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Electrochemical detection of dipeptides with selectivity against amino acids

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ABSTRACT

Electrolysis of a basic mobile phase containing biuret reagent [Cu(II) and a tartrate salt] at high (>1.2 V *vs.* Ag/AgCl) potentials modifies the glassy carbon electrode. This modified anode oxidizes dipeptides, yielding signals expected for a one-electron transfer, even at low (down to 0.7 V *vs.* Ag/AgCl) potentials and in the absence of intentionally added copper(II) ion in the reagent or mobile phase. The same modification demonstrates a selectivity to α-dipeptides over amino acids that is unprecedented. The product of the anodic reaction is reduced at a downstream cathode at low positive potentials. Sensitivities for several amino acids and dipeptides are reported under several conditions. Neither the anodic nor the cathodic signals for the buiret complex of the tripeptide Ala–Ala–Ala are significantly altered because of the modification.

INTRODUCTION

Detection strategies for chromatographically separated peptides have taken one of two courses. Either detection has been specific for an amino acid functional group or the detection has relied upon the nucleophilicity of the peptide's amine group to form a detectable derivative. An example of the former approach is the electrochemical detection of tyrosine-containing opioid peptides [1], while the fluorescence detection of the reaction product of *o*-phthalaldehyde (OPA) [2] or 2,3-naphthalenedialdehyde and peptides [3,4] are examples of the latter. The primary virtues of these methods are their low detection limits and their established nature; OPA has been used successfully and routinely for the fluorescence determination of amino acids in particular.

Although the genetically coded amino acids are only twenty species, the group of natural non-protein amino and imino acids and derivatives are probably well over 700 [5]. The theoretical number of dipeptides consisting only of protein amino acids is 400, and many of these are chromatographically similar to amino acids [6]. It is evident that routine chromatography of today is incapable of separating, for example, all of the dipeptides from all of the amino acids. A related problem is that chromatographic analysis at trace levels inevitably suffers from the presence of interfering peaks. Methods that are more molecularly selective are urgently needed to help solve both of these problems.

We have begun to develop the biuret reaction in electrochemical detection [7,8]. This reaction

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creates electroactive species from electroinactive peptides, but not from electroinactive amino acids. Thus, the electrochemical detection shows a significant selectivity for peptides over amino acids [7].

Simple pentapeptides yield stable complexes with Cu(II) in which one amine and three amide nitrogens are donors in the distorted octahedral complexes [9]. This coordination environment supports the Cu(III) oxidation state at remarkably modest potentials [9]. The potential needed to oxidize peptide-bound Cu(II) to Cu(III) becomes more positive as the number of amides becomes smaller; the resultant higher energy Cu(III) forms of smaller peptides are less stable towards intramolecular electron transfer reactions. These intramolecular reactions destroy the complex. Thus, the shorter peptides' Cu(III) complexes have short lifetimes in basic aqueous solution as evidenced by the low collection efficiency in dual-electrode electrochemical detection [8], and values of the ratio of cathodic peak current to anodic peak current in cyclic voltammetry [7]. The lifetimes of Cu(III) complexes of dipeptides are probably very short, though there is no direct chemical evidence to support this speculation. Nonetheless, we found conditions under which dipeptides are detectable using the biuret reagent [8]. The electrochemical detection cell had two electrodes, an upstream anode and a downstream cathode. Our finding was that high potential was required for the oxidation and the lifetime of the Cu(III) peptide formed in the oxidation was short, as expected. Thus, detection at both the anode and the cathode is poorer for the dipeptides than for longer peptides.

Some peptides, such as glutathione and N-acetyl–Asp–Glu [10–12], have been recognized as important to brain function. However, in general there is very little information on the concentration and distribution of dipeptides [13–22] perhaps in part due to the lack of suitable methods. Any method which is developed for peptides should, if possible, exhibit some selectivity over amino acids as the latter are expected to be generally higher in concentration than peptides. Thus, we have reinvestigated the detection of dipeptides with the ultimate purpose of finding conditions suitable for the determination of α-dipeptides in the brain and we have also looked into the selectivity of the detection for peptides in comparison to the amino acids.

EXPERIMENTAL

Chromatographic equipment. A Waters 625 pump [polyetheretherketone (PEEK)] with a 600E controller were used to pump the aqueous mobile phases through the 200×4.6 mm I.D. column of Nucleosil 5 SB (Macherey–Nagel, Düren, Germany), a 5-μm diameter silica-based anion-exchange material. The detector was a BAS LC4B with a dual glassy carbon electrode cell. The spacer thickness was 50 μm. All potentials stated are referred to the Ag/AgCl, 3 *M* NaCl electrode which was also obtained from BAS (through CMA/Microdialysis, Stockholm, Sweden). Data were recorded on a strip chart recorder. Peak areas were approximated as the product of the full width at half height and the peak height.

Mobile phases were aqueous buffers and salt solutions including 500 m*M* K_2SO_4, 500 m*M* Li_2SO_4, 500 m*M* LiCl and acetate buffers (sodium acetate, lithium acetate, acetic acid.) The LiCl was from BDH (Poole, UK), while all the rest were from Merck (analytical-reagent grade, Darmstadt, Germany). Water was Milli-Q deionized and charcoal filtered. All solutions were filtered through 0.45 μm filters (type HV; Millipore, Bedford, MA, USA). The LiCl was used both as recieved and after recrystallization from hot water. There was a significant orange color in the filtrate of the hot mother liquor which suggests the presence of iron. Indeed, after using the LiCl in the mobile phase the peaks became broader and the peaks were returned to their symmetrical shape after a couple of injections of 500 m*M* EDTA. Retention times were not noticably changed by this procedure, which was only needed once.

The Waters 625 pump uses four solvent reservoirs. The concentrations of salts in the mobile phases cited in the tables are given as final concentrations as seen by the column. However, the pH value of the individual mobile phase components is stated.

Postcolumn phases were buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES) (Sigma, St. Louis, MO, USA), and phosphate ($NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 2H_2O$), or sodium carbonate–hydrogencarbonate (the latter four compounds were analytical-reagent grade from Merck). The pH meter (Orion, Zurich, Switzerland) was calibrated daily at pH 7.00 and pH 4.00. A pH very close to 8.1 is obtained from a phosphate solution 500 m*M* in the monobasic phosphate salt and 20 m*M* in the dibasic salt; a pH near 8.0 is obtained if the latter value is 30 m*M*. A pH near 9.85 results from 100 m*M* each of sodium carbonate and hydrogencarbonate, and the pH is about 8.9 when there are 180 mmols of hydrogencarbonate to 20 mmols of carbonate per liter. A solution of $CuSO_4$ (anhydrous, Merck) analytical-reagent grade) and potassium sodium tartrate (Merck analytical-reagent grade) was prepared by making separate solutions of the two species and combining them. Molar ratios of 3:1 (tartrate to copper) and 6:1 have been used.

The electrode polishing protocol was to polish with 6 μm diamond (BAS, W. Lafayette, IN, USA) followed by vigorous squirting of the surface with deionized water, and then several minutes of ultrasonication (Juchheim Laborteknik, Seelbach, Germany) in methanol (Rathburn L.C. grade, Walkerburn, UK). Sometimes this was followed by a polish using 0.3 μm alumina (Fisher, Pittsburgh, PA, USA), and ultrasonication in methanol again.

Amino acid and peptide solutes were: from Research Plus (Bayonne, NJ, USA), Gly–Leu; from Sigma, Ala, Gly, Ser, Lys, Leu, cystine, Asp, Glu, Arg, Ala–Ala–Ala (A_3); from Bachem (Philadelphia, PA, USA), Glu–Lys, Arg–Glu, Asp–Ala, Asp–Glu, Asp–Gly, Asp–Leu. Solutions were prepared in water. They were stable for about a week when refrigerated overnight.

RESULTS

Sensitivities have been calculated as peak area per mol injected. The units are picocoulombs per picomole. For a one-electron transfer, the maximum value of the sensitivity is just Faraday's constant, about 9.65×10^4. Thin-layer flow cells of the design used usually detect a few percent of the solute that flows over the electrodes, thus a sensitivity of about 1000 C/mol is expected from an ordinary single-electron transfer detection. In a two-electrode cell, with an upstream generator and a downstream collector, the senstivity of the upstream electrode will be about 1000, while that at the downstream electrode will be about one-third of that if the species formed at the upstream generator is long-lived and survives the journey from the upstream to the downstream electrodes. In the system under discussion, the upstream generator is an anode, while the downstream collector is a cathode. Cathodic sensitivities for three representative peptides, A_3, Asp–Ala and Asp–Glu, as a function of pH and Cu(II) concentration in the postcolumn reagent are shown in Table I. The mobile phase, flowing at 1.0 ml/min, was 10 m*M* K_2SO_4, 25 m*M* pH 4.62 sodium acetate buffer. The postcolumn phases, identified in Table I, were flowing at 0.5 ml/min. Better sensitivities were recorded for the higher pH, a not unexpected result for an oxidation. However, it is surprising that the sensitivities decrease when the Cu(II) concentration is increased.

To test the effect of Cu(II) on the cathodic sensitivity, the compounds A_3 and Asp–Ala were used with a series of postcolumn phases shown in Table II. The Cu(II) seems to play no role in the generation of signal; even in the absence of Cu(II), a significant signal is obtained for the two peptides. At pH near 9 the sensitivity is as good or better than at pH near 10. However, when the postcolumn system was rinsed with a 3% solution of concentrated nitric acid in water for 90 min at 1.0 ml/min, the sensitivity with a pH 8.98 carbonate buffer postcolumn phase declined to about 5% of its initial value.

In a separate experiment, with freshly polished electrodes, the effect of pH and Cu(II) concentration were determined without changing the buffering ion. Thus, carbonate–hydrogencarbonate buffers were used at pH values near 9 and 10, and Cu(II) concentrations of 0.1 and 1.0 m*M* were used. The data for cathodic sensitivities are shown in Table III. Once again, it can be seen that the influence of Cu(II) is to decrease, rather

TABLE I

SENSITIVITIES AT THE CATHODE: EFFECT OF pH AND [Cu(II)]

Mobile phase: 1.0 ml/min 10 m*M* K_2SO_4, 25 m*M* pH 4.62 NaOAc. Postcolumn phase: 0.5 ml/min. Potassium sodium tartrate concentration is three times the stated Cu(II) concentration. Potentials: anode 1.1 V, cathode 0.0 V.

Postcolumn phase		Sensitivity (C/mol)		
		Ala–Ala–Ala	Asp–Ala	Asp–Glu
Hydrogen carbonate–carbonate	pH 9.85			
Cu(II)	0.1 m*M*	180	260	120
	1.0 m*M*	160	80	40
HEPES	pH 7.50			
Cu(II)	0.1 m*M*	80	90	110
	1.0 m*M*	20	–	–

TABLE II

SENSITIVITIES AT THE CATHODE: EFFECT OF [Cu(II)]

Mobile phase: 1.0 ml/min 10 m*M* K_2SO_4, 25 m*M* pH 4.62 NaOAc. Postcolumn phase: 0.2 *M* total (carbonate + hydrogencarbonate) as sodium salts. Potassium sodium tartrate concentration is three times the stated Cu(II) concentration. Potentials: anode 0.90 V, cathode 0.15 V.

pH	Cu(II) (m*M*)	Sensitivity (C/mol)	
		Ala–Ala–Ala	Asp–Ala
9.85	0.2	260	200
9.85	0.05	280	220
9.85	0	250	240
8.98	0	320	240
	HNO_3 rinse		
8.98	0	10	10

than increase, the signal. The sensitivities for the dipeptide Asp–Ala and for the amino acid Asp are more strongly dependent on the Cu(II) concentration than the tripeptide A_3.

A sequence of events is recorded in Table IV. The cathodic sensitivity at freshly polished electrodes and an anode potential of 1.0 V is the same in the presence and absence of bicarbonate in the Cu(II)-containing postcolumn phase (data not shown). When the anode is taken to 1.2 V, and then back to 1.0 V, there is much less of a decrement in cathodic sensitivity accompanying the potential change from 1.2 to 1.0 V when there is hydrogencarbonate in the phase. In fact, as can be seen in the table, the carbonate need not be present once the anode has been taken up to 1.2 V in its presence. The sensitivity to the dipeptides remains significant at modest potentials.

TABLE III

SENSITIVITIES AT THE CATHODE AND SELECTIVITY TO Asp–Ala OVER Asp

Mobile phase, postcolumn phase and potentials as in Table II.

pH	Cu(II) (m*M*)	Sensitivity (C/mol)			
		Ala–Ala–Ala	Asp–Ala	Asp	Asp–Ala/Asp
8.98	0.1	330	180	6	30
	1.0	270	80	0.5	160
9.88	0.1	470	110	8	14
	1.0	220	17	0.6	26

TABLE IV

SENSITIVITIES AT THE CATHODE: EFFECT OF HISTORY

Mobile phase: 1.0 ml/min 5 m*M* pH 4.62 NaOAc, 15 m*M* LiCl. Postcolumn phase: 0.5 ml/min 0.52 *M* pH 7.98 phosphate with added $NaHCO_3$ as stated, 0.1 m*M* $CuSO_4$, 0.6 m*M* potassium sodium tartrate. Potentials: anode as stated, cathode 0.0 V.

	Anode potential (V)	Sensitivity (C/mol)			
		Ala–Ala–Ala	Asp–Ala	Asp–Asp	Glu–Glu
No HCO_3^-	1.0	270	60	7	7
No HCO_3^-	1.2	240	140	18	10
No HCO_3^-	1.0	200	40	7	3
0.1 *M* HCO_3^-	1.2	290	290	13	130
0.1 *M* HCO_3^-	1.0	220	220	60	190
0.1 *M* HCO_3^-	0.95	260	180	40	60
No HCO_3^-	0.95	260	200	60	50

Table V shows anodic sensitivities (cathode not used) determined after the electrode had been anodized in a Cu(II) and bicarbonate containing mobile phase. A sampling of acidic, basic and neutral amino acids, and a selection of neutral and acidic dipeptides were tested. It can be seen that, in general, the peptides are more sensitively determined than the amino acids. Thus, the combined sensitivities of Gly and Leu is about 10, while the sensitivity of the dipeptide Gly–Leu is about 1000. Similarly, the sensitivity for the peptides Asp–Gly, Asp–Leu, Glu–Lys and Arg–Glu are significantly larger than the sum of the sensitivities of the relevant amino acids. The most sensitively detected amino acids, of the limited but representative set chosen for study, are the basic ones.

TABLE V

SELECTIVITY AT THE ANODE

Mobile phase: 1.0 ml/min 3 m*M* pH 3.72 LiOAc, 3 m*M* pH 5.72 LiOAc, 50 m*M* Li_2SO_4. Postcolumn phase: 0.5 ml/min pH 8.05 phosphate buffer. Electrode preparation: 50 min at 1.2 V in 1.0 ml/min, 3 m*M* pH 3.72 LiOAc, 3 m*M* pH 5.57 LiOAc, 100 m*M* Li_2SO_4, plus 0.5 ml/min 0.52 *M* pH 8.14 phosphate buffer, 0.1 m*M* $CuSO_4$, 0.6 m*M* potassium sodium tartrate, 0.1 *M* $NaHCO_3$. The pH of the combination is about 8. Potential: anode 0.85 V.

Solute	Sensitivity (C/mol)
Ala	2.5
Arg	220
Asp	12
Glu	7.5
Gly	2.6
Leu	7.8
Lys	34
Ser	4.8
Asp–Glu	140
Asp–Leu	300
Glu–Lys	490
Gly–Leu	900
Arg–Glu	1760
Ala–Ala–Ala	570

In some experiments it was noted that the cathodic selectivity for peptides over amino acids increased when the anodic potential was lower and the pH was lower. The effect of pH has been demonstrated in Table III. The results from a study of the anodic sensitivities are shown in Table VI. Here, a selection of neutral amino acids and peptides have been determined at two pH values and two anode potentials. It appears that the sensitivities for all the species except the tripeptide follow about the same pattern; the sensitivity decreases about a factor of three on changing the potential from 0.90 to 0.85 V at each pH, and at each potential, the sensitivity drops a factor of three in lowering the pH the stated amount. Thus the anodic sensitivity, but not the selectivity, are influenced by pH and potential in the range studied.

The potential dependences of the cathodic signal as the anodic and cathodic potentials

TABLE VI

SENSITIVITY AND SELECTIVITY AT THE ANODE: EFFECT OF pH AND POTENTIAL

Mobile phase: 1.0 ml/min 3 m*M* pH 3.72 LiOAc, 3 m*M* pH 5.72 LiOAc, 25 m*M* Li_2SO_4. Post column phase: 0.5 ml/min pH 8.05 phosphate buffer. Electrode preparation: 50 min at 1.2 V in 1.0 ml/min 3 m*M* pH 3.72 LiOAc, 3m*M* pH 5.57 LiOAc, 100 m*M* Li_2SO_4, plus 0.5 ml/min 0.52 *M* pH 8.1 phosphate buffer, 0.1 m*M* $CuSO_4$, 0.6 m*M* potassium sodium tartrate, 0.1 *M* $NaHCO_3$. The pH of the combination is about 8.

Solute	Sensitivity (C/mol)			
	Anode potential 0.9 V, pH 8.14	Anode potential 0.85 V, pH 8.14	Anode potential 0.9 V, pH 7.57	Anode potential 0.85 V, pH 7.57
Ala	8.2	2.4	3.4	0.7
Gly	6.6	2.6	2.6	0.9
Leu	23	7.8	6.4	3.2
Lys	100	30	30	8.8
Ser	16	4.8	5.6	1.6
Arg–Glu	3240	1760	1300	510
Gly–Leu	3400	750	800	80
Glu–Lys	1650	540	510	130
Ala–Ala–Ala	650	490	90	20

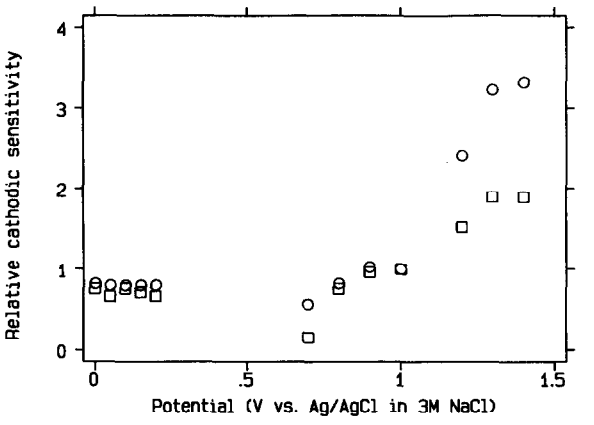

Fig. 1. Cathode sensitivities for A_3 (○) and Asp Ala (□). Data plotted from 0 to 0.2 V are taken with varying cathodic potentials and an anodic potential of 0.8 V. Conditions as in Table II. Data plotted from 0.7 to 1.0 V are also taken under conditions as in Table II, but it is the anodic potential that varies, the cathodic potential is 0.0 V. A second set of data, taken under solution conditions in Table V and with cathodic potential = 0, is plotted as the anodic potential changes from 1.0 to 1.4 V. Sensitivities from the two sets of solution conditions are normalized with respect to values found at an anodic potential of 1.0 V and a cathodic potential of 0.0 V.

change are shown in Fig. 1. This is a composite figure, so relative sensitivities to a standard condition (anode = 1.0 V, cathode = 0.0 V) are used. The right side of the diagram shows the response as the anodic potential is changed. There are two waves, one with a half-wave potential around 0.7–0.8 V and the other with a half-wave potential around 1.3 V. Our experience has been that the signal-to-noise ratio is better on the plateau around 0.9–1.0 V than at potentials above 1.4 V. On the left side of the diagram, sensitivities are shown for the cathodic potential changing with a constant anodic potential. In separate experiments we have determined that the signal is negligible when the cathodic potential is 0.5 V. Thus, although it is not well characterized in the figure, the half-wave potential is in the range of 0.4 V.

Fig. 2 shows a chromatogram of dipeptides containing the acidic amino acids. The separa-

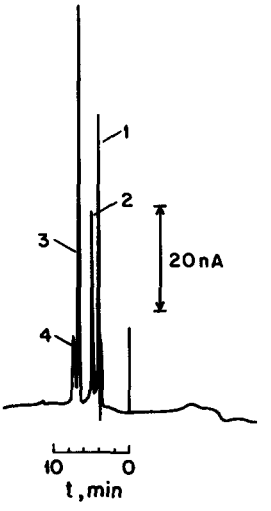

Fig. 2. Chromatogram of (1) Glu–Lys (0.49 nmol), (2) Asp–Gly (0.95), (3) Asp–Asp (0.69), (4) Glu–Glu (0.62). Mobile phase 1.0 ml/min of 6 m*M* acetate buffer pH 4.5, 75 m*M* Li_2SO_4. Postcolumn solution pH 8.14, 0.5 *M* phosphate buffer at 0.5 ml/min.

tion based largely on charge is expected of an anion exchange material.

DISCUSSION

It may be a Cu(II) complex that is detected when Cu(II) is present in the system, but if the primary signal-producing event were the oxidation of Cu(II) bound in a complex, then the law of mass action dictates that the signal must at least remain constant, or perhaps increase, as the Cu(II) concentration increases. The decrease in sensitivity caused by increasing the Cu(II) concentration in the postcolumn reagent, the presence of the signal in the absence of intentionally added Cu(II), and the disappearance of the signal when the postcolumn reactor and flow cell are rinsed with nitric acid, suggest that some surface reaction makes the electrode more sensitive to the peptides. Inspection of the electrodes shows that the anode, but not the cathode, has a film after the potential of the anode has been held at a positive potential (>1.2 V).

The data in Table III show that not only is the sensitivity dependent on the Cu(II) concentration, but the relative sensitivity of the dipeptide Asp–Ala to the amino acid Asp is influenced by Cu(II) concentration. At both high and low pH, the selectivity of the detection for the peptide is better with higher Cu(II). This is not due to a relatively greater signal from the dipeptide, but is due to a larger copper-induced decrease in the signal from the amino acid. It is well known that amino acids form complexes with Cu(II) [23]. Certainly tripeptides form the biuret complex [7,9], and, although dipeptides give a signal at the electrochemical detector under conditions which yield the biuret complex from tripeptides [8], they probably do not form a complex with a stable Cu(III) form. An investigation of a single peptide was carried out to explore this.

We have examined the cyclic voltammetry and visible absorbance spectrum of the dipeptide Gly–Leu. The cyclic voltammetry at a glassy carbon electrode in biuret reagent shows a drawn out anodic wave at around 900–1000 mV, with no cathodic wave at 200 mV s^{-1}. The visible spectra of Gly–Leu solutions in biuret reagent were recorded with the biuret reagent as a reference. The wavelength of maximum absorbance shifts slightly when the Cu(II)-to-peptide ratio is changed, from 614 nm at 1:5 to 638 nm at 5:1. Biuret complexes have λ_{max} at about 550 nm. The molar absorptivity (based on the limiting reagent) is about 50 M^{-1} cm^{-1}. The wavelength of maximum absorbance in the excess Cu(II) is consistent with the formation of a 1:1 complex (donor atoms: amine, amido nitrogens, carboxylate and hydroxide oxygens: theoretical λ_{max} = 632 nm computed according to Billo [24]).

The electrochemistry and the spectroscopy are consistent in the sense that a stable Cu(III) form is not seen on the time scale used.

The depressing effect of Cu(II) on the signal implies that coordination to Cu(II) lowers the activity of the oxidized species. This effect is more important for the amino acids and dipeptides than for the tripeptide A_3 (Tables II and III). It is noteworthy that there is no signal observed (sensitivity $\ll 1$) from N-acetyl–Asp and from N-acetyl–Asp–Glu. These observations imply that the amine group is important to the oxidation of the amino acid and dipeptides.

Recently, a catalytic oxidation by an anodized copper electrode of the amino acids and some peptides has been discussed by Luo *et al.* [25]. This approach seems very promising, as the sensitivity is good, and the electrode is long-lasting. As is the case in the current study, the sensitivity to amino acids depends upon the side chain; amino acid detection limits vary by a factor of 50 in that work. This is similar to the sensitivity variation seen in the current work. Although the data are not explicitly given, it seems that the sensitivity for small peptides with the electrode of Luo *et al.* [25] is about the same as that for the amino acids. The current work is different in two respects. First, the sensitivity to peptides is, in general, higher than for amino acids; in the few examples cited above the dipeptide's sensitivity is always significantly larger than the sum of the sensitivities of the constituent amino acids. Second, the pH required to carry out the reaction is lower. In the work of Luo *et al.* [25] the mobile phase was 0.1 *M* NaOH. In this work, a pH of 8 was shown to be compatible with detection.

CONCLUSIONS

We do not know yet what the putative electrode modification is, nor do we know why the surface reacts as it does. Nonetheless, we have shown that it is possible to detect dipeptides with some selectivity at a modest potential in weakly alkaline solutions, the latter compatible with most reversed-phase materials, for example. There is, on average, a selectivity for dipeptides over amino acids, though individual exceptions exist (Arg *vs.* Asp–Gly). Future work concerning the application of this detection technique to extracts of rat brain, and work directed towards understanding and optimizing the surface of the anode are underway.

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