



HAL
open science

Enhancing affinity purification of monoclonal antibodies from human serum for subsequent CZE-MS analysis

Tessa Reinert, Pascal Houzé, Yannis-Nicolas Francois, Rabah Gahoual

► To cite this version:

Tessa Reinert, Pascal Houzé, Yannis-Nicolas Francois, Rabah Gahoual. Enhancing affinity purification of monoclonal antibodies from human serum for subsequent CZE-MS analysis. *Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences*, 2024, 1234, pp.123974. 10.1016/j.jchromb.2023.123974 . hal-04600227

HAL Id: hal-04600227

<https://hal.science/hal-04600227>

Submitted on 4 Jun 2024

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.

Enhancing affinity purification of monoclonal antibodies from human serum for subsequent CZE-MS analysis

Tessa Reinert ^{a,b,*}, Pascal Houzé ^{b,c}, Yannis-Nicolas Francois ^a, Rabah Gahoual ^b

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

^b Université Paris Cité, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), CNRS, Inserm, Faculté de sciences pharmaceutiques et biologiques, Paris, France

^c Laboratoire de Toxicologie Biologique, Hôpital Lariboisière, Assistance Publique – Hôpitaux de Paris (AP-HP), Paris, France

ABSTRACT: Due to the separation technique employed, capillary electrophoresis coupled to mass spectrometry (CE-MS) analysis performances are significantly influenced by the chemical composition and the complexity of the sample. In various applications, that impact has prevented the use of CE-MS for the characterization and quantification of proteins in biological samples. Here we present the development and evaluation and a sample preparation procedure, based on affinity purification, for the specific extraction of the monoclonal antibody (mAbs) infliximab from human serum in order to perform subsequent proteolytic digestion and CE-MS/MS analysis. Three distinctive sample preparation strategies were envisaged. In each case, the different steps composing the protocol were thoroughly optimized and evaluated in order to provide a sample preparation addressing the important complexity of serums samples while providing an optimal compatibility with CE-MS/MS analysis. The different sample preparation strategies were assessed concerning the possibility to achieve an appropriate absolute quantification of the mAbs using CE-MS/MS for samples mimicking patient serum samples. Also, the possibility to perform the characterization of several types of post-translational modifications (PTMs) was evaluated. The sample preparation protocols allowed the quantification of the mAbs in serums samples for concentration as low as $0.2 \mu\text{g}\cdot\text{mL}^{-1}$ (2.03 nM) using CE-MS/MS analysis, also the possibility to characterize and estimate the modification level of PTMs hotspots in a consistent manner. Results allowed to attribute the effect on the electrophoretic separation of the different steps composing sample preparation. Finally, they demonstrated that sample preparation for CE-MS/MS analysis could benefit greatly for the extended applicability of this type of analysis for complex biological matrices.

1. INTRODUCTION

Since their introduction in the late 1980s, monoclonal antibodies (mAbs) have experienced increasing success in the treatment of various diseases, leading to their emergence in the mid-2000s [1,2]. The appeal of therapeutic mAbs can be explained by their favorable pharmacokinetic and pharmacodynamic properties. In addition, their ability to target a specific epitope corresponding to the antigen represents a crucial asset for the development of this type of biomolecule for therapeutic applications. Last year alone, 12 mAbs-based therapeutics were approved, and 140 are currently in clinical trials, including several for the treatment of SARS-CoV-2 [3–5]. Due to their structural complexity and their use as biopharmaceutical products, mAbs have required the development of dedicated analytical methods to provide their comprehensive structural characterization. However, almost the totality of analytical developments described in the literature focus on the characterization of mAbs in the context of their production, while methods for the analysis of mAbs in biological samples remain scarce [6,7]. Therefore, it appears essential to develop novel analytical strategies to study the outcome of mAbs after administration to patients. Bottom-up peptide-centric analysis represents a relevant methodology that, using mass spectrometry (MS), offers the possibility to obtain quantification in addition to the characterization of protein primary structure, including post-translational modifications (PTMs) [8–10].

Presently, almost all mAbs products are administered by parenteral injection into the patient's bloodstream, and therefore mAbs are present in the serum. Human serum is an extremely complex matrix composed of a wide variety of proteins, such as albumin and immunoglobulin (IgG) [11]. The presence of a large number of proteins can interfere with the MS analysis of mAbs, particularly because of the large difference in concentration between serum proteins and the administered mAbs. As a result, the analysis of a specific protein present in a biological sample requires proper sample preparation to remove other proteins and salts that would otherwise hamper the MS analysis. Sample purification can improve the reproducibility of the method and significantly reduce interferences to increase sensitivity. Indeed, the removal of albumin, which accounts for approximately 50 % of total serum protein, has been shown to improve the sensitivity of the method [12]. Various types of purification procedures, such as pellet digestion or protein precipitation, can be used to remove albumin from serum samples to decomplexify the matrix. However, their lack of specificity is a limitation for mAb isolation, as a large fraction of serum proteins are IgG. An alternative method to enhance

the specificity of sample preparation is affinity purification [13]. This type of extraction is emerging for mAb analysis, but its implementation requires particularly demanding setup and optimization [14,15].

Capillary zone electrophoresis hyphenated to tandem mass spectrometry (CZE-MS/MS) has showed to be particularly suitable for the separation and characterization of peptide mixtures [16,17]. Indeed, the electrokinetic separation provided by CZE allows the separation of a wide variety of peptides in a single experiment with respect to their chemical nature. Also, the mobility mechanism of CZE allows the baseline separation of peptides exhibiting faint PTMs from their unmodified counterparts. This feature is a strong advantage of CZE, as a lack of separation prior to MS analysis would result in overlapping isotopic profiles of peptides, preventing clear identification [18]. The diversification of CZE-MS applications was also supported by technical improvements in coupling interfaces, which have enhanced sensitivity [19–21]. As a result, CZE-MS has become particularly relevant for the characterization of biomolecules [22–24]. CZE separation is particularly sensitive to sample composition due to the limited capillary volume available and the phenomenon of electrophoresis. Samples can exhibit significant electrical conductivities relative to the background electrolyte (BGE) due to the presence of high concentrations of salts and/or organic solvents. Therefore, the composition of the sample can affect the homogeneity of the electric field, resulting in reduced separation efficiency and impaired reproducibility. In order to characterize and quantify mAbs in biological samples using CZE-MS/MS, it is necessary to develop a sample preparation that allows a relevant affinity extraction and purification of the proteins, in addition to ensuring optimal compatibility with CZE electrophoretic separation.

In the present work, we describe the development of a selective purification adapted to the analysis of the active fraction of infliximab (IFX) in human serum using CZE-MS/MS analysis. IFX is a chimeric mAb targeting tumor necrosis factor (TNF- α), used as a therapeutic treatment mainly for Crohn's disease and rheumatoid arthritis [25,26]. The affinity purification of IFX was based on the implementation of ferromagnetic beads incorporating immobilized TNF- α , which were prepared in-house. Subsequently, isolated IFX were released and subjected to trypsin digestion, followed by bottom-up analysis using CE-MS instrumentation. Different strategies regarding IFX release from functionalized beads, and buffer exchange in order to perform proteolytic digestion, and CZE separation were compared. The sequential steps that make up the different sample preparation protocols were investigated in a systematic manner

to attribute their impact on the extraction yield, IFX recovery, and the ability to maintain optimal CZE separation efficiency.

2. MATERIALS AND METHODS

2.1. Chemicals. The chemicals used were of high purity grade and purchased from Merck Sigma-Aldrich (Saint Louis, MO, USA) unless stated otherwise. The water used to prepare the buffers and sample solutions was obtained using an ELGA Purelab UHQ PS water purification system (Bucks, UK). Infliximab (IFX) samples were EMA/FDA-approved formulations (Remicade®) purchased from the manufacturer (Merck Sharp and Dohme). Stable-isotope-labeled infliximab (SIL-IFX) internal standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). RapiGest SF surfactant was purchased from Waters (Milford, MA). Streptavidin M-280 Dynabeads™ were obtained from Fisher Scientific Invitrogen (Carlsbad, CA) and biotinylated TNF- α from Biotechne (Rennes, France). Sequencing-grade modified Trypsin was purchased from Promega (Madison, WI, USA). (PBS) tablets used for reconstitution in water were purchased from Gibco Fisher. Blank sera extracted and purified from the total blood were provided by the French Institution of Blood (Paris, France).

2.2. Reference and serum samples. IFX stock solutions at a concentration of 1 g·L⁻¹ were prepared after reconstitution in Milli-Q H₂O and directly stored at -20°C until use. Two kinds of samples were prepared: IFX spiked reference samples and IFX spiked serum standards. The reference samples were prepared by introducing IFX stock solution to ammonium bicarbonate buffer (50 mM, pH 8) to a final volume of 100 μ L and concentration of 25 μ g·mL⁻¹. The standard serum samples were prepared by introducing IFX stock solution to model blank serum (initially free of IFX) to a final volume of 100 μ L and concentration of 25 μ g·mL⁻¹.

2.3. Affinity purification of IFX from human serum. The standard serum samples were purified to specifically extract IFX. First, 217 μ L of streptavidin M-280 Dynabeads™ (10 mg·mL⁻¹) was incubated with biotinylated TNF- α (148 μ L, 10 μ g·mL⁻¹ in PBS) at room temperature (RT) for 1 h. Excess biotinylated TNF- α was removed by three consecutive washing steps using 500 μ L PBS solution. Subsequently, the 100 μ L standard serum sample was added to the TNF- α -

functionalized beads. The mixture was incubated at RT for 1 h under mild agitation. Then the serum was removed, and the magnetic beads were washed twice using 500 μL of PBS.

2.4. IFX release and buffer exchange

2.4.1. Acidic dissociation followed by membrane filtration. The citrate buffer (pH 3.0) was prepared by mixing 70 μL of sodium citrate dihydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$) 100 mM with 930 μL of citric acid ($\text{C}_6\text{H}_8\text{O}_7$) 109 mM. After washing with PBS, IFX captured on the TNF- α magnetic beads was dissociated by the addition of 50 μL citrate buffer. The mixture was left at RT for 10 min, and the supernatant was collected. Another 50 μL of citrate buffer was added to the magnetic beads and incubated at RT for 10 min, followed by supernatant collection. The supernatants were pooled, and the pH of the solution was neutralized by the addition of NaOH 1 M. Consequently, buffer exchange was performed using an Amicon[®] centrifugal membrane filter provided by Merck Millipore (Molsheim, France). The filter was conditioned with 50 mM ammonium bicarbonate (pH 8.0). The sample was then subjected to two consecutive centrifugation cycles. For each centrifugation cycle, the filter was filled with 50 mM ammonium bicarbonate, and centrifugation was performed for 10 min using an Eppendorf 5424[®] centrifuge system. Finally, the sample was collected in a new tube and adjusted to a final volume of 70 μL using the ammonium bicarbonate buffer.

2.4.2. Organic solvent dissociation followed by evaporation. IFX dissociation from TNF- α magnetic beads was alternatively achieved by adding 50 μL of MeOH/H₂O/FA: 48.5/48.5/3 (v/v/v) solution to the sample, which was left at 20°C for 10 min. The supernatant was collected, and the operation was repeated once. Both supernatants were then pooled and dried for 2 h using a miVac DNA concentrator system (Genevac, NY, USA) at a temperature of 35 °C. The samples were finally reconstituted with 70 μL of 50 mM ammonium bicarbonate.

2.5. Proteolytic digestion of the IgG samples. The IFX samples (references or after purification of serum standards) are all in ammonium bicarbonate, 50 mM, pH 8. Prior to sample digestion, 4 μL of SIL-IFX stock solution at a concentration of 0.25 $\mu\text{g}\cdot\mu\text{L}^{-1}$ was added to all IFX samples. Proteolytic digestion of IFX and SIL-IFX obtained by affinity purification from serum samples was achieved following a workflow derived from a commonly used protocol for the digestion of mAbs in solution into peptides [27]. Briefly, mAbs were denatured using 5 μL of RapiGest SFTM 0.1 % and incubated at 80°C for 10 min. After cooling to RT, 10 μL of 50 mM dithiothreitol were added to the mixture, and

the sample was incubated at 80°C for 20 min. Then, 10 µL of 50 mM iodoacetamide was added, and the sample was incubated at RT in the dark for 20 min. 1 µL of trypsin at 0.25 µg·µL⁻¹ was added to the sample and left at RT for 2 h. Trypsin (1 µL) was again added, and the sample was incubated at 37°C overnight. Next, 1 µL FA (98 %) was added. The peptide digests were dried. Finally, the sample was reconstituted in 5 µL 100 mM ammonium acetate at pH 4.

2.6. Capillary zone electrophoresis – Tandem mass spectrometry for peptide analysis. Analysis of the IFX peptide digests was performed on a CESI8000[®] capillary electrophoresis system (Sciex separations, Darmstadt, Germany) coupled via a sheathless CE-ESI-MS interface to a Sciex TripleTOF 5600 mass spectrometer (Darmstadt, Germany) operated using Analyst[®] software (Sciex, Darmstadt, Germany). Total capillary volume, effective volume, and sample injection volume were calculated using CEToolbox software (available from Google Play Store) [28]. Separation was performed in a bare-fused silica capillary (total length 100 cm; 30 µm i.d.) filled with a conductive background electrolyte (BGE) consisting of 10 % acetic acid. The outlet end of the capillary was etched to obtain a 2 cm porous tip positioned inside the ESI source, and a second capillary (total length 80 cm; 50 µm i.d.) filled with the BGE was used to maintain electrical connection between the CE electrodes. Transient isotachopheresis (t-ITP) preconcentration was generated prior to CZE separation by injecting the peptide digest reconstituted in a leading electrolyte of ammonium acetate 100 mM (pH4). Hydrodynamic injection (5 psi, 100 sec) allowed 68 nL of peptide digest to be injected into the inlet of the separation capillary. A 23 kV electric field was then applied, leading the peptides to migrate toward the ESI source. The ESI voltage set was 1.5 kV, source heating temperature was 100°C, and the curtain gas value was 2. Experiments were performed in Top20 information-dependent acquisition (IDA, Sciex), in a total duty cycle of 1.9 s. Data were analyzed Skyline software developed by the University of Washington (Seattle, WA, USA) [29].

2.7. Parameters studied for protocol assessments. The process efficiency of the workflow was estimated for each protocol using Eq. (1). It corresponded to the complete recovery of IFX, from its extraction by affinity beads prior to digestion (Fig. 1). Briefly, CE-MS/MS signal ratios corresponding to IFX and SIL-IFX measured from the spiked serum samples were compared to the ratios collected for the reference sample prepared in milli-Q H₂O without purification and thus only submitted to proteolytic digestion.

$$\text{Process Efficiency (\%)} = \frac{\left(\frac{A_{IFX}}{A_{SIL-IFX}}\right)_{\text{serum}}}{\left(\frac{A_{IFX}}{A_{SIL-IFX}}\right)_{\text{reference}}} \times 100 \quad [\text{Eq. 1}]$$

where A_{IFX} and $A_{SIL-IFX}$ are the peak areas corresponding to the IFX and SIL-IFX respectively. The signals of the two proteins were monitored using the peptide LT-01 (sequence DILLTQSPAILSVSPPGER), which was beforehand selected because of its specificity for IFX and its high ionization efficiency. The same peptide was considered for SIL-IFX, including the ($^{13}\text{C}_6$; $^{15}\text{N}_4$) label for the arginine residue. The LT-01 peptide was systematically identified using MS/MS spectra from the characterization of γ and b -ions corresponding to the CID fragmentation of the peptide (Table S1). Further considerations of the performance of individual steps of IFX purification were performed by calculating the extraction recovery yield or filtration recovery yield (Fig. 1). The LOD and LOQ were calculated by considering LT-01 peptide signal. The intensity of this signal was collected as well as that of the background noise. The LOD and LOQ were assessed when the signal to noise (S/N) reached 3 and 10, respectively.

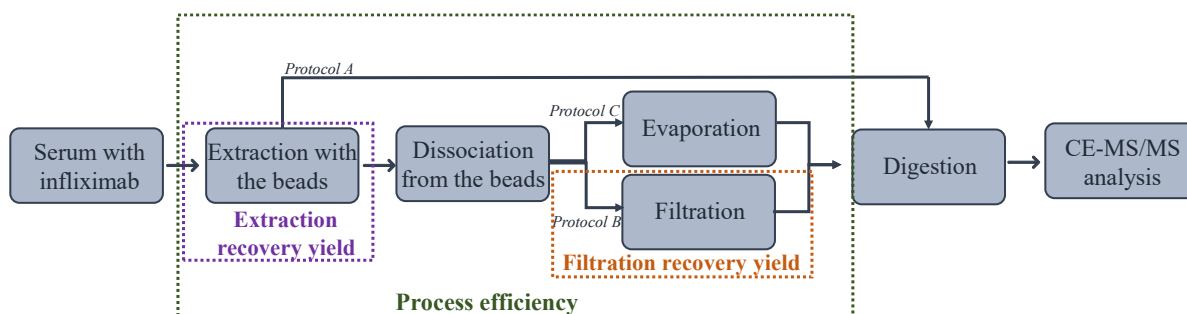


Fig. 1. Representation of the different parameters considered to evaluate the protocols of sample preparation prior CE-MS/MS analysis of peptide digest of infliximab on the analytical workflow.

2.8. Size exclusion chromatography – Multi-angle light scattering analysis. Size exclusion liquid chromatography analyses were performed using a Prominence HPLC system equipped with a SIL-10A UV absorbance detector, a RID-20A refractive index detector (Shimadzu, Marne-la-Vallée, France) and a miniDAWN Treos II multi-angle light scattering (MALS) detector acquired from Wyatt Technology (Santa Barbara, USA). Two distinctive softwares were used to pilot the SEC-UV-MALS-RI instrument: LC Solution (Shimadzu corporation) for the HPLC-UV-RI part and Astra version v7.2 (Wyatt technology, Santa Barbara, USA) for the MALS detector. SEC separations were realized using a Biozen SEC3 column (300 × 4.6 mm; 1.8 μm) provided by Phenomenex (Le Pecq, France) using a mobile phase constituted of 50 mM phosphate buffer (pH 6.8) and 300 mM NaCl. Flow rate was 200 μL·min⁻¹ and injection volume

was 20 μL . UV detection was performed at a wavelength of 280 nm. MALS detector was equipped with a 658 nm laser, and measurements were performed simultaneously at 3 different angles (49° , 90° and 131°). IFX at a concentration of $0.5 \mu\text{g}\cdot\mu\text{L}^{-1}$ in H_2O milliQ or in 50 mM citrate buffer was injected for the analyses.

2.9. Dynamic light scattering analysis. Dynamic light scattering (DLS) analyses were performed on a Zetasizer (Malvern Panalytical, Malvern, UK), using 2 angles (13° and 173°) monitored by the Zetasizer software. 400 μL of sample was analyzed. Measurements were realized at a temperature of 25°C in triplicate. For analyses, IFX was injected at a concentration of $0.5 \mu\text{g}\cdot\mu\text{L}^{-1}$ in H_2O milli-Q or in 50 mM citrate buffer.

3. RESULTS AND DISCUSSION

The reported therapeutic range for IFX (infliximab) indicates that serum concentrations after administration should fall within the range of 1 to $25 \mu\text{g}\cdot\text{mL}^{-1}$. Affinity purification is necessary to isolate low concentrations of IFX from a complex mixture composed of $1\text{--}5 \text{ mg}\cdot\text{mL}^{-1}$ of a wide variety of natural IgGs [30]. Consequently, a peptide-centric analytical strategy, derived from bottom-up proteomic analysis, was adopted as it maintained optimal sensitivity, especially considering the low-level quantification aimed at. In addition, it allowed the simultaneous characterization of the primary structure of IFX with respect to the occurrence of PTMs. Thus, tryptic digestion of purified IFX was performed as the final sample preparation step prior to CE-MS/MS analysis. Different sample preparation methods were used for IFX-spiked serum to evaluate the impact of different approaches on MS signal intensity, signal-to-noise ratio, and extraction yield, in addition to the identification of PTMs and the estimation of modification levels. The goal was to determine the most appropriate conditions to maximize the efficiency of the process and achieve optimal sensitivity while maintaining the selectivity and efficiency of the electrophoretic separation. Concerning asparagine deamidation (*deaN*) and aspartate isomerization (*isoD*), separation is mandatory for adequate identification, as they will show isotopic overlap with their unmodified counterparts in MS. Therefore, the peptide resolution was evaluated for each protocol. For analyte pre-concentration in CE, the transient isotachopheresis mode was performed, using ammonium acetate 100 mM as the leading electrolyte and acetic acid as the background electrolyte (BGE) [31]. Under these

conditions, the migration time of the peptides depends on the ITP duration, which depends on the conductivity difference between the two electrolytes. Under the same CE conditions (applied voltage, leading electrolyte, injection volume), the resolution between identical peptides should be similar. Nevertheless, we evaluated whether the different sample preparations proposed here slightly modified the ionic strength or conductivity of the sample, thus affecting the resolution between PTMs.

To support the analysis of IFX in human serum by CE-MS/MS, different sample preparation methods, shown in Fig. 2, were investigated. In the workflow referred to as protocol A, the peculiarity was that the extracted IFX was not released from the magnetic beads, and therefore the proteolytic digestion was performed *in situ*, still immobilized on the affinity purification media. This approach had the advantage of reducing the number of sequential sample preparation steps and allowing the beads to be easily placed in a buffer compatible with the digestion. In the other two workflows studied, IFX was removed from the purification beads prior to digestion, either by acidic dissociation followed by buffer exchange by membrane filtration in the case of protocol B, or by organic solvent dissociation and solvent evaporation in the case of protocol C (Fig. 2). The release of extracted IFX from the beads would potentially facilitate trypsin access to the mAbs, which could be beneficial for maximizing digestion yield. However, for both protocols, the solutions used to dissociate the IFX-immobilized/TNF- α interaction had to be removed due to their incompatibility with trypsin digestion.

3.1. Direct digestion of mAbs immobilized on beads (Protocol A). The IFX-spiked serums were subjected to affinity purification using TNF- α -functionalized magnetic beads. Then the beads were transferred to ammonium bicarbonate for digestion (Fig. 2). The CE-MS/MS results demonstrated the successful detection and quantification of IFX in spiked serums for the different concentration levels considered. The process efficiency of this sample preparation procedure was determined at 62 ± 17 % (see Fig. 1).

CE-MS/MS data also allowed the estimation of a LOQ of 1.2 ± 0.6 $\mu\text{g}\cdot\text{mL}^{-1}$ and a LOD of 0.4 ± 0.2 $\mu\text{g}\cdot\text{mL}^{-1}$, using the thresholds of $S/N > 10$ and 3, respectively (Table 1). Therefore, the method should be acceptable for the quantification of IFX in most patient serum samples (typically between 0.5 and 25 $\mu\text{g}\cdot\text{mL}^{-1}$).

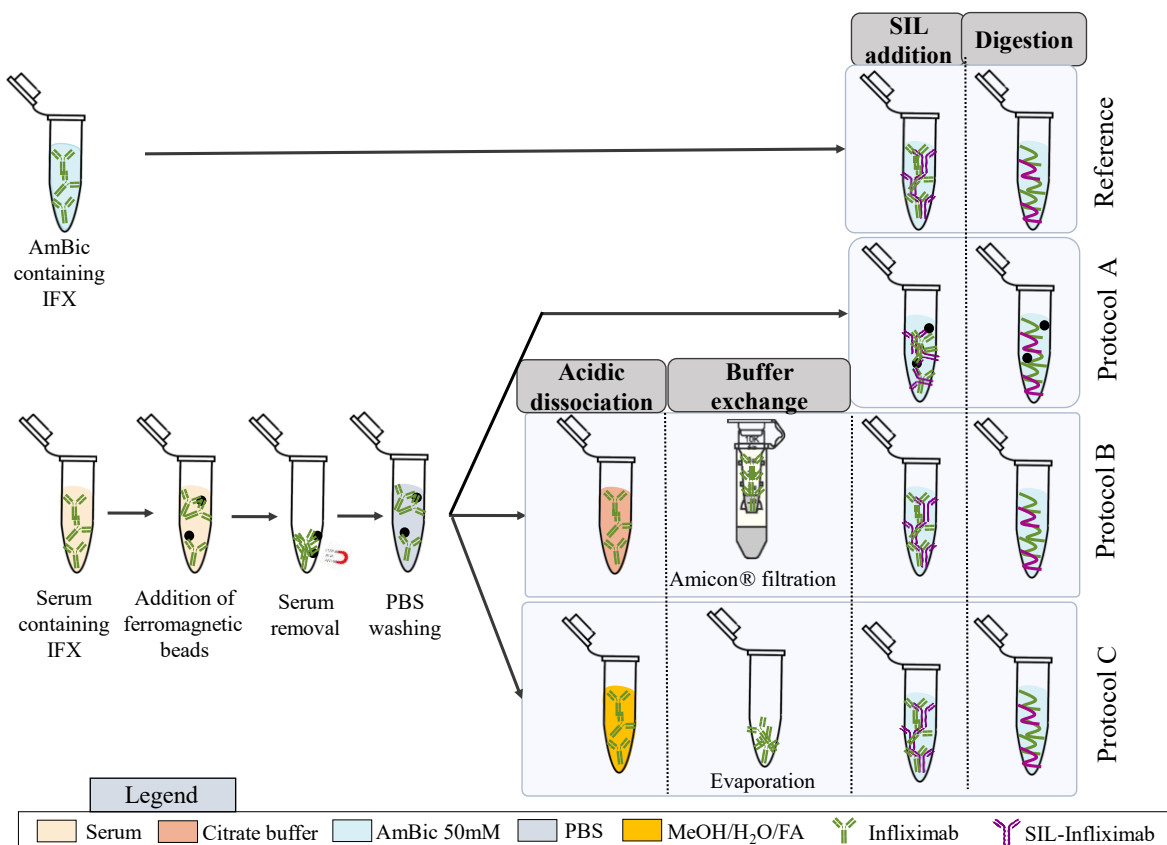


Fig. 2. Sample preparation workflows considered for IFX quantification and characterization in serum by CE-MS/MS analysis include direct digestion of bead-immobilized IFX (protocol A), acid dissociation followed by centrifugal membrane filtration (protocol B, see 2.4.1), or organic solvent dissociation and solvent evaporation (protocol C, see 2.4.2). The SIL-IFX internal standard was added prior to digestion for CE-MS/MS signal normalization. Reference samples consisted of H₂O solution spiked with IFX and SIL-IFX.

In addition, the recovery yield of bead-based IFX extraction from serum was evaluated by quantifying residual IFX using a commercial ELISA assay. Indeed, the residual IFX in the post-extraction “waste” was quantified after extraction in serum at different initial concentrations of IFX (Fig. S1). The ELISA assays showed almost complete extraction of IFX from serum at lower concentrations (>95 %). However, remaining IFX levels were significant for serums spiked at 25 µg·mL⁻¹, indicating a lower extraction yield at the highest level of concentration (80 ± 5 %). The decrease in IFX extraction yield was attributed to the capacity of the TNF-α immobilized on the ferromagnetic beads reaching its limit. Similar effects were observed for the other protocols because the affinity extraction conditions were the same (Fig. 2). This effect should not affect the performance of the absolute quantification because of the incorporation of the SIL-

IFX internal standard and because the IFX concentration allows a satisfactory MS signal sensitivity at higher concentrations.

Table 1 Comparison of the three strategies on their process efficiency, their repeatability, their detection, and quantification limits, and their PTM visibility for spiked serum with Infliximab at ($\mu\text{g}\cdot\text{mL}^{-1}$).

	Protocol A	Protocol B	Protocol C
Process efficiency	$62 \pm 17 \%$	$19 \pm 22 \%$	$58 \pm 24 \%$
LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	0.4 ± 0.2	0.8 ± 0.8	0.1 ± 0.02
LOQ ($\mu\text{g}\cdot\text{mL}^{-1}$)	1.2 ± 0.6	2.8 ± 2.5	0.2 ± 0.1

CE-MS/MS data were investigated to characterize PTM hotspots concomitantly with IFX quantification. Modified peptides usually tend to represent a small percentage compared to their unmodified counterpart; therefore, good sensitivity is required for an unambiguous estimation of the relative proportion of PTMs. As compiled in Table 2, the intensity of the MS signal corresponding to the intact peptide and its modified counterpart can be used to independently determine the level of modification for different PTMs. However, the analysis of IFX from protocol A resulted in a non-systematic characterization of the PTM level for each replicate considered (Table 2). Fig. 3 illustrates the electrophoretic separation observed for an IFX-specific peptide described to potentially exhibit an asparagine modification (*deaN57*). Indeed, the asparagine located on the amino acid sequence SINSATHYAESVK is normally deamidated at 10 % and is interesting to monitor because of its CDR position [32]. For the reference product corresponding to IFX submitted only to the digestion process (Fig. 3, a), the electropherograms showed the good separation of both peptide forms, highlighting the capacity of CZE conditions to achieve baseline separation due to the presence of the modification. In contrast, in serum samples containing IFX prepared using protocol A (Fig. 3, b), the peptides showed partial co-migration with other analytes presenting the same *m/z*. The presence of interfering compounds can induce ion competition in the electrospray ionization source, preventing the detection of lowly abundant ions. In addition, several PTMs were difficult to quantify after protocol A, due to a significant background signal. Protocol A involved the proteolytic digestion realized directly on the ferromagnetic beads (Fig. 2). Therefore, digests not only contained peptides originating from the mAb of interest, but also from TNF- α and streptavidin immobilized on the surface of the beads. As a result, the peptide mixture obtained after digestion contained additional peptides, which drastically increased the possibility of peptides interfering with the CZE separation and/or the MS analysis. Furthermore, CZE-

MS/MS electropherograms of samples obtained using protocol A showed 20 and 75-fold greater signal intensities for peptides corresponding to TNF- α and streptavidin, respectively, compared to the other protocols. Thus, this observation was also advocating for the existence of ion competition effect, which significantly hinders CE-MS/MS analysis due to the sample preparation protocol. Signal interferences also led to poor repeatability of the PTM level assessment. Nevertheless, it is important to note for peptides that CE-MS/MS analysis was not impacted by the presence of concomitant interferences, the modification levels obtained were similar to the reference samples, emphasizing that the extraction protocol does not induce artifactual PTMs.

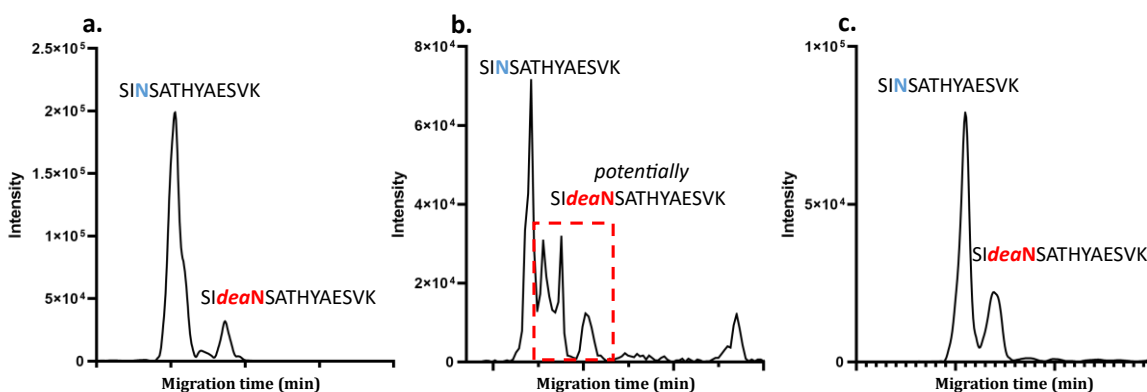


Fig. 3. Extracted Ion Electropherogram (EIE) of the m/z corresponding to peptide SINSATHYAESVK ($m/z = 703.85; 2+$) for (a) digestion of IFX marketed formulation without prior extraction (reference); (b) direct digestion of IFX immobilized on beads (protocol A) and (c) Extraction of IFX using $H_2O/MeOH$ dissociation and buffer exchange (protocol C) from spiked serum samples.

The resolution between the peptide forms for *deaN* and *isoD* was investigated as an indicator of separation efficiency (Table 2). Results showed that protocol A provided a resolution systematically superior to 1.1 for the considered PTMs. However, high values of standard deviations could be observed, which was explained by the poor repeatability of the transient isotachopheresis (t-ITP) preconcentration step performed at the beginning of the CZE-MS/MS analysis. The variability regarding t-ITP was the consequence of significant differences in ionic strength among the replicates which impacted the conductivity of the leading electrolyte (LE) composing the sample, resulting in a lower apparent mobility of the LE (Fig. S2). Thus, the application of protocol A may not completely remove ions from the treated serum or the washing buffer solution. The application of t-ITP generates a stacking effect of the sample content because of mobility differences between the BGE and the LE. For that reason, samples which contained a LE demonstrating a higher

apparent mobility provided a more efficient t-ITP pre-concentration. In addition, as mentioned previously, the digestion generated significantly more complex samples due to the presence of TNF- α and streptavidin in important quantities. That complexity influenced the ionic strength of the sample and contributed to the variability of t-ITP pre-concentration.

Table 2 Summary of PTMs characterization and modification level estimation achieved from CZE-MS/MS analysis for the different sample preparation protocols (n = 5 for each protocol) of IFX initially in serum matrix (at 25 $\mu\text{g}\cdot\text{mL}^{-1}$). CZE separation resolution was determined between modified and unmodified peptides (n.d. not distinguished).

		deaN ⁵⁷ (H)	deaN ³⁸⁷ (H)	deaN ¹³⁷ (L)	deaN ¹⁵⁸ (L)	oxiM ¹⁸ (H)	oxiM ²⁵⁵ (H)	oxiM ⁵⁵ (L)	isoD ⁴⁰² (H)	isoD ²⁸³ (H)
		HT-07	HT-38	LT-10	LT-12	HT-02	HT-22	LC-06	HC-39	HC-24
Reference	Modification level (%)	11 \pm 3 %	69 \pm 5 %	17 \pm 5 %	15 \pm 5 %	7 \pm 4 %	10 \pm 6 %	5 \pm 4 %	2 \pm 1 %	4 \pm 1 %
	Identification repeatability	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
	Separation resolution	1.8 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1	1.6 \pm 0.2	n.d.	n.d.	n.d.	1.2 \pm 0.3	1.4 \pm 0.2
Protocol A	Modification level (%)	21 \pm 14 %	62 \pm 8 %	13 \pm 4 %	15 \pm 4 %	12 \pm 3 %	n.d.	n.d.	3 \pm 4 %	3 \pm 1 %
	Identification repeatability	3/5	5/5	5/5	5/5	4/5	0/5	4/5	3/5	4/5
	Separation resolution	1.6 \pm 0.7	1.3 \pm 0.6	1.1 \pm 0.3	1.7 \pm 0.5	n.d.	n.d.	n.d.	1.1 \pm 0.3	1.0 \pm 0.3
Protocol B	Modification level (%)	15 \pm 8 %	71 \pm 16 %	26 \pm 12 %	20 \pm 3 %	10 \pm 4 %	9 \pm 4 %	9 \pm 5 %	5 \pm 5 %	4 \pm 2 %
	Identification repeatability	5/5	3/5	3/5	5/5	5/5	5/5	5/5	3/5	2/5
	Separation resolution	1.3 \pm 0.4	0.8 \pm 0.4	0.8 \pm 0.3	1.4 \pm 0.5	n.d.	n.d.	n.d.	0.9 \pm 0.2	1.4 \pm 0.4
Protocol C	Modification level (%)	8 \pm 1 %	56 \pm 2 %	14 \pm 2 %	11 \pm 2 %	4 \pm 2 %	8 \pm 4 %	7 \pm 3 %	2 \pm 1 %	3 \pm 2 %
	Identification repeatability	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	Separation resolution	1.5 \pm 0.4	1.1 \pm 0.1	1.0 \pm 0.1	1.4 \pm 0.3	n.d.	n.d.	n.d.	1.0 \pm 0.1	1.1 \pm 0.4

The development and implementation of sample preparation of protocol A allowed for the successful isolation and digestion of IFX from serum samples. Consequently, CE-MS/MS experiments demonstrated the possibility, using the sample preparation, of achieving IFX absolute quantification in this type of biological matrix over a concentration range corresponding to the levels commonly observed in treated patients. From an experimental point of view, performing proteolytic digestion directly on the ferromagnetic beads represented an interesting alternative to reduce the number of consecutive steps required to perform the protocol and limit analyte loss. However, on-beads digestion

proved to generate peptide mixtures composed of high concentrations of interferent compounds and unstable ionic strength, which dramatically hindered the characterization of PTMs.

3.2. Acidic buffer dissociation and buffer exchange filtration (protocol B). To improve the data obtained from CE-MS/MS analysis regarding the characterization and estimation of PTMs, sample preparation was modified in order to limit the presence of interferences and prevent excessive ion competition risks. An alternative consists of dissociating the interaction between the analyte of interest and the target immobilized on the surface of the ferromagnetic beads to prompt the release of analytes from the affinity purification medium. Dissociation is often performed under acidic conditions due to its efficiency in destabilizing protein–protein interactions [33]. Citrate dissociation (pH ~ 3–4) has been extensively used for mAb elution due to its efficiency [34,35]. Its low pH and ionic strength allow dissociation of the interaction of mAbs with their antigens but may alter the conformation of biomolecules. The optimal pH for such an operation is around 3, which is not suitable for tryptic digestion due to irreversible inhibition of the proteolytic enzyme. Therefore, a buffer exchange step is mandatory prior to sample digestion. The CE-MS/MS sample preparation process was modified to incorporate citrate dissociation, consequently to the affinity capture of IFX, followed by buffer exchange using ultra centrifugation filtration (protocol B, Fig. 2). Because citrate is lowly volatile, a filtration procedure was implemented for the buffer exchange step with ammonium bicarbonate. The optimization of the sample preparation was conducted by altering specific parameters to study primarily their effect on the recovery yield of intact IFX in model solutions.

Concerning centrifugation filtration, the filtration recovery yield was estimated using CE-MS/MS analysis of a water solution spiked with a fixed IFX concentration previously treated using different filter cutoffs (cf. 2.4.1.). Results showed that filtration recovery yield ranged from 0 % to 45 % depending on the type of filter used. Interestingly, 100 kDa cutoff filters did not provide good recovery of the mAb, as shown in Fig. 4. This observation could potentially be due to the denaturation of the structure of the protein under centrifugation. Therefore, the larger porosity of the filter, adapted to globular macromolecules, could not retain denatured mAbs. For subsequent experiments, a 10 kDa cutoff filter was selected as it provided optimal recovery yields. Additional centrifugation parameters were also investigated to improve recovery yield (Fig. S3). No significant differences could be observed for rotational speeds at 14,000 rcf or

20,000 rcf. Nonetheless, lower variability between replicates was observed at lower speed, which was more favorable. Two different temperatures were also used during the centrifugation; however, results did not demonstrate any beneficial effect when performing filtration at a lower temperature (Fig. S3).

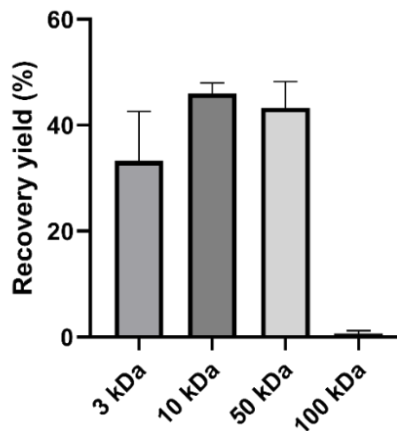


Fig. 4. Infliximab filtration recovery yield obtained using different filter size cutoff compatible with the treatment of mAbs (experiments performed in triplicates).

The recovery yield of the filtration was further compared for IFX initially spiked in citrate buffer solutions in order to simulate samples in similar conditions compared to actual samples obtained after serum affinity extraction. Results allowed for an important decrease in the filtration recovery yield for samples containing citrate buffer, as illustrated in Fig. 5A. That effect was attributed to the low pH of citrate buffer, which can alter the conformation of the mAb, potentially leading to protein denaturation and the formation of aggregates more prone to adsorption on the filter material [36]. Thus, citrate has been described as causing more aggregation than other acidic buffers [37]. Dynamic light scattering (DLS) was performed to study the denaturation and aggregate formation of IFX in the presence of citrate acidic buffer. The average hydrodynamic radius of IFX in water was found to be 9.4 ± 0.4 nm, whereas in citrate buffer, the value was increased to 11.4 ± 0.5 nm (Fig. S4). Therefore, the increase of the hydrodynamic radius is consistent with the formation of IFX aggregates. In complement, SEC-UV-MALS-RI analysis was performed for IFX previously diluted in water or citrate buffer. Chromatograms obtained for IFX solution in water exhibited a single peak presenting a retention time of 15.2 min and a molar mass of 150 kDa determined from MALS measurements, corresponding to IFX monomer (Fig. S5). However, no signal could be detected for IFX samples in citrate buffer, including

at the retention time corresponding to the exclusion volume of the stationary phase. The absence of a signal corresponding to IFX was potentially due to secondary and tertiary structure changes in the presence of citrate buffer, which made it more prone to adsorption to the SEC stationary phase. A similar adsorption phenomenon on the membrane material could be responsible for the lower recovery yield observed during the concentration step (Fig. 5A). In order to prevent the adsorption of IFX on the membrane, the filtration procedure was modified by the addition of Rapigest® surfactant to the sample and dilution in PBS (pH 6.8) prior to introduction into the centrifugation filter. The results presented in Fig. 5B showed that the addition of surfactant allowed for a significant increase in recovery yield. Therefore, the modification of the filtration procedure enabled the restoration of recovery yields comparable to the level achieved for IFX samples in water solution.

The sample preparation protocol with optimized conditions was applied for the treatment of serum samples spiked with IFX. CE-MS/MS analysis showed a global process efficiency of $19 \pm 22\%$ (Table 1). The affinity extraction step demonstrated a recovery yield ranging from 95 % at lower concentrations to 80 % for $25 \mu\text{g}\cdot\text{mL}^{-1}$ IFX spiked serum (Fig. S1). Whereas the filtration provided recovery yields typically around 25 % (Fig. 5B) showing the latter to be significantly responsible for the lower process efficiencies compared to the previous protocol. CE-MS/MS data demonstrated a calculated LOD and LOQ of 0.8 ± 0.8 and $2.8 \pm 2.5 \mu\text{g}\cdot\text{mL}^{-1}$ respectively, which might be compatible with the analysis of patient serums but revealed a significantly lower sensitivity compared to the previous workflow (Table 1). Regarding separation, t-ITP efficiency was comparable to the analysis of the IFX reference sample without extraction which was further explained by similar apparent mobilities of the LE for the two types of samples (Fig. S2). Therefore, acidic dissociation allowed to limit detrimental effects on the electrophoretic separation observed when digestion was performed directly on the beads. Consequently, this type of sample treatment provided the identification of the different PTMs hotspots of IFX, and the modification levels determined were in agreement with the levels characterized in the case of reference IFX samples (Table 2). However, it is important to note that a low recovery yield will significantly hamper the identification of PTMs presenting a faint level of modification. In addition, CE-MS/MS results obtained for samples prepared using this protocol demonstrated important variability both regarding IFX absolute quantification and the estimation of PTM modification levels. Consequently, sample treatment based on acidic

dissociation followed by buffer exchange using filtration showed the possibility of improving the compatibility with the electrophoretic separation in order to maintain optimal selectivity but presented a decreased robustness, which was not found to be favorable to the analysis of an extended number of samples originating from patients.

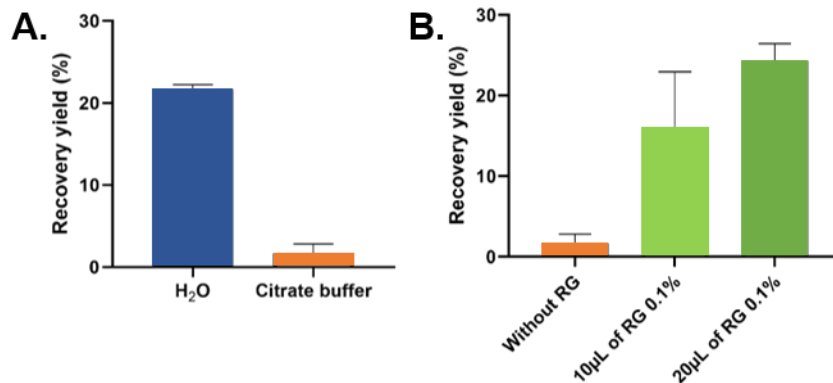


Fig. 5. Infliximab filtration recovery yield obtained using (A) H₂O or citrate buffer solutions and (B) citrate buffer containing different quantities of surfactant (SF Rapigest). Experiments were performed in triplicates.

3.3. Hydro-organic solution dissociation and evaporation (Protocol C). To address the limitations of acidic buffer dissociation previously observed, the sample preparation procedure was modified to incorporate dissociation using a hydro-organic solution followed by evaporation of the solvent before conventional trypsin digestion (Fig. 2, protocol C). The assessment of the protocol presented a process efficiency of 58 ± 16 %, similar to the performance achieved when digestion was performed directly on the beads. In addition, results further confirmed that centrifugal filtration was responsible for a large part of mAb loss during sample preparation, as described previously in the case of acidic dissociation. CE-MS/MS analysis realized for serum samples prepared using the modified protocol demonstrated an extremely sensitive detection of peptides specific to IFX used for the quantification, over the relevant concentration range. In addition, MS and MS/MS data exhibited minimal background noise due to the elimination of the dissociation solvent (Fig. 6).

LOD and LOQ of the strategy were evaluated to be 0.1 ± 0.02 and 0.2 ± 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$, systematically lower than the previous protocols and demonstrating a drastic improvement in terms of variability. Therefore, the workflow developed would be particularly appropriate for the absolute quantification of mAbs in human serum over a concentration range

corresponding to the therapeutic window of IFX and competitive with commercial ELISA quantitative assays. Regarding the electrophoretic separation, t-ITP demonstrated a significant increase of the apparent mobility of the LE, even compared to the analysis of the IFX reference sample, which indicated an optimal stacking effect and controlled complexity of the sample due to the evaporation of the dissociation solution. CE-MS/MS data recorded enabled the characterization of all monitored PTMs hotspots, in a systematic manner. Moreover, the level of each modification estimated from serum samples spiked with IFX was similar to the reference product. Therefore, it showed that the affinity extraction procedure developed did not affect the level of modification characterized (Table 2). The characterization of PTMs also emphasized the conservation of optimal CZE separation selectivity. Indeed, electropherograms showed separation of modified and unmodified peptides with performances similar to the analysis of the reference sample (Fig. 3, c), without the presence of interfering species. The results highlighted that the resolution of *deaN* and *isoD* from the unmodified counterpart is always greater than 1, which is sufficient to correctly identify the PTMs.

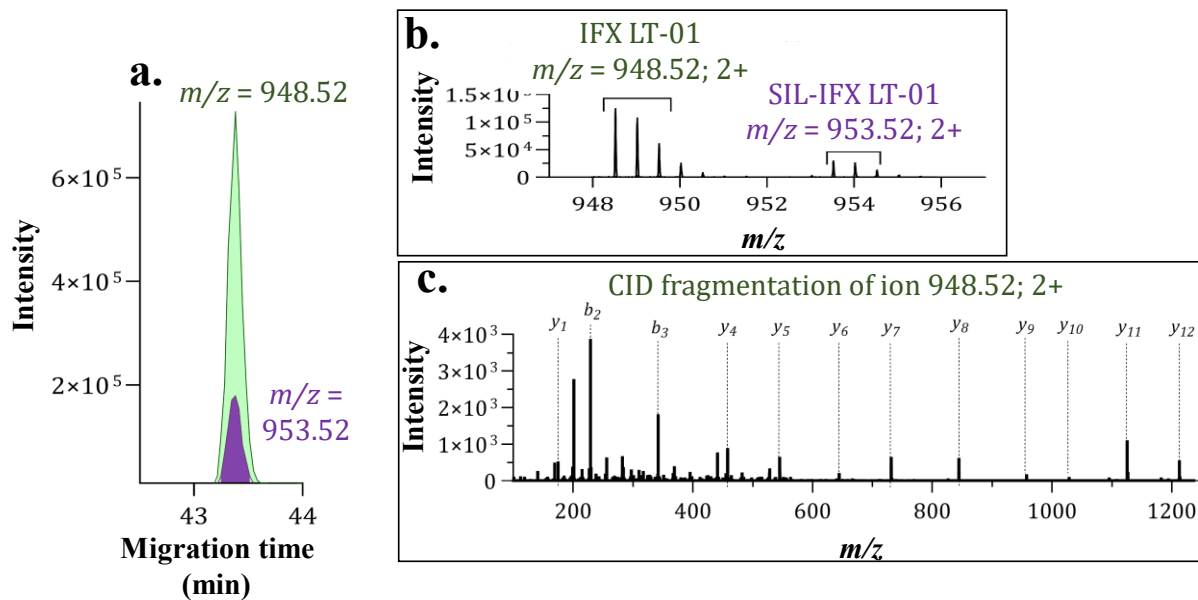


Fig. 6. Infiximab (IFX) and SIL-IFX peptide LT-01 (a) extracted ion electropherogram, (b) MS spectra ions, (c) MS/MS spectra of LT-01 ion.

4. CONCLUSION

The follow-up of patients treated using mAbs has recently appeared particularly decisive in order to adjust dosing, for instance, and more generally to understand their outcome in the serum after administration or the occurrence of an

unexpected response. Sample preparation therefore becomes key to successfully achieving the identification, characterization, and quantification of a single protein present in a complex biological matrix. Often, compromises must be maintained between practicality, selectivity of the workflow, optimal sensitivity, and compatibility with the subsequent analysis. CE separation can be affected due to the composition of the sample, which has limited its use for the analysis of biological samples such as serum.

Thus, a sample preparation workflow tailored for CZE-MS/MS analysis was developed in order to perform the affinity extraction, purification, and digestion of mAbs in serum samples corresponding to conditions observed in patients treated with that type of biopharmaceutical product. Based primarily on affinity extraction using ferromagnetic beads, different approaches were investigated in a systematic manner regarding the different steps concerning IFX dissociation and proteolytic digestion. Each preparation was assessed from the perspective of extraction efficiency, recovery yield, and CZE-MS/MS analysis performances. The different sample treatment procedures allowed us to envisage the absolute quantification of IFX in serum samples for a concentration range compatible with the analysis of patient samples and the concomitant characterization of PTMs of interest. However, significant alteration of the CZE separation could be identified when proteolytic digestion was performed directly on the beads due to the presence of a large number of interferent species, especially TNF- α and streptavidin peptides, in high concentration compared to the mAbs. As an alternative, acidic dissociation was performed, followed by buffer exchange using centrifugation filtration, in order to be in conditions compatible with the proteolytic digestion. Results demonstrated a significant loss of extracted mAbs due to denaturation of the protein in acidic citrate buffer and adsorption on filtration membrane material. The adsorption effect resulted in a dramatic reduction of signal sensitivity and an important variability in CZE-MS/MS analysis. Finally, dissociation using a hydro-organic solution followed by evaporation was implemented. Results demonstrated a maximized process efficiency and an efficient removal of the dissociation solution that allowed for the isolation of the mAbs while preventing the addition of interferent analytes such as peptides or buffer ions. Using this protocol, CZE-MS/MS analysis showed the possibility of achieving the quantification of IFX from 0.2 to 25 $\mu\text{g}\cdot\text{mL}^{-1}$. Finally, CZE separation selectivity could be maintained, which provided the separation of peptides presenting PTMs from their unmodified counterparts in the case of faint modifications such as *deaN* and *isoD*. The systematic

study of the different sample preparation workflows allowed us to further understand the critical aspects of protein sample preparation for CZE-MS/MS analysis and identify eventual bottlenecks regarding preparation efficiency and compatibility.

The sample preparation developed demonstrated the possibility to perform mAbs analysis in biological samples using CZE-MS/MS. As a consequence, the sample preparation developed provides the opportunity to open a novel field of applications for the analysis of proteins in heavily complex biological samples.

5. ACKNOWLEDGMENTS

The authors would like to thank every person that contributed to the achievement of this article. This research work was supported by the Agence National de la Recherche as part of the project MethAmAbs (project no. ANR-19-CE29-0009).

6. REFERENCES

- [1] A. Beck, T. Wurch, C. Bailly, N. Corvaia, Strategies and challenges for the next generation of therapeutic antibodies, *Nat. Rev. Immunol.* 10 (2010) 345–352, <https://doi.org/10.1038/nri2747>.
- [2] J.M. Reichert, Marketed therapeutic antibodies compendium, *mAbs.* 4 (2012) 413–415, <https://doi.org/10.4161/mabs.19931>.
- [3] H. Kaplon, J.M. Reichert, Antibodies to Watch in 2021, *mAbs.* 13 (2021) 1860476, <https://doi.org/10.1080/19420862.2020.1860476>.
- [4] D.M. Weinreich, S. Sivapalasingam, T. Norton, S. Ali, H. Gao, R. Bhore, B.J. Musser, Y. Soo, D. Rofail, J. Im, C. Perry, C. Pan, R. Hosain, A. Mahmood, J.D. Davis, K. C. Turner, A.T. Hooper, J.D. Hamilton, A. Baum, C.A. Kyratsous, Y. Kim, A. Cook, W. Kampman, A. Kohli, Y. Sachdeva, X. Graber, B. Kowal, T. DiCioccio, N. Stahl, L. Lipsich, N. Braunstein, G. Herman, G.D. Yancopoulos, REGN-COV2, a neutralizing antibody cocktail, in outpatients with covid-19, *N. Engl. J. Med.* 384 (2021) 238–251, <https://doi.org/10.1056/NEJMoa2035002>.
- [5] H. Kaplon, S. Crescioli, A. Chenoweth, J. Visweswaraiyah, J.M. Reichert, Antibodies to Watch in 2023, *mAbs.* 15 (2023) 2153410, <https://doi.org/10.1080/19420862.2022.2153410>.
- [6] X. Peng, B. Liu, Y. Li, H. Wang, X. Chen, H. Guo, Q. Guo, J. Xu, H. Wang, D. Zhang, J. Dai, S. Hou, Y. Guo, Development and validation of LC–MS/MS method for the quantitation of infliximab in human serum, *Chromatographia* 78 (2015) 521–531, <https://doi.org/10.1007/s10337-015-2866-2>.
- [7] M. El Amrani, A.A.M. Donners, C.E. Hack, A.D.R. Huitema, E.M. van Maarseveen, Six-step workflow for the quantification of therapeutic monoclonal antibodies in biological matrices with liquid chromatography mass spectrometry – a tutorial, *Anal. Chim. Acta* 1080 (2019) 22–34, <https://doi.org/10.1016/j.aca.2019.05.076>.
- [8] K. Faserl, B. Sarg, P. Gruber, H.H. Lindner, Investigating capillary electrophoresis-mass spectrometry for the analysis of common post-translational modifications, *Electrophoresis* 39 (2018) 1208–1215, <https://doi.org/10.1002/elps.201700437>.
- [9] P. Jiang, F. Li, J. Ding, Development of an efficient LC-MS peptide mapping method using accelerated sample preparation for monoclonal antibodies, *J. Chromatogr. B* 1137 (2020) 121895, <https://doi.org/10.1016/j.jchromb.2019.121895>.
- [10] E.S. Witze, W.M. Old, K.A. Resing, N.G. Ahn, Mapping protein post-translational modifications with mass spectrometry, *Nat. Methods* 4 (2007) 798–806, <https://doi.org/10.1038/nmeth1100>.
- [11] R. Pieper, C.L. Gatlin, A.J. Makusky, P.S. Russo, C.R. Schatz, S.S. Miller, Q. Su, A. M. McGrath, M.A. Estock, P.P. Parmar, M. Zhao, S.-T. Huang, J. Zhou, F. Wang, R. Esquer-Blasco, N.L. Anderson, J. Taylor, S. Steiner, The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins, *Proteomics* 3 (2003) 1345–1364, <https://doi.org/10.1002/pmic.200300449>.

- [12] C. Hagman, D. Ricke, S. Ewert, S. Bek, R. Falchetto, F. Bitsch, Absolute quantification of monoclonal antibodies in biofluids by liquid chromatography-tandem mass spectrometry, *Anal. Chem.* 80 (2008) 1290–1296, <https://doi.org/10.1021/ac702115b>.
- [13] K. Huse, H.-J. Böhme, G.H. Scholz, Purification of antibodies by affinity chromatography, *J. Biochem. Bioph. Methods* 51 (2002) 217–231, [https://doi.org/10.1016/S0165-022X\(02\)00017-9](https://doi.org/10.1016/S0165-022X(02)00017-9).
- [14] M. El Amrani, M.P.H. van den Broek, C. Göbel, E.M. van Maarseveen, Quantification of active infliximab in human serum with liquid chromatography–tandem mass spectrometry using a tumor necrosis factor alpha -based pre-analytical sample purification and a stable isotopic labeled infliximab bio-similar as internal standard: a target-based, sensitive and cost-effective method, *J. Chromatogr. A* 1454 (2016) 42–48, <https://doi.org/10.1016/j.chroma.2016.05.070>.
- [15] A.J. Kleinnijenhuis, M. Ingola, J.H. Toersche, F.L. van Holthoon, W.D. van Dongen, Quantitative bottom up analysis of infliximab in serum using protein A purification and integrated μ LC-electrospray chip IonKey MS/MS technology, *Bioanalysis* 8 (2016) 891–904, <https://doi.org/10.4155/bio-2015-0015>.
- [16] R. Gahoual, J.-M. Busnel, A. Beck, Y.-N. François, E. Leize-Wagner, Full antibody primary structure and microvariant characterization in a single injection using transient isotachopheresis and sheathless capillary electrophoresis-tandem mass spectrometry, *Anal. Chem.* 86 (2014) 9074–9081, <https://doi.org/10.1021/ac502378e>.
- [17] O.O. Dada, Y. Zhao, N. Jaya, O. Salas-Solano, High-resolution capillary zone electrophoresis with mass spectrometry peptide mapping of therapeutic proteins: peptide recovery and post-translational modification analysis in monoclonal antibodies and antibody-drug conjugates, *Anal. Chem.* 89 (2017) 11236–11242, <https://doi.org/10.1021/acs.analchem.7b03643>.
- [18] R. Gahoual, A. Beck, Y.-N. François, E. Leize-Wagner, Independent highly sensitive characterization of asparagine deamidation and aspartic acid isomerization by sheathless CZE-ESI-MS/MS: characterization of Asn and Asp PTMs by CZE-ESI-MS, *J. Mass Spectrom.* 51 (2016) 150–158, <https://doi.org/10.1002/jms.3735>.
- [19] A. Hirayama, H. Abe, N. Yamaguchi, S. Tabata, M. Tomita, T. Soga, Development of a sheathless CE-ESI-MS interface, *Electrophoresis* 39 (2018) 1382–1389, <https://doi.org/10.1002/elps.201800017>.
- [20] M.-J. Gou, G. Nys, G. Cobraiville, A. Demellenne, A.-C. Servais, M. Fillet, Hyphenation of capillary zone electrophoresis with mass spectrometry for proteomic analysis: optimization and comparison of two coupling interfaces, *J. Chromatogr. A* 1618 (2020) 460873, <https://doi.org/10.1016/j.chroma.2020.460873>.
- [21] E. Naghdi, G.E. Moran, M.E. Reinau, W. De Malsche, C. Neusüß, Concepts and recent advances in microchip electrophoresis coupled to mass spectrometry: technologies and applications, *Electrophoresis* 44 (2023) 246–267, <https://doi.org/10.1002/elps.202200179>.
- [22] L.A. Gennaro, O. Salas-Solano, S. Ma, Capillary electrophoresis–mass spectrometry as a characterization tool for therapeutic proteins, *Anal. Biochem.* 355 (2006) 249–258, <https://doi.org/10.1016/j.ab.2006.04.002>.
- [23] R. Gahoual, J.-M. Busnel, P. Wolff, Y.N. François, E. Leize-Wagner, Novel sheathless CE-MS interface as an original and powerful infusion platform for nanoESI study: from intact proteins to high molecular mass noncovalent complexes, *Anal. Bioanal. Chem.* 406 (2014) 1029–1038, <https://doi.org/10.1007/s00216-013-7226-1>.
- [24] T. Reinert, A. Beck, P. Houzé, R. Gahoual, Y. Francois, CE–MS Methods for the Characterization of Monoclonal Antibodies, in: *Capillary electrophoresis-Mass Spectrometry for proteomics and Metabolomics*. Wiley-Ramautar, R. & Chen, D. D. Y., 2022, 281–312. <https://doi.org/10.1002/9783527833092.ch11>.
- [25] C. Batot, Evaluation de l'efficacité structurale d'un anticorps monoclonal anti-tnf-alpha, l'infliximab, dans la polyarthrite rhumatoïde, (2004) 140, Thèse en médecine. Université de Nancy.
- [26] D.H. Present, L. Mayer, T. Braakman, Infliximab for the treatment of fistulas in patients with Crohn's disease, *N. Engl. J. Med.* (1999) 8, <https://doi.org/10.1056/NEJM199905063401804>.
- [27] B. Bobaly, V. D'Atri, A. Goyon, O. Colas, A. Beck, S. Fekete, D. Guillaume, Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical sample preparation, *J. Chromatogr. B* 1060 (2017) 325–335, <https://doi.org/10.1016/j.jchromb.2017.06.036>.
- [28] Y.N. François, M. Biacchi, R. Gahoual, A. Vezin, J. Pansanel, CEToolbox: specialized calculator for capillary electrophoresis users as an android application, *Electrophoresis* 42 (2021) 1431–1435, <https://doi.org/10.1002/elps.202100036>.
- [29] L.K. Pino, B.C. Searle, J.G. Bollinger, B. Nunn, B. MacLean, M.J. MacCoss, The Skyline ecosystem: informatics for quantitative mass spectrometry proteomics, *Mass Spec. Rev.* 39 (2020) 229–244, <https://doi.org/10.1002/mas.21540>.
- [30] A. Gonzalez-Quintela, R. Alende, F. Gude, J. Campos, J. Rey, L.M. Mejjide, C. Fernandez-Merino, C. Vidal, Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities: serum immunoglobulin levels in adults, *Clin. Exp. Immunol.* 151 (2008) 42–50, <https://doi.org/10.1111/j.1365-2249.2007.03545.x>.
- [31] R. Gahoual, M. Biacchi, J. Chicher, L. Kuhn, P. Hammann, A. Beck, E. Leize-Wagner, Y.N. François, Monoclonal antibodies bio-similarity assessment using transient isotachopheresis capillary zone electrophoresis-tandem mass spectrometry, *mAbs.* 6 (2014) 1464–1473, <https://doi.org/10.4161/mabs.36305>.
- [32] S. Liang, J. Dai, S. Hou, L. Su, D. Zhang, H. Guo, S. Hu, H. Wang, Z. Rao, Y. Guo, Z. Lou, Structural basis for treating tumor necrosis factor α (TNF α)-associated diseases with the therapeutic antibody infliximab, *J. Biol. Chem.* 288 (2013) 13799–13807, <https://doi.org/10.1074/jbc.M112.433961>.
- [33] A. Patton, M.C. Mullenix, S.J. Swanson, E. Koren, An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen, *J. Immunol. Methods* 304 (2005) 189–195, <https://doi.org/10.1016/j.jim.2005.06.014>.

- [34] M.A. Burich, J.P. AuBuchon, H.J. Anderson, Evaluation of rapid antibody dissociation techniques, *Immunohematology* 2 (1986) 76–80. PMID: 15945870.
- [35] D. Shukla, L. Zamolo, C. Cavallotti, B.L. Trout, Understanding the role of arginine as an eluent in affinity chromatography via molecular computations, *J. Phys. Chem. B* 115 (2011) 2645–2654, <https://doi.org/10.1021/jp111156z>.
- [36] C.J. Roberts, Therapeutic protein aggregation: mechanisms, design, and control, *Trends Biotechnol.* 7 (32) (2014) 372–380, <https://doi.org/10.1016/j.tibtech.2014.05.005>.
- [37] V. Joshi, T. Shivach, V. Kumar, N. Yadav, A. Rathore, Avoiding antibody aggregation during processing: establishing hold times, *Biotechnol. J.* 9 (2014) 1195–1205, <https://doi.org/10.1002/biot.201400052>.