



HAL
open science

Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016–2018

Antony Lechner, Jeremie Giorgetti, Rabah Gahoual, Alain Beck, Emmanuelle Leize-Wagner, Yannis-Nicolas Francois

► To cite this version:

Antony Lechner, Jeremie Giorgetti, Rabah Gahoual, Alain Beck, Emmanuelle Leize-Wagner, et al.. Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016–2018. *Journal of Chromatography B Biomedical Sciences and Applications*, 2019, 1122-1123, pp.1-17. 10.1016/j.jchromb.2019.05.014 . hal-02399570

HAL Id: hal-02399570

<https://hal.science/hal-02399570v1>

Submitted on 23 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016-2018

Antony Lechner¹, Jérémie Giorgetti¹, Rabah Gahoual², Alain Beck³, Emmanuelle Leize-Wagner¹, Yannis-Nicolas François^{1,*}

¹ Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS), Unistra-CNRS UMR 7140, Université de Strasbourg, Strasbourg, France

² Unité de Technologies Biologiques et Chimiques pour la Santé (UTCBS), Paris 5-CNRS UMR8258 Inserm U1022, Faculté de Pharmacie, Université Paris Descartes, Paris, France

³ Centre d'Immunologie Pierre Fabre, Saint-Julien-en-Genevois, France

ABSTRACT

Monoclonal antibodies (mAbs) and their related products as antibody-drug-conjugates (ADCs) or biosimilars represent a constantly growing class of molecules therapeutic proteins used as treatment against numerous diseases. These compounds can undergo several modifications which could alter the efficiency of treatments. In this context, several analytical methods were designed to deliver a comprehensive structural characterization and guarantee the quality of biotherapeutics. Capillary electrophoresis (CE) is considered today as a major technique for the analysis of biotherapeutics due to benefic characteristics as high resolution separation and miniaturized format. Different CE modes have been developed to characterize mAbs at different levels such as capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF), and capillary zone electrophoresis (CZE). Recent developments in CE-mass spectrometry (MS) coupling assessed this technology as a promising tool to obtain high level structural characterization of biopharmaceuticals. Moreover, upcoming techniques such as 2D CE-MS and microfluidic systems are now emerging to offer new possibilities beyond actual limits. This review will be dedicated to discuss the state-of-the-art CE-based methods for the characterization of mAbs and ADCs in the period 2016-2018.

Keywords: Monoclonal antibodies, Biosimilar, Antibody Drug Conjugate, Capillary Gel Electrophoresis, Capillary Zone Electrophoresis, Capillary Isoelectric Focusing, Micro-fluidic capillary electrophoresis, Mass spectrometry, Post-translational modifications

Corresponding author: Yannis-Nicolas François

E-mail address: yfrancois@unistra.fr

36

37

38

39

1. Introduction

40

Today, more than 75 mAbs and ADCs are approved as therapeutic products by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) [1]. mAbs are occupying the top selling ranks for drugs since last decade [2, 3] making it one of the most successful category of product in the pharmaceutical industry. In addition, the approval of 16 new mAbs over the last couple of years suggests that expansion will further continue [4]. The current technologies to produce recombinant proteins enable to yield different types of biomolecules derived from the mAbs format such as antibody-drug conjugates (ADCs), fusion proteins, bispecific antibodies (bsAb) or biosimilars [5-7]. Their development is currently driven by the treatment of different diseases such as various cancers, infections, autoimmune disorders or to prevent transplant rejections.

48

Due to their protein nature, these compounds are highly complex macromolecules with typical molecular mass of approximately 150 kDa. However, mAbs can undergo several chemical degradations, post-translational modifications (PTMs) or 3D conformation alterations, which may lead to the inactivation of the drug or immune reactions [8, 9]. These modifications increase the heterogeneity and the complexity of the protein. The characterization and control of protein stability are therefore crucial aspects during each phase of mAbs development, from early-stage development to production, storage, and delivery. For assessment of mAbs and ADCs, regulation agencies have introduced the concept of critical quality attributes (CQA) which describes for each therapeutic protein, the PTMs potentially involved in the alteration of the pharmacokinetic (PK) and pharmacodynamic (PD) properties of the proteins[10, 11]. Thus, stringent analytical methods for comprehensive biotherapeutics characterization are necessary to ensure the quality of this type of products.

58

Hence, a large array of separation techniques in liquid phase like high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) were developed for quality control of biopharmaceutics [12-15]. These complementary methods allow the separation of the main mAbs isoforms from the modified variants in order to provide a detailed characterization over the different level defining the structure of the protein. In the late 80's, CE has emerged as a powerful technique to analyze biomolecules, thanks to the electromigration phenomenon and the possibility to use aqueous buffers that preserve the high-order structure of proteins [16, 17]. Consequently to the introduction of mAbs, CE has therefore demonstrated to be a relevant analytical separation for the characterization and stability study of this category of protein especially due to the unique selectivity of the electrophoretic separation [14, 18, 19]. Different electrophoretic modes such as capillary gel electrophoresis (CGE), capillary iso-

66

67 electric focusing (cIEF) and capillary zone electrophoresis (CZE) are commonly used to assess mAbs. CE experi-
68 ments are generally performed utilizing optical detection such as UV or fluorescence detector. However, in order
69 to provide structural information, capillary electrophoresis hyphenated to mass spectrometry (CE-MS) has been
70 extensively developed to overcome the limitations of optical detections. Different strategies like intact, middle-
71 up, middle-down and bottom-up analysis were established to access the amino acid sequence and the major
72 PTMs of biotherapeutics [20-22]. Also, CE-MS has been recognized as a powerful tool for immunoglobulin G (IgG)
73 glycan profiling and relative quantitation.

74 Regardless, the constant introduction of novel mAbs alongside to innovative therapeutic proteins based on this
75 format still maintains the urge to develop further adapted analytical methodologies. Especially, CE has continued
76 to gain a growing interest from the scientific community. This review, following a previous work [14], is focusing
77 on the latest developments regarding the application of CE-based techniques to characterize mAbs and ADCs in
78 the period of 2016-2018. In addition, it provides some insights concerning the future developments for CE based
79 analysis of mAbs and their related formats.

80 **2. Capillary Gel Electrophoresis (CGE)**

81 **2.1. Technical considerations**

82 Based on the separation of macromolecules according to their size, CGE represents one of the reference meth-
83 ods to assess size heterogeneity and glycan profiling of mAbs and ADCs. Among CGE techniques, capillary electro-
84 phoresis-sodium dodecyl sulfate (CE-SDS) is the adaptation of the sodium dodecyl sulfate-polyacrylamide gel elec-
85 trophoresis (SDS-PAGE) methodology converted to a miniaturized capillary format. That miniaturization involves
86 the replacement of the traditional slab gel by soluble polymers employed as a replaceable molecular sieve. It
87 presents the advantages of short analysis time, to reduce the difficulty of handling, to decrease the amount of
88 sample injected and improve the reproducibility. One major concern of this methodological transfer affecting the
89 separation efficiency, consists of analyte adsorption phenomena in the inner capillary wall. To avoid this harmful
90 phenomena, the development of adapted separation conditions was realized by increasing the concentration and
91 the viscosity of the gel or the inclusion of additives to the BGE. A positive consequence of the use of additives is
92 the suppression of the electroosmotic flow (EOF). Nowadays, commercial sieving kits are available and widely
93 used in the biopharmaceutical industry. Nevertheless, other strategies have been described to reduce adsorption
94 phenomena and EOF canceling, based particularly on the neutralization of the inner surface of the capillary by
95 chemical reaction. Various commercial or home-made solutions have been reported in the literature as polyvinyl
96 alcohol (PVA) [23] or linear polyacrylamide (LPA) [24], showing the significance of this modification to maintain a
97 good reproducibility of separation.

98 Detection of analytes in CGE is classically achieved by optical techniques like UV absorption or fluorescence. In
99 most cases, UV detection of proteins is programmed at 220 nm. However, 200, 214 and 280 nm are sometimes
100 used depending on separation conditions. Laser-induced fluorescence (LIF) detection provides an higher sensitiv-
101 ity than classical UV. Generally, the excitation of analytes is performed at 488 nm and the emission signal is mon-
102 itored at 520 nm. Although, just a few molecules are naturally fluorescent which implies otherwise a derivatization
103 step for the non-fluorescent analytes. Concerning the analysis of mAb glycosylations, chemical modification based
104 on 8-Aminopyrene-1,3,6-trisulfonic-acid (APTS) reaction allows the derivation of fluorophoric group and the addi-
105 tion of three negative charges to the glycan moiety, which is considered today as one of the reference method
106 [23, 25-28]. However recently, other dye reagents like 2-aminobenzoic acid (2-AA) (excitation: 325 nm emission:
107 405 nm) [29] and Teal™ (excitation and emission: same as APTS) [28], providing respectively one and three nega-
108 tive charges, have been reported for glycan analysis. These methods revealed excellent precision and accuracy.
109 Authors comment about the potential of detecting minor glycan species with Teal™ [28]. Moreover, the Teal™ is
110 preferred for derivatization step because of (i) its higher reactivity, (ii) lower amount of derivatization compound
111 required which contain salts, and (iii) therefore an improved MS compatibility as compared to the other common
112 fluorescent dyes. It can also provide higher sensitivity detection and accurate identification of analytes for CGE-
113 LIF-MS experiments

114

115 **2.2. Applications**

116 CGE is commonly used for *N*-glycan, size heterogeneity, purity, stability and aggregation assessment of intact
117 and reduced mAbs. ADCs are also characterized by CGE to estimate the drug-to-antibody ratio (DAR). CGE is com-
118 monly employed to evaluate the similarity between original glycoproteins and their biosimilar candidates. Table 1
119 summarizes the applications of CGE for mAbs and ADCs characterization between 2016-2018.

120 **2.2.1. Size variants assessments**

121 Two strategies are established to study mAbs size heterogeneity: non-reduced and reduced conditions. Non-
122 reduced CE-SDS is classically used to control purity/aggregation whereas reduced CE-SDS is mostly used to study
123 mAbs' fragments.

124 In 2016, Xie *et al.* described a method to analyze the level of reduced disulfide bonds of mAb using non-reduced
125 CE-SDS [30]. They studied the impact of downshift cell culture pH on the formation of this modification. They
126 observed a decrease in the level of reduced disulfide bond by 31 % when lowering pH from 6.95 to 6.75 after 6
127 days incubation. In another study, Esterman *et al.* evaluated the system suitability acceptance criteria of the U.S.
128 Pharmacopeia (USP) IgG standard using non-reducing CE-SDS and reducing CE-SDS. Two protocols originating from
129 the USP and Bristol-Myers Squibb (BMS) were assessed [31]. Authors concluded that USP method is not suitable

130 for lot-release and stability testing of mAbs due to a high level of fragmentation under non-reducing conditions.
131 In 2018, Schiel *et al.* analyzed the NISTmAb by non-reduced CE-SDS to assess purity, and reduced CE-SDS to eval-
132 uate the glycan occupancies of the heavy chain (HC) alongside to the relative abundances of non-reducible species
133 [32]. Inter and intra-vial homogeneity was evaluated, and excellent standard deviation were calculated (0.053 for
134 intra-vial and 0.0137 for inter-vial). Griaud *et al.* and Miao *et al.* reported two comprehensive studies using an
135 array of orthogonal techniques and strategies including CE-SDS for biosimilarity assessment [33, 34]. They com-
136 pared the electropherograms of intact and reduced mAbs and their biosimilar candidate. Minor differences were
137 observed but generally the candidate was very close to the originator.

138 Fragmentation of mAbs represents a CQA that needs to be monitored in order to assess the purity and integrity
139 during the life cycle of the product. Studies of the fragments from stressed mAbs can bring useful information
140 notably to understand mechanism of mAbs degradation [35-37]. These investigations enable to improve mAbs
141 production and storage conditions.

142 In 2017, Dada *et al.* reported a characterization strategy that establishes the correlation between hinge region
143 fragments analyzed by CE-SDS and size exclusion chromatography (SEC) [38]. Comparative assessment of frag-
144 ments by SEC, and CE-SDS showed similar correlation with incubation time. Authors analyzed collected fractions
145 of stressed mAbs at different days. An increase of HC, HC-HC aggregates (HH) and HC-HC-LC aggregates (HHL) were
146 observed during incubation. Kubota *et al.* described a CGE method to identify and characterize an impurity of 10
147 kDa formed after incubation of sample at 25°C for 6 months [35] (Figure 1). In-gel digestion peptide mapping
148 followed by reverse phase liquid chromatography-mass spectrometry (RPLC-MS) and Gelfree 8100 fractionation
149 were used to identify the impurity corresponding to the HC1-104 fragment which is involved in the complemen-
150 tary determining region (CDR). In 2017, Li *et al.* studied the fragments of an IgG1 obtained by forced degradation
151 at 40°C for 28 days. Two unique fragments were discovered when CE-SDS was employed for the purity analysis of
152 mAbs [36]. Then, these two fragments were fully analyzed and identified to be generated by a Ser105-Ser106
153 peptide bond cleavage at CDR3 of the HC using different HPLC-MS strategies.

154 In the 90's, microfluidic transposition of capillary electrophoresis was developed to achieve fast separation for
155 characterization, development, release, and stability testing of therapeutics. Especialy, miniaturization of CGE
156 (mGE) was of great interest for mAbs analysis. Indeed, microchips, due to their intrinsic properties, enable to
157 perform high-throughput analysis while maintaining resolution and efficiency. This device is increasingly recog-
158 nized as a valuable alternative to conventional CE. In 2016, Cai *et al.* optimized mGE to study mAbs in denaturing
159 conditions [39]. The percentage of SDS was investigated to obtain totally degraded mAb. Three IgG1 and five IgG4
160 were studied. The reduced mAbs were analyzed by mGE. Electropherograms showed baseline separated peaks of
161 light chains (LC) and HC obtained in less than 30 s. The method could be successfully qualified. The authors demon-
162 strated the precision and the specificity with a linear range of 16-3000 µg/mL and a LOQ of 7.8 µg/mL. Comparison

163 between mGE and conventional CE-SDS showed that the main difference is the analysis time. Both methods pro-
164 vided similar performances in terms of separation efficiency. However, they noticed that mGE is slightly less sen-
165 sitive than CE-SDS. In 2017, Smith *et al.* validated a mGE method to study biopharmaceuticals size variants and
166 purity in reducing and non-reducing conditions [40]. Specificity, linearity, accuracy, repeatability, reproducibility,
167 limit of detection/quantification (LOD/LOQ) and robustness were assessed, and results confirmed the excellent
168 features of mGE for reduced and non-reduced mAb analysis.

169 Concerning the characterization of ADCs, critical information regarding the nature of aggregates and/or frag-
170 ments can be provided by CE-SDS following different modes of sample preparation as reduced or non-reduced.
171 Nevertheless, this method is more limited for the analysis of ADCs depending on the conjugation chemistry and
172 the attachment sites as lysines, cysteines or glycans [41-44]. Concerning lysine-conjugated ADCs, in 2016, Chen
173 and co-workers published non-reducing CE-SDS reports of trastuzumab emtansine (T-DM1) and the unconjugated
174 trastuzumab. Comparable electropherograms with a main peak corresponding to the full-length antibody and mi-
175 nor peaks representing lower and higher molecular weight species (LMW and HMW) were observed using CE-SDS
176 analysis [42]. The same year, in-depth structural characterization of T-DM1 and its biosimilar candidate were in-
177 vestigated using several orthogonal analytical methods [45]. Characterization of size variants performed with the
178 combination of SEC and CE-SDS exhibited predominant monomer contents (>95 %) in T-DM1 and the biosimilar
179 ADC sample indicating their similarity in the aspect of size heterogeneity. In 2018, Wagh *et al.* reported a compre-
180 hensive study in another lysine-conjugated ADCs [43]. Analysis of lysine-conjugated ADCs by non-reducing CE-SDS
181 confirmed a main peak of full-length conjugated antibody and few minor peaks corresponding to LMW and HMW.
182 With reduced CE-SDS, results showed prominent peaks corresponding to HC and LC and minor peaks partially
183 separated for LC with 0 to 2 drug load.

184 Concerning the interchain-cysteine modified ADCs, CE-SDS profiles look totally different as compared to lysine-
185 conjugated ADCs. Indeed, the conjugation of the cytotoxic drugs to antibodies is performed through cysteine sul-
186 fide groups which are activated by a partial reduction of interchain disulfide bonds. It means that some antibody
187 chains are no longer covalently linked by intact disulfide bonds. The presence of SDS in the sample implied the
188 formation of protein-SDS complexes and the breaking of non-covalent interactions. This involves the dissociation
189 of any antibody chains depending on the position and the number of cytotoxic drugs which then causes the pres-
190 ence of low molecular masses ADCs fragments.

191

192 **2.2.2. Glycan analysis**

193 *N*-glycan represents a CQA for biopharmaceutical as it can compromise the efficiency and the safety of mAbs.
194 O’Flaherty *et al.*, Zhang *et al.* and Hajba *et al.* reviewed HPLC, CE and MS methods for glycan analysis from mono-
195 saccharides level to intact proteins including *O*-glycans and *N*-glycans until 2016 [46-48]. *N*-linked carbohydrates
196 profiling and quantitation are carried out by CGE. Before analysis, the carbohydrates are first released from mAbs
197 using specific endoglycosidases. Then, glycans are derivatized with a fluorescent dye. Afterwards, labelled glycans
198 are analyzed by CGE-LIF. For the analysis of glycans, some polymers are present in the buffer, but the separation
199 of glycan is not based on a sieving mechanism. The polymer is used to suppress adsorption phenomena and in-
200 crease separation efficiency [49].

201 In 2016 Kovacs *et al.* reported a high-throughput *N*-glycan analysis by CGE-LIF with APTS labeled glycans [25].
202 Using a method named separation window dependent multiple injection (SWDMI) they were able to analyze 96
203 samples in 4h instead of 12h for individual separation cycle. The glycan release step has also been shortened.
204 Szigeti *et al.* developed microcolumns with immobilized PNGase F which carry out rapid and easy to automate *N*-
205 glycan release [26]. This setup allowed them to perform efficient and reproducible deglycosylation of mAb in less
206 than 10 min. More recently, Szarka *et al.* evaluated proton beam irradiation on IgG *N*-glycan to anticipate the effect
207 of a long space travel [50]. After analysis of labeled glycan by CGE-LIF, they concluded that changes occur only
208 above 10 000 Gy which is quite inferior to Galactic Cosmic Ray and Solar Particle Events. Kubo *et al.* reported a
209 new polyethyleneglycol dimethacrylate (PEGDMA) coating [51]. Different conditions for polymerization procedure
210 were optimized. They showed the effective separation of sugars released from mAbs using this coating. Authors
211 suggested that the concentration and ethylene oxide units of PEGDMA affected the range of separable molecular
212 weight.

213 In 2018, Chen *et al.* reported the use of CGE-LIF as an orthogonal method to confirm the *N*-glycan quantitative
214 results obtained by miniaturized CE-MS (mCE-MS) [27]. A DNA analyzer was used for CGE-LIF experiment. Similar
215 results, in terms of quantitation and resolution were obtained but mCE-MS provided faster separation. Quantita-
216 tion of glycans has been successfully done by Szigeti *et al.* [52] over an extended concentration range. In this work,
217 quantitation of Man5 on adalimumab has been investigated. They obtained an excellent ($R^2=0.9995$) between
218 peak area of Man5 and concentration within a range 0.01-0.5 ng/ μ L. Authors suggested this quantitation method
219 could be easily applied to other carbohydrates moieties. To further improve the identification of glycosylation
220 patterns, CE-LIF-MS coupling has been developed by Khan *et al.* [28]. This setup allowed the authors to quantify
221 and identify *N*-glycans of NISTmAb (Figure 2). The novel fluorescent dye used is Teal™ which is MS compatible and
222 provides a higher quantum yield than classic APTS. This derivative agent permits to reach qualitative and quanti-
223 tative sugars information. Results of the baseline separations of different *N*-glycans showed excellent correlation
224 between LIF and MS.

225

226 3. Capillary Isoelectric focusing (cIEF) and imagedcIEF (icIEF)

227 3.1. Technical considerations

228 cIEF represents one of the most resolute methods to separate proteins depending on their isoelectric point
229 (pI) [53]. cIEF is the well-known miniaturized analogue of IEF slab gel mainly described for the analysis of charge
230 heterogeneity of mAbs and ADCs [54-58]. Historically, the conventional protocol of cIEF follows a two steps pro-
231 cedure starting by a focusing then a mobilization step. Briefly, cIEF experiment is performed by using a alkaline
232 catholyte (NaOH) and an acid anolyte (H_3PO_4). The capillary is filled with a solution containing carrier ampholytes
233 and analytes. By applying a high voltage, a gradient of pH is established through the whole capillary and proteins
234 are focusing in the capillary until they reach their own pI . At this point, the mobility of proteins is near zero because
235 their apparent charge is neutral. Then, sample is forced to migrate toward the detector, which is placed close to
236 the capillary exit using an electrophoretic or hydrodynamic mobilization step. However, the mobilization step can
237 induce some undesired effects like a decrease of resolution or modification of migration time. To address these
238 drawbacks, whole-column imaging cIEF (icIEF) has been developed. In icIEF experiments, UV-detection is per-
239 formed by a charge coupled device (CCD) camera all along the capillary (5cm) allowing to cancel the mobilization
240 step and achieve faster separation with greater resolution, better reproducibility and reduced sample volume.
241 Commonly to cIEF and icIEF experiments, coating of inner surface capillaries is mandatory to eliminate EOF and
242 improve separation efficiency. For this purpose, neutral capillaries using dynamic or permanent coating have been
243 implemented. In cIEF, the most described coatings are LPA [54, 57, 59], hydroxypropyl methylcellulose (HPMC)
244 [60, 61] and PVA [62]. Concerning icIEF, a majority of reports in the literature described the use of a fluorocarbon
245 capillary due to manufacturer's near monopoly (Proteinsimple) [55, 63-65]. However, other manufacturer like
246 CEInfinite provide icIEF system allowing the use of a wider choice of coated capillary solution.

247 Over the last couple of years, the technical advances of cIEF and icIEF detection modes have been mainly
248 achieved using coupling with MS. Indeed, concerning UV-detection, almost every application described in cIEF and
249 icIEF are performed at 280 nm due to good absorption of proteins at this wavelength and the strong absorbance
250 of ampholytes at lower wavelengths. MS detection represents a key feature for future developments to improve
251 the performance of cIEF and icIEF in terms of sensitivity and structural characterization [66-68]. However, devel-
252 opment of the hyphenation between cIEF and MS is curbed by technical issues such as presence of salts and
253 ampholytes. Very recently, different strategies of cIEF-MS and icIEF-MS coupling have been described in order to
254 reduce or eliminate these adverse effects. In 2017, Huhner *et al.* developed an cIEF-MS methodology using an
255 innovative two dimensional CE-MS instrumentation allowing to perform cIEF or icIEF separation in the first dimen-
256 sion followed by CZE in the second dimension, with online coupling to high-resolution MS [69, 70]. In 2018, Dai *et*
257 *al.* described a new automated cIEF-MS method to separate mAb charge variants using an electrokinetically

258 pumped sheath liquid nanospray CE-MS technology [67]. These last developments showed the potential of cIEF-
259 MS coupling to become a method of interest in biopharmaceutical industries and will be deeply discussed in the
260 section 5.

261

262 **3.2. Applications**

263 cIEF and icIEF play important roles in the characterization of purity and isoelectric point (*pI*) of mAbs. These
264 methodologies are considered as reference methods to determine mAbs charge heterogeneity. Several reports
265 described these techniques for the period until 2016 [14, 15, 37, 71]. In recent years, new enzymatic digestion,
266 new matrix and fractionation method were reported. Table 1 displays cIEF and icIEF applications for mAbs analysis
267 in 2016-2018.

268 Several inter-laboratory studies to evaluate the robustness of cIEF and icIEF have been detailed these last dec-
269 ades [57, 58]. The most recent in 2018, described by Wu *et al.*, reported an interlaboratory method validation of
270 icIEF for mAbs charge heterogeneity analysis [72]. 10 laboratories and 8 companies were involved. icIEF protocol
271 was performed according to the international conference on harmonization (ICH) guidelines. The method was
272 evaluated on four mAbs. Results confirmed the good precision in *pI* determination, the satisfying separation of
273 charge variants and the high precision for suitable assessment of purity. Following icIEF method, the *pI*s and the
274 charge variants of 23 therapeutic mAbs have been reported by Goyon *et al.*[73]. Experimental *pI*s, determined by
275 icIEF, were ranged between 6.1 and 9.4. Theoretical and measured *pI*s were compared giving absolute deviations
276 always lower than 15%. The relative distributions of acidic and basic variants were also determined to range be-
277 tween 15 to 30 % using both cation exchange chromatography (CEX) and icIEF which was in agreement with the
278 literature. King *et al.* analyzed an IgG1 by hydrophobic interaction chromatography (HIC) and obtained good sep-
279 aration of two peaks [74]. The two peaks were isolated and further characterized by HPLC-MS and icIEF using
280 different enzymatic strategies. *pI* of 7.6 and 7.8 were respectively found for these peaks. Deamidation of an as-
281 paragine (deaN) residue, located in the light chain CDR3, has been highlighted. They also showed the negative
282 impact of this PTM for the antigen binding affinity of the mAb. Finally, influence of pH on the deamidation for-
283 mation was studied. The results confirmed an increasing amount of deamidation at slightly basic pH.

284 cIEF and icIEF are extensively used to study fragments from degraded mAbs and biosimilarity assessment. Xie *et*
285 *al.* studied the effect of pH shift on the production of IgG1 mAb in Chinese hamster ovary cells [30]. The *pI* and
286 absolute percentages of acidic, main and basic variants were determined by cIEF. They concluded that a pH down-
287 shift reduces the amount of acidic variants and then prevents the formation of PTMs. Further investigations
288 through tryptic peptide mapping using MS highlighted that deamination proportion of HC-Asn388 and HC-Asn394
289 or HC-Asn393 decreases while pH is lowered. In the same year, Zhang *et al.* developed a icIEF to analyze mAb

290 fragments yielded by an optimized SpeB digestion (cysteine protease) [75]. With this new strategy, they obtained
291 well resolved peaks of LC, Fd, Fc/2 fragments and their PTMs. They also carried out a study of degraded mAbs and
292 they evaluated the biosimilarity of rituximab and a candidate with SpeB proteolysis. Authors demonstrated the
293 partial removal of C-terminal lysine between the reference and the biosimilar rituximab as well as a lower level of
294 deamidation. The same year, biosimilarity assessment studies on Avastin (bevacizumab) has been reported by
295 Zhao *et al.* [76]. Several analytical methods, including icIEF, were used to compare pharmacokinetics properties of
296 Avastin and a biosimilar (Figure 3). Firstly, mAbs were prepared to obtain isolated acidic, basic and main variants.
297 Then, these fragments were analyzed by different HPLC and CE techniques. Results showed no significant differ-
298 ences in pharmacokinetics parameters. In-depth study of the biosimilarity of tocilizumab candidate was per-
299 formed by Miao *et al.* [34]. Using different methods such as icIEF, CEX, SEC and RPLC-MS to determine biological
300 and physicochemical characterization, authors illustrated the very high similarity of the biosimilar and the origi-
301 nator tocilizumab.

302 Classic denaturant like urea or formamide, used in icIEF to stabilize the molecule charge can sometimes deteri-
303 orate proteins by aggregation process. Thus, a novel matrix formula for icIEF experiment in native conditions was
304 explored and optimized by Zhang *et al.* to study charge heterogeneity of proteins [77]. This matrix, composed by
305 non-detergent sulfobetaine and taurine (NDSB-T) possesses the stabilization and separation power while main-
306 taining protein integrity. NDSB-T can separate and quantify protein charge species in native state and therefore
307 avoid partial denaturation. This matrix enables to improve the assay robustness, repeatability, precision, accuracy
308 and peak resolution for an aggregation-prone mAb. Another issue for cIEF analysis is the relatively low amount of
309 sample injected which does not permit the fractionation of variants for further characterization. Hosken *et al.*
310 proposed an optimized preparative IEF based technique which allows fractionating the charge variants of intact
311 mAb [78]. They carried out an isoelectric focusing using a free-flow electrophoresis (FFE). FFE consists of two
312 plates between a carrier ampholyte enabling the focusing of charge variants when a high voltage is applied. High
313 amount of sample can be injected and then the isolation of acidic, basic and main variants becomes possible. This
314 method was applied on 3 mAbs. Charge variants of two mAbs were highly enriched except for the variants of the
315 mAb with the highest pI which did not focus enough. The enriched fractions were sufficient for physico-chemical
316 and biological characterization and many PTMs were identified. Using another strategy, developers from CEinfinite
317 proposed new methodologies for icIEF coupled to MS and/or peak fractionation. They developed a specific pro-
318 prietary cartridge specially designed for high performance preparative icIEF using a 200 μm ID capillary followed
319 by a 50 μm ID transfer capillary. This geometry allowed great flexibility such as protein fractionation enabling direct
320 spotting process to MALDI target plate.

321 In 2018, Turner *et al.* optimized cIEF method for NISTmAb qualification [79]. Separation of basic variants, main
322 peak and acidic variants has been performed and C-terminal lysine has been identified. The authors also realized

323 the titration of each variant in a range from 0.1 to 0.6 mg/mL with $R^2 > 0.98$ and $RSD < 8\%$. A comparison with an
324 optimized CZE method allowed authors to confirm that cIEF assay is a valuable characterization tool which affords
325 information about charge variant apparent pI while CZE assay was found to be suitable for qualification as a routine
326 quality monitoring assay.

327 Focusing on ADCs analysis, isoelectric focusing methods may give significantly different information on electro-
328 phoretic profiles of ADCs [41, 42, 44]. The nature of the drug-linker, especially the charge that can be added to the
329 ADC represents a major concern on the charge profile of the protein. For example, Ji *et al.* described the charac-
330 terization of thio-succinimide hydrolysis of monomethyl auristatin E (vc-MMAE) ADC using icIEF during the formu-
331 lation development [80]. As uncharged vc-MMAE conjugation through sulfhydryl groups in the interchain-cysteine
332 residues is known not to change the net charge of the ADCs, similar charge variant profiles were expected between
333 the conjugated and the unconjugated mAb. However, overlay of icIEF vc-MMAE ADC's profiles, incubated at pH 9
334 during 0 to 48 hours, showed an important heterogeneity in the acidic region due to various negative charges of
335 the ADC gained from both deamidation and succinimide hydrolysis of the thio-succinimide linker [80]. Concerning
336 lysine-linked ADC, this type of chemistry eliminates basic sites in the proteins and changes ADCs pI s [81]. Lin *et al.*
337 described the determination of mAb-Drug Maytansinoid 4 (DM4) with an average DAR of 3.6 by icIEF [81]. pI of
338 the drug loaded species shifted toward acidic variants from the unconjugated mAb due to the increase in the
339 number of DM4 drugs conjugated. More recently, Luo *et al.* confirmed these results performing the structural
340 characterization of a mAb-DM1 ADCs using the combination of UV/vis spectroscopy, HPLC/TOF-MS and icIEF ana-
341 lytical methods [82]. Average DAR value (3.2) measured with icIEF was in good agreement with UV/vis and intact
342 mass values (3.3 and 3.1 respectively). In 2018, Wagh *et al.* reported that icIEF can be applied to measure the level
343 of unconjugated antibody and drug load distribution, but it cannot distinguish between conjugates, process inter-
344 mediates and impurities such as antibody with linker only and antibody with linker/drug conjugates. Indeed, con-
345 jugation of linker only and linker/with drug bring the same shift charge toward acidic, and therefore cannot be
346 distinguished by icIEF. [43].

347

348 **4. Capillary Zone Electrophoresis (CZE)**

349 **4.1. Technical considerations**

350 Until the first development of CZE theory, this electrophoretic mode is still the most popular for CE applications.
351 This technique leans on a separation of analytes in a background electrolyte (BGE) under an electrical field accord-
352 ing to their charge-to-size ratio [16]. Typically, the capillary is filled up with the BGE. After a sample loading in the
353 capillary via a hydrodynamic or an electrokinetic injection, a separation voltage is applied to permit the migration
354 of analytes into the capillary. The most widespread detection mode is UV detection using a single wavelength

355 detection (usually 214 nm) or a diode array detection. Besides UV, other modes are used in CZE as LIF and more
356 recently MS. CZE-MS coupling requires some optimizations of separation conditions because of the weak toler-
357 ance to salts and the need of volatile solvents. Once these parameters laid down, MS detection enhances struc-
358 tural characterization of the samples. A complete section about the CE-MS coupling will be dedicated in the sec-
359 tion 5 of this review.

360 Concerning the characterization of mAbs and ADCs, as CE-SDS and cIEF modes, the main issue of the CZE tech-
361 nique was the protein adsorption on the capillary wall. Especially for middle-up and intact mAbs analysis, dynamic
362 or permanent chemical modification of the inner surface of the capillary must be performed prior to CZE separa-
363 tion. The majority of detailed protocols in the literature concerns the use of a dynamic HPMC coated capillary
364 which is considered as a reference for mAb charge variants analysis [14, 83, 84]. However, different strategies have
365 already been described with other dynamic positive coating as Polybrene [85] or neutral coating as polyethylene
366 oxide [86, 87]. More recently, in 2017, Xiao *et al.* have developed an innovative coating inspired by a well-known
367 biological phenomenon, the blood coagulation, and worked on a fibrin coating on an open tubular column [88].
368 Apart from this parameter, the optimization of experimental conditions concerning the mAb analysis in CZE de-
369 pends enormously on the physicochemical properties of the mAbs to characterize them at the desired levels and
370 will be described in the application section 4.2.

371

372 **4.2. Applications**

373 **4.2.1. Bottom-up analysis**

374 At the bottom-up level, major concerns regarding mAbs characterization such as glycoprofiling, isomerizations
375 and amino-acid modifications, have been solved with CZE methods. Feng *et al.* have highlighted the *N*-glycosyla-
376 tion of two human IgGs with a comparison of CZE and micellar electrokinetic capillary chromatography (MEKC)
377 [23]. They worked on a PVA coated capillary to obtain a separation of 26 glycoforms referenced in the national
378 institute for bioprocessing research and training (NIBRT) glycobase in 17 minutes in CZE and 24 min in MEKC. They
379 confirmed their results with additional tests combining α -fucosidase, α -neuraminidase and β -galactosidase. To
380 further improve mAbs characterization, Griaud's team studied ranibizumab (Lucentis®) and a biosimilar
381 (razumab®) by SEC, CE-SDS and CZE methods. After optimization, they separated and identified multiple serine to
382 asparagine sequence variation sites on the mAbs. They checked the results with a MS detection and exposed a
383 difference of 27.01 Da (6-9 %). The analysis of MS/MS spectra confirmed serine to asparagine substitution on the
384 light chains [33]. The same year, disulfide isomers were separated with a high resolution. The study was focused
385 on the development of an ultra-high voltage capillary electrophoresis (UHVCE) and the analysis of 3 mAbs and 1
386 ADC. The analysis has been made with a 120 kV electric field (2 kV/cm) in a HPMC coated capillary. This system

387 enhanced resolution and separation speed allowing distinction of disulfide isomers [89]. More recently, CZE has
388 also been developed to get the best repeatability in particular with one of the key source of differentiability of
389 digestion. An integrated micro reactor has been set up to perform an in-line tryptic digestion to enhance the
390 quality control of mAbs. After an off-line optimization step of enzyme concentration and incubation time, other
391 major parameters have been adjusted in-line to determine the best nature of BGE (ionic strength, pH). The feasi-
392 bility and robustness of the project have been evaluated on three commercial mAbs: trastuzumab (Hercpetin®),
393 infliximab (Remicade®) and tocilizumab (Roactemra®). The diffusion mediated proteolysis combined with an elec-
394 trophoretic separation (D-PES) has been developed on a polyethyleneoxide (PEO) coated capillary regenerated
395 before each analysis. A specific injection pattern of mAb, trypsin and proteolytic buffer has been elaborated al-
396 lowing to produce a digest with the same efficiency as compared to the off-line protocol. This totally automated
397 method is clearly a promising solution decreasing the total analysis time by a factor of four (5 hours needed to
398 digest, separate and analyze a sample), decreasing the volume of reactants by 1000 fold and offering a great re-
399 peatability of the total ion electropherograms (TIE) [90].

400

401 **4.2.2. Intact analysis**

402 One of the highest challenges of separation methods dwells in intact proteins analysis. While CZE is not consid-
403 ered as a reference method so far, the development of this approach showed significant advances for mAb charge
404 variants analysis. Today, a classical CZE protocol from the pioneer works of He *et al.* [91, 92] which is evaluated by
405 an interlaboratory study [93], can be described as the use of a BGE composed of ϵ -aminocaproic acid (EACA),
406 triethylenetetramine (TETA) and HPMC. In 2017, a complete tutorial about a quite similar protocol, has been pub-
407 lished in video format illustrated the separation of mAbs' isoforms on a neutral capillary. These favorable condi-
408 tions increase efficiency, accuracy and repeatability while keeping undamaged capillary up to 150 injections [94].
409 Illustrating complexity to optimize the large number of parameters, Suba *et al.* described a "two-phase-four-step"
410 approach allowing a rapid, iterative method development process for CZE [95]. Based on fluorocarbon coated
411 capillaries, they set up a fast screening phase to adjust the pH and the ionic strength with the aim to get optimal
412 migration time, stability and resolving capacity of the method. Then, a fine tuning phase leaned on the addition
413 of TETA to enhance the viscosity and improve the peak resolving and the stability of the method. They concluded
414 that optimal conditions were 50 mM EACA at pH 5.1 with 1 mM TETA and 0.05% v/v HPMC. The same year, Zhao
415 *et al.* detailed the characterization of a biosimilar from Bevacizumab (Avastin®) using different methods as SEC,
416 icIEF, CEX and CZE [96]. They indicated a purity of the acidic, basic and main peaks respectively of 94 %, 82 % and
417 85 %. In addition, PK of this candidate has been studied showing that all the results are acceptable to validate it
418 as a biosimilar. However, PK and purity are not the only parameters required to allow the commercialization of

419 this kind of molecules. Additional tests with other analytical methods need to be employed to enable the charac-
420 terization at different levels. Moritz *et al.* described a design of experiments (DoE) based on differences between
421 the net charge and the hydrodynamic radius to optimize charge heterogeneity tests [97]. The DoE set up during
422 this study highlighted important parameters for CZE optimization. Described work is promising for a successful
423 development of CZE as a major method for biopharmaceuticals analysis. Alternative approach consisting of ex-
424 ploring new BGE conditions for mAb charge variants separation, has also been developed by Goyon *et al.* in 2018
425 [87]. 17 marketed mAbs have been evaluated using solely a Bis-Tris buffer (pH 7.0) and PEO dynamic coating.
426 Goyon *et al.* obtained a RSD < 5 % of migration times and the separation of acidic charge variants of 10 / 17 mAbs.
427 They also compared their methodology with the reference CZE method involving EACA, TETA and HPMC. The novel
428 CZE approach allowed separating additional charge variants (in particular more acidic variants). Conversely, a
429 higher number of basic variants were separated by the reference CZE approach for two mAbs out of 17, demon-
430 strating the complementarity of the two approaches. The relative quantitation of acidic and basic variants has
431 been established specifically 15-30 % of acidic variants and 5-15 % of basic variants (Figure 4). Authors concluded
432 that their method used to separate native mAbs offers complementary results but extend five folds the analysis
433 time and elicits a loss of sensitivity. The same year, Kahle *et al.* realized a comparison of protein charge variants
434 separated by CZE and cIEF to upgrade the quality control of batches [59]. Another approach on the development
435 of innovative coating was performed by Xiao *et al.* They reported an easy-to-use coating which gave a good sepa-
436 ration of cetuximab (Erbix[®]) and rituximab (Mabthera[®]) with respectively nine and five separated variants [88].
437 By contrast, in a bovine serum albumin (BSA) coated column, they distinguished seven and four variants for the
438 same molecules. These separations were detected by UV detection at 280 nm and gave a satisfying repeatability
439 with a deviation times < 2.42 % on day to day and run to run batches.

440 To assess the robustness and the reproducibility of CZE methods, Kubota *et al.* proposed a validation study of
441 CZE method for evaluating mAbs and ADCs [84]. Among the numerous tested parameters in this study, are repre-
442 sented identity, purity, specificity, linearity, precision, quantitation limit, repeatability, accuracy, range and robust-
443 ness of CZE to characterize biopharmaceuticals. 11 samples including 2 ADCs and 9 mAbs have been evaluated in
444 this study with different *pI*, DAR, and structures. The method showed quantitative results with high specificity,
445 separation efficiency and precision. Authors concluded that CZE appears to be a promising alternative to IEF and
446 CEX. The nearest validation step has been established by Schiel *et al.* in 2018. In two separate studies, they inves-
447 tigated the reference material (RM 8671) called NIST mAb which is a first-of-kind reference material to inform on
448 specific attributes during therapeutic proteins characterization [32, 79]. The identity, quality, stability of the NIST
449 mAb have been studied. The CZE assay was found to be suitable for qualification as a routine quality monitoring
450 assay due to excellent sensitivity, speed, simplicity, specificity, and intermediate precision. Based on their results,
451 CZE was selected for longitudinal quality monitoring of the NISTmAb Reference Material charge heterogeneity.

452

453

454 **5. Capillary electrophoresis – mass spectrometry (CE-MS)**

455 **5.1. Technical considerations**

456 The growing need of precise characterization of biotherapeutics implies the constant evolution in separation
457 methods especially with the achievement of additional structural information. As the performance of mass spec-
458 trometers improved in term of sensitivity, mass accuracy and scanning frequencies, MS became a key instrumen-
459 tation for the analysis of biomolecules. The complexity due to the large scale of heterogeneity on mAbs needs to
460 take the sensitivity into account to characterize low amount isoforms. While the different miniaturized electro-
461 phoretic modes (CE-SDS, cIEF, CZE) coupled with spectrophotometric detection are already known to be reference
462 methods for mAbs characterization, the main improvements developed these last few years were achieved in the
463 development of CE-MS methods. Since the end of the eighties, instrumental developments have been constantly
464 performed in order to optimized the hyphenation of CE with MS detection using offline or online approaches
465 depending on the nature of the MS sources: electrospray ionization (ESI) and matrix assisted laser desorption
466 ionization (MALDI) [98]. CE-MS coupling is usually implemented with ESI; however, specific CE-MALDI-MS applica-
467 tions were also described in the literature [99-101]. As the weak acceptance of salts and the need of volatile
468 solvents is known to be primary parameters for CE-MS applications, one of the main issue for CE-MS instrumen-
469 tation is known to conserve a continuous electric field during CE separation while maintaining a stable spray. This
470 last decades, a way to get around this issue has been to design innovative CE-MS interface geometries. Historical
471 developed CE-MS interfacing systems relied on a conductive sheath-liquid, connected to the outlet electrode of
472 the CE system, continuously injected to maintain the electrical field [102]. Despite a good robustness and an easy
473 setup, regarding the very low amount of sample carried in the separation capillary, the dilution effect due to the
474 sheath-liquid has a negative impact on MS sensitifity. To address this limitation, other geometries have been de-
475 veloped allowing to reduce to the minimum the flow rate or to wholly eliminate the sheath-liquid. Today some
476 commercial CE-MS solutions based on sheath liquid and sheathless interface are already fully described in the
477 literature and commercially available [98]. Nevertheless, regarding direct CE-ESI-MS, the main hurdle quite in com-
478 mon of all papers detailed in the literature consisted of the incompatibility of classical well known BGEs with MS
479 detection due to the presence of nonvolatile compounds that diminishes drastically the quality of MS data. While
480 some very recent articles describing improvement of CZE-ESI-MS or cIEF-ESI-MS applications for mAbs charge var-
481 iants characterization [67, 103, 104], another recent approach have been developed to reduce BGE impact on CE-
482 ESI-MS detection. Inspired by 2D LC-MS, the group of Prof. Neusüß worked on a promising 2D CE-MS setup, pur-
483 suing to provide interference-free mass spectra with electrophoretic separations [105]. This advanced separation
484 technique includes a first dimension which allows to perform a high resolving CE separation with nonvolatile BGEs

485 followed by a second dimension able to fully remove the ESI-interfering components from the analytes prior to
486 MS detection. For this purpose, a mechanical four-port valve with a small volume loop was utilized to lead the
487 analytes from the first dimension to the second one with limited diffusion. The main advantage of this approach
488 is the possibility to perform the 1st dimension in any CE mode (CZE, cIEF, CE-SDS) with classical conditions discussed
489 in previous sections (BGE, coating, additives, etc.). This type of instrumentation has the potential to further in-
490 crease the level of characterization achieved in a single experiment, but still needs some technical improvements
491 to make it robust and get a marketed solution.

492

493 **5.2. Applications**

494 **5.2.1. Amino acid sequence characterization**

495 MAbs are produced from a biosynthesis process in culture cells from living organisms that could induce some
496 minor differences in the amino acid sequence or PTMs during the maturation phase of the proteins. Moreover,
497 production, packaging and storage conditions of the molecules can also induce some amino acid substitutions or
498 other sort of modifications. Since 2006, Gennaro *et al.* demonstrated the potential of CZE-ESI-MS to obtained the
499 complete characterization of mAbs using a Lys-C digestion combined with HPLC-MS analysis [106]. In 2013, Ga-
500 houal *et al.* confirmed the positive impact of sheathless CZE-ESI-MS methods demonstrating the consistent char-
501 acterization of the amino acid sequence with 100% coverage and some PTMs such as *N*-glycosylations, *N*-terminal
502 glutamic acid cyclization, asparagine deamidations (deaN), methionine oxidations (oxyM), and aspartic acid isom-
503 erization (isoD) in a single analysis [107, 108]. In 2016, Said *et al.* adapted the CZE-ESI-MS method, especially with
504 the optimization of the tryptic digestion protocol, for the characterization of the primary structure of a cysteine-
505 linked ADC. Results revealed the method ability to characterize ADCs primary structure particularly with the loca-
506 tion of drug loaded peptides and the estimation of their conjugation level [109]. More recently, some aque-
507 ous/aprotic dipolar solvent mixes systems were tested by Dada *et al.* to enhance the peptide mapping on mAbs
508 and ADCs. Authors explained that *N,N*-dimethylacetamide (DMA) and *N,N*-dimethylformamide (DMF) used as BGE
509 allow to have a complete sequence coverage and improve the separation of peptides with a neutral coated capil-
510 lary at the expense of time analysis [110]. The results confirmed several advantages of CZE-ESI-MS for the analysis
511 of small hydrophilic di-/tripeptides, large hydrophobic peptides, glycopeptides, and hydrophobic drug-linked pep-
512 tides.

513

514 **5.2.2. Glycosylation characterization**

515 Glycosylation is a PTM that occurs naturally during excretion of antibodies from the expression system to the
516 extracellular medium. It only represents 2–5% of the total mass of the protein but it is subjected to extensive

517 studies due to its significant influence on effector functions of mAbs [111, 112]. As a consequence, the mAbs
518 glycosylation profile is considered as a CQA and must be thoroughly analyzed [113-115]. For over 10 years, num-
519 bers of analytical methods have been developed to better understand the synthesis, roles and consequences of
520 mAbs glycosylations [111, 116, 117]. The last development of CE-MS methods highlighted the attractiveness to
521 characterize and quantify the glycosylation heterogeneity with high accuracy and precision [107]. In 2016, a quan-
522 titative twoplex glycan analysis using heavy and light carbon stable isotopes of 2-aminobenzoic acid (2-AA) label-
523 ling have been developed to check the quality of different mAbs batches [118]. Glycosylation profiles have been
524 detected and identified with a fluorescence detector on different cetuximab batches. Comparison with orthogonal
525 techniques as HPLC-MS were realized using a released glycan approach. Results showed a good agreement and
526 the reproducibility of the two methods. In 2017, Giorgetti *et al.* evaluated the CZE-ESI-MS method to perform
527 relative quantitation of *N*-glycan species for mAbs characterization at the peptides level [119]. Validation in terms
528 of robustness and reproducibility were demonstrated through the relative quantitation of glycosylation profiles
529 for ten different mAbs and two biosimilar produced in different cell lines. A systematic comparison was performed
530 with hydrophilic interaction liquid chromatography (HILIC) reference method, obtaining very similar glycosylation
531 pattern. Some glycan differences can also be provided from a difference of linkage between two glycosylation
532 units. Sialic acid linkages differences have been observed by Kammeijer *et al.* using CZE-ESI-MS [120]. Although
533 identical fragmentation patterns obtained by collision induced dissociation (CID), authors detailed the selective
534 analysis of α 2,3 and α 2,6–sialylated glycopeptides with no additional sample preparation. The different migration
535 behavior between the two moieties was found to correlate with differences of pKa values which is consistent with
536 the selectivity of the separation. In 2018, Chen *et al.* worked on the glycoprofiling of the NIST mAb with a mCE-
537 MS interface [27]. Authors proposed a quick solution for intact mAb characterization with quantitative results on
538 protein variants. They obtained a partial separation and the relative quantitation of 18 variants including some
539 glycolytic modifications. In the last few months of 2018, Jooß *et al.* described a method based on CZE coupled to
540 drift tube ion mobility MS for the analysis of native and APTS-labeled *N*-glycans [121]. In their study, each individ-
541 ual glycan signal separated in CZE exhibited an unexpected high number of peaks observed in the IMS dimension.
542 Among the possible explanations, the type of sialic acid attached to glycans has a significant impact on the IMS
543 heat map.

544

545 **5.2.3. Middle-up analysis**

546

547 Biopharmaceutics analysis at the bottom-up level involves sample treatments as digestion protocol which is
548 known to potentially induce some artefactual modifications of amino acids implying possible over estimation of
549 PTMs. To avoid or reduce these limitations, optimizations of sample preparation were developed to study mAbs

550 at other levels such as middle-up or intact ones. Middle-up analysis consists of a prior partial and localized mAbs
551 digestion by the mean of specific enzymes and reduction reagents. Obtained fragments, usually larger than 10
552 kDa, gave the opportunity to focus on specific parts of the mAbs structure with a lower difficulty than intact mol-
553 ecules. The middle-up level has been made possible due to the development of proteolytic enzymes like *Strepto-*
554 *coccal endopeptidase* (IdeS) or papain. On contrary to bottom-up level, middle-up strategies allow to enhance the
555 sample preparation protocol by reducing the number of chemical steps and then minimizing modifications in-
556 duced by digestion.

557 Due to the limited proteolysis of mAbs which generates significantly larger peptides as compared to classical
558 trypsin or Lys-C digestions, different strategies of online and offline CE-MS coupling have been developed in the
559 last ten years. Biacchi *et al.* detailed an offline CE-MALDI/ESI-MS method for the analysis of Fc/2 and F(ab')₂ frag-
560 ments produced from the IdeS digestion of cetuximab [122]. They concluded on the baseline separation of Fc/2
561 lysine variants and the characterization of the *N*-glycosylation sites present on the mAb. In 2016, the same group
562 deepened their study by demonstrating the separation in native conditions of three Fc/2 homo- or heterodimers
563 depending on the presence of a C-terminal lysine [123]. The first study using an online CE-ESI-MS coupling for
564 mAbs separation at middle-up level was described by Han *et al.* in 2016 [124]. Authors demonstrated the baseline
565 separation of HC and LC after reduction of disulfide bridges as well as the complete separation of Fc/2, Fd and LC
566 fragments after IdeS digestion followed by a reduction step. Concerning IdeS digestion, they obtained in less than
567 20 minutes, a separation of Fc/2 lysine variants and observed mass differences between control, deamidated and
568 oxidized stressed mAbs.

569 Very recently, two major articles were published on the multi-level characterization of diverse mAbs combining
570 middle-up and intact strategies using CZE-ESI-MS methods. Belov and coworkers developed CZE-ESI-MS methods
571 to study different IgG1 at the intermediate level with an IdeS digestion but no reduction step [104]. CE conditions
572 consisted of the use of a 1-(4-iodobutyl) 4-aza-1-azoniabicyclo[2,2,2] octane iodide (M7C4I) positively coated ca-
573 pillary with a BGE composed of 50 % ethanol added to 1 % formic acid and a voltage of 20 kV. They obtained a
574 separation of Fc/2 and F(ab')₂ fragments in less than 60 minutes, with the characterization of modifications such
575 as oxidations and deamidations. In addition, they studied the same samples with a combination of IdeS digestion
576 and a TCEP reduction step. They obtained separation of respectively LC, Fc/2 and Fd fragments in nearly 40
577 minutes. Both experiments allowed to characterize different glycoforms on the Fc/2 fragments. The same year,
578 Somsen's team succeed in separating Fc/2 glycoforms by varying BGE [125]. Neutral LPA capillary has been em-
579 ployed due to the near zero EOF generated during the separation and to avoid protein adsorption on the capillary
580 wall. They discussed the effect of acetic acid concentration as BGE. A complete characterization of the different
581 Fc/2 variants has been detailed with the glycan and C-terminal Lys variability and the observation of other PTMs
582 as deamidation.

583

584 **5.2.4. Intact protein analysis**

585

586 Analysis of intact protein, which is considered particularly challenging, represents the absolute way of protein
587 characterization. Indeed, due to the reduction or even the absence of sample preparation which can introduce
588 artefactual modifications, intact protein analysis has the advantage to be more representative of the actual sam-
589 ple. However, due to the high masses and the complexity of mAbs, intact level analysis of these therapeutics is
590 still a challenge for analytical sciences. CE-MS coupling has been described as a promising technique for the char-
591 acterization of intact proteins [126] and then has been applied particularly these last years in the field of biother-
592 apeutics [14]. In 2014, Biacchi *et al.* detailed the first separation of intact mAbs using an offline CZE-MALDI-MS
593 coupling [100]. The offline property of the CE-MS coupling allows the authors to work with salt-concentrated and
594 non-volatile BGE enabling them to obtain the separation and MS analysis of intact charge variants of trastuzumab.
595 Unfortunately, limitation of MALDI-MS resolution for molecules up to 100 kDa did not allow the measurement of
596 the exact mass of these charge variants. Direct CE-ESI-MS coupling then appears as a solution to avoid this limita-
597 tion. In 2016, Han *et al.* proposed the separation and identification of free LC contained in IgG1 reference material
598 using CZE-ESI-MS method [124]. The same year, Ramsey's group developed and employed a CE-nanoESI-MS mi-
599 crochip for the analysis of intact infliximab [127]. Molecular weights were determined for five charge variants
600 separated on a 23 cm long separation channel coated with an aminopropylsilane (APS) base layer and covalent
601 modification with polyethylene glycol (PEG). Three major species corresponding to C-terminal lysine variants plus
602 some minor acidic and basic species have been characterized with an average resolution of 0.80. The same group
603 used this method for the characterization of a lysine-linked ADC [128]. Authors observed the separation of five
604 main species that differed in their respective DAR, which for each DAR displayed the same variant population
605 observed on the unconjugated mAb. They concluded that the CE-nanoESI-MS microchip method demonstrated
606 the possibility of accurately determine the DAR.

607 In 2017, Belov *et al.* have been the first group to analyze intact mAb by online native CZE-ESI-MS [22]. Working
608 on a sheathless CE-MS interface coupled with an orbitrap mass spectrometer and using neutral polyacrylamide
609 coated capillary, they obtained a partial separation of intact mAbs aggregates. Native condition as 20 mM ammo-
610 nium acetate pH 8.0 allowed them to reveal predominant forms of the intact mAb, corresponding to 2X-glycosyl-
611 ated, 1X-glycosylated and dimeric structures. They also observed other molecular species of about 101 kDa, pre-
612 sumably corresponding to the mAb with the loss of 2 light chains. The dissociated light chains have been separated
613 as monomeric and dimeric forms. In 2018, the same group worked on another mAb under denaturing conditions
614 and using a positively charged coating capillary (M7C4I) [104]. Optimized BGE condition composed of 10% isopro-
615 panol, 0.2% formic acid allows them to report the complete glycosylation profile of the mAb. Three types of mAb

616 glycans populations (2X-glycosylated, 1X-glycosylated and aglycosylated forms) have been observed (Figure 5).
617 Very soon after this paper, Haselberg *et al.* performed the heterogeneity assessment of intact trastuzumab and
618 ustekinumab using sheathless CE-MS interface coupled with a Q-TOF MS instrument [125]. For the two mAbs,
619 partial separation of charge variants has been obtained using 10% acetic acid BGE and a neutral capillary coating
620 consisting of a hydrophobic layer to protect siloxanes from hydrolysis, and a second layer of polyacrylamide provid-
621 ing a hydrophilic surface. Each peak appeared to consist of variants mixtures with different properties depending
622 of the nature of the mAb. The same year, a third paper about intact mAb separation using sheathless CZE-ESI-MS
623 has been published by Giorgetti *et al.* [103]. Using 3% acetic acid BGE, sample buffer of 30% methanol 1% formic
624 acid and a PEI positively coated capillary, separation of three world-wide health authorities approved mAbs (ritux-
625 imab, palivizumab and trastuzumab), have been evaluated. CZE-ESI-MS analysis of these three mAbs showed par-
626 tial separation obtained in less than 20 min allowing identification of mAbs isoforms. For each mAb, 2X-glycosyl-
627 ated and 1X-glycosylated structures have been identified and separated. Concerning basic and acidic variants,
628 minor differences between 0 and 2 Da have been observed suggesting potential iso-Asp modification and dea_N.
629 However, mass accuracy of the mass spectrometer added to separation performances, did not allow to conclude
630 without ambiguity on the nature of these modification.

631 As a well-known property, MS-based methods present the main drawback to be intolerant to classical salted
632 and non-volatile BGE reducing the possibility of optimizing CE methods. Indeed, in a comparison between refer-
633 ence CZE-UV and CZE-ESI-MS methods using different BGE, obtained electropherograms fitted well but lower res-
634 olutions were obtained for CZE-ESI-MS [103]. In 2017, Jooß *et al.* described a CZE-CZE-MS method for the charac-
635 terization of intact mAbs charge variants [129]. Authors achieved interference-free, highly precise mass data (de-
636 viation less than 1 Da) of trastuzumab charge variants. The mass accuracy obtained (< ppm) were discussed re-
637 garding both measured and calculated masses. The same group confirmed the advantages of their 2D CE-MS sys-
638 tem with the development of a cIEF-CZE-MS for the characterization of intact mAbs [69]. After focusing step, the
639 analytes were mobilized through two external detectors (UV and C⁴D) to select precisely analytes inside the trans-
640 fer loop and then to run the CZE dimension prior to MS detection. They established a “multiple heart-cut” ap-
641 proach considerably reducing the analysis time allowing them to perform the transfer of up to six analytes from a
642 single cIEF run and the analysis via CZE-MS in less than 3h. Application on the MS characterization of intact mAb
643 charge variants enabled them to detect a mass difference of approximately 2 Da between two variants. In 2018,
644 the same group deepened the development of the 2D CE-MS coupling to identify low abundance charge variants
645 of trastuzumab using icIEF-CZE-MS [70]. icIEF first dimension can be monitored in real-time thanks to the whole-
646 column detection. Accurate masses were attributed to deamidation, formation of succinimide or cyclisation of *N*-
647 terminal glutamic acid for the main acidic and basic variants. Despite the difficulty to achieve online cIEF-ESI-MS
648 application due to CE experimental condition, Dai *et al.* reported a new online cIEF-ESI-MS method for mAb charge

649 variant analysis using an electrokinetically pumped sheath-flow nanospray ion source with pressure-assisted
650 chemical mobilization [67]. Many critical parameters and reagents have been optimized including MS-friendly
651 analyte and catholyte, a glycerol enhanced sample mixture or ampholyte. Charge variant profiles of trastuzumab,
652 bevacizumab, infliximab and cetuximab, obtained using cIEF-MS method, were corroborated by icIEF-UV analysis.
653 Moreover, charge variants of these mAbs were characterized using the online intact MS data. More recently, the
654 same group applied their cIEF-ESI-MS method for the in-depth characterization of cetuximab charge heterogeneity
655 [68].

656

657 **6. Conclusion**

658 Due to the inherent structural complexity of the mAbs format, a wide variety of analytical techniques, including
659 liquid chromatography, electrophoresis and mass spectrometry have been implemented. Detailed characteriza-
660 tions of these therapeutic proteins have been obtained by these techniques to ensure the integrity of the drugs.
661 Therefore, the physicochemical phenomenon involved in CE separation demonstrated in numerous applications a
662 unique benefit for mAbs analysis. Conventional CE separation modes like CZE, CE-SDS or (i)cIEF are particularly
663 relevant to characterize mAbs size and charge variants thanks to CE intrinsic features which provide efficient and
664 highly-resolutive separations. Furthermore, the different inter-laboratory studies support unambiguously the con-
665 fidence of the analysis in addition to the robustness provided by the CE-based analytical methods. As a conse-
666 quence, CE has gradually become one of the reference technique in the biopharmaceutical industry to assess
667 purity and stability of mAbs. Concomitantly, CE-MS hyphenation was implemented and demonstrated to be rele-
668 vant to access precious structural information like amino acid sequences, glycosylations, PTMs and higher-order
669 structures.

670 In the most recent period, the development of analytical methodologies involving CE separation has not dimin-
671 ished, which is emphasized by the significant number of articles discussed throughout the present review. Also, it
672 is important to note that each CE separation mode is currently explored for the development of methods dedi-
673 cated to the characterization of mAbs and their related formats. Such interest is indeed attributed to an improved
674 understanding of the charge based selectivity of the electrophoretic separation in the case of mAbs, which showed
675 to be relevant to distinguish several types of micro-heterogeneities. In addition, the introduction of innovative
676 molecules based on the mAbs format like ADCs contributes to the dynamic of CE method development.

677 The implementation of CE-ESI-MS has also benefiting further from various instrumental progresses. Regarding
678 that aspect, the recent emergence of 2D CE-MS represents a significant breakthrough. Indeed, 2D CE-MS enables
679 to potentially improve separation and peak capacity in a similar manner as 2D liquid chromatography. The intro-

680 duction of microfluidic technologies has also exhibited the possibility to achieve short-time analyses while main-
681 taining a high resolution due to the reduction of diffusion effects, for instance to perform a high-throughput
682 screening of mAbs and ADCs.

683 Future prospects regarding CE based analysis of mAbs and their associated formats suggest CE-MS hold the most
684 important potential due to the complete orthogonality of both techniques and the performances of current gen-
685 eration of high resolution MS instruments. Also, by performing CZE using a MS compatible BGE over the second
686 dimension, 2D CE-MS enables to perform separation mode previously incompatible with MS coupling like CE-SDS
687 or cIEF over the first dimension. That aspect of 2D CE-MS is particularly promising because it enriches in a drastic
688 manner the applicability of CE-MS, therefore further developments is expected in that direction in the near future.
689 For instance, it is possible to envisage the use of specific CE analysis like affinity CE. The application of affinity CE
690 in conjunction with MS is particularly appealing for the characterization of mAbs. During their lifetime, mAbs are
691 interacting with targeted antigens in addition to several types proteins which influence their effector functions
692 and/or pharmacological properties. Thus, by distinguishing protein-protein/protein-ligand complexes and deter-
693 mine affinity constants using their respective electrophoretic mobilities, it could be possible to correlate structural
694 modifications with mAbs affinity opening the path to structure-function analysis. The development of affinity CE-
695 MS experiments adapted to mAbs represents a major challenge however the introduction of 2D CE-MS clearly
696 reshuffles the cards on that aspect. Finally, because native MS analysis is often limited by the absence of separa-
697 tion before the MS analysis, the development of CE-MS analysis performed could improve significantly the sensi-
698 tivity and widening the applicability of native MS. This could be applied especially for the analysis of complex
699 mixtures while maintaining therapeutic mAbs is a state as close as possible to the protein administrated to the
700 patient.

701

702 **Acknowledgements**

703 Authors would like to thank Sciex and Bruker Daltonics for the fruitful collaboration and their support. The au-
704 thors would like also to express their gratitude to Dr. E. Wagner-Rousset, μ -MC. Janin-Bussat and O. Colas (Centre
705 d'Immunologie Pierre Fabre, St Julien en Genevois, France) for helpful discussions. This work was supported by
706 the CNRS (UMR 7140), the University of Strasbourg, and a doctoral fellowship from Bruker Daltonics and the Gen-
707 eral Council of Region Grand Est to JG.

708

709

710

712

- 713 [1] Z. Elgundi, M. Reslan, E. Cruz, V. Sifniotis, V. Kayser, The state-of-play and future of antibody
714 therapeutics, *Adv Drug Deliver Rev*, 122 (2017) 2-19.
- 715 [2] Y. Lai, R. Wang, X. Chen, D. Tang, Y. Hu, J. Cai, Q. Zhang, H. Hu, Emerging trends and new developments
716 in monoclonal antibodies: A scientometric analysis (1980–2016), *Human Vaccines &
717 Immunotherapeutics*, 13 (2017) 1388-1397.
- 718 [3] A.L. Grilo, A. Mantalaris, The Increasingly Human and Profitable Monoclonal Antibody Market, *Trends
719 in Biotechnology*, 37 (2019) 9-16.
- 720 [4] H. Kaplon, J.M. Reichert, Antibodies to watch in 2018, *mAbs*, 10 (2018) 183-203.
- 721 [5] A. Beck, L. Goetsch, C. Dumontet, N. Corvaia, Strategies and challenges for the next generation of
722 antibody–drug conjugates, *Nature Reviews Drug Discovery*, 16 (2017) 315.
- 723 [6] R.C. Elgersma, R.G.E. Coumans, T. Huijbregts, W.M.P.B. Menge, J.A.F. Joosten, H.J. Spijker, F.M.H. de
724 Groot, M.M.C. van der Lee, R. Ubink, D.J. van den Dobbelsteen, D.F. Egging, W.H.A. Dokter, G.F.M.
725 Verheijden, J.M. Lemmens, C.M. Timmers, P.H. Beusker, Design, Synthesis, and Evaluation of Linker-
726 Duocarmycin Payloads: Toward Selection of HER2-Targeting Antibody–Drug Conjugate SYD985,
727 *Molecular Pharmaceutics*, 12 (2015) 1813-1835.
- 728 [7] A. Beck, T. Wurch, C. Bailly, N. Corvaia, Strategies and challenges for the next generation of
729 therapeutic antibodies, *Nat Rev Immunol*, 10 (2010) 345-352.
- 730 [8] Y. Yan, H. Wei, Y. Fu, S. Jusuf, M. Zeng, R. Ludwig, S.R. Krystek, G. Chen, L. Tao, T.K. Das, Isomerization
731 and Oxidation in the Complementarity-Determining Regions of a Monoclonal Antibody: A Study of the
732 Modification–Structure–Function Correlations by Hydrogen–Deuterium Exchange Mass Spectrometry,
733 *Analytical Chemistry*, 88 (2016) 2041-2050.
- 734 [9] S. Hermeling, D.J.A. Crommelin, H. Schellekens, W. Jiskoot, Structure-immunogenicity relationships
735 of therapeutic proteins, *Pharm Res*, 21 (2004) 897-903.
- 736 [10] H. Rahalkar, H.C. Cetintas, S. Salek, Quality, Non-clinical and Clinical Considerations for Biosimilar
737 Monoclonal Antibody Development: EU, WHO, USA, Canada, and BRICS-TM Regulatory Guidelines, *Front
738 Pharmacol*, 9 (2018).
- 739 [11] Y. Tang, F. Tang, Y. Yang, L. Zhao, H. Zhou, J. Dong, W. Huang, Real-Time Analysis on Drug-Antibody
740 Ratio of Antibody-Drug Conjugates for Synthesis, Process Optimization, and Quality Control, *Scientific
741 Reports*, 7 (2017) 7763.
- 742 [12] B. Bobály, S. Fleury-Souverain, A. Beck, J.-L. Veuthey, D. Guillarme, S. Fekete, Current possibilities of
743 liquid chromatography for the characterization of antibody-drug conjugates, *Journal of Pharmaceutical
744 and Biomedical Analysis*, 147 (2018) 493-505.
- 745 [13] V. D'Atri, A. Goyon, B. Bobaly, A. Beck, S. Fekete, D. Guillarme, Protocols for the analytical
746 characterization of therapeutic monoclonal antibodies. III - Denaturing chromatographic techniques
747 hyphenated to mass spectrometry, *Journal of Chromatography B-Analytical Technologies in the
748 Biomedical and Life Sciences*, 1096 (2018) 95-106.
- 749 [14] R. Gahoual, A. Beck, E. Leize-Wagner, Y.-N. François, Cutting-edge capillary electrophoresis
750 characterization of monoclonal antibodies and related products, *Journal of Chromatography B*, 1032
751 (2016) 61-78.
- 752 [15] S. Fekete, D. Guillarme, P. Sandra, K. Sandra, Chromatographic, Electrophoretic, and Mass
753 Spectrometric Methods for the Analytical Characterization of Protein Biopharmaceuticals, *Analytical
754 Chemistry*, 88 (2016) 480-507.

755 [16] S. Hjerten, High-performance electrophoretic counterpart of high-performance liquid-
756 chromatography, *Journal of Chromatography*, 270 (1983) 1-6.

757 [17] J.W. Jorgenson, K.D. Lukacs, Zone electrophoresis in open-tubular glass-capillaries, *Analytical*
758 *Chemistry*, 53 (1981) 1298-1302.

759 [18] R. Gahoual, E. Leize-Wagner, P. Houzé, Y.-N. François, Revealing the potential of capillary
760 electrophoresis/mass spectrometry: the tipping point, *Rapid Communications in Mass Spectrometry*,
761 DOI doi:10.1002/rcm.8238(2018).

762 [19] M.R.G. Kopp, P. Arosio, Microfluidic Approaches for the Characterization of Therapeutic Proteins, *J.*
763 *Pharm. Sci.*, 107 (2018) 1228-1236.

764 [20] R. Gahoual, A. Burr, J.M. Busnel, L. Kuhn, P. Hammann, A. Beck, Y.N. Francois, E. Leize-Wagner, Rapid
765 and multi-level characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass
766 spectrometry, *mAbs*, 5 (2013) 479-490.

767 [21] M. Biacchi, N. Said, A. Beck, E. Leize-Wagner, Y.-N. François, Top-down and middle-down approach
768 by fraction collection enrichment using off-line capillary electrophoresis – mass spectrometry coupling:
769 Application to monoclonal antibody Fc/2 charge variants, *Journal of Chromatography A*, 1498 (2017)
770 120-127.

771 [22] A.M. Belov, R. Viner, M.R. Santos, D.M. Horn, M. Bern, B.L. Karger, A.R. Ivanov, Analysis of Proteins,
772 Protein Complexes, and Organellar Proteomes Using Sheathless Capillary Zone Electrophoresis - Native
773 Mass Spectrometry, *Journal of The American Society for Mass Spectrometry*, 28 (2017) 2614-2634.

774 [23] H.T. Feng, M. Su, F.N. Rifai, P.J. Li, S.F.Y. Li, Parallel analysis and orthogonal identification of N-glycans
775 with different capillary electrophoresis mechanisms, *Analytica Chimica Acta*, 953 (2017) 79-86.

776 [24] Z. Szabo, A.s. Guttman, J. Bones, B.L. Karger, Rapid High-Resolution Characterization of Functionally
777 Important Monoclonal Antibody N-Glycans by Capillary Electrophoresis, *Analytical Chemistry*, 83 (2011)
778 5329-5336.

779 [25] Z. Kovacs, M. Szarka, M. Szigeti, A. Guttman, Separation window dependent multiple injection
780 (SWDMI) for large scale analysis of therapeutic antibody N-glycans, *Journal of Pharmaceutical and*
781 *Biomedical Analysis*, 128 (2016) 367-370.

782 [26] M. Szigeti, J. Bondar, D. Gjerde, Z. Keresztessy, A. Szekrenyes, A. Guttman, Rapid N-glycan release
783 from glycoproteins using immobilized PNGase F microcolumns, *Journal of Chromatography B-Analytical*
784 *Technologies in the Biomedical and Life Sciences*, 1032 (2016) 139-143.

785 [27] C.H. Chen, H.T. Feng, R. Guo, P.J. Li, A.K.C. Laserna, Y. Ji, B.H. Ng, S.F.Y. Li, S.H. Khan, A. Paulus, S.M.
786 Chen, A.E. Karger, M. Wenz, D.L. Ferrer, A.F. Huhmer, A. Krupke, Intact NIST monoclonal antibody
787 characterization-Proteoforms, glycoforms-Using CE-MS and CE-LIF, *Cogent Chemistry*, 4 (2018).

788 [28] S. Khan, J. Liu, Z. Szabo, B. Kunnummal, X. Han, Y. Ouyang, R.J. Linhardt, Q. Xia, On-line capillary
789 electrophoresis/laser-induced fluorescence/mass spectrometry analysis of glycans labeled with Teal™
790 fluorescent dye using an electrokinetic sheath liquid pump-based nanospray ion source, *Rapid*
791 *Communications in Mass Spectrometry*, 32 (2018) 882-888.

792 [29] H. Yamada, C. Matsumura, K. Yamada, K. Teshima, T. Hiroshima, M. Kinoshita, S. Suzuki, K. Kakehi,
793 Combination of SDS-PAGE and intact mass analysis for rapid determination of heterogeneities in
794 monoclonal antibody therapeutics, *Electrophoresis*, 38 (2017) 1344-1352.

795 [30] P.P. Xie, H.J. Niu, X.N. Chen, X.T. Zhang, S.W. Miao, X.C. Deng, X.P. Liu, W.S. Tan, Y. Zhou, L. Fan,
796 Elucidating the effects of pH shift on IgG1 monoclonal antibody acidic charge variant levels in Chinese
797 hamster ovary cell cultures, *Applied Microbiology and Biotechnology*, 100 (2016) 10343-10353.

798 [31] A.L. Esterman, A. Katiyar, G. Krishnamurthy, Implementation of USP antibody standard for system
799 suitability in capillary electrophoresis sodium dodecyl sulfate (CE-SDS) for release and stability methods,
800 *Journal of Pharmaceutical and Biomedical Analysis*, 128 (2016) 447-454.

801 [32] J.E. Schiel, A. Turner, T. Mouchahoir, K. Yandrofski, S. Telikepalli, J. King, P. DeRose, D. Ripple, K.
802 Phinney, The NISTmAb Reference Material 8671 value assignment, homogeneity, and stability, *Analytical*
803 *and Bioanalytical Chemistry*, 410 (2018) 2127-2139.

804 [33] F. Griaud, A. Winter, B. Denefeld, M. Lang, H. Hensinger, F. Straube, M. Sackewitz, M. Berg,
805 Identification of multiple serine to asparagine sequence variation sites in an intended copy product of
806 LUCENTIS (R) by mass spectrometry, *mAbs*, 9 (2017) 1337-1348.

807 [34] S. Miao, L. Fan, L. Zhao, D. Ding, X. Liu, H. Wang, W.-S. Tan, Physicochemical and Biological
808 Characterization of the Proposed Biosimilar Tocilizumab, *BioMed Research International*, 2017 (2017)
809 13.

810 [35] K. Kubota, N. Kobayashi, M. Yabuta, M. Ohara, T. Naito, T. Kubo, K. Otsuka, Identification and
811 characterization of a thermally cleaved fragment of monoclonal antibody-A detected by sodium dodecyl
812 sulfate-capillary gel electrophoresis, *Journal of Pharmaceutical and Biomedical Analysis*, 140 (2017) 98-
813 104.

814 [36] W. Li, B. Yang, D. Zhou, J. Xu, W. Li, W.-C. Suen, Identification and characterization of monoclonal
815 antibody fragments cleaved at the complementarity determining region using orthogonal analytical
816 methods, *Journal of Chromatography B*, 1048 (2017) 121-129.

817 [37] E. Tamizi, A. Jouyban, Forced degradation studies of biopharmaceuticals: Selection of stress
818 conditions, *Eur. J. Pharm. Biopharm.*, 98 (2016) 26-46.

819 [38] O.O. Dada, R. Rao, N. Jones, N. Jaya, O. Salas-Solano, Comparison of SEC and CE-SDS methods for
820 monitoring hinge fragmentation in IgG1 monoclonal antibodies, *Journal of Pharmaceutical and*
821 *Biomedical Analysis*, 145 (2017) 91-97.

822 [39] H. Cai, Y.L. Song, J. Zhang, T. Shi, Y. Fu, R. Li, N. Mussa, Z.J. Li, Optimization of microchip-based
823 electrophoresis for monoclonal antibody product quality analysis revealed needs for extra surfactants
824 during denaturation, *Journal of Pharmaceutical and Biomedical Analysis*, 120 (2016) 46-56.

825 [40] M.T. Smith, S. Zhang, T. Adams, B. DiPaolo, J. Dally, Establishment and validation of a microfluidic
826 capillary gel electrophoresis platform method for purity analysis of therapeutic monoclonal antibodies,
827 *Electrophoresis*, 38 (2017) 1353-1365.

828 [41] A. Wakankar, Y. Chen, Y. Gokarn, F.S. Jacobson, Analytical methods for physicochemical
829 characterization of antibody drug conjugates, *mAbs*, 3 (2011) 161-172.

830 [42] T. Chen, Y. Chen, C. Stella, C.D. Medley, J.A. Gruenhagen, K. Zhang, Antibody-drug conjugate
831 characterization by chromatographic and electrophoretic techniques, *Journal of Chromatography B-*
832 *Analytical Technologies in the Biomedical and Life Sciences*, 1032 (2016) 39-50.

833 [43] A. Wagh, H.T. Song, M. Zeng, L. Tao, T.K. Das, Challenges and new frontiers in analytical
834 characterization of antibody-drug conjugates, *mAbs*, 10 (2018) 222-243.

835 [44] A. Beck, V. D'Atri, A. Ehkirch, S. Fekete, O. Hernandez-Alba, R. Gahoual, E. Leize-Wagner, Y. François,
836 D. Guillarme, S. Cianféroni, Cutting-edge multi-level analytical and structural characterization of
837 antibody-drug conjugates: present and future, *Expert Rev. Proteomics*, 16 (2019) 337-362.

838 [45] L. Chen, L. Wang, H. Shion, C. Yu, Y.Q. Yu, L. Zhu, M. Li, W. Chen, K. Gao, In-depth structural
839 characterization of Kadcyca® (ado-trastuzumab emtansine) and its biosimilar candidate AU - Chen, Liuxi,
840 *mAbs*, 8 (2016) 1210-1223.

841 [46] R. O'Flaherty, I. Trbojevic-Akmacic, G. Greville, P.M. Rudd, G. Lauc, The sweet spot for biologics:
842 recent advances in characterization of biotherapeutic glycoproteins, *Expert Rev. Proteomics*, 15 (2018)
843 13-29.

844 [47] L. Zhang, S. Luo, B. Zhang, Glycan analysis of therapeutic glycoproteins, *mAbs*, 8 (2016) 205-215.

845 [48] L. Hajba, E. Csanky, A. Guttman, Liquid phase separation methods for N-glycosylation analysis of
846 glycoproteins of biomedical and biopharmaceutical interest. A critical review, *Analytica Chimica Acta*,
847 943 (2016) 8-16.

848 [49] A. Guttman, F.-T.A. Chen, R.A. Evangelista, Separation of 1-aminopyrene-3,6,8-trisulfonate-labeled
849 asparagine-linked fetuin glycans by capillary gel electrophoresis, *Electrophoresis*, 17 (1996) 412-417.

850 [50] M. Szarka, S. Szilasi, B. Donczo, D. Sarkozy, I. Rajta, A. Guttman, The effect of simulated space
851 radiation on the N-glycosylation of human immunoglobulin G1, *Electrophoresis*, 39 (2018) 2872-2876.

852 [51] T. Kubo, N. Nishimura, H. Furuta, K. Kubota, T. Naito, K. Otsuka, Tunable separations based on a
853 molecular size effect for biomolecules by poly(ethylene glycol) gel-based capillary electrophoresis,
854 *Journal of Chromatography A*, 1523 (2017) 107-113.

855 [52] M. Szigeti, J. Chapman, B. Borza, A. Guttman, Quantitative assessment of mAb Fc glycosylation of
856 CQA importance by capillary electrophoresis, *Electrophoresis*, 39 (2018) 2340-2343.

857 [53] S. Hjerten, M.D. Zhu, Adaptation of the equipment for high-performance electrophoresis to
858 isoelectric-focusing, *Journal of Chromatography*, 346 (1985) 265-270.

859 [54] D. Suba, Z. Urbanyi, A. Salgo, Capillary isoelectric focusing method development and validation for
860 investigation of recombinant therapeutic monoclonal antibody, *Journal of Pharmaceutical and*
861 *Biomedical Analysis*, 114 (2015) 53-61.

862 [55] D.A. Michels, A.W. Tu, W. McElroy, D. Voehringer, O. Salas-Solano, Charge Heterogeneity of
863 Monoclonal Antibodies by Multiplexed Imaged Capillary Isoelectric Focusing Immunoassay with
864 Chemiluminescence Detection, *Analytical Chemistry*, 84 (2012) 5380-5386.

865 [56] J. Lin, Q.Q. Tan, S.X. Wang, A high-resolution capillary isoelectric focusing method for the
866 determination of therapeutic recombinant monoclonal antibody, *Journal of Separation Science*, 34
867 (2011) 1696-1702.

868 [57] O. Salas-Solano, K. Babu, S.S. Park, X.F. Zhang, L. Zhang, Z. Susic, B. Boumajny, M. Zeng, K.C. Cheng,
869 A. Reed-Bogan, S. Cummins-Bitz, D.A. Michels, M. Parker, P. Bonasia, M.F. Hong, S. Cook, M. Ruesch, D.
870 Lamb, D. Bolyan, S. Kiessig, D. Allender, B. Nunnally, Intercompany Study to Evaluate the Robustness of
871 Capillary Isoelectric Focusing Technology for the Analysis of Monoclonal Antibodies, *Chromatographia*,
872 73 (2011) 1137-1144.

873 [58] O. Salas-Solano, B. Kennel, S.S. Park, K. Roby, Z. Susic, B. Boumajny, S. Free, A. Reed-Bogan, D.
874 Michels, W. McElroy, P. Bonasia, M.F. Hong, X.P. He, M. Ruesch, F. Moffatt, S. Kiessig, B. Nunnally,
875 Robustness of iCIEF methodology for the analysis of monoclonal antibodies: An interlaboratory study,
876 *Journal of Separation Science*, 35 (2012) 3124-3129.

877 [59] J. Kahle, H. Wätzig, Determination of protein charge variants with (imaged) capillary isoelectric
878 focusing and capillary zone electrophoresis, *Electrophoresis*, 39 (2018) 2492-2511.

879 [60] M. Kinoshita, Y. Nakatsuji, S. Suzuki, T. Hayakawa, K. Kakehi, Quality assurance of monoclonal
880 antibody pharmaceuticals based on their charge variants using microchip isoelectric focusing method,
881 *Journal of Chromatography A*, 1309 (2013) 76-83.

882 [61] T.D. Wheeler, J.L. Sun, S. Pleiner, H. Geier, P. Dobberthien, J. Studts, R. Singh, B. Fathollahi, Microchip
883 Zone Electrophoresis for High-Throughput Analysis of Monoclonal Antibody Charge Variants, *Analytical*
884 *Chemistry*, 86 (2014) 5416-5424.

885 [62] R. Bonn, S. Rampal, T. Rae, J. Fishpaugh, CIEF method optimization: Development of robust and
886 reproducible protein reagent characterization in the clinical immunodiagnostic industry, *Electrophoresis*,
887 34 (2013) 825-832.

888 [63] E.T.J. van den Bremer, F.J. Beurskens, M. Voorhorst, P.J. Engelberts, R.N. de Jong, B.G. van der Boom,
889 E.M. Cook, M.A. Lindorfer, R.P. Taylor, P.H.C. van Berkel, P.W.H.I. Parren, Human IgG is produced in a pro-
890 form that requires clipping of C-terminal lysines for maximal complement activation, *mAbs*, 7 (2015) 672-
891 680.

892 [64] Z.W.K. Glover, L. Gennaro, S. Yadav, B. Demeule, P.Y. Wong, A. Sreedhara, Compatibility and stability
893 of pertuzumab and trastuzumab admixtures in i.v. infusion bags for coadministration, *J. Pharm. Sci.*, 102
894 (2013) 794-812.

895 [65] O.O. Dada, N. Jaya, J. Valliere-Douglass, O. Salas-Solano, Characterization of acidic and basic variants
896 of IgG1 therapeutic monoclonal antibodies based on non-denaturing IEF fractionation, *Electrophoresis*,
897 36 (2015) 2695-2702.

898 [66] G.J. Zhu, L.L. Sun, P. Yang, N.J. Dovichi, On-line amino acid-based capillary isoelectric focusing-ESI-
899 MS/MS for protein digests analysis, *Analytica Chimica Acta*, 750 (2012) 207-211.

900 [67] J. Dai, J. Lamp, Q. Xia, Y. Zhang, Capillary Isoelectric Focusing-Mass Spectrometry Method for the
901 Separation and Online Characterization of Intact Monoclonal Antibody Charge Variants, *Analytical*
902 *Chemistry*, 90 (2018) 2246-2254.

903 [68] J. Dai, Y. Zhang, A Middle-Up Approach with Online Capillary Isoelectric Focusing/Mass
904 Spectrometry for In-Depth Characterization of Cetuximab Charge Heterogeneity, *Analytical Chemistry*,
905 90 (2018) 14527-14534.

906 [69] J. Hühner, K. Jooß, C. Neusüß, Interference-free mass spectrometric detection of capillary isoelectric
907 focused proteins, including charge variants of a model monoclonal antibody, *Electrophoresis*, 38 (2017)
908 914-921.

909 [70] C. Montealegre, C. Neusüß, Coupling imaged capillary isoelectric focusing with mass spectrometry
910 using a nanoliter valve, *Electrophoresis*, 39 (2018) 1151-1154.

911 [71] S. El Deeb, H. Wätzig, D.A. El-Hady, C. Sängner-van de Griend, G.K.E. Scriba, Recent advances in
912 capillary electrophoretic migration techniques for pharmaceutical analysis (2013-2015), *Electrophoresis*,
913 DOI 10.1002/elps.201600058(2016) n/a-n/a.

914 [72] G. Wu, C. Yu, W. Wang, L. Wang, Interlaboratory method validation of icIEF methodology for analysis
915 of monoclonal antibodies, *Electrophoresis*, 39 (2018) 2091-2098.

916 [73] A. Goyon, M. Excoffier, M.-C. Janin-Bussat, B. Bobaly, S. Fekete, D. Guillarme, A. Beck, Determination
917 of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies, *Journal of*
918 *Chromatography B*, 1065 (2017) 119-128.

919 [74] C. King, R. Patel, G. Ponniah, C. Nowak, A. Neill, Z.Y. Gu, H.C. Liu, Characterization of recombinant
920 monoclonal antibody variants detected by hydrophobic interaction chromatography and imaged
921 capillary isoelectric focusing electrophoresis, *Journal of Chromatography B-Analytical Technologies in the*
922 *Biomedical and Life Sciences*, 1085 (2018) 96-103.

923 [75] Z.C. Zhang, R. Perrault, Y. Zhao, J. Ding, SpeB proteolysis with imaged capillary isoelectric focusing
924 for the characterization of domain-specific charge heterogeneities of reference and biosimilar Rituximab,
925 *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 1020 (2016)
926 148-157.

927 [76] Y.Y. Zhao, N. Wang, W.H. Liu, W.J. Tao, L.L. Liu, Z.D. Shen, Charge Variants of an Avastin Biosimilar
928 Isolation, Characterization, In Vitro Properties and Pharmacokinetics in Rat, *PLoS One*, 11 (2016) 13.

929 [77] X. Zhang, L. Chemmalil, J. Ding, N. Mussa, Z. Li, Imaged capillary isoelectric focusing in native
930 condition: A novel and successful example, *Anal Biochem*, 537 (2017) 13-19.

931 [78] B.D. Hosken, C. Li, B. Mullappally, C. Co, B.Y. Zhang, Isolation and Characterization of Monoclonal
932 Antibody Charge Variants by Free Flow Isoelectric Focusing, *Analytical Chemistry*, 88 (2016) 5662-5669.

933 [79] A. Turner, J.E. Schiel, Qualification of NISTmAb charge heterogeneity control assays, *Analytical and
934 Bioanalytical Chemistry*, 410 (2018) 2079-2093.

935 [80] J.A. Ji, J. Liu, Y.N. Wang, *Formulation development for Antibody-Drug Conjugates*, Springer
936 International Publishing 2015.

937 [81] J. Lin, A.C. Lazar, Determination of charge heterogeneity and level of unconjugated antibody by
938 imaged iCIEF, Humana Press 2013.

939 [82] Q. Luo, H.H. Chung, C. Borths, M. Janson, J. Wen, M.K. Joubert, J. Wypych, Structural
940 Characterization of a Monoclonal Antibody–Maytansinoid Immunoconjugate, *Analytical Chemistry*, 88
941 (2016) 695-702.

942 [83] R. Gahoual, J. Giorgetti, A. Beck, E. Leize-Wagner, Y.-N. François, Chapter 19 - Biopharmaceutical
943 Applications of Capillary Electromigration Methods A2 - Poole, Colin F, *Capillary Electromigration
944 Separation Methods*, Elsevier 2018, pp. 453-480.

945 [84] K. Kubota, N. Kobayashi, M. Yabuta, M. Ohara, T. Naito, T. Kubo, K. Otsuka, Validation of Capillary
946 Zone Electrophoretic Method for Evaluating Monoclonal Antibodies and Antibody-Drug Conjugates,
947 *Chromatography*, 37 (2016) 117-124.

948 [85] A.L. Gassner, S. Rudaz, J. Schappler, Static coatings for the analysis of intact monoclonal antibody
949 drugs by capillary zone electrophoresis, *Electrophoresis*, 34 (2013) 2718-2724.

950 [86] Y. Shi, Z. Li, Y.B. Qiao, J. Lin, Development and validation of a rapid capillary zone electrophoresis
951 method for determining charge variants of mAb, *Journal of Chromatography B-Analytical Technologies
952 in the Biomedical and Life Sciences*, 906 (2012) 63-68.

953 [87] A. Goyon, Y.N. Francois, O. Colas, A. Beck, J.L. Veuthey, D. Guillarme, High-resolution separation of
954 monoclonal antibodies mixtures and their charge variants by an alternative and generic CZE method,
955 *Electrophoresis*, 39 (2018) 2083-2090.

956 [88] X. Xiao, W.T. Wang, Y.M. Zhang, L. Jia, Facile preparation of fibrin coated open tubular column for
957 characterization of monoclonal antibody variants by capillary electrochromatography, *Journal of
958 Pharmaceutical and Biomedical Analysis*, 140 (2017) 377-383.

959 [89] W.H. Henley, Y. He, J.S. Mellors, N.G. Batz, J.M. Ramsey, J.W. Jorgenson, High resolution separations
960 of charge variants and disulfide isomers of monoclonal antibodies and antibody drug conjugates using
961 ultra-high voltage capillary electrophoresis with high electric field strength, *Journal of Chromatography
962 A*, 1523 (2017) 72-79.

963 [90] Y. Ladner, S. Mas, G. Coussot, K. Bartley, J. Montels, J. Morel, C. Perrin, Integrated microreactor for
964 enzymatic reaction automation: An easy step toward the quality control of monoclonal antibodies,
965 *Journal of Chromatography A*, 1528 (2017) 83-90.

966 [91] Y. He, N.A. Lacher, W. Hou, Q. Wang, C. Isele, J. Starkey, M. Ruesch, Analysis of Identity, Charge
967 Variants, and Disulfide Isomers of Monoclonal Antibodies with Capillary Zone Electrophoresis in an
968 Uncoated Capillary Column, *Analytical Chemistry*, 82 (2010) 3222-3230.

969 [92] Y. He, C. Isele, W. Hou, M. Ruesch, Rapid analysis of charge variants of monoclonal antibodies with
970 capillary zone electrophoresis in dynamically coated fused-silica capillary, *Journal of Separation Science*,
971 34 (2011) 548-555.

972 [93] B. Moritz, V. Schnaible, S. Kiessig, A. Heyne, M. Wild, C. Finkler, S. Christians, K. Mueller, L. Zhang, K.
973 Furuya, M. Hassel, M. Hamm, R. Rustandi, Y. He, O.S. Solano, C. Whitmore, S.A. Park, D. Hansen, M.

974 Santos, M. Lies, Evaluation of capillary zone electrophoresis for charge heterogeneity testing of
975 monoclonal antibodies, *Journal of Chromatography B*, 983–984 (2015) 101-110.

976 [94] C.E. Espinosa-de la Garza, R.D. Salazar-Flores, N.O. Perez, L.F. Flores-Ortiz, E. Medina-Rivero,
977 Capillary Electrophoresis Separation of Monoclonal Antibody Isoforms Using a Neutral Capillary, *J. Vis.*
978 *Exp.*, DOI 10.3791/55082(2017) 7.

979 [95] D. Suba, Z. Urbanyi, A. Salgo, Method development and qualification of capillary zone
980 electrophoresis for investigation of therapeutic monoclonal antibody quality, *Journal of Chromatography*
981 *B-Analytical Technologies in the Biomedical and Life Sciences*, 1032 (2016) 224-229.

982 [96] Y. Zhao, L. Sun, M.D. Knierman, N.J. Dovichi, Fast separation and analysis of reduced monoclonal
983 antibodies with capillary zone electrophoresis coupled to mass spectrometry, *Talanta*, 148 (2016) 529-
984 533.

985 [97] B. Moritz, V. Locatelli, M. Niess, A. Bathke, S. Kiessig, B. Entler, C. Finkler, H. Wegele, J. Stracke,
986 Optimization of capillary zone electrophoresis for charge heterogeneity testing of biopharmaceuticals
987 using enhanced method development principles, *Electrophoresis*, 38 (2017) 3136-3146.

988 [98] C. Neusüß, J. Römer, O. Höcker, K. Jooß, Chapter 12 - Coupling of Capillary Electromigration
989 Techniques to Mass Spectrometry A2 - Poole, Colin F, *Capillary Electromigration Separation Methods*,
990 Elsevier2018, pp. 293-311.

991 [99] J.-M. Busnel, J. Josserand, N. Lion, H.H. Girault, Ionophoretic Fraction Collection for Coupling
992 Capillary Zone Electrophoresis with Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry,
993 *Analytical Chemistry*, 81 (2009) 3867-3872.

994 [100] M. Biacchi, R. Bhajun, N. Saïd, A. Beck, Y.N. François, E. Leize-Wagner, Analysis of monoclonal
995 antibody by a novel CE-UV/MALDI-MS interface, *Electrophoresis*, 35 (2014) 2986-2995.

996 [101] M.W. Vannatta, C.D. Whitmore, N.J. Dovichi, CE-MALDI interface based on inkjet technology,
997 *Electrophoresis*, 30 (2009) 4071-4074.

998 [102] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, Online mass-spectrometric detection for
999 capillary zone electrophoresis, *Analytical Chemistry*, 59 (1987) 1230-1232.

1000 [103] J. Giorgetti, A. Lechner, E. Del Nero, A. Beck, Y.-N. François, E. Leize-Wagner, Intact monoclonal
1001 antibodies separation and analysis by sheathless capillary electrophoresis-mass spectrometry, *European*
1002 *Journal of Mass Spectrometry*, 0 (2018) 1-9.

1003 [104] A.M. Belov, L. Zang, R. Sebastiano, M.R. Santos, D.R. Bush, B.L. Karger, A.R. Ivanov, Complementary
1004 middle-down and intact monoclonal antibody proteoform characterization by capillary zone
1005 electrophoresis – mass spectrometry, *Electrophoresis*, 39 (2018) 2069-2082.

1006 [105] J. Schlecht, K. Jooß, C. Neusüß, Two-dimensional capillary electrophoresis-mass spectrometry (CE-
1007 CE-MS): coupling MS-interfering capillary electromigration methods with mass spectrometry, *Analytical*
1008 *and Bioanalytical Chemistry*, DOI 10.1007/s00216-018-1157-9(2018).

1009 [106] L.A. Gennaro, O. Salas-Solano, S. Ma, Capillary electrophoresis–mass spectrometry as a
1010 characterization tool for therapeutic proteins, *Analytical Biochemistry*, 355 (2006) 249-258.

1011 [107] R. Gahoual, J.-M. Busnel, A. Beck, Y.-N. François, E. Leize-Wagner, Full Antibody Primary Structure
1012 and Microvariant Characterization in a Single Injection Using Transient Isotachopheresis and Sheathless
1013 Capillary Electrophoresis–Tandem Mass Spectrometry, *Analytical Chemistry*, 86 (2014) 9074-9081.

1014 [108] R. Gahoual, A. Beck, Y.-N. François, E. Leize-Wagner, Independent highly sensitive characterization
1015 of asparagine deamidation and aspartic acid isomerization by sheathless CZE-ESI-MS/MS, *Journal of*
1016 *Mass Spectrometry*, 51 (2016) 150-158.

1017 [109] N. Said, R. Gahoual, L. Kuhn, A. Beck, Y.-N. François, E. Leize-Wagner, Structural characterization of
1018 antibody drug conjugate by a combination of intact, middle-up and bottom-up techniques using

1019 sheathless capillary electrophoresis – Tandem mass spectrometry as nanoESI infusion platform and
1020 separation method, *Analytica Chimica Acta*, 918 (2016) 50-59.

1021 [110] O.O. Dada, Y.M. Zhao, N. Jaya, O. Salas-Solano, High-Resolution Capillary Zone Electrophoresis with
1022 Mass Spectrometry Peptide Mapping of Therapeutic Proteins: Peptide Recovery and Post-translational
1023 Modification Analysis in Monoclonal Antibodies and Antibody-Drug Conjugates, *Analytical Chemistry*, 89
1024 (2017) 11236-11242.

1025 [111] A. Beck, E. Wagner-Rousset, M.C. Bussat, M. Lokteff, C. Klinguer-Hamour, J.F. Haeuw, L. Goetsch, T.
1026 Wurch, A. Van Dorselaer, N. Corvaia, Trends in Glycosylation, Glycoanalysis and Glycoengineering of
1027 Therapeutic Antibodies and Fc-Fusion Proteins, *Current Pharmaceutical Biotechnology*, 9 (2008) 482-
1028 501.

1029 [112] R. Jefferis, Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of
1030 action, *Trends in Pharmacological Sciences*, 30 (2009) 356-362.

1031 [113] D. Reusch, M.L. Tejada, Fc glycans of therapeutic antibodies as critical quality attributes,
1032 *Glycobiology*, 25 (2015) 1325-1334.

1033 [114] D. Ayoub, W. Jabs, A. Resemann, W. Evers, C. Evans, L. Main, C. Baessmann, E. Wagner-Rousset, D.
1034 Suckau, A. Beck, Correct primary structure assessment and extensive glyco-profiling of cetuximab by a
1035 combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry
1036 techniques, *mAbs*, 5 (2013) 699-710.

1037 [115] M. Schiestl, T. Stangler, C. Torella, T. Čepeljnik, H. Toll, R. Grau, Acceptable changes in quality
1038 attributes of glycosylated biopharmaceuticals, *Nature Biotechnology*, 29 (2011) 310.

1039 [116] A. Beck, H. Diemer, D. Ayoub, F. Debaene, E. Wagner-Rousset, C. Carapito, A. Van Dorselaer, S.
1040 Sanglier-Cianferani, Analytical characterization of biosimilar antibodies and Fc-fusion proteins, *Trac-*
1041 *Trends in Analytical Chemistry*, 48 (2013) 81-95.

1042 [117] A. Banazadeh, L. Veillon, K.M. Wooding, M. Zabet-moghaddam, Y. Mechref, Recent advances in
1043 mass spectrometric analysis of glycoproteins, *Electrophoresis*, 38 (2017) 162-189.

1044 [118] C. Varadi, S. Mittermayr, S. Millan-Martin, J. Bones, Quantitative twoplex glycan analysis using C-
1045 12(6) and C-13(6) stable isotope 2-aminobenzoic acid labelling and capillary electrophoresis mass
1046 spectrometry, *Analytical and Bioanalytical Chemistry*, 408 (2016) 8691-8700.

1047 [119] J. Giorgetti, V. D’Atri, J. Canonge, A. Lechner, D. Guillaume, O. Colas, E. Wagner-Rousset, A. Beck, E.
1048 Leize-Wagner, Y.-N. François, Monoclonal antibody N-glycosylation profiling using capillary
1049 electrophoresis – Mass spectrometry: Assessment and method validation, *Talanta*, 178 (2018) 530-537.

1050 [120] G.S.M. Kammeijer, B.C. Jansen, I. Kohler, A.A.M. Heemskerk, O.A. Mayboroda, P.J. Hensbergen, J.
1051 Schappler, M. Wührer, Sialic acid linkage differentiation of glycopeptides using capillary electrophoresis
1052 – electrospray ionization – mass spectrometry, *Scientific Reports*, 7 (2017) 3733.

1053 [121] K. Jooß, S.W. Meckelmann, J. Klein, O.J. Schmitz, C. Neusüß, Capillary zone electrophoresis coupled
1054 to drift tube ion mobility-mass spectrometry for the analysis of native and APTS-labeled N-glycans,
1055 *Analytical and Bioanalytical Chemistry*, DOI 10.1007/s00216-018-1515-7(2018).

1056 [122] M. Biacchi, R. Gahoual, N. Said, A. Beck, E. Leize-Wagner, Y.-N. François, Glycoform Separation and
1057 Characterization of Cetuximab Variants by Middle-up Off-Line Capillary Zone Electrophoresis-
1058 UV/Electrospray Ionization-MS, *Analytical Chemistry*, 87 (2015) 6240-6250.

1059 [123] Y.-N. François, M. Biacchi, N. Said, C. Renard, A. Beck, R. Gahoual, E. Leize-Wagner, Characterization
1060 of cetuximab Fc/2 dimers by off-line CZE-MS, *Analytica Chimica Acta*, 908 (2016) 168-176.

1061 [124] M. Han, B.M. Rock, J.T. Pearson, D.A. Rock, Intact mass analysis of monoclonal antibodies by
1062 capillary electrophoresis—Mass spectrometry, *Journal of Chromatography B*, 1011 (2016) 24-32.

1063 [125] R. Haselberg, T. De Vijlder, R. Heukers, M.J. Smit, E.P. Romijn, G.W. Somsen, E. Domínguez-Vega,
1064 Heterogeneity assessment of antibody-derived therapeutics at the intact and middle-up level by low-
1065 flow sheathless capillary electrophoresis-mass spectrometry, *Analytica Chimica Acta*, 1044 (2018) 181-
1066 190.

1067 [126] R.L.C. Voeten, I.K. Ventouri, R. Haselberg, G.W. Somsen, *Capillary Electrophoresis: Trends and*
1068 *Recent Advances*, *Analytical Chemistry*, 90 (2018) 1464-1481.

1069 [127] E.A. Redman, N.G. Batz, J.S. Mellors, J.M. Ramsey, *Integrated Microfluidic Capillary Electrophoresis-*
1070 *Electrospray Ionization Devices with Online MS Detection for the Separation and Characterization of*
1071 *Intact Monoclonal Antibody Variants*, *Analytical Chemistry*, 87 (2015) 2264-2272.

1072 [128] E.A. Redman, J.S. Mellors, J.A. Starkey, J.M. Ramsey, *Characterization of Intact Antibody Drug*
1073 *Conjugate Variants Using Microfluidic Capillary Electrophoresis-Mass Spectrometry*, *Analytical*
1074 *Chemistry*, 88 (2016) 2220-2226.

1075 [129] K. Jooß, J. Hühner, S. Kiessig, B. Moritz, C. Neusüß, *Two-dimensional capillary zone electrophoresis-*
1076 *mass spectrometry for the characterization of intact monoclonal antibody charge variants, including*
1077 *deamidation products*, *Analytical and Bioanalytical Chemistry*, 409 (2017) 6057-6067.

1078 [130] A. Gerster, C. Wodarczyk, B. Reichenbacher, J. Kohler, A. Schulze, F. Krause, D. Muller, *A simple*
1079 *method to determine IgG light chain to heavy chain polypeptide ratios expressed by CHO cells*,
1080 *Biotechnology Letters*, 38 (2016) 2043-2049.

1081 [131] S. Paul, J. Connor, T. Nessor, P. Haytko, K. Boakye, M.L. Chiu, H.Y. Jiang, *An efficient process of*
1082 *generating bispecific antibodies via controlled Fab-arm exchange using culture supernatants*, *Protein*
1083 *Expr. Purif.*, 121 (2016) 133-140.

1084 [132] I. Karageorgos, E.S. Gallagher, C. Galvin, D.T. Gallagher, J.W. Hudgens, *Biophysical characterization*
1085 *and structure of the Fab fragment from the NIST reference antibody, RM 8671*, *Biologicals*, 50 (2017) 27-
1086 34.

1087 [133] A. Turner, K. Yandrofski, S. Telikepalli, J. King, A. Heckert, J. Filliben, D. Ripple, J.E. Schiel,
1088 *Development of orthogonal NISTmAb size heterogeneity control methods*, *Analytical and Bioanalytical*
1089 *Chemistry*, 410 (2018) 2095-2110.

1090 [134] O.O. Dada, Y. Zhao, N. Jaya, O. Salas-Solano, *High-Resolution Capillary Zone Electrophoresis with*
1091 *Mass Spectrometry Peptide Mapping of Therapeutic Proteins: Improved Separation with Mixed*
1092 *Aqueous–Aprotic Dipolar Solvents (N,N-Dimethylacetamide and N,N-Dimethylformamide) as the*
1093 *Background Electrolyte*, *Analytical Chemistry*, 89 (2017) 11227-11235.

1094 [135] L. Sánchez-Hernández, C. Montealegre, S. Kiessig, B. Moritz, C. Neusüß, *In-capillary approach to*
1095 *eliminate SDS interferences in antibody analysis by capillary electrophoresis coupled to mass*
1096 *spectrometry*, *Electrophoresis*, 38 (2017) 1044-1052.

1097 [136] Z. Zhang, T. Albanetti, T. Linkous, C.J. Larkin, R. Schoner, J.B. McGivney, N.J. Dovichi, *Comprehensive*
1098 *analysis of host cell impurities in monoclonal antibodies with improved sensitivity by capillary zone*
1099 *electrophoresis mass spectrometry*, *Electrophoresis*, 38 (2017) 401-407.

1101

1102

1103

1104

1105 **Caption**

1106

1107 **Fig. 1.** Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) ob-
1108 tained by CE-SDS (a) reduced and (b) non-reduced conditions. The internal standard peak, light chain peak, heavy
1109 chain peak, monomer peak, and increased peaks are indicated. Especially, the increased peak close to the internal
1110 standard is the focus of this study. Reprinted from [35]. Copyright (2017) Elsevier.

1111

1112 **Fig. 2.** Online CE-LIF-MS analysis of Teal™-labeled NISTmAb released *N*-glycans. LIF and MS base peak elec-
1113 tropherogram (m/z 620-1,800) of Teal-NISTmAb glycans using ammonium hydroxide based BGE. Reprinted from
1114 [28]. Copyright (2017) Wiley.

1115

1116 **Fig. 3.** Imaged capillary isoelectric focusing for profile of the isolated charge variants, the biosimilar product and
1117 Avastin. Reprinted from [76].

1118

1119 **Fig. 4.** Charge profiles of a mixture comprising valid bevacizumab, trastuzumab and rituximab solutions (blue
1120 trace) and expired solutions (orange trace). Reprinted from [87]. Copyright (2018) Wiley

1121

1122 **Fig. 5.** Deconvolution spectrums showing major glycosylation states of the intact mAb. Quantitative ratios of
1123 each population of the mAb (2X-glycosylated, 1X-glycosylated, aglycosylated) are specified. Reprinted from [104].
1124 Copyright (2018) Wiley.

1125

1126

1127

Table 1. Application and CE methods for the analysis of IgG, biosimilars, Fc-fusion proteins and ADCs in the years 2016-2018

Abbreviation: PBS phosphate buffer saline; EACA ϵ -amino caproic acid; TETA triethylenetetramine; BFS bare fused silica; LPA linear polyacrylamide; PVA polyvinyl alcohol; HPMC hydroxypropyl-methyl cellulose; HPC hydroxypropyl cellulose; FC fluorocarbon; PB polybren; DS dextran sulfate; PEO Polyethylene oxide; APS aminopropylsilane; APTES aminopropyltriethoxysilane

CE Mode	Detection	Sample	Application	BGE	Cap. coating	Reference
CE-SDS mGE	UV 220 nm	mAb	Size heterogeneity of reduced mAb	SDS gel separation buffer	BFS	[39]
CE-SDS	UV 220 nm	Ado-trastuzumab em-tansine & biosimilar	Biosimilarity assessment, size heterogeneity of intact and reduced mAb	SDS gel separation buffer	BFS	[45]
CE-SDS	UV 220 nm	IgG1	Disulfide bond reduction	SDS-MW gel buffer	BFS	[30]
CE-SDS mGE	UV 220 nm	IgGs mAb	Purity and stability assessment Intra and extracellular evaluation of LC:HC ratio	SDS-MW gel buffer	BFS	[31] [130]
CE-SDS	LIF 488 nm 520 nm	IgG	High-throughput N-glycan analysis	NCHO Carbohydrate Separation Buffer	NCHO separation capillary	[25]
CE-SDS mGE	LIF Indirect LIF	IgG bsAb	N-glycan analysis Size heterogeneity of reduced and intact mAb	1% PEO in 25 mM lithium acetate (pH 4.75) HT Protein Express Sample Buffer	NCHO separation capillary	[26] [131]
CE-SDS	UV 220 nm	Recombinant IgG1	Size heterogeneity of intact and reduced mAb	SDS-MW gel buffer	BFS	[38]
CE-SDS	LIF 488 nm 520 nm	Ranibizumab and biosimilar (labeled)	Biosimilarity assessment, size heterogeneity of intact mAb	0,1 M Tris-HCl/5% SDS pH 8	BFS	[33]
CE-SDS	LIF 488 nm 520 nm	mAbs	Glycan profiling	89 mM aqueous Tris-boric solution pH 8.64	PEGDMA or PAA	[51]
CE-SDS	UV 220 nm	mAb A	Size heterogeneity of reduced and intact mAb	SDS gel separation buffer (AB Sciex)	BFS	[35]
CE-SDS	UV 220 nm	Recombinant IgG1	Quantitation of impurity from degraded mAb	SDS sieving gel buffer (Beckman)	BFS	[36]
CE-SDS	UV 220 nm	IgG1	Size heterogeneity of reduced and intact mAb	SDS-MW gel buffer	BFS	[38]

CE-SDS	UV 220 nm	Tocilizumab & biosimilar	Biosimilarity assessment, size heterogeneity of reduced and intact mAb	SDS sieving gel buffer (Beckman)	BFS	[34]
CE-SDS	UV 220 nm	NIST mAb	Fab fragment characterization	SDS-MW gel buffer	BFS	[132]
CE-SDS	LIF 488 nm 520 nm	hIgG	N-glycan characterization	1% HPC (w/v), 1% HEC (w/v), 80 mM MES, 40 mM TRIS	PVA	[23]
CE-SDS	LIF 325 nm 405 nm	Rituximab, Trastuzumab & Bevacizumab	N-glycan analysis	100 mM Tris-borate buffer (pH 8.3) 5% PEG	DB-1	[29]
CE-SDS		Lys-conjugated ADC	Size heterogeneity of reduced and intact ADC			[43]
CE-SDS	LIF	hIgG	N-glycan characterization of irradiated mAb	N-CHO	BFS	[50]
CE-SDS	LIF	NISTmAb RM 8671	Glycan profiling	POP 7 polymer		[27]
CE-SDS-LIF-MS	LIF 488 nm 520 nm, LTQ-Orbitrap XL	NISTmAb RM 8671	Glycan analysis	NH4OH or AcNH4	BFS	[28]
CE-SDS mGE	LIF 488 nm 520 nm	Fc fragment of adalimumab	Glycan absolute quantification	HR-NCHO	BFS	[52]
icIEF	UV 280 nm	mAb-DM1 ADCs	Drug Load Distribution and DAR determination		FC	[82]
icIEF	UV 280 nm	3 recombinant hmAb	Charge heterogeneity of intact mAb, comparison with FFE	Amph : 3-10 Anol 80 mM H3PO4 Cath : 100 mM NaOH	Coated capillary from Convergent Bioscience	[78]
cIEF	UV 280 nm	IgG1	Charge heterogeneity, structural characterization	Amph : 3-10 Anol 200 mM H3PO4 Cath : 300 mM NaOH	eCAP™ Neutral	[30]
icIEF	UV 280 nm	Rituximab & biosimilar	Charge heterogeneity of intact mAb and mAb's fragments	Amph : 2% 5-8 & 8-10.5 Anol : 0,08 M H3PO4 Cath 0.1 M	FC	[75]

				NaOH + 0,1% MC in both		
icIEF	UV 280 nm	IgG1	Charge heterogeneity, purity assessment	Amph : 3-10 : 8-10.5 (1:1)		[76]
icIEF	UV 280 nm	23 marketed mAbs	Charge heterogeneity, pl determination	Amph : 3-10 Anol : 80 mM H3PO4 Cath : 100 mM NaOH + 0,1% MC in both	FC	[73]
icIEF	UV 280 nm	Tocilizumab & biosimilar	Charge heterogeneity of intact mAb	Amph : 3-10		[34]
icIEF	UV 280 nm	Antibody A	Charge heterogeneity of native mAb	Amph : 3-10		[77]
icIEF	UV 280 nm	IgG1, IgG2 & IgG4	Interlaboratory study Charge heterogeneity of intact mAb, purity assessment	Amph : 3-10 + 1%MC	FC	[72]
icIEF	UV 280 nm	IgG1	Charge heterogeneity of intact mAb	Anol : acid Cath : base		[74]
cIEF-MS icIEF-UV	UV 280 nm & TOF	Infliximab, Trastuzumab, Bevacizumab & Cetuximab	Charge heterogeneity of intact mAb	cIEF-MS : Amph : 3-10 Anol : 1% formic acid Cath : 0,2 N NH4OH + 15 % glycerol in both icIEF-UV : Amph : 3-10 Anol : 80 mM H3PO4 Cath : NaOH + 0,1% MC in both	cIEF-MS : Neutral coating PS1 icIEF-UV : FC	[67]
cIEF CZE	UV 280 nm	NISTmAb RM 8671	Charge heterogeneity	Amph : 3-10 : 8-10.5 (1:3) Anol 200 mM H3PO4 Cath : 300 mM NaOH	Neutral coating (Sciex PN 477441)	[79]

CZE icIEF	UV 214 nm	mAbs	Characterization of mAb variants	40 mM EACA; 2 mM TETA pH 6.0	HPMC	[59]
CZE	UV 214 nm	mAb ADC	Method validation	380 mM EACA, 1.9 mM TETA, pH 5.7 0.05% HPMC	HPMC	[84]
CZE		mAb	Method development for mAbs quality	50 mM EACA; 1 mM TETA pH 4.1	HPMC	[95]
CZE	UV 280 nm	Bevacizumab and bio- similar	Characterization of mAb charge variants	500 mM EACA	BFS	[96]
CZE	214 nm	mAb	Characterization of mAb isoforms	200 mM EACA; 30 mM lithium acetate	HPMC	[94]
CZE CE-SDS		Human IgG	Glycosylation profiling	25 mM NH4Ac, pH 4,75	PVA	[23]
CZE CE-SDS	UV 214 nm	Ranibizumab; Ra- zumab (biosimilar)	Serine to Asparagine sequence variation	400 mM EACA; 2 mM TETA pH 5.7	BFS	[33]
CZE	UV 280 nm	mAb; ADC	Charge variants and disulfide iso- mers of mAbs and ADCs	400 mM EACA; 2 mM TETA pH 5.7	HPMC	[89]
CZE	UV 214 nm	mAbs	Charge heterogeneity of mAbs	40 mM EACA; 2 mM TETA pH 6.0	HPC; HPMC	[97]
CZE	UV 280 nm	Cetuximab, Trastu- zumab, Rituximab	Characterization of mAb variants	50 mM Phosphate buffer	Fibrin	[88]
CZE	UV 214 nm	17 marketed mAbs	Characterization of mAb variants	200 mM BisTris pH 7.0	PEO; HPMC	[87]
CZE	UV 280 nm	mAbs	In-line tryptic digestion of mAbs automation	25 mM EACA; citric acid pH 5.0	PEO	[90]

CZE	UV 214 nm	NISTmAb	NISTmAb homogeneity value assignment and stability	400 mM EACA; 2 mM TETA pH 5.7; 0.03% Tween 20	HPMC	[32]
CZE	UV 214 nm	NISTmAb	Charge heterogeneity of mAbs	400 mM EACA; 2 mM TETA pH 5.7; 0.03% Tween 20	HPMC	[133]
mCE-ESI-MS	ESI-TOF-MS	IgG1, IgG2, ADC, Infliximab	Characterization of mAb variants	100 mM PBS + 10 g/L NHS-PEG	APS	[127]
CZE-MS	MALDI-TOF-MS	Cetuximab	Fc/2 dimers characterization	Inlet vial: 200 mM EACA/25 mM C ₂ H ₇ NO ₂ Outlet vial: 25 mM C ₂ H ₇ NO ₂	HPC	[123]
CZE-MS	ESI-QTOF-MS/MS	Trastuzumab	Asparagine deamidation and iso Aspartic acid isomerization	10% CH ₃ CO ₂ H	BFS	[108]
CZE-MS	ESI-TOF-MS	IgG1	Charge heterogeneity of reduced mAb Middle up analysis	10% - 30% CH ₃ CO ₂ H SL: 50% CH ₄ O/0.5% COOH	LPA	[124]
mCE-ESI-MS	ESI-QTOF-MS	ADC	Characterization of intact ADC	10% C ₃ H ₈ O + 0.2% CH ₃ CO ₂ H	PEG, APS	[128]
CZE-MS	ESI-QTOF-MS/MS	Brentuximab vedotin ADC	Intact, middle-up and bottom-up characterization	10% CH ₃ CO ₂ H	BFS	[109]
CZE-MS	ESI-QTOF-MS/MS	Cetuximab	Glycan analysis	25 mM C ₂ H ₇ NO ₂	PVA	[118]
CZE-MS	ESI-Orbitrap-MS/MS	standards proteins <i>E. Coli</i> ribosomal proteins trastuzumab	Proteins, protein complexes and organellar proteomes analysis	40 mM C ₂ H ₇ NO ₂	BFS PA	[22]
CZE-MS	MALDI-TOF-MS	Cetuximab	MAB Fc/2 charge variants analysis	Inlet vial: 200 mM EACA/25 mM C ₂ H ₇ NO ₂ Outlet vial: 25 mM C ₂ H ₇ NO ₂	HPC	[21]

CZE-MS	ESI-Orbitrap-MS/MS	ADC	Peptide mapping of therapeutic proteins	20% CH ₃ CO ₂ H 15% DMA 1% <i>m</i> -nitro-benzyl alcohol	LPA	[110]
CZE-MS	ESI-Orbitrap-MS/MS	mAb	Improvement peptide mapping of therapeutic proteins	20% CH ₃ CO ₂ H/15% DMA or DMF	LPA	[134]
CZE-MS	ESI-QTOF-MS/MS	Rituximab; palivizumab; natalizumab; nivolumab; trastuzumab; panitumumab; adalimumab; infliximab-remicade; infliximab-remsima; infliximab inflectra	Mabs N-glycosylation profiling	10% CH ₃ CO ₂ H	BFS	[119]
CZE-MS	ESI-QTOF-MS/MS	Human polyclonal IgG recombinant monoclonal IgG1	Sialic acid linkage differentiation of glycopeptides	10% CH ₃ CO ₂ H	BFS	[120]
CZE-MS	ESI-QTOF-MS/MS	IgG1	Eliminate SDS interferences in antibody analysis	1 M CH ₃ CO ₂ H SL: C ₃ H ₈ O/0.2% CH ₂ O ₂ (50:50)	PVA PB	[135]
CZE-MS	ESI-Orbitrap-MS/MS	mAb	Analysis of host cell impurities in mAbs	2 M CH ₃ CO ₂ H SL: 10% CH ₃ OH/0.5% CH ₂ O ₂	LPA	[136]
CZE-MS	ESI-Orbitrap-MS/MS	IgG1	Middle-down and intact mAb proteoform characterization	50% CH ₃ OH/1% CH ₂ O ₂ 10% C ₃ H ₈ O/0.2% CH ₂ O ₂	M7C4I PA	[104]
mCE-ESI-MS	ESI-Orbitrap-MS/MS	NIST mAb	NIST mAb proteoforms and glycoforms characterization	0.2% CH ₃ CO ₂ H/10% C ₃ H ₈ O	APS	[27]

cIEF-MS	ESI-TOF-MS	Infliximab, trastuzumab, cetuximab, bevacizumab	Intact mAb charge variants analysis	Amph: 3-10 + 5-20% glycerol Anol: 1% CH ₂ O ₂ + 15% glycerol Cath: 0.2% NH ₄ OH + 15% glycerol	Neutral PS1	[67]
cIEF-MS	ESI-TOF-MS	Cetuximab	Intact and middle-up characterization	Amph: 3-10 + 5-20% glycerol Anol: 1% CH ₂ O ₂ + 15% glycerol Cath: 0.2% NH ₄ OH + 15% glycerol	Neutral PS1	[68]
CZE-MS	ESI-QTOF-MS	Infliximab, trastuzumab, ustekinumab	Heretogeneity assessment of antibody derived therapeutics	10% CH ₃ CO ₂ H	Neutral	[125]
CZE-MS	ESI-QTOF-MS	Trastuzumab, rituximab, palivizumab	Intact mAb charge variants analysis	3% CH ₃ CO ₂ H	PEI	[103]
CZE-MS	ESI-Orbitrap-MS/MS	mAb	Glycosylation profiling	C ₂ H ₇ NO ₂ SL: 10 mM C ₂ H ₇ NO ₂ /C ₂ H ₃ N (20/80)	BFS	[28]
CZE-MS	IMS-Q-TOF-MS	mAb	Glycosylation profiling	0.1 M EACA, 0.5 M NH ₃ solved in MeOH/H ₂ O 1:1	BFS	[121]
cIEF-CZE-MS	UV; ESI Q-TOF-MS	Deglycosylated mAb	Charge heterogeneity	cIEF :Amph : 3-10 ou 6-8 Anol H ₃ PO ₄ Cath NaOH CZE : 0,2 M HCOOH	PVA both	[69]
CZE-CZE-MS	UV 214 nm ; ESI Q-TOF-MS	Trastuzumab	Intact mAb charge variants	CZE 1 : 380 mM EACA, 1,9 mM TEA and 0,05% w/w HPMC (pH	CZE 1 : BFS CZE 2 : PVA	[129]

5.7) CZE 2 : 2 M acetic acid

cIEF-CZE-MS	UV 280 nm; ESI-Q-TOF-MS	Trastuzumab	Charge heterogeneity	icIEF : Amph : AESlyte, Anol : 80 mM H3PO4, Cath : 80 mM NaOH 0.1 % MC both CZE : 1M acetic acid	PVA both	[70]
--------------------	-------------------------	-------------	----------------------	--	----------	------

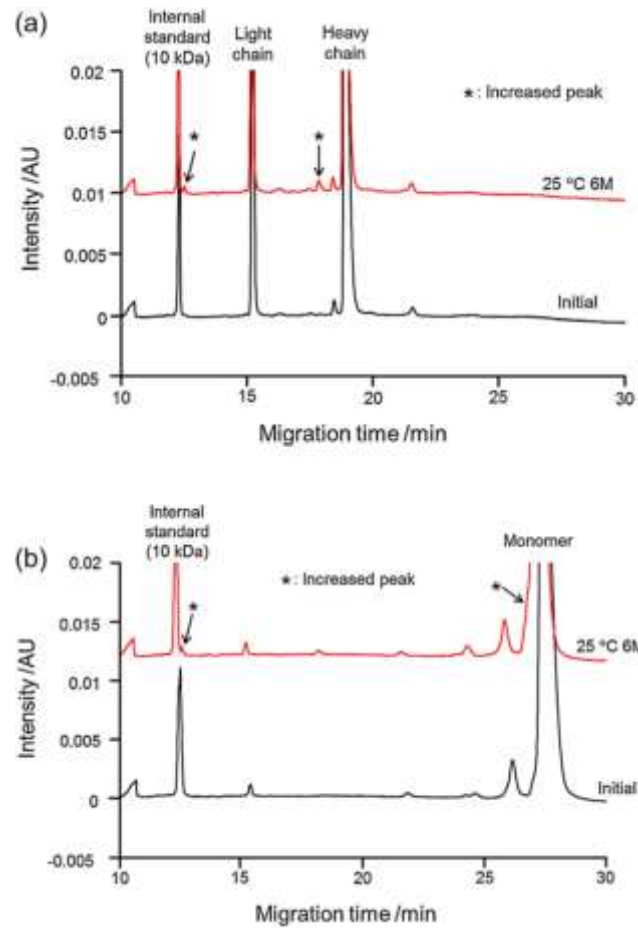


Fig. 1. Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) obtained by CE-SDS (a) reduced and (b) non-reduced conditions. The internal standard peak, light chain peak, heavy chain peak, monomer peak, and increased peaks are indicated. Especially, the increased peak close to the internal standard is the focus of this study. Reprinted from [35]. Copyright (2017) Elsevier.

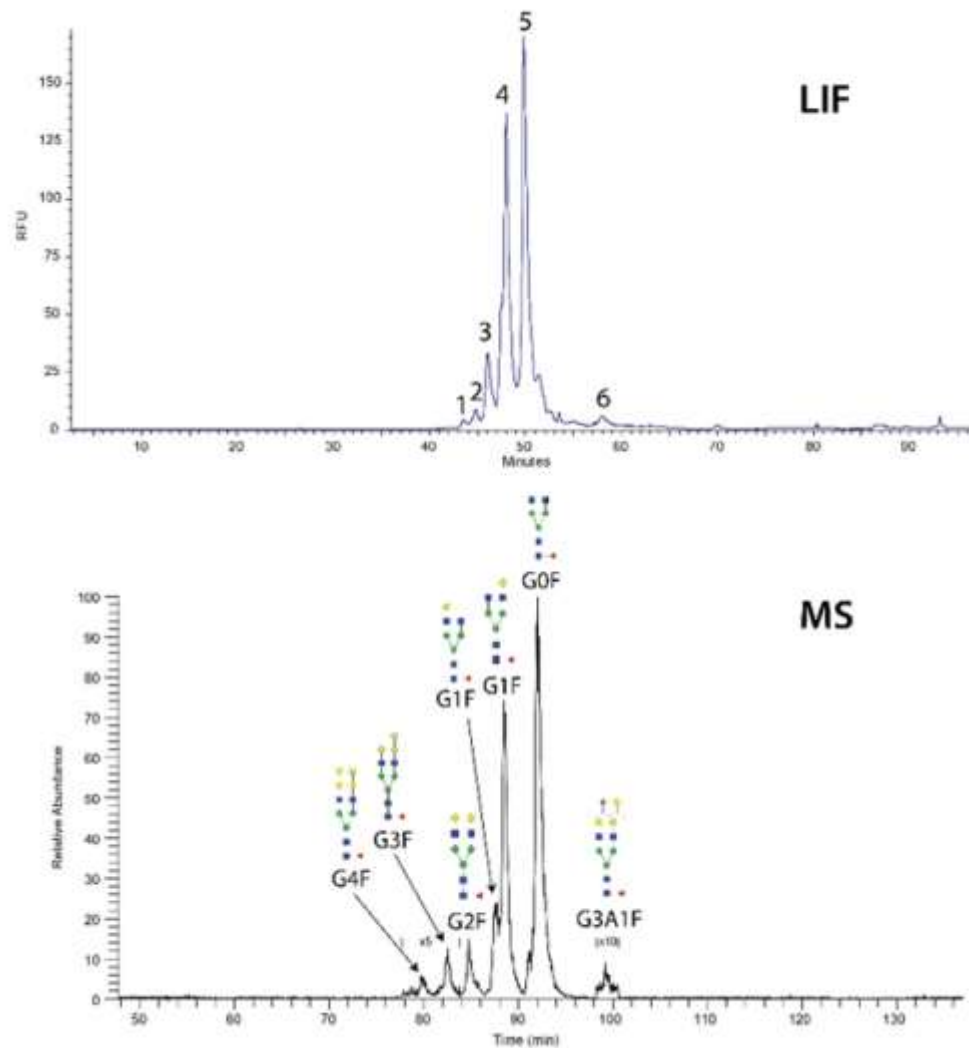


Fig. 2. Online CE-LIF-MS analysis of Teal™-labeled NISTmAb released *N*-glycans. LIF and MS base peak electropherogram (m/z 620-1,800) of Teal-NISTmAb glycans using ammonium hydroxide based BGE. Reprinted from [28]. Copyright (2017) Wiley.

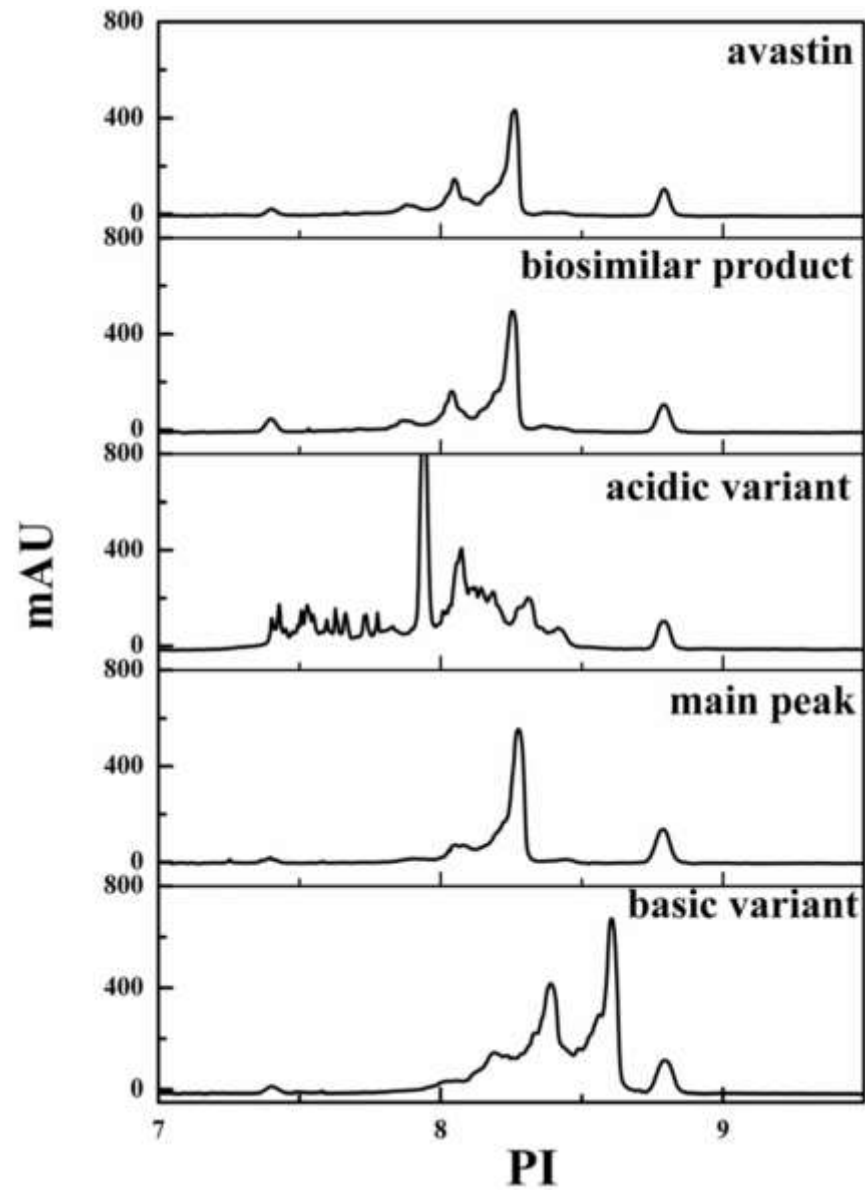


Fig. 3. Imaged capillary isoelectric focusing for profile of the isolated charge variants, the biosimilar product and Avastin. Reprinted from [76].

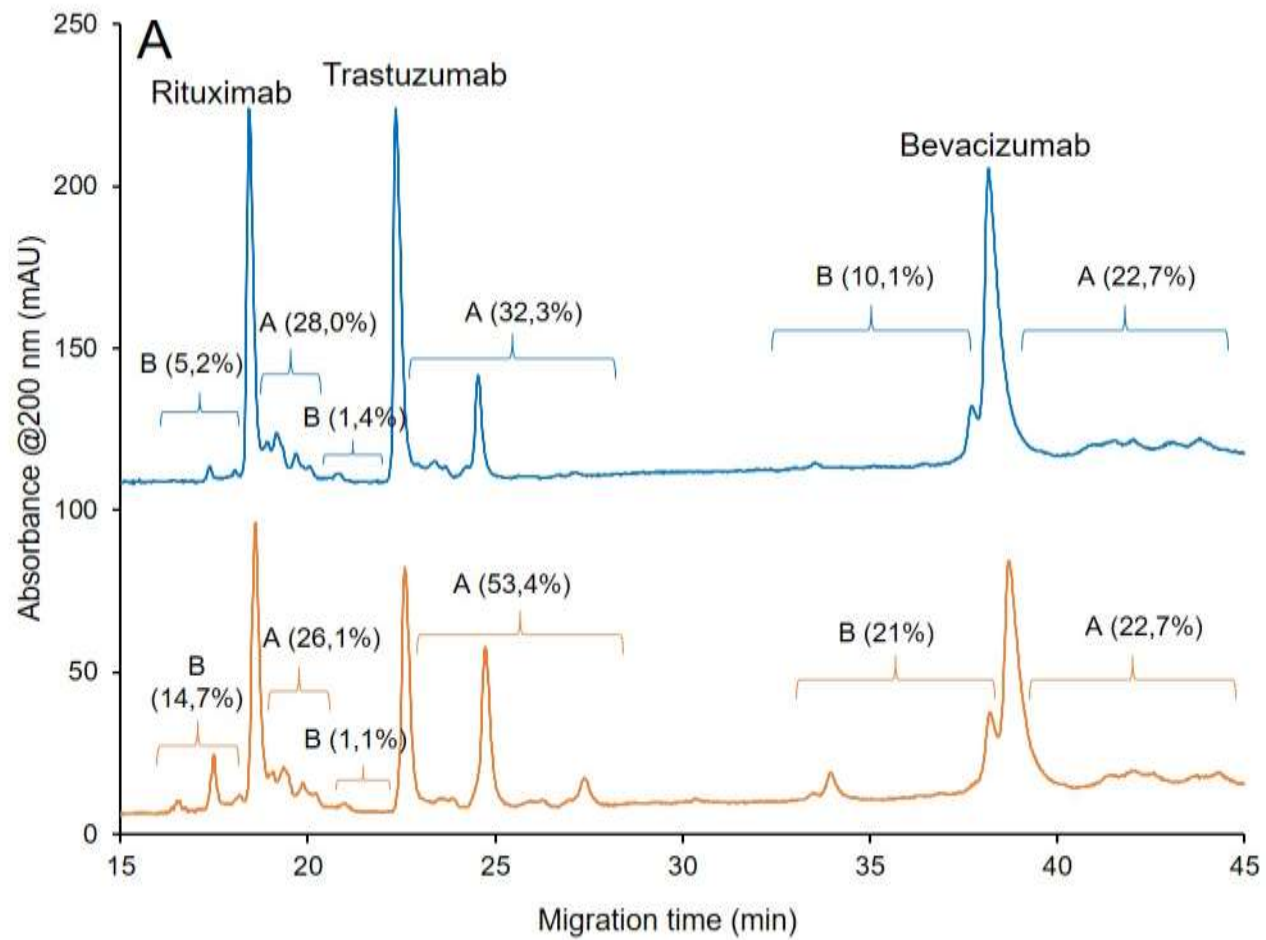


Fig. 4. Charge profiles of a mixture comprising valid bevacizumab, trastuzumab and rituximab solutions (blue trace) and expired solutions (orange trace). Reprinted from [87]. Copyright (2018) Wiley

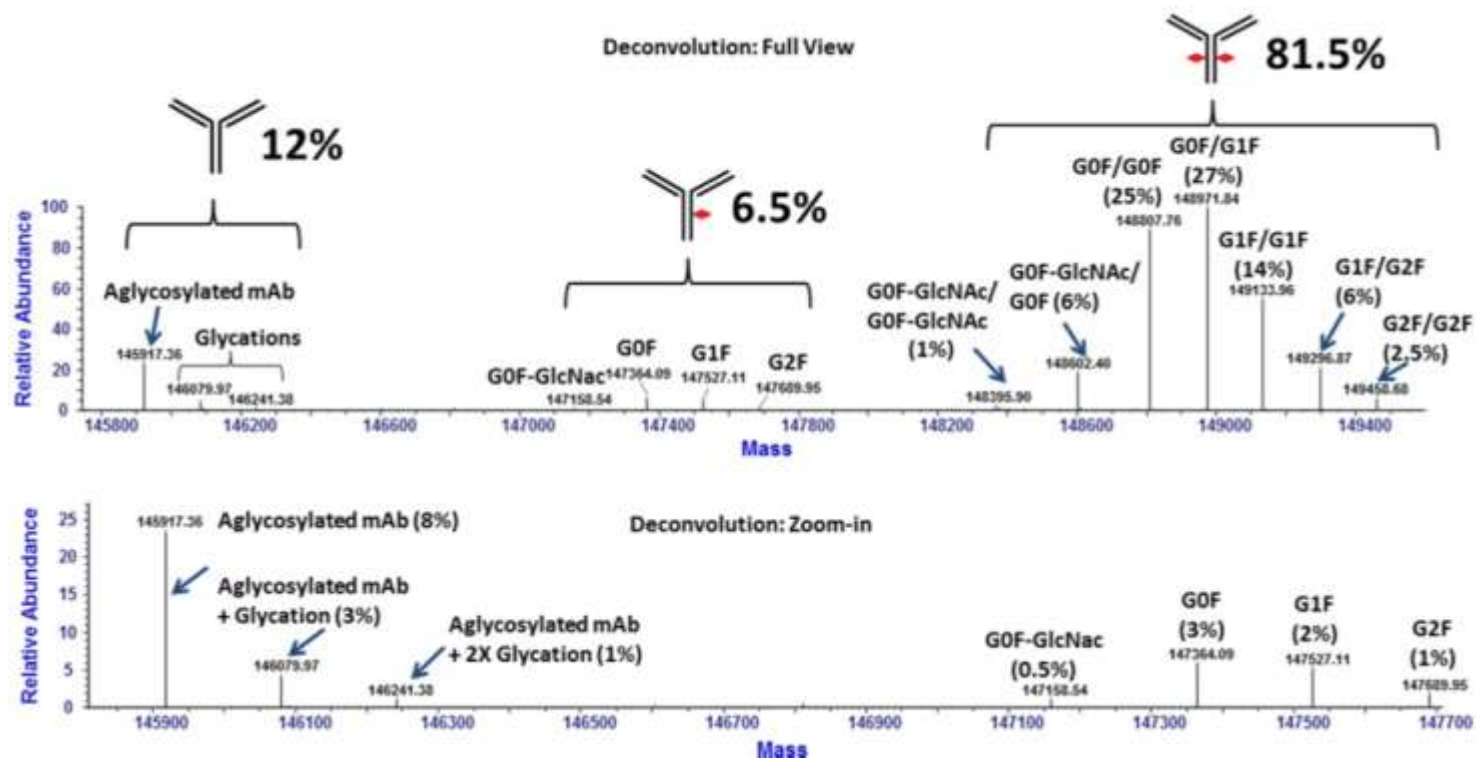


Fig. 5. Deconvolution spectrums showing major glycosylation states of the intact mAb. Quantitative ratios of each population of the mAb (2X-glycosylated, 1X-glycosylated, aglycosylated) are specified. Reprinted from [104]. Copyright (2018) Wiley.