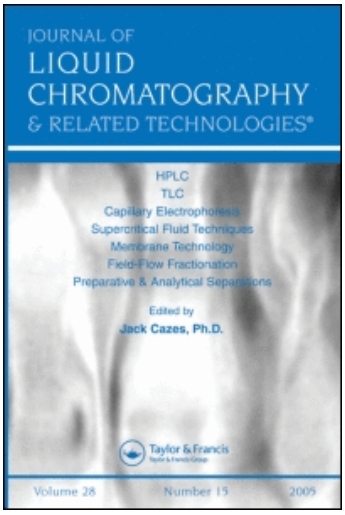

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Christian W. Klampfl[a]; Thuy Diep Thanh Vo[a]
[a] Department of Analytical Chemistry, Johannes Kepler-University Linz, Linz, Austria

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Comparison of Capillary Zone Electrophoretic Techniques Combined with Indirect UV, Direct UV, and Mass Spectrometric Detection for the Determination of Underivatized Amino Acids and Vitamin B_6 in Infusion Solutions

Christian W. Klampfl* and Thuy Diep Thanh Vo

Department of Analytical Chemistry, Johannes Kepler-University Linz, Linz, Austria

ABSTRACT

Different methods based on capillary zone electrophoresis (CZE) for the determination of underivatized amino acids and vitamin B_6 in infusion solutions are described and compared. Acidic and basic carrier electrolytes have been investigated with respect to their suitability for the separation of the selected analytes. Focusing on detection, indirect and direct UV detection at different wavelengths, as well as mass spectrometric (MS)

*Correspondence: Christian W. Klampfl, Department of Analytical Chemistry, Johannes Kepler-University Linz, Altenbergerstr. 69, A-4040 Linz, Austria; E-mail: christian.klampfl@jku.at.

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detection have been tested, with the main emphasis set on their ability to provide quantitative results for all analytes in a single run. Finally, an acidic carrier electrolyte based on phosphoric acid and direct UV detection at 195 nm, were found to be the best suited solution for the determination of the six amino acids and vitamin B_6 in the investigated real samples.

Key Words: Capillary electrophoresis; Mass spectrometric detection; Amino acids; Infusion solutions; Direct UV; Indirect UV.

INTRODUCTION

Amino acids are important organic compounds, which are routinely analyzed in a variety of different matrices. If multi-component analysis is required, as well as in the case of samples with problematic matrices, separation techniques have to be employed, not only for the resolution of the particular analytes but also for the separation of the selected solutes from interfering matrix components. Besides chromatographic techniques in particular high-performance liquid chromatography (HPLC),[1–6] electrophoretic methods like capillary electrophoresis (CE)[7–10] play an increasingly important role in the separation of these compounds within the last years. In most cases, the actual determination of these solutes, either by chromatographic or electrophoretic means, is performed after a more or less time consuming derivatization procedure. This is necessary to enhance the quality of the separation and, even more important, to obtain sufficient sensitivity in the detection of these molecules by direct UV detection or fluorescence detection. In addition to the experimental effort connected with this derivatization procedure, problems related to insufficient reproducibility of the derivatization reaction or variations in the quality of the derivatization reagent employed can also occur. For this reason, several attempts have been made regarding the determination of underivatized amino acids in different matrices within the last few years. Reports have been published describing the use of capillary zone electrophoresis (CZE) for the determination of underivatized amino acids in a variety of samples like foods and beverages,[11–15] pharmaceuticals,[16] and biological fluids.[17] Due to their zwitterionic nature, the separation of amino acids by CZE can be achieved in an anionic mode (i.e., a strongly basic carrier electrolyte and deprotonated analytes) or the cationic mode using a strongly acidic electrolyte resulting in the protonation of the solutes. Reports describing both approaches can be found in the literature.[10]

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One major advantage of acidic carrier electrolytes is the possibility to separate the isomeric pair Leu and Ile, which cannot be achieved when basic running buffers are used.[18] In addition to these investigations, different methods of detection, including indirect and direct UV detection and mass spectrometric (MS) detection have been tested with respect to their suitability for the determination of underivatized amino acids. Advantages of indirect UV detection using a carrier electrolyte with an UV absorbing probe are the more or less equal sensitivity for all amino acids, as well as the possibility to detect at higher wavelengths, which are available with almost all UV detection units commonly present in CE instruments.[11,16] If direct UV detection is chosen, low detection wavelengths (200 nm and less) have to be used, which often require special instrumentation (e.g., detection at 185 nm). This is due to the lack of suitable chromophores absorbing at higher wavelengths in the case of aliphatic amino acids.[13–15] Focusing on the sensitivity of direct UV detection, good limits of detection (LOD) can be achieved for aromatic amino acids and those carrying an additional chromophore, e.g., Arg, whereas the LODs obtained for the aliphatic species are less favorable and can be found in the range of 5–20 mg L^{-1}.

Nevertheless, employing an acidic carrier electrolyte and direct UV detection, 19 out of 20 amino acids including the pair Leu/Ile could be separated in a single CZE run.[18] Even better results could be achieved coupling CZE to MS detection using an electrospray ionization (ESI) interface.[12,17,19] This approach involves a series of benefits over the commonly used UV detection including improved certainty for peak assignment because of the additional structural information provided by the MS detector, increased sensitivity for most amino acids, and reduced effort with respect to the optimization of the separation parameters. The latter advantage is caused by the ability of the MS detection to evaluate the signals of solutes that are resolved incompletely or even co-migrating. Nevertheless, this only applies to amino acids that can be detected at different mass/charge ratios and not to isobaric substances like Leu and Ile. Unfortunately, MS detection in combination with CZE is far away from being a routine technique. The often increased effort in method development, the not yet sufficient ruggedness of this hyphenated technique, and last but not least the high costs of such systems, are still an obstacle towards the implementation of this combination in routine labs.

The aim of this work is to compare the suitability of different CZE methods employing acidic and basic carrier electrolytes in combination with direct and indirect UV detection, as well as MS detection for the determination of underivatized amino acids and vitamin B_6 in infusion solutions. A major challenge within this task are the big concentration differences between the analytes in the selected samples.

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EXPERIMENTAL

Instrumentation

The CZE instruments employed were a Quanta 4000 (Waters, Milford, MA) equipped with a positive power supply and a fixed wavelength detector (mercury lamp, 185 nm) connected to a Chromeleon data acquisition system (Dionex, Sunnyvale, CA), and a HP-3D CE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array UV detector, and a HP-Chemstation for data handling. For CE-MS experiments, a quadrupole system HP 5989B using a pneumatically assisted ESI interface HP 59987A (Hewlett Packard, Palo Alto, CA) equipped with a CE-probe and a RF-only hexapole (Analytica of Branford, Branford, CT) was coupled to the HP-3D CE instrument. The sheath liquid was delivered at a flow rate of 4 $\mu L\,min^{-1}$ by a syringe pump (Model 22, Harvard Apparatus, South Natick, MA); no spraying gas was used. Separations were carried out using fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ) with an inner diameter of 50 μm and an effective length of 70 cm (Quanta 4000), 70 cm (HP-3D CE with UV detection), and 75 cm (HP-3D CE with MS detection). In the case of UV detection, a window at a position of 8 cm (Quanta 4000) or 8.5 cm (HP-3D CE) from the end was created by burning the polyimide coating of the capillary. Injection was performed hydrostatically at the anodic side by elevating the sample at 10 cm for 15 seconds (Quanta 4000) or by applying a pressure of 50 mbar for 3 s (HP-3D CE).

Reagents and Chemicals

All chemicals, were of analytical-reagent grade. High purity water of 18 MΩ obtained from a Milli-Q System (Millipore, Marlborough, MA) was used throughout this work. Running buffers were obtained by diluting phosphoric acid with high purity water, adjusting the pH value with LiOH, and adding the appropriate amount of the alkylsulfonate. Electrolytes for MS detection were prepared by dilution of formic acid with high purity water. The sheath liquid consisted of 0.25% formic acid in 2-propanol/water (8/2). All chemicals and standard substances were obtained from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

The infusion solutions selected for this study contained six amino acids and vitamin B_6. A typical composition given on the label of such a formulation

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Table 1. Typical composition of an infusion solution included in the present study.

Peak #		Concentration ($mg\,L^{-1}$)
1	Arginine (Arg)	15,000
2	Ornithine (Orn)	5,000
3	Valine (Val)	7,000
4	Isoleucine (Ile)	8,500
5	Leucine (Leu)	9,500
6	Aspartic acid (Asp)	5,000
7	Vitamin B_6 (B_6)	120

is depicted in Table 1. As can be seen from this table, the selected analytes cover a relatively wide range of concentrations. Based on findings from previous studies dealing with the separation of underivatized amino acids, three different separation and detection systems were investigated in detail. Main emphasis was set on the quantitative determination of all selected analytes in a single run with sufficient precision.

Alkaline Carrier Electrolyte with Indirect UV Detection

Based on work published by Lee and Lin,[16] an alkaline carrier electrolyte (5 mM 4-aminosalicylic acid at pH 11) with indirect UV detection at 266 nm was tested for the determination of the selected solutes in the infusion solution. As already mentioned in the introduction to this paper, indirect UV detection of underivatized amino acids provides several benefits over direct detection, including improved LODs and similar detection sensitivity for most amino acids. Unfortunately, the concept employing a strongly basic electrolyte and the separation of these solutes in a counter-electro-osmotic mode is affected with some important disadvantages: firstly, the pair Ile/Leu (which are both present in the selected sample) cannot be separated under these conditions; secondly, Arg as a major constituent of the selected sample migrates together with the electro-osmotic flow (EOF), a fact preventing the accurate quantitative determination of this compound; and thirdly, vitamin B_6 also does not carry any charge at pH 11, so that it comigrates with Arg. Considering these facts, this method only allows the quantitative determination of Val, Orn, and Ile/Leu as a sum peak.

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Acidic Carrier Electrolyte with Direct UV Detection

Phosphate electrolytes (pH 2–2.5), together with the addition of alkylsulfonates and direct UV detection at 185 nm, have been successful in the separation and detection of 19 underivatized amino acids in a single run.[18] Focusing on the analytes of infusion solutions selected in the present work, direct UV detection is affected with a major disadvantage. Whereas, excellent sensitivity can be obtained for vitamin B_6 and also Arg, a distinctly lower detector response is obtained for the other amino acids present in this sample. This is due to the large differences in the absorbance coefficients of these compounds. Although, all analytes of interest could be separated and also detected employing this system, no quantitative determination of Arg and the other constituents in a single run was possible. Either the signal obtained for Arg was outside the linear range of the detector, or in the case of further dilution of the sample, less absorptive analytes like Val were below their limit of quantification (LOQ). Nevertheless, analyzing two different dilutions of each sample allowed the quantification of all selected solutes. A way to overcome this problem would be to use different detection wavelengths for Arg and the other analytes. Unfortunately, the instrument employed only allowed single wavelength detection either at 185, 214, or 254 nm. Switching to an instrument with diode array detection (which unfortunately does not allow detection below 195 nm) this approach could be used. It was affected with some reduction in sensitivity, especially for the aliphatic amino acids, as can be seen from the data listed in Table 2. Figure 1 shows an electropherogram obtained for a infusion solution after a 7-fold dilution with water employing direct UV detection at 195 nm. As can be seen from this figure, all analytes of interest could be determined within 16 min.

Acidic Carrier Electrolyte with Mass Spectrometric Detection

Mass spectrometric detection provides a series of benefits over the commonly employed UV detection. Especially in the case of underivatized amino acids, electrophoretic resolution of these compounds with often similar chemical structures is an important issue. Due to its capability to resolve overlapping peaks spectroscopically, MS detection often allows the use of less complicated electrolyte systems. Nevertheless, summing up the effort for the optimization of separation and detection, setting up a system with MS detection can be regarded as the more time consuming task. Focusing on the selected sample, MS detection provided shorter analysis times and in accordance with previous investigations[12,19] LODs, which are almost an order of

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Table 2. Comparison of performance at different detection wavelengths using methods[a] with direct UV detection.

	185 nm				195 nm			
	Concentration ($mg\,L^{-1}$)	Deviation[b] (%)	RSD[c] (%)	LOD[d] ($mg\,L^{-1}$)	Concentration ($mg\,L^{-1}$)	Deviation[b] (%)	RSD[c] (%)	LOD[d] ($mg\,L^{-1}$)
Arg	—[e]	—	—	1	14,750	−2	8	40
Orn	4,840	−3	3	50	5,096	+2	5	200
Val	6,728	−4	5	50	7,174	+2	3	200
Ile	8,156	−4	5	50	8,936	+5	2	250
Leu	9,152	−4	2	50	8,776	−8	5	250
Asp	5,393	+8	7	100	5,285	+6	5	500
B_6	132	+10	2	3	104	−14	8	5

[a]50 mM phosphoric acid, 35 mM octanesulfonate (pH 2.2 with LiOH).
[b]Deviation from label specifications.
[c]Three determinations.
[d]Three times noise.
[e]Signal exceeding the linear range of the detector.

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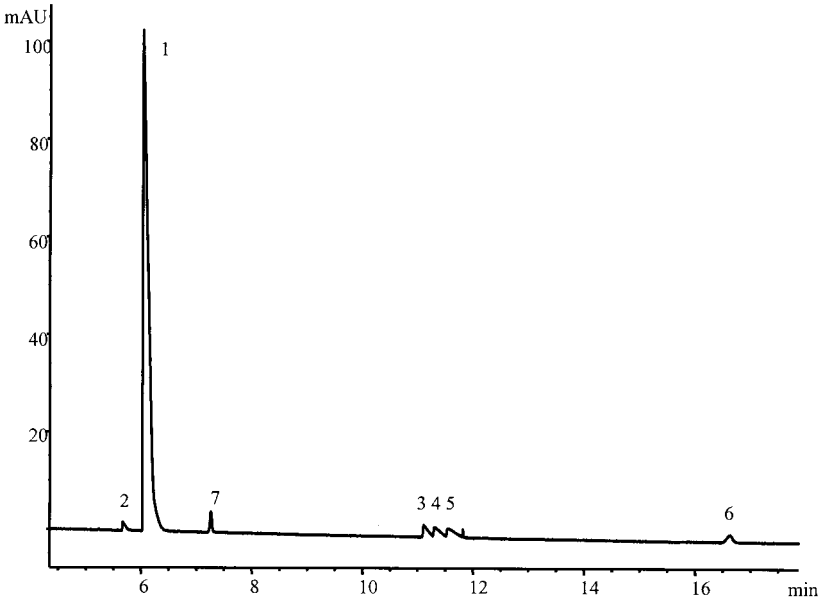

Figure 1. Electropherogram obtained for the infusion solution after a 1 : 7 dilution with deionized water employing CZE with direct UV detection at 195 nm. Experimental conditions: capillary, fused silica 70 cm × 50 µm I.D.; mobile phase, 50 mM phosphoric acid; 25 mM octanesulfonic acid, pH adjusted to 2.2 with LiOH; applied potential, 25 kV; injection, 12 s at 10 mbar; peaks, see Table 1.

magnitude better than those achieved with direct UV detection. In Fig. 2 the electropherogram obtained for the selected sample after a 50-fold dilution with water is depicted. The instrument was operated in the single ion monitoring (SIM) mode recording the mass/charge ratios for the selected analytes. The total ion current (TIC) on top of this figure can be understood as an accumulation of all SIM traces. Unfortunately, MS detection also suffers from the problem that not all analytes of interest can be determined in a single run. Dilution of the sample to an extent that allows the sufficient separation of the pair, Ile/Leu leads to a peak for vitamin B_6 below the LOD. On the other hand, in the case of less diluted samples, no resolution of Ile/Leu was obtained.

Other Tested Carrier Electrolytes

In addition to the investigations described above, several other separation and detection systems were tested. An interesting approach would be to combine

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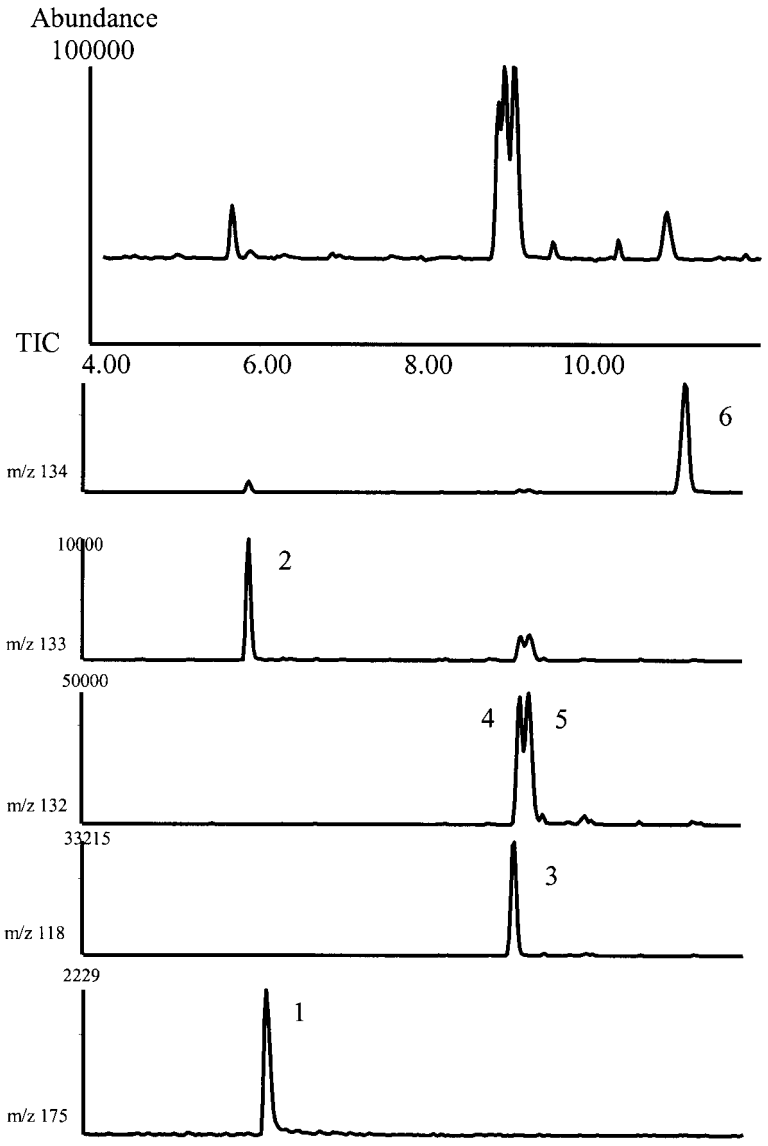

Figure 2. Capillary zone electrophoresis-mass spectrometric electropherogram obtained for the infusion solution after a 1 : 7 dilution with deionized water. Experimental conditions: capillary, fused silica 75 cm × 50 μm I.D.; mobile phase, 300 mM formic acid; applied potential, 25 kV; injection, 3 s at 50 mbar; sheath liquid, 4 μL/min of 0.25% formic acid in 2-propanol/water 80/20; peaks, see Table 1.

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the advantages of a separation system with an acidic carrier electrolyte with those of indirect UV detection. For this reason, several electrolyte systems based on different UV absorbing probes including creatinine, imidazole, 4-dimethylaminobenzaldehyde, or 3,5 diaminobenzoic acid were investigated with respect to this issue. Unfortunately, all these attempts did not lead to any acceptable results. Electropherograms obtained with these systems suffered either from excessive baseline noise, disturbing system peaks, or even from both types of interferences. For this reason none of the tested electrolytes could be regarded as suitable for the determination of amino acids in the infusion solution.

Comparison of the Investigated Methods

Table 3 lists the results obtained for the determination of selected solutes in the infusion solution employing the three different methods. Both quantitation using an external calibration and quantitative analysis employing the standard addition method were tested. Although both methods provided satisfactory results, the standard addition method was chosen finally. As can be seen from Table 3, two methods based on a separation with strongly acidic carrier electrolytes and direct UV detection at 195 nm or MS detection, respectively, allowed the quantitative analysis of all amino acids in a single run. Taking into account the additional vitamin present in the sample, only Method 2 (acidic carrier electrolyte, direct UV detection) offers data for all analytes of interest. RSD values were generally less than 10% and deviations from the manufacturers specifications were also below 10%, except for vitamin B_6, which shows a somewhat higher value. For the method employing direct UV detection at 195 nm, linearity was checked over a wide range of concentrations according to the amounts present in the actual infusion solution. As the best results could be obtained for the real sample after a 7-fold dilution, the concentration ranges investigated were 400 to 2200 mg L^{-1} for Arg, 400 to 1100 mg L^{-1} for Orn, 200 to 900 mg L^{-1} for Val, 300 to 1100 mg L^{-1} for Ile, 350 to 1300 mg L^{-1} for Leu, 500 to 1100 mg L^{-1} for Asp, and 4 to 30 mg L^{-1} for vitamin B_6.

CONCLUSIONS

The investigations described in this paper demonstrate that CZE, with either direct UV detection or MS detection, can be regarded as a suitable tool for the determination of underivatized amino acids in real samples with a similar composition as the selected infusion solutions. Regarding the samples used for this study, a strongly acidic phosphate electrolyte and direct UV detection at

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Table 3. Comparison of methods for the analysis of the infusion solution.

	Method 1[a]			Method 2[b]			Method 3[c]		
	Concentration ($mg\,L^{-1}$)	Deviation (%)[d]	RSD (%)	Concentration ($mg\,L^{-1}$)	Deviation (%)[d]	RSD (%)	Concentration ($mg\,L^{-1}$)	Deviation (%)[d]	RSD (%)
Arg	n.d.[e]	—	—	14,750	−2	8[f]	13,539	−10	6[g]
Orn	4,840	−3	10[f]	5,096	+2	5[f]	5,145	+3	3[g]
Val	7,771	+11	1[f]	7,174	+2	3[f]	6,339	−9	9[g]
Ile	17,380[h]	−8[h]	1[f,h]	8,936	+5	2[f]	7,849	−8	5[g]
Leu				8,776	−8	5[f]	9,505	0	6[g]
Asp	n.d.	—	—	5,285	+6	5[f]	4,573	−2	5[g]
B_6	n.d.	—	—	104	−14	8[f]	n.d.	—	—

[a]5 mM 4-aminosalicylate (pH 11), indirect UV detection at 266 nm.
[b]50 mM phosphoric acid, 35 mM octanesulfonate (pH 2.2 with LiOH), direct UV detection at 195 nm.
[c]300 mM formic acid, MS detection (SIM mode).
[d]Deviation from label specifications.
[e]Not determined.
[f]Three determinations.
[g]Five determinations.
[h]Sum peak of Ile/Leu.

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195 nm can be seen as the best suited solution. This method provides low RSD values, only small deviations from the concentration values specified by the manufacturer, and sufficient sensitivity for the selected type of sample. Mass spectrometric detection cannot offer sufficient benefits to justify the increased effort with respect to the instrumentation employed. Nevertheless, the situation can be different for real samples, including a larger set of amino acids like parenteral nutrition solutions where the higher resolving power of MS detection is needed, or samples where the higher sensitivity of MS detection is required.

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