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Research paper

A simple, high throughput method for the quantification of sodium alginates on oesophageal mucosa

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Abstract

Sodium alginate is a potential bioadhesive, but the lack of a convenient and suitable method for its quantification on the mucosal surface complicates the evaluation of its mucosal retentive properties. This paper develops and evaluates a spectrophotometric method for the rapid quantification of a range of sodium alginates differing in chemical composition, and investigates how quantification was influenced by the presence of oesophageal mucosa. The method, based on dye complexation with 1,9-dimethyl methylene blue (DMMB) was sensitive to alginate molecular weight and uronic acid composition, however, no significant correlations between assay performance and alginate molecular characteristics were demonstrated. The assay was also influenced by complexation time, calcium ions and mucin, but was unaffected by the presence of oesophageal tissue scrapings. The assay proved to be capable of quantifying sodium alginate with excellent linearity ($r = 0.999$), reproducibility (CV $< 3\%$) and sensitivity (0.3 g l^{-1}) and proved to be a precise, high-throughput method that may be used for quantifying the retention of sodium alginate on oesophageal mucosa.

Keywords: Alginate; Bioadhesion; Mucoadhesion; Dimethyl methylene blue; Quantification; Mucosa; Oesophagus

1. Introduction

Alginates are linear polysaccharides of 1,4 linked α-L-guluronic acid and β-D-mannuronic acid. They have recognised GRAS status and are utilised extensively as gelling and viscosity-increasing additives in pharmaceutical and food applications[1,2]. Sodium alginate is a principal component of anti-reflux medicines and is widely prescribed for symptomatic relief of gastro-oesophageal reflux disease[3]. Increasingly, it is being recognised that recurrent gastric reflux damages the lower oesophageal mucosa and may have serious long-term clinical consequences[4,5]. As a result there has been an increasing interest in developing protectants, which if retained on the oesophageal mucosal surface, would offer a novel opportunity for treatment of the local disease state[6,7]. Sodium alginate based dosage forms have been proposed as potential oesophageal protectants and have been evaluated using a novel bioadhesion testing system[8]. The retention of sodium alginate on oesophageal mucosa was tested by washing the tissue with artificial saliva and the amount of alginate eluted determined fluorometrically. Chemical assays may provide an alternative method for quantifying sodium alginate retention on the oesophageal mucosa and may allow further characterisation of the bioadhesive properties of sodium alginate dosage forms. Ideally, the quantification method should be unaffected by the presence of the mucosa and the artificial saliva used to wash the tissue. Additionally, the assay should be sensitive, precise, reproducible, simple and high-throughput. Alginates have been quantified chromatographically following acid hydrolysis, colorimetrically after uronic acid degradation[9], or by UV spectrophotometry following detergent complexation[10]. Unfortunately these methods involve either extensive sample preparation or operate over a narrow pH range. An alternative quantification method for alginate determination in polylysine/alginate microcapsules has been developed by Halle et al.[11]. This assay involves complexation between sodium alginate and a cationic dye, 1,9-dimethyl methylene blue (DMMB). The extent of complexation is dependent on alginate concentration and

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can be measured by the absorbance ratio at 520:650 nm, the absorbance maxima of bound and unbound DMMB. The present study investigated the potential for using this method to quantify the retention of sodium alginate on oesophageal mucosa. It determined the effect of alginate chemical composition on assay performance and we discuss the influence of saliva composition and the presence of oesophageal mucosa on assay response.

2. Materials and methods

2.1. Materials

Sodium alginates (Protanal®) of molecular weight (M_w) 34.7–449.3 kDa, and of mannuronate/guluronate ratio 0.44–1.38 were a gift from FMC BioPolymer AS, Drammen, Norway. They were stored at 4 °C in tightly-sealed containers and all alginate weighings were corrected for water content. DMMB, mucin type II (porcine gastric) and all other reagents (analytical grade) were obtained from Sigma-Aldrich Co Ltd, Dorset, UK. Water was Maxima HPLC grade with a maximum conductance of 18.2 MΩ/cm (USF Elga, Buckinghamshire, UK). Artificial saliva, composition 1, was 5 mM sodium bicarbonate, 7.36 mM sodium chloride, 20 mM potassium chloride, 6.6 mM sodium dihydrogen phosphate monohydrate, 1.5 mM calcium chloride dihydrate in water[12]. Artificial saliva, composition 2, was composition 1 without calcium chloride dihydrate.

2.2. Preparation of sodium alginate solutions

Artificial saliva composition 1 was used in all experiments except where the two saliva compositions were compared. A 0.5% w/v solution was prepared by completely dissolving sodium alginate in artificial saliva. This solution was then used to prepare 1 ml standard solutions over the range 0.1–4.5 g l^{-1} by diluting the solution with artificial saliva.

2.3. Alginate quantification procedure

One millilitre of 0.8 M sodium hydroxide was mixed with 1 ml standard alginate solution and neutralised after 5 min with 120 μl 2.25 M citric acid; 40 μl DMMB was then added to the sample, which was vortex mixed and incubated at room temperature for 45 min. UV-visible spectra of the solution was then obtained using a 10 mm pathlength cell in an Agilent 8453 diode array spectrophotometer (Agilent Technologies UK Ltd, Stockport, England). Absorbance was measured at 520 and 650 nm and the 520:650 nm absorbance ratio calculated.

2.4. Dependence of response on complexation time

The assay procedure was undertaken on 1 ml aliquots of 2.5 g l^{-1} alginate solution (Protanal® LF120L). The incubation time for DMMB-alginate complexation was varied between 2 and 60 min.

2.5. Effect of alginate molecular weight and uronic acid composition on assay performance

The method was applied to a range of alginates, which varied in molecular weight between 34.7 and 449.3 kDa and in mannuronate/guluronate ratio between 0.44 and 1.38 (Table 1). Incubation time was standardised at 45 min.

2.6. Effect of the presence of calcium ions in artificial saliva on assay performance

The method was applied to alginate solutions prepared in artificial saliva compositions 1 and 2. The two artificial saliva compositions differed in calcium ion concentration. Composition 1 contained a physiological level of calcium ions (1.5 mM) [12]and composition 2 was prepared without calcium ions.

2.7. Effect of mucin glycoprotein on assay performance

The method was applied on solutions of sodium alginate 2.5 g l^{-1} (Protanal® LF120L) and mucin type II (porcine

Table 1
Relationship between alginate molecular properties (molecular weight (M_w) and M/G ratio) and assay performance

Graph label	Alginate type (Protanal®)	M_w (kDa)	M/G ratio	Gradient Mean ($n = 3$) ± 1 S.D.	Intercept Mean ($n = 3$) ± 1 S.D.	Mid-point (1.3 g l^{-1}) Mean ($n = 3$) ± 1 S.D.
A	H120L	449.3	1.38	0.894 ± 0.003	0.179 ± 0.012	1.342 ± 0.009
B	SF200	387	0.449	0.553 ± 0.018	0.262 ± 0.014	0.981 ± 0.013
C	SF60L	325	1.28	0.491 ± 0.013	0.318 ± 0.011	0.956 ± 0.006
D	SF/LF	295	0.59	0.465 ± 0.007	0.148 ± 0.012	0.753 ± 0.004
E	LF120L	221	1.25	0.401 ± 0.017	0.223 ± 0.001	0.745 ± 0.022
F	SF120	195	0.44	0.449 ± 0.034	0.323 ± 0.027	0.907 ± 0.060
G	LF10L	75	1.22	0.496 ± 0.035	0.342 ± 0.035	0.987 ± 0.017
H	LFR5/60	34.7	0.56	0.214 ± 0.007	0.320 ± 0.019	0.599 ± 0.025

gastric) 2.7 g l^{-1}. Wavelength scans were performed over the range 470–680 nm.

2.8. Effect of oesophageal mucosa on assay performance

Porcine oesophagus is considered the most appropriate oesophageal model for ex vivo studies[13]. Fresh oesophagus was collected immediately after slaughter in phosphate buffered saline, and transported on ice. The musculature was removed by dissection within 1 h of slaughter, leaving a clean epithelial tissue tube. This was cut longitudinally, and the inner mucosal surface scraped with a glass microscope slide. The scrapings were dissolved overnight in 20 ml of artificial saliva and the solution used to prepare the alginate solutions for the assay.

2.9. Statistical analysis

Statistical calculations were undertaken using GraphPad InStat v.3.00 (GraphPad Software Inc, San Diego, CA, USA). Linear regression analysis, one-way analysis of variance (ANOVA), *t*-tests and variance ratio tests (*F*-test) were undertaken at a significance level (α) of 0.05 and used to evaluate method performance and the relationship with assay variables.

3. Results and discussion

3.1. Dependence of assay performance on complexation time

Fig. 1a,b shows the visible absorbance spectrum of DMMB in the absence and presence of alginate. The absorbance peak at 650 nm has been assigned to uncomplexed DMMB, the peak at 595 nm to dimerisation of DMMB and in the presence of sodium alginate the absorption peak at 530 nm represents the alginate/DMMB complex[11]. Halle et al. showed that the ratio between the absorbance intensity at 520 and 650 nm, i.e. the ratio between complexed and uncomplexed DMMB could be used to quantify alginate concentration[11]. As alginate quantification is based on the extent of complexation with DMMB it was essential to understand how varying the time allowed for complexation influenced assay performance. Fig. 2 shows how increasing the complexation time influenced the 520/650 nm absorbance ratio. The increase in the 520/650 nm absorption ratio with increasing complexation time showed that DMMB–alginate binding was time dependent. As the complexation time increased the extent of interaction between DMMB and alginate increased. Halle et al. [11]had taken ratiometric readings after 5 min complexation time, but under the conditions here the increase in complex formation, particularly up to 15 min, highlighted the need for a longer complexation time.

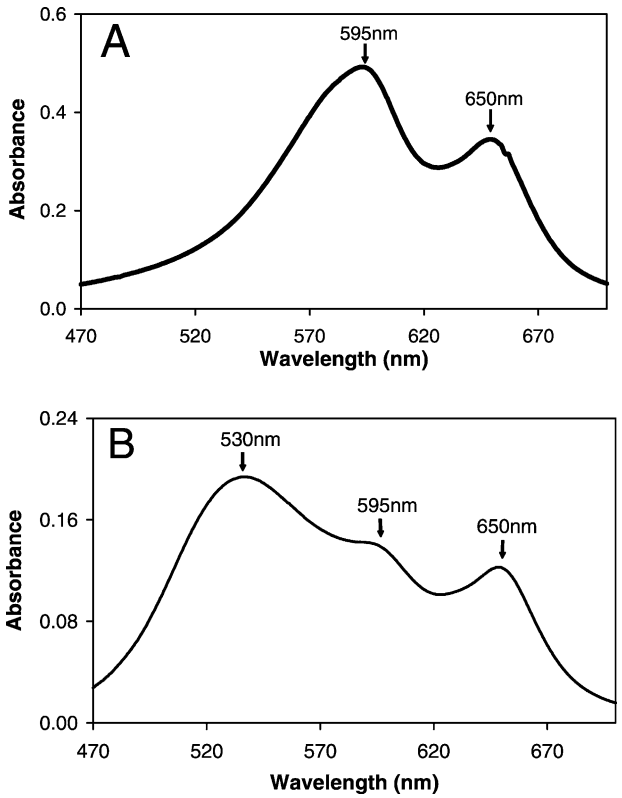

Fig. 1. Visible absorbance spectra of (A) 1 mM DMMB in artificial saliva. (B) DMMB–alginate complex (2.5 g l^{-1} sodium alginate in artificial saliva with 1 mM DMMB).

Incubation was therefore standardised at 45 min in subsequent experiments.

3.2. Effect of alginate concentration and assay variability

Fig. 3 shows the relationship between absorbance ratio (520:650 nm) and sodium alginate concentration as a mean of four independent experiments. Assay response showed excellent linearity ($r = 0.999$) within the concentration range 0.3 and 2.5 g l^{-1}, values consistent with those previously reported by Halle et al.[11]. It was also noted that the standard curve did not pass through the origin; a result of

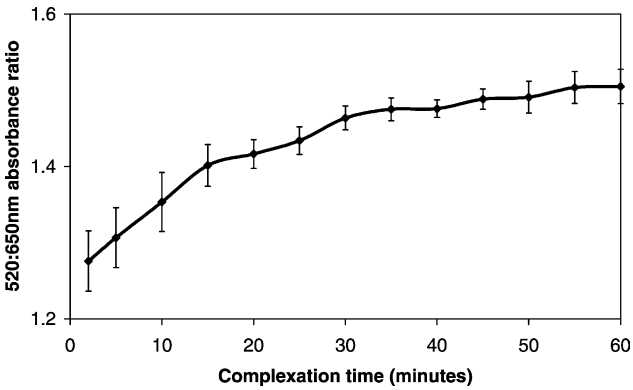

Fig. 2. Effect of DMMB–alginate complexation time on the absorbance ratio (520:650 nm). Mean ($n = 3$) ± 1 S.D.

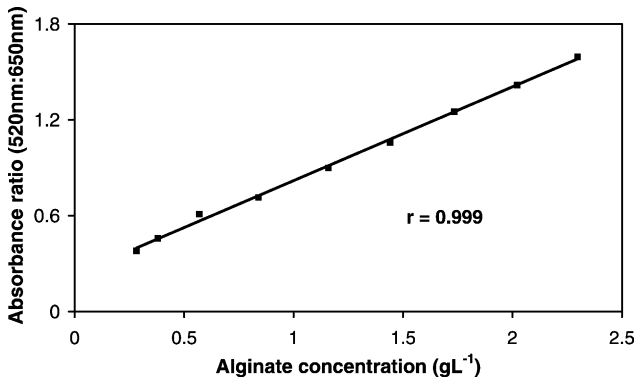

Fig. 3. Graph showing the relationship between sodium alginate concentration and absorbance ratio. Mean ($n = 4$) ± 1 S.D. (Sodium alginate: Protanal® SF200).

residual absorbance at 520 nm from the tail of the 595 nm peak (Fig. 1a). As this value might vary with the extent of DMMB–alginate complexation, no correction subtraction was made and it was accepted that the *y*-axis intercept value would always be positive. The gradient values of each of the four replicate standard curves were significantly different from zero ($P < 0.01$). Inter-experimental reproducibility expressed as a coefficient of variation was maximally 3% for single points and 3.3% for gradient values. The reproducible linear relationship and good precision provides support for this as a viable method for quantifying sodium alginate in artificial saliva.

3.3. Effect of alginate molecular weight and uronic acid composition on assay performance

Fig. 4 shows the structure of the DMMB cation, it is apparent that sodium alginate may complex with DMMB as a result of electrostatic forces between the uronic acid carboxyl groups and the positively charged cation[14]. Alginates differ in molecular weight and uronic acid composition, therefore it was important to consider the effect of alginate molecular characteristics on complexation with DMMB. Alginate molecular characteristics may influence DMMB complexation as (i) the conformational changes associated with different uronic acid sequences may present different opportunities for incoming DMMB molecules; and (ii) alginates of lower molecular weight will be associated with greater exposure of the dye to terminal end groups. In order to explore the wider applicability of the method the response to a range of different alginates was

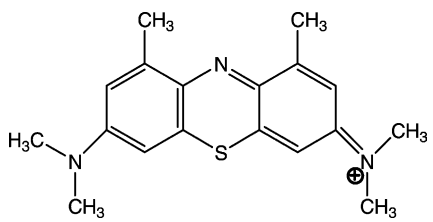

Fig. 4. Structure of 1,9-dimethyl methylene blue (DMMB).

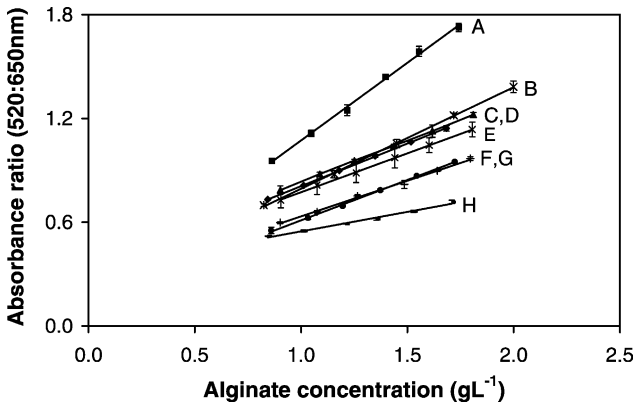

Fig. 5. Relationship between alginate concentration and absorbance ratio (520:650 nm) for a range of alginates differing in molecular weight and M:G ratio. Mean ($n = 3$) ± 1 S.D. Refer to Table 1 for details of alginates A–H.

examined. Fig. 5 shows that assay response varied considerably between different alginate types. The assay response for each alginate (A–H) was delineated by several descriptors: (i) the gradient; (ii) the *y*-axis intercept; and (iii) the mid-point absorbance ratio (520:650 nm) at 1.3 g l^{-1}. Table 1 shows these values determined for each alginate. The differences in assay performance suggest that differences exist in the binding affinity of DMMB to individual alginates. Linear correlation analysis [15] was used to assess if significant relationships existed between each of these assay performance descriptors and molecular characteristics of the alginates. The molecular characteristics investigated were: (i) molecular weight; (ii) mannuronate/guluronate ratio; and (iii) monomer uronic acid composition. A significant dependence ($P = 0.05$) was found only between gradient and mid-point values and molecular weight (Fig. 6 a,b). However, closer examination of these figures reveals that for six of the eight alginates (B–G) examined there were no significant correlations. Therefore, any significant relationship between assay performance and alginate molecular weight is at best described as tenuous.

The complexation response therefore cannot be related to the major parameters of alginate molecular structure in a simple linear way, and examination of the data did not suggest a non-linear analysis would be more enlightening. However, alginates A and H are at opposite ends of the molecular weight and uronic acid spectrum and show clearly different responses. Therefore, examination of a more diverse range of alginates may reveal a relationship between alginate molecular characteristics and assay response. In summary, the origin of the variability in the complexation response of different alginates remains unknown. The various gradient and intercept values obtained show it is necessary to perform individual calibrations with different alginates, and in every case the gradient values were significantly different from zero. Therefore, the assay method may be used to quantify any of the alginates studied.

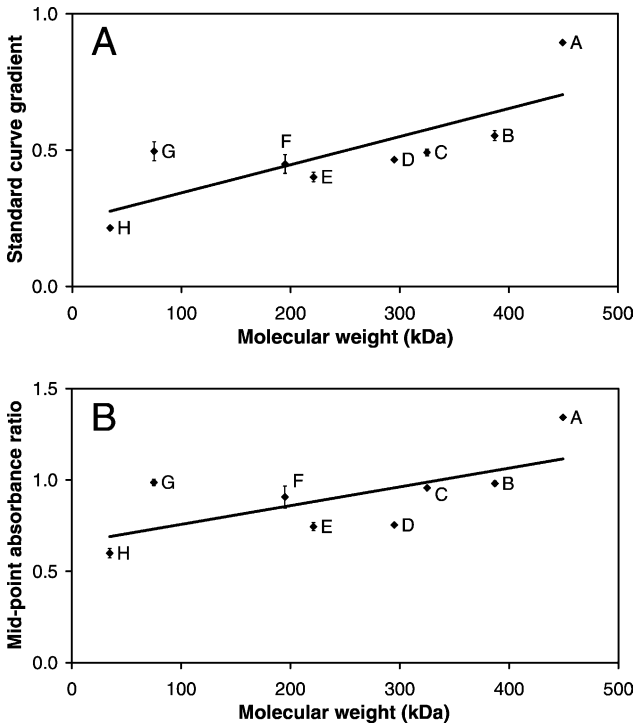

Fig. 6. Relationship between alginate molecular weight and (A) gradient of the standard curve. (B) The mid-point absorbance ratio (520:650 nm) at 1.3 g l^{-1} calculated from the standard curve. Mean ($n = 3$) ± 1 S.D. Refer to Table 1 for details of alginates A–H.

3.4. Effect of the presence of calcium ions in artificial saliva on assay performance

Having attempted to relate the assay performance of a range of alginates to molecular characteristics a satisfactory relationship could not be identified. However, the presence of a physiological level of calcium ions [12] in artificial saliva may interfere with DMMB complexation. Alginates cross-link by binding calcium ions within the hydrophilic cavity between neighbouring guluronic acid residues. Binding is greatest in alginates that contain more poly(guluronate) blocks[2]. Consequently, differences between alginates with respect to their calcium binding capacity may influence the complexation reaction between DMMB and alginate. Calcium ions may directly compete with DMMB or following calcium binding induce a conformational change that influences DMMB complexation. The binding of calcium ions may disguise any potential relationships between assay performance and alginate molecular characteristics. To investigate whether calcium ions influenced assay performance, quantification was repeated on the same range of alginates solubilised in artificial saliva without calcium ions. Table 2 illustrates how each descriptor of assay performance changed when alginate was quantified in artificial saliva that did not contain calcium ions. For all alginates removal of calcium appeared to affect the assay performance, but this effect was statistically significant only for alginates SF/LF, H120L and LF120L.

To assess if significant relationships existed between the assay performance descriptors and molecular characteristics following the removal of calcium ions, linear correlation analysis was again performed. Despite the removal of calcium ions from artificial saliva the only significant relationships found were between alginate molecular weight and standard curve gradient values and molecular weight and the absorbance mid-point value. Both these relationships existed when calcium ions were present. This suggests that calcium ions did not disguise any potential relationships between assay performance and alginate molecular characteristics.

To further analyse the response of assay performance to calcium ions, the sensitivity of assay performance to the removal of calcium ions was investigated. The sensitivity of assay performance to the removal of calcium ions has previously been described by the percentage change in each assay performance descriptor. Table 3 illustrates how the percentage change in any assay performance descriptor was related to alginate molecular characteristics. The percentage change in any assay performance descriptor due to the removal of calcium ions could not be significantly correlated with alginate molecular characteristics. Therefore, despite calcium influencing the complexation between DMMB and alginate the exact influence on assay

Table 2
Influence of the removal of calcium ions from artificial saliva upon assay performance

Graph label	Alginate type Protanal®	Influence of the removal of calcium ions from artificial saliva upon assay performance					
		% difference in mid-point	Significance (P value)	% difference in gradient	Significance (P value)	% difference in intercept	Significance (P value)
A	H120L	1.827	>0.05	−24.581	<0.05	172.954	<0.05
B	SF200	0.756	>0.05	−1.336	>0.05	6.495	>0.05
C	SF60L	2.881	>0.05	−1.078	>0.05	4.641	>0.05
D	SF/LF	9.974	<0.05	−16.638	<0.05	118.525	<0.05
E	LF120L	6.234	>0.05	−9.529	>0.05	43.148	<0.05
F	SF120	−2.951	>0.05	−5.348	>0.05	1.379	>0.05
G	LF10L	2.367	>0.05	−4.171	>0.05	14.822	>0.05
H	LFR5/60	9.133	>0.05	−2.021	>0.05	12.269	>0.05

Table 3
Significance of the relationship between sensitivity of assay performance to the removal of calcium chloride from artificial saliva and alginate molecular characteristics

		% change in assay performance descriptors due to removal of calcium ions from artificial saliva (y axis)					
		Mid-point absorbance ratio		Gradient		Intercept	
		Pearsons correlation coefficient (r)	Significance (P)	Pearsons correlation coefficient (r)	Significance (P)	Pearsons correlation coefficient (r)	Significance (P)
Molecular characteristic (x-axis)	Molecular weight	0.471	>0.05	0.557	>0.05	0.542	>0.05
	M/G ratio	0.127	>0.05	0.226	>0.05	0.329	>0.05
	F_{GG}	0.091	>0.05	0.206	>0.05	0.187	>0.05
	F_{MM}	0.258	>0.05	0.474	>0.05	0.506	>0.05
	$F_{GM.MG}$	0.382	>0.05	0.095	>0.05	0.162	>0.05
	F_{GGG}	0.110	>0.05	0.240	>0.05	0.219	>0.05
	F_{MGM}	0.396	>0.05	0.239	>0.05	0.297	>0.05

F_{XX}, fraction of uronic acid sequences.

performance could not be directly attributed to differences in alginate molecular weight or uronic acid composition.

3.5. Effect of the presence of mucin and oesophageal mucosa on assay performance

Mucin is an anionic glycoprotein found in saliva and may be capable of complexing DMMB. It was therefore necessary to determine the success of alginate quantification in the presence of artificial saliva containing mucin. The impact of including a physiological concentration of mucin (2.7 g L^{-1}) in artificial saliva [12] is shown in Fig. 7. Mucin in artificial saliva formed a complex visible as a broad absorption peak in the 530-nm region, which exceeded that of the DMMB–alginate complex. The cross-reactivity means that unless an initial separation stage is undertaken the present method may only be applicable for artificial saliva compositions free of mucin. Mucin is also found on the oesophageal surface, either as a secretion from oesophageal glands or residual salivary mucin. To assess the influence of surface mucin on assay performance, sodium alginate was quantified in artificial saliva containing scrapings from the oesophageal surface. Fig. 8 shows that the relationship between alginate concentration and absorbance ratio was not influenced by the presence of oesophageal scrapings as there were no significant ($P > 0.05$) differences between the gradient and y-axis intercept values. The insensitivity of the assay to oesophageal scrapings may be explained as the oesophageal surface, unlike other mucosa, does not have an adherent mucus gel layer covering its surface[16]. In summary, this method should work well for quantifying alginate on oesophageal mucosa, however, tissues with a higher content of surface mucin, such as gastric mucosa, may demonstrate cross-reactivity and this should be ascertained prior to use.

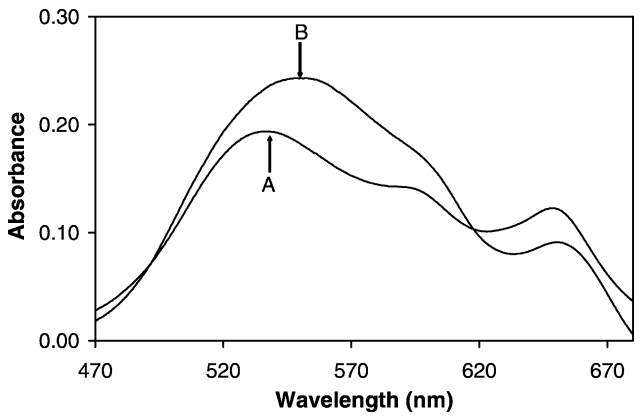

Fig. 7. Wavelength scans of sodium alginate and mucin bound to DMMB. A = 2.5 g l^{-1} sodium alginate (Protanal® LF120L) in artificial saliva with 1 mM DMMB. B = 2.7 g l^{-1} mucin type II in artificial saliva with 1 mM DMMB.

4. Conclusions

This investigation has developed and demonstrated the applicability of a method for quantifying a range of sodium alginates differing in chemical composition. The method proved to be convenient, experimentally simple and rapid, capable of quantifying sodium alginate in the presence of oesophageal mucosa. The only limitations appear to be that (i) the lower limit of quantification is 0.3 g l^{-1}; (ii) separate calibrations are advisable for different alginates; and (iii) tests for cross-reactivity should be performed, particularly against anionic species. Cross-reactivity was observed with mucin in artificial saliva, however, a similar reactivity was not observed for mucin scraped from the oesophageal surface. The sensitivity of the assay to mucin in saliva was not considered a problem as saliva compositions free of mucin could be chosen,

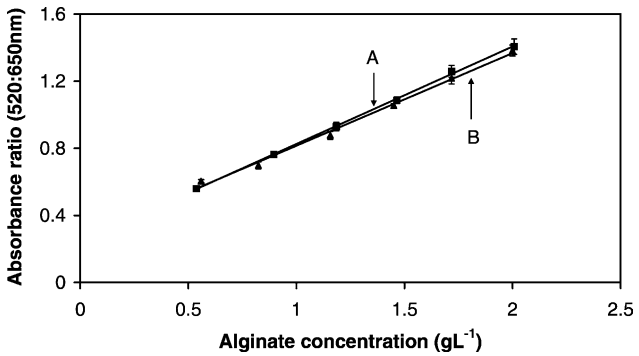

Fig. 8. Effect of oesophageal tissue scrapings on the relationship between alginate concentration and the absorbance ratio 520:650 nm. Mean ($n = 3$) ± 1 S.D. A = With oesophageal scrapings. B = Without oesophageal scrapings.

however, a method for quantification of alginates without any cross-reaction to mucin would be desirable. In conclusion this method could be used for quantifying sodium alginate retained on oesophageal mucosa and enable the characterisation and optimisation of the bioadhesive properties of sodium alginate based dosage forms.

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