ELSEVIER

Journal of Chromatography A, 913 (2001) 253–259

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Some applications of near-ultraviolet laser-induced fluorescence detection in nanomolar- and subnanomolar-range high-performance liquid chromatography or micro-high-performance liquid chromatography

N. Siméon[a], R. Myers[c], C. Bayle[a,b], M. Nertz[a], J.K Stewart[c], F. Couderc[b,*]

[a]*Picometrics, 10 Avenue de l'Europe, 31520 Ramonville, France*

[b]*Université Paul Sabatier, IMRCP-UMR 5623, 118 Route de Narbonne, 31062 Toulouse Cedex, France*

[c]*Department of Biology, Virginia Commonwealth University, Richmond, VA, USA*

Abstract

In this work we present some applications of near-UV laser-induced fluorescence (LIF) with micro-HPLC (μHPLC) and HPLC. To test the sensitivity of the detection, we used pyrene and aflatoxins, because both of these molecules exhibit native fluorescence. Then we studied catecholamines derivatized with 1,2-diphenylethylenediamine. The results show that we were able to reach better sensitivity levels than previously described in LIF studies. For catecholamines, a 50-fold increase in sensitivity compared to conventional fluorescence was obtained. These results indicate that LIF detection associated with HPLC or μHPLC can be used to detect very low concentrations of substances that can be excited in the near-UV range after labeling at nanomolar concentrations.

Keywords: Laser-induced fluorescence detection; Detection, LC; Aflatoxins; Catecholamines; Diphenylethylenediamine; Pyrene

1. Introduction

Laser-induced fluorescence (LIF) detection has become a very popular and essential detection technique in capillary electrophoresis (CE), thanks to its capability to detect substances at lower than the micromolar range, which is the limit reached by UV detection. Today there are many applications for CE–LIF [1,2]. The interesting point is that the LIF detection technique was pioneered some 20 years ago by Yeung [3], Folestad et al. [4], and Diebold et al. [5] who demonstrated its usefulness for sensitive detection in high-performance liquid chromatography (HPLC) applications. Today other alternatives, such as conventional fluorescence detection and electrochemical detection, are the two sensitive detection means most commonly used for quantitative studies in HPLC. Numerous applications have been reported in recent years. In some cases the sensitivity of these techniques was increased by use of photoactivation devices [6,7].

Because no LIF detector was commercially available, the cost of the lasers was high, and most of the analyte molecules had to be labeled with dyes that match the laser wavelength, this detection technique has been restricted to a limited number of laboratories. Moreover, the labeling reactions, which are

*Corresponding author.

E-mail address: couderc@ramses.ups-tlse.fr (F. Couderc).

0021-9673/01/$ – see front matter
PII: S0021-9673(00)01234-6

not well understood, can lead to contradictory results [8,9]. More and more analyses in the food, environmental and pharmaceutical industries require quantification at very low concentration levels. LIF detection is a very good alternative to the conventional techniques used today, particularly in pharmacokinetic studies [10].

In this paper, we report experiments that were run to test the sensitivity of the near-UV LIF detector used in our laboratory, coupled to micro-HPLC (μHPLC) or HPLC to detect molecules that have either native fluorescence or fluorescent labels.

2. Materials and methods

2.1. HPLC, μHPLC

We used an Agilent 1100 HPLC pump, equipped with an LC Packings FAMOS injector (Polymer Labs., Marseille, France), and an Accurate microflow reducer (Polymer Labs.) for μHPLC.

2.2. LIF detector

The detector is a Zetalif 325 from Picometrics (Ramonville, France) equipped with a 325 nm He–Cd laser. The power that irradiates the capillary flow cell is 6 mW. A scheme of the detector is presented in Fig. 1. Its originality is the use of a high numerical aperture sapphire ball lens that allows it to have (i) a divergent beam to illuminate all of the inner diameter of the capillary over an approximate length of 200 μm, and (ii) a high collection of fluorescence, much higher than described in the literature for other LIF detectors. Consequently, it is more sensitive.

2.3. Analysis of pyrenes

Isocratic conditions were water–acetonitrile (93:7) with a flow-rate of 4 μl/min, an LC Packings Pep Map C_{18} 3 μm μcolumn, and an injection volume of 1 μl.

2.4. Analysis of aflatoxins

Isocratic conditions were water–methanol (54:46), a YMC Pro C_{18}, 120 Å, S-5 μm, 150×3.0 mm column (YMC Europe, Schrembeck, Germany), flow-rate of 0.4 ml/min, temperature of 40°C, and an injection volume of 120 μl. The output capillary had an internal diameter of 320 μm.

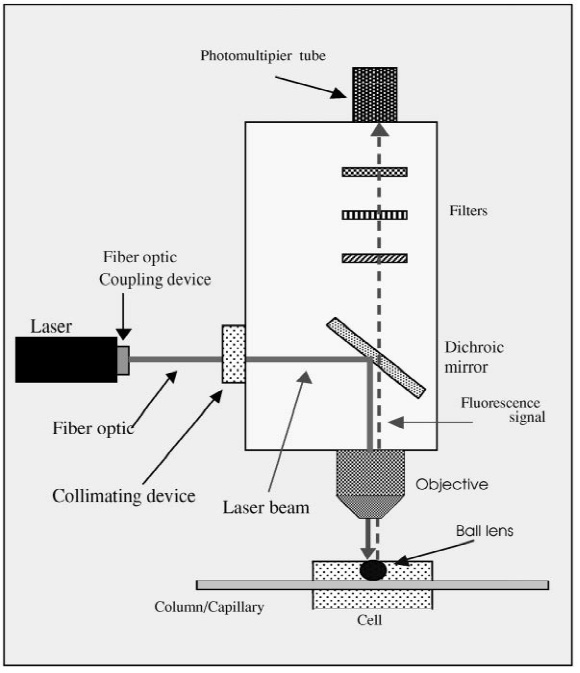

Fig. 1. Scheme of the Zetalif detector.

2.5. Analysis of catecholamines

Isocratic conditions were 62.5% of solution A (50 m*M* sodium acetate, pH 7–methanol–acetonitrile, 60:10,30) and 37.5% of solution B (50 m*M* sodium acetate, pH 7–methanol–acetonitrile, 20:20:60), a flow-rate of 4 μl/min, and an LC Packings Pep Map C_{18} 3 μm μcolumn (Polymer Labs.). The injection volume was 1 μl, and the capillary diameter was 75 μm. For derivatization of catecholamines, 20 μl of catecholamines (in water or in alumina extracts) was mixed with, 40 μl acetonitrile, 10 μl bicine (1.75 *M*, pH 7.5), 20 μl 1,2-diphenylethylenediamine (DPE, 0.1 *M* in 0.1 *M* HCl), and 4 μl potassium ferricyanide (20 m*M*) for 60 min at 37°C in a closed water bath.

2.6. Cells

Myeloid cells derived from a mouse tumor were cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 1.5% sodium hydrogencarbonate, 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1% L-glutamine, 1% minimal essential medium vitamins, 1% nonessential amino acids, 100 units/ml penicillin, and 100 mg/ml streptomycin. The cells were plated and maintained at 37°C and 5% CO_2 for 6 days. We adsorbed catecholamines in the culture media on alumina at pH 8.6 and eluted them with 1% acetic acid. Samples were maintained at −20°C before analysis.

3. Results and discussion

3.1. Adjustment of LIF detection and test of the sensitivity

Some years ago van den Beld [11] demonstrated that in LIF detection the number of molecules that are photodegraded is inversely proportional to the velocity of the fluorophore in front of the laser beam. The same phenomenon was observed by other authors as well [12]. Recently we observed that the sensitivity of the detection in HPLC depends on the inner diameter of the capillary connected to the output of the column. Consequently, at a constant flow-rate, the sensitivity depends on the velocity of the fluorophore in front of the laser beam, and the solid angle of fluorescence collection by the optical arrangement [13]. In Fig. 2 we show the behavior of LIF intensity as a function of the inner diameter (I.D.) of the capillary. The molecule that we studied was doxorubicin with an argon ion laser at 488 nm and a flow-rate of 0.5 ml/min (we injected 5 μl of a 10^{-9} *M* solution). We can see that at this flow-rate, we have an optimal I.D. at 250 μm for the intensity of fluorescence. This sigmoid curve was obtained for different molecules at different flow-rates. A general rule is that for small flow-rates of 2–10 μl/min, capillaries of 50 μm can be used, while for 200–500 μl/min, 250 to 320 μm I.D. capillaries can be used. These guidelines offer a good compromise between sensitivity and dead volumes.

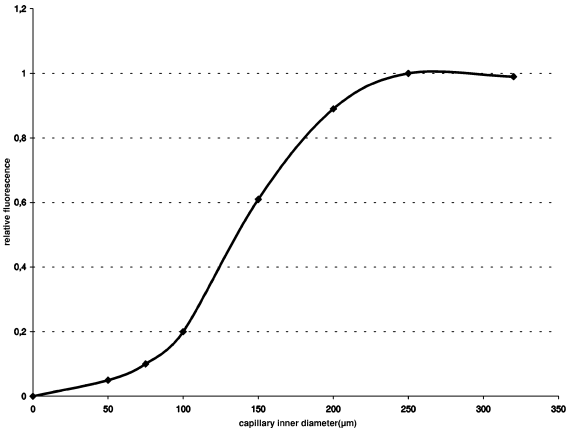

Fig. 2. Plot of relative fluorescence intensity of the peak of 10 n*M* doxorubicin excited at 488 nm, as a function of the inner diameter of the capillary. Injection volume 5 μl, flow-rate 0.5 ml/min, C_{18} column, 20 m*M* phosphoric acids, pH 4.

In flow injection experiments, at a given diameter the detector signal will increase with increasing flow-rates if photochemical degradation is a limiting factor [11]. On the other hand, if the flow-rate is fixed, an increase in diameter is expected to lead to a quadratic increase in detector volume and, thus, a quadratic increase in the number of detectable molecules. The quadratic effect is seen in the first part of the plot. The leveling off at larger diameter is due to photodegradation and to limiting detection geometry. In fact, if a larger volume is irradiated at a larger capillary diameter, the efficiency of fluorescence collection is less important than in the case of smaller capillaries. This is due to the capillary diameter, which is too long for the 2-mm diameter ball lens.

3.2. Test of sensitivity with unlabeled molecules

3.2.1. Sensitivity of pyrene analysis with μHPLC (4 μl/min flow-rate)

In this section we used μHPLC with a 0.3 mm I.D. chromatography column and a capillary of 75 μm at its output and in the detector. To test the sensitivity of the system, we injected lower and lower concentrations, and we measured the peak height. Fig. 3 presents 0.5 fmol, which is the lowest quantity we detected with a $S/N>5$ (no rise time,

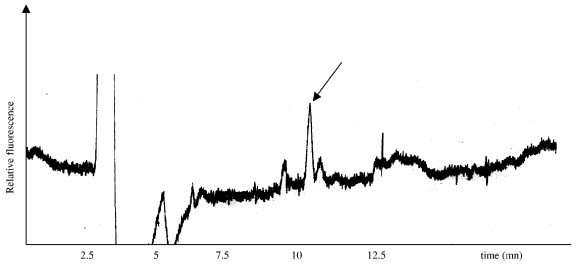

Fig. 3. Chromatogram of 0.5 fmol of pyrene (arrow) obtained by μHPLC. mn = min.

and an acquisition rate of 20 Hz). To our knowledge, it is the best reported result [14]. The calibration curve was obtained for a range of 0.5 to 50 fmol, and the equation of the curve is $y=5\cdot10^{8}x+0.2984$ (y is the area of the peak, x is the concentration in M) with $R=0.9969$.

3.2.2. Sensitivity study with aflatoxins in HPLC (400 μl/min flow-rate)

Aflatoxins are toxic metabolites of fungal origin, for which it is very important to detect the lowest amounts because of their highly toxic and carcinogenic properties. Their legal limits are very low in infant nutriment, 50 ng/l. They fluoresce when exited at 325 nm. Some analyses with CE or HPLC have been published. The CE–LIF set-up, which used a non-optimized LIF detector, exhibited a lack of sensitivity [15]. The other one used online electrochemical derivatization with a Kobra cell connected at the output of the column. The detection limits were around 50 ng/l [6].

A 3 mm I.D. C_{18} column fitted with a 200 μm I.D. capillary at its output and in the detector was used. Fig. 4 presents the separation of four aflatoxin standards G2, G1, B2, B1, at a concentration of 0.1 ppb for each aflatoxin G2 and B2 and 0.33 ppb for

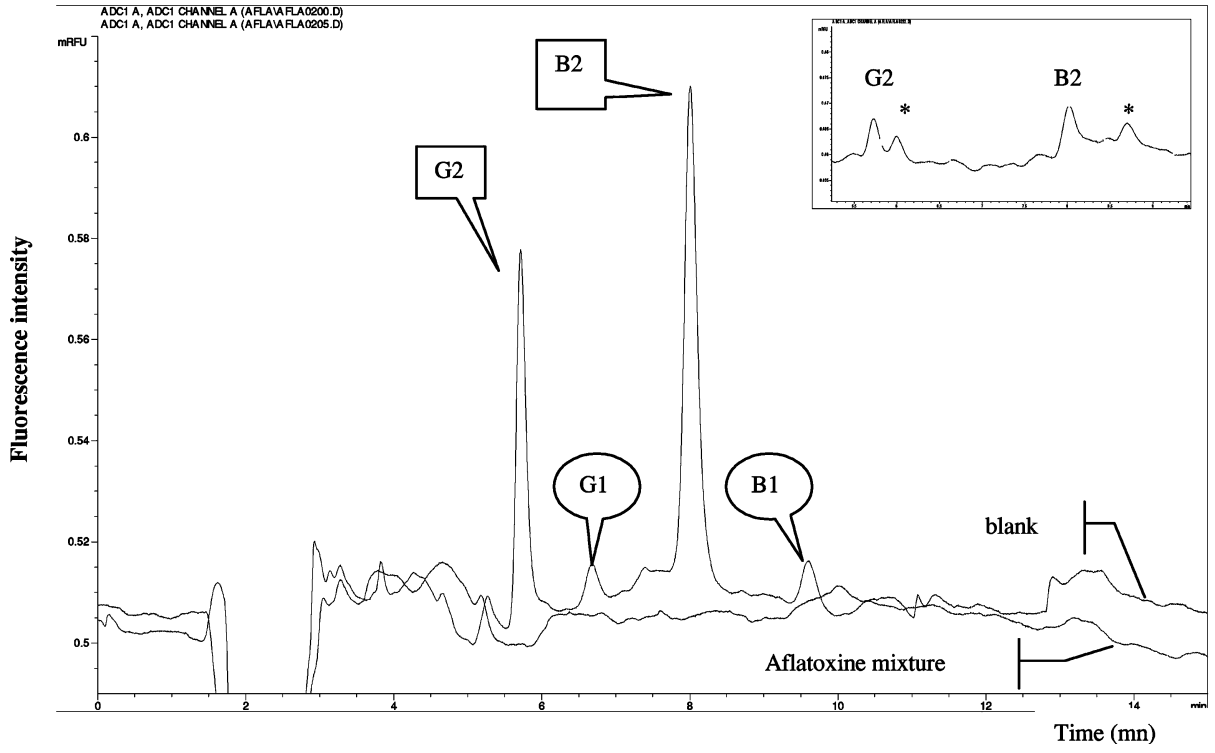

Fig. 4. Aflatoxins G1 and B1 (0.33 ppb) and G2 and B2 (0.1 ppb) diluted in water. A 100-μl volume was injected into the HPLC system with a flow-rate of 0.4 ml/min, a 2.1 mm I.D. C_{18} column, and capillary I.D. of 320 μm at the output of the column in the detector. The inset shows 0.01 ppb of G2 and B2, and * = a peak also present in the blank (rise time 2 s).

G1 and B1. The inset shows G2 and B2 at a quantity of 1.2 pg ($S/N>20$) injected. We estimate that the sensitivity is at least five-times better than the results of previous studies in which a laboratory-made LIF detector was used [5]. Aflatoxins G2 and B2 gave at least linear quantification between $3\cdot10^{-10}$ and $3\cdot10^{-11}$ *M*.

3.3. Application to labeled molecules: analysis of catecholamines

Catecholamine analysis with electrochemical detection or conventional fluorescence is well documented. The impressive results of Dr. Boomsma's laboratory (Department of Internal Medicine, Erasmus University, Rotterdam, The Netherlands) on the quantification of norepinephrine, epinephrine, and dopamine with a fluorescence detector encouraged us to use the same labeling procedure (DPE [16]) to study these catecholamines:

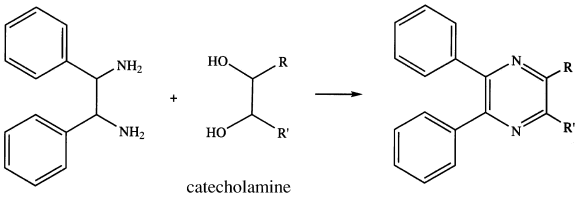

Fig. 5 presents a chromatogram of the three standard catecholamines at a concentration of 2.3 n*M*. The inset shows a chromatogram of 350 amol of norepinephrine injected. Table 1 presents the range of concentrations studied, the slopes, intercepts and correlation of the calibration curves, and the lowest quantity detected. Each catecholamine was derivatized at the concentration range mentioned. The detected quantity is 50-times lower than the one reported in the literature. We were able to label at these low concentrations, and we obtained linear calibration curves. Labeling steps at low concentrations are always the subject of heated discussions. Some authors previously noted that they were not able to label amino acids at concentrations lower than 10^{-7} *M* with 10-fold more fluoresceine-isothiocyanate or dichlorotriazinylaminofluoresceine [8]. Others have shown that they were able to label amines and amino acids at concentrations as low as 10^{-9} *M* with naphthalenedialdehyde [9]. We were able to label catecholamines at $3.5\cdot10^{-9}$ *M* with DPE. For norepinephrine and epinephrine, relative standard deviations (RSDs) were estimated to be lower than 3.2% for intra-assay analyses and lower than 8.4% for inter-assay analyses.

One of the issues encountered in labeling molecules is the reactivity of the different molecules that

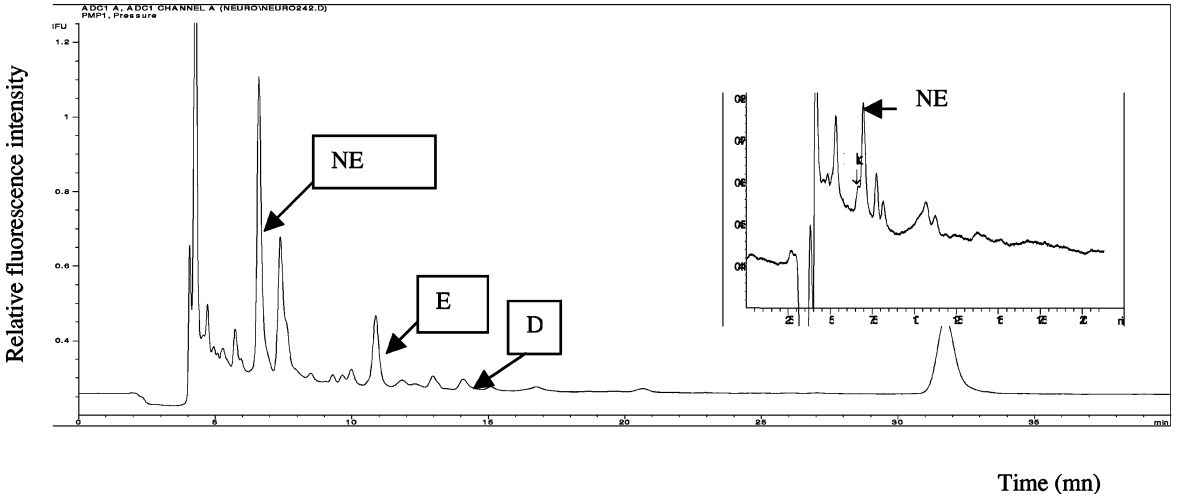

Fig. 5. Chromatogram of a mixture of catecholamine standard at a concentration of 7 n*M*, NE = norepinephrine, E = epinephrine, D = dopamine. The inset shows a chromatogram with 53 fg of NE.

Table 1
Range of concentrations studied, the slopes, intercepts and correlation of the calibration curves of the studied catecholamines[a]

	Range of concentration (n*M*)	Slope (relative area × ml/ng)	Intercept (relative area)	Correlation coefficient (*r*)
Norepinephrine standard	40–3.5	3.0225	0.508	0.9990
Norepinephrine extracted	40–3.5	1.6059	1.3511	0.9990
Epinephrine standard	35–3	0.7007	0.7879	0.9928
Epinephrine extracted	35–3	0.3473	1.1933	0.8979
Dopamine extracted	45–6	0.1073	0.9372	0.9680

[a] The curves for the standard, are obtained by diluting the catecholamine in water. The curves for the extracted catecholamines are obtained after extraction from a blank media on alumina.

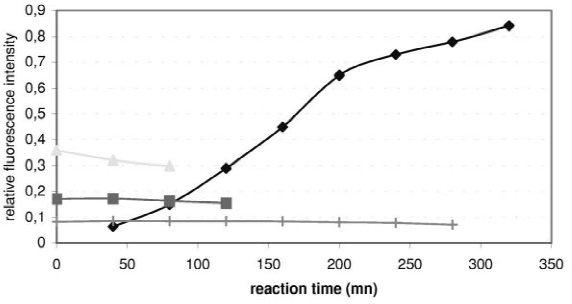

Fig. 6. Intensity of the peaks of epinephrine (■), norepinephrine (▲), dopamine (+) and dihydroxybenzoic acid (♦), following time.

can react with the dye and the stability of the resulting compounds. Fig. 6 is a kinetic comparison of the labeling rate of norepinephrine, epinephrine, and dopamine and dihydroxybenzoic acid at 10^{-8} *M*. We can see that catecholamines react rather quickly but that dihydroxybenzoic acid is much slower to react. Its reaction reaches a plateau when the other compounds begin to be unstable.

Fig. 7 presents a chromatogram of an extract of culture media removed from myeloid cells derived from a mouse tumor. We identified norepinephrine in

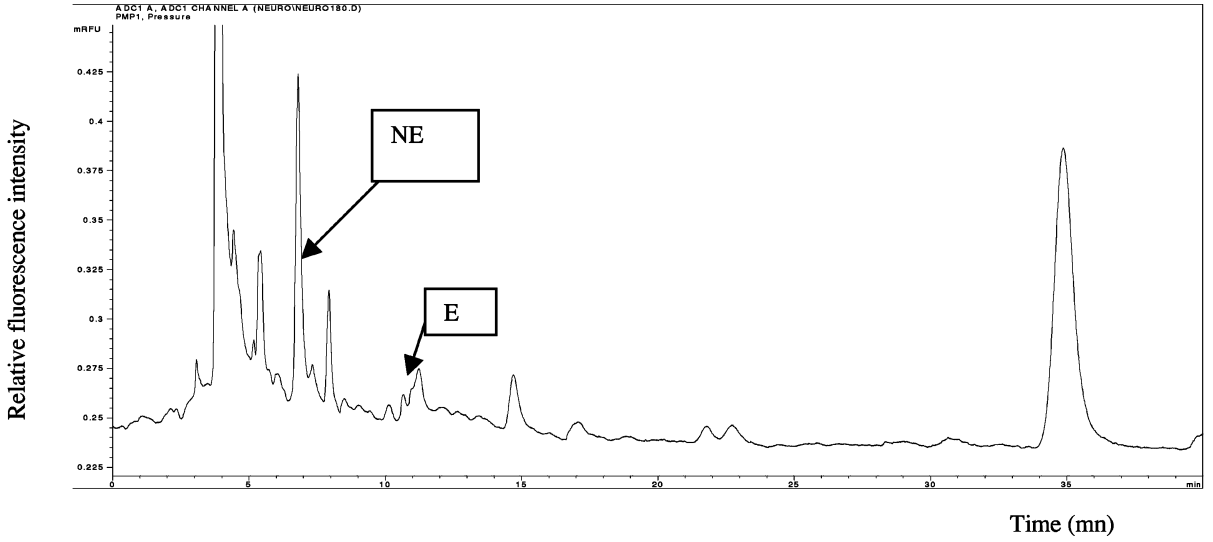

Fig. 7. Chromatogram of catecholamines in myeloid cells derived from a mouse tumor. NE = Norepinephrine (5.1 n*M*), E = epinephrine (2.0 n*M*).

this sample at a concentration of 2 ng/ml. The culture media of the cells is 15 ml for $50 \cdot 10^6$ cells. This result indicates that $1 \cdot 10^6$ cells released approximately 600 pg of NE into the media at 6 days of culture. The level of norepinephrine in the media of $1 \cdot 10^6$ cells would be difficult to detect with electrochemical detection. Moreover, the sensitivity obtained with near-UV LIF and DPE labeling has never been reported.

In conclusion, we have demonstrated that near-UV LIF detection can be used with HLPC or μHPLC. The sensitivity levels are much better than the ones observed with conventional fluorescence. Near-UV LIF detection is an appropriate detection technique to be used for very low concentrations of sample with native fluorescence or that fluoresce after derivatization.

Acknowledgements

We thank Dr. Boomsma for supplying the recrystallized 1,2-diphenylethylenediamine, and William Amoyal for improving the manuscript.

References

[1] C.E. Mac Taylor, A.G. Ewing, Electrophoresis 18 (1998) 2121.
[2] F. Couderc, E. Caussé, C. Bayle, Electrophoresis 19 (1999) 2777.
[3] E.S. Yeung, in: M.V. Novotny, D. Ishii (Eds.), Microcolumn Separations – Columns, Instrumentation and Ancillary Techniques, Elsevier, Amsterdam, 1985, p. 135.
[4] S. Folestad, B. Galle, B. Josefsson, J. Chromatogr. Sci. 23 (1985) 273.
[5] G.J. Diebold, N. Karny, R. Zare, Anal. Chem. 51 (1979) 67.
[6] K. Reif, W. Metzger, J. Chromatogr. A 692 (1995) 131.
[7] R.W. Frei, Chomatographia 15 (1982) 161.
[8] S.P.D. Lalljie, P. Sandra, Chromatographia 40 (1995) 519.
[9] F. Robert, L. Bert, L. Denoroy, B. Renaud, Anal. Chem. 67 (1995) 1838.
[10] G. Hempel, G. Blaschke, J. Chromatogr. B 675 (1996) 131.
[11] C. van den Beld, Ph.D. Thesis, Rijksuniversiteit Leiden, Leiden, 1991.
[12] R.A. Mathies, K. Peck, L. Stryer, Anal. Chem. 62 (1990) 1786.
[13] N. Simeon, E. Chatelut, P. Canal, F. Couderc, J. Chromatogr. A 853 (1999) 449.
[14] G. van de Nesse, Hoornweg, C. Gooijer, U.A.Th. Brinkman, N.H. Velthorst, Anal. Chim. Acta 281 (1993) 373.
[15] C. Maragos, J.I. Greer, J. Agric. Food Chem. 45 (1997) 4337.
[16] G. Alberts, T. Lameris, A.H. van den Meiracker, A.J. Man in't Veld, F. Boomsma, J. Chromatogr. B 730 (1999) 213.