



HAL
open science

Monoclonal antibody N-glycosylation profiling using capillary electrophoresis – Mass spectrometry: Assessment and method validation

Jeremie Giorgetti, Valentina D'atri, Julie Canonge, Antony Lechner, Davy Guillarme, Olivier Colas, Elsa Wagner-Rousset, Alain Beck, Emmanuelle Leize-Wagner, Yannis-Nicolas François

► To cite this version:

Jeremie Giorgetti, Valentina D'atri, Julie Canonge, Antony Lechner, Davy Guillarme, et al.. Monoclonal antibody N-glycosylation profiling using capillary electrophoresis – Mass spectrometry: Assessment and method validation. *Talanta*, 2018, 178, pp.530-537. 10.1016/j.talanta.2017.09.083 . hal-01981307

HAL Id: hal-01981307

<https://hal.science/hal-01981307>

Submitted on 14 Jan 2019

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 Monoclonal Antibody N-Glycosylation Profiling using 2 Capillary Electrophoresis – Mass Spectrometry: 3 Assessment and Method Validation.

4 Jérémie Giorgetti¹, Valentina D’Atri², Julie Canonge¹, Antony Lechner¹, Davy Guillarme²,
5 Olivier Colas³, Elsa Wagner-Rousset³, Alain Beck³, Emmanuelle Leize-Wagner¹, Yannis-
6 Nicolas François¹

7
8 ¹ Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS) UMR 7140 (Unistra-CNRS), Université de
9 Strasbourg, France.

10 ² School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Centre Médical Universitaire (CMU) - Rue
11 Michel-Servet 1, 1206 Geneva, Switzerland

12 ³ Centre d’immunologie Pierre Fabre; Saint-Julien-en-Genevois, France.
13

14 ABSTRACT:

15 Characterization of therapeutic proteins represents a major challenge for analytical sciences due to
16 their heterogeneity caused by post-translational modifications (PTM). Among these PTM, glycosylation
17 which is possibly the most prominent, require comprehensive identification because of their major
18 influence on protein structure and effector functions of monoclonal antibodies (mAbs). As a
19 consequence, glycosylation profiling must be deeply characterized. For this application, several
20 analytical methods such as separation-based or MS-based methods, were evaluated. However, no CE-
21 ESI-MS approach has been assessed and validated. Here, we illustrate how the use of CE-ESI-MS
22 method permits the comprehensive characterization of N-glycosylation of mAbs at the glycopeptide
23 level. Validation of the CE-ESI-MS method in terms of robustness and reproducibility was demonstrated
24 through the relative quantitation of glycosylation profiles for ten different mAbs produced in different
25 cell lines. Glycosylation patterns obtained for each mAbs were compared to Hydrophilic Interaction
26 Chromatography of 2-aminobenzamide labeled glycans with fluorescence detector (HILIC-FD) analysis
27 considered as a reference method. Very similar glycoprofiling were obtained with the CE-ESI-MS and
28 HILIC-FD demonstrating the attractiveness of CE-ESI-MS method to characterize and quantify the
29 glycosylation heterogeneity of a wide range of therapeutic mAbs with high accuracy and precision.

30
31 **Keywords:** Capillary Electrophoresis-Mass spectrometry; Monoclonal antibody; IgG glycosylation;
32 HILIC-FD; Glycopeptide

33 1. Introduction

34 Monoclonal antibodies (mAbs) were introduced for the treatment of various diseases in the late 1980
35 and they still represent the most rapidly growing category of therapeutic molecules today [1-3]. mAbs
36 are particularly interesting because of their good therapeutic efficiency, favorable pharmacokinetic and
37 pharmacodynamics, and relatively low side-effects [4]. mAbs are tetrameric glycoproteins having a
38 molecular mass of approximately 150 kDa, composed of two heavy chains and two light chains, inter-
39 linked by several disulfide bonds, and having at least one conserved N-glycosylation site located in the
40 Fc domain [3]. Glycosylation is a post-transcriptional modification (PTM) that occurs naturally during
41 excretion of antibodies from the expression system to the extracellular medium. It only represents 2-
42 5% of the total mass of the protein but it is subjected to extensive studies due to its significant influence
43 on effector functions of mAbs, especially antibody-dependent cell-mediated cytotoxicity (ADCC) and
44 complement-dependent cytotoxicity (CDC) [5-8]. As a consequence, the mAbs glycosylation profile is
45 considered as a critical quality attribute (CQA) and must be thoroughly analyzed [9-13]. The complexity
46 and heterogeneity of the glycosylation pattern is mainly due to mAbs production in living expression
47 systems [14-16] and requires a number of orthogonal analytical techniques to be fully characterized.
48 Several analytical methods have been described for the glyco-variants characterization at different
49 levels (from released glycans to intact protein level) including separative techniques (liquid
50 chromatography (LC), capillary electrophoresis (CE)) often coupled to spectrometric, amperometric
51 and mass spectrometric detection [17-21]. Recently, Reusch's group published two major articles
52 dealing with the analysis of Fc-glycosylation profiles, and comparing several separation methods
53 hyphenated or not with mass spectrometry (MS) detection [20-21]. This comprehensive comparison
54 showed an excellent precision and accuracy for all the methods. However, concerning MS-based
55 methods [22-25], a large panel of methodologies were evaluated, except the CE-ESI-MS approach.
56 Nevertheless, in 2008, Gennaro *et al.* described the development of CE-ESI-MS technology with online
57 LIF detection that allows identification of major and minor glycan species observed in the routine CE-
58 LIF assay. Despite significant instrumental development to achieve LIF and MS dual detection, their
59 strategies allowed to perform quantitative analysis provided by the on-line LIF trace and to increase
60 confidence by providing accurate mass information [26]. More recently, Gahoual *et al.* reported the
61 development of CE-ESI-MS technique for the characterization of the primary structure of mAbs
62 performed in a single injection [27]. Based on a bottom-up approach, they highlighted the benefits of
63 using electrophoretic separation in complement to chromatographic separation, which is
64 conventionally applied in this type of study. CE separation selectivity allowed to simultaneously
65 perform the amino acid sequencing and the PTMs characterization, including the N-glycosylation
66 profiling. However, concerning the latter point, the approach was not statistically validated and

67 potential bias in the obtained glycosylation heterogeneity could exist [28]. In the meantime, Heemskerk
68 *et al.* reported the use of a similar methodology for highly sensitive IgG1 glycosylation profiling as a
69 complementary method to a high-throughput nano-RPLC-MS [29]. They concluded that CE-ESI-MS
70 provide information on IgG Fc glycosylation with concentrations below the LODs of the conventional
71 methods. However, no comparison with reference method were applied to validate the obtained
72 glycosylation heterogeneity.

73 In this report, we evaluated and validated CE-ESI-MS method to characterize and quantify the
74 heterogeneity of the glycosylation pattern with high accuracy, precision and robustness. A systematic
75 characterization study of glycovariants obtained from ten different therapeutic mAbs produced in
76 different expression systems (CHO, NS0 and SP2/0), has been performed to evaluate the suitability of
77 CE-ESI-MS method, according to mAbs properties. Rituximab (chIgG1, CHO), palivizumab (hzIgG1,
78 SP2/0), natalizumab (hzIgG4, NS0), nivolumab (hulIgG4, CHO), trastuzumab (hzIgG1, CHO),
79 panitumumab (hulIgG2, CHO), adalimumab (hulIgG1, CHO), infliximab-Remicade® (chIgG1, SP2/0) as
80 well as two infliximab biosimilars, infliximab-Inflectra® (chIgG1 SP2/0) and infliximab-remsima®
81 (chIgG1 SP2/0), were selected for this study. Comparison with glyco-profiling of released and 2-AB
82 labeled glycans (used as a reference method) obtained by state-of-the-art Hydrophilic Interaction
83 Chromatography (HILIC) was methodically performed to assess the reliability of the CE-ESI-MS
84 methodology.

85

86 **2. Experimental**

87 **2.1. Chemicals.** Chemicals used were of analytical grade or high purity grade and purchased from
88 Sigma-Aldrich (Saint Louis, MO, USA). Water used to prepare buffers and sample solutions was
89 obtained using an ELGA purelab UHQ PS water purification system (Bucks, UK). RapiGest SF surfactant
90 was purchased from Waters (Milford, MA, USA). mAbs and biosimilars products were all kindly provided
91 by Pierre Fabre laboratories (Saint-Julien en Genevois, France).

92 **2.2. Sample preparation.** A volume corresponding to 100 µg of protein was used. Samples were first
93 diluted from stock solution to a concentration of 45.6 µM by using milliQ water. A second dilution to a
94 concentration of 22.2 µM was performed by using 0.1% RapiGest surfactant and incubation at 40°C for
95 10 min. Reduction of the samples was then achieved by the addition of dithiothreitol (DTT, final
96 concentration of 25 mM) and incubation at 95°C for 5 min. Once cooled down to room temperature
97 (RT), the alkylation of the cysteines (Cys) was performed to avoid the reformation of the disulphide
98 bonds. Thus, iodoacetamide (IDA, final concentration of 10 mM) was added to the samples, followed
99 by incubation at RT for 20 min in the dark. For performing the trypsin digestion, a volume of 1 µL of

100 trypsin (0.5 μ g/ μ L) was added to the samples that were left at room temperature for 3h. Then another
101 volume of 1 μ L was added afterward and digestion was performed overnight at 37°C. In order to cleave
102 the surfactant, formic acid (FA) was added to the samples at a final concentration of 1% (v/v) and
103 samples were left at RT for 2h. Samples were finally diluted to a final protein concentration of 2.2 μ M
104 using ammonium acetate 50 mM (pH 4.0).

105 **2.3. Capillary electrophoresis.** The CE experiments were performed with a CESI8000 capillary
106 electrophoresis system from Sciex Separation (Brea, CA, USA). A 32 Karat™ (Sciex Separation) was used
107 for instrument control, data acquisition and data handling. Bare fused-silica capillaries (total length 100
108 cm; 30 μ m i.d.) with characteristic 3 cm porous tip on its final end, a second capillary (total length 80
109 cm; 50 μ m i.d.) filled during experiments with BGE allows electric contact. New capillaries were flushed
110 for 10 min at 75 psi (around 5 bar) with methanol, then 10 min with 0.1 M sodium hydroxide, followed
111 by 10 min with 0.1 M hydrochloric acid and water for 20 min at 75 psi. Finally, the capillary was flushed
112 10 min at 75 psi with BGE which contains 10% acetic acid. Hydrodynamic injection (6 psi for 2 min)
113 corresponding to a total volume of 90 nL of injected sample was used. Injection volumes were
114 calculated by using the CEToolbox application (Pansanel, GooglePlay). Separations were performed
115 using a voltage of +20 kV.

116 **2.4. Mass spectrometry.** For glycopeptide analysis, the CESI system was hyphenated with a 5600
117 TripleTOF mass spectrometer (Sciex, Darmstadt, Germany). The MS instrument is equipped with a
118 hybrid analyzer composed of quadrupoles followed by a time-of-flight (TOF) analyzer. ESI source
119 parameters were set as follows: ESI voltage -1.75kV, gas supplies (GS1 and GS2) were deactivated,
120 source heating temperature 150°C and curtain gas value 5. Experiments were performed in Top15
121 information dependent acquisition (IDA), accumulation time was 250 msec for MS scans and 100 msec
122 for MS/MS scans leading to a total duty cycle of 1.75 sec. Mass/charge (m/z) range was set to 100-2000
123 in MS and 50-2000 in MS/MS. Using those parameters, the mean resolution provided by the instrument
124 is 40000 in MS (for m/z 485.251) and 25000 in MS/MS (for m/z 345.235).

125 **2.5. MS/MS data analysis.** Data obtained from the sheathless CE-MS/MS experiments were analyzed
126 using Peakview software (Sciex, San Francisco, CA). The allowed mass tolerance for search algorithm
127 identification, were set to \pm 5 ppm and \pm 0.05 Da for precursor ions and fragmentation ions, respectively.

128 **2.6. HILIC (2-AB)** Each mAb (200 μ g) was deglycosylated by incubation with 2 μ g PNGase F (500,000
129 U/ml, New England Biolabs) at 37°C for 3 h. Released glycans were labelled with 2-AB at 65°C for 3 h
130 (Glyko Signal 2-AB Labeling Kit, ProZyme), and then purified using dedicated GlikoClean S Cartridges
131 (ProZyme). Labeled glycans were washed with 96% acetonitrile (ACN), eluted from the cartridges and
132 evaporated to dryness using a speedvac, then reconstituted in 30:70 water/ACN (v/v). Analysis were
133 performed by HILIC using an Agilent AdvanceBio Glycan column (2.1 x 150 mm, 1.8 μ m) on a Waters

134 ACQUITY UPLC I-Class system equipped with a binary solvent delivery pump, an auto-sampler, a UV-
135 DAD and a fluorescence detector (FD) set at $\lambda_{\text{ex}}= 260$ nm and $\lambda_{\text{em}}= 430$ nm. The system included a flow
136 through needle (FTN) injection system with a 15 μL needle. Data acquisition, data handling and
137 instrument control were performed with Empower 2 (Waters, Milford, MA, USA). Mobile phase
138 consisted of 20 mM Ammonium Formate solution (A) and ACN (B). The column temperature was set to
139 55 °C, and injection volume was 2 μL corresponding to 0.08-0.2 μg glycan sample. The flow rate was
140 set to 0.5 mL/min, and the gradient conditions consisted of 80% to 60% B in 25 minutes, followed by a
141 3 min washing step at 20% B and a 15 min re-equilibration step. Peaks were manually integrated and
142 relative glycan compositions were calculated. For the correct identification of the labelled glycans,
143 UHPLC-MS analysis were also performed using an ACQUITY UPLC system (Waters), equipped with a
144 binary pumping system and fixed loop injector of 5 μL . This UHPLC was coupled to a fluorescence
145 detector (FD) and an electrospray time-of-flight mass spectrometer (Xevo™ Q-ToF, Waters). The mass
146 spectrometer was operated in the positive ion mode and ions were scanned over an m/z range of 500-
147 2500 with a 1 s scan rate. Capillary voltage was set to 3.0 kV, sample cone voltage to 35 V, source
148 temperature to 120 °C, desolvation gas temperature to 350 °C and gas flow to 800 L/h. The instrument
149 was calibrated using the singly charged ions produced by a 2 $\mu\text{g}/\mu\text{L}$ sodium iodide solution in 2-
150 propanol/water (1:1). Data acquisition and analysis were performed with MassLynx 4.1 (Waters). In all
151 cases, a 2-AB labelled dextran ladder standard (1 pmol/ μL) was also injected before and after a series
152 of 5 samples, to check the repeatability of the injections and verify that no drift in retention times was
153 observed.

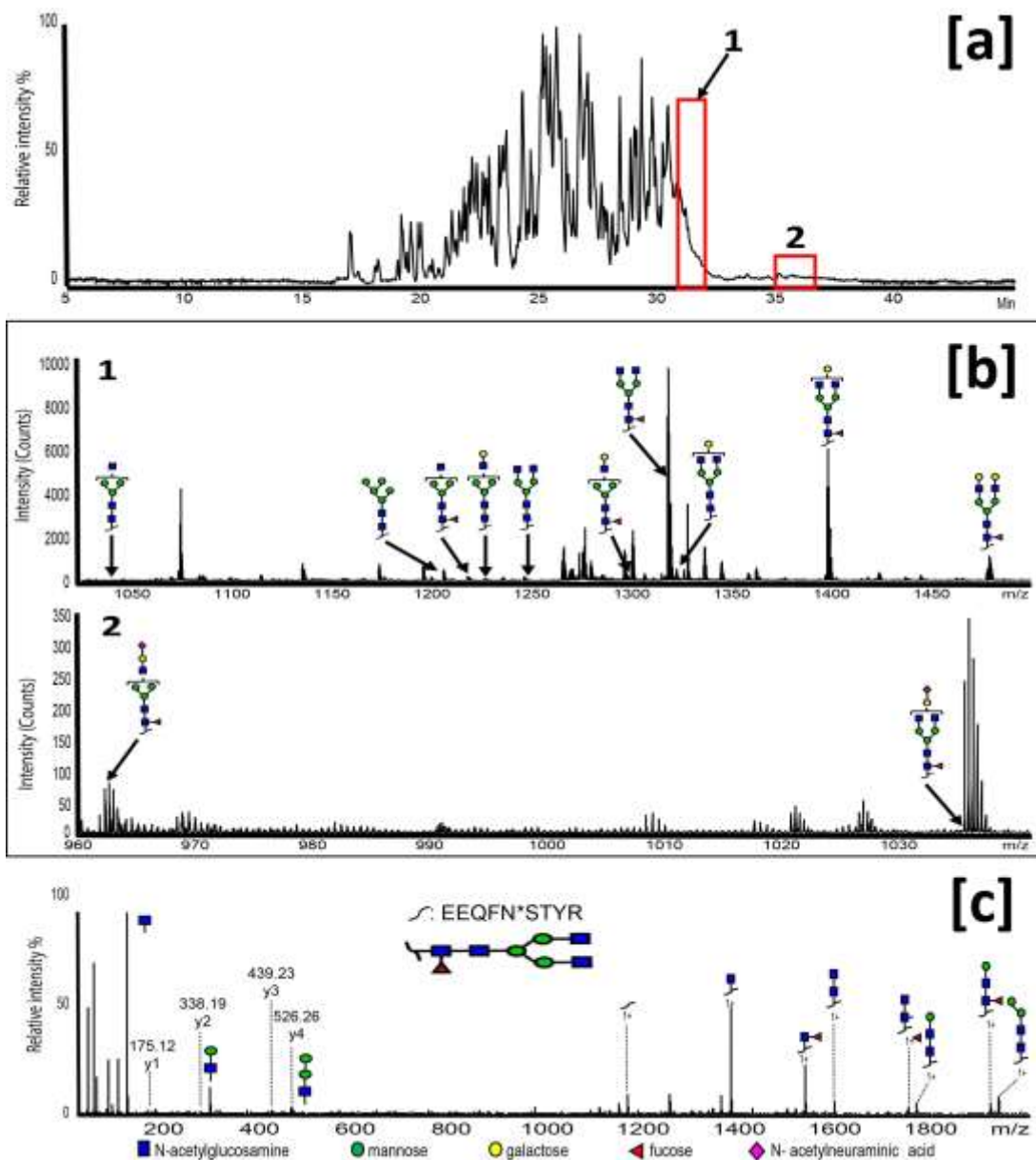
154

155 **3. Results and discussions**

156 **3.1. Characterization of N-Glycopeptide by CE-ESI-MS.**

157 mAbs are glycosylated proteins whose N-glycans are naturally incorporated in the protein during
158 secretion into the extracellular environment. Extensive glycans characterization in terms of structure
159 and relative abundance is of prime importance. The main goal of this study was to demonstrate the
160 possibility to use CE-ESI-MS method for performing glycosylation profiling of a large panel of
161 therapeutic mAbs. Therefore, we realized a systematic study of glycosylation characterization on ten
162 different mAbs produced in CHO, NS0 and SP2/0 cell lines (Table 1). To evaluate the viability of CE-ESI-
163 MS, we focused our characterization on the main Fc N-glycan species typically found in therapeutic IgG
164 mAb produced in the above mentioned cell lines and listed in Table 1. In this section, natalizumab
165 results have been selected as example to describe the CE-ESI-MS methodology allowing to assess the
166 in-depth N-glycan characterization. Fig. 1a illustrates the separation obtained for the tryptic digest of
167 natalizumab for a 200 fmol injection. The total analysis time was less than 45 min and the resulting

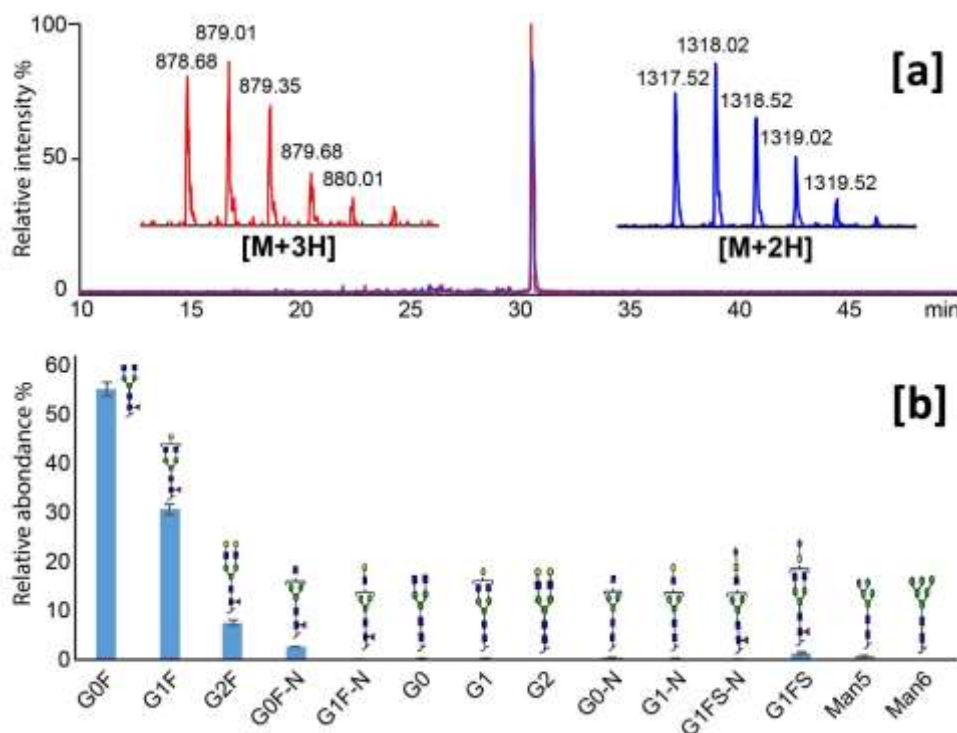
168 electropherogram showed that all peptides migrated between 15 and 40 min. As tandem MS data
 169 interpretation and peptide identification was automatically done using Mascot, to obtain fast and
 170 accurate data treatment, it was necessary to manually evaluate the CE-ESI-MS/MS data in order to
 171 identify the glyco-variants and determine their structures.



172
 173 Fig. 1. (a) Base Peak Electropherogram corresponding to the analysis by CE-ESI-MS/MS of natalizumab tryptic
 174 digest. (b) MS spectrum of 30.5 – 31.9 min and 34.9 – 36.5. (c) MS/MS fragmentation spectra of [EEQFNSTYR] +
 175 G0F. Experimental conditions described in Experimental section.

176
 177 Peak assignment of glyco-structures was performed based on accurate mass measurement in MS1,
 178 provided by high resolution MS (mass accuracy below 2 ppm) (Fig. 1b) and collision induced decay (CID)
 179 fragmentation spectra (Fig. 1c), respectively. Indeed, MS/MS spectra exhibited the fragmentation of
 180 glycan moieties present on the glycopeptide, giving structural information on the glycans along with

181 reinforcing the confidence of the identification. Furthermore, the obtained electropherogram showed
 182 the separation of several glycopeptides, demonstrating the benefit of using CE for such
 183 characterization. The charge-based CE separation allowed the baseline resolution of sialic acid and
 184 neutral glycans located on the peptide EEQFN³⁰⁰STYR. Fig. 1a represents two windows on the
 185 electropherogram, corresponding to the neutral glycans separation (between 30.5 and 31.9 min) and
 186 to the sialic acid moieties separation (between 34.9 and 36.5 min). Moreover, particular glycopeptides
 187 having a difference of only one galactose could also be baseline separated. Glycopeptides having such
 188 a small difference in mass tend to compete against each other during the ionization process, potentially
 189 interfering with relative quantification, thus the capacity to separate them is clearly an intriguing
 190 advantage. To perform the glycans profiling of each mAb, relative occurrence levels were estimated
 191 from the sum of isotopic peak intensities, considering all charge states of the ion corresponding to one
 192 glycopeptide (Fig. 2a and Table S-1). A comparison of all glycoforms abundance was then realized (Fig.
 193 2b). To validate the method reproducibility, the digestions of each mAb were performed three times
 194 using different experimenters and triplicate injections of each digested sample were carried out. The
 195 relative occurrence glycan levels reported in Table 1 were calculated for a selected mAb as the average
 196 of all relative abundances of the nine values (three digestions and three injections per digested
 197 samples), with a confidence interval of 95%.

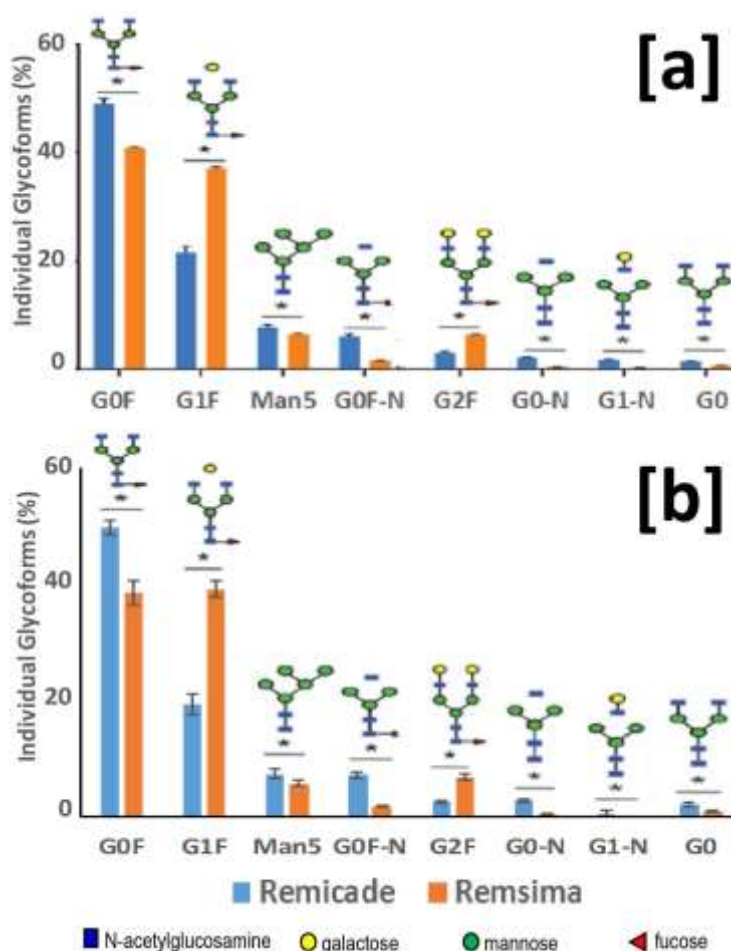


198
 199
 200
 201
 202

Fig. 2. (a) Extracted Ion Electropherogram (EIE) of m/z ratios 878.68 and 1317.52 ([EEQFN*STYR] + G0F) and corresponding MS/MS fragmentation spectra (right-hand side). (b) Glycoforms relative abundance results obtained through the CE-ESI-MS data for the natalizumab Fc glycopeptide.

203 **3.2. Evaluation of CE-ESI-MS method performance**

204 To assess the performance of the CE-ESI-MS methodology with respect to accuracy and precision, each
205 mAb glycosylation profile was compared with the reference method, namely HILIC-FD glycosylation
206 profile obtained upon release and 2-AB-labeling of the glycans. HILIC-FD acquisitions were performed
207 in triplicate and peaks on the FD chromatogram were manually integrated for estimating the relative
208 glycan compositions. The deviations observed for retention times were minimal ($RSD_{RT} = 0.39\%$,
209 calculated on G0F, for $n = 30$). Peak assignment of the 2AB-glycans was accomplished by online coupling
210 of HILIC with ESI-MS. A detailed list of the glycan composition and the theoretical masses of the
211 unlabelled/labelled glycans is shown in Table 2. The theoretical 2AB-glycan masses were used to obtain
212 the extracted ion chromatogram (EIC) of each glycan. Furthermore, GlycoMod software
213 (<http://web.expasy.org/glycomod/>) was eventually used for the prediction of the possible glycan
214 structures based on the experimentally determined masses.



215
216 **Fig. 3.** Comparison of Infliximab-Remicade® and infliximab-Remsima® glycoprofiling. (a) Quantification of N-
217 glycans adapted following the method of Pisupati et al (*adapted with permission from [30]. Copyright (2017)*
218 *American Chemical Society.* (b) Quantification of N-glycans following CE-ESI-MS analysis.

219

220 Table 1 compiles the results of CE-ESI-MS and HILIC-FD (2-AB) relative abundance values obtained for
221 each mAb. As described in the literature [20], HILIC-FD shows excellent precision with low standard
222 deviations (with the exception of Infliximab-RemSima[®] analysis). The suggested CE-ESI-MS method also
223 presents low absolute variation with values below 4% for the different glycan structures. These values
224 are comparable to those determined for other MS-based methods, such as NanoLC-ESI-MS described
225 elsewhere [21]. It is worth noticing that for each mAb, the deviations were obtained based on the
226 combination of digestions and injections performed in triplicates by different experimenters over an
227 extended period of several weeks, thus the results strongly support the performance of the method in
228 terms of robustness and reproducibility. Moreover, the relative occurrence level estimated by CE-ESI-
229 MS method were in good agreement for the values obtained with the reference HILIC-FD method. For
230 G0F and G1F, which represent at least 75% of the total glycosylation of each mAb, the relative absolute
231 difference between CE-ESI-MS means and HILIC-FD means expressed as a percentage ($100 * |\text{Mean}_{\text{CE-ESI-MS}} - \text{Mean}_{\text{HILIC-FD}}| / \text{Mean}_{\text{CE-ESI-MS}}$) showed an average of 7.5% for G0F and 7.8% of G1F. These values confirm the good
232 fit between the two methodologies for the major forms of glycosylation. Moreover, for glycosylation
233 expressed at least at 10% of the total glycoforms (i.e. G2F of Palivizumab), this value is up to 20%
234 meaning a good variability between CE-ESI-MS and HILIC (2-AB) mean amount. For glycosylation
235 representing less than 10% of the total glycoforms, the relative absolute differences between HILIC-FD
236 and CE-ESI-MS are no longer representative, because these values can be very high, due to low degree
237 of expression.
238

239 The relative quantification of mono-antennary structures, defined by the lack of N-acetylglucosamine
240 (G0F-N, G1F-N, G0-N, G1-N), is known to be problematic using MS-based methods. Indeed, in-source
241 fragmentation of bi-antennary structures resulting in the loss of one antenna can generate elevated
242 mono-antennary structures levels, along with a charge reduction that is commonly observed in MS
243 spectra and that is a consequence of a loss of the N-acetylglucosamine [21]. While only low or similar
244 levels of mono-antennary structures were detected in CE-ESI-MS for eight mAbs, higher levels of these
245 glycovariants were detected for natalizumab and nivolumab, as compared to HILIC-FD (Table S-2).
246 However, for these two mAbs, differences between the mono-antennary means obtained by CE-ESI-
247 MS and HILIC-FD were not aberrant, and no charge state reduction was observed by CE-ESI-MS,
248 meaning that the detected sum of mono-antennary structures were not over-estimated. Moreover,
249 this result confirmed that during ESI-MS analysis of glycopeptides, in source decay can efficiently be
250 avoided through the proper choice of the MS conditions and voltages, even for CE-ESI-MS method [21].

251 The sum of afucosylated species (G0, G1, G2) is a relevant parameter for antibody effector function.
252 For nine mAbs, similar levels of G0+G1+G2 were observed. Only trastuzumab showed a difference
253 between CE-ESI-MS (8.3%) and HILIC-FD (4.4%) (Table S-2). Similarly, the sum of highly mannosylated

254 species (M5, M6) was evaluated and similar levels of M5+M6 were observed for seven mAbs, along
255 with small differences concerning M5 species for adalimumab, infliximab-Remicade® and palivizumab.
256 Finally, good correlations were observed for the mean levels of sialylated structures sum (G1FS, G1FS-
257 N). Overall comparisons of results obtained by CE-ESI-MS approach and HILIC-FD reference method
258 showed very similar glycoprofiling of the ten therapeutic mAbs. CE-ESI-MS demonstrated to be a
259 valuable method to characterize and quantify with high accuracy, precision and robustness the most
260 largely expressed glycan species as well as the low abundance glycoforms.

261 Recently, Pisupati *et al.* published an important work describing a multidimensional analytical
262 comparison of infliximab-Remicade® and the biosimilar infliximab-Remsima® [30]. They performed the
263 glycoforms quantification by LC-MS using trypsin-digested products and demonstrated, for the first
264 time, significant differences in the N-Glycan distributions for infliximab-Remicade® and infliximab-
265 Remsima® (Fig. 3a). To confirm the assessment of CE-ESI-MS method in performing the relative
266 quantitation of mAbs glycopeptides, we compared our infliximab-Remicade® and infliximab-Remsima
267 means with those obtained by Pisupati *et al.* It must be highlighted that no collaboration was carried
268 out between Prof. Schwendeman's group and our laboratory, which means that samples were not from
269 the same batch, and that experimenters and instrumentations were different. Fig. 3b represents the
270 glycoprofiling of infliximab-Remicade® and infliximab-Remsima following CE-ESI-MS analysis for the
271 selected N-glycan species. Comparison between Pisupati *et al.* results and our profiles highlights a total
272 similarity between the two glycoprofilings. While the confirmation of CE-ESI-MS performance in term
273 of N-Glycan species quantification was proved, significant differences in the N-Glycan distributions for
274 infliximab-Remicade® and infliximab-Remsima® were confirmed.

275 Infliximab-Inflectra® is another biosimilar that was studied in this work and not reported by Pisupati *et*
276 *al.*. Thanks to the CE-ESI-MS methodology, we showed for the first time significant differences in the
277 N-Glycan distributions also for infliximab-Remicade® and infliximab-Inflectra® (Table 1).

278

279

280

281 **4. Conclusions**

282 To summarize, we reported here the development of a CE-ESI-MS methodology to perform relative
283 quantitation of N-glycan species for mAbs characterization at the glycopeptides level. Validation in
284 terms of robustness and reproducibility of CE-ESI-MS method were demonstrated through the relative
285 quantitation of glycosylation profiles for ten different mAbs produced in different cell lines. A

286 systematic comparison of the glycosylation patterns obtained for each mAbs was compared with that
287 obtained with the HILIC-FD reference method. Results obtained with the CE-ESI-MS approach and
288 HILIC-FD showed very similar glycoprofiling, demonstrating the attractiveness of CE-ESI-MS method to
289 characterize and quantify the glycosylation heterogeneity of a wide range of therapeutic mAbs, with
290 high accuracy and precision. Moreover, it must be mentioned that our CE-ESI-MS methodology is not
291 restricted to the sole purpose of glycopeptides characterization and quantitation, but it can also be
292 employed for the overall characterization of mAbs, including primary structure assessment with
293 complete sequence coverage as well as identification and quantification of a large number of PTMs, all
294 performed within a unique single analysis [27]. In our opinion, this work proves that CE-ESI-MS could
295 be a viable alternative to LC-ESI-MS for glycosylation profiling and should be considered as an
296 innovative approaches in MS-based proteomics applied to mAbs characterization.

297

298 **Acknowledgments**

299 Emmanuelle Leize-Wagner would like to thank Sciex for the fruitful collaboration and the support. The
300 authors would like to express their gratitude to Camille Banholzer, Jordan Kirmann and Charly Renard
301 (LSMIS, Université de Strasbourg, France), P. Hammann, Lauriane Kuhn and J. Chicher (Institut de
302 Biologie Moléculaire et Cellulaire, Strasbourg, France) and MC. Janin-Bussat (Centre d'Immunologie
303 Pierre Fabre, St Julien en Genevois, France). This work was supported by the CNRS (UMR 7140), the
304 University of Strasbourg. Davy Guillaume also wishes to thank the Swiss National Science Foundation
305 for its financial support (31003A_159494).

306

307 **References**

308

309 [1] A. Beck, T. Wurch, C. Bailly, N. Corvaia, Strategies and challenges for the next generation of therapeutic antibodies, *Nat.*
310 *Rev. Immunol.* 10 (2010) 345-352.

311 [2] J.M. Reichert, Marketed therapeutic antibodies compendium, *mAbs* 4 (2012) 413-415.

312 [3] A. Beck, H. Diemer, D. Ayoub, F. Debaene, E. Wagner-Rousset, C. Carapito, A. Van Dorsselaer, S. Sanglier-Cianferani,
313 Analytical characterization of biosimilar antibodies and Fc-fusion proteins, *Trac-Trends Anal. Chem.* 48 (2013) 81-95.

314 [4] W. Wang, S. Singh, D.L. Zeng, K. King, S. Nema, Antibody structure, instability, and formulation, *J. Pharm. Sci.* 96 (2007)
315 1-26.

316 [5] A. Beck, E. Wagner-Rousset, MC. Bussat, M. Lokteff, C. Klinguer-Hamour, JF. Haeuw, L. Goetsch, T. Wurch, A. Van
317 Dorsselaer, N. Corvaia Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion
318 proteins, *Curr. Pharm. Biotechnol.* 9 (2008) 482-501.

319 [6] A. Beck, T. Wurch, N. Corvaia Therapeutic antibodies and derivatives: from the bench to the clinic. *Curr. Pharm.*
320 *Biotechnol.* 9 (2008) 421-422.

321 [7] R. Jefferis Glycosylation as a strategy to improve antibody-based therapeutics *Nat. Rev. Drug. Discov.* 8 (2009) 226-234.

322 [8] DS. Dimitrov, JD. Marks Therapeutic antibodies: current state and future trends-is a paradigm change coming soon?
323 *Methods Mol. Biol.* 525 (2009) 1-27.

324 [9] D. Ayoub, W. Jabs, A. Resemann, W. Evers, C. Evans, L. Main, C. Baessmann, E. Wagner-Rousset, D. Suckau, A. Beck,
325 Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up,
326 middle-down and bottom-up ESI and MALDI mass spectrometry techniques, *mAbs* 5 (2013) 699-710.

327 [10] R. Gahoual, A. Burr, J.M. Busnel, L. Kuhn, P. Hammann, A. Beck, Y.N. Francois, E. Leize-Wagner, Rapid and multi-level
328 characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass spectrometry, *mAbs* 5 (2013) 479-
329 490.

330 [11] D. Reusch, M.L. Tejada, Fc glycans of therapeutic antibodies as critical quality attributes, *Glycobiology* 25 (2015) 1325-
331 1334

332 [12] M. Schiestl, T. Stangler, C. Torella, T. Cepeljnik, H. Toll, R. Grau, Acceptable changes in quality attributes of glycosylated
333 biopharmaceuticals, *Nat. Biotechnol.* 29 (2011) 310-312.

334 [13] E. Largy, F. Cantais, G. Van Vyncht, A. Beck, A. Delobel Orthogonal liquid chromatography-mass spectrometry methods
335 for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level *J.*
336 *Chromatogr. A* 1498 (2017) 128-146.

337 [14] R. Niwa, A. Natsume, A. Uehara, M. Wakitani, S. Iida, K. Uchida, M. Satoh, K. Shitara, IgG subclass-independent
338 improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn297-linked oligosaccharides, *J.*
339 *Immunol. Methods* 306 (2005) 151-160

340 [15] S.A. Brooks, Appropriate glycosylation of recombinant proteins for human use: implications of choice of expression
341 system, *Mol. Biotechnol.* 28 (2004) 241-256.

342 [16] R.G. Spiro, Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide
343 bonds, *Glycobiology* 12 (2002) 43R-56R.

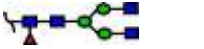
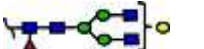
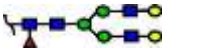









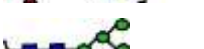
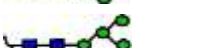
344 [17] V. Dotz, R. Haselberg, A. Shubhakar, R.P. Kozak, D. Falck, Y. Rombouts, D. Reusch, G.W. Somsen, D.L. Fernandes, M.
345 Wührer, Mass spectrometry for glycosylation analysis of biopharmaceuticals, *TrAC Trends Anal. Chem.* 73(2015) 1-9.

346 [18] L. Zhang, S. Luo, B. Zhang, Glycan analysis of therapeutic glycoproteins, *mAbs* 8 (2016) 205-215.

347 [19] M. Adamo, D. Qiu, L.W. Dick, M. Zeng, A.-H. Lee, K.-C. Cheng, Evaluation of oligosaccharide methods for carbohydrate
348 analysis in a fully human monoclonal antibody and comparison of the results to the monosaccharide composition
349 determination by a novel calculation, *J. Pharm. Biomed. Anal.* 49 (2009) 181-192.

- 350 [20] D. Reusch, M. Habegger, B. Maier, M. Maier, R. Kloseck, B. Zimmermann, M. Hook, Z. Szabo, S. Tep, J. Wegstein, N. Alt,
351 P. Bulau, M. Wuhrer, Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-
352 part 1: separation-based methods, *mAbs* 7 (2015) 167–179.
- 353 [21] D. Reusch, M. Habegger, D. Falck, B. Peter, B. Maier, J. Gassner, M. Hook, K. Wagner, L. Bonnington, P. Bulau, M.
354 Wuhrer, Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 2: Mass
355 spectrometric methods, *mAbs* 7 (2015) 732–742.
- 356 [22] A. Beck, S. Sanglier-Cianferani, A. Van Dorsselaer Biosimilar, biobetter, and next generation antibody characterization
357 by mass spectrometry, *Anal Chem* 84 (2012) 4637-4646.
- 358 [23] S. Thobhani, C.T. Yuen, M.J. Bailey, C. Jones Identification and quantification of N-linked oligosaccharides released from
359 glycoproteins: an inter-laboratory study, *Glycobiology* 19 (2009) 201-211.
- 360 [24] M. Wuhrer. Glycomics using mass spectrometry. *Glycoconj. J.* 30 (2013) 11-22.
- 361 [25] A. Banazadeh, L. Veillon, K.M. Wooding, M. Zabet-Moghaddam, Y. Mechref *Electrophoresis*, 38 (2017) 162-189.
- 362 [26] L.A. Gennaro, O. Salas-Solano, On-line CE-LIF-MS technology for the direct characterization of N-linked glycans from
363 therapeutic antibodies. *Anal. Chem.* 80 (2008) 3838-3845.
- 364 [27] R. Gahoual, J.-M. Busnel, A. Beck, Y.-N. François, E. Leize-Wagner, Full Antibody Primary Structure and Microvariant
365 Characterization in a Single Injection Using Transient Isotachopheresis and Sheathless Capillary Electrophoresis–Tandem
366 Mass Spectrometry, *Anal. Chem.* 86 (2014) 9074-9081.
- 367 [28] R. Gahoual, M. Biacchi, J. Chicher, L. Kuhn, P. Hammann, A. Beck, E. Leize-Wagner, Y.N. Francois, Monoclonal antibodies
368 biosimilarity assessment using transient isotachopheresis capillary zone electrophoresis-tandem mass spectrometry, *mAbs*
369 6 (2014) 1464–1473.
- 370 [29] A.A.M. Heemskerk, M. Whurer, J.M. Busnel, C.A.M. Koeleman, M.H.J. Selman, G. Vidarsson, R. Kapur, B. Shoenmaker,
371 R.J.E. Derks, A.M. Deelder, O.A. Mayboroda, Coupling porous sheathless interface MS with transient-ITP in neutral
372 capillaries for improved sensitivity in glycopeptide analysis. *Electrophoresis* 34 (2013) 383-387.
- 373 [30] K. Pisupati, Y. Tian, S. Okbazghi, A. Benet, R. Ackermann, M. Ford, S. Saveliev, C.M. Hosfield, M. Urh, E. Carlson, C.
374 Becker, T.J. Tolbert, S.P. Schwendeman, B.T. Ruotolo, A. Schwendeman A multidimensional analytical comparison of
375 Remicade and the biosimilar Remsima 89 (2017) 4838-4846.
- 376
- 377
- 378
- 379

380 Table 1: Comparison of mAbs glycosylation profiles by HILIC (2-AB) and CE-ESI-MS methods. Relative occurrence level of the various glycopeptides are given in percent with
 381 standard deviation in parentheses. Abbreviation: H, hexose; N, N-acetylhexosamine; F, deoxyhexose; S, N-acetylneuraminic acid; n.d., not detected

Glycan Species	Structural Scheme	Adalimumab (Humira®)		Infliximab (Remicade®)		Infliximab (Inflectra®)		Infliximab (Remsima®)		Trastuzumab (Herceptin®)	
		HILIC (2-AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF
G0F		69.3	65.7	53.7	49.7	41.6	39.3	44.6	38.6	48.7	41.6
[H3N4F1]		(0.1)	(2.4)	(0.1)	(1.3)	(0.1)	(4.0)	(5.1)	(2.0)	(0.4)	(1.1)
G1F		17.9	18.0	21.4	19.3	40.2	39.7	37.5	39.2	35.4	38.7
[H4N4F1]		(<0.1)	(3.0)	(0.1)	(1.8)	(<0.1)	(2.2)	(3.7)	(1.4)	(0.2)	(0.8)
G2F		1.3	0.8	2.1	2.5	5.7	6.4	4.7	6.8	5.3	7.2
[H5N4F1]		(<0.1)	(0.8)	(<0.1)	(0.3)	(<0.1)	(1.9)	(1.7)	(0.6)	(<0.1)	(0.6)
G0F-N		2.5	3.7	6.3	7.3	1.4	1.7	1.7	1.8	1.5	1.3
[H3N3F1]		(<0.1)	(1.0)	(<0.1)	(0.5)	(<0.1)	(0.4)	(0.6)	(0.2)	(<0.1)	(0.1)
G1F-N		1.4	0.1	2.5	3.2	0.8	0.8	0.8	0.6	1.7	0.5
[H4N3F1]		(<0.1)	(0.4)	(<0.1)	(0.4)	(<0.1)	(0.7)	(<0.1)	(0.4)	(<0.1)	(0.4)
G0		0.8	0.5	1.3	2.1	0.6	0.8	0.8	1.0	3.5	5.3
[H3N4]		(<0.1)	(0.8)	(<0.1)	(0.3)	(<0.1)	(0.1)	(0.3)	(0.1)	(<0.1)	(0.3)
G1		n.d.	0.5	0.1	0.1	0.1	n.d.	0.1	0.1	0.5	2.9
[H4N4]			(1.3)	(<0.1)	(0.3)	(<0.1)	n.d.	(<0.1)	(0.3)	(<0.1)	(0.1)
G2		n.d.	n.d.	0.3	n.d.	0.1	n.d.	0.1	0.1	0.4	0.1
[H5N4]				(<0.1)	n.d.	(<0.1)	n.d.	(<0.1)	(0.1)	(<0.1)	(<0.1)
G0-N		0.4	n.d.	1.8	2.8	0.5	0.6	0.6	0.6	0.8	0.7
[H3N3]		(<0.1)		(<0.1)	(0.3)	(<0.1)	(0.1)	(0.3)	(0.1)	(<0.1)	(0.1)
G1-N		n.d.	n.d.	0.7	0.6	0.1	0.1	0.1	0.1	n.d.	0.1
[H4N3]				(<0.1)	(0.6)	(<0.1)	(0.1)	(<0.1)	(0.1)	n.d.	(0.1)
G1FS-N		n.d.	n.d.	2.8	3.4	1.2	1.3	1.0	1.4	n.d.	n.d.
[H4N3FS1]				(<0.1)	(1.0)	(0.1)	(0.4)	(0.3)	(0.3)	n.d.	n.d.
G1FS		n.d.	n.d.	1.5	1.6	3.0	3.9	2.3	3.9	n.d.	n.d.
[H4N4FS1]				(<0.1)	(0.5)	(<0.1)	(1.2)	(0.4)	(0.8)	n.d.	n.d.
M5		4.65	8.2	5.4	7.4	4.6	5.5	5.3	5.8	1.8	1.6
[H5N2]		(<0.1)	(2.6)	(<0.1)	(0.9)	(<0.1)	(0.3)	(1.4)	(0.6)	(<0.1)	(0.1)
M6		1.57	2.57	0.1	n.d.	0.3	n.d.	0.3	0.1	0.4	n.d.
[H6N2]		(<0.1)	(0.9)	(<0.1)	n.d.	(<0.1)	n.d.	(<0.1)	(0.3)	(<0.1)	n.d.

382

Glycan Species	Structural Scheme	Palivizumab (Synagis®)		Natalizumab (Tysabri®)		Nivolumab (Opdivo®)		Rituximab (Rituxan®)		Panitumumab (Vectibix®)	
		HILIC (2- AB)	CE with Q-TOF	HILIC (2- AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF	HILIC (2-AB)	CE with Q-TOF
G0F		29.4	29.2	59.1	55.1	69.8	60.5	44.5	40.6	41.6	40.6
[H3N4F1]		(2.9)	(0.5)	(0.3)	(1.3)	(0.4)	(2.1)	(0.1)	(1.8)	(0.2)	(2.1)
G1F		44.9	43.5	30.5	30.7	23.7	28.6	42.0	44.3	39.2	36.3
[H4N4F1]		(1.5)	(2.0)	(0.1)	(1.1)	(0.2)	(1.0)	(0.1)	(1.1)	(0.2)	(2.1)
G2F		13.7	15.7	4.7	7.6	2.7	5.1	7.3	10.3	7.4	7.6
[H5N4F1]		(2.5)	(1.1)	(0.1)	(0.6)	(<0.1)	(0.5)	(0.2)	(0.9)	(0.4)	(0.6)
G0F-N		2.4	2.4	1.1	2.6	0.5	3.1	0.9	0.7	1.5	1.9
[H3N3F1]		(0.5)	(0.2)	(<0.1)	(0.1)	(<0.1)	(0.2)	(<0.1)	(0.4)	(<0.1)	(0.3)
G1F-N		3.0	3.1	0.4	0.1	0.2	0.1	1.2	0.6	1.9	0.5
[H4N3F1]		(0.2)	(0.3)	(<0.1)	(0.1)	(<0.1)	(0.1)	(<0.1)	(0.6)	(<0.1)	(0.8)
G0		0.2	0.2	0.3	0.3	1.1	1.0	1.2	0.9	0.8	1.3
[H3N4]		(<0.1)	(0.2)	(<0.1)	(0.1)	(<0.1)	(0.3)	(<0.1)	(0.5)	(<0.1)	(0.3)
G1		0.3	0.6	0.4	0.3	0.2	0.6	0.2	0.5	0.3	1.2
[H4N4]		(<0.1)	(0.2)	(<0.1)	(0.1)	(<0.1)	(0.2)	(<0.1)	(0.4)	(<0.1)	(0.3)
G2		1.2	n.d.	0.4	0.1	0.2	0.2	0.4	n.d.	n.d.	n.d.
[H5N4]		(0.1)	n.d.	(<0.1)	(<0.1)	(<0.1)	(0.2)	(<0.1)	n.d.	n.d.	n.d.
G0-N		0.7	0.8	0.4	0.5	n.d.	n.d.	0.2	0.1	0.3	0.4
[H3N3]		(0.2)	(0.2)	(<0.1)	(0.1)	n.d.	n.d.	(<0.1)	(0.1)	(<0.1)	(0.3)
G1-N		n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[H4N3]		n.d.	n.d.	n.d.	(0.2)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G1FS-N		0.5	0.6	0.3	0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[H4N3FS1]		(<0.1)	(0.2)	(<0.1)	(<0.1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G1FS		0.2	0.1	1.4	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[H4N4FS1]		(<0.1)	(0.1)	(<0.1)	(0.2)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M5		3.4	3.8	0.9	0.9	1.2	0.7	1.5	1.9	5.6	7.9
[H5N2]		(0.5)	(0.5)	(<0.1)	(0.1)	(<0.1)	(0.2)	(<0.1)	(0.4)	(<0.1)	(0.8)
M6		0.2	n.d.	n.d.	n.d.	0.3	0.2	0.5	0.1	1.5	2.3
[H6N2]		(<0.1)	n.d.	n.d.	n.d.	(<0.1)	(0.2)	(<0.1)	(0.1)	(<0.1)	(0.5)

385 Table 2: Overview of 2AB-glycans masses of monoclonal antibody

386

Glycan Species	Structural Scheme	Theo M	2-AB M	z=1 (+)	z=2 (+)	z=3 (+)
G0F [H3N4F1]		1444.534	1582.750	1583.757	792.382	528.590
G1F [H4N4F1]		1606.587	1744.803	1745.810	873.409	582.608
G2F [H5N4F1]		1768.640	1906.856	1907.863	954.435	636.626
G0F-N [H3N3F1]		1241.455	1379.671	1380.678	690.843	460.897
G1F-N [H4N3F1]		1403.507	1541.723	1542.730	771.869	514.915
G0 [H3N4]		1298.476	1436.692	1437.699	719.353	479.904
G1 [H4N4]		1460.529	1598.754	1599.752	800.380	533.922
G2 [H5N4]		1622.582	1760.798	1761.805	881.406	587.940
G0-N [H3N3]		1095.397	1233.613	1234.620	617.814	412.211
G1-N [H4N3]		1257.449	1395.665	1396.672	698.840	466.229
G1FS-N [H4N3FS1]		1710.598	1848.814	1849.821	925.414	617.178
G1FS [H4N4FS1]		1913.677	2051.893	2052.900	1026.954	684.971
M5 [H5N2]		1216.423	1354.639	1355.646	678.327	452.553
M6 [H6N2]		1378.476	1516.692	1517.699	759.353	506.571

387