TANDEM FOREST VALUES

Sweden's gift to Finland 2017

Final Report

ROle of lignin Carbohydrate complexes as Key to stable emulsions (ROCK)

1. Description of the research that has been carried out

1.1 Brief description of the project, aims, and partner universities involved in the collaboration

Wood hemicelluloses such as softwood galactoglucomanan (GGM) and hardwood glucuronoxylan (GX) are abundant polysaccharides from Nordic forest still unexploited in the industry. Preliminary studies have demonstrated the excellent stabilization capacity of GGM and GX for being applied in oil-in-water emulsion systems, especially when residual phenolic compounds, including lignin, were presented. The presence of phenolic compounds also contributed to prevent emulsified lipids oxidation. The hypothesis for the excellent capacity of GGM and GX to stabilize emulsions is that at least part of the residual lignin is chemically bonded to the hemicelluloses forming the so-called lignin-carbohydrate complexes (LCC). The resultant molecule presents both, a hydrophilic and a lipophilic region that allows simultaneous interaction of the lignocellulosic assembly with the water and oil phases in emulsion. This project aimed to investigate this hypothesis by assessing in details the structure of GGM and GX samples and investigate the chemical structure of stabilizers anchored in the emulsion interface that are active agents for emulsion stabilization.

GGM and GX samples were obtained from spruce and birch sawdust, respectively, using a pressurized hot water extraction (PHWE), within and ongoing collaboration between the University of Helsinki (UH – Finland) and the Natural Resources Institute Finland (LUKE). Dry GGM and GX samples were produced from PHWE extracts using initially two different fractionation methods, namely: spray-drying and ethanol precipitation. Subsequently, all GGM and GX fractions were characterized.

The UH and the Royal Institute of Technology (KTH – Sweden) were the partners involved in this join project. During the collaboration, the postdoctoral researcher Danila M. de Carvalho used the infrastructure and facilities of both universities. Moreover, during the whole project duration, and intensive knowledge exchange was stablished with benefits for both universities. The duration of the activities in each university was initially accorded to be one-year in each, being the first and last 6-month at KTH. Due to sanitary restrictions implemented in spring 2020, the mobility in the final 6-month of the project was not performed. This, however did not compromise the project and also allowed a systematic study on the interface of emulsions stabilized by GGM and GX using the facilities of the Department of Food and Nutrition (UH), as discussed in the subsequent sections.

1.2 Research conducted at KTH (Nov 2018 – Apr 2019)

Knowing the raw material, *i.e.*, its chemical composition, structure, and properties is crucial to understand its functionality and behavior in certain application. Therefore, the initial part of the project consisted in the assessment of the chemical properties of GGM and GX samples (obtained from spray-drying and ethanol-precipitation methods as described in section 1.1). This included the quantification of polysaccharides, lignin composition, acetyl groups, and the determination of the molar mass. More detailed information on the structural features and functional groups of GGM and GX samples was assessed by Fourier transformed infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) (using a Bruker Advance 400 Hz instrument) using the heteronuclear single quantum coherence spectroscopy (2D HSQC) technique.

For this, the postdoc got an intensive training in NMR operation and assessment of results. Prof. Martin Lawoko, an expert in assessing LCC structure using NMR, supervised this training and supported Danila's learning towards becoming independent on the assessment of such data. Important information from GGM and GX structure such as carbon-hydrogen correlation in lignin structures, lignin substructures, carbon-hydrogen correlation in polysaccharides linkages and lignin-carbohydrates bonds were obtained from the NMR results. Results indicated that the GGM and GX samples obtained by spray-dried and ethanol-precipitation of wood extracts present a heterogeneous compositions. Moreover, results also confirmed substantial reduction on the lignin content and lignin-carbohydrates bonds in GGM and GX samples due to the ethanol precipitation (Table 1). These findings motivated the investigation of the ethanol-soluble GGM and GX, fractions that were ignored in the preliminary ethanol-fractionation, but that on the light of the results appeared as interesting fractions in which lignin and LCC are potentially enriched. Indeed, after investigation, ethanol-soluble GGM and GX proved to be enriched in lignin and LCC structures, additionally to present lower values for molar mass than the ethanol-precipitated and spray-dried counterparts (Table 1). For the continuation of the project spray-dried, ethanol-soluble and ethanol-precipitated GGM and GX samples were investigated.

Table 1: Main chemical structures identified in spray-dried (sd), ethanol-soluble (es), and ethanol-precipitated (ep) GGM and GX samples.

Assessments	sdGGM	esGGM	epGGM	sdGX	esGX	epGX
Carbohydrates, %	84.6	67.1	92.6	80.1	69.6	87.3
Purity of GGM or GX	72.2	52.5	79.4	69.8	63.9	75.2
Degree of acetylation	0.23	0.27	0.27	0.46	0.40	0.48
Mn, kDa	1.7	0.7	2.6	1.2	0.6	1.6
Mw, kDa	7.1	1.5	9.3	4.2	1.2	4.9
Dispersity index	4.3	2.0	3.6	3.5	1.9	3.1
Lignin, %	15.4	32.9	7.4	19.9	30.4	12.7
LCC[a]	PG, GE	PG, GE, BE	PG, GE	PG, GE, BE	PG, GE, BE	PG, GE

[a]Structures of lignin-carbohydrate complexes (LCC) identified in GGM and GX samples, *i.e.*, PG: phenylglycoside, GE: gamma-ester, and BE: benzylether. LCC structures are presented in Figure 1.

1.3 Research conducted at UH (May 2019 – Oct 2020)

To date, the most efficient method for the identification of lignin-carbohydrate bonds in lignocellulosic assemblies is performed using 2D HSQC technique in NMR. However, this assessment can be challenging due to the low frequency of lignin-carbohydrate bonds compared to plain carbohydrate and lignin structures. To tackle this limitation, various fractionations methods were investigated aiming to concentrate LCC bonds in samples by removing carbohydrates moieties not involved in such linkage. Optimization of methods for fractionation were conducted using GGM sample. The physical, chemical, and enzymatic methods performed included anti-solvent precipitation (using ethanol/water system), fractionation in binary systems, acid hydrolysis, precipitation by pH variation, centrifugation, ultracentrifugation, enzymatic hydrolysis, and nanofiltration. Methods were applied alone or combined, as was the case of the sequential enzymatic hydrolysis → ultracentrifugation → nanofiltration. In this part of the project, the Assoc. Prof. Kirsi Mikkonen, expert in characterization of hemicelluloses and its application in advanced and functional colloidal systems, supervised Danila M. de Carvalho in carrying out such methods and assessing the resulting fractions. As a preliminary assessment, the resultant fractions were investigated by FTIR in which the increasing of the intensity of lignin peak was used for selection of potential fractions for further analysis. Those promising fractions were then assessed by NMR using the instrument Bruker Advance

850 Hz III high-definition spectrometer equipped with a cryoprobe available at UH facilities. Compared to the instrument used at KTH, the instrument at UH is much more sensitive to the chemical structure of the samples, providing a robust set of data. The training in NMR analysis obtained by Danila M. de Carvalho during her staying at KTH was of paramount importance for the assessment of the results obtained for physical, chemical, and enzymatic fractionations, especially regarding to the investigation of LCC structures. Results indicated that more promising enrichment of LCC structures were achieved by enzymatic hydrolysis, ultracentrifugation, and nanofiltration performed in sequence.

After defining the parameters for enzymatic hydrolysis, ultracentrifugation, and nanofiltration, spray-dried, ethanol-soluble and ethanol-precipitated GGM samples were subjected to these treatments and the resultant fractions were collected and analyzed. References trials in which the enzymatic hydrolysis was not performed were also investigated. A large number of fractions were obtained, including those from enzymatic hydrolysis and reference trials, pellet and supernatant from ultracentrifugation, and large and small particles separated by nanofiltration. The enzymatic and physical methods applied led to fractionation of LCC structures in the samples. Benzylether structures were identified in ethanol-soluble GGM as a positive effect of the anti-solvent treatment applied for the preparation of this sample. Overall results indicated that the enzymatic hydrolysis performed for fractionation of the samples did not improve LCC identification in fractions, indicating that the simple use of mild and inert physical methods were enough for promoting LCC fractionation and enrichment in certain fractions. The physical fractionation methods investigated, *i.e.*, ultracentrifugation and nanofiltration, proved to be efficient in concentrate benzylether structures in spray-dried and ethanol-soluble GGM fractions (Figure 1). Such results also confirmed the initial hypothesis on the existence of lignin-carbohydrate bonds in GGM samples as an explanation for their amphiphilic behavior and excellent capacity of stabilizing oil-in-water emulsion.

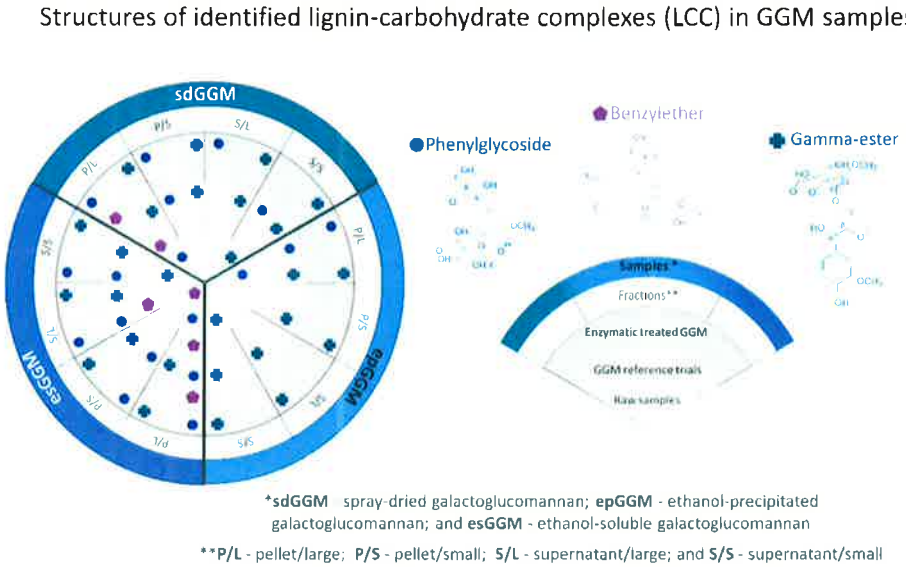

Figure 1: LCC structures identified in GGM samples and fractions.

A similar study on physical fractionation (using ultracentrifugation and nanofiltration) was also performed for spray-dried, ethanol-soluble and ethanol-precipitated GX samples. Results indicated that the fraction collected as a pellet after ultracentrifugation had only molecules larger than 500 Da and was substantially concentrated in lignin moiety. This fraction was also concentrated in benzylether structures

(Figure 2). Moreover, only larger molecules (from pellet or supernatant) presented benzylether structures after physical fractionation, indicating the possibility of various benzylether structures in the same molecule. Finally, physical fractionation proved to be an efficient strategy also for the fractionation and enrichment of LCC structures in GX samples, suggesting that physical fractionation effects do not depend on the structure of the hemicellulose, but on the lignin moiety itself.

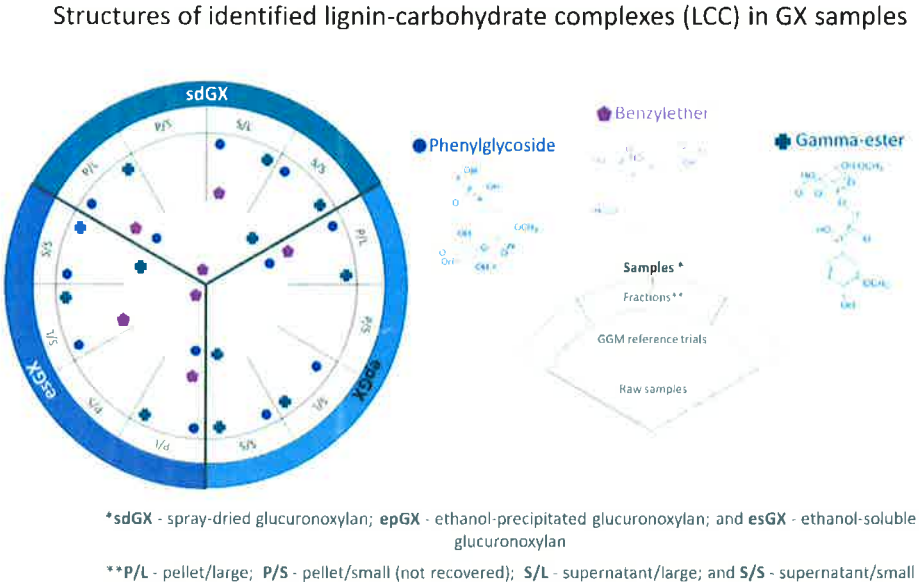

Figure 2: LCC structures identified in GX samples and fractions. After fractionation no pellet/small (P/S) fraction was formed.

After confirming that GGM and GX samples (*i.e.*, spray-dried, ethanol-soluble, and ethanol-precipitated) have a heterogeneous compositions and identifying that certain methods can promote the fractionation of GGM and GX compounds, the continuation of the project focused in investigating how such compounds are fractionated between emulsion phases. For oil droplet stabilization, amphiphilic molecules, as GGM and GX samples, are driven to the oil droplet interface. Then, the hydrophilic fraction of the molecule (mainly formed by carbohydrates) faces the water continuous phase while the lipophilic fraction (containing the lignin residues) anchored to the oil droplet. In this part of the study, the attributes of GGM and GX that facilitates the oil droplet anchoring were investigated. Oil-in-water emulsion were prepared using spray-dried, ethanol-soluble, and ethanol-precipitated GGM and GX samples as stabilizers. Next, emulsions were partitioned into cream and continuous phase (by centrifugation) and the stabilizer in each phase was recovered and analyzed. Recovering stabilizers from cream and continuous phase was crucial for identifying which structures were active in the emulsion stabilization process. Unlike to the stabilizers recovering from continuous phase that was easily performed using centrifugation, no method was available for extraction of stabilizers from cream. Therefore, a method based on liquid-extraction was developed starting with the test of fourteen reagents, including a surfactant (Tween-20) and 13 solvents (methanol, ethanol, propanol, dimethyl sulfoxide, acetone, acetonitrile, chloroform, dioxane, heptane, hexane, isooctane, toluene and water). Experiments indicated the acetonitrile as the most efficient and selective extraction agent for recovering GGM and GX stabilizers from cream. After optimization of the liquid-extraction method, acetonitrile was used for recovering stabilizers from cream. Lyophilization was used for obtaining dry stabilizers. Confirming the preliminary hypothesis for this study, the chemical structure of populations in

GGM and GX were fractionated between emulsion phases. The majority of the stabilizers used in emulsion preparation were found unadsorbed in the continuous phase (Table 2). Such unadsorbed stabilizers were also fractionated based on the chemical composition, being them depleted in lignin moiety. Moreover, certain chemical and structural attributes of stabilizers also contributed to their fractionation between interface and continuous phase. This includes the preferential adsorption of dibenzodioxocin, oxidized side chain, dehydroconyferyl alcohol, and benzylether structures in the interface. It was concluded that such structures are responsible for performing an active role on anchoring and driving the GGM and GX molecules towards the emulsion interface and, consequently, improve the stability of emulsions. Specific analysis for assessing the emulsion droplet, droplet morphology, and stability of emulsions during 1-month storage were conducted to support the findings.

Table 2: Distribution and chemical and structural aspects of GGM- and GX-stabilizers recovered from emulsion phases.

Stabilizer	Phases	Distribution, %	Lignin[a], %	Carbohydrates[a], %	Attributes[b]	LCC structures[c]
sdGGM	Interface	16.6	63.7	36.3	DBD, DCA	PG, BE, GE
	CP[d]	83.4	1.7	98.3	DCA	PG, GE
esGGM	Interface	28.0	95.0	5.0	DBD, DCA	BE, GE
	CP[d]	72.0	16.6	83.4	DCA	PG, GE
epGGM	Interface	12.7	27.0	73.0	DCA	GE
	CP[d]	87.3	0.2	99.8	-	PG, GE
sdGX	Interface	12.9	43.6	56.4	OSC, DCA	PG, BE, GE
	CP[d]	87.1	6.1	93.9	-	PG, GE
esGX	Interface	28.8	87.4	12.6	DBD, OSC, DCA	BE, GE
	CP[d]	71.2	15.2	84.8	OSC	PG, GE
epGX	Interface	17.1	15.5	84.5	-	GE
	CP[d]	82.9	0.9	99.1	-	GE

[a]Obtained from NMR spectra by semi-quantitative analysis using the aromatic G_2 (for GGM samples) and $S_{2/6}$ (for GX samples) peaks reference.

[b]Chemical and structural attributes found in samples recovered from interface and continuous phase. DBD: dibenzodioxocin; OSC: oxidized side chain, and DCA: dehydroconyferyl alcohol.

[d]CP: continuous phase.

As conclusion, this project allowed a profound understanding on the heterogeneous chemical structure of GGM and GX samples and promoted the development of efficient fractionation methods for facilitating chemical analysis and preparing of samples with target composition. Moreover, for the first time, the attributes in GGM and GX assemblies that make them active in the stabilization of oil-in-water emulsions were identified by using GGM and GX samples with varied composition and systematically combining liquid-extraction and advanced analytical NMR technique.

1.4 Communication with the other partner university when located in Finland or Sweden

During the whole project the communication was stablished by in-person meetings, online meetings, short visits, and email contact. The partners were periodically updated about the results obtained and consulted when new investigation lines needed to be decided/included. Partners also supported and

contributed during manuscripts preparation, as well as during the preparation of abstracts for conference participations.

2. Publications achieved from the project results

2.1 Articles in international scientific journals with referee practice (* = manuscripts not accepted for publication yet)

1. **Carvalho, D. M. d.,** Lahtinen, M., Bhattarai, M., Lawoko, M., Mikkonen, K. S. (2021). Active role of lignin in anchoring wood-based stabilizers to the emulsion interface. *Manuscript submitted to Green Chemistry journal.**
2. **Carvalho, D. M. d.,** Lahtinen, M., Lawoko, M., Mikkonen, K. S. (2020). Enrichment and identification of lignin–carbohydrate complexes in softwood extract. *ACS Sustainable Chemistry & Engineering* 8, 11795–11804.

3. Description of how the grant has contributed to competence building that will facilitate and strengthen long term collaboration between Finland and Sweden

Looking for the valorization of forest resources is a common aspect in the Finnish and Swedish economy development views. Indeed, the grant provided for this present collaboration intended to support initiatives that make possible such kind of valorization. Specifically in this research, wood derivatives, usually released as by-products in pre-hydrolysis treatment for it deconstruction, was used in the designing of functional interfaces that have potential to be applied in emulsion systems. Moreover an extensive chemical and structural characterization was developed and conducted, which allowed the elucidation of important attributes in wood stabilizers that make them active in the interface of emulsions. This findings were only achieved by combining specific knowledge already existent in the Finnish and Swedish partner universities, but that had not being integrated before. By this collaboration, the knowledge on wood chemistry and hemicelluloses characterization and application were put together and oriented towards promoting a deeper understanding of the mechanisms that make green-label wood stabilizers excellent local alternatives to conventional the ones. Therefore, it was proved that Finland-Sweden collaborations in science and technology can benefit both countries by using their abundant and common resources and achieving solutions that fit well in their development goals.

4. Description of research areas being started or strengthened at the departments in Finland and Sweden

Before this collaboration, the Food Materials Science Research Group led by Assoc. Prof. Kirsi Mikkonen at the Department of Food and Nutrition of the UH was conducting advanced research on the characterization and applications of hemicelluloses in colloidal systems. The available instrumentation at her department provided already detailed chemical and structural characterization of hemicelluloses, including certain functionalities. Moreover, the department also have infrastructure for the preparation and characterization of emulsion using hemicelluloses as stabilizers, which allowed exploiting the utilization of hemicelluloses in non-conventional applications. The hemicelluloses under investigation were obtained from wood through aqueous extraction, which resulted in the co-preservation of a certain amount of other carbohydrates and lignin. Although the presence of residual lignin could be identified in the hemicelluloses assemblies using the UH instrumentation, the nature of the interaction of the residual lignin with hemicelluloses (chemical or physical) was still unclear. However, this knowledge was considered as crucial for a better understanding on the application of such hemicelluloses assemblies in emulsion. This connected

The Department of Food and Nutrition of the UH with the Wallenberg Wood Science Center (WWSC) at KTH. The other partner in this research, the Prof. Martin Lawoko, based at the WWSC, is a specialist in wood chemistry and expert in the assessment of lignin structures, including in the identification of LCC structure using NMR. The WWSC offered possibilities for a detailed assessment of chemical structure of wood assemblies, useful for the characterization of the hemicelluloses extracts containing other polymers as the lignin. Specifically regarding to the lignin and LCC assessment, Prof. Martin Lawoko had a know-how for the interpretation of the data obtained by NMR. During the research, the postdoctoral researcher Danila M. de Carvalho exploited the complementary nature of the research environments, expertise, methods, and infrastructures offered by both partners' laboratories. Similarly, the professors involved in the collaboration had multidisciplinary expertise that oriented the research towards new areas of knowledge still unexploited, as for example the fundamental assessment of emulsion interfaces based on the identification of lignin attributes and LCC structures that support emulsion stabilization. The knowledge accumulated in during this research made possible de development of physical and chemical fractionation methods that improve chemical and structural characterization of lignocellulosic biomasses as well as the assessment of target functionalities required for certain application. An example was the elucidation of the chemical and structural attributes that make the hemicelluloses assemblies more active in the stabilization of emulsion interfaces. The knowledge obtained from this collaboration will definitely favor further lignocellulosic characterization in both partner's laboratories. The connection stablished between UH and KTH during this research can also facilitate further collaborations in the development of novel colloidal systems, which can be easily conducted at the UH facilities. Moreover, outcomes from this research can also favor the scientific community and industrials sectors. For example, this research provided guidelines for industries that not only orient them in the optimization of extraction processes for producing functional wood-based stabilizers, but also advance the use of such stabilizer in the formulation of novel and designed interfaces.

5. Description of how the grant has contributed to strengthening the forest sector in Finland and in Sweden

Forest-based industries have great participation in Finland and Sweden economies. Although this sector has had the pulp and paper industry as the main actor for a long time, recent efforts had oriented research towards other biorefinery processes as a way to improve forest resources utilization and expand participation in market. Spruce and birch wood were used as the initial raw material for this project. These woods represent nowadays two of the most commercially used species in Finland and Sweden, that have already tradition on their industrial utilization, but that eventually produce certain by-products still unexploited, as is the cased of hemicelluloses-rich extracts obtained from pre-hydrolysis of wood-chips. This project investigated attributes that validate the use of such hemicellulose-rich extracts in emulsion systems. Emulsion systems have a large range of application, for example, chemicals, food, and pharmaceutical and is currently a growing market. The possibility of having a local wood-based stabilizer for incorporation in emulsion systems can provide new avenues for the forest-based industry in Finland and Sweden, especially into biorefinery processes.

6. Description of communication with relevant stakeholders and end users

The main outcomes from this project were communicated to the scientific community in the publications (one of them still under revision) and in conferences (American Chemical Society – ACS, 2021 and International Polysaccharide Conference 2019). Moreover, findings were also communicated to the general public through the Food Materials Science Research Group Blog (https://blogs.helsinki.fi/foodmaterialsscience/).Posts related to the project can be found here https://blogs.helsinki.fi/foodmaterialsscience/2021/04/09/rock-building-new-knowledge-to-valorize-

forest-resources/ and here https://blogs.helsinki.fi/foodmaterialsscience/2020/08/24/links-between-lignin-and-hemicelluloses-in-spruce-extract/.

7. Financial accounting

Table 3: Financial accounting for the research conducted.

Budget category	**Estimated sum (€)**	**Amount used (€)**
Personnel costs	108963	109559
Consumables	4500	651
Subcontracting	0	0
Other	138859	138783
TOTAL	252322	248994

Helsinki, August 27th, 2021

Kirsi S. Mikkonen
Project leader

Danila Morais de Carvalho.

Danila M. de Carvalho
Postdoctoral researcher