1 **Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different**

2 **Portuguese wild mushrooms species**

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15

16 **ABSTRACT**

17 Analysis of phenolic compounds in sixteen Portuguese wild mushrooms species has
18 been carried out by high-performance liquid chromatography coupled to photodiode
19 array detector and mass spectrometer (HPLC-DAD-ESI/MS). No flavonoids were
20 detected in the analysed samples, but diverse phenolic acids namely protocatechuic, *p*-
21 hydroxybenzoic and *p*-coumaric acids, and two vanillic acid isomers were found and
22 quantified. A related non-phenolic compound, cinnamic acid, was also detected in some
23 samples, being the only compound found in *Cantharellus cibarius* (14.97 mg/Kg, dry
24 matter), *Lycoperdon perlatum* (14.36 mg/Kg) and *Macrolepiota procera* (21.53
25 mg/Kg). *p*-Hydroxybenzoic acid was found in the majority of the samples, being the
26 most abundant compound in *Agaricus silvicola* (238.7 mg/Kg). *Ramaria botrytis*
27 showed the highest phenolic acids concentration (356.7 mg/Kg) due to the significant
28 contribution of protocatechuic acid (342.7 mg/Kg).

29

30 **KEYWORDS:** Wild mushrooms; phenolic compounds, HPLC-DAD-ESI/MS.

31 ## 1. Introduction
32
33 Natural phenolics are compounds possessing one or more aromatic rings with one or
34 more hydroxyl groups and can range from simple molecules (phenolic acids,
35 phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins,
36 tannins) (Bravo, 1998). Particularly, phenolic acids can be subdivided into two major
37 groups, hydroxybenzoic acids and hydroxycinnamic acids (**Figure 1**). Hydroxybenzoic
38 acids include *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids.
39 They are commonly present in the bound form and are typically a component of a
40 complex structure like lignins and hydrolyzable tannins. They can also be found linked
41 to sugar derivatives and organic acids in plant foods. Hydroxycinnamic acids include *p*-
42 coumaric, caffeic, ferulic, and sinapic acids. In natural sources they are mainly found
43 esterified with small molecules like, e.g., quinic or tartaric acids, as well as bound to
44 cell-wall structural components such as cellulose, lignin, and proteins through ester
45 bonds (Liu, 2004).
46 Phenolic compounds, commonly found in vegetables, fruits and many plant-derived
47 foods that form a significant portion of our diet, are among the most potent and
48 therapeutically useful bioactive substances, providing health benefits associated with
49 reduced risk of chronic and degenerative diseases (Luximon-Ramma et al., 2003;
50 Luximon-Ramma et al., 2005; Soobrattee et al., 2005). Many of their biological effects
51 have been attributed to free radical scavenging and antioxidant activity (Middleton et
52 al., 2000).
53 The use of mushrooms extracts as antioxidants is becoming increasingly popular (Mau
54 et al., 2002; Lo and Cheung, 2005; Elmastas et al., 2007; Tsai et al., 2007) and our
55 research group published several studies reporting the antioxidant properties of wild
56 edible mushrooms, particularly their free radical scavenging activity and lipid

peroxidation inhibition in animal erythrocytes and in brain cells membranes (Barros et al., 2007a; Barros et al., 2008a; Barros et al., 2008b). The antioxidant properties were correlated to different antioxidant components such as tocopherols, carotenoids, ascorbic acid and total phenolics (Barros et al., 2007b; Barros et al., 2008c). However, little is known about the individual phenolic compounds present in mushroom species. A few studies concerning the analysis of the phenolic components of Portuguese wild mushrooms can be found in the literature, particularly for *Cantharellus cibarius* (Valentão et al., 2005), *Suillus bellini*, *Tricholomopsis rutilans*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *Boletus edulis*, *Tricholoma equestre*, *Suillus luteus*, *Suillus granulatus* (Ribeiro et al., 2006), and *Fistulina hepatica* (Ribeiro et al., 2007). Nevertheless, being the Northeast of Portugal one of the European regions with higher wild edible mushroom diversity, it is important to characterize the phenolic composition of other species also important and with gastronomic relevance. In this study, individual profiles of phenolic compounds in sixteen Portuguese wild mushrooms were characterised by high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC-DAD-ESI/MS).

2. Materials and methods

2.1 Samples

Sixteen mushrooms species were collected from different places in Trás-os-Montes region in the Northeast of Portugal (**Table 1**). The morphological identification of the wild macrofungi was made till species according to macro and microscopic characteristics, and following several authors (Moser, 1983; Courtecuisse and Duhem, 1995) and representative voucher specimens were deposited at the herbarium of *Escola*

82 *Superior Agrária* of *Instituto Politécnico de Bragança*. After taxonomic identification,
83 the mushrooms were immediately lyophilized (Ly-8-FM-ULE, Snijders, Holland), and
84 kept in the dark in hermetically sealed plastic bags up to analysis.

85

86 *2.2. Standards and reagents*

87 Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). All the other
88 reagents (methanol, n-hexane, ethyl acetate and diethyl ether) were of analytical grade
89 purity and were also supplied by Lab-Scan. Gallic acid was from Supelco (Bellefonte,
90 PA, USA) and the rest of phenolic standards were from Sigma Chemical Co. (St. Louis,
91 MO, USA). The Folin and Ciocalteu's reagent was purchased from Merck (Darmstadt,
92 Germany). Water was treated in a Milli-Q water purification system (TGI Pure Water
93 Systems, USA).

94

95 *2.3 Analysis of total phenolics*

96 *Sample preparation*. A fine dried mushroom powder (20 mesh) sample (~3 g) was
97 extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24 h and filtered
98 through Whatman Nº 4 paper. The residue was then extracted with two additional 100
99 mL portions of methanol, as described earlier. The combined methanolic extracts were
100 evaporated at 40 ºC to dryness and redissolved in a known concentration of methanol.
101 *Folin Ciocalteu's assay*. Briefly, the methanolic extract solution (1 mL) was mixed with
102 the Folin-Ciocalteu reagent (1 mL). After 3 min, saturated sodium carbonate solution (1
103 mL) was added to the mixture and adjusted to 10 mL with distilled water. The reaction
104 was kept in the dark for 90 min, after which the absorbance was read at 725 nm
105 (Analytikijena 200-2004 spectrophotometer, Jena, Germany). Gallic acid was used to

106 prepare the standard curve (0.01-0.4 mM; y=2.8557x-0.0021; R^2=0.9999) and the
107 results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.
108
109 *2.4. DPPH radical-scavenging activity.*
110 Various concentrations of mushroom extracts (0.3 mL) were mixed with 2.7 mL of
111 methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken
112 vigorously and left to stand for 60 min in the dark (until stable absorption values were
113 obtained). The reduction of the DPPH radical was determined by measuring the
114 absorption at 517 nm. The radical scavenging activity (RSA) was calculated as a
115 percentage of DPPH discolouration using the equation: $\% \text{RSA} = [(A_{DPPH}-A_S)/A_{DPPH}] \times$
116 100, where A_S is the absorbance of the solution when the sample extract has been added
117 at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract
118 concentration providing 50% of radical scavenging activity (EC_{50}) was calculated from
119 the graph of RSA percentage against extract concentration. BHA and α-tocopherol were
120 used as standards.
121
122 *2.5. Phenolic compounds identification and quantification*
123 *Sample preparation.* Each mushroom sample (~3 g) was extracted with acetone:water
124 (80:20; 50 mL) mixture at -20°C for 6h. The extract was put in an ultrasonic bath for 15
125 min, centrifuged at 4000*g* for 10 min, and filtered through Whatman nº 4 paper. The
126 residue was then extracted with three additional 50 mL portions of the acetone:water
127 mixture. The combined extracts were evaporated at 30 ºC to remove acetone. The
128 aqueous phase was washed with n-hexane, and then submitted to a liquid-liquid
129 extraction with diethyl ether (3 x 50 mL) and ethyl acetate (3 x 50 mL). The organic

130 phases were evaporated at 30 ºC to dryness, redissolved in water:methanol (80:20), and
131 filtered through a 0.22 µm disposable LC filter disk for HPLC analysis.
132 *HPLC-DAD-ESI/MS analyses.* The phenolic extracts were analysed using a Hewlett-
133 Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn,
134 Germany). Separation was achieved on a Spherisorb (Phenomenex, Torrance, CA)
135 reverse phase C_{18} column (3 µm, 150mm x 4.6mm i.d.) thermostatted at 25 ºC. The
136 solvents used were: (A) 2.5% acetic acid in water, (B) acetic acid 2.5%/acetonitrile
137 (90:10), and (C) 100% HPLC-grade acetonitrile. The gradient employed was: isocratic
138 100% A for 10 min, 50% A and 50% B for 10 min, isocratic 100% B for 15 min, 90% B
139 and 10% C for 10 min, 70% B and 30% C for 10 min, 50% B and 50% C for 5 min,
140 20% B and 80% C for 5 min, 100% A for 5 min, at a flow rate of 0.5 mL/min. Detection
141 was carried out in a diode array detector (DAD), using 280 nm as the preferred
142 wavelength, and in a mass spectrometer (MS) connected to the HPLC system via the
143 DAD cell outlet.
144 LC–MS analyses were performed using a Finningan™ LCQ MS detector (Thermoquest,
145 San Jose, CA, USA) equipped with an API source, using an electrospray ionisation
146 (ESI) interface. Both the sheath gas and the auxiliary gas were nitrogen at flow rates of
147 1.2 and 6 L/min, respectively. The capillary and source voltage were 10V and 3.5 kV,
148 respectively, and the capillary temperature was 175 ºC. Spectra were recorded in
149 negative ion mode between *m/z* 80 and 620. The MS was programmed to carry out a
150 series of three consecutive scans: a full mass from 150 to 1500 amu, a zoom scan of the
151 most abundant ion in a ±5 amu range, and an MS-MS scan of the most abundant ion in
152 the full mass using a normalised energy of collision of 45%.

153 The phenolic compounds present in the samples were characterised according to their
154 UV-vis spectra and identified by their mass spectra and retention times in comparison
155 with those of commercial standards. For the quantitative analysis of phenolic
156 compounds, a calibration curve was obtained by injection of different concentration of
157 protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and
158 cinnamic acid standards.

159

160 *2.6. Statistical analysis*

161 The analysis of phenolic compounds contents in each mushroom species was carried out
162 in triplicate and the results expressed as mean ± standard deviation (SD). Data were
163 analysed by one-way analysis of variance (ANOVA) followed by Tukey's HSD Test
164 with $\alpha = 0.05$, using SPSS v. 16.0 program.

165

166 **3. Results and discussion**

167

168 Total phenolic compounds in the analyzed Portuguese wild mushrooms species were
169 determined by the Folin Ciocalteu's assay. **Table 1** presents those results as also the
170 EC_{50} values (extract concentration correspondent to 50% of radical scavenging activity)
171 obtained in the assessment of the antioxidant activity of mushrooms measured by the
172 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

173 Phenolic compounds include different subclasses (flavonoids, phenolic acids, stilbenes,
174 lignans, tannins, oxidized polyphenols) displaying a large diversity of structures, some
175 of which may escape the usual methodologies of analysis, commonly carried out by
176 HPLC (High Performance Liquid Chromatography) coupled to distinct detection
177 devices. Various reasons exist for that, like the existence of isomers, difficulty for

178 chromatographic separation of some compounds, lack of commercial standards, or
179 structure not yet elucidated (Georgé et al., 2005). The method of Folin Ciocalteu's is,
180 therefore, largely used to evaluate total phenolics despite all the interferences of this
181 assay since the reagent (mixture of phosphotungstic acid and phosphomolibdic acid)
182 also reacts with other non-phenolic reducing compounds leading to an overvaluation of
183 the phenolic content. For instance, ascorbic acid is a widespread reducing agent that can
184 interfere in the Folin-Ciocalteu reaction (Georgé et al., 2005) and that was, in fact,
185 reported to be present in the studied species (Barros et al., 2007a; Barros et al., 2007b;
186 Barros et al., 2008a; Barros et al., 2008b). Other reducing substances such as some
187 sugars and amino acids could also interfere. In addition, the results have to be expressed
188 in equivalents of a particular standard compound (like catechin, gallic acid or tannin
189 acid). All these aspects make the results obtained for different authors difficult to
190 compare.
191 In previous studies of our group (Barros et al., 2007a; Barros et al., 2007b; Barros et al.,
192 2007c; Barros et al., 2008a; Barros et al., 2008b), antioxidant activity assessed in
193 mushroom extracts by different chemical and biochemical assays was correlated with
194 their contents of phenolic compounds as measured by the Folin-Ciocalteu method.
195 However, no analyses of individual phenolics were made and, therefore, the compounds
196 responsible for that antioxidant activity were unknown. In the present study we aimed to
197 identify and quantify individual compounds that may contribute to the bioactive
198 properties already found for these Portuguese wild mushroom species.
199 Three phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids) and a
200 related compound (cinnamic acid) could be positively identified and quantified in some
201 samples (**Table 2**) by comparison of their chromatographic characteristics and

202 absorption spectra with the standards compounds and confirmed by mass analysis. In
203 **Figure 2** a representative chromatogram obtained for one of the mushroom extracts
204 analysed is shown as an example. Other two compounds were also detected in the
205 samples of *L. molle* and *T. acerbum* whose UV spectra, molecular ion (*m/z* $[M-H]^-$ at
206 167) and MS^2 spectra (one fragment at *m/z* 123, $[M-44]^-$, loss of a CO_2 residue)
207 coincided with those of vanillic acid (4-hydroxy-3-methoxybenzoic acid), but that
208 showed higher retention times (40.5 and 44.0 min, respectively, in comparison with
209 30.1 min for vanillic acid). Thus, these compounds were tentatively associated to
210 vanillic acid isomers like, e.g., *o*-vanillic (i.e., 2-hydroxy-3-methoxy-benzoic acid) or
211 isovanillic acid (i.e., 3-hydroxy-4-methoxybenzoic acid), for which no standards were
212 available.
213 No phenolic acids were detected in six mushroom species: *H. fasciculare*, *L. piperatus,*
214 *L. giganteus*, *C. cibarius, L. perlatum* and *M. procera*, although the presence of
215 cinnamic acid was found in the three latter. No peaks were found in the extracts whose
216 UV spectra could be associated to hydroxycinnamic acids or their tartaric or quinic
217 esters (i.e., chlorogenic acids). Further, no detection of those compounds was made
218 when the full mass chromatograms of the samples were screened for their molecular
219 ions. Similarly, no peaks whose UV spectra or mass characteristics could be associated
220 to flavonoids were found. This fact should not be surprising since, in general, it is
221 assumed that only plants possess the biosynthetic ability to produce flavonoids and not
222 animals and fungi (Iwashina, 2000), even if some flavonoids have exceptionally been
223 reported from fungi *Aspergillus candidus* and *Phallus impudicus* (reviewed in Iwashina,
224 2000) and more recently in the edible beefsteak fungus *Fistulina hepatica* (Ribeiro et
225 al., 2007).

226 Thus, the phenolic composition of the mushrooms seems to be characterised by only the
227 presence of phenolic acids, being *p*-hydroxybenzoic acid the major compound in most
228 cases; among the species analyzed, only *L. nuda* and *R. botrytis* showed protocatechuic
229 acid as the main phenolic compound. Other authors had already reported the presence of
230 *p*-hydroxybenzoic acid in other mushroom species, such as *A. rubescens*, *T. equestre*
231 and *R. cyanoxantha* (Ribeiro et al., 2006). The same research group reported the
232 presence of *p*-coumaric acid in *C. cibarius* (Valentão et al., 2005) and *F. hepatica*
233 (Ribeiro et al., 2007). However, we could not find *p*-coumaric acid in our *Cantharellus*
234 *cibarius* sample.

235 *Ramaria botrytis* showed the highest phenolic acids concentration (356.7 mg/Kg, dry
236 matter) mostly due to the contribution of protocatechuic acid (342.7 mg/Kg). In fact,
237 this mushroom species also revealed the highest content in total phenolics as determined
238 by the Folin-Ciocalteu assay (**Table 1**) and the highest antioxidant capacity (lower EC_{50}
239 values). A low correlation between the total phenolics and phenolic acids content, was
240 obtained ($Y=0.0318X + 3.6087$; $R^2=0.4900$), which suggest that other compounds
241 different than phenolic acids are present in mushrooms and react with the Folin-
242 Ciocalteu reagent and also contribute to their antioxidant properties. This observation is
243 also emphasized by the low correlations obtained between the total phenolics ($Y=-$
244 $0.6432X + 11.6670$; $R^2 = 0.4353$) or the total phenolic acids ($Y=-0.0159X + 8.8594$; R^2
245 $= 0.1285$) present in the mushrooms extracts and their antioxidant activity, measured by
246 the DPPH assay.

247 As far as we know, this is the first report concerning the phenolic acids composition of
248 *Agaricus arvensis, Agaricus bisporus, Agaricus romagnesii, Agaricus silvicola,*
249 *Cantharellus cibarius, Hypholoma fasciculare, Lactarius deliciosus, Lactarius*

250 *piperatus, Lepista nuda, Leucopaxillus giganteus, Lycoperdon molle, Lycoperdon*
251 *perlatum, Macrolepiota procera, Ramaria botrytis, Sarcodon imbricatus,* and
252 *Tricholoma acerbum.* This study also suggests that phenolic acids analysis could be
253 useful in taxonomic studies involving mushroom species, besides their importance as
254 antioxidants for the human health. Nevertheless, further studies are required to conclude
255 about this point, as it is known that the levels of phenolic compounds depend on several
256 factors such as cultivation techniques, cultivar, growing conditions, ripening process,
257 processing and storage conditions, as well as stress conditions such as UV radiation,
258 infection by pathogens and parasites, wounding air pollution and exposure to extreme
259 temperatures (Naczk and Shahidi, 2006).
260

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264

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332

333 **Table 1**. Collection information of the wild mushroom samples, and their total phenols (by Folin Ciocalteu's assay) and antioxidant activity (by

334 DPPH assay). In each column different letters mean significant differences (p<0.05).

Species	Origin	Orchard	Date of collection	Total phenols (mg/g extract)	Antioxidant activity (EC_{50} value, mg/mL)
Agaricus arvensis	Carrazeda de Ansiães	*Pinus pinaster*	October 2006	2.75 ± 0.17 f	15.85 ± 0.27 d
Agaricus bisporus	Bragança	Grassland	October 2006	4.49 ± 0.16 e	9.61 ± 0.07 e
Agaricus romagnesii	Vinhais	*Pinus pinaster*	October 2006	6.18 ± 0.44 d	6.22 ± 0.10 gf
Agaricus silvicola	Bragança	*Quercus pyrenaica*	October 2006	6.40 ± 0.17 d	6.39 ± 0.16 f
Cantharellus cibarius	Vinhais	*Quercus pyrenaica*	June 2007	1.75 ± 0.50 g	19.65 ± 0.28 b
Hypholoma fasciculare	Bragança	*Quercus pyrenaica*	October 2006	17.67 ± 0.27 b	1.13 ± 0.03 l
Lactarius deliciosus	Bragança	*Pinus pinaster*	November 2005	3.40 ± 0.18 f	16.31 ± 0.24 c
Lactarius piperatus	Bragança	*Quercus pyrenaica*	June 2006	3.09 ± 0.12 f	20.24 ± 0.78 a
Lepista nuda	Cova de Lua	*Pinus pinaster*	November 2006	6.31 ± 0.13 d	4.41 ± 0.01 i
Leucopaxillus giganteus	Cova de Lua	*Pinus pinaster*	October 2005	6.29 ± 0.20 d	1.44 ± 0.09 l
Lycoperdon molle	Vinhais	*Quercus pyrenaica*	October 2006	11.48 ± 0.52 c	3.23 ± 0.09 k

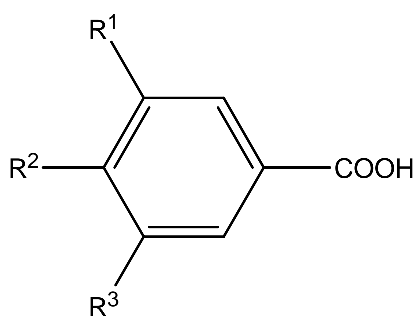
Lycoperdon perlatum	Vinhais	*Quercus pyrenaica*	October 2006	10.57 ± 0.17 c	3.95 ± 0.04 j
Macrolepiota procera	Carrazeda de Ansiães	*Quercus pyrenaica*	November 2006	3.17 ± 0.92 f	5.38 ± 0.50 h
Ramaria botrytis	Vinhais	*Quercus pyrenaica*	October 2006	20.32 ± 1.87 a	0.66 ± 0.00 m
Sarcodon imbricatus	Vinhais	*Pinus pinaster*	November 2006	3.06 ± 0.10 f	5.82 ± 0.06 g
Tricholoma acerbum	Vinhais	*Quercus pyrenaica*	October 2006	5.53 ± 0.63 d	3.60 ± 0.08 kj

Table 2. Phenolic acids found in the mushroom samples. In each column different letters mean significant differences (p<0.05).

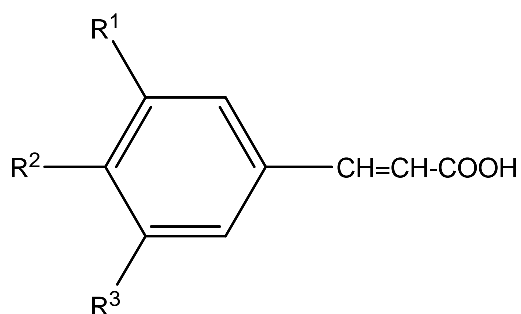
	Phenolic compounds (mg/kg, dry matter)						Other compounds (mg/kg, dry matter)
	protocatechuic acid (15.1 min)	*p*-hydroxybenzoic acid (22.9 min)	vanillic acid isomer (40.5 min)	*p*-coumaric acid (41.7 min)	vanillic acid isomer (44.1 min)	Total phenolic compounds	Cinnamic acid (51.4 min)
A. arvensis	n.d	70.13 ± 1.20	n.d	48.67 ± 3.40	n.d	118.8 ± 4.6 c	49.10 ± 8.03
A. bisporus	n.d	25.59 ± 1.55	n.d	n.d	n.d	25.59 ± 1.55 e	8.72 ± 0.71
A. silvicola	n.d	238. 7 ± 12.4	n.d	45.72 ± 1.19	n.d	284.4 ± 11.2 b	68.37 ± 11.32
A. romagnesii	n.d	32.40 ± 0.83	n.d	n.d	n.d	32.40 ± 0.83 e	49.22 ± 3.90
C. cibarius	n.d	n.d	n.d	n.d	n.d	n.d	14.97 ± 0.40
L. deliciosus	n.d	22.66 ± 0.36	n.d	n.d	n.d	22.66 ± 0.36 e	n.d
L. giganteus	n.d	n.d	n.d	n.d	n.d	n.d	n.d
L. nuda	33.47 ± 0.50	29.31 ± 1.54	n.d	3.75 ± 0.56	n.d	66.53 ± 2.62 d	n.d
L. molle	n.d	41.66 ± 0.33	35.97 ± 6.16	n.d	4.02 ± 0.55	81.65 ± 7.04 d	n.d
L. perlatum	n.d	n.d	n.d	n.d	n.d	n.d	14.36 ± 1.27
L. piperatus	n.d	n.d	n.d	n.d	n.d	n.d	n.d
M. procera	n.d	n.d	n.d	n.d	n.d	n.d	21.53 ± 1.65
H. fascicular	n.d	n.d	n.d	n.d	n.d	n.d	n.d

S. imbricatus	n.d	33.19 ± 1.92	n.d	n.d	n.d	33.19 ± 1.92 e	n.d
R. botrytis	342.7 ± 10.2	14.00 ± 0.77	n.d	n.d	n.d	356.7 ± 9.4 a	n.d
T. acerbum	n.d	29.66 ± 0.26	4.92 ± 0.72	n.d	7.81 ± 0.56	42.38 ± 1.53 e	n.d

n.d- not detected



Hydroxybenzoic acids



Cinnamic acids derivatives

Figure 1: Chemical structure of the identified phenolic acids in the wild mushroom species. Benzoic acids: *p*-hidroxybenzoic (R^1=R^3=H, R^2=OH), protocatechuic (R^1=H, R^2=R^3=OH), vanillic (R^1=CH_3O, R^2=OH, R^3=H). Cinnamic acid (R^1=R^2=R^3=H) and derivatives: *p*-coumaric (R^1=R^3=H, R^2=OH).

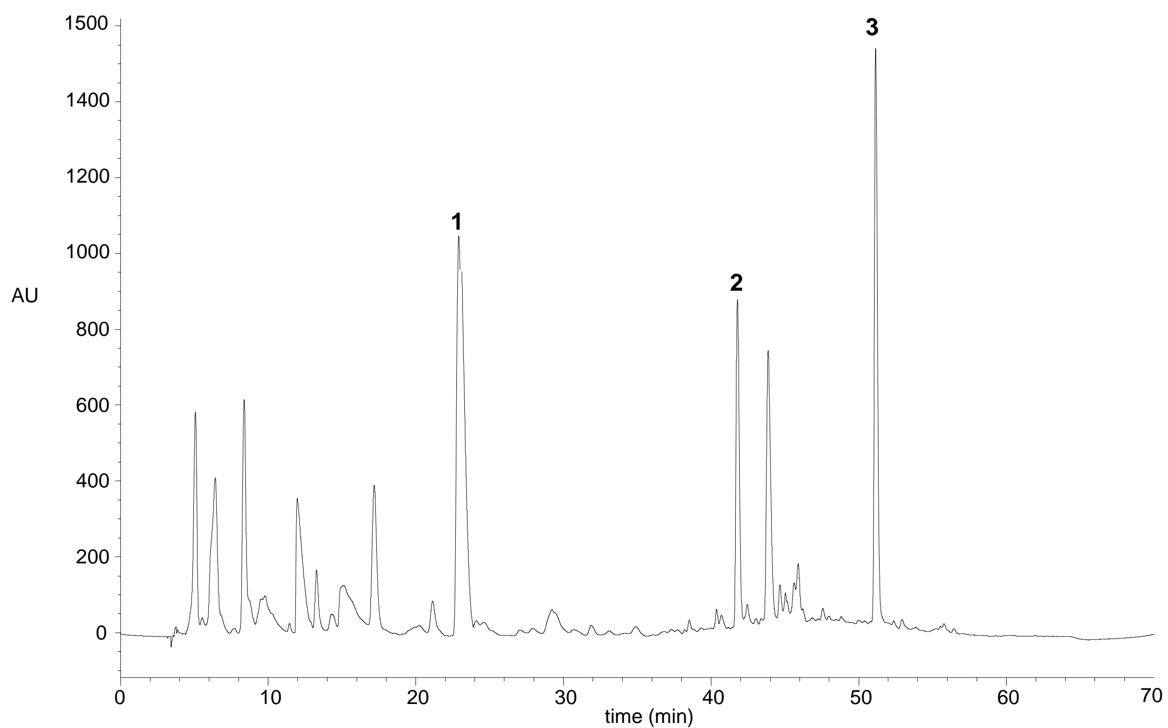


Figure 2. HPLC chromatogram recorded at 280 nm of an extract of *Agaricus silvicola*. Only peaks corresponding to phenolic compounds or related compounds are indicated: (1) *p*-hydroxybenzoic acid, (2) *p*-coumaric acid, and (3) cinnamic acid.