ELSEVIER

Journal of Chromatography A, 680 (1994) 263–270

JOURNAL OF CHROMATOGRAPHY A

Dual electrochemical detection of cysteine and cystine in capillary zone electrophoresis

Betty L. Lin*, Luis A. Colón[1], Richard N. Zare

Department of Chemistry, Stanford University, Stanford, CA 94305, USA

Abstract

A method for the simultaneous determination of cysteine and cystine at the submillimolar level is presented based on the capillary zone electrophoretic (CZE) separation of the analytes coupled to a dual electrochemical detection system. The detector uses two gold/mercury amalgam electrodes placed in series and set at −1.00 V and at +0.20 V vs. Ag/AgCl, respectively. The advantages of the proposed method for the analysis of these compounds include direct detection of both analytes, no elaborate sample preparation, no derivatization procedures, and no pathlength dependence. In addition, the small volumes required for CZE analysis make it suitable for clinical studies. The method was applied to human urine samples.

1. Introduction

The separation and detection of sulfur-containing amino acids is a topic of active interest and has medical applications. For example, hepatic cystinuria is an inherited disorder of amino acid transport characterized by high concentrations of cysteine and cystine in urine (usually >1 m*M* levels) [1]. Several analytical methods have been reported for the specific measurement of these species in urine, such as colorimetry [2,3] and high-performance liquid chromatography (HPLC) followed by spectrophotometric [4–6] or fluorimetric detection [7]. These methods usually require, however, a colorimetric or derivatizing reagent that reacts exclusively with the free sulfhydryl group and not with the disulfidic form. Therefore, cystine must be previously reduced to cysteine before detection, for example, by using dithiothreitol [7], KCN [2,4], sodium sulfite [5,6], or KBH_4 [3].

The use of HPLC with electrochemical detection has also been proposed for the determination of thiols. Initial work on this subject was performed by Rabenstein and Saetre [8], who reported the use of a mercury pool electrode. Allison and Shoup [9] developed a dual-electrode detector for the simultaneous determination of thiols and disulfides. This detector used two gold/mercury amalgam electrodes placed in series. The first electrode, or upstream electrode, set at −1.0 V vs. Ag/AgCl, reduces the disulfides to their corresponding thiols. The second electrode, or downstream electrode, set at +150 mV vs. Ag/AgCl, detects thiols that result from the reduction of disulfides at the first electrode as well as free thiols present in the sample. The main advantages of using this dual-electrode detection system are the direct detec-

* Corresponding author.

[1] Present address: Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214, USA.

SSDI 0021-9673(94)00533-F

tion of analytes, no need for reduction or derivatization before separation, and the provision of measurements independent of pathlength or cell dimensions. Moreover, the selectivity achieved when using gold/mercury amalgam electrodes is enhanced over that when using glassy carbon or gold electrodes because of the low detection potential (+ 150 mV) applied. Further applications of this dual-electrode detector coupled to HPLC have been widely reported for various thiolic and disulfidic compounds in complex matrices [10–13].

Another novel method for electrochemical detection of thiols and disulfides in HPLC is based on the formation of silver mercaptides from free sulfhydryl groups with silver ion in an ammoniacal medium [14,15]. This method again requires the conversion of disulfides into thiols with sulfite or by electroreduction. The sample preparation steps are therefore more elaborate than those with the use of gold/mercury electrodes.

As an alternative to HPLC, capillary zone electrophoresis (CZE) provides specific advantages regarding the analysis of thiols. Applications of HPLC to the detection of thiols are characterized by low column efficiency and often irreproducible results [16–19]. For clinical sample analysis, CZE is especially recommended over HPLC for the low volume of sample required, the absence of packing material, and the minimization of metallic instrumentation that would catalyze the conversion of free thiols to disulfidic forms. CZE has actually been proposed for use in the determination of thiols and disulfides coupled to absorbance [20] and to fluorescence [21] detection. A reduction of the disulfide followed by its derivatization with a fluorigenic reagent prior to CZE is again required in both methods proposed. More recently, O'Shea and Lunte [22] reported the use of gold/mercury amalgam microelectrodes for free thiol detection. Considering the previous advantages reported for CZE separations as well as for the dual electrochemical detection system, this paper presents, to our knowledge, the first time that both analytical methods are coupled together. Two gold/mercury amalgam microelectrodes are placed in series to detect simultaneously the thiolic species cysteine together with its disulfide cystine separated by CZE. Our work builds on the use of gold/mercury amalgam electrodes reported by O'Shea and Lunte combined with the use of the dual-electrode detection system proposed by Allison and Shoup for HPLC separations. The method proposed is direct, specific, and selective for the detection of cysteine and cystine. Its successful application to urine samples is also presented.

2. Experimental

2.1. Chemicals and sample preparation

Cysteine, cystine, EDTA and 2-(N-morpholinoethanesulfonic acid) (MES) were obtained from Sigma (St. Louis, MO, USA). All other compounds were chemically pure and used as received. Water purified with an Ultra-Pure water system (Millipore, Bedford, MA, USA) was used to prepare all solutions. The separation buffer consisted of a 10 m*M* MES solution adjusted at a pH of 5.5 with sodium hydroxide. The standard cysteine solutions were prepared daily in the previously mentioned MES buffer containing 2 m*M* EDTA used to prevent metal-catalyzed thiol oxidation. Cystine had to be dissolved in 10 m*M* HCl because of its poor solubility in aqueous solutions. The urine samples were diluted by a factor of ten in the separation buffer containing EDTA and stored at <4°C without further sample pretreatment. All the solutions were passed through a membrane filter (Nalgene syringe filters, 0.4-μm pore size; Baxter Diagnostic, Hayward, CA, USA) before use.

2.2. Apparatus

Capillary zone electrophoresis

The complete CZE system was built in the laboratory and has been described previously [23]. A high-voltage power supply (Hipotronics, Brewster, NY, USA) set at 20 kV was used to drive the electrophoresis in the capillary. Fused-

silica capillaries (360 μm O.D. × 75 μm I.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). A hole was drilled through the capillary (Laser Machining, Somerset, WI, USA) leaving a distance of ca. 50 cm length at one side and 1 cm on the other side. The samples were injected electrokinetically. The separation was achieved on a 10 m*M* MES buffer, pH 5.5, through which argon gas was bubbled vigorously for 30 min prior to use [22]. A gas purifier (Oxiclear; Supelco, Bellefonte, PA, USA) was used to ensure the purity of the argon before bubbling. The electrophoretic currents reached were approximately 3.5 μA. All the capillaries were cleaned with sodium hydroxide (100 m*M*) before use, followed by water and separation buffer. Whenever urine samples were injected, the cleaning procedure was repeated between runs.

Detection

Constant-potential amperometric detection in combination with CZE was performed using the end-column approach [24]. A mercury battery (Duracell, 1.4 V) provided the required voltage to the working electrode placed at the end of the separation capillary. The Faraday current was amplified using a two-electrode current amplifier (Model 428; Keithley Instruments, Cleveland, OH, USA). All potentials were referenced to the Ag/AgCl electrode (MI-402, 3 *M* KCl saturated with AgCl; Microelectrodes, Londonderry, NH, USA). Data collection was performed using a Lab Calc program. The electrochemical cell used was similar to those reported previously [25]. To decouple the electrophoretic separation field from the electrochemical detection field, three different strategies were tried: a nafion tubing joint [26], a liquid nafion joint [27], and a palladium connector device [28]. Nafion is a cation-exchange polymer membrane that allows the movement of the ions but not of the bulk solution, whereas the palladium connector is a solid-state field decoupler. The construction of each was similar to that described previously except for the palladium connector. This connector was adapted to the dual-electrode detection system as shown in Fig. 1. On one side of the decoupler was placed a 50 cm long fused-silica capillary tubing. On the other side of the decoupler was placed a capillary piece of ca. 3 cm length. This last capillary piece contains the hole at ca. 1 cm from the protruding end of the capillary. At this point, the generator electrode was built.

Dual gold/mercury microelectrode system

The downstream gold/mercury amalgam working microelectrode was constructed similar to those described previously [25] but using a 25 μm diameter gold wire (California Fine Wire, Grover City, CA, USA). This gold electrode was

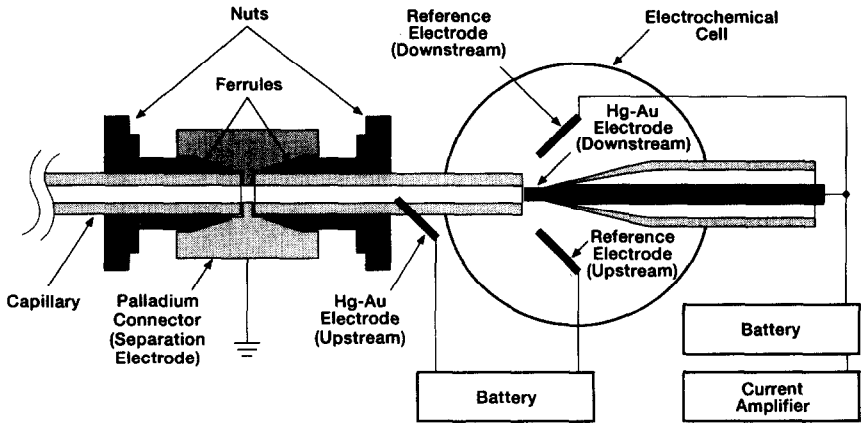

Fig. 1. Schematic diagram of the transversal cross-section of the palladium field decoupler and the dual-electrode detection system.

washed carefully with acetone and deionized water and then dipped into triple-distilled mercury (Aldrich, Milwaukee, WI, USA) for ca. 45 s.

The upstream generator electrode was constructed by inserting a ca. 1 cm piece of gold wire (25 μm diameter) through a hole drilled in the capillary. Epoxy (epoxy 907, Miller Stephenson, Danbury, CT, USA) was applied to seal the wire in place. Once cured, the rest of the protruding gold wire was attached to the end of a 5 cm length copper wire (380 μm diameter) by using conductive silver paint (Epoxy Technologies, Billerica, MA, USA) to achieve electrical connection. Once the silver paint was dry, more epoxy was applied to cover the completed connection. After an overnight curing period, the capillary was flushed with acetone and water to clean the gold electrode. The gold/mercury amalgam was prepared by electrodeposition. A 1.0 *M* KNO_3 with 5.8 m*M* mercurous ion and 0.5% HNO_3 solution was flushed through the column. A deposition potential of -230 mV vs. Ag/AgCl was applied for 5 min. The column was then thoroughly flushed with water to eliminate all traces of the mercurous solution. For the experimental measurements, this electrode was set at -1.0 V vs. Ag/AgCl by means of a mercury battery in a two-electrode configuration. The Ag/AgCl electrode was placed at the outlet buffer vial together with the detection electrodes. For reproducible results, the gold/mercury amalgam required an equilibrium period of ca. 24 h before use, in agreement with previous reports on the use of this electrode [9,22].

3. Results and discussion

3.1. Dual-electrode configuration

We first attempted to optimize the type of microelectrodes used for determining free thiols. According to the literature, gold, glassy carbon and graphite [29] coupled to HPLC have been used to measure thiols. We preferred mercury for the present determinations, however, as the optimum potential for oxidation is relatively low (<0.25 V) so that interferences from other easily oxidized species are avoided. Furthermore, gold/mercury amalgam microelectrodes were recently reported for the successful analysis of free thiols in CZE [22]. The microelectrodes we employed were similar to those reported in the literature except the gold electrode was dipped in mercury for a longer period of time.

Fig. 2 shows the hydrodynamic cyclovoltammogram of the gold/mercury microelectrode with and without cysteine in the buffer electrolyte. A potential of 0.2 V vs. Ag/AgCl was chosen for thiol determination, as it gave the maximum response while it retained the properties of the gold/mercury amalgam. Higher potentials would decrease the selectivity of the determination and remove mercury by electrooxidation.

Various designs were considered for construction of the upstream electrode. The configuration using the palladium connector was shown in Fig. 1. The configurations built at ca. 1 cm after the nafion tubing joint are shown in Fig. 3. In the configuration of Fig. 3a, the upstream electrode and a pseudoreference platinum electrode were placed facing each other on opposite sides of the CZE column. They were both connected to a battery. This first approach did not result in

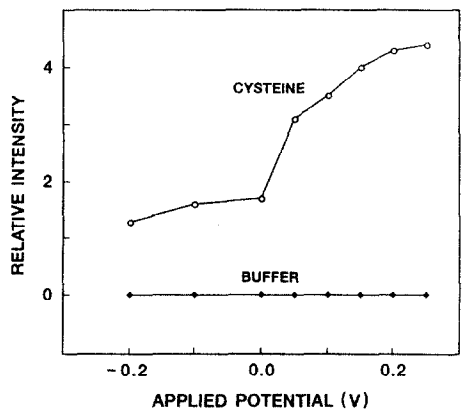

Fig. 2. Hydrodynamic cyclovoltammogram using a gold/mercury microelectrode of (◆) 10 m*M* MES (pH 5.5) and (○) 10 μ*M* cysteine in MES buffer containing 2 m*M* EDTA, CZE capillary: 52 cm × 75 μm I.D., separation voltage: 20 kV, sample injection: electrokinetically 10 s. All potentials are referenced vs. an Ag/AgCl electrode.

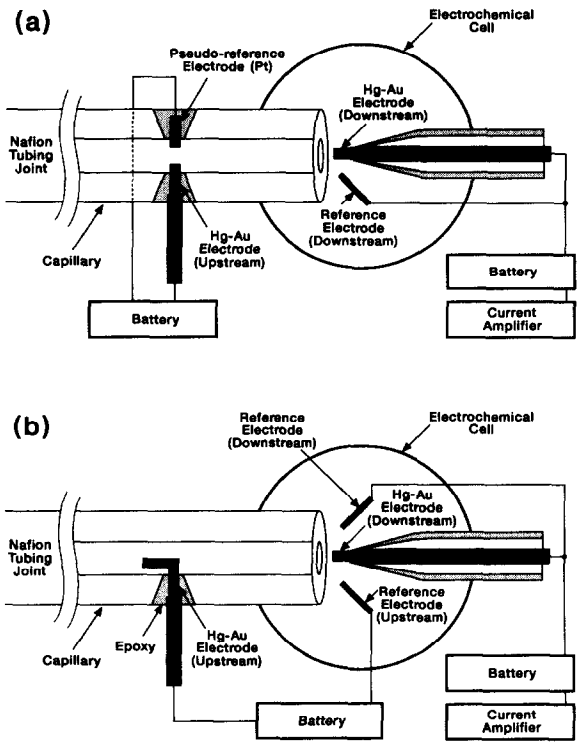

Fig. 3. Schematic diagram of two different designs for the dual-electrode detection system.

any detectable signal for cystine. Apparently, the surface of this upstream electrode was too small for the quantitative reduction of the disulfide into the corresponding thiol.

A second attempt was then made to lengthen the exposed upstream electrode surface. No reference electrode was placed inside the capillary. Instead, only the gold wire was inserted inside the capillary hole, and, with the aid of another gold wire of 50 μm diameter inserted thorough the capillary, it was pushed further inside the capillary to achieve the configuration depicted in Fig. 3b. The Ag/AgCl reference electrode for this upstream electrode was then placed outside the separation capillary, inside the electrochemical cell. This model provided reproducible signals for the determination of both cysteine and cystine and was used in subsequent measurements.

A third design was attempted that used a palladium field decoupler instead of the nafion tubing joint to simplify the handling and construction of the dual electrochemical system. This solid-state decoupler was definitely more useful for the determination of the free thiols by themselves. The selectivity, sensitivity, resolution, and reproducibility of the overall cysteine determination were similar to those of the nafion tubing joint. When this design was applied to the simultaneous determination of thiols and disulfides, however, the upstream electrode had to be placed at the second short piece of the CZE capillary, after the palladium connector and near the electrochemical cell (Fig. 1) so that the separation field would not interfere with the reduction field. All these requirements resulted in the need of at least a ca. 3 cm piece of capillary tubing that protruded through the second part of the connector. Consequently a loss of efficiency resulted from having a distance longer than 2 cm from the separation cathode to the detection cell [30]. No further attempts were therefore made in this direction.

Another approach used liquid nafion in place of nafion tubing to cover the capillary fracture. Liquid nafion was tried out on both configurations depicted in Fig. 3. The need to apply several layers of nafion, coupled with the long periods of solvent drying, made the construction of the capillary field decoupling system relatively troublesome. Moreover, if the capillary was not properly sealed to a support, the nafion could enter through the fracture and block the capillary. As a result, off-column detection using nafion tubing was chosen for the qualitative and quantitative simultaneous determination of cysteine and cystine. This design appeared to be the simplest and provided the most reproducible measurements for our purposes, and the results are reported in detail in the next section.

3.2. Qualitative and quantitative determination of cysteine and cystine

The CZE analysis of both cysteine and cystine was performed in a 52 cm long capillary using 10 m*M* MES buffer, pH 5.5. MES buffer was used because its high resistance provided low detector noise, in accordance with previous reports [30].

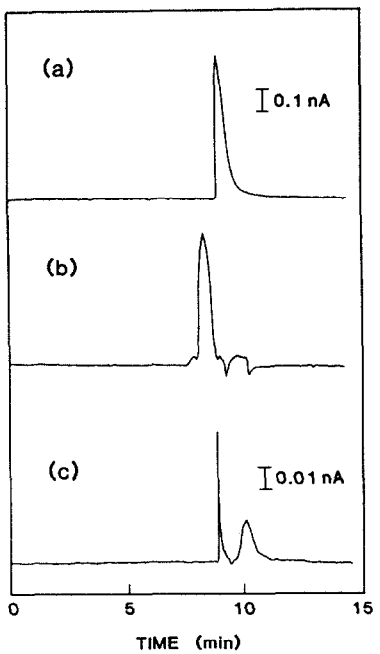

Fig. 4. Electropherograms of (a) 1 m*M* cysteine, (b) 10 m*M* cystine and (c) 10 μM cysteine and 300 μM cystine, all analytes dissolved in 10 m*M* MES pH 5.5 containing 2 m*M* EDTA. Upstream electrode : −1.0 V vs. Ag/AgCl, downstream electrode: + 0.200 V vs. Ag/AgCl. CZE conditions as in Fig. 2.

It was therefore preferred to phosphate, borate and acetate buffers, which were also tried at varying pH values ranging from 3 to 9. Fig. 4 shows the electropherograms of cysteine (a), cystine (b) and a mixture of the two (c). The migration times of the two individual peaks appeared slightly shorter than those of the mixture owing to the difference in time between the injections. After various CZE injections, the heated columns provided shorter migration times for each analyte. The different concentrations of analyte injected accounts for the different peak shapes obtained. A decrease in column voltage improved the separation although it was accompanied by a loss of efficiency and longer analysis times. A separation voltage of 20 kV was used throughout and provided the separation of both analytes in ca. 10 min. Increasing column lengths would result in a similar effect as decreasing the applied separation voltage. An average column length of 52 cm was used. The statistical data obtained from the quantitative study of cysteine and cystine following the proposed method are shown in Table 1. The poor detectability observed for cystine was caused by the low yield of cystine reduction (ca. 8%). The exposed microelectrode surface was relatively small and present only on one side of the capillary. Future work might be aimed at improving the reduction yield of the disulfide by either increasing the exposed upstream electrode surface inside the capillary or by using other dual-electrode designs.

3.3. Application to urine samples

To analyze the potential of this detector for the analysis of cysteine and cystine in real samples, urine samples from healthy volunteers were injected and evaluated. Fig. 5 shows the

Table 1
Statistical data on the quantitative determination of cysteine and cystine following the proposed method

	Cysteine	Cystine
Migration time reproducibility (R.S.D., $n = 10$ runs)	< 1%	< 1%
Migration time reproducibility (R.S.D., $n = 3$ days)	≤ 5%	≤ 5%
Peak height reproducibility (R.S.D., $n = 10$)	4.49%	5.98%
Linearity range (correlation coefficient >0.997, peak height vs. analyte concentration)	5 μM–1 m*M*	100 μM–10 m*M*
Detection limits ($S/N = 3$)	5 μM	100 μM

The specific experimental conditions are described in the text. The reproducibility tests were performed at 100 μM cysteine and 1 μM cystine concentration levels.

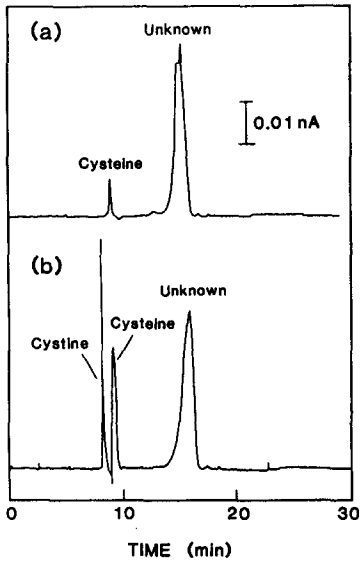

Fig. 5. Electropherogram of (a) urine diluted 1:10 and (b) the same urine sample to which 20 μM cysteine and 500 μM cystine (final concentrations) were added before injection. Conditions as in Fig. 4.

electropherogram of a tenfold dilution of human urine assayed as such (Fig. 5a) and after its spiking with cysteine and cystine at cystine concentration levels of cystinuric patients (Fig. 5b). The mean analytical recovery of spiked cysteine and cystine was 97.5% (S.D. 1.8%, $n = 3$) and 99.3% (S.D. 7.4%, $n = 3$), respectively. In this particular urine sample, a 78 μM concentration of cysteine was found, calculated by standard addition for three different determinations. This concentration is within the normal range reported [7,31]. The second compound of this electropherogram has not yet been identified. Assays from other urine samples diluted and analyzed similarly gave this unidentified peak in 50% of the volunteers. The slight shoulder observed in Fig. 5b and peak splitting observed in Fig. 5a suggests the presence of a rapidly decomposing compound or a mixture of compounds co-migrating at the same time. In any case, the analytes of interest seem to be well resolved from the unknown species. The sensitivity of the disulfide detection may be the subject of further improvement, and the application of this method to urine samples from cystinuric patients as well as to the determination of other thiolic and disulfidic species is envisaged.

Acknowledgements

B.L.L. gratefully acknowledges the Fundación Ramón Areces of Spain for a postdoctoral scholarship. The authors also thank Beckman Instruments, Inc., for support of this work.

References

[1] S. Segal and S.O. Thier in J.B. Stanbury, J.B. Wyngaarden and D.S. Frederickson, J.L. Goldstein and M.S. Brown (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1983, pp. 1774–1791.
[2] W.V. Berg and O. Kilian, *J. Clin. Chem. Clin. Biochem.*, 26 (1988) 223.
[3] S. Kelly, E. Leidhim and L. Desjardins, *Clin. Chim. Acta*, 39 (1972) 4691.
[4] J. Chrastil, *Analyst*, 115 (1990) 1383.
[5] J.C. Crawhall and D. Kalant, *Anal. Biochem.*, 172 (1988) 479.
[6] E.T. Backer, A. van den Ende and W.P. Geus, *Clin. Chim. Acta*, 165 (1987) 351.
[7] H. Birwe and A. Hesse, *Clin. Chim. Acta*, 199 (1991) 33.
[8] D.L. Rabenstein and R. Saetre, *Anal. Chem.*, 49 (1977) 1036.
[9] L.A. Allison and R.E. Shoup, *Anal. Chem.*, 55 (1983) 8.
[10] G.T. Yamashita and D.L Rabenstein, *J. Chromatogr.*, 491 (1989) 341.
[11] J.P. Richie, Jr. and C.A. Lang, *Anal. Biochem.*, 163 (1987) 9.
[12] D. Dupuy and S. Szabo, *J. Liq. Chromatogr.*, 10 (1987) 107.
[13] S.M. Lunte and P.T. Kissinger, *J. Liq. Chromatogr.*, 8 (1985) 691.
[14] T. Kuninori and J. Nishiyama, *Anal. Biochem.*, 197 (1991) 19.
[15] J. Nishiyama and T. Kuninori, *Anal. Biochem.*, 200 (1992) 230.
[16] B.L. Ling, W.R.G. Baeyens and C. Dewaele, *Anal. Chim. Acta*, 255 (1991) 283.
[17] D. Perrett and S.R. Rudge, *J. Pharm. Biomed. Anal.*, 3 (1985) 3.
[18] D. Perrett and S.R. Rudge, *J. Chromatogr.*, 294 (1984) 380.
[19] S.R. Rudge, D. Perret, P.L. Drury and A.J. Swannell, *J. Pharm. Biomed. Anal.*, 1 (1983) 205.

[20] E. Jellum, A.K. Thorsvnd and E. Time, *J. Chromatogr.*, 559 (1991) 455.
[21] B.L. Ling, W.R.G. Baeyens and C. Dewaele, *J. High Resolut. Chromatogr.*, 14 (1991) 169.
[22] T. O'Shea and S.M. Lunte, *Anal. Chem.*, 65 (1993) 247.
[23] M.J. Gordon, X. Huang, S.J. Pentoney Jr. and R.N. Zare, *Science*, 242 (1988) 224.
[24] X. Huang, R.N. Zare, S. Sloss and A.G. Ewing, *Anal. Chem.*, 63 (1991) 189.
[25] L.A. Colón, R. Dadoo and R.N. Zare, *Anal. Chem.*, 65 (1993) 476.
[26] T.J. O'Shea, R.D. Greenhagen, S.M. Lunte, C.E. Lunte, M.R. Smyth, D.M. Radzik and N. Watanabe, *J. Chromatogr.*, 593 (1992) 305.
[27] T.J. O'Shea, S.M. Lunte and W.R. LaCourse, *Anal. Chem.*, 65 (1993) 948.
[28] W.Th. Kok and Y. Sahin, *Anal. Chem.*, 65 (1993) 2497.
[29] E.G. DeMaster and B. Redfern, *Methods Enzymol.*, 143 (1987) 110.
[30] R.A. Wallingford and A.G. Ewing, *Anal. Chem.*, 59 (1987) 1762.
[31] P.R. Parvy, J.I. Bardet, D.M. Rabler and P.P. Kamoun, *Clin. Chem.*, 34 (1988) 2092.