Alternative mobile phase additives for the characterization of protein biopharmaceuticals in liquid chromatography − Mass spectrometry

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HIGHLIGHTS

- Fifteen mobile phase additives are tested for mAb analysis in RPLC- and HILIC-MS.
- A first evaluation is performed at chromatographic level by using a FLD detector.
- As alternative to TFA, four additives are selected in RPLC mode and one in HILIC mode.
- Performance of selected additives are investigated in MS after volatility assessment.

GRAPHICAL ABSTRACT
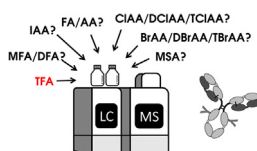
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ABSTRACT

When analyzing large complex protein biopharmaceuticals, ion-pairing agents imparting low pH are widely used as mobile phase additives to improve the chromatographic performance. However, one of the most effective additives in RPLC and HILIC, trifluoroacetic acid (TFA), is known as a strong suppressor of the MS signal and limits its use in hyphenated techniques. In this study, we evaluated a wide range of acidic additives to find alternatives to TFA that provided comparable chromatographic performance and improved MS sensitivity. It was observed that stronger acidic additives were required for intact level analysis compared to subunit level analysis and that the additive nature had a larger impact on the chromatographic performance in HILIC mode compared to RPLC. Therefore, four additives were identified as valuable alternatives to TFA in RPLC mode, namely, difluoroacetic acid (DFA), dichloroacetic acid (DClAA), trichloroacetic acid (TClAA), and methanesulfonic acid (MSA). Only one of these additives provided acceptable performance in HILIC mode, namely, TClAA.

After evaluation of the MS performance, TClAA was discarded due to the apparent loss of intensity in both RPLC-MS and HILIC-MS mode. Together, these results demonstrate that for HILIC-MS analysis TFA remains the gold standard additive. However, DFA was found as promising alternative to TFA for RPLC-MS analysis and could play an important role in the development of methods for the characterization of the increasingly complex protein biopharmaceuticals.

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1. Introduction

Over the past decades, biopharmaceuticals, and among them, monoclonal antibodies (mAbs), have emerged as an important class of therapeutics [1,2]. Thanks to their ability to specifically target antigens, mAb therapies experienced an explosive growth and have become the reference treatment for many life-threatening diseases [3]. MAbs are complex macromolecules of MW approximately 150 kDa that are produced in biological systems using recombinant DNA technology. This complex manufacturing process can generate thousands of possible variants of a given mAb product as a result of chemical and enzymatic modifications introduced during the expression, production and storage of mAbs [4]. Some of these modifications can be critical for the safety and efficacy of the drug product and are therefore known as critical quality attributes (CQAs). To ensure the quality and safety of each therapeutic agent, there is a need of state-of-the-art analytical strategies to monitor the CQAs [5]. Among them, liquid chromatography coupled to mass spectrometry (LC-MS) has become a key technique for the detailed structural characterization of mAbs [6]. In this respect, two denaturing chromatographic techniques are generally applied and coupled directly with MS, namely reversed phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC). While solute retention in RPLC is controlled principally by hydrophobic interactions [7], solute retention in HILIC is more complex and involves a combination of hydrophilic partitioning, hydrogen bonding, dipole-dipole and ionic interactions [8,9]. As a result, and contrary to RPLC, HILIC conditions enable the separation of glycoforms that are considered important CQAs of mAbs. Therefore, these two approaches are highly complementary, since their differences in retention mechanism result in differences in elution order and selectivity [10].

However, the high-molecular weight of mAbs and their numerous conformations make them challenging to characterize. A number of limitations exists and require special consideration. Strong secondary interactions between positively charged biomolecules and negatively charged residual silanols at the surface of the stationary phase are one of the major concerns when analyzing biomolecules [11]. Non-desirable interactions of proteins with the stationary phase or with the HPLC instrumentation may occur [11–13]. To prevent this, the choice of an appropriate stationary phase material with wide pore size and the use of sufficiently high column temperatures (generally 60−90 °C) is crucial [14]. At high temperatures, the mobile phase viscosity is reduced, diffusivity is improved, and adsorption/desorption kinetics are accelerated [15]. However, caution must be taken to avoid potential thermal degradation of proteins. In addition, the use of a bio-inert system can be beneficial to further reduce unwanted adsorption during analysis [14]. The large size of the mAbs also results in a slow diffusivity and mass transfer of the analytes: severe band broadening may be experienced, as well as peak tailing [16]. Together, these phenomena negatively affect the chromatographic performance. Fortunately, these effects can be limited by the addition of acidic additives to the mobile phase [7]. By acting as ion-pairing agents, acidic additives can form neutral ion-pairs with the basic groups of the protein, namely histidine, lysine and arginine and thereby enhance the hydrophobic character of the analyte [13]. This will lead to better retention and improved peak shapes during analysis. Furthermore, the use of strongly denaturing conditions (i.e. acidic pH, high temperatures, organic solvents) greatly reduces the amount of protein conformers and therefore results in sharper and more symmetrical peaks. In addition, the use of acidic additives

can reduce the amount of secondary ionic interactions between the protein and stationary phase by protonating negatively charged silanol groups and neutralizing acidic amino acids (e.g., aspartic acid, glutamic acid) [17]. As a result, peak broadening is minimized [18].

When working in LC-MS conditions, an additive is expected to provide good chromatographic performance, as well as sufficient MS sensitivity. Currently, trifluoroacetic acid (TFA) is considered as the gold standard mobile phase additive to enhance chromatographic performance of protein biopharmaceuticals. Being a strong acid ($pK_a = 0.3$), TFA is a very effective ion-pairing agent that provides symmetrical and narrow peak shapes for proteins analyzed in RPLC and HILIC. Unfortunately, TFA is also known as a strong suppressor of the MS signal [18]. Ion-pairs involving TFA are indeed hardly breakable and TFA prevents the ionisation of analytes since only charged species are detected in MS conditions [19]. In addition, TFA results in spray instabilities, due to the high conductivity and high surface tension of the eluent further hampering MS detection [18]. For these reasons, other additives have been already investigated. Formic acid (FA, $pK_a = 3.75$), which is a weaker ion-pairing agent than TFA, is often used because of its good MS detection properties. However, the chromatographic performance is reduced due to the weaker ion-pairing effect [20]. A compromise between chromatographic performance and MS sensitivity may also be found by mixing small amounts of FA and TFA [21]. Other alternative mobile phase compositions have been tested, such as perfluorinated acids, ammonium formate buffer or more recently, difluoroacetic acid (DFA) and methanesulfonic acid (MSA) [18,22−24].

In this study, we have expanded the range of possibilities for choosing the appropriate mobile phase additive for mAb characterization. We have attempted to find alternatives to TFA that can provide excellent chromatographic performance without neglecting the MS sensitivity. For this purpose, fourteen derivatives of acetic acid were considered in addition to MSA. All these mobile phase additives have a pK_a in the range of −1.9 and 4.76, possess from one to three halogen groups (i.e. chloro-, bromo-, iodo- or trifluoro-) and their similar or derived structure from TFA renders them interesting to investigate as alternative mobile phase modifiers. The additive performance was first studied and compared at the chromatographic level, in both RPLC and HILIC modes by using a fluorescence detector (FLD). For this, different mAbs were analyzed at both intact and subunit level. Then, volatility was assessed for selected additives and finally the performance was investigated in MS to evaluate their relevance in LC-MS.

2. Experimental

2.1. Materials and reagents

Rituximab, trastuzumab, bevacizumab, daratumumab, infliximab, pembrolizumab, cetuximab and pertuzumab were obtained as European Union pharmaceutical-grade drug products from their respective manufacturers. Water (art. W/0122/17) and acetonitrile (art. A/0638/17) were UPLC-MS grade and purchased from Fisher Scientific (Reinach, Switzerland). Trifluoroacetic acid (TFA, ≥99%, art. 302031), formic acid (FA, ≥98%, art. 33015), acetic acid (AA, ≥99.7%, art. 695092), difluoroacetic acid (DFA, 98%, art. 142859), dichloroacetic acid (DClAA, ≥99%, art. D54702), trichloroacetic acid (TClAA, ≥99%, art. T6399), iodoacetic acid (IAA, ≥98%, art. I4386), bromoacetic acid (BrAA, ≥99%, art. 17000), dibromoacetic acid (DBrAA, 97%, art. 242357), tribromoacetic acid (TBrAA, 99%, art.

T48208), methanesulfonic acid (MSA, ≥99%, art. 471356), TRIZMA base (≥99.8%, art. 93362) and dithiothreitol (DTT, art. 43815) were obtained from Sigma-Aldrich (Buchs, Switzerland). MS-grade difluoroacetic acid (Ionhance DFA, art. 720006578 EN) was obtained from Waters (Milford, MA, USA). Immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (*IdeS*, FabRICATOR, art. A0-FR1-250) was purchased from Genovis Inc. (Lund, Sweden).

2.2. Sample preparation

2-μL *IdeS* aliquots (67 U/μL) were initially prepared by reconstituting lyophilized *IdeS* enzyme in the appropriate volume of water and stored at − 20 °C (stable up to 6 months). 1 M DTT solution and 100 mM TRIS buffer pH 7.5 (adjusted with HCl) were freshly prepared before use. Intact sample was prepared by diluting the mAb product to 1 mg/mL with water. Digested/reduced samples were prepared according to the following procedure. MAb material was diluted with water to 10 mg/mL in 10 μl and added to 10 μL of 100 mM TRIS buffer and 68 μL of water. 2-μL *IdeS* enzyme aliquot was added and the resulting solution was incubated at 45 °C for 45 min 10 μL of 1 M DTT solution was then added to the 90-μL digested sample and incubated at 45 °C for 45 min. Final DTT concentration was 100 mM, final TRIS buffer concentration was 10 mM and final protein concentration was 1 mg/mL. The 100-μL samples were then transferred to HPLC vials for the analysis. As result of protein IdeS digestion and DTT reduction, Fd', LC and sFc fragments were obtained. The prepared samples were analyzed within 2−3 days if stored at 4 °C.

2.3. Chromatographic media

2.3.1. Instrumentation
LC analyses were performed on an ACQUITY UPLC™ H-Class system (Waters, Milford, MA, USA), equipped with an auto-sampler including a 10-μL flow-through-needle injector, a quaternary solvent delivery pump and a fluorescence detector (excitation at 280 nm, emission at 360 nm, 5 Hz, 0.2-s time constant, 2-μL detector flow-cell). LC-MS analyses were performed on an ultrahigh performance liquid chromatography (UHPLC) system (ACQUITY UPLC, Waters, Milford, MA, USA) coupled to an electrospray time-of-flight mass spectrometer (Xevo Q-ToF, Waters, Milford, MA, USA). The UHPLC system was equipped with a binary solvent delivery pump, a 1 μL fixed loop autosampler and a FLR detector (excitation at 280 nm, emission at 340 nm, 10 Hz). The mass spectrometer was operated in ESI + mode in the range 500−5500 m/z with a scan time of 1.0 s. Source temperature was 150 °C and capillary and extraction cone voltages were set 3 kV and 4 V, respectively. Desolvation temperature and gas flow were 500 °C and 1000 L/h, respectively. The system was calibrated by using sodium iodide (200 pg/μL in 50/50 ACN/water with 0.1% FA). Data acquisition and instrument control were performed by Empower Pro 3 software (Waters) or MassLynx 4.1 software (Waters), according to the used instrument, namely the UPLC™ H-Class system or the UPLC™- Xevo™ Q-ToF system. LC system lines and syringes were primed before use to ensure the elimination of possible air bubbles in the system. Columns were equilibrated for at least 10 column volumes, by using the solvent composition that was used at the beginning of the gradient elution program. In HILIC, 85:15 ACN/H2O and 40:60 ACN/H2O were used as weak and strong wash, respectively. In RPLC, both washes were 50:50 ACN/H2O.

2.3.2. RPLC and HILIC columns
In RPLC mode, the BioResolve RP mAb Polyphenyl column (2.7 μm, 150 mm × 2.1 mm, 450 Å) from Waters (art. 186009017, Milford, MA, USA) and the Halo C4 column (2.7 μm,

150 mm × 2.1 mm, 1000 Å) columns from Advanced Materials Technology (art. 92712-714, Wilmington, DE, USA) were used. In HILIC mode, the ACQUITY UPLC Glycoprotein Amide column (1.7 μm, 150 mm × 2.1 mm, 300 Å) from Waters (art. 186007963, Milford, MA, USA) was used. After use, RPLC and HILIC columns were rinsed and stored in 100% acetonitrile for the best column lifetime.

2.4. Chromatographic conditions

In RPLC and HILIC, the flow rate was set at 0.4 mL/min and the column temperature was set at 80 °C. MAbs were analyzed at both intact and subunit level. All mAb samples were concentrated to 1 mg/mL and injection volume was 1 μL. Mobile phase consisted of additive in water (A) and additive in acetonitrile (B). All experiments were performed in duplicate and identical chromatograms (perfectly overlaid without any shift in retention time or change in the peak shape) were always obtained between the two successive runs. Fluorescence detection was used to avoid additional adsorption in the detector, and FL-chromatograms were acquired at λexcitation = 280 nm and λemission = 360 nm.

2.4.1. RPLC conditions
In RPLC, the conditions included a gradient of 14%B in 12 min, followed by a 1-min washing step at 70%B and a 4.5-min re-equilibration at the initial conditions. Gradient conditions were optimised during preliminary studies depending on the additive used as mobile phase modifier. They consisted of 26−40%B for MSA and IAA, 30−44%B for TBrAA and TClAA, 27−41%B for DBrAA and DClAA, 25−39%B for BrAA and DFA, 23−37%B for FA and 28−42%B for TFA. Optimization was not achieved using AA: the tested gradient was 20−90%B followed by a 1-min washing step at 90%B.

2.4.2. HILIC conditions
In HILIC, the conditions began by a 0.2-min gradient ramp from 85%B to the initial conditions to avoid peak distortion caused by the high eluotropic strength of the aqueous sample. This fast gradient ramp was followed by a gradient of 8%B in 12 min, then by a 1-min washing step at 15%B and a 10-min re-equilibration at 85%B. Gradient conditions were optimised during preliminary studies depending on the additive used in the mobile phase. They consisted of 61-53%B for MSA, 71-63%B for TClAA and TFA, 69-61%B for DBrAA and DClAA, 60-52%B for BrAA, 66-58%B for DFA and 53-45%B for FA.

2.4.3. LC-MS conditions
For RPLC-MS analyses, 1 μL of 1 mg/mL sample was injected in partial loop mode (2-μL fixed loop). For HILIC-MS analyses, 1 μL of 1 mg/mL sample was injected in full loop mode (1-μL fixed loop) and chromatographic conditions were further optimised, consisting in 73-65%B for both TFA and TClAA.

2.5. Volatility assessment

Eluent volatility was assessed by low-temperature evaporative light-scattering detection (LT- ELSD). No chromatographic column was used. The pump of the ACQUITY UPLC™ H-Class system was used to pump the mixture of mobile phase A (additive in water) and mobile phase B (additive in acetonitrile) and was directly coupled to a SEDEX LT-ELSD Model 90LT (SEDERE, France). A negative control without additive, and a positive control with 0.1% phosphoric acid, were acquired to have reference values of a volatile and a non-volatile mobile phase, respectively. The ELSD was set as follows; gain = 8, gas pressure = 3.5 bar, evaporative tube temperature = 40 °C, acquisition rate = 10 Hz. The flow rate was 0.4 mL/min with a gradient of 5−95% B in 20 min. Between each

test, a cleaning of the system was performed with 50:50 MeOH/H$_2$O. If needed, a cleaning at 90 °C was performed to remove potential non-volatile compounds from the ELSD.

3. Results and discussions

3.1. Selection of suitable additives

With the aim to find alternative mobile phase additives providing suitable chromatographic performance and acceptable MS sensitivity compared to TFA, fourteen acidic compounds were evaluated in this study, namely FA, AA, MFA, DFA, ClAA, DClAA, TClAA, BrAA, DBrAA, TBrAA, IAA, BrDClAA, ClDBrAA, and MSA. A complete list of the investigated additives is reported in Table 1, together with information on their chemical characteristics, including among others density, purity grade, state at room temperature, pK$_a$, and pH when prepared at a concentration of 0.1% in water. In addition, safety information and price for commercial available formats have been included for sake of completeness. Already based on the information reported in Table 1, a first selection was made and some additives were discarded from further investigation. First, based on the safety information, ClAA was removed because of its acute toxicity (in particular for the hazard statement H330: fatal if inhaled). Then, ClDBrAA, BrDClAA, and MFA were discarded, since they cost more than 100 USD for less than 1 g of product and thus were not economically competitive on the basis of the amount of acid required for the preparation of the mobile phases. In addition, MFA was very difficult to handle due to its melting point (mp = 35 °C, gel at room temperature). Therefore, from the 14 additives, only 10 were considered suitable for further investigation. It is worth highlighting that besides removing some additives, a new MS-grade format of DFA was commercialized while this work was being conducted. Based on preliminary tests, the DFA purity grade showed to have a potential impact on peak shape and above all MS sensitivity, as reported in Fig. 1 for the analysis of trastuzumab in RPLC mode performed at intact and subunit level. For this reason, the LC-grade DFA was substituted for the MS-grade DFA for subsequent analyses.

3.2. Selection of a suitable RPLC column

In the last few years, there has been a significant number of advances in reversed phase chromatographic columns for protein analysis. First, it has been demonstrated that RPLC columns packed with superficially porous particles (SPP) have the most advantageous morphology for the separation of large molecules, due in part to their smaller diffusion coefficients and the reduced solute diffusion distance involved [25]. For this reason, the two RPLC columns selected in this work, namely the Halo C4 and Bioresolve RP mAb polyphenyl are packed with SPP of 2.7 μm (rho value of 0.70). Next, the pore size has to be sufficiently large to accommodate large protein species (up to 150 kDa). Therefore, the two selected stationary phases have wide pore sizes of 1000 Å and 450 Å, respectively [26]. Third, these two columns have good thermal stability, and temperatures in the range 80−90 °C can be successfully used to achieve suitable protein recovery [16]. Last, the only important difference between these two columns is the chemistry. As described elsewhere, the Bioresolve RP mAb polyphenyl is based on a high coverage phenyl bonding [27]. Due to its unique surface chemistry and large steric hindrance, this material limits silanol interactions by extensively masking the base silica particle, which is not the case of the Halo C4. This behaviour can be observed in Fig. 2, showing the analysis of intact and digested/reduced trastuzumab on the two columns using three different mobile phase additives (1% FA, 0.1% DFA and 0.1% TFA). As expected,

the observed differences between the two columns were minor or even negligible when adding 0.1% TFA to the mobile phase (blue traces on Fig. 2). This behaviour can be explained by the fact that TFA is a relatively strong ion-pairing reagent, able to mask most of the positive charges at the surface of the proteins, avoiding ionic interaction with residual silanols. On the other hand, when using 0.1% DFA, the chromatographic profiles (yellow traces on Fig. 2) were similar for the digested/reduced trastuzumab sample, but some differences can be observed at the intact level between the two columns. This was obviously attributed to the lower acidity of DFA vs. TFA and the fact that it was less able to create ion-pairs. Therefore, the presence and accessibility to residual silanols become more challenging to overcome. This was particularly critical with intact mAbs (presence of a non-negligible number of positive charges that were not adequately masked), and not with mAb subunits of 25 kDa. In this case, the two minor variants eluting after the main peak were lost in the tail of the main isoform on the Halo C4 column. Last but not least, when using 1% FA as mobile phase additive (red traces on Fig. 2), the differences between the two columns became obvious irrespective of the sample being analyzed (intact and digested/reduced mAb). Due to the weaker acidity of formic acid, the advantage of the Bioresolve RP mAb polyphenyl over the Halo C4 is clearly highlighted. However, the performance achieved with 1% FA was reduced compared to the two other additives, in particular for the intact mAb product.

Finally, to achieve the best performance with a wide range of additives, the Bioresolve RP mAb polyphenyl was selected for the rest of the study. Indeed, this stationary phase has the ability to limit ionic interactions between the positively charged proteins and the negatively charged silanols at the surface of the base particles, thanks to its coverage phenyl bonding with peculiar steric hindrance limiting the access to the silanols, as already reported elsewhere [27,28].

### 3.3. Preliminary results with selected additives

First, some experiments were carried out with both intact trastuzumab and digested/reduced trastuzumab samples in the RPLC (Bioresolve RP mAb polyphenyl) and HILIC (Glycoprotein BEH Amide) modes using the additives selected in Section 3.1. For this part of the work, the gradient conditions (initial and final compositions) were adjusted to have a suitable retention in all cases. Among the selected additives, three of them were rapidly discarded, namely 0.1% AA, 0.1% IAA and 0.1% TBrAA. With 0.1% AA, no peak was observed for the intact trastuzumab and digested/reduced trastuzumab samples both in RPLC and HILIC conditions using the generic conditions (data not shown). AA is the weakest acid of the list (pK$_a$ of 4.76) and probably has a too high pH to suppress sufficiently the ionisation of silanols or is not able to create sufficiently strong ion-pairs with proteins, leading to very strong peak distortion and adsorption of the mAb at the surface of the column. Next, the performance achieved with 0.1% IAA and TBrAA for digested/reduced trastuzumab in RPLC mode were reported in Fig. S1. As shown, the sensitivity was very low using fluorescence detection (only 1 EU) in comparison with other additives. In addition, only one peak was observed with 0.1%TBrAA, and the three peaks observed with 0.1% IAA have very uncommon shapes compared to what was observed with the sample in Fig. 2. TBrAA is a relatively strong acid (pK$_a$ of 0.72), while the IAA is less acidic (pK$_a$ of 3.18). However, these two acids have strong steric hindrance due to the presence of three bromide or one iodide atoms. Due to the non-negligible size of these atoms, it is highly probable that these two acidic additives are not able to create sufficiently strong ion-pairs with the proteins. Therefore, similarly to what happened with AA, the performance is unacceptable: peaks

Table 1
List and properties of investigated additives.

Additives	Provider	pKa	pH of 0.1% additive in water	Linear formula	Structure	Molar mass (g/mol)	State at rt	Density	Purity grade	Safety information
formic acid (FA)	Sigma-Aldrich	3.75	2.75	HCOOH		46.025	liquid	1.22	MS-grade	Danger Toxic if inhaled
acetic acid (AA)	Sigma-Aldrich	4.76	3.33	CH_3CO_2H		60.052	liquid	1.049	MS-grade	Danger
fluoroacetic acid (MFA)	Sigma-Aldrich	2.59		FCH_2CO_2H		78.042	solid	1.37	LC-grade (95%)	Danger Fatal if swallowed
difluoroacetic acid (DFA)	Sigma-Aldrich Waters	1.34	2.00	F_2CHCO_2H		96.033	liquid	1.526	LC-grade MS-grade	Danger
trifluoroacetic acid (TFA)	Sigma-Aldrich	0.3	2.04	CF_3CO_2H		114.023	liquid	1.489	MS-grade	Danger Harmful if inhaled
chloroacetic acid (ClAA)	Sigma-Aldrich	2.87		$ClCH_2CO_2H$		94.49	solid	1.58	MS-grade	Danger Fatal if inhaled, may cause respiratory irritation
dichloroacetic acid (DClAA)	Sigma-Aldrich	1.26	2.18	Cl_2CHCO_2H		128.94	liquid	1.563	MS-grade	Danger Suspected of causing cancer
trichloroacetic acid (TClAA)	Sigma-Aldrich	0.51	2.10	Cl_3CCO_2H		163.38	solid	1.62	MS-grade	Danger May cause respiratory irritation
bromoacetic acid (BrAA)	Sigma-Aldrich	2.90	2.48	$BrCH_2CO_2H$		138.948	solid	1.934	MS-grade	Danger Toxic if inhaled, may cause an allergic reaction
dibromoacetic acid (DBrAA)	Sigma-Aldrich	1.48	2.14	Br_2CHCO_2H		217.844	solid	2.382	LC-grade	Danger
tribromoacetic acid (TBrAA)	Sigma-Aldrich	0.72	2.08	CBr_3CO_2H		296.74	solid	3.098	MS-grade	Danger
iodoacetic acid (IAA)	Sigma-Aldrich	3.18	2.49	ICH_2CO_2H		185.948	solid	4.600	LC-grade	Danger
bromodichloroacetic acid (BrDClAA)	Sigma-Aldrich	0.05		$BrCCl_2CO_2H$		207.83	liquid	2.254	MS-grade	Danger Harmful if inhaled
chlorodibromoacetic acid (ClDBrAA)	Sigma-Aldrich	0.13		$ClCHBr_2CO_2H$		252.29	liquid	2.684	MS-grade	Danger

Table 1 (*continued*)

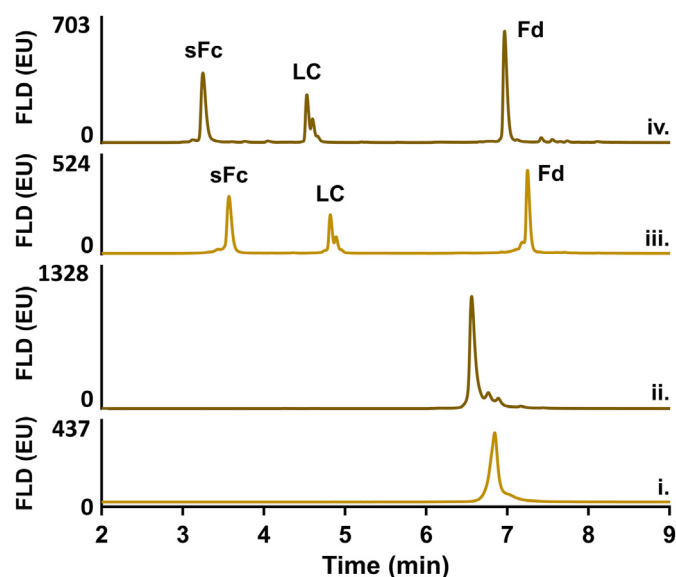| Additives	Provider	pKa	pH of 0.1% additive in water	Linear formula	Structure	Molar mass (g/mol)	State at rt	Density	Purity grade	Safety information
methanesulfonic acid (MSA)	Sigma-Aldrich	-1.90	2.04	CH₃SO₃H		96.11	liquid	1.481	MS-grade	Danger

Fig. 1. Effect of DFA grade on peak shape and sensitivity. RPLC chromatograms of trastuzumab at intact (i and ii) and subunit (iii and iv) level. Analyses were performed with LC-grade DFA (i and iii) and MS-grade DFA (ii and iv) by using the optimised conditions described in Section 2.4.1. Peaks have been labelled with the corresponding subunit name.
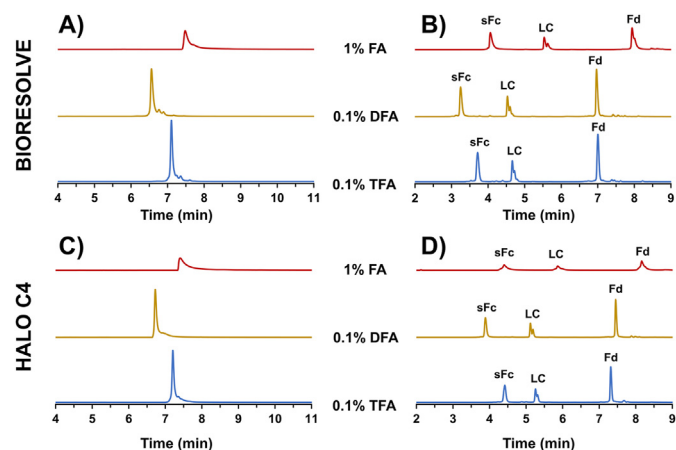

Fig. 2. Effect of RPLC columns on peak shape and sensitivity. RPLC chromatograms of intact (A−C) and digested and reduced (B−D) trastuzumab obtained by using 0.1% TFA, 0.1% DFA and 1% FA (in blue, yellow and red, respectively) in the mobile phases. Separation was performed on a BioResolve column (A−B) or a Halo C4 column (C−D) by using the optimised conditions described in Section 2.4.1. Peaks have been labelled with the corresponding subunit name. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

are distorted or not eluted and adsorption is non-negligible.

The remaining eight additives were then tested at a concentration of 0.1%, and FA was also evaluated at 1%. The corresponding results obtained in RPLC and HILIC for intact trastuzumab and digested/reduced trastuzumab samples are provided in Fig. 3. As expected, the additive nature has a stronger impact on the intact mAb (150 kDa) *vs.* the digested/reduced mAb (25 kDa fragments). This is due to the fact that there are more positive charges to mask through ion pairing, generating peak tailing effect for the largest protein species. In addition, the diffusion coefficients of larger species are smaller, leading to extra peak broadening.

In RPLC conditions (Fig. 3A and C), the worst additive was clearly 0.1% FA, followed by 1% FA and 0.1% BrAA, whatever the sample. Table 2 shows the retention times, widths, and asymmetries of the last eluted peak (Fd species) as well as the selectivity between the first two peaks (sFc and LC species). As reported, it is clear that asymmetries were particularly unacceptable with FA (8.96 with 0.1% FA and 4.37 with 1% FA), while the peak observed with 0.1% BrAA was particularly broad (45% broader than with 0.1% FA and 165% broader than with 0.1% TFA). The performance obtained with the remaining six additives (i.e. MSA, TClAA, DClAA, DBrAA, DFA and TFA) were however comparable in terms of peak width at half height (between 0.0438 and 0.0586 min) and quite close in terms of asymmetry (between 1.27 and 2.13), even if some differences are still observable in terms of selectivity (between 1.09 and 1.68).

In terms of sensitivity, considerable differences were also observed between DClAA and DBrAA. The observed differences in sensitivity could be attributed to the larger size of the bromide ions compared to the chloride ions. Thus, based on the potential steric hindrance of the two larger bromide groups and the lower electronegativity of Bromine, the use of DClAA would be better.

Besides the peak shapes, monitoring the selectivity is also important to evaluate the quality of an additive. For this reason, the selectivity between sFc and LC species was reported in Table 2, and Fig. 4 shows the zoomed chromatograms for the six different additives under RPLC conditions. As shown in Table 2, selectivity between the first two main subunit peaks in Fig. 3C was between 1.09 and 1.68. This relatively large modification of selectivity shows that the nature of the mobile phase additive has an impact on it and can be used during method development to tune selectivity. Among all the tested additives, TClAA was the one providing the lowest selectivity between these two subunits ($\alpha = 1.09$), while MSA provides the largest separation ($\alpha = 1.68$). This difference in selectivity between additives is visually observed in Fig. 4. In addition, Fig. 4 also highlights the differences in separation quality for the minor variants. From this figure, it is clear that all the minor peaks eluting between the main subunit species are better resolved with DFA, TFA, DClAA and MSA, rather than TClAA and FA. Based on the peak shapes, retention and selectivity, several additives can be successfully used for the RPLC analysis of biopharmaceutical products. Even if TFA and FA remain the most widely used, it could be interesting to evaluate several alternatives such as MSA, TClAA,
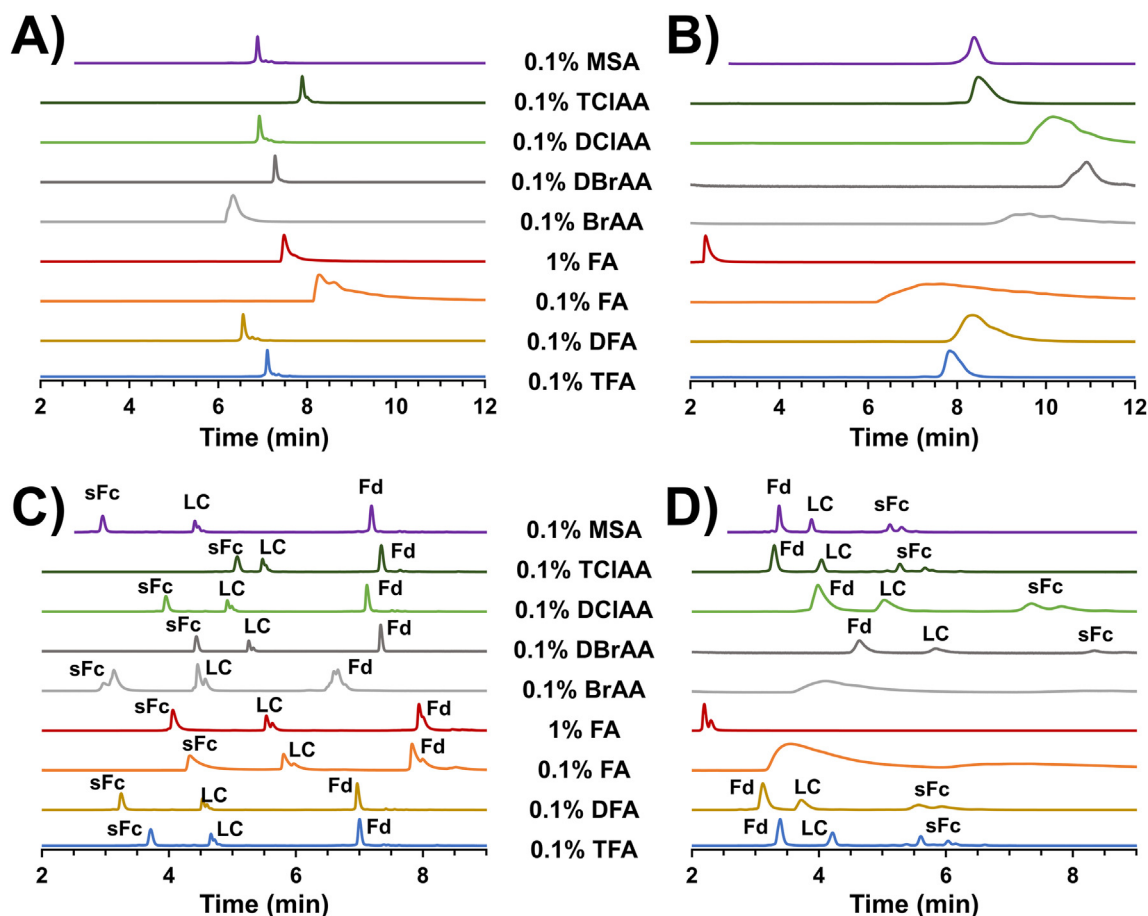
Fig. 3. Additive influence on peak shapes in RPLC and HILIC. RPLC (A–C) and HILIC (B–D) chromatograms of trastuzumab at intact (A–B) and subunit (C–D) level obtained by using the optimised conditions described in Section 2.4. Peaks have been labelled with the corresponding subunit name.

Table 2

Chromatographic parameters of trastuzumab analyzed at intact and middle-up level by HILIC and RPLC. Chromatographic conditions as from Fig. 3 t_r; retention time (min), $w_{50\%}$: peak width at half height, As: asymmetry factor @10, *n.a.* stands for not applicable, α: selectivity (defined as the ability of the method to separate two adjacent analytes from each other; it is calculated as: $\alpha = k_2/k_1$, where k_1 and k_2 are the retention factors, k, of the first and second peaks of a peak pair, while k is calculated as: $k = (t_r - t_0)/t_0$, where t_r is the retention time of the peak of interest, and t_0 is the time at which an unretained peak elutes).

	Digested/reduced trastuzumab − RPLC				Digested/reduced trastuzumab − HILIC			
	t_r	$w_{50\%}$	As	α	t_r	$w_{50\%}$	As	α
0.1% MSA	7.190	0.0531	1.27	1.68	3.373	0.0533	2.39	1.21
0.1% TClAA	7.345	0.0586	1.42	1.09	3.276	0.0883	0.91	1.34
0.1% DClAA	7.118	0.0512	1.97	1.31	3.984	0.1996	2.56	1.33
0.1% DBrAA	7.335	0.0438	1.74	1.23	4.593	0.1657	1.91	1.32
0.1% BrAA	6.661	0.1478	0.98	1.57	4.111	*n.a.*	*n.a.*	*n.a.*
1% FA	7.938	0.0914	4.37	1.45	2.165	*n.a.*	*n.a.*	1.03
0.1% FA	7.830	0.1015	8.96	1.42	3.550	*n.a.*	*n.a.*	*n.a.*
0.1% DFA	6.966	0.0520	2.13	1.53	3.116	0.1100	2.24	1.26
0.1% TFA	7.003	0.0556	1.45	1.33	3.389	0.0887	0.88	1.35

DClAA, or DFA.

In HILIC conditions (Fig. 3B and D), the conclusions are considerably different since only a very limited number of additives provided suitable chromatograms. This means that HILIC is more sensitive to ion-pairing than RPLC. Generally, for intact mAbs, the peaks observed in HILIC are broad, since the different glycoforms are only partially resolved, as already reported elsewhere [10]. In

Figs. 3B and 0.1% MSA, TFA and TClAA provided broad, but acceptable peak shapes for the analysis of intact mAb under HILIC conditions. It is important to notice that these three additives are the most acidic ones (lowest pKa values between 1.26 and 1.48), and are probably able to create the strongest ion-pairs or potentially provide more suppression of silanol group ionisation. On the contrary, peaks were very broad and distorted with 0.1% DClAA, DBrAA and DFA, which have intermediate pKa values between 1.26 and 1.48. Finally, the performance became totally unacceptable with 0.1% BrAA and FA, corresponding to the weakest acid (pKa values between 2.9 and 3.75). The use of 1% FA is a special case since the retention was strongly reduced compared to the other additives and peak shape was more symmetrical. This behaviour was due to the modification of the mobile phase ionic strength (corresponding to approx. 200 mM FA), strongly reducing ion exchange mechanism under HILIC conditions. In the end, it appears that the peak shapes in HILIC are almost exclusively governed by the acidity of the additive. Increasing the amount of additive in the mobile phase can be a good solution to improve chromatographic peak shape, but this will hamper MS detectability and selectivity can be strongly reduced, as shown in Fig. 3B. Strictly similar conclusions can be drawn for subunit analysis, as shown in Fig. 3D. Again, there is a need to have a highly acidic additive (0.1% MSA, TFA or TClAA) to obtain suitable peak shapes in HILIC. Table 2 shows the peak widths and asymmetry of the first eluted peak (Fd subunit), but only for a very limited number of additives offering acceptable peak shapes. As shown, peak widths are much broader (2 to 3-fold) than the ones observed in RPLC and asymmetries are also close to 2 or even
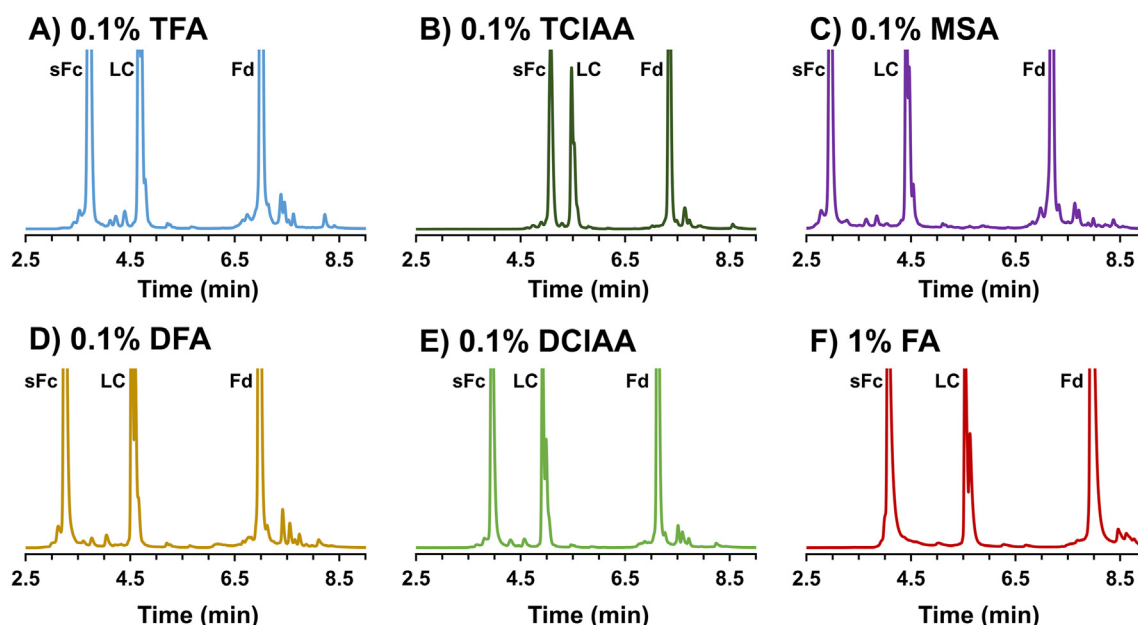
Fig. 4. Zoom on RPLC chromatograms of digested and reduced trastuzumab using selected additives. Based on Fig. 3C. Peaks have been labelled with the corresponding subunit name.

higher. Selectivity between the first two consecutive main subunits was also calculated and reported in Table 2, by using a value higher than 1.1 as acceptable criterion of goodness. As reported, selectivity was almost similar whichever additive was used ($\alpha = 1.26-1.33$), except for the 1% FA, where the selectivity becomes very low ($\alpha = 1.03$).

When analysing subunits of trastuzumab, several glycoforms can be separated on the sFc peak (last eluted peak). Therefore, we have also investigated the separation quality for the glycoforms by zooming in on the HILIC chromatograms obtained with the three most promising additives (0.1% MSA, TFA and TClAA). As shown in Fig. S2, the separation of various glycoforms can be achieved with 0.1% TFA (reference conditions). When switching to 0.1% TClAA, a lower number of glycoforms was separated. Finally, with MSA, a strong reduction of selectivity was observed for the glycoforms and all the glycoforms eluted as two main species. Based on these results, and despite the reduction of selectivity between glycoforms in some cases, three additives have the potential to be used in HILIC, namely 0.1% MSA, TFA and TClAA.

3.4. Application to wide range of monoclonal antibodies

After evaluating the chromatographic performance of the 11 different additives for the analysis of trastuzumab at intact and subunit level, six additives were selected for RPLC and three additives for HILIC mode. To further evaluate the performance of these selected additives, a wide range of mAbs (i.e., rituximab, trastuzumab, bevacizumab, daratumumab, infliximab, pembrolizumab, cetuximab, pertuzumab) was analyzed in RPLC and HILIC modes at both intact and subunit levels. By using a specific gradient for each additive, but the same gradient for all analytes, a clear comparison could be made across the different mAbs.

In RPLC conditions, consistent results were obtained for the eight different mAbs at the intact level (Fig. S3), when using five out of the six additives (i.e., TFA, TClAA, MSA, DFA and DClAA). Only when using 1% FA as mobile phase additive, substantial peak tailing was observed for all eight mAb products, causing poor overall chromatographic performance. This again, highlights the

importance of using a strong additive to mask all the protein positive charges when analysing mAbs at the intact protein level. Fig. 5 displays the corresponding chromatograms obtained after digestion and reduction of the eight mAbs. A similar trend is visible at the subunit level, where the performance of TFA, TClAA, MSA, DFA and DClAA was comparable in terms of peak shape across the subunits of each mAb. For 1% FA (Fig. 5F), peak tailing hampered the chromatographic performance, but to a lesser extent than at the intact protein level.

Besides evaluating the effect of each additive on peak shapes, we also monitored the influence of the additives on the selectivity. From the preliminary results based on trastuzumab, discussed in section 3.3, TClAA was considered to have the worst selectivity. As illustrated in Fig. 5B, this limited selectivity can be problematic when analysing multiple mAbs such as, rituximab, pembrolizumab and pertuzumab, where the sFc and LC are closely eluting. This problem could be easily overcome by switching to different additives, such as MSA, TFA, DFA and DClAA, where an improved selectivity is provided, while maintaining an acceptable peak shape. This effect demonstrates that the use of multiple additives offering various selectivities can be a valuable tool to complement an analysis strategy or to provide valuable alternatives to the use of TFA.

To obtain acceptable peak shapes in HILIC conditions, we only performed subunits analysis of the eight different mAbs using the most acidic additives (i.e., TFA, TClAA and MSA). As can be observed in Fig. 6A and B, TFA and TClAA provide highly comparable results in terms of peak shape and selectivity. This is consistent with the results obtained in the preliminary tests with trastuzumab, where only minor differences in selectivity were observed between the additives and a clear separation was found between the Fd and LC subunits (Table 2). However, for rituximab, infliximab and pembrolizumab, the Fd and LC subunits co-eluted when TFA or TClAA (Fig. 6A and B) were used as mobile phase additive. Surprisingly, when using MSA (Fig. 6C) the separation efficiency improved, and the Fd and LC species of rituximab, infliximab and pembrolizumab could be separated. This indicates that the selectivity of MSA, in HILIC conditions, is better than TClAA and TFA when separating the
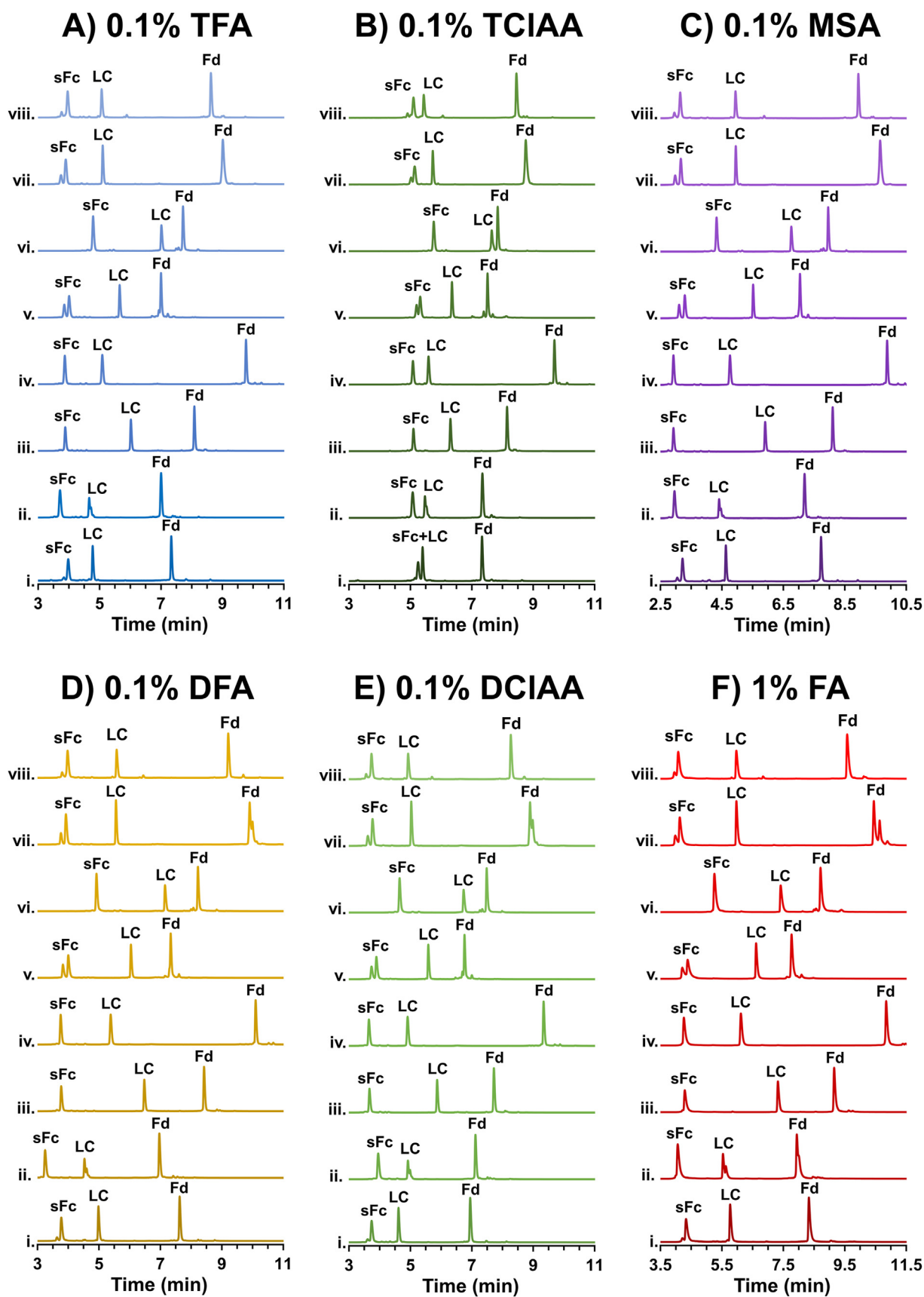
Fig. 5. RPLC analyses performed at middle-up level using selected additives. RPLC chromatographic profiles of rituximab (i), trastuzumab (ii), bevacizumab (iii), daratumumab (iv), infliximab (v), pembrolizumab (vi), cetuximab (vii), pertuzumab (viii) obtained by using the optimised conditions described in Section 2.4.1. Peaks have been labelled with the corresponding subunit name.
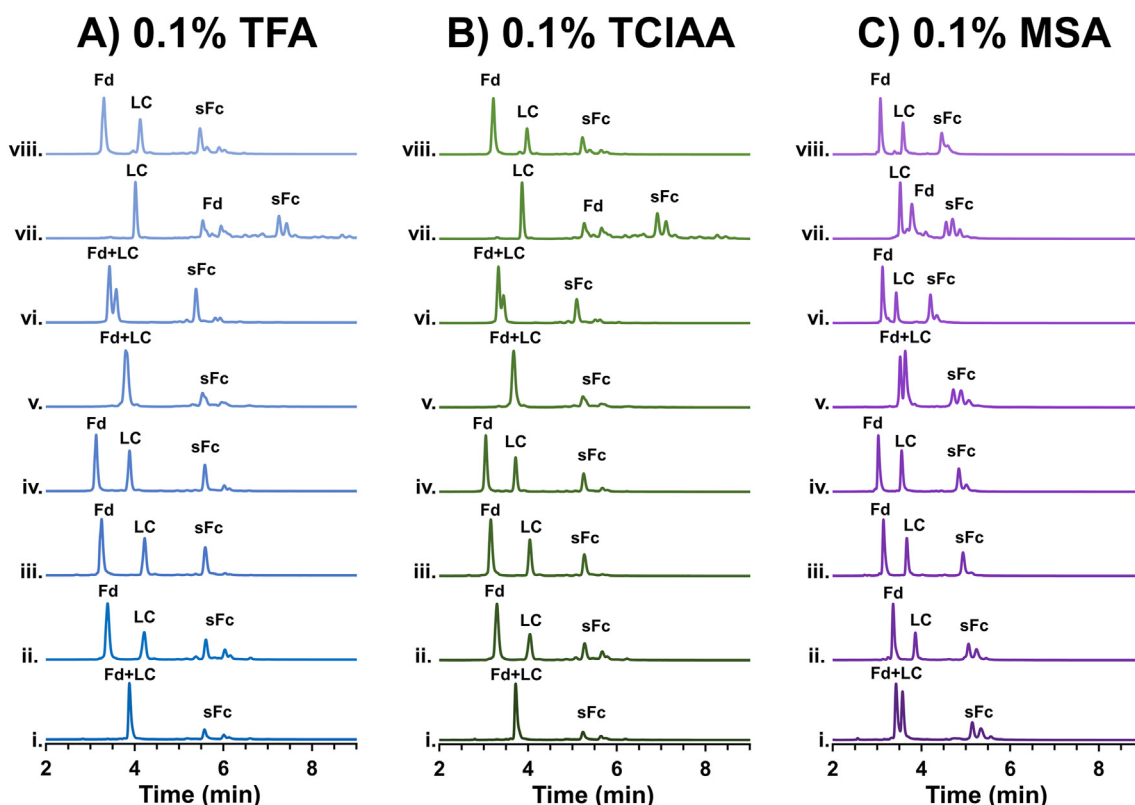
Fig. 6. HILIC analyses performed at middle-up using selected additives. HILIC chromatographic profiles of rituximab (i), trastuzumab (ii), bevacizumab (iii), daratumumab (iv), infliximab (v), pembrolizumab (vi), cetuximab (vii), pertuzumab (viii) obtained by using the optimised conditions described in Section 2.4.2. Peaks have been labelled with the corresponding subunit name.

Fd and LC subunits. However, it is worth mentioning that when focusing only on the sFc subunit bearing the different glycoforms, better selectivity was obtained when using TFA or TClAA, compared to MSA.

This can partially be explained by the loss of retention observed for the sFc subunits of each mAb when being analyzed with MSA (Fig. 6C). A clear trend is visible when comparing the retention times of the sFc subunits of the first mAb, rituximab (Fig. 6 i.), with the retention times of the sFc subunits of the last analyzed mAb, pertuzumab (Fig. 6 viii.), across the three tested additives. For TFA (Fig. 6A) and TClAA (Fig. 6B), it is observed that the main sFc glycoforms of rituximab and pertuzumab elute at approximately the same retention times. When performing a similar comparison for MSA (Fig. 6C), a substantial decrease in retention was observed for the sFc subunits of pertuzumab compared to the first injected sFc subunits of rituximab. By including the other analyzed mAbs in the comparison, a gradual decrease in retention was observed over the course of the eight injections and suggests that the exposure of the HILIC column to MSA leads to rapid alteration of the stationary phase material. To confirm the detrimental effects of MSA on the retention capacity of the HILIC column, we conducted a series of experiments analysing trastuzumab subunits (using reference conditions) after exposing the column material to 16 injections using TClAA followed by 16 injections using MSA. As shown in Fig. S4, the retention capacity of the column was sustained after exposure to TClAA, but largely reduced after exposure to MSA. It is expected that due to the strong acidic character of MSA, the additive is irreversibly adsorbed onto the stationary phase material, thus lowering the amount of possible hydrophilic interactions. This effect could lead to the observed reduction in retention of the mAb subunits when using MSA.

Based on the presented results, four additives (i.e., MSA, TClAA, DFA and DClAA) provided comparable peak shapes to TFA and therefore could be considered as valuable alternatives under RPLC conditions. In HILIC conditions, only TClAA has the potential to be used as alternative to TFA due to the detrimental effects of MSA on the retention of the mAb subunits. It is worth mentioning that, small differences were observed in selectivity among the different additives which could be used during method development. However, the chromatographic performance (e.g., peak width and asymmetry) of each additive can be considered more important, due to the many possibilities to further increase the selectivity such as the use of multi-isocratic gradients or application of negative gradient slope methods [29,30]. In addition, the MS compatibility of a given additive plays an important role during method development.

3.5. Hyphenation to mass spectrometry

From the 14 mobile phase additives initially selected for this study, only five proved to have the potential for being valuable alternatives to TFA in terms of chromatographic performance, namely FA, DFA, DClAA, TClAA and MSA. With the aim to investigate the possibility of using them for LC-MS applications, the volatility of mobile phases prepared with the aforementioned additives was assessed by low-temperature evaporative light-scattering detection (LT-ELSD). No chromatographic column was used for this test. Apart from 0.1% MSA, all mobile phases demonstrated to be volatile and therefore suitable for MS analysis. Based on these results, MSA was discarded from further investigations. LC-MS performance of the remaining 4 mobile phase additives were assessed by LC-MS using a Quadrupole-Time of Flight (Q-ToF) MS detector and *IdeS*

digested and DTT reduced cetuximab and trastuzumab as repre-sentative mAbs. It is worth mentioning that cetuximab is a particular mAb containing 2 conserved N-glycosylation sites located on the heavy chains that after digestion and reduction are present on the Fd' and sFc subunits. For HILIC-MS experiments, mobile phases containing 0.1% TFA and 0.1% TClAA were tested, while for RPLC-MS, mobile phases containing 1% FA, 0.1% TFA, 0.1% DFA, 0.1% DClAA and 0.1% TClAA were taken into consideration. Fig. 7 shows the observed MS intensities reported as signal to noise (S/N) ratio, evaluated on total ion chromatograms (TICs) of trastu-zumab Fd' and cetuximab LC subunits and also expressed as counts per seconds (cps) S/N of the most abundant charge state of each subunit. For sake of comprehensiveness, representative TICs of digested and reduced trastuzumab acquired in RPLC and HILIC modes are reported in Fig. 8 and Fig. 9, respectively, together with the associated m/z spectra. As evidenced by the S/N histograms (Fig. 7), 0.1% TClAA had the worst impact on the MS sensitivity in both HILIC and RPLC modes. In RPLC mode (Fig. 8), despite the clear improvement in chromatographic performance when using 0.1% TClAA in comparison to 1% FA, MS sensitivity suffered of an average intensity loss of 91% in cps S/N when passing from 1% FA to 0.1% TClAA mobile phases. Similarly, in HILIC mode (Fig. 9), despite the fact that chromatographic performance was comparable, MS sensitivity results were showed an average intensity loss of 88% in cps S/N when passing from 0.1% TFA to 0.1% TClAA mobile phases. Based on these observations, 0.1% TFA remains the gold standard additive for HILIC-MS analysis and the sought after MS signal improvement should be realized via other strategies (e.g., by mix-ing small amounts of FA and TFA, as already reported elsewhere) [21]. On the other hand, for RPLC-MS analyses, interesting alter-natives to 0.1% TFA were represented by 0.1% DFA, able to increase the MS sensitivity with an average gain of 50% in terms of cps S/N while keeping optimal chromatographic performance, and by 1% FA that was able to increase the sensitivity of a 68% in terms of cps S/N

but at the expense of reduced chromatographic performance.

4. Conclusions

To expand the range of mobile phase additives that can be used for the characterization of protein biopharmaceuticals, we compared a wide variety of acidic additives as ion-pairing agents in RPLC- and HILIC-MS analysis. The additives were compared to TFA, which is considered as the gold standard additive for enhancing the chromatographic performance of protein biopharmaceuticals, but is hampered by the strong ion suppressing effects in MS detection. It was demonstrated that to obtain acceptable chromatographic performance, stronger additives (e.g., MSA, TClAA, DClAA and DFA) were required at both intact protein and subunit level analysis. This effect proved to be more pronounced in HILIC analysis, indicating the importance of the strong acidic nature (e.g., MSA and TClAA) of the additives as ion-pairing reagent to prevent unwanted ionic interactions on the HILIC stationary phase material.

In RPLC conditions, MSA, TClAA, DClAA and DFA provided comparable chromatographic behaviour as TFA and therefore could be considered as valuable alternatives. For HILIC analysis, only TClAA could replace the chromatographic behaviour of TFA due to detrimental effects of MSA on the HILIC stationary phase material leading to a rapid loss of retention. Moreover, in the additive assessment using LT-ELSD it was observed that 0.1% MSA was not suitable for use with MS detection due to its limited volatility.

Subsequent evaluation of the MS performance of the remaining additives showed that the use of TClAA resulted in a substantial loss of sensitivity in both RPLC and HILIC mode. Therefore, when per-forming HILIC-MS analysis, the use of TFA is inevitable and improvement of the MS sensitivity should be realized with other solutions (e.g., mixed additive usage). For RPLC-MS analysis, a valuable alternative was found in DFA that provided comparable chromatographic performance and an improved MS sensitivity.
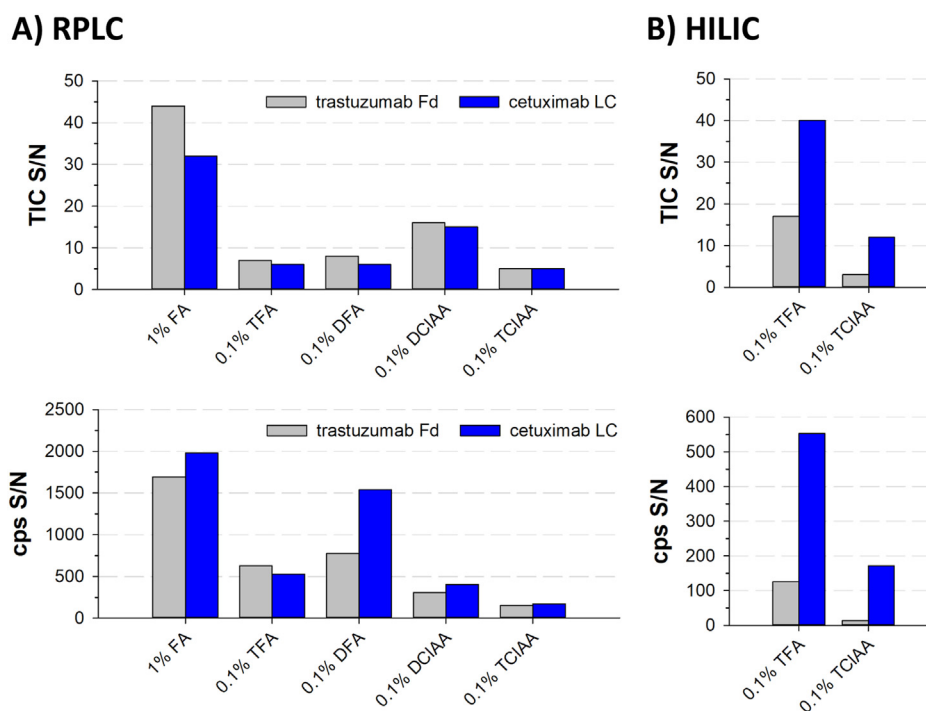

Fig. 7. Mass spectrometric S/N ratio of trastuzumab Fd (grey) and cetuximab LC (blue) subunits calculated in RPLC (A) and HILIC (B) mode when using selected mobile phase additives. S/N ratio evaluated on total ion chromatograms (TIC) and on the highest charge state of the subunits associated m/z spectra expressed in counts per seconds (cps). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

A) Trastuzumab D+R − RPLC 1% FA
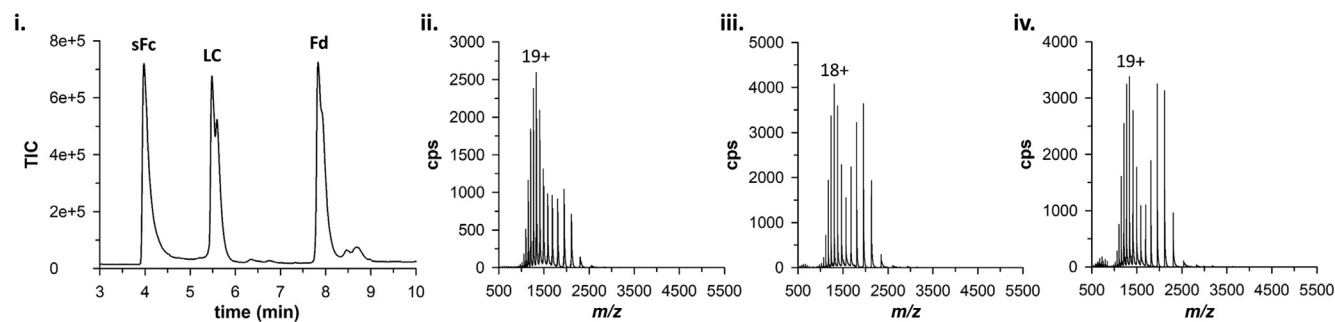
B) Trastuzumab D+R − RPLC 0,1% TClAA
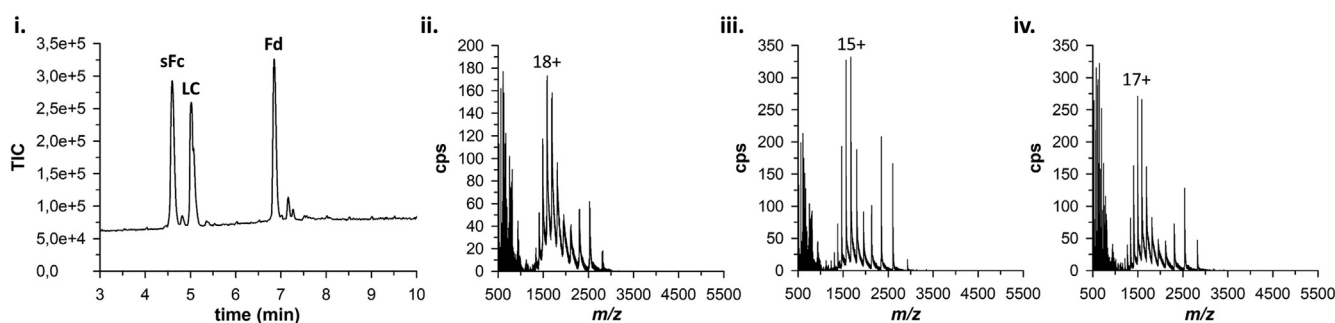
Fig. 8. Middle-up RPLC-MS analysis of trastuzumab by using representative mobile phase additives, namely 1% FA (A) and 0.1% TClAA (B). Total ion chromatograms (i) and associated *m/z* spectra of sFc (ii), LC (iii) and Fd (iv) fragments. Cps stands for counts per second.

A) Trastuzumab D+R − HILIC 0,1% TFA
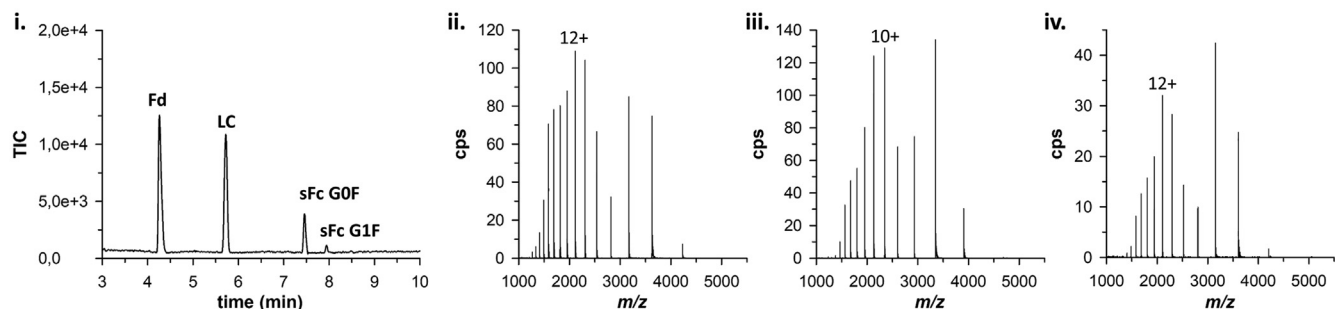
B) Trastuzumab D+R − HILIC 0,1% TClAA
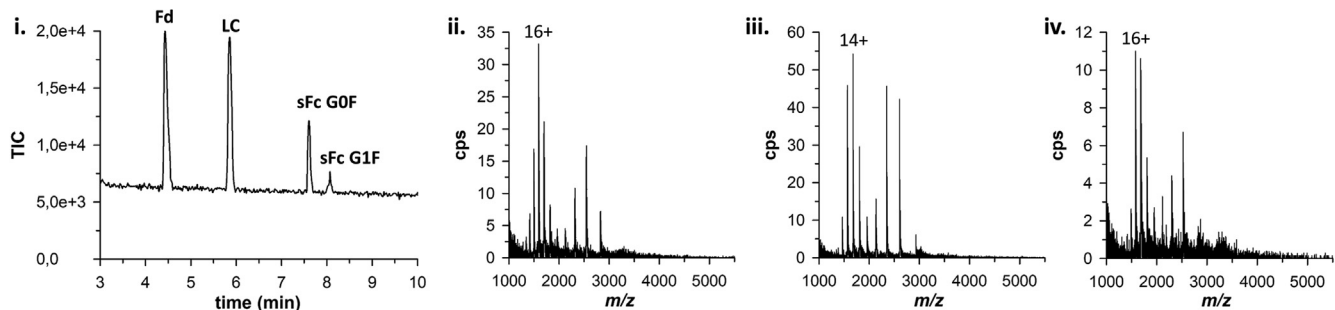
Fig. 9. Middle-up HILIC-MS analysis of trastuzumab by using 0.1% TFA (A) or 0.1% TClAA (B) as mobile phase additive. Total ion chromatograms (i) and associated *m/z* spectra of Fd (ii), LC (iii) and sFc (iv) fragments. For sake of simplicity, only the *m/z* spectra of the sFc subunit bearing the G0F glycan is shown. Cps stands for counts per second.

To conclude, this study showed that at chromatographic level multiple alternatives for TFA are available for both RPLC and HILIC conditions. By providing equivalent chromatographic behaviour, they could be used to complement the analysis strategy. However, the hyphenation to MS requires judicious additive selection by taking into account the required selectivity and MS sensitivity for

analysing the therapeutic protein of interest.

CRediT authorship contribution statement

Honorine Lardeux: Investigation, Writing − original draft, Visualization. **Bastiaan L. Duivelshof:** Writing − original draft. **Olivier Colas:** Resources. **Alain Beck:** Resources. **David V. McCalley:** Writing − review & editing. **Davy Guillarme:** Conceptualization, Supervision, Writing − original draft, Writing − review & editing. **Valentina D'Atri:** Project administration, Supervision, Writing − original draft, Writing − review & editing, Visualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338347.

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