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Impact of hydrolysis conditions on the detection of mannuronic to guluronic acid ratio in alginate and its derivatives

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ABSTRACT

Alginate is a linear and acidic polysaccharide, composed of (1 → 4) linked β-D-mannuronic acid (ManA) and α-L-guluronic acid (GulA). The ratio of ManA to GulA (*M*/*G*) is one of the most important factors for the application of alginate and its derivatives in various areas. In this work, a robust and accurate method was developed to analyze *M*/*G* using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The impact of hydrolysis conditions on the release patterns of ManA and GulA from alginate and its derivatives was investigated. The release patterns of ManA and GulA need to be considered separately to obtain an accurate *M*/*G*. Several hydrolysis conditions were established that released ManA and GulA completely and maintained these saccharide residues intact. The proper *M*/*G* of alginates from different sources and its derivatives could then be calculated by integration of the corresponding ManA and GulA peaks.

1. Introduction

Alginate is a linear copolymer composed of (1 → 4) linked β-D-mannuronic acid (ManA) and α-L-guluronic acid (GulA). Along its linear chain, there are homo-oligomeric regions of mannuronic acid (M-blocks) and of guluronic acid (G-blocks) as well as hetero-oligomeric regions (MG-blocks) (Haung, Larsen, & Smidsrod, 1966; Haug, Larsen, Smidsrod, & Painter, 1969). Alginate is a natural polysaccharide occurring in the cell wall of brown algae and also formed as a biofilm by some bacteria (Evans & Linker, 1973; Rehm & Valla, 1997; Coster, Stewart, & Greenberg, 1999). The most commercially important alginates are extracted from brown seaweeds and are widely used in different industries as gelling and texturizing agents, stabilizers, drug carriers and excipients (Murata, Sasaki, Miyamoto, & Kawashima, 2000; Stevens, Qandilo, Langer, & Shastri, 2004; Goh, Heng, & Chen, 2012). In addition to molecular weight (M_w), the ratio of mannuronic to guluronic acid (*M*/*G*) is important for the selection of the appropriate application for an alginate (Gacesa, 1988; Sen, 2011), as the *M*/*G* plays a significant role in alginate's physicochemical properties (Murata et al., 2000; Stevens et al., 2004; Pawar & Edgar, 2012). The *M*/*G* of an alginate depends on the algae species from which it was extracted and the harvest time, the harvest location and the processing of the algae (De Vos, De Haan, & Van Schilfgaarde, 1997). Thus, it is important to measure *M*/*G* accurately for determining the appropriate application of an alginate.

Nuclear magnetic resonance (NMR) spectroscopy is a widely used method to detect *M*/*G* (Grasdalen, Larsen, & Smisrod, 1979; Grasdalen, Larsen, & Smisrod, 1981; Rahelivao, Andriamanatonnina, Heyraud, & Rinando, 2013). However, NMR requires substantial amounts of sample and spectra often need to be acquired at high temperature to decrease the viscosity of the alginate solution. Thus, the applications of NMR to measure *M*/*G* of alginate and its derivatives can be challenging for small scale samples or screening many alginate samples (Wang, Yu, Zhao, Guan, & Du, 2005; Zhang et al., 2004).

The hydrolysis of alginate and its derivatives to constituent monosaccharides, followed by separation-based analysis represents another strategy for the analysis of *M*/*G*. High-performance liquid chromatography (HPLC), capillary electrophoresis (CE), gas chromatography (GC), and high-performance anion-exchange

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chromatography (HPAEC) are such separation techniques and can be efficiently applied in high sensitivity analysis (Voragen, Schols, De Vries, & Pilnik, 1982; Guttman, 1997; Rumpel & Dignac, 2006). However, most of these separation methods require derivatization following the hydrolysis of a polysaccharide to its constituent monosaccharides. Such a derivatization step requires additional assay validation and can introduce impurities. The application of HPAEC using pulsed amperometric detection (PAD) can address these concerns. HPAEC-PAD was first described at the end of the last century (Lee, 1990, 1996). Since then, it has been widely used in the analysis of various carbohydrate products (Andersen & Sorensen, 2000; Cai, Liu, Shi, Liang, & Mou, 2005; Grey, Edebrink, Krook, & Jacobsson, 2009). Recently, a new method using HPAEC-PAD was developed to analyze many different kinds of sugars directly, including ManA and GulA. This method could also be applied to more efficiently measure *M*/*G* without any required monosaccharide derivatization (Zhang, Khan, Nunez, Chess, & Szabo, 2012).

In addition to separation and detection, however, the method used for alginate hydrolysis represents an important factor in accurately determining *M*/*G*. Acidic hydrolysis is the major method used for polysaccharide analysis, however, many sugars are not stable under acidic conditions, and uronic acids are particularly labile at low pH values and at elevated temperatures (Aida, Yamagata, Watanable, & Smith, 2010; Aida et al., 2012). An optimized method to hydrolyze alginate and its derivatives to monosaccharides, while maintaining intact sugars, is critical for the accurate analysis of *M*/*G*.

In this paper, we optimize the acid hydrolysis of alginate and then use HPAEC-PAD to accurately analyze ManA and GulA. We also monitor the release patterns of ManA and GulA under different acidic hydrolysis conditions for alginate and its derivatives. The impact of the hydrolysis conditions on the accurate measurement of *M*/*G* was investigated. The hydrolysis conditions examined include the use of different acids, acid concentrations, sample concentrations, hydrolysis temperatures, and hydrolysis times.

2. Experimental

2.1. Materials

Sodium alginate (AR, 90%, 89 mPa s) extracted from *Macrocystis pyrifera* (*M.p.*) was purchased from Aladdin (Shanghai, China). Sodium alginates extracted from *Sargassum fusiforme* (*S.f.*, 570 mPa s) and *Laminaria japonica* (*L.j.*, 187 mPa s), respectively, were purchased from Haizhilin Corp. (Qingdao, China). M- and G-block were prepared in our laboratory as previously described (Zhang et al., 2006). Trifluoroacetic acid (TFA) (99%) was purchased from Energy Chemical (Shanghai, China). Sodium hydroxide solution (50%) was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Sigma-Aldrich (St. Louis, MO). High-purity water (resistivity ≥18.2 MΩ cm, 25 °C) was used throughout the study. All other chemicals and reagents were of HPLC grade.

2.2. Sample preparation

ManA and GulA were prepared by hydrolysis of M block and G blocks, respectively. Both hydrolysis reactions were carried out at 100 °C for 24 h in 2 M TFA. The hydrolyzates were purified by preparative HPLC using UV detection (210 nm) (data not shown). A semi-preparative Carbopac PA1 (20 × 250 mm, Dionex, Sunnyvale, CA) column was used at room temperature with a flow rate of 10 mL/min. The isocratic mobile phase consisted of 5 mM NaOH and 150 mM NaOAc. Purified ManA and GulA were desalted on a column (2.6 × 30 cm) packed with Sephadex G-10 (GE, Healthcare Bio-Science, Uppsala Sweden). Freshly prepared ManA and GulA were quantified using carbazole assay as previously described (Bitter & Muir, 1962). ManA and GulA standards were used to validate retention times and calibrate responses to PAD.

Table 1
Hydrolysis conditions for alginate, M- and G-block.

Sample concentration	Temperature (°C)	Acid concentration (M)	Time (h)
2.5 mg/mL and 5.0 mg/mL	100	1	2
			4h
			6
			12
			18
			24
		2	2
			4
			6
			12
			18
			24
	120	1	2
			4
			6
			12
			18
			24
		2	2
			4
			6
			12
			18
			24

Specific volumes of 1 M or 2 M TFA were added to 5 mg alginate, M-block, or G-block to afford 2.5 mg/mL or 5 mg/mL solutions. Hydrolysis reactions were carried out at 100 or 120 °C, and aliquots were taken at 2, 4, 6, 12, 18 and 24 h. The different hydrolysis conditions studied are provided in Table 1. All experiments were performed in duplicate. The reactions were monitored by TLC to determine completeness of hydrolysis (Zhang, Xie, Zhang, & Linhardt, 2007). Excess TFA was removed by rotary evaporation. Each hydrolysate was re-dissolved in the same volume of water, and its pH was adjusted to neutral using dilute aqueous NaOH. The final concentration of each solution was diluted to ~50 ppm for HPAEC-PAD analysis.

2.3. HPAEC-PAD analysis

Analysis was performed on a Metrohm 850 Professional system with a 919 IC auto-sampler plus equipped with dual pumps and with PAD (Herisau, Switzerland). Data were acquired and analyzed with MagIC Net 2.4 software (Herisau, Switzerland). A Carbopac PA1 analytical column (4 × 250 mm, Dionex, Sunnyvale, CA) was used at a flow rate of 1 mL/min and a temperature of 30 °C. The isocratic mobile phase consisted of 5 mM NaOH and 150 mM NaOAc. The recording time of data acquisition was 15 min and the injection volume was set to 20 μL.

2.4. NMR analysis

About 5 mg of each alginate was dissolved in 1–2 mL D_2O with 0.02% (w/v) 3-(trimethylsilyl) propionate-d_4 (TMSP) (Cambridge Isotope Labs, USA) as a chemical shift reference. NMR spectra were obtained with a Bruker Avance III NMR spectrometer at a ^1H frequency of 600 MHz for 96 scans. The temperatures were set at 25 °C for M and G blocks and 65 °C for alginate samples.

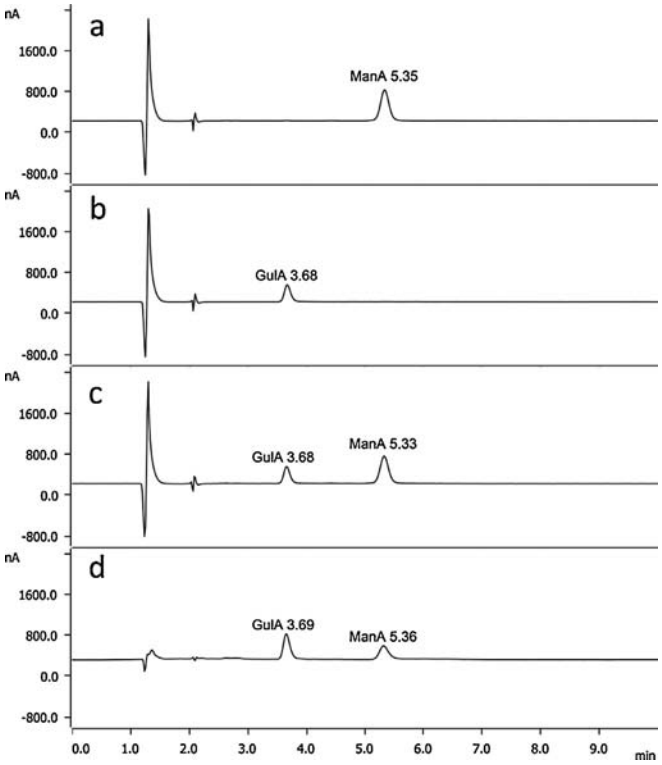

Fig. 1. The chromatograms of ManA, GulA, the mixture of ManA and GulA, and a hydrolysate from G block. (a) The chromatogram of ManA; (b) The chromatogram of GulA; (c) The chromatogram of 1:1 mixed ManA and GulA; (d) The chromatogram of a hydrolysate from G block.

3. Results

3.1. HPAEC-PAD method development

The HPAEC-PAD method had been developed to analyze acidic sugars in 2012 (Zhang et al., 2012). However, it was necessary to calibrate the PAD response for ManA and GulA, since the D-configuration of ManA and the L-configuration of GulA might lead to different aldehyde oxidizability. The same amounts of purified ManA, GulA and a 1:1 mixture of ManA and GulA were analyzed and their chromatograms are shown in Fig. 1. Peaks corresponding to ManA and GulA were observed at 3.66 and 5.33 min, respectively. The different peak integrations for the same amounts of ManA and GulA reflected their different responses to PAD. The ratio of peak integrals for identical amounts of ManA and GulA was 2.1 to 1 and this ratio was used throughout this study to calculate the actual *M*/*G*. The chromatogram of a hydrolyzed sample is shown in Fig. 1d and the hydrolyzate's retention times matched very well to those of the ManA and GulA standards.

3.2. Acid selection

Dilute sulfuric acid was reportedly used to hydrolyze PSS, a low molecular weight alginate derivative used in China as an anticoagulant, for *M*/*G* determination (Wu et al., 2014). However, under these conditions some residual oligosaccharides could still be observed on TLC when alginate, M blocks, and G blocks were hydrolyzed. The TLC images are shown in Supplementary information (SFig. 1). These results suggest that incomplete hydrolysis of alginate, M blocks, and G blocks had taken place under these conditions. Thus, TFA was selected for use in the current study to completely hydrolyze alginate and its derivatives. TFA also offers the additional advantage of being volatile so that it can be easily removed.

3.3. Pattern of M and G release from alginate (M.p.)

Alginate (*M.p.*) was hydrolyzed under a variety of different conditions (Table 1). TLC was used to monitor the completeness of hydrolysis based on the absence of oligosaccharides (Fig. 2 insets). Hydrolysis was not complete until 2.5 mg/mL alginate (*M.p.*) was treated with 1 M TFA at 100 °C for 18 h, at 120 °C for 6 h, with 2 M TFA at 100 °C for 12 h, or with 2 M TFA at 120 °C for 6 h. The density of the spot, corresponding to monosaccharide products, began to decrease after these time points, suggesting decomposition of the GulA and ManA residues was taking place. Similar results were observed at sample concentrations of 2.5 and 5 mg/mL.

The alginate (*M.p.*) hydrolysates were next analyzed with HPAEC-PAD. Integration of ManA and the calibrated integration of GulA were plotted as a function of hydrolysis time for the different conditions (Fig. 2). The hydrolysis curves obtained at sample concentrations of 2.5 and 5 mg/mL were similar.

3.3.1. Hydrolysis conditions using 1 M TFA at 100 °C

A slow increase in ManA formation was observed from beginning of hydrolysis until the aliquot taken at 18 h, after which a decrease was observed (Fig. 2a). This suggests that ManA was released from polymer completely within ~18 h, after which it started to be degraded. Another hypothesis is that the rate of release of ManA ($^{r}S_{M}$) equals the rate of degradation of ManA ($^{d}S_{M}$) at this time point. However, monosaccharide was observed as the major product on TLC (inset in Fig. 2a) at this time point, suggesting that

Table 2
The conditions to release ManA and GulA from different samples and their *M*/*G* values.

Sample	Sample concentration (mg/mL)	Temperature (°C)	Acid concentration (M)	Time integrations were considered (h)		*M*/*G*
				ManA	GulA	
Alginate (*M.p.*)	2.5	100	1	18	12	1.4
			2	12	6	1.3
	5.0	100	1	18	12	1.4
			2	18	6	1.3
M block	2.5	100	1	18	6	6.8
			2	12	6	6.8
		120	1	6	4	6.6
	5.0	100	1	18	12	6.8
			2	18	6	6.6
		120	1	12	4	6.6
G block	2.5	100	1	18	18	0.3
			2	12	6	0.3
	5.0	100	1	24	24	0.3
			2	18	12	0.3

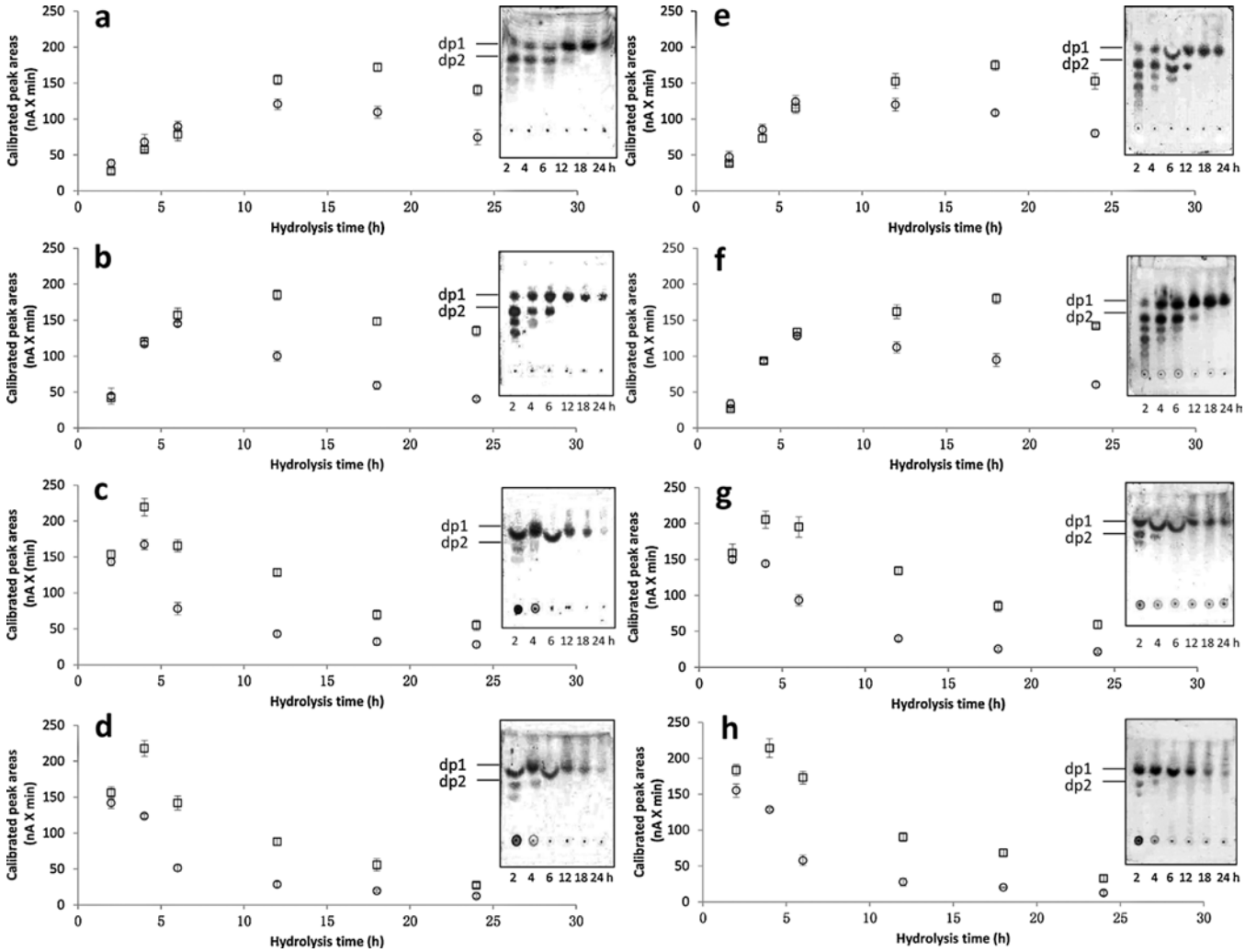

Fig. 2. The ManA and GulA release pattern from alginate (*M.p.*). (a) 2.5 mg/mL, 1 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (b) 2.5 mg/mL, 2 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (c) 2.5 mg/mL, 1 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (d) 2.5 mg/mL, 2 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (e) 5.0 mg/mL, 1 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (f) 5.0 mg/mL, 2 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (g) 5.0 mg/mL, 1 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (h) 5.0 mg/mL, 2 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h.

the most of the saccharide residues had been released, and the rate of release (^{r}S) of saccharide residue was nearly zero at this time point. Based on the second hypothesis, $^{r}S_M \approx {}^{d}S_M$ at this time point, the rate of degradation should also be nearly zero. This suggests that the degradation of released saccharide residues did not yet start at this time point, which is consistent with the first hypothesis. The consistency between these two hypotheses confirms that it is possible to select a time, at which the most of saccharide residues would be released but not degraded. The integration of ManA detected at this selected time point would then reflect the actual content of ManA in the polymer.

Interestingly, the highest integration of GulA was observed in the hydrolysate treated for 12 h (Fig. 2a). We assumed that the degradation point of GulA under this hydrolysis condition was at 12 h and that the calibrated integration of GulA detected in this aliquot would reflect the actual content of GulA in the polymer.

The integration of ManA and the calibrated integration of GulA, detected in the aliquots treated for 18 h and 12 h, respectively, were used to calculate the *M*/*G* value (Tables 2 and 3).

3.3.2. Hydrolysis conditions using 2 M TFA at 100 °C

When the TFA concentration was increased from 1 M to 2 M, the maximum amount of ManA and GulA were observed after 12 h and 6 h, respectively (Fig. 2b). Based on TLC (inset in Fig. 2b), no oligosaccharides were observed in the 12 h hydrolyzate, confirming the complete release of monosaccharide residues. The degradation point for ManA under these hydrolysis conditions was 12 h and the degradation point for GulA was 6 h. The *M*/*G* was determined from the integration of ManA and the calibrated integration of GulA after 12 h and 6 h treatment, respectively (Tables 2 and 3). The conditions used are summarized in Table 2 and the *M*/*G* values are provided in Table 3.

Higher sample concentration (5 mg/mL) afforded a degradation point for ManA to 18 h instead of the 12 h observed in 2.5 mg/mL alginate (*M.p.*) experiments (Fig. 2f and Table 2). The *M*/*G* obtained with 5 mg/mL sample concentration was also calculated and is provided in Table 3.

Table 3
The *M*/*G* value comparisons between HPAEC-PAD method and NMR analysis.

Samples	*M*/*G* values	
	HPAEC-PAD method	NMR analysis
Alginate (*M.p.*)	1.4	1.43
Alginate (*S.f.*)	0.7	0.76
Alginate (*L.j.*)	1.8	1.98
M block	6.8	7.02
G block	0.3	0.29

3.3.3. Hydrolysis conditions using 1 M TFA at 120 °C

The highest integration values for ManA and GulA were both observed at 4 h (Fig. 2c and g) when the temperature increased from 100 °C to 120 °C. However, disaccharide was still remained at this point (inset of Fig. 2c and g). This suggests that degradation had started before the release of saccharide residues was complete. Thus, the integration of ManA detected at any given time point did not reflect the actual content of ManA in polymer, and these hydrolysis conditions were not suitable for *M*/*G* analysis. Another aliquot was taken after 5 h treatment but it also did not correspond to the degradation point (data not shown).

3.3.4. Hydrolysis conditions using 2 M TFA at 120 °C

The highest integrations of ManA and GulA were observed at 4 h and 2 h, respectively, under these conditions (Fig. 2d and h). As with the previous experiment, these hydrolysis conditions were not suitable for *M*/*G* analysis (insets in Fig. 2d and h).

3.4. Pattern of M and G release from M block

As is the case for alginate (*M.p.*), the M block is primarily composed of ManA, so the release of monosaccharide residues from the M block was similar to that observed for alginate (*M.p.*). Based on the presence of oligosaccharides on TLC analysis, the hydrolysis was not complete until M block (at 2.5 mg/mL) was treated with 1 M TFA at 100 °C for 18 h or at 120 °C for 6 h, and with 2 M TFA at 100 °C for 12 h or at 120 °C for 4 h. Similar results in monosaccharide release were observed at sample concentrations of 2.5 and 5 mg/mL.

The release patterns of ManA and GulA from M block were also analyzed with HPAEC-PAD and the results are plotted in Fig. 3.

3.4.1. Hydrolysis conditions using 1 M TFA at 100 °C

The highest integration for ManA from M block was observed at 18 h, the same as observed for alginate (*M.p.*) (Fig. 3a). TLC also confirms the completeness of monosaccharide release at this time point (inset in Fig. 3a). The highest integration for GulA from M block was observed at 6 h, not the 12 h observed for alginate (*M.p.*) (Fig. 3a). The lower content of GulA residues in M block could be the reason for its more rapid release. The actual *M*/*G* of M block was determined through the integration of ManA and the calibrated integration of GulA as detected after 18 h and 6 h treatments, respectively (Tables 2 and 3).

Higher sample concentration (5 mg/mL) afforded a degradation point for GulA of 12 h instead of the 6 h, observed in the 2.5 mg/mL alginate (*M.p.*) experiments (Fig. 3e and Table 2). The *M*/*G* values obtained for the 5 mg/mL sample concentration are provided in Table 3.

3.4.2. Hydrolysis conditions using 2 M TFA at 100 °C

The degradation points for ManA and GulA were at 12 h and 6 h under these conditions (Fig. 3b and Table 2). The *M*/*G* is determined from the integration of ManA and the calibrated integration of GulA determined at their degradation points. Higher sample concentration (5 mg/mL) afforded a degradation point for ManA of 18 h, instead of the 12 h observed in the 2.5 mg/mL M block experiments (Fig. 3f and Table 2) and the *M*/*G* values obtained under these hydrolysis conditions are provided in Table 3.

3.4.3. Hydrolysis conditions using 1 M TFA at 120 °C

In contrast to the *M*/*G* of alginate (*M.p.*), the *M*/*G* of M block could be analyzed at hydrolysis temperatures under 120 °C. Degradation points for ManA were observed at 6 h for the 2.5 mg/mL sample concentration and at 12 h for the 5 mg/mL sample concentration under these conditions (Fig. 3c and g). It is speculated that the presence of a dominant ManA in M block allows it to tolerate harsher hydrolysis conditions. The *M*/*G* value is determined from the integration of ManA and calibrated integration of GulA, detected at 6 h and 4 h (2.5 mg/mL M block) or at 12 h and 4 h (5 mg/mL M block). The conditions used are summarized in Table 2 and the *M*/*G* values are presented in Table 3.

3.4.4. Hydrolysis conditions using 2 M TFA at 120 °C

High acid concentrations and high temperatures were too harsh for the proper hydrolysis of M block. TLC shows that the hydrolysis was not complete when the highest integrations of monosaccharide residues were observed (Fig. 3d and h). Thus, these hydrolysis conditions are not suitable for *M*/*G* analysis of the M block.

3.5. Pattern of M and G release from G block

In contrast to the results obtained for alginate (*M.p.*) and M block, the G block, mainly composed of GulA, showed a different hydrolysis pattern. Based on the presence of oligosaccharides on TLC analysis, the hydrolysis was not complete until G block (2.5 mg/mL) was treated with 1 M TFA at 100 °C for 18 h, at 120 °C for 6 h, or with 2 M TFA at 100 °C for 12 h, at 120 °C for 2 h. At higher sample concentrations (5 mg/mL) a longer time was required for complete hydrolysis when samples were treated at 100 °C.

The release patterns of ManA and GulA from the G block were also analyzed with HPAEC-PAD and the results are plotted in Fig. 4.

3.5.1. Hydrolysis conditions using 1 M TFA at 100 °C

GulA was observed as the major component of G block. The highest integrations for GulA and ManA were both observed after treating the G block (2.5 mg/mL) with 1 M TFA at 100 °C for 18 h (Fig. 4a). TLC also confirmed the completeness of release of monosaccharide residues at this time point (inset of Fig. 4a). The degradation points for GulA and ManA were both 18 h under these hydrolysis conditions. The degradation points for GulA and ManA were both observed after treating the G block (5 mg/mL) with 1 M TFA at 100 °C for 24 h (Fig. 4e). The actual *M*/*G* of G block could be determined from the 18 h treatment of 2.5 mg/mL G block or the 24 h treatment of the 5 mg/mL G block under these conditions (Tables 2 and 3). Observation of the degradation point for GulA under these conditions unambiguously confirms that the degradation point for GulA, assumed and used to calculate *M*/*G* values in the alginate (*M.p.*) and M block experiments, were reasonable.

3.5.2. Hydrolysis conditions using 2 M TFA at 100 °C

The highest integrations for GulA and ManA were observed at 6 h and 12 h (Fig. 4b), respectively, suggesting GulA was completely released more quickly than the ManA, although the GulA was greater than the ManA content in G block. The degradation point for GulA was assumed to be 6 h, although a small amount of disaccharide was still observed in the 6 h aliquot, (inset of Fig. 4b), which could come from the remaining M domains in the G block. TLC analysis shows that the hydrolysis was complete at 12 h, which is consistent with the time the highest integrations for ManA were observed (Fig. 4b and inset of Fig. 4b). The degradation point for ManA was then confirmed as 12 h (Tables 2 and 3). The similarity of the *M*/*G* values between these conditions and others examined in this study also demonstrate that these conditions can be used to accurately analyze the *M*/*G* value of the G block.

The degradation points for GulA and ManA are observed at 12 h and 18 h, respectively, in 5 mg/mL G block experiments (Fig. 4f, Tables 2 and 3).

3.5.3. Hydrolysis conditions using 1 M TFA at 120 °C

The highest integrations for both ManA and GulA were observed at 4 h using 1 M TFA at 120 °C (Fig. 4c and g). However, the

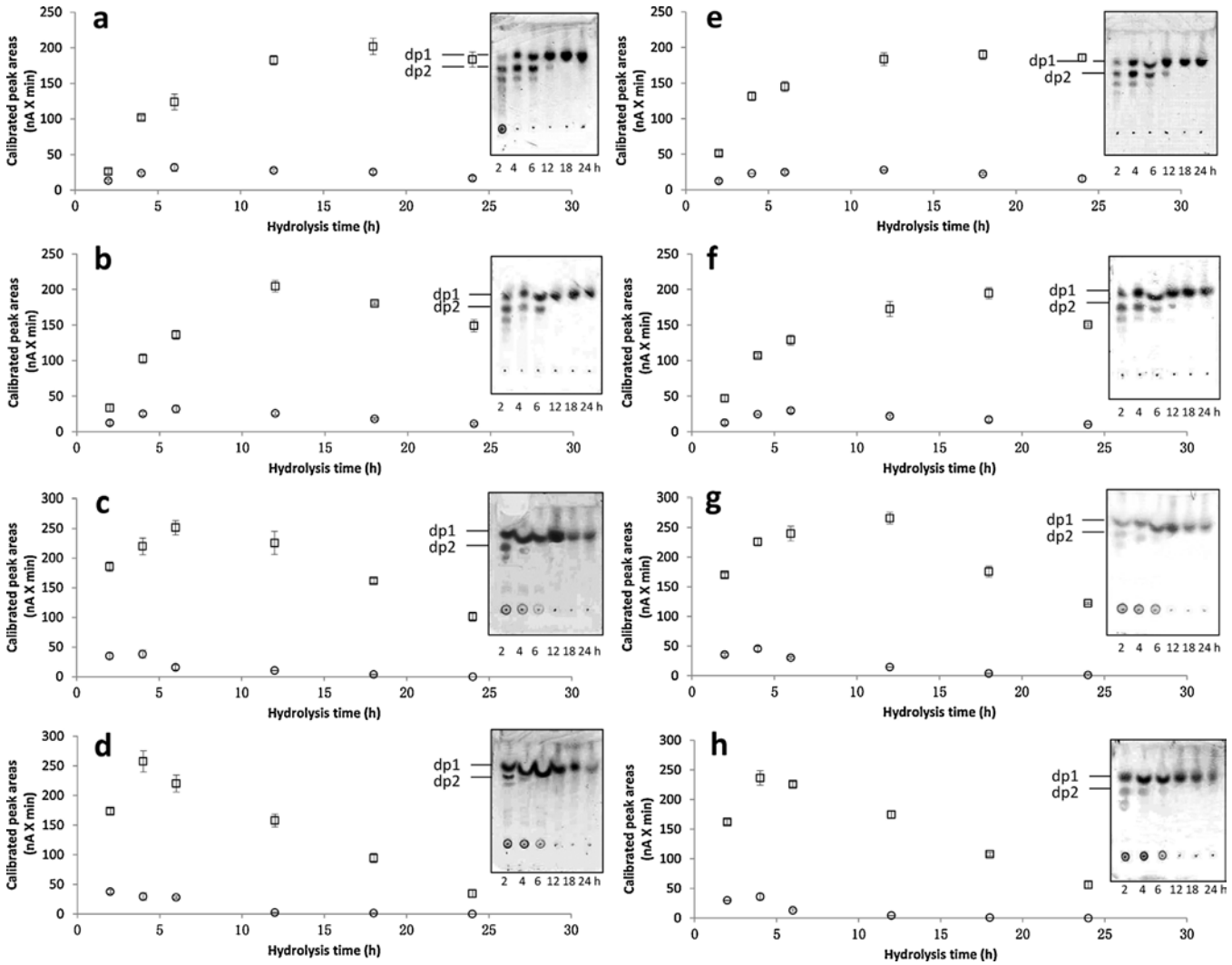

Fig. 3. The ManA and GulA release pattern from M block. (a) 2.5 mg/mL, 1 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (b) 2.5 mg/mL, 2 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (c) 2.5 mg/mL, 1 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (d) 2.5 mg/mL, 2 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (e) 5.0 mg/mL, 1 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (f) 5.0 mg/mL, 2 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (g) 5.0 mg/mL, 1 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (h) 5.0 mg/mL, 2 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h.

hydrolysis was not complete at 4 h (insets in Fig. 4c and g), suggesting that the *M*/*G* value for the G block could not be analyzed under these conditions.

3.5.4. Hydrolysis conditions using 2 M TFA at 120 °C

The use of 2 M TFA at 120 °C was too harsh to properly hydrolyze G block for *M*/*G* analysis (Fig. 4d and h). The released monosaccharide residues had already begun to degrade at 2 h.

3.6. NMR analysis of M blocks, G blocks, and alginate samples

^{1}H NMR spectra of M, G blocks and alginate samples are shown in Fig. 5. The signals of anomeric hydrogen of ManA and GulA were observed at chemical shift 4.6–4.8 ppm and 5.0–5.2 ppm, respectively. The spectra of M and G blocks (Fig. 5a and b) were obtained at room temperature. Their signals of anomeric hydrogen corresponding to ManA and GulA were assigned and integrated unambiguously. As the viscosities of alginate samples become higher, their NMR spectra needed to be acquired at an elevated temperature of 65 °C. (Fig. 5c–e) At higher temperatures, the viscosities of samples decreased; the signal of solvent (HDO) shifted to higher field, becoming better separated from the anomeric hydrogen signals; the signals of anomeric hydrogens corresponding to ManA and GulA could then be easily integrated. The *M*/*G* values from NMR analysis, corresponding to the ratios of the integrations for the signals of the anomeric hydrogens of ManA and GulA, are provided in Table 3.

4. Discussion and conclusions

It is critical to analyze the *M*/*G* value for alginate and its derivatives to determine their appropriate applications. In this work, a robust method with HPAEC-PAD analysis has been developed. The released ManA and GulA are well separated and sensitively detected without any derivatization after hydrolysis. As we hypothesized, the PAD responses for ManA and GulA were not identical. The response ratio was determined to be 2.1 to 1 and this ratio was used to calibrate integration for GulA in Figs. 2–4. In addition, these chromatograms were still of very good quality, even after hundreds of injections.

Hydrolysis is another critical factor that impacts the accurate determination of *M*/*G* values. Complete hydrolysis with the retention of intact sugar is required to accurately measure *M*/*G*. In this work, different conditions were examined using alginate and its derivatives. TFA was selected for acid hydrolysis, as it afforded complete hydrolysis of polysaccharide and TFA was easy to remove.

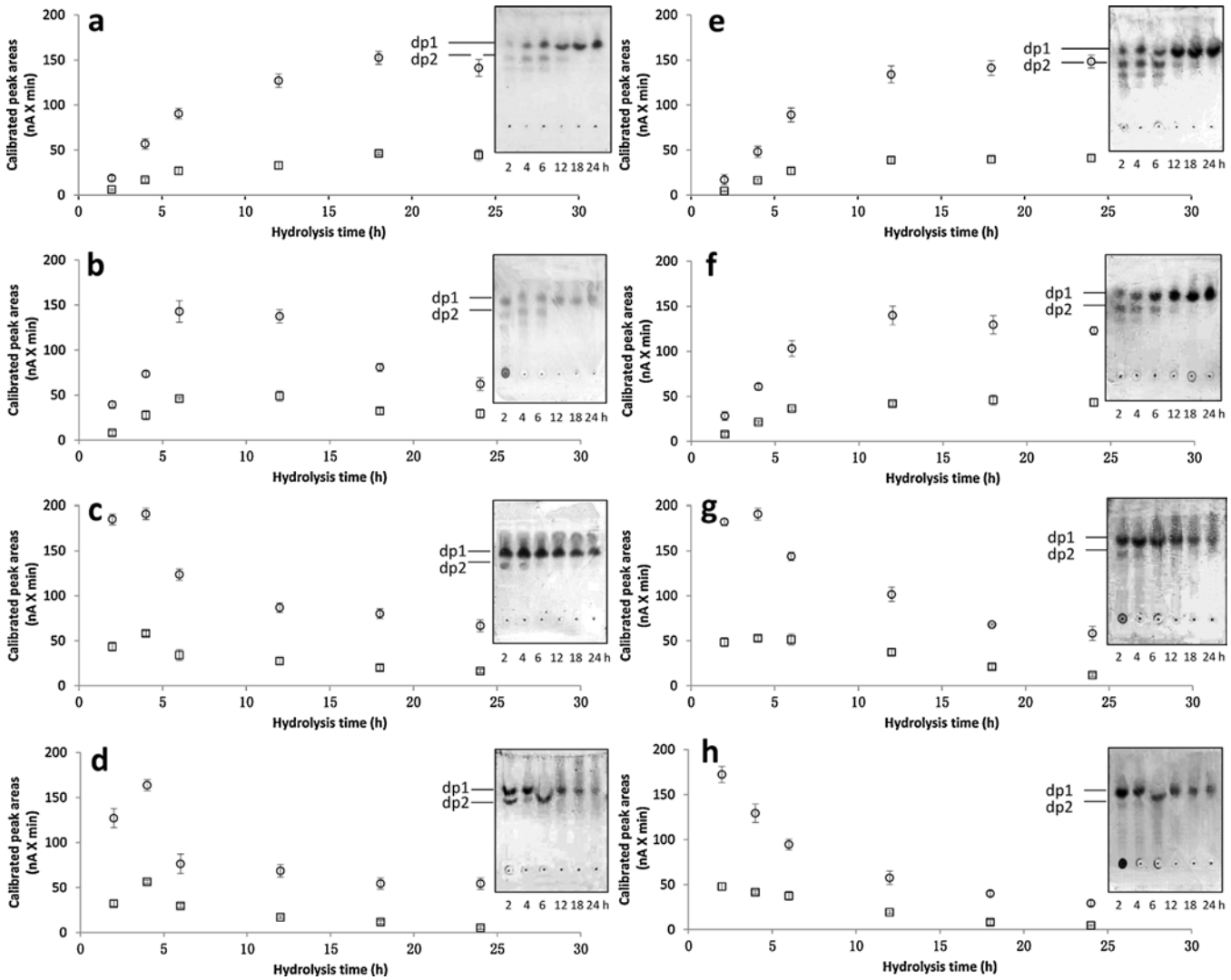

Fig. 4. The ManA and GulA release pattern from G block. (a) 2.5 mg/mL, 1 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (b) 2.5 mg/mL, 2 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (c) 2.5 mg/mL, 1 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (d) 2.5 mg/mL, 2 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (e) 5.0 mg/mL, 1 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (f) 5.0 mg/mL, 2 M TFA, 100 °C, 2, 4, 6, 12, 18, 24 h; (g) 5.0 mg/mL, 1 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h; (h) 5.0 mg/mL, 2 M TFA, 120 °C, 2, 4, 6, 12, 18, 24 h.

Nevertheless, hydrolysis conditions need to be carefully controlled, as too harsh of conditions quickly degraded the released monosaccharide residues.

Based on the results obtained, the released ManA and GulA in a single hydrolysis reaction could not be used to determine the content of ManA and GulA in the polysaccharide, as the rates of release of ManA and GulA are different. Although a higher content of ManA is present in alginate (*M.p.*), a similar or even higher amount of GulA was observed at beginning of the hydrolysis (Fig. 2), suggesting the glycosidic bonds linked to GulA are more susceptible to hydrolysis. This is also supported by the observation that the GulA was completely released more quickly than ManA even though the GulA content was greater than ManA content in the G block. Generally, GulA was released more quickly than ManA, independent of its content in the polysaccharides.

Conditions, under which both complete release and the highest integrations of monosaccharide residues were simultaneously observed, were selected as the optimal hydrolysis conditions. These conditions were defined as degradation points. The content of ManA is greater than GulA in alginate; ManA is dominant in the M block; in addition, the release rate of ManA is slower than that of GulA, so the observation of complete hydrolysis on TLC in alginate (*M.p.*) and M block experiments all corresponded to ManA. Thus, the identification of degradation points of ManA in alginate (*M.p.*) and M block was unambiguous. Whereas, the degradation points for GulA were only confirmed in some G block experiments. Fortunately, these experiments helped to confirm the degradation points for GulA that were assumed in alginate (*M.p.*) and M block experiments.

Thus, integration for ManA and the calibrated integration for GulA, observed under the optimized conditions, respectively, were used to calculate *M*/*G* values for alginate and its derivatives (M block and G block) in Table 3. The *M*/*G* value for alginate (*M.p.*) was calculated as 1.3–1.4. The hydrolysis could be carried out at a sample concentration of 2.5 or 5 mg/mL and a TFA concentration of 1 M or 2 M at 100 °C. The *M*/*G* value for M block was calculated as 6.6–6.8. The hydrolysis could be carried out at a sample concentration of 2.5 or 5 mg/mL and a TFA concentration of 1 M or 2 M at 100 °C, or at a sample concentration of 2.5 or 5 mg/mL and TFA concentration of 1 M at 120 °C. The *M*/*G* value for G block was calculated as 0.3. The hydrolysis could be carried out at a sample concentration of 2.5 or 5 mg/mL and a TFA concentration of 1 or 2 M at 100 °C.

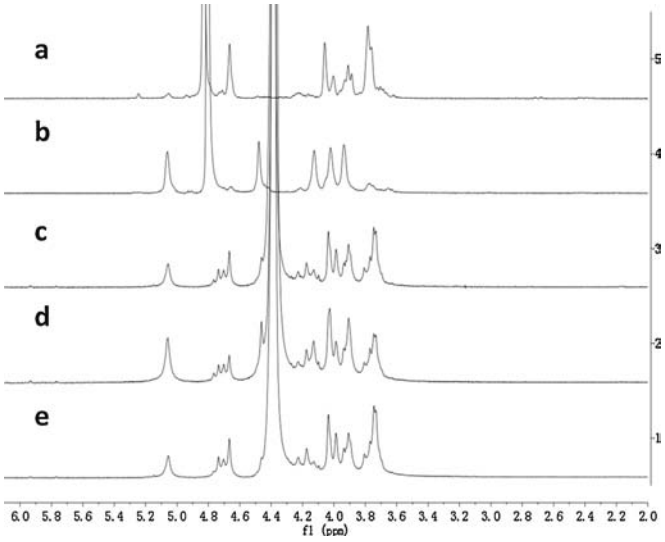

Fig. 5. ^{1}H NMR spectra of different alginate samples, M blocks and G blocks. (a) ^{1}H NMR spectrum of M block; (b) ^{1}H NMR spectrum of G block; (c) ^{1}H NMR spectrum of alginate (*M.p.*); (d) ^{1}H NMR spectrum of alginate (*S.f.*); (e) ^{1}H NMR spectrum of alginate (*S.f.*).

Integration for ManA and the calibrated integration of GulA need be considered separately to accurately determine the *M*/*G* value for alginate and its M block and G block derivatives (These conditions are summarized in Table 2 and the *M*/*G* values are provided in Table 3). The *M*/*G* values for the same sample, obtained using different hydrolysis conditions, are consistent, demonstrating that this strategy is generally applicable. The calculated *M*/*G* values of alginate (*M.p.*), M block and G block are consistent with the results from ^{1}H NMR (~1.4, ~7 and, ~0.3, respectively) as shown in Table 3, confirming the accuracy of this method.

In addition, two more alginate samples (*S.f.* and *L.j.*) were analyzed with this method. Considering the higher viscosities of these two samples, the hydrolysis were carried out at the sample concentration of 2.5 mg/mL and a TFA concentration of 2 M at 100 °C. The release patterns of ManA and GulA from these two alginate samples (*S.f.* and *L.j.*) are shown in Supplemental information (SFig. 2). The *M*/*G* values calculated based on the release patterns are listed in Table 3, which is consistent with the results from NMR analysis (Fig. 5 and Table 3).

Thus, a robust and accurate method was developed to analyze ManA and GulA; the impact of hydrolysis conditions on *M*/*G* value determination was investigated using this method; and a strategy was applied to properly determine *M*/*G* values for alginate and its derivatives.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2015.01.008.

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