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Compositional differences of β-glucan-rich extracts from three relevant mushrooms obtained through a sequential extraction protocol

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ABSTRACT

In this work, a sequential fractionation protocol (cold and hot aqueous and alkaline extractions) and detailed compositional analysis (gross composition, monosaccharide analysis, FTIR, TGA) was applied to three relevant mushrooms in terms of global production (*Grifola frondosa*, *Lentinula edodes* and *Pleurotus ostreatus*) aiming to understand what is preferentially extracted during fractionation and how β-glucan extraction is affected by mushroom source. Room temperature aqueous extracts showed highest overall yields (56.3–82%) consisting of proteins, sugars and polyphenols. β-glucan content was highest in *P. ostreatus* and was concentrated in the more soluble fractions. On the contrary, a recalcitrant β-glucan in *G. frondosa* was mainly present in the residue (7.38%). *L. edodes* showed β-glucan populations distributed along aqueous and alkaline extracts, higher abundance of non-glucan polysaccharides and higher chitin purity (47.78%) in the residue. This work sets the basis for the rational design of extraction processes aiming to valorise mushroom biomass.

1. Introduction

Edible mushrooms are a good source of interesting carbohydrates, proteins, vitamins, antioxidants and minerals with low fat levels and low caloric content, thus making them a nutritious and healthy food source (Kalač, 2012). Probably linked to the wide recognition of their nutritional and health benefits, the economic importance of mushrooms is also growing, with a considerable global production increase from 7.5 million tonnes in 2009 to 11.8 million tonnes in 2019 (Schill et al., 2021). Market requirements of uniform size, shape and colour for these type of foods result in the removal of excess stipe length and rejection of mushroom biomass with slight discoloration and gross size variations (Ramos et al., 2019). This discarded biomass could be upcycled to obtain added-value compounds, in line with current circular economy practices.

The fungal cell wall is mainly formed by two types of structural polysaccharides, a rigid chitin fibrillary structure, and a matrix-like structure consisting on β-glucans, α-glucans and glycoproteins (Ruthes et al., 2016). Fungal glucans comprise structurally different polymers of D-glucopyranose (D-Glcp), which, despite of their simple monosaccharide composition, show a large diversity regarding molecular mass, anomeric configuration, position and sequence of glycosidic bonds along the chain, branching degree, branch composition and chain conformation (de Jesus et al., 2018). According to the anomeric structure, two main groups of fungal glucans are found, i.e. α-D-glucans and β-D-glucans, the last ones being the most abundant polysaccharides in fungal cell walls (Synytsya et al., 2009). β-glucans consist of β-(1 → 3) and (1 → 6) linkages with a huge structural diversity, being lineal or branched, and amorphous or microfibrillar structures (Bai et al., 2019), which can be embedded in the crystalline chitin (Ifuku et al., 2011). The interest in fungal polysaccharides, and especially in β-glucans, derives from their proven biological activity, e.g. antioxidant, antiinflammatory, anticancer, anti-diabetic, antimicrobial, antilipidemic, hypoglycemic, and immunomodulatory activities (Bai et al., 2019), thus making them attractive as active ingredients with potential applications in food, medicine, pharmacy, cosmetics, chemical and feed industries (Mirończuk-Chodakowska et al., 2021).

Specific fungal species, such as *Grifola frondosa*, *Lentinula edodes*, and *Pleurotus ostreatus*, are even considered medicinal, being recommended for therapeutic applications (Badalyan et al., 2019). *G. frondosa* (Maitake) has been widely used as a traditional food supplement in China, Japan and Korea (Ji et al., 2019) because of the recognized health

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beneficial effects from its polysaccharides, including antioxidant and antitumor activity amongst others. Specifically, a characteristic β-glucan isolated from *G. frondosa* may serve as a macrophage activator by increasing cytokine production and TNF-α expression (Wu et al., 2021). On the other hand, β-glucans present in *L. edodes* and *P. ostreatus* (two of the most widely used and cultivated mushroom varieties), also have pharmacological activities, with proven antibacterial, antiviral and anti-inflammatory capacity amongst others (Bai et al., 2019). Other bioactive compounds such as polyphenols present in the mushroom biomasses could also contribute to the reported antioxidant or antimicrobial activity (Smolskait et al., 2015).

Of course, these bioactivities depend on the β-glucan structure which, at the same time, is influenced by source and extraction conditions. Therefore, extraction of β-glucans from different fungal biomass has been widely explored, using a plethora of methods, but mainly applying treatments based on aqueous or alkaline treatments, but without considering the inherent recalcitrance of the different biomass sources. Aqueous and alkaline extractions are the most widely used methods for β-glucan extraction (Ruthes et al., 2016). Specifically, NaOH treatments are a widely recognized simple, efficient, and low-cost purification method for obtaining water-insoluble glucan fractions with different anomeric configurations and degrees of branching (de Jesus et al., 2018). Although specific names describing bioactive β-glucan have been coined for the different species, e.g. lentinan, grifolan, pleuran, etc., these sometimes refer to fractions obtained under completely different extraction conditions, which explains great differences in the extent of their bioactive effects, both qualitatively and quantitatively (Mirończuk-Chodakowska et al., 2021). While a subject scarcely investigated, knowledge on the inherent recalcitrance of the different polysaccharide components is crucial to design protocols for the targeted extraction of bioactive polysaccharides.

In this work, a sequential fractionation protocol was applied to *G. frondosa*, *L. edodes* and *P. ostreatus* and a complete compositional analysis was performed to all fractions obtained, with emphasis on the distribution of β-glucan, chitin or other polysaccharide components and their antioxidant activity. This comparative study thus aimed at shedding some light on the inherent recalcitrance and organization of polysaccharide components in these relevant species, understanding what components were preferentially extracted along the sequential protocol, key for the design of valorisation strategies.

2. Materials and methods

2.1. Fungal biomass production

Fungal biomass from *G. frondosa*, *L. edodes* and *P. ostreatus,* grown at the Instituto de Investigación y Tecnología Agroalimentaria (IRTA, Spain), was used as feedstock (see photos in Fig. S1 from the Supplementary Material). The main characteristics of these species are also provided in the Supplementary Material and details of fruit body production are provided elsewhere (Aranaz et al., 2021). Briefly, 4 L of a standard substrate for the commercial production of edible mushrooms contained in polypropylene bags with ventilation windows (Sac O2®, Deinze, Belgium) were autoclaved at 100 °C for 2 h and then inoculated with commercial inoculum of the different species (Deinze, Belgium; https://www.mycelia.be/en). After eight weeks of incubation at 22–25 °C needed for mycelium growth, the culture bags were transferred to flowering (or fruiting) rooms at 15 ± 3 °C and 80–90 % RH to induce mushroom production and kept for another two weeks, being finally harvested.

2.2. Reagents

Reagents NaOH (pure), potassium persulfate and EtOH (96 %) were obtained from PanReac AppliChem. Lentinan (min. 30 % purity) was purchased at Carbynth Biosynth (UK). Other materials and reagents used, specifically HCl (37 %), KH_2PO_4 (≥99.0 % purity), $HNa_2O_4P{\cdot}2H_2O$ (≥99.0 % purity), shrimp shells chitin, sodium borohydride (NaBH4), Folin-Ciocalteu reagent, sodium carbonate, gallic acid, potassium persulfate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphate buffered saline (PBS), 6-hydroxy-2.5.7.8-tetramethylchromane-2-carboxylic acid (Trolox), trifluoroacetic acid (TFA), fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid were purchased from Sigma-Aldrich. Yeast β–glucan (49 % purity) was obtained from Megazyme.

2.3. Sequential extractions

For each mushroom, a fractionation protocol was performed (depicted in Fig. S2 in the Supplementary Material). 25 g of freeze-dried raw material were taken, and 300 mL of distilled water were added at room temperature (RT) under agitation for 7 h. Each sample was then centrifuged 15 min at 8000 rpm and 4 °C and the precipitate was subjected twice more to aqueous extractions at room temperature with a volume of 150 mL, under agitation for 7 h. The aqueous fractions obtained from these extractions were concentrated and then freeze-dried, resulting in fraction F1. The precipitate obtained from the F1 extraction was then subjected to an aqueous treatment at 100 °C with reflux a total of 3 times, with a volume of 300 mL of distilled water in the first incubation and twice more with a volume of 150 mL. The soluble part resulting from these 3 extractions was again concentrated and freeze-dried as the F2 fraction. The precipitate was subjected to a first alkaline treatment at room temperature overnight with 150 mL of 1 M NaOH and 0.05 % $NaBH_4$ to avoid polysaccharide oxidation or "peeling" at the reducing end (Leong et al., 2021). The suspension was centrifuged 15 min at 8000 rpm and 4°C and the supernatant was reserved for precipitation with 1:3 (v/v) ethanol to remove low molecular weight components, and subsequently neutralized by washing with 0.4 M HCl in ethanol first, followed by another ethanol washing. The neutralized precipitate was left to air dry and was subsequently freeze-dried, as fraction F3. The remaining precipitate was subjected to a second alkaline treatment, under the same conditions to promote the extraction of the less soluble β-glucans from the mushrooms, which after the first alkali treatment (F3) are expected to be more accessible. The supernatant fraction was treated as with F3 and named as F4. The final residue was named as R.

Prior to all analyses, F3, F4 and R fractions were dialyzed through a molecular weight cut-off of 100–500 Daltons (SpectrumLabs), to remove the salts used in all extraction and neutralization steps, and were finally freeze-dried.

All extractions were made in triplicate and further analyses in triplicate were performed with each of these to evaluate reproducibility of the whole extraction process.

2.4. Raw material gross characterization

Moisture content was determined gravimetrically, by weight difference before and after a freeze-drying process, resulting in the percentage of water loss (AOAC, 2022).

For the quantification of ashes, samples of known weight were placed in a muffle furnace at 550 °C until complete calcination. The difference in weight at room temperature was recorded and the result was expressed as a percentage of weight lost compared to the initial sample (AOAC, 2022).

The total nitrogen content was determined using an elemental nitrogen analyser (rapid surplus N analyser) based on the modified Dumas method. As chitin also contributes to the total nitrogen, a factor of 4.38 was applied (Kalač, 2012).

Lipid determination was based on Soxhlet extraction (Somashekar et al., 2001), starting from 5 g of biomass and using 250 mL of hexane as solvent for 8 h. The resulting extract after the treatment was dried and the lipid content estimated gravimetrically.

2.5. Antioxidant capacity

Antioxidant capacity was determined by the ABTS method (2.2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (Re et al., 1999). 0.192 g of ABTS and 0.033 g of potassium persulfate were dissolved in 50 mL of PBS (phosphate buffered saline) at pH 7.4 and left to stir overnight. Prior to the determination, $ABTS^{\cdot+}$ radical cation was diluted in PBS for an initial absorbance of about 0.70 ± 0.02 at 734 nm. The determination of the free radical scavenging activity was performed by mixing 230 μL of the $ABTS^{\cdot+}$ solution with 20 μL of each sample and measuring the absorbance after 6 min' incubation at room temperature and darkness. The experiments were done in microplates and the absorbance at 734 nm was measured in a CLARIOstar spectroscopy equipment (BMG LABTECH). A calibration curve was built using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity of the samples is expressed as μmol Trolox equivalents (TE)/g fraction. All the determinations were performed in triplicate.

2.6. Total phenolic compounds

The total phenolic content of mushrooms and their fractions were determined using the assay based on the Folin-Ciocalteu method as modified by Singleton (Singleton et al., 1999). 1000 μL of 1/10 diluted Folin-Ciocalteau reagent were mixed with 200 μL of sample and allowed to incubate for 5 min. Then, 800 μL of sodium carbonate (75 mg/mL) were added and the samples were incubated in a bath at 40 °C for 30 min. The absorbance values were measured at 760 nm. A calibration curve was performed using gallic acid as standard, and the total polyphenol concentration was expressed as mg gallic acid/g sample.

2.7. Carbohydrate composition

The monosaccharide composition was evaluated after acid hydrolysis from the raw materials and different fractions to convert the polysaccharides into monosaccharides according to (Aranaz et al., 2021). Briefly, 1–2 mg sample were added 1 mL of 2 M of trifluoroacetic acid (TFA) and kept at 120 °C for 3 h. Samples were dried with a stream of air at 40 °C for complete evaporation of the TFA and re-suspended in deionized water. The monosaccharides were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex) using a CarboPac™ PA1 column. Samples of known concentrations with mixtures of fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid were used for calibration. All experiments were carried out in triplicate.

2.8. α-Glucans and β-glucans content

According to (McCleary & Draga, 2016) the α–glucan content was determined upon enzymatic hydrolysis with amyloglucosidase plus invertase (Megazyme, Ireland). Generated glucose was quantified with a glucose determination reagent (GOPOD-glucose oxidase, peroxidase, 4-aminoantipyrine; Megazyme, Ireland). The absorbance of all solutions was measured at 510 nm against the reagent blank. The values of glucan contents were expressed as g of glucose per 100 g of dry matter. The β-glucan content was calculated by subtracting the α-glucan glucose from the total glucose content as determined by TFA hydrolysis and made in triplicate.

2.9. Chitin and protein content

To determine the chitin content, hydrolysis was carried out in 6 M HCl at 100 °C for 7 h. The hydrolysis time was set after some preliminary optimization trials (see Supplementary Material) based on the method described in (Ekblad & Nasholm, 1996) and selected conditions were found to reach maximum glucosamine recovery. In contrast to most previous works determining the released glucosamine by colorimetric methods (Vetter, 2007), glucosamine was in this case quantified by HPAEC-PAD with a CarboPac™ PA1 column.

The D-glucosamine nitrogen (NGlc) content was obtained according to:

$$NGlc\,\frac{WGlc\cdot 14}{MGlc}$$

where WGlc was the % w/w of D-glucosamine content obtained from the above-mentioned hydrolysis, and MGlc was de D-glucosamine molecular mass (Gomba et al., 2015).

The total N_2 content in all fractions was determined using an elemental nitrogen analyzer (rapid surplus N analyzer) based on the modified Dumas method. The protein nitrogen was calculated as the difference between the total nitrogen from the elemental analysis and D-glucosamine nitrogen, and by applying the correction factor for proteins in foods of 6.25.

2.10. Fourier-transform infrared (FT-IR)

Infrared analysis was performed at 21 °C using a Jasco 4100 FT-IR spectrometer. The spectra were taken at 4 cm^{-1} resolutions in a wavelength range of 600–4000 cm^{-1} with a minimum of 32 scans. The results were processed using Origin Pro 2019 software.

2.11. Thermogravimetric analysis (TGA)

Thermogravimetric curves (TG) were obtained from a made with a TGA550 from TA Instruments, equipped with a Pt/Rh furnace with a maximum temperature of 1000 °C and an autosampler. The samples were heated in platinum capsules in the range of 10 to 600 °C with a heating rate of 10 °C/min under air atmosphere. Derivative TG curves (DTG) were plotted to express the weight loss rate as a function of temperature using Origin Pro 2019 software.

2.12. Statistical analysis

Data analysis of results was carried out using Statgraphics. One-way analysis of variance (ANOVA) was done to determine the significant differences between samples, at a significance level of $P < 0.05$.

3. Results and discussion

3.1. Raw material characterization

Initially, a characterization of *G. frondosa, L. edodes* and *P. ostreatus* biomass was carried out and the results are compiled in Table 1. The moisture content was around 87–88 % with no significant differences between samples and within moisture content values (67.2 to 91.5 %) previously reported for mushrooms (Manzi et al., 2004).

The ash content ranged between 6 and 7 % (Table 1) for all three species, again without significant differences between them and in accordance to previously reported values, which are reported to vary between 5 and 12 % on a dry matter basis and usually below 10 % (Kalač, 2012).

As commented in the introduction, mushrooms generally have low lipid contents, which may range from 0.1 to 16.3 % and usually between 1 and 4 % of the dry matter (Kalač, 2012). As observed in Table 1, *P. ostreatus* was the mushroom with greatest lipid content (5.3 %), followed by *L. edodes* (4.6 %) and *G. frondosa* (4.2 %).

Regarding the protein content, the three mushrooms contained 23–26 % based on dry weight, *P. ostreatus* being again the one with greatest protein content within the margin in accordance with previous reports (González et al., 2021), and *G. frondosa* (Wu et al., 2021)

Table 1
Raw material initial characterization expressed as mean values ± standard deviations. Antioxidant capacity was expressed as μmol Trolox equivalents (TE)/g of sample, and polyphenols content as mg of gallic acid equivalents (GA)/ g of sample. Moisture is expressed as g/100 g of fresh mushroom and the other results are expressed as g/100 g of dried mushroom.

	G. frondosa	*L. edodes*	*P. ostreatus*
Moisture %	88.4 ± 2.0^{a}	87.0 ± 3.3^{a}	88.6 ± 4.1^{a}
Ash %	6.9 ± 0.2^{a}	7.2 ± 0.2^{a}	6.7 ± 0.3^{a}
Lipid %	4.2 ± 0.1^{a}	4.7 ± 0.2^{ab}	5.3 ± 0.6^{b}
Protein %	22.7 ± 2.1^{a}	24.5 ± 1.5^{a}	26.1 ± 2.4^{a}
Chitin %	5.09 ± 0.09^{a}	5.32 ± 0.06^{ab}	6.38 ± 0.30^{b}
α-glucans %	3.31 ± 0.23^{a}	0.28 ± 0.04^{b}	7.57 ± 1.01^{c}
β-glucans %	25.9 ± 1.4^{a}	23.3 ± 5.4 ^{a}	38.7 ± 2.1^{b}
Antioxidant capacity (μmol TE / g)	63.36 ± 3.57^{a}	99.81 ± 0.39^{b}	137.35 ± 9.39^{c}
Polyphenols content (mg GA/ g)	16.04 ± 0.23^{a}	18.40 ± 1.22^{b}	20.11 ± 1.00^{b}

Different letters in the same row mean that the values are significantly different.

showing the lowest (Table 1). In general, edible mushrooms are considered a good source of protein, with protein content values reported between 19 and 35 % (Ramos et al., 2019).

The amount of protein was initially determined by applying a corrective factor of 4.38 to the total nitrogen content, as previously suggested in the literature (Kalač, 2012). In order to confirm the adequacy of the factor applied, chitin content was also quantified by chromatography methods (see section 2.8) and its nitrogen contribution subtracted from the total nitrogen. The remaining nitrogen was considered to come solely from protein and a factor of 6.25 was then applied. Both calculation methods yielded results with no significant differences amongst them, thus confirming the suitability of the applied factor for estimating protein content in whole mushrooms.

In order to complete this first gross biomass characterization with other relevant compositional data, the glucans and chitin content were also evaluated for the three mushrooms. *P. ostreatus* had significantly higher α and β-glucan content and higher chitin content than *G. frondosa* and *L. edodes* (Table 1), all values within the range reported for wild mushroom species (2–8.5 dwt% for chitin, less than approximately 10 % for α-glucan and <60.8 % for β-glucan) (Vetter, 2007). This positive correlation seems logical, as it is generally considered that a large part of the β-glucans present in the cell walls of mushrooms is bound to chitin. However, for valorisation purposes, it is not only the composition that matters, but how the different components are structurally organized in the cell walls, how strong their interactions are and, thus, how easily it is to extract the fractions of interest, i.e. understanding the recalcitrance of the various biomass sources.

Finally, the total polyphenol content and antioxidant capacity of the three mushroom species were also determined. As observed in Table 1, *P. ostreatus* showed greatest polyphenol content and antioxidant capacity, while the lowest content and related antioxidant capacity was found in *G. frondosa*. The significantly greater antioxidant capacity of *P. ostreatus* could also partially be ascribed to the greater β-glucan content of this mushroom, as these biologically active carbohydrates also possess antioxidant properties (Bai et al., 2019).

With the aim of shedding some light on the inherent recalcitrance and organization of the β-glucans in these relevant mushrooms and gather useful information for process optimization and the development of future targeted extraction protocols, the three biomass sources were fractionated (see Fig. S1 in the Supplementary material) and the side streams obtained after each extraction step deeply characterized as shown below.

3.2. Extraction yields of the different fractions

As previously explained in the materials and methods section, a sequential extraction consisting on consecutive cold and hot water extraction steps (giving raise to fractions F1 and F2) followed by two alkaline extractions (F3 and F4, respectively) was applied to the three tested species (cf. Fig. S2). The biomass yields obtained after each of the extraction steps were quantified and the results are compiled in Table 2. As observed in this table, the greatest yields were obtained after the first extraction step carried out using water at room temperature (F1). In fact, 56–82 % of the mushroom biomass was easily solubilized in water without the need of applying heat treatments. Significant differences were, however observed between the three mushrooms, *G. frondosa* being much more recalcitrant than the other two species, fact which was also reflected in this mushroom having the greatest residual (R) yield (cf. Table 2).

Applying a second extraction step with hot water (F2) only seemed to be useful in the case of *G. frondosa*, again pointing out the higher recalcitrance of even water soluble components, in which around 8 % of the biomass was solubilized. For the other two mushroom species, the yields obtained were relatively low and, thus, in principle, this second step could be skipped when designing a valorisation scheme for these specific mushrooms. Direct comparison with previous research works is not straightforward as, generally, extractions were focussed on obtaining carbohydrates (Morales, Smiderle, et al., 2019; Su et al., 2017) or specific β-glucan structures (Liu et al., 2014). However, low polysaccharide yields (between ~2.5 and 3.1 % of the initial wet biomass) were also obtained from *G. frondosa* after aqueous extraction, thus confirming the recalcitrant nature of this mushroom (Su et al., 2017).

Alkali treatments are commonly applied to a variety of biomass for polysaccharide extraction, as they are known to cause a disruption of the cell wall facilitating their release (Ruthes et al., 2016). Alkaline treatments applied to extract β-glucans much differ in the literature with

Table 2
Yields and protein content *of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from G. frondosa, L. edodes and P. ostreatus*, expressed as a percentage by weight of the initial dry samples. Antioxidant capacity (in μmol Trolox equivalents (TE)/g of sample) and polyphenols content (mg of gallic acid equivalents (GA)/g of sample) in mushroom fractions.

	F1	F2	F3	F4	R
Yield (%)					
G. frondosa	56.3 ± 3.6^{a}	7.9 ± 0.2^{a}	5.6 ± 0.8^{a}	4.3 ± 1.1^{a}	15.9 ± 0.6^{a}
L. edodes	82.0 ± 4.2^{b}	5.0 ± 1.5^{b}	6.2 ± 0.7^{a}	2.2 ± 0.5^{a}	8.2 ± 1.5^{b}
P. ostreatus	76.5 ± 0.3^{b}	2.6 ± 0.5^{c}	8.3 ± 0.6^{b}	2.2 ± 0.8^{a}	8.6 ± 0.6^{b}
Antioxidant capacity (μmol TE/g)					
G. frondosa	279.95 ± 0.38^{ab}	292.94 ± 4.31^{a}	6.18 ± 3.25^{a}	6.61 ± 0.43^{a}	11.62 ± 0.08^{a}
L. edodes	250.30 ± 20.87^{a}	277.99 ± 7.84^{ab}	90.65 ± 0.27^{b}	78.01 ± 1.89^{b}	4.27 ± 1.02^{b}
P. ostreatus	294.54 ± 6.78^{b}	275.89 ± 3.06^{b}	32.13 ± 4.94^{c}	51.49 ± 1.70^{c}	12.96 ± 2.20^{a}
Polyphenols content (mg GA/g)					
G. frondosa	41.84 ± 1.80 ^{a}	44.42 ± 0.97 ^{a}	4.22 ± 0.62 ^{a}	3.21 ± 0.20 ^{a}	8.08 ± 0.21 ^{a}
L. edodes	34.35 ± 1.07^{b}	33.59 ± 0.78^{b}	17.98 ± 0.36^{b}	9.30 ± 0.23^{b}	9.25 ± 0.40 ^{a}
P. ostreatus	35.97 ± 0.92^{b}	40.10 ± 1.29^{c}	6.59 ± 0.53^{c}	9.10 ± 0.82^{b}	13.70 ± 1.10^{b}
Protein (%)					
G. frondosa	42.99 ± 3.50^{a}	22.83 ± 3.81^{ab}	1.05 ± 0.29^{a}	0.67 ± 0.04^{a}	–
L. edodes	36.55 ± 0.98^{b}	19.41 ± 5.62^{a}	1.39 ± 0.64^{a}	3.03 ± 0.35^{b}	–
P. ostreatus	36.66 ± 1.01^{b}	28.75 ± 2.33^{b}	1.57 ± 0.16^{a}	2.01 ± 0.66^{c}	–

Different letters in the same column mean that the values are significantly different. F1 and F2 are the cold and hot aqueous extracted samples, respectively. F3 and F4 are the cold and hot alkaline extracted samples and R refers to the residue.

regards to alkali concentration, temperature and time (da Silva Milhorini et al., 2022; Morales, Rutckeviski, et al., 2019). However, a substantial number of articles have dealt with alkaline extracts at room temperature as good options to obtain highly bioactive β-glucans, which would further add on cost-efficiency and justify the conditions in the present study (da Silva Milhorini et al., 2022; Morales, Rutckeviski, et al., 2019). Very interestingly, a previous work dealing with β-glucan extraction from *L. edodes* reported much larger yields in the alkaline fractions from this mushroom, which can be explained by the limited previous aqueous extraction applied (Morales, Smiderle, et al., 2019). This indicates that, in this fungal biomass most of the polysaccharides present can be extracted in water by applying longer treatments at room temperature, thus making the extraction processes greener. The recalcitrance of *G. frondosa* was again patent after the alkaline treatments as the same trend was observed with the two consecutive extraction steps, i.e. displaying the lowest yield of the three mushrooms for the F3 and the greatest yield for F4, indicating that a second alkaline treatment or harsher conditions are needed for an efficient extraction. In contrast and similarly to what was observed in the aqueous extraction steps, the F4 yields for *L. edodes* and *P. ostreatus* were very low and, thus, the process for these species could be, again, simplified.

3.3. Polyphenol content and antioxidant capacity of the different fractions

Amongst the biologically active compounds present in mushrooms, polyphenols have been widely recognized as very beneficial compounds with antioxidant and chemopreventive properties (Smolskait et al., 2015) and, thus, evaluating their content in the various fractions generated is of practical interest for valorisation purposes. Table 2 compiles the results of polyphenol content (expressed as mg of gallic acid –GA- per gram of sample) and antioxidant capacity (μmol Trolox equivalents per gram of sample) of the different fractions obtained. Most of the polyphenols were concentrated in the aqueous fractions, without significant differences between the first and second extractions (i.e. F1 and F2), and representing around 7 % of the dry extracts. This result was not surprising as the most abundant polyphenols in mushrooms are phenolic acids with good water solubility. A strong correlation has been found between the amount of polyphenols and the antioxidant activity of mushroom species, ascribed to the scavenging capacity of their hydroxyl groups (Smolskait et al., 2015). It is interesting to note that the phenolic content of the aqueous fractions was, for the three mushrooms, greater than 30 mg GA per gram of extract, being significantly greater than that from ethanolic extracts obtained from a wide range of mushrooms (Smolskait et al., 2015). This result is in agreement with a previous study dealing with the antioxidant properties of *Pleurotus citrinopileatus* extracts, where it was found that the total phenol content of the cold water extract was greater than that from an ethanolic extract and similar to the extract obtained using hot water (Lee et al., 2007). This has practical implications, showing that green and simple extractions can be applied to these mushroom species and their discards to obtain extracts with high antioxidant capacity. In fact, the antioxidant capacity values from the mushrooms aqueous extracts (all greater than 250 μmol TE per gram of extract) were very similar to the values from a range of medicinal Indian plants (with an average antioxidant capacity value of 270 μmol TE/g extract) and higher than that of some fruits with great antioxidant capacity, such as cranberries (Shahidi and Ambigaipalan, 2015). While being a simple method for quantification of antioxidant capacity, ABTS is not found naturally, so there is possible criticism that the assay is not directly relevant to any biological function (Opitz et al., 2014).

Significantly lower phenolic contents were observed in the alkaline-extracted fractions (F3 and F4). It is well-known that the reactivity of polyphenols is enhanced under alkaline conditions resulting in degradation (Honda et al., 2019). An intriguing result was, however, obtained for *L. edodes*, as its F3 fraction had a much greater phenolic content and antioxidant capacity than the other alkaline fractions. F4 from *L. edodes* also displayed a reasonable antioxidant capacity. The differences observed may be related to the different chemical structure of polyphenol as well as to the type of interaction with other cell wall components present in the three mushroom species, all of which deserves further study. *L. edodes*, for instance, has been reported to contain, amongst others, some phenolic acids, such as ferulic and cinnamic acid and flavanols like catechin which have been reported to resist major pH-induced degradation (Nam et al., 2021). Another possible explanation for the increased antioxidant capacity of F3 and F4 extracts from *L. edodes* may come from other molecules like mannogalactans (as it will be shown below), to which antioxidant properties have also been attributed (Ruthes et al., 2016).

The residual fractions (R) showed significantly lower antioxidant capacity and phenolic content. Nevertheless, the opposite tendencies in e.g. *L. edodes* and *G. frondosa*, with greater and lower polyphenol and antioxidant capacity in alkaline or residual fractions, respectively, again point out major differences in the types of polyphenols and their interactions depending on the species.

3.4. Carbohydrate compositional analysis and distribution in the different fractions

The absolute estimated amounts of the different monosaccharides present in the fractions obtained from the three mushroom species are shown in Fig. 1. Fig. 1a depicts the concentration of all detected monosaccharide units, excluding chitin contribution. For better visualization, Fig. 1b and 1c display the chitin and the rest of sugars (excluding glucose) in all sample fractions, respectively (sections 3.4.2 and 3.4.3). All these data can again be found in detail on Table S1. Further insight into these results are given in the following subsections.

3.4.1. Glucans

Not surprisingly, glucose was the most abundant monosaccharide, as the main carbohydrates present in mushrooms are glucans (Fig. 2). The much higher β-glucan content compared to the α-glucan contribution in mushrooms justifies higher glucan purity in alkaline fractions (F3 and F4), as it is generally known that β-glucans are preferentially extracted in alkaline conditions. This glucan purity ranges between 69 and 83 % for the first alkaline treatment (F3). However, a significant amount of glucose was also present in the residues from the three mushrooms, pointing out to a certain fraction of strongly bound glucans present in the three species. Although in lesser relative amounts, glucans were also present in the more abundant aqueous fractions, with significant differences among samples. As opposed to β-glucans, α-glucans are frequently non-structural carbohydrates and their content can widely vary depending on the species and developmental stage, normally being below 10 % (Avni et al., 2017). While β-glucans are preferentially extracted using alkali treatments, α-glucans, can usually be dissolved in cold or hot water depending on their degree of branching, some even needing alkaline treatments.

To have further insight into the cell wall compositional differences and the inherent recalcitrance of the different glucan structures, the α-glucan and β-glucan distribution along the different fractions was also evaluated and is depicted in Fig. 2. As expected, the overall α-glucan content accounting for 3.31 %, 0.28 % and 7.57 %, for *G. frondosa, L. edodes* and *P. ostreatus*, respectively, was much lower than the β-glucan content (Fig. 2A). Most of these minor quantities of α-glucans were concentrated in the aqueous F1 and F2 fractions in all samples. *P. ostreatus* displayed the greatest amount of α-glucans, probably with a low branching degree and, thus, easily extracted within the aqueous steps. In contrast, and despite their lower overall α-glucan content, higher quantities were present in the first alkaline fraction for *L. edodes* and *G. frondosa*, hinting towards a higher recalcitrance involving also α-glucan structures.

P. ostreatus again showed a much higher amount of β-glucans than

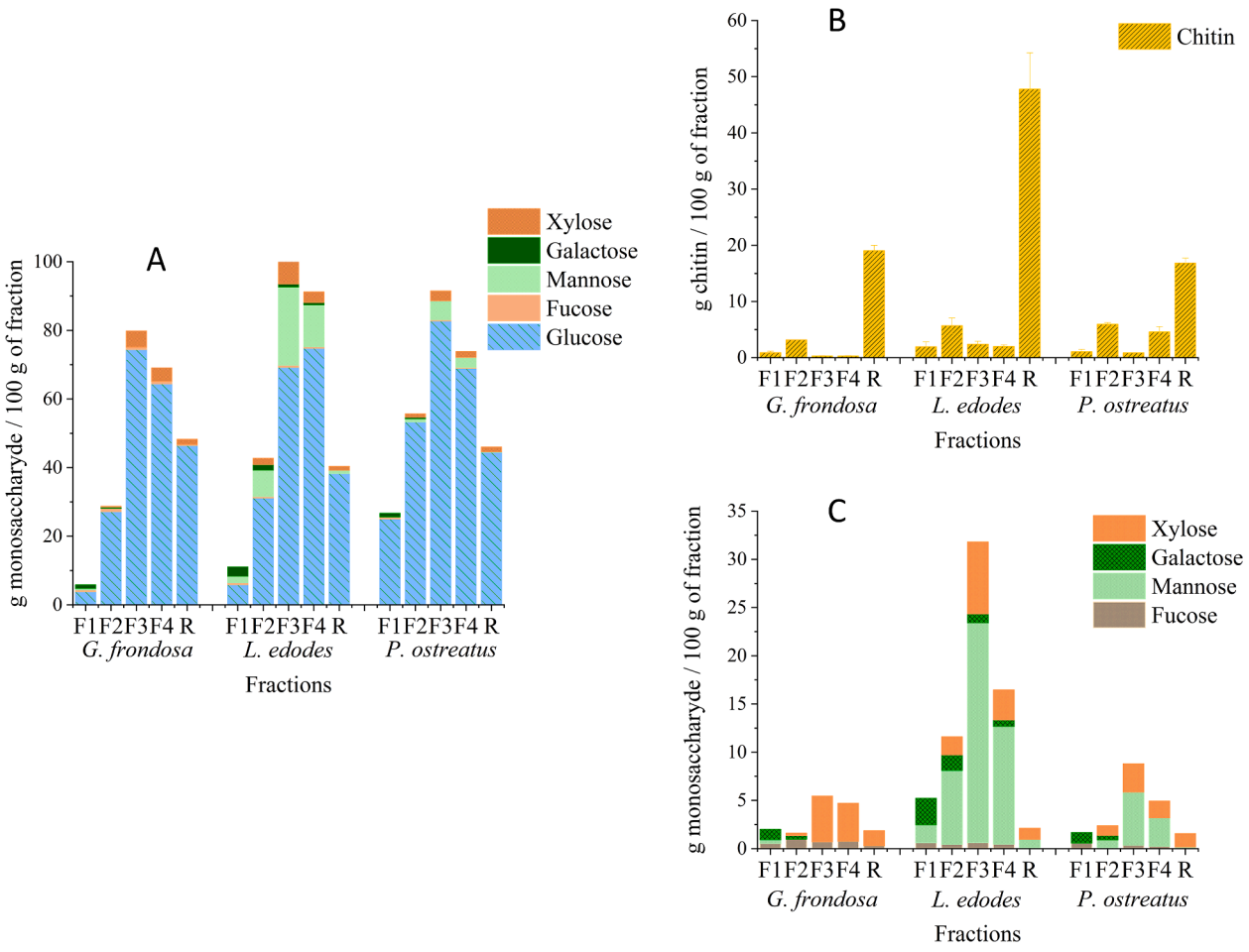

Fig. 1. Main monosaccharides of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from *G. frondosa*, *L. edodes* and *P. ostreatus*, expressed as percentage by fractions weight. A) Total monosaccharides excluding chitin; B) Chitin (*N*-acetyl glucosamine) content; and C) Total monosaccharides content excluding glucose and chitin.

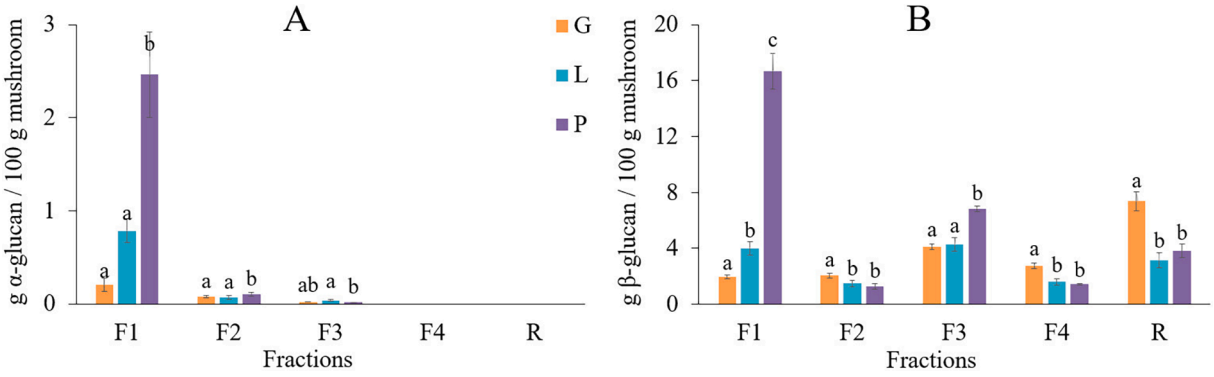

Fig. 2. Glucan content from aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) fractions obtained from *G. frondosa* (G), *L. edodes* (L) and *P. ostreatus* (P), expressed as a percentage of α and β-glucans (A and B respectively) by weight of the initial samples. Different letters in the same fractions mean that the values are significantly different.

the other two species, while no significant differences were found in the overall β-glucan content of *L. edodes* and *G. frondosa* (Table 1). However, significant differences were found in β-glucan distribution along the different fractions, depending on the species. The β-glucan in *P. ostreatus* was found to be highly soluble or accessible, with about 40–45 % of the total β-glucan present in the cold water extract (F1). Furthermore, and despite its higher overall glucan content, it showed the least β-glucan concentration in the residue. On the contrary, the lower β-glucan content in *G. frondosa* showed a remarkable recalcitrance, with >30 % of all β-glucan unextracted and present in the residue (R) even after several consecutive aqueous and 2 alkaline extractions. The relatively high yields of each second set of extractions (F2 and F4) compared to other samples further indicates a higher recalcitrance of both water and alkali soluble β-glucan fractions.

3.4.2. Chitin

In order to better understand the different accessibility of β-glucan and evaluate to what extent β-glucan-chitin interactions interfere with the release of β-glucans in the three mushroom species, the chitin content in all fractions was assessed (Fig. 1b). Common hydrolysis methods, such as TFA hydrolysis or Saeman hydrolysis, are either not aggressive enough to disrupt the crystalline chitin network or promote degradation of *N*-acetyl glucosamine, respectively. There is also discrepancy among methods used for chitin determination. Based on previous literature and preliminary optimization assays on fungal samples at different conditions (see Fig. S3 in the Supplementary material), a hydrolysis treatment with 6 M HCl at 100 °C for 7 h was selected.

As expected, chitin content was predominantly found and enriched in the solid residual fractions for all species. Interestingly, chitin was much more enriched (around 50 %) in the less abundant residual fraction of *L. edodes*, evidencing a lower recalcitrance of this species towards extraction of protein and β-glucan components. This evidences that higher chitin content is not correlated with higher recalcitrance. In fact, the higher recalcitrance in *G. frondosa* cannot be ascribed to chitin content alone, but could be better explained either by stronger β-glucan-chitin bonds or by a greater structural complexity of the β-glucans present. Although present in minor quantities in all fractions, chitin content was also relatively increased in the second round of each of the aqueous or alkali treatments (F2 and F4, especially for *P. ostreatus*), compared to the previous one, thus highlighting the harsher conditions needed for chitin extraction.

3.4.3. Other polysaccharides

The main monosaccharides detected, apart from the glucose or *N*-acetylglucosamine units from glucans or chitin, were xylose, mannose, galactose and minor amounts of fucose. These sugar units are derived from several polysaccharides known to be present in mushrooms, such as xyloglucans, xylomannans, mannogalactans or fucomannogalactans (Ruthes et al., 2016).

The results from Fig. 1c point out to differences, not only in composition, but also in recalcitrance of the three mushroom species. The significantly higher galactose, xylose and, especially, mannose contents in *L. edodes* points towards a richer content in mannogalactans and xylomannans, compared to the other species. *G. frondosa*, on the contrary, displays the lowest content in non-glucan polysaccharides, with much lower mannose content, predominance of xylose and the highest fucose contents among the three mushrooms, all of which point towards specific xyloglucan structures present in this species. These polysaccharides were predominantly enriched in the alkali fractions. The higher relative abundance of galactose in the F1 fractions points towards a higher solubility of galactomannans with higher galactose contribution. On the other hand, xylose is present in all residues and hardly represented in F1, evidencing a higher recalcitrance. As also observed from the table, the xylose-containing polysaccharides were more easily extracted in *L. edodes* and *P. ostreatus*, thus confirming the greater recalcitrance of *G. frondosa* cell walls.

3.5. Protein content of the fractions

In mushrooms there are two nitrogen sources: protein nitrogen and chitin nitrogen. The protein content was calculated as the difference between the total nitrogen from the elemental analysis and D-glucosamine nitrogen, applying the corrective factor of proteins for foods (6.25), and the results are compiled in Table 2. For all tested species, protein was predominantly concentrated in the cold aqueous fraction (F1), with a lower relative content in the hot water extract compared to other carbohydrate components. Only minor amounts of protein content were found in the alkaline extracts (F3 and F4) and no significant quantities were found in the residues. This points out to an easily extractable water soluble protein, indicating that, in these fungal species, protein is not tightly bound to other carbohydrate components, thus allowing for a cost-affordable extraction just using cold water. Interesting differences were, nevertheless, found among the relative amounts of protein in the fractions depending on the tested species. The highest relative protein content was found in the more recalcitrant *G. frondosa*, which suggests that protein does not play an important role in the structural complexity and recalcitrance of the β-glucan components in this species. On the contrary, in *P. ostreatus*, with much greater soluble β-glucan contents, a higher relative protein content was found in the hot water extract, thus pointing out to differences in the protein composition from the different mushrooms which deserves further investigation.

3.6. Fourier-transform infrared (FT-IR) spectroscopy

Fig. 3 shows the FT-IR spectra of the three mushrooms and their corresponding fractions. Lentinan and chitin controls were also included for comparison purposes. The FTIR spectra correlate very well with the previous compositional characterization. Lentinan, as a control of a relatively pure β-glucan, showed characteristic vibrational bands centred at 3450 cm^{-1} (O—H stretching), the vibrational bands corresponding to C—H stretching (2930 and 2890 cm^{-1}), and the region between 800 and 1200 cm^{-1}, typical of carbohydrates and containing highly coupled modes arising from C—C, C—O, C—H stretching and COH bending modes (Ji et al., 2019). The broad vibrational band in this region contains maximum absorbances at ~1148, 1075 and 1015 cm^{-1}, being the one around 1075 cm^{-1} mainly ascribed to β-glucan. The weaker band around 894 cm^{-1} is specific for β-glycosidic bonds (Synytsya et al., 2009). The FTIR spectrum from the chitin control, clearly shows the greater molecular order that this biopolymer has, evident from the well-defined and sharper spectral bands, displaying the N—H stretching band at 3270 cm^{-1} overlapped with the O—H stretching band. Other characteristic vibrational bands from chitin are the amide I (1660 and 1620 cm^{-1}), amide II (1560 cm^{-1}) and amide III (1306 cm^{-1}) bands and other vibrational bands from the carbohydrate backbone mainly ascribed to C—O vibrations within the *N*-acetyl-D-glucosamine units (Ifuku et al., 2011). The spectra from the three mushroom species are similar, with main contributions arising from the carbohydrates and proteins present, although certain differences are observed related to different molecular structure in their cell walls. The greater β-glucan content of *P. ostreatus* leads to the IR spectrum of this mushroom having a higher similarity with lentinan than those from the other two mushroom species. The spectra from the F1 fractions reflected that most of the proteins were extracted in this first step, with amide I and II bands clearly observed in the three mushrooms, but having variable relative intensity depending on the species. For instance, *G. frondosa*, being the more recalcitrant mushroom, shows the greatest intensity in these bands relative to the carbohydrate region, in which a small vibrational band, probably coming from low molecular weight compounds and simple sugars, is observed. In contrast, the F1 infrared spectrum from *P. ostreatus*, clearly reflects that a much greater amount of carbohydrates was obtained in this case (as seen from the 800–1200 cm^{-1} region and the relative intensity between this region and the amide I and II regions). The F1 fraction of *L. edodes* shows typical absorption bands from chitin, thus confirming that in this specific mushroom some chitin is extracted just using water at room temperature. It is known that chitin from mushrooms is γ-chitin (which appears to be a combination of the α-, β-allomorphs) (Kumirska et al., 2010) and the results obtained point out different chitin structures present in the three mushroom species, patent from the amide I shape. The F2 fractions, although similar to the F1, clearly indicate a greater prevalence of carbohydrates, as their extraction was promoted by increasing the temperature. The spectra of the F3 and F4 fractions, obtained from the alkaline extraction steps, confirm that these are mainly composed of β-glucan, being very similar to that of lentinan, and displaying the bands typically ascribed to β-glucans around 894, 990, 1040, 1080, 1100, 1162, 1317 and 1376 cm^{-1} (Synytsya et al., 2009). The spectra from the residues as well display

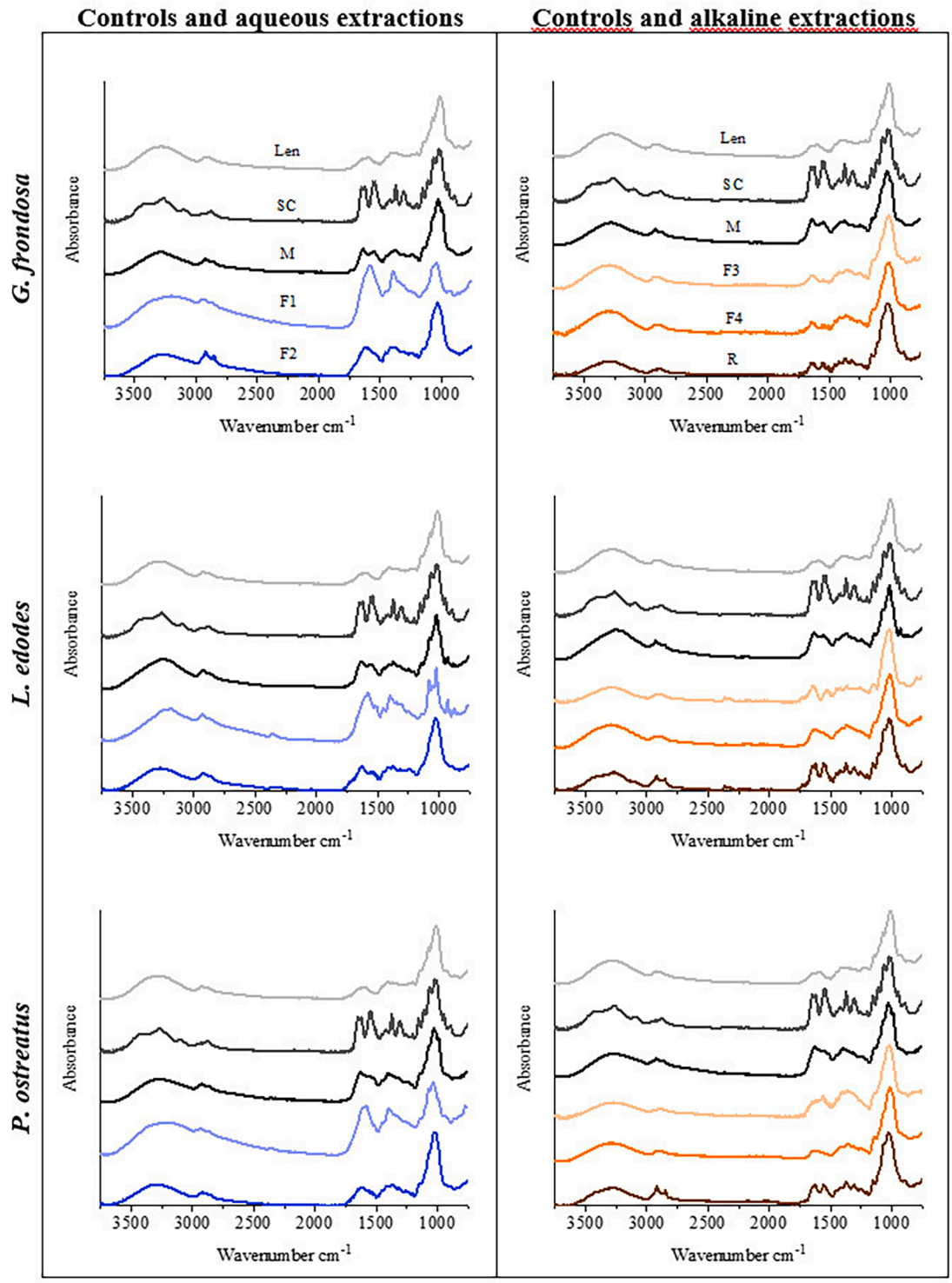

Fig. 3. FT-IR spectra of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from *G. frondosa*, *L. edodes* and *P. ostreatus*. Each graph also includes the FT-IR spectra of the whole mushroom biomass (M), shell chitin (SC) and lentinan (Len), as controls.

vibrational bands characteristic from β-glucans but also from chitin, in line with the compositional results.

3.7. Thermogravimetric analysis (TGA)

Fig. 4 shows the TGA first derivative (DTGA) graphs from the three mushroom (M) species and their corresponding fractions. As with the FTIR, the DTGA curves from chitin and lentinan controls are also included. As observed from Fig. 4, the mushroom biomass is characterized by four thermal transitions. The first one is a shoulder between 140 and 175 °C, whose intensity and main degradation temperature varies with the species, being weaker for *G. frondosa*, much more intense for *P. ostreatus* and taking place at a greater temperature for *L. edodes*. This shoulder is probably related to the loss of tightly bound water and to the degradation of low molecular weight compounds present in the mushrooms' cell walls. The second transition, around 240 °C has been

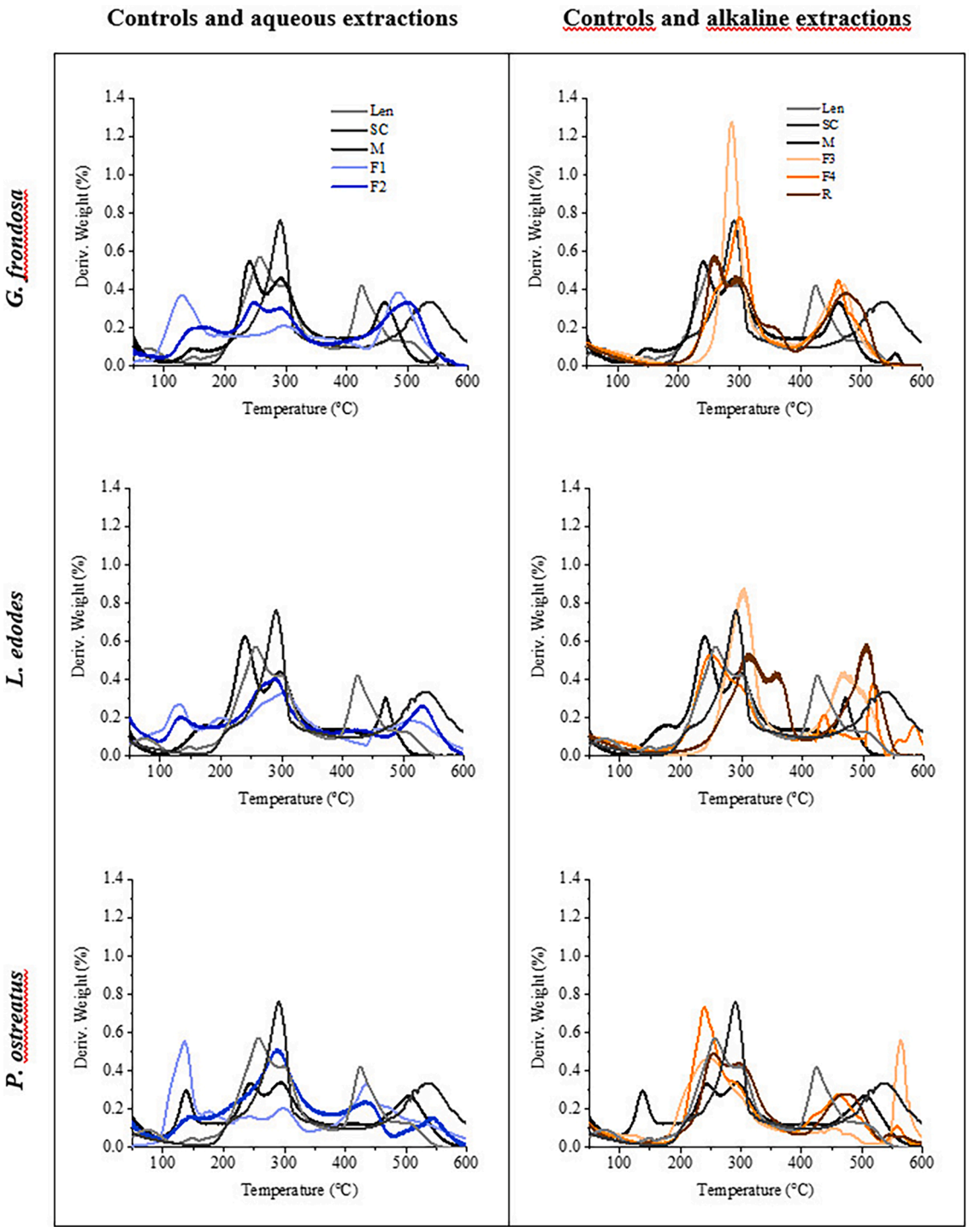

Fig. 4. Derivative thermogravimetric (DTG) curves of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from *G. frondosa*, *L. edodes* and *P. ostreatus*. Each graph also includes the DTG curves of the whole mushroom biomass (M), shell chitin (SC) and lentinan (Len) as controls.

ascribed to the degradation of polysaccharide side chains (Girometta et al., 2020) and degradation of some hemicelluloses like xylan or glucomannan (Werner et al., 2014). The third degradation peak, with a maximum between 295 and 300 °C is usually ascribed to the depolymerization of glucan chains and also associated with the cleavage of the C—C and C—O bonds in the ring structure of the sugars (Aburas et al., 2020). In fact, different polysaccharide fractions obtained from *L. edodes* have been reported to thermally degrade between 200 and 400 °C, being difficult to unambiguously ascribe the maximum degradation temperatures to specific polysaccharide structures. In fact, the DTGA of lentinan, also shows a broad degradation curve with two maximums at 256 °C and 298 °C. The fourth transition above 400 °C could be ascribed to chitin and chitin-glucan complexes degradation (Girometta et al., 2020).

The DTGAs of the aqueous fractions (F1 and F2), substantially vary depending on the mushroom species and in line with the different composition of the fractions described before. The more recalcitrant nature of *G. frondosa* is patent in the DGTA of its F1 fraction, with very little contribution from carbohydrates (small shoulder at 300 °C) and two more pronounced transitions at ~130 °C and 485 °C probably corresponding to proteins and minerals, respectively. It is interesting to

note that while F1 from *P. ostreatus* contains significantly more carbohydrates than the F1 fractions from the other two mushrooms, this is not reflected by a greater transition around 300 °C, being the contribution of the first thermal degradation transition the most significant one.

The alkaline fractions (F3 and F4), which have been seen to mainly consist on polysaccharides confirm the very different structure of the β-glucans extracted from the three mushroom biomass. While F3 from the more recalcitrant *G. frondosa* shows the sharper carbohydrate transition (maximum at 288 °C), thus indicating more homogeneous structures in this fraction, the DTGA from F3 of *P. ostreatus* shows a broader band with a maximum around 250 °C, indicating much more heterogeneous polysaccharide structures with lower thermal stability. A partial degradation upon a second alkaline treatment is observed in F4 fractions from *L. edodes* and *P. ostreatus*, reflected in a decrease in the maximum degradation temperature from the β-glucans (from 302 to 258 °C and from 250 to 240 °C, respectively), while two populations are observed for *G. frondosa*, the second one degrading at a greater temperature than F3, thus confirming the recalcitrant nature of the β-glucan present in this mushroom. Finally, the residues left after the sequential extraction steps, show a DTGA profile similar to that of the mushroom, but with the maximum degradation temperatures of each step displaced towards higher temperatures, indicating the presence of residual β-glucan and chitin of greater thermal stability.

4. Conclusions

The sequential fractionation process involving several consecutive cold or hot aqueous and alkaline treatments applied to *P. ostreatus*, *L. edodes* and *G. frondosa* revealed substantial differences in recalcitrance of these mushroom species reflected in the distribution of the different components in the various extracted fractions depending on the species. Highest yields corresponded to the aqueous treatments at room temperature (F1) for all species, with highest and lowest yields for *P. ostreatus* (82 %) and *G. frondosa* (~56 %), respectively. This fraction was the richest in sugars, proteins and polyphenols and also showed the highest antioxidant capacity. The lower recalcitrance of the cell wall architecture of *P. ostreatus* was patent in the higher relative abundance of β-glucans in this fraction (~16 g β-glucan/100 g mushroom) compared to the alkaline or residual fractions, evidencing cost-affordable valorisation possibilities for both antioxidants and glucans. *L. edodes* showed an intermediate recalcitrance, with distinct β-glucan populations distributed along aqueous or alkaline extracts, higher abundance of non-glucan polysaccharides and higher chitin purity in the residue. *G. frondosa* showed, in contrast, a remarkable recalcitrance, with highest residual content and most β-glucans remaining in the residue (~8 g β-glucan/100 g mushroom). This overall recalcitrance was further patent in the distribution of α-glucans and the higher relative yields of each second set of aqueous or alkaline extractions (F2 and F4). Furthermore, the relatively lower content on non-glucan carbohydrates was represented by xylose or fucose containing polysaccharides, pointing out to more recalcitrant xyloglucan structures compared to the higher mannose and galactose contents in *L. edodes*. These differences in the heterogeneity and recalcitrance were further evidenced in the thermal degradation patterns. These results reveal great differences in the cell wall architecture of the different mushrooms, which must be taken into account when designing appropriate fractionation or valorisation strategies. Future works could be directed towards the elimination of extraction steps depending on the species and a deeper structural analysis of these specific populations in order to unravel specific structure-bioactivity relationships.

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CRediT authorship contribution statement

Zaida Pérez-Bassart: Methodology, Investigation, Formal analysis, Writing – original draft. **Maria Jose Fabra:** Conceptualization, Writing – review & editing. **Antonio Martínez-Abad:** Conceptualization, Supervision, Writing – review & editing. **Amparo López-Rubio:** Conceptualization, Methodology, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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