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Analysis of amino acids by miniaturised isotachophoresis

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Abstract

A method allowing the miniaturised isotachophoretic analysis of amino acids has been developed. To overcome the problems of carbonate contamination which occur when performing separations at alkaline pH levels glycolate was used as the leading ion. Addition of magnesium to the leading electrolyte as a counter species was found to improve the separations. The method has been used on a poly(methyl methacrylate) microdevice with integrated on-column conductivity detectors. The behaviour of a range of common amino acids was investigated and successful separations of up to seven amino acids were made. Good linearity was observed with calibration curves for aspartic acid and phenylalanine over the range 0.063–1.0 mM. Limits of detection for these two species were calculated to be 0.060 and 0.018 mM, respectively.

Keywords: Isotachophoresis; Chip technology; Instrumentation; Miniaturisation; Amino acids

1. Introduction

The analysis of amino acids is important for many areas of chemistry and biochemistry. Traditionally such analyses have been commonly performed using chromatographic methods. However, in recent years, electrophoretic methods, particularly capillary zone electrophoresis (CZE), have found widespread application to this purpose. Such is the breadth of methodologies used, and sample matrices analysed, using CZE that they have formed the basis of a number of recent review papers [1,2].

Isotachophoresis (ITP), another form of electroseparation technique, with advantageous features such as control of separation parameters via electrolyte composition and high sample load capacity, is eminently suited to the analysis of small ions. Consequently the technique has been applied to the analysis of amino acids. A number of strategies have been employed for achieving separations of such species using capillary scale ITP. In most cases analysis has been performed on neutral and acidic amino acids as anions. A wide range of amino acids were investigated with a variety of aqueous electrolyte systems with alkaline pH levels by Everaerts et al. [3] and Hirokawa et al. [4]. The use of non-aqueous electrolytes and/or complexation reactions have also been investigated as possible means of enhancing the separability of amino acids. Non−aqueous solvents used have included formaldehyde [5] and propionaldehyde [3]. Complexing species used have included copper(II) [3,6,7], 2,4-dinitrophenol [8] and citraconic anhydride [9]. Analysis of the cationic form of amino acids is more difficult to achieve due to many of the species having, very similar, low dissociation constants. Thus such separations are only really suitable for basic amino acids such as lysine, arginine and histidine [3,10].

Miniaturised chemical analysis systems offer the prospect of a number of advantages over conventional scale equipment, such as improved analytical performance and reduced instrumentation costs. However, despite these advantages and the fact that ITP can be readily performed in a miniaturised format, there has been only a very limited application of such systems to the analysis of amino acids. The only such use known involved the chiral separation of tryptophan enantiomers using a poly(methyl methacrylate) (PMMA) chip with conductivity detection by Ölvecká et al. [11]. A similar device has also been used for coupled ITP–CZE separations of selenoamino acids [12]. A number of articles have used miniaturised technology for CZE separations of amino acids. Model mixtures have been analysed on glass chips, with amperometric detection [13,14] and laser-induced fluorescence (LIF) detection [15], and PMMA devices with contactless conductivity detection [16]. Tea and urine sam-

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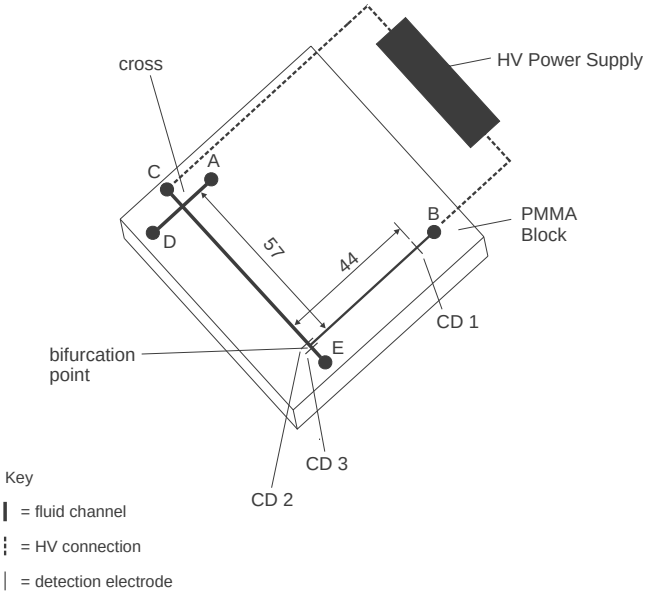

Fig. 1. Schematic diagram of the miniaturised separation device. Letters A–E refer to the wells through which fluid connections into and out of the device are made. CD1–3 refer to conductivity detectors. In this work only CD1 was used.

ples have been analysed for amino acids using respectively a PMMA device with fluorescence detection [17] and a glass device with LIF detection [15].

This paper presents a new isotachophoretic method, which can be used for analysing amino acids in a miniaturised format. The proposed electrolyte system offers a way of eliminating the carbonate contamination which occurs when performing determinations under alkaline pH conditions. The use of the method is demonstrated using a planar PMMA separation device with integrated conductivity detection electrodes. Such a device has been used to perform separations of model mixtures containing a range of amino acids.

2. Experimental

2.1. Microfabrication

Separations were performed using a directly milled, miniaturised PMMA chip device, full details of which have been previously described [18]. The device essentially consists of a 78 mm long, 78 mm wide, 6 mm thick PMMA block, sealed with 400 μm thick self−adhesive polyester laminate (Plastic Art Company, Manchester, UK) which incorporates two separation channels with integrated on-column conductivity detectors. A schematic of the layout of the device is shown in Fig. 1. In this work the two perpendicular, connected channels were used as effectively a single, long, separation column. The first channel, from the cross to the bifurcation point is 57 mm long, 300 μm wide and 300 μm deep, whereas the second, from the bifurcation point to the detector located towards well B, is 44 mm long, 200 μm wide and 300 μm deep. All of the channels intersecting at the cross are 300 μm wide by 300 μm deep. The conductivity detectors consist of pairs of 75 μm diameter platinum wire (Aldrich, Gillingham, UK) electrodes arranged in opposed configuration. Fluid connections to and from the separation device were made using 062 MINSTAC fittings (Lee, Westbrook, CT, USA).

2.2. Instrumentation

Sample injection and movement of solutions around the separation device was achieved using a hydrodynamic sample transport system based on a series of gravity feed reservoirs and solenoid actuated valves, as previously reported by the authors [19]. The constant currents used to drive the separations were provided by a PS350 5000 V–5 W high-voltage power supply (Stanford Research Systems, Sunnyvale, CA, USA), configured to produce negative voltages. Detection was achieved using a single channel capacitively coupled conductivity detector built in house.

Control of the sample transport system, high voltage power supply and dual channel conductivity detector was achieved using LabVIEW software (version 6.1) (National Instruments, Austin, TX, USA), running under the Windows XP operating system (Microsoft, Redmond, WA, USA) on a standard personal computer. The hardware interface is achieved using three National Instruments cards controlled using the NIDAQ driver (National Instruments) and programmed using LabVIEW code. The cards used were a PCI-GPIB board for the power supply, a PCI-6601 timing and digital input/output board for the detector and a PCI-6503 digital input/output card for the sample transport system.

A further LabVIEW program available in the laboratory, which determines relative step heights (RSHs) and lengths, was used for data analysis. In this work RSH was calculated using the following expression:

$$\mathrm{RSH} = \frac{f_S - f_{LE}}{f_{TE} - f_{LE}}$$

where f_{LE} is the frequency of the response produced by the leading electrolyte (Hz); f_S the frequency of the response produced by the sample (Hz); f_{TE} the frequency of the response produced by the terminating electrolyte (Hz).

2.3. Separation conditions

The miniaturised separations performed in this investigation were achieved using the two control programs shown in Table 1. In both programs the initial five steps, which move solutions around the device, are identical. The first two steps flush the device and fill the separation channels with leading electrolyte. The timing of this step is set to ensure that all traces of previously analysed samples are fully removed from the system before performing subsequent separations.

Table 1
Separation programs used for performing miniaturised ITP

Step	Program 1							Program 2						
	Time (s)	Current (μA)	Valve status					Time (s)	Current (μA)	Valve status				
			A	B	C	D	E			A	B	C	D	E
1	20	–	●	○	●	●	○	20	–	●	○	●	○	●
2	20	–	●	○	●	○	●	20	–	●	○	●	○	●
3	1	–	●	●	○	○	●	1	–	●	●	○	○	●
4	0.5	–	○	●	●	○	●	0.5	–	○	●	●	○	●
5	0.3	–	○	●	●	●	○	0.3	–	○	●	●	●	○
6	400	35	●	●	●	●	●	200	30	●	●	●	●	●
7	1000	20[a]	●	●	●	●	●	150	20	●	●	●	●	●
8								1000	15	●	●	●	●	●

[a] Unless otherwise stated, (●) closed, ○, open.

Step 3 positions the terminating electrolyte so that when the high voltage is applied the sample will be sandwiched between the leading and terminating electrolytes. Steps 4 and 5 inject the sample into the device, using the separation channel between the cross and bifurcation point as an injection loop with a volume of 5.1 μl. In program 1 the ITP separations are performed with an initial constant current of 35 μA applied between wells B and C, step 6. The current is then reduced to 20 μA (unless otherwise stated in the text) for detection in step 7. Program 2 is suitable for use with low conductivity electrolyte systems which are susceptible to bubble formation. To prevent this phenomenon happening ITP separations are performed with an initial current of 30 μA in step 6, subsequently reduced to 20 μA in step 7, with detection at 15 μA in step 8.

2.4. Chemicals

The following chemicals were used in the production of electrolyte solutions: glycolic acid (99%) from Fluka (Gillingham, Dorset, UK); magnesium sulphate (99%) and Mowiol 40–88 from Aldrich; benzylamine from Acros (Loughborough, UK); β-alanine (99%) from Sigma (Gillingham, Dorset, UK); barium hydroxide (0.05 M volumetric standard) from Riedel-de Haën (Gillingham, Dorset, UK). Amino acid solutions (0.01 M) were produced using the following: DL-alanine (Ala), L-cysteine (Cys) hydrochloride, D-leucine (Leu) and L-phenylalanine (Phe) obtained from Aldrich; L-asparagine (Asn), L-aspartic acid (Asp), L-glutamic acid (Glu), glycine (Gly), L-histidine (His), L-tryptophan (Trp) and DL-tyrosine (Tyr) obtained from Sigma; DL-*iso*leucine (Ile), DL-methionine (Met), DL-serine (Ser), DL-threonine (Thr) and L-valine (Val) obtained from BDH (Poole, UK). All amino acids had a purity of 99% or higher with the exception of Asn, Trp and Tyr which were 98% purity. The Tyr sample was dissolved in 0.1 M hydrochloric acid (1.0 M volumetric standard, Aldrich). All solutions were prepared using >18 MΩ water (Elga Maxima Ultra Pure, Vivendi Water Systems, High Wycombe, UK).

3. Results and discussion

3.1. Electrolyte system

The selection of the electrolyte systems employed in this work were based on a number of considerations. The pH of the leading electrolyte was influenced by the findings of Hirokawa et al. [4] who analysed the separation of amino acids over the pH range 8.6–9.6, and found that a maximal separation could be achieved at pH 8.64 but also that good separations could be achieved at pH 9.0. In this study, the higher of these pH levels was chosen to enable quicker separations as such conditions allowed the use of a faster, more conductive terminating electrolyte. However, despite this it was still necessary to use a slow terminating ion, β-alanine. Consequently to increase the overall conductivity across the separation device the concentration of the terminating electrolyte was set at 20 mM and the pH was adjusted to 9.7 to increase the conductivity of the terminating zone. A problem with performing isotachophoretic analyses under alkaline conditions is the migration of carbonate ions from dissolved atmospheric carbon dioxide. At certain pH levels this species can migrate with similar effective mobilities to some of the amino acids. However, at pH 9.0 carbonate has a sufficiently high effective mobility for this not to be a problem. To eliminate the unwanted carbonate zone from appearing in the results, glycolic acid, which has an effective mobility intermediate of carbonate and the most mobile amino acid, Asp, was selected as the leading ion.

Two different electrolyte systems, I and II, full details of which are shown in Table 2, were investigated. The second of these systems contained 2 mM magnesium as an additive. This species was added to see whether its presence improved the separation of amino acids. Previously Stover had employed copper(II) for a similar purpose and found some beneficial effects [6]. However, complexation with copper(II) also retarded certain species, most notably His, to such an extent that they were slower than β-alanine, the terminating ion selected for this current investigation. It was thus thought that the use of a species which would complex the amino acids less strongly may be more useful. Examination of sta-

Table 2
Composition of the electrolyte system used for performing isotachophoretic amino acid separations

Electrolyte system	I	II
Leading electrolyte	Glycolic acid (mM)	10 mM glycolic acid
Counter ion	–	2 mM Mg^{2+} (added as $MgSO_4$)
pH buffer	Benzylamine	Benzylamine
pH	9.0	9.0
Additive	1 mg ml^{-1} HEC	1 mg ml^{-1} HEC
Terminating electrolyte	20 mM β-alanine	20 mM β-alanine
Counter ion	Ba^{2+} [added as $Ba(OH)_2$]	Ba^{2+} [added as $Ba(OH)_2$]
pH	9.7	9.7

bility constants suggested zinc(II) would fulfil this criterion [20]. However, at pH 9.0 used in this work zinc is readily hydrolysed and cannot be used. Magnesium, which forms less strong complexes than zinc(II), was thus used as this species would not be hydrolysed under these conditions.

Fig. 2 shows the RSHs observed for seventeen amino acids with the two different electrolyte systems. For the majority of the species little difference in RSHs was observed between the two systems. There can however be seen that the addition of magnesium did lead to some compression of the overall range of RSHs exhibited by the amino acids. The species which were retarded significantly were the divalent ones Asp, Glu and Cys, which represent the amino acids which exhibit the highest degree of ionisation at pH 9.0. Thus, it is not known whether the change in effective mobilities is due to complexation effects or simply electrophoretic effects due to the system containing a divalent counter ion. The only other species which appeared to be retarded to any extent was His, which has a tendency to form stronger metal complexes than many of the other amino acids [20]. Previously it has been found that the addition of magnesium did prove beneficial in the separation of amino acids using CZE [21]. The prime mechanisms for this change were thought to be due to changes in electroosmotic flow (which is not a factor in the current study) and electrophoretic effects rather than complexation. Although the addition of magnesium was not found to significantly alter the separations it did lead to enhanced step definition and improved the separation of Asp, Glu and Cys. Additionally the presence of a divalent counter ion increased the conductivity of the electrolyte system. Thus, separations with electrolyte system II could be performed using separation program 1, whereas those with electrolyte system I needed to be performed using separation program 2. These benefits meant that electrolyte system II was employed for all subsequent analyses.

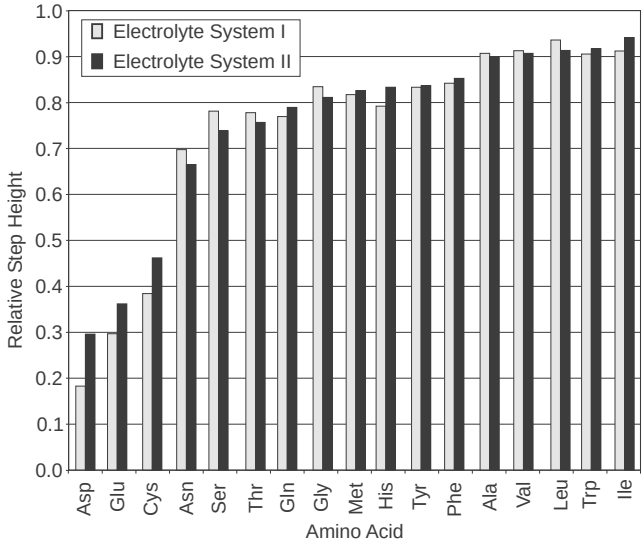

Fig. 2. Comparison of RSH values obtained using the two different electrolyte systems shown in Table 2.

3.2. Separations

Electrolyte system II was successfully used to analyse a variety of samples ranging from those containing single amino acids to multicomponent mixtures. Good reproducibility in the RSH data, which is used to for qualitative purposes in ITP, was noted. Separations of single component samples of all 17 amino acids analysed in this work yielded relative standard deviations (RSDs) in the range 0.6–6.3%, based on four determinations. Poorer reproducibility was achieved with the non-magnesium containing electrolyte system, I. With this system RSDs in the range 0.2–8.7%, based on triplicate analyses, were obtained for single component samples of all species except Glu. This later species exhibited poorer reproducibility of 11.4%.

The RSH data obtained suggested that numerous of the amino acids would co-migrate. Further investigations were made into which amino acids could be separated from one another by analysing mixtures of pairs of species with similar, but not identical, step heights. Fig. 3 shows an example of one such separation, that of a sample containing 0.2 mM Gln and Thr. The extra zone present is that of an unknown contaminant. This zone was present in all separations and was thought to arise from one of the electrolyte components. It can be seen that the complete analysis, preparation of the device and the actual separation, was achieved in just under 9 min. This represents an improvement over the analysis times possible with capillary ITP separations, where separation times alone of up to 35 min have been reported [4]. Analysis times for amino acids using ITP are generally longer than for many other applications, due to the low conductivity electrolytes required to analyse substances with very low effective mobilities. As a comparison separations of inorganic selenium species using a similar miniaturised device to that used in this work are possible in 3 min [22].

Based on the results observed with the two component mixtures it was possible to construct a separation order for the amino acids with the proposed electrolyte system, a schematic diagram of which is shown in Fig. 4. It can be seen that it is not possible to separate all of the amino acids

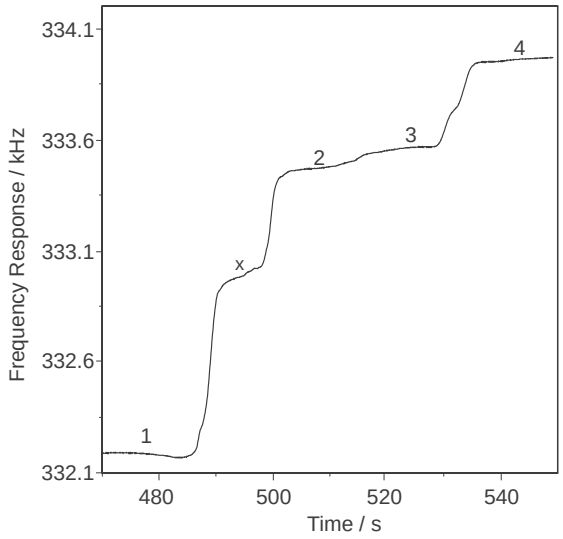

Fig. 3. Isotachopherogram produced by the separation of a sample containing 0.2 mM Gln and Thr performed with electrolyte system II using separation program 1. (1) Glycolate; (2) Thr; (3) Gln; (4) β-alanine; (×) unknown contamination.

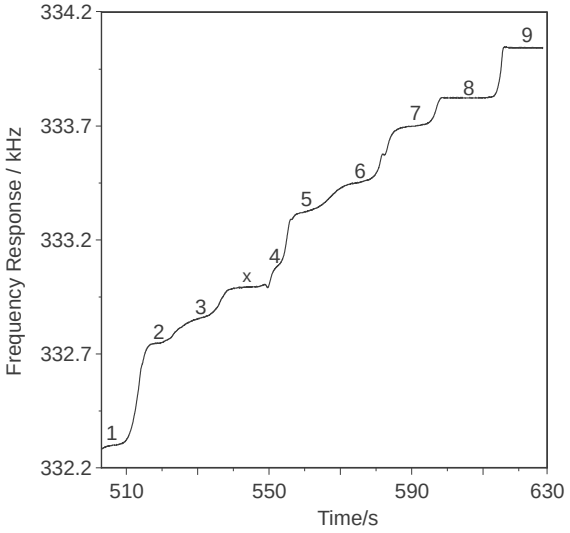

Fig. 5. Separation of a sample containing 0.1 mM Ala, Asn, Asp, Cys, Glu, Phe and Ser performed with electrolyte system II using separation program 1 with a current of 10 μA in step 7. (1) Glycolate; (2) Asp; (3) Glu; (4) Cys; (5) Asn; (6) Ser; (7) Phe; (8) Ala; (9) β-alanine; (×) unknown contamination.

studied as several mixed zones will form. The maximum number of acids which could in theory be separated with this system is 10. This figure represents a similar value to those suggested by Everaerts et al. [3] and Hirokawa et al. [4] for capillary scale systems. It was thought likely that a separation of Ala from Ile should be possible given the differences in RSH, 0.899 (±0.6%) and 0.941 (±2.1%). However, no separation was possible. It was thought that this may be due to resolution issues between Ile and the terminating ion. Thus if a different terminating electrolyte, such as proline (Pro), with a lower mobility than β-alanine was used such a separation should be possible. Such a change may also allow the resolution of Leu and Trp from Ala (but not from one another nor Ile). It should be noted that Pro ($pK_a = 10.60$ [23]) has such a low effective mobility at pH 9.0 that it cannot realistically be analysed due to the lack of a suitable terminating species. Of the other of the common amino acids found in proteins, arginine (Arg) and lysine (Lys) are better analysed as cations. These findings agree with the observation of Holloway and Pingoud, who surmised back in 1981 that the separation of all 20 such amino acids would not be possible using ITP [24].

An example of a separation of a sample containing seven amino acids, 0.1 mM Ala, Asn, Asp, Cys, Glu, Phe and Ser, is shown in Fig. 5. The separation shown in Fig. 5 was achieved

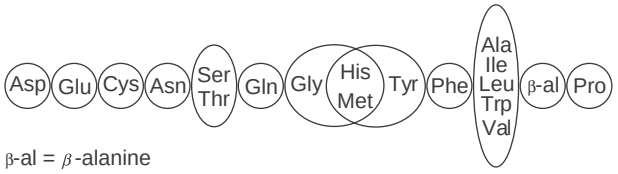

Fig. 4. Amino acid separations which can be achieved using electrolyte system II. Separations are possible between species in different circles.

using a detection current of 10 μA rather than 20 μA (step 7 in separation program 1). This change was made so that the short zones, arising from concentration of the sample components in this mixture, are more clearly visible in the resulting isotachopherogram. Reasonable reproducibility in the zone lengths, the quantitative information obtained from isotachophoretic separations, was achieved with the mixture. RSDs in the range 1.6–7.6%, based on four determinations, were observed for all of the species. Reproducibility of the RSHs of the species in the mixture was similar to that achieved with single component samples, being in the range 1.4–7.1% for all seven species.

Although in theory the electrolyte system should allow the separation of up to ten amino acids, mixed zones in readily separable species, such as Ser and Met, were observed when samples containing eight species were analysed. Thus it would appear that the separation of seven amino acids represents the maximum number that can be separated simultaneously with the current design of miniaturised separation device. It was also noted that samples containing more than six amino acids at higher concentrations than the 0.1 mM used in the example shown in Fig. 5, also lead to the formation of mixed zones. This suggests that this concentration is getting towards the upper separable limit for certain species [25].

To show that the method is suitable for quantitative work, calibration curves were produced for the two amino acids, Asp and Phe, using weighted linear regression. Details of the curves, produced based on the analysis of four replicates of seven samples with sample concentrations in the range 0.063–1 mM, can be seen in Table 3. Good linearity was observed over the range of concentrations analysed, with correlation coefficients of 0.997 and 0.998 being calculated for Asp and Phe respectively. Using the calibration equations,

Table 3
Weighted linear regression equations, of the form $y = bx + a$, for the calibration curves obtained using electrolyte system II

	LOD (mM)	$a \pm$ S.D. (s)	$b \pm$ S.D. (s mM^{-1})	r	n	Concentration range (mM)
Asp	0.060	−1.98 ± 0.58	61.9 ± 3.3	0.997	7	0.063–1.0
Phe	0.018	0.22 ± 0.45	74.5 ± 2.3	0.998	7	0.063–1.0

a: Intercept; b: slope; n: number of data points (four replications performed at each); r: correlation coefficient; LOD: limit of detection.

limits of detection, taken as the intercept (or 0 in the case of Asp which yielded a negative intercept) plus three times the standard deviation of this figure, were determined to be 0.018 mM for Phe and 0.060 mM for Asp. These detection levels represent similar performance levels to many of those achieved using miniaturised CZE [14,15]. These levels suggest that, based on the findings of previous investigations, the technique would be suitable for the analysis of beverages, like beers [26], and biological samples, such as urine [27].

4. Conclusions

The results show that miniaturised ITP can be successfully applied to the analysis of amino acids. The use of an inexpensive PMMA separation device built in-house allowed faster analysis times than those possible with conventional scale ITP equipment coupled with a good level of analytical performance. The developed electrolyte system, using glycolate as the leading ion to eliminate carbonate interference, and magnesium as a counter ion, can be used to analyse most of the common amino acids. Simultaneous determinations of up to seven amino acids were successfully attained. The proposed method should allow the determination of a greater number of species, but to achieve such a separation may require enhancements to be made to the device design. The levels of performance achieved, detection limits in the sub-mM concentration range, suggest that the method could be used to analyse samples in for example the fields of biochemistry and food chemistry.

Acknowledgements

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