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Identification and chromatographic separation of antimony species with α-hydroxy acids

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The ability of α-hydroxy acids to complex with Sb^{III} and Sb^{V} has been established in hydride generation atomic absorption spectrometric determinations of Sb^{III} and solvent extraction techniques. The nature of this complexation with Sb^{V} has yet to be rigorously investigated. NMR, electrospray ionisation mass spectrometry and HPLC–ICP-AES methods have been developed to confirm the existence of these complexes in aqueous media and to determine their molecular masses based on a mole-ratio method. It is shown that anion-exchange chromatography can be used to separate mixtures of $Sb(OH)_x(L)_n$ complexes, [L = citric, DL-malic, lactic or (±)-mandelic acid], where $n = 6 - x$.

Keywords: *Speciation; antimony; α-hydroxy acids; NMR spectroscopy; electrospray ionisation mass spectrometry; high-performance liquid chromatography–inductively coupled plasma atomic emission spectrometry*

In recent years, the determination of antimony has been dominated by spectroscopic methods utilising hydride generation–atomic absorption spectrometry or other spectrophotometric methods for total antimony as SbH_3 or $[SbCl_6]^-$.[1,2] Thus, redox reagents have been employed to transform the Sb species present in a sample into a form suitable for the detection method.

Hydride generation (HG) for the determination of Sb requires the Sb present to be in the +3 oxidation state for optimum production of stibine. Antimony(V), however, under strongly reducing HG conditions, can slowly produce SbH_3 and as such interfere in the determination of Sb^{III}. The +5 oxidation state complexes with a variety of oxygen donors,[3] and antimony salts readily form complexes with various acids in which the antimony forms the nucleus of an anion.[4]

In 1985, Sato[5] reported the complexation of Sb^{III} and Sb^{V} with mandelic acid. These complexes were then extracted into chloroform with Malachite Green (MA). The Sb^{V} used was in the form of $KSb(OH)_6$. In the absence of substantive evidence, Sato suggested that the complex anion $[Sb^{V}(MA)_2(OH)_2]^-$ was formed. Mole-ratio methods to determine this species were not possible owing to the large excess of complexing agent required.

In 1990, Mohammad *et al.*[1] used citric acid (12% m/v) to suppress hydride formation from Sb^{V} during the determination of Sb^{III}. However, the chemistry of the interaction of citrate with Sb^{V} was not elucidated, although complexation was assumed to be involved.

In later work, Hartley *et al.*[6] studied the preparation and crystal structures of Sb^{III} complexes with carboxylic acids, and more specifically the citrate complex. Coordination with the Sb was reported as being through the hydroxo oxygen and the isocarboxylic oxygen. However, this complexation was only achieved by use of lengthy refluxing of antimony(III) oxide with citric acid unlike, in contrast, the addition of citric acid to Sb^{V}, which occurs quickly at room temperature.

Although this study does not directly support Sato's work owing to the different oxidation states, the size and polycarboxylic characteristics of citric and malic acid indicate that multiple group bonding should not be ruled out. A number of studies have been reported since the original work in 1991, concerning a wider range of α-hydroxy acids.[7–9] However, to date none has tackled the question of the formulae for these complexes and their stabilities in a dilute mixed solution, especially in aqueous media.

Speciation studies employing methods already mentioned to determine Sb^{III} and Sb^{V} have gained interest owing to the relative differences in toxicities of the two oxidation states. Sb^{III} compounds can be as much as 10 times more toxic than Sb^{V} compounds, with the highly toxic SbH_3 being the most poisonous.[10] There are also differences in toxicity which depend upon the molecular form,[11] such as the difference in toxicities between organic and inorganic Sb compounds, inorganic being more toxic. A further complication is that under any given set of hydride generation conditions not all Sb compounds can form a detectable volatile species. Therefore, speciation by techniques such as HGAAS are not always sufficient, and a method to separate or isolate the dissolved Sb species is also required. There are just two common Sb compounds of differing oxidation state that are suitably soluble in aqueous media to facilitate separation studies, potassium hexahydroxyantimonate(V) and potassium antimony(III) tartrate hemihydrate. There have been studies that have successfully separated these compounds by HPLC coupled with ICP-AES and MS, but only using aqueous samples,[12,13] where the scope for more complex Sb compounds is limited. Hence the development of a chromatographic model based on just these two compounds inherently lacks robustness and may not lend itself to the separation or identification of unknown Sb compounds in a more complex sample matrix.

To aid this development, a greater range of soluble Sb species with known structures/formulae is required. α-Hydroxy acids are present in a number of environmental/biological systems and if these interact with Sb^{III} and Sb^{V} then methods need to be developed to identify and quantify these compounds.

In this study, Sb^{V} was found to complex quantitatively with citric, DL-malic and (±)-mandelic acid. The resulting complexes were analysed by NMR spectroscopy, identified by ESI-MS and separated by ion-exchange chromatography. In addition, coupling of the chromatography with ICP-AES facilitated the use of a mole-ratio method to determine the level of complexation of the ligands with Sb.

Experimental

Instrumentation

For HPLC, a ConstaMetric 3200 solvent delivery system (LDC Analytical Riviera Beach, FL, USA) and a Model 305 isocratic pump with a Model 805 manometric module (Gilson, Villiers le Bel, France) was used at 1.0 ml min^{-1}. The chromatographic column was an IonPac AS4A anion-exchange column (Dionex, Sunnyvale, CA, USA). Sample introduction was made *via* a six-port Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 100 or 20 μl stainless-steel sample loop. The ICP-AES instruments were a Liberty 200 (Varian, N. Springvale, Australia) and an Optima 3000 DV (Perkin-Elmer, Beacons-

field, Bucks., UK), The operating conditions were typically forward power 1300 W, coolant gas flow rate 15.0–16.5 l min^{-1}, auxiliary gas flow rate 0.4–1.5 l min^{-1} and nebuliser gas flow rate 0.8 l min^{-1}. The ESI-MS instrument was an LCQ mass spectrometer (Finnigan MAT, San Jose, CA, USA) with the following operating conditions: syringe pump flow rate, 5 μl min^{-1}; spray voltage, 3.43 kV; spray current, 1.02 μA; capillary voltage, 28.25 V; and capillary temperature, 210 °C. The analysis commenced 2 min after the start of infusion to allow for stabilisation. The NMR instrument used was an EX270 FT-NMR spectrometer (Jeol, Welwyn Garden City, Herts., UK).

Reagents

All reagents were of analytical-reagent grade unless stated otherwise. All working solutions of Sb^{V} were prepared by dissolving the required mass of potassium hexahydroxyantimonate(V) (Aldrich, Gillingham, Dorset, UK) in the appropriate matrix. All working solutions of citric acid, DL-malic, lactic and (±)-mandelic acid (all from Aldrich) were prepared by dissolving the required amount of each acid in the appropriate matrix. A 10–20 mM solution of NH_4Cl (BDH, Poole, Dorset, UK) was prepared by dissolving 0.5439–1.0698 g of solid in 1000 ml of water. The pH of the mobile phase was adjusted with 0.05 M HNO_3 (BDH). Water obtained using a Milli-Q system (Millipore, Molsheim, France) was used throughout.

Method development

NMR procedure

Solutions of each α-hydroxy acid (2% m/v) were prepared by dissolving 2 g of each acid in 100 ml of water. A 1% solution of Sb^{V} was prepared by dissolving 2.159 g of the Sb^{V} compound $[K(Sb(OH)_6)]$ in 100 ml of water.

Table 1 gives the volumes of each reagent added to 10 ml calibrated flasks for mixing prior to analysis by ^{1}H and ^{13}C NMR spectroscopy.

Chromatographic separation

Four solutions of 1000 mg l^{-1} Sb^{V} were prepared in 100 ml calibrated flasks together with the appropriate mass of α-hydroxy acid to complex with all or part of the Sb^{V}. These were diluted to give 10 mg l^{-1} solutions. Mixtures of these solutions were prepared in which all of the complexes were present in the matrix with Sb^{V} concentrations up to 3 mg l^{-1}. Antimony(III) was not found to react in the same way as Sb^{V} and no determinable difference was found in the retention profiles, so Sb^{III} was excluded from the latter parts of the study.

Using a mobile phase of 20 mM NH_4Cl, aliquots of the aforementioned solutions were injected on to an AS4A anion-exchange column and subsequently detected using ICP-AES, monitoring the Sb emission line at 217.581 nm. Observations were made of the effect of pH on the elution of the individual species over the desired pH range. All retention data and peak profiles were recorded.

Following this work, the chromatographic investigation of these compounds was expanded to include the monitoring of carbon in the α-hydroxy acid ligand. This was achieved by monitoring the C emission line at 193.026 nm using ICP-AES.

To assess the ion-exchange characteristics for both Sb and the ligand, a simultaneous detector was required to obtain real-time

Table 1 Typical compositions of solutions used in the NMR study

System	2% α-Hydroxyacid/ml	1% Sb^{V} solution/ml	Milli-Q water/ml	Sb : acid ratio
Citric acid	3.33	6.67	0	1 : 1
Malic acid	1.67	6.67	1.66	2 : 1
Mandelic acid	10	0	0	100% acid

Table 2 Mole ratios of solutions analysed by HPLC–ICP-AES.

Acid : Sb solution ratio	Concentration of acid/M	Concentration of Sb/M
1 : 1	0.005	0.005
2 : 1	0.0067	0.0033
3 : 1	0.0075	0.0025
4 : 1	0.008	0.002
1 : 2	0.0033	0.0067
1 : 3	0.0025	0.0075

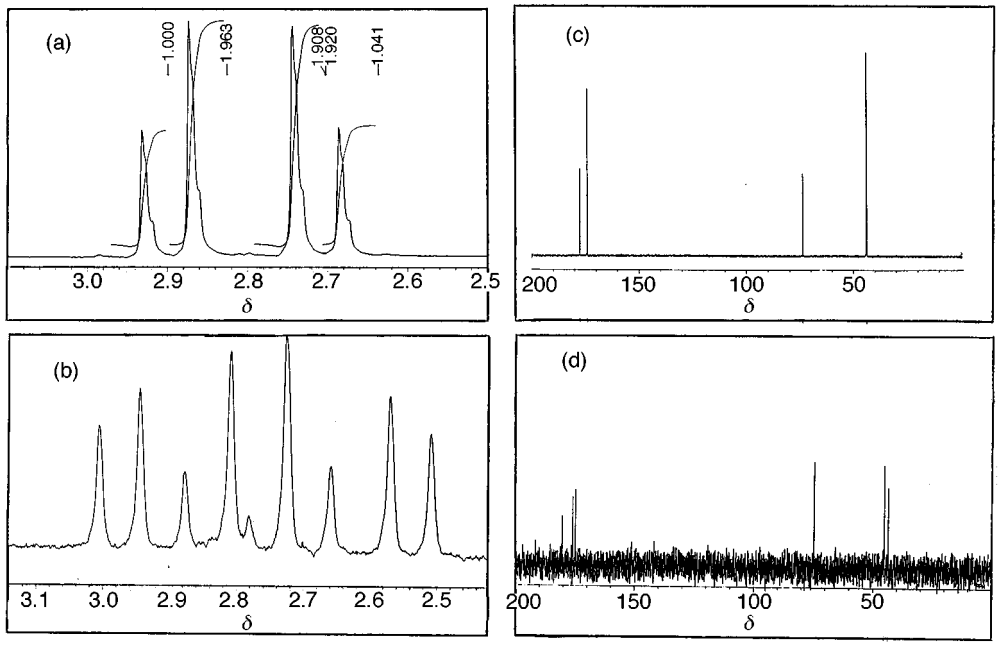
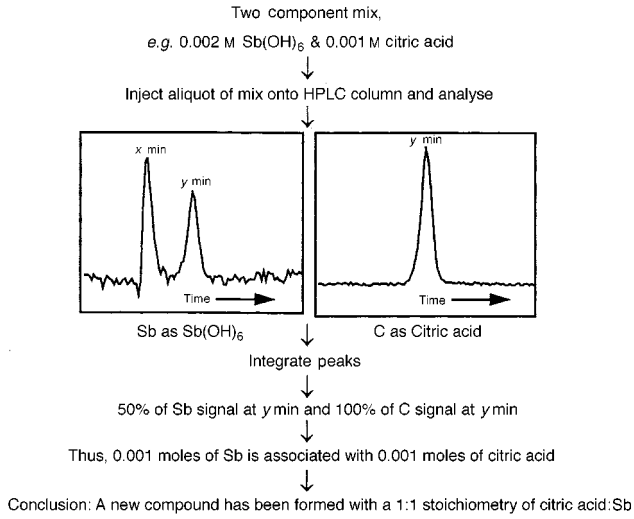


Fig. 1 ^{1}H NMR spectra of (a) 2% m/v citric acid and (b) an equimolar solution of Sb^{V} and citric acid. ^{13}C NMR spectra of (c) 2% m/v citric acid and (d) an equimolar solution of Sb^{V} and citric acid in Milli-Q water.

chromatograms under identical elution conditions. For this purpose an Optima 3000DV ICP-AES system was used to utilise the charge coupled device detector system to monitor all emission lines simultaneously.

The modelling of the level of association of α-hydroxy acid required a mole-ratio method of investigation. For this purpose, several solutions with varying mole ratios of Sb and acid were prepared as shown in Table 2. The high level of Sb present made it necessary for the less sensitive emission line at 204.597 nm to be monitored as the detector was tripped at 217.581 nm.

The model used is based on the premise that if the concentration of each component is known and the resulting peaks in the chromatogram can be identified and integrated, then the peak areas and peak area ratios allow us to assign concentration values to each peak. An example of the experimental procedure used is shown below:



The assessment of association is based on retention data and peak profiles by identifying the peak profiles for associated elements as being exactly overlapping with identical retention times. If they match both criteria then they are displaying identical ion-exchange characteristics and it is highly improbable that they are merely co-eluting. Prior to ESI-MS analysis, *m*/*z* values for these compounds were hypothesised based on the results obtained by HPLC–ICP-AES.

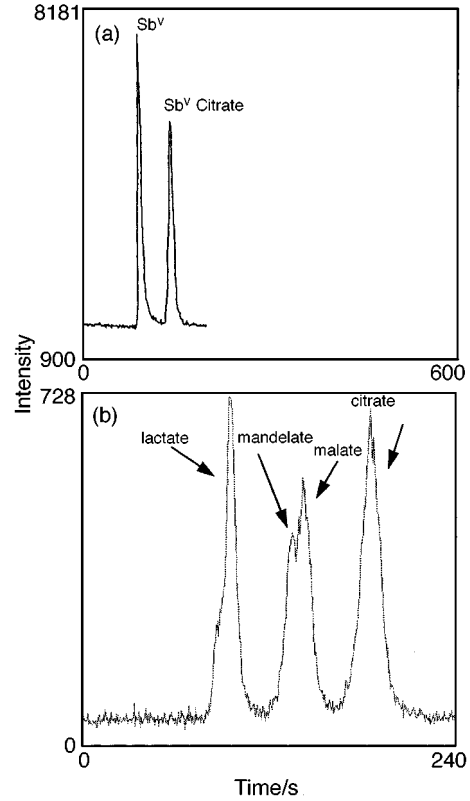


Fig. 3 Chromatograms for (a) an aliquot of Sb^{V} and Sb(citrate) highlighting resolution of peaks and (b) separation of Sb^{V} compounds associated with four different α-hydroxy acids. Concentration of Sb, 3 mg l^{-1}.

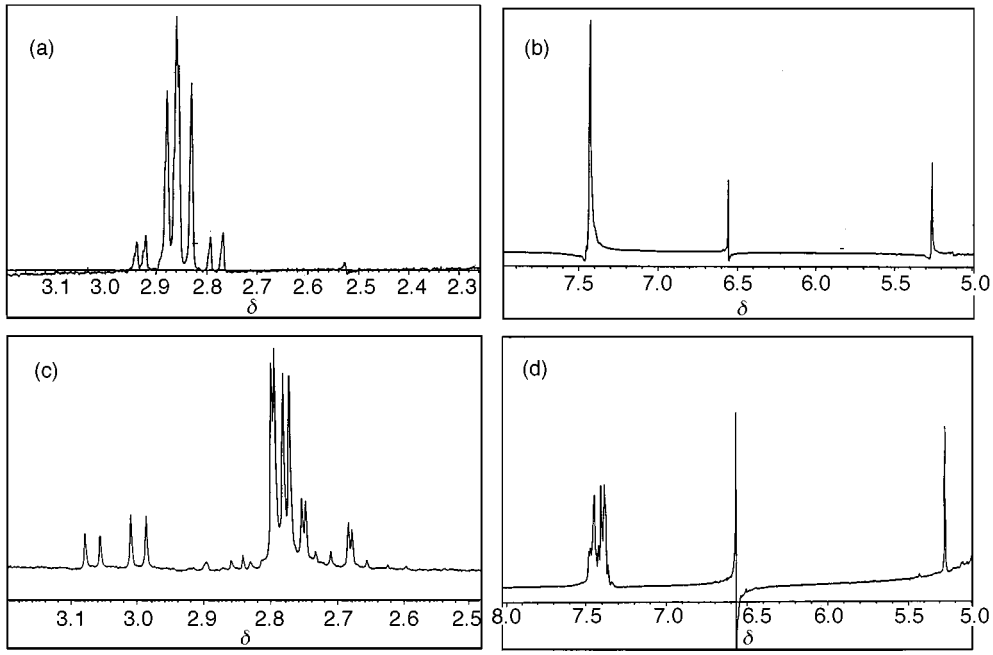


Fig. 2 ^{1}H NMR spectra of (a) 2% malic acid, (b) 2% mandelic acid, (c) an equimolar solution of Sb^{V} and malic acid and (d) an equimolar solution of Sb^{V} and mandelic acid in Milli-Q water.

ESI-MS procedure

The Sb–α-hydroxy acid solutions were infused into an LCQ ESI-MS system in order to identify the complexes formed. The Sb concentration in each solution was 500 mg l^{-1}. The concentrations of citric and malic acid were 500 mg l^{-1} and that of mandelic acid was 1000 mg l^{-1}. From this the *m*/*z* range for analysis was set from 200 to 600. Table 3 gives these *m*/*z* values and the hypothesised compounds.

Results and discussion

The ^{1}H NMR spectrum of 2% m/v citric acid (w/v) is shown in Fig. 1(a). Citric acid has a symmetrical configuration with two enantiotopic CH_2COOH groups and as such can be classified as prochiral.[14] Equivalent enantiotopic hydrogens such as the CH_2 protons in citric acid are indistinguishable in NMR so the signals for the hydrogens overlay each other but exhibit splitting due to the prochirality of the compound. The addition of Sb^V to this system was shown to affect the chemical shift of these protons. Fig. 1(b) shows the ^{1}H NMR spectrum of an equimolar solution of Sb^V–citric acid. The two sets of CH_2 protons are now clearly different. These protons can now be assigned with coupling constants, (i) 16.83 Hz and (ii) 20.28 Hz. This observation was believed to be caused by bonding of the citric acid to Sb through a terminal carboxylic acid group, which resulted in a loss of symmetry in the acid molecule. The difference in chemical shifts of the protons was attributed to the proximity of the protons to the Sb nucleus of the new complex.

The loss of symmetry was demonstrated more clearly by the ^{13}C NMR spectra for the same solutions. In Fig. 1(c), the four carbon environments expected for the 2% acid solution are easily distinguished: $–CH_2–$, 43.995; C–OH, 73.985 ppm; terminal COOH, 174.163 ppm; and iso-COOH, 177.577 ppm. With the addition of Sb, six distinct C environments were observed [Fig. 1(d)], indicating a loss of symmetry in the citric acid molecule.

Table 3 Results of model for calculation of associated peaks in the Sb–citric acid system

Concentration of associated citric acid/M	Concentration of associated Sb/M
0.005	0.0041
0.0036	0.0027
0.0023	0.0021
0.0033	0.0035

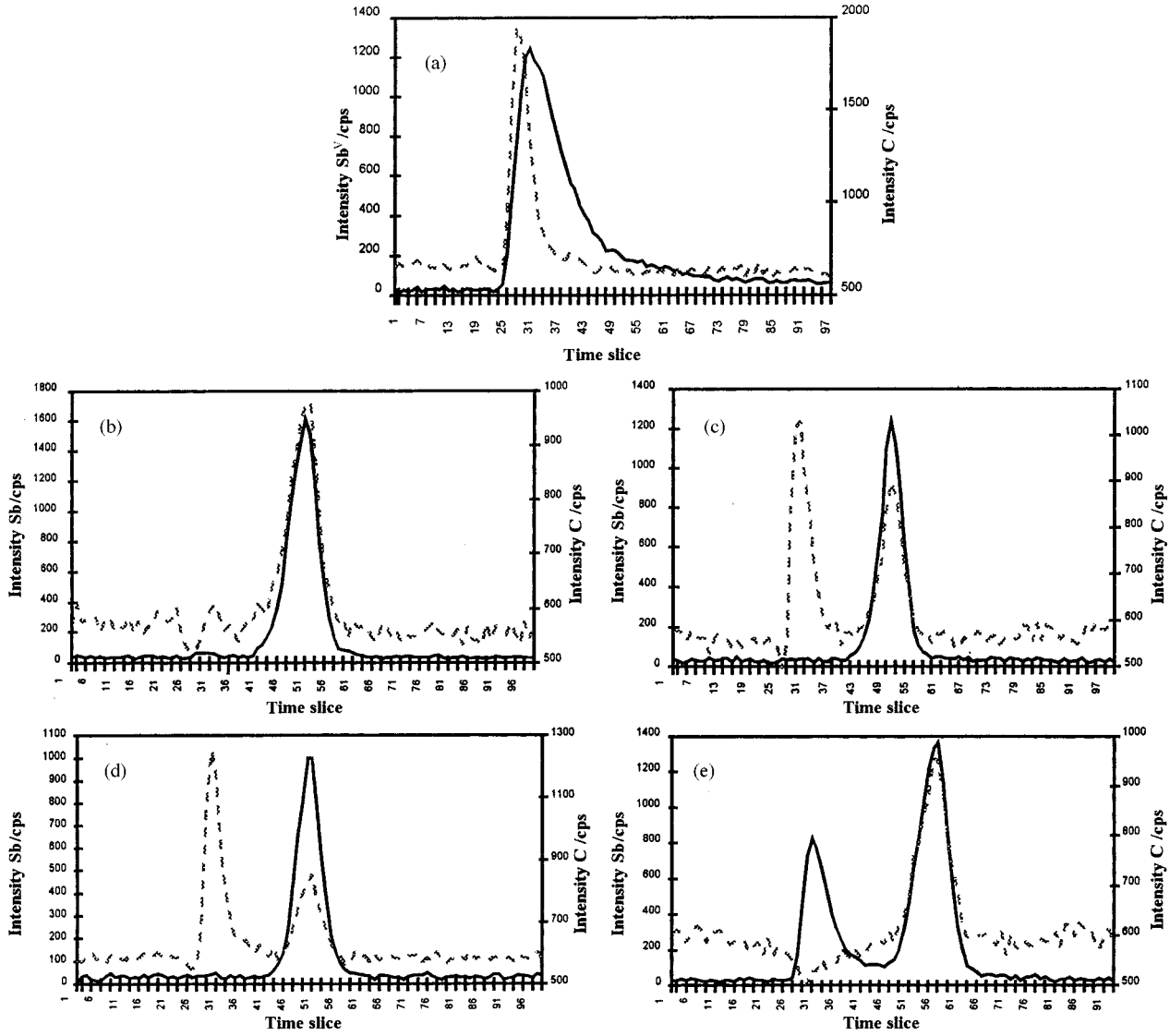


Fig. 4 Chromatograms for Sb–citric acid system. Results obtained using Optima 3000 DV ICP-AES instrument. (a) Individual components; (b) 1 : 1 ratio; (c) 2 : 1; (d) 3 : 1 ratio; and (e) 1 : 2 ratio. Solid lines, Sb 204.957 nm; dashed lines, C 193.018 nm.

The DL-malic and (±)-mandelic acid systems were slightly more complicated, with ^{1}H NMR spectra being the most difficult to interpret. However, clear differences were observed between the NMR spectra for the acid and the NMR spectra for the Sb–acid solutions (see Fig. 2).

The ^{13}C NMR spectrum of 2% malic acid showed the expected four signals; however, on addition of Sb^V, six peaks were observed. It is believed that the malic acid bonds to Sb through either carboxylic acid group and as such signals for both types of complex are observed in the NMR spectra. Hence differences in chemical shift for the most affected carbons were dependent upon which group was involved in the bonding. The ^{13}C NMR spectrum of (±)-mandelic acid supports the ^{1}H NMR spectrum because the addition of Sb produced sufficiently significant chemical shifts in the signals and as such they were better observed. The important point to note for all the systems investigated is that formal bonding of the acid molecule to Sb is strongly favoured.

HPLC–ICP-AES

In the elution protocol described previously, pH played an important role in the elution of the Sb^V–α-hydroxy acid complexes. At pH > 4 Sb–citrate did not elute at all. The other complexes were only affected in terms of retention time, *i.e.*, as the pH decreased the retention time decreased. It is thought that the retention of Sb–citrate on the column at pH values > 4 could be due to contributory anionic properties of the acid ligand itself. Strong retention of all the complexes was observed, although not as strong as for the citrate complex. Fig. 3(a) shows the chromatogram of an aliquot of a solution of $KSb(OH)_6$ and $K[Sb(OH)_x(citrate)_n]$. The peaks are clearly well resolved and the addition of citric acid to this Sb^V compound has a significant effect on its retention time and so a reaction must have taken place to change the nature of the Sb compound. This was especially pronounced when considering the effect of pH. In previous work, $[Sb(OH)_6]^-$ was observed to elute throughout the pH range. The effect of addition of the other acids was similar to that of citric acid. The optimum separation achieved is demonstrated in Fig. 3(b) with the peaks for Sb–malate and Sb–mandelate complexes being the least well resolved. Initial investigations monitoring carbon emission showed that the response was quantifiable and that the peak profiles for the associated acid were identical with those for associated Sb. Fig. 4(a)–(e) show chromatograms for the Sb–citrate solutions described in Table 2.

The peak retention profiles of $[Sb(OH)_6]^-$ and citric acid injected separately are not identical and as such do not demonstrate identical exchange characteristics on-column. However, Fig. 4(b)–(e) show that the Sb and C associated peaks have identical elution profiles and retention times for peak maxima. This was considered good evidence to suggest that the Sb and citric acid were components of one compound. These experiments supported the NMR results indicating that a reaction had taken place in solution and that a new compound had formed. Integration of these peaks and the application of the model described under Experimental showed a firm 1 : 1 relationship between citric acid and Sb. Table 3 shows this more clearly.

Results for malic and mandelic acid using this method showed 1 : 1 and an almost 2 : 1 relationship, respectively. From these results, formulae and relative molecular masses were hypothesised to aid identification by mass spectrometry.

ESI-MS

The mass spectra for the solutions investigated are shown in Fig. 5(a)–(d). The hypothesised *m*/*z* values (Table 4) are highlighted on the respective spectra.

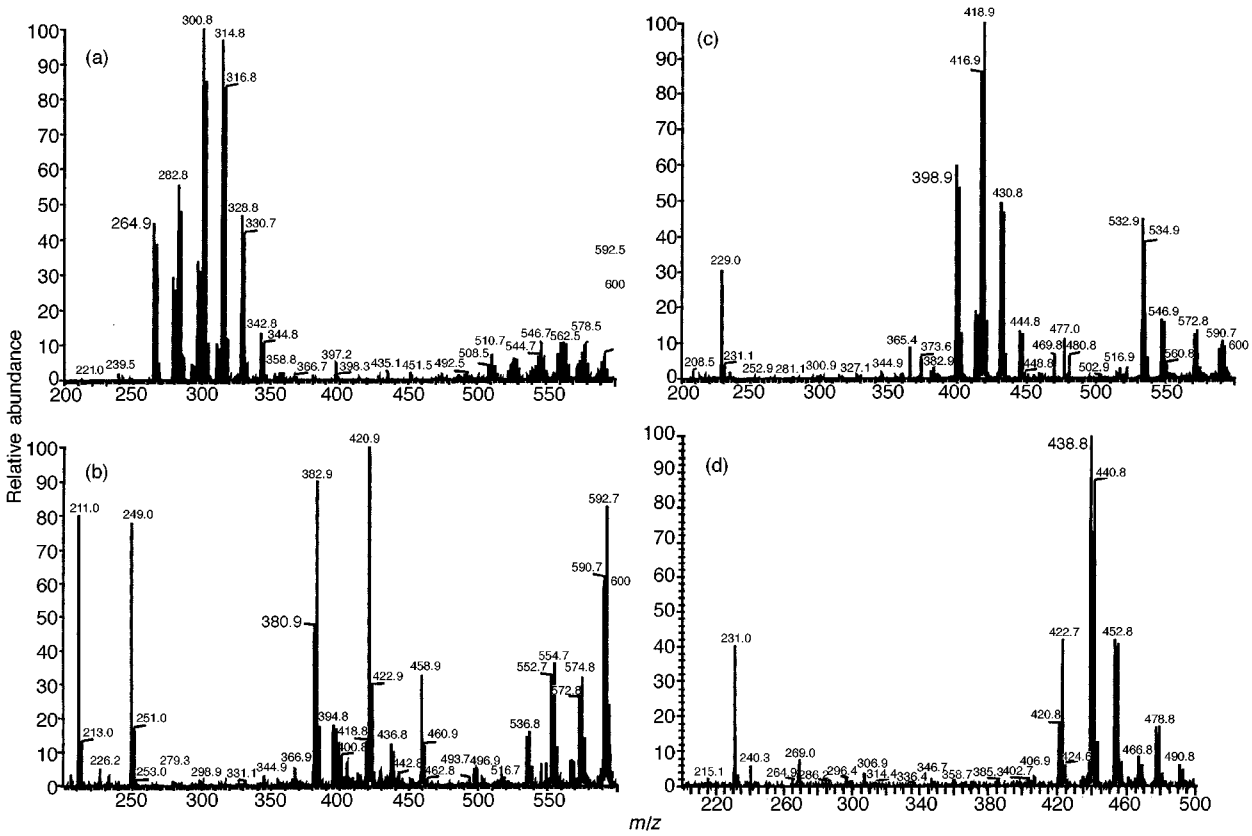


Fig. 5 ESI mass spectra for solutions described under Experimental. (a) $KSb(OH)_6$; (b) $K[Sb(OH)_x(malate)_n]$; (c) $K[Sb(OH)_x(mandelate)_n]$; and (d) $K[Sb(OH)_x(citrate)_n]$.

$KSb(OH)_6$

The extended cluster of ions in this spectrum [Fig. 5(a)] is believed to arise from the energetic ionisation process in the presence of excess MeOH coupled with the affinity of pentavalent Sb for O-atom donating ligands.[3] All the observed signals in the major cluster originate from Sb compounds as the *m*/*z* difference is 2, which reflects the two isotopes ^{121}Sb and ^{123}Sb and their relative abundances of 53% and 47%, respectively.

$K[Sb(OH)_x(malate)_n]$

Fig. 5(b) shows the complete lack of the M^+ cluster observed for $KSb(OH)_6$. The peaks observed at *m*/*z* 381–383 agree with the hypothesised value (Table 4) and that just one malic acid is associated with the Sb atom to form $K[Sb(OH)_5(malate)]$. The ion at *m*/*z* 249–251 indicates the loss of malic acid followed by a further loss of K to give *m*/*z* 211–213.

$K[Sb(OH)_x(mandelate)_n]$

Both of the hypothesised complexes (Table 4) were observed in the mass spectra for this solution [Fig. 5(c)]. The predominant M^+ cluster is associated with the peaks at *m*/*z* 397–399 with a lesser relative abundance for *m*/*z* 533. There was no *m*/*z* 265 M^+ and no evidence to support Sato's hypothesised $[Sb(OH)_2(mandelate)_2]^-$. The two complexes observed appear to be as suggested, $K[Sb(OH)_5(mandelate)]$ and $K[Sb(OH)_4(mandelate)_2]$.

$K[Sb(OH)_x(citrate)_n]$

This spectrum [Fig. 5(d)] had two main features: (i) the ion at *m*/*z* 231 due to $K(C_6H_7O_7)$ and (ii) the major ion at *m*/*z* 439–441 attributed to the presence of $K[Sb(OH)_5(citrate)]$.

The general reduction in cluster size for all the complex systems appeared indicative of stabilisation of the complex by addition of electron-rich ligands to the coordination sphere, making further addition of MeOH groups or other adducts less likely.

Conclusion

Association or complexation of three α-hydroxy acids with Sb^V has been demonstrated by NMR, ESI-MS and HPLC–ICP-AES methods. Simultaneous multi-element monitoring of, for example, Sb and C by ICP-AES coupled with chromatography has been shown to be useful for formula determinations.

The information provided by this study could prove important to the development of a robust method for the molecular speciation of Sb in real samples, by contributing to a better understanding of the separation chemistry. Further work is required to include Sb^{III} complexes and other ligands, possibly inorganic.

Although none of the complexes were isolated as solids, this study appears to be the first to identify them at low concentrations in solution.

Table 4 Hypothesised *m*/*z* values and formulae for the Sb complexes

No.	*m*/*z*	Formula
1	265	$KSb(OH)_6$
2	381	$K[Sb(OH)_5(malate)]$
3	439	$K[Sb(OH)_5(citrate)]$
4	398	$K[Sb(OH)_5(mandelate)]$
5	533	$K[Sb(OH)_4(mandelate)_2]$

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