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Intact monoclonal antibodies separation and analysis by sheathless capillary electrophoresis-mass spectrometry
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11

12 ABSTRACT:

13

14 Capillary electrophoresis mass spectrometry coupling (CE-MS) is a growing technique
15 in biopharmaceutics characterization. Assessment of monoclonal antibodies (mAbs) is well
16 known at middle-up and bottom-up levels to obtain information about the sequence, post-
17 translational modifications (PTMs) and degradation products. Intact protein analysis is an
18 actual challenge to be closer to the real protein structure. At this level, actual techniques are
19 time consuming or cumbersome processes. In this work, a 20 minutes separation method has
20 been developed to optimize characterization of intact mAbs. Thus, separation have been done
21 on a positively-charged coated capillary (PEI) with optimized volatile background electrolyte
22 (BGE) and sample buffer (SB). A sheathless interface allowed to hyphenate CE to a quadrupole-
23 time-of-flight mass spectrometer (Q-TOF) which parameters has been tuned to improve the
24 high masses detection and identification of intact mAbs. Three world-wide health authorities
25 approved mAbs have been used to set up a rapid and ease of use method. Intact trastuzumab,
26 rituximab and palivizumab isoforms have been partially separated with this method in less
27 than 20 minutes under denaturing conditions. For each mAb, 2X-glycosylated and 1X-
28 glycosylated structures have been identified and separated. Concerning basic and acidic
29 variants potential Iso-Asp modification and Asn deamidation have been observed. Accurate
30 mass determination for high-mass molecular species remains a challenge, but the progress in
31 intact mAbs separation appears very promising for biopharmaceutics characterization.

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36 **Keywords:** Capillary Electrophoresis; Mass Spectrometry; Monoclonal Antibody; micro-variant
37 separation, glycoform separation

38

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41 **Introduction**

42 Monoclonal antibodies (mAbs) are tetrameric glycoproteins having a molecular mass of
43 approximately 150 kDa, composed of two heavy chains and two light chains, inter-linked by several
44 disulfide bonds, and having at least one conserved N-glycosylation site located in the Fc domain[1]. mAbs
45 were introduced for the treatment of various diseases in the late 1980 and they still represent the most
46 rapidly growing category of therapeutic molecules today[1-3]. There are more than seventy-five mAbs
47 approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).
48 mAb are particularly interesting because of their good therapeutic efficiency, favorable
49 pharmacokinetic (PK) and pharmacodynamics (PD), and relatively low side-effects[4].

50 Several separation-based methods both on liquid chromatography and electrophoresis are used for
51 antibody characterization and homogeneity assessment[5]. These orthogonal analytical methods aim
52 particularly to separate the antibody main isoform from micro-variants[6]. Micro-variants are commonly
53 observed when mAbs are analyzed by charge-based separation techniques such as isoelectric focusing
54 gel electrophoresis (IEF), capillary IEF (cIEF), imaged cIEF (icIEF), capillary zone electrophoresis (CZE)
55 and ion exchange chromatography (CEX/ AEX)[7]. Many of the modifications leading to the formation of
56 acidic and basic species have been identified as asparagines (Asn) deamidation, methionines oxidation,
57 aspartic acid isomerization (Iso-Asp), cyclization of glutamic acid or glycosylation. mAb heterogeneity
58 explains the difficulty of separation and identification of each isoform. In a theoretical way, millions of
59 possibilities are available for only one mAb with plenty of weak masses difference between
60 proteoforms. Most of these PTMs have been localized and characterized by different techniques as
61 liquid chromatography-tandem mass spectrometry (LC-MS/MS) or capillary electrophoresis-tandem
62 mass spectrometry coupling (CE-MS/MS)[5, 8-10]. LC-MS/MS and CE-MS/MS analysis, built on a classical
63 bottom-up proteomics strategy, allowed to get a lot of information about mAbs proteoforms and their
64 PTMs but workflows can induce some modifications or degradations of therapeutic proteins.

65 This last decade, separation of intact mAbs have been studied with several electrophoretic
66 techniques such as CZE, 2D-CE-MS and CZE-MS[11-14]. He *et al* were precursor in the field of intact mAbs
67 separation using CZE-UV. They developed a rapid method using ε-amino-caproic acid (EACA) and
68 triethylenetetramine (TETA) as BGE, and hydroxypropylmethyl cellulose (HPMC) as dynamic neutral
69 coating of the capillary[11, 15]. In 2015 Moritz *et al* validated this method by an inter-laboratory study
70 allowing to consider it as the reference method for charge heterogeneity assessment of mAbs[16]. Other
71 approach using UV detection was recently described by Goyon *et al* to set up a simpler CZE method to
72 separate more acidic and basic variants for a dozen of commercial mAbs[17]. However, the composition
73 of BGEs described in these studies are not compatible with ESI-MS detection due to high salt

concentration and the presence of detergents and polymers. Since 2017, Neusüβ's group developed an original 2D-CE-MS instrumentation allowing the separation of intact mAbs by CZE-CZE-MS and icIEF-CZE-MS[18, 19]. While the first CZE dimension enabling to use the same BGE condition as the reference CZE-UV method described by He *et al*[15], the second dimension allowed the MS characterization by the use of BGEs compatible with ESI-MS detection. Jooβ *et al* illustrated their method for the detailed MS characterization of mAbs charge variants[18]. They highlighted glycosylated and deglycosylated variants and potential deamidation products for an intact antibody. While this approach appears very promising, CZE-CZE-MS setup needed heavy instrumental development and the overall analysis time is rather long. More recently, Belov *et al* developed a CZE-ESI-MS method to characterize one unknown IgG1 mAb by both middle-down and intact levels. At the intact level, CZE-ESI-MS analysis were performed under denaturing conditions and using a non-commercial positive coating (M7C4I). Baseline separation of the 2X-glycosylatd, 1X-glycosylated, and aglycosylated populations were obtained in nearly 30 minutes[20].

In this report, we developed a 20 minutes CZE-ESI-MS method for the analysis of three well-known approved mAbs at the intact level. Experiments were performed with a commercial positively-charged capillary coating of polyethyleneImine (PEI) to avoid adsorption phenomenon. Acidic background electrolyte (BGE) and acidic methanol/water sample buffer have been optimized in order to obtain micro-variants separation of intact mAbs in less than 20 minutes. Three world-wide health authorities approved mAbs: rituximab (chIgG1, CHO), palivizumab (hzIgG1, SP2/0) and trastuzumab (hzIgG1, CHO), were selected for this study. Comparison with reference CZE-UV methods have been achieved allowing potential characterization of basic and acidic variant regions. Separation of charge variants arising from 2X-glycosylated, 1X-glycosylated pattern has been identified while potential Iso-aspartic acid isomerization (Iso-Asp) and asparagine deamidation have been observed as basic and acidic variants.

Experimental

Chemicals

Chemicals used were of analytical grade or high purity grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA). Water used to prepare buffers and sample solutions was obtained using an ELGA purelab UHQ PS water purification system (Bucks, UK). mAbs were obtained as European Union pharmaceutical-grade drug product from their respective manufacturers.

106 *Sample preparation*

107 For intact mAbs analysis; trastuzumab, rituximab and palivizumab were desalted to remove all residual
108 components of the storage solution. Samples were buffer exchanged with milliQ water three times on
109 Amicon centrifugal filters with a 10 kDa cut off (Merck, Darmstadt, Germany). Each centrifugation was
110 made at 14’000 g speed during 20 min to claw back 30 µL of mAbs at a concentration of 33.3 µM.
111 Samples were led to a final concentration in protein of 6.7 µM using the desired sample buffers.

112

113 *Capillary electrophoresis*

114 All of the CE experiments have been done on a CESI8000 capillary electrophoresis system from Sciex
115 Separation (Brea, CA, USA). Bare fused-silica capillaries (total length 100 cm; 30 µm i.d.) with a porous
116 end from Sciex Separation (Brea, CA, USA) were positively coated with a commercial
117 PolyEthyleneImine (PEI) coating following the protocol provided by Sciex Separation. A second capillary
118 (total length 80 cm; 50 µm i.d.) was used to complete the electric line of the separation system. Before
119 each analysis, both capillaries were rinsed at 75 psi during 3 min with 3% acetic acid BGE. The 32
120 Karat^{TM} (Sciex Separation) software was used for instrument control and data acquisition.
121 Hydrodynamic injection (2 psi for 10 sec) corresponding to a volume of 3 nL (0.5% of the capillary
122 length) was used to inject the sample.

123

124 *Mass spectrometry*

125 The CE system was hyphenated to a maXis 4G (Bruker, Bremen, Germany) by the sheathless interface.
126 This MS instrument is equipped with a hybrid analyzer composed of hexapoles followed by a time-of-
127 flight (TOF) analyzer. Sample were run in denaturating conditions and analyzed in a m/z range from
128 2500 to 5000. The Otof control 3.4 software allowed to pilot the nano-ESI source and the settings were
129 the following ones: nanoESI voltage +1500 V, dry gas 3 L/min, ion funnels set at values of 400 and 400
130 Vpp, isCID energy at 190 eV and source temperature at 150°C. The data acquisition was made in
131 positive mode.

132

133 *Data analysis*

134 MS data have been analyzed with the dissect mode of Data Analysis 4.2 software (Bruker, Bremen,
135 Germany) with an internal S/N threshold of 3 and a maximum of 10 overlapping compounds. Cut-off
136 intensity of mass spectrum calculation was set at 0.1 %. After an automatic interpretation of the

137 results, a manual validation has been performed on the results. MS spectra have been extracted from
138 each peak represented on the BPE (Base Peak Electropherogram). Each profile has been selected and
139 deconvoluted between 140 and 160 KDa with the maximum entropy algorithm provided by Bruker's
140 software to calculate the mass of the corresponding compounds. Automatic and manual results have
141 been confronted to detect any mass modifications and get some additional information about the
142 different identified compounds.

143

144 **Results and discussion**

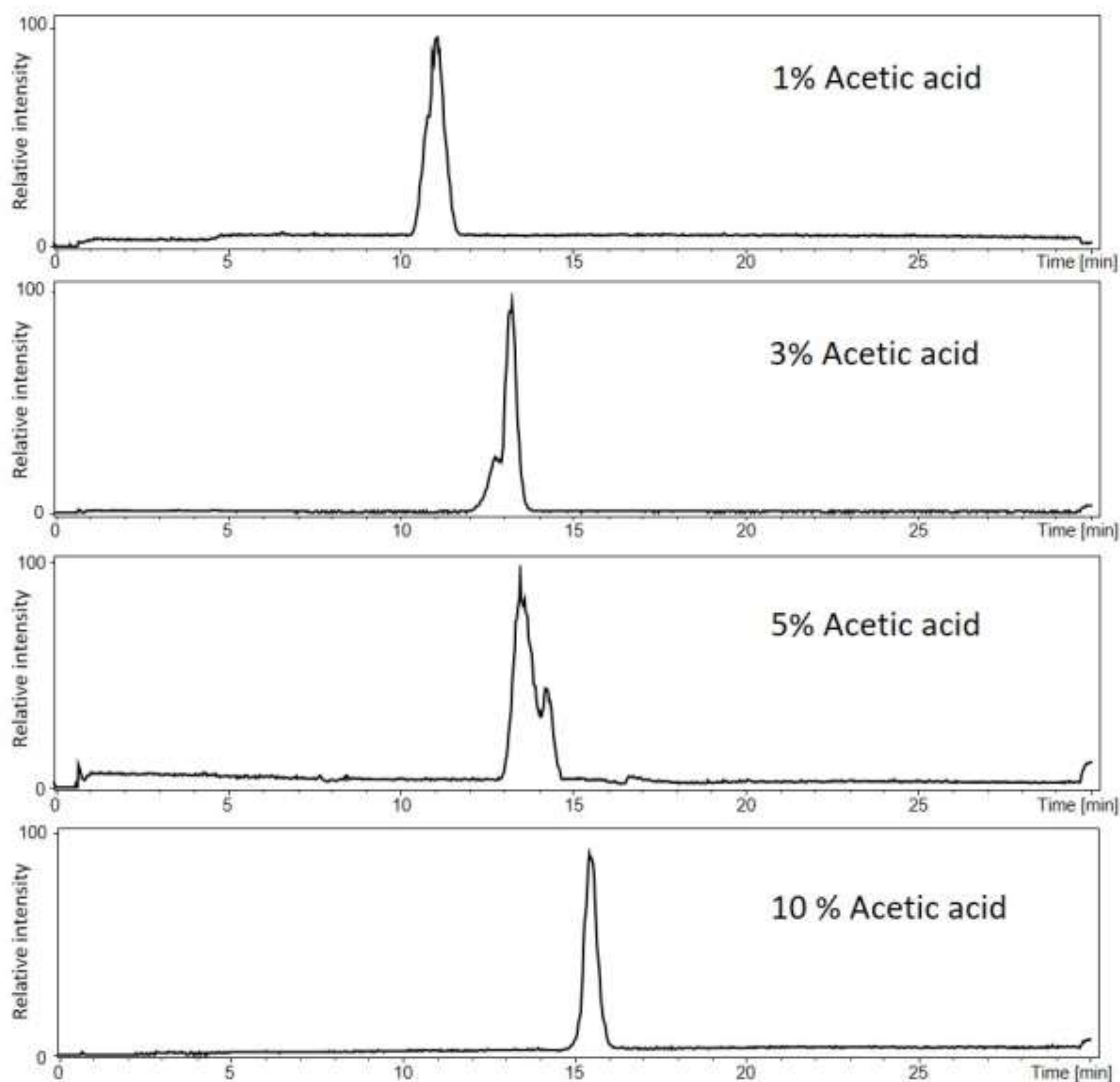
145 In this work, major separation parameters have been selected and optimized based on the
146 agreement of intact mAbs analysis and limits of CE-MS coupling. To avoid protein adsorption on the
147 inner surface of the capillary due to negative charge surface of the silanol groups, bare fused silica
148 capillaries have been previously modified with a covalent coating of PEI which confers positive charge
149 surface of the inner capillary wall and then involves a reverse electro-osmotic flow under an electric
150 field. Online CZE-ESI-MS coupling excluding the use of non-volatile salts[11], effects of volatile
151 background electrolyte (BGE), sample buffer and injection volume were optimized on the separation
152 of intact trastuzumab. This mAb can be considered as reference material for CZE-ESI-MS method
153 development due to the large number of reports describing the physicochemical properties of the
154 protein[7].

155

156 *Evaluation of BGE and sample buffer component effect on method development*

157

158 The most frequently used BGEs in CZE-ESI-MS under denaturing conditions are acetic acid and formic
159 acid because of their conductivity and their volatility allowing a good compatibility with ESI-MS
160 detection[20-23]. In our study, BGEs consisted in acetic acid and formic acid at different concentrations
161 from 1 to 10 % (v/v) and 1 to 5 % (v/v) respectively, were evaluated to keep a current intensity
162 compatible with the separation and to avoid any degradation of capillaries. No separation has been
163 observed with formic acid BGEs in the tested conditions. Concerning acetic acid, increasing
164 concentration from 1 to 10 % induced a time shift for the compounds migration (Figure 1) due to the
165 increase of ionic strength. 1% and 10% showed only one peak meaning that no separation of mAbs
166 isoforms can be observed. However, 3 and 5 % acetic acid gave partial separation of mAbs with poor
167 resolution. Indeed, obtained peaks were not thin and resolved and deconvolution spectra showed
168 partial overlapping of different isoforms. However, 3 % acetic acid BGE gave the most encouraging
169 separation of intact mAbs and was conserved to the next optimization step.



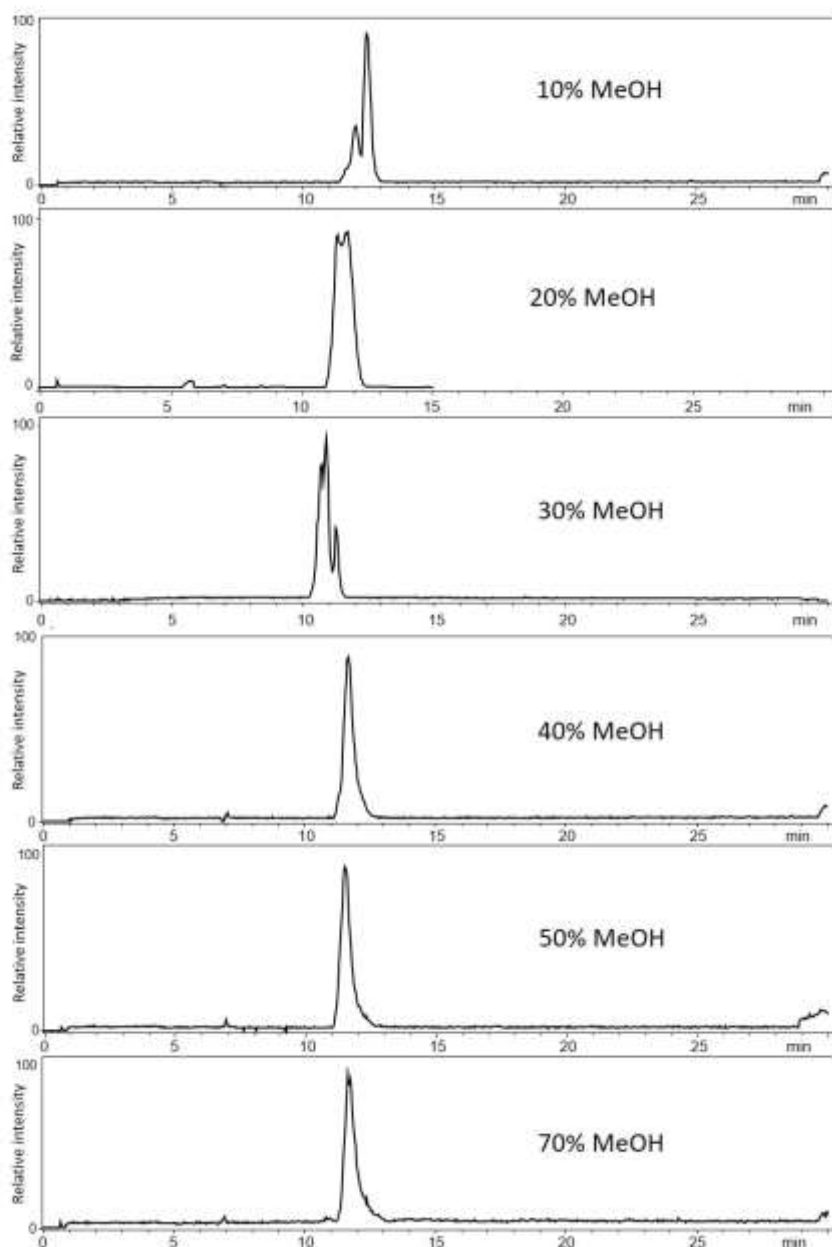
170

171 Figure 1. Effect of acetic acid concentration on the separation of trastuzumab charge variants. Acetic
172 acid proportion was set up at 1%, 3%, 5% and 10%.

173

174 Sample buffer is a parameter which can increase the performance of the separation, for instance due
175 to difference of conductivity with BGE. The first part of this optimization step consisted in testing
176 purified water, acetic acid and formic acid at different concentrations (1 to 10 % (v/v) and 1 to 5 %
177 (v/v) respectively) as sample buffer. Obtained results with purified water and acetic acid showed no
178 increase of resolution in mAbs separation. Only formic acid gave better resolution partly due to the
179 sharpness of the peaks, however, no tremendous increase of separation has been observed.
180 Nevertheless, 1% formic acid has been selected for the following steps. Based on the work of Schwer
181 *et al* who have calculated the influence on the electroosmotic velocity of adding organic solvent to the
182 electrolytes[24], we assessed the addition of methanol at different ratio (10 to 50% (v/v)) in the 1% formic

183 acid sample buffer (Figure 2). Up to 30 % Methanol, a partial separation was obtained while a total loss
184 of resolution was observed for ratio above 30%. Trastuzumab separation performed with 30 %
185 methanol, 1% formic acid sample buffer exhibited three peaks obtained in less than 15 minutes (RSD
186 < 3% on migration times (n=10)). Other organic solvents have been investigated as acetonitrile and
187 isopropanol in the same proportions, however only methanol has given some good results. Sample
188 injection volume has also been studied to subvert a capillary overloading known to affect the
189 separation of compounds. Trials were done from 1 to 20 nL corresponding to 7 to 350 fmol of mAbs.
190 Better results were obtained by injecting 3 nL (20 fmol) of trastuzumab in the PEI coated capillary.



191

192 Figure 2. Effect of methanol concentration in the sample buffer for the separation of trastuzumab
193 charge variants. Methanol proportion was set up at 10%, 20%, 30%, 40%, 50% and 70% on 1% formic
194 acid (v/v). The BGE contained 3% acetic acid.

195

196 *CZE-ESI-MS characterization of intact mAbs*

197 Since 2010, a some reports described CZE-based methods for the characterization of mAbs
198 charges variants for the quality control of biopharmaceutical companies in terms of product
199 heterogeneity[11-14, 18]. To date, two reports from Ruesch's group represents a reference for the analysis
200 of mAbs using CZE with a UV detection [11, 15]. Nevertheless, no structural information can be detailed
201 for the basic and/or acidic variants. In our study, optimized CZE-ESI-MS condition developed on
202 trastuzumab sample has been assessed on two other well-known IgG1 mAbs: rituximab and
203 palivizumab. Obtained results for these three mAbs are showed in Figure 3. Due to the reverse
204 electroosmotic mode involved by the use of PEI coated capillary, electropherograms of each mAbs
205 exhibited three peaks distributed as acidic variants in the first peak, the main variant in the second
206 peak and basic variants in the third peak. Despite the worst resolution obtained in CZE-ESI-MS as
207 compared to reference CZE-UV method which can be easily understood by the difference of BGE
208 condition (pH 5.7, presence of TETA…) and the inverted profiles of the three regions due to reverse
209 mode, obtained CZE-ESI-MS electropherograms fitted well with reference CZE-UV electropherograms.
210 Each mAbs following quite well similar behavior in appearance, trastuzumab has been chosen to
211 illustrate globally the obtained MS results. However, differences will be detailed for rituximab and
212 palituzumab. For each peak, deconvoluted mass spectra exhibited the classical glycoform pattern of
213 trastuzumab (Figure 4). Focused on the three highest abundance glycoforms of the main peak, average
214 masses of 148,057 ± 3 Da, 148,218 ± 2 Da, and 148,380 ± 3 Da were measured corresponding to
215 G0F/G0F, G0F/G1F, and G1F/G1F glycoforms respectively. Mass delta of around 162 Da between the
216 three glycoforms agreed with the theoretical mass of a galactose moiety. However, glycan structure
217 with the addition of galactose moieties does not induce a change in net charge[25-27]. Gahoual and co-
218 workers demonstrated that particular glycopeptides having a difference of one galactose could be
219 baseline separated[8] whereas Redman *et al* did not observe mobility shifts between intact mAb
220 glycoforms due to the low impact of 162 Da on the global mass of the mAb (≈0.1%)[23]. Our work follows
221 Redman *et al* observations with no separation of intact mAb variants due to differences of 2X-
222 glycosylated forms. However, in each cases, manual analysis of raw data enabled to characterize 1X-
223 glycosylated forms overlapping with the last peak meaning that separation between 2X-glycosylated
224 and 1X-glyxosylated forms are obtained for the three mAbs. This confirms the results recently
225 described by Belov *et al* on an unknown mAbs[20].

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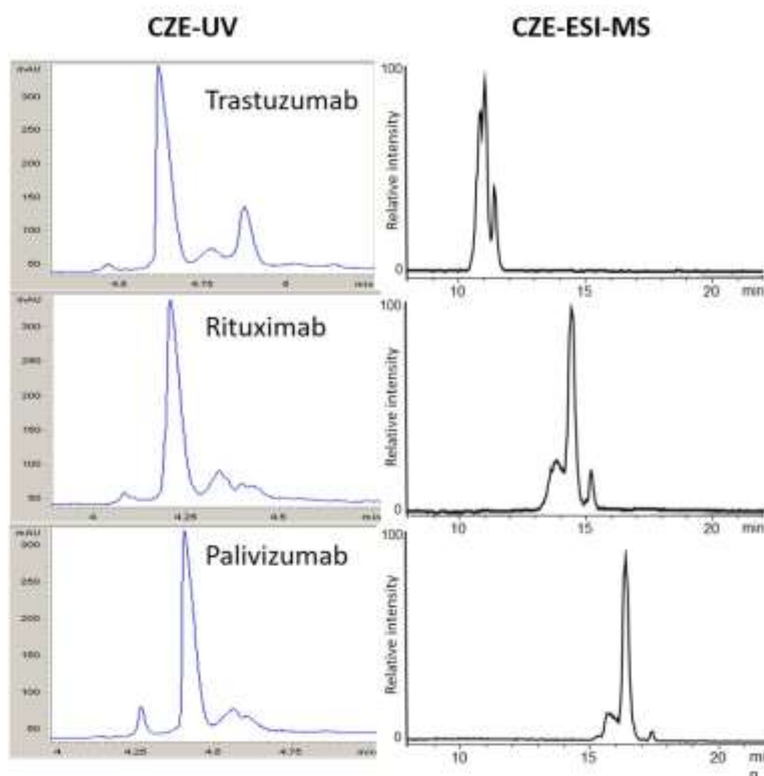

Figure 3. CZE-ESI-MS separation of intact tratuzumab, rituximab and palivizumab obtained with the optimized settings and CZE-UV profiles according to the methodology of He *et al* [15]. Reprinted from [17]. Copyright (2018), Wiley.

For each mAb, peaks corresponding to possible acidic or basic variants have been deconvoluted. Concerning basic variant, a mass difference of -1 to -2 Da compared with the main variant was observed for the three mAbs (148,217 ± 4 Da as compared to 148,218 ± 3 Da for trastuzumab), while a mass difference of -2 Da was observed only for trastuzumab (148,216 ± 4 Da) for acidic variant. As already described in the literature, the most important antibody basic charge variants are iso-Asp modification, C-terminal Lys truncation, aglycosylation, incomplete cyclization of the N-terminal glutamine (Gln) to pyroGlu or methionine oxidation; whereas sialylation, asparagine deamidation, glycation, cysteinylation are the most commonly observed acidic variants[28]. Trastuzumab possesses several Asp and Asn residues in its amino acid sequence which can be potentially iso-Asp or deamidated[10, 29]. Even if the standard deviation of mass measurement less than 4 Da doesn't allow to conclude without any ambiguity on the exact nature of the modification, we can explain the mass shift of less than 2 Da by potential iso-Asp modification for basic charge variants and potential Asn deamidation for acidic charge variants. These results are in agreement with the literature obtained for

247 the characterization of trastuzumab in a 2D-CZE-MS coupling[18]. However, while rituximab and
248 palivizumab also possess potential modified Asn, no acidic variant characterization has been observed
249 using our CZE-ESI-MS condition. Indeed, for rituximab, third peak deconvoluted mass spectrum gives
250 any masses corresponding to intact mAbs or degradation products but an unknown impurity, and for
251 palivizumab, deconvoluted mass spectra fitted with 1X-glycoform pattern. These results can
252 potentially ask the real nature of acidic variants found by the CZE-UV reference methods for these two
253 mAbs and highlights the constant need of method development with MS detection to obtained
254 structural characterization.

255

256 **Conclusion**

257 In this study, trastuzumab, rituximab and palivizumab were analyzed at the intact level by CZE-
258 ESI-MS. A rapid separation method has been developed to characterize these commercial mAbs under
259 denaturating condition. A PEI positive coating has been set up to avoid protein adsorption on the inner
260 surface of the capillary. Separation has been performed in 3% acetic acid BGE at 30 kV and sample
261 buffer has been optimized to 30% methanol, 1% formic acid with each mAbs to a final concentration
262 of 6.7 µM. CZE-ESI-MS analysis of these three mAbs showed partial separation obtained in less than
263 20 minutes allowing identification of mAbs isoforms. As a first result, CZE-ESI-MS electropherograms
264 fitted quite well with reference CZE-UV electropherograms allowing a potential characterization of the
265 basic and acidic variant regions. For each mAbs, 2X-glycosylated and 1X-glycosylated structures has
266 been identified and separated. Concerning basic and acidic variants, minor differences between 0 to 2
267 Da have been observed suggesting potential Iso-Asp modification and Asn deamidation. However,
268 mass precision didn't allow to conclude without any ambiguity on the nature of these modifications.
269 Accurate mass determination for high-mass molecular species remains a challenge, but the progress
270 in intact mAbs separation appears very promising and could be recognized as an additional step in
271 biopharmaceutics characterization.

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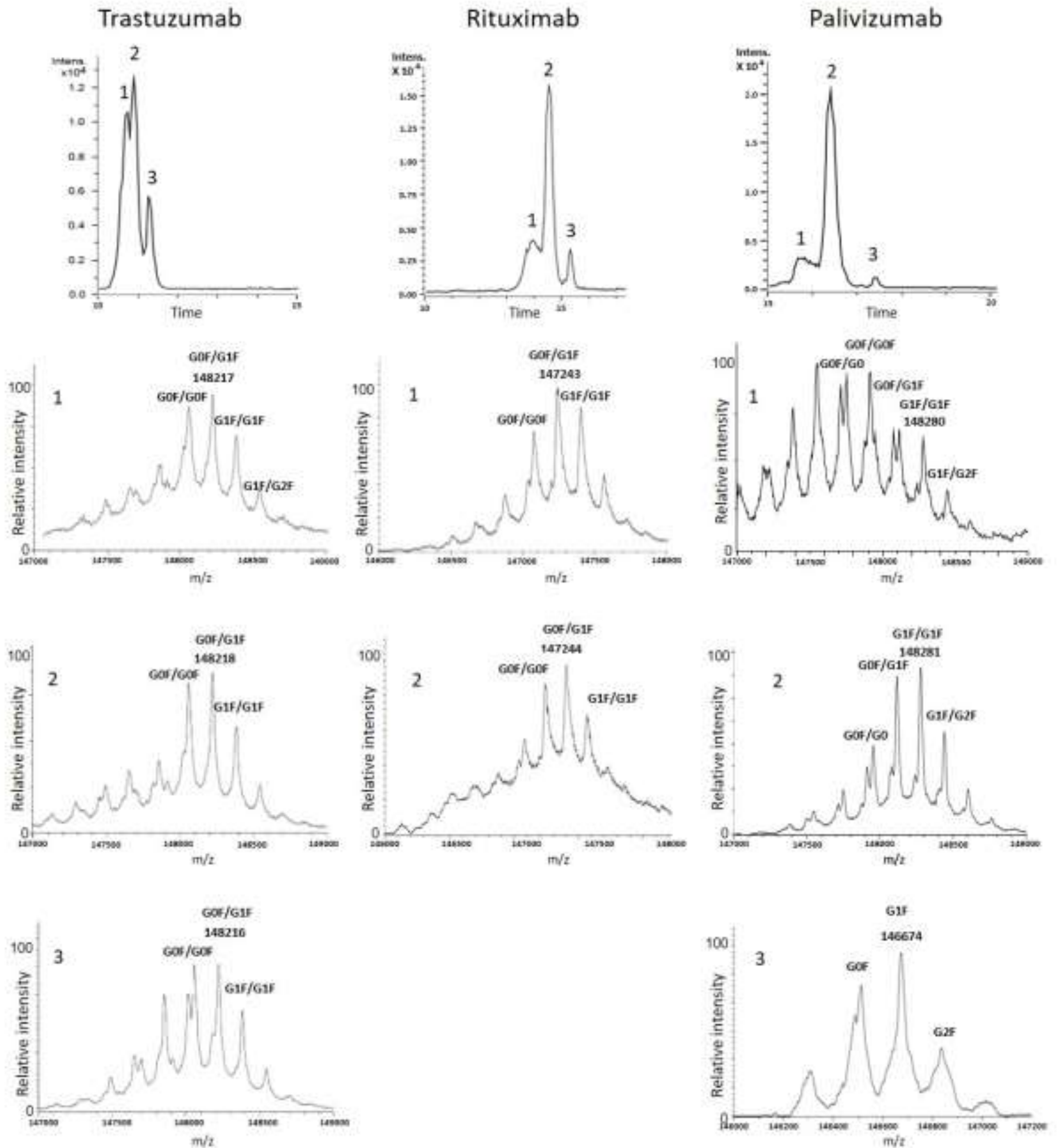

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279 Figure 4. CZE-ESI-MS separation of intact tratuzumab, rituximab and palivizumab obtained with the
280 optimized settings. For each mAbs, deconvoluted mass spectra was performed for the basic variant
281 (1), main variant (2), and acidic variant (3)

282

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