# Limits of miniaturization: Assessing ITP performance in sub-micron and nanochannels

Electronic Supplementary Information

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## 6 Additional methods

### 6.1 Fluorophore labeling of amino acids

All chemicals were acquired from Sigma-Aldrich Co. (Zwijndrecht, The Netherlands), unless noted otherwise. Labeling of glutamic acid, phenyl alanine, and leucine with fluorescein isothiocyanate (FITC) was performed as follows. To 0.5 mmol of amino acid an aqueous solution of potassium hydroxide, 1 g/ml, was added in a ratio of 1:1 (weight:volume), followed by addition of 1 ml of ethanol. Under vigorous stirring, on ice, a suspension of FITC, 1 mol/L in ethanol was added to the amino acid 1:1 (mol:mol) together with 1 ml 0.5 mol/L potassium hydroxide and 0.5 mL ethanol. This mixture was left on ice to react for 2 h in the dark under continued stirring. Purification of the reaction product was performed using a Gilson preparative HPLC system (Gilson, Inc., Middleton, USA) equipped with a Phenomex Gemini C18 column, 15x21 mm, 5 micron (Phenomenex, Torrance, USA) using an acetonitrile/water (10 mmol/L ammonium acetate, pH = 8) gradient. Purity of compounds was established with LC-UV-MS and CZE-LIF and found to be >99%. After freeze-drying, each purified FITC-amino was dissolved in dimethyl sulfoxide (DMSO), 1 mmol/L, and stored at -80 °C awaiting experiments.

FITC, having a pKa of 6.7 [1], fluoresces strongly when negatively charged at valence -2. FITC labeled biosamples are of general interest. FITC-labeled amino acids have been studied with CZE [2–4] chip ZE [5,6] and ITP in capillary [7–9], and in microchannels [10] and other techniques [11]. As unpurified reaction product is used almost exclusively in these publications, the two most abundant fluorescent by-products of this labeling are considered to be of interest and were purified and identified with LC-UV-MS (Data not shown). One reaction by-product consisted of FITC where its isothiocyanate group (-N=C=S) was degraded to an amine, which in turn reacted with FITC thus forming a fluorescent dimer (MW 737). As degraded FITC was low abundant in the reaction mixture this implied that the degradation was slower than the reaction with another FITC molecule. This dimer has an almost 4 times lower fluorescent intensity (at 488 nm excitation, 514 nm emission) compared to FITC, due in part to a shifted fluorescence wavelength optimum. The other abundant by-product (MW 436) was FITC that had reacted with ethanol (-N=C-S-O-$C_2H_5$). FITC is commonly dissolved in ethanol, but our data suggests this should be avoided.

### 6.2 Capillary electrophoresis

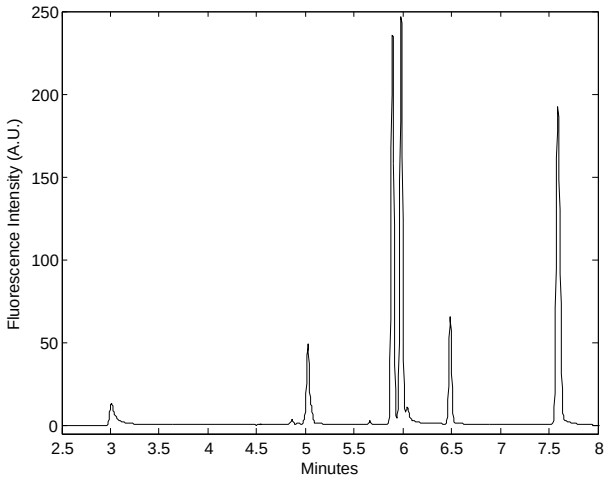


**Fig. 5** Typical CZE electropherogram of labeled amino acids and FITC by-products. From left to right first at 3 minutes the EOF marker bodipy is seen, then FITC-ethanol, leucine-FITC, phenyl alanine-FITC , fluorescein amine-FITC and glutamate-FITC, all except FITC-Ethanol at 10 µmol/L.

A PACE 5000 capillary electrophoresis apparatus equipped with laser-induced-fluorescence detection, operating at 488 nm excitation and 518 nm emission (Beckman Instruments, USA) was used for CZE. CZE was applied to determine the mobilities of the FITC-amino acids as well as establishing

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their purity for the purpose of fluorescence measurements. The temperature during all experiments was maintained at 25 °C by the instrument thermostat. The fused silica capillary (Inacom Instruments, Overberg, The Netherlands) used for CZE had a 50 µm inner diameter and a total length of 56.9 cm measuring 50.3 cm from inlet to detector. Runs were performed at 20 kV, giving a typical current of $14.4 \pm 0.1$ µA during runs. The background electrolyte was a 10 mmol/L solution of disodium tetraborate ($Na_2B_4O_7$). It was adjusted to pH = 9.50 with sodium hydroxide solution (to ensure maximum fluorescence intensity of fluorescein, as this is pH dependent pKa = 6.7[1]), verified with a Hanna HI 4521 pH meter (milli-pH unit resolution, HANNA instruments Inc. Woonsocket, USA). As a marker for the electro-osmotic flow (EOF) the uncharged fluorophore 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (bodipy), was added.

CZE of the labeled amino acids and the two reaction by-products was performed establishing their purity for fluorescence measurements (ESI Figure 5). Their calculated electrophoretic mobilities are given in Table 2. Based on their mobility phenyl alanine-FITC and glutamate-FITC were selected for the ITP protocol, as their difference increased the potential of spacing by compounds in the yeast biomatrix. As ITP electrolytes, chloride was chosen as leading ion, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) as trailing ion and sodium as the common counterion (See Table 2).

**Table 2** Electrophoretic mobilities (µ) and valences at pH 9.5 of the FITC labeled amino acids, FITC by-products and the ions in the ITP electrolytes.

| Ion | valence | µ ($10^{-9}m^2V^{-1}s^{-1}$) |
|---|---|---|
| HEPES[12],(trailing ion) | -1 | -21.8 |
| FITC-ethanol | -2 | -31.5 |
| Leucine-FITC | -3 | -38.4 |
| Phenyl alanine-FITC | -3 | -39.0 |
| Fluorescein amine-FITC | -4 | -42.0 |
| Glutamate-FITC | -4 | -47.3 |
| Chloride[13], (leading ion) | -1 | -79.1 |
| Sodium[13], (counter ion) | +1 | 51.9 |

### 6.3 Yeast pretreatment for metabolite extraction

To hydrolysate of delipided yeast biomass of *P. pastoris* (YPp(N)-HyBm1, Protein Labeling Innovation PLI, Leiden, The Netherlands) cold (-20 °C) 80:20 methanol:water was added. After placement for 30 minutes in an ultrasonic bath, this mixture was centrifuged and the supernatant collected. This extraction procedure was repeated and the combined supernatants were freeze-dried, after removal of methanol. These extracted compounds were redissolved in dimethyl sulfoxide (DMSO) to 10 mg/mL (relative to the original weight of the hydrolysate prior to pretreatment) and stored at -80 °C awaiting experiments.

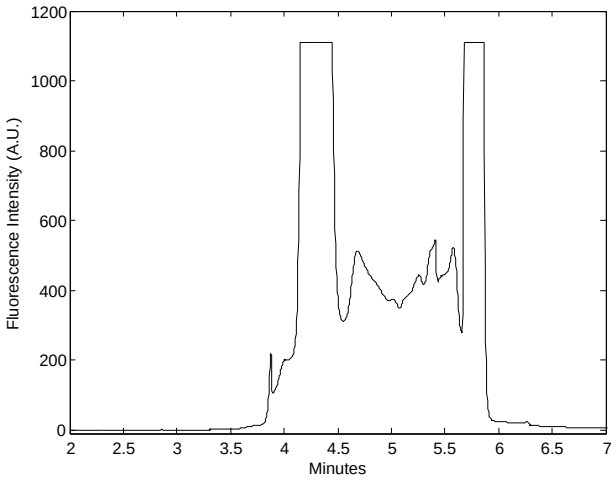


**Fig. 6** Capillary-ITP for a 0.12 µL sample of yeast biomatrix and 5 glutamate-FITC and phenyl alanine-FITC both 50 µmol/L. The measurement using LIF shows from left to right, Phe-FITC and Glu-FITC, spaced by the yeast biomatrix, with TE to the left and LE to the right of the sample.

### 6.4 Isotachophoresis protocol development in capillary

A PACE 5000 capillary electrophoresis apparatus equipped with laser-induced-fluorescence detection, operating at 488 nm excitation and 518 nm emission (Beckman Instruments, USA) was used for ITP experiments. The temperature during all experiments was maintained at 25 °C by the instrument thermostat.

The developed protocol for capillary ITP was as follows. ITP electrolytes were introduced into the fused silica capillary (27.22 cm total length, 20.38 cm from inlet to detector, 50 µm ID; Inacom Instruments, Overberg, The Netherlands) by pressure. The capillary was first flushed with trailing electrolyte (TE), 5 mmol/L of HEPES. A plug of sample was then injected, consisted of 80:20:10 TE:pretreated yeast extract:labeled amino acid solutions (v:v:v, final concentration of each amino acid 50 µmol/L) by applied pressure of 0.5 psi for 60 s which according to the Hagen-Poiseulle equation corresponds to a volume of 0.12 µL or 20% of the total capillary (6 cm). Lastly that end of the capillary was placed in a vial with leading electrolyte (LE), 10 mmol/L of NaCl, the other in one with TE. ITP was then induced by applying 5 kV. The electrolytes were prepared anew in deionized water each day, adjusted to pH 9.50 with sodium hydroxide, and stored under argon until used. Vials for experiments were covered with caps containing septa. Although sample ions migrated opposite to the EOF, at pH 9.50 the EOF was dominant. For ITP this is an advantage as more time is available to equilibrate

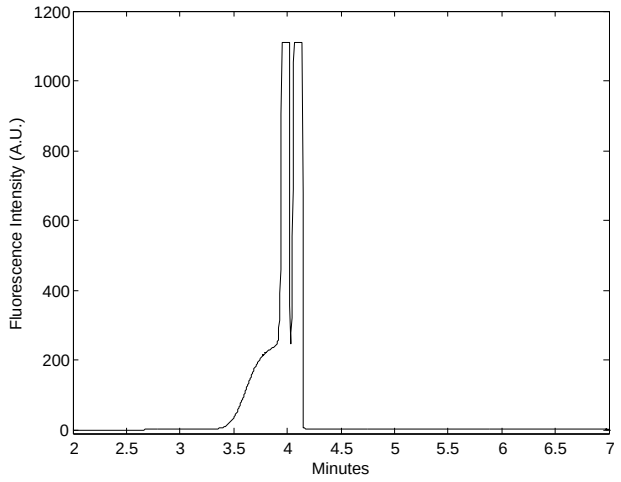


**Fig. 7** Capillary-ITP result for a 0.12 µL sample containing glutamate-FITC and phenyl alanine-FITC both 50 µmol/L. The measurement using LIF shows from left to right, Phe-FITC and Glu-FITC, shown to be concentrated so far as to saturate the detector (>250 µmol/L).

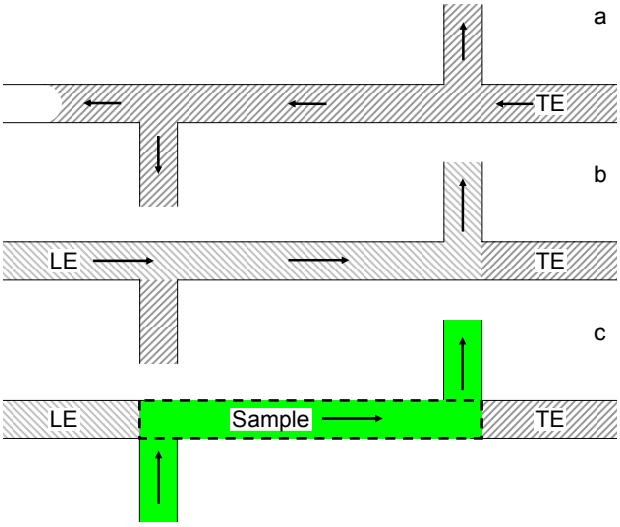


**Fig. 8** On-chip double-T injection for quantitative ITP. a) The chip is filled with trailing electrolyte by capillary filling. b) After placement of the other electrolytes in the wells, leading electrolyte is flushed towards well E, which serves as waste reservoir. c) The sample volume in the nanochannel chip (dashed box), is created by means of an exclusive EOF, from sample well D to waste E. Appropriate empirical voltage settings correspond to contanement of the fluorescence between the side channels. Settings were found to vary per channel depth. After an ITP experiment, the injection plug can be recreated by directly re-applying the sample injection voltages, e.g. those given in Figures 2 & 3.

before the analytes pass the detector.

A typical capillary ITP result is shown in Figure 6. Equilibrium formation could be concluded from the current during the run[14]. Spacing is seen between the bands of Glu-FITC and Phe-FITC. ITP of just the labeled amino acids only, does not show spacing (ESI Figure 7). ITP of yeast alone shows negligible native fluorescence (ESI Figure 11). From ITP results of each of the amino acids alone (ESI Figures 12 & 13), showing single zones, and in the presence of yeast extract (ESI Figures 14 & 15) showing added bands for Phe-FITC, it may be concluded that the fluorescence observed in the spacing is from Phe-FITC that interacted with compounds in the yeast biomatrix.

### 6.5 Chip injection protocol

Effectively translating quantitative ITP to the nano-chip format requires a well defined sample injection volume, to be interposed between a leading electrolyte (LE) and a trailing electrolyte (TE). Here, on chip T-junctions were used in combination with EOF, see Figure 1. The appropriate voltage settings on the reservoirs for sample plug formation and those for ITP, were obtained empirically by observing the effects of manual voltage changes with the fluorescence microscope, see ESI Figure 8 for details. The voltage settings used for the submicron channels are tabulated in Table 3 and those for the nanochannels in Table 3. These were incorporated in an automated protocol of the Labsmith Sequence software to execute the fast and/or simultaneous stepwise voltage changes required.

For a nano-ITP experiment first the sample plug creation step was activated. Typically after a few minutes the plug would reform and traces of fluorescence in the separation channel were removed, allowing repeated experiments. When the stable plug was observed the ITP step was initiated manually. ITP itself was automatically preceded by a pre-ITP step of 100 ms. The pre-ITP step was needed to actively regulate the electrode in well D to zero, when set to float directly the Voltage would decrease to 0 V on a time scale of a few seconds, affecting injection accuracy. Afterwards the Voltage on well A could be increased manually to increase separation, as was done in the results in Figure 2b.

Implementation of the voltage settings was non-trivial and only feasible with fast and accurate computer control of the applied voltages. Switching without losing or extracting sample into or from the sidechannels proved the most critical step, being the main source of injection errors.

**Table 3** ITP voltage steps used for 3 µm wide, 330 nm deep channels.

| Well | Sample plug Figure 2a | Pre ITP 100 ms | ITP ESI Figure 10 | ITP final Figure 2b |
|---|---|---|---|---|
| A LE | 125 V | 125 V | 100 V | 400 |
| D Sample | 100 V | 0 V | float | float |
| E TE, waste | 0 V | 0 V | float | float |
| F TE | 100 V | 100 V | -100 V | -100 |

**Table 4** ITP voltage steps used for 10 µm wide, 50 nm deep channels.

| Well | Sample plug Figure 3a | ITP Figure 3b |
|---|---|---|
| A LE | 25 V | 100 V |
| D Sample | 25 V | float |
| E TE, waste | -50 V | float |
| F TE | 25 V | -100 V |

# 7 Additional results

## 7.1 Large sample volume injections

ITP experiments were also performed on larger sample volumes, e.g. 3.2 pL (ESI Figure 9). The ITP result for this larger volume demonstrated increased peak height and spacing, compared to results with smaller sample volumes (Figure 2). This indicates that the ITP results in Figure 2b & 3b correspond to so-called peak mode ITP[15], in contrast to plateau-mode in which case more analyte leads only to broadening of the zones[13,16]. Therefore, if more absolute amount of compound would be injected, even higher equilibrium concentrations can be expected. However, this requires either higher initial concentrations (>50 µmol/L) or injecting even larger volumes.

## 7.2 ITP in submicron channels at 200 V

Figure 10 shows the result of ITP in a 330 nm deep channel for an applied Voltage of 200 V. This result is with the injection shown in Figure 2a, demonstrating that 200 V is sufficient for isotachophoretic focussing but is insufficient to separate the amino acids. By subsequently increasing the voltage during the experiment to 500 V the separation result shown in Figure 2b was achieved.

## 7.3 Movie of nano ITP in a 50 nm deep channel

NanoITP.avi, in false color, background corrected and filtered.

## 7.4 Additional capillary ITP figures

Additional ITP Figures 10-15 as referenced to in subsection 6.4.

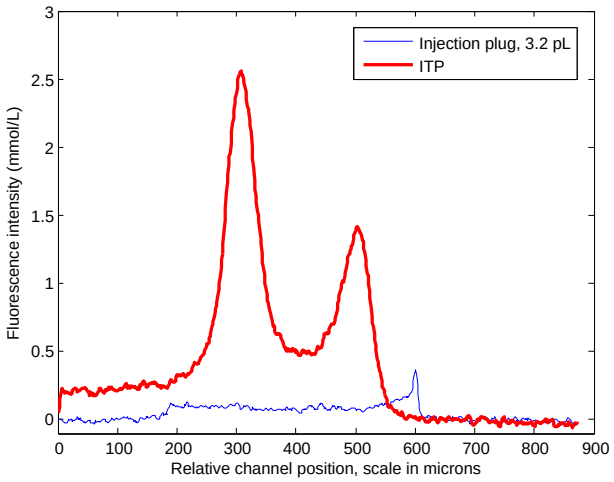


**Fig. 9** ITP result in a 330 nm deep channel. Based on the areas under the curves, the ITP result corresponds to an injection of 3.2 pL. This larger injection volume resulted in higher peaks and more spacing compared to the 0.4 pL sample in Figure 2c.

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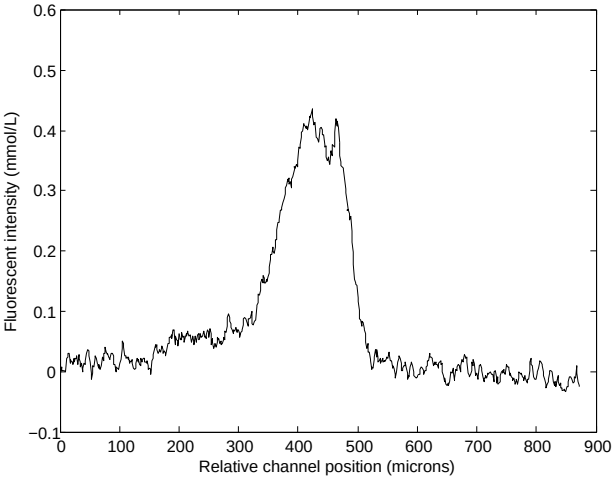


**Fig. 10** ITP foccussing result in a 330 nm deep channel, part of the same experiment as in Figure 2. The applied voltage difference of 200V (A = 100 V, F = -100 V).

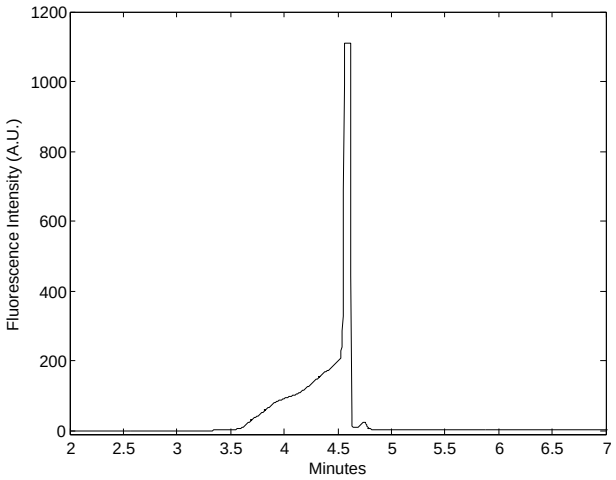


**Fig. 12** Graph showing the capillary-ITP result with 50 µmol/L phenyl alanine-FITC same protocol as used for Figure 6.

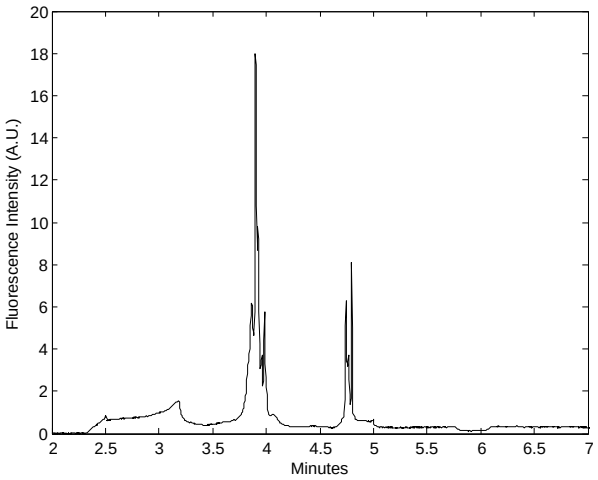


**Fig. 11** Graph showing the capillary-ITP result of yeast extract, same protocol as used for Figure 6.

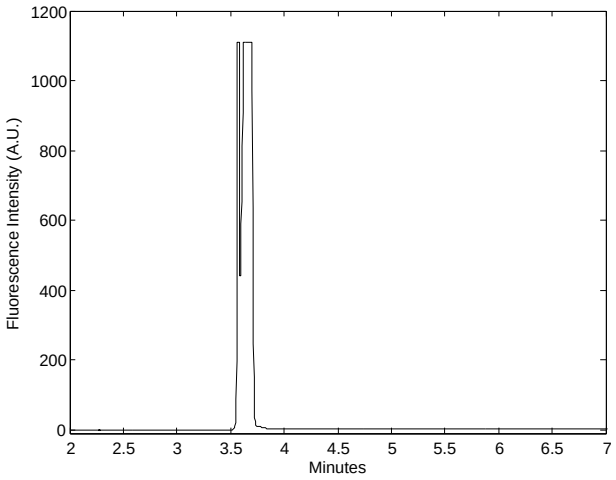


**Fig. 13** Graph showing the capillary-ITP result with 50 µmol/L glutamate-FITC same protocol as used for Figure 6.

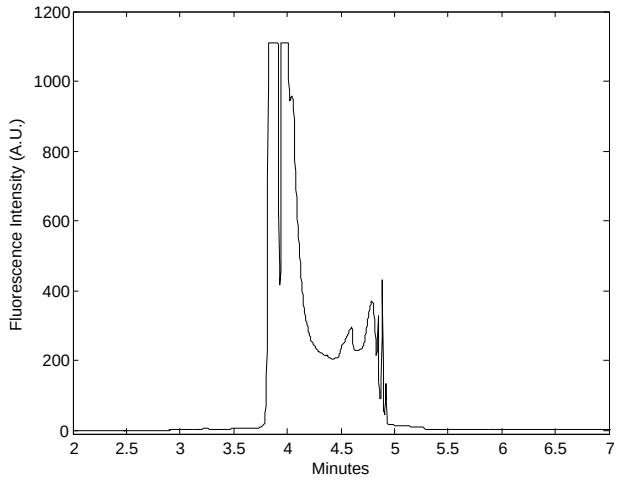


**Fig. 14** Graph showing the capillary-ITP result with 50 µmol/L phenyl alanine-FITC in presence of yeast extract, same protocol as used for Figure 6. Clearly showing the interaction of phenyl alanine-FITC with the biomatrix.

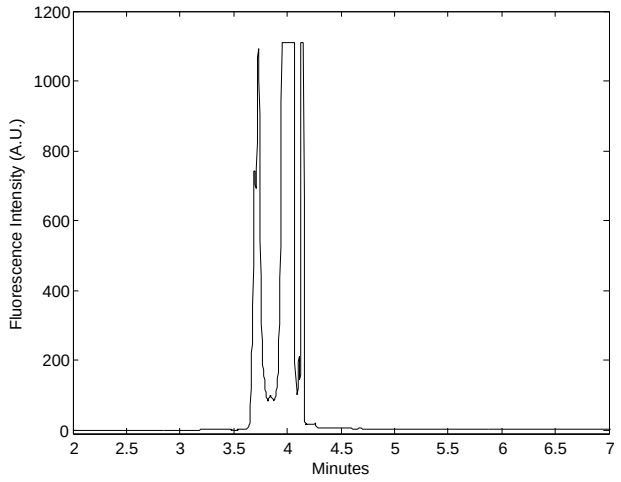


**Fig. 15** Graph showing the capillary-ITP result with 50 µmol/L glutamate-FITC in presence of yeast extract, same protocol as used for Figure 6.