Phenolic residues in spruce galactoglucomannans improve stabilization of oil-in-water emulsions

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ABSTRACT

Hypothesis. Amphiphilic character of surfactants drives them at the interface of dispersed systems, such as emulsions. Hemicellulose-rich wood extracts contain assemblies (lignin-carbohydrate complexes, LCC) with natural amphiphilicity, which is expected to depend on their chemical composition resulting from the isolation method. Lignin-derived phenolic residues associated with hemicelluloses are hypothesized to contribute to emulsions’ interfacial properties and stability.

Experiments. We investigated the role of phenolic residues in spruce hemicellulose extracts in the stabilization of oil-in-water emulsions by physical and chemical approach. Distribution and changes
occurring in the phenolic residues at the droplet interface and in the continuous phase were studied during an accelerated storage test. Meanwhile, the physical stability and lipid oxidation in emulsions were monitored.

**Findings.** Naturally associated lignin residues in GGM act as vehicles for anchoring these hemicelluloses into the oil droplet interface and further enable superior stabilization of emulsions. By adjusting the isolation method of GGM regarding their phenolic profile, their functionalities, especially interfacial behavior, can be altered. Retaining the native interactions of GGM and phenolic residues is suggested for efficient physical stabilization and extended protection against lipid oxidation. The results can be widely applied as guidelines in tailoring natural or synthetic amphiphilic compounds for interfacial stabilization.

**Keywords**

Spruce galactoglucomannans, phenolic residues, emulsion stability, lipid oxidation

**Abbreviations**

eTMP ethanol precipitated galactoglucomannan (GGM) from thermomechanical pulping, LCC lignin-carbohydrate complexes, OHB hydroxybenzoates, OHC hydroxycinnamates, PE spruce phenol extract, PHWE GGM from pressurized hot-water extraction, PR phenolic residue, PV peroxide value, sTMP spray-dried GGM from thermomechanical pulping.

**Introduction**
To deliver and protect lipophilic compounds in both technical applications and foods, lipids are preferably dispersed into polar media, most often water. This formulation — emulsion — enhances the physical properties of the product and enables efficient delivery of bioactive compounds, for example. To mix two immiscible liquids and stabilize emulsions, additional components are needed, namely, emulsifiers and stabilizers. Small-molecular amphiphilic compounds, such as phospholipids and organosulphates, adsorb readily at the emulsion interface and decrease the energy needed for emulsification. However, the interface is sensitive to environmental changes, such as the presence of other amphiphilic compounds or mechanical forces. In addition to classical surfactants, macromolecules, such as proteins and polysaccharides, are used as emulsifiers and/or stabilizers. The advantage of using polysaccharides as stabilizers is that their properties are stable under a broad pH and ion strength range, while the function of proteins is highly pH and ion strength dependent. Moreover, polysaccharide-stabilized emulsions tolerate thermal processing.

Pure polysaccharides are polar and lack a clear amphiphilic nature. Their emulsification and stabilization ability is considered to occur either via residual protein fractions that would introduce amphiphilicity, or via increment of steric repulsion and/or continuous phase viscosity. Examples include proteinous gum Arabic and corn fiber gum, and very large polysaccharides, such as pectin and xanthan. Pickering-type stabilization has also been proposed for polysaccharides. That is, nano-sized particles adsorb to the interface, creating a rigid barrier against coalescence and transfer of compounds between the dispersed and continuous phases.

The high surface area of emulsions predisposes lipids into contact with oxygen and oxidation initiators, such as transition metals. Lipid oxidation decreases the functionality and nutritional value of lipids and produces off-flavors. Moreover, oxidation causes physical changes to the emulsion structures, e.g., polymerization of lipids and eruption of emulsions. Thus, lipids should be protected against oxidation, and at the same time, the interfacial structure may be protected. The rate of oxidation is dependent on...
the amount and type of oil, emulsifiers, and stabilizers, and on the partitioning of the emulsifier and stabilizer between the interface and continuous phase.\textsuperscript{2,13–14}

Approaches to stabilizing interfaces include thickening the interface or altering its charge with multilayer coatings\textsuperscript{14–16} or incorporating antioxidants, such as polyphenols, to the interface.\textsuperscript{17} These multi-step techniques principally use electrostatic deposition of polymer layers. That is, oppositely charged polymers are layered one by one on the droplet surface. This technique is laborious and requires great control of the process parameters, such as pH and ion strength, thus leading to costly production.

Biorefineries are currently being developed for profitable isolation of various lignocellulosic fractions into precursors of bio-based chemicals and materials. Availability of potential new renewable raw materials has extended the possibilities to explore their suitability for industrial applications; however, in many cases, their applicability or sustainability may be limited due to the need for hazardous solvents or reagents in biomass derivatization. Conversion of non-food biomass into a source of energy or raw materials for technical products often involves a sequence of chemical derivatization reactions aided by solvents and reagents. Within the biorefinery approach, biomass isolation techniques are being developed to control the composition and functions of isolated fractions. In this paper, we present a green chemistry approach towards dispersing agents by exploring and exploiting the natural characteristics of softwood extracts, namely, spruce \textit{O}-acetyl-galactoglucomannans (GGM), wood-derived hemicelluloses that are still widely untapped, as multifunctional stabilizers.

We have previously shown that spruce GGM, abundant future forest biorefinery products that are relatively small, linear chain polysaccharides, adsorb at the lipid droplet interface and stabilize it against physical breakdown and lipid oxidation.\textsuperscript{18–20} However, the action mechanisms are yet to be discovered. GGM are envisioned as multifunctional, sustainable, natural stabilizers that could replace several less effective additives and be applied in various types of industrial dispersions, including food, cosmetics and pharmaceuticals after safety evaluations (Pitkänen et al., submitted) and in technical applications, e.g. coatings and chemicals.
GGM are hemicelluloses that consist of chains of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl units with single (1→6)-linked α-D-galactopyranosyl units attached to mannose. Part of the secondary hydroxyl groups of mannose units are acetylated, which introduces hydrophobic sites to the structure. Yet, the backbone of GGM lacks clear non-polar parts that would give GGM an amphiphilic nature. In softwood, hemicelluloses may be associated or partially covalently bound to lignin, forming lignin-carbohydrate complexes (LCC). Lignin-originating phenolic residues (PR) are likely to accompany hemicelluloses during the isolation process and provide additional characteristics to GGM.

The remarkable physical emulsion stabilization capacity of GGM is considered to arise from steric repulsion and/or Pickering-type stabilization by GGM assemblies. We have previously shown that GGM adsorbs at the oil droplet interface, but the driving force enabling adsorption is not known. At high GGM concentrations, stabilization also occurs via increment of viscosity. GGM also inhibits lipid oxidation both in oil-in-water emulsions and in microcapsules.

The isolation method of hemicelluloses affects both the content and composition of hemicelluloses and accompanying PR. For example, by altering the temperature and extraction time in flow-through pressurized hot water extraction, the composition and size of recovered hemicelluloses and lignin can be controlled. However, the role of PR for the functionality of GGM is largely unknown; their presence may be either beneficial or detrimental, depending on the application.

The wide availability of raw materials, the economical isolation processes, and the multifunctionality of GGM make them a highly attractive stabilizer alternative for industrial use. To understand the features of GGM and enable development of applications, knowledge of their function mechanisms is needed. The aim of this study was to demonstrate the role of naturally occurring lignin-originating PR in GGM in the stabilization of oil-in-water emulsions. Thereby we illustrate the interaction and interfacial functionality of lipophilic structures bound with hydrophilic polysaccharide tails. GGM isolated with different techniques and having either hemicellulose-phenolic compound complexes or added phenolic
extract were compared for their stabilization capacity. Partitioning of PR between the interface and continuous phase of emulsions and the changes occurring in them were determined during an accelerated storage test. Meanwhile, physical stability and lipid oxidation in emulsions was monitored. This study reveals that GGM-phenolic complexes play a key role in emulsion stabilization.

**Materials and methods**

**Materials**

GGM, a natural mixture containing variety of galactoglucomannans and wood extractives, were obtained in powder form from process water of a Finnish pulp mill in an industrial-scale isolation trial after spray-drying (sTMP) or after ethanol precipitation (eTMP)\(^{28-29}\) and as aqueous concentrate from pressurized hot water flow-through extraction (PHWE)\(^{25}\). The average molar mass of sTMP and eTMP were 20 000–30 000 g/mol\(^{18}\) and that of PHWE GGM below 10 000 g/mol\(^{19}\). Lignan-rich spruce phenol extract (PE) was isolated from spruce knotwood according to Willför et al.\(^{30}\) Rapeseed oil, consisting of 60% of monounsaturated, 31% of polyunsaturated, and 4% of saturated fatty acids (Bunge Finland Oy, Raisio, Finland), was purchased from a supermarket and purified from natural anti- and pro-oxidants by adsorption chromatography according to a previously described method.\(^{20,31}\)

**Emulsion preparation**

Emulsions containing 5 wt.-% of stripped rapeseed oil and 1 wt.-% of GGM were prepared by high-pressure homogenization according to a previously described method.\(^{20}\) For the addition of spruce phenol extract (PE) to eTMP emulsion, PE was dissolved into acetone (30 mg/mL) and then dispersed into 1 wt.-% GGM solution before emulsification to yield 5.5 mg added phenolic compounds in 100 g emulsion. The total phenol content of spruce phenol extract was estimated using the Folin-Ciocalteu
method \textsuperscript{32} and the amount of added extract was adjusted in order to double the amount of phenolic compounds in the emulsions compared to samples with plain eTMP GGM.

**Accelerated storage test**

For the accelerated storage test, 100 g of emulsion was stored in a 250-mL glass bottle at 40 \textdegree C in the dark up to 2.5 months. Stability of the emulsion was monitored several times during the first two weeks of storage and then in intervals of two weeks for a total of 11 weeks. At each sampling, a few drops of emulsion were withdrawn for determining droplet size distribution and for visual investigation with optical microscopy. At the same sampling, 0.5 g of emulsion was withdrawn for the analysis of peroxide value and polymerized lipids. For monitoring the changes occurring in PR, 30 g of emulsion was withdrawn after 0, 7 and 14 days of storage. Before each sampling, the emulsion was mixed by turning the containers upside down ten times. For the analysis of volatile oxidation products, 1.5 g of fresh emulsion was placed in 20-mL glass vials (75.5 × 22.5 mm), sealed with caps, and the vials were stored at 40 \textdegree C in the dark. At each sampling, three replicate samples were withdrawn (n = 3).

**Droplet size distribution**

The droplet size distribution was characterized by static light scattering using a Mastersizer Hydro 3000 SM (Malvern Instruments Ltd, Worcestershire, UK). Before each sampling, the emulsion was mixed gently by turning the container upside down ten times. Interfacial area was calculated from the average droplet size and volume fraction of the added oil.

**Microscopy**

The emulsion morphology was characterized using optical microscopy (AxioScope A1, Carl Zeiss Inc., Oberkochen, Germany). Before each sampling, the emulsion was mixed gently by turning the container upside down ten times.
Partitioning of emulsions

Emulsions were partitioned into aqueous and creamed phases by centrifugation.\(^{18}\) 30 g of emulsion was centrifuged at 24 000 \(g\) at RT for 15 min. The creamed phase was collected and the residue was centrifuged for additional 5 min. The second creamed phase was combined with the first one for the analysis of adsorbed GGM fraction. 1 mL of continuous phase was collected for the analysis of the non-adsorbed GGM fraction. Both the creamed phase and continuous phase were subjected to further analysis (Fig. 1).

Determination of phenolic residues by UHPLC-DAD-FLD

Distribution and changes occurring in interfacial and dispersed free and bound phenolic residues of GGM were determined in order to evaluate their possible contribution to emulsion stability.

The creamed phase was divided into two equal portions. The adsorbed GGM were precipitated with 80 mL of 80\% ethanol, which is also compatible for the extraction of free phenolic compounds (Fig. 1). Released lipids were removed from the aqueous phase by extraction with 80 mL of iso-octane. The organic phase was removed and the remaining aqueous phase was centrifuged (10 min at 24 000 \(g\)). Supernatant and precipitated GGM were collected separately. The supernatant was evaporated to dryness.

Non-adsorbed GGM in the continuous phase (1 mL) were precipitated with 4 mL of 80\% ethanol. Remaining lipids were removed by extraction with 5 mL of iso-octane. The aqueous phase and precipitate were separated by centrifugation (10 min at 3000 \(g\)). Supernatant and precipitated GGM were collected separately. The supernatant was evaporated to dryness.

The hydrolyses and analysis of PR were performed according to a previously described method using the same equipment and reagents.\(^{20}\) The analysis included fractions of ethanol-soluble free PR, ester-bound PR to ethanol-soluble GGM, ester-bound PR to ethanol-precipitated GGM and glycosidically
bound or otherwise entrapped PR in GGM (Fig. 1). All the analyses were performed in triplicates (n = 3). The results were expressed as µg/kg of emulsion. The average and standard deviation were calculated across three analytical replicates (n = 3).

Figure 1. Sample preparation for the analysis of adsorbed and non-adsorbed phenolic residues. Each creamed phase and aqueous phase provided results of six different compound groups in four different fractions. The analysis was performed in triplicates. Determination of peroxide value
The primary oxidation of the emulsions was evaluated by the total content of hydroperoxides in the lipid phase determined by the peroxide value (PV). The analysis was performed according to a previously described method\textsuperscript{20} where lipids were first released and extracted and then PV was determined according to a ferric thiocyanate method. The average and standard deviation were calculated across three analytical replicates (n = 3).

**Determination of hexanal by SHS-GC-FID**

The secondary stage of lipid oxidation in emulsions was followed in terms of hexanal formation according to a previously described static headspace gas chromatography (SHS-GC-FID) method.\textsuperscript{33} At each sampling time, three replicate vials were withdrawn for the analysis. Hexanal contents were reported as peak area per gram of emulsion (peak area/g emulsion). The average and standard deviation were calculated across three analytical replicates (n = 3).

**Determination of polymerized lipids by SEC-RI**

Lipid oxidation in emulsions was also followed in terms of polymerization. Formed oligomeric lipids and remaining monomers were determined utilizing size-exclusion chromatography (SEC) in combination with refractive index (RI) detection, as described previously.\textsuperscript{20} The results were reported as relative peak area proportions (%). The average and standard deviation were calculated across three analytical replicates (n = 3).

**Results**

**Droplet size distribution and morphology**
Physical stabilization of emulsions by studied GGM extracts were evaluated by droplet size distributions. The structures of emulsions were also visualized by optical microscopy to reveal possible breakdown.

Emulsions stabilized by PHWE GGM had a unimodal droplet size distribution with an average droplet size $D[3,2]$ of 0.4 µm (Fig. 2). The droplet size distribution remained similar during six weeks of accelerated storage test at 40 °C. In sTMP GGM-stabilized emulsions, the $D[3,2]$ was larger (2.1 µm) and increased to 3.4 µm during storage. The eTMP GGM-stabilized emulsions showed bimodal droplet size distribution and an increase in droplet size during storage. The addition of PE to eTMP GGM did not significantly influence the droplet size distribution, as the eTMP+PE emulsions formed and aged in a similar manner to those containing plain eTMP GGM, in terms of droplet size distribution. Optical microscopy images confirmed the presence of mainly very small droplets of less than 1 µm in diameter in PHWE emulsions, whereas large droplets and flocculation were seen in sTMP emulsions. In eTMP and eTMP+PE emulsions, droplets with a large size range were observed, which could be due to coalescence or Ostwald ripening (Fig. 3).

The total oil-water interfacial area of PHWE emulsion was 800 m$^2$/kg while that of sTMP emulsion was 150 m$^2$/kg (Fig. 4). No significant changes were observed in PHWE emulsion while the interfacial area of sTMP emulsion decreased to 100 m$^2$/kg during the first week of storage at 40 °C. The interfacial area of eTMP and eTMP+PE emulsions decreased continuously from 1300 m$^2$/kg to 400 m$^2$/kg during two weeks of storage.
Figure 2. Droplet size distributions of emulsions with 5 wt.-% rapeseed oil stabilized with 1 wt.-% GGM obtained from (a) pressurized hot water extraction (PHWE), (b) thermomechanical pulping after spray-drying (sTMP), (c) thermomechanical pulping after ethanol precipitation and addition of spruce phenol extract (eTMP + PE), and (d) eTMP as fresh and after 1–6 weeks storage at 40 °C. The graphs are the averages of three replicate measurements.
Figure 3. Optical microscopy images of emulsions with 5 wt.-% rapeseed oil stabilized with 1 wt.-% GGM, stored at 40 °C for 2 weeks. GGM obtained from (a) pressurized hot water extraction (PHWE), (b) thermomechanical pulping after spray-drying (sTMP), (c) thermomechanical pulping after ethanol precipitation with addition of spruce phenol extract (eTMP + PE), and (d) eTMP without additives.
Figure 4. Total interfacial area of lipid droplets (5 wt.-%) in GGM-stabilized (1 wt.-%) emulsions during accelerated storage test at 40 °C. GGM obtained from pressurized hot water extraction (PHWE), thermomechanical pulping after spray-drying (sTMP) and thermomechanical pulping after ethanol precipitation (eTMP) and addition of spruce phenol extract (eTMP + PE).

Distribution of phenolic residues in emulsions

To understand how phenolic residues (PR) in GGM affect emulsion stability, their distribution between the interface and continuous phase was determined.

In all the studied emulsions, less than 0.1% of PR were located at the interface while the majority remained in the continuous phase (Fig. 5). The content of interfacial PR in PHWE GGM-stabilized emulsion was 2.6 µg/kg emulsion, while the content in the continuous phase was 111 mg/kg emulsion. The majority of the adsorbed PR (77%) were ester bound to ethanol-soluble GGM fraction, but some of the free or weakly associated PR (21%) were located at the interface. The continuous phase of PHWE emulsion had similar distribution of ester bound and free PR as at the interface though the contents were greater. The chemical structures of the adsorbed PR and those of remaining in the continuous phase were different. While ester-bound hydroxycinnamyls (OHC) (57%) and hydroxybenzoyls (OHB) (32%) were the main PR units at the interface, ester-bound OHB (62%) and flavanols (35%) were dominant in the
continuous phase. In the free PR, the proportion of flavanols (39%) and OHC (29%) were greater at the interface than in the continuous phase (19% and 10%, respectively) while the proportion of OHB was far greater (71%) in the continuous phase than at the interface (32%).

In sTMP GGM-stabilized emulsion, the content of interfacial PR was 0.4 µg/kg emulsion and the content of continuous phase PR was 59 mg/kg emulsion (Fig. 5). PR ester bound to ethanol-soluble GGM and free PR comprised equal fractions (47%) at the interface. Free PR were the most abundant fraction (53%) in the continuous phase. Flavanols constituted the majority (65–70%) of the adsorbed and continuous phase PR followed by OHB (24–26%).

In eTMP+PE GGM-stabilized emulsion, PR of 1.7 µg/kg emulsion were adsorbed at the interface while 41 mg/kg emulsion remained in the continuous phase (Fig. 5). PR ester bound to ethanol-soluble GGM was the main fraction (54%) at the interface, but also a fraction of free (21%) and glycosidically bound (22%) PR were adsorbed. Free PR (57%) and glycosidically bound PR (39%) constituted the main fractions in the continuous phase. Flavanols constituted the majority (53–65%) of the adsorbed and continuous phase PR followed by OHB (35–47%).

As the content of PR in eTMP GGM was considered relatively low,\textsuperscript{20} that is, much lower than in sTMP GGM, the distribution of PR in eTMP emulsions were not studied in more detail.
Figure 5. Composition of adsorbed (A) and non-adsorbed (B) phenolic residues in stripped rapeseed oil (5 wt.-%) emulsions stabilized by GGM (1 wt.%). GGM obtained from pressurized hot water extraction (PHWE), thermomechanical pulping after spray-drying (sTMP) and thermomechanical pulping after ethanol precipitation and addition of spruce phenol extract (eTMP + PE). The graphs presenting hydroxybenzoates (OHB), hydroxycinnamates (OHC), flavonols and flavanols are the averages of three replicate analyses.
Changes occurring in the phenolic residues during storage

In order to understand the role of phenolic residues (PR) in the physical and oxidative stabilization of emulsions, changes occurring in the distribution and contents of PR were monitored during the first two weeks of accelerated storage test.

Changes in the contents of PR were observed both in the interfacial and continuous phases throughout the accelerated storage test (Fig. 6). In PHWE emulsion, the changes were more substantial in the continuous phase than at the interface: a decrease of 55–62% in the total PR content was observed in the continuous phase while the decrease was only 24% at the interface. Further changes were not observed after one week of storage. The changes in the continuous phase occurred mainly in PR ester bound to ethanol-soluble GGM (72%) and at the interface in free PR (55%). In the continuous phase, the decrease was more pronounced in OHB (89%) compared to flavanols (51%). At the interface, the reduction was similar for both OHB and flavanols (60–68%).

In sTMP emulsions, changes also occurred mainly in the continuous phase PR: a continuous decrease of 35% in their content was observed during two weeks. The changes occurred primarily in PR ester bound to ethanol-precipitated GGM (84%) and in PR ester bound to ethanol-soluble GGM (60%). The contents of all of the PR, OHB, OHC and flavanols decreased (51–87%).

Unlike in the other emulsions, in eTMP+PE emulsion, changes occurred only in the interfacial phenolic residues: a continuous decrease of 74% occurred over two weeks. The changes could be observed in all of the studied fractions, being most abundant in glycosidically bound PR (87%). The greatest decrease occurred in OHB and OHC (87–94%).

As the content of PR in eTMP GGM was relatively low, the changes occurring in their contents during emulsion storage were not studied in more detail.
Figure 6. Changes occurring in the adsorbed (A) and non-adsorbed (B) phenolic residues in stripped rapeseed oil (5 wt.-%) emulsions stabilized by GGM (1 wt.%) during accelerated storage test at 40 °C. GGM obtained from pressurized hot water extraction (PHWE), thermomechanical pulping after spray-drying (sTMP) and thermomechanical pulping after ethanol precipitation and addition of spruce phenol extract (eTMP + PE). The graphs are the averages of three replicate analyses.

Lipid oxidation in GGM-stabilized emulsions
In PHWE GGM-stabilized emulsion, formation of hydroperoxides was detected (PV of 61 mEq/kg) only after 9 weeks of storage at 40 °C (Fig. 7). In sTMP emulsion, hydroperoxides were detected after 2 weeks, and PV of 20 mEq/kg oil was measured after 6 weeks. eTMP GGM inhibited oxidation, but not as efficiently: hydroperoxides were detected after 4 days of storage and PV of 264 mEq/kg was measured after two weeks. Addition of spruce phenol extract to eTMP GGM enhanced the stability of lipids: hydroperoxides were detected after 9 days (10 mEq/kg oil) and PV of 228 mEq/kg oil was measured after 8 weeks of storage.

Figure 7. Formation of hydroperoxides in the stripped rapeseed oil phase (5 wt.% of GGM (1 wt.%)) stabilized emulsions during accelerated storage test at 40 °C. GGM obtained from pressurized hot water extraction (PHWE), thermomechanical pulping after spray-drying (sTMP) and thermomechanical pulping after ethanol precipitation (eTMP) and addition of spruce phenol extract (eTMP + PE). The graphs are the averages of three replicate analyses.
In PHWE and sTMP emulsions, hexanal was not detected during the first four weeks of storage (Fig. 8). As was seen in PV, spruce phenol extract added to eTMP GGM delayed the oxidation but not as efficiently as PHWE GGM and sTMP GGM. In eTMP GGM-stabilized emulsion, hexanal was detected after 2 weeks and was detected in eTMP+PE emulsions after 3 weeks. After 1.5 months of storage, the samples in headspace vials were physically separated and thus volatile oxidation products were no longer determined.

No polymerization or interaction products of the lipids were detected during the 2.5-month accelerated storage test in any of the studied emulsions.

Figure 8. Formation of hexanal in the stripped rapeseed oil phase (5 wt.%) of GGM (1 wt.%) stabilized emulsions during accelerated storage test at 40 °C. GGM obtained from pressurized hot water extraction (PHWE), thermomechanical pulping after spray-drying (sTMP) and thermomechanical pulping after ethanol precipitation (eTMP) and addition of spruce phenol extract (eTMP + PE). The graphs are the averages of three replicate analyses.
Discussion

This study clearly demonstrates that GGM containing bound phenolic residues are more efficient emulsion stabilizers than purified (ethanol precipitated) GGM alone or with incorporated phenolic compounds. This applies both for the physical stability and for the protection of dispersed lipids against oxidation. The structure and composition of residual phenolic compounds (PR) in GGM define their ability to adsorb to the oil-water interface and stabilize it. In addition, PR are responsible for the remarkable oxidative stability of the dispersed lipids. These observations will be discussed further.

Although only small portion of PR adsorbed at the interface, they had a remarkable effect on emulsion morphology and stability. According to our earlier findings, the surface load of eTMP GGM in corresponding 5% oil-in-water emulsion was 0.8 mg/m².¹⁸ This surface load corresponds to approximately 10% of GGM present in the emulsion. However, in the present study, less than 1% of the PR present in the emulsions were located at the interface. As the molar mass of the adsorbed GGM tend to be greater compared to those remaining in the continuous phase,¹⁸ greater surface load is achieved with fewer molecules. This knowledge leads us to hypothesize either that the size of GGM is of importance or that these larger molecules contain other functionalities, most likely PR, which aid their anchoring at the interface. Acetylation of hydroxyl groups alters the overall polarity of GGM, making them less polar. Acetyl groups may play a role in the functionality of GGM; however, they are not expected to carry the main responsibility for GGM’s stabilization capacity. In addition, GGM contain only negligible portions of residual proteins;³⁴ thus, their role in stabilization is considered insignificant.

PHWE GGM-stabilized emulsions had the greatest content of PR at the interface (2.6 µg/kg emulsion), especially of those that were ester bound to ethanol-soluble GGM, and at the same time had a unimodal droplet size distribution with smallest average droplet size and largest droplet surface area compared to emulsions prepared with the other types of GGM. In addition, PHWE emulsion remained extremely stable throughout the 6 weeks of the accelerated storage test. With the other types of GGM, a smaller
amount of PR (0.4–1.7 µg/kg emulsion), most of them being free or only weakly associated with GGM (i.e., extractable with ethanol), were adsorbed at the droplet interface. These emulsions had larger average droplet sizes and were less stable. More importantly, when phenolic compounds were introduced as a separate extract (in eTMP+PE), emulsification was efficient, but the long-term physical stability of emulsion was not comparable to that of PHWE emulsion. Free PR may act as emulsifiers (i.e. facilitate emulsion formation), but may destabilize the interface during storage.\textsuperscript{35} Moreover, their partitioning between the continuous and interfacial phases is highly pH dependent due to the dissociation of the acid and hydroxyl groups. Most of the ester-bound PR, on the other hand, lack the acid group and thus remain neutral at a wide pH range.

PR may be bound to the monosaccharide units of GGM either via ester or ether bonds or via glycosidic bonds.\textsuperscript{23,36} Part of these bonds may occur naturally, but they are also formed during the isolation processes. Each monosaccharide unit has several hydroxyl groups available for ester bonding, but GGM have only one site for glycosidic bonding, that is, at the reducing end of the molecule. Non-polar units attached to the polysaccharide backbone are considered to increase the amphiphilic nature of GGM and enable their anchoring to the oil droplet interface. Larger GGM chains have more possible sites available for ester linking and therefore a greater number of PR could be bound. Further increase in the amphiphilic nature would make these larger GGM molecules more susceptible to adsorb at the interface. Ester-bound PR would also enable even packaging of GGM parallel to the interface (Fig. 9) and therefore could lead to greater physical stability and protection against lipid oxidation, as shown for PHWE emulsions in this study. Glycosidically bound PR, on the other hand, may be located only at the reducing end of the GGM molecules and could therefore lead to perpendicular anchoring of GGM to the surface. Our hypotheses of orientation of GGM-PR assemblies on droplet interface are presented in Fig. 9. This orientation is hypothesized to form a thick, but porous interfacial layer due to steric hindrance caused by the GGM chains with attached galactopyranosyl side groups. It is likely that this perpendicular orientation and steric hindrance would lead to larger droplet size than if GGM chains were aligned with the interface, as
demonstrated with sTMP and eTMP emulsions containing a considerable fraction of glycosidically bound PR at the interface. In addition, a thick and porous layer could be responsible for the observed flocculation of these emulsions. Flocculation may eventually lead to coalescence and increased average droplet size. However, thick interfacial layer, if not too porous, could inhibit lipid oxidation by acting as a steric barrier between the oxidation initiators and dispersed lipids, which could explain the oxidative stability of sTMP emulsion. The presently hypothesized nature of GGM-PR assemblies at droplet interface explains the anchoring of intermediate-sized GGM tails on the droplet surface by lipophilic PR structures. This is analogic to protein anchors in gum Arabic, with the difference that the present GGM-PR assemblies were more efficient stabilizers than gum Arabic studied earlier. Such structural assemblies are suggested as guidelines in targeted isolation of bio-based hydrocolloid structures with high functionality or in derivatization or synthesis of structural elements designed for efficient interfacial stabilization.

Figure 9. Simplified illustration of the hypothetical orientation of GGM containing ester-bound (A) and glycosidically bound (B) phenolic residues at the interface of oil-in-water emulsion.

GGM may also stabilize emulsions via Pickering-type stabilization, that is, forming an interfacial layer by assemblies. Assemblies comprise parallel-orientated non-branched carbohydrate chains, which are associated via hydrogen bonding of the hydroxyl groups. Alternatively, PR may promote GGM-GGM
associations due to their polarity difference with the aqueous medium or due to the hydrogen bonding of PR. In Pickering-type stabilization, the size and orientation of the assemblies determine the droplet size and thickness of the interfacial layer.$^{10-11,19}$

The chemical structures of PR in the studied GGM samples varied from simple free aromatic phenolic acids to polymers of flavonoids. Lignin is a polymer of ester, ether, glycosidically and carbon-bound phenyl propanoid units.$^{38}$ Lignin may exist in free form or it may be bound or associated to surrounding hemicelluloses and cellulose forming LCC.$^{23,36}$ In these complexes, lignin is connected to sugar units mainly with hydroxycinnamyl or hydroxybenzyl units via glycosidic, ester or ether bonds. In base and acid hydrolys of GGM, as used in the present work, various bond types existing between the carbohydrate chain and phenolic residues and between different phenyl units in the PR are cleaved. Thus, this technique provided information about the various groups of phenyl units present in GGM, even though it did not reveal their complex native structures. As lignin fragments in LCC seem to be mainly monomers and dimers,$^{39}$ we hypothesize that the PR in the studied GGM are rather small units. The bond type and composition of released PR clearly correlated with the stabilization capacity of GGM. Hydroxycinnayls ester bound to ethanol-soluble GGM were most efficiently adsorbed to the oil droplet interface. Emulsions containing higher amounts of hydroxycinnamyls appeared to be most stable.

Previous studies have proposed that PR are more likely to interact with hemicelluloses with higher numbers of galactose side groups.$^{23}$ If the residues are bound to galactose units, this could enable PR to get in contact with the lipid surface despite the possible steric hindrance caused by rigid GGM. While attaching to the non-polar medium, the PR would simultaneously anchor GGM to the interface.

The large interfacial area in emulsions predisposes lipids into enhanced contact with oxidation initiators. In addition, flocculation may bring dispersed lipid droplets into close contact, enhancing the transfer of lipid radicals and hydroperoxides from one droplet to another. This interaction may lead to an increased oxidation rate. If the interfacial layer is sufficiently thick and/or dense, it acts as physical barrier between active initiators and lipid species, while at the same time preventing further oxidation.$^{13-}$
In addition to acting as physical barrier, GGM containing PR may protect lipids against oxidation via scavenging radicals and binding iron.

Lipids were most stable against oxidation in PHWE emulsion that had the smallest average droplet size, the largest surface area and the greatest content of PR, especially those that were bound to GGM, among the studied emulsions. In contrast, lipid oxidation was most intensive in eTMP emulsion which had larger average droplet size, smaller surface area and contained less PR compared to the other emulsions. The addition of phenolic compounds as a separate extract (eTMP+PE) improved oxidative stability, but not to the level of PHWE emulsion. Thus, PR, especially those associated with GGM, seemed to have a significant role in the stabilization of lipids against oxidation. Lipid oxidation and physical stability are also expected to be interlinked, for example, oxidized lipid droplets may be more prone to Ostwald ripening due to their increased miscibility with the continuous phase after increased polarity.

In all of the studied emulsions, decrease in the contents of PR occurred during the storage. The changes were most evident in the ethanol-soluble fractions, both in free PR and in ester-bound PR. In PHWE and sTMP emulsions, changes in the PR occurred mainly in the continuous phase. At the same time, these emulsions were most stable against oxidation. In eTMP+PE emulsion, containing only minor amount of GGM-bound PR, decrease in phenolic compounds was most apparent at the interface. In addition, lipid oxidation was greater in this emulsion compared to PHWE and sTMP emulsions. Adsorbed free phenolic compounds may have deattached from the interface during storage, leading to a decrease in their contents and possible destabilization of the interface. Reactions of PR with radicals may also promote polymerization of GGM and formation of interaction products with lipids. Even if the polymerized or interacted GGM would remain at the droplet interface, the number of antioxidant active PR would be reduced. Decrease in the coverage of the interface and reduced content of antioxidants at the site of radical formation could have led to increased lipid oxidation.
In addition to the physical stabilization of emulsions and its protective effect against lipid oxidation, GGM-bound hydroxycinnamyls located at the interface could act as antioxidants. Phenolic compounds may scavenge primary carbon-centered lipid radicals and stop the chain reaction before it escalates and leads to elevated oxidation.\textsuperscript{41} PR in the continuous phase, on the other hand, could have functioned via binding or reducing transition metals and thus inhibiting oxidation. Polysaccharides themselves may also retard lipid oxidation in emulsions.\textsuperscript{2,6} The action may be due to increment of viscosity or due to transition metal binding. Polysaccharides have also been proposed to scavenge carbon and hydroxyl radicals.\textsuperscript{42–43}

Other wood extractives, such as triterpenes and sterols, may also possess antioxidant activity. However, their contents in wood are relatively low, usually less than 1–2% in heartwood and sapwood.\textsuperscript{30} Half of this content (i.e., 0.5–1%) may have been present in the studied GGM fractions.\textsuperscript{25} If this was the case, their content could have been significant in eTMP but negligible in PHWE. Yet, PHWE emulsions were more stable against oxidation than eTMP emulsions. Knotwood extract of Norway spruce has been reported to contain considerable amounts of lignans, 7-hydroxymatairesinol being the predominant one (70–85%).\textsuperscript{44} These compounds may have been responsible for the increased oxidative stability of eTMP+PE emulsion.

Current results support the use of natural non-purified GGM fractions in emulsion stabilization. Extraction of a sufficient amount of GGM containing bound PR will give GGM superior functionality. Regarding applications, it is noteworthy that the color of GGM extracts is correlated to the content and composition of lignin residues. Obtained results provide crucial information for industry, which is currently developing non-hazardous and economical processes for the isolation of GGM. For example, with flow-through pressurized hot water extraction, the size of GGM and content of PR can be controlled.\textsuperscript{25,45} Moreover, the composition of PR and thus also the color of the extract may be adjusted by extraction temperature and time.

Conclusions
We showed that the phenolic profile in GGM contribute to their interfacial functionality. PR act as vehicles that enable the anchoring of GGM into the oil droplet interface. GGM-PR assemblies provide steric stabilization against physical breakdown and antioxidants against lipid oxidation. The composition of PR in natural GGM extracts can be tailored by sustainable and economical isolation processes. GGM are multifunctional plant-based emulsifiers and stabilizers exhibiting both physical and chemical stabilization capacity over extended storage time. According to the hypothesis, we demonstrated that by retaining interactions between isolated GGM and PR, stabilization is achieved without adding other surfactants or antioxidants. Previously known emulsifiers are typically small-molecular amphiphilic surfactants, such as Tween20,\textsuperscript{46} whereas macromolecular hydrocolloids are mainly used as viscosity-modifying stabilizers.\textsuperscript{6–9} The present work suggests that lipophilic structures bound with intermediate-sized polysaccharide tails result in highly efficient interfacial stability. Superior physical stabilization and protection against lipid oxidation compared to e.g. gum Arabic \textsuperscript{18,20} or Tween 20 \textsuperscript{46} was obtained by one-ingredient formulation and one-step emulsification process. The shelf life of PHWE GGM-stabilized emulsions, in terms of physical and oxidative stability, at room temperature would be up to 8 months. Correspondingly, the shelf life would be prolonged up to 1.5 years at cooled conditions, such as in a refrigerator. Even with less efficient GGM fractions, the emulsions would remain stable for 1–4 months. This high and long-term emulsion stability and abundant availability of raw materials makes “spruce gum” an attractive future alternative for conventionally used emulsifiers and stabilizers in various technical, pharmaceutical and food applications. Future work includes investigation of continuous phase composition and dispersed phase volume fraction on the stabilization capacity of GGM. Furthermore, understanding the functionality of LCC at the emulsion interface provides perspectives for future tailoring of both natural and synthetic compounds for efficient stabilization of dispersed systems.

Conflicts of interest
There are no conflicts to declare.

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