

Online enzyme assays in capillary electrophoresis

or

In-capillary cooking

What is it?

In online capillary electrophoresis enzyme assays the enzyme and substrate are injected separately into the capillary where they are mixed and where the enzyme reaction takes place. After certain reaction time, voltage is applied and enzyme, substrate and product(s) of the reaction are separated and detected.

Why is it useful?

The online operation is beneficial because of its high degree of automation. Automation brings the advantage of high-throughput measurements without presence of an operator. Automated mixing and operation increase repeatability of the process and minimize the risk of sample loss or contamination as well as human errors. It is particularly useful for comparison-based studies (screening of inhibitors etc.)

Enzyme kinetics studies

Studies of kinetics of enzyme reactions are an important part of research in the fields of biology, biochemistry, medicine and pharmacy. Kinetic parameters such as Michaelis constant, maximum reaction rate, pH and temperature optimum need to be determined for various reasons. One application example is screening of inhibitors of specific enzyme reactions, which is important in search for new drugs. Evaluation of inhibitory activity is also important in assessment of toxicity. For example, acetylcholinesterase inhibition is typical feature and principle of action for many venoms, poisons, nerve agents, insecticides but also medicinal drugs.

Measurement of enzyme kinetics

To monitor enzyme reaction, change of concentration of substrate and/or product(s) in time must be measured. UV/Vis spectrometry is routinely used for this purpose. The problem is that the substrate and/or product of the reaction must absorb UV/Vis radiation and their spectra must significantly differ from each other. If not, chromogenic substrates are used, with the obvious disadvantage that they are not genuine natural substrates to the enzymes.

Separation techniques in enzyme assays

In some cases when substrate and products are not distinguishable by spectrophotometry, use of separation techniques is beneficial. Typically, the enzyme reaction is performed outside the separation system and the reaction mixture is injected for separation after a given reaction time. In **Figure 1** you can see application of capillary electrophoresis to monitoring of enzyme reaction of chitotriose (trimer) that is cleaved by *N*-acetylhexosaminidase to chitobiose (dimer) and *N*-acetylglucosamine (monomer). The dimer is subsequently cleaved by the enzyme to the monomer.

Electropherogram in the **left bottom panel** of **Figure 1** shows automated monitoring of enzyme reaction where the enzyme is mixed manually with the substrate in a sample vial and then repeatedly injected to the capillary as the reaction proceeds. The **right bottom panel** shows detail of separation of the trimer, dimer and monomer.

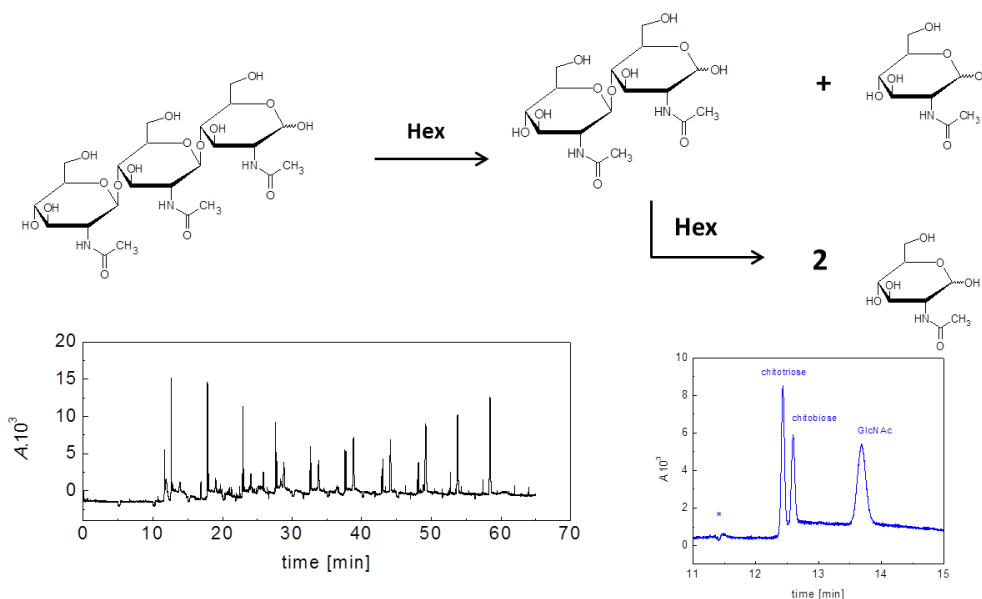


Figure 1 Application of capillary electrophoresis in offline monitoring of enzyme reaction
*Křížek et al.: Anal. Bioanal. Chem. 405 (2013) 2425

The data from the **left bottom panel** of **Figure 1** are expressed as the dependence of the concentration of the trimer, dimer and monomer on reaction time in the **figure 2**.

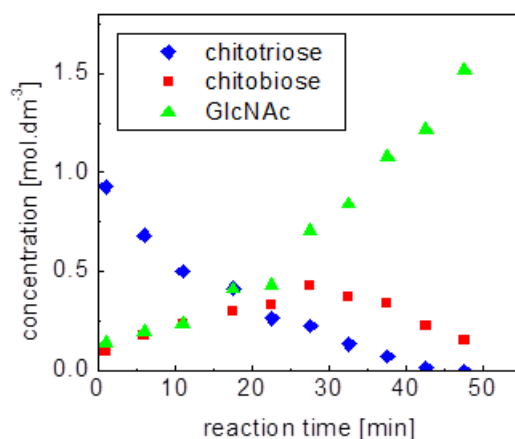


Figure 2 Dependence of concentration of reaction substrate, intermediate and product on reaction time acquired by offline capillary electrophoresis assay

Online enzyme assays in capillary electrophoresis

Online enzyme assays go one step further as they allow us to perform the enzyme reaction directly inside the separation capillary.

Injection of one reactant into solution of the other

First reported online enzyme assays were based on the capillary being filled with a background electrolyte containing enzyme, into which substrate was injected as a sample. As substrate migrated through the enzyme-containing BGE, the reaction was proceeding and the peak of the substrate was thus getting smaller. The reaction could also be performed in the other way – substrate was present in the BGE and the enzyme was injected as a sample. In both cases, this setup brought difficulties with evaluation regarding the fact that the reaction occurred continuously during the run. Resulting peaks were thus often not very sharp and evaluation was not clear and accurate.

Mixing of reactants at capillary inlet

In most recent studies different setup is used where substrate and enzyme are injected as separate zones to the inlet part of the capillary. Then they are mixed either electrophoretically or by diffusion. After a specified reaction time, the separation voltage is applied and the reactants are separated.

Electrophoretic mixing

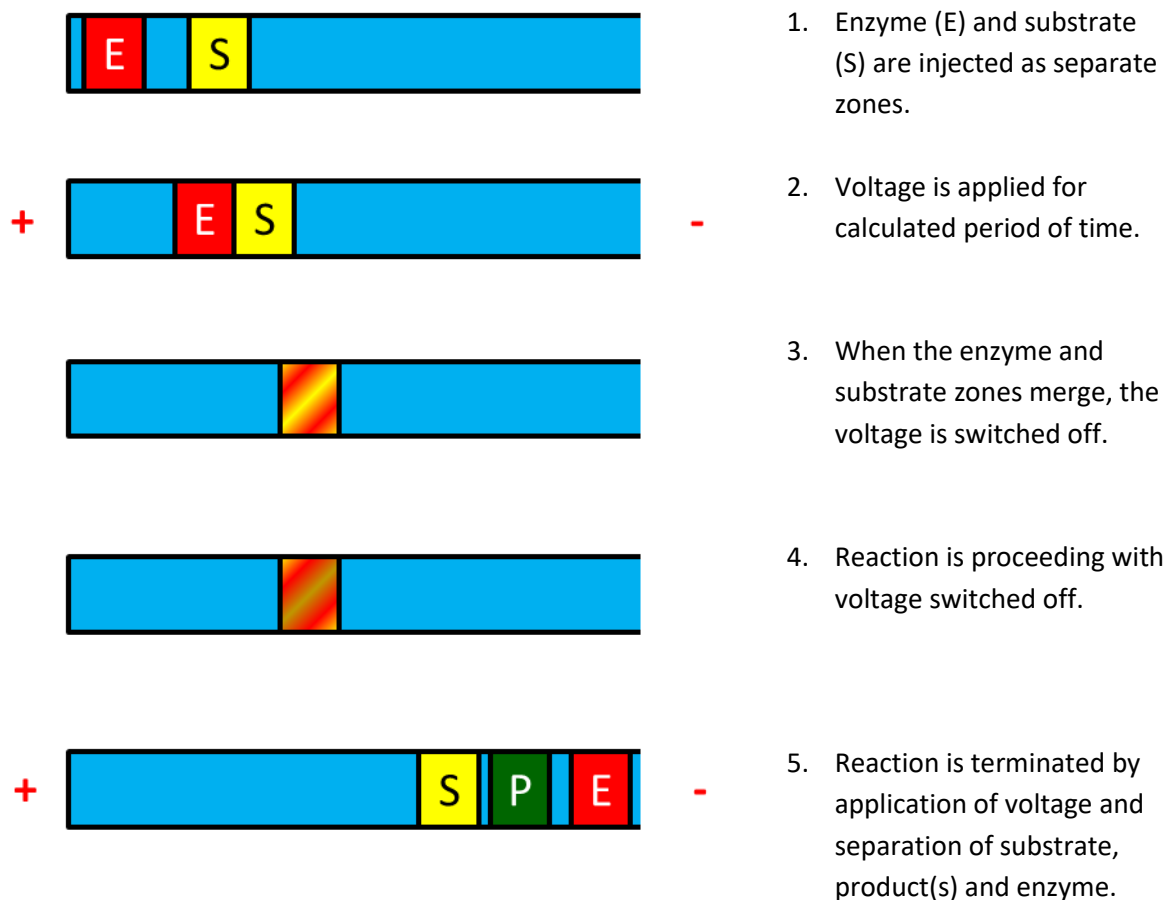
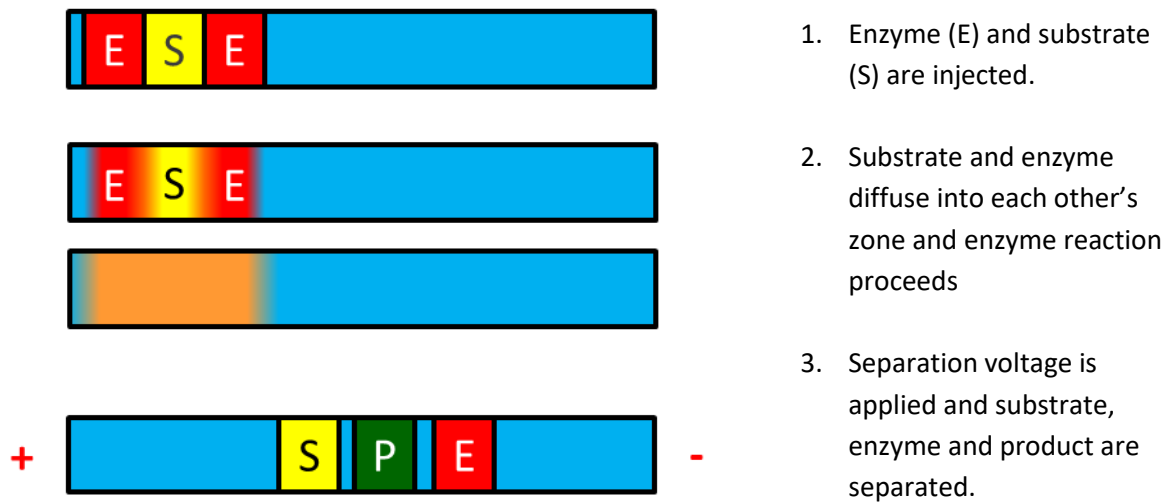


Figure 4 Electrophoretic mixing of enzyme and substrate in online enzyme assays

Advantage of this approach is that we know concentrations of reactants in the reaction zone quite exactly and we can not only compare reaction rates but also calculate kinetic parameters such as Michaelis constant.

Disadvantage is that we have to know electrophoretic mobilities in the used separation buffer to be able to calculate the time and voltage needed for the zones to merge (step 2).

Mixing by diffusion



1. Enzyme (E) and substrate (S) are injected.
2. Substrate and enzyme diffuse into each other's zone and enzyme reaction proceeds
3. Separation voltage is applied and substrate, enzyme and product are separated.

Figure 5 Mixing of reactants by diffusion in online enzyme assays

Advantage of this approach is that electrophoretic mobilities of the reactants do not need to be known as electrophoretic migration is not used for their mixing.

Disadvantage is that we don't exactly know concentrations of reactants in the reaction zone. Furthermore, the reaction runs during the mixing so the concentrations of the reactants keep changing during the reaction. For these reasons it is difficult to estimate absolute reaction rates and this approach is therefore limited for relative measurements such as inhibitor screening etc.

Task 1 – electrophoretic vs. diffusion-driven mixing of reactants

In these two articles:

J. Zhang et al.: Electrophoresis 29 (2008) 3694

Y. Martin-Biosca et al.: J. Sep. Sci. 32 (2009) 1748

Did the authors mix the reagents in the capillary using electrophoretic migration or diffusion?

Transverse Diffusion of Laminar Flow Profiles (TDLFP)

The TDLFP approach is based on online diffusion-driven mixing of the substrate and enzyme zones but it allows us to design the experiment in such a way that we can prepare a reaction mixture with known and relatively homogeneous concentrations of individual reactants. Therefore, we can calculate absolute reaction rates.

The method is based on **two presumptions**:

1. The transverse diffusion in the capillary is significantly faster than the longitudinal one, which is based on the fact that the capillary is extremely narrow (50 μm) and rather long (50 cm).
2. Pressure used to inject the zones is high enough to cause parabolic profile of the injected zones.

The process is described in **Figure 6**:

1. One of the reactants, let's say the substrate, is injected using a pressure. This results in a zone of substrate with parabolic shape. Then during very short period of time, thanks to diffusion, concentration in transverse direction becomes homogeneous. This time is not long enough for significant diffusion in the longitudinal direction.
2. In the second step, a zone of enzyme is injected. The injection pressure again deforms shapes of the peaks and, thanks to this, the enzyme (red) zone is „inserted“ into the substrate (blue) zone. After the injection, fast diffusion in transverse direction again homogenizes concentrations across the capillary.
3. If an appropriate sequence of injections is used (usually concluded by injection of background electrolyte without enzyme or substrate) we can end up with concentration profiles shown in the bottom part of the figure, where the reaction zone has practically homogeneous concentration of both reactants and this concentration can be calculated.

Task 2 – electrokinetic injection in TDLFP

What will be the difference between TDLFP mixing performed using hydrodynamic injection (by pressure) and electrokinetic injection (by voltage).

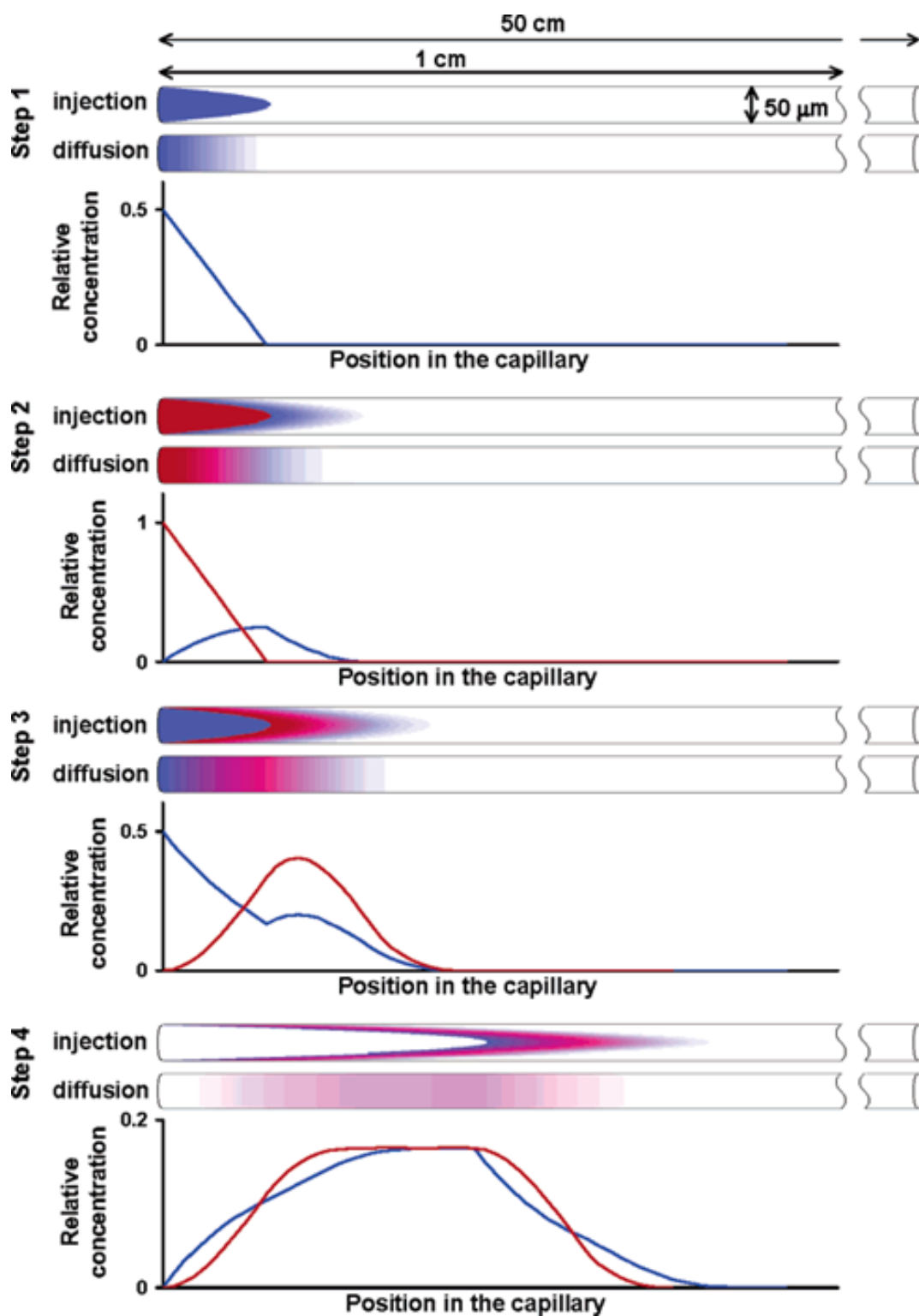


Figure 6 Principle of the Transverse Diffusion of Laminar Flow Profiles approach

*Okhonin et al.: Anal. Chem. 77 (2005) 5925.

Figure 7 shows how computer simulation of the process (**top panels**) can be used for *in silico* pre-optimization the injection sequence of the reactants. The prediction was in a good agreement with the experimentally obtained electropherograms (**bottom panels**).

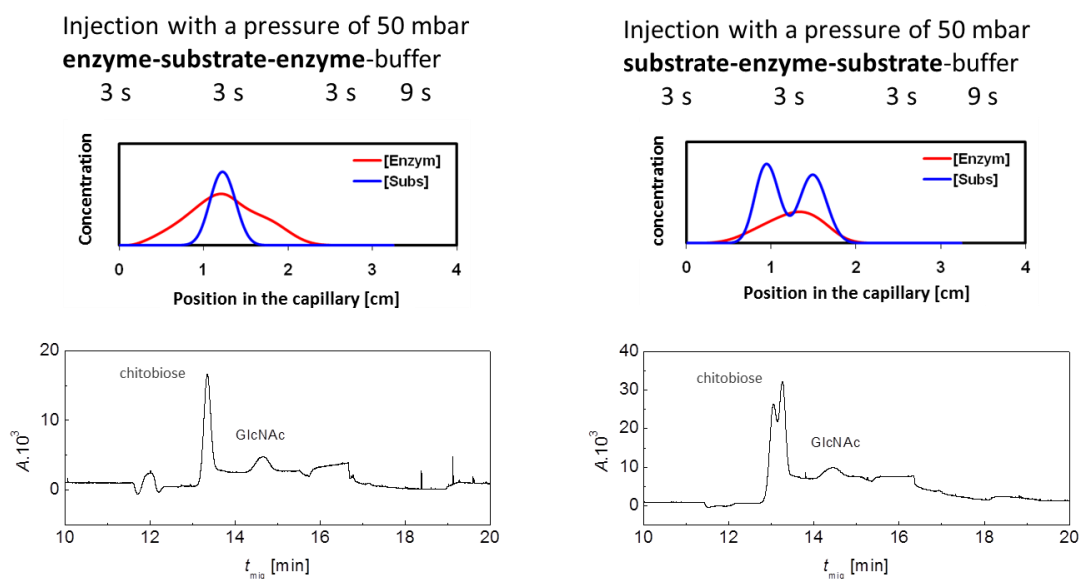


Figure 7 Application of the TDLFP approach to enzyme assay of *N*-acetylhexosaminidase with chitobiose as substrate and *N*-acetylglucosamine (GlcNAc) as product

* Křížek et al.: Anal. Bioanal. Chem. 405 (2013) 2425.

Task 3 – comparison of predicted and experimental data

Explain why in the top right panel of **Figure 7**, the left part of the twinned substrate peak is higher while in the electropherogram in the bottom right panel the right part of the substrate peak is higher.

Figure 8 shows application of the method for assessment of inhibitory effect of dimethylformamide (DMF) on the enzyme reaction.

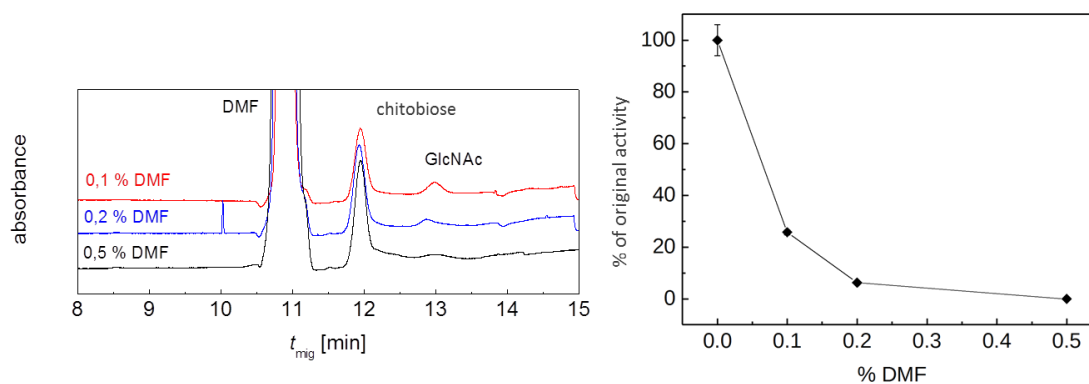


Figure 8 Application of the TDLFP method to study the inhibitory effect of dimethylformamide (DMF) on the enzyme reaction