Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species

Lillian Barros[a,b], Montserrat Dueñas[a], Isabel C.F.R. Ferreira[b,*], Paula Baptista[b] and Celestino Santos-Buelga[a,*]

[a]*Unidad de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.*

[b]*CIMO-ESAB, Instituto Politécnico de Bragança, Campus de Sta. Apolónia, 1172, 5301-855 Bragança, Portugal*

Authors to whom correspondence should be addressed (e-mail: iferreira@ipb.pt, telephone +351273303219, fax +351273325405; e-mail: csb@usal.es; telephone +34 923 294537; fax +34 923 294515).

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.

1. Introduction

Natural phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins) (Bravo, 1998). Particularly, phenolic acids can be subdivided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids (**Figure 1**). Hydroxybenzoic acids include *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids. They are commonly present in the bound form and are typically a component of a complex structure like lignins and hydrolyzable tannins. They can also be found linked to sugar derivatives and organic acids in plant foods. Hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic, and sinapic acids. In natural sources they are mainly found esterified with small molecules like, e.g., quinic or tartaric acids, as well as bound to cell-wall structural components such as cellulose, lignin, and proteins through ester bonds (Liu, 2004).

Phenolic compounds, commonly found in vegetables, fruits and many plant-derived foods that form a significant portion of our diet, are among the most potent and therapeutically useful bioactive substances, providing health benefits associated with reduced risk of chronic and degenerative diseases (Luximon-Ramma et al., 2003; Luximon-Ramma et al., 2005; Soobrattee et al., 2005). Many of their biological effects have been attributed to free radical scavenging and antioxidant activity (Middleton et al., 2000).

The use of mushrooms extracts as antioxidants is becoming increasingly popular (Mau et al., 2002; Lo and Cheung, 2005; Elmastas et al., 2007; Tsai et al., 2007) and our research group published several studies reporting the antioxidant properties of wild 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*

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

2.2. Standards and reagents

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

2.3 Analysis of total phenolics

Sample preparation. A fine dried mushroom powder (20 mesh) sample (~3 g) was extracted by stirring with 100 mL of methanol at 25 ºC at 150 rpm for 24 h and filtered through Whatman Nº 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 ºC to dryness and redissolved in a known concentration of methanol.

Folin Ciocalteu's assay. Briefly, the methanolic extract solution (1 mL) was mixed with the Folin-Ciocalteu reagent (1 mL). After 3 min, saturated sodium carbonate solution (1 mL) was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer, Jena, Germany). Gallic acid was used to

prepare the standard curve (0.01-0.4 mM; y=2.8557x-0.0021; R^2=0.9999) and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

2.4. DPPH radical-scavenging activity.

Various concentrations of mushroom extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals ($6x10^{-5}$ mol/L). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The 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 radical scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

2.5. Phenolic compounds identification and quantification

Sample preparation. Each mushroom sample (~3 g) was extracted with acetone:water (80:20; 50 mL) mixture at -20°C for 6h. The extract was put in an ultrasonic bath for 15 min, centrifuged at 4000*g* for 10 min, and filtered through Whatman nº 4 paper. The residue was then extracted with three additional 50 mL portions of the acetone:water mixture. The combined extracts were evaporated at 30 ºC to remove acetone. The aqueous phase was washed with n-hexane, and then submitted to a liquid-liquid extraction with diethyl ether (3 x 50 mL) and ethyl acetate (3 x 50 mL). The organic

phases were evaporated at 30 ºC to dryness, redissolved in water:methanol (80:20), and filtered through a 0.22 μm disposable LC filter disk for HPLC analysis.

HPLC-DAD-ESI/MS analyses. The phenolic extracts were analysed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a Spherisorb (Phenomenex, Torrance, CA) reverse phase C_{18} column (3 μm, 150mm x 4.6mm i.d.) thermostatted at 25 ºC. The solvents used were: (A) 2.5% acetic acid in water, (B) acetic acid 2.5%/acetonitrile (90:10), 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, and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

LC–MS analyses were performed using a Finnigan™ LCQ MS detector (Thermoquest, San Jose, CA, USA) equipped with an API source, using an electrospray ionisation (ESI) interface. Both the sheath gas and the auxiliary gas were nitrogen at flow rates of 1.2 and 6 L/min, respectively. The capillary and source voltage were 10V and 3.5 kV, respectively, and the capillary temperature was 175 ºC. Spectra were recorded in negative ion mode between *m/z* 80 and 620. The MS was programmed to carry out a series of three consecutive scans: a full mass from 150 to 1500 amu, a zoom scan of the most abundant ion in a ±5 amu range, and an MS-MS scan of the most abundant ion in 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

chromatographic separation of some compounds, lack of commercial standards, or structure not yet elucidated (Georgé et al., 2005). The method of Folin Ciocalteu's is, therefore, largely used to evaluate total phenolics despite all the interferences of this assay since the reagent (mixture of phosphotungstic acid and phosphomolibdic acid) also reacts with other non-phenolic reducing compounds leading to an overvaluation of the phenolic content. For instance, ascorbic acid is a widespread reducing agent that can interfere in the Folin-Ciocalteu reaction (Georgé et al., 2005) and that was, in fact, reported to be present in the studied species (Barros et al., 2007a; Barros et al., 2007b; Barros et al., 2008a; Barros et al., 2008b). Other reducing substances such as some sugars and amino acids could also interfere. In addition, the results have to be expressed in equivalents of a particular standard compound (like catechin, gallic acid or tannin acid). All these aspects make the results obtained for different authors difficult to compare.

In previous studies of our group (Barros et al., 2007a; Barros et al., 2007b; Barros et al., 2007c; Barros et al., 2008a; Barros et al., 2008b), antioxidant activity assessed in mushroom extracts by different chemical and biochemical assays was correlated with their contents of phenolic compounds as measured by the Folin-Ciocalteu method. However, no analyses of individual phenolics were made and, therefore, the compounds responsible for that antioxidant activity were unknown. In the present study we aimed to identify and quantify individual compounds that may contribute to the bioactive properties already found for these Portuguese wild mushroom species.

Three phenolic acids (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids) and a related compound (cinnamic acid) could be positively identified and quantified in some samples (**Table 2**) by comparison of their chromatographic characteristics and

absorption spectra with the standards compounds and confirmed by mass analysis. In **Figure 2** a representative chromatogram obtained for one of the mushroom extracts analysed is shown as an example. Other two compounds were also detected in the samples of *L. molle* and *T. acerbum* whose UV spectra, molecular ion (*m/z* $[M-H]^-$ at 167) and MS^2 spectra (one fragment at *m/z* 123, $[M-44]^-$, loss of a CO_2 residue) coincided with those of vanillic acid (4-hydroxy-3-methoxybenzoic acid), but that showed higher retention times (40.5 and 44.0 min, respectively, in comparison with 30.1 min for vanillic acid). Thus, these compounds were tentatively associated to vanillic acid isomers like, e.g., *o*-vanillic (i.e., 2-hydroxy-3-methoxy-benzoic acid) or isovanillic acid (i.e., 3-hydroxy-4-methoxybenzoic acid), for which no standards were available.

No phenolic acids were detected in six mushroom species: *H. fasciculare*, *L. piperatus, L. giganteus*, *C. cibarius, L. perlatum* and *M. procera*, although the presence of cinnamic acid was found in the three latter. No peaks were found in the extracts whose UV spectra could be associated to hydroxycinnamic acids or their tartaric or quinic esters (i.e., chlorogenic acids). Further, no detection of those compounds was made when the full mass chromatograms of the samples were screened for their molecular ions. Similarly, no peaks whose UV spectra or mass characteristics could be associated to flavonoids were found. This fact should not be surprising since, in general, it is assumed that only plants possess the biosynthetic ability to produce flavonoids and not animals and fungi (Iwashina, 2000), even if some flavonoids have exceptionally been reported from fungi *Aspergillus candidus* and *Phallus impudicus* (reviewed in Iwashina, 2000) and more recently in the edible beefsteak fungus *Fistulina hepatica* (Ribeiro et al., 2007).

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

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

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

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

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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

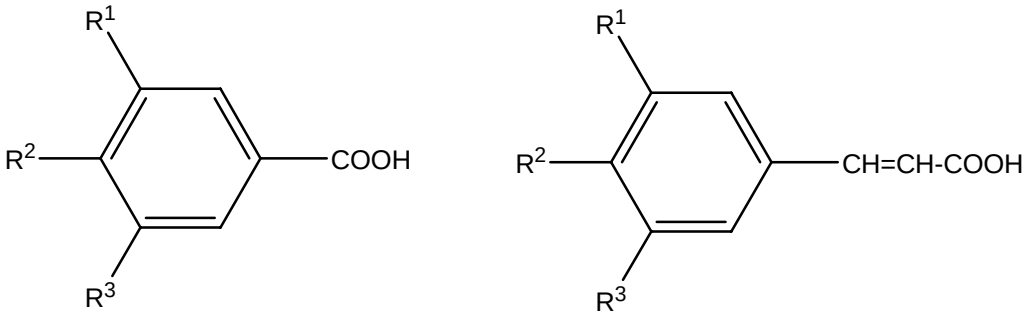
Lycoperdon perlatum	Vinhais	*Quercus pyrenaica*	October 2006	10.57 ± 0.17 c	3.95 ± 0.04 j
Macrolepiota procera	Carrazeda de Ansiães	*Quercus pyrenaica*	November 2006	3.17 ± 0.92 f	5.38 ± 0.50 h
Ramaria botrytis	Vinhais	*Quercus pyrenaica*	October 2006	20.32 ± 1.87 a	0.66 ± 0.00 m
Sarcodon imbricatus	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).

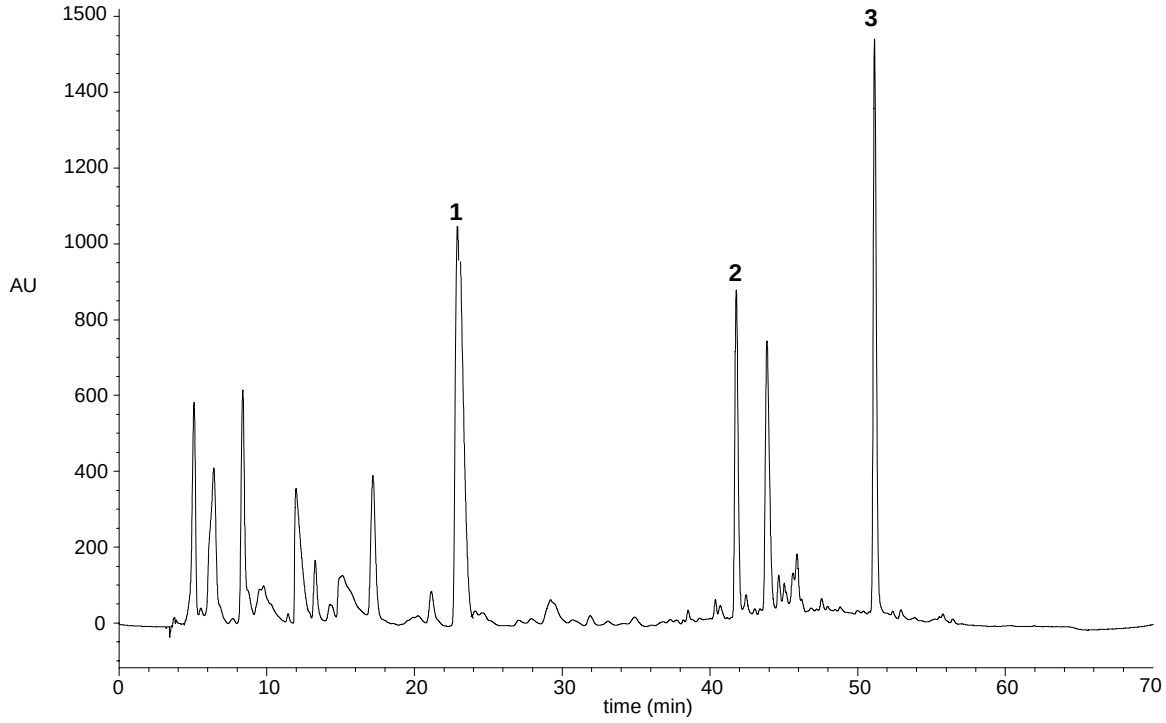


Figure 2. HPLC chromatogram recorded at 280 nm of an extract of *Agaricus silvicola*. Only peaks corresponding to phenolic compounds or related compounds are indicated: (1) *p*-hydroxybenzoic acid, (2) *p*-coumaric acid, and (3) cinnamic acid.