Separation, size and charge determination of small molecules using CE in combination with UV area imaging

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This article shows how a capillary electrophoresis instrument may be used in conjunction with UV area imaging to determine mobility, diffusion coefficient and charge of components in a mixture of small molecules. The analytes lidocaine, phenylmethanol and benzoate are chosen as representative of small molecules of different charge type: cationic, neutral and anionic respectively in the background electrolyte used, phosphate buffer at pH 7.5. A capillary with three windows is used, with detection at the first window using the Agilent 7100 CE system and at the second and third using the ActiPix D100 UV imaging detector. The separated species are characterised at the first window after short-end injection followed by pressure assisted capillary electrophoresis for 3.5 minutes. The diffusion coefficients and hydrodynamic radii of the molecules are determined from the broadening of the bands between the second and third windows during a 15 minute stage of pressure driven flow. The method allows diffusion coefficients of all species to be measured within a single run, with good repeatability (RSDs better than 2.5%, n=9). Mobilities are found from times to these windows in separate experiments using pressure assisted capillary electrophoresis. Charge follows by combining data on diffusion coefficient and mobility.

Keywords: Capillary electrophoresis, diffusion coefficient, sizing, hydrodynamic radius, Taylor dispersion analysis, mobility, ionic charge

Introduction

There is a need within the biopharmaceutical and pharmaceutical industries for techniques which allow diffusion coefficients and electrophoretic mobilities of molecules to be determined in a single experimental system. Knowing the ratio of mobility to diffusion coefficient allows the charge, or valence, of the molecule to be determined [1]. For antibodies, the higher the valence, the less the tendency for aggregation [2].

Capillary electrophoresis (CE) is a well established technique for separating compounds by application of voltage, and the method is used to measure electrophoretic mobilities with high precision [3,4]. CE instruments also allow application of pressure, and analysis of band broadening due to Taylor dispersion during pressure-driven flow in a standard CE setup provides data on diffusion coefficient and hydrodynamic radius [5-10].

There has been one report on use of a CE system with a mixture of components, using initial application of voltage to separate them followed by application of pressure to broaden the bands and allow determination of diffusion coefficients by Taylor dispersion analysis (TDA) [9].

The aim of this article is to extend this approach, using the pairing of Paraytec's ActiPix D100 UV imaging detector and the Agilent 7100 CE system. This CE system has been designed to allow use with external detectors. In previous work we have shown that use of the ActiPix UV imaging detector with two windows in a single capillary allows hydrodynamic radii to be determined with high precision in a single experiment [11,12]. Our objective in this article is to illustrate the approach and methodology for measurement of charges of individual components in a mixture of small molecules.

Experimental

Instrumentation and Materials

ActiPix D100 (Paraytec Ltd)

ActiPix cartridge for sizing application (Paraytec Ltd): ambient temperature, 23 °C.

214 nm wavelength filter (Paraytec Ltd)

Agilent 7100 CE system (Agilent Technologies)

Capillary dimensions: 75 μm nominal ID, 360 μm OD (Polymicro Technologies)

Capillary length: 101 cm total length, 8.4 cm, 35 cm & 75 cm are the positions of the three windows. The integrated Agilent 7100 detector monitors at the 1st window (short end injection) and the ActiPix D100 monitors at the 2nd and 3rd windows (40 cm between windows)

Background electrolyte (BGE): 50 mM phosphate buffer, pH 7.5; prepared by dissolving 2.5 mmol of NaH_2PO_4 in 48 mL of deionised water, adjusting to pH 7.5 with 1 M NaOH and making up to 50 mL with water.

Sample solution: ~0.2 mM sodium benzoate, 0.2 mM lidocaine and 1 mM phenylmethanol (benzyl alcohol) dissolved in BGE.

Run sequence for determination of diffusion coefficient and size

Rinse:	0.1 M NaOH; 1000 mbar, 2 min
Rinse:	water; 1000 mbar, 10 s
Rinse:	BGE; 1000 mbar, 3 min
Inject:	sample; 50 mbar, 8 s
Inject:	BGE; 50 mbar, 3 s
Run:	BGE; 20 mbar, 20 kV, 3.5 min
Rest:	BGE; 0 mbar, 0 kV, 0.5 min
Run:	BGE; 50 mbar, 15 min

The alkaline wash was applied to ensure the internal surface of the capillary was clean. A short rinse step was applied to remove hydroxide solution from the electrode and

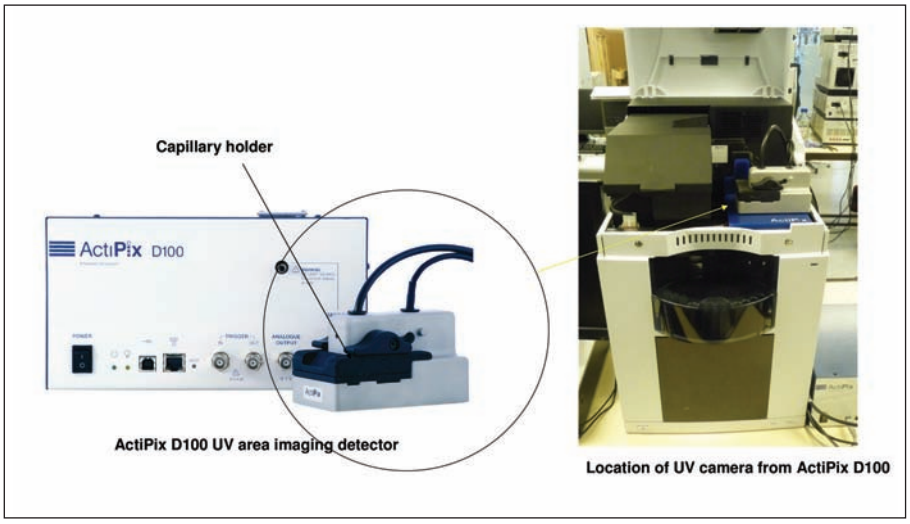

Figure 1. Integration of the ActiPix D100 UV imaging system and the Agilent 7100 CE system.

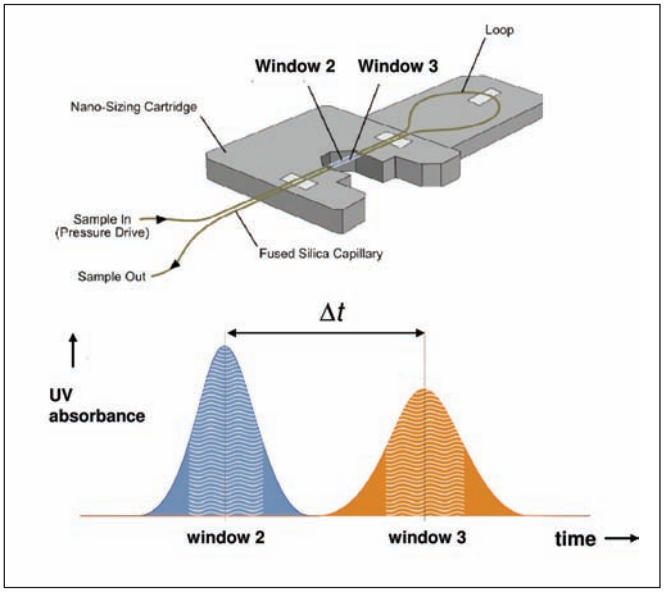

Figure 2. Schematic of ActiPix sizing cartridge and peak broadening in pressure-driven flow between second and third windows.

outside of the capillary. The capillary was re-equilibrated with BGE prior to starting data collection.

Results and discussion

For this application the capillary used has three windows. The first is at 8.4 cm from the end of the 101 cm length capillary and is used with the Agilent 7100 diode array detector following short-end injection in the CE system. The capillary then passes twice through the ActiPix D100 cartridge (see Figure 2), with the second and third windows positioned at 35 cm and 75 cm respectively. A hydrodynamic injection of a few nL is made by the CE system and a voltage is applied to separate the sample into its various components by electrophoresis. The applied voltage is removed before any of the components reach the first ActiPix detection position. A pressure is then applied to induce flow driving the sample components past the two ActiPix detection positions. UV absorption of the sample at 254 nm is recorded at the second and third detection window in order to characterise band broadening. Whilst the area of the peak is the same, the widths of both peaks are different: the signal from the third window has a greater width and lower amplitude due to Taylor dispersion, as shown in Figure 2.

Separation: voltage + pressure drive to 1st window

Pressure assisted capillary electrophoresis (20 mbar, 20 kV) for 3.5 min separates the components in the sample mixture. Pressure assisted CE [13-15] is used rather than CE alone for this separation stage, in order to assist mobilization of one of the analytes, benzoate, which has a very low net mobility at the pH of the experiment. Figure 3 shows absorbance at 214 nm output from the Agilent 7100 detector at the first window, and the UV spectrum for the 3rd peak (benzoate ion). It can be seen that there is baseline separation of all peaks.

Taylor dispersion: pressure drive between 2nd and 3rd windows

When the pressure and voltage are turned off at 3.5 minutes, the fastest component is positioned just before the second window. Joule heating raises the temperature inside the capillary while the voltage is applied; a 0.5 minute rest allows thermal equilibration to ambient temperature. Taylor dispersion analysis on the separated mixture is then carried out by applying pressure (50 mbar) which drives the component peaks past the second and third capillary windows. It should be noted that a capillary with 75 μm ID was used since this is optimal for Taylor dispersion experiments with small molecules.

Figure 4 shows an overlay of primary data obtained from 9 consecutive runs. Excellent concordance is seen between consecutive runs, demonstrating the high reproducibility of the technique. The following method is used to determine diffusion coefficient and size using TDA. Each peaks measured at the second window is convoluted with a Gaussian function; the width of the Gaussian is adjusted in the software algorithm in order to obtain the best fit between the result of the convolution and the corresponding peak measured at the third window. An example of the fitting for the third peak, benzoate, is shown in Figure 5. The standard deviation of the Gaussian convolution function, $\Delta\tau$, is used together with the measured difference in peak centre times at the second and third windows, Δt, to calculate the diffusion coefficient, D, using Equation 1

$$D = \frac{r^2 \Delta t}{24 \Delta \tau^2} \qquad (1)$$

where r is the capillary radius.

The hydrodynamic radius, R_h, is calculated using Equation 2, which is obtained by combining equation 2 with the Stokes Einstein expression linking diffusion coefficient and hydrodynamic radius.

$$R_h = \frac{4 k_B T \Delta \tau^2}{\pi \eta r^2 \Delta t} \qquad (2)$$

where k_B is the Boltzmann constant, T the absolute temperature and η the viscosity of the solution. For dilute solutions used in these experiments, the viscosity of the solution may be assumed to be that of water at that temperature. Further details concerning equations 1 and 2 and data analysis are available elsewhere [11,12].

Table 1 gives a summary of the peak width increase and hydrodynamic radii obtained from 9 consecutive runs with 8 nL injection of the sample mixture containing 0.2 mM lidocaine, 1 mM phenylmethanol and 0.2 mM sodium benzoate dissolved in the BGE. The increase in peak width can be measured very reproducibly, for example the average lidocaine peak width increase ($\Delta\tau$) is 6.99 s with a standard deviation of only 39 ms for the 9 runs. This translates to RSD for the diffusion coefficient and hydrodynamic radius of 0.9%. The precision in Taylor dispersion measurements for separated components in a mixture is considerably better than that from using a method with single point

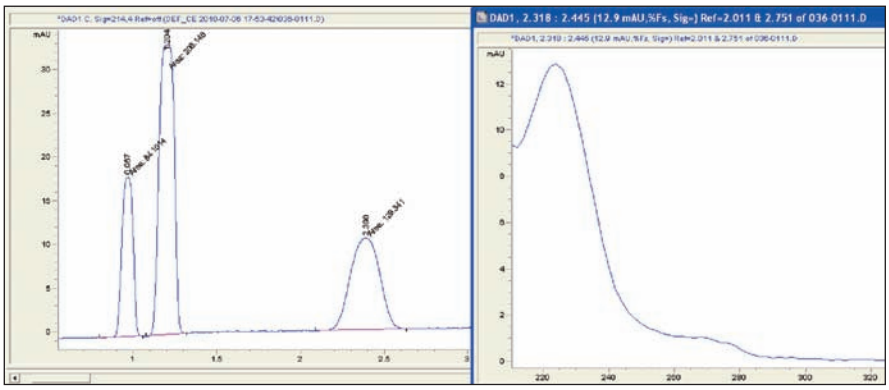

Figure 3. Absorbance at 214 nm from Agilent 7100 detector at the first window; 20 mbar, 20 kV for 3.5 min. Analyte migration order lidocaine, phenylmethanol, benzoate. Inset: UV spectrum for the 3rd peak (benzoate).

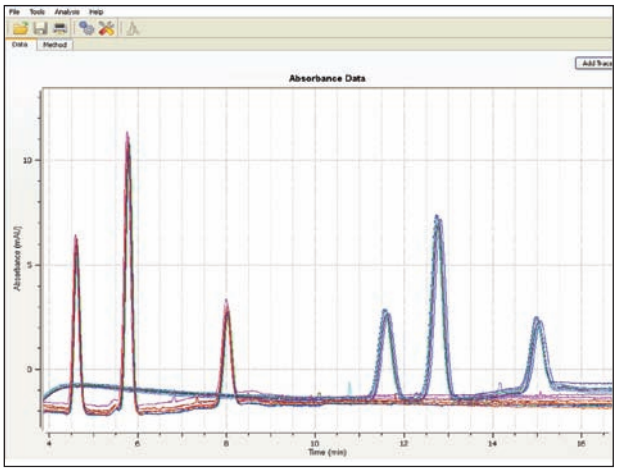

Figure 4. ActiPix D100 traces of 214 nm absorbance at window 2 (red) and window 3 (blue). Overlay of 9 consecutive runs with injection of 34 nL of the sample solution. Run sequence: (i) 20 mbar, 20 kV, 3.5 min; (ii) 0 mbar, 0 kV, 0.5 min; (iii) 50 mbar, 15 min.

	Hydrodynamic radius / nm		
Analyte	lidocaine	phenylmethanol	benzoate
Run 1	0.509	0.329	0.361
Run 2	0.502	0.325	0.355
Run 3	0.510	0.318	0.359
Run 4	0.501	0.318	0.349
Run 5	0.500	0.320	0.346
Run 6	0.500	0.320	0.367
Run 7	0.498	0.319	0.363
Run 8	0.497	0.321	0.346
Run 9	0.499	0.319	0.344
Average R_h	**0.502**	**0.321**	**0.354**
STD DEV	**0.0045**	**0.0035**	**0.0083**
RSD (%)	**0.89**	**1.1**	**2.4**
Average $\Delta\tau$/ s	**6.99**	**5.59**	**5.87**
STD DEV $\Delta\tau$/ s	**0.039**	**0.041**	**0.076**

Table 1 Repeatability and precision using Taylor dispersion analysis.

	lidocaine	phenylmethanol	benzoate
$D/10^{-10}$ m^2 s^{-1}	5.02	7.68	7.13
$\mu_{eff}/10^{-8}$ m^2 V^{-1} s^{-1}	1.29	0	-2.40
f	0.77	0	1.00
$\mu/10^{-8}$ m^2 V^{-1} s^{-1}	1.68	0	-2.40
z	0.86	0	-0.86

Table 2 . Diffusion coefficient, effective mobility, fraction in ionic form, mobility and charge for lidocaine, phenylmethanol and benzoate at 23°C in a pH 7.5 phosphate buffer, ionic strength 0.13 M

detection on an Agilent CE system [9]. Use of the ActiPix D100 detector for Taylor dispersion analysis has the inherent benefit that variance contributions of CE dispersion and injection are automatically removed when variances at the second and third windows are combined as in Figure 2. An additional benefit is that all diffusion coefficient and size measurements are made in a single run, unlike other cases where a sequence of runs at different pressures was required to separate out the different variance contributions [6-10].

Mobility: voltage + pressure drive between 2nd and 3rd windows

Whilst mobilities may be estimated from the times at the first window, the accuracy is compromised by the short length to the detector, contributions from pressure and voltage start-up characteristics, and Joule heating in the capillary [3]. An alternative approach is to use the difference in times between the second and third windows during pressure assisted CE. A series of pressure assisted (30 mbar) CE runs were carried out using a range of applied voltages (5, 10, 15 and 20 kV) to enable the electrophoretic mobility at zero power to be determined. The time taken for each peak to travel between the two windows was used to get an accurate measure of the velocities and mobilities under steady state conditions. Figure 6 shows an example of primary data.

Equation 3 was used to determine the effective mobilities, μ_{eff}, of the charged species lidocaine and benzoate, with the 2nd peak (phenylmethanol) used as neutral marker [3,4].

$$\mu_{eff} = \frac{\Delta l L}{V}\left(\frac{1}{\Delta t} - \frac{1}{\Delta t_0}\right) \quad (3)$$

where Δl is the distance between windows 2 and 3, L the total capillary length, V the applied voltage, Δt the time difference for the ionic analyte and Δt_0 the time difference for the neutral marker.

Figure 7 shows the extrapolations of measured effective mobilities to zero power.

The effective mobility is converted to the electrophoretic mobility, μ, using the relationship $\mu_{eff} = f\mu$, where f is the fraction of the analyte in the ionic form. For benzoic acid, which is fully dissociated at pH 7.5, $f = 1$ and $\mu = \mu_{eff}$. For lidocaine, which is partially in the ionic form at pH 7.5, f and μ are determined as described in the next section.

Determination of analyte charge

The analyte charge, z, is obtained by combining results for electrophoretic mobility at zero power and diffusion coefficient, D, using Equation 4 [1]

$$z = \frac{k_B T \mu}{eD} \quad (4)$$

where e is the electronic charge.

Table 2 lists diffusion coefficient, effective mobility, fraction in ionic form, electrophoretic mobility and charge for each of the three analytes lidocaine, phenylmethanol and benzoate. Values of f given in the third row were calculated from pK_a values and the composition of the background electrolyte. Benzoic acid (pK_a 4.2) is fully dissociated at pH 7.5. The composition of the pH 7.5 BGE was calculated using methodology described elsewhere [16,17] to be 9 mM $H_2PO_4^-$, 41 mM $H_2PO_4^{2-}$, 91 mM Na^+ and to have ionic strength 0.132 M. Using the same calculation method, from the pK_a for lidocaine of 7.94 at 23°C [18] a fraction 0.77 is in the acid (ionic) form at this pH and ionic strength.

The electrophoretic mobility of benzoate, -2.40×10^{-8} m^2 V^{-1} s^{-1} (Table 2) was corrected to zero ionic strength using the procedure of Nhujak and Goodall [19], giving $\mu^\circ = -3.30\times10^{-8}$ m^2 V^{-1} s^{-1} . This is in very good agreement with the literature value $\mu^\circ = -3.29\times10^{-8}$ m^2 V^{-1} s^{-1} [20], demonstrating the

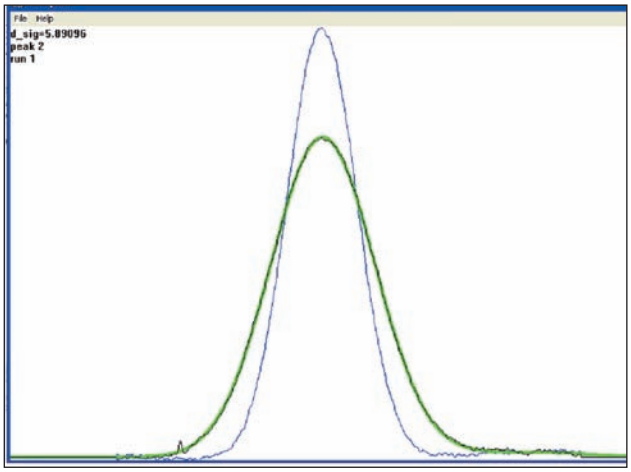

Figure 5. Example of experimental data and fitting for Taylor dispersion analysis. Blue and black lines are absorbance output traces for benzoate from the second and third windows, respectively. The green line the result of convoluting the blue line with a Gaussian function that has a standard deviation of 5.891 s.

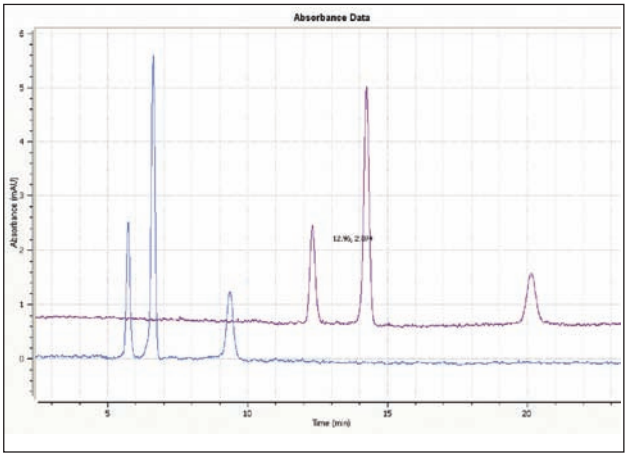

Figure 6. Traces at second and third windows (blue and red, respectively) for 30 mbar, 10 kV; ActiPix D100 detector, 214 nm. Analyte migration order lidocaine, phenylmethanol, benzoate.

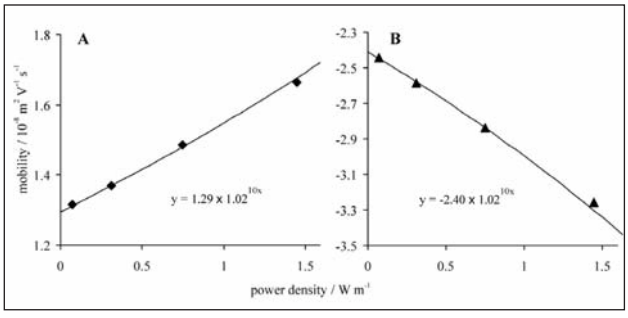

Figure 7. Measured effective mobility of A, lidocaine and B, benzoate with applied voltages of 5, 10, 15 & 20 kV plotted against dissipated power density. The fitted line extrapolated to zero gives the effective mobility at zero power. The fit assumes a mobility increase of 2% / °C and indicates an estimated temperature increase of 10 °C m W^{-1}.

benefit of the ActiPix D100 with the Agilent 7100 CE system for determination of absolute mobilities.

The charges for lidocaine and benzoic acid were expected to be 1.0 and -1.0 respectively. Measured values of charge as reported in the last row of Table 2 are 0.86 for lidocaine and -0.86 for benzoic acid. Differences from unity are most likely due to the lack of correction for effects of ionic atmospheres on the dynamics of ionic diffusion and mobility. However, the fact that the charges for fully ionised cationic and anionic species in Table 2 are equal in magnitude is encouraging and suggests that with further refinement of non-ideality effects this method combining use of the ActiPix D100 with the Agilent 7100 CE system should yield absolute values.

Conclusions

The Paraytec ActiPix D100 detector has been used in combination with an Agilent 7100 CE system to determine diffusion coefficient, hydrodynamic radius, mobility and charge of components in a mixture of small molecules. Initial separation is carried out using pressure assisted capillary electrophoresis and measurement of diffusion coefficient and size achieved after switching to pressure driven flow. A capillary with three windows is used, with detection at the first window using the Agilent 7100 and at the second and third using the ActiPix D100. The method has been applied to a mixture of three small molecules, lidocaine, phenylmethanol and benzoate, and shown to provide good repeatability (RSDs better than 2.5%, n=9) in diffusion coefficient and hydrodynamic radius. Charge follows by combining data on diffusion coefficient and mobility. The method could readily be applied with molecules in other classes, e.g. peptides and proteins.

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References

[1] J.A. Durant, C. Chen, T.M. Laue, T.P. Moody, S.A. Allison, Biophys. Chem. 101-102 (2002) 593-609.

[2] R.M. Fesinmeyer, S. Hogan, A. Saluja, S.R. Brych, E. Kras, L.O. Narhi, D.N. Brems, Y.R. Gokarn, Pharm. Res. 26 (2009) 903-913.

[3] P.D. Grossman in P.D. Grossman, J.C. Colburn (Editors), Capillary Electrophoresis - Theory and Practice, Academic Press, San Diego, CA, 1992, p. 3-43 and 111-132.

[4] D.C. Harris, Quantitative Chemical Analysis, W.H. Freeman, New York, NY, 7th Edition, 2007, Chapter 26.

[5] M.S. Bello, R. Rezzonico, P.G. Righetti, Science 266 (1994) 773-776.

[6] U. Sharma, N.J. Gleason, J.D. Carbeck, Anal. Chem. 77 (2005) 806-813.

[7] H. Cottet, J.-P. Biron, M. Martin, Anal. Chem. 79 (2007) 9066-9073.

[8] H. Cottet, M. Martin, A. Papillaud, E. Souaïd, H. Collet, A. Commeyras, Biomacromolecules 8 (2007) 3235-3243.

[9] T. Le Saux, H. Cottet, Anal. Chem. 80 (2008) 1829-1832.

[10] F. d'Orlyé, A. Varenne, P. Gareil, J. Chromatogr. A 1204 (2008) 226–232.

[11] A.J.S. Chapman, D.M. Goodall, Chromatography Today volume 1 issue 3 (2008) 22-24.

[12] Paraytec Application Note AN015. Precision and accuracy of protein size determination using the ActiPix TDA200 Nano-Sizing System.

[13] Z. Jia, T. Ramstad, M. Zhong, Electrophoresis 22 (2001) 1112-1118.

[14] H. Wan, A. Holmén, M. Någård, W. Lindberg, J. Chromatogr. A 979 (2002) 369-377.

[15] Y. Ishihama, M. Nakamura, T. Miwa, T. Kajima, N. Asakawa, J. Pharm. Sci. 91 (2002) 933-942.

[16] M.A. Survay, D.M. Goodall, S.A.C. Wren, R.C Rowe, J. Chromatogr. A 741 (1996) 99-113.

[17] I.G.R. Gutz, CurTiPot freeware for pH and acid-base equilibrium calculations, www2.iq.usp.br/docente/gutz/Curtipot_.html –

[18] M.F. Powell, Pharm. Res. 4 (1987) 42-45.

[19] T. Nhujak, D.M. Goodall, Electrophoresis 22 (2001) 117-22.

[20] T. Hirokawa, M. Nishino, Y. Kiso, J. Chromatogr. 252 (1982) 49-65.