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1 A capillary zone electrophoresis method for detection of ApolipoproteinC-III glycoforms and  
2 other related artifactually modified species

3 Coralie Ruel<sup>a,b</sup>, Marco Morani<sup>a</sup>, Arnaud Bruneel<sup>c,d</sup>, Christophe Junot<sup>b</sup>, Myriam Taverna<sup>a</sup>, François  
4 Fenaille<sup>b</sup>, Thuy Tran<sup>a</sup>

5 <sup>a</sup> Institut Galien Paris Sud, UMR 8612, Proteins and Nanotechnology in Analytical Science (PNAS), CNRS,  
6 Univ. Paris-Sud, Univ. Paris-Saclay, 5 rue Jean Baptiste Clément, 92290 Châtenay-Malabry, France

7 <sup>b</sup> CEA, UMR 0496, CEA, Institut Joliot, SPI, Laboratoire d'Etude du Métabolisme des Médicaments,  
8 MetaboHUB-Paris, Université Paris Saclay, Gif-sur-Yvette cedex, France

9 <sup>c</sup> AP-HP, Bichat University Hospital, Biochemistry, 46 Rue Henri Huchard, 75018 Paris, France

10 <sup>d</sup> INSERM UMR-1193 "Mécanismes cellulaires et moléculaires de l'adaptation au stress et  
11 cancérogène", Université Paris-Sud, 5 rue Jean Baptiste Clément, 92290 Châtenay-Malabry, France

12 corresponding author: Thuy Tran , thuy.tran-maignan@u-psud.fr, tel : 33146835903

13

#### 14 **Highlights :**

- 15 • For the first time, a CZE method was developed to analyze ApoC-III glycoforms.
- 16 • Carbamylated and sialylated forms of ApoC-III can be efficiently separated by CZE.
- 17 • Carbamylated ApoC-III species were highlighted and characterized.
- 18 • Carbamylation was evidenced in ApoC-III batches from 3 distinct commercial sources.

#### 19 **Abstract**

20 ApolipoproteinC-III (Apo C-III) is a human plasma glycoprotein whose O-glycosylation can be altered as  
21 a result of congenital disorders of glycosylation (CDG). ApoC-III exhibits three major glycoforms whose  
22 relative quantification is of utmost importance for the diagnosis of CDG patients. Considering the very  
23 close structures of these glycoforms and their tendency to adsorb on the capillary, a thorough  
24 optimization of capillary electrophoresis (CE) parameters including preconditioning and in-between  
25 rinsing procedures was required to efficiently separate all the Apo-CIII glycoforms. Permanent coatings

26 did not contribute to high resolution separations. A fast and reliable method based on a bare-silica  
27 capillary combining the effect of urea and diamine additives allowed to separate up to six different  
28 ApoC-III forms. We demonstrated by a combination of MALDI-TOF mass spectrometry (MS) analyses  
29 and CE of intact and neuraminidase-treated samples that this method well resolved glycoforms  
30 differing not only by their sialylation degree but also by carbamylation, an undesired chemical  
31 modification of primary amines. This method allowed to demonstrate the carbamylation of ApoC-III  
32 glycoforms for the first time. Our CZE method proved robust and accurate with excellent intermediate  
33 precision regarding migration times (RSDs < 0.7%) while RSDs for peak areas were less than 5%. Finally,  
34 the quality of three distinct batches of commercial ApoC-III obtained from different suppliers was  
35 assessed and compared. Quite similar but highly structurally heterogeneous ApoC-III profiles were  
36 observed for these samples.

## 37 **Keywords**

38 ApolipoproteinC-III; capillary zone electrophoresis; glycoprotein; carbamylation

## 39 **1. Introduction**

40 Glycosylation is one of the most important post-translational modifications of mammalian proteins. It  
41 corresponds to the attachment of an oligosaccharidic chain to the protein backbone by a complex  
42 enzymatic process [1]. There are two types of glycosylation, N- and O-glycosylation depending on the  
43 nature of the amino acid residue linked to the oligosaccharidic chain. Both N- and O-glycosylations can  
44 be affected in congenital disorders of glycosylation (CDGs). CDGs are inherited metabolic diseases  
45 caused by mutations of any gene coding for enzymes implied in the glycan biosynthesis [2]. CDGs  
46 affecting N-glycosylation can be defined as type I or type II according to the defective biosynthesis step  
47 [3]. The type I corresponds to a variable glycosylation site occupancy while type II is defined by the  
48 alteration of the carried N-glycan motifs. Abnormal N-glycosylation is currently clinically monitored in  
49 patients for diagnostic purposes via the analysis of plasma transferrin glycoforms either by isoelectric  
50 focusing (IEF) [4] or by capillary zone electrophoresis (CZE) [5,6]. However, O-glycosylation pathways

51 can also be affected. The detection of ApolipoproteinC-III (ApoC-III), an O-glycosylated plasma protein  
52 was first proposed by Wopereis et al (2003) [7], as a potential biological test complementary to that  
53 of transferrin [8] and to screen rather abnormal O-glycan structures [7] occurring as a consequence of  
54 mutations in genes encoding for conserved oligomeric Golgi complex [9–11].

55 ApoC-III is a 79 amino acid protein synthesized by the liver and the intestine. It is a component of  
56 circulating particles in blood rich in triglycerides and is mainly present in very-low density lipoproteins  
57 [12]. ApoC-III carries the most common form of O-linked glycans, a mucin type core 1 at threonine 74  
58 which corresponds to a galactose  $\beta$ 1-3 linked to an N-acetylgalactosamine residue. Three main  
59 glycoforms have been described [13–15], depending on the number of sialic acid residues present, i.e.  
60 from 0 to 2 (ApoCIII<sub>0</sub>, ApoCIII<sub>1</sub> and ApoCIII<sub>2</sub> forms, respectively). Recently, minor ApoC-III glycoforms  
61 containing high levels of fucosylation instead of sialylation have also been reported [16,17].

62 Several analytical techniques have been developed to analyze ApoC-III in plasma for diagnostic  
63 purposes. Electrophoresis-based methods, such as IEF [7,13,18,19] and two-dimensional  
64 electrophoresis [20–24] can be successfully used for glycoform analysis and allowed to separate the  
65 two sialylated glycoforms from the asialylated one. However, such techniques are only semi-  
66 quantitative and quite time consuming. Besides, new developments in intact ApoC-III analysis by  
67 matrix assisted laser desorption ionization (MALDI) coupled to either time of flight (TOF) [20,22,24–  
68 28] or Fourier transform ion cyclotron resonance [17] have been reported. MALDI techniques enabled  
69 the relative quantitation of glycoform ratios (ApoC-III<sub>1</sub> / ApoC-III<sub>0</sub> and ApoC-III<sub>2</sub> / ApoC-III<sub>0</sub>) but also a  
70 differentiation, in human plasma, of the ApoC-III<sub>0</sub> into two main isoforms, one without any glycan and  
71 the second bearing one asialoglycan [22,28]. The determination of those ratios allows the diagnosis of  
72 O-glycosylation related CDGs. Moreover, the sample preparation was simple as only plasma  
73 purification/delipidation and desalting steps using solid-phase extraction method were necessary. The  
74 coupling of high-performance liquid chromatography to MS using an internal standard (ApoC-III

75 deuterated on 3 alanine residues) allowed the evaluation of absolute abundances of all glycoforms  
76 [29] by performing targeted multiple-reaction monitoring detection.

77 Based on its automation, speed and ease of use, capillary electrophoresis (CE) is an alternative and  
78 quantitative technique that deserves to be considered for the analysis of glycoproteins. To the best of  
79 our knowledge, no CE method has yet been developed for the analysis of glycosylated ApoC-III in  
80 biological fluids. The main objective of the study was to implement a CZE method for the quantitative  
81 monitoring of ApoC-III glycoforms in plasma of CDG patients. In that aim, a well characterized and  
82 quantified standard solution of ApoC-III had to be defined. We therefore purchased ApoC-III (purified  
83 from human plasma) from different providers, but the first CZE analyses revealed a higher structural  
84 heterogeneity than expected (i.e. not limited to O-glycosylation). The present paper reports the  
85 numerous efforts devoted to the thorough characterization and separation of the various protein  
86 forms from three distinct batches of commercial Apo C-III obtained from different suppliers.

87

## 88 **2. Materials and methods**

### 89 *2.1 Reagents and consumables*

90 ApolipoproteinC-III (95%) provided as solution (1 mg mL<sup>-1</sup> in 10 mM ammonium bicarbonate pH 7.4)  
91 was obtained from Sigma Aldrich (St. Louis, MO, USA), from Merck (Darmstadt, Germany) and from  
92 Antibodies online GmbH (Aachen, Germany).

93 Acetonitrile, boric acid (99.5%), 1,4 diaminobutane (DAB, 99%), dimethyl sulfoxide (DMSO, 99%),  
94 neuraminidase type VIII from Clostridium perfringens (85%), sinapinic acid (99%), sodium dodecyl  
95 sulfate (SDS, 98.5%), trifluoroacetic acid (TFA), trypsin from bovine pancreas (TPCK treated) and urea  
96 (99%) were all obtained from Sigma Aldrich (St. Louis, MO, USA). Sodium di-phosphate monobasic  
97 (NaH<sub>2</sub>PO<sub>4</sub>, 99.1%) and di-sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>, 99.8%) were purchased from Thermo  
98 Fisher scientific (Waltham, MA, USA). Sodium hydroxide (1 M), hydrochloric (1 M) and acetic (99.9%)  
99 acids were obtained from VWR (Fontenay-sous-Bois, France).

100 All buffers were prepared with deionized water and were filtered through a 0.22  $\mu\text{m}$  nylon membrane  
101 before use from VWR (Fontenay-sous-Bois, France). Deionized water was prepared with a Direct-QR 3  
102 Water Purification System from Millipore (Milford, USA).

103 Bare fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA),  
104 polyvinylalcohol (PVA) and polyacrylamide (PAA) coated capillaries from Agilent Technologies (Santa  
105 Clara, CA, USA) and Sciex (Framingham, MA, USA), respectively.

### 106 *2.3 Neuraminidase digestion*

107 Neuraminidase digestion of ApoCIII was performed to remove  $\alpha$  2-3,  $\alpha$  2-6 and  $\alpha$  2-8 linked sialic acids  
108 as follows. Firstly, lyophilized neuraminidase was dissolved in 300  $\mu\text{L}$  of 50 mM sodium phosphate  
109 buffer pH 6.0. Then 20 $\mu\text{L}$  of the standard ApoC-III at 1 mg mL<sup>-1</sup> in the sample buffer (10 mM ammonium  
110 bicarbonate pH 7.4) were mixed with 2  $\mu\text{L}$  of the neuraminidase solution (0.066 units for 20  $\mu\text{g}$  of  
111 protein). The digestion was performed overnight at 37°C.

### 112 *2.4 Trypsin digestion*

113 ApoC-III was first 2-fold diluted in 100 mM ammonium bicarbonate pH 7.9 (50  $\mu\text{L}$  of ApoC-III at 1 mg  
114 mL<sup>-1</sup> mixed with 50  $\mu\text{L}$  of 100 mM ammonium bicarbonate). Trypsin digestion was performed upon  
115 the addition of 2  $\mu\text{L}$  of trypsin solution at 0.5 mg mL<sup>-1</sup> (enzyme:protein ratio of 1:50). The digestion was  
116 performed during 12 h at 37°C and was then stopped by the addition of 2  $\mu\text{L}$  of formic acid.

### 117 *2.5 MALDI-TOF MS analysis*

118 MALDI-TOF MS analysis of intact ApoC-III was performed essentially as described before [22,28], but  
119 with minor modifications. Briefly, analyses were performed on a Bruker Ultraflex extreme MALDI-  
120 TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam-II laser. Each  
121 ApoC-III sample (0.5  $\mu\text{L}$  at 1 mg mL<sup>-1</sup>) was spotted on the MALDI target and thoroughly mixed on-target  
122 with 0.5  $\mu\text{L}$  of matrix composed of a saturated solution of sinapinic acid in 50:50:0.1, Water/ACN/TFA.  
123 MS spectra of intact ApoC-III were acquired at 2 kHz laser repetition rate in the positive linear ion

124 mode, with a 20 kV acceleration voltage and an extraction delay of 250 ns. All spectra were obtained  
125 by accumulating ~1000 laser shots over the 5000–20000 m/z range.

126 MALDI-TOF MS of tryptic peptides were acquired, using  $\alpha$ -cyano-4-hydroxycinnamic acid as matrix (10  
127 mg/mL in 50% ACN containing 0.1% TFA), at 2 kHz laser repetition rate in the positive reflectron ion  
128 mode, with a 20 kV acceleration voltage and an extraction delay of 130 ns. All spectra were obtained  
129 by accumulating ~1000 laser shots over the 500–5000 m/z range. Confirmation of peptide structures  
130 can be obtained from MS/MS spectra acquired in LIFT mode, at 1 kHz laser repetition rate applying 7.5  
131 kV for initial acceleration of ions and 29.5 kV for reacceleration of fragments in the LIFT cell.

## 132 *2.6 Capillary electrophoresis analysis*

133 CE experiments were carried out with a P/ACE™ MDQ instrument (Sciex, Framingham, MA, USA)  
134 equipped with a diode array detector. UV detection was performed at 214 nm. Bare fused silica  
135 capillaries with an internal diameter of 50  $\mu$ m and an effective length to the detector of 50 cm (total  
136 length of 60.2 cm) were used. Software 32 Karat™ version 7.0 (Beckman Coulter, High Wycombe, UK),  
137 was used to control the instrument and to collect data. Fused silica capillaries were preconditioned by  
138 flushing them at 20 psi with MeOH for 10min, H<sub>2</sub>O for 10 min, 1M HCl for 10 min, H<sub>2</sub>O for 10 min, 1 M  
139 NaOH for 10 min, H<sub>2</sub>O for 10 min and background electrolyte (BGE) for 10 min. Then, the capillary was  
140 filled with 0.1 M NaOH and stored during 3 h. After this storage, the capillary was rinsed and  
141 equilibrated with the BGE (150 mM sodium borate buffer, 4M Urea, 1mM DAB pH 9.5) for 10 min and  
142 then 20 kV was applied during 3 h. A plug of sample was hydrodynamically injected from the inlet end  
143 by applying a pressure of 0.5 psi for 6 s. The separation was carried out under 20 kV at 25°C. After each  
144 run, the capillary was rinsed for 5 min with 25mM SDS solution and 5 min with H<sub>2</sub>O, and equilibrated  
145 with the running buffer for 10 min. At the end of the day, the capillary was rinsed for 5 min with 25mM  
146 SDS and 10 min with H<sub>2</sub>O and dried with air before storage at room temperature.

## 147 *2.7 Fractionation of ApoC-III by CZE and control of fractions by MALDI-TOF MS*

148 A large plug of sample (5% of the capillary volume) was injected from the inlet end by applying a  
149 pressure of 1.5 psi for 17 seconds. A first separation step was carried out under 20 kV at 25°C for 15  
150 min. Then the outlet BGE vial was removed and replaced by a vial containing 5  $\mu$ L of the sample buffer.  
151 A second separation step was then performed under the same conditions as the first one but during  
152 0.5 min for the collection of the peak of interest.

153

### 154 **3. Results and discussion**

155 The objective of this work was to develop a CZE method for separating and quantifying the different  
156 ApoC-III glycoforms. Method implementation and optimization were first conducted with  
157 commercially available plasma-derived ApoC-III. Thus, we paid particular attention to limit protein  
158 adsorption to the capillary and also to electrophoretically resolve glycoforms differing from each other  
159 by only one sialic acid residue. Classical parameters such as the pH, the ionic strength of the BGE, the  
160 nature of the capillary coating or the addition of additives to the running buffer were thoroughly  
161 examined. Also, rinsing and preconditioning procedures were carefully optimized to improve the  
162 method robustness.

#### 163 *3.1 Optimization of the CZE method for the ApoC-III glycoform separation*

##### 164 *3.1.1 Optimization of the running buffer*

165 As proteins are prone to adsorption on fused silica capillary and in order to develop a method  
166 compatible with mass-spectrometry detection, we have chosen first to work with permanent capillary  
167 coatings. We tested two commercial neutral coatings, PVA and PAA. As the isoelectric point ( $pI$ ) of  
168 ApoC-III is comprised between 4.5 and 5.0, BGEs having pHs above the  $pI$  (above pH 5) were  
169 investigated. At pH 5.0, no peak was detected with both coatings after 60 min under reverse polarity.  
170 Indeed, at pH 5.0 close to its  $pI$ , ApoC-III is globally neutral so its electrophoretic mobility may be too  
171 low to enable its migration to the detection window. At pH 7.0 peaks were observed within 20 min and



172 30 min for PAA and PVA, respectively. Although the electrophoretic resolution was sufficient ( $R_s \geq 1.68$ )  
173 in both cases, peaks were very broad (around 2 min) precluding convenient resolution improvements.  
174 The too low electroosmotic and electrophoretic mobilities led to high migration times even at higher  
175 pH.

176 We then decided to work without coatings but using repelling conditions ( $\text{pH} > \text{pI}$ ) in bare fused silica  
177 capillary and therefore under counter EOF mode. Under this configuration, high resolution is expected  
178 if the electrophoretic mobility ( $\mu_{\text{ep}}$ ) of the analytes is closed to that of the electroosmotic flow ( $\mu_{\text{eo}}$ ).  
179 We optimized the pH (from 7.0 to 10), the ionic strength (from 50 to 150mM) and the composition  
180 (sodium phosphate, sodium borate and sodium tetraborate) of BGE. Using sodium borate buffer, as  
181 soon as the ionic strength was increased, a splitting of each peak into two peaks was observed. The  
182 beneficial effect of borate can be explained by a selective complexation between borate and cis  
183 hydroxyl groups of the glycan moieties [30]. The best separation conditions were obtained with 150  
184 mM sodium borate buffer pH 9.5. Surprisingly, we observed six distinct peaks gathered into three pairs  
185 of peaks for the commercial ApoC-III sample (Fig 1A), while only three corresponding to the different  
186 glycoforms were expected. Many efforts were made first to separate those species as much as possible  
187 and then to better understand the origin and nature of these peaks (*vide infra*).

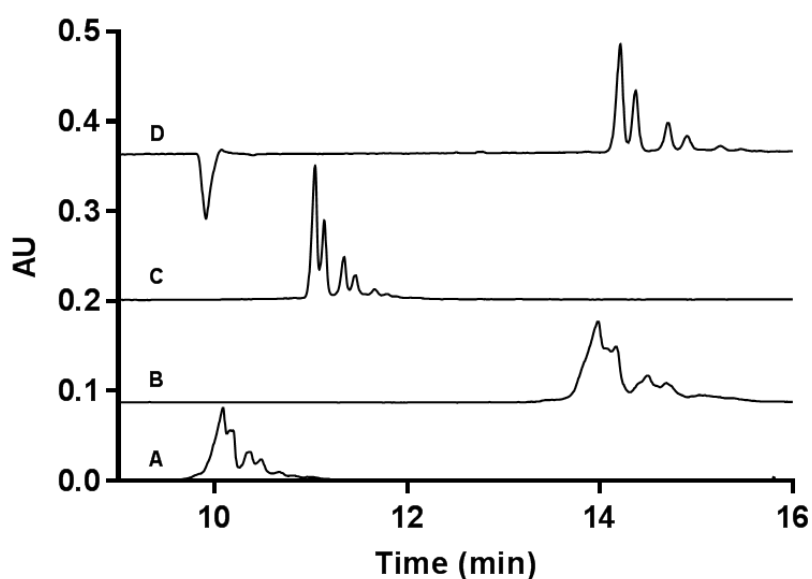
188 To increase the electrophoretic resolution, different types of additives were added to the running  
189 buffer. Firstly, the addition of diaminobutane was tested at different concentrations (from 1 mM to 10  
190 mM). It appeared that 1 mM DAB allowed increasing the resolution between each group of peaks (Fig  
191 1B) while the resolution between the first two peaks was still bad ( $R_s = 0.5$ ). DAB has been reported to  
192 prevent protein adsorption, by interacting with the negatively charged silanols groups that leads also  
193 to a decrease of the  $\mu_{\text{eo}}$ . This resolution improvement was obtained thanks to the reduced  $\mu_{\text{eo}}$   
194 intensity becoming closer to the  $\mu_{\text{ep}}$  of ApoC-III glycoforms. However, this induced an increase of  
195 migration times and peak width. Concentrations over 3 mM DAB were detrimental to the resolution  
196 due to a too low  $\mu_{\text{eo}}$ .

197 Secondly, urea was added to the separation buffer at different concentrations (from 2 M to 6 M).

198 Sharper peaks and a much higher resolution within each group of peaks and also between the first two  
199 peaks ( $R_s = 0.8$ ) were observed (Fig 1C) with 4 M of urea. Urea is a chaotropic agent that disrupts  
200 hydrogen bonds, resulting in protein unfolding which yields higher differences between charge-to-  
201 hydrodynamic volume ratios of the coexisting glycoforms in the case of ApoCIII. No noticeable effect  
202 on the resolution was observed using concentration above 4 M.

203 The profile obtained with a borate buffer pH 9.5 containing 1 mM DAB and 4 M urea showed the  
204 synergic effect of both additives with three well resolved pairs of peaks (Fig 1D). Moreover, the  
205 resolution between the first two peaks was greatly improved ( $R_s = 1.3$ ). This condition was selected  
206 for all further experiments with the ApoC-III glycoforms.

207 Finally, modifications of other parameters such as capillary length or voltage were also investigated  
208 but did not lead to significant improvements (data not shown).



209  
210 **Fig. 1.** Comparison of CZE profiles obtained for ApoC-III without additive in the BGE (A), and  
211 using different additives in the BGE as 1 mM DAB (B), 4 M urea (C) or a combination of 1 mM Dab and  
212 4 M urea (D)  
213 Sample:  $1 \text{ mg mL}^{-1}$  ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate pH  
214 9.5  
215

### 216 3.1.2 Optimization of the rinsing and preconditioning procedures

217 First experiments were performed with a classical inter-run 0.1M NaOH rinsing step and demonstrated  
218 excellent intra-day repeatability of migration times ( $T_m$ ) ( $RSD < 0.5\%$ ) but a less acceptable one for the

219 relative peak areas (rA) (RSD around 30%). This lack of repeatability was probably due to some residual  
 220 adsorption of ApoC-III onto the capillary wall. With the aim to improve the repeatability, different  
 221 rinsing procedures involving more stringent conditions were compared. It appeared that among all  
 222 tested procedures, the rinsing with 25mM SDS allowed to achieve the most satisfactory RSDs (< 1.3%  
 223 for T<sub>m</sub> and 12% for rA) (Table 1).

224

225 Table 1: Impact of the inter run rinsing and preconditioning procedures on the repeatability of the  
 226 method (n=3).

Capillary Preconditioning procedure	Inter run Rinsing procedures	RSD (%) of migration times	RSD (%) of relative peak areas
Classical*	0.1 M NaOH	0.3-0.5	27-38
Classical	25 mM SDS	1.2-1.3	3.2-11.7
Classical	25mM SDS-0.1M NaOH	4.5-7.0	19 -27
Classical + voltage**	25 mM SDS	1.4-3.0	9.3-10.7
Classical + storage 0.1M NaOH*** + voltage	25 mM SDS	0.26-0.32	0.3-3.2

227 Sample: 1 mg mL<sup>-1</sup> ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1  
 228 mM DAB, 4 M urea pH 9.5.

229 \*Classical preconditioning: MeOH (10 min), H<sub>2</sub>O (10 min), 1 M HCl (10 min), H<sub>2</sub>O (10 min), 1 M NaOH  
 230 (10 min), H<sub>2</sub>O (10 min) and BGE (10 min).

231 \*\*Storage step: capillary stored with 0.1 M NaOH during 3h.

232 \*\*\*Voltage step: capillary filled with BGE and under 20 kV during 3h.

233

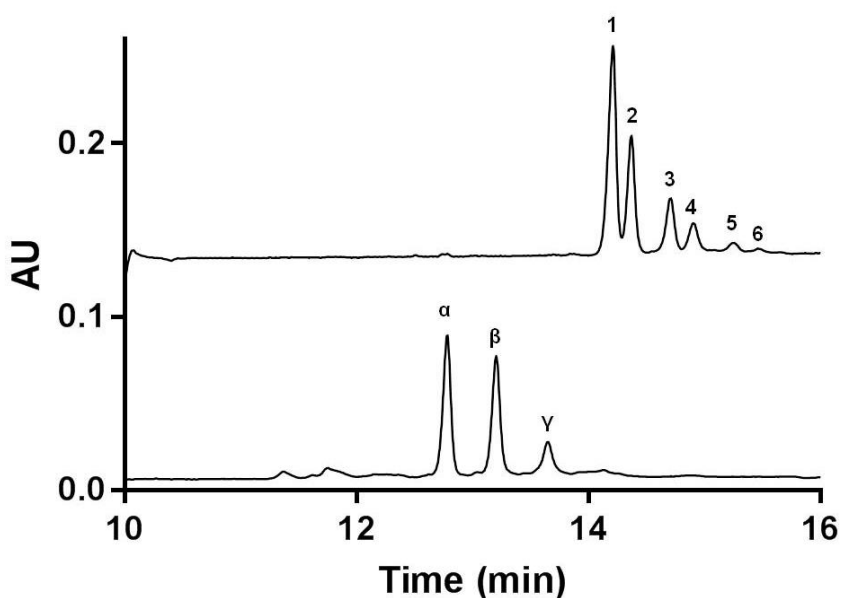
234

235 We also carefully optimized the preconditioning procedure. Each new capillary was initially  
 236 conditioned with a rather classical procedure involving different successive washing steps with  
 237 successively as described in the section 2.6. We suspected that the DAB layer was not enough stable  
 238 and homogenous to ensure sufficiently repeatable rA. Thereby, we investigated the addition of one  
 239 equilibration step with the running buffer under a 20 kV voltage application during 3 h after the  
 240 classical preconditioning. This step was expected to create a more homogenous DAB coating of the  
 241 capillary [32]. In our case, it slightly improved the repeatability of the rA (RSD < 10.7%), but at the  
 242 expense of the T<sub>m</sub> one (Table 1). We then tried to include, between the common preconditioning and

243 the equilibration step under a 20 kV voltage, another additional equilibration step under storage in 0.1  
244 M NaOH during 3 h. The combination of these two additional steps led to an excellent repeatability for  
245 both T<sub>m</sub> (RSD < 0.4%) and rA (RSD < 3.3%), rendering the method robust and reproducible.  
246 Finally, the inter-day (n=3) repeatability of the method was evaluated. An excellent intermediate  
247 precision was obtained for T<sub>m</sub> (RSD < 0.7%) and a satisfactory one for rA (RSD < 5%).

### 248 3.2 Peak identification

249 With the final optimized conditions, the CZE glycoform pattern of ApoC-III isolated from plasma,  
250 unexpectedly showed three pairs of peaks (Fig 2A) instead of three expected single peaks.



251  
252 **Fig. 2.** CZE profiles of ApoC-III before (A) and after (B) enzymatic desialylation with neuraminidase.  
253 Sample: 1 mg mL<sup>-1</sup> ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1  
254 mM DAB, 4 M urea pH 9.5.  
255

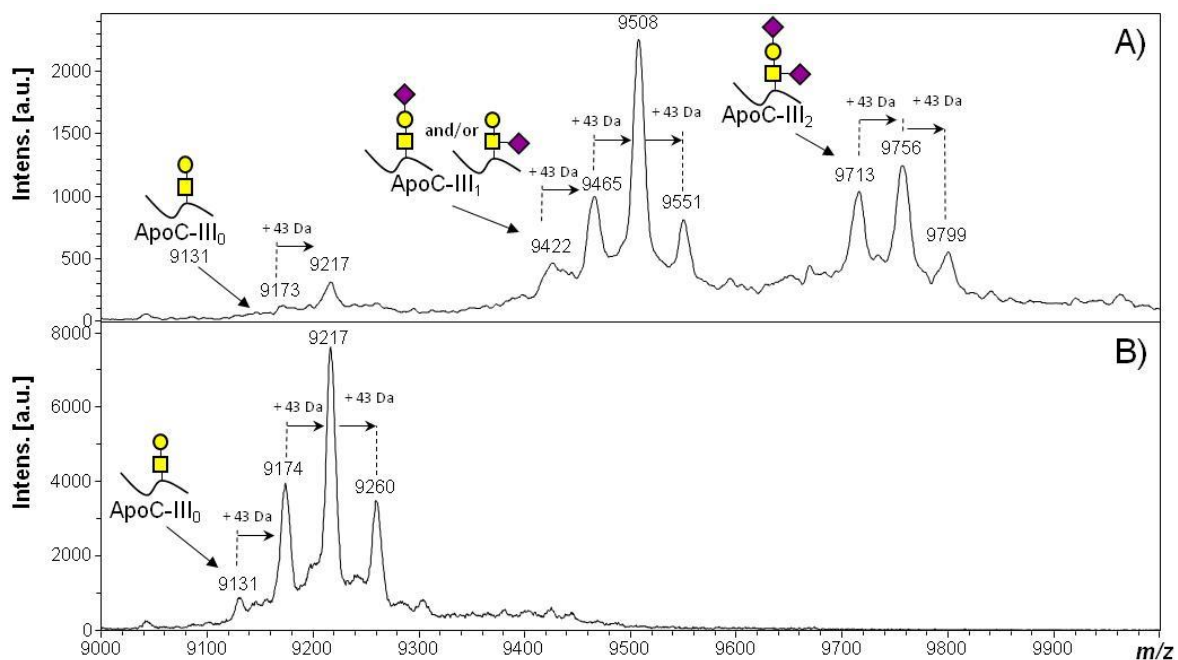
256 In order to clarify the identity of these different species, the ApoC-III sample was treated with  
257 neuraminidase to remove terminal sialic acids from the O-glycan chain (Fig 2B). Sialylated glycoforms  
258 exhibit more negative charges than their asialylated counterpart, leading to a decrease of their  
259 apparent mobility. Migration times of desialylated ApoC-III were consequently decreased. Upon  
260 neuraminidase treatment, we expected a single peak corresponding to the asialylated ApoC-III but  
261 instead we observed three distinct peaks, potentially highlighting the presence of additional post-  
262 translationally or chemically modified protein species. To investigate further the nature of this

263 modification, MALDI-TOF MS analysis of the intact and desialylated ApoC-III samples was performed.  
264 Under these conditions, three main clusters of peaks differing by 43 Da mass increments were  
265 observed for the intact ApoC-III sample and corresponded to various ApoC-III glycoforms (Fig 3A).  
266 Those ApoC-III<sub>0</sub> (m/z 9131), ApoC-III<sub>1</sub> (m/z 9422) and ApoC-III<sub>2</sub> (m/z 9713) glycoforms bear 0, 1 and 2  
267 sialic acid residues, respectively. For example, the most intense peak (m/z 9508) in Fig. 3A corresponds  
268 to the monosialylated ApoC-III with two 43Da mass increments. Such mass differences are consistent  
269 with the presence of several carbamyl groups on ApoC-III. Carbamylation is an in vitro nonenzymatic  
270 chemical modification of peptidic chains where an isocyanate group is covalently linked to the ε-amino  
271 groups of lysine residues and to the N-terminal residues of proteins/peptides [33]. This modification  
272 can be artificially introduced during sample preparation with urea (through urea degradation) [34]  
273 mimicking in vivo carbamylation. In aqueous solutions, urea can readily dissociate upon heating or  
274 after prolonged incubation or storage to form isocyanic acid and urea. Undesired protein  
275 carbamylation can hamper the process of protein analysis, especially the tryptic digestion step since  
276 carbamylated lysine will not be cleaved anymore by trypsin. Of note and as previously described by  
277 others [22,26], the ApoC-III<sub>0</sub> glycoform (m/z 9131 and related species observed in the MALDI mass  
278 spectrum might be viewed as artifactually formed by sialic acid loss, phenomenon particularly favored  
279 at high laser power. This asialylo-glycoform is almost never detected in 2D electrophoresis, and is  
280 therefore probably present at only very low levels *in-vivo* [26]. The artefactual loss of sialic acid  
281 residues observed under our MALDI conditions might also explain why the relative abundances of  
282 species bearing one or two 43 Da mass increments slightly fluctuate between mono- and di-sialylated  
283 protein species (Fig. 3A). Another potential explanation would be that the di-sialylated ApoC-III can be  
284 carbamylated to a lesser extent due to the additional steric hindrance resulting from the presence of  
285 a second sialic acid residue. Also, it might be linked to an artefactual loss of carbamyl moiety under  
286 our MALDI conditions, since such modification also seem quite labile and is rapidly lost under MS/MS  
287 conditions (*vide infra*).

288 After neuraminidase treatment, the MALDI-TOF MS spectrum (Fig 3B) showed a cluster of four peaks  
 289 corresponding presumably to the asialo-form ApoC-III<sub>0</sub> (m/z 9131) still differing by successive mass  
 290 increments of 43 Da.

291 In order to confirm the occurrence of carbamylation and identify the modified amino acid residues, a  
 292 tryptic digestion of the intact ApoC-III sample was performed and the resulting peptides analyzed by  
 293 MALDI-TOF/TOF. From these results, we deduced that the modification preferentially occurred on the  
 294 N-terminus of ApoC-III and on Lys51, and to a lesser extent on Lys17 or Lys24. Fig. S1 shows the MALDI-  
 295 TOF/TOF spectra of the two mostly (as judged from MALDI-TOF mass spectra) carbamylated peptides  
 296 (N-terminus and Lys51). As already mentioned above, such modification seems to be quite labile and  
 297 is rapidly lost under MS/MS conditions (Fig. S1).

298 Based on these results, the three peaks obtained in the CZE profile of the desialylated sample were  
 299 attributed to different carbamylated states (1C to 3C) of ApoC-III<sub>0</sub> glycoform.



300 **Fig. 3.** MALDI-TOF MS profile of the intact ApoC-III (A) and desialylated ApoC-III sample (B). Sample: 1  
 301 mg mL<sup>-1</sup> ApoC-III in 10 mM ammonium bicarbonate pH 7.4  
 302

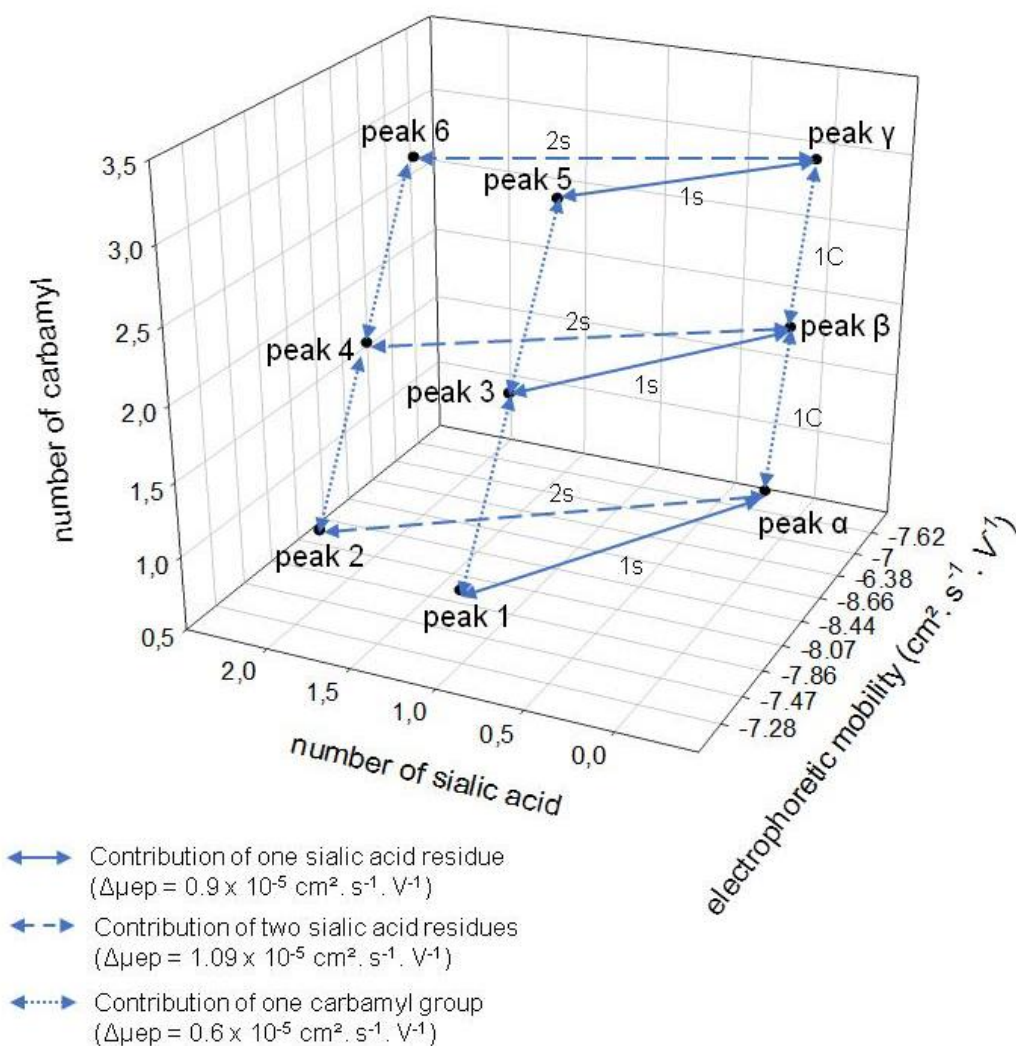
303 Altogether, these data suggest that the six peaks resolved by CZE probably correspond to a mixture of  
 304 carbamylated and sialylated forms of ApoC-III. We hypothesize that the three groups of doublet peaks

305 corresponded to three carbamylation levels whereas the two peaks composing each pair were  
306 consistent with the presence of one or two sialic acid residues on ApoC-III.

307 As mentioned above, carbamylation corresponds to the addition of an isocyanate moiety to a primary  
308 amino group of a peptidic chain, and can thereby result in an increase of both hydrodynamic volume  
309 and the overall negative charge of the protein of interest (e.g. by neutralizing the positive charge  
310 carried by lysine residues). This modification might result in a decrease of glycoform electrophoretic  
311 mobilities. Thus, glycoforms with low levels of carbamylation should migrate first while highly  
312 carbamylated species should present lower electrophoretic mobilities and appear later under our  
313 conditions. We suspected that such an artificial carbamylation could occur during the purification and  
314 extraction process of ApoC-III from human plasma. Complementary experiments proved that the high  
315 concentrations of urea present in the BGE did not induce any carbamylation of ApoC-III, which is also  
316 consistent with previous reports on 2D gel electrophoresis of carbamylated proteins [35].

317 In order to definitely assign peaks in the CZE profiles, electrophoretic mobilities of all peaks (named as  
318 1 to 6 for intact Apo-CIII and from  $\alpha$  to  $\gamma$  for desialylated ApoC-III) were plotted against the degree of  
319 carbamylation and sialylation of glycoforms in a 3-dimension graph (Fig 4). The differences of  
320 electrophoretic mobility, between two peaks 1/3, 3/5, 2/4, 4/6,  $\alpha/\beta$  and  $\beta/\gamma$  were constant ( $\approx 0.6 \times 10^{-5}$   
321  $\text{cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ ) and represented by the dotted arrows in the Fig 4. This means that this value corresponds  
322 to the  $\mu\text{ep}$  contribution of one carbamyl moiety. The CZE method developed here allowed therefore  
323 to separate glycoforms depending on their carbamylation degree. To the best of our knowledge, this  
324 work is the first one reporting an artefactual carbamylation of ApoC-III. Only *in-vivo* oxidation of the  
325 two methionine residues of ApoC-III was previously reported [36]. Some *in-vitro* carbamylated  
326 proteins, such as green fluorescent protein [37] or human serum-albumin (HSA) [38], have already  
327 been monitored by capillary IEF or CZE respectively. HSA carbamylation has been demonstrated  
328 responsible for the shift in the albumin peak symmetry which could be used to assess their  
329 carbamylation degree. However, quantification of carbamylated products could not be performed.

330 On the 3D graph, we can also observe that the  $\mu_{ep}$  differences between two peaks 1/ $\alpha$ , 3/ $\beta$  and 5/ $\gamma$  is  
 331 a constant value ( $\approx 0.9 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ ) as illustrated by the solid arrows in Fig 4). As  $\alpha$ ,  $\beta$  and  $\gamma$  present  
 332 no sialylation, it means that peaks 1, 3 and 5 have the same sialylation degree. The last difference of  
 333  $\mu_{ep}$  between two peaks 2/ $\alpha$ , 4/ $\beta$  and 6/ $\gamma$  is also constant ( $\approx 1.09 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ ) represented by the  
 334 dashed arrows in Fig 4), leading to the conclusion that all second peaks of each pair (peaks 2, 4 and 6)  
 335 have the same sialylation degree too. Finally, as the migration times are expected to increase with the  
 336 sialylation degree, the first peak of each pair was attributed to the monosialylated form whereas the  
 337 second to the disialylated glycoform.  
 338 Thus, the couple of peaks 1 and 2, 3 and 4, 5 and 6 gave after desialylation peaks  $\alpha$ ,  $\beta$  and  $\gamma$ ,  
 339 respectively.



340

341 **Fig. 4:** 3D plot of electrophoretic mobilities against the number of sialic acids and of carbamyl

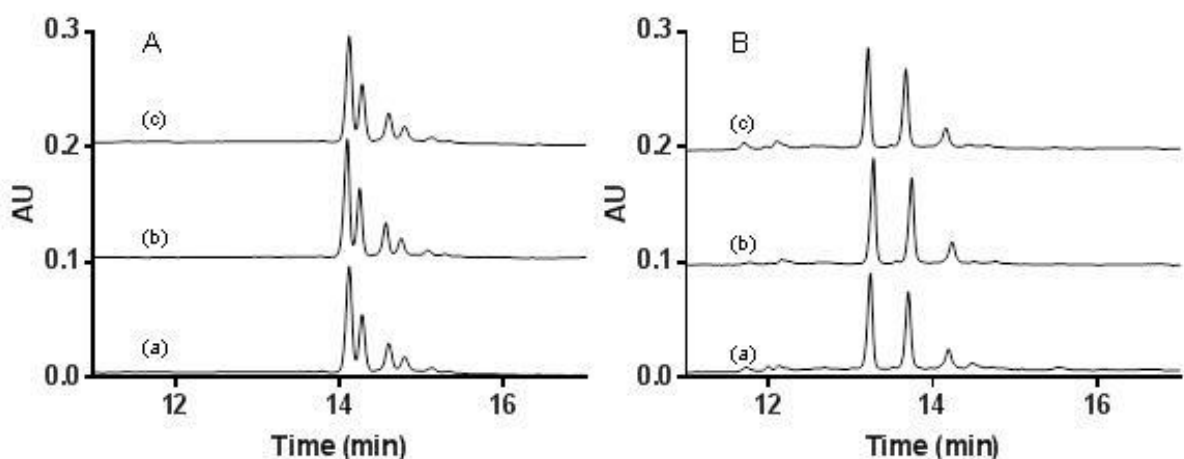


342 groups present on the glycoforms giving specific peaks in CZE.  
343 Sample: 1 mg mL<sup>-1</sup> ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1  
344 mM DAB, 4 M urea pH 9.5. The specie migrating under the peak 1 is assumed to bear one sialic acid  
345 residue and one carbamyl group and is called (1C, 1S); Peak 2 (1C, 2S); Peak 3 (2C, 1S); Peak 4 (2C, 2S);  
346 Peak 5 (3C, 1S); Peak 6 (3C, 2S); Peak  $\alpha$  (1C, 0S); Peak  $\beta$  (2C, 0S); Peak  $\gamma$  (3C, 0S)  
347

348 In a separate set of experiments, we collected some fractions from CZE runs and analyzed them by  
349 both CZE and MALDI-TOF MS. These complementary data tended to confirm our peak assignment (data  
350 not shown).

### 351 3.3 Comparison of ApoC-III batches from different suppliers

352 The optimized CZE method was then used to analyze different batches of human plasma-derived ApoC-  
353 III obtained from three different providers to see if the presence of carbamylated is a widespread  
354 phenomenon. All batches were analyzed both by MALDI-TOF MS and CZE (Fig 5A) to assess their  
355 proteoform profile, their purity being the same according to the suppliers (95%). It appeared that all  
356 commercial ApoC-III samples contained both carbamylated and sialylated forms of ApoC-III to a very  
357 similar extent. Indeed, based on the CZE profiles, all three samples were qualitatively and  
358 quantitatively homogenous, which suggests that a similar ApoC-III purification protocol was used by  
359 the different suppliers. Complementary analyses of samples obtained after desialylation further



360 confirm this observation (Fig. 5B). This absence of non-modified commercially available ApoC-III  
361 standard drastically hamper the development of a quantitative CZE method.

362 **Fig. 5:** Comparison of the three samples of intact ApoC-III before (A) and after (B)  
363 neuraminidase treatment; (a) sample from Merck, (b) sample from Antibodies online, (c) sample from  
364 Sigma Aldrich.  
365 Sample: 1 mg mL<sup>-1</sup> ApoC-III in 10 mM ammonium bicarbonate pH 7.4, BGE: 150 mM sodium borate, 1  
366 mM DAB, 4 M urea pH 9.5  
367

## 368 **Conclusion**

369 In this work, we developed for the first time a fast and accurate CZE method for the separation of intact  
370 ApoC-III glycoforms. The resolution of the separation was very satisfactory thanks to the use of an  
371 alkaline sodium borate buffer combined with the synergic effect of urea and DAB. CZE separation of  
372 sialylated ApoC-III glycoforms and their related carbamylated species proved successful. An  
373 optimization of the preconditioning and rinsing steps contributed to make the method robust. MALDI-  
374 TOF MS experiments combining with CZE of intact and neuraminidase-treated samples enabled to  
375 demonstrate for the first time the carbamylation of ApoC-III. The present method can also be used as  
376 a quality control tool for evaluating batch-to-batch consistency of plasma-derived as well as  
377 recombinantly expressed ApoC-III.

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