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Review article

Post-column derivatization for fluorescence and chemiluminescence detection in capillary electrophoresis

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Abstract

Instrumental developments and applications of post-column derivatization for fluorescence and chemiluminescence detection in capillary electrophoresis (CE) are reviewed. Various systems to merge the reagent solution with the separation medium have been developed, including coaxial capillary reactors, gap reactors and free solution or end-column systems. For all reactor types the geometry of the system, as well as the method to propel the reaction mixture (by pressure or by voltage) appeared to be critical to preserve the separation efficiency. Plate numbers of over 100000 could be realised with different reactors. The strict requirements on the rate of post-column derivatization reactions to be applied in CE limit the number of different reagents that have been used. For fluorescence detection, with laser or lamps as the excitation source, so far mainly *o*-phthalaldehyde and its naphthalene analogue have been used as reagent. Derivatization systems that are based on complexation reactions also showed good promise for application in CE. Detection limits could be obtained that were comparable to those obtained after pre-column derivatization. Various reagents for chemiluminescence detection (e.g. the luminol and peroxyoxalate systems) have been studied. The often complicated chemistry involved made application of these reagents in CE even more difficult. Results obtained so far, in terms of sensitivity, have not been up to expectation, with detection limits usually in the order of μmol l^{-1}. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Capillary electrophoresis; Post-column derivatization; Fluorescence; Chemiluminescence

1. Introduction

The rapid development of capillary electrophoresis (CE) has provided an effective tool for the analysis of complex biological samples. The applications of CE in clinical and pharmaceutical chemistry cover a wide range, including quality control of pharmaceuticals [1], chiral separations [2,3], pharmacokinetic and metabolism studies [4], the determination of drugs in body fluids [5], as well as protein–drug binding studies [6,7]. The high separation efficiency and resolution of CE often allow direct injection of body fluids such as urine and serum, without any

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extraction or other sample preparation step except the proper dilution [8]. Combined with microdialysis, it is possible to directly sample and monitor low molecular weight analytes present in the extracellular space of essential tissues from experimental animals in vivo [9]. Many researches validated CE methods for drug quantification and compared them with HPLC methods [10–12]. The results of this comparison show that CE methods provide faster analysis time and better retention time reproducibility and comparable accuracy, but sometimes poor injection precision, peak area reproducibility and lower (concentration) sensitivities are reported.

The extremely small geometry of the capillary in CE offers some advantages, such as very low mass detection limits and a low sample volume which allows the injection of single cells [13,14]. On the other hand, the small detection volume often results in poor concentration detection sensitivity. Moreover it makes it more difficult to develop the methodology for the improvement of the sensitivity in CE, which is still a major challenge.

Of all detectors applied in CE, UV detection takes a dominant place. Although the detector is of universal suitability, its disadvantage is obvious: the short optical path gives a sensitivity which is often not good enough for the analysis of (biological) samples. Laser induced fluorescence (LIF) detection has been shown to be perfectly suited for CE. The excitation light beam with its high energy can be focused on the capillary, and the collection of the resulting fluorescence can be carried out on a 'dark background'. With LIF, the detection of a few molecules appears to be possible [14]. However, compounds with intrinsic fluorescence are quite rare. Therefore, pre- or post-column derivatization is very often required for fluorescence detection.

Pre- and post-column derivatization are routinely applied in HPLC. In CE, off-line pre-column derivatization is also easily realised and therefore widely employed, most often combined with fluorescence detection [15,16]. Research efforts are focused on the high-sensitivity determination of amino acids [17,18], peptides [19] and proteins [20]. In addition, pre-column derivatization has been used with electrochemical detection of amino acids [21] and for the improvement of UV detection of compounds such as carbonyls [22], steroids [23], and metal ions [24]. Pre-column derivatization can also be performed on-line in CE in the case that off-line derivatization is not possible, such as for the analysis of single cells [25].

Pre-column derivatization can not always meet the various requirements of analysis problems. An alternative approach is post-column derivatization. When the reaction between a molecule and the fluorogenic reagent gives multiple products or unstable derivatives, post-column derivatization is a better choice. In a post-column reaction system, the delivery of the reaction reagent can be controlled automatically so that the whole analysis procedure can be automated. Another significant development for a post-column reaction system is for chemiluminescence (CL) detection. In CL detection the excitation energy to produce luminescence is provided by a chemical reaction, so that a post-column reaction system is necessary. Due to the absence of a light source, the background is extremely low and under optimum reaction conditions the sensitivity of CL detection largely depends on the quality of the photomultiplier tube.

To develop a post-column reactor for CE is not an easy task. The small volume scale of the capillary makes the construction of a post-column reaction system technically difficult. Also, because of the high separation efficiency of CE, the requirements in terms of peak broadening, not only in volume units but also in time units, are much more stringent than in HPLC. Since 1988 [26] several post-column systems have been developed. In this review, we will discuss recent developments in post-column reaction systems for fluorescence and chemiluminescence detection in CE, and show selected applications.

2. Instrumentation

2.1. Peak broadening considerations

Connecting a reactor with a separation capillary will inevitably cause extra zone broadening.

In a previous paper [27] we have discussed this zone broadening with two different approaches to propel the reaction mixture through a capillary reactor: hydrodynamic (pressure) and voltage driven systems. In a purely pressure driven system, laminar flow is the main source of peak broadening. Its effect can be described by the Taylor–Aris equation:

$$\sigma^2 = \frac{d_c^2}{96D} t_R \quad (1)$$

where σ^2 is the increase in the zone variance (in s^2), d_c the diameter of the reaction capillary, D the diffusion coefficient of the analyte and t_R the residence time in the reaction capillary. The equation shows that the peak broadening is proportional to the cross section of the reaction capillary and to the residence time. In the first instance the residence or reaction time is dictated by the reaction rate. For a reaction with fast kinetics the required reaction time can be a few seconds only, so that extensive zone broadening can be avoided. With a slow reaction a residence time may have to be used which is shorter than desired in respect to reaction yield and detection sensitivity. An alternative is to use a very narrow reaction capillary. However, this will also result in decreased sensitivity.

In a voltage driven system, the reagent is introduced by means of electroosmotic flow. An electroosmotic flow with its flat flow profile is not the cause of the peak broadening. However, a difference in the mobility between the (still) non-reacted analyte and the derivatization product in the reaction capillary will contribute to the peak broadening. For a short residence time (t_R relative to the reaction rate), the resulting zone broadening can be approximately given by:

$$\sigma^2 = \frac{1}{12}\left(\frac{\Delta\mu_{eff}}{\mu_{eff}}\right)^2 t_R^2 \quad (2)$$

where μ_{eff} is the mobility of the reaction product, and $\Delta\mu_{eff}$ the difference in mobilities between the non-reacted analyte and the product. With a residence time long enough to give complete conversion of the analyte to the (fluorescent) reaction product, the zone broadening contribution is:

$$\sigma^2 = \frac{1}{k_1}\left(\frac{\Delta\mu_{eff}}{\mu_{eff}}\right)^2 \quad (3)$$

where k_1 is the (pseudo-) first order reaction constant. Once the reaction is complete, a further increase in the residence time or length of the reaction capillary will not lead to further zone broadening. A difference in mobilities between the non-reacted and reacted molecules always exists; the effect is more serious when the reaction is between large molecules. This phenomenon was observed in a post-column affinity detection system, where a fluorophore-tagged protein was added post-column to form a binding complex with IgG Fc variants [28]. Because of the different mobilities of the binding and non-binding IgG Fc variants, peaks were distorted and negative peaks appeared when the reagent was driven by means of voltage. In some reactors pressure and voltage are used at same time. The effects of both laminar flow and differences of mobilities on zone broadening should be taken into account.

According to the discussion above, to minimise the zone broadening one should select conditions that give a high reaction rate and use narrow or short reaction capillaries for a post-column reaction system used in CE.

In HPLC, mixing of the mobile phase and the reagent in the post-column reactor is a key factor for the peak distortion and the reaction yield. Different mixer types and coiled tubes are used to provide sufficient mixing. In a capillary reactor, the choice of the mixing mode is limited. Mixing is mainly dependent on diffusion and sometimes on migration in a voltage driven reactor. Fortunately, because of the small inner diameter of the capillary, effective mixing can be obtained by diffusion alone. The mixing in a coaxial capillary reactor was examined with coloured dyes [26]. Theoretical calculations based on the Einstein equation indicated that the time required for a small molecule to diffuse from the centre to the wall of a 25 μm i.d. capillary was in the order of 0.1 s. The actual mixing time observed in the experiments was shorter, because reagent molecules also diffused radially to the centre of capillary. In a pressure driven system, convection will further speed up mixing so that a capillary

with the larger i.d. (75 μm) can be used as a reactor. In a reactor with the separation and reaction capillaries that had the same i.d., the mixing was shown to depend on diffusion rather than on convection induced by the differential flow [29]. Therefore, a narrow capillary (10 μm i.d.) was used as a reaction capillary to ensure effective mixing.

2.2. Reactor Design

In CE a post-column reaction can be carried out on-line, that is, with the electric field grounded at the end of the system, after detection, or off-line, when the high voltage is grounded in the reagent reservoir and the reaction and detection are performed afterwards. Without considering the different reagent delivering systems, post-column reactors can be classified into four types which are shown schematically in Fig. 1:

1. coaxial reactors;
2. gap reactors;
3. free solution (end column) reactors;
4. sheath flow cuvette reactors.

For the construction of a coaxial reactor the separation capillary is inserted into a reaction capillary or tubing. The derivatization reagent is delivered by means of pressure, voltage or both. Fig. 1a shows a typical coaxial reactor as developed by Nikerson et al. [30]. A separation capillary, with a narrower i.d. than the reaction capillary, has to be etched by HF so that it can be inserted into the reaction capillary. The combination of the separation and reaction capillary diameters is very important to get good separation efficiency as previously investigated in detail [26]. When the separation and reaction capillaries were of small and similar i.d. (40/50 μm), the best efficiency could be achieved. The distance from the tip of the separation capillary to the detection window is also a critical factor [30], and should be optimised according to the rate of the selected reaction. However, great care should be taken when installing such a reactor, since the thin wall of the capillary after etching will be very fragile. The coaxial reactor is often adopted in chemiluminescence detection. The fast luminescence process allows the use of a broader capillary [31] or tubing [32] as the reactor. The separation capillary then does not need to be etched and can be mounted in the detection window. Mixing of the reagent and the analytes happens in front of the PMT so that t_R for the analyte migrating the distance between the separation capillary and detection window is close to zero. Because of so called 'chemical band narrowing', the peak broadening in a CL detector can be even lower than that obtained with a UV detector without a post-column reaction system [31,33].

The first gap reactor with voltage driven reagent flow was developed by Albin et al. [34] (Fig. $1b_1$). A four-way polyacrylic connector was used to make a precise alignment of the separation and reaction capillaries with a small gap

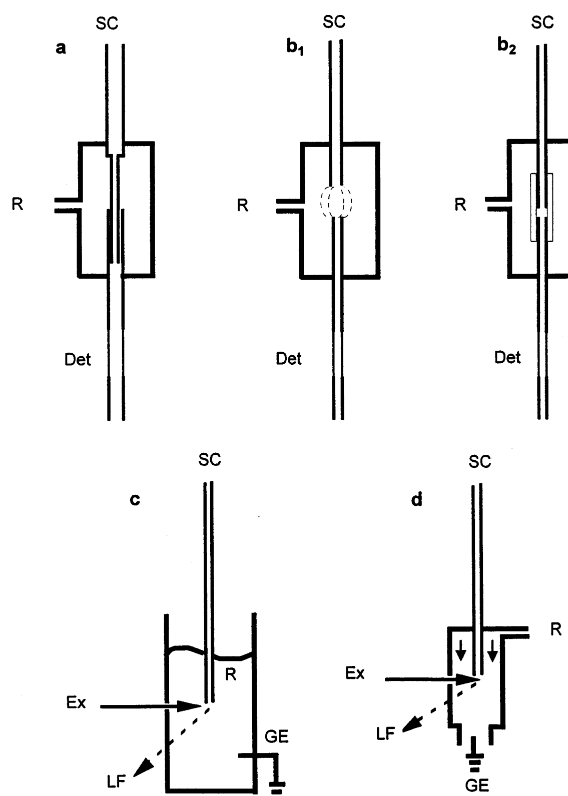

Fig. 1. Schemes of post-column reactors applied in CE. (a) A coaxial reactor, (b_1) a gap reactor driven by voltage, (b_2) a porous tube reactor driven by pressure, (c) a free solution (end column) reactor, and (d) a sheath flow cuvette reactor. Abbreviations: Det, detection window; Ex, excitation light from a laser; GE, ground electrode; LF, laser induced fluorescence; R, post-column reaction reagent; SC, separation capillary.

(from a few μm–50 μm) in between. The diameter of the reaction capillary can be the same or larger than that of the separation capillary. The high electric field is applied between the inlet buffer vial and the outlet of the reaction capillary. The electric field is continuous at the gap junction and forces the analyte ions to migrate from the separation capillary into the reaction capillary, crossing the gap. The reagent molecules diffuse into the separation buffer from the gap to react with the analytes. Gilman et al. [29] designed a similar gap reactor. They simply glued two capillaries with the same i.d. precisely into position on a glass microscope slide. The behaviour of the sample zones crossing the gap under the high electric field was observed by a CCD camera [35]. It was found that a complete mass transfer through a 50–200 μm gap largely depends on the ionic strength of the reagent and separation buffers. When a high ionic strength buffer (relative to the separation buffer) is used in the reagent solution, a slight misalignment (1 or 2 μm) between the two capillaries will induce diffusion of the sample zone out of the gap. When, as an extreme case, distilled water was used as reagent, the sample zone was drawn quickly and completely into the reaction capillary by a siphoning action. However, there is still a possibility that the analytes diffuse out of the gap, especially when large molecules are separated. To prevent the loss of the analyte (substance P), Kostel and Lunte [36] used a semipermeable membrane to cover the gap (as shown in Fig. $1b_2$). A poly-acrylonitrile microdialysis membrane (cut-off 29000) was used to connect two 50 μm i.d. capillaries with a 20–30 μm gap between them. The use of the membrane effectively prevented peptides diffusion out of the gap. The loss of sample was decreased five times compared with a normal gap reactor without a membrane. In this study, the gap reactor was compared with an etched coaxial reactor. The results showed that for large molecules the gap reactor performed better than a coaxial reactor.

Gap reactors have also been developed for off-line operation, with the HV grounded in the reagent reservoir and the reagent solution driven by pressure [27,28,37]. In a reactor developed in our laboratory (Fig. $1b_2$) [37], the separation and reaction capillaries were aligned by a porous tube with an i.d. the same as the o.d. of the capillaries, so that there was less risk of mismatching the two capillaries. Because of the roughness of the ends of the capillaries, a gap between the two capillaries of ≈ 10 μm was automatically formed. In this reactor, there is no limitation to the i.d. of the reaction capillary. A reaction capillary narrower than the separation capillary can be used to suppress the zone broadening. The air pressure to propel the reaction mixture was provided and programmed by the CE instrument. To prevent the reagent from flowing into the separation capillary, the pressure was simultaneously applied on the inlet buffer vial and the reagent reservoir during the analysis. Because the reaction mixture is propelled by pressure, it is possible to select completely different conditions for separation and reaction. The performance of the reactor with different sizes of reaction capillaries was examined. When a 50 μm i.d. reaction capillary was used, theoretical plate numbers of more than 100000 could be obtained. With a gap system Abler et al. [28] applied a vacuum at the outlet reservoir to introduce the reagent. The problem then was that both buffers in the separation capillary and the reagent reservoir were sucked into the reaction capillary, resulting in poorer resolution and lower peak heights.

The characteristic property of a free solution reactor (or end column reactor), which is shown in Fig. 1c, is the absence of a reaction capillary or tubing. The outlet buffer vial serves as the derivatization reagent reservoir. For fluorescence detection, the excitation light is provided by an optical fibre close to the capillary exit, and a PMT tube is set at one side of the reservoir to collect the fluorescence light [38]. Various factors such as illumination volume and reaction distance have been investigated. Although the convection of the effluent stream from the separation capillary will quickly remove the reacted sample from the excitation area, serious zone broadening was still observed. This type of reactor might be better used for CL detection as described by Dadoo et al. [39]. The removal of the excitation optical fibre makes the system more simple. An optical fibre leading to the PMT was immersed in the reagent

reservoir close to the tip of the separation capillary. The reaction was initiated when the sample zone emerged from the capillary. The separation efficiency was improved compared with a coaxial reactor developed earlier by the same group. However, due to the relative large detection volume, the sensitivity was lower. In addition, the slow replacement of the spent reagent in the detection zone still remained a problem.

A sheath flow cuvette reactor for CL detection [40] has been designed based on a sheath flow fluorescence detector [41] (Fig. 1d). A 200 μm square, 1.5 cm long cuvette was used as a flow chamber, and the separation capillary was held in the centre of the cuvette. The reagent was introduced by a low pressure syringe pump. The design of a flow cell providing continuously fresh reagent can overcome the problems existing in a free solution reactor. The main sources of peak dispersion are turbulence of the reagent flow and the relatively long residence time of the reaction mixture in the detection volume. The reactor was used for CL detection of isoluminol isothiocyanate labelled amino acids. Theoretical plate numbers above 100000 were obtained.

Some other types of reactors have been developed such as a cross reactor [42] and an electrochemical cell for electrogenerated CL detection [43]. Complete separation and reaction systems can be integrated on a microchip [44,45]. With such a chip, the reagent flow and reaction time can be controlled by varying the voltage applied between the mixing point and the outlet. This miniaturised system not only provides a short analysis time but also a high separation efficiency. Further development in this direction may be of interest for routine analysis in the future.

3. Fluorescence derivatization

The requirements for post-column reaction reagents in CE are similar to those in LC: the derivatization reagents should not show intrinsic fluorescence themselves, or should have distinguishably different fluorescence properties compared to the derivatives, and they should be stable under the experimental conditions. However, the requirements for the reaction time scale in CE are more strict. The reaction should be complete in a few seconds while in HPLC a reaction time up to a few minutes is possible. As a consequence, the selection of reaction types and reagents is limited.

3.1. o-Phthalaldehyde (OPA)

OPA is a well known derivatization reagent for primary amino groups. The reagent is soluble and stable in aqueous solution and the reaction is rapid. However, the reaction products, that have high fluorescence yield, are not stable. OPA is often used in CE for testing the performance of post-column reaction systems; it has also been used for real sample detection.

Rose and Jorgenson [26] used OPA and 2-mercapoethanol (2-ME) for the post-column derivatization of proteins in a coaxial reactor. A pH 9.5 buffer was used as the separation medium and for the OPA reagent buffer. With a lamp fluorescence detector, proteins could be detected in concentrations of ≈1 ppm, which was a 100-fold improvement compared with UV detection. The method was also used for the determination of amino compounds in red wine samples. A histamine peak could be clearly distinguished. Later, the reactor was modified and a He–Cd laser was used as the excitation source [30]. The separation efficiency was improved 10-fold but the LOD for proteins was not improved.

Post-column derivatization with OPA was used for the determination of non-fluorescent proteins in a single cell. Zhang and Yeung [46] constructed a coaxial post-column reactor from two narrow capillaries (15 μm i.d. for the separation and 30 μm for the reaction capillaries, respectively). The electric field was grounded in the reagent reservoir and a negative voltage was applied to the outlet buffer vial to introduce OPA/2-ME reagent. The small dimensions of the reactor allowed its use for single cell analysis. Human erythrocytes were isolated from whole blood samples and introduced into the separation capillary. The contents of haemoglobin and carbonic anhydrase I in a single cell from different blood samples, as shown in Fig. 2, was investigated. Some biological important compounds containing a primary amino

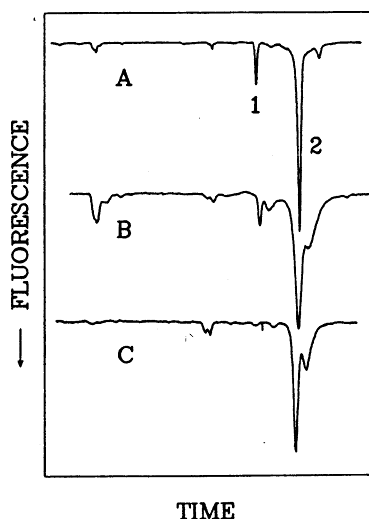

Fig. 2. Electropherograms of single human erythrocytes from (A) a normal adult, (B) a diabetic adult and (C) a newborn. Separation capillary: 15 μm i.d., 70 cm long; reaction capillary: 30 μm i.d., 12 cm long. Electric field strengths: 300 and 150 V cm^{-1} for separation and reaction capillaries, respectively. Peaks: 1, carbonic anhydrase I; 2, haemoglobin (reproduced from [46] with permission).

group were also studied. The LOD's for catecholamines, amino acids and proteins were in the range 10^{-7}–10^{-8} mol l^{-1} (3.8–100 amol) by LIF. However, for small peptides the method was not sensitive.

Amino acids could be determined in urine samples without any sample preparation by using post-column OPA derivatization [27]. Fig. 3 shows the electropherograms of a urine sample after 1:10 dilution and a standard mixture of amino acids. An off line porous-tube reactor was used for the derivatization. Fourteen amino acids were well separated in a pH 9.7, 15 mmol l^{-1} borate buffer with 10 mmol l^{-1} SDS. The optimum reagent solution was found to be a pH 10, 50 mmol l^{-1} borax buffer with 1.5 mg ml^{-1} OPA. The fluorescence detector used was a modified HPLC monitor. Because of a high background from light scattering, the LOD's for amino acids (around 10^{-6} mol l^{-1}) were not satisfactory. With another detector, especially for capillary separations, LOD's in the range of 10^{-8} mol l^{-1} were obtained [47].

3.2. Naphthalene-2,3-dicarboxaldehyde (NDA)

NDA is an analog of OPA. Similar to OPA, it reacts with compounds containing primary amino groups and has no intrinsic fluorescence itself. Normally NDA is used in the presence of cyanide as co-reagent. However, the reaction rate is too slow to be used for post-column derivatization in CE. If 2-ME is used instead of CN^{-}, the reaction rate is increased but the stability of the derivatives is decreased. One of the advantages of NDA over OPA is that the excitation wavelength of NDA derivatives matches the excitation line of an He–Cd laser (488 nm), so

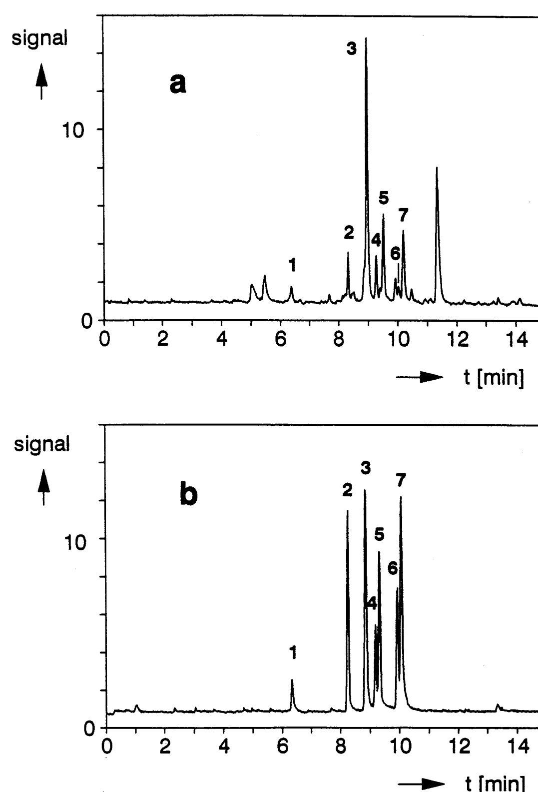

Fig. 3. Electropherograms of (a) a urine sample diluted 1:10, and (b) a 10^{-4} mol l^{-1} standard mixture of amino acids. BGE: 0.015 mol l^{-1} borate, 0.01 mol l^{-1} SDS, pH 9.7. Applied pressure 180 mbar. Separation capillary: 75 μm i.d. Reaction capillary 50 μm i.d., 22 cm long. Peaks: 1, Lys; 2, Ala; 3, Gly; 4, His; 5, Tyr; 6, Thr; 7, Ser (reproduced from [27] with permission).

that the detection sensitivity may be increased. Another advantage is that NDA can react rapidly with peptides [48], while it is well known that OPA is not suitable for peptide derivatization. A disadvantage of NDA is its low solubility in aqueous solution.

Gilman and Ewing [49] used NDA/2-ME as the post-column derivatization reagent to detect catecholamines, neuropeptides, amino acids, and proteins. A small-size gap reactor (10 μm i.d.) was used. A 50 mmol l^{-1} pH 9.5 borate buffer was used for the separation and reaction. Methanol (30%) was added in the reagent buffer to increase the solubility of NDA. With NDA, the LOD's for glycine and transferrin were twice as high as when OPA/2-ME was used [29]. The decreased sensitivity was probably caused by the high background of the NDA/2-ME reagent. The method was used for the analysis of the homogenated pedal ganglia from a pond snail. The results showed that the method can be applied for complex biological samples. The narrow capillaries used for separation and reaction allowed directly introduction of a single cell into the capillary. The contents of a single human erythrocyte were separated and detected.

One important application of the NDA/2-ME reagent is for the determination of peptides. Kostel and Lunte [36] used a membrane gap reactor in the analysis of substance P and fragments thereof. The reaction of NDA/2-ME with peptides was not very fast. For lysine containing peptides, the reaction with NDA was complete in less than 30 s at all pH ranges examined, and the highest fluorescence yield was at pH 9. However, for non-lysine containing peptides, the reaction was rather slow. The highest fluorescence was achieved in about 6 min at pH 8. 5. The detection sensitivity for lysine containing peptides was around 75–100 nmol l^{-1} and for non-lysine containing peptides 20 μmol l^{-1}. Fig. 4 shows the electropherograms of microdialysis samples obtained from a rat brain.

3.3. Fluorescamine

Fluorescamine is a well known fluorogenic reagent for peptides and proteins. The reaction of fluorescamine with peptides is very fast and produces highly fluorescent derivatives. The reagent itself is not fluorescent and hydrolyses quickly in water containing solutions. However, fluores-camine is not very soluble in aqueous solution, which limits its application in CE.

Tsuda and Kobayashi [50] installed a three-pump post-column reaction system to use fluorescamine for the detection of polyamines in urine samples. One pump was used for delivering the reaction buffer, one for delivering a fluorescamine dioxane solution and another for providing a specific pressure at the inlet buffer vial to prevent the reagent from flowing back into the separation capillary. Due to the complexity of the reaction system and the wide separation and reaction capillaries used, the separation efficiency was not very good, with plate numbers in the order of 2000.

Better separation and detection of a protein digestion mixture after post-column derivatization with fluorescamine was shown by Albin et al. [34]. Although in their work the LOD of the fluorescamine derivatives was much higher than those obtained with OPA, an 8-fold gain in sensitivity for a β-lactoglobulin digest was obtained compared with UV detection.

Recent work in our laboratory showed the possibility of applying fluorescamine to the determination of peptides [47]. Fluorescamine dissolved in acetone was used as the post-column reaction reagent. With a pure acetone reagent, problems such as an unstable current during analysis and an irregular background were observed. This was due to the low conductivity of the organic solvent for the high electric field, because the grounded electrode was inserted in the reagent reservoir. When lithium perchloride and some water (5%) were added to the reagent to increase the conductivity, it was found that both sensitivity and reproducibility were improved. The reaction between the fluorescamine and the peptides could be complete in less than 10 s. The proper reaction pH was from 8 to 10, which was provided by the separation buffer. For amino acids, OPA and fluorescamine give similar sensitivities, with LOD's in the range of

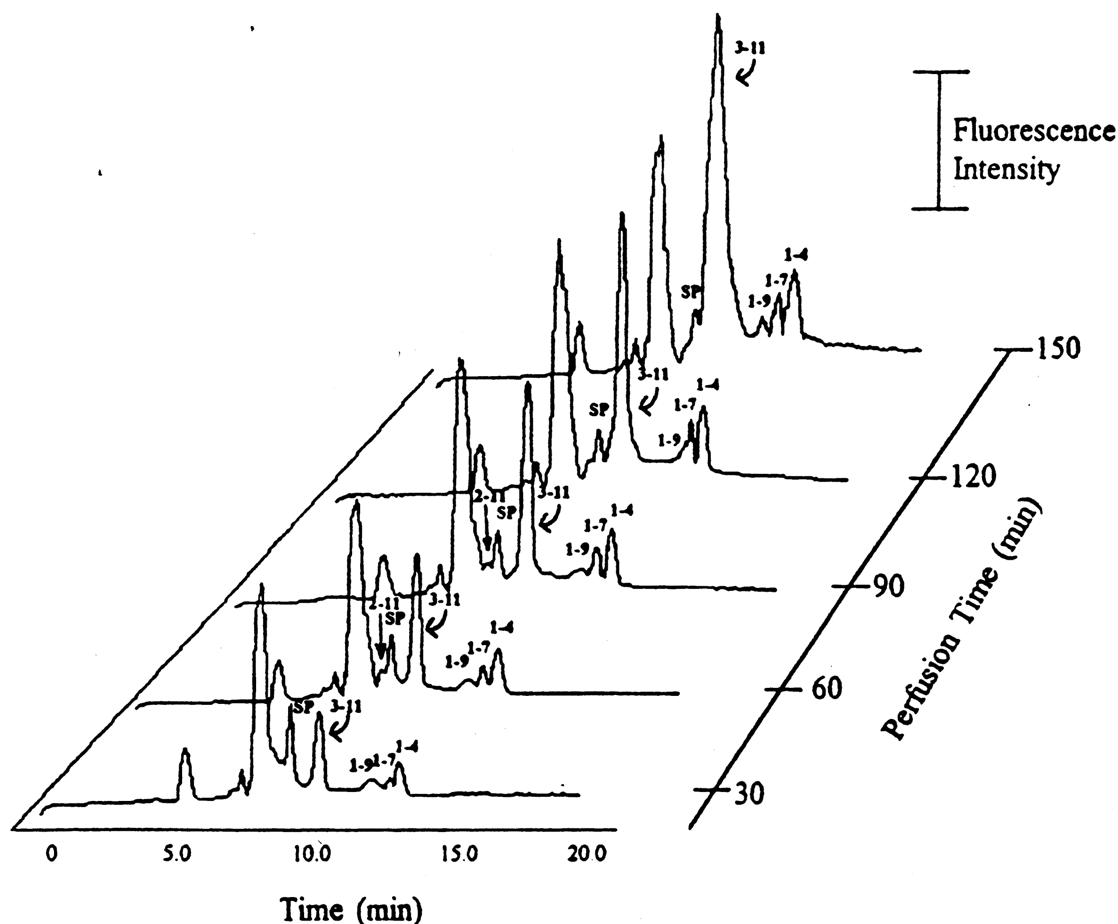

Fig. 4. Electropherograms of microdialysis samples obtained after perfusion of the striatum with 300 μmol l^{-1} substance P at 0.3 μl min^{-1}. The samples were taken every 30 min. Separation conditions: BGE, 150 mmol l^{-1} boric acid, 15 mmol l^{-1} phytic acid and 5 mmol l^{-1} SBE(IV)β-CD at pH 7.0; voltage, + 17.5 kV. Derivatization: pH 9.5; reaction time, 23 s (reproduced from [36] with permission).

10^{-8} mol l^{-1}. Fig. 5 shows that for the determination of small peptides, fluorescamine is much better than OPA. With fluorescamine detection limits of 10^{-8} mol l^{-1} for small peptides were obtained. The method was used for the detection of some tryptic protein digestion samples.

3.4. Other reagents

The requirements on the rate of the reaction to be applied post-column in CE are very strict. Therefore, complexation reactions, which are often very fast, seem to be especially suitable. An example of the use of a complexation reaction is the post-column method developed in our laboratory for the determination of catecholamines and related compounds, based on sensitized luminescence detection [37]. With a porous-tube reactor, a $TbCl_3$–EDTA mixture in a CAPS buffer of pH 11 was added to the separation buffer. At this high pH, catecholic compounds form ternary complexes with Tb^{3+} ions and EDTA. When such a complex is excited at the absorption wavelength of the catecholic compound (the donor), the Tb ion can accept its energy and the typical Tb emission bands can be observed. With a xenon–mercury lamp detector,

detection limits were in the order of 10^{-7} mol l^{-1}. Catecholic compounds could be determined in urine samples after a standard clean-up and preconcentration procedure.

Another complexation reaction used post-column in CE is that between 8-hydroxyquinoline-5-sulfonic acid (HQS) and metal ions. HQS is not fluorescent itself while many of its complexes with metal ions emit strongly. HQS has also been used as an on-column chelating reagent, i.e. as a constituent of the separation buffer [51]. However, since in this system the separation and the complexation conditions could not be optimized independently, it appeared to be difficult to obtain high sensitivity and satisfactory separation at the same time. With HQS added post-column [52], the reaction pH could be optimized independent of the separation pH. It appeared that the optimum pH depends strongly on the metal ions to be detected; for aluminium and zinc, it is around 4, while for calcium and magnesium the highest sensitivity was obtained at pH 9. Detection limits are in the ppb range.

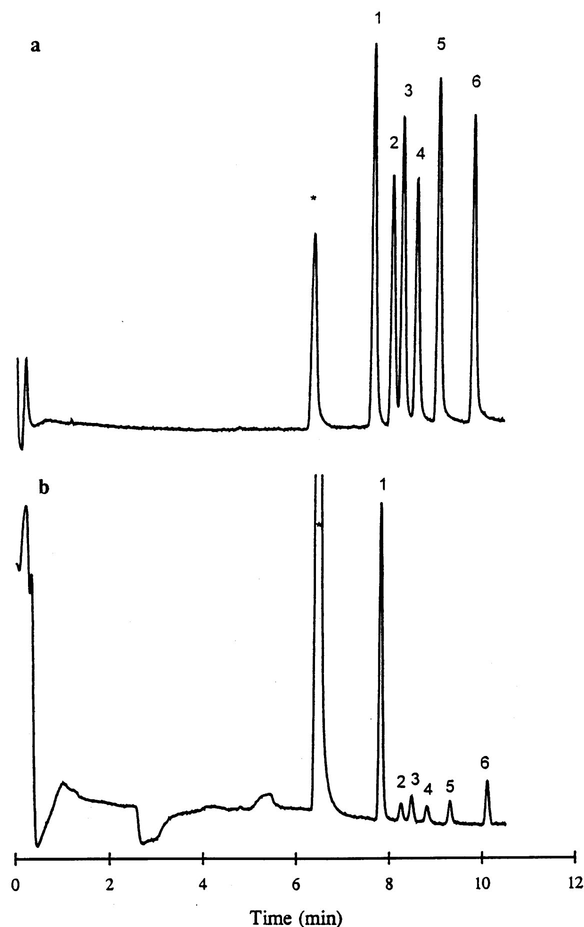

Fig. 5. Electropherograms of Gly and its oligomers derivatized with (a) fluorescamine, PCR: 1 mmol l^{-1} fluorescamine and $LiClO_4$, 5% H_2O acetone solution; and (b) OPA, PCR: 1.5 mg ml^{-1} OPA, 50 $mmol^{-1}$ borax buffer (pH 10), 5% methanol, 3.4 mmol l^{-1} 2-ME. Concentrations of the compounds 10 μmol l^{-1}. Peaks: 1, Gly; 2, GGGGGG; 3, GGGGG; 4, GGGG; 5, GGG; 6, GG and * is a marker for EOF.

3.5. Affinity complex fluorescence detection

Recently, post-column affinity fluorescence detection was applied in CE for the determination of biological macromolecules [28,53,54]. When biological macromolecules bind with a fluorescently tagged reporter protein to form affinity complexes, an enhancement of fluorescence intensity relative to the unbound protein can be observed due to the change in the microenvironment pH surrounding the tagged fluorophor. A post-column affinity detection method was developed for IgG Fc variants [28]. Fluorescein was used to label fragment B of protein A. The labelled protein BF was added post-column to bind with the IgG Fc variants. The fluorescence intensity of the complexes is higher than that of the labelled protein itself and detected by LIF. A gap reactor was used in this work. The reagent was driven into the reaction capillary by applying a vacuum to the outlet reservoir. Since large protein molecules have a low diffusion coefficient, the mixing in the reaction capillary is very slow. If a voltage was used to introduce the reagent, the mixing was not sufficient to get a high fluorescence, and negative peaks caused by different mobilities of bonded and non-bonded proteins were observed. The advantage of this post-column affinity detection method is its high selectivity. However, since the fluorophor labelled proteins produce a high background, the sensitivity was low. The same detection system was used for the determination of human immunoglobulin G subclasses (IgG $\lambda 1$, IgG 2λ and IgG 3κ) and mouse monoclonal antibody variants [53]. When UV

detection was used , all three IgG subclass peaks were observed, However, when post-column affinity fluorescence detection was employed, the peak of IgG 3κ was missing, indicating the weak binding affinity between BF and IgG 3κ. Human IgG 4 was not included because of its strong absorbtion onto the capillary wall. Mouse anti-(human-α_1-antitrypsin) variants and human follicle stimulating hormone variants were successfully resolved and determined by the same detection system. The high affinity selectivity of the method allowed the analysis of antibody variants in complex matrices. A linearity was found from 20 to 100 μg ml^{-1} between total peak areas and concentrations of mouse anti-(human-α_1-antitrypsin) in the presence of cell culture media containing 5% fetal bovine serum. Because high salt concentrations in antibody samples resulted in a decrease of resolution, all the samples were desalted before they were injected into the capillary.

Based on the specific affinity interaction of streptavidine and biotin, streptavidine labelled with fluorescein isothiocyanate (FITC) was added post-column for the determination of biotin and biotin derivatives [54]. A gap post-column reactor equipped with two high voltage supplies was used. The reagent reservoir was grounded and two separate electric fields were applied across the separation (+15 kV) and the reaction (−10 kV) capillaries. The performance of the reactor was evaluated by UV detection. All five biotin compounds could be baseline separated in a single capillary (50 μm i.d. × 70 cm) in less than 6 min with plate numbers from 170000 to 500000. When a post-column reactor was connected, losses in resolution (17%) and efficiency (31%) were acceptable. Peak areas were decreased by 45% due to the dilution. With affinity fluorescence detection the sensitivity was greatly increased. For biotin, a detection limit of 3 nmol l^{-1} was obtained. The method was applied to the analysis of biotin concentration in serum free cell culture medium. Biotin was quantified without interference. For a comparison, a pre-column affinity reaction of biotin compounds with streptavidine–FITC was conducted. No clear resolution among various biotin–streptavidine complexes was observed, and individual biotins could not be identified.

4. Chemiluminescence detection

In CL detection a chemical reaction provides energy to excite fluorophors or photoactive intermediate in the reaction system. Therefore a post-column reaction system has to be used for the coupling of CE with CL detection. Similarly to post-column fluorescence derivatization, a fast luminescence process is required in CE. Some classical chemiluminescence reactions have been applied in CE. Generally, there are at least two compounds involved in the chemical reaction apart from the analyte. In a simple reaction system there is only one reagent reservoir. Therefore, one reagent component has to be added in the separation buffer. Otherwise, two pumps have to be used to deliver the reagent solutions. Until now, much effort has been devoted to develop a suitable post- or end-column reaction system. Most often used are coaxial reactors.

4.1. Luminol–H_2O_2 system

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) reacts with hydrogen peroxide in the presence of a catalyst to form an energy-rich intermediate, with subsequent light emission by the excited aminophthalic acid. The system can be applied for the determination of luminol, H_2O_2, or catalysts such as enzymes and metals. When luminol or its derivatives *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) is tagged on important large biological molecules, the application range is significantly expanded.

A highly sensitive CL method for the determination of metal ions including Co(II), Cu(II), Ni(II), Fe(III), and Mn(II), has been developed based on their catalytic effect on luminol–H_2O_2 reaction [55]. A coaxial reactor was used. A 5 cm section of the separation capillary with 50 μm i.d. was etched and inserted into a 530 μm i.d. reaction capillary, on which a 1 cm detection window was made. When luminol and H_2O_2 in basic solution were first mixed before added post-column to the separation buffer, a steady background signal was produced, since the reaction already started when these two compounds merged, 60% of the light was lost. The detection limit for Co^{2+} was

≈ 1 μmol l^{-1}. When luminol was added to the separation buffer and only peroxide base solution was merged post-column in front of the PMT, the sensitivities were increased. With sample stacking detection limits for metal ions from 5.0×10^{-13} (Co(II)) to 8.0×10^{-6} (Mn(II)) mol l^{-1} were reported.

Gilman et al. [43] developed an electrogenerated CL method for the detection of amines labelled with ABEI which was coupled to *N*,*N*-disuccinimidylcarbonate (DSC). The reaction takes place in outlet reservoir with H_2O_2 added in the outlet buffer. An electrode was situated against the tip of the separation capillary to provide a potential from 0.65 to 1.4 V. The electrode served as the catalyst. In this way the generation of CL was isolated to a volume defined by the position of the electrode.

Dadoo et al. [39] constructed an end column (free solution) reactor for CL detection of ABEI tagged amino acids. As shown in Fig. 1c, the reaction reagent containing peroxide was added in the outlet buffer reservoir. ABEI-tagged arginine and glycine were separated in the capillary and mixed with the reagent when emerging from the end of the capillary. The excess of ABEI could not be well separated from the derivatized amino acids and hence interfered with the detection. The method was also used for ATP, using a commercially available ATP assaying reagent. CL detection of ATP normally suffers from many interferences inhibiting the reaction. CE may effectively separate the inhibitors from the analytes. One drawback of the proposed detection system is its large volume, resulting in extensive zone broadening. A maximum of 20000 plates was obtained.

Isoluminol isothiocyanate was used for labelling amino acids. The labelled compounds were detected by a sheath flow cuvette CL detector [40]. The reagent consisted of peroxide, $Fe(CN)_6^{3-}$ and NaOH and was introduced by a syringe pump. An enzymatic catalyst was dissolved in the separation buffer. The separation of amino acids was improved by adding SDS to the separation buffer. However, the CL intensity decreased dramatically with an increase of the SDS concentration, so that a relatively low SDS concentration had to be used. The LOD for the labelled amino acids was about 20 nmol l^{-1} (40 amol injected) but, because high concentrations of the amino acids were required for the derivatization, the concentration detection limits (in the sample) were poor. The performance of the reactor was good, with 100000–200000 plates for the resulting peaks.

4.2. Peroxyoxalate

The reaction between a peroxyoxalate (typically bis-2,4,6-trichlorophenyl)-oxalate (TCPO)) and H_2O_2 is able to excite a wide range of different fluorophors to get an intense luminescence under the proper reaction conditions. Unfortunately, most peroxyoxalates are not soluble in aqueous solutions, which limits their application in CE. Another limitation is that the reaction system requires a fluorescent compound; therefore, often pre-column derivatization has to be performed.

Wu and Huie [56] demonstrated the feasibility of applying the TCPO–H_2O_2 reaction for CL detection of Dns-amino acids. A coaxial reactor was adopted for this purpose. Two reagent capillaries were used to deliver H_2O_2 and TCPO solutions, respectively. TCPO, dissolved in ethyl acetate, and H_2O_2 are first mixed in a T piece and then merged with the sample zone from the separation capillary. One problem is the conductivity of the post-column reagent in organic solvent is quite different with the separation buffer. It was also found that the high voltage affected the stability of the TCPO reagent. To overcome these problems, a two step approach was used. After injection, the high voltage was applied for about 4 min to obtain a separation and then switched off. Next, a syringe pump was connected to the separation capillary supplying a dynamic flow, transporting the separated zones to the detection system. The flow rate was carefully controlled to decrease peak dispersion. The sensitivity of the detection was $\approx 10^{-7}$ mol l^{-1} (1.2 fmol) for Dns-arginine.

Hara et al. have conducted a series of studies on the CL detection of proteins by a TCPO–H_2O_2 system. TCPO and H_2O_2 dissolved in acetonitrile were added post-column. Proteins

labelled with eosin Y [57,58], dyestuffs [59], tetramethylrhodamine isothiocyanate isomer R (TRITC) [60], or fluorescamine [61] were investigated for TCPO CL detection. The best sensitivity was achieved for TRITC labelled bovine serum albumin, for which a 10^{-8} mol l^{-1} (200 amol) detection limit was obtained. In all experiments, a wide PTFE tube (500 μm i.d.) was used as the reaction capillary. The high reagent flow caused a large zone broadening.

The same reaction system was used to investigate the characteristics of dyestuff containing liposomes [62]. Two types of liposomes, multilamellar vesicle (MLV) and small unilamellar vesicle (SUV), containing eosin Y were prepared. After injection into the capillary, the liposome containing eosin Y disintegrated in the reactor. The free eosin Y was moving slower than the intact dipsomes due to the negative charge. Therefore two eosin Y peaks could be observed. The difference between two liposomes of different sizes could be clearly seen. The effect of temperature and standing time on the permeation of dyestuff in the liposome was monitored. The research showed the potential of the CE-CL method for immunoassays with liposomes.

4.3. Other CL reagents

Similar to luminol, acridinium reacts with H_2O_2 in a basic solution producing excited intermediates, followed by luminescence. Acridinium ester has been used to label peptides [63]. Most acridinium esters appeared not to be stable in pH >3 buffer except 4-(2-succinimidyl-oxycarbonylethyl) phenyl-10-methylacridinium-9-carboxylate fluorosulfonate (acridinium NHS). The tagging of peptides with acridinium NHS was conducted under basic conditions (pH 8) in ≈ 15 min. A small amount of peptides (1–5 pmol) was required for the derivatization. The reaction mixtures were stable for several weeks if preserved in a pH 6 solution at -20°C. To get an intense CL signal, the separation had to be conducted in an acidic buffer (pH 2.7) to convert acridinium NHS into a photo-active compound. Fortunately, the tagged peptides could be well separated in this pH 2.7 buffer with a cyclodextrin additive. A pH 10 peroxide solution was added post-column to provide the basic conditions for the CL reaction. The method was applied for mapping of β-casein tryptic digestions. The results was compared to the UV detection of untagged peptides. Although the separation time was much longer than for untagged peptides, the sensitivity with CL detection was obviously increased. Moreover, the separation efficiency was much better with CL detection because of ‘chemical narrowing effects’.

Permanganate reacting with certain reductants may be the most simple CL reaction system. The reaction should be carried out under strongly acidic conditions. Permanganate CL detection applied in CE was recently shown by Lee and Whang [64]. The potassium permanganate solution was added post-column. When a normal coaxial reactor was used, with the electric field grounded at the outlet vial, it was found that permanganate ions migrated through the capillary to the anode, so that no CL signal could be observed. Therefore, an off line reactor was designed and used for the detection of serotonin and catechol compounds. Still, the detection did not show good sensitivity.

Tris(2,2’-bipyridyl)ruthenium(III) ($Ru(bpy)_3^{3+}$) has drawn considerable attention because of its utility for the detection of alkylamines, amino acids, and NADH with good sensitivity [65]. The CL reaction is based on the conversion of $Ru(bpy)_3^{3+}$ to $Ru(bpy)_3^{2+}$ by reducing compounds. CL does not required fluophores in the reaction system. The more popular method is to apply situ electrogenerated $Ru(bpy)_3^{3+}$ to get a low background and high sensitivity. The CL detection mode was recently introduced in CE [66]. A 20 μm i.d. Pt/Ir electrode was inserted into the separation capillary. The high voltage was grounded before the outlet end of the capillary through a crack in the separation capillary, which was encased within cellulose acetate epoxy. A potential of $+1.25$ V was applied on the working electrode to convert $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ which then reacted with analytes from the separation capillary. A series of β-blockers was used as the model compounds. It was found that SDS and

CTAB diminished the CL signal while Triton X-100 did not. Triton X-100 was added to the buffer to separate the uncharged β-blockers. A high background was found due to the reaction of the generated $Ru(pby)_3^{3+}$ with other compounds such as OH^- ions. Detection limits obtained were in the order of only μmol l^{-1} due to this high background.

4.4. Indirect CL detection

Liao and Whang [67,68] developed an indirect CL detection method for amino acids based on the luminol reaction system. Luminol and H_2O_2 were prepared in a pH 10 carbonate buffer for the separation. A catalyst ($CuSO_4$) was introduced post-column. Because the luminol concentration was decreased in the sample zones, negative peaks proportional to the concentration of the sample compounds were observed. However, since the detection was conducted against a high background, the detection sensitivities were not as good as in direct CL detection. The method was used for the determination of the glutamate content in a commercial health drink. Compared with a standard method, similar reproducibility but less accurate results were obtained.

5. Perspectives

It has been shown that post-column derivatization is possible in CE, with good potential for the analysis of complex samples. Compared to on-column derivatization methods, it has the advantage that separation and reaction conditions can be optimized independently, and compared to pre-column derivatization, the separation can be based on the (electrophoretic) properties of the analytes themselves. Especially for very small volume samples, such as single cells or samples obtained with (in vivo) microdialysis, post-column systems are required for compounds with poor absorbance or fluorescence.

So far the number of reagents used post-column in CE is still limited. The most successful reagents are for fluorescence detection. The results obtained with chemiluminescence detection are still disappointing, with detection limits that are hardly superior to those obtained with a simple UV detector. New reactions and reaction schemes, with the required high reaction rates, should be exploited. The small dimensions of the typical CE system may be an advantage here. Expensive reagents, such as immunochemicals, can be used. Solid phase reaction systems, for instance with an enzyme immobilized on the wall of the reaction capillary [69], could be studied.

Although many types of reactors have been developed, with varying success in terms of zone broadening and the resultant detection sensitivity, more research in this direction is still necessary. It appears especially important for the progress of post-column methods that a reactor becomes (commercially) available which is easy to install and to operate, reliable and gives repeatable results.

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