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Characterization of Cetuximab $F_{c/2}$ aggregates by Off-line CZE-MS

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ABSTRACT:

Monoclonal antibody (mAb) therapeutics attract the largest concern due to their strong therapeutic potency and specificity. The Fc region of mAbs is common to many new biotherapeutics as biosimilar, antibody drug conjugate or fusion protein. Fc region has consequences for Fc-mediated effector functions that might be desirable for therapeutic applications. As a consequence, there is a continuous need for improvement of analytical methods to enable fast and accurate characterization of biotherapeutics. Capillary zone electrophoresis-Mass spectrometry couplings (CZE-MS) appear really attractive methods for the characterization of biological samples. In this report, we used CZE-MS systems developed in house and native MS infusion to allow precise middle-up characterization of $F_{c/2}$ variant of cetuximab. Molecular weights were measured for three $F_{c/2}$ charge variants detected in the CZE separation of cetuximab subunits. Two $F_{c/2}$ C-terminal lysine variants were identified and separated. As the aim is to understand the presence of three peaks in the CZE separation for two $F_{c/2}$ subunits, we developed a strategy using CZE-UV/MALDI-MS and CZE-UV/ESI-MS to evaluate the role of N-glycosylation and C-terminal lysine truncation on the CZE separation. The chemical structure of N-glycosylation expressed on the Fc region of cetuximab does not influence CZE separation while C-terminal lysine is significantly influencing separation. In addition, native MS infusion demonstrated the characterization of $F_{c/2}$ dimers at pH 5.7 and 6.8 and the first separation of these aggregates using CZE-MS.

Keywords: Capillary Electrophoresis-Mass spectrometry; Monoclonal antibody; Aggregates; nanoElectrospray

1. INTRODUCTION

Although monoclonal antibodies (mAbs) were introduced as treatments against disease in the late 1980, they currently represent the most rapidly growing category of therapeutic molecule [1-3]. MAbs are highly complex glycoproteins potentially displaying many naturally-occurring molecular micro-heterogeneities [4, 5]. The Fc region of mAbs is common to many new platforms as biosimilar, antibody drug conjugate or fusion protein. This region of the protein is conditioning the antibody isotype and particularly is responsible for effector functions such as antibody-dependent cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) like phagocytosis [6]. Additionnally it facilitates purification and can also contribute to improved solubility and stability [7]. The presence of an Fc region has consequences for Fc-mediated effector functions that might be desirable for therapeutic applications. For successful delivery of biotherapeutic to patient, one of the major issue is aggregates formation. Aggregates in formulated therapeutic products can cause unwanted immune responses and loss of biological activity [8, 9]. Literature suggests that, depending on the nature of mAbs, mAbs aggregation in human might be mediated primarily by unfolding of C_H2 domain on the Fc region [10, 11] and Fab domain [10, 12]. As a consequence, there is a continuous need for improvement of analytical methods to enable fast and accurate characterization. Mass spectrometry (MS) generally coupled with separation methods plays a key role in the characterization of therapeutic mAbs [13] . Depending on the protein structure level considered, characterization is performed following different strategies as intact molecular weight (MW) measurement, top-down, middle-up and bottom-up approaches [13-17]. Capillary zone electrophoresis (CZE) has been demonstrated to be an useful and powerful separation method for the characterization of intact proteins [18-20]. In a previous work, we developed the first analysis of intact mAb charge variant by CZE using a matrix-assisted laser desorption/ionization-MS (MALDI) detection [21]. In 2015, Redman *et al* published a remarkable paper on characterization of intact mAb variants using microfluidic CZE-ESI devices [22]. However, every studies described mAbs characterization without any aggregation phenomenon occurring. Recently, we developed a method for the middle-up characterization of cetuximab glycoforms using off-line CZE-UV/ESI-MS [23].

In this report, we used CZE-MS systems developed in house and native MS infusion to allow precise middle-up characterization of $F_{c/2}$ variant. The mAb selected was cetuximab which is human/murine chimeric IgG-1 directed against the epidermal growth factor receptor (EGFR) overexpressed in advanced-stage EGFR positive colorectal cancer. Cetuximab was approved in the US and EU in 2004 and 2005. Cetuximab contains two glycosylation sites both on the heavy chain (HC) [13]. Moreover, it has a large number of micro-heterogeneities such as PTMs including methionine oxidation, asparagine deamidation or isomerization of aspartic acid. This mAb also has one C-terminal lysine truncation.

These cumulated features make cetuximab an ideal sample for the characterization of mAbs variants by a middle-up approach. In order to cleave the protein in relatively large fragments and distribute N-glycosylation sites over different fragments generated, IdeS proteolysis was employed. IdeS is a cysteine endopeptidase enzyme naturally secreted by *Streptococcus pyogenes* [24], IdeS enzymatic reaction cleaves cetuximab between the two consecutive glycine residues present in the hinge region to obtain two types of fragments ($F_{c/2}$ and F(ab')2) each one carrying a N-glycosylation site. IdeS has demonstrated an exceptional specificity in IgG proteolysis on contrary to papain for instance, therefore it has been consequently used to ease and improve mAbs characterization [25]. IdeS characteristics regarding specificity alongside to proteolysis kinetics are explaining the success of this enzyme in mAbs characterization [26]. We developed a strategy using CZE-UV/MALDI-MS and CZE-UV/ESI-MS to evaluate the role of N-glycosylation and C-terminal lysine truncation on the CZE separation. In addition, this approach demonstrated the characterization of $F_{c/2}$ dimers at pH 5.7 and 6.8 and the first separation of these aggregates using CZE-UV off-line hyphenated to ESI-MS after Fraction collection. A better understanding of these phenomenon could improve the characterization of Fc region and their potential non-covalent interactions [27].

2. Materials and methods

2.1. Chemicals. Methanol (HPLC gradient grade) and acetic acid (100%) were obtained from VWR (Radnor, PA, USA). Ammonium acetate (>98%), sodium hydroxide, ε-Amino-caproic acid (>98%), hydroyxypropylcellulose Mw 100000 (HPC), carboxypeptidase B (CP-B) and formic acid (>98%) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Water used to prepare buffers and sample solutions was obtained using an ELGA purelab UHQ PS water purification system (Bucks, UK). IdeS (Immunoglobulin-degrading enzyme of Streptococcus pyogenes) also named FabRICATOR and IgGZERO were purchased from Genovis (Lund, Sweden). Cetuximab (Erbitux®, Merck KGaA, Darmstadt, Germany) is a sterile, preservative-free solution for intravenous infusion containing 5 mg/mL of cetuximab. Additionally to the mAbs it contains sodium chloride, glycine, polysorbate 80, citric acid monohydrate, sodium hydroxide and water for injections.

2.2. Middle-up sample preparation. Cetuximab was cleaved in the hinge region using limited proteolysis by IdeS (FabriCATOR, Genovis) to obtain two $F_{c/2}$ fragments (theoretical pI 7.74) and one $F(ab')_2$ fragment (theoretical pI 7.78). Sample was diluted using 147.25µL of 50 mM sodium phosphate, 150 mM NaCl, pH 6.60, to a final concentration of 1 µg/µL. A volume of 2.25 µL of IdeS (67 units/µL) was added to the sample which was left at 37°C for 30 min. After digestion completion, sample was desalted using Amicon centrifugal filters (cut off = 10,000 Da) in pure water at 10°C and 14,000 g for 20

min. After desalting step, sample volume recovered was about 10 µL. Sample was finally diluted to a final concentration of 5 µg/µL in a total volume of 30 µL of pure water.

2.3. Carboxypeptidase B (CP-B) treatment. Cetuximab was diluted using 145.75 µL with 10 mM phosphate buffer, 150 mM NaCl, pH 7.4, to a concentration of 1 µg/L. Volumes of 2 µL of CP-B (1mg/mL) and 2.25 µL of IdeS (67 units/µL) were added to the sample which was left at 37°C for 30 min. After digestion completion, sample was treated as described in the middle-up sample preparation.

2.4. Deglycosylation protocol. Cetuximab was diluted using 141.75 µL with 10 mM phosphate buffer, 150 mM NaCL, pH 7.4, to a concentration of 1 µg/µL. Volumes of 6 µL of IgGZERO (20 units/µL) and 2.25 µL of IdeS (67 units/µL) were added to the sample which was left at 37°C for 30 min. After digestion completion, sample was treated as described in the middle-up sample preparation.

2.5. Capillary electrophoresis. The CZE experiments were carried out on a P/ACE MDQ™ CE system from Sciex Separation (Brea, CA) equipped with a UV detection, a temperature controlled autosampler and a power supply able to deliver up to 30 kV. A 32 Karat™ 8.0 (Sciex Separation, Brea, CA) was used for instrument control, data acquisition and data handling. Polymicro bare fused-silica capillaries of 75 µm i.d., 375 o.d. (75.5 cm effective length, 82 cm total length) were obtained from Photonlines (St-Germain-en-Laye, France). New capillaries were conditioned by successive flushes with 1M and 0.1M NaOH and then with water under a pressure of 30 psi for 10 min each. The temperature in the capillary cartridge and autosampler was set to 25 °C. The acquisition rate was 10 points/s. Capillaries were rinsed with water and dried by air when not in use. UV absorbance remained fixed at 200 nm. Voltage applied at 20 kV with a ramp duration of 0.17 min and injection sample conditions were 0.5 psi for 50s. Concerning modified capillaries, capillaries were coated in house with hydroxypropylcellulose Mw 100000 (HPC) following the protocol described by Shen *et al* [28]. 5% HPC in pure water (w/v) was prepared to apply capillary coating. Coating durability is around 20 runs without recoating step. For cetuximab separation, inlet BGE 200 mM ε-Amino-caproic acid (EACA)-ammonium acetate 25 mM pH 5.70 and outlet BGE ammonium acetate 25 mM pH 5.70 have been used as separation conditions. Injection volumes have been calculated using CEToolbox application (Pansanel, GooglePlay).

2.6. CZE/Fraction Collection Interface. This interface have been described in a previous study [21]. Briefly, automated off-line coupling of CZE to MS was set up by using a homemade modified automatic spotting device Proteineer FC (Bruker Daltonics, Bremen, Germany) for the sheath flow-assisted spotting from the CZE capillary outlet onto a fraction collection target. The original set up of the UV cell in the P/ACE MDQ™ (Sciex Separation, Brea, CA) was delocalized outside the CE instrument in order to allow simultaneous UV detection and fractions collection. Hystar 3.2 (Bruker Daltonics, Bremen, Germany) was used for Proteineer FC control.

2.7. CESI-MS as nanoESI infusion platform. The infusion experiments were carried out with a PA 800 plus capillary electrophoresis (CE) system from Sciex Separation (Brea, CA). Prototype fused-silica capillaries (total length 95 cm; 30 µm i.d.) whose outlet end (about 3 cm) was etched with hydrofluoric acid were provided by Sciex Separation (Brea, CA, USA). New capillaries were initially conditioned by flushing them for 10 min with MeOH, 10 min with 0.10 M sodium hydroxide, 10 min with 0.10 M hydrochloric acid and finally with water for 20 min each flushing step being conducted at 75 psi (5.17 bar).

2.8. MALDI-TOF-MS. The matrix was prepared by dissolving 2,5-dihydroxybenzoic acid (DHB) (2g/L) in 0.1% trifluoroacetic acid/acetonitrile (TFA/ACN) (30/70)(v/v). Fraction collection was realized using Ground Steel MALDI target (Bruker Daltonics, Bremen, Germany). Mass spectra of the CZE fractions were recorded using an Autoflex II MALDI-TOF (Bruker Daltonics, Bremen, Germany), operating in reflector mode and with FlexControl software. Positively charged ions were detected and sums of 1500 single-shot spectra were acquired automatically from each sample by using the AutoXecute software. Data processing was performed with FlexAnalysis 3.0 (Bruker Daltonics, Bremen, Germany). All spectra were calibrated by external calibration using Protein calibration standard I (Bruker Daltonics, Bremen, Germany) for intact protein separation.

2.9. ESI-TOF-MS. For nanoESI-MS infusion experiments the PA800 plus system was coupled to a maxis 4G (Bruker Daltonics, Bremen, Germany). MS transfer parameters were optimized using the actual sample directly infused via the CE system using a pressure of 5 psi (340 mbar). MS parameters were optimized so that high m/z ions could be properly transferred to the TOF mass analyzer while avoiding fragmentation. Ion funnels RF were set at values of 300 Vpp and 400 Vpp. The electrospray voltage (capillary voltage) was typically ranging from -1.2 to -1.8 kV. Dry gas was set at 1.5 L/min and source temperature at 180°C. Data processing was performed with DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Deconvolution of the mass spectra was performed based on maximum entropy analysis using ESI Compass 1.3 Maximum Entropy Deconvolution Option in DataAnalysis 4.0. All spectra were calibrated by external calibration using Pepmix (Bruker Daltonics, Bremen, Germany) and cesium iodide (CsI) from Sigma-Aldrich (Saint-Louis, MO, USA).

3. Results and discussions

Cetuximab is a chimeric mouse-human IgG1 known to bear 2 N-glycosylation sites on each HC [26]. As every mAbs, cetuximab can exhibit a wide variety of micro-heterogeneities particularly due to PTMs. Furthermore, this mAb can also experience HC C-terminal lysine truncation. In a preliminary experiment, direct nanoESI-MS infusion of cetuximab IdeS digest was performed. Results proved equivalent signal abundances for $F_{c/2}$ C-terminal lysine variants which involves two distinct profiles for

the $F_{c/2}$ domains (Fig. S-1). With this information, we expected to detect at least two peaks corresponding to $F_{c/2}$ fragments due to a loss of a +1 charge associated with C-terminal lysine truncation.

Cetuximab IdeS digest was separated and characterized by offline CZE-UV/ESI-MS using an instrumental setting developed in house, described in a previous work [23]. Briefly, assymetric CZE conditions was based on inlet background electrolyte (BGE) composed of ε-amino-caproic acid (EACA) 200 mM/ammonium acetate 25 mM, pH 5.7 and outlet BGE composed of ammonium acetate 25 mM, pH 5.7. Hydroxypropylcellulose (HPC) coating was used to minimize analyte adsorption on the capillary wall and reduce the electroosmotic flow (EOF). After deposition, each fraction was collected and evaporated. Before infusion, dry samples were reconstituted in 2µL of ACN/H_20/FA (50/49/1, v/v) in order to enhance sample ionization. Thus, each fraction was infused into the MS using a flow rate of 10 nL/min in order to enhance sensitivity.

As emphasized in Fig. 1-b, CZE separation obtained shows three consecutive peaks completely separated corresponding to the $F_{c/2}$ region. Deconvoluted mass spectra of each fraction generated multiple masses corresponding to the expected presence of different neutral glycosylation on $F_{c/2}$ fragments (Fig. 1-b). In addition, the comparison with the direct infusion experiment (Fig. S-1) demonstrates that the two $F_{c/2}$ fragments corresponding to intact and with one lysine truncation ($F_{c/2}$ and $F_{c/2}$-K) are detected and separated. Deconvoluted mass spectra corresponding to peak 1 and peak 3 show a difference of 128 Da between mass peak 25359.1 Da corresponding to the most abundant $F_{c/2}$ glycoform (G0F) and mass peak 25231.1 Da corresponding to G0F for $F_{c/2}$-K. Thus, we demonstrate the complete separation of these two fragments (Fig. 1-b). Regarding deconvoluted mass spectra of peak 2 (Fig. 1-b 2), it shows a nearly equal abundance mixture of $F_{c/2}$ and $F_{c/2}$-K variants. Deconvoluted mass spectra corresponding to peak 2 lead to the same MWs as peak 1 and peak 3 and could be attributed to either an overlap during the fraction collection process or by cumulative PTMs which could lead to a variation of conformation without mass shifts. Moreover, these phenomenon could also be explained by a possible aggregation of $F_{c/2}$ fragments.

3.1. Validation of the CZE-UV/ESI-MS fraction collection process.

The fraction collection process developed in this study based on the apparent mobility of each peak, allows theoretically to recover the total quantity of each variant present in the peak. However the offline coupling strategy developed do not permit to represent the separation using MS spectra and then to observe possible diffusion. Regarding raw MS spectra (Fig. S-2) and based on the hypothesis of an equivalent ionization efficiency of all $F_{c/2}$ variants, potential carryover effect due to peak broadening

or excessive diffusion should be eliminated. However, in the aim to confirm the absence of overlap during the fraction collection, experiments using CZE-UV/MALDI-MS coupling have been performed. Same experimental conditions described previously have been implemented. Only deposition process has been changed to allow a fraction collection directly on a MALDI target plate with a deposition time interval of 1 minute between each fraction. Fraction collection was performed between 43 and 55 minutes according to the electropherogram represented in Fig. 2-a. Fig. 2-b emphasizes the reconstruction of the separation using MS spectra of each fraction. First of all, the CZE/MALDI-MS reconstruction exhibits three distinct peaks corresponding to the CZE separation. MS spectra of each peak in CZE show a value of 25 kDa corresponding to the expected $F_{c/2}$ variants. However due to presence of other sources of micro-heterogeneities and the limited resolution of the MALDI-MS instrument used, it was impossible to deduce the average mass of the charge variants confidently. In the other hand, the CZE/MALDI-MS profile (Fig. 2-b) confirms the good agreement between the UV detection time and the deposition time. Based on the difference of protein apparent mobilities described by the equation:

$$\mu_{app} = \frac{\mathrm{L\,l}}{\mathrm{t}_m\,\mathrm{V}}$$

with μ_{app} the apparent mobility which is the sum of effective mobility and residual electroosmotic flow, L and l the total capillary length and length to detection window, respectively, V the applied voltage and t_m the migration time. Deposition time t_d is then calculated by the equation:

$$t_d = \frac{L^2}{\mu_{app} V}$$

The localization of each $F_{c/2}$ variants shows a time shift between the UV detections and the spotting process due to UV localization at 6.5 cm from the capillary outlet. These results confirm without any remaining ambiguity the absence of carryover effect and diffusion phenomenon during the fraction collection process, demonstrating the accuracy and robustness of the instrumental setting developed. As a consequence, characteristics of the analytical strategy developed to characterize $F_{c/2}$ fragments cannot be doubted. Therefore it is necessary to focus on cetuximab structure and potential modifications on the fragments generated during IdeS proteolysis in order to conclude on the CZE separation described previously (Fig. 1-a).

3.2. Influence of N-glycosylation on $F_{c/2}$ variants separation

An important class of modification that can significantly impact the immunogenic properties of cetuximab in terms of pharmacokinetic and pharmacodynamic is glycosylation [26]. Cetuximab

contains two sites of glycosylation on the HC: one is located in the $F_{c/2}$ domain (Asn^{299}) and the second is located in the $F(ab')_2$ domain on Asn^{88} [13]. Cetuximab glycosylation profile has been extensively described in particular as it is expressing glycoforms responsible for immunogenic reaction [26]. Regarding $F_{c/2}$ domain, branched glycan structures are primarily composed of fucose, N-acetylglucosamine, and mannose but can differ in the number of terminal galactose, mannose or N-acetylglucosamine residues. Glycan structure with the addition of galactose moieties involves a mass increase of 162 Da but does not induce a change in net charge [29, 30]. In a previous work we demonstrated that concerning glycoform with a difference of one galactose residue, middle-up approach does not allow to observe baseline separations [23]. The mass of galactose moiety accounts for only ≈0.6% of the $F_{c/2}$ domain and ≈0.2% of the $F(ab')_2$ domain. Thus mass difference should be greater or involve a significant conformation change to enable a significant effective mobility shift. To confirm these theoretical considerations, analysis of deglycosylated sample of cetuximab by CZE-UV/ESI-MS was performed in order to evaluate the role of glycans in the separation process. Before IdeS digestion of cetuximab, a deglycosylation step using IgGZERO has been added to the digestion protocol. The aim is to remove all glycans in the Asn^{299}. IgGZERO is an endoglycosidase specific for N-glycans on the Fc domain of IgGs which are cleaved after the first Glc-NAc. Following IgGZERO and IdeS treatment, the most intense ion series is corresponding to expected cetuximab $F_{c/2}$ and $F_{c/2}$-K subunits with GlcNac-Fuc remaining (Fig. 4-b and Table S-1). CZE separation, represented by Fig. 3-B, contains the three peaks corresponding to $F_{c/2}$ subunits with the same profile in terms of migration times and resolution compared to the original separation of the sample non treated with IgGZERO enzyme (Fig. 3-A). With these results, we can conclude that glycan nature expressed on Asn^{299} residue does not impact significantly the CZE separation of $F_{c/2}$ variants. Effect of others microheterogeneities like asparagine deamidation or methionine oxidation are quite difficult to observe on the intact protein or using limited proteolytic digestion because of the number of these modifications, their localization and the difficulty to control the level of modification in the proteins. Indeed, some parameters like storage conditions or digestion pH can induce endogenous asparagine deamidation or methionine oxidation.

3.3. Influence of C-terminal lysine truncation on $F_{c/2}$ variants separation

The presence of the lysine in HC C-terminal position is essential to understand the separation of $F_{c/2}$ variants. Indeed, in this work, we demonstrated the separation of the two $F_{c/2}$ fragments corresponding to intact and with one lysine truncation ($F_{c/2}$ and $F_{c/2}$-K). Peak 1 and peak 3 (Fig. 3-A) correspond to the most abundant $F_{c/2}$ glycoforms and $F_{c/2}$-K glycoforms respectively. However for peak 2, a nearly equal abundance mixture of $F_{c/2}$ and $F_{c/2}$-K have been observed. To evaluate the role of lysine truncation in the separation process, IdeS digestion protocol has been modified to perform a total truncation of C-

terminal lysine of cetuximab. Sample was treated by carboxypeptidase B (CP-B), removing systematically the C-terminal lysine residues from the two HCs of the antibody [31], it was added prior to IdeS digestion step. After CP-B and IdeS treatment, the most intense ion series corresponds to the expected glycoforms of $F_{c/2}$-K variants (Fig. 4-c). Moreover, as compared to sample treated only with IdeS (Fig. 4-a), we observe in the deconvoluted mass spectrum the complete loss of $F_{c/2}$ glycoforms profile which confirms the total removing of C-terminal lysine on the HC. CZE separation, represented on Fig. 3-C, contains only one peak corresponding to $F_{c/2}$ subunits. This peak has been characterized as $F_{c/2}$-K variants. Moreover, we observe the absence of the two first peaks compared to the original separation. Despite the fact that the loss of the first peak is totally justified by the unique presence of $F_{c/2}$ variant trimmed from both HC C-terminal lysine, the loss of the second peak proves that the presence of C-terminal lysine residue plays an active role in the separation process of $F_{c/2}$ variants. These results also allow to reject hypothetic effects on effective mobilities due to several cumulative PTMs leading to modification of the mAb without MS resolved mass shift. The second peak of the separation of sample without CP-B treatment (Fig. 3A) has been characterized by a nearly equal abundance mixture of $F_{c/2}$ and $F_{c/2}$-K variants. Absence of this peak in cetuximab separation after CP-B+IdeS digestion (Fig. 3-C) and the fact that no $F_{c/2}$-K variants have been detected at that migration time involves a strong relation between $F_{c/2}$ and $F_{c/2}$-K variants.

3.4. Role of $F_{c/2}$ aggregates on CZE separation

In this work, we described a protocol based on nanoESI-MS infusion of the different fractions using CESI-MS interface as nanoESI platform. After CZE separation, each collected fraction was systematically dried by air and reconstituted in 2µL of ACN/$H_2$0/FA (50/49/1, v/v) to enhance ionization. However, infusion conditions differed from CZE conditions at pH 5.7 and are denaturing by the presence of acetonitrile. Indeed, in these experiments, noncovalent interactions between $F_{c/2}$ variants can influence CZE separation at pH 5.7 but protein-protein interaction cannot actually be observed due to the denaturing nanoESI-MS conditions used. To evaluate these phenomena, nanoESI-MS infusion of cetuximab IdeS digest have been prepared using denaturing conditions and using CZE conditions as well. For denaturing conditions, sample has been diluted in 2µL as stated previously while for CZE conditions, sample has been diluted in ammonium acetate 25mM, pH 5.7. Each sample has been reconstituted to a concentration of 1µM. Each sample was infused into the MS using a flow rate of 10 nL/min. Deconvoluted mass spectra in denaturing conditions generated multiple masses corresponding to the expected presence of neutral glycosylation variants of $F_{c/2}$ fragments (Fig. 5-a and Table S-1). Mass peak 25359.2 Da corresponding to G0F for $F_{c/2}$ and mass peak 25231.1 Da corresponding to G0F for $F_{c/2}$-K are in agreement with theoretical masses of these glycoforms (Table S-

1). Concerning CZE conditions, charge variant deconvoluted mass spectrum generated multiple masses between 50.4 kDa and 51.2 kDa (Fig. 5-b). These peaks correspond to the formation of protein-protein aggregates due to noncovalent interactions between $F_{c/2}$ subunits. Indeed, mass peak 50463.4 Da corresponds to the mass of $F_{c/2}$-K subunits homodimer, mass peak 50591.8 Da corresponds to the heterodimer between $F_{c/2}$ and $F_{c/2}$-K subunits and mass peak 50721.2 Da corresponds to $F_{c/2}$ subunit homodimer (Table S-2). Moreover, other mass peaks between 50.4 kDa and 51.2 kDa correspond to the expected presence of the combination of neutral glycosylation variants of these different dimers (Table S-2). In addition, no deconvoluted mass spectrum have been observed at 25 kDa that excludes the presence of free $F_{c/2}$ variants in ammonium acetate 25mM at pH 5.7. These results allow the explanation of the presence of three different peaks corresponding to $F_{c/2}$ variants in CZE separation. Indeed, peak 1 (Fig. 1-a) corresponds to $F_{c/2}$ homodimer, peak 2 corresponds to $F_{c/2}$/$F_{c/2}$-K heterodimer and peak 3 correspond to $F_{c/2}$-K homodimer. To our knowledge, this is the first time that aggregates of cetuximab $F_{c/2}$ subunits have been separated and characterized. Finally, a last study consisted to infuse the same sample in condition classically described in the literature as native conditions [32-35]. To be in agreement with CZE condition, 25 mM ammonium acetate at pH 6.8 was selected. Obtained MS profiles were exactly the same as those observed using CZE conditions. This confirms the presence of $F_{c/2}$ dimers in native conditions and exclude a possible major role of EACA in the protein-protein aggregation process. Results obtained from CZE-UV/MALDI-MS and CZE-UV/ESI-MS experiments clearly demonstrate the formation of $F_{c/2}$ fragments following IdeS proteolytic digestion of mAbs. This observation is particularly important from an analytical chemistry point of view. As the outstanding selectivity of IdeS has favored its wide use among the scientific community and the formation of aggregates, even if reversible, it should be taken into account in the development of innovative analytical methods.

4. Conclusions

To summarize, we report here the use of off-line CZE-UV/ESI-MS for the middle-up characterization of $F_{c/2}$ variant of cetuximab. To validate the method, developed CZE offline instrumental setting was implemented to perform direct deposition of collected fractions on a MALDI target and used to characterize IdeS proteolytic digest fragments by MALDI-TOF MS. Results obtained demonstrated successful CZE-UV/MALDI-MS characterization. MS spectra were in complete agreement with the separation observed by the intermediate of the UV detection. Additionally data generated allowed to exclude overlapping during the collection and deposition process validating the instrumental approach regarding accuracy and robustness. In order to investigate further the separation of three different species corresponding to $F_{c/2}$ variants, CZE-UV/ESI-MS experiments were performed on cetuximab

treated consequently with igGZERO and IdeS enzymes to permit the removal of N-glycosylation present in the Fc region of the protein. Results showed that chemical structure of N-glycosylation expressed on the Fc region of cetuximab does not influence CZE separation as both revealed similar UV electropherograms. A similar analysis was then realized on cetuximab treated successively with CP-B and IdeS in order to systematically remove C-terminal lysine from the $F_{c/2}$ fragments generated. Results from the CZE separation exhibited the absence of two peaks compared to the same separation using cetuximab solely treated with IdeS. As expected from the CZE conditions employed, C-terminal lysine is significantly influencing separation. Finally direct nanoESI-MS infusion of the collected fractions from CZE separation were performed using different buffering systems, one which is similar to CZE background electrolyte composed of ammonium acetate 25 mM, pH 5.70, and in parallel in denaturing conditions favoring ionization efficiency and signal sensitivity. In similar conditions as CZE separation, MS allowed to identify without ambiguity the nature of each analyte separated by CZE. MS spectra obtained revealed the first specie to be $F_{c/2}$ homodimer while the second specie detected was $F_{c/2}$/$F_{c/2}$-K heterodimer and the last one being $F_{c/2}$-K homodimer. Results demonstrated to be in agreement regarding the influence of C-terminal lysine on the separation but additionally showed the formation of $F_{c/2}$ aggregates as a consequence of IdeS proteolytic digestion. Results from the different experiments demonstrated the relevance of this CZE-UV/ESI-MS analytical method developed to perform the comprehensive middle-up characterization of mAbs. Indeed it was possible to conclude of the formation of $F_{c/2}$ aggregates generated consequently from cetuximab IdeS treatment. As this enzyme recently tends to be widely used, due its exceptional specificity, for mAbs characterization, aggregation related to sample treatment appears as a new insight which should be taken into account during method development. The implementation of an electrophoretic separation prior to the MS analysis showed its interests in this work as separation efficiency provided by CZE allowed to completely separate the different types of aggregates while showing the possibility to maintain these aggregates. Moreover, the Fc region of mAbs is common to many new platforms and these results could improve the characterization of Fc region of biotherapeutics and their potential non-covalent interactions.

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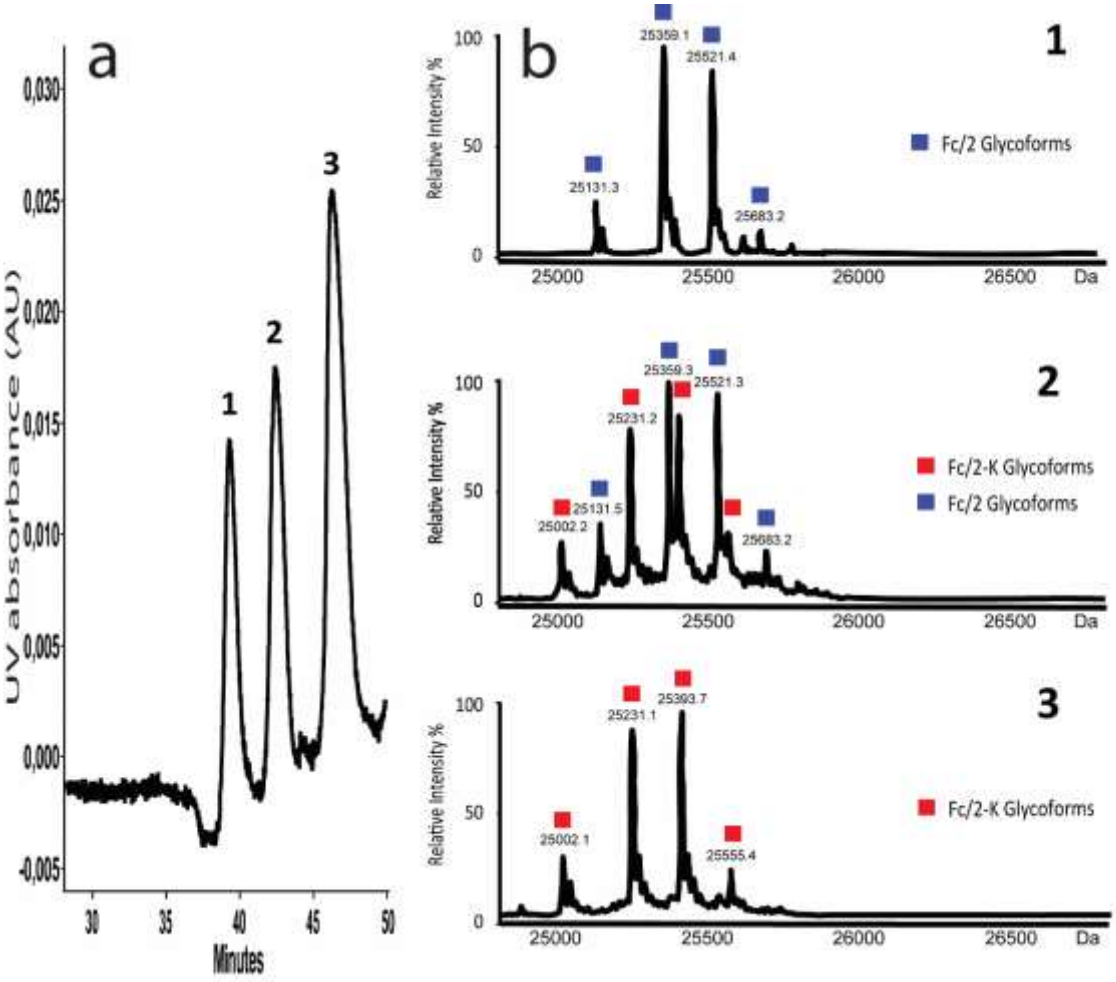

Fig. 1. (a) Off line CZE-UV/ESI-MS separation of middle-up cetuximab charge variants. (b) Deconvoluted mass spectra for each $F_{c/2}$ variant. The MS peak were labeled with the correspondent glycoform. Experimental conditions: Inlet BGE: EACA 200 mM ammonium acetate 25 mM pH 5.70 and outlet BGE: ammonium acetate 25 mM pH 5.70; HPC-coated capillary, total/effective length 82/75.5 cm x 75 µm i.d.; voltage, 20 kV; UV absorbance at 200 nm; MS condition: See Experimental section; sample, IdeS digest of cetuximab (5 µg/µL); sample injection 0.5 psi 50s.

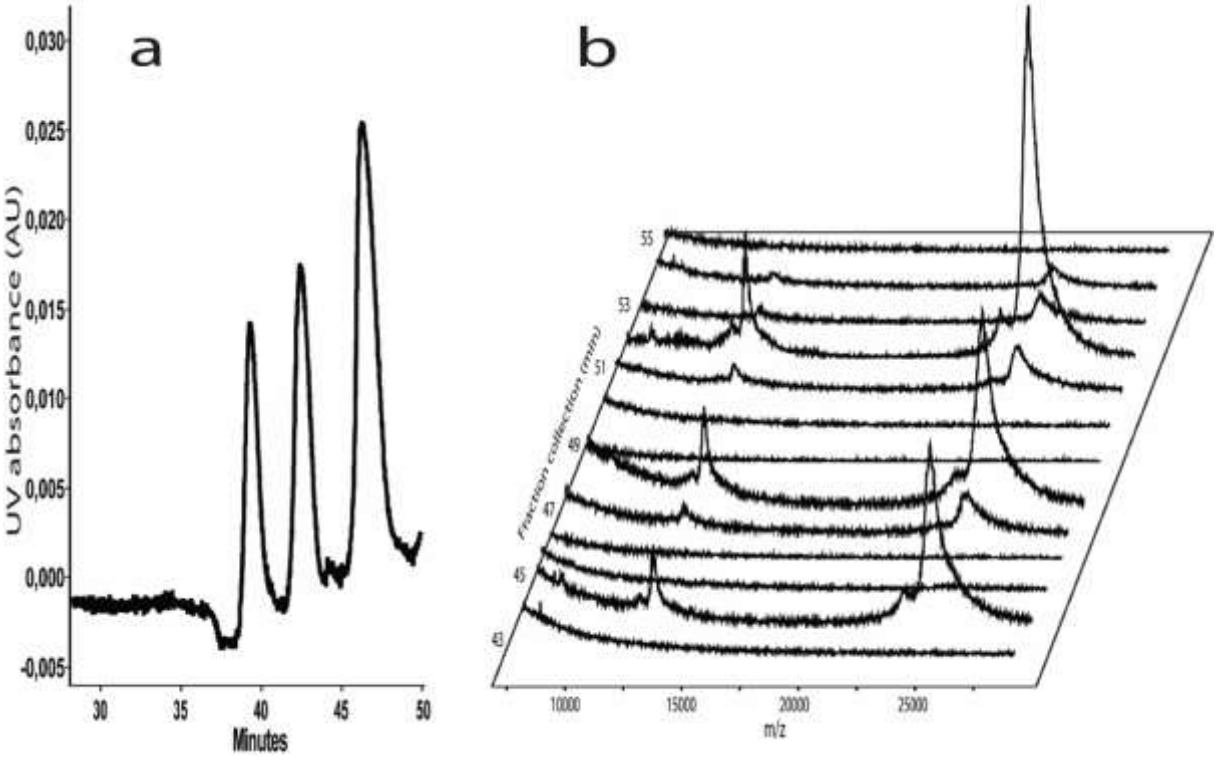

Fig. 2. CZE-UV off-line coupled to MALDI-MS via fractionation for a middle-up characterization of Cetuximab $F_{c/2}$ variants. (a) CZE-UV electropherogram. (b) Analysis of CZE-UV fractions by MALDI-MS. CE Experimental conditions: See Fig. 1; MS condition: See Experimental section; sample, IdeS digest of cetuximab (5 µg/µL)

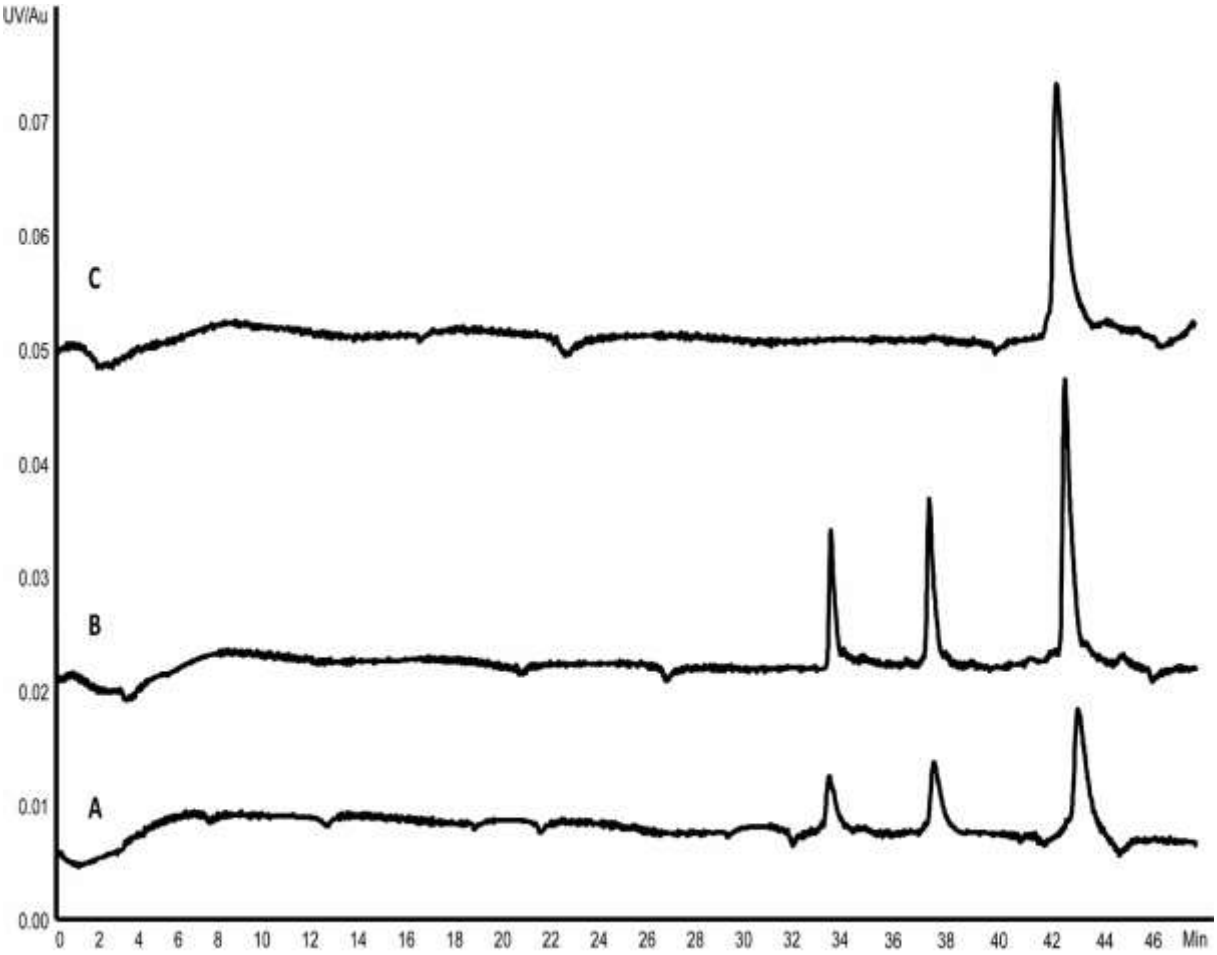

Fig. 3. Impact of glycosylation and lysine truncation on electrophoretic resolution. Electrophoretic separation of (A) cetuximab with IdeS treatment, (B) cetuximab with IgGZERO+IdeS treatment and (C) cetuximab with CP-B+IdeS treatment. Inlet BGE composed by a mixture of EACA 200 mM and ammonium acetate 25 mM pH 5.70 and outlet BGE by ammonium acetate 25 mM at pH 5.70.

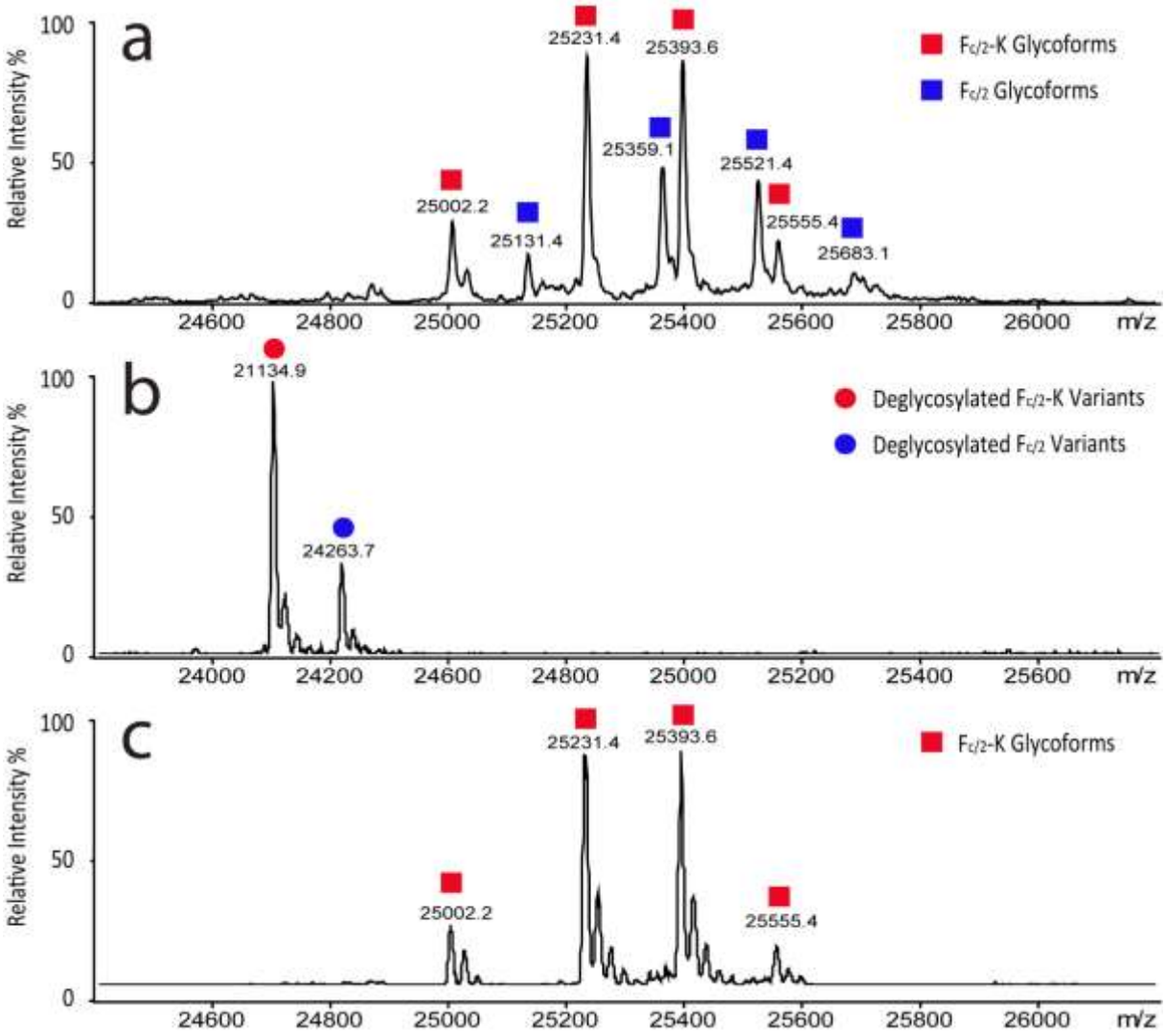

Fig. 4. $F_{c/2}$ variants deconvoluted mass spectra of (a) IdeS digest cetuximab, (b) IgGZERO+IdeS digest cetuximab and (c) CP-B+IdeS digest cetuximab. Experimental conditions: 50%/49%/1%, acetonitrile/water/formic acid (v/v/v); sample concentration 1 µM.

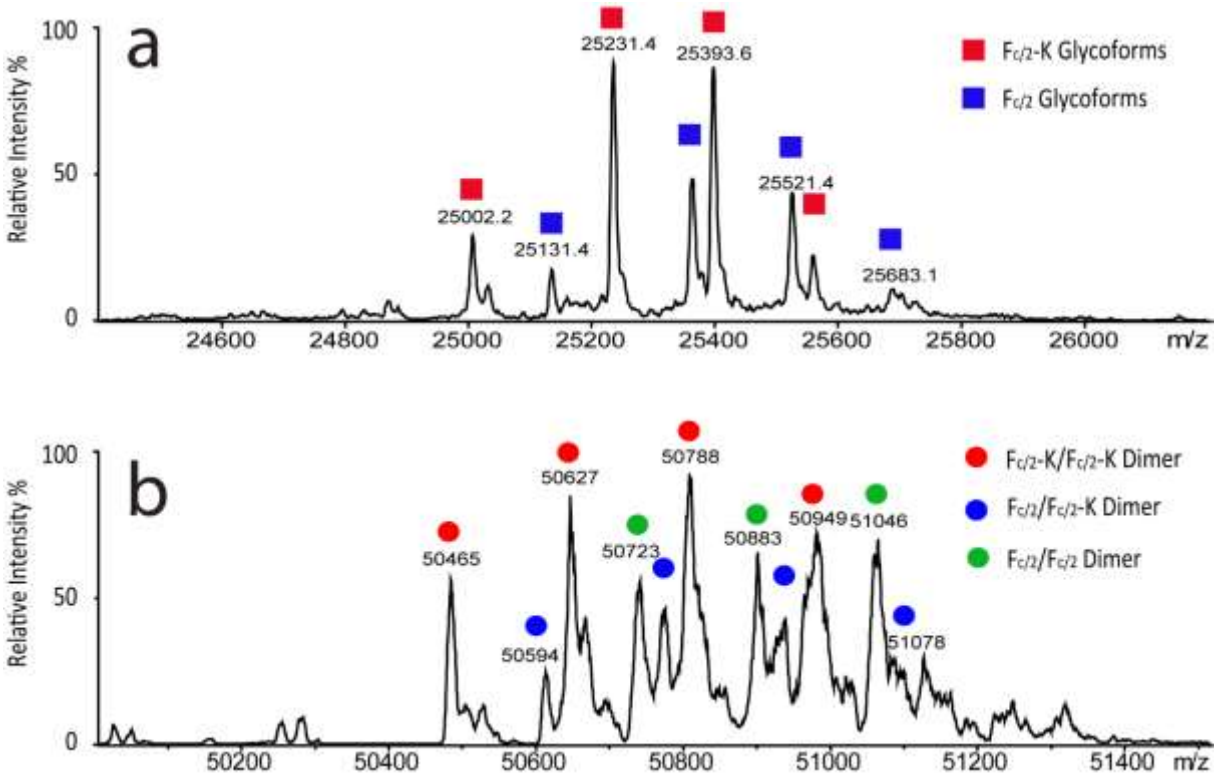

Fig. 5. Cetuximab $F_{c/2}$ variants deconvoluted mass spectra in (a) denaturing and (b) CZE conditions. Experimental conditions: denaturing conditions, 50%/49%/1%, acetonitrile/water/formic acid (v/v/v); CZE conditions, ammonium acetate 25mM, pH 5.7; sample concentration 1 µM.

Caption

Fig. 1. (a) Off line CZE-UV/ESI-MS separation of middle-up cetuximab charge variants. (b) Deconvoluted mass spectra for each $F_{c/2}$ variant. The MS peak were labeled with the correspondent glycoform. Experimental conditions: Inlet BGE: EACA 200 mM ammonium acetate 25 mM pH 5.70 and outlet BGE: ammonium acetate 25 mM pH 5.70; HPC-coated capillary, total/effective length 82/75.5 cm x 75 µm i.d.; voltage, 20 kV; UV absorbance at 200 nm; MS condition: See Experimental section; sample, IdeS digest of cetuximab (5 µg/µL); sample injection 0.5 psi 50s.

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