DEVELOPMENT OF INNOVATIVE LIPID-BASED MATERIALS FOR ENCAPSULATION OF **BIOACTIVE INGREDIENTS**

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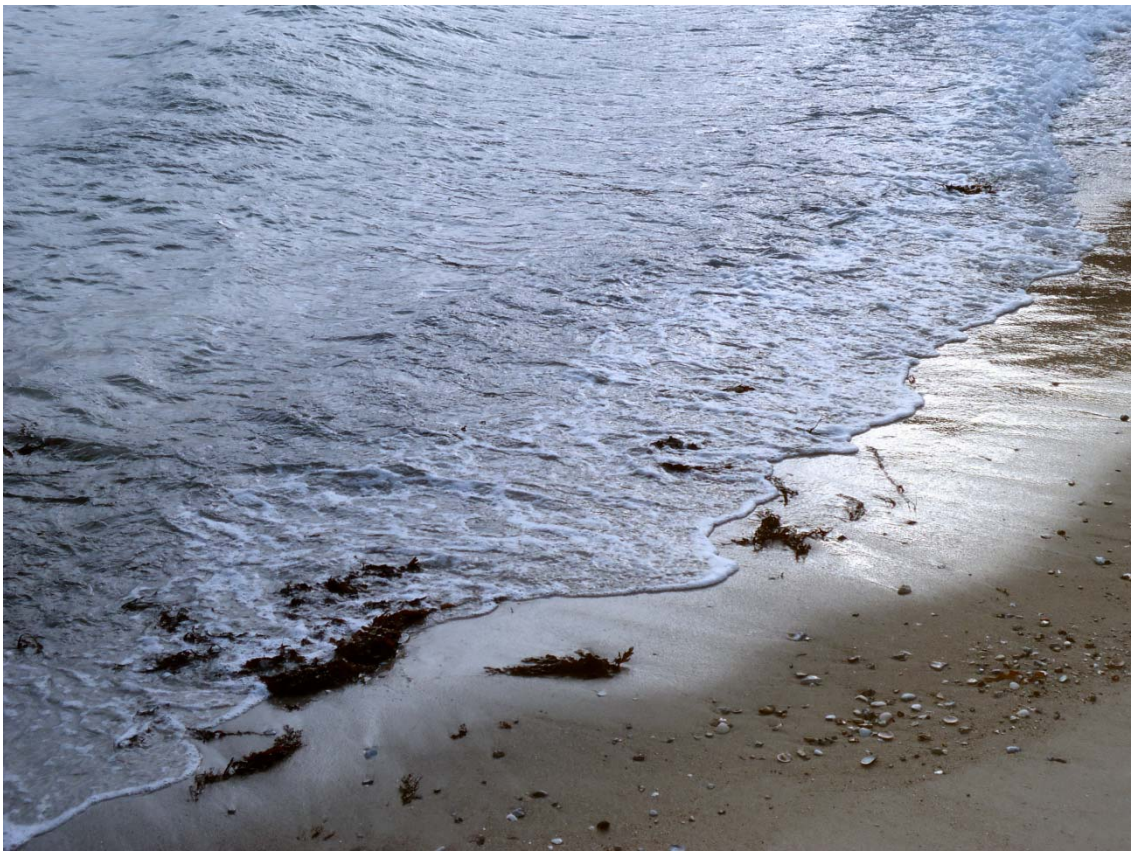

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Keywords: Biobased products, biotechnology, functional lipids, lipid technology, material synthesis, sustainable technologies.

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Abstract

This report is based on studies that were aimed at developing innovative emulsifiers for use in emulsion-based systems, which can deliver omega-3 rich oils into food products.

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Work is ongoing to modify the alginate saccharides for the production of emulsifiers, which will be applied in the formation of oil-in-water emulsions. These emulsions will be characterized, with special focus on their physical stability and the oxidative stability of the emulsified oil.

Summary

This report summarizes the progress of the first half of my Ph.d. study at Department of Engineering, Aarhus University. The project is aiming at developing innovative emulsifiers for encapsulation purposes. The experimental work was initiated in October 2011, and was originally focused on the synthesis of glycophospholipids. The results from this work have been submitted to Biomaterials entitled "Stabilising Nanoliposomes by Synthetic Biomimetic Phosphatidylsaccharides". The main focus of the Ph.d. project is on the synthesis and application of glycoesters synthesized from saccharides originating from *alginate*; a cheap and abundant polysaccharide, which has been shown to have antioxidant properties. The applications of these glycoesters of interest in this project are in the stabilization of marine oil emulsions, for use as systems for delivery of sensitive omega-3 rich oils into food products. The strategy is to enzymatically depolymerize alginate, followed by modification of the alginate saccharides trough ester-bonding with hydrophobic moieties. Two types of hydrophobic modifications are in focus: Type A; esterification of the carboxylic group(s) of alginate saccharides with fatty alcohols, and type B; esterification of the hydroxyl group(s) of alginate saccharides with fatty acids. For each type, a range of emulsifiers will be synthesized by varying the carbon chain length and the degree of esterification. The resulting emulsifiers will be characterized, and applied in oil-in-water emulsions. These emulsions will be characterized, with special focus on their physical stability and the oxidative stability of the emulsified oil.

During 2012, work has been focused on the enzymatic depolymerization of alginate, and characterization of the resulting alginate oligosaccharides, with special focus on their antioxidative properties. The majority of this work has been completed, and is expected to be presented in an article entitled "Preparation of Alginate Oligosaccharides through Enzymatic Depolymerization and Characterization of their Antioxidant Properties". A suitable method for the synthesis of alginate-based glycoesters type A has been found, and using this method, a range of compounds will be synthesized and characterized. These results are expected to be presented in an article entitled "Synthesis of Alginate Oligosaccharide Esters and Characterization of their Surface Activity and Antioxidant Properties". Part B of the Ph.d. studies will be focused on developing a suitable method for the synthesis of type B glycoesters, as well as on the application the emulsifiers in the formation of oil-in-water emulsions.

During my work I have received excellent guidance from my main supervisor Zheng Guo, Department of Engineering, and previously Xuebing Xu, Department of Engineering. My co-supervisor Marianne Glasius, Department of Chemistry, has been very helpful in providing practical assistance with specialized analyses. My work has been guided by Ling-Zhi Cheong, Department of Engineering. Master student Carlo Gianfico from University of Rome, 'Tor Vergata', assisted in the experimental work during the spring semester 2012. The project is partly funded by "Innovationskonsortiet: Multifunktionelle indkapslingssystemer - Multicaps" in collaboration with Danish Technological Institute, DuPont, Marine Bioproducts, and University of Southern Denmark.

Contents

1. Background

Marine oils have a high content of long chain omega-3 (n-3) polyunsaturated fatty acids (PUFAs). Extensive research has shown that increased consumption of these n-3 PUFAs has a number of beneficial health effects, and the importance of these fatty acids in human nutrition and disease prevention is scientifically recognized (Narayan *et al.* 2006; Ruxton *et al.* 2007; Shahidi & Miraliakbari 2004; Shahidi & Miraliakbari 2005). It has been suggested that the typical western diet, which is high in n-6 fatty acids but low in n-3 fatty acids, may not supply the appropriate balance of PUFAs for proper biological function of the body. This imbalance is believed to cause a variety of diseases, including cardiovascular diseases and inflammatory disorders (Din *et al.* 2004; Wall *et al.* 2010). For this reason, there is an interest for producing regularly-consumed food products enriched with marine oils (Jacobsen & Nielsen 2007; Kolanowski *et al.* 1999; Taneja & Singh 2012). Problems occur as n-3 PUFAs are highly sensitive and prone to oxidation due to their high degree of unsaturation. Lipid oxidation is initiated by metal ions, heat, or free radicals from e.g. proteins, which can be present in food products, which causes the unsaturated fatty acids to form alkyl radicals, which form peroxyl radicals in the presence of oxygen. Peroxyl radicals react with unsaturated fatty acids to form lipid hydroperoxides and new lipid radicals, which can continue the free radical chain reaction (Schaich 2005; Shahidi & Zhong 2010). The primary products of lipid oxidation, the hydroperoxides, are taste- and odorless (Jacobsen & Nielsen 2007), but they decompose into a variety of secondary oxidation products, which can be harmful, and cause unacceptable off-flavors in the food product (Jacobsen *et al.* 2008; Jacobsen & Nielsen 2007). It is hence important to stabilize the PUFAs when producing food products enriched with marine oil. This can be done by the use of antioxidants, which work as free radical scavengers, as metal chelating agents, or as reducing agents (Jacobsen *et al.* 2008; Jacobsen & Nielsen 2007; Shahidi & Zhong 2010). In addition to, or in combination with, the use of antioxidants, microencapsulation of marine oil has been shown to be effective in protecting the PUFAs from oxidation (Taneja & Singh 2012). Microencapsulation can also enhance the compatibility of the marine oil with the food product. The majority of the currently applied techniques for microencapsulation of marine oil require the formation of an oil-in-water emulsion (Taneja & Singh 2012). The most commonly used microencapsulation technique involves drying of the emulsified oil, e.g. by spray-drying, using a suitable carrier material in which the emulsified oil will be embedded (Barrow *et al.* 2007; Desai & Park 2005). Spray-dried emulsions have been applied in short shelf-life products such as bread (Barrow *et al.* 2007). The spray-drying technique is less suitable for liquid food products, as the carrier materials usually are water-soluble (Sagalowicz & Leser 2010). Simple oil-in-water emulsions, which have not been dried, can be used to deliver marine oils into certain food products, such as milk, yoghurt and salad dressings (Let *et al.* 2007), and processed cheese (Ye *et al.* 2009). Modifications of the simple emulsion by layer-by-layer electrostatic deposition have been shown to further stabilize the emulsified marine oil (Guzey & McClements 2006). Common for all the mentioned delivery systems is the formation of a stable emulsion. To prepare a physically stable emulsion, the addition of an emulsifier during the emulsification process is required. Emulsifiers are surface active molecules with amphiphilic properties, which can reduce the surface tension at the oil-water interface. Compared to bulk oil, the susceptibility of lipid oxidation in an oil-in-water emulsion is increased, due to the large interfacial area (Jacobsen & Nielsen 2007; Shahidi & Zhong 2011). To protect emulsified oil from oxidation, special focus

needs to be placed on the oil-water interface where oxidation is initiated, and on the location of antioxidants in the emulsion. Regarding the latter, antioxidants present at, or near, the oil-water interface provides better protection, than antioxidants present in the aqueous phase (Coupland & McClements 1996; Shahidi & Zhong 2011; Yuji *et al.* 2007). The type and quantity of emulsifier in the system can change the effect of antioxidants. The emulsifier saturates the oil-water interface, thus leaving less interfacial area available for antioxidants. If the emulsifier is present in high quantity, micelles form in the aqueous phase, which may entrap antioxidants (Shahidi & Zhong 2011). Following these considerations it was hypothesized, that in order to optimally protect emulsified marine oil from oxidation, an emulsifier was required which, in addition to physically stabilizing the oil-in-water emulsion, also functioned as an antioxidant. Based on this, the aim of this project is to develop innovative emulsifiers, designed to have optimal amphiphilic structures for stabilizing oil-in-water emulsions, and to have antioxidative properties, protecting the emulsified oil from oxidation. The emulsifiers will be developed by derivatization of alginate, which has been found to have antioxidative properties. It is expected that emulsifiers derived from alginate will be antioxidative, too. In the following section, the use of alginate is introduced, and the work performed regarding the depolymerization of alginate and characterization of the alginate oligosaccharides is presented.

2. Alginate Depolymerization

2.1. Introduction

This project focuses on developing new emulsifiers by derivatization of alginate. Alginate is a linear polysaccharide composed of α-L-guluronate (G) and its C5 epimer β-D-mannuronate (M), arranged as homopolymeric blocks (polymannuronate and polyguluronate), and heteropolymeric blocks (strictly alternating GM blocks and random G/M blocks) (Benvegnu & Sassi 2010; Lee & Mooney 2012; Pawar & Edgar 2012). Guluronate and mannuronate are uronates with carboxylate groups at the C5 positions, the configuration of which represents the difference between the two pyranoses. Alginate can be obtained from both algal and bacterial sources; the commercial available alginate is currently extracted from algal sources only. Approximately 30,000 tons of alginate is produced annually, which is estimated to be less than 10% of the total amount of biosynthesized alginate (Benvegnu & Sassi 2010; Pawar & Edgar 2012). Alginate can hence be considered an unlimited, renewable resource of biomaterial. Currently, alginate has a broad range of applications owing to its low cost, low toxicity, and biocompatibility (Lee & Mooney 2012). Based on the properties and abundance of alginate, there is a potential for expanding its use as a sustainable material for the production of a range of bioproducts, including emulsifiers for use in food products.

Alginate lyases catalyze the depolymerization of alginate by breaking the glycosidic linkages between the mannuronate and/or guluronate units by an endo-active β-elimination mechanism. The resulting oligosaccharides will have *4-deoxy-erythro-hex-4-enopyranosyluronate* at their non-reducing ends. The outcome of the depolymerization reaction is a mixture of unsaturated oligosaccharides (Gacesa 1992; Kim *et*

al. 2011; Wong *et al.* 2000). An illustration of sodium alginate oligosaccharides with 4-deoxy-erythro-hex-4-enopyranosyluronate at their non-reducing ends is given in Figure 1. The configuration of the C5 carboxylate group(s) in the reducing ends depends on the nature of saccharide (guluronate or mannuronate).

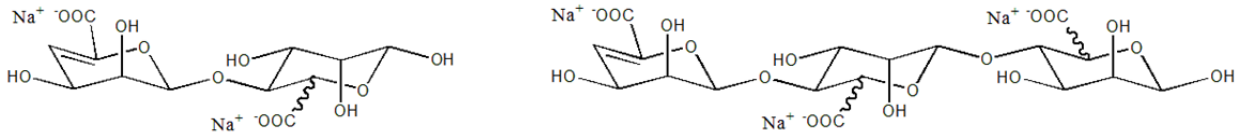

Figure 1: Sodium alginate oligosaccharides (dimer and trimer).

Based on literature, it is expected that alginate oligosaccharides prepared by enzymatic depolymerization have antioxidant properties. Zhao *et al.* (Zhao *et al.* 2012) and Wang *et al.* (Wang *et al.* 2007) have shown that enzymatically depolymerized alginate of varying molecular size can scavenge free radicals; however, when analyzing lipid peroxidation in an emulsion system, inhibition activity was shown only by Zhao *et al.*, and not by Wang *et al.*. Some reports have shown that the molecular weight of alginate influences its antioxidant activities. Lower-molecular weight fractions produced by enzymatic depolymerization (Zhao *et al.* 2012) or by radiation-induced degradation (Sen 2011) have higher antioxidant activity, than higher-molecular weight fractions. No reports exists regarding the antioxidative properties of an alginate oligosaccharide equilibrium mixture prepared by complete depolymerization of sodium alginate by a β-eliminating lyase; or of the monomeric forms of alginate prepared by complete acid hydrolysis (mannuronate and guluronate). In this study, the antioxidative properties of alginate oligosaccharides are characterized by various assays, and compared to ascorbic acid. Comparative studies are made on the polymeric, oligomeric, and monomeric forms of alginate; on mannuronate- and guluronate-rich fractions; and on acid and salt forms of the saccharides, in order to elucidate the mechanism of the antioxidant activities.

2.2. Materials

Sodium alginate (Grindsted® Alginate FD 170) was provided by Danisco, Brabrand, Denmark. This alginate originated from brown algae, and the ratio of α-L-guluronate units to β-D-mannuronate units was 40-60. Alginate lyase S derived from *sphingobacterium* was provided by Nagase Enzymes, Kyoto, Japan. Amberlite® 200 Na^+ strong acidic cation exchanger resin, Tween® 20 non-ionic detergent, phosphate buffered saline (PBS) 0.01M pH 7.4 foil pouch, trichloroacetic acid, ascorbic acid, D-glucuronic acid, and thiobarbituric acid (TBA), were from Sigma-Aldrich. Linoleic acid 96% was from Zhongchuan Biotechnology Co Ltd. Anqing, China. All solvents were of chromatographic purity.

2.3. Methods

2.3.1. Enzymatic Depolymerization of Sodium Alginate

Sodium alginate was depolymerized in 0.05M ammonium acetate at a concentration of 2% w/v sodium alginate, using alginate lyase S at a concentration of 5% w/w of sodium alginate, at 35˚C and 200 rpm. Aliquots of 200 µL were withdrawn periodically in which the lyase was inactivated by heat denaturation at 110°C for 15 min. The absorbance at 234 nm of the properly diluted supernatant after lyase inactivation was

measured (Varian Cary 50Bio UV-visible spectrophotometer, quartz cuvette), using 0.05M ammonium acetate as blank. After 72 hours, the lyase was denatured by incubation at 110°C for 15 min, and removed by centrifugation. The alginate oligosaccharides were purified and dried by repeated evaporation of water and ammonium acetate at 20 mbar and 65°C.

2.3.2. Acidification of Alginate Oligosaccharides

For production of alginic acid oligosaccharides, alginate oligosaccharides were dissolved in water to a concentration of 200 mg/mL and passed over Amberlite® 200 resins, which had been activated by 1M HCl. The acidified oligosaccharides were purified and dried by repeated evaporation of water and excess ions at 20 mbar and 65°C. The acidification procedure was confirmed by FTIR, and the composition of the acidified oligosaccharides was determined by thin layer chromatography (TLC).

2.3.3. Preparation of Polymannuronate and Polyguluronate

Sodium alginate was partially hydrolyzed according to the modified procedure by Chandía *et al.* (Chandia *et al.* 2001), as follows. Three g sodium alginate in 300 mL deionized water was heated at reflux for 20 min with 9 mL 3M HCl. The cooled suspension was centrifuged at 10,000 g for 20 min, and the precipitate was suspended in 300 mL 0.3M HCl and heated at reflux for 2 hours. The cooled suspension was centrifuged at 10,000 g for 20 min, and the precipitate was neutralized with 1M NaOH. The pH was adjusted to 2.85 ± 0.05 with 1M HCl. After centrifugation at 10,000g for 15 min, the soluble polymannuronate fraction and the insoluble polyguluronate fraction were collected and neutralized with 1M NaOH. The pH of both fractions was adjusted to 2.85 ± 0.05, centrifuged, and the collected fractions were neutralized with 1M NaOH. The saccharides were then precipitated in ethanol, washed with ethanol, and dried. FTIR was used to determine the degree of separation of the homopolymeric mannuronate and guluronate fractions.

2.3.4. Characterization of Alginate Oligosaccharides

TLC analyses were performed on silica plates (Merck Silica Gel 60, glass plates, 20x20 cm) using 1-butanol:formic acid:water 4:6:1 v:v:v as developing solvent. The saccharides were visualized by spraying with 10% v/v sulphuric acid in ethanol followed by heating at 110°C for 10 min. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Bruker micrOTOF-Q III mass spectrometer in negative mode. Sodium alginate oligosaccharides were dissolved in deionized water and infused into the ESI source, using the following settings: capillary voltage 4.00 kV, nebulizer pressure 3.4 bar, gas flow rate 10.0 L/min, and temperature 180°C. The resulting mass spectra were analyzed using Bruker Daltonics DataAnalysis 3.4 software. Fourier-transformed infrared (FTIR) spectra were recorded in absorbance mode in the 4000-650 cm^{-1} region at a resolution of 4 cm^{-1} using a Qinterline QFAflex spectrometer equipped with a deuterium triglycine sulfate (DTGS) detector. The samples were placed in their pure solid form in a Pike attenuated total reflectance (ATR) device thermostated at 25°C. The spectra were ratioed against a single-beam spectrum of the clean ATR crystal. The resulting spectra were analyzed using GRAMS/AI software.

2.3.5. Effect on Lipid Peroxidation in Emulsion

The ability of the saccharides to inhibit iron-induced lipid oxidation in a model emulsion system was evaluated by the thiobarbituric acid reactive species (TBARS) assay. The saccharides were dissolved in PBS buffer in the concentration range 0-175 mg/mL. A model emulsion was prepared by mixing 1 mL linoleic acid and 0.5 mL Tween® 20 in 48.5 mL PBS buffer. Fifty µL of this emulsion was combined with 1.5 mL saccharide in PBS buffer and 250 µL 25 mM $FeSO_4$. The mixture was incubated under magnetic stirring at 400 rpm for 15 min at room temperature, after which 0.5 mL trichloroacetic acid and 1 mL 0.7% TBA in 0.05M KOH was added. The samples were incubated at 100°C for 15 min, cooled to room temperature and centrifuged at 4,000g for 1 min. The absorbance at 534 nm was then determined in a Varian Cary 50Bio UV-visible spectrophotometer. For each sample, at each concentration, a negative control was prepared by replacing the emulsion with pure PBS. The absorbances of these samples were withdrawn the absorbances of the respective samples with emulsion, to account for the absorbance of the saccharides, and other substances, at 534 nm. This difference in absorbance, at a given saccharide concentration, is notated $A_{c,}$ and the percentage inhibition at each concentration of each saccharide is calculated according to equation 1, in which A_o refers to the absorbance of a sample in which pure PBS was added in replacement of saccharide solution.

$$Inhibition = \frac{A_o - A_c}{A_o} * 100 \qquad \text{(Eq. 1)}$$

Samples were analyzed in duplicates, and duplicate spectrophotometric measurements were made for each sample. Ascorbic acid was analyzed for comparison.

2.4. Results and Discussion

Sodium alginate was enzymatically depolymerized using alginate lyase S, and the formation of unsaturated oligosaccharides was followed by measuring the absorbance at 234 nm. The time course of the depolymerization reaction is shown in Figure 2.

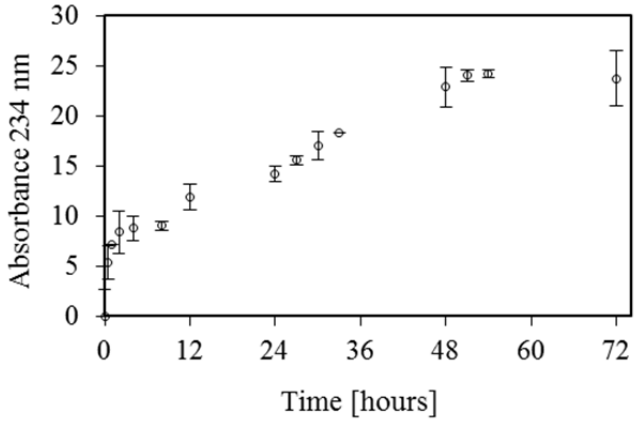

Figure 2: Increase in absorbance at 234 nm during 72 hours of sodium alginate depolymerization. The error bars indicate double standard variations around the mean values obtained from triplicate analyses with duplicate spectrophotometric measurements.

Approximately one third of the final absorbance was reached within 2 hours, after which the rate of depolymerization decreased, indicating that the lyase had a higher activity towards the starting material (sodium alginate) than towards the oligosaccharide intermediates. No further increase in absorbance ($p<0.05$) was observed after 48 hours, indicating that no more unsaturated saccharides formed.
Samples from hours 0, 1, 5, and 23 were analyzed by TLC, along with glucuronic acid (monomer standard). The results are shown in Figure 3, which shows that the depolymerization reaction progressed through endo-cleaving activity of the lyase, by which the large polymers were internally cleaved into smaller units, until the mixture contained only dimers, trimers, tetramers, and undetectable amounts of higher oligosaccharides.

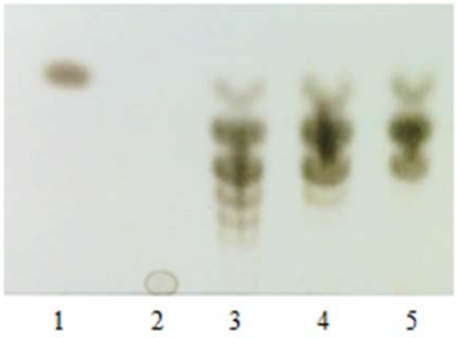

Figure 3: ***Lane 1****: Glucuronic acid.* ***Lanes 2-5****: Sodium alginate after 0, 1, 5, and 23 hours of depolymerization, respectively.*

For production of alginate oligosaccharides for use in the synthesis of emulsifiers, the depolymerization reaction was terminated after 72 hours. A portion of the alginate oligosaccharides obtained after 72 hours of depolymerization were acidified by ion exchange. The compositions of alginate oligosaccharides and acidified oligosaccharides after 72 hours were analyzed by TLC, and the results are presented in Figure 4.

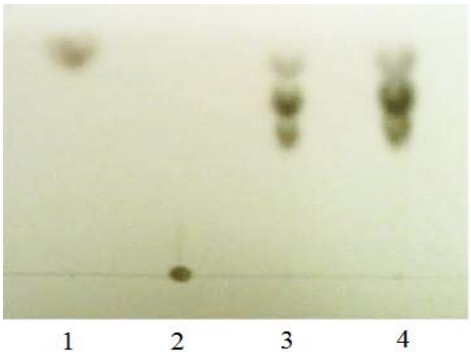

Figure 4: ***Lane 1****: Glucuronic acid.* ***Lane 2****: Sodium alginate.* ***Lane 3****: Depolymerized sodium alginate.* ***Lane 4****: Depolymerized alginate acidified by ion exchange.*

Only dimers, trimers, and tetramers were detected by TLC after 72 hours of depolymerization. Comparison of lanes 3 and 4 in Figure 4 reveals that the acidification using ion exchanger resin did not change the composition of the oligosaccharides.
The composition of alginate oligosaccharides was confirmed by ESI-MS, as presented in Figure 5.

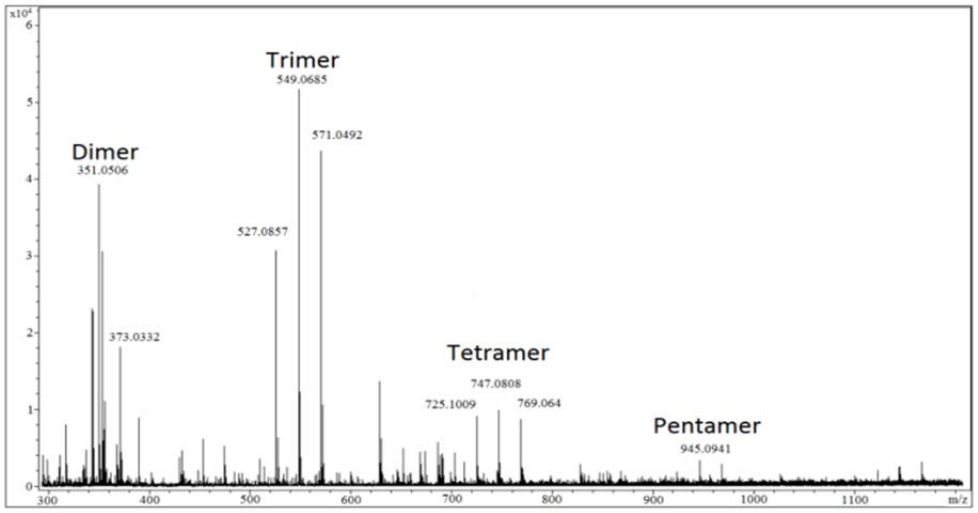

Figure 5: ESI-MS spectrum of depolymerized sodium alginate: Signal intensity x10^4 vs. *m/z.*

ESI-MS confirmed that the oligosaccharides were composed of dimers, trimers, and tetramers; this technique was able to also detect a very low amount of pentamers, which were undetectable by TLC. Each oligomer occurs as several signals in the MS-spectra, depending on the ionization of the carboxyl group. The dimer occurs as signals 373.03 m/z ($M - 1Na^+$) and 351.05 m/z ($M+H^+ - 2NA^+$); the trimer occurs as signals 571.05 m/z ($M - 1Na^+$), 549.07 m/z ($M+H^+ - 2NA^+$), and 527.09 m/z ($M+2H^+ - 3NA^+$); the tetramer occurs as signals 769.06 m/z ($M - 1Na^+$), 747.08 m/z ($M+H^+ - 2NA^+$), 725.10 m/z ($M+2H^+ - 3NA^+$), and 703.12 m/z ($M+3H^+ - 4NA^+$) (low signal, not indicated in Figure 5); where M corresponds to the respective sodium alginate oligomer.

As TLC and ESI-MS are unable to give quantitative information, the molar- or weight percentage distribution of dimers, trimers, and tetramers could not be determined using these methods. An HPLC method using a normal-phase amino column and acetonitrile-water as mobile phase is currently being developed, which can provide quantitative information about the composition of the oligosaccharides.

The FTIR spectra of sodium alginate, sodium oligosaccharides, and acidified oligosaccharides, are shown in Figure 6.

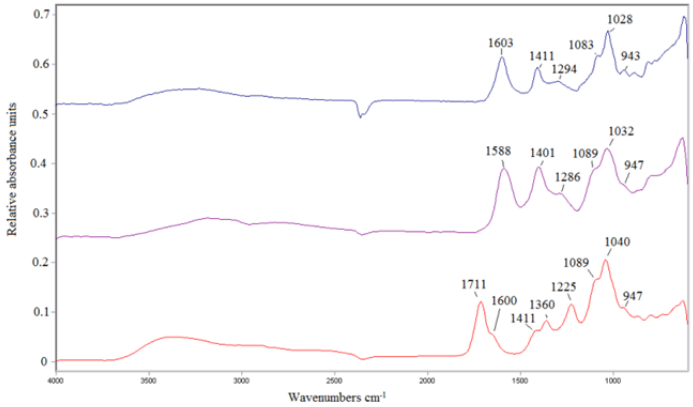

Figure 6: FTIR spectra of sodium alginate (top), sodium oligosaccharides (mid), and acidified oligosaccharides (bottom).

The strong bands in the sodium alginate and sodium oligosaccharides spectra at 1603 cm^{-1} and 1588 cm^{-1}, respectively, are assigned to the asymmetric stretching vibrations of the C=O carboxylate (Gomez-Ordonez & Ruperez 2011; Leal *et al.* 2008; Yadav 2005). The bands at 1411 cm^{-1}, 1401 cm^{-1}, and 1411 cm^{-1} in the spectra are assigned to the C–OH deformation vibrations. In the top two spectra, these bands (1411 cm^{-1} and 1401 cm^{-1}) are with contribution from the symmetric stretching vibrations of the C=O carboxylate. Due to strong hydrogen bonding, carboxylic acids exist as dimers, giving it a center of symmetry. Thus, in the bottom spectrum, the symmetric C=O stretching vibration do not absorb in the infrared. The decrease in double bond character of the C=O by resonance is greater in the carboxylate than in the carboxylic acid, causing the C=O asymmetric stretching vibration of the carboxylic acid to occur at higher wavenumbers than the C=O asymmetric stretching vibration of the carboxylate (Yadav 2005). In the spectrum of the acidified oligosaccharides, the band at 1711 cm^{-1} is hence assigned to the C=O asymmetric stretching vibration of the carboxylic acid (Gomez-Ordonez & Ruperez 2011; Yadav 2005). Some absorbance in the ~1600 cm^{-1} region can be observed in the spectrum of the acidified oligosaccharides, suggesting that some of the carboxyls may still be on carboxylate form. The band at 1225 cm^{-1} in the acidified oligosaccharide spectrum is assigned to the C–O stretching vibration of the carboxylic acid (Yadav 2005). In each spectrum, two bands at around 1080 cm^{-1} and 1030 cm^{-1} occur. These have in the literature been assigned to the C–O and C–C stretching vibrations of pyranose ring (Gomez-Ordonez & Ruperez 2011; Leal *et al.* 2008). Sakugawa *et al.* (Sakugawa *et al.* 2004) suggested a method for determining the relative content of mannuronate and guluronate in alginate, based on the ratio of absorbance at the bands at 1080 cm^{-1} and 1030 cm^{-1}, as will be discussed later. The band at 943 cm^{-1} in the alginate spectrum is assigned to the 1→4 glycosidic linkage (Chandia *et al.* 2001). In the depolymerized alginate samples, the majority of these bands have been broken, and the band is, as expected, nearly undetectable in the bottom two spectra. All the saccharides show the characteristic broad absorbance band in the region 3200-3500 cm^{-1}, assignable to the hydroxyl stretching vibrations (Yadav 2005). The acidified oligosaccharides further show the very broad band at 2500 – 3300 cm^{-1}, assignable to the O–H stretching vibration of the carboxylic acid (Yadav 2005).

In conclusion, sodium alginate was successfully depolymerized into alginate oligosaccharides, composed of dimers, trimers, and tetramers of mannuronate and guluronate. Acidification of the oligosaccharides using ion exchanger did not change the composition of the oligomers.

To prepare isolated mannuronate and guluronate fractions, a method consisting of partial acid hydrolysis of alginate followed by pH adjustment was used. The method was originally reported by Haug *et. al.* (Haug *et al.* 1966), and the method and variations is frequently used for the preparation of polyguluronate and polymannuronate fractions of alginate (Chandia *et al.* 2001; Fenoradosoa *et al.* 2010; Sakugawa *et al.* 2004). It is based on the higher resistance to acid hydrolysis of the homopolymeric blocks compared to the heteropolymeric blocks. It follows that the heteropolymeric fractions can be removed by partial acid hydrolysis of the alginate, followed by centrifugation to recover the insoluble homopolymeric fraction. The homopolymeric fraction can then be separated into polymannuronate and polyguluronate by adjusting the pH to 2.85, at which polymannuronate is soluble and polyguluronate is insoluble. Haug *et al.* (Haug *et al.* 1966) reported that the homopolymeric fractions prepared this way were 80-90% pure. For each 1,000 mg alginate

hydrolyzed, 200-300 mg polyguluronate and 270-370 mg polymannuronate was isolated. The FTIR spectra of isolated polymannuronate and polyguluronate and of sodium alginate in the range 2000-650 cm^{-1} are shown in Figure 7.

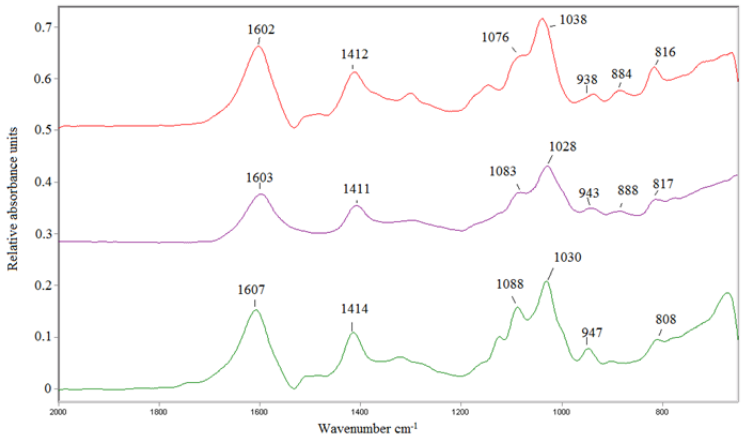

Figure 7: FTIR spectra of polymannuronate (top), sodium alginate (mid), and polyguluronate (bottom).

As described previously, the bands at ~1600 cm^{-1} are assigned to the asymmetric stretching vibration of the carboxylate C=O. All the carboxyl groups in these samples were hence present as carboxylates and not carboxylic acids. The bands at ~1080 cm^{-1} and ~1030 cm^{-1} can be used to estimate the relative contents of mannuronate and guluronate, as suggested by Sakugawa *et al.* (Sakugawa *et al.* 2004). Following the methodology of Sakugawa *et al.*, the ratio of absorbance intensities in these bands was plotted *versus* the content of mannuronate in each sample, assuming the homopolymeric fractions were 90% pure. As in the report by Sakugawa *et al.*, a linear relationship was found (R^2 = 0.940), with the ratio being highest for polymannuronate, and lowest for polyguluronate. This indicates successful fractionation of the sodium alginate into sodium polyguluronate and sodium polymannuronate. However, the standard curve constructed by Sakugawa *et al.* was based on calcium alginate. Due to the lack of pure sodium polymannuronate and sodium polyguluronate standards, no standard curve of the ratio of absorbance at 1030 cm^{-1} and 1080 cm^{-1} could be constructed in this work, and more precise estimations of the relative contents of mannuronate and guluronate can hence not be made based on this method. The band at 884 cm^{-1} was assigned to the anomeric C–H deformation vibration of β-mannuronate residues (Chandia *et al.* 2004; Chandia *et al.* 2001; Gomez-Ordonez & Ruperez 2011; Leal *et al.* 2008). In this work it is indeed observed, that the absorbance in this band is highest in the polymannuronate sample and undetectable in the polyguluronate sample.

Taken together, the FTIR data indicates that the fractionation of alginate into polymannuronate and polyguluronate has been successful. It is expected, that the HPLC-method under development can give a more accurate estimate on the relative contents of mannuronate and guluronate in these fractions. Additionally, HPLC can give information regarding the molecular sizes of the homopolymeric oligosaccharides.

The commonly used TBARS assay was used to determine the antioxidant activities of the alginate saccharides. This assay was found suitable, as it examines lipid oxidation in an emulsion system, which is the expected area of application of the saccharides. The lipid hydroperoxides, which form during the iron-

induced oxidation of the linoleic acid, decomposes into a variety of secondary oxidation products, including malondialdehyde. Under acidic conditions, TBA reacts with malonaldehyde, and other hydroperoxide decomposition products, to form a colored complex. The substances which reacts with TBA is termed TBA-reactive substances (TBARS), and include 2-alkenals and 2,4-alkadienals, in addition to malondialdehyde. The formation of the colored complex can be followed by spectrophotometric measurements (Guillen-Sans & Guzman-Chozas 1998; Jacobsen 2008). In this work, the absorbance of the saccharides was taken into account; both the absorbance of the saccharides themselves, and the absorbance that may occur due to interaction with TBA. Inhibition percentages were then calculated as the percentage difference in absorbance between samples incubated with and without saccharides, at varying concentrations. The oxidative status of the unsaturated fatty acid was tested by measuring the TBARS content of the emulsion system directly. It was found that the emulsion system itself contained no TBARS. Furthermore, no increase in the content in TBARS was found in the model system without incubation with ferrous sulphate.

As shown in Figure 8, the alginate oligosaccharides were found to have excellent antioxidative properties, in a concentration-dependent manner. The alginate oligosaccharides were able to completely inhibit the ferrous-induced peroxidation, in contrast to ascorbic acid, which at its highest was able to inhibit 89%.

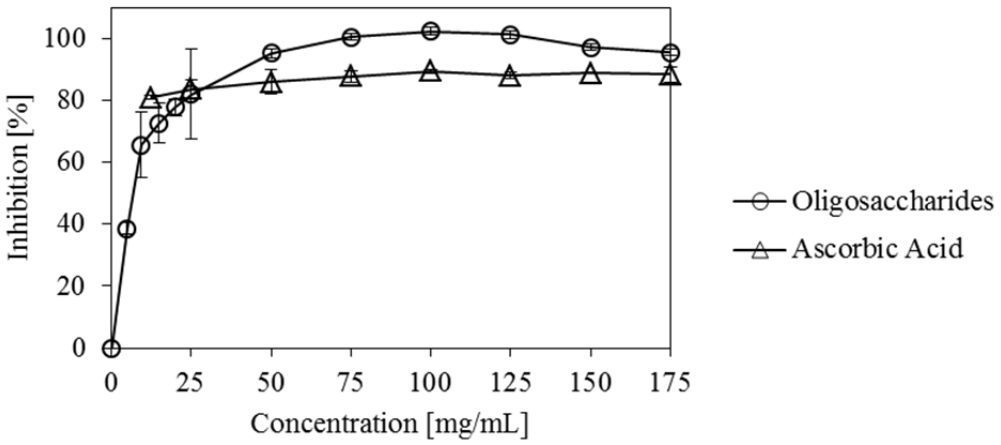

Figure 8: Percentage inhibition of lipid peroxidation of alginate oligosaccharides and ascorbic acid as a function of their concentrations. The error bars indicate double standard variations around the mean values obtained from duplicate analyses with duplicate spectrophotometric measurements.

The mechanisms by which the oligosaccharides exert their antioxidant activity are currently under evaluation. It has been suggested by Zhao *et al.* (Zhao *et al.* 2012) and Wang *et al.* (Wang *et al.* 2007) that alginate oligosaccharides with molecular sizes below 10 kDa, and 4388 Da, respectively, can scavenge free radicals. To study this, the use of the DPPH* free radical method will be used, according the method suggested by Brand-Williams (Brand-Williams *et al.* 1995). Zhao *et al.* (Zhao *et al.* 2012) and Wang *et al.* In addition to acting as a free radical scavenger, Rupérez *et al.* (Ruperez *et al.* 2002) showed that polymeric alginate-rich fractions of polysaccharides had reducing power. The ferric reducing antioxidant power (FRAP) assay will be used to determine the reducing power of the alginate oligosaccharides in this study, according to the methods suggested by Benzie *et al.* (Benzie & Strain 1996).

To analyze if the depolymerization reaction had an influence of the antioxidative properties, the TBARS assay was performed on polymeric and oligomeric alginate for comparison. The characterization of the antioxidative properties of polymeric alginate by the TBARS assay was made difficult by the high viscosity of the sample solution (sodium alginate in PBS buffer), and data points could only be collected at lower concentrations (0-50 mg/mL). The results are presented in Figure 9.

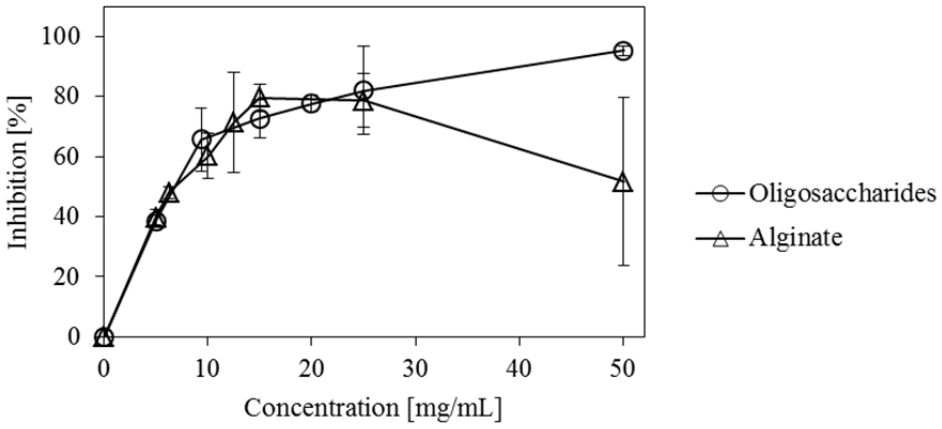

Figure 9: Percentage inhibition of lipid peroxidation of oligomeric and polymeric alginate as functions of concentrations. The error bars indicate double standard variations around the mean values obtained from duplicate analyses with duplicate spectrophotometric measurements.

At lower concentrations (< 25 mg/mL), no difference in antioxidant activity between polymeric and oligomeric alginate was observed. At increasing concentrations it is indicated, that the antioxidative activity of polymeric alginate in decreasing, oppositely to oligomeric alginate.

The polymannuronate and polyguluronate fractions of alginate prepared by acid hydrolysis and pH adjustment were subjected to TBARS analysis, and the results are presented in Figure 10. It shows that the antioxidant activity of alginate does not depend on the relative content of mannuronate and guluronate.

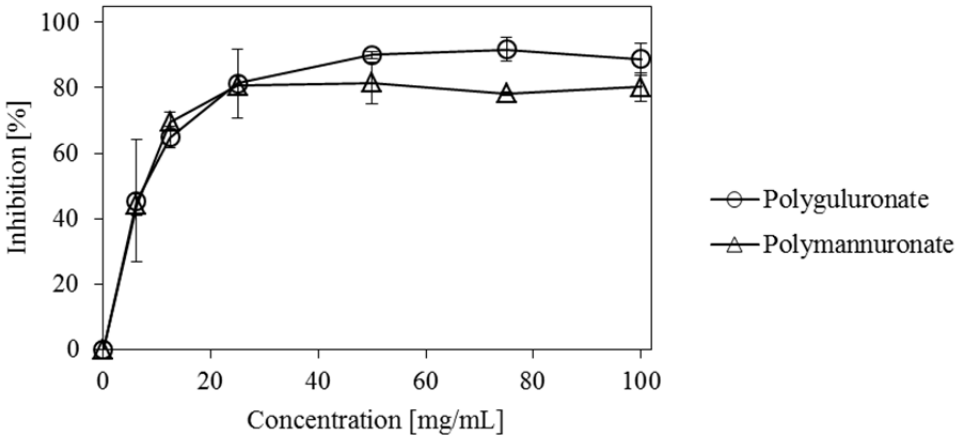

Figure 10: Percentage inhibition of lipid peroxidation of polyguluronate and polymannuronate as functions of concentrations. The error bars indicate double standard variations around the mean values obtained from duplicate analyses with duplicate spectrophotometric measurements.

Monomeric forms of alginate (mannuronate/mannuronic acid and guluronate/guluronic acid) can be prepared by complete acid hydrolysis of the polymannuronate and polyguluronate fractions, according to the methods suggested by Chandía *et al.* (Chandia *et al.* 2001). With this method it is, however, difficult to obtain monomers on the gram-scale, as needed for analysis of antioxidant activity. Alternatively, an analogue to mannuronic acid and guluronic acid, glucuronic acid, which is commercially available, was analyzed by the TBARS assay in this study. This saccharide was found to act pro-oxidatively in a linear concentration-dependent manner ($R^2 = 0.96$), as shown in Figure 11. Mannuronic acid and guluronic acid are expected to have a similar effect on the lipid peroxidation.

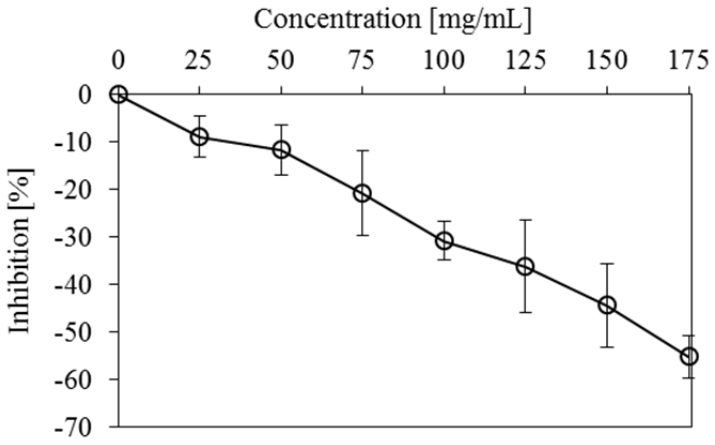

Figure 101: Percentage inhibition of lipid peroxidation of glucuronic acid as a function of concentration. The error bars indicate double standard variations around the mean values obtained from duplicate analyses with duplicate spectrophotometric measurements.

It is expected, that mannuronic acid and guluronic acid, due to the structure similarity, will act in a similar way as glucuronic acid. The mechanisms of this pro-oxidative activity will be elucidated by the DPPH* and FRAP assays.

2.5. Summary and Future Works

Sodium alginate has been successfully depolymerized by an alginate lyase, and the composition of the alginate oligosaccharides has been determined by TLC and ESI-MS. An HPLC method is currently under development, which can give quantitative information about the composition of the alginate oligosaccharides. The alginate oligosaccharide were found to completely inhibit iron-induced lipid peroxidation in a oil-in-water emulsion, and are hence considered excellent starting materials for the development of emulsifiers for microencapsulation of marine oil. The elucidation of the mechanisms of the antioxidative properties of alginate oligosaccharides will continue in part B. This will include; analysis of the polymannuronate- and polyguluronate-rich fractions, analysis of the acidified oligosaccharides, and formation and analysis of alginate monomers. Some or all of the mentioned saccharides will be analyzed by the TBARS, and the FRAP and DPPH* assays.

3. Fatty Alcohol Esters of Alginate Oligosaccharides

3.1. Introduction

Two approaches for hydrophobically modifying the alginate oligosaccharides are being examined in this study. This section describes the work done regarding the esterification of the carboxyl group(s) of alginate oligosaccharides with fatty alcohols. The reaction is illustrated in Figure 12. The saccharide part of this reaction can be trimers and tetramers as well as the illustrated dimer. The position of esterification is chosen arbitrarily and can vary depending on the esterification method, including the possibility of multiple esterifications.

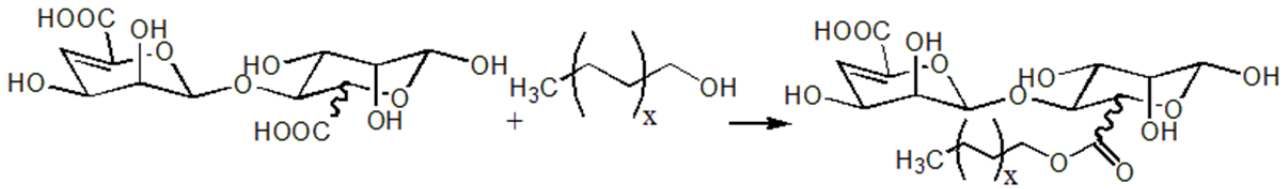

Figure 112: Esterification of acidified dimer with fatty alcohol.

Yang *et al.* (Yang *et al.* 2012) modified polymeric sodium alginate with dodecanol trough ester functions using chemical catalysis. In this study, their method was applied to the synthesis of octanol esters of alginate oligosaccharide esters, as described in the following.

3.2. Materials

Depolymerized sodium alginate (dimers, trimers, and tetramers) was prepared by enzymatic depolymerization as described in Section 2. Octanol was from Fluka, and formamide, dimethylformamide, toluenesulfonic acid, 4-(N,N-dimethylamino) pyridine (DMAP), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), were from Sigma-Aldrich.

3.3. Methods

3.3.1. Synthesis of Fatty Alcohol Esters of Alginate Oligosaccharides

Sodium alginate oligosaccharides prepared by enzymatic depolymerization (1.0 g) were partly protonated in 35 mL formamide:dimethylformamide 10:9 v:v containing 0.50 g toluenesulfonic acid, under magnetic stirring at 55°C for 30 min. 2.93 g octanol was added, along with 0.380 g EDCI and 0.475 g DMAP, and the reaction was continued for 30 hours at 45°C. The reaction mixture was cooled, and the saccharide esters and unreacted saccharides were precipitated in 4 volumes of ethanol. The ethanol precipitate was either dried under nitrogen for FTIR analysis, or dissolved in deionized water for TLC analysis.

3.3.2. Characterization of Fatty Alcohol Esters of Alginate Oligosaccharides

FTIR spectra were recorded in absorbance mode in the 4000-650 cm^{-1} region at a resolution of 4 cm^{-1} using a Qinterline QFAflex spectrometer equipped with a DTGS detector. The samples were placed in their pure solid form in a Pike ATR device thermostated at 25°C. The spectra were ratioed against a single-beam spectrum of the clean ATR crystal. The resulting spectra were analyzed using GRAMS/AI software.

TLC analyses were performed according to the method by Kou and Xu (Kou & Xu 1998). Samples were spotted onto a silica-gel plate (10 cm x 10 cm) and the plate was developed for 2.2 cm in chloroform:methanol:water 64:10:1 v:v:v. The slightly dried plate was then developed on 7.5 cm with hexane:diethyl ether:acetic acid 70:30:1 v:v:v. The products were visualized by spraying with 10% v/v sulphuric acid in ethanol followed by heating at 110°C for 10 min.

3.4. Results and Discussion

Sodium alginate oligosaccharides were chemically modified by esterification of the carboxyl groups with octanol, and the resulting material was analyzed by TLC and FTIR. TLC analysis confirmed that the ethanol precipitate was completely free of unreacted octanol, and did not contain remains of DMAP or EDCI (data not shown). The product appeared as a smear on the TLC plate, indicating a range of similar, but not identical, products had formed, as expected from the reaction scheme.

The FTIR results are presented in Figure 13, which shows the spectra of the oligosaccharide esters (top), octanol (middle) and sodium oligosaccharides (bottom).

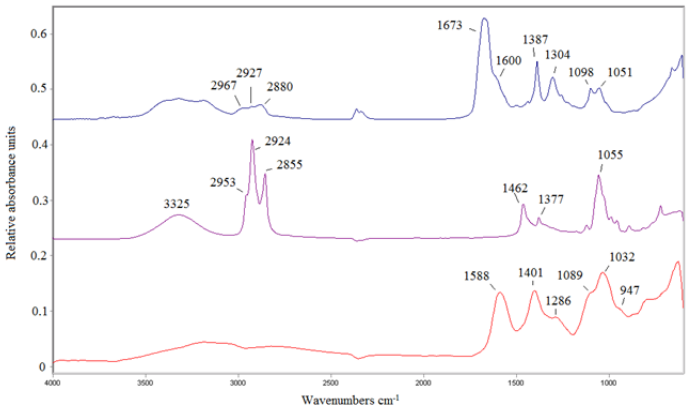

Figure 123: FTIR spectra of oligosaccharide ester (top), octanol (mid), sodium oligosaccharides (bottom).

The identification of absorbance bands in the alginate oligosaccharide spectrum has been done previously (Figure 6). The spectrum of pure octanol contains the broad band centered at 3325 cm^{-1}, assignable to the hydroxyl stretching vibration. The three characteristic peaks at 2953, 2924, and 2855 cm^{-1} are assignable to the C–H stretching vibrations of the alkane moiety. The bands at 1462 cm^{-1} and 1377 cm^{-1} are assignable to C–H deformation bending vibrations in the alkane moiety. The band at 1055 cm^{-1} is assignable to the alcohol C–O stretching vibration (Yadav 2005).

The top spectrum is that of the ethanol precipitate (alginate oligosaccharide esters, possibly with unreacted alginate oligosaccharides). The asymmetric stretching vibrations of the carbonyl at 1588 cm^{-1} in the alginate oligosaccharides shift to higher wavenumbers when ester bonds are formed. The band at 1673 cm^{-1} is hence assigned to the asymmetric stretching vibrations of the C=O in the esters, with contribution from the analogue vibration from the unreacted acids. The signals for the alkane moiety is present in the spectrum of the oligosaccharide esters; they are shifted to higher wavenumbers, 2967, 2927, and 2880 cm^{-1}, as are the signals indicating the presence of the C–O and C–C stretching vibrations of pyranose ring (1098 and 1089, and 1051 and 1032 cm^{-1}, respectively). The band at 1304 cm^{-1} is expected to be the C–O–C stretching vibration of the ester.

Based on the FTIR absorption data, it is expected that the synthesis method based on the use of DMAP and EDCI results in the formation of the desired product. More structural information in the form of NMR and MS data are needed to fully confirm the structure of the formed product.

3.5. Summary and Future Works

A method based on chemical catalysis has been found to successfully synthesize fatty alcohol esters of alginate oligosaccharides. More structural data are needed to fully confirm the structure of the formed compounds. Chromatographic methods are expected to be developed, which can give information regarding the composition of the formed product. Once the structures have been finally confirmed, the method will be applied in the synthesis of a range of fatty alcohol esters of alginate oligosaccharides, by varying the fatty alcohol moiety and the degree of esterification. A method based on the use of gas chromatography suggested by Pelletier *et al.* (Pelletier *et al.* 2000) is suggested to be used to determine the degree of esterification of the products. The final products will be characterized in terms of their surface-active properties, and their antioxidative properties, using a method similar to the TBARS assay presented in Section 2. Later, they will be applied in the formation of oil-in-water emulsions, as will be outlined in Section 6.

4. Fatty Acid Esters of Alginate Oligosaccharides

4.1. Introduction

The second type of hydrophobic modification of alginate oligosaccharides of interest in this project is the esterification of the hydroxyl groups with fatty acids. To develop a suitable method for this esterification, glucuronic acid has been used as a model compound for alginate oligosaccharides. The reaction scheme of glucuronic acid and fatty acid is shown in Figure 14. The position of esterification is chosen arbitrarily, and this position can vary depending on the reaction method, including the possibility of multiple ester-formations in one glucuronic acid molecule.

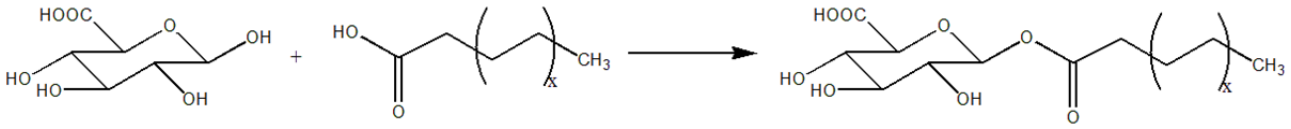

Figure 134: Esterification of glucuronic acid with fatty acid.

Several reports in literature demonstrate the successful synthesis of saccharide fatty acid esters. In this project, a range of different methods have been screened to find a suitable method for the synthesis of the desired saccharide-ester in high yield. The enzymatic methods include: reaction in organic solvent; reaction in solvent free systems; reaction in organic solvent with immobilization of substrate; reactions using "activated" fatty acids; and reaction in ionic liquids. Additionally, a method using chemical catalysis was examined. A detailed account of the materials and methods is given in sections 4.2 and 4.3, and the results obtained to date are presented and discussed in section 4.4.

4.2. Materials

Lipase Novozym 435 was from Novozymes. D-glucuronic acid; molecular sieves, 3Å, 8-12 mesh; hexanoic anhydride; iron(III) chloride hydrate, sodium hydrogen carbonate, and solvents, were from Sigma-Aldrich. Palmitic acid >97%, and Silica gel 60 were from Fluka. Oleic acid 60% was from Merck, and lauric acid >98% was from SAFC. The ionic liquid: methyl trioctyl ammonium trifluoro acetate, >98%, was from IoLiTec Ionic Liquids Technologies, Germany.

4.3. Methods

4.3.1. Reactions in Organic Solvents

The modified method of Kou and Xu (Kou & Xu 1998) was used, as follows: Specified amounts of oleic acid and glucuronic acid was combined with 100 mg freshly activated molecular sieves in 3 mL 2-butanone at 65°C. One hundred mg Novozym 435 was added and the reactions were continued under magnetic stirring at 300 rpm for 7 days. As positive control, a reaction using glucose as the saccharide part was performed. A reaction with no enzyme was included as negative control. An additional reaction in *tert*-butanol (*t*-BuOH) using lauric acid as fatty acid part was performed. Samples were withdrawn daily and diluted 1-1 in chloroform:methanol 1:1 v:v and centrifuged. The supernatant was analyzed by TLC and ESI-MS. The molar ratios of substrates were varied according to Table 1. In reaction 3, glucuronic acid was added stepwise by adding equal amounts at days 0, 3, and 6 (2.0 mmol in total).

Table 1: Molar ratios of substrates.

Reaction	Glucuronic acid [mmol]	Oleic acid [mmol]
1	1.0	1.0
2	2.0	1.0
3	2.0* stepwise	1.0
4	5.0	1.0
5	1.0	5.0
6	1.0* glucose	1.0
7* no lipase	1.0	1.0
8* in t-BuOH	5.0	1.0* lauric acid

4.3.2. Reaction in Solvent Free System

The modified method of Ye *et al.* (Ye *et al.* 2010) was used, as follows: Twenty mmol molten lauric acid at 80˚C was combined with 5 mmol glucuronic acid. Two hundred mg Novozym 435 was added, and the reaction was continued for 7 days at 80˚C under magnetic stirring at 500 rpm. Additional reactions were performed, in which 5 g acetone or 5 g 2-butanone was included in the reaction mixture. The containers were open to atmosphere allowing for free solvent evaporation, which according to literature was completed within 24 hours (Ye *et al.* 2010). In an additional reaction, 5 g acetone was included as above, but the lipase was added after solvent evaporation (24 hours). Samples were withdrawn daily and diluted 1-1 in dichloromethane and centrifuged. The supernatant was analyzed by TLC.

4.3.3. Substrate Immobilization

1.40 mmol oleic acid and 0.35 mmol glucuronic acid were combined in 2 mL hexane. One hundred mg molecular sieves and/or 136 mg silica gel (2-1 w/w of glucuronic acid) was added, and the reactions were initiated by the addition of 100 mg Novozym 435, and continued for 7 days at 60˚C under magnetic stirring at 200 rpm. A fourth reaction with no addition of either molecular sieve or silica gel was performed. Samples were withdrawn daily and diluted 1-1 in chloroform:methanol 1:1 v:v and centrifuged. The supernatant was analyzed by TLC.

4.3.4. Reaction with Anhydride

One mmol hexanoic anhydride, 1.0 mmol glucuronic acid, and 1.0 mmol sodium hydrogen carbonate were combined with 100 mg molecular sieves in 5 mL dry 2-butanone. The reaction was continued for 48 hours at 50˚C under magnetic stirring at 250 rpm. Samples were withdrawn from the reaction mixture and analyzed immediately by TLC.

4.3.5. Reaction in Ionic Liquids

One hundred mg glucuronic acid was combined with 2 mL ionic liquid and stirred at >1000 rpm at 80˚C for one hour. Undissolved saccharide was removed, and 4 mL molten lauric acid was added under high speed stirring (>1000 rpm) at 80˚C. The temperature was adjusted to 60˚C and the stirring speed to 600 rpm. The

reaction was initiated by the addition of 250 mg Novozym 435 and continued for 5 days, after which the reaction mixture was analyzed by TLC and ESI-MS.

4.3.6. Chemical Catalysis

The modified method of Komura *et al.* (Komura *et al.* 2008) was used, as follows: Equimolar amounts of glucuronic acid and palmitic acid (6 mmol) were dissolved in 40 mL aromatic hydrocarbon solvent (mesitylene, xylene, or toluene) combined with 2 mL dimethyl sulfoxide (DMSO), and the reaction was initiated by the addition of catalytic amounts (0.6 mmol) of $FeCl_3$-$6H_2O$. The reaction was continued for 24 hours under solvent reflux and magnetic stirring at 200 rpm, after which the reaction mixture was analyzed by ESI-MS.

4.3.7. Analytical Methods

TLC analyses were performed according to the method by Kou and Xu (Kou & Xu 1998). 2 µL diluted sample was spotted onto a silica-gel plate (10 cm x 10 cm) and the plate was developed for 2.2 cm in chloroform:methanol:water 64:10:1 v:v:v. The slightly dried plate was then developed on 7.5 cm with hexane:diethyl ether:acetic acid 70:30:1 v:v:v. The products were visualized by spraying with 10% v/v sulphuric acid in ethanol followed by heating at 110°C for 10 min.

ESI-MS was performed using a Bruker micrOTOF-Q III mass spectrometer in negative mode. Samples were infused into the ESI source using the following settings: capillary voltage 4.00 kV, nebulizer pressure 3.4 bar, gas flow rate 10.0 L/min, and temperature 180°C. The resulting mass spectra were analyzed using Bruker Daltonics DataAnalysis 3.4 software.

4.4. Results and Discussion

Several reports exist regarding enzymatic synthesis of saccharide fatty acid esters, and the subject is extensively reviewed, e.g. by Gumel *et al.* (Gumel *et al.* 2011), Chang & Shaw (Chang & Shaw 2009), Kobayashi (Kobayashi 2011), and Kennedy *et al.* (Kennedy *et al.* 2006). With inspiration from the existing literature, a range of esterification methods using lipase as catalysts have been examined in this study. A central challenge was to find a reaction medium in which the saccharide and the fatty acid both were soluble, while the enzyme retained its activity. Kou and Xu (Kou & Xu 1998) successfully synthesized a range of saccharide esters in *tert*-butanol using an excess of fatty acid. A modified version of their method was examined in this study. Figure 15 shows the results from TLC analysis of the reaction mixtures (RM) 1 and 6, defined in Table 1, with glucuronic acid and glucose, respectively, in substrate ratio 1-1, after 0 and 48 hours of reaction. Figure 15 additionally shows the results from TLC analysis of RM 7, performed with no lipase. According to Kou and Xu (Kou & Xu 1998), the retention factor of saccharide monoesters is approximately one third of the retention factor of fatty acid, while for diesters the retention factor is approximately two thirds of the fatty acid. The retention factor of the formed product(s) in reaction 1 (esterification of glucuronic acid, lane 5) indicates that monoester(s) have formed in very low yield. Possibly a mixture of monoesters have formed, which could be a mixture of positional isomers. The esterification of glucose, lane 6, results in the formation of a mixture of products with retention factors close to, or less than,

one third of the fatty acid. These products are assumed to be a mixture of glucose monoesters. Opposing to glucuronic acid, glucose has both primary and secondary hydroxyl group(s) in its structure, and esterification at the different positions is expected to result in formation of products with varying retention factors in TLC. Lane 7 in Figure 15 shows that no formation of saccharide ester can be detected when no lipase is included in the reaction. Lanes 3 and 4 show that no product is present in the reaction mixtures at initiation.

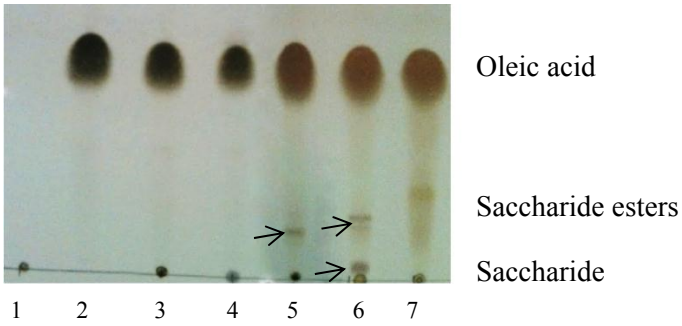

Figure 145 : TLC analysis of: ***Lane 1:*** *Glucuronic acid.* ***Lane 2:*** *Oleic acid.* ***Lane 3:*** *RM 1 at 0h.* ***Lane 4:*** *RM 6 at 0h.* ***Lane 5:*** *RM 1 at 48h.* ***Lane 6:*** *RM 6 at 48h.* ***Lane 7:*** *RM 7 at 48 hours.*

It was then evaluated if an excess of one of the substrates would result in a higher yield of glycoester. The result from the TLC analysis is shown in Figure 156.

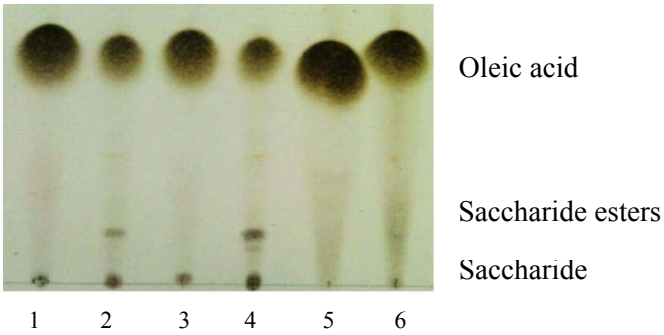

Figure 156: TLC analysis of: ***Lanes 1 and 2:*** *RM 1 at 0 and 7 days.* ***Lanes 3 and 4:*** *RM 4 at 0 and 7 days.* ***Lanes 5 and 6:*** *RM 5 at 0 and 7 days.*

As expected, after 0 days of reaction, no product is present in the reaction mixtures (lanes 1, 3, and 5). Increasing the ratio of glucuronic acid to oleic acid increased the yield of saccharide ester (lane 4 compared to lane 2). Using an excess of fatty acid inhibits the reaction, and no detectable products form (lane 6). Multiple products can be detected in lane 4, which are expected to be positional isomers of the saccharide ester.

In this reaction system, an excess of glucuronic acid is concluded to lead to an increased product molar yield, yet the conversion of saccharide is very low. Stepwise addition of the glucuronic acid did not lead to a detectable higher yield of the saccharide ester (reaction 3 compared to reaction 2 in Table 1, data not shown). The reaction system using five-times molar excess of glucuronic acid was repeated in *tert*-butanol, using lauric acid as fatty acid part (reaction 8 in Table 1). The presence of glucuronic acid fatty acid esters in the reaction mixtures 4 and 8 was confirmed by ESI-MS. The results are presented in Figure 17 and 18.

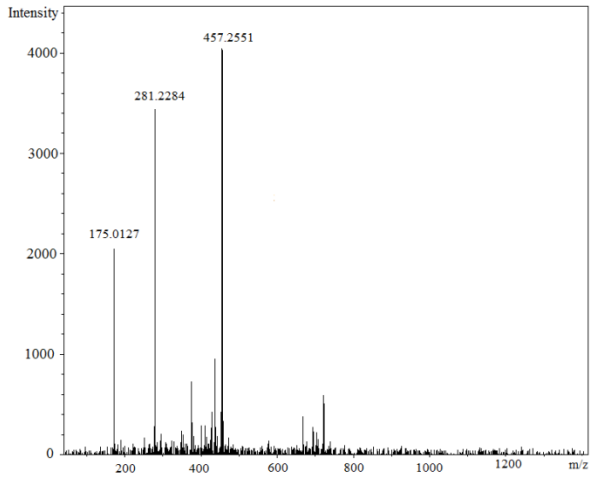

Figure 16: ESI-MS spectrum of reaction 4 – glucuronic acid oleate.

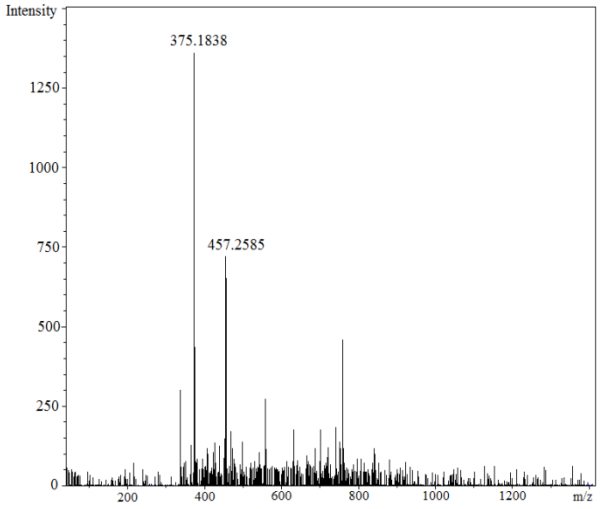

Figure 17: ESI-MS spectrum of reaction 8 – glucuronic acid laureate.

The ESI-MS spectrum in Figure 17 confirmed the presence of glucuronic acid oleate with exact mass 458.26 (457.26 + $1H^+$). The reaction mixture also contained oleic acid with exact mass 282.28 (281.23 +$1H^+$). The ESI-MS spectrum in Figure 18 confirmed the presence of glucuronic acid laureate with exact mass 376.21 (375.18 + $1H^+$). The ionization parameters were adjusted to focus on the glycoester, and lauric acid with exact mass 200.18 is not visible in the presented spectrum. The signal at 457.26 in Figure 18 corresponds to "carry-over" of the glucuronic acid oleate previously analyzed on the same equipment. Both glycoesters were fragmented in MS-MS, and the fragments were found to be glucuronic acid, and oleic acid and lauric acid, respectively (data not shown).

The degrees of conversion in the presented reactions were all very low. One reason for this is the very low solubility of glucuronic acid in organic solvent, and the low reactivity of the secondary hydroxyl groups in glucuronic acid. Attempts were made to increase the conversion, including immobilization of glucuronic acid onto silica gel, reaction in solvent free system and in ionic liquid, and the use of fatty anhydride instead of fatty acid. No product formation could be detected in any of these systems using TLC and ESI-MS. A

discussion of the ideas behind each of the used methods is given in the following, including suggestions for why they were not successful.

Ye *et al.* (Ye *et al.* 2010) synthesized fructose oleate in a solvent-free system, performing the reactions at elevated temperature at which the fatty acid could function as solvent. They included acetone in their reactions, which was allowed to evaporate from the reaction mixture. After evaporation, the reaction continued as a solvent-free. A similar method to this was examined in this project, studying the use of both acetone and 2-butanone as the "initial solvent", as well as a system which was completely solvent free. As acetone is known to inactivate lipases, an additional reaction was performed using 5 g acetone as the initial solvent, but adding the lipase after solvent evaporation. No formation of glucuronic acid ester product was detected by analysis with TLC (data not shown). The use of excess fatty acid is believed to be the cause, as this was previously found to result in very low product formation (Figure 16).

A method using substrate immobilization in apolar solvent was studied. In apolar solvents, polar substrates such as glucuronic acid can adsorb to the enzyme, leading to a decrease in reaction rate. This can be overcome by including silica gel in the reaction mixture. Polar substrates will adsorb to the silica gel instead of the enzyme, and as the concentration of free substrate decreases due to the progress of reaction, the polar substrate will be released from the silica (Castillo *et al.* 1997; Torres & Otero 2001). This approach was applied to the synthesis of glucuronic acid esters in this study, yet no product formation could be detected. A large excess of fatty acid was used in accordance with the literature. The excess of fatty acid, combined with unsuccessful immobilization of glucuronic acid onto the silica gel, are hypothesized to be the causes.

The higher reactivity of anhydrides compared to acids was exploited by reacting glucuronic acid with hexanoic anhydride in 2-butanone, yet again, no formation of product could be detected by analysis with TLC (data not shown). The very low reactivity of the hydroxyls on glucuronic acid is believed to be the main cause.

Ha *et al.* (Ha *et al.* 2010) have successfully used ionic liquids as reaction medium for the synthesis of saccharide fatty acid esters. In this study, glucuronic acid was found to have a very low solubility in the ionic liquid, and the reaction in ionic liquid did not lead to the formation of product. The complicated reaction mixture could not be applicably analyzed by TLC, but direct inject of the reaction mixture into the ESI-MS confirmed that no product had formed, as shown in Figure 19.

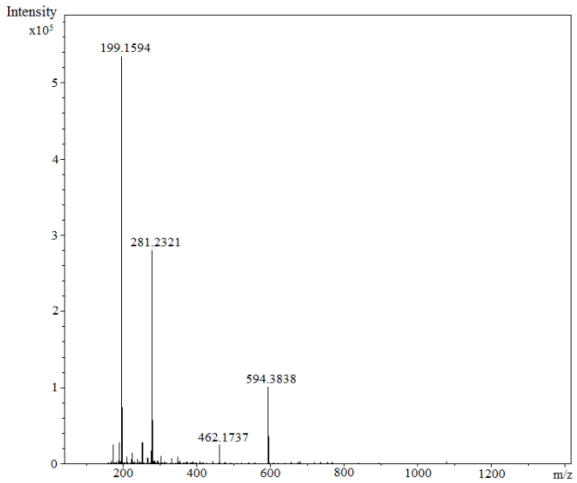

Figure 189: ESI-MS spectrum of reaction mixture in ionic liquid.

The glycoester product has an m/z of 376.21, and no compounds with this mass could be detected in the ESI-MS spectrum. The signal at 199.16 m/z origins from the substrate lauric acid. The peak at 281.23 is expected to be oleic acid which had been "carried over" in the ESI-MS equipment from previous analyses.
In conclusion, the use of lipases did not result in synthesis of the desired glucuronic acid ester product in high yield. With inspiration from the synthesis methods reported by Komura *et al.* (Komura *et al.* 2008), a method based on the use of chemical catalysis was examined. Komura *et al.* esterified various steroid alcohols with fatty acids in a one-step reaction in aromatic hydrocarbon solvents. They found that the use of ferrous chloride and mesitylene as catalyst and solvent, respectively, resulted in the highest degree of esterification. It was hypothesized that their method, appropriately modified, could be used to synthesize the desired glycoester product. The method reported by Komura *et al.* used aromatic hydrocarbons as solvents; however, glucuronic acid has very low solubility in these solvents. For that reason, DMSO was included in the reaction mixture. The final reaction mixture was analyzed by ESI-MS, which revealed that a complex mixture of products had formed, none of which was the desired glycoester (data not shown). It was suggested that the reason for this was the decomposing of the saccharide during reaction due to the high temperatures. The products were expected to be a mixture of these decomposition products and their fatty acid esters. Lower-boiling point aromatic hydrocarbon was used to replace mesitylene (xylene and toluene), but the glycoester product did not form.

4.5. Summary and Future Work

Several methods have been screened in order to find a suitable method for synthesizing fatty acid esters of glucuronic acid. Only reactions performed in amphiphilic organic solvents, *tert*-butanol and 2-butanone, led to the formation of the desired ester product, the structure of which was confirmed by ESI-MS (which positional isomers formed was not confirmed). However, the conversion of saccharide was very low, and the low yield did not allow for purification of the product. Work will continue regarding the synthesis of this compound in Part B of the Ph.d. studies. It is suggested that the use of alkyl succinic anhydrides using enzymatic catalysis, with inspiration from the methods reported by Song *et al.* (Song *et al.* 2006) and Xu *et al.* (Xu *et al.* 2012) will be successful. The final saccharide ester is expected to have distinctive properties due to the presence of the carboxylic acid group in the saccharide head group.

5. Synthesis of Glycophospholipids

The "Multicaps"-funded project at Aarhus University focuses on developing a range of new emulsifiers, which can be used for microencapsulation. In addition to the alginate-based esters, which are the primary focus of this Ph.d. project, work has also been done regarding the synthesis of glycophospholipids. In 2011, Ph.d. student Shuang Song developed a method for the synthesis of phosphatidyl-glucose from phosphatidyl-choline and glucose, catalyzed by phospholipase D. This work was presented in Food Chemistry in 2012 (Song *et al.* 2012). During the first three months of my Ph.d. study, I assisted Shuang Song with the larger-

scale synthesis (500 µmol phosphatidyl-choline per batch) and purification of the glycophospholipids, using the methods she had established during her work. Shortly, the glycophospholipids were synthesized in a single-step enzymatic reaction in a biphasic reaction system, in which phosphatidyl-choline was dissolved in an organic solvent phase, and the saccharide was dissolved in a buffer phase. The transphosphatidylation took place at the interface, catalyzed by phospholipase D. At the end of the reaction, the lipids were extracted and purified using thin layer chromatography. Various analyses of the synthesized compounds were then performed, including structural determinations by NMR and ESI-MS. From the purified glycophospholipids, nano-liposomes were formed and characterized in terms of their physical properties and stability during storage and dehydration processes. My contributions to this project lead to a co-authorship of the resulting article entitled "Stabilising Nanoliposomes by Synthetic Biomimetic Phosphatidylsaccharides", which has been submitted to Biomaterials.

6. Part A Summary and Part B Planning

Part A of my Ph.d. studies has been focused on depolymerization of sodium alginate, and characterization of the resulting alginate oligosaccharides. Special focus has been placed on characterizing the antioxidative properties of the oligosaccharides, and this work will continue in Part B. The TBARS analyses, which have been initiated in part A, will be finalized in part B, and will be supplemented with analyses for reducing power using the FRAP assay, and analyses for radical scavenging activity using the DPPH* assay. It is expected, that this work will result in a publication entitled: "Preparation of Alginate Oligosaccharides through Enzymatic Depolymerization and Characterization of their Antioxidant Properties", and the suggested list of authors is: Mia Falkeborg, Ling-Zhi Cheong, Carlo Gianfico, Kasper Kristensen, Marianne Glasius, Xuebing Xu, and Zheng Guo. According to the plan, this work will be finalized before April 2013.

Initial experiments have shown the possibility of esterifying the alginate oligosaccharides with fatty alcohols using the chemical method presented in Section 3. In part B of the Ph.d. studies, this method will be used to synthesize a range of alginate oligosaccharide esters with varying fatty alcohol chain length, and varying degree of esterification. The structures of the resulting compounds will be identified using FTIR, NMR, and MS methods. It is suggested that a method based on the use of gas chromatography can be used to determine the degree of esterification, which can be varied by varying the reaction time. The resulting emulsifiers will be characterized in terms of their surface-activity, thermal profiles, and antioxidative properties. This work is expected to be completed by May 2013, resulting in a publication entitled: "Synthesis of Alginate Oligosaccharide Esters and Characterization of their Surface Activity and Antioxidant Properties", with the suggested list of authors: Mia Falkeborg, Ling-Zhi Cheong, Marianne Glasius, Xuebing Xu, and Zheng Guo.

As described in Section 4, the synthesis of fatty acid esters of glucuronic acid has been difficult, and so far, no suitable method has been found which can synthesize this compound in high yield. During Part B, work will continue regarding the development of a suitable synthesis method. Once found, it is expected that the

method can be applied to alginate saccharides, either in monomeric form (guluronic acid and mannuronic acid) or in oligosaccharide form.

Starting from May 2013, the emulsifiers will be applied in marine oil-in-water emulsions. It is hypothesized, that the fatty esters of alginate oligosaccharides can form physically stable emulsions due to their amphiphilic nature, while providing oxidative stabilization to the emulsified marine oil due to their antioxidative properties. Experimental work in this section includes formation of oil-in-water emulsions (with varying ratio of ingredients) by homogenization, and characterization of the physical properties of the resulting emulsions including; particle size, particle size distribution, surface charge, morphology, amount of surface oil, and stability during storage at varying temperatures. The oxidative stability of the emulsified oil will be evaluated over time, and it is expected, that the use of the emulsifiers developed in this project will lead to an increased oxidative stability, compared to commercial emulsifiers. This work will be ongoing during summer 2013. During autumn/winter 2013-2014 I will be a guest Ph.d student at Professor Jonathan Dordick's lab at the Rensselaer Polytechnic Institute in New York, USA. During this stay I am expected to work with the development of new biomaterials functionalized with cell lytic enzymes to kill pathogenic bacteria. It is expected, that this work will result in a publication. The work regarding formation and characterization of oil-in-water emulsions, using the emulsifiers developed during the project, can continue after my return from USA. The final Ph.d. thesis should be submitted no later than August 31st 2014.

References

Barrow, C. J., Nolan, C. and Jin, Y. (2007). *"Stabilization of Highly Unsaturated Fatty Acids and Delivery into Foods."* Lipid Technol **19** (5). 108-111.

Benvegnu, T. and Sassi, J. F. (2010). *"Oligomannuronates from Seaweeds as Renewable Sources for the Development of Green Surfactants"*. In: Carbohydrates in Sustainable Development I: Renewable Resources for Chemistry and Biotechnology. A. P. Rauter, P. Vogel and Y. Queneau. **294**. 143-164.

Benzie, I. F. F. and Strain, J. J. (1996). *"The Ferric Reducing Ability of Plasma (Frap) as a Measure of "Antioxidant Power": The Frap Assay."* Analytical Biochemistry **239** (1). 70-76.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995). *"Use of a Free-Radical Method to Evaluate Antioxidant Activity."* Food Science and Technology **28** (1). 25-30.

Castillo, E., Dossat, V., Marty, A., Condoret, J. S. and Combes, D. (1997). *"The Role of Silica Gel in Lipase-Catalyzed Esterification Reactions of High-Polar Substrates."* Journal of the American Oil Chemists Society **74** (2). 77-85.

Chandia, N. P., Matsuhiro, B., Mejias, E. and Moenne, A. (2004). *"Alginic Acids in Lessonia Vadosa: Partial Hydrolysis and Elicitor Properties of the Polymannuronic Acid Fraction."* Journal of Applied Phycology **16** (2). 127-133.

Chandia, N. P., Matsuhiro, B. and Vasquez, A. E. (2001). *"Alginic Acids in Lessonia Trabeculata: Characterization by Formic Acid Hydrolysis and FT-IR Spectroscopy."* Carbohydrate Polymers **46** (1). 81-87.

Chang, S. W. and Shaw, J. F. (2009). *"Biocatalysis for the Production of Carbohydrate Esters."* New Biotechnology **26** (3-4). 109-116.

Coupland, J. N. and McClements, D. J. (1996). *"Lipid Oxidation in Food Emulsions."* Trends in Food Science & Technology **7** (3). 83-91.

Desai, K. G. H. and Park, H. J. (2005). *"Recent Developments in Microencapsulation of Food Ingredients."* Drying Technology **23** (7). 1361-1394.

Din, J. N., Newby, D. E. and Flapan, A. D. (2004). *"Science, Medicine, and the Future - Omega 3 Fatty Acids and Cardiovascular Disease - Fishing for a Natural Treatment."* British Medical Journal **328** (7430). 30-35.

Fenoradosoa, T. A., Ali, G., Delattre, C., Laroche, C., Petit, E., Wadouachi, A. and Michaud, P. (2010). *"Extraction and Characterization of an Alginate from the Brown Seaweed Sargassum Turbinarioides Grunow."* Journal of Applied Phycology **22** (2). 131-137.

Gacesa, P. (1992). *"Enzymatic Degradation of Alginates."* International Journal of Biochemistry **24** (4). 545-552.

Gomez-Ordonez, E. and Ruperez, P. (2011). *"FTIR-ATR Spectroscopy as a Tool for Polysaccharide Identification in Edible Brown and Red Seaweeds."* Food Hydrocolloids **25** (6). 1514-1520.

Guillen-Sans, R. and Guzman-Chozas, M. (1998). *"The Thiobarbituric Acid (TBA) Reaction in Foods: A Review."* Critical Reviews in Food Science and Nutrition **38** (4). 315-330.

Gumel, A. M., Annuar, M. S. M., Heidelberg, T. and Chisti, Y. (2011). *"Lipase Mediated Synthesis of Sugar Fatty Acid Esters."* Process Biochemistry **46** (11). 2079-2090.

Guzey, D. and McClements, D. J. (2006). *"Formation, Stability and Properties of Multilayer Emulsions for Application in the Food Industry."* Advances in Colloid and Interface Science **128**. 227-248.

Ha, S. H., Hiep, N. M., Lee, S. H. and Koo, Y. M. (2010). *"Optimization of Lipase-Catalyzed Glucose Ester Synthesis in Ionic Liquids."* Bioprocess and Biosystems Engineering **33** (1). 63-70.

Haug, A., Larsen, B. and Smidsrod, O. (1966). *"A Study of Constitution of Alginic Acid by Partial Acid Hydrolysis."* Acta Chemica Scandinavica **20** (1). 183-&.

Jacobsen, C. (2008). *"Measuring Oxidative Stability in Functional Lipids"*. In: Handbook of Lipid Enzymology. X. Xu, C R C Press.

Jacobsen, C., Let, M. B., Nielsen, N. S. and Meyer, A. S. (2008). *"Antioxidant Strategies for Preventing Oxidative Flavour Deterioration of Foods Enriched with n-3 Polyunsaturated Lipids: A Comparative Evaluation."* Trends in Food Science & Technology **19** (2). 76-93.

Jacobsen, C. and Nielsen, N. S. (2007). *"Optimization of Oxidative Stability of Omega-3 Enriched Foods"*. In: Long-Chain Omega-3 Specialty Oils. H. Breivik. 197-217.

Kennedy, J. F., Kumar, H., Panesar, P. S., Marwaha, S. S., Goyal, R., Parmar, A. and Kaur, S. (2006). *"Enzyme-Catalyzed Regioselective Synthesis of Sugar Esters and Related Compounds."* Journal of Chemical Technology & Biotechnology **81** (6). 866-876.

Kim, H. S., Lee, C. G. and Lee, E. Y. (2011). *"Alginate Lyase: Structure, Property, and Application."* Biotechnology and Bioprocess Engineering **16** (5). 843-851.

Kobayashi, T. (2011). *"Lipase-Catalyzed Syntheses of Sugar Esters in Non-Aqueous Media."* Biotechnology Letters **33** (10). 1911-1919.

Kolanowski, W., Swiderski, F. and Berger, S. (1999). *"Possibilities of Fish Oil Application for Food Products Enrichment with Omega-3 PUFA."* International Journal of Food Sciences and Nutrition **50** (1). 39-49.

Komura, K., Ozaki, A., Leda, N. and Sugi, Y. (2008). *"$FeCl_3 \cdot 6H_2O$ as a Versatile Catalyst for the Esterification of Steroid Alcohols with Fatty Acids."* Synthesis-Stuttgart (21). 3407-3410.

Kou, X. F. and Xu, J. L. (1998). *"Enzymatic Synthesis of Saccharide Fatty Acid Esters"*. In: Enzyme Engineering Xiv. A. I. Laskin, G. X. Li and Y. T. Yu. **864**. 352-358.

Leal, D., Matsuhiro, B., Rossi, M. and Caruso, F. (2008). *"FT-IR Spectra of Alginic Acid Block Fractions in Three Species of Brown Seaweeds."* Carbohydrate Research **343** (2). 308-316.

Lee, K. Y. and Mooney, D. J. (2012). *"Alginate: Properties and Biomedical Applications."* Progress in Polymer Science **37** (1). 106-126.

Let, M. B., Jacobsen, C. and Meyer, A. S. (2007). *"Lipid Oxidation in Milk, Yoghurt, and Salad Dressing Enriched with Neat Fish Oil or Pre-Emulsified Fish Oil."* Journal of Agricultural and Food Chemistry **55** (19). 7802-7809.

Narayan, B., Miyashita, K. and Hosakawa, M. (2006). *"Physiological Effects of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) - a Review."* Food Reviews International **22** (3). 291-307.

Pawar, S. N. and Edgar, K. J. (2012). *"Alginate Derivatization: A Review of Chemistry, Properties and Applications."* Biomaterials **33** (11). 3279-3305.

Pelletier, S., Hubert, P., Lapicque, F., Payan, E. and Dellacherie, E. (2000). *"Amphiphilic Derivatives of Sodium Alginate and Hyaluronate: Synthesis and Physico-Chemical Properties of Aqueous Dilute Solutions."* Carbohydrate Polymers **43** (4). 343-349.

Ruperez, P., Ahrazem, O. and Leal, J. A. (2002). *"Potential Antioxidant Capacity of Sulfated Polysaccharides from the Edible Marine Brown Seaweed Fucus Vesiculosus."* Journal of Agricultural and Food Chemistry **50** (4). 840-845.

Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A. and Millington, K. J. (2007). *"The Health Benefits of Omega-3 Polyunsaturated Fatty Acids: A Review of the Evidence."* Journal of Human Nutrition and Dietetics **20** (3). 275-285.

Sagalowicz, L. and Leser, M. E. (2010). *"Delivery Systems for Liquid Food Products."* Current Opinion in Colloid & Interface Science **15** (1-2). 61-72.

Sakugawa, K., Ikeda, A., Takemura, A. and Ono, H. (2004). *"Simplified Method for Estimation of Composition of Alginates by FTIR."* Journal of Applied Polymer Science **93** (3). 1372-1377.

Schaich, K. M. (2005). *"Lipid Oxidation: Theoretical Aspects"*. In: Bailey's Industrial Oil and Fat Products, John Wiley & Sons, Inc.

Sen, M. (2011). *"Effects of Molecular Weight and Ratio of Guluronic Acid to Mannuronic Acid on the Antioxidant Properties of Sodium Alginate Fractions Prepared by Radiation-Induced Degradation."* Applied Radiation and Isotopes **69** (1). 126-129.

Shahidi, F. and Miraliakbari, H. (2004). *"Omega-3 (n-3) Fatty Acids in Health and Disease: Part I - Cardiovascular Disease and Cancer."* Journal of Medicinal Food **7** (4). 387-401.

Shahidi, F. and Miraliakbari, H. (2005). *"Omega-3 Fatty Acids in Health and Disease: Part 2 - Health Effects of Omega-3 Fatty Acids in Autoimmune Diseases, Mental Health, and Gene Expression."* Journal of Medicinal Food **8** (2). 133-148.

Shahidi, F. and Zhong, Y. (2010). *"Lipid Oxidation and Improving the Oxidative Stability."* Chemical Society Reviews **39** (11). 4067-4079.

Shahidi, F. and Zhong, Y. (2011). *"Revisiting the Polar Paradox Theory: A Critical Overview."* Journal of Agricultural and Food Chemistry **59** (8). 3499-3504.

Song, S., Cheong, L. Z., Guo, Z., Kristensen, K., Glasius, M., Jensen, H. M., Bertelsen, K., Tan, T. W. and Xu, X. B. (2012). *"Phospholipase D (PlD) Catalyzed Synthesis of Phosphatidyl-Glucose in Biphasic Reaction System."* Food Chemistry **135** (2). 373-379.

Song, X., Chen, Q., Ruan, H., He, G. and Xu, Q. (2006). *"Synthesis and Paste Properties of Octenyl Succinic Anhydride Modified Early Indica Rice Starch."* Journal of Zhejiang University Science B **7** (10). 800-805.

Taneja, A. and Singh, H. (2012). *"Challenges for the Delivery of Long-Chain n-3 Fatty Acids in Functional Foods"*. In: Annual Review of Food Science and Technology, Vol 3. M. P. Doyle and T. R. Klaenhammer. **3**. 105-123.

Torres, C. and Otero, C. (2001). *"Part III. Direct Enzymatic Esterification of Lactic Acid with Fatty Acids."* Enzyme and Microbial Technology **29** (1). 3-12.

Wall, R., Ross, R. P., Fitzgerald, G. F. and Stanton, C. (2010). *"Fatty Acids from Fish: The Anti-Inflammatory Potential of Long-Chain Omega-3 Fatty Acids."* Nutrition Reviews **68** (5). 280-289.

Wang, P., Jiang, X. L., Jiang, Y. H., Hu, X. K., Mou, H. J., Li, M. and Guan, H. H. (2007). *"In Vitro Antioxidative Activities of Three Marine Oligosaccharides."* Natural Product Research **21** (7). 646-654.

Wong, T. Y., Preston, L. A. and Schiller, N. L. (2000). *"Alginate Lyase: Review of Major Sources and Enzyme Characteristics, Structure-Function Analysis, Biological Roles, and Applications."* Annual Review of Microbiology **54**. 289-340.

Xu, J., Zhou, C. W., Wang, R. Z., Yang, L., Du, S. S., Wang, F. P., Ruan, H. and He, G. Q. (2012). *"Lipase-Coupling Esterification of Starch with Octenyl Succinic Anhydride."* Carbohydrate Polymers **87** (3). 2137-2144.

Yadav, L. D. S. (2005). *"Infrared (IR) Spectroscopy"*. In: Organic Spectroscopy. L. D. S. Yadav, Kluwer Academic Publishers. 52-106.

Yang, J. S., Jiang, B., He, W. and Xia, Y. M. (2012). *"Hydrophobically Modified Alginate for Emulsion of Oil in Water."* Carbohydrate Polymers **87** (2). 1503-1506.

Ye, A., Cui, J., Taneja, A., Zhu, X. and Singh, H. (2009). *"Evaluation of Processed Cheese Fortified with Fish Oil Emulsion."* Food Research International **42** (8). 1093-1098.

Ye, R., Pyo, S. H. and Hayes, D. G. (2010). *"Lipase-Catalyzed Synthesis of Saccharide-Fatty Acid Esters Using Suspensions of Saccharide Crystals in Solvent-Free Media."* Journal of the American Oil Chemists Society **87** (3). 281-293.

Yuji, H., Weiss, J., Villeneuve, P., Lopez Giraldo, L. J., Figueroa-Espinoza, M. C. and Decker, E. A. (2007). *"Ability of Surface-Active Antioxidants to Inhibit Lipid Oxidation in Oil-in-Water Emulsion."* Journal of Agricultural and Food Chemistry **55** (26). 11052-11056.

Zhao, X., Li, B. F., Xue, C. H. and Sun, L. P. (2012). *"Effect of Molecular Weight on the Antioxidant Property of Low Molecular Weight Alginate from Laminaria Japonica."* Journal of Applied Phycology **24** (2). 295-300.

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