Universidade de Aveiro

Ano 2020

Departamento de Química

INÊS MARIA VAZ SILVA

Potencial hipocolesterolémico *in vitro* de polissacarídeos marinhos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica – Ramo de Bioquímica Alimentar, realizada sob a orientação científica do Doutor Filipe Manuel Coreta-Gomes e da Doutora Cláudia Sofia Cordeiro Nunes, Investigadores do Departamento de Química da Universidade de Aveiro.

This research was funded by Fundação para a Ciência e a Tecnologia (FCT/MCTES) supporting the research units LAQV-REQUIMTE (UIDB/50006/2020) and to the Portuguese NMR Network; Moreover, it was also supported by the projects PTDC/QUI-OUT/29373/2017 and PTDC/BII-BIO/31032/2017 through national funds (OE) and co-financed by the FEDER, by the Operational Program of Competitiveness and Internationalization (POCI), within the PT2020 Partnership Agreement.

Cofinanciado por:

FCT Fundação para a Ciência e a Tecnologia

COMPETE 2020 PROGRAMA OPERACIONAL COMPETITIVIDADE E INTERNACIONALIZAÇÃO

PORTUGAL 2020

UNIÃO EUROPEIA
Fundo Europeu de Desenvolvimento Regional

Dedico este trabalho aos meus pais.

o júri

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agradecimentos

Agradeço aos meus orientadores Doutor Filipe Coreta-Gomes e Doutora Cláudia Nunes por toda a disponibilidade, paciência, apoio e conhecimentos transmitidos.
À Doutora Ana Moreira por todos os ensinamentos e ajuda.
Aos meus colegas de laboratório pela ajuda e companheirismo.
Aos meus primos Ana, Carla e Leandro pelo incentivo e companhia apesar da distância.
Ao Lisandro por todo o apoio e encorajamento.
Aos meus avós pelo carinho.
Aos meus pais por sempre me apoiarem e acreditarem em mim.
A todos aqueles que, de um modo ou de outro, tornaram possível a realização da presente dissertação.

palavras-chave

Polissacarídeos, quitosana, fucoidana, *Saccharina latissima*, bioacessiblidade do colesterol, ácidos biliares

resumo

A prevalência de elevados níveis de colesterol no sangue da população traduz-se num índice de mortalidade e morbilidade com elevados custos humanos e económicos, sendo muito importante encontrar novas estratégias para mitigar este problema. Uma forma de diminuir os níveis de colesterol no sangue é diminuir a sua bioacessibilidade, ou seja, diminuir a quantidade que é emulsificada no lúmen intestinal com o auxílio de ácidos biliares e, portanto, disponível a ser absorvida.

No presente trabalho, polissacarídeos de origem marinha com diferentes características estruturais foram estudados quanto ao seu efeito hipocolesterolémico, assim como possíveis mecanismos de ação, num sistema modelo intestinal *in vitro* simplificado. Da alga castanha *Saccharina latissima* foram fracionados e caracterizados estruturalmente dois tipos de polissacarídeos, as laminaranas (neutras) e os polissacarídeos sulfatados contendo fucose (carregados negativamente). Adicionalmente, estudou–se a quitosana (um polissacarídeo carregado positivamente), obtida por desacetilação da quitina, que normalmente é obtida a partir de cascas de crustáceos.

Os resultados obtidos mostraram que a quitosana de baixo peso molecular foi o polissacarídeo mais promissor, seguido da fucoidana, na redução da bioacessibilidade de colesterol promovida pela sequestração de ácidos biliares, sendo que os restantes polissacarídeos não demonstraram um efeito significativo. Por forma a entender se outros mecanismos de ação estariam envolvidos no efeito hipocolesterolémico dos polissacarídeos de origem marinha, avaliou-se a viscosidade dos polissacarídeos mais efetivos na diminuição da solubilidade de colesterol, verificando-se que a quitosana apresentou a maior viscosidade em comparação com a fucoidana comercial nos regimes de concentração estudados. Contudo, o coeficiente de difusão obtido por DOSY, comparando a solução na presença e ausência de quitosana não mostrou alterações significativas à concentração usada, sendo indicativo que este polissacarídeo não afeta significativamente a difusão das micelas do ácido biliar com colesterol no lúmen intestinal, descartando este mecanismo. O potencial zeta permitiu verificar que ao pH fisiológico, usado nos ensaios de solubilidade de colesterol *in vitro,* a quitosana possuía carga positiva, reforçando a possibilidade de sequestração de ácidos biliares carregados negativamente por interação electroestática, corroborado por quantificação por RMN. A fucoidana apesar do seu carácter negativo conferido pelos grupos sulfatos e ácidos urónicos também mostrou sequestrar ácidos biliares, contrariamente ao observado com as laminaranas (neutras), realçando a possibilidade de interações hidrofóbicas entre os ácidos biliares e os polissacarídeos, dependendo da sua natureza estrutural. O presente trabalho identifica alguns polissacarídeos de origem marinha, nomeadamente a quitosana e as fucoidanas, com potencial para serem usados como agentes hipocolesterolémicos, detalhando possíveis mecanismos de ação que poderão ser úteis no design de alimentos funcionais com propriedades na redução de absorção de colesterol e consequentemente poderão ter elevado impacto na sociedade, nomeadamente na saúde e economia.

keywords

Polysaccharides, chitosan, fucoidan, *Saccharina latissima,* cholesterol bioaccessibility, bile acids

abstract

The prevalence of high cholesterol levels in the blood of the population translates in a mortality and morbidity index with high human and economic costs, being highly important to find new strategies to mitigate this problem. One way to reduce cholesterol levels in blood is to diminish its bioaccessibility, this is, decrease the amount that is emulsified in the intestinal lumen with the help of bile salts and thus available to be absorbed.

In this work marine origin polysaccharides with different structural characteristics were explored, evaluating its hypocholesterolemic potential in an *in vitro* simplified intestinal model, as well as possible mechanisms of action. From brown algae *Saccharina latissima*, two types of polysaccharides were fractioned and structurally characterized, the laminarans (neutral) and fucose-containing sulphated polysaccharides (negatively charged). Chitosan (a positively charged polysaccharide) was also studied, which is a polysaccharide obtained from the deacetylation of chitin from crustaceous shells.

The obtained results showed that the low molecular weight chitosan was the most promising polysaccharide, followed by the commercial fucoidan, in the reduction of cholesterol's bioaccessibility, promoted by the sequestration of bile salts, whereas the other polysaccharides did not show a significant effect. To understand if other mechanisms of action were involved in the hypocholesterolemic effect of the marine origin polysaccharides, the viscosities of the most effective polysaccharides in reduction of cholesterol solubility were evaluated. In the concentration ranges studied, chitosan revealed higher viscosity compared to the commercial fucoidan. However, the diffusion coefficient evaluated by DOSY, which compared the solution in the presence and absence of chitosan, did not show significant alterations at the concentration used. This indicate that chitosan does not affect significatively the diffusion of bile salts micelles with cholesterol in the intestinal lumen, discarding this mechanism. Zeta-potential allowed to verify that at the physiologic pH used in the *in vitro* cholesterol solubility assays, the chitosan had positive charge, reinforcing the possibility of negatively charged bile salts sequestration by electrostatic interaction, corroborated by NMR quantification. Despite the negative character of fucoidan due to the sulphates groups and uronic acids, it has also capacity to sequestrate bile salts, contrary to what was observed with the laminarans (neutral), highlighting the possibility of hydrophobic interactions between bile salts and polysaccharides, depending on their structural nature. The present work allowed to identify that chitosan and fucoidans as potential to be used as hypocholesterolemic agents, detailing the possible mechanisms of action which can be useful for the design of functional foods with properties in reduction of cholesterol absorption that can have high impact in the society, namely in health and economy.

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List of abbreviations

Abbreviation	Designation
ABC	ATP- binding cassette
ACAT	Acyl-CoA:cholesterol acyltransferase
AIR	Alcohol insoluble residue
ATP	Adenosine triphosphate
BACS	Bile acid:CoA synthetase
BAT	Bile acid:amino acid transferase
CMC	Critical micellar concentration
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol-7-alpha-hydroxylase
CYP7B1	25-hydroxycholesterol 7-alpha-hydroxylase
CYP8B1	Sterol 12-alpha-hydroxylase
DCA	Deoxycholic acid
DLS	Dynamic light scattering
DOSY	Diffusion ordered spectroscopy
FXR	Farnesoid X Receptor
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GLCA	Glycolithocholic acid
GLCAS	Glycolithocholic sulphate acid
HDL	High-density lipoproteins
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
IDL	Intermediate-density lipoproteins
LCA	Lithocholic acid
LDL	Low-density lipoproteins
Man	Mannose
MVD	Mevalonate diphosphate decarboxylase
MVK	Mevalonate kinase
NMR	Nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-like 1
PCSK9	Proprotein convertase subtilisin/kexin type 9
PMVK	Phosphomevalonate kinase
PUFA	Polyunsaturated fatty acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TLCA	Taurolithocholic acid
TLCAS	Taurolithocholic sulphate
VLDL	Very-low density lipoproteins

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Chapter 1 – Introduction

Cholesterol is essential to human life, however its presence in excess can threat health [1]. Due to the high prevalence of high cholesterol levels in humans, finding new functional foods with hypocholesterolemic activity is mandatory. The ingestion of certain food ingredients allows to reduce the amount of cholesterol absorbed. As pharmacologic treatments can draw adverse secondary effects and/or being expensive, the aim is to develop effective functional foods that can replace or reduce the use of drugs.

Polysaccharides have been known to interact with bile salts, which are essential to cholesterol solubilization and its further absorption, resulting in a hypocholesterolemic effect due to the reduction of cholesterol bioaccessibility. Different mechanisms of action based on polysaccharide's characteristics, such as charge and viscosity, are reported and more than one mechanism can be responsible for the hypocholesterolemic potential of polysaccharides.

Seas and oceans cover the majority of Earth's surface and contain a large biodiversity. From the several life forms existing in marine environments, algae present themselves as a good source of biomolecules such as polysaccharides with a wide range of potential biological activities [2–7]. Brown algae have three characteristic polysaccharides: laminarans, alginate, and fucose-containing sulphated polysaccharides. These polysaccharides have different structural characteristics such as molecular weight, sugar composition and charge (laminarans are neutral and the other two are negatively charged). Furthermore, chitin is an abundant polysaccharide that constitutes the exoskeleton of crustaceous. Chitin is usually deacetylated to enhance its solubility and range of applications, giving rise to chitosan which is a positively charged polysaccharide.

The focus of this study is to evaluate the potential of several polysaccharides form marine origin as hypocholesterolemic food ingredients, evaluating possible mechanisms such as: 1) sequestration of a bile salt; 2) changes on viscosity medium; or 3) diffusion coefficients which can have impact on cholesterol's solubility and absorption.

1.1. Polysaccharides of marine origin

Marine environment is a great source of polysaccharides, namely algae (e.g. Brown Algae) and crustaceous. The distinct characteristics of marine polysaccharides, such as charge, molecular weight and monosaccharide composition, can give them different biological activities.

1.1.1. Algae polysaccharides

Algae are photosynthetic organisms that gained several importance due to theirs broad spectrum of bioactive compounds, as well as their nutritional properties [8]. There are three types of macroalgae (Figure 1): red (Rhodophyta), green (Chlorophyta) and brown (Phaeophyta) [9,10]. Red and green algae belong to the kingdom Plantae and brown algae to the kingdom Chromista. The distinctive color of the different types of seaweed are due to the different pigments present. Green seaweed contains chlorophyll *a* and *b* in the same proportions of land plants, red seaweed contains mainly the pigments phycoerythrin and phycocyanin (containing also chlorophyll *a* and β-carotene), and brown seaweed gets its color from the pigment fucoxanthin, having also lower contents of chlorophyll *a* and *c*, β-carotene, and xanthophylls.

a) **b)** **c)**

Figure 1. Three types of macroalgae: a) green (*Ulva lactuca*) [11], b) red (*Palmaria palmata*) [12] and c) brown (*Saccharina latissima*) [13].

Seaweeds or macroalgae have been traditional consumed in Asian countries. However, its consumption has been increasing in the western world due to its nutritional aspects, namely due to the low caloric content and its potential as functional foods [14]. Macroalgae contain high quantities of carbohydrates [15], that can make up to almost 70 % of the seaweed dry weight, being considered dietary fiber since are non-degraded by human gastric system. Compared to land food sources, macroalgae can contain higher levels of fiber (Table 1) with a serving size of 8 g of seaweeds can satisfy up to 12.5 % of the daily recommended dose of fiber [8]. Macroalgae also contain proteins, lipids, vitamins and minerals. In terms of minerals, 8 g of seaweeds can contain more iron than 100 g of steak and are also a good source of copper and iodine [8]. Due to the wide range of seaweeds, there are a lot of bioactive compounds, such as polysaccharides, lipids, diterpenes and phlorotannins [9], with biological activities, as anti-inflammatory [2,3], antidiabetic [4], antitumoral [5,6] and antihyperlipidemic [7].

Table 1. Comparison between seaweed and other foods in terms of total fiber and carbohydrates [8].

Food type	Total fiber	Carbohydrates
Seaweed (g/100 g wet weight)		
Ascophyllum nodosum	8.8	13.1
Laminaria digitata	6.2	9.9
Himanthalia elongata	9.8	15.0
Undaria pinnatifida	3.4	4.6
Whole food (g/100 g wet weight)		
Brown rice	3.8	81.3
Cabbage	2.9	4.1
Carrots	2.6	7.9
Apples	2.0	11.8
Bananas	3.1	23.2

Different algae contain different polysaccharides with different structure, sugar composition and molecular weight. Typical polysaccharides from brown algae include alginates, laminarans and fucose-containing sulphated polysaccharides

whose main characteristics are present in Table 2. Even within the same species, the polysaccharides can vary due to diverse growth conditions such as water salinity, season, location and nutrients availability. For instance, high water salinity enhances the production of fucose-containing sulphated polysaccharides in order to strength the cell wall for a higher resistance to osmotic stress [16]. Furthermore, fucoidan composition can also change between species in terms of monosaccharides or sulphate composition and quantities [17].

Table 2. Main characteristics of typical polysaccharides from brown algae (adapted from Zvyagintseva *et al.* [18]).

	Alginates	**Laminarans**	**Fucose-containing sulphated polysaccharides**
Yield (% dry mass)	10 – 40	Up to 35	1 – 20
Molecular weight (kDa)	100 – 1900	3 – 6	7 – 2379
Composition	Mannuronic acid and guluronic acid	Glucose (can have mannitol at the reducing end)	High quantities of fucose and sulphate. Can also contain galactose, mannose, glucose, xylose, and uronic acids

Polysaccharides from brown algae have shown a wide range of applications, as well as biological activities, such as anticoagulant, antioxidant, antitumoral and anti-inflammatory [17,19].

1.1.1.1. Alginate

The main polysaccharide in brown algae is alginate, which confers flexibility to the algae. Its abundance depends on the species, as well as cultivation conditions, varying generally from 10 to 40 % of the dry weight (Table 2) [20]. Alginate is a linear polysaccharide composed of β-D-mannuronic acid (M blocks) and α-L-guluronic acid (G blocks) with (1→4) linkages (Figure 2), conferring negative charge to the polymer. Its molecular weight ranges from 100 up to 1900 kDa. It is widely used in food industry as thickening and stabilizer agent and in the

biomedical field due to its biocompatibility and low toxicity [21]. The ratio between L-guluronic and D-mannuronic acids influences the polysaccharide properties, namely the thickening properties, since M blocks segments confer flexibility with linear structures while G blocks confer rigidness with folded structures.

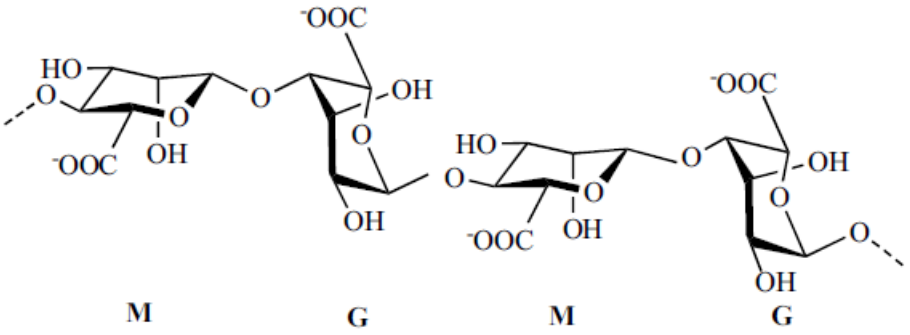


Figure 2. Structure of alginate (adapted from Cardoso *et al.* [22]).

1.1.1.2. Laminarans

Laminarans (also known as laminarins) are homopolymers composed of D-glucose units linked by (β1→3) linkages with an average length of 20 to 25 sugar residues. Usually they have low molecular weight from 3 to 6 kDa. They can represent up to 35 % of the dry algae mass and are found mainly in algae from the *Laminaria* and *Saccharina* genus [19,23]. Laminarans can be branched with (β 1→6) linkages, which influence their solubility. Highly branched laminarans are water-soluble at low temperature, while laminarans with few branches are only soluble in warm water. The chains of laminaran can contain mannitol (M) at the reducing end (Figure 3). The content of mannitol varies between species and some laminarans do not show the presence of mannitol. Due to its composition, laminarans are not digested by the human digestive system, reaching to the colon intact, being considered dietary fiber [24].

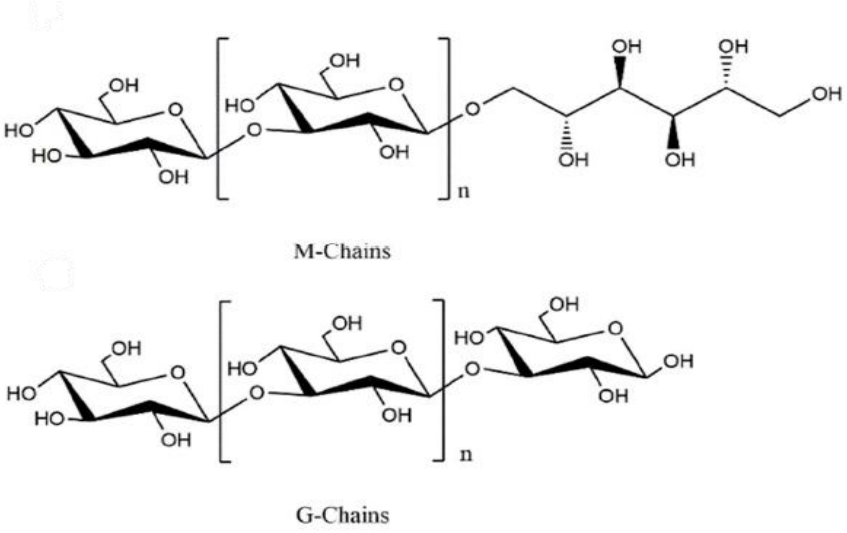


Figure 3. Structure of laminaran with mannitol (M-Chains) or glucose (G-Chain) in the reducing end [25]

1.1.1.3. Fucose-containing sulphated polysaccharides

There are two main types of fucose-containing sulphated polysaccharides in brown algae: fucans and fucoidans. Fucans are polysaccharides with a backbone mainly composed of L-fucose with sulphate groups, that can be branched with neutral sugars or uronic acids [17]. There are mainly two types of fucans: type I, which contain high quantities of α-L-fucopyranose with (1→3) linkages, and type II, containing (1→3) and (1→4) linkages of α-L-fucopyranose (Figure 4). The term fucoidan refers to heteropolymers with high quantities of fucose and sulphate groups but with diverse backbones containing neutral sugars, such as galactose, mannose, and xylose, as well as uronic acids [17,26]. Galactose residues are usually present in significant amounts and may have sulphate groups in different locations, for instance (1→6) linkages have been reported to be sulphated at C3 [27,28], disulphated at C2 and C3 or C3 and C4 [29]. Fucans and fucoidans can represent 1 to 20 % of the algae dry mass [20] and their molecular weight ranges from 7 to 2379 kDa (Table 2) [30].

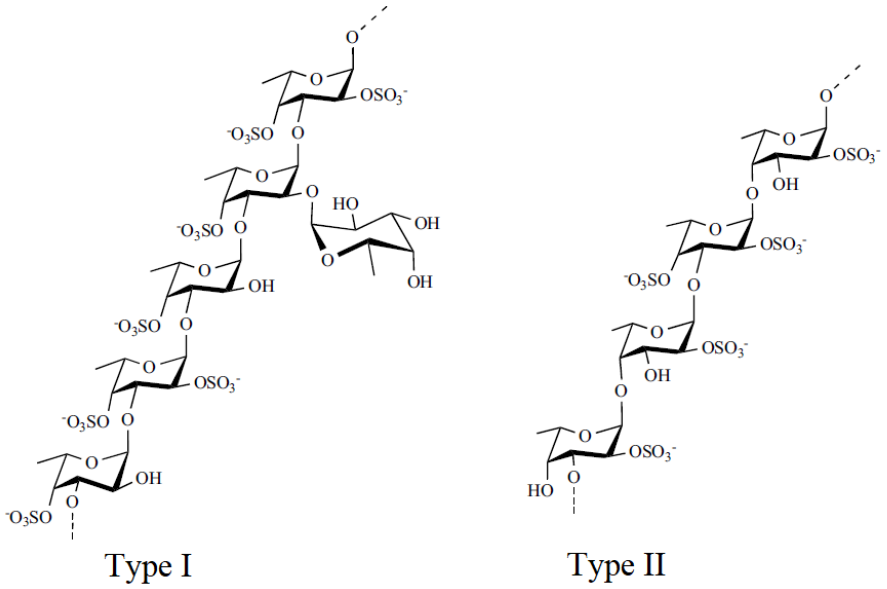


Figure 4. Structure of type I and type II fucans (adapted from Cardoso *et al* [22]).

Both fucoidans and fucans usually contain high quantity of sulphate esters mainly in *O*2, *O*3 and/or *O*4 of fucose residues [23,26], which are associated with an enhanced biological activities, such as anti-inflammatory, antioxidant and anticancer [17]. These polysaccharides also showed hypocholesterolemic activity in mice fed with diets rich in cholesterol [31–35]. These biological activities vary with their structure, molecular weight, degree of sulphation and sulphate groups location. As can be observed in Table 3, different species have different yields and structural characteristics, although containing fucose and sulphate in high proportion. The low toxicity combined with a broad spectrum of biological activities made fucoidans attractive polysaccharides to be applied in the biomedical field [36].

Table 3. Yield and composition variation of fucans from brown macroalgae for different species [27,33,34,37].

		Composition (%)								
Specie	**Yield (% dry weight)**	**Fuc**	**Gal**	**Xyl**	**Man**	**Glc**	**Ara**	**Rha**	**Uronic acids**	**Sulphate**
Fucus evanescens	6.1	58.7	1.6	1.6	-	-	-	-	-	46.5
Saccharina latissima	1.1	35.6	8.1	0.9	0.7	0.2	-	-	2.5	36.8
Laminaria japonica	1.3	59.9	-	-	-	-	7.9	0.1	-	28.1
Kjellmaniella crassifolia	3.8	32.0	23.5	3.4	7.1	11.8	-	1.4	7.8	20.0

1.1.2. Chitosan

Chitosan, obtained through the deacetylation of chitin, is soluble in diluted acids and has lower viscosity compared to chitin which enables a wider range of applications. It is composed mostly of D-glucosamine residues with (β1→4) linkages with variable contents of *N*-acetyl-D-glucosamine depending of the deacetylation degree (Figure 5). Chitosan has several biological activities, such as antioxidant [38] and antimicrobial [39], and it is biocompatible and biodegradable, allowing to be used both in medical and food industries.

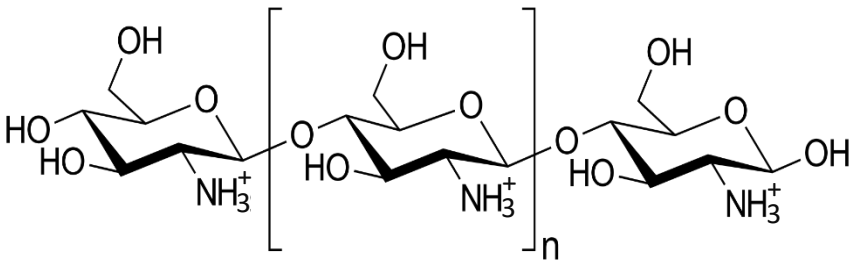


Figure 5. Chitosan's structure.

Chitosan oligosaccharides are obtained by depolymerization of chitosan and have a molecular weight of 10 kDa or less [40], which makes them less viscous and,

unlike chitosan, soluble in water. Biological activities of chitosan oligosaccharides include antimicrobial, antioxidant, and anti-tumoral [40,41]. Chitosan as shown promising results in *in vivo* [42–44] and *in vitro* [44,45] trials as a hypocholesterolemic agent. Diet supplementation with chitosan oligosaccharides decreases cholesterol levels *in vivo* [46,47], having an higher effect than chitin and chitosan *in vitro* [45]. The mechanism by which these oligosaccharides affect cholesterol will be detailed further in this work.

1.1.3. Marine origin polysaccharides as functional foods

Functional foods provide health benefits beyond basic nutrition and their market grows each year [48]. Brown seaweeds polysaccharides, chitosan and its oligosaccharides, which have been described to possess several biological activities, have potential as functional ingredients. This way, these polysaccharides could be used to add to food products or even used as nutraceuticals which are physiologically active compounds present in food with disease prevention potential or health promotional that can be consumed under the form of supplements.

Prevalence of elevated cholesterol levels in humans can lead to severe consequences that reduce health and life quality and most of the times leading to death [1]. There are several strategies to lower cholesterol such as drugs and food ingredients with hypocholesterolemic effect. The use of cholesterol-lowering drugs can carry secondary effects and/or elevated costs. Moreover, the discovery of new functional ingredients with hypocholesterolemic activity could decrease drug dependence without carrying unwanted secondary effects or minor their effect. Since marine environments have a wide range of different polysaccharides, they could be a good source of polysaccharides with hypocholesterolemic activity. Chitosan has already shown hypocholesterolemic effect in humans [43] as well as alginate [49], however the other brown algae polysaccharides remain to be subjected to human trials to confirm that they are indeed functional ingredients.

1.2. Cholesterol Homeostasis and Metabolism

Cholesterol is an essential molecule for human life, which is involved in the regulation of the phospholipidic membrane fluidity and synthesis of steroid hormones, vitamin D and bile acids [50,51]. However, hypercholesterolemia is a serious threat to human health being responsible for 4,5 % of total death worldwide and 2 % of morbidity cases according to the World Health Organization [1]. The presence of high quantity of cholesterol transported in the bloodstream and its accumulation in the blood vessels is associated with the incidence of atherosclerosis, one of the major causes of death across the world [52].

Cholesterol's quantity in the body is maintained through: i) its endogenous synthesis at liver, ii) intestinal absorption (including the bioaccessibility and bioavailability) at lumen, and bile excretion at large intestine and iii) blood circulation by lipoproteins.

1.2.1. Cholesterol synthesis

Cholesterol is synthetized mainly in the liver, although some minor contributions from other tissues are reported [53]. This endogenous production in the liver accounts for two thirds of the daily cholesterol absorption of a total 1.8 g, being the remaining supplied by the diet [54].

The process of cholesterol synthesis, whose main steps are shown in Figure 6, begins with the formation of acetoacetyl-CoA from the reaction of two acetyl-CoA molecules catalyzed by the acyl-CoA:cholesterol acyltransferase (ACAT). The action of the enzyme HMG-CoA synthase originates 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is converted to mevalonate. The enzyme responsible for the reaction that gives rise to mevalonate is HMG-CoA reductase. This enzyme can be inhibited by statins, a common pharmacological strategy used to reduce the endogenous cholesterol synthesis at liver. Although severe secondary side effects are reported to these compounds, such as muscle and hepatic disorders [55].

Furthermore, the inhibition of HMG-CoA reductase will affect the synthesis of bile salts, which can affect the emulsion process at the intestinal lumen, and the production of important hormones that have cholesterol as precursor.

The sequential action of the enzymes mevalonate kinase (MVK), phosphomevalonate kinase (PMVK) and mevalonate diphosphate decarboxylase (MVD) converts mevalonate into isopentenyl diphosphate. The following action of isopentenyl-PP isomerase gives rise to 3,3-dimethylallyl diphosphate, which will be condensed with isopentenyl-PP to form geranyl diphosphate. The addition of another isopentenyl-PP culminates in farnesyl diphosphate, a molecule with 15 carbon atoms. The fusion of two molecules of farnesyl diphosphate, catalyzed by farnesyl-diphosphate farnesyltransferase 1 (FDFT1), will originate the 30 carbons molecule squalene. Squalene oxidases originate squalene 2,3-epoxi, which is converted to lanosterol by the action of the enzyme oxido squalene lanosterolcyclase. After 19 more reactions, lanosterol originates cholesterol.

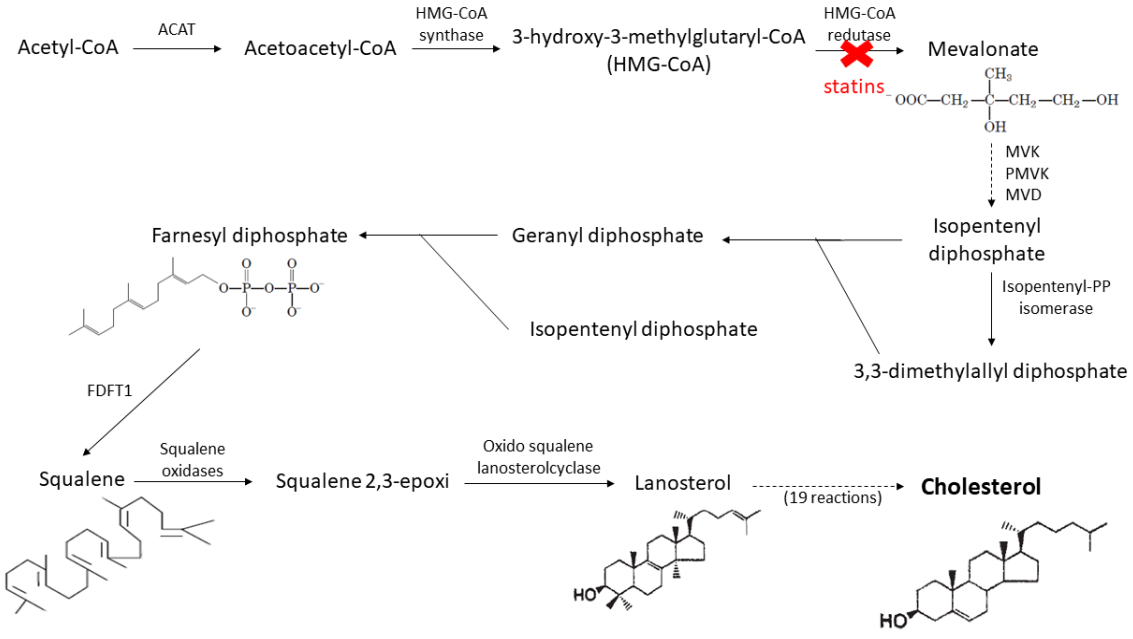


Figure 6. Cholesterol biosynthesis [56].

1.2.1.1. Bile acids synthesis

Bile salts are water soluble and can form micelles, being involved in the emulsification processes that aid the solubilization of hydrophobic molecules and their synthesis begins with cholesterol.

The two primary bile acids in humans are cholic acid (CA) and chenodeoxycholic acid (CDCA) [51]. For the synthesis of these primary bile acids two pathways can occur. The classic pathway, in which most bile acids are synthetized, begins with the action of the enzyme cholesterol-7-alpha-hydroxylase (CYP7A1). The following action of sterol 27-hydroxylase (CYP27A1) gives rise to the primary bile acids but in the case of CA there is the need to a previous action of the enzyme sterol 12-alpha-hydroxylase (CYP8B1). The other pathway is an acidic one, which consists in the oxidation of the side chain by CYP27A1 followed by the action of 25-hydroxycholesterol 7-alpha-hydroxylase (CYP7B1). Before being secreted into the bile, the resultant bile acids from either pathway will be conjugated with glycine or taurine, with the action of bile acid:CoA synthetase (BACS) and bile acid:amino acid transferase (BAT), to reduce their toxicity and enhance solubilization (due to reduction of bile acid pKa) [51,57] (Figure 7).

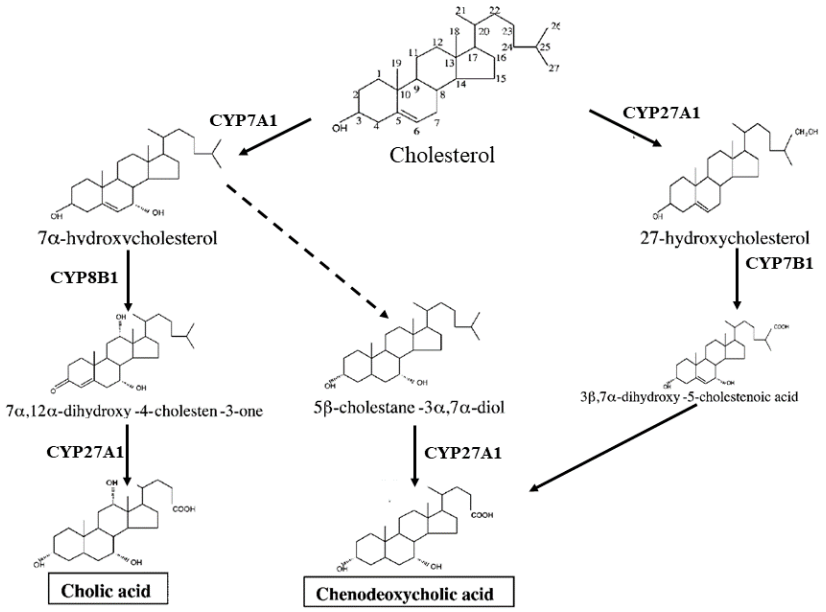


Figure 7. Bile acid synthesis through neutral (left) and acidic (right) pathways [51].

Secondary bile acids result from the action of intestinal bacteria after the deconjugation of glycine or taurine from CA and CDCA [51,57]. A portion of CA and CDCA will originate deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Portions of CA, CDCA and DCA will be absorbed and sent back to the liver while the majority of LCA will be eliminated in the feces. The reabsorbed bile acids will move from the intestine to the liver in a process called enterohepatic circulation which happens after each meal.

The transcription of the rate-limiting enzyme CYP7A1 is regulated by bile acids as well as other molecules. The regulation of this enzyme is made by negative feedback by two complementary mechanisms whose initial step occurs in the liver or in the intestine. In the mechanism starting in the liver, the bile acids will bind to the Farnesoid X Receptor (FXR), activating it. FXR will then induce the nuclear receptor Small Heterodimer Partner and it will suppress CYP7A1 gene expression.

A way of reducing cholesterol levels is enhancing bile acids synthesis due to the need for it to be catabolized during this process. Blocking enterohepatic recirculation or the addition of sequestrants of bile salts will lead to an increase

synthesis and consequently an enhance cholesterol catabolism, which will lower cholesterol levels.

1.2.2. Bioaccessibility of cholesterol

Bioaccessibility is defined as the portion of a substance that has potential to be absorbed by the organism [58]. Due to the low solubility of cholesterol in aqueous environment reported to be in the range of 0.025 [59] to 0.09 mg/L [60], it must be first emulsified in bile salt micelles in order to diffuse to the enterocyte's membrane and get absorbed [54].

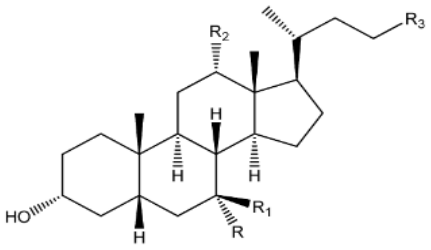
After the major meals, the gallbladder excretes bile which is mainly composed of bile salts, phospholipids, and free cholesterol. The dietary mixed micelles formed by the bile components will trap hydrophobic molecules (*e.g.* cholesterol, free fatty acids, phospholipids) in their core, aiding their solubilization.

1.2.2.1. Emulsification processes

Bile salts are biological detergents that are not only involved in cholesterol solubilization but also in the solubilization of other lipophilic nutrients such as some vitamins and fats. They are surfactants, which means that reduce surface tension, promoting solubilization of hydrophobic molecules. Contrary to regular surfactants that usually have a hydrophobic alkyl chain and a polar head group, bile salts are amphiphilic molecules that have a hydrophobic and a hydrophilic surface.

The major bile salts present in human bile are CA, CDCA and DCA. Bile salts can be conjugated either with glycine or taurine (Figure 8). However, in the human species the bulk of bile salts are glycine conjugated. Conjugation of bile salts with glycine, enable a lowering in pKa which will prevent their precipitation at physiological pH and reduce its interaction with the hydrophobic membranes, reducing toxicity. Conjugated bile salts commonly found in humans include

glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), taurodeoxycholic acid (TDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycolithocholic acid (GLCA), glycolithocholic sulphate acid (GLCAS), taurolithocholic acid (TLCA) and taurolithocholic sulphate (TLCAS), whose molar percentages can be found in Table 4 [61].



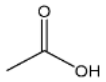
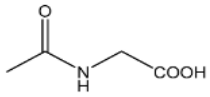
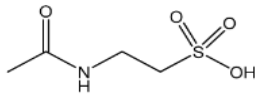
Bile acid	R	R_1	R_2
CA	-OH	-H	-OH
CDCA	-OH	-H	-H
DCA	-H	-H	-OH
	unconjugated		
R_3			
	glycine-conjugated		
R_3			
	taurine conjugated		
R_3			

Figure 8. Structure of the main bile acids (adapted from Steiner et al. [62]).

Table 4. Content (mol%) of bile salts present in the intestinal lumen [61].

Bile salt	Conjugated with glycine	Conjugated with taurine
CA	24	12
CDCA	24	12
DCA	16	8
LCA	0.7	0.3
LCAS	3	1
Total	67.7	33.3

Once the bile salts are present in solution at a concentration above the critical micellar concentration (CMC) they aggregate and form micelles. Above CMC the more bile salts in solution the more micelles are formed. The presence of phospholipids and fatty acids decrease de CMC of the bile salts micelles, allowing aggregation phenomena at lower concentrations, which will lead to a higher capacity to solubilize cholesterol in the so-called dietary mixed micelles. Concerning the three most common bile acids, GCA has a higher CMC than GDCA and GCDCA, which can be explained by the fact that GCA possesses an extra hydroxyl group as compared with the other two bile salts, increasing its solubility as monomer in aqueous solution to a higher concentration. Due to the higher CMC, GCA monomers only start to aggregate and forming micelles at an higher concentration than GDCA and GCDCA [63]. In contrast GDCA is the one with the lowest CMC which is associated with its higher hydrophobicity. In terms of cholesterol solubility, GDCA is the bile salt which solubilizes more cholesterol in contrast with GCA.

1.2.3. Bioavailability of cholesterol

Bioavailability of cholesterol corresponds to the fraction that reaches the plasma, being available to exert functions in the organism [58].

1.2.3.1. Cholesterol absorption

Cholesterol absorption takes place in the small intestine, more specifically in the duodenum and proximal jejunum. Cholesterol uptake by enterocytes can occur via passive diffusion (not considered in this work) or mediated transport, which will be briefly detailed.

Transmembrane protein NPC1L1

The mediated transport requires the transmembrane protein Niemann-Pick C1-like 1 (NPC1L1). NPC1L1 is located on the cell membrane lipid rafts which are regions rich in gangliosides, cholesterol and receptors and is highly expressed in the small intestine and in the liver. For cholesterol transport across the cell membranes NPC1L1 needs gangliosides which are important to the formation of the membrane's domains where cholesterol is transported [64]. The NPC1L1's location alternates between the cell membranes and the intercellular components depending on cholesterol availability [65]. If low amounts of cholesterol are available, the NPC1L1 will relocate in the cell surface, a process that can be reversed by the addiction of more cholesterol. NPC1L1 can absorb other sterols (for example, phytosterols), but has a higher affinity for cholesterol.

The drug ezetimibe targets NPC1L1, however the exact mechanism by which it blocks the cholesterol transport is not yet elucidated. Firstly, it was thought that ezetimibe targeted the NPC1L1's ability to perform endocytosis [66], but more recently was discovered that the cholesterol uptake by this protein is independent of endocytosis [67]. More studies need to be done to better understand the inherent mechanisms.

Ezetimibe and statins are often prescribed together for a simultaneous lowering of cholesterol absorption and synthesis, respectively. However, the consumption of drugs can cause hepatotoxicity and statins inhibit cholesterol synthesis but also other compounds that arise from cholesterol (for example bile

acids). Thus, it is important to find new alternatives to cholesterol-lowering drugs that minimize the secondary effects [55].

ABCG5 and ABCG8 transporters

ATP-binding cassette transporters (ABC transporters) are a group of transporters that require adenosine triphosphate (ATP) as source of energy for the translocation of molecules across cellular membranes. Two ABC transporters are known to be involved in cholesterol excretion: ABCG5 (sterolin-1) and ABCG8 (sterolin-2).

ABCG5 and ABCG8 transport phytosterols and non-esterified cholesterol across the cell membrane to the intestinal lumen, preventing its accumulation. The malfunction of these transporters will cause accumulation of high quantities of phytosterols [68], which will lead to a pathology called sitosterolemia.

Studies with mice models have shown that loss of ABCG8 functionality will lead to a decrease in cholesterol excretion to the bile although sitosterol excretion was not affected, which leads to the possibility that ABCG8 plays a major role in biliary cholesterol excretion [69].

1.2.4. Cholesterol Blood Circulation in Lipoproteins

Cholesterol is transported in lipoproteins which are complex of transporter proteins (apolipoproteins) with different proportions of phospholipids, cholesterol, cholesterol esters and triglycerides (Table 5). Lipoproteins have a spherical form, being the hydrophobic lipids placed in the core.

Table 5. Properties of lipoproteins [70].

	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g.cm^{-3})	<0,95	<1,006	1,006-1,019	1,019-1,063	1,063-1,210
Protein (%)	1,5-2,5	5-10	15-20	20-25	40-55
Phospholipids (%)	7-9	15-20	22	15-20	20-35
Free Cholesterol (%)	1-3	5-10	8	7-10	3-5
Triglycerides (%)	84-89	50-65	22	7-10	3-5
Cholesteryl esters (%)	3-5	10-15	30	35-40	12

Absorption of cholesterol takes place in the intestine and it is esterified before being joined with triglycerides to form chylomicrons. Chylomicrons are the least dense and bigger lipoproteins and will enter the circulation towards the peripheric tissues. After losing the bulk of its triglycerides, phospholipids and free cholesterol are transferred to high-density lipoproteins (HDL) [71]. The remnants chylomicrons move through the blood flow to the liver where release their cholesteryl esters and are degraded.

Cholesterol synthetized in the liver is incorporated in very-low-density lipoproteins (VLDL). VLDL interacts with lipoprotein lipases which provokes the release of triglycerides and a consequent shrinkage (and density enhancement) happens, originating intermediate-density lipoproteins (IDL) [71]. The excess of cholesterol and phospholipids are transferred to HDL. VLDL loses most of the remaining triglycerides and loses some apolipoproteins, giving rise to low-density lipoproteins (LDL) which will take cholesterol to the liver and extrahepatic cells. A schematic representation can be found in Figure 9.

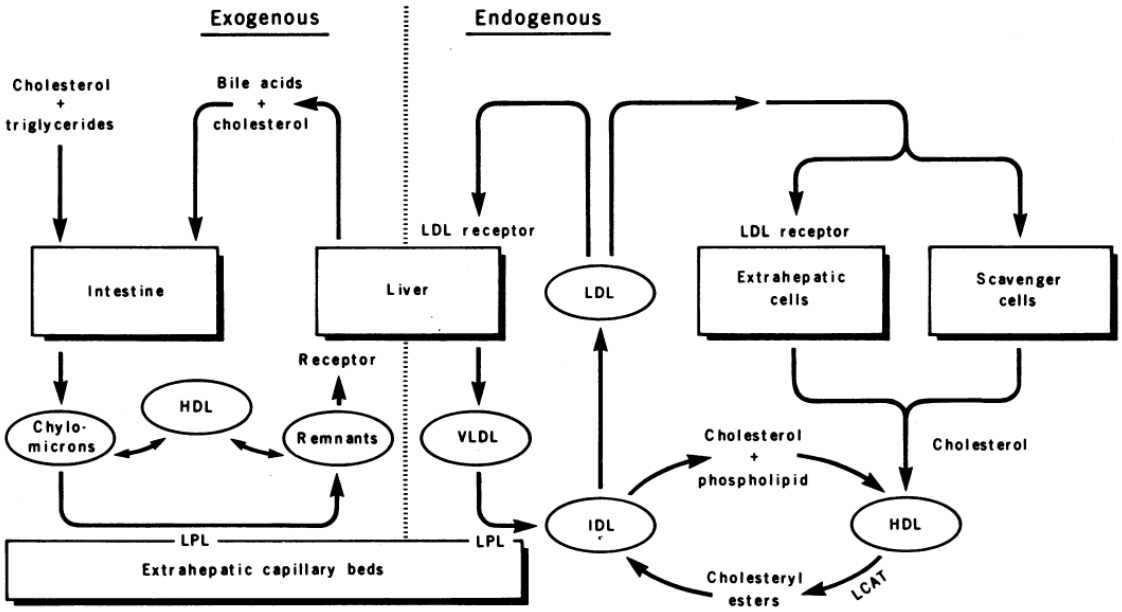


Figure 9. Transport of cholesterol in lipoproteins [71].

High levels of cholesterol increase the risk of atherosclerosis, which starts with the accumulation of cholesterol in arterial intima, leading to an inflammatory process that culminates in the blockage of the arteries [52]. Briefly, the oxidation of LDL (to OxLDL) and its accumulation in the arterial walls will contribute to the process of plaque formation which is accompanied by the expression of leukocyte adhesion molecules by the endothelial cells [72]. This event will result in the migration of monocytes and T cells which will bind to the endothelium and infiltrate the arterial wall, beginning the inflammation process. In the final stages of atherosclerosis, the number of macrophages and other inflammatory cytokinin infiltrated in the wall will be higher, matrix metalloproteinases will be secreted and will occur the degradation of collagen fibers of the extracellular matrix [73]. These events will culminate in the blockage, bleeding and rupture of the arteries resulting in a cardiovascular event that may lead to death.

1.2.4.1. LDL-receptors

The LDL carry cholesterol and delivery it to the peripheric cells. The cholesterol uptake by the cells requires LDL-receptors which will internalize LDL that will be dissociated from the receptor inside the cell [74]. The LDL-receptors can be recycled back to the cell's surface where they will capture more LDL particles to be internalized. However, if proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to an LDL-receptor it will not be recycled back to the cell surface.

The concentration of LDL-receptors in the cells is modulated by the enzyme PSCK9. When PCSK9 binds to an LDL receptor, this will promote their lysosomal degradation in the cells. Inhibition of PCSK9 will prevent degradation of LDL receptors and more of these receptors will be recycled back to the hepatocyte membranes, capturing more LDL and thus reducing the quantity that circulate in the blood. There are drugs available (Evolocumab and Alirocumab) to inhibit PSCK9, however these drugs are very expensive and are only administered in hospital scenarios.

1.2.5. Hypocholesterolemic strategies

Regarding cholesterol incorporation in micelles, this process can be affected by other molecules present in the intestinal lumen such as polysaccharides. Various mechanism can occur and the reduction of bioaccessibility can be due to more than one mechanism at the same time (Figure 10).

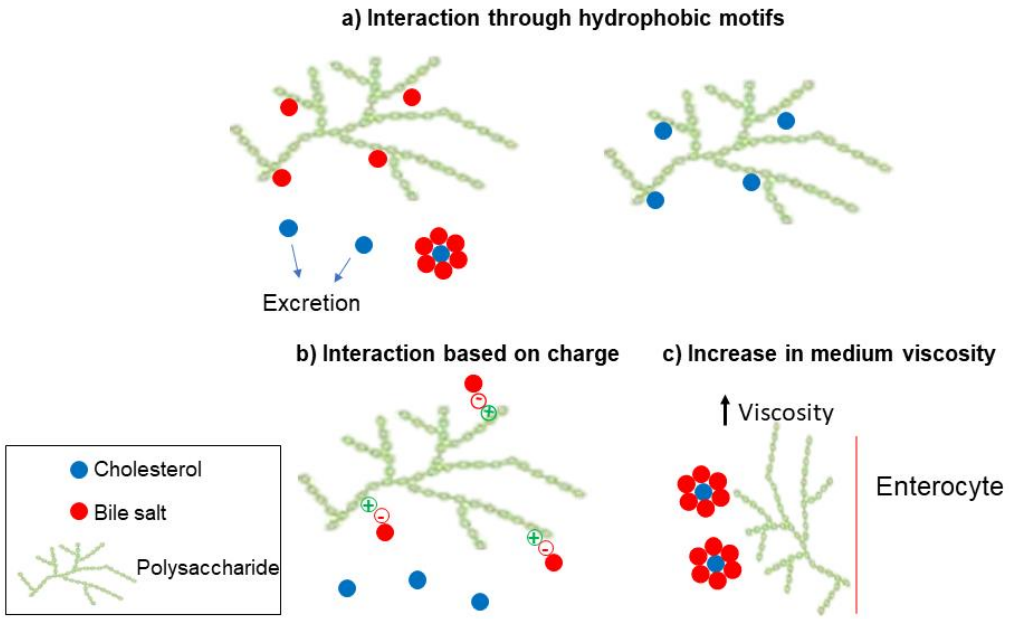


Figure 10. Hypocholesterolemic mechanisms of polysaccharides: a) interaction through hydrophobic motifs, b) interaction based on charge and c) increase in medium viscosity.

One of the possible mechanisms of action by which polysaccharides can affect cholesterol bioaccessibility is the sequestration of bile salts through adsorption by its hydrophobic motifs or even by the interaction of cholesterol itself (Figure 10a). Linear β-glucans (neutral polysaccharides) formed by (1→3) and (1→4) linkages are known to reduce cholesterol. One possible mechanism described is the interaction with bile salts [75]. Pectin, which is negatively charged, also interacts with bile salts most likely due to the interaction with the hydrophobic motifs [76].

The presence of positively charged molecules can affect cholesterol incorporation in micelles due to the interaction of the cationic compounds with the negatively charged bile salts (Figure 10b). The use of cationic resins, such as colestipol [77], decreases cholesterol absorption and, at the same time, increases bile salts excretion, increasing cholesterol catabolism to give rise to new bile salts. The seas are a great source of polysaccharides that can be used as bile salts sequestrants such as the positively charged chitosan and its oligosaccharides. Regarding chitosan's mechanisms of action, properties such as degree of

deacetylation and molecular weight did not show conclusive correlation with its hypocholesterolemic potential *in vitro*, although smaller particle size had a better effect [44]. Although several studies have shown the potential hypocholesterolemic effect of chitosan [42–45] and its oligosaccharides [45–47], interaction based on charged does not seem to be the only mechanism by which it acts since other factors such as higher viscosity can also contribute to the effectiveness [44,45].

Viscosity in the intestinal lumen can affect the diffusion rate of the micelles and interfere with cholesterol uptake by the enterocytes, which will increase the excretion of cholesterol (Figure 10c). The brown algae polysaccharide alginate reduces cholesterol uptake possibly due to an increase in viscosity which will interfered in the cholesterol delivery to the enterocytes [49,78]. Rats feed with laminaran from *Laminaria digitata* have shown an increase in mucus acidity which makes it more viscous, thus influencing the membrane crossing [79].

Other way to reduce cholesterol's absorption relies on the ingestion of other sterols like phytosterols. Phytosterols can affect cholesterol solubilization by two processes: co-solubilization or co-precipitation. In the co-solubilization mechanism, the phytosterols will compete with cholesterol for the incorporation in the micelles and this way preventing the incorporation of cholesterol in micelles [80]. On the other hand, co-precipitation happens when the interaction between both sterols causes their precipitation [81]. Both mechanisms can occur at the same time. However, the co-solubilization mechanism can affect the solubility of other hydrophobic compounds such as lipid-soluble vitamins and consequently decrease their absorption. Fucosterol is the main sterol in algae and has shown potential to reduce cholesterol bioaccessibility due to interaction with micelles [82]. Nevertheless, the elucidation if fucosterol is capable of co-solubilization and/or co-precipitation would be important to understand if it could interfere with other hydrophobic compounds absorption.

Sterols are not the only lipids with hypocholesterolemic properties. Phospholipids have been reported to interfere with cholesterol absorption being one explanation the fact that excess of phospholipids can prevent the hydrolysis of phospholipids which is required for cholesterol uptake [83,84]. Polyunsaturated fatty

acids (PUFAs) reduce cholesterol levels thrown an enhancement of its excretion [85]. Oleic acid (a monosaturated fatty acid) has shown to decrease cholesterol solubility [63]. Since lipids that exist in brown algae have shown potential as cholesterol-lowering agents, these macroalgae have potential to reduce cholesterol levels.

Regarding bioavailability, this process can also be affected. As previously mention, cholesterol is converted in cholesteryl esters by ACAT before being incorporated in chylomicrons. Phytosterols can inhibit ACAT activity resulting in a reduction of cholesteryl esters formed and consequent decrease in cholesterol incorporation in chylomicrons [86]. Non-esterified cholesterol will be sent back to the intestinal lumen, by the transporters ABCG5 and ABCG8, and then excreted.

To maintain human health new hypocholesterolemic strategies are needed to reduce cholesterol levels. Although pharmacologic options are available in the market, functional foods with hypocholesterolemic effect can prevent diseases without the secondary effect of pharmaceutical drugs. Polysaccharides have shown potential to reduce cholesterol through distinct mechanisms however the quantification of its effectiveness is also important to better understand which characteristics are more efficient.

1.2.6. Methods to evaluate cholesterol bioaccessibility

It is important to quantify the amount of cholesterol solubilized in micelles to understand the effectiveness of polysaccharides in lowering cholesterol's bioaccessibility. Several methods have been described in the literature to perform these quantifications *in vitro*. Matsuoka *et al*. [87] followed the solubilization of cholesterol in micelles through the incubation of cholesterol and bile salts followed by filtration and quantification of cholesterol using an enzymatic kit. Although this methodology is simple, the filtration step can affect micelles stability.

To evaluate the polysaccharides potential in lowering cholesterol bioaccessibility, usually the fibers are incubated with cholesterol and bile salts and

the bile salts are quantified enzymatically after centrifugation [45,88,89]. Xu *et al*. [45] used centrifugation to quantify the amount of bile salts sequestrated by chitin, chitosan and oligosaccharides of chitosan (soluble in water) isolated from lobster shells. The oligosaccharides were more effective than chitin and chitosan in sequestration of the more hydrophobic bile salts tested. However, the efficacy of the polysaccharides tested is significatively lower than the cationic resin (cholestyramine) used has positive control. Liu *el al.* [44] also tested chitosan but cholesterol was quantified instead of bile salts and oleic acid was added to the bile salts and cholesterol mixture, which can alter the solubilization process making results comparation with the work of Xu *et al*. [45] unreliable.

In the works done by Kim *et al*. [88], Camire *et al*. [89] and Deng *et al*. [90] different polysaccharides were incubated with diverse bile salts, followed by centrifugation and enzymatic quantification of bile salts, and the binding capacity was calculate in function to the one showed by cholestyramine. Although the methodology followed by the previously mentioned studies were similar, the concentration of polysaccharides and bile salts and the bile salts used were different, which makes comparation between studies difficult. Nevertheless, the comparation with cholestyramine, which is used a hypocholesterolemic agent, elucidate the efficacy of the polysaccharides studied as bile salts sequestrants. It is also important to mention that the centrifugation step can also disrupt micelles equilibrium.

Although the previously mentioned methodologies to evaluate cholesterol bioaccessibility are simple they can affect micelles stability. The use of nuclear magnetic resonance (NMR) to quantify solubilized cholesterol and bile acid sequestration is expensive but is a good approach since this technique does not perturb micellar equilibrium [91]. To perform the quantification via NMR it is needed bile salts and cholesterol enriched in ^{13}C (to be able to quantify cholesterol due to its low concentration and isotopic abundance compared to bile salts), which are incubated with the compounds under study and then through NMR it is possible to quantify the solubilized cholesterol in bile salt micelles and the quantity of bile acids sequestered [92]. However, large aggregates such as crystals of cholesterols are not seen [91]. Although cholesterol enriched in ^{13}C is expensive it could be also

used to follow the absorption of cholesterol from endogenous production (unlabeled) and the one from the diet, allowing to follow the metabolism of cholesterol. The methodology mentioned that used NMR was already used to follow the emulsification processes in the presence of phytosterols, tocopherol, saturated and unsaturated fatty acids, and polysaccharides [63,92]. In this work the same approach will be used to evaluate the sequestration of bile salts by polysaccharides and within the same measurement the effect on cholesterol solubility.

Regarding bioavailability, the usage of cell models is a possible method to determine if the presence of specific polysaccharides can affect the permeation of cholesterol across cell membranes *in vitro*. Monolayers of Caco-2 cells on a permeable filter are often use to determine permeability of drug-like substance and predict intestinal absorption [93]. Caco-2 cells could be used to predict if cholesterol's absorption is limited by presence of determined substance.

1.3. Aims of this work

The aim of this work is to evaluate the *in vitro* capacity of polysaccharides from sea origin to decrease cholesterol's solubility as a measure of their effect on its bioaccessibility. The polysaccharides from macroalgae (i.e. *S. latissima*) and crustaceous residues were fractionated and chemically characterized to allow a relation between the structure and the biological function. This work provides a better understanding of the hypocholesterolemic properties of polysaccharides of marine origin and the influence of their physical-chemical characteristics, allowing to know their potential as functional food ingredients.

Chapter 2 – Material and Methods

2.1. Samples

Two commercial polysaccharides were used in this work, a fucoidan (Shandong Jiejing Group Corporation) and a chitosan soluble in water (Carbosynth). These commercial polysaccharides were further purified by dialysis in distilled water using membranes with a cutoff of 12-14 kDa. The water was changed five times and the retained was lyophilized and analyzed.

2.2. Extraction and fractionation of polysaccharides from *S. latissima*

Polysaccharides extracted from the brown algae *Saccharina latissima* were also analyzed. The biomass of *S. latissima* was cultivated in offshore and collected in May of 2017. The seaweed was frozen, lyophilized and milled.

For hydration of the lyophilized *S. latissima*, 80 mL of distilled water were added to 10 g of biomass and stirred for 10 min at room temperature. Then, 320 mL of absolute ethanol were added and the mixture was left under agitation for 15 min at 80 °C. The solution was filtered with a funnel with porous plate and a 110 nm filter under vacuum. An alcohol insoluble residue (AIR) was obtained and washed with ethanol 96 % and acetone. The AIR was dried in a Petri dish covered with aluminum foil at room temperature before being used in the upcoming steps.

In a glass cup, 280 mL of distilled water were added to 2.5 g of AIR and kept at 90 °C for 1 h under agitation. The solution was filtered under vacuum with a porous plate and the water extract was used in the further fractionation steps.

For alginate precipitation with $CaCl_2$ 2 %, 3.8 g of $CaCl_2$ were added to 380 mL of the water extract obtained and solubilized during 25 min at room temperature. The precipitation occurred in the cold chamber for 2 h and the solution was centrifuged at 15000 rpm for 20 min at 4 °C. The supernatant and the precipitate (containing the alginates) were dialyzed against distilled water using membranes with a cutoff of 12-14 kDa, with a few drops of toluene and chloroform to prevent proliferation of microorganisms. The dialysis was completed when the conductivity

of dialysis water was the same as distilled water and the samples were then lyophilized. This supernatant was used to continue the extraction.

The mixture of polysaccharides from *S. latissima* in the supernatant (mixture S.l.) was further fractionated in an anionic exchange column equipped with a resin DEAE-Trisacryl (Sigma). Approximately 100 mg of sample were introduced in the column with a flux of 0.5 mL/min. The first fraction, which contained the laminarans, was eluted with hydrochloric acid (HCl) 0.05 M and two fractions containing fucoidans (F1 and F2) were eluted with sodium chloride (NaCl) 1M and 2M. For the laminarans 35 mL were collected and for F1 and F2 40 mL. All the fractions were then dialyzed as described before and lyophilized.

2.3. Structural characterization of polysaccharides

2.3.1. Neutral sugar and glucosamine analysis

Neutral sugar composition of the polysaccharides was accessed using the alditol acetates method [94]. Polysaccharides were firstly hydrolyzed to give rise to monosaccharides which were then reduced (to prevent the structure from closing) and acetylated (originating alditol acetates) to be further analyzed using gas chromatography with a flame ionization detector (GC-FID). For the determination of neutral sugars and glucosamine of the chitosan the hydrolysis conditions employed were different and the analysis was made using GC-MS.

Hydrolysis conditions used were different for the different polysaccharides. For determination of glucosamine in the chitosan samples the hydrolysis was performed by weighting 1-2 mg of sample in a glass tube previously washed with nitric acid, followed by the addiction of 1 mL of HCl 1M and incubation at 100 °C for 24 h. In the case of the neutral sugars of chitosan, the hydrolysis consisted in the addition of 0.5 ml of TFA 2M and incubation at 121 °C for 1 h followed by evaporation of the TFA in a speedvac. For the commercial fucoidan and the fractions of *S. latissima* a pre-hydrolysis was made by adding 200 µL of sulfuric acid (H_2SO_4) 72 % to 1-2 mg of sample, weight in a glass tube previously washed with nitric acid,

and incubating at ambient temperature for 3 h. After the pre-hydrolysis, 2.2 mL of distilled water were added (to reach a concentration of 1M H_2SO_4) and incubated at 100 °C for 2.5 h.

After the hydrolysis, the tubes were cooled in ice before the addition of 2-deoxyglucose (1 mg/mL) as internal standard. In the case of the commercial fucoidan and samples of *S. latissima* hydrolysis 1 mL was transferred to a new tube before the neutralization with NH_3 25 % and in the case of chitosan the neutralization was made in the same tube used for the hydrolysis. The reduction was then carried through the addition of 100 µL of a $NaBH_4$ 15 % (w/v) solution prepared in 3 M NH_3. In the neutral sugar analysis of chitosan 300 µL of NH_3 2 M and 20 mg of $NaBH_4$ were added instead. Reduction occurred in a bath at 30 °C for 1 h. After the reduction, the tubes were cooled in an ice bath and 50 µL of glacial acetic acid were added two times in order to eliminate the BH_4^- in excess.

For the acetylation, 300 µL were transferred to new tubes with teflon caps (to prevent contaminations). In an ice bath, 450 µL of 1-metilimidazol and 3 mL of acetic anhydride were added and then incubated at 30 °C for 30 min. After, 3 mL of distilled water and 2.5 mL of dichloromethane were added to the tubes in an ice bath and then agitated manually. To separate the two phases, a centrifugation at 3000 rpm for approximately 30 s was made. The aqueous phase was then aspirated by suction with a Pasteur pipette and the additions repeated. Two more washings with 3 mL of distilled water were made and the aqueous phase removed as described previously. The dichloromethane was evaporated in a speedvac and 1 mL of anhydrous acetone was added two times and evaporated.

The alditol acetates of the commercial fucoidan and the polysaccharides from *S. latissima* were analyzed by GC-FID using a column DB-225 (30 m long, 0.25 mm diameter, and 0.15 µm thickness). The samples were dissolved in 30 to 50 µL of anhydrous acetone and 2 µL were injected in a chromatograph (Perkin Elmer – Clarus 400). The temperature of the injector and detector were 220 °C and 230 °C, respectively. The temperature's program used had an initial temperature of 200 °C. The first ramp increased 40 °C per minute until the temperature reached 220 °C,

which was maintained during 7 min. The second ramp increased the temperature by 20 °C per minute until reached 230 °C, which was maintain by 1 min.

The alditol acetates of the water-soluble chitosan were determined by GC-MS using a HT-5 column (30 m long, 0.25 mm diameter, 0.10 µm thickness). The samples were dissolved in anhydrous acetone as described previously and were injected in a GC-MS (SHIMADZU GCMS-QP2010 Ultra). The temperature of the injector and detector were 350 °C and 250 °C, respectively. The oven initial temperature was 140 °C and reached a temperature of 180 °C for 1 min with a rate of 5 °C/min, followed by an increase of 5 °C/min until 250 °C and a second increase at a rate of 100 °C/min until 350 °C.

All the samples were analyzed in triplicate and the sugars were identified based on their retention time.

2.3.2. Uronic acids

For determination of uronic acids the colorimetric method described by Coimbra *et al.* was used [94]. For the hydrolysis, 1 to 2 mg of sample were weighted into a glass tube and incubated with 200 µL of H_2SO_4 72 % for 3 h at room temperature. Then, 2.2 mL of distilled water were added and incubated at 100 °C for 1 h. After the hydrolysis, the sample was diluted using 3 mL of distilled water.

Concentrations of galacturonic acid ranging from 0 to 80 µg/mL were prepared from a standard and each added to 3 test tubes (1 blank and 2 replicas). In the case of the sample, 0.5 mL were added to each of the 3 test tubes of the sample. All the tubes were placed in an ice bath and 3 mL of 200 mM sodium borate prepared in concentrated H_2SO_4 was added. The tubes were agitated and covered in aluminum foil before being placed for 10 min in a bath at 100 °C. After cooling the tubes in an ice bath, 100 µL m-phenylphenol (MFF) 0.15 % (w/v) prepared in sodium hydroxide (NaOH) 0.5 % (w/v) was added to 2 of the 3 tubes of each standard and sample. The tubes were stirred manually and let to react in the dark for 30 min. Then, the tubes were again stirred and the absorbances were measured at 520 nm,

using the tube without MFF as blank. The uronic acids concentration was determined using the calibration curve constructed with the standards.

2.3.3. Sulphate esters determination

Sulphate concentration was determined for the commercial fucoidan using the barium chloride–gelation method [95]. Gelatin solution was prepared by dissolving 1 g of gelatin in 200 mL of hot water (60 to 70 °C) and was left to rest overnight at 4 °C. The gelation solution was then divided equally by two cups and 0.5 g of barium chloride were added to one of the cups and left still for 2 to 3 h. Different solutions with SO_4^{2-}concentration ranging from 0.1 to 1 mg/mL were prepared from a K_2SO_4 standard solution. Regarding the samples, 2 to 4 mg were dissolved in 1 mL of 1 M HCl and incubated at 105 to 110 °C for 5 h. The tubes were cooled down before opening.

A volume of 0.2 mL of the samples or standards was transferred to test tubes (in duplicate) containing 3.8 mL of trichloroacetic acid 3 % (w/v) and 1 mL of barium chloride–gelatin was added. In the blanks the gelatin added did not contained barium chloride. The test tubes were agitated and transferred to a microplate were the samples rested for 15 to 20 min before the reading at 360 nm. Sulphate concentration was determined using the calibration curve constructed with the different concentrations of the standard solution.

For the samples extracted from *S. latissima* sulphate esters determination was made by elemental analysis. Two replicas of approximately 2 mg were analyzed in a Truspec 630-200-200 equipment using a furnace temperature of 1075 °C and afterburner temperature of 850 °C. The sulphate detection was made by infrared absorption.

2.3.4. Methylation analysis

For the methylation of the polysaccharides 1-2 mg of sample were weighted into tubes with a septum and left under vacuum overnight. Then, 1 mL of anhydrous dimethyl sulfoxide (DMSO) was added and left under agitation overnight. Approximately 40 mg of crushed NaOH were added and agitated for 30 min at room temperature. With a syringe, 80 µL of CH_3I were added and left with vigorous agitation for 30 min. The addition of CH_3I was performed two more times before the dissolution of the samples in 3 mL of a solution of $CHCl_3$:MeOH (1:1, v/v). Dialysis against a solution of equal parts of ethanol and distilled water was performed and the EtOH:H_2O was changed three times. The methylation procedure was repeated beginning in the addition of DMSO.

The methylated polysaccharides were hydrolyzed using 500 µL of TFA 2M at 121 °C for 1 h. The TFA was then evaporated in a speedvac. The reduction was performed by adding 300 µL of NH_3 2M and 20 mg of $NaBD_4$ and incubated at 30 °C for 1 h. The acetylation was performed as described for the neutral sugar analysis.

The partially methylated alditol acetates were dissolved in 30-50 µL of anhydrous acetone and 0.5-1 µL were injected in a GC-MS (SHIMADZU GCMS-QP2010 Ultra) equipped with a HT-5 column (30 m long, 0.25 mm diameter, 0.10 µm thickness). Helium was the carrier gas and had a flow rate of 1.84 mL/min. The temperature of the injector and detector was 250 °C. The initial temperature of the oven was 80 °C, followed by an increase of 7.5 °C/min until it reached 140 °C, which was maintained for 5 min, and a second increase of 15 °C/min until it reached 250 °C which was kept for 5 min. Linkages were identified based on their retentions time and mass spectra (Annexes Table 14).

To determine the sulphate group locations, desulphation as describe by Miller *el al.* [96] was carried out before methylation. In a glass tube, 10 mg of the polysaccharide was dissolved in 1.8 mL of DMSO. Then, 0.1 mL of pyridine, 0.013 g of pyromellitic acid, 0.012 g of sodium fluoride and more 0.2 mL of pyridine were added. The stirred mixture was left at 120 °C for 3 h and 1 mL of sodium hydrogen

carbonate ($NaHCO_3$) 3 % was added. The mixture was dialyzed in distilled water until the conductivity was the same as distilled water. After lyophilization the desulphated polysaccharide was subjected to methylation analysis and the results compared to the ones obtained with methylation without desulphation.

2.4. Protein content of extracts

Protein content of the samples of *S. latissima* and the commercial fucoidan was determined through elemental analysis. The content in nitrogen (% N) was converted to protein by multiplying the percentage of nitrogen by the conversion factor of 4.37 for *S. latissima* and 4.17 for commercial fucoidan [97]. For elemental analysis, a Truspec 630-200-200 equipment was used with a furnace temperature of 1075 °C and afterburner temperature of 850 °C, being the nitrogen detected by thermal conductivity. For each sample two duplicates were made with approximately 2 mg of sample.

2.5. Viscosity of polysaccharides

An Ostwald viscometer (Cannon-Fenske) size 50 with a constant of 0.004 mm^2/s^2 and viscosity range of 0.8-4 mm^2/s^2 was used to determine the kinematic viscosity of the polysaccharides. Solutions of each polysaccharide with concentrations ranging from 1 to 20 mg/mL were prepared in distilled water. The measurements were made with the viscometer immersed in a water bath at 25 or 37 °C. For each assay 10 mL of solution were inserted in the viscosimeter and allowed to reach the desired temperature before the solution being pulled to the top mark. The time needed for the solution to reach the bottom mark was counted and this procedure was repeated three times for each solution.

Density was calculated by measuring the mass of 1 mL of each solution at the temperature at which the experiments were made. Dynamic viscosity was obtained by multiplying the density by the kinematic viscosity.

2.6. Particles size of aggregates in the *in vitro* intestinal model

Size of particles in solution were measure by dynamic light scattering (DLS). Solutions used in the *in vitro* cholesterol solubility assays were filtered after preparation using a syringe and filters of 10 µm, 450 nm and 200 nm sequentially. The size of the aggregates present in the samples were determined using a Malvern Zetasizer Nano ZSP apparatus, using a scattering angle of 173°. Experiments were carried at 37 °C, considering a refraction index of 1.33 and a viscosity of 1.064 cP for commercial fucoidan, 2.361 cP for water-soluble chitosan and 0.684 cP for GDCA and laminaran solutions. Autocorrelation curves were fitted with two mono-exponential curves and the radius was obtained with the Stokes-Einstein equation [98].

2.7. Zeta-potential of chitosan sample

A solution of water-soluble chitosan 0.5 mg/mL was prepared in distilled water. Zeta-potential of the solution was measure using a Malvern Zetasizer Nano ZSP apparatus. The measures were performed at different values of pH (4-9.4) which was adjusted with the addition of alkaline solution of NaOH with a different range of concentrations enabling pH variation.

2.8. Diffusion Ordered Spectroscopy (DOSY)

DOSY was performed on 3 samples: i) GDCA; ii) GDCA and cholesterol; iii) GDCA, cholesterol and water-soluble chitosan. The experiments were made using

a Bruker 400 MHz NMR spectrometer. The diffusion time was 0.05 s, the gradient length was 0.07 s and the gradient strength varied from 0.68 to 32.35 G/cm (16 scans).

2.9. *In vitro* cholesterol solubility assays

In order to assess the potential of different polysaccharides in the reduction of cholesterol's solubility, mixtures of the polysaccharides with GDCA where added to [4-^{13}C] cholesterol films [91]. GDCA was used due to their prevalence in real scenario at intestinal lumen and because it is one of the bile salts that solubilize higher amount of cholesterol. Labelled cholesterol was necessary due to low concentration of cholesterol in comparation with GDCA and low natural abundance of ^{13}C.

To obtain a final concentration of 3.5 mM, the required volume of the solution of [4-^{13}C] cholesterol was poured in a glass tube and the chloroform:methanol (87:13, v/v) azeotropic solution evaporated by passing nitrogen over the heated solution. The tubes with the films were placed under a vacuum for 30 min at room temperature to remove traces of the organic solvents.

The GDCA solution was prepared in an aqueous buffer, to mimetize intestinal lumen conditions, containing Tris-HCl 10 mM (pH 7.4), NaCl 0.15M, ethylenediaminetetraacetic acid (EDTA) 1 mM and sodium azide (NaN_3) 0.02 % in deuterated water (D_2O), using 3-(Trimethylsilul)propionic-2,2,3,3-d4 acid sodium salt (TSP) as an internal standard for quantification. This solution was then pre-heated in a water bath at 37 °C and added to tubes containing the polysaccharides in a concentration of 5 mg/mL, being the cationic resin colestipol used as positive control. Then, the pre-heated mixture of the GDCA with the polysaccharide was added to the cholesterol films and these mixtures were left under agitation at 100 rpm, 37 °C for 24 h.

The amount of cholesterol solubilized and polysaccharides in solution was determined by NMR. ^{13}C NMR spectra were acquire using a 90° pulse, at 37 °C,

spectral width of 25252 Hz, acquisition time of 1.3 s, relaxation delay of 5 s and 2040 acquisition scan. Proton decoupling was accomplished by using a WALTZ-16 decoupling sequence. Nuclear Overhauser Enhancement (NOE) ^{13}C-^{1}H was obtained through the comparation between ^{13}C spectra with full proton decoupling and with proton decoupling only during acquisition. ^{1}H NMR spectra were acquired at 37 °C, with a 90° pulse, spectral width of 7500 Hz, acquisition time of 1 s, relaxation delay of 5 s and 128 acquisition scans. The NMR experiments were performed using a Bruker 500 MHz NMR spectrometer and spectra were processed using the software MestreNova 6.1.1 (Mestrelab Research, Santiago de Compostela, Spain).

Statistical significance was determined using analysis of variance (ANOVA) and Tukey's test with $\alpha=0.1$, using the Excel (Microsoft, Seattle, WA, America).

Chapter 3 – Results and Discussion

3.1. Marine origin polysaccharides structural characterization

A structural characterization was performed for the polysaccharides analyzed in the *in vitro* cholesterol solubility assays: a commercial fucoidan, a water-soluble chitosan, and the polysaccharides from *S. latissima* (laminarans and the extract containing the mixture of polysaccharides (laminarans+F1+F2)). The extract (Mixture S.I.) was further fractionated and the fractions analyzed, which gives a more accurate structural characterization of this fraction.

Neutral sugar analysis was performed using GC-FID for the commercial fucoidan and *S. latissima* polysaccharides and GC-MS for water-soluble chitosan. Sugars were identified based on their retention time and quantified using an internal standard. A typical chromatogram, obtained for commercial fucoidan as an example, is presented in Figure 11 with the sugars identification.

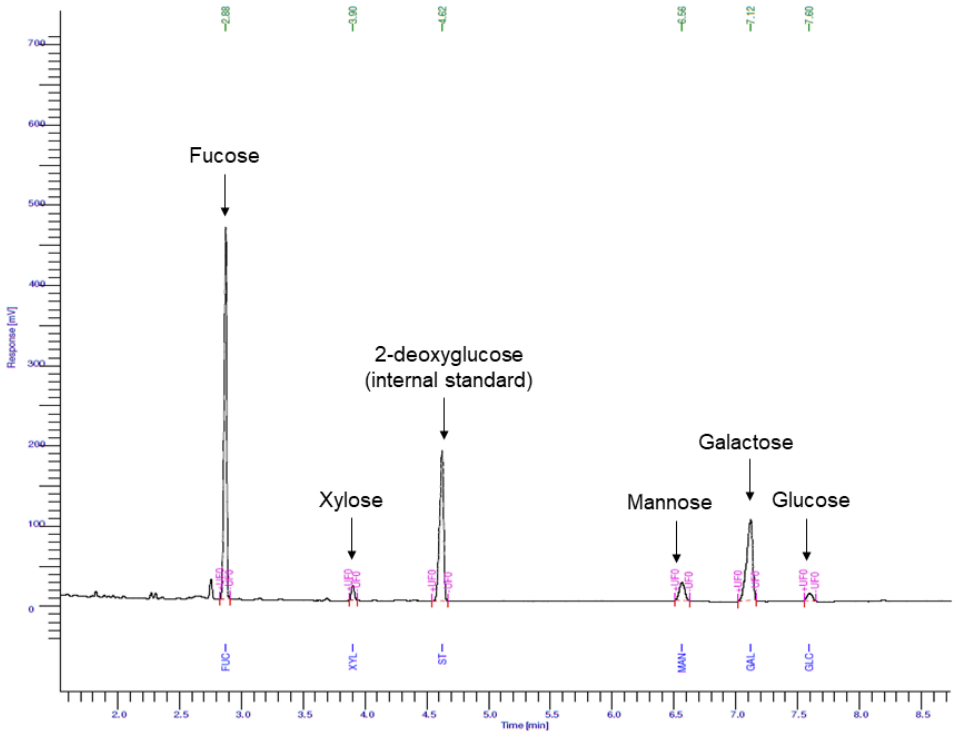


Figure 11. Chromatogram obtained in GC-FID for quantification of neutral sugars of commercial fucoidan. Sugars are identified in the chromatogram.

Different hydrolysis conditions were performed to the commercial fucoidan to determine the optimize condition for this type of polysaccharide. The differences in the sugar yields and sugars composition are shown in Table 6. The hydrolysis with H_2SO_4 1M yield a higher amount of total sugars (357 mg/g of sample), in comparison with TFA 1M (176 mg/g) and TFA 2M (275 mg/g), which indicates a higher efficiency of the hydrolysis. Comparing the hydrolysis with H_2SO_4 1M and TFA 2M, the last yield a lower amount of fucose, which can lead to differences in sugar compositions when performing neutral sugars and methylation analysis since different hydrolysis are used for these procedures. As the presence of sulphate groups can interfere with sugar hydrolysis, sugar analysis using hydrolysis with H_2SO_4 1M and TFA 2M were performed in desulphated FC (Table 7). After sulphate removal the yield per original sample was lower which indicates degradation of sugar residues during hydrolysis. A major discrepancy was found between fucose quantity using H_2SO_4 1M and TFA 2M since the last yield a lower amount of fucose, which shows that the hydrolysis H_2SO_4 1M is better to analyze fucoidans. These differences in sugar content and composition with hydrolysis conditions show that this step is crucial to optimize the analysis of sulphated polysaccharides.

Table 6. Neutral sugar composition w/w (mg/g sample) of commercial fucoidan using different hydrolysis conditions.

Hydrolysis	Fuc	Xyl	Man	Gal	Glc	Total
H_2SO_4 1 M, 100°C, 2.5 h	203.5 ± 5.9	10.4 ± 1.2	22.7 ± 2.0	111.0 ± 7.9	9.7 ± 1.7	357.3 ± 15.0
TFA 2 M, 121°C, 1 h	108.8 ± 1.12	9.1 ± 0.04	27.4 ± 12.64	115.4 ± 5.26	14.5 ± 5.91	275.2 ± 22.7
TFA 1 M, 121°C, 1 h	67.4 ± 2.5	6.8 ± 0.3	13.8 ± 2.0	80.7 ± 8.1	7.2 ± 1.0	175.9 ± 12.3

Table 7. Neutral sugar composition w/w (mg/g sample) of desulphated commercial fucoidan using different hydrolysis conditions.

Hydrolysis	Fuc	Xyl	Man	Gal	Glc	Total (mg/g desulphated sample)	Total (mg/g original sample)
H_2SO_4 1M, 100°C, 2.5 h	231.3 ± 22.5	16.7 ± 0.4	18.7 ± 2.0	179.5 ± 5.5	16.8 ± 0.6	463.1 ± 31.0	265.33
TFA 2M, 121°C, 1 h	152.0 ± 28.6	12.3 ± 1.8	18.0 ± 2.1	154.0 ± 30.2	11.2 ± 0.7	347.5 ± 59.2	233.12

The commercial fucoidan (FC) was dialyzed in a sleeve with a cutoff of 12-14 kDa. After hydrolysis conditions selection, both the native sample and the retained fraction were analyzed in terms of sugar composition and sulphate content. The native FC had high content of sugars (77 %, w/w), mainly composed by fucose (22 mol%), sulphate esters (48 mol%), uronic acids (15 mol%), and galactose (11 mol%), with low content of mannose (2 mol%), xylose (1 mol%), and glucose (1 mol%) (Table 8). This sample also revealed a low content in protein (31.1 mg/g). These results are consistent to the usual composition of fucoidans reported in literature which have high quantities of fucose, sulphate, and galactose, together with minor amounts of other monosaccharides [17,99]. Regarding the retained fraction obtained after dialysis, a slightly higher quantity of polysaccharides was determined (79.8 %) with an approximately similar sugar composition, except the lower mannose content (Table 8). These results allowed to infer that native FC had a high content of polysaccharide with high purity.

Table 8. Total sugar content (%, w/w) and neutral sugar composition, uronic acids and sulphate content (mol%) and percentage of native and dialyzed commercial fucoidan.

Commercial fucoidan	mol %							Total (%, w/w)
	Fuc	Xyl	Man	Gal	Glc	UA	SO_3	
Native	21.9 ± 0.6	1.2 ± 0.1	2.2 ± 0.2	10.8 ± 0.8	0.9 ± 0.2	15.3 ± 3.1	47.7 ± 0.7	77.2
Dialyzed	24.5 ± 4.6	1.0 ± 0.3	0.9 ± 0.4	12.2 ± 3.5	0.8 ± 0.2	17.5 ± 3.1	43.0 ± 1.4	79.8

The sugar compositions of the extract containing the mixture of polysaccharides from *S. latissima* (Mixture S.l.) and the fractions obtained after the anionic exchange chromatography are presented in Table 9. Mixture S.l. had a high content of sugars (80.3 %), mainly composed by glucose (57.2 mol%), probably due to the high content in laminarans, and also had fucose (15.3 mol%), sulphate (14.6 mol%), galactose (5.9 mol%) and uronic acids (5.6 mol%), due to the presence of fucans and fucoidans. This sample also had lower amounts of xylose (2.3 mol%), mannose (1.3 mol%) and ribose (0.8 mol%). This fraction also contains a low content of protein (112 mg/g). The fraction eluted from the column with buffer (without ionic force) had a total content of polysaccharides of 90.4 % consisting mainly of glucose (91.3 mol%), which is the building block of the laminarans [19].

Fraction eluted with NaCl 1M (F1) had a total content in polysaccharides of 46.7 % which had high quantities of uronic acids (34.1 mol%), fucose (26.4 mol%), and sulphate (18.7 mol%), together with minor amounts of ribose (3.5 mol%), mannose (4.7 mol%), glucose (5.6 mol%) and galactose (7.1 mol%). In addition, F1 also had protein (207 mg/g). The fraction eluted with the strongest ionic force (F2) showed a total content of polysaccharides of 61.6 %, which was composed by fucose (38.6 mol%), sulphate (36.8 mol%) and galactose (14.6 mol%), containing also uronic acids (7.0 mol%), mannose (1.6 mol%), glucose (1.1 mol%). A low content of protein was also detected in this fraction (62 mg/g).

The sugar composition of F2, with high percentage of fucose, sulphate and galactose, has a composition comparable to FC. However, F2 had higher amounts of fucose and lower content in uronic acids and sulphate. F1 differs from FC due to lower amount of fucose, sulphate and galactose.

Table 9. Total content of sugar (%, w/w) and of neutral sugar composition, uronic acids and sulphate content (mol%) and protein content (mg/g) of extract from brown algae (mixture S.l.) and the fractions separated in an anion exchange column (Laminarans, F1, and F2).

Fraction	η* (%)	mol %								Total Sugars (%, w/w)	Protein (mg/g)
		Fuc	Xyl	Man	Gal	Glc	Rib	UA	SO_3		
Mixture S.l.		15.3±1.2	2.3±0.3	1.3±1.2	5.9±04	57.2±0.6	0.8±0.1	5.6±5.0	14.6±0.1	80.3	112.0±0.8
Laminaran	29	1.3± 1.0	-	3.6±0.03	-	91.3±2.5	-	3.8±1.5	-	90.4	25.9±1.6
F1	19	26.4±0.1	-	4.7±0.1	7.1±0.1	5.6±0.4	3.5±0.1	34.1±0.1	18.7±1.7	46.7	207.4±2.2
F2	18	38.6±0.4	-	1.6±0.1	14.9±0.4	1.1±0.1	0.1±0.1	7.0±0.8	36.8±4.2	61.6	62.2±0.2

* Yield in relation to the amount of mixture S.l. inserted in the column.

Methylation analysis allowed, not only the determination of the glycosidic linkages present in the different polysaccharides, but also the location of sulphate groups based on the comparation between the original and the desulphated

samples. The species were identified based on retention time and mass spectra (Figure 12).

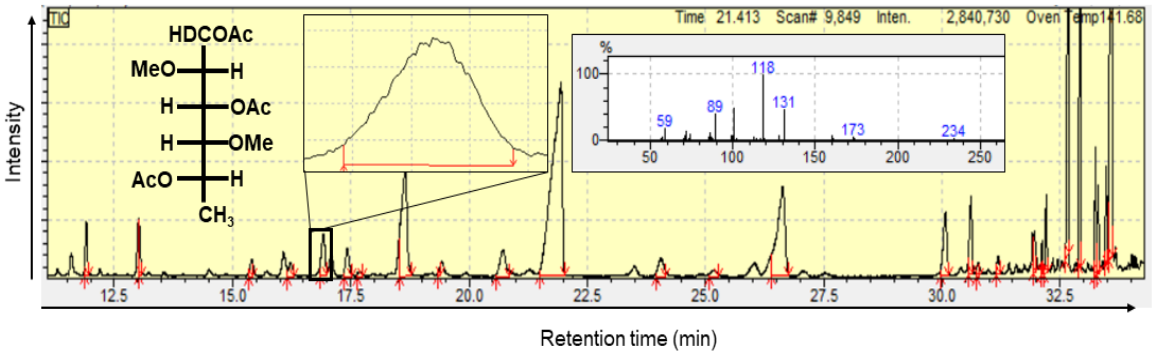


Figure 12. Chromatogram obtained for methylation analysis of commercial fucoidan. Insert shows an example of a mass spectra corresponding to the (1→3)-Fuc linkage (1,3,5-Tri-O-acetyl-1-deuterio-6-deoxy-2,4-di-O-methyl-L-galactitol).

FC showed an increase in terminal fucose residues, from 0.8 % to 5.1 %, after removal of the sulphate groups which indicates presence of sulphate in the terminal residue (Table 10). A decreased of (1→2,3,4)-Fuc was verified after desulphation together with an increase of (1→3)-Fuc, which indicates that sulphate groups were present in *O*2 and *O*4 of Fuc, and smaller increases in (1→4) and (1→2)-Fuc, which indicates presence of sulphates in *O*3, *O*2 or *O*4. The appearance of (1→2,3)-Fuc after desulphation indicates that this is a branching residue, that was sulphated at *O*4. Since fucoidans have usually (1→3)-Fuc linkages or (1→3) and (1→4)-Fuc linkages alternated [26], the (1→3,4)-Fuc residue can be (1→3)-Fuc with ramification at *O*4 or (1→4)-Fuc with ramification at *O*3. Regarding galactose linkages in FC, (1→6)-Gal was the major linkage after desulphation (19.8 %) and it also contained (1→3,6)-Gal followed by (1→3)-Gal, t-Gal and (1→4)-Gal (Table 10). FC has ramifications probably at *O*3 of (1→6)-Gal linkages (due to the presence of (1→3,6)-Gal) which can be sulphated at *O*2 and *O*4 due to the notorious reduction of (1→2,3,4,6)-Gal and (1→3,4,6)-Gal. The (1→6)-Gal linkages also contain sulphate groups at *O*3, *O*4 and/or *O*2 due to the reduction of the previously referred species, which is in agreement with the literature [29]. No major differences were observed after sulphate removal in glucose and mannose linkages which indicates low amount of sulphate in these sugar residues. Only terminal xylose was found

both in the original and desulphated FC which shows that there are not sulphate groups in these terminals. Overall, the number of terminals of all types of sugar residues of FC were significatively lower than the number of ramifications which may indicate the presence of uronic acids in the terminals. In literature is reported a linear backbone composed mainly of (1→6)-Gal with lower amounts of (1→2)-Man, (1→3) and (1→4)-Gal, and with branching consisting of (1→3) and/or (1→4)-Fuc, (1→4)-GlcA and t-Xyl [29]. This is in agreement with the linkages found in commercial fucoidan since its main linkage is (1→6)-Gal, it contains (1→3) and (1→4)-Gal linkages, (1→3)-Fuc, (1→4)-Fuc, and terminal xylose (Table 10) and it also has uronic acids.

F1 was composed mainly by uronic acids followed by fucose and hexoses (Table 9). Fucoidans with high quantity of uronic acids and hexoses usually have them in the backbones and the other sugars in the branches [26]. Terminal fucose was the major type of fucose linkage in F1 sample (Table 10), which indicates that t-Fuc may be linked to other sugars besides fucose, which is in agreement with literature [27] that reported terminal fucose linked to galactose residues in *S. latissima*. Besides t-Fuc, F1 also contains (1→2)-Fuc and (1→3)-Fuc in similar amounts and (1→4)-Fuc in minor amounts (Table 10), without relevant differences after desulphation, which mean that Fuc residues were not sulphated. F1 showed a decreased of (1→3,4,6)-Gal after desulphation together with an increase of (1→4)-Gal (Table 10), which indicates the presence of (1→4)-Gal linkages with sulphates in *O*3 and *O*6. (1→4)-Gal linkage is the main linkage (15.6 %) and some (1→3)-Gal and (1→6)-Gal were also present. The backbone of this fraction is probably constituted by uronic acids, (1→4)-Gal and (1→2)-Man. Differences before and after desulphation were not very noticeable for glucose and mannose residues which indicates low sulphate groups in these sugar residues. In relation to ribose, only terminal residues were found before and after desulphation, showing that these terminals are not sulphated. Furthermore, the presence of (1→3)-Glc can be due to the presence of laminarans that were also eluted in this fraction.

F2 had major quantities of t-Fuc followed by (1→3)-Fuc (Table 10). The presence of (1→3,4)-Fuc and (1→2,3)-Fuc could be due to the presence of a

backbone with (1→3)-Fuc linkages with ramifications in *O*4 and *O*2, respectively. This is in agreement with a fucan containing as main chain (1→3)-Fuc, reported for *S. latissima* [27]. Some minor quantities of (1→2)-Fuc and (1→4)-Fuc were also found in this fraction. F2 also showed a decrease in (1→3,4,6)-Gal but the major increase was in (1→6)-Gal linkages followed by a smaller increase of (1→3)-Gal, which indicates (1→6)-Gal sulphated at *O*4 and sulphated or branched at *O*3, together with some (1→3)-Gal linkages sulphated or branched in *O*4 and *O*6. These results agree with the structure of a fucoidan from *S. latissima* reported [27] which consisted of a backbone of (1→6)-Gal with branching at *O*4 and sulphate at *O*3. This (1→6)-Gal linkage sulphated at *O*3 has also been determined in other brown algae [28].

Few differences were found before and after removal of sulphate groups from fucose residues in F1 and F2, indicating low abundance of sulphate in these residues which is not expected because fucose residues usually contain one or two sulphate groups [26,27]. Structures reported for *Saccharina* genus have sulphate at *O*4 and/or *O*2 of the (1→3)-Fuc linkages [27,28,100,101]. In relation to the fucose linkages, F1 and F2 differ from the FC in terms of number of terminal and the FC has more ramifications and sulphates linked to fucose residues. Nevertheless, FC and F2 have higher amounts of (1→3)-Fuc. Galactose linkages of F2 and FC are similar due to the high presence of (1→6)-Gal, percentage of galactose terminals and presence of (1→3)-Gal, which differ from F1 since it has mainly (1→4)-Gal as described above. FC and F2 are the most similar fucoidans so FC was used in the cholesterol solubility assays due to higher amount of sample available.

Laminarans were mostly composed by glucose and had mainly (1→3)-Glc linkages (Table 10). The linkage (1→3,6)-Glc indicates ramification at C6 of the linear backbone of (1→3)-Glc, which is an agreement with the reported composition of laminarans [19]. Laminarans had high quantity of t-Glc (20 %) which indicates that this polysaccharide has small chains.

Table 10. Relative abundance of the partially methylated alditol acetates present before and after desulphation (DS) of commercial fucoidan (FC), laminarans (L), F1 and F2.

Sugar derivative	FC	FC DS	L	F1	F1 DS	F2	F2 DS
t-Fuc	0.8%	5.0%		9.7%	13.3%	11.1%	11.8%
2-Fuc	0.5%	2.6%		6.0%	2.9%	6.3%	1.2%
3-Fuc	1.7%	8.3%		4.8%	2.5%	4.4%	6.7%
4-Fuc	0.6%	2.7%		0.4%	1.5%	0.3%	3.2%
3,4-Fuc	7.2%	8.5%		2.3%	2.6%	2.5%	4.0%
2,4-Fuc	1.8%	1.7%					
2,3-Fuc		4.1%		1.4%		1.2%	3.9%
2,3,4-Fuc	22.7%	2.3%		0.7%	1.7%	1.1%	0.7%
Total Fuc	**35.3%**	**35.3%**		**25.3%**	**24.5%**	**26.9%**	**31.5%**
t-Gal	0.1%	5.7%		0.2%	1.9%	0.6%	5.1%
3-Gal		6.0%		2.6%	2.6%	2.6%	7.5%
4-Gal	7.8%	4.2%		4.2%	15.6%	4.6%	3.0%
6-Gal	2.2%	19.8%		3.8%	6.7%	4.1%	24.4%
3,4-Gal	10.9%			4.2%		4.1%	
2,3-Gal	0.4%	0.3%				0.9%	0.5%
3,6-Gal		7.7%		2.9%		3.3%	6.2%
2,6-Gal	1.3%						
3,4,6-Gal	7.1%			12.0%	3.9%	12.8%	1.8%
2,3,6-Gal	1.6%			0.2%		0.3%	0.5%
2,3,4,6-Gal	24.2%	3.2%		5.0%	10.0%	3.7%	2.2%
Total Gal	**55.7%**	**46.9%**		**35.1%**	**40.7%**	**41.3%**	**51.9%**
t-Glc	1.2%	2.3%	20.2%	3.3%	2.8%	2.7%	0.9%
3-Glc			64.5%	7.8%	4.2%	4.9%	2.0%
4-Glc	0.5%	2.2%		0.3%	0.5%	0.3%	1.7%
6-Glc		1.4%	4.7%				
2,4-Glc	0.4%	0.2%		3.7%	4.3%		
3,6-Glc			7.1%			1.3%	1.0%
4,6-Glc	0.8%						
2,3,4-Glc				0.4%	0.1%		
Total Glc	**2.9%**	**6.0%**	**96.5%**	**15.5%**	**11.9%**	**9.3%**	**5.6%**
t-Man	0.2%	0.5%		0.8%	1.3%	0.8%	0.5%
2-Man	1.2%	4.7%		4.7%	8.1%	5.2%	5.4%
4-Man			2.0%		0.2%		
6-Man		0.7%					
2,4-Man		0.9%	0.1%				
2,3-Man		1.2%	0.1%	0.8%	0.8%		1.8%
3,4-Man			0.1%				
3,6-Man		0.4%		1.6%	0.6%	1.4%	0.3%
4,6-Man	0.5%						
2,3,4-Man				1.2%			
2,3,6-Man	1.9%			4.7%		4.6%	0.9%
2,3,4,6-Man	1.1%	1.7%	1.1%	0.5%	1.8%	0.5%	0.3%
Total Man	**4.9%**	**10.0%**	**3.4%**	**14.3%**	**12.8%**	**12.4%**	**9.2%**
t-Rib				9.8%	10%	10.1%	1.8%
t-Xyl	1.3%	1.8%					

The water-soluble chitosan shown to be only composed by glucosamine (1080.4 ± 178.4 mg/g) and no neutral sugar were detected, which is in agreement to the literature [40]. The linkage analysis revealed the presence of (1→4)-GlcN (79 %) and t-GlcN (8 %), typical linkages of chitosan. The ratio between 4-GlcN and t-GlcN is near 10, meaning that chitosan has small size chains (average 10 residues) which is probably the reason for this polysaccharide to be soluble in water. However, (1→4)-Glc and t-Glc were also detected (Table 11). Since the neutral sugar analysis did not detected neutral sugars, the glucose present can come from the dialysis membrane that is constituted by cellulose acetate.

Table 11. Relative abundance (mol %) of glucose and glucosamine linkages present in water-soluble chitosan.

Sugar derivative	Water-soluble chitosan (mol %)
t-Glc	0.4 ± 0.2
4-Glc	12.2 ± 3.7
t-GlcN	8.4 ± 1.1
4-GlcN	79.0 ± 2.8

3.2. *In vitro* cholesterol solubility assays

The effect of the polysaccharides previously characterized, commercial fucoidan, polysaccharides extracted from brown algae (laminarans and mixture of polysaccharides), and chitosan, in the sequestration of GDCA bile salt and reduction of cholesterol emulsification were evaluate by quantitative NMR. Figure 13 shows a typical ^{13}C NMR spectra were several resonances of GDCA are identified.

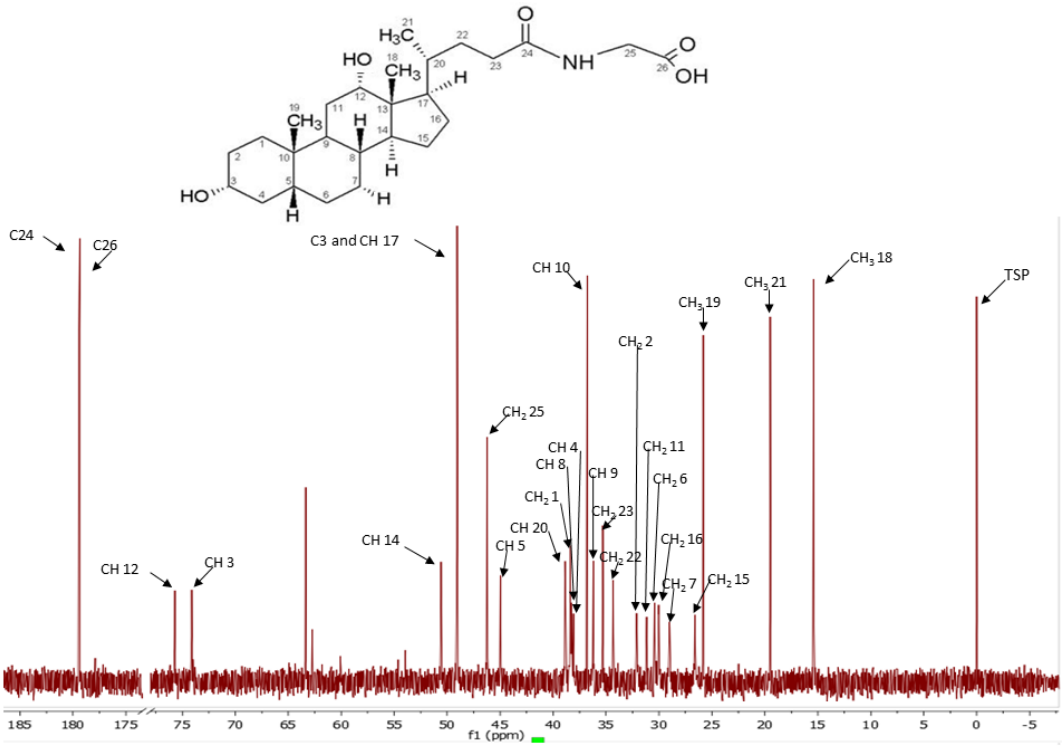


Figure 13. ^{13}C NMR spectra of GDCA with the resonances identified.

Cholesterol solubility was affected along with changes in GDCA content by the addition of the polysaccharides, namely chitosan (Figure 14), which is an indication of sequestration of bile salts. Moreover, a chemical shift in several resonance was also seen, indicating that changes in chemical environment are being sensed by the bile salts in the presence of the polysaccharides (Figure 14). Bile salts chemical shifts in the presence of β-glucans has been reported and indicates a direct interaction between polysaccharides and bile salts [75].

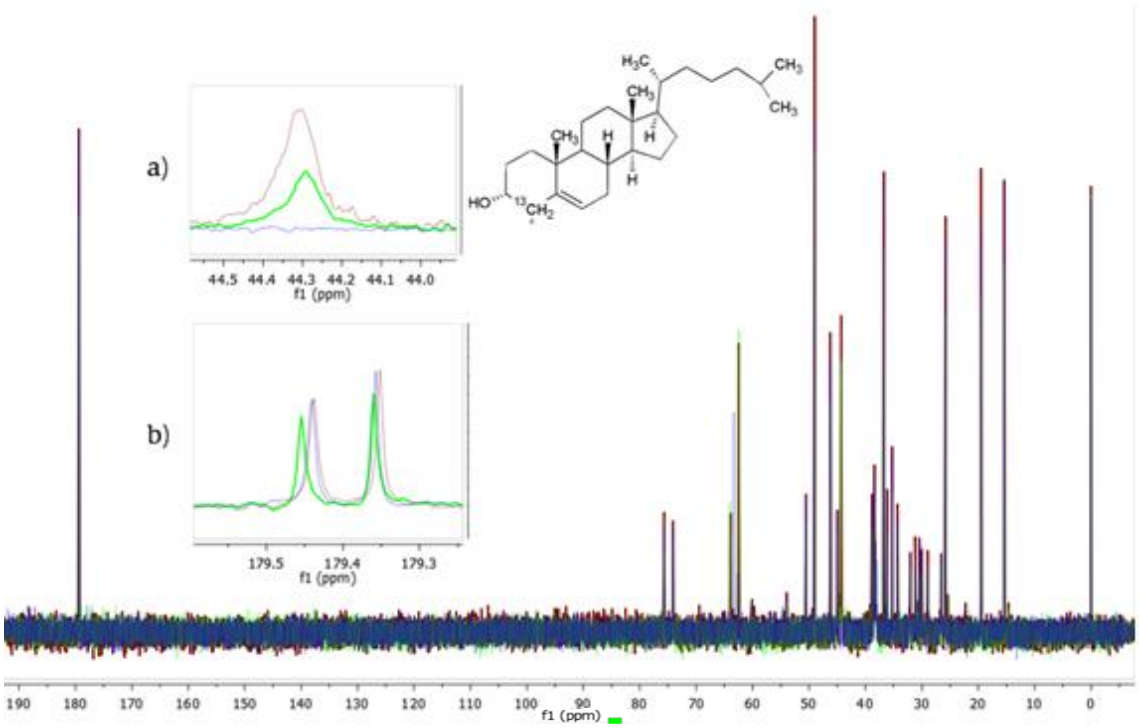


Figure 14. Superposition of GDCA (blue), GDCA with [4-^{13}C] cholesterol (red) and GDCA with [4-^{13}C] cholesterol and water-soluble chitosan (green) NMR spectra. Inserted graphs: a) Cholesterol peak at 44 ppm which decreases in the presence of water-soluble chitosan; b) Chemical shifts in the presence of water-soluble chitosan.

The water-soluble chitosan and FC showed to significatively decrease the amount of GDCA in solution compared to the control group (GDCA with cholesterol) (Figure 15). The other polysaccharides tested (Brown algae extract and Laminaran) revealed no statistical differences in comparison with control. Chitosan was the most effective polysaccharide and its ability to sequestrate GDCA was not statistically different from the cationic resin (a positive control). Fucoidan (FC) was statistical less effective than the chitosan and the cationic resin. Cholesterol solubility decreased significatively in the presence of the water-soluble chitosan and commercial fucoidan (Figure 16). The quantity of solubilized cholesterol was proportional to the amount of GDCA in solution (Figure 15), which indicates that the mechanism behind the reduction of cholesterol solubilization is the sequestration of bile salts by the polysaccharides.

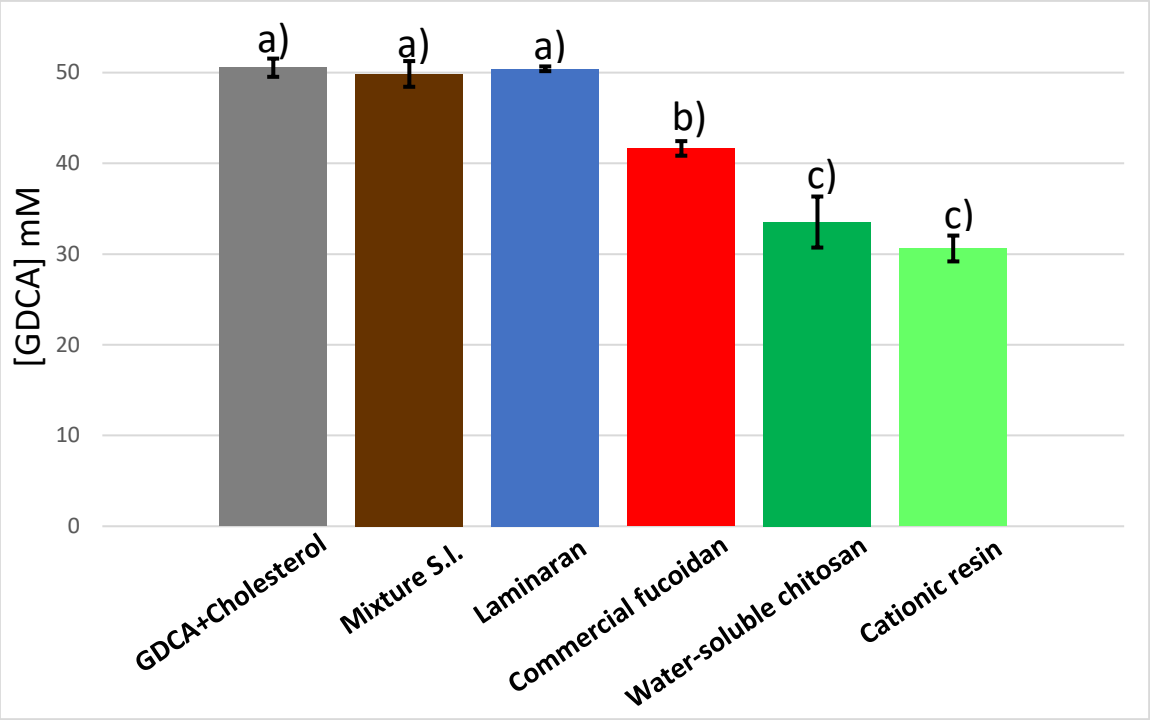


Figure 15. Sequestration of bile salts by the polysaccharides studied. Different letters indicate significant statistical difference between groups with α=0.1.

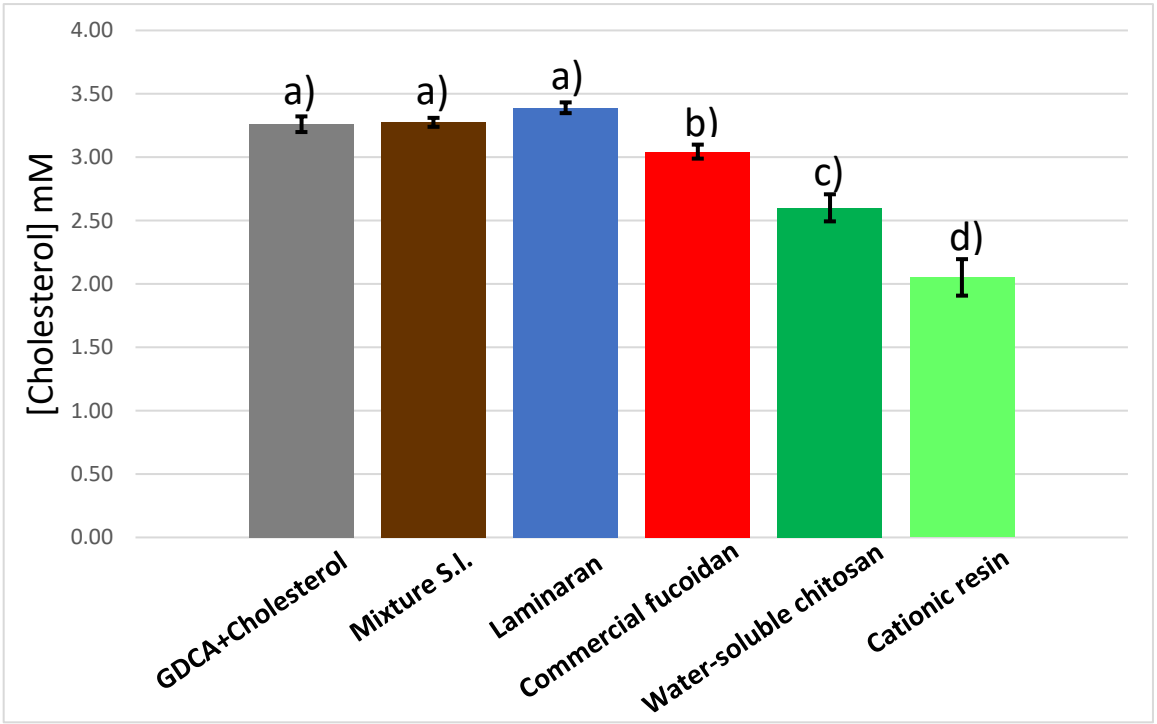


Figure 16. Reduction of cholesterol solubilized in the presence of different polysaccharides. Different letters indicate significant statistical difference between groups with α=0.1.

Commercial fucoidan as shown a significant effect in the reduction of GDCA in solution compared with the control group besides its negative charge. However, polysaccharides composed by uronic acids have been reported to interact with bile acids despite their anionic charge [76,90,102], possibly due to interaction between hydrophobic motifs. Therefore, it is also expected that the effect of the commercial fucoidan could be based on this type of interaction. As F2 and commercial fucoidan have some structural similarities (Tables 9 and 10), it is probable that F2 also has the capacity to sequestrate bile salts. Since F1 has even fewer negative charges (due to be eluted with a weaker ionic strength and have a lower content of sulphate groups compared to F2) it is expected to have a better effect, since it has less repulsive charges.

Laminarans did not shown effect neither in the sequestration of GDCA nor cholesterol solubility. Although other polysaccharides containing glucose, namely the β-glucans, have been known to decrease cholesterol solubility the structure is different. β-glucans which showed hypocholesterolemic activity have (β1→4)-Glc in addition of (β1→3)-Glc linkages, whereas laminarans have (β1→3) and (β1→6)-Glc. Furthermore, glucans are expected to be more viscous than laminarans since the last have short chains. Furthermore, the extract from the brown algae *S. latissima* (Mixture S.l.) did not shown effect on cholesterol solubility since has a high content of Glc (57 mol%) comparing with Fuc (15 mol%), indicating that is mainly constituted by the laminarans (Table 9).

Since the negatively charged fucoidan sequestrated bile salts it was expected that laminarans (neutral) also had potential to interact with bile salts, however the laminarans did not showed effect on the sequestration of bile salts. One hypothesis that could explain the effect of the fucoidan is that its negative charges and hydroxyl groups will face towards water forming a hydrophobic cavity more exposed which could allow the interaction with both amphiphilic and hydrophobic character of bile salts, contrary to laminarans which showed that bile salts preferred to be in solution probably as a reflection of the hydrophobic core being less exposed.

Water-soluble chitosan was the most promising polysaccharide to lower cholesterol emulsification, being the effect similar to the obtained with the cationic resin used as positive control. This effect can be due to the positive charges of the chitosan which can facilitate the sequestration of negatively charged bile salts. In accordance with these results, studies have reported the bile salt sequestrating effect of chitosan and showed that better results were obtained using chitosans with smaller particle sizes [44] and smaller molecular weight [45].

3.3. Possible hypocholesterolemic mechanisms

Several experiments were carried out to understand other possible hypocholesterolemic mechanisms of the polysaccharides under study. The particles size of laminarans, commercial fucoidan, and water-soluble chitosan were determined. The viscosities of the two most promising polysaccharides for decreasing cholesterol solubility, commercial fucoidan and water-soluble chitosan, were measured and the polysaccharide with higher viscosity was used in DOSY assays to determine if diffusion coefficients of the species in solution was altered. Moreover, the chitosan's charge at physiological pH was analyzed by zeta-potential, to evaluate if electrostatic interactions between polysaccharide and bile salt can be one mechanism of the effect on cholesterol homeostasis.

3.3.1. Particles size

The particles size was obtained by DLS, through analysis of autocorrelation curves which were fitted with two mono-exponential equations (Figure 17). The mean size of the particles in the major populations (over 94 %) of the samples are present in Table 12. Major population of GDCA and GDCA plus cholesterol solutions had a mean diameter of 4.14 and 3.8 nm, respectively. These values correspond to the bile salts micelles and are in agreement with the 2.2 to 4.2 nm of diameter reported in the literature for these kind of micelles [91,103,104]. The size of micelles

in the presence of cholesterol do not show a significant difference when compared with its absence, which is in agreement with the literature [91]. The sizes obtained in the samples of commercial fucoidan and laminaran are close to the ones determine for the micelles (4.46 and 4.86 nm, respectively). In the case of water-soluble chitosan, the average size determined was lower than bile salt micelles size, which could be due to a high content of polysaccharide species that can be masking the small content of bile salt micelles which have a similar size. The approach used assumes that the species are spheres. However, micelles are oblates and polysaccharides can have a more linear structure. The results obtained are an approximation to their size and therefore the methodology is not optimized to calculate non-spherical particles size. Although the micelles size determined was in agreement to the literature, the value obtained for the water-soluble chitosan did not correspond to the micelles size.

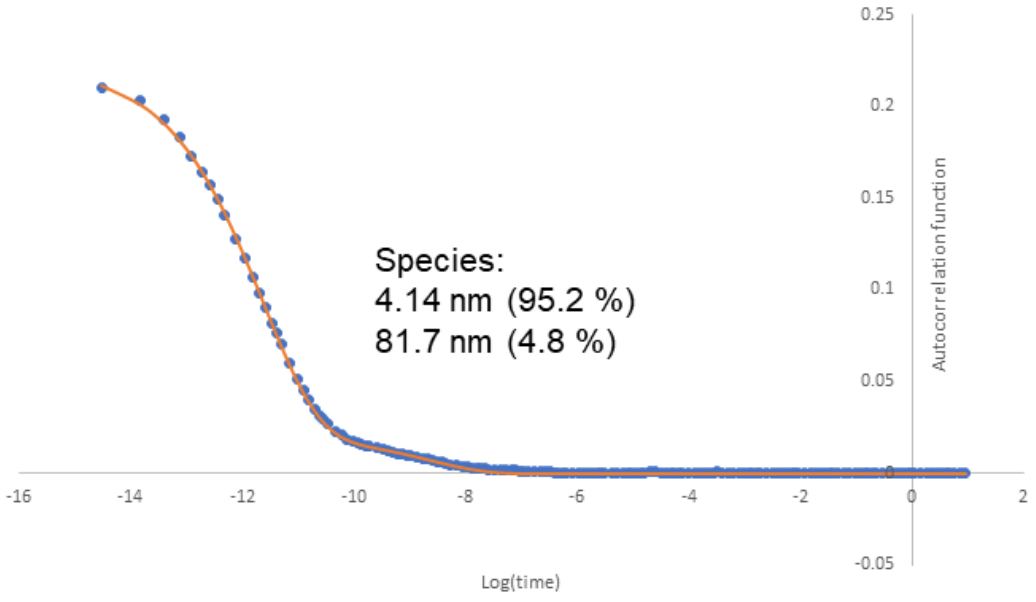


Figure 17. Example of an autocorrelation curve obtained for the determination of particles size of a GDCA sample.

Table 12. Major population diameter obtained for the different samples analyzed.

Sample	Diameter (nm)
GDCA	4.14 ± 0.01
GDCA + cholesterol	3.8 ± 1.0
Commercial Fucoidan + GDCA + cholesterol	4.46 ± 0.06
Laminaran + GDCA + cholesterol	4.86 ± 0.6
Chitosan + GDCA + cholesterol	1.12 ± 0.34

3.3.2. Viscosity

Dynamic viscosity measures the fluid's resistance to flow when an external force is applied. Kinematic viscosity measures the fluids inherent resistance to flow without the action of an external force, except gravity, and it is obtained by dividing the dynamic viscosity by the density.

Kinematic (Figure 18) and dynamic viscosities (Figure 19) were evaluated for FC and chitosan. It was not possible to determine viscosities of the polysaccharides from *S. latissima* due to the insufficient amount mass of sample available. At the concentration and temperature used in the solubility assays (5 mg/mL at 37 °C), the kinematic and dynamic viscosities of FC were 1.04 mm^2/s and 1.06 cP, respectively, whereas the water-soluble chitosan had kinematic (2.25 mm^2/s) and dynamic viscosities (2.36 cP) two times higher than FC.

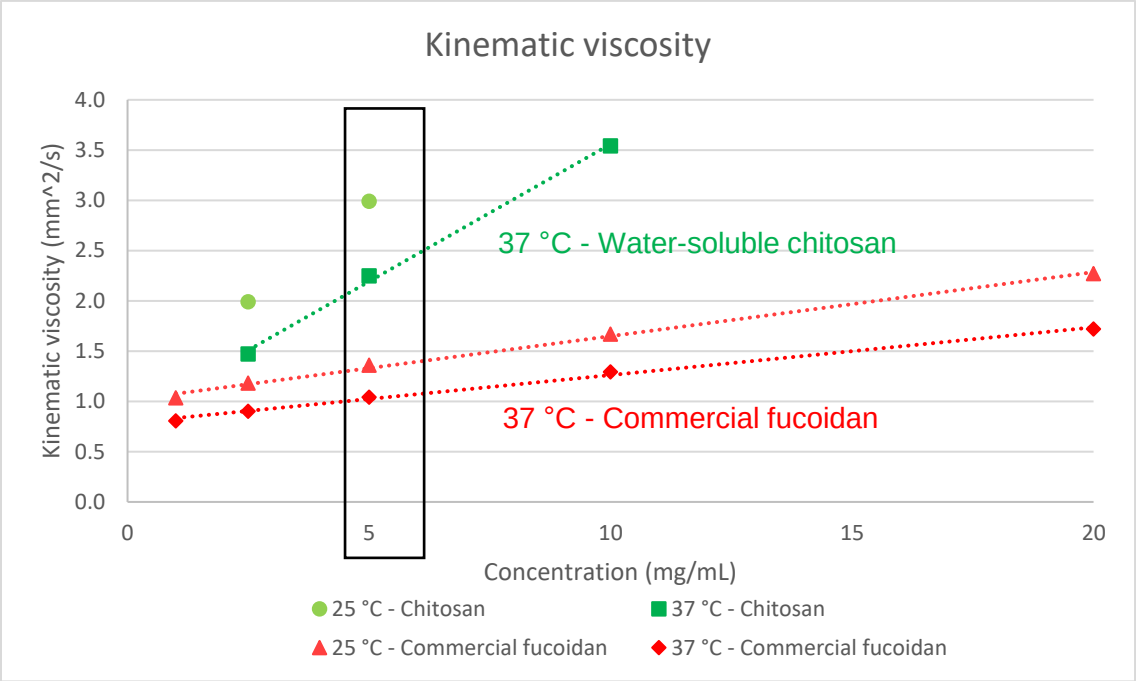


Figure 18. Kinematic viscosity of commercial fucoidan and water-soluble chitosan obtained for different concentrations at 25 and 37 °C.

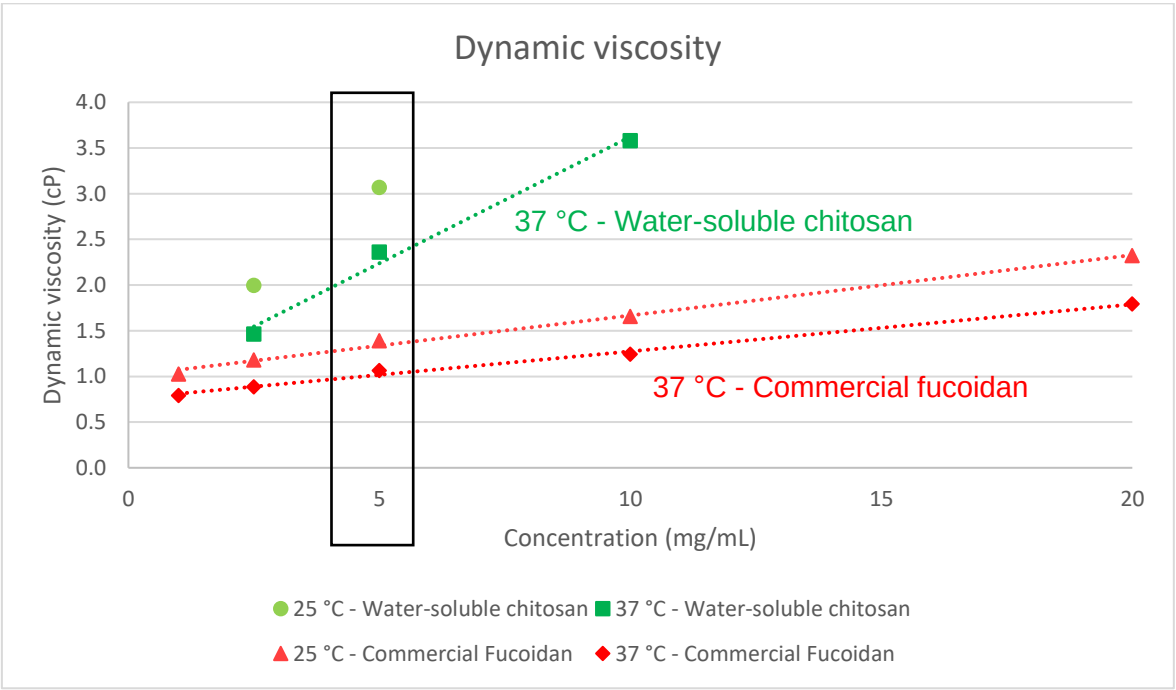


Figure 19. Dynamic viscosity of commercial fucoidan and water-soluble chitosan obtained for different concentrations at 25 and 37 °C.

The literature describes the fucoidans solutions has having a low viscosity comparing to other polysaccharides namely alginate, which seem to be affected by molecular weight, sulphate content and branching degree [105]. On the other hand, the dynamic viscosity of a chitosan with similar molecular weight (1.5 kDa) has been reported as 1.2 cP (25 °C, 0.5 %) [106], which is lower than the 3.0 cP obtained for the water-soluble chitosan studied in the same conditions. This difference may arise from the different solvents used, since 1.2 cP was obtained with samples diluted in aqueous acetic acid solution and the water-soluble chitosan used in this work was dissolved in distilled water.

Several studies have related higher viscosity with a higher capacity of restriction mobility of bile salts [107,108], even in polysaccharide with negative charge [76]. At the conditions used in the solubility assays the chitosan had twice the viscosity of the commercial fucoidan. This higher viscosity of the chitosan could make changes in the environment and decrease mobility of species at intestinal lumen. In order to evaluate this effect, DOSY assays were carried using this polysaccharide to evaluate their effect on the coefficient of diffusion of the different species in solution.

3.3.3. Diffusion Ordered Spectroscopy (DOSY)

DOSY experiments were performed with and without the presence of the water-soluble chitosan to compare the diffusion coefficients of the different species mimetizing intestinal lumen content. If diffusion coefficients of bile salts are altered by the presence of polysaccharides it is an indication that the mobility is restrained. DOSY spectrum of the GDCA in the buffer allowed to determine the diffusion coefficients of water, buffer solution, TSP and GDCA (Figure 20). The different compounds were identified by the proton spectra.

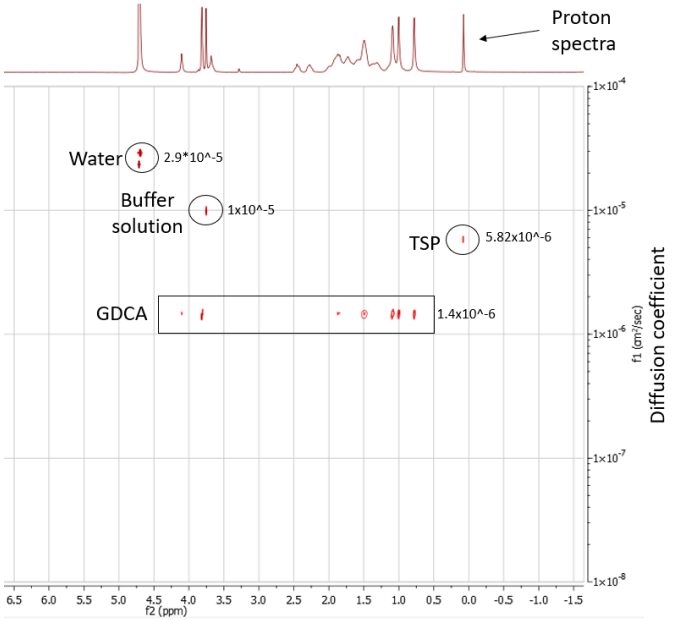


Figure 20. DOSY spectrum of GDCA in buffer solution. The different species present in solution and their respective diffusion coefficient are identified.

DOSY spectrum of GDCA and cholesterol in buffer solution was also performed (Figure 21). No specific resonance allowed the cholesterol identification. However, as cholesterol is solubilized in bile salt micelles, its diffusion coefficient should be similar to the one obtained for GDCA.

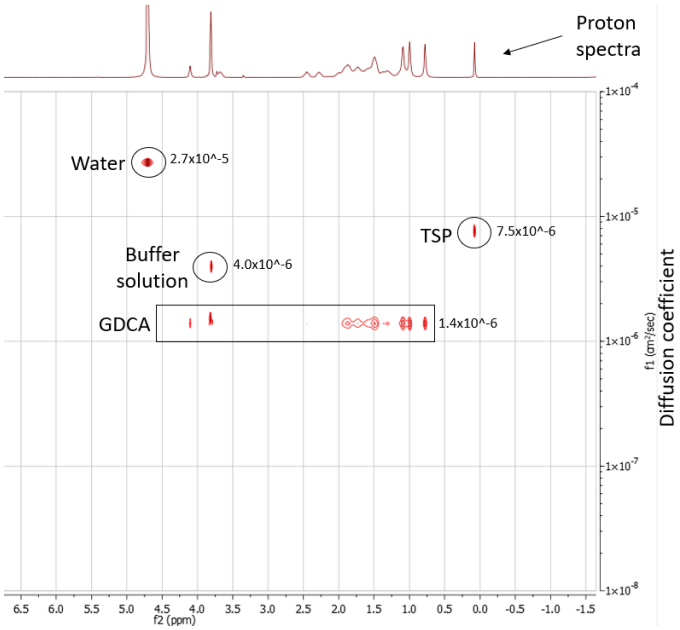


Figure 21. DOSY spectrum of a solution of GDCA and cholesterol in buffer solution. The different species present in solution and their respective diffusion coefficient are identified.

The DOSY spectrum of water-soluble chitosan, GDCA, cholesterol and buffer showed a specie with a distinct diffusion coefficient that was not present in the previous experiments (Figure 22). This new specie was identified as the water-soluble chitosan and had a diffusion coefficient of $2.2x10^{-6}$ cm^2/s.

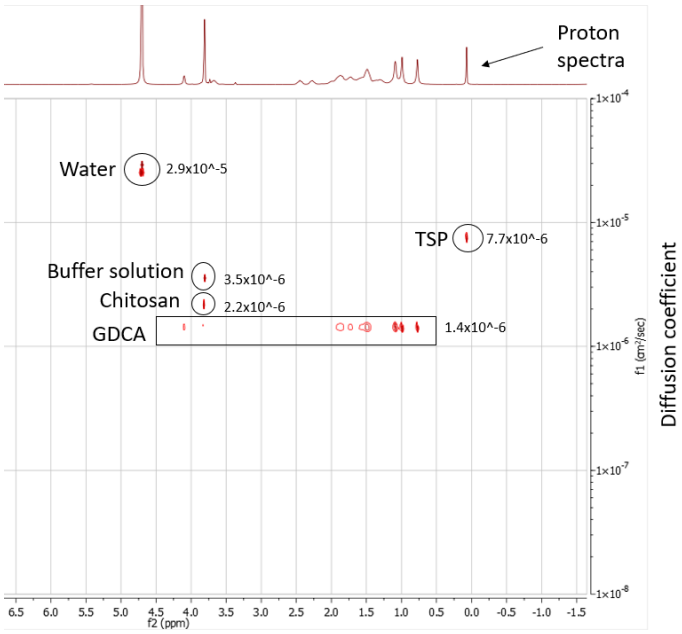


Figure 22. DOSY spectrum of a solution of GDCA, cholesterol and water-soluble chitosan in buffer solution. The different species present in solution and their respective diffusion coefficient are identified.

The diffusion coefficients obtained for the different species are present in Table 13. The diffusion coefficient obtained for GDCA ($1.4x10^{-6}$ cm^2/s) is in agreement with the $1.5x10^{-6}$ cm^2/s reported by Oh *et al.* [103]. Considering that the chitosan used in this study had on average of 10 units of glucosamine per chain (determined by methylation analysis, Table 11) and each glucosamine unit has a molecular weight of 179.17 g/mol, the water-soluble chitosan has a molecular weight of 1792 g/mol. The expression used by Kuroiwa *et al.* [109] gives the diffusion coefficient (D) by multiplying $8.76x10^{-9}$ to the molecular weight (M) raised to -0.48 ($D = 8.76x10^{-9}xM^{-0.48}$). The substitution of the molecular weight of water-soluble

chitosan in the expression resulted in a diffusion coefficient of $2.4x10^{-6}$ cm^2/s, in agreement with the experimental value of $2.2x10^{-6}$ cm^2/s.

Table 13. Diffusion coefficients obtained for each specie in the different DOSY experiments.

	Diffusion coefficient (cm^2/s)			**Literature values**
Mixture / **Specie**	GDCA	GDCA + Cholesterol	GDCA + Cholesterol + Water-soluble chitosan	
Water	$2.9x10^{-5}$	$2.7x10^{-5}$	$2.9x10^{-5}$	$2.7x10^{-5}$ cm^2/s [110]
TSP	$5.8x10^{-6}$	$7.5x10^{-6}$	$7.7x10^{-6}$	
Buffer	$1x10^{-5}$	$4x10^{-6}$	$3.5x10^{-6}$	
GDCA	$1.4x10^{-6}$	$1.4x10^{-6}$	$1.4x10^{-6}$	$1.5x10^{-6}$ cm^2/s [103]
Water-soluble chitosan			$2.2x10^{-6}$	$2.4x10^{-6}$ cm^2/s*

*Value calculated from the formula in Kuroiwa *et al* [109] considering 10 units of glucosamine per chain (molecular weight = 1791.7 g/mol).

In the presence of chitosan the diffusion coefficient of GDCA remained similar ($1.4x10^{-6}$ cm^2/s). This result allowed to infer that the viscosity of the water-soluble chitosan at the concentration used is not enough to decrease the diffusion of the species in solution. Nevertheless, the presence of chemical shifts indicates interaction between the bile salts and the water-soluble chitosan which could be due to the interaction between hydrophobic or electrostatic interaction. To verify this late hypothesis the charge of water-soluble chitosan at the physiologic pH was evaluated by zeta-potential analysis.

3.3.4. Zeta-potential

Zeta-potential was performed for the water-soluble chitosan with a concentration of 0.5 mg/mL at different pH values. It was observed that the zeta-potential decrease with the pH increment (Figure 23). At pH 7.4, the pH of the *in vitro* cholesterol solubility assays, the polysaccharide still was positively charged (Figure 23). It was also possible to estimate the pKa of the chitosan by calculation

the medium point of the curve that was 6.4, which is in agreement with the values from 6.17 to 6.51 reported in the literature [111]. In this assay the polysaccharide concentration was 10 times inferior to the one used in the cholesterol solubility assays. In these assays, it is expected that the zeta potential was higher due to the higher number of positive charges present in the solution [112], since the concentration is higher.

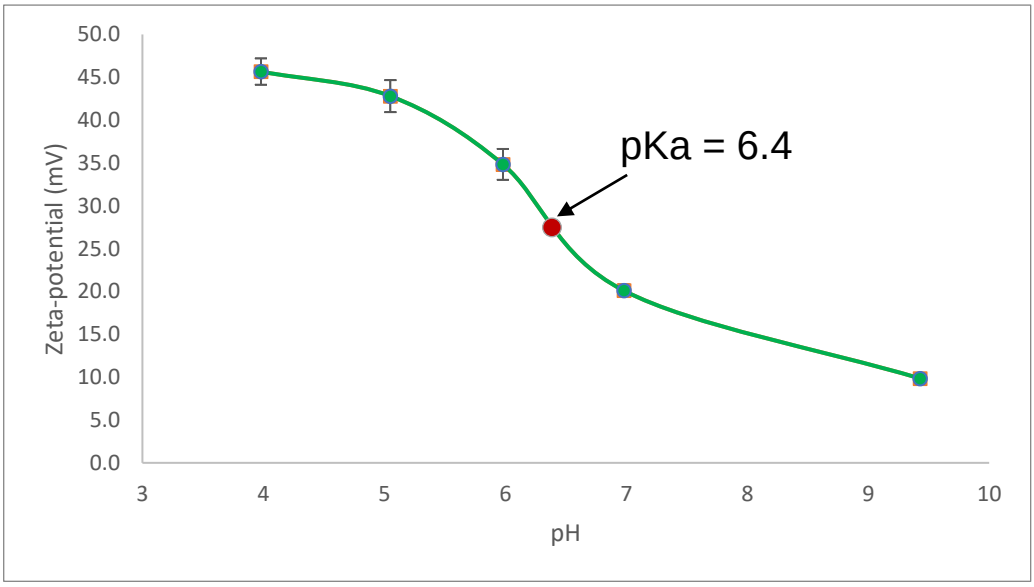


Figure 23. Zeta-potential of water-soluble chitosan.

GDCA has a pKa of 4.77 [113] so at pH 7.4 it is negatively charged. Since the chitosan still retains positive charge at pH 7.4, it can interact electrostatically with the negative charges of the GDCA, leading to a sequestration and consequent reduction of the cholesterol emulsification.

Chapter 4 – Conclusions

4. Conclusions

In this work polysaccharides from marine origin with different structural characteristics were evaluated as potential agents to affect cholesterol bioaccessibility. The polysaccharides used were: 1) commercial fucoidan; 2) water-soluble chitosan; 3) laminarans; and 4) extract from *S. latissima* with a mixture of laminarans and fucose-containing sulphated polysaccharides. The laminarans extracted from brown algae were mainly composed of (1→3)-Glc with some ramifications (1→3,6)-Glc, with high content of terminals revealing small polymeric chains. Commercial fucoidan had (1→3)-Fuc, with sulphate at *O*2 and *O*4, and (1→3,4)-Fuc as the major fucose linkages. Furthermore, fucoidan was also constituted by (1→6)-Gal, (1→3,6)-Gal and (1→3)-Gal, with sulphates at *O*2, *O*4 and *O*3/*O*6. The fucose-containing sulphated polysaccharides extracted from brown algae and obtained with a higher ionic strength in the anionic exchange chromatography (F2) was the most similar in terms of structure (sugar composition, and glycosidic linkage) to commercial fucoidan. The fucoidan obtained with a lower ionic strength (F1) has a distinct structure comparing with the other fucoidans (commercial and F2), since the major linkage was (1→4)-Gal and had lower galactose terminals content. Both F1 and F2 did not show the presence of sulphate esters in the fucose residues in contrast with the commercial fucoidan. The water-soluble chitosan was composed of small chains of glucosamine (approximately 10 residues), explaining the solubility in water. This set of characterized polysaccharides allowed to have different structures and properties, namely charge and sugar composition, that could explain the effect on cholesterol bioaccessibility.

In vitro cholesterol solubility assays demonstrated that water-soluble chitosan was the most promising polysaccharide followed by commercial fucoidan. However, the fraction enriched in laminarans and the extract containing the mixture of polysaccharides from *S. latissima* (mainly composed by laminarans) did not show a significant effect. As the fraction of *S. latissima* enriched in fucoidan (F2) was structurally similar to the commercial fucoidan, which had shown a significant effect on cholesterol solubility, allow to reinforce that this fraction should be explored as hypocholesterolemic agent in future. In addition, since F1 was eluted with weaker

ionic strength, it has fewer negative charges than F2, highlighting that this fraction can have also potential as sequestration agent due to its lower negative character.

The viscosity of the solutions can affect diffusion of molecules from distal intestinal lumen epithelium, limiting the available soluble cholesterol in micelles to be absorbed at enterocytes. The water-soluble chitosan was more viscous than the commercial fucoidan at the concentration and pH used for the *in vitro* cholesterol solubility assays. However, DOSY experiments showed no differences in the diffusion coefficients of the species in solution used in the intestinal *in vitro* model, indicating that viscosity of the chitosan was not enough to decrease species diffusion. Therefore, this mechanism of action did not explain the effect in cholesterol solubility by chitosan. However, water-soluble chitosan at the physiological pH used in the cholesterol solubility assays still had positive charge character. This supports that one of the mechanisms that explain the higher effect of this polysaccharide is the interaction between its positive charge and the negative charge of bile salts. Furthermore, the hydrophobic interaction should not be discarded due to changes in chemical shifts observed in several resonances of bile salts in presence of the polysaccharides. Moreover, since commercial fucoidan is negatively charged its interaction with the bile salts should be most likely due to interaction between hydrophobic motifs which should not occur in the case of the neutral polysaccharide laminaran, highlighting that polysaccharide structures (sugar composition and branching degree) have an impact on the interaction with bile salts.

Water-soluble chitosan capacity of sequestration was close to the capacity demonstrated by the cationic resin, which demonstrates the potential of chitosan soluble in water to be used as nutraceuticals with hypocholesterolemic effect in future. Although in less extent fucoidan also shown to have hypocholesterolemic effect reinforcing the possibility of the use of polysaccharides obtained from algae as hypolipidemic agents.

Chapter 5 – Future Works

5. Future works

This work focused on cholesterol bioaccessibility however cholesterol metabolism and homeostasis are complex.

Since commercial fucoidan showed potential as hypocholesterolemic agent and F2 had structural similarities with this polysaccharide, F2 could also interact with the bile salts. Furthermore, F1 has fewer negative charges which could lead to a better sequestration of bile salts. This way, both F1 and F2 from *S. latissima* should be tested on their potential to sequestrate bile salts and decrease cholesterol emulsification *in vitro*,

The next step would be investigating if these polysaccharides influence cholesterol's bioavailability *in vitro*. One away to evaluate cholesterol's bioavailability would be using cell lines, such as Caco-2, to evaluate if cholesterol can cross the cell layer in the presence of determine polysaccharides.

In this work were not considered polysaccharide's modifications that could arise from digestion. The passage of the polysaccharides through the human digestive system can lead to alterations due to interaction with gastric enzymes and microbiome. The *in vitro* testing of cholesterol solubilization reduction of modified polysaccharides would be more similar to what happens in the human system.

Also, testing a wider range of polysaccharides would help to understand which characteristics are more efficient in lowering cholesterol absorption.

Chapter 6 – References

6. References

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Chapter 7 – Annexes

7. Annexes

Table 14. Partially methylated acetate alditols (PMAAs) and corresponding linkage patterns. Fragments that allowed the identification of the PMAAs are also present.

Partially methylated acetate alditols (PMAAs)	Fragments (diagnostic ions, m/z)	Linkage patterns
1,5-di-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-ribitol	118, 117, 101, 102, 162	t-Rib
1,5-di-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-xylitol	101, 102, 118, 117, 161, 162	t-Xyl
1,5-di-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-fucositol	118, 115, 102, 131, 89, 175	t-Fuc
1,4,5-tri-O-acetyl-1-deuterio-3,4-di-O-methyl-fucositol	131, 89, 130, 100, 115, 190	2-Fuc
1,3,5-Tri-O-acetyl-1-deuterio-6-deoxy-2,4-di-O-methyl-L-galactitol	118, 131, 101, 89, 16, ,234	3-Fuc
1,4,5-tri-O-acetyl-1-deuterio-2,3-di-O-methyl-fucositol	118, 101, 143,102, 203	4-Fuc
1,3,4,5-tetra-O-acetyl-1-deuterio-2-O-methyl-fucositol	118, 87, 113, 99, 129, 173	3,4-Fuc
1,2,4,5-tetra-O-acetyl-1-deuterio-3-O-methyl-fucositol	143, 130, 101, 88, 190, 203	2,4-Fuc
1,2,3,5-tetra-O-acetyl-1-deuterio-6-deoxy-4-O-methyl-L-galactitol	131, 128, 89, 262, 202	2,3-Fuc
1,2,3,4,5-penta-O-acetyl-1-deuterio-fucositol	129, 171, 115, 99, 231	2,3,4-Fuc
1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-glucitol	102, 118, 129, 145, 162, 205	t-Glc
1,2,5-tri-O-acetyl-1-deuterio-3,4,6-tri-O-methyl-D-glucitol	129, 130, 87, 161, 190	2-Glc
1,3,5-tri-O-acetyl-1-deuterio-2,4,6-tri-O-methyl-D-glucitol	118, 129, 101, 161, 234	3-Glc
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-1-deuterio-glucitol	118, 113, 99, 102, 233, 129	4-Glc
1,5,6-tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-glucitol	102, 118, 129, 99, 87, 189	6-Glc
1,2,4,5-tetra-O-acetyl-1-deuterio-3,6-di-O-methyl-D-glucitol	130, 113, 87, 190, 233	2,4-Glc
1,3,5,6-tetra-O-acetyl-1-deuterio-2,4-di-O-methyl-D-glucitol	118, 129, 87, 189, 234	3,6-Glc
1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-1-deuterio-D-glucitol	118, 102, 99, 127, 261	4,6-Glc

1,2,3,4,5-penta-O-acetyl-1-deuterio-6-O-methyl-D-glucitol	129, 115, 87, 140, 157, 185	2,3,4-Glc
1,5-di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-mannitol	102, 129, 145, 162, 161, 205	t-Man
1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-1-deuterio-D-mannitol	129, 130, 161, 88, 87, 190	2-Man
1,4,5-tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-mannitol	118, 102, 113, 233	4-Man
1,5,6-tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-mannitol	102, 118, 129, 99, 189, 162	6-Man
1,2,4,5-tetra-O-acetyl-1-deuterio-3,6-di-O-methyl-D-mannitol	130, 190, 87, 113, 88, 233	2,4-Man
1,2,3,5-tetra-O-acetyl-1-deuterio-4,6-di-O-methyl-D-mannitol	129, 161, 101, 128, 262	2,3-Man
1,3,4,5-tetra-O-acetyl-1-deuterio-2,6-di-O-methyl-D-mannitol	118, 129, 87, 185, 305	3,4-Man
1,3,5,6-tetra-O-acetyl-1-deuterio-2,4-di-O-methyl-D-mannitol	118, 129, 87, 189, 234	3,6-Man
1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-D-mannitol	118, 102, 127, 85, 261	4,6-Man
1,3,4,5,6-penta-O-acetyl-1-deuterio-2-O-methyl-D-mannitol	118, 139, 97, 333	2,3,4-Man
1,2,3,5,6-penta-O-acetyl-4-O-methyl-1-deuterio-D-mannitol	129, 87, 128, 189, 100	2,3,6-Man
1,2,3,4,5,6-hexa-O-acetyl-1-deuterio-D-mannitol	115, 103, 140, 188, 218	2,3,4,6-Man
1,5-di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-glucitol	102, 118, 129, 145, 161, 162, 205	t-Gal
1,3,5-Tri-O-acetyl-1-deuterio-2,4,6-tri-O-methyl-D-galactitol	118, 129, 161, 101, 234	3-Gal
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-1-deutério-D-galactitol	118, 113, 102, 99, 87, 233	4-Gal
1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-1-deuterio-D-galactitol	118, 102, 99, 129, 87, 162, 189	6-Gal
1,3,4,5-tetra-O-acetyl-1-deuterio-2,6-di-O-methyl-D-galactitol	118, 129, 87, 143, 305	3,4-Gal
1,2,4,5-tetra-O-acetyl-3,6-di-O-methyl-1-deuterio-D-galactitol	130, 88, 190, 113, 99, 233	2,4-Gal
1,2,3,5-tetra-O-acetyl-1-deuterio-4,6-di-O-methyl-D-galactitol	129, 161, 101, 86, 262	2,3-Gal

1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-1-deuterio-D-galactitol	118, 129, 87, 101, 189	3,6-Gal
1,2,5,6-tetra-O-acetyl-1-deuterio-3,4-di-O-methyl-D-galactitol	130, 129, 190, 189, 100, 87	2,6-Gal
1,3,4,5,6-penta-O-acetyl-2-O-methyl-1-deuterio-D-galactitol	118, 139, 97, 59	3,4,6-Gal
1,2,3,5,6-penta-O-acetyl-4-O-methyl-1-deuterio-D-galactitol	129, 87, 189, 86, 100, 262	2,3,6-Gal
1,2,3,4,5,6-hexa-O-acetyl-1-deuterio-D-galactitol	115, 128, 103, 145, 187, 157	2,3,4,6-Gal
1,5-di-O-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,4,6-tri-O-methyl-D-glucitol	117, 159,145, 75, 129	t-GlcN
1,4,5-tri-O-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3,6-di-O-methyl-D-glucitol	117, 159, 75, 233	4-GlcN