ELSEVIER

Journal of Chromatography B, 733 (1999) 273–279

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

# Capillary zone electrophoresis of potassium in human vitreous humour: validation of a new method

Franco Tagliaro[b], Giulia Manetto[a,*], Francesca Cittadini[b], Daniela Marchetti[b], Federica Bortolotti[a], Mario Marigo[a]

[a]*Institute of Forensic Medicine, University of Verona, Policlinico, 37134 Verona, Italy*
[b]*Institute of Forensic Medicine, Catholic University of the Sacred Hearth, Largo Francesco Vito 1, 00168 Rome, Italy*

**Abstract**

The analysis of potassium in the vitreous humour has long been regarded as an important tool in medicolegal and forensic toxicological investigation, particularly for the determination of the post-mortem interval. The present work was aimed at the optimisation and validation of a reliable, simple and fast capillary electrophoresis method for potassium analysis in the human vitreous humour with indirect UV detection at a wavelength of 214 nm. Electrophoretic separations were carried out in a running buffer comprising 5 m*M* imidazole, 5 m*M* 18-crown-6 ether and 6 m*M* D,L α-hydroxybutyric acid (HIBA), adjusted to pH 4.5. Constant voltage runs were carried out by applying a voltage of 500 V/cm at 25°C. The samples were injected in the hydrodynamic mode at the anodic end of the capillary (0.5 p.s.i. for 10 s; 1 p.s.i.=6894.76 Pa). The method showed good linearity in the concentration range from 6.5 m*M* to 16.25 μ*M*, with an $r^2$value of 0.9994. The limit of detection, based on a signal-to-noise ratio of three, was 9.0 μ*M*. Absolute intra-day RSDs of migration times were <0.40%, while the day-to-day values were ⩽1.72%. Absolute peak area reproducibility was always better than 2.50%. A comparison of capillary electrophoresis with flame photometry on twelve real autopsy samples showed an excellent correlation with an $r^2$ value of 0.9333. A preliminary application to real cases (20 subjects) was carried out plotting vitreous humour potassium vs. post-mortem interval with a resulting $r^2$ of 0.904 and a *Y*-intercept of 4.75 m*M*, in agreement with the existing literature.
© 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Vitreous humour; Potassium

## 1. Introduction

The analysis of vitreous humour has long since been regarded as an important tool in medicolegal and forensic toxicological investigation. In particular, among the most significant post-mortem chemical modifications, the rise of potassium concentration in the vitreous humour has been reported by several authors as one of the main parameters to determine the time since death [1]. In fact, because of its anatomic confinement, vitreous humour has been regarded as the ideal extracellular fluid to measure the post-mortem release of intracellular potassium (mainly from the retina) consequent to the energy breakdown and the related cessation of active transport and selective membrane permeability. A large body of literature on this subject has accumulated since the middle of the 1960s showing an undoubted correlation between potassium concentrations in the vitreous body and the post-mortem interval (PMI). However, marked discrepancies were observed between different investigators when testing the vitre-

*Corresponding author.

0378-4347/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00338-2

ous potassium–PMI relationship, in terms of slope and intercept of the regression line [2–4]. Moreover, some other authors demonstrated significant between-eye differences in the electrolyte levels, which seem to further undermine the potential usefulness of the potassium determination in the vitreous humour for the evaluation of time since death [5].

In reality, several factors, including sampling procedures, sample storage, environmental temperature, ante-mortem pathologies, analytical factors etc., may affect the final results.

Concerning the analytical aspects, non-separative methods largely used in clinical chemistry such as flame photometry and, more recently, ion-selective electrodes have generally been adopted for potassium determination, which may account for at least a part of the large variability of data reported in the literature. This outlines the need for the development and validation of rapid, but reliable and accurate, separative analytical methods by which the potassium concentration in the vitreous humour can be determined directly, also in the presence of large variations in the medium composition, as may happen due to post-mortem biochemical changes and putrefaction.

A separation technique that has recently developed into a wide array of microanalytical applications is capillary electrophoresis (CE), which is widely appreciated for its high efficiency, resolution, selectivity, simplicity, speed and low buffer consumption. CE has proved to possess the accuracy typical of separation methods, but in a manner different from that of chromatographic methods, is also inherently insensitive to contamination from matrix components, which can frequently be encountered in real forensic casework due to putrefaction or other post-mortem changes.

In recent years, CE has been successfully introduced into the analysis of low-molecular-mass ionic species [namely, capillary ion analysis (CIA)], as is evident from a large and steadily expanding body of literature [6]. For inorganic ions, which generally have no optical absorption, indirect on-column detection is mostly applied. Interesting applications of CIA have been reported in different matrices including drinking water, high purity water [7], waste water [8], and biological specimens such as serum [9] and plasma [10]. Quite recently, a method for the determination of potassium in the human vitreous humour has been published highlighting the applicability of CIA for this purpose [11]. Unfortunately, this method is based on a commercial reagent (UV-CAT-1, Waters, Milford, MA, USA) of unknown composition, hampering its inter-laboratory transferability, which is fundamental for admissibility in court.

The present work was aimed at the optimisation and validation of a reliable, simple and fast CIA method for potassium analysis in the human vitreous humour, entirely based on laboratory-made separation buffers, with indirect UV detection. In addition, a preliminary application to real cases was carried out in order to verify the advantages that a microanalytical separative technique such as CE could offer in this field.

## 2. Experimental

### 2.1. Standards and chemicals

All chemicals were of analytical-reagent grade. Imidazole (99% pure) was obtained from Sigma (St. Louis, MO, USA), and 18-crown-6 ether (99% pure) and D,L α-hydroxybutyric acid (HIBA) (99% pure) were from Aldrich (Milan, Italy). Standards solutions of $K^+$, $Ba^{2+}$, $NH_4^+$, $Na^+$ and $Ca^{2+}$ were prepared from AnalaR salts (Merck, Darmstadt, Germany). $K^+$ standard was checked versus a control serum for clinical chemistry analysers from Boehringer Mannheim (Mannheim, Germany) containing 6.5 m*M* $K^+$. Water used for the preparation of the buffer electrolyte and for sample dilution was of HPLC grade (Carlo Erba, Milan, Italy). The electrophoretic buffer pH was adjusted to the desired pH with 1 *M* acetic acid. Any buffer and rinsing solution was filtered and degassed under vacuum before use through a 0.22 μm membrane filter (Millipore, Vimodrone, Italy).

### 2.2. Instrumentation

All experiments were carried out using a P/ACE 5500 automated capillary electropherograph (Beckman, Fullerton, CA, USA) equipped with a filter UV absorbance detector. The capillary was thermostated

with a perfluorinated coolant circulating in the capillary cartridge. Throughout all of the experiments, untreated fused-silica capillaries (75 μm I.D., 50 cm effective length; Beckman) were used. The detection window was 200×100 μm. The detector time constant was 0.1 s and the data acquisition rate was 5 Hz. A Beckman P/ACE Station (version 1.0) was used for instrument control, data acquisition and processing.

### 2.3. Electrophoretic conditions

The electrophoretic separations were carried out in a running buffer composed of 5 m*M* imidazole, 5 m*M* 18-crown-6 ether and 6 m*M* HIBA, adjusted to pH 4.5. Constant voltage runs were carried out in all experiments by applying a voltage of 500 V/cm at 25°C with a resulting current of about 20 μA. Detection was by indirect UV absorbance at a wavelength of 214 nm. The solutes were injected at the anodic end of the capillary in the hydrodynamic mode by applying nitrogen pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for 10 s. New uncoated fused-silica capillaries were washed with a solution containing 1 *M* sodium hydroxide (5 min) followed by 0.1 *M* sodium hydroxide (5 min), water (5 min) and were finally conditioned with the running buffer (10 min). Between consecutive runs, the capillary was washed with water (1 min) and then with the electrolyte buffer (4 min) to improve the reproducibility of the electroosmotic flow and the migration time of the analytes. Water blanks were routinely checked for contamination by trace amounts of cations.

### 2.4. Sample preparation

Stock standards solutions of $NH_4^+$, $K^+$, $Ca^{2+}$, $Na^+$ and $Ba^{2+}$ were prepared in polypropylene vials by dissolving appropriately weighed amounts of salts in water (2 mg/ml). Working standard solutions of the above substances were prepared daily to the required concentrations by dilution in water. Cation concentrations in the analytical process were expressed in μg/ml to make handling of the solution easier, but the results in real samples were converted into corresponding m*M* values to allow comparisons with the existing literature.

Duplicate samples of vitreous humour (about 1.5 ml each) from both eyes were independently collected at autopsy by needle puncture of the posterior chamber, by gentle sucking with a 2.5-ml plastic syringe. All samples were then diluted 1:100 with a 40-μg/ml aqueous solution of barium, the internal standard (I.S.) used. Until analysis, samples were stored in a refrigerator at +4°C.

## 3. Results and discussion

As is well known, for potassium determination by CE using standard instrumentation (universally fitted with UV detectors), indirect UV radiation absorption detection is required since alkali metals and alkaline earth metals have no UV absorbance. Imidazole has widely been reported as one of the most suitable co-ions for the detection of ammonium, alkali, alkaline earth and transition metal cations by CIA, because its electrophoretic mobility matches that of the analytes. Thus, an imidazole-based buffer has been used since the start of this work at a pH of 4.5, where imidazole is completely protonated.

In addition, it is well known from the literature [12–17] that the separation of metal ions with nearly the same electrophoretic mobilities is only possible using chemical complex formation equilibria with different stabilities. For this reason, 18-crown-6 ether and HIBA were used as complexing agents at optimised concentrations of 5 m*M* for each electrolyte. The introduction of 18-crown-6 ether provided an easy way of separating ammonium and potassium peaks, while the addition of 6 m*M* HIBA effected the complete resolution of all of the cations studied. The importance of a complete separation of potassium from ammonium in real samples is fundamental for avoiding interferences from large excesses of ammonium, which can be present in putrefied bodies. Under the optimised experimental conditions, the electroosmotic flow was in the same direction as the electrophoretic mobility of the analytes and cations migrated in peaks that were completely resolved from each other and from matrix components. Peak asymmetry, due to a slight mobility mismatch between analytes and the buffer additive, did not hinder quantification. Typical electropherograms of a standard mixture containing $NH_4^+$

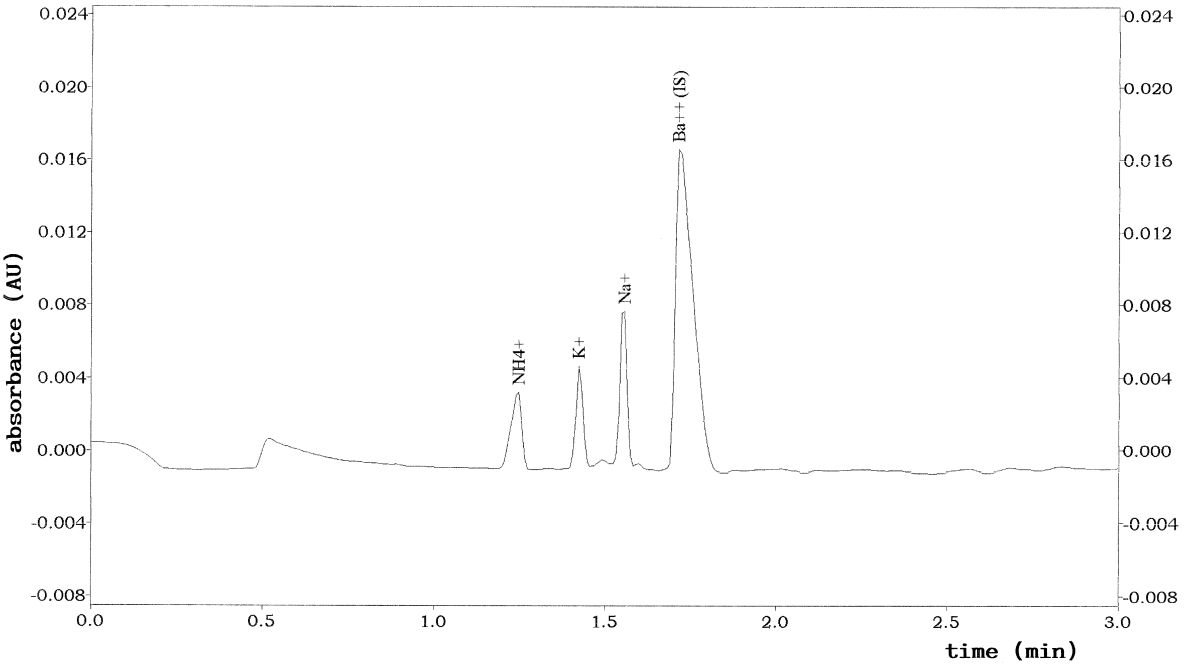


Fig. 1. Electropherogram of a mixture of pure cations containing: (a) $NH_4^+$ (5 μg/ml), (b) $K^+$ (5 μg/ml), (c) $Na^+$ (15 μg/ml) and (d) $Ba^{2+}$ (120 μg/ml) analysed under the following conditions: injection, 0.5 p.s.i. ($=3.44 \cdot 10^7$ Pa) for 10 s; separation, 5 m*M* imidazole, 5 m*M* 18-crown-6 ether and 6 m*M* HIBA, pH 4.5; electric field, 500 V/cm; temperature, 25°C; detection, indirect UV absorbance at 214 nm.

(5 μg/ml), $K^+$ (5 μg/ml), $N_4^+$ (15 μg/ml) and $Ba^{2+}$ (120 μg/ml) and of a human vitreous humour are depicted in Fig. 1 and Fig. 2, respectively. Peak identification for $NH_4^+$, $K^+$, $Ca^{2+}$, $Na^+$ and $Ba^{2+}$ ions was carried out by sample spiking.

It is worth noting that even a great excess of $Na^+$ did not interfere with potassium analysis, but did occasionally partially overlap with the $Ca^{2+}$ peak, which otherwise is not relevant to the present study. It is also worth mentioning that $NH_4^+$ is well separated and does not interfere with $K^+$ determination.

A calibration curve was obtained by plotting peak areas of potassium against analyte concentration. Five decreasing concentrations of a potassium control serum in the range from 6.50 m*M* to 16.25 μ*M* were used. Concentrations and peak areas correlated linearly in the examined range, as described by the following equation: $y=356.1x-131$ ($r^2=0.9994$) ($y$=peak area; $x$=concentration). Under the described conditions, the limit of detection (LOD) of potassium in a standard mixture, based on a signal-to-noise ratio of three, was 9.0 μ*M*.

Analytical precision, in terms of both migration times and peak areas, was evaluated intra-day and day-to-day. Quantification was carried out by external standardisation and by internal standardisation, using barium as the I.S. The latter method, however, did not improve analytical precision. For this reason, external standardisation was preferred and was adopted in all further experiments. Concentrations of 0.25 and 6.50 m*M* were used to check repeatability with six repeats in intra-day precision tests, which were repeated on five different days. The results of this series of experiments are displayed in Table 1. The absolute intra-day RSDs of migration times were always $<0.40\%$, while the day-to-day RSDs were $\leq 1.72\%$. Absolute peak area reproducibility was always better than 2.50%.

Analytical precision was also verified in a real case by repeated analysis on three different days of the same sample of human vitreous humour, after

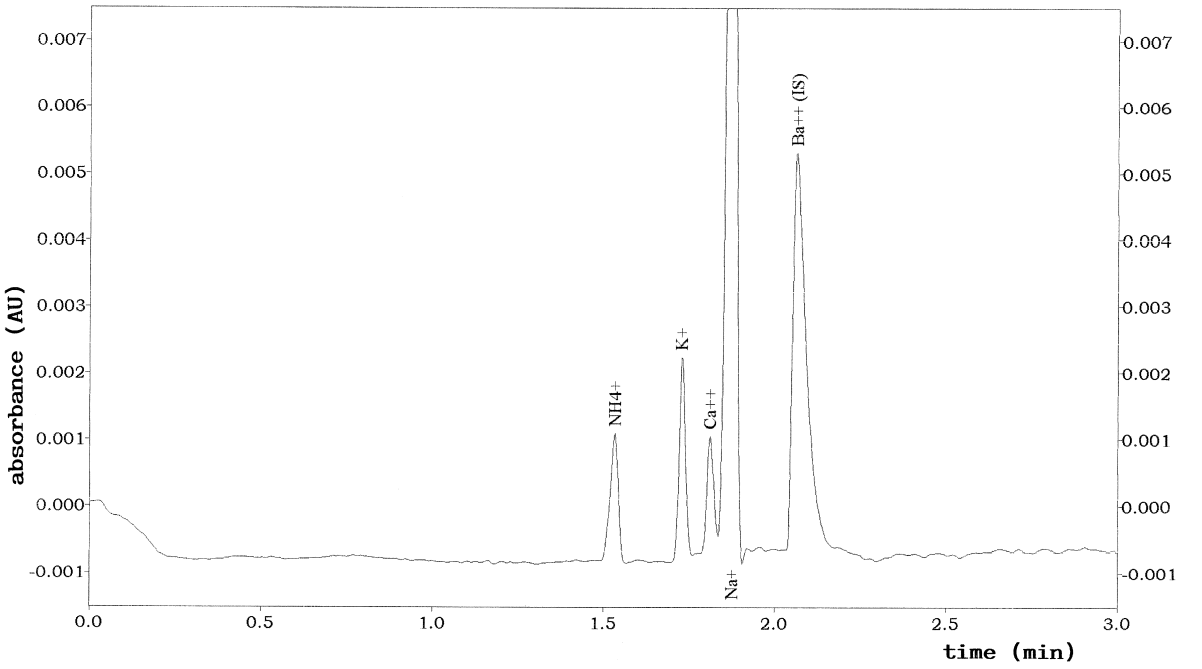


Fig. 2. Typical electropherogram of a human vitreous humour sample with a $K^+$ concentration of 28 m*M* (analytical conditions as in Fig. 1).

Table 1
Analytical reproducibility (RSD%) of CZE analysis of potassium in a standard solution ($n$=6)

| Concentration (mmol/l) | Migration times | | Peak areas | |
|---|---|---|---|---|
| | Intra-day | Day-to-day | Intra-day | Day-to-day |
| 0.25 | 0.39 | 1.19 | 2.45 | 2.12 |
| 6.50 | 0.31 | 1.72 | 0.81 | 1.59 |

dilution (1:100, v/v) with water containing the I.S. (40 μg/ml). The reproducibility of migration times and peak areas are displayed in Table 2 in terms of both absolute and relative values. Variation of absolute migration times intra-day and day-to-day was comparable to that of the former experiment, while peak areas were not as precise as in pure solution (RSDs ⩽7.59 and 10.33%, respectively), but overall precision was still acceptable for practical application. The introduction of an I.S. improved the analytical precision, but not dramatically.

Table 2
Analytical reproducibility (RSD%) of CZE analysis of potassium in vitreous humour ($n$=6)

| | Migration times | | Peak areas | |
|---|---|---|---|---|
| | Intra-day | Day-to-day | Intra-day | Day-to-day |
| Absolute | 0.28 | 1.84 | 7.59 | 10.33 |
| Relative | 0.26 | 0.72 | 7.42 | 6.66 |

Analytical accuracy was investigated with a dilution test performed on a sample of vitreous humour with a high potassium concentration (28 m*M*). Five serial dilutions were carried out from 1:10 to 1:400 (v/v) in water and potassium concentrations were determined in each dilution. Regression analysis showed an excellent linearity, described by the equation: $y=288\,706x+114.58$ ($r^2=0.9998$) ($y$= peak area; $x$=dilution factor).

The determinations of potassium with the present method in 12 vitreous humour specimens (ranging from 3.9 and 28.2 m*M* potassium concentration) collected at autopsy were compared to those obtained with flame photometry, using a model 943 automated flame photometer from Instrumentation Laboratory (Milan, Italy). The results were well correlated,

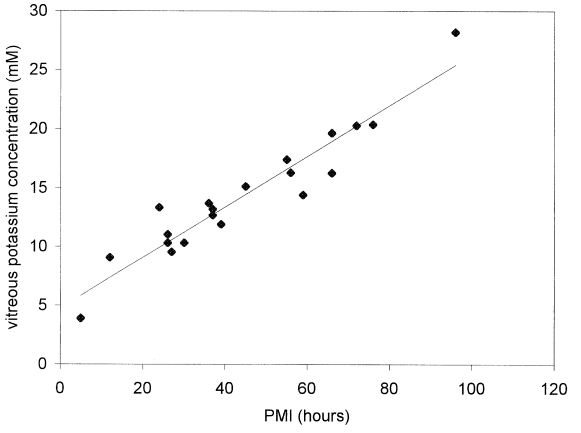


Fig. 3. Correlation between PMI and vitreous $K^+$ concentration determined in 20 cases of acute violent death. Equation: $y=0.215x+4.755$ ($r^2=0.904$), in which $y=K^+$ concentration (m$M$); $x$=PMI (h).

according to the equation $y=0.9303x+0.1393$ ($r^2=0.9333$) ($y$=m$M$ concentration from CIA; $x$=m$M$ concentration from flame photometry).

A preliminary study on the correlation between PMI and potassium levels in the vitreous humour was conducted on 20 subjects, who died from different violent causes, whose time since death was reasonably well established in the range between 5 and 96 h. The resulting equation was $y=0.215x+4.755$ ($r^2=0.904$), in which $y=K^+$ m$M$ concentration; $x$=PMI) (see also Fig. 3). The slope and $y$ intercept of the regression line were in good agreement with those reported by Forman and Butts [18], confirming the reliability of the proposed CIA method in real casework.

## 4. Conclusion

The use of potassium changes in the ocular fluids has shown great potential for the estimation of the time since death, but the available literature, based on traditional clinical chemistry methods for ion analysis, is partially discordant on the reliability of this methodology [19].

The proposed method for potassium analysis in the vitreous humour by CIA has undergone a full validation, showing accuracy, precision and sensitivity values that are suitable for forensic application. The simplicity and automation of the technique look competitive with the current methods, such as flame photometry or ion-selective electrodes, and the separative mechanism on which CIA is based offers intrinsically superior reliability in the analysis of complex matrices, such as post-mortem samples. A further potential advantage of CE is the minimum amount of sample required, which allows microsampling of ocular fluids. This will permit detailed studies to be carried out on the changes in potassium concentration after death in the different ocular compartments, which could offer new perspectives of improved accuracy to the PMI estimation based on vitreous humour analysis.

After the assessment of a close linear relationship with the PMI, potassium determination in the ocular fluids, in principle, could also offer a tool for investigating intoxications by potassium, for which blood concentrations of the ion are unsuitable, being heavily affected by post-mortem haemolysis.

## Acknowledgements

The authors feel obliged to thank Dr. Romolo Dorizzi, Clinical Biochemistry Laboratory, Hospital of Verona, for carrying out the potassium determinations by flame photometry.

## References

[1] J.I. Coe, Forensic Sci. Int. 42 (1989) 201–213.
[2] B.A.W. Balasooriya, C.A. St. Hill, A.R. Williams, Forensic Sci. Int. 26 (1984) 85–91.
[3] B. Madea, C. Henssge, W. Honig, A. Gerbracht, Forensic Sci. Int. 40 (1989) 231–243.
[4] B. Madea, N. Herrmann, C. Henbge, Forensic Sci. Int. 46 (1990) 277–284.
[5] D.J. Pounder, D.O. Carson, K. Johnston, Y. Orihara, J. Forensic Sci. 43 (3) (1998) 604–607.
[6] M. Macka, P.R. Haddad, Electrophoresis 18 (1997) 2482–2501.
[7] J.P. Romano, J.A. Krol, J. Chromatogr. 640 (1993) 403–408.
[8] S.A. Oehrle, R.D. Blanchard, C.L. Stumpf, D.L. Wulfeck, J. Chromatogr. A 680 (1994) 645–652.
[9] J.M. Monaghan, K. Cook, D. Gara, D. Crowther, J. Chromatogr. A 770 (1997) 143–149.
[10] A.M. Leone, P.L. Francis, P. Rhodes, S. Moncada, Biochem. Biophys. Res. Commun. 200 (1994) 951–957.

[11] K.E. Ferslew, A.N. Hagardorn, M.T. Harrison, W.F. McCormick, Electrophoresis 19 (1998) 6–10.
[12] Q. Yang, Y. Zhuang, J.S. Verbeke, D.L. Massart, J. Chromatogr. A 706 (1995) 503–515.
[13] C. François, Ph. Morn, M. Dreux, J. Chromatogr. A 706 (1995) 535–553.
[14] C. François, Ph. Morn, M. Dreux, J. Chromatogr. A 717 (1995) 393–408.
[15] Y.-H. Lee, T.-I. Lin, J. Chromatogr. A 675 (1994) 227–236.
[16] Y. Shi, J.S. Fritz, J. Chromatogr. A 671 (1994) 429–435.
[17] S.A. Oehrle, J. Chromatogr. A 745 (1996) 87–92.
[18] D.T. Forman, J. Butts, Clin. Chem. 26 (1980) 1042.
[19] B. Madea, C. Henssge, in: C. Henssge, B. Kinght (Eds.), Estimation of Time since Death in Early Postmortem Period, Arnold Edward, London, 1995, pp. 106–135.