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Analysis of biological samples by capillary electrophoresis with laser induced fluorescence detection

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ABSTRACT

In this paper an overview is provided on practical difficulties as well as applications of capillary electrophoresis coupled to laser induced fluorescence detection methods in the field of analysis of biological samples. Various methodological approaches elaborated for determination of small molecules, peptides and proteins are outlined. Besides giving an overview on detection based on native fluorescence, immune and enzyme assays, the main focus is the problematics of sample derivatization and achievable detection sensitivities in the analysis of real biological samples. The characteristics and applicability of the most commonly used labeling reagents are discussed in details.

1. Introduction

Analysis of biological samples has its own difficulties, because of the usually limited amount of sample specimens, the low analyte concentration, the complex sample matrix, etc. While capillary electrophoresis requires small sample volumes, and has high efficiency to separate considerable number of sample components, it may suffer from the poor concentration sensitivity of the most widely used UV absorbance detection and the deteriorating effect of the sample matrix on separation efficiency. There are strategies developed to overcome or alleviate these difficulties, *e.g.* using a more sensitive detection mode or application of some sample clean-up methods or on-capillary concentration. Compared to UV absorbance, laser induced fluorescence detection offers better sensitivity, besides it is regarded to be more selective (for review see [1–5]). Although there are various applications of CE-LIF in the analysis of biological samples demonstrating these advantages, this method also has its own limitations and cannot be applied easily for all kinds of analytes and biological samples.

In this review it is intended to outline the use of CE-LIF in the analysis of endogenous small molecules, drugs, peptides and proteins in biological samples, giving examples of excellent applications, but mainly focusing on some of the difficulties. Analysis of nucleic acids and carbohydrates is not included into the present discussion.

2. Determination of analytes having intrinsic fluorescence

The advantages of CE-LIF can mainly be achieved when analytes with native fluorescence are to be determined, although these applications comprise a smaller proportion of CE-LIF determinations. Commonly three excitation wavelengths are used for detection of sample components having intrinsic fluorescence. These are 257/266/275/284 nm range, 325 nm and 488 nm wavelength of the common laser sources. The excitation wavelength is a fundamental determinant of the achievable detection sensitivity. At higher wavelength fewer compounds can be detected, thus the selectivity increases and sensitivity improves. Besides interfering peaks from the biological sample matrix, the quantum yield and the proper fit of the wavelength of excitation and the laser source are the main determinants of the detection limits.

UV lasers: solid state diode lasers, frequency doubled Argon-ion lasers and Krypton-ion laser are available as excitation sources at 266 nm, 257/275 nm and 284 nm, respectively. They have been used for the determination of biologically active amines and some drugs, as well. In low UV range relatively high number of analytes can be excited, however the noise from the sample matrix, and light

Abbreviations: CBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; CFSE, carboxyfluorescein succinimidyl ester; DTAF, 5-(4,6-dichrolotriazinyl) aminofluorescein; ERK, extracellular signal-regulated protein kinase; FITC, fluorescein isothiocyanate; FQ, 3-(2-furoyl)quinoline-2-carboxaldehyde; GABA, γ-amino-butyric acid; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NDA, naphthalene dicarboxaldehyde; OPA, *ortho*-phtalaldehyde; SAMF, 6-oxy-(*N*-succininmidyl acetate)-9-(2′-methoxycarbonyl) fluorescein; SIFA, *N*-hydroxysuccinimidyl fluorescein-O-acetate.

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scattering can limit the detection performance. In biological samples interference derives from the native fluorescence of proteins, peptides, nucleotides and other aromatic compounds resulting in rather complex electropherograms while impairing sensitivity analyzing real samples.

Serotonin, catecholamines and their metabolites could be measured in various biological samples with 10–100 nM detection limits [6,7]. On-capillary preconcentration methods [6,8] or selective sample extraction [9] was used to further improve detection sensitivity to the subnanomolar concentration range needed to analyze urine samples. Based on the native fluorescence in deep UV range propranolol and methylenedioxymethamphetamine (Ecstasy) have been determined in plasma and urine samples, respectively. However, the reported detection sensitivity was in the 10^{-7} M range, which is only slightly better than that of conventional UV detection [10,11].

HeCd laser source also can be used in the UV range at 325 nm wavelength. This higher wavelength is accompanied by a better selectivity due to less interfering compounds in the biological matrices resulting in improved sensitivity.

Impressive detection limit of 10^{-10} M concentration range has been reported for pteridines, probable cancer biomarkers; although aqueous standard solutions were used for the calibration and no data were provided on analyte concentration in the urine samples analyzed [12]. Several drugs, like fluoroquinolones [13,14], triamteren [15], phenprocoumon [16], tramadol [17], carvedilol [18], zaleplon [19] zopiclone [20] and salicylates [11,21] also exhibit native fluorescence fitting the lower wavelength of HeCd laser. In real biological samples, the improvement in detection sensitivity was 10–100 times compared to UV detection.

The application of UV laser induced native fluorescence detection is not really widespread so far, because the commercially available CE instruments are not equipped with these types of expensive laser sources.

The most widely used is the argon-ion laser having a 488 nm excitation wavelength that perfectly fits the detection of flavins and anthracyclines. Because of the low interference at the visible wavelength, 10^{-10} to 10^{-9} M detection limits were routinely achieved for flavin vitamers in plasma [22] and various tissues [23]. Terabe and co-workers combined LIF detection and dynamic pH junction-sweeping preconcentration method to further decrease the limit of detection by two orders of magnitude [24,25].

In case of anthracycline anticancer drugs, like doxorubicin, daunorubicin and idarubicin 10^{-9} to 10^{-8} M detection limit in human serum samples was achieved [26]. In a recent work liposome enclosed doxorubicin was separated from the free drug to assay the stability of liposomal preparation during various conditions, including in plasma samples. Due to quenching effect of the liposomes the fluorescence was more than five times less intense resulting in similarly poorer detection limit [27].

3. Detection of analytes without fluorofore

As considerably less compounds possess native fluorescence compared to those having UV absorbance, this detection mode usually requires sample derivatization, or indirect detection can be performed. This latter approach is hardly applicable in case of biological samples as it does not provide the needed selectivity since all the sample components are detected. Its sensitivity is much less compared to the direct methods, the detection limits are similar to that of the direct UV detection and are in the micromolar concentration range [28,29]. As the direct methods are considerably more sensitive, sample derivatization with a fluorofore or a fluorogenic tag is used in majority of cases, although this is not without difficulties, as is discussed in the next paragraphs.

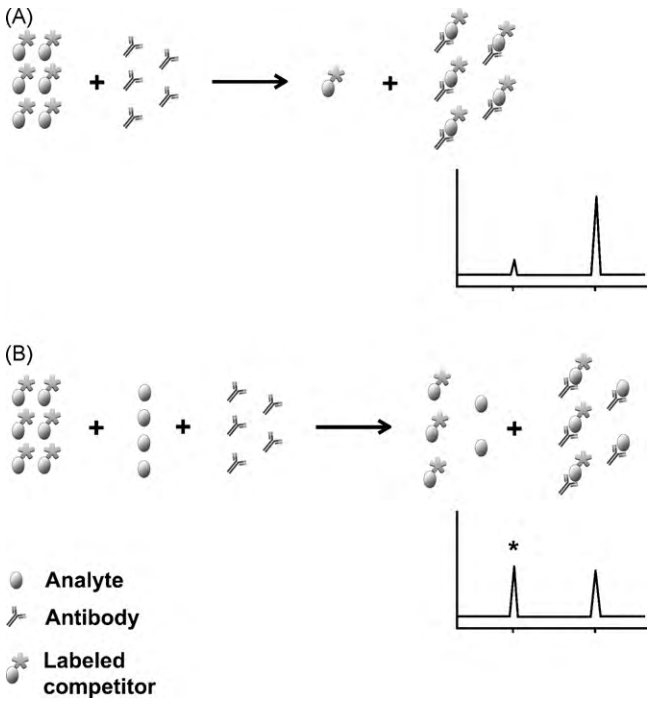

Fig. 1. Scheme of competitive immunoassay in the absence (A) and presence (B) of anlyte. The labeled competitor and the analyte are reacted with limited amount of antibody followed by the separation of the formed immunecomplex from the excess antigen. Asterisk indicates the peak corresponding to excess competitor. The increase of this peak correlates with analyte concentration.

Beside covalent derivatization, interaction with a fluorofore probe is another possibility to use for determination of the analytes, like dynamic labeling of proteins [4,30] or DNA [31–34] and immune and enzyme assays [35]. The advantage of this latter approach is that a preformed labeled probe is used, which is either commercially available or can be prepared at high concentration followed by purification. At high concentration the labeling reaction is more reliable and as the high excess of the labeling reagent and side products can be removed, there are no interfering peaks deriving from the derivatization reaction. Using prelabeled probes the electropherograms are less complex, resulting in easier separation and peak identification as well as higher sensitivity due to better selectivity. The same applies for monitoring enzyme reactions, when the separation of the two fluorescent sample components, the substrate and the product is usually easy to perform. The listed advantages allow achieving detection sensitivity in the subnanomolar concentration range.

3.1. Competitive immunoassays

Some of the immunoassays based on the competition between the analyte and its fluorescently labeled derivative for a limited amount of antibody. In this case the labeled antigen should be separated from its complex formed with the antibody. The amount of the competing analyte is proportional to the increase of peak corresponding to the labeled antigen (Fig. 1). Using this competitive immunoassay arrangement, proteins and peptides have been determined in biological samples. Method has been developed to assess prion protein in blood as a potential clinical diagnostic tool for spongiform encephalopathy [36]. Recombinant hirudin has been assessed in plasma samples with 20 nM detection limit [37]. Vasopressin has been measured in cerebrospinal fluid down to nanomolar concentrations using FITC-labeled vasopressin as fluorescent probe [38]. Methionine-enkephalin has been determined in the plasma and increased level has been found in cancer

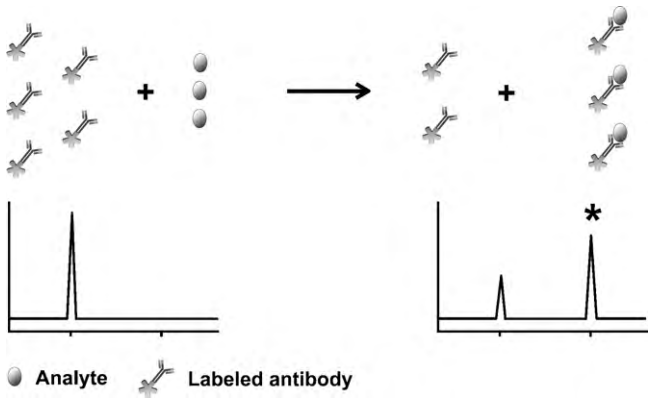

Fig. 2. Scheme of non-competitive immunoassay. The analyte is reacted with excess amount of labeled antibody followed by the separation of the formed immunecomplex from the excess antibody. Asterisk indicates the peak corresponding to immunecomplex. The calculation of analyte concentration is based on this peak.

patients compared to healthy controls. The LOD of the method has not been reported, but methionine-enkephalin level measured in plasma samples was in the low nanomolar range [39]. Kennedy and co-workers have developed several methods to measure insulin content and secretion from islets of Langerhans [40,41]. The method has been extended to simultaneous monitoring of glucagon secretion [42] then a multi-channel microfluidic device has been constructed allowing high-throughput immunoassays to perform [43]. The LOD values reported were in the 0.3–10 nM range.

Small drugs and hormones were also measured in biological fluids with competitive assays. Several methods have been developed for screening of various drugs, like opioids [44–47], amphetamines [46,48] and clenbuterol [49] as well as testosterone [50] in human urine. Competitive immunoassay has been also used for determination of serum levels of digoxine [51], theophylline [52] and other commonly used drugs [53] as well as several hormones, like cortisol [54], thyroxine [55], estrone [56] and estriol [57] with detection limits in the range of 10^{-10} to 10^{-8} M. The results of CE immunoassay measurements were in agreement with those of standard ELISA methods. In case of small molecule analytes usually their protein conjugates, most commonly with bovine serum albumin, are used to induce the production of antibodies, because relatively small molecules are not recognized by the immune system. The conjugates are also used as the labeled probes.

A combination of immunoaffinity extraction and competitive immunoassay has also been reported for the determination of testosterone. Limited amount of antibody was immobilized on a monolithic capillary packing to capture the analyte and the labeled probe from the mixture. After washing the excess, the bound probe was eluted and quantitated. In this case the separation of the probe from the immune complex was not necessary and the antibody packing was reusable [58].

These competitive immunoassay methods have been shown robust and sensitive, thus in addition to research they can find place in clinical laboratories replacing more expensive diagnostic assays.

3.2. Non-competitive immunoassays

Recently the feasibility of non-competitive immunoassays in CE was also presented. In this type of assay the antibody is labeled fluorescently and added in excess to the sample containing the antigen. After incubation the immune complex and the excess of the labeled antibody is to be separated. The quantitation is based on the peak heights or area of the formed complex (Fig. 2). Similarly to other antibody based methodologies sometimes not the analyte specific antibody but a secondary antibody is labeled. In this case a ternary complex, containing the analyte, the primary and the secondary antibody is formed.

The major advantage of non-competitive assays is the commercial availability of labeled antibodies. However, the separation of two proteins, the antibody and its adduct with the analyte, or three proteins in case of using labeled secondary antibody (the secondary antibody, and its complex with primary antibody and the ternary complex also containing the analyte) is a challenge. In free zone electrophoresis the proteins may not considerably differ in their charge to mass ratio resulting in low separation selectivity, and the interaction with the capillary wall may further decrease the resolving power. Determination of benzo(a)pyrene diol epoxide-DNA adducts in cells was reported by LeBlanc et al. using non-competitive immunoassay. Separation of the ternary complex containing DNA from the excess of antibodies was relatively easy due to the high negative charge of the nucleic acid resulting in considerable difference in charge to mass ratio of the analytes. Elevated level of DNA-adducts has been demonstrated when glutathion synthesis was blocked in leukocytes [59]. Ye et al. could solve the separation problem by addition of SDS into the running buffer when carcinoembryonic antigen was assayed in serum using glass microfluidic device. The SDS may alter both the hydrophobicity and the charge of the proteins as well as can reduce the interaction with the capillary wall resulting in improved separation performance [60]. The size-exclusion phenomenon is also exploitable in separation of large molecules, especially when the size difference is significant. *In situ* polymerized polyacrylamide membrane in microchip-based electrophoresis was used by Reichmuth et al. for separation of swine influenza virus containing immune complex from the unbound labeled antibody. Besides separation, the porous membrane also allowed the concentration of the immune complex resulting in a four fold increase in sensitivity compared to open-channel method [61]. An interesting approach was proposed to capture the excess of labeled antibody before separation by antigen bound covalently to the inner wall of the capillary [62]. Although, the large peak in the antigen blank sample indicates that the antibody removal was only partial. This can be the consequence of either the insufficient binding capacity of antigen attached to the wall or the slow kinetics of the binding. However, these aspects have not been discussed in the paper.

3.3. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays conventionally used in slab gel format have been adopted in capillary electrophoresis. This method allows the analysis of specific interaction of proteins with nucleic acids. The principles are the same as in case of noncompetitive immunoassays. The protein sample is incubated with a labeled DNA probe followed by electrophoretic separation of the formed complex from the excess of the labeled probe (Fig. 3). Laser induced fluorescence detection is perfectly suitable for the selective and sensitive detection of the labeled nucleic acid. The method is mainly used for studying the DNA binding affinity of transcription factors [63–67]. The feasibility of the electrophoretic shift method for study interaction between phospholipids and proteins has also been shown [68].

3.4. Enzyme assays

CE-LIF enzyme assays are based on the quantification of the fluorescently labeled product after separation from the labeled substrate. In majority of cases the enzyme reaction runs before the injection to ensure an appropriate incubation time (Fig. 4). However, on-capillary assays have also been reported.

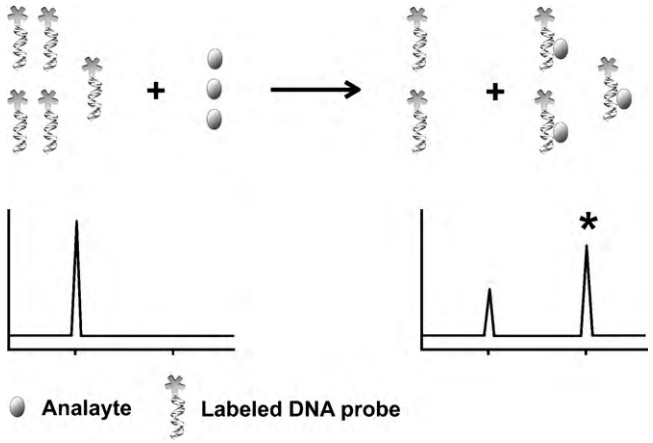

Fig. 3. Scheme of electrophoretic mobility shift assay. The analyte, usually a transcription factor, is reacted with excess amount of labeled DNA probe followed by the separation of the formed compex from the excess probe. Asterisk indicates the peak corresponding to DNA-protein adduct. The calculation of analyte concentration is based on this peak.

Phosphorylation reactions are very important in cell signaling and regulation of cell function. The activity of phosphorylating (*i.e.* kinases) and dephosphorylating (*i.e.* phosphatases) enzymes are thus important to be determined. A fluorescently labeled peptide substrate for extracellular signal-regulated protein kinase (ERK) has been used to assess its activity. In a microchip-based method the phosphorylated product and the non-phosphorylated substrate were separated within 20 s. The reported calibration range was 22–300 ng/mL, which was appropriate to measure ERK activity in endothelial cell extract [69]. The same authors have further developed the method in a 96-lane capillary electrophoresis format allowing high-throughput enzyme assay [70]. Using similar principals, a method for determination of phosphatase activity of calcineurin in cell extract has been elaborated [71], and the activity of sphingosine kinase in cell lysate has also been determined [72]. Cyclic adenosine-monophosphate, an important second messenger in cell signaling is formed by the adenylyl cyclase enzyme after activation of various receptors. Using its fluorescently labeled precursor, adenosine-triphosphate, a sensitive assay has been developed and used for measurement of the enzyme activity in cells in the presence of receptor agonists or antagonists [73]. Microbial content of soil samples was characterized by determination

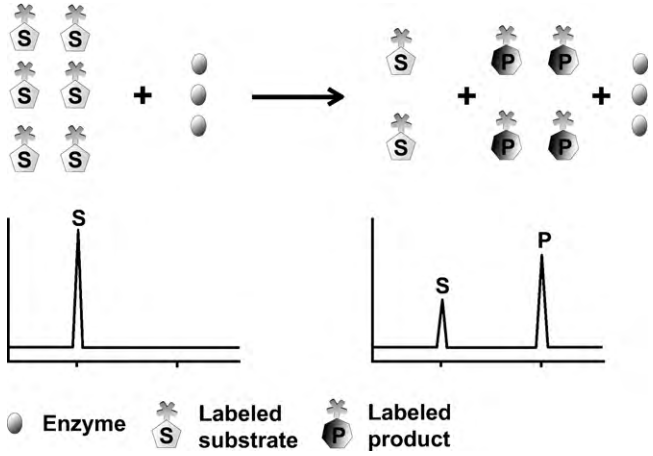

Fig. 4. Scheme of enzyme assay. The enzyme is incubated in the presence of excess amount of labeled substrate. After appropriate reaction time the formed labeled product is separated from the excess of labeled substrate. **S** and **P** indicate the peaks corresponding to the substrate and product, respectively.

of β-glucosidase enzyme activity using fluorescein labeled glucose. Fluorescein released by the enzyme could be determined with quantitation limit in the low nanomolar range [74].

Cleavage products of collagen were separated by capillary gel electrophoresis to follow the activity of various matrix metalloproteases. Dynamic fluorescent labeling of the protein substrate and products with NanoOrange was used for detection. This assay has been shown to be useful for screening potential enzyme inhibitors [75].

Using fluorogenic substrate only the product possesses fluorescence, thus no separation is required under these circumstances. Various phosphatases from marine bacteria could be assessed in a single electrophoresis run using on capillary enzyme reaction. The fluorogenic substrate was included in the separation buffer and the sample contained the mixture of phosphatases. After applying electric field the isoenzymes were separated and detected *via* the separation of their fluorescent products [76].

The feasibility of miniaturized enzyme assays in single cell fashion has been also demonstrated. Single cell analysis has potential advantage in studying heterogeneous cell populations such in case of tumor biology. Two sampling approaches have been shown. Both ones includes the loading the cell with a cell permeable labeled substrate. Afterwards the cell is lysed either in the capillary [77] or in a vessel [78] and after appropriate incubation time the products are measured by CE-LIF. While the advantage of the first approach is that the enzyme reaction takes place in the normal intracellular environment, the other one allows repeated sampling of the enzyme reaction.

3.5. Sample derivatization

In majority of CE-LIF applications the analytes should be labeled to possess fluorescence required for detection. There are several fluorogenic and fluorofore tags available for sample derivatization. Majority of them react with primary and/or secondary amino groups of the analytes, but thiol reagents or sometimes carboxy reagents are also used. The derivatization itself raises a wide range of problems especially in case of biological samples.

Selectivity, one of the claimed advantages of fluorescence detection methods, is rather impaired when the sample is derivatized, since biofluids contain lots of compounds labeled alongside with the analytes of interest. Interfering peaks also derive from the derivatizing reagent either as decomposition products or in case of fluorofore labels as the high excess of the reagent itself. The concentration of these interfering compounds and side products usually exceeds that of the analyte and this difference sometimes can reach several orders of magnitude. The impaired selectivity is accompanied by impaired sensitivity as the latter depends on the signal-to-noise ratio rather than the signal itself. The reported LOD values for analytes in biological matrix usually significantly higher compared to those in aqueous solutions [79–82]. Analysis of biological samples is further complicated when the composition of the sample matrix varies sample by sample resulting in differences in interfering peaks and/or their relative peak size [82,83]. The complex samples require carefully designed separation conditions to ensure appropriate resolution and peak capacity. Several buffer additives, like organic solvents, detergents, EOF modifiers, cyclodextrins are commonly used simultaneously, to improve selectivity and widen separation time window [84–89]. All the buffer compositions are based on trial-and-error method, since the appropriate composition can hardly be predicted. The complicated separation buffer may question the robustness of these methods. Identification and quantification of small peaks surrounded by a plethora of considerably higher ones can be very difficult. Musenga et al. reported that it was necessary to inject blank solution before each sample to ensure reliable peak identi-

fication [83]. Sample clean-up methods may alleviate the matrix effect and improve the selectivity and sensitivity when complex biological samples are analyzed [90–92].

The other major claimed advantage, the high sensitivity of LIF detection is also limited by the labeling reaction itself. Although the derivatized analytes can be very sensitively detected, the derivatization reaction cannot be performed reliably at low sample concentration [93]. In the early reports the derivatization was carried out at micromolar or even higher analyte concentration followed by dilution and analysis. This way LOD values down to picomolar concentration range were reported for standard solutions [89,94–98]. However, these data are informative only about the intrinsic sensitivity of the LIF detector (the dilution also masks the deteriorating effect of interfering peaks by reducing the relative peak size difference) and are far better than achievable in real sample analysis. Sample derivatization at the submicromolar concentration suffers from the slower reaction rate and the consequentially increased competition with hydrolysis reaction of the labeling reagent as well as the adsorption of analyte to the surface of the reaction vessel [99]. The consequence of the incomplete derivatization of highly dilute analyte solution is the loss of linear correlation between the concentration and peak area or height [87]. The majority of papers reporting method development intend to prove its applicability in wide dynamic calibration range of two to three orders of magnitude. However, this can raise a bias because of the overrepresentation of the higher concentration points on the calibration curve, since the regression coefficient is determined by the absolute deviation of the measured data points from the fitted line. In the low concentration part of the curve thus even a large relative deviation (*i.e.* the deviation in percentage) only slightly affects the *r*-value of the entire calibration line. Due to this bias a 0.995 value of regression coefficient itself does not grant accurate determination in the lower concentration part of the calibration range. Being aware of the above discrepancy the estimated LOQ values can be unreliable. Determination of LOQ values is thus especially crucial to be based on the accuracy data. In line with the above discussed problem of the quantitative derivatization at low sample concentration, the appropriately determined quantification limits may differ considerably from the limit of detection (the difference can be two orders of magnitude) [79,100].

Another practical problem during derivatizing biological sample is the calculation of the appropriate derivatizing reagent concentration. To ensure quantitative derivatization a proper ratio of labeling reagent to analyte should be found, which is usually in the range of 50–1000. On the other hand too high excess of the reagent should be avoided otherwise the separation is impaired by the more pronounced interference. When aqueous standard solutions are labeled in the method development phase, the excess of reagent required can be easily determined. However, the biological samples contain lots of derivatizable components in addition to the analytes of interest and their concentration can hardly be estimated. Using biological sample matrix during optimization of derivatization is thus substantial [83].

The derivatization also has a great impact on the electrophoretic properties and thus the separation of the analytes, especially when several similar sample components are to be determined. The labeling tag alters the mass as well as the charge of the compounds. In case of small molecules the large fluorescent label significantly reduces the difference in the charge-to-mass ratio making the separation more difficult. Derivatization of an amine usually results in the loss of a chargeable group. On the other hand the label itself can carry one or more, usually negative charge that also substantially defines the separation conditions. In case of compounds possessing more than one derivatizable groups, *e.g.* proteins, peptides and polyamines, multiple labeled products may be formed. This further complicates the separation and may interfere with the sensitive and accurate quantification [86,101–104].

In the next paragraphs the most widely used labeling reagents and their applications in the analysis of biological samples are overviewed. Reagents suitable for detection with commercially available Argon-ion, HeCd and red and near infrared diode lasers are included.

3.5.1. Fluorescein based reagents

Argon-ion laser (emitting at 488 nm) is the most commonly used laser source with commercially available CE instruments. (Application using various labeling reagent and Argon-ion laser are summarized in Table 1.) The excitation wavelength of fluorescein based labeling reagents exactly fits this laser. Fluorescein also possesses high absorptivity and quantum yield ideal for highly sensitive detection. Although giving numerous fluorofore hydrolysis products makes its great disadvantage. The photostability of fluorescein and its derivatives is rather limited, thus all manipulation should be performed in the dark.

Fluorescein isothiocyanate (FITC) was one of the first amine reactive probes used. It reacts with both primary and secondary amines, but labeling reaction requires rather long time, conventionally overnight at room temperature [94,99,105], although shorter reaction periods at higher temperatures have been also reported [97,105–108]. Sometimes 0.1% pyridine is used as a catalyst in labeling reaction, but its advantage is not unequivocally demonstrated [92,105]. Due to the plethora of derivatization side products the separation requires delicate buffer composition and significant dilution of the sample (up to 100 fold) before injection.

Fully validated method using FITC has been developed for the determination of illicit amphetamine and ephedrine derivatives in human blood and urine after solid phase extraction as sample clean-up. The accuracy of the method has been demonstrated at the low nanomolar concentration range and its applicability has been shown for spiked biofluids [92]. Raggi and co-workers have developed several validated methods for measurement of various drugs in plasma and urine samples using different fluorescent labels including FITC. The accuracy of the assays has been presented at about 35 and 500 nM for sertraline [85] and pramipexole [91], respectively. Several papers deal with amino acid analysis in various biological samples. Determination of different amino acids, including γ-amino-butyric acid (GABA), glutamate and aspartate, in brain homogenate [105] and microdialysis [106] samples has been reported. The applicability of the methods to real samples has been demonstrated at low micromolar concentration levels, although nanomolar and subnanomolar LOD values for aqueous standards have been claimed. Amino acids in urine [79] and plasma [108] samples have been analyzed and micromolar quantification limits have been reported. The relatively high LOQ values are likely due to insufficient labeling efficiency at lower concentrations [79].

Several on-capillary sample concentration methods have been applied for determination of FITC labeled peptides in cerebrospinal fluid and saliva samples. These papers could demonstrate the 10–100 fold increase of peak height depending on the preconcentration method used [109,110]. Although the application of these techniques can be useful, the original problem of efficient derivatization at low concentration cannot be solved this way.

To overcome some disadvantages of FITC, several other fluorescein derivatives are also used for amine labeling. Carboxyfluorescein succinimidyl ester (CFSE) is regarded as a more reactive agent. Banks and co-workers have compared the derivatization of amino acids at low concentration by FITC and CFSE. They have found that contrary to FITC, in case of CFSE the rate constant for amine derivatization exceeds that of its hydrolysis reaction, resulting in more efficient sample derivatization at low concen-

Table 1
CE-LIF determinations of various analytes using Argon-ion laser.

Analyte	Sample matrix	Labeling reagent	Sensitivity	Reference
Amphetamines	Spiked urine	FITC	LOD: 200 ng/mL with SPE	[90]
Ephedrines, amphetamines	Spiked urine or blood	FITC	LOD: 0.2 ng/mL, with SPE	[92]
Sertraline and desmethyl-sertraline	Plasma	FITC	LOD: 1.5 and 2 ng/mL, with SPE	[85]
Pramipexol	Urine	FITC	LOD: 10 ng/mL, with LLE	[91]
Amino acids	Brain homogenate	FITC	LOD: 2.1×10^{-11} to 6.3×10^{-10} M; calibration from 10^{-8} M	[105]
Amino acids	Brain microdialysate	FITC	LOD: 0.05–0.1 μM	[106]
Amino acids	Urine	FITC	LOD: 160–330 nM, LOQ ~10 μM	[79]
Taurine	Plasma	FITC	LOD: ~10 μM	[108]
Bradykinin-related peptides	Saliva, CSF	FITC	LOD: 0.02–0.04 nM with SPE and stacking	[109]
Peptide hormones	CSF	FITC	LOD: 0.04–0.2 nM with SPE and pseudo-tITP	[110]
Glutamate, aspartate	Microdialysate	CFSE	LOD: ~0.7–0.8 nM, calibration from 0.02 μM	[112]
Aminoglycosides	Plasma	CFSE	LOD: 14–24 nM, calibration from 0.15 μM	[80]
Baclofen	Plasma	CFSE	LOD: 0.1 μM	[81]
Vigabatrin	Plasma	CFSE	LOD: 2 μg/mL, calibration from 10 μg/mL	[111]
Biogenic amines	HeLa cells and fish samples	SAMF	LOD: 0.25–2.5 nM, accuracy shown at 10 nM	[103]
Short-chain aliphatic amines	Serum, cells	SIFA	LOD: 0.02–0.1 nM, accuracy shown at 200 nM	[113]
Duloxetine	Plasma	DTAF	LOD: 1 ng/mL with LLE	[83]
Bradykinin	Saliva, plasma	DTAF	LOD: 0.1–0.3 pM, with tITP	[115]
Homocysteine and other thiols	Plasma	IAF	LOD: 0.25 μM, calibration from 1 μM	[117]
Thiol compounds	Plasma, cells	IAF	LOD: 1 μM	[118]
D-Penicillamine	Plasma	IAF	LOD: 0.1 μM	[119]
Glutathione	Bacteria, Cells	IAF	LOD: 0.5 ng/mL LOD: 4 μM	[121] [122]
Thiouracil	Spiked urine	IAF	LOD: 2 nM, calibration from 0.1 μM	[123]
Captopril	Urine	IAF	LOD: 0.5 ng/mL	[124]
Cadaverine, lysine	Saliva	NBD-F	LOD: 0.3–0.5 μM, calibration from 1 μM	[101]
Histamine, polyamines	Plant extract	NBD-F	LOD: 0.02–0.05 μM	[127]
Dimethyl arginine, arginine	Plasma	NBD-F	LOD: 0.1 μM	[129]
Serine (chiral)	Brain tissue	NBD-F	LOD: 0.3 μM, calibration from 1 μM	[130]
Various amines	Plasma, saliva, urine	NBD-F	LOD: 5–58 nM, calibration from 75 nM	[131]
Amino acids	Plant extract	NBD-F	LOD: 7–42 nM, calibration from 80 nM	[87]
Amino acid transmitters	Microdialysate, on-line derivatization	NBD-F	LOD: 5–85 nM	[133]
Proteins	Cell extract, single cell	FQ	LOD: 5–30 nM	[136–138]
Aminophospholipids	Cell extract	FQ	LOD: 10^{-9} M, calibration from 0.6 μM	[140]
Aminocyclopropane-1-carboxylic acid	Apple extract	FQ	LOD: 10 nM, calibration from 50 nM	[141]
Amino acid transmitters	Microdialysate	FQ	Calibration from 25 nM	[142]
Amino acids	Plasma, on-capillary derivatization	FQ	LOD: 23–50 nM, calibration from 0.1 μM	[143–144]
Amino acids	CSF, microdialysate	CBQCA	LOD: 0.29–100 nM	[146–147]
Amino acids	Organ perfusate	CBQCA	LOD: 30–600 nM	[148]
Carnosine related peptides	CSF	CBQCA	LOD: 4–5 nM, LOQ: 0.1 μM	[150]

tration while less side products are generated [99]. Originally overnight derivatization was used with this label too, however recently as short as 30 min derivatization time at room temperature has also been reported [80,111]. CFSE has been used for derivatization of low concentration of amino acids in microdialysis samples, but the accuracy of the method has not been shown [112]. In other reports although nanomolar detection limits could be achieved for aqueous drug standards using CFSE labeling, their determination in plasma samples could be performed only at 0.1–50 μM level, probably due to the matrix effect [80,81,111]. Other fluorescein succinimidyl ester derivatives, *e.g.* 6-oxy-(*N*-succininmidyl acetate)-9-(2′-methoxy-carbonyl) fluorescein (SAMF) and *N*-hydroxysuccinimidyl fluorescein-O-acetate (SIFA) have been developed by the group of Wang and Zhang. These reagents provide the advantage of more rapid labeling reaction, while other characteristics are similar to those of CFSE. Using SAMF derivatization (10 min at 20 °C), biogenic amines have been determined in cells and fish samples. Accuracy of the method in real samples has been shown at 10–30 nM analyte concentration and LOD was between 0.25 and 2.5 nM [103]. SIFA derivatized short-chain aliphatic amines (labeled for 30 min at 45 °C) have been measured in cells and serum samples. While LOD values in 0.02–0.1 nM range have been reported the accuracy has been demonstrated only at 200 nM concentration [113].

5-(4,6-Dichrolotriazinyl)aminofluorescein (DTAF) is another fluorescein derivative that is claimed to react more rapidly than FITC with primary and secondary amino groups [114]. Only two reports analyzing real biological sample have been found in the literature. Duloxetine has been determined with high sensitivity in human plasma; both LOD and LOQ values in the nanomolar concentration range have been reported [83]. In another study bradykinin levels were measured in human saliva and plasma samples using transient isotachophoresis preconcentration to further improve detection sensitivity. Impressive, subpicomolar detection limits have been achieved, but the concentration of the analytes used in the derivatization reaction and the accuracy of the method have not been reported [115]. Zone passing mode of in-capillary derivatization of amines and amino acids with DTAF has been described and validated for aqueous standards but was not used for biological samples [116].

Fluorescein derivatives for thiol labeling are also available. The most widely used of them is 5-iodoacetamidofluorescein. Its first application in capillary electrophoresis has been shown by Coudercand co-workers for quantification of homocysteine, glutathione,

cysteinylglycine and cystationine in plasma. The calibration was performed at 1–200 μM concentration and the reported LOD was 0.25 μM. The results for plasma homocysteine measurements were in agreement with those of other established methods [117]. Shorter derivatization time of 10 min for these thiols has been shown by Zinellu et al. They have used *N*-methyl-*D*-glucamine as an EOF modifier to improve separation efficiency. Similar quantitative results have been achieved, presenting accuracy in the micromolar concentration range [118]. This group has presented several applications of their method including determination of *D*-penicillamine in plasma [119] and improving sensitivity by sample stacking [120]. Similar methods for measurement of cellular glutathione concentration have also been published [121,122]. Besides endogenous thiols, thiol drugs and drug residues in biological samples could be determined in the same way. Pérez-Ruiz et al. have shown the applicability of these methods for determination of thiouracil and captopril in spiked samples at micromolar concentration range [123,124].

3.5.2. Other labeling reagents fitting the Argon-ion laser

4-Fluoro- or 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-F or NBD-Cl) are fluorogenic derivatizing agents for compounds containing primary or secondary amino groups. Although they are fluorogenic their hydrolysis products show fluorescent properties. Anyway, compared to fluorescein based dyes much clearer electropherograms can be expected because of the less number of side products and lack of impairing effect of the reagent excess. They react rapidly with the amines at relatively high temperature (55–65 °C) [125] and NBD-F is claimed to be ten times more reactive than NBD-Cl. In contrast to the majority of labels, NBD derivatives show constant fluorescence in the pH range of 2–9 [126] allowing more flexible optimization of separation conditions. Monolabeled di- or polyamine analytes possessing chargeable amino group(s) have been separated in their cationic form at acidic pH resulting in selective analysis [101,127]. On the other hand the fluorescence intensity is sensitive to the solvent polarity, being more intense in apolar environment [126]. In capillary electrophoresis non-ionic surfactant, Brij 35 has been shown to increase the fluorescence signal by about 3 times, although a more pronounced increase of the reagent peaks has also been observed [89,128]. For biological samples LOQ values around 10^{-7} to 10^{-6} M are reported most likely due to the problem of the quantitative derivatization with NBD-F at lower analyte concentration [101,127,129–132]. Barett and co-workers have reported that calibration curves for NBD labeled amino acids became non-linear below 80 nM concentration [87].

As a rapidly reacting label it can be used for on-line derivatization after microdialysis sampling. NBD-F has been applied in a hyphenated setting where the reagent was mixed to the microdialysis effluent then introduced into the separation capillary. Sixteen amino acids have been separated and small changes in the amino acid levels could be detected [133]. In-capillary derivatization with NBD-F has also been performed in the zone passing mode, however the applicability to biological samples was not presented [134].

3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) is an analog of *ortho*-phtalaldehyde (OPA) and naphthalene dicarboxaldehyde (NDA) but the fluorescence spectrum of its derivatives fit the wavelength of Argon-ion laser. It is a fluorogenic label that reacts only with primary amines in the presence of cyanide as co-reagent. Compared to OPA or NDA the labeling reaction with FQ requires higher temperature and at least several minutes [135], but is still much shorter compared to the fluorescein based labels. High excess of this fluorogenic derivatizing agent can be used without high interference signal in the electropherogram. It has been introduced by Dovichi's workgroup and they used extensively for protein labeling. Denatured standard proteins and cell extracts have been labeled within 5 min at 65 °C. LOD values of 5–30 nM have been reported but quantification limit and accuracy data have not been presented [136]. Because of the short labeling reaction time they could also perform on-capillary denaturation and derivatization (5 min at 90 °C) followed by capillary gel electrophoresis separation. The method allowed single cell protein analysis [137,138]. Recently, Veledo et al. used similar on-capillary derivatization method for protein fingerprint analysis of bacterium species [139]. Low molecular weight compounds have also been labeled and analyzed using FQ. Aminophospholipid components in cell extracts have been determined at micromolar concentration range [140]. FQ derivatives of biogenic amines were analyzed in tobacco leaf, the reported LOD values are in the nanomolar range, but the applicability for quantification has been demonstrated at 10^{-7} to 10^{-6} M concentration level [84]. Similar quantitative performance has been reported for determination a specific amino acid, 1-aminocyclopropane-1-carboxylic acid in apple [141]. Amino acid neurotransmitters have been analyzed in microdialysate samples, quantitative derivatization down to 25–220 nM analyte concentration have been demonstrated and quoted as LOQ values rather then LOQ based on signal-to-noise ratio estimation [142]. On capillary derivatization of amino acids in plasma samples after deproteinization has also been shown with LOD around 10^{-8} M concentration, but calibration and determinations have been performed in the 10^{-7} to 10^{-5} M range [143,144].

A similar fluorogenic reagent 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) has been shown to react with primary amines at room temperature in the presence of cyanide. The derivatives can be excited with both the 442 nm line of HeCd and the 488 nm line of Ar-ion laser sources [145]. The reaction time for labeling is longer for this reagent, requires a couple of hours to complete. One of the first applications to real biological samples came from the group of Bergquist. Fifty labeled peaks in human cerebrospinal fluid samples have been detected and ten of them have been identified and quantitated. The LOD values for the determined amino acids were between 0.29 and 100 nM [146]. They also have analyzed amino acids in as low as 1 μL brain microdialysate samples with similar quantification performance [147]. Even lower volume (0.5–1 μL) of organ perfusate has been derivatized for determination of amino acid content. Seventeen amino acids including D-serine and D-aspartate have been resolved and quantitated at micromolar levels [148]. Derivatization with CBQCA at low concentrations (40 nM) of amino acids in human plasma has been demonstrated and plasma amino acid profiles in normal subjects and aminoacidopathy patients have been compared [149]. Dipeptides in cerebrospinal fluid have been measured with an LOQ of 0.1 μM which is significantly different from the instrumentation LOD of 4–5 nM due to the presence of background peaks in the biological sample [150]. CBQCA has also been used in assessing botulinum toxin enzyme activity to screen combinatorial peptide libraries for potential inhibitors. A synthetic peptide is used as substrate for this proteolytic enzyme, the substrate and products were labeled after the enzyme reaction in the micromolar concentration range [151].

3.5.3. Labels for other laser sources

3.5.3.1. NDA for HeCd laser sources. NDA is close to be an ideal derivatization reagent as it is flouorogenic, reacts with primary amines very rapidly at room temperature and there are no fluorofore side products. It was developed from OPA, but it has the advantage of giving more stable derivatives as well as more favorable excitation spectrum. While OPA derivatives should be excited in the UV range, those of NDA in higher wavelengths, between 400 and 450 nm. Visible excitation results in more sensitive detection compared to UV due to less interference and light scattering [152]. The major limitation of its more widespread use is the requirement of the expensive and less commonly available HeCd laser for excita-

Table 2
CE-LIF determinations of various analytes using NDA labeling.

Analyte	Sample matrix	Excitation source	Sensitivity	Reference
Catecholamines	Microdialysate	HeCd laser (442 nm)	LOD: 10^{-9} M	[154,158]
Excitatory amino acid transmitters	Microdialysate, on-line derivatization	HeCd laser (442 nm)	LOD: 2.3–2.6 nM, calibration from 130–160 nM	[155–156,158]
GABA	Microdialysate	HeCd laser (442 nm)	LOD: 3 nM	[157–158]
Vigabatrin	Microdialysate	HeCd laser (442 nm)	LOD: 1 nM, accuracy shown at 0.5 μM	[88]
Amino acid transmitters	Microdialysate, in-capillary derivatzation	HeCd laser (442 nm)	LOD: 0.1 μM, calibration from 1 μM	[159]
Amino acid transmitters	Microdialysate, on-line derivatization	HeCd laser (442 nm)	LOD: 0.01 and 0.1 μM, calibration from 0.1 and 1 μM	[160–161]
Amino acid transmitters (chiral)	Single cell	HeCd laser (442 nm)	LOD: 0.1 μM	[163]
Phosphorilated amino acid	Protein hydrolysate	HeCd laser (442 nm)	LOD: 5–7 nM, accuracy shown at 4 μM	[166]
Enterostatin	CSF	HeCd laser (442 nm)	LOD: 4.8 μM	[167]
Sustance P related peptides	Microdialysate	HeCd laser (442 nm)	LOD: 2.5–26 nM	[168]
	Postcolumn dericvatization		LOD: 74–100 nM	[168,174]
Tryptophan (chiral)	Urine, brain tissue, CSF	HeCd laser (442 nm)	LOD: 33 nM, calibration from 0.1 μM	[169]
Glutamate (chiral)	Brain tissue, single neuron	HeCd laser (442 nm)	LOD: 0.57 μM	[170]
Baclofen (chiral)	Spiked plasma	HeCd laser (442 nm)	LOD: 10 ng/mL, calibration from 0.1 μM	[171–172]
Glutathion (without cyanide)	Cell extract	Argon-ion laser (488 nm)	Calibration from 0.16 μM	[173]
Proteins, peptides (with mercaptoethanol)	Cell extract, post-column derivatization	Argon-ion laser (488 nm)	LOD: 8–32 nM, calibration from 0.39 μM	[175]
Aminoglycosides (with mercaptoethanol)	Milk, post-column derivatization	Diode laser (473 nm)	LOD: 7–20 ng/mL, calibration from 20 ng/mL	[176]
Catecholamines and amino acid transmitters	Microdialysate	Diode laser (410 nm)	LOD: 0.5–3 nM	[179]
Amino acid transmitters	Serum, CSF	Diode (425 nm)	LOD: 21–23 nM	[180]
Amino acids	CSF	Diode (405 nm)	LOD: 3–7 nM with stacking, calibration from 20 nM	[181–182]
Proteins	Urine	Diode (405 nm)	LOD: 0.59–4.22 nM with stacking	[183]
Octopamine	Spiked plasma	Diode (405 nm)	LOD: 5 nM, calibration 10 nM with off-line preconc.	[184]

tion of the derivatives at 442 nm. Although recently, the much less expensive violet light emitting diodes and diode lasers have also been used successfully for detection of NDA derivatives. (Applications using NDA labeling are summarized in Table 2.) Similarly to OPA and other aldehyde reagents, a nucleophilic co-reagent is necessary to the derivatization reaction. Cyanide is the most commonly used one as it results in derivatives with high fluorescence intensity and stability, however thiol containing compounds can also be used [152]. The first CE-LIF application of NDA has been published by Hernandez et al. in 1993 for analysis of excitatory amino acids in rat brain dialysate [153]. The group of Renaud and Denoroy has shown the capability of NDA to label catecholamines at as low as 10^{-9} M concentration [154]. They extended their method for analysis of excitatory amino acids and further developed by coupling microdialysis to continuous flow derivatization [155,156]. Later on they also measured GABA [157] and vigabatrin [88] concentration in microdialysate and elaborated an on-line device for continuous sampling, derivatization and injection to CE [158]. Recently they have also developed in-capillary derivatization using sandwich method, although this approach has been accompanied by impaired detection sensitivity [159]. On-line derivatization of microdialysis sample with NDA has also been described by Zhou et al. with 10^{-7} M detection limit for glutamate and aspartate [160]. Subminute separation of 17 amino acid derivatives following on-line continuous derivatization and periodic injection of microdialysate has been performed by Shou et al. They have reported LOD in the 10^{-8} M range and demonstrated the applicability to real samples where 0.1–3 μM analyte concentrations were found [161]. On column derivatization has been also used for single cell analysis [162,163] as well as analysis of a single secretory vesicle [164] and subcellular compartments [165]. Phosphorylated amino acids in protein hydrolysate have been determined as their NDA derivatives at the submicromolar level, however the reproducibility of the method has been presented at much higher concentrations [166]. Peptides have been measured in various samples at micromolar concentrations; enterostatin in cerebrospinal fluid [167] and substance P metabolites in brain microdialysate [168]. A moderate enhancement of maximum fluorescence signal of NDA derivatives has been observed using separation buffers containing cyclodextrins [98]. Using this type of buffer additives allowed the separation of enantiomers of amino acids [163,165,169,170] and the chiral drug, baclofen [171,172]. The reported quantification limits for the enantiomer separations were about 10^{-7} M. Glutathion can be very selectively derivatized with NDA in the absence of cyanide, as in addition to the derivatizable amino group it also contains a thiol group serving as the co-reagent needed for the ring closure. The fluorescence spectrum of the derivatization product has shifted to the higher wavelength range allowing the use of Argon-ion laser for excitation. Changes in glutathion content of cultured cells on oxidative stress could be measured in 10^{-7} to 10^{-6} M concentration range [173].

As a rapidly reacting and fluorogenic agent NDA can be used for post-column labeling as well. This way of derivatization allows the separation of analytes in their unlabeled form that can be particularly useful when the charge of the compound is lost by derivatization or multiple labeling of proteins results in broad or multiple sample peaks. At the same time, post-column derivatization requires specific instrumentation and the achievable sensitivity is usually inferior compared to pre-column labeling. The reported detection limits for brain metabolites of substance P were 74–100 and 2.5–26 nM with post- and pre-column labeling, respectively [168,174]. Problems deriving from multiple labeling of proteins [175] and aminoglycosides [176] could be avoided by performing post-column labeling. Microchip-based integration device for cell-immobilization, electrophoretic separation and post-column derivatization has been described for monitoring dopamine release from PC 12 cells, however the reported LOD value of 70 μM is far from competitive [177].

Several light emitting diodes with wavelength between 405 and 425 nm have been used for fluorescence detection of NDA derivatized compounds. The first application of a violet LED (peak emission at 410 nm) as excitation source was published in 2003

for the measurement of NDA labeled dopamine. The detection limit was far behind the previously reported ones using HeCd laser, but using preconcentration with sweeping, near 2 orders of magnitude improvement could be achieved [178]. Couderc and co-workers have used a 410 nm diode laser and found that it provides comparable detection sensitivity to HeCd laser, while it is less expensive and has a much longer lifetime. They have determined catecholamines and amino acids in microdialysate samples at low nanomolar concentration levels [179]. Wang et al. used a laboratory-built instrument and found the 425 nm wavelength of the violet diode as optimal for the detection of NDA labeled amino acids. They have presented the applicability for serum and cerebrospinal fluid samples at 10^{-7} M levels [180]. Chang and co-workers have introduced a 405 nm LED for detection of NDA labeled amino acids [181,182] and proteins [183], and acceptable detection limits in the low nanomolar range could be achieved by using sample stacking with poly(ethylene oxide). Octopamine in human plasma has been determined by using a similar LED, and 10^{-8} M quantitation limit has been reported after a ten fold concentration during sample clean-up [184].

Although there are some applications of OPA for CE-LIF determination, the unfavorable spectral characteristics (excitation in the UV range), the expensive laser source and the limited stability of the labeled analytes results in poor detection sensitivity. Based on these disadvantages better options are available and chosen to use.

3.5.3.2. Red and near infrared dyes. Fluorescence detection in the far red and near infrared wavelength range is claimed to be ultrasensitive due to the low background noise deriving from the high selectivity and lack of light scattering. However the excess of derivatizing reagent and its hydrolysis products as well as the simultaneously labeled components of biological samples can cause interference similarly to other labeling agents. The traditional gas laser sources are now largely replaced by the less expensive diode lasers. LIF detection in this wavelength range was first introduced and is used mainly in the field of nucleic acid analysis [185]. Only a couple of applications for determination of small molecules or proteins in biological samples has been reported. Cyanine based dyes are used for labeling of primary and secondary amino groups. Similarly to CFSE they possess a succinimidyl ester as reactive moiety providing appropriate reactivity to ensure rapid derivatization at room temperature. Amantadine in human plasma has been determined after liquid–liquid extraction providing off-line sample concentration and derivatization with Cy5.29. Quantification in the 10^{-8} to 10^{-6} M range has been demonstrated [186]. Simultaneous determination of residues of various aminoglycosides in bovine milk could be performed following sample clean-up with SPE and labeling to Cy5 derivatives. Formation of multiple derivatives has been found. The precision of the method has been demonstrated at 10^{-7} M concentration levels after 5 fold off-line sample preconcentration [187]. The same group has reported sensitive determination of herbicides in soil samples using Cy5 for derivatization [188]. The near infrared dye MeCy5 has been applied for derivatization of polyamines for their determination in human erythrocytes. Careful optimization of separation conditions was important because of the multiply labeled polyamines, which could be analyzed in the 10^{-8} to 10^{-6} M range [86].

3.5.4. "Recombinant labeling"

A special labeling method has been presented by Yoon et al. The fusion protein of the analyte, ERK2 and green fluorescent protein has been recombinantly expressed in cells. Following cell lysis the phosphorylated and dephosphorylated ERK2 could be separated and detected [189,190].

3.5.5. Immunoaffinity capillary electrophoresis

Specific sample clean-up can be achieved by immunoaffinity capturing the analyte after or before labeling. Using this method the interfering sample components and reagent excess can be removed before separation. Applying before sample derivatization trace amount of analytes can be concentrated allowing the improvement of derivatization efficiency. Philips and co-workers have extensively studied the possibility of immunoaffinity sample clean-up. They have covalently bound the antibodies to a portion of the inner surface of the capillary and after selective extraction the analytes are washed, eluted and separated. They have analyzed various cytokines and other inflammatory mediators in cell secrete [191–193], body fluids [194,195] and tissue samples [196] as well as intracellular regulatory proteins in cell extracts [197] and brain-derived neurotrophic factor in skin biopsies [198]. Chip-based system has been designed for assessing hormones in body fluids [199] and alpha-fetoprotein in serum samples [200]. These assays have been found providing fast, accurate and precise measurements.

4. Concluding remarks

Following an enthusiastic period, the era of real evaluation of CE-LIF technique has arrived. The method could not fulfill the expectation of becoming a routinely applied technique in clinical laboratories or pharmaceutical industry. This is justified by the limited number of real applications compared to HPLC methods. The reasons are the relatively high cost of instrumentation, the lack of ready to use applications and the need for careful design of method development requiring skilled and experienced analyst. However, being aware of the advantages and shortcomings of CE-LIF, it can find its place as an important and valuable complementary analytical technique.

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