CHEMICAL COMPOSITION OF WILD EDIBLE MUSHROOMS AND ANTIOXIDANT PROPERTIES OF THEIR WATER SOLUBLE POLYSACCHARIDIC AND ETHANOLIC FRACTIONS

Josiana A. Vaz[a,b,c,d], Lillian Barros[a,e,f], Anabela Martins[e], Celestino Santos-Buelga[f], M. Helena Vasconcelos[b,c], Isabel C.F.R. Ferreira[a,e*]

[a]*Mountain Research Centre (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal*.

[b]*Faculty of Pharmacy, University of Porto, Portugal.*

[c]*Cancer Biology Group, IPATIMUP - Institute of Molecular Pathology and Immunology of the University of Porto, Portugal*.

[d]*CEQUIMED-UP - Center of Medicinal Chemistry- University of Porto, Portugal*.

[e]*Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal*.

[f]*Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.*

* Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt telephone +351-273-303219; fax +351-273-325405).

Abstract

Mushrooms have become attractive as functional foods and as a source of physiologically beneficial bioactive compounds. Herein, we describe and compare the chemical constituents (phenolic compounds, macronutrients, sugars, fatty acids, tocopherols and ascorbic acid) of four wild edible mushrooms widely appreciated in gastronomy: *Armillaria mellea* (Vahl) P. Kumm, *Calocybe gambosa* (Fr.) Donk, *Clitocybe odora* (Fr.) P. Kumm., *Coprinus comatus* (O.F. Müll.) Pers. Furthermore, the antioxidant activity of their water soluble polysaccharidic and ethanolic fractions was studied by three different *in vitro* assays. *Coprinus comatus* revealed the highest concentrations of sugars (43.23 g/100 g dry weight), PUFA (77.46%), phenolic compounds (45.02 mg/Kg), tocopherols (301.03 μg/100 g) and, among all of the fractions tested, its ethanolic fraction showed the highest antioxidant activity (EC_{50}<2.6 mg/ml). *Clitocybe odora* revealed one of the highest ascorbic acid (172.65 mg/100 g) contents and its water soluble polysaccharidic fraction showed the best antioxidant properties (EC_{50}<3.6 mg/ml) among the polysaccharidic fractions. The studied mushrooms species could potentially be used in well-balanced diets and as a source of bioactive compounds.

*Keywords***:** Wild edible mushrooms; Bioactive compounds; Phenolic compounds; Polysaccharides; Chemical composition

1. Introduction

Reactive oxygen species (ROS) production occurs during normal cell metabolism, both in animals and plants. Excess of ROS leads to oxidative stress, resulting in oxidative DNA damage which is implicated in the pathogenesis of numerous disorders, e.g. cardiovascular, atherosclerosis, reperfusion injury, cataractogenesis, rheumatoid arthritis, inflammatory disorders and cancer (Halliwell & Gutteridge, 1999; Valko et al., 2007). Mushrooms contain many different dietary nutrients with strong antioxidant capacities, such as phenolic compounds and vitamins (Ferreira, Barros, & Abreu, 2009). In recent years, increased interest in human health, nutrition and disease prevention has enlarged consumer demand for functional foods. In fact, mushrooms have become attractive as functional foods and as a source of bioactive compounds (Chang, 1996; Wasser, 1999; Lindequist, Niedermeyer, & Julich, 2005; Poucheret, Fons, & Rapior, 2006; Zhang, Cui, Cheung, & Wang 2007).

Armillaria mellea is an edible, medicinal mushroom also known as honey fungus. It has been reportedly used in treating geriatric patients with palsy, dizziness, headache, neurasthenia, insomnia, numbness in limbs and infantile convulsion, while also reportedly exerting neuroprotective effects (Gao, Yang, Wang, & Liu, 2001; Colak, Sahin, Yildirim, & Sesli, 2007; Kim et al., 2010). The substance distilled from *A. mellea* has a similar pharmacological activity and clinical curative effect to that of *Rhizoma Gastrodiae* and has no poisonous or side effects. The substance has shown anti-tumor, anti-inflammation, anti-radiation and immunomodulation functions (Sun, Liang, Zhang, Tong, & Liu, 2009).

Calocybe gambosa, commonly known as St. George's mushroom, can be eaten raw, dry or pickled. It has revealed antibacterial activity towards *Bacillus subtilis* and

70 *Escherichia coli* (Keller, Maillard, Keller, & Hostettmann, 2002) and an ability to
71 reduce blood sugar levels (Brachvogel, 1986).
72 *Clitocybe odora*, also known as the aniseed toadstool, is an edible mushroom and its
73 caps can be dried, used as a condiment, or used fresh for flavouring. Polysaccharides
74 extracted from the mycelial culture of *C. odora* and administered intraperitoneally into
75 white mice at a dosage of 300 mg/kg have been shown to inhibit the growth of Sarcoma
76 180 and Ehrlich solid cancers by 70% and 60%, respectively (Ohtsuka et al., 1973).
77 *Coprinus comatus* (Shaggy mane) is a delicious, highly nutritious edible fungus and is
78 also considered a source of valuable medicinal compounds. It has been reported to have
79 various bioactive effects such as immunomodulation, hypoglycaemic, hypolipidemic,
80 antitumor and antibacterial (Yu et al., 2009).
81 The aim of the present work is to obtain quantitative data on the chemical constituents
82 of the mentioned mushrooms widely appreciated in gastronomy, including on
83 antioxidants such as phenolic compounds, reducing sugars, tocopherols and ascorbic
84 acid. Furthermore the antioxidant activity of two different fractions (water soluble
85 polysaccharidic and ethanolic fractions) obtained from each species was compared.

2. Material and methods

2.1. Mushroom species

Samples of *Armillaria mellea* (Vahl) P. Kumm, *Calocybe gambosa* (Fr.) Donk, *Clitocybe odora* (Fr.) P. Kumm. and *Coprinus comatus* (O.F. Müll.) Pers. were collected in Bragança (Northeast Portugal) in the autumn of 2009. Taxonomic identification of sporocarps was made according to other authors (Bon, 1988; Courtecuisse & Duhem, 2005) and online keys (http://www.mycokey.com/) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. All samples were lyophilised (Ly-8-FM-ULE, Snijders, Holland) and reduced to a fine dried powder (20 mesh).

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 well as other individual fatty acid isomers, ascorbic acid, tocopherols, sugars and phenolic standards (gallic, protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, and cinnamic acids). 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 used in the studies was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

110 *2.3. Evaluation of antioxidant activity*

111 *Preparation of the fractions.* The water soluble polysaccharidic and ethanolic fractions
112 were prepared following the procedure described by Cheng, Lin, Lur, Chen, & Lu
113 (2008) with some modifications. Polysaccharides were extracted from lyophilized
114 mushrooms (~1.5 g) with boiling water (50 ml) for 2 h under agitation (150 rpm; Velp
115 Are magnetic stirrer) before being subsequently filtered through Whatman No. 4 paper.
116 The residue was then extracted with two further portions of boiling water over a total 6
117 h extraction. The combined extracts were lyophilized before 95 % ethanol (10 ml) was
118 added and polysaccharides were precipitated overnight at 4 ºC. The precipitated
119 polysaccharides were collected after centrifugation (Centorion K24OR- 2003
120 refrigerated centrifuge) at 3,100*g* for 40 min followed by filtration, before being
121 lyophilized, resulting in a crude polysaccharidic sample. The ethanolic supernatant was
122 evaporated at 40 ºC under reduced pressure (rotary evaporator Büchi R-210), giving the
123 ethanolic fraction. The crude polysaccharidic samples were re-dissolved in water while
124 the ethanolic extracts were re-dissolved in ethanol, at final concentrations of 50 mg/ml,
125 for the antioxidant activity assays.

126

127 *DPPH radical-scavenging activity.* This assay was performed in 96-well microtiter
128 plates using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction
129 mixture in each of the 96-wells of the plate consisted of one of the different
130 concentrations of the extracts (30 µl) and aqueous methanolic solution (80:20, v/v, 270
131 µl) containing DPPH radicals ($6x10^{-5}$ mol/l). The mixture was left to stand for 60 min in
132 the dark. Reduction of the DPPH radical was determined by measuring the absorption at
133 515 nm. Radical scavenging activity (RSA) was calculated as a percentage of DPPH

discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration.

Reducing power. This assay was also performed using microtiter plates and the Microplate Reader described above. Different extract concentrations (0.5 ml) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 ºC for 20 min and trichloroacetic acid (10% w/v, 0.5 ml) added. This mixture (0.8 ml) was then poured into the wells of a 48-well microplate, also containing deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml) and the absorbance was measured at 690 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration.

Inhibition of β-carotene bleaching. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40ºC under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg) and distilled water (100 ml) were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing different concentrations of the extracts (0.2 ml). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm

158 (Analytikjena 200-2004 spectrophotometer). A blank, devoid of β-carotene, was
159 prepared for background subtraction. β-Carotene bleaching inhibition was calculated
160 using the following equation: (β-carotene content after 2h of assay/initial β-carotene
161 content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was
162 calculated by interpolation from the graph of β-carotene bleaching inhibition percentage
163 against extract concentration.
164
165 *2.4. Phenolic compounds*
166 Each sample (~3 g) was extracted with acetone:water (80:20, v/v; 30 ml) at -20°C for
167 6h. After sonication for 15 min, the extract was centrifuged at 4,000*g* for 10 min, and
168 filtered through Whatman nº 4 paper. The residue was then extracted with two
169 additional 30 ml portions of the acetone:water mixture. Combined extracts were
170 evaporated at 40 ºC under reduced pressure to remove acetone. The aqueous phase was
171 washed with n-hexane, and then submitted to a liquid-liquid extraction with diethyl
172 ether (3 × 30 ml) and ethyl acetate (3 × 30 ml). The organic phases were evaporated at
173 40 ºC to dryness, re-dissolved in water:methanol (80:20, v/v; 1 ml), followed by
174 filtering through a 0.22 µm disposable LC filter disk for HPLC analysis. The analysis
175 was performed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent
176 Technologies) as previously described (Barros, Dueñas, Ferreira, Baptista, & Santos-
177 Buelga, 2009). Separation was achieved on a Spherisorb S3 ODS-2 (Waters) reverse
178 phase C_{18} column (3 µm, 150 × 4.6 mm) thermostated at 25 ºC. The solvents used were:
179 (A) 2.5% acetic acid in water, (B) 2.5% acetic acid:acetonitrile (90:10, v/v), and (C)
180 100% HPLC-grade acetonitrile. The gradient employed was: isocratic 100% A for 10
181 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 of dry weight.

2.5. Macronutrients

The 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; ash content was determined by incineration at 600 ± 15ºC. Carbohydrates were calculated by difference: Carbohydrates = 100 – (g protein + g fat + g ash). Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein +g carbohydrate) + 9 × (g lipid).

2.6. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as previously described by the authors (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and RI detector (Knauer Smartline 2300). Dried sample powder (1.0 g) was spiked with raffinose as internal standard (IS, 5

mg/ml) and was extracted with 40 ml of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged at 15,000 *g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 ml of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 ml and filtered through 0.2 µm nylon filters. Chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 µm, Knauer) operating at 30ºC (7971 R Grace oven). The mobile phase was acetonitrile:deionized water, 7:3 (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 was analysed using Clarity 2.4 Software (DataApex). Quantification was made by internal normalization of the chromatographic peak area and the results expressed in g per 100 g of dry weight.

2.7. Fatty Acids

Fatty acids were determined by gas chromatography with flame ionization detection (GC-FID) as described previously by the authors (Barros et al., 2008) and after the following trans-esterification procedure: fatty acids (obtained after Soxhlet extraction) were methylated with 5 ml of methanol:sulphuric acid 95%:toluene 2:1:1 (v/v/v), for at least 12 h in a bath at 50 ºC and 160 rpm; to obtain phase separation 3 ml of deionised water were added; the fatty acids methyl esters (FAME) were recovered by shaking in a vortex with 3 ml of diethyl ether, and the upper phase was passed through a micro-column of anhydrous sodium sulphate to eliminate the water. The sample was recovered in a vial with Teflon and filtered through a 0.2 µm Whatman nylon filter. The fatty acid profile was analyzed with a DANI model GC 1000 instrument equipped with a

split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m × 0.32 mm ID × 0.25 µm d_f). The oven temperature program followed was an initial column temperature of 50 ºC, held for 2 min, followed by a 10ºC/min ramp to 240 ºC for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 ml/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. For each analysis 1 µl of the sample was injected in GC. 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 1.7 software (DataApex 1.7) and expressed as a relative percentage of each fatty acid.

2.8. Tocopherols

Tocopherols content was determined following a procedure previously described by the authors (Barros et al., 2008). Butylated hydroxytoluene, BHT solution in hexane (10 mg/ml; 100 µl) and IS solution in hexane (tocol; 50 µg/ml; 400 µl) were added to the sample prior to the extraction procedure. Samples (~500 mg) were homogenized with methanol (4 ml) by vortex mixing (1 min). Subsequently, hexane (4 ml) was added and again vortex mixed for 1 min. Saturated NaCl aqueous solution (2 ml) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4,000 *g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with n-hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 ml of n-hexane, dehydrated with anhydrous sodium sulphate and filtered through 0.2 µm nylon filters and transferred into a dark injection vial. Analyses were performed by the HPLC system (described above) connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330

254 nm. Chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm)
255 normal-phase column from YMC Waters operating at 30ºC. The mobile phase used
256 consisted of a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1
257 ml/min, with an injection volume of 20 µl. The compounds were identified by
258 chromatographic comparisons with authentic standards. Quantification was based on the
259 fluorescence signal response, using the IS method. Tocopherol contents in the samples
260 were expressed in µg per 100 g of dry sample.
261
262 *2.9. Ascorbic acid*
263 A fine dried powder (20 mesh; 150 mg) was extracted with metaphosphoric acid (1%,
264 10 ml) for 45 min at room temperature and filtered through a Whatman Nº 4 filter paper.
265 The filtrate (1 ml) was mixed with 2,6-dichloroindophenol (9 ml) and the absorbance
266 measured within 30 min at 515 nm against a blank. Ascorbic acid content was
267 calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006-0.1
268 mg/ml) and the results expressed as mg per 100 g of dry weight.
269
270 *2.10. Statistical analysis*
271 All sample assays were carried out in triplicate. The results are expressed as mean
272 values and standard deviation (SD). The results were analyzed using one-way analysis
273 of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment
274 was carried out using SPSS v. 16.0 program.
275

3. Results and discussion

Some literature refers to a number of bioactive chemical constituents of *Armillaria mellea* from the Czech Republic (Kalac, 2009), Turkey (Ouzouni et al., 2009; Yilmaz, Solmaz, Turkekul, & Elmastas, 2006) and US (Cox, Scherm, & Riley, 2006) and to antioxidant properties of *Armillaria mellea* from China (Yu et al., 2009) and *Coprinus comatus* from Taiwan (Tsai, Tsai, & Mau, 2007) but there are no such reports on Portuguese samples of these mushrooms.

A sequential extraction with boiling water and ethanol was performed in order to obtain extracts with high molecular weight compounds such as polysaccharides and low molecular weight compounds such as phenolic compounds. Both kinds of compounds play important roles in mushrooms, including medicinal functions (Ferreira, Vaz, Vasconcelos, & Martins, 2010). The extraction yields obtained for ethanolic fractions were lower than the yields for water soluble polysaccharidic fractions (**Table 1**).

To evaluate the antioxidant properties of both fractions, three different assays were carried out: scavenging activity on DPPH radicals, reducing power and inhibition of lipid peroxidation. Water soluble polysaccharidic fractions revealed a higher antioxidant activity than ethanolic fractions, unless for *Coprinus comatus* (**Table 1**). Moreover, the ethanolic fraction of this species showed the highest DPPH radical scavenging activity (EC_{50} value 2.56 mg/ml). This observation is in agreement with its higher content in phenolic compounds compared to the other mushrooms (**Table 2**). The ethanolic fraction here studied gave better results (reducing power 1.61 at 5 mg/ml; DPPH scavenging activity 79.92% at 5 mg/ml, data not shown) than ethanolic (reducing power 0.45 at 5 mg/ml; DPPH scavenging activity 84.5% at 5mg/ml) and hot water (reducing

299 power 0.25 at 5 mg/ml; DPPH scavenging activity 58.9% at 20 mg/ml) extracts of
300 *Coprinus comatus* from Taiwan (Tsai et al., 2007).
301 The water soluble polysaccharidic fraction of *Clitocybe odora* showed the lowest EC_{50}
302 value for reducing power (0.94 mg/ml) and β-carotene bleaching inhibition (0.27
303 mg/ml). The ethanolic fraction of *Calocybe gambosa* showed higher EC_{50} values (i.e.,
304 lower antioxidant activity) than a commercial sample previously studied by us (7.14
305 mg/ml, 4.31 mg/ml and 2.77 mg/ml for DPPH scavenging activity, reducing power and
306 β-carotene bleaching inhibition, respectively). Nevertheless, that sample was a crude
307 methanolic extract obtained at room temperature (Queirós, Barreira, Sarmento, &
308 Ferreira, 2009) and not a fractionated procedure with boiling water and ethanol like that
309 used in the present work.
310 Up to three phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids)
311 and a related compound (cinnamic acid) could be identified and quantified in the
312 different samples analysed by HPLC-DAD (**Table 2**). *Coprinus comatus* showed the
313 highest concentration of phenolic acids (80.33 mg/Kg, dry weight), due to the
314 contribution of *p*-hydroxybenzoic and *p*-coumaric acids, whereas *Calocybe gambosa*
315 revealed the highest concentration of cinnamic acid (17.69 mg/Kg). Several phenolic
316 compounds were identified and quantified in wild mushrooms from Finland, India,
317 Korea and Portugal (Ferreira et al., 2009) but not in the studied species. However, to the
318 best of our knowledge this is the first report on individual phenolic compounds of
319 *Armillaria mellea*, *Calocybe gambosa*, *Clitocybe odora* and *Coprinus comatus*.
320 Nonetheless, Signore, Romeo, & Giaccio (1997) had previously reported total phenolics
321 in *A. mellea* and *C. odora* from Italy, measured by the Folin Ciocalteu assay.
322

323 The results of the moisture, macronutrients composition, individual sugars and
324 estimated energetic value obtained for the four wild edible mushrooms are shown in
325 **Table 3**. Significant differences (p<0.05) were observed in the moisture (90.92 g/100 g)
326 and ash (13.89 g/100 g dry weight) contents between the different mushrooms, with
327 *Calocybe gambosa* revealing the highest values, whilst no differences were found for
328 carbohydrates and proteins (~70 g/100 g and ~16 g/100 g, expressed in dry weight,
329 respectively), which were the most abundant macronutrients. Fat was the less abundant
330 macronutrient, being lower than 5.6 g/100 g, dw. *Armillaria mellea* showed the highest
331 energetic contribution (400.68 Kcal/100 g, dw) mainly due its higher fat values. A
332 sample of this same mushroom species from Greece (Ouzouni et al., 2009) revealed
333 similar moisture (87.17 g/100g, dw) and ash (7.95 g/100 g, dw) contents, but higher
334 protein concentration (24.47 g/100 g, dw), and lower fat (2.10 g/100 g, dw) and
335 carbohydrate (65.47 g/100 g, dw) levels. An *A. mellea* sample from Poland (Kalac,
336 2009) showed a quite different macronutrient composition from the sample from Greece
337 and the sample here studied, particularly regarding carbohydrate content (16.4 g/100 g,
338 dw). Regarding *Calocybe gambosa*, a commercial sample previously analysed by our
339 group (Barros, Tomé, Baptista, Estevinho, & Ferreira, 2008) revealed higher protein
340 (47.22 g/100 g, dw) and lower carbohydrate (43.01 g/100 g, dw) contents than the wild
341 sample now studied. The variability among samples of different origin might be related
342 to environmental temperature, relative humidity during growth and relative amount of
343 metabolic water produced or utilised during storage, as well as to the industrial
344 processes to which the commercial mushrooms are submitted (Ouzouni et al., 2009). To
345 the best of our knowledge, this is the first report on *Clitocybe odora* and *Coprinus*
346 *comatus* macronutrient composition.

347

348 In relation to sugar composition (**Table 3**), the edible mushrooms yielded trehalose as
349 the main sugar. The *Coprinus comatus* sample showed the highest total sugars
350 concentration (43.23 g/100 g, dw), mostly due to trehalose (42.82 g/100 g, dw). The
351 highest values of mannitol (5.45 g/100 g, dw) were found in the *Armillaria mellea*
352 sample, and arabinose was only detected in this sample (0.78 g/100 g, dw). Sugar
353 composition of the wild *Calocybe gambosa* was very similar to the results obtained for a
354 commercial sample (mannitol 0.27 g/100 g, trehalose 8.01 g/100 g and total sugars 9.13
355 g/100 g, expressed in dry weight basis), although in that case melezitose was also
356 detected but could not be found in the wild sample (Barros et al., 2008).

357

358 The results for fatty acid composition, total saturated fatty acids (SFA),
359 monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of the
360 wild edible mushrooms analysed are given in **Table 4**. Up to twenty-two fatty acids
361 were detected and quantified. The major fatty acid found was oleic acid (C18:1n9) for
362 *Armillaria mellea* and *Clitocybe odora*, and linoleic acid (C18:2n6) for *Calocybe*
363 *gambosa* and *Coprinus comatus*, contributing to the prevalence of PUFA in the latter
364 two species. The studied species also contained palmitic acid (C16:0) as a major fatty
365 acid. Linoleic acid was also described as a major fatty acid in a sample of *Coprinus*
366 *comatus* from Turkey (25.8% in fruit body and 59.5% in stem; Yilmaz et al., 2005) but
367 at a lower percentage than that found in the present study (74.86%). The major fatty
368 acid found in samples of *Armillaria mellea* from Turkey (49.6% in fruit body and
369 2.56% in stem; Yilmaz et al., 2005) and US (39.6%; Cox et al., 2006) was linoleic acid
370 and not oleic acid as here obtained. A commercial sample of *Calocybe gambosa*

371 revealed a similar profile to that obtained for the wild sample, but with the lowest
372 MUFA (19.05 %) and highest PUFA (58.42%) contents (Barros et al., 2008). Low
373 calorie and low fat diets are recommended for people with high blood cholesterol, and
374 oils with high linoleic and oleic acid levels are known to help preventing
375 atherosclerosis. In addition to their low calories and low fat, mushrooms are also rich in
376 these fatty acids, which allows considering them healthy foods.
377
378 Vitamin (tocopherols and ascorbic acid) contents in the four wild edible mushroom
379 species analysed are quoted in **Table 5**. γ-Tocopherol was the major compound in all
380 cases, whereas β-tocopherol was not detected in any of the studied samples and δ-
381 tocopherol was not detected in *Armillaria mellea*. The *Coprinus comatus* sample
382 presented the highest content of tocopherols (301.03 μg/100 g of dry weight), while
383 *Clitocybe odora* (172.65 mg/100 g, dw) and *Calocybe gambosa* (180.47 mg/100g)
384 revealed the highest levels of ascorbic acid without significant statistical differences. To
385 the best of our knowledge, this is the first report on antioxidant vitamins in these four
386 mushroom species. These compounds are known to be important in safeguarding
387 against free-radical-mediated tissue injuries. Vitamin E is a major antioxidant in
388 biological systems acting as a powerful chain-breaking agent through the scavenging of
389 peroxyl radicals, and terminating the chain reaction of lipid peroxidation in membranes
390 and lipoproteins (Shirpoor et al., 2007).
391
392 Overall, *Coprinus comatus* revealed the highest concentrations in sugars, PUFA,
393 phenolic compounds, tocopherols and, among all the tested extracts, its ethanolic
394 fraction showed the highest antioxidant activity. *Clitocybe odora* revealed one of the

highest ascorbic acid contents, and its water soluble polysaccharidic fraction showed the best antioxidant properties, among the polysaccharidic extracts. All the studied mushrooms species can be considered as suitable foods to be included in well-balanced diets due to their high proteins and carbohydrates contents, and low fat levels. Furthermore, these species should be further studied as a source of bioactive compounds, including high molecular weight (*e.g.* polysaccharides) and low molecular weight compounds (*e.g.* phenolic compounds, tocopherols and ascorbic acid)..

Acknowledgements

The authors acknowledge Maria João Sousa and Sandrina Heleno for the collection and identification of the mushroom species. The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) and COMPETE/QREN/UE (research project PTDC/AGR-ALI/110062/2009) for financial support. L. Barros (BPD/4609/2008) and J.A. Vaz (BD/43653/2008) also thank FCT. IPATIMUP (FCT, I&D 4040/2007) is an Associate Laboratory of the Portuguese Ministry of Ministry of Science, Technology and Higher Education and is partially supported by FCT, the Portuguese Foundation for Science and Technology. The GIP-USAL is financially supported by the Spanish *Ministerio de Ciencia e Innovación* through the *Consolider-Ingenio 2010* Programme (FUN-C-FOOD, CSD2007-00063), and *Junta de Castilla y León* (Grupo de Investigación de Excelencia, GR133).

References

AOAC (1995). *Official methods of analysis* (16th Ed.). Arlington VA, USA: Association of Official Analytical Chemists.

419 Barros, L., Cruz, T., Baptista, P., Estevinho, L.M., Ferreira, I.C.F.R. (2008). Wild and
420 commercial mushrooms as source of nutrients and nutraceuticals. *Food and*
421 *Chemical Toxicology*, *46*, 2742–2747.
422 Barros, L., Dueñas, M., Ferreira, I.C.F.R., Baptista, P., & Santos-Buelga, C. (2009).
423 Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different
424 Portuguese wild mushrooms species. *Food and Chemical Toxicology*, *47*, 1076-
425 1079.
426 Bon, M. (1988). *Guia de campo de los hongos de Europa*. Ediciones Omega, Barcelona.
427 Brachvogel, R. (1986). Reduction of blood sugar by *Calocybe gambosa* Fr. Donk
428 *Zeitschrift für Mykologie*, *52*, 445.
429 Chang, R. (1996). Functional properties of edible mushrooms. *Nutrition Reviews*, *54*,
430 S91-93.
431 Cheng, J.-J., Lin, C.-Y., Lur, H.-S., Chen, H.-P., & Lu, M.-K. (2008). Properties and
432 biological functions of polysaccharides and ethanolic extracts isolated from
433 medicinal fungus, *Formitopsis pinicola*. *Processes in Biochemistry*, *43*, 829-834.
434 Colak, A., Sahin, E., Yildirim, M., & Sesli, E. (2007). Polyphenol oxidase potentials of
435 three wild mushroom species harvested from Lişer High Plateau, Trabzon. *Food*
436 *Chemistry*, *102*, 1426-1433.
437 Courtecuisse, R., & Duhem, B. *Guía de los hongos de la Península Ibérica*, Europa y
438 Norte de África. Ediciones Omega, Barcelona, 2005.
439 Cox, K.D., Scherm, H., & Riley, M.B. (2006). Characterization of *Armillaria spp*. from
440 peach orchards in the southeastern United States using fatty acid methyl ester
441 profiling. *Miycological Research*, *110*, 414-422.

442 Ferreira, I.C.F.R., Barros, L., & Abreu, R.M.V. (2009). Antioxidants in wild
443 mushrooms. *Current Medicinal Chemistry*, *16*, 1543-1560.
444 Ferreira, I.C.F.R., Vaz, J.A., Vasconcelos, M.H., & Martins, A. (2010). Compounds
445 from wild mushrooms with antitumor potential. *Anti-cancer Agents in Medicinal*
446 *Chemistry*, 2010, *10*, 424-436.
447 Gao, J.M., Yang, X., Wang, C.-Y., & Liu, J.K. (2001). Armillaramide, a new
448 sphingolipid from the fungus *Armillaria mellea*. *Fitoterapia*, *72*, 858-864.
449 Halliwell, B., & Gutteridge, J.M.C. (1999). Free radicals in Biology and Medicine (3rd
450 ed.), Oxford University Press, Oxford, UK.
451 Kalac, P. (2009). Chemical compositions and nutritional value of European species of
452 wild growing mushrooms: a review. *Food Chemistry*, *113*, 9-16.
453 Keller, C., Maillard, M., Keller, J., & Hostettmann, K. (2002). Screening of European
454 fungi for antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free-
455 radical scavenging activities and subsequent isolation of bioactive compounds.
456 *Pharmaceutical Biology*, *40*, 518-25.
457 Kim, Y.S., Im, J., Choi, J.-N., Kang, S.-S., Leec, Y.J., Lee, C.H., Yun, C.-H., Sond,
458 C.G., & Han, S.H. (2002). Induction of ICAM-1 by *Armillariella mellea* is
459 mediated through generation of reactive oxygen species and JNK activation.
460 *Journal of Ethnopharmacology*, *128*, 198–205.
461 Lindequist, U., Niedermeyer, T.H.J., & Julich, W.D. (2005). The pharmacological
462 potential of mushrooms. *eCAM*, *2*, 285-299.
463 Ohtsuka, S., Ueno, S., Yoshikumi, C., Hirose, F., Ohmura, Y., Wada, T., Fujii, T., &
464 Takahashi, E. (1973). Polysaccharides having an anticarcinogenic effect and a
465 method of producing them from species of Basidiomycetes. UK Patent 1331513.

466 Ouzouni, P.K., Petridis, D., Koller, W.-D., & Riganakos, K.A. (2009). Nutritional value
467 and metal content of wild edible mushrooms collected from West Macedonia and
468 Epirus, Greece. *Food Chemistry*, *115*, 1575-1580.
469 Poucheret, P., Fons, F., & Rapior, S. (2006). Biological and Pharmacological Activity of
470 Higher Fungi: 20-Year Retrospective Analysis. *Mycologie*, *27*, 311-333.
471 Queirós, B., Barreira, J.C.M., Sarmento, A.C., & Ferreira I.C.F.R. (2009). In search of
472 synergistic effects in antioxidant capacity of combined edible mushrooms.
473 *International Journal of Food Sciences and Nutrition*, *60*, 1-13.
474 Shirpoor, A., Ansari, M.H.K., Salami, S., Pakdel, F.G, & Rasmi, Y. (2007). Effect of
475 vitamin E on oxidative stress status in small intestine of diabetic rat. *World Journal*
476 *of Gastroenterology*, *13*, 4340-4344.
477 Signore, A.D., Romeo, F., & Giaccio, M. (1997). Content of phenolic substances in
478 basidiomycetes. *Mycological Research, 101*, 552-556.
479 Sun, Y., Liang, H., Zhang, X., Tong, H., & Liu, J. (2009). Structural elucidation and
480 immunological activity of a polysaccharide from the fruiting body of *Armillaria*
481 *mellea*. *Bioresource Technology, 100,* 1860–1863.
482 Tsai, S.-Y., Tsai, H.-L., & Mau. J.-L. (2007). Antioxidant properties of *Agaricus blazei*,
483 *Agrocybe cylindracea*, and *Boletus edulis*. *LWT, 40,* 1392-1402.
484 Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., & Telser, J. (2007). Free
485 radicals and antioxidants in normal physiological functions and human disease.
486 *International Journal of Biochemistry and Cell Biology, 39*, 44-84.
487 Wasser, S.P. (1999). Medicinal properties of substances occurring in higher
488 Basidiomycete mushrooms: current perspective (review). *International Journal of*
489 *Medicinal Mushrooms, 1*, 31-62.

Yilmaz, N., Solmaz, M., Turkekul, I., & Elmastas, M. (2006). Fatty acid composition in some wild edible mushrooms growing in the middle Black Sea region of Turkey. *Food Chemistry, 99*, 168-174.

Yu, J., Cui, P.-J., Zeng, W.-L., Xie, X.-L., Liang, W.-J., Lin, G.-B., & Zeng, L. (2009). Protective effect of selenium-polysaccharides from the mycelia of *Coprinus comatus* on alloxan-induced oxidative stress in mice. *Food Chemistry*, *117*, 42–47.

Zhang, M., Cui, S.W., Cheung, P.C.K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology, 18*, 4-19.

Table 1. Extraction yields and antioxidant activity (EC_{50} values[a]) of two fractions obtained from wild edible mushrooms (mean ± SD; n=3). In each row different letters imply significant differences ($p<0.05$).

Species	*Armillaria mellea*		*Calocybe gambosa*		*Clitocybe odora*		*Coprinus comatus*	
Fraction	Ethanolic	Water soluble polysaccharidic	Ethanolic	Water soluble polysaccharidic	Ethanolic	Water soluble polysaccharidic	Ethanolic	Water soluble polysaccharidic
Extraction yield (g/100 g dry weight)	10.91 ± 0.39 c	39.66 ± 1.49 a	14.89 ± 0.51 c	37.92 ± 2.09 a	1.26 ± 0.10 e	18.68 ± 0.23 b	5.56 ± 0.18 d	12.54 ± 0.61 c
DPPH scavenging activity (mg/ml)	17.13 ± 0.67 b	3.95 ± 0.16 d	34.60 ± 0.44 a	7.08 ± 0.12 c	6.77 ± 0.05 c	3.56 ± 0.13 d	2.56 ± 0.31 e	7.31 ± 0.22 c
Reducing power (mg/ml)	7.53 ± 0.10 b	0.98 ± 0.00 g	11.46 ± 0.18 a	2.38 ± 0.02 e	3.63 ± 0.14 d	0.94 ± 0.01g	1.47 ± 0.01 f	4.67 ± 0.04 c
β-carotene bleaching inhibition (mg/ml)	8.94 ± 0.02 a	0.87 ± 0.01 e	7.57 ± 0.09 c	8.17 ± 0.34 b	1.36 ± 0.08 d	0.27 ± 0.00 f	1.26 ± 0.01 d	7.43 ± 0.02 c

[a]Concentration of extract providing 50% of antioxidant activity in DPPH scavenging activity and β-carotene bleaching inhibition assays, and 0.5 of absorbance in reducing power assay.

Table 2. Contents of phenolic compounds and cinnamic acid (mg/Kg of dry weight) measured by HPLC in the four wild edible mushroom species (mean ± SD; n=3). In each row different letters imply significant differences ($p<0.05$).

	Armillaria mellea	*Calocybe gambosa*	*Clitocybe odora*	*Coprinus comatus*
Protocatechuic acid	nd	2.58 ± 0.49	nd	nd
p-Hydroxybenzoic acid	4.00 ± 0.72 c	38.40 ± 3.14 b	27.93 ± 2.79 b	61.53 ± 1.19 a
p-Coumaric acid	nd	4.04 ± 0.29 b	1.81 ± 0.09 c	18.79 ± 0.92 a
Total phenolic acids	4.00 ± 0.72 d	45.02 ± 3.34 b	28.83 ± 1.51 c	80.33 ± 0.27 a
Cinnamic acid	8.67 ± 0.03 c	17.69 ± 0.21 a	13.77 ± 0.59 b	12.58 ± 0.12 b

nd- not detected.

Table 3. Moisture (g/100 g of fresh weight), macronutrients (g/100 g of dry weight) and energetic value (Kcal/100 g of dry weight) in the four wild edible mushroom species analysed (mean ± SD; n=3). In each row different letters imply significant differences (p<0.05).

	Armillaria mellea	*Calocybe gambosa*	*Clitocybe odora*	*Coprinus comatus*
Moisture	88.27 ± 0.60 c	90.92 ± 1.08 a	88.49 ± 3.03 b	85.19 ± 0.50 d
Ash	6.78 ± 1.28 c	13.89 ± 1.41 a	9.55 ± 0.68 b	12.85 ± 0.42 a
Proteins	16.38 ± 1.34 a	15.46 ± 0.24 a	17.33 ± 1.37 a	15.67 ± 0.23 a
Fat	5.56 ± 0.53 a	0.83 ± 0.11 c	2.46 ± 0.04 b	1.13 ± 0.05 c
Carbohydrates	71.28 ± 1.06 a	69.83 ± 1.22 a	70.66 ± 1.09 a	70.36 ± 0.26 a
Energy	400. 68 ± 5.50 a	348.58 ± 3.58 c	374.12 ± 1.81 b	354.27 ± 1.18 c
Mannitol	5.45 ± 0.04 a	0.29 ± 0.01 c	0.59 ± 0.02 b	0.40 ± 0.04 d
Trehalose	9.33 ± 0.04 b	7.96 ± 0.28 b	7.77 ± 0.30 b	42.82 ± 2.59 a
Arabinose	0.78 ± 0.04	nd	nd	nd
Total sugars	15.66 ± 0.04 b	8.26 ± 0.29 c	8.36 ± 0.32 c	43.23 ± 2.62 a

nd- not detected.

Table 4. Relative percentages of fatty acids in the four wild edible mushroom species analysed (mean ± SD; n=3). In each row different letters imply significant differences ($p<0.05$).

	Armillaria mellea	*Calocybe gambosa*	*Clitocybe odora*	*Coprinus comatus*
C6:0	nd	0.27 ± 0.03	0.04 ± 0.00	0.05 ± 0.00
C8:0	0.11 ± 0.01	0.25 ± 0.02	0.03 ± 0.00	0.05 ± 0.00
C10:0	0.09 ± 0.00	0.13 ± 0.01	0.02 ± 0.00	0.09 ± 0.00
C12:0	0.65 ± 0.00	0.15 ± 0.01	0.07 ± 0.00	0.16 ± 0.00
C14:0	0.27 ± 0.00	0.39 ± 0.02	0.20 ± 0.01	0.41 ± 0.02
C15:0	0.27 ± 0.02	0.36 ± 0.02	0.54 ± 0.01	0.35 ± 0.01
C16:0	11.04 ± 0.06	13.57 ± 0.50	12.46 ± 0.25	10.56 ± 0.44
C16:1	6.36 ± 0.01	0.65 ± 0.03	0.17 ± 0.03	0.59 ± 0.02
C17:0	0.03 ± 0.00	0.26 ± 0.05	0.10 ± 0.00	0.20 ± 0.02
C18:0	3.53 ± 0.01	3.24 ± 0.12	3.46 ± 0.26	1.90 ± 0.13
C18:1n9c	47.74 ± 0.35	32.54 ± 1.37	46.07 ± 0.17	6.27 ± 0.03
C18:2n6c	27.71 ± 0.32	43.88 ± 0.31	34.90 ± 0.68	74.86 ± 0.95
C18:3n3	0.04 ± 0.00	0.93 ± 0.01	0.06 ± 0.00	1.90 ± 0.14
C20:0	0.15 ± 0.00	0.47 ± 0.06	0.39 ± 0.02	0.11 ± 0.00
C20:1c	0.10 ± 0.01	0.13 ± 0.02	0.05 ± 0.00	0.10 ± 0.00
C20:2c	0.01 ± 0.05	0.06 ± 0.00	0.02 ± 0.00	0.37 ± 0.02
C20:3n3+C21:0	nd	0.07 ± 0.01	0.03 ± 0.00	0.32 ± 0.03
C20:5n3	0.01 ± 0.00	0.14 ± 0.04	0.04 ± 0.00	nd
C22:0	0.26 ± 0.01	0.72 ± 0.04	0.46 ± 0.03	0.43 ± 0.01
C23:0	0.02 ± 0.00	0.69 ± 0.03	0.24 ± 0.01	0.21 ± 0.03
C24:0	0.81 ± 0.02	1.04 ± 0.18	0.57 ± 0.00	0.91 ± 0.02
C24:1	0.81 ± 0.01	0.07 ± 0.00	0.09 ± 0.00	0.16 ± 0.02
SFA	17.23 ± 0.07 c	21.54 ± 1.62 a	18.57 ± 0.55 b	15.42 ± 0.55 c
MUFA	55.01 ± 0.36 a	33.38 ± 1.42 c	46.39 ± 0.14 b	7.12 ± 0.02 d
PUFA	27.76 ± 0.29 d	45.07 ± 0.20 b	35.04 ± 0.70 c	77.46 ± 0.57 a

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); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1c); Eicosadienoic acid (C20:2c); Eicosatrienoic acid + Heneicosanoic acid (C20:3n3+C21:0); Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0); Nervonic acid (C24:1). nd- not detected.

Table 5. Vitamin contents in the four wild edible mushroom species analysed (mean ± SD; n=3). In each row different letters imply significant differences ($p<0.05$).

	Armillaria mellea	*Calocybe gambosa*	*Clitocybe odora*	*Coprinus comatus*
α-tocopherol	9.06 ± 0.15 c	15.81 ± 0.01 b	18.24 ± 1.03 b	22.75 ± 2.67 a
γ-tocopherol	57.96 ± 0.60 b	119.42 ± 0.03 a	116.66 ± 6.37 a	124.27 ± 0.74 a
δ-tocopherol	nd	24.70 ± 5.78 b	54.46 ± 2.82 b	154.01 ± 23.30 a
Total tocopherols (μg/100 g dry weight)	67.02 ± 0.46 c	159.93 ± 5.74 b	189.36 ± 2.53 b	301.03 ± 26.71 a
Ascorbic acid (mg/100 g dry weight)	148.02 ± 6.64 b	180.47 ± 23.96 a	172.65 ± 19.77 a	132.88 ± 11.35 b

nd- not detected.