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Soraya CHAPEL[a], Florent Rouvière[a], Pierre Guibal[b], Delphine Mathieu[b], Sabine Heinisch[a]*

[a]*Université de Lyon, Institut des Sciences Analytiques, UMR 5280, CNRS, 5 rue de la Doua, 69100, Villeurbanne, France*

[b]*Sanofi Aventis R&D, 1 impasse des Ateliers, 94400 Vitry-sur-Seine, France*

(*)Corresponding author: Tel: +33 437 423 551, E-mail: sabine.heinisch@univ-lyon1.fr

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Abstract

This study details the development of on-line 2D-LC methods combining cation-exchange chromatography (CEX) and reversed-phase liquid chromatography (RPLC) for the separation of the charge variants of a lysine-linked antibody-drug conjugate (ADC). This combination gives an excellent example of the potential benefits of 2D-LC approaches for the analysis of such complex protein formats. CEX is considered the reference technique for the separation of protein charge variants but its retention mechanism usually requires the use of a high concentration of non-volatile salts, which impedes its compatibility with MS detection. In this context, the use of an on-line 2D-LC-MS approach not only allows on-line desalting and indirect coupling of CEX with MS detection but it also provides increased and complementary information within a single analysis. The first part of this study was devoted to the choice of stationary phases and the optimization of chromatographic conditions in both dimensions. Based on the results obtained in 1D-CEX with ultraviolet detection (UV) and 1D-RPLC with UV and high-resolution mass spectrometry (HRMS) detections, an on-line comprehensive two-dimensional liquid chromatography method combining CEX and RPLC was developed. The last part of this study was devoted to the identification of the separated species using HRMS detection and in the comparison of three ADC samples exposed to different durations of thermal stress.

1. Introduction

Antibody-drug conjugates (ADCs) belong to a new and fast-growing class of highly potent biotherapeutic drugs targeted for the treatment of cancer, autoimmune, and inflammatory diseases [1]. Structurally speaking, an ADC is composed of a monoclonal antibody (mAb) covalently linked to small molecule drugs via a chemical linker. Monoclonal antibodies are highly complex "Y-shaped" tetrameric glycoproteins with molecular weights near 150 kDa that are composed of two identical heavy chains and two identical light chains held together by disulfide bonds [2,3]. They are thus large and structurally complex molecules.

Product heterogeneity, introduced during manufacturing or storage, is very common for these compounds but can influence biological activity, product stability, and product safety [4,5]. For this reason, a comprehensive characterization of ADCs throughout their research and development process is mandatory. The analytical characterization of ADCs requires the investigation and monitoring of numerous critical quality attributes (CQAs), including drug distribution, average drug-to-antibody ratio, amount of unconjugated mAb, site occupancy, glycosylation patterns, protein aggregation, size variants, and charge variants [6]. In practice, such a thorough characterization often involves multiple complementary methods based on electrophoresis, liquid chromatography (LC), and mass spectrometry (MS) [6–11].

Over the past few years, two-dimensional liquid chromatography coupled with mass spectrometry detection (2D-LC-MS) has emerged as an attractive analytical approach to address the challenges associated with the characterization of these complex molecules [12–14]. They have been proven to be rapid and efficient analytical tools: (i) to provide a very high resolving power by increasing the overall peak capacity compared to conventional one-dimensional liquid chromatography (1D-LC) and (ii) to allow MS coupling with chromatographic techniques that are not compatible with MS detection to facilitate structural elucidation. The characterization of ADCs can be achieved from three different and complementary approaches: top-down (analysis of intact protein), middle-up (analysis after protein cleavage into large subunits between 25 and 100 kDa) or bottom-up (analysis after protein cleavage into peptides < 7 kDa), each of them giving specific information on the protein.

Because of their potential influence on biological activity and product stability, charge variants are considered as critical quality attributes of ADCs. Cation-exchange chromatography (CEX) is the gold-standard technique to separate protein charge variants in liquid chromatography but it usually requires a large amount of salt which makes it incompatible with MS detection. In CEX, the use of volatile buffers such as ammonium acetate was reported for mAb analysis [15–19]. The main problem is the salt concentration able to combine good chromatographic resolution and high MS sensitivity. CEX was also successfully combined with reversed-phase liquid chromatography (RPLC) as

second dimension in order to (i) desalt prior to MS and (ii) provide additional selectivity for in-depth characterization of mAbs and related biomolecules [20–23] Such a combination not only allows the direct assignment of ^{1}D-CEX peaks by MS detection but it also greatly extends, in a single analysis, the level of information about the compounds present in the sample. Both the multiple heart-cutting mode (mCEX-RPLC) and the selective comprehensive mode (sCEX x RPLC) were applied to mAbs and/or ADCs using top-down and middle-up approaches [20,21,24]. The comprehensive mode (CEX x RPLC) was also applied to mAbs using top-down and middle-up approaches [22,24,25], and to both mAbs and ADCs using a bottom-up approach (i.e. analysis at the peptide level) [20,23]. Until now, on-line CEX x RPLC has never been applied to the characterization of ADCs in a top-down or a middle-up approach.

The objective of the current work was to develop an on-line comprehensive CEX x RPLC method, operating in less than one hour, for in-depth characterization of the charge variants of a lysine-linked antibody-drug conjugate belonging to the immunoglobulin G1 (IgG1) subclass. Herein, the focus was put on the analysis of the protein samples after cleavage into large subunits following limited proteolysis. In this work, the investigated ADC will be referred to as ADC-S01.

2. Experimental section

2.1. Chemical and reagents

Acetonitrile (ACN, LC-MS grade) was obtained from Sigma-Aldrich (Steinheim, Germany). Water was purified and deionized by an Elga Purelab Classic UV purification system (Veolia water STI, Le Plessis Robinson, France). Formic acid (FA, LC/MS grade), trifluoroacetic acid (TFA, LC/MS grade), sodium chloride (NaCl), 2-(N-morpholino) ethanesulfonic acid (MES), sodium hydroxide (NaOH), monosodium phosphate (NaH_2PO_4), and disodium phosphate (Na_2HPO_4), all analytical grade, were obtained from Fischer scientific (Illkirch, France). IdeS protease enzyme (FabRICATOR) was obtained from Genovis (Lund, Sweden).

The investigated samples of ADC-S01 were provided by Sanofi Aventis R&D (Vitry sur Seine, France) and were formulated at 5 mg/mL. Thermally-stressed samples were obtained by exposing ADC-S01 to elevated temperature (40°C) for either two weeks or four weeks. In this work, the unstressed, stressed for two weeks, and stressed for four weeks samples will be referred to as ADC-t0, ADC-t2, and ADC-t4, respectively.

2.2. Sample preparation

The antibody-drug conjugate studied was a lysine-linked ADC incorporating an antibody of the immunoglobulin G1 (IgG1) isotype with isoelectric points ranging from 8.5 for the naked mAb to 7.1 for most conjugated species. ADC samples were injected at their formulated concentration, without any pre-treatment nor prior dilution. Partially digested ADC samples were obtained using IdeS protease enzyme (FabRICATOR) following the instructions provided by the manufacturer (i.e. one unit of enzyme/µg of ADC incubated at 37°C for 30 min). Partial digestion with IdeS cleaves the protein below the hinge region of the heavy chain, which leads to the formation of two Fc/2 subunits and one F(ab')2 subunit [26].

2.3. Columns

Two columns were evaluated for the first CEX dimension: BioResolve SCX (100 x 2.1 mm, 3 µm) from Waters (Milford, MA, USA) and Bio MAb NP5 (250 x 2.1 mm, 5 µm) from Agilent Technologies (Waldbronn, Germany).

Five columns were evaluated for the second RPLC dimension: BioResolve RP mAb polyphenyl (50 x 2.1 mm, 2.7 µm) and Acquity BEH C4 (50 x 2.1 mm, 1.7 µm) from Waters (Milford, MA, USA), Biozen intact XB-C8 (50 x 2.1, 3.6 µm) from Phenomenex (Torrence, CA, USA), PLRP-S (50 x 2.1mm, 5 µm) and AdvanceBio RP-mAb diphenyl (50 x 2.1mm, 3.5 µm) from Agilent Technologies (Waldbronn, Germany).

2.4. Instrumentation

1D-LC-UV column evaluations were carried out with an Acquity UPLC liquid chromatography system from Waters (Milford, MA, USA). The instrument includes a high-pressure binary solvent delivery pump, a sample manager with a 20-µL loop, a column manager equipped with a column oven with a maximum temperature of 90°C, and a diode-array detector equipped with a 0.5-µL flow-cell withstanding pressure up to 70 bar. The dwell volume and extra-column volume for this entire system were measured using a zero-dead volume union connector in place of the column and were respectively 110 µL and 12 µL.

Other 1D-LC-UV-HRMS experiments were carried out using the first dimension of a 1290 Infinity series 2D-LC system (see description of the instrument below).

On-line LC x LC experiments were carried out using a 1290 Infinity series 2D-LC system from Agilent Technologies (Waldbronn, Germany). The instrument includes two high-pressure binary solvent delivery pumps, an autosampler with a flow-through needle of 20 µL equipped with a 160-µL

extension loop when needed, two thermostated column compartments with a maximum temperature of 100 °C equipped with low-dispersion preheaters, and two diode-array detectors equipped with 0.6-µL flow-cells.

The interface connecting the two dimensions consisted of a 2-position/4-port duo valve, equipped with two identical 80-µL loops. To minimize dispersion, the valve was configured in back-flush injection mode. A pressure release kit placed between the ^{1}D-outlet and the interface was used to minimize the pressure downstream induced by the switch of the 2D-LC valve to protect the flow-cell and avoid artefacts in the signal. The measured dwell volumes and extra-column volumes of this 2D-LC system were respectively 170 µL and 22 µL in ^{1}D, and 80 µL and 8.5 µL in ^{2}D (loop volume at the interface excluded).

The 2D-LC system was hyphenated to an Agilent G6560B Q-TOF mass spectrometer, equipped with a JetStream electrospray ionization (ESI) source. For the LC x LC experiment, a diverter valve was placed between the 2D-LC and MS instruments to redirect part of the ^{2}D-effluent to the waste following each injection and prevent contamination of the MS source with non-volatile NaCl.

Data acquisition and instrument control were performed using Agilent OpenLab software for 2D-LC and MassHunter software for MS.

MS data processing was performed using Agilent MassHunter qualitative analysis software for MS data and Agilent BioConfirm software for deconvolution of the MS spectra. 2D-LC data were processed using an in-house script developed on Matlab.

2.5. Chromatographic and detection conditions

The analytical conditions used in 1D-CEX and 1D-RPLC for developing and optimizing the 2D-LC methods are specified in the respective figure captions and in the text.

The conditions for the optimized CEX x RPLC method are given in Table 1.

3. Results and discussion

3.1. Development and optimization of 2D-LC conditions

A 2D-LC separation is the combination of two 1D-LC experiments. As part of method development, each dimension (i.e. CEX on one hand and RPLC on the other hand) was individually optimized with a view to maximize the separation power. The investigated parameters were: the nature of the

stationary phase, the nature and the composition of the mobile phase, the gradient conditions, and the injection conditions.

3.1.1. Choice of stationary phase and mobile phase buffer in 1D-CEX

The first step of this study aimed at finding a suitable CEX method for separating charge variants in the first dimension. For this purpose, we evaluated several stationary phases and mobile phase buffers. In the literature, the Agilent Bio MAb NP5 column was exclusively used in ^{1}D-CEX for the 2D-LC separation of mAbs and related products [20–22], with either MES [20,21], phosphate [20] or ammonium acetate [22] as mobile phase buffers.

In addition to this column, we evaluated the BioResolve SCX column from Waters. Both columns are packed with non-porous polymeric-based particles and are specifically designed for the separation of the charge isoforms of biotherapeutic proteins. Non-porous polymeric particles are usually preferred for protein analysis in CEX. They enable to work across a wide range of mobile phase pH and provide better chromatographic performance by limiting trans-particle mass transfer resistance [27], which can be a critical issue for large proteins. The Bio MAb NP5 column contains weak cation-exchange sites (WCX), whereas the BioResolve SCX column contains strong cation-exchange ones (SCX). Because SCX phases employ strong acids, the number of charges on their surface is expected to remain constant over a broad pH range, which makes them attractive in CEX. In contrast, WCX phases employ weak acids that gradually lose their ionization when the pH decreases below pH 5 [28]. It is important to be aware of such limitations during method development. However, it should be pointed out that, in our study, the conditions were operated over a small pH range between 6.5 and 7.6, in which both columns are expected to deliver optimal performance.

In CEX, protein elution may be performed with either salt gradients, pH gradients or salt-mediated pH gradients (combined salt/pH gradients) [28–30]. In this work, we used a classical salt gradient for the separation, as this is the most frequently used mode for the separation of protein charge variants [28]. Sodium chloride (NaCl) was used for the salt gradient while two different mobile phase buffers were evaluated for the separation (MES and phosphate). In CEX, the separation mechanism relies on the binding of positively charged analytes on a negatively charged stationary phase. Because a positive charge is removed from the molecule when a lysine is conjugated with a drug, increasing drug conjugation is expected to lead to decreasing retention. For this reason, the influence of the mobile phase pH on the separation was also investigated. Analyte retention was found to be insufficient above pH 7.6 and the best separation was achieved with pH 6.5 (Fig. S1), which was

expected given the isoelectric point range (7.3 < pI < 8.5) of the expected isoforms (8.5 corresponding to the non-conjugated mAb).

Evaluation of the two sets of stationary phase and mobile phase buffers was performed on IdeS-treated ADC-S01 sample at pH 6.5. The results obtained for this comparison are shown in Fig. 1, which draws attention to the effect of the buffer for a given column (Bio MAb in Fig. 1a and BioResolve in Fig. 1b). As can be seen, overall, the separations were quite similar with the two columns. Although the Bio Mab NP5 column gave slightly better peak separation and resolution than the BioResolve SCX column, no significant differences were found between the two columns. Therefore, we made a decision based on column dimensions and selected the BioResolve SCX column to keep the analysis time as short as possible in 2D-LC (shorter column length allowing shorter gradient time in ^{1}D). As shown in Fig. 1b, the MES buffer gave slightly better separation than the phosphate buffer and was thus selected for the ^{1}D-CEX. These results are in line with the work of Baek et al. [31] who reported a better separation for model proteins using MES buffer compared to phosphate buffer at the same pH. Those results were attributed to the zwitterionic nature of MES. This latter buffer allows for better control of pH at the surface of the column resin. It also removes interactions with protein charges, unlike the phosphate buffer.

With a view to minimize the dilution factor in ^{1}D, a brief study of the effect of the injected volume on the separation was conducted. No deterioration in peak shape was observed up to an injection volume of 80 µL (see Fig S2). This is mainly due to the excellent focusing at the head of the column when injecting in CEX a sample diluted in water.

3.1.2. Choice of stationary phase and mobile phase additive in 1D-RPLC

For the analysis of ADCs in top-down and/or middle-up, the use of a ^{2}D-RPLC separation after a ^{1}D-CEX separation provides two benefits: (i) it allows the indirect on-line coupling of CEX with MS detection and (ii) it potentially brings an additional and complementary separation to the ^{1}D-CEX.

Because the cytotoxic drugs are hydrophobic, the process of drug conjugation increases the hydrophobicity of the molecule. A separation by increasing drug load or drug-to-antibody (DAR) can thus be expected in RPLC. In this work, RPLC conditions were optimized keeping two main objectives in mind: (i) maximizing peak separation and (ii) allowing sufficient mass spectrometric sensitivity for peak identification.

In RPLC-MS analysis of proteins, the selection of the mobile phase additive is a key parameter during method development. Due to its strong hydrophobic, acidic, and ion-pairing characters,

trifluoroacetic acid (TFA) is well-known to provide the best chromatographic results for the separation of proteins in RPLC [32–34]. However, it is also well-known to provide a dramatic decrease in MS detection sensitivity due to analyte ionization suppression and spray instability. Decreasing the concentration of TFA in the mobile phase was demonstrated to be an effective strategy to preserve the chromatographic separation while maintaining enough MS sensitivity [35]. In this work, the influence of the concentration of TFA in the mobile phase on the separation was investigated on intact and IdeS-treated ADC-S01 samples. Fig. S3 shows 1D-RPLC-UV separations of both samples using 0.1% TFA (Fig. S3a) or 0.05% TFA + 0.1% FA (Fig. S3b) in the mobile phase. Decreasing the concentration of TFA in the mobile phase from 0.1% to 0.05% had no significant impact on chromatographic performance while significantly increasing MS sensitivity. It was reported that below 0.05%, the recovery of intact proteins decreased due to adsorption on the stationary phase [36]. We therefore selected a concentration of 0.05% TFA in the mobile phase for the rest of this study.

Several stationary phases were investigated for the second dimension. Except for the non-porous polymeric-based PLRP-S column, all columns contained silica-based particles with either C4 alkyl- (Acquity BEH C4), C8 alkyl- (Biozen intact XB-C8), diphenyl- (AdvanceBio RP-Mab diphenyl) or polyphenyl ligands (BioResolve RP mab Polyphenyl) bonded to superficially porous particles. Non-porous and superficially porous particles are usually preferred for protein analysis in RPLC as they limit mass transfer resistance [37]. Fig. 2 shows a comparison of RPLC-UV separations of intact and IdeS-treated ADC-S01 samples obtained with each of these stationary phases. From a chromatographic standpoint, the best results were obtained with the Bioresolve RP mAb column (Fig. 2a). The Agilent PLRP-S column (Fig. 2b) also showed good performance and could have been attractive to prevent high temperature-related silica bleeding in ^{2}D as it is a polymeric-based column. However, the very low pressure limit of this column (< 200 bar) makes it incompatible with the high flow rates that are required in ^{2}D. The Acquity BEH C4 column was also evaluated but showed poorer performance than the BioResolve RP mAb column. The comparison between these two columns can be found in Fig. S4.

3.1.3. Determination of the useful separation space in RPLC

To better optimize the second RPLC dimension, IdeS-treated ADC-S01 sample was analyzed in 1D-RPLC using both UV and HRMS detections. As shown in Fig. 3a (UV) and Fig. 3b (MS), the area of interest in the chromatogram (i.e. the time interval during which the species of interest elute) was found to be very short relative to the entire separation space. The useful separation space ranged

from about 2.5 min to 4.4 min with a gradient elution ranging from 20% to 95% B in 8.8 min. This area of interest was delimited after identifying the separated peaks based on their exact masses using HRMS detection. The peaks of interest are labelled from 1 to 7 in Fig. 3b and the corresponding assignments are given in Table 2. Assignments were made by comparing experimentally the measured masses with the expected theoretical masses. Species corresponding to Fc/2 subunits with 1 or 2 drugs were identified in the first part of the area of interest, whereas F(ab')2 subunits with drug loads from 0 to 5 were found in the second part. Several peaks with very close masses but different retention times were observed (e.g. peak #3, #4, #5, and #6a), suggesting the presence of isomeric compounds. The presence of distinct subunits with identical drug load in RPLC could be explained by drug conjugation at different lysine residues. Similar results were reported for the analysis of the commercialized lysine-conjugated ADC ado-trastuzumab emtansine (Kadcyla®) in mCEX-RPLC [20].

In the two samples that were analyzed (i.e. intact ADC and IdeS-treated ADC), the presence of numerous interfering peaks that could correspond to various polymer excipients was highlighted in the second half of the chromatogram (label #8 in Fig. 3b). Excipients were non-ionic surfactants used to prevent protein aggregation. In our study, those compounds were found to be strongly retained in RPLC due to the lipophilic part which exhibits strong hydrophobicity. Most of them were more retained than the species of interest. However, some of the earliest eluted excipients were found to coelute with the latest eluted species of interest and appeared to create significant ion suppressions in MS.

3.2. On-line CEX x RPLC analysis of IdeS-digested ADC-S01

In the context of top-down and/or middle-up analysis of mAb and ADC, both on-line multiple heart-cutting mCEX-RPLC (for ADC) [20] and full comprehensive CEX x RPLC (for mAb) [21,22] were reported in the literature.

Before fully optimizing the conditions in CEX x RPLC, we performed an on-line CEX x RPLC analysis to study the retention of the excipients in CEX and determine whether or not these compounds could be eliminated in ^{1}D. Fig. 4 shows the contour plot obtained for the analysis of the IdeS-treated ADC sample using MS detection. As seen, most of the excipients eluted before the delimited area of interest in ^{1}D-CEX (i.e. before 5 min) whereas only a small part of them (highlighted by a black circle) coeluted with the species of interest. It should be pointed out that, contrary to the observations

made in 1D-RPLC-HRMS (Fig. 3b), the different excipients elute all along the RPLC separation making MS signal intensity too low to unambiguously identify F(ab')2 species in ^{2}D-RPLC.

As previously underlined, the separation space was quite small in ^{1}D-CEX. All compounds eluted before 12 min with a gradient elution ranging from 1% to 40% B in about 40 min. Considering the reduced elution range in ^{1}D, the conditions in CEX was later further optimized by decreasing the upper composition range to decrease the gradient slope and hence improve the separation.

Conditions were further optimized in full comprehensive mode, keeping three main objectives in mind: (i) removing interfering excipients as much as possible, (ii) finding sub-hour conditions, and (iii) maximizing MS-detection sensitivity. Given the limited elution range in CEX, the gradient time was set at 20 min with gradient compositions ranging from 1% to 17% B (total analysis time = 31.3 min). The sampling time and hence the analysis time in ^{2}D was set at 0.78 min to maintain 2 to 3 fractions per ^{1}D-peak and minimize undersampling [38]. The strategy used to avoid MS signal suppression arising from coelutions with excipient species was to take advantage of their low retention in ^{1}D-CEX and remove them as much as possible before starting to send the fractions to ^{2}D-RPLC. As highlighted in Fig. S5, to improve the chromatographic separation while keeping a short gradient time, the gradient composition range was reduced from 30-95% B to 30-50% B. The resulting optimized conditions for this comprehensive 2D-LC method are given in Table 1. The gradient time in CEX with this column size (i.e. 10 cm) was increased as much as possible (i.e. 20 min), resulting in a total analysis time of 30 min. An increase in the gradient time up to 60 min resulted in a decrease in sensitivity without a significant gain in peak capacity.

Fig. 5 shows the resulting on-line CEX x RPLC separations of IdeS-treated ADC-S01 sample using MS detection. The separated peaks are labelled from 1 to 21 and the proposed assignments for each peak are given in Table 3. As aforementioned, the 2D-LC conditions were optimized in each dimension to maximize the peak capacity and eliminate the excipient species that were found to impact MS sensitivity. The composition range was reduced in both dimensions to fit the elution range of the species of interest regardless of the excipients. These species could be removed from the separation space of interest by starting to send the fractions in ^{2}D from 4 min or 5 min depending on the conditions (see Table 1).

As seen in Fig. 5, this comprehensive method led to a good separation of Fc/2 and F(ab')2 species in both dimensions. It is interesting to point out the good complementarity of the two chromatographic techniques for the separation of these species. Multiple peaks are observed on the contour plot, some of which being well separated in CEX but not in RPLC, and vice-versa. For example, peaks #7 and #15 have very similar retention times in ^{1}D which means that these two compounds would have

coeluted in a single 1D-CEX separation. The two peaks are, on the other hand, easily separated in ^{2}D. Similarly, peaks #7 and #8 mainly coelute in ^{2}D but are well separated in ^{1}D. This underlines that different and complementary selectivities are provided by the two dimensions.

Several peaks are observed on the contour plot, which indicates a large number of isoforms in the analyzed sample. Distinct species with the same measured mass that were identified as Fc/2 subunits with one drug were found to be separated in one or both dimensions (e.g. peaks #1 to #8). In addition, F(ab')2 fragments with drug loads from 1 to 3 could be identified in the second part of the contour plot. Similarly to the Fc/2 species, a few isobars identified as F(ab')2 with the same number of drugs were separated in CEX x RPLC (e.g. peaks #13 and #18 or peaks #16, #19 and #20). As pointed out before, some of these species could correspond to subunits with the same number of drugs but conjugated at different lysine residues. Differences in conjugated sites could explain differences in retention in RPLC for subunits displaying the same drug load (e.g. peaks #3, #5, and #7). However, such an explanation does not work for species separated in ^{1}D-CEX since such differences should not impact the net charge of the molecule (e.g. peaks #7, #8, and #9). For those compounds, the different peaks exhibiting the same mass that we observe could be the result of post-translational modifications (PTMs) that would change the charge and/or hydrophobicity of the molecule while creating very small mass differences (barely detectable in MS), such as deamidation, isomerization or racemization among others [10,22].

To summarize, as indicated in Table 3, Fc/2 subunits with 1 and 2 drugs, as well as F(ab')2 subunits with 0, 1, 2, and 3 drugs could be unambiguously identified using HRMS detection. Unlike in 1D-RPLC, F(ab')2 subunits with 4 and 5 drugs could not be identified in CEX x RPLC due to the limited MS signal obtained for these large fragments. Based on its retention times in both dimensions (peak least retained in CEX but most retained in RPLC), we suspected peak #21 to be a F(ab)2 fragment with a drug load of 4. However, the signal was too low for proper MS spectrum deconvolution. Considering the retention time of peak #21, it is most likely that any F(ab')2 fragments with a drug load of 5 eluted before the first analyzed ^{1}D-fraction (i.e. before 5 min), which explains their absence in the chromatogram. It should be pointed out that some very large mass differences were sometimes observed between experimental and expected theoretical masses (cf. Tables 2 and 3). Those differences can be attributed to post-translational modifications, especially oxidation or linker and drug modification due to thermal stress.

To improve MS sensitivity, we tried to increase the injected volume in ^{1}D-CEX (from 40 µL to 80 µL). As expected, increasing the injected volume from 40 µL (Fig. S6a) to 80 µL (Fig. S6c) resulted in a 2-fold increase in peak intensity in UV in the second dimension without affecting the separation.

However, as seen in Figs. S6b vs. S6d, poorer results were obtained in MS detection due to a significant increase of background noise that counteracted the expected increase of signal-to-noise (S/N) ratio. Consequently, increasing the injected volume in ^{1}D-CEX did not facilitate MS identification in ^{2}D-RPLC as expected but rather made it more difficult. This increase in background noise in ^{2}D when doubling the injected volume in ^{1}D is probably the result of increased matrix effects.

Fig. 5 also highlights the species that were identified in the IdeS-digested ADC-S01 sample in on-line CEX x RPLC. As can be expected, ^{2}D-RPLC allowed separating Fc/2 species from F(ab')2 species on one hand, and Fc/2 subunits according to their drug load on the other hand. F(ab')2 subunits with different drug load were poorly separated in RPLC while well separated in ^{1}D-CEX. As can be expected, the most retained F(ab')2 fragment in CEX was the one with the lowest drug load (0 in this case). Conversely, RPLC was very useful to discriminate F(ab')2 fragments exhibiting a close mass, whereas Fc/2 fragments exhibiting a close mass were better separated in CEX. Each black rectangle in Fig. 5 includes one or several peaks identified as F(ab')2 fragments with the same drug load. Interestingly, the most retained isoform in CEX was also the most retained one in RPLC, although the shift in retention was very subtle in CEX. This suggests that those additional variants might be the result of post-translational modifications (PTMs) that would simultaneously increase the net negative charge and the hydrophobicity of the protein.

3.3. Application of developed method to stability study

The stability of biotherapeutic antibodies is a critical quality attribute that is essential to assess to support formulation development, understand degradation pathways, and evaluate product shelf life, among other objectives [39]. The applicability of the developed on-line CEX x RPLC-UV-HRMS method to stability studies was investigated by analyzing three different IdeS-treated ADC samples: a non-stressed sample (ADC-t0), a sample stressed at 40°C for two weeks (ADC-t2), and a sample stressed at 40°C for four weeks (ADC-t4).

Fig. 6 shows the contour plots obtained in UV detection for the three samples. First of all, the 2D method was found to be highly repeatable between analyses (in terms of retention times), which allowed easy and fast comparison of the 2D maps. As mentioned before, the two dimensions were very complementary for the separation of these species. The direct visual comparison of the 2D maps showed marked differences between the three IdeS-digested samples. The most striking differences are indicated by black dotted circles (see Table 3 for corresponding identifications). For the large majority of peaks, intensities decrease from t0-IdeS to t4-IdeS (i.e. #1-6, 9, 10, 11, 13, and 17), until even completely disappearing for some peaks (e.g. #9, 10, and 11). These peaks correspond to Fc/2

with 1 drug (#1-6 and 9), Fc/2 with 2 drugs (#10 and 11), F(ab')2 with 1 drug (#18 and 19), and F(ab')2 with 2 drugs (#13 and 17). Other peaks, initially absent in t0-IdeS, progressively appear in the chromatogram (i.e. #22, which could not be identified). Lastly, for other species (i.e. # 16, 18, 19, and 21), the peak intensity increases from t0-IdeS to t2-IdeS but decreases from t2-IdeS to t4-IdeS. Those results emphasize the great potential of using on-line LC x LC for stability studies during ADC research and development.

4. Conclusion and future prospects

This work has led to the development of on-line CEX x RPLC-UV-HRMS method for an extensive characterization of the charge variants of a lysine-linked antibody-drug conjugate in less than 30 min.

The good complementarity between the two combined dimensions (CEX and RPLC) provided much insight into the different isoforms present in this sample and highlighted the existence of numerous species with very similar masses, possibly resulting from drug conjugations on different lysine residues or post-translational modifications. Fc/2 sub-units with 1 and 2 drugs on one hand, and F(ab')2 sub-units with drug loads from 0 to 5 drugs could be identified in the IdeS-digested sample. Direct comparison of 2D maps obtained from samples subjected to temperature-induced stress provided valuable information on possible modifications resulting from sample degradation.

One of the major issue encountered in this work was the limited MS detection sensitivity, which impeded deconvolution of some MS spectra, and thus peak identification. This limited sensitivity was ascribed to (i) the formation of highly multi-charged ions reducing peak intensity, (ii) the presence of TFA in the mobile phase, creating ion suppression, (iii) the omnipresence of excipients also creating ion suppression, and (iv) the high-salt content coming from ^{1}D-CEX, which might impact ionization efficiency despite the use of a diverted valve before MS (as suggested by the large amount of white deposits observed on the source cone at the end of each analysis). Possible solutions to alleviate the latter issue could consist in using more volatile buffers as the buffer combinations reported for the analysis of mAbs in 1D-CEX-MS [15–19] or in on-line CEX x RPLC-MS [22].

Besides an extensive optimization of the ESI-MS ionization conditions, other options to improve MS sensitivity could involve (i) a further reduction of the flow entering MS employing larger flow splitting ratios [40], (ii) the introduction of a make-up flow prior to MS in order to decrease TFA concentration without impacting the chromatographic separation [40,41] or (iii) the use of alternative mobile phase additives as was recently done for mAbs [42]. The three options were discussed by d'Atri et al. [37] in a recent review, focusing on current trends in RPLC analysis of therapeutic proteins.

To further improve both separation and detection sensitivity, the potential of other chromatographic combinations such as HILIC and RPLC could be investigated. The interest of this combination was previously demonstrated for the separation of partially digested mAbs [25] and peptides [23,43]. HILIC requires less MS-incompatible salts for the separation compared to CEX, which could be attractive for reducing ion suppression. However, this combination is also known to be very challenging for the separation of peptides and proteins due to injection issues arising from solvent strength mismatch in both dimensions [23,25,43]. Finally, the separation power should be improved by increasing the gradient time in ^{1}D but at the cost of longer analysis times and larger sample amounts to maintain similar sensitivity.

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Figure captions:

Fig. 1: 1D-CEX-UV (280 nm) analyses of IdeS-treated ADC-S01 using water + 20 mM MES at pH 6.5 or water + 10 mM phosphate at pH = 6.5 as solvent A using either (a) the Bio MAb NP5 (250 x 2.1 mm, 5 µm) column or (b) the BioResolve SCX (100 x 2.1 mm, 3 µm) column . Conditions: solvent A + 300 mM NaCl as solvent B, gradient 1% to 40% B in 20.28 min (BioResolve SCX) or 50.7 min (Bio MAb NP5), flow rate = 0.2 mL/min, temperature = 30°C, injected volume = 5 µL.

Fig. 2: 1D-RPLC-UV (280 nm) analyses of intact and IdeS-treated ADC-S01 with (a) BioResolve RP mAb polyphenyl (50 x 2.1 mm, 2.7 µm), (b) PLRP-S (50 x 2.1 mm, 5 µm), (c) Biozen intact XB-C8 (50 x 2.1 mm, 3.6 µm) and (d) AdvanceBio RP-mAb diphenyl (50 x 2.1 mm, 3.5 µm) columns. Conditions: water + 0.05% TFA + 0.1% FA as solvent A and ACN + 0.05% TFA + 0.1% FA as solvent B, gradient 20% to 95% B in 8.8 min or 7 min (PLRP-S), flow rate = 0.6 mL/min, temperature = 80°C, injected volume = 8 µL.

Fig. 3: (a) 1D-RPLC-UV (280 nm) and (b) 1D-RPLC-HRMS (TIC, ESI+) analyses of IdeS-treated ADC-S01 with BioResolve RP mAb polyphenyl (50 x 2.1 mm, 2.7 µm) column. The two dotted horizontal lines delimit the area of interest of the separation. Numbers 1 to 8 indicate the identified species in Table 2. Conditions: flow rate = 0.3 mL/min. Same other conditions as in Fig. 2.

Fig. 4: Contour plot (TIC, ESI+) of the on-line CEX x RPLC analysis of IdeS-treated ADC-S01 used for method development. Conditions in ^{1}D: flow rate = 0.1 mL/min, gradient 1-40% B in 40.56 min, V_i = 5 µL. Conditions in ^{2}D: flow rate = 1.2 mL/min, gradient 20-95% B in 1.1 min, sampling time = 1.5 min (loop volume = 180 µL). Same other conditions as for optimized CEX x RPLC in Table 1. The dotted square indicates the area of interest, the straight line indicates the time below which most of the excipients are eluted in ^{1}D-CEX and the circle shows the excipients coeluting with the species of interest in ^{1}D-CEX.

Fig. 5: Contour plots (TIC, ESI+) of optimized on-line CEX x RPLC analysis of IdeS-treated ADC-S01 sample: (a) MS detection (TIC, ESI+) and (b) UV detection (280 nm). See conditions in Table 1. Numbers from 1 to 21 labelled in (a) indicate the species identified in Table 3.

Fig. 6: Comparison of on-line CEX x RPLC-UV separations of IdeS-treated ADC samples with different levels of stress: (a) non-stressed sample, (b) sample stressed for two weeks, and (c) sample stressed for four weeks. UV detection (280 nm). Conditions are given in Table 1. Numbers from 1 to 22

636 indicate the species identified in Table 3. Main differences between samples highlighted by black
637 dotted circles.

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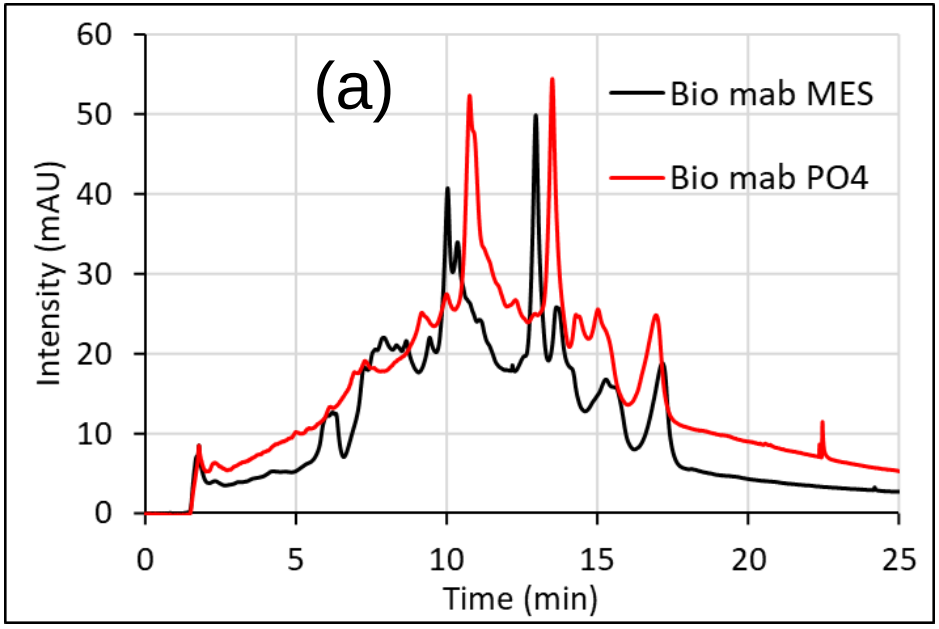
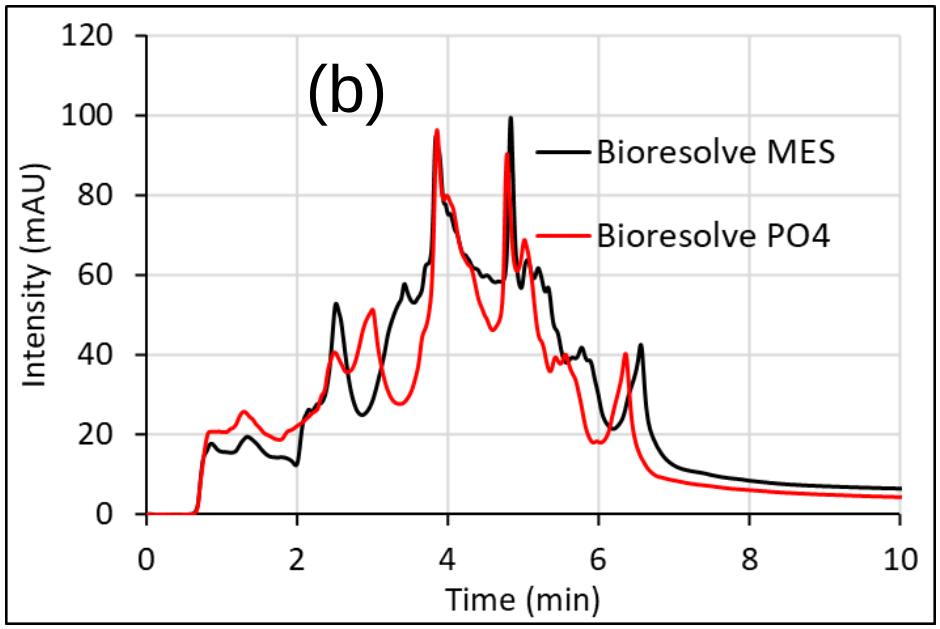

(a)
Bio mab MES
Bio mab PO4
Intensity (mAU)
Time (min)
0
10
20
30
40
50
60
0
5
10
15
20
25


(b)
Bioresolve MES
Bioresolve PO4
Intensity (mAU)
Time (min)
0
20
40
60
80
100
120
0
2
4
6
8
10

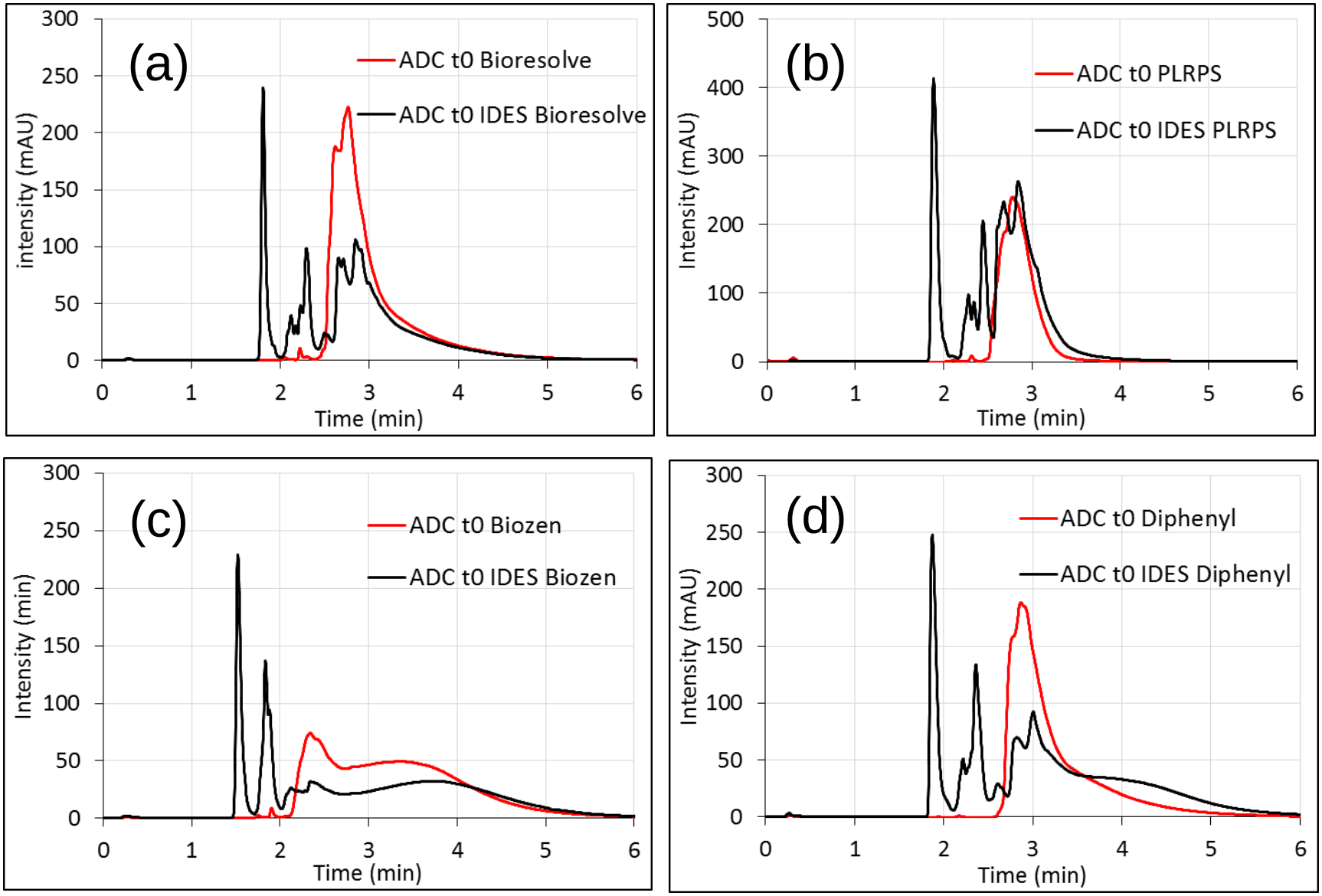

(a)
ADC t0 Bioresolve
ADC t0 IDES Bioresolve
intensity (mAU)
Time (min)
(b)
ADC t0 PLRPS
ADC t0 IDES PLRPS
Intensity (mAU)
Time (min)
(c)
ADC t0 Biozen
ADC t0 IDES Biozen
Intensity (min)
Time (min)
(d)
ADC t0 Diphenyl
ADC t0 IDES Diphenyl
Intensity (mAU)
Time (min)

Fig 2

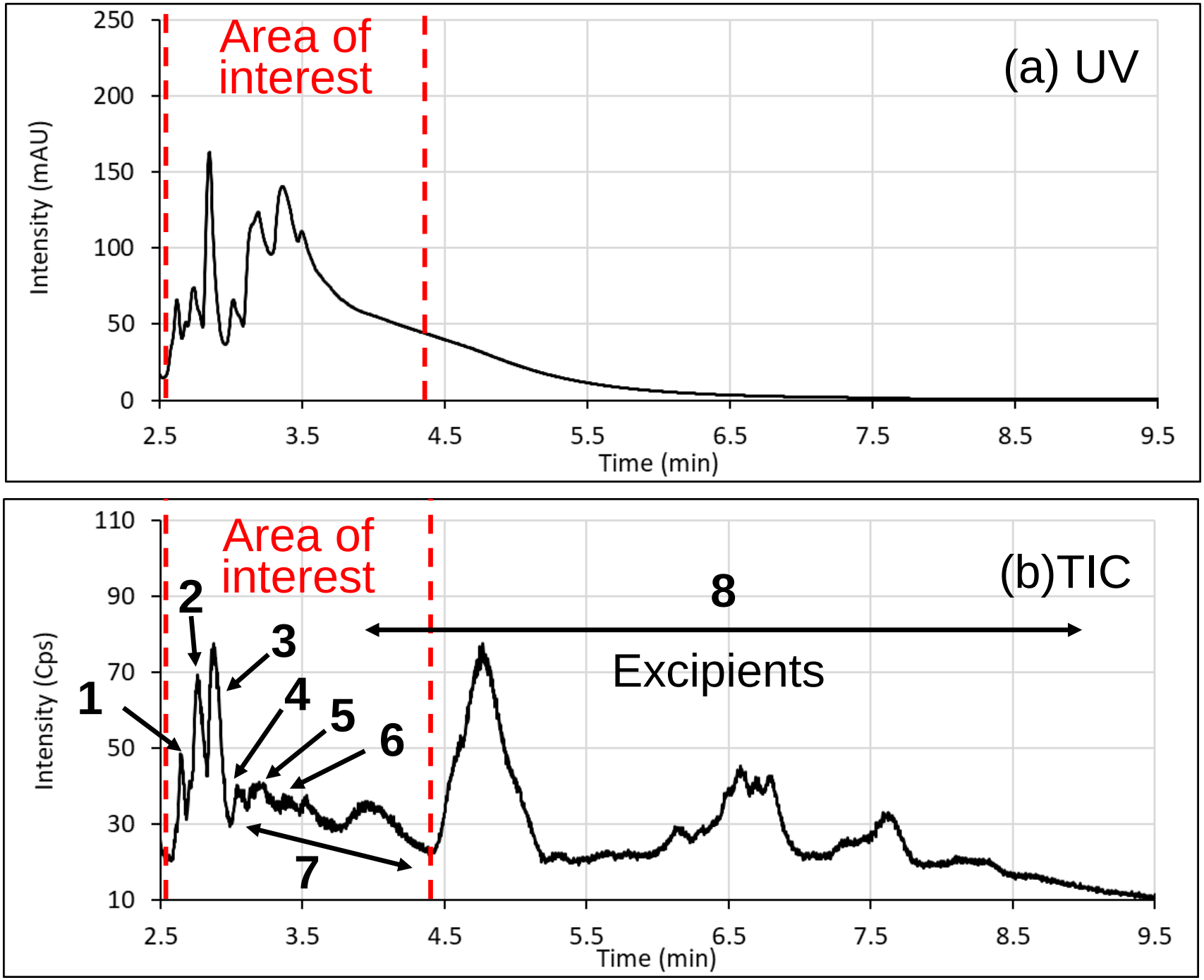

Area of interest
(a) UV
Intensity (mAU)
250
200
150
100
50
0
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
Time (min)
Area of interest
(b)TIC
8
Excipients
1
2
3
4
5
6
7
Intensity (Cps)
110
90
70
50
30
10
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
Time (min)

Fig 3

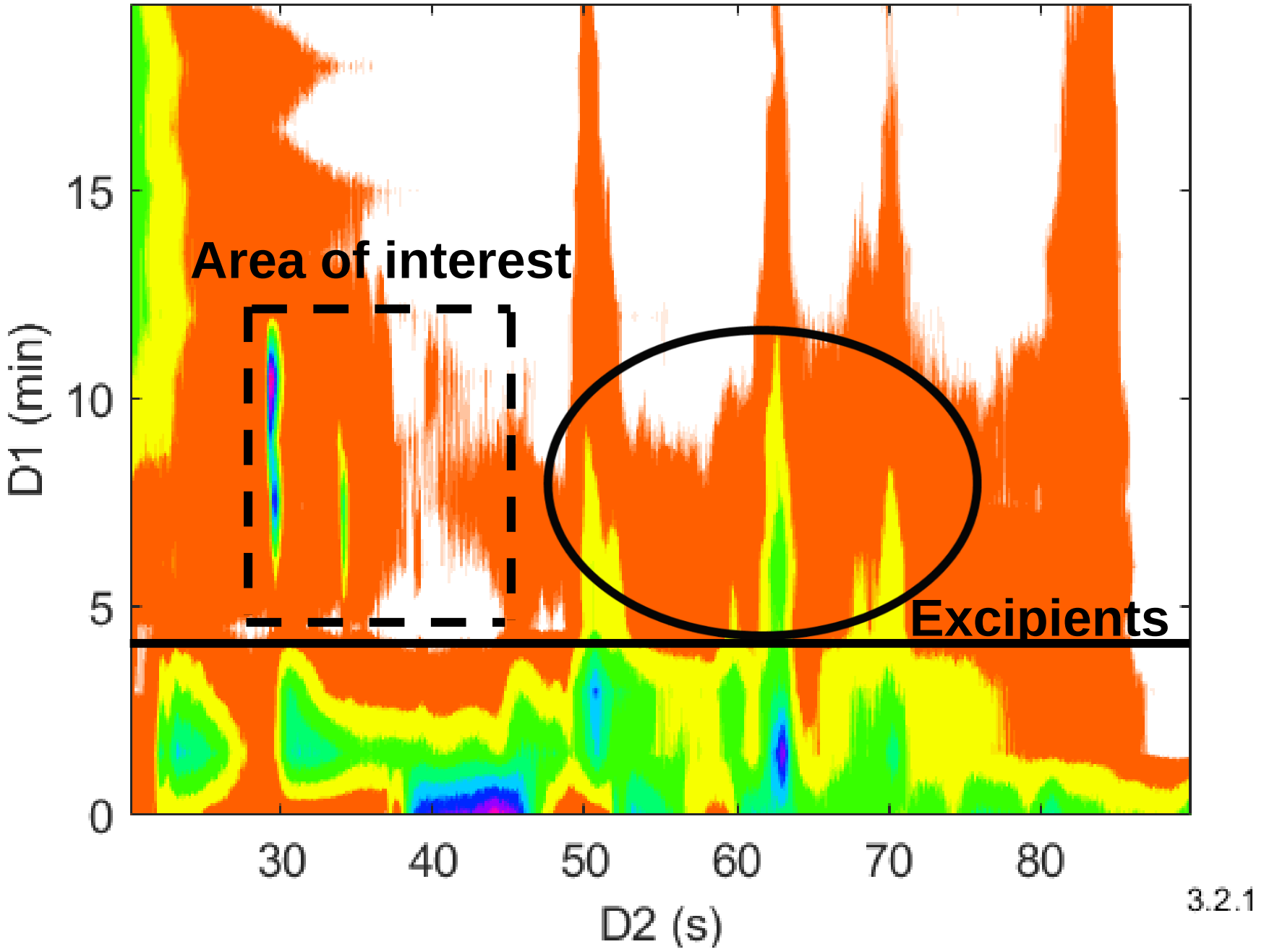

Area of interest
Excipients
D1 (min)
D2 (s)
0
5
10
15
30
40
50
60
70
80
3.2.1

Fig 4

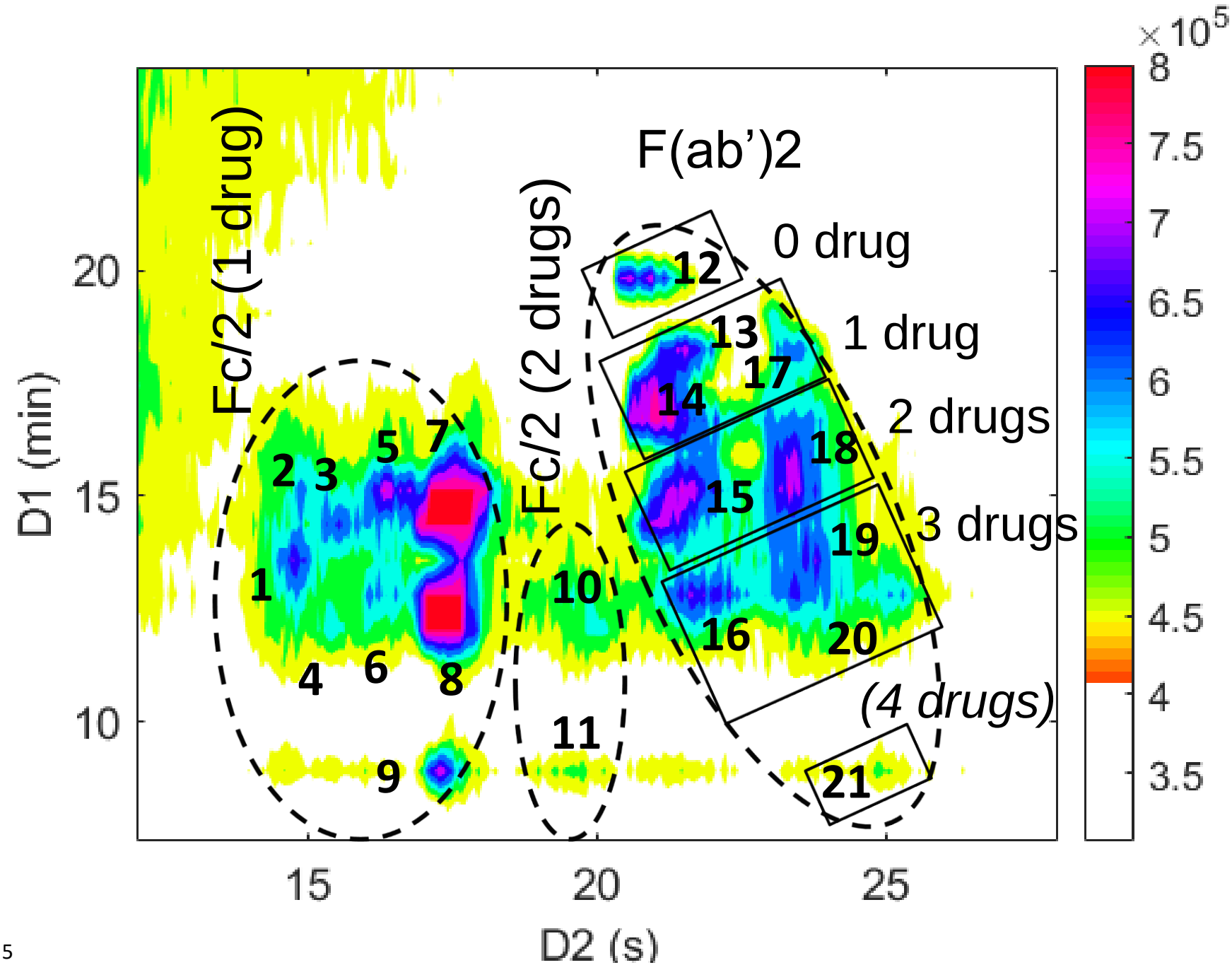

Fc/2 (1 drug)
Fc/2 (2 drugs)
F(ab')2
0 drug
1 drug
2 drugs
3 drugs
(4 drugs)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
D1 (min)
D2 (s)
10
15
20
15
20
25
×10^5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5

Fig 5

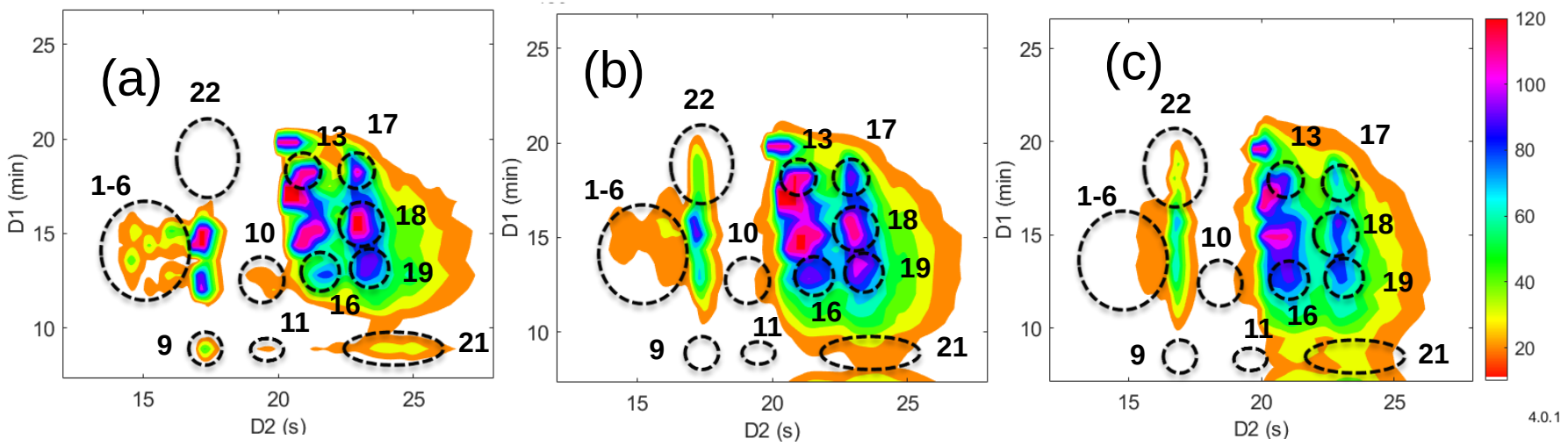


Fig 6

Table 1: Experimental conditions for the optimized CEX x RPLC –UV/HRMS method.

	CEX x RPLC
First dimension (^{1}D)	
Injection volume	40 µL
Injection solvent	Water
Stationary phase	Bioresolve SCX
Column geometry	(100 x 2.1 mm, 3 µm)
Temperature	30°C
Mobile phase	A: water + 20 mM MES pH = 6.5 B: A + 300 mM NaCl
Flow rate	75 µL/min
Gradient	1-17-1-1% B in 0-20-22.77-31.3 min
Post-column split	/
Modulation	
Loop size	80 µL
Sampling time	0.78 min Starting from 5 min (t0-IdeS and t2-IdeS) or 4 min
Second dimension (^{2}D)	
Stationary phase	Bioresolve RPmAb pk
Column geometry	(50 x 2.1 mm, 2.7 µm)
Temperature	80°C
Mobile phase	A: 0.1% FA + 0.05% TFA in water B: 0.1% FA + 0.05% TFA in ACN
Flow rate	1700 µL/min
Gradient	30-50-30-30% B in 0-0.49-0.55-0.78 min
Post-column split	1:1.5
Diverter valve	Flow to the waste: from 0 to 0.1 min Flow to MS: from 0.1 min to the end
UV detection	280 nm (40 Hz in ^{1}D and 80 Hz in ^{2}D)

HRMS detection	
Ionization mode	ESI+
Mass range (Da)	1200-5000
Scan rate (spectra/s)	10
Gas temp (°C)	325
Drying gas (L/min)	13
Nebulizer (psi)	40
Sheath gas (°C)	350
Sheath gas flow (L/min)	12
Capillary voltage (V)	4000
Nozzle voltage (V)	300
Fragmentor (V)	185
Oct 1 Rf Vpp (V)	750
Quad AMU (V)	600

Table 2: Proposed species for the peaks labelled in Fig. 3b after comparison between experimental and theoretical masses.

#	Difference between theoretical and experimental masses (Da)	Proposed specie
1	-1	Fc/2 + 1 drug
2	-1	Fc/2 + 1 drug
3	-1	Fc/2 + 1 drug
4a	-1	Fc/2 + 1 drug
4b	-2	Fc/2 + 2 drugs
5a	+2	(Fab')2 + 0 drug
5b	+2	(Fab')2 + 1 drug
5c	+2	(Fab')2 + 2 drugs
6a	+2	(Fab')2 + 1 drug
6b	+1	(Fab')2 + 2 drugs
6c	0	(Fab')2 + 3 drugs
6d	+1	(Fab')2 + 4 drugs
7a	+3	(Fab')2 + 0 drug
7b	+2	(Fab')2 + 1 drug
7c	+54	(Fab')2 + 2 drugs
7d	0	(Fab')2 + 3 drugs
7e	-1	(Fab')2 + 4 drugs
7f	-1	(Fab')2 + 5 drugs

Table 3: Proposed species for the peaks labelled in Figs. 5 and 6.

#	Difference between theoretical and experimental masses (Da)	Proposed specie
1	-3	Fc/2 + 1 drug
2	-3	Fc/2 + 1 drug
3	-3	Fc/2 + 1 drug
4	-1	Fc/2 + 1 drug
5	-3	Fc/2 + 1 drug
6	-2	Fc/2 + 1 drug
7	-3	Fc/2 + 1 drug
8	-3	Fc/2 + 1 drug
9	-3	Fc/2 + 1 drug
10	-4	Fc/2 + 2 drugs
11	-1	Fc/2 + 2 drugs
12	-7	(Fab')2 + 0 drug
13	-4	(Fab')2 + 1 drug
14	-9	(Fab')2 + 1 drug
15	+3	(Fab')2 + 2 drugs
16*	+405	(Fab')2 + 3 drugs
17*	+198	(Fab')2 + 1 drug
18	+11	(Fab')2 + 2 drugs
19	+7	(Fab')2 + 3 drugs
20	/	/
21	/	/
22	/	/

*:Given the large mass difference between theoretical and experimental deconvoluted masses, these two assignments were made based on the relative positions of the peaks in the contour plot.

Lysine-linked ADC

Intact ADC
~ 150 kDa

IdeS

Partially digested ADC

F(ab')2
~ 100 kDa

+

Fc/2 (x 2)
~ 25 kDa

Analysis

On-line CEX x RPLC-HRMS
In 30 min