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1                   Efficient extraction of intact HSA-A $\beta$  peptide complexes from sera: toward  
2                   albuminome biomarker identification

3  
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11  
12  
13                  **Abstract**

14  
15                  The performances of three commercial albumin extraction methods for the isolation of intact  
16                  albumin-amyloid beta peptide (HSA-A $\beta$ ) complexes from serum were compared using different  
17                  analytical approaches. To determine the extraction yield, the repeatability and the selectivity of  
18                  the extraction procedures, a capillary electrophoresis coupled to UV detection method was  
19                  developed. For the evaluation of the specificity and integrity of the extracted HSA-A $\beta$   
20                  complexes, SDS-PAGE, hybrid and ultra-sensitive ELISA experiments were conducted. All the  
21                  extraction methods showed different characteristics depending on their chemical binding  
22                  affinities toward albumin. The ProteoExtract Albumin Depletion kit extracted albumin with a  
23                  high repeatability but was not efficient for the extraction of intact HSA-A $\beta$  complexes. The  
24                  PureProteome Albumin magnetic beads showed a high specificity toward HSA thanks to the  
25                  grafting of anti-HSA antibodies on their surface but tended to dissociate HSA from A $\beta$  peptides.  
26                  The Pierce Albumin depletion kit showed a high extraction yield, no selectivity towards the  
27                  different albumin proteoforms and proved to be the most efficient method for the extraction of  
28                  intact HSA-A $\beta$  complexes from serum.

29  
30                  **Keywords**

31  
32                  Albuminome  
33                  Sample preparation  
34                  Biomarker  
35                  Amyloid beta peptide  
36                  Capillary electrophoresis  
37                  ELISA

## 43 1. Introduction

44 Human Serum Albumin (HSA) is the most abundant protein in human serum with a concentration of  
45 640  $\mu\text{M}$  [1]. HSA is well known to regulate the osmotic pressure in blood but also to bind and transport  
46 a large variety of endogenous (like fatty acids for example) and exogenous compounds (drugs) [2,3].  
47 HSA can also bind small metabolites, peptides and proteins [4,5]. Proteins and peptides bound to HSA  
48 have been called “albuminome” for the first time by Gundry’s group in 2005 [6]. Since that, more and  
49 more studies have been conducted on albumin and its bound proteins and peptides [7–9]. Because HSA  
50 has a serum half-life of 16-20 days, the albuminome can be protected from the renal clearance and  
51 directly reflect the actual physiological state of a patient but also disease stages, which is not always the  
52 case for other “free” biomarkers that have shorter half-life [10]. The HSA bound proteins and peptides  
53 have indeed been demonstrated as a source of biomarkers and have been used through their proteomic  
54 signatures for the diagnosis of different diseases such as cancers [11] or heart diseases [12,13]. Among  
55 the proteins bound to HSA, we find the amyloid beta peptide 1-42 ( $\text{A}\beta$  1-42), one of the three validated  
56 biomarkers of Alzheimer Disease (AD) in the cerebrospinal fluid (CSF) [14–16]. The interaction of  
57 HSA with  $\text{A}\beta$  peptides has been first demonstrated in 1996 by Biere et al [17]. Since then, different  
58 groups have further characterized this interaction and studied the influence of HSA on  $\text{A}\beta$  peptides  
59 aggregation in the case of AD [18–20].

60 The  $\text{A}\beta$  1-42 peptide can be quantified in CSF for AD diagnosis thanks to immune enzymatic techniques  
61 (ELISA) [16,21,22] or mass spectrometry (MS) [15,23,24] and it has been shown that AD patients have  
62 lower concentrations of this peptide in their CSF than controls [22]. However, the CSF sampling is  
63 invasive and is performed only at the hospital, making it difficult to perform at regular intervals of time  
64 to follow the evolution of AD. In addition, it precludes the early detection of this disease as only patients  
65 suffering already from cognitive impairment or memory loss attend memory centers or go to a  
66 neurologist consultation at the hospital.

67 In this way, scientists have shown more and more interest in blood as an alternative matrix for AD  
68 diagnosis. They tried to quantify the peptide  $\text{A}\beta$  1-42 in plasma and serum, but this peptide has not been  
69 clinically validated yet [25–27]. Possible reasons are the low concentration of this peptide in plasma  
70 ( $<0.5$  nM) and the presence of components in the blood that can cause interferences in the  
71 immunoassays. But another explanation is that 90-95% of amyloid peptides are bound to HSA [17,18],  
72 so would not be considered in those quantifications. To solve this problem and increase the accuracy  
73 and reliability of blood tests, several studies emerged during the last few years. First, to overcome the  
74 issues related to the low  $\text{A}\beta$  peptides concentration in plasma, different studies have developed methods  
75 to preconcentrate it before its quantification [28–30] or have proposed ultra-sensitive immunoassays  
76 able to detect it [31]. To lower the interferences of other plasma components, different studies have  
77 treated the plasma before analysis [32,33]. Finally, to also consider the amyloid beta bound to other  
78 plasma proteins in the quantification, recent works have put some efforts on the treatment of the plasma

79 before analysis by immunoassays [34,35]. However, these studies did not focus on the fraction of A $\beta$  1-  
80 42 peptides specifically bound to HSA. Yamamoto et al. [36] focused for the first time on the complex  
81 formed between A $\beta$  1-42 and HSA in serum by developing a homemade hybrid ELISA. They showed  
82 that low serum A $\beta$  1-42-HSA complexes level was associated with a higher AD prevalence and this  
83 could be used to monitor its progression.

84 To perform a relevant albuminome investigation, the HSA with its partners has first to be extracted  
85 while preserving the interaction between them. The extraction of HSA can be performed using different  
86 treatment methods. The most common one is the precipitation with ethanol [37,38], which enables the  
87 isolation of HSA without effective cost, but the precipitation is time consuming and requires the use of  
88 chemicals that can break the interaction between HSA and its bound proteins. In 2017, Liu et al [9]  
89 proposed a two-steps precipitation method to extract HSA with its albuminome through the use of  
90 formaldehyde as a crosslinker to maintain the interaction between HSA and its bound proteins. However,  
91 the release of the covalently bound molecules requires a boiling step that is not adapted in our case for  
92 A $\beta$  peptides. A common method to enrich HSA is the use of immunoaffinity or dye-based depletion  
93 kits. Since few years, different works have exploited these different depletion procedures to extract the  
94 albuminome from plasma [7,9,13,39]. In 2013, Holewinski [8] et al compared several methods to isolate  
95 albumin and its bound proteins from serum. Their goal was to select the most interesting one in terms  
96 of efficiency and repeatability. They then characterized the isolated HSA bound proteins by proteomic  
97 approaches (LC-MS/MS) without releasing a priori the bound proteins. So, no evidence was provided  
98 in this study on the stability of the isolated HSA-proteins complexes during the extraction procedures.  
99 More recently, Pisanu et al [40] and De Jesus et al [41] have compared different depletion procedures  
100 but this time to remove the highest abundant proteins for biomarker discovery studies. In conclusion,  
101 none of these studies has focused on the isolation of intact HSA complexes and on maintaining as much  
102 as possible the interaction between HSA and its partners.

103 The objective of our study was therefore to investigate commercial HSA depletion methods as new tools  
104 for intact HSA complexes extraction to quantify bound A $\beta$  peptides in blood. Our goal was to select the  
105 one allowing an efficient extraction of HSA, maintaining as much as possible the HSA-A $\beta$  complexes  
106 and producing a good repeatability and specificity toward HSA. In addition, as HSA exists as a mixture  
107 of several proteoforms (native, cysteinylated, glycosylated, carbamylated,...) [42,43] and considering that  
108 the glycosylated HSA proteoform has been demonstrated to bind molecules with different affinities  
109 compared to native HSA [44], a particular attention was paid to evaluate the ability of these extraction  
110 methods to preserve the natural abundance of each of them during the depletion process. To allow this  
111 evaluation, we developed a quantitative capillary electrophoresis method to determine not only the  
112 extracted HSA amount but also its proteoform profile after extraction. This is the first time that albumin  
113 proteoform abundances were quantified and compared before and after an extraction process. To ensure  
114 that the selected extraction methods did not alter the HSA-A $\beta$  complexation, two different approaches

115 were employed to analyse the extraction fraction. The first one consists in the quantification of the  
116 complex by the hybrid ELISA developed by Yamamoto et al [36]. The second consists in the  
117 quantification of A $\beta$  1-42 peptides released from HSA during the extraction procedure.

## 118 **2. Experimental**

### 119 120 *2.1 Chemicals and Reagents*

121 Plasma derived Human Serum Albumin (HSA) (lyophilized powder, essentially globulin free,  $\geq 99\%$ ),  
122 phosphate buffered saline 10X concentrate (PBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
123 (HEPES), sodium phosphate monobasic, sodium thiocyanate, sodium dodecyl sulfate (SDS), Blocking  
124 Reagent for Elisa, trizma base and glycine hydrochloride were provided by Sigma Aldrich (St. Louis,  
125 MO, USA). Anhydrous di-sodium hydrogen orthophosphate was obtained from Fisher Scientific  
126 (Hampton, NH, USA). 1M sodium hydroxide, 1M hydrochloric acid and Nanosep centrifugal device  
127 with 3 kDa Omega membrane were acquired from VWR (Fontenay sous bois, France). Pierce Albumin  
128 Depletion kit was purchased from Thermo Scientific (Waltham, MA, USA), ProteoExtract Albumin  
129 Removal Kit from Calbiochem (San Diego, United States) and PureProteome Albumin Magnetic Beads  
130 from Merck (Darmstadt, Germany). Mini Protean TGX gels (10%, 15 well, 15  $\mu$ L) and Coomassie  
131 Brilliant Blue R-250 were acquired from Bio-Rad (Hercules, CA, USA). Pre-stain proteins ladder was  
132 obtained from Euromedex (Souffelweyersheim, France). The Human  $\beta$  amyloid (1-42) high sensitive  
133 Elisa kit was obtained from Wako (Osaka, Japan). The Neurology 3-Plex A from Quanterix (Lexington,  
134 MA, USA) was used as well as the following reagents: Simoa Discs, Simoa Cuvettes, Simoa Sealing  
135 Oil and SIMOA Buffer (1&2). Deionized water was prepared using a Direct-QR 3 Water Purification  
136 System from Millipore (Billerica, MA, USA). All buffers were prepared using deionized water and  
137 filtered through a 0.22  $\mu$ m nylon membrane (VWR) before use.

### 138 *2.2 Biological samples*

139 Horseradish peroxidase (HRP) conjugated goat anti human albumin antibody was obtained from  
140 Euromedex (Souffelweyersheim, France). For the specificity and integrity with the hybrid ELISA  
141 studies, serum samples were obtained from Zen-Bio. They were collected from 25 consented healthy  
142 adult donors in a United States FDA licenced Blood Bank, aliquoted upon reception and stored at -20°C  
143 before use. For the integrity study with the SIMOA ELISA, serum samples originate from the ethically  
144 approved Montpellier Neurobank #DC-2008-417 at the certified NFS 96-900 CHU resource center BB-  
145 0033-00031, [www.biobanques.eu](http://www.biobanques.eu). Authorization to handle personal data was granted by the French  
146 Data Protection Authority (CNIL) under the number 1709743 v0.d.

### 147 *2.3 Albumin extraction methods*

148 A commercial plasma derived HSA solution at 40 mg/mL in water, called the “HSA standard solution”  
149 was first prepared. HSA was then extracted from human serum or from the HSA standard solution

150 according to the manufacturer's instructions with little modifications for the three albumin extraction  
151 kits.

### 152 2.3.1 *Extraction of HSA with Pierce Albumin Depletion Kit*

153 First, the depletion column was prepared by adding 400  $\mu\text{L}$  of the slurry resin into a spin column. The  
154 column was then centrifuged with a micro star 17 R centrifuge (VWR) for 1 min at 12,000 g to remove  
155 the excess liquid. 200  $\mu\text{L}$  of binding/wash solution (included in the kit) was then added to activate the  
156 resin after centrifugation (12,000 g for 1min). 15  $\mu\text{L}$  of serum or HSA standard solution was applied  
157 onto the column, incubated for 2 minutes at room temperature and the column was then centrifuged  
158 (7000 g for 1 minute). The flow through was reapplied onto the column. After a second incubation and  
159 centrifugation in the same conditions than before, the column was washed 4 times with 50  $\mu\text{L}$  of  
160 binding/wash solution each. To elute the extracted albumin and its bound partners, the resin was washed  
161 4 times with 200  $\mu\text{L}$  of a solution composed of 20 mM sodium phosphate and 250 mM sodium  
162 thiocyanate, pH 7.2. The elution fractions were collected separately in 2mL Eppendorf tubes before  
163 being pooled for further analysis.

### 164 2.3.2 *Extraction of HSA with ProteoExtract Albumin Depletion Kit*

165 The column was first prepared by removing the storage solution and then activated by adding 1 mL of  
166 the ProteoExtract Albumin Binding Buffer using a gravity flow. 30  $\mu\text{L}$  of serum or HSA standard  
167 solution was diluted in the binding buffer to have a total volume of 350  $\mu\text{L}$  which was then applied onto  
168 the column. The column was then washed twice with 600  $\mu\text{L}$  of binding buffer. To elute the extracted  
169 albumin, the column was washed twice with 600  $\mu\text{L}$  of a solution containing 20 mM sodium phosphate  
170 and 250 mM sodium thiocyanate, pH 7.2. The elution fraction was then analysed directly by CZE-UV  
171 or SDS-PAGE.

### 172 2.3.3 *Extraction of HSA with PureProteome Albumin Magnetic Beads*

173 The beads were first resuspended by vortexing the tube for 10 seconds. 750  $\mu\text{L}$  of the resuspended beads  
174 were then pipetted and added in a 2 mL Eppendorf tube. The tube was then placed in a magnetic stand  
175 to allow the beads migrating to the magnet, then the storage buffer was removed. This step was  
176 performed every time to remove any liquid from the beads. The beads were then washed twice with 500  
177  $\mu\text{L}$  of PBS. For each step, PBS was added to the beads and the tube was vortexed for 10 seconds before  
178 removing the PBS solution. Then, 30  $\mu\text{L}$  of serum or HSA standard solution diluted in 70  $\mu\text{L}$  of PBS  
179 was applied on the beads and incubated for one hour with a continuous end-over-end rotation. The tube  
180 was then placed in the magnetic stand to remove the HSA-depleted serum. The beads were then washed  
181 three times with 500  $\mu\text{L}$  PBS. To recover the extracted HSA, the beads were resuspended three times  
182 with 120  $\mu\text{L}$  of 200 mM Glycine-HCl, pH 2.0. All the elution fractions were then neutralized with 80

183  $\mu\text{L}$  of 1M Tris, pH 8.0 (to get a final volume of 200  $\mu\text{L}$  for each elution fraction) before being pooled  
184 for further analysis.

## 185 2.4 Capillary zone electrophoresis coupled to UV detection (CZE-UV)

### 186 2.4.1 Instrumentation

187 Capillary electrophoresis experiments were conducted on a P/ACE MDQ Instrument (Sciex,  
188 Framingham, MA, USA), coupled to a diode array detection.

### 189 2.4.2 Method

190 UV detection was set at 214 nm. Polyvinyl alcohol (PVA) capillaries, purchased from Sciex with an  
191 internal diameter of 50  $\mu\text{m}$  and a total length of 60.2 cm (effective length 50 cm) were employed.  
192 Software 32 Karat<sup>TM</sup> version 7.0 (Sciex) was used to control the instrument and to collect the data. The  
193 background electrolyte (BGE) consisting of 75 mM HEPES at pH 8.0, containing 0.5 mM SDS was  
194 prepared by adding 0.894 g of HEPES and 7.21 mg of SDS to deionized water. The pH was then adjusted  
195 to 8.0 with 1 M NaOH before adding water to a final volume of 50 mL. Before the first use, the capillary  
196 was rinsed at 30 psi during 10 minutes with water and BGE. Between each analysis, the capillary was  
197 flushed at 20 psi during 5 minutes with water and BGE. The sample was hydrodynamically injected  
198 from the inlet by applying a pressure of 0.5 psi for 15 seconds. The separation was then conducted at -  
199 25 kV at 25°C. At the end of the day, the capillary was rinsed with water (3 min, 30 psi) and BGE (3min,  
200 30 psi) before being stored at 4°C with the ends in water.

### 201 2.4.3 Validation

202 The developed CZE-UV method for the separation of the HSA proteoforms was validated using a  
203 plasma derived HSA standard sample at 1 mg/mL in water. For the intraday study, the sample was  
204 analysed six consecutive times, the same day and using the same capillary. For the intermediate  
205 precision study, the sample was analysed over three days using the same capillary. For the inter-capillary  
206 study, the sample was analysed twice on three PVA capillaries over different days (n=6). The relative  
207 standard deviation (RSD) of the migration times ( $t_m$ ), total peak area (tPA) (sum of the peak areas of all  
208 HSA proteoforms) and relative peak area (rPA) (percentage of each HSA proteoform peak, related to  
209 the total peak area) were then calculated. For the linearity study, plasma-derived HSA standard solutions  
210 at 5 concentrations were prepared (1.0;1.5;2.0;4.0;5.0 mg/mL) and analysed three times with the  
211 developed CZE-UV method. The tPA was then calculated for each albumin concentration. Fisher's tests  
212 were then performed to validate the linear model between the HSA concentration and the tPA. The limit  
213 of detection (LOD) and limit of quantification (LOQ) were determined using the slope of the calibration  
214 curve [45].

215

#### 216 2.4.4 Quantification

217 To quantify HSA, a calibration curve was created by measuring tPA for different HSA concentrations  
218 (1.0-5.0 mg/mL) using the developed CZE-UV method. The HSA from the HSA standard solution was  
219 then extracted using one of each depletion kit and the extraction yield could be determined using the  
220 calibration curve. By knowing the amount of HSA employed for each extraction method and the  
221 resulting amount of HSA in each elution fraction, the HSA extraction yield (HSA EY) could be easily  
222 determined using eqn (1):

$$223 \quad \text{eqn (1) : HSA EY (\%)} = \frac{\text{amount of HSA in the elution fraction} \times 100}{\text{amount of HSA treated}}$$

#### 224 2.5 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

225 SDS-PAGE was used to analyse the elution fractions resulting from the depletion treatments of serum  
226 with the three kits. 10  $\mu\text{L}$  of elution fraction was mixed with 2  $\mu\text{L}$  of a sample buffer, composed of 60%  
227 glycerol, 300 mM Tris pH 6.8; 6mM EDTA, 12% SDS, 864 mM mercaptoethanol-2 and 0.05% of  
228 bromophenol blue. The obtained 12  $\mu\text{L}$  of sample were mixed and heated at 95°C for 10 minutes. The  
229 samples were then loaded on the 10% Precast TGX gel. Protein weight markers were also analysed. The  
230 electrophoretic separation was performed at 100 V for 5 min and then 130 V for 50 min. The gels were  
231 then washed with deionized water for 15 minutes, stained with Coomassie Brilliant Blue for 45 minutes  
232 and bleached overnight in a solution of 7% acetic acid in 23% ethanol. Gels were read on a Chemic doc  
233 imaging system from BioRad and the images were processed with ImageLab software (BioRad,  
234 Hercules, CA, USA). The specificity of each extraction method was estimated by dividing the HSA  
235 band intensity in the elution fraction lane by the total band intensities.

#### 236 2.6 Elisa

##### 237 2.6.1 Hybrid ELISA

238 To detect specifically the HSA-A $\beta$  complex, we used the Human  $\beta$  amyloid (1-42) high sensitive Elisa  
239 kit and the HRP conjugated anti-HSA antibody. 100  $\mu\text{L}$  of samples (diluted serum, PBS or diluted  
240 elution fractions) were dispensed in the wells of the anti-human A $\beta$  1-42 antibody (BAN50) coated  
241 microplate, which was previously blocked for one hour at room temperature with 200  $\mu\text{L}$  of 1:10 diluted  
242 Blocking Reagent. The microplate was then covered with a plate seal and refrigerated overnight (4°C).  
243 After this, the solutions were discarded from the wells and the microplate was washed five times with  
244 300  $\mu\text{L}$  of washing buffer. After this washing step, 100  $\mu\text{L}$  of HRP-conjugated anti human albumin  
245 polyclonal antibody, diluted 1:30000 in a solution of 1:25 diluted Blocking Reagent, was added in the  
246 different wells. The microplate was covered with a plate seal and was then incubated at 4°C for one  
247 hour. The antibody solutions were then removed from the wells and the microplate was washed five  
248 times with the washing solution. After washing, 100  $\mu\text{L}$  of 3,3',5,5'-tetramethylbenzidine substrate  
249 (TMB) were added to the wells. The microplate was then covered with an aluminium paper and left in



250 the dark for 30 min. To stop the reaction, 100  $\mu$ L of acidic stop solution was added into each well.  
251 Absorbances were read at 450 nm with an EL800 instrument from Biotek (Winooski, Vermont, USA).  
252 Measurements of all samples were carried out in triplicates. For the HSA-A $\beta$  complex quantification, a  
253 calibration curve was created by serial dilution of the initial serum in PBS. The calibration curve was  
254 performed in duplicates with serum dilution factor ranging from 1:4000 to 1:128000. The HSA-A $\beta$   
255 complex extraction yields (HSA-A $\beta$  EY) were then calculated thanks to eqn (2):

$$256 \quad \text{eqn (2) : HSA - A}\beta \text{ EY (\%)} = \frac{\text{estimated dilution factor of the treated serum} \times 100}{\text{theoretical dilution factor of the treated serum}}$$

257 where the estimated dilution factor of the treated serum is the one determined by using the standard  
258 calibration curve, and the theoretical one is determined by considering the dilutions made during the  
259 different steps of the extraction procedure.

## 260 2.6.2 SIMOA (Quanterix) for amyloid beta peptide 1-42 quantification

### 261 2.6.2.1 Sample preparation method

262 After extraction with the Pierce Albumin Depletion kit or PureProteome Albumin Magnetic Beads, the  
263 elution fractions were filtered and dissolved in PBS in order to concentrate and quantify the free  
264 amyloid-beta peptide 1-42. For that, after having washed a 3 kDa Nanosep membrane with 2x500  $\mu$ L  
265 of deionized water, the elution fraction was applied onto the membrane and centrifuged at 4,000 g until  
266 approximately 20  $\mu$ L of sample was left in the retentate. Then 500  $\mu$ L of PBS was added onto the  
267 membrane and the obtained sample was centrifuged at 4,000 g until 70  $\mu$ L was left onto the membrane.  
268 This fraction was then analysed. Two duplicates per extraction methods were prepared.

### 269 2.6.2.2 Analysis of the free amyloid-beta peptide 1-42

270 Concentration of human amyloid beta peptide 1-42 was measured with the Single Molecule Array  
271 (SIMOA ) technology (Quanterix, Lexington, MA, USA), comprising a SIMOA HD-1 analyzer [46].  
272 This method is approximately 1000-fold more sensitive than common ELISA methods. The monoclonal  
273 anti A $\beta$  capture antibody was attached to the surface of paramagnetic beads. The presence of the antigen  
274 was recognized by biotinylated monoclonal anti A $\beta$  1-42 detection antibodies. After the formation of  
275 sandwich complexes on magnetic micro beads, streptavidin- $\beta$ -galactosidase was added to bind to the  
276 biotinylated detection antibody, when present. Resorufin  $\beta$ -d-galactopyranoside, which acts as a  
277 substrate, becomes fluorescent when it comes into contact with the streptavidin- $\beta$ -galactosidase fixed  
278 on the beads that had captured the antigen. The beads were then loaded into arrays of 216,000 femtoliter-  
279 sized wells, which held only one bead per well. The wells were sealed with oil. The number of  
280 fluorescent wells was counted. The ratio of wells containing a fluorescent bead to the total number of  
281 wells is proportional to the concentration of the antigen. The free A $\beta$  1-42 peptide samples were diluted  
282 4-fold with the Quanterix SIMOA sample diluent buffer before analysis. Controls for SIMOA  
283 performance were obtained with serial measurement of quality control (QC) samples; this analysis

284 confirmed the low intra-assay variation (CV ranged from 1 to 6%) and the good inter-assay variation  
285 (CV ranged from 4 to 18%).

### 286 **3. Results and discussion**

#### 287 *3.1 Optimization and validation of the CZE-UV method for the quantification of human serum albumin*

288 In order to compare the performances of different HSA depletion approaches aimed at extracting HSA  
289 complexed to its partners, we developed first a reliable quantitative method for HSA analysis. Most  
290 current methods for HSA quantification measure the total HSA concentration. However, as this plasma  
291 protein exhibits several proteoforms, we searched for a method allowing not only the total concentration  
292 determination but also the relative abundance of each proteoform. In order to avoid HSA adsorption  
293 onto the capillary wall, several neutral coated capillaries were tested. According to our previous work  
294 on CE-UV of HSA [42], homemade PEO coated capillary was first used with a BGE of 75 mM HEPES,  
295 pH 7.4 containing 0.5 mM SDS. As expected, a separation of three HSA proteoforms into glycosylated  
296 (peak1), native (peak 2) and cysteinylated forms (peak 3), was obtained (figure 1A). However, the  
297 regeneration of this semi-permanent coating each day and between runs was necessary to obtain a good  
298 reproducibility, thus increasing the total analysis time. A commercial permanent PVA coated capillary  
299 avoiding tedious regeneration steps, was then tested under the same electrophoretic conditions. A similar  
300 and well-resolved separation of the three HSA proteoforms was achieved (figure 1B) with that  
301 alternative coating. The resolution between peaks 1 and 2 ( $R_{S1,2}$ ), or peaks 2 and 3 ( $R_{S2,3}$ ) and the  
302 asymmetry factors (0.80-1.2) were similar with both capillaries. Moreover, the analysis duration with  
303 the PVA capillary was significantly shortened. The BGE was also optimized before the method  
304 validation to improve resolution between peaks 2 and 3 for better quantification accuracy. By increasing  
305 the pH of the BGE from 7.4 to 8.0, this resolution was greatly improved. At pH 8.0, both resolutions  
306 ( $R_{S1,2}$  and  $R_{S2,3}$ ) (figure 1C) were satisfactory. Considering the significant reduction of the total analysis  
307 time, the PVA capillary has been selected for the rest of our studies.

308 Before applying the CZE-UV method to real samples after HSA extraction from serum, the validation  
309 of the method was carried out in terms of intra-day, inter-day and inter-capillary repeatability and  
310 linearity. The RSD obtained for  $t_m$ , tPA and rPA of the three main peaks (native, glycosylated and  
311 cysteinylated proteoforms) are reported in *Table 1*. The intra-day study showed the excellent  
312 repeatability for  $t_m$  and very satisfactory repeatabilities for tPA and rPA. For the inter-day experiments,  
313 the RSD of the different parameters slightly increased but remained satisfactory with values less than  
314 5% for tPA and rPA. The RSD of the tPA and rPA of the inter-capillary study were very satisfactory,  
315 especially when considering it was conducted on different days. All these results showed the excellent  
316 reproducibility of the method. The quantification performances of the method were also evaluated by  
317 analysing plasma derived HSA standard solutions in water at five concentrations and by plotting the  
318 total peak area against the concentration. It showed that the method was linear in the range of 1.0 to 5.0

319 mg/mL ( $y = 77321x - 5616$ ) with a good correlation coefficient (0.98). A linearity test was performed  
320 and validated the linearity of the developed method ( $F_{\text{exp}} = 0.044 < F_{\text{limit}} = F_{5\% (3,10)}$ ). The LOD and the  
321 LOQ were determined to be 0.31 and 1.0 mg/mL respectively [45]. These results indicated that the  
322 method is suitable for the quantification of HSA in the elution fractions from the different enrichment  
323 methods.

### 324 *3.2 Comparison of the performances of the different extraction methods*

325 To select the most efficient HSA extraction method to enrich HSA and its partners from plasma, three  
326 HSA extraction methods based on different affinity mechanisms were compared in terms of yield and  
327 repeatability of extraction, selectivity toward the different HSA proteoforms, specificity regarding other  
328 plasma proteins and integrity of the isolated complex. The HSA standard solution at 40 mg/mL was  
329 treated to assess the three first criteria whereas serum was rather used for specificity and integrity of the  
330 methods.

#### 331 *3.2.1 Extraction Yield*

332 The extraction yield of each HSA extraction method was determined by analysing the elution fractions  
333 resulting from the treatment of the HSA standard solution. The analysis was performed with the  
334 developed CZE-UV method. Six replicates of each extraction methods were prepared, and the six  
335 corresponding elution fractions were analysed the same day to determine the mean yield of extraction  
336 and its standard deviation. The extraction yields obtained were  $83 \pm 17\%$ ,  $71 \pm 5\%$  and  $77 \pm 10\%$  for  
337 Pierce Albumin Depletion kit, ProteoExtract Albumin Depletion kit and PureProteome Albumin  
338 Magnetic Beads, respectively. Pierce Albumin Depletion kit is thus the method extracting the highest  
339 amount of HSA but also showing the less satisfactory repeatability. On the contrary, the ProteoExtract  
340 Albumin Depletion kit extracts slightly less HSA than the two others but exhibits the highest  
341 repeatability. The high extraction yield of Pierce extraction method could be explained by the fact that  
342 centrifugation is used to recover the different elution fractions from the resin so that sample loss is less  
343 likely to occur. On the contrary, for ProteoExtract extraction method, a gravity flow is employed to  
344 recover the elution fraction, so it is possible that some sample stays stuck onto the resin.

#### 345 *3.2.2 Selectivity for a particular HSA proteoform*

346 The selectivity of each HSA extraction method was evaluated by comparing the abundance of the three  
347 HSA proteoforms in the HSA standard solution before and after 6 treatments performed using each  
348 enrichment method. The initial solution and elution fractions were analysed by the CZE-UV method.  
349 By comparing the abundances and the associated standard deviation of the three main HSA proteoforms  
350 in the elution fractions and in the HSA standard solution, the Pierce Albumin Depletion kit and the  
351 PureProteome magnetic beads did not exhibit selectivity for a particular HSA proteoform (Figure 2). On  
352 the contrary, after treatment with the ProteoExtract Albumin Depletion kit, the glycosylated form was

353 slightly enriched whereas the cysteinylated form was depleted. To ascertain the observed selectivity of  
354 the ProteoExtract Depletion kit for these two proteoforms, a Mann-Whitney test was performed. The  
355 glycosylated form abundance in the HSA standard was significantly different from that in the ProteoExtract  
356 elution fractions ( $p$  value  $< 0.05$ ). The same result was obtained for the cysteinylated form. Thus, this  
357 statistical test confirmed that the ProteoExtract led to an enrichment of the glycosylated at the expense of  
358 the cysteinylated HSA proteoforms. The selectivity tendency of ProteoExtract could be explained by  
359 the interaction mode involved between the solid support and HSA. Indeed, ProteoExtract is composed  
360 of a resin which captures HSA through electrostatic interactions. HSA proteoforms have different  
361 hydrodynamic volumes, and do not expose thus the same charges at their surfaces. Their resulting  
362 electrostatic interaction with the resin can therefore be different. Thus, it is possible that the resin of  
363 ProteoExtract interacts preferentially with the glycosylated form. Although Pierce extraction method is also  
364 based on a resin substrate, this specificity could not be observed due to its low repeatability (see 3.2.3).

### 365 3.2.3 HSA extraction repeatability

366 The repeatability of each extraction method was evaluated the same day by extracting six times the same  
367 batch of HSA standard solution. The RSD of the extraction yield and proteoform abundances in the  
368 elution fractions for the different procedures are reported in *Table 2*. The Pierce Albumin Depletion kit  
369 and PureProteome Albumin magnetic beads led to less repeatability. For Pierce, several elution fractions  
370 were collected separately before being pooled together for analysis. Thus, it is possible that HSA was  
371 not fully recovered from the different Eppendorf tubes and that the volume of the pooled elution fraction  
372 was not exactly the same between replicates. For PureProteome Albumin magnetic beads, the elution  
373 fractions were collected through the capture of the beads by a magnetic stand, so recovered volume  
374 variation may also occur between replicates. The ProteoExtract Albumin Depletion kit showed the most  
375 interesting results in terms of repeatability as elution fractions were recovered once in one tube, without  
376 any centrifugation or magnetic stand.

### 377 3.2.4 HSA extraction specificity

378 The specificity of each HSA isolation procedure was assessed by analysing the extracted proteins present  
379 in the elution fraction by SDS-PAGE with coomassie staining (Figure 3) and calculating the percentage  
380 of albumin in the corresponding elution fraction. PureProteome Albumin Magnetic Beads and  
381 ProteoExtract Albumin Depletion kits were the most specific methods, extracting 93.5% and 92.2% of  
382 albumin respectively. For the Pierce Albumin Depletion kit, other bands at 150 kDa and ~ 80 kDa for  
383 instance which may correspond to Immunoglobulin G and transferrin respectively were present in the  
384 elution fraction, which led to a less satisfactory specificity (60.3%). The specificity difference between  
385 the HSA isolation methods studied is mainly due to the different binding mechanisms involved. Indeed,  
386 the PureProteome Albumin Magnetic Beads kit isolates HSA through an antibody ligand, specific to  
387 this protein whereas Pierce and ProteoExtract Depletion kits rely rather on electrostatic interaction of

388 HSA with resins, which are known to be less specific than antibodies. The new resin proposed by  
389 ProteoExtract appeared however more specific than Cibacron Blue (Pierce).

### 390 3.2.5 Integrity of the HSA-A $\beta$ complex

391 The interaction between albumin and its protein/peptide partners must be preserved during the extraction  
392 procedure as our objective is to study the HSA bound A $\beta$  peptides. Thus, the preservation of the HSA-  
393 A $\beta$  complex is the most important criteria for our purpose. To evaluate the capacity of the three  
394 extraction methods to maintain intact the complex after extraction, two different approaches were used.  
395 The first one consisted in the quantification of the HSA-A $\beta$  complexes in the elution fractions by a  
396 hybrid ELISA recently reported [36] which combines a capture anti-A $\beta$  and a detection anti-HSA  
397 antibodies. The second approach relied on the determination of the free A $\beta$  1-42 peptide in the elution  
398 fraction by an ultra-sensitive SIMOA ELISA to ensure that the HSA bound A $\beta$  1-42 peptide was not  
399 released from HSA during the elution step. For the first approach, we used the calibration curve resulting  
400 from the analysis of diluted sera. By applying serum diluted from 1:4000 to 1:128000, a linear  
401 decreasing of the signal was observed ( $y= 1575x + 0.0469$ ,  $r^2=0.9938$ ). The elution fractions resulting  
402 from the three albumin extraction methods and the HSA standard solution were then diluted the same  
403 way in PBS for hybrid ELISA analysis. The HSA standard solution signal was slightly higher than the  
404 PBS blank one (by 1.2) but lower than that of the three different elution fractions (by  $\sim 1.5$ ). This proves  
405 that all the elution fractions contained HSA-A $\beta$  complexes. We can however not exclude that some extra  
406 HSA could be non-specifically adsorbed on the surface of the ELISA microplate, leading to an  
407 overestimation of the signal. However, since the aim of this experiment was only to compare the amount  
408 of HSA-A $\beta$  complexes in different elution fractions which were expected to contain the same quantity  
409 of HSA (similar extraction yields, see 3.2.1), the hybrid assay could still be used for a comparative  
410 study of HSA-A $\beta$  complexes integrity. The extraction yields of total HSA (which includes also HSA-  
411 A $\beta$  complexes) calculated based on the calibration curve were  $85 \pm 16\%$ ,  $18 \pm 5\%$  and  $48 \pm 9\%$  ( $n=3$ )  
412 for Pierce, ProteoExtract and PureProteome kits, respectively. Considering that the yield achieved with  
413 ProteoExtract was too low, this extraction method was not retained for the rest of the experiments. For  
414 Pierce and PureProteome methods, the second approach was then applied to investigate if the difference  
415 in the complex extraction yields was due to a different affinity toward the HSA-A $\beta$  complex or a  
416 tendency of PureProteome to dissociate more the extracted complex during the elution step. The  
417 concentration of free A $\beta$  1-42 peptide in the initial serum, determined by SIMOA, was 16.2 pg/mL.  
418 After its extraction with Pierce and PureProteome methods, A $\beta$  1-42 peptide found in the elution  
419 fractions was concentrated and diluted in PBS before SIMOA analysis. Its concentration in the elution  
420 fraction after extraction with the Pierce method was 3.2. pg/mL (representing 20% of the initial one).  
421 Using the PureProteome method, the concentration reached 34.9 pg/mL (representing 215% of the initial  
422 concentration). This much higher amount of A $\beta$  1-42 peptide found in the PureProteome elution fraction  
423 indicates that this extraction method partly dissociates the interaction between this peptide and HSA

424 during the elution step. The complexes dissociation tendency of PureProteome method can be explained  
425 by the rather acidic conditions (pH 2) required to elute albumin and its partners from the magnetic beads.  
426 On the contrary the elution step of Pierce method is performed under milder conditions (pH 7.2), leading  
427 likely to less complexes dissociation. The A $\beta$  1-42 peptide detected in both elution fractions could  
428 however originate also to a certain extent from its nonspecific binding to the resin/beads, especially for  
429 Pierce Albumin Depletion kit which showed the lowest specificity towards albumin. However, the A $\beta$   
430 1-42 peptide nonspecific adsorption on Pierce resin was assumed to be very low considering that its  
431 concentration in the elution fraction was five times lower than its initial serum one. The overall results  
432 proved that the HSA-A $\beta$  complexes can be extracted from serum and that the Pierce Albumin depletion  
433 kit appears as the most efficient method for this purpose.

#### 434 **4. Conclusions**

435 Extraction of albumin and its bound partners represents a real challenge and also the most critical step  
436 of an albuminome study. In this work, three albumin extraction methods were compared in order to  
437 isolate and study specifically the HSA-A $\beta$  complexes. Based on the five criteria investigated (extraction  
438 yield, selectivity, repeatability, specificity, and integrity), all the three methods presented interesting but  
439 different characteristics. The Proteoextract method presented the most interesting results in terms of  
440 repeatability but this method tended to change the natural abundances of HSA proteoforms during the  
441 process and it extracted only a small quantity of HSA-A $\beta$  complex. The PureProteome Albumin  
442 magnetic beads method extracted more specifically HSA with a high extraction yield but tended to  
443 disrupt the interaction between albumin and its bound A $\beta$  peptides. Finally, the best extraction efficiency  
444 of intact HSA-A $\beta$  complexes was obtained using the Pierce Albumin Depletion Kit. Indeed, if the  
445 extraction method used dissociates even partly the interaction between albumin and its partners, a  
446 specific albuminome study is then made impossible. Thus, Pierce Albumin Depletion kit can be  
447 considered as the best serum sample preparation method to extract HSA bound A $\beta$  peptides for the study  
448 of AD biomarkers.

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640 **Illustrations and tables captions**

641

642 **Figure 1:** Comparison of CZE-UV profiles obtained for plasma derived HSA standard using PEO and  
643 PVA capillaries with different BGE pHs: A) PEO capillary with BGE at pH 7.4; B) PVA capillary  
644 with BGE at pH 7.4; C) PVA capillary with BGE at pH 8.0. Peaks assignment: 1: glycosylated HSA; 2:  
645 native HSA; 3: cysteinylated HSA

646 Conditions: Sample: 1mg.ml<sup>-1</sup> HSA in water, BGE: 75 mM HEPES with 0.5 mM SDS, capillary: 50  
647  $\mu$ m, 50 cm (effective length), voltage: -25kV, temperature: 25°C, injection: 0.5 psi for 15 s.

648

649 **Figure 2:** Comparison of the main HSA proteoform abundances in the elution fractions resulting  
650 from the treatment of HSA standard solution (40 mg/mL) by three different enrichment methods and  
651 in a non-treated HSA standard solution. Plotted values represent the mean $\pm$ SD for 5 replicates (HSA  
652 standard) and 6 replicates (elution fractions). Asterisks \* indicate statistically significant difference  
653 between HSA standard solution and ProteoExtract elution fraction according to Mann-Whitney test (p  
654 value <0.05)

655

656 **Figure 3:** SDS-PAGE of the molecular weight markers (M) and the elution fractions from Pierce (1),  
657 ProteoExtract (2) and PureProteome (3) kits

658

659 **Table 1:** Relative Standard Deviations of the different parameters studied for the intra-day, inter-day  
660 and inter-capillary repeatability of the CZE-UV method. Conditions: as in fig 1C

661 **Table2:** Repeatabilities of the extraction yield and proteoform abundances in the elution fractions  
662 resulting from the three HSA enrichment procedures

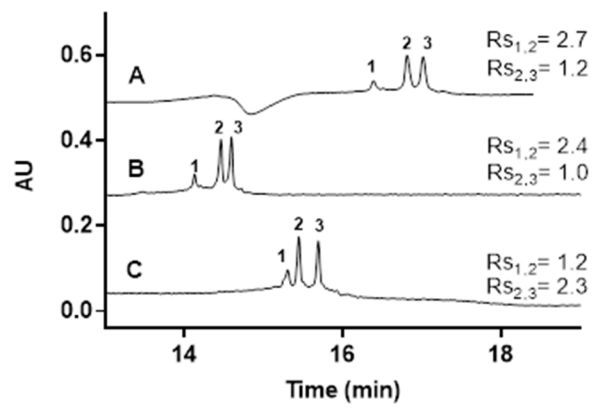


Fig1

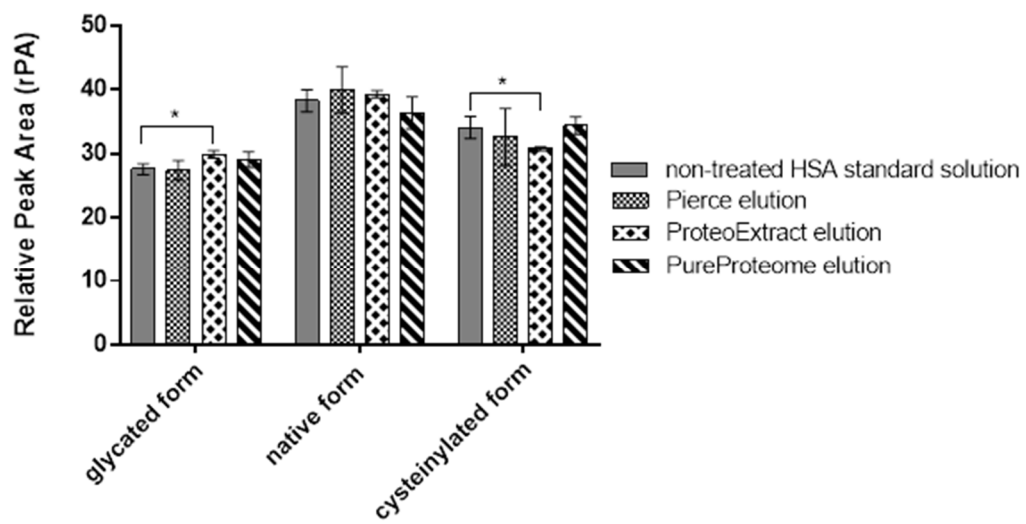


Fig2

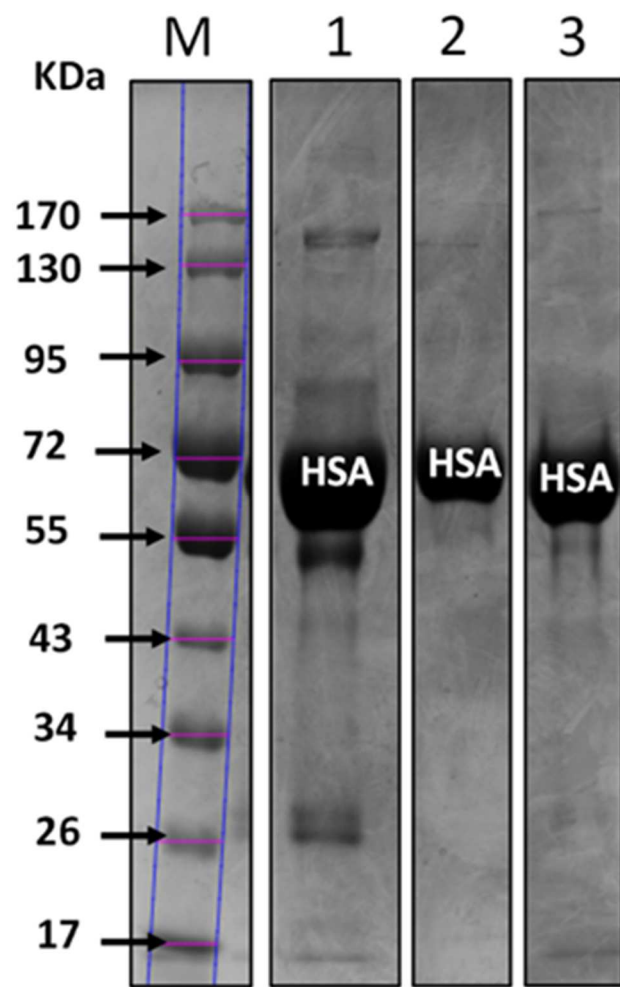


Fig3

	RSD of $t_m$ (%)	RSD of tPA* (%)	RSD of rPA* (%)
Intra-day	<0.1	<2.5	<3.0
Inter-day	<2.0	<2.6	<4.6
Inter-capillary and -day	<2.0	<7.0	<7.0

\* Peak areas were measured using the baseline integration mode. The total area is the sum of the peaks areas of all HSA proteoforms. Relative Peak Area (rPA) is the percentage of each HSA proteoform over all proteoforms.

	Pierce Albumin Depletion Kit	ProteoExtract Depletion kit	PureProteome Magnetic beads
Extraction yield RSD* (%)	20	7.1	12
Proteoform abundances RSD* (%)	5.6-14	1.0-2.0	4.0-6.9

\*Extraction yield and proteoform abundances were determined for each method by performing six replicates of the HSA standard solution extraction and by analysing the elution fractions by CZE-UV.



