Biotechnology Progress 30.2 (2014): 391-400

DOI: http://dx.doi.org/10.1002/btpr.1865

Pressurized water extraction of β-glucan enriched fractions with bile acids-binding capacities obtained from edible mushrooms

Marimuthu Palanisamy*, Laila Aldars- García, Alicia Gil-Ramírez, Alejandro Ruiz-Rodríguez, Francisco R. Marín, Guillermo Reglero, Cristina Soler-Rivas

Department of Production and Characterization of Novel Foods, CIAL – Research Institute in Food Science (UAM+CSIC), C/ Nicolas Cabrera 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain

***Corresponding author.** Tel:+34-910017918 (Office); Fax: +34-910017905

E-mail address: marimuthu.palanisamy@uam.es

Running title: Bile acids-binding β-glucans from mushrooms

ABSTRACT

A pressurized water extraction (PWE) method was developed in order to extract β-glucans with bile acids-binding capacities from cultivated mushrooms (Agaricus bisporus, Lentinula edodes and Pleurotus ostreatus) to be used as supplements to design novel foods with hypocholesterolemic properties. Extraction yields were higher in individual than sequential extractions being the optimal extraction parameters: 200ºC, 5 cycles of 5 min each at 10.3 MPa. The crude polysaccharide (PSC) fractions, isolated from the PWE extracts contained mainly β-glucans (including chitooligosaccharides deriving from chitin hydrolysis), α-glucans and other PSCs (hetero-/proteo-glucans) depending on the extraction temperature and mushroom strain considered. The observed bile acids-binding capacities of some extracts were similar to a β-glucan enriched fraction obtained from cereals.

Keywords: Accelerated solvent extraction, β-glucans, bile acids, Oyster mushroom, shiitake mushroom, white button mushroom, cholesterol

Introduction

Certain polysaccharides (PSC) present in plants and particularly those from cereals have recently gained attention because of their potential beneficial effects for human health. The (1 → 3),(1 → 4)-β-glucans are believed to perform many biological functions such as *i.e.* modeling of immune response[1] or reducing of cholesterol levels in serum.[2]

Direct binding of bile acids and cholesterol (from ingested food) and increasing their faecal excretion has been hypothesized as a possible mechanism by which the water-insoluble polysaccharides lower cholesterol.[3] By binding bile acids, they prevent their reabsoption and stimulate plasma and liver cholesterol conversion to additional bile acids.[4,5] The cholesterol-lowering effect of water-soluble dietary fibers seems to be due to several mechanisms being the increase in viscosity (water binding capacity in the chyme) the main effect. This leads to a reduced diffusion rate of bile acids, which cannot be reabsorbed by the body being then excreted.[3]

Edible mushrooms also contain interesting polysaccharides[6] such as other β-glucans, which are structurally different from plants since their branches are (1 → 3) and (1 → 3) (1 → 6) but share biologically important properties including hypocholesterolemic effects[7], and chitin, a water-insoluble β-(1→4)-glucan of N-acetylglucosamine monomers, also considered as dietary fiber. However, not all the fungal β-glucans show all the beneficial biological activities, the immunomodulatory and antitumor properties seemed to be more related to the water soluble fraction[8] including the oligomers and low molecular weight polymers generated from chitin hydrolysis (LMWC or chitooligosaccharides)[9] while their effect as prebiotic might be due to their water-insoluble β-glucans (chitins and protein-bound glucans).[10] Obviously, their solubility in water depends, above all, on their molecular structure largely influenced by their monomer composition, type and degree of branching, conformation (single or triple helix), molecular weight, N-acetylation degree etc.[11] and,

according to recent review, there is insufficient information to establish a broad generalization correlating structure with function.[12]

A wide variety of polysaccharides with different structures and biological activities have been obtained from edible mushrooms using different extraction processes. The traditional methods include the use of boiling water, sodium hydroxide, ammonium oxalate and more complex protocols,[13] while the more advanced technologies included ultrasonic-assisted (UAE)[14,15] and microwave assisted (MAE)[16] extractions or combinations,[17] supercritical carbon dioxide[18] and pressurized solvent extraction using water[19] or ethanol[20] as extraction solvents.

The work presented in this paper, describes the optimization of a specific procedure to obtain β-glucan enriched fractions from the three most commonly consumed edible mushrooms using an accelerated solvent extraction (ASE) device, which enable the use of pressurized water as extraction solvent (PWE). Several PWE conditions and procedures were tested to investigate whether the mushrooms could be fractionated separating in one/some fraction/s those β-glucans with bile acid-binding activities to use them as food-grade supplements to design novel foods with hypocholesterolemic properties. Then, the obtained fractions were submitted to an *in vitro* digestion model to determine whether they are able to bind bile acids as the β-glucans from a cereal foodstuff.

Materials and methods

Biological material

Mushroom strains used in this investigation were *Agaricus bisporus* L. (Imbach), *Lentinus edodes* S. (Berkeley) and *Pleurotus ostreatus* (Jacq.Ex Fr.) Kummer. Mushroom strains were cultivated at CTICH (Research Center for Mushroom Science and Technology, Autol, Spain) and harvested at the optimal developmental stage for consumption. Fruiting bodies

were dehydrated and ground into fine powder as described by Ramírez-Anguiano et al. (2007)[21] and stored at -18ºC until further use.

Commercially available breakfast cereals containing oat, rice, wheat and barley β-glucans bearing the claim `containing β-glucans able to reduce cholesterol levels´ were obtained from a local supermarket. All the experiments were performed from the same package.

Reagents

Ethylenediaminetetraacetic acid, sea sand and methanol HPLC grade were purchased from Panreac (Barcelona, Spain). D (+)-glucose, ferric chloride, ammonium sulphamate, sodium nitrite, 3-methyl-2-benzothiazolone hydrazone hydrochloride, phenol, chitin (from shrimp), glucose, pancreatin, standard bile acids and porcine bile extract, cellulose, cholestyramine and bovine serum albumin (BSA) were procured from Sigma-Aldrich Co. (Steinheim, Germany). All other reagents and solvents were used of analytical grade.

Pressurized water extractions to obtain PSC enriched fractions

Mushroom powder (1 g) were mixed with washed sea sand (4 g) and submitted to pressurized water extraction (PWE) at 10.68 MPa using an Accelerated Solvent Extractor (ASE) (Dionex Corporation, ASE 350, Sunnyvale, CA, USA). Extraction procedure per cycle was as follows: firstly, the sample was loaded into 11 ml extraction cells. Then, the cell was filled with solvent (MilliQ water), heat-up time applied and static extraction was carried out with all system valves closed. Afterwards, the cell was rinsed, solvent was purged from cell with N_2 gas and depressurization took place. Several parameters such as solvent, static extraction time or cycles number and temperature were changed in order to optimize the extraction method.

A sequential extraction procedure was also tested by mixing the mushroom powders (1 g) with sea sand (4 g) into the ASE extraction cells. Then, the samples were submitted to 5 extraction cycles of 5 minutes each at 25ºC. Afterwards, the solvent was removed and another extraction was carried out for 5 cycles of 5 min each at 50ºC. These processes were repeated at 100, 150 and 200ºC.

Obtained fractions were immediately frozen, lyophilized and stored at -18ºC until further analysis. Extracted dry matter content was measured to calculate the extraction yields. A minimum of two fractions were collected from each type of sample.

Standardized extraction method to obtain PSC enriched fractions

Mushroom powders (1 g) and powdered dehydrated cereals (1 g) were mixed with MilliQ water (100 ml), heated at 120 °C for 20 min and cooled down to 4ºC following the method reported by Jeurink et al. (2008)[22] The crude polysaccharide fraction was isolated as described below.

Isolation of the PSC fractions

The polysaccharides (PSCs) from the extracts obtained by both standardized and PWE methods were isolated by adding two volumes of cold ethanol, vigorous stirring and allowing polysaccharide precipitation overnight at 4°C. The precipitated PSCs were collected by centrifugation (10000g for 30 min at 4°C) (Thermo Scientific Heraeus Multifuge, Fisher scientific, Madrid, Spain), suspended in Milli-Q water and dialyzed with membrane tubing (MWCO 3500 Da; Medicell International Ltd London, UK) against distilled water to remove low molecular weight (LMW) compounds during 24 h at 5ºC with three or four changes of the distilled water. After the dialysis, the fraction remaining inside

the membrane (mw>3.5 kDa) was lyophilized, weighed and stored at −18°C until further use.[22] This extract was called `crude PSC fraction´.

Total polysaccharide determination

The polysaccharide concentration of the obtained extracts was evaluated as their total carbohydrate content with the modified phenol–sulphuric acid method as described by Fox & Robyt (1991).[23] Glucose was used as standard for quantification.

Determination of β-glucans

The β-glucan content of the obtained mushroom extracts (50 mg) was evaluated by a β-glucan determination kit specific for mushrooms and yeasts (Megazyme, Barcelona, Spain) following the instructions of the user´s manual. Absorbance at 510 nm was measured using a spectrophotometer (Evolution 600, Fisher scientific, Madrid, Spain).

The procedure calculates the β-glucan concentration directly from mushroom powder by subtracting the values obtained from a first assay in which the content of total glucans (α-glucan + β-glucan), D-glucose in oligosaccharides, sucrose and free D-glucose are quantified, to a second assay in which the content of α-glucans (glycogen and starch), D-glucose in sucrose and free D-glucose are quantified. Therefore, if these assays are carried out using the isolated crude PSC fractions (obtained after dialysis), mono- di- and oligosaccharides as well as broken or small polysaccharides are lacking. Thus, the values obtained by carrying out the first assay should be mainly due to the total glucans and in the second assay to α-glucans. Therefore, in these types of samples total, α- and β-glucans can be quantified.

Determination of chitin and chitooligosaccharides

The N-glucosamine containing β-glucans were determined in the crude PSC fractions following the method described by Vetter (2007)[24] adjusted from a previous method.[25] This method is usually utilized to determine chitin, however, if it is applied to extracts obtained with water (or pressurized water) where chitin is not extracted, it evaluates the amount of N-glucosamine-containing residues present in the fraction determining in this case, the amount of chitooligosaccharides, also referred as LWMC (low molecular weight chitin-hydrolysis products), which are depolymerized fractions showing values of n > 20 (n = monomers number) with increased water solubility.[9] Chitin from shrimp was used as standard for quantification.

Determination of total protein

The total protein concentration of the samples (2 mg/ml) was determined using the Bradford method reagents (cat. num. B6916, Sigma-Aldrich, Madrid, Spain) according to the Instruction Manual. BSA was used as standard for protein quantification.

In vitro digestion of the PSC fractions

Mushroom PSC fractions (isolated after PWE or the standard extraction methods), cereal PSC fractions, cellulose (negative control for bile acid-binding properties) and cholestyramine (positive control) were submitted to *in vitro* digestion following the procedure described by Kahlon, Chapman & Smith (2007)[4] with a few modifications. Briefly, the PSC extracts were digested in 1 ml of 0.01M HCl for 1 h at 37ºC with gentle agitation (Orbital incubator S150, Stuart, Stone, UK). Afterwards, the sample pH was adjusted to 6.3 with 0.1 ml of 0.1 M NaOH. Then, 4 ml of bile extract (14.3 mg in tris-maleate buffer pH 6.3) and 5 ml of pancreatin (10 mg in tris-maleate buffer pH 6.3) were added and the mixture was incubated for 1 hr at 37 ºC. After digestion, the samples were

filtered through filter paper and the bile acids isolated from the filtrate. A control sample was prepared following the same digestion protocol but without the addition of PSC fractions.

Quantification of bile acids by HPLC

Isolation of the bile acids was carried out using C_{18} Sep-pak cartridges and resulting extracts were injected (20 µl) in a HPLC system (Varian Pro-star 330, Madrid Spain) equipped with a C_{18} column (Microsorb-MV 100-5 Varian, 25cm × 4.6 mm and 5 µm particle size) and a PDA detector according to Pang et al. (1990).[26]

The chromatograms from the utilized bile extract (before and after digestion) showed four major peaks in concordance with those described by Qiao et al. (2011)[27] for pig bile extract and identified as glycoursodeoxycholic acid (RT = 7.3 min) (GUDCA), glycohyodeoxycholic acid (RT= 7.7 min) (GHDCA), taurochenodeoxycholic acid (RT =10.9 min) (TCDCA) and taurohyodeoxycholic acid (RT= 11.8 min) (THDCA). The bile acid-binding capacity of the PSC extracts was determined by subtracting the total bile acid concentration found in the control samples (digestion with no PSC fractions) and the digested PSC-containing samples. The total bile acid concentration considered was the sum of the four major bile acids present in the utilized porcine bile extract accounting for almost 93% of the total bile acids.[27]

Statistical analysis

One way analysis of variance (ANOVA) was performed using a Statgraphics® Plus 3.1 for Windows software (Statistical Graphics Corporation, Rockville, MD, USA). The mean comparison test used was Fisher's least significant differences procedure (LSD).

Results and discussion

The pressurized water extraction (PWE) procedure was initially optimized for only one of the mushroom species (*Lentinula edodes*) in order to define the parameters inducing larger variations in the extraction yields to be taking into consideration for the extractions of the other mushroom species.

Extractions of L.edodes PSC using different temperatures

The temperature of the extraction water could be the most critical parameter to define the distribution of the extracted β-glucans within the fractions obtained at different temperatures since their solubility in water depends on their molecular structure and it increases with temperature.[8] Indeed, the extraction yields of the PWE extracts and of the crude PSC fraction obtained from these extracts exponentially increased with the temperature (Table 1). However, when the results of the PWE extractions were compared with those obtained after a standard PSC extraction method, the latter yielded higher crude PSC fraction (48.6%) than the best PWE condition at 200ºC (28.3%) but, it contained 39% PSCs while the PWE extract (at 200ºC) almost doubled the amount of PSCs (74.2%) pointing PWE as a more selective procedure to extract PSCs.

Extractions of L.edodes PSC using different extraction times

There are two parameters that can be adjusted in PWE to modify the extraction time, one is the time that the solvent is in contact with the sample in the extraction cell and the other is the number of cycles of extraction. Therefore, temperature was fixed at 200ºC and the influence of both parameters in the extraction yields was investigated.

No significant differences were found neither in the levels of material extracted by PWE (approx. 80% of dry weight) nor in the levels of the crude PSC fraction extracted (219-285

mg/g mushroom powder) if the extraction time of each cycle was set from 1 to 5 min (with a total of 5 cycles). However, the amount of extracted PSCs slightly increased after 4 min (from on average 187.7 mg/g after 1, 2 or 3 min up to 206.9 mg/g) pointing 5 min as the best extraction time (255.7 mg/g). Moreover, 1, 5, 10 and 15 cycles of 5 min each one at 200ºC extracted from 627.6 to almost 890 mg dry matter with a concomitant increase of the crude PSC fraction (from 215 up to 303.4 mg/g) however, no significant PSC increase was observed from 5 to 15 cycles, apparently all the extractable PSCs were already obtained after 5 cycles.

Sequential extractions of L.edodes PSC

A sequential extraction of *L. edodes* powder was carried out by gradually increasing temperatures from 25 to 200ºC (Fig. 1). The first extraction carried out at 25ºC extracted only 5.6% of dry weight and the second extraction (at 50ºC) yielded higher amount (9.7%) however, apparently in these extractions mostly low molecular weight material was extracted since their PSC content was very low. The third extraction showed a low yield thus most of the extracted PSCs were obtained after the forth (150ºC) and particularly the fifth (200ºC) extraction where 30.3% of material was extracted containing only 5.4% of PSCs. Thus, sequential extractions did not improve the yields obtained by individual extractions and if the data from the complete set of obtained fractions were added together, by sequentially performing the extractions not all the mushroom PSCs were extracted. Thus, sequential extraction was discarded as a method to fractionate the β-glucans from mushrooms.

Extractions of PSC from other mushroom species

The results obtained after submitting *L. edodes* to PWE extractions indicated that the highest PSC yields were obtained by carrying out individual extractions, setting 5 cycles of 5 min as extraction time and 200ºC being the extraction temperature the most crucial parameter. Therefore, PWE extractions of two other cultivated mushrooms such as *Agaricus bisporus* and *Pleurotus ostreatus* were carried out at different temperatures maintaining other parameters as established above.

The observed increase in extraction yields with the temperature for both mushroom species was similar to the one observed for *L. edodes* being 200ºC the optimal temperature to obtain approx. 78% of the dry matter for both *A. bisporus* (Table 2) and *P. ostreatus* (Table 3). Also from these two other species, a higher amount of the crude PSC fractions could be extracted using the standard PSCs extraction method, however they contained less PSCs than those obtained using pressurized water extractions at 200ºC pointing PWE also for these mushrooms as a more specific method for fungal polysaccharide extraction.

Pressurized water extraction (PWE) have already been utilized to extract polysaccharide enriched fractions from mushrooms such as *Ganoderma* sp.[19] using a similar ASE device and conditions (120ºC, 10.7 MPa, and 2 cycles of 5 min) because the total amount of extracted PSC was higher than those using sonication (water 100ºC 30 min). But, no optimization of the extraction process was carried out and no more detailed information was given about the obtained yield. Moreover, the filtrate obtained from the culture broth where *L. edodes* mycelium was growing was also submitted to PWE in order to isolate bioactive fractions.[20] However, precipitation of the polysaccharides from the filtrates with ethanol was carried out before PWE was applied thus in this case, PWE was not used for extraction but to further fractionate isolated polysaccharides obtained by a standard method therefore, no previous information on the yields of fungal polysaccharide extractions by PWE was found in the literature. But, PWE of β-glucans from barley using temperatures

from 135 – 180ºC (15 – 75 min total extraction times) generated fractions with 23 – 54 % β-glucans being the optimal condition at 157.5ºC (45 min).[28] These values indicated that the yields obtained for fungal polysaccharides at 150ºC were similar to those obtained from barley. However, if higher temperatures were utilized, barley β-glucans were partially hydrolyzed therefore, the presence of hydrolyzed β-glucans in the obtained PWE extracts and the presence of other compounds was determined.

Composition of the PWE extracts

As expected, the obtained PWE extracts showed higher amounts of proteins and peptides in the fractions obtained at temperatures below 100ºC and higher PSC concentrations above that temperature. However, a different distribution was observed depending on the mushroom specie considered (Figure 2). The PWE extracts obtained from *L. edodes* and *A. bisporus* at 50ºC showed the largest protein concentrations while more proteins were extracted from *P. ostreatus* with only 25ºC. The fraction of the latter mushroom obtained at 200ºC also showed high protein levels. Since proteins precipitate at temperatures close to 100ºC these values might be due to proteins or fragments tightly bound to PSCs forming proteoglucans, glucoproteins or other protein-glucan complexes.

A. bisporus PWE extracts contained less PSCs and β-glucans than *L. edodes* and *P. ostreatus* (Fig. 2b) probably because this mushroom contained significantly lower levels of these compounds (respect. 12.30 mg β-glucans/100 mg *A. bisporus*, 38.18 mg/100 mg *L. edodes* and 41.79 mg/100 mg *P. ostreatus* mushroom powders dw).

L. edodes PWE extractions carried out at low temperatures (Fig. 2a) yielded larger amounts of hydrolyzed β-glucans (or β-glucan oligomers, with monomers number (n) between 2-10, molecular weight < 3.5 kD) than the other two mushroom species. Their concentration was increasing with the temperature up to 100ºC, above that temperature β-glucans of higher

molecular weight were extracted. At 200ºC other polysaccharides beside β-glucans were also extracted since the total PSC concentration was significantly higher than the amount of higher molecular weight β-glucans (> 3.5 kD, β-glucan polymers). The distinction between β-glucan oligomers and polymers could be calculated because the β-glucan levels were determined in the complete PWE extracts and in the separated (though a dialysis membrane with a MWCO 3.5 kD) crude PSC fractions obtained from them.

The *A. bisporus* and *P. ostreatus* PWE extracts obtained at temperatures higher than 100ºC showed β-glucan oligomer concentrations that were absent at lower temperatures suggesting that in these cases, degradation of the β-glucan polymers might have occurred probably as effect of the pressure and temperature as occurred with barley β-glucans.[28]

The *P. ostreatus* extracts obtained at 200ºC contained other PSC that were not β-glucans (Fig. 2c) as it was previously observed for *L. edodes.* Thus, a more detailed study on the type of polysaccharides present in the PWE extracts was carried out.

Polysaccharides in the crude PSC fractions

The composition of the crude PSC fractions (>3.5 kD) obtained from PWE extracts was compared with the crude fraction obtained from a standard PSC extraction method and results indicated that they contained different type of polysaccharides (Figure 3).

In order to distinguish between β-glucans derived from chitin hydrolysis (chitooligosaccharides) and other type of β-glucans, the N-glucosamine concentration was determined in the fractions. Chitins levels in the selected mushrooms were 4.6, 4.9 and 3.3 mg/100 mg for respectively *L. edodes*, *A. bisporus* and *P. ostreatus* mushrooms so these values were in concordance with previous determinations (Vetter 2007)[24]. However, these molecules cannot be present in the PWE fractions because they are not soluble in water and they request heavy extraction methods to solubilize them.[29] Thus, the N-glucosamine levels

measured in the PWE fractions corresponded to the so called chitooligosaccharides or LMWC (low molecular weight chitin derivatives obtained after its hydrolysis) which were pointed as water soluble compounds with therapeutic properties.[9]

The amount of chitooligosaccharides in the crude PSC fractions obtained from the PWE extracts of *L. edodes* (Fig 3a) was increasing with the temperature up to 150ºC following a similar tendency than the β-glucan oligomers determined in the same PWE extracts (Fig 2a), suggesting that these fractions might be rich in monomers and oligomers considered as immunomodulators, with antimicrobial, antitumor activities etc.[9] Indeed, the crude PSC fraction from the PWE extract obtained at 50ºC showed very interesting immunomodulatory activities in macrophages that were lacking in the fraction obtained at 200ºC.[30] The reason could be because in the latter fraction, the major PSCs were other β-glucans rather than those chitooligosaccharides (Fig 3a). Most of these β-glucans and a few α−glucans (glycogen and starch) were also present in the crude fraction obtained using the standard PSC extraction method. However, besides these glucans, the fraction obtained from the PWE extract at 200ºC contained other polysaccharides probably with different structures since they were only detected by the total PSCs determination test, thus, they might be heteroglucans, proteoglucans or others with specific configurations.[11] These other polysaccharides were extracted in larger concentrations with the increase of temperature and they were the responsible for the higher PSC extraction yield obtained in the PWE extracts at 200ºC (74.2%) compared to the one obtained using the standard method (39%).

The PSC composition of the crude fractions obtained from PWE extracts isolated from the other two mushroom species showed higher amount of chitooligosaccharides than the fractions obtained by the standard PSC extraction method but similar content of other β-glucans and α−glucans (Fig. 3b and c). The main difference between these mushrooms was that *P. ostreatus* contained larger amounts of other polysaccharides while in *A. bisporus*

most of the extracted PSC were β-glucans as in the fraction obtained using the standard PSC extraction method. Since the protein concentration in the *P. ostreatus* PWE extract at 200ºC was so high (Fig 2c) these other polysaccharides might be proteoglucans or other protein-polysaccharide complexes.

Bile acid-binding capacity by crude PSC fractions

Direct binding or scavenging (because of their gel forming properties) of bile acids during digestion have been postulated as the main mechanisms of action for respectively the water-insoluble and water-soluble cereal β-glucans to reduce cholesterol from serum[3] and even some reports indicate that the cholesterol-reducing effect of water-insoluble fraction is rather low compared to the water-soluble polysaccharides.[3] However, since the crude PSC fractions obtained from both the PWE extracts and the fractions obtained using the standard PSC extraction method contained both water-soluble and –insoluble material (depending on the water temperature utilized for their extractions), the fractions were submitted to an *in vitro* digestion model and their bile acid-binding capacity determined by quantifying the bile acids which were not retained by the PSC fractions independently whether this retention was by binding or scavenging of the bile acids. Thus, the PSCs effect will be referred as their ¨bile-acid binding activity¨ since some studies also suggested that direct binding forces between soluble polysaccharides and bile acids might also take place.[3,31]

The crude PSC fractions were digested in the presence of bile extracts in ratios 1:10 (bile extract: PSC extract), 1:30, 1:50 1:75 and 1:100 but, although a slight reduction of GUDCA, GHDCA, TCDCA and THDCA were observed compared to the control digestion with increasing concentrations of the cereal and mushroom PSCs, they were only significant when the ratio was 1:100. Digestion of cellulose showed no reduction at any of

the selected concentrations while the positive control, cholestyramine, showed a remarkable binding capacity already at ratios 1:10 (w/w).

Thus, the crude PSC fraction obtained from the cereals mixture (as positive control) and applied at ratio 1:100 was able to bind almost 40% of the four major bile acids showing no preference or binding affinity for any of the specific bile acids since their concentrations were almost equally reduced. The crude PSC fractions isolated from the PWE extracts obtained using temperatures lower than 200ºC showed none or insignificant bile acid-binding capacity however, those obtained from the PWE extracts at 200ºC of *A. bisporus* and *L. edodes* showed similar binding capacity than the cereal. *P. ostreatus* showed lower binding capacity (23%) although still significantly higher than the negative control (Figure 4). When the crude PSC fractions obtained by the standard PSC extraction method were similarly digested (ratio 1:100) no significant differences were observed respect to their corresponding PWE extracts except for *L. edodes*. A slight reduction on the binding capacity of *L. edodes* and *A. bisporus* extracts (std. method) respect to the cereal fraction could be noticed.

Chitin and their chitooligosaccharides enhanced excretion of triglycerides in feces but showed no cholesterol-lowering properties[9] thus, the fact that the crude PSC fractions from the PWE extracts obtained at 25 – 150ºC showed no bile acid-binding properties was not surprising since most of the β-glucans present in these fractions were the water-soluble chitooligosaccharides deriving from chitin hydrolysis. Only chitosan (a de-N-acetylated derivative of chitin) and its generated chitooligosaccharides, showed hypocholesterolemic action[32] however, no acid/alkaline treatments were given to the PWE extracts to induce chitin deacetylation or transformation into chitosan hydrolysis products and therefore the chitooligosaccharides detected were probably deriving only from chitin. Moreover, the fractions obtained from PWE extracts at 200ºC from *L. edodes* and *P. ostreatus* showing

bile acid-binding activities contained very low chitooligosaccharides levels pointing to the rest of polysaccharides as the responsible for the observed activities. Within these other polysaccharides, the fraction from *P. ostreatus* PWE extract at 200ºC showed similar concentration of `non-chitooligosaccharides or -chitins´(or deaminated β-glucans, named in the figures as ¨other β-glucans¨) than the fraction obtained using the standard PSC extraction method and higher amount of other polysaccharides (tentatively proteo-glucans) but both fractions showed the same bile acid-binding capacity pointing those deaminated β-glucans as the responsible for the activity as occurred in cereals.[28] The crude PSC fraction obtained from cereals using the standard method and used as control contained 47.9 mg β-glucans/100 mg PSC fraction, the one obtained from *L. edodes* PWE extract at 200ºC contained 41.7 mg deaminated β-glucans/100 mg and the one obtained using the standard extraction method 29.3 mg/100 mg PSC fraction. The bile acid-binding capacity of the latter fraction was lower than the PWE extract and the cereal extract suggesting again that these compounds could be the responsible for the observed activities.

However, apparently not all the β-glucans from the three species were equally efficient in the binding capacity since both *A. bisporus* extracts (obtained from the PWE and std method) contained respect. 24.6 and 19.21 mg/100 mg PSC fraction while *L. edodes* extracts almost double their β-glucan content and still both mushroom extracts showed bile acid-binding activities similar to each other and to those observed for the cereal extract.

Conclusions

It can be concluded that pressurized water extraction can be used to obtain β-glucans-rich fractions from edible mushrooms with different biological activities and compositions depending on the selected temperature. If the extraction is carried out at 200ºC (5 cycles of 5 min) the obtained fractions contain higher percentage of PSC than those usually obtained

by a standardized PSC extraction method facilitating their incorporation into food matrices for their technological manufacture (faster extraction, higher PSC concentration can be added with less extract, more homogeneous chemical composition etc.). Their bile acid-binding capacities were similar to the fractions obtained by the standard PSC extraction method and they were also in the range of the β-glucans obtained from a cereal foodstuff commercially available and claiming to be able of reducing cholesterol levels. Thus, the obtained mushrooms extracts will further be tested to define whether they can be used as bioactive ingredients to functionalize foods with hypocholesterolemic properties.

Acknowledgments

The research was supported by the European Union within the 7th framework programme (7FM-PEOPLE-2009-IIF project 251285), ALIBIRD-CM S2009/AGR-1469 regional program from the Community of Madrid (Spain) and AGL2010-21537 national R+D program from the Spanish Ministry of Science and Innovation. CTICH is also acknowledged for the cultivation and supplying of the mushrooms fruiting bodies.

Notation

GHDCA = glycohyodeoxycholic acid

GUDCA = glycoursodeoxycholic acid

LMWC = low molecular weight chitin derivatives or chitooligosaccharides

MWCO = molecular weight cut off

PSC = polysaccharides

PWE =pressurized water extraction

TCDCA = taurochenodeoxycholic acid

THDCA = taurohyodeoxycholic acid

Literature cited

1. Schepetkin IA, Quinn, MT. Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *Int Immunopharmacol.* 2006;*6*:317-333.
2. Drozdowski LA, Reimer RA, Temelli F, Bell RC, Vasanthan T, Thomson ABR. β-Glucan extracts inhibit the in vitro intestinal uptake of long-chain fatty acids and cholesterol and down-regulate genes involved in lipogenesis and lipid transport in rats. *J Nutr Biochem.* 2010;21:695-701.
3. Zacherl C, Eisner P, Engel, KH. *In vitro* model to correlate viscosity and bile acid-binding capacity of digested water-soluble and insoluble dietary fibres. *Food Chem.* 2011;126:423-428.
4. Kahlon TS, Chapman MH, Smith GE. *In vitro* binding of bile acids by okra, beets, asparagus, eggplant, turnips, green beans, carrots, and cauliflower. *Food Chem.* 2007;103:676-680.
5. Chen ZY, Ma KY, Liang Y, Peng C, Zuo Y. Role and classification of cholesterol-lowering functional foods. *J FuncFoods.* 2011;3:61-69.
6. Roupas P, Keogh J, Noakes M, Margetts C, Taylor, P. The role of edible mushrooms in health: Evaluation of the evidence. *J Func Foods.* 2012;4:687-709.
7. Chen J, Seviour R. Medicinal importance of fungal β-(1→3), (1→6)-glucans. *MycolRes.* 2007;*111:*635-652.
8. Rop O, Mlcek J, Jurikova T. Beta-glucans in higher fungi and their health effects. *Nutr rev*. 2009;67:624-631.
9. Prashanth KVH, Tharanathan RN. Chitin/chitosan: modifications and their unlimited application potential – an overview. *Trends Food Sci Tech*. 2007;18:117-131.
10. Aida FMNA, Shuhaimi M, Yazid M, Maaruf AG. Mushroom as a potential source of prebiotics: a review. *Trends Food Sci Tech*. 2009;20:567-575.

11. Synytsya A, Novak M. Structural diversity of fungal glucans. *Carbohyd Polym*. 2013;92:792-809.
12. Ramberg JE. Nelson ED, Sinnott RA. Immunomodulatory dietary polysaccharides: a systematic review of the literature. *Nutr J*. 2010;9:54-76.
13. Zhang M, Cui SW, Cheung PCK, Wang Q. Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and activity. *Trends Food Sci Tech*. 2007;18:4-19.
14. Cheung YC, Siu KC, Liu YS, Wu JY. Molecular properties and antioxidant activities of polysaccharide–protein complexes from selected mushrooms by ultrasound-assisted extraction. *Process Biochem.* 2012;47:892-895.
15. Che W, Wang WP, Zhang HS, Huang Q. Optimization of ultrasonic-assisted extraction of water-soluble polysaccharides from *Boletus edulis* mycelia using response surface methodology. *Carbohyd Polym.* 2012;87:614-619.
16. Zeng WC, Zhang Z, Gao H, Jia LR, Chen WY. Characterization of antioxidant polysaccharides from *Auricularia auricular* using microwave-assisted extraction. *Carbohyd Polym*. 2012;89:694-700.
17. Huang SQ, Ning ZX. Extraction of polysaccharide from *Ganoderma lucidum* and its immune enhancement activity. *Int J BiolMacromol*. 2010;47:336-341.
18. Wang BJ, Won SJ, Yu ZR, Su CL. Free radical scavenging and apoptotic effects of *Cordyceps sinensis* fractionated by supercritical carbon dioxide. *Food Chem Toxicol.* 2005;43:543-552.
19. Di X, Chan KKC, Leung HW, Huie CW. Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography. *J Chromatogr A*. 2003;1018:85-95.

20. Lo TCT, Tsao HH, Wang AY, Chang, CA. Pressurized water extraction of polysaccharides as secondary metabolites from *Lentinula edodes*. *Journal of Agr Food Chem.* 2007;55: 4196-4201.
21. Ramírez-Anguiano AC, Santoyo S, Reglero G, Soler-Rivas C. Radical scavenging activities, endogenous oxidative enzymes and total phenols in edible mushrooms commonly consumed in Europe. *J Sci Food Agr.* 2007;87:2272-2278.
22. Jeurink PV, Noguera CL, Savelkoul HFJ, Wichers HJ. Immunomodulatory capacity of fungal proteins on the cytokine production of human peripheral blood mononuclear cells. *Int Immunopharmacol*. 2008;8:1124-1133.
23. Fox JD, Robyt JF. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal Biochem.* 1991:195: 93-96.
24. Vetter J. Chitin content of cultivated mushrooms *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*. *Food Chem.* 2007;102:6-9.
25. Smith RL, Gilkerson E. Quantitation of glycosaminoglycan hexosamine using 3-methyl-2-benzothiazolone hydrazone hydrochloride. *Anal Biochem.* 1979;98:478-480.
26. Pang CP, Mok SD, Lam PK, Varma J, Li AKC. Simultaneous determination of conjugated bile acids in human bile. *Clin Biochem* .1990;23:85-90.
27. Qiao X, Ye M, Pan DL, Miao WJ, Xiang C, Han J. Differentiation of various traditional Chinese medicines derived from animal bile and gallstone: simultaneous determination of bile acids by liquid chromatography coupled with triple quadrupole mass spectrometry. *J Chromatogr A.* 2011;1218:107-117.
28. Benito-Román O, Alonso E, Cocero MJ. Pressurized hot water extraction of β-glucans from waxy barley. *J Supercrit Fluid.* 2013;73:120-125.

29. Jeon YJ, Shahidi F, Kim SK. Preparation of chitin and chitosan oligomers and their applications in physiological functional foods. Food Reviews International. 2000;16:159-176.
30. Gil-Ramírez A, López de las Hazas MC, Soler-Rivas C, Ruiz-Rodriguez A, G. Reglero G, S. Santoyo S. Immunomodulatory Properties of edible mushroom extracts obtained by pressurized solvent technologies. *Proc 6th Int Symposium on High Pressure Processes Technol* 2013;
31. Kritchevsky D. *In vitro* binding properties of dietary fibre. *Eur J Clin Nutr.* 1995;49:113-115.
32. Sugano M, Watanabe S, Kishi A, Izume M and Ohtakara A. Hypocholesterolemic action of chitosan with different viscosity in rats. Lipids 1988; 23:187-191.

Table 1. Extraction yields (total dry matter extracted in the PWE extracts and in the crude PSC fractions isolated from those PWE extracts) and PSC concentrations obtained after submitting *Lentinula edodes* powder to pressurized water extractions at different temperatures.

Extraction temperature (°C)	PWE extract (mg/g mushroom dw)	Extraction yield (%)	Crude PSC fraction obtained in PWE extracts (mg/g mushroom)	Extraction yield (%)	Crude PSC fraction in PWE extracts (%)	Total PSCs obtained in PWE extracts (mg/g mushroom)	Extraction yield (%)	Total PSCs in the crude PSC fraction (%)	Total PSCs in PWE extract (%)
25	201±35[a]	20.1	48.0±20.4[a]	4.8	23.9	15.1±1.2[a]	1.51	31.5	7.5
50	291±48[a]	29.1	42.6±19.0[a]	4.3	14.7	12.1±1.8[a]	1.21	28.5	4.2
100	248±92[a]	24.8	44.0±14.2[a]	4.4	17.7	19.6±4.1[a]	1.96	44.6	7.9
150	301±48[a]	30.1	85.7±13.7[b]	8.6	28.5	47.1±17.8[b]	4.71	54.9	15.6
200	823±34[b]	82.3	282.7±73.8c[c]	28.3	34.4	209.7±28.0[c]	21.0	74.2	25.5
Std PSC ext. method			485.5±72.8[d]	48.6		189.2±27.5[c]	18.9	39.0	

[a,b,c,d] Denotes statistically significant differences ($P < 0.05$) among values from the same column

Table 2. Extraction yields (total dry matter extracted in the PWE extracts and in the crude PSC fractions isolated from those PWE extracts) and PSC concentrations obtained after submitting *Agaricus bisporus* powder to pressurized water extractions at different temperatures.

Extraction temperature (°C)	PWE extract (mg/g mushroom dw)	Extraction yield (%)	Crude PSC fraction obtained in PWE extracts (mg/g mushroom)	Extraction yield (%)	Crude PSC fraction in PWE extracts (%)	Total PSCs obtained in PWE extracts (mg/g mushroom)	Extraction yield (%)	Total PSCs in the crude PSC fraction (%)	Total PSCs in PWE extract (%)
25	267±20.2[a]	26.7	48.5±12.0[a]	4.8	18.2	14.8±3.3[a]	1.5	30.6	5.6
50	240±12.9[a]	24.0	42.5±4.9[a]	4.3	17.7	6.9±3.3[a]	0.7	16.2	2.9
100	317±21.8[b]	31.7	36.0±11.3[a]	3.6	11.4	14.1±2.1[a]	1.4	39.1	4.4
150	597±19.0[c]	59.7	89.5±12.0[b]	9.0	15.0	30.9±3.5[b]	3.1	34.5	5.2
200	783±88.2[d]	78.3	211.0±38.2[c]	21.1	26.9	103.7±7.1[c]	10.4	49.2	13.2
Std PSC ext. method			290.0±83.4[c]	29.0		83.5±6.1[c]	8.4	28.8	

[a,b,c,d] Denotes statistically significant differences ($P < 0.05$) among values from the same column

Table 3. Extraction yields (total dry matter extracted in the PWE extracts and in the crude PSC fractions isolated from those PWE extracts) and PSC concentrations obtained after submitting *Pleurotus ostreatus* powder to pressurized water extractions at different temperatures

Extraction temperature (°C)	PWE extract (mg/g mushroom dw)	Extraction yield (%)	Crude PSC fraction obtained in PWE extracts (mg/g mushroom)	Extraction yield (%)	Crude PSC fraction in PWE extracts (%)	Total PSCs obtained in PWE extracts (mg/g mushroom)	Extraction yield (%)	Total PSCs in the crude PSC fraction (%)	Total PSCs in PWE extract (%)
25	154±26.2[a]	15.4	36.0±5.7[a]	3.6	23.4	19.9±4.4[a]	2.0	55.2	12.9
50	148±36.7[a]	14.8	48.5±6.4[a]	4.9	32.8	28.9±8.7[a]	2.9	59.6	19.5
100	198±31.8[a]	19.8	58.0±17.0[a]	5.8	29.3	40.5±12.3[a]	4.1	70.0	20.5
150	293±87.0[a]	29.3	75.0±26.9[a]	7.5	25.6	43.5±9.3[a]	4.4	58.0	14.8
200	786±272.2[b]	78.6	187.5±78.5[b]	18.8	23.9	153.0±30.0[b]	15.3	81.6	19.5
Std PSC ext. method			494.0±28.3[c]	49.4		209.6±58.7[b]	21.0	42.4	

[a,b,c] Denotes statistically significant differences ($P < 0.05$) among values from the same column

Figure Captions

Fig. 1. Extraction yields (PWE extracts and crude PSC fractions) and PSC concentration obtained after a PWE sequential extraction of *Lentinula edodes* (5 cycles, 5 minutes). 1st ext: extraction at 25ºC; 2nd ext: extraction at 50ºC; 3rd ext: extraction at 100ºC; 4th ext: extraction at 150ºC and 5th ext: extraction at 200ºC.

Fig. 2: Proteins (and peptides), polysaccharides and β-glucans (oligomers and polymers) of the PWE extracts obtained at different temperatures (5 cycles of 5 min) from a) *Lentinula edodes* b) *Agaricus bisporus* and c) *Pleurotus ostreatus.*

Fig. 3: Polysaccharides distribution within the crude PSC fractions obtained after PWE at different temperatures (5 cycles of 5 min) and after extraction using a standard PSC extraction method from a) *Lentinula edodes* b) *Agaricus bisporus* and c) *Pleurotus ostreatus.*

Fig. 4: Bile acid binding capacity of cellulose, cholestyramine and crude PSC fractions obtained from a cereals mixture and mushrooms by a standard PSC extraction method and by PWE at 200ºC after an *in vitro* digestion model (mixed in a ratio 1:100 bile extract: PSC fractions except for cholestyramine which was 1:10). * Denotes statistically significant differences ($P < 0.05$) between samples extracted by PWE and the standard PSC extraction method

Fig. 1

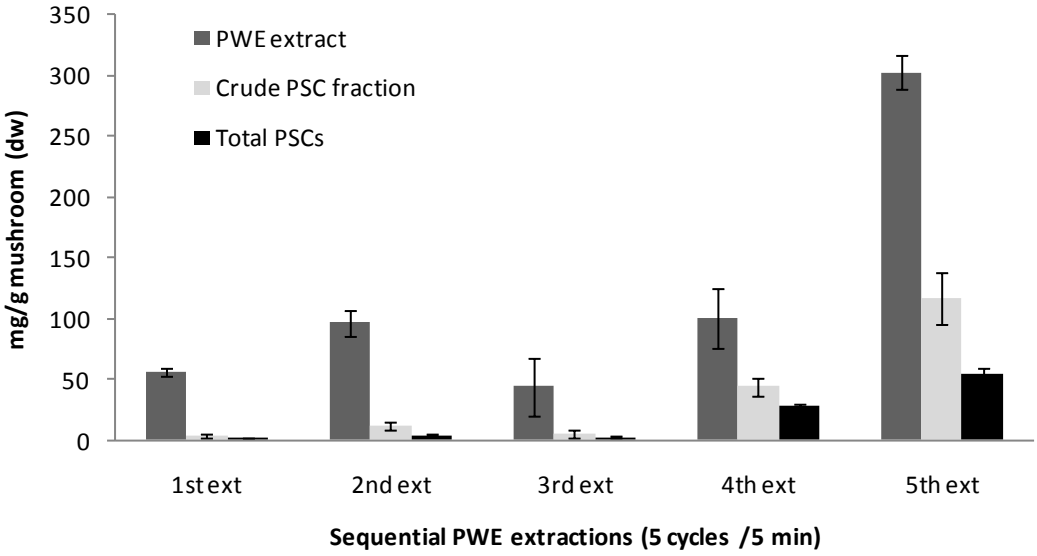
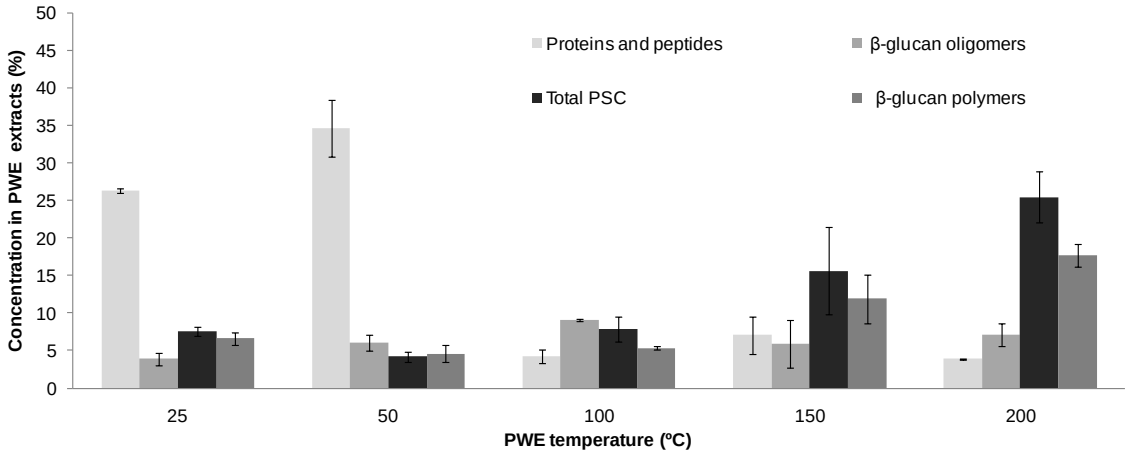
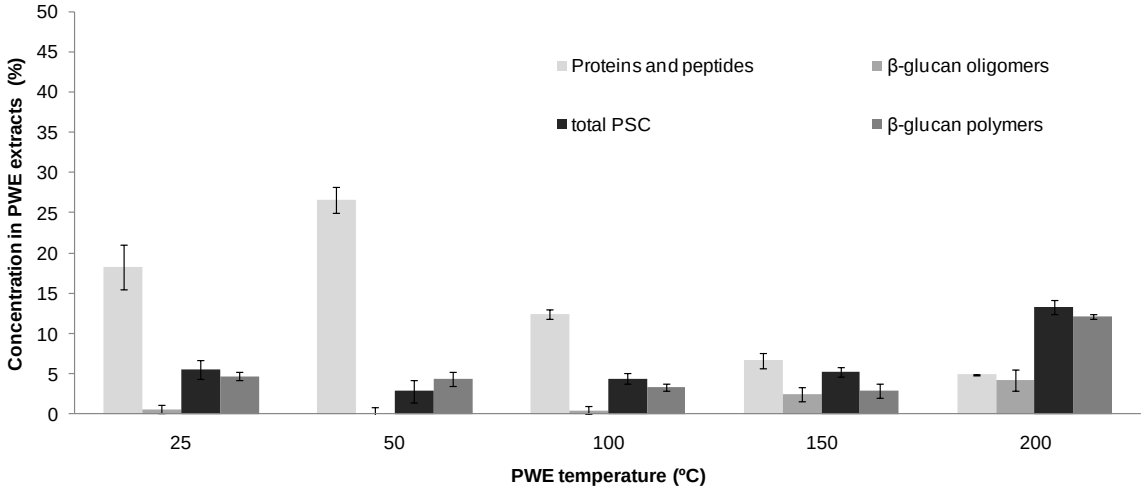

350
300
250
200
150
100
50
0
mg/g mushroom (dw)
PWE extract
Crude PSC fraction
Total PSCs
1st ext
2nd ext
3rd ext
4th ext
5th ext
Sequential PWE extractions (5 cycles /5 min)

Fig. 2:

a)


Proteins and peptides
β-glucan oligomers
Total PSC
β-glucan polymers
Concentration in PWE extracts (%)
PWE temperature (ºC)
25
50
100
150
200

b)


Proteins and peptides
β-glucan oligomers
total PSC
β-glucan polymers
Concentration in PWE extracts (%)
PWE temperature (ºC)
25
50
100
150
200

c)

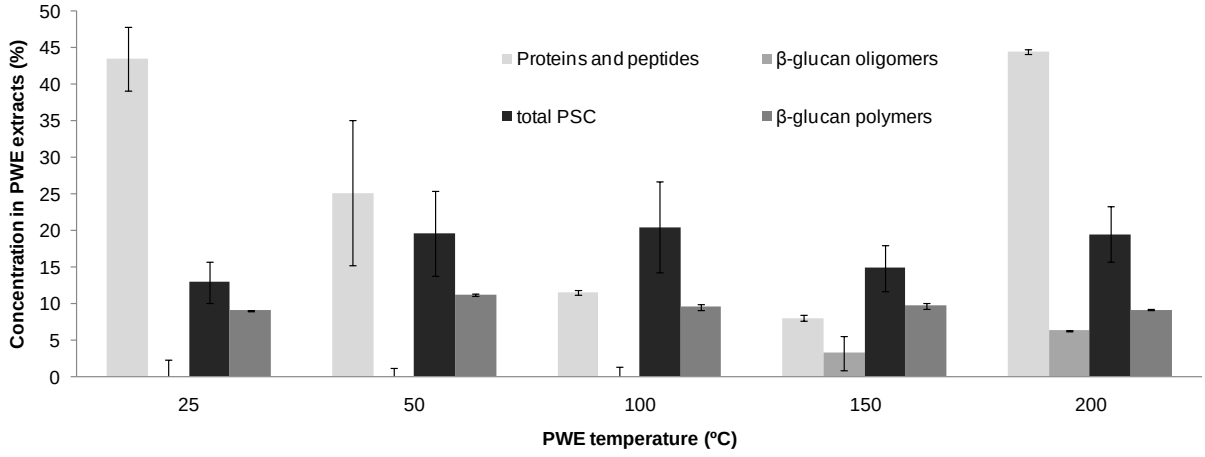
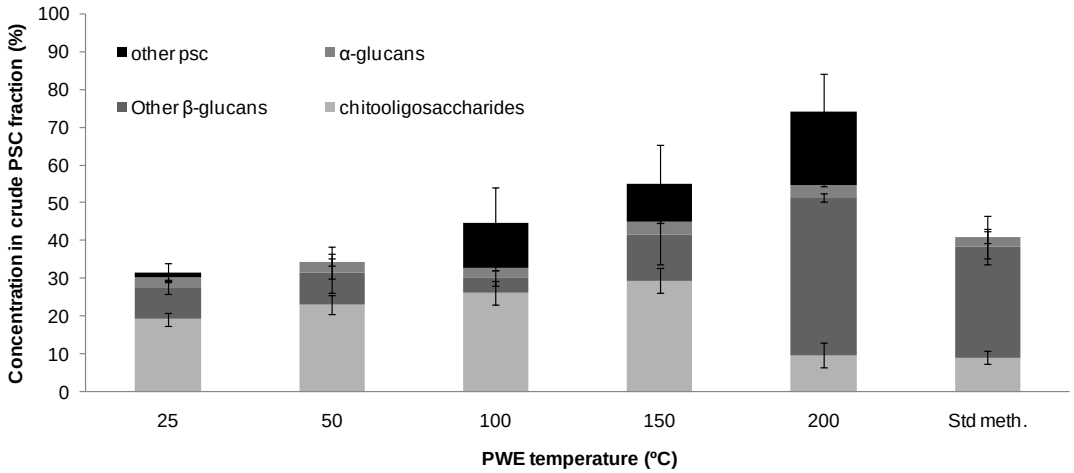
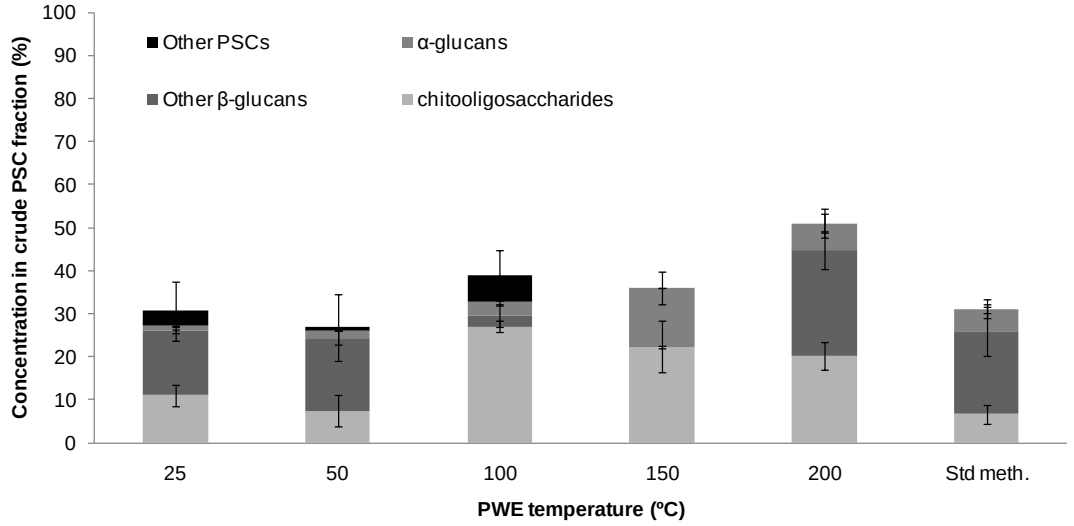

Proteins and peptides
β-glucan oligomers
total PSC
β-glucan polymers
Concentration in PWE extracts (%)
PWE temperature (ºC)
25
50
100
150
200

Fig. 3:

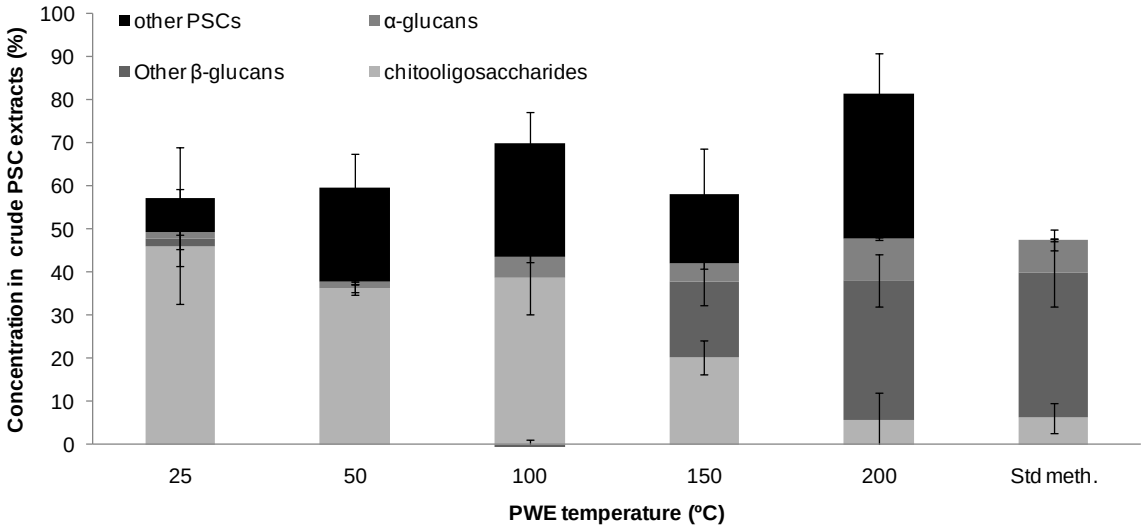
a)


other psc
α-glucans
Other β-glucans
chitooligosaccharides
Concentration in crude PSC fraction (%)
PWE temperature (ºC)
25
50
100
150
200
Std meth.

b)

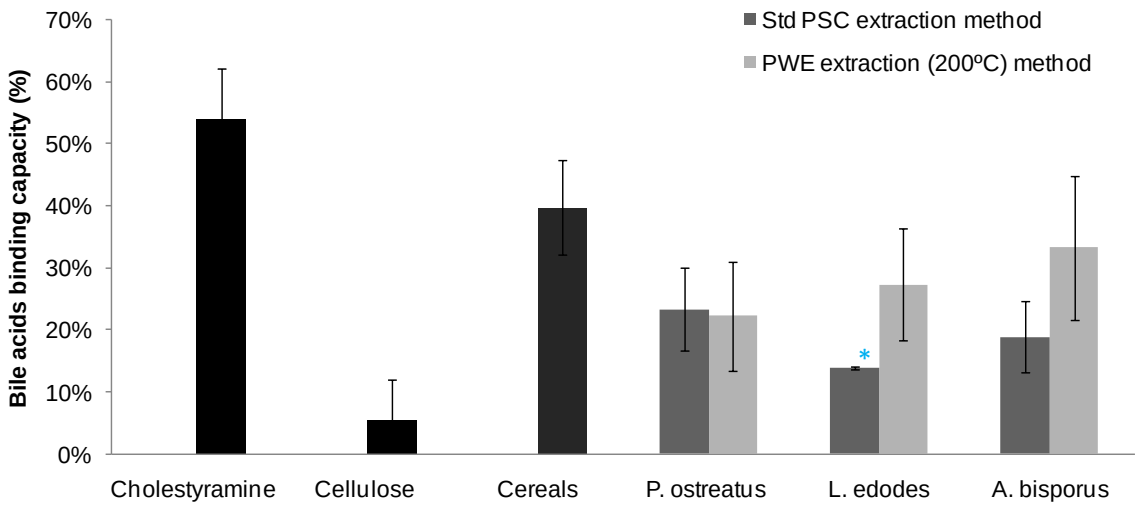

Other PSCs
α-glucans
Other β-glucans
chitooligosaccharides
Concentration in crude PSC fraction (%)
PWE temperature (ºC)
25
50
100
150
200
Std meth.

c)


other PSCs
α-glucans
Other β-glucans
chitooligosaccharides
Concentration in crude PSC extracts (%)
PWE temperature (ºC)
25
50
100
150
200
Std meth.

45

46 **Fig. 4:**



47
48