

## Multi-compound electrophoretic assays for biocatalytic activity with the ActiPix D100 UV area imaging detector

**Keywords:** EMMA, biocatalysis, capillary electrophoresis, on-line capillary enzyme assay, substrate specificity, tyramine oxidase,

### Summary

This application note describes how the ActiPix UV area imaging detector can be used to test biocatalyst substrate specificity towards a mixture of UV active compounds using a continuous engagement electrophoretically mediated microanalysis (EMMA) assay method.

A plug of the putative substrates is injected into a fused silica capillary containing the background electrolyte and dissolved enzyme. The components are separated in the initial part of the run, avoiding the competitive binding and inhibition problems that can occur in standard enzyme assays. The peaks corresponding to each of the separated components are measured at multiple time points along the looped capillary, providing intrinsic self-referencing and allowing the identification of good substrates as those components that show decreased peak areas over time.

### Instrumentation and Materials

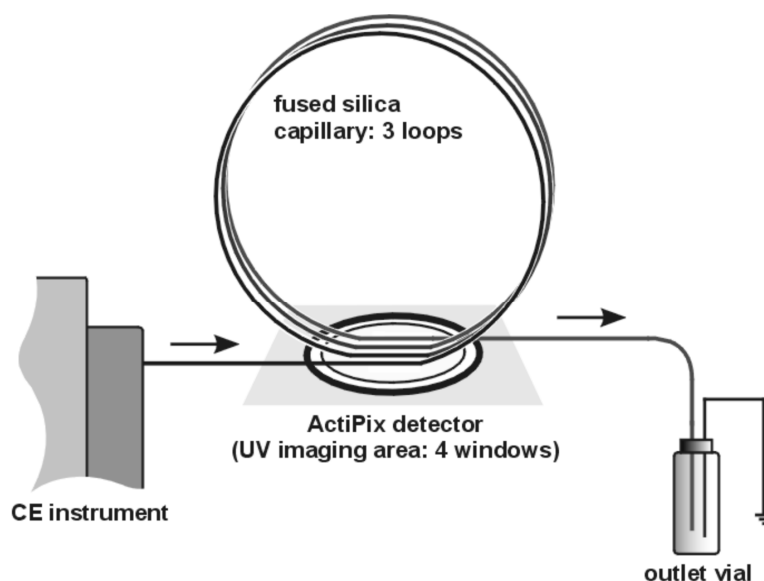
- |   |                          |
|---|--------------------------|
| ✓ ActiPix UV imaging system                             | (Paraytec D100)          |
| ✓ 144 cm 75 x 193 µm fused silica capillary             | (Polymicro Technologies) |
| ✓ Commercial capillary electrophoresis system*          |                          |
| ✓ HPLC grade water                                      | (Fisher Scientific)      |
| ✓ Tyramine oxidase (TAO)                                | (Sigma-Aldrich)          |
| ✓ 25 mM ammonium acetate solution at pH 7.0**           | (Sigma-Aldrich)          |
| ✓ agmatine sulphate                                     | (Sigma-Aldrich)          |
| ✓ tyramine base   | (Sigma-Aldrich)          |
| ✓ benzylamine   | (Sigma-Aldrich)          |
| ✓ serotonin hydrochloride                               | (Sigma-Aldrich)          |
| ✓ primaquine diphosphate                                | (Sigma-Aldrich)          |
| ✓ 2-phenylethylamine                                    | (Sigma-Aldrich)          |
| ✓ benzyl alcohol  | (Sigma-Aldrich)          |
| ✓ NAD (β-nicotinamide adenine dinucleotide sodium salt) | (Sigma-Aldrich)          |

\* e.g. PrinCE capillary electrophoresis system

\*\* prepared by adjusting the pH of the solution with concentrated ammonium hydrogencarbonate

### Method

Assemble setup similar to the schematic shown in Figure 1. Note that the method will work with most commercially available CE instruments, provided that a cartridge bringing the capillary outside the instrument is available (e.g. that for MS). Note the capillary length will need to be modified for other systems.



**Figure 1:** Schematic representation of CE arrangement with 3 loops and 4 detection windows (with effective lengths of 40, 70, 100 and 130 cm) for on-capillary reaction monitoring using the ActiPix.<sup>2</sup>

## Procedure

Background electrolyte (BGE) solutions containing 25 mM ammonium acetate (adjusted to pH 7.0 with concentrated ammonium hydrogencarbonate) were prepared using HPLC grade water and various concentrations of tyramine oxidase (TAO), up to a maximum of 15  $\mu\text{l}$  of a 1.4  $\text{mg ml}^{-1}$  (5U  $\text{ml}^{-1}$ ) of TAO stock solution per 4 ml of BGE.

Multi-compound reaction mixtures containing 5.48 mM agmatine, 0.455 mM tyramine, 0.560 mM benzylamine, 0.588 mM serotonin, 0.275 mM primaquine, 0.495 mM 2-phenylethylamine, 0.601 mM benzyl alcohol and 0.183 mM NAD were dissolved in the various BGE solutions.

At the beginning of each run, the separation capillary is preconditioned using the following

- 1.5 bar, 2 min, 0.1 M NaOH,
- 1.5 bar, 2 min, water,
- 1.5 bar, 3 min, BGE

The capillary electrophoresis (CE) run was then started, with components added in the following sequence:

- 30 mbar, 6 s, BGE without enzyme
- 30 mbar, 6 s, reactant mixture
- 30 mbar, 6 s, BGE without enzyme

This was followed by the application of voltage (normally 25 kV) and pressure (30 mbar) at ambient temperature (25 °C).

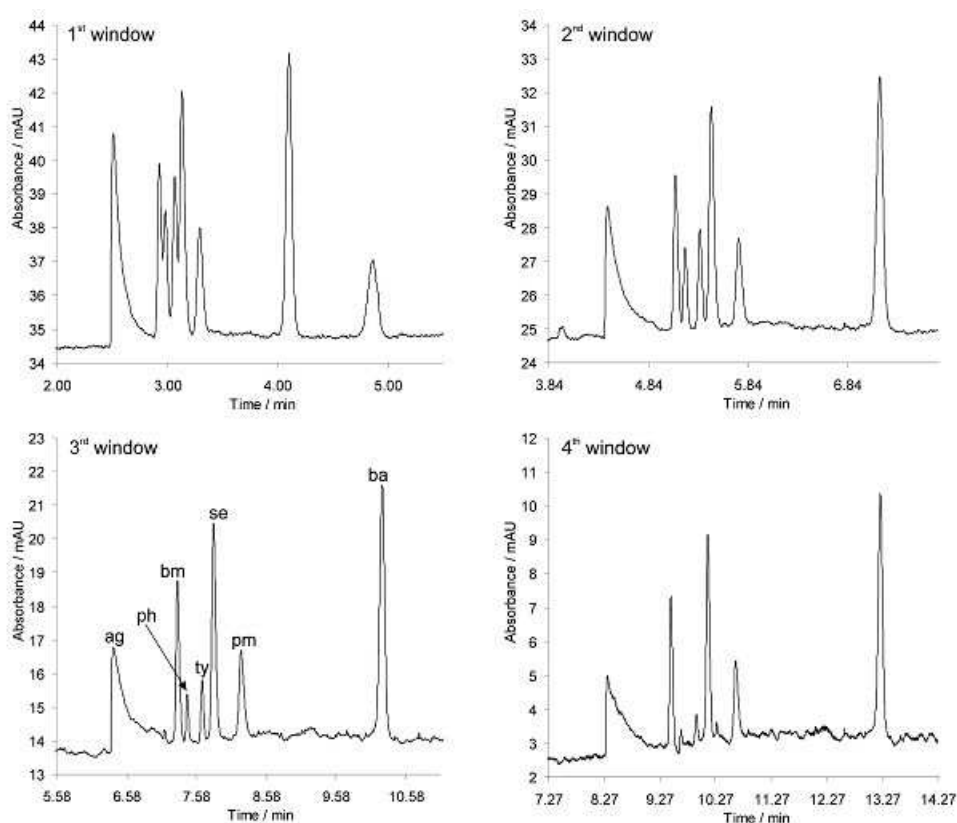
## Principles

- A plug of the multi compound reaction solution is injected at the capillary inlet and driven by the application of external pressure along the capillary.

- Continuous engagement EMMA allows the interaction between the enzyme and substrates to occur throughout the entire CE run, unlike plug-plug EMMA methods that have only a short overlap period between the substrate and enzyme zones. This permits much lower concentrations of enzyme to be used.
- The ActiPix detector and loop arrangement enables the progress of the enzyme-catalyzed reactions to be followed at the four spatially resolved windows.
- The peak area, which corresponds to the amount of compound present, can be calculated using commercially available software.
- A compound can be regarded as a good substrate if a decrease in peak area is observed as the peak traverses the four windows.
- As peak separation is often incomplete by the first window, competitive binding could occur and limit the turnover rate of active substrates in the initial capillary section.

## Results and Discussion

Figure 2 shows the absorbance versus time plot of the injected compounds as they pass the four spatially resolved windows. The peak areas, and therefore concentration, of 2-phenylethylamine (ph) and tyramine (ty) decrease as the reaction passes the four windows, indicating they are both good substrates for TAO.



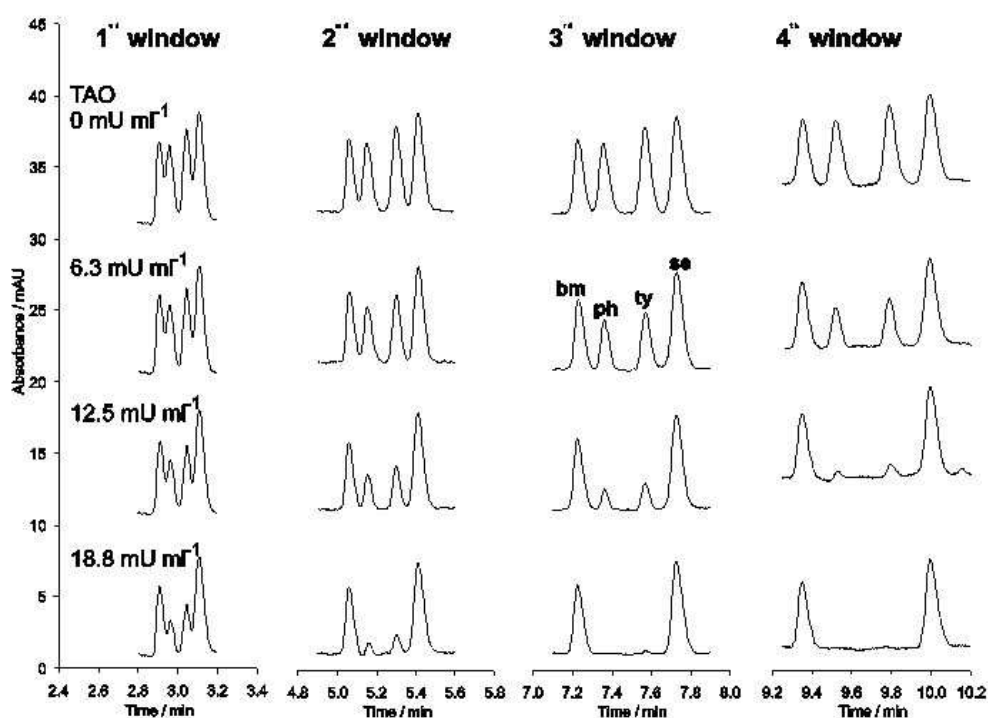
**Figure 2:** Normalised electropherograms showing time-dependent reaction for ph and ty as the compounds pass all four capillary windows. BGE containing  $12.5 \text{ mU ml}^{-1}$  TAO. Separation conditions, 25 kV + 30 mbar. Labels: ag, agmatine; bm, benzylamine; ph, 2-phenylethylamine; ty, tyramine; se, serotonin; pm, primaquine; ba, benzyl alcohol.

The system is pressure driven, causing the peak profiles to broaden as the compounds travel the length of the capillary. The application of this external pressure has been found to have an influence on the observed conversion, with the peak broadening increasing the volume of enzyme and oxygen accessing the substrate band and leading to increased conversions.

Conversion rates for the tyramine and 2-phenylethylamine substrates are calculated as a function of decreasing peak area over time as 5.0 and 7.1  $\text{fmol s}^{-1}$  respectively.

These values compare favourably with the expected conversion rate for tyramine that is calculated to be 7.8  $\text{fmol s}^{-1}$  at 37°C.

Figure 3 shows the effect of changing the concentration of TAO in the BGE while keeping the concentrations of the putative substrates constant.



**Figure 3:** Effect of change of enzyme concentration in continuous engagement EMMA showing 1 min sections from electropherograms at each of the four detection windows at various TAO concentrations. Labels: bm, benzylamine; ph, 2-phenylethylamine; ty, tyramine; se, serotonin.

The results show that the method is amenable to working with low enzyme activities,  $\text{mU ml}^{-1}$ . This is of vital importance when working with expensive enzymes or those that have just been purified and are of limited availability.

## Conclusions

- Multi-compound continuous engagement EMMA assay using the ActiPix and a capillary with three loops has been shown to enable multi-component substrate specificity analysis for the tyramine oxidase enzyme. To date, this has not been possible using existing systems.
- UV absorption peaks due to all compounds in a multi-compound mixture were monitored at four windows, enabling the ready identification of tyramine and 2-phenylethylamine as the only reactive components.
- After the components have separated in the initial part of the run there are no competitive binding or inhibition problems as would occur in a standard enzyme assay with all components incubated together.
- Imaging the same peak of a separated compound at multiple time points provides intrinsic self-referencing, since the method is free from any uncertainties due to sampling which are present in other enzyme assay methods.
- Only components that have a peak area that decreases over time are good substrates.

## Further Applications

- This method provides a quick, easily integrated technique for assessing enzyme-substrate specificity using a multi-compound mixture of putative substrates and has the potential to be of broad applicability to other UV active compounds.
- Use of the ActiPix with a multi-looped capillary could readily be extended to other enzyme classes, e.g. kinases.
- The technique offers an alternative to enzyme assays that involve the separate steps of incubation, sampling at a number of time points, separation and detection.

## References

- 1 [Pawel L. Urban, David M. Goodall, Alexandre Z. Carvalho, Edmund T. Bergström, Ann Van Schepdael, Neil C. Bruce; \*Multi-compound electrophoretic assays for tyramine oxidase with a UV area detector imaging multiple windows on a looped capillary\*, Journal of Chromatography A, 2008, in press.](#)
- 2 Reprinted from reference 1 with kind permission from Elsevier.