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Review

Carrageenans: Biological properties, chemical modifications and structural analysis – A review

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ABSTRACT

Carrageenans are sulphated linear polysaccharides of D-galactose and 3,6-anhydro-D-galactose extracted from certain red seaweeds of the *Rhodophyceae* class. They have been extensively used in the food industry as thickening, gelling and protein-suspending agents, and more recently by the pharmaceutical industry as excipient in pills and tablets. Besides the well-known biological activities related to inflammatory and immune responses, carrageenans are potent inhibitors of herpes and HPV viruses and there are indications that these polysaccharides may offer some protection against HIV infection. Thus, this review describes important aspects of carrageenans related to their industrial/therapeutic applications, physicochemical properties and structural analysis. Moreover, chemical modifications of carrageenans that can lead to prototypes with potential application for the treatment of several diseases, such as herpes, HPV and AIDS, will be outlined.

1. Introduction

Over the last years, marine microorganisms such as bacteria, microalgae and seaweeds have represented a large source of valuable materials (DeLong, 1997; Rasmussen & Morrissey, 2007). The various unique carbohydrate residues that are found in marine organisms are especially interesting and emphasize the importance of obtaining further knowledge in this area (Michel, Nyval-Collen, Barbeyron, Czjzek, & Helbert, 2006). So far, the three commercially exploited carbohydrate polymers from marine organisms are: (1) alginates, the mannuronic-acid- and guluronic-acid-containing polymers from brown seaweeds; (2) agar, the D-galactose and 3,6-anhydro-L-galactose-containing polymers that are isolated from red seaweeds; and (3) carrageenans (De Ruiter & Rudolph, 1997; Selby & Whistler, 1993).

Carrageenan is a generic name for a family of polysaccharides, obtained by extraction from certain species of red seaweeds (*Rhodophyta*) (Van De Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). The word carrageenan is derived from the colloquial Irish name for this seaweed, carrageen, which means "little rock". The aqueous extraction of red seaweeds to obtain these hydrophilic colloids is known in Ireland since 1810 (Stanley, 1987). All seaweeds that produce carrageenan as their main cell-wall material belong to *Rhodophyta*, but there is almost no information available on the biosynthesis of carrageenans and the genetics of the seaweed plant cell wall (De Ruiter & Rudolph, 1997).

In the food industry, carrageenans are widely utilized due to their excellent physical functional properties, such as thickening, gelling and stabilizing abilities, and have been used to improve the texture of cottage cheese, to control the viscosity and texture of puddings and dairy desserts, and as binders and stabilizers in the meat-processing industry for the manufacture of patties, sausages and low-fat hamburgers.

The food industry accounts for 70–80% of the total world production, estimated at about 45,000 metric tonnes per year, of which about 45% goes to dairy products and 30% to meat and meat derivatives. The total market of carrageenans has been estimated as US $300 million/year (McHugh, 2003).

Carrageenans are also used in various non-food products, such as pharmaceutical, cosmetics, printing and textile formulations (Imeson, 2000). Carrageenans stabilize toothpaste preparations, absorb body fluids when formulated in wound dressings and interact with human carotene to give soft skin and silky hair in hand lotions and shampoos, respectively. They have proved to be useful as tableting excipients due to the good compatibility, high robustness and persistent viscoelasticity of the tablet during compression. These interesting properties indicated that carrageenans are suitable excipients for sustained-release formulations (Bhardwaj, Kanwar, Lal, & Gupta, 2000).

Therefore, considering the extensive applications of carrageenans, the aim of this review article is to present a comprehensive

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overview of recent and relevant aspects related, principally, to their biological activities, chemical modifications and structural analysis.

2. Source, structure and physicochemical properties

Carrageenans are obtained from different species of Rhodophyta: *Gigartina, Chondrus crispus, Eucheuma* and *Hypnea* (Stanley, 1987). These polysaccharides are traditionally split into six basic forms: Iota (ι)-, Kappa (κ)-, Lambda (λ)-, Mu (μ)-, Nu (ν)- and Theta (θ)- carrageenan. This nomenclature is relevant both for their chemical classification (as discussed below) and to the commercial production, since the different carrageenans subtypes are extracted from distinct weed sources.

κ-Carrageenan is predominantely obtained by extraction of the tropical seaweed *Kappaphycus alvarezii*, known in trade as *Eucheuma cottonii* (or simply cottonii) (Rudolph, 2000). *Eucheuma denticulatum* (trade name *Eucheuma spinosum* or simply spinosum) is the main species for the production of ι-carrageenan. The seaweeds are usually extracted with alkali at high temperatures to transform the biological precursors μ- and ν-carrageenans into commercial κ- and ι-carrageenans. λ-Carrageenan is obtained from different species of the *Gigartina* and *Chondrus* genera. The sporophytic plants of these seaweeds produce λ-carrageenan (Mccandless, West, & Guiry, 1982), while the gametophytic plants produce a κ/ι-hybrid type of carrageenan. These κ/ι-hybrid type carrageenans, also known as "kappa-2" or "weak-gelling kappa carrageenans", consist of mixed polysaccharide chains containing both κ- and ι-units and range from almost pure ι-carrageenan to almost pure κ-carrageenan.

Carrageenans belong to the family of hydrophilic linear sulphated galactans. They mainly consist of alternating 3-linked β-D-galactopyranose (G-units) and 4-linked α-D-galactopyranose (D-units) or 4-linked 3,6-anhydro-α-D-galactopyranose (DA-units), forming the disaccharide repeating unit of carrageenans (Fig. 1). The letter codes assigned to the residues correspond to the simplified denomination developed by Knuten and coworkers (Table 1) (Knutsen et al., 1994). Sulphated galactans are classified according to the presence of the 3,6-anhydro-bridge on the 4-linked-galactose residue and the position and number of sulphate groups. Commercial carrageenans have an average molecular mass ranging between 100 and 1000 kDa. Besides galactose and sulphate, other carbohydrate residues can be present in carrageenan preparations, such as xylose, glucose and uronic acids, as well as some substituents, for example, methyl ethers and pyruvate groups (Van De Velde, Knutsen et al., 2002).

Carrageenans are traditionally identified by a Greek prefix. The three commercial most important carrageenans are Iota (ι-), Kappa (κ-) and Lambda (λ)-carrageenan and their corresponding names, based on IUPAC nomenclature and on the letter codes (Table 1) are carrageenase 2,4′-disulphate (G4S-DA2S), carrageenase 4′-sulphate (G4S-DA), and carrageenan 2,6,2′-trisulphate (G2S-D2S,6S), respectively. Besides these major carrageenan types, two other types, called Mu (μ) and Nu (ν) carrageenan (G4S-D2S,6S and G4S-D6S) are often encountered in commercial samples (Van De Velde, Peppelman, Rollema, & Tromp, 2001). They are the biological precursors of κ- and ι-carrageenan, respectively (Fig. 1), which are formed in vivo by the action of a sulfohydrolase (De Ruiter et al., 2001). The κ-, ι- and λ-carrageenan dimers have one, two and three sulphate ester groups, respectively, resulting in correspondent calculated sulphate contents of 20%, 33% and 41% (w/w). Typically, commercial κ-carrageenan contains 22% (w/w) of sulphate, ι-carrageenan 32% (w/w) and λ-carrageenan 38% (w/w), although large variations can occur owing to differences between seaweed species or batches (De Ruiter & Rudolph, 1997). Considering that each natural carrageenan is a complex galactose-based polysaccharide that has different quantities of sulphate esters at different positions and with different distributions, the term disaccharide repeating unit refers to the idealized structure (Van De Velde, Knutsen et al., 2002). The official method for determining the

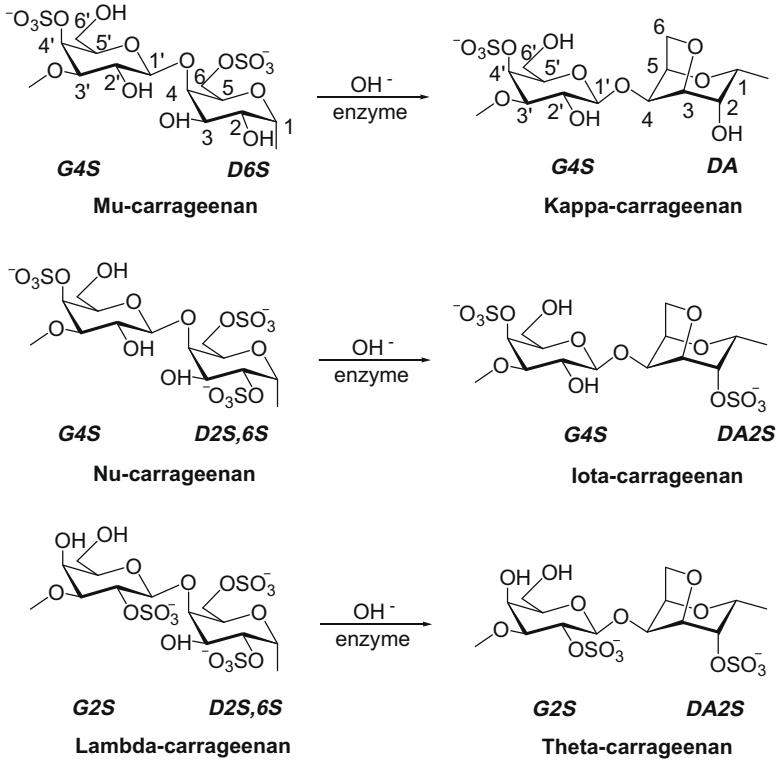

Fig. 1. Schematic representation of the different structures of the repeating dimeric units of commercial carrageenans and related structures.

Table 1
Alternative letter code for the different sugar units found in carrageenans, as developed by Knutsen, Myladobodski, Larsen, and Usov (1994).

Letter Code	Carrageenan	IUPAC name
G	β	3-Linked β-D-galactopyranose
D	Not found	4-Linked α-D-galactopyranose
DA	κ, β	4-Linked 3,6-anhydro-α-D-galactopyranose
S	κ, ι, λ, μ, ν, θ	Sulphate ester (O-SO_3^-)
G2S	λ, θ	3-Linked β-D-galactopyranose 2-sulphate
G4S	κ, ι, μ, ν	3-Linked β-D-galactopyranose 4-sulphate
DA2S	ι, θ	4-Linked 3,6-anhydro-α-D-galactopyranose 2-sulphate
D2S,6S	λ, ν	4-Linked α-D-galactopyranose 2,6-disulphate
D6S	μ	4-Linked α-D-galactopyranose 6-sulphate

sulphate content of carrageenans is based on the selective hydrolysis of the sulphate ester by acid and subsequent selective precipitation of the sulphate ions as barium sulphate, which is then measured by weighing or by turbidimetry as described by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (Food and Agricultural Organization, 1992). However, this method shows disadvantages: it is laborious and requires large quantities of samples. Some alternatives to this method have been described, including elemental analysis, infrared (IR) spectroscopy (Rochas, Lahaye, & Yaphe, 1986) methylation analysis (Stevenson & Furneaux, 1991) and nuclear magnetic resonance (RMN) (Knutsen & Grasdalen, 1992; Van De Velde, Knutsen et al., 2002).

Several carrageenans contain additional substituents. The most common are *O*-methyl groups in the position 6 of D unity, as occurs with κ-carrageenan extracted from *Kappaphycus alvarezii* and several others polysaccharides (Chiovitti et al., 1998). Pyruvate groups are also found mainly in λ-carrageenans, which forms a cyclic acetal at the positions 4 and 6 of 3-linked-galactose units (Fig. 2). Small quantities of terminal xylose have also been described in carrageenans, but their precise location has not been determined (Van De Velde et al., 2001).

All carrageenan fractions are water soluble, being insoluble in organic solvents, oil or fats. However, their water solubility depends essentially on the levels of sulphate groups (very hydrophilic) and on their associated cations. The main ionizable cations found in carrageenans are sodium, potassium, calcium and magnesium, but other ions can also occur at lower frequency (Pardonche, 1985). Consequently, the proportion of sulphate fractions and the equilibrium of cations in the water solution determine the viscosity of solutions or strength of gels formed by carrageenans, representing the major characteristics explored by the food and pharmaceutical industries in the use of carrageenans as thickening, gelling, and stabilizing agents.

Gelation of carrageenans, especially kappa, involves two separate and successive steps; coil-to-helix transition upon cooling and subsequent cation-dependent aggregation between helices. The presence of suitable cation, typically potassium or calcium, is an absolute requirement for gelation to proceed. For both ι- and κ-carrageenans, the alkali metal ions (Li^+, Na^+, K^+, Rb^+, Cs^+) are all capable of inducing gelation, but K^+ and Rb^+ are considerably more effective than other ions in inducing gelation at much lower concentrations of both the cation and the carrageenan (Funami et al., 2007; Ramakrishnan & Prud'homme, 2000). The viscosity of carrageenans depends on concentration, temperature, the presence of other solutes, the type of carrageenan and its molecular weight, and increases exponentially with the increase in their concentration, being a typical behaviour of linear polymers with charge, or polyelectrolytes. However, the increase of viscosity can occur by two different mechanisms: (i) interaction between the linear chains, with a decrease of the free space or increase of the excluded volume; (ii) formation of a physical gel caused by "crosslinking" between chains. In the first case, the increase of the macromolecule concentration allows a major interaction between the chains and the presence of salts can decrease the viscosity by reducing the electrostatic repulsion among the sulphate groups. For instance, this is what occurs for lambda fraction. The second mechanism of viscosity increase is particular from kappa, iota and hybrid kappa-2 fractions. For these carrageenans, in small concentrations of salt and low temperature, the carrageenan solutions can gelify, with increase of the apparent viscosity. The viscosity of the carrageenan solution decreases reversibly with the increase of temperature (Anderson, 1968).

Among commercial carrageenans, κ- and ι- are gel-forming carrageenans, whereas λ-carrageenan is characterized only as a thickener agent. The difference in rheological behaviour between κ- and ι-carrageenan on one side and λ-carrageenan on the other side results from the fact that the anhydrogalactose units (DA) of the gelling ones have $^1C_4$ conformation (Fig. 3) and the D-galactopyranosil units (D) in λ-carrageenan do not. The $^1C_4$ conformation of the 3,6-anhydro-D-galactopyranosil units in κ- and ι-carrageenan allows a helicoidal secondary structure, which is essential for the gel-forming properties. It's important to note that the μ- and ν-carrageenans, natural precursors of κ- and ι-carrageenans, with the D-galactopyranosil units in the $^4C_1$ conformation (Fig. 3), are also non-gelling carrageenans. The occurrence of disaccharide units without the 3,6-anhydro ring and having a $^4C_1$ conformation causes "kinks" in the regular chain, preventing the formation of helical strands and as a result, prevents gelation of the carrageenan (Van De Velde, Knutsen et al., 2002).

Kappa- and iota-carrageenans form a network of three-dimensional double helices (Fig. 4), resulting from the "crosslinking" of the adjacent spiral chains that contain sulphate groups oriented towards their external part. In λ-carrageenan, the 2-sulphate group is oriented towards the internal part, thus avoiding this "crosslinking" (Morais, Do Valle, & Pizzinatto, 1989).

The presence of considerable quantities of precursors units in the commercial preparations of carrageenans (κ- and ι-) has a negative effect in the functional properties of the product (gel

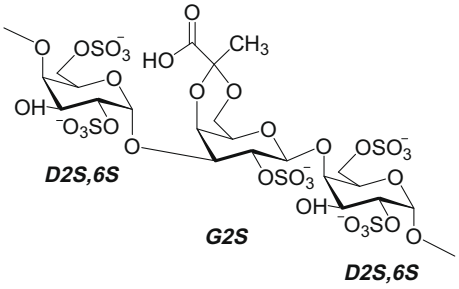

Fig. 2. Pyruvate substituent in λ-carrageenan.

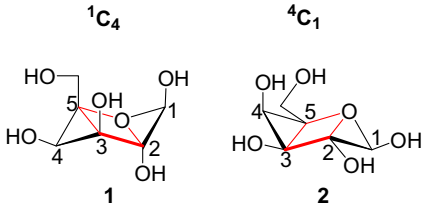

Fig. 3. Representation of $^1C_4$ and $^4C_1$ conformations for β-D-galactopyranose. *The conformations are defined by numerals indicating ring atoms lying above or below a defined reference plane (red) containing C-2, C-3, C-5 and O atoms. In structure **1**, C-1 is above the plane and C-4 is below, and thus, the symbol is $^1C_4$. Likewise, structure **2** is $^4C_1$.

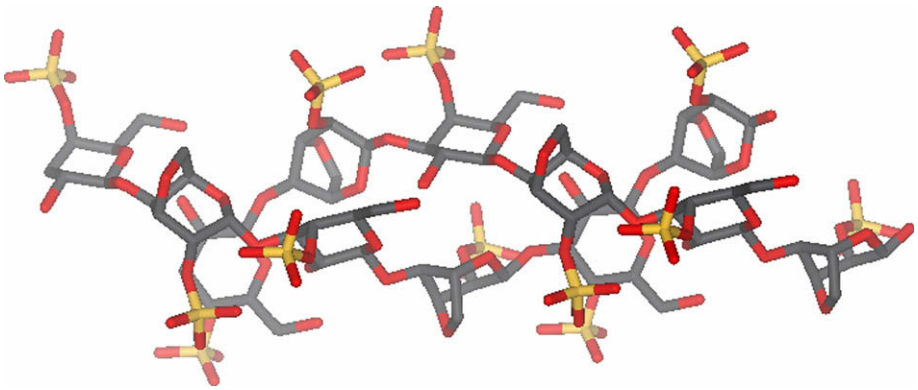

Fig. 4. Double helix model of ι-carrageenan. Structure downloaded from Protein Data Bank (PDB code 1CAR) (www.rcsb.org/pdb).

formation). Thus, in industrial processing, previous to use, crude carrageenan is submitted to alkaline treatment or alkaline extraction, catalyzing the cyclization reaction with hydroxide (OH^-) to generate the 3,6-anhydro-bridge (Fig. 5) (Van De Velde, Knutsen et al., 2002). The reaction occurs when 6-sulphated-α-galactose units are present. Thus, by heating the polysaccharide in strong alkaline media, the free 3-OH group is ionized and produces an intramolecular nucleophilic displacement of the sulphate group at position 6. Another requirement for the helix formation is the ions present in solution. Potassium ions are able to be introduced between double helices and, as they neutralize the charges of sulphate groups, facilitate the approach between them. Potassium has also the property of stabilizing the double helix. The diameter of sodium ion, considerably bigger (hydrated radius), does not cause the same effect. Divalent cations also decrease the viscosity when present at high levels, but increase it at lower concentrations (Morais et al., 1989).

3. Biological activities

Carrageenans are classically used as agents for the induction of experimental inflammation and inflammatory pain (Morris Christopher, 2003). They have also shown several potential pharmaceutical properties including antitumor, immunomodulatory (Zhou et al., 2004), antihyperlipidemic (Panlasigui, Baello, Dimatangal, & Dumelod, 2003) and anticoagulant activities (Caceres, Carlucci, Damonte, Matsuhiro, & Zuniga, 2000). Some *in vitro* studies suggest that carrageenans may also have antiviral properties, inhibiting the replication of herpes and hepatitis A viruses (Carlucci, Scolaro, & Damonte, 1999; Girond, Crance, Van Cuyck-Gandre, Renaudet, & Deloince, 1991; Gonzalez, Alarcon, & Carrasco, 1987; Marchetti et al., 1995).

More recently, Schiller and coworkers demonstrated that carrageenan is an extremely potent infection inhibitor of a broad range of genital human papillomaviruses (HPVs) and there are indications that carrageenan-based sexual lubricant gels may offer protection against HPV transmission (Buck et al., 2006; Roberts et al., 2007).

Researchers from the Population Council, a non-profit research organization in the USA, conducted a clinical trial investigating whether a carrageenan-based sexual lubricant was effective as a topical microbicide by blocking HIV infection in women. The study was conducted from March 2004–2007 in South Africa and enrolled more than 6000 volunteers. The results demonstrated that the experimental microbicide gel was safe for use but does not provide protection against HIV (Population Council, 2008). These results are consistent with "*in vitro*" assays since carrageenan was active against HIV only at concentrations about 1000 times higher than those required to inhibit papillomaviruses (Buck et al., 2006). However, carrageenans may serve as models for designing novel anti-HIV agents, improving their therapeutic properties through chemical modifications.

Questions about the safety of carrageenan-containing foods were raised by Tobacman (2001). In a review of 45 studies using animal models (rats, mice, rabbits and guinea pigs), low molecular weight (degraded) carrageenans were associated with induction and promotion of intestinal neoplasms and ulcerations. These results are a warning to potential problems associated with carrageenan consumption in humans, such as inflammatory bowel disease and gastrointestinal malignancy.

At the same time, the safety of carrageenans for use in foods was confirmed at the 57th meeting of the JECFA-Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives (JECFA, 2001). According to the JECFA, only degraded carrageenans were associated to adverse effects and should not be used as food additives.

However, in a wider perspective, it is true that the exposure to non-degraded carrageenan is inevitably accompanied by exposure to degraded carrageenan. Six years later, the JECFA reviewed their position and declared the use of carrageenans in infant formula as not recommended, suggesting new studies on the consumption of these polysaccharides in the diet (JECFA, 2007).

Recently, Tobacman and coworkers demonstrated that the exposure of ingested carrageenans on human intestinal epithelial cells resulted in increased cell death, reduced cell proliferation and cell cycle arrest when compared to unexposed control cells (Bhattacharyya, Borthakur, Dudeja, & Tobacman, 2008). According

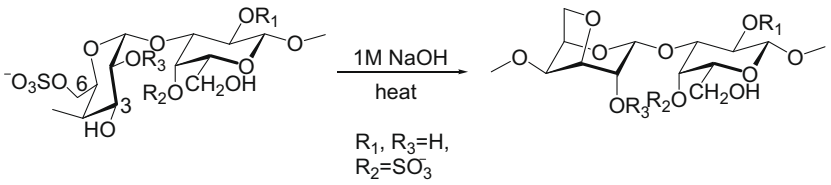

Fig. 5. Cyclization reaction catalyzed by alkaline treatment applied to carrageenans.

to these data, the extensive use of carrageenans as food additives should be reconsidered.

4. Chemical modifications

Several chemical modifications have been proposed to modulate physicochemical properties of carrageenans. Guiseley (1978) proposed the synthesis of κ-carrageenan-containing hydroxyalkyl groups. Aqueous κ-carrageenan gels can display an undesirable large extent of syneresis (the extraction of a liquid from a gel) when subjected to mechanical deformation or aging, a natural process that affects the mechanical, magnetic and rheological properties of the gels. Gels prepared from hydroxyalkyl κ-carrageenan derivatives demonstrated decreased syneresis and could contribute to a wider industrial use of these polysaccharides. Recently, Tari and Pekcan (2008) demonstrated that the association of κ-carrageenan with $CaCl_2$ can change the swelling properties of carrageenan gels, emphasizing the commercial relevance of carrageenans chemical modifications.

Probably the best described chemical reactions of carrageenans are related to alkali modifications. λ- and κ-Carrageenans undergo cyclization in the presence of 1 M sodium hydroxide at 80 °C. The formation of 3,6-anhydro-α-D-galactose units from α-D-galactose 6-sulphate (as previously illustrated in Fig. 5) follows a pseudo first-order kinetics and is 20–60 times faster for κ-carrageenans than for those of the λ-family (Ciancia, Noseda, Matulewicz, & Cerezo, 1993). The cyclization of homogeneous ι/ν-hybrid carrageenan (71% ι- and 21% ν-) under the above described conditions is about 210 times faster than that of a λ-carrageenan. Consequently, the alkaline treatments used industrially to increase the gelling properties of crude ι/ν-hybrid carrageenans could be carried out under milder conditions, avoiding degradation and increasing the gel strength (Viana, Noseda, Duarte, & Cerezo, 2004). When different reaction variables were tested, a higher rate of cyclization was observed at the 2 M sodium hydroxide concentration and the use of microwave heating accelerated in 30–60 times the rate of λ-carrageenans reactions (Navarro & Stortz, 2005).

Hosseinzadeh, Pourjavadi, Mahdavinia, and Zohuriaan-Mehr (2005) prepared a κ-carrageenan hydrogel with a very high absorptivity in saline through polyacrylamide "crosslinking" followed by alkaline hydrolysis (Fig. 6). The hydrogel displayed water absorbance properties similar to those of κ-carrageenan copolymerized with acrylic acid (Pourjavadi, Harzandi, & Hosseinzadeh, 2004). By using γ-irradiation as an initiator and "crosslinking" agent at the same time, the swelling behaviour of these synthesized hydrogels could also be improved (Rezanejade Bardajee, Pourjavadi, Sheikh, & Sadegh Amini-Fazl, 2008).

Grafting of κ-carrageenan with methyl methacrylate was carried out by Prasad, Meena, and Siddhanta (2006) employing microwave irradiation. The resultant crystalline product could be obtained rapidly (2 min). The copolymerization of κ-carrageenan with acrylic acid and 2-acrylamido-2-methylpropanesulfonic acid led to the development of biodegradable hydrogels with potential use for novel drug delivery systems (Pourjavadi, Barzegar, & Zeidabadi, 2007). These carrageenan hydrogels are very promising for industrial immobilization of enzymes since the immobilization procedures result in increases of the storage and thermal stability of the enzymes necessary for use in continuous systems (Tumturk, Karaca, Demirel, & Sahin, 2007).

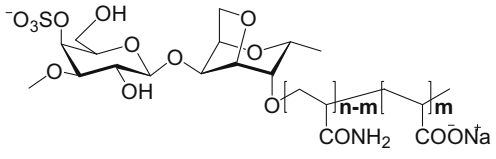

Fig. 6. Structure of partially hydrolyzed κ-carrageenan hydrogel copolymerized with acrylamide. In structure, *n* represents the total number of acrylamide units grafted on κ-carrageenan in the presence of *N,N'*-methylene bisacrylamide and *m* represents the number of these residues that underwent partial hydrolyzes by the action of NaOH and heating.

In the biomedical area, the use of modified carrageenans has also been explored. Porous nanocomposites were prepared by coprecipitation of calcium phosphates into a κ-carrageenan matrix (Daniel-da-Silva, Lopes, Gil, & Correia, 2007), whose resulting porosity and morphology were suitable for application in bone tissue engineering. The association between carrageenan, nanohydroxyapatite and collagen resulted in an injectable bone substitute biomaterial, suitable for bone reconstruction surgery (Gan & Feng, 2006).

Yuan et al. (2005) synthesized oversulphated, acetylated and phosphorylated derivatives of κ-carrageenan. Sulphation was carried out using chlorosulphonic acid ($HClSO_3$) in *N,N*-dimethylformamide (DMF), acetylation employed acetic anhydride (Ac_2O) and pyridine (Py) in DMF, and phosphorus oxychloride ($POCl_3$)/Py in formamide (FA) followed by hydrolysis were applied for phosphorylation (Fig. 7). All derivatives exhibited an antioxidant activity higher than that of κ-carrageenan, suggesting that chemical modification can enhance antioxidant activity of carrageenans (Yuan et al., 2006). Previously, Jones, Colfen, and Antonietti (2000) had shown that κ-carrageenan acts *in vitro* as an efficient stabilizer of iron oxides even up to very high pH values. Mou, Jiang, and Liu (2007) prepared κ-carrageenan oligose sulphonate, by combining enzymolysis and chemical modification, and demonstrated significant antioxidant activity of the products obtained.

He, Cheng and Lu demonstrated that synthetic κ-selenocarrageenans may inhibit the proliferation of breast cancer cells (Fig. 8) (He, Cheng, & Lu, 1997). Hu, Jiang, Aubree, Boulenguer, and Critchley (2006) used κ-carrageenases extracted from the marine bacterium Cytophaga MCA-2 to produce low molecular weight κ-carrageenans. These derivatives showed much higher *in vivo* tumor inhibition than the parent compounds, and the observed weight increase of immune organs such as the thymus suggested that the antitumor mechanism of the carrageenan oligosaccharides may be initiated via organ-mediated defense reactions. These findings are complementary to the studies of Mou, Jiang, and Guan (2003) in which depolymerized κ-carrageenans were sulphated by the use of chlorosulfonic acid in formamide and the resulting oligosaccharides showed *in vitro* antitumor activity.

Low molecular weight carrageenans and their sulphated derivatives were prepared by depolymerization in the presence of ferrous ions or ferrous ions plus ascorbic acid by Yamada et al. (1997). All compounds demonstrated anti-HIV activities and sulphation of κ-carrageenan also increased the biological effects. In a later study these depolymerized carrageenans were acylated by carboxylic acid anhydrides using DMF as solvent and 4-dimethylaminopyridine (DMAP)/tributylamine (TBA) as catalysts (Fig. 9) (Yamada, Ogamo, Saito, Uchiyama, & Nakagawa, 2000). The acylation of low molecular weight carrageenans resulted in potentiation of anti-HIV activity although the anticoagulant activity of the compounds decreased.

Jiang and Guo (2005) achieved the synthesis of an *O*-maleoyl derivative of κ-carrageenan by reaction of tetrabutylammonium salt of the anionic polysaccharide fragments with maleic anhydride, 4-dimethylaminopyridine and tributylamine under homogeneous conditions in *N,N*-dimethylformamide. In contrast to the acylation described by Yamada et al. (2000), that took place at all free hydroxyl groups of κ-carrageenan, the maleoylate substitution resulted in the formation of a monoester derivative at the C2 of the G4S unit (Fig. 10). The design of the *O*-maleoyl derivative increased the density of the negative charge and rigidity of the carrageenan molecule, two attributes that enhances the anti-HIV activity.

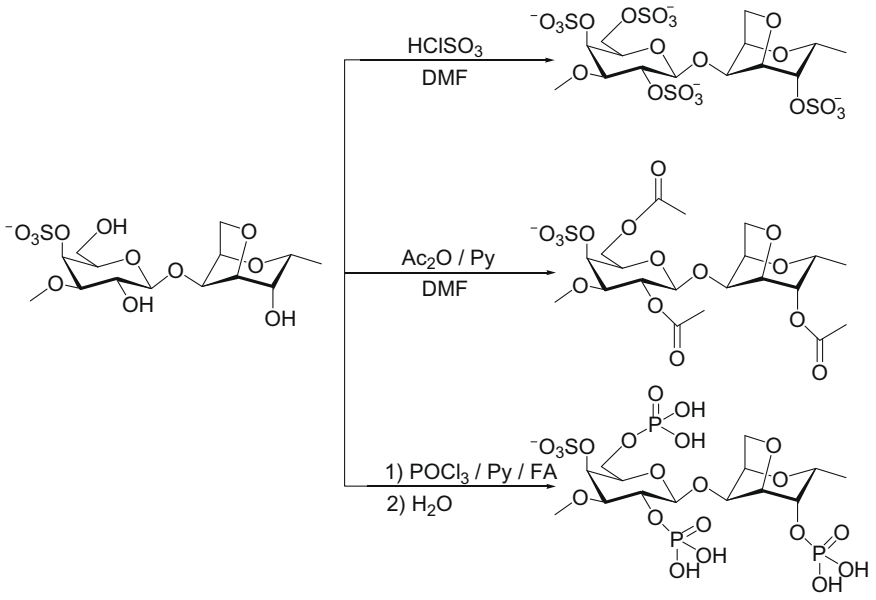

Fig. 7. Sulphation, acetylation and phosphorylation of κ-carrageenan.

O-Succinyl derivatives of ι-carrageenan have also been synthesized by carrageenan depolymerization in mild hydrochloric acid condition followed by acylation with succinic anhydride in *N*,*N*-dimethylformamide and in the presence of 4-dimethylaminopyridine/tributylamine under homogeneous conditions (Jiang, Guo, & Chen 2007). In this reaction, the succinyl substitution took place at both free hydroxyl groups of the ι-carrageenan G4S unit.

Vlieghe et al. (2002) described for the first time the association between κ-carrageenan and AZT (Azidothymidine). A succinate diester spacer was used to attach one molecule of AZT to a tirade of κ-carrageenan residues (Fig. 11) and synergistic activity of the two drugs was demonstrated when MT-4 cells were pre-incubated with the conjugate prior to HIV-1-infection. The κ-carrageenan–AZT conjugate allowed the reduction of the individual doses, avoiding AZT toxicity and preventing the emergence of drug-resistant virus strains.

5. Structural analysis of carrageenans

There are not enough suitable analytical techniques to determine the quantity, the polydispersity and the purity of carrageenans in food products and raw materials. Moreover, the relationship of their biological activities with their substitution pattern (position of sulphate group) is still uncertain because of the lack of analytical methods for the determination of the fine structure of carrageenans at the polysaccharide level (Aristotelis, Patrick, William, & Michel, 2007). Thus, proper analytical tools are needed in order to characterize such complex mixtures.

Colorimetry and immunoassays, HPLC and electrophoresis are some examples of different techniques and approaches used for this type of analysis (Roberts & Quemenery, 1999). The chemical analysis to reveal the detailed molecular structure of carrageenans

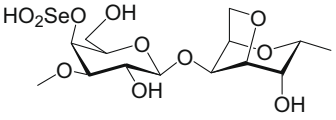

Fig. 8. κ-Selenocarrageenan.

is mostly done on isolated and purified carrageenan samples. Initially, the chemical modification and degradation methods were time-consuming and laborious techniques, but, since 1970s a great progress was made by the introduction of Nuclear Magnetic Resonance (RMN). At present, NMR spectroscopy (both ^{1}H and ^{13}C) is one of the standard tools for the determination of the chemical structure of carrageenan samples, being faster and practical (Roberts & Quemenery, 1999). Besides NMR spectroscopy, other analytical techniques, such as sulphate content, monosaccharide composition and methylation analyses are being applied. Infrared (IR) spectroscopy has been used for the qualitative characterization of carrageenans (Pascal, Richard, & Christelle, 2004), in terms of purity and composition, and recently, electrospray ionization mass spectrometry (ESI-MS) has proven to be a valuable tool for the characterization of sulphated oligosaccharides (Antonopoulos, Favetta, Helbert, & Lafosse, 2005; Antonopoulos, Hardouin, Favetta, Helbert, & Lafosse, 2005; Aristotelis et al., 2007; Guangli et al., 2006). In most cases, it is advisable to use more than one technique, simultaneously, for the identification of carrageenan constituents. The evaluation of rheological parameters is also frequently employed for commercial carrageenans.

5.1. NMR spectroscopy of main types of carrageenans

In general, carrageenan samples are sonicated prior to NMR analysis and the NMR experiments are carried out at elevated temperatures to reduce the viscosity of the solution (high viscosity results in line broadening). Nevertheless this causes alterations in the signals chemical shifts. In the case of ^{1}H NMR, the effect is greater for protons directly linked to carbons involved in glycosidic linkages. An important factor is that the signal correspondent to the residual water (around 4.8 ppm at room temperature) suffers displacement to high field, being around 4.2 ppm when the spectra are obtained at elevated temperatures. Due to the low natural abundance of the ^{13}C isotope, samples for ^{13}C NMR are prepared at relatively high concentrations (5–10% w/w in D_2O). Both the ^{1}H and ^{13}C NMR spectra reveal that it's difficult to have "pure" carrageenan samples, being common to find units of ι- in κ-carrageenan and vice versa, as well as low percentages of carrageenan precursor units (Van De Velde, Knutsen et al., 2002).

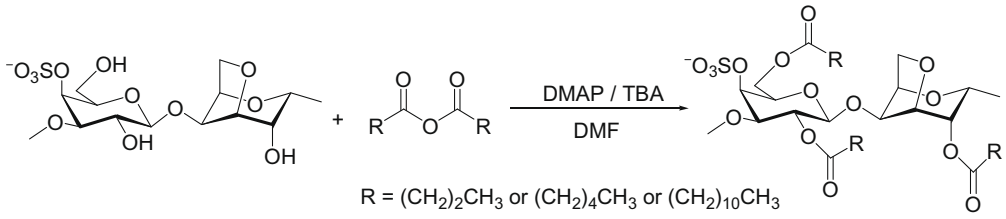

Fig. 9. Total *O*-acylation of κ-carrageenan with three different acid anhydrides: butanoic, hexanoic and dodecanoic.

5.1.1. ^{1}H NMR spectroscopy

Quantification of different carrageenan types in a sample by ^{1}H NMR spectroscopy is based on the position and intensity of the resonances of the α-anomeric hydrogens of the repeating D- and DA-units in the region from 5.1 to 5.7 ppm (Table 2 and Figs. 8–10). On the other hand, the signals of α-anomeric hydrogens of the G-units are less suitable for either identification or quantification purposes, considering that the β-galactose residues are identical in κ- and ι-carrageenans (G4S unit), giving signals at the same regions (Van De Velde, Knutsen et al., 2002) (Figs. 12–14).

^{1}H NMR spectroscopy has more advantages if compared to ^{13}C NMR, such as sample concentration and shorter time for analysis. In particular, it is more suitable for a quantitative approximation of the different types of carrageenans, although quantitative results of great accuracy are very difficult to be obtained. Nonetheless, there is a possibility to obtain specific fragmentations, using enzymes, which could generate fragments that in solution would not alter the viscosity, thus turning enable to get spectra with reliable integrals. One difficulty for this strategy is the hybrid nature of carrageenans that, containing different disaccharide units and being resistant to enzymolysis, do not produce equivalent fragments.

Regarding the analysis of carrageenan fragments by ^{1}H NMR spectroscopy, the knowledge about the different oligosaccharides in the different fractions can be useful for determination of the structure of the original carrageenan sample. In this fragment analysis approach, ^{1}H NMR spectroscopy is used due to its high sensitivity and the possibility of resolving the fine structure of carrageenan oligosaccharides. Carrageenan oligosaccharides can give higher and lower molecular mass fractions, which are separated by precipitation methods and further fractionated by gel permeation chromatography (GPC). Subsequently, high field ^{1}H NMR spectroscopy is used to elucidate the detailed molecular structure of the different di-, tetra- and oligosaccharides (Van De Velde, Knutsen et al., 2002; Van De Velde et al., 2001).

Carrageenan preparations, mixtures of carrageenans and commercial carrageenan blends can qualitatively and quantitatively be characterised by NMR techniques, which allow the identification of various carrageenan forms and the determination of the molar ratios and the content of the individual components in the mixture. Both ^{1}H and ^{13}C NMR spectroscopy are applicable for these purposes, but ^{1}H NMR has the advantage of a relatively high sensitivity: ^{1}H NMR spectra of samples with low carrageenan concentration (0.5–1.0% w/w) can be recorded in a couple of minutes (Van De Velde, Knutsen et al., 2002; Van de Velde, Pereirac, & Rollemab, 2004).

5.1.2. ^{13}C NMR spectroscopy

^{13}C NMR spectroscopy is recommended for distinguishing the polysaccharides of the agar and carrageenan groups. This technique allows to differentiate D-galactose signals (present in carrageenans) from those of L-galactose (particular of agar), especially notably for anomeric carbon resonances. The anomeric carbon of 3,6-anhydro-D-galactopyranose generate signals with a difference of 3 ppm in high field if compared to the anomeric signal of 3,6-anhydro-L-galactopyranose (Usov, Yarotskii, & Shashkov, 1980). The ^{13}C NMR spectra of gel-forming κ- and ι-carrageenans were studied using synthetic models of monosaccharide or oligosaccharides (Greer, Rochas, & Yaphe, 1985; Usov, 1984). The structures were then confirmed by various 2D NMR experiments, being the assignments successfully used to identify the corresponding polysaccharides isolated from new sources (Usov & Shashkov, 1985). The well resolved spectrum of λ-carrageenan $[G2S\text{-}D2S,6S]_n$ was obtained later due to some technical difficulties, which are usually explained by the high viscosity of λ-carrageenan solutions (Falshaw & Furneaux, 1994).

Most carrageenans differ only in 3,6-anhydrogalactose content, degree of sulphation and positions of sulphate groups. An attempt was made to calculate the ^{13}C NMR spectra of many possible carrageenan structures, including those that were not isolated from natural sources, utilizing spectral features of known polysaccharides (Storz & Cerezo, 1992). However, the amount and specific position of charged sulphate groups may result in conformational changes of polysaccharide chains, which are not taken into consideration by additive schemes in calculations of chemical shifts (Van De Velde, Knutsen et al., 2002).

Considering that ^{13}C NMR spectroscopy has low sensibility, it is not possible to determine small proportions of minor substituents in carrageenans. Thus, negative results do not exclude the presence, for example, of α-L-galactose and/or hybrid components D/L or even agarans as components (Estevez, Ciancia, & Cerezo, 2000). Nevertheless, ^{13}C NMR spectroscopy can be useful to determine the contaminants and additives in carrageenan samples, as for example, galactomannans or mannans of some higher plants (Imeson, 2000), which are added to carrageenans to increase their gelling properties. Since the ^{13}C NMR spectra of galactomannans are well-known (Grasdalen & Painter, 1980), the corresponding spectra of mixed preparation recorded at elevated temperatures (above the melting points of gels) can be used to detect the presence of galactomannan additives and calculate their content relative to carrageenan. Pyruvic acid is another common component of many complex carrageenans that can be detected by ^{13}C NMR. It forms a cyclic acetal at positions 4 and 6 of 3-linked-galactose

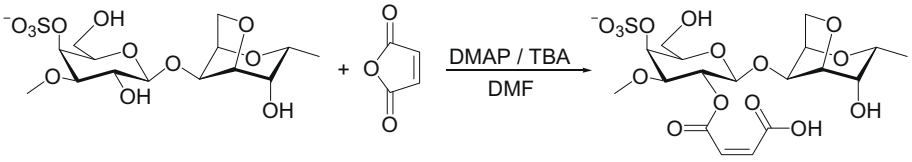

Fig. 10. Synthesis of *O*-maleoyl κ-carrageenan derivative.

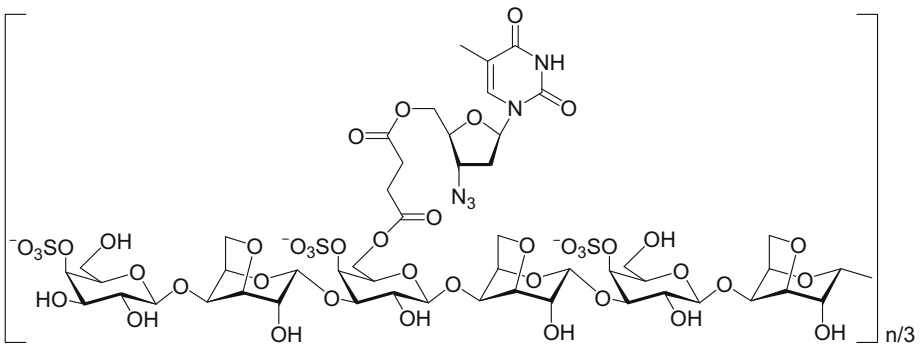

Fig. 11. Structure of κ-carrageenan-AZT conjugate. In structure, *n* represents the total number of κ-carrageenan units in the polymer.

residues and can be identified by characteristic signals of its carbons together with specific substitution effects on the corresponding carbon atoms of 3-linked D-galactose (Chiovitti et al., 1998; Falshaw & Furneaux, 1995).

5.2. Colorimetric methods

Several colorimetric methods for the determination of total carrageenans or, more specifically, sulphated polyanions, have been described in the literature (Cundall, Phillips, & Rowlands, 1973; Soedjak, 1994; Yabe, Ninomiya, Tatsuno, & Okada, 1991). In general, colorimetric methods use some type of cationic dye which dynamically complexes with polyanions. The binding event then changes either the spectrophotometric or spectrofluorimetric properties of the dye molecule which can be measured with an appropriate optical detection system. Considering that carrageenans are highly sulphated, they are good binding partners with these dyes. Furthermore, the sulphate group remains anionically charged even at a very low pH, being the manipulation of the pH an important way to obtain higher specificity of other polyanions, such as carboxylated polymers that are present in the sample. All colorimetric methods are sensitive to the degree of sulphation and, therefore, give more or less different responses for kappa-, iota- and lambda-carrageenan types (Roberts & Quemenery, 1999).

The colorimetric method based on the polyanion binding to the cationic dye, methylene blue (Soedjak, 1994), has been considered as one of the most advantageous colorimetric method reported so far. In this method, it is necessary to work at very dilute assay concentrations and, upon binding to a polyanion such as carrageenans, methylene blue undergoes an absorption maximum shift from 610/664 to 559. As expected, the dye responds linearly to the molar sulphate content of carrageenans and, therefore, as a different response factor for κ-, ι- or λ-carrageenan. This method has proved to be very sensitive to carrageenans, with capacity of determination between 0.2 and 2×10^{-3}% of sample.

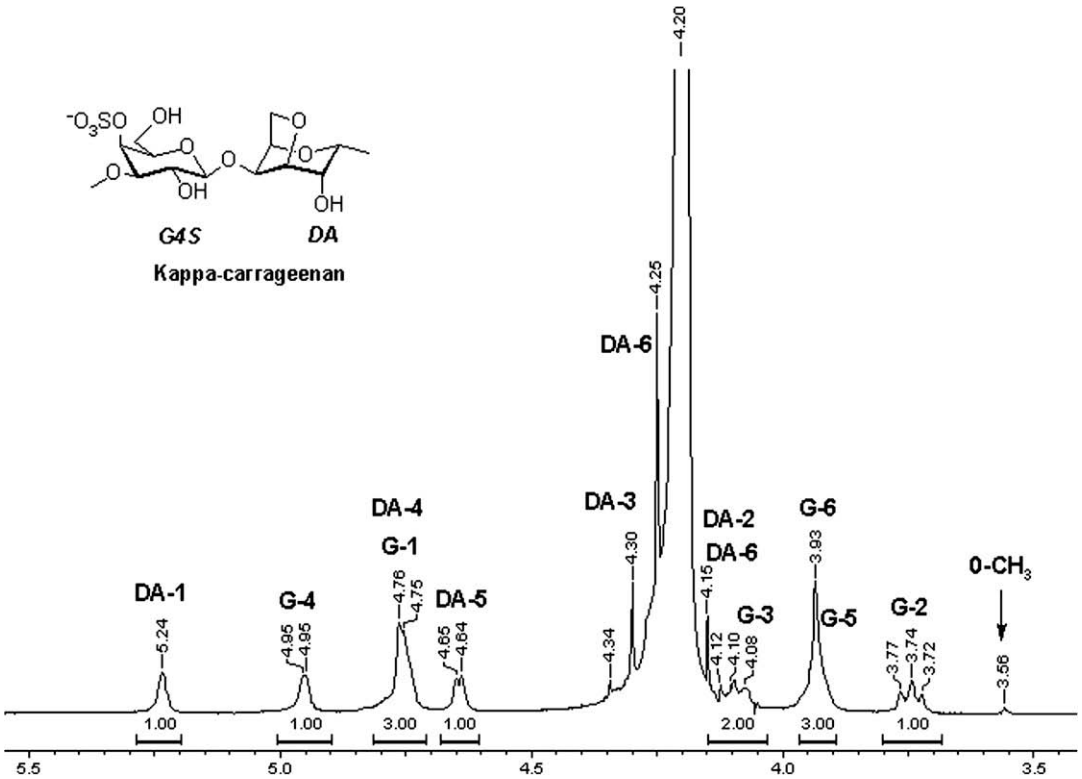

Fig. 12. ^{1}H NMR spectra of κ-carrageenan in deuterium oxide (D_2O). The analysis was performed on a Bruker DPX 400 MHz spectrometer at 80 °C.

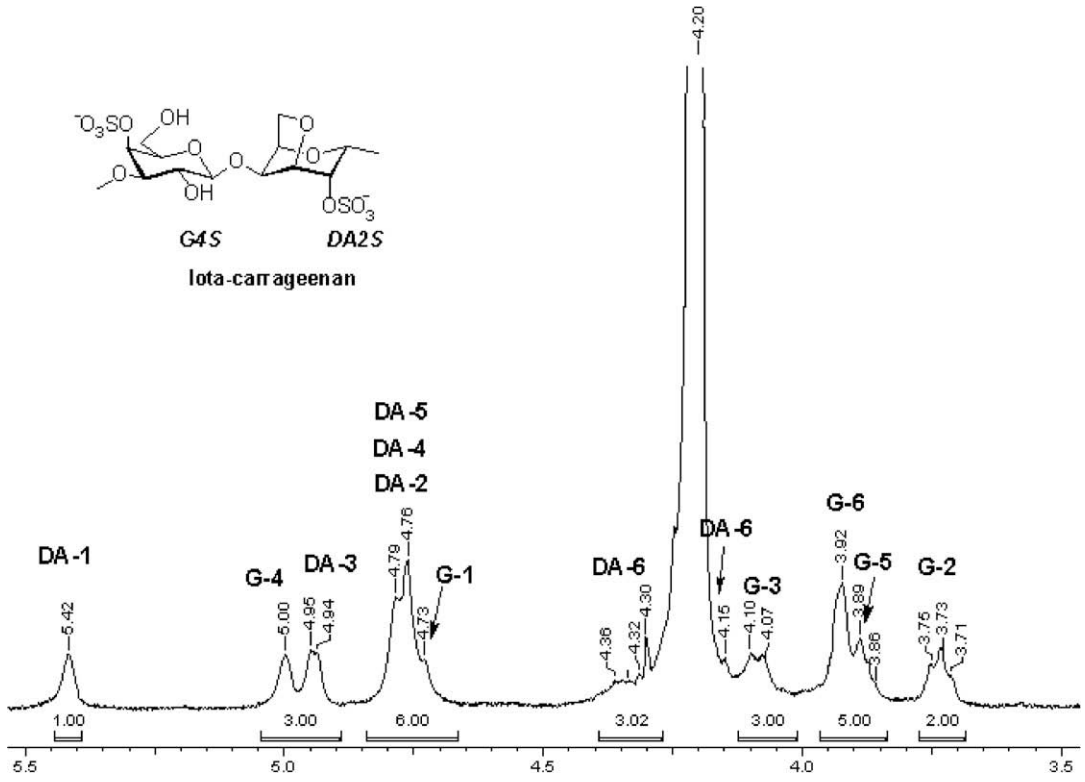

Fig. 13. ^{1}H NMR spectra of ι-carrageenan in deuterium oxide (D_2O). The analysis was performed on a Bruker DPX 400 MHz spectrometer at 80 °C.

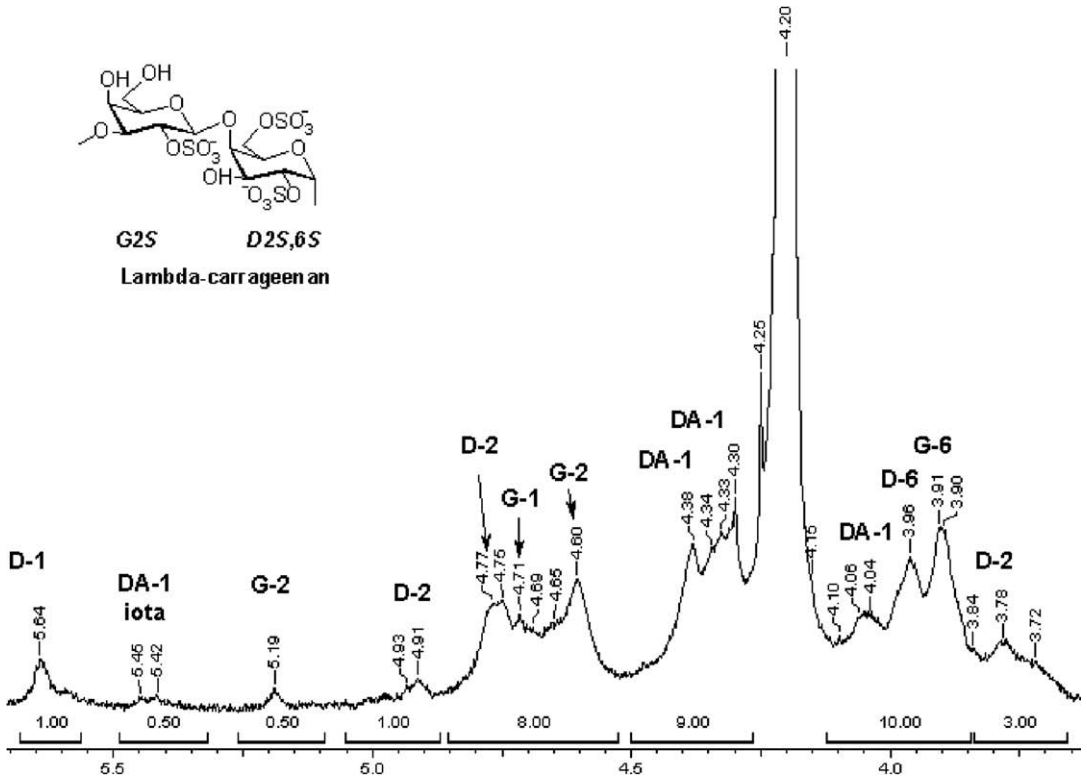

Fig. 14. ^{1}H NMR spectra of λ-carrageenan in deuterium oxide (D_2O). The analysis was performed on a Bruker DPX 400 MHz spectrometer at 80 °C.

Table 2
Chemical shifts (ppm) assignment in the ^{1}H NMR spectra of kappa-, iota- and lambda-carrageenans obtained at 80 °C.

Carrageenan	Residue G					
	H-1	H-2	H-3	H-4	H-5	H-6,6′
κ	4.75	3.74	4.10	4.95	3.93	3.93
ι	4.73	3.73	4.07	5.00	3.89	3.92
λ	4.71	4.60	–[a]	–	–	–
	Residue DA or D					
	H-1	H-2	H-3	H-4	H-5	H-6_{exo}/H-6_{endo}
κ	5.24	4.12	4.30	4.75	4.65	4.25/4.15
ι	5.42	4.76	4.95	4.76	4.76	4.25/4.15
λ	5.64	4.77	–	–	–	–

[a] Difficult identification.

5.3. Chromatographic methods

5.3.1. Gas chromatography (GC) and high-performance liquid chromatography (HPLC)

Gas chromatography (GC) has been used to analyse small saccharides which have undergone derivatization; however, it is not directly applied to larger oligosaccharides or polysaccharides. In general, polysaccharides have to be hydrolyzed and the released monosaccharides reduced to alditols or oxidized (to eliminate the anomeric center), followed by their conversion into volatile compounds (Roberts & Quemenery, 1999). Both steps, hydrolysis of the polysaccharide and reduction of the constituent monosaccharides into their corresponding alditols must be carefully evaluated in order to optimize the analysis with respect to reliability, reproducibility and sensitivity, resulting in a rapid, sensitive, and accurate procedure (Jol, Neiss, Penninkhof, Rudolph, & De Ruiter, 1999). The derivatization techniques employed have included trimethylsilylation, acetylation, and trifluoroacetylation, being the alditol acetates the most popular derivatives for GC analysis (Bradbury & Halliday, 1984).

This combination of mild acid degradation, monosaccharide derivatization, and subsequent separation by GC can be used for the analysis of carrageenans, as well as many other industrial gums (Al-Hazmi & Staufer, 1986; Clingman, Nunn, & Stephen, 1957; Lawrence & Iyengar, 1985). The hydrolysis of gum samples can be performed using 0.5 M trifluoroacetic acid (TFA) for 4 h at 100 °C, followed by a two step derivatization procedure to aldonitrile acetates. In case of carrageenans, additional care has to be taken during the degradation using acid in order to protect the acid-sensitive 3,6-anhydrogalactosyl residues used as a marker for the parent polysaccharide (Clingman et al., 1957).

High-performance liquid chromatography (HPLC) analysis of carrageenans has also been performed after various depolymerization methods, either directly on commercial products or for their detection in a food matrix Heyraud and Rochas (1982). Voragen, Schols, and Pilnik (1982) have analysed different commercial anionic gums using methanolysis and subsequent reversed-phase HPLC for the quantification of 3,6-anhydro-galactose dimethylacetal released from carrageenans (as agars). Methanolysis was carried out in methanolic 0.15 N HCl for 16 h at 80 °C. The molar ratio between sulphate and anhydrogalactose contents was used for the characterization of carrageenan preparations, permitting the differentiation of mixtures of κ- with some ι-carrageenan, of ι- with some κ- carrageenan and of κ-, ι- and λ-carrageenans. However, the relatively poor efficiency of the Lichrosorb 10 RP 18 column used in these experiments did not allow the simultaneous determination, in a single run, of both the anhydrogalactose and galactose constituents of carrageenan preparations studied.

The methanolysis of galactans from red algae under various experimental parameters (time, temperatures and acidic strength), followed by reversed-phase HPLC using water as the eluent, was investigated by Quemener, Lahaye, and Metro (1995). Carrabiose- and agarobiose-dimethyl acetal produced from carrageenan and agar, respectively, using very mild methanolysis (0.01 M HCl, 100 °C, 1 h) were well separated on a Superspher C18 cartridge (25 × 0.4 cm). Thus, these conditions may be used for the identification of carrageenan in the presence of agar. The same group also compared methanolysis–HPLC with reductive hydrolysis coupled to high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC–PAD) (Quemener & Lahaye, 1998). By this technique, optimal hydrolysis conditions involved a reaction at 120 °C with 1 M sulfuric acid for 120 min prior to neutralization at pH 7.0 (Koswig, Fuchs, Hotsommer, & Graefe, 1997), using the Carbopac MA1^R column. Both of the techniques compared provided accurate and similar measurements of the amount of κ- and ι-carrageenans and were therefore termed complementary techniques.

5.3.2. Molecular weight analysis through size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) has been extensively used in the food industry for the determination of molecular weight distributions (MWD) and quality control of polysaccharides (Beri, Walker, Reese, & Rollings 1993; Fishman, Pfeffer, Barford, & Doner 1984). The understanding of carrageenans molecular mass (MM) and their distribution give important information about chemical composition, chain branching and kinetics of degradation reactions (Eremeeva, 2003).

SEC proved to be useful for the characterization of carrageenans. For instance, oligomers of κ-carrageenan were fractionated using modified silica packing material; intermediate and higher molecular weight carrageenans were separated on sepharose (Slootmaekers, Dijk, Varkevisser, Bloys van Treslong, & Reynaers, 1991). However, accurate measurement of molecular size distribution can only be obtained under conditions in which aggregation of the sulphated chains does not take place. This has been achieved by operating both column and eluent at high temperature and by adding iodide salts of non-gelling cations [e.g., Li^+, Na^+, $N(CH_3)_4^+$, etc.] to the solvent, since these salts induce an ordered conformation without further aggregation (Hjerde, Smidsrød, & Christensen, 1999; Sworn, Marrs, & Hart, 1987).

Based on the principle that intrinsic viscosity is related to the hydrodynamic volume of the polymers, various detector systems have been used in conjunction with SEC for the analysis of carrageenans. They comprise refractive index (RI) (Ekeberg, Knutsen, & Sletmoen, 2001; Ekström & Kuivinen, 1983; Karlsson & Singhb, 1999; Knutsen, Moe, Larsen, & Grasdalen, 1993; Knutsen et al., 2001; Montolalu, Tashiro, Matsukawa, & Ogawa, 2007; Spichtig & Austin, 2008; Sworn et al., 1987), low-angle laser light scattering (LALLS) (Hjerde et al., 1999), multi-angle light scattering (MALS) (Girod, Baldet-Dupy, Maillols, & Devoisselle, 2001) and vacuum-ultraviolet inductively coupled plasma-atomic emission spectrometry (ICP) (Uno et al. 2001). Triple detector system (RI-viscometer-light scattering) (Myslabodski, Stancioffh, & Heckertta, 1996) and dual detector systems: RI and LALLS (Slootmaekers et al., 1991), RI and light scattering (Singh & Jacobsson, 1994; Viebke, Borgström, & Piculell, 1995), RI and MALS (Marcelo, Saiz, & Tarazona, 2005) have also been described.

One of the main focuses of SEC method for carrageenans analysis are related to the study of products degradation, either highlighting the potential health risks associated with the consumption of low molecular weight (LMW) carrageenans (Uno et al., 2001) or to the impact on their functionality as gel-forming or viscosity-enhancing polymers in foods (Karlsson & Singhb, 1999). Spichtig and Austin (2008) used high performance SEC for the measurement of LMW carrageenans in food ingredients and

finished products and demonstrated that they had a little increase during food processing in the LMW fraction when pH levels below 4.0 were avoided. Strong mineral acids are known to hydrolyze carrageenans and the treatment of κ-carrageenan with simulated gastric fluid (pH 1.0) showed significant breakdown, as measured by SEC (Ekström, 1985). Under closely controlled pH and temperature conditions, the acid-catalyzed changes in MWD determined by SEC can be described by a first-order hydrolysis process involving selective attack at carrageenan glycosidic linkages (Myslabodski et al., 1996).

Hydrolysis of carrageenan is sometimes desirable to control physical properties of solutions (e.g., the viscosity) or gels (such as rigidity and the stress at fracture), which are determined by MWD. Van de Velde, Rollema et al. (2002) employed SEC for analysis of oligomers produced by the alkaline treatment of ι-carrageenan, whilst Zúniga, Matsuhiro, and Mejías (2006) used SEC on the analysis of agaran–carrageenan hybrid polysaccharides produced by free radical depolymerization reaction. The use of carrageenases, enzymes which cleave specifically the glycosidic linkage of carrageenans, also have promising applications for food industry since they reduce drastically the viscosity of a concentrated solution of carrageenan and the integrity of the native chemical structure is well preserved (Guibeta, Kervarecb, Génicota, Chevolota, & Helbert, 2006). The association between these enzymes and SEC has also been described (Knutsen et al., 2001).

5.4. Methylation

Methylation analysis is a technique applied to complex polysaccharides that allows the determination of the substitution pattern of isolated monomers. It is important to mention that NMR spectroscopy does not enable a differential analysis of monomers. Thus, this method has been employed for elucidation of the structures of several sulphated polysaccharides including carrageenans (Ciancia, Matulewicz, & Cerezo, 1997; Stevenson & Furneaux, 1991). The procedure offers a reasonable confirmation of chemical structure aspects, such as the kind of linkage (1-3 or 1-4) and, especially, of the monomers substitution pattern. The technique consists of the methylation of carrageenans with methyl iodine in a strong basic media (Hakomori, 1964). The methylated product is then submitted to an acid hydrolysis reaction, which must be able to break the glycosidic bonds without degrading the anhydro bridge of its residues. Stevenson and Furneaux (1991) performed the reductive hydrolysis, utilizing 4-methylmorpholine–borane acid to reduce the extremities of 3,6-anhydrogalactose groups released during hydrolysis. In order to minimize the problems related to incomplete methylation and desulfation of carrageenans, the polysaccharide in the form of tetraethylamonium salt is employed. The alditols produced after hydrolysis are then acetylated and these derivatives are analysed, in general, by gas chromatography (GC) and gas chromatography coupled to mass spectrometer (GC–ME) (Ciancia et al., 1997).

5.5. Mass spectrometry

In the last decade, mass spectrometry has become increasingly important for analysis of carbohydrates, including fast screening (Chai, Luo, Lim, & Lawson, 1998; Dell & Morris, 2001) and detailed sequence analysis due to its high sensitivity, high accuracy and fast processing compared to NMR and various chromatographic methods (Chai, Piskarev, & Lawson, 2002; Chai, Piskarev, Zhang, Lawson, & Kogelberg, 2005; Chai et al., 2006; Harvey, 2005; Kogelberg, Piskarev, Zhang, Lawson, & Chai, 2004; Zhang et al., 2006). Carbohydrates, however, are difficult to analyse because of their structural heterogeneity, labile substituents and linkage isomerism.

For carrageenan analysis, strong analytical tools are needed in order to gain insight into the complexity of different carrageenan structures. After enzymatic depolymerisation, methods providing molecular weight information, fast screening, sensitivity and accuracy, through direct mass spectrometric analysis, are mandatory (Antonopoulas, Hardouin et al., 2005; Antonopoulos, Favetta et al., 2005). In this respect, electrospray ionization mass spectrometry (ESI-MS) (Ekeberg et al., 2001) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Ackoo, Terlouw, Ruttink, & Burgers, 2001; Dai et al., 1997; Fukuyama et al., 2002) are the best-known techniques employed for the analysis of carrageenan samples. ESI-MS creates multiply charged ions (Kebarle & Ho, 1997) making the interpretation of a mixture of highly charged oligosaccharides through direct ESI-MS analysis a rather complex task (Zaia & Costello, 2001). For this reason, high-performance liquid chromatography (Kuberan et al., 2002) and size-exclusion chromatography (Zaia & Costello, 2001) are usually coupled to ESI-MS in order to simplify the sample entering the ESI-MS instrument. However, highly sulphated oligosaccharides give very poor ESI-MS response because of their extremely polar (ionic) character; thus, it is difficult to produce intact gas-phase ions for MS analysis (Juhasz & Biemann, 1995). In contrast, MALDI-MS (Harvey, 1999) is an alternative to the ESI-MS (Zaia, 2004) technique for the analysis of sulphated oligosaccharides owing to its different ionization principle. In addition, the fact that most of the time MALDI yields mono-charged ions means that molecular weight information from a mass spectrum of a complex sample is easier to interpret.

The use of soft ionization techniques, such as MALDI and ESI, in the positive and negative modes, together with MS/MS fragmentation, have provided important strategies for determination of sulphate substitution patterns from different types of κ-carrageenan (Aguilan et al., 2003; Antonopoulos, Favetta, Helbert, & Lafosse, 2004; Fukuyama et al., 2002). The presence of sulphate substituents in κ-carrageenan makes it amenable to analysis by MALDI and ESI-MS in the negative mode. Antonopoulos, Favetta et al. (2005) and Antonopoulas, Hardouin et al. (2005) demonstrated for the first time the characterization of κ- and ι-carrageenans (extracted from *Kappaphycus alvarezii* and *Eucheuma denticulatum*, respectively), enzymatically digested with the correspondent κ- and ι-carrageenases, using the LC/ESI-MS method, which is based on ion-pair liquid chromatography coupled to ESI-MS in the negative-ion mode. Even though this method can indirectly determine and verify the exact number of sulphate groups, as well as the molecular weight of the primary backbone structure of each oligosaccharide in the mixture, determination of sulphate position is hindered (Aristotelis et al., 2007). A hybrid sample of ι-/ν-carrageenans (extracted from *E. denticulatum*) enzymatically digested with ι-carrageenase was also subsequently characterized by LC/ESI-MS (Antonopoulas, Hardouin et al., 2005; Antonopoulos, Favetta et al., 2005). Enzymatically digested oligosaccharides of κ-, ι- and hybrid ι/ν-carrageenans were recently analysed by Antonopoulos et al. (2005) with MALDI-MS in the negative-ion mode, giving characteristic mass spectral fingerprints, particularly for the κ-carrageenans.

Another technique that has being explored for the analysis of carbohydrates is nanoelectrospray ionization mass spectrometry (nano-ESI-MS) (Karas, Bahr, & Dülcks, 2000; Schmidt, Bahr, & Karas, 2001). This technique offers high sensitivity with a detection limit down to 10^{-8} M (Wilm & Mann, 1996). In addition, it is a soft ionization technique that is ideal for polysaccharides with labile substituents. Since nano-ESI requires only a small amount of sample, a number of experiments such as high-resolution and collision-induced dissociation (CID or MS/MS) determinations can be performed with 1 pmol (Karas et al., 2000). Nano-ESI can be coupled to different mass analysers such as quadrupole time-of-flight

(qTOF,) ion trap, triple quad and Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) (Park & Lebrilla, 2005; Zamfir, Seidler, Kresse, & Peter-Katalinic, 2003). FTICR-MS is advantageous because it can analyse large compounds and is able to achieve high mass resolution with a mass error of less than 1.5 ppm (Hofstadler, Griffey, Pasa-Tolic, & Smith 1998; Park & Lebrilla, 2005). Sustained off-resonance irradiation (SORI) is a CID method that is often used with FTICR (Harvey, 2005; Park & Lebrilla, 2005). SORI-CID involves irradiation of the ion slightly off its resonance frequency causing the acceleration and deceleration of ions during the radiofrequency (RF) pulse at a constant pressure of collision gas. Aguilan, Dayrit, Zhang, Niñonuevo, and Lebrilla (2006) described the structural analysis of κ-carrageenan oligosaccharide standards, with a polymerization degree (DP) between 1 and 5, by positive mode using nano-ESI-FTMS and MS/MS analysis using SORI-CID. It was verified that the high mass accuracy and sensitivity of nano-ESI-FTMS together with the mild collision conditions of SORI-CID proved to be very effective for the analysis of sulphated carbohydrates like carrageenans. Thus, this technique can also be applied to the analysis of other carbohydrates that are difficult to be evaluated using other MS methods.

6. Concluding remarks

Carrageenans have been widely used by food industry and may represent, in the future, a valuable and low cost source of new drugs. Current research goals are focused on identifying more potent and specific compounds with antitumor, immunomodulatory, antihyperlipidemic, anticoagulant and antiviral activities. Therapeutic applications of low molecular weight carrageenans have been limited because of their gastrointestinal toxic side effects. In this way, parenteral formulations of carrageenans have also proved to be efficient against HPV transmission and may be very promising against HIV after chemical modification or in association with other antivirals agents. The increasing knowledge of structural analysis and chemical modifications enables the use of carrageenans as probes for investigation of their mechanisms against important diseases, such as cancer and AIDS. These polysaccharides can also be employed as prototypes for the design of novel therapeutic agents, more effective and less toxic than current chemotherapeutic agents. Lastly, the diversity of analytical techniques employed for structural analysis of carrageenans has been gradually increasing, with NMR spectroscopy (both ^{1}H and ^{13}C NMR) being regarded as a powerful tool for the qualitative and quantitative analysis of carrageenans, and more recently, mass spectrometry (ESI-MS and MALDI-MS) has proven to be of utmost importance to gain insight into the complexity of different carrageenan structures.

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