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Diode laser-induced fluorescence detection in capillary electrophoresis after pre-column derivatization of amino acids and small peptides

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Abstract

The use of diode laser-induced fluorescence (DIO-LIF) detection in the field of capillary electrophoresis (CE) is examined. A simple but sensitive detection system was constructed. The performance of the system was evaluated with respect to design factors and its sensitivity was compared with the theoretically achievable sensitivity. To enhance the applicability of direct DIO-LIF detection in CE, a derivatization method for amines was developed. A red-absorbing label, consisting of a dicarbocyanine fluorophore with a succinimidyl ester functionality, was synthesized for this purpose. After derivatization of 1×10^{-6} *M* glycine, a detection limit of 0.1 amol was observed for the labeled glycine. Similar detection limits were observed for other amino acids. To show that derivatization preserves the separation efficiency of CE for the analytes examined, 18 amino acids and tyramine were separated with micellar electrokinetic chromatography after labeling. In addition, even labeled peptides, including structurally related enkephalin-type compounds, were separated from each other with zone electrophoresis. To test the applicability of the derivatization method to biological samples, tyramine was determined in urine before and after the consumption of cheese.

1. Introduction

Diode laser-induced fluorescence (DIO-LIF) detection is considered a very promising technique [1]. Diode lasers have several advantageous characteristics: they are small, have a low flicker noise (<0.05%), and the available wavelengths are in the red (>630 nm) to near-infrared region, where the light scattering and fluorescence background are generally low. Diode lasers emitting at 670 nm have been applied in analytical chemistry, especially in combination with liquid chromatography (LC) [2]. Recently, DIO-LIF detection has been combined with capillary electrophoresis (CE) [3].

The low linear velocity (0.01–1 cm s^{-1}) used in CE is favorable for fluorescence detection because it allows multiple excitation of the analytes during the passage through the illuminated volume. In combination with the picoliter-to-nanoliter (pl–nl) volumes of typical CE flow cells, 100 mW of laser light can already result in an irradiance sufficient for excitation saturation

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and/or photodegradation of the analyte, which ultimately determines the detection limit.

The miniaturization of the detection volumes has some disadvantages as well, such as an increased background signal due to scattering and flow-cell fluorescence [4,5]. At longer wavelengths, contributions from these two sources of background are reduced, so that DIO-LIF detection in the red region of the spectrum should be more sensitive than LIF detection at shorter wavelengths. However, because few analytes absorb at wavelengths longer than 630 nm, the direct applicability of DIO-LIF detection is limited. The purpose of this paper is to confirm that DIO-LIF detection is very sensitive and that applicability can be broadened by the introduction of derivatization with a newly developed label for primary and secondary amines.

Higashijima et al. [3] showed the possibility of labeling amino acids with a red-absorbing thiazine-based succinimidyl ester. However, labeling was performed with a very large excess of amino acid compared to the label and only arginine and glycine were present in the reaction mixture. The detection limits for the derivatized compounds were at the 10 pmol level. Fuchigami et al. [6] recently performed labeling with a pyronin succinimidyl ester that is fluorescent in the deep-red region. They reported detection limits for derivatized amino acids of 0.8–4.5 amol using CE with DIO-LIF detection. Separation of the derivatives of arginine, alanine, glycine, glutamic acid and aspartic acid was examined, but broadening and overlap of the peaks interfered with quantitation of the individual compounds.

Therefore, in order for DIO-LIF detection in CE to be useful, the separation power of CE should remain intact and the derivatization reaction should be reasonably efficient even at concentrations lower than those reported before. To achieve this goal we prepared a fluorescence label with a dicarbocyanine fluorophore and a single succinimidyl ester functionality (Fig. 1) [7], which is selective for primary and secondary amino group under slightly alkaline conditions. The absorption and emission maxima of this label lie at 667 ($\epsilon = 187\,000$ l mol^{-1} cm^{-1}) and

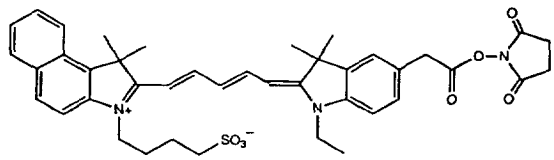

Fig. 1. Chemical structure of the dicarbocyanine label.

689 nm, respectively. The fluorescence quantum yield of the label is 0.23; all values were measured in methanol–water (50:50, v/v). The spectral characteristics of labeled analytes are similar to those of the unreacted label.

The concentration at which the analytes are derivatized here is almost 2 orders of magnitude lower than those reported before. However, due to the limited reactivity of succinimidyl esters, derivatization of the analytes still has to be performed at a much higher concentration than the achievable detection limit for the labeled analytes with CE–DIO-LIF. However, as far as we know, this problem is common to all derivatization-based detection schemes [8].

2. Experimental

2.1. Reagents

Boric acid, sodium hydroxide and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). The amino acids and peptides were purchased from Sigma (St. Louis, MO, USA). Sodium dodecylsulfate (SDS) and all other chemicals were obtained from Aldrich (Milwaukee, WI, USA). Solutions were filtered through a 0.2-μm pore size membrane filter before use. The fluorescence label (Fig. 1) was prepared and purified at the Free University (Amsterdam, Netherlands).

2.2. Derivatization

All analytes, except tyramine in urine, were labeled in acetonitrile–borate buffer (pH 9.0, 25 mM). A 75-μl aliquot of the sample solution was mixed with 25 μl borate buffer (pH 9.0, 0.10 M), after which 100 μl of a 5×10^{-4} M solution

of the label in acetonitrile was added. The reaction was performed for 20 min at 65°C. Because succinimidyl esters tend to hydrolyze in aqueous solutions, especially at a higher pH, the concentration of the analyte has to be 2×10^{-7} *M* or higher to ensure quantitative reaction with the label. Below this concentration the reaction yield decreases quickly.

For the determination of tyramine in urine, some pretreatment of the sample was required [9]: 0.5 ml 6 *M* hydrochloric acid was added to 5 ml urine and the mixture was heated at 100°C for 30 min to hydrolyze tyramine–*o*-sulphate [10]. To this solution 5 ml diethyl ether was added and the mixture was mechanically shaken for 10 min and centrifuged at 2000 rpm for 5 min. The organic layer was discarded. The aqueous solution was adjusted to pH 9.6 with borate buffer. Then, 5 ml diethyl ether and 3 g anhydrous sodium sulfate were added in succession. The mixture was shaken and centrifuged as described before. The organic fraction was transferred to a glass centrifuge tube and evaporated to dryness with a stream of nitrogen. The sample was then redissolved in 0.2 ml acetonitrile containing 0.5% triethylamine and 2.5×10^{-4} *M* of the label. The reaction was performed for 45 min at 60°C. Because no water is present in the reaction mixture, tyramine could be derivatized at concentrations as low as 5×10^{-8} *M*.

2.3. Electrophoresis

CE was performed in a 75-cm long fused-silica capillary with an outer diameter of 350 μm and an internal diameter (I.D.) of either 33 or 50 μm (Polymicro Technologies, Phoenix, AZ, USA). The polyimide coating of the capillary was burned off to form a window 45 cm from the injection end (grounded anode end) of the capillary.

Vacuum siphoning was used to inject 2 nl of the sample solution. The injection volume was calculated according to the equation described by Rose and Jorgenson [11]. A run typically consisted of 10-min flush with the running buffer, followed by vacuum injection of the sample and CE at a constant voltage of 30 kV. Both the injection system and the high-voltage power supply were taken from a commercial CE apparatus (ISCO Model 3140, Lincoln, NE, USA).

2.4. DIO-LIF detection

A simple and inexpensive fluorometer designed by us [12] was used as a platform for our DIO-LIF detection system. Because diode lasers are available for less than $100, the laser no longer forms the major part of the costs of the system. To optimize the DIO-LIF detection system, some specific features of diode lasers have to be reckoned with [13]. In contrast to most lasers, diode lasers emit an elliptical beam with a Gaussian intensity profile. For optimum excitation, the major axis of the beam should be oriented along the capillary. This allows the largest amount of light to be coupled into the flow cell. Furthermore, in this geometry the incident laser light is *p*-polarized, which ensures the highest transmission of laser light through the flow cell material.

Diode lasers normally show astigmatism. Because this is in the order of 10–20 μm for index-guided diode lasers, no corrective optics are required for most capillaries. For gain-guided diode lasers this value is 4 times higher and therefore such devices should be avoided. According to Larson et al. [13] the illuminated volume in a 50-μm capillary can be as small as 1.5 pl. In our case, the size of the laser beam was 20×10 μm upon entering the capillary. Although the beam was focused in the center of the capillary, deformation by the capillary wall and astigmatism of the laser beam make an illuminated volume of 10 pl a reasonable estimate [14,15].

To our original design of the fluorometer [12], a number of light shields were added and a *f*/1 biconvex lens, with a 1-cm focal length, is installed to focus the fluorescence collected by the 20 × microscope objective onto the photomultiplier (PMT) (Fig. 2). The diode laser is a 10 mW 670 nm LAS200-670-10 diode laser (Lasermax, Rochester, NY, USA), equipped with a 10 × microscope objective. The excitation filter consists of a combination of a 670 ± 5 nm

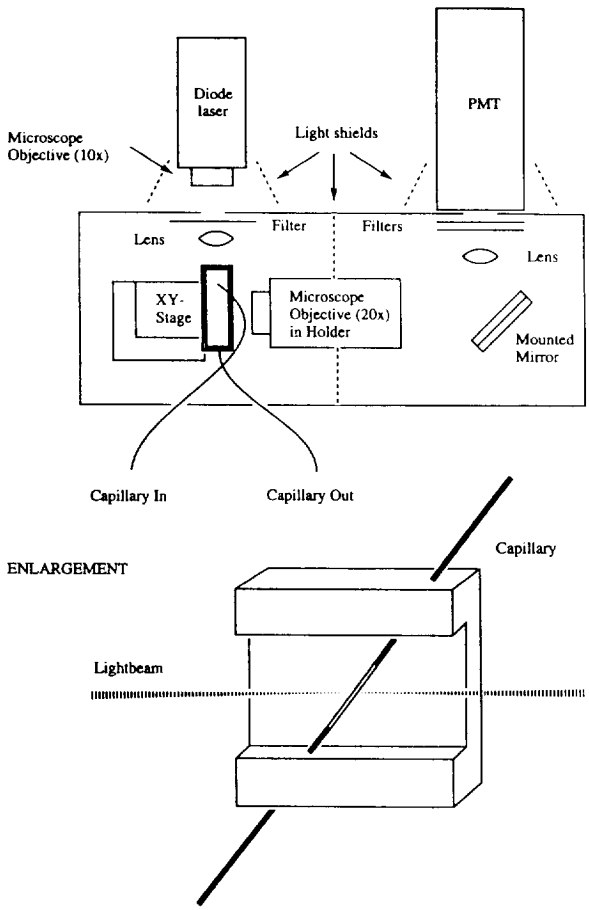

Fig. 2. Schematic diagram of the DIO-LIF detector for CE, including an insert that shows the relative position of the capillary to the excitation beam.

bandpass filter (70% max. transmission) supplied by Omega Optical (Brattleboro, VT, USA) and a standard polarization filter to remove spontaneously emitted light. As a result of the collimating optics and the filters, the excitation power at the capillary is reduced to 3.5 mW.

A 690 ± 5 nm bandpass filter (70% max. transmission, Omega) and a 4-mm RG695 Schott-filter form the emission filter set. A cooled C31034 (RCA, Lancaster, UK) PMT, operated at 1750 V, is used in combination with a SR400 photon counter (Stanford Research Systems, Palo Alto, CA, USA). The data are collected at 5 Hz and stored on the hard disk of a Macintosh Classic computer. Other parts of the DIO-LIF detection represented in Fig. 2 are described in Ref. [12].

3. Results and discussion

3.1. DIO-LIF detection

To determine the sensitivity of the CE–DIO-LIF detection system, 1.0×10^{-6} *M* glycine was labeled and diluted before detection in a 50-μm capillary. The CE conditions were similar to those used in Fig. 3 (see below), but 20 kV were applied across the capillary instead of 30 kV. The labeled glycine and the excess label showed migration times of 20 min and 25 min, respectively. The observed background signal was 1.9×10^{5} counts (in 0.2 s), with a (peak-to-peak) noise level of 2.0×10^{3} counts. Because the power fluctuations in the output of the diode laser (within 10 min) are less than 0.05%, detection is shot-noise limited.

Fluorescence from the capillary walls is negligible. Raman scattering from the solvent has no recognizable contribution either: its intensity is very low in the red- to near-infrared region of the spectrum and the major peaks of the Raman spectrum of methanol and water lie outside the emission spectral window. Consequently, the background signal can be assumed to consist entirely of elastically scattered light. The addition of another 695-nm bandpass filter reduced both the background signal and the emission collection efficiency by a factor 4. Because detection is shot-noise limited, the observed signal-to-noise ratio was a factor 2 worse. The same negative effect was observed for adding other long-pass filters.

The detection limit for the labeled glycine was 5×10^{-11} *M* or 0.1 amol injected (signal-to-noise ratio = 3, noise = peak-to-peak noise). The signal for labeled glycine was examined for concentrations between 5×10^{-11} *M* and 5×10^{-8} *M*. The slope of the corresponding linear calibration curve was 1.2×10^{14} counts l mol^{-1} with a correlation coefficient of 0.996 (9 data points). This shows that the detector has good linearity

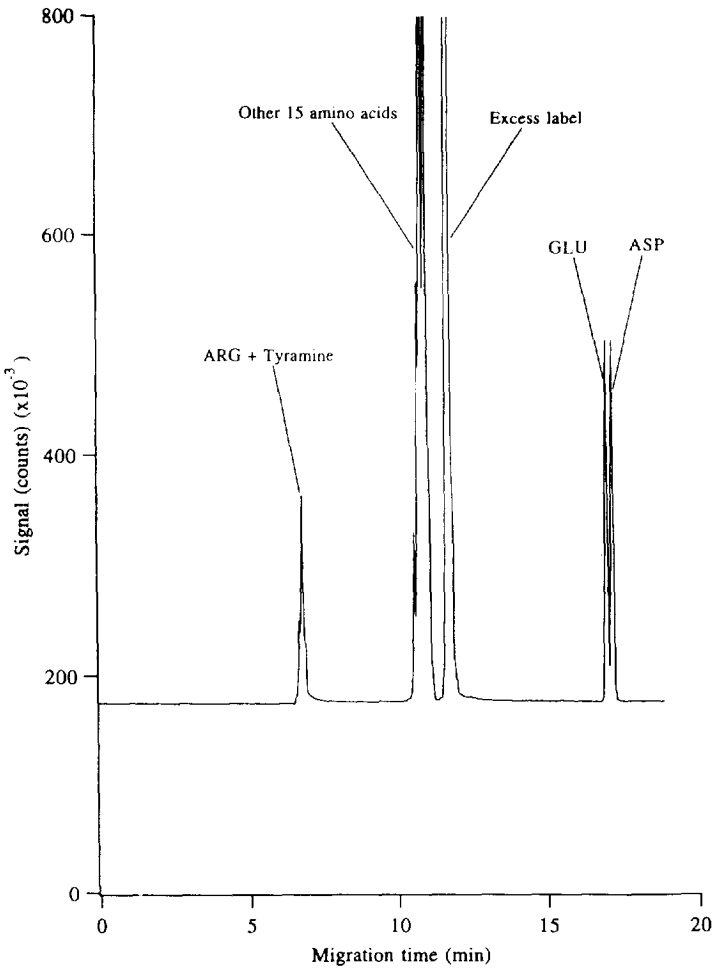

Fig. 3. Electropherogram of a mixture of 18 amino acids plus tyramine, obtained after derivatization. CE conditions: 75 cm capillary (50 μm I.D.); 30 kV; buffer: borate (pH 9.0, 75 mM)–methanol (72.5:27.5, v/v).

over a concentration range of at least 3 orders of magnitude. The detection limits observed for the same labeled analyte in 33 μm I.D. capillaries were 1.5-fold worse, due to the difference in pathlength. The observed background signal was similar. Clearly, CE with DIO-LIF detection allows the reliable detection of very low concentrations of red and near-infrared absorbing analytes.

Under the conditions used, dilution on the 50 μm I.D. capillary is negligible and the concentration of the labeled glycine in the migrating sample is the same as the one injected: the migration time of the labeled glycine is 20 min, corresponding to a distance of 45 cm traveled. This indicates a migration velocity of 0.04 cm s^{-1}. At this migration rate, the 3-s observed peak width (at half height) corresponds to 0.12 cm or 2 nl.

To show the different factors that determine the sensitivity of the DIO-LIF detection system, a theoretical estimate can be made of the amount of signal produced per 1-s interval (n_f):

$$n_f = 2.3\epsilon[C_{analyte}]L\Phi_f PC \quad (1)$$

The molar absorptivity (ϵ) of labeled glycine is assumed to be 187 000 l mol^{-1} cm^{-1}, the analyte

concentration ($[C_{analyte}]$) is 5×10^{-11} M at the detection limit, the optical pathlength (L) is 5.0×10^{-3} cm, the fluorescence quantum yield (Φ_f) is 0.23, and the incident radiant power (P) is 1.2×10^{16} photons s^{-1}. C is the collection efficiency of the optical system of the detector, which is limited by three factors: the lens geometric factor (0.042) [16] the filter transmission (0.032), and the PMT quantum efficiency (0.20). Other factors, such as transmission through capillary walls, the lenses and the PMT window, decrease the collection efficiency only 25%. Combining these effects results in a value for C of 2×10^{-4}.

According to Eq. 1, n_f should be equal to 1.2×10^4 counts per 0.2 s (data collection interval) for labeled glycine at the detection limit. This value is only a factor of 2 from the experimental signal of 6×10^3 counts, as derived from the slope of the calibration curve.

Approximating the laser beam cross-section to a 20×10 μm rectangle with a uniform intensity distribution, the formulas described in Ref. [17] can be used to show that every molecule that passes through the detection volume will emit 5.0×10^4 photons. At a concentration of 5×10^{-11} M labeled glycine, only 300 detectable molecules are present in the detection volume. In addition, with a constant migration rate of 0.04 cm s^{-1}, this detection volume is refreshed 4 times per 0.2-s interval. Therefore, a signal of 4 times 300 times 5.0×10^4 photons, or 6.0×10^7 photons, will be produced in the detection volume if the concentration is maintained during the 0.2 s. Correcting for the emission collection efficiency ($C = 2 \times 10^{-4}$) results in an observed signal of 1.2×10^4 counts in 0.2 s, confirming the previously calculated value.

Obviously, the assumptions made concerning the beam shape are not entirely correct, since the actual beam is elliptical and has a Gaussian intensity distribution (see formula 2 in Ref. [13]). In addition, the illuminated volume is somewhat overestimated, because the beam is focused inside the capillary. However, the calculations presented still enable a reasonable estimate of the signal to be expected.

Under the present experimental conditions, photodestruction is not observed. Using flow injection of 5×10^{-10} M labeled glycine and linear velocities between 0.01–0.2 cm s^{-1}, no significant differences in signal height were observed. It should be noted that even if either excitation saturation or photodestruction becomes limiting, a wider excitation beam could be used. With the present 10-μm wide spot on the capillary wall less than one in four of the molecules passing through the capillary actually enters the illuminated volume.

On the detection side, the use of a microscope objective with a larger numerical aperture would obviously improve signal collection. Because detection is shot-noise limited, an equivalent increase in collection efficiency for the background and the signal would still result in a better detection limit. Obviously, sharper bandpass filters may improve the detection limit as a result of the better rejection of scattered light (without a large reduction in the emission collection efficiency). However, such filters are expensive and few analytes have to be detected at a concentration lower than 1×10^{-10} M directly.

3.2. Detection of amino acids

We used the newly developed dicarbocyanine label with a succinimidyl ester functionality to extend the applicability of CE–DIO-LIF detection. Due to the limited reactivity of the functionality, labeling of the amino acids has to be performed at concentrations above 2×10^{-7} M, when aqueous samples are studied. Therefore, derivatization was performed at an analyte concentration of 1×10^{-6} M; detection limits were then determined from serial dilution of the sample after derivatization.

For all amino acids tested, detection limits for the labeled analogs were $(0.5–1.5) \times 10^{-10}$ M. To determine to what extent the separation power of CE was affected by the labeling procedure, a mixture of 18 amino acids (involved in protein synthesis) and tyramine was labeled and subsequently separated by CE. Proline and cysteine were not included: proline did not give an acceptable peak shape and cysteine formed

sulfur–sulfur bonds under the conditions used for labeling.

All electropherograms were recorded by applying 30 kV across the capillary unless mentioned otherwise. By using a 50 μm I.D. capillary and borate (pH 9.0, 75 m*M*)–methanol (72.5:27.5, v/v) as the buffer, only 5 peaks were observed: labeled arginine and tyramine, 15 labeled monofunctional amino acids, the hydrolyzed label, labeled glutamic acid and labeled aspartic acid (Fig. 3). Arginine and tyramine are not negatively charged at this pH. The 15 labeled amino acids with one carboxylic acid moiety are not well resolved owing to their similar molecular structure. The hydrolyzed label is somewhat smaller than the labeled analytes and has a higher charge-per-size ratio, resulting in a longer migration time. Aspartic acid and glutamic acid contain two carboxylic acid groups that are largely negatively charged. Higher methanol percentages seriously decreased the migration time of the labeled analytes and were avoided for that reason.

For pH values between 8.0–10.0 no change in elution order was observed. Increasing the pH to 11 resulted in a higher effective charge on tyramine and tyrosine due to their phenolic substituent. As a result, separation of the labeled amino acids in the mixture is slightly improved (Fig. 4), although labeled aspartic acid and labeled glutamic acid show the same migration time under these conditions.

Micellar electrokinetic chromatography (MEKC) is often applied to improve the separation of amino acids [18]. However, because the attachment of the label will affect the partition coefficient of the amino acid between the aqueous buffer and the micelle, it is hard to predict the effect of an ionic surfactant on the CE separation of the derivatized amino acids. In practice, the addition of SDS to the borate (pH 9.0, 20 m*M*)–methanol (72.5:27.5, v/v) buffer

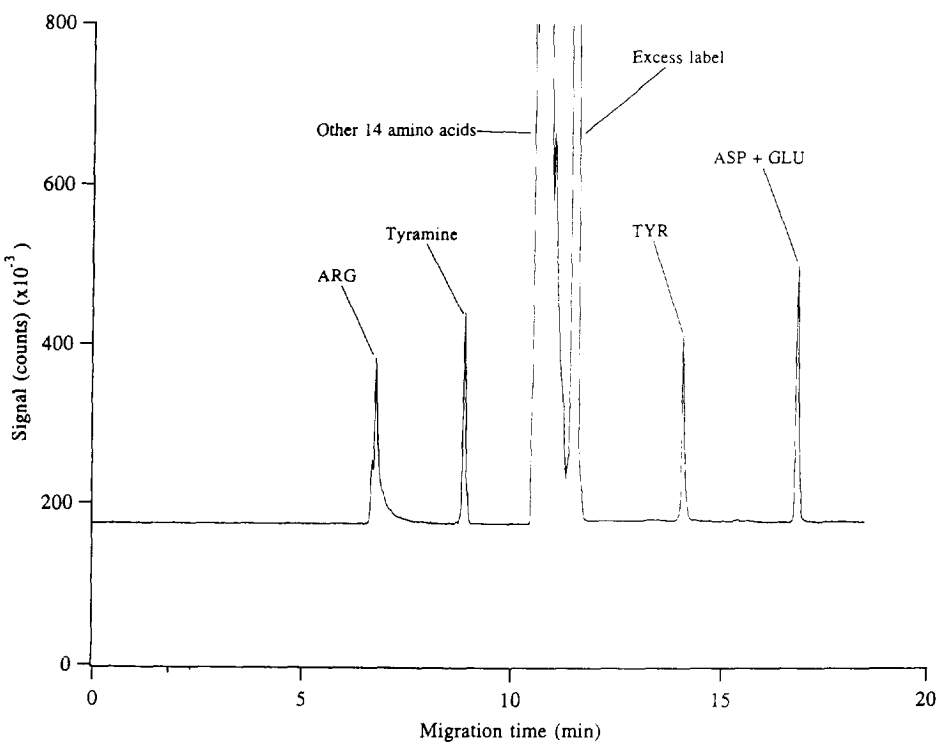

Fig. 4. Electropherogram of a mixture of 18 amino acids plus tyramine, obtained after derivatization. CE conditions: 75 cm capillary (50 μm I.D.); 30 kV; buffer: borate (pH 11.0, 75 m*M*)–methanol (72.5:27.5, v/v).

had a very large effect. Using 17.5 m*M* SDS the labeled amino acids are well separated on a 33 μm I.D. capillary (Fig. 5), except for labeled tyrosine and labeled glutamine, which have a similar migration time. Changes in pH or SDS concentration allowed separation of the latter two compounds, but resulted in similar migration times for other labeled analytes. Under the conditions used, the excess label is well separated from the analytes. The elution order of the different labeled analytes is almost reversed compared to that observed with conventional CE, because the compounds with a higher charge spent less time in the micelles than the less charged ones. As a result, labeled serine and labeled threonine, which contain hydrophilic residues, migrate faster that labeled glycine, which is obviously smaller. However, the size of the aliphatic part of the amino acid has a small effect as well: labeled valine migrates slower than labeled alanine, which migrates slower than labeled glycine.

It should be noted that only a very small peak is visible for labeled lysine, which contains two primary amine groups. The marked peak represents the lysine that is labeled only once. As was observed for *o*-phthaldehyde (OPA)-labeled analytes previously [19], the multi-labeled lysine is not visible, probably as a result of severe fluorescence quenching. There are two sources of background contamination in the electropherogram. The peaks in the region between 41 and 43 min are associated with the reagent blank. The remaining unmarked peaks are associated with labeled impurities in the amino acids.

The addition of SDS resulted in increased migration times, but all compounds were eluted past the detection window within 45 min. If more methanol is added, the same separation efficiency can only be achieved with an increased

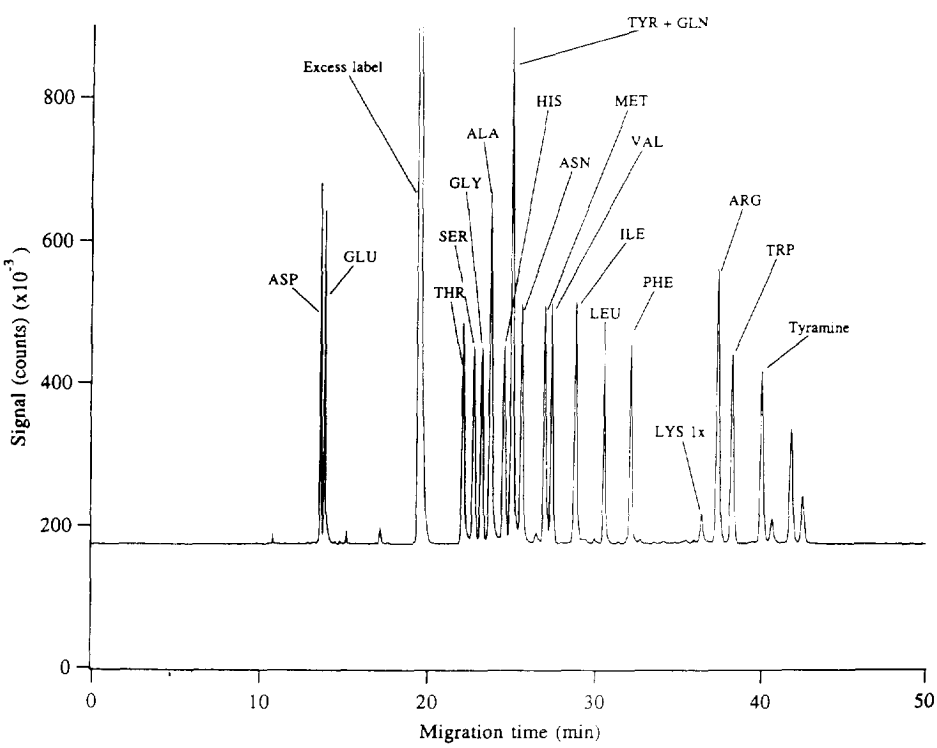

Fig. 5. Electropherogram of a mixture of 18 amino acids plus tyramine, obtained after derivatization. CE conditions: 75 cm capillary (33 μm I.D.); 30 kV; buffer: borate (pH 9.0, 20 m*M*)–methanol (72.5:27.5, v/v) and 17.5 m*M* sodium dodecylsulfate.

SDS concentration. A similar separation in 50 μm I.D. capillaries resulted in somewhat lower resolution. Apparently, the reduced joule heating in the 33 μm I.D. capillary enhanced the separation.

Addition of cyclodextrins to the MEKC buffer has been shown to induce separation of the D- and L-isomers of amino acids [18], even though the size and charge of the isomers are the same. Attempts to separate D- and L-amino acids (DL-alanine, DL-phenylalanine, DL-leucine and DL-methionine were tested) by the addition of α- and β-cyclodextrin in MEKC did not influence the relative migration of the different amino acids or result in any observable enantiomeric separation. Only when γ-cyclodextrin was used, the relative migration of the amino acids was influenced, but still no separation of the D- and L-isomers could be observed. Obviously, the fluorescence label does not allow a selective interaction of the amino acid enantiomers with the cyclodextrins tested [18].

3.3. Detection of peptides

The separation of peptides with CE is largely based on differences in size instead of charge, especially if the same end-groups are present. To examine whether the size of the label hinders the CE separation of small peptides, a mixture of glycine-based peptides was derivatized at 1×10^{-6} *M*. Peptides containing 1–6 glycines were first studied individually to determine their migration times. Baseline separation was achieved in a 33-μm capillary with a buffer consisting of borate (pH 9.0, 75 m*M*)–methanol (65:35, v/v) (Fig. 6). Addition of surfactant did not improve the separation: the smaller peptides are separated more efficiently, but Gly-Gly-Gly-Gly-Gly and Gly-Gly-Gly-Gly-Gly-Gly are still resolved from each other.

Based on this positive result, the separation of two very similar enkephalin-type peptides and related fragments was attempted. These opioid peptides, leucine–enkephaline (Tyr-Gly-Gly-Phe-Leu or T-G-G-P-L) and methionine–enkephaline (Tyr-Gly-Gly-Phe-Met or T-G-G-P-M), represent an important class of neurotransmitters [20]. In the literature, a number of articles related to the analysis of these compounds has appeared using HPLC in combination with precolumn derivatization [21] and CE with UV detection [22]. However, separation of the two related enkephalins was difficult to achieve. Leucine–, methionine–enkephalin and a number of their fragments were derivatized individually at a concentration of 1×10^{-6} *M*. After identification of the peaks, a mixture of these compounds was derivatized.

Because not all fragments of the enkaphalin-type compounds contain a tyrosine unit (with a phenolic substituent), separation was examined at a high pH. With borate (pH 11.3, 75 m*M*)–methanol (65:35, v/v) buffer, most of the labeled fragments are well separated from each other (Fig. 7). Even labeled Leu–and Met–enkephalin can be identified, although baseline separation is not achieved for these two analytes. As can be seen from the electropherogram a significant number of unidentified peaks is present, which originate from impurities and degradation products in the peptide solution.

3.4. Detection of tyramine

Tyramine (4-hydroxyphenethylamine) is an indirectly acting sympathomimetic amine found in cheese, fermented foods and red wine [23]. Previously, tyramine has been determined in urine by HPLC coupled with fluorimetric [10] or electrochemical detection [24]. To show that the combination of derivatization with CE–DIO-LIF detection can also be applied to real samples, it was used to determine the tyramine concentration in the urine of a healthy volunteer before and after eating 100 g of cheddar cheese.

Tyramine is converted to tyramine–*o*-sulphate before it is excreted in urine, which necessitates pretreatment with hydrochloric acid at elevated temperatures. The combination of dilution as a result of this treatment and the fact that many other amines are present in urine make it impossible to perform the labeling prior to the extraction. Pretreatment and derivatization was therefore performed as described in the Experimental section. As can be observed in the

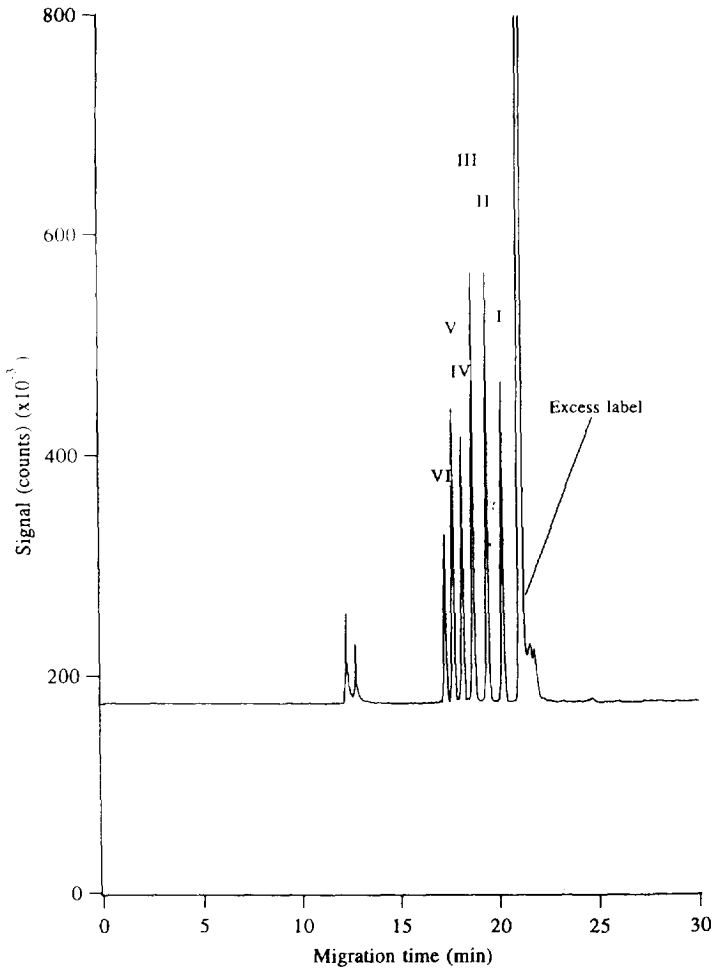

Fig. 6. Electropherogram of a mixture of small peptides [Gly (I), Gly-Gly (II), Gly-Gly-Gly (III), Gly-Gly-Gly-Gly (IV), Gly-Gly-Gly-Gly-Gly (V) and Gly-Gly-Gly-Gly-Gly-Gly (VI)] obtained after derivatization. CE conditions: 75 cm capillary (33 μm I.D.); 30 kV; buffer: borate (pH 9.0, 75 mM)–methanol (65:35, v/v).

corresponding electropherograms, the concentration of tyramine increases almost 2-fold (from 2.5 to 4.7×10^{-6} M) as a result of eating the cheese (Fig. 8). This change is similar to that observed by Koning et al. [10]. Identification was checked by spiking urine with $(1–25) \times 10^{-6}$ M tyramine, followed by the sample preparation procedure described in the Experimental section.

A plot of the peak area versus the concentration showed a linear relation with a correlation coefficient of 0.989 (9 data points). The relative standard deviation of the determination of 5×10^{-6} M tyramine ($n = 5$), 5×10^{-9} M injected, was 4.1%. Clearly, the method described can be applied to the reproducible determination of tyramine in urine. However, it should be noted that the concentrations that are determined here are rather high and further work is necessary to determine the general applicability of derivatization in combination with CE–DIO-LIF detection in other real samples.

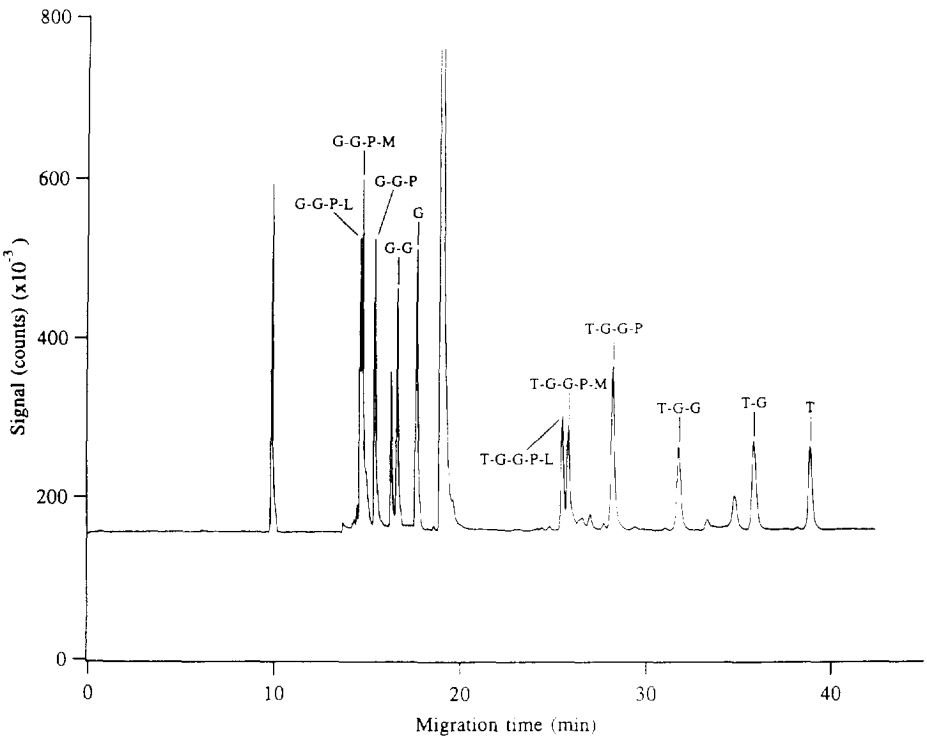

Fig. 7. Electropherogram of a mixture of enkephalines and related fragments, obtained after derivatization. CE conditions: 75 cm capillary (33 μm I.D.); 30 kV; buffer: borate (pH 11.0, 50 mM)–methanol (60:40, v/v).

4. Conclusions

A sensitive DIO-LIF detection system was built for use in CE. The detection limits achieved with this device were compared with theoretical values to identify the factors which influence its performance. To improve the applicability of the system, a derivatization method was developed for primary and secondary amines. A detection limit of 5×10^{-11} M (0.1 amol) was achieved for labeled glycine and similar values were found for other labeled amino acids. It should be noted that due to the limited reactivity of the succinimidyl ester functionality, the analytes were derivatized at a concentration of 1×10^{-6} M in aqueous solutions.

Although the separation power of conventional CE was not sufficient to obtain resolved peaks for all 18 labeled amino acid, micellar electrokinetic chromatography greatly enhanced the separation and nearly all labeled amino acids could be quantified. With conventional (zone) CE a number of labeled peptides with the same end-groups could be separated, despite the large size of the label. Even similar enkephalin-type compounds (and fragments of these compounds) were separable by zone electrophoresis, indicating that labeling does not seriously compromise the high resolving power of CE. Finally, tyramine was determined in urine to show the applicability of the combination of derivatization and CE–DIO-LIF detection to real samples.

It is clear that CE–DIO-LIF detection is a sensitive alternative for the detection of red- to near-infrared absorbing compounds. However, for wide acceptance of the technique its applicability should be broadened. Although the presented results with an amine-selective label

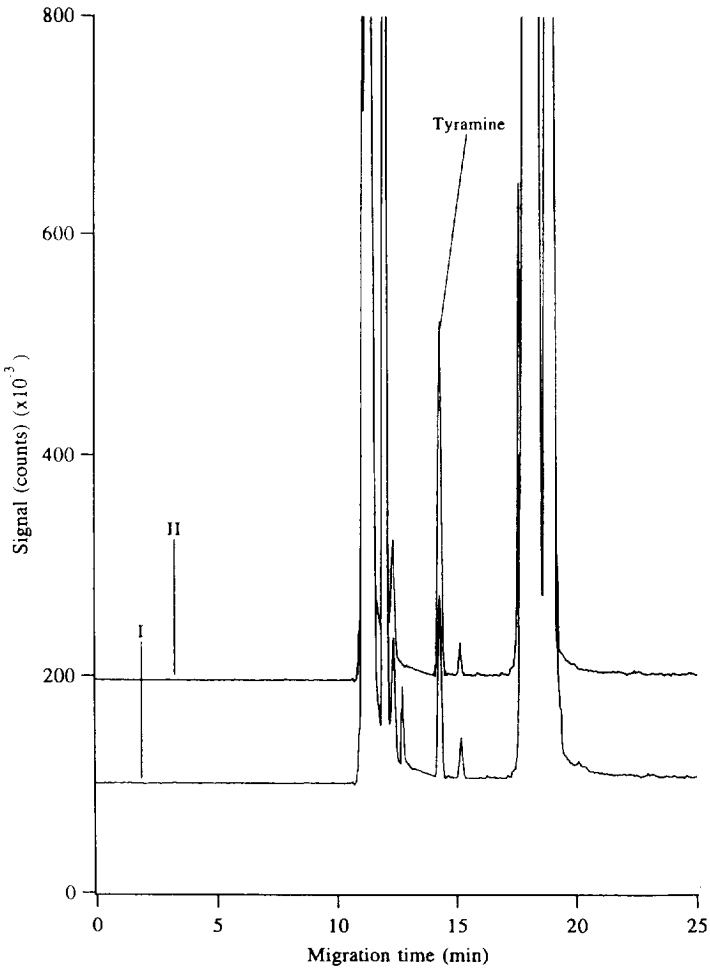

Fig. 8. Electropherograms of urine samples, before (I) and after (II) eating 100 g of cheddar cheese. Both were obtained after extraction and derivatization. CE conditions: 75 cm capillary (50 μm I.D.); 30 kV; buffer: borate (pH 11.0, 50 mM)–methanol (60:40, v/v).

are promising, new labels are required that allow derivatization at much lower concentrations of analyte. The dicarbocyanine fluorophore possesses most of the characteristics required for detection at very low levels, such as a high extinction coefficient at a long wavelength and a high fluorescence quantum yield. However, the succinimidyl ester reactive group is not reactive enough and hydrolyses easily. A more reactive functionality would allow the labeling reaction to be performed with yet lower concentrations of analyte.

At the same time, miniaturization of the derivatization procedure described in this paper would improve the usefulness of the technique as well. Detection of analytes in smaller volumes, such as in single cells, has become possible with the introduction of CE. Cells often contain high concentrations of interesting compounds ($>1 \times 10^{-6}$ M), but they are normally lysed before derivatization is performed. As a result, the concentration in the reaction mixture is much lower. If derivatization could be performed in a very small volume or even inside the cell [25], this problem could be avoided. Investigations involving both the introduction of different re-

active groups on red-absorbing labels and the miniaturization of the derivatization process are being continued.

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