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Choice of different dyes to label tyrosine and nitrotyrosine

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Abstract

In this work, we will present some attempts to analyze tyrosine and nitrotyrosine using capillary electrophoresis and either UV–Visible detection or laser-induced fluorescence (LIF) detection. An argon ion (488 nm) laser is used for fluorescein isothiocyanate (FITC) and 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F). A near infrared (780 nm) laser is used for NIR 780 derivatives. The UV–Visible limit of detection is 2.5 μ*M* whereas it is in the range of 30 n*M* for LIF detection.

Keywords: Dyes; Tyrosine; Nitrotyrosine

1. Introduction

Nitrotyrosine (Ntyr) is an amino acid which is found at low μ*M* range in some biological fluids and in proteins. It comes from the reaction of peroxynitrite ($ONOO^-$) on tyrosine (Tyr). Such nitroaromatic amino acids can be ''markers'' of $NO^{\cdot}$-dependent oxidative damage. It seems that 3-nitrotyrosine is a good indicator of NO production in cells. Most of the time, nitrotyrosine is analyzed by high-performance liquid chromatography (HPLC) and electrochemical detection [1] (as reported in the literature, this detection technique adds artifacts to the chromatograms making their interpretation more difficult [2]), gas chromatography–mass spectrometry (GC–MS) of 6-methyl-*tert*.-butyldimethylsilyltrifluoroacetamide derivatives using electron impact ionization [3], GC–MS using electron capture ionization [4] and immunodetection [5].

Nitrotyrosine in protein hydrolysates was detected at μ*M* levels by HPLC and conventional fluorescence detection after derivatizing with 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) [6]. Most of the analytical methods to analyze Ntyr are reviewed in Ref. [7]. Our experience with capillary electrophoresis (CE) in the analysis of biological media led us to study these aromatic amino acids with this technique. The purpose of this work is to test different ways to quantify nitrotyrosine and tyrosine in biological media such as synovial fluids or sinus washes. These biological fluids which contain large amounts of high molecular mass polymers are very complex and viscous media. Consequently, samples must be diluted before analyzing them. The issue resulting from the dilution is that tyrosine and nitrotyrosine are difficult to detect at sub-μmolar levels; even though this concentration is not very low.

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In this paper, we will report different strategies that we developed to analyze Ntyr and Tyr standards using CE–UV or CE coupled to laser-induced fluorescence (LIF) and evaluate the detection sensitivity on the different derivatives we used. We will take a look at UV detection without derivatization and LIF detection using different dyes to label these amino acids.

Even though the detection selectivity with CE–UV absorbance detection is good because the nitrotyrosine aromatic nucleus absorbs around 400 nm, we will show that it is not effective to detect sub-μ*M* concentrations. CE–LIF has demonstrated its effectiveness in detecting other amino acids in biological samples [8,9]. Based on this experience, we have adapted this technique to tyrosine and nitrotyrosine analysis.

Various dyes, including fluorescein isothiocyanate (FITC) and 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) were used to label these aromatic amino acids. We detected these derivatives with a laser-induced fluorescence detector featuring a 488-nm argon ion laser as the excitation source. FITC did not provide satisfactory results for nitrotyrosine analysis. Nitrotyrosine and tyrosine derivatives using NBD-F as a dye, fluoresce in organic media only. Non-aqueous capillary electrophoresis must be used, resulting in large peaks for the derivatized samples and making the separation difficult. The limit of detection was around 30 n*M*. Then, we used a novel cyanine near-infrared (NIR) fluorescent dye (λ_{exc} = 780 nm) which isothiocyanate function reacts with the amino group of the amino acids. The fluorescent derivative was detected with a LIF detector equipped with a 785-nm laser diode. The lowest concentration we detected was in the 30-n*M* range for samples diluted in water (45 n*M* in sinus wash after a dilution of 100 of the derivatized sample). However, the separation was much better than the one obtained with NBD-F derivatives.

2. Experimental

NIR 780 comes from Fluka (St. Quentin Fallavier, France), all other chemicals come from Sigma (St. Quentin Fallavier, France). A Prince Technology (Emmen, The Nederlands) CE system was used to run the different experiments, the fused-silica capillary (Polymicro Technology, Phoenix, AR, USA) characteristics were 75 μm I.D., 375 μm O.D., 75 cm total length, 40 cm effective length. A multi wavelengths scanning UV detector (Fast Scanning Detector UV206) from Thermo Separation was used for UV detection.

A Zetalif 488 LIF detector (Picometrics) equipped with a 488-nm argon ion laser of 20 mW and the appropriate filter kit was used for the FITC and NBD-F labeled amino acids. A ZETALIF 785 LIF detector equipped with a 785-nm diode laser of 50 mW and the appropriate filter kit was used for the cyanine near-infrared (NIR) labeled amino acids.

2.1. FITC derivatization

The derivatization procedure was performed as previously described [10,11]. A 100-μl aliquot of 100 μ*M* of Tyr or Ntyr, is added to 50 μl carbonate buffer (0.3 *M*, pH 9.5) and alkalinized with 10 μl of sodium hydroxide (5 *M*). After vortex mixing, 50 μl of FITC solution was added. The solution of FITC isomer I (fluorescein-5-isothiocyanate) was prepared by dissolving 1 mg of FITC in 1 ml acetone and the reaction was performed in the dark overnight at ambient temperature. Samples were diluted in water prior to injection.

2.2. NBD-F derivatization

First, 100 μl of 2.4 μ*M* Tyr or Ntyr in water added to 50 μl of 0.3 *M* bicarbonate buffer pH 9.5 and 50 μl 1.5 m*M* NBD-F solution in isopropanol 20 min at 60 °C and diluted 50× prior to injection in acetonitrile–methanol (84:16, v/v).

2.3. NIR780 derivatization

First, 100 μl of Ntyr or Tyr 1 m*M* in water were added to 100 μl carbonate buffer and 50 μl of NIR780 dye at 1 mg in 500 μl DMSO, 1 h at 45 °C and diluted 1000× prior to injection.

CE–UV experiments were run using a boric acid

40 m*M*, CAPS 20 m*M* pH 9.5 buffer and 6s injection at 50 mbar. The voltage was 20 kV.

CE–LIF experiments using FITC were run using a buffer made of boric acid 50 m*M*, CAPS 20 m*M* adjusted at pH 10 with NaOH pellets. The voltage was 30 kV, injection time 6 s, 50 mbars.

CE–LIF experiments using NBD-F were run using different separation media: (i) aqueous medium: boric acid 50 m*M*, CAPS 20 m*M* adjusted at pH 10 with NaOH pellets, (ii) non-aqueous medium: acetonitrile–methanol–ammonium acetate 25 m*M* (83:15:2, v/v), each experiments were run at 25 kV with 6 s, 50 mbars injection.

CE–LIF experiments using NIR780 were run at the same conditions as the ones for FITC-labeled compounds.

3. Results and discussion

3.1. Tyrosine and nitrotyrosine study using CE and UV detection

The UV visible spectra of nitrotyrosine shows a strong absorption at 235, 280 nm and a very large band centered around 420 nm, in water at pH 8. The spectrum is not influenced by pH changes in the range of pH 8–5. A detection limit of 2.5 μ*M* of nitrotyrosine (injected in CE) was achieved at 235 nm. The literature mentions that the concentration of nitrotyrosine in such media is below 1 μ*M*. Fig. 1A is an electropherogram of a blank synovial liquid after centrifugation and analysis by CE–UV (235 nm). Fig. 1B is an electropherogram of the same synovial liquid which was spiked with nitrotyrosine at a final concentration of 1 m*M*. The results indicate that no nitrotyrosine was identified in this media. Therefore the detection sensitivity is not high enough. These results led us to try LIF detection.

3.2. FITC labeling

The 488-nm argon ion laser is a convenient laser wavelength to use because it is one of the most popular and reliable laser available on the market.

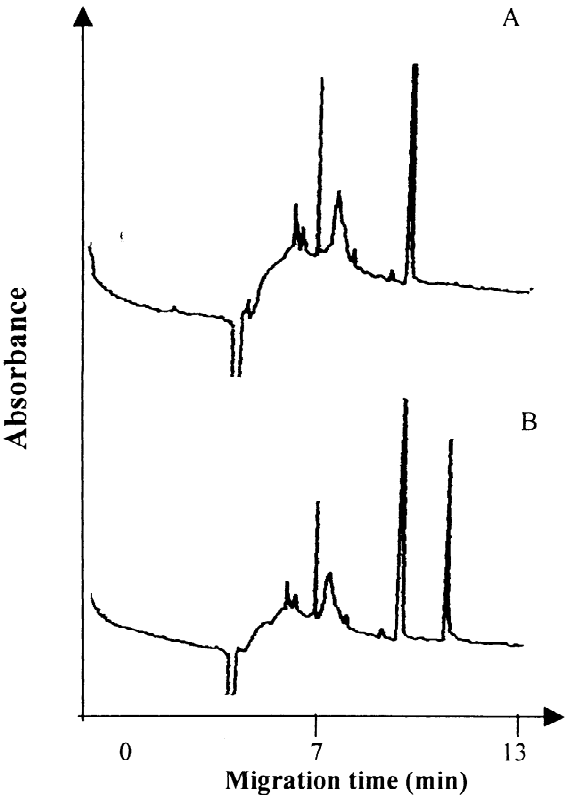


Fig. 1. CE–UV analysis of synovial liquid after centrifugation and injection in CE–UV (235 nm) (A). Detection of the liquid spiked with nitrotyrosine at a final concentration of 1 m*M* (B). Buffer, boric acid 40 m*M*, CAPS 20 m*M* pH 9.5, 20 kV.

Therefore, we labeled tyrosine and nitrotyrosine with FITC. Below is the chemical reaction:

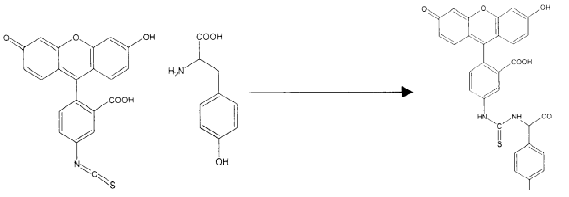


Fig. 2 shows the separation of tyrosine and nitrotyrosine standards with CE–LIF. No nitrotyrosine peak was identified. We believe that it is due to a quenching effect between the nitrotyrosine and the fluorescein nucleus. The UV–Visible spectrum shows the recovery of nitrotyrosine and fluorescein

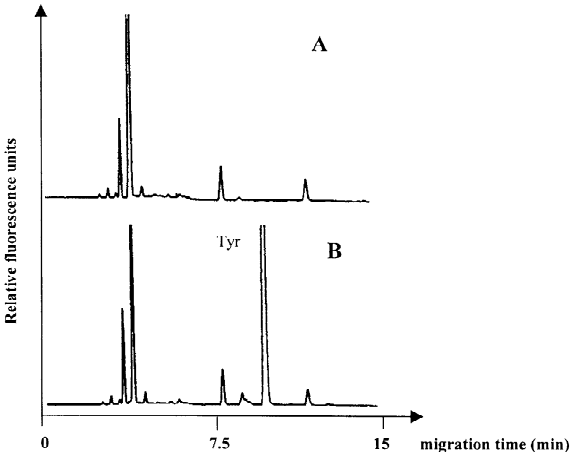
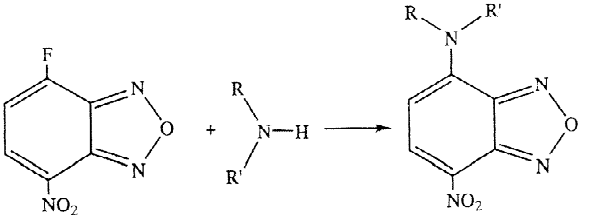


Fig. 2. CE–LIF analysis of tyrosine and nitrotyrosine. (A) Blank, (B) 500 μ*M* tyrosine and nitrotyrosine labeled with FITC and diluted 1000 times prior to injection. Buffer: boric acid 50 m*M*, CAPS 20 m*M* adjusted at pH 10 with NaOH pellets, capillary 75 μm I.D., 75 cm length, 30 kV, injection time 3 s, 350 mbar.

visible spectra, which can result in a decrease of the fluoresceine–thiocarbamyl–nitrotyrosine fluorescence yield. Therefore, fluorescein isothiocyanate cannot be used.

3.3. NBD-F labeling

We tried another less popular than FITC, but more reactive dye which absorbs at 488 nm: the 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) (for a review, see Ref. [12]):



In the past, this dye was used for the detection and quantitation of nitrotyrosine [6] by HPLC and conventional fluorescence. We determined the limit of detection for tyrosine and nitrotyrosine. A separation of 0.3 μ*M* NBD-tyrosine and NBD-nitrotyrosine sample with a conventional aqueous buffer was realized. No signal was observed for NBD-nitrotyrosine (data not shown). We analyzed the same

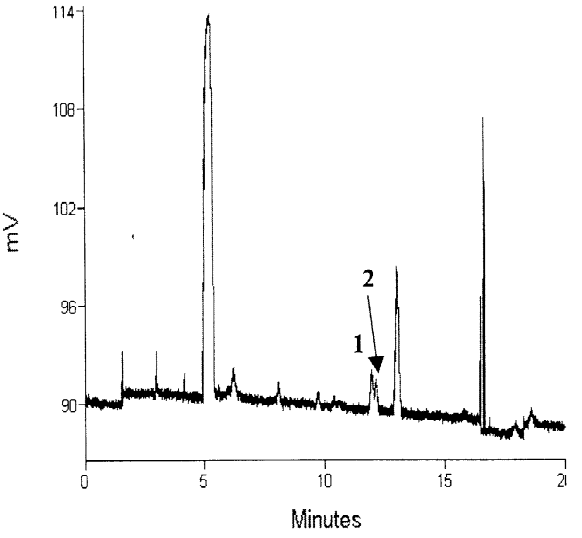


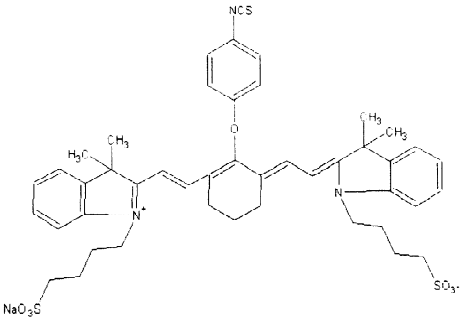
Fig. 3. CE–LIF analysis of tyrosine and nitrotyrosine using NBD-F labeling (1 = Tyr, 2 = Ntyr). The analysis is run in an acetonitrile–methanol–ammonium acetate 25 m*M* (83:15:2, v/v) buffer, analysis 25 kV, 6 s injection at 50 mbar. The sample is diluted prior to injection in acetonitrile–methanol (85:15, v/v), the final concentration of Tyr and Ntyr is 13 n*M*.

NBD derivatives using an acetonitrile–methanol–20 m*M* ammonium acetate (83:15:2, v/v) buffer. The electropherogram is shown in Fig. 3, the sample was diluted 50 times in a 85:15 (v/v) acetonitrile–methanol mixture. The peaks of the derivatized tyrosine and nitrotyrosine can be detected, but the separation is not obvious. Moreover when the sample is diluted in water, no separation between these two species can be done. In the labeling reaction, where we have salts in large quantity, the conductivity of the injected plug is bigger than the analytical buffer. Because of the lack of sensitivity of the derivatized sample we were not able to dilute the sample any longer in organic media prior to injection (the dilution in organic solvent would decrease the plug conductivity). Consequently, the stacking process which would improve the separation is limited. The limit of concentration detected in such conditions was 10 n*M*.

3.4. NIR 780 labeling

Fluka supplies a cyanide isothiocyanate dye (NIR

780). This dye can be excited at 785 nm and emits at 810 nm:



NIR 780 is particularly well adapted to CE–LIF analysis because of its charge, its good fluorescence quantum yield and its high absorbency coefficient. However, the other function can be hydrolyzed easily, which may prevent a good labeling yield. The purity of the dye is reported to be in the range of 75%, which limits its interest for biological studies.

Applications of this dye have been described recently by Patonay and co-workers [13,14]. In our case, this dye, solved the quenching process we observed with FITC which in the end led to a better sensitivity. Linear calibration curves were done in the range of 0.1–0.01 μ*M* injected. Fig. 4 presents a separation of standards of these amino acids at a concentration of 0.3 μ*M* before injecting. The insert corresponds to a concentration of 30 n*M* injected.

In experiments in which a 10^{-6} *M* solution of NIR780 was flushed in the capillary, we noticed that this dye was 24 times less sensitive (data not shown) than the NIR780 of Li-Cor (Lincoln, NE, USA) (an S atom takes the place of the O phenyl ether in the chemical structure). Absorption coefficients and fluorescence quantum yields of these dyes being quite identical, we assume that the purity of these NIR dyes might explain this effect [15].

In Fig. 5A, we present a sinus wash (100FL) which was filtered on a 0.5-μm filter and labeled by NIR780, using the same procedure than the one for standards. Then prior to injection the sample is diluted 100 times in water. Peak 1 is identified as tyrosine by spiking a solution of labeled tyrosine (data not shown). Peak 2 is not identified. An addition of Ntyr at a final concentration of 500 μ*M* in the filtrated sinus wash is shown in Fig. 5B. Ntyr elutes very close to peak 2, but the migration time is

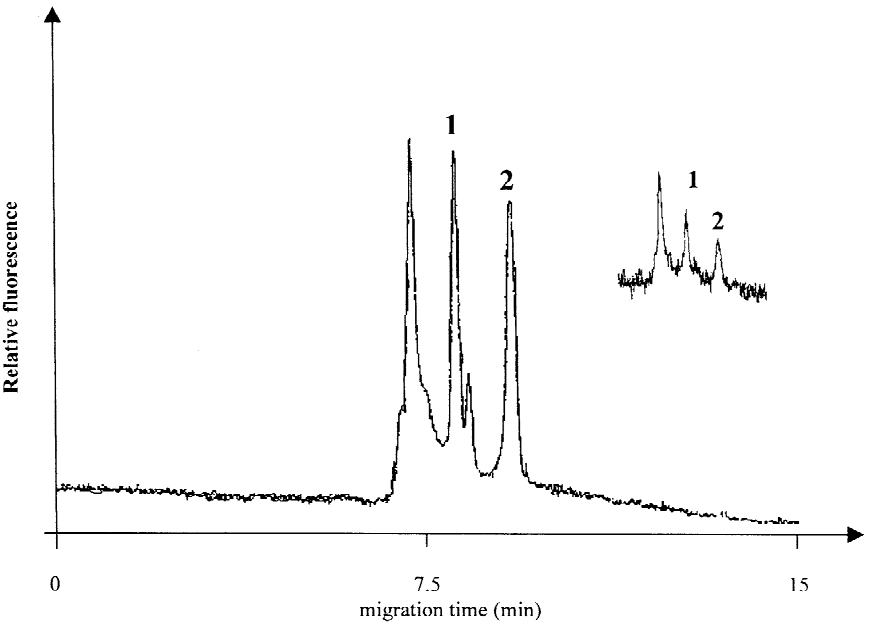


Fig. 4. Separation of a solution of 0.3 μ*M* of tyrosine (1) and nitrotyrosine (2) labeled with NIR 780 and detected with LIF at 785 nm using a diode laser. The insert shows a limit of detection of 30 n*M*. Separation conditions as in Fig. 2. (1 is Tyr, 2 is Ntyr).

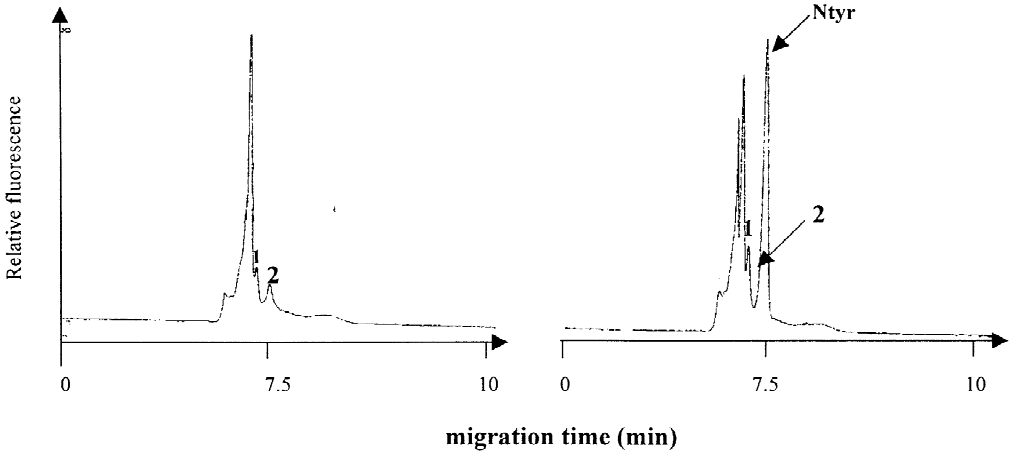


Fig. 5. CE–LIF analysis of a sinus wash labeled by NIR780 using a 785-nm diode laser. (A) Sinus wash filtered on a 0.5-μm filter and labeled with NIR 780 and diluted 100 times prior to injection. (B) Same sinus wash added with 500 μ*M* Ntyr, filtered and labeled with Ntyr and diluted 100 times prior to injection. Separation conditions as in Fig. 2. The Ntyr peak presents a concentration of 5 μ*M*. 1 is Tyr, 2 an unknown.

slightly different because peak 2 is in the foot of the added Ntyr. The injected sample was diluted 100 times in water prior to injection, showing a final concentration of Ntyr of 5 μ*M*. The calculated limit of detection in the sinus wash is 45 n*M* ($S/N=3$), which is slightly higher than with a standard diluted in water.

4. Conclusion

Nitrotyrosine in biological media presents two main difficulties for the analytical chemist. Because this analyte is present in very complex media, diluting the samples prior to injection is mandatory. UV detection is not sensitive enough to be used for quantitation purposes. In the case of LIF detection, FITC derivatives exhibit poor fluorescence yield, which limits the sensitivity. NBD derivatives imply using non-aqueous CE, which limits the stacking process to increase sensitivity. The lowest detected concentration was 10 n*M*. Because the NIR 780 dye is highly fluorescent in the near infra red spectrum, quenching is limited. The lowest detected concentration was 30 n*M*. A higher purity of this dye would certainly lead to sensitivity improvements. In a sinus wash, the estimated limit of detection was about 45 n*M*.

References

[1] H. Oshima, I. Celan, L. Chazotte, B. Pignatelli, H.F. Mower, Nitric Oxide 3 (1999) 132.
[2] H. Kaur, L. Lyras, P. Jenner, B.J. Halliwell, Neurochemistry 70 (1998) 2220.
[3] M.T. Frost, B. Halliwell, K.P. Moore, Biochem. J. 345 (2000) 453.
[4] J.P. Gaut, J. Byun, J. Tran, D. Hung, J.W. Heinecke, Anal. Biochem. 300 (2002) 252.
[5] I. Girault, A.E. Karu, M. Schaper, M.H. Barcellos-Hoff, T. Hagen, D.S. Vogel, B.N. Ames, S. Christen, M.K. Shigenaga, Free Radic. Biol. Med. 31 (2001) 1375.
[6] Y. Kamisaki, K. Wada, K. Nakamoto, Y. Kishimoto, M. Kitano, T. Itoh, J. Chromatogr B 685 (1996) 343.
[7] C. Herce-Paglia, S. Kotecha, D.E. Shuker, Nitric Oxide 2 (1998) 324.
[8] E. Causse, P. Malatray, R. Calaf, P. Charpiot, M. Candito, C. Bayle, P. Valdiguie, R. Salvayre, F. Couderc, Electrophoresis 21 (2000) 2074.
[9] E. Causse, N. Siri, H. Bellet, S. Champagne, C. Bayle, P. Valdiguie, R. Salvayre, F. Couderc, Clin. Chem. 45 (1999) 412.
[10] G. Nouadje, H. Rubie, E. Chatelut, P. Canal, M. Nertz, P. Puig, F. Couderc, J. Chromatogr. 717 (1995) 293.

[11] G. Nouadje, N. Siméon, F. Dedieu, N. Nertz, P. Puig, F. Couderc, J. Chromatogr. A 765 (1997) 337.
[12] S. Uchiyama, T. Santa, N. Okiyama, T. Fukushima, K. Imai, Biomed. Chromatogr. 15 (2001) 295.
[13] J. Sowell, R. Parihar, G. Patonay, J. Chromatogr. B 752 (2001) 1.
[14] J. Sowell, K.A. Agrew-Heard, J.C. Mason, C. Mama, L. Strekowsky, G. Patonay, J. Chromatogr. B 755 (2000) 91.
[15] S. Daehne, U. Resh-Genger, O.S. Wolfbeis (Eds.), NATO ASI Series 3: High Technology, Vol. 52, Kluwer, Dordrecht, 1999.