Solution-state behaviour of algal mono-uronates evaluated by *pure shift* and *compressive sampling* NMR techniques

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1 Highlights

- Mono-dimensional *pure shift* ^{1}H and *compressive sampling* ^{1}H-^{13}C HSQC NMR spectroscopic methods were used, for the first time, to unambiguously assign the ^{1}H and ^{13}C spectra of the algal mono-uronic acids: D-mannuronic acid (**HManA**) and L-guluronic acid (**HGulA**)

- The acidity constants of different forms of the acids were determined:
  $pK_a(^{1}C_4$-*α*-L-gulopyranuronic acid) = 3.45 ± 0.03
  $pK_a(^{1}C_4$-*β*-L-gulopyranuronic acid) = 3.44 ± 0.03
  $pK_a(^{4}C_1$-*α*-D-mannopyranuronic acid) = 3.33 ± 0.03
  $pK_a(^{4}C_1$-*β*-D-mannopyranuronic acid) = 3.21 ± 0.03

- The $^{1}C_4$-*β*-L-gulopyranose and $^{4}C_1$-*α*-D-mannopyranose forms of the uronic acids were found to be the most stable at all pD values from 1.4 to 7.5.

- Both **HManA** and **HGulA** form furanurono-6,3-lactones at pD 1.4, in an analogous manner to D-glucuronic acid (**HGlcA**). **HManA** was found to lactonise more readily than **HGulA**.

2 Abstract

Sodium salts of the algal uronic-acids, D-mannuronic acid (**HManA**) and L-guluronic acid (**HGulA**) have been isolated and characterised in solution by nuclear magnetic resonance (NMR) spectroscopy. A suite of recently-described NMR experiments (including *pure shift* and *compressive sampling* techniques)

were used to provide confident assignments of the pyranose forms of the two uronic acids at various pD values (from 7.5 – 1.4). The resulting high resolution spectra were used to determine several previously unknown parameters for the two acids, including their pK_a values, the position of their isomeric equilibria, and their propensity to form furanurono-6,3-lactones. For each of the three parameters, comparisons are drawn with the behaviour of the related D-glucuronic (**HGlcA**) and D-galacturonic acids (**HGalA**), which have been previously studied extensively. This paper demonstrates how these new NMR spectroscopic techniques can be applied to better understand the properties of polyuronides and uronide-rich macroalgal biomass.

3 Keywords

- *Pure shift* NMR spectroscopy
- *Compressive sampling*
- Uronic acids
- Mannuronic / mannuronate
- Guluronic / guluronate
- Macroalgae / kelp / seaweed
- Hydrothermal lactonisation

4 Introduction

As researchers have sought to develop new routes to carbon-based fuels and chemicals from renewable feedstocks, kelps (large brown seaweeds) have been identified as promising sources of biomass starting materials owing to their high carbohydrate content and high photosynthetic efficiency.[1] In particular, furan-2-carbaldehyde (furfural) is a highly desirable commercial platform molecule and consequently, optimisation of its production from biomass has been a key target for researchers.[2] In this context, it is now well-established that both the thermal and hydrothermal treatment of alginic acid, as well as that of other uronide-rich biopolymers such as hemicellulose (containing glucuronic acid, **HGulA**)[3,4] and pectin (containing galacturonic acid, **HGalA**),[3–5] can be used to prepare furfural through a pathway of depolymerisation, decarboxylation, and dehydration. Kelps are ideally placed as a sustainable starting material for the manufacture of furfural since they are comprised of significant quantities of alginic acid. Indeed, present in its salt form (alginate), it comprises up to 40 wt.% of kelp's dry weight.

Alginic acid is a copolymer of 1,4-linked $^{4}C_{1}$-*β*-D-mannopyranuronic (**HManA**) and $^{1}C_{4}$-*α*-L-gulopyranuronic acid (**HGulA**) as depicted in Figure 1.[6–8] A possible reaction pathway for the thermal degradation of polyuronides is *via* internal esterification of the free mono-uronic acids to yield a furanurono-6,3-lactone (see Figure 2), a process that is known to occur readily during the hydrothermal treatment of **HGlcA**.[3] Whilst lactones of **HManA** and **HGulA** are known to exist, their formation from the free acids has not been studied in detail. Indeed, over the many decades since their original isolation,[9–13] surprisingly little has been reported on the solution state behaviour of **HManA** and **HGulA**, despite their potential importance as chemical feedstocks for the production of furfural and other valuable compounds.[14] NMR spectroscopy is a valuable tool for studying such complex aqueous reactions, although to date investigations of the algal uronic acids has been

impeded by their lack of commercial availability and the complexity of their ^{1}H NMR spectra.

In this paper, we provide a straightforward and detailed methodology for preparing purified solutions of **NaManA** and **NaGulA** from alginic acid, and report on the analysis of solutions of these salts by state-of-the-art NMR spectroscopic techniques. Notably, recently reported *pure shift* and *compressive sampling* NMR techniques can be used to increase resolution and simplify spectra, enabling rapid and confident characterization of the algal mono-uronates across a range of pH levels. Using these types of spectral analysis techniques, it was possible to assess the relative stabilities of the configurational and conformational isomers of **ManA** and **GulA**, and the respective acid dissociation constants of individual species present in solution. The data obtained also allows for the comparison of the lactonisation behaviour of **HManA** and **HGulA** relative to that of **HGlcA** and **HGalA**, under a range of conditions of relevance to the hydrothermolysis of alginic acid. It Is anticipated that the results reported here will not only serve to enhance the understanding of the solution state properties of **HManA** and **HGulA**, but also provide a point of reference for the analysis of uronate-rich solutions (such as kelp hydrolysates) by NMR spectroscopy of relevance in the production of platform chemicals from sustainable feedstocks.

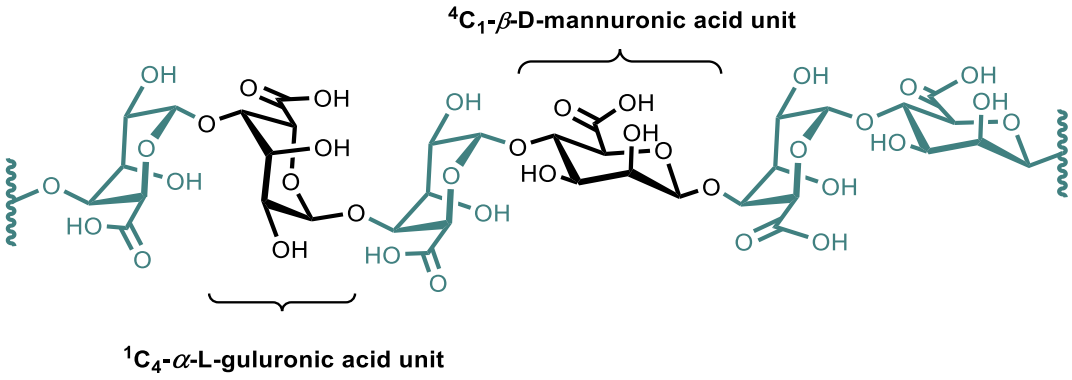

Figure 1 Algal mono-uronic acids (L-guluronic and D-mannuronic acids) as found in their parent kelp polysaccharide alginic acid.

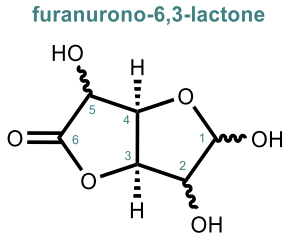

Figure 2 The generic structure of a bicyclic furanurono-6,3-lactone.

5 Results and discussion

5.1 NMR spectroscopic characterisation of HManA and HGulA by *pure shift* and *compressive sampling* experiments

Samples of **HManA** and **HGulA** were first isolated from the hydrolysate of alginic acid from a kelp (*Macrocystis pyrifera*), according to the methods reported in ESI Section 2.1. Subsequent analysis of the two uronic acids by NMR spectroscopy is complicated by signal overlap. This problem was solved using two complementary methods. Firstly, homonuclear multiplets were collapsed into singlets using a technique known as *pure shift* NMR spectroscopy,[15] which essentially increases the effective resolving power of a spectrometer several times.[16] An example of the *pure shift* experiment applied to analysis of a solution of **HManA** is shown in Figure 3. Here signals are resolved using a mono-dimensional PSYCHE *pure shift* ^{1}H technique.[17]

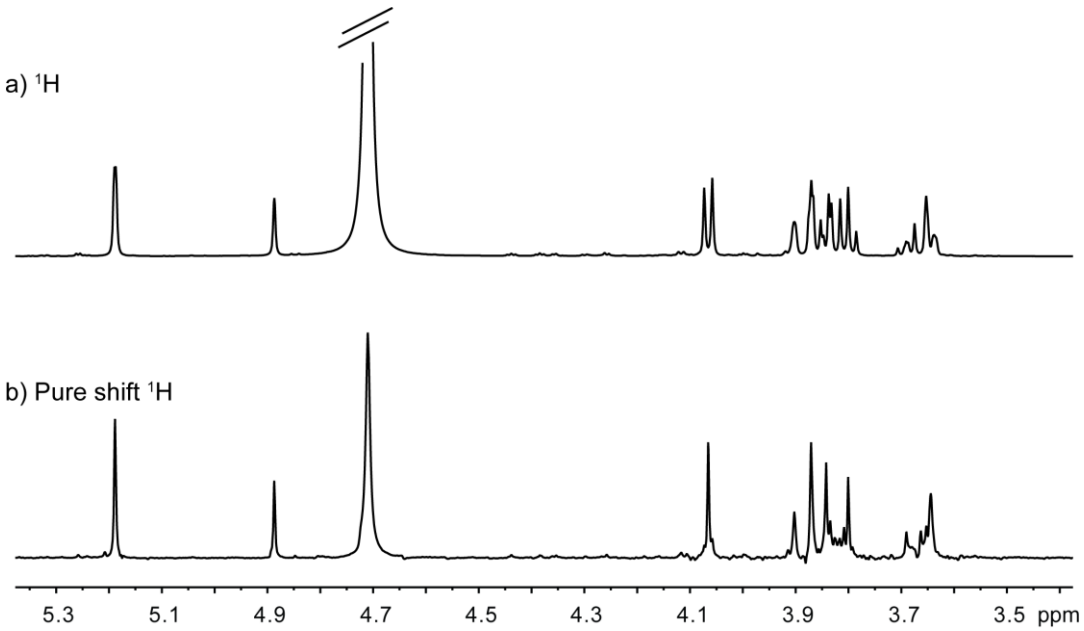

Figure 3 600 MHz ^{1}H NMR spectra of **HManA** at pD 4.60 dissolved in D_2O at 25 °C. (a) Overlapping signals in the standard ^{1}H NMR spectrum. (b) Resolution of overlapping signals achieved by collapsing multiplets into singlets using a PSYCHE *pure shift* technique. The determination of specific chemical shifts is easier in (b) than in (a).

Whilst there were clear improvements achieved by using *pure shift* NMR techniques relative to standard ^{1}H NMR approaches, sometimes the *pure shift spectra* did not give sufficient resolution for both **HManA** and **HGulA** across the pD ranges studied. This

limitation was overcome by employing multidimensional NMR spectroscopic experiments. However, multidimensional experiments such as ^{1}H-^{13}C HSQC are much slower (typically 40 minutes for 512 increments, with two scans per increment) and therefore impractical when analysis of many titration points is required. To circumvent this problem, fewer increments can be used, however the spectral resolution is significantly degraded (Figure 4). Fortunately, it is possible to reduce experimental times associated with multidimensional NMR spectroscopic techniques without reducing resolution by exploiting *compressive sampling* theory. This theory states that objects that are sparse or compressive, such as NMR spectra, can be reconstructed from fewer data points than the traditional Shannon-Nyquist theorem requires by collecting a set of incoherent measurements and then reconstructing the object (spectrum) using an algorithm that minimises the ℓ_1-norm of the object.[18–21] A detailed account of this method can be found elsewhere.[22] Here it was possible, using this approach, to reduce the acquisition time of the ^{1}H-^{13}C HSQC experiment (1024 increments, 2 transients per increment, 14 Hz/point) employed for the analysis of **HManA** and **HGulA** from around 80 minutes to 10 minutes. As an example, Figure 4 shows a comparison between a traditionally-acquired ^{1}H-^{13}C HSQC (10 min) of **HManA** at pD 3.4, and the same experiment acquired using *compressive sampling* (also 10 min). In the traditional experiment, the resolution is not sufficient to resolve overlapping signals, but these were resolved using the *compressive-sampling* HSQC approach. Additionally, the use of *compressive sampling* also allows for the chemical shift of the carbon atoms to be determined with minimal time commitment, and therefore using this approach also enabled full ^{13}C signal assignments across the pD range.

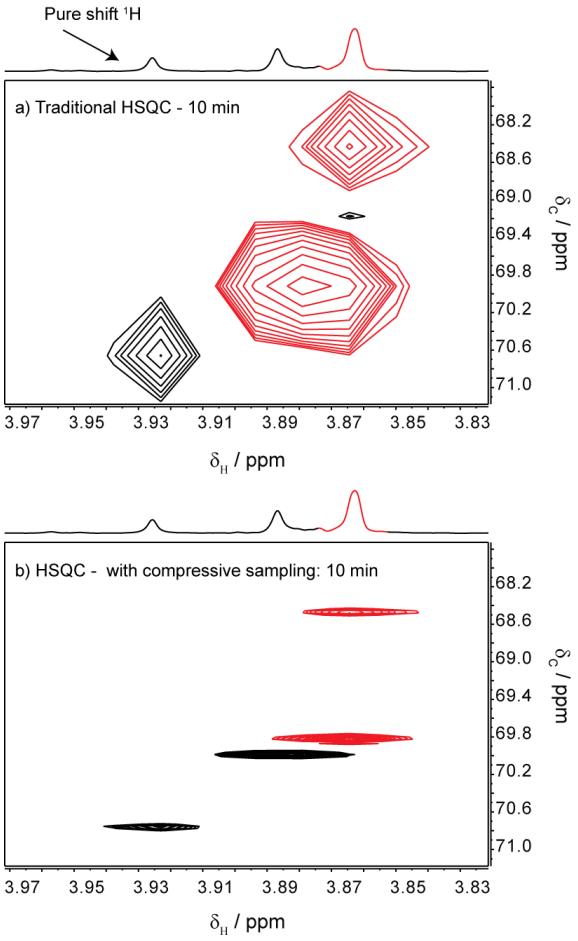

Figure 4 600 MHz ^{1}H-^{13}C HSQC spectra of **HManA** at pD 3.39 dissolved in D_2O at 25 °C. At this pD, signals overlap so much that not even *pure shift* methods can resolve some signals. ^{1}H-^{13}C HSQC can be used to resolve these signals. (a) Traditional sampling methods do not provide sufficient resolution in short acquisition times. (b) Compressive sampling allows increased resolving power without increasing experimental times.

In addition to the above experiments, **NaManA** and **NaGulA** pD titrations were studied using ^{13}C{^{1}H} and ^{1}H-^{1}H COSY, whilst a ^{1}H-^{13}C HMBC NMR experiment was also used at pD 7.4 – 7.6 at 295 K to help resolve connectivity problems encountered. Full ^{1}H and ^{13}C NMR spectral assignments of **NaManA** and **NaGulA** at pD 7.4 – 7.6 are reported in Table 1 and Table 2, respectively, with detailed explanations being provided in the ESI (Sections 3.1 – 3.4).

Whilst the assignments of the ^{1}H NMR spectra of **NaGulA** and **NaManA** reported in Table 1 are largely consistent with the few previously reported accounts,[23–28] the enhanced resolution of the current spectra allows for the clarification of some important details. Most significantly, the signal corresponding to **NaGulA**-αH5 is measured here at 4.55 ppm, having been previously mis-assigned (see ESI Section 3.1).[24] Similarly, the complex spectral

region between 3.74 and 3.95 ppm in the ^{1}H NMR spectrum of **NaManA** has evaded full deconvolution in past analyses, but can be readily interpreted here.

Table 1 ^{1}H NMR spectroscopic assignments of sodium mono-uronate salts in D_2O (0.26 ± 0.02 M) at 600 MHz, pD 7.4 – 7.6, 295 K (relative to $C_6\underline{H}_6$ in C_6D_6, δ = 7.15 ppm).

δ (ppm) J (Hz)[a]	Na-D-mannopyranuronate α	 β	Na-L-gulopyranuronate α	 β
δH1	**5.17, d**	**4.87, s**	**5.17, d**	**4.84, d**
$J_{1,2}$	1.7	n.d.[b]	3.7	8.4
δH2	**3.86, *pt***	**3.89, d**	**3.91, *pt***	**3.61, dd**[c]
$J_{1,2}$	2.0	n.d.[b]	3.7	n.d.
$J_{2,3}$	3.3	3.2	4.0	n.d.
δH3	**3.83, dd**	**3.63, dd**	**3.99, *pt***	**4.05, m**
$J_{2,3}$	3.3	3.2	3.8	n.d.
$J_{3,4}$	9.2	8.9	4.3	n.d.[d]
δH4	**3.78, *pt***	**3.67, *pt***	**4.15, dd**	**4.05, m**
$J_{3,4}$	9.2	9.4	4.3	n.d.[d]
$J_{4,5}$	9.4	9.6	1.8	n.d.
δH5	**4.03, d**	**3.61, d**	**4.55, d**	**4.31, d**
$J_{4,5}$	9.3	9.6	1.8	0.8

Key: d = doublet, dd = doublet of doublets, *pt* = *pseudo*-triplet, m = multiplet, n.d. = not determined

[a] Scalar coupling constants (J) quoted to a precision of ± 0.1 Hz.

[b] Whilst $J_{1,2}$ is not resolved for β-**NaManA** at 600 MHz, at 400 MHz a value of $J_{1,2}$ = 1.1 Hz was measured.

[c] Additional splitting due to virtual coupling is also visible. At pD 1.4, βH3 and βH4 are sufficiently separated to remove the effects of virtual coupling to βH2 to give a well resolved dd with $J_{1,2}$ = 8.4 Hz and $J_{2,3}$ = 3.3 Hz

[d] A value for $J_{3,4}$ could not be measured for **NaGulA** at pD 7.6 as βH3 and βH4 are superimposed. However, at pD 1.4 the two signals are sufficiently separated to measure their mutual coupling as $J_{3,4}$ = 3.7 Hz.

Table 2 ^{13}C NMR spectroscopic assignments of sodium mono-uronate salts in D_2O (0.26 ± 0.02 M) at 600 MHz, pD 7.4 – 7.6, 295 K (relative to $\underline{C}_6D_6$ in C_6D_6, δ = 128.06 ppm).

δ (ppm)	Na-D-mannopyranuronate α	 β	Na-L-gulopyranuronate α	 β
δC1	94.29	93.99	93.26	93.89
δC2	70.81	71.49	64.82	69.14
δC3	70.49	73.26	71.46	71.64
δC4	69.39	68.97	71.32	71.46
δC5	73.33	76.73	67.75	74.77
δC6	177.41	176.67	176.98	176.29

5.2 Configurational equilibria of algal uronic acids in neutral and acidic solutions

The ^{1}H NMR spectra of the algal uronates at pD ≈7.5 contain groups of signals with areas in the ratios 100:50:6:4 (**NaManA**) and 100:22:6:3 (**NaGulA**), corresponding to the presence of various configurational isomers. Following assignments of the signals, the population distributions were calculated and are reported in Figure 5. It can be seen that the

[pyranose]:[furanose] ratio is approximately 13.5:1 for **NaGulA** and 15:1 for **NaManA**, noticeably lower than values reported for their neutral counterparts L-gulose[29] (32:1) and D-mannose[29] (111:1). For D-mannuronate, the ratio drops further to 10:1 as the pD is brought below the pK_a (see Section 5.4), whilst for L-guluronate it remains virtually unchanged. Understanding the stability of pyranuronates relative to furanuronates is important for modelling the mechanisms of lactone formation and degradation (see Section 5.5).

With regards to the pyranose configurations, D-mannuronate shows a two-fold preference for the α-anomer over the β-form (close to the $[\alpha]/[\beta]$ ratio of 1.90 previously reported for D-mannopyranose).[29] In contrast, the α-anomer of L-gulopyranuronate is heavily disfavoured, with $[\alpha]/[\beta]$ being 0.22 (again, similar to that observed for L-gulopyranose of 0.20).[29] These $[\alpha]/[\beta]$ ratios are quite invariant towards pD changes, with only modest deviations observed on transitioning from neutral to acidic conditions. Given the apparent instability of the α-pyranose configuration of L-guluronate, it is interesting to question why this form is found in alginic acid in place of the lower energy β-anomer. It is likely that the preferential metal-binding properties of the α-pyranose form of L-guluronate confer advantages to the kelp, which is the topic of an accompanying investigation.

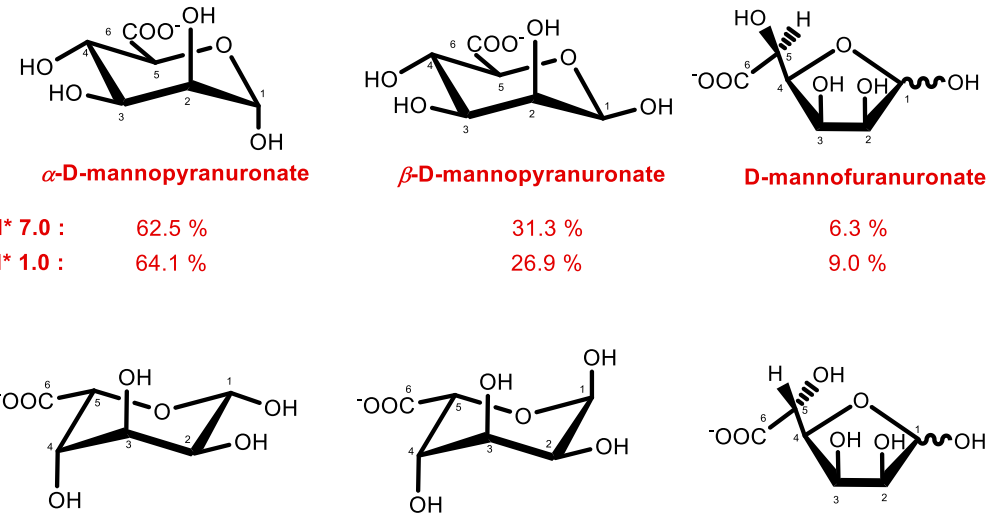

Figure 5 Speciation of sodium D-mannuronare (top) and L-guluronate (bottom) in neutral and acidic D_2O solutions (0.26 ± 0.02 M, 295 ± 2 K). Standard error ± 0.5 %.

5.3 Changes to the chemical shifts of HManA and HGulA with varying pD

The ^{1}H and ^{13}C NMR chemical shifts (δ) from analysis of **NaManA** and **NaGulA** (D_2O, 0.26 ± 0.02 M, 295 K) were recorded in the presence of increasing concentrations of DCl (from pD 7.5 to pD 1.4). The changes in δ ($\Delta\delta$) with decreasing pD for both anomers of the pyranoid forms of the two uronates are shown in Figures 6 - 9, in an analogous manner to those determined for Na-D-galacotpyranuronate and Na-D-glucopyranuronate by Jaques *et al*.[30] In the cases of the ^{13}C NMR spectra of **NaManA** and **NaGulA**, C6 (the carboxylate carbons) undergo the greatest changes upon acidification, with $\Delta\delta$ values of –3 to –4 ppm (similar to those determined for **NaGalA** and **NaGlcA**).[30]

The $\Delta\delta$ values determined for the ring-carbon nuclei of **HGulA** and **HManA** upon changing pD roughly decrease with increasing through-bond distance from the carboxylate moiety ($\Delta\delta$ C5 > C4 > C3 ≈ C2), mirroring the trend observed for **NaGalA** and **NaGlcA** (including the positive value of $\Delta\delta$ C1).[30] For the analogous analysis of $\Delta\delta$ in the ^{1}H NMR spectra of **NaGulA** and **NaManA** on lowering the pD, *pure shift* experiments were found to be particularly helpful, as they facilitated rapid measurement of the centre of a particular resonance without the need to deconvolute splitting patterns. The data in Figures 7 and 9 show that, unlike the ^{13}C NMR experiments, the ^{1}H $\Delta\delta$ values from all protons in both pyranose anomers of **NaManA** and **NaGulA** are positive with increasing [D_3O^+], corresponding to a deshielding of these nuclei. The magnitudes of the $\Delta\delta$ values again follow the order of distance of the proton from the carboxylate group, such that approximately $\Delta\delta$ H5 > H4 > H1 > H3 ≈ H2, in further agreement with the work of Jaques *et al.* for the **NaGlcA** and **NaGalA** systems.[30]

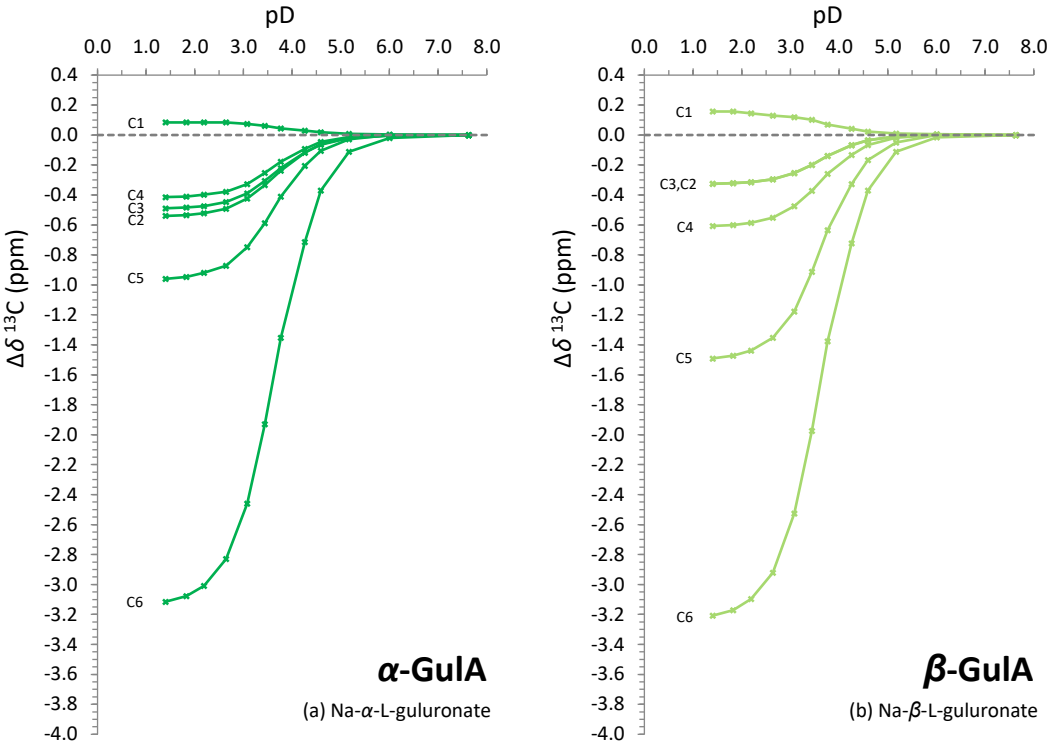

Figure 6 Plots of Δδ as a function of pD in the ^{13}C NMR spectra *versus* pD for the sodium salt of (a) α- and (b) β-L gulopyranuronate in D_2O (0.26 ± 0.02 M) at 600 MHz, and 295 K. The starting pD is 7.60, which is lowered by addition of small volumes of DCl. Horizontal error bars of ± 0.03 pD units omitted for clarity.

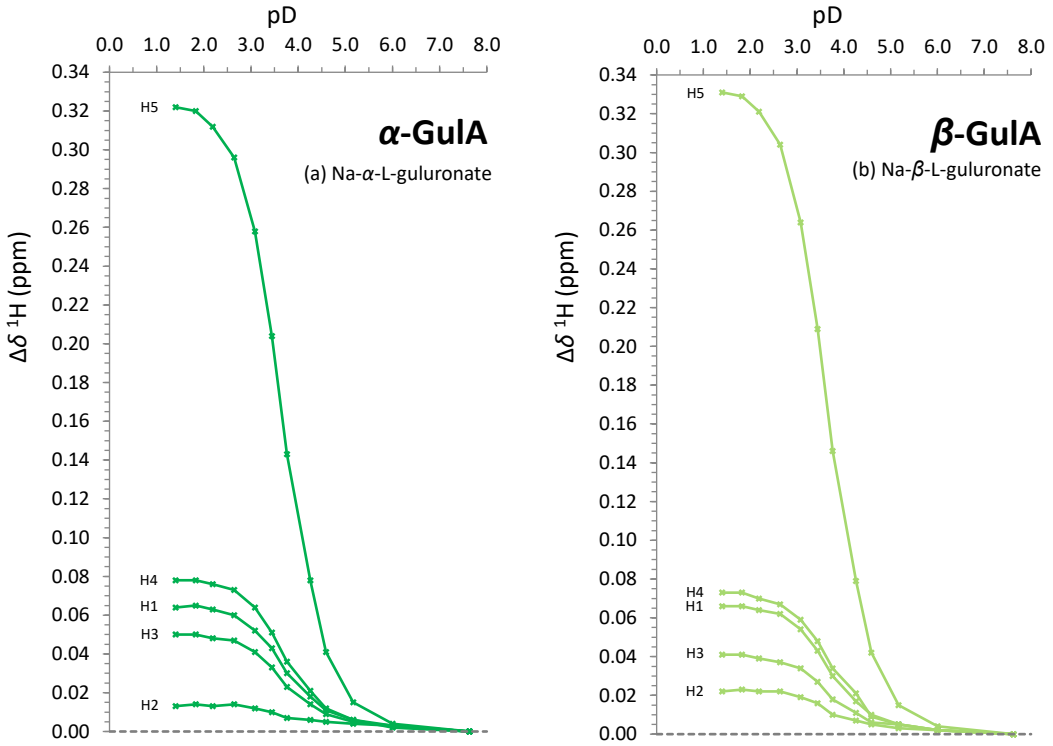

Figure 7 Plots of Δδ as a function of pD in the ^{1}H NMR spectra for the sodium salt of (a) α- and (b) β-L-gulopyranuronate in D_2O (0.26 ± 0.02 M) at 600 MHz, and 295 K. The starting pD is 7.60, which is lowered by addition of small volumes of DCl. Horizontal error bars of ± 0.03 pD units omitted for clarity.

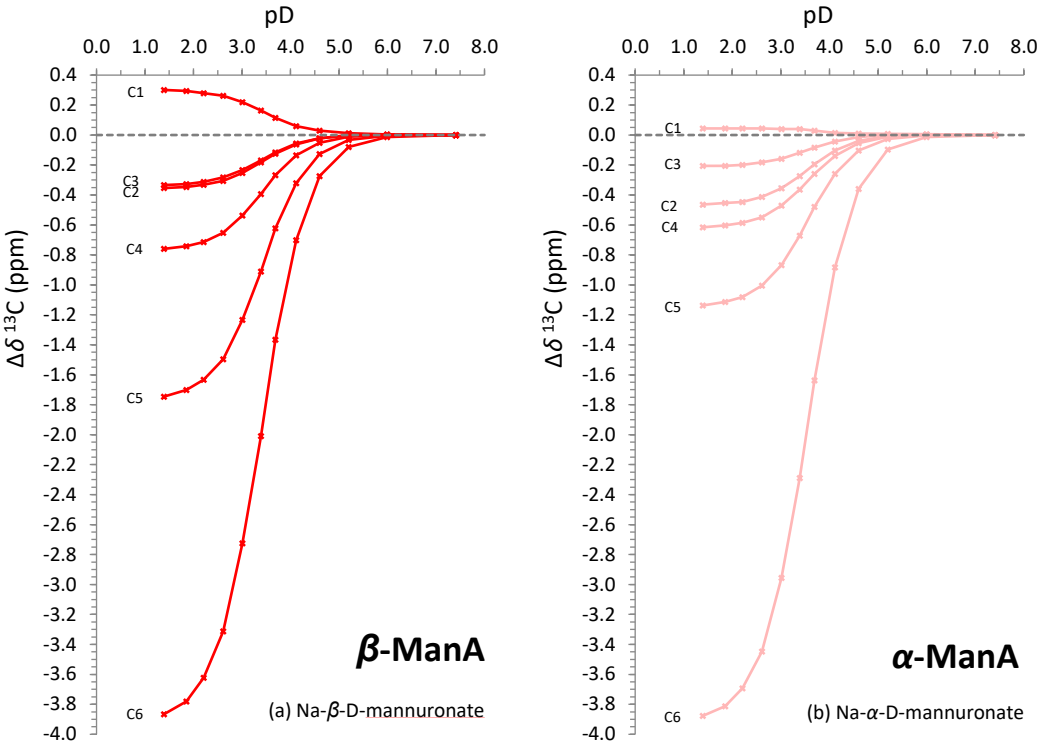

Figure 8 Plots of Δδ as a function of pD in the ^{13}C NMR spectra for the sodium salt of (a) β- and (b) α-D-mannopyranuronate in D_2O (0.26 ± 0.02 M) at 600 MHz, and 295 K. The starting pD is 7.41, which is lowered by addition of small volumes of DCl. Horizontal error bars of ± 0.03 pD units omitted for clarity.

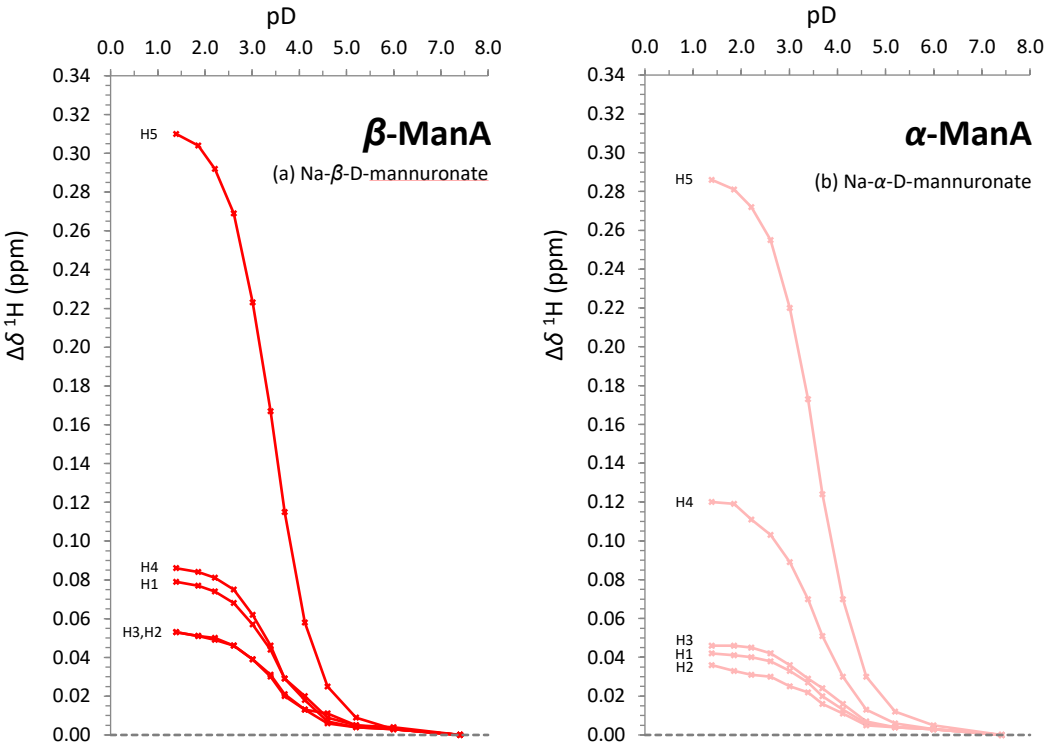

Figure 9 Plots of Δδ as a function of pD in the ^{1}H NMR spectra for the sodium salt of (a) β- and (b) α-D-mannopyranuronate in D_2O (0.26 ± 0.02 M) at 600 MHz, and 295 K. The starting pD is 7.41, which is lowered by addition of small volumes of DCl. Horizontal error bars of ± 0.03 pD units omitted for clarity.

5.4 Determination of pK_a of algal uronic acids

By plotting the $\Delta\delta$ values determined by $^{13}C\{^1H\}$ NMR spectroscopy for C6 (the carboxylate carbon) of a particular uronate against pH or pD, a corresponding measure of the pK_a of that species can be calculated.[31] This NMR titration-based methodology enables the determination of individual pK_a values for each of the species in solution, in contrast to values derived by potentiometric methods, from which only a weighted average can be determined.[32] Furthermore, Krężel and Bal have derived semi-empirical methods to convert a measured pK_a(H*) (the pK_a of a compound as measured in D_2O using the uncorrected reading of an electrode calibrated in H_2O) into an equivalent pK_a(H) (the pK_a of a compound in H_2O).[33] The results of the measurements of pK_a(H*), pK_a(H), and pK_a(soln) (the pK_a obtained by calculating an average of the individual pK_a(H) values of all species in solution weighted by their relative concentration) are reported in Table 3 for both anomers of the pyranose forms of **HManA** and **HGulA**.

The results presented in Table 3 indicate that, whilst the two anomers of L-gulopyranuronic acid have virtually the same pK_a(H) (approximately 3.25), the *β*-anomer of D-mannopyranuronic is a slightly stronger acid than its *α*-counterpart by between 0.07 to 0.19 pK_a units. From the calculated pK_a(H) values, the corresponding pK_a(soln) values for **HGulA** and **HManA** were found to be 3.44 ± 0.03 and 3.29 ± 0.03, respectively, slightly lower than those derived by potentiometric titration by Haug *et al.* (3.65 for **HGulA** and 3.38 for **HManA**).[34] Interestingly, Wang *et al.*, who performed similar NMR-based titration experiments on *α*- and *β*-glucopyranuronic acids, also reported slightly lower values than those that they had determined by potentiometric means under similar conditions.[32] These variations are expected, since pK_a values vary with ionic strength as well as with the nature of the medium in which they were undertaken for a particular study. Regardless of the absolute values, **HManA** is found to be the stronger acid in all cases, with a pK_a(soln) lower than **HGulA** by 0.15 ± 0.06 units. It is noteworthy that the two constituent monomers of alginic acid (*α*-**HGulA** and *β*-**HManA**) have a pK_a(H) difference of between 0.24 ± 0.06, which is large enough to substantially influence the properties (especially hydrothermal behaviour) of kelps possessing alginates with differing mannuronate:guluronate ratios.[34–39]

Table 3 pK_a values for individual anomers of algal mono-pyranuronic acids and the weighted average pK_a of their resulting solutions.

Uronic acid	Relative concentration[a]	pK_a (H*)[b]	pK_a (H)[c]	pK_a (soln)[d]	pK_a (soln)-lit[34]
$^1C_4$-α-L-gulopyranuronic acid	18 %	3.26	3.45	3.44	3.65
$^1C_4$-β-L-gulopyranuronic acid	82 %	3.25	3.44		
$^4C_1$-α-D-mannopyranuronic acid	70 %	3.13	3.33	3.29	3.38
$^4C_1$-β-D-mannopyranuronic acid	30 %	3.00	3.21		

[a] Determined at a pD value close to the pK_a
[b] Measured in D_2O using an uncorrected pH value
[c] Converted to an equivalent value in H_2O using the equation of Krężel and Bal[33]: $pK_a(H) = 0.929pK_a(H^*) + 0.42$. Standard error ± 0.03
[d] Weighted average of $pK_a(H)$ values from all species in solution, omitting small contributions from furanuronic acids. Standard error ± 0.03.

5.5 Lactonisation of algal uronic acids

While the ability of **HGlcA** to form a furanurono-6,3-lactone (see Figure 2) under thermal and hydrothermal conditions is well documented, there is also evidence to suggest that algal uronic acids behave in a similar manner. In order to compare the saccharides directly, solutions of 50 mM **NaGlcA**, **NaManA**, **NaGulA**, and **NaGalA** (the latter being stereochemically forbidden from forming a 6,3-lactone) were subjected to various heating regimes, and the uronate:lactone ratio monitored by standard ^{1}H NMR spectroscopy (see Table 4).

It is found that no lactones form in the uronate solutions at pD ≈ 7.5, even upon heating for 12 hours at 60 °C (Experiment **A**, Table 4). Upon lowering the pD to 1.4 at room temperature, NMR signals corresponding to lactones were observable after 24 hours (full characterisation provided in ESI). Allowing the solutions to reach equilibrium at room temperature (Experiment **B**) yielded uronate:lactone ratios of 80:20 (**HManA**), 96:4 (**HGulA**), and 83:17 (**HGlcA**), with the latter value being in good agreement with previous measurements.[3] Subsequent heating of the solutions at 60 °C (Experiment **C**) and 90 °C (Experiment **D**) pushes the equilibrium increasingly towards lactone formation in all but solutions of **HGalA**, which remains unchanged (see ESI Figure S.13). The data from

Experiments **C** and **D** (Table 4) are also in good agreement with those from previous polarimetry studies, which determined a uronate:lactone ratio of 60:40 for a sample of **HManA** heated at 80 °C for 19.5 hours (but at an unreported pH),[12] and a ratio of 40:60 for a sample of **HGlcA** heated at 100 °C for 2 hours at pH 2.5 – 3.5.[3] As the solutions of **NaGlcA**, **NaManA**, **NaGulA**, and **NaGalA** were heated more severely, a yellowing was observed coupled with the evolution of a furfural-type odour, and the precipitation of brown polymeric compounds. Together, these observations are all indicative of irreversible thermolysis taking place. At the end of Experiment **D** (Table 4), the samples were left to stand at room temperature; the remaining uronates returned to the same equilibrium positions as was found following Experiment **B**. Neutralisation of the solutions after Experiment **D** induced complete hydrolysis of any lactones still present.

Table 4 Lactonisation of mono-uronic acids under various conditions

	Experiment	**A**	**B**	**C**	**D**
	Duration (hours)	12	Eq[a]	12	60
	Temperature (°C)	60	RT[b]	60	90
	pD	7.5	1.4	1.4	1.4
Constitution of solution (uronate : lactone)	**HGlcA**	100:0	83:17	64:36	48:52[c]
	HManA	100:0	80:20	68:32	48:52[c]
	HGulA	100:0	96:4	83:17	71:29[c]
	HGalA	100:0	100:0	100:0	100:0[c]

[a] Solutions stood at room temperature until no further changes were detected
[b] RT = room temperature
[c] Yellowing of the solutions observed, along with the formation of brown precipitate and furfural odour

Analysis of the data in Table 4 demonstrates the similarities in lactonisation behaviour between **HGlcA** and **HManA**, while **HGulA** proves to be less susceptible to lactone formation. Whilst lactonisation is believed to be an important preliminary step in the thermolysis of uronides,[40,41] it is clearly only of relevance when the carboxylate is protonated, and when cyclisation is stereochemically feasible. The degradation of **HGalA**, which is stereochemically unable to form a lactone, is evidence of the presence of competing degradation mechanism(s) that warrant future investigation.

6 Conclusions and outlook

In this work, solutions of L-guluronate and D-mannuronate were isolated and studied by NMR spectroscopy. The assignments of ^{1}H NMR spectra across a range of pD values (7.5 – 1.4) are reported with high-confidence, with ambiguities from previously-reported data clarified. The *pure shift* technique was found to be especially useful in this task and will be a valuable tool for simplifying the NMR spectroscopic analysis of carbohydrates more widely. From the data obtained it was possible to measure pK_a values for all anomers of the pyranose forms of the algal uronic acids, as well as their relative concentrations in solution. It was found for **HGulA** that the β-pyranose form is the more stable, and has a similar pK_a to α-**HGulA**, whilst for **HManA**, the α-pyranose form is the most stable form and has a lower pK_a than that of the β-anomer. Furthermore, it was demonstrated that the lactonisation of uronic acids can only occur under acidic conditions, with **HManA** forming lactones to roughly the same extent as **HGlcA**, while **HGulA** is more resistant to lactonisation.

The analysis reported in this paper will better enable the study of complex product mixtures arising from thermochemical treatment of alginate-rich algal biomass. The results also enhance the current understanding of the solution state behaviour of algal uronic acids, which will help in the modelling and design of processes and catalysts employed in the upgrading of these fundamental biomass units.

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8 References

1 J. S. Rowbotham, P. W. Dyer, H. C. Greenwell and M. K. Theodorou, *Biofuels*, 2012, **3**, 441–461.

2 X. Li, P. Jia and T. Wang, *ACS Catal.*, 2016, **6**, 7621–7640.

3 C. A. Marsh, in *Glucuronic Acid Free and Combined*, Elsevier, 1966, vol. 35, pp. 3–136.

4 R. M. de Lederkremer and C. Marino, in *Adv. Carbohydr. Chem. Biochem.*, 2003, vol. 58, pp. 199–306.

5 F. Delbecq, Y. Wang, A. Muralidhara, K. El Ouardi, G. Marlair and C. Len, *Front. Chem.*, 2018, **6**, 146.

6 E. D. T. Atkins, I. A. Nieduszynski, W. Mackie, K. D. Parker and E. E. Smolko, *Biopolymers*, 1973, **12**, 1865–1878.

7 D. W. Drummond, E. L. Hirst and E. Percival, *J. Chem. Soc.*, 1962, 1208–1216.

8 E. D. T. Atkins, I. A. Nieduszynski, W. Mackie, K. D. Parker and E. E. Smolko, *Biopolymers*, 1973, **12**, 1879–1887.

9 L. H. Cretcher and W. L. Nelson, *Science*, 1928, **67**, 537–538.

10 W. L. Nelson and L. H. Cretcher, *J. Am. Chem. Soc.*, 1929, **51**, 1914–1922.

11 W. L. Nelson and L. H. Cretcher, *J. Am. Chem. Soc.*, 1930, **52**, 2130–2132.

12 W. L. Nelson and L. H. Cretcher, *J. Am. Chem. Soc.*, 1932, **54**, 3409–3412.

13 F. G. Fischer and H. Dörfel, *Hoppe-Seyler´s Zeitschrift für Physiol. Chemie*, 1955, **302**, 186–203.

14 A. Meillisa, H.-C. Woo and B.-S. Chun, *Food Chem.*, 2015, **171**, 70–77.

15 K. Zangger, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2015, **86**–**87**, 1–20.

16 J. A. Aguilar, S. Faulkner, M. Nilsson and G. A. Morris, *Angew. Chemie Int. Ed.*, 2010, **49**, 3901–3903.

17 M. Foroozandeh, R. W. Adams, N. J. Meharry, D. Jeannerat, M. Nilsson and G. A. Morris, *Angew. Chemie Int. Ed.*, 2014, **53**, 6990–6992.

18 E. Candès and J. Romberg, *Inverse Probl.*, 2007, **23**, 969–985.

19 E. J. Candes and M. B. Wakin, *IEEE Signal Process. Mag.*, 2008, **25**, 21–30.

20 D. L. Donoho, *IEEE Trans. Inf. Theory*, 2006, **52**, 1289–1306.

21 E. J. Candes, J. Romberg and T. Tao, *IEEE Trans. Inf. Theory*, 2006, **52**, 489–509.

22 J. A. Aguilar and A. M. Kenwright, *Magn. Reson. Chem.*, 2018, **56**, 983–992.

23 H. Grasdalen, F. Mo, J. Olav Bjørgum and I. R. Siddiqui, *Carbohydr. Res.*, 1990, **203**, 281–286.

24 C. A. Steginsky, J. M. Beale, H. G. Floss and R. M. Mayer, *Carbohydr. Res.*, 1992, **225**, 11–26.

25 Konino, M.; Takashi, O., JP4897310 (B2), *JPO*, 2007, 1–11.

26 A. Matsumoto, S. Hosoyama, K. Higashi and T. Toida, *Carbohydr. Res.*, 2012, **358**, 82–88.

27 B. Larsen, D. M. S. A. Salem, M. A. E. Sallam, M. M. Mishrikey and A. I. Beltagy, *Carbohydr. Res.*, 2003, **338**, 2325–2336.

28 A. Penman and G. R. Sanderson, *Carbohydr. Res.*, 1972, **25**, 273–282.

29 S. J. Angyal, in *Advances in Carbohydrate Chemistry and Biochemistry*, 1984, pp. 15–68.

30 L. W. Jaques, J. B. Macaskill and W. Weltner, *J. Phys. Chem.*, 1979, **83**, 1412–1421.

31 J. Bezençon, M. B. Wittwer, B. Cutting, M. Smieško, B. Wagner, M. Kansy and B. Ernst, *J. Pharm. Biomed. Anal.*, 2014, **93**, 147–155.

32 H. M. Wang, D. Loganathan and R. J. Linhardt, *Biochem. J.*, 1991, **278**, 689–695.

33 A. Krężel and W. Bal, *J. Inorg. Biochem.*, 2004, **98**, 161–166.

34 A. Haug, *Acta Chem. Scand.*, 1961, **15**, 950–952.

35 F. Delben, À. Cesaro, S. Paoletti and V. Crescenzi, *Carbohydr. Res.*, 1982, **100**, C46–C50.

36 C. Rey-Castro, P. Lodeiro, R. Herrero and M. E. Sastre de Vicente, *Environ. Sci. Technol.*, 2003, **37**, 5159–5167.

37 A. Haug, S. Myklestad, B. Larsen, O. Smidsrød, G. Eriksson, R. Blinc, S. Paušak, L. Ehrenberg and J. Dumanović, *Acta Chem. Scand.*, 1967, **21**, 768–778.

38 A. Haug, B. Larsen, B. Samuelsson, J. Sjövall and J. Munch-Petersen, *Acta Chem. Scand.*, 1963, **17**, 1653–1662.

39 E. Fourest and B. Volesky, *Appl. Biochem. Biotechnol.*, 1997, **67**, 215–226.

40 R. Wang, T. Kobayashi and S. Adachi, *J. Appl. Glycosci.*, 2009, **56**, 181–184.

41 R. Wang, T. L. Neoh, T. Kobayashi, Y. Miyake, A. Hosoda, H. Taniguchi and S. Adachi, *Biosci. Biotechnol. Biochem.*, 2010, **74**, 601–605.