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# Production of agaro- and carra-oligosaccharides by partial acid hydrolysis of galactans

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**Abstract:** Agaro- and carra-oligosaccharides were produced by partial acid hydrolysis of commercial agarose and kappa-carrageenan. Di- and tetrasaccharides were purified by gel filtration chromatography and characterized by NMR (1D and 2D) spectroscopy and ESIMS. The following oligosaccharides were obtained: agarobiose, agarotetraose, kappa-carrabiose and kappa-carratetraose. Agarobiose and agarotetraose were used as standards to develop a high performance size exclusion chromatography (HPSEC) method which was utilized to study the hydrolysis rate of agarose and oligosaccharide production. Six hours of hydrolysis (0.1 M TFA, 65 °C) produced mainly di- and tetrasaccharides. The methodology for oligosaccharide production and evaluation developed in the present work shows good potential for the production of bioactive oligosaccharides.

## Introduction

Red seaweeds produce linear galactans as the principal constituent of the extracellular matrix. Chemically, these polymers consist of alternating (1→3)-linked β-D-galactopyranose and (1→4)-linked α-galactopyranose units. Frequently, the 4-linked units are in the 3,6-anhydro form. These polymers are classified according to the stereochemistry of the 4-linked units into agarans (L-enantiomer) and carrageenans (D-enantiomer) (Painter, 1983; Craigie, 1990). Recently, a third group, denominated D/L-hybrids and containing D and L enantiomers of the α-Gal*p* units (Zibetti et al., 2005; Zibetti et al., 2009) has emerged. Agarans and carrageenans can present complex substitution patterns due to the presence of sulfate, methyl, 4,6-*O*-(1'-carboxyethylidene) and glycosyl groups (Craigie, 1990).

Galactan oligosaccharides can be obtained through partial acid (Yu et al., 2002; Gonçalves et al., 2010; Ciancia et al., 2005) or enzymatic hydrolysis (Guibet et al., 2006; Young et al., 1978; Rochas et al., 1994). Usually, the partial acid hydrolysis methods (Figure 1) promote the specific cleavage of 3,6-anhydro-α-galactosidic bonds to produce the reducing di- or tetrasaccharides **2** and **4**, containing the unusual anhydro galactose in hydrated aldehyde form as the terminal unit. These oligosaccharides can be isolated and characterized (Miller et al., 1982; Fatema et al., 2010) and even used as starting materials to synthesize carbohydrate building blocks (Ducatti et al., 2009). However, due to the instability of 3,6-anhydro-galactose units to harsh acidic conditions, partial hydrolysis with strong and concentrated acids can also produce the reducing trisaccharide **3** containing galactose as the terminal unit (Yang et al., 2009). A good alternative for preserving the anhydro units is the addition of reducing (Usov & Elashvili, 1991; Gonçalves et al., 2002) or oxidizing (Penman & Rees, 1973) agents during the hydrolysis step to produce alditol or aldonic oligosaccharides, respectively.

Galactan oligosaccharides and their derivatives can exhibit significant antioxidant (Chen & Yan, 2005), antitumoral (Haijin et al., 2003) and antiangiogenic (Chen et al., 2007) activities. In particular, oligosaccharides and compounds containing the reducing 3,6-anhydro-L-galactose unit, such as agarobiose, agarotetraose and agarohexaose can suppress nitric oxide, prostaglandin E2 and pro-inflammatory cytokine production *in vitro* (Enoki et al., 2010; Kobayashi et al., 2003). The presence of the anhydro unit at the reducing terminal end appears to play a crucial role in these activities.

Due to the potential applicability of

oligosaccharides in the pharmaceutical and food industries, production protocols (Enoki et al., 2007) have been developed. In addition, chromatographic methods are utilized to control the hydrolysis process (Chen et al., 2004; Kazlowski et al., 2008), being useful for monitoring bioactive oligosaccharide production.

In this paper we describe the production and characterization of agaro- and carra-oligosaccharides via the partial acid hydrolysis of commercial agarose and kappa-carrageenan. Agarobiose and agarotetraose, purified from agarose, were used as standards to evaluate the hydrolytic process by HPSEC.

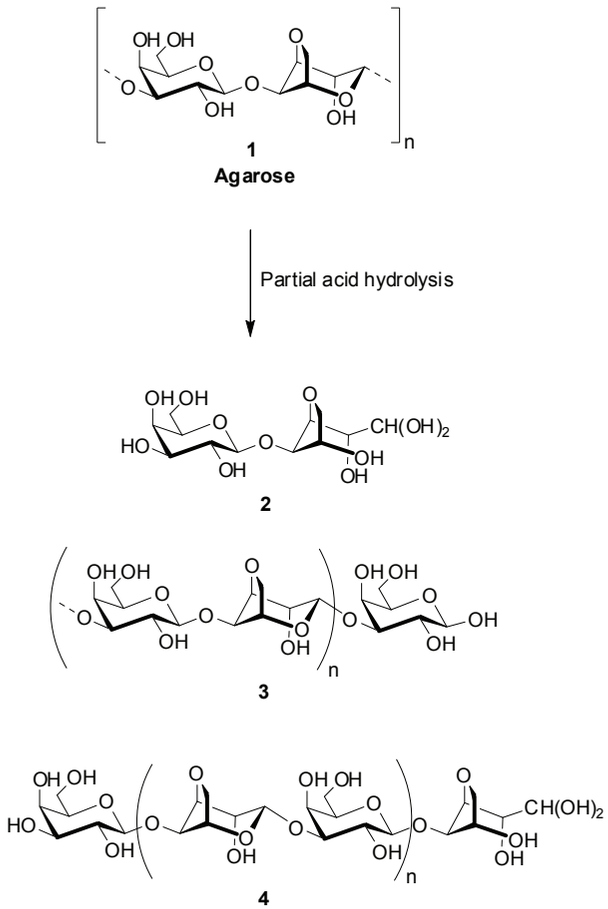


**Figure 1.** Production of reducing agaro-oligosaccharides through partial acid hydrolysis of agarose.

## Materials and Methods

*Chemical and reagents*

Sample 1 corresponded to a commercial agar purchased from Vetec Química (Rio de Janeiro, RJ, Brazil), sample 2 was a commercial agarose purchased from Sigma-Aldrich (St. Louis, MO, USA) and sample 3 was a low melting point agarose purchased from Invitrogen (Carlsbad, CA, USA). Kappa-carrageenan (KWS) was obtained as previously described (Gonçalves et al., 2005). Trifluoracetic acid, sulfuric acid, and phenol were high purity reagents from Merck. MilliQ Water with a specific resistance higher than 18.2 MΩ.cm was utilized for HPLC analysis.

*Preparation of agarobiose and agarotetraose*

Sample 1 (150 mg) was first dissolved in hot (~90 °C) $H_2O$ (13.5 mL) and then 1 M TFA solution (1.5 mL) was added in one portion. The resulting mixture was heated at 65 °C for 2 h, cooled to room temperature, diluted with $H_2O$ (15 mL), and then concentrated under vacuum. The resulting residue was coevaporated with toluene three times to give a syrup. This material was dissolved in $H_2O$ (2 mL) and applied on a Bio-Gel P2 column (70 x 1.5 cm). Oligosaccharide detection was performed by the phenol-sulfuric acid method (Dubois et al., 1956) and TLC. The TLC was carried out on silica gel 60 (2:2:1 BuOH-AcOH-H2O) with detection by charring with 0.5% orcinol in EtOH-$H_2SO_4$ (20:1). The fractions AA and AB were concentrated and freeze-dried to give agarobiose (15 mg) and agarotetraose (13 mg), respectively.

*Preparation of kappa-carrabiose and kappa-carratetraose*

KWS (150 mg) was first dissolved in hot (~90 °C) $H_2O$ (13.5 mL) and then 1 M TFA solution (1.5 mL) was added in one portion. The resulting mixture was heated at 65 °C for 3 h, cooled to room temperature, diluted with $H_2O$ (15 mL), and then concentrated under vacuum. The resulting residue was coevaporated with toluene three times to give a syrup. This material was dissolved in $H_2O$ (2 mL) and applied on a Sephadex G-25 column (100 x 1.5 cm). Oligosaccharide detection was performed as described for agarobiose preparation. The fractions KA and KB were concentrated and freeze-dried to give kappa-carrabiose (31 mg) and kappa-carratetraose (12 mg), respectively.

*Production rate of agarobiose and agarotetraose from agarose by HPSEC analysis*

Samples of commercial agar 1 (10 mg) were first dissolved in TFA 0.1 M. These mixtures were hydrolyzed at 65 °C for 1, 2, 4, 6 and 8 h (n=3 for each time). After hydrolysis, the solutions were concentrated and freeze-dried. The resulting hydrolysates (1 mg) were diluted in ultrapure $H_2O$ (1 mL) and analyzed by HPSEC. The chromatographies were performed with a Shimadzu equipment using a RI detector operating at 40 °C. The chromatographic separation was achieved with an Ultra-hydrogel (Waters) 120 (7.8 x 300 mm) column. Elution

was carried out with ultrapure water at 30 °C with a flow rate of 0.4 mL.min$^{-1}$. Samples were injected manually with a Rheodyne 7725i injector (50 µL sample loop). Calibration curves were obtained by injecting increasing concentrations (0.25 to 2.0 mg.mL$^{-1}$) of agarobiose and agarotetraose (n=3 for each concentration).

*Monosaccharide composition analysis of the galactans by gas chromatography coupled to mass spectrometry (GC-MS)*

Monosaccharide compositions of polysaccharide samples were performed by reductive hydrolysis (Stevenson & Furneaux, 1991). GC-MS analyses were carried out with a Varian 3800 chromatograph equipped with a fused-silica capillary column (30 m x 0.25 mm) coated with DB-225MS (Durabond). The chromatograph was programmed to run at 50 °C for 1 min, then 50-215 °C at 40 °C.min$^{-1}$, using helium as carrier gas at 1 mL.min$^{-1}$.

*Nuclear magnetic resonance (NMR) spectroscopy*

1D- and 2D-NMR spectra were acquired on a Bruker Advance DRX 400 spectrometer equipped with a 5 mm wide bore probe, operating at 400 MHz for $^{1}$H and 100 MHz for $^{13}$C. Samples were exchanged with deuterium by repeated evaporations in $D_2O$. Analyses were performed in $D_2O$ at 30 °C for oligosaccharides and 70 °C for polysaccharides. The spectra were internally referenced using acetone (δ=2.224 ppm for $^{1}$H and δ=30.20 ppm for $^{13}$C).

*Electrospray-ionization mass spectrometry (ESI-MS)*

The ESIMS equipment used was a Micromass Quattro LC-MS/MS triple quadrupole mass spectrometer. Data acquisition and processing were performed using Maslynx 3.5 software. Mass spectrometry was carried out in the negative and positive-ion modes. Samples (0.125 mg.mL$^{-1}$) were injected in a 70:30 Acetonitrile/water mixture by a syringe pump (KD Scientific Inc.) flowing at 60 µL.min$^{-1}$. ESI conditions were as follows: N2 was used as nebuliser (87 L.h$^{-1}$) and desolvation gas (429 L.h$^{-1}$). The source was operated at 80 °C with a desolvation temperature of 130 °C. The electrospray capillary voltage in the negative-ion mode was 2.79 kV and the cone voltage was 71 V. In the positive-ion mode, the capillary voltage was 1.98 kV and cone voltage was 171 V. The RF lens was set at 0.30. The mass scan range was 2-1500 u, for 1 min total scan time, with 3 s scan time and 0.1 s interscan time.

## Results and Discussion

*Production of agarobiose and agarotetraose from agarose*

We analyzed the composition of three different commercial samples of agarose by NMR and GC-MS techniques. The monosaccharide compositions of sample 1 and 2 were similar, with galactose and 3,6-anhydro-galactose as the principal constituents (Table 1). Small amounts of 2-*O*-methyl-3,6-anhydro-galactose and 6-*O*-methyl-galactose were also observed and the signals of the methyl protons of these units were assigned at 3.51 and 3.42 ppm, respectively, in the $^{1}$H-NMR spectra (Figure 2) (Mazumder et al., 2002). Sample 3 is a low melting point agarose with a lower 3,6-anhydro-galactose content than samples 1 and 2 and a high amount of natural methylated sugars. The $^{13}$C-NMR spectra of all samples showed a resonance at 97.8 ppm, which was assigned to C-1 of 3,6-anhydro-α-L-galactopyranose. The signal corresponding to C-1 of 2-*O*-methyl-3,6-anhydro-α-L-galactopyranose was observed at 98.4 ppm for sample 3 (Figure 2). The NMR and GC-MS analyses of samples 1 and 2 were similar, indicating that both samples are good sources of agarose with a high degree of purity.

**Table 1.** Monosaccharide composition[a] of commercial agarose samples (1-3) and kappa-carrageenan (KWS).

| Samples[b] | Monosaccharide (mol%)[c] | | | | |
|---|---|---|---|---|---|
| | Gal | 3,6-AnGal | 2Me-AnGal | 6Me-Gal | 2Me-Gal |
| 1 | 57.0 | 38.5 | 2.2 | 2.1 | 0.3 |
| 2 | 54.5 | 42.0 | 2.2 | 1.4 | 0 |
| 3 | 49.1 | 29.6 | 9.1 | 4.9 | 7.3 |
| KWS | 44.2 | 50.4 | 0 | 4.7 | 0.7 |

[a]Compositional analyses determined after total reductive hydrolysis. [b]Samples are defined in the Material and Methods section. [c]Gal corresponds to galactosyl units; 3,6-AnGal to 3,6-anhydro-galactosyl units; 2Me-AnGal to 2-*O*-methyl-3,6-anhydro-galactosyl units; 6Me-Gal to 6-*O*-methyl-galactosyl units; 2Me-Gal to 2-*O*-methyl-galactosyl units.

The instability of 3,6-anhydro-galactose residues under acidic conditions has been known since the first structural studies on carrageenans and agarans (O'Neill, 1955; Araki & Hirase, 1953). Stevenson & Furneaux (1991) showed that the anhydro units can resist mildly acidic conditions and then be quantified, after derivatization, by GLC. Thus, we submitted agarose (sample 1) to partial acid hydrolysis (0.1 M TFA, 65 °C, 2 h) to promote the cleavage of 3,6-anhydro-galactosidic linkages. The oligosaccharide mixture was purified on a Bio-Gel P2 column, yielding two distinct fractions, AA (10%) and AB (9%).

$^{13}$C-NMR analysis of the fraction AA (Figure 3a) showed a spectrum characteristic of a disaccharide with two signals in the anomeric region. The assignments for agarobiose **2** (Table 2 and Figure 1) are in agreement with those previously reported (Miller et al., 1982). Reducing oligosaccharides with 3,6-anhydro-galactose as the terminal unit have a simple $^{13}$C NMR spectrum because the

reducing end does not present mutarotation. Indeed, the typical signal of C-1 of 3,6-anhydro-galactose in the hydrated aldehyde form was observed at 89.9 ppm. ESIMS analysis in the positive-ion mode confirmed the structure of **2** (data not shown). Two principal peaks corresponding to the aldehyde form ($[M + Na]^+$ at *m/z* 347) and the hydrated aldehyde form ($[M + H_2O + Na]^+$ at *m/z* 365) were observed.

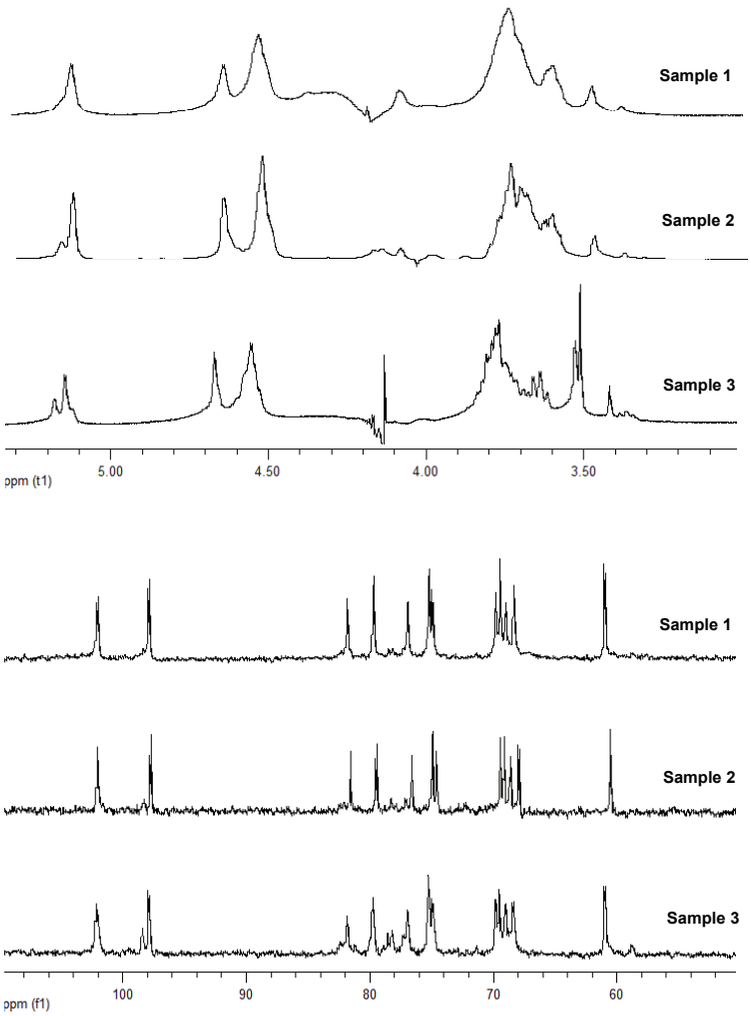


**Figure 2.** $^{1}H$- and $^{13}C$-NMR spectra of commercial agarose samples.

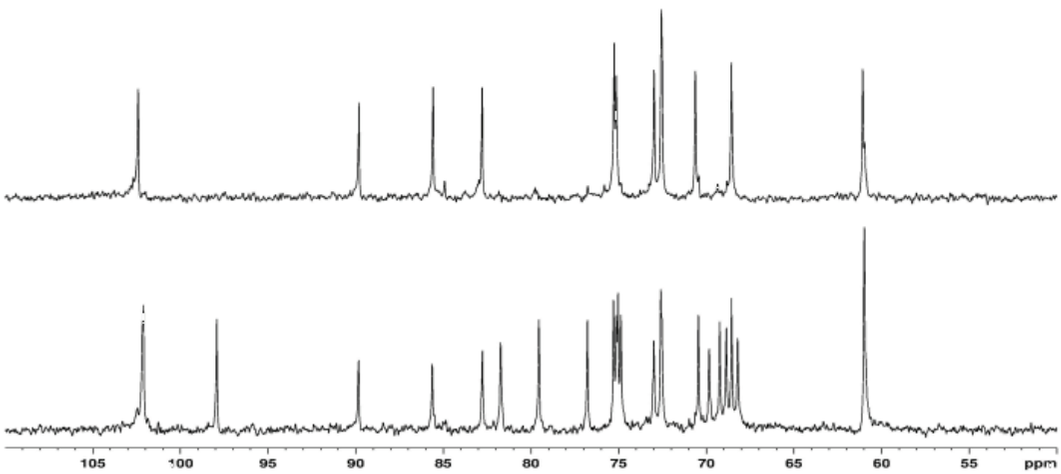


**Figure 3.** $^{13}C$-NMR spectra of fractions AA (a) and AB (b).

**Table 2.** $^{1}H$- and $^{13}C$-NMR assignments of agaro- and carra-oligosaccharides.

| Unit | | Agarobiose | | Agarotetraose | | κ-carrabiose | | κ -carratetraose | |
|---|---|---|---|---|---|---|---|---|---|
| | | $^{1}H$ | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ | $^{13}C$ |
| | 1 | 4.56 | 102.4 | 4.56 | 102.1 | 4.56 | 103.0 | 4.60 | 102.7 |
| | 2 | 3.53 | 70.5 | 3.50 | 70.5 | 3.57 | 70.7 | 3.51 | 70.6 |
| | 3 | 3.66 | 72.5 | 3.65 | 72.6 | 3.82 | 71.4 | 3.80 | 71.6 |
| β-D-Gal*p* (ext.) | 4 | 3.93 | 68.4 | 3.94 | 68.6 | 4.67 | 76.3 | 4.68 | 76.4 |
| | 5 | 3.71 | 75.2 | 3.74 | 75.3 | 3.86 | 74.5 | 3.85 | 74.4 |
| | 6 | 3.78 | 60.9 | 3.77 | 61.0 | 3.82 | 60.9 | 3.80 | 60.8 |
| | 6’ | 3.78 | | 3.77 | | 3.82 | | 3.80 | |
| | 1 | | | 5.16 | 98.2 | | | 5.10 | 94.2 |
| | 2 | | | 4.14 | 69.3 | | | 4.14 | 69.1 |
| | 3 | | | 4.56 | 79.6 | | | 4.52 | 78.7 |
| 3,6-An-α-Gal*p* (int.) | 4 | | | 4.66 | 76.8 | | | 4.61 | 77.8 |
| | 5 | | | 4.57 | 74.9 | | | 4.65 | 76.2 |
| | 6 | | | 4.03 | 68.9 | | | 4.06 | 69.0 |
| | 6’ | | | 4.24 | | | | 4.21 | |
| | 1 | | | 4.61 | 102.2 | | | 4.59 | 102.2 |
| | 2 | | | 3.66 | 69.9 | | | 3.67 | 69.2 |
| | 3 | | | 3.82 | 81.8 | | | 4.00 | 77.6 |
| β-D-Gal*p* (int.) | 4 | | | 4.14 | 68.2 | | | 4.84 | 73.2 |
| | 5 | | | 3.74 | 75.1 | | | 3.85 | 74.4 |
| | 6 | | | 3.77 | 61.0 | | | 3.80 | 60.9 |
| | 6’ | | | 3.77 | | | | 3.80 | |
| | 1 | 5.02 | 89.9 | 5.01 | 89.8 | 5.01 | 89.8 | 5.00 | 89.8 |
| | 2 | 3.74 | 72.5 | 3.73 | 72.5 | 3.65 | 72.7 | 3.66 | 72.7 |
| | 3 | 4.08 | 82.8 | 4.07 | 82.8 | 4.09 | 82.3 | 4.09 | 82.4 |
| 3,6-An-Gal (red.) | 4 | 4.38 | 85.6 | 4.39 | 85.6 | 4.29 | 86.9 | 4.28 | 86.9 |
| | 5 | 4.41 | 75.1 | 4.41 | 75.2 | 4.49 | 75.3 | 4.49 | 75.4 |
| | 6 | 3.86 | 72.8 | 3.86 | 73.0 | 3.86 | 72.7 | 3.84 | 72.8 |
| | 6’ | 3.99 | | 4.00 | | 4.03 | | 4.02 | |

$^{13}C$-NMR analysis of the fraction AB (Figure 3b) indicated the presence of a tetrasaccharide. The spectrum of agarotetraose **4** (Figure 1, n=1) has signals in the anomeric region at 98.2 ppm (C-1 of 3,6-anhydro-α-L-Gal*p*) and 102.1 ppm (C-1 of β-D-Galp), in agreement with previously reported data (Rochas et al., 1994). Two-dimensional NMR experiments were performed to complete the $^{13}C$ and $^{1}H$ assignments (Table 2). The structure of agarotetraose was also confirmed by ESIMS analysis. In the positive-ion mode, two molecular ions were observed at *m/z* 653 and 671 corresponding to the tetrasaccharide in the aldehyde and hydrated aldehyde forms, $[M + Na]^+$ and $[M + H_2O + Na]^+$, respectively.

*Production of kappa-carrabiose and kappa-carratetraose from kappa-carrageenan*

A water-soluble polysaccharide fraction (KWS) was obtained from *Kappaphycus alvarezii* as previously described (Gonçalves et al., 2005). The monosaccharide composition of KWS showed galactose and 3,6-anhydro-galactose as the principal constituents (Table 1). $^{13}C$- and $^{1}H$-NMR analyses presented spectra typical of kappa-carrageenan (Van de Velde et al., 2002). In the $^{1}H$-NMR spectrum (not shown), the signal corresponding to H-1 of the 3,6-anhydro-α-D-Galp units was observed at 5.10 ppm. These results confirmed the presence of kappa-carrageenan as the principal constituent of KWS.

Kappa carrageenan **5** (KWS) was submitted to partial acid hydrolysis (Figure 4) for 3 h to give a mixture of sulfated oligosaccharides. This mixture was purified by gel filtration chromatography on a Sephadex G-25 column to give two principal fractions, KA (21%) and KB (8%).

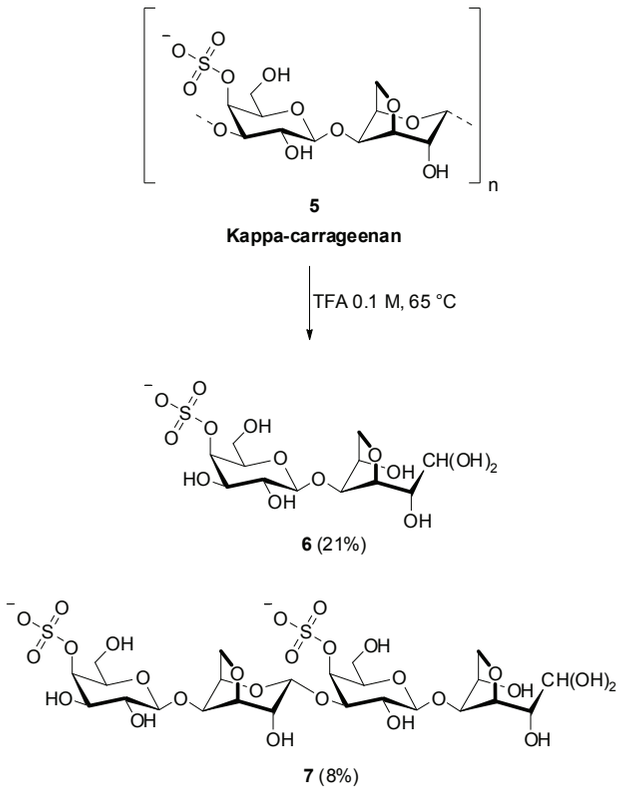


**Figure 4.** Production of carra-oligosaccharides via partial acid hydrolysis of kappa-carrageenan.

The $^{1}$H-NMR spectrum of KA exhibited a doublet at 5.01 ppm (*J 1,2*=6.0 Hz) that was assigned to H-1 of the reducing 3,6-anhydro-galactose units. The $^{13}$C-NMR spectrum of KA (Figure 5a) was attributed to the kappa-carrabiose disaccharide **6** (Figure 4), in agreement with data previously reported (Miller et al., 1982). The resonances at 103.0 and 76.6 ppm were assigned to C-1 and C-4 of the β-D-Gal*p*-4-sulfate unit, respectively (Table 2). ESIMS analysis in the negative-ion mode confirmed the structure of 6 in the fraction KA. Two molecular ions were observed at m/z 403 and 421, corresponding to the aldehyde and hydrated aldehyde forms $[M - H]^-$ and $[M - H + H_2O]^-$, respectively.

The $^{13}$C-NMR spectrum of KB (Figure 5b) showed three signals in the anomeric region, which were assigned to C-1 of the terminal β-D-Galp 4-sulfate (102.7 ppm), the internal β-D-Galp 4-sulfate (102.2 ppm) and the 3,6-anhydro-β-D-Galp (94.2 ppm) units. The reducing 3,6-anhydro-galactose in the hydrated aldehyde form was also observed via the C-1 signal at 89.8 ppm. Complete $^{1}$H- and $^{13}$C-NMR assignments (Table 2) and ESIMS analysis confirmed the sulfation at C-4 for both β-D-Galp units and the presence of kappa-carratetraose 7 (Figure 4) in the fraction KB. All the oligosaccharides isolated presented good stability for months when stored in the freezer.

*Agarobiose and agarotetraose production rates determined by HPSEC analysis*

HPLC has emerged as an important tool for carbohydrate analysis (Ascencio et al., 2006; Givry et al., 2007). Facilities such as the lack of a need for derivatization of the sample and the utilization of water as solvent led us to develop a high performance size exclusion chromatographic (HPSEC) method for evaluating the rate of production of agarobiose and agarotetraose from agarose. The partially depolymerized (4 h of hydrolysis) agarose samples, *i.e.*, agarobiose (AA) and agarotetraose (AB), were used to determine the best chromatographic conditions for oligosaccharide separation. Ultra-hydrogel 120, 250 and 500 columns were utilized individually or coupled at several temperatures and with different buffers as mobile phases. Good resolution for separation and quantification were found by using an ultra-hydrogel 120 column eluted with ultrapure water at 30 °C at a flow rate of 0.4 mL.min$^{-1}$ (Figure 6).

HPSEC analyses of fractions AA and AB confirmed the high degree of purity of the oligosaccharides inferred from the $^{1}$H-NMR spectra. Therefore, these oligosaccharides were used as standards to construct calibration curves. Calibration curves were obtained for oligosaccharide concentrations ranging from 0.25 to 2.0 mg.mL$^{-1}$. The correlation coefficients of the graphs were 0.9999 for agarobiose and 0.9998 for agarotetraose.

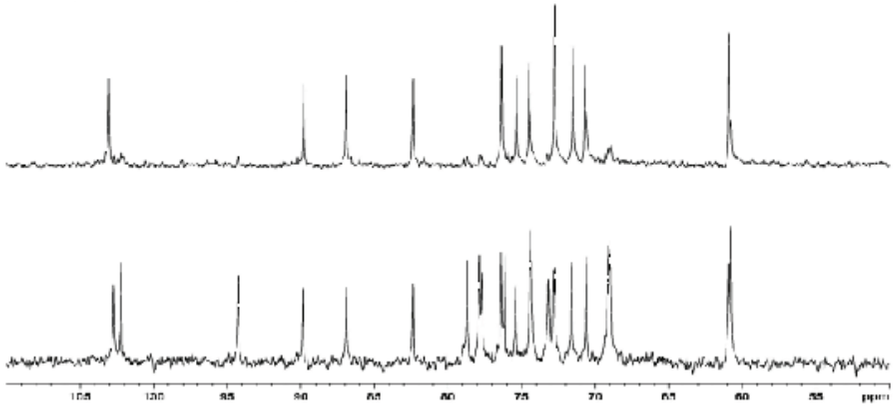

**Figure 5.** $^{13}$C-NMR spectra of fractions KA (a) and KB (b).

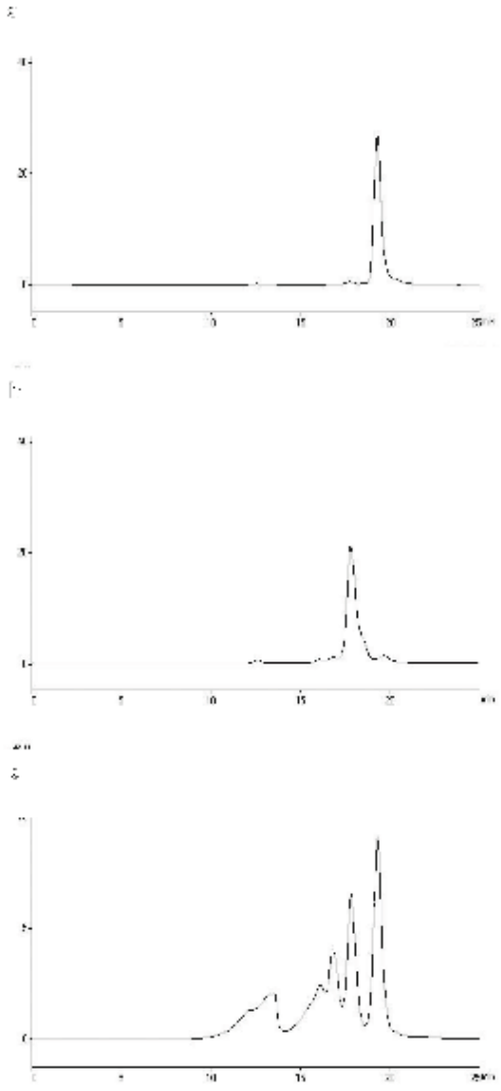

**Figure 6.** HPLC chromatograms of agarobiose (a), agarotetraose (b) and partially depolymerized agarose (c).

Agarose (sample 1) was submitted to acid hydrolysis for different times and the oligosaccharide mixtures were analyzed by HPSEC. The rates of production of agarobiose and agarotetraose were measured with the assistance of the calibration curves and the results are depicted in Figure 7. The agarobiose content did not exceed that of agarotetraose until four hours of hydrolysis. Although the disaccharide content increased with hydrolysis time, the agarotetraose content remain constant (at aprox. 19%) between four and eight hours. This suggests that the rates of agarotetraose formation from high molecular mass oligosaccharides and of its hydrolysis to yield agarobiose maintained a constant ratio during these times. At six hours of hydrolysis, about 50% of the depolymerization products were di- and tetrasaccharides, indicating a rapid hydrolysis of agarose, as expected for a non-sulfated galactan. This rapid rate of hydrolysis can be explained by the lack of substituent groups that might stabilize the galactan 3,6-anhydro-galactosidic bonds.

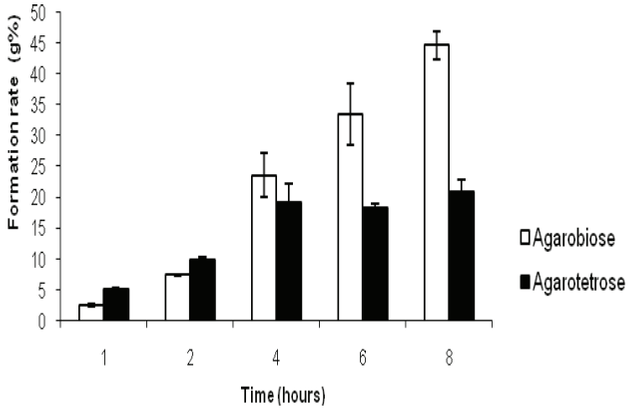


**Figure 7.** Production of agarobiose (□) and agarotetraose (■) at different hydrolysis times as determined by the HPSEC method.

The preparative gel permeation chromatography of hydrolyzed agarose (2 h of partial hydrolysis) on Bio-Gel P2 provided agarobiose (10%) and agarotetraose (9%). These yields are in good agreement with those determined by HPSEC analysis at the same two hours of hydrolysis time.

In conclusion, stable reducing neutral and acidic (sulfated) oligosaccharides with 3,6-anhydro-galactose at the reducing terminal end were obtained by applying partial acid hydrolysis to agarose and kappa-carrageenan. A rapid HPSEC method was developed to estimate the formation of agarobiose and agarotetraose by partial acid hydrolysis from a commercial sample of agarose. Theses methodologies of oligosaccharide production and evaluation show good potential for the production of bioactive galactan oligosaccharides.

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