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Micellar electrokinetic capillary chromatographic analysis of platelet activating factor in human blood by indirect ultraviolet detection

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Summary. - In this paper, 1-O-hexadecyl and 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphorylcholine (C_{16}-AGEPC and C_{18}-AGEPC) in the platelet activating factor of human blood were analyzed by micellar electrokinetic capillary chromatography (MECC) with indirect ultraviolet absorption detection at 254 nm. The optimum running buffer for the separation contained 50 mmol/l sodium dodecy sulphate, 20 mmol/l potassium hydrogen phthalate, 10 mmol/l borax and 3 mol/l urea (pH 6.8). The separation was completed within 10 min. The detection limits of C_{16}-AGEPCand C_{18}-AGEPC were the same, i.e. 60 ng/ml (k = 3). The analytical precision (n = 6) was 2.8-3.0% and 1.4-1.7% for the determination (peak height mode) and for the measurement of the migration times, respectively. The application of this method to the clinical samples was demonstrated.

Key words: micellar electrokinetic capillary chromatography, indirect UV detection, platelet activating factor.

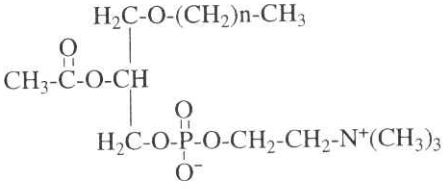
Riassunto *(Cromatografia capillare elettrocinetica micellare degli attivatori di piastrine nel sangue umano con determinazione UV indiretta).* - In questo lavoro gli attivatori di piastrine nel sangue umano 1-O-exadecyl e 1-O- octadecyl-2-acetyl-sn-glicero-3-fosforilcolina (C_{16}-AGEPC e C_{18}-AGEPC) sono stati analizzati tramite cromatografia capillare elettrocinetica micellare (CCEM) con rivelazione indiretta in assorbimento ultravioletto a 254 nm. Il miglior tampone di lavoro per la separazione è risultato essere quello che contiene 50 mmol/l di sodio dodecilsolfato, 20 mmol/l di biftalato di potassio, 10 mmol/l di borace e 3 mmol/l di urea (pH 6,8). La separazione è stata completata in 10 min. I limiti di rivelabilità della C_{16}-AGPEC e della C_{18}-AGEPC coincidono, vale a dire 60 ng/ml (k = 3). La precisione analitica (n = 6) è stata 2,8-3,0% e 1,4-1,7% per la determinazione in altezza di picco e per la misura dei tempi di migrazione, rispettivamente. E' stata evidenziata l'applicabilità del metodo a campioni clinici.

Parole chiave: cromatografia capillare elettrocinetica micellare, determinazione UV indiretta, attivatore delle piastrine.

Introduction

Platelet activating factor (PAF) is a natural glyceral phospholipid and is an important component of the biomembrane. It has spread bioactivity in the human body and its metabolic level in it is closely related with the occurrence and development of many diseases in the human being. The analysis of PAF has an important significance in the research of medicine.

There are mainly two types of PAF in human blood: 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine (C_{16}-AGEPC) and 1-O-octadyl-2-acetyl-sn-glycero-3-phosphorylcholine (C_{18}-AGEPC). Their contents in human blood are between the ranges of pg and ng [1]. Their molecular formula is:



The difference between them is on the lengths of their alkylic chain at C-1 position. GC-MS [2, 3], radioimmunoassay [4] and chromatography [1, 5] methods have all been used in the analysis of PAF, but there are many defects in these methods, such as

expensive equipments, radioactive contamination, complicated operations and expending too much time, etc.

Micellar electrokinetic capillary chromatography (MECC) is the potent tool used in biochemistry analyses and has very high discrimination power, offering separations with a large number of theoretical plates, and sufficient selectivity under properly chosen conditions on neutral and charged material [6-10]. Using MECC, we have successfully separated two types of PAF in 10 min in this separating system: anion surfactant-sodium dodecyl sulphate (SDS) used as the micellar phase; some borax and urea were added into the aqueous phase; potassium hydrogen phthalate was selected as the probe ion used for indirect absorption detection. The two types of AGEPC were detected by indirect ultraviolet absorption mode at 254 nm. The change of peak height with the concentration of both AGEPC was linear in the range of 5-80 ng/ml. The determinated precisions (CV) of peak heights (n = 6) were less than 3.0% and the detection limits were 60 ng/ml (k = 3) for both.

Materials and methods

Apparatus and reagents

A model 1229 capillary electrophoresis system with 254 nm UV detector (New Technology Application Institute of Beijing) was employed. MECC was performed in a 70 cm x 50 μm ID fused silica capillary tube (Hebei Yongnian Optical Fiber Factory in PRC) where 53 cm was the effective length for separation. The signals were transmitted to a X-Y recorder (Sichuan Meter Factory in PRC). All experiments were carried out at ambient temperature (ca. 24-26 °C).

The standard reagents of C_{16}-AGEPC and C_{18}-AGEPC were purchased from Sigma. All other reagents came from China and were analytical-reagent grade. All the solutions were prepared by sub-boiling distilled water.

Procedure

Each kind of solution was filtered through 0.3 μm diameter tiny hole filter membrane before going into the capillary separating column. The injection was at the positive end in hydrostatic mode for 10 s (the altitude was 10 cm). To obtain good separation, the capillary was cleaned according to the following procedure each time the buffer solution was changed: the capillary was purged for 5 min with 0.1 mol/l KOH; for 2 min with water and then for 2 min with the new buffer solution. In addition, the capillary was purged for 1 min with the working buffer before each injection.

Dispose of human blood samples

Three ml venous blood were injected into the tube held with 15 ml methanol and mixed immediately on vortex vibrator, then centrifuged (4000 rpm, 15 min) to get rid of proteins and other impurities. The supernatant was drew out and some chloroform and water added, then mixed waiting two layers to be seen clearly, the chloroform phase was taken out and dried with N_2, 30 μl methanol were added to resolve it again and waiting to be measured.

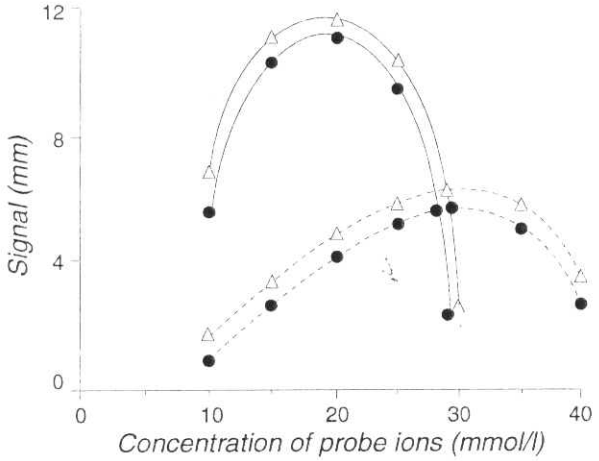


Fig. 1. - Effect of probe ions on the sensitivity of indirect absorption detection, potassium hydrogen phthalate (—); salicylic acid (----) 20 μg/ml C_{16}-AGEPC (●) and 20 μg/ml C_{18}-AGEPC (Δ). Running buffer: 50 mmol/l SDS, 10 mmol/l borax, 3 mol/l urea (pH 6.8). Applied voltage: 25 kV, detection wavelength: 254 nm; injection: h = 10 cm, t = 10 s.

Results and discussion

Selection of the indirect absorption probe ions

The ultraviolet wavelengths absorbed of C_{16}-AGEPC and C_{18}-AGEPC are between 204-206 nm [11]. If the two types of phosphorylcholine are to be detected at 254 nm, the indirect absorption detection must be employed. The compounds having strength absorption at 254 nm are used to serve as the background electrolyte, when the absorption electrolyte was replaced by the separated PAF constituent, the background absorption strength was decreased, and the target signal is negative peak. These ions that can produce strength absorption background are named as indirect absorption probe ions. In the system (pH 6.8) of 50 mmol/l SDS, 10 mmol/l $Na_2B_4O_7$ and 3 mol/l urea, a series of concentrations of solicylic acid and potassium hydrogen phthalate were added into the mixed standard solution of 20 μg/ml C_{16}-AGEPC and C_{18}-AGEPC, respectively, doing the sensitivity test. The results are shown in Fig. 1. When potassium hydrogen

phthalate is used as probe ion, it has higher detection sensitivity for both PAF than salicylic acid. There were two possibile causes: one was that there were two carboxylic radicals on the phenyl in the molecule of potassium hydrogen phthalate, which made the strength absorption peak (β band) of phenyl red shift to 254 nm. Potassium hydrogen phthalate has more strength absorption than that of salicylic acid, which contain only one carboxylic radical [12]. The larger is the molar absorption coefficient of the probe ion, the more stable is the absorption background and the smaller is the noise. The lower background noise improves indirect detection sensitivity [13, 14]. We also found that there was faint basic line in the system of potassium hydrogen phthalate. The other cause was that the molecular weight of potassium hydrogen phthalate is bigger than that of salicylic acid and its molecular weight is more close to that of PAF. The similar size of molecule weight between sample and probe ions is favourable to increase their replacement ratio and improve detection sensitivity [13].

Fig. 1 also showed that the concentration of the probe ions can obviously influence the detection sensitivity. If the concentration of the probe ions is over high, which is unfavourable to detect the small signal under large background even though it can produce higher absorption background [13]. And also accompanied by the increase of the probe ions concentration, the separating electric current enlarged evidently, producing Joule heat and noise increase too, so that the detection sensitivity fell. But if the concentration of the probe ions is too low, the background absorption will be unstable and the noise will increase; meanwhile, the replacement ratio of the composition in the samples to the probe ions will decline, both causes can lead the detected signal to decline and to be unstable. According to our results, 20 mmol/l potassium hydrogen phthalate was selected to serve as the probe ion for detecting PAF by indirect absorption detection at 254 nm.

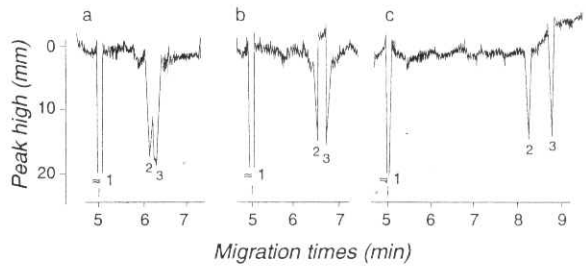


Fig. 3. - Effect of borax and urea on the resolution. Running buffer: a) 50 mmol/l SDS-20 mmol/l potassium hydrogen phthalate (pH 6.8); b) added 10 mmol/l borax into a); c) added 3 mol/l urea into b). The other conditions as in Fig. 1. Peak 1: methanol, peak 2: C_{16}-AGEPC, peak 3: C_{18}-AGEPC.

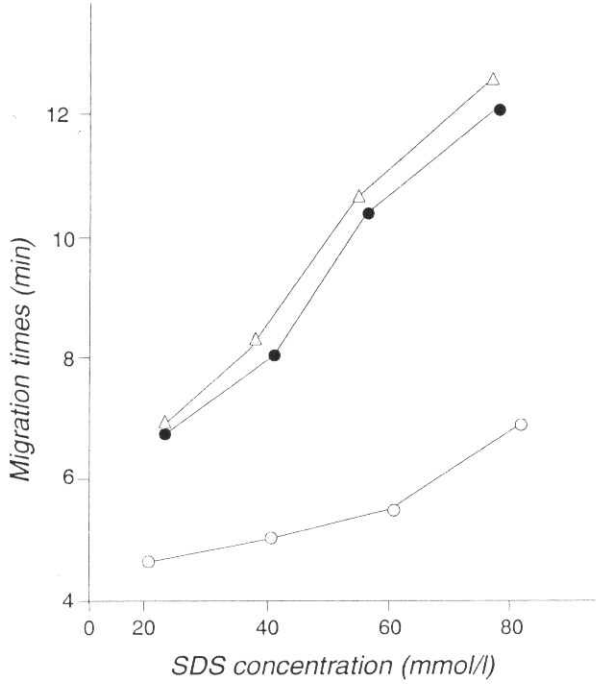


Fig. 2. - Effect of SDS concentration on the electroosmotic flow (O) and the migration times of C_{16}-AGEPC (●) and C_{18}-AGEPC (Δ). Running buffer: 20 mmol/l potassium hydrogen phthalate, 10 mmol/l borax, 3 mol/l urea (pH 6.8). Other conditions as in Fig. 1.

Effect of SDS concentration

The separation principle of MECC is based on the different distribution of solute between in the micellar phase and the aqueous phase. The concentration of the micellar affects directly the separation efficiency. The anion surfactant SDS used as micellar phase and methanol used as electroosmotic flow symbol, the influences of the concentration of SDS on the electroosmotic flow and the retention time of the samples are shown in Fig. 2. As the concentration of SDS increases, the viscosity of the buffer system increased, resulting in a decrease in the electroosmotic flow, as described in Fig. 2 the retention time of methanol extended. On the other hand, at higher SDS concentration, the phase ratio of the micellar to the aqueous phase in the system increased as well as the sample's distribution in the micellar, i.e. the capacity factor increased, so that the retention time of the samples increased too (Fig. 2). This results were identical with the report in some theses [6, 7]. The two types of PAF molecule, however, have a similar hydrophobic hydrocarbonic chain and a hydrophilous pole, both of them have very similar solubilization in the SDS micellar. So only increasing SDS concentration can not improve the resolution of the two kinds of AGEPC (Fig. 3a). However, the concentration of SDS was over 50 mmol/l, the electroosmotic flow became smaller and also the detection time window, so the resolution had a little

decline [6]. The lower is the micellar concentration, the larger is the electroosmotic flow, the components retention time of PAF shortened and both components cannot be separated entirely. So 50 mmol/l was used as micellar phase concentration in this experiment.

Effects of borax and urea

Various modifier composition in the aqueous phase can effectively enhance the separation selectivity of MECC system [8, 9]. Fig. 3 shows the effects of borax and urea in the buffer solution (pH 6.8). Borax is a typical deficient electron compound and is liable to complex with O, N, P etc. elements which can supply electrons. After borax complex with these two PAF molecules, their resolution was improved obviously, probably due to the changes in the solubilization degree of C_{16}-AGEPC and C_{18}-AGEPC in the SDS micellar. In order ensure lower ionic strength and lower noise level in the separation system, the concentration of borax was not over 10 mmol/l in the aqueous phase.

Three mol/l urea were added to the 10 mmol/l borax solution, the system separating current did not increase, but the retention time of both PAF delayed evidently and the peak shape became sharp; dilatory tail reduced, and the separating efficiencies were distinctly improved. But in the system which included urea solutions without borax, the retention time of two types of PAF molecule can be delayed, but cannot be fully separated from each other. So, we can see that the role of urea is to make the retention time of the SDS micellar delayed and further enlarge the time window of separating period.

The addition of borax allows the two molecular forms of PAF to be satisfactorily separated. The presence of urea, however, can make the peaks even sharper, this facilitating the analysis e.g. of human blood. The working solution thus contains both 3 mol/l urea and 10 mmol/l borax.

Effect of pH

The changes of the electroosmotic flow and the retention time of the components as the pH changes in the system are described in Fig. 4. The figure shows that as the pH value increased, the electroosmotic flow enlarged, i.e. the retention time of the methanol was shorter. At the same time, as the pH was higher, the separating current increased at the same applied voltage, so the retention times of the samples were shortened. A lower pH value is advantageous to use the higher separating voltage and obtain the higher separation efficiency. For both of the PAF, we found that the average efficiencies (N) were 1.15×10^5 (pH 4.8) and 3.0×10^5 (pH 6.8), theoretical plates/meter, respectively. The N value was lower at the lower pH, probably due to the separated band broadening with the increasing retention time. Finally, we selected pH 6.8 to separate the system acidity.

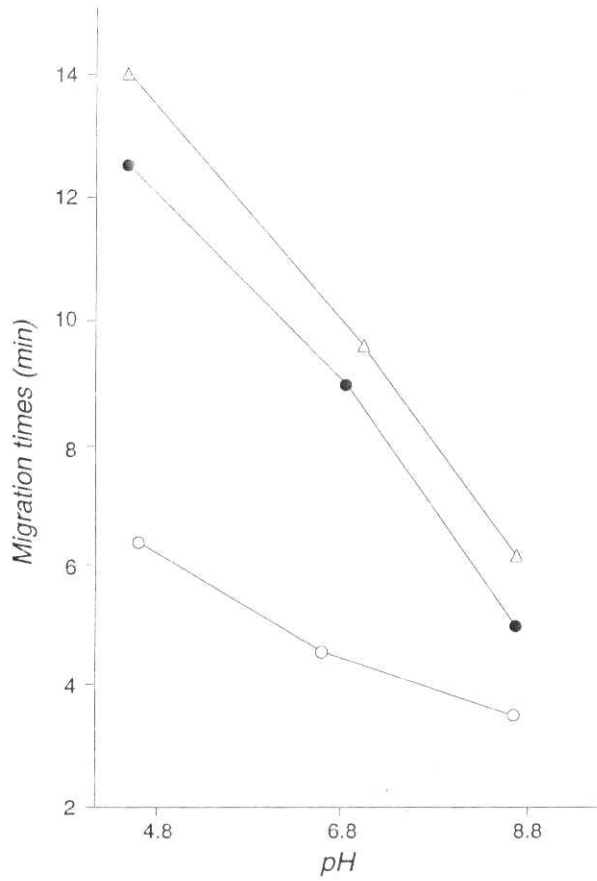


Fig. 4. - Effect of pH on the electroosmotic flow time (O) and the migration times of C_{16}-AGEPC (●) and C_{18}-AGEPC (Δ). Running buffer: 50 mmol/l SDS, 20 mmol/l potassium hydrogen phthalate, 10 mmol/l borax, 3 mol/l urea. Other conditions as in Fig. 1.

Precision, linearity and recovery

On the basis of the above optimized parameters, the retention times of C_{16}-AGEPC and C_{18}-AGEPC were 8' 10" and 8' 42" respectively in the system of 50 mmol/l SDS, 20 mmol/l potassium hydrogen phthalate, 10 mmol/l borax and 3mol/l urea (pH 6.8), the applied voltage was 25 kV and the current 20 μA (Fig. 3c). The mixed standard solution contained 40 μg/ml of C_{16}-AGEPC and 40 μg/ml of C_{18}-AGEPC were measured 6 times, the analytical precisions of the measurement were 3.0% (C_{16}-AGEPC) and 2.8% (C_{18}-AGEPC); and the precisions of the retention time were 1.4% (C_{16}-AGEPC) and 1.7% (C_{18}-AGEPC). This system gave linear plots for the against concentration up to 80 μg/ml: r = 0.9958 for C_{16}-AGEPC, r = 0.9962 for C_{18}-AGEPC (n = 10). Before each injection of the same sample, the capillary tube must be washed for one minute in the order of 0.1 mol/l KOH, sub-boiling distilled water and the working buffer solution, if not,

there were very big variations in the retention times. With an injection time of 10 s, peak height was 10 cm and the detection limits for both compounds were 60 ng/ml (k = 3). Once the injection time increased, the bands broadened, so that the linear relationships between the peak height and the concentration were damaged. In the other hand, taking ten venous blood samples, then every samples was divided into two aliquots and 450 ng standards of C_{16}-AGEPC and 400 ng C_{18}-AGEPC were added into one of the aliquot as the determination of the recovery, the disposal process of the samples of recovery test was the same as mentioned in the part of experimental. The recoveries of C_{16}-AGEPC and C_{18}-AGEPC were the same and the ranges were 90%-95% (n = 10).

Determination of blood samples

The method of added standard into blood samples was used for detecting PAF in blood so as to reduce the drift of the components' retention time when they were separated from each other. Ten µl standard solution contained 20 µg/ml of C_{16}-AGEPC and 20 µg/ml of C_{18}-AGEPC, respectively, were added into the 10 µg/ml sample and then were measured under our optimum condition, these results were recorded as C_{16} and C_{18}; two PAF molecules' true concentration (C) in human blood samples were calculated in the light of the following formula:

$$C = (C'-C'')\ 1/50\ (\mu g/ml)$$

The concentration range for the C_{16}-AGEPC was 0.61-0.16 µg/ml (the mean value was 0.35 µg/ml); for the C_{18}-AGEPC was 0.52-0.12 µg/ml (the mean value was 0.27 µg/ml). Our results were identical to those obtained by other authors. Fig. 5 shows the typical human blood separating graph under optimum condition.

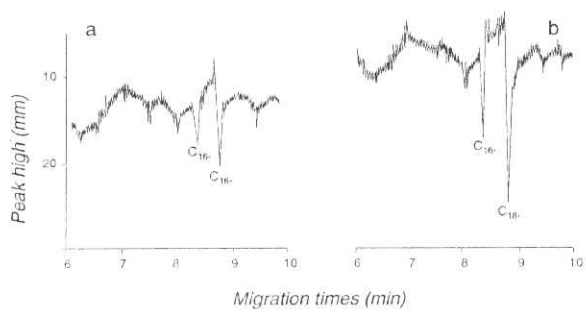


Fig. 5. - Electropherograms of (a) human blood and (b) human blood with 20 µg/ml mixed standard solution, other conditions as in Fig. 3c.

Conclusions

In the PAF, the various phosphocholines have similar structure and similar chemical properties, and all have no optical activity in the area of visibile light. The expected concentrations in the body are very low. Thus, a quick accurate and sensitive method to separate and measure PAF is a challenge. We first used the high performance capillary electrophoresis to separate and quantify the two kinds of PAF molecule in human blood. The method is simple and quick; the preparation of samples is simple and the components' loss very little, the amount of used samples is very small, so this method is very suitable for clinical medical research.

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REFERENCES

1. MITA, H., YASUEDA, H., HAYAKAWA, T. & SHIDA, T. 1989. Quantitation of platelet-activating factor by high performance liquid chromatography with fluorescent detection. *Anal. Biochem.* **180:** 131-135.

2. RAMESHA, C.S. & PICKETT, W.C. 1986. Measurement of subpicogram quantities of platelet activating factor (AGEPC) by gas chromatography negative ion chemical ionization mass spectrometry. *Biomed. Environ. Mass Spectrom.* **13:** 107-109.

3. CLAY, K.L., STENE, D.O. & MURPHY, R.C. 1984. Quantitative analysis of activating factor (AGEPC) by fast atom bombardment mass spectrometry. *Biomed. Mass Spectrom.* **11:** 47-49.

4. JAENERO, D.R. & BURGHARDT, C. 1990. Solid-phase extraction on silica cartridges as an aid to platelet activating factor enrichment and analysis. *J. Chromatogr.* **526:** 11-14.

5. LI, H.P., ZHANG, R. & ZHANG, J. 1993. Measurement of platelet activating factor in human blood by high performance thin layer chromatography and its clinical application. *J. Tongji Medical University* **13**(4): 226-230.

6. TERABE, S., OTSWKA, K., ICHIKAWA, K., TSUCHIYA, A. & ANDO, T. 1984. Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* **56:** 111-113.

7. KHALEDI, M.J., SMITH, S.C. & STRASTERS, J.K. 1991. Micellar electrokinetic capillary chromatography of acidic solutes: migration behavior and optimization strategies. *Anal. Chem.* **63:** 1820-1830.

8. WALLINFORD, R.A. & EWING, A.G. 1989. Separation of serotonin from catechols by capillary zone electrophoresis with electrochemical detection. *Anal. Chem.* **61:** 98-100.

9. GOZEL, P., GASSMAN, E., MICHELSON, H. & ZARE, R.N. 1987. Electrokinetic resolution of amino acid enantiomers with copper (II) - aspartame support electrolyte. *Anal. Chem.* **59:** 44-49.

10. TOMITA, M., OKUYAMA, T., SATO, S. & ISHIZU, H. 1993. Simultaneous determination of nitrazepam and its metabolites in urine by micellar electrokinetic capillary chromatography. *J. Chromatogr.* **621:** 249-255.

11. ANDRIKOPOULOS, N.K., DEMOPOULOS, C.A. & KAPADAI, A.S. 1986. High performance liquid chromatography analysis of platelet activating factor on a cation-exchange column by direct ultraviolet detection. *J. Chromatogr.* **363:** 412-417.

12. TONG, H.D. 1992. *Distinguish spectrogram of organic compounds.* X.D. Zhu (Ed.). Beijing University Press, Beijing, p. 214-215.

13. KUHR, W.G. & YEUNG, E.S. 1988. Optimization of sensitivity and separation in capillary zone electrophoresis with indirect fluorescence detection. *Anal. Chem.* **60:** 2642-2646.

14. WANG, T. & HARTWICK, R.A. 1992. Binary buffers for indirect absorption detection in capillary zone electrophoresis. *J. Chromatogr.* **589:** 307-313.