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

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ABSTRACT

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

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1. Introduction

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

Analysis of intact mAbs is attractive as information on the global structure will be obtained with almost no sample preparation. However, this approach provides incomplete information about some microheterogeneities and their localization. Bottom-up and middle-up approaches give complementary information to

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intact mAb analysis. They provide access to more details on the sequence and/or post-translational modifications but require multiple sample preparation steps. The middle-up approach offers a simple investigation of mAbs with less complicated sample preparation than bottom-up and more accurate characterization than the intact approach. Multi-attribute assessment, like glycosylation and charge variants, can be done from a single run with high accuracy [12,13]. Nevertheless, the off-line sample preparation of established methods presents several drawbacks related to the manual handling (multisteps of pipetting and transfer, time and reactants consumption) that lead to the increase of the risks of contamination, errors, and loss of reactants by contact to surfaces or evaporation. Moreover, sample preparation can introduce artifacts by increasing deamidation and oxidation or desialylation [14].

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

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

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

(ii) Direct enzymatic digestion using enzymes with high specificity for a single cleavage site: papain [23], IgdE, and SpeB [18] cleave over the hinge region yielding F(ab) and Fc subunits. IdeS (Immunoglobulin degrading enzyme of *Streptococcus pyrogenes*) cleaves under the hinge region of mAbs to obtain $F(ab)_2$ (MW ≈ 100 kDa) and Fc/2 (MW ≈ 25 kDa) subunits [24]. IdeS is the most popular for the characterization of mAbs due to its fast and efficient enzymatic activity. It has been used to characterize Fc/2 glycoforms, charge variants, and Fc/2 dimers by CE [8,25,26]. Recently, we developed an in-line methodology for this approach with simplified procedure, temperature control, and high resolution of subunits separation under non-denaturing MS-compatible conditions [27].

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

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

2. Materials and methods

2.1. Chemicals

Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, ≥98%), sodium hydroxide (NaOH, ≥98%), hydrochloric acid (HCl, ≥37%), polyethylene oxide (PEO, MW = 1,000,000), bovine serum albumin (BSA), and sinapic acid (≥99%) were purchased from Sigma-Aldrich (Germany). Ammonium bicarbonate (NH_4HCO_3, ≥99.5%) and trifluoroacetic acid (TFA) were purchased from Fluka Analytical (USA). Ammonium acetate (≥98%), acetonitrile (ACN, ≥99%) and water for mass spectrometry analysis were purchased from Carlo Erba (France). Glacial acetic acid was purchased from Prolabo (France). All reagents were of analytical grade.

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

2.2. Capillary coating, BGE, and reactant solutions

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

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

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

2.3. Instrumentation and operating conditions

CE-UV analyses were performed on a Beckman-Coulter PA800 capillary electrophoresis system (Beckman, USA). Polymicro (Molex, USA) fused silica capillaries (id = 30 µm, L_{eff} = 50 cm, L = 60 cm) were used for enzymatic reactions and electrokinetic separations. Temperatures of the autosampler and capillary cartridge were set to 4 and 37 °C, respectively. All rinsing operations

were performed under 75 psi. The capillary pre-conditioning was performed by successive rinses with 1 M NaOH and water (15 min each). Before every run, capillaries were rinsed successively with 1 M NaOH, water, and 0.1 M HCl (3 min each). The coating of capillaries was then performed by rinsing with PEO coating solution for 6 min, followed by 5 min water rinsing. Eventually, the capillary was rinsed with appropriate BGE for 5 min. Reactants and samples were injected hydrodynamically from the anodic side. Separations were achieved under +15 kV and the positively charged mAb or subunits migrated toward the cathode. UV detection was performed at 214 nm.

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

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

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

2.4. Off-line reduction

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

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

For MALDI-MS analyses of off-line samples assays, the matrix was prepared by dissolving sinapic acid in water/ACN-0.1% TFA (50/50) (v/v) to a final concentration of 10 g.L^{-1}. Samples were diluted by a factor of ½ in the matrix before spotting. Calibration for high *m/z* ratios was performed before each analysis with BSA at 1 mg.L^{-1} in water, based on the multiply charged species and non-specific dimer.

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

2.6. In-line and off-line assays

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

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

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

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

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

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

Different BGE were used depending on the implemented reaction:

- In-line reduction: separation with acetic acid-ammonium acetate pH 4.0 IS 25 mM BGE.
- In-line digestion: separation with acetic acid-ammonium acetate pH 5.0 IS 75 mM (non-denaturing BGE) or acetic acid 20% (v/v) (denaturing BGE). For analyses with denaturing BGE, plugs of 200 mM ammonium bicarbonate were injected at both sides of the in-line digestion or simultaneous digestion-reduction injection scheme. The volume of each NH_4HCO_3 plug was 5.1 nL (0.5 psi, 40 s).

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

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

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

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 ($C_{TCEP,final}$ = 10 mM) using off-line protocols based on literature [18,28]. Entire reduction of adalimumab was confirmed by MALDI-MS analyses (Fig. 1A).

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 (2 psi, 5 s)	2.6 nL (2 psi, 5 s)	0 nL
In-line digestion/ non-denaturing BGE	2.6 nL (2 psi, 5 s)	0 nL	2.0 nL (2 psi, 4 s)
In-line simultaneous digestion-reduction / non-denaturing BGE	2.6 nL (2 psi, 5 s)	2.6 nL (2 psi, 5 s)	2.0 nL (2 psi, 4 s)
In-line digestion / denaturing BGE	5.1 nL (4 psi, 5 s)	0 nL	4.1 nL (4 psi, 4 s)
In-line simultaneous digestion-reduction / denaturing BGE	5.1 nL (4 psi, 5 s)	5.1 nL (4 psi, 5 s)	4.1 nL (4 psi, 4 s)

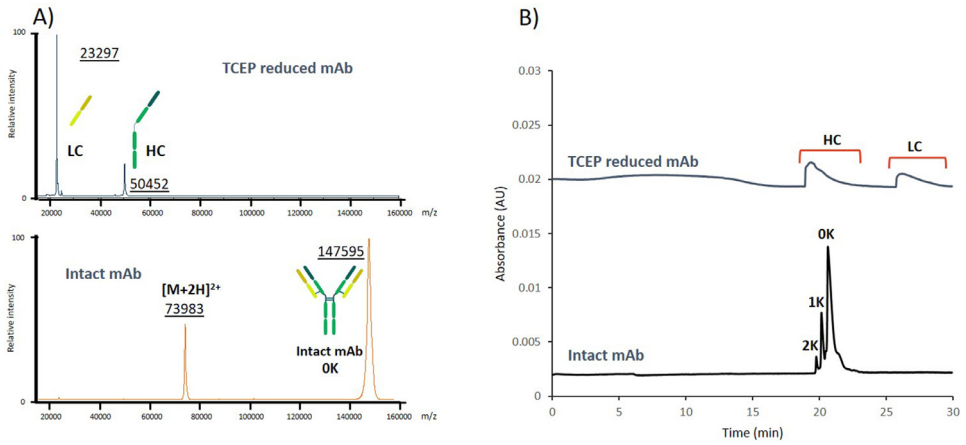


Fig 1. Off-line TCEP reduction of adalimumab. (A) MALDI-MS spectra of intact and reduced adalimumab, (B) Electrophoretic UV profiles of intact and TCEP reduced adalimumab. Experimental conditions: (A) Intact and off-line reduced adalimumab (1 g.L^{-1}) (see Section 2.4). MALDI-MS analyses (see Section 2.5). Matrix: sinapic acid 10 g.L^{-1} in water/ACN-0.1% TFA (50/50, v/v), *m/z* 10,000–160,000. (B) Intact and off-line reduced adalimumab (1 g.L^{-1}) (see Section 2.4). Injection volume = 10.3nL, BGE: acetic acid–ammonium acetate pH 5.0 IS 25 mM, T = 37 °C, and voltage = 15 kV.

Only masses of the light chain ($MW_{LC} \approx 23$ kDa) and heavy chain ($MW_{HC} \approx 50$ 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 (0 K).

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

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

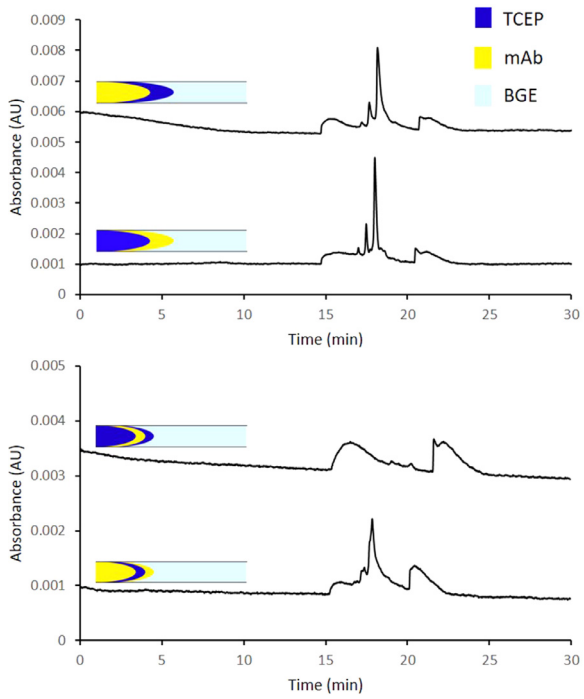


Fig 2. Effect of injection schemes on the yield of in-line TCEP reduction of adalimumab: Top: plug/plug mode. Bottom: sandwich mode. Experimental conditions: Adalimumab (1 $g.L^{-1}$), TCEP 100 mM in water, injection plug volumes: 2.5 nL of mAb and 1.3 nL of TCEP, BGE: acetic acid–ammonium acetate pH 5.0 IS 25 mM, incubation time: 10 min.

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

3.1.3. Application to other mAb

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

3.2. In-line simultaneous digestion and reduction

As the IdeS enzyme does not contain disulfide bonds [30], digestion and reduction steps can be combined to decrease $F(ab)_2$ subunit structural complexity. This approach yields Fc/2, Fd and LC subunits in the 23 to 26 kDa mass range, allowing subunit-specific characterization [36].

First, previously optimized reduction conditions were used for in-line simultaneous digestion-reduction. However, to preserve the IdeS activity in the in-line methodology, the pH of adjacent IdeS plugs has to be in the pH range 5.1–7.6 [41]. Therefore, 100 mM TCEP solution was prepared in 200 mM ammonium bicarbonate (pH 8.0). Since TCEP is efficient under a wide pH range [18], its activity should be preserved.

3.2.1. Separation under non-denaturing conditions

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

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

To investigate the extent of off-line simultaneous digestion-reduction and identify the products of reaction, MS experiments were performed by nano-ESI-MS as it allows analysis under non-denaturing conditions. Analysis with low activation energy (Fig. 5) showed 48.9 and 50.4 kDa masses resulting from the non-covalent association of LC and Fd into F(ab) and two Fc/2 into Fc, respectively. This was confirmed by applying high collision energy in the mass spectrometer to dissociate non-covalent interactions. Results showed the decrease of MS spectra intensity of F(ab) and Fc subunits decreased and the appearance of MS spectra with lower *m/z*.

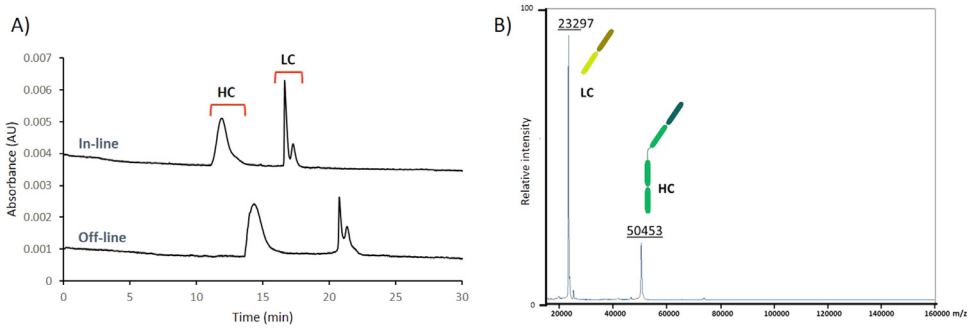


Fig 3. In-line and off-line reduction of adalimumab under optimized conditions: (A) CZE-UV analyses (B) MALDI-MS analysis of off-line sample Experimental conditions: (A) CZE-UV analyses: Off-line reduced adalimumab (see Section 2.6), In-line reduction: adalimumab (1 g.L^{-1}), TCEP 100 mM in water, injection scheme: TCEP/mAb/TCEP, injection volumes (Table 1), BGE: acetic acid–ammonium acetate pH 4.0 IS 25 mM, incubation time: 25 min. (B) Off-line reduced adalimumab MALDI-MS analysis (Section 2.5 and 2.6): sinapic acid matrix in water/ACN-0.1% TFA (50/50) (v/v) 10 g.L^{-1}, *m/z* 10,000–160,000.

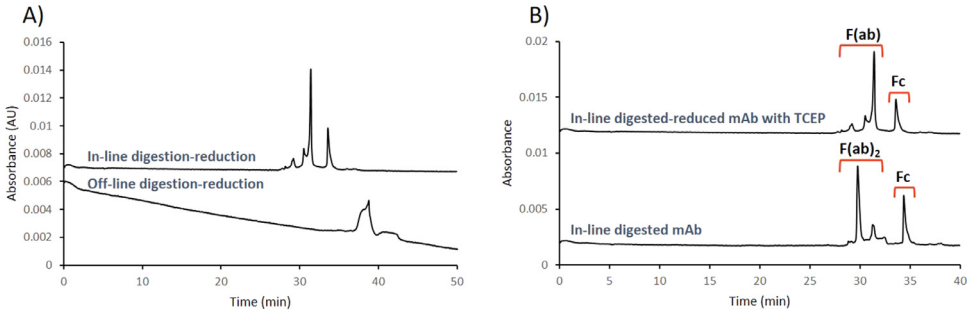


Fig 4. Simultaneous digestion-reduction of adalimumab under non-denaturing conditions (A) Comparison of off-line and in-line assays, (B) Electropherograms in-line digested and simultaneously digested-reduced adalimumab. Experimental conditions: (A) Off-line simultaneously digested-reduced adalimumab (Section 2.6), injection volume: 11.8 nL, injection scheme: off-line sample (B) Adalimumab (1 g.L^{-1}), TCEP 100 mM prepared in 200 mM NH_4HCO_3, IdeS (0.2 g L^{-1}), injection schemes: IdeS/mAb/IdeS (digestion), TCEP/IdeS/mAb/IdeS/TCEP (digestion-reduction), injection conditions (Table 1). (A,B) BGE: acetic acid–ammonium acetate pH 5.0 IS 75 mM.

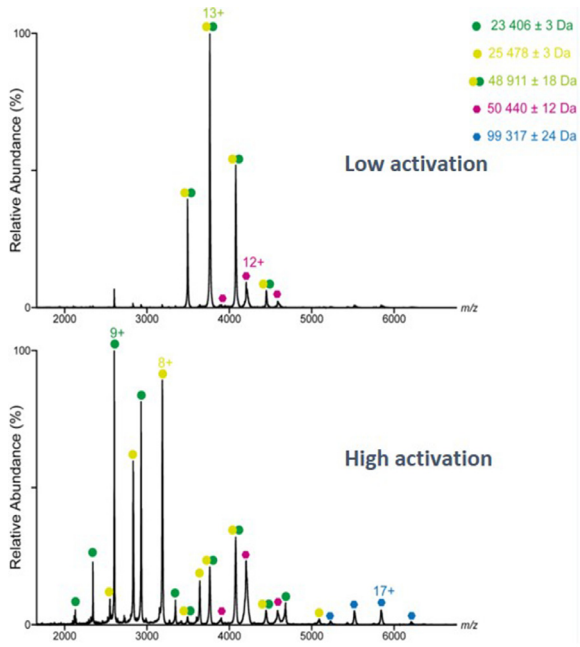


Fig 5. Mass spectra of nano-ESI-MS infusion of off-line simultaneously digested-reduced adalimumab with TCEP in 200 mM NH_4HCO_3. Experimental conditions: off-line simultaneously digested-reduced adalimumab (see Section 2.6), Nano-ESI-MS: *m/z* 1000–7000, collision energy 30 V for low activation condition and 150 V for high activation condition. LC (green circle), Fd and Fc/2 (yellow circle), F(ab) (green-yellow circles), Fc (purple), non-specific complexes (blue) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

The deconvolution of the appeared MS spectra showed masses corresponding to the dissociated subunits 23 kDa (LC) and 25 kDa (Fd and Fc/2). The observed mass 99.3 kDa may correspond to a non-specific complex of F(ab) and Fc. Intact mAb mass was not detected, supporting the evidence of complete digestion/reduction.

As a consequence, these results suggest that the two main peaks observed in CZE profile of digested-reduced adalimumab emanated from the association of the subunits (LC, Fd and Fc/2) by non-covalent interactions to form F(ab) and Fc subunits during the migration process under non-denaturing separation conditions (i.e. pH 5.0). In addition, the last peak would correspond to Fc subunit as it results also from digestion. So, digestion-reduction of adalimumab was achieved in the in-line assay. These results are in agreement with the results described in the literature. Indeed, LC and Fd association to F(ab) has been previously reported under native conditions in cIEF-MS [12,42], and Fc/2 association to form Fc has been reported in native CZE-ESI-MS analyses [8,27].

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

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

The methodologies were applied to other mAbs. Different electrophoretic profiles were obtained for digested and simultaneously digested-reduced mAb (Fig. 6). Peaks with similar electrophoretic mobilities and shapes are observed (*) in the electropherograms of digested mAbs and simultaneously digested-reduced mAbs that

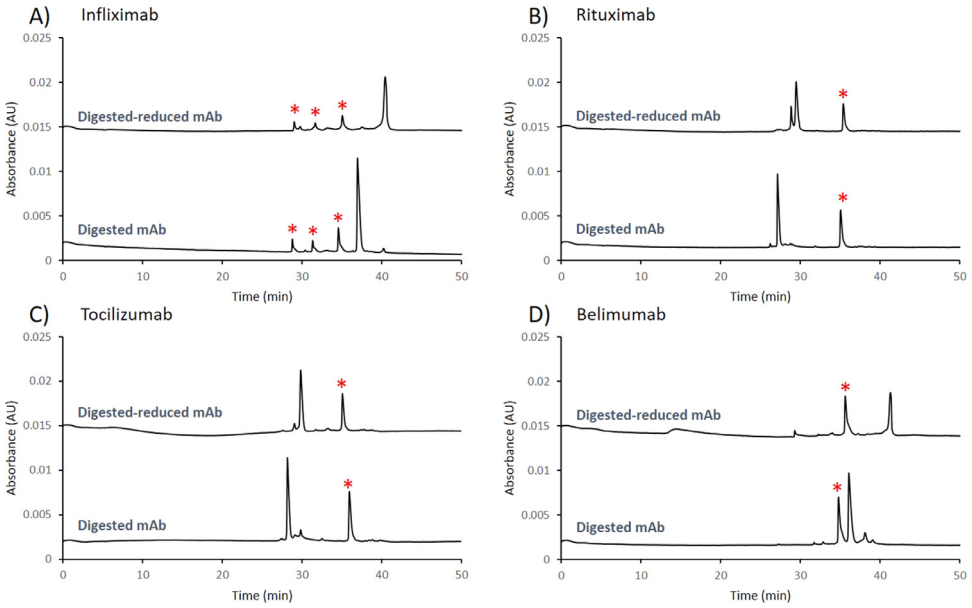


Fig 6. Comparison of in-line digested and simultaneously digested-reduced mAbs, (A) infliximab, (B) rituximab, (C) tocilizumab, and (D) belimumab. (*) corresponds to Fc subunits. Experimental conditions: mAbs (1 g L^{-1}), TCEP 100 mM prepared in 200 mM NH_4HCO_3, IdeS (0.2 g L^{-1}), NH_4HCO_3 (200 mM), injection schemes: IdeS/mAb/IdeS (digestion) or TCEP/IdeS/mAb/IdeS/TCEP (digestion-reduction), injection volumes (Table 1), BGE: acetic acid–ammonium acetate, IS = 75 mM pH 5.0.

could be attributed to the Fc subunit. In the case of infliximab, the first three peaks in the electropherograms of digested infliximab and simultaneously digested-reduced infliximab were observed with the same migration times. The three peaks would correspond respectively to the C-terminal lysine variants 2, 1, and 0 K of Fc subunit resulting from differential truncation of lysine residue carrying one positive charge [28]. The separation of C-terminal lysine variants has been reported at the middle-up level for cetuximab [8] and infliximab [27] under non-denaturing conditions.

3.2.3. Separation under denaturing conditions

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

However, the pH of this BGE is not compatible anymore with the enzymatic reaction. It may affect IdeS activity by mixing with reactants plugs. For this reason, plugs of 200 mM ammonium bicarbonate were injected on both sides of the reactant injection sequence (TCEP/IdeS/mAb/IdeS/TCEP) to prevent interferences of BGE with the enzymatic reaction [47,48]. Thus, the resulting injection scheme was the following: NH_4HCO_3/TCEP/IdeS/mAb/IdeS/TCEP/NH_4HCO_3. These extra ammonium bicarbonate plugs were injected by low pressure (i.e. 0.5 psi) to achieve flat profiles minimizing transverse diffusion with BGE. The influence of ammonium bicarbonate concentration and plugs volume on the digestion-reduction reaction and subunits separation was carefully studied.

The injected volume of each ammonium bicarbonate plug was increased from 0 to 10.3 nL and resulted in various electrophoretic profiles. Fig. 7 shows the dependence of digestion efficiency on ammonium bicarbonate plugs volume. Similar electrophoretic profiles were observed at a volume equal or higher than 5.1 nL, indicating an equilibrium state. Similar profiles for off-line (also analyzed by MALDI-MS (data not shown)) and in-line assays under optimal conditions were obtained (Fig. 8A), showing that adalimumab was well digested-reduced with the in-line methodology even in the presence of denaturing BGE. Three major peaks were obtained that correspond likely to Fc/2, Fd and LC subunits as acidic BGE disrupts non-covalent interactions. The analysis of TCEP following the scheme (NH_4HCO_3/TCEP/NH_4HCO_3) did not show any peak confirming the absence of TCEP interference with the electrophoretic profile of digested-reduced mAb (data not shown).

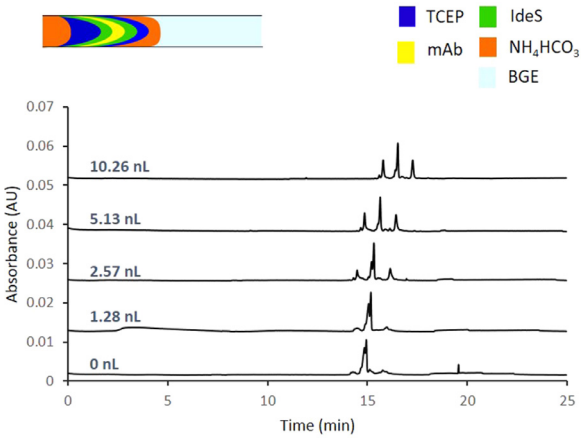


Fig 7. In-line digestion-reduction of adalimumab: effect of ammonium bicarbonate plugs volume. Experimental conditions: Adalimumab (1 g.L^{-1}), TCEP 100 mM prepared in 200 mM NH_4HCO_3, IdeS (0.2 g.L^{-1}), NH_4HCO_3 (200 mM), injection scheme: NH_4HCO_3/TCEP/IdeS/mAb/IdeS/TCEP/NH_4HCO_3, injection volumes (Table 1). BGE: acetic acid 20% (v/v) IS 8.2 mM pH 2.1.

The in-line assay provided good repeatability (RSD less than 0.8% for migration time and less than 3.84% for corrected peak areas). The in-line electrophoretic profiles of intact, digested, and simultaneously digested-reduced adalimumab were compared. Reactant concentrations and injection conditions are given in Table 1.

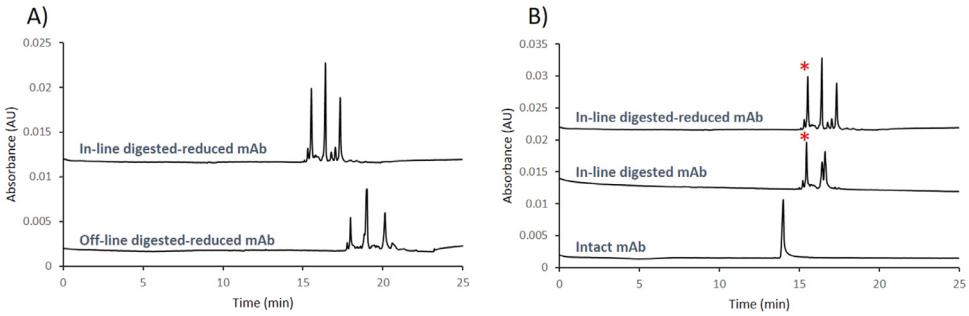


Fig 8. Simultaneous digestion-reduction of adalimumab under denaturing conditions (A) Comparison of off-line and in-line assays, (B) Electropherograms of intact, in-line digested and simultaneously digested-reduced adalimumab. (*) correspond to Fc/2 subunits. Experimental conditions: (A) Off-line simultaneously digested-reduced adalimumab (Section 2.6), injection volume: 23.6 nL, injection scheme: NH_4HCO_3/off-line sample/NH_4HCO_3 (B) mAb (1 $g.L^{-1}$), TCEP 100 mM prepared in 200 mM NH_4HCO_3, IdeS (0.2 $g.L^{-1}$), NH_4HCO_3 (200 mM), injection schemes: NH_4HCO_3/IdeS/mAb/IdeS/NH_4HCO_3 (digestion), NH_4HCO_3/TCEP/IdeS/mAb/IdeS/TCEP/ NH_4HCO_3 (digestion-reduction), injection volumes (Table 1). (A,B) BGE: acetic acid 20% (v/v) pH 2.1 IS 8.2 mM.

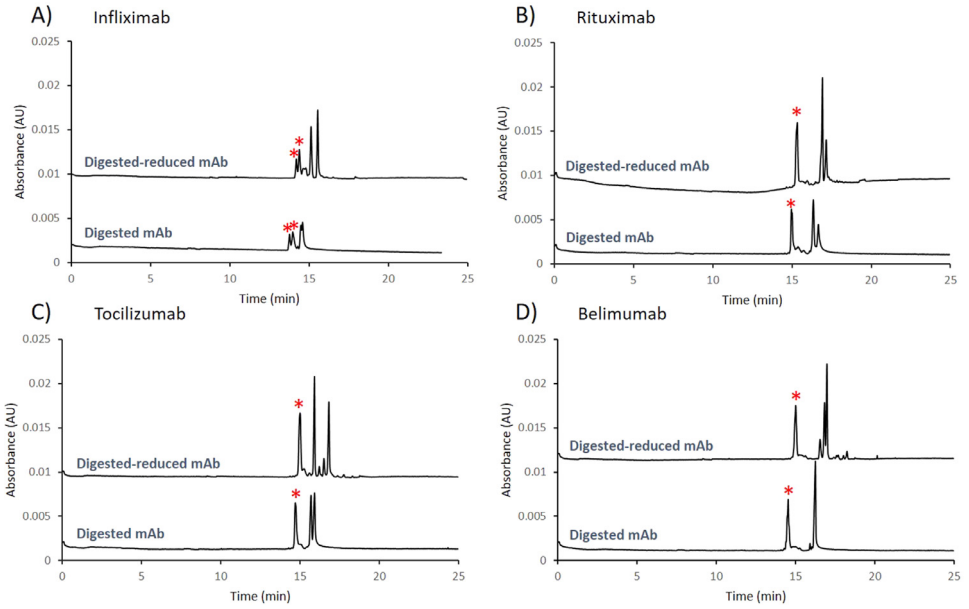


Fig 9. Comparison of in-line digested and simultaneously digested-reduced mAbs: (A) infliximab, (B) rituximab, (C) tocilizumab, and (D) belimumab. (*) corresponds to Fc/2 subunits. Experimental conditions: mAbs (1 $g.L^{-1}$), TCEP 100 mM prepared in 200 mM NH_4HCO_3, IdeS (0.2 $g.L^{-1}$), injection schemes: NH_4HCO_3/IdeS/mAb/IdeS/NH_4HCO_3 (digestion), NH_4HCO_3/TCEP/IdeS/mAb/IdeS/TCEP/ NH_4HCO_3 (digestion-reduction), injection volumes (Table. 1).

The differences observed in electrophoretic profiles (Fig. 8B) demonstrate that in-line enzymatic reactions were well carried out. The contribution of TCEP to the enzymatic cleaving process was shown in the differences between digested and digested-reduced electrophoretic profiles. Electrophoretic mobility of first migrating peak (*) appears to be similar for both digested and digested-reduced mAb. This peak could be attributed to Fc/2 subunit, which is a shared subunit of both reactions.

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

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

4. Conclusion

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Meriem Dadouch: Investigation, Methodology, Writing – original draft. **Yoann Ladner:** Methodology, Writing – review & editing. **Claudia Bich:** Methodology, Writing – review & editing. **Jérôme Montels:** Resources. **Catherine Perrin:** Methodology, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462213.

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