CE/LIF from Apogee to Slow Cruising Speed, for a Nice Future

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In this short review, we will summarise through examples the most important applications developed in CE/LIF over the last twenty years for both small and large molecules. Though the number of publications using CE/LIF is decreasing, we will see that we can be confident in the developments of this technique in the future.

I. Introduction.

Laser induced fluorescence (LIF) or light emitting diode (LED) induced fluorescence (LEDIF) are well-known detectors for capillary electrophoresis (CE) studies. LIF was initially designed for micro HPLC detection, but since 1988 it was further developed and used for CE. Historically, LIF detection was first introduced as a CE detector, with different optical arrangements (collinear, orthogonal, sheath flow…) which were built in the laboratory, and the obtained sensitivity had a real importance, i.e. femtomolar detection was a goal. Then, LIF was presented as a detection method to obtain good performances in separation since samples could be diluted in water prior to injection, making sample stacking possible. CE/LIF was both selective and sensitive, and was overcoming the optical pathlength limits of the detector using UV-visible absorption. In consequence, many applications were developed, mainly for DNA separations or analysis of amino acids, proteins and sugars. Figure 1 presents the number of publication concerning CE over the last thirty years, as well the publications concerning CE/LIF and CE/LEDIF. CE began in the 90ies, with the first CE commercial instruments (Spectra Physics, Beckman). LIF took off a couple of years afterwards, first with the Beckman instrument, followed by the Zeta Technology one. After 2005, due to the introduction of high intensity LEDs, the number of CE/LEDIF publications increased. At this time, CE reached its apogee, even if a LEDIF detector was available (Picometrics). After a slow decrease in CE-focused publications, a cruising speed has now been found and the amount of CE/LIF or CE/LEDIF articles has been decreasing from 9% of the total amount of CE studies in the 2000s to 5% today. While it is a slow decrease, it remains less important than the decrease of CE studies since 2008, which shows that CE/LIF (or CE/LEDIF) stays proportionally important for analysts. This article will summarise the three main optical arrangements used in most of the publications, before examining the most important CE/LIF applications for sugars, amino acids, proteins and nucleic acids.

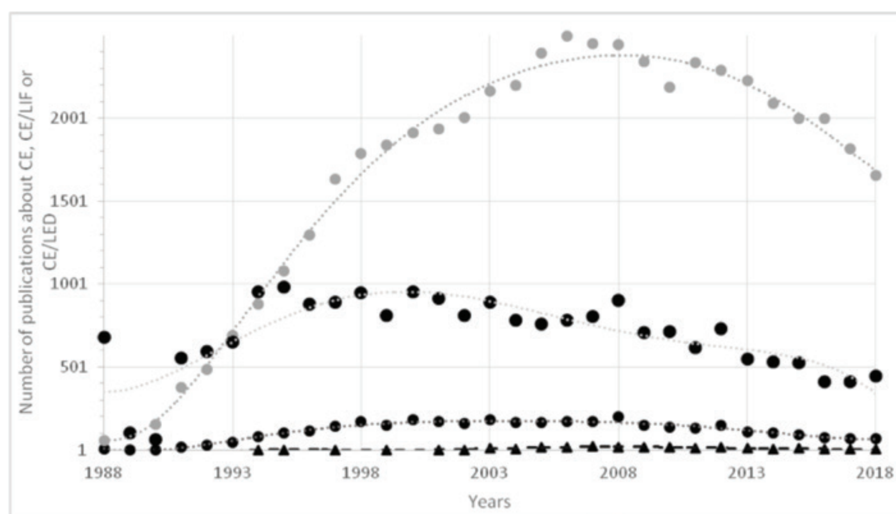


Figure 1: The number of publications containing CE (small grey dots), CE/LIF (small black dots) of CE/LED (triangles) in the title or the abstract (Source: Web of Knowledge). The graph shows that 2003 was the optimum of publications concerning LIF (maximum 180) while it is 2007 for LED (maximum 24). The big black dots presents the ratio (x10⁴) of CE/LIF or LEDIF versus CE.

II. The LIF detectors.

In the current applications, there are three main optical arrangements that are most frequently found:

a) The Sciex (formerly Beckman-Coulter) LIF arrangement.

As the first commercial instrument in the 90's it underwent many modifications. Currently, the excitation consists in one or two optic fibers (two if two excitation wavelengths are used) that illuminate the capillary. A parabolic mirror concentrates the fluorescent light emitted from the capillary in the direction of detection. The light is then collected by a ball lens and transmitted to a photo multiplier tube (PMT) (Figure 2 I), through a high pass filter.

b) The 'ball lens' ZETALIF confocal arrangement.

Described in 1995, the ball lens LIF detector [1] was developed as a collinear arrangement, where a 2 mm silica ball

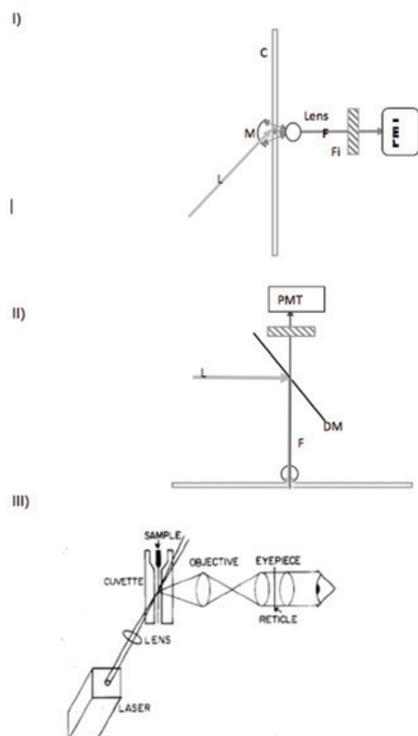


Figure 2: Optical schemes of the three main LIF detectors. I) Sciex arrangement (C capillary, F fluorescence, Fi filters, L laser, M Mirror), II) Ball lens arrangement (ZETALIF), C Sheath flow cuvette arrangement III) Sheath flow cuvette [5].

lens replaces the objective of a collinear microscope, i.e. a dichroic mirror and a high pass filter are used. The ball lens allows to focus the laser light inside to illuminate the capillary. Because of its high numeric aperture (higher than an objective), it collects a large part of the fluorescence emitted in volume ball lens/capillary. It is as sensitive as a 60x microscope objective but is much easier to work with as it is less sensitive to the position of the capillary in front of the laser beam (Figure 2 II). LED were adapted on this optical arrangement, thanks to the ball lens which concentrate the very divergent light of the LED emerging from the optical fibre inside the capillary without loss of sensitivity when compared to a laser [2]. LEDs have the advantage of a lower slow noise than lasers even though they have stronger rapid noise, which -after filtering this rapid noise with a 1s rise time- gives a better baseline than the laser ones, moreover LEDs are cheaper. This optical arrangement was also studied with UV-pulsed lasers [3], and capillaries containing a bubble in the detection window [4]. The larger irradiated volume allows a better detection sensitivity, which is limited by the photodegradation processes depending on the fluorescent dye.

c) The sheath flow cuvette arrangement.

It is the most sensitive LIF detector, developed by Zarrin and Dovichi in 1985 [5] and applied to CE in 1988 for FITC- labelled amino acid analysis. 6000 real molecules of FITC-Ala were detected [6]. Figure 2 III shows a diagram of this simplified detector which is an end column detector [7]. Ten years ago, the use of PDMS cuvette was attempted, which could make the industrialisation of this kind of scheme easier, since the moulding process can be readily adapted to fabricate multichannel detectors [8].

d) How to get a better LIF sensitivity?

The work with detectors was completed by studies on how to reach a better sensitivity with LIF detection. Mathies et al. [9] insisted on general mathematical expressions of the emitted photons by a fluorescent molecule as a function of the intensity and the duration of laser illumination. These equations could help to find the optimal expressions for detecting fluorescent molecule in the presence of ground-state depletion and photochemical reactions as photobleaching. Bayle et al. [10] illustrated this by describing the variation of fluorescence in a capillary, depending on the migration velocity. Johnson and Landers (2004) showed that all the optical schemes allow to use an excitation and emission volume that match to the separation system and keep scattering and luminescence background to a minimum [11]. A work concerning these approaches and all the reported literature was recently

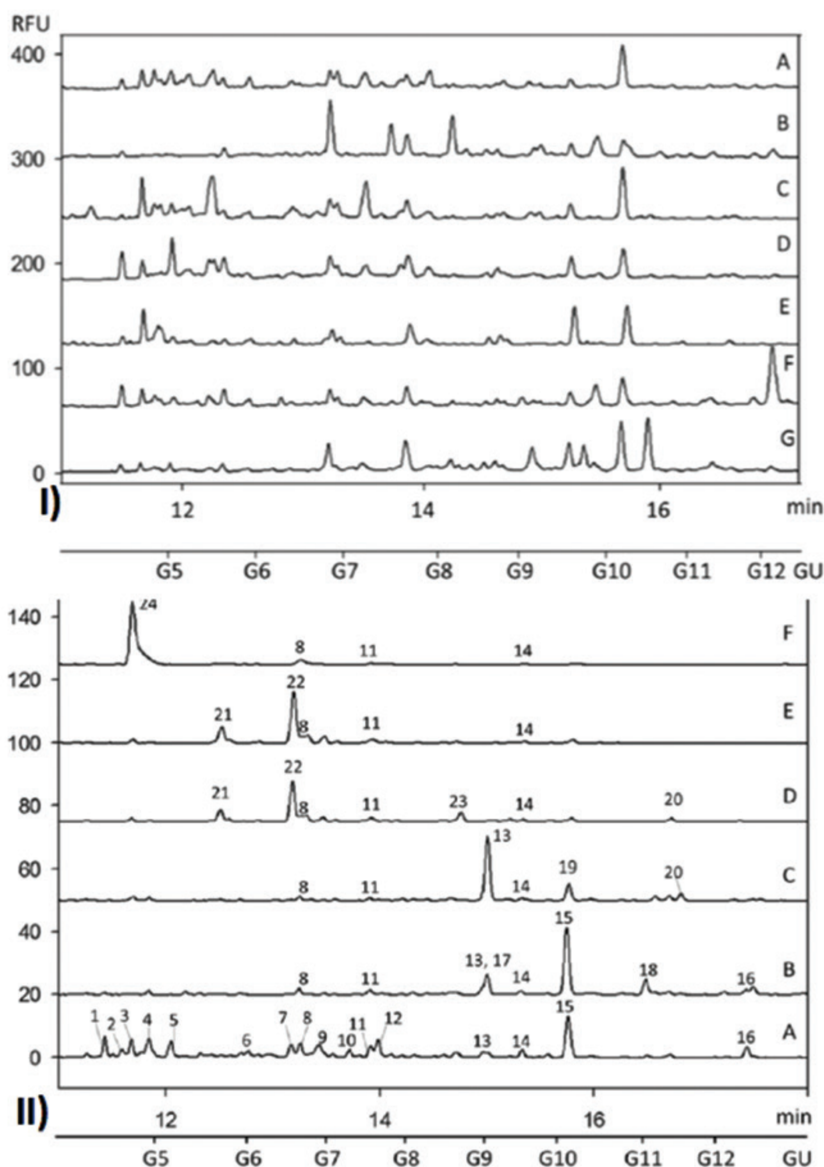
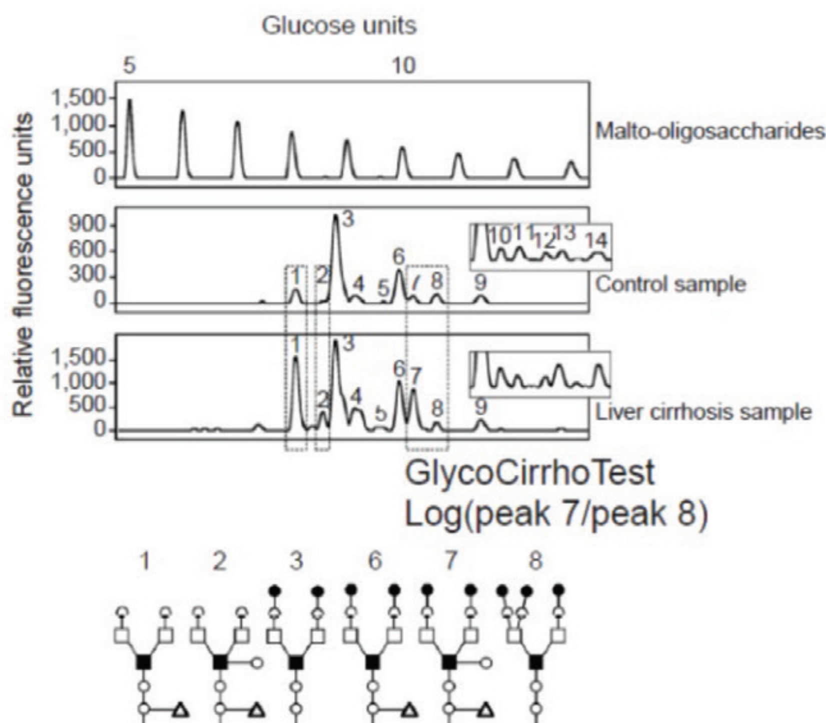


Figure 3: Separations of APTS labelled glycans. I): APTS N-glycans from formalin fixed paraffin embedded mouse tissue specimens. A lung, B brain, C heart, D spleen, E liver, F kidney, G intestine. Separation conditions 60cm NCHO separation capillary and the NCHO gel (both Sciex), 50 cm effective length 50 µm id) -30kV.The X scale id given function of migration times or the corresponding Glucose Units (GU) of maltooligosaccharides. II).Exoglycosidase array based sequencing of mouse lung N-glycans. A non treated, B sialidase, C sialidase + fucosidase, D sialidase + fucosidase+ galactosidase, E sialidase + fucosidase+ galactosidase+ galactosidase, F sialidase + fucosidase+ galactosidase+ galactosidase+ hexoaminidase reaction mixture treated. [23].



○, β-linked GlcNAc;●, β-linked galactose , □, α-linked mannose; ▪, β-linked mannose; Δ, α-1,6-linked fucose ,

Figure 4: Separations of APTS labelled glycans. Top, maltooligosaccharide reference. Middle, typical electropherogram of desialylated N-glycans derived from proteins in control serum sample. Nine peaks are clearly visible in the full detection range, with five more in the ×10 blow-up of the latter part of the electropherogram. Bottom, representative electropherogram obtained from cirrhosis case. Structures of N-glycans of relevance to this study are shown below the panels; peaks that are important for fibrosis/ cirrhosis markers are boxed [24].

published [12]. For LIF studies pulsed laser can be used. Two articles summarise the photodegradation processes using these high pulsated power lasers. The first mathematically describe the process of the pulsed photodegradation [13] and the second shows that photodegradation will drive to get non-linear calibration curves when the range of concentrations is of two decades [14].

III. The main applications.

Many applications are described in the literature; however, the focus will be on the ones considered most important.

a) Glycans.

The review articles of Mantovani et al. [15] and Lu et al. [16] underlined the use of LIF detection for glycan applications, particularly regarding the glycan part of proteins, especially for N-glycosylation. For these molecules, the different steps were greatly optimised. As an example, the first step for N-glycan release from the glycoprotein was run using immobilised PNGase F [17]. The obtained glycans are labelled by reductive amination, with a charged fluorophore containing a primary amine for example the 8-aminopyrene-1,3,6-trisulfonate (APTS)- which reacts with the aldehyde group at the reducing end of the glycan structures. The Schiff base thus formed is reduced with sodium cyanoborohydride to form a stable conjugate [18]. To minimise the loss of sialic acid, authors have proposed a simple protocol to label the glycans from 100 µg of a glycoprotein in a sample containing THF that evaporates slowly during the derivatisation at 60°C. Using these conditions, the authors demonstrated an increase of sialilated species by a factor two [19]. In another study, carboxyl-coated magnetic microparticles (COOH-beads) were reported to specifically bind polysaccharides and were used for a simple sample preparation for automated analysis. The excess of APTS was removed and the saccharides could be concentrated [20].

Recently, a catalytic hydrogen transfer from formic acid catalysed by water-soluble iridium (III)-phosphine complexes was proposed as an alternative to the cyanoborohydride [21] in an effort to prevent HCN formation.

One of the most impressive results on this matter is presented in Figure 3 I and II, APTS N-glycans from formalin-fixed, paraffin-embedded mouse tissue specimens, were analysed [22] and an attempt at glycome sequencing was realised using different exoglycosidases [23].

Earlier, Callewaert et al. [24] were able to help liver cirrhosis diagnosis by using CE/ LIF in quite the same conditions as the ones described above [25], the log of the ratio of peak 7 and 8 from Figure 4 allowing a diagnostic of the pathology.

Mass spectrometry can be used as detector after the CE/LIF system. To minimise the differences in migration times, the LIF detector can be connected just before the ESI source [26].

b) Amino acids and biogenic acids.

This application has been regularly reviewed since 2001, first by Prata et al. [27], then by Poinsot et al. [28]. Because most of amino acids (AA) are not native fluorescent, a labelling step is necessary. If all the AA and biogenic amines have to be studied, FITC or 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) can be used and the derivatives easily separated. Both labels are be excited with a 488nm Argon laser or a LED at 480 nm [29] to get the same sensitivity. The main limitations with FITC are its natural fluorescence and its impurities making the identification of the labelled molecules at low concentrations more difficult, because they could migrate with the impurities. Using MEKC, highly resolved specific separations can be obtained [30] and AA can be identified as taurine [31] (Figure 5 I) or dimethyl arginine [32].

CBQCA is a fluorogenic dye with low levels of impurities, however it cannot label secondary amines e.g. proline (Pro). In addition, Perquis et al. demonstrated that the fluorescent yield of the CBQCA-Trp derivative was 50 times less important than CBQCA-Trp, making Tryptophan (Trp) a difficult AA to be identified [33]. However, very good separations can be obtained using micellar electrokinetic chromatography (MEKC) [34] (Figure 5 II).

A good molecule candidate for better more selection and identification of thiol-containing AA, such as cysteine (Cys) or homocysteine (Hcy) [35], is the iodoacetamido fluorescein. It was used in clinical studies to measure the concentration of Hcy in plasma (Figure 6), to help diagnosis of cardiovascular disease [36,37]. Trp, Tyrosine (Tyr) and their metabolites can be selectively detected using UV pulsed lasers [38].

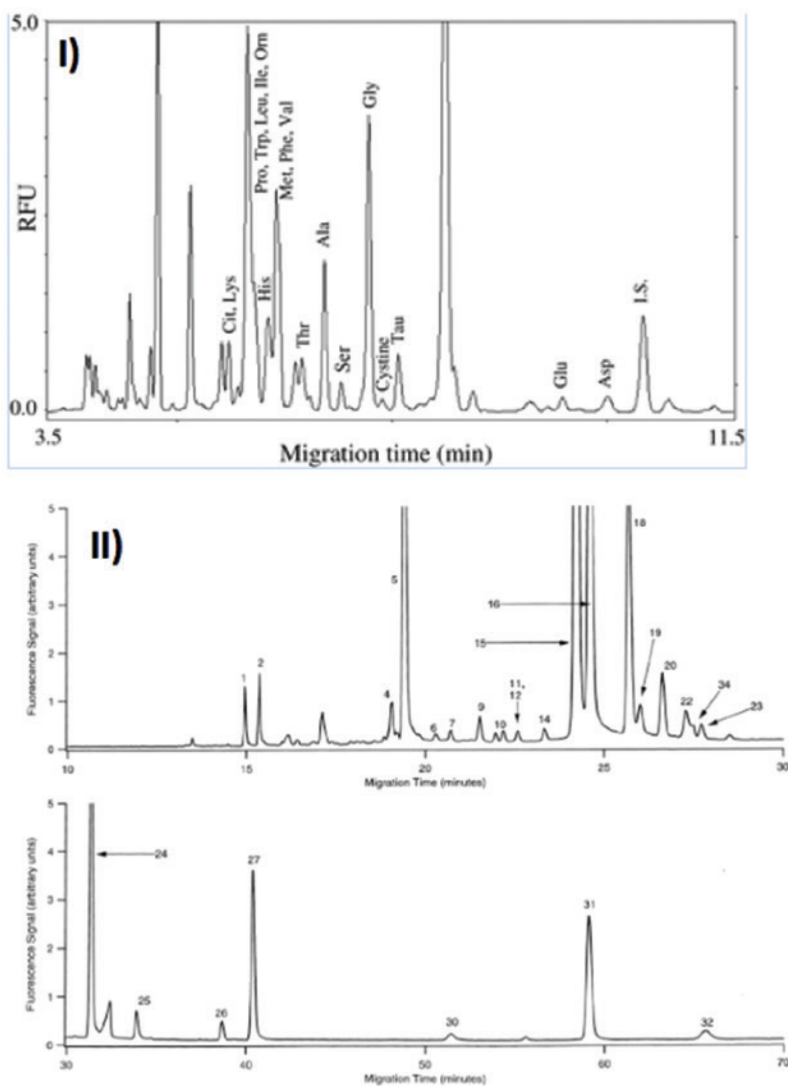


Figure 5: CE/LIF of AA and biogenic amines.

I) Electropherogram of a plasma sample labelled by FITC where Taurine (Tau) can be identified. Separation conditions: 20 mmol/L tribasic sodium phosphate pH 11.8, 23°C, 22 kV [31]. II) Separation of CBQCA-AAs in a normal plasma sample of a child. (1 and 2): Lys; (4): Cit; (5): Gln; (6): Ans; (7): Asn; (9): Tyr; (10): Orn; (11): b-Ala; (12): Carnosine; (14): Ser; (15): Gly; (16): Ala; (18): Tau; (19): L-a-amino-butyric acid; (20): Val; (22): Met; (23): Thr; (24): N-Val; (25): Ile; (26): Phe; (27): Leu; (30): Glu; (31): Arg; (32): Asp. BGE:160 mM borate, 130 mM SDS, 7.5 mM g-CD and 20 mM NaCl at a pH of 9.5; capillary: 67 cm length (60 cm to detector) and 50 µm id. 30 kV [34].

c) Proteins.

The main problem in using CE/LIF to study proteins is that only Trp and Tyr are fluorescent, and those AA are not abundant in proteins. In consequence, very few studies can be run using CE/LIF without labelling the proteins.

As for AA analysis, different dyes can be used: 5-carboxy-tetramethylrhodamine succinimidyl ester (5-TAMRA.SE, excitation at 488 or 532 nm), CBQCA (excitation at 488 nm), 3-(2-furoyl)-quinoline-2-carboxyaldehyde (FQ) (excitation at 488 nm) or 2, 3-naphthalenedialdehyde (NDA). NDA is a fluorogenic dye, excited at 442 nm using Helium Cadmium Laser, or a 450 nm LED can also be used. These labelling reagents react mainly on the lateral chain of Lysine. Since this AA is quite abundantly present in proteins, many different labelled species can be seen, which will cause enlargements of the peaks in the electropherograms.

The two main applications concerning CE/LIF applications were the applications concerning recombinant IgG purities and proteomic approaches using this technique to quantify proteins.

The impurities from pharmaceutical recombinant IgG were studied by Hunt and Nashabeh [39]. They used a purification step after the IgG labelling with 5-TAMRA.SE using a NAP-5 column (Pharmacia) which allowed them to remove excess dye. The separation was run using a non-gel sieving media called Biorad SDS buffer (no longer available but can be replaced by SDS-CGE buffer from SCIEX). Figure 7 shows a separation of denaturated and native IgG, and a comparison with the conventional slab gel. This work spurred a lot of different developments of this application for recombinant IgG quality control [40]. A protocol to identify 0.5% of an impurity profiling in a recombinant antibody sample was precisely detailed by Le Potier et al. [41].

Good 2D separations were achieved by Zhu et al. The sample underwent separation in a first-dimension capillary by sieving electrophoresis (SE). Fractions were periodically transferred across an interface into a second-dimension capillary, where components were further resolved by MEKC. Five SE and five MEKC capillaries allowed separation of five samples in parallel. The FQ-labelled samples were injected into the five first-dimension capillaries, fractions were transferred across an interface to five second-dimension capillaries, and the analytes were detected in a sheath-flow cuvette containing five capillaries. A complex protein mixture of an A549 cell-line homogenate was separated in such manner [42].

d) Nucleic acids.

Since the work of Drossman et al. [43], where the authors claimed to have rapidly separated fluorescently labelled DNA fragments generated in sequencing reactions, DNA sequencers were developed by different companies (Promega, Thermo Fisher Scientific...). In the last ten years, Berezovski et al. [44] proposed a method called 'non-equilibrium capillary electrophoresis of equilibrium mixtures' (NECEEM), to select aptamers from a DNA library, involving repetitive steps of partitioning without amplification between them. While the authors contributed to a real, major innovation in aptamers selection, the problem remains that for now it is impossible to detect the peak of the selected aptamer. Nevertheless, it can be collected and sequenced using the new generation of DNA sequencers as Illumina [45.

Currently, there is an ongoing effort to replace Northern-blot electrophoresis and autoradiography by CE/LIF. As an example, the separation of RNA fragments ranged from 100 to 10,000 nucleotides (nt) in polyethylene glycol (PEG) and polyethylene oxide (PEO) solutions with different molecular weight and different concentrations has been developed. The separation of small RNA fragments (<1000 nt) was improved with the increase of polymer concentration, whereas the separation performance for the large ones

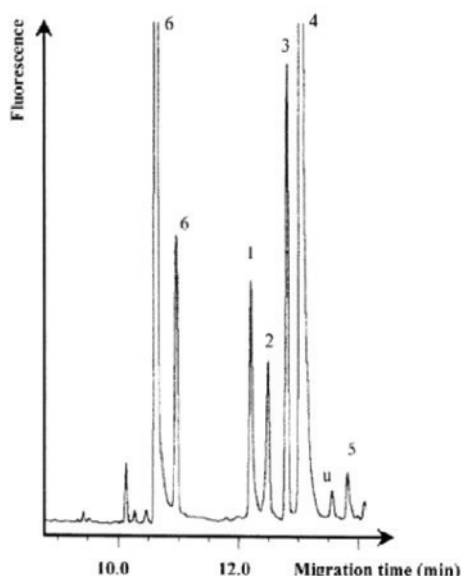


Figure 6: Example of separation using CE/LIF of 6-IAF-labelled thiols in a pathological sample (1) homocysteine (Hcy), (2) cysteinyl–glycine (CysGly), (3) N-acetylcysteine, (4) cysteine, (Cys), (5) glutathione (GSH), (6) 6-iodoacetamidofluorescein (IAF), (u) unknown. Pathological sample: Hcy, 32 mM; Cys–Gly, 31 mM; Cys, 280 mM; G-SH, 4.1 mM. Separation buffer 50 mM boric acid, 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 10. 30 kV, 50 µm id capillary 85/50 cm are total and effective length [35].

(>4000 nt) deteriorated in PEG or PEO solutions when the concentration was above 1.0% and 0.6%, respectively [46].

Microchips from the Agilent bioanalyser were used to measure the ratio of 28S/18S rRNA in Diamond-Blackfan anaemia patients and could become a diagnostic tool [47].

It can be seen that using CE/LIF is becoming more important in RNA work. However, it is only the beginning of this kind of work, which involves mainly biologists, that are less inclined to perform analytical assays and protocol developments on the CE/LIF instruments than analytical chemists are.

IV. The future.

The final question to ask: is LIF or LEDIF really of use in today's science? What will the future research axes and applications be?

First, in the major developments on DNA sequencing with dedicated instruments and separation kits, it can be seen that CE/LIF has had many successes.

For twenty years the analysis of pharmaceutical recombinant monoclonal antibodies was largely performed using LIF or LEDIF. It has become a significant technique in pharmaceutical companies for analysing the polysaccharide part of glycoproteins.

The main issue now is the continued development of simple applications using CE/LIF. This is now the case for polysaccharides, since it has been seen that the CE/LIF/MS experiments are very important to polysaccharides identification [26]. Labelling and separation kits are available, mainly from Sciex.

For the future, the selection of aptamers is a matter of considerable importance and that CE/LIF/Illumina can be developed to help. The main problem, however, is the development of the required bioinformatic analysis tools to identify the selected aptamers and the kits needed to allow scientists to easily run selection experiments using CE/LIF.

Finally, for use in clinical studies, while homocysteine has shown that it can be easily detected using this device, it still lacks automation and applications on other small molecules. The dimethyl arginines (with CBQCA or napthtalene dialdehyde (NDA) labelling) could be good candidates. Now, though, few clinical laboratories have developed such analysis methods. Generally speaking, clinical biochemistry laboratories need quick, robust and kit-based methods, only a few of their protocols use CE/LIF.

In conclusion, while CE/LIF and CE/LEDIF developments in the literature have been decreasing, its future remains promising.

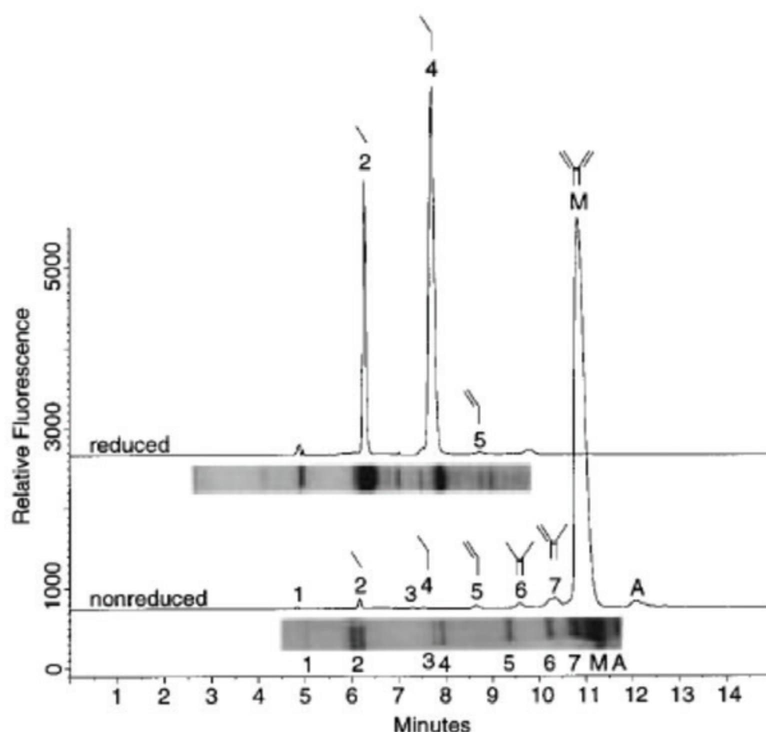


Figure 7: CE-SDS-nongel sieving media separations of nonreduced and reduced preparations of a 5-TAMRA.SE-labelled IgG. Bio-Rad SDS running buffer capillary, untreated fused silica, 50 µm i.d. effective length, 19.4 cm; injection, 15 s at 417 V/cm, applied electric field, 625 V/cm, temperature, 20°C. Detection was performed with laser-induced fluorescence using a 3.5 mW argon ion laser, 488 nm excitation. Insets show silver-stained SDS-PAGE traces of unlabelled sample preparations [39].

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