographic peak area and the results are expressed in g per 100 g of dry weight (dw).

2.3.4. Tocopherols. Tocopherols content was determined following a procedure previously optimized and described by the authors (Heleno et al., 2010), using tocol as internal standard. The HPLC system described above was connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters operating at 30ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min, and an injection volume of 20 µl. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the IS method. Tocopherol contents in the samples were expressed in µg per g, dw.

2.3.5. Ascorbic acid. A fine dried powder (20 mesh; 150 mg) was extracted with metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through a Whatman Nº 4 filter paper. The filtrate (1 ml) was mixed with 2,6-dichloroindophenol (9 ml) and the absorbance was measured after 30 min at 515 nm against a blank (Analytikjena 200 spectrophotometer). A calibration curve of authentic L-ascorbic acid (0.006-0.1 mg/ml) was used, and the results were expressed as mg per g, dw.

179 *2.4. Secondary metabolites*

180 *2.4.1. Extraction procedure.* Each sample (~3 g) was extracted with acetone:water
181 (80:20; 30 ml) at -20°C for 6h, following a procedure previously described by the
182 authors (Barros et al., 2009). After sonication for 15 min, the extract was centrifuged at
183 4,000*g* for 10 min, and filtered through Whatman nº 4 paper. The residue was then
184 extracted with two additional 30 ml portions of the acetone:water mixture. The
185 combined extracts were evaporated at 40 ºC under reduced pressure to remove acetone
186 (rotary evaporator Büchi R-210). The aqueous phase was washed with n-hexane, and
187 then submitted to a liquid-liquid extraction with diethyl ether (3 × 30 ml) and ethyl
188 acetate (3 × 30 ml). The organic phases were evaporated at 40 ºC to dryness, re-
189 dissolved in methanol (at a concentration of 50 mg/ml) for bioactivity assays and total
190 phenolics quantification, and in water:methanol (80:20), followed by filtering through a
191 0.22 µm disposable LC filter disk for HPLC analysis of phenolic acids.

192

193 *2.4.2. Total phenolics.* Phenolic quantification was performed by *Folin-Ciocalteu* assay.
194 The extract methanolic solution (1 ml) was mixed with *Folin-Ciocalteu* reagent (5 ml,
195 previously diluted with water 1:10, v/v) and sodium carbonate (75 g/l, 4 ml). The tubes
196 were vortex mixed for 15 s and allowed to stand for 30 min at 40 °C for colour
197 development. Absorbance was then measured at 765 nm. Gallic acid was used to obtain
198 the standard curve (0.0094 – 0.15 mg/ml), and the results were expressed as mg of gallic
199 acid equivalents (GAE) per g of extract.

200

201 *2.4.3. Free phenolic acids.* The equipment was a Hewlett-Packard 1100 series liquid
202 chromatograph (Agilent Technologies) as previously described (Barros et al., 2009).

Separation was achieved on a Spherisorb S3 ODS-2 (Waters) reverse phase C_{18} column (3 μm, 150 × 4.6 mm) thermostated at 25 ºC. The solvents used were: (A) 2.5% acetic acid in water, (B) 2.5% acetic acid:acetonitrile (90:10, v/v), and (C) 100% HPLC-grade acetonitrile. The gradient employed was: isocratic 100% A for 10 min, 50% A and 50% B for 10 min, isocratic 100% B for 15 min, 90% B and 10% C for 10 min, 70% B and 30% C for 10 min, 50% B and 50% C for 5 min, 20% B and 80% C for 5 min, 100% A for 5 min, at a flow rate of 0.5 ml/min. Detection was carried out in a diode array detector (DAD), using 280 nm as the preferred wavelength. The phenolic compounds were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results were expressed as mg per Kg, dw.

2.5. In vitro assays to evaluate antioxidant activity

In vitro assays already described by the authors (Heleno et al., 2010), were used to evaluate the antioxidant activity of the samples: DPPH radical-scavenging activity, reducing power, and inhibition of β-carotene bleaching in the presence of linoleic acid radicals. Different concentrations of the extracts (20 to 0.25 mg/ml) were employed to find EC_{50} values (extract concentration providing 50% of antioxidant activity or 0.5 of absorbance in reducing power assay). These values were calculated from the graphs of antioxidant activity percentages (DPPH and β-carotene bleaching assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

2.6. Statistical analysis

For each mushroom species three samples were assayed and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 16.0 program.

3. Results and discussion

3.1. Primary metabolites

The results of the macronutrients composition and estimated energetic value (expressed on dry weight basis) obtained for the studied edible *Boletus* sp. are shown in Table 2. It was not observed any significant ($p<0.05$) difference between their moisture contents (~90 g/100 g). Ash was more abundant in *B. reticulatus* (19.72 g/100 g) and less abundant in *B. edulis* (5.53 g/100 g). Protein was found in relatively high levels and varied between 17.86 g/100 g in *B. aereus* and 22.57 g/100 g in *B. reticulatus*. Fat was the less abundant macronutrient being lower than 2.55 g/100 g. Carbohydrates, calculated by difference, were the most abundant macronutrients and were higher than 55.16 g/100 g. Reducing sugars were only a small part of carbohydrates due to the abundant presence of polysaccharides in fungi. The highest energetic values were obtained in *B. edulis* (Table 2). As far as we know, this is the first report on *B. reticulatus* macronutrients composition. The analysed *B. aereus* and *B. edulis* samples revealed a profile similar to samples from Greece, but with lower levels of proteins and fat, and higher contents in ash and carbohydrates (Ouzouni & Riganakos, 2007; Ouzouni, Petridis, Koller, & Riganakos, 2009). The same profile was also observed between the studied wild *B. edulis* and samples from Taiwan (Tsai et al., 2008) but with

lower levels of ash, fat and reducing sugars, and higher levels of proteins and carbohydrates. Furthermore, the studied wild *B. edulis* revealed higher levels of proteins, but lower contents in fat and carbohydrates than a commercial sample studied in a previous report (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008).

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of the studied wild *Boletus* sp. are given in Table 3. Up to twenty-six fatty acids were detected in most of the samples. The major fatty acid found was oleic acid (C18:1n9) for *B. edulis*, *B. reticulatus* and *B. satanas*, and linoleic acid (C18:2n6), for *B. aereus*, *B. purpureus* and *B. rhodoxanthus* contributing to the prevalence of PUFA in the last two species. The studied species also revealed palmitic acid (C16:0) as a major fatty acid. The UFA oleic and linoleic acids have also been reported as main fatty acids in *B. edulis* from India (Kavishree, Hemavathy, Lokesh, Shashirekha, & Rajarathnam, 2008) and in a Portuguese sample collected in 2005 by other research group in a *Castanea sativa* orchard (Ribeiro, Pinho, Andrade, Baptista, & Valentão, 2009). But for the fact that not edible *Boletus* species revealed higher levels of SFA, no other relevant differences were observed between the fatty acids profiles of edible and not edible species. The edible species might be regarded as health foods, owing to their low fat contents and the high relative levels of polyunsaturated fatty acids, particularly the essential fatty acid linoleic, making them suitable to be recommended in the diets of people with high blood cholesterol (Kavishree et al., 2008).

In relation to sugar composition (Table 4), the three edible *Boletus sp.* and *B. rhodoxanthus* gave trehalose as the main sugar, while mannitol predominated in *B. purpureus* and *B. satanas*. The edible *B. edulis* and the poisonous *B. rhodoxanthus* revealed the highest total sugars content (~15 g/100 g), with the highest levels of trehalose (12.40 g/100 g) and mannitol (3.95 g/100 g), respectively. A third sugar, arabinose, was only detected in *B. rhodoxanthus* (1.12 g/100 g). The chromatogram obtained for this sample is presented in Fig. 1.

The total sugars contents determined are in agreement with those reported by Tsai et al. (2008) for *B. edulis* from Taiwan (15 g/100 g). Nevertheless, the amount found in the wild sample now analysed was higher than total sugars determined in a commercial sample of *B. edulis* (13.46 g/100 g), previously studied (Barros et al., 2008). The sugars content of the other five *Boletus* species is herein reported for the first time. Total sugars determined by HPLC-RI (Table 4) were higher than reducing sugars obtained by DNS method (Table 2) due to the contribution of non-reducing sugars such as trehalose.

Vitamins (tocopherols and ascorbic acid) contents in the studied wild *Boletus* species are also given in Table 4. The values obtained in the analysis of the different samples revealed significant differences in what concerns tocopherols composition. γ-Tocopherol was the major compound in all the species, β-tocopherol was not detected in any of the studied *Boletus* sp., and δ-tocopherol was not detected in *B. reticulatus* (Fig. 2) and *B. satanas*, either.

The edible *B. reticulatus* presented the highest content of tocopherols (25.33 μg/g of dry weight), while the poisonous *B. rhodoxanthus* and *B. satanas* revealed the lowest contents without statistical differences ($p<0.05$). As far as our knowledge, this is the

first report on tocopherols composition of *B. aereus*, *B. reticulatus*, *B. purpureus*, *B. rhodoxanthus* and *B. satanas*. Tocopherols have been determined in *B. edulis* from Taiwan (Tsai et al., 2007), but the values were expressed in the extract mass and not in a dry weight basis and, therefore, they cannot be compared; nevertheless, those authors describe α-tocopherol as the main vitamin E isoform. Vitamin E is known to provide valuable antioxidant properties, probably preventing the destruction of vitamin A and unsaturated fatty acids in biological membranes by free radical reactions (Dewick, 2002).

Ascorbic acid was the most abundant vitamin in all the species (Table 4). Jaworska & Bernas (2009) reported the quantification of ascorbic acid in *B. edulis* from Poland, but the results were expressed in a fresh weigh basis.

3.2. Secondary metabolites

In the present study, free phenolic acids were determined by HPLC-DAD. Up to three phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids) and a related compound (cinnamic acid) could be identified and quantified in the different samples (Table 5). *B. aereus* showed the highest concentration of phenolic acids + cinnamic acid (49.49 mg/Kg, dry weight), while *B. satanas* revealed the highest concentration of phenolic acids (25.49 mg/Kg) mostly due to the contribution of protocatechuic (15.92 mg/Kg) and *p*-hydroxybenzoic (9.57 mg/kg) acids. Ribeiro et al. (2008) could not find phenolic compounds in *B. edulis* from Portugal also collected in *Quercus pyrenaica* habitat. Those authors used an extraction methodology with boiling water. In our study heat was avoided due to the fact that phenolic compounds are unstable and readily become non-antioxidative under heating and in the presence of antioxidants (Barros et

al., 2009). Puttaraju, Venkateshaiah, Dharmesh, Urs, & Somasundaram (2006) reported the presence of tannic and protocatechuic acids in methanolic and water extracts of *B. edulis* from India, and caffeic and coumaric acids only in the water extracts. Tannic or caffeic acids could not be detected in the here studied *B. edulis* or any other of the analysed samples.

Total phenolics were also measured by the colorimetric Folin Ciocalteu assay (Table 5), and the results were expressed gallic acid equivalents (GAE) by extract weight for a direct comparison with antioxidant properties. The edible *B. aereus* revealed the highest content (46.05 mg GAE/g extract), while the not edible *B. purpureus* and the poisonous *B. rhodoxanthus* showed the lowest values (8.20 and 8.38 mg GAE/g extract, respectively). The *B. edulis* extract gave slightly lower phenolic content (28.56 mg GAE/g extract) than ethanolic extracts of *B. edulis* samples from Taiwan (36.24 mg GAE/g extract, after conversion according to extraction yield; Tsai et al., 2007), and methanolic extracts of *B. edulis* samples from Turkey (31.64 mg GAE/g extract; Sarikurkcu, Tepe, & Yamac, 2008) and from India (30 mg GAE/g extract, after conversion according to extraction yield; Puttaraju et al., 2006). As far as we know, phenolic composition of the other five *Boletus* sp. was not available in literature.

As a source of these secondary metabolites and taking advantage on the synergistic and/or additive effects of all the antioxidants present, the edible mushrooms could be directly used in the human diet to contribute to combat oxidative stress, while inedible species could represent a source of extractable phenolic compounds to be used as additives in the food industry or as components in pharmaceutical and cosmetic formulations.

346 *3.3. Antioxidant activity*

347 Three different assays were carried out for the *in vitro* evaluation of the antioxidant
348 properties of *Boletus* wild species: scavenging activity on DPPH radicals, reducing
349 power, and inhibition of lipid peroxidation. The results are shown in Table 5. Highest
350 antioxidant values (lowest EC_{50} values) were found for the edible *B. aereus*, which is in
351 agreement to its higher total phenolic content (measured by Folin Ciocalteu assay), and
352 phenolic acids + cinnamic acid content (measured by HPLC-DAD). The poisonous *B.*
353 *rhodoxanthus* presented the lowest antioxidant properties (highest EC_{50} values), which
354 are compatible to its lower total phenolic content, despite its relatively high
355 concentration of phenolic acids (Table 5).

356 The studied sample of *B. edulis* revealed lower EC_{50} value for DPPH scavenging
357 activity (0.43 mg/ml) than *B. edulis* samples from India (1.40 mg/ml; Puttaraju et al.,
358 2006), Taiwan (~1.5 mg/ml; Tsai et al., 2007) and Turkey (~0.5 mg/ml; Sarikurkcu et
359 al., 2008), but higher than a Portuguese sample analysed by other group (0.184 mg/ml;
360 Ribeiro et al., 2008). Concerning the reducing power, the sample here studied gave
361 better results (EC_{50} = 1.16 mg/ml) than a sample from Turkey (~2 mg/ml; Sarikurkcu et
362 al., 2008), and similar to a sample from Taiwan (Tsai et al., 2007). For β-carotene-
363 linoleic acid assay, the obtained result was worst (EC_{50} = 2.46 mg/ml) than for a sample
364 from Turkey (<0.5 mg/ml; Sarikurkcu et al., 2008).

365

366 In conclusion, the results document for the first time primary and secondary metabolites
367 composition in different wild Boletus species (*B. aereus*, *B. reticulatus*, *B. purpureus*,
368 *B. rhodoxanthus* and *B. satanas*), as well as in wild *B. edulis* that could be compared to
369 samples from other countries. Beside primary metabolites, i.e., proteins, carbohydrates,

370 fatty acids, mainly linoleic acid, sugars, mainly mannitol and trehalose, and vitamins
371 (tocopherols and ascorbic acid), they also possess relevant secondary metabolites, such
372 as phenolic acids, and good antioxidant properties, that could help preventing or
373 fighting oxidative stress and that have applications in food, cosmetic and
374 pharmaceutical industries.
375
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383
384 **References**
385 Alessio, C.L. (1985). *Boletus Dill. ex L. Fungi Europaei*. Vol. 2. Libreria Editrice
386 Giovanna Biella, Saronno.
387 AOAC. (1995). *Official methods of analysis* (16th Ed.). Arlington VA, USA:
388 Association of Official Analytical Chemists.
389 Barros, L., Cruz, T., Baptista, P., Estevinho, L.M., & Ferreira, I.C.F.R. (2008). Wild and
390 commercial mushrooms as source of nutrients and nutraceuticals. *Food and*
391 *Chemical Toxicology, 46*, 2742–2747.
392 Barros, L., Dueñas, M., Ferreira, I.C.F.R., Baptista, P., & Santos-Buelga, C. (2009).
393 Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different

394 Portuguese wild mushrooms species. *Food and Chemical Toxicology*, *47*, 1076–
395 1079.
396 Bon, M. (1988). *Guia de campo de los hongos de Europa*. Ediciones Omega, Barcelona.
397 Courtecuisse, R., & Duhem, B. (2005). *Guía de los hongos de la Península Ibérica*,
398 Europa y Norte de África. Ediciones Omega, Barcelona.
399 Dewick, P.M. (2002). *Medicinal Natural Products*. John Wiley & Sons, Lda.
400 Ennamany, R., Lavergne, J.P., Reboud, J.P., Dirheimer, G., & Creppy, E.E. (1995).
401 Mode of action of bolesatine, a cytotoxic glycoprotein from Boletus satanas Lenz.
402 Mechanistic approaches. *Toxicology*, *100*, 51-55.
403 Fang, Y.-Z., Yang, S., & Wu, G. (2002). Free radicals, antioxidants, and nutrition.
404 *Nutrition*, *18*, 872-879.
405 Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., & Ferreira, I.C.F.R. (2009). Study
406 and characterization of selected nutrients in wild mushrooms from Portugal by gas
407 chromatography and high performance liquid chromatography. *Microchemical*
408 *Journal*, *93*, 195–199.
409 Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., & Ferreira, I.C.F.R. (2010).
410 Tocopherols composition of Portuguese wild mushrooms with antioxidant
411 capacity. *Food Chemistry, 119*, 1443–1450.
412 Isaac, S. (1997). Fungi naturally form many diverse biochemical products, some of
413 which are now commercially important; how and why do they do this? *Mycologist*,
414 *11*, 182-183.
415 Jaworska, G., & Bernas, E. (2009). The effect of preliminary processing and period of
416 storage on the quality of frozen *Boletus edulis* (Bull: Fr.) mushrooms. *Food*
417 *Chemistry*, *113*, 936-943.

418 Kavishree, S., Hemavathy, J., Lokesh, B.R., Shashirekha, M.N., & Rajarathnam, S.
419 (2008). Fat and fatty acids of Indian edible mushrooms. *Food Chemistry*, *106*, 597-
420 602.
421 Mattila, P., & Hellstrom, J. (2007). Phenolic acids in potatoes, vegetables, and some of
422 their products. *Journal of Food Composition and Analysis*, *20*, 152-160.
423 Moser, M. (1983). *Keys to Agarics and Boleti (Polyporales, Boletales, Agaricales,*
424 *Russulales)*. Mad River Pr Inc, London.
425 Ouzouni, P.K., Petridis, D., Koller, W.-D., & Riganakos, K.A. (2009). Nutritional value
426 and metal content of wild edible mushrooms collected from west Macedonia and
427 Epirus, Greece. *Food Chemistry*, *115*, 1575-1580.
428 Ouzouni, P.K., & Riganakos, K.A. (2007). Nutritional value and metal content of Greek
429 wild edible fungi. *Acta Alimentaria*, *36*, 99-110.
430 Puttaraju, N.G., Venkateshaiah, S.U., Dharmesh, S.M., Urs, S.M.N., & Somasundaram,
431 R. (2006). Antioxidant activity of indigenous edible mushrooms. *Journal of*
432 *Agricultural and Food Chemistry*, *54*, 9764-9772.
433 Ribeiro, B., Lopes, R., Andrade P.B., Seabra, R.M., Gonçalves, R.F., Baptista, P.,
434 Quelhas, I., & Valentão, P. (2008). Comparative study of phytochemicals and
435 antioxidant potential of wild edible mushroom caps and stipes. *Food Chemistry*,
436 *110*, 47-56.
437 Ribeiro, B., Pinho, P.G., Andrade, P.B., Baptista, P., & Valentão, P. (2009). Fatty acid
438 composition of wild edible mushrooms species: A comparative study.
439 *Microchemical Journal*, *93*, 29-35.
440 Sarikurkcu, C., Tepe, B., & Yamac, M. (2008). Evaluation of the antioxidant activity of
441 four edible mushrooms from the Central Anatolia, Eskisehir – Turkey: *Lactarius*

deterrimus, *Suillus collitinus*, *Boletus edulis*, *Xerocomus chrysenteron*. *Bioresource Technology*, *99*, 6651-6655.

Tsai, S.-Y., Tsai, H.-L., & Mau, J.-L. (2007). Antioxidant properties of *Agaricus blazei*, *Agrocybe cylindracea*, and *Boletus edulis*. *LWT- Food Science and Technology*, *40*, 1392-1402.

Tsai, S.-Y., Tsai, H.-L., & Mau, J.-L. (2008). Non-volatile taste components of *Agaricus blazei*, *Agrocybe cylindracea* and *Boletus edulis*. *Food Chemistry*, *107*, 977-983.

Wang, H., Ng, T.B., & Ooi, V.E.C. (1998). Lectins from mushrooms. *Mycological Research*, *102*, 897-906.

Table 1. Information about the wild *Boletus* species analysed.

Scientific name	English name	Edibility	Habitat	Date of collection	Ecology
Boletus aereus Bull.	Black porcino	Edible	Mixed stands	29-10-2009	Mycorrhizal
Boletus edulis Bull.: Fr.	King bolete	Edible	*Quercus pyrenaica*	04-11-2009	Mycorrhizal
Boletus reticulatus Schaeff.	Summer cep	Edible	*Castanea sativa*	08-11-2009	Mycorrhizal
Boletus purpureus Fr. & Hök	Not found	Not edible	Mixed stands	29-10-2009	Mycorrhizal
Boletus rhodoxanthus (Krombh.) Kallenb.	Not found	Poisonous	*Castanea sativa*	08-11-2009	Mycorrhizal
Boletus satanas Lenz	Devil's bolete	Poisonous	*Castanea sativa*	08-11-2009	Mycorrhizal

Table 2. Moisture, macronutrients, and energetic values in wild edible *Boletus* species. In each line different letters mean significant differences ($p<0.05$).

	Boletus *aereus*	*Boletus edulis*	*Boletus reticulatus*
Moisture (g/100 g fw)	91.65 ± 1.04 a	89.15 ± 0.90 a	91.10 ± 2.21 a
Ash (g/100 g dw)	8.87 ± 0.10 b	5.53 ± 0.23 c	19.72 ± 0.25 a
Proteins (g/100 g dw)	17.86 ± 0.96 b	21.07 ± 0.66 a	22.57 ± 2.08 a
Fat (g/100 g dw)	0.44 ± 0.08 b	2.45 ± 0.09 a	2.55 ± 0.01 a
Carbohydrates (g/100 g dw)	72.83 ± 0.90 a	70.96 ± 0.66 a	55.16 ± 2.03 b
Reducing sugars (g/100 g dw)	2.77 ± 0.22 b	2.87 ± 0.20 b	3.61 ± 0.12 a
Energy (Kcal/100 g dw)	366.69 ± 0.66 b	390.11 ± 2.58 a	333.87 ± 1.00 c

Table 3. Relative percentages of fatty acids in wild *Boletus* species. In each line different letters mean significant differences ($p<0.05$).

	B. aereus	*B. edulis*	*B. reticulatus*	*B. purpureus*	*B. rhodoxanthus*	*B. satanas*
C6:0	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.05 ± 0.00	0.01 ± 0.00
C8:0	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.07 ± 0.00	0.01 ± 0.00
C10:0	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.10 ± 0.01	0.01 ± 0.00
C12:0	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.30 ± 0.02	0.02 ± 0.00
C14:0	0.15 ± 0.01	0.08 ± 0.00	0.39 ± 0.00	0.26 ± 0.03	0.77 ± 0.03	0.15 ± 0.02
C15:0	0.23 ± 0.00	0.23 ± 0.01	0.30 ± 0.00	0.36 ± 0.03	1.27 ± 0.07	0.33 ± 0.03
C16:0	12.47 ± 0.03	9.57 ± 0.15	11.00 ± 0.05	17.49 ± 0.41	17.41 ± 0.38	15.24 ± 0.25
C16:1	0.58 ± 0.00	0.55 ± 0.02	0.56 ± 0.01	2.12 ± 0.02	3.13 ± 0.05	1.91 ± 0.05
C17:0	0.25 ± 0.00	0.17 ± 0.00	0.25 ± 0.00	0.08 ± 0.00	0.50 ± 0.02	0.08 ± 0.00
C17:1c	0.03 ± 0.00	0.01 ± 0.00	0.12 ± 0.01	0.01 ± 0.00	nd	0.01 ± 0.00
C18:0	3.80 ± 0.02	3.11 ± 0.00	4.25 ± 0.05	1.83 ± 0.01	3.39 ± 0.13	3.03 ± 0.01
C18:1n9c	36.72 ± 0.01	42.05 ± 0.18	47.20 ± 0.09	24.18 ± 0.18	7.21 ± 0.15	44.53 ± 1.13
C18:2n6c	43.83 ± 0.01	41.32 ± 0.08	32.83 ± 0.01	51.22 ± 0.12	62.34 ± 0.90	32.18 ± 1.01
C18:3n6	0.07 ± 0.00	0.03 ± 0.00	0.09 ± 0.00	0.32 ± 0.00	nd	0.05 ± 0.00
C18:3n3	0.02 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.17 ± 0.00	0.21 ± 0.00	0.08 ± 0.01
C20:0	0.53 ± 0.00	0.45 ± 0.01	0.67 ± 0.00	0.18 ± 0.01	0.21 ± 0.00	0.26 ± 0.01
C20:1c	0.18 ± 0.01	0.38 ± 0.03	0.32 ± 0.02	0.12 ± 0.02	0.22 ± 0.01	0.16 ± 0.00
C20:2c	0.12 ± 0.00	0.18 ± 0.00	0.11 ± 0.01	0.16 ± 0.01	0.48 ± 0.01	0.09 ± 0.00
C20:4n6	0.03 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.01 ± 0.00	nd	0.01 ± 0.00
C20:3n3+C21:0	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	nd	0.03 ± 0.00
C20:5n3	0.04 ± 0.00	0.01 ± 0.00	0.37 ± 0.01	0.01 ± 0.00	nd	0.15 ± 0.01
C22:0	0.34 ± 0.00	0.37 ± 0.02	0.48 ± 0.01	0.46 ± 0.00	0.53 ± 0.06	0.66 ± 0.05
C22:1n9	0.14 ± 0.00	0.21 ± 0.01	0.14 ± 0.00	0.13 ± 0.01	nd	0.02 ± 0.00
C23:0	0.03 ± 0.00	0.37 ± 0.03	0.06 ± 0.01	0.06 ± 0.00	0.44 ± 0.06	0.13 ± 0.01
C24:0	0.21 ± 0.01	0.32 ± 0.01	0.47 ± 0.02	0.50 ± 0.00	1.12 ± 0.04	0.65 ± 0.06
C24:1	0.12 ± 0.01	0.33 ± 0.00	0.16 ± 0.00	0.27 ± 0.02	0.26 ± 0.01	0.22 ± 0.01
SFA	18.08 ± 0.02 d	14.77 ± 0.19 e	17.95 ± 0.06 d	21.27 ± 0.32 b	26.15 ± 0.67 a	20.57 ± 0.37 c
MUFA	37.78 ± 0.01 d	43.53 ± 0.18 c	48.50 ± 0.09 a	26.84 ± 0.20 e	10.83 ± 0.23 f	46.85 ± 1.38 b
PUFA	44.14 ± 0.01 c	41.71 ± 0.01 d	33.55 ± 0.03 e	51.89 ± 0.12 b	63.03 ± 0.90 a	32.58 ± 1.01 e

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); *cis*-10-Heptadecenoic acid (C17:1); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); γ-Linolenic acid (C18:3n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1c); *cis*-11,14-Eicosadienoic acid (C20:2c); Arachidonic acid (C20:4n6); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); *cis*-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); Behenic acid (C22:1n9); Tricosanoic acid (C23:0); Lignoceric acid (C24:0); Nervonic acid (C24:1). nd- not detected.

Table 4. Sugars and vitamins in wild *Boletus* species. In each line different letters mean significant differences ($p<0.05$).

	B. aereus	*B. edulis*	*B. reticulatus*	*B. purpureus*	*B. rhodoxanthus*	*B. satanas*
Mannitol	1.34 ± 0.11 d	2.45 ± 0.14 c	2.93 ± 0.03 b	2.34 ± 0.01 c	3.95 ± 0.33 a	2.09 ± 0.01 c
Trehalose	4.65 ± 0.24 c	12.40 ± 0.41 a	3.92 ± 0.08 c	0.32 ± 0.00 d	10.42 ± 0.92 b	0.69 ± 0.04 d
Arabinose	nd	nd	nd	nd	1.12 ± 0.14	nd
Total sugars (g/100 g dw)	5.99 ± 0.35 b	14.85 ± 0.55 a	6.85 ± 0.06 b	2.66 ± 0.00 c	15.49 ± 1.10 a	2.78 ± 0.05 c
α-tocopherol	0.14 ± 0.02 b	0.12 ± 0.00 cb	0.10 ± 0.01 cd	0.23 ± 0.01 a	0.03 ± 0.00 e	0.08 ± 0.00 d
γ-tocopherol	5.66 ± 0.15 c	5.17 ± 0.22 c	25.23 ± 1.16 a	12.00 ± 0.27 b	2.10 ± 0.15 d	3.18 ± 0.04 d
δ-tocopherol	0.29 ± 0.06 a	0.51 ± 0.03 a	nd	0.30 ± 0.01 a	0.27 ± 0.14 a	nd
Total tocopherols (μg/g dw)	6.09 ± 0.23 c	5.80 ± 0.26 c	25.33 ± 1.18 a	12.54 ± 0.46 b	2.40 ± 0.00 d	3.26 ± 0.04 d
Ascorbic acid (mg/g dw)	3.71 ± 0.35 b	5.32 ± 0.35 a	2.70 ± 0.02 c	1.90 ± 0.68 dc	2.17 ± 0.58 dc	1.58 ± 0.14 d

nd- not detected.

Table 5. Phenolic acids and related compounds, total phenolics and antioxidant activity EC_{50} values of wild *Boletus* species. In each line different letters mean significant differences ($p<0.05$).

	B. aereus	*B. edulis*	*B. reticulatus*	*B. purpureus*	*B. rhodoxanthus*	*B. satanas*
Protocatechuic acid	nd	2.02 ± 0.32 b	nd	nd	1.72 ± 0.03 b	15.92 ± 0.94 a
p-Hydroxybenzoic acid	13.36 ± 0.11 b	6.55 ± 0.29 d	12.08 ± 1.54 b	nd	22.57 ± 0.65 a	9.57 ± 0.83 c
p-Coumaric acid	4.53 ± 0.42 a	1.17 ± 0.00 b	nd	nd	nd	nd
Total phenolic acids (mg/Kg dw)	17.89 ± 0.53 b	9.74 ± 0.61 d	12.08 ± 1.54 c	nd	24.29 ± 0.67 a	25.49 ± 0.12 a
Cinnamic acid (mg/Kg dw)	31.60 ± 2.72 a	3.72 ± 0.00 c	2.83 ± 0.63 d	3.25 ± 0.04 c	9.46 ± 0.14 b	1.99 ± 0.30 e
Total phenolics (mg GAE/g extract)	46.05 ± 0.06 a	28.56 ± 0.01 d	42.62 ± 0.03 b	8.20 ± 0.58 e	8.38 ± 0.94 e	37.85 ± 1.84 c
DPPH scavenging activity (mg/ml)	0.25 ± 0.02 e	0.43 ± 0.03 c	0.38 ± 0.01 d	0.58 ± 0.06 b	0.79 ± 0.04 a	0.43 ± 0.03 c
Reducing power (mg/ml)	0.47 ± 0.04 e	1.16 ± 0.07 c	0.96 ± 0.04 d	1.55 ± 0.05 b	1.88 ± 0.04 a	1.01 ± 0.08 d
β-carotene bleaching inhibition (mg/ml)	0.60 ± 0.02 f	2.46 ± 0.13 c	1.62 ± 0.07 d	3.05 ± 0.05 a	2.74 ± 0.07 b	1.27 ± 0.01 e

nd- not detected

Figure 1. Individual sugars chromatogram of *Boletus rhodoxanthus*. **1**. arabinose **2.** mannitol; **3.** trehalose; **4.** IS (raffinose).

Figure 2. Individual tocopherols chromatogram of *Boletus reticulatus*. **1**. α-tocopherol; **2.** BHT; **3.** γ-tocopherol; **4.** IS (Tocol).

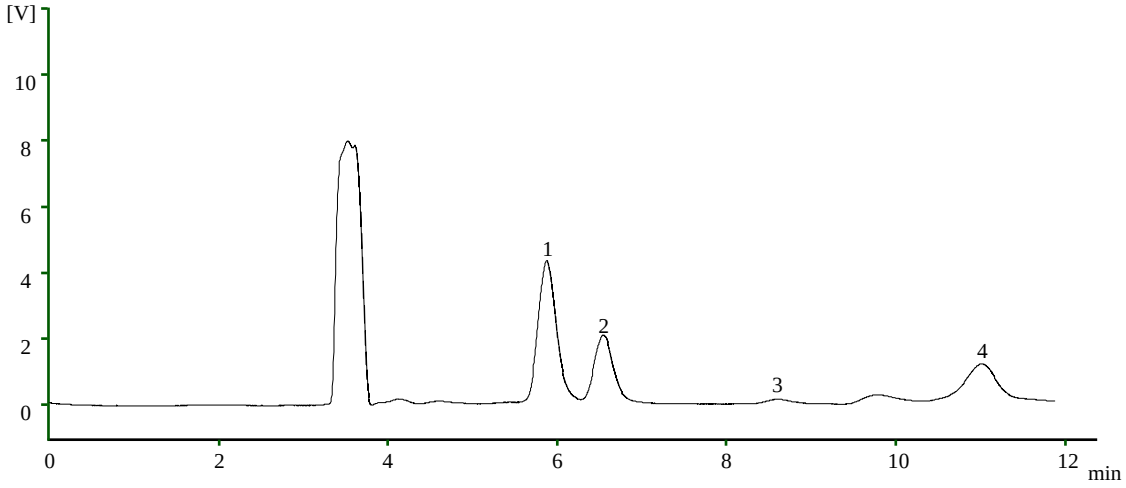

Figure 1.

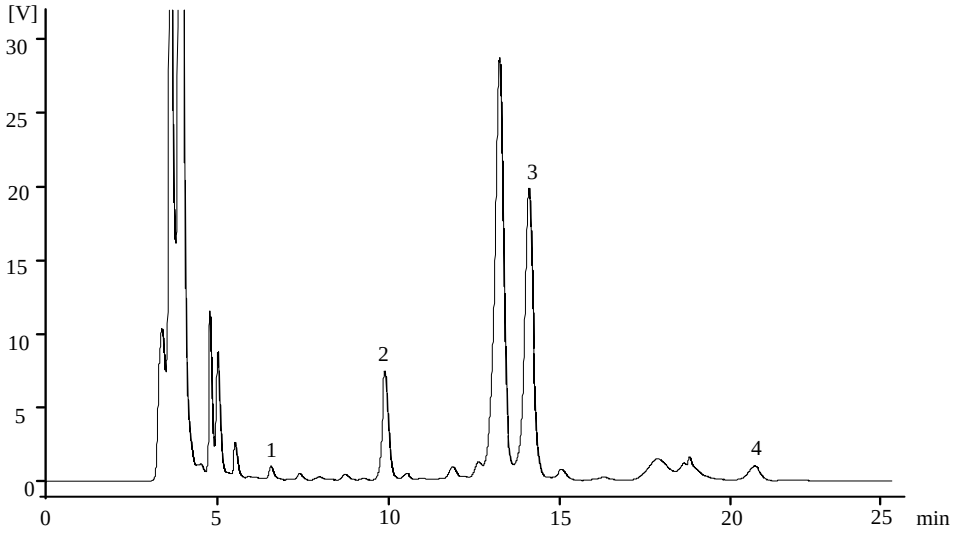

Figure 2.