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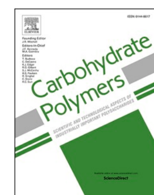

Estimation of alginate purity and M/G ratio by methanolysis coupled with anion exchange chromatography

Hylenne Bojorges [a], Amparo López-Rubio [a,b], María José Fabra [a,b], Antonio Martínez-Abad [a,b,*]

[a] Food Safety and Preservation Department, Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain
[b] Interdisciplinary Platform for Sustainable Plastics towards a Circular Economy- Spanish National Research Council (SusPlast-CSIC), Madrid, Spain

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ABSTRACT

Alginates are industrially relevant polysaccharides widely used in the food and biomedical industries for their excellent gelling properties. The growing emphasis on the valorization of marine resources has evidenced the need for alternative methods for the determination of both alginate content and the M/G ratio. This study describes the application of acid methanolysis and separation by anion exchange chromatography. Five samples, including alginates extracted from *Saccharina latissima*, *Ascophyllum nodosum*, a certified standard, and two polyuronates (Poly-M and Poly-G), were analysed for their M/G ratio and alginate content at different treatment conditions, and compared with other conventionally used or reference methods (NMR, FTIR, and colorimetric methods). Quantitative estimation of alginate was relatively accurate at optimum conditions (4 h at 100 °C), as compared with the certified standard or with other colorimetric methods. M/G ratios were not significantly different from those determined after the reference method (^{1}H NMR) or compared to FTIR protocols. The results evidence that methanolysis may be applied to simultaneously estimate the purity and M/G ratio of alginate-rich samples in a single analysis.

1. Introduction

Alginates are industrially relevant polysaccharides extracted from brown seaweed (Phaeophyceae) and widely used as thickeners, stabilisers and gelling agents in the food, pharma, or cosmetic industries. Alginates can be described as copolymers of (1–4) linked β-D-mannuronic acid (ManA or M) and α-L-guluronic acid (GulA or G). These polysaccharides are typically described by their M/G ratio, molecular weight and distribution of M and G units, as M, MG or G blocks along the chain, as these parameters highly determine the biological and physical properties of alginate (Haug, Myklestad, Larsen, & Smidsrød, 1967). The alginate contents, as well as its extractability or recalcitrance within the cell wall architecture, its molecular weight, and sequential structure, all greatly vary, depending on the brown algae species, season, growing stage, and geographic location (Haug, Larsen, & Smidsrød, 1974; Indergaard, Skjåk-Bræk, & Jensen, 1990). A growing focus towards a "blue bioeconomy" and the valorisation of ocean resources has prompted research and industrial efforts to deeper study the composition and potential applications of brown algae, evidencing the lack of straightforward methods for the determination of both alginate content and M/G ratio.

The estimation of alginate content is usually based on colorimetric methods, among which many different possibilities have been proposed and modified through many years and are still used by different authors. These methods mostly involve the formation of a coloured complex in the presence of uronic acids with reagents such as resorcinol, carbazole, *m*-hydroxydiphenyl, etc. (Kumar & Kumar, 2017). The most widely used chemometric method for the quantitative determination of uronic acids is the method developed by Blumenkrantz and Asboe-Hansen (1973). Although very suitable for pectin or other plant samples, the original method was not tested or developed for its application on alginates and may present certain limitations. A further modification of this method, involving the addition of sulfamate to *m*-hydroxydiphenyl, was described by Fillissetti-Cozzi and Carpita, which aimed at reducing interference from neutral sugars, and was applied to both polygalacturonates as well as to alginic acid (Filisetti-Cozzi & Carpita, 1991). There is, however, no generally accepted or reference method for alginate analysis.

The reference method for the determination of the M/G ratio is liquid-state nuclear magnetic resonance (NMR) spectroscopy, based on extensive work performed in the late 70s and early 80s (ASTM F2259-10, 2012; Grasdalen, 1983; Grasdalen, Larsen, & Smidsrød, 1979).

* Corresponding author at: Food Safety and Preservation Department, Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain.
E-mail address: conaba@iata.csic.es (A. Martínez-Abad).

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NMR not only gives an estimate of the overall M/G ratio, but also information about the block distribution, which also plays a crucial role in the rheological behaviour. This analysis is, however, complex, due to the need of a pre-hydrolysis step to enhance solubility and reduce viscosity. NMR must also be done at increased temperature (80–90 °C) for resolved peaks allowing integration. Solid-state ^{13}C NMR is another powerful non-destructive method to determine the alginate's M/G ratio. However, this technique may exhibit lower signal sensitivity than liquid-state NMR, making it difficult to obtain information about M- and G-blocks (Ishii, Yesinowski, & Tycko, 2001; Salomonsen, Jensen, Larsen, Steuernagel, & Engelsen, 2009; Shimba et al., 2004). In addition, using this technique often requires a considerably large calibration model or prior knowledge of the M/G ratio in the calibration samples (Salomonsen et al., 2009). Therefore, it implies the need to use advanced analysis and processing techniques to calculate the M/G ratio, which can be complex and require specialized expertise, increasing analysis time and driving this technique less attractive in terms of efficiency.

Owed to the industrial need of estimating M/G ratio in a rapid and non-destructive manner, vibrational spectroscopy, e.g. infrared (IR), Raman and near infrared (NIR) spectroscopy have also been optimized in combination with chemometrics and proven useful for quantitative estimation of the M/G ratio of alginates (Jensen, Larsen, & Engelsen, 2015; Salomonsen, Jensen, Stenbæk, & Engelsen, 2008). These approaches are, however, designed for relatively pure alginate samples, not giving a quantitative estimation of alginate content, and also other compounds might overlap with the signals of interest. Other techniques explored for determining alginate's M/G ratio are circular dichroism (CD). However, CD has some disadvantages, such as lower sensitivity compared to other analytical methods, such as NMR, which could affect obtaining high-quality spectra and will depend on the purity and homogeneity of the alginate sample (Morris, Rees, & Thom, 1980). Likewise, interpreting CD spectra can be challenging, especially when dealing with mixtures of different monomer compositions or conformational variations in the alginate structure (Donati et al., 2003, 2005).

Chromatographic methods allowing separation of the different monomeric units for their quantification would, in principle, allow for direct estimation of both alginate content and M/G ratio, regardless of the purity or the presence of other compounds. The hydrolysis step to transform the polysaccharide into their monomers is challenging, because of the acid lability of alginate monomers, prone to degradation. For a quantitative determination of alginate monomers, uronate linkages must be quantitatively hydrolysed, while preserving the native structure of the monomers to be chromatographically separated. Previous studies on alginate samples using trifluoracetic acid (TFA) or formic acid, for example, have evidenced the narrow balance between quantitative scission of all glycosidic bonds and degradation of the monomeric units (Chandía, Matsuhiro, Mejías, & Moenne, 2004; Lu et al., 2015). Methanolysis is performed in the absence of water with anhydrous acidic methanol and has been applied to plant hemicelluloses presenting significant quantities of uronic acids, such as glucuronoxylans, as these conditions result in higher selectivity in the recovery of the uronic acids compared to TFA or sulfuric acid (Saeman) hydrolysis (Bertaud, Sundberg, & Holmbom, 2002; Willför et al., 2009). All this raised a hypothesis about whether methanolysis could be an alternative method for carbohydrate analysis in alginate-rich samples and provide results comparable to those of current NMR or chemometric methods. Therefore, this study aimed at exploring the application of acid methanolysis followed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for the quantitative determination of the carbohydrate composition, alginate purity, and M/G ratio of alginate-rich samples, simultaneously. Different methanolysis conditions were applied based on previous works on plant polysaccharides and the results are critically discussed and compared to colorimetric methods, IR spectroscopy, or NMR.

2. Materials and methods

2.1. Alginate samples used in the study

Five alginate-rich samples were used in this study. A certified reference sodium alginate (Ref. PHR1471; Lot#: LRAB5524; referred to as REF) with a reported purity of 87.6 ± 0.2 % was purchased from Merck. Poly-guluronic acid (Poly-G) and poly-mannuronic acid (Poly-M) were supplied by Carbosynth, Biosynth (UK). Furthermore, two alginate samples were produced in the lab from the brown seaweeds *Ascophyllum nodosum* (ASC) and *Saccharina latissima* (SAC). Both seaweeds were harvested in February 2017 and September 2018, respectively, and kindly supplied as a dry powder by Porto-Muiños S.L. (Cerceda, A Coruña, Spain). The algae were submitted to the acid-alkaline treatment conventionally used to extract sodium alginate at industrial scale as described in Fig. S1 of the suppl. Material. D_2O (≥99.9 atom% D), TTHA (Triethylenetetramine-N,N,N′,N″,N‴,N‴-hexaacetic acid, T7633, Sigma, Japan), and 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium (TSP-d4, ≥98 atom% D) were purchased from Sigma-Aldrich.

2.2. Proximate composition

The total nitrogen content of the samples was measured using an Elemental Analyzer Rapid N Exceed (Paralab S.L., Spain). Around 250 mg of alginate samples were compressed into pellets and subjected to analysis using the Dumas method, which involves combusting the sample and detecting the resulting release of N_2 gas (Wiles et al., 1998). The total protein content was calculated by multiplying a factor of 5 (Angell, Mata, de Nys, & Paul, 2016). The ash content was estimated by calcination in a muffle furnace, following the standard method (AOAC, 1990). Approximately 3 g of freeze-dried alginate and alginate-based fractions powder were calcined at 500 °C. The lipid content of the samples was quantified through a gravimetric analysis using the Soxhlet method (Method 920.39; AOAC, 1990). These analyses were performed in triplicate All data were expressed as a percentage of dry weight (DW).

2.3. Methanolysis and HPAEC-PAD analysis

The carbohydrate composition, including neutral sugars and uronic acids of the alginates and poly-uronates was determined after acid methanolysis and separation by HPAEC-PAD. Freeze-dried samples (2 mg) were incubated with 1 mL of 2 M hydrochloric acid (HCl) in dry methanol at different times at 80 °C or 100 °C. The samples were neutralized with pyridine, dried under a stream of air, and further hydrolysed with 2 M trifluoroacetic acid (TFA) at 120 °C for 40 min. The samples were again dried under a stream of air, re-dissolved in ultrapure water, and filtered (0.2 μm). The monosaccharides were analysed using HPAEC-PAD with an ICS-6000 system (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex). Neutral monosaccharides were separated at 30 °C isocratically with 8 mM NaOH for 15 min after a conditioning step before each run of 7 min with 250 mM NaOH and 70 mM sodium acetate (NaAc) and 6 min equilibration time before injection. Uronic acids were separated at 30 °C with 30 mM NaOH and a 100–180 mM NaAc gradient over 20 min. The eluents were filtered and degassed with helium and kept under helium pressure during the analysis. Control samples of known concentrations of mixtures of fucose (Fuc), rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man), galacturonic acid (GalA), glucuronic acid (GlcA), all purchased at Merck, as well as mannuronic acid (ManA) and guluronic acid (GulA), purchased at Carbosynth, Biosynth (UK), were used for calibration. A typical chromatogram of uronic acids from an alginate sample is shown in Fig. S2. The M/G ratio was calculated by dividing the mannuronic acid concentration between the guluronic acid concentration. All determinations were carried out at least in triplicate.

2.4. Nuclear magnetic resonance (NMR) spectroscopy

NMR analysis was carried out following the method described by Jensen et al. (2015). ^{1}H liquid-state NMR measurements were performed on a Bruker AV400 spectrometer operating at a frequency of 400 MHz for ^{1}H nuclei using a BBI ATM 1H/BB inverse multinuclear probe (5 mm). Prior to analysis, the samples were depolymerized by a mild partial hydrolysis. 0.1 % (*w/v*) sodium alginate solutions were adjusted to 5.6 using 0.1 M HCl and heated at 100 °C for 1 h. After cooling to room temperature, the pH was re-adjusted to 3.8 with 0.1 M HCl and incubated at 100 °C for 30 min. Subsequently, samples were neutralized (pH 7) with 0.1 M NaOH and freeze-dried overnight. The samples were dissolved in 5 mL of D_2O (99.9 %) and freeze-dried again in order to minimize the presence of non-deuterated water. 10 mg of the samples were then dissolved in 1 mL of D_2O (99.9 %) and 700 μL of the solution were added into NMR tubes along with 20 μL of the TTHA solution. The TTHA was used as a chelating agent to prevent traces of divalent cations from interacting with the alginates. The NMR spectra were recorded at 80 °C with a 30° pulse angle, relaxation delay 2 s, and acquisition time of 4.096 s. 128 scans were accumulated for signal averaging. The chemical shifts and calculations followed to calculate the M/G ratio and the frequencies of diads and triads of uronic blocks are described in section 1.3 of the supplementary material. The obtained spectra were processed using Mnova NMR software (Mestrelab Research, Spain).

2.5. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were collected on a Cary 630 FTIR spectrometer (Agilent, USA) using the attenuated total reflectance (ATR) sampling method. The IR spectra were recorded at 4 cm^{-1} resolution in a wavelength range between 400 and 4000 cm^{-1} and averaging a minimum of 64 scans. Spectra were analysed with Origin pro 2019 software (OriginLab Corporation, USA). All the samples were prepared in the form of KBr pellets and alginic acid was used as standard. Triplicate KBr pellets were employed to estimate the sample's M/G ratio, by calculating the mean value of absorbance observed at the relevant bands. The M/G ratio of the samples was estimated according to the ratio of the intensities of the bands assigned specifically to mannuronic acid (M) and guluronic acid (G) at approximately 1030 cm^{-1} and 1080 cm^{-1}, respectively (Sakugawa, Ikeda, Takemura, & Ono, 2004). The baseline method was employed to determine the transmittance at the above wavenumbers in the average IR spectra (Rochas, Lahaye, & Yaphe, 1986). Then, the M/G ratio was calculated by comparing the absorbance at the corresponding wavenumbers using the following equation:

$$A = log T_b / T_p \quad (1)$$

where A stands for Absorbance and T for transmittance for baseline (b) and peak (p).

2.6. Size exclusion chromatography (SEC-MALLS)

The molar mass distribution of the samples was determined (Bojorges et al., 2023) using size exclusion chromatography (SECcurity 1260, Polymer Standard Services, Mainz, Germany coupled to a multiple-angle laser light scattering detector -MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York), and a refractive index detector (SECcurity 1260, Polymer Standard Services, Mainz, Germany) thermostated at 45 °C. The eluent was 0.1 M $NaNO_3$ containing 5 mM NaN_3 at a 0.6 mL/min flow rate. Samples were dissolved in the eluent phase at 1 mg/mL concentration. Sample injections of 100 μL were injected into a combined column set-up with a SUPREMA pre-column, a SUPREMA 1000 Å, and two SUPREMA 3000 Å analytical columns (PSS, Mainz, Germany). A comprehensive description of the procedure, calibration, and parameter settings can be found in section 1.4 of the Supplementary Material.

2.7. Statistical analysis

The statistical analysis of experimental data was performed using IBM SPSS Statistics software (version 23, IBM Corp., USA). One-way analysis of variance (ANOVA) was done to determine if differences between sample means were significant at a significance level of $p < 0.05$. The mean tests were performed using the Least Significant Difference (LSD) Test.

3. Results

3.1. Gross composition and molecular weight of the tested alginates

In order to indirectly assess the purity of the five alginate samples and the nature of other components other than alginate, the samples were first analysed on their protein and ash content (Table 1). The very low content of protein and lipids (not significantly present in any of the samples) reflects the high purity of the tested alginate samples. Nevertheless, as all samples were isolated as sodium alginate salts, and other salts and minerals can also be co-extracted and enriched in the final alginate fractions, significant ash content was expected to be found in all samples. This ranged between 20 and 25 %, with the partially digested poly-G and poly-M having significantly higher amounts. Alginates produced from *A. nodosum* and *S. latissima* following conventional alginate extraction methods presented minor amounts of protein, while the rest of commercially available purified samples only showed negligible or trace amounts. The downstream purification processes or digestion steps to further purify these commercial samples (REF, POLY-M, and POLY-G) might also explain their significantly lower molecular weight, as compared to ASC and SAC samples. It should be emphasized that all samples' polydispersity was >1, showing high Mw dispersity, particularly in the REF sample.

Superscripts with different letters in the same row denote significant differences between samples (p < 0.05). The abbreviations Mw, Mn, and Đ denote the molecular weight, number molecular weight, and polydispersity, respectively.

3.2. Methanolysis of alginates

In order to investigate the optimum conditions for the methanolysis of alginates into their monomeric units (M and G), samples were subjected to acid methanolysis at 100 °C or 80 °C, and aliquots were taken after 3, 4, 5 and 6 h or after 14, 16 and 18 h, respectively. Fig. 1 shows the total uronic acid content and M/G ratio of the tested alginates under increasing methanolysis time and under the two different temperature conditions. The range of time and temperature conditions was selected based on conditions already optimized for uronic acid-rich plant polysaccharides (Bertaud et al., 2002; Martínez-Abad, Giummarella, Lawoko, & Vilaplana, 2018; Martínez-Abad, Jiménez-Quero, & Wohlert, 2020; Willför et al., 2009). The range of aggressiveness in the treatments allowed observing two opposite tendencies with increasing methanolysis time, showing a balance between achieving adequate hydrolysis and avoiding further degradation. First, a slight increase in uronic acid yields observed in some samples may be attributed to enhanced

Table 1
Gross composition and molecular weight of the alginate samples.

	ASC	SAC	REF	POLY-M	POLY-G
Protein (%)	3.3 ± 0.3^{b,c}	2.9 ± 0.3^{b}	<0.1^{a}	<0.1^{a}	<0.1^{a}
Ash (%)	19.4 ± 0.1^{a}	21.3 ± 0.0^{c}	20.0 ± 0.0^{b}	22.6 ± 0.5^{d}	25.5 ± 0.3^{e}
Lipids (%)	<0.1	<0.1	<0.1	<0.1	<0.1
Mw (kDa)	241.90	169.40	68.34	4.08	3.79
Mn (kDa)	157.40	74.74	19.24	3.95	3.62
Đ	1.54	2.27	3.55	1.03	1.04

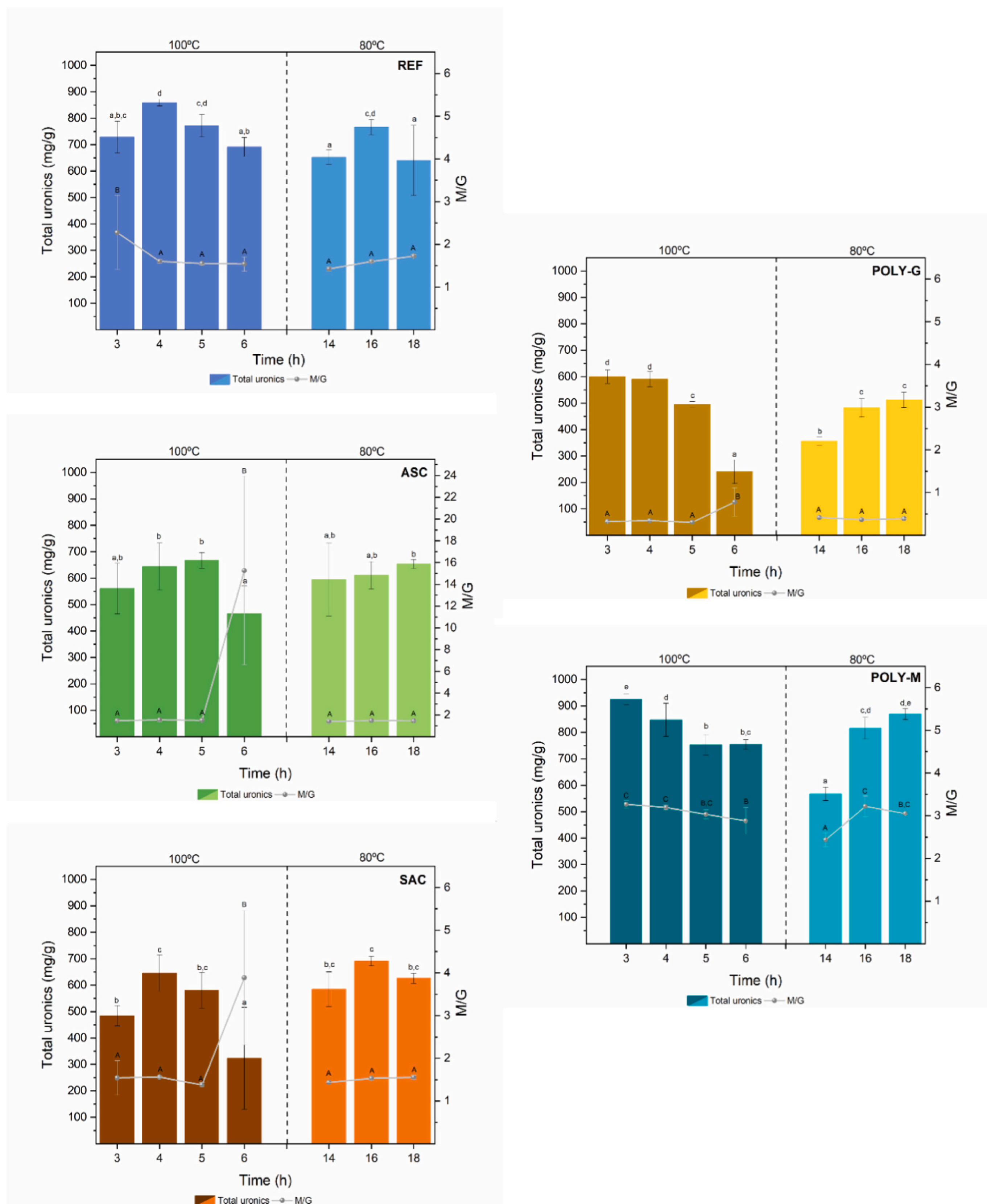

Fig. 1. Total uronic acid content (bars) and M/G ratio (lines) of the tested samples determined by HPAEC-PAD after methanolysis (3–6 h; 100 °C or 14-18 h 80 °C, separated by a dashed line). Total uronic acid content was calculated as the sum of guluronic, mannuronic, and glucuronic acids. Glucuronic acid was only present in minor amounts (<15 mg/g) and galacturonic acid was not detected in any of the samples tested. Different lowercase letters indicate significant differences ($p < 0.05$) in the total uronics content. Different uppercase letters indicate significant differences ($p < 0.05$) in M/G ratio.

efficiency in the cleavage of glycosidic bonds. At prolonged times, however, a decrease in uronic acid yields and a significant increase in the M/G ratio both indicate degradation of the alginate monomers, with proclivity for the more labile G units. As commented in the introduction, chromatographic determination of alginate monomers after acid treatment is challenging. First, a complete scission of all glycosidic linkages has to take place. Here, previous studies have ascertained a higher lability of G-M linkages (Holtan, Zhang, & Strand, 2006). Furthermore, acid conditions can degrade the alginate monomers, M and G, which would reduce their recovery through chromatographic separation. Previous studies have also noted reduced recovery of G units at acidic conditions, which clearly impacts the M/G ratio (Lu et al., 2015).

When methanolysis was performed at 100 °C, alginate samples obtained from *A. nodosum* and *S. latissima* following the conventional industrial extraction and purification, showed increasing uronic acid content from 3 h to its maximum at 4 or 5 h methanolysis, although differences in this 4–5 h range were not significant. 6 h methanolysis resulted in a decrease in uronic acid yield and a significant increase in the M/G ratio, pointing at degradation. The reference alginate showed the highest response in uronic acid content after 4 h methanolysis at 100 °C, with values around 850 mg/g. These values denote high accuracy, in accordance with the certified specifications (876 mg/g; see section 2.1). For the digested and purified Poly-M and Poly-G alginate fractions, with molecular weight < 5 kDa, highest yields were observed with only 3 h methanolysis at 100 °C. This might be explained by the significantly lower molecular weight of these commercial uronate samples. Very low molecular weight samples, however, might not be the target of routine analysis, so 4 h could be selected as the best balance between recovery of alginate monomers and degradation. Typical chromatograms for the 5 tested samples are shown in Fig. S4.

When methanolysis was performed at 80 °C but longer times (14–18 h), maximum yields were observed for 16 h or 18 h, depending on the sample. For the higher molecular weight alginates (SAC and ASC) yields were comparable to the ones observed after 4 h at 100 °C, at all selected time points (14–18 h), where 16 h was the optimum treatment time at 80 °C for the more labile REF and Poly-uronate samples. These results illustrate the complexity of accurately determining alginate and M/G ratio depending on the origin and structural features of the samples tested, owed to a difficult balance between cleavage and degradation. Methanolysis at 80 °C for 16 h did not show any significant differences in the maximum recovery of monomers compared to 100 °C for 4 h, suggesting both approaches would be equally useful, so the latter was selected for further comparison with other methods. The total uronic acid content and M/G ratio of the samples at these conditions is summarized in Fig. S5.

3.3. Carbohydrate composition

A chromatographic approach not only allows for the estimation of alginate monomers, but also enables identification and estimation of the whole carbohydrate composition, including other neutral sugars or polysaccharides present in the sample. This might be of special interest in less pure alginate samples or brown seaweed samples including other interesting polysaccharides, such as fucans or fucoidan, laminaran, etc. Although all neutral (Fuc, Ara, Glc, Gal, Rha, Man, Xyl, etc) and acid (GalA, GlcA, ManA, and GulA) monomers may be separated in a longer single run, we found it practical to create two separate gradients to check for either neutral sugars or uronic acids (section 2.3). Table 2 summarizes the carbohydrate composition of the five alginate samples. The carbohydrate composition confirms the relatively high purity of all samples, consisting mainly of alginate GulA and ManA units, and only very minor amounts of Fuc and Xyl were found in SAC and ASC, probably arising from fucoidan.

In order to compare the results from methanolysis coupled with HPAEC-PAC with other typically used methods for the determination of alginate, their uronic acid content was further analysed following the

Table 2
Carbohydrate composition and uronic acid content of the samples (dry wt%).

	ASC	SAC	REF	POLYM	POLYG
Fucose[1]	2.8 ± 1.2 [b,c]	1.1 ± 0.7[b]	<0.2 [a]	<0.2 [a]	<0.2 [a]
Xylose[1]	1.9 ± 0.7[b,c]	0.2 ± 0.2[a]	1.1 ± 0.1[a,b]	<0.2 [a]	<0.2 [a]
GulA[1]	26.6 ± 4.5[b,c]	24.2 ± 3.3[b]	30.2 ± 2.2[c]	18.6 ± 0.9[a]	38.1 ± 1.0[d]
GlcA[1]	0.3 ± 0.0[a]	0.2 ± 0.0[a]	<0.2[a]	<0.2[a]	<0.2[a]
ManA[1]	39.7 ± 0.4[b]	33.5 ± 5.0[b]	46.9 ± 3.9[c]	56.6 ± 4.0[d]	11.5 ± 0.3[a]
Uronic acids[1]	66.6 ± 4.9[b]	57.7 ± 8.3[b]	77.1 ± 6.1[c]	75.2 ± 4.9[c]	49.6 ± 1.0[a]
Uronic acids[2]	20.0 ± 3.9[b]	32.2 ± 9.9[b]	41.5 ± 2.8[c]	31.9 ± 10.2[c]	23.3 ± 2.1[a]
Uronic acids[3]	64.7 ± 2.8[b]	85.7 ± 6.2[b]	82.7 ± 1.1[c]	75.6 ± 3.5[c]	64.9 ± 5.0[a]

Gal, Glc, Ara, Man, Rha, GalA were not detected or only in trace amounts (<2 mg/g).
Values with different letters in the same line are significantly different ($p \leq 0.05$).
[1] determined as the sum of GulA, ManA and GlcA by the methanolysis/HPAEC-PAD suggested in this work.
[2] determined colorimetrically after Blumenkrantz and Asboe-Hansen (1973).
[3] determined colorimetrically after Filisetti-Cozzi and Carpita (1991).

colorimetric method described by Blumenkrantz and Asboe-Hansen, later modified by Filisetti-Cozzi and Carpita, using GalA as standard for the calibration curve. The former research study clearly showed a drastic difference in the colour response depending on the monosaccharide used for calibration, e.g. mannuronic acid compared to glucuronic or galacturonic acid (Blumenkrantz & Asboe-Hansen, 1973). We further confirmed that also GulA gave a significantly lower response, following both colorimetric methods (Fig. S6 and S7). The low colour response of M and G units may result in underestimation of alginate content in some cases. This was especially patent when applying the Blumentkrantz method (Blumenkrantz & Asboe-Hansen, 1973), which although frequently reported to determine uronic content in alginates, was not originally designed for alginate samples, thus showing significant discrepancies with the other tested methods (Table 2). However, if ManA or GulA were used for calibration, results were overestimated, most probably due to the higher lability of these monomers to concentrated sulfuric acid (Table S2). The results obtained from both the methanolysis/HPAEC-PAD method and the colorimetric method proposed by Filissetti-Cozzi, and Carpita are comparable for ASC, REF and POLY-M samples, but differ in SAC and POLY-G samples. In this case, we might hypothesize that the HPAEC-PAD method underestimates high G content samples. Although both analytical methods have their inherent limitations, it is important to note that the HPAEC-PAD method provides a more comprehensive mass balance than the colorimetric methods, considering factors such as ash and protein content, and aligns with the reference M/G ratio obtained by NMR.

3.4. M/G ratio

As outlined in the introduction, the estimation of the M/G ratio in alginate samples is of utmost importance, as this ratio has a strong influence in both their biological and technological properties. NMR gives not only an estimate on the overall M/G ratio, but also crucial information as to the block distribution of M and G units. The suggested method does not aim to replace or improve the reference method, but explores a potential alternative for a simultaneous estimation of the M/G ratio, purity and carbpohydrate composition of alginate-rich samples. This might be interesting in samples of lesser alginate purity, where information on total alginate and other polysaccharide content might be more useful than a detailed alginate characterization. Table 3

Table 3
Mannuronate (M)/guluronate (G) ratio of the alginate and poly-uronate samples tested in this study through different methods.

Sample	M/G ratio		
	NMR	HPAEC-PAD	ATR-FTIR
ASC	$1.5 \pm 0.1^{a,B,C}$	$1.5 \pm 0.1^{a,B}$	$1.7 \pm 0.1^{a,B}$
SAC	$1.4 \pm 0.0^{a,B}$	$1.4 \pm 0.1^{a,B}$	$1.5 \pm 0.1^{a,A}$
REF	$1.7 \pm 0.1^{a,C}$	$1.6 \pm 0.1^{a,B}$	$1.9 \pm 0.1^{a,C}$
POLYM	$4.0 \pm 0.0^{c,D}$	$3.0 \pm 0.2^{b,C}$	$2.5 \pm 0.1^{a,D}$
POLYG	$0.2 \pm 0.0^{a,A}$	$0.3 \pm 0.0^{b,A}$	$1.3 \pm 0.0^{c,A}$

summarizes the M/G ratio of the five tested samples resulting from methanolysis/HPAEC-PAD under the selected conditions (4 h at 100 °C), compared with the results from the reference method, liquid-state ^{1}H NMR, and with estimation by ATR-FTIR. The frequency of GulA and ManA, their diads, triads (Table S1) and the ^{1}H NMR spectra of all tested samples (Fig. S8) are available in section 2.3 of the supplementary material. For all methods, the hierarchical order is the same, with M/G ratio of REF > ASC > SAC for the alginate samples, while Poly-M and Poly-G had the highest and lowest M/G ratio, respectively. This indicates that either of the non-reference methods would be suitable to compare among samples. The M/G ratio values given by all three different methods were not significantly different for the reference and higher molecular weight alginates. However, significant differences were found for uronate samples, with higher values of Poly-M with NMR and higher Poly-G values through FTIR analysis, as compared with the other two methods. Nevertheless, it seems logical that differences become more pronounced when the difference between M and G is greater or values are closer to threshold or baseline detection values. This withstanding, the present results show that methanolysis/HPAEC-PAD may be a suitable method to evaluate differences in the M/G ratios of different alginate samples, with quite comparable results to the reference or other spectrometric methods.

Results are expressed as mean values plus standard deviation of $n = 6$ from three independent analyses performed in duplicate. Different lowercase letters in the same row indicate significant differences ($p < 0.05$) for methods. Different uppercase letters in the same column indicate significant differences ($p < 0.05$) for samples.

4. Conclusions

Methanolysis followed by separation by HPAEC-PAD was investigated as a means to simultaneously analyse the carbohydrate composition, alginate purity, and M/G of different alginate and poly-uronate samples. Five samples, including alginates extracted from *S. latissima, A. nodosum*, a certified standard and two poly-uronates were tested, as to encompass a relevant application range. The selectivity of methanolysis to cleave glycosidic bonds without incurring in major degradation, already used for uronic-rich plant polysaccharides, was successfully applied with optimal response at methanolysis conditions of 100 °C for 4 h. The methanolysis/HPAEC-PAD gave plausible results, considering the lack of protein, lipids, and contribution of ashes in the samples. As for the M/G ratio, the same tendencies and differences were observed for all samples, following estimation by either NMR, methanolysis, or ATR-FTIR. M/G ratios were not significantly different in the alginate samples when estimated by methanolysis compared to liquid ^{1}H NMR (reference method), except for low molecular weight uronate samples. The method does not involve a pre-hydrolysis step or expensive equipment, such as with NMR, and offers the opportunity of estimating alginate content, M/G ratio, and carbohydrate composition in a single analysis. This method may therefore constitute a useful analytical tool for the characterization of alginate-rich samples of different purity and origin.

CRediT authorship contribution statement

Hylenne Bojorges: Data curation, Software, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Amparo López-Rubio:** Resources, Funding acquisition, Data curation, Writing – review & editing, Project administration, Funding acquisition. **María José Fabra:** Validation, Resources, Writing – review & editing, Supervision. **Antonio Martínez-Abad:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2023.121285.

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