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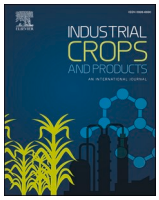

Enzyme-assisted nanofiltration to enrich tannins from softwood bark extract

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ABSTRACT

Softwood bark is a rich source of renewable chemicals, including phenolic compounds known as tannins. Tannins can be extracted from bark with hot water, but the yield can be relatively low, leading to dilute extracts. Next to tannin, a considerable amount of extracted dry matter comprised of carbohydrates, and bark carbohydrates limit the applicability of the extract in applications that demand high purity. This study used membrane filtration to refine and concentrate tannins produced from hot water extracts of softwood bark. Enzymatic hydrolysis of bark carbohydrates prior to membrane filtration was assessed as means to improve the separation of bark sugars from bark phenols. Enzymatic hydrolysis using an optimised enzyme mixture liberated 55% of bark carbohydrates mainly by degradation of pectin and other polysaccharides in spruce unrefined tannins. Separation of bark phenols from the enzyme-treated extract by precipitation at acidic pH and subsequent microfiltration produced low tannin yield. Instead, keeping bark phenols soluble at alkaline pH allowed us to concentrate them 15-fold with good and stable flux using nanofiltration. Additionally, enzymatically liberated sugars permeated well through the nanofiltration membrane, decreasing the free sugar content of the concentrated tannin from 31% to 7.4%. However, diafiltration did not seem to decrease the proportion of free sugars in the concentrate further. This enzyme-assisted nanofiltration concept was successfully upscaled to a pilot scale which was a promising result for improved industrial applicability of softwood bark tannins in the future.

1. Introduction

Softwood bark is a major forest industry co-stream in the Nordic countries. Nowadays, it is mainly used as an energy source, meaning that 10–15% of the dry weight of a tree has only a low-value use (Kemppainen, 2015). Softwood bark is a rich source of renewable chemicals, such as tannins, constituting 10% of bark. Tannin-rich effluents are also produced in debarking at pulp mills, but today they are directed to wastewater treatment plants (Leiviskä et al., 2012). Plant-based tannins are traditionally used in leather tanning, but they also hold potential as biobased chemicals in various applications, such as in replacing phenols in thermosetting resins, like wood adhesives and flocculants in water purification (Pizzi, 1982; Sánchez-Martín et al., 2010; Kayugusuz et al., 2018). They also hold potential in food and beverage, cosmetic and pharmaceutical applications since tannins exhibit antimicrobial, antioxidant and antiviral properties (Burda and Oleszek, 2001; Orlowski et al., 2014; Raitanen et al., 2020; Tapia-Quirós et al., 2022; Amândio et al., 2022; Granato et al., 2022; Pizzi, 2008).

Tannins can be divided into three main groups: proanthocyanidins (also known as condensed tannins), gallo- and ellagitannins and phlorotannins (Quideau et al., 2011). Condensed tannins are comprised of flavan-3-ol units and are found in softwood (Bianchi, 2016). As the term 'tannin' covers a wide range of macromolecules with a molecular weight from 500 to 20,000 Da (Cassano et al., 2003; Romero-Dondiz et al., 2015), bark phenols and bark carbohydrates are referred to in this study for the composition of softwood bark extracts. Bark phenols include various phenolic compounds, mono-, oligo- and polyphenols typically associated with tannins, while bark carbohydrates include various mono-, oligo- and polysaccharides found in bark extracts.

Hot water extraction is a straightforward process widely studied for extracting tannins from softwood bark. The extraction's downside is low bark yield, leading to dilute extracts. For example, in a pilot-scale extraction of industrial bark from spruce (*Picea abies*), a 12% extraction yield was achieved, and 50% of the extract was found to comprise

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tannin (Kemppainen et al., 2014). In addition, to dilute solutions, a considerable amount of extracted dry matter comprises bark carbohydrates that limit the applicability of the extract (Bianchi, 2016). The high carbohydrate content limits the use of the extract in applications where the application is based on the reactivity of the phenolic tannin (Kilpeläinen et al., 2023). Covalently linked carbohydrates block reactive groups in tannins, reducing tannin reactivity in resin or adhesive preparations (Pizzi, 1983). The suitability of bark extracts in various applications would benefit if efficient methods existed to produce refined tannin fraction, i.e. by liberation of bound sugars, fractionation of bark sugars to a separate fraction and concentration of bark phenols.

Covalently bound carbohydrates present in unrefined tannins originate from typical softwood bark polysaccharides, such as hemicelluloses (galactoglucomannans, arabino-4-O-methylglucuronoxylans, galactans, arabinogalactans and arabinans),while the primary polymer released in hot water extraction is pectin, consisting of considerable amounts of galacturonic acid building blocks (Krogell et al., 2012; Le Normand et al., 2012; Kemppainen et al., 2014; Bianchi et al., 2015; Jinze et al., 2023). The association of carbohydrates with tannins in softwood bark is still not fully discovered. Tannins are known for their ability to make complexes with cellulose, pectins and proteins (Haslam, 1988), but studies on the glycosylation of tannins are rare. Every second procyanidin unit of tannin oligomers from spruce bark is estimated to be covalently bound to glucose (Zhang and Gellerstedt, 2008). Spruce bark also contains stilbene glucosides and stilbenes that can be extracted with hot water (Jyske et al., 2014; Gabaston et al., 2017; Halmemies et al., 2022).

Enzymatic hydrolysis can be adopted for the degradation of bark polysaccharides and tannin-associated carbohydrates. Fungal enzymes involved in plant polysaccharide degradation are the best candidates for the cleavage of various carbohydrates and are available on the market. Nevertheless, batch application of commercial cellulase and pectinase cocktails might not be efficient enough to improve yields of monosaccharides in bark hot water extracts (Kemppainen, 2015). Polysaccharide degradation requires combining diverse types of enzymes specific to certain linkages and carbohydrates (Van den Brink and de Vries, 2011). Enzymatic treatment is, nevertheless, challenging due to the inhibition of enzymes by tannin (Tejirian and Xu, 2011) and its ability to bind and precipitate proteins (Soares et al., 2012). It was shown that the presence of negatively charged polysaccharides, such as xanthan, pectin and gum arabic, restrain the formation of protein-tannin aggregates (Mateus et al., 2003). The degradation of polymeric carbohydrates in unrefined tannins decreases their solubility, which is a disadvantage for further processing. A vast majority of data published on the enzymatic treatment of tannins is related to tannases (Mingshu et al., 2006; Bhoite et al., 2015; Lekshmi et al., 2021), while the enzyme-mediated release of carbohydrates associated with tannins is published mainly for stilbenes and flavonoids (Mandalari et al., 2006; Drovou et al., 2015; Mulat et al., 2014; Gabaston et al., 2017; Kornpointner et al., 2022). In this aspect, a detailed study of the treatment of bark hot water extracts with a set of enzymes having a wide range of activities gives an overview of enzymatic modification and further purification of tannins from unrefined hot water extract.

Though hot water extraction is widely known for extracting tannins from softwood bark, less is known about the concentration and purification of tannins. Tannins can be purified using adsorbent to remove carbohydrates (Jyske et al., 2022), but considering life cycle assessment, filtration is showing to be the most promising concentration and purification method of tannins from hot water extracts (Carlqvist et al., 2020; Ding et al., 2017). It was noted decades ago that phenolic compounds' solubility increases with increasing pH (Yazaki, 1987), meaning that low pH can be applied for the precipitation of bark phenols. Still, only 61% recovery of the bark phenols at pH 1.2 was obtained. Additionally, the requirement of substantial amounts of concentrated HCl to decrease the pH, and the instability of polymers in acid, made the purification of bark phenols by acid precipitation impractical. At the time, also microfiltration (MF) assisted ultrafiltration (UF) having a pore size of 10 kDa was tested on a small scale for impurity removal from softwood bark extracts to produce a uniform quality of the bark phenols (Yazaki, 1985). Results showed the good potential of UF to purify and concentrate high molecular weight bark phenols, but improvements, e.g. for the recovery of low molecular weight components, are still needed.

Both UF and nanofiltration (NF) are considered for the tannin enrichment concept, but research regarding membrane filtration is commonly related to the treatment of exhausted tannin liquors from the leather industry. Romero-Dondiz et al. (2016) compared UF membranes having molecular weight cut-off (MWCO) rates of 25 and 50 kDa and thin film composite polyamide NF membranes MWCO of 150–300 kDa for reuse of vegetable tannin liquor and found UF membranes to produce higher flux decline in comparison with NF membranes. Conidi et al. (2017) applied UF flat-sheet membranes for the purification of biologically active phenolic compounds of clarified pomegranate juice from a permeate stream containing mainly glucose and fructose. The concept, which included 150 kDa UF pre-filtration, 2 kDa concentration and diafiltration, was suggested for phenolic compounds recovery. The yield of polyphenols in the concentrate stream was 85%, and the recovery efficiency on the permeate side for glucose and fructose was 90% and 93%, respectively. Only some research has been conducted for membrane enrichment of tannins from wood extracts. Pinto et al. (2017) carried out the recovery of polyphenols and carbohydrates from Eucalyptus bark extract. UF and diafiltration, as well as adsorption and desorption, were proposed to obtain enriched fractions of polyphenols from Eucalyptus bark, with the additional possibility of ethanol and water recovery in both diafiltration and adsorption/desorption steps.

Enzymatic hydrolysis can be used for the liberation of softwood bark sugars, followed by separation from bark phenols via membrane filtration methods. Sugars are naturally in the form of monosaccharides and disaccharides with Mw of 150–350 Da, meaning 1–2 sugar units. The size of sugars directs the selection of membrane pore size for separation. No information is available on enzymatically aided membrane filtration, which targets the separation of softwood bark phenols from bark carbohydrates. In this study, a membrane-based concept was designed to enrich tannins from enzymatically treated hot water extracts of softwood bark.

2. Material and methods

Hot water extracts from softwood bark were enzymatically treated prior to membrane filtration to enable enhanced purification of the tannin-rich extract from excess sugars (Fig. 1). The starting material was the hot water extract, which contained bark phenols and carbohydrates, called 'unrefined tannins' in this study. Enzymes and a membrane system were first selected for the enrichment concept in laboratory-scale screening trials. The best enzymatic hydrolysis and membrane conditions obtained from the laboratory scale were validated in the pilot scale, where tannins were diafiltered to decrease sugar content further. The final stage in piloting was spray drying.

2.1. Softwood bark

The bark residues for the first pilot scale trial were obtained from a Finnish sawmill. The spruce bark contained 20% of wood, and the pine bark contained 30%. Both barks were ground at the mill (Atrex-mill G160, Megatrex Oy) prior to extraction. The bark residues for the following pilot scale work were a new batch of ground Norway spruce bark obtained from a Finnish pulp mill, ground similar to the earlier batch.

2.2. Hot water extraction of unrefined tannins

Three pilot-scale hot water extractions were conducted in batches for Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) bark. The

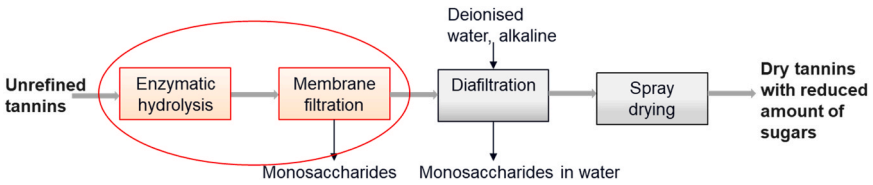

Fig. 1. Schematic picture of the validated pilot process. The research focus was on enzymatic hydrolysis and membrane filtration.

extracts were characterised for total solids (TS), total bark carbohydrates, total bark phenols and average molecular weight of tannin. In the first pilot scale trial, spruce and pine bark were extracted in an 1800 L steel reactor with water at 90 °C for 2 h at 5% concentration (Alakurtti et al., 2018). Residual bark was separated from the aqueous tannin extract using a decanter centrifuge and a bag filter having 100 μm pore size. The dilute solution was then concentrated by evaporation at 0.2 bar at 50 °C applying an evaporation efficiency of 170 L/h. The concentrated tannin extracts were stored at 4°C and used later in the lab scale screening to develop the enzymatic treatments and membrane filtration strategy. For the following pilot scale trials, a new batch of ground Norway spruce bark from a Finnish pulp mill was extracted for 2 h at 110 °C in water (Kilpeläinen et al., 2023). The extract was analysed for tannin content, and 100 l was stored at − 20 °C for further fractionation.

2.3. Selection of enzymes

All the enzymes used in the study were commercial preparations. The preparations are referred to here by their trade names and major enzymatic activities (AB Enzymes): ROHALASE® SEP (β-glucanase, xylanase), Flashzyme® Plus 200 (cellulase, xylanase), Flashzyme® G775 (β-glucosidase), ECOPULP® TX-800 A (endo-1,4-xylanase), ECOPULP® ARL (endo-1,4-glucanase), ROHAPECT® DA12L (pectinase, arabinase), ROHAPECT® B1L (pectinase, cellulase, mannanase), ROHAPECT® PTE 100 (pectin lyase), ROHALASE® BXL (β-1,3-glucanase, β-1,6-glucanase), ROHAVIN® L (pectinase, arabinase), ROHAPECT® MA Decanter (pectinase, arabinase), ROHAPECT® UF (pectinase, arabinose) and ROHAPECT® Classic (pectin lyase) (AB Enzymes, Germany). All the enzymes were tested for their hydrolytic activity on unrefined tannin extracts at optimal conditions, and the best candidates were selected for treatment prior to membrane filtration. The protein concentration of the preparations was measured with the DC Protein Assay (Bio-Rad, USA) using bovine serum albumin (BSA) as a standard and referred to as enzyme dosage further on.

Enzymatic treatments were performed on never-dried tannin extracts. Small-scale tests (1–10 ml) for enzyme selection were conducted in 50 mM sodium acetate at pH 5.0% and 2.5% TS using an enzyme dosage of 1–20 mg protein/g TS. Reactions were run in triplicates for 2 h at 50°C, stopped by the addition of 10 μl of 10 M NaOH and centrifuged at 4000 rpm. The concentration of released carbohydrates was measured in the supernatant using an assay of reducing sugars (Lever, 1972).

Lab-scale enzymatic treatment for membrane selection was performed at optimal hydrolysis conditions in 50 mM sodium acetate at pH 5.0% and 1% TS using an enzyme dosage of 5 mg protein/g TS. The enzyme mixture was composed of ROHAPECT® UF, ROHAPECT® Classic and ROHALASE® BXL in a % ratio 2:2:1. Enzymatic treatment was performed in 3 l volume at room temperature (22 °C) with slow mixing and then divided into two batches. For enzymatically treated spruce tannins, the pH of the batches was adjusted to 12 and 2 after treatment using 0.1 M sodium hydroxide and 40 mM sulphuric acid as final concentrations, respectively, which stopped the enzymatic reaction. Enzymatic treatment for pine tannins was performed as described, but at mild alkaline conditions, pH 9. 25 mM sodium hydroxide as a final concentration was, in this case, used to stop the enzymatic reaction. All the samples were stored at 4 °C prior to membrane filtration.

2.4. Pilot scale enzymatic treatment of unrefined tannins

Spruce bark hot water extract, 100 L, TS 1.19% (Table 2), was thawed at 4 °C prior to use. The pilot scale enzymatic treatment was performed in three batches using a Zirco reactor (40 l) equipped with a blade mixer at 50 °C and pH 5.1. The treatment was conducted for 2 h with slow mixing (20 rpm) in a 50 mM sodium acetate buffer and an enzyme dosage of 10 mg/g TS. The optimised enzyme mixture contained 1.2 g of ROHALASE® BXL, 5 g of ROHAPECT® Classic and 4.95 g of ROHAPECT® UF in 1 l of 0.1 M sodium acetate buffer at pH 5.0, which was also divided into three batches accordingly. The enzymes were inactivated by adding NaOH to obtain the final concentration of 25 mM, which produced pH 8.9 at 50 °C. The hydrolysate was then frozen prior to membrane filtrations.

2.5. Membrane selection

Membrane selection was performed using a rotation-assisted laboratory scale filtration test cell, model XFUFO7601 (Millipore, USA). The effective membrane area in the Millipore device was 30 cm^2, the maximum batch volume was 300 ml, and the driving over pressure was created by air, with a maximum pressure of 6 bar. At acidic pH, the tannin was supposed to be precipitated; thus, MF membranes were tested to achieve the best recovery concept for enzymatically treated tannin employing membrane filtration (Fig. 2). At alkaline pH, the tannin was supposed to be in dissolved form. NF membranes were tested at alkaline conditions since the molecular weight of tannin could be as low as 500 Da, as described earlier.

Two MF membranes, MV020 and MP005 (Microdyn-Nadir, Germany), were studied at pH 2 for rejection of suspended solids (SS), i.e. precipitates of spruce tannin extract. MV020 is an MF membrane having a pore size of 0.2 μm. MP005 is classified as an MF membrane, but the pore size, 0.05 μm, is similar to the pore size of loose UF membranes. The NF membranes NP010 and NP030 (Microdyn-Nadir, Germany) were studied for the enzymatically treated spruce tannin at pH 12 and subsequently enzymatically treated pine tannin at pH 9. These membranes have chemical stability over the full (0−14) pH range; thus, pH 12 could also be used. NP010 is classified as an NF membrane, but the pore size, 1 kDa, is similar to tight UF membranes. NP030 is an NF membrane with a pore size of 500–600 Da. All the membranes were characterised before tannin filtrations by determining pure water flux and salt rejection and comparing the results to the information obtained from the manufacturer (Table 1).

In tannin filtrations, the pressure used depended on the membrane, being 0.5 bar and 1 bar for MF membranes MV020 and MP005 and 6 bar

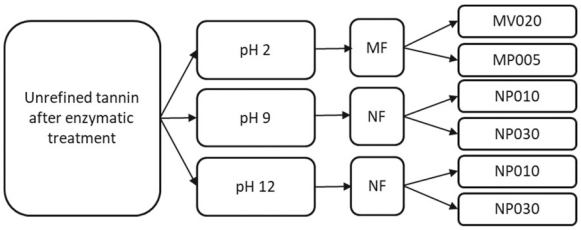

Fig. 2. Scheme of membrane testing strategy.

Table 1
Membranes used in the study.

Membrane	Material	Pore size	Permeability from manufacturer LMH/bar	Na_2SO_4 rejection from manufacturer %	Measured permeability LMH/bar	Measured Na_2SO_4 rejection %
MV020	PVDF	0.2 μm	>700		5500	
MP005	PES	0.05 μm	>285		1660	
NP010	PES	1000–1200 Da	>5*	35–75*	9 ± 3**	62 ± 4**
NP030	PES	500–600 Da	>1*	80–95*	2.1 ± 0.1**	73 ± 3**

*Measured at 40 bar; ** Measured at 6 bar

for NF membranes, respectively. Filtrations were performed at room temperature, 22 °C. Lab scale filtrations were short to control fouling in rejection tests of the membranes studied. The performance of each filtration was estimated based on the achieved permeabilities, i.e. flux divided by pressure, and based on the rejection of phenols, analysed as described below.

2.6. Pilot filtration

Based on the membrane selection tests, enzymatically treated spruce tannin having pH 9 was chosen for the pilot filtration. The selected pH enabled the usage of thin film polyamide membranes (PA-TFC) with a pH range from 2 to 10 to be used for tannin concentration and purification by diafiltration. MF was carried out prior to NF to ensure SS removal from the feed, hence eliminating SS fouling. The tannin sample was filtered by 5 μm nominal rating depth cartridges in Watman FP3 9 3/4 housing. Concentration and diafiltration of the MF permeate were performed using an NFG-3-2540HM spiral wound element, which has a PA-TFC membrane with MWCO of 600–800 Da (Synder Filtration, USA). The membrane element has a spacer thickness of 1.168 mm (46 mil), resulting in an effective membrane area of 1.95 m^2. Membrane characterisation of the element was done by pure water flux and salt rejection test using 2000 ppm $MgSO_4$ at 7.6 bar (110 psi) and 25 °C before concentration and diafiltration, resulting in 123 LMH pure water flux and 48.2% rejection, respectively. The function of the element was acceptable since the membrane manufacturer informs an average $MgSO_4$ rejection of 50% and typical operating fluxes in the range of 93-102 LMH (55-60 GFD).

Membrane concentration was conducted using a pressure of 6 bar and a temperature of 50 °C. The temperature was the maximum for the membrane, and it was used to have as low a viscosity as possible in the water phase containing the tannin during concentration. The concentration performance was evaluated based on the flux or permeability, achieved tannin concentration and tannin concentrate quality. The final volume reduction factor (VRF) was calculated based on the water recovery (*WR*), i.e. the per cent recovery of permeate from the feed. Diafiltration of the concentrate was carried out by adding deionised water with pH 9, adjusted using NaOH, at the same rate as the permeate flux.

After membrane treatment, the tannin was dried using a rotary atomiser spray dryer, where the feed was centrifugally accelerated to high velocity in the atomiser wheel before being discharged into hot drying gas. Tannin drying was carried out using a Niro P-6.3 Spray Dryer (GEA Process Engineering A/S, Denmark) at a feed capacity of 21 kg/h with temperatures of 180 °C in and 80 °C out.

2.7. Analyses

The sugar composition of softwood bark extracts was analysed with acid methanolysis (Sundberg et al., 1996) using methods described by Kilpeläinen et al. (2014). Briefly, extract samples were frozen, freeze-dried and then depolymerised by acid methanolysis at 105 °C for 3 h. Samples were silylated and analysed with gas chromatography using a flame ionisation detector (GC-FID, Shimadzu GC-2010, Kyoto, Japan). Method was used to analyse glucose (Glu), mannose (Man), galactose (Gal), xylose (Xyl), arabinose (Ara), glucuronic acid (GlcA), galactouronic acid (GalA), rhamnose (Rha) and 4-O-methyl-glucuronic acid (MeGlcA) in samples. Monomeric sugars in the extracts were analysed using the same GC-FID system without prior acid methanolysis (Raitanen et al., 2020). In small-scale tests, the concentration of liberated sugars was estimated by reducing sugar assay (Lever, 1972). In the pilot scale, enzymatic treatment total carbohydrates were analysed with high-performance liquid chromatography (HPLC) after acid hydrolysis, including only neutral sugars. Liberated monosaccharides were analysed as such, as described in Kemppainen et al. (2014).

A UV-280 method was used to measure the concentration of bark phenols. The extracts were analysed by direct UV measurement at 280 nm for the total aromatic material (Antoine, 2004) using a reference standard curve with Tannino QS-SOL (Silvateam S.p.A., Italy). Bark phenols for the first estimation of filtration performance were analysed using a spectrophotometer DR3900 and cuvette tests LCK345 (phenols 0.05–5 mg/l) or LCK346 (phenols 5–200 mg/l), wherein the presence of an oxidising agent ortho- and meta-substituted phenols form coloured complexes with 4-aminoantipyrine (AAP). The values obtained with the method were smaller than those obtained with the UV-280 method since the UV-280 method measures total aromatic material.

The molar mass measurements for the bark phenols were performed by size exclusion chromatography (SEC) with UV-detection and analysed as described in Jääskeläinen et al. (2017). TS content of the fractions was analysed using standard method SFS 3008, in which the solids content was determined as a ratio of weights obtained before and after the drying process in an oven at a temperature of 105 °C until a steady mass was obtained. TS content of the spray-dried product was analysed using Mettler Toledo HR73 Halogen Moisture Analyzer.

3. Results

3.1. Characterisation of softwood bark extracts

The unrefined tannins from spruce and pine bark had a high content of carbohydrates (Table 2). Approximately half of the dry solids in spruce bark extract consisted of carbohydrates, whereas in pine bark extract, two-thirds consisted of carbohydrates. These results are consistent with the research of Bianchi et al. (2015), where a high proportion of carbohydrates in hot water extracts of Scots pine bark was found when comparing the compositions of bark extracts from European softwood species. In addition, the extraction yield from spruce bark extract 1 was two times higher than the extraction yield from the pine bark extract. The average molecular weight of tannin was found to be similar, ca. 2000–3000 Da, in the spruce bark extract 1 and pine bark extract, while for spruce bark extract 2, it was almost two times higher.

Due to a higher extraction yield and higher polyphenol (tannin) content in the spruce bark extract 1 compared to the pine bark extract, the work was continued with spruce bark. Spruce bark extraction was repeated to provide material for pilot scale enzyme treatment and filtration trials, obtaining spruce bark extract 2. Nevertheless, the spruce bark extraction 2 provided a considerably lower extraction yield than extraction 1, 7.2% and 10.9%, respectively. This was most likely due to the extraction's too-fine bark particle size. The small particle size was found to cause dense packing of the extracted bark, which had a

negative impact on filtration efficiency and, thus, the extraction yield. Extraction temperature could also promote condensation of polyphenols and form furfurals that reduced overall extraction yield.

3.2. Enzymatic treatment

The spruce bark extract 1 and pine bark extract were treated first at a small scale with 13 commercial enzyme preparations used for the liberation of bark sugars. The aim was to degrade oligo- and polysaccharides found in unrefined tannin extracts and release sugars covalently bound to bark phenols to assist permeation of liberated small sugar molecules, mostly monosaccharides having an Mw less than 200 Da, in subsequent membrane filtration. This was supposed to enable the enrichment of bark phenols from refined tannin fraction. The used commercial enzymes covered most of the enzymatic activities required for bark polysaccharide degradation, according to the sugar profile of spruce and pine bark hot water extracts (Raitanen, 2020). The best enzymes in polysaccharide degradation were pectin lyases and pectinases on both extracts, while cellulases, xylanase and beta-glucosidase were inefficient on bark extracts (Fig. S1). However, the enzymes with identical major activities had, at times, different effects on unrefined tannins (Fig. 3). This was observed when two studied pectin lyases performed differently, ROHAPECT® Classic and ROHAPECT® PTE 100. ROHAPECT® Classic liberated 34% of the total bark carbohydrates when using maximum enzyme dosage, while only 2.5% of the total bark carbohydrates were released by ROHAPECT® PTE 100 (Fig. 3A). A similar result was observed for glucanases, ROHALASE® BXL and ROHALASE® SEP, obtaining 16% and 2% yields, respectively (Fig. 3A). Among pectinases, ROHAPECT® MA Decanter was the most efficient, producing 32% yield, while ROHAPECT® B1L released only 2% of total carbohydrates (Fig. 3A).

The concentration of sugars liberated by means of enzymatic treatment was slightly different for spruce and pine unrefined tannins, 34% and 37% of the total bark carbohydrates, %, respectively. Increasing enzyme dosage was more efficient with pine. At the same time, with spruce, the plateau of liberated was reached already at 20 mg of enzyme per 1 g TS (Fig. 3), indicating that the limiting step for the efficiency of enzymatic treatment was probably the structural differences of bark phenols and bark carbohydrates for these two wood species. For example, glucanase ROHALASE® BXL performed better than pectinase ROHAPECT® DA12L for unrefined pine tannins, suggesting easier degradation of glucan moieties and less inhibition of the enzyme by pine tannins. In contrast, the formed plateau of liberated sugars indicated the high inhibitory effect of spruce tannins.

Pectin lyase ROHAPECT® Classic was the most efficient on both bark extracts, followed by pectinases ROHAPECT® MA Decanter and ROHAPECT® UF. Pectin-active enzymes have broad industrial applications, including extraction and clarification of fruit juices and wines (Kohli and Gupta, 2015), which are known to be rich in condensed tannins (Watrelot et al., 2017). Softwood bark hot water extracts, however, are much more recalcitrant than fruits, and a combination of several enzymes was needed to increase sugar yields. To improve the yields of liberated sugars, different combinations of enzymatic activities were tested. Their synergistical action was needed to successfully cleave off bound sugars in soluble and insoluble pectins and arabinans when present in spruce bark extracts. The most promising combinations were pectinase together with pectin lyase.

In the optimised mixture, pectin-degrading enzymes were supported by glucanase ROHALASE® BXL to ensure the liberation of monosaccharides (MW < 200 Da), which was meant to improve the removal of bark sugars from bark phenols by means of nanofiltration. The optimally designed enzyme mixture contained 46% of pectin lyase ROHAPECT® Classic, 44% of pectinase ROHAPECT® UF and 11% of glucanase ROHALASE® BXL. The hydrolysis reactions of spruce bark extract 1 were tested with an optimised enzyme mixture loading 5 mg/g TS and 10 mg/g TS. The liberated sugars were 40% and 50% of the total carbohydrates, respectively. Thus, the optimised enzyme mixture showed at least a 20% increase in sugar yield compared to pectin-active enzymes acting alone (Fig. 3A). This optimised mixture was used for membrane screening and pilot-scale treatment of unrefined spruce tannins.

3.3. Membrane selection

The spruce bark extract 1 and pine bark extract (Table 2) were used for selecting adequate membranes to concentrate and fractionate the extracts. Filtration time in membrane selection was kept short to prevent

Table 2
Characterisation of hot water extracts from Norway spruce and Scots pine bark. The spruce bark extract 1 and pine bark extract were used to screen appropriate enzymes and membranes. The spruce bark extract 2 was used for piloting the selected enzyme treatment and membrane filtration concept.

	Spruce bark extract 1	Pine bark extract	Spruce bark extract 2
Extraction yield from bark (%)	10.9	5.1	7.2
Total solids (g/l)	50.0	48.5	11.9
Bark carbohydrates (g/l)	17.2	21.1	5.1
Bark phenols (g/l)*	22.5	15.1	6.8
Tannin molecular weight, average (Da)	2790	2080	4980

* Bark phenols were measured by UV-280 method and referred as tannins

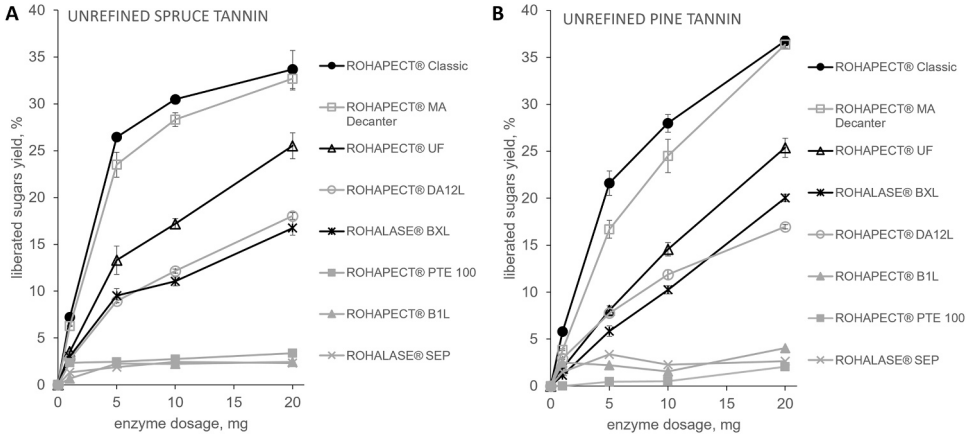

Fig. 3. Sugars liberated from A) unrefined spruce tannins and B) unrefined pine tannins during a 2 h enzymatic treatment by commercial enzymes preparations at 50 °C and pH 5, expressed as a percentage of total bark carbohydrates. Commercial enzymes selected for the optimally designed enzyme mixture are coloured in black.

fouling from interfering with the rejection results. Both studied MF membranes produced stable fluxes and permeabilities in short filtrations. The more open membrane MV020 produced double permeability (Fig. 4) but lower rejections of precipitated bark phenols at pH 2 than the MP005 membrane (Table 3). The bark phenol rejection was 58% for the MV020 membrane and 66% for the MP005 membrane.

In the case of spruce tannin extract 1 at pH 12, low permeabilities and clear permeates were true, especially with the NP030 membrane, and good rejections were obtained for both NF membranes (Fig. 5, Table 3). Additionally, the permeabilities were stable in short filtrations. The permeability of NP010, 3.3 LMH/bar, was one-tenth of the most open membrane MV020 and three times higher than that of the NP030 membrane. Bark phenols rejections were 94% and 98% for NP010 and NP030, respectively. In the case of pine bark extract at pH 9, low permeabilities, very clear permeates, and good rejections were obtained using both NF membranes (Fig. 5, Table 3). The permeabilities were lower for the pine bark extract at pH 9 when the bark carbohydrates concentration in the feed extract was higher and the bark phenols concentration lower (Table 2) than that of spruce bark extract at pH 12. Higher carbohydrate content created higher fouling potential since enzymatically liberated monosaccharides (Mw < 200 Da) were supposed to permeate the membrane. A high concentration of compounds inside the membrane increased the potential for adsorption; thus, some fouling occurred. Additionally different pH may have somewhat affected the permeabilities. Bark phenols rejection was slightly higher for NP030 than NP010, 97% and 94%, respectively.

Bark phenols rejections were clearly better when using NF membranes for bark extracts at alkaline pH, than when using MF membranes for partly precipitated bark extracts at acidic pH. For this reason, adjustment to alkaline pH after enzymatic treatment and NF membranes were selected conditions for pilot filtration. Spruce was preferred for pilot tests due to the possibility of higher yield and lower bark sugar contents, which could also lower membrane fouling. Since enzymatically treated spruce bark extract 1 at pH 9 behaved nearly as well as the same extract at pH 12 regarding fluxes and permeate qualities of NF, extract pH 9 was selected to pilot filtrations. Employing pH 9 would mean less chemical usage in the treatment concept, thus fewer costs when carrying out the tannin purification and concentration at a larger scale. Lower pH is also easier for the membrane filtration concept since more membranes are available for pH 9 instead of pH 12. For example, most polyamide thin film NF membranes do not stand a process pH of 12. NP030 having a nominal cut-off rate of 500–600 Da, produced better permeate quality than NP010, having a nominal cut-off rate of 1000 Da, thus better rejection of bark phenols. Since there was no explicit option for an NF membrane for the pilot test, an NF membrane having a pore size of 600–800 Da, in between the pore sizes of NP010 and NP030, was selected for piloting.

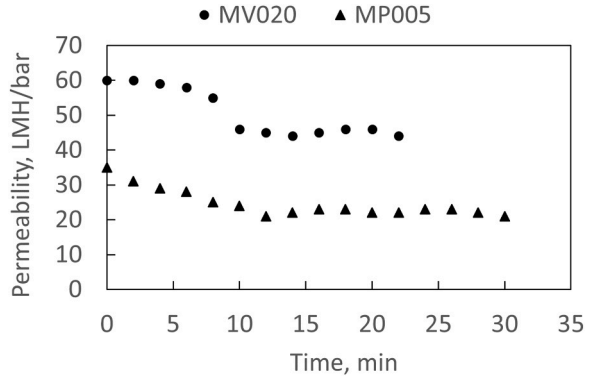

Fig. 4. MF permeabilities in short filtrations of the enzymatically treated spruce bark extract 1 at pH 2.

Table 3
Bark phenol concentrations of the enzymatically treated spruce bark extract 1 as feeds and obtained permeates.

pH	Sample	Bark phenol mg/l	Bark phenol rejection %
2	Feed	265	
	MV020 permeate	110	58
	MP005 permeate	91.2	66
12	Feed	266	
	NP010 permeate	15.8	94
	NP030 permeate	4.8	98
9	Feed	196	
	NP010 permeate	11.3	94
	NP030 permeate	6.2	97

3.4. Pilot scale enzymatic treatment

In the pilot scale, bark extract 2 was treated with the optimised enzyme mixture, and dosage was chosen based on small-scale results. About 55% of bark carbohydrates were liberated as monosaccharides in the pilot-scale enzyme treatment of unrefined spruce tannin. The major sugars released were galacturonic acid, arabinose, glucose and galactose (Fig. 6). Galacturonic acid constitutes the backbone of pectin, while arabinose might be a part of pectin or originate from arabinoglucuronoxylan from wood or inner bark part of industrially processed bark samples (Jinze et al., 2023). Glucose could also originate from starch. Almost complete liberation of galacturonic acid confirmed degradation of pectin in spruce bark hot water extracts.

Interestingly the average Mw and polydispersity of bark phenols increased for the enzymatically treated fraction (Table S1), which might be caused by polymerisation or agglomeration of tannins happening along with enzymatic degradation of bark carbohydrates. Freezing of the enzymatically treated extract resulted in spontaneous fractionation of the minor amount of high molecular weight tannins (Table S1), which were not included in pilot filtration.

3.5. Pilot scale nanofiltration

For pilot filtration, the enzyme-treated unrefined spruce bark extract 2 was first pre-treated by MF to remove SS before filtration with spiral wound NF element, NFG-3-2540HM. The selected polyamide-based NF membrane worked well in the filtration of extract 2 at pH 9 regarding the obtained flux and *WR* (Fig. 7). The flux at the start was 42 LMH meaning the permeability of 7 LMH/bar, which is good for NF. Additionally, a very high *WR* 90%, thus a high VRF 10, was obtained, which, together with good flux, indicates low fouling. The flux at *WR* 90% was 5 LMH meaning the permeability was 0.83 LMH/bar. The decrease seemed to occur mainly due to a concentration increase in the feed; thus, osmotic pressure increased. The flux in pilot NF was even better than those obtained at lab-scale membrane screening using either PES membranes. It is noteworthy, that the TS content of the extract was lower, and Mw was higher in piloting (Table 2) than in membrane screening which might have improved membrane performance in piloting. The permeability increased in diafiltration to 2.7 LMH/bar.

The rejection of bark phenols when using the NF membrane was good, 96%. Thus, the bark phenols concentration of the concentrate was 15 times higher than the bark phenols concentration in the 5 μm filtrate, which was feed to NF (Table 4). By contrast, the rejection of free sugars was poor, 58%; thus, they were separated well from rejected polyphenols. The NF feed contained 31% of free sugars and permeate 91% of free sugars, while the NF concentrate contained only 7.4% free sugars (Table 4, Fig. 8). Diafiltration did not decrease further the content of free sugars in the NF concentrate. Mass balance calculation and bark phenols analysis indicated some losses at each separation step. The loss of bark phenols in MF pre-treatment was 20%, in NF concentration 3.5% and in diafiltration 1.0% (Table 4). Thus, the loss was the highest during pre-

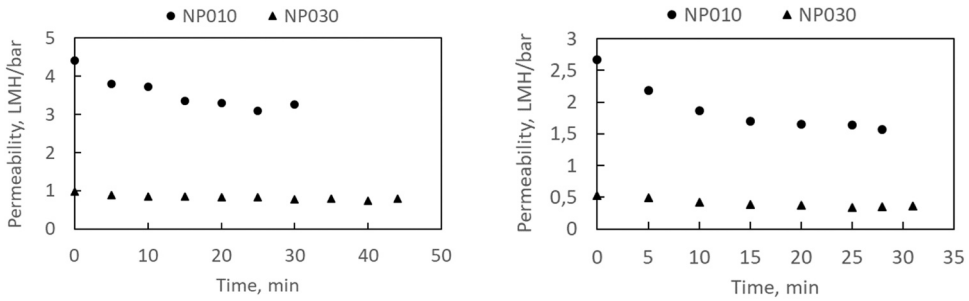

Fig. 5. NF permeabilities in short filtrations for enzymatically treated spruce bark extract 1 at pH 12 (left) and pine bark extract at pH 9 (right).

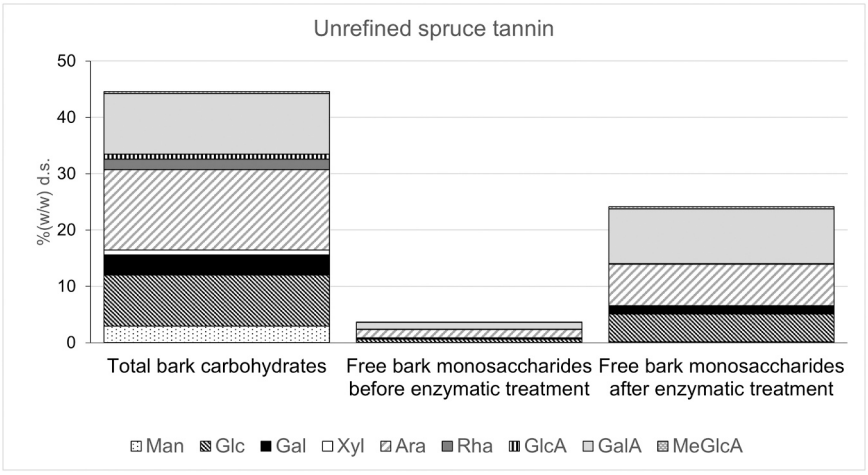

Fig. 6. Carbohydrate composition of unrefined spruce tannins before and after enzymatic treatment at pilot scale.

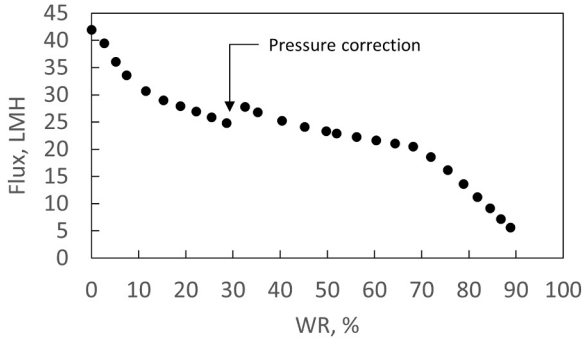

Fig. 7. NF flux during concentration of piloting.

treatment. Finally, 8.9 kg of enriched tannin was obtained from 100 kg of unrefined tannins. After the membrane concept, tannin was spray dried to the dry solids content of 96%, when the yield was 500 g dried tannins with a reduced amount of sugars.

4. Discussion

The results clearly show that pine and spruce bark extracts are different, and pine extract is easier material for the enzymatic liberation of bark sugars compared to spruce bark extract. This is likely explained by the higher content of bark phenols in spruce bark extracts and higher Mw of the spruce tannins, causing more potent inhibition of the enzymes in the current study. Nevertheless, the overall yield of liberated sugars was promising, and the enzymatic treatment of spruce bark extract was successfully upscaled. The number of liberated sugars was increased more than four times by enzymatic treatment.

Selected commercial enzyme preparations are typically used in grape processing and advanced maceration of pome fruits, especially targeting grape skin rich in tannins. The resistance of the enzymatic cocktails to precipitation by tannins probably was an essential characteristic for an efficient enzymatic treatment of spruce bark extracts. It is known that tannins form complexes with proteins (Haslam, 1974), and the protein precipitation method (Hagerman and Butler, 1978) has been widely used for the quantitative determination of tannins for over 40 years. The severity of precipitation might depend on enzyme properties, i.e.

Table 4
MF and NF results from pilot filtration.

Sample description	Mass kg	pH	Total solids g/l	Bark phenols g/l	Total bark carbohydrates g/l	Free bark monosaccharides g/l
Enzyme-treated extract	100.5	8.6	18.0	4.9	5.2	2.87
Suspended solids from MF	3.7	8.9	54.9	26.5	7.0	0.09
5 μm filtrate	96.3	8.6	162	2.2	6.2	2.61
NFG concentrate	10.0	8.4	85.4	33.6	42.3	5.63
NFG permeate	85.8	8.6	8.1	0.2	1.3	1.20
NFG wash concentrate	8.9	8.2	84.8	31.5	39.4	3.96
NFG wash permeate	17.6	8.3	6.2	0.2	1.3	2.03

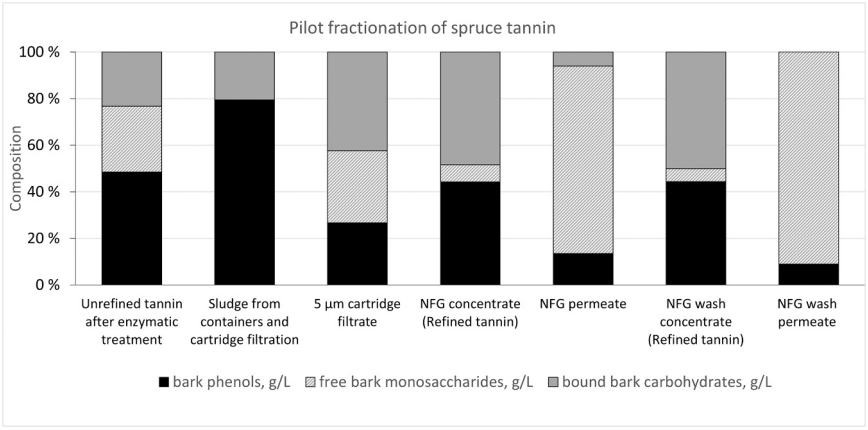

Fig. 8. Composition of spruce tannin fractions during pilot fractionation.

surface-exposed amino-acid composition (Adamczyk et al., 2011). Additionally, the composition of bark extracts and Mw of tannins affect enzymes differently, as was shown in enzymatic treatment experiments carried out side-by-side for spruce and pine bark. Additives used in commercial enzyme preparations might also prevent the binding of tannins to the enzymes. This may explain why enzymatic preparations with a similar type of action had a different effect on unrefined tannins. Previously the saccharification of spruce bark hot water extraction residue was reported by Kemppainen et al. (2012), who showed the efficiency of cellulase and pectinase activities. However, no data on the enzymatic treatment of liquid fraction is available to date.

Since the structure of polysaccharides and their conjugates with bark phenols released into solution during hot water treatment are unknown, it is hard to prove whether enzymes managed to cleave off external water-exposed sugar units in tannins or just degraded pectic polysaccharides not bound to tannins. Based on the results, most liberated sugars came from the degradation of pectin and hemicelluloses. Along with the hydrolytic activity of an enzymatic cocktail targeting sugar liberation, one of its components, pectin lyase, might initiate the polymerisation of phenolic units via radical coupling with possibly oxidised sugar intermediate. A more detailed analysis of tannin structure is required to propose the enzymatic mechanism of tannin polymerisation, which was not in the scope of this paper.

In general, the 5 µm filtrate was considered a feed to NF and purified tannin with enriched bark phenols and reduced free sugar content was successfully obtained via enzymatic treatment. Additional investigation on enzymatic modification of spruce bark hot water extracts is needed to find more efficient enzymes with reduced cost to liberate bark sugars for a feasible industrial application.

Precipitation at low pH and subsequent MF produced low recovery of bark phenols. They only partly precipitated at pH 2, and the concept based on tannin precipitation should include an additional rejection step for soluble tannin. Instead, NF rejected polyphenols well, 98%, when the membrane's pore size was 600 Da and pH was 12. For pilot filtrations, a bit larger pore size was used to increase the flux, pH was lowered to 9, enabling a polyamide NF membrane to be used for concentration, and tannin concentrate was diafiltered at the same pH to obtain even purer and more concentrated tannin fraction than obtained in one-step NF. However, diafiltration did not improve either purity or concentration. Thus, liberated sugars could already permeate well enough through the membrane when concentrating bark phenols up to a high VRF, 10. Bark phenols concentration increased even more than the VRF; hence 15 times higher concentration was achieved in the NF concentrate than in the feed. This was probably due to additional concentration via evaporation when filtering at 60 °C.

Membrane fouling is a challenge in the filtration of tannin-rich solutions. With careful selection of membrane pore size and filtration circumstances, the problems related to fouling can be reduced. Low MWCO membranes have a less severe fouling phenomenon than high MWCO membranes (Romero-Dondiz et al., 2015). The greater the adsorption of tannin on the membrane, the greater the flux decline. Additionally, the adsorption of tannin can gradually decline the rejection of tannin (Guo et al., 2010). Oligomeric carbohydrate impurities can also cause problems since they have a molecular size close to that of tannins (Pinto et al., 2014). Membrane performance is also influenced by membrane morphological structure, charge, surface roughness and porosity (Pinto et al., 2017; Guo et al., 2010). In this study, no severe fouling or decline of rejection was observed caused by the adsorption of bark phenols on the NF membrane or the permeation of free sugars through the NF membrane. The choices of membrane and parameters for piloting produced better fluxes with the polyamide NFG spiral wound membrane than those obtained in membrane screening. However, a longer pilot period is needed in order to see the frequency need for washing cycles and the membrane lifetime.

Good flux in bark phenols concentration was obtained after removing SS by cartridge MF as a pre-treatment. However, bark phenols (tannin) loss can be high during pre-treatment if SS content is high, as seen in this study. High SS content could have come from freezing the sample before filtration. Bark phenols should have been concentrated straight after the enzymatic hydrolysis. Here we can also speculate that enzymatic removal of covalently linked carbohydrates increases tannin reactivity and promotes aggregation. The effect of temperature is also essential. If tannin extract contains a lot of SS, cartridge MF is not the most suitable pre-treatment; instead, e.g. a pressure filter can be recommended.

The enzyme-assisted nanofiltration concept, built up to increase bark phenols concentration and reduce free sugars concentration in refined tannin, widens the applicability of tannin extract for different applications. Diafiltration, by contrast, seems not to improve the tannin applicability further as it did not increase the removal of free sugars in proportion to bark phenols. Thus, it is not a suggested step in the tannin enrichment concept. Instead, spray drying was proven to be a suitable technology for producing dry tannin powder for use.

5. Conclusions

Softwood bark is a rich source of renewable chemicals, including condensed tannin. The yield of tannin extracted from softwood bark is typically low, leading to dilute extracts. In addition, a considerable amount of extracted dry matter comprises carbohydrates that limit the applicability of the extract. The suitable composition of enzymes studied here improved bark sugars liberation for subsequent removal from tannin by membrane filtration. Bark phenols could be concentrated at an alkaline pH 15-fold by pilot scale NF with good fluxes. Additionally,

liberated sugars permeated well through the NF membrane, decreasing the free sugar content of the concentrated tannin, meaning improved applicability of tannin extract for applications.

CRediT authorship contribution statement

Hanna Kyllönen: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Anna Borisova**: Methodology, Investigation, Writing – original draft, Writing – review & editing. **Juha Heikkinen:** Investigation, Writing – original draft. **Petri Kilpeläinen**: Methodology, Writing – review & editing. **Jenni Rahikainen**: Conceptualization, Project administration, Writing – review & editing. **Christiane Laine**: Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

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

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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.2023.117441.

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