Carbohydrate Polymers 217 (2019): 135-143

DOI: https://doi.org/10.1016/j.carbpol.2019.04.051

Strengths and weaknesses of the aniline-blue method used to test mushroom (1→3)-β-D-glucans obtained by microwave-assisted extractions

Alicia Gil-Ramírez*[1]; Fhernanda Ribeiro Smiderle[2]; Diego Morales[3]; Marcello Iacomini[4] and Cristina Soler-Rivas[3]

[1]Lund University, Department of Chemistry, Centre for Analysis and Synthesis, P.O. Box 124, SE-22100, Lund, Sweden

[2]Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdades Pequeno Príncipe, CEP 80250-060, Curitiba, PR, Brazil.

[3]Department of Production and Characterization of Novel Foods, Institute of Food Science Research-CIAL (UAM + CSIC), c/Nicolas Cabrera 9, Campus de Cantoblanco, 28049 Madrid, Spain.

[4]Department of Biochemistry and Molecular Biology, Federal University of Parana, CP 19046, Curitiba, Parana, Brazil.

*Corresponding author: aliciagilramirez@gmail.com

Co-authors email addresses: fhernandas@gmail.com, diego.morales@uam.es, iacomini@ufpr.br, cristina.soler@uam.es

Keywords: microwave-assisted extraction; mushrooms; (1→3)-β-D-glucans; polysaccharides; fluorimetric assay; MAE

Abbreviations: PEF, polysaccharide-enriched fractions; MAE, microwave-assisted extraction; TCH, total carbohydrate; PLE, pressurized-liquid extraction; UAE, ultrasound-assisted extraction; MP, mushroom powder; PSC, polysaccharide; PUL, 1,3-1,6-β-D-glucan from *P. pulmonarius*.

Abstract

The parameters to extract polysaccharide-enriched fractions (PEF) from mushrooms using MAE (microwave-assisted extraction) were adjusted following a full factorial 3^2 experimental design. The highest yield and total carbohydrate values, using *Lentinula edodes* as model mushroom, were obtained at 180 ºC and 30 min. Several mushroom species were submitted to MAE and their PEF yields ranged between 12.1-44.2%. (1→3)-β-Glucans determination using a conventional fluorimetric method changed depending on the standard utilized. NMR analyses of PEF indicated that the presence of other polysaccharides in the extracts or their specific folding, might impair the proper determination of (1→3) linkages by the fluorophore. Mushrooms from Cantharellales order contained (1→3)-β-glucans but they were not detected with the fluorimetric method. Therefore, although the method (after adjustments) was sensitive enough to detect their presence in many mushroom extracts, it cannot be used for all species and it is also not recommended for quantitative determinations.

1. Introduction

Mushroom polysaccharides showed many beneficial properties for human health. They were described as immunomodulatory, antibacterial, antidiabetic, anti-inflammatory, hypocholesterolemic agents etc. (Roncero-Ramos & Delgado-Andrade, 2017). Although mushrooms can synthesize a wide variety of polysaccharides, β-D-glucans are those pointed as responsible for most of the biological activities.

β-D-Glucans are present in all mushroom species since they are the major constituents of fungal cell walls contributing to their structure (Ruthes, Smiderle, & Iacomini, 2015). Although linear β-D-glucans were also isolated from certain species, their molecular structures mainly contain a (1→3)-linked backbone chain with substitutions at O-6 by single units of β-D-glucopyranose (Ruthes et al., 2015). Previous studies suggested that the biological properties of these compounds are related to their chemical structure. Thus, characteristics such as the linkage type, polymerization level (Wang et al., 2017) or degree of branching (Lehtovaara & Gu, 2011) of such molecules might determine not only their biological but also their physic-chemical properties (*e.g.* solubility) (Lehtovaara & Gu, 2011; Moreno et al., 2016; Thompson, Oyston, & Williamson, 2010). Zhang, Li, Xu, & Zheng (2005) reported that the complexity of the helical conformation (*i.e.* single or triple) determined the ability of the (1→3),(1→6)-β-D-glucans to inhibit the tumour growth (Meng, Liang, & Luo, 2016). In addition, modified pachymaran, a (1→3),(1→6)-β-D-glucan, also showed antitumor properties, after β-(1→6) side chain removal (Wang et al., 2017). These evidences indicated that preserving the structural integrity of fungal β-D-glucans (mostly (1→3),(1→6)-β-D-glucans) during the extraction and analysis processes, might be essential to maintain intact their bioactivities.

Protocols including hot water extractions are the most common methods to obtain fungal polysaccharides (Ruthes et al., 2015; Wang et al., 2017). However, polysaccharides with complex conformations require more aggressive extraction methods such as hot alkali solutions and the use of chloroacetic or concentrated sulphuric acids, but they might also induce undesirable structural modifications (Alzorqi, Singh, Manickam, & Al-Qrimli, 2017; Eskilsson & Bjorklund,

2000; Zhu, Du, & Xu, 2016). Alternatively, new extraction technologies using water such as pressurized liquid extraction (PLE), ultrasound assisted extraction (UAE) or microwave assisted extraction (MAE) were also studied as effective tools for harmless polysaccharide extractions (Smiderle et al., 2017). MAE uses microwaves energy to heat directly the extraction solvent without a heat transfer from the vessels to the solvent. It maintains thermal gradients to minima inducing a more homogeneous temperature through the sample (Eskilsson & Bjorklund, 2000). The high pressure generated during the extraction process maintains water in its liquid state at temperatures higher than its boiling point, accelerating mass transfer of compounds (Li, Dobruchowska, Gerwig, Dijkhuizen, & Kamerling, 2013; Ruthes et al., 2015). Several studies used MAE to extract total polysaccharides from mushrooms (Chen, Shao, Tao, & Wen, 2015; Wang & Li, 2010) however, only a few studied the β-D-glucans content of the obtained extracts (Ruthes et al., 2015; Zheng, Zhang, Gao, Jia, & Chen, 2012).

A common method used to study β-D-glucans is based on the sirofluor ability to bind polysaccharides with (1→3)-β-D-glucan branches (Alzorqi et al., 2017; Evans, Hoyne, & Stone, 1984; Ko & Lin, 2004). Some reports claimed that such fluorimetric method is selective for (1→3)-β-D-glucan binding but, it showed certain restrictions that were not always considered, for instance, the polymerization degree, presence/absence of substituents and their tridimensional conformation might modulate the fluorimetric determination (Evans et al., 1984; Ruthes et al., 2015).

Therefore, a three level factorial experimental design was carried out to find the most suitable MAE conditions to obtain β-D-glucans-enriched extracts using *Lentinula edodes* as mushroom model. Afterwards, a screening of other species was performed using similar extraction conditions. The influence of the (1→3)-β-D-glucan structure on the fluorimetric assay was studied (using also NMR) to remark its limitations and the method was adjusted to improve its accuracy for the detection of β-D-glucans in MAE extracts.

2. Experimental

2.1. Biological material, reagents and standard compounds

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies with a particle size lower than 0.5 mm and a moisture content less than 5% were purchased from Glucanfeed S.L. (La Rioja, Spain). Dried fruiting bodies, commercially available in local markets, from *Auricularia judea* (Bull. Ex St.Amans) Berck,, *Cantharellus cibarius* (Fr.) *Cantharellus tubaeriformis* (Fr.), *Cantharellus lutescens* (Hierve.) Fr., *Cantharellus cornucopoides* (L. Ex Fr.) Pers,, *Boletus edulis* (Bull. Ex Fr.), *Lactarius deliciosus* (Fr.), *Pleurotus pulmonarius* (Fr.) Quel., *Pleurotus eryngii* (D.C. Ex Fr.) Quel, *Morchella conica* (Pers.), *Agrocybe aegerita* (Briganti) Singer, *Amanita caesarea (*Scop. Ex Fri.) Pers. Ex Schw. and *Hypsizygus marmoreus* (Peck) H.E. Bigelow were purchased and ground using a Grindomix GM200 Retsch mill (VERDER Group, The Netherlands) as described by Ramirez-Anguiano, Santoyo, Reglero, & Soler-Rivas (2007). Powdered mushrooms (MP) were stored at -20 °C under darkness until further use.

Absolute ethanol was purchased from Panreac (Barcelona, Spain) as well as concentrated sulfuric acid. Phenol, sodium borohydride, sodium hydroxide pellets, glycine, aniline blue diammonium salt 95% and hydrochloride acid 37% were obtained from Sigma-Aldrich (Madrid, Spain). Compounds used as standards were two linear β-D-glucans: curdlan (a (1→3)-β-D-glucan from *Alcaligenes faecalis*, Sigma-Aldrich, Madrid, Spain) and a (1→6)-β-D-glucan isolated from *A. bisporus* (named B6G) (Smiderle et al., 2013); and two branched (1→3),(1→6)-β-D-glucans: schizophyllan from Contipro Biotech (Dolni Dobrouc, Czech Republic) and a chemically characterized glucan (PUL, previously named B1316PP) extracted from *P. pulmonarius* (Smiderle et al., 2008). Moreover, a heteropolysacharide (mannogalactan, MG) isolated from *P. pulmonarius* (Smiderle et al., 2008) and commonly found in Basidiomycetes, was also used to compare with the β-D-glucans.

2.2. Microwave-assisted extractions (MAE)

Polysaccharides-enriched fractions (PEFs) were obtained using an automated microwave extraction system coupled to a MAS 24 auto-sampler (Monowave 300, Anton Paar GmbH, Graz, Austria). Extractions were performed at 1:30 mushroom powder:water ratio, 850W power, 2455 MHz frequency and 30 bar pressure as was described by Smiderle et al. (2017). After MAE, the samples were centrifuged and the pellet discarded. An aliquot of the supernatant (200 µL) was

used to estimate the total carbohydrate content (TCH) in the MAE extracts. Afterwards, the polysaccharides were precipitated with ethanol under cold conditions following the procedure of Smiderle et al. (2017). Obtained polysaccharide-enriched fractions (PEF) were collected, freeze-dried, weighted and kept at -20 °C until further use (equation 1, Figure 1).

$$(1)\ \text{PEF yield (\%)} = \frac{\text{weight of polysaccharide enriched fraction after MAE precipitation (g)}}{\text{weight of mushroom powder (g)}} \times 100$$

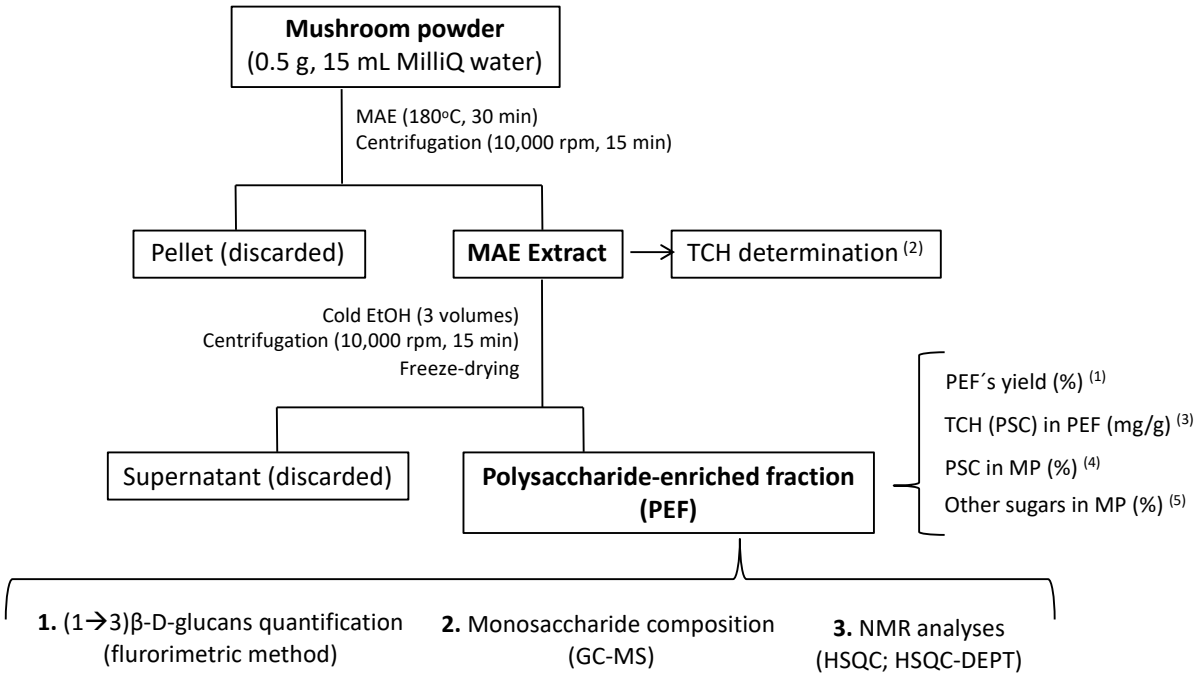

Figure 1. Workflow of the experiments developed in this work. Superscripts 1-5 correspond to the specific equations used to determine the corresponding values.

2.3. Design of experiment (DoE) approach for MAE of Lentinula edodes

In order to explore the efficiency of MAE to obtain PEFs, a full factorial three level experimental design (3^k) was selected using *L. edodes* as mushroom model. Two factors (*k*), extraction temperature and time, were studied and their ranges were set according to the equipment limitations and results reported for other mushroom species (respectively 50 – 180 °C and 5 - 30 min) (Smiderle et al., 2017). PEF yield (%, g PEF/ 100 g MP) and TCH (mg equivalents

of glucose/ g MP) were selected as response variables. Eleven randomized extractions were performed following the parameters indicated in Table 1, *i.e.* three levels per factor (3^2) with two additional central points. Most convenient MAE conditions achieved for *L. edodes* were selected to obtain PEFs from the other mushroom species.

2.4. Determination of carbohydrates from MAE extracts

The total carbohydrate content (TCH) in MAE extracts and PEFs (obtained after precipitation of MAE extracts) (Figure 1) was measured using the phenol-sulfuric acid method, as detailed by Smiderle et al. (2017). Since only traces of monosaccharides or oligosaccharides might remain in obtained PEFs, in this case, the TCH values indicated the total polysaccharide (PSC) concentration. Calculations were performed as follow (including unit conversions),

(2) $$\text{TCH in MAE extracts (mg/g)} = \frac{\text{equivalents of glucose in MAE extracts (mg)}}{\text{weight of MP (g)}}$$

(3) $$\text{TCH (or total PSC) in PEFs (mg/g)} = \frac{\text{equivalents of glucose in PEF (mg)}}{\text{weight of PEF (g)}}$$

(4) $$\text{PSC in MP after MAE ppt. (\%)} = \frac{\frac{\text{equivalents of glucose in PEF (mg)}}{\text{weight of PEF (g)}} \times \frac{\text{weight of PEF (g)}}{\text{weight of MP (g)}}}{10\ (\text{unit conversion})}$$

(5) $$\text{Other sugars in MP after MAE ppt. (\%)} = \frac{\text{TCH in MAE extracts}}{10\ (\text{unit conversion})} - \text{PSC in MP by MAE precipitation}$$

Supernatants obtained after MAE (equation 2, Figure 1) were diluted for all species as 1:15 (extract: water) except for *L. edodes* (1:5) and for *A. judea* and *P. eryngii* (1:30). TCH in PEFs (equation 3, Figure 1) were diluted for all species as 1:5. Samples were analyzed in triplicate and glucose was used as standard.

2.5. Determination of (1 → 3)-β-D-glucans from PEF

β-D-Glucans were determined by the fluorimetric method firstly described by Evans et al. (1984). This method uses an impurity from the aniline blue stain (sirofluor) as fluorochrome because of its ability to bind polysaccharides with (1 → 3)-β-linkages (Figure 1). Thus, the presence of (1 → 3)-β-D-glucans was determined in freeze-dried MAE polysaccharide fractions (PEF) using the method reported by Ko & Lin (2004) with some modifications concerning the sample preparation and the analytical procedure. Briefly, fractions were solubilized in 0.05 M

NaOH with 1% $NaBH_4$ (0.02 mg/mL) to preserve the polysaccharides integrity. Then, samples (300 μL) were mixed with 30 μL of 6 M NaOH and 630 μL of a dye solution (0.1% aniline: 1 M HCl: 1 M glycin/NaOH buffer pH 9.5 33:18:49, v/v/v) and incubated at 50 °C for 30 min in a water bath. Each mixture (250 μL) was transferred to a 96-well plate and analyzed using a M200 Plate Reader (Tecan, Mannedorf, Swtzerland) with excitation and emission wavelengths of 398 nm and 502 nm respectively. The buffer was freshly prepared before use to avoid degradation.

Fluorescence signals relative to the β-D-glucans content in PEF were obtained at 0.002, 0.01 and 0.02 mg/mL for all previously mentioned species. The standards for β-D-glucans were used in a range of 0-0.02 mg/mL for calibration curves and a solution of 0.05 M NaOH with 1% $NaBH_4$ was used as blank. Determinations were carried out in triplicate. The selection of the adequate standard for the detection of (1 → 3)-β-D-glucans in a mushroom extract was dependent on recorded fluorescence slopes.

2.6. Analysis of monosaccharide composition by GC-MS

The polysaccharide-enriched fractions (PEF) from MAE extracts (1 mg) were hydrolyzed with 2 M TFA at 100 ºC for 8 h, followed by evaporation to dryness (Figure 1). The dried carbohydrate samples were dissolved in distilled water (100 μL) and 1 mg $NaBH_4$ was added. The solution was held at room temperature overnight to reduce aldoses into alditols (Sassaki et al., 2008). The product was dried, the excess of $NaBH_4$ neutralized by the addition of acetic acid and removed with methanol (x 2) under a compressed air stream. Alditols acetylation was performed in pyridine–Ac_2O (200 μL; 1:1, v/v), for 30 min at 100 ºC. The pyridine was removed by washing with 5% $CuSO_4$ solution and the resulting alditol acetates were extracted with $CHCl_3$. The resulting derivatives were analyzed by GC-MS (Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer), using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C/min, with He as carrier gas. The obtained monosaccharides were identified by their typical retention time compared to commercially available standards. Results were expressed as mol%, calculated according to Pettolino, Walsh, Fincher, & Bacic (2012).

2.7. Nuclear magnetic resonance spectroscopy

NMR spectra (HSQC and HSQC-DEPT) from PEFs (Figure 1) were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 ºC and the samples (30 mg) were dissolved in D_2O (400 µL). Chemical shifts are expressed in ppm (δ) relative to resonance of acetone at δ 30.2 and 2.22 corresponding to ^{13}C and ^{1}H signals, respectively. NMR signals were assigned on the basis of 2D NMR experiment (HSQC) and literature data.

2.8. Statistical analysis

In order to set the experimental conditions and detect the optimal MAE parameters, analysis of variance (two-way ANOVA test) was carried out using the Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA). The confidence level was set at 95% ($p < 0.05$) for all cases.

3. Results and discussion

3.1. Response surface study of microwave assisted extractions from L. edodes.

Powdered fruiting bodies from *Lentinula edodes* were submitted to MAE following a full factorial 3^2 experimental design. Two factors, extraction time and temperature, were tested to estimate the more convenient combination to obtain high TCH values and PEF yields (Table 1).

Table 1. Full factorial 3^2 experimental design for *L. edodes* MAE. [1]TCH, total carbohydrates content in MAE extracts; [2]mg equiv. Glc/g MP, milligrams of equivalent glucose per gram of mushroom powder. 3 PEF yield, polysaccharide yield of the enriched fraction (PEF) obtained from MAE extracts.

Exp. Number	Factors		Response variables	
	Temperature (°C)	Time (min)	TCH in MAE[1] extracts (mg equiv. Glc/g MP)[2]	PEF yield[3] % (w/w)
1	180	5	263.21 ± 18.4	15
2	50	5	107.52 ± 5.8	4.6
3	115	30	88.62 ± 3.12	4.4
4	50	17.5	107.36 ± 8.5	4
5	115	17.5	83.33 ± 2.0	4
6	115	5	98.65 ± 31.0	5
7	115	17.5	86.21 ± 31.4	3.4
8	50	30	116.80 ± 4. 9	2.8
9	180	30	290.43 ± 20.6	15.4
10	180	17.5	238.76 ± 12.5	16.4
11	115	17.5	80.38 ± 19.2	3.6

Similar trends in the response surface plots for both variables were obtained (Figure 2), higher TCH and yields were reached at the highest tested temperatures. The model showed an adequate fitting to experimental data supported by the elevated variability percentage in TCH and yield explained by the model (99.4 and 98.8% respectively). The regression equations fitting to the data were:

$$y_1 = 13.1 - 0.22x_1 - 0.12x_2 + 1.3 \cdot 10^{-3}x_1^2 + 6.7 \cdot 10^{-4}x_1x_2 + 6.0 \cdot 10^{-4}x_2^2$$

$$y_2 = 281.08 - 4.12x_1 - 3.89x_2 + 0.02x_1^2 + 5.5 \cdot 10^{-3}x_1x_2 + 0.1x_2^2$$

where the linear and quadratic effects of both factors (x_1 = temperature and x_2 = time) as well as the interaction between them were included for y_1 (yield) and y_2 (TCH) prediction. Only 'linear' and 'temperature' quadratic terms showed a statistically significant influence on the TCH and yield, following the analysis of the variance results (ANOVA, $p<0.05$). The factor 'time' as well as the interaction between both factors, were defined as statistically insignificant. However, according to the Pareto charts for each variable response (Supplementary Figure 1), the term 'quadratic time' affected more to TCH content than to the yield. This effect can be easily visualized in the respective response surface plotting by the curvature along time (Figure 2a).

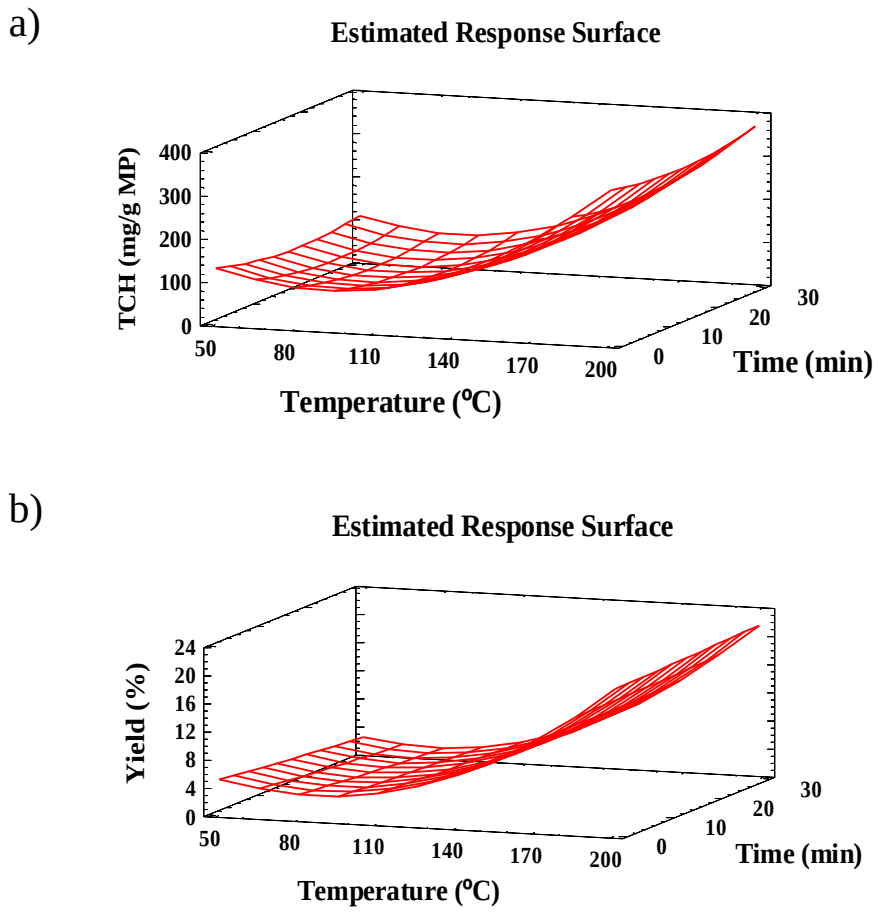

Figure 2. Estimated response surfaces plots for a) TCH content and b) PEF yields after MAE from *L. edodes*.

The temperature and time values that maximize the TCH and PEF yields of MAEs, within the selected conditions ranges, were 180 °C and 30 min. These values were similar to those previously described for other mushroom species such as *Pleurotus ostreatus* and *Ganoderma lucidum* after a similar extraction procedure (Smiderle et al., 2017). The desirable linear regression obtained by the plot observed vs. predicted values for each variable response validated the model (Supplementary Figure 2). Nevertheless, to verify it, three additional microwave-assisted extractions from *L. edodes* were performed at 180 °C and 30 min. Only slightly lower values were observed for TCH (224.4 ± 6.1 mg equiv. Glc/g MP) and higher values for PEF yield (19.1 ± 0.3%, w/w) were noticed compared to the software predicted values (respectively 278 mg equiv. Glc/g and 15% w/w). Therefore, the predictive model fitted the experimental behavior.

Substantially higher PEF yields were observed after MAE compared to the recoveries previously reported for conventional hot water extractions, *i.e.* 5.3% were obtained after seven extractions at 100 ºC for 200 min (Zhu, Nie, Liang, & Wang, 2013). The higher MAE efficiency could be explained by the more effective disruption of analytes-matrix interactions and an improved mass transference at high temperatures and pressures, resulting in larger recoveries (Plaza & Turner, 2015). However, their higher solubilization might also indicate that MAE could generate some physical changes in the tertiary conformations of the polysaccharides enhancing their extractability (Wang et al., 2017) and/or that some chemical changes such as partial hydrolysis might occur as indicated by other studies (Synytsya et al., 2014; Ookushi, Sakamoto, & Azuma, 2006). Nevertheless, since the extraction yield steadily increased and no drastic changes were observed/predicted at least up to 180 °C, the latter possibility seemed unlikely.

3.2. Microwave-assisted extractions from other edible mushrooms

MAE were also carried out at 180 °C and during 30 min for the other selected species since with these parameters, the highest TCH and PEF yields were obtained for *L. edodes* and, according to Smiderle et al. (2017), they were also appropriate for other two mushrooms. The yields of obtained PEFs from the selected species ranged between 12.1 (*Cantharellus cibarius*) and 19.1 % (*L. edodes*) except for *Auricularia judea* that showed extremely high yields (Table 2). However, the MAE extracts that showed higher TCH values besides *Auricularia judea* were *Pleurotus eryngii, Lentinula edodes* and *Boletus edulis* indicating that the latter two species contain higher levels of monosaccharides and oligosaccharides than the rest of species. In the case of *A. judea,* results indicated that almost half of the dry matter from the mushroom powder could be extracted using MAE obtaining PEFs with 96.4% polysaccharides. *L. edodes* yields were in the range of the previously observed values (15.4%) as also noticed for the other species *i.e. Armillaria luteovirens* (8.40 - 8.34%) (Chen et al., 2015), *G. lucidum* (11.2%) (Smiderle et al., 2017), *Fomitopsis ulmaria* (8.36%) (Zhao, Tang, Liu, & Zhang, 2014) etc. Other mushrooms such as *Morchella conica* showed PEF yields (16.5%) slightly higher than other MAE obtained extracts previously reported (5.86%) (Xu et al., 2018) and *Pleurotus pulmonarius* showed lower

values than other related species such as *P. ostreatus* (32.4%) (Smiderle et al., 2017). However, the different extraction conditions such as shorter times and/or different mushroom varieties or cultivation conditions, might be the reason for the noticed differences.

The TCH content determined in PEFs indicated their polysaccharides concentration (since only traces of monosaccharides or oligosaccharides might be present after the precipitation procedure). Results indicated that, indeed, PEFs were fractions with high polysaccharide content except for *Craterellus cornucopioides* (40.3%). PEF obtained from mushrooms such as *Amanita caesarea, Agrocybe aegerita* or *Hypsizygus marmoreus* contained 63.6 to 68.2% PSCs. The rest of the PEF weight might be proteins (perhaps bound to polysaccharides as glucoproteins or proteoglucans) as some of them might also precipitate with the ethanol concentrations utilized.

For certain fungal species, MAE seems to be a more efficient method to obtain polysaccharides than conventional methods or other advanced techniques but not for all. Pressurized water extractions (PWE) extracted higher polysaccharide amounts than MAE from *P. ostreatus* but not significant differences were noticed when the polysaccharides were extracted from *G. lucidum* (Smiderle et al., 2017). Moreover, *L. edodes* showed a 21% PSCs extraction yield using PWE at higher temperatures (200 ºC and 10.7 MPa) but 4.71% was recovered when extraction was carried out at 150 ºC (Zhu et al., 2013), values very similar to MAE where a 19.1% yield was noticed. Ultrasound-assisted extractions (UAE) were also tested using several mushrooms however, they were usually less effective than MAE, for instance UAE from the latter mushroom extracted 9.75% PSCs, meaning 1.6 fold increase compared to conventional hot water extraction but still lower than observed using MAE (Zhao, Yang, Liu, Zhao, & Wang, 2018).

Table 2. Total carbohydrate contents (TCH) in MAE extracts (mg/g), yields of obtained polysaccharide-enriched fraction (PEF) after MAE precipitation, TCH (mg/g) in PEF, PSC (%) in PEF and PSC (%), as well as other sugars, after MAE precipitation. *n=3 independent colorimetric measurements for TCH determination, † n=2 independent MAE extractions.

Species	TCH in MAE extracts (mg/g)*	PEF yield % (w/w)†	TCH in PEF (mg/g)*	PSC in PEFs (%)	PSC in MP after MAE precipitation (%)	Other sugars in MP after MAE precipitation (%)
A. judea	485.5 ± 11.0	44.2 ± 1.3	964.6 ± 65.5	96.4	42.64	5.91
A. aegerita	175 ± 3.9	17.6 ± 0.3	682.7 ± 42.5	68.2	12.02	5.48
A. caesaria	180.6 ± 5.5	13.9 ± 1.2	636.4 ± 37.2	63.3	8.85	9.21
B. edulis	226.9 ± 12.8	12.3 ± 0.1	588.8 ± 8.8	58.8	7.24	15.45
C. cibarius	140.3 ± 31	12.1 ± 0.3	567.5 ± 49.6	56.7	6.87	7.16
C. cornucopioides	87.19 ± 7.9	15.3 ± 0.6	403.4 ± 1.7	40.3	6.17	2.55
C. lutescens	157.4 ± 18.9	15.7 ± 0.1	608.8 ± 40.7	60.8	9.56	6.18
C. tubaeformis	91.3 ± 3.4	16.4 ± 0.1	511.1 ± 34.8	51.1	8.38	0.75
H. marmoreus	184.8 ± 1.0	12.8 ± 1.4	681.5 ± 79.2	68.1	8.72	9.76
L. deliciosus	140.8 ± 11.0	17.8 ± 0.1	561.2 ± 26.5	56.1	9.99	4.09
L. edodes	290.43 ± 20.6	15.4 ± 0.4	563.7 ± 33.6	56.3	8.68	20.32
M. conica	143.4 ± 3.5	16.5 ± 0.9	552.4 ± 53.1	55.2	9.11	5.23
P. eryngii	301.2 ± 16.0	16.8 ± 0.2	593.8 ± 30.1	59.3	9.98	20.14
P. pulmonarius	166.6 ± 3.1	15.4 ± 0.3	585 ± 10.6	58.5	9.01	7.65

3.3. *Adjustments of the fluorimetric method for fungal (1→3)-β-D-glucans determination*

The fluorimetric method is based on the sirofluor preference for binding to (1→3)-β-D-glucans. Although an increased signal emission intensity can be expected with higher number of these branches, there is not a direct correlation since the fluorescence intensity is affected by the polysaccharide structure (Evans et al., 1984). Ko & Lin (2004) also indicated the influence of the structure and conformation of (1→3)-β-D-glucans to the fluorescence profiles of nine standards, *e.g.* pachymanan, yeast glucans, curdlan. Therefore, the maintenance of the β-D-glucan native structure during their extraction process is critical for a reliable identification and quantification. However, in previous studies, insufficient attention was given to sample preparation to keep the integrity of these molecules, usually a conventional protocol was followed (Ko & Lin, 2004). Thus, the fluorimetric method used to determine fungal (1→3)-β-D-glucans was adapted at several stages, from the sample preparation to the selection of the adequate standard compound for the β-D-glucan quantification.

The PEFs obtained with MAE were not completely soluble in water at room temperature but in alkalis. However, a careful post-extraction treatment of the samples should be carried out, avoiding drastic changes that could compromise the chemical integrity of the β-D-glucans. Treatment with sodium hydroxide improve β-D-glucans separation from impurities and facilitate their quantification but could modify their helical 3-D conformations (Lehtovaara & Gu, 2011; Young, Dong, & Jacobs, 2000) and in high concentrations could damage even the primary structure. Therefore, a solution of 0.05 M instead 1 M NaOH (Ko & Lin, 2004) was used to dissolve β-D-glucans since the lower concentration was able to dissolve the samples and standards. $NaBH_4$ (1% w/v) was also added to the NaOH solution to protect the polysaccharide chains from degradation (Whistler & BeMiller, 1958). Indeed, addition of $NaBH_4$ enhanced 17 % the fluorescence intensity of curdlan compared to a similar solution without the reducing agent. Moreover, previous studies remarked the importance of the incubation step at 80 °C for 30 min to enhance the complex between sirofluor and the polysaccharides with (1→3)-β-D-glucopyranose-linkages (Ko & Lin, 2004). However, if lower temperatures were tested (down to 50°C), no differences in fluorescence intensity were noticed therefore, fifty degrees were selected to protect the polysaccharides from thermic de-polymerization.

Isolated polysaccharides including the β-D-glucans-linkages frequently described in mushrooms were selected as representative standards to adjust the fluorimetric method. When these β-D-glucans were treated according to the modified method, linear (1→3)-β-D-glucans such as curdlan emitted intense fluorescence, but moderate fluorescence was noticed for branched (1→3)-(1→6)-β-D-glucans (Figure 3a). Schizophyllan and PUL were both (1→3)-β-D-glucans with (1→6)-β-D-Glc unit branching respectively every 3 and 2.7 residues of the backbone chain (Zhang, Kong, Fang, Nishinari, & Philips, 2013; Smiderle et al., 2008) and therefore, they showed slight differences in their degree of branching (DB) (0.33 for schizophyllan and 0.37 for PUL). Moreover, when β-glucans from *Saccharomyces cerevisiae* were analyzed (DB = 0.03-0.2) (Synytsya & Novak, 2013), an intermediate fluorescence was noticed between curdlan (DB = 0) and schizophyllan suggesting that higher DB might fold the molecules more compact or tighter, impairing the binding of the fluorophore to the (1→3)-linkages of the backbone chain. This effect

might result in a fluorescence reduction and indeed, PUL showed a slightly lower fluorescence intensity than schizophyllan. Computer modeling studies using glucans with different configurations (α or β) and linkages, suggested that (1→6)-β-D-glucans might show highly flexible 3D conformation due to an easy rotation freedom between glucose residues, while (1→3)-β-D-glucans showed helical and flexible conformations (including also glucans with O-6-branches) (Zhang, Cui, Cheung, & Wang, 2007). These observations indicated that the helical conformation was essential for the sirofluor fluorescence stimulation. Thus, a linear (1→6)-β-D-glucan (B6G) and a mannogalactan (MG) were also tested under the adjusted conditions and both lacked fluorescence. This result confirmed that still after the protocol modifications, sirofluor was binding to β-D-glucans with helical conformations including linear or/and branched (1→3)-β-D-glucans.

Since each mushroom species synthesize its own particular set of β-D-glucans, a wide variety of primary structures, degrees of branching and conformations are described (Synytsya & Novak, 2013). When the PEF obtained by MAE from several selected species were analyzed to detect the fluorescence of their β-D-glucans, a range of responses was noticed (Figure 3b), leading to slightly similar slope values. The lowest and highest data were respectively for *M. conica* and *A. caesarea* (Table 3) showing fluorescence values closer to PUL than to schizophyllan or curdlan. Obviously, the extracts contained many β-D-glucans showing several structures and conformations that were all contributing to the fluorescence intensity while signals from curdlan and schizophyllan are exclusive. However, only curdlan (linear (1→3)-β-D-glucan) is frequently used as standard for the β-D-glucan quantification regardless the mushroom source and most of the edible species (basidiomycetes) contain larger amounts of branched (1→3),(1→6)-β-D-glucans than (1→3)-β-D-glucan (more common in ascomycetes) (Ruthes et al., 2015). Thus, by using curdlan as standard, the β-D-glucan concentration of many mushroom species might be underestimated or missunderstood. For instance, the β-D-glucan content for *A. aegerita* PEF were 140.1 mg/g using curdlan and 522.3 mg/g using a (1→3),(1→6)-β-D-glucan (PUL) as standards (Table 3). For most of the tested species, results obtained using curdlan or PUL were significantly different (one-way ANOVA analysis ($p<0.05$) (Table 3). Thus, since the major compounds in

basidiomycetes are branched (1→3),(1→6)-β-D-glucans, it should be expected a higher contribution of these structures to the total polysaccharide fractions than linear glucans. Therefore, PUL might be a more suitable compound to be used as standard than curdlan, even schizophyllan could also be adequate and more convenient since it is commercially available and would induce a lower quantification error than curdlan (Figure 3b).

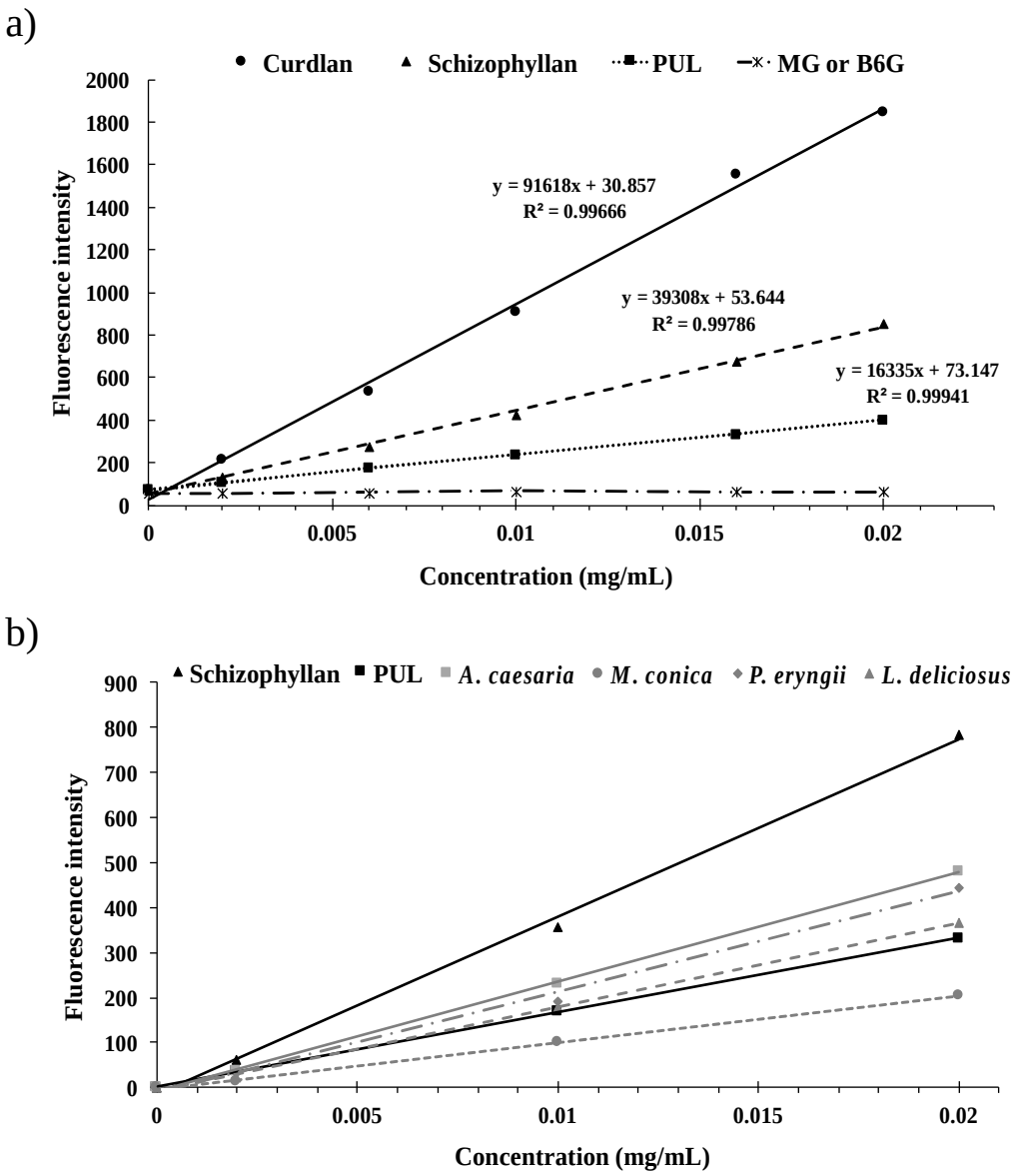

Figure 3. Fluorescence intensity of a) standard β-D-glucans and a mannogalactan (MG) b) the PEFs extracted by MAE from several mushroom species. The regression equation for the standards are included.

For the same reason, depending on whether PUL or curdlan are used as standard (Table 3), the PEFs composition might be completely different. For instance, the (1→3)-β-D-glucans

represented 14.8% of the polysaccharides from *A. judea* PEF when curdlan is used as standards while using PUL, 63.2% of the PEF polysaccharides were (1→3)-β-D-glucans. Similarly, the β-glucan percentages found in PEFs seemed to be highly underestimated in other species such as *A. agerita, H. marmoreus, M. conica* etc. These results could be supported by those previously reported by Synytsya & Novak (2013) and Smiderle et al. (2011, 2017) where β-D-glucans were found in higher concentrations than other polysaccharides such as α-glucans, chitins, heteropolysaccharides etc. If β-D-glucans are quantified using curdlan their contribution to the total polysaccharide values is quite low to be the predominant compound within the polysaccharide fractions.

However, quantification using PUL could also overestimate their contribution to the polysaccharides content because in the case of species such as *A. caesarea, P. eryngii* and *B. edulis* the estimation of β-D-glucans was almost 2-fold the amount of total polysaccharides. Nevertheless, the phenol-sulfuric acid method estimates the PSC concentration mainly because glucose residues are generated with the digestion, if other monosaccharides (xylose, mannose, galactose, etc.) are generated, the method precision decreases. These results bring a suspicious about the suitability of the fluorimetric method for the quantification of β-D-glucans in a complex polysaccharide matrix.

On the other hand, the fluorimetric determinations (independently of the utilized standard) indicated that the polysaccharides detected in PEFs obtained from mushrooms belonging to the Cantharellale order (*Cantharellus lutescens, Cantharellus cibarius, C. cornucopioides* and *C.tubaeformis*) (Table 2) might be different than (1→3)-β-D-glucans, or contain also other polysaccharides interfering in the sirofluor complexation, as no fluorescence was detected (Table 3).

Table 3. Fluorescence (emission at 503 nm) of PEFs extracted by MAE from several edible mushrooms and their (1→3)-β-D-glucans concentration depending on the standard utilized (curdlan or PUL). n=2 MAE extracts per species; n=2 fluorimetric measurements per PEF; n.d. no signal detected. The linear fitting of the curves (R^2) was 0.99 for all the mushroom species. *

Data statistically different (one-way ANOVA, $p > 0.05$, 95% confidence level) between standards for the same species.

Species	Fluorescence results (AU)				(1→3)-β-D-glucans (mg/g) in PEFs		(1→3)-β-D-glucans of PSC (%)	
	0.002 mg/mL	0.01 mg/mL	0.02 mg/mL	Curve slope	Curdlan	PUL	Curdlan	PUL
A. judea	30.25 ± 11.5	159.5 ± 56.6	295 ± 86.3	14829	142.7 ± 49.3	610.0 ± 234.1	14.8	63.2
A. aegerita	18.7 ± 6.6	135.7 ± 11.1	298.2 ± 15.3	14637	140.15 ± 8.62*	522.6 ± 37.0*	20.5	76.5
A. caesarea	37 ± 7.7	231 ± 43.3	481.5 ± 78.0	24266	245.94 ± 43.1*	1249.9 ± 241.6*	38.6	196.4
B. edulis	37.7 ± 10.6	207.5 ± 55.0	446.2 ± 110.6	22401	229.21 ± 63.2	1020.3 ± 300.1	38.9	173.3
C. cibarius	n.d.	n.d.	n.d.		n.d.	n.d.	-	-
C. cornucopioides	n.d.	n.d.	n.d.		n.d.	n.d.	-	-
C. lutescens	n.d.	n.d.	n.d.		2.12 ± 2.12	n.d.	0.3	-
C. tubaeformis	n.d.	n.d.	n.d.		n.d.	n.d.	-	-
H. marmoreus	17.2 ± 4.3	115 ± 10.0	250.7 ± 33.0	12643	120.0 ± 18.5*	543.6 ± 13.8*	17.6	79.8
L. deliciosus	22.7 ± 5.3	178 ± 19.6	368.5 ± 22.1	18716	184.7 ± 12.4*	713.7 ± 53.5*	32.9	127.2
L. edodes	23.5 ± 12.0	135 ± 0	311.5 ± 10.6	15593	152.7 ± 5.9*	647.1 ± 28.7*	27.1	114.8
M. conica	15.2 ± 5.7	102 ± 23.6	204 ± 42.3	10325	94.5 ± 23.1	400.5 ± 129.5	17.1	72.5
P. eryngii	32 ± 4.9	192.7 ±18.0	445 ± 37.1	22349	226.4 ± 20.3*	1140.5 ± 113.8*	38.1	192.1
P. pulmonarius	18.7 ± 4.8	156.7 ± 14.4	337.7 ± 36.2	17153	167.2 ± 20.3*	639.4 ± 87.5*	28.6	109.3

3.4. *Chemical characterization of the PEFs extracted by MAE*

In order to study the real β-D-glucans contribution to the fluorescence intensity noticed (and to the total polysaccharide values of the PEFs) as well as the method quantification accuracy, the monosaccharide composition and structure of PEFs were monitored.

The PEFs that exhibited fluorescence contained more than 64% glucose in their monosaccharide composition (Table 4). Species that did not show fluorescence, belonging to the genus *Cantharellus*, showed lower glucose content (51.4 - 42.9%) but considerable amounts of mannose (29.1 – 38 %) and xylose comparing to the other species. The mushroom from the Boletales order (*B. edulis*) showed higher galactose levels than the rest of selected species. Fucose monosaccharide was only found in few species at levels below 3%. Moreover, methyl-hexose was detected in Pleurotaceae mushrooms (*P. eryngii* and *P. pulmonarius*) and in *B. edulis* in low

amounts. Therefore, the slight differences observed in the monosaccharide composition are not consistent enough to explain the different slopes noticed in the fluorescence regression equations. For example, *P.eryngii* and *H.marmoreus* showed similar monosaccharide composition (~82% glucose, ~5% galactose and ~10% mannose) however, the regression slope of the first one was 1.8 fold the other, suggesting that sugar moieties might not be highly involved in the fluorescence observed (Tables 3 and 4). Wise to take into consideration was the fact that those mushrooms showing high β-glucan contents (even higher than their PSC values) *i.e. A. caesarea* or *P. eryngii* also showed high glucose contents (above 80%). The PSC levels of the Chantarelle family might also be underestimated because they contained large amounts of other monosaccharides that are not glucoses (*i.e. C. lutescens* contains 38% mannose and 13.7 % xylose) adding larger errors to the colorimetric determination.

Table 4. Monosaccharide composition (%) of PEFs obtained by MAE. [a] % of peak area relative to total peak areas, determined by GC–MS; [b] Trace amounts ≤ 0.5%.

Species	Monosaccharides (%)[a]					
	Fucose	**Xylose**	**Methyl-Hexose**	**Mannose**	**Galactose**	**Glucose**
A. judea	-	3.4	-	14.3	-	82.3
A. aegerita	2.3	-	-	3.6	5.3	88.8
A. caesarea	1.8	-	-	5.8	5.2	87.2
B. edulis	Tr.[b]	2.3	Tr.[b]	22.8	10.1	64.2
C. cibarius	Tr.[b]	12.7	-	35.9	-	51.2
C. cornucopioides	Tr.[b]	15.8	-	33.5	5.9	44.5
C. lutescens	Tr.[b]	13.7	-	38.0	5.3	42.7
C. tubaeformis	Tr.[b]	21.8	-	29.1	4.7	44.1
H. marmoreus	Tr.[b]	1.8	-	9.9	5.5	82.7
L. deliciosus	Tr.[b]	3.1	-	12.0	6.5	78.2
L. edodes	1.6	2.1	-	14.3	4.9	77.0
M. conica	-	-	-	10.3	4.0	85.7
P. eryngii	-	-	2.5	10.8	4.9	81.8
P. pulmonarius	Tr.[b]	1.3	2.6	8.9	6.8	79.8

Signals observed in the HSQC spectra from PEFs were in concordance with the monosaccharides determined for each species. The most representative spectra are compiled in Figure 4, while the others are supplied as supplementary material (Supplementary Figure 3). With exception of *M. conica,* spectra from *A. caesarea, C. lutescens, L. edodes* (Figure 4a, b and c), *A. aegerita* or *B. edulis* (Supplementary Figure 3), showed intense signals at δ 102.8/4.52 and 102.8/4.72 relative to C-1 of β-D-Glc*p*; at δ 85.0/3.75 relative to C-3 *O*-substituted and; at δ 69.0/4.19 and 69.0/3.86 relative to CH_2 *O*-substituted of the same units. These data suggest the presence of β-D-glucans (1→3)-(1→6)-linked (de Jesus et al., 2017). The O-6 substitution was confirmed by inversion of CH_2 signals of DEPT-HSQC experiment (data not shown). Linear (1→3)-β-D-glucans signals could be overlapped to the branched β-D-glucans, therefore a separation process might be required to quantify each of them. Besides, signals at δ 99.8/5.36 and δ 77.7/3.64 were intense in *L. edodes* (Figure 4c) and *M. conica* (Figure 4d) spectra, characteristic of C-1 of α-D-Glc*p* and C-4 *O*-substituted, indicating the presence of glycogen (α-1,4-1,6-D-glucan), the energy stock of fungi (Synystya & Novak, 2013). Small intensity signals of α-D-Gal*p* were observed in all spectra at δ 98.0/4.98 and signals of β-D-Man*p* were observed mainly in *C. lutescens*, *B. edulis*, and *L. edodes* at δ ~101.6/5.14-102.3/5.11. Species from genus *Cantharellus* showed the higher xylose content, that is not commonly observed in mushrooms and its presence was confirmed by signals at δ ~103.6/4.42 in the spectra of all *Cantharellus* species (Figure 4b and Supplementary Figure 3). Mannogalactans, fucomannogalactans and xylomannans were already isolated from other basidiomycetes such as *P. pulmonarius*, *Amanita muscaria*, and *Flammulina velutipes* (Smiderle et al., 2008; Ruthes et al., 2013; Smiderle et al., 2006), which confirms the findings in this study. Considering that β-D-glucans are able to complex with sirofluor and *Cantharellus* species did not show fluorescence, it is possible that polysaccharides containing xylose and mannose (present in high amounts in these species) may influence the β-D-glucans 3D conformation present in *Cantharellus* PEFs. These types of polysaccharides were also previously pointed as indirect scavengers of smaller molecules because

of their gelling properties so, they could partially attach to sirofluor provoking a lower fluorescence emission (Ngwuluka, Ochekpe, & Aruoma, 2016). Furthermore, *M. conica* showed low fluorescence and its HSQC spectrum presented more intense glycogen signals than signals of β-D-glucans in comparison to the other species, indicating that glycogen might also interfere the proper binding of the fluorochrome to the β-D-glucans structures.

Hence, the fluorimetric method seemed to be sensitive enough for the detection of small amounts of (1→3)-β-D-glucans in polysaccharide mixtures such a MAE extracts, with limits of detection bellow 2 μg/mL. So, it might be used as a fast and easy alternative to determine the presence/absence of (1→3)-β-D-glucans in complex mixtures, being tentatively applicable to other biological sources. However, to be sure that these linkages are not present in the analyzed samples when lack of fluorescence is observed, more exhaustive qualitative techniques (i.e. NMR) must be performed. No specific correlation was obtained between the fluorescence intensity noticed in the PEFs and their chemical composition suggesting that the fluorescence test should not be recommended for an accurate and trustable quantification of the (1→3)-β-D-glucans content for any mushroom species. The presence of α-glucans and other polysaccharides, their particular interactions and possible complexing between them or the sirofluor, might under/overestimate their real concentrations depending on the species and standard considered.

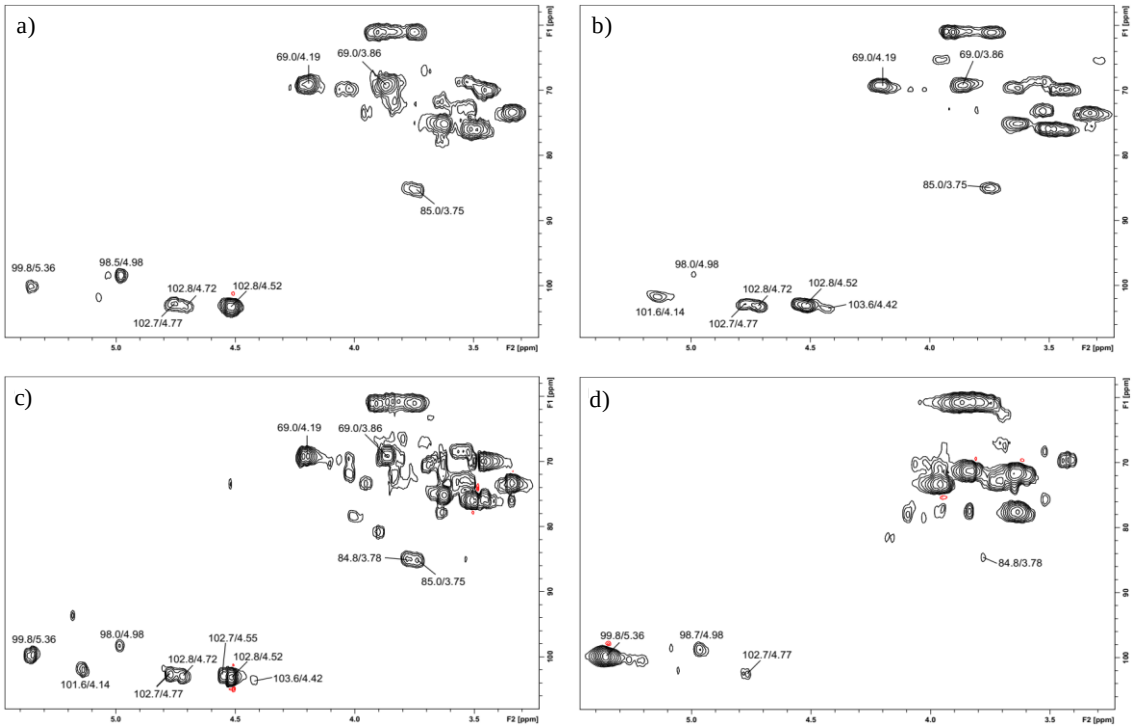

Figure 4. HSQC NMR spectra of a) *Amanita caesarea*, b) *Cantharellus lutescens*, c) *Lentinula edodes* and d) *Morchella conica.* Experiments were performed in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

4. Concluding remarks

Microwave-assisted extraction is a friendly environmental technology that could be used to obtain polysaccharide-enriched fractions from edible mushrooms. The most suitable extraction conditions seemed to be applicable to many different species obtaining interesting β-D-glucan yields. Although the fluorimetric method resulted appropriate for β-D-glucan detection, this technique must not be used for quantification analysis and a few details should be taken into consideration when carrying it out. For instance, modifications such as reduction of the alkali concentration utilized for experiments, decrease of the incubation temperature and addition of $NaBH_4$ improved the polysaccharide stability. Utilization of branched (1→3),(1→6)-β-D-glucans (particularly PUL or the commercially available schizophyllan) instead of linear (1→3)-β-D-glucans such as curdlan lowered the experimental error because the fluorescence intensity was influenced by the degree of branching (DB) of the β-D-glucans and most of the mushrooms showed (1→3),(1→6)-branched structures in high levels. Moreover, in the case that no

fluorescence is observed using the aniline-blue method for mushroom extracts a more exhaustive analysis (i.e. NMR) must be carried out before discarding the sample to avoid misinterpretations (as noticed in the *Cantharellus* genus). Perhaps the presence of other polysaccharides and/or their particular tridimensional configurations negatively affected the preference of fluorophore to bind (1→3),(1→6)-β-D-glucans. Deeper studies on the tridimensional folding possibilities of (1→3),(1→6)-β-D-glucans and their complexing with other polysaccharides are required to clarify this artefact before using the method for these mushroom species.

Acknowledgements

This research was supported by national R+D program from the Spanish Ministry of Science and Innovation (Project AGL2014-56211-R) and the regional program from the Community of Madrid, Spain (S2013/ABI-2728). The authors would also like to thank the Brazilian funding agencies CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico*), CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*) and the Fundação Araucária.

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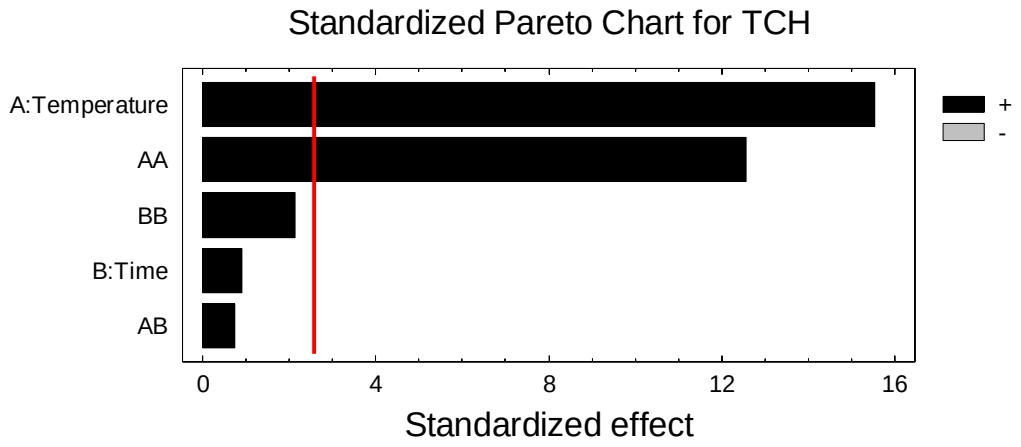
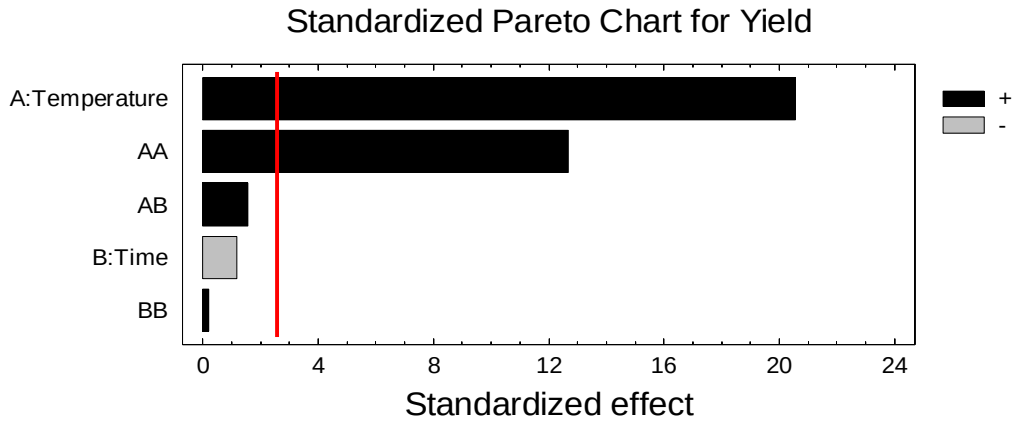
Supplementary information

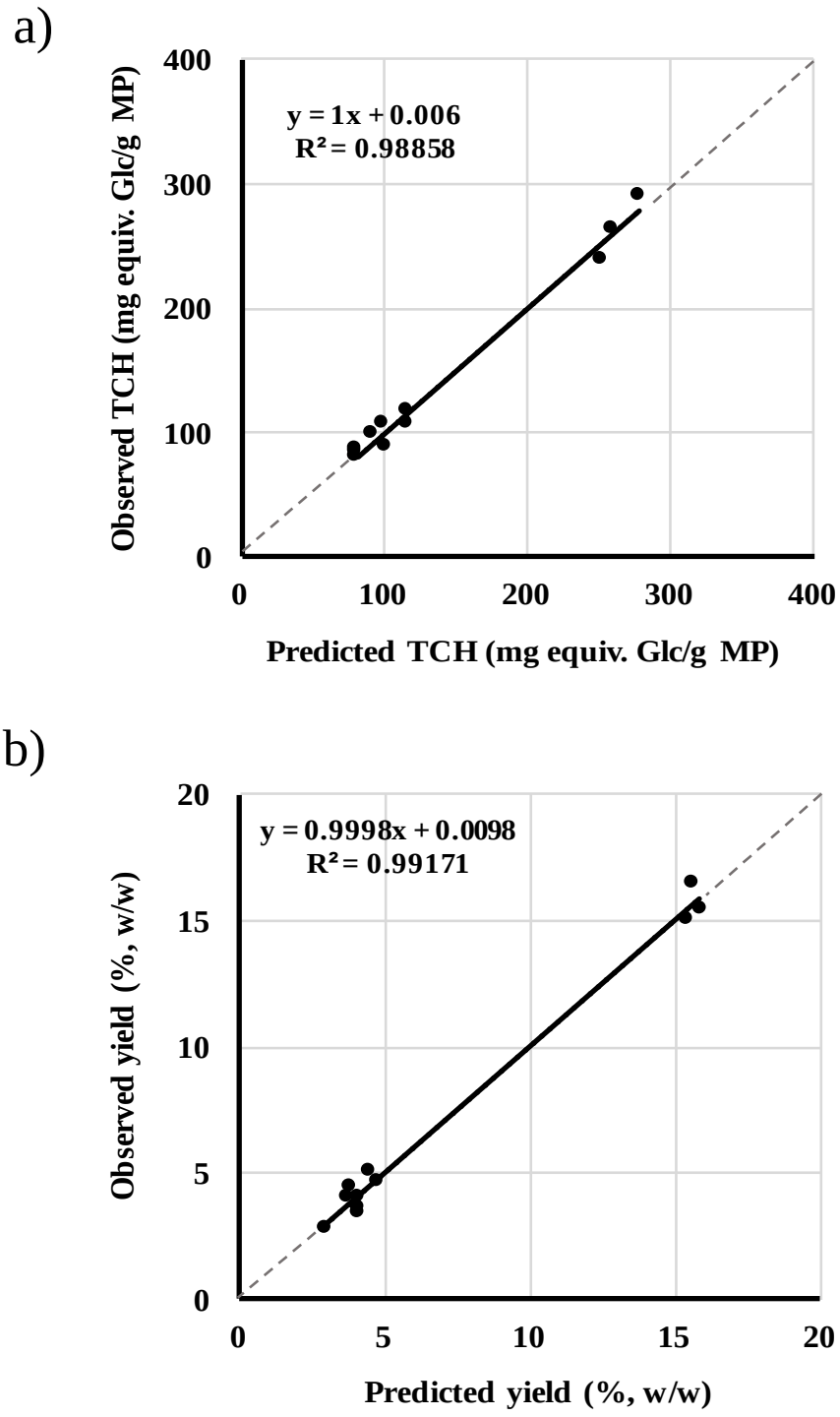
a)



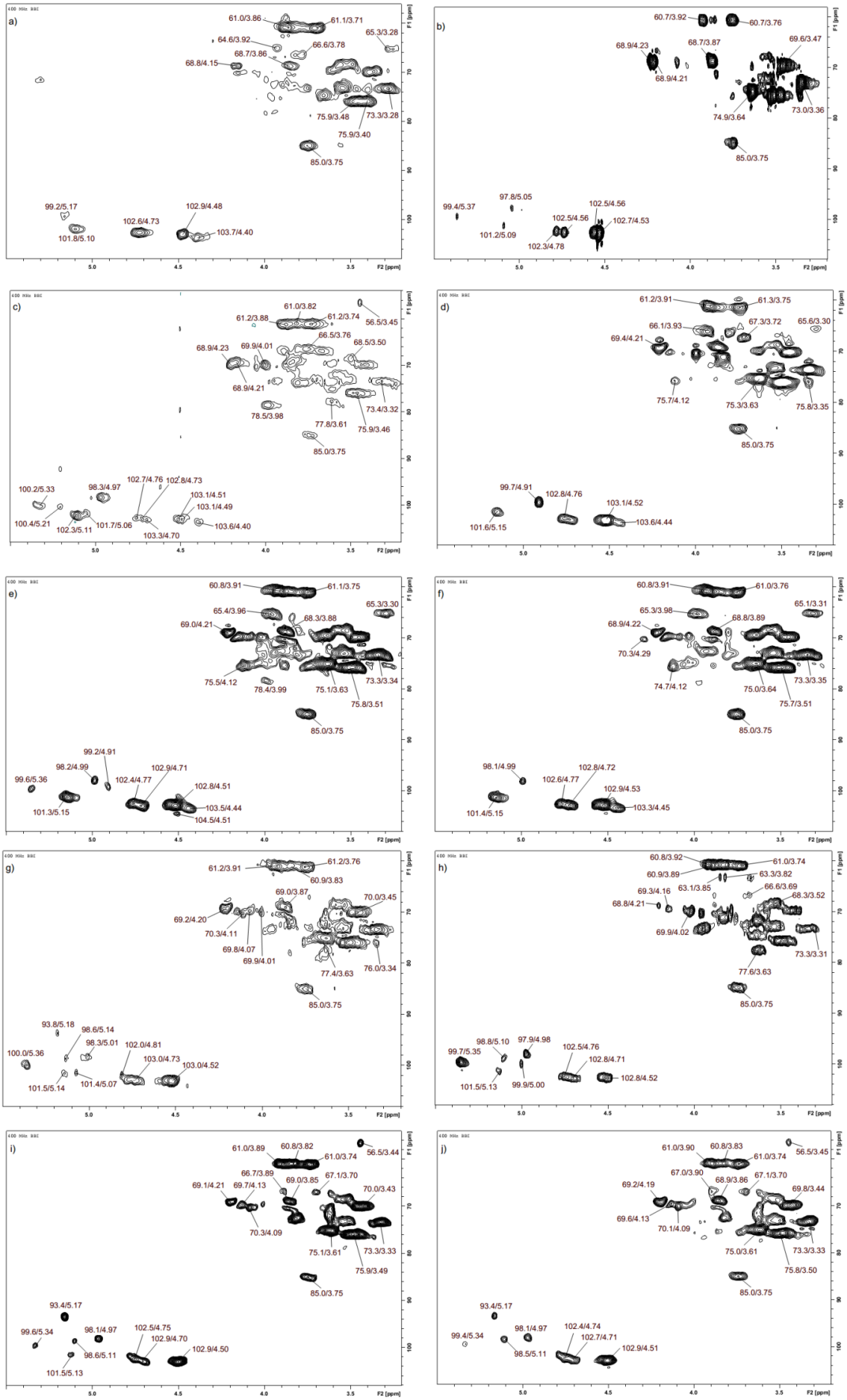
b)



Supplementary Figure 1. Predicted effect of factors ´temperature´ and ´time´ and their interactions on a) TCH content and b) the PEF yield, shorted in decreasing order of importance. Red line represents p=0.05.



Supplementary Figure 2. Observed vs. predicted values of a) TCH content and b) PEFs yield from *L. edodes* after MAE. Dashed lines correspond to the perfect fit line (y-intercept=0, slope=1).


a)
61.0/3.86
61.1/3.71
65.3/3.28
64.6/3.92
66.6/3.78
68.7/3.86
68.8/4.15
75.9/3.48
73.3/3.28
75.9/3.40
85.0/3.75
99.2/5.17
102.9/4.48
102.6/4.73
103.7/4.40
101.8/5.10
b)
60.7/3.92
60.7/3.76
68.9/4.23
68.7/3.87
69.6/3.47
68.9/4.21
74.9/3.64
73.0/3.36
85.0/3.75
99.4/5.37
97.8/5.05
102.5/4.56
102.5/4.56
102.7/4.53
101.2/5.09
102.3/4.78
c)
61.0/3.82
61.2/3.88
61.2/3.74
56.5/3.45
66.5/3.76
68.5/3.50
68.9/4.23
69.9/4.01
68.9/4.21
73.4/3.32
78.5/3.98
77.8/3.61
75.9/3.46
85.0/3.75
100.2/5.33
98.3/4.97
102.7/4.76
102.8/4.73
103.1/4.51
103.1/4.49
100.4/5.21
102.3/5.11
101.7/5.06
103.6/4.40
103.3/4.70
d)
61.2/3.91
61.3/3.75
66.1/3.93
67.3/3.72
65.6/3.30
69.4/4.21
75.7/4.12
75.3/3.63
75.8/3.35
85.0/3.75
99.7/4.91
102.8/4.76
103.1/4.52
103.6/4.44
101.6/5.15
e)
60.8/3.91
61.1/3.75
65.3/3.30
65.4/3.96
68.3/3.88
69.0/4.21
75.5/4.12
78.4/3.99
75.1/3.63
73.3/3.34
75.8/3.51
85.0/3.75
99.2/4.91
98.2/4.99
102.9/4.71
99.6/5.36
102.4/4.77
102.8/4.51
103.5/4.44
101.3/5.15
104.5/4.51
f)
60.8/3.91
61.0/3.76
65.1/3.31
65.3/3.98
68.8/3.89
68.9/4.22
70.3/4.29
74.7/4.12
73.3/3.35
75.0/3.64
75.7/3.51
85.0/3.75
98.1/4.99
102.8/4.72
102.6/4.77
102.9/4.53
101.4/5.15
103.3/4.45
g)
61.2/3.91
61.2/3.76
60.9/3.83
69.0/3.87
70.0/3.45
69.2/4.20
70.3/4.11
69.8/4.07
69.9/4.01
77.4/3.63
76.0/3.34
85.0/3.75
93.8/5.18
98.6/5.14
98.3/5.01
102.0/4.81
100.0/5.36
103.0/4.73
103.0/4.52
101.5/5.14
101.4/5.07
h)
60.8/3.92
61.0/3.74
60.9/3.89
63.3/3.82
63.1/3.85
66.6/3.69
69.3/4.16
68.3/3.52
68.8/4.21
69.9/4.02
73.3/3.31
77.6/3.63
85.0/3.75
98.8/5.10
97.9/4.98
99.7/5.35
102.5/4.76
102.8/4.71
102.8/4.52
101.5/5.13
99.9/5.00
i)
61.0/3.89
60.8/3.82
56.5/3.44
61.0/3.74
66.7/3.89
69.0/3.85
67.1/3.70
69.1/4.21
69.7/4.13
70.0/3.43
70.3/4.09
75.1/3.61
73.3/3.33
75.9/3.49
85.0/3.75
93.4/5.17
99.6/5.34
98.1/4.97
102.5/4.75
102.9/4.70
102.9/4.50
98.6/5.11
101.5/5.13
j)
61.0/3.90
60.8/3.83
56.5/3.45
61.0/3.74
67.0/3.90
68.9/3.86
67.1/3.70
69.2/4.19
69.8/3.44
69.6/4.13
70.1/4.09
75.0/3.61
73.3/3.33
75.8/3.50
85.0/3.75
93.4/5.17
99.4/5.34
98.1/4.97
102.4/4.74
102.7/4.71
102.9/4.51
98.5/5.11
F1 [ppm]
F2 [ppm]

Supplementary Figure 3. HSQC NMR spectra of a) *Auricularia judea*, b) *Agrocybe aegerita*, c) *Boletus edulis*, d) *Cantharellus cibarius*, e) *Cantharellus cornucopioides*, f) *Cantharellus tubaeformis*, g) *Hypsizygus marmoreus*, h) *Lactarius deliciosus*, i) *Pleurotus eryngii* and j) *Pleurotus pulmonarius.* Experiments were performed in D_2O at 70 °C (chemical shifts are expressed in δ ppm).