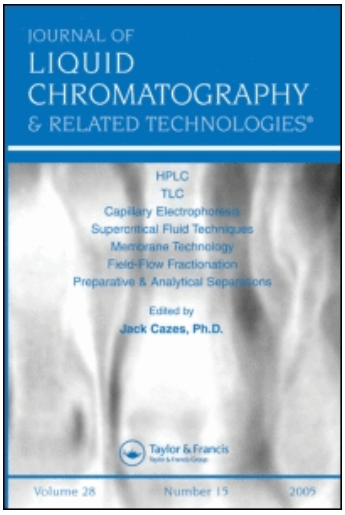

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DETERMINATION OF THE DEGREE OF POLYMERIZATION OF AGAR-TYPE POLY-SACCHARIDES BY A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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ABSTRACT

A reversed-phase HPLC methods in combination with Diode Array Detection is described for separation of polysaccharides in acid hydrolysates from many different agar-type polysaccharides. Temperature is optimized at 30°C. The detection limit is about 5 pmol. Sugars show a linear response in a wide concentration range. This technique is able to resolve the different oligomers according to the respective degree of polymerization (DP) in a very rapid, easy, reliable and reproducible way.

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INTRODUCTION

The molecular weigth distribution is very important for characterization of natural and synthetic polymers in biochemistry, agriculture and the food industry. Given that the molecular weight of polysaccharides seem not be controlled during biosynthesis, a large range of components of different molecular weight will be formed (1).

Molecular weights and different structures of agar-type polysaccharides are specially important since the gel strength, gelling and melting temperatures are determinant properties for its use in biochemical techniques (immunodiffusion and diffusion; electroforesis of proteins, nucleic acids and polysaccharides; immunoelectrophoresis, electrofocusing, affinity chromatography, chromatofocusing, ...) and microbiological techniques (culture media) (2,3).

Several types of methods have been employed for determining molecular weights. Classical methods based on osmotic pressure, viscosity, light scattering and streaming biofringance measurements are not used appreciably at present. More recently new tecniques based in partition chromatography on cellulose or ion-exchange resins, separation of saccharides in the form of borate complexes on anion exchangers, gel filtration on cation exchangers or polyacrylamide gels and chromatography on silica with chemically bonded amino phases have been used (4), but only if the molecular weight of the polysaccharides are reasonably large can be effectively carried out (5,6).

The use of refractive index (IR) detectors (7,8) and UV detectors (4) are widely employed. In this study a reversed-phase HPLC in combination with Diode Array detection has been used for the separation and quantitative

determination of the different hydrolyzed oligomers forming agar polymers. The simplest and ideal methods of detection are those, that require none chemical reaction of the sugars, being detection of separated sugars without previous derivatization.

MATERIALS AND METHODS

Reagents

The oligosaccharides have been separated using water distilled and deionized water, filtered through GS Millipore membranes (0.22 μm pore size; Molshem, FRANCE). Samples were filtered through GV-Millex 013 Millipore membranes (0.22 μm pore size; Bedford, MA, USA).

Galactose, sucrose and maltooligosaccharides were purchased by Sigma. Agarose and Agar of *Gelidium* used as black control have been furnished by Hispanagar S.A. as aditional material for this research.

Apparatus

A 1090 HP Hewlett-Packard Liquid Chromatograph system (Waldbroon, Germany) equipped with a Diode Array Detector System, a Rheodyne loop injector of 20 μl and a Supelcosil LC-18 column, 15 cm x 4.6 mm (5 μm packing) and a W/guard (Supelguard LC-18, 2 cm x 4.6 mm, 5 μm packing; Supelco, Inc., Bellefonte, PA, USA) columns were used.for the chromatographic separation of the derivatives. A Hewlett-Packard System

consisting in a Workstation plus a Operation Software was employed to continuous on-line quantification of chromatographic peaks. UV wavelength for detection was of 190 ± 4 nm.

Biological samples

Gelidium sesquipedale (Clem.) Born et Thur., a red algae, has been the biological material for the extraction of the cell-wall polysaccharides. Algal treatment and cell-wall polysaccharides extraction was carried out as reported previously by Santos and Doty (9) modified by Torres et al. (10).

Sample preparation

Polysaccharides (0.015 g) were hydrolysed with hydrochloric acid (0.01 M, 1 ml) for 10 and 24 hours in a water bath (100°C). The resulting solution was keep to reach room temperature and then filtered through GV-Millex 0.13 Millipore membranes.

Chromatographic conditions were described by Vratny, P., et al. (4) with minor modifications, omitting the following addition of absolute ethanol to allow precipitation of polisaccharides. The flow rate was 1.2 ml/min. Analysis were carried out with 20 μl of sample.

Chromatographic conditions (Vratny, P., 1983):

Equipment for liquid chromatography manufactured by Laboratorní Prístroje (Prague, Rep. Czech) was used in the experiments. The instrument

consisted of a high-pressure HPP 4001 linear pump, an LCI-02 septum injection valve and an LCI-20 stop-flow valve with and RIDK 101 refractometric detector. A Supelcosil LC-18 column, 25 cm x 4.6 mm (5 μm packing) and a W/guard (Supelguard LC-18, 2 cm x 4.6 mm, 5 μm packing) columns were used.

To preparate the sample, add 1 g or starch to 10 ml of 0.2 M trifluoroacetic acid. Heat at 100 ^{o}C for one hour. Cool, then add 90 ml or absolute ethanol and store at -70 ^{o}C to allow precipitaded saccharides to settle. Decant the supernatant and remove all remaining traces of ethanol by evaporation under nitrogen (ethanol interferes with retention of the larger, DP5, oligomers). Redisolve the dried residue in 10 ml of deionized water and filter through a 0.45 μm filter. Store frozen samples until analysis. Glucose oligomers were obtain from corn syrup by precipitating them with ethanol as described above.

RESULTS

Oligosaccharides have been separated by a reversed-phase liquid chromatography method, in a very easy, rapid and reproducible manner, resolving the different compounds according to the respective DP (11,12). After injection of a polysaccharide-free sample there is disturbance of the detection baseline a which has been identified agreeing with Vratny as a peak of solvent. The monomeric compounds of galactose are detected from the second peak.

The separation of oligosaccharides has been achieved by optimizing the column temperature at 30°C, at this temperature the rate of interconversion between the α- and β-anomers of polysaccharides increase the presence of double peaks.

The standard calibration curve of galactose and sucrose units is showed in figure 1. Sugars showed a linear response in the concentration range 20 pmol-12000 nmol. The detection limit obtained by this method was about 5 pmol.

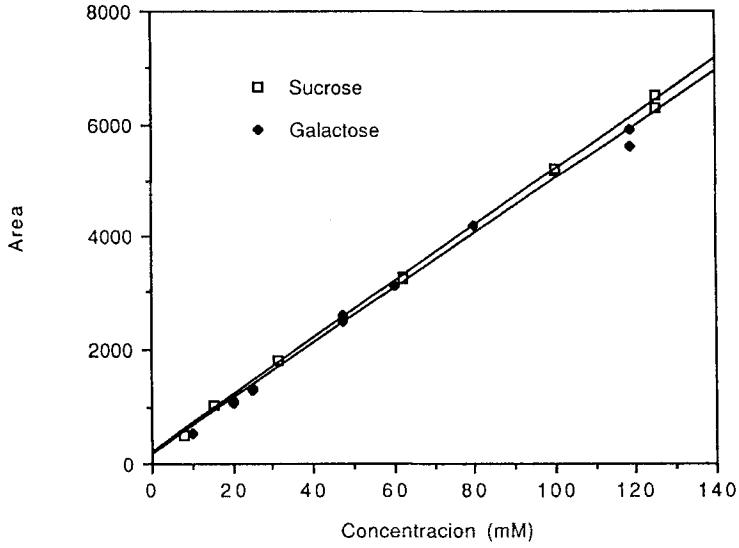

FIGURE 1: Standard calibration curve of galactose and sucrose units. Both of them are very similar, and showed a linear response in the concentration range 20 pmol-1200 nmol.

In table I appears the calculated concentration of degrees of polymerization corresponding to samples hydrolysed for 10 hours, from thallus, apical tips, unpurified agar from *Gelidium*, Bacto agar, agar and agarose from *Gelidium*. High concentrations of monomers and DP12 are observed, and couple monomers but no odd chain oligomers are detected.

Figure 2 shows a typical chromatogram obtained from maltooligosaccharides. The chromatographic retention times for the oligomers DP 4 to DP 9 were from 1.5 until 7 minutes.

Chemical hydrolysis for b-C1-C4 link is easier than a-C1-C3 (2), by this reason oligosaccharides are detected. In figure 3, appears the concentration at

TABLE 1

Concentration (mM) of Degrees of Polymerization Corresponding to Samples, after 10 Hours of Hydrolysis, from Agarosa from *Gelidium*, Agar, Bacto Agar, Tallus, Apical Tips and Unpurified Agar (Whole Plant). Retention Times Are Expresed + S.E.M., from at least 4 Different Assays.

DP	T.Ret.(min)	Agarose	Agar (Hispanagar)	Bacto Agar	Agar (Tallus)	Agar (Apical)	Agar (Whole plant)
1	1.45±0.03	988.77	1012.19	1100.78	1064.75	1061.27	1108.15
2	1.83±0.02	3.27	2.50	13.60	2.99	6.61	4.03
3	1.92±0.01						
3	2.09±0.12	13.60	10.00	30.91	10.00	6.89	32.09
4	2.28±0.04						
4	2.39±0.01	8.03	6.62	7.76	9.32	49.57	21.99
6	3.51±0.05						
6	3.83±0.05	2.90	8.50	2.89	2.03	2.88	60.73
8	5.81±0.20						
8	6.05±0.20	34.99	12.88	9.46	14.34	10.80	7.95
10	10.97±0.75						
10	12.57±0.18	–	1.33	–	0.70	–	–
12	12.98±0.30						
12	15.24±0.45	1430.45	1093.36	1005.61	1004.37	1452.91	1398.88

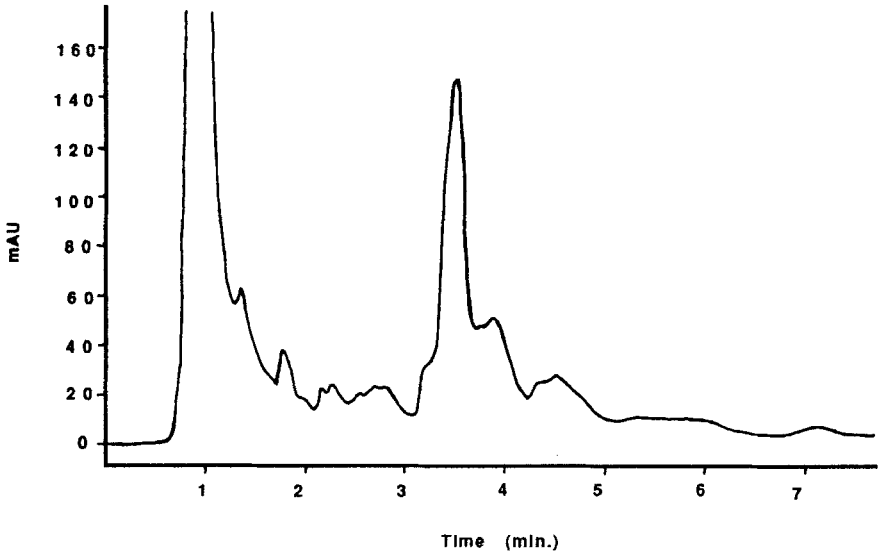

FIGURE 2: Chomatogram from maltooligosaccharides. We can see the typical double peaks for each respective degree of polymerization (DP). Retention times are expressed in table 1.

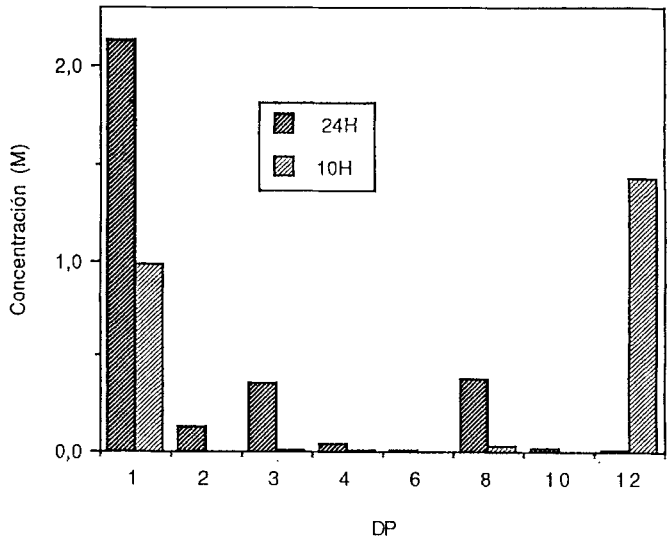

FIGURE 3: Concentration of the different DP after 10 and 24 hours of acid hydrolysis. Larger oligosaccharides are present at the minor hydrolysis time. Mainly oligosaccharides couples monomers are obtained in both of them, 10 and 24 hours.

different degree of polimerization after 10 and 24 hours of acidic hydrolysis. The appearance of that larger oligosaccharides is formed early in the analisis.

Each oligomer elution time has been calculated using five different samples. The variation coefficient (C.V.) was less than 5%. The sensitivity of the technique was similar than those reported by other authors (4).

DISCUSSION

The main effort was directed towards an exhaustive study of separation and quantification of agar by HPLC technique. Using the HPLC procedure described, we resolved DP1 to DP12 galactose oligomers without pre- or post-derivation. The used column provides several advantages, greater efficiency, faster separations and better resolution of the larger oligomers than other methods (11).

Agarose links are very resistant to enzymatic hydrolysis by bacteriane α- and β-agarases, so, acidic hydrolysis has been used to obtain the structural monomerics. To avoid interferences with retention times of oligomers larger than DP5 the latter addition of ethanol, was eliminated. UV detectors are very sensitive at low wavelengths (190 nm) and remain unaffected by temperature and pressure variations. In this method two additional important advantages are the use of a pressure-stable stationary phase, and a neutral and non-toxic cheap aqueous eluent.

In summary, our method allows the separation and the quantitative determination of the DP1-DP12 oligomers from galactose agar-type polysacharides. Thus, molecular size can be deduce and subsequent rheological properties could be established.

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