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Hydrolysis and Speciation of Al Bound to Pectin and Plant Cell Wall Material and Its Reaction with the Dye Chrome Azurol S

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Hydrolysis of aluminum (Al) in solution increases at pH ≥ 4 and with an Al concentration. Pectin, an important anionic polysaccharide of plant cell walls, adsorbs Al, but this phenomenon is poorly understood. This study showed that Al^{3+} hydrolysis results in binding of Al to pectin in excess of the stoichiometric equivalent, leading to oversaturation of the pectin with Al. However, the degree of pectin methyl-esterification did not affect the extent of Al hydrolysis. Binding of Al to purified cell wall material also resulted in Al hydrolysis in a pH- and soluble Al concentration-dependent manner, but the source of cell wall material had no effect at fixed pH. Staining of Al-treated pectin and cell wall material from wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus* L.) with the Al-specific dye, chrome azurol S (CAS), resulted in the formation of a purple color, with the intensity related to the extent of Al hydrolysis.

KEYWORDS: Pectin; cell wall; aluminum; adsorption; speciation; hydrolysis; *Helianthus annuus*; *Triticum aestivum*; *Vigna unguiculata*

INTRODUCTION

The trivalent cation aluminum (Al^{3+}) has a small ionic radius and high affinity for oxygen-containing ligands. Therefore, Al reacts with organic acids and carbohydrates present in soil and plant roots and causes toxic effects in plant roots (*1*). Soluble Al is prevalent in soils at acidic pH because of dissolution of Al-containing clay minerals, and Al undergoes hydrolysis at pH ≥ 4 (*2*). The simple hydrolytic reactions of Al^{3+}, leading to species such as $AlOH^{2+}$ and $Al(OH)_2^{+}$, are reasonably well-understood, but polymerization reactions of Al leading to the formation of $Al_2(OH)_2^{4+}$, $Al_2(OH)_5^{+}$, $Al_8(OH)_{20}^{4+}$, $AlO_4Al_{12}(OH)_{24}^{7+}$ (Keggin Al13), and numerous other Al−hydroxy polymers are less well-understood (*2*−*9*). It was recently suggested (*8*) that gradual hydrolysis of Al solution results in monomeric Al−OH species, which lead to "core and link" Al−hydroxy species that may rearrange into planar polymers, e.g., $Al_{13}OH_{30}^{9+}$ and $Al_{54}OH_{144}^{18+}$. These species have no tetrahedrally coordinated Al atoms and are therefore not detectable by ^{27}Al nuclear magnetic resonance (NMR); however, they can be detected indirectly by electrospray ionization time-of-flight mass spectrometry (*9*). The Al−OH sheets dissolve or rearrange and form ellipsoid Keggin $Al_{13}OH_{24}^{7+}$ molecules with a centrally located tetrahedrally coordinated Al atom, which can be detected by ^{27}Al NMR. Eventually, the "core and link" Al−hydroxy species and Keggin Al13 may slowly rearrange into crystalline $Al(OH)_3$ (*2*, *8*, *10*). Several polymeric Al species were detected even in dilute (1−10 μM) Al solutions that had not been pH-adjusted (*9*).

The effects of organic ligands on the hydrolysis and polymerization of Al in soil solution is still debated (*11*−*13*). Low-molecular-weight organic acids, such as malic and citric acids, are considered to prevent Al hydrolysis (*14*−*16*) or induce depolymerization of Keggin Al13 (*15*), whereas the inorganic anions sulfate and silicate either prevent the formation of Keggin Al13 (*17*, *18*) or lead to its precipitation (*19*). High-molecular-weight organic acids, such as humic acids and pectin, appear to induce depolymerization of Keggin Al13 or precipitation (*20*). Notably, plant roots contain high-molecular-weight acids (pectin) in the cell walls and exude small organic acids as part of Al-tolerance mechanisms, and this may affect the formation of Keggin Al13 in the cell wall.

The concentration of Al in soil solution is controlled by the complexation of Al with organic matter rather than the dissolution of Al-containing minerals (*21*−*23*). The most toxic Al species is Keggin Al13, which is toxic at ≤1 μM. While Keggin Al13 can be formed by the addition of either acid or alkali to Al solutions (*24*), an increase in temperature and pH (i.e., OH/Al ratio) greatly increases Keggin Al13 formation (*25*); however, Keggin Al13 may even be present in undersaturated (i.e., dilute) Al solutions at pH 3.3−4.0 (*13*). The toxicity of polymeric Al−hydroxy species other than Al13 is uncertain, partly because of a lack of sensitive methods to detect these Al species (*8*).

The effect of plant roots and their constituent components on the formation of mono- and polymeric Al−hydroxy species is

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unknown. Roots adjust the pH of the apoplast (i.e., the cell wall) and rhizosphere irrespective of the bulk solution pH, and cowpea roots (*Vigna unguiculata* L.) were shown to trigger the formation of Al13 in the bulk solution (*26*). Detection of Al13 bound to plant cell walls was not achieved convincingly because the bound Al13 may not react or change the kinetics of the reaction with the ferron reagent (*25*). The formation of Al13 has also been postulated in soils and aquatic bodies (*11*, *27*, *28*). Because Al13 is a heptavalent cation, sorption of Al13 will be much stronger than that of Al^{3+} or La^{3+}. Although Keggin Al13 forms at 10−100 μM Al in solution (*27*, *29*), the detection limit for Keggin Al13 by ^{27}Al NMR is 200 μM (*27*), highlighting the difficulty in detecting Keggin Al13 by NMR at environmentally relevant Al concentrations.

Colorimetric methods for the detection of monomeric Al^{3+} species have been described. While their ability to detect Al−hydroxy species is uncertain, reagents, such as chrome azurol S (CAS) and ferron, react with small Al−hydroxy species (*30*−*32*). The colorimetric assay using CAS has been used to determine the Al concentration in plant material (*33*), and our results indicate that CAS can be used to detect Al bound to plant roots.

Therefore, the aim of this study was to first determine the factors governing the formation of Al−hydroxy species, specifically addressing the observation that Al hydrolysis increases with the Al concentration. Second, we aimed to determine the reaction of Al−hydroxy species with the model cell wall component, pectin, and to determine the reaction of CAS with Al−hydroxy species bound to pectin and cell wall material. This reaction may permit the screening of plant roots for Al sensitivity and improve our understanding of why Al is toxic to plant roots.

MATERIALS AND METHODS

General Methods. Stock solutions (10 mM) of $LaCl_3$ or $AlCl_3$ were prepared from analytical-grade reagents in 0.05 M NaCl as the background electrolyte. Working solutions containing 0.1−5 mM Al were obtained by dilution with 0.05 M NaCl. The solution pH was adjusted between pH 3 and 5 by titration with either 20 mM HCl or 20 mM NaOH, and the pH was measured with a combination pH electrode (TPS Ionode, Springwood, Australia).

The formation of Al−hydroxy species was determined by mixing Al solutions or gels (see below) with NaF (50 mM in 0.05 M NaCl) to a F/Al ratio of 3 and backtitrating the solution to the initial pH. The number of hydroxyl groups displaced from Al was calculated from the titer after correction for water−pectin−NaF blank titers and adjustment for the number of carboxyl groups of pectin in the blank and sample. The Al concentration was determined by inductively coupled plasma−optical emission spectroscopy (ICP−OES), from which the experimentally determined OH/Al ratio was calculated. The ICP−OES analyses were performed on a Varian Vista-Pro multi-charged coupled device (CCD) instrument, using the 167.02 nm line for Al. The coolant flow rate was 15 L min^{-1}, and the flow rate in the Teflon spray chamber was 0.75 mL min^{-1}.

The time course of Al hydrolysis was determined by mixing pectate solution with sufficient Al chloride solution to yield 100 or 200% Al saturation. The gels were stored at 4 °C for 16 h prior to adjustment to pH 4, 4.5, and 5 and the time noted. Aliquots of the Al−pectate gels were withdrawn at various times, mixed with 50 mM NaF, and the released OH groups were backtitrated to the initial pH with HCl. The OH/Al ratio was determined from the titer and divided by the amount of Al added. Alternatively, the pH change of the gels was determined by mixing pectate with NaOH to give an initial pH 8.3, and an aliquot of pectate was mixed with a requisite amount of $AlCl_3$ (not pH adjusted) to give a gel with final pH 4.7 and a charge saturation of 100%. The resultant weak gel was vigorously mixed, and the pH was recorded over 0.2−5 min.

Staining of Al with CAS and Ferron. Colorimetric assays for Al^{3+} and Keggin Al13 were performed with CAS (3″-sulpho-2″,6″-dichloro-3,3′-dimethyl-4-hydroxyfuchson-5,5′-dicarboxylic acid) and ferron (8-hydroxy-7-iodo-5-quinoline-sulfonic acid). For the CAS assay, CAS dye (60% purity) was made up in 10 mM Na−acetate buffer at pH 4.5 (apart from experiments testing the effect of pH) and used within 5 days. The concentration of the reagent was 0.04 mM. The reaction mixture contained 3 mL of CAS in buffer and 0.1 mL of sample or Al standard, and the absorbance spectrum was recorded after 15−30 min at 25−26 °C. The ferron assay was conducted as described by Parker and Bertsch (*34*). Briefly, 2.85 mM ferron and 0.5 mM phenanthroline were prepared in deionized water as were separate solutions of 4.3 M Na−acetate and 1.44 M hydroxylamine reagent. The working reagent consisted of 25 mL of ferron reagent, 10 mL of Na−acetate solution, and 10 mL of hydroxylamine reagent, adjusted if necessary to pH 5.2. The reagent was kept in darkness for at least 7 days prior to use. For the assay, 50 μL of Al standard or sample was mixed with 2 mL of ferron working reagent at 25−26 °C and the starting time noted. The absorbance at 363 nm was measured for 10 min, and the concentration of Keggin Al13 was calculated from the slope of the 5−10 min segment of the absorbance reading over time.

Preparation of Al−Pectate Gels. Citrus pectin was obtained from the Sigma Chemical Co., and the degree of esterification was determined by titration and varied by alkaline de-esterification (*35*). Gels of Al−pectate were prepared by mixing pectic acid [100 μmol of galacturonic acid (GalA)/3 mL; approximately pH 2.5] with a calculated volume of 10 mM $AlCl_3$ (approximately pH 3.5) to give 50−800% nominal Al saturation. The pH of the pectin and Al chloride solution was not adjusted prior to mixing to avoid the formation of Al−OH artifacts. The pH of the resultant Al−pectate gel dropped to pH 2.2−2.4 and was slowly adjusted to the desired pH over 2−3 days.

The Al saturation (percent charge neutralization) of the Al−pectate gel was determined by measuring the Al content of an aliquot of gel after drying and weighing. The dry residue was digested with 10 mL of acid (five parts nitric acid plus one part perchloric acid) in capped polypropylene tubes at room temperature (25 °C) for 2−3 weeks or until completely clear, and Al was quantified by ICP−OES. The concentration of bound Al was multiplied by 3 (the charge of Al) and divided by the concentration of GalA determined on another aliquot of the gel by the 3-phenylphenol assay (*36*) using GalA as the standard. Experiments were designed as randomized complete block design, repeated at least twice, and data were analyzed by Proc GLM in SAS. Where appropriate, treatment differences were considered significant at the 5% level of probability.

Preparation of Cell Wall Material. Seeds of cowpea (*V. unguiculata* L. cv. Red Caloona), sunflower (*Helianthus annuus* L. cv. Hysun), and wheat (*Triticum aestivum* L. cv. Kennedy) were germinated at room temperature in a paper towel soaked with 1 mM Ca and 5 μM boric acid. The 1 cm tip section of the roots were collected on ice and homogenized with a Ten Broek homogenizer on ice. The homogenized cell wall material was washed with ice-cold acetone, isopropanol, and deionized water on a 64 μm steel mesh. The resulting pellet was stored in 0.05 M NaCl solution at 4 °C for up to 5 days or frozen at approximately −20 °C and thawed for later use. An aliquot of the cell wall material was mixed with increasing volumes of 2 mM $AlCl_3$ in 0.05 M NaCl, incubated for 18−48 h at 4 °C, and the pH was repeatedly adjusted to pH 3−5 until constant. Cell wall material without Al addition was treated in an identical fashion as the control. The slurry was centrifuged (3000*g* for 10 min), and aliquots of the pellet and supernatant were used to determine the OH/Al ratio with NaF. An aliquot was dried (80 °C, 4 days); the Al pellet was digested with acid as outlined above (Preparation of Al−Pectate Gels); and the bound cations were determined by ICP−OES and expressed relative to the cation-exchange capacity of the cell wall material.

RESULTS

The hydrolysis of Al as determined by the OH/Al ratio (concentration of hydroxyl groups bound to Al divided by the concentration of Al) increased with the amount of Al bound to pectin (**Figure 1**). If the Al saturation of the sorbent was below 50% (expressed on a charge basis), no hydrolysis of Al was observed. When the sorbent was 100% saturated, however, the OH/ratio reached 0.6, and at 200% charge saturation, the OH/Al ratio was near 1.5 (**Figure 1**).

The concentration OH displaced from Al was calculated from the titer after NaF addition and corrected for NaF−pectin blank titers. If the measured titer was very low, occasional negative OH

concentrations were calculated after subtraction of the blank NaF−pectin titer because of experimental variability, resulting in negative OH/Al ratios.

Cell wall material mixed with 100% Al and adjusted to various pH values showed hydrolysis of Al in the supernatant fraction. As expected, the hydrolysis of Al in the supernatant fraction increased with pH; it was noteworthy that no differences were observed between sources of cell wall material (**Figure 2a**). No Al hydrolysis was evident at pH ≤ 4 but increased markedly to an OH/Al ratio of 2 at around pH 5.

The Al bound to the pellet underwent hydrolysis as the pH increased (**Figure 2b**). Unlike the supernatant fraction (**Figure 2a**), hydrolysis of Al bound to the cell wall material was evident at a lower pH (pH > 3.5 in cell wall material versus pH > 4.2 in the supernatant). There were no marked differences in the OH/Al ratio at a given pH for wheat and cowpea, and the OH/Al ratio increased linearly to pH 5. The OH/Al ratio of sunflower material was initially higher than that of the other two plant materials but appeared to level off at pH > 4.2.

Time Course of Al Hydrolysis. The OH/Al ratio of Al−pectate gels with 100 and 200% nominal Al saturation ranged from 0.4 for 100% Al-saturated pectate at pH 5 to 1.6 for 200% Al-saturated pectate at pH 5. However, there were no clear indications that the OH/Al ratio changed from 10 min to 120 h (**Figure 3**). To further study the time course of hydrolysis reactions, Al−pectate gels with 100% charge neutralization were prepared and the change of pH was recorded over 0−8 min. The pH decreased from pH 4.73 at 0.25 min to pH 4.26 after 5 min and then remained unchanged up to 10 min.

Effect of the Degree of Esterification on Al Hydrolysis. The reaction of Al with pectin adjusted to a range of degrees of esterification did not show clear trends. At pH 4, the OH/Al ratio was approximately 0, irrespective of esterification, whereas at pH 4.5, the OH/Al was approximately 0.6, again without a clear trend relating to esterification (**Figure 4**). The gels had an Al saturation ranging from 66 to 104% at pH 4 and 73−131% at pH 4.5, and the saturation was independent of the degree of esterification of the pectin.

Reaction of Hydrolyzed Al with CAS Dye. CAS in solution had a broad absorption peak around 470 nm, which shifted to higher wavelengths when Al was added (**Figure 5**). The location of the peak at 545 nm was independent of the pH in the range of pH 3−5, and the absorbance was stable for at least 5 h (data not shown).

There was a shift in the maximum adsorption wavelength from 545 nm (magenta) to 572 nm (purple) as the Al/CAS ratio

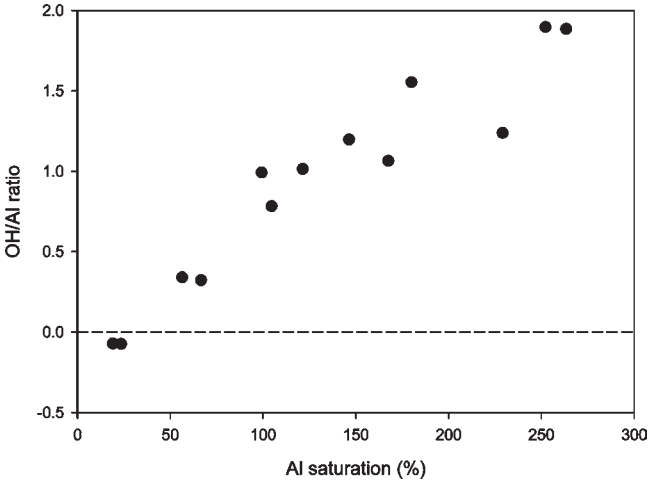


Figure 1. Measured OH/Al ratio of Al−pectate gels at pH 4.5 as affected by Al saturation. The concentration of OH groups bound to Al was determined from the OH groups displaced by NaF and divided by the concentration of Al ions.

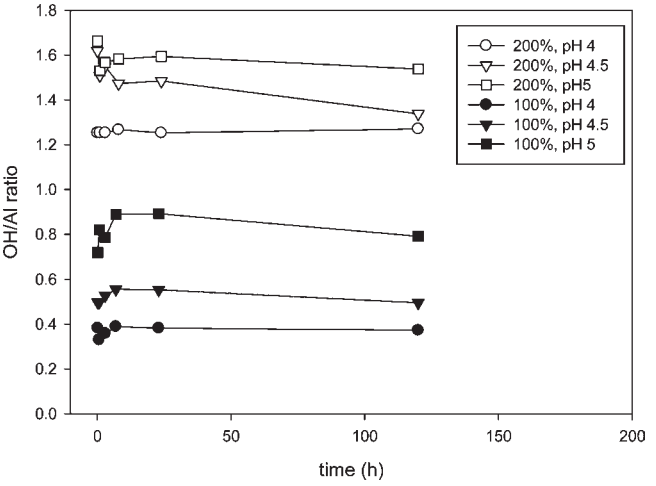


Figure 3. Time course of hydrolysis of Al bound to pectin from 10 min to 120 h. Al−pectate gels with 100 and 200% Al saturation were prepared; the pH was adjusted; and the OH/Al ratio of the gels was determined. The experiment was repeated twice, and for the sake of clarity, the results of a single replicate is shown here.

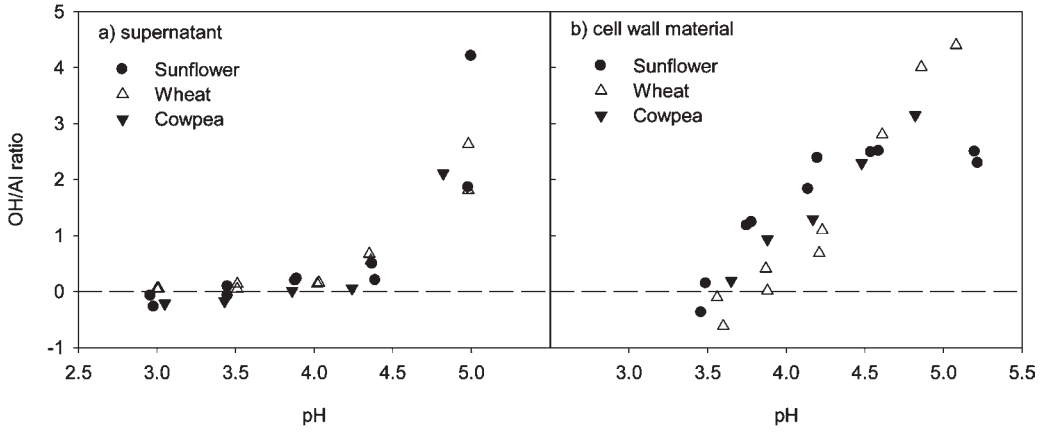


Figure 2. OH/Al ratio of the (a) supernatant and (b) pellet fraction of Al-saturated cell wall material adjusted to various pH values. Cell wall material was isolated from sunflower, wheat, and cowpea roots. Values are the combined results from two experiments.

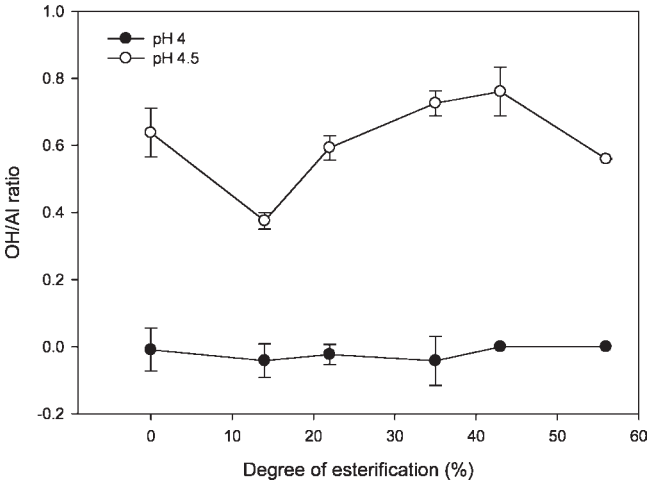


Figure 4. Effect of the degree of esterification of pectin on the measured OH/Al ratio of bound Al at pH 4 and 4.5. Values are the mean of three determinations, with standard deviations shown if not obscured by the symbols.

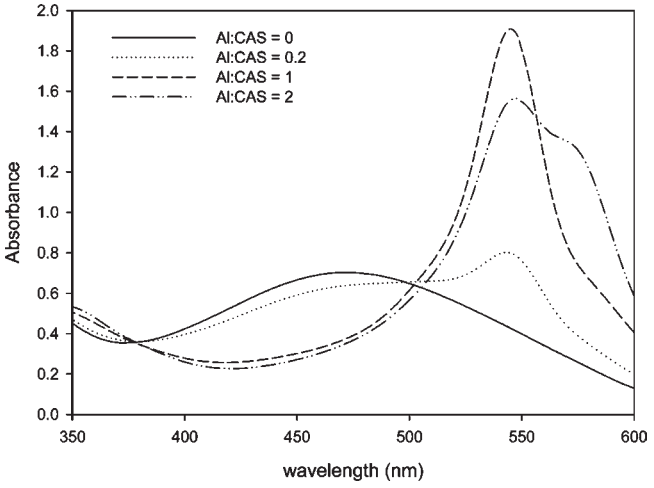


Figure 5. Influence of the Al/CAS ratio on the absorbance spectra of Al−CAS complexes. The Na−acetate buffer (10 mM, pH 4.5) contained 40 μM CAS and 0, 8, 40, or 80 μM Al to give Al/CAS ratios of 0, 0.2, 1, and 2, respectively.

increased (**Figure 4**). The wavelength shift was not directly affected by the pH of the solution, although the precipitation of Al or the formation of Al13 decreased the CAS-reactive Al fraction, thereby changing the Al/CAS ratio. A comparison between the ferron and CAS assays revealed that both assays gave similar results for monomeric Al but CAS did not react with Al13 (data not shown).

Al−pectate gels with varying Al saturation and OH/Al ratios were stained with CAS (**Table 1**). We assessed visible color rather than absorbance wavelength, because the absorbance of the gels could not be accurately measured on a standard spectrophotometer and we had no diffuse reflectance spectrophotometer available.

The correlation between Al saturation and the OH/Al ratio of the gel was weak (R^2 = 0.397), but the OH/Al ratio was significantly correlated with the pH of the gel (R^2 = 0.742) (**Table 2**). Further, the color (stainability) of the gels was strongly correlated with the OH/Al ratio (R^2 = 0.808) but only weakly correlated with the Al saturation of the gel (R^2 = 0.284) (**Table 2**).

Cell wall material from wheat and sunflower with varying Al saturation was also stained with CAS. With increasing Al saturation, the cell wall material developed a purple color (**Table 3**), which was strongly correlated with both the Al saturation of the cell walls and the calculated OH/Al ratio (**Table 4**).

Table 1. Effect of Al Saturation, OH/Al Ratio, and pH of Al−Pectate Gels on the Reaction of the Gels with CAS

Al saturation (%)	OH/Al	pH	color
25	0	3.1	colorless
37	0	3.7	colorless
42	0	3.3	colorless
67	0.4	4.0	colorless
77	0	3.5	colorless
63	1.2	4.9	light pink
76	1.2	4.9	light pink
93	0	3.4	light pink
170	0.2	3.5	pink
155	2.2	4.5	purple
155	1.6	5.1	purple
256	2.0	4.3	purple
284	2.2	5.1	purple
1158	2.6	4.9	purple

Table 2. Pearson Linear Correlation Coefficients between Al Saturation of the Pectate Gel and the Measured OH/Al Ratio, pH of the Gel, and Its Color after CAS Staining[a]

	Al saturation	OH/Al	pH
Al saturation			
OH/Al	0.397 (p = 0.016)		
pH	0.185 (p = 0.125)	0.742 (p < 0.001)	
color	0.284 (p = 0.050)	0.808 (p < 0.001)	0.577 (p = 0.002)

[a] The probability values are shown in parentheses.

Table 3. Effect of Al Saturation, OH/Al Ratio, and pH of Al-Saturated Cell Wall Material on the Reaction with CAS

species	Al saturation (%)	OH/Al	pH	color
wheat	64	0	3.0	light pink
	64	0	4.0	light pink
	70	0	5.0	light pink
	223	1.7	3.0	light pink
	209	1.6	4.0	light pink
	212	1.6	5.0	light pink
	532	2.4	3.0	purple
	582	2.5	4.0	purple
	575	2.5	5.0	purple
sunflower	14	0	3.0	colorless
	25	0	4.0	colorless
	27	0	5.0	colorless
	136	0.8	3.0	light pink
	135	0.8	4.0	light pink
	152	1.0	5.0	light pink
	447	2.3	3.0	purple
	442	2.3	4.0	purple
	333	2.1	5.0	light purple

DISCUSSION

The hydrolysis of Al increased with both an increase in pH and the concentration of Al bound (**Figure 1**). The hydrolysis of Al in solution with increasing pH is controlled by the stability constant for the Al−OH complexes, and the hydrolysis of Al is known to increase with pH > 4 (panels **a** and **b** of **Figure 2**). The extent of Al hydrolysis in the supernatant solution of mixtures containing plant cell walls was similar. Because the hydrolysis of Al and the formation of Al−OH complexes in solution are determined by

Scheme 1

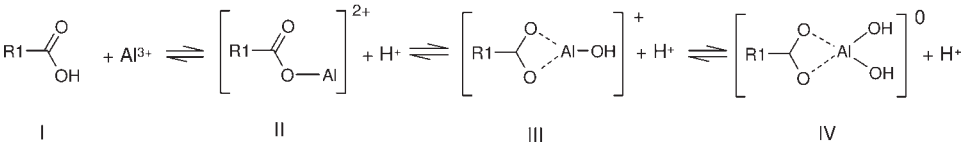


Table 4. Pearson Linear Correlation Coefficients between Al Saturation of Wheat and Sunflower Cell Wall Material, Measured OH/Al Ratio, and the Color after CAS Staining[a]

	Al saturation	OH/Al
wheat		
Al saturation		
OH/Al	0.847 ($p < 0.001$)	
color	0.802 ($p = 0.001$)	0.657 ($p = 0.008$)
sunflower		
Al saturation		
OH/Al	0.976 ($p < 0.001$)	
color	0.822 ($p < 0.001$)	0.866 ($p = 0.003$)

[a] The probability values are shown in parentheses.

the formation constants, which are an intrinsic property of Al, it was not expected that the OH/Al ratio of the supernatant would be affected by the source of plant material.

The measured and calculated OH/Al ratio for Al−pectate gels at pH 4.5 was in good agreement and confirms the validity of the NaF method to determine the OH/Al ratio (data not shown). Hydrolysis of Al bound to pectic acid increased near-linearly with an increase in Al saturation; the OH/Al ratio increased from approximately 0.3 at 60% Al saturation to approximately 1.5 at 200% Al saturation (**Figure 1**). In cell wall material, the extent of hydrolysis increased with pH (**Figure 2b**) and there was no clear difference in the extent of Al hydrolysis with plant species, despite the fact that wheat has a different cell wall composition from dicots, such as cowpea and sunflower (*37*). At a given pH, Al hydrolysis was greater when Al was bound to cell wall material than when free in solution (compare panels **a** and **b** of **Figure 2**). This may be attributed to the fact that the binding sites in cell wall material and pectic acid consist predominantly of carboxyl groups of GalA, and this may increase hydrolysis of Al to yield complexes such as GalA−AlOH and GalA−$Al(OH)_2$ (molecules III and IV in **Scheme 1**). These GalA−Al−hydroxy complexes would be more stable than a GalA−Al complex (molecule II) because of multidentate complexation in the former complexes. Further precipitation or complexation of Al ion onto the bound GalA−Al−hydroxy complexes cannot be excluded, however, and may result in OH/Al ratios increasing to OH/Al > 2.

The measured OH/Al ratios did not permit identification of the exact Al−OH complex that may have formed, because the values are bulk average OH/Al values and may consist of several Al−OH species, many of which have similar OH/Al ratios. For instance, an OH/Al ratio of 2.0 can be attributed to a combination of species, such as $AlOH^+$, $Al_7(OH)_{16}^{5+}$, and $Al_{10}(OH)_{24}^{6+}$. Indeed, Sarpola et al. (*9*) have described numerous Al−OH species, depending upon the Al concentration and solution pH, which differ little in the OH/Al ratio but have widely differing ionic charge and structure.

Only tetrahedral Al, which is present in Al13, can be determined directly by ^{27}Al NMR (*19*). We have observed Al13 in solution using ^{27}Al NMR, but the sensitivity of the method was too low to detect Al13 in pectate gels and cell wall material (data not shown). Likewise, Masion and Bertsch (*25*) could not detect Al13 in cell wall material, whereas Xia and Rayson (*38*) have detected Al13 in plant cell walls. Kopittke et al. (*26*) found that root-induced rhizosphere acidification can lead to Al13 formation in the rhizosphere, an unlikely event in this study because we used isolated cell wall material. Determination of Al13 with alternative methods, such as mass spectrometry (*9*), is unsuitable for gels or cell wall samples.

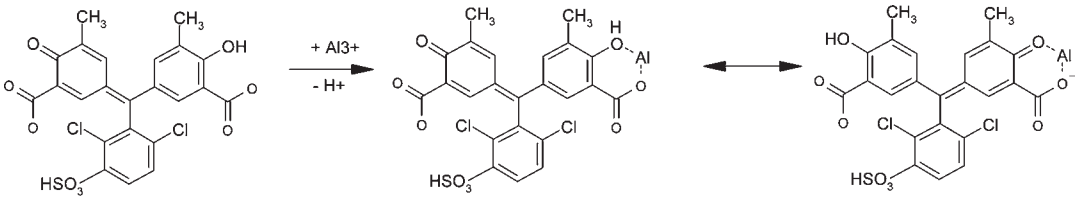
Time Course of Al Hydrolysis. The measured OH/Al ratio of Al−pectate gels did not change substantially from 10 min to 120 h (**Figure 3**). Again, caution is needed in this interpretation because the measured OH/Al ratio is the bulk average; i.e., the value comprises several Al−hydroxy species with differing OH/Al ratios, which is not detected by this method. Furthermore, if Al−hydroxy complexes polymerize because of aging, the formed species may have a very similar OH/Al ratio. For instance, the species $Al(OH)_2^+$, $Al_2(OH)_4^{2+}$, and $Al_6(OH)_{12}^{6+}$ all have OH/Al ratio = 2. Thus, polymerization may occur but cannot be detected by measuring the OH/Al ratio. Likewise, Sarpola et al. (*9*) could not detect significant changes in Al speciation by mass spectrometry in samples aged for 14 days.

Alternatively, Al−pectate gels with 100% charge neutralization were prepared, and the change of pH was recorded over 0−5 min. In this case, the pH decreased from pH 4.73 at 0.25 min to pH 4.26 after 5 min and then remained unchanged up to 10 min, which may suggest that hydrolysis of Al bound to pectate is complete in 5 min. Therefore, either Al hydrolysis is complete within 5 min or the Al−hydroxy species are metastable over the time frame investigated. The Al ion is considered to be a sluggishly labile ion (*20*, *39*), with comparatively slow establishment of equilibrium and half-life for Keggin Al13 reported to be several hundred hours (*7*). Bi et al. (*8*) proposed that the conversion of core-link Al13 to Keggin Al13 releases protons and results in a drop of pH. However, this conversion would result in a change of the calculated OH/Al from 2.3 for core-link $Al_{13}OH_{30}$ to 1.8 for Keggin $Al_{13}OH_{24}$. Because we have not observed a marked change in OH/Al ratios over time (**Figure 3**), the contribution of Al13 to the overall OH/Al may be small in this study.

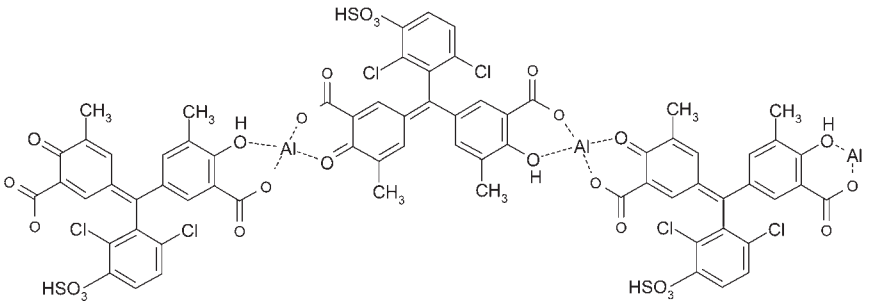
Effect of the Degree of Esterification on Al Hydrolysis. The increased Al hydrolysis with Al saturation of pectin may indicate that Al hydrolyses when Al ions are held in close proximity [i.e., if the mononuclear wall is exceeded (*2*)]. By implication, if the anionic charges on pectin are closely spaced, the Al ions bound to GalA are also in closer proximity. Thus, a pectin molecule with a low degree of esterification has few intervening methoxyl groups and may have greater Al hydrolysis than a high-ester pectin. However, the reaction of Al with pectin with varying esterification did not show clear trends. At pH 4 (66−104% Al saturation), the OH/Al ratio was approximately 0, irrespective of the degree of esterification (**Figure 4**), whereas at pH 4.5 (73−131% Al saturation), the OH/Al ratio was approximately 0.6, again without a clear trend relating to esterification (**Figure 4**). Therefore, the spacing of Al ions on the pectin molecule does not appear to influence Al hydrolysis.

Notably, Mimmo et al. (*40*) did not find an effect of esterification on Al binding by pectate either. It may be possible that the charges on pectin are too far apart to enable an interaction between neighboring Al ions. However, bound Al may act as a nucleation site for other Al ions, with Al "precipitates" clustered along the pectin molecule at discrete sites determined by the presence of carboxyl groups. A similar localized precipitation reaction has been described for ruthenium red binding to pectin (*41*).

Scheme 2



Scheme 3



Numerous, both positively and negatively charged, polymeric Al−OH species have been detected by mass spectrometry in dilute Al solution; for a list, see ref *9*. It is possible that the positively charged Al species bind to pectin, followed by alternate negatively and positively charged species.

Reaction of Hydrolyzed Al with CAS Dye. Monomeric Al can be detected by colorimetric reagents, such as pyrocatechol violet, aluminon, morin, and hematoxylin, and it has been suggested that small Al-hydroxy species apart from Keggin Al13 can be stained with CAS (*31*). Our experience is that the dyes develop intensely colored complexes with Al, in both freshly neutralized Al solution and Al bound to pectin or plant material. Because the color development may be due to a pH effect on the dye rather than Al−hydroxy species formation, we investigated the reaction of Al and Al−pectate with Al-specific dyes at several pH values. Initially, we investigated aluminon, solochrome cyanine R, eriochrome cyanine R, and CAS. Of these dyes, only CAS showed sufficient sensitivity and linearity to warrant further investigation. Because of the pH sensitivity of the absorbance spectrum (*42*, *43*), a 10 mM Na−acetate buffer at pH 4.5 was used. Acetate may inhibit Al13 formation (*14*, *16*), but because CAS does not detect Al13 in any case, we considered the effect of acetate on the colorimetric assay unimportant.

In solution, CAS (molecule V in **Scheme 2**) showed a broad absorption peak around 470 nm, which shifted to higher wavelengths when Al was added (**Figure 5**). This shift is due to resonance developing between molecules VIa and VIb (**Scheme 2**) (*43*). The location of the peak at 545 nm was independent of the pH in the range of pH 3−5, and the absorbance was stable for at least 5 h (data not shown).

The ratio of Al/CAS determines the location of the absorbance maxima of the Al−CAS complex; if CAS is in excess (i.e., Al/CAS < 1), aggregates form in which two or more CAS molecules are bridged by Al ions, presumably forming linear aggregates (*43*) in the form CAS−Al−CAS−Al−CAS−... (**Scheme 3**), which absorb at 545 nm. At low pH (pH 3−3.5), protonated complexes (H−CAS−Al) may occur, which absorb at 455−465 nm.

If Al is in excess, monomeric complexes (Al−CAS−Al) are dominant, which absorb at 470−472 nm, manifesting as a shoulder or split peak on the absorbance spectra (*44*) (**Figure 5**). This wavelength shift leads to a visible color change from magenta to purple. We speculate that CAS favors binding of $AlOH^+$ or $Al(OH)_2^+$ species to satisfy local binding site electroneutrality, a suggestion supported by the proposal of Kennedy and Powell (*31*) that CAS reacts with small Al−hydroxy species.

The wavelength shift between 545 and 572 nm was not directly affected by the pH of the solution, although the precipitation of Al or the formation of Al13 decreases the CAS-reactive Al fraction, thereby changing the Al/CAS ratio, which may result in the wavelength shift. A comparison between the ferron and CAS assays revealed that both assays gave similar results for monomeric Al but CAS did not react with Al13 (data not shown), confirming the results of Kennedy and Powell (*31*).

Next, we prepared Al−pectate gels and correlated their stainability with CAS to their Al saturation, extent of Al hydrolysis, and pH values. The color of the stained Al−pectate was strongly correlated with the OH/Al ratio of the gel but only to a lesser extent with the Al saturation (**Table 3**). Likewise, staining of Al-treated cell wall material from wheat and sunflower showed a strong correlation with the OH/Al ratio and the Al saturation of the cell wall material. This indicates the formation of an Al species, possibly species III or IV (**Scheme 1**), which reacts with CAS. Initially, CAS is added to cell wall material in excess, but upon destaining in water, the concentration of CAS decreases, while the concentration of Al remains constant, resulting in an increased Al/CAS ratio and development of a purple color. The color developed on both Al−pectate gels and Al−cell wall material was stable for several weeks at 4 °C, with no significant destaining over that period. This suggests that CAS is strongly complexed with Al and that Al is bound tightly to pectate or cell wall material.

The development of a purple color on Al−pectate and cell wall material indicates a high Al concentration bound to the sorbents with consequential hydrolysis of Al. Hence, a purple color developing on stained Al-treated roots does not only indicate a high Al concentration but also hydrolysis of the bound Al.

ABBREVIATIONS USED

CAS, chrome azurol S; GalA, galacturonic acid; ICP−OES, inductively coupled plasma−optical emission spectroscopy; NMR, nuclear magnetic resonance.

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LITERATURE CITED

(1) Wehr, J. B.; Blamey, F. P. C.; Menzies, N. W. Aluminum. In *Encyclopedia of Soil Science*; Lal, R., Ed.; Taylor and Francis: New York, 2007; pp 1−6.

(2) Bertsch, P. M.; Parker, D. R. Aqueous polynuclear aluminum species. In *The Environmental Chemistry of Aluminum*; Sposito, G., Ed.; CRC Lewis Publishers: Boca Raton, FL, 1996; pp 117−168.

(3) Akitt, J. W.; Greenwood, N. N.; Khandelwal, B. L.; Lester, G. D. ^{27}Al nuclear magnetic resonance studies of the hydrolysis and polymerisation of the hexa-aquo-aluminium(III) cation. *J. Chem. Soc., Dalton Trans.* **1972**, *1972*, 604–610.

(4) Hayden, P. L.; Rubin, A. J. Determination of monomeric and polynuclear aluminum(III) species and hydrolysis constants. *Sep. Sci. Technol.* **1987**, *21*, 1009–1024.

(5) Mesmer, R. E.; Baes, C. F. Acidity measurements at elevated temperatures. V. Aluminum ion hydrolysis. *Inorg. Chem.* **1971**, *10*, 2290–2296.

(6) Ye, C.; Wang, D.; Shi, B.; Ge, X.; Qu, J. Formation and transformation of Al_{13} from freshly formed precipitate in partially neutralized Al(III) solution. *J. Sol-Gel Sci. Technol.* **2007**, *41*, 257–265.

(7) Casey, W. H. Large aqueous aluminum hydroxide molecules. *Chem. Rev.* **2005**, *106*, 1–16.

(8) Bi, S. P.; Wang, C. Y.; Cao, Q.; Zhang, C. H. Studies on the mechanism of hydrolysis and polymerization of aluminum salts in aqueous solution: Correlations between the "Core-links" model and "Cage-like" Keggin-Al_{13} model. *Coord. Chem. Rev.* **2004**, *248*, 441–455.

(9) Sarpola, A. T.; Hietapelto, V. K.; Jalonen, J. E.; Jokela, J.; Ramo, J. H. Comparison of hydrolysis products of $AlCl_3 \cdot 6H_2O$ in different concentrations by electrospray ionization time of flight mass spectrometer (ESI TOF MS). *Int. J. Environ. Anal. Chem.* **2006**, *86*, 1007–1018.

(10) Bourrie, G. Deux voies de formation des hydroxydes alumineux en fonction du comportement des complexes polynucleaires d'aluminium: Voie lixiviee a gibbsite et boehmite et voie confinee a gels et bayerite. *C. R. Acad. Sci. Paris* **1990**, *310*, 1221–1226.

(11) Toma, M.; Hiradate, S.; Saigusa, M. Chemical species of Al in a gypsum-treated Kitakami andosol. *Soil Sci. Plant Nutr.* **1999**, *45*, 279–285.

(12) Hiradate, S.; Taniguchi, S.; Sakurai, K. Aluminum speciation in aluminum−silica solutions and potassium chloride extracts of acidic soils. *Soil Sci. Soc. Am. J.* **1998**, *62*, 630–636.

(13) Boudot, J. P.; Maitat, O.; Merlet, D.; Rouiller, J. Occurrence of nonmonomeric species of aluminium in undersaturated soil and surface waters: Consequences for the determination of mineral saturation indices. *J. Hydrol.* **1996**, *177*, 47–63.

(14) Thomas, F.; Masion, A.; Bottero, J. Y.; Rouiller, J.; Genevrier, F.; Boudot, D. Aluminum-III speciation with acetate and oxalate a potentiometric and aluminumn-27 NMR study. *Environ. Sci. Technol.* **1991**, *25*, 1553–1559.

(15) Masion, A.; Thomas, F.; Tchoubar, D.; Bottero, J. Y.; Tekely, P. Chemistry and structure of Al(OH)/organic precipitates. A small-angle X-ray scattering study. 3. Depolymerization of the Al_{13} polycation by organic ligands. *Langmuir* **1994**, *10*, 4353–4356.

(16) Masion, A.; Bottero, J. Y.; Thomas, F.; Tchoubar, D. Chemistry and structure of Al(OH)/organic precipitates. A small-angle X-ray scattering study. 2. Speciation and structure of the aggregates. *Langmuir* **1994**, *10*, 4349–4352.

(17) Kerven, G. L.; Larsen, P. L.; Blamey, F. P. C. Detrimental sulfate effects on formation of Al-13 tridecameric polycation in synthetic soil solutions. *Soil Sci. Soc. Am. J.* **1995**, *59*, 765–771.

(18) Perry, C. C.; Shafran, K. L. The sytematic study of aluminium speciation in concentrated aqueous solutions. *J. Inorg. Biochem.* **2001**, *87*, 115–124.

(19) Bertsch, P.; Layton, W.; Barnhisel, R. I. Speciation of hydroxy-aluminum solutions by wet chemical and aluminum-27 NMR methods. *Soil Sci. Soc. Am. J.* **1986**, *50*, 1449–1454.

(20) Yamaguchi, N.; Hiradate, S.; Mizoguchi, M.; Miyazaki, T. Disappearance of aluminum tridecamer from hydroxyaluminum solution in the presence of humic acid. *Soil Sci. Soc. Am. J.* **2004**, *68*, 1838–1843.

(21) Adams, M. L.; Hawke, D. J.; Nilsson, N. H. S.; Powell, K. J. The relationship between soil solution pH and Al^{3+} concentrations in a range of South Island (New Zealand) soils. *Aust. J. Soil Res.* **2000**, *38*, 141–153.

(22) Skyllberg, U. pH and solubility of aluminium in acidic forest soils: A consequence of reactions between organic acidity and aluminium alkalinity. *Eur. J. Soil Sci.* **1999**, *50*, 95–106.

(23) Lofts, S.; Woof, C.; Tipping, E.; Clarke, N.; Mulder, J. Modelling pH buffering and aluminium solubility in European forest soils. *Eur. J. Soil Sci.* **2001**, *52*, 189–204.

(24) Parker, D. R.; Zelazny, L. W.; Kinraide, T. B. Comparison of three spectrophotometric methods for differentiating mononuclear and polynuclear hydroxy-aluminum complexes. *Soil Sci. Soc. Am. J.* **1988**, *52*, 67–75.

(25) Masion, A.; Bertsch, P. M. Aluminium speciation in the presence of wheat root cell walls: A wet chemical study. *Plant Cell Environ.* **1997**, *20*, 504–512.

(26) Kopittke, P. M.; Menzies, N. W.; Blamey, F. P. C. Rhizotoxicity of aluminate and polycationic aluminium at high pH. *Plant Soil* **2004**, *266*, 177–186.

(27) Furrer, G.; Trusch, B.; Muller, C. The formation of polynuclear aluminum under simulated natural conditions. *Geochim. Cosmochim. Acta* **1992**, *56*, 3831–3838.

(28) Parker, D. R.; Bertsch, P. Formation of the "Al13" tridecameric polycation under diverse synthesis conditions. *Environ. Sci. Technol.* **1992**, *26*, 914–921.

(29) Brown, P. L.; Sylva, R. N.; Batley, G. E.; Ellis, J. The hydrolysis of metal ions. Part 8. Aluminium(III). *J. Chem. Soc., Dalton Trans.* **1985**, *1985*, 1967–1970.

(30) Feng, C.; Shi, B.; Wang, D.; Li, G.; Tang, H. Characteristics of simplified ferron colorimetric solution and its application in hydroxy-aluminum speciation. *Colloids Surf., A* **2006**, *287*, 203–211.

(31) Kennedy, J. A.; Powell, H. K. J. Colorimetric determination of aluminium(III) with chrome azurol S and the reactivity of hydrolysed Al species. *Anal. Chim. Acta* **1986**, *184*, 329–333.

(32) Kinraide, T. B. Reconsidering the rhizotoxicity of hydroxyl, sulphate, and fluoride complexes of aluminium. *J. Exp. Bot.* **1997**, *48*, 1115–1124.

(33) Jansen, S.; Robbrecht, E.; Beeckman, H.; Smets, E. Aluminium accumulation in the Rubiaceae: An additional character for the delimitation of the subfamiliy Rubioideae? *IAWA J.* **2000**, *21*, 197–212.

(34) Parker, D. R.; Bertsch, P. M. Identification and quantification of the "Al13" tridecameric polycation using ferron. *Environ. Sci. Technol.* **1992**, *26*, 908–914.

(35) Wehr, J. B. Reactions of cations with pectin and root cell walls. Ph.D. Thesis, The University of Queensland, St. Lucia, Queensland, Australia, **1998**.

(36) Blumenkrantz, N.; Asboe-Hansen, G. New method for the quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484–489.

(37) Carpita, N. C.; Gibeaut, D. M. Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **1993**, *3*, 1–30.

(38) Xia, H.; Rayson, G. D. Investigation of aluminum binding to a *Datura innoxia* material using ^{27}Al NMR. *Environ. Sci. Technol.* **1998**, *32*, 2688–2692.

(39) Orvig, C. The aqueous coordination chemistry of aluminum. In *Coordination Chemistry of Aluminum*; Robinson, G. H., Ed.; VCH: New York, 1993; p 234.

(40) Mimmo, T.; Marzadori, C.; Gessa, C. Does the degree of pectin esterification influence aluminium sorption by the root apoplast? *Plant Soil* **2009**, *314*, 159–168.

(41) Hanke, D. E.; Northcote, D. H. Molecular visualization of pectin and DNA by Ruthenium Red. *Biopolymers* **1975**, *14*, 1–17.
(42) Pakalns, P. Spectrophotometric determination of aluminium with chrome azurol S. *Anal. Chim. Acta* **1965**, *32*, 57–63.
(43) Hawke, D. J.; Powell, K.; Sjöberg, S. A potentiometric and spectrophotometric study of the system $H^{+}-Al^{3+}-$chrome azurol s. *Polyhedron* **1995**, *14*, 377–385.
(44) Hawke, D. J.; Powell, H. K. J.; Simpson, S. L. Equilibirium modelling of interferences in the visible spectrophotometric determination of aluminium(III): Comparison of the chromophores chrome azurol S, eriochrome cyanine R and pyrocatechol violet, and stability constants for eriochrome cyanine R−aluminium complexes. *Anal. Chim. Acta* **1996**, *319*, 305–314.

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