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## **In-situ protein determination to monitor contamination in a centrifugal partition chromatograph**

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40

## 41 Introduction

42 Centrifugal liquid-liquid chromatography, including counter-current chromatography (CCC) and centrifugal  
43 partition chromatography (CPC), is a chromatographic separation technique based on the partition of solutes  
44 between two immiscible liquid phases with no solid support (1-3). One of the two immiscible phases is retained  
45 in the column by centrifugal force fields; it is called the stationary phase. The other phase is the mobile phase; it  
46 percolates through the stationary one. CCC and CPC have numerous advantages such as a high loading capacity  
47 and no loss of solute since it is always possible to recover any material trapped in a liquid phase. These  
48 advantages were used over the past years to purify biomolecules, especially proteins, by CCC and CPC modes  
49 (4, 5). Aqueous Two Phase Systems (ATPS) were found very efficient for protein purification (6, 7). As their  
50 name says, ATPSs are composed by two immiscible aqueous phases. This is obtained either dissolving two  
51 polymers in water or dissolving a polymer and a salt or an ionic liquid and a salt. ATPSs combine a high  
52 biocompatibility and selectivity for biomolecules (8). These solvent systems were proved effective in  
53 biopurification due to their high water content and low interfacial tension, which make them gentle towards  
54 proteins (9, 10). However, CCC was found unable to retain efficiently an aqueous liquid stationary phase likely  
55 due to the low ATPS interfacial tension (3). CPC, with its constant centrifugal field and its rotor of  
56 interconnected chambers was able to retain ATPSs allowing for protein purification (1-3).

57 One of the issues when working with proteins is the risk of contamination of the CPC equipment. Indeed  
58 proteins can easily adsorb on the rotor material. Adhesion of proteins to solid surface can occur under various  
59 conditions and cause problems for biotechnology manufacturers. For example, in case of food manufacturing,  
60 proteins can form a fouling which is an unwanted deposit on the equipment surface (11). Thereby, an  
61 insufficient cleaning may result in the development of bacteria and biofilm formation (12). Moreover, the  
62 adherent proteins may pose a cross contamination risk (13). For these safety reasons, the cross-contamination  
63 risks are strictly controlled through governmental organizations, such as the U.S Food and Drugs Administration  
64 (FDA). These Agency documents clearly establish the required expectation for cleaning procedure validation  
65 (14). CPC is no exception. So, one of the most important issues for its development in the protein industry is to  
66 ensure the cleanliness of the equipment after a purification in order to avoid cross-contamination and hence to be  
67 able to detect protein contamination.

68 A cleaning method was specifically developed by Chollet (15) for CPC rotors. This method consists in  
69 alternate rinsing steps of water, 0.5M sodium hydroxide solution and sulfuric acid solution at  $2.10^{-5}M$ , repeated  
70 twice. While it follows the FDA standards and the Good Manufacturing Practice (GMP), this method is only  
71 available in French. The validity of the cleaning procedure is controlled either by visual criterion opening the  
72 rotor and swiping the disks surface, or by the protein determination in the various rinse solutions via the  
73 Bradford method (16). Unfortunately, the ultimate rinsing solutions are highly diluted, and their analysis requires  
74 a highly sensitive detection system. Although the validation of the cleaning protocol by swiping is soundproof, it  
75 is only accessible to instrument suppliers, as it requires the mechanical opening of the rotor. The industrial users  
76 do not have the facilities to tighten disks and to equilibrate weights after closure of this rotating device.

77 In the present work we propose an in-situ strategy to determine the state of protein contamination inside the rotor  
78 without opening the machine. This in-situ method derived from the Amino Density Estimation by Colorimetric  
79 Assay (ADECA) method (17). This method was established to rapidly quantify grafted proteins on a solid  
80 support such as 96-well plates. It is based on the affinity of a dye, the Coomassie Brilliant Blue (CBB), with  
81 protonated amino groups. The ADECA method consists in three steps: first a fixation step (or staining) ensures  
82 that the dye is bound to the surface material by an  $N^+$ -dye complex formation. Next a washing step removes any  
83 unbound dye. Last, the dye bounded to proteins is eluted by a pH switch which breaks the  $N^+$ -dye complex and  
84 the quantification of grafted protonated groups is directly related to the amount of released CBB dye. Thereby,  
85 this method should accurately indicate if any traces of proteins remain in a rotor after a full cleaning. The CBB  
86 dye would stick to such traces and any blue color seen during the acid wash would point remaining proteins. To  
87 set up this method in CPC instruments, we applied the ADECA protocol to a commercial 25 mL stainless steel  
88 rotor and the optimal conditions were determined. A graduated range of protein contamination was simulated  
89 using bovine serum albumin.

90

## 91 **Experimental**

### 92 *Materials*

93 The dye Coomassie Brilliant Blue CBB-G250 (CBB, >99%, C<sub>45</sub>H<sub>44</sub>N<sub>3</sub>NaO<sub>7</sub>S<sub>2</sub>, MW= 854 g/mol, a  
94 triphenylmethane dye with two benzene-sulfonic acid and three amine groups), potassium carbonate (>99.5%;  
95 MW=138.2 g/mol), potassium bicarbonate (>99.5%; MW=100.12 g/mol) and phosphate buffer saline (PBS) as  
96 well as the protein bovine serum albumin (BSA 96%, MW= 66463, pI=4.7) were from Sigma Aldrich (Saint-  
97 Quentin Fallavier, France). Ethanol absolute was from ThermoFisher (Villebon-sur-Yvette, France).

98 The pH measurements are performed in the overall hydroorganic solution. The effective hydrogen activity in  
99 aqueous/organic solutions can be only estimated using water calibrated pHmeter and will be stated as “apparent  
100 pH”.

101

### 102 *Instrumentation*

103 The CPC instrument is a hydrostatic apparatus model, FCPC-A from Kromaton Rousselet-Robatel (Annonay,  
104 France) with interchangeable rotors. A stainless steel 316 rotor with a volume of 25 mL was mainly used in this  
105 study. For comparative assays, two prototype rotors were assessed: a stainless steel 316 rotor with a volume of  
106 80 mL and a titanium rotor with a volume of 46 mL. The internal surface was calculated thanks to the cell and  
107 channel dimensions provided by the manufacturer and was evaluated at 0.38 m<sup>2</sup> for the 25 mL rotor, 0.61 m<sup>2</sup> for  
108 the 80 mL rotor and 0.31 m<sup>2</sup> for the 46 mL titanium rotor.

109 A Spot Prep II integrated system from Armen Instruments (Saint-Avé, France, Gilson USA) was used. This  
110 equipment is the assembly of a quaternary pump, an automatic loop injection valve fitted with a 1 mL sample  
111 loop, a UV/Vis spectrophotometer dual wavelength set up at 259 nm and 280 nm and a fraction collector.

112

### 113 *Rotor cleaning procedure*

114 After protein impregnation and/or ADECA implementation, the used rotors were fully cleaned according to  
115 Common Industrial Protocol, i.e. alkaline solution pH 14, for the equivalent of 3 column volumes.

116

### 117 *Preparation of solutions*

118 Solutions for the staining step were prepared by dissolving 500 mg of CBB in 100 mL of ethanol and 50 mL of  
119 glacial acetic acid (CH<sub>3</sub>COOH) and stirred. After complete dissolution, deionized water was added up to a final  
120 volume of 1 Liter. The final composition of the staining solution was 0.05% (w/v) CBB, 10% ethanol, 5%  
121 CH<sub>3</sub>COOH and 85% H<sub>2</sub>O (v/v). The apparent pH is 2.4.

122 The composition of the washing solution was the same as that of the staining solution, i.e. 10% ethanol, 5%  
123 CH<sub>3</sub>COOH and 85 % H<sub>2</sub>O (v/v) but with no CBB. For pH studies in the range 2.4 to 12, acetic acid or potassium  
124 carbonate was added until the desired target pH was reached.

125 The composition of the elution solution was 50% (v/v) EtOH and 50% carbonate buffer pH 12.

126

### 127 *Extent of proteins contamination in various rotors*

128 Five BSA proteins standard solutions were prepared in the range of 0 mg/mL to 200 mg/mL in phosphate buffer  
129 saline (PBS) pH=7.4 or in carbonate buffer pH=9 and pH=12. The simulated contaminations were performed on  
130 a clean rotor by injecting 1 mL of protein solution in the rotor using the chromatographic system then rinsing by  
131 the buffer solution. It was previously checked that no significant adsorption happens in PEEK tubings and in the  
132 injection device. Blanks were performed by injecting phosphate buffer without protein.

133

### 134 *Implementation of a dynamic ADECA method in a CPC rotor*

135 The ADECA method developed to quantify the amount of grafted proteins on a surface contains three different  
136 steps. To transfer this static method performed in 96-well plates to a dynamic method in CPC rotor, the three  
137 ADECA steps were adapted as follows: **A-The staining step** was carried out pumping the staining solution at 10  
138 mL/min during 15 min; i.e a volume of 150 mL staining solution; **B-The washing step** was achieved with a flow  
139 rate of 10 mL/min during 18 min unless stated otherwise. **C-The elution step** was realized by pumping the  
140 elution solution at 10 mL/min during 15 min through the rotor.

141 During all these different steps the rotor was not set in rotation and detection was performed at 259 nm which  
142 corresponds to the maximum of the dye absorbance in the UV region.

143

#### 144 *Detector calibration*

145 To relate the CBB peak area to the amount of proteins to which CBB molecules were bound, a calibration of the  
146 UV detector is necessary. First of all the detector was calibrated with different proteins solutions with an  
147 increasing concentration of CBB in the range 0.6- 200 mg/mL injecting 1 mL each time. Then it was calibrated  
148 by the CBB solution in the range 0.1-13.5 mg/mL in the acidic blue form (pH 2.4, 10% ethanol/5% acetic acid)  
149 and in the range 0.1-25 mg/mL in its basic redish form (pH 12, carbonate/ethanol (50/50 v/v) in order to  
150 determine the linearity range of the detector for these solutions. Each injection was repeated three times.

151

## 152 **Results and Discussions**

### 153 1. *Extent of proteins contamination*

154 Protein adsorption occurs when a protein solution comes in contact with a solid surface (18). Centrifugal  
155 partition chromatography is a preparative downstream process, handling very concentrated protein solutions (g/L  
156 to hundredth of g/L range). During method developments, rotor contaminations by proteins were noted by users.  
157 To check these observations, a 25 mL commercially available rotor made of stainless steel was intentionally  
158 stained by proteins at different pHs. The surface contamination can be linked to solid surface properties, solution  
159 conditions and proteins properties (19). Stainless steel surfaces are known to form an oxide layer covered by  
160 hydroxyl groups whose charge may strongly affects adsorption properties. According to the literature (20), the  
161 stainless steel surface has positive charge above pH 8.5 and is neutral above this value. In order to study the pH  
162 effect of the surface nature on the extent of protein contamination, different amounts of BSA were introduced at  
163 different pHs from 7.4 (physiological pH) to 12. Figure 1 shows the quantity of residual proteins (ADECA  
164 method) inside the 25 mL stainless steel rotor in regards to the quantity of injected proteins at different pH  
165 values.

166 The residual protein quantity was determined by subtracting the amount of proteins eluting from the rotor after  
167 washing with one column volume. The contamination is linearly related to the amount of injected proteins (Fig.  
168 1). Surprisingly, saturation of the surface is not attained with the BSA protein reaching density values as high as  
169 100 mg BSA/m<sup>2</sup>, while for the same 316 stainless steel it was said that saturation occurred around 3 mg BSA/m<sup>2</sup>  
170 stainless steel (20). What is more surprising is that the adsorption lines have a slope around 0.19 that does not  
171 depend on pH (Fig. 1). The BSA isoelectric point being 4.7, the protein is overall negatively charged for all  
172 experiments. At pH 7.4, the stainless steel surface is positively charged and BSA would be supposed to adsorb  
173 through charge-charge interactions through carboxyl groups. However, at pH 9 and 12, the stainless steel surface  
174 has a zero charge density, thus proteins contamination should be lesser. Since no difference was observed, it  
175 confirms that the binding between stainless steel and proteins is not only controlled by the stainless steel surface  
176 charge but also through dipole-dipole interactions, hydrogen bonding and coordination bonding.

177 All these results show that contamination of CPC rotors with BSA is indeed happening. Therefore a method to  
178 detect proteins contamination should be welcome by users. For further study, the BSA staining was done at pH  
179 7.4 which corresponds to the physiological pH.

### 180 2. *ADECA based on the N<sup>+</sup>-dye interaction.*

181 The three steps of the ADECA method have to be optimized to ensure a reproducible protocol. The staining  
182 solution should maximize the CBB-protein interaction. The dye CBB exists under three different forms: cationic

183 form at pH below 3, neutral form at pH between 3 and 12 and anionic form at pH above 12 (21, 22). In order to  
184 allow the N<sup>+</sup>-CBB interaction, the protein net charge should be positive and the CBB sulfonic acid groups  
185 should be negatively charged. The first condition is better fulfilled for pH below 4.7 (BSA isoelectric point). In  
186 order to quantitatively relate the amount of CBB to the amount of proteins, the pH condition was selected so that  
187 only one site NH<sup>+</sup> is bounded to the CBB molecule. This is possible at pH below 3, so staining was achieved at  
188 pH 2.4 (17). Besides, for staining to be complete, the dye was injected in excess and solubilized in 10% ethanol.

189 The washing step must remove the dye located in the void volume and also the background staining due to  
190 nonspecific binding on native rotor material. The free dye must be highly soluble in the washing solution that  
191 should not disrupt the N<sup>+</sup>-CBB interactions on rotor material. In order to maintain N<sup>+</sup>-CBB interactions, the pH  
192 of the washing solution should be the same as that of the staining step, i.e. pH 2.4.

193 The elution step must dissociate quantitatively the N<sup>+</sup>-CBB interactions. To allow this dissociation, the charge of  
194 the CBB or of the proteins has to be modified. At pH 12.4, both BSA and CBB become negatively charged,  
195 which generates ionic repulsion. However the pink basic CBB form is less soluble than the blue form (23).  
196 Thereby to avoid any CBB precipitation, the ethanol percentage was increased. According to Coussot (17) the  
197 maximum usable percentage of ethanol is 50% because the dye can also precipitate above this value.

198

### 199 *3. Application of a dynamic ADECA method in a stainless steel CPC rotor*

200 In order to simulate a protein contamination in a CPC rotor, an injection of 300 mg of BSA at pH 7.4 was  
201 performed in a commercial 25 mL stainless steel rotor and a subsequent ADECA protocol was performed to  
202 quantify the BSA staining. Figure 2 represents the UV detection signal at 259 nm that was recorded during the  
203 experiment. After the protein injection, a one-column volume of phosphate buffer saline is introduced at 10  
204 mL/min, removing the non-adsorbed proteins. This elution out of the rotor produces a UV signal as a Gaussian  
205 peak, the shape being due to the Poiseuil dispersion through the column. As the detector was calibrated with  
206 known amounts of proteins in the same operating conditions, the eluting BSA can be quantitated and it can be  
207 deduced by subtraction that, after this phosphate buffer rinse step, a 67 mg amount of BSA or 22% of the 300 mg  
208 injected remains adsorbed in the rotor. In order to in-situ quantitate the extent of contamination, the dynamic  
209 ADECA method is implemented. The staining step corresponds to the introduction of the CBB dye in the 25 mL  
210 rotor at a concentration of 500 mg/L. A fixed volume of 150 mL (15 min elution at 10 mL/min) of staining  
211 solution allowed the saturation of the interacting sites. The excess dye that does not interact with N<sup>+</sup> elutes out  
212 of the rotor, which is translated to an increase of the UV signal at 259 nm (part A in Figure 2). The staining step  
213 was optimized both in terms of thermodynamic aspects (concentration of the dye and its overall quantity) and on  
214 the kinetic aspects (contact time, flow rate of introduction). The flow rate had no effect on the quantity of  
215 bounded CBB.

216 The washing step must remove any CBB molecule that is not bound to BSA, without breaking the N<sup>+</sup>-dye  
217 interaction. As the rotor is filled with the staining solution containing CBB dye, introducing a solution without  
218 any absorbing molecule results in a decrease of UV signal (part B in Figure 2). The baseline return ensures that  
219 all unbound dye has been washed off. This extensive washing corresponds to 10 times the volume of the rotor.

220 Finally the elution step is performed by the introduction of a pH 12 organic-carbonate solution which breaks the  
221 interaction between CBB and N<sup>+</sup>. The CBB release is almost instantaneous as pH changes. This result in a CBB  
222 peak that moves at the elution solution velocity (part C in Figure 2). The peak area is related to the amount of  
223 released CBB, hence to the amount of stainless steel bound BSA.

224 In order to check if the peak signal from step C is not due to some protein release after the basic pH switch, a  
225 blank experiment was performed injecting 100 mg of proteins in the 25 mL stainless steel rotor followed by the  
226 ADECA protocol without any CBB dye. Since no UV absorption was observed in the elution step, it means that,  
227 to the extent of our detection level, no protein was released at that stage and hence the quantification of peak C  
228 relates only to CBB eluting molecules.

229 The ADECA method was also implemented in a clean rotor, that is to say, in a rotor onto which no protein was  
230 injected and after an extensive CIP cleaning procedure. The results are shown in Figure 2b. During the elution

231 step C, a peak is present, suggesting the presence of either CBB fouling in dead volumes or non-specific  
232 interactions. In order to make sure that the retained CBB dyes are not just hold in any dead volume, the operating  
233 parameters of CPC rotor, i.e. flow rate and rotation speed, were studied to evaluate their impact on the non-  
234 specific peak present in the step C. Indeed, in CPC rotor, the flow path is governed by hydrodynamic (24, 25).  
235 Thereby to create a powerful mobile phase spray that can extensively reach any part of the cell chamber, the  
236 rotation speed was set at 1800 rpm and the flow rate up to 35 mL/min during washing step. This did not affect  
237 the presence of the released CBB peak on blank experiments. Hence, the main cause of the background staining  
238 is due to the nonspecific CBB binding with native materials. Indeed, the staining step taking place at pH 2.4, the  
239 stainless steel rotor has a positive charge at its outer layer surface while the CBB is negatively charged. Thereby,  
240 ionic interactions are generated between the rotor surface and the CBB during staining step (step A) and  
241 maintained during washing step (step B, same pH 2.4). The basic switch in elution step (step C, pH 12) releases  
242 CBB by changing the material charge density, explaining the presence of an elution peak in a clean rotor.

243 The non-specific interaction between CBB and the stainless steel surface can be strongly reduced by controlling  
244 the charge density of the surface (Figure 3A). When the washing step was performed at pH 2.4, 10% ethanol,  
245 the dye density due to non-specific interactions is estimated to be  $9.0 \pm 0.4$  mg/m<sup>2</sup>. Introducing washing  
246 solutions with higher pHs does not significantly improve the situation, till the pH reaches the value of 8.6. At  
247 this value, the stainless steel hydroxide layer becomes neutral and hence the interaction with the anionic CBB  
248 becomes weaker. The density of CBB was measured to be only  $1.2 \pm 0.3$  mg/m<sup>2</sup>. A further increase of pH up to  
249 12 did not change the extent of non-specific interactions.

250 Because CBB is soluble in ethanol, we tried to further decrease the background staining by increasing the  
251 ethanol content in the washing solution. As shown in Figure 3B, this had a significant effect on reducing the non-  
252 specific interactions: a washing solution with 30% ethanol and pH 8.6 lead to the complete elimination of CBB-  
253 metal interaction.

254 Unfortunately, when implementing the ADECA protocol with a 30% ethanol, pH 8.6 washing step on a soiled  
255 rotor, the results showed that the specific CBB-protein binding was affected and hence the quantification was no  
256 longer reliable. This was also the case with a 10% ethanol, pH 8.6 washing solution. Indeed, at pH 8.6, the BSA  
257 is negatively charged and we suspect that ionic repulsion may occur between the negative dye and the protein.

258 Hence the accurate determination of protein fouling in a CPC rotor has to be performed with a pH switch from  
259 2.4 to 12 and the background staining has to be taken into account.

260 Linearity, repeatability, quantification and limit of detection were studied under these conditions by creating a  
261 range of controlled stainings on the 25 mL stainless steel rotor. Five proteins standard solutions were prepared in  
262 phosphate buffer solution in the range of 0 to 200 mg/mL and 1 mL injected led to a fouling extent in the range  
263 0-70 mg (Figure 1), i.e a protein density on the stainless steel surface in the range 0-180 mg/m<sup>2</sup>. After each  
264 contamination, the ADECA protocol was conducted and the released CBB peak area was monitored (Figure 4).  
265 The rotor was then extensively washed before the next contamination. The linearity between the quantity of  
266 residual proteins in the column and the area of released CBB molecules was verified in the studied range with  
267  $r^2 > 0.965$ . The limits of detection and quantification were 0.9 mg and 3.1 mg respectively.

268 The same experiment was also set in a larger stainless steel rotor in order to study the influence of the available  
269 surface. A 80 mL prototype rotor from Kromaton, with cells four times larger than the commercialized 25 ml  
270 rotor, but made of the same 316 stainless steel quality, was submitted to the very same controlled contamination.  
271 The calculated internal surface is 0.61 m<sup>2</sup>, to be compared to the 0.38 m<sup>2</sup> of the commercialized 25ml rotor.  
272 Surprisingly, the limits of detection and quantification were respectively 0.9 mg and 3.1 mg, the same as those of  
273 the 25 mL rotor. It is possible that the significant background staining plays an important role in these elevated  
274 values.

275

276 *4. Application of a dynamic ADECA method in a prototype titanium CPC rotor*

277

278 Titanium is a favored material in biotechnology because it is known to be easily cleaned at high pHs compared  
279 to stainless steel (20). Using a very similar BSA staining process, the possibility to implement the ADECA  
280 procedure in a 46 mL titanium prototype rotor was investigated to assess the in-situ extent of contamination.  
281 However, the preliminary results showed that the titanium rotor was unexpectedly prone to protein adsorption.  
282 Titanium exhibits an isoelectric point of 4.5. The existence of this electric point suggests that the surface could  
283 be positively charged below pH 4.5 and negatively charged above pH 4.5 ( $-\text{Ti}_2\text{O}^-$ ). Ionic interactions between  
284 titanium surface charges and protonated amino groups on aminoacid residues have already been observed (19).  
285 Working at pH 7.4, the net BSA charge is weakly negative, so a large number of amino sites are available for  
286 interactions. Fouling is close to linear in regards to the injected amounts and the slope is similar to the one  
287 observed for contamination on stainless steel. Nonetheless, when calculating the fouling density, the values were  
288 out of range and saturation was reached at  $160 \text{ mg/m}^2$ , which value does not match at all the reported value of  $4$   
289  $\text{mg/m}^2$  (20). Hence we suspect that the manufacturing process for rotor engraving that differs from stainless  
290 steel, generates a rough surface and/or a high specific area, as confirmed by a noted ruggedness of the rotor  
291 titanium surface. The calculated  $0.31 \text{ m}^2$  surface of the 46 mL rotor is likely largely underestimated, while the  
292 effective internal surface of this titanium rotor is unfortunately not accessible.

293 The ADECA profiles for cleaned and contaminated titanium rotor were highly similar as the ones observed on  
294 stainless steel (Figure 2) and are not reproduced here. However, the non-specific interactions of CBB molecules  
295 on titanium surface were found much higher than on stainless steel with a much less accurate quantification.

296

## 297 **Conclusion**

298 A method has been developed for the in-situ determination of protein fouling inside metallic CPC rotors, based  
299 on the interaction between proteins and Coomassie Brilliant Blue dye as initially introduced as the ADECA  
300 method. This method is linear for stainless steel rotor up to a  $70 \text{ mg}$  BSA fouling concentration. Its limit of  
301 detection is around  $1 \text{ mg}$ . This makes the method suitable for contamination warning at a preparative scale.  
302 However, a significant CBB interaction with metal surface was noted, so other protein dyes should be  
303 investigated in regards of non-specific interactions with metals. The method showed that there was a significant  
304 background staining of CPC rotors. This needs to be drastically reduced for this CPC purification method to be  
305 suitable in an industrial validation environment.

306

## 307 **Aknowlegement**

308 KF and FB thanks the professor Alain Berthod for the support in this study.

309

310

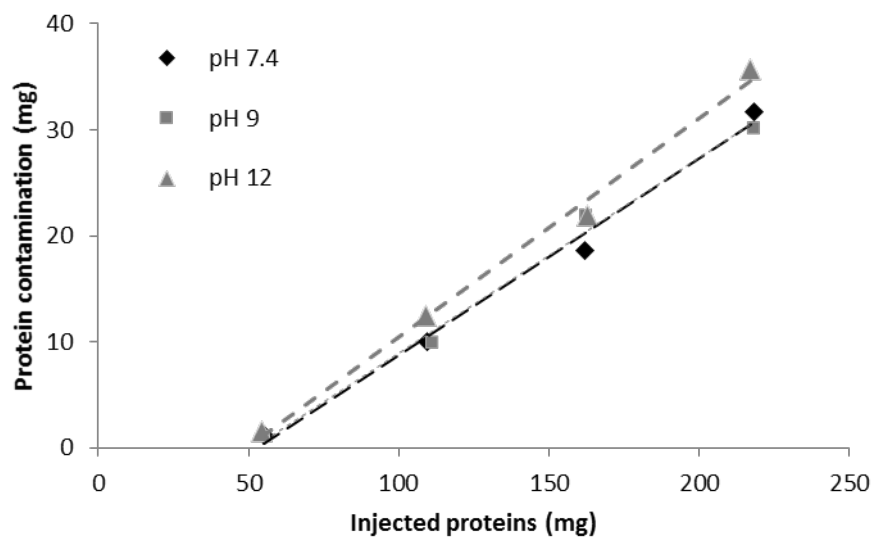
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316 **Figure 1.** Relationship between the quantity of proteins injected in the stainless steel rotor and the remaining  
317 proteins after a one-column buffer rinse at various pHs.

318

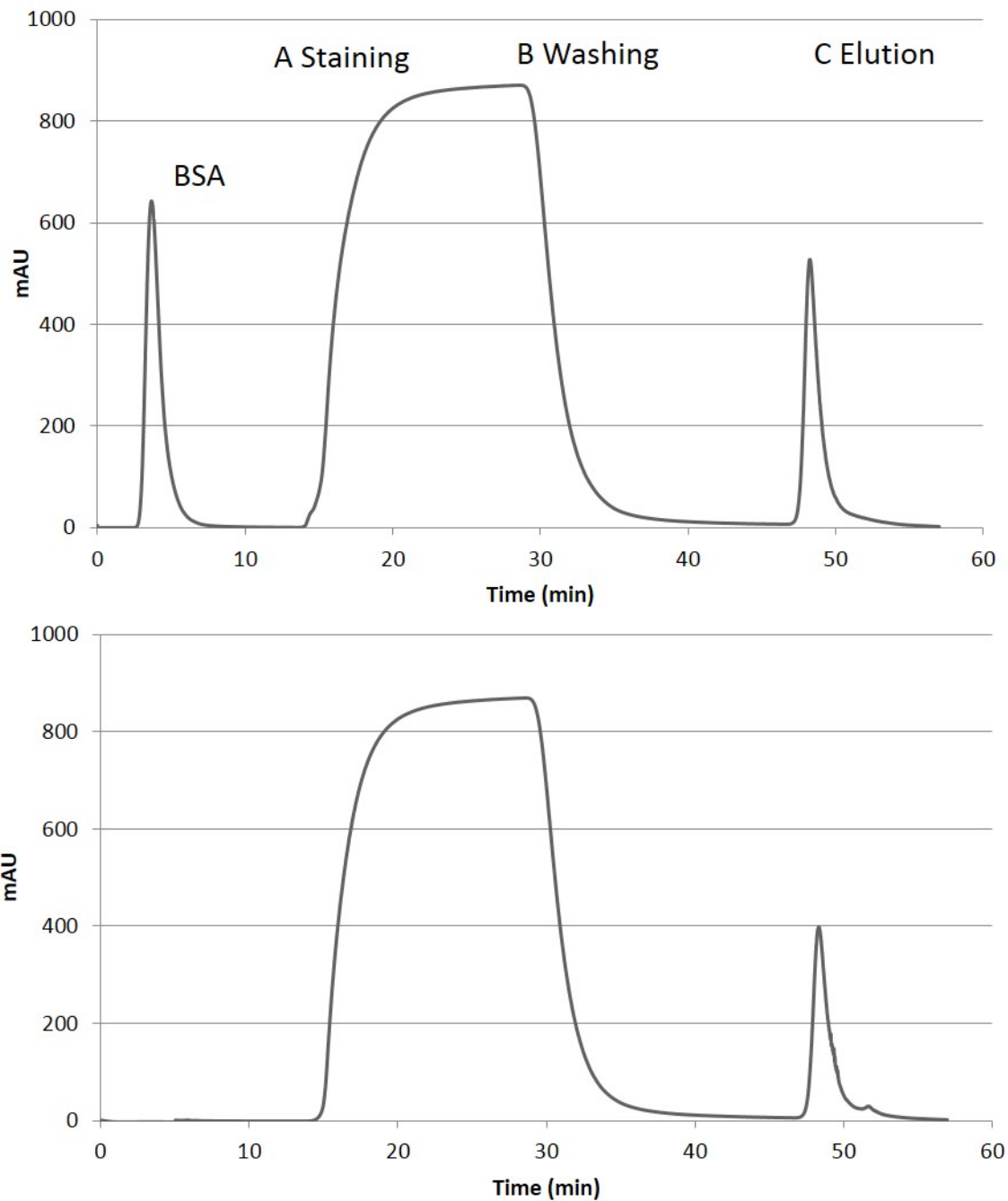
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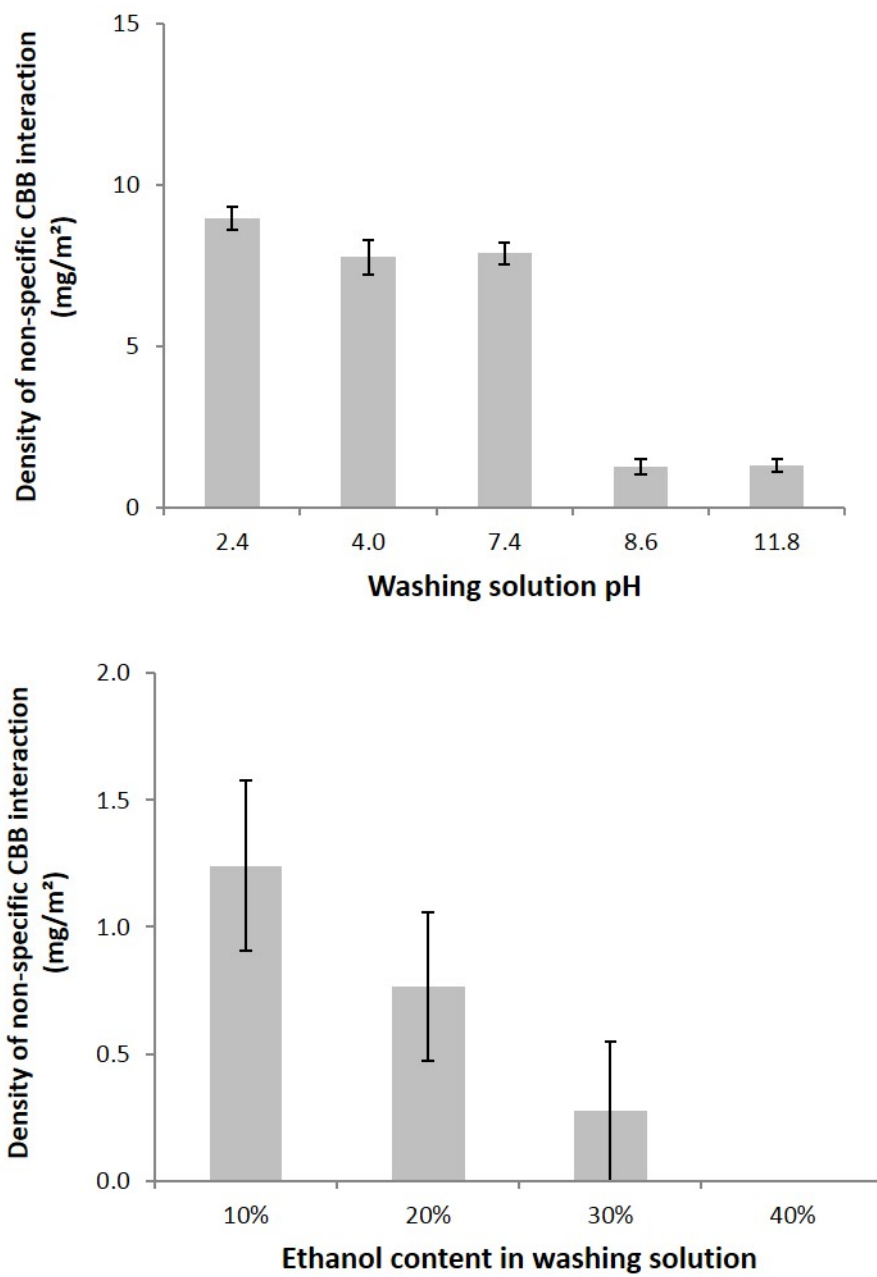


324

325 **Figure 2.** UV signal of the different ADECA steps on a 25 mL stainless steel rotor, (top) contaminated with  
 326 BSA protein and (bottom) the 25 mL clean rotor with no protein contamination.  
 327 (top) at 0 min, injection of 300 mg BSA pH 7.4 and one-column volume rinse by phosphate buffer saline  
 328 solution pH 7.4; (both) A- between 15 and 30 min, staining step with 0.05% CBB, 10% ethanol, pH 2.4; B-  
 329 between 30 and 45 min, washing step with 10% ethanol pH 2.4; C- at 45 min, CBB elution step 50% ethanol pH  
 330 12. Every dynamic step is performed at 10 mL/min 0 rpm. Detection 259 nm.  
 331

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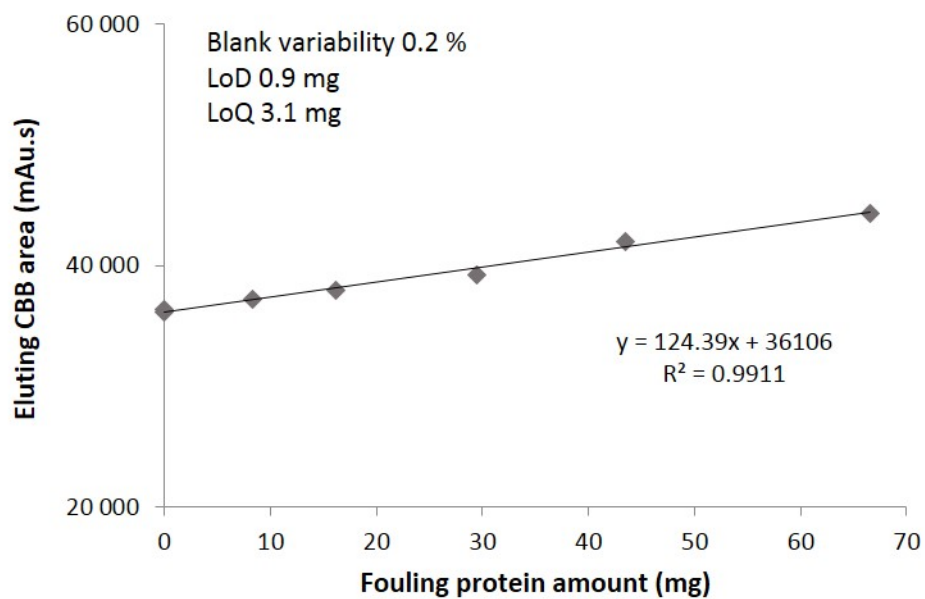
334

335 **Figure 3** Evaluation of the non-specific CBB interaction expressed as the amount of CBB released after the  
336 washing step done with (top) various pHs and 10% EtOH, (bottom) various ethanol contents at pH 8.6.

337

338

339



340

341 **Figure 4** Calibration curve relating the amount of proteins in the stainless steel rotor and the CBB dye area  
342 during elution step. Protocol as described in the Figure 2 caption.

343

344

345

346

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