Screen-printed amperometric biosensors for the rapid measurement of L- and D-amino acids

The Analyst

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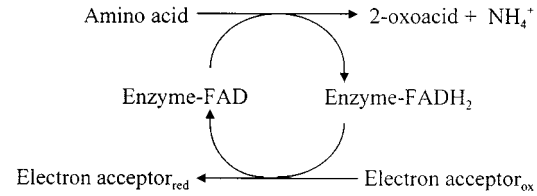
Screen-printed three-electrode amperometric sensors incorporating L- and/or D-amino acid oxidase for the general purpose measurement of L- or D-amino acids is described. The working electrode incorporates rhodinised carbon, to facilitate hydrogen peroxide oxidation at a decreased operating potential, and immobilised enzyme. The devices responded to all 20 common L-amino acids and all of the D-amino acids examined, the exceptions being L- and D-proline. Linear response profiles were observed for L-leucine, L-glycine and L-phenylalanine with limits of detection of 0.47, 0.15 and 0.20 mM respectively. The devices were reproducible and exhibited stability over a 56 d test period. The biosensor compares favourably with a standard photometric amino acid test and was used to monitor milk ageing effects. The assay is cheap, simple to perform and rapid, requiring only buffer–electrolyte and a small sample volume.

Introduction

L- and D-amino acids are common constituents of many fermented products such as beers, cheese and yoghurt. D-Amino acid levels are a useful indicator of microbial contamination of milk[1] and of cheese age.[2] Amino acid levels can also be used to monitor the state of fermentation processes[3] and amino acids are a major nutrient source for yeast cell growth.[4] Various medical conditions are also associated with elevated amino acid levels[5] and defects in amino acid metabolism.[6] Simple, rapid and reliable assay methods for the monitoring of amino acids would therefore benefit a wide range of industries, notably in the food, biotechnology and medical sectors.

Traditional analytical techniques for amino acid measurement are complex laboratory procedures requiring a number of assay steps, additional chemicals and/or complex instrumentation. Individual or bulk amino acid levels are commonly measured through direct reaction with colorimetric agents such as ninhydrin[7,8] or *via* redox dyes.[9,10] Mixtures of amino acids can be resolved by liquid chromatography if individual amino acid quantification is required. Pre- or post-column analyte derivatisation is usual since most amino acids are not amenable to direct optical or electrochemical detection. Exceptions include the electrochemically oxidisable sulfur-containing amino acids[11] and tryptophan which is photometrically active.

Biosensors, incorporating amino acid-specific enzymes coupled to electrochemical transducers, have also been developed for the direct, rapid measurement of amino acids in solution. Such devices are cheap and simple to fabricate, can be highly specific and sensitive and, by virtue of their speed of measurement and simplicity, have potential niche roles as monitoring tools in the nutritional and medical fields. Typically, amino acid analytes are enzymatically oxidised using a specific or broad-spectrum amino acid oxidase (AAO) according to the reaction scheme outlined below.



The re-oxidation of reduced electron acceptor may be measured amperometrically at a working electrode.[7,12–14] A number of sensors operate by measuring peroxide activity. In these cases, dioxygen present in the liquid sample acts as the electron acceptor with the concomitant production of peroxide.[3,15–17] The use of metallised carbons to allow the electrochemical oxidation of peroxide detection at reduced operating potentials has been widespread in such systems.[18–21] Earlier approaches focused on the direct potentiometric measurement of the enzymatically generated ammonium ions rather than monitoring by-product oxidation.[22,23] Amino acid biosensors have been reviewed recently.[24]

Stability has been an issue with many electrochemical amino acid sensors, with significant response decreases being observed within 1 month of preparation.[7,14,16,17] The use of stable enzyme electrodes to measure individual amino acid species in solution is attractive for reasons of simplicity, rapidity, economy, portability and a minimum sample size requirement. The aim of this work was to develop more stable, cheap, mass-producible screen-printed electrochemical sensors for the rapid and reproducible measurement of single D- and L-amino acid species in sample solutions. Electrocatalytic rhodinised carbon was used to allow the non-mediated detection of hydrogen peroxide at low potentials. The performance of the device was compared with that of a standard photometric procedure and its application to monitoring milk ageing effects is described.

Experimental

Reagents

A buffer of 0.1 M KH_2PO_4–K_2HPO_4 (pH 7.8) was used. Buffer–electrolyte solutions contained 0.1 M KCl. L-Amino acid oxidase (L-AAO, EC 1.4.3.2, from *Crotalus adamateus*, specific activity 0.31 U mg^{-1}), D-amino acid oxidase (D-AAO, EC 1.4.3.3, from porcine kidney, specific activity 0.8 U mg^{-1}) and all L- and D-amino acids were obtained from Sigma–Aldrich (Gillingham, Dorset, UK) and were prepared in buffer–electrolyte as required. Polyethylenimine (PEI) and glutar-

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aldehyde were also obtained from Sigma–Aldrich. Hydroxyethyl cellulose (HEC) was purchased from Fluka (Buchs, Switzerland). Solutions were prepared using reverse osmosis water.

Sensor fabrication

Three-electrode devices were mass manufactured in-house by a multi-stage screen-printing process using a DEK 248 machine (DEK, Weymouth, UK) and screens with appropriate stencil designs (60 per screen) fabricated by DEK Precision Screen Division. The stainless steel screen mesh was mounted at 45° to the print stroke with 77 wires cm^{-1} and emulsion thicknesses of 13 and 18 μm for the solvent and water-resistant screens, respectively.

Devices were printed on to 250 μm thick polyester sheet (Cadillac Plastic, Swindon, UK). The circular electrocatalytic working electrode (planar area 0.16 cm^2) was fabricated from MCA 4a, a commercially available carbon powder containing 5% rhodium plus promoters (MCA Services, Cambridge, UK), made into a screen-printable paste by mixing 1:4 in 2% m/v HEC in buffer–electrolyte. PEI-modified electrodes were prepared with the same HEC mixture, but also containing 1% m/v PEI. The reference electrode ink contained 15% silver chloride in silver paste (MCA). The counter electrode and basal tracks were fabricated from I45R carbon ink (MCA). The basal tracks were insulated from the measurement solution using 242-SB epoxy-based protective coating ink (Agmet ESL, Reading, UK). The electrodes were then heat treated at 125 ºC for 2 h to cure the epoxy resin and to stabilise the electrocatalytic pad to allow prolonged use of the device in aqueous solutions.[25]

The electrocatalyst–HEC complex forms a thick, porous pad that is a suitable base for immobilisation of biocomponents.[26,27] Aliquots (10 μl) containing various amounts of L- and/or D-AAO in 2% m/v glutaraldehyde in buffer were pipetted on to working electrodes, dried for 12–16 h at 4 ºC, washed in buffer and stored at 4 ºC in air until required.

Test procedure

The test procedure was controlled using an Autolab Electrochemical Analyser with GPES3 software (Ecochemie, Utrecht, The Netherlands). A 1.1 cm diameter Whatman 114 filter disc (Whatman, Maidstone, UK) was placed over the three-electrode assembly which, when wetted with sample, completed the electrochemical circuit. A 40 μl sample was deposited on the filter-paper and the working electrode poised at a potential of +400 mV *versus* an Ag/AgCl reference electrode. The system was allowed to equilibrate in the presence of sample, according to the method of Kröger *et al.*,[26] and the current value was noted at 4 min. Tests were performed at ambient temperature on PEI electrodes unless stated otherwise. Amino acid tests solutions were prepared in buffer–electrolyte. Milk samples were diluted five-fold with buffer–electrolyte before testing.

Ninhydrin test method

The test reagent was 150 ml of glycerol, 0.625 g of ninhydrin, 18.387 g of citric acid and 133.3 μl of 150 mM $MnSO_4$, made up to 250 ml in water. The test reagent (2 ml) was added to 0.1 ml of sample diluted 10-fold in water. The mixture was heated at 100 ºC for 12 min., diluted two-fold in water and the absorbance was recorded at 570 nm. The method works for all amino acids except proline.

Tests on real samples

Milk, fruit juice and urine samples were diluted five-fold in buffer–electrolyte solution and measured according to the test procedure. Full-fat milk samples (~4% fat) were aged by storage at room temperature and sampled at 12 h intervals for 48 h.

Results and discussion

Optimisation of enzyme loading activity

Initial tests were focused on determining the minimum enzyme activity required to generate a maximum current response from the system. L-Leucine and D-alanine were chosen as substrates since *Crotalus adamateus* L-AAO and porcine kidney D-AAO exhibit high k_m values for these substances.[16,28] In order to ensure that the amino acid concentration was not a limiting factor in the enzyme-electrode response, the concentration at which each of these substrates saturated the corresponding electrode-immobilised AAO enzyme was determined by measuring the substrate concentration at which no current increase was observed. These tests were performed under excess enzyme activity conditions (100 mU of each enzyme per electrode). Complete saturation of electrode-immobilised L-AAO and D-AAO was achieved with 8 mM L-leucine and 12 mM D-alanine, respectively.

A series of tests were then performed under saturating L-leucine and D-alanine concentrations in which the loaded enzyme activity was progressively decreased and the current response of the system monitored. A decrease in signal output was observed at L-AAO activities of < 25 mU per electrode and D-AAO activities of < 20 mU per electrode. Accordingly, in subsequent experiments, the L- and D-AAO enzyme activities were fixed at 5 mU above these levels, namely 30 mU (96.8 μg) L-AAO and 25 mU (31.0 μg) D-AAO per electrode.

AAO electrode performance

Since the k_m values of the L- and D-AAO enzymes vary according to amino acid type, each substrate will yield a different calibration profile. An apparently linear relationship between current response and amino acid concentration was observed for L-leucine, L-glycine and L-phenylalanine across the concentration range 0–1 mM, as shown in Fig. 1. The correlation coefficients for these three amino acids were 1.000,

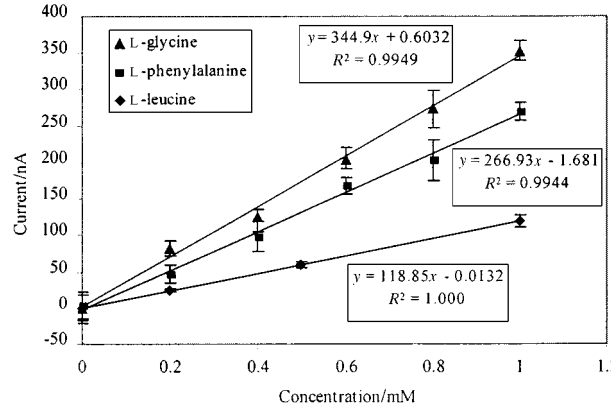

Fig. 1 Calibration curves for L-leucine, L-glycine and L-phenylalanine from 0 to 1 mM in 0.1 M phosphate buffer (pH 7.8) with a rhodinised carbon–HEC–PEI electrode containing immobilised L-AAO. Error bars = *s*, $n = 3$.

0.9949 and 0.9944, respectively. The equations for lines of best fit shown in Fig. 1 were used to calculate device limits of detection (LOD) for the three amino acids. Using the criterion of LOD = 2.5 × blank SD, values of 0.47 mM for L-leucine, 0.15 mM for L-glycine and 0.20 mM for L-phenylalanine were recorded.

The response of the device to 0.1 M concentrations of the 20 common L-amino acids and six important D-amino acids is shown in Table 1. The sensor responded to all of the amino acids tested except L- and D-proline, the observed differences in current being a function of the amino acid side-chain. Current densities of the order of microamps per square centimetre of the screen-printed working electrode were recorded, with repeatability, expressed as relative standard deviation (RSD), varying from 0.4 to10.6%. The response of the device to the L- and D-amino acids tested was standardised against L- and D-phenylalanine respectively, the comparative data being shown in column 5 of Table 1.

Kacaniklic *et al.*[29] reported a flow injection amperometric biosensor for amino acid detection, incorporating PEI-immobilised AAO dispersed throughout a graphite mixture. Co-immobilised peroxidase enzyme allowed the reduction current due to hydrogen peroxide product to be measured at −50 mV *vs*. an Ag/AgCl reference electrode. The results, relative to L-phenylalanine, are also given in Table 1. The two types of system yield different relative responses for the 20 test L-amino acids. For example, L-tryptophan, L-glycine and L-alanine yield responses greater than L-phenylalanine using the screen-printed sensor, whereas L-methionine and L-leucine yielded responses greater than L-phenylalanine for the sensor of Kacaniklic *et al.*[29] Generalisations regarding the responses of both sensors to the range of amino acids tested cannot be made since assay performance is a function of many factors, including enzyme–substrate affinity, the enzyme immobilisation protocol, diffusion effects and the effects of the electrode matrix on substrate diffusion and enzyme behaviour.

Enzyme electrode stereospecificity

Co-immobilisation of L- and D-AAO on the electrode is advantageous in that the device could serve as a generic tool for the quantification of single L- or D-amino acid species in solution. This is credible provided that the L- and D-forms of the enzyme do not exhibit significant reactivity towards the opposite stereoisomeric amino acid form. Evidence for stereospecificity was examined electrochemically using L-AAO, D-AAO and L- + D-AAO electrodes. The results are given in Table 2.

The device exhibited minimum non-stereospecificity for the range of amino acids tested except D-arginine, where 30% of the D-AAO electrode response was observed using the L-AAO electrode. However, this finding should be viewed in the context of the low D-AAO electrode response to D-arginine as shown in Table 1. These initial findings suggest that the use of a co-immobilised L- + D-AAO electrode as a flexible, generic tool for the rapid measurement of individual D- and L-amino acids is possible. However, further work is required to ensure that enzymatic stereospecificity exists for all of the common L- and D-amino acids.

Polyethylenimine and enzyme electrode stability

It has been shown that the presence of polyethylenimine (PEI) in working electrode pastes can significantly improve the response of alcohol sensors to ethanol, in some cases by more than 50-fold.[30,31] Similar work has led to enhanced responses of L-AAO -electrodes to 1 mM L-phenylalanine.[29] These findings were attributed to improved enzyme immobilisation characteristics. The effect of PEI on screen-printed L-AAO electrodes was assessed during the course of this study.

The presence of PEI in the rhodinised carbon–HEC working electrode was found to have no significant effect on device

Table 1 L- and D-AAO electrode response to the 20 common L-amino acids and six important D-amino acids, respectively. Volumes of 40 μl of 0.1 M amino acid in buffer–electrolyte were applied to the sensor. Device responses were recorded after 4 min. Data are also expressed relative to L- or D-phenylalanine and compared with the results of Kakaniklic *et al.*[29]

Amino acid	Mean response/ nA	RSD (%) (n = 3)	Current density/ μA cm^{-2}	Response relative to L- or D-phenylalanine	
				This work	Kacaniklic *et al.*
L-Alanine	290	9.1	1.81	1.07	0.20
L-Arginine	226	8.8	1.41	0.83	0.28
L-Asparagine	178	0.4	1.11	0.65	0.22
L-Aspartic acid	112	14.4	0.70	0.41	0.14
L-Cysteine	250	5.6	1.56	0.92	*Ox*[a]
L-Glutamic acid	206	6.8	1.29	0.76	0.29
L-Glutamine	43	4.9	0.27	0.16	0.28
L-Glycine	356	8.0	2.23	1.31	0.20
L-Histidine	56	3.3	0.35	0.21	0.38
L-Isoleucine	205	4.6	1.28	0.75	0.46
L-Leucine	119	8.2	0.74	0.43	1.13
L-Lysine	202	1.5	1.26	0.74	0.34
L-Methionine	254	5.6	1.59	0.94	1.33
L-Phenylalanine	272	1.1	1.70	1.00	1.00
L-Proline	0	—	0	0.00	0.33
L-Serine	92	1.3	0.58	0.34	0.36
L-Threonine	167	7.8	1.04	0.61	0.27
L-Tryptophan	592	5.6	3.70	2.18	0.65
L-Tyrosine	226	1.7	1.41	0.83	0.92
L-Valine	127	1.6	0.79	0.46	0.35
D-Alanine	282	9.9	1.76	1.58	—
D-Arginine	98	10.6	0.61	0.55	—
D-Methionine	162	4.7	1.01	0.91	—
D-Phenylalanine	179	5.6	1.19	1.00	—
D-Proline	0	—	0	0.00	—
D-Valine	298	8.8	1.86	1.66	—

[a] Directly oxidised.

response to 0.1 M L-phenylalanine. Similar mean response values of ~300 nA were found for both PEI- and non-PEI-modified working electrodes. Interestingly however, the PEI-modified electrodes were found to be more stable over time than the unmodified electrodes, losing ~40% of the original current response compared with ~70% for the unmodified electrodes after 56 d of storage in air at 4 °C. The modified electrodes also yielded more reproducible data. Fig. 2 compares the stability and reproducibility characteristics of the two types of working electrode.

The stability of the PEI-modified electrodes is significantly better than most of the electrochemical amino acid sensors reported in the literature. Rivas and Maestroni,[7] using an iridium-dispersed carbon paste electrode, found a 40% decrease in sensor response over an 8 d period (electrode stored in air at 4 °C), whilst Chi and Dong[14] quoted a 'stable' response over 2 weeks for a D-AAO sensor prepared by electrochemical co-deposition of the enzyme into a Prussian blue film (electrode stored in buffer, pH 5.5, at 4 °C). A 50% decrease in electrode response was found by Albery *et al.*[16] using an L-AAO enzyme immobilised on an oxygen electrode (electrode stored in buffer at 4 °C). Cooper and Schubert[15] reported a 36% decrease in sensor activity over 31 days using polytyramine-immobilised L-AAO (no storage details). Many publications do not quote sensor stability characteristics.

Sensor performance *versus* standard assay method

The performance of the L-AAO electrode assay was compared with that of the ninhydrin test, a standard photometric assay for the determination of free amino acids in solution.[32] An L-leucine stock standard solution was diluted and assayed in triplicate across the concentration range 0–2.5 mM by this method. An apparently linear relationship between L-leucine concentration and absorbance was recorded (correlation coefficient 0.9927) with a slope of 0.235 mM L-leucine (absorbance)$^{-1}$. A comparison between the electrochemical and photometric methods is shown in Fig. 3. The correlation coefficient of 0.9895 indicates a close agreement between the methods and a linear slope of 516 nA (absorbance)$^{-1}$ was recorded for L-leucine. The reproducibilities of the two methods are similar, as indicated by the size of standard error bars in Fig. 3.

The electrochemical method, whilst having similar analytical performance to the photometric assay, exhibits certain operational advantages. It is simple to perform, requiring only the addition of measurement solution, whereas the photometric procedure requires a complex chemical mixture and a heating stage. Furthermore, the electrochemical sensor approach is cheap, portable and, with a total assay time of 4 min, is more rapid than the photometric method (>12 min). However, the electrochemical method is only able to quantify single amino acid species in solution.

Table 2 Background-corrected current responses of L-AAO, D-AAO and L- + D-AAO electrodes to 1 mM L- and D-amino acids in buffer–electrolyte

	Current response/nA		
Amino acid	L-AAO electrode	D-AAO electrode	L + D-AAO electrode
L-Cysteine	240	15	250
L-Valine	124	12	127
D-Valine	39	285	304
D-Arginine	23	77	98
D-Phenylalanine	13	159	179
L-Phenylalanine	273	27	256

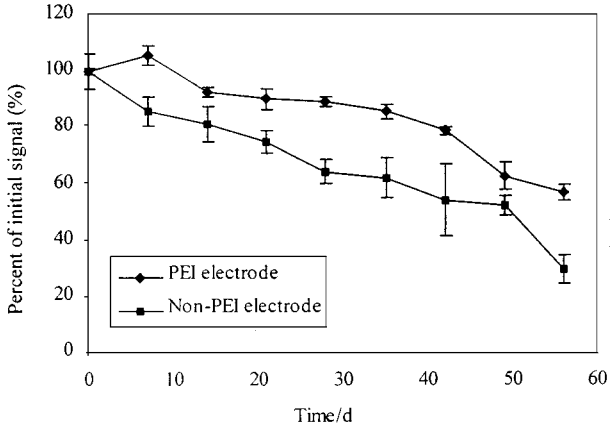

Fig. 2 Relative responses of rhodinised carbon–HEC L-AAO electrodes with and without PEI, tested over a 56 d period. Data are reported as the mean percentage response of the device *versus* the device response at time zero, 0.1 M L-phenylalanine being used as the test analyte. Error bars = *s*, *n* = 3.

Amino acid measurement in real samples

Gandolfi *et al.*[1] reported that certain D-amino acids, notably D-alanine, can act as indicators of microbial milk contamination with D/D + L ratios increasing from 3–4% to >50% after milk storage at 4 ºC for 1 month. The performance of the screen-printed L- + D-AAO electrode as a means of monitoring milk contamination was assessed during this study and compared with ninhydrin tests performed in parallel. Increases in signal responses were observed for both methods when testing room temperature-aged milk samples, as shown in Fig. 4. Fig. 5 shows the relationship between the two assay methods. Unlike the tests performed on spiked buffer solutions, a non-linear correlation was observed. It appears that the enzyme electrode approach is comparable to the photometric method as an indicator of milk contamination, whilst being appreciably simpler and more rapid to perform.

L- + D-Amino acid sensors have potential niche applications in the food quality and medical fields. As an illustration, samples of fresh milk, fruit juice and urine were tested using the enzyme electrode and ninhydrin assay methods and the results compared with L-leucine calibration curves established for each method. The results in Table 3 show similar trends between the two methods, illustrating that the enzyme electrode has some potential as a rapid screening tool for examining samples

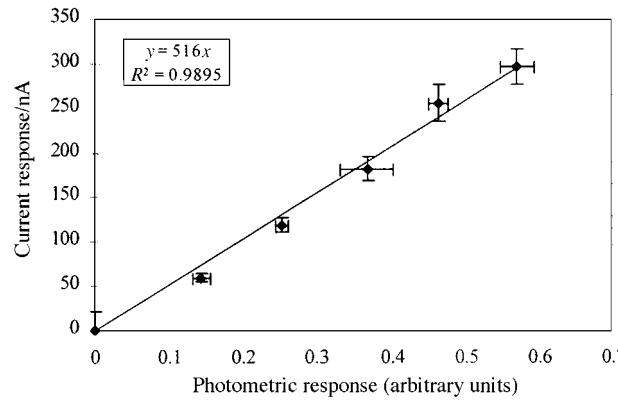

Fig. 3 Correlation between L-AAO electrode and standard photometric assay response for the measurement of 0–2.5 mM L-leucine samples. The background current of 745 nA was subtracted from the electrochemical response values.

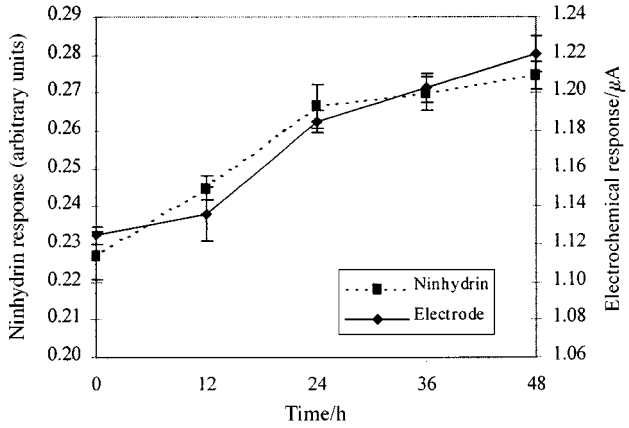

Fig. 4 Response of L- + D-AAO electrodes and ninhydrin assay method to aged milk samples. The milk was left to age at room temperature over a 48 h period. Error bars = *s*, *n* = 3.

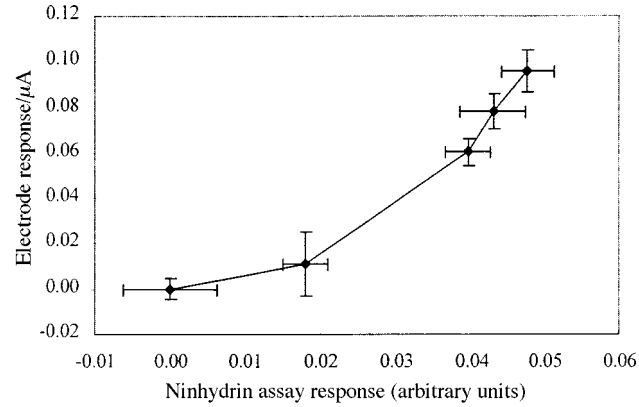

Fig. 5 Correlation between L- + D-AAO electrode and ninhydrin assay response data during milk ageing studies. Error bars = *s*, *n* = 3.

Table 3 Comparison of results with real samples using the L- + D-AAO electrode and measurements by standard assay for free amino acid, with RSD values in parentheses ($n = 3$).

Sample	LD electrode response/nA[a]	Ninhydrin response (absorbance)[b]	Concentration relative to L-leucine/nM	
			Sensor	Ninhydrin
Milk	332 (10.9)	0.377 (9.5)	13.45	16.1
Fruit juice	65 (12.0)	0.044 (13.2)	2.63	1.87
Urine	196 (9.2)	0.112 (9.8)	7.94	4.76

[a] 1:5 diluted sample. [b] 1 : 10 diluted sample

containing amino acid mixtures. Samples exceeding a predetermined threshold level could then be analysed by a more specific instrumental method. The presence of electroactive compounds in these real samples (such as uric acid in urine) was accounted for by subtraction of enzyme-free electrode responses from equivalent L- + D-AAO electrode responses. Since the screen-printed electrodes are mass producible at low cost, the devices can be used once only and then disposed of, thus minimising the problems associated with biosensor re-use and electrode fouling effects.

Conclusion

This work has demonstrated the use of cheap, mass-producible screen-printed electrodes incorporating L- and/or D-amino acid oxidase immobilised on rhodinised carbon working electrodes as a viable means of measuring L- and D-amino acids. The L-AAO electrode responded to all of the 20 common L-amino acids tested, the exception being L-proline, with limits of detection down to 0.15 mM. The D-AAO electrode responded to five of the six D-amino acids tested, the exception again being proline. The non-additive nature of the individual amino acid responses limits usage of the device to sample solutions containing single amino acid species or as a basic screening tool for amino acid mixtures. The co-immobilisation of both stereospecific forms of the enzyme leads to a more generic D/L-amino acid probe for use in a number of situations, requiring only 96.8 and 31.0 μg of L- and D-AAO respectively.

The addition of PEI to the working electrode paste resulted in improved device stability and reproducibility, although no increases in device response were noted. The PEI-modified sensor retained 60% activity over a 56 d test period, which compared favourably with AAO sensors reported in the literature. The protocol was simple and rapid, requiring only a sample dilution step in electrolyte–buffer and a 4 min test step. The bi-enzyme device was used successfully to monitor changes in the amino acid content of aged milk samples and yielded similar response values to a standard ninhydrin photometric test when measuring amino acid levels in milk, fruit juice and urine, relative to the L-leucine response. Overall, the electrochemical method compared favourably with the standard photometric method for amino acid determination, as shown in Table 4.

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Table 4 Comparison of the standard ninhydrin method and amperometric bi-enzyme sensor for measurement of amino acids

Factor	Electrochemical method	Photometric method
Assay complexity	Sample diluted in electrolyte–buffer and applied to electrode	Numerous sample + reagent handling steps, heat (see text)
Linear range	0.47–2.5 mM (L-leucine) 0.20–2.0 mM (L-glycine)	< 0.2 –2.5 mM
Sample volume	10 μl	100 μl usual
Time	< 5 min	< 15 min
Costs	Low labour, disposables and reagent costs	Higher labour costs, several reagents required
Limitations	Single amino acid measurement, 40% activity loss after 56 d	Complex laboratory procedure Non-portable
Benefits	Cheap, rapid, portable, simple	Total amino acid measurement Stable

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