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# THE DETERMINATION OF THE URONIC ACID COMPOSITION OF ALGINATES BY ANION-EXCHANGE LIQUID CHROMATOGRAPHY

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## ABSTRACT

An isocratic, anion-exchange, liquid chromatography system has been developed to separate and quantify the uronic acids (D-mannuronic and L-guluronic acids) that are present in acid hydrolysates of alginates. The sensitivity of the technique will allow 10 $\mu$g of uronic acid to be detected. The method has the advantages of speed (typical analysis time, 20 min) and a small-sample requirement (routine analysis, 140 $\mu$g of hydrolysate), whilst retaining the accuracy of the traditional method.

## INTRODUCTION

Alginates are (1→4)-linked glycuronans made up of residues of $\beta$-D-mannosyluronic acid (M) and $\alpha$-L-gulosyluronic acid (G) arranged in block structures[1] that can be homopolymeric [poly($\beta$-D-mannosyluronic acid) (MM) and poly($\alpha$-L-gulosyluronic acid) (GG)] or heteropolymeric [often occurring[2] as alternating blocks (MG)]. These polysaccharides are synthesised as cell-wall components by such brown seaweeds as *Ascophyllum nodosum*. These algae provide the traditional source of this commercially important polysaccharide. Alginates are also produced by certain species of bacteria, *e.g.*, *Azotobacter vinelandii* and *Pseudomonas aeruginosa*.

The gelling characteristics of alginates are influenced by the uronic acid composition, and the M/G ratio is an index of the nature of the gel that is formed in the presence of divalent cations[3]. In particular, alginates having a low M/G ratio give strong, brittle gels, whereas alginates having a high M/G ratio form more elastic gels[4,5].

The standard method for determining M/G ratios for alginate samples involves acid hydrolysis followed by analysis of the resulting uronic acids by gradient-elution anion-exchange chromatography[1]. Although reliable, the published methods require several hours for each analysis[6–8] and are not suited to the analysis of a large number of samples. An alternative approach involves the differential effect of borate in the carbazole reaction[9], although the method consistently overestimates L-guluronate[4]. A g.l.c. procedure has also been described[10], but this

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required derivatisation of the uronic acids. Recently, more sophisticated and non-destructive methods based on circular dichroism[11] and n.m.r.[4,12] have been applied to the analysis of alginates; these methods rely on the availability of expensive equipment and are not suitable as general laboratory procedures.

We now report the development of a rapid, isocratic, anion-exchange, liquid chromatography (l.c.) system for the separation and analysis of $\beta$-D-mannuronic and $\alpha$-L-guluronic acids, and its application to acid hydrolysates of alginate samples.

## EXPERIMENTAL

The liquid chromatograph consisted of the following components: a Waters 6000A pump, a Rheodyne 7125 loop-injector fitted with a 20-$\mu$L loop (Jones Chromatography Ltd.), a Whatman Partisil 10-SAX anion-exchange column (250 $\times$4.6 mm i.d.), a Cecil CE2112 variable-wavelength u.v. detector, and a Philips PM 8251 chart-recorder.

D-Glucurono-6,3-lactone, D-mannurono-6,3-lactone, sodium D-glucuronate, and D-galacturonic acid were commercial materials (Sigma). Sodium L-guluronate was prepared by the acid hydrolysis of sodium alginate and purified by anion-exchange chromatography[1]. The alginates were commercial Manucol DH (Cambrian Chemicals Ltd.), Alginic acid (BDH Ltd.), and Manugel DMB (a gift from Alginate Industries Ltd.). Two samples of sodium alginate were extracted from *Ascophyllum nodosum* by standard methodology[13].

Chromatographic standards were prepared from uronic acids or lactones and were adjusted to a final concentration of 1% (v/v) triethylamine 10 min prior to analysis, in order to hydrolyse lactones[14]. Standards were eluted isocratically with 0.02M $KH_2PO_4$ (Aristar grade, pH 4.6) containing 5% (v/v) of methanol, at a flow rate of 1.0 mL.min$^{-1}$. Uronic acids were detected by absorption at 210 nm. Alginate samples were hydrolysed with $H_2SO_4$, and the hydrolysates were neutralised and filtered to remove any particulate matter, as described by Haug and Larsen[1]. These samples were treated with triethylamine (1% v/v) and chromatographed in the same way as the standards. For comparison, the ratio of D-mannuronate to L-guluronate (M/G ratio) in the various hydrolysates was determined independently by the standard method of Haug and Larsen[1], using the multiplication factor of 0.66 to correct for the preferential destruction of L-guluronate and the different reactivities of the two uronic acids to the orcinol reagent. Uronic acids were analysed by the differential carbazole method[9], and by descending paper chromatography (p.c.) on Whatman No. 1 paper with pyridine–ethyl acetate–acetic acid–water (5:5:1:3)[15] and detection with alkaline silver nitrate[16].

## RESULTS AND DISCUSSION

Chromatography of D-mannuronate and L-guluronate established that the

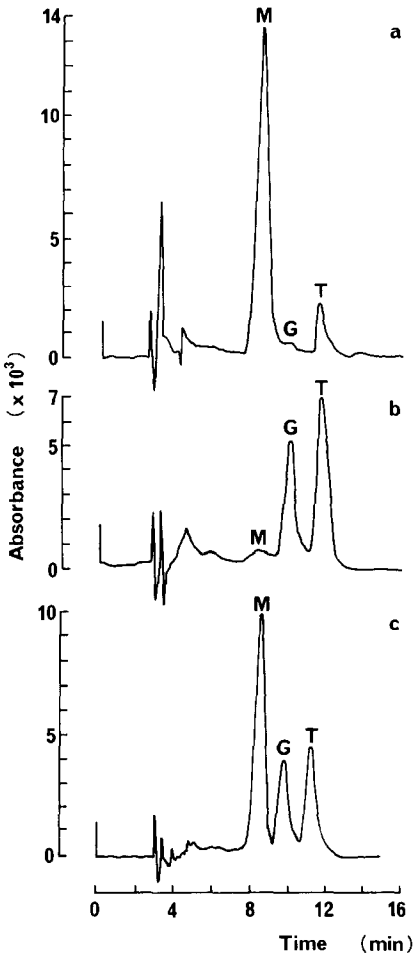


Fig. 1. Chromatography of (*a*) D-mannuronate (M), (*b*) L-guluronate (G), and (*c*) D-mannuronate + L-guluronate on Partisil 10-SAX eluted with 20mM $KH_2PO_4$ (pH 4.6) containing 5% (v/v) of methanol at a flow rate of 1.0 mL.min$^{-1}$. T connotes triethylamine.

optimum conditions of ionic strength and pH for the selective retention of uronic acids on a column of Partisil 10-SAX were 20mM $KH_2PO_4$ (pH 4.6) containing 5% (v/v) of methanol. Under these conditions, the D-mannuronate was eluted at 8.1 min ($k'$ 2.9) followed by L-guluronate at 9.6 min ($k'$ 3.4) (see Fig. 1). The D-mannuronolactone standard contained a small amount of L-guluronate (Fig. 1*a*). Similarly, the L-guluronate sample was contaminated by a small quantity of D-mannuronate (Fig. 1*b*). The addition of triethylamine to the sample was essential to eliminate lactones. Samples not treated with alkali remained in the lactone form, which was eluted considerably earlier than the free acid (Table I). The lactone form of D-mannuronate is particularly stable and, although the use of triethylamine obviated this problem, it did introduce a u.v.-absorbing peak that was eluted after the L-

TABLE I

RETENTION TIMES OF URONIC ACIDS ON PARTISIL 10-SAX[a]

| *Carbohydrate* | *Retention time (min)*[b] | | | |
|---|---|---|---|---|
| | *5mM Buffer (0.4 mL/min)* | *5mM Buffer (1.0 mL/min)* | *20mM Buffer (0.6 mL/min)* | *20mM Buffer (1.0 mL/min)* |
| D-Glucuronate | 43.2 (2.0) | 15.7 (0.9) | 12.0 (0.8) | 7.4 (0.4) |
| D-Mannuronate | 45.0 (2.2) | 16.2 (1.0) | 12.3 (0.8) | 8.1 (0.6) |
| D-Galacturonate | 49.5 (1.7) | 17.7 (1.7) | 13.8 (1.5) | 8.9 (1.1) |
| L-Guluronate | 53.0 (2.3) | 19.0 (0.9) | 14.4 (0.6) | 9.6 (0.4) |
| D-Glucurono-6,3-lactone | n.d.[c] | n.d. | n.d. | 7.4 (0.4) |
| D-Mannurono-6,3-lactone | n.d. | n.d. | n.d. | 8.1 (0.6) |

[a]Standards of uronic acids (typically, 20 μg) were applied to the column, and eluted at the flow rate indicated with $KH_2PO_4$ buffer (pH 4.6) containing 5% (v/v) of methanol. [b]Peak width at half peak-height indicated in parenthesis. [c]Not determined.

TABLE II

COMPARISON OF RETENTION TIME WITH $pK_a$ VALUES OF URONIC ACIDS[a]

| *Carbohydrate* | *Buffer, pH 3.5* | *Buffer, pH 4.6* | $pK_a$[17] |
|---|---|---|---|
| D-Glucuronate | 7.3 (0.7) | 7.4 (0.4) | 3.20 |
| D-Mannuronate | 7.0 (0.7) | 8.1 (0.6) | 3.38 |
| D-Galacturonate | 6.7 (0.7) | 8.9 (1.1) | 3.42 |
| L-Guluronate | 8.1 (0.4) | 9.6 (0.4) | 3.65 |

[a]Standards of uronic acids (typically, 20 μg) were applied to the column, and eluted (1.0 mL.min$^{-1}$) with 20mM $KH_2PO_4$ containing 5% (v/v) of methanol. Results are expressed as indicated in Table I.

guluronate (Fig. 1). This impurity did not interfere with the quantification of the uronic acids, and it was noted that the height of its peak was inversely proportional to the quantity of lactone hydrolysed.

Other uronic acid standards were chromatographed under the conditions described above. The results in Table I show that it was not possible to separate all the uronic acids from each other with baseline resolution. In particular, the C-2 epimers, D-mannuronate and D-glucuronate, were not completely resolved by using a flow rate of 1.0 mL.min$^{-1}$ and 20mM phosphate buffer. Furthermore, D-galacturonate was eluted as a comparatively broad peak. No improvement in the separation was achieved either by lowering the flow rate or by reducing the ionic strength of the buffer. However, a combination of low flow-rate (0.4 mL.min$^{-1}$) and low buffer strength (5mM) did separate D-glucuronate and D-mannuronate sufficiently to allow identification of the individual uronic acids.

The elution sequence of the various uronic acids was not as might have been predicted from the published $pK_a$ values of the carboxylate groups[18] (Table II). This result indicates that the interaction of the uronic acids and Partisil 10-SAX is

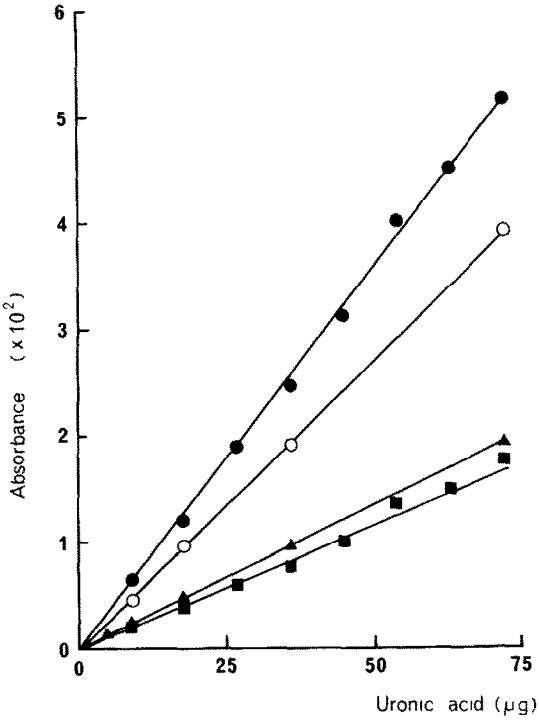


Fig. 2. Correlation between absorbance at 210 nm and the amount of D-glucuronate (—●—), D-mannuronate (—○—), D-galacturonate (—■—), and L-guluronate (—▲—) applied to the column. Elution conditions are as described in Fig. 1.

not solely a function of the $pK_a$ value, but could involve the partial molar volumes of the uronates or intramolecular bonding, as has been suggested for organic acids[19].

Analysis of each of the uronic acid standards showed that there was a linear relationship between peak height and the amount of material present (Fig. 2). Separate calibration curves were necessary, because the detector response was different for each of the uronic acids; the relative responses were D-glucuronate, 100%; D-mannuronate, 76%; L-guluronate, 35%; and D-galacturonate, 33%. With the system employed in this work, the minimum amount of D-glucuronic acid that could be detected was ~10 μg.

To establish that the u.v.-absorbing peaks eluted from the column correlated with uronic acids, standard solutions of D-mannuronate and L-guluronate were chromatographed [20mM $KH_2PO_4$ (pH 4.6), 5% (v/v) methanol], and the eluant corresponding to each peak was collected for further analysis. Analysis by the differential carbazole method showed that the u.v.-absorbing peaks corresponded to D-mannuronate and L-guluronate. Furthermore, descending p.c. of the samples showed that the eluted material co-chromatographed with the standard samples of

TABLE III

DETERMINATION OF M/G RATIOS IN ACID HYDROLYSATES OF ALGINATES[a]

| *Sample* | *I. c* | *Standard estimation* |
|---|---|---|
| *Ascophyllum nodosum* Preparation I | 1.05 | 1.15 |
| Alginic acid (BDH) | 0.94 | 0.82 |
| Manucol DH | 1.10 | 1.12 |
| Manugel DMB | 0.49 | 0.56 |
| *Ascophyllum nodosum* Preparation II | 7.50 | 7.69 (see ref. 17) |
| Alternating-block structure | 1.40 | 1.50 (see ref. 10) |

[a]Samples of alginate were hydrolysed with acid, and analysed for D-mannuronate and L-guluronate by l.c. and by the standard method[1]. The M/G ratios obtained by l.c. have been multiplied by a factor of 0.85 to compensate for the preferential destruction of L-guluronate during acid hydrolysis[1]. The two uronic acids have different reactivities with the orcinol reagent[1], and a correction factor of 0.66 was used to offset both effects in the standard estimation. Experimental conditions are described in the text.

uronic acids. It is concluded, therefore, that the u.v.-absorbing peaks corresponded to D-mannuronate and L-guluronate.

In extending the method to the analysis of acid hydrolysates of alginates, six different samples of alginate were hydrolysed to determine the M/G ratio[5]. Four of these samples were analysed independently by the method of Haug and Larsen[1]. Representative chromatograms of two of the samples are shown in Fig. 3, and the results of the other samples are given in Table III. There is a good correlation between the l.c. results and those obtained by the classical method. The chromatogram in Fig. 3*a* is for an alginate sample containing 90% of D-mannuronate but, even at this M/G ratio, the L-guluronate peak is not obscured and it is possible to estimate M/G ratios as high as 10. In addition to the D-mannuronate and L-guluronate peaks, there were a number of other u.v.-absorbing components, but they did not interfere with the quantification of D-mannuronate and L-guluronate. As the alginates contain only D-mannuronate and L-guluronate, the column can be eluted with 20mM $KH_2PO_4$ (pH 4.6) containing 5% (v/v) of methanol at a flow rate of 1.0 mL.min$^{-1}$, enabling the analysis to be completed within 25 min. However, to check for the presence of other uronic acids, a separation procedure using 5mM $KH_2PO_4$ (pH 4.6) containing 5% (v/v) of methanol at a flow rate of 0.4 mL.min$^{-1}$ would be advantageous.

Thus, isocratic anion-exchange l.c. is suitable for the analysis of acid hydrolysates of alginates. The M/G ratios obtained by this procedure are comparable with those obtained by conventional methods of analysis, but the l.c. technique permits a faster analysis on considerably less material without sacrificing accuracy.

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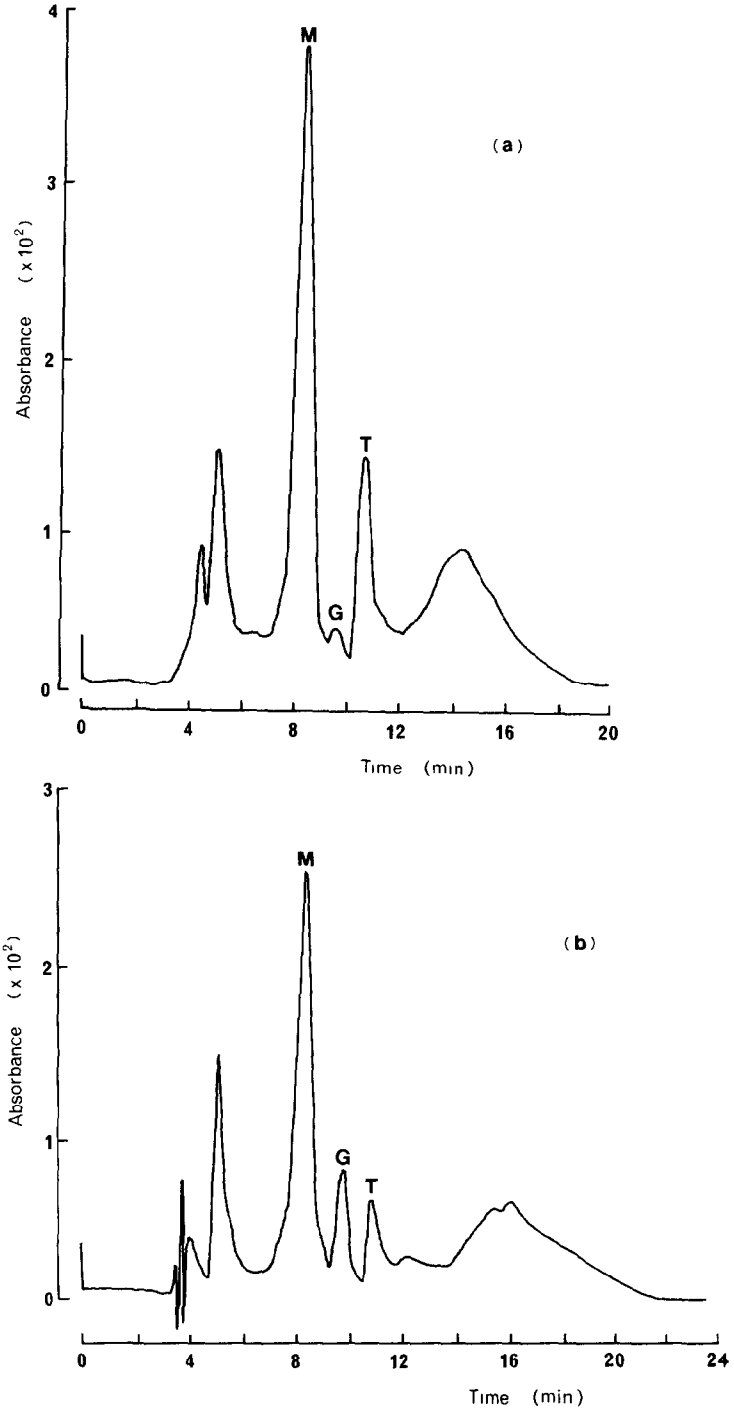


Fig. 3. Chromatography of acid hydrolysates of (a) *Ascophyllum nodosum* Preparation II, and (b) Manucol DH. Elution conditions are as described in Fig. 1.

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