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Optimization of a capillary electrophoresis–electrospray mass spectrometry method for the quantitation of the 20 natural amino acids in childrens blood

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Abstract

This paper describes useful information on the capillary electrophoresis–electrospray ionization mass spectroscopy (CE–ESIMS) interfacing for the analysis of amino acids (AAs) in standard mixtures and in child plasma blood serum. The developed procedure allows quantitation of the 20 natural AAs, in a single run, without any derivatization. Limits of detection as low as 3–20 μmol/l (5–30 pg injected) per analyte were obtained with an efficiency of about 100 000 plates and a peak area relative standard deviation below 4%.

Keywords: Amino acids

1. Introduction

Since its first demonstration by Jorgenson and Lukacs in the early 1980s [1–3], capillary electrophoresis (CE) has become a routine technique in many laboratories, as it permits the analysis of a broad range of mineral and organic compounds with high resolution and low sample and solvent consumption.

Amino acids (AAs) were the first compounds investigated by CE [4] due to the ability of these components to form ions in solution. Their analysis has formed an important area of research [5–8]. Sandra and co-workers [9] published the first complete separation of all 20 natural AAs, reporting a detailed derivatization procedure for improving analyte absorption coefficients in the UV-visible range.

Although two modes of CE are adequate for AAs analysis, i.e. capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), the latter technique is not pertinent in CE–ESIMS due to the introduction of the non-volatile micellar phase into the ESI source.

The aim of CE–ESIMS is to overcome the lack of sensitivity of UV detection and the difficulty in analyzing all 20 AAs in a single run.

Several derivatizing agents [5], enhancing the detection response, have been investigated for AAs. Dansylation is most commonly used. The compound stability, solubility, the reaction rate, the variation in amino acid properties and structures and the com-

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plexity of the analysis are the main limiting factors of pre-, on-, or post-column derivatization. This is why new detection strategies have been investigated, such as direct [10,11] and indirect UV absorption [12], indirect fluorescence [13], proton nuclear magnetic resonance (NMR) [14], and thermooptical [15], electrochemical [16] and mass spectrometric [17] detection.

The development of on-line CE–MS was pioneered by Olivares et al. [18], Smith et al. [19], Lee et al. [20] and Johansson et al. [21] and a few reviews have been published [22–25]. Several ionization methods have been used for CE–MS: electrospray ionization, ion spray or pneumatically assisted electrospray and continuous-flow fast atom bombardment (CF-FAB). The electrospray ionization (ESI) technique developed by Mack et al. [26] and more recently described by Yamashita and co-workers [27] has become the method of choice for coupling CE to MS, because it is a soft ionization mechanism. Several approaches have been designed to interface CE and ESI, in order to maintain a stable electrical contact. Suggested designs are either a coaxial sheath flow with [28] or without the use of an additional nebulizing gas [21,24], or a metal-coated [29] sharpened CE column outlet, or a gold wire electrode [30] inserted into the CE column outlet, or a liquid junction interface [20].

The only report of the analysis of AAs by CE–MS in the literature is from Lu and co-workers [31] which is concerned with four natural AAs. This paper reports a procedure developed in order to analyze directly the 20 underivatized natural amino acids in one single run based on CE optimization and on the ESIMS capabilities.

2. Experimental

All experiments were performed on a P/ACE system 2100 (Beckman Instruments, Palo Alto, CA, USA) coupled to a Quattro II (Micromass UK, Altrincham, UK) tandem quadrupole mass spectrometer. Instrument control, data acquisition and data processing were carried out using the GOLD software package for CE and the MASSLYNX software for ESIMS.

CE separations were carried out at 23°C on a 50 μm I.D., 375 μm O.D., 105 cm long capillary column purchased from Thermo Separation Products (Les Ulis, France). The UV detector, located 20 cm from the capillary injection end, was operated continuously at 200 nm for coarse control of analyte migration. The analytical buffer was 50 m*M* aqueous formic acid, pH 2.5. The applied voltage was 30 kV. CE buffer solutions were filtered through a 0.2 μm filter to prevent blockage of the CE capillary and for degassing. New capillaries were flushed successively for 20 min with 0.1 *M* NaOH, water and the buffer solution. Between analyses, capillaries were usually rinsed with two column volumes of buffer for total reconditioning. Rinsing and conditioning were performed by pressurizing the inlet vial to 20 psi ($1.38 \cdot 10^5$ Pa). Samples were introduced hydrodynamically into the capillary at 0.5 psi ($3.45 \cdot 10^3$ Pa). The injection time was varied between 5 and 20 s.

The CE column extremity was positioned in a CE–ESIMS probe (Micromass UK) consisting of three concentric capillaries. The CE capillary extended fully to the probe tip through a stainless-steel sheath capillary carrying a sheath solvent. Around the sheath liquid capillary the nitrogen gas flowed to the probe tip through a nebulizer capillary.

A syringe pump (Harvard Apparatus, South Natick, MA, USA) was used to provide the sheath-liquid flow at a flow-rate of 10 μl/min.

The ESI source was operated in the positive ionization mode, and optimal performance was produced by applying a 3.2 kV voltage to the probe tip with a 20 V cone voltage. The source temperature was maintained at 80°C, and nitrogen was used for both nebulizing (35 l/h) and drying (100 l/h).

For full-scan mass spectra, data were acquired for 60 s over the range m/z 70 to 210 with a 1 s scan time at unit mass resolution. For MS–MS spectra, argon was used as the collision gas at $3.2 \cdot 10^{-3}$ mbar with 20 eV collision energy.

For calibration, individual stock solutions of the 20 common AAs were prepared in LC grade water at a concentration of around 10 000 μmol/l. Due to its lack of solubility, tyrosine was prepared at 2200 μmol/l. A mixture of the 20 AAs was prepared from aliquots of each individual stock solution at 500 μmol/l (Tyr 110 μmol/l). Then eight fresh working solutions were prepared by serial dilution, ranging from 500 to 20 μmol/l, with the addition of valine-

d_8 at a constant 200 μmol/l concentration as internal standard.

L-AAs 99% and formic acid were purchased from Aldrich (Milwaukie, WI, USA), methanol and LC grade water from Sigma (St. Louis, MO, USA) and L-valine-d_8 from Cambridge Isotope Laboratories (Innerberg, Switzerland).

Other experimental details are described in the text.

3. Results and discussion

The procedure was optimized by studying the influence of a set of operating parameters: the various capillary distances from the probe tip, the sheath liquid composition and flow-rate, the ESI tuning parameters and gas flow-rates, the CE injection and separation protocol and the sample solvent composition. Criteria for evaluating the optimum parameters were the peak shape and intensity, the migration times and the electrophoretic and electrospray current intensities.

The sheath flow interface used in this study was specific for the material provided by the supplier. The optimization of the geometrical settings of the different coaxial capillaries has already been described [32] and is mandatory for obtaining a stable spray and an intense signal, which are controlled on the terminal peak checking window from the mass peak shape. The CE column tip was set 0.2 mm protruding from the sheath liquid tube to avoid both sample dilution and column retroelution. Retroelution is observed when the nitrogen flow induces an outlet overpressure, which drastically counterbalances the electrophoresis column flow. Retroelution is detected by UV from the increase in analyte migration time. The sheath liquid tube end was positioned 0.5 mm out of the sheath gas tube to form droplets without sputtering. Both the probe and MS earths were grounded to avoid arcing. The CE column outlet was set at the same height as the CE–ESIMS probe to prevent siphoning.

A 50 m*M* formic acid buffer pH 2.5 was selected after testing classical volatile solvents such as acetic acid and ammonium acetate usually used for coupling CE to ESIMS. This slight acid addition improves AA protonation, thus leading to stronger peaks.

The sheath liquid is required to maintain a stable electrical contact at the probe tip, as the CE fused-silica column is not suitable for electrically grounding the setting and to supplement the inadequately low CE flow. Typical sheath liquid mixtures usually contain volatile acids, with a composition preferentially the same as that of the CE buffer in order to avoid the formation of a moving boundary [33] with backmigration of the sheath liquid counterions into the CE capillary. Organic solvents are also added to improve the desolvation process and thus enhance the stability of the electronebulization.

Our experiments on a test mixture of two AAs (Fig. 1) show that, in our configuration, pure methanol does not lead to smooth migration peaks even when formic acid is added, although it was recommended by Wheath et al. [34]. The peak shape improved drastically when both water and formic acid were added, probably due to better miscibility with the CE buffer and the sample water matrix. The

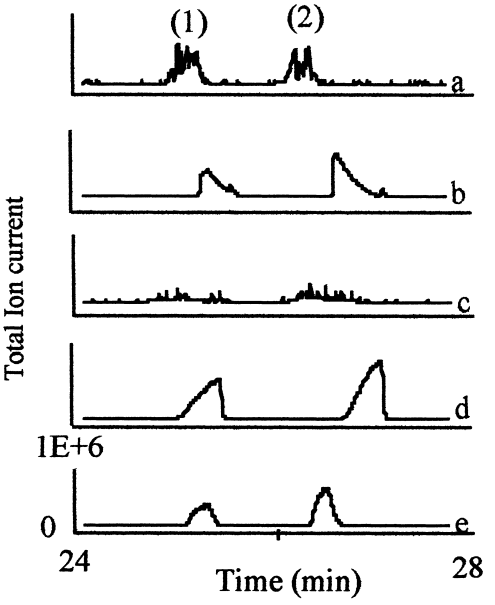

Fig. 1. Influence of sheath liquid composition on the CE–ESIMS peak shape. Total ion electropherogram of a 0.5 mg/ml aqueous mixture of (1) glycine and (2) alanine. Sheath liquid composition: (a) MeOH, (b) H_2O–MeOH (50:50), (c) MeOH + 1% formic acid, (d) H_2O–MeOH (50:50) + 1% formic acid, (e) H_2O–MeOH (50:50) + 0.1% formic acid.

optimum peak profile was obtained for a 50:50 water–MeOH mixture with 0.1% formic acid.

The optimum flow-rate was set at 10 μl/min (Fig. 2b) in all subsequent experiments. A too low sheath flow-rate leads to a lack of liquid feeding in the spray, resulting in a sputtering effect (Fig. 2c). Increasing the sheath flow-rate to 20 μl/min results in peak intensity and shape degradation (Fig. 2a), probably due to the desolvation degradation because of the overfeeding of the spray with solvent. Solvent mixing was improved by removing a few millimeters of the polyamide outlet coating of the CE capillary column.

The operating parameters of CE and MS should be adjusted individually when coupling both techniques.

Optimization of ESIMS critical parameters, i.e. capillary probe, counter electrode, extraction cone voltages and gas flow-rates, was achieved in the full-scan mode (scanning from m/z 70 to 210) by infusing a 20 AAs mixture through the CE capillary column at a pressure of 20 psi ($1.38 \cdot 10^5$ Pa) without any CE separation (i.e. absence of any CE voltage). A 20 V cone voltage was set to produce predominantly $[M+H]^+$ ions with little evidence of analyte fragmentation, with a 3.2 kV probe voltage and 0.4 kV HV lens voltage.

A temperature of 80°C was suitable for water desolvation. Low-mass resolution and high-mass resolution settings were fixed to a value of 15, which permits the achievement of a higher resolution and lower peak width. The drying gas flow-rate, which is often set at 300 l/h, was set at 100 l/h in order to overcome the possible contribution to retroelution through a backpressure effect [35]. The coaxial nebulizer gas was optimized at 35 l/h and the ion energy was maintained at 0.5 V.

Leucine and isoleucine and lysine and glutamine appear at the same mass on the infusion mass spectrum of the mixture. However, when using CE–ESIMS in the SIR mode, a specific electropherogram is recorded for each selected $[M+H]^+$ ion (Fig. 3) with an improved limit of quantitation. Each AA is

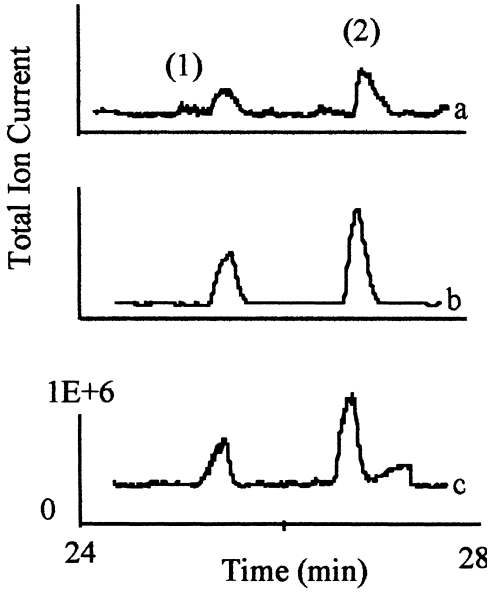

Fig. 2. Influence of the sheath liquid flow-rate on the CE–ESIMS peak shape. Sheath liquid flow-rate: (a) 20 μl/min, (b) 10 μl/min, (c) 5 μl/min. (1) and (2) as in Fig. 1.

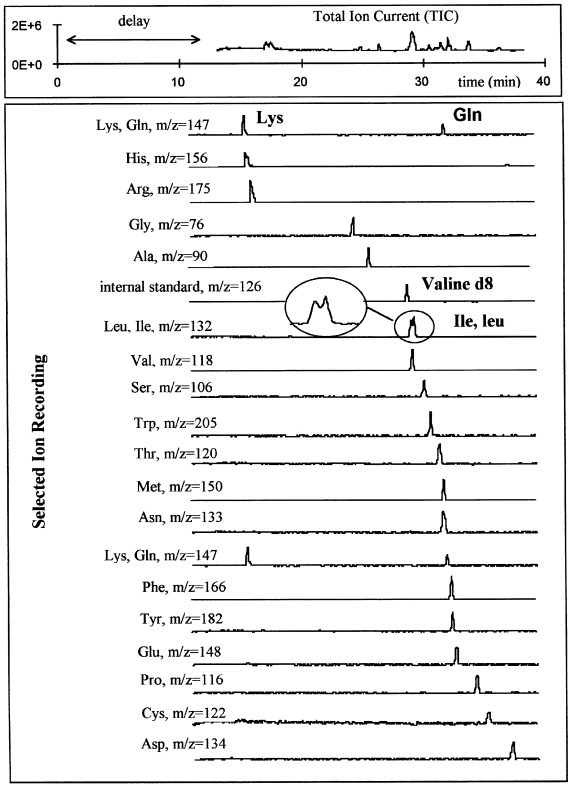

Fig. 3. Total ion current (TIC) and mass electropherograms (SIR) of a 500 μmol/l mixture of the 20 AAs (tyrosine at 100 μmol/l). Internal standard: valine-d_8 at 200 μmol/l.

thus identified from both its migration time and its characteristic ion. Operating at 40 V cone voltage permits the differentiation of coeluting leucine and isoleucine from the immonium-NH_3 fragment at m/z 69, which only appears for leucine. Lysine and glutamine appear at different migration times on the m/z 147 mass electropherogram (Fig. 3).

In our experiments, the UV detector was operated continuously at 200 nm for checking AA migration, and thus the absence of critical retroelution. Hydrodynamic injection was chosen to preserve representative aliquots of the sample to be analyzed and to avoid analyte discrimination. The injection time was set at 10 s to obtain symmetrical peaks. The applied voltage was increased to 30 kV (8 μA) to shorten the time of analysis without compromising the resolution, although this parameter is not of prime importance. The effective voltage applied along the CE capillary was the difference between the CE running voltage (30 kV) and the probe voltage (3.2 kV). Classical electrophoretic migration was continuously assisted by application of a slight overpressure (0.5 psi $= 3.45 \cdot 10^3$ Pa) to reduce the time of analysis and diffusion, leading to a better peak shape (Fig. 4). An attempt at increasing the speed of analysis by suspending electrophoretic migration and pressurizing the column for 1 min was totally unsuccessful as it resulted in critical band broadening (Fig. 4c).

Pure water was preferred in the preparation of standard solutions in order to benefit from the stacking effect [36].

It should also be noted that it is not wise to set the nebulization and drying gases during hydrodynamic injection and to immediately switch the ESI voltage on [35], because it can either decrease the introduced sample volume by decreasing the column pressure or create a reverse flow, which offsets the flow induced by the hydrodynamic overpressure. Low migration time components are affected more, as exemplified in Fig. 5, their peak intensity thus becomes smaller and their migration time longer.

A solvent delay (13 min) in the ESIMS acquisition protocol was necessary as the source voltage, gas and liquid sheath flows were only switched on after AA UV detection (5 min). During the delay, a small amount of the CE liquid electrolyte reaches the ESI source volume, which then has to be pumped out before recording clean mass data.

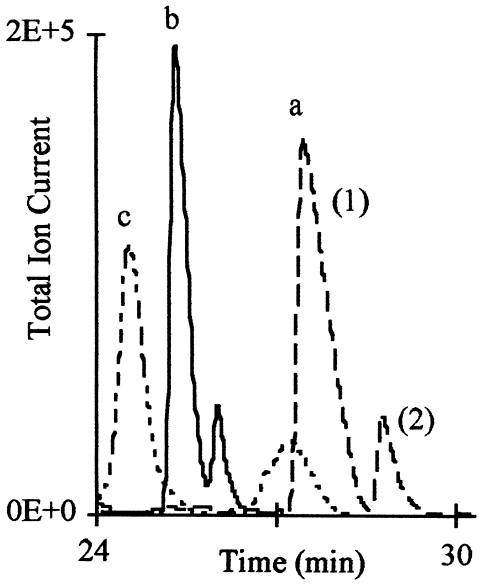

Fig. 4. Influence of pneumatic assistance of classical electrophoretic migration at 30 kV voltage. (a) Voltage only; (b) voltage + pneumatic assistance; (c) 5 min voltage + pneumatic assistance + 1 min high pressure assistance (20 psi) + return to (b); (1) and (2) as in Fig. 1.

The efficiency, reproducibility, linearity, and limit of quantitation of the method were first evaluated and then the accuracy of the method was verified using real samples.

The efficiency of the CE method, calculated from each ion electropherogram, for a mixture containing the 20 AAs at a concentration of 500 μmol/l lies between 29 000 and 820 000 plates per meter, which corresponds to typical efficiency values obtained for small molecules in CE. Slower components are affected more by band broadening due to diffusion

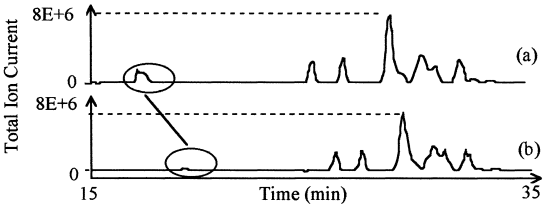

Fig. 5. Comparison of operating protocols (a) with and (b) without turning on gas, sheath liquid and voltage. Total ion current pattern. Sample as in Fig. 3.

effects, thus exhibiting significantly lower plate numbers.

Addition of an internal standard was required in order to normalize variations in both migration times — electroosmotic flow and apparent mobilities — and CE peak areas due to the injection volume inaccuracy and to minimize differences between standard and real sample matrices. Valine-d_8 (m/z 126) was used as the internal standard in all the AAs mixtures because of its similar solubility and behavior to the 20 AAs, its chemical stability, and its absence in real samples.

Table 1 clearly shows that the relative standard deviations (RSDs) of the migration times and peak areas are smaller when reporting data relative to the corresponding internal standard data.

RSD values of peak areas are $<4\%$. This value shows that MS is adequate as a reproducible quantitative CE detector. The major factors which were difficult to control in our experimental design and which may explain the data variation were the inaccuracy of the injection system, the lack of continuity in the temperature regulation system along the column and sample adsorption on the column walls. The use of an internal standard [38] is an elegant alternative to minimize these effects on data reproducibility.

Four replicate analyses of each of the eight fresh working solutions were carried out for calibration purposes with injection times ranging from 5 to 20 s in order to improve the S/N ratio for diluted compounds, and to obtain more symmetrical peaks for concentrated compounds. Calibration curves were then plotted according to injected quantities and not according to injected concentrations and were established on relative peak areas.

All curves were found to be linear over the 20–500 μmol/l concentration range (with regression

Table 1
Quantitation procedure for the validation of the 20 AAs: reproducibility after eight replicates of a 120 μmol/l mixture and limits of detection are defined at $S/N = 3$ are defined

AA	$[M+H]^+$	Reproducibility (%RSD)				LOD pg injected (μmol/l)
		t_m [a]	Relative [b] t_m	Peak area	Relative [b] peak area	
Gly	76	3.52	0.17	33.37	2.08	5.2 (7)
Ala	90	4.68	0.36	35.23	3.81	7.9 (9)
Ser	106	4.56	0.24	34.62	2.64	8.1 (8)
Pro	116	3.99	0.20	45.20	1.01	23.7 (20)
Val	118	5.10	0.20	38.67	2.56	10.3 (9)
Thr	120	3.55	0.24	33.53	2.06	10.3 (9)
Cys	122	3.39	0.34	53.41	1.92	24.9 (20)
Leu	132	–	–	–	–	270 (200)
Ile	132	–	–	–	–	270 (200)
Asn	133	4.75	0.40	46.78	2.65	27.2 (20)
Asp	134	2.99	0.36	32.60	3.16	27.4 (20)
Lys	147	4.31	0.41	47.73	2.74	15.0 (10)
Gln	147	4.1	0.39	53.73	3.23	30.0 (20)
Glu	148	3.36	0.49	43.18	3.27	30.3 (20)
Met	150	4.15	0.38	31.78	2.16	15.3 (10)
His	156	4.28	0.40	57.75	1.66	5.3 (3)
Phe	166	4.13	0.44	35.69	2.31	14.5 (9)
Arg	175	4.37	0.47	49.84	1.83	15.3 (9)
Tyr	182	3.59	0.28	33.49	2.87	7.4 (4)
Trp	205	3.53	0.23	32.84	1.95	7.0 (3)

[a] t_m, migration time.
[b] Relative to the internal standard.

coefficient between 0.990 and 0.998). Experimental individual limits of detection (LOD) for the 20 AAs at $S/N = 3$ varied from 5 to 30 pg (3–20 μmol/l) injected (Table 1). They depend on the ESI proton affinity of each AA. Measured LODs are mostly adequate for real sample AAs quantitation, and may be improved by pre-concentration techniques.

For leucine and isoleucine, quantitation by EC–ESIMS–MS from m/z 131 to 69 transition monitoring, the LOD deteriorates by a factor of 10 as compared to EC–ESIMS quantitation of other AAs due to the low ion transmission rate (10%) in the different quadrupoles.

The accuracy of the optimized CE–ESIMS method was tested on the quantitation of AAs in child plasma sera.

Deproteinization of samples prior to analysis was accomplished by a 1:10 (v/v) addition of 35% 5-sulphosalicylic (SSA) acid to the plasma, then cooling of the mixture to 4°C for 1 h and centrifugation for 15 min in a high-speed centrifuge to spin down the precipitate. The supernatant was removed and filtered through a 0.2 μm filter. This procedure removed the proteins but also deteriorated tryptophan. Samples were analyzed on a Biochrom 20 (Amersham Pharmacia Biotech) LC system with UV detection after ninhydrin derivation before CE–ESIMS analysis [37]. LC separation was achieved on a standard high-performance cation exchange column, 200 mm long, 4.6 mm I.D., using six successive lithium and lithium hydroxide buffers. The time of analysis was 90 min.

The deuterated internal standard was added to each sample before CE–ESIMS analysis and concentrations were recalculated for dilution correction.

A mass spectrum of the infused plasma sample was recorded beforehand in order to screen the sample composition. No AAs were found in the full-scan mode due to the lack of sensitivity of that mode. Using the developed protocol with selected ion recording, all plasma serum AAs were selectively detected and quantified, although the total ionic current (TIC) electropherogram is of no help. No peak was detected for cysteine (m/z 122), perhaps because of its oxidation to cystine, and no peak was detected for tryptophan (m/z 205), because of its degradation by the deproteinization procedure.

An extra rinse with 0.1 *M* sodium hydroxide followed by an extensive rinse with the rinsing electrolyte was necessary between each run to remove undesirable compounds adsorbed on the column walls. Before any new CE–ESIMS run, column reconditioning was checked by running pure electrolyte and verifying that no CE peak was detected.

Routine LC quantitation data and our CE–ESIMS results obtained as the average of four replicate analyses are compared in Table 2. The discrepancy between individual LC and CE–ESIMS data is always less than three times the CE–ESIMS relative standard deviation (RSD), except for aspartate, the concentration of which lies close to the limit of quantitation.

CE–ESIMS is competitive with the commonly used LC procedure as it provides faster analysis: a CE–ESIMS run is only 30 min compared to 90 min for a LC run. CE requires significantly less sample (20 nl) than LC (20 μl). Furthermore, the LC procedure necessitates the tedious use of six consecutive lithium buffers to elute all AAs. Finally, solvent consumption is much lower with CE (about 10 μl/min buffer) than for LC (0.4 ml/min solvent), which also requires post-column reaction of the analytes with ninhydrin.

4. Conclusion

Mass spectrometric detection combined with capillary electrophoresis allows to accept a loss in electrophoretic separation efficiency, as mass spectrometric detection is capable of high selectivity. Although CE–ESIMS is not considered to be a routine technique, this paper describes the precautions necessary to produce a rather easy interfacing of both techniques. It also demonstrates that the limits of detection of CE–ESIMS are adequate for the quantitative analysis of amino acids in biological samples. CE–ESIMS can compete with classical LC in terms of time of analysis and sample and fluid consumption. Furthermore, an on-line preconcentration procedure can be used for enhancement of the method limit of quantitation. In the very near future, CE–ESIMS will benefit from improvements in the ESI probe and atmospheric pressure ionization

Table 2
Comparison between LC and CE–ESIMS results ($n = 4$)[a]

AA	LC conc. (μmol/l)	CE–ESIMS conc. (μmol/l)	CE–ESIMS RSD (%)	Discrepancy |LC–CE–ESIMS| (%)
Gly	178	172	2.1	3.4
Ala	219	207	2.2	5.8
Ser	82	81	1.8	1.2
Pro	154	146	1.8	5.3
Val	180	173	1.3	4.0
Thr	135	132	1.7	2.2
Cys	72	nd	–	–
Leu	105	nd	–	–
Ile	58	nd	–	–
Asn	65	63	1.2	3.1
Asp	5	6	2.6	18.2
Lys	152	143	0.9	6.1
Gln	648	630	1.2	2.8
Glu	47	48	2.0	2.1
Met	29	28	2.5	3.5
His	87	87	2.5	0
Phe	45	43	1.5	4.5
Arg	66	63	2.2	4.6
Tyr	83	81	1.8	1.2
Trp	Not measured	–	–	–

[a] RSD, relative standard deviation; CE–ESIMS, capillary electrophoresis–electrospray mass spectrometry; nd, not detected.

source designs, such as size diminution, nano-flow compatibility and better column temperature control.

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