Capillary Electrophoresis with Laser-Induced Fluorescence Detection (CE LIF)

Theoretical part

A. Capillary Electrophoresis

Capillary Electrophoresis (CE) is a modern high performance separation method. The separation is based on different migration of analytes in a capillary over which a high voltage (typically 10-30 kV) is applied. Typical inner diameters of commonly used capillaries are in the range of 25-75 μm and the detection is usually on-column.

Capillary zone electrophoresis (CZE) is one of the CE modes that is characterized by the use of a single background electrolyte (BGE) (isocratic), which enables the generation of a constant electrical field. The detector response in the end of the capillary (the electrophoregram), has a characteristic peak profile that is a function of many factors (type of sample, injection, detection, sorption, mobility differences, etc.). The qualitative characteristics are related to the migration time of the peaks and the quantitative characteristics are represented by the peak height or the peak area.

Electrically charged particles are moving in the applied electrical field in a direction determined by its charge and the field orientation. Positively charged particles are moving towards the negative pole and negatively charged particles are moving towards the positive pole.

A charged particle is characterized by an *electrophoretic mobility* μ defined as:

$$\mu = \frac{v}{E} = v.\frac{L}{U} \qquad \left[\frac{\mathrm{m}^2}{\mathrm{Vs}}\right]$$

where E is the intensity of the electrical field, U is the applied voltage, L is the total distance between the electrodes (the total length of the separation capillary).

The relationship between the mobility and the charge of the particle follows from the balance between the electric driving force and the retarding friction force. The electric force is given by the product of the particle charge and electric field intensity; the friction force is given by the Stokes relationship $F = 3\pi\mu dv$, where μ is the liquid viscosity, d is the hydrodynamic particle diameter and v is the particle velocity. The electrophoretic mobility is conventionally defined positive for cations and negative for anions.

The electrophoretic mobility is calculated from the migration time t_m, which is the time during which the analyte migrate from the injection end of the capillary to the detector:

$$\mu = \frac{v}{E} = \frac{l_{ef}}{t_m E} = \frac{l_{ef} L}{t_m U}$$

where l_{ef} is the effective length of the capillary (distance between the injection end of the capillary and the detector).

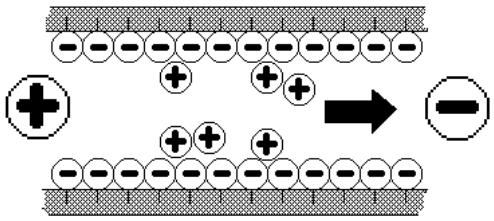
In the case of commonly used fused silica capillaries, basic electromigration is influenced by the *electroosmotic flow* (EOF*;* Fig. 1). EOF is independent on the charge of the analytes and the resulting mobility μ can be defined as:

$$\vec{\mu} + \vec{\mu}_0 = \frac{l_{ef} L}{t_m U} \qquad \Rightarrow \qquad \mu = \frac{l_{ef} L}{U}\left(\frac{1}{t_m} - \frac{1}{t_0}\right)$$

where t_0 is the migration time of neutral (non-charged) particles.

Figure 1. Electroosmotic flow

The inner surface of the fused silica capillary carries fixed negative charges (dissociated silanol groups). In consequence of the law of electroneutrality conservation, there is an excess of positively charged ions in the BGE near the inner capillary wall. Movement of these cations towards the cathode generates a flow of bulk liquid inside the capillary in this direction.



Many analytes that can be separated by CE has an acid-base character. Because the ionization equilibrium is established in a much shorter time compared to the electromigration time, all forms of a certain ion are moving with the same speed in a single zone. The resulting mobility, the *effective mobility*, is the sum of the electrophoretic mobilities μ_i of each ion form multiplied by the respective molar fraction x_i:

$$\mu_{ef} = \sum x_i \mu_i$$

Analytical chemists usually solve the task how to separate all analytes (or the major analytes at least) efficiently. The chemists try to optimize the separation, which usually means finding the conditions at which the differences of the ion mobilities of the analytes are maximal. The parameters to optimize are: p*H*, ionic strength and polarity of BGE, voltage and current during analysis, injection process, etc. Complexation with ligands e.g. crown-ethers or cyclodextrins or enhanced modes of CE, such as micellar electrokinetic chromatography (MEKC) can be used to improve separation efficiency. This is especially useful for separation of neutral compounds (by MEKC, where separation is based on differential distribution into micelles) or enantiomers (by use of cyclodextrin. Enantiomers interact differentially with cyclodextrins), which are not separated by conventional CE. Modifiers of capillary walls, such as organic solvent, gel or linear polymer can be used to suppress sorption of analytes to the capillary wall and suppress or even reverse the direction of the EOF. For instance, addition of a cationic surfactant cetyltrimethylamoniumbromid (CTAB) forms a positively charged layer on the silica surface resulting in a reversed EOF.

B. Instrumentation - theory

Capillary zone electrophoresis

The basic instrumental setup for CZE contains a separation capillary, power supply, detector and device for recording, storing and processing of analytical signals (Fig. 2). The voltage is typically in the range of 10 – 30 kV and the BGE buffer concentration is in the range of 10 – 50 mM. The CE analysis is usually performed in a fused silica capillary, and rarely in Teflon or glass capillaries. Silica capillaries are covered with a thin layer of polyimide on its outer side to protect the capillary from getting brittle and break. The typical inner diameter (*i.d.)* of common capillaries is in the range of 25 – 75 μm, and the outer diameter (*o.d.*) is in the range of 150 – 400 μm. In practice, the length of the separation capillary is in the range 30 – 70 cm and the volume is a few μl.

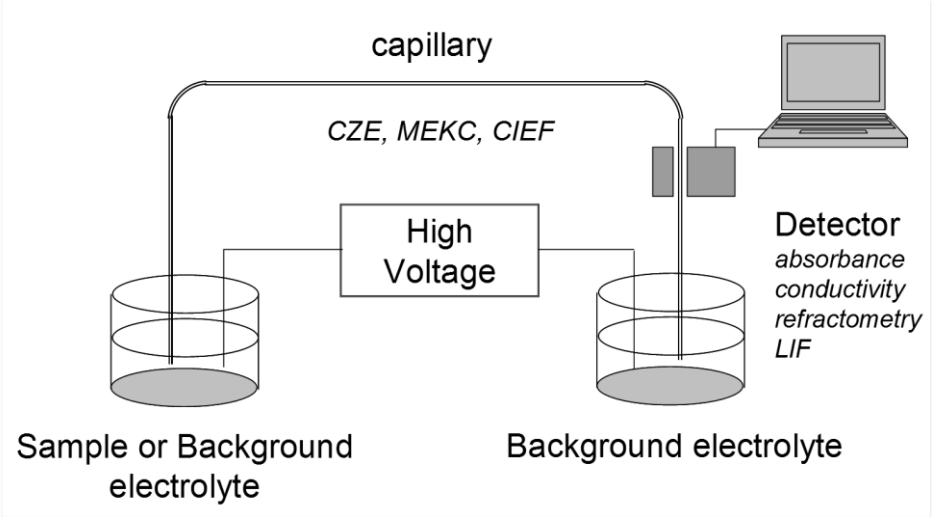


Figure 2. Scheme of the instrumental setup of capillary electrophoresis

Injection

Injection of a sample into the separation capillary is performed either hydrodynamically or elektrokinetically.

Hydrodynamic injection is achieved by applying an overpressure on the injection (input) side of the capillary or using reduced pressure on the detection (output) side of the capillary. The pressure difference can be accomplished with compressed gas, vacuum pump or by elevation of the injection vial relatively to the output vial.

Electrokinetic injection is accomplished by the application of an injection voltage for a short period of time (usually a few seconds). The injected amount of analytes, during the electrokinetic injection, depends on the EOF as well as on the electromigration of each analyte. Thus, this type of injection is discriminating: it prefers the ions with higher mobility. If the conductivity of the sample solution is lower than that of the BGE, the analyte ions are pre-

concentrated at the front of the sample plug (a phenomenon called sample stacking). Compared to the hydrodynamic injection, the electrokinetic injection is usually less reproducible.

Detection

Detection is most often based on absorbance or fluorescence of analyte zones flowing through the detection window of the capillary. Laser-induced fluorescence (LIF) benefits from the superior properties of laser light; especially the directional characteristics of the laser beam, which makes it compatible with on-column capillary detection. The narrow laser beam is characterized by minimal divergence and hence it can be focused between the inner walls of the capillary (the width of the focused laser beam is in the range of units or tens of μm). Hence, due to nearly 100% utilization of laser power, even lasers with relative low output (~ a few mW), can be used for efficient excitation. One limitation of laser applications is the lack of inexpensive lasers with tunable wavelength. Usually, the only possibility is to select specific lasers with a discrete wavelength suitable for target application. Common lasers applied in CE or HPLC and their wavelengths are listed in the following table:

Laser	**Wavelength (nm)**
HeNe	543, 632
Ar+	488, 514, UV
Diode	400 – 1000

A disadvantage of fluorescence or absorbance detection is that not all analytes contain chromophores with sufficient fluorescence or absorbance in the common UV-VIS region (180-800 nm). For these analytes, direct detection is not possible. Other options for such analytes include derivatization of the analytes with a label containing a suitable chromophore, use of another detection method (refractometry, amperometry, conductance, MS) or operation in the indirect detection mode. A disadvantage with the use of fluorescent labeling of proteins is that it is cumbersome to achieve a reproducible and uniform labeling of the proteins.

C. Instrumentation – setup (Fig. 3)

The instrument is equipped with a DPSS Nd:YAG laser (*diode pumped solid state neodymium-doped yttrium aluminum garnet*, 1064 nm) with a frequency doubler emitting light with the wavelength 532 nm. The laser beam is focused with a quartz lens (with the focal length of 10 mm) into the center of the capillary held in an adjustable clamp positioned on micrometric xy stage. Fluorescent radiation is collected with a microscope objective (60×) and directed through an optical slit (spatial light filtration) towards a photomultiplier tube (PMT). Two filters are inserted in front of the PMT to assure optical filtration.

***Note**: If you remove the shielding tube placed between the microscopic objective and PMT and place a piece of white paper in front of the optical slit, four interfaces (air/outer capillary wall, inner capillary wall/liquid, liquid/inner capillary wall and outer capillary*

wall/air) should be visible. The slit should be positioned between the second and the third interface to transmit the (orange) fluorescence and block scattered (green) light.

The entire optical setup is covered with a light-protective cover (shield). The carousel with injection and BGE vials and the high voltage electrodes is placed in a protective plastic cover equipped with a safety switch, which turns off the HV if the protective cover is removed to protect operators from electrical shocks. The electrical current from the PMT is digitized with a 16-bit A/D (Analog/Digital) converter in a personal computer. The acquired data (PMT current as a function of time) are recorded and stored into a file in the ASCII format with a program written in LabVIEW environment. A simple spreadsheet editor (MS Excel) can be used for data processing and graphical presentation of the resulting electrophoregrams.

Reaching the lowest detection limits is based on efficient excitation, as well as sensitive detection and noise reduction. Efficient excitation is accomplished only with proper focusing of the laser into the center of the capillary. In addition, sensitive detection is based on utilization of an efficient collector of emitted fluorescent radiation and a detector with great gain (i.e. the PMT). There is a variety of noise sources, which require application of a range of tools to suppress noise:

a) scattering of the laser light
   - insertion of optical filters and slits in front of the PMT
   - insertion of light protective shields into the optical part of the instrument
   - black, non-fluorescent painting of the box, shields, and all holders of the optics

b) penetration of daylight into the optical compartment of the instrument
   - covering the optical setup with the light protective cover (shield)
   - blocking of all optical gaps, openings and slits presented in the cover

c) electrical interference, induction of signal generated with electrical power network
   - application of an RC filter for suppression of noise with frequency above ~10 Hz
   - integration of signal for the time corresponding to any multiple of the electrical power line period 20 ms (50 Hz) – proper integration period in the LabVIEW program.

CE

An unmodified fused silica capillary with the effective length of 30 cm and the total length of 37 cm is inserted into the CE LIF instrument. The inner diameter of the capillary (*i.d.*) is 50 μm and the outer diameter (*o.d.*) is 360 μm.

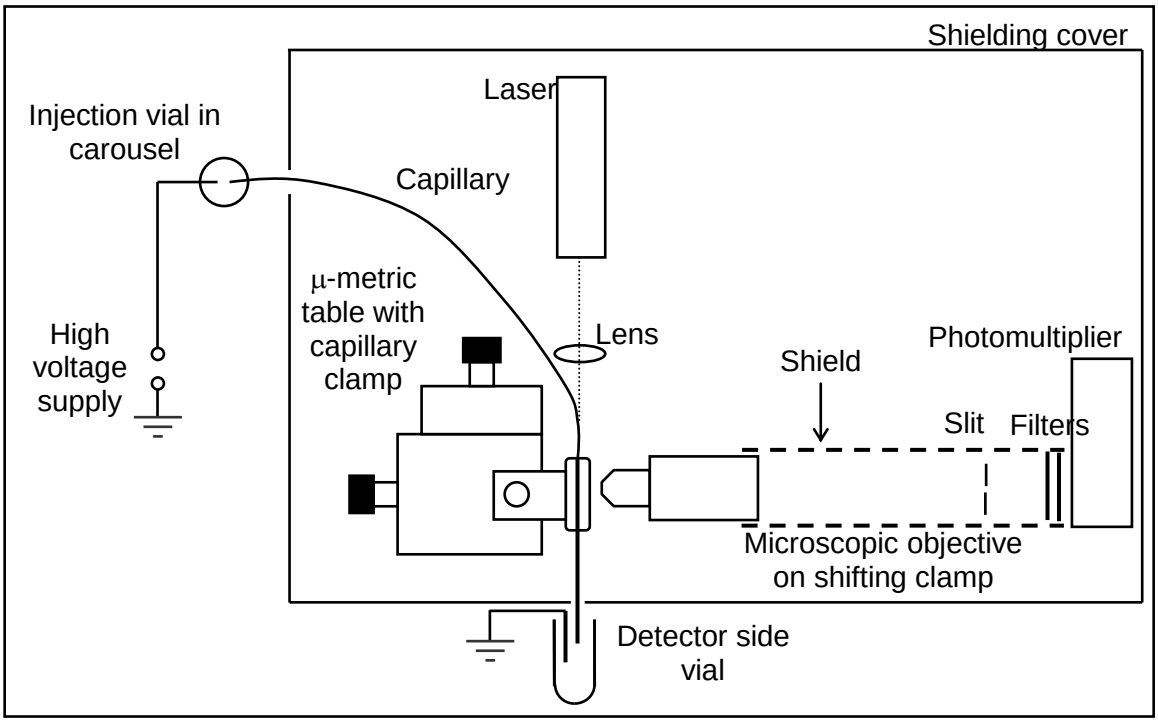


Figure 3. Scheme of CELIF instrumentation.

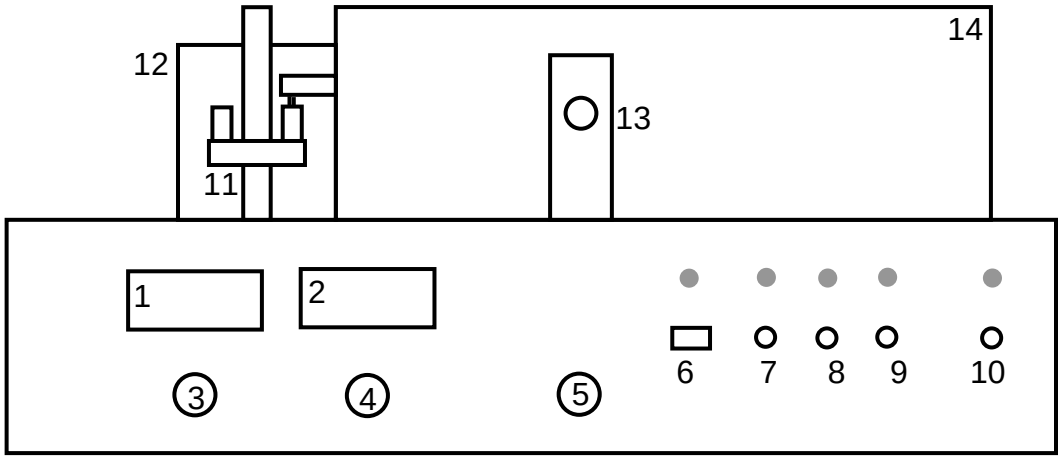


Figure 4. Description of the CELIF instrument

1 High voltage display
2 Electrical current display
3 High voltage knob (local)
4 Electrical current limiter knob (set approximately on 25 %)
5 High voltage knob for PMT (set on value “650 pcs”)
6 Local/Remote switch (pushed = local/pulled = remote)
7 High voltage switch (local)
8 PMT high voltage switch (local)
9 Laser switch (local)
10 Power (main) switch
11 Carousel with injection vials
12 Protective cover of the carousel
13 Detection side vial
14 Main protective cover (shield)

CE LIF program for instrument control and data recording

The CE LIF program was created in LabVIEW development system. The program is designed for control of the CE LIF instrument and data acquisition using a 16-bit A/D converter. Using the program, experiments can be performed in manual control mode (local) or in automatic control mode (remote). You will operate the CE LIF instrument only in the automatic control mode (remote), which designed for common use. In the remote mode, the laser, the PMT, high voltage (for injection and analysis) and the maximum time of the analysis are controlled by the program. Open the CE LIF program by clicking on the icon "CELIF latest version" on the desktop of the computer. Before beginning an experiment, enter a name of the output file (with ".txt" file extension) including directory, total time of analysis (typically 15 minutes) and sampling frequency (typically 4 Hz, maximum is 10 Hz).

***Warning**: Make sure that the directory exists, or create a new one if it does not exist. (If the directory does not exist or the entered path is wrong, no data are stored during such experiment.)*

Then, enter the time and the voltage for injection and value for the separation voltage. Before you run the program, it is advised to clear the display from previously recorded data (move the cursor of the mouse to the display, click on the right button of the mouse and select "Data operations » Clear chart" command in the menu).

After entering all parameters of the experiment you can start the CELIF program with the white arrow ("Run") placed in the upper left corner of the program's window. The laser and the PMT are controlled with the appropriate buttons in the lower left bottom corner of the program's window. Injection voltage is switched on by clicking on the button "Injection", and the injection voltage is switched off automatically after the entered period of the injection. High voltage and data recording during the separation are started with the "Start" button; separation is ended after the entered max. time or manually using the "Stop" button.

WARNING

You may switch on high voltage (HV) only with the plastic protective cover placed over the sampling carousel with samples!

You may switch on the PMT only with the main protective cover in place!

During operation with laser beam do not look straight into the laser! Be careful about reflections of the laser beam from steel clamps, watch, jewelry, etc.

Experimental part

A. Instrumental optimization.

Determination of limit of detection (LOD) of rhodamine 6G.

Before separation experiments, it is necessary to verify the alignment of the CELIF instrument and check its performance. In this task you should become familiar with the CELIF instrumentation and verify a proper setup by determination of the LOD for rhodamine 6G.

1. Preparation of solutions

Prepare 100 ml background electrolyte buffer (BGE), 50 mM citric acid in 10 % (v/v) ethanol (EtOH) and titrate it to p*H* = 2.5 with a solution of concentrated ammonia.

Fill 2 vials with BGE buffer for the CELIF instrument. Prepare a set of vials with the calibration samples of rhodamine 6G. Concentrations of samples should be 10^{-8} M, 10^{-9} M, 10^{-10} M and 10^{-11} M in BGE buffer.

The stock solution of rhodamine 6G is 2 mM. Dilute the samples gradually in several steps (e.g. 10x, 100x, 1000x) in small final volumes (100-1000 μl). To improve precision of pipetting, do not pipette less than 1 μl. Use the small (200 μl) microvials for CELIF samples . The volume of each sample should not be lower than 150 μl.

Remove air from the BGE and sample solutions in an ultrasonic bath (for 3 minutes).

2. Verification of the proper alignment and function of the CELIF instrument with determination of the limit of detection (LOD) of rhodamine 6G

Filling of the capillary

Fill the capillary with BGE using the injection syringe with adapter and assure that no bubbles get into the capillary. Fill from the detection end of the capillary and press gently at least 3 drops of BGE through the capillary into a small tissue held close to the injection end of the capillary. Elevate the vial with BGE on the injection side (in carousel) and continue pressing BGE through the capillary for a few seconds. Quickly but carefully remove the syringe from the capillary on the detection side and replace it with a vial of BGE. Place the plastic protective cover on the sampling carousel. Start the CELIF program and set the high voltage to 15 kV. Run a blank analysis with no injection and observe the progress for a few minutes. If arcing occurs in the sampling carousel (it produces sharp snappy sound) it is necessary to interrupt the experiment and clean up the carousel space. If everything is in order, record the value of the electrical current (in μA) and observe it for ~30 s. In case that the value of the current falls more than about 10 %, it is necessary to fill the capillary with BGE again. The next step is to observe the properties of the detection system (laser, PMT). End previous experiment and switch on the laser and the PMT (do not change the value set on the PMT knob) using the CELIF program. Record the PMT signal using the CELIF program with the high voltage set to 15 kV. Specify variation of the PMT signal – from minimum to maximum values of the signal during the blank

experiment (without injection of a sample). The optimal value of the variation, from min. to max., is about 10.

Injection of sample and analysis of Rhodamine 6G

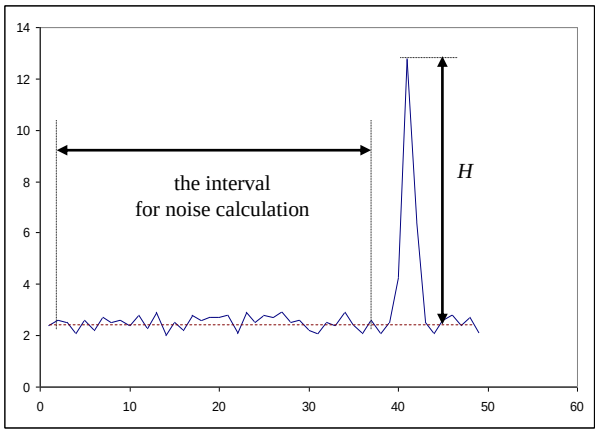
After the capillary is filled with BGE properly, the first sample may be injected. In the window of the CELIF program write down a name of the output file, the value of the voltage during separation U = 15 kV, the time of analysis (15 minutes) and the sampling frequency (4 Hz). With the HV switched off, replace the vial of BGE with the vial containing rhodamine 6G at the lowest concentration (10^{-10} M) using the sampling carousel (push down, turn and pull up). Inject the sample into the capillary at 5 kV for 10 s using the CELIF program. Replace the vial of the sample by the vial of BGE. Then, immediately click on the start button in the CELIF program and start the analysis. Record the value of the electrical current at start of the analysis and monitor its value during the entire experiment and check that no significant change occurs during the experiment. Repeat this experiment with the other rhodamine 6G concentrations in the set (10^{-8} M, 10^{-9} M and 10^{-10} M) and do not forget entering a new file name for every new experiment. After you finish all experiments, fill both vials and the capillary with fresh BGE buffer solution.

Evaluation of results

Process the acquired data using the MS Excel program. Import the ASCII data into a sheet and construct a graph with all recorded electrophoregrams. In a table, show migration time, peak height, peak width at half maximum (FWHM) and number of theoretical plates for all concentrations of the analyte in the set. Construct calibration graphs, i.e. linear function of peak height and peak area vs. analyte concentration. Estimate a concentration limit of detection (mol.l^{-1}):

$$LOD = \frac{3 \cdot s \cdot c}{H}$$

where H is the height of the peak of rhodamine 6G, c is the rhodamine 6G concentration and s is the standard deviation of the noise signal intensity (use at least 20 values for its calculation).



3. Questions

1. *Two optical filters, selected from the set of filters with transmission spectra shown in Fig. 5a, are placed in front of PMT. The emission spectrum of rhodamine 6G is in Fig. 5b. Which two filters would you select? Why?*

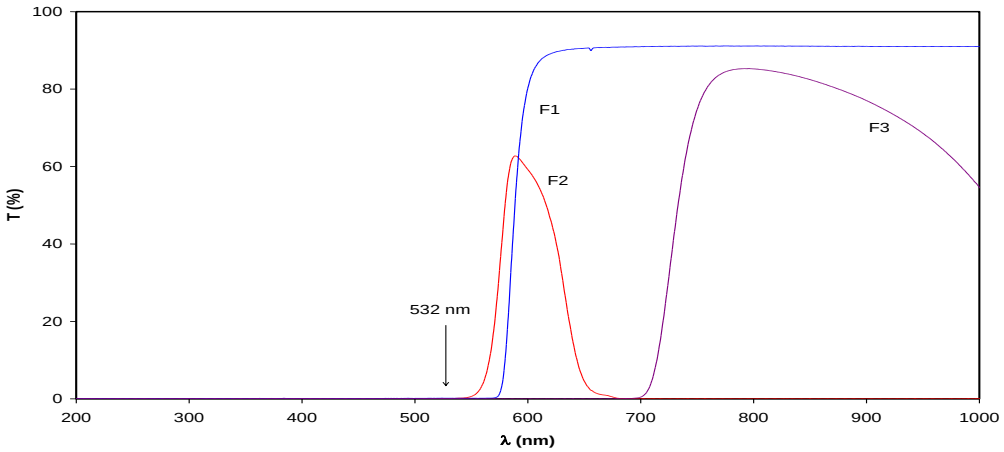


Figure 5a. Transmission spectra of selected optical filters

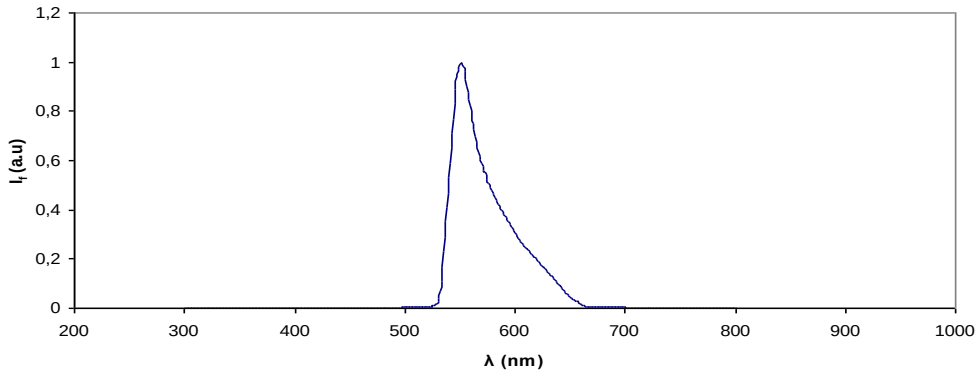


Figure 5b. Emission spectrum of rhodamine 6G

2. *A non-zero value of signal–to-noise could be observed even in the case only water or BGE was present in the capillary. Explain this observation.*
3. *Explain the "peak tailing" in the electrophoregrams of rhodamine 6G. How was the peak tailing suppressed during the experiments? Describe other ways of suppressing peak tailing in CE.*
4. *Compare the calculated values of number of theoretical plates in experiments with various concentrations of rhodamine 6G. Explain the differences.*
5. *Increased diffusion of the analyte zones is frequently observed at Joule's heat higher than 1 W per meter of capillary. What power (in the form of Joule's heat) was produced in the capillary during your CELIF experiment? Could production of heat in your experiment cause zone (peak) broadening? Suggest ways of decreasing excessive capillary heating.*
6. *It is possible that some experiments did not provide results in accordance with your expectation. Describe the difference from your assumption and try to explain it.*
7. *Calculate velocity and effective ion mobility of the rhodamine 6G zone during the separation.*

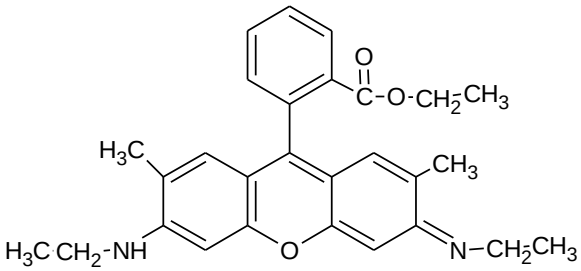
B. Separation of rhodamine dyes

Flush the capillary with BGE solution for at least 5 minutes and check the current stability at a voltage of U = 15 kV.

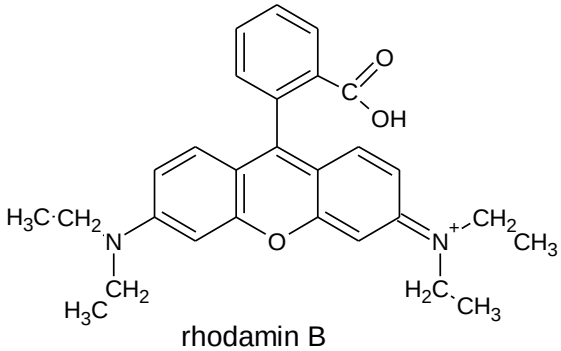
Prepare a sample mixture of rhodamine 6G, rhodamine 123 and rhodamine B with concentrations of 1 nM, 100 nM and 10 nM, respectively. Use the BGE for sample dilution.

Stock solutions of the rhodamine-based dyes were prepared with dissolution of the dyes (Sigma-Aldrich) in mixed water/ethanol solvent and concentrations of the dyes in the stock solutions are:

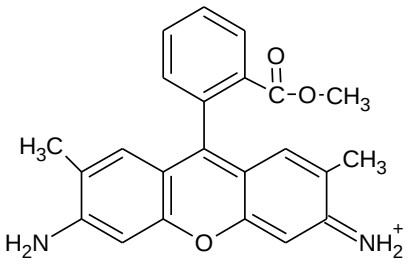
rhodamine 6G	2 mM
rhodamine 123	300 μM
rhodamine B	1 mM



rhodamin 6G



rhodamin B



rhodamin 123

Figure 6: Structure of the rhodamine dyes

Inject the sample electrokinetically at 5 kV applied for 10 s. Use the BGE: 50 mM solution of citric acid in 10 % EtOH (v/v) and add concentrated ammonia solution to adjust pH = 2.5. Perform electrophoretic separation at 15 kV. Using MS Excel program construct an electrophoregram of the separation. After you finish all experiments, fill both the injection and detector vials as well as the capillary with fresh BGE solution.

Questions

1. *Compare migration times of peaks in this experiment with the migration time of the rhodamine 6G peak in Experiment A. Which peak in this experiment corresponds to rhodamine 6G?*
2. *How many peaks can you observe in the electrophoregram? Is this number different from your hypothesis? In case it is different provide an explanation.*
3. *You know the size of all dyes (from its structural formula) and the charge of the dyes at pH ~ 2.5. Determine which peaks correspond to rhodamine B and rhodamine 123.*
4. *Write the migration times, calculated effective migration times and number of theoretical plates of each analyte into a table.*
5. *Suggest options to improve separation efficiency (number of theoretical plates).*
6. *According to the calibration graph obtained in Experiment A calculate the concentration of rhodamine 6G in the mixed sample and compare it with the expected value.*

C. Laboratory report

In laboratory reports, write down the date, all names of participants, and titles of the experiments. One report for the entire group is sufficient, preferably in electronic format (Word document). Copy neither the principle nor the instructions from this manual. On the other hand, write down all experimental conditions (sample preparations, dilutions, injections, capillary pretreatment, electrical current in capillary, etc.), if your experiments would be reproduced. Present your results in a clear and well-arranged form (inserted Excel graphs and tables). Answer all questions stated at the end of every task (experiment).

Finally, review critically the experiments you made. Discuss reproducibility and sensitivity of the method and importance of the method for practice. We appreciate all comments and proposals leading to improvement of this laboratory course.