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Optimization of indirect photometric detection of anions in high-performance capillary electrophoresis

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ABSTRACT

Optimization of the indirect photometric detection in high-performance capillary electrophoresis is demonstrated. The influences of background electrolyte (BGE) concentration, capillary diameters, linear polymers, pH of BGE and types of BGE have been studied and assessed in terms of theoretical predictions. The experimental results fit the theoretical deduction very well. At optimized conditions, sub-femtomoles of simple anions can be detected, offering a 500-fold improvement compared to previous studies, and being close to the predicted limit of detection.

INTRODUCTION

High-performance capillary zone electrophoresis (HPCZE) has been recognized to be a modern separation technique which offers higher resolution with decreased analysis time than high-performance liquid chromatography (HPLC). However, a detection method with wide applicability is urgently needed for the advancement of HPCZE. UV–visible absorbance has been utilized as a detection method for HPCZE and still remains the most popular method of detection [1–5]. This method has several shortcomings: (1) samples separated by HPCZE are required to absorb light in the UV or visible region; (2) the detection limit and linearity are impaired due to the changes in the absorption coefficients (ε) of the analytes in different samples. The ones having lower absorption coefficients could not be detected at lower concentrations (*e.g.* femtomole range). Therefore, this detection method is mainly used for aromatic compounds [6], peptides [7], proteins [8], and nucleotides [9]. Fluorescence is a sensitive and most easily adapted detection method for HPCZE [10–12]. However, it can only be used to detect the samples which fluoresce or can be chemically modified to a fluorescent derivative. Chemical derivatization is an undesirable complication because of possible error in the chemistry and irreproducible operation, which can drastically affect quantitation. Mass spectrometry (MS) has been developed as a detector for HPCZE [13–15]. It is sensitive and can offer structural information of the samples. The high expense of a mass spectrometer, however, limits the widespread use of this technique in practice. Also, the detection of large molecules (MW > 100 000) by MS can be difficult even though some demonstrations have been made [16].

Indirect fluorometric detection has been demonstrated to be a universal, sensitive detector for HPCZE [17–20]. The remarkable aspect of this technique is the potential application to solutes with few other detectable properties. With laser excitation of the background fluorophore, typical detection limits (LOD) are in the attomole range which compares favorably with most other detection techniques. However, the impressive LOD is due to the high intensity of the excitation laser beam, which highly increases the cost of the instrumentation setup.

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Indirect photometric detection is another universal detection technique for HPCZE, which has a similar detection mechanism to that of the indirect fluorometric detection method. Detection of rare earth metals [21], cyclodextrins [22] and anions [23] has been demonstrated. However, the conditions were never optimized. The LOD at present (300–500 femtomoles) has not reached the level that should be attainable. Recently, Nielen [24] has done some quantitative studies on indirect UV absorbance detection in HPCZE. The author used a series of alkylsulphate surfactants as a model system to study the repeatability of the mobilities, response factors and linearity of the detection signal. However, other factors which may influence the separation and detection were not mentioned.

A strategy is presented in this paper for the optimization of indirect photometric detection of anions in HPCZE. Since the detection of trace amount of common anions, such as Cl^-, Br^-, NO_3^-, and SO_4^{2-}, is always of ongoing interest, the development of a rapid and sensitive detection method is very practical. The influence of capillary diameter, buffer pH, linear polymers, concentration of background electrolyte (BGE), and different kinds of BGE is described.

THEORETICAL

In the indirect photometric detection method for HPCZE, the detection limit can be described:

$$C_{lim} = C_{BGE}/(RDr) \quad (1)$$

where C_{lim} is the minimum analyte concentration of detection, C_{BGE} is the concentration of BGE, R is the displacement ratio (which is defined as the number of BGE molecules replaced by one analyte species), and Dr is dynamic reserve (which is defined as the ratio of the background signal to the noise level). Eqn. 1 reveals that the detection limit can be maximized by adjusting C_{BGE} and Dr, R is almost constant for a fixed separation system.

From the Lambert Beer law:

$$A_{BGE} = \log (I_0/I) = \varepsilon_{BGE}\, bC_{BGE} \quad (2)$$

where I_0 is the beam intensity before striking the cell, I is the beam intensity coming out the sample cell, ε_{BGE} is the molar absorption coefficient of BGE, and b is the optical path length of the beam I. Substituting eqn. 2 into eqn. 1:

$$C_{lim} = A_{BGE}\varepsilon_{BGE}bRDr \propto C_{BGE}\varepsilon_{BGE}bRDr \quad (3)$$

The absorbance detectors currently used in HPCZE exhibit a noise level of *ca.* $1 \cdot 10^{-4}$ absorbance units (a.u.). The displacement ratio is usually 1 for anions [25]. If we use 50-μm capillaries for the separation, the b in eqn. 3 will be $5 \cdot 10^{-3}$ cm. The Dr can reach 500 if we stabilize the background absorbance signal. If we choose a BGE with ε = 10 000 l/mol cm, and use the detection limit at signal-to-noise (S/N) = 5 ($A_{BGE} = 5 \cdot 10^{-4}$ a.u.), then the C_{lim} can be estimated:

$$C_{lim} = 5 \cdot 10^{-4}/(10\,000 \cdot 5 \cdot 10^{-3} \cdot 500) = 2 \cdot 10^{-8}\ M$$

If a 5-nl sample is injected, the mole detectability will be 0.1 fmol.

Because of the optical medium, a non-linear response is usually observed for direct absorbance measurement. Higher concentrations of analytes will lead to non-linear calibration plots due to self-absorption, self-quenching and detector saturation. In indirect absorbance detection, the response is not based on the analyte itself and a linear response should be, theoretically, easier to obtain.

EXPERIMENTAL

Equipment

The HPCZE system used was purchased from ISCO (Lincoln, NE, USA; Model 3850). A negative high voltage was applied to the capillary (*i.e.* the injection end was maintained at negative high voltage while the other end was held at ground potential). The data were collected with a Datajet computing integrator (Spectra Physics, Mountain View, CA, USA). The capillary column (Polymicro Technologies, Phoenix, AZ, USA) was 60 cm long. The outer diameter (O.D.) of the columns was always 150 μm, while the inner diameter (I.D.) ranged from 10 to 100 μm. The polymer coating was burned off 25 cm from the anodic end of the capillary to form the detection window.

Reagents

All chemicals were reagent grade unless otherwise noted. Deionized water was obtained using a

Milli-Q system (Millipore, Bedford, MA, USA).

HPLC-grade ethanol, benzoic acid, *o*-benzoyl benzoic acid, 2-sulfobenzoic acid, phthalic acid and hydroxylpropylmethyl cellulose (HMC) (4000 cP at 25°C for 2% solution) were purchased from Aldrich (Milwaukee, WI, USA). Other inorganic chemicals were purchased from Sigma (St. Louis, MO, USA).

RESULTS AND DISCUSSION

Fig. 1 demonstrates the influences of different kinds of BGE on the separation and detection of anions. It is shown that under the same concentration of BGE the detector response of separated anions was different. Even though *o*-benzyl benzoic acid has a much larger absorption coefficient (ε = 19 000 l/molcm) than benzoic acid (ε = 11 900 l/molcm) (at 228 nm), the peak heights of the anions are almost the same. The ε of the 2-sulfobenzoic acid (ε = 40 000 l/molcm) is 3 times larger than that of phthalic acid (ε = 13 000 l/molcm) at 228 nm. However, the detector responses of the anions are also almost the same. It also can be seen that NO_3^- gives a very weak signal when 2-sulfobenzoic acid is used as a BGE. All these phenomena suggest that with indirect photometric detection, the response of the analyte is not totally proportional to the absorption coefficient of the BGE, but also dependent on the displacement ratio (*R*). Also, for different BGEs, the dynamic reserve (*Dr*) is different. This is due to the variation of Joule heating from one BGE

TABLE I

THE ELECTROOSMOTIC VELOCITIES FOR DIFFERENT BGEs

Conditions: 0.02 *M* for each BGE; pH 6.5; other conditions as in Fig. 1.

BGE	μ_{eo} ($10^{-3}cm^2V^{-1}s^{-1}$)[a]
Benzoic acid	0.510 ± 0.13%
Phthalic acid	0.709 ± 0.15%
2-Sulfobenzoic acid	0.532 ± 0.13%
o-Benzylbenzoic acid	0.874 ± 0.14%

[a] The mean values of eight analyses ± relative standard deviation.

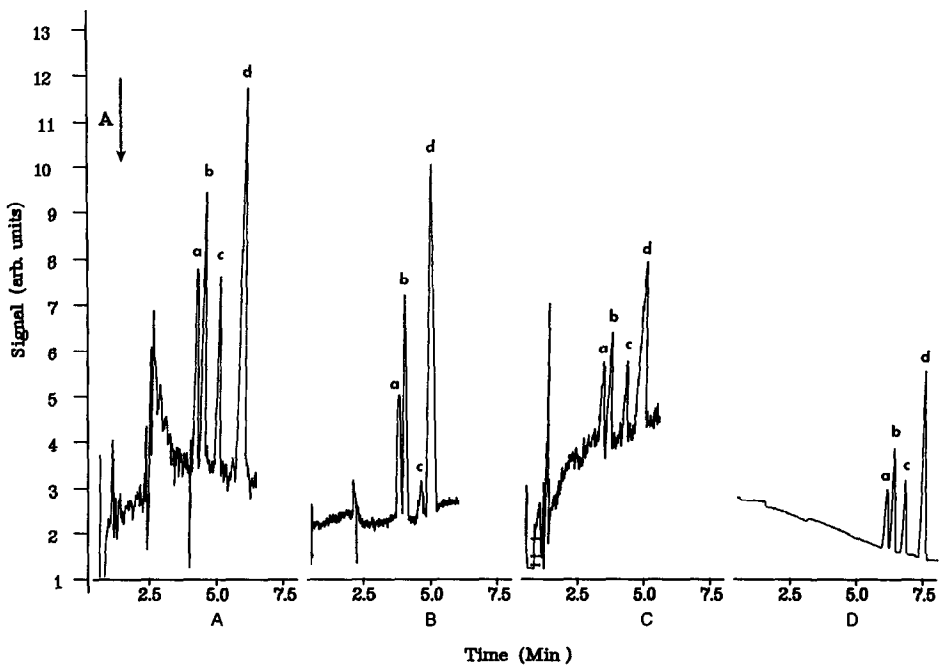

Fig. 1. The influences of different kinds of background electrolytes (BGE) on the separation and detection of anions. BGE used: (A) phthalic acid; (B) 2-sulfobenzoic acid; (C) benzoic acid; (D) *o*-benzyl benzoic acid. BGE concentration: 0.02 *M*. pH of BGEs: 6.5. A 3-s, −20-kV injection of 100 μM of each anion followed by electrophoresis at −20 kV in a 60-cm (75 μm I.D.) capillary column. Detection wavelength: 228 nm. Peak identification: a = bromide; b = chloride; c = nitrate; d = sulfate.

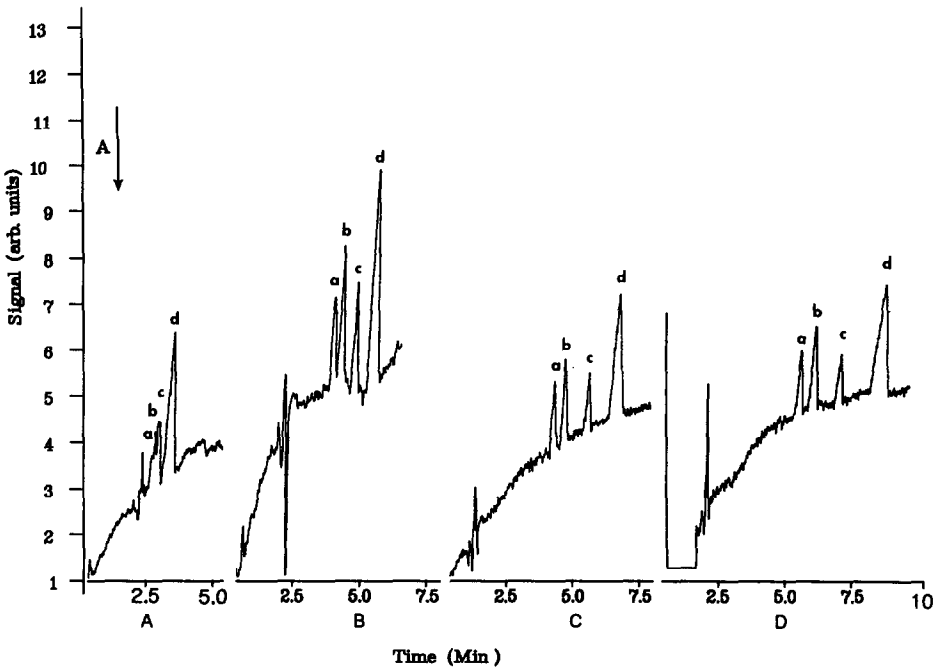

Fig. 2. The influence of the pH of the BGE solution. BGE used: 10 mM phthalic acid. The pH tested: (A) pH = 4.3; (B) pH = 6.5; (C) pH = 7.7; (D) pH = 9.2. Injection time: 2 s. The concentration of the anions: 50 μM each. All other conditions and peak identifications are the same as those of Fig. 1.

to another, which causes the variation of baseline drifting. In this case, phthalic acid would be a good choice. We noticed that the migration times of the analytes varies from one BGE to another. This is because of the changes of electroosmotic flow with BGEs, even though the pH values of the BGE were kept same. The μ_{eo} values for different BGEs are tabulated in Table I.

Fig. 2 demonstrates the influence of the pH of the BGE solution (the pH influences are very similar for four different BGEs studied). It is clear that when the pH of the BGE solution is too low, the separation of the anions becomes worse and the signal becomes smaller. The reason is that the BGE molecules are not charged at low pH, the replacement ratio is smaller, and the signal becomes smaller. At lower pH, the electroosmotic flow, migrating oppositely from that of anions, is also smaller, causing the effective mobility and resolution of the analytes to change which also affects the separation. This phenomenon has been described earlier [18]. At higher pH, the separation of the anions becomes better. However, the migration time is longer and the peaks are broader. This is understandable, because the electroosmotic flow at higher pH is much larger.

Fig. 3 shows the influence of the linear polymers. We found that the analyte peaks are sharper and elute faster with the addition of 0.3% hydroxylpropylmethyl cellulose (HMC) in the running buffer, comparing to that containing no HMC. This is due to the decreases of electroosmotic flow by additive HMC. However, addition of 0.5% of HMC in the running buffer causes the analyte peaks to be smaller, more broad and take longer to elute than that of 0.3% HMC. The reason for this is due to: (1) the increases of viscosity of the running buffer solution which decrease the migration rate of analyte ions; (2) the pH change (from pH 6.5 to pH 7.8). These changes will change the separation behavior and displacement ratio. The μ_{eo} values, pH and viscosity for different amount of HMC (in the running buffer) are listed in Table II.

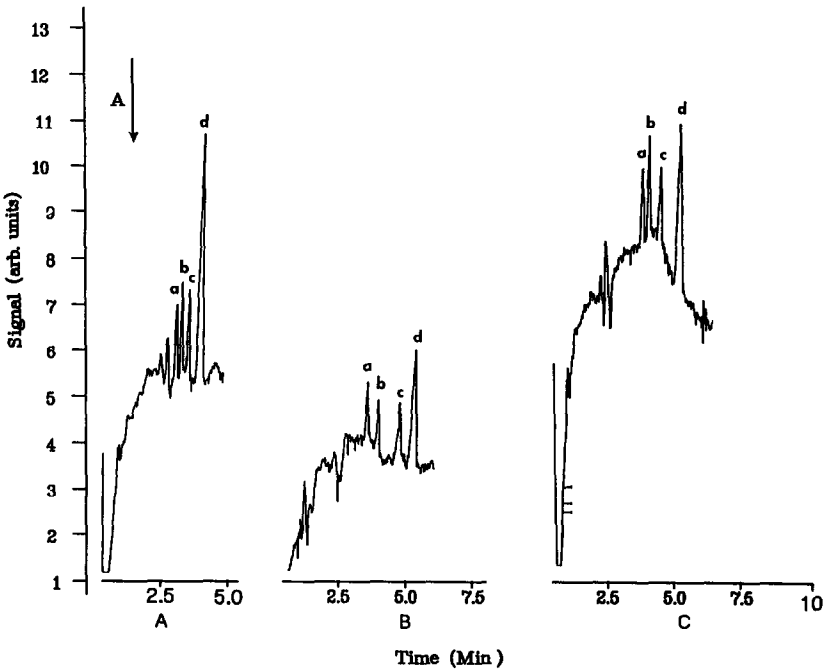

Fig. 3. The influences of the linear polymers (additive). BGE used: 10 m*M* phthalic acid (pH = 6.5). Linear polymer: hydroxylpropyl methyl cellulose (HMC). Polymer concentration: (A) 0.3%; (B) none; (C) 0.5%. Injection time: 2 s. The concentration of the anions: 50 μM each. All other conditions and peak identifications are the same as those of Fig. 1.

The influence of the capillary diameter is shown in Fig. 4. We found that the peak areas of the analytes were smaller and the peaks were sharper in 25 μm I.D. capillary column than those of 75 μm I.D. when the injection time and the other parameters were kept the same. Based on these experiments, the detection limits for both diameters were almost same. This suggests that with a narrow range of column diameters, the column diameter is not the major contribution to the limit of detection. The explanation for this is that when column diameter was changed, Joule heating (increasing with column diameter) [26–28], *Dr*, and velocity of electroosmotic flow would change also. The μ_{eo} values and *Dr* for

TABLE II

THE μ_{eo} VALUES, pH AND VISCOSITY FOR DIFFERENT HMC IN THE RUNNING BUFFER

Conditions: 10 m*M* phthalic acid; the measurements of viscosity were carried out at 25°C and atmospheric pressure; other conditions as in Fig. 1.

% of HMC	μ_{eo} (10^{-3}cm^{2}V^{-1}s^{-1})[a]	pH	viscosity(η)[b] (cP)
0	0.713 ± 0.14%	6.5	0.8940 ± 0.07%
0.3	0.405 ± 0.15%	6.9	1.7391 ± 0.06%
0.5	0.408 ± 0.13%	7.8	2.1739 ± 0.06%

[a] The mean of eight analyses ± relative standard deviation.
[b] The mean of three measurements ± relative standard deviation.

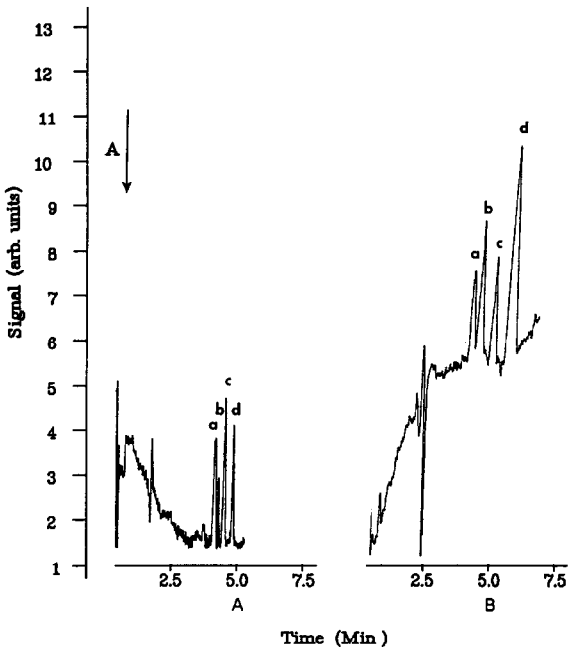

Fig. 4. The influence of the capillary diameters. BGE used: 10 mM phthalic acid (pH = 6.5). Capillary diameters: (A) 25 μm; (B) 75 μm. Injection time: 2 s for both. The concentration of the anions: 50 μM each. All other conditions and peak identifications are the same as those of Fig. 1.

different diameter of columns are listed in Table III. However, when a 10 μm-I.D. capillary was used, the LOD worsened dramatically (chromatogram is not included). This was found to be due to the inability to focus all the light onto a 10-μm capillary. When a 100 μm I.D. capillary was used, the large increase of Joule heating caused a serious fluctuation of baseline, which decreased the Dr significantly. The Joule heat generated for different diameter of columns (W/cm) is listed in Table IV.

The influence of concentration of the BGE was also investigated. We found that there were no analyte peaks observed when the BGE concentration was smaller than $1.0 \cdot 10^{-5}$ M. This was due to the very low background absorbance signal, low dynamic reserve (Dr) which was caused by serious baseline shifting and high noise level. Other authors have found the same problem [24,29,30], and the reasons for the baseline problem at very low concentration of BGE have been well explained. All these factors made the replacement signal negligible. When BGE concentration was smaller than ~ 1 mM, the broader analyte peaks were observed and the separation was very poor. This can be explained by laminar flow effect [31]. According to the Kohlrausch theory [32], a narrow zone of analytes can be formed when a long plug of low-concentration buffer containing analytes is injected into a column filled with a high-conductivity buffer. However, at low concentration of BGE, since the local electroosmotic flow in the sample plug is smaller than the bulk electroosmotic flow of the running buffer, the pressure difference caused by the mismatch in electroosmotic flow will generate a laminar flow inside the column which will broaden the sharp zone generated by the stacking process and sharply reduce the resolution. The details on this aspect have been reported by Burgi and Chien [33,34]. We also found that 5 to 15 mM BGE was the best concentration range to work with. In addition, the BGE concen-

TABLE III

THE μ_{eo} VALUES AND Dr FOR DIFFERENT DIAMETERS OF COLUMNS

Conditions: 10 mM phthalic acid; pH 6.5; other conditions as in Fig. 1.

Capillary diameter	μ_{eo} (10^{-3}cm^{2}V^{-1}s^{-1})[a]	Dr
25 μm	0.705 ± 0.14%	741
50 μm	0.709 ± 0.13%	638
75 μm	0.713 ± 0.14%	474

[a] The mean of eight analysis ± relative standard deviation.

TABLE IV

THE JOULE HEAT GENERATED FOR DIFFERENT DIAMETERS OF COLUMN

Conditions: 10 mM phthalic acid; pH 6.5; capillary length: 60 cm; voltage applied: −20 kV; the molar conductivity of phthalic acid: 382 Ω^{-1}cm^{2}mol^{-1}.

Column diameter (μm)	Joule heat generated (V$^2\Omega^{-1}$cm^{-1}) or (Watt/cm)
10	$1.06 \cdot 10^{-4}$
25	$6.63 \cdot 10^{-4}$
50	$2.65 \cdot 10^{-3}$
75	$5.97 \cdot 10^{-3}$
100	$1.06 \cdot 10^{-2}$

TABLE V

THE REPEATABILITY OF MOBILITIES AND DETECTION SIGNALS

Conditions: 10 m*M* phthalic acid; pH 6.5; other conditions as in Fig. 1.

Component	Migration time[a] (min)	Peak area[a] (10^5 counts)
Br^-	4.15 ± 0.69%	3.87 ± 7.3%
Cl^-	4.42 ± 0.82%	4.92 ± 4.9%
NO_3^-	4.86 ± 0.91%	3.99 ± 4.3%
SO_4^-	5.31 ± 0.96%	15.21 ± 6.8%

[a] The mean values of eight analysis ± relative standard deviation.

tration needed to be increased when the analyte concentration was high.

The repeatability of the mobilities and detection signals have been evaluated. The mean values and relative standard deviations based on eight times of analysis are listed in Table V. It can be concluded that the mobilities and detection signals are very constant based on statistic rules (at 90% confidence level).

The detection limit of the anions was demonstrated at optimized conditions. The detection limit for four common anions is shown in Fig. 5 . Sub-femtomoles of each anion were detected, which fits the theoretical prediction very well.

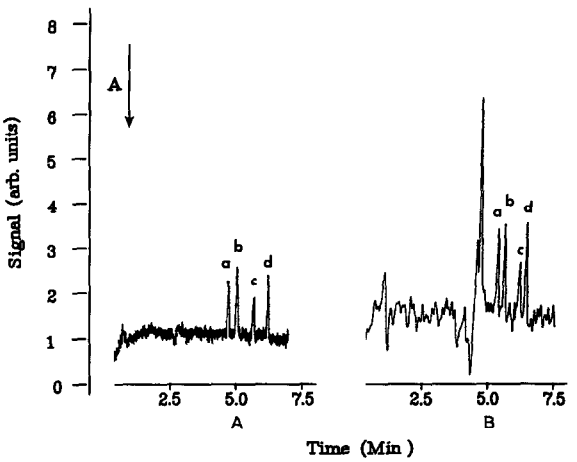

Fig. 5. The detection limits of anions. BGE used: 10 m*M* phthalic acid (pH = 6.5). Capillary diameter: (A) 25 μm; (B) 75 μm. Concentration of anions: $[Br^-] = [NO_3^-] = 2.0 \cdot 10^{-6}$ *M*; $[Cl^-] = 1.0 \cdot 10^{-6}$ *M*; $[SO_4^{2-}] = 2.0 \cdot 10^{-7}$ *M*. Injection time: (A) 5 s; (B) 2 s. Moles injected: 0.3 fmol and 1.1 fmol of Cl^- were injected for (A) and (B) individually. All other conditions and peak identifications are the same as those of Fig. 1.

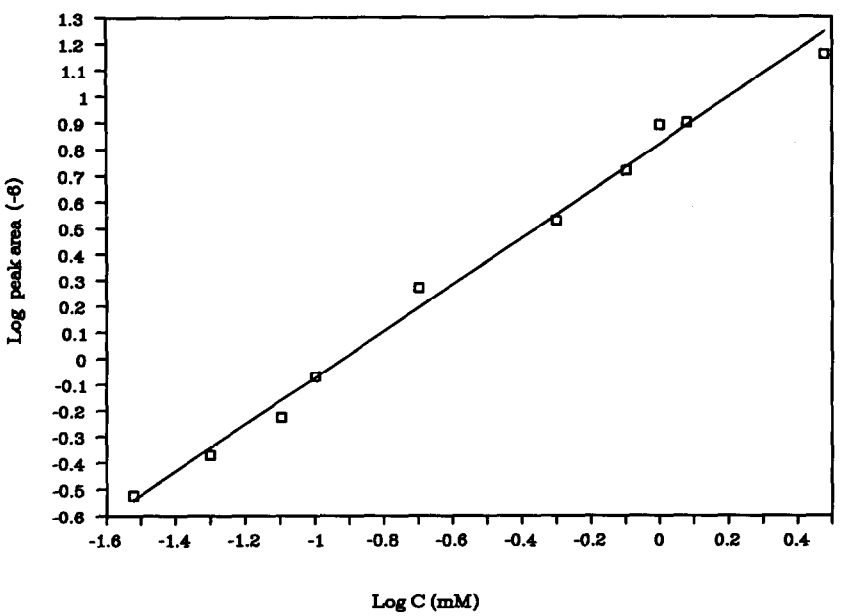

Fig. 6. The linearity for indirect photometric detection based on peak areas. The test anion is chloride.

Finally, the linearity for indirect photometric detection is shown in Fig. 6. A linear response range with two orders of magnitude was obtained for chloride, which would be impossible for direct UV absorption measurement.

CONCLUSION

The theoretical and practical strategies for optimization of indirect photometric detection in HPCZE have been presented in this paper. The influences of background electrolyte (BGE) concentration, capillary diameters, free flow linear polymers, pH of the BGE and type of BGE have been studied with our HPCZE system. The experimental results fit the theoretical deduction very well. Under optimized conditions, sub-femtomoles of simple anions were detected, offering a 500-fold improvement compared to previous studies [23,35], and approaching the predicted limit of detection. In addition, the linearity for indirect photometric detection was studied. Over two orders of magnitude of linearity were observed based on the peak area calculations.

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