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Amperometric determination of underivatized amino acids at a nickel-modified gold electrode by anion-exchange chromatography

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Abstract

A nickel-based composite electrode obtained by anodic electrodeposition of nickel (III) oxyhydroxide film on the gold electrode substrate was characterized as an amperometric sensor and successfully applied to the determination of underivatised amino acids in flow-through systems. The electrodeposition of nickel oxyhydroxide films was obtained by cycling a gold electrode between 0.0 V and +1.0 V vs. a saturated calomel electrode in a 80 μM Ni^{2+} solution buffered at pH 10 with $NaHCO_3/Na_2CO_3$. The resulting Au–Ni composite electrode exhibits good stability in alkaline medium and can be used as an amperometric sensor of underivatised amino acids at a fixed applied potential (+0.55 V vs. Ag/AgCl). The detection limits ($S/N=3$) for all investigated compounds ranged between 5 and 30 pmol injected, while the linear ranges spanned over two or three orders of magnitude. The contents of several free amino acids in two sample cheeses from different brands were evaluated by calibration graphs.

Keywords: Amperometric detection, LC; Cheese; Food analysis; Amino acids

1. Introduction

The determination of amino acids and their related compounds is of great significance in clinical, nutritional and biotechnological fields, and many efforts have been devoted to devise simple and sensitive analytical methods. Several instrumental techniques such as UV–visible absorption, fluorescence and mass spectrometry have been utilised for the detection of these compounds following their chromatographic separation. Although amino acids are readily separated from matrix interference by using high-performance liquid chromatography (LC), their detection is hindered by the absence of a strong chromophore, and formation of derivatives is required to enhance the absorbivity. Indeed, photometric detection of amino acids upon chemical derivatisation with *o*-phthaladehyde [1], ninhydrin [2] and phenyl isothiocyanate [3] are very sensitive. Nevertheless, different detection strategies which do not require derivatising reagents, when available, are generally preferred for simplicity and economical convenience.

Electrochemical detection following liquid chromatography (LC–ED) represents a very attractive detection possibility for underivatised amino acids [4,5]. The unique serious drawback of amperometric sensors based on noble metal electrodes is that they are subject to rapid surface fouling, so that pulsed potential waveforms are required for a stable long-

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term operation. Just very recently Clarke et al. [6] have reported the pulsed amperometric detection of amino acids in conjunction with anion-exchange chromatography. An integrated amperometry waveform, which requires up to six potential pulses repeating every 600 ms at a gold working electrode, was employed. Alternatively, several transition metal-based electrodes have been proposed as amperometric sensors for the determination of amino acids at constant applied potential [7–11], and the development of new electrode materials for direct amperometric detection of electroactive aliphatic compounds is a growing area of electroanalytical chemistry. In this respect, the modification of traditional electrode surfaces provides an attractive way of confining active catalytic species to the effective spatial region, and combines the experimental advantages of heterogeneous catalysis with the benefits of a three-dimensional distribution of catalytic centres typically characteristic of homogeneous catalysis. The feasibility of deposit active metallic particles onto an electrodic substrate provides a good physical dispersion of the catalyst with subsequent increase of the active electrode surface. Highly dispersed metal oxide/hydroxide particles on the noble metals or graphitic substrates are usually prepared by electrochemical techniques [12–15]. Evenly distributed deposits can be prepared and the final morphology and texture can be easily modulated by means of the various parameters affecting the electrodeposition process such as supporting electrolyte, solvent, electrolysis time, applied potential, etc.

Recently, we have proposed some chemically modified electrodes (CMEs) based on nickel catalyst, which have been successfully employed for the determination of carbohydrates [16,17] and alcohols [18] in liquid chromatography. In order to extend the range of analytes, which can be detected at the nickel-modified electrode, the determination of amino acids in LC–ED is described in the following sections. A gold electrode substrate modified by anodic deposition of a thin film of nickel(III) oxyhydroxide, designed as Au–Ni, was prepared and characterised by cyclic voltammetry (CV) towards the electrooxidation of underivatised amino acids in alkaline medium. Flow injection analysis (FIA) and anion-exchange chromatography were used to characterise the amperometric sensor at constant potential operation for the determination of amino acids. The large analytical signal observed for these compounds, as well as the resistance of this composite electrode to fouling, makes it excellent candidate for application as an amperometric sensor of amino acids in complex mixtures following their separation by anion-exchange chromatography. Examples of simple determination of amino acids in real matrices (i.e., cheese), avoiding derivatisation procedures pre- or post-column, are reported.

2. Experimental

2.1. Reagents

Solutions were prepared from analytical-reagent grade chemicals without further purification and by using twice distilled and deionized water. Individual solutions of amino acids (Aldrich Chemie) were prepared daily in distilled water. Appropriate dilutions were made with 0.1 *M* NaOH solutions and distilled water in FIA and LC, respectively. Unless otherwise specified, the experiments were performed in 0.1 *M* NaOH as supporting electrolyte. All experiments were carried out at ambient temperature. Before injection in column, samples of cheese were treated in 100 ml of boiling distilled water and sonicated for a few minutes. The suspension was filtered by passage through filter paper, the resulting solution was diluted 1:100 with distilled water and then injected. The concentrations of amino acids were determined by a standard addition method. The samples were spiked after the relevant extraction process (with boiled water) and were analyzed in triplicate.

2.2. Apparatus

Cyclic voltammetry was performed by an EG&G Princeton Applied Research (PAR) Model 273 potentiostat/galvanostat. Data acquisition and potentiostat control were accomplished with a 486/50 MHz IBM-compatible computer running the M270 electrochemical research software (EG&G). All experiments were carried out at room temperature in a standard three-electrode glass cell using the Au–Ni as a working electrode, a SCE as a reference

electrode and a platinum foil as a counter-electrode. The gold substrate electrode used in CV (geometric area, 0.125 cm^2) was purchased from PAR. All current densities are quoted in terms of mA/cm^2 of apparent geometric area of the gold electrode.

Amperometric measurements in flowing streams were performed using a PAR Model 400 electrochemical detector and a flow-through thin-layer electrochemical cell consisting of the Au–Ni as working electrode, an Ag/AgCl (4 *M* KCl) reference electrode and a stainless steel counter electrode. The output signal was recorded by a Model Servogor 120 BBC recorder. A Varian 2510 pump equipped with a Model 7125 Rheodyne injector using a 50 μl sample loop was used for experiments in FIA and LC. The mobile phase was purged from oxygen by an on-line degasser system (Hewlett-Packard Series 1050).

Chromatographic separations were performed using a CarboPac PA1 (Dionex) anion-exchange column (250×4 mm I.D.) coupled with a CarboPac PA1 (50×4 mm I.D.) guard column.

2.3. Electrode preparation

Prior to each electrode modification, traces of nickel species were removed from the gold surface by soaking the electrode in concentrated nitric acid (70%, w/w) for a few minutes. The electrode was then polished with 0.05 μm α-alumina powder on a polishing micro-cloth and washed with doubly distilled water. Films of nickel oxyhydroxide were obtained by voltage cycling (50 mV s^{-1}) between 0.0 V and +1.0 V vs. a saturated calomel electrode (SCE) in a non deoxygenated 80 μ*M* $Ni(NO_3)_2$ solution buffered at pH 10 with $NaHCO_3/Na_2CO_3$ ($NaHCO_3+Na_2CO_3=0.1$ *M*). Subsequently, the modified electrode (Au–Ni) was washed with twice distilled water and conditioned by continuous CV cycling the potential between −0.1 V and +0.65 V for ca. 15 min at 50 mV s^{-1} in 0.1 *M* NaOH.

The surface concentration of nickel sites (Γ_{Ni}) was evaluated by integration of the cathodic wave centred at about +0.35 V corresponding to the $Ni^{III}\rightarrow Ni^{II}$ reduction process, assuming that all the nickel redox sites are electroactive on the voltammetric time scale. Unless otherwise specified, all experiments were performed with nickel surface loadings at the Au–Ni composite electrode of 4–7 nmol cm^{-2}.

3. Results and discussion

3.1. Voltammetry

Fig. 1 shows the rapid growth of a nickel oxyhydroxide film on a gold electrode substrate during continuous potential cycling between 0.0 and 1.0 V vs. SCE in freshly prepared solution containing 80 μ*M* $Ni(NO_3)_2$ in 0.1 *M* $NaHCO_3/Na_2CO_3$ at pH 10. As characteristic of conducting film formation, the nickel oxyhydroxide deposit remains electroactive during prolonged electrodeposition so that the redox transition waves a_1 and c_1 grow with the number of cycles. Such a film on the gold surface exhibits good electrical properties as the peak current and peak potential relevant to c_2 wave (relevant to gold oxide reduction) are virtually identical to those observed at the bare gold electrode. In addition to the gold electrochemical behaviour, the Au–Ni composite electrode exhibits an anodic peak a_1 at ca. +0.75 V due to the oxidation of Ni^{II} to Ni^{III}, which precipitates on the electrode surface, presumably as NiOOH. On the reverse scan, the cathodic peak c_1 corresponds to the reduction of Ni^{III}. As proposed by Tench and Warren [12], the overall reaction of nickel precipitation can be schematically represented as follows:

$$Ni^{2+} \rightarrow Ni^{3+} + e^-$$
$$\downarrow + 2OH^-$$
$$NiOOH + H^+ + e^- \Leftrightarrow Ni(OH)_2$$

Upon cycling the nickel deposit between NiOOH and $Ni(OH)_2$, both peak currents (a_1/c_1) increase as additional Ni^{2+} present in solution is oxidised and deposited on the gold substrate. The surface concentration of electrodeposited nickel (Γ_{Ni}) linearly increased on increasing the number of cycles up to 40–50. Note that a similar behaviour was observed when glassy carbon and platinum were employed as electrodic substrates.

It is well known that in alkaline medium the electrogenerated NiOOH species show a powerful electrocatalytic activity towards the oxidation of polar aliphatic organic compounds [14–20]. For this reason, the electrocatalytic oxidation of amino acids

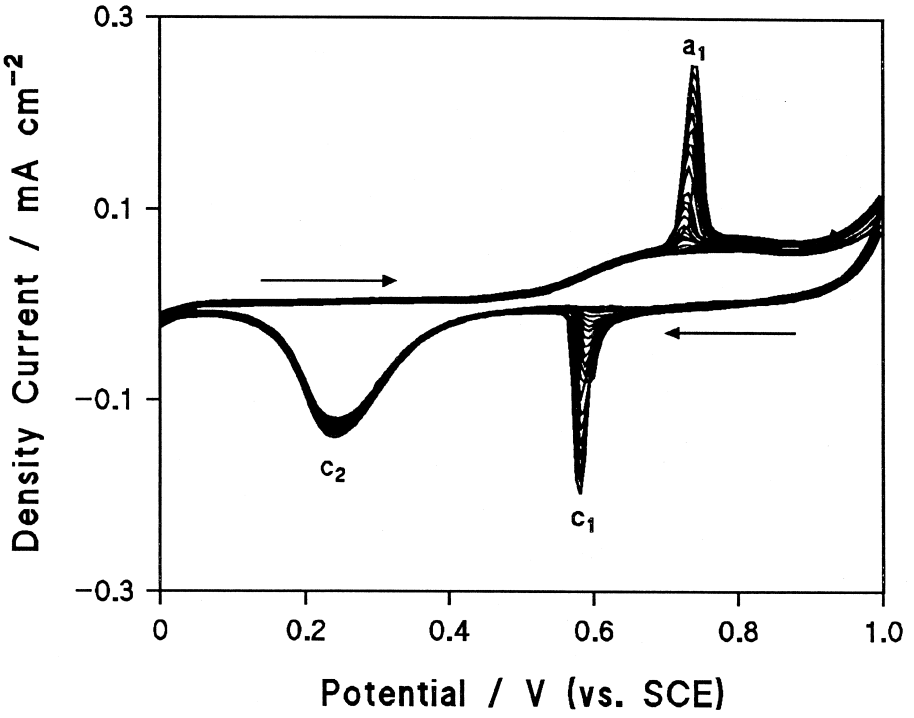

Fig. 1. Deposit of $Ni(OH)_2$/NiOOH growth on a gold electrode substrate in 0.1 *M* $NaHCO_3/Na_2CO_3$ solution buffered at pH 10 containing 80 μ*M* $Ni(NO)_3$. The potential was cycled continuously at 50 mV s^{-1} between 0.0 V and +1.0 V vs. SCE.

at the Au–Ni electrode in 0.1 *M* NaOH solution was investigated. Representative CVs obtained at the Au–Ni electrode in alkaline solution are illustrated in Fig. 2. Addition of cysteine (Fig. 2A) results in a large anodic wave comprised between +0.15 V and 0.6 V. Such an electrooxidation process appears to be sustained by both gold oxide and nickel oxyhydroxide species. The total absence of peak c_1 in the return scan suggests that nickel oxyhydroxide species (NiOOH) involved in the electrocatalytic oxidation process are also most likely subject to complexing reactions with cysteine or its reaction products. Fig. 2B displays the voltammetric profile obtained in the presence of asparagine. A significant electrooxidation current is observed only in the region of potentials comprised between +0.4 V and +0.65 V where the electrooxidation process is attributed to formation of NiOOH. It has to be mentioned that an oxidation current is also present at lower potentials (i.e., 0.28–0.34 V) where the oxidation power may be attributed to the gold substrate. In the case of threonine (Fig. 2C), two independent oxidation waves at low and high potentials were observed. It is conjectured that while in the potential region comprised between −0.05 V and +0.15 V the electrooxidation process is sustained by the involvement of adsorbed hydroxyl radicals on the Au–Ni composite electrode [15,21], the oxidation current observed between +0.45 V and +0.65 V is attributed to the catalytic action of Ni(III)–oxyhydroxide. The anodic peak currents linearly increased on increasing the amino acid concentration up to 8 to 12 m*M*. Virtually, the same voltammetric profile of asparagine or threonine was observed for other investigated amino acids, including methionine, proline, leucine, alanine and tryptophan. As can be seen in Fig. 2B and C, the electrochemical behaviour of the sensing electrode can be considered as the sum of the gold and nickel activities toward the electrooxidation of electroactive compounds. Indeed, the bimetallic character of the modified electrode leads to a greater extension of the potential window in which there are significant oxidation currents in presence of threonine (see Fig. 2C) and other amino acids such as alanine,

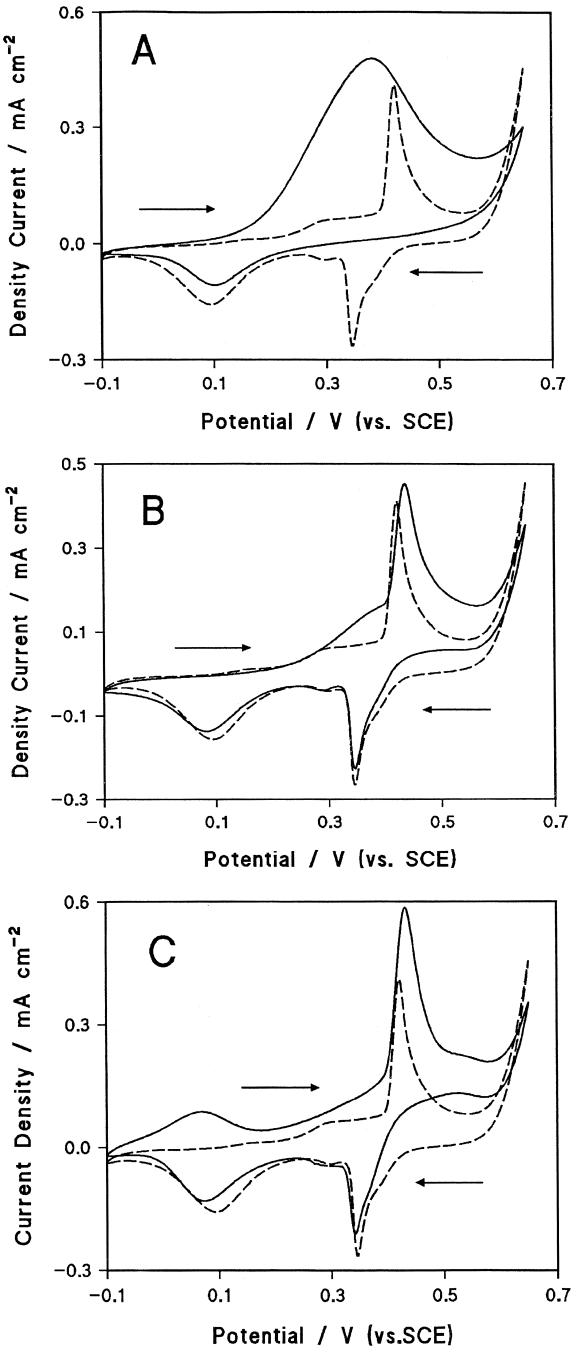

Fig. 2. Cyclic voltammograms (5th cycle) at a Au–Ni composite electrode (dashed curves) obtained in 0.1 *M* NaOH solution. (A): 6.0 m*M* cysteine; (B): 2.0 m*M* asparagine; (C): 4.0 m*M* threonine. Sweep rate 50 mV s^{-1}.

methionine, proline etc. Although no synergistic interactions of the gold substrate and nickel oxohydroxide species in alkaline medium were observed, the concomitant presence on the electrode surface of $Au(OH)_{ads}$ and NiOOH, leads to a significant extension of the potentials range where the electrode can be used as an amperometric sensor. In addition, the bimetallic behaviour of the Au–Ni electrode is in agreement with earlier findings [15], where the conducting properties of the nickel hydroxide layer does not produce any significant screening effects on the electrocatalytic activity of the gold substrate. Thus, the suitability of the Au–Ni composite electrode as an amperometric sensor in flow injection analysis and liquid chromatography using constant (DC) and pulse-applied potential (PAD) was successfully explored for the determination of sugar compounds [22].

3.2. Amperometric flow measurements

While the use of a constant applied potential at a conventional gold electrode results in rapidly decreasing sensitivity as products from the oxidation reaction coat and poison the electrode surface, a different behaviour was observed with the Au–Ni composite electrode. It was tested as amperometric sensor for the determination of amino acids in a flow-through system using 0.1 *M* NaOH as carrier electrolyte. Following an initial decrease of 3–5% in the first 15 injections of 0.1 m*M* glycine, the peak current decrease by only 2.5% over a subsequent 80 consecutive injections. Thus it was concluded that surface fouling of the Au–Ni composite electrode does not occur. In order to optimise the applied potential towards the electrooxidation of amino acids, the hydrodynamic voltammograms of glycine, asparagine and arginine were investigated at 50 mV increments between +0.35 V and +0.60 V vs. Ag/AgCl. Fig. 3 shows the resulting hydrodynamic voltammograms. In agreement with the electrocatalytic behaviour of the nickel-based catalyst in alkaline medium [14–20], in DC mode the maximum responses were observed between +0.50 V and +0.55 V where the presence of NiOOH on the electrode surface sustains the electrooxidation process. At potential values higher than +0.55 V, the hydrodynamic voltammograms exhibit a noticeable

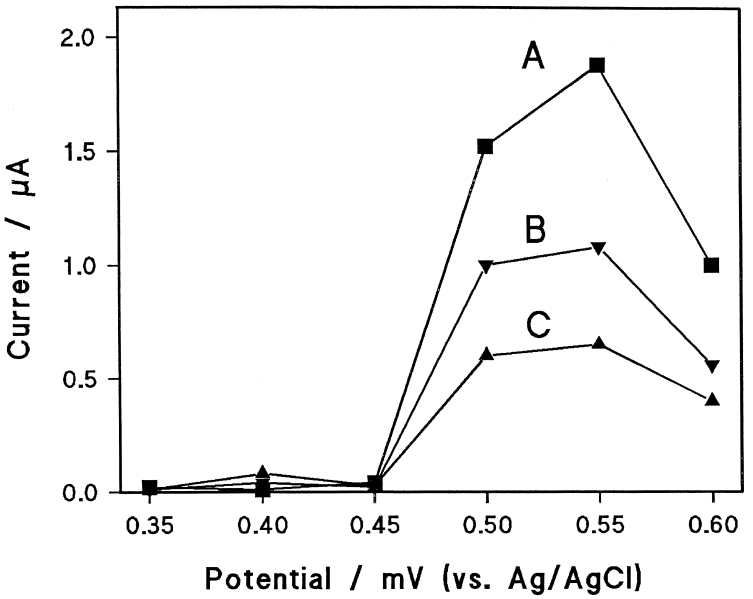

Fig. 3. Hydrodynamic voltammograms for (A) 25 μ*M* glycine; (B) 46 μ*M* asparagine; (C) 28 μ*M* arginine. Conditions: mobile phase, 0.1 *M* NaOH; flow-rate, 1.0 ml min^{-1}.

decrease for all the investigated analytes. All amino acids exhibited almost the same behaviour as a function of potential except that the amplitudes were different. This most likely occurs because of the effects of different molecular size or side chain substitution within the sample molecule. Therefore, an operating potential of +0.55 V vs. Ag/AgCl was chosen in the following flow-through measurements as it represents the best compromise between maximum current response and minimum background current (usually comprised between 0.3 and 0.8 μA).

The effect of flow-rate on detector response was tested by flow injection analysis using glycine and asparagine at the concentration of 0.1 m*M*. At an applied potential of +0.55 V vs. Ag/AgCl and in the range of flow-rate comprised between 0.2 and 4.0 ml/min, the amperometric signal was found to be inversely related to flow-rate. This behaviour suggests that, the catalytic process on the surface electrode is relatively slow to produce appreciable currents during fast passage of the sample plug. In these cases, the mass transfer of analytes has a negligible effect on the overall electrooxidation process.

The molar sensitivities of some amino acids derived from the calibration curves in flow-injection are compared in Table 1. As can be seen, the amperometric response is related to (i) the number of amino group per molecule (see valine and asparagine), (ii) the presence on the molecule of other electroactive groups such as –OH or –SH (see serine, threonine, tyrosine and cysteine), and (iii) the molecular size (see glycine, alanine, valine and leucine). Note that, a drastic lowering in sensitivity

Table 1
Sensitivity of various amino acids evaluated in flow injection using the Au–Ni composite electrode[a]

Compound	Sensitivity (μA mM^{-1})	Compound	Sensitivity (μA mM^{-1})
Alanine	2.7	Lysine	21.3
Arginine	24.5	Methionine	10.1
Asparagine	24.0	Phenylalanine	6.1
Cysteine	166.2	Proline	5.5
Glycine	77.5	Serine	56.7
Glutamine	9.5	Threonine	32.6
Histidine	20.4	Tryptophan	34.5
Isoleucine	8.7	Tyrosine	43.1
Leucine	6.5	Valine	6.3

[a] Experimental conditions: applied potential, +0.55 V vs. Ag/AgCl; flow-rate, 1.0 ml/min; carrier electrolyte, 0.1 *M* NaOH.

on increasing the molecular size was observed. On differently prepared nickel-based electrodes a similar behaviour was observed for the electrooxidation of aliphatic alcohols and amines in strongly alkaline solutions [18,23]. Thus, stereochemical effects on the kinetics of oxidation may be due to a need for the electroactive analytes to be correctly oriented on the catalytic sites of the Au–Ni composite electrode, and a strong adsorption/orientation process of the molecules onto the electrode surface occurs before the electrooxidation process.

3.3. Liquid chromatography

An alkaline medium is required to enhance the electroactivity of transition metals such as Cu, Co, Ni, Au, when used as amperometric sensors of electroactive organic compounds [4,5,9–11,14–23]. Therefore it was not unexpected that the electrochemical activity of Au–Ni composite electrodes for amino acids oxidation is pH-dependent, occurring with greater ease in solutions with higher hydroxide concentration. Under such experimental conditions, an anion-exchange column was employed for the separation of several common amino acids.

Fig. 4 displays the hydroxide concentration effect on the retention time of lysine (a), glutamine (b), valine (c) and alanine (d). As can be seen, each of these chromatographic peaks was shifted to shorter times at higher hydroxide concentration leading to a lower resolution. An eluent of 0.1 *M* NaOH was therefore chosen as being a good compromise between chromatographic resolution and electrode sensitivity. Fig. 5 shows a typical chromatogram of a standard mixture of some representative amino acids, obtained by using a CarboPac PA1 column and a flow-rate of 0.3 ml/min. The limit of detection (LOD), linear range, sensitivity, and repeatability are summarised in Table 2. The LODs range from 4 pmol for glycine and serine to 20 pmol for valine, alanine and methionine, while a linear response was observed over a range of approximately two or three orders of magnitude. The precision expressed as percent relative standard deviation (% RSD) of twelve repetitive chromatographic experiments (about 4 h of operation time), ranged from 3.5% of valine to 9.2% of glycine. The analytical perform-

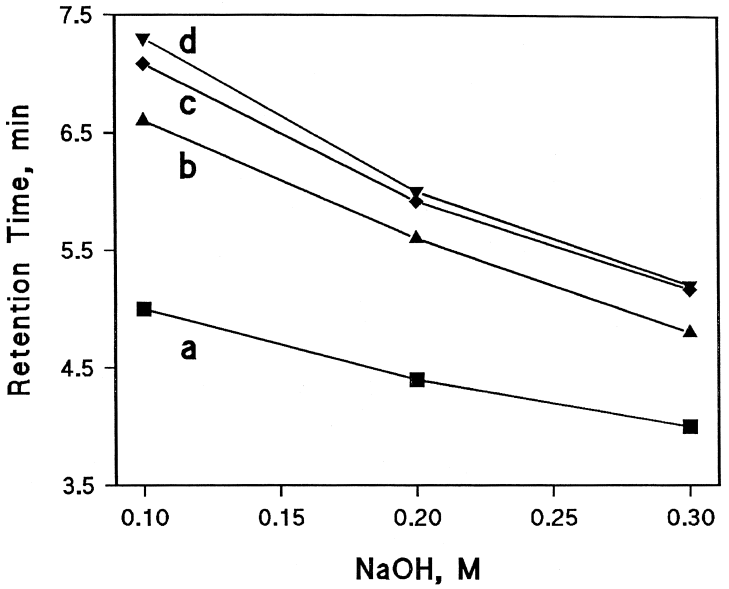

Fig. 4. Effect of hydroxide concentration on the retention times of (a) lysine; (b) glutamine; (c) valine; (d) alanine. Column: Carbopac PA1; flow-rate, 0.8 ml min^{-1}; applied potential, +0.55 V vs. Ag/AgCl.

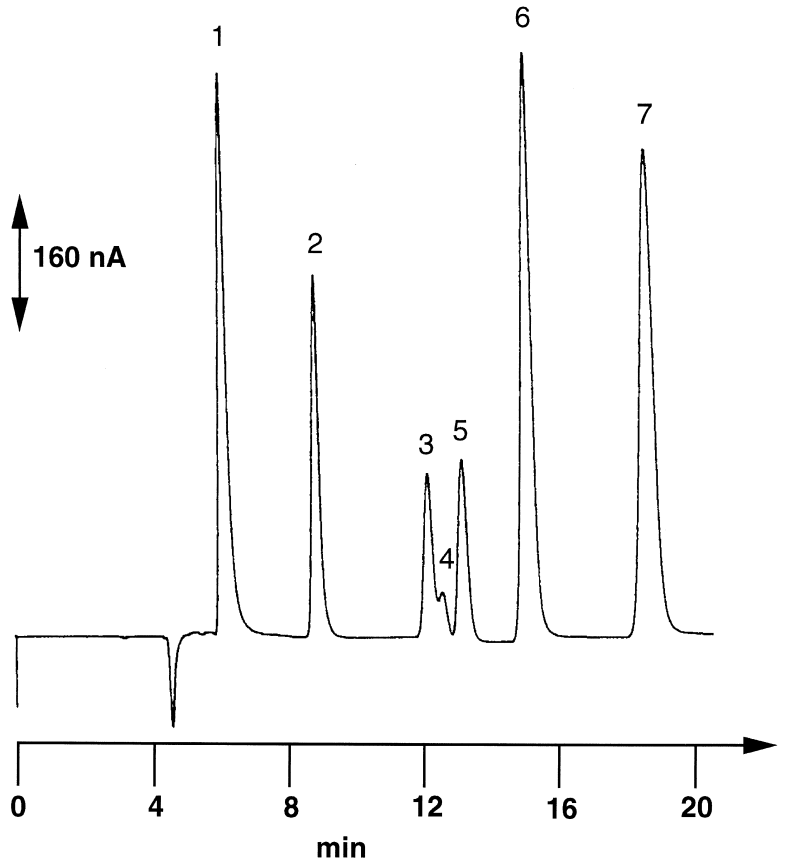

Fig. 5. Anion-exchange chromatographic separation with electrochemical detection at a Au–Ni electrode of a standard mixture containing (1) 0.12 m*M* histamine (used as internal standard), (2) 0.15 m*M* lysine, (3) 0.18 m*M* glutamine, (4) 0.24 m*M* valine, (5) 0.15 m*M* alanine, (6) 0.071 m*M* glycine, (7) 0.10 m*M* serine. Experimental conditions: column, carbopac PA1 (250×4 mm I.D) coupled with a CarboPac PA1 (50×4 mm I.D.) guard column; isocratic elution with 0.1 *M* NaOH as mobile phase; flow-rate, 0.3 ml min^{-1}; applied potential, +0.55 V vs. Ag/AgCl.

ances, expressed in terms of LOD and linear range, are generally better or comparable with those obtained by other amperometric sensors based on single metal- or biosensor systems [7,10,24–26].

Similarly with other nickel-based chemically modified electrodes used as amperometric sensors in alkaline medium [14–17,22,23], the Au–Ni sensor used here shows an acceptable temporal stability; a freshly prepared nickel deposit after more than 24 h of continuous use exhibited a signal decrease of about 20%. Under such circumstances it is possible to correct the electrode sensitivity by using a proper internal standard.

3.4. Selected analytical applications

The importance of peptides and free or bonded amino acids in cheese taste is widely recognised [27]. Such compounds impart a pleasant or unpleasant flavour depending upon the predominance of one or more of them. In order to ascertain the potential analytical performances of the Au–Ni composite electrode as an amperometric sensor in LC, the determination of free amino acids present in two different cheeses, such as ‘‘Parmiggiano Reggiano’’ and ‘‘Emmenthal’’, was performed. For the amino acids extraction, a suspension of finely ground

Table 2
Quantitative determination of amino acids in anion-exchange chromatography using the Au–Ni composite electrode as an amperometric sensor[a]

Amino Acid	LOD[b] (pmol)	Linear range (μM)	Sensitivity[c] ($\mu A\ mM^{-1}$)	Repeatability[d] (%)
Alanine	20	1.5–500	1.3	8
Arginine	9	0.2–600	8.0	6
Asparagine	25	3.1–800	3.2	5
Glutamine	9	1.5–700	1.1	7
Glycine	4	0.8–800	9.6	9
Lysine	25	6.2–500	2.6	9
Methionine	22	3.0–800	0.8	6
Serine	4	1.5–600	5.1	6
Threonine	12	3.0–800	2.0	5
Valine	30	6.0–800	0.2	4

[a] CarboPac PA1 column; flow-rate, 0.3 ml/min; isocratic elution with 0.1 *M* NaOH; sample loop, 50 μl; applied potential, +0.55 V vs. Ag/AgCl. Thin-layer cell with an Au–Ni electrode.
[b] LOD determined for $S/N=3$ from the lowest injected concentration.
[c] From least-squares analysis of eighteen data points spaced over the linear range. Correlation coefficients were always greater than 0.998.
[d] The repeatability was expressed as percent relative standard deviation of twelve repetitive chromatographic injections at the 0.1 m*M* concentration of each analyte.

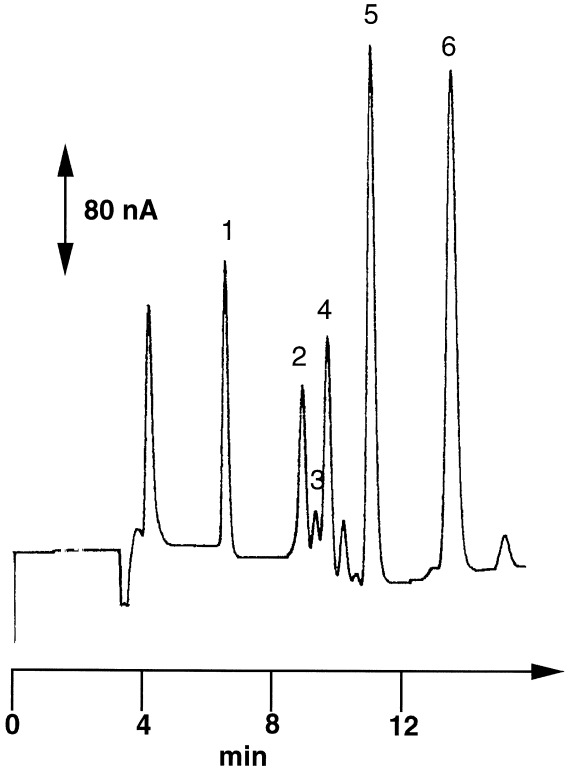

Fig. 6. Chromatographic separation of a sample of ''Parmiggiano Reggiano'' cheese: 10 g of finely ground cheese were sonicated in 100 ml of water, the suspension was filtered and the resulting solution was diluted 1:100. (1) lysine, (2) glutamine, (3) valine, (4) alanine, (5) glycine, (6) serine. Flow rate, 0.4 ml min^{-1}, other experimental conditions as in Fig. 5.

cheese, 10 g in 100 ml of boiled water was sonicated for 10 min. Then, the suspension was filtered by passage through a filter circle and the relevant solution was diluted 1:100 with pure water. The chromatograms are shown in Figs. 6 and 7, Parmiggiano Reggiano and Emmenthal samples, respectively. Peak identification was based on the retention time of amino acids and was confirmed by spiking authentic standard solutions to the sample extract. The relevant quantitative results are listed in Table 3. The precision, estimated as % RSD by three repetitive chromatographic injections of the sample extract was comprised between 5% and 9% for valine and alanine, respectively. Good reproducibility was obtained for separations on repetitive injections, and no apparent deterioration of the catalytic activity was observed during consecutive analysis. The good correlation coefficients of the regression lines (>0.999) for the calibration curves confirm the apparent absence of any fouling effect on the catalytic performance of the modified electrode.The mean recoveries obtained by triplicate experiments of each spiked sample ranged from 92% to 97%.

4. Conclusion

A chemically modified electrode composed of nickel-oxyhydroxide on a gold electrode substrate

Table 3
Quantitative results of the free amino acids content in finely ground cheeses

Sample cheese	Amino acid (μmol g^{-1})[a]					
	Alanine	Glutamine	Glycine	Lysine	Serine	Valine
Parmiggiano Reggiano	45	17	33	130	56	77
Emmenthal	–	9	10	29	11	–

[a] The concentrations were evaluated by the standard addition method (four additions).

coupled with an anion-exchange column has been used for the quantitative analysis of several common amino acids. Electrochemical detection in constant applied potential allows for their direct and sensitive determination following anion-exchange chromatography using an alkaline eluent. The present method appears to be very useful for the analytical determination of several common amino acids in real matrices without using any derivatisation procedure. Work is in progress to optimise separation efficiency of amino acid compounds and to extend the analytical applications of the Au–Ni amperometric system to other real samples.

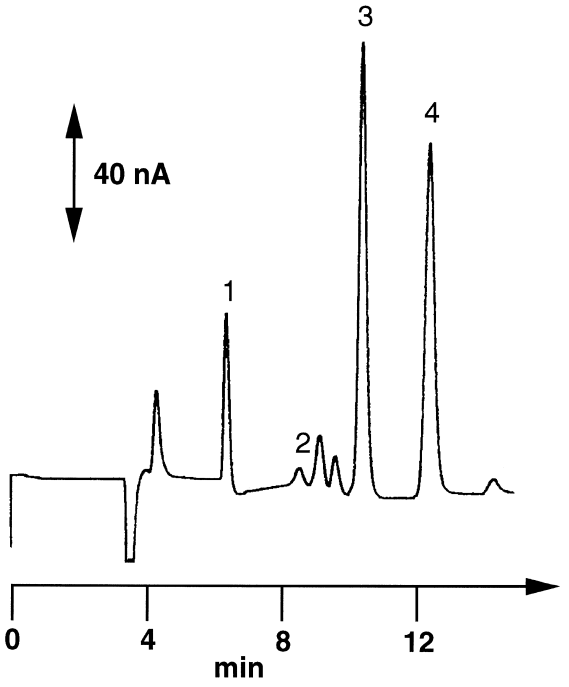

Fig. 7. Chromatographic separation of a sample of ''Emmenthal'' Swiss cheese: 10 g of finely ground cheese were sonicated in 100 ml of water, the suspension was filtered and the resulting solution was diluted 1:100. (1) lysine, (2) glutamine, (3) glycine, (4) serine. Experimental conditions as in Fig. 6.

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