ELSEVIER

Journal of Chromatography A, 671 (1994) 375–382

JOURNAL OF CHROMATOGRAPHY A

# Applications of capillary zone electrophoresis in clinical chemistry
# Determination of low-molecular-mass ions in body fluids

W. Buchberger*, K. Winna, M. Turner

*Department of Analytical Chemistry, Johannes-Kepler-University, A-4040 Linz, Austria*

**Abstract**

Investigations were carried out on parameters influencing separation selectivity in the capillary zone electrophoresis of inorganic cations. Copper sulphate can be recommended as a carrier electrolyte salt for the separation of alkali and alkaline earth metal ions. Its separation selectivity is different from that of the widely used aromatic amines and it is compatible with indirect UV detection at 214 nm. The addition of organic solvents to the electrolyte results in a general increase in the migration times of divalent cations relative to monovalent cations. Ion-pairing reagents such as sodium dodecyl sulphate were found to exhibit specific effects on some ions (especially strontium and barium), but are less useful owing to interferences with the separation of alkali metal ions by sodium introduced with the ion-pairing reagent. Applications to the determination of cations relevant in clinical chemistry are demonstrated for serum samples. Further, the determination of anions in serum was investigated using chromate as electrolyte. Generally, the advantage of capillary zone electrophoresis over ion chromatography can be seen in the fact that proteins need not be removed from the sample and do not interfere with the separation of low-molecular-mass ions.

## 1. Introduction

The determination of inorganic cations (alkali and alkaline earth metal ions) and low-molecular-mass anions in body fluids is of considerable importance for diagnostic purposes and part of the routine tasks in clinical laboratories. Ion chromatographic methods, although capable of separating all of the relevant cations or anions in one run, have not found widespread acceptance. This may be partly due to problems with the lifetime of separation columns if proteinaceous samples such as serum or plasma are injected. Therefore, proteins should be removed before injection, but this requires additional sample preparation procedures, which can be time consuming or result in incomplete recoveries. Recently, the use of a new stationary phase with a semi-permeable surface allowed the direct injection of body fluids for the separation of anions by ion-interaction chromatography [1]. Nevertheless, problems still exist in separating early-eluting ions such as fluoride or organic acids from the proteins eluting in the void volume.

Capillary zone electrophoresis (CZE) should be capable of overcoming several of the drawbacks of chromatography. It is a highly efficient separation method for ionized species based on the combined effects of electrophoresis and electroosmosis and can handle proteinaceous

* Corresponding author.

0021-9673/94/$07.00 
*SSDI* 0021-9673(94)00206-O

samples much better owing to the absence of a stationary phase. Denaturation and precipitation of proteins can easily be avoided by choosing a physiologically compatible carrier electrolyte. Further, this technique is likewise suited if only a limit amount of sample (*e.g.*, a few microlitres of tear fluid) is available.

This paper reports results from investigations into the applicability of CZE to the routine determination of several cations and some anions in different body fluids. Special attention was paid to the parameters affecting the separation selectivity of cations. The influence of different carrier electrolyte salts, of organic solvents and of ion-pairing reagents was investigated in detail.

## 2. Experimental

The CZE instrument employed was a Quanta 4000 (Waters, Milford, MA, USA) equipped with a negative and a positive high-voltage power supply and interfaced to a Hewlett-Packard Model 3359 data acquisition system. Separations were carried out using an AccuSep fused-silica capillary (52 cm effective length × 75 $\mu$m I.D.) (Waters). Injection was performed hydrostatically by elevating the sample at 10 cm for a specified time. Indirect UV detection at 214 or 254 nm was used.

The carrier electrolytes for cation separations were prepared from copper sulphate, copper chloride or imidazole (adjusted to pH 4.5 with hydrochloric acid) and the carrier electrolyte for anion separation from sodium chromate (all chemicals obtained from Merck, Darmstadt, Germany) and OFM BT Anion (obtained from Waters). The electrolytes were prepared either in water purified with a Milli-Q system (Millipore) or in water–ethylene glycol mixtures. Samples were prepared in Milli-Q-purified water.

Electroosmotic flow mobilities were determined by injecting mesityl oxide or benzyl alcohol as a neutral marker.

Reference serum samples with certified values were obtained from Nycomed (Oslo, Norway) and Behringwerke (Marburg, Germany).

## 3. Results and discussion

### *3.1. Effect of nature of carrier electrolyte on cation separation*

The separation of several alkali and alkaline earth metal ions relevant for clinical analysis is hampered by the fact that some of them have almost identical mobilities (such as sodium and magnesium). Weston *et al.* [2] have shown that the employment of a complexing agent such as citric acid or $\alpha$-hydroxyisobutyric acid as an additive to the carrier electrolyte can solve this problem owing to changes in the effective charge of the analyte ions on establishing complexation equilibria.

An alternative way of adjusting the separation selectivity was reported by Beck and Engelhardt [3]. They used imidazole as a carrier electrolyte instead of the more commonly used aromatic amines. In this way they claimed to be able to separate the sodium–magnesium peak pair. Unfortunately, during our experiments we were not able to confirm these results. Nevertheless, it seemed worthwhile to investigate further electrolytes with respect to different separation selectivities. The choice is restricted owing to the prerequisites of a reasonable UV absorption in order to allow indirect UV detection and also a mobility similar to that of the analyte ions. Papers on ion chromatography of cations with indirect UV detection had suggested the use of cerium(III) or copper(II) salts as mobile phases [4]. These electrolytes should also meet all the requirements of CZE. Owing to the better baseline stability at 214 nm, copper(II) was chosen for our work instead of cerium(III), although the latter has also recently been reported to be useful for several applications with indirect UV and fluorescence detection [5,6].

The behaviour of cations in a copper(II) electrolyte turned out to be dependent on the counter ion of the copper. Copper(II) chloride yielded almost the same separation selectivity as the imidazole electrolyte (which in turn gave the same separation selectivity as aromatic amines in their protonated form). Pronounced changes in migration order were observed when using cop-

Table 1
Relative migration times of cations in different carrier electrolytes (sodium = 1)

| Ion | 5 m*M* $CuSO_4$ | 5 m*M* $CuCl_2$ | 5 m*M* imidazole (pH 4.5) |
|---|---|---|---|
| Caesium | 0.71 | 0.73 | 0.74 |
| Rubidium | 0.73 | 0.73 | 0.74 |
| Potassium | 0.76 | 0.76 | 0.80 |
| Sodium | 1.00 | 1.00 | 1.00 |
| Barium | 1.09 | 0.90 | 0.90 |
| Strontium | 1.11 | 0.94 | 0.92 |
| Calcium | 1.13 | 0.95 | 0.93 |
| Magnesium | 1.18 | 1.00 | 1.00 |
| Lithium | 1.21 | 1.17 | 1.09 |
| Nickel | 1.22 | 1.00 | 1.00 |
| Manganese | 1.23 | 1.01 | 1.00 |
| Zinc | 1.23 | 1.00 | 1.00 |

Conditions: voltage, 25 kV; indirect UV detection at 214 nm.

per(II) sulphate instead of copper(II) chloride. Table 1 shows the migration order (given as relative migration times normalized to the migration time of sodium) of several alkali, alkaline earth and transition metal ions.

As can be seen from Table 1, the most obvious changes occurred with the divalent cations, whose migration times were increased with respect to the monovalent ions. One might speculate on association equilibria between divalent cations and sulphate leading to a decreased effective charge and therefore longer migration times. This idea is supported by the decrease in electrophoretic mobilities, $\mu_{ephor}$ (which are the observed mobilities minus the electroosmotic mobility), of divalent cations on increasing the copper sulphate concentration (Fig. 1). An estimation of the magnitude of the interaction between the cations and sulphate can be carried out in a way analogous to the determination of dissociation constants of acids described by Cleveland *et al.* [7]. The association constant $K_{ass}$ for a metal cation and sulphate can be found from the equation

$$\log c_{SO_4^{2-}} = \log \frac{1}{K_{ass}} - \log \frac{\mu_{ephor}}{\mu^0_{ephor} - \mu_{ephor}} \quad (1)$$

where $\mu^0_{ephor}$ is the electrophoretic mobility of the cation in an electrolyte without sulphate ions. The association constants calculated for calcium, magnesium and nickel were in the range 25–50 $mol^{-1}\ l^{-1}$. It should be remembered that these calculations give just a rough estimation of the magnitude of the interactions and, among other things, do not take into account changes in the size of the species.

### 3.2. Effects of organic modifier on cation separation

Work carried out earlier [8] on the separation of anions by CZE has demonstrated that the

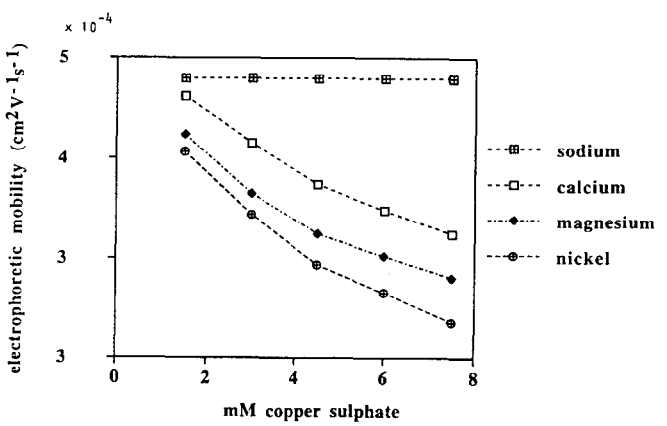


Fig. 1. Effect of copper sulphate concentration in the carrier electrolyte on the migration order of cations. Conditions: voltage, 20 kV; indirect UV detection at 214 nm.

addition of organic solvents to the carrier electrolyte can change the migration order considerably. Therefore, in a series of experiments the influence of ethylene glycol at levels up to 20% was investigated. As can be seen from the results in Fig. 2 for the copper sulphate electrolyte, there are some general trends resulting from the organic modifier, such as the increase in migration times of divalent ions relative to the migration times of the monovalent ions sodium and lithium. Similar results were obtained with the imidazole electrolyte. This effect might be attributed to the changes in the hydration of ions on addition of an organic solvent resulting in changes in migration times. Increasing the amount of organic modifier in an imidazole electrolyte up to 60% even made possible the separation of some transition metal ions, as can be seen from Fig. 3. Unfortunately, the increase

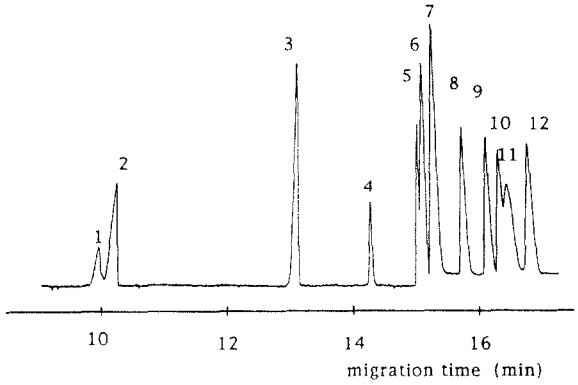


Fig. 3. Separation of a standard mixture of cations using a 5 m*M* imidazole carrier electrolyte (pH 4.5) containing 60% ethylene glycol. Peaks: 1 = rubidium; 2 = caesium, potassium; 3 = sodium; 4 = barium; 5 = strontium; 6 = calcium; 7 = magnesium; 8 = manganese; 9 = cobalt; 10 = nickel; 11 = zinc; 12 = lithium. Conditions: voltage, 20 kV; indirect UV detection at 214 nm.

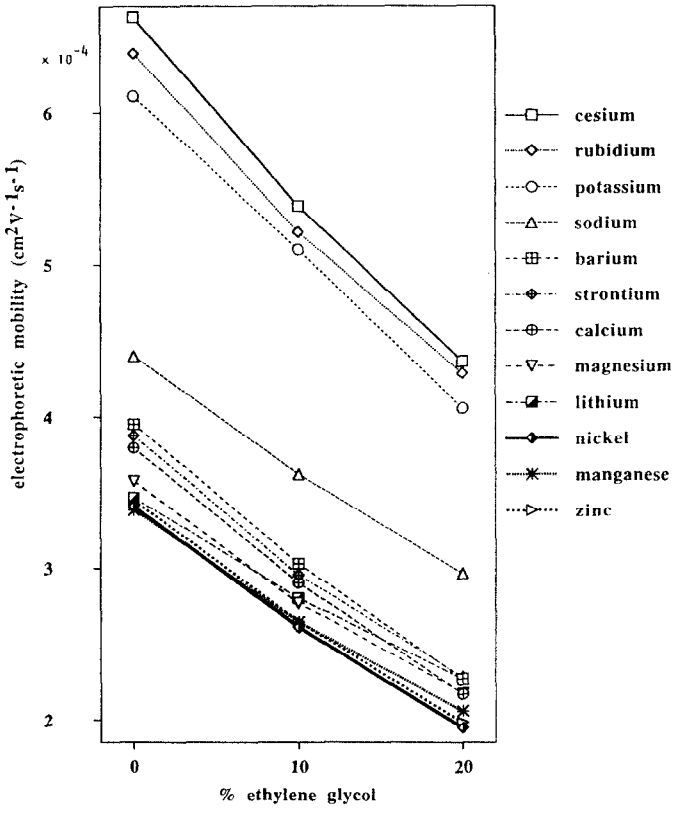


Fig. 2. Effect of ethylene glycol in the carrier electrolyte on the migration order of cations. Conditions: voltage, 20 kV; indirect UV detection at 214 nm.

in the amount of organic modifier causes a decrease in the electroosmotic flow, which was $0.96 \cdot 10^{-4}$, $0.56 \cdot 10^{-4}$ and $0.50 \cdot 10^{-4}$ $cm^2$ $V^{-1}$ $s^{-1}$ in 5 m*M* $CuSO_4$ with 0, 10 and 20% ethylene glycol, respectively. Therefore, higher voltages are recommended in order to avoid too long migration times.

### 3.3. *Effects of ion-pairing reagents on cation separations*

Ion-pairing reagents have been successfully applied to the optimization of anion separations, but so far this approach has not yet been used in the separation of cations. In this work, sodium dodecyl sulphate (SDS) was investigated as an ion-pairing reagent. Unfortunately, in an imidazole electrolyte even an SDS concentration as low as 0.5 m*M* leads to pronounced peak tailing. On the other hand, a copper sulphate electrolyte with up to 10 m*M* SDS resulted in well shaped peaks. As can be seen from Fig. 4, the most striking effect is the much slower migration of strontium and barium. Their migration times are considerably lower than those of several transition metal ions if an SDS concentration between 5 and 10 m*M* is used, and became even longer than that of the neutral marker. This suggests some equilibrium between these cations and SDS adsorbed on the inner surface of the capillary. The electroosmotic flow increased considerably when SDS was added to the carrier electrolyte. The electroosmotic mobilities in 5 m*M* copper sulphate were $0.96 \cdot 10^{-4}$ $cm^2$ $V^{-1}$ $s^{-1}$ without

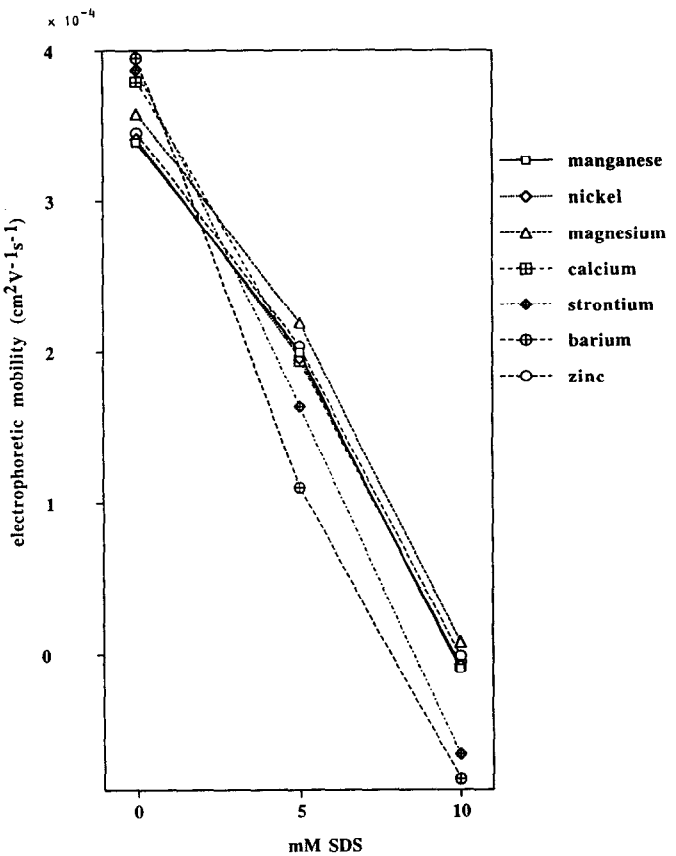


Fig. 4. Effect of sodium dodecyl sulphate in the carrier electrolyte on the migration order of cations. Conditions: voltage, 20 kV; indirect UV detection at 214 nm.

SDS and $2.65 \cdot 10^{-4}$ $cm^2$ $V^{-1}$ $s^{-1}$ with 5 or 10 m*M* SDS. Unfortunately, the presence of sodium ions from SDS in the electrolyte caused system peaks that interfered with the determination of alkali ions.

### *3.4. Application to samples of biological material*

Biological samples such as serum may contain a high concentration of sodium (*ca.* 140 m*M* in serum). This can be expected to have adverse effects on the efficiency of the separation. Generally, the electrical conductance of the sample should be considerably lower than that of the electrolyte in order to obtain sample focusing effects called "sample stacking". Obviously, this demand cannot be met by biological samples, because their electric conductances even at a 10- or 20-fold dilution may still be equal to or higher than that of the electrolytes normally used. Fortunately, this disadvantage can be overcome by making use of "sample self-stacking" recently described by Gebauer *et al.* [9]. If there is one analyte ion in excess, all other analyte ions having lower mobilities can be focused as long as the electrolyte has a (slightly) lower mobility than all analyte ions. Copper fulfils this criterion for serum samples, as it has a mobility slightly lower than those of the alkali and alkaline earth metals of interest in serum. In addition, its mobility is still high enough to avoid tailed peaks which will occur if there is an excessive mismatch between electrolyte and analyte ions. The focusing effect can be expected not to apply to potassium, which migrates faster than sodium. Nevertheless, the results given below demonstrate that the peak shape of potassium in serum samples is still acceptable.

Considering sensitivity and sample loading, a 20-fold dilution of serum samples and an injection time of 20 s were found to be appropriate. Care must be taken to avoid exceeding the linear range for sodium. Generally, the dynamic range is limited by the concentration of the carrier electrolyte, as changes in the detector signal can be expected only as long as there are enough UV absorbing ions in the carrier. It might be assumed that 1 mol of sodium should displace 0.5 mol of copper; in fact, its transfer ratio is even lower, as analyte ions having higher electrophoretic mobilities than electrolyte ions exhibit lower transfer ratios than calculated from a one-to-one equivalent displacement [10]. The experimental data confirmed that in a 3 m*M* copper sulphate electrolyte the response for sodium is linear up to at least 250 ppm (corresponding to 5000 ppm in the sample before dilution). This covers the whole range of normal and pathological serum samples. It would even allow a lower dilution of the sample in order to increase the sensitivity for potassium, calcium and magnesium. Unfortunately, in this case the resolution decreases considerably.

For quantification purposes, an internal standard was added to the samples and to the standard solutions to correct for eventually changing injection volumes. Lithium was chosen as the internal standard, as it migrates closely after the peaks of interest and its normal concentration in biological samples is negligible.

During a series of injections for the determination of cations with a copper electrolyte, it was noticed that the migration times of all ions increased considerably from run to run. Obviously, this was due to the proteins in the samples, which tended to adsorb to the inner surface of the capillary, thereby reducing the $\zeta$-potential and the electroosmotic flow. This problem could be overcome by flushing the capillary with 0.1 *M* sodium hydroxide solution between runs. This procedure resulted in stable migration times. No interfering peaks from proteins were observed in the electropherograms.

Fig. 5 shows the chromatogram of a serum sample. The reproducibility (relative standard deviation) for the determination of cations was *ca.* 7% for potassium, 1% for sodium, 5% for calcium and 7% for magnesium. This reproducibility might seem poor, but nevertheless could be sufficient for several routine purposes. One of the obvious advantages of CZE is its ability to handle biological samples available only in small amounts, such as tear fluid, which was also successfully analysed by this technique.

The accuracy of the mean was checked by

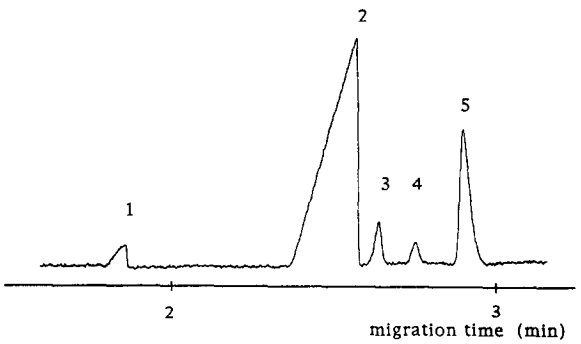


Fig. 5. Electropherogram of cations in a serum sample (1:20 dilution) using a 5 m*M* copper sulphate electrolyte. Peaks: 1 = potassium; 2 = sodium; 3 = calcium; 4 = magnesium; 5 = lithium (internal standard). Conditions: voltage, 30 kV; indirect UV detection at 214 nm.

using a reference serum with certified values. Table 2 compares the results of the CZE method and the certified values. The agreement between these data seems fairly satisfactory. In addition, it should be pointed out that calcium partly exists as a protein-bound species in serum. Obviously, the CZE method measures the total amount of calcium, which means that any bound calcium is released from proteins after injection.

Another series of experiments were carried out on the determination of anions in serum samples. Of the anions present in serum, chloride, sulphate, phosphate, carbonate acetate and lactate are in a concentration range suitable for CZE. Typical electrolytes described in the literature for the CZE of anions include chromate, phthalate and benzoate [11]. Recently, we found that Cu(II) $EDTA^{2-}$ is another electrolyte with interesting prospects for anion separations, as it can selectively detect monobasic carbonic acids in mixtures with dibasic acids. For serum samples, 5 m*M* chromate containing 0.5 m*M* OFM BT Anion as an electroosmotic flow modifier gave the best performance. Serum samples were diluted 20-fold before injection. No changes in migration times for anions in proteinaceous samples were observed (contrary to the situation for cation determinations as described above). Probably adsorption of proteins on the inner surface is suppressed by the use of the electroosmotic flow modifier, which itself adsorbs to the surface.

Table 2
Comparison of results for a serum reference sample obtained by CZE with certified values

| Element | Concentration (mg $l^{-1}$) | |
|---|---|---|
| | CZE | Certified value[a] |
| Potassium | 178 | 174 |
| Sodium | 2915 | 2970 |
| Calcium | 103.5 | 98 |
| Magnesium | 19.1 | 20.1 |

[a] Certified values were obtained by atomic emission spectrometry.

Similarly to cation determination, an internal standard was used for quantification. Malonic acid (concentration 10 ppm), which migrates between sulphate and phosphate, turned out to be useful for this purpose. Generally, the reproducibility was less satisfactory for some of the anions owing to the occurrence of interfering negative peaks and to sensitivity problems in the case of sulphate. The accuracy of the means could not be checked as certified reference materials were not available.

In conclusion, CZE was found to be adequate for the determination of a range of cations relevant in the clinical analysis of biological samples and several anions. The most important advantage seems to be that proteins do not interfere (in contrast to ion chromatography) so that sample preparation can be kept to a minimum. More sensitive detection methods would help to overcome some of the drawbacks of the proposed methods and open up CZE to the determination of trace elements in blood, serum and similar matrices. Post-separation reaction detectors and electrochemical detectors might have some prospects with respect to sensitivity and selectivity for complex samples, but are not yet ready for routine application.

## References

[1] W. Buchberger, H. Malissa and E. Mülleder, *J. Chromatogr.*, 602 (1992) 51.

[2] A. Weston, P.R. Brown, P. Jandik, W.R. Jones and A.L. Heckenberg, *J. Chromatogr.*, 593 (1992) 289.
[3] W. Beck and H. Engelhardt, *Chromatographia*, 33 (1992) 313.
[4] J.H. Sherman and N.D. Danielson, *Anal. Chem.*, 59 (1987) 490.
[5] K. Bächmann, J. Boden and I. Haumann, *J. Chromatogr.*, 626 (1992) 259.
[6] K. Bächmann, I. Haumann and T. Groh, *Fresenius' J. Anal. Chem.*, 343 (1992) 901.
[7] J.A. Cleveland, M.H. Benko, S.J. Gluck and Y.M. Walbroehl, *J. Chromatogr. A*, 652 (1993) 301.
[8] W. Buchberger and P.R. Haddad, *J. Chromatogr.*, 608 (1992) 59.
[9] P. Gebauer, W. Thormann and P. Bocek, *J. Chromatogr.*, 608 (1992) 47.
[10] M.W.F. Nielen, *J. Chromatogr.*, 588 (1991) 321.
[11] P. Jandik and W.R. Jones, *J. Chromatogr.*, 546 (1991) 431.