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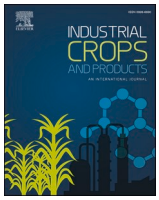

Comparative extraction of bioactive compounds from spent mushroom substrates of *Lentinula edodes* and *Pleurotus ostreatus* using subcritical water and pressurized ethanol

Afrina Akter [a,1], Sarah J. Klausen [a,1], Luis A. Romero-Soto [a,b], Francisco Díaz [c], Herminia Domínguez [c], Shaojun Xiong [d], Knut O. Strætkvern [a], Carlos Martín [a,e,*]

[a] Department of Biotechnology, University of Inland Norway, Hamar N-2317, Norway
[b] Facultad de Ingeniería y Arquitectura, Universidad Privada Boliviana, La Paz, Bolivia
[c] Department of Chemical Engineering, University of Vigo (Campus Ourense), Ourense 32004, Spain
[d] Department of Forest Materials and Technology, Swedish University of Agricultural Sciences, Uppsala SE-901 83, Sweden
[e] Department of Chemistry, Umeå University, Umeå SE-901 87, Sweden

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ABSTRACT

This study aimed to assess subcritical water extraction (SWE) and pressurized ethanol extraction (PEE) for recovering bioactive compounds from spent mushroom substrate (SMS) derived from shiitake and oyster mushrooms. The extraction of bioactive compounds can facilitate the valorisation of SMS, an increasingly important waste stream whose management and disposal pose challenges for both mushroom producers and the environment. A 3^2 experimental design, using three temperatures (125, 150, and 175 °C) and three holding times (0, 15, and 30 min) was used in SWE. PEE was run at a single temperature (175 °C) and two holding times (0 and 15 min). The experimental results revealed that the highest extraction yield was achieved in shiitake SMS using SWE at 175 °C and 15 min. In most SWE conditions, shiitake SMS yielded higher concentrations of total phenolic compounds, total carbohydrates, and β-glucans compared to oyster mushroom SMS. Ascorbic acid, vanillic acid, protocatechuic acid, and gallic acid were the most abundant phenolic acids identified in both extracts. The SWE extracts of shiitake SMS exhibited higher DPPH and FRAP antioxidant activity than those of oyster mushroom SMS, and this activity increased with increasing temperature and time. PEE extracts exhibited higher DPPH and TEAC antioxidant activity for oyster mushroom SMS compared to shiitake SMS and showed a negative trend with increasing extraction time at 175 °C for both SMS. Our results show that SWE and PEE are viable methods for extracting bioactive compounds from SMS.

1. Introduction

Mushrooms have become an increasingly popular food choice due to their rich nutrient content and health-promoting bioactive compounds (El-Ramady et al., 2022). In 2013, the total global mushroom production was 34 million tons, with the genera *Lentinula* and *Pleurotus* counted among the three most cultivated edible mushrooms worldwide (Royse et al., 2017). For every kg of mushrooms harvested, 4–5 kg of spent mushroom substrate (SMS) is generated as a by-product (Umor et al., 2021). This by-product consists of lignocellulosic material that has not been fully broken down during fungal growth, along with mycelium that has colonized the substrate. As the mushroom industry increases, the SMS generation also increases, resulting in substantial amounts of waste residues (Atallah et al., 2021). Although SMS still contains valuable components of exploitation interest, it is currently regarded as waste and is typically disposed of through landfilling, composting, or incineration. These disposal methods cause unwanted environmental issues, such as foul odours, greenhouse gases, and leachate contamination of groundwater, and leave a potential biorefinery feedstock unused (Martín et al., 2023). Valorising SMS is therefore crucial to both utilising this bioresource and creating a sustainable, circular economy for the mushroom industry.

It has been shown that bioactive compounds extracted from the fruiting bodies of shiitake (*Lentinula edodes* P.) and oyster mushrooms

* Corresponding author at: Department of Biotechnology, University of Inland Norway, Hamar N-2317, Norway.
E-mail address: carlos.medina@inn.no (C. Martín).
[1] A.A. and K.S.J share the first authorship.

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(*Pleurotus ostreatus* K. and other *Pleurotus spp*.) exhibit antioxidant, antimicrobial, immunomodulatory, antitumor, hypoglycemic, anti-inflammatory and prebiotic effects (Venturella et al., 2021). Some of the bioactive molecules typical of mushrooms can be found in the SMS as a consequence of secretion during fungal growth or as constituents of the mycelium (Lin et al., 2013). Furthermore, the SMS can contain bioactive compounds of lignocellulosic origin, including phytochemicals derived from the substrate and oligomers formed during the degradation of polysaccharides and lignin (Petraglia et al., 2024). As the SMS is rich in bioactive and beneficial substances, such as β-glucans, phenolics, sterols, and vitamins, and it also contains cellulose and hemicelluloses, it is a valuable source of bioactive compounds (Qin et al., 2023) that can be extracted, and sugars than can be released by saccharification and used in microbial fermentations (Klausen et al., 2023). Therefore, SMS is a potential biorefinery feedstock, allowing for several valorisation routes (Martín et al., 2024).

The extraction of bioactive compounds from plant and waste sources has gained increased interest over the years, as it can provide valuable substances to the food, pharmaceutical, and biomedical industries. An extraction technique that has attracted considerable attention is subcritical water extraction (SWE), also referred to as superheated water or pressurized hot water extraction (Aminzai et al., 2024). Subcritical water extraction refers to extractions using water as a solvent at temperatures above the boiling point (100 °C) but below the critical point (374 °C), with pressure up to 22.1 MPa to maintain the solvent in a liquid state during extraction (Aminzai et al., 2024). SWE offers several advantages over conventional extraction techniques, such as Soxhlet, maceration, and percolation, by greatly reducing extraction time and energy cost and by utilising water instead of toxic or expensive solvents. Pressurized liquid extraction (PLE) employing aqueous solutions of solvents instead of pure water offers similar benefits as SWE (Wijngaard et al., 2012). While the extraction of bioactive compounds from mushroom fruitbodies is well studied, their recovery from SMS remains a relatively novel area. Although some non-conventional methods—such as ultrasound-assisted extraction, microwave-assisted extraction, accelerated solvent extraction, and supercritical fluid extraction—have been reported for extracting bioactive substances from the residues of various edible fungi (Qin et al., 2023), no previous studies have reported the use of subcritical water extraction or pressurized liquid extraction for recovering bioactive compounds from SMS from *P. ostreatus* and *L. edodes*. The present study aims to address the existing knowledge gap.

This group previously investigated the extraction of bioactive compounds from *P. ostreatus* SMS using various conventional and non-conventional extraction techniques (Klausen et al., 2023). It was found that SWE holds great potential for extracting bioactive compounds with antioxidant activity from *P. ostreatus* SMS. Additionally, Soxhlet extraction using a 40:60 ethanol:water solution as the solvent resulted in a high extraction yield.

As a continuation of our previous work, the present study evaluates subcritical water extraction and pressurized ethanol extraction for recovering bioactive compounds with antioxidant activity from the spent substrate of oyster and shiitake mushrooms using water and aqueous ethanol as solvents.

2. Materials and methods

2.1. Materials

The mushroom farm Husbonden AS (Disenå, Norway) supplied the oyster mushroom spent substrate, which was derived from the culture of a wild strain of *P. ostreatus* isolated in Norway. The cultivation was performed using Husbonden's proprietary method on an initial substrate consisting of approximately 60 % water, 0.08 % $CaCO_3$, 8 % wheat bran (*Triticum aestivum*), and 32 % oak sawdust (*Quercus robur*).

SMS from shiitake mushroom was supplied by the Swedish University of Agricultural Sciences (Umeå, Sweden). Shiitake cultivation was performed as previously described on an initial substrate consisting of 60 % water and 40 % dry plant biomass (Chen et al., 2022a). The plant biomass material was birch sawdust (80 %) and wheat bran (20 %).

The SMS samples were placed in a ventilation hood and dried at room temperature for 4–5 days until the dry matter (DM) content exceeded 90 % (w/w). After that, they were shredded using a Robot Coupe R3 cutter with serrated knives (SNC, Montceau-Bourgogne, France) to reduce the particle size according to the requirements of further experiments. The resulting material was sieved into different particle-size fractions. The fraction with a size below 1 mm was used in the characterisation, while the fraction with a size between 1 and 2 mm was used in the extraction experiments. Until further use, the material was kept at room temperature in ziplock plastic bags.

All the chemicals were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used in the experiments as received unless otherwise stated.

2.2. Characterization of the SMS

The content of dry matter, mineral components, extractive compounds, structural polysaccharides, and lignin was determined in both raw and extract-free SMS using standard methods. Each analysis was carried out in triplicates, and the mean values and standard deviations were reported.

Dry matter was determined gravimetrically using a moisture analyzer (Mettler-Toledo GmbH, China). The mineral content was determined as ash following the methodology established by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008a). Two grams (dry weight (DW)) of the material was incinerated in tared porcelain crucibles at 575 °C for 5 h in a Carbolite CWF 1100 muffle furnace (Carbolite Gero, Sheffield, UK).

The content of extractive compounds was determined using a sequential Soxhlet extraction method with distilled water and 96 % ethanol as solvents, following the NREL standard methodology (Sluiter et al., 2005). Ten grams of dry weight (DW) biomass and 250 mL of solvent, with a solvent-to-biomass ratio (SBR) of 25:1 (v/w), were used in the extraction.

Water extraction was conducted for 13 h, whereas ethanol extraction was conducted for 6 h until no more colour was seen in the extract in the extraction chamber. Following the extraction process, the volume of the extract was measured using a volumetric cylinder. After that, 10-mL samples were collected in Falcon tubes and stored frozen until further analyses. The extract-free solid residue was air-dried at room temperature until constant weight. To quantify the content of extractives, 1-mL aliquots of the extracts were evaporated overnight at 105 °C in a Termaks TS4057 oven (Bergen, Norway), followed by weighing using an analytical balance (Sartorius). The content of extractives was determined as the mass percentage of dry extract relative to the mass of the SMS sample subjected to extraction.

The determination of structural carbohydrates and lignin was performed using analytical acid hydrolysis followed by HPLC according to a relevant protocol (Sluiter et al., 2008b). Aliquots of 300 mg of extract-free solids were suspended in 3 mL of 72 % sulfuric acid in glass tubes and hydrolyzed at 30 °C for one hour in a water bath. Next, the samples were moved to 100-mL screw-top flasks, and 84 mL of distilled water was added to decrease the H_2SO_4 concentration to 4 %. A second hydrolysis step was carried out at 121 °C for 1 h in a CV-EL 18 L autoclave (CertoClav Sterilizer GmbH, Leonding, Austria). Subsequently, the flasks containing the hydrolysates were cooled in an ice bath, and distilled water was added to restore the original weight. The hydrolysates were then separated from the solid residue by vacuum filtration using previously dried and weighed glass filters. The Klason lignin content was measured gravimetrically after the sample was dried overnight at 105 °C in an oven (Termaks). One-mL hydrolysate samples were stored frozen for further assessment by HPLC.

2.3. Subcritical water extraction

The SMS samples were subjected to extraction with subcritical water and pressurized ethanol according to the scheme presented in Fig. 1.

A suspension of 15 g (DW) of each SMS in 285 mL of distilled water was heated in a pressurized Parr 4520 reactor (Parr Instrument Company, Moline, IL, USA). A 3^2 experimental design with the temperature (125 °C, 150 °C, and 175 °C) and the holding time (0 min, 15 min, and 30 min) as independent variables was used for the extraction. The variables and their levels were chosen based on a previous study, where subcritical water extraction was applied to SMS of oyster mushrooms (Klausen et al., 2023). The selected extraction times enabled one set of experiments to be conducted under non-isothermal conditions (i.e., with no holding time at the target temperature), and two other sets under partially isothermal conditions (i.e., with isothermal periods of 15 or 30 min, in addition to the non-isothermal phases associated with heating and cooling). In general, the heating-up period lasted 10–15 min, and the cooling-down period lasted 10–15 min, depending on the working temperature (Fig. S1). The experimental run at the center-point conditions (150 °C, 15 min) was performed in triplicate.

The severity factor (SF) during extraction was calculated as the logarithm of a reaction ordinate incorporating time and temperature, as shown in the equations below, where t represents the holding time (in minutes), and Tr represents the temperature (in °C) (Ilanidis et al., 2021).

$$SF = Log\ Ro \tag{1}$$

$$Ro = t \quad \times \exp \quad \left(\frac{Tr - 100}{14.75}\right) \tag{2}$$

To facilitate the calculation, a heating profile (Fig. S1) was built by recording the temperature at five-minute intervals.

After extraction, the reaction vessel was cooled to 30 °C, and the slurry was separated by vacuum filtration. A portion of the extracts (10 mL) was transferred to 15-mL Falcon tubes, and the rest was placed in 500-mL plastic bottles. Both fractions were stored frozen. The extract-free solid residue was washed thoroughly with distilled water and dried at room temperature until constant weight. After determining the dry matter content of the extract-free solids, their content of structural carbohydrates and lignin was determined. The remaining material was stored in ziplock plastic bags for further use beyond the current study.

2.4. Pressurized ethanol extraction

Pressurized ethanol extraction (PEE) of bioactive compounds from shiitake SMS and oyster mushroom SMS was performed in a Parr reactor. For both SMS types, 15 g of material was suspended in 285 mL of a solvent mixture consisting of 40 % (v/v) ethanol and 60 % (v/v) distilled water. The extraction was conducted at 175 °C, with holding times of either 0 min or 15 min at the target temperature. The composition of the solvent mixture was based on a previous study (Klausen et al., 2023), whereas the temperature and holding time were selected according to the results of the subcritical water extraction performed in this work. The extraction operations, including the separation of the extracts and extract-free solids, were performed as described for subcritical water extraction (see Section 2.3).

2.5. Analytical methods for determining the composition of the extracts

For both subcritical water extraction (SWE) and pressurized ethanol extraction (PEE), the extraction yields were determined gravimetrically after drying 1-mL aliquots of the extracts overnight at 105 °C. Aliquots of the extracts were also used to determine the concentrations of total carbohydrates, phenolic compounds, and protein. Extraction yield and total phenolic content were analysed in triplicate, while total carbohydrate content and proteins were analysed in duplicate.

The concentration of total carbohydrates was determined spectrophotometrically using a modification of the phenol-sulfuric acid protocol (DuBois et al., 1956). Glucose was used as a calibration standard, and the absorbance was read at 490 nm using a UV-3100PC spectrophotometer (VWR, Leuven, Belgium). The concentration of total phenolics was determined with the Folin–Ciocalteau method (Sánchez-Rangel et al., 2013). The calibration standard consisted of gallic acid dissolved in a 10 % ethanol solution. The absorbance was measured at 765 nm using a UV-3100PC spectrophotometer (VWR). The results were expressed as gallic acid equivalents (GAE). Protein was determined by the Bradford method using bovine serum albumin (BSA) as a calibration standard and measuring the absorbance at 595 nm (Bradford, 1976).

Monosaccharides and oligosaccharides in the extracts were determined by HPLC (see Section 2.6). To determine the oligosaccharides, the extracts were subjected to analytical acid post-hydrolysis before HPLC analysis. Post-hydrolysis was performed with 4 % H_2SO_4 for 60 min at 121 °C in an autoclave (CertoClav).

For the determination of β-glucans, approximately 200 mL of the samples were lyophilized for 48 h in a Christ Alpha 1–4 freeze drier (Medizinischer Apparatebau, Germany). The determination of β-glucans was performed using the Megazyme Mushroom and Yeast β-Glucan assay kit (Megazyme International, Bray, Ireland) according to the manufacturer's protocol, but with freeze-dried extract instead of mushroom powder used during the determination. The determination of β-glucans was performed indirectly by first determining the total and α-glucans in the freeze-dried extracts. The content of β-glucans was calculated by subtracting the content of α-glucans from that of total glucans.

2.6. Methods for determining the antioxidant properties of the extracts

The antioxidant properties of the extracts were determined by the ferric-ion reducing antioxidant power (FRAP) method (Guo et al., 2003), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

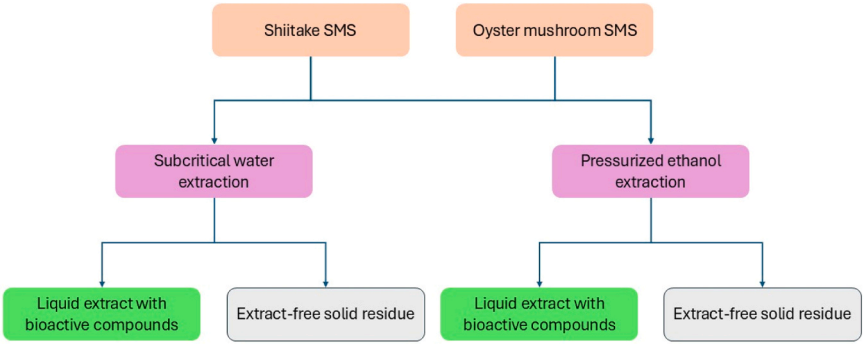

Fig. 1. Schematic layout of the study and experimental procedure followed for the extraction of bioactive compounds from SMS.

(Sharma and Bhat, 2009), and the Trolox equivalent antioxidant capacity (TEAC) (Re et al., 1999).

Fresh FRAP reagent was prepared before each use by combining 10 mM tripyridyltriazine (TPTZ) dissolved in 40 mM 37 % HCl with 20 mM $FeCl_3 \cdot 6\ H_2O$ dissolved in water and acetate buffer (pH 3.6) in a 1:1:10 ratio. The samples (30 μL) were mixed with 1350 μL of the FRAP reagent and 150 μL of distilled water, subjected to successive treatments at 37 °C, and the absorbance was measured at 595 nm in UV-3100PC spectrophotometer (VWR). The antioxidant activity was expressed as FRAP equivalent (mM Fe^{2+}), using ferrous iron (Fe^{2+}) as standard.

A 0.1 mM DPPH solution was made by diluting a 10 mM stock solution in 100 % ethanol, and the solution was protected from light. The sample (10 μL) was mixed with 500 μL of absolute ethanol and 500 μL of a 0.1 mM DPPH solution and then incubated for 30 min in darkness at room temperature. Then, the absorbance was measured using a UV-3100PC spectrophotometer (VWR) at 520 nm, with chlorogenic acid at 0.10 mg/mL in 60 % ethanol serving as the calibration standard. The antioxidant activity was expressed as a percentage of inhibition of DPPH free radicals.

A TEAC working solution was made in phosphate-buffered saline (PBS) (pH 7.4) containing ABTS and potassium persulfate. The solution was immediately wrapped in aluminium foil, shaken for 16 h, and then used for analysis or stored frozen in the dark. To measure the TEAC activity, 40 mL of PBS was mixed with 3 mL of TEAC working solution, and adjustments were made to achieve an absorbance at 734 nm between 0.70 and 0.72. Then, 10 μL of the samples were mixed with 1 mL of diluted TEAC mixture, and incubated at 30 °C for 6 min, followed by reading the absorbance at 734 nm using PBS as blank.

2.7. HPLC determination of sugars and phenolic acids

An Ultimate 3000 HPLC system (Dionex Softron, Germering, Germany) was used to quantify the sugars in the extracts, analytical acid hydrolysates, and post-hydrolysates, as well as the phenolic acids in the extracts. The sugars (xylose, glucose, arabinose) in the SMS extracts were separated using a Rezex-RPM Monosaccharide Pb^{+2} column (Phenomenex, Torrance, CA, USA) heated to 85 °C and detected with a Series 200 refractive index (RI) detector (Showa Denko). Distilled water, filtered through a 0.45-μm membrane filter (GE Healthcare, Buckinghamshire, United Kingdom), was used as eluent. An elution rate of 0.6 mL/min was applied for 40 min per sample. Sugars in the analytical acid hydrolysates and post-hydrolysates were separated using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) set at 60 °C and detected with a refractive index (RI) detector (Showa Denko). The mobile phase was 5 mM sulfuric acid (VWR Chemicals, France), eluted at a flow rate of 0.6 mL/min. The phenolic acids were separated on a Kinetex 5 μm C18 100Ax4.6 mm column (Phenomenex). Before HPLC analysis of phenolic acids, all standards were appropriately dissolved in a 50 % acetonitrile diluted mobile phase and then filtered through 0.45-μm Nylon syringe filters (VWR, Radnor, PA, USA). A UV detector (Dionex Softron) was used at 330 nm, 280 nm, and 260 nm. The mobile phase consisted of 95 % 0.1 % trifluoroacetic acid (FLUKA Chemie AG, Buchs, Switzerland) and 5 % acetonitrile (VWR Chemicals, France) eluted at a flow rate of 1.5 mL/min at 25 °C for 18 min per sample.

2.8. Statistical analysis

The statistical significance ($\alpha = 0.05$) was calculated in Microsoft Excel (Office LTSC 2021, version 2502) using one-way and two-way ANOVA with replication and two-tailed Student's t-test assuming equal variances with a hypothesized mean difference of 0. Correlations between the different response factors (extraction yield, total phenolic content, identified phenolic acids, total carbohydrate content, protein content, β-glucan content and antioxidant activity) were also determined using Microsoft Excel (version 2502), and the R^2, Pearson's correlation coefficient and *p*-value were calculated. Microsoft Excel, Statgraphics Plus 5.0 for Windows (Manugistics Inc., Rockville, MD, USA), and MODDE 11.0 (Umetrics AB, Umeå, Sweden) were used for processing the results.

2.9. Molecule drawing

Chemical structures of phenolic acids were drawn using the free online platform MolView (https://molview.org).

3. Results and discussion

3.1. Characterization of the spent mushroom substrate

A thorough characterization of the chemical composition of the spent mushroom substrates (SMS) from shiitake (Sh-SMS) and oyster mushrooms (Oy-SMS) was performed before starting the extraction experiments. Standard methods typically used for biomass analysis were applied to characterize SMS.

3.1.1. Determination of the main components of the spent mushroom substrate

The characterization of the SMS from shiitake and oyster mushrooms started by removing the extractive compounds to quantify them further. The extractive compounds were removed from the SMS by successive extractions with water and ethanol. In biomass analysis, exhaustive extraction is a mandatory operation to avoid interferences in the downstream analysis of cell-wall structural constituents (Sluiter et al., 2005), especially regarding lignin quantification (Sluiter et al., 2008b). After removing the extractives, the structural carbohydrates and lignin in the extract-free biomass were determined using a two-step analytical acid hydrolysis procedure followed by HPLC and gravimetric analyses. The results are presented in Table 1.

The compositional analysis revealed that cellulose was the main component in both SMS types (Table 1). Cellulose content was higher in Sh-SMS (34.9 ± 1.9 % (w/w)) than in Oy-SMS (28.1 ± 1.5 %), but there was little evidence for a significant difference ($p = 0.06$, Student's *t*-test). Xylan content was comparable in Sh-SMS (13.8 ± 2.5 %) and Oy-SMS (12.8 ± 0.5 %). Arabinan was the less abundant carbohydrate component, which is a consequence of its low content in hardwood (Fengel and Wegener, 1989), the main ingredient of the initial substrates for mushroom cultivation. Arabinan content was 2.6 ± 0.3 % and 1.7 ± 0.4 % for Sh-SMS and Oy-SMS, respectively.

The lignin content, measured as Klason lignin, was almost two-fold higher in Oy-SMS (18.0 ± 0.6 % (w/w)) than in Sh-SMS (10.9 ± 0.4 %), ($p < 0.04$, Student's *t*-test) (Table 1). This trend is consistent with the values previously reported in a study comparing SMS of shiitake and oyster mushroom (Klausen et al., 2025). Furthermore, the lignin content in the Oy-SMS is comparable with previously reported values for SMS of two different species of oyster mushrooms (Klausen et al., 2023).

Table 1
Content of major components in raw spent substrates from shiitake and oyster mushrooms. Values are means ± standard deviations from triplicate measurements.

Component, % (w/w)	Shiitake SMS	Oyster mushroom SMS
Cellulose[a]	34.9 ± 1.9	28.1 ± 1.5
Xylan	13.8 ± 2.5	12.8 ± 0.5
Arabinan	2.6 ± 0.3	1.7 ± 0.4
Lignin[b]	10.9 ± 0.4	18.0 ± 0.6
Water extractives	25.6 ± 0.3	14.6 ± 0.1
Ethanol extractives	1.8 ± 0.1	2.6 ± 0.7
Ash	5.1 ± < 0.1	3.5 ± <0.1

[a] Determined as glucan
[b] Determined as Klason lignin

The higher lignin content in the Oy-SMS might suggest that lignin degradation was lower during oyster mushroom cultivation than during shiitake cultivation. That can be a consequence of the different cultivation times for shiitake and oyster mushrooms. Shiitake cultivation in sawdust typically requires between 8 and 10 weeks, whereas oyster mushroom cultivation can be completed in 6–7 weeks (GroCycle, 2025). In addition to being a method for preparing biomass for structural component analysis, the extractions with water and ethanol are also an approach for quantifying non-cell wall material. The non-cell wall material is determined as extractive compounds, which include water extractives (i.e., water-soluble compounds) and ethanol extractives (i.e., ethanol-soluble compounds). The extraction results revealed that both SMS types contained a large share of non-cell wall material, as indicated by the high content of total extractives (Table 1).

The content of total extractives was higher for shiitake SMS (27.4 % (w/w)) than for oyster mushroom SMS (17.2 % (w/w)) (Table 1). For both SMS types, the content of water extractives was higher than that of ethanol extractives ($p < 0.02$, Student's *t*-test). Water extractives constituted 25.6 ± 0.3 % (w/w) in Sh-SMS, which is approximately 75 % higher compared to the content in Oy-SMS (14.6 ± 0.1 % (w/w)). This difference is significant ($p < 0.01$). The higher content of total extractives and water extractives in Sh-SMS than in Oy-SMS confirms the previously reported trend for these SMS types (Klausen et al., 2025). On the other hand, the content of ethanol extractives in Oy-SMS (2.6 ± 0.7 % (w/w)) and Sh-SMS (1.8 ± 0.1 % (w/w)) showed no significant difference. This result on ethanol extractives differs from the values and trend reported by Klausen et al. (2025), which might be attributed to the use of different strains of oyster mushrooms in both studies.

The content of mineral components, expressed as ash, was higher in Sh-SMS (5.1 % (w/w)) than in Oy-SMS (3.5 % (w/w)). The higher ash content of Sh-SMS compared to Oy-SMS is in agreement with previously reported results (Klausen et al., 2025). The different content of mineral components might be related to the use of different biomass materials in the formulation of the initial substrates used in the cultivations.

3.1.2. Investigation of the main organic functionalities in the extractive compounds

To gather more information about the possible bioactive compounds contained in the water-soluble and ethanol-soluble fractions of the extractives, a group analysis was performed to determine the total carbohydrates, total phenolic compounds, β-glucans, and proteins. The results are presented in Table 2.

The group analysis of organic functionalities revealed that total carbohydrates were the major group in both extractive fractions of both SMS types (Table 2). The overall concentration of extractable carbohydrates, i.e., the sum of total carbohydrates quantified in both water and ethanol extractives, was almost twice as high in the oyster mushroom SMS (54.8 mg/g SMS) as in the shiitake SMS (30.3 mg/g SMS). Most of the carbohydrates were found in the water-soluble fractions. The content of total carbohydrates in the water-soluble fraction of the shiitake SMS was 28.9 mg per gram of initial SMS, corresponding to 95 % of the overall extractable carbohydrate content. In the oyster mushroom SMS, the total carbohydrate content in the water-soluble fraction was 52.6 mg/g, corresponding to 96 % of the overall content. Total carbohydrates were also detected in the ethanol-soluble fraction, but their content was lower than in the water-soluble fraction. Oy-SMS showed significantly higher carbohydrate content in the ethanol extracts compared to Sh-SMS ($p < 0.001$, Student's *t*-test).

Phenolic compounds were the second most important organic functionality quantified in the extractives (Table 2). The overall content of phenolics was almost twice as high in the Sh-SMS (19.7 mg GAE/g SMS) than in the Oy-SMS (11.2 mg GAE/g SMS). As for carbohydrates, the content of total phenolic compounds was higher in the water extractives than in the ethanol extractives ($p < 0.01$, Student's *t*-test). However, the share of phenolics in the ethanol extractives was not as low as that observed for carbohydrates. In the ethanol-soluble fraction of the shiitake SMS, the content of total phenolic compounds was 2.1 ± < 0.3 mg GAE/g SMS, which is equivalent to 10 % of the overall content of phenolics. In the oyster mushroom SMS, the total phenolics in the water-soluble fraction was 3.0 mg GAE/g SMS, corresponding to 27 % of the overall content. It is noteworthy that the content of total phenolic compounds in the ethanol extracts was higher than that of total carbohydrates. Furthermore, total phenolic compounds were quantified in higher amounts in the ethanol-soluble fraction of Oy-SMS than in that of Sh-SMS ($p < 0.02$).

The content of β-glucans was only determined in water extracts, considering that the mushroom β-glucans are water-soluble. The water solubility of mushroom β-glucans is a consequence of their molecular structure, which features glucose molecules linked together by β-(1→3) glycosidic bonds with branching through β-(1→6) bonds, creating a more flexible structure that facilitates water solubility (Sun et al., 2022). The β-glucan concentration was higher in shiitake SMS (7.0 mg/ g SMS) than in oyster mushroom SMS (5.6 mg/g SMS).

The protein content in water extracts was comparable for both SMS (Table 2), but Oy-SMS showed higher protein content in the ethanol extracts than Sh-SMS ($p < 0.05$, Student's *t*-test). While the water and ethanol extracts were comparable in Oy-SMS, a significant difference was observed in Sh-SMS ($p < 0.04$).

Table 2
Chemical composition and antioxidant activity of water (WE) and ethanol (EE) extractives from spent substrates of shiitake and oyster mushrooms. Values are means ± standard deviations from triplicate measurements[a].

Content and antioxidant activity (per gram of SMS)	Shiitake SMS			Oyster mushroom SMS		
	WE	EE	Overall content	WE	EE	Overall content
Total carbohydrates, mg	28.9 ± < 0.1	1.4 ± <0.1	30.3	52.6 ± 0.3	2.2 ± <0.1	54.8
Total phenolic compounds, mg GAE	19.7 ± 0.6	2.1 ± 0.3	21.8	11.2 ± 0.1	3.0 ± 0.1	14.2
β-Glucans, mg	7.0	N.D.[b]	7.0	5.6	N.D.[b]	5.6
Proteins, mg	5.1 ± 0.6	4.0 ± 0.1	9.1	5.2 ± 0.5	4.6 ± 0.3	9.8
DPPH inhibition, %	27.1 ± 3.1	28.3 ± 1.6	-	29.8 ± 1.5	76.5 ± 0.8	-
FRAP antioxidant capacity, mM Fe^{b+} equivalents	2.6 ± < 0.1	0.2 ± < 0.1	-	1.3 ± 0.6	0.4 ± < 0.1	-

[a] β-Glucans were determined in single measurements
[b] N.D., not determined.

3.1.3. Antioxidant activity of the extractive compounds

The fruitbodies of edible and medicinal mushrooms contain bioactive compounds responsible for various functional properties (Gargano et al., 2017). Some of the bioactive molecules typical of mushrooms can be found in the SMS as a result of secretion during fungal growth or as constituents of the mycelium. Furthermore, the SMS can contain bioactive compounds of lignocellulosic origin, such as phytochemicals derived from the substrate and oligomers formed during the degradation of polysaccharides and lignin.

In a recent study by this group, antioxidant activity was detected in extracts of oyster mushroom SMS (Klausen et al., 2023). As a continuation of this research line, the antioxidant properties of extracts from shiitake and oyster mushrooms are assessed in this work. The antioxidant activity was investigated using the FRAP (ferric reducing antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays.

The water extracts of shiitake and oyster SMS showed comparable antioxidant activity as indicated by the percentage of inhibition of the DPPH radical. The DPPH inhibition was slightly higher for the water extracts of oyster mushroom SMS (29.8 % ±1.0) than for those of shiitake SMS (27.1 % ± 3.1), but the difference was not statistically

significant (Table 2). On the other hand, the percentage of inhibition of the DPPH radical observed for the ethanol extract of Oy-SMS (76.5 % ± 0.8) was almost three times higher than the value observed for Sh-SMS ethanol extract (28.3 % ± 1.6) ($p < 0.01$, Student's *t*-test). Comparing the percentage of DPPH radical inhibition for both extracts from each SMS, it is evident that for Sh-SMS, the water and ethanol extracts exhibited comparable values. In contrast, for Oy-SMS, the ethanol extract showed significantly higher inhibition than the water extract ($p < 0.01$).

Meanwhile, the FRAP assay presented a different picture, with higher antioxidant capacity in the water extracts than in the ethanol extracts (Table 2). The antioxidant capacity of the water extract was two-fold higher for shiitake SMS (2.6 ± <0.1 mM Fe^{2+} equivalents) compared to Oy-SMS (1.3 ± <0.6 mM Fe^{2+} equivalents) ($p < 0.01$, Student's *t*-test). On the other hand, the FRAP capacity in the ethanol extract of Oy-SMS (0.4 ± 0.1 mM Fe^{2+} equivalents) was two-fold higher than that in that of Sh-SMS (0.2 ± <0.1 mM Fe^{2+} equivalents) ($p < 0.05$).

In conclusion, it can be stated that the ethanol extracts exhibit a higher percentage of inhibition of the DPPH radical, while the water extracts show a higher FRAP antioxidant capacity. These results reveal the antioxidant potential of the ethanol extracts of oyster mushroom SMS and the water extracts of shiitake SMS. It should be noted, however, that the composition and properties of the extracted bioactive compounds may have been influenced by the initial substrates used in the mushroom cultivation. A study by this group is currently underway to elucidate the respective contributions of fungal metabolism and the initial substrate to the profile of bioactive compounds.

3.2. Subcritical water extraction of bioactive compounds from spent mushroom substrate

In previous research on the extraction of bioactive compounds from oyster mushroom SMS, subcritical water extraction (SWE) has shown good potential compared to other methods (Klausen et al., 2023). In that research, SWE was performed at 150 °C using both non-isothermal and partially isothermal heating modes. A temperature holding time of 10 min was used in the partially isothermal heating mode. Based on the results of that study, it was decided to perform SWE at three temperatures (125, 150, and 175 °C), to keep the non-isothermal (NI) mode, and to try two holding times (15 and 30 min) in the partially isothermal (PI) mode. A two-factor, three-level (a 3^2) experimental design was applied to each SMS. The center point condition (150 °C, 15 min) was performed in triplicate and used as the basis for calculating the standard error of the experiment.

3.2.1. Yield of extractive material and extract-free solids after SWE

As a result of the subcritical water extraction, extractable material was transferred from the raw SMS to the aqueous phase. After separating the extract-containing solvent from the biomass suspension, a solid material, hereafter referred to as extract-free residue, was generated.

The extraction yield, i.e., the yield of extracted material, was significantly higher for shiitake SMS than for oyster mushroom SMS across all SWE runs ($p < 0.0001$, two-way ANOVA). For example, in the extractions at 125 °C, the extraction yield ranged from 25.1 % (w/w) to 28.5 % (w/w) for Sh-SMS (Fig. 2-A), while the range for Oy-SMS was between 12.6 % (w/w) and 16.2 % (w/w) (Fig. 3 B). The same pattern continued at 150 °C with higher extraction yields for Sh-SMS (27.5 – 32.0 % (w/w)) than for Oy-SMS (17.5 – 24.7 % (w/w)) and at 175 °C with 31.9 – 35.0 % (w/w) for Sh-SMS and 24.2 – 29.1 % (w/w) for Oy-SMS.

In the experiments at 125 °C, the extraction yield increased proportionally with the extraction time; however, the correlation was not as apparent at other temperatures. For example, extending the extraction time from 15 min to 30 min at 150 °C resulted in a clear increase of the extraction yield for Oy-SMS from 17.5 % to 24.2 % ($p < 0.001$, Student's *t*-test; Fig. 2-B). In contract, no significant increase was observed for Sh-SMS under the same conditions (Fig. 3-A). At 175 °C, increasing the extraction time led to a significant decrease in the extraction yield for Sh-SMS, from 35.0 % to 31.9 % ($p < 0.001$, one-way ANOVA), while the yield remained largely unchanged for Oy-SMS. The extraction yields in both SMS consistently increased with the temperature, while the increase with time was restricted to extractions at 125 °C and 150 °C. At 175 °C, the extraction yield decreased when the extraction time exceeded 15 min. The standard error for the yield of extractive material was 0.17 for shiitake SMS and 1.0 for oyster mushroom SMS.

For both SMS types, the yield of extract-free residue recovered after extraction decreased continuously with increasing temperature and time (Fig. 2). The yield of extract-free residue for shiitake SMS ranged from 76.6 % in the non-isothermal SWE at 125 °C (NI-125 in Figs. 2-A) to 53.9 % in the partially isothermal SWE at 175 °C (PI30–175). A comparable decreasing trend was observed for Oy-SMS, with yields of extract-free residues ranging from 91.2 % in the NI-125 run to 62.8 % in the PI-175 run (Fig. 2-B). However, a plateau was observed in the experiments with a partially isothermal heating mode at 175 °C for both SMS types and at 150 °C for Sh-SMS. The yield of extract-free residue was lower overall for Sh-SMS than for Oy-SMS, which corresponds to the observed trend for the extraction yields. The standard error for yield of extract-free solid was 2.3 for shiitake SMS and 0.7 for oyster mushroom SMS.

According to the Pareto chart for shiitake SMS (Fig. 3-A), the temperature was the only parameter exerting a statistically significant effect on the extraction yield. The time, its interaction with temperature, the quadratic term of time, and the quadratic term of temperature also exerted some effects on the extraction yield. Still, all of these effects were below the significance threshold. Using multiple regression

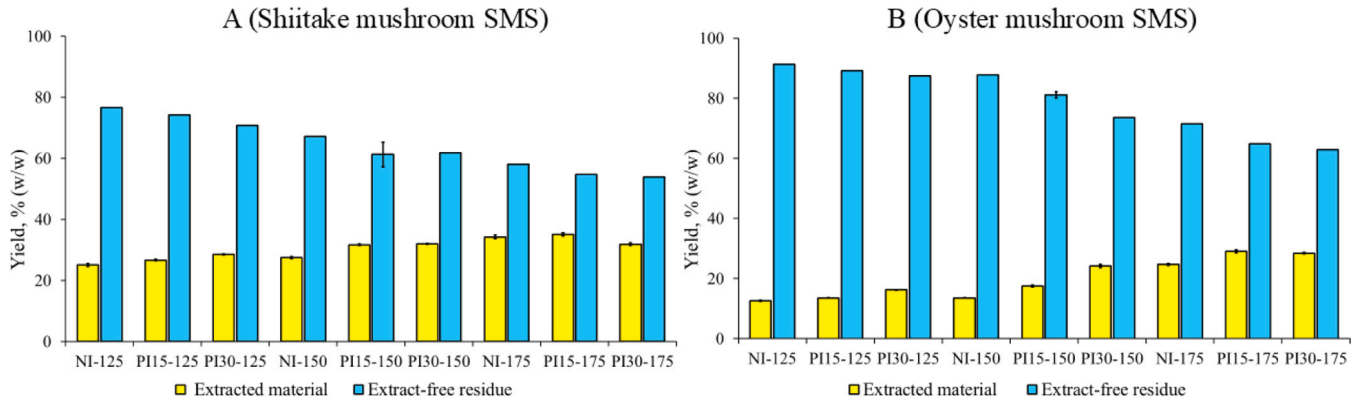

Fig. 2. Yields of extracted material and extract-free residue after subcritical water extraction of shiitake SMS (A) and oyster mushroom SMS (B). Error bars for extracted material indicate standard deviations (n = 3). Residue yields are from single experiments, except the central point (150 °C, 15 min), performed in triplicate.

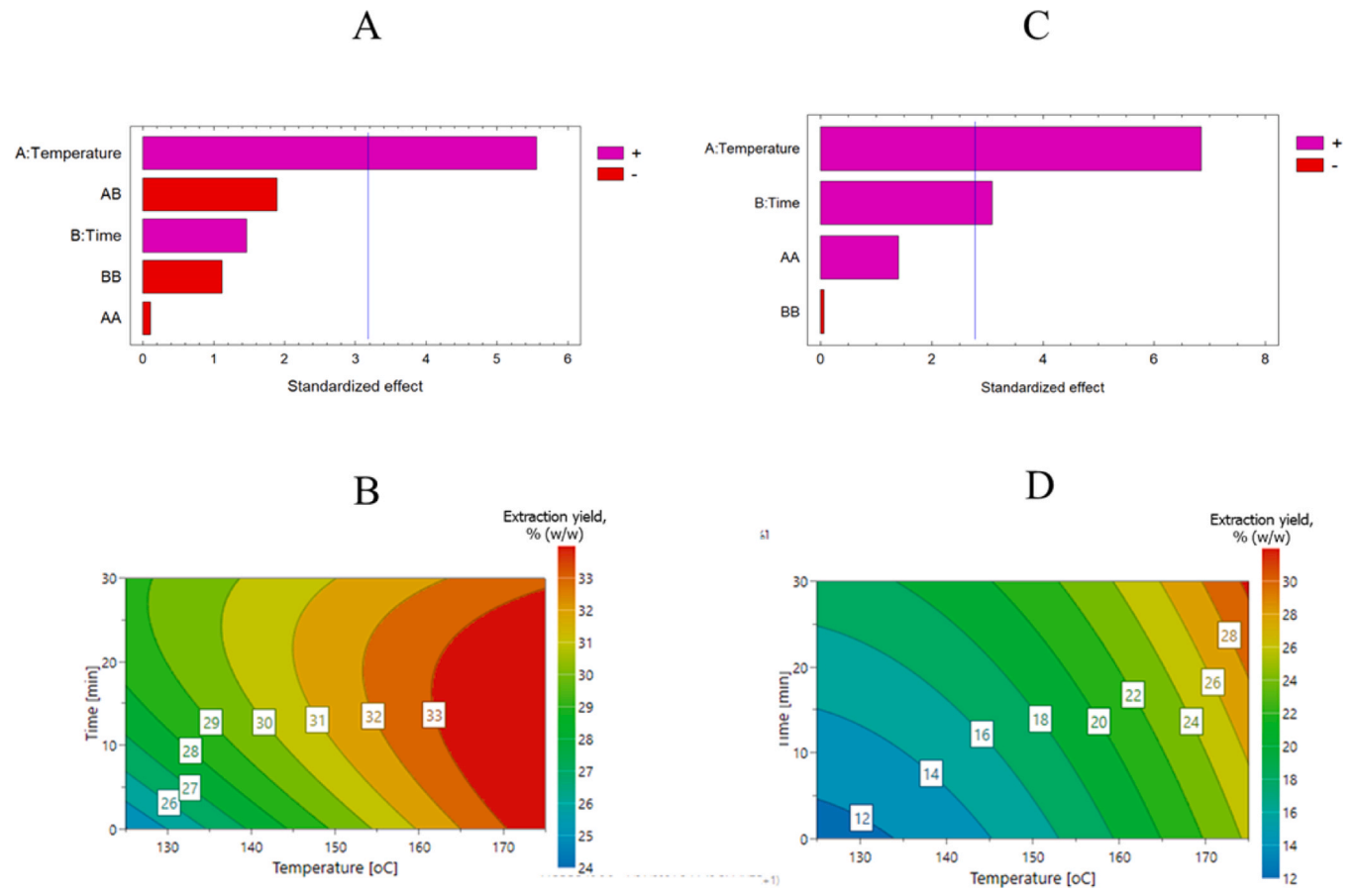

Fig. 3. Pareto charts of standardized effects (A, C) and response contour plots (B, D) for the extraction yield from SMS of shiitake (A, B) and oyster mushroom (C, D).

analysis of the experimental results, a model ($R^2 = 0.96$) was built to show the effect of operational conditions on the extraction yield (Eq. 3).

$$EY = 31.18 + 6.97 \times T + 1.83 \times t - 0.03 \times T^2 - 2.9 \times T \times t - 2.43 \times t^2 \quad (3)$$

where EY is the extraction yield, T is the temperature (in °C), and t is the extraction time (in min).

The positive signs in the model indicate the direct effects of the factors on the extraction yield, while the negative signs show inverse effects. The model predicts that a maximum yield within the tested conditions can be achieved through extraction at 175 °C for 11.7 min. These results can be visualized using a response contour plot (Fig. 3-B), which reveals the proximity of an area of optimal extraction under moderate extraction times paired with the highest tested temperature.

The Pareto chart for oyster mushroom SMS indicates that, like shiitake SMS, the temperature was the factor exerting the strongest effect on extraction (Fig. 3-C). However, what is particular to oyster mushroom SMS is that the extraction yield was also significantly affected by the time. The response contour plot shows that the extraction yield continued to increase with temperature and time without approaching an area of optimal conditions within the investigated range of the independent factors (Fig. 3-D).

3.2.2. *Concentration of total phenolic compounds in the subcritical-water extracts*

Phenolic compounds are a diverse group of secondary metabolites found in plants and fungi, known for their antioxidant properties (Fogarasi et al., 2024). Phenolics are to be expected in spent mushroom substrate (SMS) both from the fungal mycelium and substrate phytochemicals and because of the fungal degradation of lignin. A recent study evaluated the phenolic compounds in shiitake SMS and their antioxidant and antibacterial effects (Baptista et al., 2023). However, no comparative studies on the phenolic compounds in SMS from both shiitake and oyster mushrooms have been published to date.

The concentration of total phenolic compounds in the extracts produced by subcritical water extraction of the spent substrates of shiitake and oyster mushrooms in the experiment is presented in Fig. 4. The results reveal two main trends, namely, one regarding how the concentrations are affected by the fungal species and another one regarding how they are affected by the SWE conditions.

A two-way ANOVA revealed that under all conditions, the extracts from shiitake SMS contained significantly higher concentrations of total phenolic compounds than those of oyster mushroom SMS ($p < 0.0001$; Fig. 4). For example, in the extracts from the SWE at 125 °C, the concentrations were 0.8–1.0 mg GAE/mL for shiitake SMS and 0.4–0.6 mg GAE/mL for oyster mushroom SMS. The same trend was observed for the extractions at 150 and 175 °C.

For both SMS types, increasing the temperature and extending the time resulted in higher concentrations of total phenolics. As an example, the total phenolic concentration in shiitake SMS extracts from SWE lasting 15 min increased from 1.0 mg GAE/mL in the experiment at 125 °C to 1.5 mg GAE/mL in the experiment at 175 °C (Fig. 4). Statistical analysis using one-way ANOVA confirmed that the observed increase was significant ($p < 0.03$). Similarly, for the experiments at a given temperature, the concentrations increased from the extracts resulting from extractions under non-isothermal heating mode (experiments with 0-min holding time) to the extracts from experiments with 30-min holding time. For example, a significant increase ($p < 0.001$, one-way ANOVA) of the concentrations of shiitake SMS extracts from SWE at 150 °C (from 1.1 mg GAE/mL in the experiment with a 0-min holding time to 1.3 mg GAE/mL in the experiment lasting 30 min) was observed.

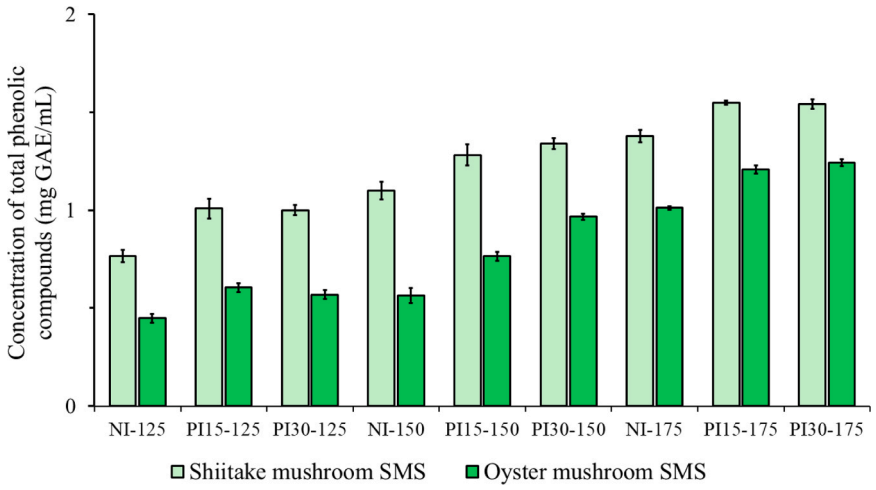

Fig. 4. Concentration of total phenolic compounds (mean of triplicates) in subcritical water extracts of spent substrates from shiitake and oyster mushrooms. Horizontal axis codes: NI, non-isothermal extraction; PI15 and PI30, partially isothermal extraction with holding times of 15 and 30 min, respectively; 125, 150, and 175 °C indicate extraction temperatures.

The same trend can be observed for the extracts of oyster mushroom SMS.

Based on the concentrations in the extracts (Fig. 4), the yields of total phenolic compounds from the raw SMS were calculated. Pareto charts and counterplots were constructed using the extraction yields for different SWE conditions for both SMS types. The Pareto chart shows that the temperature was the experimental factor exerting the strongest effect on the extraction yield of total phenolic compounds in the SWE extracts produced from both SMS types (Fig. 5-A, C). The extraction time also exerted a significant effect. Neither the interaction between temperature and time nor their quadratic terms had a significant effect on the extraction yield of total phenolics. The response contour plot for the shiitake SMS extracts indicates the vicinity of a region of maximum yield in the corner corresponding to the highest temperature (175 °C) and time (25.5 min) (Fig. 5-B). However, for the SWE extracts of oyster mushroom SMS, the response contour plot provided no indications of a possible optimum within the investigated range of the independent variables (Fig. 5-D).

The extraction efficiency was calculated by expressing the extraction yields as a percentage of the total phenolic content determined during the characterization of the SMS (Table 2). For shiitake SMS, the extraction efficiency of total phenolics ranged from 64.2 % to 82.5 % in

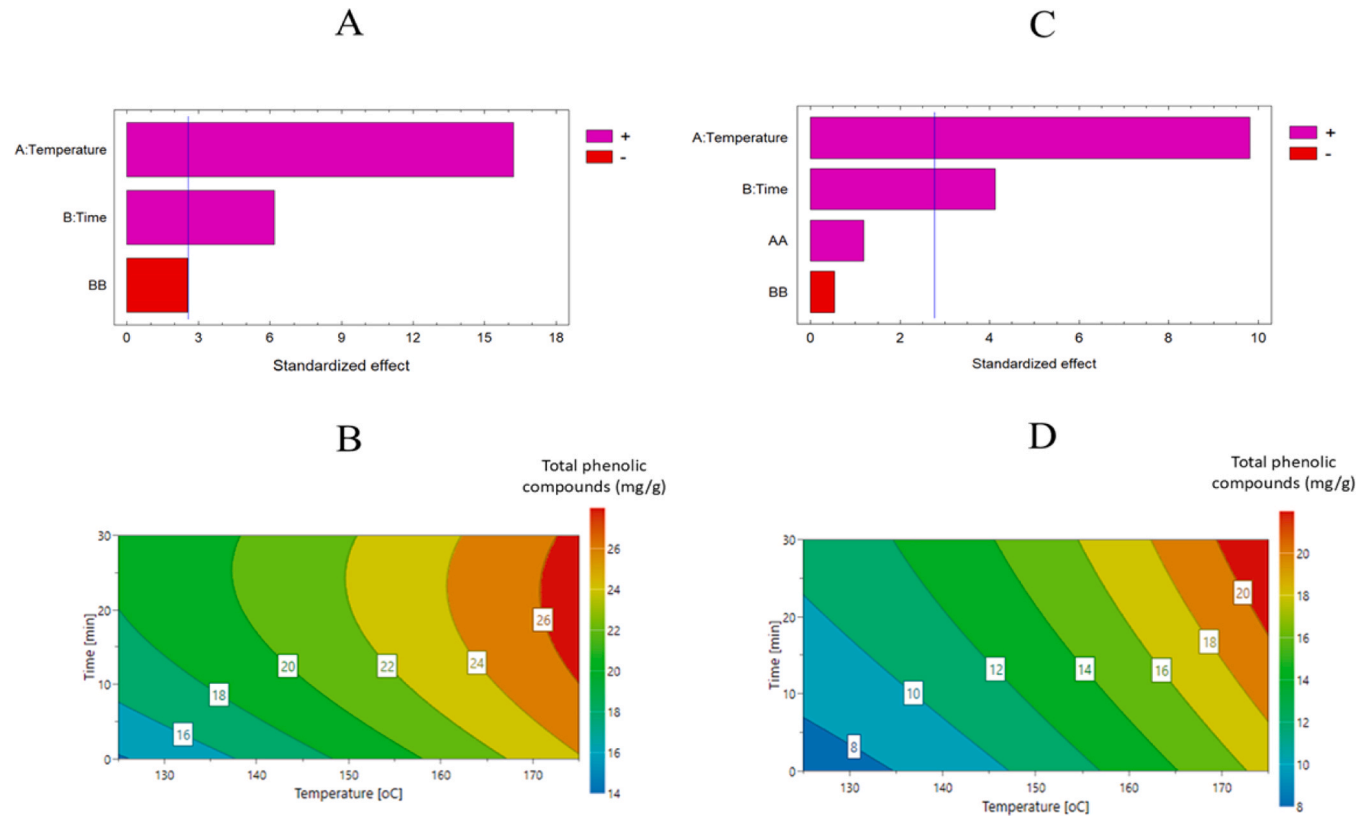

Fig. 5. Pareto chart of standardized effects (A, C) and response counter plots (B, D) for the extraction yield of total phenolics (mg GAE/g SMS) in the subcritical water extracts of spent substrates of shiitake (A, B) and oyster mushrooms (C, D).

the SWE experiments at 125 °C and from 86.8 % to 98.7 % for temperature holding times of 0 and 15 min at 150 °C. It exceeded 100 % in the partially isothermal run of 30 min at 150 °C and all experiments at 175 °C. For oyster mushroom SMS, the calculated efficiency ranged from 53.1 % to 70.2 % in the experiments at 125°C and from 66.5 % to 91.9 % in the 0- and 15-minute runs at 150°C. It exceeded 100 % in the 30-minute run at 150°C and in all runs at 175 °C. These results reveal that phenolic compounds are more easily extracted from shiitake SMS than from oyster mushroom SMS. The extraction efficiencies exceeding 100 % suggest that, under severe SWE conditions, phenolics from sources other than just the extractive fraction are extracted.

3.2.3. Quantification of individual phenolic compounds in the subcritical-water extracts

The extracts from the SWE at 175 °C were subjected to HPLC analysis to identify and quantify individual phenolic compounds. The analysis revealed the presence of eight phenolic acids in the SWE extracts from both SMS (Fig. S2).

Ascorbic acid and vanillic acid were the compounds found in higher concentrations in most of the extracts (Table 3). The concentrations of ascorbic acid were higher in the Sh-SMS extracts (15.9 –16.6 mg/L) than in Oy-SMS extracts (10.7–14.8 mg/L) ($p < 0.001$, two-way ANOVA). On the other hand, vanillic acid was predominant in Oy-SMS extracts, where its concentration ranged between 12.0 mg/L and 20.4 mg/L, whereas in the Sh-SMS extracts, it was only 3.7–4.0 mg/L ($p < 0.001$). For Sh-SMS extracts, the concentrations of ascorbic acid and vanillic acid were relatively higher in the extracts from extractions lasting up to 15 min compared to those from the 30-minute extraction. In contrast, Oy-SMS extracts showed an increasing trend in the concentrations of ascorbic acid ($p < 0.03$, one-way ANOVA) and vanillic acid ($p < 0.001$) with longer extraction times. The trend of these two major phenolic acids aligns well with the observed trend for total phenolic compounds in SWE extracts of Sh-SMS (Fig. 5-B) and Oy-SMS (Fig. 5-D).

ND, not detected

The extracts were also relatively rich in protocatechuic- and gallic acid (Table 3). The concentrations of protocatechuic acid were higher for Oy-SMS extracts (11.1–16.2 mg/L) compared to Sh-SMS extracts (3.0–3.8 mg/L). A similar trend was observed for gallic acid, with concentration ranges of 7.5–8.7 mg/L for Oy-SMS extracts and 3.3–7.7 mg/L for Sh-SMS. The concentrations of protocatechuic acid correlated with extraction time, following the same trend observed for ascorbic acid and vanillic acid. Conversely, for gallic acid, a decreasing trend in concentration was observed with longer extraction times. This decrease could be attributed to the faster degradation of gallic acid compared to the other phenolic acids (Khuwijitjaru et al., 2014).

The next compound in decreasing order was caffeic acid, which ranged between 3.6 mg/L and 4.0 g/L (Table 3). Caffeic acid concentration was comparable for the extracts of both SMS types (ranging from 3.6 mg/L to 4.0 mg/L), and no distinguishable dependence on the extraction conditions was detected.

Ferulic acid and chlorogenic acid were found at lower concentrations than the previously discussed acids. Both of them displayed higher concentrations in Sh-SMS extracts than in Oy-SMS extracts. The concentration ranges in Sh-SMS extracts were 3.1–4.8 mg/L for ferulic acid and 2.9–4.1 mg/L for Oy-SMS, whereas for chlorogenic acid, the ranges were 2.4–4.1 mg/L and 1.6–2.1 g/L, respectively. For both, an increasing trend in concentration was detected with longer extraction times. Gentisic acid was detected only in the extract of shiitake SMS from the SWE run lasting 30 min, whereas it was below the detection level in the other extracts (Table 3). Some other prominent unidentified peaks of potential interest for future studies were also visible in the chromatograms.

Ascorbic acid has previously been detected in shiitake fruitbodies (Jiang et al., 2010), while vanillic acid is one of the most abundant polyphenolic constituents in oyster mushrooms (Matkovits et al., 2024). We have previously identified vanillic acid and gallic acid as the most abundant phenolic acids in SWE extracts of the oyster mushroom SMS (Klausen et al., 2023), whereas no studies have reported the presence of ascorbic acid in SMS to date. The observed vanillic acid concentration in this study aligns closely with our previous report (16.4 ± 0.2 mg/L). However, in our earlier work, we found a higher gallic acid concentration (11.1 ± 0.5 mg/L) than what we report in this study.

Table 3
Concentration of phenolic acids (mg/L) in selected subcritical water extracts of spent substrates from shiitake and oyster mushrooms. Values are means ± standard deviations from duplicate analyses.

Compound	NI-175		PI15-175		PI30-175	
	Sh-SMS	Oy-SMS	Sh-SMS	Oy-SMS	Sh-SMS	Oy-SMS
Ascorbic acid	16.1 ± 1.0	10.7 ± 0.3	16.5 ± 0.2	12.9 ± 1.2	15.9 ± 0.1	14.8 ± 0.2
Vanillic acid	4.0 ± 0.2	12.0 ± 0.4	4.0 ± 0.1	17.7 ± 0.3	3.7 ± 0.1	20.4 ± 0.1
Protocatechuic acid	3.6 ± 0.4	11.1 ± 0.8	3.8 ± 0.4	14.4 ± 0.5	3.0 ± 0.1	16.2 ± 0.4
Gallic acid	7.7 ± < 0.1	8.4 ± 0.5	5.7 ± 0.6	8.7 ± 0.5	3.3 ± 0.1	7.5 ± 0.1
Caffeic acid	3.6 ± < 0.1	3.6 ± 0.1	4.0 ± 0.1	3.8 ± < 0.1	4.0 ± < 0.1	3.7 ± < 0.1
Ferulic acid	3.1 ± 0.1	2.9 ± < 0.1	3.9 ± < 0.1	3.8 ± < 0.1	4.8 ± 0.1	4.1 ± 0.1
Chlorogenic acid	2.4 ± 0.	1.6 ± < 0.1	3.3 ± 0.1	2.1 ± 0.1	4.1 ± 0.1	2.3 ± < 0.1
Gentisic acid	ND	ND	0.7 ± 0.4	ND	2.5 ± < 0.1	ND

3.2.4. Quantification of total carbohydrates in the subcritical-water extracts

The spectrophotometric quantification of total carbohydrates revealed that the concentrations vary depending on the SWE conditions and the type of SMS. For both SMS types, the concentration of total carbohydrates in the extracts increased with increasing temperature and time (Fig. 6-A). The values were generally higher in the extracts from shiitake SMS compared to those from oyster mushroom SMS, especially for the experiments at 125 °C and 150 °C ($p < 0.03$, two-way ANOVA). As the temperature increased, the concentration of total carbohydrates increased more in the Oy-SMS extracts (1.9–9.3 mg/mL) than in the Sh-SMS ones (3.2–9.1 mg/mL). In the experiments at 175 °C, for the partially isothermal SWE, no significant differences ($p = 0.9$) were detected between the concentration values of the extracts from shiitake and oyster mushroom (SMS) for both 15-min and 30-min holding times. This observation suggests that under mild and moderate SWE conditions, shiitake SMS is more susceptible than oyster mushroom SMS to release carbohydrates, while under more severe conditions, both SMS are equally susceptible.

The extraction yields of total carbohydrates from the raw SMS were calculated and used to construct Pareto charts, allowing for the assessment of the effect of independent factors on the extraction yields. The Pareto charts show that for shiitake SMS, temperature was the only factor significantly influencing the extraction yield of total carbohydrates (Fig. 6-B). For oyster mushroom SMS, both temperature and time exerted significant effects (Fig. 6-C). A model based on the experimental results predicted optimal extraction of total carbohydrates by SWE at 175 °C for 17 min. For oyster mushroom SMS, no optimum is predicted within the investigated range of the independent variables.

The calculation of the extraction efficiency of total carbohydrates revealed values exceeding 100 % for all conditions in Shiitake SMS and for experiments at 150 °C and 175 °C in oyster mushroom SMS. This finding suggests that carbohydrates are extracted from sources beyond the extractive fraction. To elucidate other sources of carbohydrates, the concentrations of monosaccharides and oligosaccharides in the extracts were analysed using a protocol that combined sulfuric acid post-

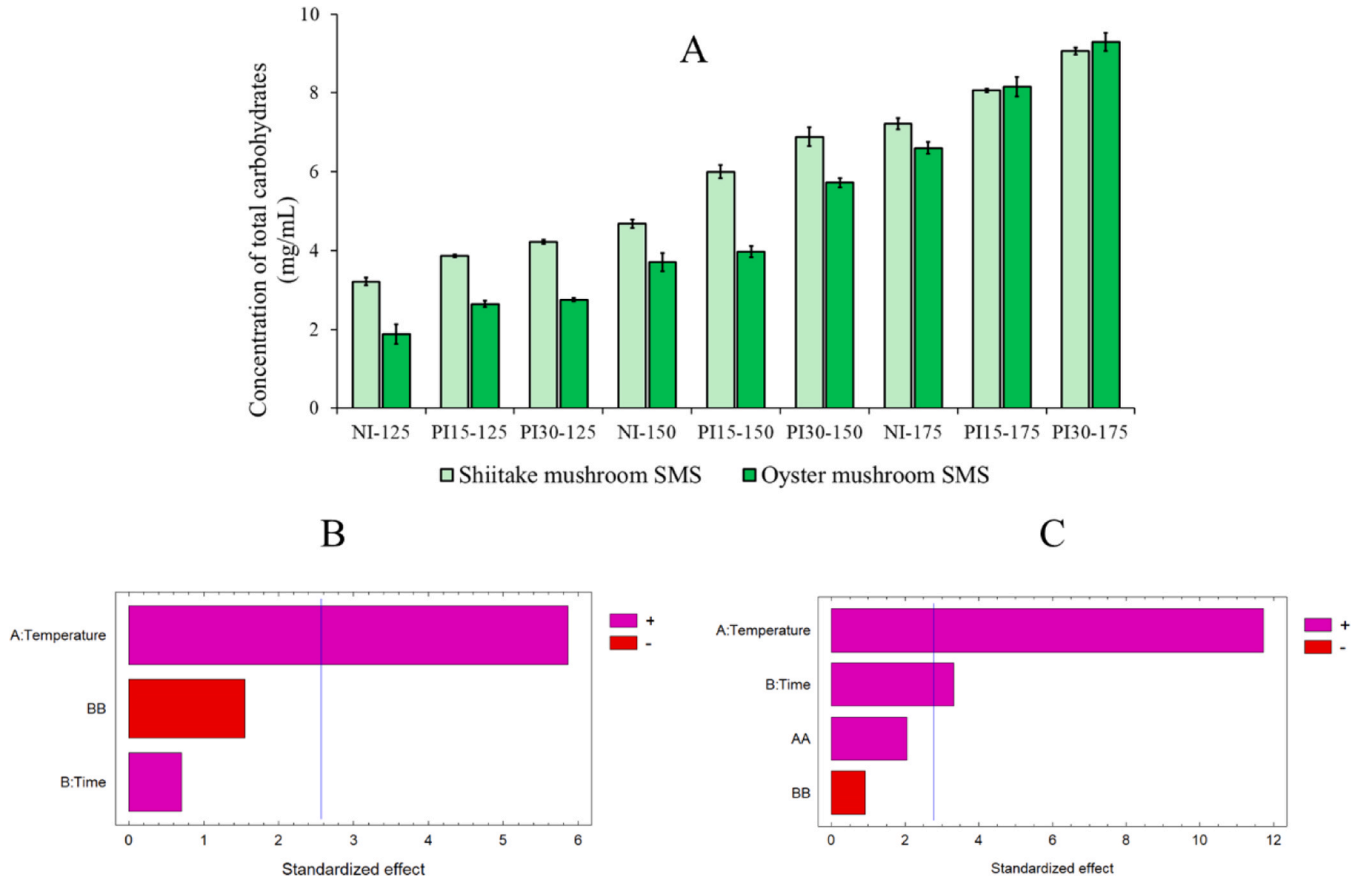

Fig. 6. Concentration of total carbohydrates in the subcritical water extracts (A) and Pareto charts of standardized effects for total carbohydrate extraction yields from spent substrates of shiitake (B) and oyster mushrooms (C). Codes on the horizontal axis in panel A are explained in Fig. 4. Values in Panel A are means from duplicate measurements.

hydrolysis and HPLC. The analysis revealed relatively high concentrations of xylose, glucose, and their corresponding oligosaccharides (Fig. S3).

Xylose and xylo-oligosaccharides can originate from hemicelluloses, considering that xylans are essential hemicellulosic components of the hardwood species used for mushroom cultivation (Chen et al., 2022b). Glucose and gluco-oligosaccharides can come from either the cellulose and hemicelluloses from the substrate or β-glucans from the mycelium. So, the presence of xylose saccharides in the extracts of shiitake SMS and oyster mushroom SMS (Fig. S3) suggests that hemicelluloses are a possible source of a part of the total carbohydrates identified spectrophotometrically. On the other hand, although glucose saccharides can originate from different sources, hemicelluloses are an important source whose contribution should not be ignored, considering their higher solubility and reactivity compared to cellulose.

For both SMS types, the concentration of xylose and xylo-oligosaccharides was higher compared to glucose and gluco-oligosaccharides. In addition, the concentration of oligosaccharides in the extracts was higher than that of monosaccharides. This indicates that under SWE conditions, polysaccharides, primarily hemicelluloses, are solubilized rather than hydrolyzed. This phenomenon is typically observed during the hydrothermal pretreatment of lignocellulosic materials (Martín et al., 2022), a process in which biomass is treated with hot liquid water, analogous to SWE.

3.2.5. *Quantification of β-glucans in the subcritical-water extracts*

The quantification of β-glucans by subcritical water extraction revealed higher concentrations in all extracts from shiitake SMS compared to the extracts from oyster mushroom SMS (Table 4). Since the initial β-glucan content is higher in Sh-SMS compared to Oy-SMS (Table 2), this is a logical result. The extraction efficiency in Sh-SMS exceeded 100 % in all runs from PI30–125, whereas the extraction efficiency for Oy-SMS ranged from 42.9 % to 86.7 % at 125 °C and was

Table 4
Concentration of β-glucans and proteins in subcritical water extracts of spent substrates of shiitake and oyster mushrooms. Experimental run codes are explained in Fig. 4. Protein concentrations are presented as means ± standard deviations from triplicate measurements. β-Glucans were analyzed in single measurements due to time and equipment restraints.

Experimental run	β-Glucans, mg/mL		Proteins, mg/L	
	Shiitake SMS	Oyster mushroom SMS	Shiitake SMS	Oyster mushroom SMS
NI−125	0.26	0.14	137.1 ± 5.1	57.6 ± 7.7
PI15–125	0.34	0.18	166.0 ± 12.8	91.1 ± 1.3
PI30–125	0.55	0.27	175.1 ± 5.1	45.8 ± 6.4
NI−150	0.49	0.22	228.4 ± 3.8	64.9 ± 2.6
PI15–150	1.53	0.45	260.1 ± 15.0	108.8 ± 6.3
PI30–150	1.08	0.57	304.4 ± 6.4	100.2 ± 3.8
NI−175	1.39	0.59	251.9 ± 9.0	141.8 ± 1.3
PI15–175	1.48	0.88	165.1 ± 6.4	41.3 ± 2.6
PI30–175	1.45	0.98	128.1 ± 12.8	13.2 ± 3.8

65.2 % in NI-150 before exceeding 100 % in all subsequent runs. This result indicates that, under the conditions used in this study, shiitake β-glucans are more susceptible to being extracted than oyster mushroom β-glucans.

The concentration of β-glucans correlated in a slightly different manner with the SWE conditions, depending on the SMS type (Table 4). For shiitake SMS, the concentration increased proportionally with the extraction time only in the experimental runs conducted at 125 °C. In contrast, at 150 °C and 175 °C, a plateau was reached, and no significant differences were observed as the extraction conditions were varied (p=0.3, two-way ANOVA). On the other hand, for the oyster mushroom SMS, the concentration of β-glucans increased continuously with both temperature and time across the entire experimental matrix ($p < 0.01$).

3.2.6. Quantification of total protein in the subcritical-water extracts

The protein concentration in the subcritical water extracts of the spent substrates from shiitake and oyster mushrooms was determined using the Bradford assay. For all the SWE conditions, the protein concentration was significantly higher in the extracts of Sh-SMS (128.1–304.4 mg/L) than in those of Oy-SMS (13.2–141.8 mg/L) ($p < 0.01$, two-way ANOVA; Table 4). Furthermore, the protein concentration trends varied slightly depending on the SWE conditions for each SMS. For both SMS types, the protein concentration in the extracts increased gradually with the temperature and time, reached a maximum, and then started to decrease. For Sh-SMS, the maximum was reached in the experimental run at 150 °C with partially isothermal heating and a 30-min holding time, whereas for Oy-SMS, the maximum was reached at 175 °C under non-isothermal heating (0-min holding time). The decrease in protein concentration at high temperatures can be attributed to thermal degradation, hydrolytic reactions (Rogalinski et al., 2008), or phenomena of aggregation and disaggregation (Marcet et al., 2016). Apparently, at low temperatures, an equilibrium exists between protein recovery and degradation, with some minor recovery being evident. After reaching certain conditions, the degradation reactions outweigh the extraction process, resulting in an apparent decrease in protein recovery. Since the temperatures used in the experiment were well above the denaturation temperature of most proteins, and edible mushrooms are not thermophilic organisms, one can be tempted to attribute the results to analytical bias caused by the interference of monosaccharides and oligosaccharides contained in the extracts (Banik et al., 2009). However, extracting proteins with subcritical water at high temperatures has already been reported with different matrices (Álvarez-Viñas et al., 2021). In addition, it is assumed that the temperatures required for extracting proteins are higher than those for extracting oligosaccharides, as proteins are generally more structurally complex and more tightly bound within the biomass matrix. This contrasts with oligosaccharides, whose glycosidic linkages are more readily solubilized under milder conditions (Rogalinski et al., 2008).

Although the concentrations can have been overestimated, the calculation of the extraction efficiency, based on the protein content of the SMS (Table 2), revealed that only 53.8 % of the protein contained in Sh-SMS and 25.8 % of the protein in Oy-SMS was extracted. These results suggest that, although subcritical water has previously been shown to be effective for protein extraction from soybeans (Ndlela et al., 2012), the operational conditions used in the current study are not suitable for recovering proteins from SMS. Instead, the used conditions might be suitable for producing protein hydrolysates and peptides, as demonstrated in subcritical water extraction and hydrolysis processes applied to various agro-food wastes and algae (Álvarez-Viñas et al., 2021).

3.2.7. Characterization of the solid residues of the subcritical water extraction

The extract-free residues from all the SWE runs were subjected to two-step analytical acid hydrolysis to analyze their chemical composition. The composition of the residues from the three extractions conducted at 175 °C (runs NI-175, PI15–175, and PI30–175) and for the three extractions with 30-min holding time (runs PI30–125, PI30–150, and PI30–175) is presented in Table 5. The analysis revealed that cellulose was the primary component of the extract-free residues, with a consistently higher content in oyster mushroom SMS compared to shiitake SMS. For both Sh-SMS and Oy-SMS, the cellulose content generally increased with temperature and time. The increase in cellulose content over time was more pronounced for Sh-SMS than for Oy-SMS.

Xylan content was higher in the SWE residues of Oy-SMS than in those of Sh-SMS (Table 5) ($p < 0.02$, two-way ANOVA). For Oy-SMS, xylan content consistently decreased over time at all temperatures, whereas for Sh-SMS, the decreasing trend was less pronounced. The decrease in xylan content can be attributed to the solubilization of hemicelluloses due to the relatively high SWE temperature. This assumption can be confirmed by the disappearance of arabinan, which was detected only in the extract-free residues of some of the least severe conditions (data not shown), and the relatively high concentrations of xylose and xylo-oligosaccharides in the extracts (Fig. 9). The lignin content increased with temperature and time for both SMS types ($p < 0.01$). The Oy-SMS residue displayed consistently higher lignin values than the Sh-SMS one ($p < 0.02$), which is an expected result given the higher lignin content in the raw Oy-SMS compared to raw Sh-SMS (Table 1).

The increase in the content of cellulose and lignin is a consequence of the solubilization of extractive compounds and a part of the hemicelluloses as reported for biomass hydrothermal pretreatment (Martín et al., 2022), a process similar to SWE. As a consequence of the adjustments under severe SWE conditions, the contents of cellulose, xylan, and lignin in the extract-free residues from the extraction at 175 °C with a partially isothermal heating mode and a 30-min holding time were comparable for Sh-SMS and Oy-SMS (Table 5).

3.3. Pressurized ethanol extraction of bioactive compounds from spent substrates of shiitake and oyster mushroom

Considering the high antioxidant activity observed in the ethanol extracts from the Soxhlet extraction used in the characterization of the SMS (Table 2) and the effectiveness of subcritical water extraction (SWE) to recover phenolics and carbohydrates (see Sections 3.2.2 and 3.2.4), pressurized ethanol extraction (PEE) was applied to the spent substrates of shiitake and oyster mushrooms. PEE combines the benefits of a pressurized liquid extraction system, similar to SWE, with the use of a slightly less polar solvent, such as ethanol. The PEE experiment was run at 175 °C with either non-isothermal or partially isothermal heating modes. The operational conditions were established based on the trends observed in the SWE for the yield of extracted material (Fig. 3), the recovery of phenolics (Fig. 5) and carbohydrates (Fig. 6). Aqueous ethanol, namely a 40:60 (v/v) ethanol-water mixture, was used as

Table 5
Content of major components in extract-free residues from subcritical water extraction of spent substrates of shiitake (Sh-SMS) and oyster mushrooms (Oy-SMS). Values are means ± standard deviations from triplicate measurements.

Experimental run[1]	Cellulose, % (w/w)		Xylan, % (w/w)		Lignin, % (w/w)	
	Sh-SMS	Oy-SMS	Sh-SMS	Oy-SMS	Sh-SMS	Oy-SMS
PI30–125	30.4 ± 6.9	35.5 ± 0.5	9.3 ± 1.6	14.3 ± 0.3	12.8 ± 0.3	20.2 ± 0.2
PI30–150	43.6 ± 1.3	43.1 ± 1.1	9.3 ± 0.1	13.1 ± 0.3	17.8 ± 0.1	24.6 ± 0.2
NI−175	30.5 ± 1.5	42.2 ± 0.1	7.0 ± 1.8	9.9 ± 0.1	15.8 ± 0.4	25.6 ± 2.0
PI15–175	40.8 ± 3.1	46.8 ± 0.4	4.6 ± 0.9	6.6 ± 0.1	21.0 ± 0.7	25.3 ± 1.0
PI30–175	54.0 ± 0.7	53.1 ± 5.2	6.7 ± 0.6	6.0 ± 0.7	23.6 ± 0.5	27.8 ± 0.2

[1] The codes of the experimental runs are explained in Fig. 4

solvent based on the effectiveness shown by this solvent system in reflux extraction of bioactive compounds from oyster mushroom SMS (Klausen et al., 2023).

3.3.1. Yield of extracted material and extract-free solids after PEE

The PEE resulted in a higher extraction yield for shiitake SMS compared to oyster mushroom SMS (Fig. 7-A) ($p < 0.02$, Student's *t*-test). For Sh-SMS, the extraction yield increased from 30.5 ± 0.5 % in the PEE experiment under non-isothermal heating mode (i.e., with 0-min holding time at 175 °C) to 32.8 ± 0.3 % in the partially isothermal PEE (i.e., with 15-min holding time at 175 °C), whereas for Oy-SMS, the yields increased from 23.0 ± 0.1 % to 30.4 ± 0.5 %, correspondingly. This reveals that the difference in extraction yield between the two SMS types was more pronounced under non-isothermal heating than under partially isothermal heating and that for both SMS types, the extraction yield increased with holding time ($p<0.002$, two-way ANOVA). The yield of extract-free residue followed a trend that logically matched the observed trend for the yield of extracted material.

3.3.2. Quantification of total phenolic compounds and phenolic acids in the pressurized ethanol extracts

The pressurized ethanol extraction resulted in a higher concentration of total phenolic compounds in the experiment under the partially isothermal mode than in the experiment with the non-isothermal heating mode for both SMS (Fig. 7-B) ($p<0.01$, two-way ANOVA). These results show the same trend as for subcritical water extraction (SWE), namely an increase in total phenolic compounds with longer extraction times. The concentration of total phenolic compounds was higher in the extracts of Sh-SMS (1.8–2.1 mg GAE/mL) than in those of Oy-SMS (1.2–1.6 mg GAE/mL).

Eight phenolic acids were identified in the PEE extracts (Table 6).

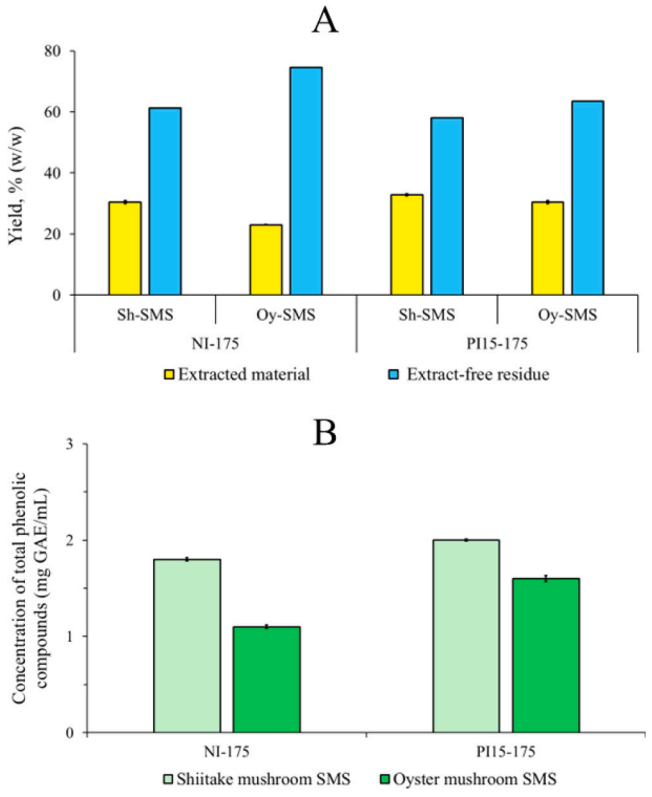

Fig. 7. Pressurized ethanol extraction of spent substrates from shiitake (Sh-SMS) and oyster mushrooms (Oy-SMS) at 175 °C under non-isothermal heating (NI-175) and partially isothermal heating with a 15-min holding time (PI15–175). (A) Yield of extracted material and extract-free residue; (B) Concentration of total phenolic compounds in the extracts.

Table 6
Concentration of phenolic acids (mg/L) in pressurized ethanol extracts of spent substrates from shiitake (Sh-SMS) and oyster mushrooms (Oy-SMS), mg/L. Values are means ± standard deviations from duplicate analyses.

Compound	NI-175		PI15-175	
	Sh-SMS	Oy-SMS	Sh-SMS	Oy-SMS
Vanillic acid	4.4 ± 0.1	13.4 ± 0.3	4.5 ± 0.2	18.0 ± 0.3
Gallic acid	12.4 ± 0.2	11.9 ± 0.1	16.0 ± 0.1	14.8 ± 0.1
Ascorbic acid	11.4 ± 0.2	13.4 ± 0.1	13.0 ± 1.9	9.4 ± 0.1
Protocatechuic acid	N.D.	7.5 ± 0.3	N.D.	8.6 ± 0.5
Chlorogenic acid	3.3 ± 0.1	1.8 ± < 0.1	3.5 ± 0.1	2.1 ± 0.1
Caffeic acid	4.0 ± 0.1	3.5 ± < 0.1	3.8 ± 0.1	3.5 ± < 0.1
Ferulic acid	3.9 ± < 0.1	3.3 ± < 0.1	4.1 ± 0.1	4.4 ± < 0.1
Gentisic acid	0.7 ± 0.4	N.D.	N.D.	N.D.

N.D., Not Detected

The most noteworthy result was the difference between the extracts of oyster mushroom SMS and shiitake SMS regarding their content of vanillic acid, which was the most abundant phenolic acid in Oy-SMS extracts (13.4–18.0 mg/L), whereas its concentrations in Sh-SMS extracts were relatively modest (4.1–4.5 mg/L). Gallic acid was the main phenolic acid in Sh-SMS extracts (12.4–16.0 mg/L) and the second most abundant in Oy-SMS extracts (11.9–14.8 mg/L). Ascorbic acid was abundant in the extracts of both SMS types, with concentration ranges of 11.4–13.0 mg/L in Sh-SMS extracts and 9.4–13.4 mg/L) in Oy-SMS extracts. The other phenolic acids were found in lower concentrations.

For the Oy-SMS PEE extracts, the concentrations of vanillic-, gallic-, and ferulic acid increased with the holding time at 175 °C ($p<0.01$, Student's *t*-test; Table 6), as well as the gallic acid concentration in Sh-SMS extracts ($p<0.01$). Ascorbic acid concentration showed no significant change over time in the Sh-SMS extracts ($p=0.4$), while the trend was decreasing for Oy-SMS extracts ($p<0.001$). In addition, no clear changes over time were observed in the concentrations of chlorogenic acid and caffeic acid.

3.3.3. Quantification of carbohydrates in the pressurized ethanol extracts

The concentrations of total carbohydrates in the pressurized ethanol extracts under non-isothermal heating (0-min holding time at 175 °C) were higher for shiitake SMS than for oyster mushroom SMS ($p<0.02$, Student's *t*-test; Fig. 8-A). However, in the extractions with partially isothermal heating (15-min holding time), they were comparable for both extracts. This trend is similar to that observed in subcritical water extraction under the same operational conditions (Fig. 6-A). The concentrations of total carbohydrates in the PEE extracts increased over the holding time for both SMS types ($p<0.05$, two-way ANOVA). The change was more notable in the Oy-SMS extract, which increased from 5.32 ± 0.17 mg/mL in the extraction with 0-min holding time (experiment NI-175) to 7.50 ± 0.02 mg/mL in the PI15–175 experiment (15-min holding time).

Among the carbohydrate components, β-glucans, monosaccharides and oligosaccharides were identified. The concentration of β-glucans was higher in the shiitake SMS extracts (0.8–1.5 mg/mL) compared to oyster mushroom SMS extract (0.4–0.9 mg/mL) (Fig. 8-B). The concentration of β-glucans in both extracts increased over the holding time at 175 °C.

The concentration of oligosaccharides was higher than that of monosaccharides, with both being higher in Sh-SMS extracts than in Oy-SMS extracts (Fig. 13-C). The only exception was observed for xylo-oligosaccharides in the extract from the PI15–175 experiment, where the concentration in the Sh-SMS extract (4.1 mg/mL) was comparable to that of the Oy-SMS extract (4.3 mg/mL). For both SMS types, the concentration of xylo-oligosaccharides was higher than that of gluco-oligosaccharides, with a trend of increasing concentration observed over the holding time. In all extracts, xylose concentrations were consistently higher than those of glucose. The concentrations of both xylose and glucose were lower in the PEE extracts (Fig. 8-C,D) than in

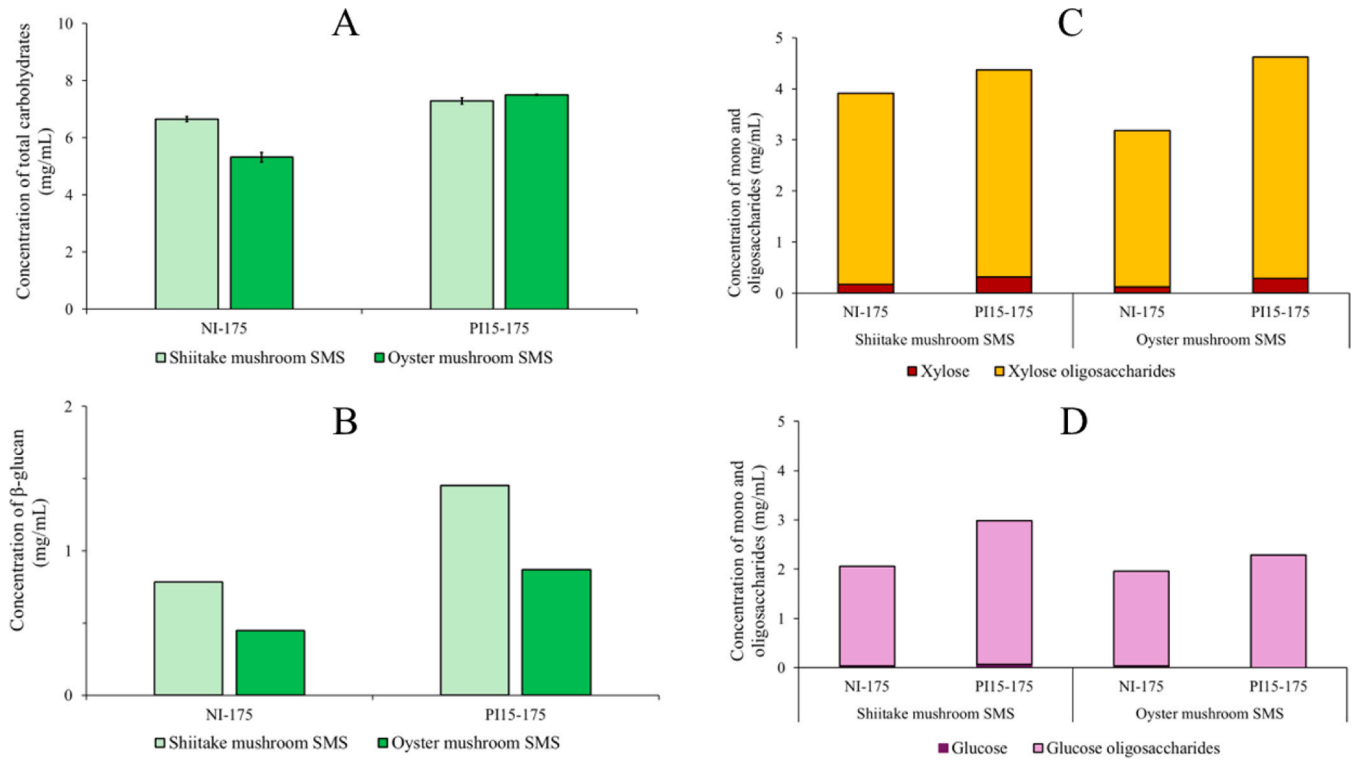

Fig. 8. Concentration of carbohydrates in pressurized ethanol extracts of spent substrates from shitake and oyster mushrooms. (A) Total carbohydrates; (B) β-Glucans; (C) Xylose and xylo-oligosaccharides; (D) Glucose and gluco-oligosaccharides. Extractions were performed at 175° C under non-isothermal (NI-175) and partially isothermal (PI15–175) conditions. Panel A shows means from duplicate measurements; panels B and C are single measurements.

the SWE extracts (Fig. S3), indicating that hemicelluloses were less hydrolyzed in PEE than in SWE.

3.4. *Antioxidant activity of extracts produced by subcritical water extraction and pressurized ethanol extraction of spent substrates of shiitake and oyster mushrooms*

The antioxidant activity of biological and chemical molecules is routinely assessed in food, pharmaceutical, and biological research using several *in vitro* tests. The Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging capacity are among the most extensively used antioxidant assays. These three methods were employed in this study to evaluate the antioxidant properties of extracts produced at 175 °C using both subcritical water extraction (SWE) and pressurized ethanol extraction (PEE).

The experiments revealed different trends for the two SMS types depending on the extraction method. For example, for subcritical water extraction, the shiitake SMS extracts displayed a better DPPH radical scavenging capacity than the oyster mushroom extracts ($p < 0.0001$, two-way ANOVA; Fig. 9-A). On the other hand, for the pressurized ethanol extraction, the Oy-SMS showed a higher percentage of inhibition of the DPPH radical than the values shown by the Sh-SMS extracts ($p<0.02$).

In the SWE extracts, DPPH radical inhibition increased with the holding time for both SMS, reaching 85.6 % for Sh-SMS and 60.3 % for Oy-SMS in the partially isothermal extraction with a 30 min holding time (experiment PI30–175) (Fig. 9-A). When compared to the water extract in the characterization (Table 3), the PI30–175 SWE extracts showed a 315 % increase in DPPH radical inhibition for Sh-SMS and a 202 % increase for Oy-SMS. This increase could be attributed to the enhanced extraction of antioxidant compounds, such as phenolic compounds or β-glucans, by subcritical water at high temperatures compared to boiling-point water used in the Soxhlet extraction during characterization. Furthermore, the formation of neo-antioxidants during subcritical water extraction, as a consequence of thermo-oxidation, Maillard reactions, and caramelization, is a possibility that has been previously demonstrated (Plaza et al., 2010).

In the PEE extracts, a different trend was observed (Fig. 9-B). For both SMS, the DPPH radical inhibition decreased with increasing holding time ($p < 0.01$, two-way ANOVA). It is noteworthy that under the same conditions, Oy-SMS extracts showed higher DPPH inhibition in PEE than in SWE, while Sh-SMS showed lower DPPH inhibition in PEE compared to SWE. The better results of the Oy-SMS PEE extracts align well with the results of the initial characterization of the SMS, where Oy-SMS ethanol extract displayed considerably higher DPPH radical inhibition compared to Sh-SMS extract (Table 2).

The results of the TEAC assay were consistent with those of the DPPH assay (Fig. 9). For the PEE extracts, the TEAC results followed a similar trend to that of the DPPH radical inhibition, with Oy-SMS extracts showing higher activity than Sh-SMS extracts, and a decrease in activity observed over the holding time ($p<0.02$, two-way ANOVA) (Fig. 9-D). In the SWE extracts, except for the PI15–175 experiment, TEAC activity was higher in Sh-SMS extracts compared to Oy-SMS extracts ($p<0.001$) (Fig. 9-C). The highest activity for Oy-SMS (2.7 mg Trolox equivalent/mL extract) was observed in the extract produced by SWE with a 15-minute holding time at 175 °C (experiment PI15–175). In comparison, the highest activity for Sh-SMS (4.3 mg Trolox equivalent/mL extract) was observed after a 30-minute holding time (experiment PI30–175). As expected, Oy-SMS showed higher TEAC activity in PEE than SWE under the same conditions ($p<0.02$). However, TEAC activity was lower for SH-SMS in PEE compared to SWE ($p<0.03$).

The FRAP activity in the PEE extracts exhibited a different trend from that observed in the DPPH and TEAC assays, with Sh-SMS showing higher activity than Oy-SMS and the activity increasing over time for both SMS types ($p<0.01$, two-way ANOVA; Fig. 9-F). The activity in the PEE extracts was higher than that in the SWE extracts under similar conditions for both SMS but was most pronounced at NI-175. For the

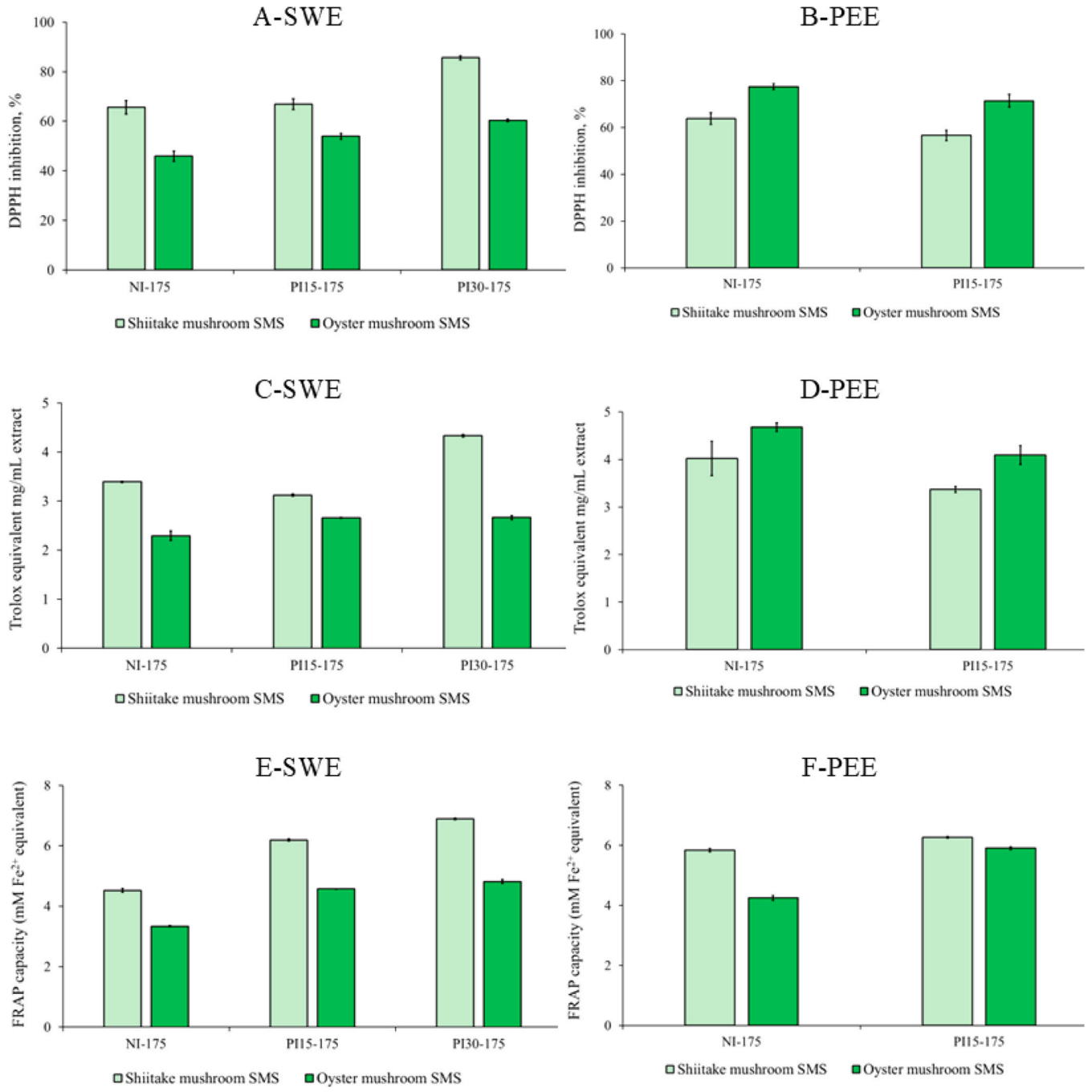

Fig. 9. Antioxidant properties of extracts obtained by subcritical water extraction (left) and pressurized ethanol extraction (right), assessed by DPPH (A, B), TEAC (C, D), and FRAP (E, F) assays. Mean values are from triplicate measurements for DPPH and FRAP, and duplicate measurements for TEAC.

SWE extracts, the FRAP results displayed a similar trend to that of the DPPH radical inhibition, with Sh-SMS (4.5–6.9 mM Fe^{2+}) showing higher activity than Oy-SMS (3.3–4.8 mM Fe^{2+}), and the activity increasing over time ($p < 0.0001$) (Fig. 9-E). In general, these results for the SWE and PEE extracts align well with the characterization results, where Soxhlet water extracts exhibited higher FRAP activity than Soxhlet ethanol extracts, and Sh-SMS extracts showed higher activity than Oy-SMS extracts (Table 2).

The comparable results obtained from the DPPH (Fig. 9-A) and TEAC (Fig. 9-B) assays for the extracts from Sh-SMS and Oy-SMS produced by subcritical water extraction and pressurized ethanol extraction can be explained by the fact that both methods assess free radical scavenging activity. Although the results of the DPPH and TEAC assays may vary slightly due to the different radical species used, the general antioxidant capacity of a sample, as measured by both assays, tends to align closely (Apak et al., 2018). Similarly, the differing results between the FRAP assay, on the one hand, and the DPPH and TEAC assays, on the other hand, can be attributed to their distinct methods of assessing antioxidant capacity. While the DPPH and TEAC assays measure radical scavenging, the FRAP method assesses electron donation and the reduction of metal ions, specifically the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}).

3.5. *Correlation of the antioxidant activity of the extracts with their phenolic acids*

The antioxidant properties of the extracts were correlated with the

concentrations of phenolic compounds. Among the antioxidant assays used in this work, the FRAP method was initially chosen to establish correlations, enabling comparison with previously generated correlations for extracts of spent substrates from various *Pleurotus* spp. strains, where the FRAP method was also employed (Klausen et al., 2023).

Initially, the correlation between the FRAP activity of the extracts and their total phenolic content was confirmed. For example, in the Oy-SMS extracts, it was found that there is a direct correlation between the FRAP activity (in mM Fe^{2+}) and the concentration of total phenolic compounds (TPC, in g/L) described by the following model:

$$FRAP = 4.3648 \times TPC - 0.8369 \quad (4)$$

The Pearson correlation coefficient (PCC) of the relationship was 0.98, which shows its strength and linearity. The strength and statistical significance of the correlation are confirmed by its high coefficient of determination ($R^2 = 0.96$) and low p-value ($p<0.01$). This result is consistent with previous findings, which show a strong correlation between the FRAP activity in extracts of oyster mushrooms and their total phenolic content (Klausen et al., 2023).

After confirming the strong correlation between FRAP activity and the content of total phenolic compounds, the relationship with the concentrations of phenolic acids in the extracts was assessed. First, the phenolic acids found in higher concentrations in the extracts, i.e., vanillic acid in Oy-SMS extracts and gallic acid in Sh-SMS extracts (Table 6), were examined. Still, no strong correlations were found between the FRAP antioxidant activity and the other parameters. This observation implies that while these phenolic acids are present in significant amounts, their direct contribution to antioxidant activity may not be as strong as expected or that other compounds present in the extracts might have a more dominant role in the antioxidant properties. Therefore, the next step in the study was to assess the effect of phenolic acids found in lower amounts. It was found that ferulic acid displayed the best correlation (PCC = 0.96, $R^2 = 0.92$, p-value < 0.01) with the FRAP activity of the Oy-SMS extracts. Ferulic acid also correlated well with the FRAP antioxidant activity of the Sh-SMS extracts (PCC = 0.97, $R^2 = 0.94$, p-value < 0.01). However, the strongest correlation with the FRAP activity of the Sh-SMS extracts was observed with chlorogenic acid (PCC = 0.98, $R^2 = 0.96$, p-value < 0.01).

Despite being present in very low amounts in the spent substrates of shiitake and oyster mushrooms, these results indicate that ferulic acid and chlorogenic acid make a significant contribution to the antioxidant properties of extracts produced from these SMS by either subcritical water extraction or pressurized ethanol extraction. However, this study is limited to a reduced number of compounds, and it does not imply that other compounds do not contribute to antioxidant activity or that interactions between different bioactive compounds, including those beyond phenolic acids, are not important contributors. The interaction between different bioactive compounds in SMS extracts might be more important than the concentration of any single compound. That will be the focus of future research efforts by this group. The results of the DPPH radical inhibition and TEAC activity were also correlated with different phenolic acids; however, surprisingly, no strong correlations were found.

3.6. Comparative summary of the subcritical water extraction and pressurized ethanol extraction of bioactive compounds from spent substrates of shiitake and oyster mushrooms

For shiitake SMS, subcritical water extraction (SWE) resulted in higher extraction yields (approximately 3–4 % higher on average) than pressurized ethanol extraction (PEE) under both non-isothermal and partially isothermal conditions. On the other hand, for oyster mushroom SMS, the extraction yield was somewhat comparable for SWE and PEE.

For both SMS types, the extraction of total phenolics was more effective with PEE than with SWE, whereas the extraction of total carbohydrates showed the opposite trend. The extraction of β-glucans from Sh-SMS was always better with SWE than with PEE. However, for Oy-SMS, SWE was more effective than PEE under non-isothermal conditions, while both methods showed comparable performance under partially isothermal conditions, i.e., when the extraction temperature was maintained for an extended period.

4. Conclusion

This study evaluated the suitability of subcritical water extraction and pressurized ethanol extraction for recovering bioactive compounds from the spent substrate of shiitake and oyster mushrooms. It was demonstrated that pressurized ethanol extraction is an effective method for extracting phenolic compounds from spent mushroom substrate, whereas subcritical water extraction is more effective for extracting total carbohydrates and β-glucans. Pressurized ethanol extraction yielded extracts with higher antioxidant activity than those produced by subcritical water extraction.

Ethics statement

This study does not contain any human and animal trials. The study was conducted in compliance with relevant codes and legislation and in an ethical manner. The study does not contain any private databases or biological specimens.

CRediT authorship contribution statement

Afrina Akter: Writing – original draft, Visualization, Investigation, Formal analysis. **Sarah J. Klausen:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Luis A. Romero-Soto:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Francisco Diaz:** Investigation, Formal analysis. **Herminia Dominguez:** Writing – review & editing, Supervision. **Shaojun Xiong:** Writing – review & editing, Conceptualization. **Knut O. Strætkvern :** Writing – review & editing, Supervision, Methodology. **Carlos Martin:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.121750.

Data availability

Data will be made available on request.

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Glossary

ABTS: 2,2´-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid)
DPPH: 2,2-Diphenyl-1-picrylhydrazyl
FRAP: Ferric reducing antioxidant power
GAE: Gallic acid equivalent
NI: Non-isothermal
Oy-SMS: Oyster mushroom SMS
PEE: Pressurized ethanol extraction
PI: Partially isothermal

PLE: Pressurized liquid extraction
Sh-SMS: Shiitake SMS
SMS: Spent mushroom substrate
SWE: Subcritical water extraction
TEAC: Trolox equivalent antioxidant capacity