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Potentiometric, FTIR and NMR studies of the complexation of metals with theaflavin

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Aluminium(III) complexes of theaflavin (LH) were studied by potentiometric, UV-Vis, NMR and FTIR spectroscopic methods. The stoichiometries of the main species formed in the aluminium(III)–theaflavin systems are [AlL], [AlL_2], [AlL_3], [AlL_3H_{-1}] and [AlL_3H_{-2}]. AlL, AlL_2 and AlL_3 correspond to complexes formed between Al(III) and the benzotropolone ring of one, two and three theaflavins, respectively. The species [AlL_3H_{-1}] and [AlL_3H_{-2}] could be accounted for by deprotonation of free phenol groups in the complexed theaflavin. ^{1}H NMR and HMQC experiments provided structural information on the complexes and allowed the determination of the metal coordination site. FTIR analysis of model aluminium complexes together with the aluminium theaflavin complexation corroborated the coordination site and allowed further spectral analysis of the aluminium–theaflavin system. The following complex stability constants have been evaluated from potentiometric analysis; $\log\beta_{AlL}$= 7.80 (± 0.07), $\log\beta_{AlL_2}$= 15.00 (± 0.12), $\log\beta_{AlL_3}$ = 22.46 (± 0.13), $\log\beta_{AlL_3H_{-1}}$ = 16.98 (± 0.1), $\log\beta_{AlLH_{-2}}$ = 10.19 (± 0.1). UV-Vis spectroscopy confirmed in part the stoichiometric coordination of the aluminium theaflavin complex. The complexation of Mn(II) with theaflavin was also investigated potentiometrically. The stoichiometry of the major complex formed in the manganese(II)–theaflavin system is [$MnLH_{-1}$] and the equilibrium constant has been evaluated as $\log\beta_{MnL}$ = 4.80 (± 0.03).

Introduction

Tea drinking is widespread throughout the world. The invigorating properties of tea have long been known. More recently, the possible health benefits of tea have received considerable attention and these have been attributed primarily to the presence of flavonoid phenolics present in tea.[1] The evidence for these benefits rests on the results obtained from numerous *in vitro* antioxidant experiments but also from short-term intervention[2–5] and epidemiological studies.[6]

A group of polyphenolic compounds unique to black tea are the theaflavins. These are benzpyran-substituted benzotropolones, formed during tea processing by the enzymatic oxidative condensation of fresh leaf catechins (flavan-3-ol) or their gallates. The theaflavins make a non-dominant contribution to the taste, colour (brightness) and antioxidant capacity of the tea liquor and therefore play a role in conferring quality and health properties to tea. They are a group of flavonoid polyphenols that have been extensively investigated.[7–10]

There are, in addition, possible adverse effects of polyphenolic compounds associated with tea drinking. They can for example react with numerous metal ions through a variety of mechanisms thereby reducing their bioavailability. There is some evidence that some tea constituents can have a negative association with measures of iron status in a number of different populations.[11–14] However a more recent study has been suggested that in the well balanced diet of western populations this effect is negligible.[15] Polyphenols, together with other metal chelators have also been reported to have a positive influence on the treatment of iron overloading in humans.[16,17] Metal ions have been reported to form stable metal complexes with 1,2-diolate chelate rings[18–20] and it has been suggested that the galloyl group in polyphenols is especially responsible for the inhibitory effects of iron absorption.[21] The antioxidant properties of theaflavins are also related to their galloyl groups due to the enhanced iron scavenging ability.[22]

Tea is naturally high in aluminium. However, studies have consistently demonstrated that the beverages prepared by traditional infusion methods contains considerably less aluminium than the original leaves[23] and that the bioavailability of aluminium from tea infusions is low. A study by the British Biological Research Association[24] has demonstrated that consumption of tea resulted in the same blood plasma level of aluminium as consumption of water. This finding, based on measurements in human volunteers, is in line with other work by St. Thomas′ Hospital.[25] which noted that " ··· only a small proportion of the aluminium in tea is even potentially available for absorption through the small bowel". The low biological availability of aluminium has also been highlighted in recent detailed reviews[26,27] and this may possibly be related to the strength of the binding of the numerous polyphenolics and organic acids to the aluminium ions.

Tea is also a potentially important source of dietary manganese as one litre (4 cups) of tea is estimated to contain 1.8–5.2 mg Mn.[28–31] Manganese is an essential element for a number of key enzymes including liver pyruvate carboxylases, arginase and most notably, Mn-dependent superoxide dismutase (MnSOD).[32] The bioavailability of the naturally present manganese could however be affected by its complexation with tea polyphenolics.

The interactions between tea polyphenols and metal ions is thus an important area of study and in this work the reaction between theaflavin and the metals Al(III) and Mn(II) have been investigated using potentiometric titrimetry. The complexation of aluminium with theaflavins has also been investigated using UV-Vis, FTIR and NMR spectroscopy.

Experimental

Purification of theaflavin

All solvents were of analytical grade (Fisher) and used as supplied. Methylbutanol and acetic acid were supplied by Sigma. Tannase (tannin acylhydrolase, EC.3.1.1.20) was purchased from Kikkoman, Japan. The enzyme was stored at 4 °C. Mixed theaflavin was obtained from black tea, as detailed elsewhere.[8] Pure theaflavin was prepared by the enzymatic de-gallation of this fraction.[8] Tannase, with an optimum temperature of 35 °C and a pH of 6.0,[33] is sufficiently active at slightly lower pH values (pH = 4.0) to enable the hydrolysis of gallated theaflavins

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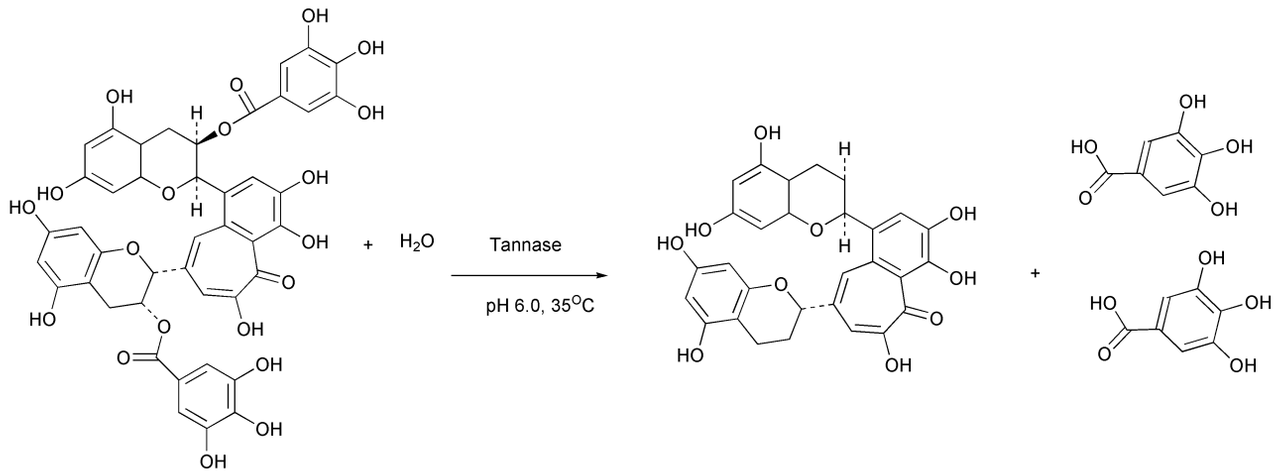

Scheme 1

according to Scheme 1. Theaflavin extract (1 mg ml^{-1}) was hydrolysed at a tannase concentration of 2 mg ml^{-1} at a temperature of 35 °C.[34] The process of degallation was monitored using cellulose TLC plates (Merck) and the solvent systems of methybutanol–acetic acid–water (2 : 2 : 1). The tannase hydrolysis reaction reached completion after about 20 min with complete disappearance of the gallated theaflavins. Detection was carried out using ferric chloride reagent.[35] The crude reaction mixture was lyophilised and separated on a Sephadex LH-20 column (40 cm × 4.8 cm i.d.).[8,36] The solvent system of acetone–water (40 : 60) facilitated the separation of the gallic acid from the theaflavin. TLC was again used to confirm elution of gallic acid into the different fractions using the above solvent system. Pure theaflavin fractions were concentrated and lyophilised. The purity of the theaflavin was confirmed by TLC and ^{1}H NMR spectroscopy.[8]

Titrations

High purity reagents were used throughout. Aluminium trichloride ($AlCl_3$ from Aldrich) and manganese chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$ from Sigma) solutions were purified or obtained of the highest purity as detailed elsewhere.[37,38] Titrations were carried out in a jacketed titration vessel supplied by Metrohm through which water at 25 °C was circulating using a DMS 716 Titrino. A series of perchloric acid solutions in the range 1.0–5.0 × 10^{-3} M, adjusted to ionic strength of 0.5 M with $NaClO_4$ were titrated potentiometrically against standard NaOH (0.096 M). The endpoints of the titration were determined using the Gran method.[39] In this manner the hydrogen ion concentration at any point on the titration curve could be calculated using a computer programme entitled GRAN1.[39] Hence, the pH meter reading could be directly related to the actual hydrogen ion concentration present in the solution. In the titration vessel 50 ml of electrolyte solution (0.5 M $NaClO_4$ in all cases) was combined with aliquots of standardised aluminium (0.1–0.2 mM) and theaflavin (0.5–0.8 mM). Each titration consisted of a forward titration with 40–120 points collected over 24 h. An equilibrium time of 8 min was allowed for the addition of each aliquot. No attempts were made at back titrations due to the inherent instability of theaflavins in alkaline environment.[40] The pH instability of theaflavins was checked by acidifying alkaline solutions of theaflavin (titrated against NaOH to pH 9.00) and re-titrating against NaOH. The logβ values were calculated from triplicate determinations. The data from each titration were imported into the program HYPERQUAD2000[41] and treated by non-linear least square refinement. This program and the algorithms employed are dealt with in detail elsewhere.[42] All equilibrium constants were defined as cumulative formation constants, β_{mlh} according to eqns. (1) and (2). Theaflavin was treated as a monoprotonated ligand (LH) with the deprotonated theaflavin as (L^-). The manganese system was treated in a similar fashion.

$$m\,Al^{3+} + l\,L^- + h\,H^+ \rightleftharpoons Al_mL_lH_h^{3m-l+h} \quad (1)$$

$$\beta_{mlh} = \frac{[Al_mL_lH_h]}{[Al]^m[L]^l[H]^h} \quad (2)$$

The pK_a values of the theaflavin were investigated previously using spectrophotometry.[40] It was only possible to obtain an accurate value for one phenolic moiety, due to their instability in alkaline environments. The value obtained previously for the proton on the tropolone moiety of 8.7 was employed during the calculations of this study. Species distribution diagrams were constructed using the WINCOMICS2000 program.[43] UV-Vis spectra were recorded on a Perkin Elmer Lambda 35 UV/VIS spectrometer.

NMR Spectroscopy

^{1}H NMR spectra were measured at 400 MHz on a Bruker AMX400 spectrometer using a multinuclear 5 mm inverse probe at 300 K. The solvent used was ^2H_2O and the internal reference was TSP (sodium 3-(trimethylsilyl)tetradeutereopropionate). The pD values were obtained on the basis of the relationship pD = pH_{measd} + 0.45. pH_{measd} was determined using a thin combination electrode calibrated by with standard aqueous buffers.[44] Heteronuclear Multiple Quantum Coherence (HMQC) is one of the simplest techniques designed to correlate proton and carbon nuclei. HMQC spectra were run at 300 K using the multinuclear 5-mm inverse probe. Unlike most other metals, extremely useful structural information can be obtained on the coordination of aluminium organic compounds through the use of ^{1}H NMR spectroscopy.[45]

FTIR Spectroscopy

In the preparation of IR samples, metal ion was added to aqueous solutions of theaflavin to obtain a final solution, which was 1 mM in theaflavin and 0.5 mM in aluminium. The pH was adjusted using NaOH (0.096 M). Prior to spectroscopy all samples were lyophilised at pH 4.0. The FTIR spectra were recorded from dried powder using a Bio-Rad FTS-6000 spectrometer (Bio-Rad Laboratories, Cambridge MA, USA) and a Golden-Gate single reflection diamond ATR unit (Specac Inc., Smyrna GA, USA). A spectral resolution of 2 cm^{-1} was used in all measurements and 1000 interferograms were co-added before Fourier transformation. These values were chosen to achieve a sufficient number of data points and signal-to-noise ratio for deconvolution enhancement[46,47] of the apparent resolution in the spectra. A peak width narrowing factor (K

factor) of 2 and a half width of 16 cm^{-1} were used in the deconvolution procedure. In addition to this the resultant peak positions were checked by calculating the second derivative of the original spectrum. The mathematical transformations were performed in absorbance mode using Win-IR Pro Version 2.97 (Bio-Rad). The same software was used for recording the spectra. The deconvoluted spectra were then transformed to GRAMS/32 (Galactic Industries, USA) for publication. The aluminium tropolone complex was synthesised as detailed elsewhere.[48]

Results and discussion

Fig. 1 illustrates the UV-Vis spectra obtained for the addition of aluminium aliquots to theaflavin at a pH of 4.1. The presence of isosbestic points at 479, 425, 380 and 348 nm indicate the existence of two absorbing species in equilibrium with the smooth conversion from theaflavin to metal–theaflavin product. Only one complex is observed during UV-Vis spectroscopic analysis, on the addition of a large excess of aluminium aliquots. Major peaks are present in the spectrum at 525, 460, 400 and 360 nm.

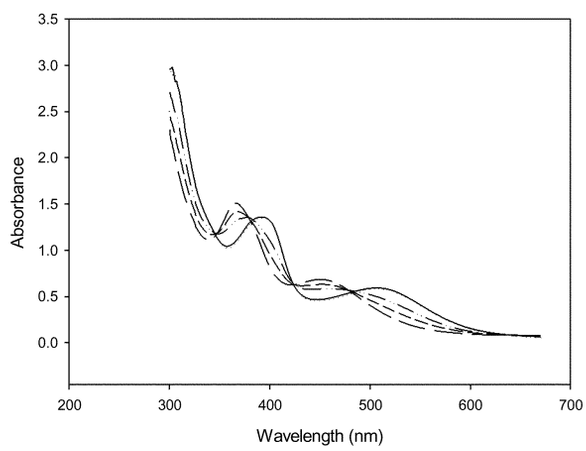

Fig. 1 UV-Vis spectra obtained for the addition of aluminium aliquots (0.2 ml of 1 mM) aluminium against theaflavin (2 ml of 0.1 mM) at a pH of 4.50; 0.05 M Despen buffer, GFS Chemicals.

Stoichiometric coefficients can be easily determined using the molar ratio method on a spectrophotometric titration carried out in methanol (Fig. 2) and confirm the suspected formation of the 1 : 1 species, with the addition of aluminium

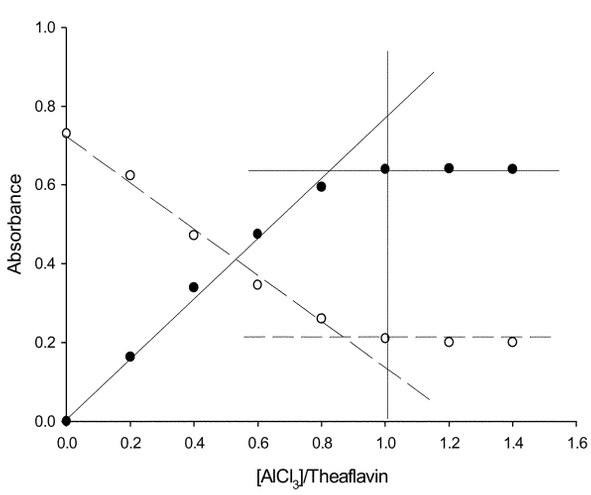

Fig. 2 Determination of the stoichiometry of complexation of aluminium to theaflavin in methanol using the molar ratio method. Titration was carried out by the addition of 0.05 ml aliquots of aluminium (2.5 mM) to 2 ml of 0.5 mM theaflavin.

to the theaflavin solution. Theaflavin has three different rings (Fig. 3) with a number of potential coordinating sites. This could possibly give rise to polynuclear species in solution depending on the concentrations employed, hence during the potentiometric analysis theaflavin was always used in large excess. The coordination site of the species formed with aluminium was investigated by ^{1}H NMR spectroscopy in order to determine all the coordination sites. A complete assignment of the ^{1}H NMR signals of theaflavin in [$^2H_6$]dimethyl sulfoxide has been published.[8] Fig. 4 shows the benzotropolone region of a ^{1}H NMR spectrum recorded prior to and following the addition of aluminium. The three peaks in the spectrum of the 'free' theaflavin (A) correspond to the g, e and c protons,[8] while the protons in the complexed theaflavin (B) are indicated by g1, e1 and c1. In the spectrum of a metal–polyphenol complex the coordination site can be easily located.

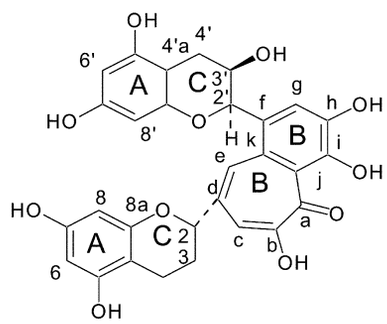

Fig. 3 Structure of theaflavin, together with numbering employed in the assignment of spectra.[8]

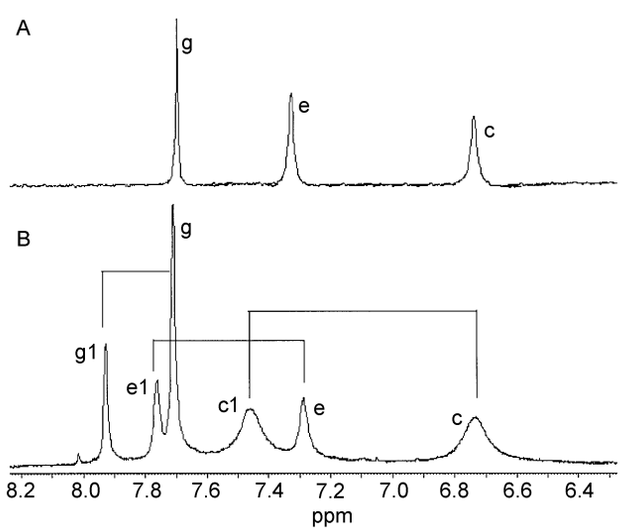

Fig. 4 ^{1}H NMR spectra of theaflavin with and without aluminium: theaflavin = 3 mM, aluminium = 10 mM, pD =3.7; A = theaflavin, B = complex.

The downfield shift observed for the C–H proton signal of the benzotropolone ring in the complexed theaflavin, suggests that complexation is reducing the electronic density of the oxygen (phenolic and carbonyl) and consequently the densities of the aromatic carbon atoms, so that the aromatic C–H signal shift downfield. There are some small shifts evident for the protons of the A ring, however this is more likely due to a disruption of the known interactions between the theaflavin rings.[7] The 'stacking phenomena' or self-association of theaflavins in solution is well characterised and can result in concentration dependent chemical shifts of the measured spectra.[7] This quite simple approximation of associating chemical shift with proximity to coordination site is mainly valid for larger molecules, as in the case of small ligands such as citric acid all proton and carbon nuclei are close enough to the donor atoms.[49] The

Table 1 Assigment of proton peaks of the benzotropolone ring after the addition of aluminium[a]

	δ_H	
Proton	Al–Theaflavin complex	Theaflavin
g	7.8	7.62
e	7.68	7.29
c	7.40	6.80

[a] Only benzotropolone protons shown.

numbering scheme in the assignment of the ^{1}H NMR spectra of free theaflavin is as assigned previously.[8] ^{1}H NMR spectra were recorded of theaflavin solutions prior to and following the addition of aluminium in ^2H_2O. The most significant changes occur in the benzotropolone region of the spectrum, with the appearance of three new distinct peaks. The data are shown in Table 1. HMQC spectra indicate that the major shift on complexation arises with the proton in the "c" position (Fig. 5(a) and 5(b)). This is to be expected from its position regarding the hydroxyl carbonyl moiety. The peak shift in the ^{1}H NMR of the complex relative to the ligand correlates quite well to proximity to the coordination site. The smallest shift is for the proton in position "g" in the complexed theaflavin. This would be expected if complexation was occurring on the tropolone ring and not involving the phenolic groups in position "h" or "i". There is no evidence of the formation of polynuclear species, even when such an excess of aluminium is employed. There is no evidence of the metal having any significant effect on A or C rings of the compound (Fig. 5(a)). ^{1}H NMR spectral changes were monitored over a 24 h period. The only significant change in the spectrum was the disappearance of some of the A-ring peaks. The two A-ring proton signals weaken gradually with time, as a result of deuteration (H-6 and H-6′).[7] This deuterium substitution occurs in the presence of acidic ^2H_2O. This is observed, even in the absence of any metal. There is no indication of any further spectral changes occurring over time. ^{1}H NMR spectra were recorded of solutions containing 5.0 mM theaflavin to 1.0 mM aluminium at pH 4.5 and 6.0. There was no indication of the involvement of the diphenolic group of the theaflavin in complexation. There was no evidence of the formation of any polynuclear aluminium species.

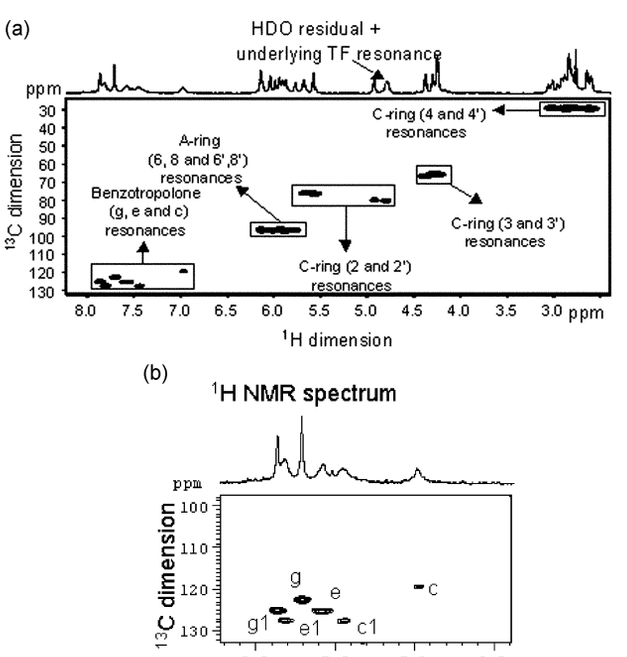

Fig. 5 (a) HMQC spectrum of a solution which is 3 mM theaflavin and 10 mM aluminium recorded in 10% aqueous DMSO. (b) HMQC spectrum of a solution which is 3 mM theaflavin and 10 mM aluminium recorded in 10% aqueous DMSO (benzotropolone region).

IR spectroscopy has led to some success in the characterisation of organic phenolic based polymeric structures.[50,51] IR spectroscopy has been rarely used to characterise or investigate the interactions of metal ions with organic material. Some success has been achieved in the characterisation of metal–humic-acid interactions.[51] The theaflavins contain a number of characteristic structural moieties, which have been well characterised in species of lower molecular weight using vibrational spectroscopy,[52] and hence FTIR spectroscopy offered the opportunity of investigating these systems and providing corroborating evidence for other techniques utilised. Of extreme importance in this analysis is the region from 1750 to 1550 cm^{-1} as the characteristic peaks of tropolonic compounds are most evident in this region. The IR spectra of tropolone has been previously ascribed together with a number of its chelated complexes.[53,54] The characteristic bands of interest for tropolone complexes are present in two main regions 1624–1605 cm^{-1} and 1570–1538 cm^{-1}, which correspond to the CO and CC stretching regions of the benzotropolone ring. The lowering of the carbonyl peak to 1615 cm^{-1} from the more common region at 1700 cm^{-1} has been attributed by Koch to ring size.[55] He suggested that a five-membered ring will result in a strengthening of the CO bond and a weakening of the C–C bonds of the ring while seven-membered aromatic rings show the reverse effect with a weakening of the CO bond and a strengthening of the ring. The carbonyl frequency falls further to 1590 cm^{-1} in the copper complex of tropolone, and hence illustrates the importance of applying this technique.

The carbonyl peak arises in the spectra of both theaflavin (1630 cm^{-1}) and purpurogallin (2,3,4,6-tetrahydroxy-5*H*-benzocyclohepten-5-one) in quite similar regions (1624 cm^{-1}). The benzotropolone peak for CC stretching is strongly evident in the other phenolic compounds analysed arising at 1588 cm^{-1} in purpurogallin and 1601 cm^{-1} in theaflavin. Any disruption to the benzotropolone ring as might occur on metal complexation should be clearly evident from changes to these vibrational modes.

Fig. 6 presents the FTIR data on theaflavin in the absence and presence of Al^{3+} together with the aluminium tropolone complex, focussing on the 1750–1550 cm^{-1} region, in which C=O stretching frequencies occur. The complex was prepared from samples as illustrated in the Experimental section. Examination of this spectral region shows the appearance of an extra band at lower energy to the carbonyl peak at 1590 cm^{-1}, an effect, which is quite similar to that obtained for the copper tropolone complex[52] while the band at 1601 cm^{-1} has shifted slightly to 1605 cm^{-1}. FTIR analysis of the aluminium tropolone complex shows that the carbonyl peak for tropolone at 1613 cm^{-1} has completely disappeared, having been replaced by a new peak at 1590 cm^{-1} in the complex, which is a clear indication that the coordination modes of the complexes in this investigation are quite similar. Analysis of the complexation of metals with carboxylic acid groups in humic substances, resulted in the appearance of a band for the –COO–M asymmetric stretch frequency *ca.* 60 cm^{-1} higher in energy than the original carbonyl frequency.[54] These results and shifts in the bands of both CO and COO on complexation with metals are again consistent with that data.

Equilibrium studies

The spectral, acid–base and redox properties of theaflavins have been studied by pulse radiolysis in aqueous solution.[40] The pH instability of theaflavins enabled the determination of only the first pK_a, which arose from removal of the tropolone proton. The assignment of this pK_a value was made on the basis of the existence of resonance charge-separated forms in benzocycloheptenone, where the energy needed for charge separation is

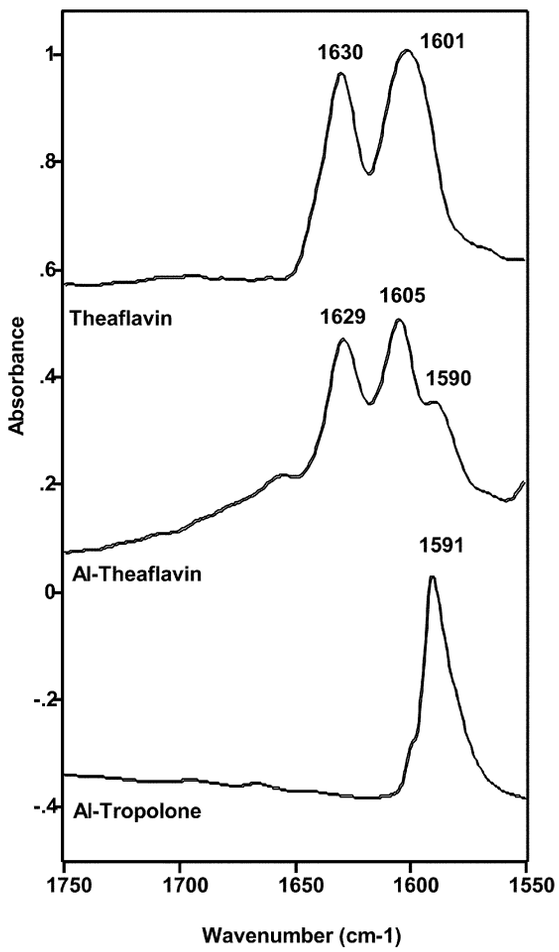

Fig. 6 FTIR spectra obtained of the 1750–1550 cm^{-1} region of aluminium tropolone, free theaflavin and a sample lyophilised from aluminium theaflavin samples.

partially compensated by energy gained by resonance of the tropylium cation.[40] There is no detailed work available in the literature on the interaction of metals with this family of polyphenols. Potentiometric analysis of metal–phenol complexes has proven to be extremely successful, even in extremely complicated systems as highlighted by the work by Öhman *et al.*[56–59] Titrations confirmed that the decomposition of theaflavins did not occur during the course of the pH range investigated during this study (pH < 9.0). All experiments were recorded at 25 °C with a large excess of theaflavin to alleviate the formation of polynuclear species in solution. Only mononuclear species were detected during all of the titrations. Equilibrium studies were carried out on systems involving varying concentrations of aluminium to theaflavin from 1 : 3 to 1 : 6. No studies were carried out with the metal in excess due to the difficulties encountered due to the hydrolysis of aluminium and possible polymeric aluminium complexes. Solutions of variable ratio C_L/C_M (total ligand to total metal concentration) were neutralised by NaOH (0.096 M). Qualitative analysis of the shape of the titration curve allows the stoichiometry of the main complexes to be determined quite easily with sharp inflection points corresponding to the main species in solution.

For the system under investigation a number of hydrolytic aluminium species need to be considered $Al(OH)^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_3$, $Al(OH)_4^{-}$, $Al_2(OH)_2^{4+}$ and $Al_3(OH)_4^{5+}$. The values used are as reported previously.[56] On addition of aluminium, complex formation occurs immediately, as can be seen from the sudden drop in pH. Data sets of 40–120 points, up to a pH of 9.0 were collected and refinement of the individual data sets was successful when a number of aluminium complexes were considered. The final refinement was carried out using all the data sets at different concentrations simultaneously. The main

Table 2 Results of potentiometric study of complex formation between M^{n+} and HL in aqueous 0.5 M $NaClO_4$ at 25 °C (reactions $mM^{n+} + lL^{-} + hH^{+} \Leftrightarrow M_mL_lH_h^{n-l+h}$)

Species	Logβ^a
$[AlL]^+$	7.80 (± 0.07)
$[AlL_2]^+$	15.00 (± 0.12)
$[AlL_3]$	22.46 (± 0.13)
$[AlL_3H_{-1}]^-$	16.98 (± 0.1)
$[AlL_3H_{-2}]^{2-}$	10.19 (± 0.1)
$[MnL]^+$	4.80 (± 0.03)

[a] Averages (± standard deviations) for three titrations.

aluminium complexes detected in solution are AlL, AlL_2, AlL_3, AlL_3H_{-1} and AlL_3H_{-2} where L^- is the deprotonated theaflavin (Table 2). There was no indication of precipitation in the systems investigated as the ligand to metal ratio was always at least in three-fold excess. Under alkaline conditions two species were detected which have been assigned as AlL_3H_{-1} and AlL_3H_{-2}. AlL_3H_{-1} is as a result of the deprotonation of AlL_3 at one of the hydroxy phenols and AlL_3H_{-2}, which arises due to deprotonation of a second hydroxy phenol (eqns. (3) and (4)).

$$AlL_3 \rightleftharpoons AlL_3H_{-1} + H^+ \quad (3)$$

$$AlL_3H_{-1} \rightleftharpoons AlL_3H_{-2} + H^+ \quad (4)$$

The sum of the two dissociations would suggest a pK_a value in the region of 6.10 for the phenolic proton dissociating. This is not unreasonable when compared with pK_a values reported previously for similar phenolic compounds. It is also quite similar to the iron theaflavin system where similar complexes were detected. A similar situation where "isolated" protons become dissociated has arisen previously in the investigation of the interaction of Al(III) with 3-hydroxy-2(1*H*)-pyridinone.[56] The pK_a of the pyridinic proton (>13) was found to have a value of 9.26 in the complex. The lowering of the pK_a for dissociation resulted in the formation of similar species to the present system, *i.e.* AlL_3H_{-1}. While it is was possible to determine the pK_a values of all the phenolic groups of theaflavin using either potentiometric or spectrophotometric techniques[40] due to their pH instability the addition of the metal could change the overall electronic distribution in the ring and hence allow deprotonation to occur. The pK_a values of the phenolic groups in pulse radiolysis generated theaflavin radicals can be as low as 4.3,[40] due to the difference in electronic distribution of the daughter radical, hence it could be suggested that the disruption to the electronic distribution on the benzotropolone ring arising from complexation of a metal could lead to a similar result. The species AlL_3H_{-3} was not detected. This species may occur under more alkaline conditions than those measured under present circumstances (<9.0). Fig. 7 shows a distribution diagram obtained for a solution containing a five-fold excess of theaflavin to aluminium. The proposed structures of the aluminium species detected are shown in Fig. 8. The assignment of the structures for the species $AlLH_{-1}$ and $AlLH_{-2}$ are tentative, as either of the uncomplexed phenolic groups could become deprotonated in an alkaline environment. On comparison with literature data on similar polyphenols, the phenolic groups on the benzonoid moiety of the benzotropolone ring would be most readily lost in an alkaline environment.[56] The metal complexation on the benzotropolone ring would have the greatest effect on the electronic distribution of relevance to these groups. The formation of polynuclear complexes in solution was further investigated through isolating model complexes of aluminium purpurogallin (3,4,5-trihydroxybenzocycloheptenone), from solutions of similar concentration ratios to the present study and their analysis using LC–MS. Only mononuclear complexes were obtained.[60]

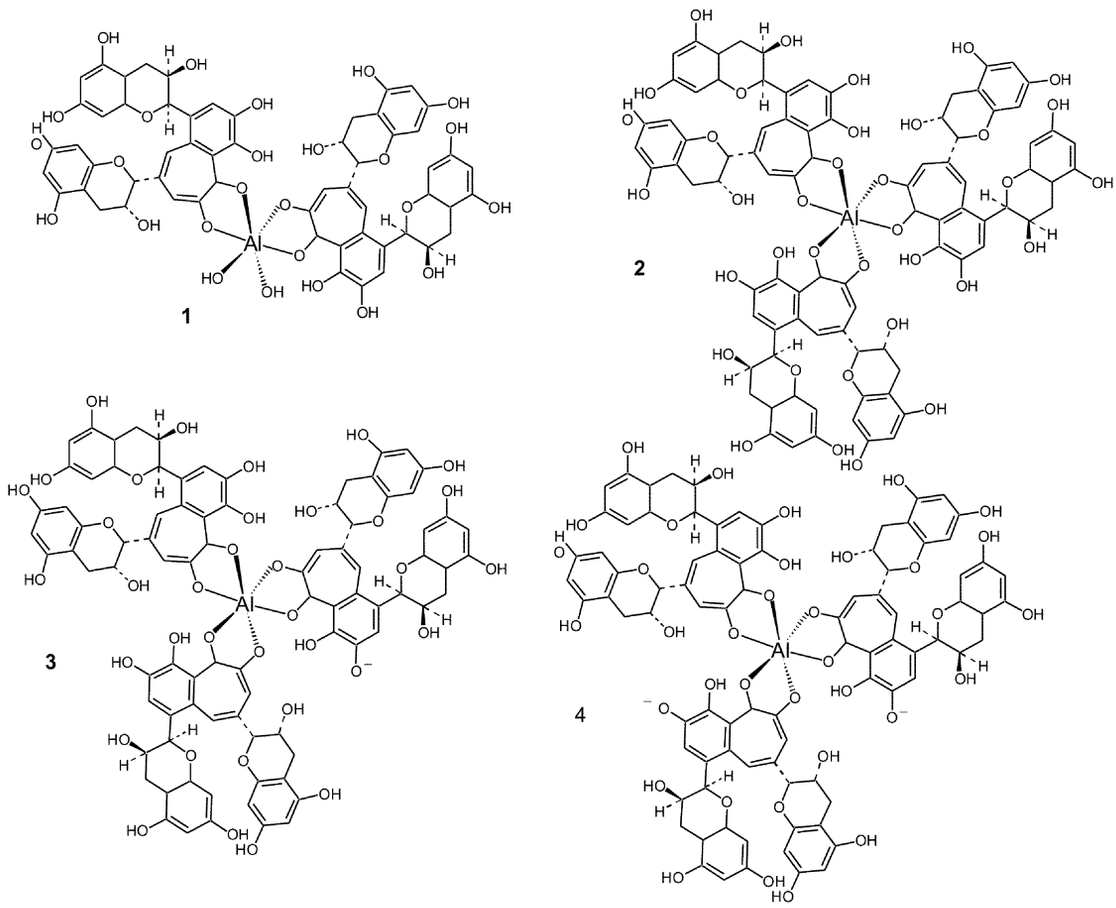

Fig. 8 Proposed structures for the aluminium theaflavin complexes AlL_2, AlL_3 AlL_3H_{-1}, AlL_3H_{-2}.

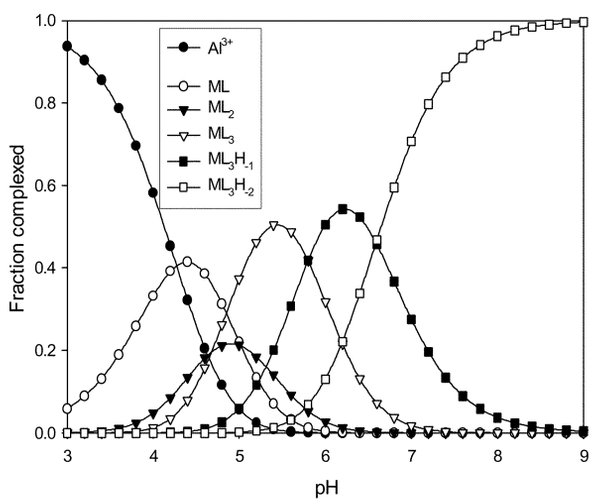

Fig. 7 Distribution diagram obtained for the reaction of aluminium with theaflavin: theaflavin = 0.4 mM, aluminium = 0.1 mM.

Tea is a major source of manganese in the diet.[61] The binding of manganese to theaflavins was thus of interest. Mn^{2+} forms complexes of the least stability with theaflavin. When Mn^{2+} is added to theaflavin solutions there is not as significant a drop in pH as was evident from the additions of aluminium. Analysis of the reaction of manganese with theaflavin indicated the formation of only one major species in solution, a 1 : 1 monomeric species. The $\log\beta$ value for the 1 : 1 complex was calculated at 4.44. The excellent fit to the data would suggest that the position of complexation is quite similar to aluminium. This would indicate that theaflavin would have very little impact on the binding of manganese to its preferred option of lower molecular weight organic acids such as oxalic acid, which are present at much higher concentrations.

It has been suggested that theaflavins inhibit the ability of cells to oxidise low-density lipoprotein and also behave as strong antioxidants through their ability to chelate metals.[22] This has been partly ascribed to their strong iron chelating capability. The gallated theaflavins were suggested as the most effective metal chelators. It is clear that theaflavin digallate (Scheme 1) should have enhanced metal chelation capability, based on the addition of the galloyl groups. The interaction of iron with theaflavin has not been investigated in this study, however the structural investigations of the aluminium and manganese theaflavin systems would suggest a great deal of similarity in the complexation.

Conclusion

Both aluminium and manganese form complexes with the benzotropolone moiety of theaflavins. This is clearly highlighted by the analytical techniques employed in this study. The preference for the benzotropolone moiety might be explained by the existence of the resonance charge-separated form in the B-ring, where the energy needed for charge separation is partially compensated by the energy gained by resonance of the tropylium cation.[40] The long term stability of the complexes formed at neutral to alkaline pH is questionable due to the inherent instability of theaflavins in this region and the formation of negatively charged complexes, which may over longer time periods than investigated in this study lead to polymerisation. There was no evidence of the formation of any polynuclear complexes from potentiometric or NMR analysis. All the complexes investigated in this study are quite large with a molecular weight greater than 600. The lack of bioavailability and difficulty in removal of metaI ions from tea could in terms of the complexes investigated in this study be interpreted on the basis of (1) the stability of each metal polyphenolic complex and (2) the size of each complex. The stability constants at

25 °C compare quite favourably to those of chelators recommended for the treatment of metal toxicity,[62] recorded at similar temperature. Bioavailability is defined as the percentage of absorbed dose of a drug, which reaches the systemic blood circulation.[60] Complexes of molecular weight above 500 Da have very poor absorption.[63] The major body of evidence on the speciation of metals in tea suggests complexes of this magnitude.[26] ^{1}H and ^{13}C NMR spectroscopy are quite useful in providing information for metal complexes involving small organic ligands. The assignment becomes more difficult for larger species, but can be overcome by the application of two-dimensional techniques as illustrated above. Catechins are another major component of tea. On comparison of the equilibrium constants determined for their reaction with aluminium[17,18] with those of theaflavin, it is clear that (assuming similar concentrations) the aluminium theaflavin complexation will be favoured at pH values <5.0. These results, together with investigations carried out by other authors highlight the stability of the complexes tea polyphenols are capable of forming with metals such as aluminium.[18,19,56] The nature of a number of complexes suggested by this study, which are mainly coordinatively unsaturated and hence could be open to smaller organic acids such as oxalate, which is widely available in tea.[10] This work also adds further strength to the work of Ghisalberti in using plant phenolics as a natural alternative for the treatment of metal poisoning.[16,17]

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