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ORIGINAL PAPER

Optimization of dynamic pH junction for the sensitive determination of amino acids in urine by capillary electrophoresis

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Abstract A capillary electrophoresis method with UV-absorbance detection was studied and optimized for the determination of underivatized amino acids in urine. To improve concentration sensitivity the utility of in-capillary analyte stacking via dynamic pH junction was investigated with phenylalanine (Phe) and tyrosine (Tyr) as model amino acids. Before sample injection, a plug of ammonium hydroxide solution was injected to enable analyte concentration. Samples were 1:1 (*v*/*v*) mixed with background electrolyte (1 M formic acid) prior to injection. The effect of the injected sample volume, and the injected ammonium hydroxide volume and concentration on analyte stacking and separation performance was investigated. The optimal volume of ammonium hydroxide depended on the injected sample volume. Using a dynamic pH junction good resolution (1.4) was obtained for a sample injection volume of 10% of the capillary (196 nl) with Phe and Tyr dissolved in water. Limits of detection (LODs) were 0.036 and 0.049 μM for Phe and Tyr, respectively. For urine samples, the optimized procedure comprised a 1.7-nl injection of 12.5% ammonium hydroxide, followed by a 196-nl injection of urine spiked with Phe and Tyr. Satisfactory resolution was obtained and amino acid peak widths at half height were only 1.6 s indicating efficient stacking. Calibration plots for Phe and Tyr in urine showed good linearity (R^2>0.96) in the concentration range 10–175 μM, and LODs for Phe and Tyr were 0.054 and 0.019 μM, respectively. RSDs for peak area and migration time for Phe and Tyr were below 7.5% and 0.75%, respectively.

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Introduction

The determination of amino acids in biological samples is important for metabolic profiling and the screening of deficiencies in amino acid metabolism. Capillary electrophoresis (CE) has shown to be highly suitable for this purpose, often combined with derivatization to allow UV-detection of all amino acids including those that lack intrinsic chromophores [1–3]. CE with laser-induced fluorescence detection of derivatized amino acids has also been widely applied [4–6]. More recently, capillary electrophoresis–mass spectrometry (CE-MS) has become an established technique, which overcomes the need for derivatization and enables selective and sensitive compound detection, discriminating between analytes and matrix compounds [7–9].

CE is particularly suited for the analysis of amino acids in biomedical samples since it allows analysis of small sample volumes, and elaborate sample preparation is often not required. Besides, CE analysis times are usually shorter than those for liquid chromatography (LC), and CE analysis consumes very small amounts of solvents. Separation of amino acids with CE is based on the charge-to-mass ratio of analytes and is, thus, complementary to reversed-phase (RP) LC. CE is suited for the analysis of highly polar ionogenic compounds which may be difficult to separate with RPLC. Recent reviews on amino acids analysis by CE provide an adequate overview of the potential of CE in this field [10, 11].

The sensitivity of CE with UV-absorbance detection is limited due to the very small injection volumes and short optical pathway for detection. Therefore, in order to allow analysis of low concentrations of amino acids in biological samples, analyte preconcentration will often be needed. For amino acids, in-capillary stacking using a pH junction might be a useful approach to improve detection limits [9, 12–14]. Using this technique, analytes are concentrated in a discontinuous electrolyte system containing solutions with different pHs. Various approaches have been described in literature, and nomenclature varies among authors. In pH-mediated stacking, also called moving chemical reaction boundary, a low-conductivity sample zone is created by titrating ions of the background electrolyte (BGE) with a strong base or acid from the sample zone. In the resulting low-conductivity zone, charged analytes will have a high velocity until they reach the high-conductivity BGE, where they slow down and accumulate at the interface between sample and BGE. In dynamic pH junction, the pH of sample and BGE are chosen in a way that the mobility of the analytes in these two media are opposing, and the analytes stack at the interface between low and high pH [8, 13]. It is also possible to adjust the pH of the sample solution to that of the BGE. A plug of solution with deviant pH is introduced in front of the sample and analytes will stack at the interface between the sample and the injected plug. Since the use of acidic or alkaline sample conditions may not be applicable to certain types of analytes, i.e., to those that are labile in these conditions, in this way one can select stacking conditions suitable for the sample.

A fivefold preconcentration of zwitterionic peptides by dynamic pH junction using CE-UV was first described by Aebersold et al. [15]. The pH of the low concentrated samples was raised by addition of ammonium hydroxide while electrophoresis was performed in citrate buffer (pH 2.5). The zwitterionic peptides had opposing mobilities in sample solution and electrophoresis buffer and stacked at the border between those solutions. Limits of detection (LODs) for the peptides were below 1 ng/ml. Schwer and Lottspeich sandwiched a sample solution between a zone of sodium hydroxide and phosphoric acid to concentrate four peptides in the resulting low-conductivity zone [16]. The method was not named pH-mediated stacking by the authors, but described as a variant of isotachophoresis. Dynamic pH junction was applied for the detection of epinephrine in dental anesthesia using CE-UV by Britz-McKibbin et al. [17]. Quantification was not hindered by presence of NaCl, methylparaben and large quantities of lidocaine, because a basic BGE was used so that the matrix compounds migrated with the electroosmotic flow (EOF), while the zwitterionic epinephrine accumulated at the border between acidic sample solution (pH 3–3.5) and basic BGE (pH 10.1). The LOD for epinephrine was 50 μM. Subsequently, different pH-mediated strategies have successively been applied for many purposes [13, 18–22], including environmental, clinical, and pharmaceutical applications.

For the determination of amino acids several applications have been published in recent years. Wang et al. published a CE-MS method with pH-mediated stacking method for the quantification of 19 amino acids in urine [23]. LODs varied from 0.0116 μg/ml for glutamic acid to 2.38 μg/ml for serine. The method was applied for the analysis of urine of bladder cancer patients and healthy subjects. Mayboroda et al. [7] efficiently concentrated amino acids from urine using a dynamic pH junction in CE–time-of-flight MS, distinguishing healthy volunteers from patients with osteoarthritis. Stacking was achieved by the pre-injection of a small volume of ammonium hydroxide before sample injection. Apart from pH adjustment of the samples, no pretreatment was applied. Ramautar et al. used a similar approach for the analysis of amino acids in human urine by CE–time-of-flight MS [9]. This method was used to stack analytes from a 100-nl injection of spiked urine using a BGE of 1 M formic acid. LODs for amino acids were down to 20 nM, however, the utility of dynamic pH junction for the determination of amino acids in urine had not been evaluated systematically.

In this study, we aimed to optimize dynamic pH junction in terms of maximal percentage of capillary volume that can be filled with sample and the amount of ammonium hydroxide needed for full sample stacking without causing negative effects on the separation. First, conditions for aqueous samples were studied. Subsequently, optimization was directed towards urine samples and sensitivity was assessed.

Experimental

Chemicals

Formic acid and ammonium hydroxide (25%) were from Merck (Darmstadt, Germany). Amino acids (phenylalanine, and tyrosine), polybrene (hexadimethrine bromide, PB), and 25% (*m*/*v*) aqueous solution of poly(vinylsulfonate) (PVS) sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). BGE solution was prepared by dissolving formic acid in Milli-Q water (1 M, pH 1.8). Aqueous solutions of amino acids were prepared with water taken from a Milli-Q water purification system (Millipore, Bedford, MA, USA), and 1:1 mixed with BGE. Human urine samples were provided by a healthy volunteer and

stored at −18 °C until analysis. Prior to analysis, the urine samples were thawed, centrifuged (4,600 rpm) for 1 min, spiked with amino acids and 1:1 (*v*/*v*) mixed with BGE.

Instrumentation and CE conditions

CE was performed on a Agilent Technologies G1600 HPCE (Agilent Technologies, Waldbronn, Germany), equipped with an on-column diode-array detector and a temperature control system. CE Chemstation (Agilent Technologies) was used for instrument control, data acquisition and data handling. Electropherograms were monitored at 200 nm (bandwidth 16 nm). Fused-silica capillaries with an internal diameter of 50 μm had a total length of 100 cm and an effective length of 91.5 cm. Injections were performed hydrodynamically. Injection volumes were calculated using CE Expert 9.0 (version 1.0, Beckman Coulter, Brea, CA, USA). The separation voltage was 30 kV and the capillary was thermostated at 25 °C. For large volume sample injections applying a dynamic pH junction, prior to sample injection a 0.7–21 nl plug of 12.5% ammonium hydroxide was introduced by applying a 50 mbar pressure for 1–25 s. All samples were 1:1 (*v*/*v*) mixed with BGE before injection. Sample injection was performed at 50 mbar for 6.0 s for injecting 0.25% of capillary volume, up to 296.6 s for injecting 13% of capillary volume.

To maintain reproducible migration times, a PB-PVS bilayer capillary coating was applied, which provides a relatively high and pH-independent EOF [9, 24]. These coatings have been shown to be compatible with MS detection [24, 25]. New bare fused-silica capillaries were rinsed with water for 5 min followed by 1 M sodium hydroxide for 15 min, and water for 5 min. All rinses were performed at 930 mbar. Coating was performed by rinsing for 30 min with 10% (*m*/*v*) PB and successively with water for 5 min. Subsequently, the capillary was flushed with 5% (*v*/*v*) PVS for 30 min, and again with water for 5 min. At the start of the day, coated capillaries were flushed with water for 1 min and with BGE for 2 min. Between runs, the coated capillaries were flushed with 5% (*v*/*v*) PVS for 2 min and with BGE for 5 min.

Results and discussion

Proof of principle

For method development, two UV-absorbing amino acids, phenylalanine (isoelectric point, 5.91) and tyrosine (isoelectric point, 5.63), were chosen as test compounds. Formic acid was selected as BGE as it was shown to be useful for the CE analysis of amino acids and compatible with MS detection [23, 26–28]. To allow efficient stacking, a formic acid concentration of 1 M was chosen (pH 1.8), to ensure that all amino acids potentially present in samples were positively charged.

Amino acids were first analyzed without applying a stacking procedure to define a starting point. A mixture of phenylalanine (Phe) and tyrosine (Tyr) was prepared in water and 1:1 (*v*/*v*) mixed with BGE. This solution was injected hydrodynamically to fill 0.25 to 1.0% of the capillary volume. Upon separation at 30 kV, Phe migrated at 6.4 min, followed by Tyr at 6.5 min (Fig. 1). When the injection volume was increased, the peaks became wider, starting at 1.2 s for the 0.25% injection rising to 4.0 s for the 1.0% injection. The tops of the peaks started to level off for the 0.75% and 1.0% injections (i.e., 14.7 and 19.6 nl, respectively) and resolution deteriorated.

Subsequently, the effects of a pH junction on stacking efficiency and resolution were investigated. For comparison reasons, we did not alter sample composition, injected sample volume, and separation conditions. Prior to a 19.6-nl sample injection, 2.6 nl of 12.5% (*v*/*v*) ammonium hydroxide was injected, thus creating a pH discontinuity in the capillary. The ammonium hydroxide solution was highly concentrated to keep required volumes small and injection times short. Migration times of the amino acids became 0.5 min longer due to the stacking procedure. In the resulting electropherogram (Fig. 2), the peak areas were comparable to those obtained without pH junction (Fig. 1), whereas peaks were considerably more narrow and three times higher. Since the difference in migration time between the model amino acids hardly changed, resolution was increased from 0.85 to 2.1. Peak widths became smaller upon applying a dynamic pH junction, leading to improved LODs (Table 1). The applied dynamic pH junction proved to be a promising method to enhance concentration sensitivity.

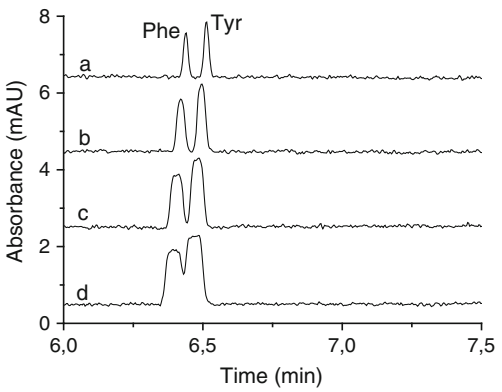

Fig. 1 CE-UV of a mixture of Phe and Tyr (20.0 μM each) using an injection volume of *a* 4.9, *b* 9.8, *c* 15, and *d* 19 nl. Experimental conditions: BGE, 1 M formic acid; injection pressure, 50 mbar; injection time, *a* 6.0, *b* 11.9, *c* 17.9 s, and *d* 23.8 s. For further conditions, see the "Experimental" section

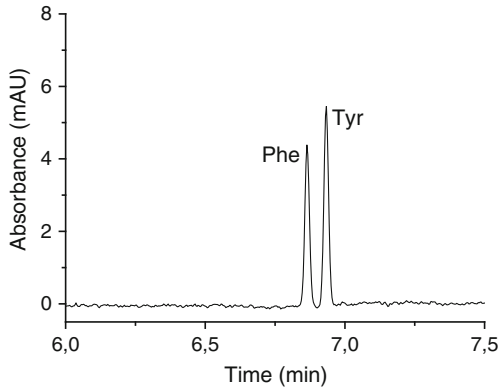

Fig. 2 CE-UV of a mixture of Phe and Tyr (20 μM each) with application of a dynamic pH junction. Experimental conditions: BGE, 1 M formic acid; injection pressure, 50 mbar; injection time, 23.8 s;12.5% ammonium hydroxide injection volume, 2.6 nl. For further conditions, see the "Experimental" section

Large volume injections

Ramautar et al. [9] filled 4% of the total capillary volume with urine sample, and narrow amino acid peaks were obtained after a dynamic pH junction procedure was applied. To investigate the maximum sample injection volume that still can be efficiently concentrated using a dynamic pH junction, we performed CE-UV measurements with sample injections filling 5–15% of the capillary volume. Amino acids were dissolved in water and 1:1 (*v*/*v*) mixed with BGE. The total injected amount of Phe and Tyr was kept constant for all injections, to facilitate comparison. Prior to sample injection, a volume (1.7–21 nl) of 12.5% ammonium hydroxide was brought into the capillary. The injected volume of ammonium hydroxide needed for efficient stacking of amino acids appeared to be dependent on the volume of the injected sample. Moreover, the amount of injected ammonium hydroxide influenced migration times, and resolution of the two model amino acids.

When a sample injection of 10% of capillary volume was performed without prior ammonium hydroxide injection, the amino acids were not concentrated and appeared in the electropherogram as a broad band with a width at half height of 32 s (data not shown). When a sample injection of 10% of capillary volume was performed preceded by 1.7 nl of ammonium hydroxide solution, two peaks could be observed for Phe and Tyr followed by an elevated baseline (Fig. 3a). Some analyte concentration occurs; however, a significant part of the amino acids has not been stacked due to the small ammonium hydroxide volume and consequently migrates as a broad band. Upon increasing the volume of ammonium hydroxide to 3.4–6.9 nl, the broad band diminishes and stacking efficiency improves, resulting in higher peaks for Phe and Tyr (Fig. 3b–d). For injected volumes of ammonium hydroxide of 8.6 nl and higher, the peaks of Phe and Tyr start to overlap and eventually fully coincide (Fig. 3e–i). Moreover, both peaks approach a large disturbing band that migrates with the EOF after 7.4 min and even start to overlap with this band when 15 nl of ammonium hydroxide is used.

From these experiments, it was concluded that the optimal stacking volume of 12.5% ammonium hydroxide for a 10% capillary volume injection (i.e. 196 nl of sample) was 6.9 nl (Fig. 3d). For sample injections of 5–15% of the capillary volume, a very similar dependence on ammonium hydroxide volume was observed (Table 2). Upon injecting less than the optimal volume of ammonium hydroxide, low peaks and broad bands for the amino acids were observed. Too-high volumes of ammonium hydroxide solution caused deteriorated resolution.

For the determination of LODs, peak heights obtained for Phe and Tyr using 6.9 nl ammonium hydroxide solution for 196 nl of sample, were measured in the range 0.1–

Table 1 Limits of detection (μM) for amino acids dissolved in water

Conditions	Phe	Tyr
1% of capillary volume without dynamic pH junction	3.2	2.7
1% of capillary volume with dynamic pH junction	1.4	1.1
10% of capillary volume with dynamic pH junction	0.036	0.049

LOD was calculated for *S*/*N*=3. For dynamic pH junction, 2.6 (for 1%) or 6.9 nl (for 10%) of 12.5% ammonium hydroxide was injected prior to sample injection. Injected sample volume, 19.6 nl for 1% of capillary volume, and 196 nl for 10% of capillary volume

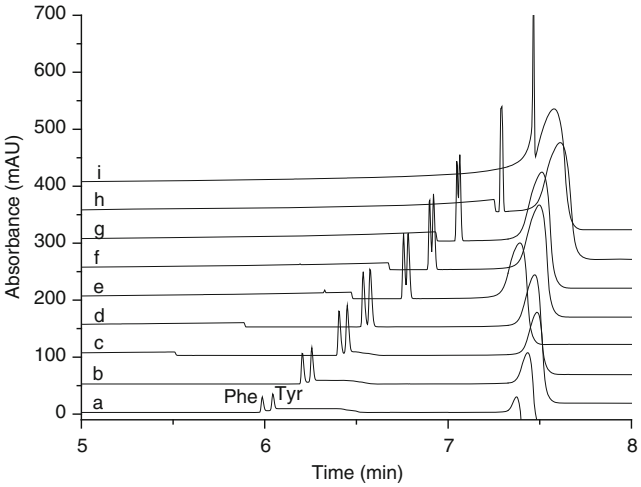

Fig. 3 CE-UV of a mixture of Phe and Tyr using a dynamic pH junction applying an ammonium hydroxide volume of *a* 1.7, *b* 3.4, *c* 5.2, *d* 6.9, *e* 8.6, *f* 10, *g* 12, *h* 14, and *i* 15 nl. Experimental conditions: BGE, 1 M formic acid; amino acid concentration, 42.0 μM each; injection pressure, 50 mbar; sample injection time, 228.2 s. For further conditions, see the "Experimental" section

Table 2 Required volume of 12.5% ammonium hydroxide for efficient preconcentration

Percentage of capillary volume filled, injected sample volume	Volume of 12.5% ammonium hydroxide
5% (98 nl)	4.2 nl
10% (196 nl)	6.9 nl
15% (294 nl)	18 nl

Samples were prepared in water and 1:1 (*v*/*v*) mixed with BGE. Total injected amount of Phe was 4.1 nmol

2.0 μM (Table 1). The established LODs were considerably lower than those obtained for sample injections of 1% of capillary volume. The gain in sensitivity is higher than the factor 10 that can be expected considering the larger sample volume only. This can be explained by the peak narrowing that occurs during the dynamic pH junction phase. Peak widths for Phe and Tyr for sample injections of 1% of capillary volume without application of the dynamic pH junction were 4.0 s (Fig. 1d). In sample injections of 10% of capillary volume, applying a dynamic pH junction resulted in peak widths of 0.80 s and 1.0 s for Phe and Tyr, respectively (Fig. 3d). These data demonstrate that the procedure for in-capillary concentration is efficient.

Spiked urine

The suitability and performance of the dynamic pH junction method was further investigated for the analysis of amino acids in urine. The required volume of 12.5% ammonium hydroxide to efficiently stack the amino acids from urine, without causing deteriorative effects on resolution, was investigated. Urine spiked with Phe and Tyr (42.0 μM each) was 1:1 (*v*/*v*) mixed with BGE for pH adjustment, and injected in the capillary (10% of the capillary volume) preceded by different volumes of 12.5% ammonium hydroxide solution. When no ammonium hydroxide was injected (Fig. 4a), no amino acid peaks could be discerned. Standard addition showed that both Phe and Tyr had a migration time of about 6.8 min. Upon injecting 1.7 nl ammonium hydroxide (Fig. 4b), the test amino acids were efficiently stacked and baseline resolution was achieved. Surprisingly, the required volume of ammonium hydroxide solution was much smaller than the volume needed for efficient concentration of amino acids in water. When 3.4 nl ammonium hydroxide solution was injected (Fig. 4c), the separation of Phe and Tyr became worse, i.e., their resolution was reduced from 1.7 for 1.7 nl ammonium hydroxide to 1.1 for 3.4 nl.

The injection of larger ammonium hydroxide volumes also resulted in a smaller separation window in which components can be detected (Fig. 4). When the volume of ammonium hydroxide injections exceeded 3.4 nl, the separation window became increasingly smaller, reducing the amino acid resolution even more. So it appeared that 1.7 nl 12.5% ammonium hydroxide solution was optimal for a 196-nl spiked urine injection (10% of capillary volume). The required volume of 12.5% ammonium hydroxide solution was also investigated for sample injections exceeding 10% of capillary volume. For a 216-nl injection (11% of capillary volume), the optimal volume of ammonium hydroxide was 2.6 nl, and for a 255-nl injection (13% of capillary volume) it was 3.4 nl ammonium hydroxide. For these large sample volumes and required larger ammonium hydroxide volumes, a noticeably smaller separation window was observed. Resolution for Phe and Tyr for sample injections of 11 and 13% of capillary volume were 1.2 and 1.0, respectively, compared to 1.7 for sample injections of 10% of capillary volume.

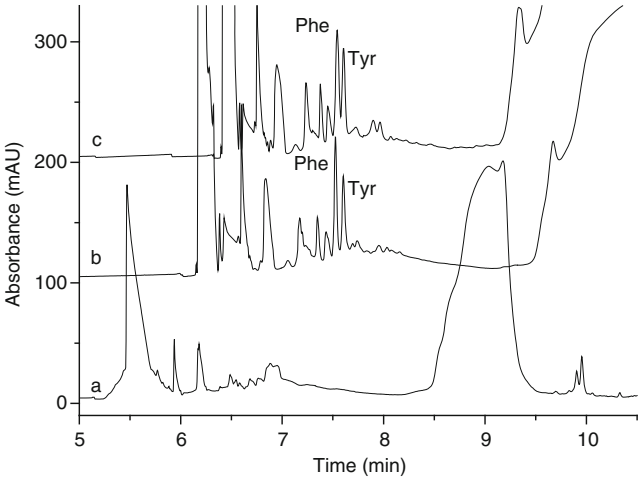

Fig. 4 CE-UV of urine spiked with Phe and Tyr (42 μM each) using an ammonium hydroxide injection volume of *a* 0, *b* 1.7, and *c* 3.4 nl. Experimental conditions: BGE, 1 M formic acid; injection pressure, 50 mbar; sample injection time, 228.2 s. For further conditions, see the "Experimental" section

For Phe and Tyr dissolved in water, peak widths were 0.80 and 1.0 s (Fig. 3d), respectively. For urine samples, the Phe and Tyr peaks were wider, namely both 1.6 s (Fig. 4b). Water-dissolved amino acids also undergo stacking due to

Table 3 Limits of detection (μM) for amino acids in spiked urine

Conditions	Phe	Tyr
1% of capillary volume without dynamic pH junction	1.8	1.3
1% of capillary volume with dynamic pH junction	0.73	0.72
10% of capillary volume with dynamic pH junction	0.054	0.019

LOD was calculated for *S*/*N*=3. For dynamic pH junction, 0.85 (for 1%) or 1.7 nl (for 10%) of 12.5% ammonium hydroxide was injected prior to sample injection. Injected sample volume, 19.6 nl for 1% of capillary volume, and 196 nl for 10% of capillary volume

lower conductivity of the sample compared to that of the BGE. This field-amplified sample stacking-effect is absent or less potent with urine, which is a highly conductive matrix.

Validation

For optimal stacking via a dynamic pH junction in CE-UV of urine a 196-nl injection (10% of capillary volume) and a preceding plug of 12.5% ammonium hydroxide solution of 1.7 nl was selected. Using these conditions, calibration plots for Phe and Tyr in urine were constructed in the concentration range 10–175 μM, yielding determination coefficients (R^2) of 0.964 and 0.983 for Phe and Tyr, respectively. RSDs for peak areas were 7.3% and 6.4%, for Phe and Tyr in urine samples, respectively (42.0 μM each). RSDs for migration times for the same samples were 0.74% and 0.65%, respectively (n=4). For the determination of LODs in urine, peak heights of Phe and Tyr were measured in the range 0.025–20 μM and corrected for endogenous Phe and Tyr levels. The corrected peak heights were used for calculating the LODs as the concentration producing a signal-to-noise ratio (S/N) of 3. When the injected sample volume was 1% of capillary volume, stacking via dynamic pH junction improved the LODs with a factor 2 to 2.5, as was also the case for amino acids in water (Table 3). Upon injecting a ten times larger sample volume, the LODs decreased to 0.054 and 0.019 μM for Phe and Tyr, respectively. As was the case for samples in water, LODs were even more advantageous than expected. The applied pH junction seems to be more effective for larger samples, resulting in lower LODs. The observed differences in LODs between urine and water are small (Tables 1 and 3). Apparently, the CE system provides an efficient separation of the target amino acids from matrix components, so that LODs in urine are similar to those obtained in water.

Conclusion

In this work, the utility of a dynamic pH junction for the on-line concentration of amino acids was evaluated. Sample pretreatment was confined to 1:1 (v/v) mixing with BGE. The efficiency of the stacking process was greatly influenced by the amount of ammonium hydroxide injected prior to sample injection. Upon injecting samples of 10% of capillary volume peak widths for Phe and Tyr in spiked urine, which contains considerable amounts of endogenous amino acids, increased to 1.6 s, still indicating efficient concentration of the amino acids. In this way, analysis down to 19 nM Tyr and 54 nM Phe in urine samples is possible, compared to 1.3 μM Tyr and 1.8 μM Phe without dynamic pH junction. These LODs are quite favorable when considering that sample pretreatment was minimal (1:1 mixing with BGE) and simple conventional UV-absorbance detection was applied without derivatization of the amino acids. For CE-MS of underivatized amino acids, LODs have been reported to be in the 0.1–14 μM range [28]. It would be interesting to apply the presented pH junction preconcentration in CE-MS to improve sensitivity. Using pre-column derivatization with fluorescent labels (e.g. FITC) in combination with special laser-induced fluorescence detectors, LODs for amino acids can be brought down to 0.01 nM [10]. Common amino acid analysis encompasses ion-exchange liquid chromatography with post-column derivatization yielding LODs in the picomolar range [29]. This can partly be attributed to the relatively large injection volumes in LC. However, this methodology is rather complex and laborious, and cannot be combined easily with ESI-MS.

This study shows that stacking of analytes via a dynamic pH junction is a promising procedure to enhance the sensitivity of CE. More research needs to be done on the large difference in required volume of 12.5% ammonium hydroxide between water-dissolved samples and spiked urine. In the future, the described procedure may also be applied to other biological samples like CSF. The necessary volume ammonium hydroxide needs to be optimized again for other matrices. The method is compatible with MS detection as volatile BGE components were selected. A great advantage of MS detection above UV-absorbance detection is the possibility to detect all amino acids including those that lack chromophores. Sample injections exceeding 10% of capillary volume may also be possible, since MS detection is selective and full amino acid resolution is not required.

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