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RESEARCH PAPER

Structural identification and absolute quantification of monoclonal antibodies in suspected counterfeits using capillary electrophoresis and liquid chromatography-tandem mass spectrometry

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Abstract

Monoclonal antibodies (mAbs) represent a major category of biopharmaceutical products which due to their success as therapeutics have recently experienced the emergence of mAbs originating from different types of trafficking. We report the development of an analytical strategy which enables the structural identification of mAbs in addition to comprehensive characterization and quantification in samples in potentially counterfeit samples. The strategy is based on the concomitant use of capillary zone electrophoresis analysis (CZE-UV), size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) and liquid chromatography hyphenated to tandem mass spectrometry (LC–MS/MS). This analytical strategy was applied to the investigation of different samples having unknown origins seized by the authorities, and potentially incorporating an IgG 4 or an IgG 1. The results achieved from the different techniques demonstrated to provide orthogonal and complementary information regarding the nature and the structure of the different mAbs. Therefore, they allowed to conclude unequivocally on the identification of the mAbs in the potentially counterfeit samples. Finally, a LC–MS/MS quantification method was developed which specificity was to incorporate a different mAbs labeled with stable isotopes as internal standard. The LC–MS/MS quantification method was validated and thus demonstrated the possibility to use common peptides with the considered IgG in order to achieve limit of quantification as low as 41.4 nM. The quantification method was used to estimate the concentration in the investigated samples using a single type of internal standard and experimental conditions, even in the case of mAbs with no stable isotope labeled homologues available.

Keywords Monoclonal antibodies · Mass spectrometry · Capillary electrophoresis · Size exclusion chromatography · Absolute quantification · Multi-angle light scattering

Introduction

Monoclonal antibodies (mAbs) and their related formats are meeting an undeniable success as therapeutic agents in different fields like oncology, immune disorder, and

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more recently the treatment of SARS-CoV-2 virus [1]. The interest of mAbs used as therapeutic treatment can be explained by their excellent specificity for the targeted antigen in addition to particularly favorable pharmacokinetic and pharmacodynamic properties. As a consequence, mAbs currently represent a category of therapeutic agents experiencing one of the most rapid growth in terms of market share and revenues [2]. At present, more than 100 mAbs are approved for therapeutic use, with the approval of 10 novel mAbs in 2020 which shows the infatuation for this type of protein [3]. Different types of biopharmaceutical agents are derived from mAbs like antibody fragments (fusion proteins), bispecific antibodies [4], glycoengineered antibodies, or antibody–drug conjugates (ADC) [5]. On the sidelines, the irrepressible expansion of therapeutic mAbs and their significant market value have quite recently led to the emergence of trafficking activities regarding this type of molecule, likewise already observed in the case of some pharmaceutical products [6, 7]. The development of fraudulent activities regarding such regulated therapeutic agent as mAbs therefore urges for the implementation of analytical strategies which allow to perform a comprehensive characterization of suspicious samples [8]. Thus, it is crucial to provide a degree of characterization which allows to prove unequivocally the presence and the nature of mAbs in the sample content. Also, it should provide a comprehensive comparison of the structure of the mAbs contained in the investigated samples compared to the original product, in addition to enable an accurate quantification of the sample content.

mAbs represent tetrameric glycoproteins potentially exhibiting a wide variety of post-translational modifications (PTMs) [9]. To address the complexity of mAbs due to their inherent structure and the occurrence of PTMs, extensive research activities have been conducted in order to develop analytical methodologies able to provide a detailed characterization [10]. Therefore, mAbs characterization requires a panel of orthogonal analytical methods in order to cover the different aspects and levels defining the structure of the protein. They include reversed-phase liquid chromatography (RP-LC) [11, 12], hydrophilic liquid chromatography (HILIC) [13], ion-exchange chromatography (IEC) [14], and size exclusion chromatography (SEC) [15]. Capillary electrophoresis (CE) has also demonstrated to be particularly relevant for the characterization of mAbs [16, 17]. Especially, the implementation of capillary zone electrophoresis (CZE) [18], capillary isoelectric chromatofocusing (cIEF) [19], or micellar electrokinetic chromatography (MEKC) appeared relevant for mAbs characterization [20]. Indeed, the electrokinetically driven separation of CE showed to be suitable for the analysis of polar and charged macromolecules like mAbs in addition to provide relevant specificities [21]. Mass spectrometry (MS) has also quickly gained a key role in the characterization of therapeutic proteins [22, 23]. This is explained by the outstanding specificity and sensitivity provided by current MS instrumentation and the possibility to obtain structural information using dedicated methodologies. MS can be used for the characterization of peptides as well as intact proteins maintained in pseudo-native conditions [24], which shows the possibility to use MS on the different levels defining the structure of mAbs.

In the present work, we developed an analytical strategy in order to investigate the presence of two different types of mAbs in samples suspected to be originating from biopharmaceutical product trafficking. The samples considered were previously seized by the authorities. In order to prove unambiguously the presence of the expected mAbs, the analytical strategy relied on the concomitant application of capillary zone electrophoresis (CZE-UV), size exclusion chromatography hyphenated to multi-angle light scattering (SEC-MALS), and reversed-phase liquid chromatography coupled to tandem MS (LC–MS/MS). The data obtained from the different analyses were crossed and compared to their respective original product in order to obtain concordant information regarding the identification and the structure of the mAbs potentially present in the sample. Finally, a novel LC–MS/MS method was developed in order to enable the absolute quantification of the different mAbs. The characteristic of the methods was the implementation of a stable-isotope labeled infliximab used as a surrogate internal standard. This approach allowed to use a common internal standard for the quantification of two different types of mAbs which suitability was demonstrated by the intermediate of method statistical validation.

Experimental section

Chemicals used were systematically of analytical grade or high purity grade. Ultra-pure water used to prepare buffers and sample solutions was obtained using a Milli-Q reference A + water purification system purchased from Merck Millipore (Billerica, MA). LC–MS grade H_2O and acetonitrile (ACN) used for UPLC-MS/MS experiments were purchased from VWR chemicals (Fontenay-sous-Bois, France). Hydropropyl methylcellulose (HPMC), ε-aminocaproic acid, triethylenetetramine (TETA), dithiotreitol (DTT), and iodoacetamide (IAM) were purchased from Sigma-Aldrich (Breda, The Netherlands). Trypsin enzyme was purchased from Promega (Madison, WI). The original products corresponding to the EMA/FDA-approved samples of mAb1 (mAb1 reference) and mAb2 (mAb2 reference) were provided by their respective manufacturer.

Capillary electrophoresis

The CZE-UV experiments were performed using a PACE/MDQ capillary electrophoresis system (Sciex separations, Darmstadt, Germany) equipped with a UV detector, a temperature-regulating system, and a power supply able to deliver up to 30 kV. Separations were performed using bare fused silica capillary (total length 45 cm, effective length 35 cm) coated with polyimide (50-µm i.d., 375-µm o.d.) purchased from Polymicro Technologies (Phoenix, AZ, USA). Capillary total volume, effective volume, and sample injection volume were calculated using CEToolbox software (available through Google Play Store) [25]. The background electrolyte (BGE) was composed of 600 mM ε-amino caproic acid with 2 mM triethylenetetramine (TETA) and 1% (v/v) hydroxymethyl propyl cellulose (pH 5.6). New capillaries were flushed for 10 min at 50 psi (5.17 bar) with 0.1 M hydrochloric acid followed with flushing with BGE at 50 psi (5.17 bar) for 10 min. Afterward, an electric field of + 20 kV was applied for 10 min and the capillary was finally flushed for 5 min at 50 psi with the BGE. Prior to each analysis, the capillary was rinsed for 3 min with the BGE. Hydrodynamic injection (37.5 mbar for 10 s) corresponding to a total volume of 15 nL of sample injected was used. Separations were performed using a voltage of + 20 kV using normal polarity. The analysis time was 20 min with UV absorbance detection at a wavelength of 214 nm. After each analysis, the capillary was rinsed with the BGE for 3 min using a pressure of 50 psi followed by 3 min using 0.1 HCl. At the end of each day and for long-time storage, the capillary was flushed for 10 min with phosphate buffer 10 mM (pH 6.9) and the extremities of the capillary were left immerged in Milli-Q H_2O.

Size exclusion chromatography-multi-angle light scattering

Intact mAbs samples were characterized by size exclusion liquid chromatography (SEC) using a Biozen SEC column (300 × 4.6 mm, 1.8 µm) purchased from Phenomenex (Le Pecq, France). Experiments were performed using a Prominence HPLC system (Shimadzu; SIL-20A autosampler; 2 × LC-20AD solvent delivery 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) instrument acquired from Wyatt Technology (Santa Barbara, USA). The MALS instrument was equipped with a 658-nm laser and light scattering measurements were performed simultaneously at 49°, 90°, and 131°. The MALS was also equipped with a COMET ultrasonic actuator (Wyatt Technology) which enables sonication of the MALS flow cell between each analysis in order to prevent any deposit formation. The mobile phase used for SEC-UV/RI-MALS analysis was composed of 50 mM phosphate buffer (pH 6.8) and 300 mM NaCl using a flowrate of 200 µL/min. An injection volume of 20 µL was used, the UV absorbance detection was performed at a wavelength of 280 nm, and the analysis time was 35 min. Data were collected and processed using the ASTRA® software V7.2 (Wyatt Technology).

Sample preparation and tryptic digestion of monoclonal antibodies for MS analysis

The sample preparation protocol used was derived from anterior experiments performed in our research group [23]. The investigated samples used for the characterization of the primary structure were diluted to a concentration of 1 mg/mL whereas the samples used for mAbs quantification were at a concentration of 50 µg/mL. The different types of samples were treated in the same manner. A volume of 10 µL of 0.1% RapiGest surfactant (Waters, Manchester, UK) was added to the samples followed by incubation at 40 °C for 10 min. A solution of DTT was added to the mixture to a final concentration of 10 mM and the samples were incubated at 80 °C for 20 min. The samples were then cooled to room temperature (RT) and IAM was added to a final concentration of 10 mM. Afterward, the samples were incubated in the dark at RT for a duration of 20 min in order to allow alkylation of the previously reduced thiol residues. A volume of 1 µL of trypsin (0.5 µg/µL) was added to the mixtures which were incubated at RT for 3 h. Another volume of 1 µL was added then and proteolytic digestion was performed overnight at 37 °C. Following digestion, a volume of 1 µL of formic acid (FA) 98% was added and the samples were left at room temperature for 1 h. Digested samples were stored at 5 °C prior to LC–MS/MS analysis.

Peptide digest analysis using liquid chromatography-tandem mass spectrometry

The LC–MS/MS analysis conditions were derived from a method developed previously in our research group [23]. The analysis of the peptide mixtures generated from proteolytic digestion was performed using an ACQUITY ultra-high-performance liquid chromatography (UPLC) system (Waters, Manchester, UK) equipped with a C18 stationary phase (BEH C18 300 Å, 1.7 µm, 2.1 × 150 mm) purchased from Waters (St Quentin-en-Yvelines, France) directly coupled with a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). LTQ Orbitrap XL MS was equipped with heated electrospray ionization source (HESI-II) from Thermo Scientific (Bremen, Germany). The mobile phases were composed of 0.1% FA in water (mobile phase A) and 0.1% FA in ACN (mobile phase B). The volume of sample injected was systematically 10 µL. Peptide separation was carried out using a gradient from 5 to 60% B in

38 min and maintained at 60% B for 3 min, at a flowrate of 100 µL/min. ESI source parameters were set as follows: ESI voltage − 4.0 kV, normalized sheath gas flowrate value was set 40, and an auxiliary gas flowrate value of 12. ESI nebulizer temperature was set to 300 °C. Capillary voltage and tube lens were set to 35 V and 90 V, respectively. MS/MS experiments were performed in a data-dependent acquisition (DDA) composed of one full MS scan over the mass/charge (*m*/*z*) range 150–2000 followed by five sequential MS/MS realized on the five most intense ions detected at a minimum threshold of 500 counts. Full MS scans were collected in profile mode using the high-resolution FTMS analyzer ($R = 60{,}000$). For mAbs primary structure characterization, a full scan AGC target of 1E6 and microscans = 1 was selected. In the case of LC–MS/MS quantification experiments, full scan AGC target was set to 3E6 and a maximum accumulation time of 500 ms; otherwise, other MS parameters were equivalent. The linear ion trap mass analyzer was used to analyze MS/MS fragments, in centroid mode at normal scan rate. MSn AGC target was set to 1E4 with three microscans. Ions were selected for MS/MS using an isolation window of 2 Th, then activated by collision-induced dissociation (CID) using normalized CID energy of 35, an activation Q of 0.25, and an activation time of 30 ms. Parent ions were excluded from MS/MS experiments for 60 s in case ion triggered an event twice in 15 s using an exclusion mass width of ± 1.5 Th. The instruments were controlled using Xcalibur 2.1.0 SP1 Build 1160 (Thermo Scientific, Bremen, Germany).

MS/MS data analysis

For the characterization of the amino acid sequence of mAbs, data treatment was performed similarly to [23] and MS/MS data obtained from LC–MS/MS experiments were analyzed using Xcalibur Qual Browser 2.2 SP1.48 (Thermo Scientific, Bremen, Germany). Purely proteolytic peptides (no miscleavages or PTMs except from cysteine carbomidomethylation) were determined in silico considering mAb1 and mAb2 amino acid sequences following the digestion specificities of trypsin [26] and carbomidomethylation of cysteine (+ 57.0215 Th) considered as a systematic modification. Peptide identifications were achieved manually from the conjunction of intact peptide mass measurements in full MS and MS/MS peptide fragments attribution using a mass tolerance inferior to 5 ppm in MS and 0.05 Th in MS/MS.

Data analysis of mAbs absolute quantification experiments was realized using Skyline software developed in the University of Washington (Seattle, WA, USA) [27]. For the quantification of mAb1, the peptides 1LT-07 (sequence DSTYSLSSTLTLSK; charge states 1 +, 2 +, 3 +) and 1HT-02 (sequence DTLMISR; charge states 1 +, 2 +) were considered. In the case of mAb2, the peptides selected were peptide 2HT-01 (sequence GPSVFPLAPSSK; charge state 2 +) and peptide 2HT-21 (sequence TTPPVLDSDGSFFLYSK; 1 +, 2 +, 3 +). For each LC–MS/MS quantification experiment, the peak area was measured for each charge state using systematically the first three isotopes of the peptide. If applicable, the sum of the peak areas corresponding to the different charge states detected was taken into account for the quantification.

LC–MS/MS quantification method validation

The reference products corresponding to mAb1 and mAb2 were used in order to prepare the calibration samples and quality controls. The method was validated with regard to its specificity, linearity, repeatability, reproducibility, and accuracy. The sensitivity of the method was evaluated by the measurement of the limits of detection (LOD) and quantification (LOQ) for each mAb, using the Eqs. 3.3 SD/slope and 10 SD/slope deduced from the equation of the calibration curves. The specificity of the method is assessed by the targeted and specific selected peptides (c.f. "7" section). Linearity was evaluated from 20 to 100 µg/mL using a calibration curve prepared as follows: first, a stock solution was prepared by dilution of the reference product to a concentration of 250 µg/mL using milli-Q H_2O. Afterward, the stock solution was diluted in order to obtain a volume of 45 µL at different concentrations (20, 50, 75, and 100 µg/mL) using milli-Q H_2O. Accuracy, intraday precision, and interday precision of the method were evaluated by assaying quality control (QC) solution, prepared independently from the solution used for calibration curve. The stock solution was diluted using milli-Q H_2O to obtain a sample volume of 45 µL at a concentration of 30 µg/mL (QC-A), 60 µg/mL (QC-B), and 80 µg/mL (QC-C) respectively. A volume of 5 µL of stable-isotope labeled infliximab SIL (SiluMAB™, Sigma-Aldrich, St Quentin Fallavier, France) at a concentration of 250 µg/mL was added to calibration and QC samples before tryptic digestion. The ratio of areas between analyte and internal standard was used as signal during the validation procedure. Each QC level was assayed three times in 1 day, and the validation procedure was repeated on three different days. For each QC level, accuracy was evaluated as the mean percentage of the ratios between observed back-calculated concentration and the theoretical concentration on 3 days ($n = 9$/QC level); intraday precision and interday precision were calculated by analysis of variances (single-way ANOVA) on back-calculated concentrations ($n = 3$/QC level/day). The method was considered validated if a maximum of 10% deviation was observed for each parameter.

Results and discussion

For this study, a total of 6 different samples (SC-01, SC-02, SC-03, SC-04, and SC-05), all of them seized by the authorities, were undergoing a comprehensive characterization in order to identify without any ambiguity the presence of the specified mAbs, in the allegedly counterfeit samples. In case of positive identification, the secondary objectives of the study were to perform a quantification of the mAbs in the samples and to attribute eventual signs of degradation which could have been induced by inappropriate storage. Based on the indication of the packaging, samples SC-01 and SC-02 were supposedly incorporating mAb1. The biopharmaceutical product mAb1 is an IgG 4 first approved in 2014 for the treatment of different advanced forms of cancer. Therefore, the protein has the ability to target the programmed cell death 1 (PD-1) receptor and it represents a mean molecular mass of 147,757 Da. The samples SC-03, SC-04, and SC-05 were potentially incorporating mAb2. The product referred as mAb2 is an IgG 1 first approved by the FDA in 2011 used as a therapeutic agent in oncology for the treatment of various forms of cancer, including advanced melanoma. The protein mAb2 has the specificity to target the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and represents an average molecular mass of 146,860 Da. From their external aspect, the packaging of the investigated samples was similar to their respective marketed products. It is worth noting that the languages present on the packaging were suggesting various geographical origins of the different investigated samples. Finally, the vials composing the investigated samples exhibited a transparent liquid without any traces of visible particles.

Monoclonal antibodies sample characterization using capillary zone electrophoresis

The investigated samples and the corresponding reference products were characterized by CZE-UV analysis. Indeed, CZE-UV previously demonstrated the possibility to perform the separation of mAbs charge variants [28, 29]. Therefore, it allows to achieve a straightforward and robust characterization of this type of mAbs isoforms [30]. The composition of the background electrolyte (BGE) regarding the concentration of ε-aminocaproic acid in addition to the pH was adjusted using the reference samples in order to provide a suitable separation of the mAbs charge variants (cf. "Experimental section"). As emphasized in Fig. 1A, the electropherogram corresponding to the CZE-UV analysis of mAb1 reference product showed the detection of a main mAb1 variant having a migration time of 11.1 min. Different mAb1 acidic charge variants having migration times of 11.6 and 12.1 min could also be separated. In addition, a basic charge variant was identified exhibiting a migration time of 10.4 min. The presence of charge variants regarding mAbs is explained by the intrinsic microheterogeneity of this type of therapeutic protein. For instance, charge variants can be originating from the heavy chain (HC) C-terminal lysine clipping. Due to the basic nature of their side chain ($pK_r = 10.67$), lysine residues are positively charged considering the mildly acidic pH of the BGE. Therefore, the loss of C-terminal lysine residues modifies significantly the electrophoretic mobility leading to the separation of the charge variants [31] [32, 33]. In a similar manner, the deamidation of asparagine residues into aspartic acid is described to involve a significant modification of the electrophoretic mobility of mAbs [34]. The CZE-UV analysis of the samples

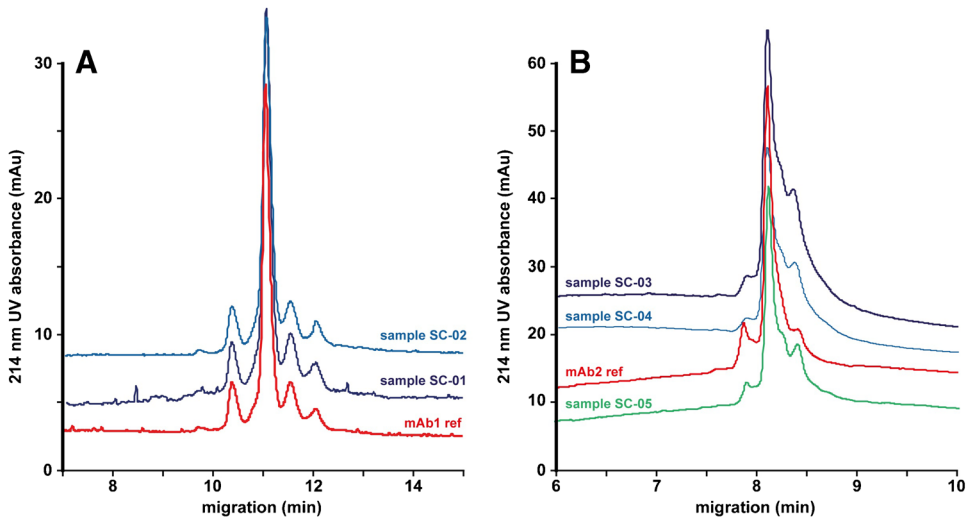

Fig. 1 Electropherograms obtained from CZE-UV analysis of (**A**) mAb1 reference product, SC-01 and SC-02 samples. (**B**) mAb2 reference product, SC-03, SC-04, and SC-05 samples. Conditions: BGE 600 mM EACA, 2 mM TETA, 1% HPMC (pH 5.6); sample concentration 1 mg/mL, 214-nm UV absorbance detection

SC-01 and SC-02 allowed to obtain highly similar electropherograms compared to mAb1 reference product (Fig. 1A) which showed a comparable charge variant profile between the different samples.

In parallel, the reference product mAb2 was analyzed using the CZE-UV method. The main charge variant of mAb2 showed a migration time of 8.0 min. In addition, a single basic mAb2 variant could be detected with a migration time of 7.8 min alongside two types of acidic variants demonstrating migration times of 8.1 min and 8.3 min (Fig. 1B). The CZE-UV analysis of the samples SC-03, SC-04, and SC-05 allowed to obtain similar electropherograms regarding the migration times of the separated species. In this case as well, the mAbs charge variants separated and detected in the investigated samples are showing completely similar migration times as mAb2 reference product and no additional peaks could be identified (Fig. 1B). Considering the conditions used for the CZE separation, the mobility of the protein is mainly influenced by the isoelectric point (pI) and the hydrodynamic radius of the macromolecule. Thereby, the pI is determined by the amino acid sequence of the mAbs which explains for example the different migration time of the main variant of mAb1 (11.0 min) and mAb2 (8.0 min). As a consequence, the results obtained from the CZE-UV analysis are strongly suggesting that the investigated samples are containing the same mAbs as their corresponding reference products.

In order to further advance the characterization of the samples, the peak areas corresponding to the different charge variants separated in CZE-UV analysis were exploited to estimate the charge variant profiles. In the case of samples SC-01 and SC-02, the profiles obtained demonstrated to be similar to the reference product mAb1 with the main variant representing from 70.1% (ref. mAb1) to 70.5% (SC-01). The comparison of the different samples only enabled to indicate a decrease in the proportion of basic variants to the profit of an increase regarding the proportion of acidic variants (Fig. 2A). For each sample, CZE-UV experiments were performed in triplicate and it is important to note that like for the migration times, the distribution obtained demonstrated low variability emphasizing the robustness of the analytical method. In addition, the possibility to obtain strictly comparable charge variant profiles between the investigated samples and the reference mAb1 also advocates in favor of the presence of mAb1 in SC-01 and SC-02 samples. The charge variant profiles were also deduced from CZE-UV data for the samples corresponding to mAb2 as emphasized in Fig. 2B. The profiles obtained demonstrated a majority of main variant ranging from 64.9% (SC-03) to 74.8% (ref. mAb2). In terms of relative abundance, the acidic variant systematically demonstrated to be more prominent compared to the basic variants. Thus, the investigated samples globally demonstrated similar distribution among the different charge variants. However, in comparison, the mAb2 reference product exhibited significantly higher proportion of basic and main variants, which mechanically resulted in lower proportion of acidic variants. This observation was attributed to the occurrence of lysine clipping from HC C-terminal extremity and the level of deamidation of asparagine residues. Indeed, the mAbs variants presenting lysine residues of both HC C-terminal section should represent the most basic variant, therefore exhibiting the shorter migration time (7.8 min) due to an increased electrophoretic mobility. The loss of one or both C-terminal lysine residues is lowering the electrophoretic mobility of the mAbs which leads to longer migration times in this case of 8.0 and 8.3 min (Fig. 1B). Although naturally occurring in vivo, HC C-terminal lysine cleavage can be favored depending on the storage conditions. Also, asparagine residues deamidation in aspartic acid results in higher abundance of acidic variants exhibiting longer migration times [35]. As a consequence, the differences observed in the case of mAb2 regarding charge variant distribution can be explained by endogenous C-terminal lysine clipping and deamidation, probably generated by inappropriate storage. Also, the charge variant profiles achieved from CZE-UV data showed consistent distributions among the investigated samples which highlights that samples stored in non-appropriate conditions together exhibited similar differences compared to the marketed reference product, stored in appropriate conditions.

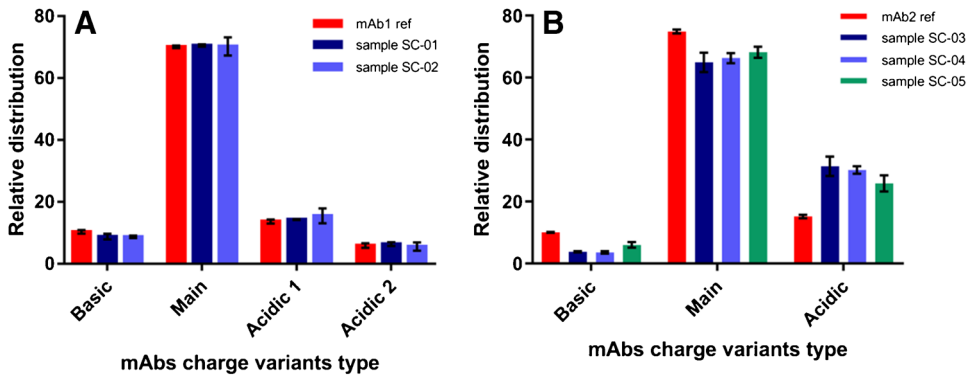

Fig. 2 mAbs charge variant distribution calculated from CZE-UV analysis of (**A**) mAb1 reference product, SC-01 and SC-02 samples. (**B**) mAb2 reference product, SC-03, SC-04, and SC-05 samples. Conditions: BGE 600 mM EACA, 2 mM TETA, 1% HPMC (pH 5.6); sample concentration 1 mg/mL, 214-nm UV absorbance detection

Thus, CZE-UV analysis was performed on the investigated samples and the reference products in each case in order to enable back-to-back comparison. As expected, the CZE-UV method enabled the separation of the mAbs charge variants. However, results have showed that the mobility of the therapeutic protein is to a great extent influenced by the primary structure. Using CZE-UV data, the comparison regarding the migration time and the charge variant distribution demonstrated to be systematically similar between the investigated samples and their corresponding reference. Therefore, the cross correlation of charge variants nature, respective migration times, and charge variant distribution allowed to conclude that the investigated samples contained effectively mAb1 in the case of SC-01 and SC-02 samples and mAb2 for SC-03, SC-04, and SC-05 samples. CZE-UV results allowed to demonstrate that the electrophoretic separation provided by CZE can be implemented as an effective identification marker for mAbs, even enabling to discriminate the two types of mAbs. It is important to consider the charge variant distribution in complement to migration times in order to improve the confidence of the identification. The CZE-UV method demonstrated to be particularly robust which allowed to perform a direct comparison of the electropherograms corresponding to the investigated samples and their respective reference products. In addition, the analysis did not require any sample treatment prior to the analysis which offers a relevant analytical tool for rapid confirmation regarding the nature of the product.

Monoclonal antibodies sample aggregation characterization using size exclusion chromatography-multi-angle light scattering

Following CZE-UV analysis, the samples were analyzed using size exclusion liquid chromatography coupled to multi-angle light scattering (SEC-MALS). The analytical conditions implemented for SEC-MALS experiments (cf. "2") potentially enable to separate mAbs oligomeric forms like dimer, trimer, and high molecular weight species (HMWS) from mAbs monomer, based on the chromatographic separation provided by the stationary phase [36]. In addition, MALS analysis performed online allows to confidently estimate the molecular mass of the separated species using light scattering analysis [37]. Therefore, SEC-MALS analysis represents a relevant tool for the characterization of mAbs aggregation [38].

Regarding SEC-MALS analysis of the mAb1 reference product, the chromatogram showed a main peak having a retention time of 19.1 min (peak 3) as emphasized in Fig. 3A, which is consistent with a 150-kDa protein based on the characteristics of the stationary phase. This peak was attributed to mAb1 monomer based on the SEC retention time and the MALS signal (Table 1). Before the main peak, the chromatogram presented two consecutive peaks exhibiting retention times of 16.7 min (peak 1) and 17.6 min (peak 2) which should correspond to significantly larger species compared to mAb1 monomer. However, MALS analysis enabled to determine a molecular mass close to the

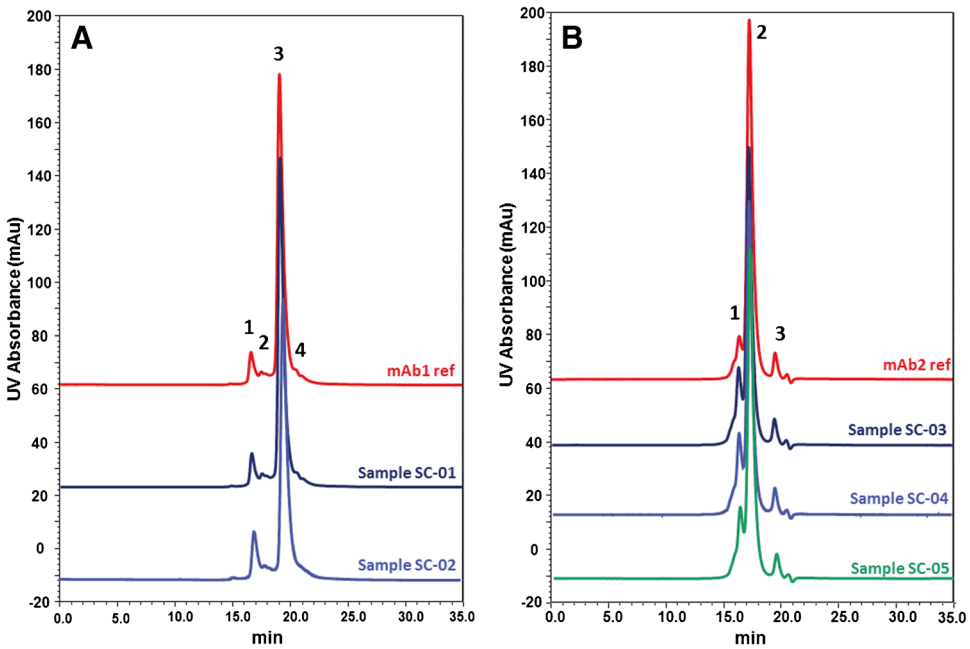

Fig. 3 Comparison of the chromatogram obtained for the SEC-UV/RI-MALS analysis of (**A**) mAb1 reference product, SC-01 and SC-02 samples. (**B**) mAb2 reference product, SC-03, SC-04, and SC-05 samples. Conditions: Biozen SEC-3 (300 × 4.6 mm) stationary phase, mobile phase 50 mM phosphate buffer (pH 6.8); 300 mM NaCl, flowrate 200 µL/min, injection volume 10 µL

Table 1 Molecular mass calculated from MALS measurements for the different peaks observed during SEC-MALS analysis for mAb1 reference, SC-01 and SC-02 samples. Conditions: cf. "Experimental section» (*n.d.* not determined)

	16.7 min	17.6 min	19.1 min	20.3 min
Reference mAb1	133.7 kDa ± 3.0%	123.9 kDa ± 4.0%	150.5 kDa ± 0.5%	n.d
Sample SC-01	134.8 kDa ± 6.3%	118.7 kDa ± 8.5%	152.5 kDa ± 0.8%	n.d
Sample SC-02	138.7 kDa ± 4.9%	124.8 kDa ± 9.0%	145.5 kDa ± 1.1%	n.d

Table 2 Molecular mass calculated from MALS measurements for the different peaks observed during SEC-MALS analysis for mAb2 reference, SC-03, SC-04, and SC-05 samples, respectively. Conditions: cf. "Experimental section" (*n.d.* not determined)

	16.3 min	17.2 min	19.5 min
Reference mAb2	150.4 kDa ± 11.7%	146.6 kDa ± 1.2%	n.d
Sample SC-03	136.1 kDa ± 6.8%	142.1 kDa ± 2.1%	n.d
Sample SC-04	155.7 kDa ± 4.7%	153.0 kDa ± 1.8%	n.d
Sample SC-05	140.4 kDa ± 1.7%	145.9 kDa ± 1.1%	n.d

monomeric form, especially for peak 1 (Table 1). Similar results have been previously described by Ehkirch et al. They attributed early peaks to changes in the conformation of the mAbs, which resulted in significantly higher hydrodynamic diameter but did not affect the molecular mass of the mAbs variant [36]. Finally, the SEC chromatogram allowed to observe minor peaks corresponding to lower molecular mass species like free light chain (LC) with retention time around 20.3 min (Fig. 3A). The analysis of the sample SC-01 enabled to obtain a chromatogram extensively similar to the results achieved for the reference product. The main peak demonstrated a retention time of 19.1 min which indicates that the sample SC-01 contains a molecule compatible with the size of a mAb. Also, MALS measurement enabled to determine a molecular mass of 150 kDa for this particular peak. Regarding peaks 1 and 2 eluting prior to the main species, retention times remained consistent and MALS measurement provided a molecular mass close to the mAbs monomer similarly to the result obtain for mAb1 reference product (Table 1). The SEC-MALS analysis of the sample SC-02 provided a chromatogram equivalent to the reference product as presented in Fig. 3A. In this case as well, the analysis showed that the principal compound present in the sample SC-02 is compatible with the size of a mAb based on both retention time and MALS measurements (Table 1).

Concerning the SEC-MALS analysis of mAb2 reference product, the chromatogram presented in Fig. 3B showed a main peak exhibiting a retention time of 17.2 min. The MALS measurement corresponding to this peak allowed to determine a molecular mass of 146.6 kDa, therefore attributed to mAb2 monomer. The chromatogram demonstrated the separation of higher molecular mass species with a retention time of 16.3 min which mass was estimated to 150.4 kDa based on MALS measurement (Table 2). This peak was therefore attributed to a change in the conformation of the mAbs because no significant differences in molecular mass could be identified from MALS analysis. In addition, a peak corresponding to lower molecular mass species was detected at 19.5 min; unfortunately MALS signal was not sufficient to determine the mass of these species. It is interesting to note that the retention times for mAb1 and mAb2 are not strictly the same which suggests that the hydrodynamic diameter of the proteins in solution may be significantly different. As emphasized in Fig. 3B, the analysis of the investigated SC samples demonstrated systematically similar SEC-MALS chromatograms compared to mAb2 reference product. As a consequence, it was possible to conclude that the investigated samples contained a main compound which molecular mass was comparable to the

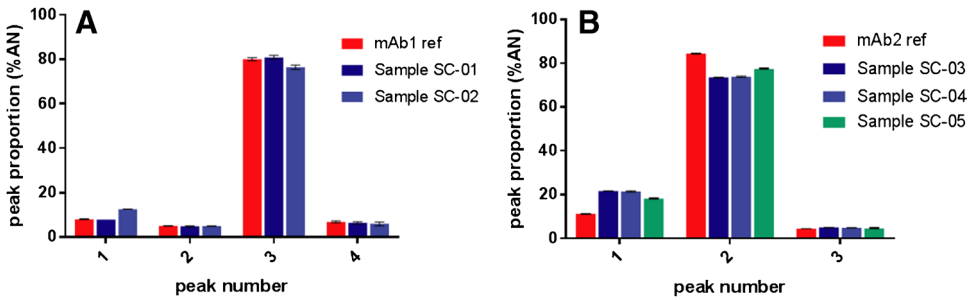

Fig. 4 mAbs size variant distribution calculated from SEC-UV/RI-MALS analysis of (**A**) mAb1 reference product, SC-01 and SC-02 samples. (**B**) mAb2 reference product, SC-03, SC-04, and SC-05 samples. Conditions: Biozen SEC-3 (300 × 4.6 mm) stationary phase, mobile phase 50 mM phosphate buffer (pH 6.8); 300 mM NaCl, flow-rate 200 µL/min, injection volume 10 µL

mass of mAbs based concomitantly on the retention time observed in SEC chromatography and MALS measurements.

SEC-MALS data were further used to determine the distribution of the size variants for the two sets of samples. In the case of mAb1, Fig. 4A shows that the distribution is consistent with the results achieved for the analysis of samples SC-01 and SC-02. In the case of sample SC-02, the proportion of monomer showed lower abundance (peak 3) compared to mAb1 reference product to the profit of early eluting variants (peak 1). This result could potentially be originating from inappropriate storage which gradually generated a conformational change of the protein. Concerning the samples related to mAb2, in this case also the proportion of monomer was found to be marginally lower compared to the reference product to the benefit of the early eluting variants (Fig. 4B).

Regarding the characterization of the SC samples, SEC-MALS cannot strictly provide an absolute identification of the protein constituting the different samples. As a consequence, SEC-MALS could not provide information regarding the nature of the mAbs effectively present in the different samples investigated. However, SEC-MALS data enabled to demonstrate for the different samples that the main compound detected was compatible with the presence of a mAb considering the SEC retention times and MALS measurements. In addition, the SEC chromatograms exhibited high similarities compared to their respective reference products. Therefore, like CZE-UV experiments, the data generated from SEC-MALS clearly suggest that SC-01 and SC-02 samples effectively contained mAb1 while SC-03, SC-04, and SC-05 samples were composed of mAb2. Also, SEC-MALS results showed a potential alteration of the mAbs content regarding size variants, which was attributed to inappropriate storage and regarding that aspect was in agreement with CZE-UV analysis.

Primary structure characterization using liquid chromatography hyphenated to tandem mass spectrometry

Alongside the characterization performed using CZE-UV and SEC-MALS analysis, the samples suspected to be counterfeit were also characterized by LC–MS/MS analysis. The methodology applied is derived from bottom-up proteomic analysis; therefore, the samples were undergoing enzymatic digestion in order to generate peptides. Afterward, the peptide mixture was separated and fully characterized using LC–MS/MS in order to characterize the amino acid sequence of the protein contained in the samples and to perform sequence matching compared to the sequence of mAb1 and mAb2. The identification of the peptides was based on high-resolution MS measurements achieved by the MS instrumentation in addition to the fragmentation of the peptides observed in MS/MS spectra. This analytical methodology enables to provide an unambiguous characterization of the amino acid sequence of the protein and offers the possibility to distinguish faint changes such as single amino acid differences which may occur for instance when the mAbs are produced in different expression systems [39].

Concerning the mAb1 reference sample, the UPLC-MS/MS data allowed to achieve a sequence coverage of 94.6% for the HC and 95.9% in the case of the LC (Table S1). The mAb1 peptides that could not be identified demonstrated to be systematically short peptides (1–4 amino acids). This observation was explained by poor retention which hampered detection due to their co-elution in the dead volume. As emphasized in Fig. 5A, the characterization of the SC-01 and SC-02 samples allowed to identify an extensive part of the amino acid sequence mAb1. The identified peptides exhibited a retention time variation systematically inferior to 0.15 min compared to their homologue peptides, observed in the case of mAb1 reference sample experiment, further

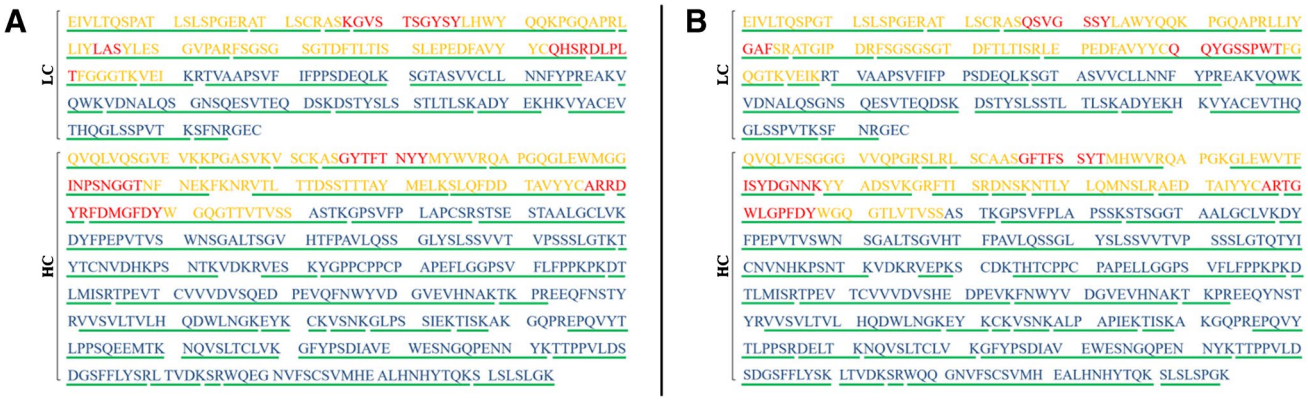

Fig. 5 Detailed amino acid sequence coverage achieved using LC–MS/MS analysis for (**A**) samples SC-01 and SC-02 containing mAb1, and (**B**) samples SC-03, SC-04, and SC-05 composed of mAb2. Variable domain and complement determining regions are represented in orange and red, respectively. Constant domains are represented in blue (conditions cf. "Experimental section»)

confirming their identical nature (Table S2). Also, the peptides composing the variable domain of the protein could be entirely identified apart from 5 amino acids (Fig. 5A). Therefore, MS data allowed to characterize the highly specific peptides corresponding strictly to mAb1 like peptides HT-05 and LT-04 (Table S2), further advocating on the positive identification of mAb1 constituting the samples SC-01 and SC-02. Also, it is important to mention that the peptides which escaped from the identification were common to mAb1 reference sample analysis (Table S2–S3). In complement, database search identification in a similar manner as shotgun bottom-up proteomic analysis was performed using the UPLC-MS/MS data. Database searches did not enable the identification of other significant proteins except from IgG-related identification due to common peptides present in the constant domain.

For the analysis of mAb2 reference sample, similarly to the previous set of experiment, MS data enabled to identify 93.3% of the amino acid sequence corresponding to mAb2 HC and 95.9% to the LC of the protein (Table S4). Therefore, the results demonstrated the possibility to characterize almost entirely the amino acid sequence of the protein in a consistent and robust manner. Regarding the analysis of the samples of interest, the experiments enabled to identify nearly the entire amino acid sequence of mAb2 for samples SC-03, SC-04, and SC-05. As represented in Fig. 5B, the characterization achieved led to similar sequence coverage for the three different samples concerning the nature of the identified peptides. As a consequence, more than 98% of the variable domain, containing the proteotypic peptides of mAb2, could be successfully identified demonstrating without any ambiguity that samples SC-03, SC-04, and SC-05 effectively contained mAb2 (Table S5–S7). In this case as well, short peptides exhibited to be difficult for identification which still represents a minor portion of the amino acid sequence.

As a consequence, the characterization of the primary structure of the protein composing the different samples allowed to successfully demonstrate the identification of mAb1 in the samples SC-01 and SC-02, and in a similar manner the identification of mAb2 in samples SC-03, SC-04, and SC-05. The quality and precision of MS data allowed to achieve an extensive sequence coverage that improved the confidence of the identification. Furthermore, the information provided by the LC–MS/MS analysis showed a relevant complement to the results obtained from CZE-UV and SEC-MALS experiments. Indeed, LC–MS/MS analysis enabled an extensive characterization of the mAbs primary structure by the intermediate of peptide identification, which was determinant but implied important proteolytic sample treatment. In parallel, CZE-UV and SEC-MALS allowed to ensure that the intact protein was also corresponding to the investigated mAbs with the advantage of minimal sample treatment, while maintaining relevant specificity.

Absolute quantification of monoclonal antibodies content using liquid chromatography-mass spectrometry

The counterfeiting of regulated substances like pharmaceutical products may in practice adopt different forms. For instance, the sample could contain a molecule relatively close to the original product however produced non-GMP environment. As a consequence, such sample could potentially contain remaining reagents, residual solvents, side products, and/or degradation products which compromise dramatically the safety of the substance. Also, counterfeiting can reside in the concentration of the active molecule which is intentionally lower to the specified concentration using simple dilution. That type of product alteration may be particularly interesting in the case of mAbs trafficking because of, on the one hand, the important commercial value of the product and, on the other hand, the difficulty to produce the protein independently without highly advanced dedicated facilities.

Following the demonstration of the presence of mAb1 and mAb2 in the different samples of interests, the absolute quantification of the mAbs was performed in order to attribute any alteration of the product regarding the concentration of active substance. Thus, the analytical method developed for the mAbs quantification consisted on performing proteolytic digestion of the sample content. The peptide mixture generated was consequently separated and analyzed using LC–MS/MS analysis. Absolute quantification using MS analysis requires the implementation of an internal standard generally adopting the form of homologous molecules labeled with stable heavy isotopes. Thereby, the LC–MS methods recently described for the quantification of mAbs commonly incorporate the exact same mAbs incorporating stable heavy isotopes as internal standard [40, 41]. Stable-isotope labeled standard corresponding to mAb1 and mAb2 are not available. In order to tackle that limitation, the characteristic of the developed method is the implementation of a stable-isotope labeled infliximab (SIL) as a surrogate internal standard. SIL possesses the advantage to be cost-effective and readily available. Therefore, the peptides considered for the quantification were the ones common to SIL and mAb1 on one side, and common to SIL and mAb2 on the other side. For each mAb, the quantification method was submitted to statistical validation in order to determine the performance of the analysis.

In the case of mAb1, two peptides on the HC and the LC were considered, 1HT-02 and 1LT-07, respectively, in order to give the opportunity to confirm the quantification. The calibration curve demonstrated to be consistently

Table 3 Compilation of the results obtained for the statistical validation of absolute quantification of mAb1 and mAb2 using the LC–MS/MS method developed

Peptide id	mAb1		mAb2	
	1LT-07	1HT-02	2HT-01	2HT-21
Sequence	DSTYSLSSTLTLSK	DTLMISR	GPSVFPLAPSSK	TTPPVLDSDGSFFLYSK
Retention time (min)	17.65 ± 0.12	4.21 ± 0.05	17.60 ± 0.09	21.8 ± 0.04
Concentration range (µg/mL)	20–100	20–100	20–100	20–100
Equation	$6.05\times10^{-2}x - 0.024$	$8.79\times10^{-2}x - 0.265$	$6.73\times10^{-2}x - 0.097$	$6.51\times10^{-2}x - 0.084$
R^2	0.998	0.998	0.997	0.998
LOD	5.31	9.64	1.99	4.11
LOQ	16.08	29.22	6.04	12.45
Accuracy ($n = 9$)				
QC-A	101.9%	97.3%	93.3%	94.6%
QC-B	94.7%	92.7%	103.4%	98.8%
QC-C	109.4%	108.9%	100.1%	96.7%
Intraday precision ($n = 9$)				
QC-A	3.7%	6.8%	4.2%	2.6%
QC-B	1.2%	4.8%	4.6%	2.7%
QC-C	1.0%	7.0%	2.4%	2.4%
Interday precision ($n = 9$)				
QC-A	4.5%	7.0%	4.5%	3.4%
QC-B	4.1%	6.1%	6.8%	4.0%
QC-C	8.1%	8.7%	4.6%	2.6%

linear in the calibration range as emphasized in Table 3 and Fig. S1. In addition, the variability related to the equation of the calibration curve was relatively limited which showed the consistency of the MS signal obtained. Considering the peptide 1LT-07, the LOD achieved was 5.3 µg/mL (35.9 nM) and the LOQ was 16.1 µg/mL (109.0 nM), respectively. The analysis of the QC samples showed the appropriate accuracy of the method. Similarly, the intraday precision and interday precision demonstrated to be particularly adequate, with a value systematically below 10%, as emphasized in Table 3. Thereby, the characteristics of the LC–MS/MS method determined from the statistical validation demonstrated the validity of the quantification. Indeed, results obtained during the validation showed that considering common peptides between SIL surrogate internal standard and mAb1 allows to compensate the variability originating from the experimental conditions such as the ESI ionization efficiency impacting MS signal intensity and the yield of the proteolytic digestion process. The investigated samples SC-01 and SC-02 were diluted to a theoretical concentration of 50 µg/mL based on the indication of the packaging. Afterward, the quantification of mAb1 was performed for both samples using the LC–MS/MS method. Results allowed to determine that the quantity of mAb1 in sample SC-01 was equal to 96.8 ± 0.8% compared to the concentration mentioned on the product labelling. Regarding the sample SC-02, the concentration of mAb1 measured was corresponding to 90.1 ± 0.6% in regard to the indication of the product. Therefore, LC–MS/MS quantification enabled to determine that the concentration of mAb1 is relatively close to the value mentioned on the product. This observation is consistent with the identification of mAb1 in the studied samples and a minor degradation identified from CZE-UV and SEC-MALS characterization, probably originating from inadequate storage conditions.

Regarding the quantification of mAb2, two peptides (2HT-01 and 2HT-21) were also considered to perform the quantification. It is important to note that the selected peptides were common to mAb2 and SIL. However, they could not correspond to any peptide generated from the digestion of mAb1 in order to prevent any sort of mismatch during the quantification. Results recorded from the statistical validation are compiled in Table 3. As emphasized, the calibration curve generated demonstrated consistent linearity over the concentration range (Fig. S2) and low variability. Similarly to the mAb1 quantification, the accuracy achieved demonstrated to be consistently in the 90–110% range. Considering the peptide 2HT-01, the LOD achieved was 2.0 µg/mL (13.6 nM) and the LOQ was 6.1 µg/mL (41.5 nM), respectively. The analysis of QC samples allowed to determine that both intraday precision and interday precision were consistently inferior to 10% (Table 3). Therefore, in this case as well, the analytical method could be validated for the absolute quantification of mAb2. The samples SC-03, SC-04, and SC-05 were analyzed using the developed method in

order to quantify mAb2. MS data allowed to determine that the concentration of mAb2 in sample SC-03 was equal to 91.3 ± 0.4% in regard to the nominal concentration indicated. Results of the quantification performed for sample SC-04 exhibited a value of 90.2 ± 1.2% compared to the designated value on the product. In the case of the sample SC-05, the data obtained for the quantification experiments allowed to determine actual mAb2 concentration corresponding to 106.3 ± 3.8% of the mentioned value. Thus, the quantification of mAb2 performed for the different samples demonstrated that the concentration was relatively close to the presented value. This observation is further attesting that the investigated samples are corresponding to the original product diverted from its normal distribution pipeline through trafficking.

The analytical method developed in order to perform the quantification of mAb1 and mAb2 in their respective samples demonstrated a relevant accuracy, repeatability, and robustness throughout an extensive method validation. Especially, the implementation of SIL as a surrogate internal standard showed to be relevant. In particular, it gives the opportunity to perform the quantification of mAbs even in the case when the stable-isotope labeled homologues are not available. In the context of this study, the use of the SIL enabled to envisage the quantification of two different mAbs using the same molecule as internal standard. To the best of our knowledge, this is the first time that the quantification of mAbs has been successfully performed using a surrogate internal standard. Considering the fast-paced development of this category of biopharmaceutical products with the approval of about 10 products each year, the possibility to use a surrogate internal standard allows to broaden the applicability of LC–MS/MS analysis for the specific quantification of mAbs. Regarding the investigated samples, the quantification experiments enabled to measure mAbs concentration close to the amounts specified on the different product packaging. Therefore, results of the quantification of mAb1 and mAb2 reinforced also the conclusion of the previous characterization, to which the investigated samples are corresponding to the conventional products.

Conclusion

In the present work, we could develop an analytical strategy in order to provide the identification, the characterization, and the quantification of different types of mAbs in samples with unknown contents. The analytical characterization implemented concomitantly on CZE-UV, SEC-MALS, and LC–MS/MS analysis to provide complementary information concerning the nature and the structure of the sample contents. Thus, the selectivity provided by the electrokinetic separation of CZE-UV emphasized the possibility to identify confidently the different mAbs based on their respective mobilities and charge variant distribution. Concomitantly, the SEC-MALS experiments enabled to study an eventual presence of other types of proteins apart from the identified mAbs, in addition to provide the characterization of the mAbs aggregation and heavy/light chain dissociation. Therefore, SEC-MALS analysis allowed to determine in a straightforward manner the stability of the different samples regarding aggregation and mAbs dissociation. The chromatographic separations and MALS measurements showed complete similarity between the different samples investigated and their respective reference products which further advocated for the positive identification of the two mAbs in the unknown samples. In order to complete the identification, the characterization of primary structure was performed by proteolytic digestion followed by LC–MS/MS analysis. Results showed in each case the possibility to characterize nearly the complete amino acid sequence of the protein contained in the samples in a robustness manner. The extensive sequence coverage allowed to characterize the highly specific portion of the mAbs which succeeded to prove the presence of the different mAbs in their respective samples. Thereby, the implementation of different analytical techniques that use several physico-chemical mechanisms enabled to systematically identify mAb1 and mAb2 in their investigated samples, respectively. Therefore, using this approach provided a maximum confidence regarding the identification as it was established from independent data like primary structure characterization and intact protein analysis. Indeed, it allowed to tackle in a relevant manner the structural complexity inherent to mAbs in order to obtain an identification without false positive and/or biases. In addition, experimental results illustrated the complementarity of the different analytical techniques employed and the possibility to investigate at the same time an eventual degradation of the product. Finally, the quantification of the two different mAbs was realized in their respective samples using LC–MS/MS analysis and a common stable isotope labeled mAb implemented as internal standard. The results of the method validation demonstrated for each type of mAbs studied the possibility to perform an accurate and robust quantification using the same internal standard while maintaining an appropriate level of specificity. As a consequence, the LC–MS/MS method described opens the way to perform the quantification using LC–MS/MS analysis even in the case of analytes which labeled homologues are not yet available.

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Declarations

Conflict of interest The authors declare no competing interests.

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