



## UvA-DARE (Digital Academic Repository)

### Online multimethod platform for comprehensive characterization of monoclonal antibodies in cell culture fluid from a single sample injection - Intact protein workflow

Sadighi, R.; de Kleijne, V.; Wouters, S.; Lubbers, K.; Somsen, G.W.; Gargano, A.F.G.; Haselberg, R.

**DOI**

[10.1016/j.aca.2023.342074](https://doi.org/10.1016/j.aca.2023.342074)

**Publication date**

2024

**Document Version**

Final published version

**Published in**

Analytica Chimica Acta

**License**

CC BY-NC-ND

[Link to publication](#)

**Citation for published version (APA):**

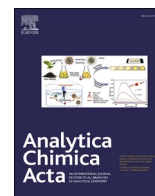
Sadighi, R., de Kleijne, V., Wouters, S., Lubbers, K., Somsen, G. W., Gargano, A. F. G., & Haselberg, R. (2024). Online multimethod platform for comprehensive characterization of monoclonal antibodies in cell culture fluid from a single sample injection - Intact protein workflow. *Analytica Chimica Acta*, 1287, Article 342074. <https://doi.org/10.1016/j.aca.2023.342074>

**General rights**

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, P.O. Box 19185, 1000 GD Amsterdam, The Netherlands. UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>). You will be contacted as soon as possible.



# Online multimethod platform for comprehensive characterization of monoclonal antibodies in cell culture fluid from a single sample injection - Intact protein workflow

Raya Sadighi<sup>a,b,\*</sup>, Vera de Kleijne<sup>a</sup>, Sam Wouters<sup>c</sup>, Karin Lubbers<sup>d</sup>, Govert W. Somsen<sup>a,b</sup>, Andrea F.G. Gargano<sup>b,e</sup>, Rob Haselberg<sup>a,b</sup>

<sup>a</sup> Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1108, 1081 HZ, Amsterdam, the Netherlands

<sup>b</sup> Centre for Analytical Sciences, Amsterdam, the Netherlands

<sup>c</sup> Agilent Technologies, Hewlett-Packard-Str. 8, Waldbronn, 76337, Germany

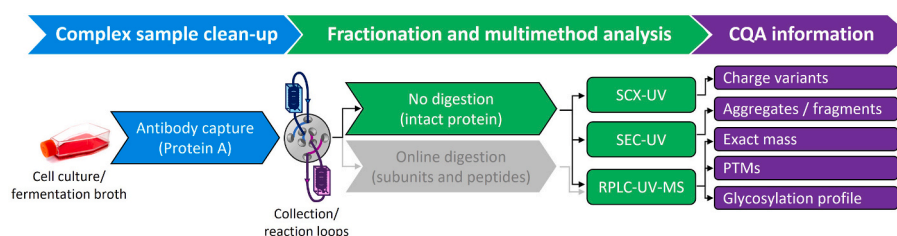
<sup>d</sup> Polpharma Biologics Utrecht B.V., Yalelaan 46, 3584 CM, Utrecht, the Netherlands

<sup>e</sup> Analytical Chemistry Group, van't Hoff Institute for Molecular Sciences, University of Amsterdam, PO Box 94720, 1090 GE, Amsterdam, the Netherlands

## HIGHLIGHTS

- A multi-method platform for automated intact mAb characterization is presented.
- Multiple CQAs can be determined from a single injection of cell culture fluid.
- Significantly reduced analysis times due to on-line purification and analysis.
- The system performance is comparable to established off-line methods.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Keywords:

Protein A affinity chromatography  
mAb  
Critical quality attributes  
Multi-dimensional characterization  
Protein liquid chromatography

## ABSTRACT

**Background:** Therapeutic monoclonal antibodies (mAbs) comprise a large structural variability with respect to charge, size and post-translational modifications. These critical quality attributes (CQAs) need to be assessed during and after the production of mAbs. This normally requires off-line purification and sample preparation as well as several chromatographic selectivities, which makes the whole process time-consuming and error-prone. To improve on this, we developed an integrated and automated multi-dimensional analytical platform for the simultaneous assessment of multiple CQAs of mAbs in cell culture fluid (CCF) from upstream processes.

**Results:** The on-line system allows mAb characterization at the intact level, combining protein A affinity chromatography (ProtA) with size-exclusion, ion-exchange, and reversed-phase liquid chromatographic modes with UV and mass spectrometric detection. Multiple heart cuts of a single mAb elution band from ProtA are stored in 20- $\mu$ L loops and successively sent to the multimethod options in the second dimension. ProtA loading and elution conditions and their compatibility with second-dimension LC modes were studied and optimized. Subsequently, heart-cutting and valve-switching schemes were investigated to achieve effective and reproducible analyses. The applicability of the developed workflow was demonstrated by the direct analysis (i.e. not requiring off-line

\* Corresponding author. Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1108, 1081 HZ, Amsterdam, the Netherlands.

E-mail address: [a.sadighi@vu.nl](mailto:a.sadighi@vu.nl) (R. Sadighi).

<https://doi.org/10.1016/j.aca.2023.342074>

Received 1 September 2023; Received in revised form 23 November 2023; Accepted 25 November 2023

Available online 30 November 2023

0003-2670/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

sample preparation) of a therapeutic mAb in CCF, obtaining useful information on accurate molecular mass, glycosylation, and charge and size variants of the mAb product at the same time and in just over 1 h.

**Significance:** The developed multidimensional platform is the first system that allows for multiple fractions from a single ProtA band to be characterized using different chromatographic selectivities in a single run allowing direct correlation between CQAs. The performance of the system is comparable to established off-line methods, fully compatible with upstream process samples, and provides a significant time-reduction of the characterization procedure.

## 1. Introduction

Monoclonal antibodies (mAbs) have offered new possibilities in therapies for severe diseases and have conquered the pharmaceutical market with applications ranging from the treatment of cancers to autoimmune disorders [1]. Most mAbs are tailor-made immunoglobulin G (IgG) molecules, which are tetrameric protein complexes of about 150 kDa molecular mass [2]. A pharmaceutical mAb has an intrinsic heterogeneity that comprises of differences in N-glycosylation, N- and C-terminal motifs, amino acid composition, local charges, disulfide bonds, fragmentation, and/or aggregation [3],[4]. Each of these modifications can influence the stability, efficacy, and safety of the product, thereby affecting overall mAb quality [5]. To ensure the desired product quality, these structural properties (*i.e.* critical quality attributes or CQAs) have to meet specific requirements and, therefore, should be monitored during mAb development and subsequent production.

Since recombinant mAbs are produced in cell cultures, a purification step is often mandatory before assessment of a CQA is possible. Protein A-based affinity chromatography (ProtA) is most commonly used as a sample pre-treatment step for mAb isolation [6], as this bacterial protein can be efficiently immobilized on solid supports and selectively binds the Fc region of IgGs. ProtA not only separates the antibody from ubiquitous and potentially interfering cell-culture components, but can also be used to determine antibody concentrations or titer when combined with UV absorbance detection [7]. For the determination of a particular chemical CQA, typically an analytical method with a specific separation selectivity is required. For example, the characterization of size variants of mAbs resulting from aggregation, fragmentation or truncation, requires molecular-size-based resolution as provided by size-exclusion chromatography (SEC) [8]. Charge variants (acidic and basic) of mAbs can be separated and monitored using protein ion-exchange chromatography (IEC), where usually strong cation-exchange chromatography (SCX) is used [9]. The heterogeneity of mAbs originating from post-translational modifications (PTMs), such as glycosylation and oxidation, is frequently assessed among others by reversed-phase liquid chromatography (RPLC) combined with high-resolution mass spectrometry (MS) [10]. Consequently, for each CQA, often a dedicated method and corresponding experimental setup is needed, which makes comprehensive mAb characterization quite time-consuming and prone to experimental errors due to the many analytical steps required.

Over the last decade, multidimensional LC has emerged as a powerful tool for the analysis of highly complex samples, and its usefulness for protein separation in top-down proteomics and the biopharmaceutical field has been demonstrated [11–34]. However, often the purification of mAbs from crude samples, is performed offline. In order to achieve a full integration and automation of the analytical workflow and methods needed for CQA assessment of intact antibodies in cell cultures, sample preparation (*i.e.* mAb isolation from cell culture fluid (CCF)) has to be included in the online scheme. So far this has been reported only in a handful of papers in different types of combinations. Amand et al. coupled ProtA to IEC [26], and Williams et al., coupled ProtA to SEC<sup>27</sup> for the separation and quantification of charge and size variants, respectively, using inline fractionation devices. Dunn et al. coupled ProtA to SEC for determination of titer and aggregation [25]. Sandra et al. reported on a platform for the online combination of ProtA

with either SEC, IEC, or RPLC-MS using a heart-cutting approach to transfer the mAb band from ProtA to the respective second LC dimension [34]. This approach was refined by adding hydrophobic interaction chromatography (HIC) to the second LC dimensions and using SEC as a third desalting dimension facilitating MS detection [21]. Recently, Camperi et al. introduced an automated method for sample clean up from bioreactors with ProtA prior to online bottom-up and intact charge variant analysis [35]. Liu et al. developed an online purification platform where, after cell removal and ProtA purification, the mAbs were analysed via SEC or online bottom-up proteomics for PTM analysis [36]. Still, as one ProtA heart cut was taken per sample, only a single second dimension LC analysis at a time could be performed per injection, which implies that multiple sample injections are required to assess several CQAs of one sample.

Advanced two-dimensional (2D) LC equipment provides heart-cutting options that would allow a first-dimension LC peak to be split into multiple cuts, which then could be subjected to several second LC-dimension analyses. In the present study we took advantage of these possibilities to develop an online multidimensional platform that enables assessment of multiple CQAs on the intact protein level from a single sample injection. This is highly useful in situations where many cell lines have to be screened in parallel and making fast decisions on what cell lines are sufficiently promising is essential. Moreover, as the lifespan of a ProtA column is limited by the number of samples loaded, costs for sample treatment are significantly reduced. We used ProtA as the first dimension (<sup>1</sup>D) LC mode to capture/purify mAb from a crude sample of interest and divided the desorbed mAb zone into three heart cuts which were subsequently stored in loops. The cuts were then successively analysed by different second-dimension (<sup>2</sup>D) LC modes, *viz.* SEC, SCX, and RPLC-MS, in order to reveal size-, charge- and glycosylation variants, respectively. The performance of the proposed system was assessed by comparison to offline combinations of ProtA with LC. Heart-cutting of the ProtA peak was optimized, and the scheme and repeatability of the loop filling and valve switching was evaluated. Compatibility of the ProtA eluent with the <sup>2</sup>D LC modes was studied and optimized, and the performance of the respective ProtA-LC combinations was examined and established using therapeutic mAb. Finally, the potential of the platform to simultaneously attain several CQAs in less than 65 min was evaluated by analyzing CCF samples.

## 2. Experimental

### 2.1. Reagents and chemicals

Acetonitrile (ACN), formic acid (FA), glacial acetic acid (AA) and trifluoroacetic acid (TFA) were purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate buffered saline (PBS), sodium chloride, glycine, cytochrome C, monosodium phosphate, disodium phosphate, dithiothreitol (DTT) and uracil were obtained from Sigma (Amsterdam, The Netherlands). CX-1 pH Gradient Buffer A pH 5.6 and CX-1 pH Gradient Buffer B pH 10.2 were purchased from Thermo Scientific (Waltham, MA, USA). Ultrapure water was obtained from a Milli-Q Purification system (Merck Millipore, Burlington, MA, USA). Two sets of therapeutic mAb samples (purified mAb1 and mAb1 in CCF) were obtained from Polpharma Biologics (Utrecht, The Netherlands). Purified mAb1 (5.2 mg mL<sup>-1</sup>) was diluted in water to 1 mg mL<sup>-1</sup> prior to analysis,

whereas CCF samples were used as supplied.

## 2.2. LC and MS systems

LC experiments were performed using a multidimensional LC system (Agilent Technologies, Waldbronn, Germany) comprising a 1290 Infinity II High Speed Pump for the first LC dimension (<sup>1</sup>D) and a 1260 Infinity II Bio-Inert Quaternary Pump for the second LC dimension (<sup>2</sup>D). Sample injection was performed using a 1290 Infinity II Multisampler. Two 1290 Infinity II Diode Array Detectors were used as <sup>1</sup>D and <sup>2</sup>D UV-absorbance detector, respectively. The <sup>2</sup>D columns were situated in a 1290 Infinity II Multicolumn Thermostat with a column divider allowing the setting of column-specific temperatures. A 10-port valve, typically used in a 2D setup, was used here as an “interface” valve. Two 13-port valves were used for the <sup>2</sup>D solvent A and B selection, and a 6-port valve was used as an “intact” valve holding 20- $\mu$ L loops to store the heart cuts between the two LC dimensions. An “analytical” column-selection valve (8-position valve) was used to select and switch between the respective <sup>2</sup>D LC columns. Fig. 1 shows the schematic of the system in more detail. This set-up was coupled to a timsTOF instrument from Bruker (Bremen, Germany) equipped with an electrospray-ionization (ESI) source. The ESI settings were as follows: capillary voltage, 4.5 kV; nebulizer gas, 1.2 bar; dry gas flow, 8.0 L/min; drying temperature, 220 °C; ion energy, 5 eV; collision energy, 10 eV; in-source collision-induced dissociation, 120 eV. The mass spectrometer was operated in positive mode and tims off (no ion mobility) using a *m/z* range of 500–7000. Ion funnels 1 and 2 were set at values of 350 Vpp and 600 Vpp, respectively. Data analysis was performed using Bruker Compass DataAnalysis. The UV data was processed by Agilent Chemstation software.

## 2.3. LC conditions

<sup>1</sup>D: ProtA.

A Bio-Monolith Protein A column (4.95  $\times$  5.2 mm) from Agilent Technologies was used at room temperature for mAb capture using an injection volume of 20  $\mu$ L. The loading buffer was PBS (pH 7.4; solvent A). The optimized elution buffer was 0.01 M FA (pH 2.9; solvent B). The loading and elution conditions are summarized in Table 1.

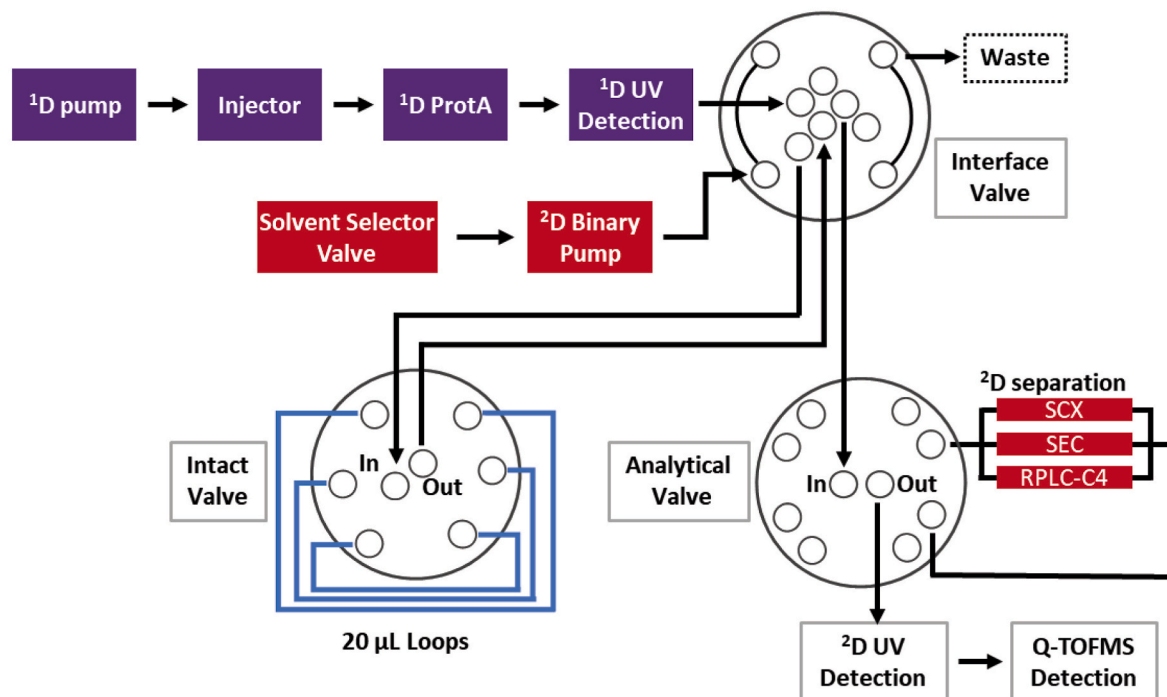
<sup>2</sup>D: SEC, SCX and RPLC.

RPLC was performed using a 2.1  $\times$  50 mm ACQUITY UPLC Protein BEH C4 column (Waters, Milford, MA, USA). SEC was performed using a 4.6  $\times$  150 mm TSKgel UP-SW Aggregate column supplied by TOSOH (Tokyo, Japan). SCX was performed using a 4.6  $\times$  150 mm Proteomix SCX-NP3 column (Sepax Technologies, DE, USA). The eluent conditions are summarized in Table 1.

## 3. Results and discussion

For the assessment of CQAs, such as aggregation, glycosylation, charge variants, titer, and exact molecular weight, of mAb from CCF and formulated samples, an integrated multi-dimensional platform was developed. Fig. 1 depicts the schematics of the platform. During development of the platform, mAb1 was used as a model protein. The heterogenous glycans of this mAb mainly consist of galactose, mannose, N-acetylglucosamine, and fucose. Due to lysine clipping, oxidation, and deamidation, charge heterogeneity is also expected.

This mAb1 was isolated from cell culture via ProtA in the first LC dimension (Fig. 1, purple boxes). The trapped mAb is desorbed and via the interface valve directed towards the intact valve where sampling of the mAb occurs in three 20- $\mu$ L loops, dividing the ProtA peak into three fractions. These heart cuts are then transferred via the analytical valve towards the respective <sup>2</sup>D separation modes. The heart cut sent to RPLC-MS (wide pore C4 stationary phase) provides the exact mass of the antibody and information about the glycosylation profile. The second heart cut is transferred to SEC to detect potential mAb fragments and aggregates, whereas the third heart cut is analysed by SCX to reveal charge variants.



**Fig. 1.** Schematics of the multi-dimensional platform to characterize CQAs of mAbs from cell cultures. The purple boxes show the parts needed for the mAb purification via ProtA in the first-dimension. The interface valve is used to direct the desorbed mAb to the intact valve where three 20- $\mu$ L loops can store heart cuts from the ProtA peak. After storage, the second-dimension flow is directed to the intact valve via the interface valve and successively the heart cuts are moved to the LC column of choice (indicated in red) in the second dimension using the analytical valve. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**

Overview of <sup>1</sup>D and <sup>2</sup>D LC conditions as used in the multidimensional approach to study CQAs of intact mAbs.

<sup>1</sup> D LC	ProtA		
Solvent A	50 mM PBS, pH 7.4		
Solvent B	10 mM FA, pH 2.9		
Flow rate	0.25 mL/min		
Loading/ elution	0–5min: 0 % B		
5–8min:	0–100 % B		
8–10 min:	0 % B		
Temperature	23 °C		
Injection volume	20 µL		
Detection wavelength	280 nm		
<sup>2</sup> D LC	SEC	RPLC	SCX
Solvent A	Water-ACN-FA-TFA (80:20:0.1:0.1, v/v/v/v)	Water-FA-TFA (99.8:0.1:0.1, v/v)	1:1 dilution of 2X concentrated pH gradient 5.6 in water
Solvent B	–	ACN-FA-TFA (99.8:0.1:0.1, v/v)	1:1 dilution of 2X concentrated pH gradient 10.2 in water + 400 mM NaCl
Flow rate	0.35 mL/min	0.3 mL/min	0.8 mL/min
Gradient	–	0–5 min: 5 % B	
5–10 min:			
0–95 % B			
10–11 min:			
95 % B			
11.0–11.1 min:			
95–5% B			
11.1–15 min:	0–4 min: 0 % B		
5 % B			
4–15 min:			
25 % B			
15–18 min:			
100 % B			
17–18 min:			
0 % B			
Temperature	40 °C	75 °C	30 °C
Injection volume	20 µL	20 µL	20 µL
Detection wavelength	254 and 280 nm	254 and 280 nm	254 and 280 nm

In order to develop the proposed workflow, online ProtA purification of mAb and its compatibility with the <sup>2</sup>D LC modes was evaluated and optimized. Subsequently, heart cutting of the ProtA peak and transfer of the respective heart cuts to the <sup>2</sup>D separations was studied in order to achieve continuous analysis. Finally, the performance and repeatability of the overall established workflow was evaluated by the direct analysis of mAb1 in cell culture.

### 3.1. Online ProtA performance and compatibility with LC

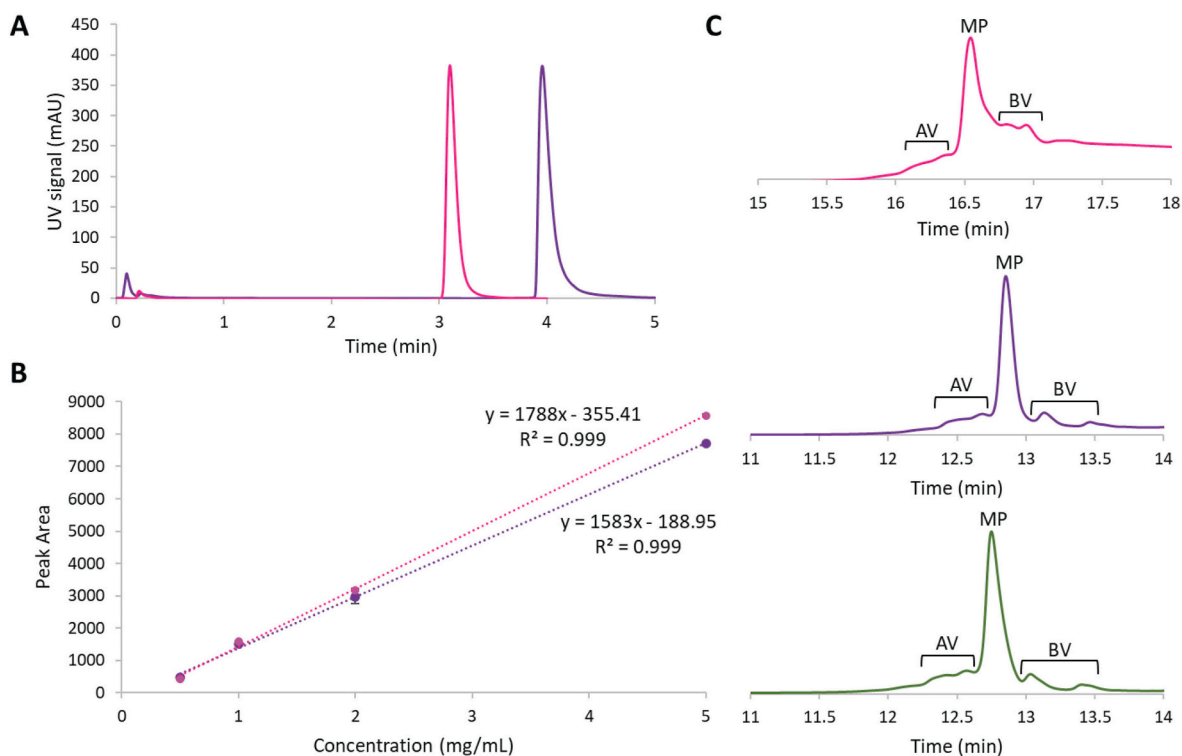
ProtA is commonly used for the selective isolation of mAbs from multi-component samples, due to its specificity and high binding affinity for the Fc region of IgG antibodies [37]. Loading of CCF containing mAbs on ProtA, usually occurs at neutral pH using 50 mM PBS (pH 7.4) as mobile phase. After 3.0 min, a 1-min gradient from 0 % to 100 % of a low-pH buffer is applied to elute the isolated mAb from the ProtA column. A conventional elution solvent (as often prescribed by ProtA column vendors) is 500 mM acetic acid (AA; pH 2.6) yielding good mAb recoveries (>95 %). Fig. 2A (pink line) shows the result after loading 20 µL of mAb1 (3.5 mg mL<sup>-1</sup>) to ProtA followed by elution with 500 mM AA. Loading various amounts of this mAb, yielded a linear response (Fig. 2B pink) demonstrating adequate performance of ProtA in isolating the mAb. However, to allow proper LC analysis after ProtA isolation, the

elution buffer needs to be compatible with the three <sup>2</sup>D LC modes. Therefore, the effect of AA on the performance of each second-dimension separation was investigated by dissolving the mAb in 500 mM AA and injecting it on the RPLC, SEC, and SCX columns. RPLC presented no compatibility issues when using AA, as the mAb was trapped on the head of the RPLC column and salts were flushed off the column at the start of the applied gradient (Fig. S1). Using the AA-containing injection solvent in SEC also yielded no interferences with the eluting mAb exhibiting the expected retention time profile (Fig. S2). Unfortunately, using 500 mM AA as injection solvent in SCX caused a disturbed elution profile, complicating the distinction of the acidic and basic charge variants (Fig. 2C, top). Lowering the AA concentration did not improve the resolution. However, by substituting AA for formic acid (FA; 10 mM) as injection solvent, satisfactory SCX profiles were obtained (Fig. 2C, middle). These were very similar to those obtained when injecting mAb1 in water (Fig. 2C, bottom), resolving acidic and basic variants from the parent mAb. Injection of this mAb in 10 mM FA also provided good RPLC and SEC performance (Figs. S3–4). Using 10 mM FA as elution solvent in ProtA gave satisfactory results (Fig. 2A and B, purple line), which were comparable to what was obtained with AA. At relatively high mAb concentrations, the recovery obtained with 500 mM AA was slightly higher, most probably because relative to 10 mM FA, it provides a somewhat lower pH and higher ionic strength, enhancing mAb desorption. Overall, we concluded that selecting 10 mM FA as ProtA elution solvent provides both good mAb-isolation performance and excellent compatibility with SCX, RPLC and SEC.

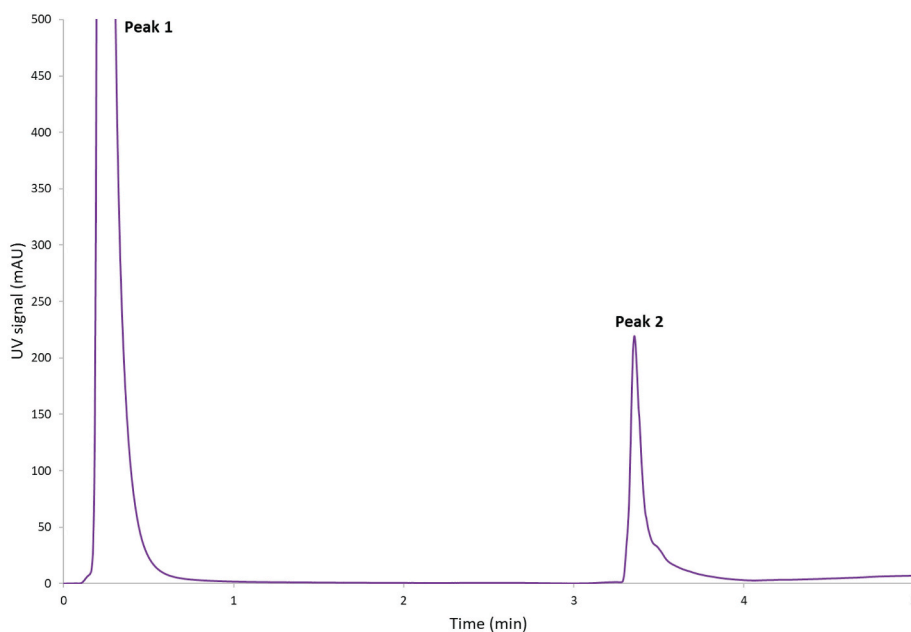
The optimized ProtA method was applied to the isolation of mAb1 from CCF. For this, 20 µL of CCF was loaded on to the ProtA column at neutral pH using 50 mM PBS (pH 7.4) at 0.5 mL/min. After 3.0 min a 1-min gradient from 0 % to 100 % 10 mM FA (pH 2.9) at 0.5 mL/min was applied to induce mAb elution. The resulting chromatogram (Fig. 3) shows that most CCF components, which exhibit no ProtA affinity, elute within 1 min, whereas mAb1 is firstly retained and then elutes at approximately 3.5 min under acidic conditions. Using the <sup>1</sup>D UV absorbance detector, the online setup can be readily used to determine the mAb titer (a CQA) of CCF as well. Based on the UV absorbance detection signal, the titer of the CCF was determined to be 3.5 mg mL<sup>-1</sup> using the constructed external calibration curve for mAb1 (Fig. 2B purple). With the conventional method using 500 mM AA as desorbing solution, the determined titer was almost equivalent, deviating less than 2 %.

### 3.2. Heart cutting and valve configuration

To enable heart cutting of the peak resulting from mAb1 ProtA purification, a 10-port interface valve was used. The function of this valve is to cut the purified peak in multiple fractions and direct each to a 20 µL loop installed on the intact valve for storage (Fig. 4). Injection of 20 µL mAb1 on the ProtA column and subsequent elution using 10 mM FA, resulted in a peak volume of approximately 115 µL. In the 'Loading' position (Fig. 4A), the flow (0.5 mL/min) from the <sup>1</sup>D pump carrying the isolated mAb enters the transfer valve (pink flow path). From there the flow goes to the intact valve, where the flow enters the first loop and after 8 s, it switches to the second loop and subsequently to the third loop, creating three heart cuts from the eluting ProtA mAb peak. In this position, the <sup>2</sup>D flow (indicated in blue) enters the transfer valve and from there to the analytical valve. This valve includes an 8-column selection valve which holds the SCX, SEC and RPLC columns. In the loading position, the second-dimension flow goes through a bypass line and from there to waste. In the 'Analyzing' position (Fig. 4B), the second-dimension pump enters the interface valve and from there is directed to the intact valve. One by one, the heart cuts are transferred by the <sup>2</sup>D flow to the analytical valve and the different columns that are installed. Simultaneously, the first-dimension flow switches to the waste.



**Fig. 2.** Effects of different elution buffers on ProtA performance and SCX separation for mAb1. (A) ProtA chromatograms using 500 mM AA (pink) or 10 mM FA (purple) as elution solvent. (B) mAb1 response curves when using 500 mM AA (pink) or 10 mM FA (purple) as elution solvent in ProtA. (C) SCX chromatograms obtained for mAb1 when using 500 mM AA (pink, top), 10 mM FA (purple, middle) or water (green, bottom) as injection solvent. MP: main peak, AV: acidic variants, BV: basic variants. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

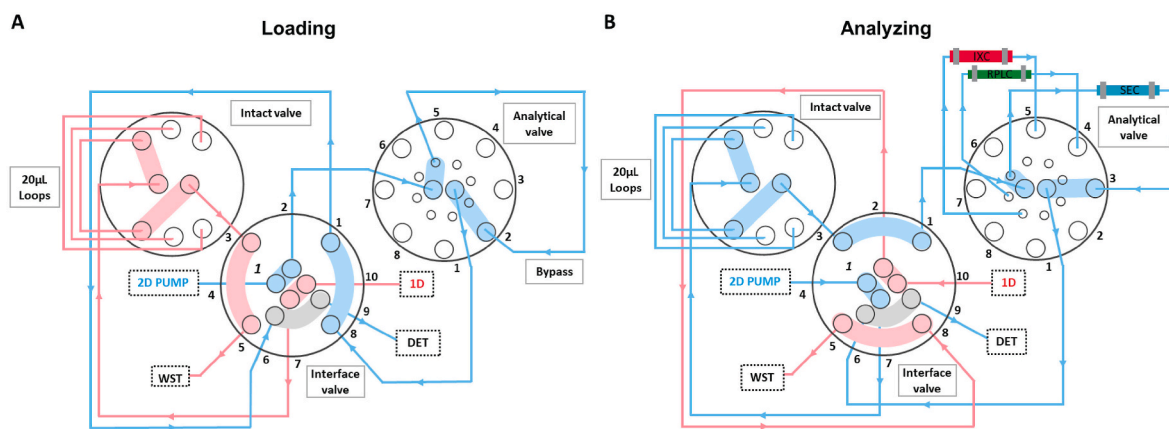


**Fig. 3.** ProtA-UV chromatogram obtained when analyzing mAb1 from CCF. Loading buffer (solvent A), 50 mM PBS; elution buffer (solvent B), 10 mM FA; flow rate, 0.5 mL/min. Mobile phase gradient, 0–3 min: 0 % B, 3–4 min: 0%–100 % B, 4–8 min 0 % B. Peak 1 is caused by non-retained CCF components and Peak 2 is the isolated mAb.

Initially, a flow of 0.5 mL/min was used for ProtA using the <sup>1</sup>D pump. However, when switching the Intact Valve (Fig. 1) for loop filling, back pressure spikes were observed, which caused run-to-run variations in the obtained ProtA elution times of the isolated mAb. This problem was solved by reducing the ProtA flow rate to 0.25 mL/min and adjusting the

gradient and switching times accordingly (Table 1), allowing to obtain consistent heart cuts.

The loops (1, 2 and 3) located on the intact valve can be filled and emptied in different orders. Initially, filling the loops in the 1–2–3 order was chosen followed by emptying in the 3–1–2 order for the <sup>2</sup>D analysis.



**Fig. 4.** Loading and analyzing configurations of the intact valve (6-port valve with storage loops) and the interface valve (10-port valve) facilitating the injection of ProtA heart cuts into the <sup>2</sup>D LC columns. **(A)** Loading position; The red trace follows the <sup>1</sup>D flow that carries the purified mAb from the ProtA to the intact valve where three 20- $\mu$ L loops are installed. The blue trace follows the <sup>2</sup>D flow. This flow is transferred to a bypass position where it will go the second-dimension detector. **(B)** Analyzing position; The second-dimension flow (blue trace) is directed to the intact valve where it will carry one by one the heart cuts to the desired separation. The <sup>1</sup>D flow (pink trace) is then transferred to waste. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

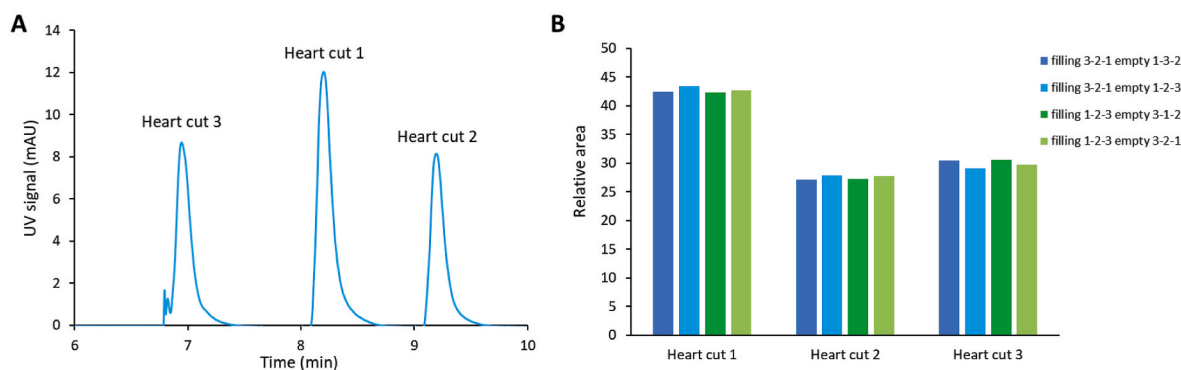
This means that the first cut is stored in loop 1 which is then emptied as second in order, the second cut is stored in loop 2 and emptied as last, and the third cut is stored in loop 3 and emptied as first. To evaluate the repeatability of the heart cuts and study potential carryover, the effect of the order of filling and emptying the loops was investigated. For this, a test solution of purified mAb1 was directly injected (i.e., omitting the ProtA column; see Fig. 1) and detected using the <sup>1</sup>D detector. Subsequently, three successive heart cuts of the mAb1 band were stored in the three respective 20- $\mu$ L loops, positioned on the “Intact valve”. Then, the three loops were successively emptied and their content was directed to the <sup>2</sup>D detector. For loop filling, two switching sequences were tested: clockwise (1-2-3) and anticlockwise (3-2-1). The LC hardware dictates that the loop filled last, has to be emptied first. The next two loops can be emptied in arbitrary order. Direct injection of the mAb1 solution yielded a broad asymmetric (tailing) band profile from which the three heart cuts were taken. The first heart cut comprised the peak apex and therefore the highest mAb concentration. This is also clear from Fig. 5A, which shows the resulting profiles of the three heart cuts after filling the loops clockwise (1-2-3) and emptying them in the 3-1-2 order. Four different loop filling/emptying combinations were evaluated and for each heart cut profile the peak area was determined (Fig. 5B). Regardless of the loop filling or emptying order, the peak area of each specific heart cut remained very similar (CV < 4 %). From this experiment we conclude that the order of filling and emptying the loops causes no bias,

and that the switching system works properly with no observable carryover. The initial loop filling/emptying order of 1-2-3/3-1-2 was selected and used for further analyses.

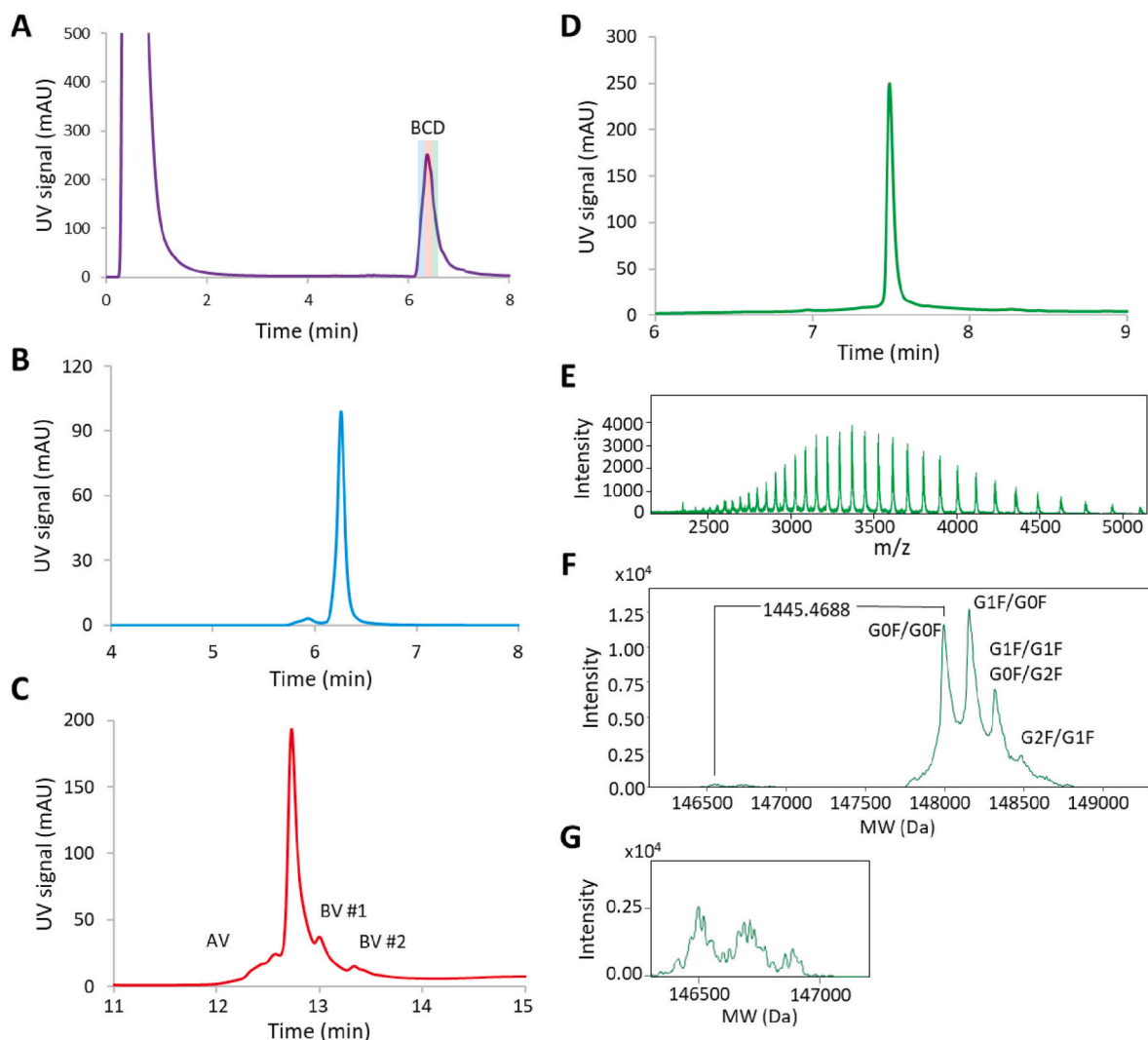
### 3.3. Online ProtA-SEC/SCX/RPLC-MS of intact mAb directly from CCF

After evaluating and solving some technical aspects, the ability of the developed platform to determine multiple mAb CQAs from a single injection of culture medium was studied. Fig. 6 shows the results when the entire online ProtA-LC system was applied to the direct analysis of CCF taken from mAb1 production. The mAb band resulting from ProtA purification was divided into three fractions, which were subsequently analysed with SCX-UV, SEC-UV, and RPLC-MS. The entire process was carried out online and automated, thus requiring no manual intervention. From the obtained data, quantitative information on CQAs was determined, as summarized in Table 2.

The ProtA chromatogram (Fig. 6A) shows the isolation of mAb1 from the non-binding components of the CCF (eluting around 1 min). The mAb elutes around 6 min in a volume of about 118  $\mu$ L with a determined concentration of 3.5 mg mL<sup>-1</sup>. The three successive fractions of the mAb peak, collected in the 20- $\mu$ L loops, are highlighted by color (Fig. 6A). The first fraction (in blue) was diverted to the SEC column for analysis of potential size variants. SEC separation of the fraction yielded a main band at 6.3 min preceded by a relatively small peak at 5.8 min (Fig. 6B)



**Fig. 5.** Evaluation of filling and emptying the three loops on the ‘Intact valve’ with heart cuts of an injection of a solution of mAb1 (no chromatography in the first and the second dimension). Four sequences of loop filling/emptying were tested: 3-2-1/1-3-2, 3-2-1/1-2-3, 1-2-3/3-1-2, and 1-2-3/3-2-1. **(A)** Emptying profiles when the order of filling the loops with mAb1 was 1-2-3 and the order of emptying was 3-1-2. **(B)** Relative peak areas measured on the <sup>2</sup>D detector for each collected heart cut at indicated sequences of loop filling/emptying.



**Fig. 6.** Online ProtA-SEC-UV/SCX-UV/RPLC-MS analysis of mAb1 in CCF. **(A)** ProtA-UV analysis showing the three heart cuts taken from the mAb1 peak. **(B)** SEC-UV analysis of the first fraction. **(C)** SCX-UV analysis of the second fraction with acidic (AV) and basic variants (BV) annotated. **(D)** RPLC-MS analysis of the third fraction. **(E)** Mass spectrum and **(F)** deconvoluted mass spectrum of mAb1 obtained during RPLC-MS analysis of this third fraction with the observed glycoforms annotated. **(G)** A low intensity signal with a deconvoluted mass around 146500 Da shows the absence of a G0F glycan from one of the heavy chains.

**Table 2**

Quantitative CQAs of mAb1 in CCF determined by online integrated ProtA-SEC-UV/SCX-UV/RPLC-MS and by offline combinations of ProtA with separate SEC-UV, SCX-UV and RPLC-MS systems.

CQAs	Online analysis	Offline analysis
<b>SEC-UV</b>		
Dimer	1.8 %	2.3 %
Monomer	98.2 %	97.7 %
<b>SCX-UV</b>		
Acidic variants	12.6 %	13.3 %
Main peak	73.1 %	72.1 %
Basic variants #1	11.2 %	11.4 %
Basic variants #2	3.1 %	3.1 %
<b>RPLC-MS</b>		
Exact mass main glycoform	147992.6	147990.2
Ratio G0FG0F/G1FG0F	0.90	0.89
Exact mass partially glycosylated	146546.5	146544.5

which could be attributed to the monomeric mAb and its dimer, respectively. Based on relative peak area the dimer content was estimated to be 1.8 %.

The second ProtA fraction (in red) was diverted to the SCX column to

reveal possible presence of acidic and basic variants of mAb1. In the SCX-UV chromatogram (Fig. 6C) next to the main peak of native mAb1 (at 12.8 min), convoluted peaks of more acidic (12.2–12.6 min) and more basic (12.9–13.4 min) mAb species are clearly observed. Based on relative peak area, the acidic and basic variants accounted for 13.3 % and 15.7 %, respectively. The SCX method used in the presented platform is not compatible with MS, and therefore, it was not possible to assess the identity of the variants at the intact level. However, fractionation of the peaks resulting from SCX followed by offline trypsin digestion and LC-MS of the generated peptides revealed that the acidic variants are the result of deamidation in both the Fc and F(ab') region (data not shown). The basic variants could be attributed to the conversion of N-terminal glutamic acid into glutamine and the presence of residual C-terminal lysines (data not shown).

The third ProtA fraction (in green) was sent to the RPLC column which was hyphenated to high-resolution MS. RPLC-MS yielded one peak (at 7.5 min), indicating that no additional impurities or fragments of different overall hydrophobicity than mAb1 were present (Fig. 6D). The mass spectrum obtained in the apex of the peak (Fig. 6E) shows a typical charge state distribution of an intact mAb centered around  $[M+43H]^+_{43+}$ . Deconvolution yielded a molecular weight of 147990.8 Da for the most abundant species (Fig. 6F). This mass agrees well (5.4

ppm) with the theoretical mass of the most abundant glycoform of mAb1 bearing G0F and G1F glycans on its heavy chains. Additional masses were observed in the deconvoluted spectrum, which could be assigned to G0F/G0F, G1F/G1F or G0F/G2F, and G2F/G1F glycoforms. Moreover, a low intensity signal around 146500 Da was observed (1.3 % of signal intensity), which corresponds to a mAb species that lost a G0F glycan (mass difference of 1445.5 Da).

For a standard mAb1 sample, the CQAs were determined with the new integrated platform and compared with those obtained with conventional analysis, in which ProtA was combined offline with SEC-UV, SCX-UV and RPLC-MS, respectively (Table 2). For SEC it can be observed that online and offline analysis produce slightly different results with respect to dimer percentages. It is known that ProtA may yield monomer-dimer separations, but we have excluded this as cause (see Section 3.4). The difference was attributed to variation in the number of freeze-thaw cycles that the standard mAb was exposed to in between injections and the different batches of standard mAb that were used. For SCX, the offline and online analyses showed no significant difference in relative amounts of basic variants and only a slight difference for the acidic variants. For the RPLC-MS the observed exact masses were within the experimental error and comparable glycosylation profiles were obtained. Overall, it is concluded that the multidimensional platform does not lead to a CQA bias compared to the standard offline approach. Notably, the data was gathered in 65 min using the multidimensional platform. For offline analyses, this is normally achieved in 3–4 h for small scale productions and up to 8 h for (larger) process batches. The integrated system clearly provides a substantial reduction of analysis time.

### 3.4. Evaluation of ProtA bias and platform repeatability

Previous work on ProtA of mAbs reports that partial separation of mAb species can occur on the affinity column [38]. This depends on the nature of the mAb and the gradient used for ProtA purification. In our set up, such selectivity is not desirable, as it might lead to bias in CQA results depending on which loop is used in combination with which LC mode. To determine whether the three heart cuts of the ProtA peak of the mAb provide consistent CQA information, they were also all analysed by the same LC mode. This was tested for SEC, SCX, and RPLC-MS, and for each mode the CQA results of the three heart cuts were highly similar (Table 3), indicating that ProtA does not distinguish among the mAb proteoforms. This most probably is due to the steep gradient used

**Table 3**

Quantitative CQAs with RSDs of mAb1 in CCF determined by online integrated ProtA-SEC-UV, ProtA-SCX-UV, and ProtA-RPLC-MS. In each run, three heart cuts of the ProtA peak (Fractions 1–3) were analysed by one LC mode.

	Fraction 1	Fraction 2	Fraction 3	RSD (% , n = 3)	
				Elution time	CQA
<b>SEC</b>					
Dimer	2.3 %	1.8 %	2.2 %	0.92	12.6
Monomer	97.7 %	98.2 %	97.8 %	0.72	0.27
<b>SCX</b>					
Acidic variant	13.3 %	13.1 %	13.6 %	0.04	1.9
Main peak	71.1 %	72.0 %	72.0 %	0.02	0.7
Basic variant #1	11.7 %	11.6 %	11.3 %	0.05	1.8
Basic variant #2	3.9 %	3.3 %	3.1 %	0.06	12.1
<b>RPLC-MS</b>					
Exact mass main glycoform	147991.8	147991.8	147991.7	0.21	0.00004 (0.4 ppm)
Ratio G0FG0F/G1FG0F	0.87	0.90	0.89		1.7
Exact mass partially glycosylated mAb	146545.6	146545.3	146545.9		0.0002 (2 ppm)

for eluting the ProtA column, which prevents potential separation of mAb1 on the ProtA stationary phase. This might be different for a mAb of other nature or when using another gradient, and therefore, a user of this approach in principle would have to check that the ProtA step is not causing a separation of, for example, dimer/monomer or charge variants of the analysed mAb.

From the obtained data also repeatability data could be derived (Table 3). Within one run (i.e., one sample injection, three heart cuts), relative standard deviations (RSDs) for almost all CQAs monitored were less than 2 %. The exceptions were the mAb dimer content as determined from SEC (12.6 %) and the basic variants #2 as determined from the SCX (12.1 %). These higher RSDs can be related to the low intensity of the related peaks and resulting variability in peak integration. For the LC elution times, RSDs were always (much) less than 1 %, indicating stable performance of the LC systems within the entire platform.

For the evaluation of day-to-day repeatability, the same sample was analysed by ProtA-RPLC-MS, ProtA-SEC-UV, and ProtA-SCX-UV on five different days spread over 5 weeks. RSD values for RPLC were 0.16 %, and 0.00006 % (0.6 ppm) and 1.8 % for elution time, exact mass, and glycoform ratio, respectively. For SEC, the RSD values for elution time of the monomer and dimer were 0.6 % and 1.1 %, whereas the dimer content had an RSD of 14.3 %. For SCX, the RSD for the acidic variant percentage was 0.7 % and for the basic variants between 0.6 % and 0.8 %. These RSDs are slightly higher as compared to within-run RSD values but still very acceptable, indicating that the performance of the platform is stable and reliable.

## 4. Conclusion

This work demonstrates the use of a multidimensional LC system for the fast determination of multiple CQAs of a therapeutic mAb from a single injection of CCF in a fully online and automated fashion. The integrated platform employs valve loops to store three heart-cut fractions of a mAb band resulting from ProtA purification. Each fraction can subsequently be sent to a separation mode of choice. Using SEC-UV, SCX-UV and RPLC-MS the platform provides valuable information related to dimer content, charge variants, and exact molecular mass and glycosylation pattern of the targeted mAb, respectively. Implementation of a UV absorbance detector between ProtA column and storage loops also allows simultaneous determination of the mAb titer of the analysed CCF. The performance of the platform was shown to be repeatable (within and between days) and was benchmarked against conventional methods employing ProtA in offline combination with separate LC systems employing the respective modes of separation. Highly similar CQA values were obtained with the online multidimensional system as compared to the common methodology, but at a significantly reduced analysis time (i.e., 65 min vs. 4–8 h for the assessment of several different CQAs). A substantial part of the time gain can be attributed to the fully automated online purification step prior to the LC analyses. Sampling bias is also avoided because the CQAs are determined from a single injection.

From an application perspective - when fully qualified/validated - the new platform shows great potential for bioprocess monitoring, where sample numbers are limited, but a high degree of (chemical) information is essential. During a mAb production process (on average 10–14 days), the quality of a product should be monitored as closely as possible. As the presented system allows direct CCF characterization with short analysis times, it can help in early decision making on e.g. a production stop or process adjustment when the product quality starts deviating. The used separation methods (ProtA, SEC, RPLC, and, to a lesser degree, SCX) are quite universally applicable, that is, it will mostly require no or only simple adjustments in eluent conditions to allow for optimal analysis of any particular mAb.

The platform also shows attractive options for extending the CQA coverage. Introduction of HIC or hydrophilic interaction chromatography (HILIC) as <sup>2</sup>D protein separation modes could provide detailed

information on e.g. oxidation, conjugation and glycosylation of mAbs when MS detection is used. For that, active-solvent modulation (ASM) between the ProtA and LC dimensions would be needed to achieve eluent compatibility, but that is very well feasible in modern 2D-LC setups [39]. Another attractive option is the use of the multidimensional system for middle-up and bottom-up characterization approaches involving enzymatic mAb digestion into subunits or peptides. As we will demonstrate in the near future, this can be accomplished by incorporation of immobilized enzyme reactors into the online integrated platform (Sadighi et al., manuscript in preparation).

### CRedit authorship contribution statement

**Raya Sadighi:** Investigation, Methodology, Visualization, Writing – original draft. **Vera de Kleijne:** Investigation, Methodology. **Sam Wouters:** Resources, Writing – review & editing. **Karin Lubbers:** Resources, Writing – review & editing. **Govert W. Somsen:** Supervision, Writing – review & editing. **Andrea F.G. Gargano:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Rob Haselberg:** Conceptualization, Project administration, Supervision, Visualization, Writing – review & editing.

### Declaration of competing interest

The authors declare the following financial interests/ which may be considered as potential competing interests: Govert W. Somsen reports equipment was provided by Agilent Technologies Inc.

### Data availability

The data that has been used is confidential.

### Acknowledgements

This research was supported by Agilent Technologies through an ACT-UR grant 4475 “MAP4mAb: Multi-Attribute Platform for Monoclonal AntiBodies”. We thank Sascha Lege for technical assistance and discussions on the RPLC hardware.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2023.342074>.

### References

- [1] S. Fekete, A.L. Gassner, S. Rudaz, J. Schappler, D. Guillaume, Analytical strategies for the characterization of therapeutic monoclonal antibodies, *TrAC - Trends Anal. Chem.* 42 (2013) 74–83, <https://doi.org/10.1016/j.trac.2012.09.012>.
- [2] W. Wang, S. Singh, D.L. Zeng, K. King, S. Nema, Antibody structure, instability, and formulation, *J. Pharm. Sci.* 96 (1) (2007) 1–26.
- [3] F. Cymer, H. Beck, A. Rohde, D. Reusch, Therapeutic monoclonal antibody N-glycosylation – structure, function and therapeutic potential, *Biologicals* 52 (2018) 1–11.
- [4] J.R. Birch, A.J. Racher, Antibody production, *Adv. Drug Deliv. Rev.* 58 (5–6) (2006) 671–685, <https://doi.org/10.1016/j.addr.2005.12.006>.
- [5] N. Alt, T.Y. Zhang, P. Motchnik, R. Taticek, V. Quarby, T. Schlothauer, H. Beck, T. Emrich, R.J. Harris, Determination of critical quality attributes for monoclonal antibodies using quality by design principles, *Biologicals* 44 (5) (2016) 291–305, <https://doi.org/10.1016/j.biologicals.2016.06.005>.
- [6] G. Dhandapani, E. Wachtel, I. Das, M. Sheves, G. Patchornik, Purification of antibody fragments via interaction with detergent micellar aggregates, *Sci. Rep.* 11 (1) (2021) 1–11, <https://doi.org/10.1038/s41598-021-90966-1>.
- [7] H.F. Liu, J. Ma, C. Winter, R. Bayer, Recovery and purification process development for monoclonal antibody production, *mAbs* 2 (5) (2010) 480–499, <https://doi.org/10.4161/mabs.2.5.12645>.
- [8] S. Fekete, A. Beck, J.L. Veuthey, D. Guillaume, Theory and practice of size exclusion chromatography for the analysis of protein aggregates, *J. Pharm. Biomed. Anal.* 101 (2014) 161–173, <https://doi.org/10.1016/j.jpba.2014.04.011>.
- [9] S. Fekete, A. Beck, J.L. Veuthey, D. Guillaume, Ion-exchange chromatography for the characterization of biopharmaceuticals, *J. Pharm. Biomed. Anal.* 113 (2015) 43–55, <https://doi.org/10.1016/j.jpba.2015.02.037>.
- [10] B. Bobaly, V. D’Atri, A. Goyon, O. Colas, A. Beck, S. Fekete, D. Guillaume, Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – enzymatic and chemical sample preparation, *J. Chromatogr. B* 1060 (2017) 325–335.
- [11] D. Stoll, J. Danforth, K. Zhang, A. Beck, Characterization of therapeutic antibodies and related products by two-dimensional liquid chromatography coupled with UV absorbance and mass spectrometric detection, *J. Chromatogr. B* 1032 (2016) 51–60.
- [12] J. Camperi, A. Goyon, D. Guillaume, K. Zhang, C. Stella, Multi-dimensional LC-MS: the next generation characterization of antibody-based therapeutics by unified online bottom-up, middle-up and intact approaches, *Analyst* (2021), <https://doi.org/10.1039/d0an01963a>.
- [13] A. Ekhkirch, V. D’Atri, F. Rouviere, O. Hernandez-Alba, A. Goyon, O. Colas, M. Sarrut, A. Beck, D. Guillaume, S. Heinisch, S. Cianferani, An online four-dimensional HIC×SEC-IM×MS methodology for proof-of-concept characterization of antibody drug conjugates, *Anal. Chem.* 90 (3) (2018) 1578–1586, <https://doi.org/10.1021/acs.analchem.7b02110>.
- [14] A. Ekhkirch, A. Goyon, O. Hernandez-Alba, F. Rouviere, V. D’Atri, C. Dreyfus, J.-F. Haeuw, H. Diemer, A. Beck, S. Heinisch, D. Guillaume, S. Cianferani, A novel online four-dimensional SEC×SEC-IM×MS methodology for characterization of monoclonal antibody size variants, *Anal. Chem.* 90 (23) (2018) 13929–13937, <https://doi.org/10.1021/acs.analchem.8b03333>.
- [15] A. Bathke, D. Klemm, C. Gstottner, C. Bell, R. Kopf, Rapid online reduction and characterization of protein modifications using fully automated two-dimensional high performance liquid chromatography-mass spectrometry, *LCGC North Am.* 36 (2018) 18+.
- [16] C. Gstöttner, D. Klemm, M. Haberer, A. Bathke, H. Wegele, C. Bell, R. Kopf, Fast and automated characterization of antibody variants with 4D HPLC/MS, *Anal. Chem.* 90 (3) (2018) 2119–2125, <https://doi.org/10.1021/acs.analchem.7b04372>.
- [17] A. Goyon, L. Dai, T. Chen, B. Wei, F. Yang, N. Andersen, R. Kopf, M. Leiss, M. Mølhoj, D. Guillaume, C. Stella, From proof of concept to the routine use of an automated and robust multi-dimensional liquid chromatography mass spectrometry workflow applied for the charge variant characterization of therapeutic antibodies, *J. Chromatogr. A* 1615 (2020), 460740, <https://doi.org/10.1016/j.chroma.2019.460740>.
- [18] L. Verscheure, A. Cerdobbel, P. Sandra, F. Lynen, K. Sandra, Monoclonal antibody charge variant characterization by fully automated four-dimensional liquid chromatography-mass spectrometry, *J. Chromatogr. A* 1653 (2021), 462409.
- [19] J. Camperi, I. Grunert, K. Heinrich, M. Winter, S. Özipek, S. Hoeltherhoff, T. Weindl, K. Mayr, P. Bulau, M. Meier, M. Mølhoj, M. Leiss, D. Guillaume, A. Bathke, C. Stella, Inter-laboratory study to evaluate the performance of automated online characterization of antibody charge variants by multi-dimensional LC-MS/MS, *Talanta* 234 (2021), 122628.
- [20] A. Goyon, M. Kim, L. Dai, C. Cornell, F. Jacobson, D. Guillaume, C. Stella, Streamlined characterization of an antibody-drug conjugate by two-dimensional and four-dimensional liquid chromatography/mass spectrometry, *Anal. Chem.* (2019), <https://doi.org/10.1021/acs.analchem.9b02454>.
- [21] L. Verscheure, G. Vanhoenacker, S. Schneider, T. Merchiers, J. Storms, P. Sandra, F. Lynen, K. Sandra, 3D-LC-MS with 2D multimethod option for fully automated assessment of multiple attributes of monoclonal antibodies directly from cell culture supernatants, *Anal. Chem.* 94 (17) (2022) 6502–6511, <https://doi.org/10.1021/acs.analchem.1c05461>.
- [22] J. Camperi, L. Dai, D. Guillaume, C. Stella, Fast and automated characterization of monoclonal antibody minor variants from cell cultures by combined protein-A and multidimensional LC/MS methodologies, *Anal. Chem.* 92 (12) (2020) 8506–8513, <https://doi.org/10.1021/acs.analchem.0c01250>.
- [23] M. Alvarez, G. Tremintin, J. Wang, M. Eng, Y.-H. Kao, J. Jeong, V.T. Ling, O. V. Borisov, On-line characterization of monoclonal antibody variants by liquid chromatography–mass spectrometry operating in a two-dimensional format, *Anal. Biochem.* 419 (1) (2011) 17–25.
- [24] J. Camperi, D. Guillaume, C. Stella, Targeted bottom-up characterization of recombinant monoclonal antibodies by multidimensional LC/MS, *Anal. Chem.* 92 (19) (2020) 13420–13426, <https://doi.org/10.1021/acs.analchem.0c02780>.
- [25] Z.D. Dunn, J. Desai, G.M. Leme, D.R. Stoll, D.D. Richardson, Rapid two-dimensional protein-A size exclusion chromatography of monoclonal antibodies for titer and aggregation measurements from harvested cell culture fluid samples, *mAbs* 12 (1) (2020), 1702263, <https://doi.org/10.1080/19420862.2019.1702263>.
- [26] St Amand, M. M., B.A. Ogunnaike, A.S. Robinson, Development of at-line assay to monitor charge variants of mAbs during production, *Biotechnol. Prog.* 30 (1) (2014) 249–255.
- [27] A. Williams, E.K. Read, C.D. Agarabi, S. Lute, K.A. Brorson, Automated 2D-HPLC method for characterization of protein aggregation with in-line fraction collection device, *J. Chromatogr. B* 1046 (2017) 122–130.
- [28] D.R. Stoll, D.C. Harnes, J. Danforth, E. Wagner, D. Guillaume, S. Fekete, A. Beck, Direct identification of rituximab main isoforms and subunit analysis by online selective comprehensive two-dimensional liquid chromatography–mass spectrometry, *Anal. Chem.* 87 (16) (2015) 8307–8315, <https://doi.org/10.1021/acs.analchem.5b01578>.
- [29] R.E. Birdsall, H. Shion, F.W. Kotch, A. Xu, T.J. Porter, W. Chen, A rapid on-line method for mass spectrometric confirmation of a cysteine-conjugated antibody-drug-conjugate structure using multidimensional chromatography, *mAbs* 7 (6) (2015) 1036–1044, <https://doi.org/10.1080/19420862.2015.1083665>.
- [30] K. Sandra, G. Vanhoenacker, I. Vandenhede, M. Steenbeke, M. Joseph, P. Sandra, Multiple heart-cutting and comprehensive two-dimensional liquid chromatography hyphenated to mass spectrometry for the characterization of the antibody-drug conjugate ado-trastuzumab emtansine, *J. Chromatogr. B* 1032 (2016) 119–130.

- [31] M. Sorensen, D.C. Harmes, D.R. Stoll, G.O. Staples, S. Fekete, D. Guillaume, A. Beck, Comparison of originator and biosimilar therapeutic monoclonal antibodies using comprehensive two-dimensional liquid chromatography coupled with time-of-flight mass spectrometry, *mAbs* 8 (7) (2016) 1224–1234, <https://doi.org/10.1080/19420862.2016.1203497>.
- [32] M. Sarrut, A. Corgier, S. Fekete, D. Guillaume, D. Lascoux, M.-C. Janin-Bussat, A. Beck, S. Heinisch, Analysis of antibody-drug conjugates by comprehensive on-line two-dimensional hydrophobic interaction chromatography x reversed phase liquid chromatography hyphenated to high resolution mass spectrometry. I – optimization of separation conditions, *J. Chromatogr. B* 1032 (2016) 103–111.
- [33] J.J. Gilroy, C.M. Eakin, Characterization of drug load variants in a thiol linked antibody-drug conjugate using multidimensional chromatography, *J. Chromatogr. B* 1060 (2017) 182–189.
- [34] K. Sandra, M. Steenbeke, I. Vandenheede, G. Vanhoenacker, P. Sandra, The versatility of heart-cutting and comprehensive two-dimensional liquid chromatography in monoclonal antibody clone selection, *J. Chromatogr. A* 1523 (2017) 283–292, <https://doi.org/10.1016/j.chroma.2017.06.052>.
- [35] S. Dahotre, L. Dai, K. Kjenstad, C. Stella, J. Camperi, Real-time monitoring of antibody quality attributes for cell culture production processes in bioreactors via integration of an automated sampling technology with multi-dimensional liquid chromatography mass spectrometry, *J. Chromatogr. A* 1672 (2022), 463067.
- [36] Y. Liu, C. Zhang, J. Chen, J. Fernandez, P. Vellala, T.A. Kulkarni, I. Aguilar, D. Ritz, K. Lan, P. Patel, A. Liu, A fully integrated online platform for real time monitoring of multiple product quality attributes in biopharmaceutical processes for monoclonal antibody therapeutics, *J. Pharm. Sci.* 111 (2) (2022) 358–367.
- [37] G.R. Bolton, K.K. Mehta, The role of more than 40 Years of improvement in protein A chromatography in the growth of the therapeutic antibody industry, *Biotechnol. Prog.* 32 (5) (2016) 1193–1202.
- [38] H. Pan, K. Chen, M. Pulisic, I. Apostol, G. Huang, Quantitation of soluble aggregates in recombinant monoclonal antibody cell culture by PH-gradient protein A chromatography, *Anal. Biochem.* 388 (2) (2009) 273–278, <https://doi.org/10.1016/j.ab.2009.02.037>.
- [39] R.S. van den Hurk, M. Pursch, D.R. Stoll, B.W.J. Pirok, Recent trends in two-dimensional liquid chromatography, *TrAC Trends Anal. Chem.* 166 (2023), 117166.