Voltammetric Sensing of Amino Acids in the Presence of Cu(II) in Acidic and Alkaline Solutions

Rasa Pauliukaite,[a] *Edilson M. Pinto*,[a] *Aziz Amine*,[b] *Christopher M. A. Brett**[a]

[a] Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3004–535 Coimbra, Portugal
tel/fax: +351239835295
[b] Faculty of Science and Techniques, University Hassan II Mohammedia, BP 146 Mohammedia 20650, Morocco
*e-mail: brett@ci.uc.pt

Received: December 28, 2011
Accepted: March 17, 2012

Abstract
A number of amino acids have been determined at carbon film electrodes in the presence of copper. Strongly acidic, 0.1 M HCl, in the presence of 0.1 mM Cu(II), as well as alkaline, 0.1 M NaOH, solution permit successful measurement of individual amino acids, clearer separation between oxidation of Cu and Cu-complexes occurring in alkaline solution. Electrochemical impedance showed that Cu(II) facilitates charge transfer, particularly in alkaline medium. Square wave voltammetry with preconcentration increased the response compared to linear sweep voltammetry. Protein hydrolysis rates were monitored through determination of amino acids produced by decomposition, injecting samples into alkaline electrolyte solution.

Keywords: Amino acid determination, Cu(II) complex, Electrochemical impedance, Protein hydrolysis

DOI: 10.1002/elan.201100733

1 Introduction

Amino acids are main components of all proteins, and which exist in all living organisms, amino acids (and peptides) being released during decomposition as well as during acid hydrolysis. The determination of amino acids is important in different fields, particularly in food and biotechnology [1,2]. They are often added to foods, as fortifiers, in order to correct for possible dietary deficiencies [1,3–5], some of which can cause allergic or other collateral effects [5].

Since amino acids have at least two active functional groups (depending on pH): $-NH_2$ and $-COOH$, they have the ability to complex some transition metals. One of the easiest metal ions to complex is the Cu(II) ion, can form mixed cation or mixed anion complexes at different pH with H^+ or OH^- in the presence of amino-acid ligand [6].

Recently, a number of researchers have been attracted towards investigating the processes and methods for the detection of amino acids with the aim of applying this to the food industry; new strategies and new materials are being developed for this purpose [4,7,8].

Electrochemical determinations have been performed at Cu electrodes in alkaline medium due to easier formation of the complexes with Cu(II) [9]. Cu(II) can form complexes with amino acids either in bulk solution or at the electrode surface (Cu electrodes) in alkaline medium [10,11]. The amino acids most determined electrochemically by Cu(II) complexation were glycine (Gly) [10,12], cysteine (Cys) [11], histidine (His) [13] all in neutral or alkaline solutions. There are only a few reports concerning the electrochemical determination of amino acids, such as Cys [14], phenylalanine (PhAla) [15], and tyrosine (Tyr) [14,15], in neutral solution without Cu or any other metal ion. Furthermore, there is a lack of reports on amino acid determination in acidic solutions. One paper has been published on the determination of alanine (Ala), His and arginine (Arg) at Cu electrodes modified with polypyrrole film doped with glutamate [16], in the pH range 2 to 6.

In this work the determination of several amino acids was studied in the presence of Cu(II) in strongly acidic and alkaline solutions. Carbon film electrodes were used for the determination of amino acids by cyclic voltammetry and square wave anodic stripping voltammetry in the presence of Cu(II) in 0.1 M HCl and 0.1 M NaOH solutions. Electrochemical impedance spectroscopy was used to investigate the interactions between Cu(II) and tryptophan (Trp) in acidic and basic media. Hydrolysis of bovine serum albumin was performed in order to determine the amino acids released using electrochemical methods in the presence of copper ions. Since protein hydrolysis is usually performed in strong acidic solution, a rapid and simple method is needed for the determination of amino acids in strong acidic solution in order to monitor the rate of hydrolysis.

2 Experimental

2.1 Apparatus

A PalmSens micropotentiostat connected to a personal computer was used with PSLite 1.7.3. software. Cyclic voltammetry (CV) experiments were done using a scan rate of 50 $mV\,s^{-1}$; and square wave anodic stripping voltammetry parameters (SWASV) were: deposition potential −1.0 V (in acidic medium) or −0.7 V (in alkaline medium), deposition time 60 s, pulse frequency 25 Hz, amplitude 25 mV, and step potential 2 mV.

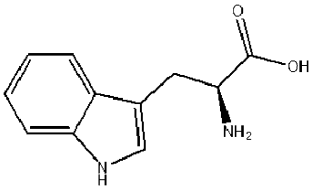
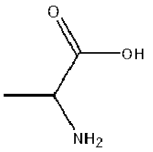
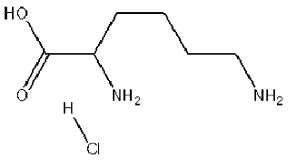
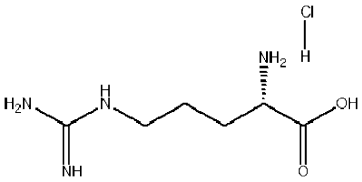
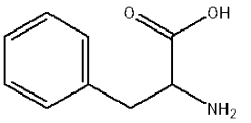
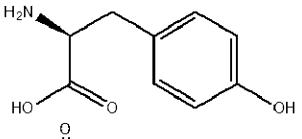
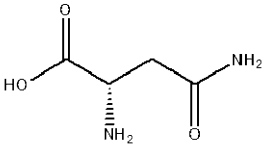
Carbon film cylinder electrodes (geometric area 0.20 cm^2) were used as working electrodes, a Pt spiral as counter electrode and a saturated calomel electrode (SCE) as reference. The carbon film electrodes were prepared from carbon film electrical resistors as described in [17].

Electrochemical impedance spectroscopy was performed using the same three-electrode electrochemical cell and a 1250 Frequency Response Analyser, coupled to a Solartron 1286 Electrochemical Interface (Ametek, UK) controlled by ZPlot software (Scribner Associates Inc., USA). The frequency range used was 65 kHz to 0.1 Hz with 10 frequencies per decade, and integration time 60 s.

The pH measurements were done with a CRISON 2001 micro pH-meter (Crison, Spain).

All experiments were performed at room temperature, 25 ± 1 °C.

Table 1. Chemical structure of the amino acids studied.

Amino acid	Chemical structure
L-Tryptophan	
DL-Alanine	
DL-Lysine monohydrochloride	
L-Arginine monohydrochloride	
DL-Phenylalanine	
L-Tyrosine	
L-Asparagine	

2.2 Chemicals

Stock solutions of 10 mM of L-tryptophan, DL-a-alanine, DL-lysine monohydrochloride, L-arginine monohydrochloride, DL-phenylalanine, L(-)-tyrosine (Merck, Germany), and L-asparagine (Riedel-de Haen, Germany) were prepared in 0.1 M acetate buffer pH 3.0. The chemical structures are presented in Table 1. Electrolyte solutions were prepared from reagents obtained from Riedel-de Haen, Germany: HCl, H_2SO_4, NaOH, and acetate buffer ($NaCH_3COO$ and CH_3COOH). $CuSO_4$ was purchased from Panreac, Spain. BSA was obtained from Sigma, Germany. All solutions were prepared with Millipore Milli-Q nanopure water (resistivity ≥ 18 MΩ cm).

2.3 Cu Deposition

Cu was electrochemically preplated on the working electrodes. Prior to electrodeposition, they were pretreated by application of a fixed potential of +1.8 V vs. SCE for 120 s followed by −1.8 V vs. during another 120 s in 0.1 M KCl. The electrodeposition of thick copper films was performed from 3 mM $CuSO_4$ in 2.2 M H_2SO_4 at −0.4 V vs. SCE for 900 s.

2.4 Protein Hydrolysis

An amount of 1 $mg\,mL^{-1}$ of BSA in 1 M HCl was placed in a thermocontrolled incubator Multiplace (J. P. Selecta, Spain) at a constant temperature of 100 °C for 4 h. Samples were taken after 0 min, 30 min, 1 h, 2, h, 3 h, and 4 h of hydrolysis. They were cooled down to room temperature and analysed by injecting of 50 μL of sample into 0.1 M HCl or NaOH solution in the presence of 0.1 mM Cu(II).

3 Results and Discussion

3.1 Carbon Film Electrodes for Amino Acid Determination with Addition of Cu(II) to Solution

3.1.1 Acidic Medium

3.1.1.1 Optimisation of Supporting Electrolyte

Since amino acids can easily complex Cu(II), a thick Cu film was deposited on the electrodes, using the procedure described in section 2.3, necessary because of the loss of

copper from the electrode surface if and when soluble complexes are formed.

Experiments with amino acids were carried out in 0.1 M acetate buffer solution pH 4.0. Tryptophan was studied first, since it is an electrochemically active amino acid. Cyclic voltammograms (not shown) demonstrate Cu oxide formation and Cu-Trp complex formation, leading to a decrease in response with the number of scans due to insoluble products of Cu-Trp interaction on the electrode surface, most probably insoluble $CuTrp_2$ [18]. Other amino acids were also tested but the signals were not stable and decreased dramatically with cycling under these conditions. It was thus decided to investigate amino acid determination at lower pH.

Cu-coated carbon film electrodes were tested in HNO_3 and H_2SO_4 solution; however, under these conditions the Cu film is blocked by surface oxides and by complexes [19]. Therefore, further experiments were performed in 0.1 M HCl in the presence of 0.1 mM Cu(II) instead of preplating Cu film onto the electrodes.

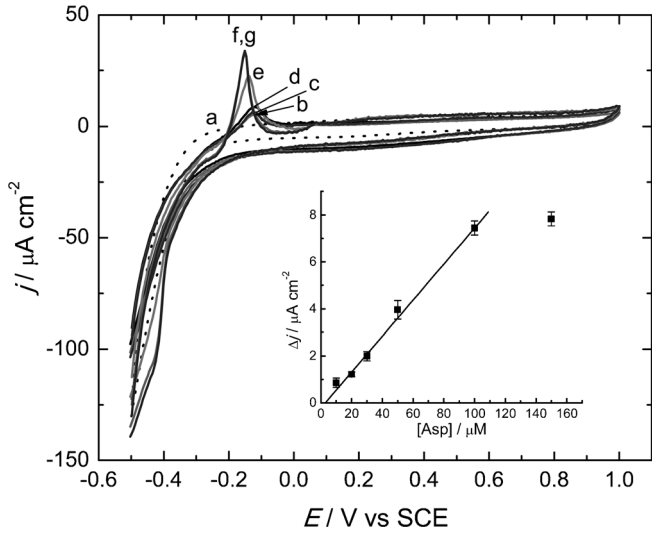


Fig. 1. Determination of Asp at C film electrode in 0.1 M HCl in the presence of 0.1 mM of Cu(II) by CV. Asp concentration: (a) 0; (b) 10; (c) 20; (d) 30; (e) 50, (f) 100; and (g) 150 μM. Potential scan rate 50 $mV s^{-1}$. Inset: Calibration curve calculated from CV data after Cu(II) signal subtraction.

CV and SWV measurements were carried out with the amino acids arginine (Arg), asparagine (Asp), lysine (Lys), Trp, and (Tyr) in 0.1 M HCl. No response was found for Tyr, probably due to an electrochemically inactive protonated form, since it can be determined in neutral solutions and without any complexing cation [14,15].

Figure 1 shows CVs at a carbon film electrode in electrolyte (curve a) and after addition of 0.1 mM Cu(II) with different concentrations of Asp. After Asp addition, no new peaks were observed but the Cu(II) oxidation peak increased linearly in height with concentration up to 100 μM of Asp and then remained stable (Inset of Figure 1). The same saturation behaviour was found with all the other amino acids studied that responded under these conditions. The sensitivity to various amino acids is in the sequence $Asp > Trp \geq Arg > Lys$, see Table 2, although this method is not specific for any particular amino acid. The stability constants, β_2, of these Cu(II)-amino acid complexes are: 14.9 Asp, 14.9 Trp, 13.74 Arg and 13.7 Lys in KCl or KH_2PO_4 solutions [19], suggesting a dependence of the sensitivity on the Cu-amino-acid complex stability. The reproducibility is sufficiently good, with RSD 3.2%. The detection limit can also depend on the stability of the Cu(II) complex: a higher complex stability leads to a lower limit of detection [10].

Further measurements were performed with Trp in order to clarify the nature of the electrochemical process. The CV results obtained at different potential scan rates showed a linear dependence of the oxidation current on the square root of scan rate up to 25 $mV s^{-1}$, indicating a diffusion-controlled process, with a slope of 0.034 $\mu A V^{-1/2} s^{1/2}$.

3.1.1.2 Optimisation of Square Wave Parameters

To enhance sensitivity, SWV measurements were made starting at −1.0 V, where Cu(II) is deposited, (Figure 2A) since, at more negative potentials, hydrogen evolution occurred simultaneously. Preconcentration was done prior to recording the voltammograms to increase sensitivity; the effect of preconcentration potential on the SWV response in Figure 2A shows that −1.0 V gives the best sensitivity. Preconcentration has been used previously to determine Cu(II) at a Cys-modified electrode at open circuit [20], at −0.5 to −1.0 V vs. Ag/AgCl for Cys determi-

Table 2. Analytical parameters for the determination of some amino acids in 0.1 M HCl in presence of 0.1 mM Cu(II).

Amino acid	Method	E_p (V vs.SCE)	Linear range (μM)	Sensitivity ($\mu A \mu M^{-1}$)	*LOD* (μM)	*RSD* (%)[a]
Arg	CV		10–160	0.013	9	6
	SWASV	−0.05	5–60	0.595	3	4
Asp	CV		10–100	0.077	2	5
	SWASV	−0.05	5–40	2.272	1	3
Lys	CV		30–200	0.011	21	8
	SWASV	−0.05	5–60	0.311	3	3
Trp	CV		10–40	0.063	3	2
	SWASV	0.98	5–200	0.137	2	2

[a] *RSD* for $n = 5$ assays

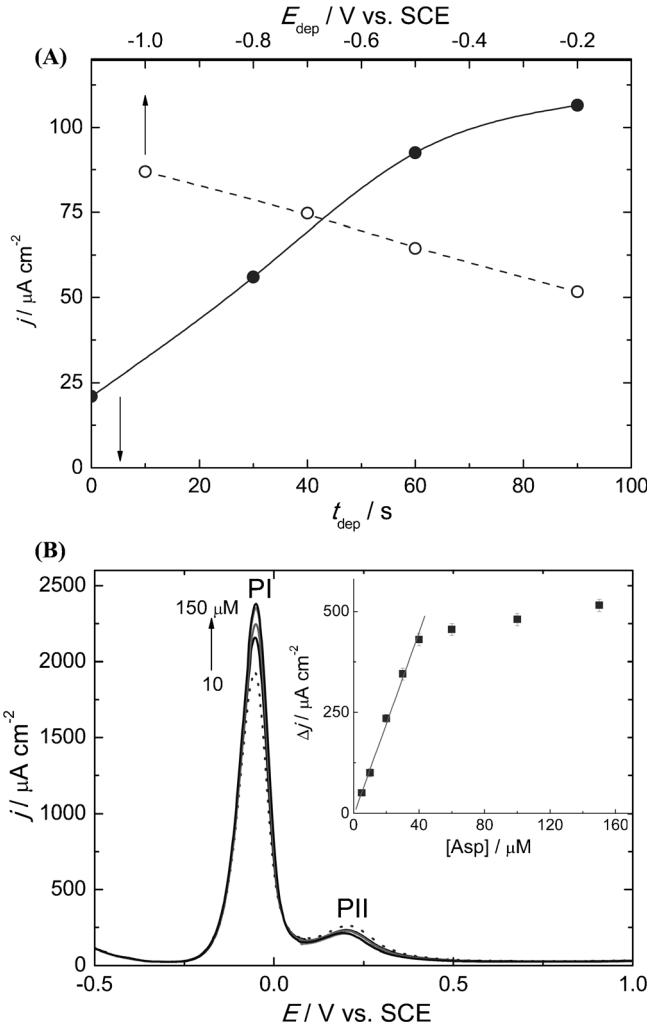


Fig. 2. (A) Dependence of the Cu-Trp oxidation peak height at C film electrodes on (○) preconcentration potential and (●) preconcentration time. Solution composition 0.1 M HCl, 0.1 mM Cu(II), 10 μM Trp. (B) SWVs of Asp in 0.1 M HCl in the presence of 0.1 mM of Cu(II). Asp concentration: (a) 0; (b) 30; (c) 100; (d) 150 and (e) 200 μM. Inset: calibration curve calculated from peak PI (0.04 V) after Cu(II) signal subtraction.

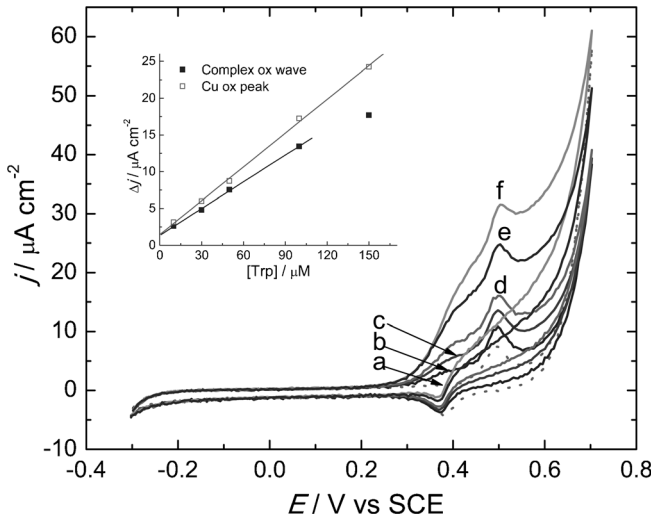


Fig. 3. Determination of Trp at C film electrodes in 0.1 M NaOH in the presence of 0.1 mM Cu(II) by CV. Asp concentrations: (a) 0; (b) 10; (c) 30; (d) 50, (e) 100; and (f) 150 μM. Potential scan rate 50 mV s^{-1}. Inset: Calibration curve calculated from CV Cu oxidation peak (■) and Cu-Trp complex oxidation wave (□) after Cu(II) signal subtraction.

nation at Hg films in the presence of Cu(II) [11], or at −0.2 V for albumin determination at electrodes modified with carbon nanotubes and copper microparticles [13]. Figure 3A also shows the dependence of peak height on preconcentration time. The peak current increases with increase in deposition time, but after 60 s begins to saturate, so 60 s was chosen for further measurements in this medium.

3.1.1.3 Calibration Plots of Amino Acids

Typical SWVs for amino acid determination after addition to a solution of Cu(II) in 0.1 M HCl solution are presented in Figure 2B, with Asp as example. All amino acids studied (except Trp) gave similar profiles under these conditions with two peaks, PI and PII. The oxidation peak of Cu, PI, is at −0.05 V vs. SCE, increases with the concentration of amino acid in solution and depends linearly on the concentration of amino acid added. This can be attributed to formation of a Cu(II) complex near the surface following preadsorption of the amino acid causing the amount of Cu that is oxidised to Cu(II) to increase [21–23]. This can easily disproportionate chemically and go back to a Cu(II)-Trp complex plus metallic Cu, which is then oxidised at the electrode surface.

Peak PII at +0.22 V is much smaller and the dependence on amino acid concentration is not that evident. A similar behaviour in Cu(II) amino acid solution (when the stoichiometric amount amino acid is kept constant) was observed by differential pulse voltammetry in [21], where the first peak is attributed to a stripping of deposited Cu and the second peak is oxidation of Cu-amino acid complexes present in acidic solutions [21,22].

In the case of Lys or Arg, PI and PII also increase with amino acid concentration, PI in a linear fashion. Adding Trp to the Cu(II) solution an additional peak PIII appears at 0.98 V which indicates oxidation of the Cu-Trp complex (not shown).

Calibration data for the amino acids obtained from SWV are presented in Table 2. The sensitivity towards the amino acids from SWV is higher than that by CV for all amino acids by at least a factor of two, and the highest is to Asp, as in the case of CV.

A mixture of two amino acids was also tested under the same conditions; Asp and Trp were injected into the same 0.1 M HCl solution containing 0.1 mM Cu(II). The additional peak, PII, did not appear at any concentration studied and no linear increase in the peak current of PI was obtained. Consequently, acidic media cannot be easily used for the determination of amino acids in pro-

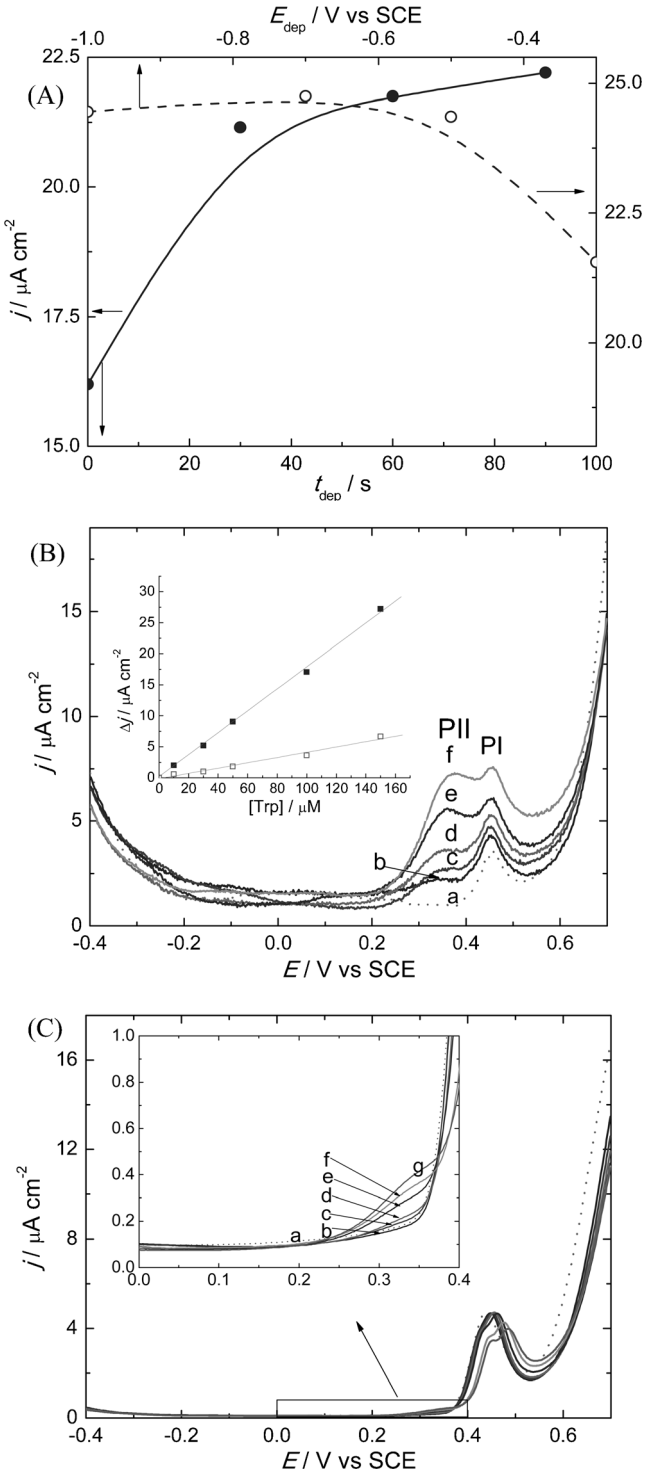


Fig. 4. (A) Dependence of the Cu-Trp oxidation peak height on (○) preconcentration potential and (●) preconcentration time. Solution composition 0.1 M NaOH, 0.1 mM Cu(II), 150 μM Trp. (B) SWVs of Trp in 0.1 M NaOH in the presence of 0.1 mM Cu(II). Trp concentration: (a) 0 (b) 10 (c) 30 (d) 50, (e) 100 (f) 150 μM. Inset: Calculated calibration curve after Cu(II) signal subtraction of peak PII (0.35 V) (■) and PI (0.46 V) (□). (C) SWVs of Asp and Trp in 0.1 M NaOH in the presence of 0.1 mM Cu(II). Asp and Trp concentrations: (a) 0 (b) 10 (c) 30 (d) 50 (e) 100 (f) 150 and (g) 200 μM.

teins, but can be used for some amino acids in samples where only one of these amino acids is present.

3.1.2 Alkaline Medium

The determination of amino acids at Cu electrodes in alkaline solution due to easy complex formation between Cu, amino acids and OH^- is well known [10–13]. Thus, the electrochemical behaviour of the amino acids Ala, Asp, PhAla, and Trp was tested in 0.1 M NaOH solution in the presence of 0.1 mM Cu(II). As in strong acidic solution, measurements were performed using CV and SWV. An example of CVs under these conditions is given in Figure 4 after addition of various aliquots of Trp. A response to all amino acids was obtained but with varying sensitivity. The Cu oxidation peak was at 0.50 V and reduction at 0.39 V in 0.1 M NaOH solution. Addition of amino acids caused an increase in the Cu oxidation peak, which was used for analytical quantification, as well as the appearance of a new oxidation wave between 0.38 and 0.39 V vs. SCE for Trp, PhAla, and Ala, which shifts to slightly more positive values with higher amounts of amino acid. This additional oxidation wave is due to complex formation between Cu(II), amino acid and hydroxyl ions and was well-defined for Trp, but not for the other amino acids, probably due to the Trp indole moiety.

The calibration curves in the Inset of Figure 3 were calculated for the Cu oxidation peak (empty symbols) and for the oxidation wave of the Cu-Trp complex. The calibration parameters in 0.1 M NaOH are presented in Table 3. The sensitivity in most cases is lower than that in strongly acidic solution.

3.1.2.1 Optimisation of Square Wave Parameters and Calibration Data

SWVs were recorded in 0.1 M NaOH for all amino acids, after selection of preconcentration potential and time, see Figure 4. The peak current increases with more negative deposition potential, from −0.3 to −0.7 V, then remaining almost constant down to −1.0 V; thus, −0.7 V vs. SCE was selected for further experiments. The peak current response to Trp rose dramatically on increasing the deposition time up to 30 s, above which there were no significant changes. The determination of the other amino acids was thus performed by SWV using preconcentration at −0.7 V for 30 s.

Unlike in acidic medium, an additional oxidation wave due to the Cu-amino-acid complex appeared for all amino acids studied, except PhAla; however, its shape varied. The most clearly visible oxidation wave of the complex, as well as the highest sensitivity, was in the case of Trp (Table 3). PI and PII increased with addition of amino acid, but PI (Cu oxidation) was more sensitive to all amino acids, except Trp. In the case of Trp, the size of the oxidation wave of the Cu-Trp complex, PII, increased faster. The PII potential was similar in all cases. Alkaline

Table 3. Analytical parameters for the determination of some amino acids in 0.1 M NaOH in presence of 0.1 mM Cu(II).

Amino acid	Method	E_p (V vs. SCE)	Linear range (μM)	Sensitivity ($\mu A\,\mu M^{-1)}$	*LOD* (μM)	*RSD* (%)[a]
Ala	CV	0.51	10–150	0.015	8	6
	SWV	0.48	5–60	0.035	3	4
Asp	CV	0.52	10–200	0.020	6	5
	SWV	0.49	5–60	0.020	3	4
PhAla	CV	0.51	30–200	0.010	14	8
	SWV	0.48	5–60	0.014	2	4
Trp	CV	0.50	10–150	0.153	7	3
	CV	0.38	10–100	0.121	4	3
	SWV	0.46	5–150	0.016	2	4
	SWV	0.35	10–60	0.041	9	4

[a] *RSD* for $n = 5$ assays

solution is more selective for distinguishing different amino acids than acidic medium.

Measurements were performed with a mixture of Asp and Trp, in 0.1 M NaOH electrolyte solution, chosen because the positions of the wave due to the complex differ significantly and their sensitivities also differ. Figure 5C shows a voltammogram after addition of the mixture of Asp and Trp, with an oxidation peak due to Asp at 0.30 V and a peak for Trp at 0.45 V, as a shoulder of the Cu oxidation peak. This shoulder increases in height with increase in Trp concentration while the main Cu oxidation peak at 0.47 V decreases. The sensitivity to these acids in mixtures was significantly less than when analysed separately and it was not possible to calculate the response to Trp due to overlap its peak with the Cu oxidation peak. Nevertheless, these data demonstrate that it is possible to distinguish between two different amino acids in alkaline solution but that the response in mixtures is different from that in separate solutions. Unfortunately, voltammetry of mixtures of other amino acids without Trp did not show any separate oxidation peaks.

The analytical parameters of this new methodology can be compared with those in the literature. For example, the sensitivity of the amperometric determination of some amino acids at 0.0 V in 0.01 M NaOH solution at a CNT-paste electrode modified with Cu nanoparticles [13] was significantly lower than here: Ala 134 $\mu A\,M^{-1}$, Asp 270 $\mu A\,M^{-1}$, Cys 2880 $\mu A\,M^{-1}$, PhAla 166 $\mu A\,M^{-1}$, and Trp 91 $\mu A\,M^{-1}$. The lowest sensitivity in [13] was to Trp, in contrast to this work with Cu(II) in solution.

3.2 Electrochemical Impedance Spectroscopy of Tryptophan in the Presence and Absence of Cu(II)

Electrochemical impedance spectra were recorded in order to follow the changes at the electrode/solution interface during Cu(II) and Trp interactions in both acidic and alkaline media. Examples of the spectra obtained are shown in Figure 5a in acidic medium and in Figure 5b in alkaline medium.

The applied potentials were chosen according to the cyclic voltammograms recorded in each medium: 1) in 0.1 M HCl at −0.40 V (Cu deposition), at −0.15 V (Cu oxidation peak) and at 0.0 and 0.30 V (no redox process); 2) in 0.1 M NaOH at −0.40 V (Cu deposition), 0.0 V (no redox process), +0.38 V (reduction of surface CuO), at +0.40 V (oxidation of Cu(II)-Trp complex) and at +0.50 V (oxidation of Cu). At each potential, impedance spectra were recorded in blank electrolyte solution (0.1 M HCl or 0.1 M NaOH), electrolyte +30 μM of Trp, electrolyte +100 μM of Cu(II), and electrolyte +30 μM of Trp +100 μM of Cu(II). In order to investigate the influence of amino acid concentration on the electrode/solution interface, a further 30 μM of Trp was added to the last of these solutions.

3.2.1 Acidic Medium

Figure 5a1 and a2 show complex plane spectra in 0.1 M HCl solution at 0.0 V and at −0.40 V. The spectra were fitted to the same equivalent electrical circuit at all potentials, which consists of the cell resistance (R_Ω) in series with a parallel combination of charge transfer resistance, R_{ct}, and constant phase element (CPE) as non-ideal capacitance. Here $CPE = -1/(Ci\omega)^{\alpha}$, where the capacitance C describes the charge separation at the double layer interface, ω is the angular frequency and the α exponent is due to the heterogeneity of the surface.

In the absence of Cu(II), with or without Trp, the shape of the spectra was similar at −0.15, 0.0 (Figure 5a) and +0.30 V. Addition of Cu(II) decreases R_{ct}, and further addition of Trp increases the charge transfer resistance due to complex formation between Cu(II) and Trp on the electrode surface. A second addition of Trp hardly changes the impedance spectrum. At −0.40 V a similar tendency was found except that impedance values were smaller due to easier charge transfer: when Trp is added to electrolyte solution charge transfer is more difficult but Cu(II) addition improves charge transfer by around a factor of 6 (from 7.6 to 1.2 $k\Omega\,cm^2$). On the other hand, addition of Trp to in electrolyte solution that already contains Cu(II) increases R_{ct} from 1.2 to 1.9 $k\Omega\,cm^2$ due to complex formation. Thus, the order of addition of the two components is important and in

The values obtained by fitting show that in all cases R_{ct} decreases significantly after addition of Cu(II) and in-

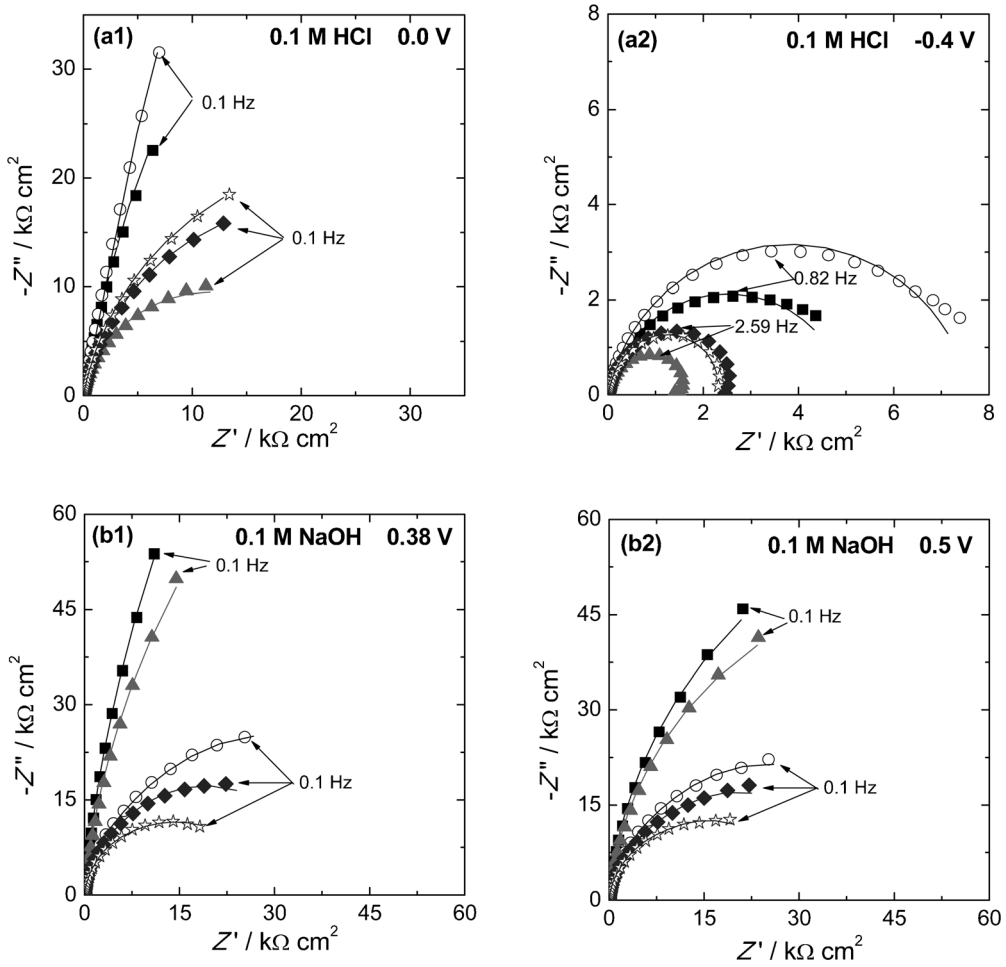


Fig. 5. Complex plane electrochemical impedance spectra at carbon film electrode in (a) 0.1 M HCl and (b) 0.1 M NaOH electrolyte solutions at different potentials. Solution compositions: (■) electrolyte; (○) electrolyte +30 μM Trp; (▲) electrolyte +100 μM Cu(II); (◆) electrolyte +100 μM Cu(II) +30 μM Trp; (☆) electrolyte +100 μM Cu(II) +60 μM Trp. Lines indicate equivalent electrical circuit fitting.

creases in the presence of Trp, probably due to adsorption of protonated amino acid on the electrode surface. The exponent α is almost in all cases around 0.89, except at −0.4 V in the presence of Cu(II) with or without of Trp when it is almost 1 showing that deposited Cu reduces the electrode surface non-uniformity. R_Ω was always ~4 Ω cm^2.

3.2.2 *Alkaline Medium*

Spectra of Cu(II) in the presence or absence of Trp recorded in 0.1 M NaOH electrolyte solution were different to those in acid solution and three equivalent circuits were needed to fit to the spectra at all the potentials studied. The spectra in blank and Cu(II) solutions at all potentials, except 0.0 V and −0.40 V, were analysed using the same equivalent circuit as in acidic medium. At 0.0 V (except Trp in electrolyte) a simpler circuit comprising R_Ω and CPE as non-ideal capacitance in series were used (reflecting the fact that there is no redox process), the spectra being straight lines, showing just charge separation, independent of solution composition. At −0.40 V the equivalent circuit was extended with a second *R*-CPE parallel combination, suggesting formation of a surface film or surface adsorption, the spectra being almost the same for any solution composition. R_Ω was around 6 Ω cm^2 at all potentials and the exponent α in all cases was ~0.9.

The dependence on solution composition at +0.38 and +0.50 V is shown in Figure 5b1 and b2. The spectra at +0.38 and +0.40 V were almost identical. Interestingly, Trp addition decreased R_{ct} significantly at the electrode interface under these conditions: from 436 to 47 kΩ cm^2 without Cu(II) and from 269 to 40 kΩ cm^2 with Cu(II) in solution. Trp is deprotonated at this pH and is more electroactive than in acidic medium and so facilitates charge transfer. Since Cu(II) forms oxides at these potentials, impedance values decrease only slightly after its addition. Addition of Trp after Cu(II) decreases the impedance values significantly to values lower than in just Trp solution without Cu(II). Addition of another aliquot of Trp to the same solution decreases the impedance values further.

Table 4. Summary of Trp determination in the presence of 0.1 mM Cu(II) in 0.1 M HCl and 0.1 M NaOH solutions.

Technique	Parameter	Acidic medium	Alkaline medium
CV			
	Peak position	−0.1 V	+0.38 V, +0.50 V
	Sensitivity	0.063 μA μM^{-1}	0.153 μA μM^{-1}
	LOD	3 μM	4 μM
	RSD	3 %	3 %
	Determination in presence of other amino acids and proteins	Not possible	Possible
SWV			
	Peak position	−0.07 V, +0.20 V	+0.35 V, +0.46 V
	Sensitivity	0.137 μA μM^{-1}	0.041 μA μM^{-1}
	LOD	2 μM	2 μM
	RSD	2 %	4 %
	Determination in presence of other amino acids and proteins	Not possible	Possible
EIS			
	R_Ω	4 Ω cm^2	6 Ω cm^2
	Influence of Trp on charge transfer	Makes easier	Makes more difficult
	Influence of Cu(II) on charge transfer in absence of Trp	Makes more difficult	Makes easier
	Change in R_{ct} with increase in [Trp] in presence of Cu(II)	Almost does not change	Decreases

This shows that impedimetric measurements at constant frequency (in the low frequency region) between +0.38 and +0.50 V can be used for Trp determination.

Additionally, EIS confirms that, in acidic and in alkaline media, Cu(II)-Trp complexation and amino acid determination occur by different mechanisms. In acidic medium, as discussed above, a Cu(I)-Trp complex is formed (more easily than in alkaline solution) and exists as $[CuTrp]^+$ [21–23]. This can easily disproportionate chemically and go back to a Cu(II)-Trp complex, which is further oxidised at the electrode surface:

$$2[CuTrp]^+ \rightarrow Cu + [CuTrp_2]_{ad}{}^{2+} \quad (1)$$

$$[CuTrp_2]_{ad}{}^{2+} + 2e^- \rightarrow Cu^0{}_{ad} + 2Trp \quad (2)$$

However, in alkaline solution Cu^0 obtained by reduction of the complex $CuTrp_2$ [21,22,24], which exists in neutral, and probably alkaline, form, facilitates Trp oxidation:

$$CuTrp_2 + 2e^- \rightarrow Cu^0 + 2Trp^- \quad (3)$$

The data obtained clearly demonstrate that Cu(II) is necessary to increase the sensitivity of the amino acid determination.

3.3 Comparison Between Acidic and Alkaline Media

The main data for determination of Trp in acidic and alkaline media are summarised in Table 4, which sheds light on choosing the best conditions for amino acid determination by voltammetry. LODs are similar in both media, but the sensitivity depends on the method used: higher in acidic medium for SWV, and higher in alkaline medium for CV. Trp might be determined in presence of other amino acids and proteins only in alkaline medium. There is some evidence that even the impedimetric method might be used for such analysis.

3.4 Hydrolysis of BSA

To measure the protein hydrolysis rate, 1 mg mL^{-1} of BSA was hydrolysed in 1 M HCl at 100 °C for several hours and samples were taken after 0 min, 30 min, 1, 2, 3, and 4 h. The samples were analysed in acidic and alkaline media adding 50 μL of the sample to 10 mL of electrolyte solution. Analysis of the samples was performed by CV and by SWV using the standard addition method (adding Trp).

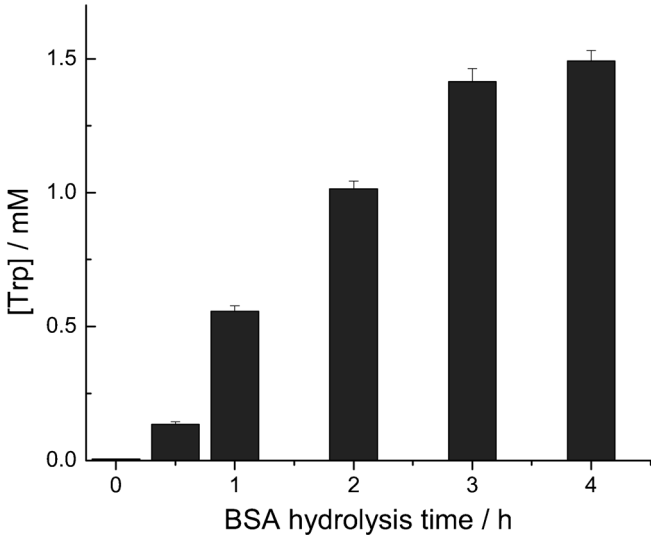


Fig. 6. Trp concentration dependence on BSA hydrolysis time obtained by standard addition method in 0.1 M NaOH electrolyte solution in the presence of 0.1 mM Cu(II) by CV at potential scan rate of 50 mV s^{-1}.

In acidic medium a large interference was obtained from the complex sample matrix and it was not possible to detect the amino acids formed during BSA hydrolysis. On the other hand, a clear and well-defined response was obtained in alkaline medium, better by CV than by SWV because the latter was found to be more sensitive to matrix effects in this case. The shape of the voltammograms (not shown) after sample injection was the same as in the case of Trp (Figure 3). The results, recalculated for the original sample, are presented in Figure 6: BSA hydrolysis under the conditions described above takes ~3 h, giving a response already after half an hour. This method allows following the BSA hydrolysis rate without additional neutralisation of the hydrolysis solution. Although it is not selective to a particular amino acid, as seen from the results of analysis of the hydrolysis products, it can be used for control of the hydrolysis rate.

4 Conclusions

Amino acids can be successfully determined separately at carbon film electrodes in strongly acidic 0.1 M HCl solution in the presence of 0.1 mM Cu(II). The determination of amino acids in acid solution in the presence of large molecules such as proteins was not possible due to electrode blocking or to irreversible Cu(II) complexation by proteins. However, amino acid determination in alkaline solution, 0.1 M NaOH, in the presence of 0.1 mM Cu(II) was possible for individual amino acids and also in the presence of proteins. The sensitivity to amino acids was slightly higher in strongly acidic than in alkaline medium. The *RSD* for amino acid determination is similar in both media and it varies in the range of 2–8% depending on the amino acid.

Impedance spectra in acidic solution showed that Cu(II) facilitates charge transfer and Trp makes it more difficult, whereas in alkaline medium the opposite effect was observed. The best effect of Cu(II) and Trp on R_{ct} is visible from 0 to -0.4 V and from $+0.38$ to $+0.5$ V in acidic and alkaline medium, respectively.

The hydrolysis rate of BSA can be followed by determining the amino acids formed, by adding the partially hydrolysed samples to 0.1 M NaOH and 0.1 mM Cu(II) solution and using cyclic voltammetry. In this way, after appropriate fundamental studies, the decomposition of protein-containing foods can also be examined.

Acknowledgements

This work was supported by the *European Commission* 7th Framework Programme Marie Curie Actions People IRSES N°230815 NANOSENS, by *Fundação para a Ciência e a Tecnologia* (*FCT*), PTDC/QUI/65732/2006, POCI 2010 (co-financed by the European Community Fund FEDER) and *CEMUC* (Research Unit 285), Coimbra, Portugal. R. Pauliukaite thanks *FCT* for a postdoctoral fellowship (SFRH/BPD/27075/2006).

References

[1] H. D. Belitz, W. Grosch, R. Schieberle, *Food Chemistry*, 3rd ed., Springer, Berlin **2004**.
[2] M. I. Mohamed, C. Cordle, *Dev. Food Sci.* **2000**, *41*, 181.
[3] M. G. Valdés, A. C. V. González, J. A. G. Calzón, M. E. Díaz-García, *Microchim. Acta* **2009**, *166*, 1.
[4] J. Saurin, S. Hernández-Cassou, E. Fàbregas, S. Alegret, *Analyst* **1999**, *124*, 733.
[5] D. H. Backer, *Amino Acids*, **2009**, *37*, 29.
[6] H. Masuda, A. Odani, T. Yamazaki, T. Yajima, O. Yamauchi, *Inorg. Chem.* **1993**, *32*, 1111.
[7] A. R. Fiorucci, E. T.G. Cavalheiro, *J. Pharm. Biomed. Anal.* **2002**, *28*, 909.
[8] A. A. Ensafi, R. Hajian, *Anal. Chim. Acta* **2006**, *580*, 236.
[9] J. G. Chen, S. J. Weber, *Anal. Chem.* **1995**, *67*, 3596.
[10] C. F. B. Coutinho, L. F. M. Coutinho, L. H. Mazo, *Quim. Nova* **2009**, *32*, 228.
[11] P. A. M. Farias, A. L. R. Wagener, A. A. Junqueira, A. A. Castro, *Anal. Lett.* **2007**, *40*, 2032.
[12] H. Heli, M. Hajjizadeh, A. Jabbari, A. A. Moosavi-Movahedi, *Anal. Biochem.* **2009**, *388*, 81.
[13] G. L. Luque, N. F. Ferreyra, G. A. Rivas, *Talanta* **2007**, *71*, 1282.
[14] M. Vasjari, A. Merkoçi, J. P. Hart, Salvador Alegret, *Microchim. Acta* **2005**, *150*, 233.
[15] N. Ya. Mokshina, O. A. Pakhomova, S. I. Niftaliev, *J. Anal. Chem.* **2007**, *62*, 965.
[16] B. A. Deore, H. Shiigi, T. Nagaoka, *Talanta* **2002**, *58*, 1203.
[17] C. M. A. Brett, L. Angnes, H. D. Liess, *Electroanalysis* **2001**, *13*, 765.
[18] C. C. Wagner, E. J. Baran, *Acta Farm. Bonaerense* **2004**, *23*, 339.
[19] E. P. Grishina, A. M. Udalova, E. M. Rumyantsev , *Russ. J. Electrochem.* **2002**, *38*, 1041.
[20] A. C. Liu, D. C. Chen, C. C. Lin, H. H. Chou, C. H. Chen, *Anal. Chem.* **1999**, *71*, 1549.
[21] N. Arulsamy, B. Srinivas, P. S. Zaeharias, *Transition Met. Chem.* **1990**, *15*, 309.
[22] G. Thomas, P. S. Zacharias, *Polyhedron* **1985**, *4*, 811.
[23] C. K. Siu, Y. Ke, Y. Guo, A. C. Hopkinson, K. W. M. Siu, *Phys. Chem. Chem. Phys.* **2008**, *10*, 5908.
[24] S. Çakir, E. Biçer, *Trans. Met. Chem.* **2001**, *26*, 89.

