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Fluorescence detection system for capillary separations utilizing a liquid core waveguide with an optical fibre-coupled compact spectrometer

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Abstract

A fluorescence detection system for capillary liquid separation methods is described. The system is based on a silica capillary coated with a low refractive index fluoropolymer Teflon AF that serves both as a separation channel and as a liquid core waveguide (LCW). A fibre-coupled laser excites separated analytes in a detection point and arising fluorescence is collected at one end of the LCW capillary into the other optical fibre which brings it to a compact charge-coupled device (CCD) array spectrometer installed in a desktop computer. No additional components such as focusing optics or filters are necessary. This system was used for detecting isoelectrically focused fluorescent low-molecular-mass p*I* (isoelectric point) markers and fluorescein isothiocyanate (FITC) labelled proteins. The ability of the system to acquire fluorescent spectra is also demonstrated.

Keywords: Fluorescence detection; Liquid core waveguide; Capillary liquid separation methods; Isoelectric focusing; p*I* standards; CCD spectrometer

1. Introduction

Currently, capillary microseparation methods including μ-HPLC, zone electrophoresis (CZE) [1], capillary isoelectric focusing (cIEF) [2] and electrochromatography (CEC) [3] rank among the most efficient separation techniques. Moreover, the small inner diameter of the capillary tube is well adapted for the analysis of nanolitre sample volumes. However, given the small amount of molecules injected into the system, high-sensitivity detectors are often required for these techniques. In this respect, the laser-induced fluorescence detection (LIF) is of particular interest since very low detection limits of down to 10^{-18}–10^{-21} mol can be reached and various molecules such as derivatized amino acids, peptides, proteins and carbohydrates can be detected. On the other hand, these limits of detection can only be reached with expensive, bulky and cumbersome devices. Hence, alternative and simpler systems with not only comparable performance but also with a capacity for miniaturizing are being developed [4]. One possible solution is based on the utilization of liquid core waveguides (LCW) [5].

LCWs enable light propagation within a liquid medium when the core liquid has a higher refraction index (RI) than the surrounding solid tubing. Then the light is guided through the LCW and can be collected on its outlet.

Fujiwara et al. [6,7] described the first application of a liquid core capillary cell in fluorescence spectrometry. They used a laser to axial excitation of fluorophores and the emitted light was scanned on the other tip of the LCW. As there was no coating or tubing material with the RI value lower than that of water, this LCW was made from quartz (RI 1.46) and carbon disulfide (RI 1.63) was used as a liquid core.

In the area of LCW development, great progress was made by the introduction of a new amorphous fluoropolymer based on 2,2-bis(trifluoromethyl)-4,5-difluoro-1,3-dioxole whose RI of 1.29 is lower than that of water (RI 1.33). This polymer is now available as a copolymer with tetrafluorethylene, commercially named Teflon AF. At this time, not only Teflon AF

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tubes but also fused silica capillaries with Teflon AF coatings are available.

The first report about good performance of Teflon AF LCWs with transverse excitation for measurement of fluorescence in aqueous solutions was presented by Dasgupta et al. [8]. Lately, they presented several configurations that utilize LCWs for the fluorometric and absorbance detection of ammonia, CO_2, NO_2, and other compounds based on their various chromogenic reactions [9]. The same group used light-emitting diodes as an excitation source and optical fibre was used to collect fluorescence emission. They also utilized a charge-coupled device (CCD) spectrometer for the detection of the light in their portable absorbance, fluorescence and chemiluminiscence flow analyzer [10]. A similar spectrometer was also used by Byrne et al. This group presented a compact spectrofluorometer/spectrophotometer flow system using the LCW for the determination and quantification of quinine sulfate and chlorophyll-a [11].

The Teflon AF-coated fused silica capillaries with various detection configurations were also used as separation channels [8,12]. Hanning et al. demonstrated four-color DNA sequencing with the LCW based detection system [13,14]. Lately, Olivares et al. [15] employed the LCW to image both DNA electrophoresis separations and isoelectric focusing of derivatized proteins. In the same paper, they also reported the monitoring of the separation process through the excitation of separated analyte bands via moving the excitation optical fibre along the capillary. Fluorescence was collected at the LCW capillary end by an optical fibre-coupled photomultiplier. Recently, Pawliszyn et al. [16] continued their early works dealing with the monitoring of whole column imaging detection (WCID) using an axially illuminated LCW and a CCD camera for scanning the emitted light.

In the present work, we combine some of the principles mentioned above. We have used the LCW for both the separation and the detection of analytes. However, we use a fibre-coupled CCD array spectrometer for the detection of fluorescence emission. Analytes are excited in the LCW by the orthogonally attached optical fibre carrying excitation energy from a light source. The fluorescence emission is collected from one end of the LCW by the other optical fibre joined to a compact CCD array spectrometer installed in a desktop. Such detection offers simple arrangement and easy optical alignment. The capability of the spectrometer to resolve a signal from a broad range of wavelengths permits to reject the excitation wavelength without the need of optical filters and other optical components. Described configuration also offers a significant potential of miniaturization.

2. Experimental

2.1. Instrumental set up

A schematic drawing of the instrument is presented in Fig. 1. The basic component is a 100-μm i.d./375 μm o.d. fused silica capillary coated with Teflon AF (TSU 100375, Polymicro Technologies, Phoenix, AZ, USA) serving as a liquid core waveguide. The injection end of the LCW is fitted into the inlet vial (anolyte reservoir), the detection end then to the polymethylmethacrylate (PMMA) T-piece that is used for connecting both the 400-μm core diameter scanning fibre-optic (FVP400440480UVM, Polymicro Technologies) and the outlet vial (catholyte reservoir). The gap between the LCW capillary and the scanning fibre is about 0.3 mm. All separations are driven by high-voltage supply (CZE 1000R, Spellman, Planview, NY, USA). The argon ion laser (2201-15SLB, Cyonics, San Jose, CA, USA) is used as a source of excitation light which is led via optical fibre to the excitation point. The distance between the excitation point and the end of separation capillary was 30 mm. For its transparentness, it is unnecessary to remove the Teflon AF coating from the capillary. The fluorescence emission is collected by the scanning optical fibre and detected by the CCD array spectrometer (S2000, Ocean Optics, Dunedin, FL, USA). The signal is evaluated by OOIBase32 software.

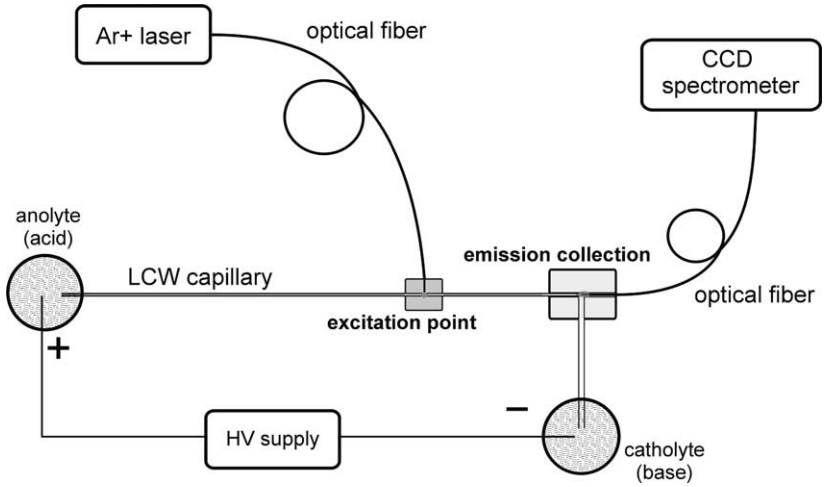

Fig. 1. Schematic diagram of the instrumental setup.

2.2. Chemicals

For the modification of inner capillary walls γ-glycidoxypropyltrimethoxysilane (GOPTMS) and acryl amide (AA) were purchased from Sigma (St. Louis, MO, USA); *N*,*N*,*N*′,*N*′-tetramethylethylendiamin (TEMED) and ammonium persulfate (APS) were obtained from Merck (Darmstadt, Germany). The electrophoretic buffers and hydroxypropylmethylcelulose (HPMC) were also from Sigma. The carrier ampholytes in pH ranges 3–10 and 6–8 were purchased from Bio-Rad (Hercules, CA, USA). Fluorescein was from Pliva-Lachema (Brno, Czech Republic), hemoglobin, thrombin and fluorescein isothiocyanate were from Fluka (Buchs, Switzerland).

2.3. Isoelectric point markers (pI markers)

p*I* markers are substances that are used for isoelectric point determination and for the pH gradient monitoring in the capillary or on the gel. The labelled native proteins (e.g. albumin) are often used as p*I* markers. This type of marker has some drawbacks like instability in water solutions or tendency to precipitate in the place of its focusing. Another possibility is to use labelled peptides (e.g. with dansyl or rhodamine) [17]. The low-molecular-mass standards (Mr ∼ 400 g mol^{-1}) can eliminate some of the disadvantages related with the above-mentioned types [18,19]. These compounds are good ampholytes ($\Delta pK_a \leq 4$), well soluble in water and also able to be excited by visible light. The fluorescein-based derivatives display very intensive fluorescence. Moreover, their absorption maxima correspond with the wavelength of the Ar^+ laser, which makes them attractive for the LIF detection. Structural and spectral similarities enable them to be used in combination with FITC derivatives of proteins.

The 10^{-6} M standard solutions of p*I* markers 5.4, 5.7, 6.0 and 6.6 in deionized water were prepared. These solutions were diluted to desirable concentrations by 4–6% (w/v) aqueous solution of carrier ampholytes.

2.4. Modification of the inner surface

In cIEF, it is necessary to obtain sufficient time to focus analytes, so the EOF should be reduced or even eliminated. The reduction of the EOF and protein adsorption can be accomplished by using appropriate coatings. We use two procedures for the modification of the inner wall – a static modification with γ-glycidoxypropyltrimethoxysilane and that with polyacrylamide (PAA) [20–22].

In both cases capillaries were firstly etched with 1 M sodium hydroxide for 30 min, rinsed with 0.1 M hydrochloric acid and acetone.

In the modification with GOPTMS, the capillary was then rinsed with methanol and subsequently a 10% (v/v) methanol solution of GOPTMS was slowly forced through the capillary for about 2 h. The column was purged with nitrogen gas at 80 °C for 2 h. After this process, the capillary was rinsed with 2 ml of methanol and 2 ml of water. Thus, prepared capillaries were filled with a 4% (w/v) carrier ampholytes solution and left to be treated overnight at laboratory temperature.

In the PAA modification, the capillary was pre-treated as mentioned above and then filled with a 50% (v/v) solution of 7-oct-1-enyltrichlorsilane in toluene and kept for 1 h. Afterwards the same operation was repeated once more. The capillary was finally rinsed with toluene (2 min), acetone (10 min) and water (10 min). one millilitre of 0.15 M AA solution and 69.8 μl of 10^{-2} M TEMED were prepared and well degassed by stripping with helium for 30 min. 46.5 μl of well-degassed 10^{-2} M APS were then added and the mixture was intensely stirred and then slowly forced through the capillary. After 1 min, both ends of the capillary were plugged and the reaction was allowed to pass overnight. The unbonded polymer was then rinsed out with water. Thus the modified capillaries were conditioned with a 4% (w/v) solution of carrier ampholytes.

The results were obtained on one LCW capillary modified with GOPTMS and two modified with PAA (I and II).

2.5. Electrolyte systems and sample preparation

NaOH (20 mM), acting as the catholyte, and H_3PO_4 (100 mM) as the anolyte were used for all the cIEF experiments. The 0.15–0.25% and 0.05–0.15% (w/v) of HPMC were added to the catholyte or anolyte, respectively. Before their use the electrolytes were well-degassed to prevent bubble formation in the LCW capillary. The capillaries were rinsed with carrier ampholytes for 5 min before each analysis and then filled with the catholyte. Samples were injected hydrodynamically by 70–150 mm height difference between the reservoirs. Injection times and particular conditions of each analysis are mentioned below in figure captions.

The process of FITC derivatization was similar to what was described previously [16]. A 10^{-5} M solution of proteins was prepared by dissolving appropriate amounts of each protein in 20 mM borate buffer. Three hundred microlitres of this solution was then mixed with 25 μl of 5 mM fluorescein isothiocyanate solution (prepared in acetone with addition of pyridine). The mixture was homogenized for 30 s in ultrasonic bath, heated for 10 min at 50 °C and cooled down. The FITC-derived proteins were prepared daily.

3. Results and discussion

3.1. Characterization of detection system

The fluorescence detection system presented in this work is based on utilization of liquid core waveguides (Fig. 1). Compared with the similar previously described systems it offers design simplicity. The whole system is composed of few components that can be easily assembled and adjusted without high requirements on operator's technical skills. The excitation energy is brought to the excitation point by

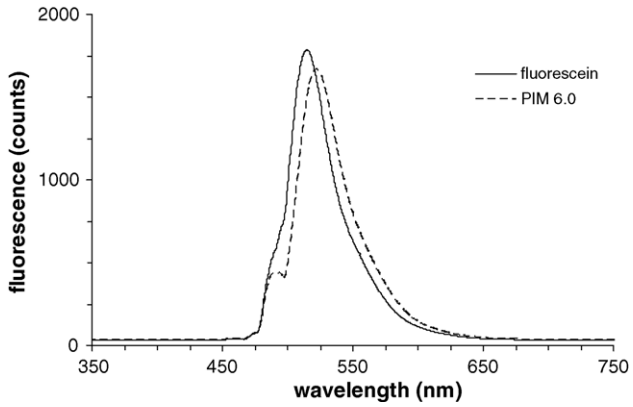

Fig. 2. Emission spectra of fluorescein and p*I* marker.

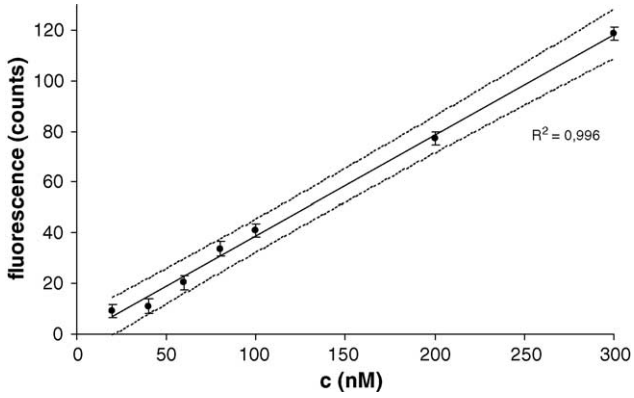

Fig. 3. Calibration of the detection system.

the optical fibre. This brings two advantages. Firstly, the quick exchange of excitation sources with different excitation wavelengths is facilitated. Secondly, a simple Plexiglas holder can be used to bring the excitation energy into a precisely defined point of the separation capillary (LCW) without any need for special positioners.

The important part of the system is the fibre-coupled compact CCD spectrometer, serving for detection of fluorescence emission. It allows eliminating special optical elements such as filters or microscope objectives as well as parts for their accurate adjusting. Moreover, the control software of the CCD spectrometer enables a quick change of the emission wavelength without any changes in the detection part of the system. In the spectrometer used it is possible to detect fluorescence in the range from 380 to 1000 nm. The selection of the emission wavelength is important in the case of analytes with different emission maxima as in Fig. 2, or for stray light suppression. The simultaneous detection of several wavelengths can also be employed, only with respect to some coincidences between emission spectra of different analytes or with spectra of excitation sources.

The scattered light could cause some problems in the meaning of the excitation of fluorophores that are present at any place of the capillary. This fact could increase background signal. However, we conclude that in our case this problem is negligible. According to the literature [9,13], during transverse illumination only a minimal portion of the excitation light is transferred into the LCW capillary. Furthermore, scattered light is preferentially conducted by silica wall and not by a liquid core. We also didn't observe increase of the background signal during the system calibration. Such increase would be expressed as a positive deviation from the calibration curve linearity; however the curve is linear (Fig. 3).

3.2. Calibration of the system response

The described fluorescence detection system was tested by fluorescein solutions of various concentrations and the obtained calibration curve is showed in Fig. 3. The individual points were obtained as follows. The signal was measured every 100 ms for one minute while the fluorescein solution was slowly flushing through the LCW to prevent photobleaching. The blank was measured in the same manner. Acquired data were averaged and then the blank signal was subtracted. The calibration curve with the 95% confidence interval and standard deviations of each point is shown in Fig. 2. As could be seen from this figure, the response is almost linear (characterized by correlation coefficient $r^2 = 0.99$) in given range of concentrations.

The limits of detection (LODs) were calculated according to Graham [23] to be 21×10^{-9} M (X_D^{α}) and 57×10^{-9} M (X_D^{β}). X_D^{α} represents detection limit protected against Type I error (accepting a hypothesis that the analyte is present when it is in actually absent) and X_D^{β} minimizes risk of making a Type II error (accepting the alternative hypothesis that the analyte is absent when it is in actuality present). In comparison with these results, we also calculated LOD from standard deviation of the blank sample (noise). LOD calculated as S/N = 3 was 9×10^{-9} M.

Such sensitivity seems to be rather week, but it is counterbalanced by constructional and operational simplicity. Moreover, the performance of the described model could be improved substantially. A higher fluorescence signal could be gained by bringing more laser energy into detection domain. Relatively high noise could be decreased by using some more sophisticated CCD-spectrometer.

3.3. Capability of the detection system to measure fluorescence spectra

Utilizing the CCD-spectrometer as a detector of fluorescence emission has some significant benefits. The ability to detect fluorescence selectively from a broad range of wavelengths allows excluding all other wavelengths. That makes the use of excitation and emission filters unnecessary. A unique feature of CCD based spectrometers is their capability to acquire fluorescence spectra. This ability is useful for measuring spectra from low sample volumes and also for determination of optimum wavelength for separation. An

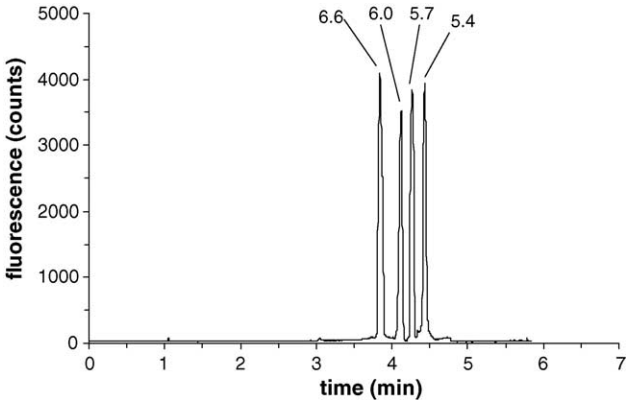

Fig. 4. Isoelectric focusing of p*I* markers. Conditions: LCW capillary, 100 μm i.d./360 μm o.d.; total length, 17 cm; excitation point (detection), 14 cm; inner coating, GOPTMS; catholyte, 20 mM NaOH, 0.15% (w/v) HPMC; anolyte, 100 mM H_3PO_4, 0.05% (w/v) HPMC; voltage, 19 kV; sample, p*I* markers (10^{-7} M) in 3% (w/v) ampholine (3–10); injection, height difference at the siphoning injection, 80 mm; injection time, 20 s.

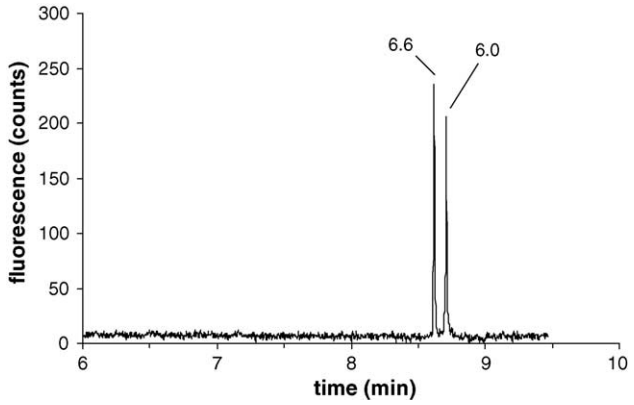

Fig. 6. Isoelectric focusing of p*I* markers. Conditions: LCW capillary (I), 100 μm i.d./360 μm o.d.; total length, 20 cm; excitation point (detection), 17 cm; inner coating, PAA; catholyte, 20 mM NaOH, 0.25% (w/v) HPMC; anolyte, 100 mM H_3PO_4, 0.15% (w/v) HPMC; voltage, 12 kV; sample, p*I* markers (10^{-8} M) in 6% (w/v) ampholine (6–8); injection, height difference at the siphoning injection, 100 mm; injection time, 50 s.

illustration of this ability is presented in Fig. 2, where the emission spectrum of fluorescein and that of a p*I* marker are depicted. As can be seen from the figure, the p*I* marker spectrum shows a slight shift (about 10 nm) to the longer wavelengths. There can also be seen a small peak around 490 nm that belongs to the scattered light from the Ar^+ excitation laser. The emission wavelength was set at 522 nm, so the scattered light was cut-off by the spectrometer.

3.4. Application of the detection system in capillary isoelectric focusing

The application of the described system as a fluorescence detector for a capillary separation method was demonstrated on capillary isoelectric focusing. Low-molecular mass fluorescent p*I* markers were chosen as testing analytes. The p*I* markers are often used for pH gradient calibration and for determination of isoelectric points of analytes. They are also very suitable as model analytes for checking and optimization of the instrumentation functionality. The separation of p*I* markers is shown in Fig. 4. As can be seen in Fig. 5, the pH gradient (characterized by correlation coefficient $r^2 = 0.99$) was almost linear.

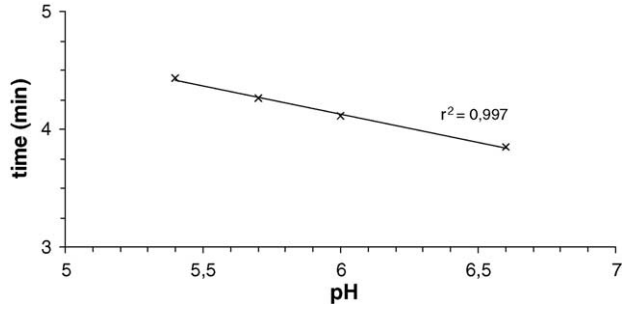

Fig. 5. Linearity of pH gradient.

Although the sample concentration of p*I* markers injected into the capillary was 10^{-7} M, the focusing process brings about 10–20 times higher concentration in the focused zone, so the concentration in the detector is about one order of magnitude higher. The isoelectric focusing in the LCW capillary with different inner coating (PAA instead of GOPTMS) and ampholytes with narrower pH-gradient are shown in Fig. 6. The p*I* markers 6.0 and 6.6 in 10^{-8} M concentration were used. The capillary with the PAA coating shows better efficiency and about three times slower EOF compared with the capillary coated with GOPTMS. The only problem was that of inner surface stability. After about 15 runs, the EOF gradually increased and the efficiency decreased. This was probably caused by the uncovering of some parts of the PAA coating. These spots of bare silica can contribute to the EOF inhomogenities or adsorption to the walls. In such case, it was

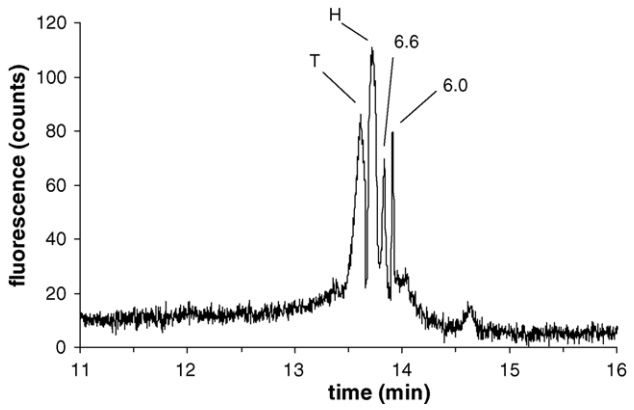

Fig. 7. Isoelectric focusing of p*I* markers and FITC-labeled proteins. Conditions: LCW capillary (II), 100 μm i.d./360 μm o.d.; total length, 20 cm; excitation point (detection), 17 cm; inner coating, PAA; catholyte, 20 mM NaOH, 0.25% (w/v) HPMC; anolyte, 100 mM H_3PO_4, 0.15% (w/v) HPMC; voltage, 12 kV; sample, p*I* markers (10^{-8} M), FITC derivatives of (T) thrombin and (H) hemoglobin (10^{-7} M) in 6% ampholine (6–8); injection, height difference at the siphoning injection, 100 mm; injection time, 35 s.

necessary to use a new capillary. The isoelectric focusing of FITC labelled proteins was also performed. The separation is illustrated in Fig. 7. Peaks of proteins in the figure are rather broad however that is caused by imperfect focusation process and not due to the detection part. For better separation performance the CIEF conditions have to be further optimized.

4. Conclusions

A simple fluorescence detection system suitable for microseparation methods based on liquid core waveguides is described. A fibre-coupled CCD spectrometer installed in a PC is used in this system for detecting emitted light. The functionality of this system was demonstrated on isoelectric focusing of low-molecular-mass p*I* markers and FITC derivatized proteins. The ability to acquire emission spectra was presented. Although the described system shows a higher limit of detection compared to more sophisticated LIF detectors, it offers some very useful features. The construction, assembling and optical alignment are surprisingly effortless and no other optical components such as filters or microscope objectives are necessary. The system could easily be tuned for the detection of analytes with different spectral characteristics only by changing the excitation source and without any modification in the detection part. Described detection system also offers a high degree of miniaturization that is in conformity with present-day trends.

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