



**HAL**  
open science

# Critical parameters for highly efficient and reproducible polyelectrolyte multilayer coatings for protein separation by capillary electrophoresis

Laura Dhellemmes, Laurent Leclercq, Alisa Höchsmann, Christian Neusüß, Jean-Philippe Biron, Sébastien Roca, Hervé Cottet

## ► To cite this version:

Laura Dhellemmes, Laurent Leclercq, Alisa Höchsmann, Christian Neusüß, Jean-Philippe Biron, et al.. Critical parameters for highly efficient and reproducible polyelectrolyte multilayer coatings for protein separation by capillary electrophoresis. *Journal of Chromatography A*, 2023, 1695, pp.463912. 10.1016/j.chroma.2023.463912 . hal-04247515

**HAL Id: hal-04247515**

**<https://hal.science/hal-04247515v1>**

Submitted on 18 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



15 **HIGHLIGHTS**

- 16 ● High repeatability and intra- and inter-capillary precision were achieved
- 17 ● Cross-contamination between polyelectrolyte vials lowers repeatability and efficiency
- 18 ● PDADMAC/PSS coatings are durable over 100 runs and can be regenerated
- 19 ● Average retention factors of  $4 \times 10^{-2}$  quantify low residual adsorption
- 20 ● Proteins must be stored separately in water to avoid degradation

21 **ABSTRACT**

22

23 Since the introduction of polyelectrolyte multilayers to protein separation in capillary  
24 electrophoresis (CE), some progress has been made to improve separation efficiency by  
25 varying different parameters, such as buffer ionic strength and pH, polyelectrolyte nature and  
26 number of deposited layers. However, CE is often overlooked as it lacks robustness compared  
27 to other separation techniques. In this work, critical parameters for the construction of  
28 efficient and reproducible Successive multiple ionic-polymer layers (SMIL) coatings were  
29 investigated, focusing on experimental conditions, such as vial preparation and sample  
30 conservation which were shown to have a significant impact on separation performances. In  
31 addition to repeatability, intra- and inter-capillary precision were assessed, demonstrating  
32 the improved capability of poly(diallyldimethylammonium chloride) / poly(sodium styrene  
33 sulfonate) (PDADMAC / PSS) coated capillaries to separate model proteins in a 2 M acetic acid  
34 background electrolyte when all the correct precautions are put in place (with run to run  
35  $\%RSD(t_m) < 1.8\%$ , day to day  $\%RSD(t_m) < 3.2\%$  and cap to cap  $\%RSD(t_m) < 4.6\%$ ). The approach  
36 recently introduced to calculate retention factors was used to quantify residual protein  
37 adsorption onto the capillary wall and to assess capillary coating performances. 5-layer  
38 PDADMAC / PSS coatings led to average retention factors for the five model proteins of  
39  $\sim 4 \times 10^{-2}$ . These values suggest a relatively low residual protein adsorption leading to  
40 reasonably flat plate height vs linear velocity curves, obtained by performing electrophoretic  
41 separations at different electrical voltages (-10 to -25kV).

42

43

44 **Keywords:** SMIL coatings, protein separation, separation efficiency, intermediate precision,  
45 residual adsorption

46

## 47        **1. Introduction**

48

49        As a highly efficient separation technique, capillary electrophoresis (CE) finds applications  
50 notably in the pharmaceutical field, where it can be used to separate molecules according to  
51 their size and charge [1]. Indeed, therapeutic compounds such as monoclonal antibodies  
52 (mAbs) and fusion proteins attract particular interest for the treatment of specific diseases  
53 and therefore require adequate analysis. Among different separations techniques, CE-based  
54 methods are pertinent to separate protein size and charge variants, and include capillary gel  
55 electrophoresis (CGE), capillary isoelectric focusing (CIEF) and free solution capillary zone  
56 electrophoresis (CZE) [2-3]. CZE offers the advantages of simplicity and good compatibility  
57 with mass spectrometry (MS), which enables the identification of separated variants.  
58 However, it has received less attention for the analysis of therapeutic drugs since positively  
59 charged proteins tend to adsorb onto the capillary wall which presents negatively charged  
60 silanol groups [4]. Indeed, in the ideal case, close to one million theoretical plates should be  
61 reached for a typical globular protein (~3 nm hydrodynamic radius) analyzed by CE.  
62 Experimentally, this number rarely exceeds 100 000 [5]. This may be explained by additional  
63 dispersion sources, such as capillary coiling, electromigration dispersion, temperature  
64 gradients, or residual protein adsorption [6]. Various methods of reducing protein adsorption  
65 onto the capillary wall have been investigated, including the use of extreme pH and ionic  
66 strengths [7] [8], zwitterionic additives [9], and capillary coatings. The latter are particularly  
67 interesting, as they are easy to put in place and can be finetuned to fit specific needs. Capillary  
68 coatings can be divided into three types, namely permanent, semi-permanent, and dynamic  
69 coatings [10]. Among these, semi-permanent coatings, which are physically adsorbed onto  
70 the capillary wall, are the simplest to achieve, as they do not require chemical reactions like  
71 covalently bonded permanent coatings, and remain compatible with MS, unlike dynamic  
72 coatings where the coating reagent present in the background electrolyte (BGE) can interfere  
73 with the detection. These semi-permanent coatings can be made up of a single neutral or  
74 charged layer, or successive multiple ionic-polymer layers (SMIL) which provide a more stable  
75 base [11].

76        Since their introduction in CE by Katayama [11], SMIL coatings have been widely studied  
77 as a tool to limit protein adsorption onto the capillary wall. The polyelectrolyte multilayers

78 are generated by flushing the capillary with alternating polycation and polyanion solutions  
79 until the desired number of layers is reached. Various polymers have been used as coating  
80 agents, the most common polycations being polyethylene imine (PEI) [12, 13], polybrene (PB)  
81 [5, 11, 14-16], poly(allylamine hydrochloride) (PAH) [12, 17], and  
82 poly(diallyldimethylammonium chloride) (PDADMAC) [12, 18, 19], and the most common  
83 polyanions being dextran sulfate (DS) [5, 11, 13, 16], poly(methacrylic acid) (PMA) [5, 12, 17,  
84 20], and poly(sodium styrene sulfonate) (PSS) [5, 12, 17-19]. Coatings with a final polycationic  
85 layer are well-suited to separate positively charged compounds, while those ending with a  
86 polyanion are preferred for negatively charged analytes. Polyelectrolyte nature has been  
87 shown to have a significant impact on separation efficiency as well as stability of the  
88 electroosmotic flow (EOF), with plate numbers ranging from 4000 plates/m with a PEI-DS-PEI  
89 coating [13] to over 800 000 plates/m with a 13-layer PDADMAC/PSS coating in a 20mM  
90 phosphate buffer (pH 4) [18] for the separation of model proteins. Other elements, such as  
91 the coating buffer, the BGE, the number of deposited layers, and the physicochemical  
92 characteristics of the polyelectrolytes, may have a great impact on coating performances, as  
93 was described in a recent review [20].

94 Despite the abundance of studies on the subject, comparing different SMIL coatings and  
95 separation conditions remains challenging, as different proteins migrate at different speeds  
96 and the electroosmotic mobility changes the migration times on different coatings. To this  
97 end, the recently developed approach based on calculating protein retention factors  $k$  from  
98 separation efficiencies, enables a quantitative measurement of residual adsorption, and  
99 therefore a simple way of ranking capillary coatings [19]. Determining retention factors in CE  
100 is atypical, since they are calculated from separation efficiencies rather than migration times  
101 like in chromatography. Whereas repeatability is often evaluated for SMIL coatings,  
102 reproducibility and intermediate precision are less studied, though they are crucial for the  
103 comparison of different conditions and coatings. Bekri et al. performed such a study for a 5-  
104 layer PDADMAC/PLC coating, obtaining a 1.2% RSD on migration times for three capillaries  
105 [12]. Similarly, Qu et al. found run-to-run, day-to-day, and column-to-column reproducibilities  
106 all below 2% for a PDADMAC/graphene oxide coating [21]. Reproducibility of the EOF may  
107 also be considered, Liu et al. finding that a PB/DS coated capillary stored in water overnight  
108 had a day-to-day RSD of 9.2% ( $n=3$ ), which was greatly improved by rinsing the capillary with

109 a buffer solution containing 0.01% w/v DS, reaching 0.8% ( $n=3$ ) [22]. Likewise, Graul et al.  
110 tested several 13-layer PDADMAC/PSS coatings which had RSDs on the EOF below 2% [18].  
111 Capillary-to-capillary intermediate precision or reproducibility in CE has been more  
112 investigated for silica capillaries compared to SMIL coatings. Intermediate precision and  
113 reproducibility problems in CE may be linked to unequal levels in the buffer vials, high current,  
114 insufficient rinsing between runs, or low injection pressures [24, 25]. Thus, it is clear that  
115 reproducibility studies for SMIL coatings in CE need to be further developed.

116 In this work, critical parameters for the construction of SMIL coatings used for protein  
117 separations by CE are examined with the aim of improving the experimental protocol and  
118 maximizing SMIL coating performance and reproducibility. Among these, sample preparation  
119 and conservation, contamination between polyelectrolyte vials, endurance and durability of  
120 the capillary coatings are investigated. Repeatability and intermediate precision are evaluated  
121 through run-to-run, day-to-day and cap-to-cap analyses, focusing on 5-layer PDADMAC/PSS  
122 coatings used to separate five model intact proteins in a 2 M acetic acid BGE. Separation  
123 performances are assessed by calculating retention factors for each protein, which enables  
124 an evaluation of the residual adsorption of proteins onto the capillary coating and provide a  
125 quantitative basis for the ranking of capillary coatings in 2 M acetic acid conditions. Overall,  
126 this work may serve as a guide to the obtention of efficient and performant SMIL coatings.

127

## 128 **2. Materials and Methods**

### 129 *2.1. Chemicals & Vial Preparation*

130 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid (HEPES) and acetic acid were  
131 purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The model proteins, Carbonic  
132 Anhydrase I from human erythrocytes (CA, purity not indicated by the supplier), Trypsin  
133 Inhibitor from Flycine max (soybean) (TI, purity not indicated by the supplier), Myoglobin from  
134 equine skeletal muscle (Myo, purity  $\geq 95\%$ ), Ribonuclease A from bovine pancreas (RNAse A,  
135 purity  $\geq 60\%$ ), and Lysozyme from chicken egg white (Lys, purity  $\geq 90\%$ ) were purchased from  
136 Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was obtained using a MilliQ  
137 system from Millipore (Molsheim, France). Poly(diallyldimethylammonium chloride)  
138 (PDADMAC, high molecular weight:  $M_w 4 \times 10^5 - 5 \times 10^5$  Da) 20% w/w in water was purchased

139 from Aldrich (Lyon, France). Poly(styrene sulfonate) (PSS,  $M_w$   $7 \times 10^5$  Da) was purchased from  
140 Acros Organics (Geel, Belgium).

141 2 M acetic acid was used as the background electrolyte (BGE), adding 64 mL of pure acetic  
142 acid to a 500 mL volumetric flask and completing with pure water. The measured pH was 2.2  
143 and the solution was kept in the fridge. The vials were prepared by adding 1 mL of BGE into  
144 glass vials. The level should be the same for the inlet and outlet vials to ensure stable current.  
145 HEPES solution was used as the construction buffer. For a 1 L solution of HEPES at 20 mM,  
146 with 10 mM NaOH, add 4.8 g of HEPES and 10 mL of a 1 M NaOH solution to a 1 L volumetric  
147 flask and complete with water. The measured pH was 7.4 and the solution was kept in the  
148 fridge. The vials were prepared by adding 1 mL of HEPES buffer into glass vials.

149 Polyelectrolyte solutions (3 g/L PDADMAC and PSS) were prepared by weighing the  
150 desired amount of polymer in a 5 mL Eppendorf®, adding the corresponding amount of HEPES  
151 buffer for a 3 g/L solution. Both must be prepared one night before the first use. PDADMAC  
152 solution must be kept in the freezer. Indeed, PDADMAC solution freshness was shown to have  
153 an impact on the quality of the separations. To prepare the vials, 200  $\mu$ L of the polyelectrolyte  
154 solution were added into a 1 mL polypropylene vial, making sure that the level of the liquid  
155 was below the electrode (in the case of Agilent CE equipment with long electrodes). The  
156 dangers of cross-contamination between polyelectrolyte vials will be detailed later in the  
157 paper.

158 The protein sample was prepared from individual solutions of proteins stored in water. To  
159 prepare these, proteins were weighed in 5 mL Eppendorfs® and the corresponding amount of  
160 water was added for 2 g/L solutions, which were stored in the freezer. Right before  
161 conducting the analyses, the stock solutions of proteins were thawed and 10  $\mu$ L of each one  
162 were added into a 1 mL Eppendorf®, adding another 50  $\mu$ L of BGE at double the concentration  
163 (4 M acetic acid) for a 100  $\mu$ L total solution, with each protein at 0.2 g/L in BGE. After heat  
164 treatment at 37°C for 30 min, the vial was prepared by adding 25  $\mu$ L of this aliquot to a  
165 polypropylene conical vial, an amount which is enough for 25 injections. The rest of the  
166 aliquot may be kept in the freezer over a short period (no degradation was observed over one  
167 week).



168 DMF was used as the EOF marker and injected before the proteins in BGE. A first solution  
169 was prepared by diluting 10  $\mu$ L of DMF in 5 mL of water (in a 5 mL Eppendorf®) to create a  
170 0.2% solution. A second solution was made by diluting 50  $\mu$ L of the first solution in 5 mL of  
171 water (in a 5 mL Eppendorf®) for the 0.002% solution in water. Finally, 1 mL of the second  
172 solution was mixed with 1 mL of BGE for a 0.001% DMF solution in BGE (1 M acetic acid). To  
173 prepare the vial, 25 to 100  $\mu$ L of the final solution was added into a polypropylene conical vial.

174

## 175 *2.2. Coating Procedure*

176 The SMIL coatings were prepared by preconditioning the silica capillary with 1 M NaOH  
177 for 10 min, then flushing with water for 5 min and HEPES for 10 min. Next, the polyelectrolyte  
178 solutions were flushed for 7 min each, followed by 3 min of HEPES, starting with the  
179 polycation and alternating with the polyanion, for a total of 5 layers. After the last HEPES  
180 flush, wait 5 min and flush with water for 3 min, with BGE for 10 min and wait for another 5  
181 min before starting analyses. All flushes were performed at 930 mbar. Different HEPES vials  
182 were used after the polycation and the polyanion in order to limit cross-contamination.

183

## 184 *2.3. Capillary Electrophoresis*

185 Analyses were performed on an Agilent 7100 CE in a 2 M acetic acid BGE at pH 2.2. Fused  
186 silica capillaries of 50  $\mu$ m in diameter and 40 cm total length (31.5 cm to the detector) were  
187 used. Applied voltages were typically between -25kV and -10kV. The capillary was flushed for  
188 5 min with BGE before each run. A 0.001% solution of DMF in BGE was injected first as the  
189 EOF marker (30 mbar, 3 s), and the protein mixture next (30 mbar, 4 s), with CA, TI, Myo,  
190 RNase A and Lys each at 0.2 g/L in BGE. The temperature was set at 25°C and detection  
191 wavelength was 214 nm.

192 Calculations of separation efficiencies were done with CEval software [25] available at  
193 [<https://echmet.natur.cuni.cz/>] as will be detailed in the results and discussion section. The  
194 capillary total ( $L_t$ ) and effective ( $l$ ) lengths must be entered in the software, as well as the half  
195 ramp time ( $t_{1/2}$  = 0.6 s on Agilent instruments). The linear migration velocity  $u$  of each protein  
196 is given by Eq. 1:

197

198

$$u = \frac{l}{t_{apex} - t_{1/2}} \quad (1)$$

199

where  $t_{apex}$  corresponds to the migration time at the peak apex written *peak max at x* in CEval.

200

### 3. Results and discussion

201

#### 3.1. Experimental determination of protein separation efficiency and retention factors on

202

#### SMIL coated capillaries

203

204

205

206

207

208

209

210

Before studying the critical experimental parameters that are controlling the separation performances of SMIL coatings, it was important to set the experimental methodology for calculating the retention factors of the proteins which represent a quantitative estimation of the residual adsorption of the protein onto the capillary coating (and thus an estimation of coating performance). Traditionally, separation efficiency in CE, as well as chromatography, is determined from the total variance of the peak, which is related to the full width at half height  $\delta$  in the case of gaussian peak [26]. The plate number can therefore be expressed with the following equation (method 1):

211

$$N = 5.54 \left( \frac{t_{apex}}{\delta} \right)^2 \quad (2)$$

212

213

214

215

216

217

218

However, perfectly Gaussian peaks are rarely observed because of extra-column processes [27], which raises the question of how to best calculate separation efficiency. To this end, other methods were investigated and compared, starting with the formal expression of the plate number which relies on the moments of the peak, shown in Eq. 3, where  $m_1$  is the first moment which is equal to the average migration time, and  $m_2$  is the peak variance, or second moment of the peak (method 2):

219

$$N = \left( \frac{m_1^2}{m_2} \right) \quad (3)$$

220

221

222

223

224

This value of  $N$  corresponds to the  $N_{asym}$  value given by the CEval software. This approach has already been used to determine plate numbers in HPLC and compared to other methods (based on peak area, peak tangents and peak half-width) on tailing peaks [28]. For moderately tailing peaks, all methods gave good approximations of the plate number, with

225 errors compared to the true value below 4%. However, only the method with the moments  
 226 gave satisfying results when peak tailing was more important.

227 In addition, a third method using the Haarhoff-van der Linde (HVL) function, a nonlinear  
 228 regression engine included in the CEval software (see Eq. 4), was considered. This method is  
 229 useful to fit asymmetrical peaks that can be observed in CE in the case of electromigration  
 230 dispersion, as it takes the form of a distorted Gaussian peak (see Eq. 5). The parameters  
 231  $x_{mean,HVL}$  and  $\sigma_{mean,HVL}$  are given by CEval and are numerically calculated from  $a_0, a_1, a_2, a_{3\delta}$ ,  
 232 which correspond to the peak area, the correct migration time of the analyte, the diffusional  
 233 broadening of the peak, and the peak distortion, respectively [29].

$$N_{asym} = \left( \frac{x_{mean}}{\sigma_{mean}} \right)_{HVL}^2 \quad (4)$$

235

$$HVL_{\delta}(t; a_0, a_1, a_2, a_{3\delta}) = \frac{\frac{a_0}{a_2 a_{3\delta} \sqrt{2\pi}} \exp\left[-\frac{1}{2} \left(\frac{t - a_1}{a_2}\right)^2\right]}{\frac{1}{\exp(a_{3\delta}) - 1} + \frac{1}{2} \left[1 + \operatorname{erf}\left(\frac{t - a_1}{\sqrt{2} a_2}\right)\right]} \quad (5)$$

238 Then, plate heights may be obtained from plate numbers using Eq. 6:

$$H = \frac{l}{N} \quad (6)$$

240 As was shown by Schure et al. [30], the different sources of peak broadening may be  
 241 summed, leading to the expression of plate height as a function of the linear velocity  $u$ ,  
 242 diffusion coefficient  $D$ , capillary internal diameter  $d_c$ , film thickness  $d_f$ , diffusion coefficient in  
 243 the stationary phase  $D_s$ , and retention factor  $k$ :

$$H = \frac{2D}{u(1+k)} + \frac{d_c^2 u}{D} \frac{k^2}{16(1+k)} + \frac{2 d_f^2 u}{3 D_s} \frac{k}{1+K} + \text{constant} \quad (7)$$

245

246 The first term corresponds to axial diffusion, the second one to the resistance to mass  
 247 transfer in the mobile phase and the third one to the resistance to mass transfer in stationary  
 248 phase. The latter may be ignored as the film thickness is small compared to the capillary  
 249 internal diameter ( $\sim 10$  nm vs  $50$   $\mu$ m). The addition of a constant allows a better fit of the  
 250 experimental data and includes additional, extra-column dispersion sources, such as the Joule  
 251 effect and electromigration dispersion. This equation may then be solved for  $k$  and the  
 252 constant with the Excel solver using the experimental data and the known parameters. The

253 diffusion coefficients  $D$  were experimentally obtained by Taylor Dispersion Analysis for each  
254 protein in the BGE (see Table SI1 and Fig. SI1).

255 The three methods described previously for the determination of  $N$  were compared  
256 for the separation of five model proteins using a 5-layer PDADMAC/PSS coated capillary in  
257 Fig. 1. It can be seen that methods 1 and 3 give quite similar results with plate heights below  
258 10  $\mu\text{m}$  and small error bars, indicating good precision. Method 2, however, leads to much  
259 higher plate numbers and slopes and the points are not as well aligned on the theoretical  
260 curve, showing that the formal method with the moments is not well adapted to these  
261 experiments. This may be due to some protein impurities appearing at the basis of the peak  
262 which make difficult the determination of the first two moments by peak integration. The HVL  
263 method does not seem to bring much improvement, as the analyzed peaks are already quite  
264 symmetrical. Finally, the first method using the width at half height will be used for the rest  
265 of the calculations (see Fig. SI3 and Table SI3).

266 It should be noted that the range of electric fields was chosen as to obtain points that  
267 were in the linear part of the  $H=f(u)$  curve since that corresponds to the contribution of  
268 adsorption to the plate height. At lower velocities, plate heights were shown to increase as  
269 predicted by the theoretic curve due to axial diffusion, but were not included in the current  
270 study (see Fig. SI4).

### 271 **3.2. Protein sample preparation and conservation**

272 Protein analysis requires proper preparation of the samples and conservation  
273 techniques in order to obtain consistent and efficient separations. Indeed, CE is generally  
274 performed in mild conditions, avoiding organic solvents and high salt concentrations in order  
275 to prevent protein degradation [31]. Moreover, it has been shown that acetic acid may  
276 partially denature protein structure, RNase A in particular being folded in aqueous solutions  
277 and becoming unfolded in 40% acetic acid [32]. These considerations are relevant in view of  
278 the experimental results obtained for a protein sample analysed over time. Initial experiments  
279 were conducted by mixing four model proteins (TI, Myo, RNase A, Lys) in the BGE, 2 M acetic  
280 acid, leading to the separations shown in Fig. 2A on a 5-layer PDADMAC/PSS coated capillary.  
281 The superposition of the five electropherograms at -10kV shows very repeatable results and  
282 thin peaks that indicate good separation efficiency (see Fig. SI2 and Table SI2 for the data on

283 all proteins). The protein mix was then stored in the freezer and used for analyses over several  
284 months. Five months later, the analysis was repeated in the same conditions using the aged  
285 protein mix, returning much larger peaks and poor return to the baseline between proteins  
286 (see Fig. 2B). This indicates the proteins have degraded over time, which is confirmed by the  
287 preparation of a fresh protein mix in BGE which yields thin, repeatable peaks very comparable  
288 to the initial test (see Fig. 2C and Fig. S15).

289 Several options were considered to address this issue of protein conservation. The  
290 protein solutions in water were mixed and then frozen or lyophilized to test storage of the  
291 protein mix in water and dry storage, both of which still led to peak deformation over time,  
292 most likely due to protein interactions. Then, proteins were prepared in individual solutions  
293 in water at higher concentrations (2 g/L) and kept in the freezer, the mix being done right  
294 before the analysis by mixing equal parts of each protein solution and acetic acid at a higher  
295 concentration so as to keep the final concentration of each protein in BGE constant (see  
296 section 2.1). This method was successful in properly conserving the proteins without any  
297 evolution of peak shape or width over time and was used for the rest of the experiments. In  
298 addition to protein conservation, the polycation used for the SMIL coating was also seen to  
299 deteriorate over time if not kept in the freezer (data not shown).

300 Another important point to consider is the medium of the injected samples. Generally,  
301 protein separation in CE is conducted with the proteins in an aqueous solution, yet this may  
302 not be the most favourable option, therefore, two sample mediums were tested and  
303 compared.

304 Initially, the injected samples (protein mix and DMF) were in an aqueous medium,  
305 leading to the curves shown in Fig. 3 A and an average retention factor of  $k(10^{-2}) = 5.59 \pm 1.31$   
306 ( $\pm$  one standard deviation for the 5 proteins). In contrast, injecting the samples in BGE resulted  
307 in the slopes shown in Fig. 3 B, with much flatter slopes in the linear part of the curve, which  
308 is reflected by a lower average retention factor:  $k(10^{-2}) = 3.33 \pm 0.93$ . Therefore, using BGE as  
309 the injection sample medium is advantageous, since the separation efficiency has less  
310 dependence on the electric field. It is important to note that both the protein and the DMF  
311 medium must be modified. This difference may be due to a stacking effect: to preserve the  
312 continuity of the current, the less conductive water leads to a higher electric field and  
313 therefore higher temperature, causing more diffusion [33]. At low electric field, the stacking

314 effect prevails, leading to lower plate heights, as can be seen in Fig. 3, and at high electric  
315 field, the thermal heating prevails, leading to higher plate heights, which explains the higher  
316 slope for the separations in water (see Table SI4 and Fig. SI6).

### 317 **3.3 Influence of polyelectrolyte cross-contamination during SMIL construction**

318 Another aspect to take into account while building SMIL coatings is vial preparation and  
319 potential contamination. During capillary conditioning and injection, successive rinsing steps  
320 cause solutions to be deposited on the outer capillary surface and electrodes, which may lead  
321 to carryover between vials [34]. For this reason, it was ensured that the level of the  
322 polyelectrolytes in the vials stayed below the electrodes on Agilent machines (see section  
323 2.1). Other steps such as burning the ends of the capillary so as to reduce adherence to the  
324 capillary surface and rinsing with fresh buffer between injections may be put in place. In the  
325 case of SMIL coatings, the presence of polyanion and polycation vials which are deposited  
326 one after the other, with a construction buffer rinsing step in between, constitutes an  
327 additional risk of contamination. Indeed, the stability of SMIL coatings is based on  
328 electrostatic interactions between polyanionic and polycationic layers [35], which may be  
329 disrupted if one of the polyelectrolyte solutions contains traces of the other.

330 In order to confirm the importance of this effect, polyelectrolyte vials were voluntarily  
331 contaminated with 10% v/v of the oppositely charged polyelectrolyte. Fig. 4 shows a  
332 comparison between the two contamination cases and the normal one, with both  
333 contaminated PSS (Fig. 4 A) and contaminated PDADMAC (Fig. 4 B) vials causing a loss in  
334 repeatability as well as separation efficiency: on average for all proteins,  $k(10^{-2}) = 6.21 \pm 1.51$   
335 for contaminated PDADMAC and  $k(10^{-2}) = 8.20 \pm 1.78$  for contaminated PSS (see Table SI5,  
336 Fig. SI7 and Fig. SI8 in Supporting Information), whereas without contamination, the average  
337 retention factor was  $k(10^{-2}) = 3.33 \pm 0.93$  in the best case (Cap 2 in Table SI6). The  
338 contaminated PDADMAC seems to be especially detrimental to the repeatability of the  
339 separations, with %RSD( $t_m$ ) at -10kV averaging at 8.5% for all proteins, compared to 5.6% for  
340 the contaminated PSS, and 0.13% for normal, unpolluted polyelectrolytes. To limit this  
341 outcome, sufficient amounts of polyelectrolyte were added to the vials and different buffer  
342 vials were used to flush the capillary after each oppositely charged layer (see section 2.1).

### 343 **3.4. Intra- and inter-capillary reproducibility**

344 In addition to repeatability, which was tested by repeating each separation 5 times leading  
345 to run-to-run RSDs, it is important to confirm the reproducibility and intermediate precision  
346 of the analyses. Strictly speaking, reproducibility refers to inter-laboratory comparisons,  
347 which are relatively rarely investigated either on silica capillaries [34] or for neutral coatings  
348 [36], since they are time consuming. Such investigations are also lacking for SMIL coatings in  
349 CE. Intra- and inter-capillary Intermediate precisions can be more easily evaluated, and are  
350 useful to evaluate method robustness. Figure 5 depicts the concepts of inter-capillary  
351 precision, which was measured by repeating the coating procedure on three different  
352 capillaries and comparing the separations obtained on each one (giving cap-to-cap RSDs), and  
353 intra-capillary precision, which was evaluated by repeating five series of measures on the  
354 same capillary coating. One series was made up of five runs at five different electric fields, for  
355 a total of 25 runs. This is equivalent to calculating day-to-day RSDs, while also testing the  
356 coating's durability.

357 Overall, the 5 series performed on the same 5-layer PDADMAC/PSS coating each showed  
358 very good repeatability, with RSDs on migration times all below 0.9% at -10kV and below 1.8%  
359 at -25kV (see Table 1). Moreover, intra-capillary precision was also satisfying, with a maximum  
360 RSD of 2.0% at -10kV and 3.2% at -25kV. Regarding the plate height, which even more  
361 sensitive compared to migration times, the first four series lead to quite similar plate heights  
362 for Myo, and only start to deviate for the fifth series, as can be seen in Fig. 6. This proves  
363 satisfying precision and durability over 100 runs. Similar results were found for the other  
364 proteins in the mix (see Fig. SI9). In terms of inter-capillary precision, RSDs on migration times  
365 were all below 4.6% with an average value of 4.2% at -10kV and 4.5% at -25kV, which is very  
366 satisfactory (see Table 1). RSDs on the retention factors and on the constant term in Eq. (7)  
367 were much higher, with an average value for all proteins of 12% and 16% respectively, which  
368 is understandable knowing the sensitivity of separation efficiency in general (see Table 2).  
369 Still, the retention factors obtained on the different capillaries are similar (within 10-20%) and  
370 validate the protocol for the determination of the retention factor as an estimation of residual  
371 protein adsorption. Plate numbers for the separations at -10kV vary between 51 000 and 82  
372 000 on average (see Table SI7).

### 373 **3.5. Capillary coating regeneration**

374           Regeneration possibilities were also investigated, as this simplifies the experimental  
375 procedure and increases the lifetime of each capillary. First, a used capillary stored in BGE  
376 vials over one month was tested, showing slightly lower separation efficiency with an  
377 average  $k(10^{-2}) = 7.48 \pm 1.68$  and a modified profile of the  $H$  versus  $u$  curves (see Fig. 7 A).  
378 Next, this same capillary was flushed with a 2 M NaCl, 0.1 M NaOH aqueous solution for 10  
379 min, followed by the usual coating procedure, and tested once more for the separation of  
380 the model proteins. This led to lower retention factors,  $k(10^{-2}) = 5.55 \pm 1.69$  on average, and  
381 the same profile of plate heights as the initial experiments on new capillaries, showing that  
382 regeneration can be an option to revive old capillaries which have been properly stored (see  
383 Fig. 7). Rinsing with other solvents, such as a 2% v/v Hellmanex solution followed by a 2 M  
384 NaCl, 1 M NaOH solution, or 1 M HCl followed by 1 M NaOH, before regeneration of the  
385 PDADMAC/PSS coating also gave satisfying results, with average retention factors of  $k(10^{-2})$   
386  $= 4.31 \pm 1.05$  and  $k(10^{-2}) = 3.94 \pm 0.98$  respectively (see Fig. SI10 and Fig. SI11).

387

## 388 **Conclusions**

389           In this work, 5-layer PDADMAC/PSS coatings were used to separate a mix of model  
390 proteins in 2 M acetic acid BGE. The parameters having the most impact on separation  
391 performances were determined, paying particular attention to experimental conditions, such  
392 as vial preparation, and evaluating reproducibility and intermediate precision on migration  
393 times. First, different calculation methods for plate heights were compared, using the width  
394 at half heights, the moments, and CEval's HVL function, leading to the conclusion that the  
395 classic method with the width and half height is adapted to these experiments as the peaks  
396 are quite symmetrical. Next, protein sample conservation was shown to be a crucial aspect,  
397 as the proteins tend to alter over time if stored in BGE and/or in a mix prior to analysis, which  
398 leads to broader peaks and lower separation efficiency. The injection vial medium was also  
399 significant, as injecting samples in water rather than BGE led to a stacking effect, which  
400 increased plate height dependence on electric field and may lead to wrong evaluation of  
401 residual adsorption via the determination of the retention factor. Moreover, polyelectrolyte  
402 vial contamination must be avoided, as it was shown to be detrimental to the repeatability  
403 and efficiency of the analyses. Throughout these experiments, protein retention factors were



404 determined as a measure of residual adsorption onto the capillary wall, enabling a  
405 quantitative evaluation of the most important points to look out for. They varied between  
406  $3.1 \times 10^{-2}$  and  $5.6 \times 10^{-2}$  for different proteins on a fresh PDAMDAC/PSS coating. Repeatability  
407 and intermediate precision of the separations were also assessed looking at run-to-run, day-  
408 to-day and cap-to-cap RSDs on migration times, which reached 1.3%, 3.2% and 4.6%,  
409 respectively. Overall, the aspects investigated in this paper have greatly improved protein  
410 separation efficiency and reproducibility through the identification of several critical  
411 parameters, which are highly relevant to control for the successful implementation of  
412 performant SMIL coatings in CE.

413

#### 414 **Declaration of competing interest**

415 The authors declare no conflict of interest.

416

#### 417 **Acknowledgments**

418 This work was realized within the scope of a PRCI in collaboration with German partners  
419 from the University of Aalen, Germany (ANR-DFG SMIL E, ANR-20-C E 92-0021-01).

420

#### 421 **Supporting Information**

422 Please find supplementary material provided with this file.

423

#### 424 **References**

425 [1] J. Cai, J. Henion, Capillary electrophoresis-mass spectrometry, *J. Chromatogr. A.* 703  
426 (1995) 667–692.

427 [2] R. Gahoual, A. Beck, E. Leize-Wagner, Y.-N. François, Cutting-edge capillary  
428 electrophoresis characterization of monoclonal antibodies and related products, *Journal of*  
429 *Chromatography B.* 1032 (2016) 61–78. <https://doi.org/10.1016/j.jchromb.2016.05.028>.

430 [3] S. Štěpánová, V. Kašička, Recent applications of capillary electromigration methods  
431 to separation and analysis of proteins, *Analytica Chimica Acta.* 933 (2016) 23–42.  
432 <https://doi.org/10.1016/j.aca.2016.06.006>.

- 433 [4] C.A. Lucy, A.M. MacDonald, M.D. Gulcev, Non-covalent capillary coatings for protein  
434 separations in capillary electrophoresis, *Journal of Chromatography A*. 1184 (2008) 81–105.  
435 <https://doi.org/10.1016/j.chroma.2007.10.114>.
- 436 [5] L. Leclercq, M. Morvan, J. Koch, C. Neusüß, H. Cottet, Modulation of the  
437 electroosmotic mobility using polyelectrolyte multilayer coatings for protein analysis by  
438 capillary electrophoresis, *Anal. Chim. Acta*. 1057 (2019) 152–161.  
439 <https://doi.org/10.1016/j.aca.2019.01.008>.
- 440 [6] B. Gaš, E. Kenndler, Dispersive phenomena in electromigration separation methods,  
441 *Electrophoresis*. 21 (2000) 3888–3897. [https://doi.org/10.1002/1522-](https://doi.org/10.1002/1522-2683(200012)21:18<3888::AID-ELPS3888>3.0.CO;2-D)  
442 [2683\(200012\)21:18<3888::AID-ELPS3888>3.0.CO;2-D](https://doi.org/10.1002/1522-2683(200012)21:18<3888::AID-ELPS3888>3.0.CO;2-D).
- 443 [7] H.H. Lauer, Douglass. McManigill, Capillary zone electrophoresis of proteins in  
444 untreated fused silica tubing, *Anal. Chem.* 58 (1986) 166–170.  
445 <https://doi.org/10.1021/ac00292a041>.
- 446 [8] J.S. Green, J.W. Jorgenson, Minimizing adsorption of proteins on fused silica in  
447 capillary zone electrophoresis by the addition of alkali metal salts to the buffers, *Journal of*  
448 *Chromatography A*. 478 (1989) 63–70. [https://doi.org/10.1016/0021-9673\(89\)90006-X](https://doi.org/10.1016/0021-9673(89)90006-X).
- 449 [9] M. Bushey, W. Jorgenson, OF ZWITTERIONIC SALTS, (n.d.) 10.
- 450 [10] L. Hajba, A. Guttman, Recent advances in column coatings for capillary  
451 electrophoresis of proteins, *TrAC, Trends Anal. Chem.* 90 (2017) 38–44.  
452 <https://doi.org/10.1016/j.trac.2017.02.013>.
- 453 [11] H. Katayama, Y. Ishihama, N. Asakawa, Stable Cationic Capillary Coating with  
454 Successive Multiple Ionic Polymer Layers for Capillary Electrophoresis, *Anal. Chem.* 70  
455 (1998) 5272–5277. <https://doi.org/10.1021/ac980522l>.
- 456 [12] S. Bekri, L. Leclercq, H. Cottet, Polyelectrolyte multilayer coatings for the separation  
457 of proteins by capillary electrophoresis: Influence of polyelectrolyte nature and multilayer  
458 crosslinking, *Journal of Chromatography A*. 1399 (2015) 80–87.  
459 <https://doi.org/10.1016/j.chroma.2015.04.033>.
- 460 [13] F. Kitagawa, M. Kamiya, Y. Okamoto, H. Taji, S. Onoue, Y. Tsuda, K. Otsuka,  
461 Electrophoretic analysis of proteins and enantiomers using capillaries modified by a  
462 successive multiple ionic-polymer layer (SMIL) coating technique, *Anal. Bioanal. Chem.* 386  
463 (2006) 594–601. <https://doi.org/10.1007/s00216-006-0438-x>.
- 464 [14] R. Haselberg, G.J. de Jong, G.W. Somsen, Capillary electrophoresis of intact basic  
465 proteins using noncovalently triple-layer coated capillaries, *J. Sep. Sci.* 32 (2009) 2408–2415.  
466 <https://doi.org/10.1002/jssc.200900164>.
- 467 [15] T. Isemura, F. Kitagawa, K. Otsuka, Separation of complex mixtures of fluorobenzoic  
468 acids by capillary electrophoresis, *J. Sep. Sci.* 32 (2009) 381–387.  
469 <https://doi.org/10.1002/jssc.200800549>.

- 470 [16] K.E. Swords, P.B. Bartline, K.M. Roguski, S.A. Bashaw, K.A. Frederick, Assessment of  
471 polyelectrolyte coating stability under dynamic buffer conditions in CE: Electrodriven  
472 Separations, *J. Sep. Sci.* 34 (2011) 2427–2432. <https://doi.org/10.1002/jssc.201100044>.
- 473 [17] R. Nehmé, C. Perrin, H. Cottet, M.-D. Blanchin, H. Fabre, Stability of capillaries coated  
474 with highly charged polyelectrolyte monolayers and multilayers under various analytical  
475 conditions—Application to protein analysis, *J. Chromatogr. A.* 1218 (2011) 3537–3544.  
476 <https://doi.org/10.1016/j.chroma.2011.03.040>.
- 477 [18] T.W. Graul, J.B. Schlenoff, Capillaries Modified by Polyelectrolyte Multilayers for  
478 Electrophoretic Separations, *Anal. Chem.* 71 (1999) 4007–4013.  
479 <https://doi.org/10.1021/ac990277l>.
- 480 [19] L. Leclercq, C. Renard, M. Martin, H. Cottet, Quantification of Adsorption and  
481 Optimization of Separation of Proteins in Capillary Electrophoresis, *Anal. Chem.* 92 (2020)  
482 10743–10750. <https://doi.org/10.1021/acs.analchem.0c02012>.
- 483 [20] S. Roca, L. Dhellemmes, L. Leclercq, H. Cottet, Polyelectrolyte Multilayers in Capillary  
484 Electrophoresis, *ChemPlusChem.* 87 (2022). <https://doi.org/10.1002/cplu.202200028>.
- 485 [21] Q. Qu, C. Gu, Z. Gu, Y. Shen, C. Wang, X. Hu, Layer-by-layer assembly of  
486 polyelectrolyte and graphene oxide for open-tubular capillary electrochromatography, *J.*  
487 *Chromatogr. A.* 1282 (2013) 95–101. <https://doi.org/10.1016/j.chroma.2013.01.055>.
- 488 [22] C. Liu, J. Kang, Improved capillary electrophoresis frontal analysis by dynamically  
489 coating the capillary with polyelectrolyte multilayers, *J. Chromatogr. A.* 1238 (2012) 146–  
490 151. <https://doi.org/10.1016/j.chroma.2012.03.043>.
- 491 [23] J.P. Schaeper, M.J. Sepaniak, Parameters affecting reproducibility in capillary  
492 electrophoresis, *Electrophoresis.* 21 (2000) 1421–1429. [https://doi.org/10.1002/\(SICI\)1522-  
493 2683\(20000401\)21:7<1421::AID-ELPS1421>3.0.CO;2-7](https://doi.org/10.1002/(SICI)1522-2683(20000401)21:7<1421::AID-ELPS1421>3.0.CO;2-7).
- 494 [24] T. Faller, H. Engelhardt, How to achieve higher repeatability and reproducibility in  
495 capillary electrophoresis, *Journal of Chromatography A.* 853 (1999) 83–94.  
496 [https://doi.org/10.1016/S0021-9673\(99\)00382-9](https://doi.org/10.1016/S0021-9673(99)00382-9).
- 497 [25] P. Dubský, M. Ördögová, M. Malý, M. Riesová, CEval: All-in-one software for data  
498 processing and statistical evaluations in affinity capillary electrophoresis, *Journal of*  
499 *Chromatography A.* 1445 (2016) 158–165. <https://doi.org/10.1016/j.chroma.2016.04.004>.
- 500 [26] D.S. Burgi, R.Ling. Chien, Optimization in sample stacking for high-performance  
501 capillary electrophoresis, *Anal. Chem.* 63 (1991) 2042–2047.  
502 <https://doi.org/10.1021/ac00018a028>.
- 503 [27] W.E. Barber, P.W. Carr, Graphical method for obtaining retention time and number  
504 of theoretical plates from tailed chromatographic peaks, *Anal. Chem.* 53 (1981) 1939–1942.  
505 <https://doi.org/10.1021/ac00235a052>.
- 506 [28] J.J. Kirkland, W.W. Yau, H.J. Stoklosa, C.H. Dilks, Sampling and Extra-Column Effects in  
507 High-Performance Liquid Chromatography; Influence of Peak Skew on Plate Count

508 Calculations, *Journal of Chromatographic Science*. 15 (1977) 303–316.  
509 <https://doi.org/10.1093/chromsci/15.8.303>.

510 [29] P. Dubský, M. Dvořák, L. Múllerová, B. Gaš, Determination of the correct migration  
511 time and other parameters of the Haarhoff-van der Linde function from the peak geometry  
512 characteristics: General, *Electrophoresis*. 36 (2015) 655–661.  
513 <https://doi.org/10.1002/elps.201400463>.

514 [30] M.R. Schure, A.M. Lenhoff, Consequences of wall adsorption in capillary  
515 electrophoresis: theory and simulation, *Anal. Chem.* 65 (1993) 3024–3037.  
516 <https://doi.org/10.1021/ac00069a015>.

517 [31] R. Haselberg, G.J. de Jong, G.W. Somsen, Capillary electrophoresis-mass  
518 spectrometry for the analysis of intact proteins 2007-2010, *Electrophoresis*. 32 (2011) 66–  
519 82. <https://doi.org/10.1002/elps.201000364>.

520 [32] J.P. López-Alonso, M. Bruix, J. Font, M. Ribó, M. Vilanova, M.A. Jiménez, J. Santoro, C.  
521 González, D.V. Laurents, NMR Spectroscopy Reveals that RNase A is Chiefly Denatured in  
522 40% Acetic Acid: Implications for Oligomer Formation by 3D Domain Swapping, *J. Am. Chem.*  
523 *Soc.* 132 (2010) 1621–1630. <https://doi.org/10.1021/ja9081638>.

524 [33] L.L. Garcia, Z.K. Shihabi, Sample matrix effects in capillary electrophoresis I. Basic  
525 considerations, (1993) 5.

526 [34] B.X. Mayer, How to increase precision in capillary electrophoresis, *Journal of*  
527 *Chromatography A*. 907 (2001) 21–37. [https://doi.org/10.1016/S0021-9673\(00\)01057-8](https://doi.org/10.1016/S0021-9673(00)01057-8).

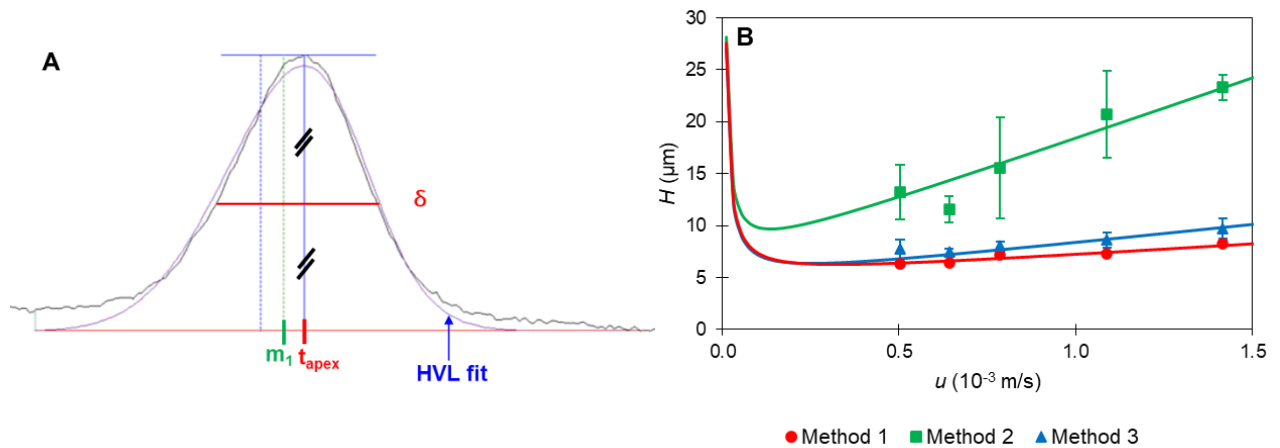
528 [35] R. Nehmé, C. Perrin, H. Cottet, M.D. Blanchin, H. Fabre, Influence of polyelectrolyte  
529 coating conditions on capillary coating stability and separation efficiency in capillary  
530 electrophoresis, *Electrophoresis*. 29 (2008) 3013–3023.  
531 <https://doi.org/10.1002/elps.200700886>.

532 [36] C. Wenz, C. Barbas, Á. López-González, A. Garcia, F. Benavente, V. Sanz-Nebot, T.  
533 Blanc, G. Freckleton, P. Britz-McKibbin, M. Shanmuganathan, F. de l'Escaille, J. Far, R.  
534 Haselberg, S. Huang, C. Huhn, M. Pattky, D. Michels, S. Mou, F. Yang, C. Neusuess, N.  
535 Tromsdorf, E.E.K. Baidoo, J.D. Keasling, S.S. Park, Interlaboratory study to evaluate the  
536 robustness of capillary electrophoresis-mass spectrometry for peptide mapping:  
537 Electrodriven Separations, *J. Sep. Science*. 38 (2015) 3262–3270.  
538 <https://doi.org/10.1002/jssc.201500551>.

539  
540  
541  
542  
543  
544

545

FIGURES

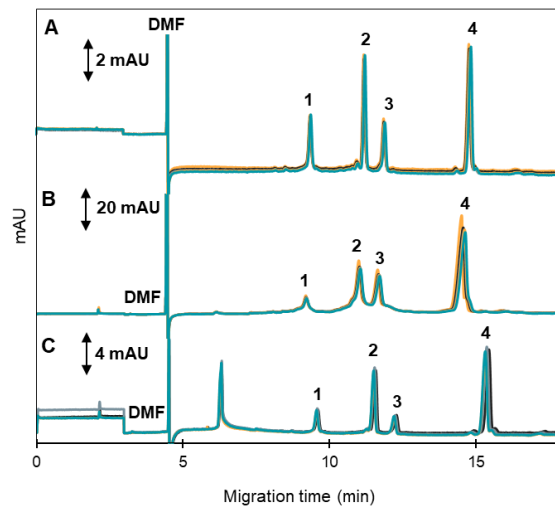


546

547

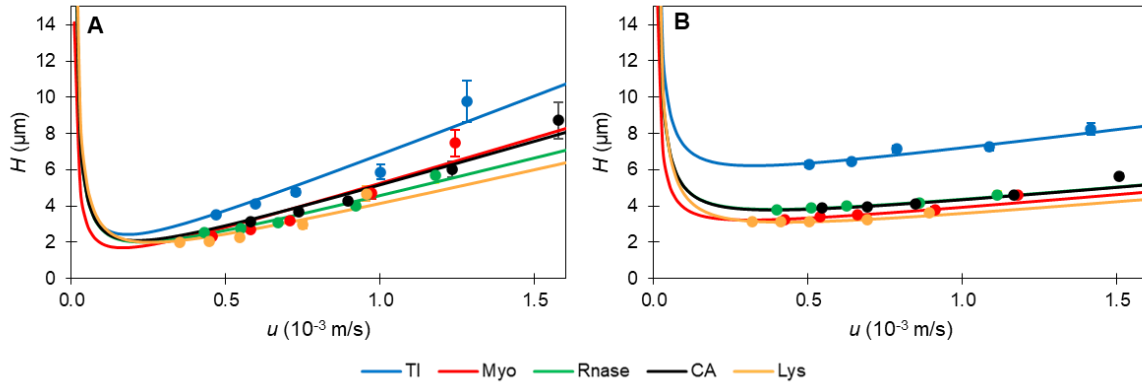
548 **Figure 1.** Different methods of calculating plate heights shown on a TI peak analyzed on a 5-layer  
 549 PDADMAC/PSS coating (A) and corresponding representations of  $H$  as a function of  $u$ . Method 1 in red  
 550 (eq. 2), method 2 in green (eq. 3) and method 3 (eq. 4) in blue (B). Experimental conditions: 5-layer  
 551 SMIL coated capillary terminating with the polycation PDADMAC. Capillary: 40 cm (31.5 cm to the  
 552 detector) x 50  $\mu\text{m}$  I.D. BGE: 2 M acetic acid, pH 2.2. Flush before each run: BGE 1 bar, 5 min.  
 553 Hydrodynamic injection: 30 mbar, 4 s. Sample mixture: 0.2 g/L of each protein in BGE. Hydrodynamic  
 554 co-injection of 0.02% DMF in BGE: 30 mbar, 3 s. Temperature: 25°C. For the coating procedure, see  
 555 section 2.2.

556



557

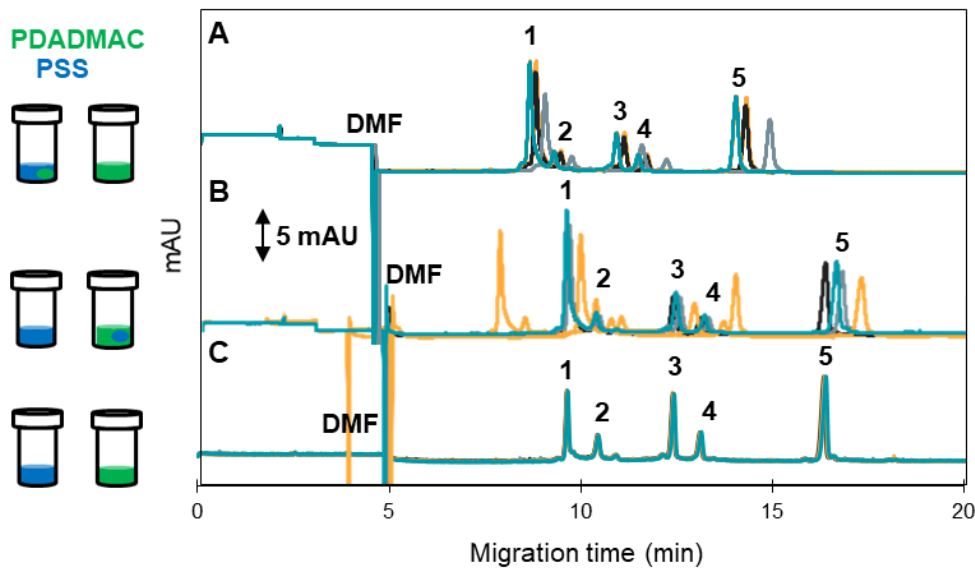
558 **Figure 2.** Superposition of five repetitions of electropherograms of 4 model protein mix at -10kV on 5-  
 559 layer PDADMAC/PSS SMIL coatings for initial fresh protein mix in BGE (A), the same mix after 5 months  
 560 storage in the freezer (B), and freshly prepared sample (C). Experimental conditions: 5-layer SMIL  
 561 coated capillary terminating with the polycation PDADMAC. Capillary: 40 cm (31.5 cm to the detector)  
 562 x 50  $\mu\text{m}$  I.D. Electrolyte: 2 M acetic acid, pH 2.2. Flush before each run: BGE 1 bar, 5 min. Peak  
 563 identification: TI (1), Myo (2), RNase A (3), Lys (4). Hydrodynamic injection: 30 mbar, 4 s. Sample  
 564 mixture: 0.2 g/L of each protein in BGE. Hydrodynamic co-injection of 0.02% DMF in BGE: 30 mbar, 3  
 565 s. Temperature: 25°C. For the coating procedure, see section 2.2.



566

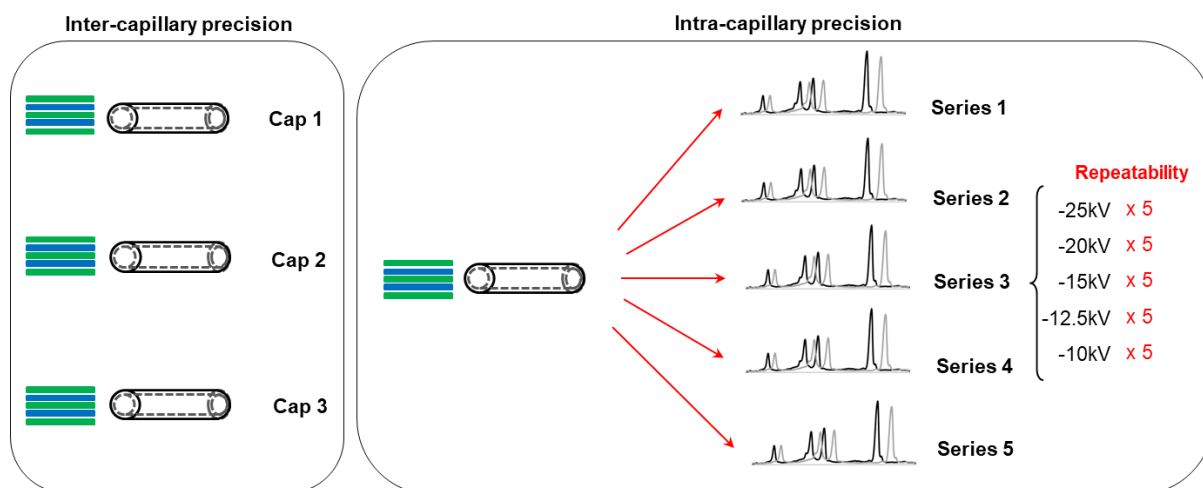
567 **Figure 3.** Influence of the sample matrix (water (A) and BGE (B)) on the  $H$  vs  $u$  representations for the  
 568 separation of five model proteins on a 5-layer PDADMAC/PSS SMIL coating. Sample mixture: 0.2 g/L  
 569 of each protein in water (A) or BGE (B). Hydrodynamic co-injection of 0.01% DMF in water (A) or BGE  
 570 (B). Error bars are  $\pm$  one SD on  $n=5$  repetitions. Other experimental conditions as in Fig. 1. (A) CA:  $k(10^{-2}) = 3.77 \pm 0.06$ ,  $cst(\mu\text{m}) = 0$ ; TI:  $k(10^{-2}) = 7.34 \pm 0.23$ ,  $cst(\mu\text{m}) = 0.01 \pm 0.03$ ; Myo:  $k(10^{-2}) = 5.04 \pm 0.20$ ,  
 572  $cst(\mu\text{m}) = 0$ ; RNase A:  $k(10^{-2}) = 5.79 \pm 0.27$ ,  $cst(\mu\text{m}) = 0.08 \pm 0.18$ , Lys:  $k(10^{-2}) = 5.99 \pm 0.23$ ,  $cst(\mu\text{m}) =$   
 573 0. (B) CA:  $k(10^{-2}) = 1.88 \pm 0.66$ ,  $cst(\mu\text{m}) = 4.71 \pm 0.12$ ; TI:  $k(10^{-2}) = 4.29 \pm 0.12$ ,  $cst(\mu\text{m}) = 4.71 \pm 0.12$ ;  
 574 Myo:  $k(10^{-2}) = 3.08 \pm 0.23$ ,  $cst(\mu\text{m}) = 2.03 \pm 0.16$ ; RNase A:  $k(10^{-2}) = 3.50 \pm 0.08$ ,  $cst(\mu\text{m}) = 2.61 \pm 0.04$ ,  
 575 Lys:  $k(10^{-2}) = 3.90 \pm 0.16$ ,  $cst(\mu\text{m}) = 1.78 \pm 0.06$ .

576



577

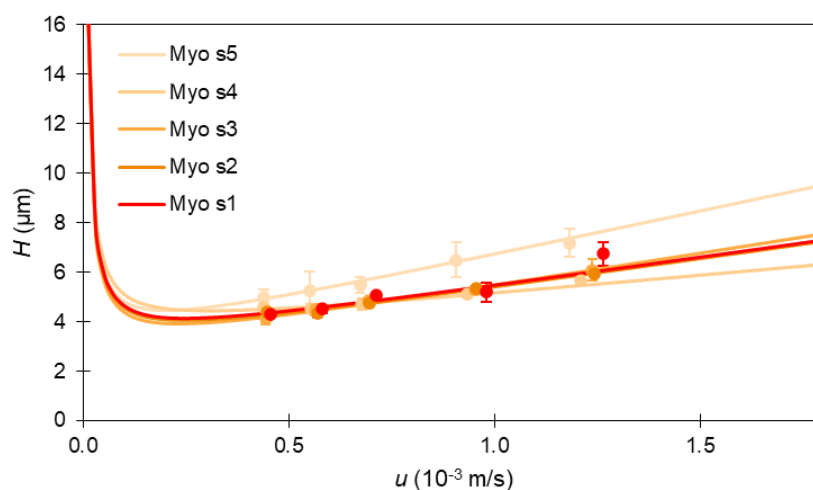
578 **Figure 4.** Superposition of five repetitions of electropherograms of five model proteins at -10kV on 5-  
 579 layer PDADMAC/PSS SMIL coatings for PSS-contaminated PDADMAC coating solution (A), PDADMAC-  
 580 contaminated PSS coating solution (B), and non-contaminated polyelectrolyte solutions (C). Peak  
 581 identification: CA (1), TI (2), Myo (3), RNase A (4), Lys (5). Other experimental conditions as in Fig. 1.



582

583 **Figure 5.** Principles of inter-capillary and intra-capillary intermediate precision.

584



585

586 **Figure 6.** Superposition of five successive series of separations of five model proteins on a single 5-  
 587 layer PDADMAC/PSS SMIL coating, showing the  $H$  vs  $u$  curves for Myo. Error bars are  $\pm$  one SD on  $n=5$   
 588 repetitions. Experimental conditions as in Fig. 1.

589

590

591

592

593

594 **Table 1.** Values of %RSDs given for each protein on migration times at -10kV and -25kV. Run-to-run  
 595 RSDs are calculated on five runs, day-to-day RSDs are calculated on the five series and cap-to-cap RSDs  
 596 on three capillaries. Experimental conditions as in Fig. 1.

		%RSD( $t_m$ ) at -10kV			%RSD( $t_m$ ) at -25kV		
		run to run	day to day	cap to cap	run to run	day to day	cap to cap
CA	s1	0.12	1.3	4.3	1.0	2.2	4.5
	s2	0.19			0.04		
	s3	0.57			0.11		
	s4	0.12			0.95		
	s5	0.74			0.90		
TI	s1	0.18	1.4	4.1	1.07	2.2	4.3
	s2	0.19			0.05		
	s3	0.58			0.12		
	s4	0.11			1.01		
	s5	0.77			0.96		
Myo	s1	0.13	1.6	4.4	1.3	2.6	4.6
	s2	0.23			0.06		
	s3	0.55			0.15		
	s4	0.13			0.98		
	s5	0.82			1.02		
RNase A	s1	0.14	1.7	3.9	1.4	2.7	4.5
	s2	0.24			0.06		
	s3	0.57			0.17		
	s4	0.14			0.98		
	s5	0.84			1.04		
Lys	s1	0.11	2.0	4.3	1.8	3.2	4.4
	s2	0.30			0.09		
	s3	0.57			0.19		
	s4	0.16			0.98		
	s5	0.90			1.3		

597  
 598  
 599  
 600  
 601  
 602  
 603  
 604  
 605



606 **Table 2.** Values and RSDs of calculated retention factors and constants.  $k$  and  $cst$  are given by the  
607 average of the values calculated by the Jackknife method  $\pm$  one standard deviation ( $n=5$ ). Day-to-day  
608 RSDs are calculated on the five series and cap-to-cap RSDs on three capillaries. Experimental  
609 conditions as in Fig. 1.

		$k (10^{-2})$	Average $k$ in $10^{-2}$ (RSD)		$cst (\mu\text{m})$	Average $cst$ in $\mu\text{m}$ (RSD)	
			day to day	cap to cap		day to day	cap to cap
CA	s1	$3.1 \pm 0.2$	3.1 (11%)	2.7 (27%)	$2.9 \pm 0.2$	3.0 (10%)	2.7 (18%)
	s2	$3.0 \pm 0.2$			$2.8 \pm 0.4$		
	s3	$3.0 \pm 0.2$			$3.2 \pm 0.4$		
	s4	$3.2 \pm 0.3$			$2.8 \pm 0.5$		
	s5	$3.8 \pm 0.2$			$3.5 \pm 0.5$		
TI	s1	$4.9 \pm 0.4$	5.1 (7.4%)	4.7 (8.1%)	$6.0 \pm 0.3$	6.3 (14%)	5.4 (12%)
	s2	$4.7 \pm 0.04$			$6.1 \pm 0.1$		
	s3	$5.6 \pm 0.3$			$5.9 \pm 0.4$		
	s4	$5.3 \pm 0.4$			$5.8 \pm 0.6$		
	s5	$5.3 \pm 0.6$			$7.8 \pm 0.5$		
Myo	s1	$3.8 \pm 0.3$	3.5 (12%)	3.6 (14%)	$2.7 \pm 0.2$	3.0 (10%)	2.3 (14%)
	s2	$3.4 \pm 0.1$			$2.9 \pm 0.1$		
	s3	$3.6 \pm 0.1$			$2.7 \pm 0.1$		
	s4	$2.9 \pm 0.2$			$3.4 \pm 0.2$		
	s5	$4.1 \pm 0.1$			$3.2 \pm 0.1$		
RNAse A	s1	$4.1 \pm 0.3$	4.2 (5.4%)	3.9 (10%)	$3.5 \pm 0.2$	3.7 (6%)	3.0 (15%)
	s2	$4.2 \pm 0.3$			$3.7 \pm 0.2$		
	s3	$4.3 \pm 0.4$			$3.4 \pm 0.3$		
	s4	$3.9 \pm 0.4$			$3.8 \pm 0.2$		
	s5	$4.5 \pm 0.3$			$3.9 \pm 0.2$		
Lys	s1	$4.9 \pm 0.3$	4.9 (10%)	4.6 (13%)	$2.5 \pm 0.1$	2.5 (13%)	2.2 (17%)
	s2	$4.9 \pm 0.2$			$2.4 \pm 0.1$		
	s3	$5.7 \pm 0.3$			$2.0 \pm 0.3$		
	s4	$4.3 \pm 0.2$			$2.8 \pm 0.2$		
	s5	$4.7 \pm 0.1$			$2.7 \pm 0.1$		

610

611

612

613

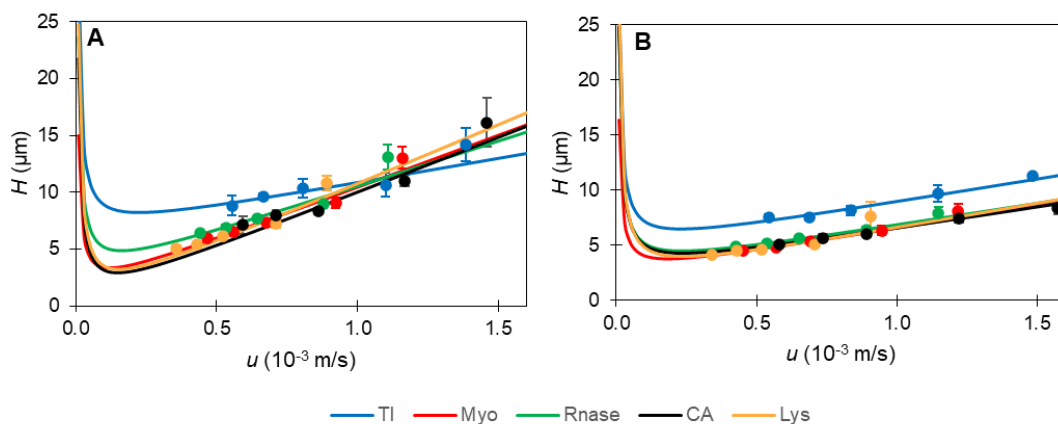
614

615

616

617

618



619

620 **Figure 7.** Representations of plate heights  $H$  as a function of  $u$  on a 5-layer PDADMAC/PSS SMIL coating  
 621 after storage for one month in BGE (A) and after regeneration (rinsed with a 2 M NaCl, 0.1 M NaOH  
 622 solution for 10 min and then the normal coating procedure) (B). Other experimental conditions as in  
 623 Fig. 1.

624

625

### LIST OF CAPTIONS

626

627 **Figure 1.** Different methods of calculating plate heights shown on a TI peak analyzed on a 5-  
 628 layer PDADMAC/PSS coating (A) and corresponding representations of  $H$  as a function of  $u$ .  
 629 Method 1 in red (eq. 2), method 2 in green (eq. 3) and method 3 (eq. 4) in blue (B).  
 630 Experimental conditions: 5-layer SMIL coated capillary terminating with the polycation  
 631 PDADMAC. Capillary: 40 cm (31.5 cm to the detector) x 50  $\mu\text{m}$  I.D. BGE: 2 M acetic acid, pH  
 632 2.2. Flush before each run: BGE 1 bar, 5 min. Hydrodynamic injection: 30 mbar, 4 s. Sample  
 633 mixture: 0.2 g/L of each protein in BGE. Hydrodynamic co-injection of 0.02% DMF in BGE: 30  
 634 mbar, 3 s. Temperature: 25°C. For the coating procedure, see section 2.2.

635 **Figure 2.** Superposition of five repetitions of electropherograms of 4 model protein mix at -  
 636 10kV on 5-layer PDADMAC/PSS SMIL coatings for initial fresh protein mix in BGE (A), the same  
 637 mix after 5 months storage in the freezer (B), and freshly prepared sample (C). Experimental  
 638 conditions: 5-layer SMIL coated capillary terminating with the polycation PDADMAC.  
 639 Capillary: 40 cm (31.5 cm to the detector) x 50  $\mu\text{m}$  I.D. Electrolyte: 2 M acetic acid, pH 2.2.  
 640 Flush before each run: BGE 1 bar, 5 min. Peak identification: TI (1), Myo (2), RNase A (3), Lys  
 641 (4). Hydrodynamic injection: 30 mbar, 4 s. Sample mixture: 0.2 g/L of each protein in BGE.  
 642 Hydrodynamic co-injection of 0.02% DMF in BGE: 30 mbar, 3 s. Temperature: 25°C. For the  
 643 coating procedure, see section 2.2.

644 **Figure 3.** Influence of the sample matrix (water (A) and BGE (B)) on the  $H$  vs  $u$  representations  
 645 for the separation of five model proteins on a 5-layer PDADMAC/PSS SMIL coating. Sample  
 646 mixture: 0.2 g/L of each protein in water (A) or BGE (B). Hydrodynamic co-injection of 0.01%  
 647 DMF in water (A) or BGE (B). Error bars are  $\pm$  one SD on  $n=5$  repetitions. Other experimental  
 648 conditions as in Fig. 1. (A) CA:  $k(10^{-2}) = 6.32 \pm 0.12$ ,  $cst(\mu\text{m}) = 0$ ; TI:  $k(10^{-2}) = 7.34 \pm 0.23$ ,

649  $cst(\mu\text{m}) = 0.01 \pm 0.03$ ; Myo:  $k(10^{-2}) = 5.04 \pm 0.20$ ,  $cst(\mu\text{m}) = 0$ ; RNase A:  $k(10^{-2}) = 5.79 \pm 0.27$ ,  
650  $cst(\mu\text{m}) = 0.08 \pm 0.18$ , Lys:  $k(10^{-2}) = 5.99 \pm 0.23$ ,  $cst(\mu\text{m}) = 0$ . (B) CA:  $k(10^{-2}) = 3.98 \pm 0.31$ ,  
651  $cst(\mu\text{m}) = 2.23 \pm 0.23$ ; TI:  $k(10^{-2}) = 4.29 \pm 0.12$ ,  $cst(\mu\text{m}) = 4.71 \pm 0.12$ ; Myo:  $k(10^{-2}) = 3.08 \pm$   
652  $0.23$ ,  $cst(\mu\text{m}) = 2.03 \pm 0.16$ ; RNase A:  $k(10^{-2}) = 3.50 \pm 0.08$ ,  $cst(\mu\text{m}) = 2.61 \pm 0.04$ , Lys:  $k(10^{-2})$   
653  $= 3.90 \pm 0.16$ ,  $cst(\mu\text{m}) = 1.78 \pm 0.06$ .

654 **Figure 4.** Superposition of five repetitions of electropherograms of five model proteins at -  
655 10kV on 5-layer PDADMAC/PSS SMIL coatings for PSS-contaminated PDADMAC coating  
656 solution (A), PDADMAC-contaminated PSS coating solution (B), and non-contaminated  
657 polyelectrolyte solutions (C). Peak identification: CA (1), TI (2), Myo (3), RNase A (4), Lys (5).  
658 Other experimental conditions as in Fig. 1.

659 **Figure 5.** Principles of repeatability and intermediate precision.

660 **Figure 6.** Superposition of five successive series of separations of five model proteins on a  
661 single 5-layer PDADMAC/PSS SMIL coating, showing the H vs u curves for Myo. Error bars are  
662  $\pm$  one SD on n=5 repetitions. Experimental conditions as in Fig. 1.

663 **Figure 7.** Representations of plate heights  $H$  as a function of  $u$  on a 5-layer PDADMAC/PSS  
664 SMIL coating after storage for one month in BGE (A) and after regeneration (rinsed with a 2  
665 M NaCl, 0.1 M NaOH solution for 10 min and then the normal coating procedure) (B). Other  
666 experimental conditions as in Fig. 1.

667