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# 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.



## 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

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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 (Alessio, 1985; Bon, 1988; Courtecuisse & Duhem, 2005; Moser, 1983), 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 °C 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 (1995) procedures. 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 ± 15 °C. Carbohydrates were calculated by difference: Carbohydrates = 100 − (g protein + g fat + g ash). Reducing sugars were determined by the DNS (dinitrosalicylic acid) method. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g lipid).

#### 2.3.2. Fatty acids

Fatty acids were determined by gas chromatography with flame ionization detection (GC-FID) as described previously by the authors (Heleno et al., 2009). The equipment was a DANI model GC 1000 instrument equipped with a split/splitless injector, a FID (at 260 °C) and a Macherey–Nagel column (30 m × 0.32 mm ID × 0.25 μm $d_f$). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow rate was 4.0 ml/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW DataApex 1.7 software and expressed in relative percentage of each fatty acid.

#### 2.3.3. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as previously described by the authors (Heleno et al., 2009), using

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

| Scientific name | English name | Edibility | Habitat | Date of collection | Ecology |
|---|---|---|---|---|---|
| *Boletusaereus* 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 |