



HAL
open science

In-capillary (electrophoretic) digestion-reduction-separation: A smart tool for middle-up analysis of mAb

Meriem Dadouch, Yoann Ladner, Claudia Bich, Jérôme Montels, Jacques Morel, Cherine Bechara, Catherine Perrin

► To cite this version:

Meriem Dadouch, Yoann Ladner, Claudia Bich, Jérôme Montels, Jacques Morel, et al.. In-capillary (electrophoretic) digestion-reduction-separation: A smart tool for middle-up analysis of mAb. *Journal of Chromatography A*, 2021, 1648, pp.462213. 10.1016/j.chroma.2021.462213 . hal-03231054

HAL Id: hal-03231054

<https://hal.science/hal-03231054>

Submitted on 24 May 2023

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.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 In-capillary (electrophoretic) digestion-reduction-separation: a smart tool for middle-up
2 analysis of mAb.

3 Meriem Dadouch¹, Yoann Ladner¹, Claudia Bich¹, Jérôme Montels¹, Jacques Morel², Cherine
4 Bechara^{3,4}, Catherine Perrin^{1*}

5 ¹ Institut des Biomolécules Max Mousseron (IBMM), UMR 5247-CNRS-UM-ENSCM, Université de
6 Montpellier, Montpellier, France

7 ² Département de Rhumatologie, Université de Montpellier, Hôpital Lapeyronie, 34295, Montpellier
8 Cedex 5, France

9 ³ Institut de Génomique Fonctionnelle (IGF), Université de Montpellier, CNRS, INSERM, Montpellier,
10 France

11 ⁴ Institut Universitaire de France (IUF)

12 * Corresponding author: catherine.perrin@umontpellier.fr / + 33 4 11 75 95 92

13 Highlights

- 14 - Automated simultaneous TCEP reduction and IdeS digestion of mAb are implemented.
- 15 - High resolution of separation under denaturing and non-denaturing conditions.
- 16 - Good performance and versatility of the methodologies are demonstrated.

17 Abstract

18 Comprehensive characterization of physicochemical properties of monoclonal antibodies (mAbs) is a
19 critical process to ensure their quality, efficacy, and safety. For this purpose, mAb analysis at
20 different levels (bottom-up, middle-up) is a common approach that includes rather complex
21 multistep sample preparation (reduction, digestion). To ensure high analysis performance, the
22 development of fully integrated methodologies is highly valuable. Capillary zone electrophoresis is a
23 particularly well-adapted technique for the multistep implementation of analytical strategies from
24 sample preparation to detection. This feature was employed to develop novel integrated
25 methodologies for the analysis of mAb at the middle-up level. Multiple in-line reactions
26 (simultaneous reduction and digestion) were performed for the first time in the separation capillary.
27 Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was used as an effective reducing agent under
28 a broad pH range and IdeS (Immunoglobulin degrading enzyme from *Streptococcus*) as a highly
29 specific enzyme for mAb digestion. Transverse diffusion of laminar flow profile (TDLFP) was applied
30 for reactants mixing. Both in-line sample preparation and separation parameters were optimized
31 under non-denaturing and denaturing conditions. The developed in-line methodologies provided
32 good reproducibility and higher peak efficiencies comparing with off-line assays. They were
33 successfully applied to different mAbs.

34 Keywords

35 Capillary electrophoresis; Transverse Diffusion of Laminar Flow Profile; Monoclonal antibodies;
36 Middle-up approach; In-line reduction-digestion.

37

1 1. Introduction

2 Monoclonal antibodies (mAbs) have revolutionized cancer and autoimmune disorders management
3 and dominated the biopharmaceutical sector [1]. mAbs are inherently heterogeneous in structure
4 due to numerous post-translational modifications (PTMs) and degradation reactions occurring at all
5 production steps. Therefore, a comprehensive characterization of mAb microheterogeneities along
6 with structural integrity is necessary to guarantee their quality, efficacy, and safety and satisfy
7 regulatory requirements [2]. The physicochemical characterization of mAbs involves a set of
8 separation techniques that provide complementary information about critical quality attributes such
9 as glycosylation, deamidation, and oxidation. Much effort has been invested in developing
10 chromatographic methods with different modes (reverse-phase, ion-exchange, hydrophilic
11 interaction...) and dimensions (one or two-dimensional formats) [3,4]. Recently, alternative
12 separation techniques have shown improved/complementary separation abilities in particular
13 capillary zone electrophoresis (CZE) [5]. Thanks to its high flexibility and cost-effectiveness, CZE is a
14 promising technique for characterizing mAbs at the intact, middle-up/down, and bottom-up levels.
15 Furthermore, analyses can be performed under native or denaturing conditions. Native CZE analyses
16 (i.e. neutral pH and high ionic strength buffer) provide high resolution of mAb variants separation
17 [6,7] and insights about inter- and intra-molecular interactions [8–10]. This allows the investigation
18 of mAb structure and stability (structural modifications, aggregation). Analyses under denaturing
19 conditions (i.e. acidic solution with or without organic solvent in the limit of mAbs solubility) are well
20 suited for microheterogeneity characterization [11].

21 Analysis of intact mAbs is attractive as information on the global structure will be obtained with
22 almost no sample preparation. However, this approach provides incomplete information about some
23 microheterogeneities and their localization. Bottom-up and middle-up approaches give
24 complementary information to intact mAb analysis. They provide access to more details on the
25 sequence and/or post-translational modifications but require multiple sample preparation steps. The
26 middle-up approach offers a simple investigation of mAbs with less complicated sample preparation
27 than bottom-up and more accurate characterization than the intact approach. Multi-attribute
28 assessment, like glycosylation and charge variants, can be done from a single run with high accuracy
29 [12,13]. Nevertheless, the off-line sample preparation of established methods presents several
30 drawbacks related to the manual handling (multisteps of pipetting and transfer, time and reactants
31 consumption) that lead to the increase of the risks of contamination, errors, and loss of reactants by
32 contact to surfaces or evaporation. Moreover, sample preparation can introduce artifacts by
33 increasing deamidation and oxidation or desialylation [14].

34 Recent developments have highlighted the high potential of automation of CZE for the bottom and
35 middle-up analysis since enzymatic reactions can be performed inside the capillary with the
36 possibility of temperature control [15]. Two mixing modes can be implemented in CZE: (i)
37 electrophoretically-mediated microanalysis (EMMA) that allows efficient mixing based on
38 electrophoretic migration. Its implementation is however, rather difficult for the mixing of more than
39 two reactants; (ii) transverse diffusion of laminar flow profile (TDLFP), a generic method that is
40 based on the hydrodynamic mixing of more than two reactants [16]. In-line sample preparation
41 enables integrating all analysis steps (sample preparation, separation, and detection) to minimize
42 errors, artifacts, time and costs. Our group had applied the TDLFP successfully for the bottom-up
43 analysis of mAbs [17].

44 Middle-up approach can be operated *via* different protocols:

1 (i) Partial reduction of disulfide bonds resulting in heavy (HC) (MW \approx 50 kDa) and light (LC) (MW \approx 25
2 kDa) chains. Reducing agents such as dithiothreitol (DTT) and tris (2-carboxyethyl)phosphine (TCEP)
3 are commonly used for mAb reduction [18]. DTT is active at neutral conditions (pH 6 to 8) and widely
4 used because the reaction can be quenched by acidification. Unlike DTT, TCEP is odorless, less
5 susceptible to oxidation, active under a large pH range (about 2 to 8), and unreactive toward other
6 functional groups of proteins [19]. Furthermore, TCEP is compatible with electrospray ionization at
7 moderate concentrations [20]. High separation resolution of HC and LC have been reported in CE
8 with acidic BGE and neutral coated capillaries [21,22];

9 (ii) Direct enzymatic digestion using enzymes with high specificity for a single cleavage site: papain
10 [23], IgdE, and SpeB [18] cleave over the hinge region yielding F(ab) and Fc subunits. IdeS
11 (Immunoglobulin degrading enzyme of *Streptococcus pyrogenes*) cleaves under the hinge region of
12 mAbs to obtain F(ab)₂ (MW \approx 100 kDa) and Fc/2 (MW \approx 25 kDa) subunits [24]. IdeS is the most
13 popular for the characterization of mAbs due to its fast and efficient enzymatic activity. It has been
14 used to characterize Fc/2 glycoforms, charge variants, and Fc/2 dimers by CE [8,25,26]. Recently, we
15 developed an in-line methodology for this approach with simplified procedure, temperature control,
16 and high resolution of subunits separation under non-denaturing MS-compatible conditions [27].

17 (iii) Enzymatic digestion followed by reduction to decrease mAbs subunits complexity and enhance
18 electrospray ionization by reducing their molecular weight. IdeS digestion, followed by reduction
19 with DTT or TCEP was reported [28]. The resulted subunits (LC, Fd, and Fc/2, MW \approx 25 kDa) were
20 partially separated using positively charged capillary coating and acidic BGE. In another study,
21 subunits were baseline separated using a neutral capillary coating and acidic BGE [29]. As the IdeS
22 enzyme does not contain disulfide bonds [30], the digestion and reduction steps can be combined
23 without affecting the IdeS structure and activity. This decreases the time required for incubation and
24 makes the sample preparation more straightforward.

25 Here, we describe the implementation of novel enzymatic microreactors for different in-line
26 electrophoretic middle-up approaches using TDLFP. In-line TCEP reduction and simultaneous IdeS-
27 TCEP digestion-reduction were implemented using adalimumab as model mAb. Adalimumab, a fully
28 human antibody inhibitor of tumor necrosis factor-alpha, is widely used to treat several chronic
29 inflammatory diseases (rheumatoid arthritis, Crohn disease, psoriasis...) [31]. It ranked the top-selling
30 list of biopharmaceuticals since 2012 together with infliximab, trastuzumab, and rituximab [32,33].
31 Different parameters were carefully considered during the development: nature of BGE, mixing of
32 reactants, incubation, separation of reaction products, analysis time, reproducibility, and
33 applicability. Particular attention has been paid to the study of non-covalent interactions between
34 mAb subunits and their dependence on electrophoretic conditions (denaturing and non-denaturing
35 conditions). Eventually, these developed methodologies were applied to analyze different mAbs.

36

1 2. Materials and methods

2 2.1. Chemicals

3 Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, ≥98%), sodium hydroxide (NaOH, ≥98%),
4 hydrochloric acid (HCl, ≥37%), polyethylene oxide (PEO, MW = 1000000), bovine serum albumin
5 (BSA), and sinapic acid (≥99%) were purchased from Sigma-Aldrich (Germany). Ammonium
6 bicarbonate (NH₄HCO₃, ≥99.5%) and trifluoroacetic acid (TFA) were purchased from Fluka Analytical
7 (USA). Ammonium acetate (≥98%), acetonitrile (ACN, ≥99%) and water for mass spectrometry
8 analysis were purchased from Carlo Erba (France). Glacial acetic acid was purchased from Prolabo
9 (France). All reagents were of analytical grade.

10 Pharmaceutical formulations of Adalimumab (Humira®), Belimumab (Benlysta®), Tocilizumab
11 (RoActemra®), Infliximab (Remicade®), Rituximab (MabThera®) and Trastuzumab-Emtansine
12 (Kadcyla®) were provided by the Pharmacy of the University Hospital of Montpellier (CHRU,
13 Montpellier, France). IdeS enzyme was purchased from Genovis (Sweden).

14 2.2 Capillary coating, BGE, and reactant solutions

15 All aqueous solutions were prepared with double-distilled water. PEO stock solution was prepared by
16 dissolving 100 mg of PEO in 45 mL of water by heating at 95°C and then stored at 4°C. PEO coating
17 solutions were prepared by adding 0.5 mL of 1 M HCl to 4.5 mL of PEO stock solution.

18 Solutions of (i) acetic acid-ammonium acetate at different pH (3, 4, 4.5 and 5 +/- 0.1 pH units) and
19 various ionic strengths (IS) (25, 50, 75, 100 and 150 mM) and (ii) acetic acid at 20% (v/v) at pH 2.1 and
20 IS 8.2 mM were used as BGEs. Exact pH was measured by potentiometry (Mettler Toledo, France).

21 100 mM TCEP solutions were prepared in water or 200 mM ammonium bicarbonate.

22 2.3. Instrumentation and operating conditions

23 CE-UV analyses were performed on a Beckman-Coulter PA800 capillary electrophoresis system
24 (Beckman, USA). Polymicro (Molex, USA) fused silica capillaries (id = 30 μm, L_{eff} = 50 cm, L = 60 cm)
25 were used for enzymatic reactions and electrokinetic separations. Temperatures of the autosampler
26 and capillary cartridge were set to 4 and 37 °C, respectively. All rinsing operations were performed
27 under 75 psi. The capillary pre-conditioning was performed by successive rinses with 1 M NaOH and
28 water (15 min each). Before every run, capillaries were rinsed successively with 1 M NaOH, water,
29 and 0.1 M HCl (3 min each). The coating of capillaries was then performed by rinsing with PEO
30 coating solution for 6 min, followed by 5 min water rinsing. Eventually, the capillary was rinsed with
31 appropriate BGE for 5 min. Reactants and samples were injected hydrodynamically from the anodic
32 side. Separations were achieved under +15 kV and the positively charged mAb or subunits migrated
33 toward the cathode. UV detection was performed at 214 nm.

34 CE expert lite software (Sciex, USA) was used for injection volumes calculation. 32 Karat™ 8.0
35 software (Sciex, USA) for direct control of capillary electrophoresis instrument as well as data
36 acquisition and analysis.

37 A MALDI-MS rapiflex instrument with a time of flight analyzer (TOF) (Bruker, Germany) was used to
38 perform off-line mass spectrometry analyses. FlexControl 4.0 software was used for data acquisition
39 and FlexAnalysis 4.0 was used for data processing.

40 A Synapt G2-Si instrument equipped with a nano-ESI source (Waters Corporation, UK) was used to
41 perform native MS analysis. MassLynx software was used for data acquisition and processing.

1 2.4. Off-line reduction

2 The reduction of adalimumab was performed using TCEP. Mixtures of mAb (final concentration (C_f),
3 1 g.L^{-1}) and TCEP ($C_f = 10 \text{ mM}$) were prepared in water. The samples were then incubated at 37°C for
4 60 min. A sample volume of 10.3 nL was injected and analyzed by CZE-UV.

5 2.5. MALDI-MS and nano-ESI-MS analyses

6 For MALDI-MS analyses of off-line samples assays, the matrix was prepared by dissolving sinapic acid
7 in water/ACN-0.1% TFA (50/50) (v/v) to a final concentration of 10 g.L^{-1} . Samples were diluted by a
8 factor of $\frac{1}{2}$ in the matrix before spotting. Calibration for high m/z ratios was performed before each
9 analysis with BSA at 1 mg.L^{-1} in water, based on the multiply charged species and non-specific dimer.

10 Prior to nano-electrospray analyses, samples were buffer exchanged in 200 mM ammonium acetate
11 buffer (Sigma, Germany) using Bio-Spin microcentrifuge columns (Bio-Rad Laboratories, USA). Nano
12 ESI-MS spectra were recorded on a Synapt G2-Si instrument modified for high mass analysis and
13 operated in ToF mode. Samples were introduced into the ion source using borosilicate emitters
14 (Thermo Scientific, USA). Optimized instrument parameters were as follows: capillary voltage 1.4 kV ,
15 sampling cone 80 V , source offset 50 V , transfer collision energy 5 V and argon flow rate 3 mL/min .

16 2.6. In-line and off-line assays

17 In-line reduction or in-line simultaneous digestion and reduction were carried out using reducing
18 agent 100 mM TCEP in water or 200 mM ammonium bicarbonate, IdeS enzyme (0.2 g.L^{-1}) in water,
19 and mAb in water (1 g.L^{-1}) as substrate. Analyses were performed as follows (capillary is initially filled
20 with BGE):

21 1/ Injection of the different reactants (for optimal volume and injection conditions, see table 1):

22 - In-line reduction: reducing agent (TCEP in water) and mAb were hydrodynamically injected
23 following the injection scheme TCEP/mAb/TCEP;

24 - In-line digestion: IdeS and mAb were hydrodynamically injected following the injection scheme
25 IdeS/mAb/IdeS;

26 - In-line simultaneous digestion and reduction: reducing agent (TCEP in 200 mM ammonium
27 bicarbonate), IdeS and mAb were hydrodynamically injected following the injection scheme
28 TCEP/IdeS/mAb/IdeS/TCEP;

29 2/ Injected plugs were pushed to the thermostated zone of the capillary (6 cm from the capillary
30 inlet) with BGE at 0.5 psi for 5.5 min to establish a temperature control (i.e. 37°C) of the enzymatic
31 reaction;

32 3/ The plugs were incubated for different times (0 to 60 min) without application of pressure or
33 voltage;

34 4/ A 15 kV voltage was applied to separate the reaction products.

35 Different BGE were used depending on the implemented reaction:

36 - In-line reduction: separation with acetic acid-ammonium acetate $\text{pH } 4.0$ IS 25 mM BGE.

37 - In-line digestion: separation with acetic acid-ammonium acetate $\text{pH } 5.0$ IS 75 mM (non-denaturing
38 BGE) or acetic acid 20% (v/v) (denaturing BGE). For analyses with denaturing BGE, plugs of 200 mM
39 ammonium bicarbonate were injected at both sides of the in-line digestion or simultaneous
40 digestion-reduction injection scheme. The volume of each NH_4HCO_3 plug was 5.1 nL (0.5 psi , 40 s).

1 In-line assays were compared with off-line assays. Off-line sample preparation conditions were
2 adjusted to in-line assays conditions, i.e., reactants concentration, dilution factor, temperature and
3 incubation time.

4 Off-line reduction was realized by mixing mAb (1 g.L⁻¹) and 100 mM TCEP solutions prepared in water
5 to achieve final concentrations of 0.33 g L⁻¹ and 66.7 mM respectively. Sample was then incubated at
6 37°C for 25 min. A volume of 7.7 nL corresponding to the sum of the injected reactants in in-line
7 assay was injected and analyzed by CZE-UV.

8 For off-line simultaneous digestion and reduction, a mixture of adalimumab (1 g.L⁻¹), IdeS (0.2 g.L⁻¹),
9 and 100 mM TCEP solutions prepared in 200 mM ammonium bicarbonate was prepared to achieve
10 final concentrations of 0.22 g.L⁻¹, 0.07 g.L⁻¹, and 43.5 mM, respectively. The sample was then
11 incubated at 37°C for 25 min. A volume of 11.8 nL or 23.6 nL corresponding to the sum of the
12 injected reactants in in-line assay (under non-denaturing or denaturing conditions) was injected and
13 analyzed by CZE-UV.

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

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
36
37
38
39
40
41
42
43
44
45

3. Results and discussion

We have previously developed an in-line methodology for IdeS digestion. The present study aimed to develop novel in-line methodologies for other middle-up approaches, i.e., the reduction and simultaneous IdeS digestion-reduction of therapeutic mAbs with high separation resolution. A neutral coating based on polyethylene oxide (PEO) was used to avoid adsorption. Analyses were performed with acetic acid/ammonium acetate BGE, suitable for mAb analyses under both denaturing and non-denaturing conditions [34]. Adalimumab was chosen as model mAb for the methodological developments.

3.1. In-line mAb reduction

3.1.1. Off-line experiments

As mentioned earlier, TCEP was used as a reducing agent for mAb analysis at the middle-up level due to its large pH range activity and compatibility with mass spectrometry analysis. Adalimumab was reduced with TCEP in water ($C_{\text{TCEP,final}} = 10 \text{ mM}$) using off-line protocols based on literature [18,28]. Entire reduction of adalimumab was confirmed by MALDI-MS analyses (Fig. 1A). Only masses of the light chain ($MW_{\text{LC}} \approx 23 \text{ kDa}$) and heavy chain ($MW_{\text{HC}} \approx 50 \text{ kDa}$) were observed.

The off-line reduced adalimumab sample was then analyzed by CZE-UV. Based on previously developed conditions [27], acetic acid/ammonium acetate pH 5.0 was used as BGE. The ionic strength of BGE was optimized to allow the separation of HC and LC subunits of reduced mAb. IS 25 mM provided the best resolution of separation. Using these electrophoretic conditions, different electrophoretic profiles were obtained for TCEP reduced and intact adalimumab, suggesting that the reduction can be monitored with CZE-UV (Fig. 1B). Analysis of TCEP 10 mM showed no peak with normal polarity (Fig. S1), suggesting that the TCEP does not interfere with reduced mAb peaks. This reduced mAb electropherogram was considered as a reference electrophoretic profile for implementing in-line reduction inside the capillary. The profile of intact mAb showed three partially separated peaks that correspond to the charge variants due to differential clipping of C-terminal lysines [35,36]. This variation induces a mass shift of 128 Da that is not detectable by MALDI-MS due to its limited resolution, accuracy, and low charge state (+1 or +2) of ionization. Also, spotting the sample without prior separation leads to ionization competition between sample compounds. Consequently, the MS spectrum peaks correspond to the most abundant variant with two lysines clipping (OK).

3.1.2. Implementation in-line reduction and optimization

In order to achieve an efficient mixing by TDLFP, the plugs (sample and reactant) were injected with a high pressure (i.e. 2 psi) and short injection time (i.e. 5 s), so that they have a parabolic profile necessary for optimal transverse diffusion [16]. Next, a BGE plug was added to push all reactants in the thermostated part of the capillary and incubation was performed in 10 min [37]. The effect of injection sequences with 100 mM TCEP in water were studied to optimize plug mixing: (i) plug/plug mode (TCEP/mAb or mAb/TCEP), (ii) sandwich mode (TCEP/mAb/TCEP or mAb/TCEP/mAb).

Whatever the injection scheme, in-line electrophoretic profiles (Fig. 2) were clearly different from the expected profile (Fig. 1B). Intact mAb traces were observed, suggesting an incomplete reduction of adalimumab. This can be explained by insufficient mixing efficiencies and/or incubation time. Indeed, the local concentration of each reactant inside the microreactor depends on (i) injection conditions (reactants concentrations, injection scheme, pressure, and time) affecting the shape and the volumes of the plugs and (ii) reactant diffusion (diffusion coefficient) inside the capillary by TDLFP [16,17,27]. So, the injected TCEP concentration and the injection conditions have to be optimized for

1 in-line reaction. The sandwich mode TCEP/mAb/TCEP was selected as it provided more advanced
2 reduction.

3 In order to enhance the reduction yield, different parameters were optimized such as the TCEP
4 concentration in water (from 10 to 150 mM) and the injection conditions (pressure and time).
5 Optimal conditions are displayed in table 1. Different incubation times (from 0 to 60 min) were then
6 tested (Fig. S2). For 5 and 15 min incubation time, peaks of intact mAb or intermediate compounds of
7 reduction were observed, suggesting incomplete reaction. Identical profiles to the reference profile
8 of reduced adalimumab were obtained for 25 and 60 min. However, the broadening of reduced mAb
9 peaks may hide traces of unreduced mAb. Thus, improvement of peak shapes and resolution was
10 necessary to evaluate the progress of the reduction reaction. Eventually, the pH of the BGE was
11 optimized for off-line sample. Improved peak efficiencies and resolution were achieved with acetic
12 acid/ammonium acetate pH 4.0 IS 25 mM (Fig. S3). In-line analysis at pH 4.0 confirmed the complete
13 reduction from 25 min of incubation time. The TCEP was analyzed under the same conditions as in-
14 line reduction. No peak was observed, suggesting the absence of TCEP interference with the
15 electrophoretic profile of reduced mAb (data not shown).

16 Optimized reduction conditions and BGE pH of in-line assay were implemented for off-line assay (see
17 section 2.6) for direct comparison. Similar electrophoretic profiles were obtained (Fig. 3A),
18 confirming the feasibility of in-line reduction assay. Longer migration times for off-line assay were
19 observed as the injected plug was not pushed to the thermostated zone (longer effective separation
20 length). MALDI-MS analysis (Fig. 3B) of off-line assay sample indicated only masses of the light chain
21 ($MW_{LC} \approx 23$ kDa) and heavy chain ($MW_{HC} \approx 50$ kDa), confirming that adalimumab was well reduced. A
22 third peak appeared in the electropherogram and its height increased by increasing the incubation
23 time (data not shown). Additional MS experiments showed that the second and the third peaks
24 correspond to the LC (Fig. S4). Increased intensity and charge state of MS spectra was observed in
25 the case of the third peak. Besides, the mass difference between the two peaks was about 2 Da. This
26 confirms the conformational change due to the additional reduction of intra-chain disulfide bonds
27 and increases the hydrodynamic size [28,38,39]. RSD values (n=5) were less than 0.28% for migration
28 time and less than 5.48% for corrected peak areas showing good repeatability of the in-line
29 methodology.

30 3.1.3. Application to other mAb

31 Several mAbs (infliximab, rituximab, tocilizumab, belimumab) were reduced under previously
32 optimized conditions and compared with their intact profiles in order to demonstrate the
33 applicability of in-line reduction (Fig. S5). Reduced mAbs presented different profiles compared to
34 their intact profiles. HC and LC were not separated in the case of infliximab, partially separated in the
35 case of tocilizumab and belimumab, baseline separated in the case of rituximab. These differences
36 could be interpreted by structural (amino-acid sequence) and formulation composition differences of
37 the mAbs [40]. Off-line samples prepared in consistent conditions with in-line experiments were also
38 analyzed and produced similar electrophoretic profiles. MALDI-MS experiments of off-line samples
39 confirmed the complete reduction of the mAbs (data not shown).

40 3.2. In-line simultaneous digestion and reduction

41 As the IdeS enzyme does not contain disulfide bonds [30], digestion and reduction steps can be
42 combined to decrease F(ab)₂ subunit structural complexity. This approach yields Fc/2, Fd and LC
43 subunits in the 23 to 26 kDa mass range, allowing subunit-specific characterization [36].

44 First, previously optimized reduction conditions were used for in-line simultaneous digestion-
45 reduction. However, to preserve the IdeS activity in the in-line methodology, the pH of adjacent IdeS

1 plugs has to be in the pH range 5.1-7.6 [41]. Therefore, 100 mM TCEP solution was prepared in
2 200 mM ammonium bicarbonate (pH 8.0). Since TCEP is efficient under a wide pH range [18], its
3 activity should be preserved.

4 3.2.1. Separation under non-denaturing conditions

5 IdeS, TCEP, and mAb plugs were injected according to the sandwich mode:
6 TCEP/IdeS/mAb/IdeS/TCEP. The injection of two IdeS plugs next to the mAb plug guarantees
7 successful mixing and fast enzymatic reaction. The previous study [27] showed that 1 min of
8 incubation with IdeS in the thermostated part of the capillary is sufficient to achieve complete
9 enzymatic reaction, while the reduction kinetic is slow, i.e. 25 min of incubation in the thermostated
10 part (see section 3.1.2). This could be advantageous as the IdeS digestion may render the disulfide
11 bonds more accessible for reduction and accelerate reaction reduction. Acetic acid-ammonium
12 acetate pH 5.0 was used for separation due to its compatibility with IdeS activity for in-line reaction,
13 as shown in our previous study [27]. Indeed, during the TDLFP process in the thermostated part of
14 the capillary, the plugs of TCEP/IdeS/mAb/IdeS/TCEP penetrates in the BGE filling the capillary. So, in
15 this case, the BGE maintains an adequate pH for the enzyme. The BGE ionic strength at pH 5.0 was
16 optimized to improve separation efficiency. 75 mM was selected as it provided higher peak
17 efficiencies (Fig. S6).

18 Off-line and in-line simultaneous digestion-reduction assays were compared (Fig. 4A). Different
19 electrophoretic profiles were observed: higher peak efficiencies and short migration time were
20 obtained for in-line assay, demonstrating high in-line assay performance. This can be explained by
21 the successive injection of plugs with different compositions that allows in-line stacking of
22 adalimumab subunits. In order to show the contribution of TCEP to the enzymatic cleaving process,
23 in-line digestion and simultaneous digestion-reduction experiments were carried out. Reactant
24 concentrations and injection conditions are given in table 1. For digestion experiments, TCEP plugs
25 were removed from the injection sequence (IdeS/mAb/IdeS). The comparison of in-line digested and
26 simultaneously digested-reduced mAb showed different profiles (Fig. 4B), confirming the successful
27 occurrence of digestion and reduction. The RSD values (n=5) were less than 2.4 % for migration time
28 and less than 2.9% for the corrected peak areas, confirming good repeatability of the in-line
29 methodology. These results demonstrate the successful implementation of the microreactor under
30 non-denaturing conditions.

31 To investigate the extent of off-line simultaneous digestion-reduction and identify the products of
32 reaction, MS experiments were performed by nano-ESI-MS as it allows analysis under non-
33 denaturing conditions. Analysis with low activation energy (Fig. 5) showed 48.9 kDa and 50.4 kDa
34 masses resulting from the non-covalent association of LC and Fd into F(ab) and two Fc/2 into Fc,
35 respectively. This was confirmed by applying high collision energy in the mass spectrometer to
36 dissociate non-covalent interactions. Results showed the decrease of MS spectra intensity of F(ab)
37 and Fc subunits decreased and the appearance of MS spectra with lower m/z. The deconvolution of
38 the appeared MS spectra showed masses corresponding to the dissociated subunits 23 kDa (LC) and
39 25 kDa (Fd and Fc/2). The observed mass 99.3 kDa may correspond to a non-specific complex of F(ab)
40 and Fc. Intact mAb mass was not detected, supporting the evidence of complete digestion/reduction.

41 As a consequence, these results suggest that the two main peaks observed in CZE profile of digested-
42 reduced adalimumab emanated from the association of the subunits (LC, Fd and Fc/2) by non-
43 covalent interactions to form F(ab) and Fc subunits during the migration process under non-
44 denaturing separation conditions (i.e. pH 5.0). In addition, the last peak would correspond to Fc
45 subunit as it results also from digestion. So, digestion-reduction of adalimumab was achieved in the

1 in-line assay. These results are in agreement with the results described in the literature. Indeed, LC
2 and Fd association to F(ab) has been previously reported under native conditions in cIEF-MS [12,42],
3 and Fc/2 association to form Fc has been reported in native CZE-ESI-MS analyses [8,27].

4 MALDI-MS analysis of off-line assay sample was also conducted to confirm the complete digestion-
5 reduction of mAb. The MS spectrum showed masses corresponding to the expected subunits (LC, Fd
6 and Fc/2) (data not shown). F(ab) and Fc subunits were not detected by MALDI-MS because MALDI
7 analysis is based on mixing the sample with a denaturing matrix (sinapic acid, acetonitrile and
8 trifluoroacetic acid). Therefore, the MALDI process will disrupt potential non-covalent interactions.

9 3.2.2. Application of non-denaturing in-line analyses to other mAbs

10 The methodologies were applied to other mAbs. Different electrophoretic profiles were obtained for
11 digested and simultaneously digested-reduced mAb (Fig. 6). Peaks with similar electrophoretic
12 mobilities and shapes are observed (*) in the electropherograms of digested mAbs and
13 simultaneously digested-reduced mAbs that could be attributed to the Fc subunit. In the case of
14 infliximab, the first three peaks in the electropherograms of digested infliximab and simultaneously
15 digested-reduced infliximab were observed with the same migration times. The three peaks would
16 correspond respectively to the C-terminal lysine variants 2K, 1K, and 0K of Fc subunit resulting from
17 differential truncation of lysine residue carrying one positive charge [28]. The separation of C-
18 terminal lysine variants has been reported at the middle-up level for cetuximab [8] and infliximab
19 [27] under non-denaturing conditions.

20 3.2.3. Separation under denaturing conditions

21 In order to dissociate the non-covalent interactions between LC, Fd and Fc/2 subunits forming F(ab)
22 and Fc subunits as observed in part 3.2.1, the use of an acidic BGE was evaluated after simultaneous
23 digestion-reduction of adalimumab. Decreasing the pH should cause protein unfolding by increasing
24 protonation and charge repulsion [43–45]. Therefore, non-covalent interactions such as electrostatic
25 bonds should be disrupted. Haselberg *et al.* reported that non-covalent interactions between Fc/2
26 subunits were fully dissociated using acetic acid at concentrations higher than 10% (v/v) [26]. Also,
27 acetic acid has low conductivity [46]. For these reasons, acetic acid 20% (v/v) (pH ~ 2.1) was chosen
28 as denaturing BGE.

29 However, the pH of this BGE is not compatible anymore with the enzymatic reaction. It may affect
30 IdeS activity by mixing with reactants plugs. For this reason, plugs of 200 mM ammonium
31 bicarbonate were injected on both sides of the reactant injection sequence
32 (TCEP/IdeS/mAb/IdeS/TCEP) to prevent interferences of BGE with the enzymatic reaction [47,48].
33 Thus, the resulting injection scheme was the following:
34 $\text{NH}_4\text{HCO}_3/\text{TCEP/IdeS/mAb/IdeS/TCEP}/\text{NH}_4\text{HCO}_3$. These extra ammonium bicarbonate plugs were
35 injected by low pressure (i.e. 0.5 psi) to achieve flat profiles minimizing transverse diffusion with
36 BGE. The influence of ammonium bicarbonate concentration and plugs volume on the digestion-
37 reduction reaction and subunits separation was carefully studied.

38 The injected volume of each ammonium bicarbonate plug was increased from 0 to 10.3 nL and
39 resulted in various electrophoretic profiles. Figure 7 shows the dependence of digestion efficiency on
40 ammonium bicarbonate plugs volume. Similar electrophoretic profiles were observed at a volume
41 equal or higher than 5.1 nL, indicating an equilibrium state. Similar profiles for off-line (also analyzed
42 by MALDI-MS (data not shown)) and in-line assays under optimal conditions were obtained (Fig. 8A),
43 showing that adalimumab was well digested-reduced with the in-line methodology even in the
44 presence of denaturing BGE. Three major peaks were obtained that correspond likely to Fc/2, Fd and
45 LC subunits as acidic BGE disrupts non-covalent interactions. The analysis of TCEP following the

1 scheme ($\text{NH}_4\text{HCO}_3/\text{TCEP}/\text{NH}_4\text{HCO}_3$) did not show any peak confirming the absence of TCEP
2 interference with the electrophoretic profile of digested-reduced mAb (data not shown).

3 The in-line assay provided good repeatability (RSD less than 0.8% for migration time and less than
4 3.84% for corrected peak areas). The in-line electrophoretic profiles of intact, digested, and
5 simultaneously digested-reduced adalimumab were compared. Reactant concentrations and
6 injection conditions are given in table 1. The differences observed in electrophoretic profiles (Fig. 8B)
7 demonstrate that in-line enzymatic reactions were well carried out. The contribution of TCEP to the
8 enzymatic cleaving process was shown in the differences between digested and digested-reduced
9 electrophoretic profiles. Electrophoretic mobility of first migrating peak (*) appears to be similar for
10 both digested and digested-reduced mAb. This peak could be attributed to Fc/2 subunit, which is a
11 shared subunit of both reactions.

12 3.2.4. Application of denaturing in-line analysis to others mAbs

13 In order to evaluate the versatility and applicability of the in-line methodology, different mAbs
14 (infliximab, rituximab, tocilizumab and belimumab) were analyzed using the in-line methodology
15 under the optimal conditions defined previously (Fig. 9). Similarly to adalimumab, the electrophoretic
16 profiles of digested-reduced mAbs were different from their digested electrophoretic profiles. Each
17 mAb presented a specific profile at the middle-up level. The differences in the electrophoretic
18 profiles between mAbs can be explained by their physicochemical properties: amino-acids sequence,
19 microvariations and isoelectric point. Similarly to adalimumab, the first peak (*) of digested and
20 simultaneously digested-reduced infliximab, rituximab, tocilizumab and belimumab exhibited the
21 same migration time and could be attributed to Fc/2 subunits.

22 4. Conclusion

23 In this work, the development of novel automated methodologies for monoclonal antibodies quality
24 control at the middle-up level is described. In-line TCEP reduction of disulfide bonds and
25 simultaneous IdeS digestion-TCEP reduction were successfully integrated inside the capillary. The
26 subunits obtained were then separated with a high resolution of separation and short analysis time.
27 The in-line methodologies yielded good repeatability, higher performance than off-line assays in
28 terms of reactants consumption, analysis time, and versatility through a successful application to
29 several mAbs. Therefore, this study confirmed the utility of CZE to integrate multiple reactions of
30 sample preparation and separation steps as well as its high flexibility that allows analyses under
31 diverse conditions. It is worth noting that the developed conditions are compatible with mass
32 spectrometry analysis requirements. Therefore, the next step will rely on the transposition of the
33 methodologies to sheathless CZE-MS for full mAb characterization.

34 Conflicts of interest

35 There are no conflicts to declare.

36 Acknowledgements

37 The authors gratefully thank the Algerian Ministry of Higher Education and Scientific Research
38 (MESRS) for the scholarship of excellence to Meriem Dadouch and would like to thank the regional
39 funds (FEDER, Région Occitanie) for the Synapt instrument used for intact mass spectrometry
40 analyses performed at the Functional Proteomics Platform of Montpellier, Languedoc-Roussillon.

41

1 Bibliography

- 2
- 3 [1] G. Jagschies, Selected Biotherapeutics Overview, in: *Biopharm. Process. Dev. Des. Implement.*
4 *Manuf. Process.*, Elsevier, 2018: pp. 59–72. doi:10.1016/B978-0-08-100623-8.00003-7.
- 5 [2] Y. Xu, D. Wang, B. Mason, T. Rossomando, N. Li, D. Liu, J.K. Cheung, W. Xu, S. Raghava, A.
6 Katiyar, C. Nowak, T. Xiang, D.D. Dong, J. Sun, A. Beck, H. Liu, Structure, heterogeneity and
7 developability assessment of therapeutic antibodies, *MAbs.* 11 (2019) 239–264.
8 doi:10.1080/19420862.2018.1553476.
- 9 [3] S. Fekete, D. Guillarme, P. Sandra, K. Sandra, Chromatographic, Electrophoretic, and Mass
10 Spectrometric Methods for the Analytical Characterization of Protein Biopharmaceuticals,
11 *Anal. Chem.* 88 (2016) 480–507. doi:10.1021/acs.analchem.5b04561.
- 12 [4] A. Beck, F. Debaene, H. Diemer, E. Wagner-Rousset, O. Colas, A. Van Dorsselaer, S. Cianfèrani,
13 Cutting-edge mass spectrometry characterization of originator, biosimilar and biobetter
14 antibodies, *J. Mass Spectrom.* 50 (2015) 285–297. doi:10.1002/jms.3554.
- 15 [5] R. Gahoual, A. Beck, Y.-N. François, E. Leize-Wagner, Independent highly sensitive
16 characterization of asparagine deamidation and aspartic acid isomerization by sheathless CZE-
17 ESI-MS/MS., *J. Mass Spectrom.* 51 (2016) 150–158. doi:10.1002/jms.3735.
- 18 [6] Y. He, C. Isele, W. Hou, M. Ruesch, Rapid analysis of charge variants of monoclonal antibodies
19 with capillary zone electrophoresis in dynamically coated fused-silica capillary, *J. Sep. Sci.* 34
20 (2011) 548–555. doi:10.1002/jssc.201000719.
- 21 [7] S. Carillo, C. Jakes, J. Bones, In-depth analysis of monoclonal antibodies using microfluidic
22 capillary electrophoresis and native mass spectrometry, *J. Pharm. Biomed. Anal.* (2020)
23 113218. doi:10.1016/j.jpba.2020.113218.
- 24 [8] Y.-N. François, M. Biacchi, N. Said, C. Renard, A. Beck, R. Gahoual, E. Leize-Wagner,
25 Characterization of cetuximab Fc/2 dimers by off-line CZE-MS., *Anal. Chim. Acta.* 908 (2016)
26 168–176. doi:10.1016/j.aca.2015.12.033.
- 27 [9] A.M. Belov, R. Viner, M.R. Santos, D.M. Horn, M. Bern, B.L. Karger, A.R. Ivanov, Analysis of
28 Proteins, Protein Complexes, and Organellar Proteomes Using Sheathless Capillary Zone
29 Electrophoresis - Native Mass Spectrometry, *J. Am. Soc. Mass Spectrom.* 28 (2017) 2614–
30 2634. doi:10.1007/s13361-017-1781-1.
- 31 [10] V. Le-Minh, N.T. Tran, A. Makky, V. Rosilio, M. Taverna, C. Smadja, Capillary zone
32 electrophoresis-native mass spectrometry for the quality control of intact therapeutic
33 monoclonal antibodies, *J. Chromatogr. A.* 1601 (2019) 375–384.
34 doi:10.1016/j.chroma.2019.05.050.
- 35 [11] K.M.E.D.-V.G.W.S.R. Haselberg, Middle-Up Characterization of the Monoclonal Antibody
36 Infiximab by Capillary Zone Electrophoresis–Mass Spectrometry, *LCGC Eur.* 32 (2019) 130–
37 137. [http://www.chromatographyonline.com/middle-characterization-monoclonal-antibody-](http://www.chromatographyonline.com/middle-characterization-monoclonal-antibody-infiximab-capillary-zone-electrophoresis-mass-spectrometry)
38 [infiximab-capillary-zone-electrophoresis-mass-spectrometry](http://www.chromatographyonline.com/middle-characterization-monoclonal-antibody-infiximab-capillary-zone-electrophoresis-mass-spectrometry) (accessed April 25, 2019).
- 39 [12] J. Dai, Y. Zhang, A Middle-Up Approach with Online Capillary Isoelectric Focusing/Mass
40 Spectrometry for In-Depth Characterization of Cetuximab Charge Heterogeneity, *Anal. Chem.*
41 90 (2018) 14527–14534. doi:10.1021/acs.analchem.8b04396.
- 42 [13] D. Firth, L. Bell, M. Squires, S. Estdale, C. McKee, A rapid approach for characterization of
43 thiol-conjugated antibody–drug conjugates and calculation of drug–antibody ratio by liquid

- 1 chromatography mass spectrometry, *Anal. Biochem.* 485 (2015) 34–42.
2 doi:10.1016/J.AB.2015.06.001.
- 3 [14] M. Dadouch, Y. Ladner, C. Perrin, Analysis of Monoclonal Antibodies by Capillary
4 Electrophoresis: Sample Preparation, Separation, and Detection, *Separations*. 8 (2021) 4.
5 doi:10.3390/separations8010004.
- 6 [15] Y. Ladner, S. Mas, G. Coussot, J. Montels, C. Perrin, In-line tryptic digestion of therapeutic
7 molecules by capillary electrophoresis with temperature control, *Talanta*. 193 (2019) 146–
8 151. doi:10.1016/J.TALANTA.2018.09.090.
- 9 [16] S.M. Krylova, V. Okhonin, S.N. Krylov, Transverse diffusion of laminar flow profiles - a generic
10 method for mixing reactants in capillary microreactor, *J. Sep. Sci.* 32 (2009) 742–756.
11 doi:10.1002/jssc.200800671.
- 12 [17] Y. Ladner, S. Mas, G. Coussot, K. Bartley, J. Montels, J. Morel, C. Perrin, Integrated
13 microreactor for enzymatic reaction automation: An easy step toward the quality control of
14 monoclonal antibodies, *J. Chromatogr. A.* 1528 (2017) 83–90.
15 doi:10.1016/J.CHROMA.2017.10.066.
- 16 [18] B. Bobaly, V. D’Atri, A. Goyon, O. Colas, A. Beck, S. Fekete, D. Guillarme, Protocols for the
17 analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical
18 sample preparation, *J. Chromatogr. B.* 1060 (2017) 325–335.
19 doi:10.1016/j.jchromb.2017.06.036.
- 20 [19] V. Domkin, A. Chabes, Phosphines are ribonucleotide reductase reductants that act via C-
21 terminal cysteines similar to thioredoxins and glutaredoxins, *Sci. Rep.* 4 (2015) 5539.
22 doi:10.1038/srep05539.
- 23 [20] G. Wang, I.A. Kaltashov, Approach to characterization of the higher order structure of
24 disulfide-containing proteins using hydrogen/deuterium exchange and top-down mass
25 spectrometry, *Anal. Chem.* 86 (2014) 7293–7298. doi:10.1021/ac501789e.
- 26 [21] Y. Zhao, L. Sun, M.D. Knierman, N.J. Dovichi, Fast separation and analysis of reduced
27 monoclonal antibodies with capillary zone electrophoresis coupled to mass spectrometry,
28 *Talanta*. 148 (2016) 529–533. doi:10.1016/J.TALANTA.2015.11.020.
- 29 [22] M. Han, B.M. Rock, J.T. Pearson, D.A. Rock, Intact mass analysis of monoclonal antibodies by
30 capillary electrophoresis-Mass spectrometry., *J. Chromatogr. B. Analyt. Technol. Biomed. Life*
31 *Sci.* 1011 (2016) 24–32. doi:10.1016/j.jchromb.2015.12.045.
- 32 [23] Y. Shi, Z. Li, Y. Qiao, J. Lin, Development and validation of a rapid capillary zone
33 electrophoresis method for determining charge variants of mAb, *J. Chromatogr. B Anal.*
34 *Technol. Biomed. Life Sci.* 906 (2012) 63–68. doi:10.1016/j.jchromb.2012.08.022.
- 35 [24] J. Sjögren, F. Olsson, A. Beck, Rapid and improved characterization of therapeutic antibodies
36 and antibody related products using IdeS digestion and subunit analysis, *Analyst*. 141 (2016)
37 3114–3125. doi:10.1039/C6AN00071A.
- 38 [25] M. Biacchi, R. Gahoual, N. Said, A. Beck, E. Leize-Wagner, Y.-N. François, Glycoform Separation
39 and Characterization of Cetuximab Variants by Middle-up Off-Line Capillary Zone
40 Electrophoresis-UV/Electrospray Ionization-MS, *Anal. Chem.* 87 (2015) 6240–6250.
41 doi:10.1021/acs.analchem.5b00928.
- 42 [26] R. Haselberg, T. De Vijlder, R. Heukers, M.J. Smit, E.P. Romijn, G.W. Somsen, E. Domínguez-
43 Vega, Heterogeneity assessment of antibody-derived therapeutics at the intact and middle-up
44 level by low-flow sheathless capillary electrophoresis-mass spectrometry, *Anal. Chim. Acta*.

- 1 (2018). doi:10.1016/J.ACA.2018.08.024.
- 2 [27] M. Dadouch, Y. Ladner, C. Bich, M. Larroque, C. Larroque, J. Morel, P.A. Bonnet, C. Perrin, An
3 in-line enzymatic microreactor for the middle-up analysis of monoclonal antibodies by
4 capillary electrophoresis, *Analyst*. 145 (2020) 1759–1767. doi:10.1039/c9an01906e.
- 5 [28] A.M. Belov, L. Zang, R. Sebastiano, M.R. Santos, D.R. Bush, B.L. Karger, A.R. Ivanov,
6 Complementary middle-down and intact monoclonal antibody proteoform characterization by
7 capillary zone electrophoresis - mass spectrometry, *Electrophoresis*. 39 (2018) 2069–2082.
8 doi:10.1002/elps.201800067.
- 9 [29] M. Han, B.M. Rock, J.T. Pearson, D.A. Rock, Intact mass analysis of monoclonal antibodies by
10 capillary electrophoresis—Mass spectrometry, *J. Chromatogr. B*. 1011 (2016) 24–32.
11 doi:10.1016/j.jchromb.2015.12.045.
- 12 [30] K. Wenig, L. Chatwell, U. von Pawel-Rammingen, L. Björck, R. Huber, P. Sondermann,
13 Structure of the streptococcal endopeptidase IdeS, a cysteine proteinase with strict specificity
14 for IgG., *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 17371–6. doi:10.1073/pnas.0407965101.
- 15 [31] V. Azevedo, L. Dela Coletta Troiano Araujo, natalia Bassalobre Galli, alais kleinfelder, nathan
16 marostica Catolino, paulo cesar martins Urbano, Adalimumab: a review of the reference
17 product and biosimilars, *Biosimilars*. Volume 6 (2016) 29–44. doi:10.2147/BS.S98177.
- 18 [32] P. Norman, Humira: the impending patent battles over adalimumab biosimilars, *Pharm. Pat.*
19 *Anal.* 5 (2016) 141–145. doi:10.4155/ppa-2016-0002.
- 20 [33] M. Kesik-Brodacka, Progress in biopharmaceutical development, *Biotechnol. Appl. Biochem.*
21 65 (2018) 306–322. doi:10.1002/bab.1617.
- 22 [34] L. Konermann, Addressing a Common Misconception: Ammonium Acetate as Neutral pH
23 “Buffer” for Native Electrospray Mass Spectrometry, *J. Am. Soc. Mass Spectrom.* 28 (2017)
24 1827–1835. doi:10.1007/s13361-017-1739-3.
- 25 [35] F. Füssl, A. Trappe, K. Cook, K. Scheffler, O. Fitzgerald, J. Bones, Comprehensive
26 characterisation of the heterogeneity of adalimumab via charge variant analysis hyphenated
27 on-line to native high resolution Orbitrap mass spectrometry, *MAbs*. 11 (2019) 116–128.
28 doi:10.1080/19420862.2018.1531664.
- 29 [36] J. Giorgetti, A. Beck, E. Leize-Wagner, Y.N. François, Combination of intact, middle-up and
30 bottom-up levels to characterize 7 therapeutic monoclonal antibodies by capillary
31 electrophoresis – Mass spectrometry, *J. Pharm. Biomed. Anal.* 182 (2020).
32 doi:10.1016/j.jpba.2020.113107.
- 33 [37] S. Gattu, C.L. Crihfield, G. Lu, L. Bwanali, L.M. Veltri, L.A. Holland, Advances in enzyme
34 substrate analysis with capillary electrophoresis, *Methods*. 146 (2018) 93–106.
35 doi:10.1016/J.YMETH.2018.02.005.
- 36 [38] G. Rajpal, P. Arvan, Disulfide Bond Formation, in: *Handb. Biol. Act. Pept.*, Elsevier Inc., 2013:
37 pp. 1721–1729. doi:10.1016/B978-0-12-385095-9.00236-0.
- 38 [39] H. Liu, K. May, Disulfide bond structures of IgG molecules, *MAbs*. 4 (2012) 17–23.
39 doi:10.4161/mabs.4.1.18347.
- 40 [40] V. Hebber, S. Chattopadhyay, A.S. Rathore, High performance liquid chromatography (HPLC)
41 based direct and simultaneous estimation of excipients in biopharmaceutical products, *J.*
42 *Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1117 (2019) 118–126.
43 doi:10.1016/j.jchromb.2019.04.022.

- 1 [41] ‡ Bjarne Vincents, §,|| Ulrich von Pawel-Rammingen, § and Lars Björck, ‡ Magnus
2 Abrahamson*, Enzymatic Characterization of the Streptococcal Endopeptidase, IdeS, Reveals
3 That It Is a Cysteine Protease with Strict Specificity for IgG Cleavage Due to Exosite Binding†,
4 (2004). doi:10.1021/BI048284D.
- 5 [42] Z. Zhang, R. Perrault, Y. Zhao, J. Ding, SpeB proteolysis with imaged capillary isoelectric
6 focusing for the characterization of domain-specific charge heterogeneities of reference and
7 biosimilar Rituximab, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. (2016).
8 doi:10.1016/j.jchromb.2016.03.031.
- 9 [43] B. Zimmermann, H.M. Grey, Noncovalent Interactions between Immunoglobulin Polypeptide
10 Chains. Stability to Dissociation by Dnaturants, Biochemistry. 11 (1972) 78–84.
11 doi:10.1021/bi00751a014.
- 12 [44] K.A. Sharp, B. Honig, Electrostatic interactions in macromolecules: Theory and applications,
13 Annu. Rev. Biophys. Biophys. Chem. 19 (1990) 301–332.
14 doi:10.1146/annurev.bb.19.060190.001505.
- 15 [45] K.A. Dill, Dominant Forces in Protein Folding, Biochemistry. 29 (1990) 7133–7155.
16 doi:10.1021/bi00483a001.
- 17 [46] Y. Zhao, L. Sun, M.M. Champion, M.D. Knierman, N.J. Dovichi, Capillary Zone Electrophoresis–
18 Electro spray Ionization-Tandem Mass Spectrometry for Top-Down Characterization of the
19 *Mycobacterium marinum* Secretome, Anal. Chem. 86 (2014) 4873–4878.
20 doi:10.1021/ac500092q.
- 21 [47] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Michaelis-Menten analysis of bovine plasma
22 amine oxidase by capillary electrophoresis using electrophoretically mediated microanalysis in
23 a partially filled capillary, Electrophoresis. 22 (2001) 1436–1442. doi:10.1002/1522-
24 2683(200105)22:7<1436::AID-ELPS1436>3.0.CO;2-8.
- 25 [48] P. Nowak, M. Woźniakiewicz, P. Kościelniak, An overview of on-line systems using drug
26 metabolizing enzymes integrated into capillary electrophoresis, Electrophoresis. 34 (2013)
27 2604–2614. doi:10.1002/elps.201300108.
- 28
29
30
31
32
33
34
35
36
37
38
39

1 Table 1 : Optimal injection conditions of the different reactants for in-line analyses

Injection conditions	mAb (1 g.L ⁻¹)	TCEP (100 mM)	IdeS (0.2 g.L ⁻¹)
In-line reduction	2.6 nL (2psi, 5s)	2.6 nL (2psi, 5s)	0 nL
In-line digestion/ non-denaturing BGE	2.6 nL (2psi, 5s)	0 nL	2.0 nL (2psi, 4s)
In-line simultaneous digestion-reduction / non-denaturing BGE	2.6 nL (2psi, 5s)	2.6 nL (2psi, 5s)	2.0 nL (2psi, 4s)
In-line digestion / denaturing BGE	5.1 nL (4psi, 5s)	0 nL	4.1 nL (4psi, 4s)
In-line simultaneous digestion-reduction / denaturing BGE	5.1 nL (4psi, 5s)	5.1 nL (4psi, 5s)	4.1 nL (4psi, 4s)

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

1 Figures captions

2 **Figure 1:** Off-line TCEP reduction of adalimumab. (A) MALDI-MS spectra of intact and reduced
3 adalimumab, (B) Electrophoretic UV profiles of intact and TCEP reduced adalimumab.

4

5 Experimental conditions:

6 (A) Intact and off-line reduced adalimumab (1 g.L⁻¹) (see section 2.4). MALDI-MS analyses (see section
7 2.5). Matrix: sinapic acid 10 g.L⁻¹ in water/ACN-0.1 % TFA (50/50, v/v), m/z 10000-160000.

8 (B) Intact and off-line reduced adalimumab (1 g.L⁻¹) (see section 2.4). Injection volume = 10.3nL, BGE:
9 acetic acid–ammonium acetate pH 5.0 IS 25 mM, T = 37 °C, and voltage = 15 kV.

10

11 **Figure 2:** Effect of injection schemes on the yield of in-line TCEP reduction of adalimumab: Top:
12 plug/plug mode. Bottom: sandwich mode.

13

14 Experimental conditions:

15 Adalimumab (1 g.L⁻¹), TCEP 100 mM in water, injection plug volumes: 2.5 nL of mAb and 1.3 nL of
16 TCEP, BGE: acetic acid–ammonium acetate pH 5.0 IS 25 mM, incubation time: 10 min.

17

18 **Figure 3:** In-line and off-line reduction of adalimumab under optimized conditions: (A) CZE-UV
19 analyses (B) MALDI-MS analysis of off-line sample

20

21 Experimental conditions:

22 (A) CZE-UV analyses: Off-line reduced adalimumab (see section 2.6), In-line reduction: adalimumab
23 (1 g.L⁻¹), TCEP 100 mM in water, injection scheme: TCEP/mAb/TCEP, injection volumes (table 1), BGE:
24 acetic acid–ammonium acetate pH 4.0 IS 25 mM, incubation time: 25 min.

25 (B) Off-line reduced adalimumab MALDI-MS analysis (section 2.5 and 2.6): sinapic acid matrix in
26 water/ACN-0.1% TFA (50/50) (v/v) 10 g.L⁻¹, m/z 10000-160000.

27

28 **Figure 4:** Simultaneous digestion-reduction of adalimumab under non-denaturing conditions (A)
29 Comparison of off-line and in-line assays, (B) Electropherograms in-line digested and simultaneously
30 digested-reduced adalimumab.

31

32 Experimental conditions:

33 (A) Off-line simultaneously digested-reduced adalimumab (section 2.6), injection volume: 11.8 nL,
34 injection scheme: off-line sample

35 (B) Adalimumab (1 g.L⁻¹), TCEP 100 mM prepared in 200 mM NH₄HCO₃, IdeS (0.2 g L⁻¹), injection
36 schemes: IdeS/mAb/IdeS (digestion), TCEP/IdeS/mAb/IdeS/TCEP (digestion-reduction), injection
37 conditions (table 1).

38 (A,B) BGE: acetic acid–ammonium acetate pH 5.0 IS 75 mM.

39 **Figure 5:** Mass spectra of nano-ESI-MS infusion of off-line simultaneously digested-reduced
40 adalimumab with TCEP in 200 mM NH₄HCO₃.

41

42 Experimental conditions: off-line simultaneously digested-reduced adalimumab (see section 2.6),
43 Nano-ESI-MS: m/z 1000-7000, collision energy 30 V for low activation condition and 150 V for high
44 activation condition. LC (green circle), Fd and Fc/2 (yellow circle), F(ab) (green-yellow circles), Fc
45 (purple), non-specific complexes (blue).

46

47 **Figure 6:** Comparison of in-line digested and simultaneously digested-reduced mAbs, (A) infliximab,
48 (B) rituximab, (C) tocilizumab, and (D) belimumab. (*) corresponds to Fc subunits.

49

1 Experimental conditions: mAbs (1 g L⁻¹), TCEP 100 mM prepared in 200 mM NH₄HCO₃, IdeS (0.2 g L⁻¹),
2 NH₄HCO₃ (200 mM), injection schemes: IdeS/mAb/IdeS (digestion) or TCEP/IdeS/mAb/IdeS/TCEP
3 (digestion-reduction), injection volumes (table 1), BGE: acetic acid–ammonium acetate, IS = 75 mM
4 pH 5.0.

5

6 **Figure 7:** In-line digestion-reduction of adalimumab: effect of ammonium bicarbonate plugs volume.

7

8 Experimental conditions: Adalimumab (1 g.L⁻¹), TCEP 100 mM prepared in 200 mM NH₄HCO₃, IdeS
9 (0.2 g.L⁻¹), NH₄HCO₃ (200 mM), injection scheme: NH₄HCO₃/TCEP/IdeS/mAb/IdeS/TCEP/NH₄HCO₃,
10 injection volumes (table 1). BGE: acetic acid 20% (v/v) IS 8.2 mM pH 2.1.

11

12 **Figure 8:** Simultaneous digestion-reduction of adalimumab under denaturing conditions (A)
13 Comparison of off-line and in-line assays, (B) Electropherograms of intact, in-line digested and
14 simultaneously digested-reduced adalimumab. (*) correspond to Fc/2 subunits.

15

16 Experimental conditions:

17 (A) Off-line simultaneously digested-reduced adalimumab (section 2.6), injection volume: 23.6 nL,
18 injection scheme: NH₄HCO₃/off-line sample/NH₄HCO₃

19 (B) mAb (1 g.L⁻¹), TCEP 100 mM prepared in 200 mM NH₄HCO₃, IdeS (0.2 g.L⁻¹), NH₄HCO₃ (200 mM),
20 injection schemes: NH₄HCO₃/IdeS/mAb/IdeS/NH₄HCO₃ (digestion),
21 NH₄HCO₃/TCEP/IdeS/mAb/IdeS/TCEP/ NH₄HCO₃ (digestion-reduction), injection volumes (table 1).

22 (A,B) BGE: acetic acid 20% (v/v) pH 2.1 IS 8.2 mM.

23

24 **Figure 9:** Comparison of in-line digested and simultaneously digested-reduced mAbs: (A) infliximab,
25 (B) rituximab, (C) tocilizumab, and (D) belimumab. (*) corresponds to Fc/2 subunits.

26

27 Experimental conditions: mAbs (1 g.L⁻¹), TCEP 100 mM prepared in 200 mM NH₄HCO₃, IdeS (0.2 g.L⁻¹),
28 injection schemes: NH₄HCO₃/IdeS/mAb/IdeS/NH₄HCO₃ (digestion),
29 NH₄HCO₃/TCEP/IdeS/mAb/IdeS/TCEP/ NH₄HCO₃ (digestion-reduction), injection volumes (table.1).

30

31

32

33

34

















