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Determination of size variants by CE-SDS for approved therapeutic antibodies: Key implications of subclasses and light chain specificities

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ABSTRACT

In the present work, a generic non-reducing capillary electrophoresis sodium dodecyl sulphate (nrCE-SDS) method was tested for a wide range of 26 FDA and EMA approved monoclonal antibodies (mAbs) and 2 antibody drug conjugates (ADCs) as well as for the NISTmab, in a QC environment (e.g. testing quality requirements for batch manufacturing or batch release). This method allows obtaining rapidly and accurately the amount of size variants in drug products within about 40 min and may be used for batch release and consistency as well as for stability and shelf-life. First, the method repeatability was found to be excellent in terms of relative migration times and relative proportions of fragments (average RSD values of 0.3 and 0.2 %, on relative migration times and relative percentages of fragments, respectively), thanks to the addition of an internal standard. A panel of chimeric, humanized and human mAbs were tested, belonging to different subclasses (heavy chain gamma 1, 2, 2/4 and 4) and light chain types (κ or λ) and produced in different cell lines (CHO, NS0 and SP2/0). For all these biopharmaceutical products, the amount of H2L2 species was comprised between 90.9 % and 97.7 %, except for the two mAbs belonging to the IgG1λ subclass, namely avelumab and belimumab, which were prone to partial reduction during the sample preparation at 70 °C. Based on the CE-SDS results obtained for a diverse panel of therapeutic antibodies investigated in this study, and covering a wide range of structural and physico-chemical properties, a specification on the intact antibody content (H2L2) greater than 90 % can be achieved.
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1. Introduction

For the first time, either the Food and Drug Administration (FDA) or the European Medicine Agencies (EMA) has approved a two-digit numbers of antibodies in 2018 [1]. More than 80 mAbs have been approved by regulatory agencies over the last 30 years and this number is expected to double in the next 15 years, taking into account the number of 570 antibodies currently evaluated at various stages in clinical trials and a success rate of 17–25% from phase 1 to approval [1]. To ensure the quality and safety of drug product, various antibody variants such as size, charge and hydrophobicity variants have to be assessed at various stages of the drug lifecycle, during production and shelf-life [2]. Size-variants are potential Critical Quality Attributes (pCQAs) that are often considered as one of the most important CQA in quality assessment studies [3] and for which specifications have to be set for batch release and during shelf-life.

According to the monograph 129 of the United States Pharmacopeia (USP), size exclusion chromatography (SEC) is considered a robust method for the determination of high molecular weight species (HMWS) in native conditions while CE-SDS is more reliable for the quantification of low molecular weight species (LMWS) in denaturing conditions [4]. CE-SDS is a key Quality Control (QC) method for size-variant quantification of antibodies and related products, [5,6] as demonstrated in a recent inter-laboratory study involving biopharmaceutical companies and regulatory authorities [7]. Of particular interest, CE-SDS has been shown to provide additional information in comparison to SEC, for the separation of the

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sFc and Fd fragments for example, which could not be achieved by SEC [8].

In order to attain an efficient antibody coating with SDS to achieve size-based separations, a sample preparation is required to denature the antibody at the intact or reduced level. In order to increase sample throughput, generic and automated sample preparation methods have been proposed by biopharmaceutical companies [9]. Non-reduced CE-SDS (nrCE-SDS) is used to assess the amount of intact species (main peak) and LMWS resulting from partial reduction of fragmentation products (*e.g.* Lys-Pro motives). Alternatively, a reducing agent can be added to the sample gel buffer to perform the CE-SDS separation at antibody chains level. Reduced CE-SDS (rCE-SDS) is commonly performed to quantify the relative amounts of light and heavy chains (around 33 % and 67 % expected) as well as the determination of non-glycosylated heavy chain (NG−HC) that may be present in the sample [10,11].

However, there is no clear specification requested in regulatory guidelines of monographies, [12] meanwhile artefacts induced by the sample preparation reducing agent, heating time and temperature have been reported in several studies. In particular, artificial fragmentation is commonly observed in nrCE-SDS, due to insufficient alkylation of the free sulfhydryl groups by iodoacetamide or the sample tray room temperature indicated in the USP method [4,12]. When validating nrCE-SDS methods, the sample incubation temperature and time have to be carefully optimized and the method robustness *versus* these parameters should be assessed during method development. In particular, the temperature has to be ideally set at 65−70 °C, to limit artificial fragmentation occurring at temperatures higher than 90 °C and avoid improper peak shapes that can be observed at 45 °C [7]. Finally, the nature of the detergent can also become critical when analysing some challenging proteins and therefore, sodium dodecyl sulphate (SDS) was replaced by sodium hexadecyl sulphate (SHS) to increase the peak resolution [13].

To our knowledge, there is very scarce data published on quantitative results obtained by CE-SDS for Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved antibodies. Following previous comprehensive studies on icIEF, CEX, [14] CZE, [15] SEC-UV [16] and SEC-MS, [17] we report here a generic CE-SDS method illustrating its potential with a wide panel of antibodies approved by the regulatory agencies. The specificity of the tested antibodies investigated in this work are the following: i) engineered format (chimeric, humanized and human); ii) subclasses (IgG1, 2, 2/4 and 4); iii) light chain types (κ or λ) and iv) produced in different cell lines (CHO, NS0 or SP2/0). In addition, several case studies are presented with the comparison of nrCE-SDS profiles for i) the originator infliximab *vs.* two EMA-approved biosimilar products; ii) as control method during process optimization and for iii) lysine-linked and hinge cysteine-linked antibody-drug conjugates (ADCs).

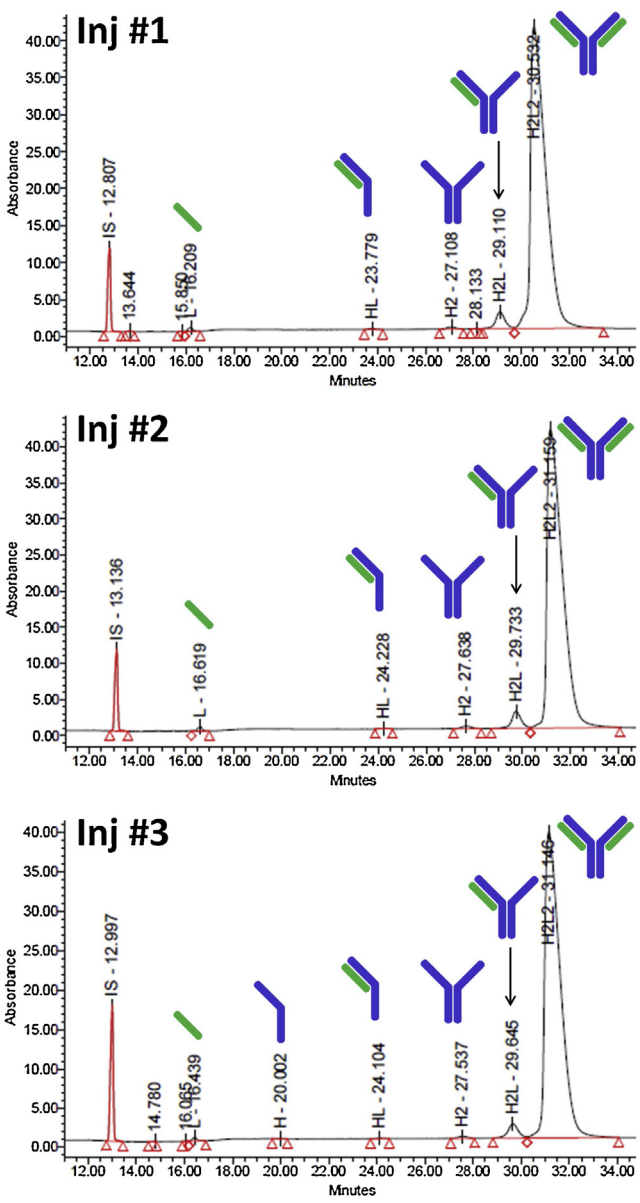

Fig. 1. Triplicate analysis of trastuzumab (hzIgG1κ) using generic nrCE-SDS-UV conditions.

2. Material and methods

2.1. Chemicals and reagents

NrCE-SDS experiments were performed using a Maurice™ CE-SDS application kit from ProteinSimple (San Jose, CA, USA), containing CE-SDS cartridges, vials, caps and 96-well plates, as well as CE-SDS separation matrix, different buffers, wash and conditioning solutions and internal standards. Samples were prepared using iodoacetamide (IAM) (≥99 %) purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

FDA and EMA approved therapeutic antibodies were obtained as European Union pharmaceutical-grade drug product from their respective manufacturers. The NISTmAb was obtained from the National Institute of Standards & Technology (Gaithersburg, MD, USA). A summary of the biopharmaceutical proteins used in this study and their isotypes and production systems can be found in Table 1.

2.2. nrCE-SDS analysis

IgGs were analyzed in non-reduced conditions using a Maurice™ system (Protein Simple) equipped with the Compass™ system according to the manufacturer instructions. Samples were diluted in 1X Maurice CE-SDS Sample Buffer to a final concentration of 1 mg/mL, from which 50 µL aliquot samples were made. Then, 2 µL of Maurice 25X Internal Standard was added to each sample. 2.5 µL of a 250 mM stock solution of the alkylating agent IAM was added to each 50 µL sample to block disulfide scrambling or exchange. Samples were denatured at 70 °C for 10 min, cooled on ice for 5 min and mixed by vortex. Each sample was then transferred to a 96-well plate and spun down in a centrifuge for 10 min at 1000 x g. All samples were run in triplicates (for a single sample preparation, three replicate injections were

Table 1
Summary of investigated therapeutic antibodies throughout this study. Average % of intact form and fragments experimentally determined by nrCE-SDS-UV (n = 3) for 28 FDA and EMA approved products, consisting of 26 mAbs and 2 ADCs plus NISTmab reference material.

Isotype	Sample	Cell	%H2L2	%H2L	%H2	%HL	%H	%L
chIgG1κ	rituximab	CHO	90.9	5.7	1.3	0.5	0.3	1.3
	infliximab (Remicade)	Sp2/0	95.7	2.3	1.1	0.1	0.3	0.4
	infliximab (Remsima)	Sp2/0	92.4	4.9	1.3	0.4	0.2	0.7
	infliximab (Inflectra)	Sp2/0	92.1	4.9	1.4	0.4	0.2	1.0
	cetuximab *(Fab N-glycosylated)*	Sp2/0	95.1	3.0	1.2		0.4	0.3
hzIgG1κ	palivizumab	NS0	96.2	2.1	1	0.2	0.2	0.3
	obinutuzumab *(N glyco-engineered, low fucose)*	CHO	95.8	2.7	0.7	0.2	0.2	0.3
	bevacizumab	CHO	95.6	2.6	0.8	0.1	0.2	0.4
	trastuzumab	CHO	95.6	3.3	0.5	0.1	0.1	0.4
	elotuzumab	NS0	95.1	3.3	0.8	0.2	0.1	0.3
	pertuzumab	CHO	94.5	3.3	0.8	0.2	0.2	0.6
	atezolizumab *(N298A, a-glycosylated)*	CHO	94.4	3.4	0.7	0.4	0.3	0.5
hzgG1κ	NISTmab	NS0	97.7	1.6	0.4	0.2	0.2	0.2
huIgG1κ	adalimumab	CHO	96.2	2.6	0.4	0.2	0.1	0.5
	ramucirumab	CHO	96.1	2.5	0.7	0.2	0.1	0.4
	ipilimumab	CHO	96.5	2.2	0.7	0.2	0.2	0.3
	ofatumumab	NS0	95.9	2.2	1.1	0.1	0.4	0.4
huIgG1λ	belimumab	NS0	75.7	13.2	5.5	0.2	0.6	2.2
	avelumab	CHO	69.4	17.4	7.7	0.4	0.6	3.1
	ixekizumab *(S227 P)*	CHO	95.8	3.0	0.3	0.3	0.1	0.4
hzIgG4κ	natalizumab	NS0	95.3	1.6	0.4	2.2	0.1	0.2
	pembrolizumab *(S228 P)*	CHO	94.6	3.9	0.1	0.5	0.1	0.5
	reslizumab	NS0	93.7	1.6	0.2	3.9	0.2	0.3
huIgG4k	nivolumab *(S221 P)*	CHO	96.3	2.5	0.4	0.1	0.1	0.3
hzIgG2/4κ	eculizumab	NS0	96.9	2.6				0.3
hzIgG2κ	denosumab	CHO	96.0	3.4	0.2	0.1	0	0.4
	panitumumab	CHO	94.6	4.6	0.1	0.2	0.1	0.5
ADCs	trastuzumab emtansine	CHO	95.9	3.0	0.5	0.1	0.1	0.4
	brentuximab vedotin	CHO	8.1	25	15.5	21.3	13.1	13.1

performed within the same day) and electrokinetically injected into the cartridge capillary by applying 4600 V for 20 s before separation by electrophoresis at 5750 V during 35 min. Electropherograms were analyzed with the Empower™ (Waters) data software.

3. Results and discussion

Detecting the fine fragmentation of a mAb is critical for determining both manufacturing and product stability consistency. In this study, we show that fragmentation species can be experimentally monitored in their original state, by applying a generic CE-SDS method under non-reducing conditions (nrCE-SDS). In this context, fragmentation species are mainly native antibody subunits represented by light chain (L, 25 kDa), heavy chain (H, 50 kDa), heavy-light (HL, 75 kDa), heavy-heavy (H2, 100 kDa) and heavy-heavy-light (H2L, 125 kDa) that are separated from the intact mAb (H2L2, 150 kDa) and identified based on their relative migration time (RMT).

First, the ability of the method to detect and resolve molecular fragments of a common mAb, namely trastuzumab, was confirmed and repeatability was assessed using a single sample preparation and three replicate injections within the same day. Molecular subunits were efficiently resolved and corresponded to L, HL, H2, H2L fragments, along with the intact mAb (H2L2) and the internal standard peak (IS). The excellent precision of the assay was demonstrated, with an average RSD of 9.6 % for the relative percentage of fragments and an average RSD of 1.1 % for RMT, which was dropping to 0.3 % when supported by the use of the IS. Fig. 1 shows the three electropherograms of trastuzumab injections used for the precision test.

The generic nrCE-SDS method was then applied to a wider range of FDA and EMA approved products, consisting of 27 mAbs and 2 ADC. All non-reduced samples were run in triplicates and separated within 35 min (see Materials and Methods for details). The average percentages of experimentally determined intact forms and fragments are reported in Table 1, which highlights that the amount of H2L2 was found to be higher than 90 % for the majority of the mAbs. The only exception was represented by the huIgG1λ belimumab and avelumab and the ADC brentuximab vedotin that will be discussed in further details in sections 3.2 and 3.6, respectively. It is worth mentioning that the generic nrCE-SDS method was not able to baseline resolve the non-glycosylated (NG) H2L2 species, and therefore the relative percentages of these non-glycosylated species, when present, were evaluated as part of the relative percentages of the main peak H2L2. No method optimisation was performed to gain in peak resolution, since quantification aspects related to NG species where out of the scope of this work and can be easily achieved under reducing conditions.

For sake of convenience, all data were also graphically summarized in the form of histograms to emphasize the percentage of intact forms with respect to the different mAb subclasses (Fig. 2) and the subunit pattern of each mAb (Fig. 3).

3.1. Contribute to demonstrate biosimilarity by nrCE-SDS

Fig. 4 shows the electropherograms corresponding to three chIgG1κ infliximab products, the innovator Remicade (Fig. 4a) and two EU biosimilar products, namely Remsina (Fig. 4b) and Inflectra (Fig. 4c) analyzed under the generic nrCE-SDS conditions. The three samples were run in triplicate and the precision of the method was again evidenced by a remarkable average RSD of 0.4 % for the RMT

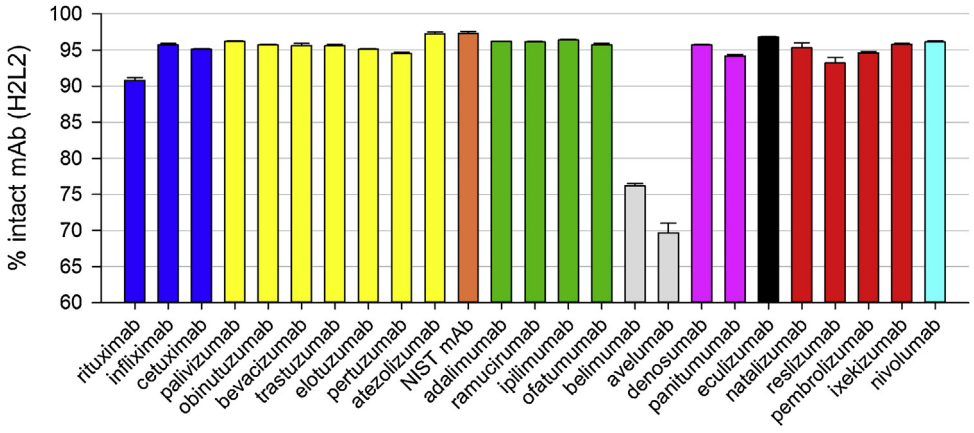

Fig. 2. Experimental measurement of % intact forms for 25 FDA/EMA approved mAbs, measured by nrCE-SDS-UV. Bar color refers to different mAb subclasses, namely chIgG1κ (blue), hzIgG1κ (yellow), hzgG1κ (orange), huIgG1κ (green), huIgG1λ (grey), hzIgG2κ (pink), huIgG2/4κ (black), hzIgG4κ (red), and huIgG4κ (cyan). Error bars were reported even though were found to be negligible. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

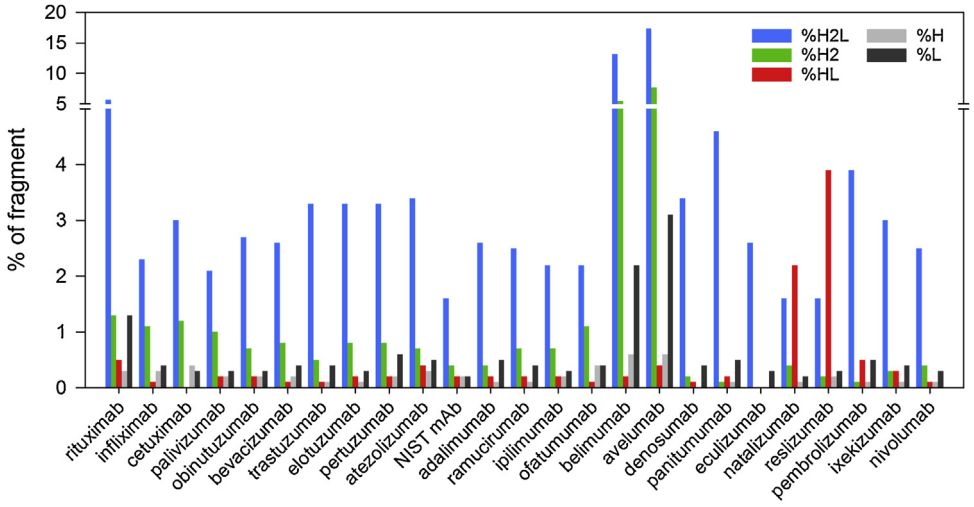

Fig. 3. Experimental measurement of the different fragments observed in the 25 commercial mAb products, measured by nrCE-SDS-UV. Blue, green, red, grey and black bars correspond to %H2L, %H2, %HL, %H and %L fragments, respectively. Note that the % of fragment axis is discontinuous and scaling is changing after the gap. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

of each peak and an average RSD of 2.2 % (Remicade), 5.5 % (Remsina) and 2.3 % (Inflectra) for the relative percentages of fragments. The amount of H2L2 was comprised between 92.1 % and 95.7 % for these three mAbs and overall, the biosimilar electropherograms mirrored the one of the reference product to a very high degree, by showing a very similar fragmentation pattern, with H2L species representing the main subunit. Interestingly, the relative percentages of H2L and L fragments of the biosimilar products were slightly higher than that of the innovator product (Table 1), specifically 4.9 % *vs.* 2.3 % for the H2L and 0.7 % (Remsina) and 1 % (Inflectra) *vs.* 0.4 % (Remicade) for the L species. Despite this, the sum of all relative peak areas other than the principal peak was always lower than 8 % for all samples, suggesting a possible acceptance criterion for biosimilarity assessment in nrCE-SDS conditions [18–20].

3.2. Behaviour of IgG1 subclass with respect to κ or λ light chain type

The generic nrCE-SDS method was applied to investigate the behaviour of IgG1 subclass, with respect to their light chain type (κ or λ). The main structural difference between these species is that the Cys residue in the light chain responsible for the interchain binding with the heavy chain is located at the end of the amino acid sequence in IgG1κ, whereas it is followed by a Ser residue in IgG1λ.

Fig. 5 shows the electropherograms corresponding to two huIgG1κ (adalimumab and ofatumumab) and two huIgG1λ (avelumab and belimumab) that were investigated for this scope. As reported in Table 1, the relative amount of H2L2 in huIgG1κ was equal to 95.9 % (ofatumumab) and 96.2 % (adalimumab), whereas it was significantly lower for the huIgG1λ, namely 75.7 % and 69.4 for belimumab and avelumab, respectively (Fig. 2). As shown in Fig. 3, the main fragment of the huIgG1κ products was represented by the H2L (relative amount between 2.2 % and 2.6 %), while all the other fragments were observed in much lower amounts (between 0.1 % and 1.1 %). A completely different scenario was offered by the huIgG1λ products, that presented a fragmentation pattern in which the main subunits were represented by H2L (>13 %), H2 (>5 %) and L (>2 %), while the other subunits were in agreement with the general tendency of HL and H fragments (between 0.2 % and 0.4 % for HL and 0.6 % for H). Despite the evidence of the experimental data, the unexpected and unusual increasing of the relative percentages of the H2L, H2 and L subunits of the huIgG1λ subclass could not properly reflect a native fragmentation pattern, but more probably be due to thermal artefacts originating from the sample preparation conditions (denaturation step at 70 °C for 10 min). In

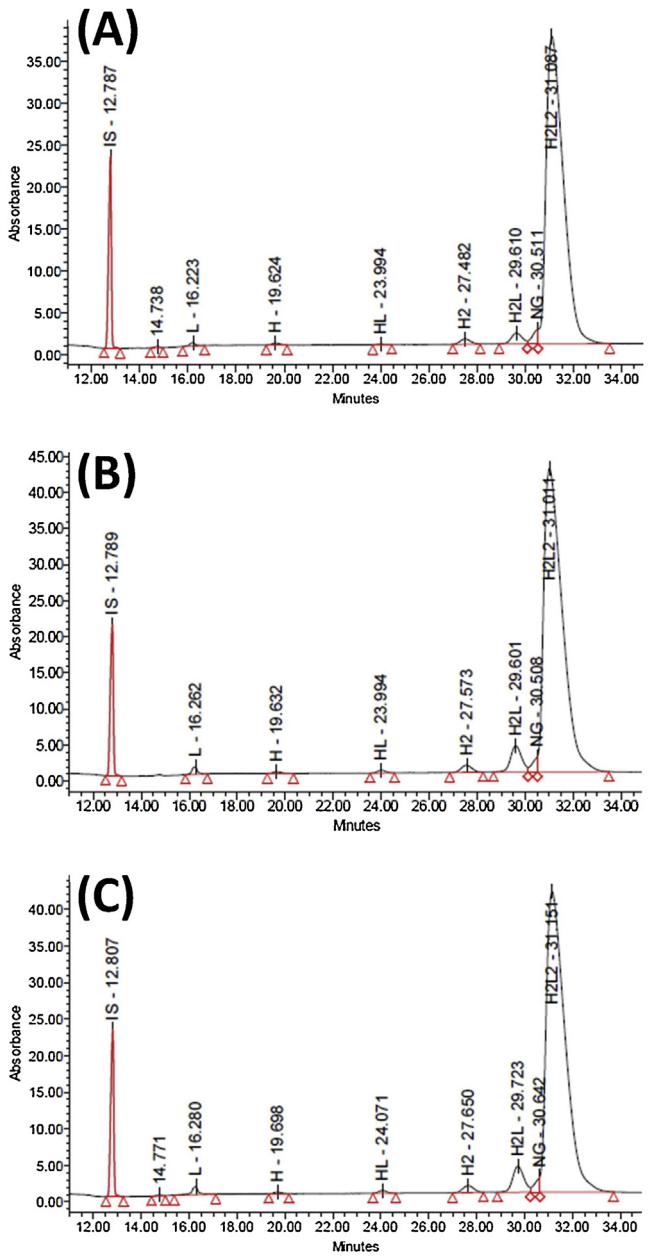

Fig. 4. nrCE-SDS electropherograms corresponding to: (A) originator infliximab, chIgG1κ (Remicade), and two EU biosimilars products, namely (B) Remsima and (C) Inflectra.

fact, as reported by Liu et al. [21], the presence of the terminal Ser in IgG1λ has a significant impact on the stability of the interchain L-H binding, making this bond weaker than the disulfide bond of the interchain H—H binding. All together these evidences are in line with our results. In fact, a fragmentation pattern in which the main subunits are represented by exceptionally higher relative percentage of H2L, H2, and L fragments may be interpreted as a direct consequence of fragility and rupture of the H–L binding with the consequent loss of L and releasing of HH species (H2L and H2 in this case). By taking note of this behaviour, the application of the generic nrCE-SDS method for the analysis of huIgG1λ may be biased by the sample preparation step that would require further optimization to be successfully applied to this peculiar subclass.

3.3. Behaviour of IgG2 subclass products in nrCE-SDS

Fig. 6 shows the electropherograms corresponding to two humanized IgG2κ (i.e., panitumumab and denosumab), and one humanized IgG2/4κ (eculizumab; CH1 and hinge domains from an IgG2; CH2 and CH3 from an IgG4). Again, the generic method allows a direct evaluation of the amount of H2L2 and their fragments in less than 40 min. As reported in Table 1, the amount of H2L2 was comprised between 94.6 and 96.9 % for these three mAbs. The main fragment found in these products was the H2L, having a mass of about 125 kDa (relative amount between 2.6 and 4.6 %). The other fragments were observed in much lower amounts (between 0 and 0.5 % only).

Another observation that can be made from Fig. 6 is that the H2L2 peak of panitumumab was separated as a doublet and exhibits therefore a specific heterogeneity. This peak splitting is probably related to the presence of molecular species with different covalent structures, as previously reported in the literature for IgG2 subclass. Indeed, Guo et al. have also highlighted the presence of two main peaks for IgG2 subclass in nrCE-SDS, which were not an artefact or incomplete denaturation. They showed that these two isoforms reflect different hinge isomers, corresponding to different disulfide bond-related species [22]. To better understand this observation, it is important to come back to the structure of IgG. Although all subclasses (IgG1, IgG2 and IgG4) share more than 90 % of homology in their amino acid sequences, the structure of their hinge regions and the disulfide bond connectivity differ significantly. In particular, IgG1 and IgG4 contain two disulfide bridges in the hinge region linking the heavy chains, while IgG2 contains four disulfide bridges in the hinge region linking the two heavy chains together [23]. Therefore, some authors have hypothesized that the consensus structural model of human IgG2 is only a minor form, among several disulphide-related structural isoforms (isoforms having different cysteine connectivity) [24]. The resolution achieved by nrCE-SDS technique was not sufficient to baseline separate the two isoforms present in the samples, but these species were partially separated (presence of a doublet), related to the fact that the two isoforms possess identical mass, but different conformations that could be differentiated by nrCE-SDS.

A similar behaviour was also detected with denosumab (Fig. 6). However, in this case, a doublet was not observed on the H2L2 peak, due to limited selectivity, but the peak was quite large and comparable to the one of panitumumab (1 min width at half height), and much broader than the average H2L2 peaks observed for the other therapeutic mAbs (0.6 min width at half height in average). This confirms that several species might coexist below the H2L2 peak of panitumumab, which could correspond again to different disulfide bond-related species.

The case of eculizumab (Fig. 6) was quite different, since this therapeutic antibody has been created from the combination of IgG2 and IgG4 heavy chain sequences to form a hybrid constant region unable to bind Fc receptors or to activate the complement cascade [25]. In this sample, the hinge region is similar to the one of IgG1 or IgG4, with only two disulfide bridges linking the heavy chains. Therefore, this sample was not prone to the heterogeneity of the disulfide bond-related species and only one single peak was observed for the H2L2 species (0.6 min half height width).

In conclusion, the results obtained for the size-based variants of IgG2 subclass products can be considered as reliable, and the isoforms partially separated on the H2L2 species can be considered as one single peak for calculation of relative fragments percentages. It is also important to keep in mind that nrCE-SDS has potentially the ability to differentiate disulfide bond-related species of IgG2, but further optimization of the analytical conditions would be required, as the selectivity remains too limited with the generic conditions employed in this study.

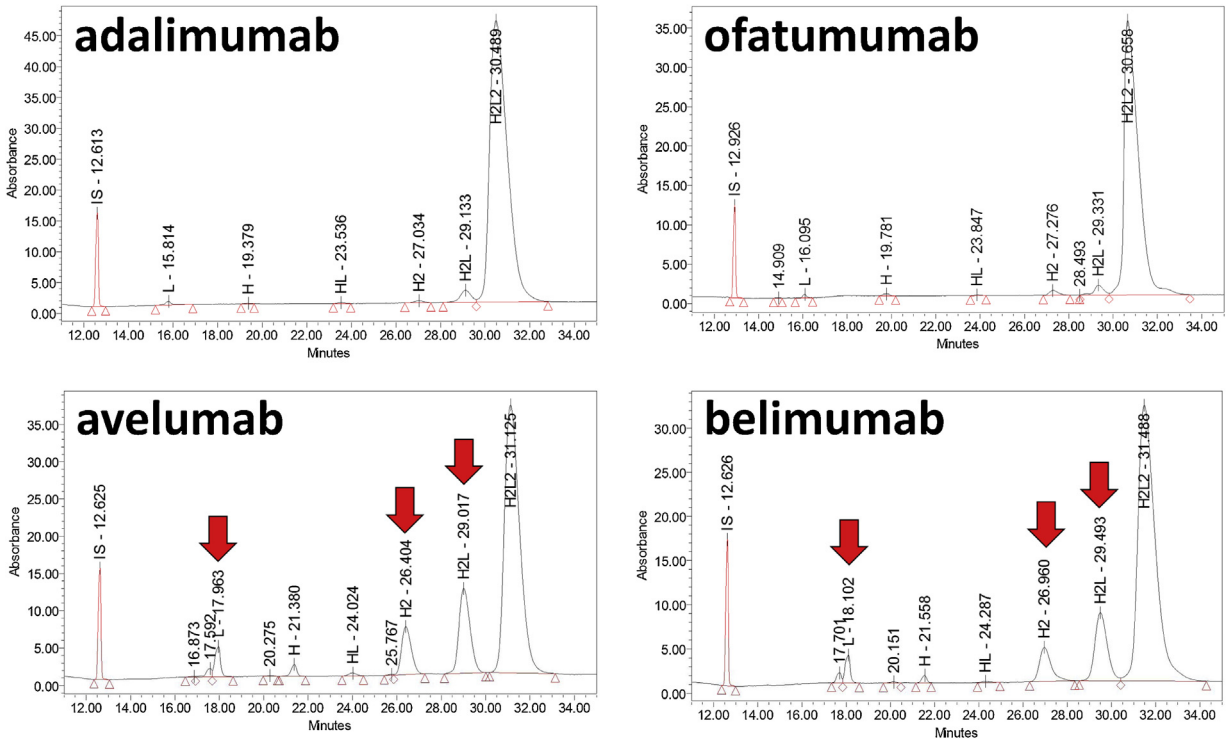

Fig. 5. huIgG1κ (adalimumab, ofatumumab) vs huIgG1λ (avelumab, belimumab).

3.4. Behaviour of IgG4 subclass with respect to hinge stabilized formats

Next to the cases of IgG1 and IgG2, IgG4 were also investigated using the generic nrCE-SDS method. For this purpose, four different mAb products belonging to the IgG4 subclass were selected, including two wild type HzIgG4k (natalizumab and reslizumab) and three hinge-stabilized hzIgG4k (ixekizumab, pembrolizumab) or huIgG4k (nivolumab) biopharmaceutical products.

As shown in Table 1, the generic method is useful to characterize the IgG4 products, and the amount of H2L2 species varies between 93.7 and 96.3 %. Similarly, to what was observed for the other IgG subclasses, the main fragment found in these five products was the H2L having a mass of about 125 kDa (relative amount between 1.6 and 3.9 %). The other fragments were observed in very low amount (between 0.1 and 0.5 % only). The main difference compared to the other investigated therapeutic mAbs was related to the %HL species, which was unusually high, and equal to 2.2 and 3.9 %, for the two wild types IgG4, namely natalizumab and reslizumab, respectively. This differentiation between wild type and hinge stabilized IgG4 products can be rapidly and simply monitored by a visual inspection of the electropherograms reported in Fig. 7. Indeed, the peak migrating at about 23 min, which corresponds to the HL species was clearly visible for the wild type IgG4 (Fig. 7, upper panels) and almost invisible for the hinge stabilized IgG4 (Fig. 7, bottom panels).

As reported for example by Labrijn et al. [26], wild type IgG4 products can form half mAbs (one heavy and one light chain, 75 kDa) *in vitro* and *in vivo* through a process termed Fab-arm exchange, when they are not stabilized by a serine-to-proline mutation in the hinge region [26]. This characteristic of wild type IgG4 may be attributed to: i) the flexibility associated with the IgG4 hinge region, ii) the relatively labile disulfide bonds between the two heavy chains in the hinge region and, iii) the non-covalent interactions between the two CH3 domains of the two heavy chains, thus facilitating the H–L pair exchange between two IgG4s [27].

In conclusion, the developed nrCE-SDS method also allows an easy evaluation of the amount of size variants in IgG4 subclass products, and the differentiation between wild type and hinge stabilized IgG4 can be rapidly assessed, simply after a visual inspection of the electropherograms (Fig. 7).

3.5. nrCE-SDS used during process optimization case study

nrCE-SDS is also a key QC method during mAb production process optimization. Fig. 8shows nrCE-SDS of two different batches of the same antibody (HzIgG1k) produced in CHO cells in two different culture conditions. Batch #1 shows a size-variant profile consistent with other therapeutics mAbs. Batch #2 shows many LMWs identified by off-line mass spectrometry as H, HL and H2L species. Such profiles have been reported by *Du et al.* during large-scale mAb manufacturing and caused by disulfide bond partial reduction [28]. Many investigations have been conducted across the biopharmaceutical industry to identify the reasons (*e.g.* cellular reducing components or depletion of dissolved oxygen) and diverse strategies have been proposed to mitigate these issues both during upstream and downstream processing. Clearly, nrCE-SDS combined to mass spectrometry are mandatory methods that should be used all along the development phase.

3.6. Behaviour of ADC products in nrCE-SDS

The applicability of the generic nrCE-SDS method has been studied for two types of second-generation ADCs, namely for hinge cysteine conjugated brentuximab vedotin and for lysine conjugated trastuzumab emtansine.

In hinge cysteine conjugated ADCs, reduced inter-chain disulphides yielding free thiol groups are conjugated for example to maleimide drug-linker payloads (LPs).These ADCs are characterized by certain drug load distribution (DLD), drug-to-antibody ratio (DAR) and by an H2L2 structure that is maintained only by electrostatic interactions occurring between the chains. For example, in

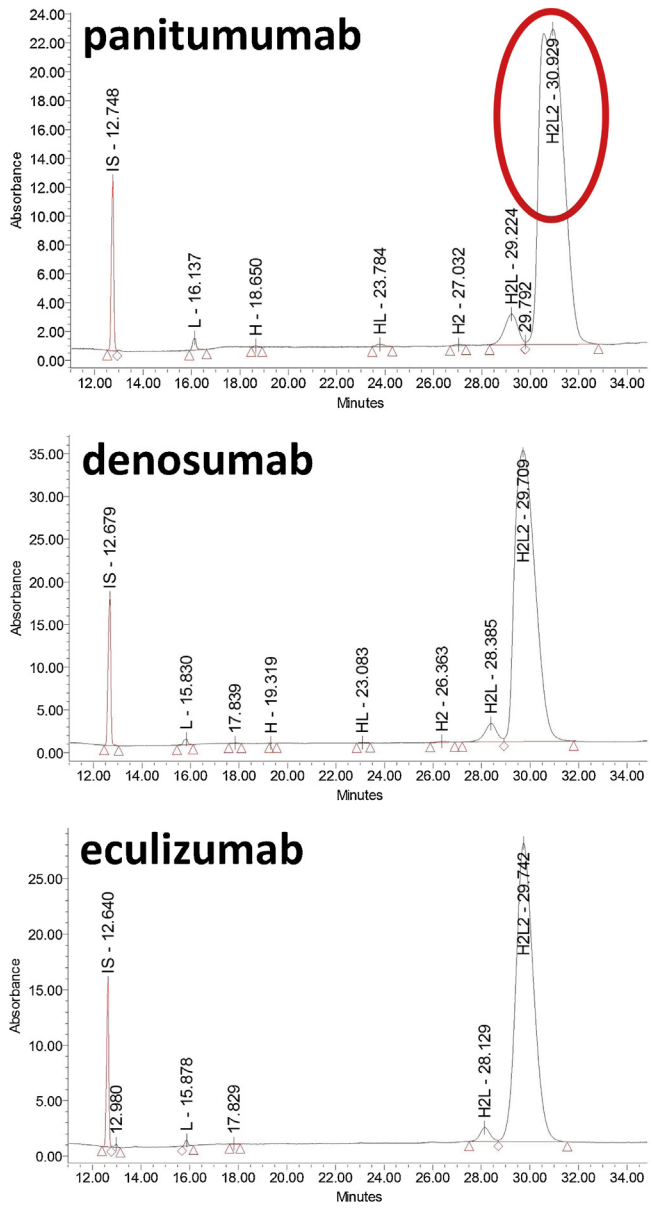

Fig. 6. huIgG2k (panitumumab, denosumab) and hzIgG2/4κ (eculizumab).

the case of DAR8 ADCs trastuzumab deruxtecan and sacituzumab govitecan (the latter currently in clinical phase III), there are no remaining interchain disulphide bridges which is not deleterious for manufacturing, safety and PK/PD. For such ADCs, DLD and properties related to the native ADC are preferably measured by using mild non-denaturing conditions. In the case of brentuximab vedotin reported here (Table 1), only 8.1 % H2L2 species are found that is consistent with the amount of naked antibody assessed by HIC or native-MS. The L (+ 1 linker-payload, PL), H (+ 3 LPs), HL (+ 2 LPs), H2 (+ 2 LPs), H2L (+ 1 LP) and H2L2 species can be separated with baseline resolution (Fig. 9a). NrCE-SDS is not the method of choice to analyse hinge cysteine linked ADCs DLD and DAR but may be used as identification method and for batch consistency. Hydrophobic interaction chromatography (HIC), native mass spectrometry, or reversed phase liquid chromatography (RPLC) in combination with disulfide reduction or size exclusion chromatography (SEC) are more frequently used as QC methods for batch release or characterization [29].

Trastuzumab emtansine is a lysine conjugated ADC which consists of a humanized anti-HER2 IgG1 and emtansine (DM-1) payloads conjugated *via* non-cleavable linkers. Lysine is a prevalent amino acid present in mAb sequences. Among several possible conjugation sites of trastuzumab, only around 20 are highly solvent accessible and capable of conjugation. The resulting mixture of conjugated species is unprecedentedly heterogeneous and shows drug load distribution from 0 to 8. However, with lysine conjugations, the H2L2 structure of those ADCs is maintained by the inter-chain disulphide bridges. Therefore nrCE-SDS can be a useful tool to determine and separate monomer and fragmented species as illustrated in Fig. 9b. All the L, H, HL, H2, H2L and H2L2 species are well separated. The H2L2 native monomeric form is present in about 96 %, like in trastuzumab (the parent naked antibody).

It is worth mentioning that currently the so called third generation ADCs are based on engineered mAbs possessing conjugation sites at defined positions (not on cysteines of hinge region inter-chain disulphides), enabling the production of much more homogeneous ADCs [30]. For those ADCs – similarly to lysine linked conjugations - nrCE-SDS is a powerful tool to analyse molecular fragments and determine the amount of H2L2.

4. Conclusion

The goal of the present work was to highlight the applicability of a generic nrCE-SDS method for the determination of size-based variants (H2L2 and LMWs) of a wide range of therapeutic antibodies having different isotypes and light chain specificities. For this purpose, 26 FDA and EMA approved mAbs and two ADCs were selected.

From the results obtained in this study, it is clear that the repeatability was excellent, both in terms of relative migration times and relative percentages of fragments *vs.* intact mAb. The method has been successfully applied to IgG1, IgG2 and IgG4 subclasses.

For IgG1, it appears that only the mAb products belonging to the specific IgG1λ subclass were prone to denaturation during the sample preparation at 70 °C and cannot be optimally analyzed with the generic nrCE-SDS conditions developed here.

For IgG2, some isoforms can be partially separated on the H2L2 species, corresponding to different disulfide bond-related species. The selectivity of the generic method employed here is too low to clearly discriminate these isoforms, but this is out of the scope of the present work. Importantly, the species contained below the H2L2 peak, can be considered as one single species for accurate calculation of relative fragments percentages.

For IgG4, the developed nrCE-SDS method allows an easy differentiation between wild type and hinge stabilized IgG4, simply after a visual inspection of the electropherograms.

For ADC samples, the developed strategy was successfully applied for lysine linked ADC products (trastuzumab emtansine), and for Cysteine hinge conjugated ADC (brentuximab vedotin).

In the end, we can conclude that this method can be used for release, stability, batch consistency and shell life of biopharmaceutical products in a QA/QC environment. Based on the results observed for 28 biopharmaceutical products, the specification for intact IgG (H2L2 species) with kappa light chain in therapeutic mAbs can be fixed at not less than 90 %.

CRediT authorship contribution statement

Elsa Wagner: Methodology, Investigation. **Olivier Colas:** Methodology, Investigation. **Stéphane Chenu:** Methodology, Investigation. **Alexandre Goyon:** Writing - original draft. **Amarande Murisier:** Writing - original draft. **Sarah Cianferani:** Conceptualization. **Yannis François:** Conceptualization. **Szabolcs Fekete:** Writing - original draft. **Davy Guillarme:** Supervision, Writ-

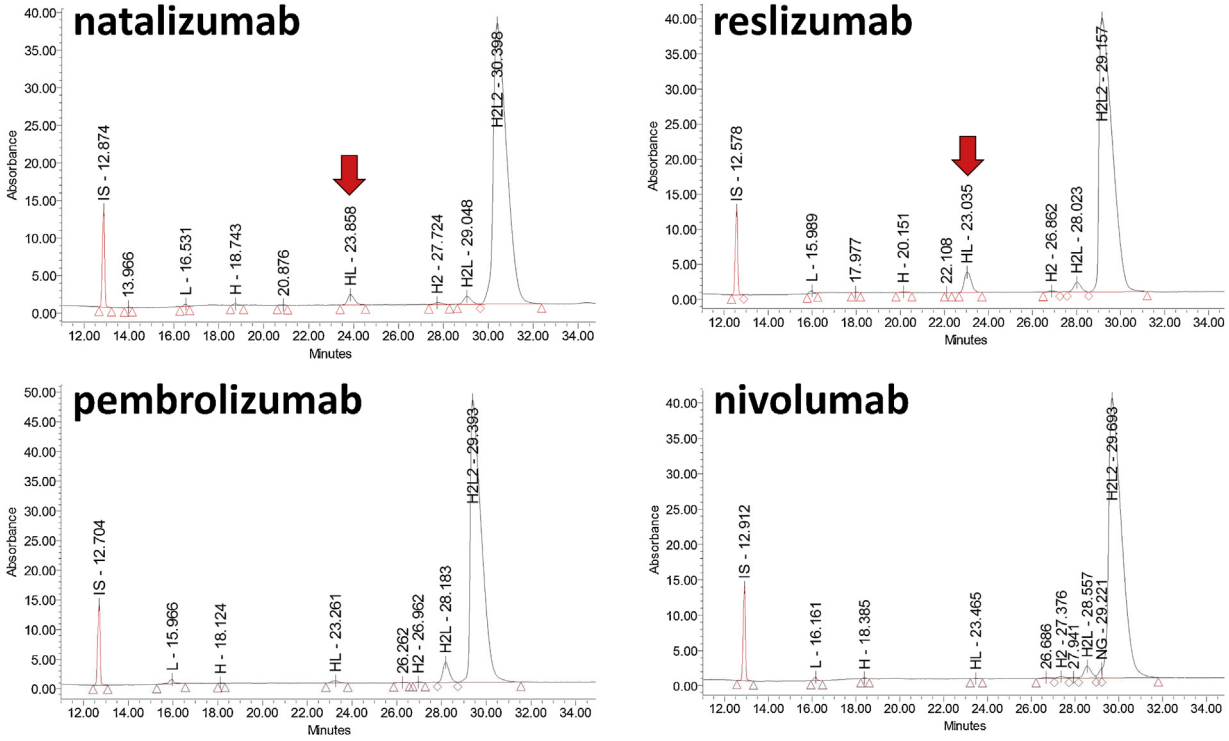

Fig. 7. Wild type hzIgG4κ (natalizumab, reslizumab) and hinge stabilized hzIgG4κ (pembrolizumab) and huIgG4κ (nivolumab).

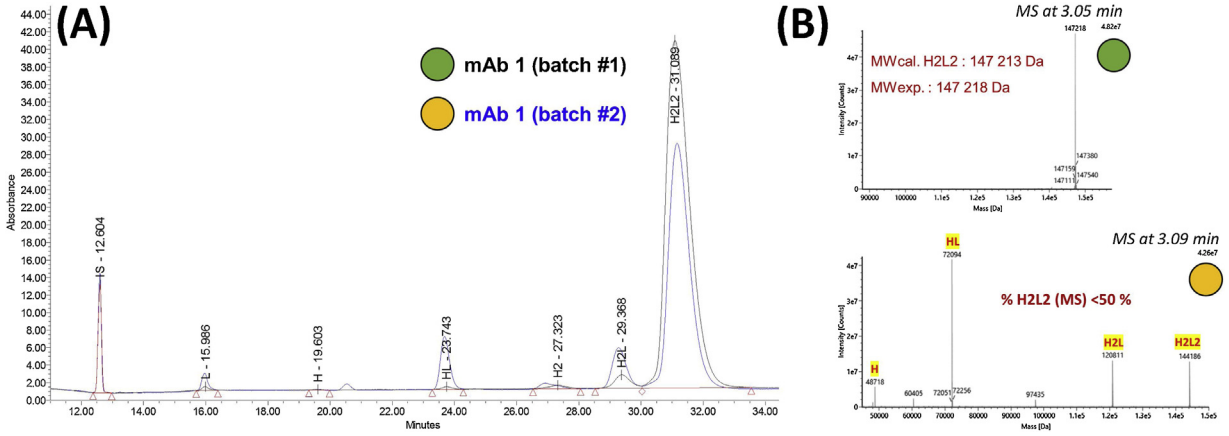

Fig. 8. (A) QC method involving nrCE-SDS-UV for two batches of the same partially reduced mAb product. (B) Mass spectrometry structure assessment QC.

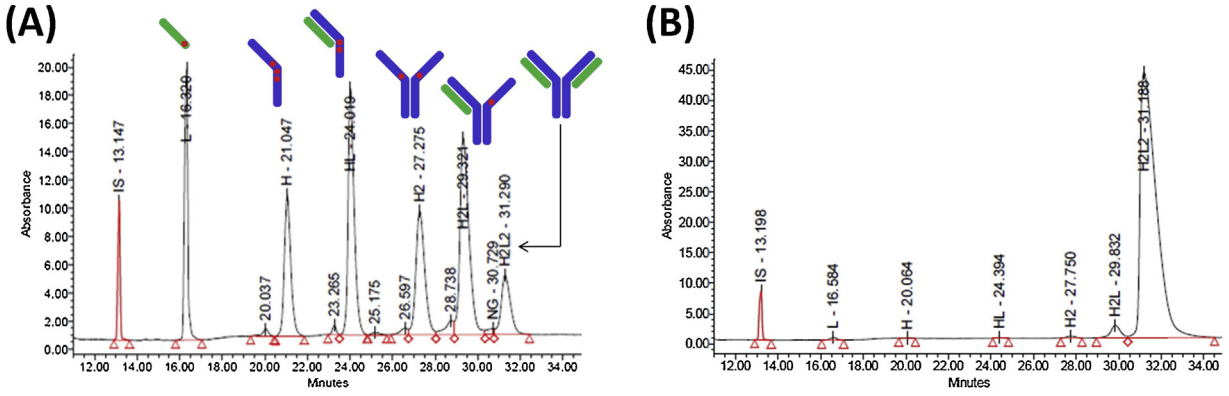

Fig. 9. nrCE-SDS-UV electropherograms corresponding to two commercial ADC samples: (A) chIgG1κ Cys hinge conjugate ADC (brentuximab vedotin) and (B) hzIgG1κ Lys conjugate ADC (trastuzumab emtansine).

ing - review & editing. **Valentina D'Atri:** Visualization, Writing - review & editing. **Alain Beck:** Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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