

Aromatic amino acids in high selectivity bismuth(III) recognition

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The three aromatic amino acids, tyrosine, tryptophan and phenylalanine, play different physiological roles in life processes. Metal ions capable of binding these amino acids may aid in the reduction of effective concentration of these amino acids in any physiological system. Here we have studied the efficacy of some heavy metals for their complexation with these three amino acids. Bismuth has been found to bind selectively with these aromatic amino acids and this was confirmed using spectrofluorimetric, spectrophotometric and cyclic voltammetric studies. The series of heavy metals has been chosen because each of these metals remains associated with the others at very low concentration levels and Bi(III) is the least toxic amongst the other elements. So, selective recognition for Bi(III) would also mean no response for the other heavy elements if contaminants are present even at low concentration levels. The affinity towards these amino acids has been found to be in the order tryptophan < phenylalanine < tyrosine. The association constants of these amino acids have been calculated using Benesi–Hildebrand equations and the corresponding free energy change has also been calculated. The values of the association constants obtained from BH equations using absorbance values corroborate with the Stern–Volmer constants obtained from fluorimetric studies. The evidence for complexation is also supported by the results of cyclic voltammetry.

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Introduction

The aromatic amino acids (AAA), tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), are responsible for the fluorescence emission in proteins and for this reason they have been used as an intrinsic fluorescence probe for the structural and conformational studies of proteins.^{1–3} Tryptophan fluorescence is very sensitive to the chromophore environment and a lot of information can be obtained at different pH, ionic strength, *etc.* More recently tyrosine has attracted much attention as an interesting fluorescence intrinsic probe.^{4–6} The decrease of tyrosine fluorescence due to the binding of Cu²⁺ ions has been previously detected in tyrosine and glycytyrosine.^{7,8} Metal ions can bind to amino acids in several ways, depending on the pH and stoichiometry and the involvement of oxygen from carboxylate groups and nitrogen from amino groups has been demonstrated.^{9,10}

Tyrosine is a precursor to the neurotransmitter, dopamine, and increases the level of dopamine in blood plasma.¹¹ A number of studies have found tyrosine to be useful during conditions of stress, cold, and fatigue.¹² It has also been found that manic patients do better in tyrosine depleted diet. So it is

quite necessary to suppress tyrosine in blood plasma of patients suffering from mania. Metal ion complexation may result in blockage of the active site of tyrosine and hence a suppression in its concentration.

Phenylketonuria (PKU) is a metabolic genetic disorder characterized by inefficient conversion of phenylalanine (Phe) to the amino acid tyrosine. Early cases of PKU were treated with a low-phenylalanine diet. Recent research shows that diet alone may not prevent elevated phenylalanine levels. Optimal treatment involves maintaining blood Phe levels in a safe range while monitoring diet and cognitive development.^{13,14} There is no cure for PKU till date except for the proper diet management. Metal ion complexation may lead to structural changes in Phe and subsequently reduce its toxic effect.

Tryptophan (Trp) helps in vitamin B3 synthesis in our body. It also serves as a precursor for serotonin, a neurotransmitter that helps the body regulate appetite, sleep patterns, and mood. “Carcinoid syndrome” is a malignant condition where tryptophan gets converted to 5-hydroxy indole acetic acid (HIAA) in excess causing cardiac abnormality, intestinal obstruction, *etc.* In such cases suppression of tryptophan in blood plasma may bring out an effective therapeutic condition. Metal ions capable of binding these amino acids may help in the reduction of these amino acids in the blood plasma. There are reports on metal amino acid complexes which may have antibacterial effects. Nickel(II)–tyrosine and Cu(II)–tyrosine complexes have been reported.^{15,16}

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Liver failure is associated with hepatic encephalopathy (HE). An imbalance in plasma levels of AAA and branched chain amino acids (BCAA) and their ratio has been suggested to play a crucial role in HE. The changes in plasma levels are thought to be caused by an increase in BCAA catabolism and a decrease in AAA breakdown in the failing liver. The increase in plasma AAA contributes to increased influx of AAA in brain and this in turn leads to HE. Complexation of AAA with a single metal ion may reduce the total AAA concentration in the plasma if administered in a measured dose.¹⁷

Certain heavy metals like bismuth and thallium in spite of their toxicity at certain concentrations have wide applications towards beneficial use. Bismuth and its salts can cause kidney and liver damage, however the degree of such damage is usually mild.¹⁸ Industrially it is considered as one of the less toxic heavy metals. Certain Bi compounds like bismuth subcarbonate and bismuth subnitrate are used in medicine. Bismuth subsalicylate is used as an antidiarrhoeal and is used in the treatment of some other gastro-intestinal diseases.¹⁹

The present article reports for the first time on the selective complexation of Bi³⁺ with aromatic amino acids. This metal ion at trace concentrations may be used to modify the structure and properties of the fluorescent amino acids. We have tried to compare the ability of complexation of this metal ion with other heavy metals such as Pb(II), Hg(II), Tl(III) and Sb(III) salts with an objective to establish high selectivity towards Bi(III) which could eliminate the possibility of interference from these associated elements.

Experimental

Materials

The amino acids tyrosine (E. Merck), tryptophan and L-phenylalanine (Sisco Research Laboratories) were used as obtained. The Bi(NO₃)₃, Tl(NO₃)₃, Pb(NO₃)₂, HgCl₂, and K₂Sb₂(C₄H₂O₆)₂ salts were of analytical grade. All other chemicals used were of analytical grade.

Apparatus

A double beam Perkin-Elmer LS-55 with a 1% attenuator was used for spectrofluorimetry studies; we have also used Horiba Jobin Yvon Fluorocube 01-NL and 291 nm Horiba nanoLED, IBH DAS-6 decay analysis software for Time Correlated Single Photon Counting (TCSPC) lifetime spectroscopy. A double beam Perkin-Elmer Lambda 25 UV-visible spectrophotometer was used for UV visible spectral studies. A digital pH meter Systronics (type no. 335) was used to measure and adjust pH of different solutions. Cyclic voltammetric (CV) measurements were done using a Bioanalytical System EPSILON electrochemical analyzer. The measurements were carried out in aqueous medium, with a three-electrode assembly comprising a glassy carbon disk working electrode, a platinum auxiliary electrode and an aqueous Ag/AgCl reference electrode.

Methods

1 Spectral studies. The tyrosine, tryptophan and L-phenylalanine solutions (1 mL, 1 mmol L⁻¹ each) were prepared.

Bi(NO₃)₃, Tl(NO₃)₃, Pb(NO₃)₂, HgCl₂ and K₂Sb₂(C₄H₂O₆)₂ solutions (1 mmol L⁻¹ each) were prepared in triple distilled water. The two solutions were mixed in varying proportions and the volume was made up to 4 mL using suitable buffers. The solutions were subjected to UV visible and fluorimetric studies for monitoring the complexation. To identify the optimum pH of complexation, different buffer solutions were prepared *viz.* pH 3 (0.1 mol L⁻¹ sodium citrate-citric acid buffer), 5.6 and 6.7 (0.1 mol L⁻¹ phosphate buffer). The amino acid solutions were prepared in the respective buffers, metal salts were added and the volume was made up to 4 mL using the same buffer as in Table 1.

2 Time Correlated Single Photon Counting (TCSPC) lifetime spectroscopy. Fluorescence lifetimes of tyrosine, tryptophan and L-phenylalanine-Bi complexes were determined by the method of time correlated single-photon counting (TCSPC) using a nanosecond diode laser as the light source at 291 nm (for tyrosine and tryptophan) and 280 nm (for phenylalanine). The IBH DAS-6 decay analysis software was used to deconvolute the fluorescence decays.

The mean fluorescence lifetimes for the decay curves were calculated from the decay times and the relative contribution of the components using the following equation:

$$\tau_{av} = \sum a_i \tau_i^2 / \sum a_i \tau_i$$

where τ_i represents the decay time of the related species, τ_{av} represents the average decay time and a_i represents the coefficient of the i^{th} component. Tyrosine and tryptophan (1 mL, 1 mmol L⁻¹ each) and L-phenylalanine (1 mL, 10 mmol L⁻¹) solutions were mixed with different stoichiometric ratios of Bi (1 mmol L⁻¹ for Tyr and Trp, 10 mmol L⁻¹ for Phe); the volume was made up to 4 mL using pH 3 citrate buffer (0.1 mol L⁻¹ for Tyr and Trp and 1 mol L⁻¹ for Phe) and the average life time of the complex species was identified.

3 Cyclic voltammetry. The solutions for electrochemical analysis were prepared in a similar citrate buffer (0.1 mol L⁻¹ and pH 3 for all the cases) with amino acid to metal in the molar ratio maintaining at Tyr : Bi = 1 : 2, Phe : Bi = 1 : 2 and Trp : Bi = 1 : 1. These were obtained from Benesi-Hildebrand equations obtained from the spectral studies. The CV of 1 mmol L⁻¹ solution of Bi(NO₃)₃ in the same citrate buffer was also taken for comparison.

Table 1 Variations in the mixing proportions of metal with amino acids

Tyrosine/tryptophan/ phenylalanine (1 mmol L ⁻¹) (mL)	Bi(NO ₃) ₃ /Tl(NO ₃) ₃ /Pb(NO ₃) ₂ / HgCl ₂ /K ₂ Sb ₂ (C ₄ H ₂ O ₆) ₂ solution (1 mmol L ⁻¹) (mL)	Buffer (0.1 mol L ⁻¹) (mL) citrate buffer pH 3
1	0	3
1	0.2	2.8
1	0.4	2.6
1	0.6	2.4
1	0.8	2.2
1	1	2
1	1.2	1.8
1	1.4	1.6

Results and discussion

UV-visible spectra

The UV-visible spectra of tyrosine ($\lambda_{\max} = 274$ nm), tryptophan ($\lambda_{\max} = 279$ nm), and L-phenylalanine ($\lambda_{\max} = 258$ nm) were recorded for the complexation studies with metal salts at different pHs. The spectral data confirm that all the aromatic amino acids form complexes with bismuth at pH 3. At higher pHs there was considerable precipitation in the solutions making them unsuitable for further analysis. In addition, dissolution of bismuth nitrate in any aqueous medium is accompanied by its hydrolysis to poorly soluble bismuth oxy- and hydroxynitrates. Unless dissolved in high concentration of HNO_3 , it slowly precipitates out. Only in citrate buffer (pH 3) it remains in solution as a Bi-citrate complex. An attempt to perform the experiment in any other buffer medium results in the precipitation of bismuth itself. Therefore all the complexation studies were done in citrate buffer medium. The Bi^{3+} complex of the amino acids was found to absorb at the same wavelength of maximum absorption as the amino acid itself. With increase in the metal concentration, the absorbance values were found to increase (Fig. 1). Similar studies with other metals *viz.* Hg^{2+} , Pb^{2+} , Tl^{3+} and Sb^{3+} show no other absorption maximum and the absorbance values at the same λ_{\max} values of the amino acids themselves were also not found to increase much (Fig. 2–5). With this precise information, further studies of metal-amino acid complexation were carried out with Bi^{3+} using pH 3 buffer.

The UV-vis spectra indicate complexation of the aromatic amino acids with Bi^{3+} selectively amongst the heavy metals studied. The stability constants of the Bi^{3+} -amino acid complexes were obtained from the absorbance values obtained by varying Bi(III) concentrations at pH 3 using Benesi-Hildebrand (BH) equations.

$$\frac{1}{A - A_0} = \frac{1}{A_1 - A_0} + \frac{1}{(A_1 - A_0)K[M]} \quad (1)$$

$$Y = A + BX \quad (2)$$

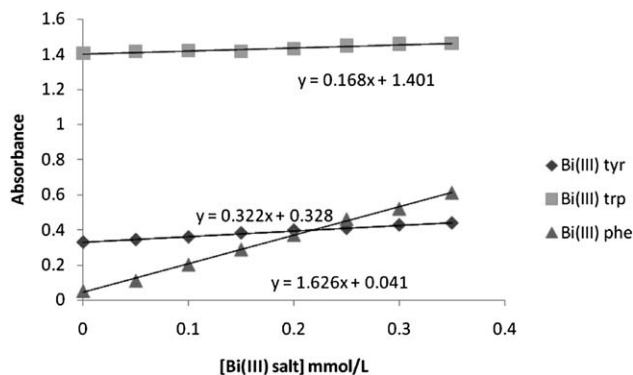


Fig. 1 Plot of absorbance vs. concentration of $\text{Bi(NO}_3)_3$ at pH 3 maintained with citrate buffer.

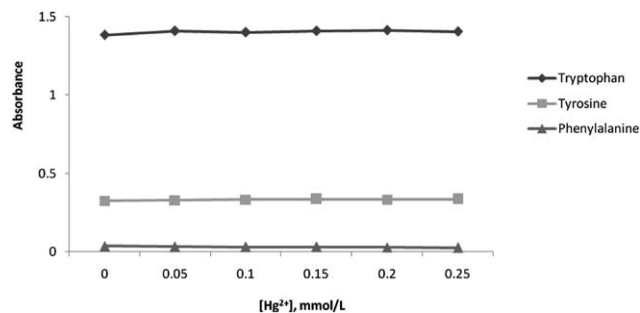


Fig. 2 Plot of absorbance vs. concentration of HgCl_2 at pH 3 maintained with citrate buffer.

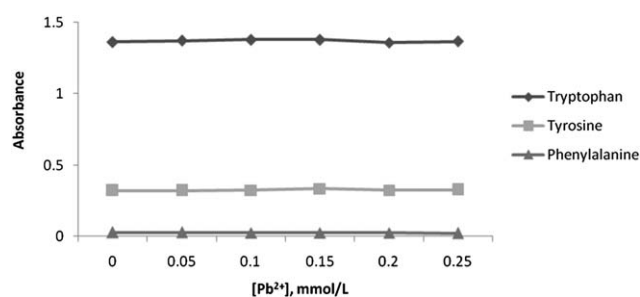


Fig. 3 Plot of absorbance vs. concentration of $\text{Pb(NO}_3)_2$ at pH 3 maintained with citrate buffer.

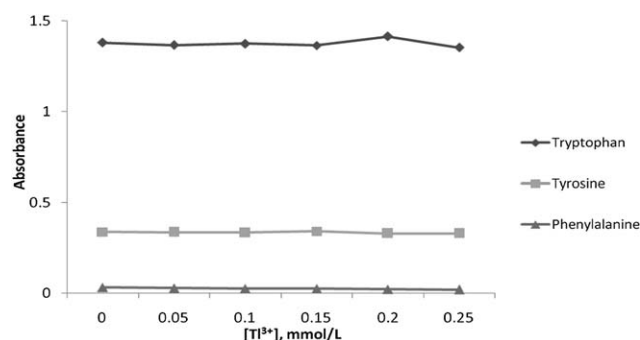


Fig. 4 Plot of absorbance vs. concentration of $\text{Tl(NO}_3)_3$ at pH 3 maintained with citrate buffer.

where A_0 is the absorbance of the amino acid only; A_1 is the absorbance when amino acid is completely bound with the metal; A is the absorbance of the amino acid at varying concentrations of the metal; $[M]$ is the metal ion concentration; K is the binding/association constant.

Eqn (1) can be presented in a simple format of eqn (2). A plot of $1/(A - A_0)$ vs. $1/[M]$ appears to be a straight line and the intercept/slope of the line reveals the corresponding binding constant. The free energy change (ΔG) for the process was also calculated at temperature (T) = 298 K using the following equation:

$$\Delta G = -RT \ln K \quad (3)$$

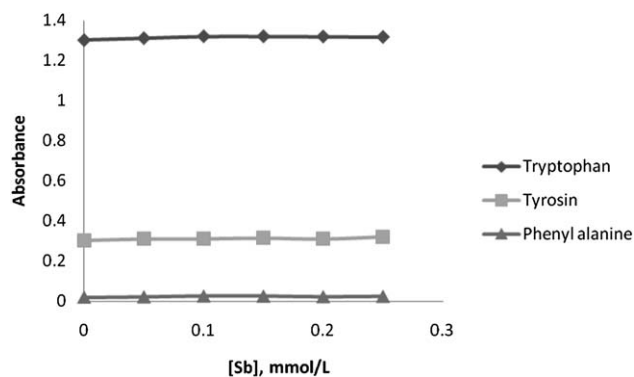


Fig. 5 Plot of absorbance vs. concentration of $K_2Sb_2(C_4H_2O_6)_2$ at pH 3 maintained with citrate buffer.

The BH plots of Tyr-Bi³⁺, Trp-Bi³⁺ and Phe-Bi³⁺ (Fig. 6–8 respectively) indicate the stability constants and the stoichiometric ratios of the respective complexes and are tabulated in Table 2. The formation of a 1 : 2 amino acid-Bi³⁺ complex in the case of Tyr and Phe is indicated by the BH plots. Fig. 6 represents a plot of $1/(A - A_0)$ vs. $1/[Bi^{3+}]^2$ which is linear having a positive intercept as per eqn (1) compared to the one

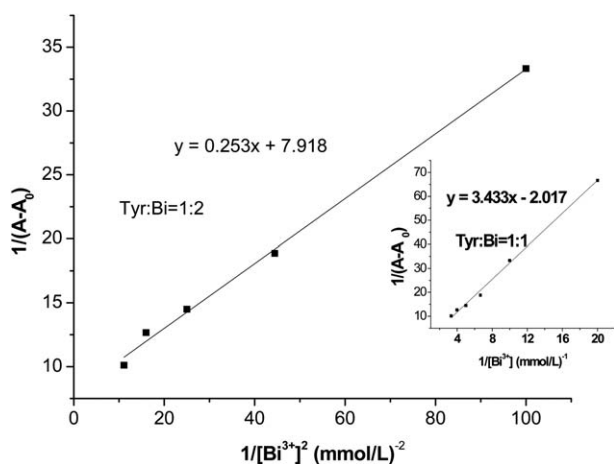


Fig. 6 Benesi-Hildebrand plot of the tyrosine-bismuth(III) complex at pH 3.

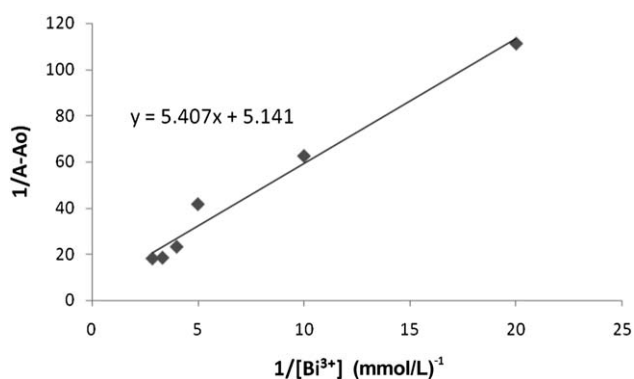


Fig. 7 Benesi-Hildebrand plot of the tryptophan-bismuth(III) complex at pH 3.

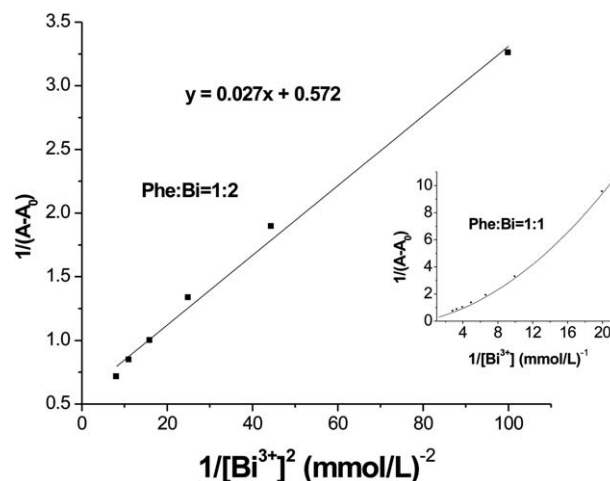


Fig. 8 Benesi-Hildebrand plot of the phenylalanine-bismuth(III) complex at pH 3.

Table 2 Values of binding constants and Stern-Volmer constants as obtained from the BH-plot and Stern-Volmer plot respectively

Complex	Binding constant (K)	ΔG (kJ mol^{-1})	Stoichiometric ratio	Stern-Volmer constant (K_{SV})
Tyrosine-Bi ³⁺	3.1296×10^7 (mol L^{-1}) ⁻²	-42.76	1 : 2	1158 M^{-1}
Tryptophan-Bi ³⁺	9.508×10^2 (mol L^{-1}) ⁻¹	-16.99	1 : 1	520 M^{-1}
Phenylalanine-Bi ³⁺	2.1185×10^7 (mol L^{-1}) ⁻²	-41.80	1 : 2	—

when a plot of $1/(A - A_0)$ vs. $1/[Bi^{3+}]$ is drawn (shown in the inset) with a negative intercept. This indicates a higher possibility of 1 : 2 Tyr : Bi³⁺ complexation. This observation may be explained due to the formation of dimeric bismuth citrate ion $[Bi_2(cit_2)]^{2-}$ in citrate buffer medium at pH 3.²⁰ At this pH, the amino acids are below their isoelectric points and the $-NH_3$ group remains protonated giving an overall positive charge to the amino acid. The ionic interactions resulting under these conditions are responsible for complexation. Aqueous chemistry of Bi(III) being very versatile, it exhibits peculiar complexes and the coordination numbers for Bi(III) are highly variable, ranging from 3 to 10, with irregular coordination geometries.²¹ The expected formation of $[Bi_2(cit_2)]^{2-}$ in citrate buffer medium is responsible for an outer sphere complex with the protonated amino acid and the residual charge neutralization is possible with H^+ ions at this acidic pH. The same explanations also hold good for the Bi³⁺-Phe complex as the BH plot (Fig. 8) is linear for $1/[Bi^{3+}]^2$ and nonlinear for $1/[Bi^{3+}]$ (shown in the inset). However, for the Trp-Bi³⁺ complex (Fig. 7) the BH plot indicates a 1 : 1 complex which means that there are two Trp molecules for charge neutralization of $[Bi_2(cit_2)]^{2-}$ at the outer sphere of the complex. The ΔG values for the formation of the three Bi³⁺-amino acid complexes are presented in Table 2 which also indicates that the probability of formation of the Tyr-Bi³⁺ complex is the highest followed by that of Phe-Bi³⁺ and

Trp-Bi³⁺ complexes. The low value of stability constant and the high value of ΔG for the Trp-Bi³⁺ complex are explainable by the fact that two Trp molecules having a heterocyclic moiety interact with the [Bi₂(cit₂)²⁻] system in this case. Since ΔG also reflects the chemical potential that is minimized when a system reaches equilibrium and is a convenient criterion of spontaneity for processes at constant pressure and temperature, its more negative value indicates higher complexation probability.^{23,24}

Because the aqueous chemistry of Bi(III) is very versatile, it exhibits peculiar complexes and the coordination numbers for Bi(III) are highly variable, ranging from 3 to 10, with irregular coordination geometries. In citrate medium it is reported to remain as dimeric [Bi₂(cit₂)²⁻] species which are expected to form an outer sphere complex with the protonated amino acid below its isoelectric pH (pH 3).^{21,22} Tyr and Phe have similar structures, the only difference being the presence of an -OH group at the *para* position in Tyr. However in Trp, the structure is slightly different in having an indole functional group. The binding constants of the 1 : 2 complexes of Tyr-Bi(III) and Phe-Bi(III) are very high and comparable in the range 10⁷ (mol L⁻¹)⁻²; however that of the 1 : 1 complex of Trp-Bi(III) has lower stability as the binding constant is 9.508 × 10² (mol L⁻¹)⁻¹ only (Table 2). The presence of a phenyl group in both Tyr and Phe contributes to lesser steric effect in forming a 1 : 2 complex as compared to the indole group containing Trp which forms a 1 : 1 complex having a lesser stability constant. The situations get even more complicated with the fact that Bi(III) forms various polymeric structures with citrate ion and attains variable three dimensional structures. The elucidation of the exact structural composition of these complexes is a subject of further research.

The fluorescence of the aromatic amino acids in this study is of particular interest for many years. This property was also exploited to explore the type of complexation of these amino acids with Bi(III). The steady state and excited state fluorescence spectra of Tyr, Trp and Phe-Bi³⁺ complexes are shown in Fig. 9–11 respectively. The Stern–Volmer plot of the Tyr-Bi³⁺ complex (Fig. 9) suggests that the K_{SV} value is 1158 M⁻¹.

The K_{SV} value was calculated using the following equation:

$$F_0/F = 1 + K_{SV}[M].$$

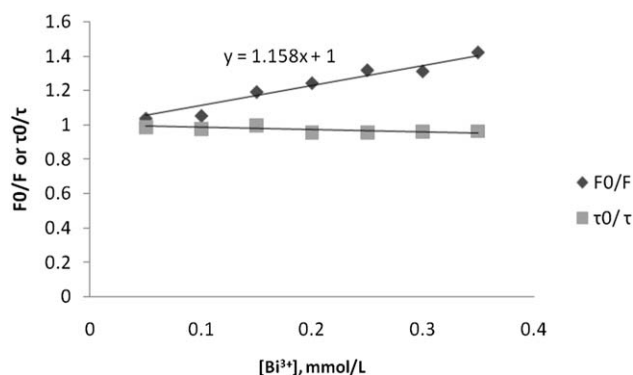


Fig. 9 Stern–Volmer plot of the tyrosine–Bi(III) complex at pH 3.

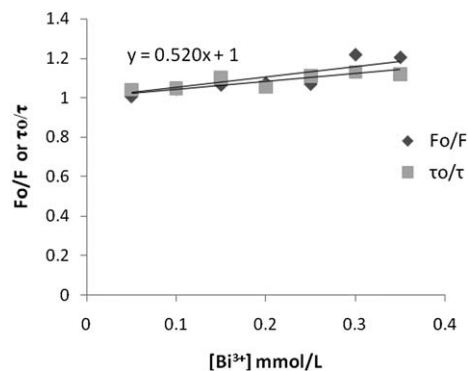


Fig. 10 Stern–Volmer plot of the tryptophan–Bi(III) complex at pH 3.

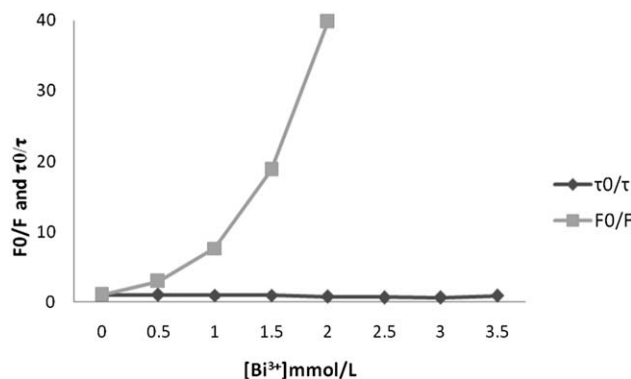


Fig. 11 Stern–Volmer plot of the phenylalanine–Bi(III) complex at pH 3.

The F_0/F value (where F_0 and F are the steady state fluorescence intensities in the absence and in the presence of the quencher) shows a considerable change with the quencher concentration. On the other hand the ratio of lifetimes τ_0/τ (without and with quencher ions) does not vary with increasing quencher concentration. This suggests that the quenching of Tyr fluorescence with addition of Bi(III) is not dynamic collisional quenching.²⁰ Rather the steady state fluorescence quenching indicates that there are molecular interactions in the ground state. For the Trp-Bi³⁺ complex (Fig. 10) the slope of F_0/F versus metal concentration is slightly less, indicating a lower value of K_{SV} for this complex. The nature of the ratio of life times is almost the same as with the Tyr-Bi³⁺ complex. The nature of the Phe-Bi complex is slightly different as revealed by Fig. 11. This may be due to both collisional and steady state interactions of Phe and Bi³⁺ in the complex.

The cyclic voltammograms of the Bi(III) salt in citrate buffer and those of the Tyr-Bi³⁺, Trp-Bi³⁺ and Phe-Bi³⁺ complexes are presented in Fig. 12–15 respectively. Fig. 12 reveals a peak at an oxidation potential of 0.162 V for the production of Bi(III) and a peak at 0.641 V for further oxidation to Bi(V). During the reduction process, two peaks are again observed at 0.387 V for Bi(V) reduction and 0.131 V for the reduction of Bi(III). The low difference in the oxidation and reduction potentials (<0.060 V) of Bi(III) reveals a reversible mechanism in the electrochemistry of Bi(III) in citrate medium. This trend is altered in the case of

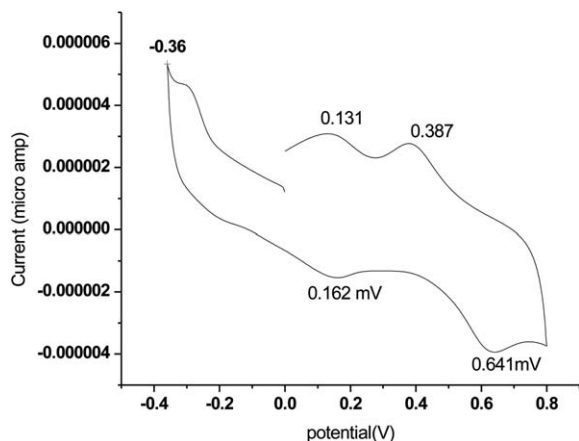


Fig. 12 Cyclic voltammogram of Bi(III) in citrate buffer.

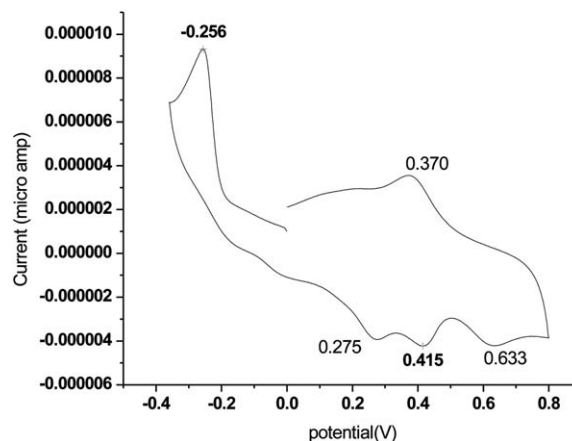


Fig. 15 Cyclic voltammogram of the Phe-Bi(III) complex.

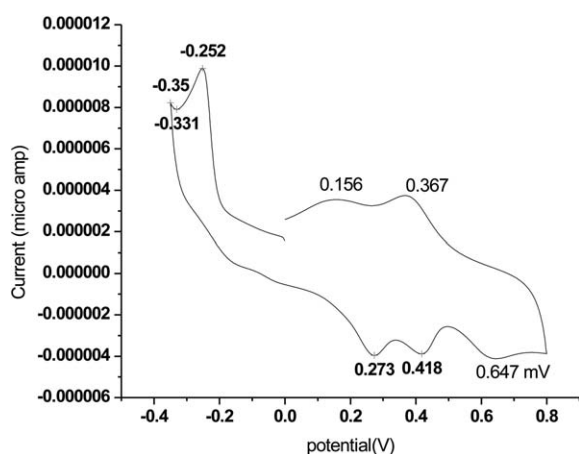


Fig. 13 Cyclic voltammogram of the Tyr-Bi(III) complex.

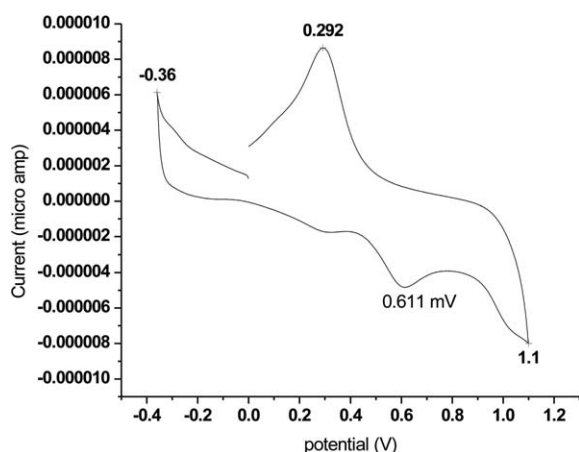


Fig. 14 Cyclic voltammogram of the Trp-Bi(III) complex.

Table 3 Potential values in the cyclic voltammograms of Bi³⁺ and its amino acid complexes

Species	Bi ³⁺ -Citrate	Tyr-Bi ³⁺	Trp-Bi ³⁺	Phe-Bi ³⁺
Oxidation potential (V)	0.162	0.273	0.611	0.275
	0.641	0.418		0.415
Reduction potential (V)	0.131	0.156	0.292	0.633
	0.387	0.367		0.370

peak at 0.367 V and a small peak at 0.156 V. As the differences in the oxidation and reduction potentials are more than 0.060 V the electrochemical process for this complex seems to be irreversible.²⁵ A similar observation was found for the Phe-Bi³⁺ complex which shows a peak at oxidation potentials 0.275 V, 0.415 V and 0.633 V and a prominent reduction potential at 0.370 V which is again indicative of an irreversible electrochemical process (Fig. 15). The CV pattern for the Trp-Bi³⁺ complex is however different and there is only one peak at an oxidation potential of 0.611 V corresponding to the formation of Bi(V) and a peak at a reduction potential of 0.292 V indicating the formation of an irreversible electrochemical system (Fig. 14). The irreversible electrochemical systems clearly indicate complexation of Bi(III) with the aromatic amino acids. The potential values for all the complexes are tabulated in Table 3.

In addition to the UV-vis spectra, the fluorescence and cyclic voltammetric studies of aromatic amino acid complexation with the other heavy elements (Hg²⁺, Pb²⁺, Tl³⁺, Sb³⁺) were also found to be in agreement with the absorbance data and show no significant changes after metal ion treatment (data not shown). This strengthens the fact that amongst the heavy metal series as well as group, the selectivity of AAA for Bi(III) is very prominent. This can be established equally using the absorbance, fluorescence and cyclic voltammetric studies.

metal-amino acid complexes. Here also we find a similarity between the Tyr-Bi³⁺ and Phe-Bi³⁺ complexes. For Tyr-Bi³⁺, three peaks in the oxidation curve at 0.273 V, 0.418 V and 0.647 V are observed (Fig. 13). The reduction curve shows a prominent

Conclusion

The extraordinary response of the aromatic amino acids towards Bi(III) is established in the current report. This article

describes the formation of a 1 : 2 complex of Tyr–Bi³⁺ and Phe–Bi³⁺ and a 1 : 1 complex of Trp–Bi³⁺ at pH 3 of citrate buffer. The complexation takes place at the lower end of the isoelectric pH for all the three amino acids where they exist as protonated species. Bi(III) remains as a dimeric citrate anion and associates with the cationic species of the amino acids. The complexes are well suited for the modification of amino acids and may have potential applications in solving physiological problems associated with them as discussed in the Introduction section.

Acknowledgements

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