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1 Optimization of limit of detection in Taylor Dispersion

2 Analysis: application to the size determination of

3 vaccine antigens

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15 Abstract

16 The development of a new vaccine requires the precise characterization of all the
17 physicochemical parameters of the vaccine antigens, which are the molecules that induce the
18 immune response. Taylor dispersion analysis (TDA) is a promising alternative technique for the
19 determination of diffusion coefficients and hydrodynamic radii of proteins, macromolecules and
20 nanoparticles. In this work, TDA was used to determine the hydrodynamic radius distribution and
21 its average value of four antigens: diphtheria toxoid (DT), tetanus toxoid (TT), hepatitis B surface
22 antigen (HBsAg) and polyribosyl-ribitol phosphate conjugated to tetanus toxoid (PRP-T). The
23 robustness of the results obtained was investigated on bare fused silica capillary and
24 hydroxypropylcellulose coated capillary. The impact of operational parameters on the limit of

25 detection (LOD) and limit of quantification (LOQ) were studied from both theoretical and
26 experimental points of view. The influence of the diameter and the length of the capillary on the
27 LOD and LOQ were studied as well as the impact of the mobilization pressure. General guidelines
28 for the choice of the initial operating conditions are given for the development of future TDA
29 methods.

30 **Keywords:** Taylor dispersion analysis, proteins, vaccines, limit of detection, limit of
31 quantification, quantitative analysis.

32

33 1. Introduction

34 Taylor Dispersion Analysis (TDA) is a straightforward method for the determination of the
35 diffusion coefficient and, thus, the hydrodynamic radius of nano-objects. TDA is based on the
36 dispersion of a solute plug in an open tube under a laminar Poiseuille flow.^{1,2} The dispersion is due
37 to the combined action of the dispersive parabolic velocity profile and the molecular diffusion that
38 redistributes the molecules in the cross-section of the capillary. When the conditions of validity of
39 Taylor dispersion are fulfilled, the elution profile recorded as a function of time for a
40 monomolecular sample is a Gaussian peak.³ The determination of the temporal variance of the
41 elution profile σ_t^2 allows to directly calculate the molecular diffusion coefficient D_m and the average
42 hydrodynamic radius R_h by using the Stokes-Einstein equation. TDA can also provide both the
43 individual hydrodynamic radius R_h and the relative amounts of a mixture of two or three analytes.⁴⁻
44 ⁶ It can thus be considered as a separation method for which the selectivity is based on dispersion
45 rather than on retention. Moreover, an original approach for the data analysis of polydisperse
46 samples, based on Constrained Regularized Linear Inversion (CRLI), allows to obtain the size
47 distribution of the nano-objects from the experimental taylorgrams.⁷

48 TDA does not require any calibration and the knowledge of the sample concentration is not
49 needed for the size determination. Moreover, it is suitable for the analysis of low-abundant samples,
50 as the injected volume is very small (only a few nL are typically injected). TDA is applicable to
51 samples of different natures (small molecules⁸, proteins⁹, polymers¹⁰, liposomes¹¹,
52 microemulsions^{12,13}), either in aqueous or nonaqueous liquid phases, with hydrodynamic radius
53 (R_h) from 0.1 nm to about 150 nm.¹⁴ This method has also recently shown promise for the study of
54 vaccine antigens¹⁵ and lipid nanoparticles for mRNA delivery.¹⁶

55

56 Vaccines have shown their effectiveness throughout history on the occurrence of infectious
57 diseases.^{17,18} In addition to preventing deaths, vaccines also massively reduce complications and
58 disabilities.¹⁹ Yet many diseases still lack effective treatment and new epidemics are frequently
59 emerging.²⁰ Furthermore, vaccines have today the potential to prevent or treat different types of
60 diseases such as cancer²¹ and neurodegenerative disorders²². In vaccines' products, antigens are the
61 substances that trigger the immune response. The development of a new vaccine, and the release
62 of commercial lots, requires characterizing the physicochemical properties of antigens in details,
63 including their size.

64 The first part of this work was focused on the development of a method allowing to
65 characterize the size of four vaccine antigens: two toxoids, namely diphtheria toxoid (DT) against
66 diphtheria and tetanus toxoid (TT) against tetanus, a subviral particle named Hepatitis B surface
67 Antigen (HBsAg) for the prevention of *Hepatitis B* infection and a glycoconjugate named
68 polyribosyl-ribitol phosphate conjugated to tetanus toxoid (PRP-T) for the prevention of
69 *Haemophilus influenzae type b* infection.

70 As antigens are often at low concentrations in vaccine formulations, the limit of detection
71 (LOD) of the TDA method should be as low as possible. The second part of this work aims at
72 finding the optimal conditions to obtain the lowest LOD in TDA. Three main operating parameters,
73 namely the capillary radius, the applied pressure and the capillary total length were studied for the
74 optimization.

75

76 **2. Material and methods**

77 **2.1. Chemicals and Materials.** Diphtheria toxoid (DT) and tetanus toxoid (TT) were provided
78 at 18.5 g/L and 20.7 g/L in a 10 mM PBS, 200 mM glycine and 154 mM NaCl mixture buffered at
79 pH 6.9 by Sanofi (Marcy-l'Étoile, France). Hepatitis B surface antigen (HBsAg) was provided at
80 1.6 g/L in a 10 mM PBS and 154 mM NaCl mixture buffered at pH 7.4 by Sanofi (Marcy-l'Étoile,
81 France). Polyribosyl-ribitol phosphate conjugated to tetanus toxoid (PRP-T) was provided at 0.56
82 g/L in a 10 mM TRIS and 8% (w/w) sucrose mixture buffered at pH 7.4 by Sanofi (Marcy-l'Étoile,
83 France). Tris(hydroxymethyl)aminomethane (TRIS, $(\text{CH}_2\text{OH})_3\text{CNH}_2$, $M_w = 121$ g/mol),
84 Phosphate-Buffered Saline tablets (PBS), glycine ($\text{NH}_2\text{CH}_2\text{COOH}$, $M_w = 75.07$ g/mol), sucrose
85 ($\text{C}_{12}\text{H}_{22}\text{O}_{11}$, $M_w = 342.30$ g/mol) and hydroxypropyl cellulose (HPC, $M_w = 1 \times 10^5$ g/mol) were
86 purchased from Merk (Darmstadt, Germany). Bare fused silica capillaries were purchased from
87 Molex Polymicro Technologies (Phoenix, USA). Deionized water was further purified with a Milli-
88 Q system from Millipore (Molsheim, France).

89 **2.2. Sample preparation.** Stock solutions of DT, TT, HBsAg and PRP-T were diluted to 0.4
90 g/L in their respective analytical buffer. HBsAg was further diluted until 0.01 g/L in its analytical
91 buffer. Final solutions were homogenized by manual stirring before analysis.

92 **2.3. Capillary coating.** HPC capillaries coatings were performed based on a previously
93 published protocol.²³ Bare fused silica capillaries of 75 μm i.d. \times 75 cm total length (66.5 cm to the
94 detector) were used. HPC was dissolved in water at room temperature to 5% (w/w) final
95 concentration. The capillaries were flushed 30 min at 1 bar with the polymer solution using
96 capillary electrophoresis equipment and then heated in a gas chromatography oven (GC-14 A,
97 Shimadzu, France) under a nitrogen stream of 30 kPa. Temperature program was: 60°C for 10 min,
98 then 5°C/min gradient from 60°C to 140°C and finally, 140°C for 20 min. Before use and between

99 two samples, the coated capillaries were rinsed 2 min with water and then 2 min with the analysis
100 buffer at 1 bar.

101 **2.4. Taylor Dispersion Analysis (TDA).** All experiments were carried out on a 1600 CE
102 Agilent system (Santa Clara, USA). This system is equipped with a diode array detector (DAD).
103 Capillaries dimensions and injections conditions are stated in each figure caption. The elution peaks
104 obtained by TDA were fitted by the sum of Gaussians, according to equation (1) using a home-
105 developed Excel spreadsheet:

$$106 \quad S(t) = \sum_{i=1}^2 \frac{A_i}{\sigma_{t,i} \sqrt{2\pi}} \exp \left[-\frac{(t-t_0)^2}{2\sigma_{t,i}^2} \right] \quad (1)$$

107 where $S(t)$ is the absorbance signal, $\sigma_{t,i}^2$ is the temporal variance, A_i is a constant that depended on
108 the response factor and the injected quantity of solute and t_0 is the average elution time. t_0 is directly
109 obtained from the position of the maximum of absorbance and σ_i , and A_i are adjusting parameters
110 obtained by nonlinear least square regression using Excel solver.

111 The temporal variance $\sigma_{t,i}^2$ allows to calculate the molecular diffusion coefficient $D_{m,i}$
112 according to equation (2):

$$113 \quad D_{m,i} = \frac{R_c^2 t_0}{24\sigma_{t,i}^2} \quad (2)$$

114 where R_c is the capillary radius. Stokes–Einstein equation (3) allows then to determine the
115 hydrodynamic radius $R_{h,i}$:

$$116 \quad R_{h,i} = \frac{k_B T}{6\pi\eta D_{m,i}} \quad (3)$$

117 where k_B is the Boltzmann constant, T is the temperature, and η is the eluent viscosity.

118 The validity of TDA and equation (2) is conditioned to the assessment of two requirements.³
 119 First, the axial (longitudinal) diffusion must be negligible compared to the dispersion due to the
 120 parabolic velocity profile. Second, the average elution time must be longer than the characteristic
 121 time of diffusion of the analyte in the cross section of the capillary. For a relative error on the
 122 determination of the molecular diffusion coefficient (D_m) lower than 3%²⁴, the two conditions lead
 123 to equations (4) and (5):

$$124 \quad P_e = \frac{u R_c}{D_m} \geq 40 \quad (4)$$

125 where P_e is the Péclet number and u is the linear mobile phase velocity.

$$126 \quad \tau = \frac{D_m t_0}{R_c^2} \geq 1.25 \quad (5)$$

127 where τ is an adimensional parameter inversely proportional to the characteristic time of diffusion
 128 across the capillary section.

129 To get the size distribution, the elution profile was fitted using a second approach based on the
 130 Constrained Regularized Linear Inversion (CRLI) algorithm⁷ according to equation (6):

$$131 \quad S(t) = \int_0^\infty CM(D_m) \rho(D_m) \sqrt{D_m} \exp\left[-\frac{12(t-t_0)^2 D_m}{R_c^2 t_0}\right] dD_m \quad (6)$$

132 where C is an instrumental constant, $M(D_m)$ and $\rho(D_m)$ are the molar mass and the molar
 133 concentration in the injected sample of the objects with the diffusion coefficient D_m , respectively.

134 **2.5. Calculation of the limit of detection (LOD) and limit of quantification (LOQ).**

135 The LOD was calculated according to equation (7):

$$136 \quad LOD = \frac{3G}{a} \quad (7)$$

137 where G is the standard deviation of the blank signals and a is the slope of the calibration curve,
138 *i.e.* the plot of the peak height *vs.* the analyte concentration in the injected sample, in given
139 operating conditions.

140 In the same way the LOQ was determined according to equation (8):

$$141 \quad LOQ = \frac{10G}{a} \quad (8)$$

142 **3. Theoretical background**

143 **3.1. Optimization of the limit of detection (LOD) in TDA.** In the following, we assume
144 that the taylorgram of a single analyte has a Gaussian shape:

$$145 \quad S(t) = S_0 \exp\left[-\frac{(t-t_0)^2}{2\sigma_t^2}\right] \quad (9)$$

146 where S is the detector signal at time t , S_0 the maximum value of the signal at the average analyte
147 elution time t_0 and σ_t the temporal standard deviation of the signal. As the detector is assumed to
148 provide a signal proportional to the concentration, c , of the analyte in the detector, then:

$$149 \quad S(t) = k c(t) \quad (10)$$

150 where k is the detector response factor for the analyte. Let m be the amount of analyte injected in
151 the capillary. As in TDA, the whole injected analyte is flowing through the detector, m becomes
152 equal to the integral over the time of the product $c(t) Q$, where Q is the volumetric flow-rate of the
153 carrier liquid, *i.e.*, with help of the above equations:

$$154 \quad m = \int_t c(t) Q dt = Q \int_t c(t) dt = Q c_0 \sigma_t \sqrt{2\pi} = Q \frac{S_0}{k} \sigma_t \sqrt{2\pi} \quad (11)$$

155 where c_0 is the analyte concentration in the detector at time t_0 . This amount is a mass or a number
156 of moles if the concentration $c(t)$ is a mass or molar concentration, respectively.

157 **3.2. LOD expressed as an amount of analyte.** The concept of detection or quantification
158 limit is a rather complex topic.^{25,26} In the following, we consider, as previously done in liquid
159 chromatography²⁷, that this limit is based on the signal-to-noise ratio and corresponds to the
160 minimum amount of analyte, m_{lim} , that must be injected to provide a peak height $S_{0,\text{lim}}$ equal to an
161 arbitrarily selected multiple, λ , of the noise level, G , defined, as noted above, as the standard
162 deviation of the blank signal, expressed in the same unit as the signal (usually in millivolt). In the
163 study, λ is fixed equal to 3 for the LOD and 10 for the LOQ. Hence the limit corresponds to the
164 minimum analyte concentration, $c_{0,\text{lim}}$, at the peak top equal to:

$$165 \quad c_{0,\text{lim}} = \frac{\lambda G}{k} \quad (12)$$

166 The temporal standard deviation, σ_t , is related to the spatial standard deviation, σ_z , when the signal
167 reaches the detector as $\sigma_t = \sigma_z/u$, where u is the mean velocity of the carrier liquid, equal to
168 $Q/(\pi R_c^2)$. Hence, the LOD can be expressed as:

$$169 \quad m_{\text{LOD}} = Q \sigma_t \sqrt{2\pi} \frac{\lambda G}{k} = \pi R_c^2 \sigma_z \sqrt{2\pi} \frac{\lambda G}{k} \quad (13)$$

170 As in chromatography, the spatial standard deviation is related to the plate height, H , as $\sigma_z = \sqrt{HL_d}$
171 , where L_d is the length of the capillary from the inlet to the detector. In Taylor conditions³, the
172 dispersion arising from the nonuniform (parabolic) flow profile dominates the one due to axial
173 diffusion, so the plate height becomes equal to:

$$174 \quad H = \frac{R_c^2 u}{24 D_m} \quad (14)$$

175 Furthermore, when TDA is performed with a capillary electrophoresis equipment using an *in situ*
 176 optical detector, the response factor, which is proportional to the optical path length, *i.e.* equal to
 177 the capillary diameter, can be expressed as:

$$178 \quad k = \kappa R_c \quad (15)$$

179 where κ is a parameter depending on the analyte attenuation coefficient, but not on the capillary
 180 dimensions. Then, the expression of the LOD becomes:

$$181 \quad m_{LOD} = \pi \sqrt{\frac{\pi}{12}} R_c^2 \sqrt{u L_d} \frac{\lambda G}{\kappa \sqrt{D_m}} \quad (16)$$

182 It appears that the LOD expression depends on a numerical constant, on a central group of operating
 183 parameters (here R_c , u and L_d) which can be adjusted to optimize the LOD, and on a last fraction
 184 which can be considered as a constant for a given analyte and a given detector (assuming that the
 185 noise level does not significantly depend on operating conditions).

186 Alternatively, one may express the LOD as a function of the mean sojourn time, t_0 , or of the applied
 187 pressure drop, ΔP , along the capillary of length L_c . Noting that $u = L_d/t_0$ and $u = (R_c^2/8\eta) (\Delta P/L_c)$
 188 according to the Poiseuille law, this gives:

$$189 \quad m_{LOD} = \pi \sqrt{\frac{\pi}{12}} \frac{R_c^2 L_d}{\sqrt{t_0}} \frac{\lambda G}{\kappa \sqrt{D_m}} = \sqrt{\frac{\pi}{12}} \frac{V_d}{\sqrt{t_0}} \frac{\lambda G}{\kappa \sqrt{D_m}} \quad (17)$$

190 where V_d is the volume of the capillary from inlet to detection point. Finally, one gets:

$$191 \quad m_{LOD} = \frac{\pi}{4} \sqrt{\frac{\pi}{6}} R_c^3 \sqrt{\frac{L_d \Delta P}{L_c}} \frac{\lambda G}{\kappa \sqrt{\eta D_m}} \quad (18)$$

192 **3.3. LOD expressed as a concentration of analyte in the injected sample.** The above
 193 expressions give the minimum amount of analyte that must be injected to detect or quantify the
 194 analyte by TDA. It may be interesting to express the LOD in terms of the minimum concentration,
 195 $c_{inj,LOD}$, of the analyte in the injected sample. The larger the injection volume, the larger the amount
 196 of analyte injected in the capillary and the lower the required analyte concentration in the sample,
 197 but also the larger the contribution of the injection process to the standard deviation of the peak,
 198 which can lead to unacceptable error in the determination of the diffusion coefficient of the analyte
 199 by TDA. That is why in TDA, the injection volume V_{inj} is often limited to a fraction, θ , of the
 200 capillary volume, V_d , up to the detection point (generally θ is selected equal to 1%):

$$201 \quad V_{inj} = \theta V_d = \theta \pi R_c^2 L_d \quad (19)$$

202 Thus, $c_{inj,LOD}$ is related to m_{LOD} as:

$$203 \quad c_{inj,LOD} = \frac{m_{inj,LOD}}{\theta V_d} = \frac{m_{inj,LOD}}{\theta \pi R_c^2 L_d} \quad (20)$$

204 Using equations (16)-(18), the minimum concentration can be expressed in terms of the various
 205 operating parameters as:

$$206 \quad c_{inj,LOD} = \sqrt{\frac{\pi}{12}} \sqrt{\frac{u}{L_d}} \frac{\lambda G}{\theta \kappa \sqrt{D_m}} \quad (21)$$

$$207 \quad c_{inj,LOD} = \sqrt{\frac{\pi}{12}} \sqrt{\frac{1}{t_0}} \frac{\lambda G}{\theta \kappa \sqrt{D_m}} \quad (22)$$

$$208 \quad c_{inj,LOD} = \frac{1}{4} \sqrt{\frac{\pi}{12}} R_c \sqrt{\frac{\Delta P}{L_d L_c}} \frac{\lambda G}{\theta \kappa \sqrt{\eta D_m}} \quad (23)$$

209 The central groups of parameters in the right-hand-side term of these equations reflect how the
 210 LOD, that is expressed as a concentration in the sample, is affected by any change in the operating
 211 parameters.

212 **3.4. Dilution.** The amount of analyte injected into the capillary depends on the concentration, c_{inj} ,
 213 of the analyte in the solution injected and the volume injected, V_{inj} :

$$214 \quad m_{inj} = c_{inj} V_{inj} \quad (24)$$

215 Using equations (11), (13) and (24), we can deduce that the dilution factor of the analyte resulting
 216 from the migration in the capillary, represented by the ratio of the concentration of the analyte in
 217 the sample to the concentration at the top of the detected peak, is equal to:

$$218 \quad \frac{c_{inj}}{c_0} = \frac{Q \sigma_t \sqrt{2\pi}}{V_{inj}} = \frac{\pi R_c^2 \sigma_z \sqrt{2\pi}}{V_{inj}} \quad (25)$$

219

220 **3.5. Influence of operating parameters on the LOD expressed in terms of**
 221 **concentration.** Simplifying the previous expressions and dropping the numerical factors and the
 222 parameters that are considered to be constant (λ , G , κ , η , D_m) in order to better understand the
 223 influence of the operating parameters that are easily modifiable (R_c , L_d , u or t_0), equation (25) can
 224 be written:

$$225 \quad c_{inj} \propto \frac{R_c^2 \sigma_z c_0}{V_{inj}} \quad (26)$$

226 where the sign \propto indicates proportionality rather than equality. In the case where the concentration
 227 at the top of the peak, c_0 , corresponds to the minimum concentration to allow detection or
 228 quantification, the concentration of the injected sample becomes, using equation (12):

$$229 \quad c_{inj,LOD} \propto \frac{R_c^2 \sigma_z}{k V_{inj}} \quad (27)$$

230 This relationship is general. The standard deviation σ_z when the peak top passes in front of the
 231 detector is equal to $\sqrt{H L_d}$ and, under Taylor conditions using equation (14), σ_z becomes
 232 proportional to $R_c \sqrt{u L_d}$, i.e. $R_c L_d / \sqrt{t_0}$. Thus, the equation becomes:

233

$$234 \quad c_{inj,LOD} \propto \frac{R_c^3 \sqrt{u L_d}}{k V_{inj}} \propto \frac{R_c^3 L_d}{k V_{inj} \sqrt{t_0}} \quad (28)$$

235

236 Expression (28) includes the response factor k and the volume injected, V_{inj} . Several cases are to
 237 be considered depending on the way in which the detection is carried out and the option chosen for
 238 V_{inj} .

239 When the detection takes place in the capillary, the optical path of the detector is proportional to
 240 the radius of the capillary (equation (15)), k is proportional to R_c , and the LOD becomes:

$$241 \quad c_{inj,LOD} \propto \frac{R_c^2 \sqrt{u L_d}}{V_{inj}} \propto \frac{R_c^2 L_d}{V_{inj} \sqrt{t_0}} \quad (29)$$

242

243 **3.6. On-capillary detection at constant V_{inj}/V_d (constant injected percentage).** In this
 244 case, the injected volume is proportional to the volume of the capillary entering the detector, which
 245 keeps the ratio of the length of the injected area to the length of the capillary to the detector constant
 246 in order to maintain the contribution of the dispersion injection within acceptable limits. Then, V_{inj}
 247 is given by equation (19) with θ constant. Equation (29) becomes:

$$248 \quad c_{inj,LOD} \propto \sqrt{\frac{u}{L_d}} \propto \frac{1}{\sqrt{t_0}} \quad (30)$$

249 In that case, (i) the LOD does not depend on the capillary radius, (ii) the LOD varies as \sqrt{u} at
250 constant L_d or as $1/\sqrt{t_0}$, and therefore only depends on t_0 , and (iii) the LOD varies as $1/\sqrt{L_d}$ at
251 constant u , since then V_{inj} increases faster than σ_z . These three statements were verified in the
252 experimental part.

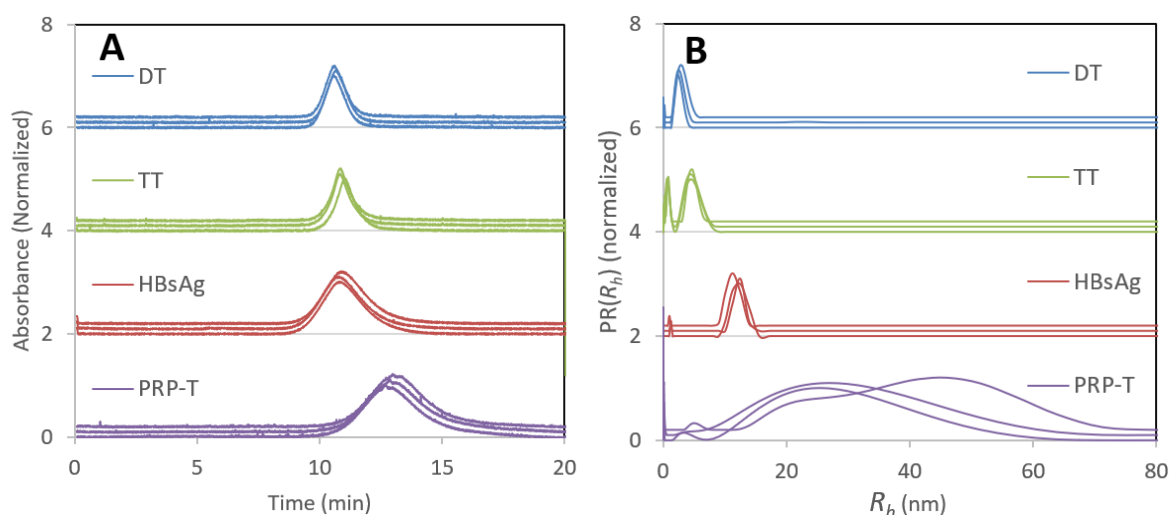
253

254 **4. Results and discussion**

255 **4.1. Analysis of different types of vaccine antigens by TDA.**

256 The conditions of validity of TDA depend on the molecular diffusion coefficient (D_m) (see
257 equations (4) and (5)). To be able to size nano-objects from 0.2 to 90 nm in radius¹⁴, while
258 preserving the validity of Taylor conditions, a bare fused silica capillary of 75 cm total length (66.5
259 cm to the UV detector) \times 75 μ m i.d. with a mobilization pressure of 40 mbar was used. Moreover,
260 in order to reduce the adsorption of the studied antigens on the bare fused silica capillaries walls,
261 the capillaries were presaturated by performing a preconditioning step consisting of injecting the
262 sample to be analyzed for 2 min at 960 mbar, in order to saturate the possible sites of interactions.
263 The taylorgrams obtained on the four antigens are presented in Figure 1, they were next fitted with
264 the sum of two Gaussians giving access to the average hydrodynamic radius (R_h) of the antigens.
265 The Gaussian fit was performed only on the left part of the elution peaks to avoid any impact of
266 peak tailing on the size measurement. Two-Gaussian fit was required due to the presence of UV-
267 absorbing small molecules in the sample, appearing as the small sharp peak on the top of the signal.
268 The Gaussian fits are presented in Figure SII and the average hydrodynamic radii (R_h) obtained
269 are reported in Table 1. DT and TT are the smaller antigens with 3.8 and 5.4 nm respectively, while
270 HBsAg and PRP-T are much larger ones with 13.3 and 33.9 nm respectively. The RSD on the
271 average hydrodynamic radius is typically lower than 5%, as already reported for other polydisperse

272 samples²⁸. The same taylorgrams were fitted by Constrained Regularized Linear Inversion (CRLI)
 273 to obtain the size distribution of the antigens presented in Figure 1B. The excellent CRLI fits are
 274 presented in Figure SI2. The PRP-T antigen appeared to be highly polydisperse, while the three
 275 other antigens have much lower polydispersity index as presented in Figure 1B. The PRP-T antigen
 276 appeared to be highly polydisperse with a PI of 0.25, while the 3 other antigens have much lower
 277 PI comprised between 0.01 and 0.07 in agreement with the distributions presented in Figure 1B.
 278 As in intermediate conclusion, TDA is a simple and straightforward method allowing determining
 279 the average hydrodynamic radius and the mass-weighted size distribution of different types of
 280 antigens, without calibration.



281
 282 **Figure 1:** Three repetitions of experimental taylorgrams obtained for four antigens (A), diphtheria toxoid
 283 (DT), tetanus toxoid (TT), Hepatitis B surface antigen (HBsAg) and Polyribosyl-ribitol phosphate
 284 conjugate (PRP-T), and the corresponding size distributions obtained by CRLI (B). Experimental
 285 conditions: bare fused silica capillaries of 75 cm total length (66.5 cm to the UV detector) \times 75 μ m i.d.
 286 Buffers: PBS 10 mM, glycine 200 mM, NaCl 154 mM, pH 6.9, $\eta = 0.9 \times 10^{-3}$ Pa.s for DT and TT ; PBS
 287 10 mM, NaCl 154 mM, pH 7.4, $\eta = 0.9 \times 10^{-3}$ Pa.s for HBsAg, and TRIS 10 mM, sucrose 8% (w/w), pH
 288 7.4, $\eta = 1.1 \times 10^{-3}$ Pa.s³ for PRP-T. Capillary presaturation: Sample for 10 min at 40 mbar. Capillary
 289 preconditioning: water for 2 min at 960 mbar followed by 2 min buffer at 960 mbar. Injections: 20 mbar, 6
 290 s (0.46% of the capillary volume to the detector). Mobilization pressure: 40 mbar. Antigen concentration
 291 in sample: 0.4 g/L. UV detection: 215 nm. Temperature: 25°C.

292

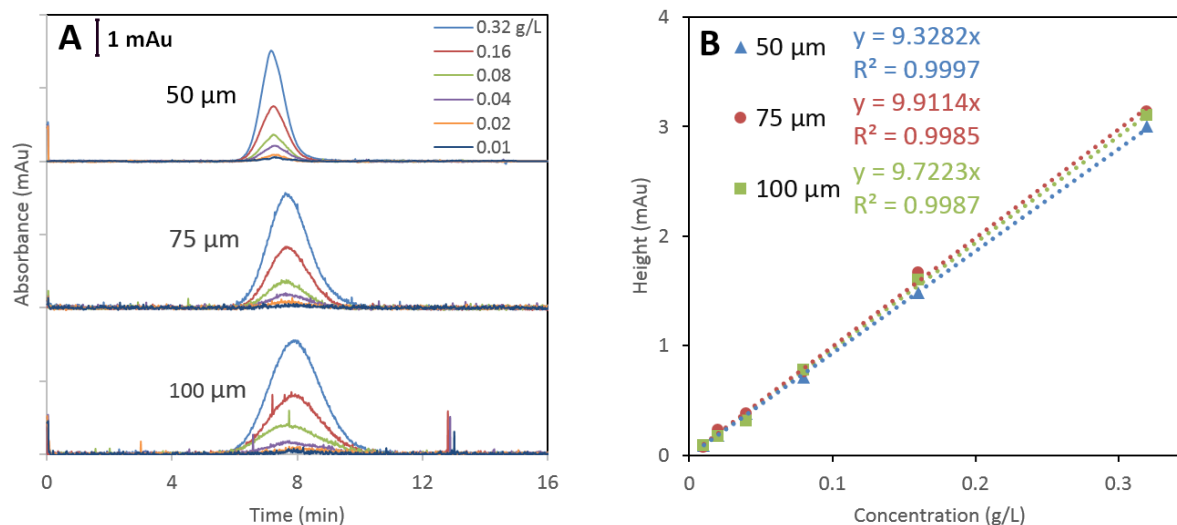
293 To test the robustness of the R_h values obtained by TDA on a bare fused silica capillary, the
294 experiments were repeated on coated capillaries. Slight adsorption was observed on the right side
295 of the taylorgrams on the bare fused silica capillaries, as can be seen on the Gaussian fit in Figure
296 SI1. Thus, neutral hydroxypropylcellulose (HPC) coated capillaries were tested. The taylorgrams
297 obtained are presented in Figure SI3A. A bare fused silica capillary of lower I.D. (50 μm) was also
298 tested to verify how robust the R_h values are regarding a change on operating conditions. The
299 taylorgrams obtained are presented in Figure SI3B. The R_h results are reported in Table 1. No major
300 difference was observed concerning the values obtained on the three capillaries, which confirms
301 the robustness of the results, in good agreement with a previous study showing that all TDA
302 experiments should lead to similar results as long as the operating conditions verify the conditions
303 of validity of the TDA method.²⁴ It is worth noting that the presence of significant adsorption on
304 the capillary wall would impact the R_h by increasing the apparent value due to additional peak
305 dispersion. As many different combinations of operating parameters (capillary diameter and length,
306 mobilizing pressure) allow to fulfill Taylor conditions of validity, it is sometimes difficult to choose
307 the initial parameters to develop a new TDA method, especially in view to improve the limits of
308 detection (LOD) / quantification (LOQ).

309

310 **Table 1:** Average hydrodynamic radii (R_h) of the four antigens studied obtained by Gaussian fit of the TDA
 311 elution profiles. The standard deviation was calculated over three repetitions. Experimental conditions: the
 312 same as in Figure 1.
 313

	Average R_h (nm)			
	DT	TT	HBsAg	PRP-T
Fused Silica 75 μm	3.8 ± 0.2	5.4 ± 0.3	13.3 ± 0.3	33.9 ± 0.3
HPC 75 μm	3.7 ± 0.1	5.4 ± 0.3	12.8 ± 0.2	34.2 ± 1.5
Fused Silica 50 μm	3.9 ± 0.1	5.5 ± 0.1	12.2 ± 0.5	35.2 ± 0.8

314 **4.2. Optimization of the limit of detection (LOD).** The LOD is a limiting factor in vaccines
 315 analysis as vaccine antigens are often present at very low concentrations in formulations. The
 316 possibility of finding a specific set of operating parameters that will allow to lower the LOD by
 317 TDA is thus desirable. This question is very general and may be also relevant for any other solute
 318 / sample. First, the impact of the internal diameter of the capillary on the LOD was studied. The
 319 same six solutions (0.32, 0.16, 0.08, 0.04, 0.02 and 0.01 g/L) of HBsAg antigen in PBS buffer were
 320 analyzed by TDA on three capillaries of different internal diameters using the same linear velocity
 321 of the eluent, the same capillary length and at constant V_{inj}/V_d (constant injected length) (Figure
 322 2A). The calibration curves allowing the determination of the LOD are presented in Figure 2B. The
 323 different conditions tested and the LOD obtained are summarized in Table 2. It appeared that the
 324 LOD did not significantly depend on the diameter of the capillaries, thus confirming the theoretical
 325 result derived from equation (30). The larger optical path in larger diameter capillaries is
 326 compensated by a higher dispersion of the molecules in these capillaries. Thus, taking a capillary
 327 with a larger diameter did not provide better sensitivity in TDA. Therefore, using a 50 μm capillary
 328 is generally recommended, as it consumes less product. However, the diameter of the capillary may
 329 also have an impact on the adsorption of the molecules on the capillary wall, however no visible
 330 impact of the capillary diameter on the adsorption was observed in this study.



331

332 **Figure 2:** Impact of the capillary internal diameter (i.d.) on the limit of detection (LOD). Experimental
 333 taylorgrams obtained on three capillaries of different internal diameters (i.d.) 50 μm , 75 μm and 100 μm
 334 (A) and the associated calibration curves representing the height of the obtained peaks as a function of the
 335 concentration of the analyzed samples. Experimental conditions: bare fused silica capillaries of 50 cm total
 336 length (41.5 cm to the UV detector). Sample: Hepatitis B surface antigen (HBsAg). Buffer: PBS 10 mM,
 337 NaCl 154 mM, pH 7.4, $\eta = 0.9 \times 10^{-3}$ Pa.s. Capillary presaturation: Sample for 10 min at 40 mbar. Capillary
 338 preconditioning: water for 2 min at 960 mbar followed by 2 min buffer at 960 mbar. Injections: 20 mbar 6
 339 s (0.5% of the capillary volume to the detector) on 50 μm i.d. capillary, 18 mbar 3s (0.5% of the capillary
 340 volume to the detector) on 75 μm i.d. capillary, 10 mbar 3s (0.5% of the capillary volume to the detector)
 341 on 100 μm i.d. capillary. Mobilization pressure: 50 mbar on 50 μm i.d. capillary, 22 mbar on 75 μm i.d.
 342 capillary, 12.5 mbar on 100 μm i.d. capillary. UV detection: 215 nm. Temperature: 25°C.
 343

344 **Table 2:** Influence of the operating conditions on the LOD or LOQ of HBsAg antigen.

	Capillary diameter (μm)	Mobilization pressure (mbar)	Total capillary length (cm)	Injection ^a	LOD (g/L)	LOQ (g/L)
Figure 2: Impact of capillary internal diameter	50	50	50	20 mbar 6s (0.5%)	0.021	0.070
	75	22	50	18 mbar 3s (0.5%)	0.018	0.061
	100	12.5	50	10 mbar 3s (0.5%)	0.019	0.062
Figure 3: Impact of mobilization pressure	50	50	50	20 mbar 6s (0.5%)	0.021	0.070
	50	30	50	20 mbar 6s (0.5%)	0.016	0.052
	50	20	50	20 mbar 6s (0.5%)	0.013	0.044
	50	10	50	20 mbar 6s (0.5%)	0.010	0.033
Figure 4: Impact of capillary length	50	22	40	12 mbar 6s (0.5%)	0.019	0.063
	50	28	50	20 mbar 6s (0.5%)	0.016	0.054
	50	39	70	41 mbar 6s (0.5%)	0.014	0.047
	50	50	90	42 mbar 10s (0.5%)	0.013	0.042

345 ^a The percentage of the injected volume relative to the detection volume is given in parenthesis.

346

347 In a second time, the impact of the pressure (and thus of the average elution time) on the LOD was

348 studied by TDA at constant V_{inj}/V_d (constant injected length). Different mobilization pressures of

349 10, 20, 30 and 50 mbar were tested on a same capillary (50 μm × 50 cm bare fused silica capillary,

350 Figure 3A). The calibration curves allowing the determination of the LOD are presented in Figure

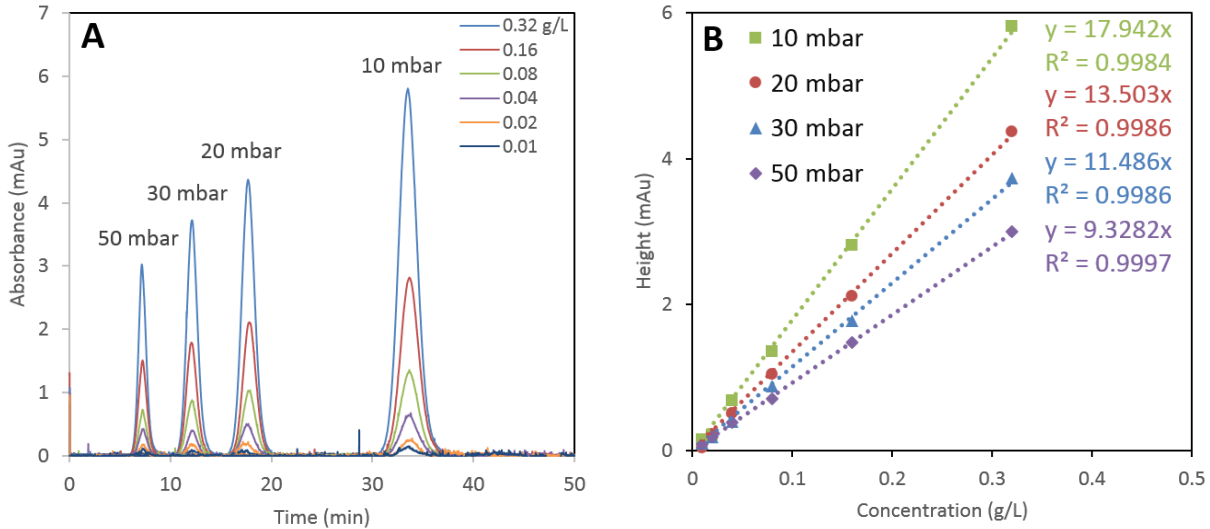
351 3B and the linear function representing the limit of detection (LOD) as a function of $1/\sqrt{t_0}$ is

352 presented in Figure 3C, in good agreement with eq. (28). The lower the pressure, the higher t_0 , the

353 more sensitive the method to the detriment of the analysis time. The right balance between the

354 duration of analysis and the sensitivity must therefore be found.

355

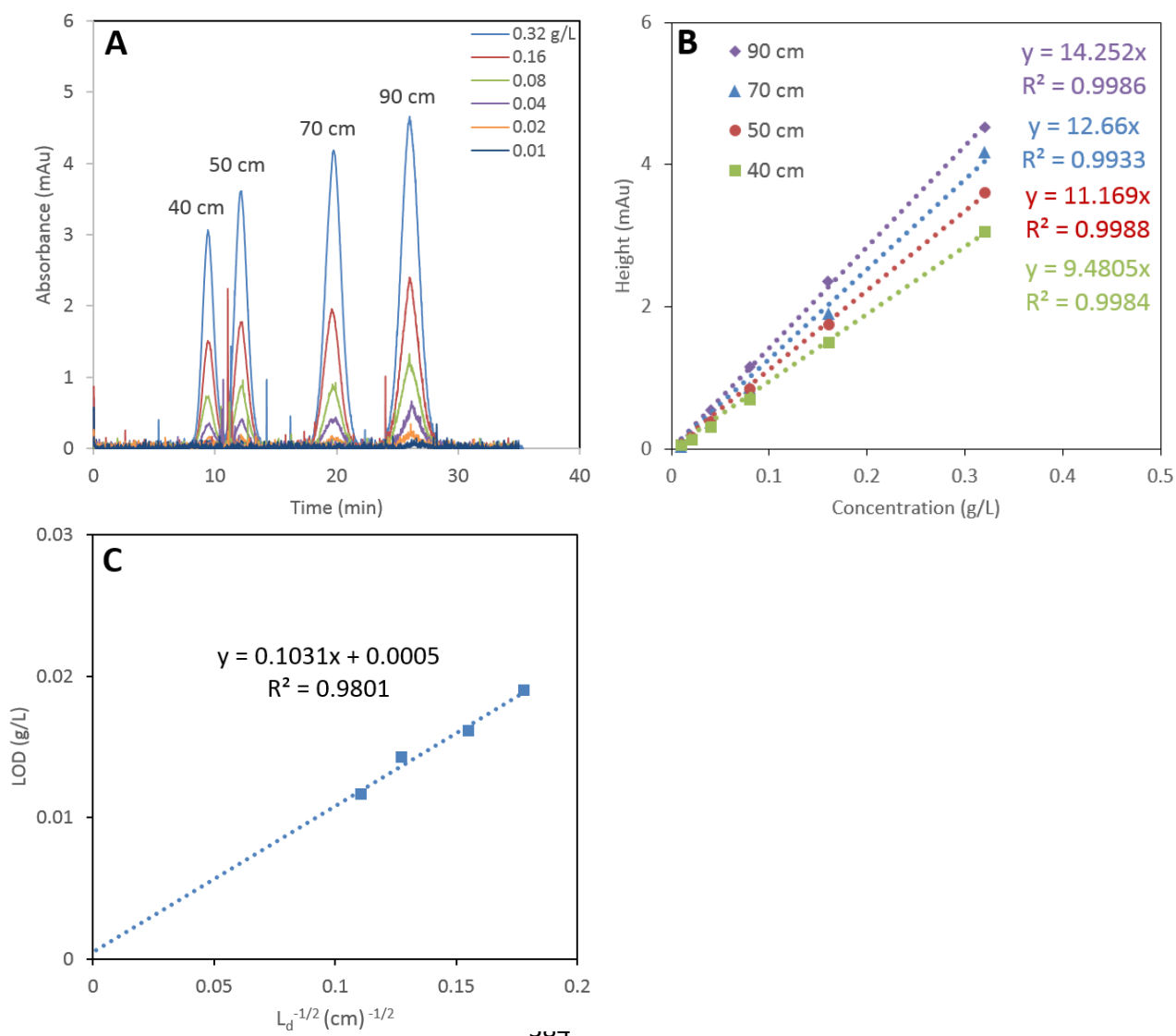


356

366 **Figure 3:** Impact of the mobilization pressure on the limit of detection (LOD) of HBsAg antigen.
367 Experimental Taylorgrams obtained on a same capillary using different mobilization pressures 50, 30, 20
368 and 10 mbar (A), the associated calibration curves representing the height of the obtained peaks as a function
369 of the concentration of the analyzed samples (B) and the linear correlation of the limit of detection (LOD)
370 as a function $1/\sqrt{t_0}$ (C). Experimental conditions: bare fused silica capillaries of 50 cm total length (41.5
371 cm to the UV detector) \times 50 μ m i.d. Injections: 20 mbar, 6 s (0.5% of the capillary volume to the detector).
372 Other experimental conditions: as in Figure 2.

373

374



385 **Figure 4:** Impact of the capillary length on the limit of detection (LOD) of HBsAg antigen. Experimental
 386 Taylorgrams obtained on four capillaries of different total lengths 40, 50, 70 and 90 cm (A), the associated
 387 calibration curves representing the height of the obtained peaks as a function of the concentration of the
 388 analyzed samples (B) and the linear correlation representing the limit of detection (LOD) as a function of
 389 $1/\sqrt{L_d}$ (C). Experimental conditions: bare fused silica capillaries of 50 μm i.d. The detection window is
 390 located 8.5 cm from the capillary end ($L_c=L_d+8.5$ in cm). Injections: 12 mbar 6 s (0.49 % of the capillary
 391 volume to the detector) on the 40 cm capillary, 20 mbar 6 s (0.49 % of the capillary volume to the detector)
 392 on the 50 cm capillary, 41 mbar 6 s (0.49 % of the capillary volume to the detector) on the 70 cm capillary
 393 and 42 mbar 10 s (0.49 % of the capillary volume to the detector) on the 90 cm capillary. Mobilization
 394 pressure: 22 mbar on the 40 cm capillary, 28 mbar on the 50 cm capillary, 39 mbar on the 70 cm capillary
 395 and 50 mbar on the 90 cm capillary. Other experimental conditions: as in Figure 2.

396 Finally, the impact of the capillary length on the LOD was studied. Different total capillary lengths
 397 of 40, 50, 70 and 90 cm were studied by TDA at constant linear velocity of the eluent and at

398 constant V_{inj}/V_d (Figure 4A, $L_c=L_d+8.5$ in cm). Note that constant V_{inj}/V_d means increasing injected
399 length on capillaries of increasing total length. The calibration curves allowing the determination
400 of the LOD are presented in Figure 4B and the linear function representing the LOD as a function
401 of $1/\sqrt{L_d}$ are presented in Figure 4C. At constant V_{inj}/V_d , the longer the capillary the lower the
402 LOD. As for the influence of the mobilization pressure at constant V_{inj}/V_d , the LOD increased as
403 the analysis time lengthened scaling as $1/\sqrt{t_0}$. A 60 cm capillary may be a good starting choice for
404 the development of a new TDA method.

405 406 **Conclusions**

407
408 This work first showed that TDA is a suitable method for the determination of the average
409 R_h , the polydispersity and the size distribution of different types of vaccine antigens such as
410 toxoids, glycoconjugates and subviral particles, as far as the size of the antigen remains in the
411 typical sizing range of TDA (i.e. from 0.1 to 300 nm). Moreover, the results obtained were
412 consistent whatever the diameter and the capillary coating used, showing the robustness of the
413 TDA methodology. Since the limit of detection (LOD) is a limiting factor of the analytical methods
414 for vaccine antigen characterization due to their low concentration in vaccine formulations, the
415 impact of operational parameters on the LOD was investigated. A very good correlation between
416 theory and experiment was observed. It has been shown that at constant injected percentage relative
417 to the detection volume, the LOD did not depend on the capillary radius. Then, the impact of the
418 mobilization pressure on the LOD was studied on a same capillary at constant linear velocity of
419 the eluent. It appeared that the LOD varies as $1/\sqrt{t_0}$ as predicted by the theory. The lower the
420 pressure, the more sensitive the TDA method, but to the detriment of the analysis time. It is often
421 beneficial to start the development of a method with a not too low mobilization pressure to be able
422 to do a large number of analyzes in limited time and decrease the pressure afterwards to increase

423 the LOD as needed. Finally, the impact of the length of the capillary was studied, the LOD varies
424 as $1/\sqrt{L_d}$ at constant V_{inj}/V_d and constant u . Taking a longer capillary therefore allows to reduce
425 the LOD but the analysis time is also longer. Last but not least, to decrease the LOD, it is also
426 possible to slightly adjust the percentage injected by approaching 1% V_d . Following these results,
427 starting with a 50 μm i.d. \times 60 cm total length capillary and applying 50 mbar mobilization
428 pressure is recommended to analyze molecules from 0.5 to 125 nm remaining under TDA validity
429 conditions. Typical RSD about 1 to 5% (3% in average) were obtained for the R_h determination of
430 vaccine antigen at concentrations (0.4 g/L) well above the LOQ. The conclusion from this work
431 about how improving the LOD in TDA is very general and can be of course applied to the analysis
432 of any sample in TDA.

433

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435
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438

439 **Declaration of competing interest**

440 The authors declare no conflict of interest. Camille Malburet, Jean-François Cotte and Jérôme
441 Thiebaud are Sanofi employees and may hold shares or stocks in the company.

442

443 **Supporting Information.**

444 Gaussian fits of the TDA elution profiles allowing to calculate the average hydrodynamic radii.
445 CRLI fits of the TDA elution profiles allowing to calculate the size distributions. Experimental
446 taylorgrams of four antigens obtained of different capillaries.

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