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Journal of Chromatography A, 807 (1998) 3–26

JOURNAL OF
CHROMATOGRAPHY A

Review

Derivatization in capillary electrophoresis

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Abstract

In recent years capillary electrophoresis (CE) has been developed into a versatile separation technique, next to gas and liquid chromatography (LC), well suited for the determination of a wide variety of e.g., pharmaceutical, biomedical and environmental samples. The main advantages of CE over chromatographic separation techniques are its simplicity and efficiency. It is well recognized, however, that the sensitivity and selectivity of the detection are relatively weak points of CE. One way to overcome these limitations is the conversion (derivatization) of the analytes into product(s) with more favourable detection characteristics. Although, in principle, almost any detection mode can be combined with a derivatization procedure, in practice, fluorescence monitoring is favoured in most cases. This paper aims to give a short overview on the various reagents that can be used for pre-, post- and on-column derivatization in CE. First, a short introduction is given on CE as an analytical technique, followed by a discussion of the pros and cons of the various modes of derivatization, a comparison of derivatizations in CE with derivatizations in LC, the principles of fluorescence and prerequisites for a good fluorophore and the potential of using diode lasers in combination with a labelling procedure. With respect to the derivatization reagents the emphasis is on the labelling of amino, aldehyde, keto, carboxyl, hydroxyl and sulfhydryl groups.
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Keywords: Reviews; Derivatization, electrophoresis; Amines

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PII: S0021-9673(98)00230-1

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1. Introduction

A comparison between liquid chromatography (LC) and capillary electrophoresis (CE) shows that CE methods, in general, are faster, possess a better reproducibility of the migration times and an accuracy which is comparable [1–3]. In contrast, the injection precision, peak area reproducibility and concentration sensitivities are sometimes unfavourable compared with LC. Because in CE, capillaries with relatively small internal diameters are used the mass detection limits are rather low (values in pmol to fmol range are reported), but because of the small detection volume (nl range) the resulting concentration detection limits are relatively high [4,5]. This means that improvement of the detectability is still the major challenge in CE.

So far, detection in CE is mainly performed by using ultraviolet (UV) absorbance detection, but because of the relatively short optical pathlength the sensitivity is limited. An increase of the detectability can be obtained by using either fluorescence, laser-induced fluorescence (LIF), chemiluminescence or electrochemical detection. The fact that a laser beam can be perfectly focused on a small surface, for example a capillary, means that this technique is the most promising detection mode in CE. The limitation of the above mentioned sensitive detection principles is their inherent selectivity which means that frequently a derivatization reaction should be incorporated into the analytical procedure [6,7]. By incorporating a derivatization procedure and LIF detection, concentration detection limits in the zmol range have been described [8].

Although, in principle, almost any detection mode can be combined with a derivatization procedure, normally fluorescence (or LIF) monitoring is favoured. The obvious reason is that fluorescence detection is more selective and sensitive than UV absorbance detection, is applicable in a wider variety of solvent systems compared with electrochemical detection and is somewhat more robust than chemiluminescence and electrochemical detection. An additional problem using electrochemical detection can be contamination of the electrodes when complex matrices should be analyzed.

1.1. Capillary electrophoresis

CE separations are based on the different electrophoretic mobility of analyte ions present in an electrophoretic medium which is inside of a narrow-bore capillary [9].

CE can be performed in various modes. The most frequently applied modes are: capillary zone electrophoresis (CZE); capillary gel electrophoresis (CGE); micellar electrokinetic capillary chromatography (MECC); capillary electrochromatography (CEC); capillary isoelectric focusing (cIEF) and capillary isotachophoresis (cITP).

The existence of these different modes is due to the fact that most CE techniques and principles have been developed by combining one or more chromatographic and electrophoretic techniques. While in most cases liquids (aqueous separation buffers) are used as the separation medium, in some cases a gel, a chromatographic sorbent or a coating is present in the capillary in addition to the separation buffer [8].

Free solution CE, or CZE, nowadays is the most commonly applied technique in electrophoresis. In this case separation of the solute ions is based on their charge-to-mass ratio. The separation of neutral

molecules is normally performed using MECC, in which separation is due to the difference in distribution of analytes between an aqueous eluent and an micellar pseudo-stationary phase.

One of the most important differences between CZE and LC is the plug flow profile in CZE, because of the electroosmotic flow, versus the parabolic flow profile in pressure-driven systems like LC. The result is a significantly increased efficiency in free solution CE systems, with the inherent advantages of reduced band broadening and improved resolution.

1.2. Derivatization reactions, why, how and when?

Although improved detectability is an important reason to perform a derivatization procedure, there can be other reasons as well. These goals include (i) improved separation from interfering compounds, (ii) improved electrophoretic behaviour (peak shapes), (iii) prevention of decomposition during the separation procedure or (iv) more convenient sample preparation. Contrary to LC, improvement of the separation by means of an electrophoretic process is an important reason. This because mainly charged compounds can be efficiently separated by CE, which means that neutral components have to be manipulated prior to the separation. This manipulation can be achieved via a derivatization procedure introducing a charged moiety into the analyte.

The second important reason is, as mentioned above, improvement of the detectability by converting the analyte into a highly detectable product. For this purpose compounds are labelled with a chromophore, electrophore or fluorophore.

Two types of derivatization reactions can be distinguished: labelling and non-labelling reactions [10]. In labelling procedures a covalent bond is formed between the analyte and the labelling reagent. All other types of reactions, e.g., ion-pair formation, photolysis, redox and electrochemical reactions are called non-labelling reactions.

Another classification of derivatization reactions is based on their place in the final analytical set-up: before (pre-column), during (on-column) or after (post-column) the electrophoretic separation. The most suitable approach depends on the reason why a derivatization procedure is introduced, the number of samples that have to be analyzed, the physicochemical properties of the analyte and of the reagent, etc. Contrary to LC where the pre-column mode is the most popular and the post-column approach is the less popular, in CE the on- and post-column modes are also of importance. It should be realized that post-column and on-column derivatization reactions are invariably carried out in the on-line mode, which makes these techniques well suited for use in automated systems. Pre-column techniques, on the other hand, are normally employed in the off-line mode. The on-column approach is rather favourable in combination with CE, and this can be explained by the fact that in this case no interfering interactions with the separation sorbent are possible, while in the post-column mode the reagents can be added by means of simple devices without the need for additional pumps or other advanced equipment.

The main advantages and disadvantages or pre- and post-column derivatization procedures have repeatedly been discussed in the literature. Therefore, they are only briefly outlined here. The advantages of post-column derivatization techniques are: (i) the formation of side products plays a minor role, the reaction does not need to be complete and the reaction products do not need to be stable. The only requirement is reproducibility. (ii) The analytes are separated in their original form, which often permits the adoption of known separation conditions.

On the other hand, there are also disadvantages, which are: (i) the limited choice of reaction conditions with respect to both the solvent composition and the reaction time. (ii) The requirement of additional equipment, especially for reagent addition. (iii) The extra-column peak broadening caused by the reactor which may diminish the chromatographic resolution. (iv) The presence of a large excess of reagent possessing the same detection properties as the derivatives.

When working in the pre-column mode, the main advantages are: (i) a relatively free choice of the reaction conditions with, e.g., the possibility to select solvents which are not compatible with the CE system. (ii) The virtual absence of restrictions with respect to the reaction kinetics, provided a single derivative is formed with the analyte. (iii) The relative ease with which multi-step operations can be performed.

The main disadvantages of pre-column procedures

are: (i) the formation of side products and derivatives of other sample constituents, which is especially detrimental in trace level studies. (ii) The ''open'' nature of the, often, multi-step procedures, which can cause interfering peaks in the electropherograms due to, e.g., contamination of the sample from the laboratory air or solvent constituents, analyte losses during evaporation of the solvent or by adsorption to the walls of the vials used, as well as a loss in sensitivity due to the injection of aliquots only.

With respect to pre-column derivatization procedures it should be mentioned that these reactions can be performed either in the off-line (manual) or in the at-line (automated) mode. Using this approach not only the detectability of the analyte(s), but also the separation properties will be changed. This means that, for example, the peak shape, peak height, efficiency, selectivity, resolution will be changed either positively or negatively [11].

The majority of the probing reactions in CE are performed in this mode because of (i) the large flexibility in optimizing in the optimum reaction conditions, (ii) the fact that the conditions during the derivatization should not be compatible the electrophoretic buffer, (iii) the availability of a wide variety of labelling reagents, and (iv) the fact that no complex instrumentation is needed [12,13]. Disadvantages of this approach are that in a number of cases the excess of the probe should be removed from the reaction mixture before the actual separation and that the derivatives are not always sufficiently hydrolytic or thermally stable [13]. However, contrary to derivatizations in LC, separation of the derivatives from the excess of the probe is normally not a major problem, because in CE separation is not primarily based on the polarity of the molecule but on the charge-to-mass ratio.

The advantages of post-column derivatization procedures are outlined above, but the most important ones are that the reaction only should be reproducible and that multiple products may be formed. The result is that this type of labelling is normally applied when non-stable derivatives are formed or when the analyte contains multiple derivatization sites [12,13].

The inevitable disadvantages of post-column reactions are that (i) the choice of the reaction conditions is rather critical and should be compatible with the required separation conditions, the (ii) reaction times are relatively short which means that the detectability normally is lower in comparison with pre-column reactions, that (iii) special equipment is necessary, and that (iv) the detection properties of the derivatives should be different than of the reagent [12].

Several post-electrophoretic reactor designs have been described [14]. In most cases the fluid-gap reactor type is applied. Other approaches are the sheath-flow cuvette, the free-solution reactor and reactor designs based on a pressure driven introduction of the derivatization reagent through coaxially coupled capillaries or cross connectors. Because of the extremely low flow-rates used in CE no pumps are needed to add the different reagents, hydrostatic pressure normally is sufficient to add the reagents. However, reproducibility and robustness of these devices is still somewhat disappointing.

The latest development applying derivatization procedures in CE is the use of on-column approaches. In principle, the front-end of the capillary is used as the reaction chamber. The result is that, in comparison with the post-column mode, sample dilution is reduced to a minimum. As a result, this approach is especially suitable for the determination of analytes present in extremely small sample volumes, such as single-cell analysis [14].

In spite of these positive features there are also some limitations. The derivatization reaction should be fast and quantitative, no interfering side products should be formed and the derivatives should be stable. So far this procedure is mainly applied in combination with fluorescence detection and *o*-phthaldialdehyde (OPA) as the derivatization reagent. Fluorophoric reagents have the disadvantage of native fluorescence and unless the excess reagent is separated from the derivatives – because of a difference in electrophoretic mobility – removal of the excess of the probe is necessary before detection can be performed. Because this is rather difficult in the on-column mode, mainly fluorogenic reagents, not possessing native fluorescence, are applied.

1.3. Principles of fluorescence and prerequisites for a good fluorophore

The theory and applications for the determination

of organic solutes by means of fluorescence detection have been described, for LC, in numerous textbooks [15,16]. Therefore, only a brief survey concerning those aspects that are important in fluorescence derivatization, for CE, is presented here.

Fluorescence is the emission of light accompanying the transition of an electronically excited molecule in a singlet state to its electronic ground state (S_0). The excitation of a molecule is achieved by the absorption of a light quantum of an appropriate wavelength promoting, in a simplified view, a π or non-bonding (n) electron to a π^* anti-bonding orbital. The process of an excited molecule returning to its ground electronic state usually starts with a vibrational relaxation and internal conversion to the lowest excited singlet level (S_1). However, only a limited number of molecules arriving in the S_1 state will emit fluorescence light, because of the competition of radiation-less decay processes, i.e., internal conversion and intersystem crossing. In other words, for fluorescence to be dominant, the rate constant for radiative transition should be large relative to those for non-radiative transitions, i.e., the molecules possess a high fluorescence quantum yield (ϕ_f). Hence, to be a good fluorophore, a molecule should not contain functional groups or structural features that enhance the rates of these radiationless transitions. In addition to a high ϕ_f, a molecule should possess a high molar absorptivity (ε).

In general, parent aromatic hydrocarbons and their heteroatomic analogues which possess a rigid planar structure, fulfil these requirements. Their fluorescence is strongly influenced by the presence of certain functional groups and by the nature and degree of their interaction with the solvent(s) used [16]. Although some rules can be derived for the influence of substituents on the fluorescence of the compounds mentioned above, it should be noted that there are many exceptions and that it is difficult to provide a complete set of rules. Aromatic hydrocarbons and their heteroatomic analogues with electron donating, e.g., $-NH_2$, –NHR, $-NR_2$, –OH, $-OCH_3$ functional groups, seem to be favourable, since the presence of these functional groups normally results in a shift of the excitation maximum to longer (more selective) wavelengths, as well as to a higher ϕ_f [17]. On the other hand, electron withdrawing groups, e.g., –CHO, –COOH, $-NO_2-$, –F, –Cl, have a negative effect on the fluorescence ϕ_f. Furthermore, heavy atoms such as bromine and iodine will cause a higher intersystem crossing rate and, as a result, a diminished fluorescence sensitivity. For fluorescence derivatization purposes it will be clear that a reactive group must be present, that can be used for derivatization with the analyte.

With respect to the influence of the solvent(s), dipolar and hydrogen-bonding interactions are particularly important [18]. The effects can be dramatic; for instance, the emission wavelength of the dansyl derivative of phenylalanine is 490 nm and 550 nm in chloroform and water, respectively. Furthermore, the fluorescence intensity decreases by a factor of ca. 50, going from chloroform to water [19]. Coumarin derivatives of carboxylic acids, on the other hand, are only strongly fluorescent in aqueous solvents [20]. Another possibility to increase the fluorescence sensitivity is to transfer the fluorescent solutes to a micellar environment by applying micellar separations. In LC this has lead to 50-fold improved detection limits [21] or to more favourable excitation and emission wavelengths [22]. The fluorescence properties of fluorophores containing acidic or basic functional groups can be strongly dependent on the pH of the solvent. For instance, *N*-(1-naphthyl)ethylene diamine, which can be applied for the labelling of carboxylic acids, is only fluorescent between pH 4 and 9 [23].

Changes in temperature affect the number of collisions of the molecules of the fluorophore with solvent molecules and, hence, the bimolecular decay processes. For most compounds, a 1–2% decrease in fluorescence per 10°C increase in temperature is observed.

1.4. (Diode) laser-induced fluorescence detection

LIF nowadays is accepted as a selective and sensitive detection mode in LC and CE. The principles of LIF have been explained in a previous monograph in this series [24] and some recent reviews [25–27].

In particular in miniaturized systems, like CE, laser-based techniques have some advantages. This because the fluorescence signal is not only proportional to the detection pathlength, as in absorbance,

but also to the irradiance (W/cm^2). This explains why in CE a gain of four-orders of magnitude can be obtained comparing laser-based with conventional techniques, just by focusing the beam on a spot small enough to fit inside a CE capillary. In LC this gain normally is less than 100-fold. However, it should be kept in mind that the absolute laser power is certainly not the most critical parameter. For example, detection limits of 10^{-8} M, in CE, can be obtained using an excimer laser with an average laser power of 1 W, which results in 100 mW after passing a dye lase and 5 mW after frequency doubling, and by using only 10% of the light to prevent damage of the capillary.

The positive features of LIF, using visible or UV lasers, are selective excitation, small detection volumes and high signals. The most important limitations are that this technique is limited to fluorescing compounds and that the traditionally used lasers are expensive and relatively unstable.

Recently, infrared (IR) and near-IR diode lasers have become available [6,7,28]. Diode lasers, emitting radiation with wavelengths over 620 nm, are stable (intensity fluctuations <0.02% and with feedback stabilization <0.003%), cheap (< US$100.00 for a 10 mW laser emitting at 670 nm), small (<10 mm^3 for the total package) and have a lifetime of over 25 000 h. In addition, background signal due to fluorescence from optical impurities in the eluent is negligible and the contribution of scatter to the background signal is relatively small. Rayleigh and Raman scatter are proportional to λ^{-4}, and as a result 50-fold lower at 670 nm compared with 250 nm. This means that applying excitation at 670 nm, Raman scatter of eluent components (e.g., acetonitrile, methanol, water) is primarily observed at wavelengths over 800 nm.

The main limitation is that the applicability, using wavelengths over 600 nm, is even more limited than by applying visible or UV lasers. The result is that a combination of diode laser-induced fluorescence (DIO-LIF) and a pre-column or post-column derivatization procedure allows the labelling of non-absorbing compounds and this by using a red- to near-IR absorbing label which means almost no matrix interferences, but the limitation is that at the moment hardly any commercial labels are available.

2. Derivatization reactions in capillary electrophoresis

2.1. Introduction in fluorescence derivatization

Because in combination with CE mainly fluorescence detection and covalent labelling reactions have been applied so far, only those reactions in which a non-fluorescent compound is converted into a fluorescent derivative will be overviewed. In these type of derivatizations, a reactive function of the label reacts with a specific group of the analyte.

When choosing a suitable label for a certain application, criteria playing a decisive role are: (i) the required reaction conditions. The analyte must be stable under these conditions and the sample should easily dissolve in the required reaction solvent. When an organic solvent has to be used, most biological, as well as many environmental samples, usually cannot be subjected directly to the derivatization. (ii) The occurrence of side reactions. This plays a role mainly in pre-column labelling reactions, because side products may cause interfering peaks in the chromatogram. (iii) The fluorescence sensitivity of the derivative. (iv) The difference in spectroscopic properties between the label and its reaction products. This is especially important in post-column reactions, because of the fact that the label, present in a large excess compared with the analyte, can cause an undesirably high fluorescence background. (v) The purity, stability, commercial availability, toxicity and price of the label. In most on-line post-column derivatization procedures, reagent consumption is relatively high and the use of expensive labels should be avoided.

In an ideal situation, a derivatization reaction proceeds rapidly (seconds to minutes) under mild conditions (room temperature), forming a single, stable and highly fluorescent derivative, whereas the label itself is non-fluorescent. It is obvious that it is almost impossible to fulfil all these requirements in real analytical situations, which explains the existence of many labels for the derivatization of analytes with one specific functional group [11].

For the fluorescence labelling of amines OPA, naphthalene-2,3-dicarboxyaldehyde (NDA) (primary amines) and 9-fluorenylmethyl chloroformate

(FMOC) (primary and secondary amines) are suitable labels, since they react within 1 min at room temperature under basic conditions in aqueous environment. In addition, they are commercially available, their derivatives are intensely fluorescent and show large Stokes-shifts. OPA derivatives however, are normally not very stable and should be analyzed immediately after derivatization of the sample. Because OPA itself is non-fluorescent, post-column labelling of primary amines forms a useful alternative.

Maleimides are suitable labels for thiols. When the reactions are carried out at a pH of about 8, they are particularly selective, because the reaction with amines usually requires a higher pH. Furthermore, maleimides are not appreciably fluorescent until after reaction with thiols. *N*-(7-Dimethylaminoamino-4-methylcoumarinyl)maleimide and *N*-(1-pyrenyl) maleimide react fast at room temperature. From the thiol selective benz-2-oxa-1,3-diazoles, only 4-fluoro-7-(*N*,*N*-dimethylaminosulfonyl)-benz-2-oxa-1,3-diazole probably is a fast reacting probe. The bimanes monobromotrimethyl-aminobimane (water soluble) and monobromobimane (MBBr), also are interesting labels for thiol modification, showing fast reactions at room temperature, while the labels possess no native fluorescence.

Fluorescence labelling of carboxylic acids with alkyl halides usually requires the use of organic solvents and phase transfer, because the reactivity in aqueous solution is very low [29]. Alkyl bromides, e.g., panacyl-Br and 4-bromomethyl-7-methoxycoumarin, are commonly applied. A disadvantage of these reagents is their poor selectivity. Diazo labels generally are not very pure and stable (e.g., 9-anthryldiazomethane), react slowly and may form side products. Hence they are not the labels of choice for the derivatization of carboxylic acids. More selectivity can be obtained with indirect labelling procedures. In indirect derivatization procedures, the carboxylic acid function is first activated by a carbodiimide, viz. dicyclohexyl-cardodiimide, or the water-soluble *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride, or 2-bromo-1-methylpyridinium iodide and is subsequently reacted with a fluorescent alcohol or amine [30].

Derivatization of alcohols usually is carried out in aprotic solvents and reactions are base catalyzed. Generally, the selectivity is rather poor and reaction kinetics are not fast. In fact, FMOC or its analogue (+)/(−)-1-(9-Fluorenyl)ethyl chloroformate (FLEC) (for chiral analytes), which are also rather reactive towards amines, may be a good choice for alcohol derivatization; an aqueous medium (pH of about 10) can be used and the reaction is very fast, even at room temperature.

Hydrazine labels, e.g., 5-dimethylaminonaphthalene-1-sulfonylhydrazine (DNSH) and 4-hydrazino-7-nitrobenz-2-oxa-1,3-diazole, are used for labelling of aldehydes and ketones, but aldehydes are far more reactive. Interesting labels are 1,3-cyclohexadione and 1,2-diamino-4,5-methylenedioxybenzene, which are non-fluorescent until after reaction and are extremely selective towards aldehydes and α-dicarbonyls, respectively.

2.2. (Diode) laser-based fluorescence derivatization

The combination of a covalent derivatization procedure using a red-absorbing, and fluorescing probe, offers interesting possibilities to improve both the selectivity and sensitivity of an analytical procedure. However, nowadays the number of organic solutes with adequate fluorescent properties is rather limited and are hardly commercially available. Furthermore, the spectroscopic properties of the labels and the corresponding derivatives are about the same, which means that only pre-column derivatization procedures can be performed [28].

Indirect detection was one of the first applications of red-absorbing labels, since until recently hardly any chromophores/fluorphores were available with suitable functionalities that could be applied for pre-separation derivatization purposes in either LC or CE [28]. When indirect detection is applied DIO-LIF probably is the best choice, since diode lasers have all the characteristics of a perfect light source in the red or near-IR region, while interferences from matrix components are strongly reduced in this wavelength region.

A second possibility of using this type of probes is by forming ion-pairs, which is an example of a non-labelling derivatization procedure. For example, charged solutes can be extracted into an organic

phase after the addition of a near-IR absorbing compound as the counter ion. Compounds that can be used for this purpose are rhodamine 800, oxazine 750, thionine, nile blue, or methylene blue. Detection limits that can be obtained are in the region of $1 \cdot 10^{-7}$ *M* [31].

For pre-column derivatization purposes mainly (dicarbo)cyanine and squaraine derivatives have been synthesized and applied so far [28]. These solutes have excitation maxima of 620–720 nm, emission maxima of 640–780 nm, molar absorptivities of at least 100 000 L/cm mol and quantum yields of fluorescence (ϕ_f) of over 0.1 in water–acetonitrile and water–methanol mixtures.

Large shifts in the excitation maxima can be obtained by extending the polymethine chain in a cyanine with one double bond. Dicarbocyanines have excitation maxima at ca. 670 nm, while tricarbocyanines have excitation maxima close to 780 nm. Smaller spectral shifts can be obtained by changing the substituents on the heterocyclic rings [X,Y=O, S or $C(CH_3)_2$].

2.3. Post-column derivatization

The main problem in post-column derivatization in CE is peak broadening because the separation capillary should be connected with a reagent capillary (reactor) and because one or more reagents should be added. In most cases either a pressure or a voltage driven device is used for reagent addition [32]. In both cases the residence time of the analyte in the reactor determines the amount of band broadening, and this parameter is influenced by the laminar flow and differences in the mobility of the free and derivatized analyte. A general conclusion given by Zhu and Kok [32] is that to minimize band broadening the conditions should be chosen in such a way that a high reaction rate is obtained and that narrow or short reaction capillaries are used.

The problem of mixing in a post-column CE reactor is of a different dimension compared with mixing in a post-column LC reactor. Because of the relatively small internal diameter of the capillaries used, normally mixing by means of a diffusion process is sufficient. Just like in LC a number of reactor devices are in use in CE. The most frequently applied reactors are: (i) coaxial reactor, (ii) gap reactor, (iii) free solution reactor and (iv) sheath-flow reactor.

An overview on the advantages and limitations can be found in a review paper of Zhu and Kok [3].

3. Derivatization of amines

A wide variety of reagents are available for the derivatization of the amines. In particular the labelling of primary and secondary amines is well described in the literature. Just like in LC, the tagging of amines can be performed, as mentioned before, in the pre-, on- and post-column mode. Contrary to LC, in CE mainly amino acids, amino sugars, peptides and proteins are derivatized prior to absorbance or fluorescence detection. An overview of the most frequently applied derivatization reagents, the most suitable separation and detection modes the analytes that can be labelled, the sensitivity, and the sample in which the analytes are present is given in Tables 1–4. In all Tables the limit of detection (LOD) values are calculated assuming an injection volume of 1 nl.

3.1. Pre-column derivatization of amines

In Table 1 the reagents for pre-column, and mainly off-line, derivatization reactions are summarized. With respect to the LOD values it should be taken into consideration that in some cases these values are determined by derivatizing the analytes at a relatively high concentration and subsequent dilution of this mixture. Normally derivatization reactions can be performed quantitatively at concentrations of 10^{-8} *M* or higher, but at lower concentration most reactions are far from quantitative. Interesting features from this Table are that sensitive peptide mapping of relatively large proteins using benzoin, fluorescamine and OPA has been performed using either CZE or MECC. Furthermore, it can be seen that the number of ‘‘real life’’ applications still is rather limited.

In the next paragraphs some of the procedures mentioned in Table 1 will be discussed in somewhat more detail.

Isothiocyanates react with primary and secondary amines forming thioureas which can be detected

Table 1
Pre-column derivatization of amines

Reagent	CE mode	Detection	Compounds	LOD	Ref.
AEQC	CZE/MECC	UV	Amino acids	150 amol	[33]
	CZE/MECC	LIF	Amino acids	0.1 amol	[33]
AQC	CZE	UV	Synthetic peptides	15 fmol	[34]
	CZE	F	Synthetic peptides	10 fmol	[34]
Benzoin	CZE	LIF	Arginine containing peptides	270 amol	[35]
CBQCA	MECC	LIF	Amino acids	9 zmol	[36]
	MECC	LIF	Pepides	5–70 amol	[37]
	MECC	LIF	Amino sugars	240 amol	[38]
	CZE/CGE	LIF	Amino acids		[39]
	MECC	LIF	Amine containing compounds	5–900 amol	[40]
	MECC	LIF	Aminated sugars	75 zmol	[41]
CTSP	CZE	LIF	Amino acids	5 amol	[42]
DCC	MECC	LIF	Amino acids/small peptides	0.1 amol	[43]
DCCS	CZE	LIF	Amino acids	100 amol	[44]
FITC	CZE	LIF	Amino acids	zmol range	[8] [45]
	CZE	LIF	Peptides		[46]
Fluorescamine	MECC	LIF	Peptides		[37]
	CZE	LIF	Marine toxins		[47]
	CZE	LIF	Amino acids	25 fmol	[48]
	MECC	F	Polyamines	200 fmol	[49]
	CZE	UV	Amino acids/peptides/ proteins		[50] [51]
	MECC	UV/LIF	Proteins	100 amol	[52]
FMOC	CZE/MECC	F/LIF	Amino acids	1 fmol	[48]
	MECC	LIF	Amino acids	0.2 amol	[53]
	MECC	LIF	Secondary amino acids		[54]
IDA	MECC	UV	Amino acids	2 fmol	[55]
NBD-F	MECC	LIF	Dipeptides	70 amol	[56]
NDA	CZE	LIF	Amino acids	12 amol	[57]
	NGSCE	UV/LIF	Proteins	10–60 amol	[52]
	CZE	LIF	Jeffamine polymers	58	
	CZE	ED	Amino acids	40–90 fmol	[59]
	CZE	UV	Amino acid neuro-transmittors	40–60 fmol	[60]
	CZE	UV	α-Difluoromethylornithine	7.5 fmol	[61]
OPA	MECC	UV	Peptides	62	
	CZE	LIF	Marine toxins	0.1 amol	[47]
	CZE	LIF	Amino acids	80 amol	[57]
	NGSCE	UV/LIF	Proteins	5–100 amol	[52]
	CZE/MECC	F	Amino acids	300 fmol	[63]
OPA/FMOC	MECC	LIF	Amino acids	54	
PITC	CZE	UV	Amine containing compounds		[64]
TRITC	MECC	LIF	Amino acids	1 zmol	[65]

Table 2
Post-column derivatization of amines in combination with fluorescence detection

Reagent	Detection	Reactor	Compounds	LOD	Ref.
Fluorescamine	UV/F/LIF	Fluid gap	Amino acids	30 fmol	[48]
NDA	F	Fluid gap	Amino acids/ peptides/proteins		[69]
	F	Membrane gap	Peptides		[70]
OPA	UV/F/LIF	Fluid gap	Amino acids	5 fmol	[48]
	F	Coaxial	Amino acids/ peptides	amol	[71]
	LIF	On-column	Amino acids	fmol	[72]
	LIF	Coaxial	Myoglobine	45 amol	[73]
	F	Free solution	Glycine		[74]
	UV/LIF	Fluid gap	Amino acids/	10–200 amol	[75]
	LIF	Coaxial	Amino acids/proteins /catecholamines	5–100 amol	[76]
	F	Porous tube	Amino acids	100 amol	[32]

Table 3
Post-column derivatization of amines in combination with chemiluminescence detection

Reagent	Chemiluminesence system	Reactor	Compounds	LOD	Ref.
ABEI	Luminol	Capillary	Amines		[77]
		Free solution	Amino acids		[78]
ABEI	Isoluminol	Sheath flow	Amino acids	40 amol	[79]
IITC	Isoluminol	Sheath flow	Amino acids	500 amol	[79]
TCPO	Peroxyoxalate	Capillary	Dns-amino acids	1 fmol	[80]
	Peroxyoxalate	Capillary	Labelled proteins	200 amol	[81]

either by absorbance or fluorescence detection. The most popular reagent in this category is fluorescein isothiocyanate (FITC) because it can used in combination absorbance, conventional-induced fluorescence and LIF detection using the 488.0 nm emission line of the argon-ion gas laser. Using LIF detection LOD values in the zmol range can be obtained for amino acids. In addition to FITC, tetra-

Table 4
On-column derivatization of amines

Reagent	CE mode–detection	Compound	Details	LOD	Ref.
NDA	cITP–LIF	Amino acids	Automated on-column reaction cell		[82]
	CZE–LIF	Dopamine/ amino acids	Single mammalian cells	0.2–5 fmol/cell	[83]
	CZE–LIF	Aspartate/ glutamate	In vivo in rat brain	5 fmol	[84]
OPA	cITP–LIF	Amino acids	Automated on-column reaction cell		[82]
	MECC–LIF	Amino acid enantiomers		130 amol	[85]

methylrhodamine isothiocyanate (TRITC) can also be used for LIF detection. Labelling of amino acids with this probe results in LOD values in the zmol range [65]. Especially in the case of FITC the pH of the running buffer is of major importance for the sensitivity, since this reagent is only fluorescent at relatively high pH values.

Another isothiocyanate that has been used is phenyl isothiocyanate (PITC). PITC is the classical reagent used in the Edman degradation and the derivatives can be detected by UV absorbance detection. To ensure single labelling of the *N*-terminal α-amino group of lysine containing peptides, the solutes are taken through a single cycle of the Edman degradation reaction [46]. Therefore, the peptides are first reacted with PITC resulting in a labelling of all primary and secondary amino groups. During the subsequent Edman degradation step the *N*-terminal amino acids are cleaved, whereafter the unreacted primary amino groups are derivatized with FITC. Finally the derivatives are determined by means of CZE–LIF. PITC has been applied also for the derivatization of compounds with a reactive amino group resulting from a Maillard reaction [64].

TRITC and FITC are modified Edman degradation reagents. TRITC strongly absorbs in the green part of the spectrum and emits in the red. This reagent was used by Zhao et al. [65] in combination with MECC–LIF for the labelling of amino acids. The LOD values were in the low zmol range.

Succinimidyl esters are used in combination with diode laser excitation. These lasers have some advantages over solid-state or gas lasers in LIF detection. Because they emit in the red or near-IR part of the spectrum, scattering and background fluorescence is limited, and they are rather small, stable, cheap.

A few red-absorbing labels have been developed for the labelling of primary and secondary amines followed by diode-LIF detection. Labels that have been described are 7-(diethylamino)coumarin-3-carboxylic acid succinimidyl ester (DCCS) [44], 9-cyano-*N*,*N*,*N*′-triethyl-*N*′-(5′-succinimidyloxycarbonylpentyl)pyroninchloride (CTSP) [42] and a label with a dicarbocyanine fluorophore and a single succinimidyl functionality (DCC) [43]. Due to hydrolysis, the reaction product formed immediately degrades into two products: the fluorescent derivative and an *N*-hydroxysuccinimide. The fluorescence of the latter does normally not interfere with the fluorescence of the derivative [34]. A major limitation of this type of derivatization is the relatively large amount of reagent that is needed to obtain a quantitative reaction. This is caused by hydrolysis of the succinimidyl ester in an aqueous solution, especially at higher pH values, and the limited reactivity of the ester. However, detection limits in the amol range have been observed.

De Antonis et al. [34] used a fluorescent succinimidyl ester, 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AQC) for the derivatization of prothrombin leader peptides. Applying either UV absorbance detection (185 nm or 254 nm) or fluorescence detection with and excitation wavelength of 250 nm (emission at 395 nm) resulted in LOD values of about 10 fmol.

Derivatization with cyanine-based labels in combination with CE is rather promising for the quantitation of amino acids and small peptides. Despite the bulky derivatization reagent, 18 amino acids can be separated at the amol level after labelling with a dicarbocyanine label containing the succinimidyl ester functionality, but the derivatization should be performed at concentrations of $2.5 \cdot 10^{-7}$ *M* or higher.

This because in aqueous solutions hydrolysis of the label competes with the derivatization procedure [43]. The MECC separation is performed with a 75 cm×33 μm I.D. capillary, a potential of 30 kV and a run buffer of methanol–20 m*M* borate buffer (pH 9.0) (28:72, v/v) containing 17.5 m*M* sodium dodecyl sulphate.

Not only amino acids can be separated in this way, but also small peptides (e.g., enkephalins). A positive feature of CE over LC is that in this case, depending on the pH of the run buffer, all tyrosine-containing enkephalin fragments can be found in a particular part of the electropherogram [43]. Even labelled Leu- (Tyr–Gly–Gly–Phe–Leu) can be separated from Met-enkephalin (Tyr–Gly–GLy–Phe–Met).

Tyramine (4-hydroxyphenethylamine) is an indirectly acting sympathomimetic amine found in cheese, fermented foods and red wine [66]. The relative standard deviation of the determination of $5 \cdot 10^{-6}$ *M* tyramine in urine is 4.1% ($n=5$) using the same analytical procedure as described for peptides and amino acids.

Chloroformates react rather rapidly with both primary and secondary amines forming stable carbamates. A well-known representative of this group of labels is FMOC [48,53,54]. Other chloroformates are 2-(9-anthryl)ethyl chloroformate (AEOC) [33] and FLEC [67]. After MECC separation and LIF detection the LOD of a number of FMOC derivatives of amino acids is less than 5 fmol [48]. Another nice feature of chloroformate labels is that they can be used for the separation of enantiomers, both for direct and indirect chiral separation.

By far the most frequently applied derivatization reagent for primary amines is OPA [47,48,52,57,62,63]. Dialdehydes or aroylaraldehydes react relatively fast with primary amines resulting in highly fluorescent isoindoles (Fig. 1). Since these reagents are normally fluorogenic, they can be applied in the pre-, on- and post-column mode.

OPA reacts quantitatively with primary amines, within 30 s, in the presence of a reducing agent, such as 2-mercaptoethanol or another thiol [63]. The only problem in using OPA is the limited stability of some of its derivatives because of oxidation and/or photodecomposition. Although the choice of the thiol is of some influence on the stability, another fluorogenic reagent NDA can be used to improve the stability [52,57–61]. An additional advantage of NDA over OPA is that a longer excitation wavelength should result in an improved signal-to-noise ratio for the derivatives.

The most recently introduced isoindole-forming fluorogenic reagent is 3-(4-carboxybenxoyl)-2-quinoline carboxaldehyde (CBQCA) [36–41]. This reagent forms rather stable derivatives and can also be used in combination with LIF detection. The detection limits of some amino acids after MECC–LIF are ca. 10 zmol [36].

In addition to the reagents discussed above there a number of other agents that have been applied in combination with a pre-column CE derivatization procedure. Fluorescamine, or 4-phenylspiro[furan-2(3H)-1′-phthalan]-3,3′-dione, is a widely used fluorogenic reagent in CE [37,47–52] as well as in LC. It provides highly fluorescent pyrrolinones only after reaction with primary amines, while secondary amines do not react. The reaction is rather fast (<1 s) in aqueous solutions and the excess of the reagent is hydrolyzed into a non-fluorescent product. Because of the instability of most of the derivatives, this procedure is in particular suited for on- and post-column derivatization reactions.

Benzoin can be used for the selective labelling of arginine-containing peptides as well as for peptide mapping [35]. 2-Mercaptoethanol is used to stabilize the derivatives and sodium sulphite is added to

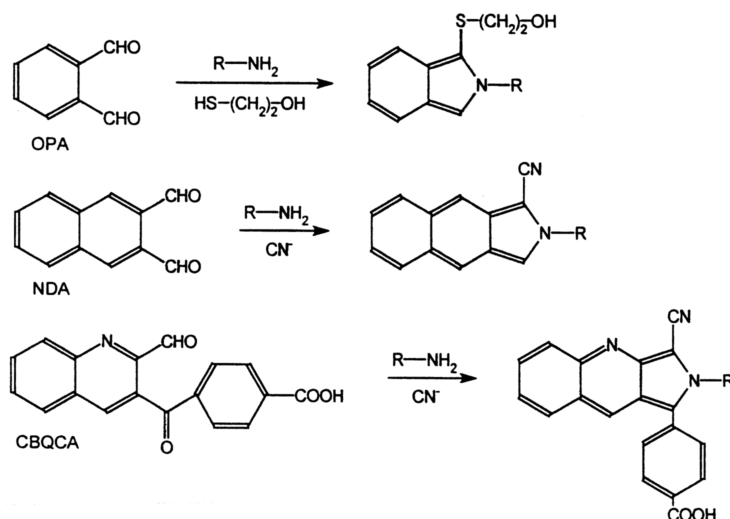

Fig. 1. Derivatization of primary amines with aldehydes derivatives forming isoindole derivatives.

suppress the background fluorescence. The derivatives can be detected either by UV absorbance or by LIF detection resulting in LOD values of 270 amol.

In order to find a derivatization reagent suitable for the detection of dipeptides with an argon-ions gas laser (output wavelength is 488 nm) a number of fluorophores – i.e., FITC, CBQCA, 5-carboxyfluorescein succinimidyl ester, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) – can be used. Of these reagents, NBD-F gave a good response combined with a relatively short derivatization time and a rather clean electropherogram [55]. However, in order to obtain a quantitative reaction the label should be present in a large excess and the reaction mixture should contain at least 15% of 2-propanol. The sensitivity can be improved by adding a micellar agent to the background electrolyte. The chiral purity of amino acids can be determined by means of a comparable procedure [68]. The indolizine label 1 - methoxycarbonylindolizine - 3,5 - dicarbaldehyde (IDA) has been synthesized for the derivatization of primary amines [55]. The derivatives can be detected by means of fluorescence detection and are stable for at least 24 h. Wright et al. [47] tried to separate polar marine toxins with CZE–LIF after derivatization with DNSH. Although the separate toxins gave a single sharp peak, a mixture of different toxins could not be separated. However, the fluorescamine and OPA derivatives of these toxins could be separated rather easily.

3.2. Post-column derivatization of amines

The number of reagents that react sufficiently fast to be used in the post-column mode is rather limited (Tables 2 and 3). OPA, NDA and fluorescamine are the most frequently applied tagging reagents in this mode. In the pre-column mode the instability of the derivatives can be a problem, but in the post-column mode this is of far less importance.

A number of post-column reactor designs have been used in combination with OPA labelling reactions. The principle of the fluid-gap reactor is based on diffusion for the addition and mixing of the derivatizing reagent and the analyte(s) [48,75]. Mixing is performed in a 25 μm gap formed in a 10 μm I.D. (285 μm O.D.) capillary.

The created gap reservoir (1.5 ml) is filled with the derivatization reagent by means of a syringe, and the connecting reaction and separation capillaries are filled independently with the running buffer. The LOD for human transferrin after labelling with OPA is 5 amol. This means that the detection limits obtained with this system are comparable with those obtained with OPA after a pre-column labelling. In addition to this reactor device, two more or less related set-ups are described: the coaxial capillary reactor [71,73] and the on-column capillary reactor [72]. A last possibility that has been described is the use of a free-solution reactor for the labelling of glycine with OPA [74].

An interesting application is the determination of proteins in a single cell by means of a coaxial post-column reactor [76]. This reactor consists of two narrow-bore capillaries: a separation capillary with an I.D. of 15 μm and a reaction capillary with I.D. of 30 μm. Grounding of the electric field was performed in the reagent container and the negative voltage was applied to the outlet buffer container. The latter container was used to add the OPA reagent. Using this procedure human erythrocytes can be isolated from whole blood samples and introduced into the separation capillary. The LOD values for catecholamines, amino acids and proteins are in the range of 5 to 100 amol using LIF detection.

In addition to OPA its structure analogue NDA can be used. The most important advantages of using NDA over OPA are the excitation wavelength of 488 nm of the derivatives which matches the He–Cd laser and its relatively fast reaction with peptides. A limitation may be the decreased solubility in aqueous solutions. A combination of NDA and mercaptoethanol has been used for the determination of substance P in a microdialysate [70] (Fig. 2). In this case of membrane-gap reactor is used. The most important conclusion for this study is that the reaction is relatively fast for lysine-containing peptides and relatively slow for non-lysine containing peptides.

Chemiluminescence can also be applied in combination with a CZE separation. The advantage of this detection mode is that no external light is needed, which means a simpler set-up and an improved signal-to-noise ratio because of a lower background signal. On the other hand, mixing of the analyte and

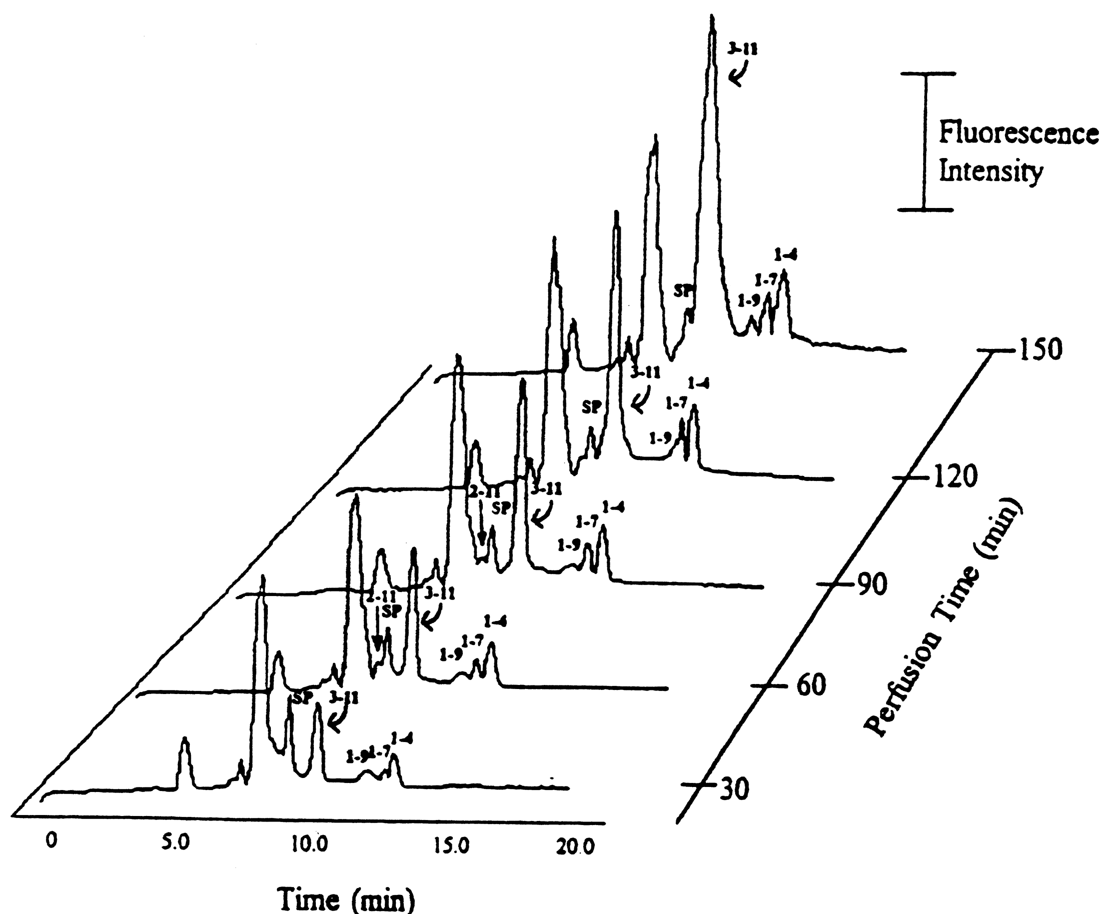

Fig. 2. Electropherograms of microdialysis samples obtained after perfusion of the striatum with substance P. Substance P was labelled with NDA using a membrane-gap reactor [70].

the reagent streams is rather critical and consequently the flow system is rather complex. The use of a sheath-flow cuvette provides satisfactory results, but the detection limits are in the high amol range.

The two most frequently applied chemiluminescence systems are the luminol and the peroxyoxalate reaction systems. An interesting example is the determination of ATP which is labelled with *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) [78]. The required hydrogen peroxide is added via a free-solution reactor. In a slightly different procedure using a sheath-flow cuvette and isoluminol isothiocyanate as the labelling reagent the detection limit of amino acids is about 40 amol [79].

The peroxyoxalate system has been applied for the quantitation of amino acids which are, for example, labelled with dansylchloride [80] or proteins labelled with TRITC [81].

The limitations of the peroxyoxalate system are that most peroxyoxalates are hardly soluble in aqueous solutions and that analyte should be derivatized with a suitable fluorophore before detection can be performed. A disadvantage of both the luminol and the peroxyoxalate systems is that relatively large volumes and/or high reagent flows should be used which results in significant band broadening.

3.3. On-column derivatization of amines

The latest development in derivatization procedures for CE is the use of the on-column approach.

Especially in the case when only small sample volumes are available (e.g., single-cell analysis) this seems to be an interesting mode because the whole procedure can be automated and continuous monitoring is possible. In this set-up the front-end of the capillary is used as the reaction chamber. The analyte and reagents plugs are injected separately and are mixed because of their different electrophoretic mobilities. The most pronounced positive feature is the small volume of the reaction chamber, which means that dilution is minimum. Just like in post-column labelling reactions the disadvantage is that, in principle, only fluorogenic reagents can be used. In Table 4 a short overview is given of the reagents that have been applied so far for the on-column derivatization of primary amine containing solutes.

Glutathione and a number of amino acids are determined using an automated on-column reaction cell [82]. A combination is made between the preconcentration potential of ITP and the separation efficiency of CZE. The analyte(s) are concentrated in the focusing step, derivatized with either OPA or NDA in the ITP step and subsequently separated in the CZE step using LIF detection.

This approach has also been used for the enantiomeric separation of D- and L-amino acids [85]. A buffered sample and a mixture of the derivatization reagent OPA and a chiral thiol, which are both dissolved in acetonitrile, are injected consecutively into the capillary as two discrete plugs. Due to differences in the mobility of the plugs on-column mixing occurs. The result is a derivatization, separation (MECC) and detection (LIF) of amino acids in a single procedure with a sensitivity in the order of 150 amol (Fig. 3).

One of the few real applications is described for the determination of dopamine and some amino acids in single mammalian cells [83]. In the first part of the capillary the cell is lysed and derivatized with NDA. After completion of the labelling reaction the derivatives are separated by CZE and detected by LIF. Solutes from individual rat pheochromocytoma cells can be quantitated at the fmol–amol level.

A development that certainly will gain in popularity is the combination of CZE–LIF with a microdialysis sampling system [83]. This combination of techniques allows the real-time or near real-time analysis of endogenous compounds (e.g., aspartate, glutamate) in anaesthetized or awake, free moving animals. The beauty of this combination of techniques is that no special interfaces are needed because the flow-rates are compatible.

The system consists of a microdialysis probe, an on-line reactor, a simple injection interface and a detection unit. Combining this set-up with an NDA-based tagging procedure results in LOD values of about 5 fmol.

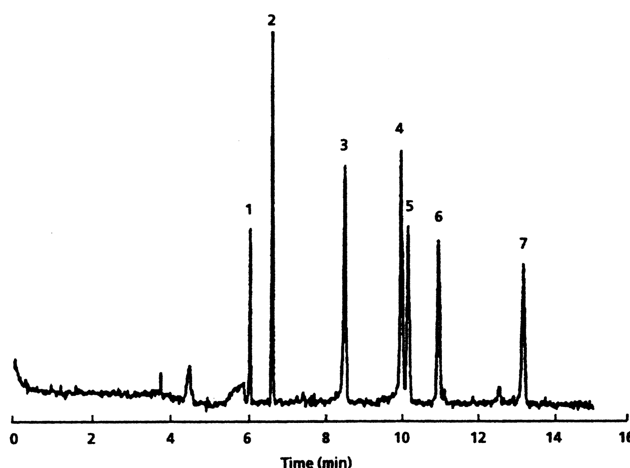

Fig. 3. MECC separation of OPA/TATG on electrophoretically derivatized amino acids using an additional stacking procedure after injection of the sample and reagent (TATG=2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose) [85].

4. Derivatization of reducing compounds

The derivatization of the reducing aldehyde and/or keto groups in carbohydrates can be performed in several ways (i.e., reductive amination, condensation with 1-phenyl-3-methyl-5-pyrazolone). An overview of the most frequently applied procedures is given in Table 5. So far, only pre-column labelling procedures have been described for reducing agents in combination with an electrophoretic separation.

4.1. Reductive amination of reducing compounds

Reductive amination can be performed in two ways: (i) derivatization of the reducing group with the amino function of an reagent followed by

Table 5
Derivatization of reducing groups

Reagent	CE mode–detection	Compound	LOD	Ref.
ABN	CZE/MECC–UV	Aldoses/ketoses/uronic acids	1 fmol	[86]
AC	MECC–LIF	Sugars/polysaccharides		[87]
	MECC–LIF	Branched oligosaccharides		[88]
7-ANDA	CZE–UV	Malto-oligosaccharides		[89]
	CZE–LIF	Oligosaccharides		[90]
2-/5-ANSA	CZE–UV	Malto-oligosaccharides		[89]
ANTS	CZE–LIF	Oligosaccharides		[89]
	CZE–UV/LIF	Malto-oligosaccharides		[91]
	CZE–LIF	Charged oligosaccharides		[92]
AP	CZE–LIF	Oligosaccharides		[87]
	CZE–UV	Monosaccharides	10 pmol	[93]
	CZE–UV	Malto-oligosaccharides		[94]
	CZE–UV/F	Oligosaccharides		[95]
	CZE–UV	Oligosaccharides		[96]
	CZE–UV	Saccharides		[97]
APTS	CZE–LIF	Mono/oligosaccarides	2 pmol	[98]
AQ	CZE–LIF	Charged oligosaccharides		[91]
	CZE–UV	Oligosaccharides		[95]
CBQCA	CZE–LIF	Mono/oligosaccharides	0.5 amol	[99]
	CZE/CGE–LIF	Aldose oligosaccharides		[100]
	CGE–LIF	Oligosaccharides	85 fmol	[101]
EPAB	CZE–UV	Saccharides	5 fmol	[96]
	CZE–UV	Aldoses/ketoses/uronic acids	10 fmol	[102]
PABA	CZE–UV	Saccharides	15 fmol	[103]
	CZE–UV	Aldoses/ketoses/uronic acids	15 fmol	[104]
	CZE–UV	Monosaccharides	15–30 fmol	[105]
PMP	CZE–UV	Aldoses		[106]
	CZE–UV	Monosaccharides		[107]
	CZE–UV	Unsaturated disaccharides		[108]
TRSE	MECC–LIF	Monosaccharides	17 zmol	[109]

reductive amination to a secondary amine. (ii) Reductive amination of the reducing group followed by derivatization with a primary amine.

4.1.1. Derivatization of reducing compounds followed by reductive amination

The general reaction scheme of a labelling procedure followed by a reductive amination is given in Fig. 4. First, the reducing group reacts with the primary amino group of the label forming a Schiff base. The next step is reduction of the Schiff base to a secondary amine using sodium cyanoborohydride forming a stable end product. This procedure is the most frequently applied procedure for the derivatization of carbohydrates and other reducing polyalcohols. This can also be found in Table 5, where all reagents except CBQCA and 1-phenyl-3-methyl-5-pyrazolone (PMP) react via this reaction principle.

Using on-column UV absorbance detection at 240 nm allows the detection of aldoses and uronic acids, at the low pmol level, after derivatization with 2-aminopyridine (AP) [84]. A representative electropherogram after labelling reducing sugars is given in Fig. 5. The disadvantage of AP is, however, that only solutes containing a free aldehyde function can be derivatized.

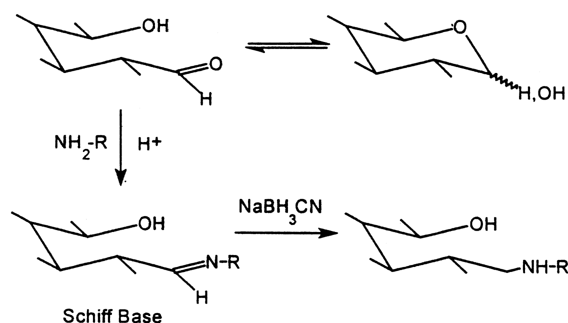

Fig. 4. Derivatization of a reducing agent with the amino group of a reagent followed by reductive amination to a secondary amine.

More recently, labelling reagents like *p*-aminobenzoic acid (PABA) [97,104,105], ethyl-*p*-aminobenzoate (EPAB) [96,102], 2-aminoacridone (AC) [87,88] and *p*-aminobenzonitrile (ABN) [88] have been introduced also enabling the derivatization of

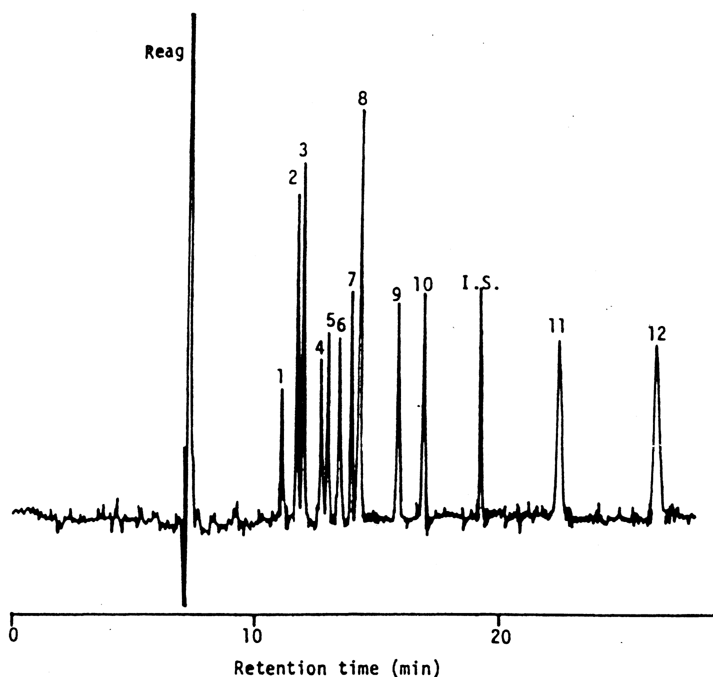

Fig. 5. Separation of pyridylaminated reducing sugars by CZE as borate complexes [93]. Peaks: Reag=reagent; 1=*N*-acetylgalactosamine; 2=lyxose; 3=rhamnose; 4=xylose; 5=ribose; 6=*N*-acetylglucosamine; 7=glucose; 8=arabinose; 9=fucose; 10=galactose; I.S. (internal standard)=cinnamic acid; 11=glucuronic acid; 12=galacturonic acid.

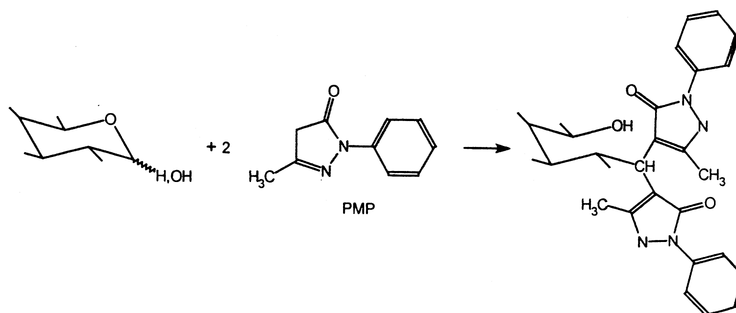

Fig. 6. Derivatization of aldehyde with 1-phenyl-3-methyl-5-pyrazolone (PMP).

ketoses. Normally carbohydrates are separated by means of CZE, but after derivatization with ABN or AC MECC is a good alternative because of reduced migration times and an improved selectivity [83]. A favourable feature of this procedure is that the excess of the fluorophore, which is not charged, can not interfere with the analysis because it is trapped inside of the micelle. The result is a significantly longer migration time of the probe in comparison with the migration time of the negatively charged derivatives [87,88].

4.1.2. Derivatization of reducing compounds after reductive amination

The second approach is derivatization of the analyte after reductive amination. CBQCA can be used to label reducing monosaccharides and oligosaccharides before a CZE separation and LIF detection [99–101].

Prior to a reaction with the fluorescent label, reductive amination of the reducing group is performed by means of ammonium ions in the presence of sodium cyanoborohydride. The resulting 1-amino-1-deoxyalditol reacts in the last step with the CBQCA in the presence of cyanide ions.

The derivatization of aldehydes, present in reducing carbohydrates, can also be performed by means of a condensation reaction between the active hydrogens of PMP and the aldehyde functionality using slightly basic conditions (Fig. 6). The formed bis-PMP derivatives can be separated with free-solution CE and UV absorbance detection [106–108]. Using the described procedure the detectability of glycosaminoglycans is in the order of 10 fmol.

By replacing sodium borate by alkaline earth metal salts the PMP derivatives of isomeric aldopentoses can be completely separated because of the difference in interaction between the various pentoses and the metal ions [107].

5. Derivatization of other functional groups

An overview of the most frequently applied reagents for the derivatization of functional groups, other than the amino group or reducing groups, is given in Table 6. In case the detection limits are not given in absolute amounts, they are calculated by assuming that the injection volume is 1 nl. As discussed before relevant information on the used separation and detection mode as well as information on the sensitivity is summarized for functionalities like the 1-chloroalkyl, carbonyl, carboxyl, dicarbonyl, hydroxyl and sulfhydryl group.

5.1. Derivatization of the 1-chloroalkyl group

The anticancer drug prospidin can be determined quantitatively in human papilloma using CZE after labelling of the analyte with diethyldithiocarbamic acid (DDTC) [110]. The disadvantage of this procedure is that a rather large excess of the reagent is required and that for a quantitative reaction about 1.5 h is needed. Another problem may be that prospidin, and its derivatives, will be adsorbed onto the capillary wall during the separation. To minimize this effect at least 50% of methanol is added to the running buffer and a pH of 11.2 is used. A positive feature is that using absorbance detection at 254 nm the LOD is about 5 fmol.

Table 6
Derivatization of various functional groups

Reagent	Function	CE mode–detection	Compound	LOD	Ref.
DDTC	1-Chloralkyl	CZE–UV	Prospidin	4 fmol	[110]
DNSH	Carbonyl	CZE–UV/F	Aldehydes/ketones	1 fmol	[111]
DNPH	Carbonyl	MECC–UV	Aldehyde sugars		[112]
MPD	Carbonyl	CZE–LIF	Tyr-containing peptides	400 amol	[36]
AAF	Carboxyl	MECC–LIF	Phenoxy acid herbicides	3 amol	[112]
7-ANDA	Carboxyl	CZE–UV/F	Acidic monosaccharides	1–5 fmol	[113]
		CZE–UV/F	Sialogangliosides		[114]
		CZE/F	Sialo-oligosaccharides	0.5 fmol	[115]
		CZE–UV/F/LIF	Phenoxy acid herbicides		[116]
PDAM	Carboxyl	CZE–LIF	Dicarboxylic acids	amol	[117]
SA	Carboxyl	CZE–UV	Acidic monosaccharides	10 fmol	[113]
	Carboxyl	CZE–UV	Sialogangliosides		[114]
OPD	Dicarbonyl	CZE–UV	Dicarbonyl sugars	0.1 pmol	[118]
DBTA	Hydroxyl	CZE–UV	Anticoagulant drugs		[119]
FLEC	Hydroxyl	CZE–UV/F	Carnitine		[120]
FMOC	Hydroxyl	CZE–UV/F	Carnitine		[120]
CY5.3a.IA	Sulfhydryl	CZE–LIF	Captopril	40 fmol	[121]
CY5,4a.IA	Sulfhydryl	CZE–LIF	Captopril	40 fmol	[121]
MBBr	Sulfhydryl	CZE–UV	Glutathion		[122]
SBD-F	Sulfhydryl	CZE–UV	Thiols	pmol	[123]
NDA	2-(Sulfhydryl-methyl)carboxyl	CZE–LIF	Glutathion	amol	[124]

5.2. Derivatization of the (di)carbonyl group

Hydrazines can react with carbonyls, using acidic conditions, forming fluorescent hydrazones. The most prominent representative of this group, for the labelling of aldehydes and ketones, is DNSH [111]. A comparison between absorbance and fluorescence detection, however, shows that in many cases fluorescence detection does not provide more sensitivity than UV absorbance detection. Another label that can be used is 2,4-dinitrophenylhydrazine (NDPH). The corresponding derivatives can be detected either electrochemically or by means of absorbance detection. The latter reagent was used by Deyl et al. [64], in combination with PITC derivatives, for the separation and characterization of Maillard reaction products of reactive amino groups (Fig. 7).

Tyrosine containing peptides can be derivatized selectively using the fluorescence probe 4-methoxy-1,2-phenylenediamine (MPD) [36]. In combination with LIF detection the LOD for enkephaline is ca. 400 amol, which corresponds with a gain in sensitivity of about four-orders of magnitude compared with LC with conventional fluorescence detection.

A selective labelling reagent for dicarbonyl compounds is *o*-phenylenediamine (OPD) [118]. In this case dioxo solutes are converted into their corresponding quinoxalines. Although the reaction takes 2 h at 40°C (pH) and the resulting quinoxalines should be extracted with *n*-butanol and recrystallised from ethanol, LOD values of ca. 100 fmol can be obtained. Especially for catecholamines this reaction, followed by fluorescence detection, is a good alternative for direct amperometric detection of the parent compounds.

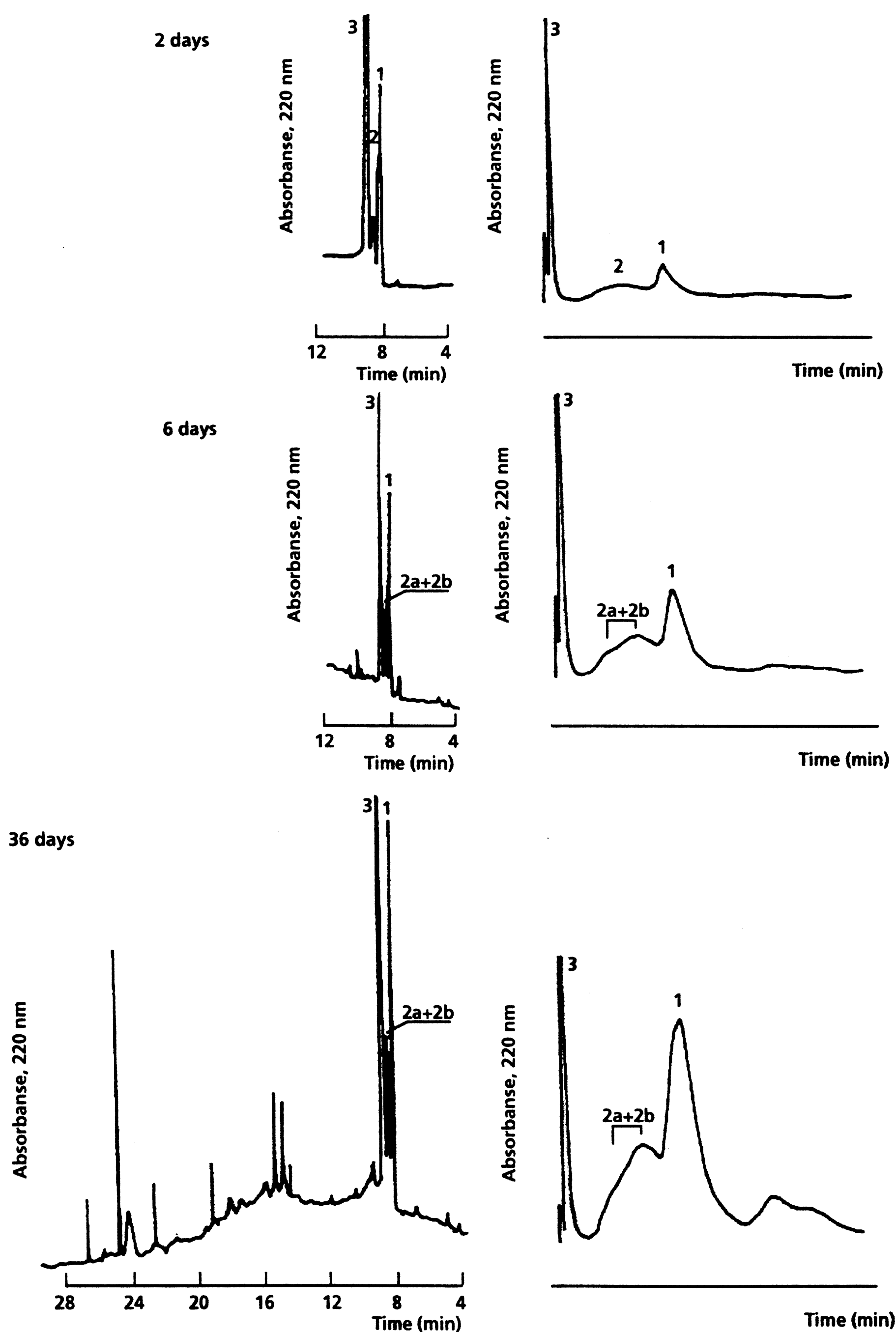

Fig. 7. Electrophoretic (left) and LC (right) profiles of ribose–glycine Maillard reaction products [64].

5.3. Derivatization of the carboxyl group

Carboxylic acids can be probed by means of a condensation reaction between the carboxyl group and an primary amine function of a suitable reagent (Fig. 8). During the reaction an amide bond is formed by an acid catalyzed removal of water in the presence of a carbodiimide. This reaction principle is

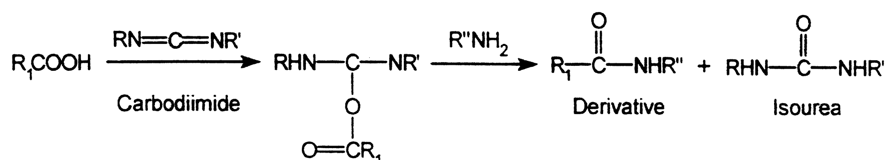

Fig. 8. Condensation reaction of carboxylic acids with primary amines in the presence of a carbodiimide.

used for the labelling of phenoxyacid herbicides with 7-aminonaphthale-1,3-disulfonic acid (7-ANDA) [116]. The resulting derivatives are separated by CZE using either absorbance or LIF detection. In the latter case, LODs are in the pmol to amol range. The separation of phenoxyacid herbicides with MECC has been described. All tested phenoxyacid herbicides are separated simultaneously with an LOD of approx. 3 amol.

The above described reaction mechanism is also used for the derivatization of acidic monosaccharides, sialoganggliosides and sialo-oligosaccharides derived from sialoganggliosides. In this case 7-ANDA [113–115] and sulfanilic acid (SA) [113,114] are used as the derivatization reagents.

The labelling of a dicarboxylic acid with 1-pyrenyldiazomethane (PDAM) can be performed without the addition of carbodiimide [117]. However, due to steric hindrance only the 1-pyrenylmethyl monoester is formed. Using CZE–LIF the detectability is in the sub-amol range.

5.4. Derivatization of the hydroxyl group

The enantioselective separation of D- and L-carnitine is of major importance because L-carnitine is important in the metabolism of long chain fatty acids and D-carnitine is toxic for biochemical processes. After a chiral derivatization with (+)-FLEC the diastereomeric derivatives can be separated with CZE [125]. (−)-FLEC [120] and FMOC can be used instead of (+)-FLEC. Using this last reagent cyclodextrins are used to separate the enantiomers.

5.5. Derivatization of the sulfhydryl group

In spite of the fact that LIF detection normally provides a better sensitivity than conventional fluorescence detection there are also, as mentioned before, some limitations. Some of these can be circumvented by using diode lasers, but the problem in using diode lasers is that only wavelengths over 630 nm are available. CI5.3a.IA and CI5.4a.IA are examples of red-absorbing cyanine-based labels that can be used, because of the presence of a iodoacetamido group, for the selective labelling of thiols (Fig. 9). Captopril, a specific angiotensin-converting enzyme inhibitor which is used for the treatment of congestive heart failure, can be probed with these reagents and after a straightforward CZE separation the LOD is about 40 fmol [121]. This reaction can also be used for the direct derivatization of thiols in urine and deproteinized plasma/serum samples. Separation is performed using a 85 cm×75 μm I.D. capillary. The run buffer consists of a mixture of methanol–100 m*M* borate buffer (pH 11.1) (50:50). A voltage of 27 kV is applied.

Captopril and some other thiol-containing solutes (e.g., glutathione, cysteine) can be derivatized with the fluorogenic reagent SBD-F (7-fluoro-2,1,3-benzoxadiazole-4-sulfonate) [123]. Both the reagent itself and the corresponding derivatives contain a permanently charge sulfonic acid groups which facilitates the electrophoretic separation between the excess of the probes and the derivatives.

A rather sophisticated application is the determination of glutathione in single human erytrocytes [122]. Using an in vitro micro-derivatization procedure with MBBr and CZE–LIF separation–detection, the detectability, of intracellular components, is at the low amol level.

The most interesting aspect of this derivatization

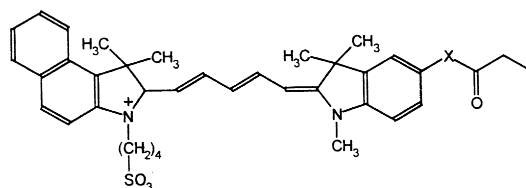

Fig. 9. Structures of CY5.3a.IA (X=NH–) and CY5.4a.IA (X=–CH_2NH–).

procedure is that the small and non-polar MBBr molecules can pass the semipermeable membrane of the cell, and as a result are labelled in the ''reaction vessel'' formed inside of the cell.

In addition to simple thiols 2-(sulfhydrylmethyl)carboxyl groups can also be derivatized. As discussed before, NDA, in the presence of cyanide ions, is used for the derivatization of primary amines. However, glutathione and its precursor (γ-glutamylcysteine) react rapidly with NDA, without the necessity of adding an additional nucleophile [124]. The in vitro labelling of thiols which are present in mammalian cells can be performed, by this reaction, under physiological conditions.

6. Abbreviations

AAF	5-(Aminoacetamido)fluorescein
ABEI	*N*-(4-Aminobutyl)-*N*-ethylisoluminol
ABN	4-Aminobenzonitrile
AC	2-Aminoacridone
AEOC	2-(9-Anthryl)ethyl chloroformate
7-ANDA	7-Aminonaphthalene-1,3-disulfonic acid
2-ANSA	2-Aminonaphthalene-1-sulfonic acid
5-ANSA	5-Aminonaphthalene-2-sulfonic acid
ANTS	8-Aminonaphthalene-1,3,6-trisulfonic acid
AP	2-Aminopyridine
APTS	9-Aminopyrene-1,4,6-trisulfonic acid
AQ	6-Aminoquinoline
AQC	6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
CBQCA	3-(4-Carboxybenzoyl)-2-quinoline carboxaldehyde
CE	Capillary Electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
cITP	Capillary isotachophoresis
CTSP	9-Cyano-*N*,*N*,*N'*-triethyl-*N'*-(5-succinimidyloxycarbonylpentyl)-pyronin-chloride
CZE	Capillary zone electrophoresis
DCC	Dicarbocyanine reagent
DCCS	7-(Diethylamino)coumarin-3-carboxylic acid succinimidyl ester
DDTC	Diethyldithiocarbamic acid
DNSH	5-(Dimethylamino)naphthalene-1-sulfonehydrazide (dansylhydrazine)
DNPH	2,4-Dinitrophenylhydrazine
EPAB	Ethyl-*p*-aminobenzoate
F	Fluorescence
FITC	Fluorescein isothiocyanate
FLEC	(+)/(−)-1-(9-Fluorenyl)ethyl chloroformate
FMOC	9-Fluorenylmethyl chloroformate
LC	Liquid chromatography
IDA	1-Methoxycarbanylindolizine-3,5-dicarbaldehyde
IITC	Isoluminol isothiocyanate
IR	Infrared
ITP	Isotachophoresis
LIF	Laser-induced fluorescence
LOD	Limit of detection
MBBr	Monobromobimane
MECC	Micellar electrokinetic capillary chromatography
MPD	4-Methoxy-1,2-phenylenediamine
NBD-F	4-Fluoro-7-nitro-2,1,3-benzoxadiazole
NDA	Naphthalene-2,3-dicarboxyaldehyde
NGSCE	Non-gel sieving capillary electrophoresis
OPA	*o*-Phthaldialdehyde
OPD	*o*-Phenylenediamine
PABA	*p*-Aminobenzoic acid
PDAM	1-Pyrenyldiazomethane
PITC	Phenyl isothiocyanate
PMP	1-Phenyl-3-methyl-5-pyrazolone
SA	Sulfanilic acid
SBD-F	7-Fluoro-2,1,3-benzoxadiazole-4-sulfonate
SP	Substance P
TATG	2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucpyranose
TRSE	5-Carboxytetramethylrhodamine succinimidyl ester
TRITC	Tetramethylrhodamine isothiocyanate
UV	Ultraviolet

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