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applications based on their unique intrinsic properties. Nowadays NMR spectroscopy (both $^1$H- and $^{13}$C-NMR) is one of the standard tools for the determination of the chemical structure of carrageenan samples. The NMR spectroscopy of carrageenans is used in industry and for research with different approaches corresponding to different ultimate goals or interests. In the first case this technique is seen as a screening tool to determine the possible commercial or industrial value of raw extracts obtained from still unexploited red seaweeds and also used to control the quality of carrageenan products and to study the influence of processing parameters, such as temperature and pH, on the structure. A different approach is used when the interest is centred in the system of carrageenans biosynthesised by the seaweed or in the fine structure of a carrageenan or a carrageenan fraction. This review gives an overview of NMR-spectroscopy as a powerful tool for the analysis of carrageenan samples.

Structure of carrageenans

Carrageenans are a family of water soluble, linear, sulfated galactans. They are composed 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 (see Fig. 1). The letter codes in Fig. 1 refer to the nomenclature developed by Knutsen, Myslabodski, Larsen, and Ussov (1994) (see Box 1). The sulfated 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 sulfate groups. For commercial carrageenan the sulfate content falls within the range from 22 to 38% (w/w). Commercial (food-grade) carrageenans have a weight average molecular mass ($M_w$) ranging from 400 to 600 kDa. Besides galactose and sulfate, other carbohydrate residues (for example xylose, glucose and uronic acids) and substituents (for example methyl ethers and pyruvate groups) can be present in carrageenan preparations. Since natural carrageenan is a mixture of nonhomologous polysaccharides, the term disaccharide repeating unit refers to the idealised structure.

The most common types of carrageenan are traditionally identified by a Greek prefix. The three commercial most important carrageenans are called ρ-, κ-, and λ-carrageenan, the corresponding IUPAC-inspired names and letter codes are carrageenose 3,4-disulfate (G4S-
Box 1. Nomenclature of carrageenans

Traditionally carrageenans are identified by a Greek prefix, indicating the major component of the sample. This nomenclature is used universally in trade, science, and legislation. However, this system is not suitable to describe more complex polymers unambiguously. To describe more complex structures, Knutsen, Myslabock, Larsen, and Usov (1994) proposed an alternative nomenclature for carrageenan and agar. This letter code based nomenclature (see Table 1) is founded on the worldwide accepted IUPAC nomenclature (McNaught, 1997) and allows a systematic description of complex polymer molecules. The structure based on a perfect alternating sequence of \( \beta-(1\to3) \)-linked \( \alpha \)-galactopyranose residues and \( \alpha-(1\to4) \)-linked 3,6-anhydro-\( \alpha \)-galactopyranose residues is called the 'ideal' structure. Based on the Knutsen-nomenclature, the names and letter codes of the dimeric structures of \( \kappa \), \( \iota \), and \( \lambda \)-carrageenan are carrageenose 4'-sulfate (G45-DA), carrageenose 2,4'-disulfate (G45-DA2S), and carrageenan 2,6,2'-trisulfate (G2S-D2S,6S), respectively.

This letter code nomenclature is gaining importance in scientific publications dealing with carrageenans and agars. Most authors describing complex structures adopt this nomenclature. In addition to the notation (S) for the sulfate ester distribution, methyl ethers (M), pyruvate acetals (P), and glycosyl units such as xylose (X) are introduced to describe carrageenans with different substituents (Miller, 1998). Agars differ from carrageenans as they have the \( \iota \)-configuration for the 4-linked galactose residue. Incidentally this configuration is observed in carrageenans (Storz & Cerezo, 2000) and in the corresponding letter codes the letter \( \iota \) is replaced by the letter \( l \).

<table>
<thead>
<tr>
<th>Letter code</th>
<th>Found in carrageenans</th>
<th>IUPAC namea</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>( \beta )</td>
<td>3-linked ( \beta )-galactopyranose</td>
</tr>
<tr>
<td>D</td>
<td>Not foundb</td>
<td>4-linked ( \alpha )-galactopyranose</td>
</tr>
<tr>
<td>DA</td>
<td>( \kappa ), ( \lambda )</td>
<td>4-linked 3,6-anhydro-( \alpha )-galactopyranose</td>
</tr>
<tr>
<td>S</td>
<td>( \kappa ), ( \lambda ), ( \mu ), ( \nu )</td>
<td>Sulfate ester (O-SO3)</td>
</tr>
<tr>
<td>G2S</td>
<td>( \lambda )</td>
<td>3-linked ( \delta )-galactopyranose 2-sulfate</td>
</tr>
<tr>
<td>G4S</td>
<td>( \kappa ), ( \lambda ), ( \mu ), ( \nu )</td>
<td>3-linked ( \delta )-galactopyranose 4-sulfate</td>
</tr>
<tr>
<td>DA2S</td>
<td>( \lambda ), ( \nu )</td>
<td>4-linked 3,6-anhydro-( \alpha )-galactopyranose 2-sulfate</td>
</tr>
<tr>
<td>D2S,6S</td>
<td>( \lambda ), ( \nu )</td>
<td>4-linked ( \alpha )-galactopyranose 2,6-disulfate</td>
</tr>
<tr>
<td>D6S</td>
<td>( \mu )</td>
<td>4-linked ( \alpha )-galactopyranose 6-sulfate</td>
</tr>
</tbody>
</table>

a International Union of Pure and Applied Chemistry nomenclature as recommended in 1996 (McNaught, 1997).
b Not found in natural occurring carrageenans, but can be found in desulfated carrageenan samples.

DA2S), carrageenose 4'-sulfate (G4S-DA), and carrageenan 2,6,2'-trisulfate (G2S-D2S,6S). Besides these three major carrageenan types, two other types, called \( \mu \)- and \( \nu \)-carrageenan (letter code G4S-D2S,6S and G4S-D6S, respectively), are often encountered in commercial carrageenan samples. \( \mu \)- and \( \nu \)-carrageenan are the biological precursors of respectively \( \kappa \)- and \( \iota \)-carrageenan. The different types of carrageenan are obtained from different species of the Rhodophyta. \( \kappa \)-Carrageenan is predominantly 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 \( \iota \)-carrageenan. For the production of the \( \kappa \)- and \( \iota \)-carrageenan, mixtures of plants of both sexual stages of seaweeds of the above species are used. The seaweeds are usually extracted with alkali at elevated temperatures to transform the biological precursors, \( \mu \)- and \( \nu \)-carrageenan into \( \kappa \)- and \( \iota \)-carrageenans (see below). \( \lambda \)-Carrageenan is obtained from different species from the *Gigartina* and *Chondrus* genera. The sporophytic plants of these seaweeds produce \( \lambda \)-carrageenan (McCullers, West, & Guiry, 1982), whereas the gametophytic plants produce a \( \kappa \)-/\( \iota \)-hybrid type of carrageenan. These \( \kappa \)-/\( \iota \)-hybrid carrageenans consist of a mixed chain containing both \( \kappa \)- and \( \iota \)-units (Van de Velde, Peppelman, Rollema, & Tromp, 2001) and range from almost pure \( \iota \)-carrageenan to almost pure \( \kappa \)-carrageenan (Bixler, 1996). The production of \( \lambda \)-carrageenan implies the selection of samples in the sporophyte stage while the extraction can be carried out with hot water as the cyclization, in alkaline medium, to \( \theta \)-carrageenan is difficult (Ciancia, Noseda, Matulewicz, & Cerezo, 1993) and this product has essentially the same properties as \( \lambda \)-carrageenan (Glicksman, 1983).

\( \kappa \)- and \( \iota \)-carrageenan are gel forming carrageenans, whereas \( \lambda \)-carrageenan is a thickener. The difference in rheological behaviour between \( \iota \)- and \( \kappa \)-carrageenan on one side and \( \lambda \)-carrageenan on the other side results from the fact that the DA-units of the gelling ones have the \( 1 \)-conformation and the D-units in \( \lambda \)-carrageenan do not. The \( 1 \)-conformation of the 3,6-anhydro-\( \alpha \)-galactopyranosyl units in \( \iota \)- and \( \kappa \)-carrageenan allows a helical secondary structure, which is essential for the gel forming properties. The natural precursors of \( \iota \)- and \( \kappa \)-carrageenan, \( \nu \)- and \( \mu \)-carrageenan, are also non-
gelling carrageenans with the D-units in the $^{13}$C-conformation. Occurrence of disaccharide units without the 3,6-anhydro ring and having a $^{13}$C-conformation causes "kinks" in the regular chain and prevents the formation of helical strands and by consequence prevents gelation of the carrageenan. In vivo, $\alpha$- and $\kappa$-carrageenan are formed enzymatically from their precursors, by a sulfohydrolase (Wong & Craigie, 1978, De Ruiter et al., 2000). The usual presence of considerable amounts of $\alpha$-units in commercial carrageenan preparations has a strong negative effect on the functional (e.g. gel-forming) properties of the carrageenan (Hansen, Larsen, & Grøndal, 2000; Van de Velde et al., in press). Therefore, in industrial processing, prior to use crude carrageenan is submitted to an alkaline treatment or alkaline extraction, catalysing the cyclisation reaction with OH$^-$ (Therkelsen, 1993; Van de Velde & De Ruiter, 2002) (Table 1).

Analysis of carrageenans
At present, there is still lack of adequate analytical techniques to determine the amounts, the polydispersity and the purity of carrageenans in food products and raw materials. Different techniques and approaches used for this type of analysis are, for example colorimetry and immunoassays, HPLC and electrophoresis (Roberts & Quemener, 1997). To reveal the (detailed) molecular structure of carrageenans, a chemical analysis is mostly done on isolated and purified carrageenan samples. In the beginning, chemical modification and degradation methods were time-consuming and tedious analytical techniques. In the mid 1970s a real boost was given by the introduction of NMR spectroscopy. Nowadays NMR spectroscopy (both $^1$H- and $^{13}$C-NMR) is one of the standard tools for the determination of the chemical structure of carrageenan samples (Hansen, Larsen, & Grøndal, Van de Velde & De Ruiter, 1999; Usov, 1998). In addition to NMR spectroscopy, other analytical techniques, such as sulfite content analysis, monosaccharide composition analysis (Jol, Neiss, Penninkhof, Rudolph, & De Ruiter, 1999) and methylation analysis (Falshaw, Bixler, & Johndro, 2001; Falshaw & Furneaux, 1994) are applied.

$^{13}$C-NMR spectroscopy of the major carrageenan types
In general, carrageenan samples are sonicated prior to recording the spectra and the NMR experiments are carried out at elevated temperature to reduce the viscosity of the solution (high viscosity results in line broadening). 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$_2$O) compared to $^1$H-NMR samples (0.5-1.0% w/w in D$_2$O). Overviews of the $^1$H and $^{13}$C-NMR spectra of the most important carrageenan types are shown in respectively, Figs. 2 and 3. Both the $^1$H- and $^{13}$C-NMR spectra reveal that "pure" carrageenan samples are rare. The $\kappa$- and $\iota$-carrageenan samples may contain extraneous units of $\iota$- and $\kappa$-carrageenan respectively, which is quite well known for carrageenans extracted from K. alvarezii and E. denticulatum. Samples containing precursor carrageenans are always hybrids containing low percentages of the precursor units.

$^{13}$C-NMR spectroscopy
Proton-decoupled $^{13}$C-NMR spectra of highly regular red algal galactans resemble the spectra of corresponding substituted disaccharides. Since the pairs of diads G-D and G-L, as well as G-DA and G-LA, are diastereoisomeric, they give different spectra, the differences being especially noticeable for the anomeric carbon resonances (Table 2). This observation made it possible to recommend the $^{13}$C-NMR spectroscopy as the simplest tool for distinguishing the polysaccharides of the agar and carrageenan groups (Bhattacharjee, Yaphe, & Hamer, 1978; Yareysky, Shashkov, & Usov, 1977).

The $^{13}$C-NMR spectra of gel forming $\kappa$- and $\iota$-carrageenan were studied, using model synthetic monosaccharide derivatives (Usov, 1984) or oligomeric polysaccharide fragments (Greer, Rochas, & Yaphe, 1985; Rochas, Renaudo, & Vincendon, 1983) and interpreted, as indicated in Table 3. Later on this interpretation was confirmed by various 2D NMR experiments (Chiovitti et al., 1997; Falshaw et al., 1996). These assignments were successfully used to identify the corresponding poly-

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### Table 2. $^{13}$C-NMR chemical shifts of basic agar and carrageenan structures

<table>
<thead>
<tr>
<th>Diad</th>
<th>Unit</th>
<th>C-1 (ppm)</th>
<th>C-2 (ppm)</th>
<th>C-3 (ppm)</th>
<th>C-4 (ppm)</th>
<th>C-5 (ppm)</th>
<th>C-6 (ppm)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>G</td>
<td>104.7</td>
<td>70.0</td>
<td>81.0</td>
<td>68.9</td>
<td>75.6</td>
<td>61.4</td>
<td>Lahaye, Yaphe and Rochas (1985)</td>
</tr>
<tr>
<td>G</td>
<td>L</td>
<td>100.9</td>
<td>69.4</td>
<td>71.0</td>
<td>79.3</td>
<td>72.2*</td>
<td>61.2</td>
<td>Lahaye, Yaphe, Viet, and Rochas (1989)</td>
</tr>
<tr>
<td>CA</td>
<td>G</td>
<td>102.4</td>
<td>70.4</td>
<td>82.2</td>
<td>68.8</td>
<td>75.1</td>
<td>61.4</td>
<td>Lahaye, Yaphe, Viet, and Rochas (1989)</td>
</tr>
<tr>
<td>CA</td>
<td>L</td>
<td>98.3</td>
<td>69.9</td>
<td>80.1</td>
<td>77.4</td>
<td>75.7</td>
<td>69.4</td>
<td>Usov, Yareysky, and Shashkov (1990)</td>
</tr>
<tr>
<td>GD</td>
<td>G</td>
<td>100.8</td>
<td>70.6</td>
<td>78.9</td>
<td>65.8</td>
<td>75.4</td>
<td>61.1</td>
<td>Usov, Yareysky, and Shashkov (1990)</td>
</tr>
<tr>
<td>GD</td>
<td>D</td>
<td>98.4</td>
<td>69.3</td>
<td>71.0</td>
<td>78.7</td>
<td>79.5</td>
<td>61.5</td>
<td>Usov, Yareysky, and Shashkov (1990)</td>
</tr>
<tr>
<td>GDA</td>
<td>G</td>
<td>102.5</td>
<td>69.5</td>
<td>80.4</td>
<td>66.4</td>
<td>75.1</td>
<td>61.1</td>
<td>Usov, Yareysky, and Shashkov (1990)</td>
</tr>
<tr>
<td>GDA</td>
<td>D</td>
<td>94.7</td>
<td>71.2</td>
<td>79.4</td>
<td>78.8</td>
<td>76.8</td>
<td>69.5</td>
<td>Usov, Yareysky, and Shashkov (1990)</td>
</tr>
</tbody>
</table>
Fig. 2. $^1$H-NMR spectra of the major carrageenan types from bottom to top: $\kappa$-carrageenan from Kappaphycus alvarezii (with permission of Elsevier adopted from Van de Velde et al., 2001); $\iota$-carrageenan from Eucheuma denticulatum (with permission of Elsevier adopted from Van de Velde et al., 2001); $\nu$-$\kappa$-hybrid carrageenan containing 25% $\nu$-carrageenan (spectrum published with permission of CP Kelco); $\lambda$-carrageenan from Inulæa undulosa (with permission of Elsevier adopted from Storz et al., 1994).

Table 3. $^{13}$C-NMR chemical shifts for the main structural units of commercial carrageenans

<table>
<thead>
<tr>
<th>Carrageenan</th>
<th>Unit</th>
<th>Chemical shifts (ppm)</th>
<th>$^{13}$C-5</th>
<th>$^{13}$C-6</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\kappa$</td>
<td>G4S</td>
<td>90.5 69.9 78.4 74.1 74.0 61.3</td>
<td>Usov and Shashkov (1985)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>95.1 69.9 79.2 78.3 76.8 69.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\iota$</td>
<td>G4S</td>
<td>90.2 69.3 76.6 72.2 74.8 61.3</td>
<td>Usov and Shashkov (1985)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA2S</td>
<td>92.3 75.9 77.8 78.3 77.0 69.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda$</td>
<td>G2S</td>
<td>93.4 77.4 75.8 64.2 74.2 61.3</td>
<td>Talshaw and Turner (1994)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA5S</td>
<td>94.6 74.8 69.5 80.1 68.7 68.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3. $^{13}$C-NMR spectra of the major carrageenan types from bottom to top: κ-carrageenan from *Kappaphycus alvarezii* (with permission of Elsevier adopted from Van de Velde et al., 2001); ν-carrageenan from *Eucheuma denticulatum* (with permission of Elsevier adopted from Van de Velde et al., 2001); ν-κ hybrid carrageenan containing 25% ν-carrageenan (spectrum published with permission of CP Kelco); κ-carrageenan from *Iridaea undulosa* (with permission of Elsevier adopted from Storz et al., 1994).

Saccharides isolated from new sources (Usov & Shashkov, 1985). The well-resolved spectrum of λ-carrageenan [G2S-D2S-6S], was obtained much later (Falshaw & Furname, 1994; Storz, Bacon, Cherniak, & Cerezo, 1994) due to some technical difficulties, which are usually explained by the high viscosity of λ-carrageenan solutions. Assignment of anomic resonances in the spectra of the biological precursors μ- and ν-carrageenan was made by Bellion, Brugaud, Prone, and Boeck (1983), whereas the complete interpretation of these spectra was published much later (Ciancia, Matulewicz, Finch, & Cerezo, 1993; Storz et al., 1994) (Table 4).

It should be noted that the biological precursors mentioned above and many other possible carrageenan diads are usually found only as components of hybrid polymeric molecules (see the spectra of μ- and ν-carrageenan containing samples in Figs. 2 and 3 and Refs. Ciancia et al., 1993; Storz et al., 1994). Interpretation of signals in the spectra of such hybrid carrageenans was usually made in a tacit assumption that minor diads are
arranged in blocks, e.g. the given diad has the neighbours of the same structure on its right and left. Evidently, this assumption may not be valid for many real polysaccharides, and hence, the signal assignment for minor diads suggested in the literature [see, for example Ciancia et al. (1993)] should be regarded only as tentative.

Most carrageenans differ only in 3,6-anhydrogalactose content, degree of sulfation and positions of sulfate groups. An attempt was made to calculate the $^{13}$C-NMR spectra of many possible carrageenan structures, including those, which were not isolated at that time from natural sources, using spectral features of the known polysaccharides (Storz & Cerezo, 1992). However, the experimental spectra of $\alpha$- and $\beta$-carrageenan, showed insufficient coincidence with the predicted values. Evidently, the amount and specific position of charged sulfate groups may result in conformational changes of polysaccharide chains, which are not taken into consideration by additive schemes in calculations of chemical shifts.

$^{1}$H-NMR spectroscopy

The $^{1}$H NMR spectra of monomeric methyl $\beta$-galactopyranoside and methyl 3,6-anhydro-\(\alpha\)-galactopyranoside have initially been used as the basis of the interpretation of the proton spectra of the gelling $\kappa$- and $\iota$-carrageenan (Welty, 1977). Later on these assignments have been confirmed by the spectra of the neocarrabiose oligosaccharides (Knutsen & Grasdalen, 1992a). For the biological precursor units, $\nu$- and $\mu$-carrageenan, only the $\alpha$-anomeric protons were assigned (Ciancia et al., 1993; Storz et al., 1994), due to the complexity of the hybrid samples containing these precursors. The viscosity of $\lambda$-carrageenan samples allows only the assignment of the $\alpha$-anomeric protons in a pure sample (Storz et al., 1994). Nowadays, quantification of different carrageenan types in a sample by $^{1}$H-NMR spectroscopy is based on the resonances of the $\alpha$-anomeric protons (D- and DA-units) in the region from 5.1 to 5.7 ppm (see Table 5). The signals for the $\beta$-anomeric protons (G-units) are less suitable for either identification or quantification purposes.

NMR spectroscopy of minor substituents in carrageenans

Several carrageenans have additional substituents, which may be identified by NMR spectroscopy. Thus, a small amount of 3-linked 6-O-methyl-$\beta$-galactose residues was found in $\kappa$-carrageenan from Kappaphycus alvarezii (Bellion et al., 1983) and in several other polysaccharides (Chiovitti et al., 1998). These residues give specific signals for OMe at 59.0, for the substituted C-6 at 71.8, and for the neighbouring C-5 at 73.3 ppm in $^{13}$C-NMR. Small amounts of terminal xylose residues were also detected in many carrageenans, but their location usually remains uncertain. In the case of an unusual polysaccharide $\left[\text{G4,6S-DA}\right]_{n}$ from Phaseolus vulgaris (Liao et al., 1996) NMR spectral data indicated the attachment of xylose residues at position 3 of some 4-linked $\alpha$-d-galactose residues. In $\iota$-carrageenan from Eucheuma denticulatum this monosaccharide probably occupies position 6 of 3-linked $\beta$-d-galactose residues (Usov and Knutsen, unpublished).

<table>
<thead>
<tr>
<th>Carrageenan</th>
<th>Monosaccharide</th>
<th>Chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>D2S</td>
<td>5.32</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>DA</td>
<td>5.11</td>
</tr>
<tr>
<td>$\iota$</td>
<td>D2S,6S</td>
<td>5.39</td>
</tr>
<tr>
<td>$\mu$</td>
<td>D2S,6S</td>
<td>5.52</td>
</tr>
<tr>
<td>$\nu$</td>
<td>D6S</td>
<td>5.26</td>
</tr>
</tbody>
</table>

Table 4. $^{13}$C-NMR chemical shifts for the most common carrageenan structural units encountered in hybrid molecules

<table>
<thead>
<tr>
<th>Carrageenan</th>
<th>Unit</th>
<th>Chemical shifts (ppm)</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>G</td>
<td>102.7 69.6 81.9 66.9 75.3 61.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Falshaw et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>D2S</td>
<td>94.7  75.4 78.1 78.3 77.1 70.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liao, Kraft, Munro, Craik, &amp; Bacic (1993)</td>
</tr>
<tr>
<td>$\beta$</td>
<td>G</td>
<td>102.7 69.7 80.5 66.5 75.5 61.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Falshaw &amp; Furneaux (1994)</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>94.7  70.3 79.5 78.3 77.0 69.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liao et al. (1996)</td>
</tr>
<tr>
<td>$\theta$</td>
<td>G2S</td>
<td>100.3 77.6 77.2 67.8 74.7 61.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Falshaw &amp; Furneaux (1994)</td>
</tr>
<tr>
<td></td>
<td>DA2S</td>
<td>95.6  74.7 77.4 79.5 77.0 69.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Usov and Shashkov (1985)</td>
</tr>
<tr>
<td>$\omega$</td>
<td>G6S</td>
<td>102.7 60.4 80.3 66.0 72.9 67.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liao et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>94.7  70.1 79.5 78.5 76.9 69.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ciancia et al. (1993)</td>
</tr>
<tr>
<td>$\iota$</td>
<td>G45S</td>
<td>102.8 69.6 78.9 74.0 72.7 68.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Storz et al. (1994); Ciancia et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>D6S</td>
<td>95.3  69.9 79.5 78.9 77.0 69.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Storz et al. (1994); Ciancia et al. (1993)</td>
</tr>
<tr>
<td>$\mu$</td>
<td>G4S</td>
<td>105.3 70.7 78.8 73.8 75.4 61.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Falshaw et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>D6S</td>
<td>98.2  68.9 70.7 79.5 68.9 68.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liao et al. (1996)</td>
</tr>
<tr>
<td>$\nu$</td>
<td>G4S</td>
<td>105.3 70.7 80.4 73.8 75.4 61.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ciancia et al. (1993); Storz et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>D2S,6S</td>
<td>98.8  76.8 68.9 79.5 68.9 68.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liao et al. (1996)</td>
</tr>
</tbody>
</table>

Table 5. $^{1}$H-NMR chemical shifts of the $\alpha$-anomeric protons of carrageenans (Ciancia et al., 1993; Storz et al., 1994)
Pyrusric acid is a common component of many complex carrageenans. It forms a cyclic acetal at positions 4 and 6 of 3-linked galactose residues and may be found in diads related to 3- and 0-carrageenans. This substituent 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) (Table 6). The pyruvic acid ketals are also detected in the 1H-NMR spectra by methyl proton resonances with a chemical shift of 1.45 ppm (Chiovitti et al., 1997). This signal does not overlap with other proton resonances in these carbohydrate derivatives. In addition, specific substitution effects on the chemical shifts of the other protons are observed in the 1H-NMR spectra (Chiovitti et al., 1997).

According to the structure of sulfated galactans, red algae are traditionally divided into agarophytes and carrageenophytes. However, galactan sulfates of intermediate structure, which may be termed as D/L-hybrids, have been found recently in many species. They seem to be characteristic to representatives of Cryptonemiales and Rhodymeniales, may be isolated from several species of Ceramiales, and surprisingly are also present as minor components in many species of Gigartinales. These D/L-hybrids are no more carrageenans, and their structural analysis will not be discussed here, but it should be noted that their 13C-NMR spectra are rather complex and, as a rule, can not be interpreted unambiguously without additional evidence obtained by chemical methods (Estevez, Ciancia, & Cerezo, 2001).

NMR spectroscopy of contaminants and additives in carrageenan samples

Carrageenan preparations may contain admixtures of two different origins. Some of them are components of red algal biomass, which were not removed in the carrageenan isolation procedure (Rudolph, 2000; Van de Velde & De Ruiter, 2002). Other compounds (inorganic salts, sucrose, galactomannans) can be added by manufacturers to improve and/or control some functional properties of carrageenan samples (solubility, viscosity, gel strength, etc.). Small contaminations and additives, such as inorganic salts and sugars, can be removed by dialysis, prior to recording the spectra.

Floridean starch, a branched (1→4,1→6)-β-D-glucan structurally related to plant amyllopectins and animal glycogens, is a reserve polysaccharide of red seaweeds. It is soluble in water and can accompany carrageenans in the extraction and precipitation steps. Apart from the detection of glucose in an acid hydrolysate, the presence of floridean starch can be confirmed by the well-known set of signals of 4-linked β-D-glucopyranose residues in the 13C-NMR spectrum (see Table 7) (Colson, Jennings, & Smith, 1974; Knutsen & Grasdalen, 1987). In the 1H-NMR spectra, floridean starch is detected by the signal of the anomeric proton of the α(1→6)-linked β-D-glucopyranosyl at 5.37 ppm (Knutsen & Grasdalen, 1987). The resonance of the anomeric proton of the α(1→6)-linked β-D-glucopyranosyl appears at 4.98 ppm. Floridean starch can be removed by starch degrading enzymes or by any procedure of separation of acid and neutral polysaccharides.

Galactans of the agar group may be present in carrageenans, when mixed algal populations containing both agarophytes and carrageenophytes are used for carrageenan manufacture. As in the case of D/L-hybrids, unambiguous identification of agar-like structures may require chemical evidence to complement the 13C-NMR spectral data. Some red algae contain water-soluble sulfated xylomannans and neutral xylans, but these polysaccharides are usually absent from the common
carrageenophytes and only accidentally may be found in carrageenans. Red algal xylans are abundant in representatives of the orders Nemaliales and Palmariales, whereas sulfated xylomannans were found only in several species of the order Nemaliales. $^{13}$C-NMR spectra of both types of polysaccharides were published (Kolender, Pujol, Damonte, Matelewicz, & Cerezo, 1997; Kovac, Hirsch, Skashkov, Usov, & Yarotsky, 1997).
Additives such as sucrose and glucose are often used to adjust the viscosity of the commercial carrageenan preparations. Sucrose interferes with colorimetric procedures of determination of carrageenans based on specific color reactions of 3,6-anhydrogalactose, since fructose and 3,6-anhydrogalactose behave similarly in

Fig. 5. $^{13}$C-NMR spectra of the major carrageenan variants, sucrose and glucose. From bottom to top: K-carrageenan from Kappaphycus alvarezii (100 MHz, 75°C), ε-carrageenan from Eucheuma denticulatum (100 MHz, 75°C), sucrose (10% w/w, 150 MHz, 80°C) and glucose (10% w/w, 150 MHz, 80°C).

1980; Ussov & Dobkina, 1991) and may be used for their identification.
Analysis and quantification of the composition of carrageenan blends by NMR spectroscopy

In industrial applications the composition of carrageenan blends is very important with respect to the desired functionality. The type of carrageenan present in a commercial preparation and possible additives can to a large extent determine the functional properties of the final product. Carrageenan preparations, mixtures of carrageenans and commercial carrageenan blends can qualitatively and quantitatively be characterised by NMR spectroscopy. NMR techniques 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. $^1$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. The analysis of the $^1$H-NMR spectra is based on the position and intensity of the resonances of the $\alpha$-anomeric protons of the repeating unit (see Table 5). $^{13}$C-NMR has the advantage of a high information content. Due to the high chemical shift dispersion, in a $^{13}$C-NMR spectrum each carbon atom of the repeating unit of a carrageenan variant gives rise to one single signal and the various carrageenan variants show unique and characteristic patterns (see Tables 3 and 4). Quantitative determination of the composition of a mixture of carrageenans can be based on the intensity of the resonances of the anemic carbons of both rings of the repeating unit. A disadvantage of $^{13}$C-NMR is its low sensitivity. For the recording of $^{13}$C-NMR spectra samples with high carrageenan concentration (7–10% w/w) have to be used. Even then a $^{13}$C-NMR experiment takes approximately 12–18 h to reach a reasonable signal to noise ratio (S/N).

In most cases $^1$H-NMR will suffice for the characterisation of a carrageenan blend. In cases where initially $^1$H-NMR is not conclusive and additional identification is required, $^{13}$C-NMR spectroscopy can be applied. Additives such as sucrose and glucose which are often used to adjust the viscosity of the carrageenan preparations, do not disturb the NMR analysis and can in general be detected in addition to the different carrageenan forms (see Figs. 4 and 5). Only the resonance of the anomeric proton of glucose overlaps with the resonance of $\mu$-carrageenan in $^1$H-NMR. If necessary, glucose and sucrose together with inorganic salts can be removed by dialysis or ethanol precipitation.

The gelling properties of carrageenans can be considerably improved by addition of several galactomannans or mananns of some higher plants (Imeson, 2000; Therkelsen, 1993). NMR studies were used to elucidate the possible modes of interaction between polysaccharide components in these blends (Rochas, Taravel, & Turquois, 1990). Since the $^{13}$C-NMR spectra of galactomannans are well-known (Grasdalen & Painter, 1980), the corresponding spectra of mixed preparations recorded at elevated temperatures (above the melting points of gels) can be used to detect the presence of galactomannan additives and to calculate their content relative to carrageenan. In $^1$H-NMR spectra the anomeric proton of the $\alpha$-d-galactopyranose is observed at 5 ppm and that of the $\beta$-d-mannopyranose at 4.8 ppm (Grasdalen & Painter, 1980).

### Table 8. Longitudinal relaxation times of a number of $^{13}$C- and $^1$H-NMR resonances of $\kappa$-carrageenan, $\tau$-carrageenan, sucrose and glucose

<table>
<thead>
<tr>
<th>Compound</th>
<th>Resonances</th>
<th>Frequency (MHz)</th>
<th>$T_1$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\kappa$- and $\tau$-carrageenan</td>
<td>$^{13}$C-NMR anomeric carbons</td>
<td>300</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>$^1$H-NMR anomeric protons</td>
<td>600</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>$^1$H-NMR all</td>
<td>600</td>
<td>4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>$^1$H-NMR anomeric proton</td>
<td>600</td>
<td>0.45</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
different carbons. However in the case of carrageenans all carbon atoms of the repeating unit have one proton attached. Based on this one would expect little variance in the NOE effect for the different carbons. Experiments with a mixture of kappa- and iota-carrageenan using continuous composite pulse decoupling and gated decoupling produced apart from S/N, the same result for the intensity ratio of the anomic carbon resonances (Rollema, unpublished).

Table 9 illustrates that reasonable agreement is obtained between results obtained by 'H- and 13C-NMR spectroscopy with respect to the determination of the quantitative composition of carrageenan mixtures. It should be mentioned that the relatively low S/N of 13C-NMR spectra implicates that minor components can either not be detected or if their signals are detectable, the content determined by 13C-NMR will have a limited accuracy.

Finally if use is made of an appropriate internal or external reference, using 1H-NMR spectroscopy the molar ratios and the absolute contents of carrageenan forms and added mono- or di-saccharides can be determined in a single experiment.

Table 9. Comparison of the composition of three different carrageenan preparations obtained by 13C- and 1H-NMR spectroscopy

<table>
<thead>
<tr>
<th>Carrageenan form(s)</th>
<th>Sample 1 (1C-NMR)</th>
<th>Sample 1 (1H-NMR)</th>
<th>Sample 2 (1C-NMR)</th>
<th>Sample 2 (1H-NMR)</th>
<th>Sample 3 (1C-NMR)</th>
<th>Sample 3 (1H-NMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1: neutrally extracted carrageenan from Sarcothalia crispata. Sample 2: neutrally extracted carrageenan from Eucheuma denticulatum. Sample 3: commercial carrageenan sample, called λ-carrageenan.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample 1: neutrally extracted carrageenan from Sarcothalia crispata. Sample 2: neutrally extracted carrageenan from Eucheuma denticulatum. Sample 3: commercial carrageenan sample, called λ-carrageenan.

<table>
<thead>
<tr>
<th></th>
<th>13C-NMR</th>
<th>1H-NMR</th>
<th>13C-NMR</th>
<th>1H-NMR</th>
<th>13C-NMR</th>
<th>1H-NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>κ</td>
<td>44.8</td>
<td>40</td>
<td>47</td>
<td>58</td>
<td>37.2</td>
<td>36.6</td>
</tr>
<tr>
<td>τ</td>
<td>21.2</td>
<td>23.2</td>
<td>80.6</td>
<td>73.8</td>
<td>26.7</td>
<td>23.3</td>
</tr>
<tr>
<td>μ + ν</td>
<td>23.8</td>
<td>25.5</td>
<td>11.4</td>
<td>16.9</td>
<td>20.6</td>
<td>21.8</td>
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<tr>
<td>λ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>14.7</td>
</tr>
<tr>
<td>Unidentified</td>
<td>10.3</td>
<td>11.3</td>
<td>3.4</td>
<td>3.5</td>
<td>6.6</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Fig. 6. The most useful 'H-region of neocarrabioside type disaccharides represented by 500 MHz spectra at 25°C of the two tetrasaccharides G4SβD4Gα and G4SβD4Gβ. Please note that due to space DA residues are denoted as A only and the position of the residues is denoted by reducing end side (α) or non-reducing end side (β) exemplified by Anr4G4SβAr. G4Sα and where appropriate α or β. Arrows are indicating isotope-induced shifts. Results presented in Knutsen, 1992, and partly in Knutsen & Gudstuen, 1992a.
1H-NMR spectroscopy of carrageenan fragments

Knowledge about the different oligosaccharides in the different fractions can be used to deduce the structure of the original carrageenan sample. In this fragment analysis approach, 1H-NMR spectroscopy is used for its high sensitivity and possibilities to resolve the fine structure of carrageenan oligosaccharides. Carrageenan oligosaccharides are prepared by chemical or enzymatic hydrolysis. The higher and lower molecular mass fractions are separated by precipitation methods and further fractionated by gel permeation chromatography (GPC) or size exclusion chromatography (SEC), see Box 2. High field 1H-NMR spectroscopy is then used to elucidate the detailed molecular structure of the different di-, tetra-, and oligosaccharides.

NMR-elucidated sequence information

A sulfation of a certain carbon will influence not only the chemical shift on its attached proton but also the protons on other ring carbon atoms and even neighboring sugar residues. Therefore the identity of the actual carrageenan residue as well as some sequence information can be obtained. Minimal sequence information for the partly desulfated carrageenan from alkali treated Furcellaria lumbricalis was obtained by 400 MHz 1H-NMR spectroscopy, documented by an approximately 0.02 ppm upfield shift of the resonance of A1H-G compared to A1H-GAS (Knutsen, Myslobodski, & Grasdalen, 1990). Sequential information regarding the distribution of O-methyl groups (G4M) in agar from Porphyra umbilicalis had much earlier been demonstrated with 90 MHz 13C-NMR spectroscopy (360 MHz for 1H) (Morrice, McLean, Long, & Williamson, 1983). Even at a very low field strength of 2.35 T, the effect of a desulfated reducing end neighbour of 3,6 anhydrogalactose i.e. DAgC (94.6 ppm) as compared to DAgC-G4S (95.2 ppm) (Knulsen & Grasdalen, 1987) were noticed. However it is likely that some sequence information beyond this might be obtained by observing the anomerie region characteristic for both the 3-linked and 4-linked residues in well resolved spectra at.

Box 2. Preparation of carrageenan fragments

Depolymerization

Chemical depolymerisation

In general a slight degradation of the molecular weight will reduce the viscosity in a sample and hence improve its NMR-spectrum especially due to less line broadening. This can be achieved by non-specific methods such as ultrasonic degradation (Van de Velde et al., 2001) and acid hydrolysis (Caram-Lelham, Sundelof, & Andersson, 1995; Rochas & Heyraud, 1981). The latter is difficult to control since selective degradation of 3,6-anhydro units (DA2S or DA) and removal of sulfate groups is most likely to occur. A strategy based on autohydrolysis (Storz & Cerezo, 1991) has been developed with quite promising results but has so far not been used by many workers. Another depolymerisation tool, which might be useful in combination with NMR, is oxidative radical depolymerisation. Treatment of a polysaccharide solution with ferrous ions plus ascorbic acid at room temperature will induce random depolymerisation (Hjerde, Kristiansen, Stokke, Smidsrd, & Christensen, 1994; Yamada et al., 1997). The use of chemical fragmentation must be done very carefully, taken into account the possibility of lateral reactions concomitant with the lytic ones, i.e. autohydrolysis will produce the splitting of the α-(3,6-anhydrogalactosidic) linkages flanked by sulfate groups (Ciancia, Matulewicz, Storz, & Cerezo, 1991) but also will produce the hydrolysis of the β-galactose 2-sulfate, as a result of the loss of this sulfate group the resonance of the α-galactose 2,6-sulfates will displaced from ≈92.0 ppm to ≈94.7 ppm (Noseda, 1994; Noseda & Cerezo, 1993).

Enzymatic depolymerisation

As for polysaccharides in general, the use of specific enzymes is an ultimate tool for structural analysis of carrageenans. By selecting a suitable enzyme, i.e. carrageenase, well-defined oligosaccharides with characteristic structural elements can be obtained in reasonable high quantities (Bellion et al., 1983; Greer, Shomer, Goldeinstein, & Yaphc, 1984; Rochas & Heyraud, 1981). On the other hand molecules enriched with uncommon irregularities or nor carrageenan constituents might be retained and subsequently isolated by a proper fractionation technique (see below). In this way, cryptic structures, that otherwise would be masked below the noise level in the 13C-NMR spectra can be identified. Carrageenases are endo-β-galactanases, cleaving the internal β-(1→4) linkages with sulfate substitution pattern related specificity, reviewed by De Ruiter & Rudolph (1997). Oligosaccharides of the neocarbose series (DA-G4S), or (DA2S-G4S), are the main products from molecules or molecular regions that possess structural regularities of a certain length. By far the most popular enzyme is k-carrageenase, which might be obtained from several bacteria. It has successfully been produced in a stable form by large-scale fermentation of Pseudo

continued on next page
Fractionation
Separation based on solubility in ethanol

Carrageenans are never occurring structurally pure but with a varying ratio of characteristic structural elements as mentioned in Fig. 1. In order to isolate specific structural motives occurring in low quantity the use of specific carrageenases combined with some fractionation procedure is required. For the subsequent analysis NMR-spectroscopy is the most preferred tool (see below). Adding alcohol to an aqueous hydrolysate has been used to precipitate the high polymeric weight fraction, often containing a high content of irregular structures such as 6-sulfated precursor units or 3,6-anhydro units with “wrong” sulfate substitution on itself or its neighbouring residues. The term enzyme resistant fraction (ERF) was introduced for such fractions (Bellion et al., 1983).

In principle it is possible to produce pure oligosaccharides of a certain type, i.e. (DA2S-G4S)_n or (DA-G4S)_n provided such sequences occur in separate molecules or in long blocks. At this point it must be noted that if irregularities along the carrageenan chain occur close to the linkage subjected to enzymatic hydrolysis, but not so close to hinder an enzymatic attack on the actual beta 1-4 linkage, oligosaccharides carrying both precursor or unsulfated 3-linked units might be produced and hence solubilised in the 60–80% ethanol fraction (Knutsen & Grasdalen, 1992b). So far there is no literature describing enzymes with capability to specifically remove all corresponding sugar units from a certain carrageenan sample.

Nevertheless, alcohol precipitation might be used for a rough estimate of the apparent kappa-content in different samples after a treatment with κ-carrageenase (Fig. 9). The results of such a fractionation combined with the gross composition of the different major structural elements as obtained with 1H-NMR, some indication of the occurrence of sequences of a certain length (block size) could be obtained. As opposed to precipitation, leaching of a previously dried hydrolysate into increasingly water diluted ethanol solutions was shown to give more defined fractions of oligosaccharide, which easily can be subjected to NMR analysis (Knutsen et al., 1995).

![Fig. 9.](image)

The apparent (DA-G4S)_n character of some carrageenans as estimated after the digestion with κ-carrageenase. The content is estimated as the percentage of oligosaccharides as compared to the alcohol insoluble fraction. ERF was estimated gravimetrically after precipitation in 70% ethanol, dialysis and lyophilization. Where EC: a commercial extract from *Kappaphycus alvarezii* (LITEX 171505), AP: a commercial extract from *Furcellaria lumbricalis* (LITEX AP641), FLHI: the KCI-insoluble fraction of a hot water extract from *Furcellaria lumbricalis*, FLCS: the KCl-soluble fraction of a cold-water extract of *Furcellaria lumbricalis*, EG: the hot water extract of *Eucheuma gelesilnoqua*, EM: the hot water extract of *Eucheuma muriatata* and iota: a commercial extract from *Eucheuma denticulatum* (SIGMA). Unpublished results from (Knutsen, 1992).

Separation based on chromatography

In order to obtain pure fractions, or hopefully to isolate the individual oligosaccharides obtained by the enzymatic hydrolysis, some chromatographic step is needed. At first Size Exclusion Chromatography (SEC) was performed on Sephadex (McLean & Wilhelmsson, 1979) and Bio Gel type materials (Knutsen & Grasdalen, 1992b; Rochis & Heyraud, 1981). However, at present materials such as Superdex (Pharmacia) (Knutsen & Grasdalen, 1992b; Careri-Zelham et al., 1995), which can
The additional information obtained from spectra recorded from oligosaccharide samples as compared to polymeric samples (Van de Velde et al., 2001; Wehl, 1977) is related to resolved resonances from reducing and non-reducing end residues. This is only achieved as long as the level of residual water is minimised by repeated freeze drying in D2O. In general by applying carrageenases and a suitable fractionation, the resulting oligosaccharide spectra are less complex due to the pre-eminence of neocarrabiose type oligosaccharides, giving non-reducing end 3,6-anhydrogalactose and 3-linked galactose on the reducing end respectively. Furthermore, some of the H-resonances of a DA-unit neighbouring the 3-linked reducing end unit will be split due to the anomic equilibrium and may be used as diagnostic peaks in high field spectra. Although in some cases for polymer spectra a few resonances may be easily assigned by their characteristic coupling constants, the spectra obtained from oligosaccharides are more resolved and some very useful diagnostic H-signals can be found. H-NMR assignments and coupling constants of di-tetra- and hexasaccharides of the DA-G type, and the effect of desulfation are presented elsewhere (Knutsen & Grasdalen, 1992a). Assignments of the similar region for (DA2S-G4S)n, or iota-type oligosaccharides is given elsewhere (Knutsen et al., 2001). In practice some selected peaks in the spectral region from approximately 3.3 ppm to 5.3 ppm can be used to characterise (DA-G4S)n or kappa-type oligosaccharides with respect to sulfation and chain length. The effect of a desulfation of the reducing end residue is demonstrated by Fig. 6 showing this selected H-spectral region of tetrasaccharides purified from a κ-carrageenase hydrolysate of Kappaphycus alvarezii and Fucellaria carrageenan. Some useful resonances for neocarrabiose oligosaccharides and some observed splitting due to spin-spin coupling are given in Tables 10 and 11, respectively. The co-existence of oligosaccharides with either DA-G or DA-G4S type non reducing end sequence co-occurring in a SEC fraction (Bio Gel P4 see Knutsen & Grasdalen, 1992b) of a Fucellaria-carrageenan hydrolysate is demonstrated by the resonances of H5 (~4.41 ppm) and H3 (~4.35 ppm) of the DA-unit in Fig. 7. The lack of reducing end residues in addition to presence of the -G4Snn resonance at 5.32 ppm demonstrate the purity with respect to neocarrabiose.
The signals for H5 of 3,6 anhydrogalactose units appear as broad peaks. For actual shift positions see Table 10 and for all coupling constants see Knutsen & Grasdalen (1992a). Values for DA2S unpublished.

<table>
<thead>
<tr>
<th>Proton</th>
<th>In residue/sequence</th>
<th>Splitting (Hz)</th>
<th>Contributing coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>-G4S- and G4Sβ</td>
<td>7.9</td>
<td>1,2</td>
</tr>
<tr>
<td>H4</td>
<td>-G4Sα and G4Sβ</td>
<td>3.9</td>
<td>1,2</td>
</tr>
<tr>
<td>H1</td>
<td>-G4Sβ</td>
<td>3.1</td>
<td>1,2</td>
</tr>
<tr>
<td>H1</td>
<td>-G4Sα</td>
<td>2.5</td>
<td>1,2</td>
</tr>
<tr>
<td>H1</td>
<td>DA</td>
<td>2.4</td>
<td>1,2</td>
</tr>
<tr>
<td>H2</td>
<td>DA2S</td>
<td>2.3</td>
<td>1,2</td>
</tr>
<tr>
<td>H3</td>
<td>DA</td>
<td>2.4</td>
<td>1,2</td>
</tr>
<tr>
<td>H4</td>
<td>DA</td>
<td>5.4</td>
<td>1,2</td>
</tr>
<tr>
<td>H5</td>
<td>DA</td>
<td>1.9</td>
<td>Broad</td>
</tr>
</tbody>
</table>

A hyphen denotes the remaining chain whereas Greek letters specify the anomeric form. Positions in the chains do only affect shift value. The signals for H5 of 3,6 anhydrogalactose units appear as broad peaks. For actual shift positions see Table 10 and for all coupling constants see Knutsen & Grasdalen (1992a). Values for DA2S unpublished.

Table 11. Some of the characteristic splittings due to spin-spin couplings in 1H-NMR spectra of carrageenan oligosaccharides

Outlook and perspectives
The role of NMR spectroscopy of carrageenans in industry and research

For industrial purpose NMR-spectra are obtained without further purification and or fractionation of the sample, as primarily the overall composition is important. Resonances in the anomeric region of the 13C- and 1H-NMR spectra reflect the main diad components, which determine the type of the possible major carrageenans in the sample. It is important to remember that a pair of correlated resonances determines a diad. The spectrum is a superposition of the resonances of these diads and, therefore, represents only an average composition of the structural units present in the sample, without any indication whether these units are present in the same molecule or in different ones (see for example Van de Velde et al., 2001). When substituents occur that do not affect characteristic chemical shift pattern of the anomeric region, for 13C or 1H-NMR spectroscopy, they might be identified by typical resonances in other regions of the spectra.

1H-NMR spectroscopy with all its advantages, such as low sample concentrations and short analysis times in particular is suitable for the quantification of the different carrageenan types. The α-anomeric protons of the major carrageenans types (κ, λ, μ, and ν) give signals in the region 5.1–5.7 ppm, which are easily separated in a high-field instrument. Routine rheological analysis of the raw extract completes the commercial-value analysis of the original or alkali-treated carrageenan.

In summary, the NMR spectra (1H and 13C) of raw extracts give good identification of the major type of carrageenans produced by the seaweed when compared with spectra of “model” carrageenans (Turquois et al., 1996). The availability of equipment with enhanced resolution will promote the 1H-NMR spectroscopy for this use even more. It is possible that the spectra show minor or trace signals originating from non-precursor “kinks” (i.e. non-sulfated α-1, 3-galactose units) which without changing the “type” of carrageenan can modulate its rheological behaviour.

A different approach is used when one focusses on the system of carrageenans biosynthesised by the seaweed
The 1H-region of the 500 MHz-spectra of a mixture of partly desulfated neocarrabee type oligosaccharides from Furcellaria-carrageenan recorded at 25°C. Only selected assignments are denoted on the figure. For comparison a spectrum of the same sample recorded at 90°C is included. R is indicating a remaining part of an oligosaccharide chain towards the non-reducing end and R' towards the reducing end. Presented only in (Knutsen, 1992).

or in the determination of the fine structure of a carrageenan or a carrageenan fraction. The identification of a carrageenan mixture produced by a seaweed requires an elaborate process of extraction and fractionation. The composition and yield of the fractions obtained indicate the presence of minor components, the dispersion of structures in the system and the strength of the interaction of these molecules in the seaweed tissue. Subfractionation, when necessary, gives the different “pieces” of a jigsaw puzzle, the solution of which will show an overall picture of the carrageenan system synthesised by the seaweed. In the study of this puzzle,
NMR spectroscopy plays an important but not always a conclusive role as in the industrial approach. The main drawback of this methodology in the determination of fine structural details in carrageenans is due to the complexity of these polymers, their molecular interactions and their high molecular weight. Due to its low sensitivity, $^{13}$C-NMR spectroscopy cannot detect low percentages of minor components. Negative results do not exclude the presence of minor components, for example the presence of $\alpha$-l-galactose and/or 3,6-anhydro $\alpha$-l-galactose and the corresponding $\delta$/l-hybrids and/or agarans (Estevez et al., 2001). The $^1$H-NMR spectra of complex polysaccharides usually show broad resonances, sometimes overlapping and difficult to integrate. In the case of carrageenans the diads can be recognised by the resonances of the $\alpha$-anomeric protons (Feldman, Storz, Vigna, & Cerezo, 1994).

A new approach to evaluate $^{13}$C-NMR spectroscopical data for the structural analysis of red algal galactans was suggested recently (Miller & Blunt, 2000a,b). This approach is based on the availability of a large number of $^{13}$C-NMR spectra of different well characterised galactans. The $^{13}$C-NMR spectrum of a polysaccharide with an unknown structure is assigned by a mathematical procedure that determines the best fit between the experimental spectrum and the elements of the database of spectra of characterised galactans. In spite of rather complicated mathematical terminology used, in principle the approach seems to be fruitful, but has some marked experimental limitations, since it is difficult to carry out desulfation and methylation steps quantitatively. Incomplete chemical modifications may, of course, result in undesirable complication of spectra. Nevertheless, several examples of successful application of this approach to the elucidation of structures of complex galactans, including $\delta$/l-hybrids (Miller, 2001a; Miller & Blunt, 2000c) and an unusual carrageenan (Miller,2001b), are found in the literature.

Most of these problems can be overcome by selective modification of the molecule (i.e. desulfation) or by selective, if not specific, fragmentation of the carrageenan molecule. These fragmentations can be carried out through enzymatic or chemical methods (see Box 2). By degrading carrageenans with structure-specific enzymes a series of sulfated oligosaccharides of known structure is formed. However, since carrageenan molecules in general are hybrids and also may contain "abnormal" units interspersed in the backbone some larger fragments, resistant to enzymolysis and with a size depending on in which sequence they occur will be enriched. These are suitable samples for NMR analysis as they concentrate these usual minor details. However this might be a challenging task, since complex molecules strongly deviating from a regular sequence might occur, especially in carrageenan precursor rich molecules. In such cases the application of $^1$H-NMR spectroscopy, will result in higher sensitivity, more reliable integration of the signals and hence the best possible quantitative results. In most cases some complementary methods for identification of constituents in the sample like methylation analysis (Falshaw et al., 2001; Falshaw & Furneaux, 1994) or reductive hydrolysis (Jol, Neiss, Penninkhof, Rudolf, & DeRuiter, 1999) or the use $^{13}$C NMR or 2D-NMR techniques must be performed simultaneously. Finally if high resolved $^{13}$C-NMR spectra can be obtained from such fractions some crucial insight into the sequential nature of a carrageenan and its biosynthesis might be obtained.

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