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Automated on-capillary isotachophoretic reaction cell for fluorescence derivatization of small sample volumes at low concentrations followed by capillary zone electrophoresis

N.J. Reinhoud, U.R. Tjaden*, J. van der Greef

Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, Netherlands

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Abstract

An automated on-line reaction cell is described combining the concentrating properties of isotachophoresis (ITP), the separation efficiency in capillary zone electrophoresis (CZE) and the detection sensitivity of laser-induced fluorescence (LIF) detection. The applicability of the reaction cell is demonstrated for an automated derivatization procedure of glutathion and several amino acids with *o*-phthaldialdehyde (OPA) and naphthalene-2,3-dicarboxaldehyde. Nanolitres analyte, at nanomolar concentration level, were concentrated and derivatized in capillary ITP. The derivatives were analyzed by CZE with LIF detection. The entire procedure was automated and took place in the same capillary using single capillary ITP–CZE. Furthermore, an easy applicable on-capillary OPA derivatization is described for CZE without ITP step.

1. Introduction

The on-line coupling of capillary zone electrophoresis (CZE) with other analytical separation techniques has resulted in increased selectivity and separation power. Improvements in detection techniques has resulted in detection of extremely low amounts of analyte [1–3]. In combination with electrophoretic stacking or isotachophoretic preconcentration methods the corresponding determination limits are impressive [4].

Small injection volumes, typically in the nanolitre range, are characteristic for CZE. However, in most analytical sample pretreatment procedures the smallest volumes that can be handled are in the microlitre range. This implies that in most cases more than 99% of the sample is wasted. In situations where analytes are chemically modified before analysis a more efficient use of reagents is achieved by reduction of the reaction cell volume. This is especially relevant in cases where expensive reagents are involved such as enzymes or antibodies. The reaction cell described in this paper has at least a factor 100 lower reagent consumption compared to a conventional 100-μl reaction vial. The applicability of the reaction cell is demonstrated for a fluorescence introducing derivatization reaction.

The use of on-line fluorescence derivatization procedures puts several demands on the analytical method. Fluorophoric derivatization reagents, where the reagent shows fluorescent

* Corresponding author.

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properties similar to that of the derivatization product, are difficult to apply in an on-line procedure because the reagent has to be separated from the derivative before detection takes place. Therefore, fluorophoric derivatization reagents are usually applied in the pre-column mode, sometimes in combination with an additional step for removal of the excess of reagent. Fluorogenic reagents, where the reagent does not have fluorescent properties similar to that of the derivatization product, are considerably easier to apply in on-line procedures [5–7].

Several applications of CZE with laser-induced fluorescence (LIF) detection and pre-column derivatization have been described [1–3,7]. Recently, Houben *et al.* [8] described an automated derivatization procedure for absorbance detection of amino acids. Combination of CZE with an on-capillary enzyme reactor has been described by Avila and Whitesides [9]. Chang and Yeung [10] described an on-column protein digestion with pepsin followed by CZE with native LIF detection.

In this paper the applicability of the capillary reaction cell is demonstrated for an automated on-capillary derivatization of low concentrations of analyte in single capillary isotachophoresis (ITP)–CZE. The analyte is concentrated in the focusing step, derivatized in the ITP step and analyzed in the CZE step. The method takes place in a commercially available CZE system without any hardware modifications. In previous papers we have described automated procedures for ITP–CZE in a single capillary for cationic and anionic separations [11–13]. Furthermore, an on-capillary *o*-phthaldialdehyde (OPA) derivatization is described for CZE without an ITP step of amines at higher analyte concentrations.

2. Theory

2.1. Derivatization reactions

The OPA reagent in combination with a nucleophile such as β-mercaptoethanol (ME) forms N-substituted 1-alkyl-isoindole derivatives. The derivative is formed within 1 min and slowly decomposes (Fig. 1A). Therefore, it is important that the reaction times are kept constant. This makes the OPA reagent only suitable for pre-capillary derivatization if the procedure is automated so that the derivatization times are kept constant as is the case in the described on-capillary derivatization methods. Derivatization of peptides with the OPA reagent is only possible if the peptide contains lysine, due to the high reactivity of the ε-amine function [14,15].

The naphthalene-2,3-dicarboxaldehyde (NDA) reagent in presence of a nucleophile such as cyanide forms stable derivatives with primary amines, including the terminal amine function of peptides. As a result of the formation of side products and the derivatization time needed (10

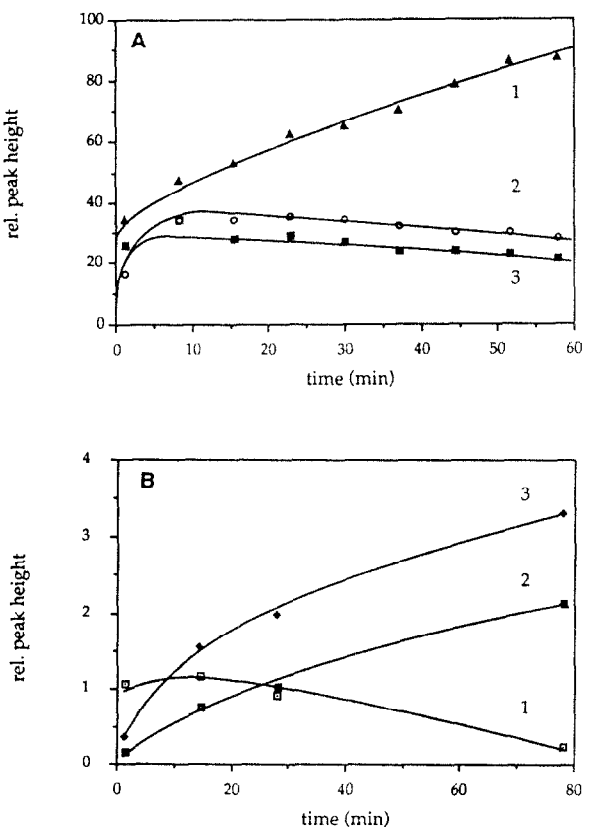

Fig. 1. Relative peak height measured at different times after derivatization of GSH (1), Asp (2) and Glu (3) with (A) OPA–ME and (B) NDA–CN reagent. Concentrations analyte were respectively 100, 100 and 200 ng/ml for the OPA derivatization and 1.0 μ*M* each for the NDA derivatization.

min for peptides, Fig. 1B) it is mainly used as pre-capillary derivatization reagent. NDA in combination with ME forms a less stable product but in combination with fast reaction times (1 min for peptides) it is suitable as post-capillary derivatization reagent [16].

OPA and NDA are also used in derivatization procedures for electrochemical detection [17], for NDA derivatives a selective peroxyoxalate chemiluminescence detection procedure has been described [18]. Certain analytes such as histidine [19], histamine [19], spermidine [20], serotonin [21] and glutathione [22,23] react with OPA without addition of a nucleophile which increases the selectivity of the derivatization reaction considerably.

2.2. ITP–CZE

CZE has become an important separation technique complementary to high-performance liquid chromatography (HPLC). One of the challenges in the application of CZE separations in the analysis of biological samples is to reach for relevant determination limits. Electrophoretic analyte focusing techniques provide a way to increase the loadability and concentration detection sensitivity in CZE. The coupling of ITP with CZE has been described by several authors [4]. Improvements in determination limits by a factor of 1000 have been reported.

In ITP a discontinuous buffer is used and only anions or cations can be analyzed in one run. In anionic separations the capillary and the anode vial are filled with leading buffer. The leading buffer contains anions with a mobility higher than the mobility of the analyte ions. The cathode vial is filled with terminating ions which have the lowest mobility. The pH is set with the buffering counterions. In the steady state, when the analytes are separated, they migrate as consecutive zones ordered by their electrophoretic mobilities. A characteristic of ITP is its concentrating property. The concentrations of analyte ions are adapted to the concentration of the leading ions according to the Kohlraush regulation function (see ref. 13). The analyte concentrating property makes the technique attractive as preconcentration technique for CZE.

2.3. On-capillary reactions in ITP–CZE

An on-capillary reaction cell in ITP–CZE puts several demands on the system that is used. The reagent is added to the ITP buffer, therefore it should not be reactive to buffer components and the buffers should be of a high purity. In case of reactions with primary amines the number of buffers that can be used is limited. When the reagent is ionic, care must be taken that the ITP conditions are not disturbed and that mixing of the reagent with sample zones takes place. The analyte is derivatized under ITP conditions. To maintain sharp analyte zones the analyte and derivative should be within the ITP separation window. These conditions are all fulfilled with the OPA and NDA derivatization of primary amines.

The combination of single capillary ITP–CZE and an on-capillary reaction offers several advantages. Reaction conditions such as the temperature can be chosen easily, most of the commercially available CZE equipment offers the possibility of temperature control. The reaction time can be chosen as long as necessary. We already demonstrated that the ITP time does not influence the efficiency in single capillary ITP–CZE [11]. When the analyte zone is concentrated and fixed on a certain position in the capillary it stays there until the hydrodynamic pressure is reduced. In an automated ITP–CZE procedure the reaction time will be constant which is important in case of instable reaction products.

Using single capillary ITP–CZE adds an interesting feature to reaction kinetics. Not only the initial concentrations of reactants can be set but also the supply of fresh reagent can be varied while the analyte concentration is kept constant. In principle only the length of the underivatized analyte zone will reduce under ITP conditions until the derivatization is complete. The mixing of the reacting species is done in a very controlled way. The velocity of the neutral reagent is precisely known and can be manipulated by the applied electrical field strength during the

ITP step. The velocity of the analyte is zero and because of the concentrating properties of ITP the analyte concentration will be high resulting in favourable reaction kinetics. The excess of reagent will automatically be removed before the CZE is started. All reaction products that are outside the separation window with a mobility lower than the terminator ions, are discarded during the ITP step. Another aspect is that in ITP–CZE using narrow-bore capillaries, reactions in picolitre volumes can be carried out.

2.4. The reagent molar mass flow

One of the attractive features of the on-capillary ITP reaction cell is the possibility of controlling the reagent molar mass flow in the analyte zone. The reagent molar mass flow is calculated by deriving an equation for the velocity of neutrals during the ITP step. A simple buffer system is assumed, consisting of only one type of univalent cations and anions (i.e. L^- and R^+, T^- and R^+ for leading and terminating buffer).

In single capillary ITP–CZE a hydrodynamic pressure is applied during the focusing and ITP step to keep the front boundary of the sample zone (Fig. 2, B1) on a fixed position in the capillary. The hydrodynamic flow velocity counter balances the velocity of the leading ions so that the total velocity of the leading ions is zero. In the focusing step the terminating boundary of the sample zone (B2) migrates towards B1 resulting in a concentration of the analytes until the ITP state is reached. Under ITP conditions the velocities of all anions are the same. This distinguishes the focusing step from the ITP step.

The molar mass flow of a neutral reagent in the ITP step (J in mol/s) is determined by the reagent velocity, the cross-sectional area of the capillary and the reagent concentration and is given by

$$J = v\pi r^2 c \quad (1)$$

where v is the velocity of the neutral reagent under ITP conditions with a counterbalancing pressure, c is the reagent concentration and r is the capillary radius.

When the velocity of the leading ions is

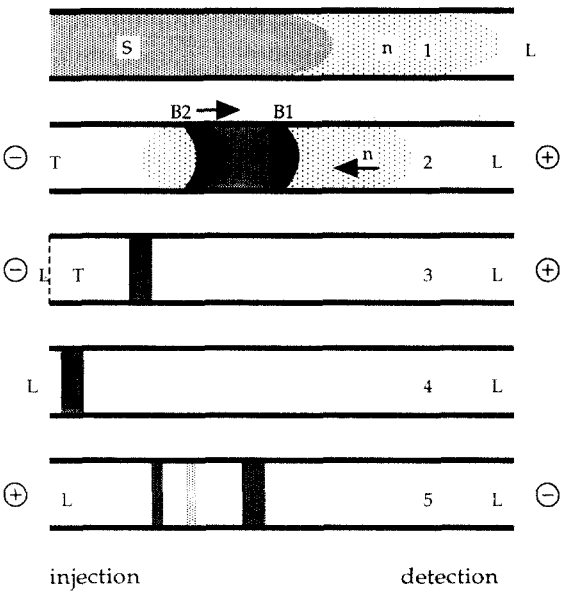

Fig. 2. Schematic representation of the on-capillary derivatization during the ITP step in single capillary ITP–CZE. In step 1 the neutral reagent (n, in leading buffer) is injected before the sample (S, in terminating buffer). In the focusing step 2 electrophoretic and hydrodynamic velocities are balanced so that the boundary B1 is kept on a fixed position in the capillary. Boundary B2 moves to boundary B1. The neutral derivatization reagent is moving through the sample zones into the direction of the cathode and the derivatization takes place. The reagent is supplied by injection as a zone with a defined length or continuously supplied by addition to the leading buffer vial. See Experimental for further explanation.

counterbalanced by a pressure-induced flow, the electrophoretic velocity of the leading ions will be zero. Fig. 3 gives a schematic vector representation of the velocities during electrophoresis. Under ITP conditions the velocity of the neutrals is determined by the hydrodynamic and electroosmotic velocity. During electrophoresis a leading anion with an electrophoretic velocity (vector 1) will migrate to the cathode with a total velocity (vector 3) due to a high electroosmotic velocity (vector 2). Neutrals are carried with the electroosmotic flow and have a velocity (vector 2). When a counterbalancing pressure is applied (vector 4), the velocity of the anion (vector 3) is reduced to zero. Under these conditions the velocity of the neutrals (vector 5) is also reduced. The velocity of neutrals under conditions

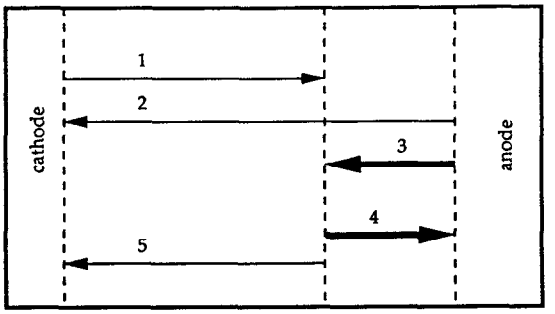

Fig. 3. Velocities during electrophoresis (1–3) and during electrophoresis under ITP conditions (4,5). The vectors represent the electrophoretic velocity of the leading ions (1), the velocity of the electroosmotic flow (2), the net velocity of the leading ion (3) which is the sum of 1 and 2, the velocity of the counterbalancing hydrodynamic flow (4) and the velocity of the neutrals in presence of the counterbalancing hydrodynamic flow during the ITP step (5). For further explanation see Theory.

with a counterbalancing pressure (vector 5) is the same as the electrophoretic velocity of the leading anions without a counterbalancing pressure (vector 1), in the opposite direction.

The velocity of the neutrals is then given by [13]

$$v = -v_{el,L} \quad (2)$$

where $v_{el,L}$ is the electrophoretic velocity of the leading ions under ITP conditions without a counterbalancing pressure.

The supply of reagent can be manipulated by the electric field strength. Using $v_{el,L} = \bar{m}_L E_L$, the reagent molar mass flow is given by

$$J = \bar{m}_L E_L \pi r^2 c \quad (3)$$

where $\bar{m}_L$ is the effective electrophoretic mobility of the leading ions and E_L is the electrical field strength in the leading buffer zone. Analogous to Eq. 3 the molar mass flow in the terminator zone is given by $J = \bar{m}_T E_T \pi r^2 c$. In the ITP steady state the electrical field strength in the leading buffer zone (E_L) is by definition lower than in the terminating buffer zone (E_T). In ITP it holds that $E_T \bar{m}_T = E_L \bar{m}_L$ which means that the molar mass flow of the neutral reagent in the terminator zone is the same as in the leading buffer zone. In a similar way it can be shown that in ITP the molar mass flow in the sample zones is the same as in the leading buffer zone.

The capillary reaction cell is not only restricted to neutrals. Charged reagents can be used as long as the focusing and ITP process are not disturbed. In case of anionic separations cationic reagents or anionic reagents with a low electrophoretic mobility can be used as well. Ampholytic reagents (enzymes, antibodies) can be used at a pH near the isoelectric point where the nett charge is zero. For on-capillary NDA derivatization of amino acids cyanide is used which has a small anionic charge at the working pH (pK_a of cyanide is 9.5). When a counterion is used as a reagent the velocity and concentration in the leading and terminating zone can be calculated using the well known moving boundary equation.

3. Experimental

Untreated fused-silica capillaries (SGE, Ringwood, Australia) were used. A programmable injection system for capillary electrophoresis (PRINCE; Lauerlabs, Emmen, Netherlands) equipped with a reversible polarity power supply and possibility for pressurised and electrokinetic injection was used for the analyte focusing process. The LIF detection system has been described previously [11]. Excitation of OPA derivatives took place at 351.1 and 363.8 nm and for emission a 450-nm bandpass filter (10 nm bandwidth, type 53830; Oriel, Stratford, CT, USA) was used. The NDA derivatives were detected using the 457.9-nm laser line for excitation and a 515-nm long-pass filter (Oriel) for emission.

3.1. Chemicals

Sodium cacodylate, triethanolamine (TEtOHA) (97%) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) were purchased from Janssen Chimica (Beerse, Belgium). Hydroxypropylmethylcellulose (HPMC) with a viscosity for a 2% aqueous HPMC solution of 4000 cP, came from Sigma (St. Louis,

MO, USA). Acetic acid (HAc), sodium borate, phosphoric acid, barium hydroxide, potassium cyanide (KCN), glutamic acid (Glu), aspartic acid (Asp), arginine (Arg), lysine (Lys), tryptophan (Trp), phenylalanine (Phe), threonine (Thr), serine (Ser), 3,4-dihydroxyphenylalanine (Dopa) and bromophenol blue were from Merck (Darmstadt, Germany). OPA and ME were from Aldrich Chemie (Steinheim, Germany). NDA came from Molecular Probes (Eugene, OR, USA). In all experiments deionised water was used (Milli-Q system; Millipore, Bradford, MA, USA).

3.2. Analyte focusing with on-capillary derivatization

The ITP–CZE procedures were used as described previously [11,12], with exception of the first step (Fig. 2). In step 1 the capillary is filled with leading buffer containing the derivatization reagent. Then the injection of sample takes place hydrodynamically at a pressure of 100 mbar. In step 2 the analyte focusing starts by applying a voltage in conjunction with a hydrodynamic pressure. After 5–20 min of focusing and derivatization, depending on the injected volume, a voltage of −20 kV is applied without a hydrodynamic pressure (step 3). The zone of terminating buffer is removed isotachophoretically out of the capillary. At the time that the sample zone is approaching the capillary inlet the terminator buffer vial is replaced for a vial containing the CZE background electrolyte (step 4), the voltage is reversed and the CZE run is started (step 5).

The current is monitored for precise timing of the moment to switch from ITP to CZE. When a constant voltage is applied the current increases as long as the terminating ions leave the capillary. The CZE equipment can be programmed so that at a defined threshold of the current the switching takes place automatically. In principle all ions with mobilities below that of the terminating ion, including the counter ions, are removed by the described procedure.

3.3. ITP–CZE buffer systems

System I

The leading buffer consisted of 10 m*M* HAc set at pH 8.0 with TEtOHA and was also used as CZE background electrolyte. The terminating buffer was 10 m*M* HEPES set at pH 8.0 with TEtOHA. The capillary dimensions were 590 × 0.100 mm, detection took place at 520 mm from the inlet.

System II

The leading buffer was a 10 m*M* sodium phosphate buffer at pH 9.4 and was also used as CZE background electrolyte. To the leading buffer 0.05% (w/v) of HPMC was added. The terminating buffer consisted of 10 m*M* sodium cacodylate set at pH 9.4 with $Ba(OH)_2$ and contained 0.05% HPMC (w/v). The capillary dimensions were 700 × 0.100 mm, detection took place at 600 mm from the inlet.

System III

The CZE buffer consisted of a 40 m*M* borate buffer at pH 9.5 and contained 0.1 mg/ml OPA and 0.1% (v/v) ME. In initial experiments 1.0 mg/ml OPA was used. The capillary dimensions were 730 × 0.075 mm, detection took place at 665 mm from the inlet.

3.4. Derivatization

Pre-column OPA derivatives were made by 1:1 addition of the analyte in leading buffer to an OPA solution of 1 mg/ml in leading buffer containing 0.1% ME. The NDA derivatives were made by 1:1 addition of the analyte in leading buffer to a NDA solution of 2 m*M* in leading buffer containing 1 m*M* potassium cyanide. The NDA reagent was diluted from a stock solution of 100 m*M* in MeOH and mixed with KCN in leading buffer just before the derivatization was started.

For on-capillary derivatization reactions in ITP–CZE the reagent was added to the leading buffer. The OPA reagent was used at a concentration of 0.1 mg/ml and 0.01% ME and was

added to the leading buffer vial. This buffer vial was used during the entire ITP–CZE procedure. The NDA and KCN concentrations were 0.1 m*M*. The NDA reagent was supplied by injecting a zone of leading buffer containing the derivatization reagent before sample injection for immediate start of the derivatization during the focusing step. For NDA derivatization with continuous supply of reagent only during the ITP step the leading buffer vial was switched after the focusing step for a leading buffer vial containing the NDA reagent. Glutathion is unstable under alkaline conditions and was added to the terminator buffer just before injection.

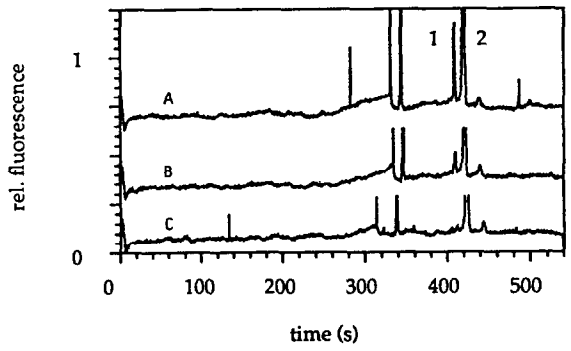

Fig. 4. On-capillary NDA derivatization and ITP-CZE of 3.0 (A) and 1.0 (B) n*M* GSH (1) and the blank (C). Peak 2 is the internal standard fluorescein. Other peaks are system peaks.

4. Results and discussion

Several aspects of the on-capillary reaction cell are investigated. The linearity and reproducibility of the method is investigated for the selective derivatization of glutathion (GSH) with NDA at nanomolar analyte concentration levels. Supplying the reagent immediately after analyte injection is compared with supplying the reagent from the leading buffer vial after the focusing step, during the ITP step. A less selective and more widely applicable derivatization procedure of amino acids with NDA in conjunction with CN is investigated.

The loadability and linearity over a large concentration range is investigated for the OPA–ME derivatization in ITP–CZE of some amino acids. Finally, the linearity and efficiency of a rapid on-capillary derivatization of amino acids in CZE without ITP step is investigated.

4.1. On-capillary ITP derivatization with NDA

In Fig. 4 the electropherograms are shown of the on-capillary reaction of GSH with NDA at nanomolar concentration level. System I is used for ITP–CZE (see Experimental). The capillary is filled for 13.5% with leading buffer containing NDA, followed by a 27% injection of terminator buffer containing GSH. The focusing step took place at a voltage of −10 kV, in the CZE step 25 kV is used. The electrical field strength in the leading buffer zone under ITP conditions is −12 kV/m (for calculation see ref. 13, conductivity ratio leading and terminating buffer is 0.39, fractional leading zone length 0.73). When an effective electrophoretic mobility of acetate of $-42 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} is assumed, the velocity of the neutral NDA is 0.5 mm/s (Eq. 2). The reagent molar mass flow is $3.92 \cdot 10^{-13}$ mol/s under these conditions (Eq. 3, $c = 0.1$ m*M*, $v = 0.5$ mm/s). The time for the NDA zone to leave the capillary is 372 s.

The linearity of the reaction of NDA with GSH is investigated at concentrations just above the detection limit from 1 to 11 n*M* (Table 1). The correlation is considerably improved by

Table 1
Unweighted linear regression data of the on-capillary derivatization in ITP–CZE of GSH with NDA in the range 1–11 n*M*

	a ± S.D.	*b* ± S.D.	*r*
Height	248 ± 152	151 ± 22	0.960
Height ratio	0.18 ± 0.10	0.20 ± 0.015	0.989
Area ratio	−0.022 ± 0.084	0.17 ± 0.012	0.990

Six calibration points were used with concentration intervals of 2 n*M*. Analyte injection volumes of 1.25 μl were used corresponding to 27% of the capillary volume. The intercept *a* and the slope *b* are given with the standard deviation. The correlation coefficient is *r*.

using fluorescein as internal standard. The relative standard deviation in CZE migration times of the ITP–CZE derivatization procedure is 1.4% ($n = 5$).

When in the ITP–CZE procedure, the capillary is filled with leading buffer containing the neutral reagent, the derivatization starts immediately after injection of the analyte. For the considered derivatization reaction this is convenient because it reduces the total analysis time. However, in cases where precise control of the reaction parameters are needed, the starting time of the reaction should be better defined. Therefore, the possibility of performing a derivatization reaction under ITP conditions was demonstrated in Fig. 5. The same ITP–CZE buffers are used as in Fig. 4 except that the capillary was filled with leading buffer without NDA reagent. The underivatized GSH was focused for 10 min and than the leading buffer vial was replaced for a leading buffer vial containing the reagent. The GSH is kept isotachophoretically at a fixed position in the capillary and the velocity of the neutral NDA reagent is 1.0 mm/s at an ITP voltage of −20 kV ($E_L = -24$ kV/m). After 10 min of derivatization under ITP conditions the CZE is started. The remaining NDA reagent migrates with the velocity of the electroosmotic flow which is seen as an increase in the baseline until $t = 400$ s. At 750 s the derivative of GSH appears.

This example illustrates the flexibility of the on-capillary reaction cell. The analyte zones are kept isotachophoretically between the leading and terminating buffer. Using a hydrodynamic counter flow the position of the analyte zones along the capillary is fixed. By switching buffer vials cationic or neutral reagents can be supplied; as long as the ITP conditions are not disturbed the analyte zones remain concentrated. The reaction speed can be increased with the reagent molar mass flow. This can be done by choosing a leading ion with a high electrophoretic mobility, by increasing the electrical field strength during ITP or by using higher reagent concentrations (Eq. 3).

4.2. On-capillary ITP derivatization with NDA–CN

In the derivatization reaction of amines with NDA in presence of CN stable cyanobenz[*f*]isoindole (CBI) adducts are formed. However, also several side products are formed, even when no amine-containing analyte is present [18]. The on-capillary derivatization and ITP–CZE analysis of GSH, Glu and Asp is demonstrated in Fig. 6. At low analyte concentrations the analysis is hindered by background peaks. Most of the background peaks appeared also in the blank

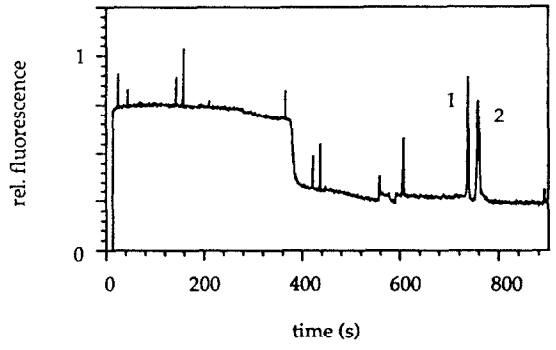

Fig. 5. ITP-CZE of 5 n*M* GSH (1) with NDA reagent in the leading buffer vial. Derivatization of GSH occurred after focusing during the ITP step. The remaining NDA reagent caused an increased background in the CZE step. Peak 2 is fluorescein.

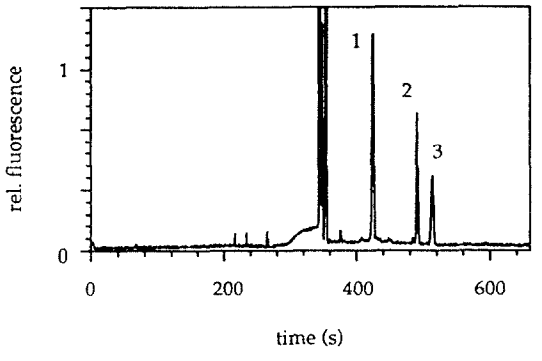

Fig. 6. On-capillary NDA–CN derivatization and ITP-CZE of 50 n*M* GSH (1), Glu (2) and Asp (3). Other peaks are system peaks.

Table 2
Unweighted linear regression data of the on-capillary derivatization in ITP–CZE of Glu and Asp with OPA–ME in the range 50–2000 ng/ml

	$a \pm$ S.D.	$b \pm$ S.D.	r
Glu (height)	150 ± 160	3.4 ± 0.17	0.995
Asp (height)	−31 ± 100	4.4 ± 0.11	0.999
Glu (area)	137 ± 1700	36 ± 1.6	0.997
Asp (area)	−19 ± 1500	54 ± 1.6	0.998

Six calibration points were used with concentrations 50, 100, 200, 500, 1000 and 2000 ng/ml. Analyte injection volumes of 0.53 μl were used corresponding to 9.6% of the capillary volume.

electropherogram and were formed at the moment that KCN and NDA were mixed. Therefore, quantitative analysis with NDA–CN derivatization is difficult at low analyte concentration levels.

4.3. On-capillary ITP derivatization with OPA–ME

The linearity in on-capillary ITP–CZE derivatization is investigated for the reaction of OPA–ME with Glu and Asp in the range 50–2000 ng/ml. Although an internal standard was not used all correlation coefficients were higher than 0.99 (Table 2).

The determination limit of Glu and Asp is 40 pg/ml based on 10 times the signal-to-noise ratio (Fig. 7A). The noise is calculated as one fifth of the peak-to-peak noise, measured at a high sensitivity. The determination limit of Glu and Asp in CZE with conventional pre-capillary derivatization is 7 ng/ml (Fig. 7B). In both cases a derivatization time of approximately 10 min is used. The improvement in the determination limit by a factor of 175 is mainly due to the concentrating ITP step. The analyte loadability

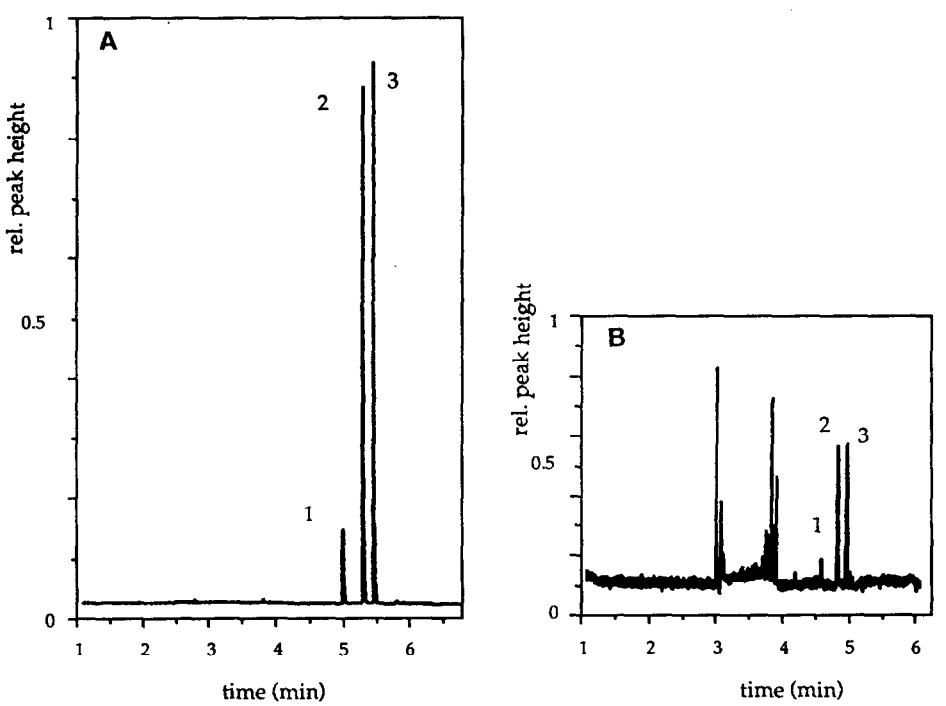

Fig. 7. Derivatization of 100 ng/ml GSH (1), Glu (2) and Asp (3) with OPA–ME with (A) on-capillary derivatization using ITP–CZE and with (B) CZE with conventional off-line derivatization. Injection volumes are (A) 1800 nl and (B) 13 nl. Improvement in S/N ratio in (A) is mainly due to the high loadability and the ITP concentration step.

in ITP–CZE is increased by a factor 135 in comparison with CZE. In CZE several system peaks resulting from the OPA reagent are visible between 3–4 min (Fig. 7B). In ITP–CZE these side products migrate outside the ITP window and do not appear in the electropherogram (Fig. 7A).

In Fig. 8 the effect of the loadability on the peak height is shown. The increase in peak height is linear with the loadability until the injection zone length is 60% of the total capillary length. At higher injection volumes, longer ITP and focusing times are used and degradation of the derivatives results in a decreased signal. The zone width is the same at all injection volumes. Similar results have been obtained in ITP–CZE of these amino acids with pre-capillary derivatization [11].

At high sensitivity determinations precautions are taken to avoid memory effects. Because ITP–CZE takes place without waste of buffers, in principle all injected analytes maintain within the separation system until all buffers are refreshed. In conventional CZE this is not a problem because all analyte ions are migrating in the same direction from injection to detection. However, in ITP–CZE the voltage is reversed in the ITP step which makes it in principle possible for analyte ions to be concentrated several times within the ITP window resulting in an increase of the background signal. In practice this means that the capillary is flushed between runs and that the buffers are refreshed several times a day.

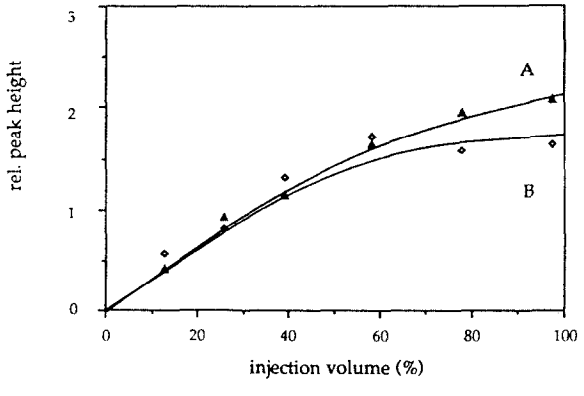

Fig. 8. Loadability in on-capillary derivatization of 100 ng/ml Glu (A) and Asp (B) with OPA–ME using ITP–CZE.

4.4. On-capillary derivatization for CZE

In cases where the analyte concentrations are high enough the ITP step is not always necessary. In Fig. 9B and C an on-capillary OPA derivatization method for CZE without ITP step is demonstrated. Simply by adding the OPA reagent to the background electrolyte (system III, see Experimental), a nanolitre (or picolitre, if smaller-I.D. capillaries are used) reaction cell is created.

This is demonstrated in Fig. 9B. A mixture of underivatized amino acids was electrokinetically injected and the CZE was started within 20 s. Plate numbers in this on-capillary derivatization procedure (Fig. 9B) were varying from $1.5 \cdot 10^5$ to $2.5 \cdot 10^5$. This was approximately 15% lower than in CZE with conventional off-line derivatization (Fig. 9A). This indicates that the OPA derivatization takes place before the electrophoresis is started. Otherwise zone broadening would occur because of small differences in electrophoretic mobility of the amino acids and the OPA derivatives of the amino acids.

The reaction times are easily kept constant in automated CZE procedures and can be increased if necessary, without zone broadening. In Fig. 9C the time between injection and the start of the CZE was 10 min. Plate numbers are the same as in Fig. 9B. The fluorescence background signal increased by a factor 2 after addition of 1 g/l OPA and 0.1% (v/v) ME to the background electrolyte. The detection limit for Phe was 10 ng/ml based on a S/N ratio of 3. This was 5 ng/ml for CZE with conventional off-line derivatization of Phe under similar CZE conditions. When a ten times lower reagent concentration is used in the electrophoresis buffer the derivatization reaction still takes place and the increase in fluorescence background is less than 20% compared to buffer only. Fluorescent reaction products, including contaminants in the buffer, decompose in time. Therefore, the OPA reagent was mixed with the electrophoresis buffer a day before use.

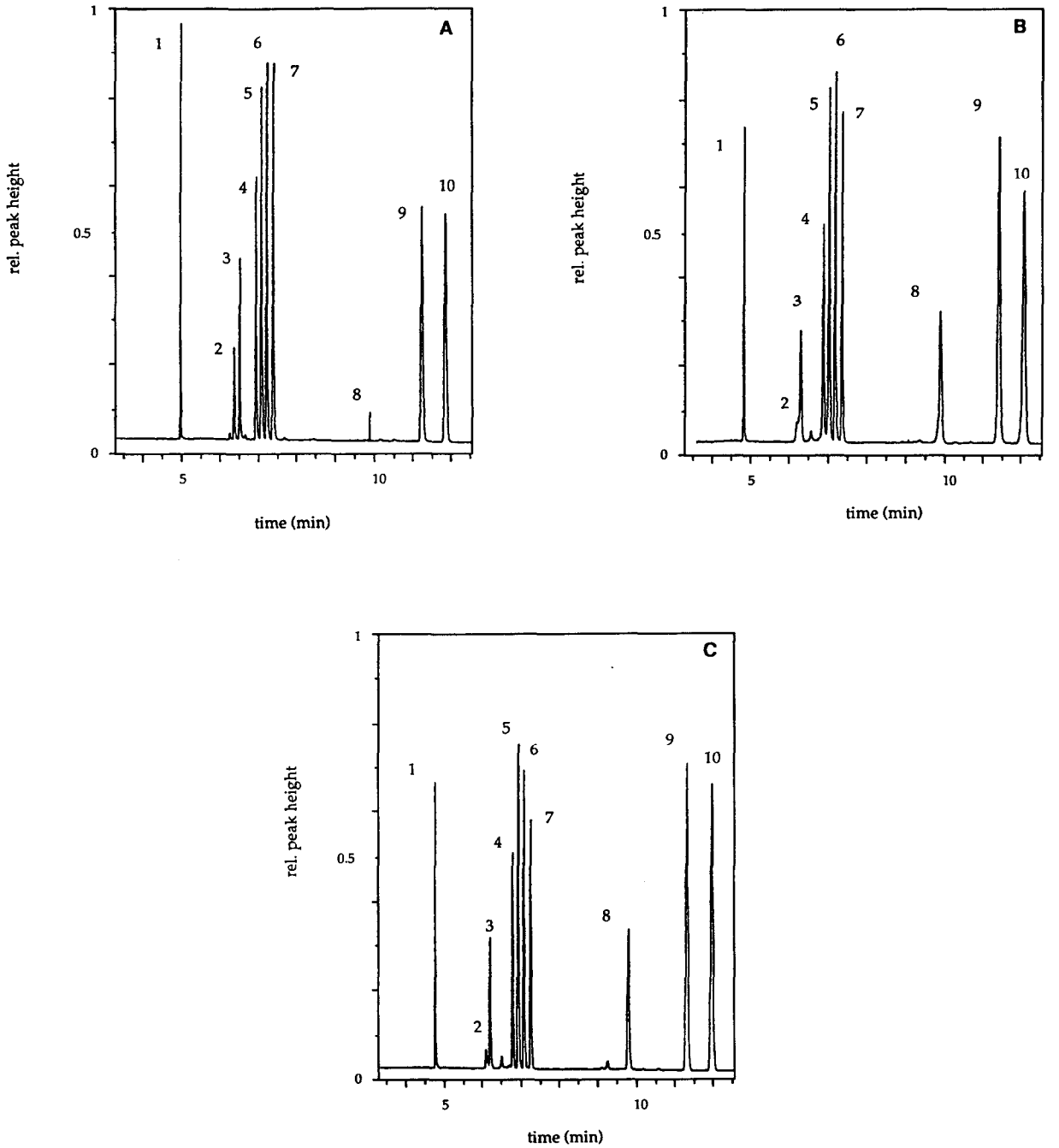

Fig. 9. On-capillary derivatization using CZE without ITP step. Separation of Arg (1), Lys (2, 3), Trp (4), Phe (5), Thr (6), Ser (7), L-Dopa (8), Glu (9) and Asp (10). (A) CZE after 10 min off-line derivatization compared with (B) on-capillary derivatization using CZE immediately started after injection or (C) started 10 min after electrokinetic injection. All concentrations were 5 μg/ml except Lys (50 μg/ml), Asp and Glu (12 μg/ml).

Table 3
Unweighted linear regression data of the on-capillary derivatization in CZE of amino acids with OPA–ME in the range 0.040–240 μg/ml

	$a \pm$ S.D.	$b \pm$ S.D.	r
Arg	202 ± 149	0.149 ± 0.002	0.9996
Lys	205 ± 125	0.0048 ± 0.00015	0.997
Trp	118 ± 68	0.0746 ± 0.0007	0.9997
Phe	130 ± 73	0.128 ± 0.0008	0.9999
Thr	150 ± 94	0.121 ± 0.0009	0.9998
Ser	−169 ± 245	0.145 ± 0.003	0.9990
Glu	−251 ± 321	0.039 ± 0.002	0.997
Asp	−161 ± 215	0.030 ± 0.001	0.997

Seven calibration points were used with concentrations 0.040, 0.200, 0.800, 4.00, 20.0, 80.0 and 240 μg/ml. Electrokinetic injection was applied at 3 kV for 3 s, corresponding to 5–15 nl injection volume depending on the analyte mobility.

The linearity of the on-capillary derivatization method for CZE was studied in the range of 0.040–240 μg/ml for eight amino acids. All correlation coefficients are higher than 0.99 (Table 3).

5. Conclusions

An on-capillary reaction cell is described for nanolitre volumes at nanomolar concentration levels. When reactions with expensive reagents are involved the costs are reduced with the reduction of the reaction cell volume. The applicability is demonstrated for on-capillary fluorescence derivatization reactions. Requirements for the ITP reaction cell are described, including the equations necessary to calculate the electrophoretic supply of a neutral reagent.

The described procedure combines the concentrating properties of ITP, the separation efficiency of CZE and the detection sensitivity of LIF. The method is automated and quantitative at nanomolar concentration level for the reaction of GSH with NDA. Depending on the concentration of analyte and the required determination limit a choice can be made for on-capillary OPA–ME derivatization with or without ITP step before CZE.

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