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**CHITOSAN HYDROLYSIS, QUATERNIZATION, AND
COUNTERION EXCHANGE FOR ENHANCED ANTIOXIDANT
ACTIVITY**

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ABBREVIATIONS

¹³C NMR – Carbon-13 nuclear magnetic resonance

¹H NMR – Hydrogen-1 nuclear magnetic resonance

2MNACA – HACC with 2-mercaptopnicotinic acid

2MNATMC – TMC acylated with 2-mercaptopnicotinic acid

2MPAHAC – HACC with 2-mercaptopropionic acid

2MPATMC – TMC with 2-mercaptopropionic acid

A-ComCh – Commercial chitosan electrostatic complex with ascorbate

A-COS – COS electrostatic complex with ascorbate

A-QChit2 – QChit2 electrostatic complex with ascorbate

CFA – Caffeic acid

CMC – Carboxymethyl cellulose

CNF – Cellulose nanofibrils

COS – Chito-oligosaccharide(s)

COSY – Correlation Spectroscopy (2D NMR)

CUPRAC – Cupric ion-reducing antioxidant capacity

D – Diffusion coefficient

Đ – Dispersity index

DA – Degree of acetylation

DLS – Dynamic light scattering

DMA – N,N-Dimethylacetamide

DMSO – Dimethyl sulfoxide

DP – Degree of polymerization

DPPH• – 2,2-diphenyl-1-picrylhydrazyl radical

DQ – Degree of quaternization

ELS – Electrophoretic light scattering

FRAP – Ferric reducing/antioxidant power

FTIR – Fourier transform infrared spectroscopy

G-ComCh – Commercial chitosan electrostatic complex with gallate

G-COS – COS electrostatic complex with gallate

GLA – Gallic acid

GlcN – deacetylated glucosamine unit

GlcNAc – *N*-acetylated glucosamine unit

GTEAC – Glycidyltriethylammonium chloride

GTMAC – Glycidyltrimethylammonium chloride

HACC – Hydroxypropyl trimethylammonium chloride chitosan quaternary ammonium salt

HAT – Hydrogen atom transfer

HCl – Hydrochloric acid

HSQC – Heteronuclear Single Quantum Coherence (2D NMR)

HTCC – *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride

IC₅₀ – Half maximal inhibitory concentration

***i*-PrOH** – Isopropanol

M_n – Number average molar mass

MP – Molded pump (container)

MW – Molecular weight

O-HTCC – *O*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride

PA – Pattern of acetylation

PBA – Phenylboronic acid

PCA – Protocatechuic acid

PVA – Polyvinyl alcohol

Q02H – Quaternized COS

QChit1 and QChit2 – Quaternized commercial chitosan

ROS – Reactive oxygen species

SD – Degree of substitution

SEC – Size-exclusion chromatography

SEM – Scanning electron microscopy

TMC – *N,N,N*-trimethyl chitosan chloride

TMCSAs – TMC and ascorbate complex

TMCSGa – TMC and gallate complex

TRAP – Total radical absorption potentials

UV-Vis – Ultraviolet-Visible spectroscopy

XRD – X-ray diffraction

INTRODUCTION

Antioxidants are materials capable of delaying, preventing, or eliminating oxidative damage to the target molecule. They range from small molecules, for example, ascorbic acid, to proteins like superoxide dismutase [1]. Currently, antioxidant research has been steadily on the rise. Significant attention has been devoted to enhancing the antioxidant activity of selected materials and adapting well-established antioxidants for different fields, ranging from agriculture to medicine [18]. Chitosan, a partly deacetylated derivative of chitin, is yet another potential antioxidant. This abundant biomaterial has demonstrated antioxidant activity attributed to its nucleophilic groups present on the glucosamine ring: C3-OH, C6-OH, and C2-NH₂. However, several limitations restrict its broader application. Firstly, chitosan is soluble only in acidic environments, making it difficult to apply under physiological pH. Secondly, its physicochemical and antioxidant properties are often inconsistent. Chitosan properties vary based on many factors, like size, degree of acetylation, or biological and processing origin. Thus, biomedical applications of materials with an inconsistent chitosan profile may not meet the required quality. Thirdly, even when dissolved, acidic chitosan solutions are viscous, which complicates the handling of such solutions, slows reactions, and limits efficient mixing.

The modification of chitosan can improve both its physicochemical and antioxidant properties. For example, chitosan may be functionalized with hydrophobic or hydrophilic groups. The introduction of hydrophobic groups can lead to the formation of amphiphilic derivatives, which are favorable for nanoparticles or micelles. In contrast, hydrophilic groups on chitosan enhance the solubility of chitosan and improve cation coordination. Therefore, depending on the application context, chitosan can be modified in many ways to achieve the desired functional properties. Certain chitosan modifications are a great tool for antioxidant activity enhancement as well. For example, chito-oligosaccharides have been shown to exhibit improved free radical scavenging than chitosan [58]. Therefore, chitosan hydrolysis to generate chito-oligosaccharides is a powerful tactic to improve the antioxidant activity. Quaternization of chitosan further improved solubility with enhanced antioxidant performance of reactive functional groups [73]. Electrostatic complexes with natural strong antioxidants like polyphenols or vitamins were demonstrated to work synergistically. Overall, chitosan hydrolysis, quaternization, and counterion exchange offer several advantages that are worth exploring and applying. This study aims to find a profound relationship between the structure of synthesized chitosan compounds and their antioxidant activity.

The aim is to investigate the chemical transformations occurring during chitosan hydrolysis and chito-oligosaccharides (COS) hydrolysate concentration, and to evaluate the effects of chitosan and COS quaternization and counterion substitution on antioxidant activity.

Tasks:

1. To synthesize chito-oligosaccharides (COS) of different molecular weights by acidic hydrolysis of chitosan, to perform their fractionation, and to characterize their structural features.
2. To prepare quaternized derivatives of commercial chitosan and selected COS fractions and to evaluate their degree of quaternization.
3. To perform counterion exchange of quaternized chitosan and COS derivatives with biologically active ascorbate and gallate anions and to evaluate their structural modifications.
4. To determine the antioxidant activity of the synthesized compounds.

1. LITERATURE REVIEW

1.1 Typical Oxidants in the Environment and Their Use *In Vitro* Antioxidant Activity Assays

The term “oxidative stress” is a change of balance between oxidant and antioxidant, the oxidant being in favor. It is usually related to reactive oxygen species (ROS) produced by living organisms due to cellular respiration. At high concentrations, they harm various biomolecules, such as lipids, proteins, and DNA [1]. ROS may kill pathogens as well as produce pyrimidine oxidation products, induce DNA-protein interactions, and methylate DNA bases, resulting in DNA damage [2], [3]. Therefore, oxidative stress is involved in many pathologies, such as neurological disorders, diabetes, cancer, and hypertension [1]. An antioxidant is referred to as a material that can prevent, delay, or eliminate oxidative damage to a target molecule [4]. Aerobic organisms have both enzymatic and nonenzymatic antioxidant systems to counteract the effects of ROS. The enzymatic ROS scavenging includes superoxide dismutases, catalases, and glutathione peroxidases – the most important enzymes. Nonenzymatic antioxidants can be grouped into antioxidants found in blood plasma (ferritin, albumin, transferrin, and ceruloplasmin) and other dietary antioxidants. These are mainly low-molecular-weight molecules, such as vitamins and flavonoids [1], [5], [6]. In more detail, the mechanism of antioxidant action involves processes such as hydrogen atom transfer, single electron transfer, sequential proton loss followed by electron transfer, and the chelation of transition metals [7]. All in all, oxidants have a huge impact on any organism’s state and well-being. Their effect is so powerful that multiple ways of regulation have been established.

The most frequent ROS generation is through particular enzymatic activity, but these compounds might also develop from glyceraldehyde, FADH₂, neurotransmitters, and hormones’ auto-oxidation [6]. ROS are divided into two groups: free radicals and nonradicals. Free radicals contain one or more unpaired electrons; meanwhile, nonradicals are composed of two free radicals sharing a pair of electrons. The most important ROS include hydrogen peroxide (H₂O₂), hydroxyl radical (•OH), and superoxide anion (O₂•⁻) [1], [8], [9], [10]. Hydroxyl radicals are the most reactive ROS, primarily generated by the metal-catalyzed reactions. The antioxidant activity against them is often studied *in vitro* using Fenton and Fenton-like reactions [8], [11]. Another group of ROS – superoxide anions – is usually generated via the mitochondrial electron transport chain. These species are alternatively produced by NAD(P)H oxidase in macrophages during phagocytosis to fight bacteria. With the help of superoxide dismutases, O₂•⁻ is converted to H₂O₂, which easily crosses plasma membranes [1]. O₂•⁻ can be monitored by testing external antioxidants involving electrochemical assays [12]. The amount and reactivity of hydrogen peroxide are expanded by additional sources of formation, such as peroxisomes. The classical antioxidant detection method involving H₂O₂ is UV measurement at 230 nm. However, there are also additional, colorimetric, and fluorescence methods [13], [14].

Other ROS are peroxy radicals ROO•, like HOO•, involved in lipid peroxidation chain reactions, and DPPH•, a widely used ROS *in vitro*. The scavenging mechanism of DPPH• involves the reduction of methanolic DPPH• solution when a hydrogen-donating antioxidant is present, leading to the formation of the non-radical form, DPPH-H, through the color-changing reaction [9], [15] (Figure 1). There are many other antioxidant assays, for example, ferric reducing/antioxidant power (FRAP), cupric ion-reducing antioxidant capacity (CUPRAC), or total radical absorption potentials (TRAP).

Still, the DPPH• scavenging assay is one of the most popular ones, because of its simplicity, speed, effectiveness, and relatively low price [16].

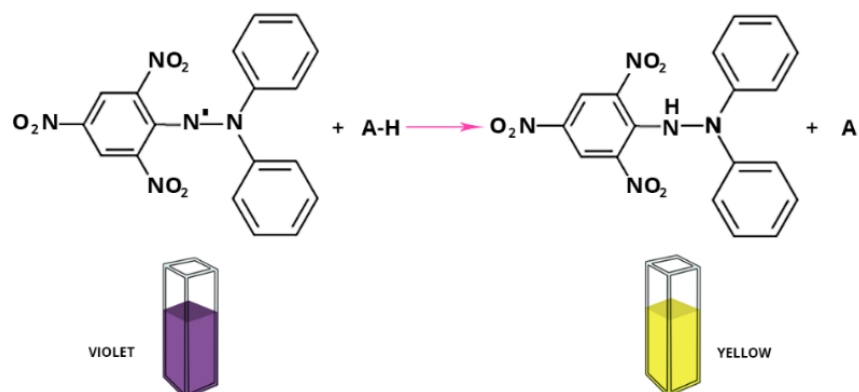


Figure 1. DPPH• scavenging colorimetric assay principle [17].

1.2 New emerging antioxidants

Nowadays, research aims to investigate new sources of effective and natural antioxidants. The interest revolves around food applications, medicine, and other related fields [18]. Food packaging is one of the areas where antioxidant coatings might be adopted for food spoilage prevention. Enhanced coatings might protect their contents from ultraviolet radiation, mechanical, or microbiological damage. At present, nonbiodegradable materials based on petroleum are usually used, offering good protective barrier qualities – chemical stability and mechanical strength. Functional biopolymer films, composed of polysaccharides/lipids/proteins, are eager to change this situation. They are a promising alternative due to sustainability, eco-friendliness, and biodegradability. To overcome their limitations – low sturdiness and poor barrier performance – scientists incorporate various functional additives, often with antimicrobial and/or antioxidant qualities. One of such examples is a molded pump (MP) container of cellulose nanofibrils (CNF) and carboxymethyl cellulose (CMC) paste [19]. It was shown that CNF/CMC-coated MP trays are resistant to grease, air, and moisture. Their structure was more uniform (SEM analysis), had a higher crystallinity (XRD analysis), and was proven to repel oils (standardised TAPPI-T559 grease resistance testing) compared to highly porous MP trays. In medicine, hydrogels with antioxidant properties arise. Hydrogels can be made solely of antioxidative materials, so-called ‘self-antioxidant hydrogels’. Alternatively, antioxidants can be incorporated into the hydrogel's backbone, which does not contain antioxidative action. ‘Self-antioxidant hydrogels’ are both synthetic and natural. Natural ones consist of typically marine-derived polysaccharides, like fucoidan from brown algae. Fucoidan-based alginate hydrogels were tested against pancreatic insulinoma β -cells of rats and were shown to reduce intracellular oxidative stress. Additionally, synthetic polymers, like polyvinyl alcohol (PVA) or phenylboronic acid (PBA), are being modified to receive antioxidant action. PVA modified with vanillin or catechol demonstrated promising results in therapeutic areas like wound healing and epithelial regeneration [20].

In addition, efforts are put into chemically modifying natural antioxidants to enhance their properties. As for medicine, one of the examples is lysozyme-loaded sulphate-modified chitosan hydrogels. They had a greater free radical scavenging activity than sole chitosan hydrogels, which is thought to be due

to lysozyme influence on chitosan degradation and the formation of small molecular mass chitosan particles [20]. Polysaccharides with insufficient biological activity are being actively modified by acetylation, sulfation, selenylation, phosphorylation, and carboxymethylation. It may not only enhance, but also add new beneficial functions. Upon selenium introduction to the *Atractylodes macrocephala* polysaccharide structure, both direct free radical neutralization and antioxidant enzyme activity were enhanced. Inclusion of an additional functional group can change the pattern of receptor/enzyme binding affinity by exposing or suppressing certain polysaccharide groups [21]. Out of the scope of many natural polymeric antioxidants, chitin and chitosan seem like one of the great perspectives to investigate.

1.3 Antioxidant potential and general modification of chitosan

Chitin (*N*-acetyl glucosamine, GlcNAc) and chitosan (glucosamine, GlcN, and *N*-acetyl glucosamine) are natural and unique antioxidant potential-containing compounds [22], [23]. Chitin is a linear polysaccharide composed of GlcNAc units linked through β -1,4-glycosidic bonds, whereas chitosan comes from the deacetylation of chitin (Figure 2). Chitin is a homopolymer having 3 polymorphic modifications and is the second most abundant natural polymer after cellulose [24]. Polymorphic modifications of chitin arise from its complex biosynthesis; alpha modifications represent antiparallel polymer chains, beta – parallel, and gamma – mixed antiparallel and parallel polymer chains. They differ depending on the organism's nature and function [25]. Chitin has a lot of hydrogen bonds, both intramolecular and intermolecular. That is the reason for its poor solubility and chemical reactivity [24]. Chitosan, on the other hand, does not have as many acetyl groups, capable of hydrogen bonding. Consequently, it is more flexible, soluble, reactive, and applicable [26]. Due to the reported biocompatibility, chitosan (as a base material) is applicable in biomedical sciences, finding a role in tissue regeneration or drug delivery, connected to pH responsiveness. Chitin and chitosan are known for their anti-inflammatory [27], antimicrobial [28], antitumor [29], wound healing [30], and cholesterol-lowering effects [31].

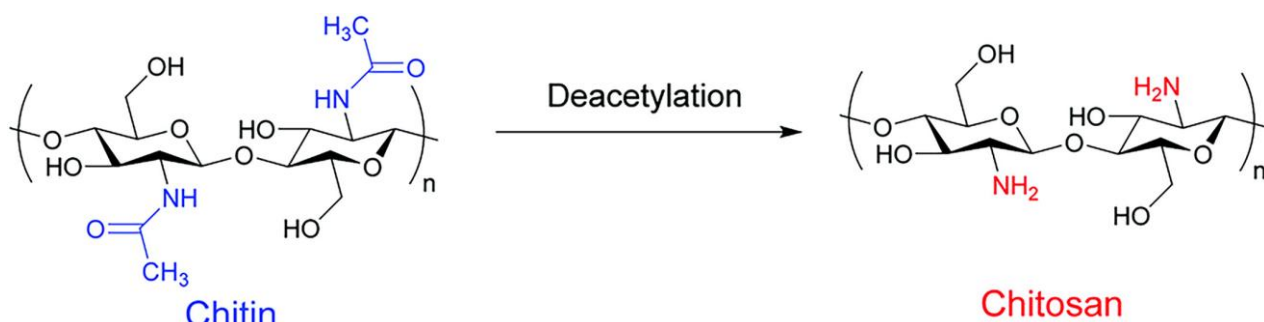


Figure 2. Visual representation of a full chitin deacetylation [32]. Chitin's degree of acetylation is commonly 100 %, making it a homopolymer, while chitosan is a random copolymer with DA varying from 2 to 45 % [24].

Both chitin and chitosan are prospective antioxidants for several reasons. One is their abundance and accessibility. Chitin is present in many commonly found natural sources like insect cuticles, crustacean shells, and fungal walls; it is further reported that living organisms produce from 10^{10} to 10^{12} tons of chitin every year [24]. Its deacetylation is usually harsh and energy-consuming; nevertheless, sustainable methods are being developed, allowing the green synthesis of chitosan.

Biological chitosan generation is possible with chitin deacetylase found in fungi, but currently it is too expensive and inconsistent (regarding degree of acetylation (DA)) for the industry [26]. Nonetheless, chitosan is a tempting possibility for innovation because of chitin as an unused food industry waste; the production of chitosan may change depending on the chitin source, like fungi or snails, that could provide more opportunities for the advancement of green technologies [24]. The secondary use of chitosan is an eco-friendly solution that may be applied very creatively. That point brings to the second reason, which is versatility, that, apart from medicine, includes the use of those polymers in nanotechnology [29], wastewater treatment [33], food [23], [33], and the textile industry [30].

Lastly, it is their direct antioxidant action that contributes the most to their perspectives. In recent years, there has been increased attention towards chitin and chitosan's antioxidative abilities. According to the research [23] chitosan and its derivatives effectively neutralize a variety of oxygen radicals, including DPPH•, hydroxyl, alkyl (like methyl, CH₃•), and superoxide *in vitro*. Their scavenging effect is furthermore associated with molecular weights (MW) and DA. The best antioxidant activity was noticed with the lower MW (≤ 30 kDa) and lower DA (10 %) [23]. Although the exact mechanism is not completely clear, many scientists support the idea that hydroxyl and amino groups exhibit significant reactivity towards ROS [9], [22], [34]. Noticeably, the pattern may change depending on the hydrogen bonding. It may block these groups from the full exposition to ROS, but alternatively, it might also stabilize the structure for a reaction [35]. That is speculated as a cause of why native chitosan sometimes does not perform well in antioxidant studies [22], [34]. Apart from a direct effect, chitosan was shown to regulate antioxidant enzymes' activities and decrease lipid peroxidation [36].

Although chitosan is a promising material with many advantages, it has several drawbacks. First of all, unmodified chitosan has a limited solubility at higher pH values. It is a polycationic polymer only at acidic-slightly neutral conditions (chitosan pKa is 6.3-6.5) [37], [38]. The physiological pH, which is around 7-7.4 for most tissues [39], causes chitosan amino group deprotonation and aggregation [38], reduced mucoadhesion [40], and antioxidant activity [41]. Importantly, solubility not only includes the effects of pH; it is additionally influenced by DA, molecular weight, temperature, and crystallinity [42]. DA is one of the most important physicochemical properties of chitosan. It is defined as the ratio of *N*-acetylglucosamine monomers to the total number of polymer units and is usually less than 50 %. Depending on DA, chitosan can have variable solubilities and charge densities upon protonation. Acetyl groups are not capable of ionization, and the more monomers they occupy, the more soluble the polymer is at acidic pH. Apart from DA, chitosan is also described in terms of degree of polymerization (DP) and the pattern of acetylation (PA). DP is related to DA and covers the size of chitosan composition in terms of units. PA, unfortunately, does not have a suitable determination analysis yet [24]. Therefore, pH and DA may significantly alter chitosan's solubility and reactivity.

Secondly, pH, DA, solvent composition, and ionic strength have a huge impact on the viscosity [42], [43], another quality of chitosan that is not favorable. High viscosity results in difficult management [42]. Also, it affects drug delivery performance (slow diffusion and release) and slow interaction with other materials or tissues [44], [45]. High viscosity that is related to polymer entanglement can also result in non-uniform reactions/biological effects since the entanglements can be distributed non-uniformly in the solution [46]. Thirdly, a lot of factors influence the reproducibility, which is yet

another limitation in chitosan applicability. Already mentioned factors like pH, solvent, ionic strength, and even the source of chitosan can dramatically change chitosan's behaviour. It is especially important in medicine, where the quality of a material should be controllable [47].

The introduction of new moieties into the chitosan may improve its properties and applications. Most commonly, the derivatization of chitosan is acquired by direct modification, enzymatic reactions, and chemical grafting. Chitosan contains three nucleophilic functional groups: a primary hydroxyl group at C6, a secondary hydroxyl group at C3, and a primary amine group at C2 (Figure 3) [22]. These groups act as both hydrogen bond donors and acceptors and react as nucleophiles. In this way, hydrophilic (sulphates, sulphonates, carboxylic, and phosphate) and hydrophobic groups (acylation with fatty acids, alkylation with hydrocarbon chains) can be introduced. Hydrophilic groups can make a compound more soluble at neutral pH and provide a better cation coordination. Hydrophobic groups may provide amphiphilic derivatives, applicable in micelles or nanoparticles [24].

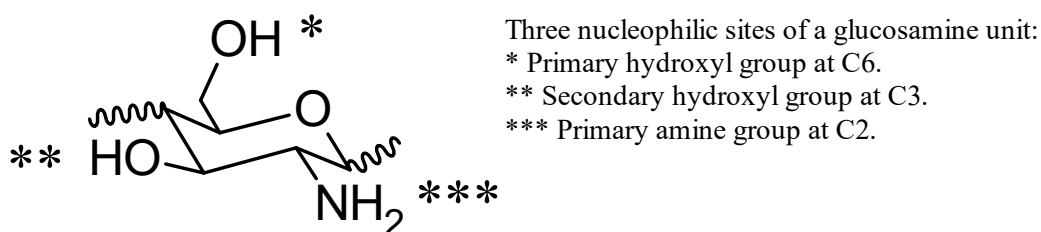


Figure 3. Nucleophilic groups of a glucosamine unit.

Chitosan modification faces regioselective and chemoselective issues that result in mixed derivatives, limiting reproducibility and usefulness. To control the situation, robust chemistry has to be applied, together with homogeneous conditions, as heterogeneous ones significantly lower the substitution and generate by-products. An important consideration of homogeneity is pH, where aqueous acidic conditions, which help to dissolve chitosan, may induce water competition against nucleophilic chitosan groups to provide lower modification yields. It can also cause chitosan decomposition and a lower substitution due to ammonium ions. Therefore, it requires controlled chemistry and even activation strategies. A big advantage of chitosan modification is the higher reactivity of amino groups when compared with hydroxyl groups. The amino group may participate in alkylation, acylation, Michael addition, and reductive amination (two past reactions together with imine formation are completely chemoselective) (Figure 4) [24]. Depending on the synthesis process, groups can be temporarily protected to facilitate the desired modification. For example, to achieve amino functionality, hydroxyl groups are converted to silyl ethers [22], [48]. To achieve hydroxyl functionality, amino groups are protected with the phthaloyl group or its derivatives.

The final products of chitosan modification can improve greatly in antioxidant efficiency because of their enhanced structure-activity relationships [49], [50]. As it will be discussed later, a high importance belongs to changes in molecular mass and DA. Additionally, the antioxidant effect strongly depends on the substitution degree, position, and conformation [51]. Polysaccharide modification with sulphation, for example, increased OH• scavenging. It was proposed that sulfated derivatives donated hydrogen atoms to OH• and led to the formation of stable radicals [36], [52]. Antioxidant effect also increases greatly when the additional antioxidant potential containing compound is introduced, such as gallic acid [22]. Adding aromatic compounds (benzene or pyridine) provides a hydrogen atom that stabilizes radicals. Overall, by changing the electronic or steric

characteristics of amino and hydroxyl groups present in polysaccharides, it is possible to achieve greater antioxidant action.

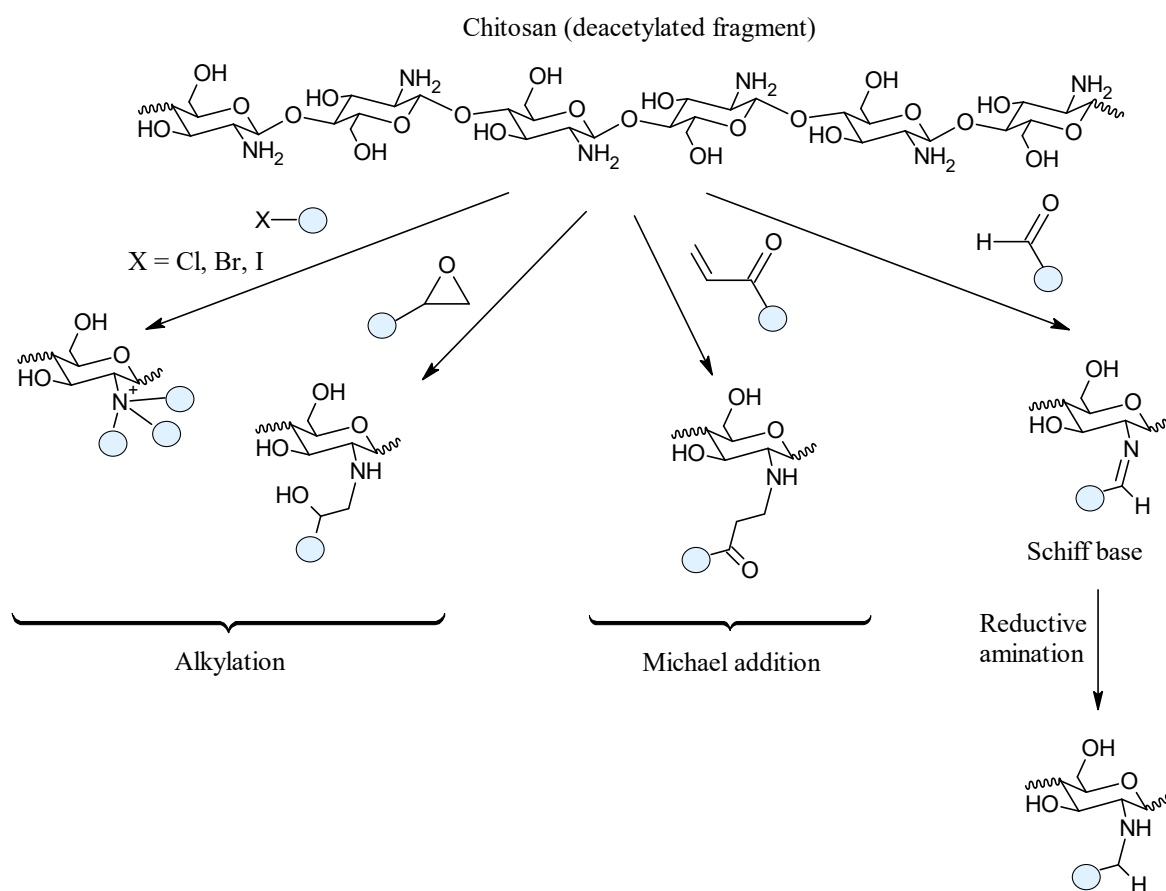


Figure 4. One of the possible reactions of amino groups present on chitosan: alkylation (nucleophilic substitution, resulting in possible peralkylation, and nucleophilic epoxide opening), Michael addition, and reductive amination. Adapted from Petroni and colleagues [24].

1.4 Chito-oligosaccharides and their derivatives

Generally, chitosan chains that have a degree of polymerization (DP) less than 20 and an average MW of less than 3.9 kDa are referred to as chito-oligosaccharides (COS) [53]. But this definition is not strict, as some authors assign chito-oligosaccharides to larger fragments (1-10 kDa). COS are divided into homo-chito-oligosaccharides of either acetylated or deacetylated glucosamine units and hetero-chito-oligosaccharides, which are the varying DP, DA, and PA oligomer combinations. Important consideration is PA – even in homo-chito-oligosaccharides, it can differ [54]. Macromolecules with the same DP can exhibit significant differences in their properties due to variations in the degree of acetylation and the specific arrangement of acetyl groups along the polymer chain [55]. Qualities that distinguish COS from chitin and chitosan are water solubility, low viscosity, alleviated absorption, and cell membrane permeability. Due to those qualities and antioxidant, antitumor, anti-inflammatory, and antimicrobial action, COS have numerous positive effects on bone-marrow/neuronal/embryonic stem cell growth support, inflammatory response reduction, asthma treatment, hydrogels, and other medical applications [53], [54]. Apart from medicine, they have already found their way in cosmetics (due to absorption through epidermal cells), food, and agriculture [54], [56], [57].

The antioxidant activity of COS was studied both *in vitro* and *in vivo* [58]. It was reported that COS effectively scavenges superoxide radicals and protects DNA from $\bullet\text{OH}$ [54]. In general, various oligosaccharides' antioxidant activity is tightly connected to their structure, and chito-oligosaccharides are not an exception [59]. COS with a DP of 10 – 12 or low MW (1-5 kDa) exhibited the best antioxidant activity, as well as COS with a lower DA [58], [60]. Antioxidant activity was also validated in 5 – 87.7 kDa COS [54]. Higher molecular weight chitosan may be inefficient due to the formation of strong intramolecular hydrogen bonds between the amino and hydroxyl groups. A high-MW chitosan has a dense polymer structure, which promotes closer proximity of functional groups within the macromolecule. Consequently, the formation of hydrogen bonds limits the flexibility, reactivity, and availability of these functional groups with ROS [60]. DA, as can be seen from Figure 5, is not to the advantage of the antioxidant activity. It serves as a block for amino groups and provides a carbonyl oxygen that provides more hydrogen bond interactions. However, some research did not find a notable DA effect [9], [60], [61]. A high-fat diet combined with COS additionally increased glutathione peroxidase, catalase, and superoxide dismutase levels, meaning COS can restore the activity of these major enzymatic antioxidants [55]. In general, COS has antioxidative activities due to free radical scavenging or enzymatic induction; it highly depends on COS structural features.

Other chitosan-based derivatives involve biomolecules like gallic acid, ferulic acid, caffeic acid, cinnamic acid, coumaric acid, chlorogenic acid, salicylic acid, and catechin. These are mostly described as plant polyphenols – secondary plant metabolites having an aromatic moiety with at least one hydroxyl group [57], [58]. Phenolic acids like gallic acid, ferulic acid, and chlorogenic acid have gained the most interest in recent years because of their enhanced antioxidant properties. Ferulic acid, for example, decreases lipid peroxidation and enhances the level of glutathione [62], while catechin is known for the activity enhancement of superoxide dismutases and catalases [63]. A combination of these two antioxidant-potency-containing molecules (chitosan and phenolic acid) is often synergistic and holds a part in future investigations [22].

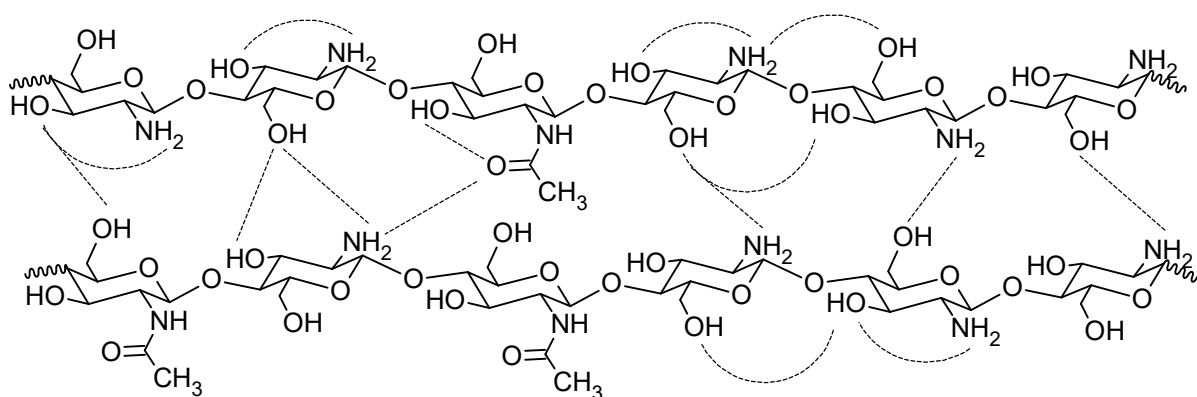


Figure 5. Visual representation of possible intramolecular and intermolecular hydrogen bonds in chitosan and COS. Adapted from [64].

COS antioxidant activity, even if better than that of chitosan, is still not sufficient. Their IC_{50} values are higher than those of other antioxidants. To solve the issue, COS are supplemented with functional groups by chemical modification or conjugated with plant polyphenols [58]. Potential COS conjugates involve gallic acid-conjugated COS and sulfated COS. These conjugates have managed to show a higher antioxidative effect relative to COS in macrophage and pancreatic β cells studies

[22]. Recently, phenolic-acid-grafted COS demonstrated great results in the food industry for cherry tomato preservation. Protocatechuic acid (PCA), caffeic acid (CFA), and gallic acid (GLA), attached to COS via amide linkages, showed great results in DPPH• scavenging. Their antioxidant effect was situated as follows: GLA > PCA > CFA conjugates, when GLA showed the best results; the grafting itself helped to enhance the antioxidant activity by approximately 14 % [57]. Other modifications of COS include carboxylation and quaternary ammonium group introduction. This topic is discussed in more detail in the next chapter (1.5). These modifications ought to further enhance the antioxidant and antimicrobial, drug-like properties of COS – positive charge is capable of attaching to negative charge possessing bacterial cell membranes or biomolecules, like DNA [65], [66].

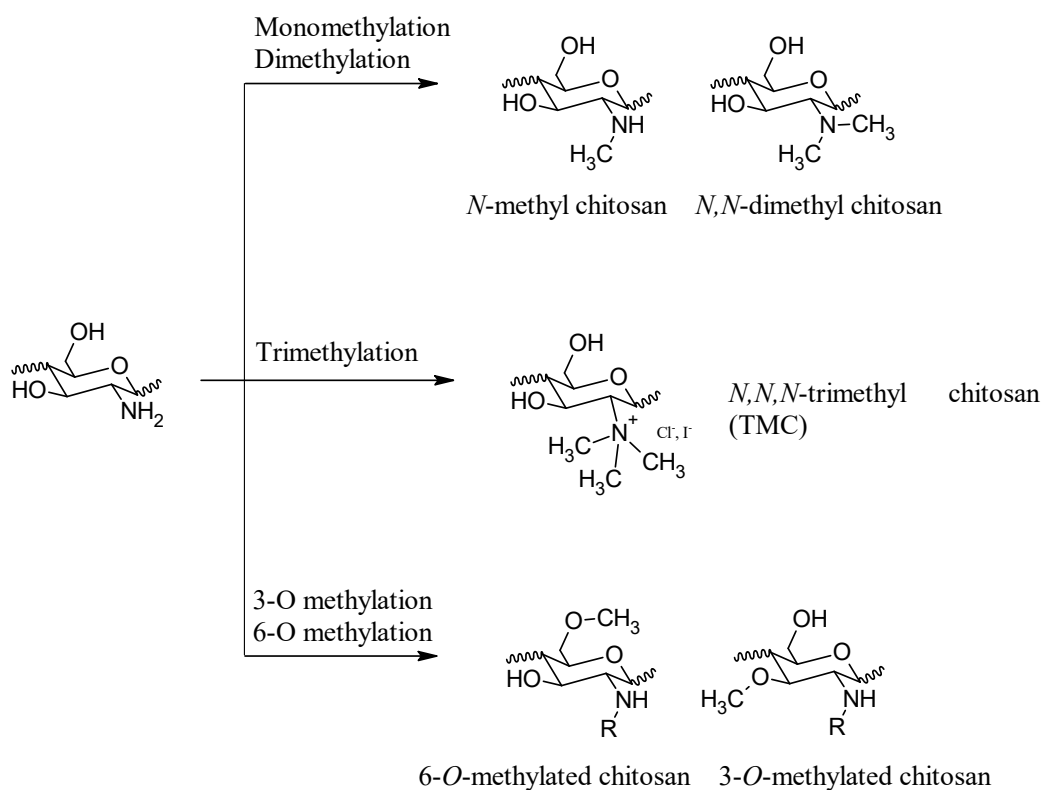
1.5 Quaternary chitosan derivatives

Chitosan derivatives with a positive permanent charge are often achieved either by direct quaternization of chitosan's amino group or by establishing quaternary ammonium groups to any of the three reactive sites (primary or secondary -OH or -NH₂, present on chitosan) [48]. Studies demonstrate that quaternization enhances chitosan's antioxidant efficacy, particularly in aqueous environments where native chitosan shows limited activity, although a minority of research groups did not receive a significant antioxidative action enhancement [67], [68]. The exact mechanism of quaternary chitosan's antioxidative action is not yet known due to the complex research area. Factors like reaction conditions, structure, and degree of substitution may change antioxidant activity [68]. However, several points have been established. Quaternized chitosan exhibits antioxidant properties compared to native chitosan due to several structural and chemical factors that enhance its interactions with ROS and free radicals. The main reasons include: **1) increased water solubility; 2) electrostatic interaction with radicals; 3) accessibility of reactive sites.** The positive charge significantly improves chitosan's solubility in water across a wide pH range. Increased hydrophilicity ensures better accessibility of reactive centers for interaction with water-soluble radicals, such as hydroxyl radicals. Quaternary ammonium group enhances the electrostatic attraction to negatively charged reactive oxygen species, such as the superoxide anion [69]. Moreover, permanent charges give rise to electrostatic repulsions between chitosan chains; lateral alkyl groups increase these gaps sterically even more. In both cases, the hydrogen bond network is disrupted, and it leads to the unfolding of chitosan chains [67]. It was reported that the type and length of the substituent groups can have a great impact on chitosan's antioxidative action [68]. A key advantage of chitosan quaternization is that it occurs on chitosan's backbone functional groups. As a result, new functionalities are introduced while retaining the inherent physicochemical/biological chitosan properties [67]. This explains the widespread use of quaternized chitosan in biomedical and food-related applications [67], [70].

Antioxidant activity is related to antibacterial activity. Polyphenols (generally antioxidants) like (-)-Gallocatechin-3-gallate, malvidin, or peonidin cause antimicrobial activity by ROS production. The antioxidative or oxidative behavior of polyphenols might change depending on the structure and environment [71]. Quaternized chitosan is not an exception. In a study, several compounds were tested to examine their antimicrobial activity against *E. coli* and *S. aureus* in different pH media. The research concluded [72] that the mere addition of a quaternary ammonium group to chitosan is not enough to achieve antimicrobial activity; the crucial factor is the optimal placement of the positive charge relative to the polymer backbone [22], [72]. Therefore, to earn the optimal chitosan antibacterial and antioxidant action, it is important to draw attention to the structure and position of the quaternary ammonium group. As an example, *N*-quaternised derivatives show higher DPPH• scavenging activity than *O*-quaternised chitosan, meaning that the hydroxyl group plays a more important role than amine in DPPH• scavenging [73]. The same researchers have also described how

modified chitosan can participate in reactions with $\bullet\text{OH}$ and $\bullet\text{O}_2^-$. $\bullet\text{OH}$ is scavenged by hydroxyl groups in an H-abstraction reaction. $\bullet\text{OH}$ and $\bullet\text{O}_2^-$ can also react with amino groups, forming stable macromolecule radicals. In addition, $\bullet\text{OH}$ and $\bullet\text{O}_2^-$ scavenging relate to the number of active hydroxyl groups present on a molecule [73], [74].

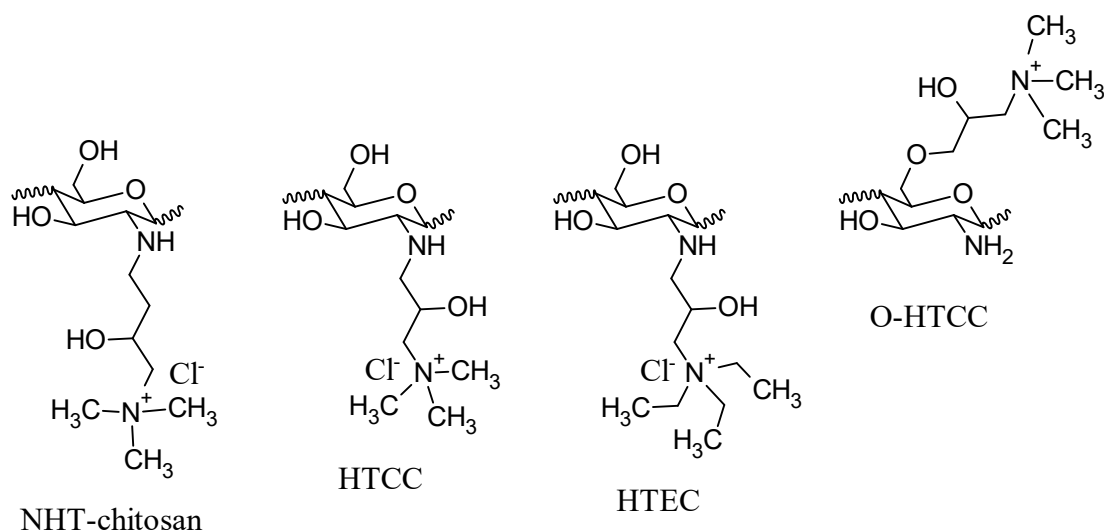
One of the most promising quaternary chitosan derivatives, an example of a direct chitosan amino group quaternization, is *N,N,N*-trimethyl chitosan (TMC). It is the most studied and simple form of quaternary ammonium chitosan [75]. TMC is usually synthesized by methyl iodide in the presence of sodium hydroxide [48]. This compound is soluble in both neutral and alkaline media, and the solubility is influenced by DA and DQ (degree of quaternization). Higher DQ means more positive charge, leading to better solubility. On the other hand, the quaternization of chitosan does not always result in higher solubility. The prolonged synthesis results in the production of *N,N*-dimethyl chitosan and *O*-methylated chitosan, which are insoluble and will hence diminish the overall solubility of TMC (Scheme 1) [75]. The authors showed that high charge density is necessary for TMC to significantly improve the paracellular permeability, which means that the transport enhancement across intestinal epithelia is improved with higher DQs [76]. Trimethyl chitosan displayed penetration enhancement towards the intestinal [77], nasal [78], corneal [79] and bronchial epithelia [80], and buccal mucosa [81]. Recently, it was actively used in nanodelivery systems [82], [83], [84]. Skin collagen peptides encapsulated in TMC, for example, alleviated neuronal damage in Alzheimer's disease, associated with oxidative stress damage [83].



Scheme 1. Possible products of TMC production (methylation) from chitosan. Even if rare, these by-products are not desired as they result in unintended properties [75].

The antioxidant activity of quaternized *N,N,N*-trimethyl chitosan with various anionic counterions, including iodide, citrate, acetylsalicylate, ascorbate, and gallic acid, has been evaluated [77]. Authors

have reported that both *N,N,N*-trimethyl chitosan with ascorbate (TMCSAs) and gallate (TMCSGa) anions exhibited excellent antioxidant activity, demonstrating strong DPPH radical scavenging (> 95 % even at the lowest test concentration) and effective superoxide radical neutralization (up to 100 % at 0.4 mg/mL). In terms of reducing power, TMCSGa was found to be the most effective reducing agent, with absorbance values reaching around 4 A across various test concentrations. TMCSAs also displayed strong reducing power, with absorbance values reaching 4 A at concentrations exceeding 0.4 mg/mL. The other prevalent method for quaternizing chitosan involves attaching a quaternary ammonium group outside the chitosan backbone by hydroxypropyl trimethyl ammonium chloride [49], [85], [86], or also glycidyl trialkylammonium chloride (Scheme 2) [85], [87], [88].



Scheme 2. *N*-(2-hydroxy-3-trimethylammonium) propyl chitosan [85], *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan (HTCC), *N*-(2-hydroxypropyl)-3-triethylammonium chitosan (HTEC), and *O*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (O-HTCC) [73].

In the publication [73] three novel water-soluble quaternized chitosans were synthesized by directly introducing quaternized glycidyl trimethylammonium chloride (GTMAC) and glycidyl triethylammonium chloride (GTEAC) groups onto chitosan of different molecular weights (400 and 1240 kDa). These derivatives included *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (400-HTCC and 1240-HTCC), *N*-(2-hydroxypropyl)-3-triethylammonium chitosan chloride (400-HTEC and 1240-HTEC), and *O*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (400-O-HTCC). Their antioxidant activity was evaluated *in vitro* using three oxidant assays, with consideration given to factors such as MW, *O*- and *N*-substitution patterns, and the type of introduced functional groups. As a result, all of the derivatives had an antioxidant activity. 400-O-HTCC showed an antioxidant effect similar to 1240 kDa commercial chitosan, and their order of scavenging ability was $\bullet\text{O}_2^- > \bullet\text{OH} > \text{DPPH}\bullet$. 400-HTCC and 400-HTEC exhibited better $\text{DPPH}\bullet$ and worse $\bullet\text{O}_2^-$ scavenging.

$\text{DPPH}\bullet$ scavenging assay revealed that 400-HTCC, 400-HTEC, and 1240-HTEC reached > 70 % antioxidant activity, which decreased in the order of their listing. 1240-HTCC had < 60 %, and 400-O-HTCC had no more than 5 % antiradical activity. Thus, it was concluded that hydroxyl groups are crucial for inactivating $\text{DPPH}\bullet$; smaller fragments were more relevant, HTCC being at an advantage.

Interestingly, 1240-HTEC was superficial over 1240-HTCC. As 1240-HTEC had a lower DQ, researchers suggested that steric hindrance is an important consideration here.

As for $\bullet\text{OH}$ scavenging, it depends on compounds' concentration – scavenging rate is directly proportional to the amount of available hydroxyl and amino groups. Comparing the highest concentrations, 1240 commercial chitosan was the best ($> 80\%$). It was followed by 1240-HTCC ($\sim 47\%$), 400-O-HTCC ($\sim 30\%$), and 400-HTCC, 1240-HTEC, and 400-HTEC (all had a lower than 22% scavenging activity). Namely, amino groups are important for the reaction with $\bullet\text{OH}$, as higher MW *N*-substituted chitosans had a lower antioxidative activity than chitosan itself, and only 1240-HTCC was better than 400-O-HTCC. The fact that 1240-HTCC was significantly better than 1240-HTEC was justified by a lower steric hindrance of the positive charge, capable of antioxidative action. 1240-HTEC had a lower degree of quaternization (74.7% ; 1240-HTCC DQ was 84.6%).

Lastly, superoxide radical neutralization was the best using 1240 kDa chitosan and 1240-HTEC, around 97% at maximum concentration. Like in the case of $\bullet\text{OH}$, scavenging was dependent on different concentrations. Among 400 kDa fragments, HTEC was the best ($\sim 93\%$), and HTCC was the worst ($\sim 43\%$). The activity of 1240-HTCC was around 85% , making both 1240 kDa compounds superior to those of 400 kDa. It is related to $\bullet\text{O}_2^-$ being dependent on the content of hydroxyl and amino groups – bigger MW results in a greater effect. Also, HTEC, either small or big, was better than HTCC. The explanation was that HTEC had a smaller DQ, allowing the utilization of more -OH and $-\text{NH}_2$ groups present on chitosan. 400-O-HTCC was better than 400-HTCC, showing the importance of the amino group. Overall, due to different radical scavenging mechanisms, quaternary chitosan compounds show contrasting results. Lower MW HTCC worked best for DPPH $\bullet$, while higher MW HTCC was the most effective with $\bullet\text{OH}$ and higher MW HTEC – with $\bullet\text{O}_2^-$.

Based on the results from various antioxidant assays (DPPH radical scavenging, superoxide anion radical scavenging, and reducing power assays), the antioxidant activity of hydroxypropyl trimethylammonium chloride chitosan (HACC) and *N,N,N*-trimethyl chitosan iodine (TMC) was evaluated in comparison to their acylated derivatives and different cationic chitosan salts [49]. Both HACC and TMC demonstrated enhanced DPPH radical scavenging activity (76.75% and 68.51% , respectively) compared to pure chitosan ($\sim 35\%$) at 1.6 mg/ml concentration. Acylation of these chitosan derivatives further improved DPPH radical scavenging activity, particularly for sulfhydryl ($-\text{SH}$)-bearing derivatives. For instance, HACC acylated with 2-mercaptopyruvic acid (2MPA) achieved near 100% scavenging efficiency at 1.6 mg/mL (Figure 6). Moreover, acylation exhibited superior superoxide anion radical scavenging activity compared to pure chitosan, HACC, and TMC. Specific acylation with compounds such as 2-amino-3-mercaptopropanoic acid significantly enhanced the interaction with superoxide anions. TMC salts like 2MPATMC (TMC with 2-mercaptopyruvate anion) and 2MPATMC reached 92.78% and 98.70% , respectively (Figure 6). Chitosan itself demonstrated negligible reducing power; HACC and TMC displayed moderate improvement. Meanwhile, further acylation augmented this property, with certain derivatives achieving substantially higher absorbance values, such as 2MPAHAC (2.50 A at 1.6 mg/mL) and 2MPAACA (2.35 A at 1.6 mg/mL) (Figure 6). These findings suggest that HACC and TMC acylation, either by amide bond or electrostatic interaction, significantly enhances their antioxidant capacity, making them promising scaffolds for further investigation. By selecting functional groups, the selectivity of chitosan derivatives toward various scavenging activities can be optimized. For

example, the reducing power of chitosan derivatives can be improved by incorporating electron-donating functional groups.

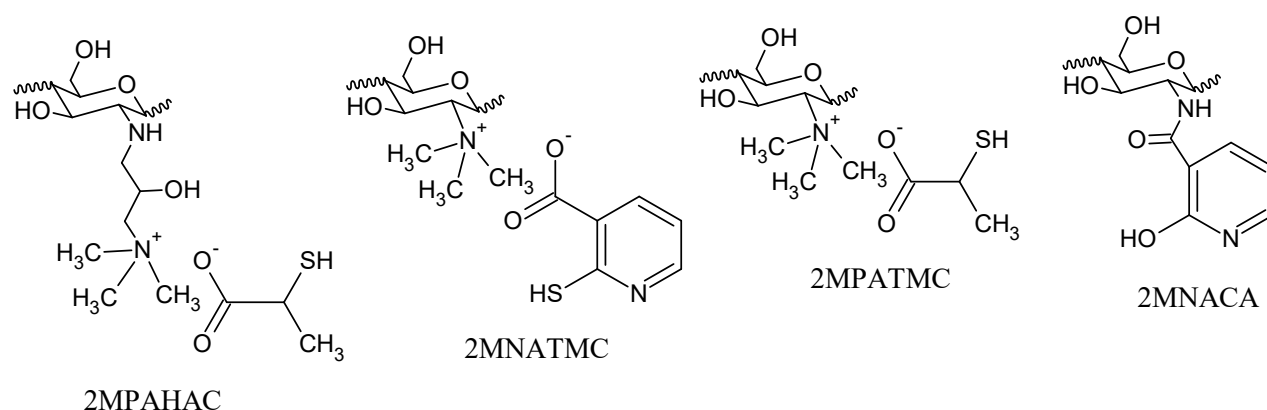


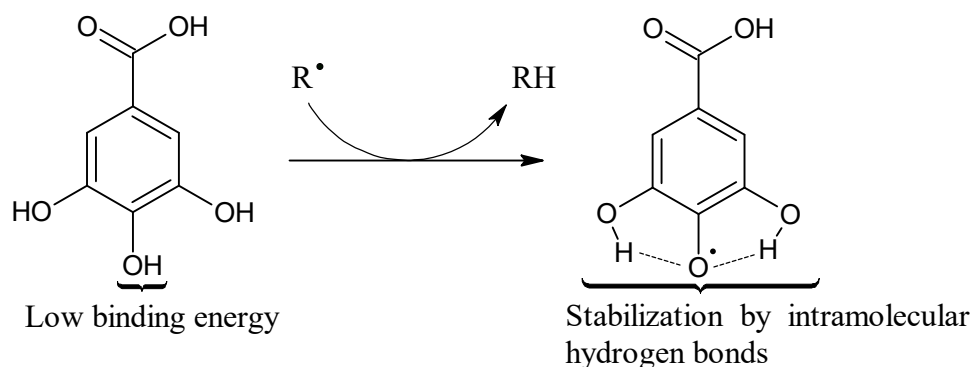
Figure 6. Different HACC and TMC derivatives [49].

Other (not quaternary) promising chitosan derivatives include carboxymethyl chitosan (amphoteric polymer), hydroxypropyl chitosan, esterified chitosan, and many more [89]. One of the recently described derivatives of chitosan is with the selenourea group. These compounds have demonstrated > 90 % superoxide anion elimination activity at ≥ 0.4 mg/ml concentration, universal reducing power improvement, and > 50 % hydroxyl radical elimination efficiency for almost all synthesized compounds at 0.2 mg/ml concentration [50]. Another recent publication was exploring pyridinium sulphonate chitosan derivatives, acquired by amidation [90]. Their antioxidative action was better than that of chitosan, and increased with concentration. The position of a nitrogen in the pyridine ring was noted as a very important factor for radical scavenging, as well as its degree of substitution. At a 0.8 mg/ml concentration, 100 % DPPH radical scavenging rate was achieved by pyridinium sulfoxide with a hydroxyl group. All in all, the quest to improve the antioxidant effects of chitosan remains popular. Numerous chemical modification methods and antioxidation assessment methods enable the exploration of an increasing number of possible chitosan combinations.

1.6 Gallic and ascorbic acid antioxidant mechanisms

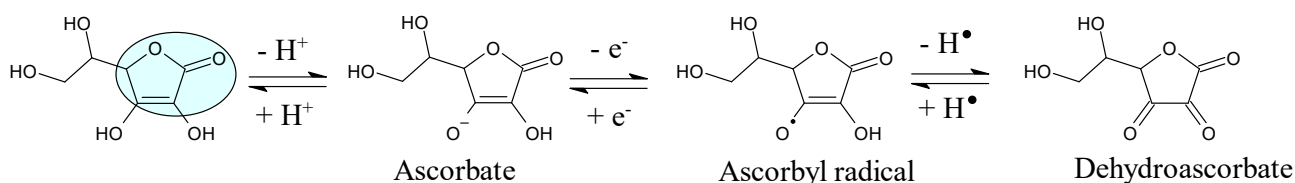
As was described before, plant polyphenols are one of the strategies to improve chitosan's antioxidant activity [22]. One of them is gallic acid (3,4,5-trihydroxybenzoic acid). It occurs naturally and has an antioxidative action against peroxy, hydroxyl, and superoxide radicals, as well as non-radicals like hypochlorous acid or hydrogen peroxide. This planar molecule is capable of scavenging radicals by hydrogen bond stabilization and other structure-related factors (Scheme 3). The major part of the activity is prescribed to hydroxyl groups; 3 of those groups make gallic acid one of the most potent antioxidants among many polyphenols [91]. The radical scavenging mechanism is favored in a polar medium. Computational analysis revealed that gallic acid performs both hydrogen atom transfer (HAT) and transition metal chelation mechanisms [92]. In this current study, HAT is considered the main mechanism since no transition metals were used or formed. Authors [92] have also specified that gallic acid forms a radical at the third oxygen atom because this oxygen has the lowest bond order with its hydrogen. Moreover, oxygen radicals achieve stability, caused by nearby hydrogen groups and their impact on hydrogen bonding [91]. An important note is that gallic acid's scavenging mechanism depends on pH [93]. At highly alkaline conditions (pH 11), gallic acid demonstrates

single electron transfer as its hydroxyl groups are deprotonated. At pH 9, gallic acid undergoes HAT [93]. Overall, as the carboxyl group of gallic acid does not participate in radical scavenging, its deprotonated form with chitosan was a promising candidate for the synthesis.



Scheme 3. Gallic acid mechanism of radical scavenging (HAT). Adapted from [91].

Vitamin C or ascorbic acid, even if not a plant polyphenol, is a hydroxyl acid [77]. According to the publication [94] this lactone is widely popular among food protection strategies. It reduces the enzymatic browning of fruits and vegetables. Additionally, it inhibits lipid peroxidation and keeps the desired color of meat. Its antioxidative mechanism, as in gallic acid, involves hydroxyl groups. To be concrete, alkaline conditions deprotonate ascorbic acid (generates ascorbate), which sequentially undergoes electron (generates ascorbyl radical) and then hydrogen radical loss (generates dehydroascorbate) (Scheme 4) [95], [96]. Proposed mechanisms for radical neutralization are HAT, sequential proton loss electron transfer, and single electron transfer. Computational analysis of ascorbic acid and its various derivatives showed that the main group participating in antioxidative action is 3,4-dihydro-furan-2-one [95]. This suggests that ascorbate should perform well in radical scavenging assays, as the ascorbate is generated through deprotonation of one of its hydroxyl groups.



Scheme 4. Ascorbic acid neutralization mechanism (adapted from [95]). The light blue circle indicates the responsible structure of antioxidative action, 3,4-dihydro-furan-2-one.

1.7 Chitosan derivatives as food packaging material

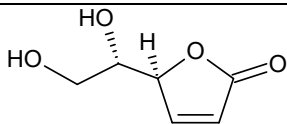
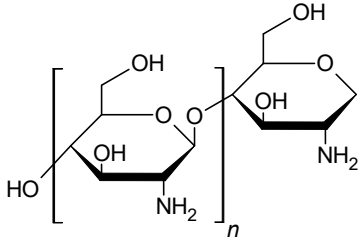
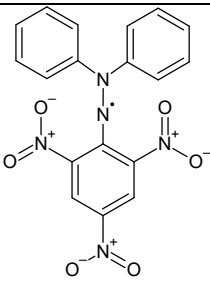
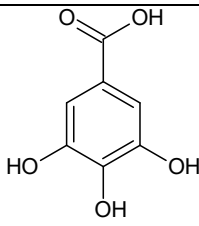
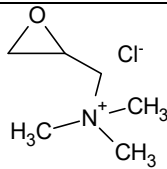
Bioactivity, biocompatibility, biodegradability, and film-forming qualities make chitosan so attractive for food/plant applications [97], [98]. In 2025, COS had already emerged as a good fruit and vegetable packaging material [98]. The COS preservation mechanism was divided into extracellular and intracellular modes. Extracellular mode is about COS acting as a protective barrier that slows metabolic activity and postpones senescence by reducing moisture evaporation and oxygen transfer. Intracellularly, penetrated COS trigger plant defense mechanisms and enhance the activity of antioxidative enzymes. In a study that was examining antioxidant effect in soy bean sprouts by DPPH• [99], COS of 1.0, 2.0, and 3.0 kDa were tested. Their concentrations varied between 0 to 1%. As it turned out, 1.0 kDa 0.01 % COS had the best radical scavenging activity. Berries were also

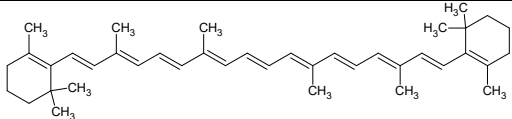
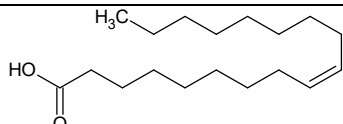
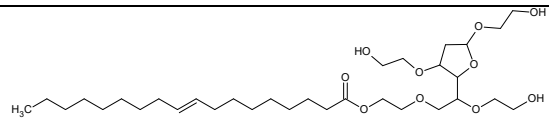
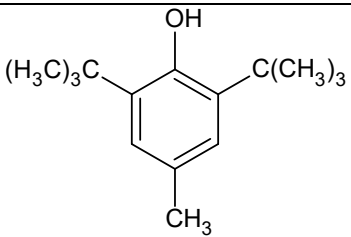
subjected to COS enhancements [98]. COS, combined with lysine by the Maillard reaction, enhanced the preservation of fresh-cut kiwi fruits. A 10-minute hold of 0.5 % conjugate solution managed to maintain a higher amount of ascorbic acid in kiwi fruits [100].

Quaternary-ammonium chitosan provides intermolecular hydrogen bonding and crystalline phase region formation – qualities that help to create a packaging material. It can be further improved with plasticizers like glycerol or sorbitol [101]. In the study of 2020, HACC and PVA were blended to obtain an antifogging and antibacterial coating [102]. HACC/PVA composite coating achieved a great antibacterial activity (up to 99 % against pathogenic bacteria) and an almost 98 % of transmittance ratio. Quaternary chitosan, merged with other polymers like gelatin or polylactic acid, also showed good properties as an alternative for food packaging [101]. Another paper was analysing modified HACC for tropical fruit preservation [103]. They created a multifunctional material of HACC, quaternary phosphonium salt, and PVA. The result, HA-CS-NP/PVA composite film, had strong water barrier (up to 110 g/m²h water vapor permeability) and mechanical (up to 20 MPa of tensile strength) qualities. All in all, many various chitosan modification strategies and their success suggest that chitosan will be widely used as a food packaging material in the future.

2. METHODS

2.1 Materials and reagents

Material/reagent name	Properties	Formula
Acetic acid (Honeywell Fluka™)	M = 60.05 g/mol $d_4^{20} = 1.049$ g/mL $T_b = 118-119$ °C	CH ₃ COOH
Ascorbic acid (Sigma-Aldrich®)	M = 176.12 g/mol $d_4^{20} = 1.65$ g/mL $T_m = 191$ °C	
Commercially available low molecular weight chitosan (Sigma-Aldrich®)	$M_v = 50 - 190$ kDa DD = 75 - 85% $T_m = 102.5$ °C	
Dialysis bags (Cellulose membrane; 1000 Da, 3000 Da, 6-8 kDa cut-off) (Merck KGaA)	-	-
DPPH• (powder) (Sigma-Aldrich®)	M = 394.3 g/mol	
Gallic acid (Sigma-Aldrich®)	M = 170.12 g/mol $d_4^{20} = 1.7$ g/mL $T_m = 258 - 265$ °C	
Glycidyl trimethylammonium chloride (GTMAC) (Sigma-Aldrich®)	M = 151.63 g/mol $d_4^{20} = 1.1$ g/mL	
Hydrochloric acid (UAB Eurochemicals)	M = 36.46 g/mol	HCl
Iron (III) chloride (Sigma-Aldrich®)	M = 162.20 g/mol $d_4^{20} = 2.8$ g/mL $T_m = 304$ °C	FeCl ₃
Isopropanol (UAB Eurochemicals)	M = 60.10 g/mol $d_4^{20} = 0.785$ g/mL $T_b = 82.3$ °C	(CH ₃) ₂ CHOH

Methanol (PanReac AppliChem ITW Reagents®)	M = 32.04 g/mol $d_4^{20} = 0.792 \text{ g/mL}$ $T_b = 64 \text{ °C}$	CH_3OH
Potassium carbonate (Merck KGaA)	M = 138.205 g/mol $d_4^{20} = 2.29 \text{ g/mL}$ $T_m = 899 \text{ °C}$	K_2CO_3
Potassium ferricyanide (Reahim)	M = 329.24 g/mol $d_4^{20} = 1.89 \text{ g/mL}$	$\text{K}_3\text{Fe}(\text{CN})_6$
Sephadex G-15 slurry	Dextran cross-linked with epichlorohydrin, 40-120 μm beads	-
Sodium hydroxide (Sigma-Aldrich®)	M = 40.00 g/mol $d_4^{20} = 2.13 \text{ g/mL}$ $T_m = 323 \text{ °C}$	NaOH
Trichloroacetic acid (Carl Roth GmbH)	M = 163.38 g/mol $d_4^{20} = 1.62 \text{ g/mL}$ $T_b = 195.5 \text{ °C}$	CCl_3COOH
Heavy water (deuterium oxide) (Sigma-Aldrich®)	M = 20.03 g/mol $d_4^{20} = 1.11 \text{ g/mL}$ $T_b = 101.4 \text{ °C}$	D_2O
Deuterated dimethyl sulfoxide (Carl Roth GmbH)	M = 84.17 g/mol $d_4^{20} = 1.19 \text{ g/mL}$ $T_b = 189 \text{ °C}$	$\text{DMSO-}d_6$
β -carotene (Sigma-Aldrich®)	M = 536.87 g/mol $d_4^{20} = 1.00 \text{ g/mL}$	
Oleic acid (Merck KGaA)	M = 282.47 g/mol $d_4^{20} = 0.89 \text{ g/mL}$ $T_b = 360 \text{ °C}$	
Tween 80 (Merck KGaA)	M = 1310 g/mol $d_4^{20} = 1.06 - 1.09 \text{ g/mL}$	
Chloroform (ChemPur)	M = 119.38 g/mol $d_4^{20} = 1.49 \text{ g/mL}$ $T_b = 61.2 \text{ °C}$	CHCl_3
Butylated hydroxytoluene (BHT) (Honeywell Fluka™)	M = 220.35 g/mol $d_4^{20} = 1.05 \text{ g/mL}$	

2.2 Synthesis methods

2.2.1 Hydrolysis of chitosan

Chitosan acid hydrolysis reactions were performed using 2 methodologies [104], [105]. Overall, 10 (1H-10H) hydrolysis reactions were performed with different acids at varying concentrations. The first two hydrolysis reactions (according to A. Bočkuvienė and J. Sereikaitė [105]) were prepared from 0.8 g of commercially available chitosan, mixed with either 25 % acetic acid and 2 M HCl or 8.2 M HCl. Reactions were carried out at 70 °C for 2 hours, and the products were purified using a Sephadex G-15 column (height – 76.5 cm, radius – 1.5 cm). The other consequential hydrolysis reactions were performed according to K. Li and colleagues [104]. They included 10 g of commercially available chitosan mixed with 6 M or 3 M HCl and distinct purification methods by either dialysis (1000 Da) or Sephadex G-15 column. Reactions were conducted in an oil bath at 70 °C for 2 hours. In all cases, the hydrolizate was neutralized with sodium hydroxide to a pH 4 or pH 5 before concentration with a rotary evaporator. After concentration, samples were purified and lyophilized for further use.

¹H-NMR (400 MHz, D₂O): δ [ppm] = 8.2-8.3 (1H, **H**-C=N, azomethine bond) 4.8-5.3 (1H, -O-**CH**-O-, H₁), 3.0-4.0 (5H, -O-**CH**(CH₂OH)-**CH**(O)-**CH**(OH)-, H₃-H₆), 2.6-3.0 (1H, -**CH**-NH₂, H₂), 1.9-2.0 (3H, -NH-C(=O)-**CH**₃). ¹³C-NMR (400 MHz, D₂O): δ [ppm] = 172 (1C, H-C=N, azomethine bond), 99 (1C, -O-CH-O-, C₁), 78-72 (3C, -O-CH(CH₂OH)-CH(O)-CH(OH)-, C₃-C₅), 60 (1C, -O-CH(CH₂OH)- C₆), 56 (1H, -CH(OH)-CH(NH₂)- C₂), 21 (1C, -NH-C(=O)-CH₃). FTIR (ATR, cm⁻¹): 3260-3340 (b, O-H, N-H of glucosamine), 2870-2890 (s, C-H of glucosamine), 1610 (s, -NH₂), 1518 (s, -NH₃⁺), 1021-1064 (s, C-O of glucosamine).

2.2.2 Quaternization of chitosan in *i*-PrOH

Quaternization of a commercial chitosan (synthesis of N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (QChit1)) was performed using glycidyl trimethylammonium chloride (GTMAC) in isopropanol according to the methodology [49] with modifications. Before quaternization, commercial chitosan (0.1 g) with isopropanol (2.48 ml) was kept at 80 °C for a day. The GTMAC water solution was added slowly (dropwise) to the reaction (dissolved chitosan with K₂CO₃ to keep pH 7-8) in an ice bath. The glycidyl: chitosan chains molar ratio was 3:1, reaction time 2 hours, temperature 60 °C. After the alkylation, the products were dialyzed with a 3000 Da cut-off dialysis bag and lyophilized.

¹H-NMR (400 MHz, D₂O): δ [ppm] = 4.5 (1H, -O-**CH**-O-, H₁), 4.3 (-**CH**(OH)-CH₂-, H_b), 3.5-4.0 (5H, -O-**CH**(CH₂OH)-**CH**(O)-**CH**(OH)-, H₃-H₆), 3.4 (-CH(OH)-CH₂-, H_c), 3.2 (9H, -N(CH₃)₃⁺, H_d), 3-2.5 (2H, -NH-CH₂-CH(OH)-, H_a), 2.5 (1H, -**CH**-NH-, H₂), 2.0 (3H, -NH-C(=O)-CH₃). FTIR (ATR, cm⁻¹): 1488 (CH₃ of -N(CH₃)₃⁺).

2.2.3 Quaternization of chitosan in water

Quaternization of a commercial chitosan (synthesis of N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (QChit2)) and COS-5H-5 (Q02H) was performed using glycidyl trimethylammonium chloride (GTMAC) in distilled water according to the methodology [106] with

modifications, when 3 grams of lyophilized sample was mixed with 30 ml of distilled water and left at 55 °C to mix for a day. The GTMAC solution was added slowly (dropwise) to the reaction (dissolved chitosan with K₂CO₃ to keep pH 7-8) in an ice bath. The glycidyl: chitosan chains molar ratio was 5:1, reaction time 5 hours, temperature 85 °C. After the reaction, products were dialyzed with a 3000 Da cut-off dialysis bag and lyophilized.

¹H-NMR (QChit2, 400 MHz, D₂O): δ [ppm] = 4.5 (1H, -O-CH-O-), 4.3 (-CH(OH)-CH₂-, H_b), 3.5-4.0 (5H, -O-CH(CH₂OH)-CH(O)-CH(OH)-, H₃ – H₆), 3.4 (2H, -CH(OH)-CH₂-, H_c), 3.2 (9H, -N(CH₃)₃⁺, H_d), 3.1 (1H, -CH-NH₂), 3-2.5 (2H, -NH-CH₂-CH(OH)-, H_a), 2.5 (1H, -CH-NH-, 2H), 2.0 (3H, -NH-C(=O)-CH₃). ¹³C-NMR (QChit2, 400 MHz, D₂O): δ [ppm] = 102 (1C, -O-CH-O-, C₁), 79-70 (3C, -O-CH(CH₂OH)-CH(O)-CH(OH)-, C₃-C₅), 69 (1C, (-C(OH)-CH₂-, C_c), 65 (-CH(OH)-CH₂-, C_b), 62 (1C, -CH-NH₂, C₂⁺), 60 (1C, -O-CH(CH₂OH)-, C₆), 54 (N(CH₃)₃⁺, C_d), 52 (1C, -NH-CH₂-CH(OH)-, C_a). FTIR (QChit2, ATR, cm⁻¹): 1475 (CH₃ of -N(CH₃)₃⁺).

2.2.4 Chloride counterion substitution to ascorbate

Counterion substitution to ascorbate was conducted solely by dialysis under an inert atmosphere [107], [108]. For 0.5 g of commercially available chitosan, 6-8 kDa cut-off dialysis bags were used (product – chitosan ascorbate, A-ComCh), as for 1 g of its quaternized substitute (QChit2, product – quaternized chitosan ascorbate, A-QChit2). For 0.2 grams of hydrolysed 5.6 kDa chitosan, 1000 Da cut-off dialysis bags were used (product – chito-oligosaccharide ascorbate, A-COS). The ascorbic acid solution in excess was prepared separately in water and added later in a dialysis bag already containing modified or unmodified chitosan and deionized water (all under an inert atmosphere, N₂).

¹H-NMR (A-ComCh and A-COS, 400 MHz, D₂O): δ [ppm] = 4.5 (1H, -CH-CH(OH)-CH₂OH, ascorbic acid lactone proton, H_a), 4.0 (1H, CH-CH(OH)-CH₂OH, ascorbic acid glycol proton, H_b), 3.70 (2H, CH-CH(OH)-CH₂OH, ascorbic acid glycol protons, H_c). ¹H-NMR (A-QChit2, 400 MHz, D₂O): 4.5 (1H, -CH-CH(OH)-CH₂OH, ascorbic acid lactone proton, H_a), 4.0 (1H, CH-CH(OH)-CH₂OH, ascorbic acid glycol proton, H_b), 3.70 (2H, CH-CH(OH)-CH₂OH, ascorbic acid glycol protons, H_c), 3.4 (2H, -CH(OH)-CH₂-, 2-hydroxypropyl trimethyl ammonium chloride methylene protons, H_c⁺), 3.2 (9H, -N(CH₃)₃⁺, 2-hydroxypropyl trimethyl ammonium chloride methyl protons, H_d⁺), 3.0-2.8 (2H, -NH-CH₂-CH(OH)-, 2-hydroxypropyl trimethyl ammonium chloride methylene protons, H_a⁺). FTIR (ATR, cm⁻¹): 1720 (s, C=O of ascorbic acid).

2.2.5 Chloride counterion substitution to gallate

Counterion substitution with gallate was conducted similarly to that described in 2.2.4. [107], [108]. The gallic acid powder in excess was added later in a 6-8 kDa cut-off dialysis bag already containing 2 % w/v chitosan in HCl (product – chitosan gallate, G-ComCh) or a 1000 Da dialysis bag containing hydrolysed 1.1 kDa chitosan (product – chito-oligosaccharide gallate, G-COS).

¹H-NMR (G-ComCh, 400 MHz, D₂O): δ [ppm] = 6.9 (1H, CH-C-COOH of gallic acid). FTIR (G-ComCh, ATR, cm⁻¹): 1525 (b, -NH₃⁺), 1462 (-COO⁻ of gallic acid), 2925/2851 (s, C-H), 1391/1396/1230 (s, phenolic C-O, aromatic C-C), 732/720 (O-H, phenolic). FTIR (G-COS, ATR, cm⁻¹): 1529 (b, -NH₃⁺), 1460 (-COO⁻ of gallic acid), 2925/2851 (s, C-H), 1391/1260/1230 (s, phenolic C-O, aromatic C-C), 732/720 (O-H, phenolic).

2.3 Analysis methods

2.3.1 NMR Spectroscopy

Both ^1H and ^{13}C spectra were recorded using a Bruker 400 Ascend™ 400 MHz NMR spectrometer. The experiments were run at 23 °C using D_2O or $\text{DMSO-}d_6$ solvents. To enable accurate determination of compounds, internal standards were either acetone or $\text{DMSO-}d_6$. Chemical shifts of the signals were expressed in parts per million (ppm).

The degree of quaternization (DQ) of QChit1 and QChit2 was determined by ^1H NMR spectroscopy. DQ was calculated from NMR spectra by the equation:

$$\text{DQ} = \text{AR} \cdot \frac{\int 3.17}{\int 2.00}, \quad (1\text{A})$$

where the acetylation ratio (AR) is defined as the molar ratio of GlcNAc to GlcN units; $\int 3.17$ and $\int 2.00$ are integrals of these signals assigned to the N,N,N-trimethyl signal at δ 3.17 ppm and the methyl protons of the acetyl group of N-acetylglucosamine units, respectively. The quaternization degree of Q02H was calculated from ^1H NMR spectra based on the equation below (1B):

$$\text{DQ} = \frac{\int 3.3-3.49}{\int 2.9-3.2} \times 100\% \quad (1\text{B})$$

where $\text{N}(\text{CH}_3)_3^+$ is the integral of the trimethyl substitute proton signal at approximately 3.3 – 3.4 ppm and H_2 – the integral of the proton signal in position 2 of glucosamine units at approximately 2.9–3.2 ppm.

The relative amount of imine groups was estimated from the integral ratio between the imine proton signal at 8.2 ppm and the CH_2 signal at the C2 position of the glucosamine unit (Figure S1):

$$\text{Imine index} = \frac{\int 8.2}{\int 3.0} \quad (2)$$

The integral areas in ^1H NMR spectra were used to quantify the substitution degrees of ascorbate (SD) in chito-oligosaccharide ascorbate, and the value was calculated using the following equation (3) [109]:

$$\text{SD} = \frac{\int 4.5}{\int 2.9} \quad (3)$$

Meanwhile, in the case of commercial chitosan and its quaternized compounds, the substitution degrees of ascorbate were estimated by equation (4):

$$\text{SD} = 3 \cdot \text{AR} \cdot \frac{\int 4.5}{\int 2.1} \quad (4)$$

where the acetylation ratio (AR) is defined as the molar ratio of GlcNAc to GlcN units; $\int 4.5$ and $\int 2.1$ are integrals of these signals assigned to lactone C-H_a in ascorbate and the methyl protons of the acetyl group of N-acetyl glucosamine units, respectively. $\int 2.9$ is the integral of the H_2 signal in the chito-oligosaccharide spectrum.

2.3.2 FTIR Spectroscopy

FTIR spectra were acquired with a Perkin-Elmer Frontier 65 spectrometer using Universal ATR Sampling Accessory. The instrumental settings were as follows: 500 scans, a resolution of 2 cm⁻¹, and a scan range of 550–4500 cm⁻¹.

2.3.3 SEC measurement

The polymer characteristics, such as number average and weight average molecular weights, and dispersity, when a dispersity $\bar{D}$ equals the weight average molecular weight divided by the number average molecular weight, were analyzed by size exclusion chromatography, SEC. The eluent for the chitosan sample (10-15 mg/ml) was the 250 mM sodium acetate buffer (pH 4.5). The analysis was conducted with an A6000 M General Mixed 300x8.0 mm column at 30 °C with a flow rate of 0.5 ml/min. Samples were detected by the Viscotek TDMax (Malvern) detection system.

2.3.4 Determination of antioxidant activity by DPPH• scavenging

The antioxidant activity of synthesized compounds was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay [15], [110]. 2 mg/ml solutions of the lyophilized sample were prepared in small round-bottomed flasks. Then, 2 ml of the latter solution was mixed with 2 ml of 0.10 mM DPPH• solution in methanol. After 30 minutes of incubation at room temperature, the mixtures were measured at 517 nm (A_1). If needed, they were additionally filtered before the measurement. In the blank group (A_0), lyophilized samples were exchanged for distilled water. The results were calculated as follows (5):

$$\text{Antioxidant activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (5)$$

2.3.5 Reducing power

The reducing power analysis was performed by the established method with some modifications [111]. First, different reagents were prepared: 5 % w/v TCA, 0,05 % w/v FeCl₃, 0,5 % w/v K₃Fe(CN)₆, and 0,05 M phosphate buffer (pH = 6.6). For the analysis, 0.6 mg/ml of the lyophilized solution of samples was prepared using phosphate buffer as a solvent. Then, 0.5 ml of this solution was mixed with 1.25 ml of phosphate buffer and 1.25 ml of K₃Fe(CN)₆ solution. Mixtures were incubated at 50 °C for 20 minutes. After that, 1.25 ml of TCA solution was added to each of the reaction vials. It was followed by filtration with 0.2 µm filters. 3 ml of the filtered solution was then mixed with 250 µl FeCl₃ solution, and after 10 minutes, measured at 700 nm (A_1). In the blank group (A_0), lyophilized samples were exchanged for distilled water. The results were calculated as follows (6):

$$\text{Reducing power (\%)} = \frac{A_1 - A_0}{A_0} \times 100 \quad (6)$$

2.3.6 β-carotene bleaching method

The β-carotene bleaching method was carried out according to the report [112]. 0.5 mg of β-carotene was mixed with 25 µl of oleic acid and 200 µl of Tween 80. Then, the mixture was supplemented

with 1 ml of chloroform. At 25 °C, this solution was evaporated (under vacuum) using a rotary evaporator. Right before the analysis, the concentrate was dissolved in distilled water. All actions were done under an inert atmosphere (N₂). For analysis, 2.5 ml of the resulting β-carotene solution was mixed with 0.5 ml of the sample; samples were measured at 470 nm (A₀). Mixtures were incubated at 50 °C for 1 hour and then measured again at 470 nm (A₁). The blank group contained distilled water instead of the sample (A₀' before 1 hour, A₁' after 1 hour of incubation). Positive control was BHT. The β-carotene bleaching inhibition was calculated according to formula (7):

$$\beta - \text{carotene bleaching inhibition (\%)} = 1 - \frac{A_0 - A_1}{A_0' - A_1'} \times 100 \quad (7)$$

2.4 Modified chitosan compounds application to berries

To evaluate the preservation efficiency of synthesized compounds, they were tested on the local berries – blueberries (*Vaccinium*) and raspberries (*Rubus idaeus*), purchased at the local market in Vilnius, Lithuania. Both kinds of berries were treated with 2 % aqueous solution of the sample. For the analysis, four replicates of every treatment were used. Each berry was immersed in a distinct solution and left at room temperature for weight evaluation. Weight was taken at the beginning of the treatment and after 3 and 5 days.

3. RESULTS AND DISCUSSION

3.1 Preparation of chito-oligosaccharides

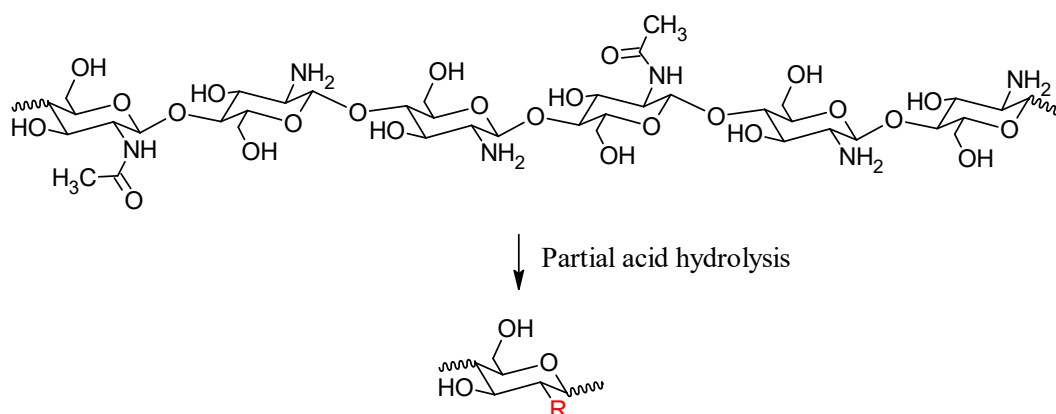
3.1.1 Acidic hydrolysis of chitosan

In the present part of the study, commercial chitosan was hydrolyzed using 6 M and 3 M hydrochloric acid. The obtained neutral hydrolysates of chito-oligosaccharides (COS) were transferred to gel filtration on Sephadex G-15. Although Sephadex G-15 is commonly applied for desalting, under suitable conditions, it enabled controlled fractionation of low-molecular-weight COS. Molecular weights of the products were determined by a size-exclusion chromatograph. COS products were analyzed by UV-Vis, NMR, and FTIR spectroscopies.

A general view of chitosan hydrolysis is depicted in Scheme 5 [113], [114]. Chitosan, depending on the acid hydrolysis conditions, can be hydrolyzed to partly or fully deacetylated oligosaccharides/chito-oligosaccharides, having various polymerization degrees. Chitosan hydrolysis was performed under acidic conditions using 6 M HCl with a chitosan concentration of 10 % at 70 °C for 2 hours according to the Li method [104] (Table 1). Moreover, the hydrolysis of chitosan was carried out at subtle conditions at 3 M HCl. To maintain proper conditions, the reaction mixture was stirred constantly, and the temperature was monitored accordingly.

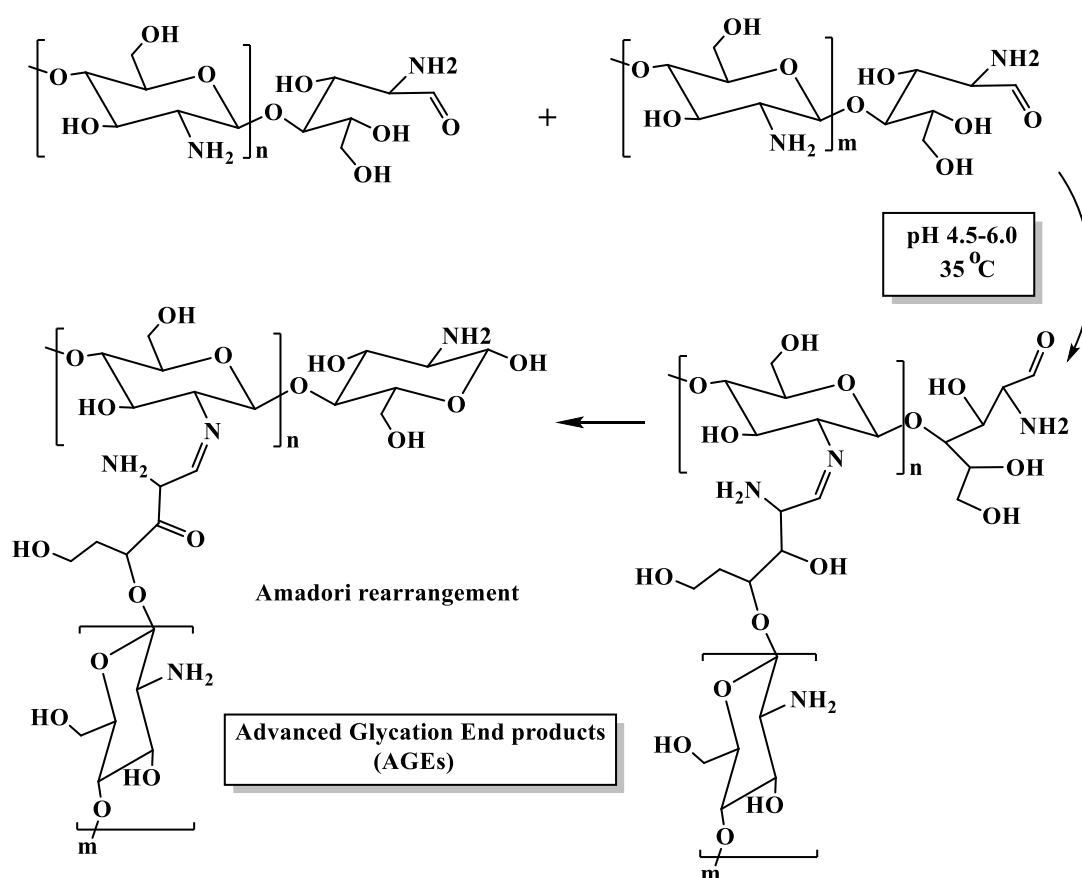
Table 1. Chitosan hydrolysis cases: 10 % at at 70 °C for 2 hours.

Sample ID	HCl, M	Preparation before purification	Purification method
1H	6	Adjusted to pH 4	Sephadex G-15 column
2H	6	Adjusted to pH 5; Concentrated under reduced pressure (20 mbar) at 35°C for 30 min	
3H	6	Adjusted to pH 5; concentrated under reduced pressure (20 mbar) at 35 °C for 60 min	
4H	3	Adjusted to pH 5; concentrated under reduced pressure (20 mbar) at 35 °C for 60 min	
5H	3		Dialysis tubes



Scheme 5. The visual representation of chitin partial hydrolysis. R = NH₂ or NHAc.

Before the purification with a sorbent, COS hydrolyzates were neutralized with sodium hydrocarbonate under cooling (Table 1). Moreover, in all cases after the neutralization, partial precipitation of COS occurred. The precipitates were isolated by centrifugation, redissolved, and dialyzed against deionized water. The supernatants of 2H-4H cases were concentrated by rotary evaporator. It is notable that when the neutralized hydrolysis products were concentrated using a rotary evaporator, browning/partial blackening of the mixture was observed. Such color development is commonly associated with Maillard-type reactions occurring between amino groups and carbonyl groups (Scheme 6). In acidic hydrolysates, short COS fragments may contain reducing ends capable of acting as carbonyl donors, while residual amino groups of chitosan serve as nucleophiles. Their interaction may lead to Schiff base formation and subsequent browning [115], [116]. The intensity of browning is influenced by the degree of polymerization and pH, which determine the balance between early-stage imine formation and further rearrangement products.



Scheme 6. The Maillard reaction and Amadori rearrangement during chitosan hydrolysis solution browning.

In the final stage, chito-oligosaccharides were purified by gel filtration using Sephadex G-15. The sorbent enables both desalting and collecting fractions of a neutral COS hydrolysate. The Sephadex G-15 molecular weight exclusion limit is 1.500 kDa, and thus this molecular sieve was suitable for further fractionation of peptides in the range of 1–2 kDa [117]. Therefore, this sorbent is suitable for the fractionation of low-MW COS. Four fractions were obtained (test tubes: 7, 8–10, 11–12, and 13–14), and the corresponding elution profile is depicted in Figure 7. The volumes of fractions were 150, 200, 230, and 260 ml, respectively.

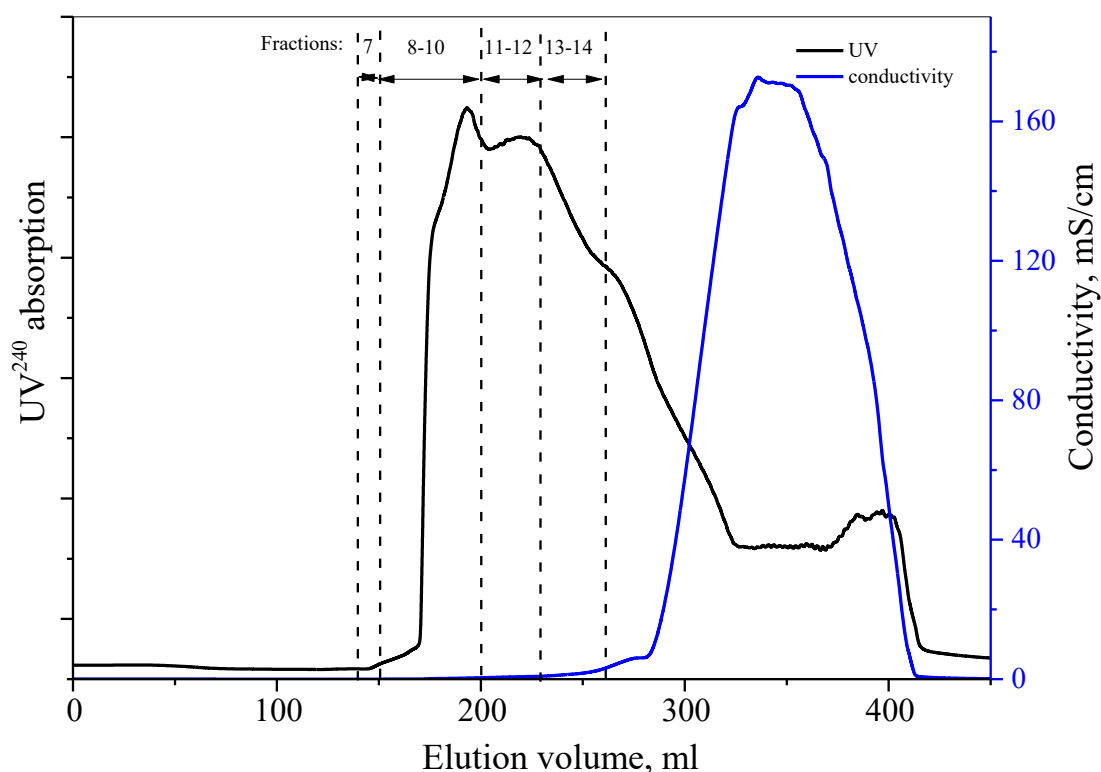


Figure 7. Gel filtration profile of hydrolysed COS-1H adjusted at pH 4 and carried out on a Sephadex G-15 packed column.

Figure 8 presents the MW distribution curves of hydrolyzates 1H and 3H. They exhibit a broad MW range from 0.5 to 10.7 kDa, suitable for choosing fragments of desired molecular weight for further tasks. They also contain low dispersity indexes ($\bar{D}$) from 1.1 to 1.3. In cases of COS-1H-10 and COS-3H-3, $\bar{D}$ are a little higher, 1.5 and 1.7. In contrast, the purification of COS-5H using only dialysis tubes with different cut-offs (cut-offs 8.0 and 1.0 kDa) resulted in two fractions with MW values of 11.2 and 2.8 kDa and higher $\bar{D}$ of 1.6 and 2.2, respectively, than those obtained after Sephadex G-15 fractionation (Table 2). The obtained COS fractions were suitable for structural analysis and antioxidant evaluation, and presented for further modification.

Generally, chitosan products' molecular weight is reduced using higher acid concentrations, temperatures, and longer times [118]. However, some authors report the wide range of molecular masses received from this type of reaction. For example, it is stated that the same conditions can result in very different depolymerization and deacetylation patterns of structurally similar structures. During hydrolysis, the reagent does not have uniform access to all glycosidic bonds. Effects of temperature, crystallinity, and moisture play a far more significant role than was originally believed [119]. Others mention that even if harsh acidic conditions perform depolymerization efficiently, chito-oligosaccharides' purification and separation are complex [114]. In this study, on the other hand, the fractionation of COS with Sephadex G-15 was uncomplicated and allowed to gather different COS fractions.

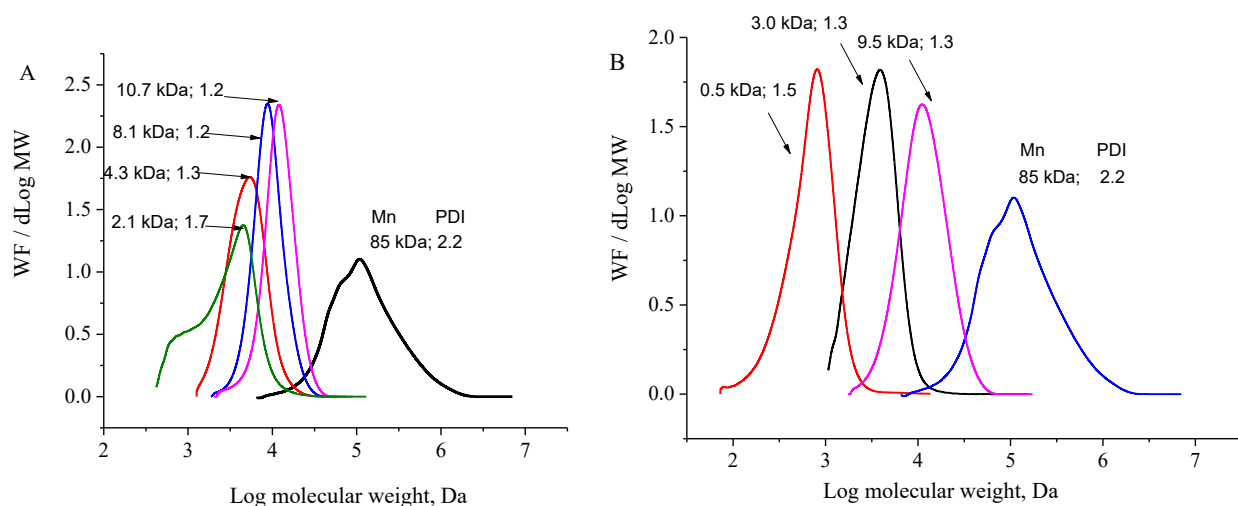


Figure 8. Molecular weight distributions after the fractionation of hydrolysate 1H (A) and 3H (B).

Table 2. Results of acidic hydrolysis and COS fractionation.

	Sample ID	Mn, kDa	Đ	DP	Imine index
	ComCh	84.7	2.2	384	-
1	¹ COS-1H-25	5.5	1.3	25.0	-
	COS-1H-36	8.1	1.2	36.7	-
	COS-1H-19	4.3	1.3	19.5	-
	COS-1H-10	2.1	1.7	2.8	0.004
2	¹ COS-2H-25	5.3	1.2	25	-
	COS-2H-16	3.7	1.3	16.8	0.2
	COS-2H-5	1.1	1.3	5.1	0.1
3	¹ COS-3H-43	9.5	1.2	43.0	-
	COS-3H-13	3.0	1.3	13.6	0.1
	COS-3H-4	0.88	1.2	4.0	0.032
	COS-3H-3	0.6	1.5	2.7	-
4	COS-4H-8	1.8	1.5	8	-
	COS-4H-4	0.9	1.36	4	-
15	COS-5H-51	11.2	1.6	50.8	-
	COS-5H-13	2.8	2.2	12.6	-

¹the dialysis against deionized water was performed.

Some NMR spectra exhibit a small signal at approximately 8.2 ppm, possibly corresponding to an imine proton. The relative amount of imine groups was estimated from the integral ratio between the imine proton signal at 8.2 ppm and the CH₂ signal at the C2 position of the glucosamine unit. Among the investigated samples, a series of COS-3H exhibited the highest imine indices, reaching 0.032 and 0.10, respectively (Table 2).

The additional evidence of Schiff base formation is shown in Figure 9. COS-3H-4 and COS-1H-19 showed two distinct bands in the 250–280 nm and 300–330 nm regions. According to Jarzabek and coworkers [120] Schiff base model compounds in methanol/chloroform/DMA solvents exhibited two absorbance bands at 250–280 nm (λ_1) and 320–380 nm (λ_2). Moreover, Hassan [121] reported clear differences between chitosan and its corresponding Schiff base derivatives in UV–Vis spectra.

Chitosan coupled with indole-3-carboxaldehyde (Schiff base I) and chitosan coupled with 4-dimethylaminobenzaldehyde (Schiff base II) showed significantly increased absorbance at approximately 247 nm and 361 nm. Importantly, 2 peaks should be visible of early Maillard reaction products. As the reaction proceeds, the absorbance shifts to 320 nm, as observed for COS-3H-13.

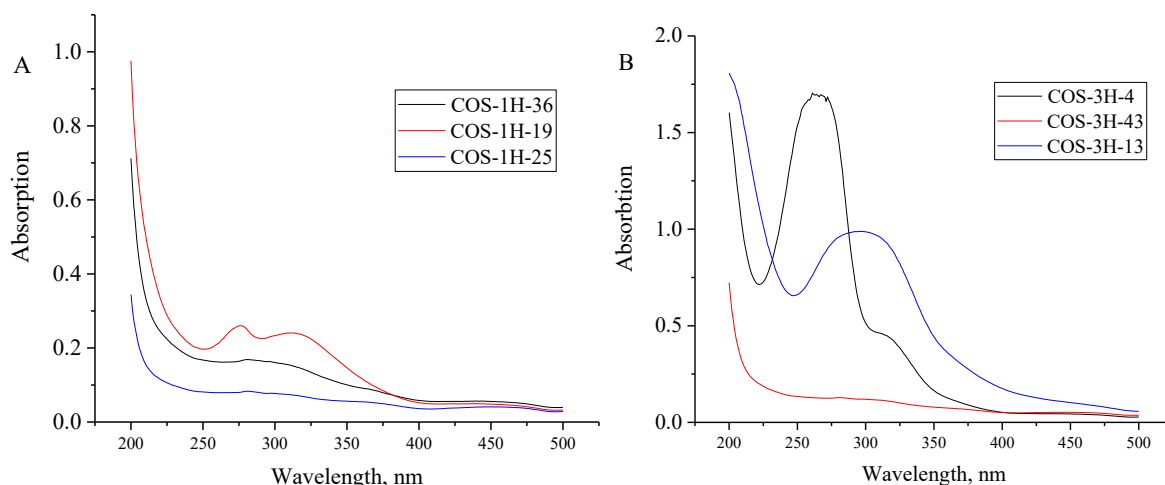


Figure 9. UV-Vis spectra of COS-1H (A) and COS-3H (B) with different molecular weights of COS. [COS-nH] = 0.5 mg/ml; n 1 or 3.

The ^1H NMR spectrum (Figure S1A) showed signals between 3.2–4.0 ppm, assigned to H2–H6 of GlcN and GlcNAc units. A doublet at 5.29 ppm (coupling constant $J = 3.0$ Hz) corresponds to the anomeric H α proton of COS with β -1 $\rightarrow$ 4 glycosidic linkages [122]. DMSO- d_6 was selected as a solvent to improve the solubility of imine derivatives and unsaturated substituents. However, the ^1H NMR spectrum recorded in DMSO- d_6 shows broad signals for COS-1H-36 (Figure S1B). This broadening is attributed to the higher viscosity of DMSO- d_6 and slower molecular motion compared with D_2O . A broad resonance is observed between 8.0 and 5.5 ppm. It may arise from the hydrogen bonding of hydroxyl groups of sugars and slow H/D exchange in an organic solvent [123]. Both spectra exhibit a small signal at approximately 8.2 ppm, possibly of an imine group [124]. The ^{13}C NMR spectrum (Figure S1C) shows a signal at 98.9 ppm, assigned to the anomeric carbon. Signals at 78.0, 75.5, and 71.2 ppm correspond to C4, C5, and C3, respectively. The resonance at 60.2 ppm is assigned to C6 (methylene carbon), and the signal at 56.3 ppm corresponds to C2. Additional signals at 21.4 and 172.4 ppm are attributed to the methyl and carbonyl carbons of GlcNAc. After COS fractionation, the low-MW samples retained the same characteristic chemical shifts as the original chitosan. However, due to the increased molar fraction of terminal units relative to internal glucosamine residues, the signals corresponding to both non-reducing and reducing (α and β) terminal units became clearly distinguishable in the NMR spectra. In the ^1H – ^{13}C HSQC spectrum of COS-3H-4 (Figure S2), anomeric cross-peaks were observed at $\delta\text{H}/\delta\text{C}$ 5.25/88.5 ppm and 4.70/98.8 ppm, corresponding to the α - and β -hemiacetal configurations of the reducing terminal units. The stronger signal is attributed to the predominant α -form. An additional correlation at $\delta\text{H}/\delta\text{C}$ 2.92/54.0 ppm was assigned to H2/C2 of α -terminal glucosamine bearing a free amino group, further confirming the presence of reducing ends and distinguishing them from internal residues.

The spectra of COS-3H-4 and COS-3H-3 are not shown in this work; however, they exhibit the same spectral profile as COS-3H-4. Signals at 4.46, 3.95, and 3.67 ppm are assigned

to vicinal hydroxylated sugar protons. In the ^1H - ^{13}C HSQC spectrum, these resonances correlate with carbons in the 70–75 ppm region, characteristic of C3 and C4 in glucosamine units. Sequential ^1H - ^1H COSY cross-peaks confirm their mutual coupling and establish the 3–4 backbone connectivity typical of $\beta(1\rightarrow4)$ structures. After COS fractionation, the relative intensity of these correlations increases due to the higher molar fraction of terminal units, allowing clearer identification of the vicinal diol system. The proposed structure of low MW COS is consistent with the model shown in Figure 10, where reducing and non-reducing terminal units are present, together with a vicinal diol, an imine group, and a double bond within the glucosamine backbone.

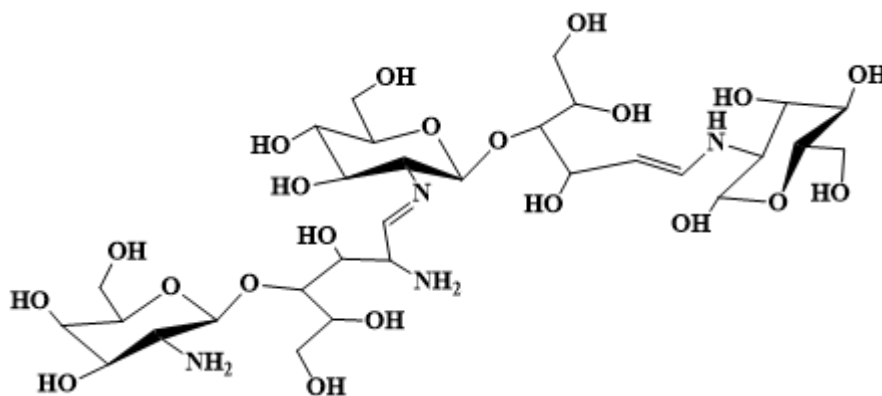


Figure 10. Proposed structure of low molecular weight COS.

FTIR spectra confirmed the structure of chito-oligosaccharides (Figure 11B). Compared to commercially available chitosan (Figure 11A), both of the structures have a sharp peak at 1029-1064 cm^{-1} (representing C-O (C6)/C-O-C stretching; around 1027–1073 cm^{-1} peaks are also attributed to C3-OH and C6-OH [125], 2871–2891 cm^{-1} (representing C-H symmetric and asymmetric stretching of methylene), and a broad peak at approximately 3300 cm^{-1} (representing O-H and N-H stretching). Chito-oligosaccharide peaks of N-H and O-H vibrations, compared to chitosan, are relatively more intense, indicating changes in hydrogen bonding. In this case, it may be due to the amine and hydroxy groups, exposed after the molecular weight reduction [126], [127]. Moreover, spectra show peaks at 1610 cm^{-1} (amino group) and 1517–1520 cm^{-1} (protonated ammonium group) [128]. Chito-oligosaccharides (Figure 11B) have more protonated ammonium groups than chitosan.

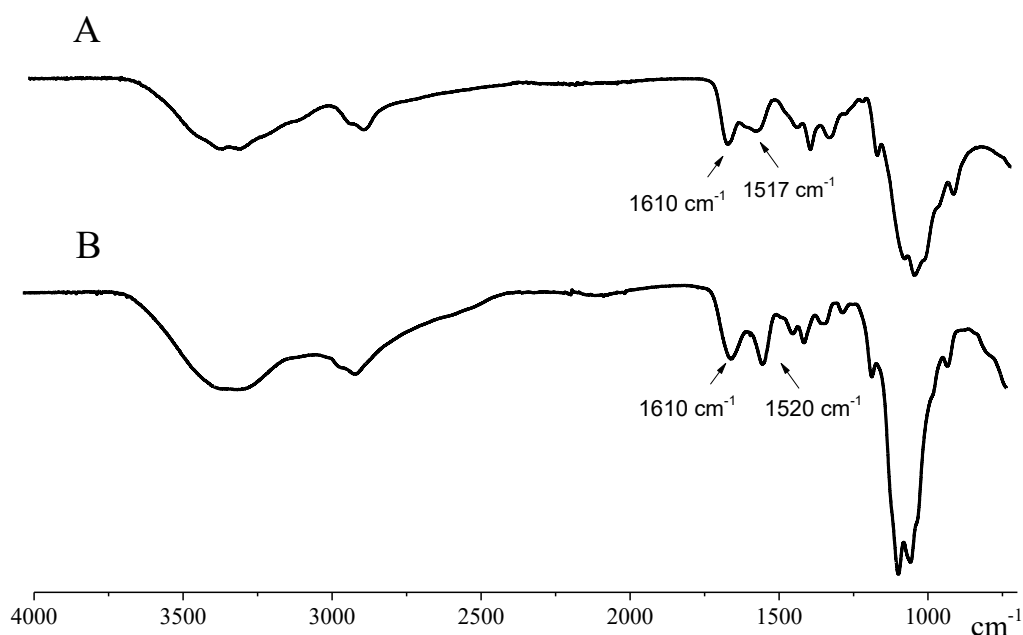


Figure 11. FTIR spectra of chitosan (A) and chito-oligosaccharide (B, COS-5H-13).

3.1.2 COS antioxidant activity and reducing power

Small COS, as previously reported in a literature review, usually show a higher antioxidant activity [58]. The antioxidant activity and reducing power of chito-oligosaccharides are shown in Figure 12. Both antioxidant activity (Figure 14A) and reducing power (Figure 14B) were the best of COS-3H-13 (81 % and 86 %, respectively) and COS-3H-4 (77 % and 58 %, respectively). These compounds, as it was described before, are Schiff bases. Schiff bases are capable of several antiradical mechanisms and were shown to provide excellent antioxidant activity [135]. Importantly, COS-3H-4 is speculated to form AGEs (Scheme 6, Figure 9). In our case, AGEs were less effective than primary Maillard reaction products. Looking back at imine indexes (Table 2) COS-3H-13 had a higher imine index among tested compounds (0.1) while COS-3H-4 had a lower index, 0.032. Therefore, the imine groups, present on the COS backbone, directly affect antioxidant activity. Regarding the DPPH radical scavenging, COS-4H-4 and COS-4H-8 (34 % and 41%, respectively) were better than commercial chitosan (22 %) and COS-5H-13 (13 %). COS-5H-13 is a bigger chito-oligosaccharide than both COS-4H chito-oligosaccharides and has a higher polydispersity index. The reasons why COS-5H-13 has lower activity than commercial chitosan require more studies, but it is possibly affected by other factors (pH, temperature, ionic strength). Reducing power of commercial chitosan (7.8 %), COS-5H-13 (5.2 %), COS-4H-4 (16 %), and COS-4H-8 (9.8 %) was also distributed unevenly. COS-4H-4, compared to COS-4H-8, is more effective at reducing power; experiments indicate that it may be more effective in other radical studies ($O_2^{\bullet-}$, H_2O_2).

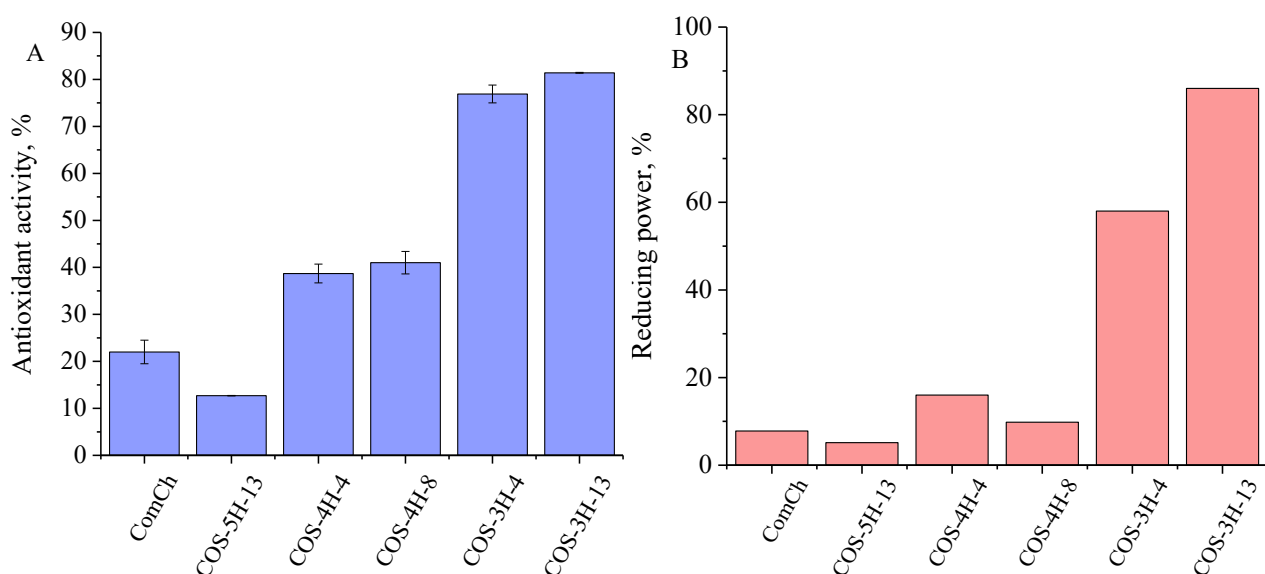


Figure 12. Antiradical activity (AA) (A) and reducing power (RP) (B) of commercial chitosan and COS derivatives were evaluated by the DPPH assay (2 mg/mL) and reducing power assay (0.6 mg/mL), respectively.

Other COS antioxidant activity and reducing power are depicted in Figure 13. The highest antioxidant (Figure 13A) and reducing power (Figure 13B) activity was of the smallest COS, COS-2H-16, 46.6 % and 128 %, respectively. Higher DP COS were not effective antioxidants, as described in the literature review. COS with DP less than 20 has significantly higher antioxidant activity, which is what we see in our results. Interestingly, COS-2H-25 showed good reducing power activity. However, it is significantly lower than the lowest tested DP COS and continues to decrease with increasing DP.

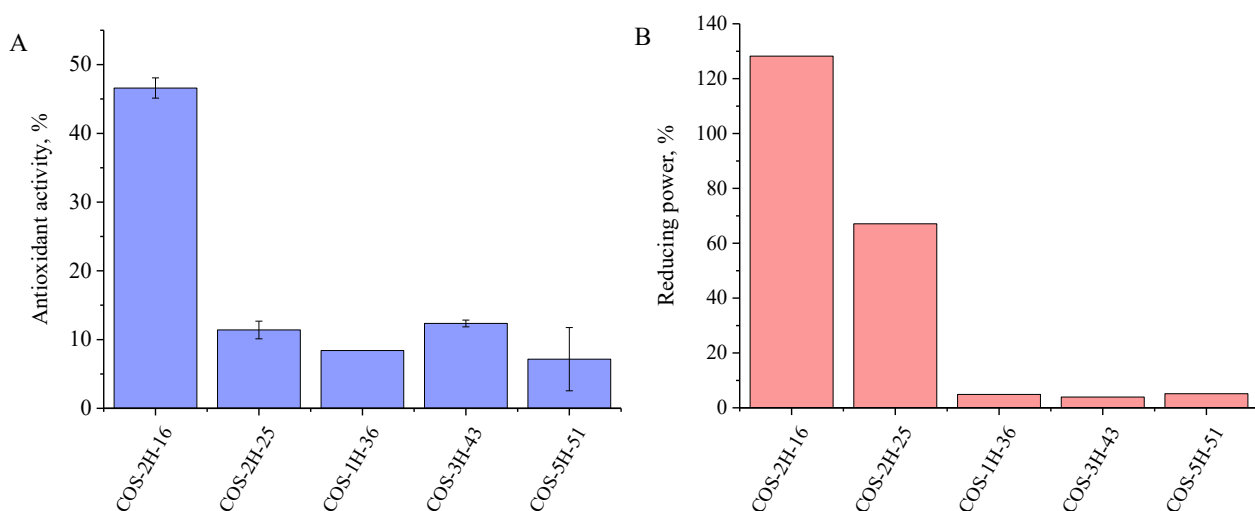
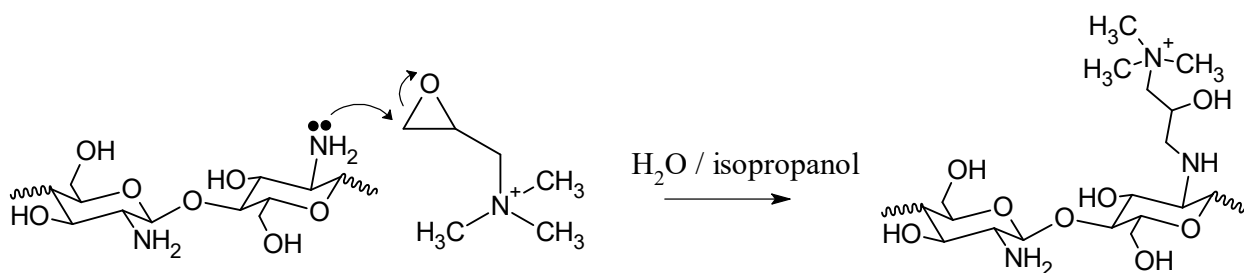


Figure 13. Evaluation of antiradical activity of chito-oligosaccharides with different DP (COS-2H-16, COS-2H-25, COS-1H-36, COS-3H-43, and COS-5H-51) by the DPPH radical scavenging method: sample concentration 2 mg/ml (A) and evaluation of reducing power, sample concentration 0.6 mg/ml (B).

3.2 Synthesis of quaternized chitosan and COS derivatives with selected counterions

3.2.1 Quaternization of chitosan and chito-oligosaccharides

In the next part of the study, quaternized chitosan and its low-MW derivatives were synthesized. The resulting product with *N*-(2-hydroxy-3-trimethylammonium)propyl] glucosamine units is less sensitive to pH changes due to the permanently charged quaternary ammonium groups and is ionized at any pH [75]. During quaternization, commercial chitosan was mixed slowly with glycidyl trimethylammonium chloride (GTMAC) to obtain QChit1 and Q-COS2H-25 (abbreviated as Q02H) in deionized water [49], and QChit2 in *i*-PrOH [106] under different conditions, as described in the methodology part 2.2.3 method and 2.2.2. method, respectively. Q02H was synthesized from fractionated COS-2H-25 (Table 1). Quaternization of glucosamine units proceeds through nucleophilic attack of the free amino group on the GTMAC epoxide ring (Scheme 7) [129]. Potassium carbonate was added to maintain the amino groups in their unprotonated form. It is worth mentioning that *O*-substitution is possible but less favorable [75]. The reaction parameters are summarized in Table 3.



Scheme 7. The mechanism of quaternization with GTMAC.

In summary, Table 3 represents the quaternization conditions and outcomes of all three quaternization products. Solvent, temperature, and molar ratio were adopted from the literature [49], [106]. An alternative quaternization method using ionic liquid (EMIMOAc) was also examined [130]. Unfortunately, the reaction did not proceed homogeneously. The resulting reaction mixture exhibited extremely high viscosity, suggesting possible interchain crosslinking of chitosan.

According to Table 3, the number-average molecular weight (*M_n*) decreased significantly after quaternization, especially for QChit1, given that the initial *M_n* of commercial chitosan was 85 kDa. A similar trend has been reported for *N*-trimethyl chitosan chloride [131], where elevated temperatures and a strong alkaline environment contributed to polymer chain degradation. Although our used potassium carbonate is a weaker base than sodium hydroxide, it may still promote partial depolymerization under prolonged or elevated-temperature conditions. Another group of scientists [132] methylated chitosan and its derivatives with iodomethane and sodium hydroxide in *N*-methyl pyrrolidone. The methylation condition also resulted in a decrease in molecular weight, which was attributed to oxidative degradation and alkaline depolymerization. Furthermore, the degree of quaternization (DQ) showed an inverse relationship with *M_n*, especially at extended reaction times. For the QChit1 product, *M_n* reduction was more pronounced in isopropanol than in water. The lower polarity of isopropanol may limit polymer solubility and increase susceptibility to alkaline degradation. In addition, the swelling temperature of chitosan reached 85 °C, which likely promotes chain scission of chitosan.

Nevertheless, Mn reduction was not a critical problem since the aim was to achieve a high degree of quaternization rather than preserving the initial molecular weight. In the future, the investigation of the reduction of Mn would be beneficial for improved reaction control. However, further steps of the study will therefore focus on QChit2, as its high molecular weight ensures a clear separation from Q02H and other COS.

Due to this structural difference, the DS of QChit1, QChit2 was calculated using equation 1A (2.3.1 method part), whereas equation 2B was applied for a low MW COS. Degrees of quaternization (DQ) of the glucosamine unit in QChit1 and QChit2 were close to 1, indicating quaternization at the amino groups, as shown in Scheme 7. In contrast, the Q02H case is more complex, having 3 available substitutions: one amino and two hydroxyl groups. Although *O*-substitution is possible, it is less favorable under typical reaction conditions. The Q02H sample has a low molecular weight, is more soluble, and more flexible than the commercial chitosan. As a result, its nucleophilic functional groups are more accessible for quaternization [133].

Table 3. Quaternization conditions and parameters of QChit1, QChit2, and Q02H

Sample	[Glycidyl]/[Gln unit] molar ratio	Solvent	Temperature, °C	Mn, kDa	DQ of Gln unit
¹ QChit1	3/1	<i>i</i> -PrOH	60	³ 10.9	1.12
² QChit2	5/1	H ₂ O	85	⁴ 63.3	1.15
Q02H	5/1	H ₂ O	85	-	3.00

¹[49]

²[106]

³Commercial chitosan was kept at 80°C for 24 hours before quaternization.

⁴Commercial chitosan was kept at 55°C for 24 hours before quaternization.

To examine quaternized chitosan QChit2, its ¹H NMR spectrum was compared with that of commercial chitosan (Figure 14). The signals in the range of δ 4.0 – 3.4 ppm correspond to the glucosamine ring protons, H₃-H₆. A sharp signal at 2.0 ppm is attributed to the methyl protons of the acetyl group of *N*-acetylglucosamine units. The formation of quaternized chitosan is primarily confirmed by the appearance of the characteristic *N,N,N*-trimethyl signal at δ 3.17 ppm [103], [134], [135], [136]. In addition, quaternized chitosan derivatives (Figure 14B) exhibit characteristic signals at δ 4.51 ppm, assigned to the anomeric protons (H₁) of substituted glucosamine units, δ 4.28 ppm, corresponding to the methylene protons of 2-hydroxypropyl trimethyl ammonium chloride (H_b), and δ 3.36 ppm, reflecting other methylene protons of a substituent (H_c). Due to the restricted mobility of the (2-hydroxypropyl) trimethylammonium substituent, the methylene protons (H_a) are split into two distinct signals at δ 2.90 and 2.75 ppm [134]. Some signals, for example, assigned to protons H_b and H_c, are downfield shifted compared to the previously reported data [134], which may be due to the solvent, temperature, and co-units' distribution [137].

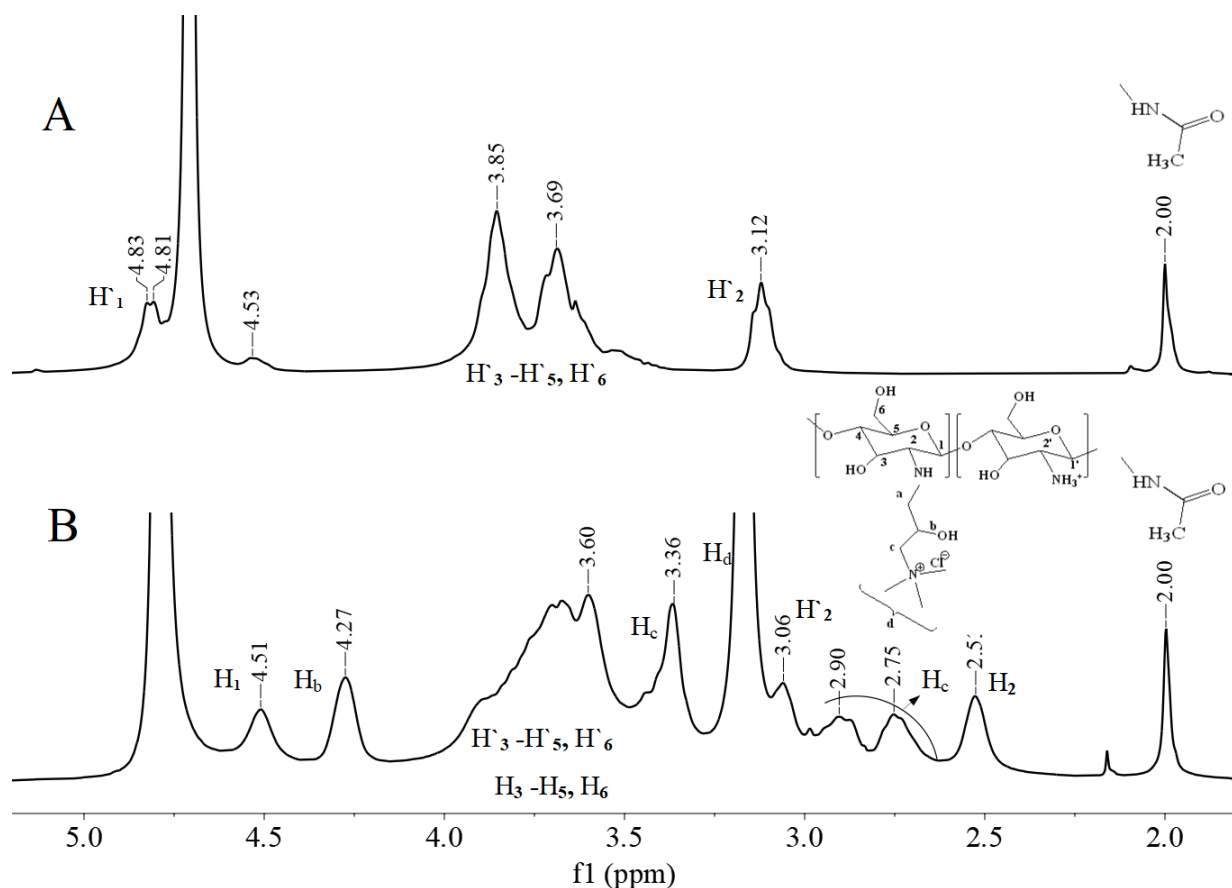


Figure 14. ^1H NMR spectra of commercial chitosan (A) and QChit2 (B).

To confirm the final assignments of ^1H NMR, 2D NMR analysis was performed using ^1H - ^1H Correlation (COSY) and ^1H - ^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectroscopies of QChit2. In the ^1H NMR spectrum (Figure 14), two distinct signals can be observed as the methylene protons a (H_a), which undergo diastereotopic splitting due to hindered rotation. This assignment is supported by the HSQC spectrum (Figure 15), where both signals correlate with the same carbon at δ 51.6 ppm. A similar trend is observed for the H6 protons (H'_6 and H6) of the glucosamine ring, which correlate with a carbon signal at δ 60.1 ppm. In the HSQC spectrum, in the anomeric region, two anomeric cross-peaks are observed at $\delta\text{H}/\delta\text{C}$ 4.74/97.9 and 4.50/101.5 ppm. It is also observed in the ^1H - ^1H COSY spectrum (Figure S3) that a cross-peak appears between the anomeric proton H_1 at δ 4.50 ppm and a signal at δ 2.53 ppm, which can be assigned to the H_2 proton of a glucosamine unit substituted with a (2-hydroxypropyl)trimethylammonium group. In addition, the HSQC spectrum shows a correlation at $\delta\text{H}/\delta\text{C}$ 3.06/55.6 ppm, which is attributed to the H_2 proton of glucosamine with an unsubstituted amino group. Despite the successful assignment of substituent signals, the signals corresponding to the chitosan backbone were less clearly resolved and broadened. This behavior is consistent with previous NMR studies of chitosan derivatives, where increasing substitution leads to signal broadening due to reduced molecular mobility and increased molecular weight [138]. This is likely due to the introduction of side chains, which increase the molecular weight and reduce molecular mobility. As a result, shorter transverse relaxation times (T_2) lead to signal broadening and lower resolution. In addition, signal overlap further complicates the analysis, especially at higher degrees of substitution.

The signals corresponding to the (2-hydroxypropyl) trimethylammonium substituent were integrated. However, the obtained integral ratios for H_a, H_b, H_c, and H_d (2.63 : 1.0 : 20 : 90) did not match the theoretical proton ratio (2 : 1 : 2 : 9). Notably, the signals are assigned to H_c and H_d, corresponding to the methylene and methyl protons of the outside quaternary ammonium group, were significantly more intense, indicating their dominant contribution to the spectrum. Therefore, the DQ calculated using signals of protons H_a, H_b, as reported in the literature [134], may not be reliable in this case. Instead, DQ was determined by integrating the protons of the residual acetyl groups of N-acetylglucosamine units and the trimethylammonium group, providing a more accurate estimation.

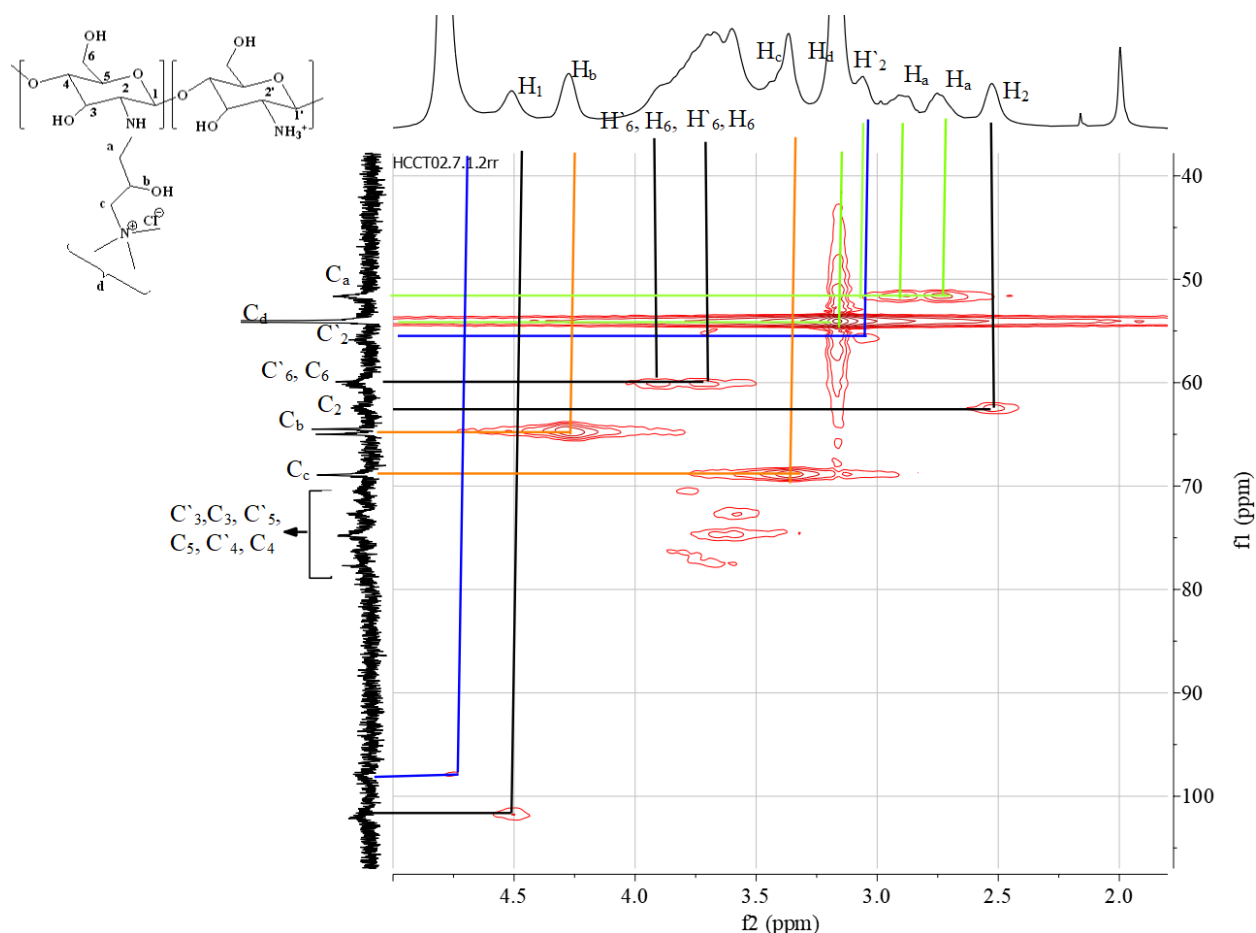


Figure 15. ^1H - ^{13}C HSQSC spectrum of QChit2.

In addition to the data of FTIR spectra of QChit1 and QChit2 were evaluated. As shown in Figure 16, the spectra of QChit1 and QChit2 were compared with the FTIR spectrum of commercial chitosan. Apart from the main structural similarities described earlier in 3.1.1 chapter, the spectra of QChit exhibited a characteristic band at around 1480 cm^{-1} , corresponding to the trimethyl group of the quaternary ammonium moiety [103], [125], [139]. Peaks of 1488 cm^{-1} and 1474 cm^{-1} identify the trimethyl group of a quaternary ammonium, attached to the chitosan chain.

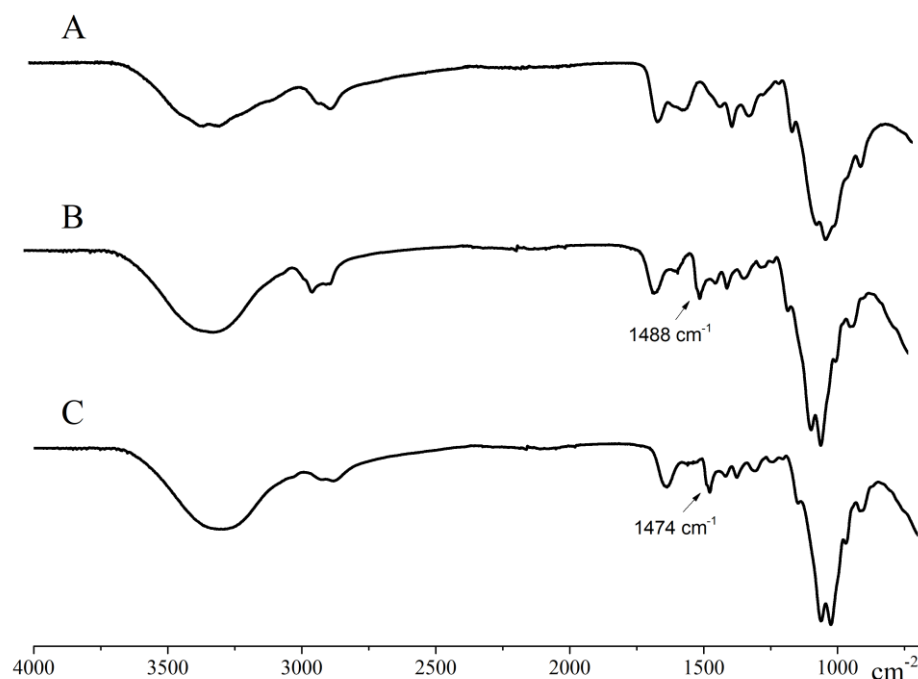
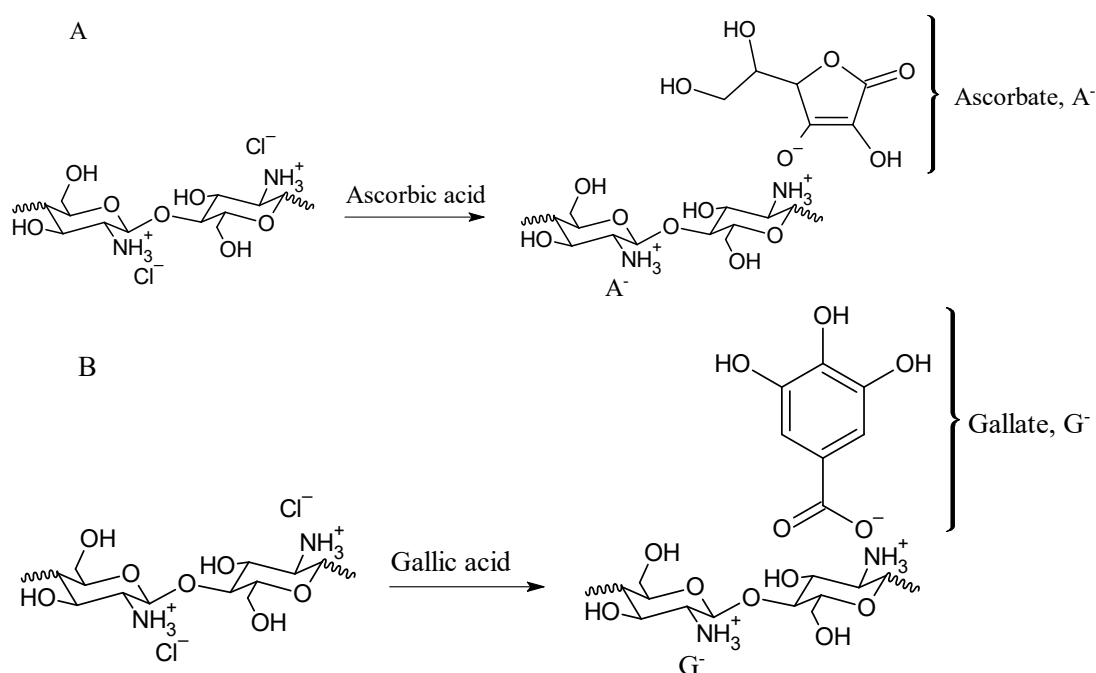


Figure 16. FTIR spectra of commercial chitosan (A), QChit1 (B), and QChit2 (C).

3.2.2 Counterion substitution features

Counterion exchange with molecules containing excellent antioxidant properties, such as ascorbate and gallate, was performed to enhance the antioxidant potential of chitosan and COS derivatives through electrostatic complexation. Ascorbic acid or vitamin C is a widely known antioxidant, and chitosan ascorbate complexes have been investigated in several papers [107], [108], [140], [141], [142]. In this study, chitosan ascorbates were synthesized during dialysis against deionized water based on the electrostatic interaction between protonated ammonium groups in the chitosan backbone and the acidic hydroxyl group of ascorbates using the excess of ascorbic acid ions (Scheme 8A) according to approved methodologies [107], [108]. The precursor type, Substitution Degree of Ascorbate (SDA), and Mn are shown in Table 4. The Mn increased after substitution in all samples, although absolute values may be affected by counterion exchange with acetate during SEC analysis. The SD of COS was calculated using 3 and 4 equations, represented in the methodology part (2.3.1 method). Chito-oligosaccharide ascorbate, A-COS-25, exhibited a higher degree of substitution (up to 46 mol%) compared to commercial chitosan ascorbate, A-ComCh (up to 20 mol%). It can be attributed to their higher solubility and greater accessibility of reactive sites. In the case of quaternized chitosan, A-QChit2, the SD reached 100 %. It has been reported that *N,N,N*-trimethyl chitosan exhibits a higher degree of permanent positive charges, leading to more effective ionic interactions and stronger complexation compared to native chitosan [143].



Scheme 8. Representations of counterion substitution to ascorbate (A) and gallate (B).

Table 4. Indications of substituted counterion complexes.

Samples	Name of Precursor	Precursor, $\text{Mn} \cdot 10^3$	$\text{Mn} \cdot 10^3$	SD, mol %
¹ A-COS-25	COS-5H-25	5.6	6.4	46
¹ A-ComCh	ComCh	84.7	89.6	20
¹ A-QChit2	QChit2	63.3	84.0	100
² G-COS-5	COS-5H-5	1.1	-	100
² G-ComCh	ComCh	84.7	-	27

¹Contains A, ascorbate

²Contains G, gallate

Gallic acid is a plant-derived polyphenol with strong antioxidant properties. While numerous studies with covalent conjugates of chitosan with gallic acid through ester and amide linkages have been widely represented [144], [145], [146], [147], [148], [149]. However, only a few studies have focused on the synthesis of electrostatic complexes between gallate and chitosan [77], [150], [151]. Therefore, it was interesting to examine these complexes. COS/commercial chitosan gallates (as ascorbates) were synthesized by electrostatic interactions between the chitosan protonated ammonium groups and gallates, using aqueous gallate solution without any organic reagents in excess (Scheme 8B). In solution at pH over 4.5, a carboxyl group of gallic acid deprotonates to the carboxylate groups (the $\text{pK}_{\text{a}1}$ of the carboxylic acid group is ~ 4.5 , but gallate was still very poorly soluble in water). Gallic acid is prone to oxidation, resulting in color and solubility changes [93]. It forms auto-oxidation products in alkaline conditions and the presence of O_2 . From pH 4 to pH 9, a gradual color from orange to green is recorded [150]. To keep the low pH, additional ions of a strong acid or a buffer solution have to be introduced to the system. It is a disadvantage in our established dialysis system because these anions compete for the quaternary ammonium cation with gallate. Hence, quaternary chitosan compounds failed to be subjected to gallate ion exchange. To maintain unoxidized gallate,

more acid would be required than in the case of commercial chitosan or COS. Nonetheless, COS and chitosan were shown to be suitable for this counter-ion exchange (Table 4). The substitution degree of COS gallate (G-COS, abbreviated G-COS) reached 100%, significantly higher than that of commercial chitosan gallate (G-ComCh, 27%). This behavior is similar to that of ascorbates; it can be attributed to the lower molecular weight and higher chain flexibility of COS. A very high G-COS substitution degree can be attributed to its low precursor molecular mass, 1.1 kDa.

The NMR spectra of ascorbates (A-ComCh, A-COS-25, and A-QChit2) enabled the identification of significant peaks and thus important structural characteristics (Figure 17). It was particularly important to justify ascorbate's presence near the C2 ammonium ion. According to Tan and coworkers [109], chitosan ascorbates containing protonated amino groups exhibit characteristic signals at approximately δ 4.51 ppm (lactone C-H_a, next to glycol moiety), 3.99 ppm (C-H_b of glycol), and 3.71 ppm (C-H_c of glycol), whereas a pure ascorbic acid shows signals at δ 4.721-5 ppm (H_a), 3.733-4.1 ppm (H_b), and 3.45-3.7 (H_c) ppm. Therefore, while in a complex with chitosan, ascorbate's H_a peak shifts upfield (becomes more shielded) and H_c shifts downfield (becomes more deshielded). All three of the peaks can be identified in the ¹H NMR spectra of A-ComCh (Figure 19A), A-COS-25 (Figure 19B), and ¹A-QChit-02 (Figure 17C) obtained in this study. In all three cases, a distinct H_a signal is observed at δ 4.51 ppm. The H_b signal appears at δ 4.02 ppm, 4.00 ppm, and 3.99 ppm for A-ComCh, A-COS, and A-QChit-02, respectively. H_c signal is not clearly resolved in the A-ComCh spectrum, likely due to the high viscosity of the solution; however, in the cases of A-COS and A-QChit2, it is δ 3.74 ppm. Additionally, the A-QChit-02 spectrum exhibits H'_a – H'_c signals, corresponding to methylene groups of the attached quaternary ammonium moiety (Figure 17C).

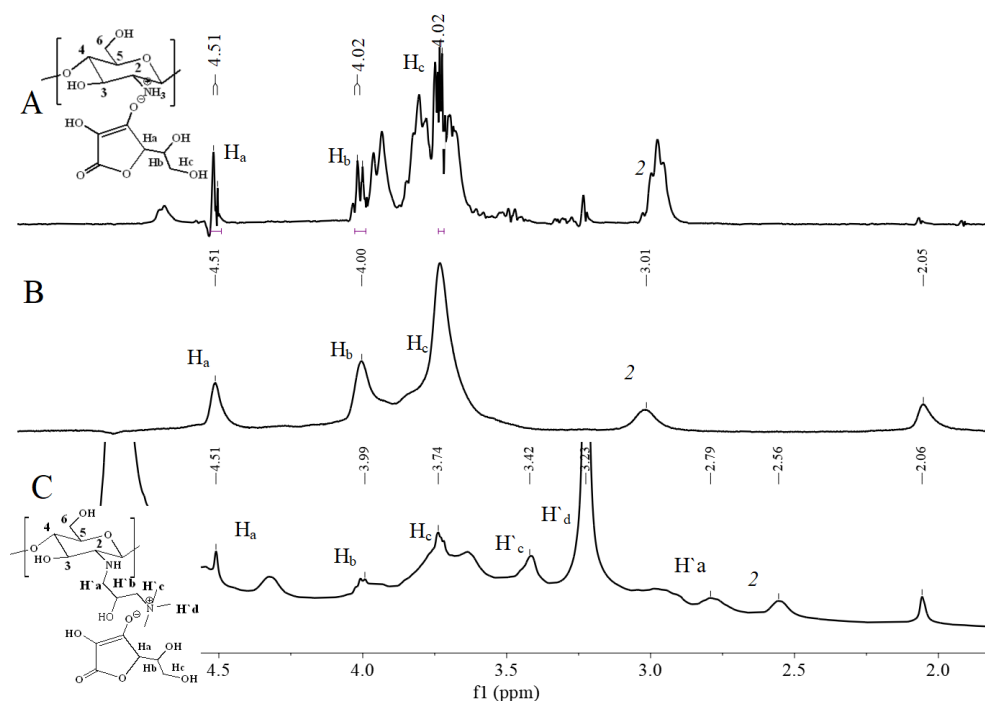


Figure 17. ¹H NMR spectra of A-COS-25 (A), A-ComCh (B) or A-QChit2 (C).

Figure 18 shows FTIR spectra of A-ComCh, A-COS-25, and A-QChit2. The broad band at around 3350 cm⁻¹ is attributed to the stretching vibrations of –NH₂ and –OH groups, associated with a

hydrogen bond. Its shape changes according to the compound: A-ComCh exhibits the broadest band, whereas A-QChit2 shows the most pronounced and narrow band among the three samples. Hydrogen bonding alters hydroxyl/amino group vibration frequencies and creates a distribution of different environments [152]. The band assigned to ascorbate is typically observed around 1720 cm^{-1} , corresponding to the stretching vibration of the carbonyl group of the lactone group [153], [154]. In our case, ascorbate identifying peaks appear at 1725 cm^{-1} for A-COS, 1717 cm^{-1} for A-ComCh, and 1721 cm^{-1} for A-QChit2. Additionally, 1672 cm^{-1} is assigned to $\text{C}=\text{C}$ ascorbate stretches [154]. In this case, they are hardly separated from other peaks, located at $1560\text{--}1640\text{ cm}^{-1}$. Those peaks regard amine and protonated ammonium [126], [127]. Even if it was noticed that 1601 cm^{-1} reflects primary amine vibrations, that is not entirely true for $^+\text{N}\text{--H}$ bonds vibrations, as these shift to $1555\text{--}1521\text{ cm}^{-1}$ [155]. This trend mostly appears in A-ComCh regarding the achieved spectrum. Speculatively, it can be due to a bigger molecular mass and a higher number of $^+\text{N}\text{--H}$ bonds. Apart from the main peak characteristic of ascorbate, A-QChit2 has the quaternization identifying peak at 1480 cm^{-1} , already discussed in 3.4. chapter.

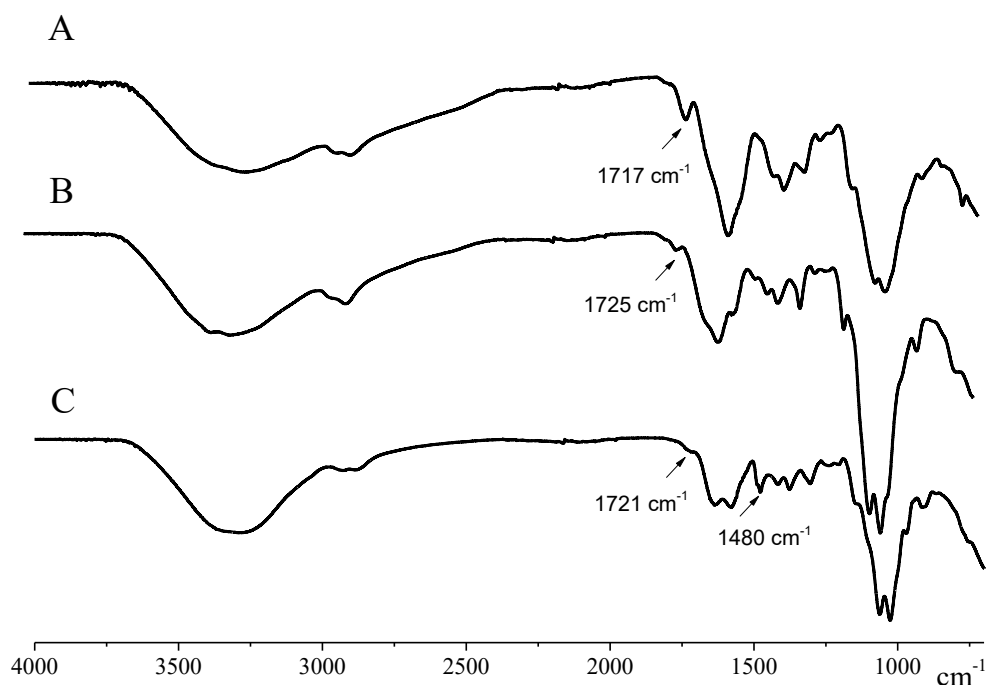


Figure 18. FTIR of A-ComCh (A), A-COS (B), and A-QChit-02 (C).

Its structure was confirmed by the ^1H -NMR (Figure 19). Phenyl protons of gallic acid appear at around $\delta\ 6.92\text{--}7.63\text{ ppm}$ [133], [144], [156]. Figure 19 demonstrates these protons (as a new peak) as well, at around $\delta\ 6.9\text{ ppm}$, consistent with gallate anion resonance, as previously reported [77]. The same identification is noticed in G-COS (Figure S4). The slight upfield position of this signal may reflect the mildly alkaline conditions required to increase gallate solubility [150]. Some authors also mention the H2-6 peaks' shift during gallic acid placement near the chitosan backbone. Similarly, here, chitosan gallate glucosamine protons shift upfield ($\delta\ 3.34\text{--}3.84\text{ ppm}$ instead of $\delta\ 3.5\text{--}4.0\text{ ppm}$; $\delta\ 2.84\text{ ppm}$ instead of $\delta\ 3.1\text{--}3.4\text{ ppm}$), revealing that gallate ions may be substituted at all possible ways (C2, C3, and C6).

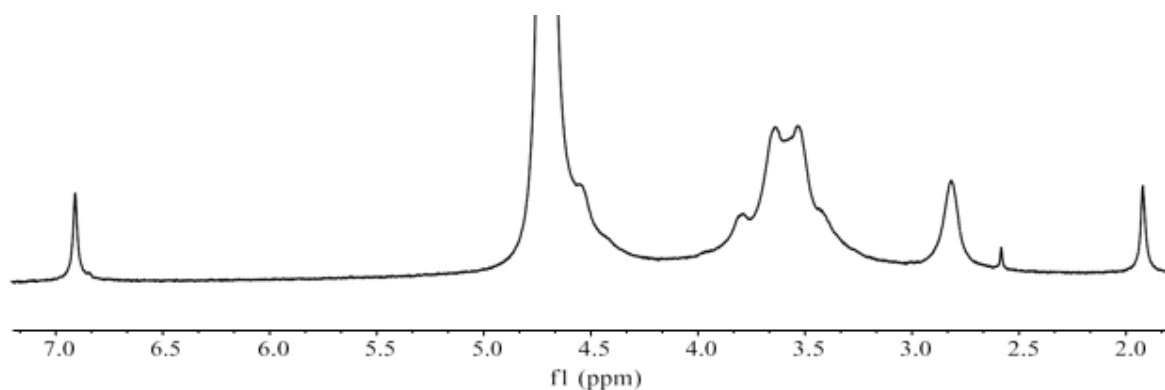


Figure 19. ^1H NMR spectra of G-ComCh.

In the FTIR spectrum of lyophilized chito-oligosaccharide hydrochloride, two characteristic absorption bands corresponding to amino and protonated ammonium groups are observed at approximately 1610 and 1518 cm^{-1} , respectively [128]. In contrast, in the spectra of G-ComCh (Figure 20A) and G-COS (Figure 20B), a broad band is observed at 1525 and 1529 cm^{-1} , respectively. This band arises from the overlapping contributions of protonated ammonium groups of COS and the asymmetric stretching vibrations of the carboxylate group of gallate. The significant shift of this band toward higher wavenumbers compared to ammonium hydrochloride indicates a change in the ionic environment of the $-\text{NH}_3^+$ groups and strongly supports the formation of electrostatic interactions between the protonated amino groups of chitosan and COS and the carboxylate groups of gallate. The band at approximately 1462 cm^{-1} is attributed to the symmetric stretching vibration of the carboxylate group, in agreement with literature data for gallate salts [157]. The bands at 2920 and 2850 cm^{-1} correspond to asymmetric and symmetric stretching vibrations of aliphatic CH_2 groups characteristic of both chitosan and COS gallates. The increased intensity of these bands in both chitosan and COS gallates may reflect changes in the local molecular environment and intermolecular interactions induced by the incorporation of gallate moieties. Additional bands observed at 1391 and 1366 cm^{-1} , as well as at 1260 and 1230 cm^{-1} , are assigned to coupled vibrational modes involving aromatic ring stretching, phenolic $\text{C}-\text{O}$ stretching, and contributions from $\text{C}-\text{H}$ and $\text{C}-\text{OH}$ bending vibrations [158]. These features further confirm the presence of aromatic gallate structures within the polymer matrix. Furthermore, strong absorption bands appearing at 732 and 720 cm^{-1} are associated with the out-of-plane bending vibrations of the $\text{O}-\text{H}$ group, and this is also characteristic of the spectra of alcohols and phenols.

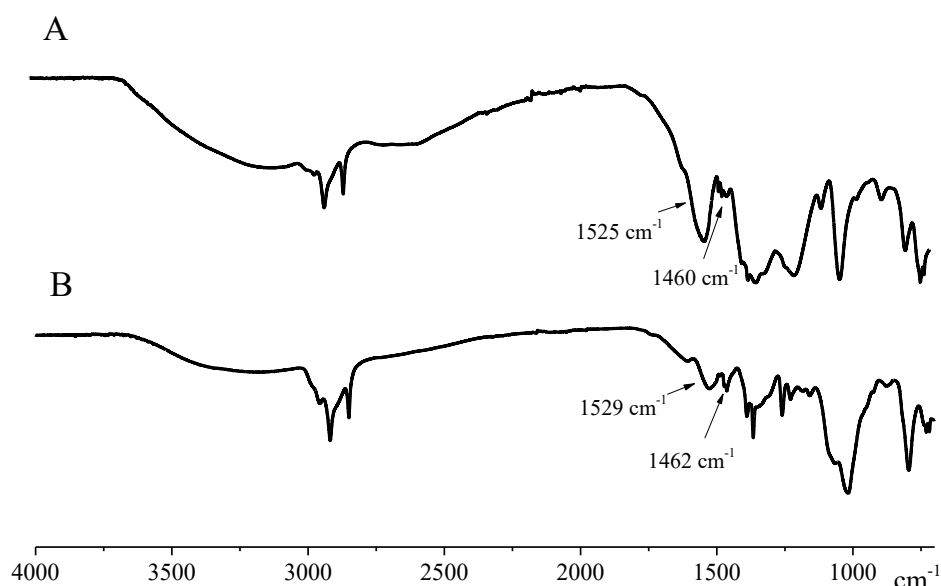


Figure 20. FTIR of G-ComCh (A) and G-COS (B).

Our synthesized G-ComCh shows an absorption peak at ~ 216 nm and ~ 260 nm in the UV-Vis spectra (Figure 21). According to the literature, gallic acid exhibits absorption bands at 212 and 262 nm due to the benzene ring π system [144]. Ascorbate complexes (A-ComCh and A-COS), on the other hand, absorb light at around 240-290 nm and have the λ_{max} of ~ 265 nm. Reports state that ascorbic acid shows absorption peaks at 230-280 nm [159], [160] when λ_{max} in a particular case was 265 nm [161]. A-ComCh had a visibly higher absorption than A-COS, mainly due to its larger size. The absorption of gallate complexes is comparatively much higher. It is possible because of the gallate's nature – benzene ring. In contrast, compounds without antioxidant counterions do not absorb light in these regions (COS-2H-25, COS-1H-25, and QChit2).

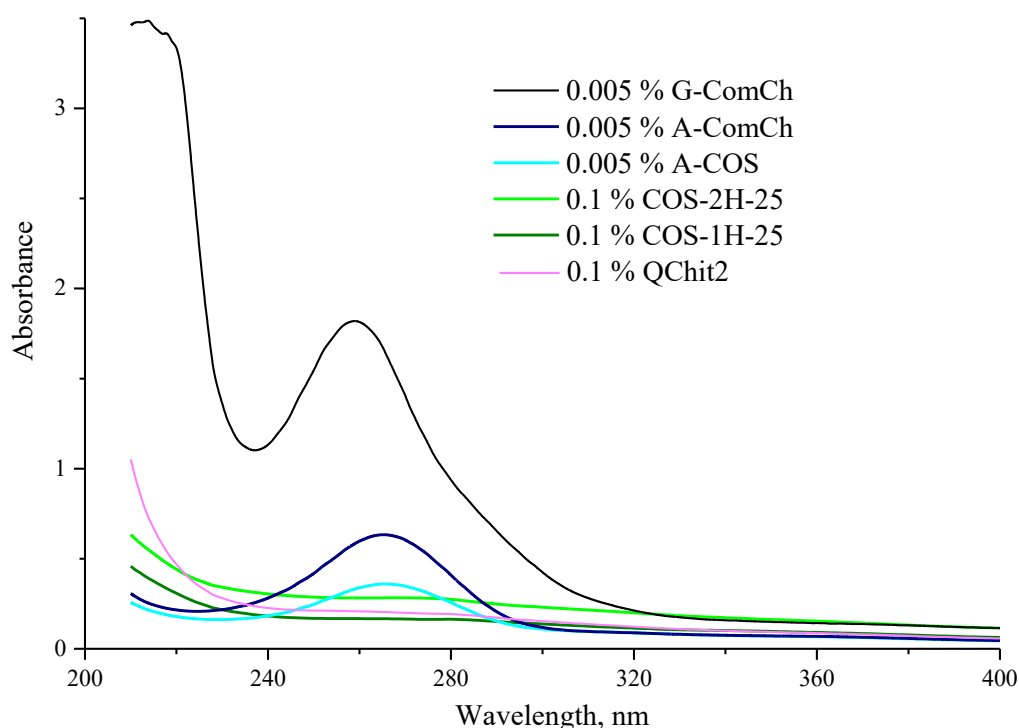


Figure 21. UV-Vis of different synthesized compounds (COS, gallates, ascorbates, QChit2).

3.3. Antioxidant properties of synthesized compounds

ComCh, A-ComCh, G-ComCh, A-QChit2, and QChit2 antioxidant activities and reducing power are shown in Figure 24. Ascorbates contain the best antioxidant activity results (Figure 22A). A-QChit2 had the best activity (78 %) compared to QChit2 (20 %), commercial chitosan (22 %), A-ComCh (66 %), and G-ComCh (60 %). High A-QChit2 activity is expected, since their SD is 100 %. A-ComCh and G-ComCh had similar results. Even though G-ComCh has a higher SD, A-ComCh showed slightly better results. It was mentioned before that gallic acid is prone to fast, uncontrolled oxidation. Therefore, its antioxidant activity could be moderated by environmental conditions, like temperature. On the other hand, it could be moderated by radical scavenging mechanisms. G-ComCh reducing power (electron-donating) activity (Figure 21B, 127 %) was better than the reducing power activity of A-ComCh (Figure 22B, 115 %). Therefore, G-ComCh could be more keen on other ROS scavenging. Interestingly, QChit2 does not show better DPPH radical scavenging activity than commercial chitosan. As QChit had a DS of 1, all amino groups could be substituted with 2-hydroxypropyl trimethyl ammonium chloride, and it is known that amino (together with hydroxy) groups play an important part in radical scavenging [22]. Considering its good overall reducing power capacity (Figure 24B), our synthesized quaternized chitosan compounds could be worse at scavenging DPPH radicals, particularly. The reducing power of QChit2 was approximately 7 times higher than that of commercial chitosan (ComCh – 8 %, QChit2 – 55 %). Thus, more experiments on stabilizing different radicals need to be performed.

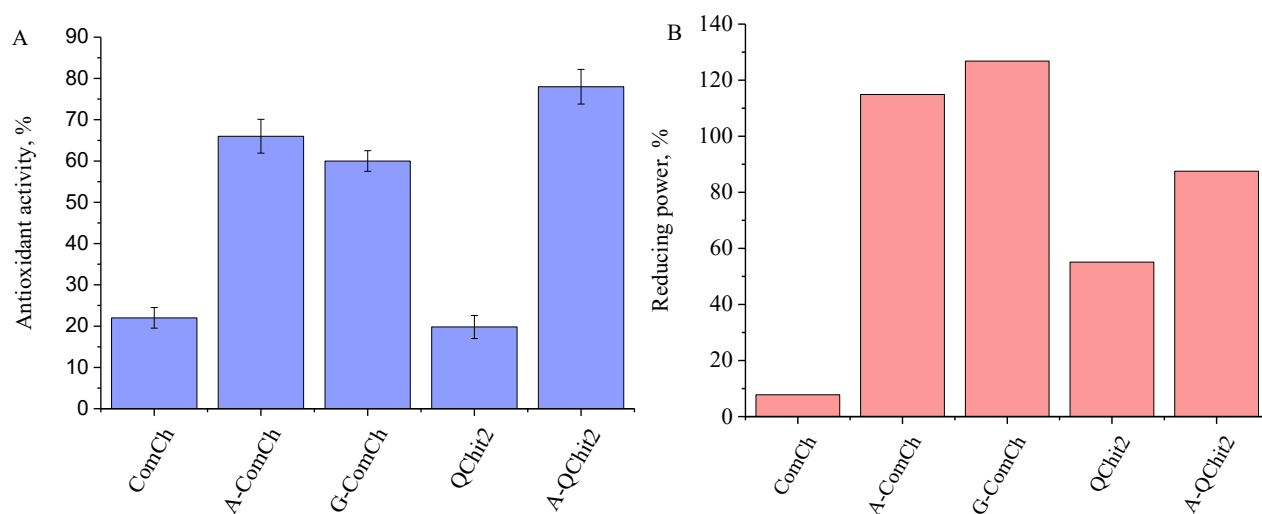


Figure 22. Evaluation of antiradical activity of commercial and modified chitosan compounds (ComCh, A-ComCh, G-ComCh, QChit2, and A-QChit2) by the DPPH radical scavenging method: sample concentration 2 mg/ml (A) and evaluation of reducing power, sample concentration 0.6 mg/ml (B).

Moreover, the antioxidant activity of COS has highly favored electrostatic complexes. According to the DPPH radical scavenging assay results (Figure 23A), G-COS and A-COS had the highest antioxidant activity (66.8 % and 66.9 %, respectively). It is followed by Q02H (53.5 %), COS-1H-19 (15.6 %), and COS-1H-25 (3.99 %). A similar pattern is observed in the reducing power graph (Figure 23B), where the highest reducing power activity belongs to G-COS (86.4 %) and A-COS (85.6 %),

and the lowest activity belongs to COS-1H-19 (14.4 %) and COS-1H-25 (12.8 %). Q02H has a value between these compounds (35.9 %). Counterion substitution products, as before (Figure 22), performed well, synergistically matching chito-oligosaccharide and gallate/ascorbate antioxidant activity. Q02H had a higher tendency to scavenge DPPH radicals rather than reducing Fe^{3+} . Q02H contains a bulky quaternary ammonium group, which may repel positive charge-containing iron ions. Consequently, it can result in poor metal coordination and accessibility to reactive sites. The same tendency is true for COS-1H-19. This compound, however, does not contain charge-repelling groups but has an imine bond (Scheme 6, Figure 9). However, its imine index was too small to be presented (Table 2). Schiff bases favor a hydrogen-transfer mechanism, whereas Fe^{3+} to Fe^{2+} reduction requires a single-electron-transfer mechanism [162]. Lastly, the COS-1H-25 size and antioxidative action were worse than those of COS-1H-19. These findings coincide with the literature [104].

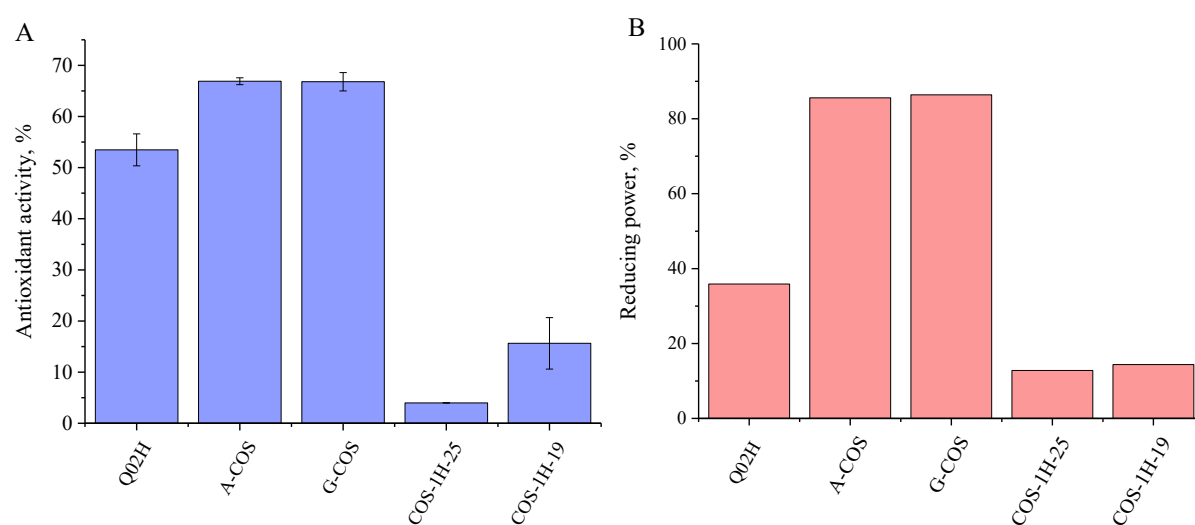


Figure 23. Evaluation of antiradical activity of commercial and modified chitosan compounds (Q02H, A-COS, G-COS, COS-1H-25, and COS-1H-19) by the DPPH radical scavenging method: sample concentration 2 mg/ml (A) and evaluation of reducing power, sample concentration 0.6 mg/ml (B).

β -carotene bleaching inhibition outcomes are shown in Figure 24. Among all the tested compounds, only gallate electrostatic complexes were able to demonstrate satisfactory repeatable results. G-COS (82.9 %) and G-ComCh (84 %) protected β -carotene from oxidation better than BHT (73.4 %). Also, they were approximately 5 times better than commercial chitosan (15.9 %). Speculatively, gallic acid is a less hindered polyphenol, and thus gallate complexes show better results.

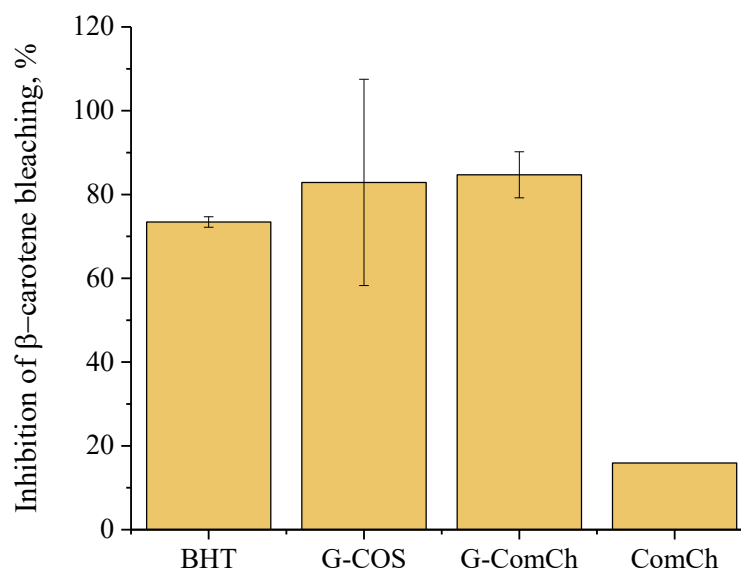


Figure 24. Results of β -carotene bleaching inhibition assay regarding BHT (positive control), G-COS, G-ComCh, and commercial chitosan.

3.4. Synthesized compounds application for berry preservation

Ascorbates and chito-oligosaccharides were subjected to berry (raspberry and blueberry) preservation. Gallate electrostatic complexes were not used since they have a tendency for fast, uncontrollable oxidation. Each berry was immersed into 2 % aqueous sample solution and kept at room temperature; their mass was marked right after the application, after 3 days, and after 5 days. The results are shown in Figure 25. All tested compounds showed an activity compared to berries with no treatment (black line). To be more precise, next to the legend of the plots indicated the slope of each graph. No treatment berries graph has the lowest slope (-0.53), while the COS-3H-13 has the highest (-0.22). Ascorbate, commercial chitosan, and A-ComCh had almost identical slopes (from -0.32 to -0.32), resulting in a similar berry protection pattern. Better than these compounds, apart from COS-3H-13, were A-QChit2 (-0.28) and COS-1H-25 (both had slopes of -0.28), and worse – A-COS (-0.38). COS-3H-13 and A-QChit2 both had very good antioxidant activities, and therefore, these compounds were expected to show good results. Commercial chitosan being as protective as ascorbate is also reasonable, since chitosan has antioxidant activity, related to its functional groups (Figure 22). Unfortunately, A-ComCh and A-COS did not demonstrate great results. Their antioxidant activities were higher than those of commercial chitosan (Figure 22, Figure 23), and thus, it is speculated that the raspberry protection relies on more factors than sole antioxidative activity. COS-1H-25 had a better effect than commercial chitosan, but worse than Schiff base, COS-3H-13. This result is consistent with its physicochemical background (flexible, soluble chains with exposed functional groups).

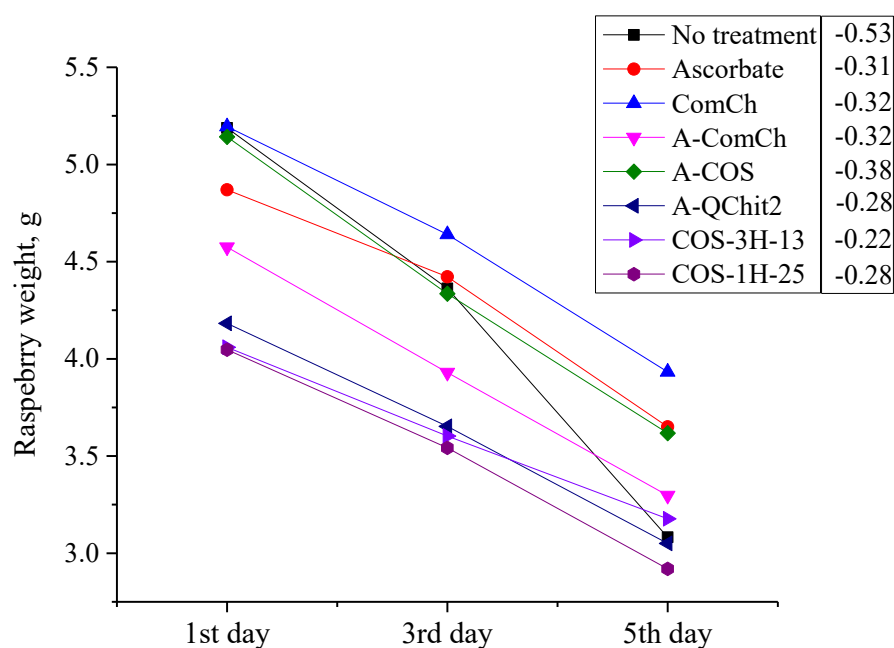


Figure 25. Raspberry weight change immediately after treatment with 2% of the sample (1st day), after 3 days (3rd day), and after 5 days (5th day). The numbers next to the legend indicate the slope coefficient of each curve.

Regarding blueberries' mass change (Figure 26), the effect of the tested compounds was not as prominent as in the case of raspberries (Figure 25). Slopes of each graph (Figure 26) did not indicate major differences. A control group with no treatment had a slope of -0.087, and compounds with a higher slope (smaller mass change) were ascorbate (-0.083), commercial chitosan (-0.076), A-ComCh (-0.083), COS-3H-13 (-0.067), and COS-1H-25 (-0.081). A-COS (-0.099) and A-QChit2 (-0.094) showed the opposite effect than expected, providing worse berry protection results than the initial control. Again, A-COS and A-QChit are good antioxidant activity-bearing compounds. It may be that these compounds have reduced film-forming properties, attributed to chitosan itself. In fact, it showed second-best results after COS-3H-13. Overall, COS-3H-13 demonstrated the best protective barrier properties in both blueberries and raspberries. Ascorbate, A-ComCh, and COS-1H-25 performed uniformly, thus further emphasizing that the protection of berries from environmental conditions does not depend on the replacement of the chitosan counterion. Moreover, it is important that each berry can change the effect drastically. Although the berries were picked in a similar size and condition, they may have had damage in their internal structure and may be more or less affected by growing, transportation, and storage conditions.

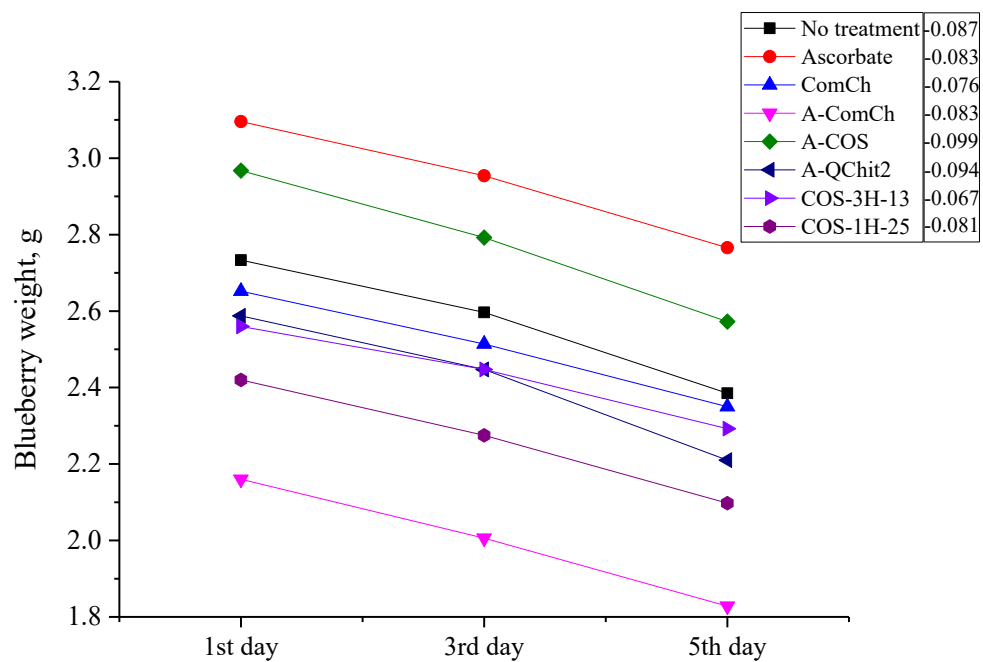


Figure 26. Blueberry weight change immediately after treatment with 2% of the sample (1st day), after 3 days (3rd day), and after 5 days (5th day). The numbers next to the legend indicate the slope coefficient of each curve.

CONCLUSIONS

1. Chito-oligosaccharides (COS) of different molecular weights were successfully synthesized by acidic hydrolysis of chitosan, also by neutralization and controlled fractionation. The principal structural changes occurred during hydrolysate neutralization and concentration. Low molecular weight COS contained reducing and non-reducing terminal units, a vicinal diol system, imine groups, and unsaturation within the glucosamine backbone. UV–Vis spectroscopy confirmed the formation of early Maillard reaction products with absorption bands and a bathochromic shift to 320 nm.
2. Sephadex G-15 gel filtration, typically applied for hydrolysate desalting, was successfully employed for the controlled separation of low molecular weight COS fractions. Sephadex G-15 gel filtration enabled the isolation of COS fractions with a degree of polymerization (DP) from 40 to 4. The isolated fractions exhibited narrow molecular weight distributions, with a dispersity index of 1.2, indicating effective fractionation.
3. Low molecular weight COS (DP < 13) containing imine groups exhibited significantly enhanced antioxidant activity and reducing power compared to commercial chitosan and COS without imine groups. The highest activities were observed for COS (antioxidant activity 80%, and reducing power 85%), which also showed the highest imine index (0.10).
4. The molecular weight of commercial chitosan (85 kDa) decreased to 63.3 kDa (QChit2) and 10.9 kDa (QChit1) during the quaternization process. Reduction in MW is attributed to the long reaction time and harsh reaction conditions, particularly under alkaline catalysis. The degree of substitution was approximately 1 for QChit1 and QChit2, and approximately 3 for quaternized COS. The introduction of the quaternary ammonium group was confirmed by NMR and FTIR analyses.
5. Counterion exchange was successfully performed, yielding substitution degrees ranging from 20 to 100 mol% and producing A-ComCh, A-COS, A-QChit2, G-ComCh, and G-COS. G-QChit2 was not obtained due to the low solubility of gallic acid and rapid, uncontrolled oxidation.
6. The best antioxidant activity was of gallate and ascorbate complexes (> 60 %). Similar values were recorded for G-COS and A-COS (66.8–66.9%) and for G-ComCh and A-ComCh (60–66%).

SUMMARY

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Chitosan Hydrolysis, Quaternization, and Counterion Exchange for Enhanced Antioxidant Activity

Chitosan ((1→4)-2-amino-2-deoxy-d-glucose) is a natural polymer with promising applications in the food industry, cosmetics, and medicine. Its advantages include antimicrobial, antioxidative, and film-forming properties, as well as biocompatibility and biodegradability. Various modification strategies are currently being actively explored. Modifications such as alkylation or Schiff base formation allow improved applicability, enhanced antioxidant activity, and tailored physicochemical properties.

This study focused on the analysis of specific chitosan modifications (acid hydrolysis, quaternization, and counterion exchange). Sephadex G-15 gel filtration, commonly used for desalting hydrolysates, was successfully applied for the controlled separation of low molecular weight COS fractions. Spectroscopic data showed that acid hydrolysis products, chito-oligosaccharides (COS), formed Schiff bases, unsaturated structures, and the vicinal diol system after the neutral hydrolysate concentration process. These compounds demonstrated exclusive antioxidant and reducing power activity of more than 100 %.

Quaternization of COS and chitosan was performed using glycidyl trimethylammonium chloride, attaching a 2-hydroxypropyl trimethylammonium group on each glucosamine unit. Substitution degrees were approximately 1 for quaternized chitosan and 3 for quaternized COS. The introduction of the quaternary ammonium group was confirmed by ^1H NMR, ^{13}C NMR, and FTIR analyses. Quaternized compounds with COS were further subjected to counterion exchange to gallate or ascorbate. The formation of their electrostatic complex was confirmed not only with ^1H NMR and FTIR but also with UV-Vis spectroscopy. Among quaternized chitosan, gallate, and ascorbate complexes, the best antioxidant activity was of ascorbates and gallates. The best protective layer properties were prescribed to chito-oligosaccharides forming Schiff bases. A 2% solution of these compounds was a significantly better alternative to the ascorbate solution.

SANTRAUKA

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Chitozano modifikavimas hidrolizės, kvaternizavimo ir priešjonių keitimo būdu siekiant padidinti antioksidacinį aktyvumą

Chitozanas ((1→4)-2-amino-2-deoksi-d-gliukozė) yra natūralus polimeras, turintis daug potencialo maisto pramonėje, kosmetikoje ir medicinoje. Jis turi antimikrobinį, antioksidacinį poveikį, yra biologiškai skaidus, geba formuoti plėvelę ir yra biologiškai suderinamas. Šiuo metu aktyviai tiriamos įvairios šio polimero modifikavimo strategijos. Tokios modifikacijos kaip alkilinimas arba Šifo bazės formavimas leidžia pagerinti chitozano fizikines, chemines ir antioksidacines savybes.

Šiame tyrime daugiausia dėmesio skirta specifinių chitozano modifikacijų (rūgštinės hidrolizės, kvaternizacijos ir priešjonių mainų) analizei. „Sephadex G-15“ gelio filtravimas, dažniausiai naudojamas hidrolizatų nudruskinimui, buvo sėkmingai pritaikytas kontroliuojamam mažos molekulinės masės COS frakcijų atskyrimui. Spektroskopiniai duomenys parodė, kad po neutralaus hidrolizato koncentravimo proceso rūgštinės hidrolizės produktai, chito-oligosacharidai, sudarė Šifo bazes, nesočiąsias struktūras ir vicinalinę diolių sistemą. Šie junginiai pasižymėjo išskirtiniu antioksidaciniu ir redukciniu aktyvumu, viršijančiu 100 %.

COS ir chitozano kvaternizavimas buvo atliktas naudojant glicidiltrimetilamonio chloridą, prijungiant 2-hidroksipropiltrimetilamonio grupę prie kiekvieno gliukozamino vieneto. Kvaternizuoto chitozano pakeitimo laipsniai buvo maždaug 1, o kvaternizuoto COS – 3. Ketvirtinės amonio grupės įvedimas buvo patvirtintas ^1H BMR, ^{13}C BMR ir FTIR analize. Kvaternizuotų junginių priešjoniai (chloridai) buvo toliau keičiami į galatą arba askorbatą. Elektrostatinį kompleksų susidarymas buvo patvirtintas ne tik ^1H BMR ir FTIR, bet ir UV-Vis spektroskopija. Tarp kvaternizuotų chitozano, galato ir askorbato kompleksų geriausią antioksidacinį aktyvumą turėjo askorbatai ir galatai. Geriausios apsauginio sluoksnio savybės buvo priskirtos chito-oligosacharidams, sudarantiems Šifo bazes. Dviejų procentų šių junginių tirpalas buvo žymiai geresnė alternatyva askorbato tirpalui.

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APPENDICES

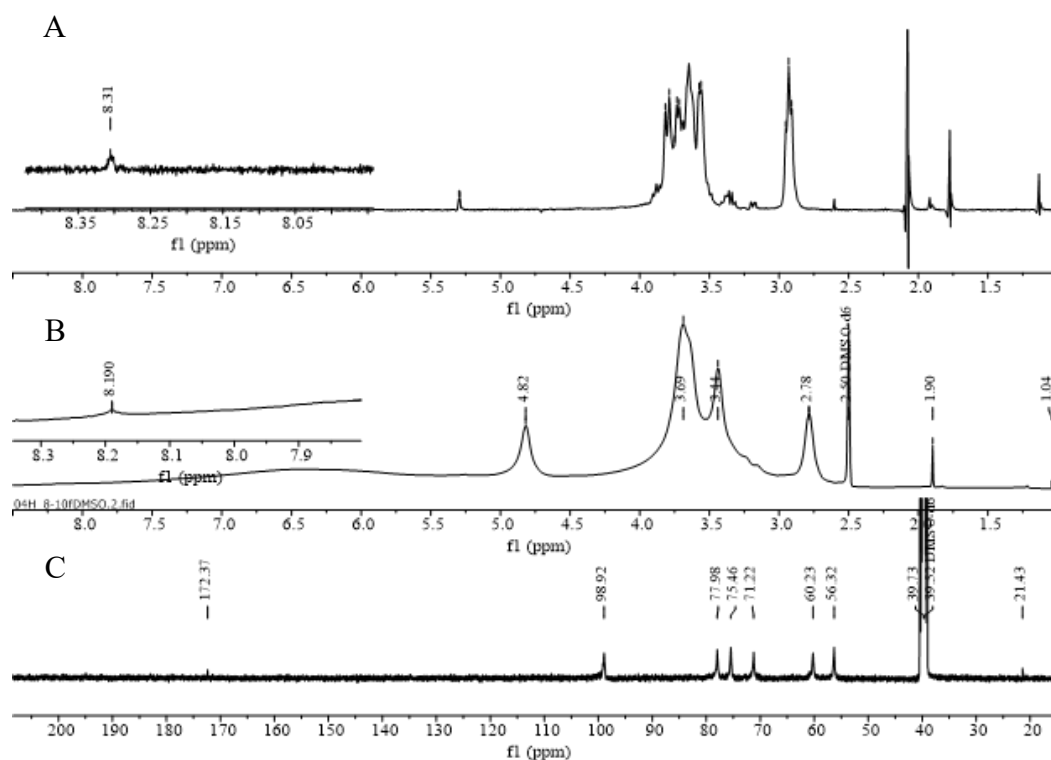


Figure S1A. ^1H NMR spectra of COS-1H-36 in D_2O (A), $\text{DMSO}-d_6$ (B) and ^{13}C BMR spectrum of COS-1H-36 $\text{DMSO}-d_6$ (C).

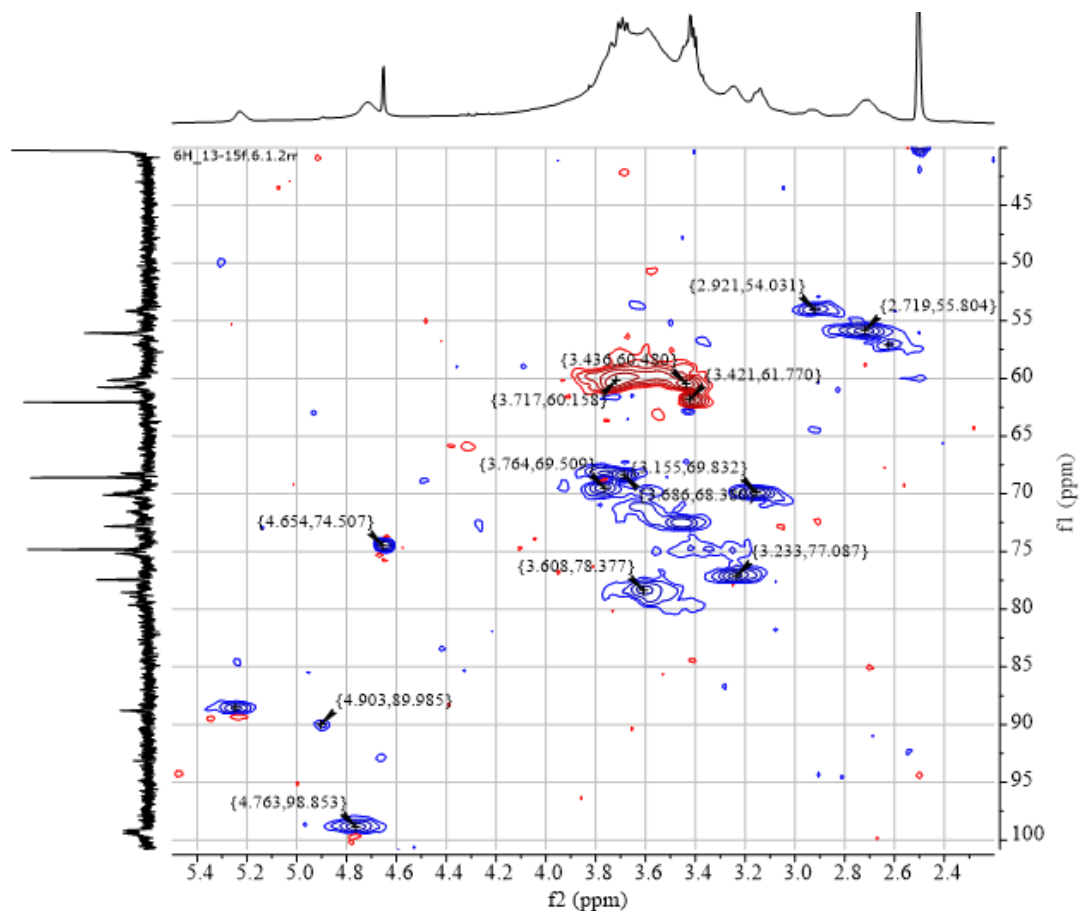


Figure S2. ^1H - ^{13}C HSQC spectrum of COS-3H-4.

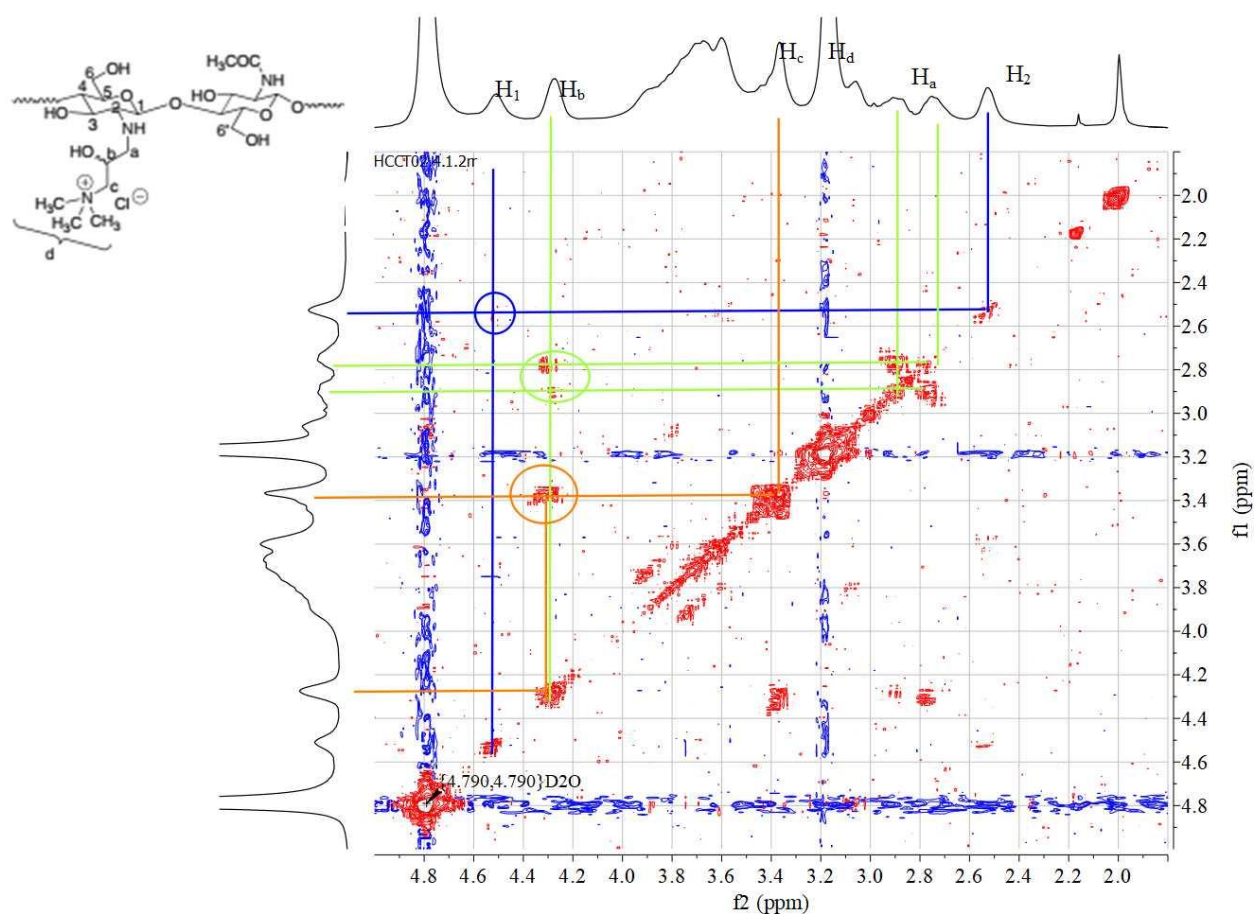


Figure S3. ^1H - ^1H COSY spectrum of QChit2.

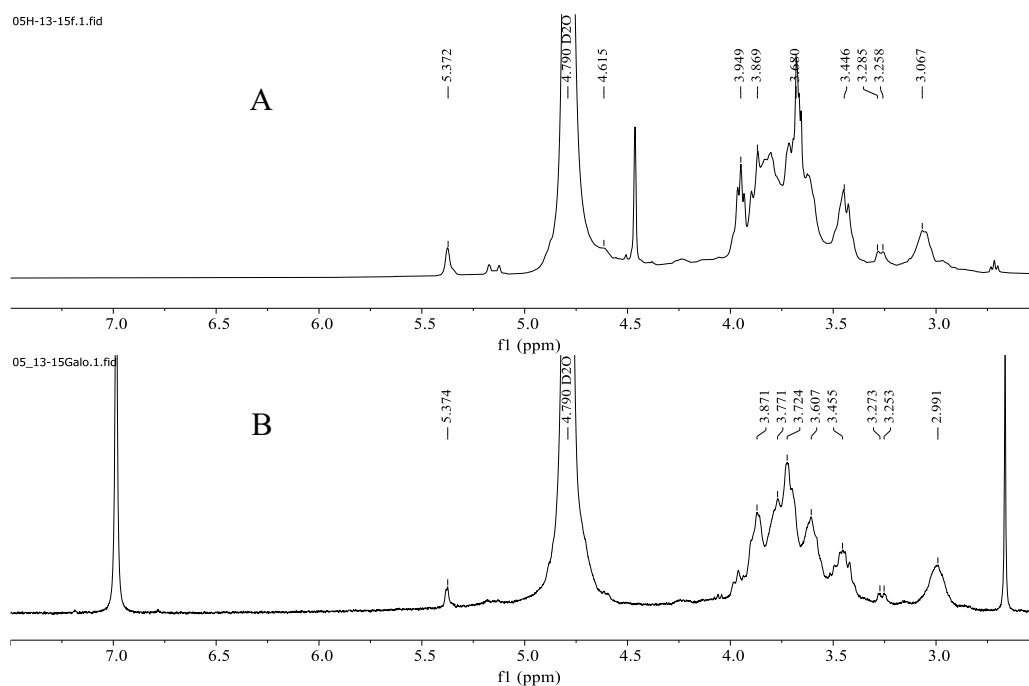


Figure S4. ^1H NMR spectra of COS-2H-5 (A), and its gallate, G-COS (B).