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Title: Evaluation of additives on reversed-phase chromatography of monoclonal antibodies using a 1000 angstrom stationary phase.

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Abstract: A wide pore (1000 Å) diphenyl stationary phase was evaluated for the analysis of monoclonal antibodies (mAbs), comparing a conventional mobile phase of acetonitrile-water containing overall 0.1 % trifluoracetic acid (TFA) with a similar mobile phase incorporating in addition 5 % butanol. Alternatively, TFA was replaced by ammonium formate (AF) buffer (pH 3.0) and by methane sulfonic acid. Addition of 5% butanol to the mobile phase reduces the minimum temperature at which suitable UV analysis of the mAbs can be obtained from about 70 oC with TFA alone to about 60 oC thus potentially improving column lifetime and reducing the possibility of sample degradation. AF buffers produce satisfactory UV sensitivity at 70 oC and have the advantage of reducing signal suppression in mass spectrometry (MS). Some peak tailing was noted in comparison with TFA separations. Methane sulfonic acid at the same molar concentration as TFA produced the best chromatographic peaks, maintaining reasonable UV sensitivity down to 50 oC, also giving acceptable results even at only 3 mM concentration of the additive. The good results with this additive were attributed to its stronger acidity and consequent suppression of the ionisation of column silanols. Surprisingly, peak response (as measured by the size of the peaks) was rather poorly correlated with the peak capacity of the gradient analysis. A possible explanation is self-deactivation of active column sites by a portion of the sample.

Cover letter

***Response to Reviewer Comments**

***Highlights (for review)**

- Diphenyl bonded 1000 Å column evaluated for separation of monoclonal antibodies.
- Rituximab and Bevacizumab selected as difficult probes.
- Column was tested with trifluoroacetic acid and a variety of additives.
- Ammonium formate is mass spectrometer friendly, with acceptable results at 70 ^{o}C.
- Methanesulfonic acid gives best peak capacity, and reasonable results at 50 ^{o}C.

Evaluation of additives on reversed-phase chromatography of monoclonal antibodies using a 1000Å stationary phase.

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Abstract

A wide pore (1000 Å) diphenyl stationary phase was evaluated for the analysis of monoclonal antibodies (mAbs), comparing a conventional mobile phase of acetonitrile-water containing overall 0.1 % trifluoracetic acid (TFA) with a similar mobile phase incorporating in addition 5 % butanol. Alternatively, TFA was replaced by ammonium formate (AF) buffer (pH 3.0) and by methane sulfonic acid. Addition of 5% butanol to the mobile phase reduces the minimum temperature at which suitable UV analysis of the mAbs can be obtained from about 70 ^{o}C with TFA alone to about 60 ^{o}C thus potentially improving column lifetime and reducing the possibility of sample degradation. AF buffers produce satisfactory UV sensitivity at 70 ^{o}C and have the advantage of reducing signal suppression in mass spectrometry (MS). Some peak tailing was noted in comparison with TFA separations. Methane sulfonic acid at the same molar concentration as TFA produced the best chromatographic peaks, maintaining reasonable UV sensitivity down to 50 ^{o}C, also giving acceptable results even at only 3 mM concentration of the additive. The good results with this additive were attributed to its stronger acidity and consequent suppression of the ionisation of column silanols. Surprisingly, peak response (as measured by the size of the peaks) was rather poorly correlated with the peak capacity of the gradient analysis. A possible explanation is self-deactivation of active column sites by a portion of the sample.

1. Introduction.

Monoclonal antibodies (mAbs) represent an increasingly large proportion of world-wide pharmaceutical sales, which for some compounds are in the range of billions of dollars per annum. As with all pharmaceuticals, quality control of formulations is essential. In addition, many mAbs are coming off-patent, and much effort has been expended on the preparation of biosimilars, which have equivalent properties to the original drug, or biobetters that may have improved efficacy and safety [1]. Careful assessment of these materials e.g. for comparability with the original drug, is required. Characterisation may involve a number of approaches and techniques such as separation by charge (e.g. by ion-exchange chromatography, capillary electrophoresis); hydrophobicity/hydrophilicity (e.g. RPLC, hydrophilic interaction chromatography); size (size exclusion chromatography); and affinity (affinity chromatography) [2]. Some of these techniques can be combined to give high resolution 2-dimensional separations of the proteins [3, 4].

Much information can be derived by cleaving mAbs with specific reagents to produce characteristic sub-units that can be more easily analysed. In addition, the whole protein can also be digested with enzymes such as trypsin to give peptides that characterise the protein. However, there is interest in the separation of intact mAbs by RPLC (and other LC separation mechanisms), as the structure and properties of the entire drug are retained [5, 6]. Superficially porous ("shell") particles would seem an ideal choice for the analysis of these large molecules (MW~150,000 Da) as mass transfer in and out of the porous fraction of these particles is facilitated by the small diffusion distance required. This factor, while especially beneficial for large molecules due to their low diffusion coefficients, may not be important for small molecule compounds (MW<500 Da) where other considerations, possibly the better packing qualities of shell compared with totally porous materials may be more significant [7]. However, it is necessary to use materials with sufficient pore size to accommodate these large species-otherwise size exclusion effects could dominate the separation. According to Guiochon and co-workers, the pore size of the stationary phase should be at least three times the protein size e.g. at least 300 Å for immunoglobulins [8]. Kirkland and co-workers [9] recommended the use of shell particles with 1000 Å pores for the analysis of mAbs. Such large pore size materials also prevent the hindered diffusion that may be experienced with smaller pore materials, leading to peak shape problems. The authors reported narrower peak widths for the Sigma test mAb on a 2.7 μm 1000 Å C4 phase compared with a 1.7 μm 300 Å C4 phase, using

an ACN gradient with 0.1% difluoroacetic acid as additive. The improvement was attributed to mass transport limitations in the smaller pore size material. For small molecules analysed in the isocratic mode, the large pore C4 phase also gave good performance with reduced plate height h~2.1. Fekete and co-workers [10] recently evaluated a 2.7 μm 450 Å pore size high coverage triphenyl phase (Waters) for the analysis of mAbs using mainly 0.1 % trifluoroacetic acid (TFA) as additive in an ACN – water gradient. The authors stressed the importance of using elevated temperatures (80-90 ^{o}C) to obtain good "recoveries" of intact proteins. (Recovery was measured as the response at a given temperature compared with that at the highest temperature used, 90 ^{o}C) . At lower temperatures, proteins may adsorb too strongly onto the stationary phase, giving poor peak shapes and above all, very limited sensitivity. Conversely, elevated temperatures may result in sample degradation and also negatively affect the column lifetime. The authors therefore emphasised the importance of inclusion of temperature as a variable in column evaluation studies. It is well-known that elevated temperature can lead to improvements in separation for proteins beyond those attainable for small molecules. Raising the temperature may also reduce the number of protein conformers (likely by denaturing the protein into its unfolded state) hence reducing peak broadening [6, 11] .

It has been known for many years that TFA produces good chromatographic results for the separation of peptides and proteins [12], although it is possible that other additives might give chromatographic improvements over TFA. With TFA, ion pair formation with basic groups on side chains enhances the hydrophobic character of the peptide or protein, enhancing suitability for RP analysis [13]. Ion pairing may also reduce ionic interactions of the solute with ionised silanols. However, as the ion pairs may not be broken, signal suppression may result in electrospray mass spectrometry. Furthermore, surface tension effects and high conductivity of the eluent may cause spray instability. In the end, TFA may lead to sensitivity loss up to an order of magnitude compared with more favourable methods [14]. In some cases, post-column addition of counteracting agents (e.g. propionic acid) has been advocated to improve sensitivity [15]. Formic acid can give reasonable peak shapes for peptides while maintaining good MS detection properties, but its chromatographic performance for large proteins is doubtful, leading to significant peak broadening or even irreversible adsorption compared with use of TFA [10, 16]. Another problem however with TFA is that restoring the LCMS system to highest sensitivity operation after use may take a considerable period of flushing (e.g. 24 hours with 50:50 ACN/water containing 1% acetic acid [17]. In this context, the use of ammonium formate

(AF) buffers adjusted to acid pH with FA may provide a compromise, giving acceptable chromatographic performance while maintaining good MS sensitivity. A 5 fold average increase in sensitivity was reported for therapeutic proteins and their fragments [12, 13] using AF buffers at pH 3 compared with TFA.

The aims of the present study were:

a) To evaluate a new shell 1000 Å pore size diphenyl stationary phase, comparing its performance for mAbs with a C4 phase synthesised on the same base material.
b) To compare the performance of different mobile phases on the diphenyl phase, with the aim of obtaining the best chromatographic results at the lowest possible temperature.
c) To determine whether it is possible to improve chromatographic performance for mAbs using alternatives to 0.1 % TFA.

2. Experimental

Experiments were performed using a 1290 ultra-high performance liquid chromatograph (UHPLC, Agilent, Waldbronn, Germany) comprising a binary pump, autosampler and photodiode array UV detector set at 280nm (0.6 μL flow cell). The gradient delay of the UHPLC system was measured as 150 μL by running a gradient from 100 % water to 100 % water plus 10 ppm of a UV absorbing substance (uracil) using an empty tube (10 cm x 0.05mm internal diameter) to generate a backpressure for normal pump performance. At a flow of 0.4 mL/ min. all peaks with a retention of > 0.375 min (thus all the peaks in the present study) emerged under gradient (rather than isocratic) conditions. Sample injection volume was 2 μL and flow rate was 0.4 mL/min. ACN (gradient UV grade), formic acid, methanesulfonic acid (MSA), trifluoroacetic acid (TFA) and ammonium formate (AF) were purchased from Fisher (Loughborough, U.K.). Rituximab (Mabthera) and Bevacizumab (Avastin) were obtained from the School of Pharmaceutical Sciences, University of Geneva. Columns used were Halo diphenyl and Halo C4 (10 x 0.21 cm, 2.7 μm particle size, 1000 Å pore size (Advanced Materials Technology, Wilmington, USA). Peak capacity in gradient elution was determined using the equation:

$$P = 1 + t_G/1.699w_{0.5}$$

where t_G is the gradient time, and $w_{0.5}$ the peak width at half height [12]. This equation was used rather than its equivalent using peak width at base due to the difficulty of reproducible measurement of this quantity at base. The mobile phase gradient was typically 30:70 ACN-water to 45:55 ACN-water in 5 min. The concentration of the various additives (e.g. 0.1 % TFA) was maintained constant throughout the gradient by inclusion in both mobile phase reservoirs. An equilibration time of 15 min was allowed between each run. mAb samples were prepared at a concentration of 500 mg/L dissolved in 0.1 % aqueous TFA.

3. Results and Discussion.

3.1 Column and antibody selection.

We first evaluated two different 1000 Å stationary phases (diphenyl and C4) from the same manufacturer for the determination of the mAbs Rituximab and Bevacizumab (approx. 0.5 mg/mL concentration) using a 5 minutes gradient of 30-45 % ACN containing 0.1 % TFA (v/v) at 0.4 mL/min. From a previous work of Bobaly et al., Rituximab and Bevacizumab produced the 20th and 21st lowest values of the chromatographic behaviour parameter B_C among the 23 mAbs tested where

$$B_C = P_{rel} \times R_{rel}$$

P_{rel} is the peak capacity in gradient elution and R_{rel} the peak area both at 60 ^{o}C relative to results obtained at 90 ^{o}C. Results were obtained using an ACN gradient with 0.1 % TFA as the additive [16]. These two mAbs therefore represent amongst the most chromatographically demanding probes of performance. As reported elsewhere, Rituximab can give particular problems with ionic interactions with the stationary phase due to its high pI of 9.4, whereas Bevacizumab can undergo strong hydrophobic interactions as illustrated by its high retention in hydrophobic interaction chromatography [18, 19]. They are both important biopharmaceutical products, each having current annual sales of > $7 billion. Rituximab is currently used for the treatment of non-Hodgkin's Lymphoma, whereas Bevacizumab is used for ovarian, lung, colon and rectal cancers. With a column temperature of 80 ^{o}C, reasonably good results with peaks of acceptable symmetry were obtained both on the C4 (Fig.1) and diphenyl (Fig. 2a) columns. Note the Y axis scale was constant with a maximum value of 90 mAU in each of Figures 1 and 2. However, column lifetime is enhanced at lower temperatures, and degradation of mAbs may be reduced. Less aggressive conditions are also a goal for mAb separations, as the protein may exist

fully or partially in a “native” state, retaining or partially retaining its 3D structure. Folded proteins are more compact and the interior amino acids are less prone to be charged than those at the surface. Thus “native” proteins have a narrower charge state envelope in electrospray MS, meaning the signal intensity is distributed over fewer charged states, potentially leading to increased sensitivity [20]. However, sufficiently non-aggressive conditions to allow native protein analysis remain an ambition in RP separations. With the diphenyl column, 70 ^{o}C appears to be an acceptable temperature in terms of response and peak shape for both mAbs using TFA. In comparison this lower temperature gave reduced response for both mAbs on the C4 column. At 60 ^{o}C, the diphenyl column gave reduced sensitivity (only ~40 % of the response compared with higher temperatures), but the C4 column failed to elute Bevacizumab and gave only a very small peak for Rituximab. At 50 ^{o}C, the diphenyl column gave a very small response with both mAbs, while the C4 column showed no response at all. Guillarme and co-workers also reported that higher temperatures (85-90 ^{o}C) were necessary to achieve similar performance for intact mAbs on a C4 column compared with a triphenyl column operated at 75 ^{o}C [10]. In view of its superior performance, further work concentrated on the diphenyl phase.

3.2 Evaluation of UV response with different additives.

We chose peak height as a measure of response rather than peak area, as the former is less susceptible to the presence of isoforms present in the standard mAbs, which may/may not be completely separated from the main peak under the various conditions used. For example, Fig. 2d (0.013 M MSA) shows the presence of several minor variants in the chromatogram of Rituximab and Bevacizumab, whereas these peaks are merged with the major peaks in Fig. 2c (5mM AF pH 3) preventing the assessment of these isoforms. Use of peak area would unfortunately skew the results in favour of poorer separations, as the peak area of the major peak would be increased by inclusion of these isoforms. Note that while peak height varies considerably with retention in isocratic analysis, peak height is approximately independent of retention time in gradient elution when the flow rate, the column, the gradient time and the gradient programme are all maintained constant, as is the case for all the results in the present study shown in Figs. 2-4 [21].

Figure 2b shows the effect of incorporating 5 % n-butanol to each channel while maintaining the concentration of TFA in each at 0.1% and the ACN gradient constant. It has previously been suggested that incorporating additives such as propanol or butanol into a TFA containing mobile phase can improve the response at lower temperatures. It is

possible that these alcohols may shield silanols from detrimental ionic interaction with the protein [22] . Comparison with Fig 2a shows that addition of butanol clearly has some beneficial effect, with greater response to both mAbs at 60 and 50 ^{o}C compared with use of 0.1% TFA alone. Fig. 3a and 3b show plots of peak height obtained for Rituximab and Bevacizumab respectively at temperatures from 50-80 ^{o}C using different mobile phase additives. The graphs illustrate a more quantitative summary of the results under different conditions compared with mere visual inspection of the chromatograms in Fig. 2. Fig. 3 shows more clearly that increases in peak height were obtained with butanol addition at 60 ^{o}C of ~65% and ~57 % for Rituximab and Bevacizumab respectively compared with use of TFA alone. However this effect is rather limited as demonstrated by the poor response of both mAbs at 50 ^{o}C, even in the butanol containing mobile phase.

In an effort to improve MS sensitivity, attempts have been made to replace some of the mobile phase TFA content with FA, as FA alone gives poor chromatographic results (see above). The presence of acidic silanols can result in pronounced (probably cationic) adsorption of protein. Residual silanols are increasingly ionised at higher mobile phase pH. The true thermodynamic $_{s}^{s}$ pH of 0.013M FA in 60 % ACN is around 3.5, whereas that of the same molar concentration of TFA is about 2.0, and undoubtedly the milder acidity of the former gives rise to more deleterious effects [23]. The very low ionic strength of 0.013M FA at 60 % ACN (~0.3 mM) compared with that of TFA (~11mM) may also lead to greater possibilities of overloading with the former additive. Unfortunately, a study where half of the TFA concentration was replaced with FA resulted in significant recovery decrease for six representative mAbs [16], indicating that this approach was not profitable, although greater success was obtained for the analysis of mAb sub-units. Similarly poor results were found with FA alone in the analysis of basic peptides [12]. However, much improved results were obtained when using ammonium formate buffers both for peptides [12] and also for some proteins [13]. Thus, we attempted the separation using ammonium formate buffers (AF, 5 mM $_{w}^{w}$ pH 3). We chose a low buffer concentration as it limits MS signal suppression. It has also been reported that chromatographic peak shapes were worse for mAbs at higher buffer concentration, perhaps due to salting out and irreversible adsorption [13]. While AF buffers cannot allow exploration of the beneficial low pH region provided by TFA, they at least solve the problem of low ionic strength of the mobile phase, as the salt is completely dissociated even in 60 % ACN. Ammonium ions should also compete beneficially with sample molecules for cationic sites on the column. Fig. 2c shows that it is indeed possible to obtain reasonable results with AF/FA, without using fluorinated acids that are detrimental to MS sensitivity. However, clearly some of the resolution of the

impurities/heterogeneities in the standards is lost when using AF/FA compared with TFA (Fig 2a), with these various minor variants merging into the tail of the major peak. Fig. 3a and 3b confirm that for AF/FA, there is some improvement in response by using 80 rather than 70 ^{o}C. However, response drops off considerably at lower temperatures. For Rituximab, the response using AF/FA at 60 ^{o}C was reduced to 29 % compared with that obtained using the same mobile phase at 80 ^{o}C, whereas it was only 2 % for Bevacizumab. At 50 ^{o}C, neither mAb could be detected using this mobile phase. Despite this poorer behaviour at lower temperatures, over 80% of the average response for the two mAbs was maintained using AF/FA compared with that using 0.1 % TFA when analysis was carried out at 80 ^{o}C.

Methane sulfonic acid (MSA) has also been demonstrated as a useful additive in a study of the separation of peptides. Kadar et al. [24] reported improvements in retention and efficiency for peptides in HILIC analysis when compared with TFA. The improved retention in HILIC was attributed to ion-pairing of peptide with MSA. MSA has slightly stronger acidity than TFA at the same molar concentration even when the pH is measured in the presence of high concentrations of ACN as used in HILIC separations, and provides similar ionic strength to TFA [23]. Fig. 2d indicates this acid, when used at the same molar concentration as 0.1% v/v TFA (0.013M), gave superior response for both Rituximab and Bevacizumab. The superior response is particularly apparent at lower temperatures (50-60 ^{o}C) where peak shape and response is maintained to a greater extent compared with that at higher temperatures using MSA. Fig. 3a indicates that the response for Rituximab at 60 and 50 ^{o}C using MSA was 92 and 44% of the response at 80 ^{o}C compared with considerably lower values of 67 and 8 % for the same compound using 0.1% TFA/butanol. Clearly, there are even greater gains in response using MSA compared with TFA alone as additive. All of these results were mirrored using Bevacizumab (Fig. 3b). Note that the selectivity for variants/isoforms shown using TFA was maintained when using MSA.

The present study uses exclusively UV detection. Indeed, for large proteins, the sensitivity of UV detection is better than for MS detection (whereas the opposite is true for the detection of small molecules or peptides) due to the large envelope of MS ions produced from these large molecules (see above). Furthermore, MS detection in quality control of biopharmaceuticals is rarely used due to complexity and cost. Particularly for good sensitivity in MS detection however, the concentration of additives should be maintained at the lowest possible value consistent with preservation of the chromatographic peak shape. MSA was reported to be MS compatible [24], but at higher concentrations led to signal suppression, at least in the HILIC mobile phases used in that

study which contained a high proportion of ACN. Clearly, further investigation of the MS properties of MSA is required and will be the subject of a further study. Fig. 2e demonstrates that good results can even be obtained with 0.0033M MSA i.e. at one quarter of the previous concentration. Note that despite the low gradient retention times of the peaks in Fig. 2e (1-2 min.), all peaks eluted under gradient conditions after the gradient delay (0.375 min.-see Experimental), allowing comparisons to be made with the other mobile phase conditions. Use of the more dilute MSA concentration resulted in a loss of peak height and peak capacity of about 25% compared with 0.013M MSA, but it appears that the good results can still be obtained at 60 ^{o}C using the lower concentration of acid. Some small loss of selectivity for the isoforms can be seen at this lower concentration of MSA.

3.4 Effect of additive and temperature on Peak Capacity.

Figs. 4a and 4b show the peak capacity of both mAbs (calculated for a gradient of 30-60 % ACN in a time of 10 min.) using each of the 4 mobile phases over the same range of temperatures (50-80 ^{o}C). Particularly for Bevacizumab, peak capacities are highest when using MSA and lowest when using AF/FA. However, comparison of Figs. 3 and 4 does not show a close correlation between peak response and peak capacity. For example, Rituximab shows a rather small decrease in peak capacity at 60 ^{o}C compared with that at 80 ^{o}C, using AF/FA (Fig. 4a) but a much larger decrease in response (Fig. 3a). Undoubtedly, results for peak capacity with AF/FA are optimistic, because peak widths were recorded at half height rather than at the base, as the latter method has problems of reproducibility. This means that the peak tailing evident in Fig. 2d is not taken into account. However, observation of the chromatograms (e.g Fig. 2a using 0.1 % TFA alone) indicates that peak shape is largely maintained as the temperature is lowered, even as the response decreases considerably. It seems more likely that some fraction of the injected protein sample becomes irreversibly adsorbed to the stationary phase when held at low temperature, providing a more inert surface for the chromatography of the remaining sample. Indeed it was observed that running a blank sample at 70-80 ^{o}C immediately after a run at 50 ^{o}C using AF/FA resulted in elution of “ghost” peaks with surprisingly good shape at the appropriate retention time (results not shown).

Fig. 5 shows the variation of peak capacity with gradient time over the range 5-60 min. using 0.013M MSA as additive and a column temperature of 70 ^{o}C. The limiting peak capacity for both Rituximab and Bevacizumab appears to be of the order of ~300. At higher gradient times, the experimental conditions approximate more closely to those in an

isocratic separation, with similar band broadening processes involved, which explains the levelling off shown.

4. Conclusions

A 1000 Å diphenyl phase was found to give good results for the analysis of monoclonal antibodies using a gradient of 0.1 % TFA in acetonitrile. The two commercial mAbs Rituximab and Bevacizumab were chosen as probes, because they are generally considered to be difficult to analyse as they undergo strong ionic and hydrophobic interaction respectively with RP materials. Investigations of various alternative additives were performed with the objective of obtaining the best chromatographic performance at the lowest possible temperature. Lower temperatures increase the lifetime of the column and reduce the possibility of sample degradation. Addition of butanol to the 0.1% TFA mobile phase lowers the useful analysis temperature from about 70 ^{o}C to 60 ^{o}C; possibly the alcohol blocks active column sites. AF buffers, which are known to give better MS detection properties than TFA, produce reasonable chromatographic results at 70 ^{o}C or above, although peak shape was slightly inferior to those obtained with TFA. More significant chromatographic benefits can be obtained by use of methanesulfonic acid which reduces the feasible analysis temperature to around 50 ^{o}C, even when low concentrations of additive are utilised (~3 mM). The stronger acidity of MSA may be beneficial in reducing detrimental ionised silanol interactions. Further studies are necessary to assess the MS compatibility of this additive. Surprisingly, peak capacity (a measure of peak narrowness in gradient elution) correlated rather poorly with response (as measured by the size of peaks) when using any of the different additives. It is possible that a portion of the sample is adsorbed in active sites at low temperature, allowing some sort of self-deactivation of the column surface.

5. Acknowledgements

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6. Legend to Figures

Fig. 1 Separation of Rituximab (first eluted) and Bevacizumab on wide pore C4 column. Mobile phase gradient 30-45 % ACN containing 0.1 % TFA. Column temperature from 50-80 ^{o}C. Gradient time t_G =5 min. Flow rate 0.4 mL/min. Injection volume: 2 μL. UV detection at 280 nm.

Fig. 2 Separation of mAbs on wide pore diphenyl column using additives in 30-45 % ACN gradient, t_G=5 min. Mobile phases: a) 0.1 % TFA; b) 0.1 % TFA and 5% butanol; c) 5mM AF pH3 ; d) 0.013 M MSA; e) 0.0033M MSA. Column temperature, flow rate, injection volume, detection wavelength as Fig. 1

Fig. 3 a) Effect of temperature (Black 80 ^{o}C; Red 70 ^{o}C; Green 60 ^{o}C; Blue 50 ^{o}C) on response of Rituximab using 4 different mobile phases as in Figs 2a-2d.b) Same for Bevacizumab. Column temperature, flow rate, injection volume, detection wavelength as Fig. 1.

Fig. 4 a) Effect of temperature on peak capacity using 4 different mobile phases (as Figs. 2a-d) for Rituximab. b) Same for Bevacizumab. Column temperature, flow rate, injection volume, detection wavelength as Fig.1.

Fig. 5 Effect of gradient time on peak capacity for diphenyl column. Column temperature 70 ^{o}C. Mobile phase gradient 30-60 % ACN 0.013M MSA with gradient times 5-60 min. Column temperature, flow rate, injection volume, detection wavelength as Fig.1.

7. References

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Figure 1

Fig. 1

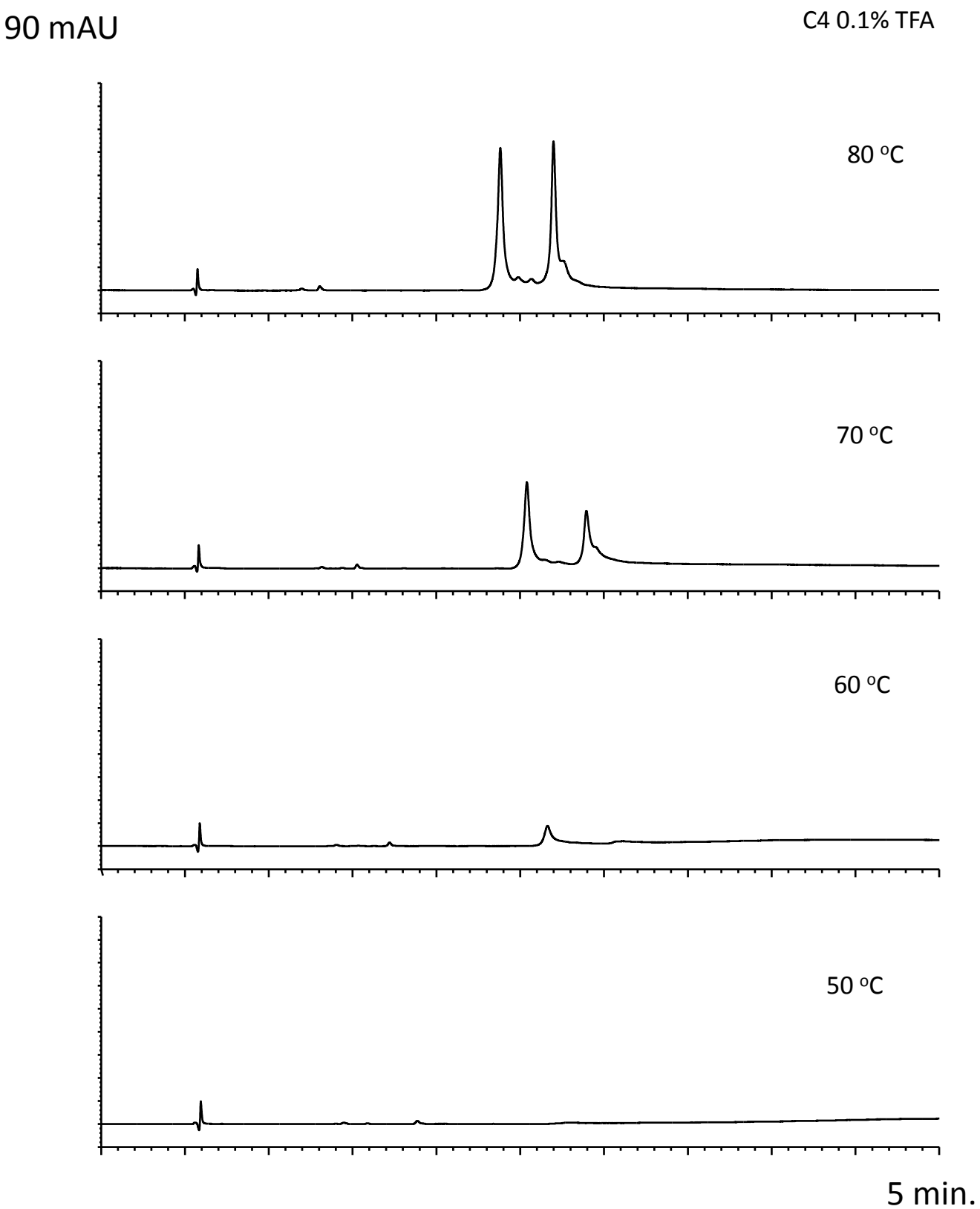


Figure 2a

Fig. 2a

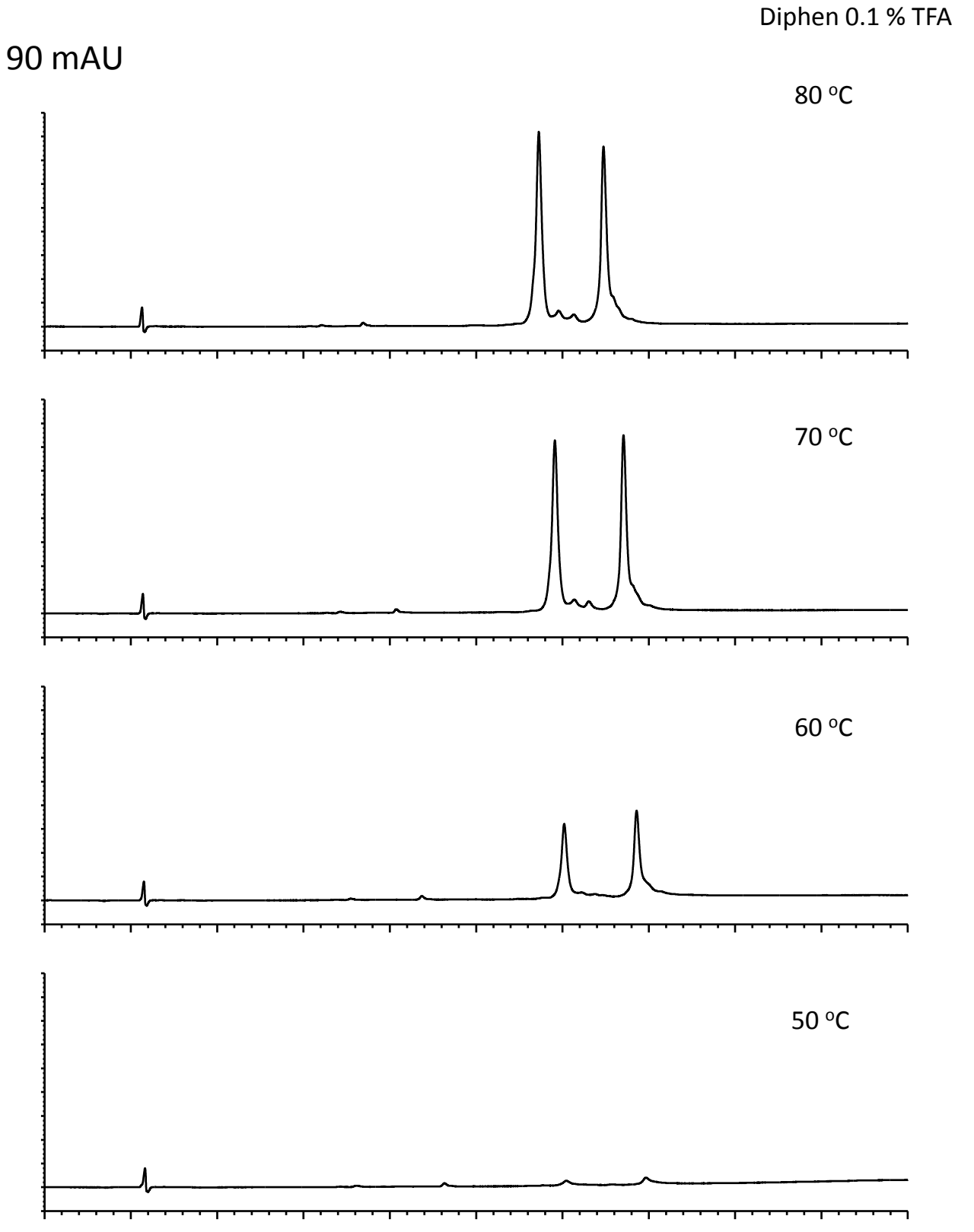


Figure 2b

Fig. 2b

5% butanol 0.1 % TFA

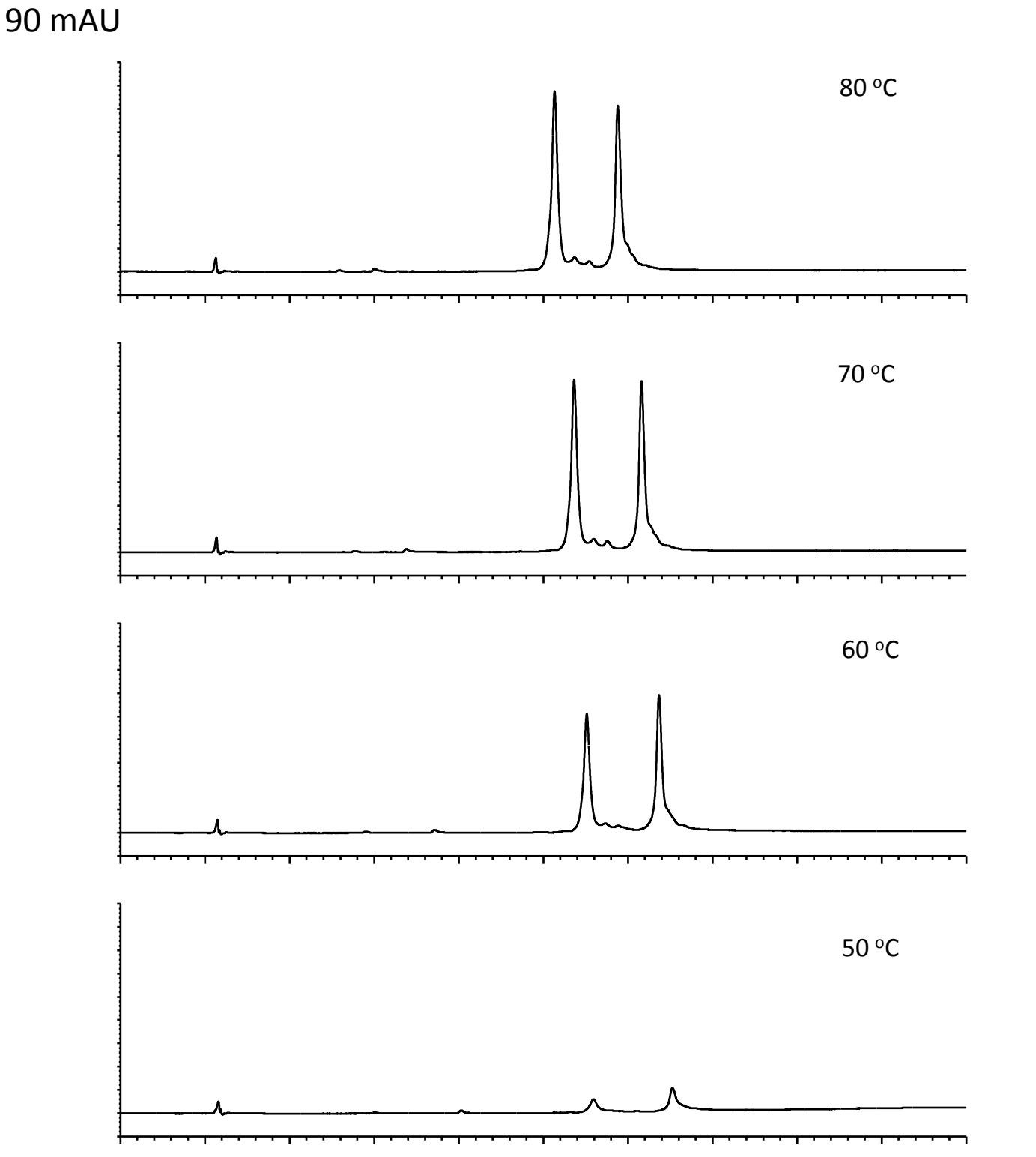


Figure 2c

Fig. 2c

5mM AF pH 3

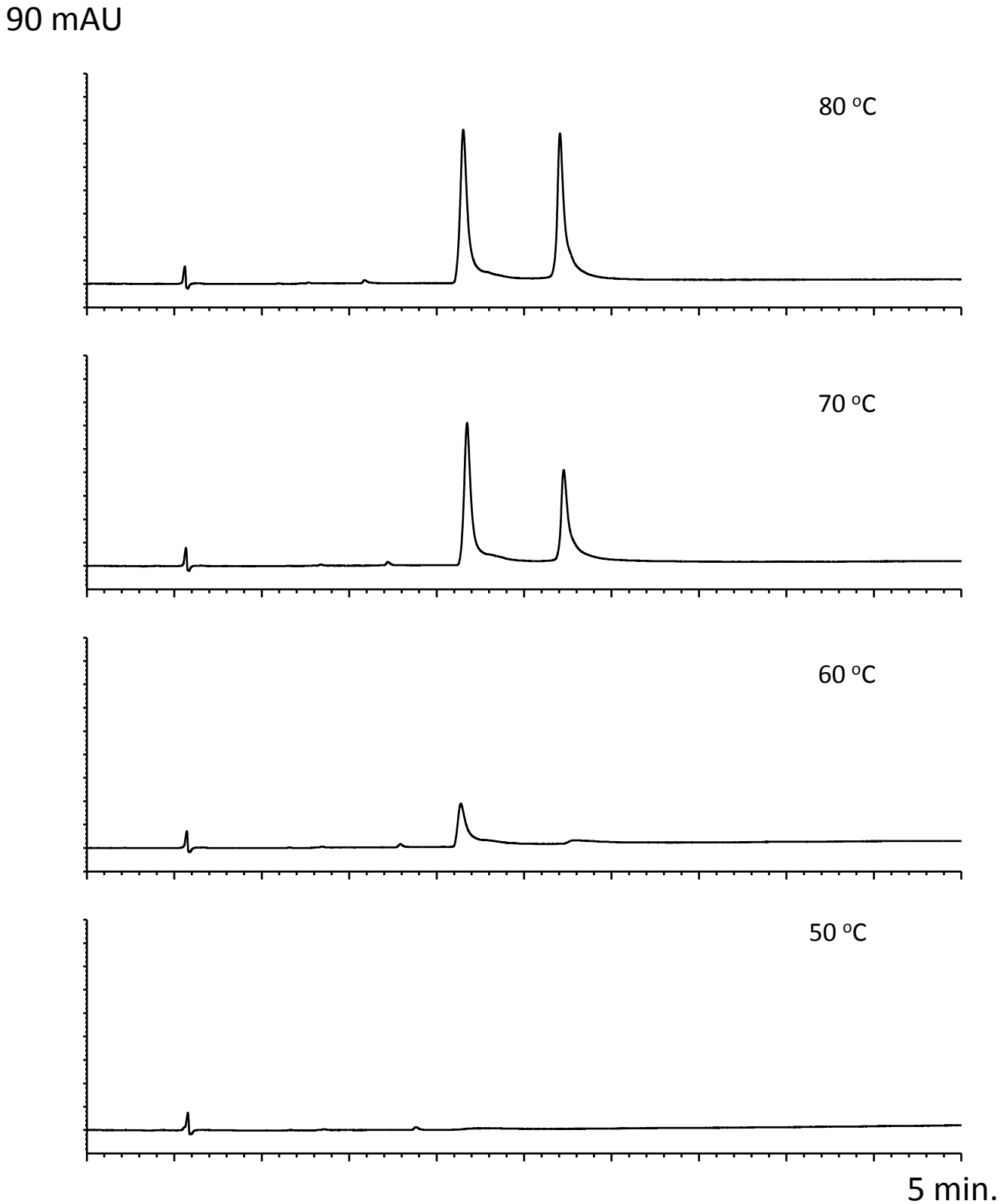


Figure 2d

Fig. 2d

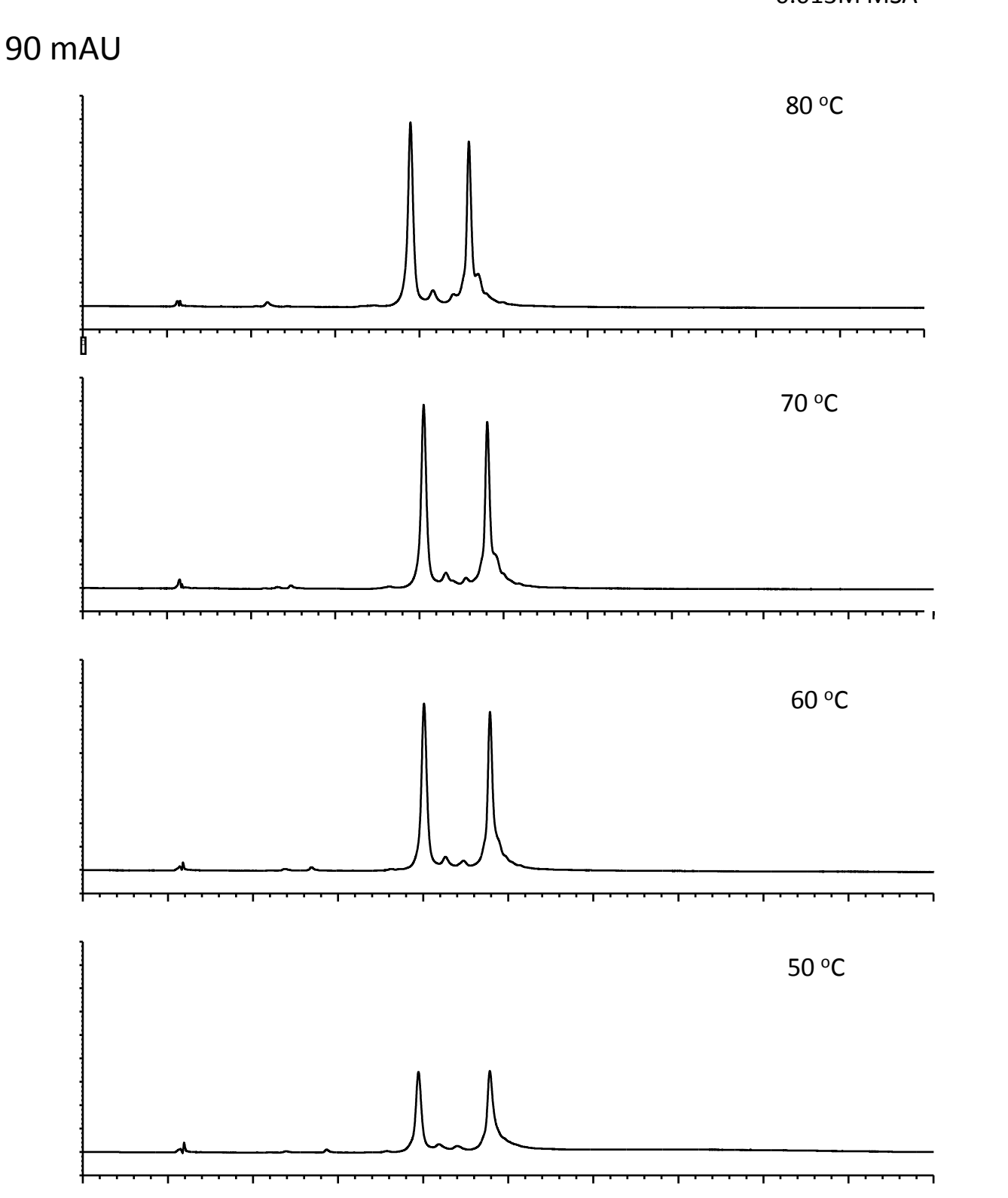


Figure 2e

Fig. 2e

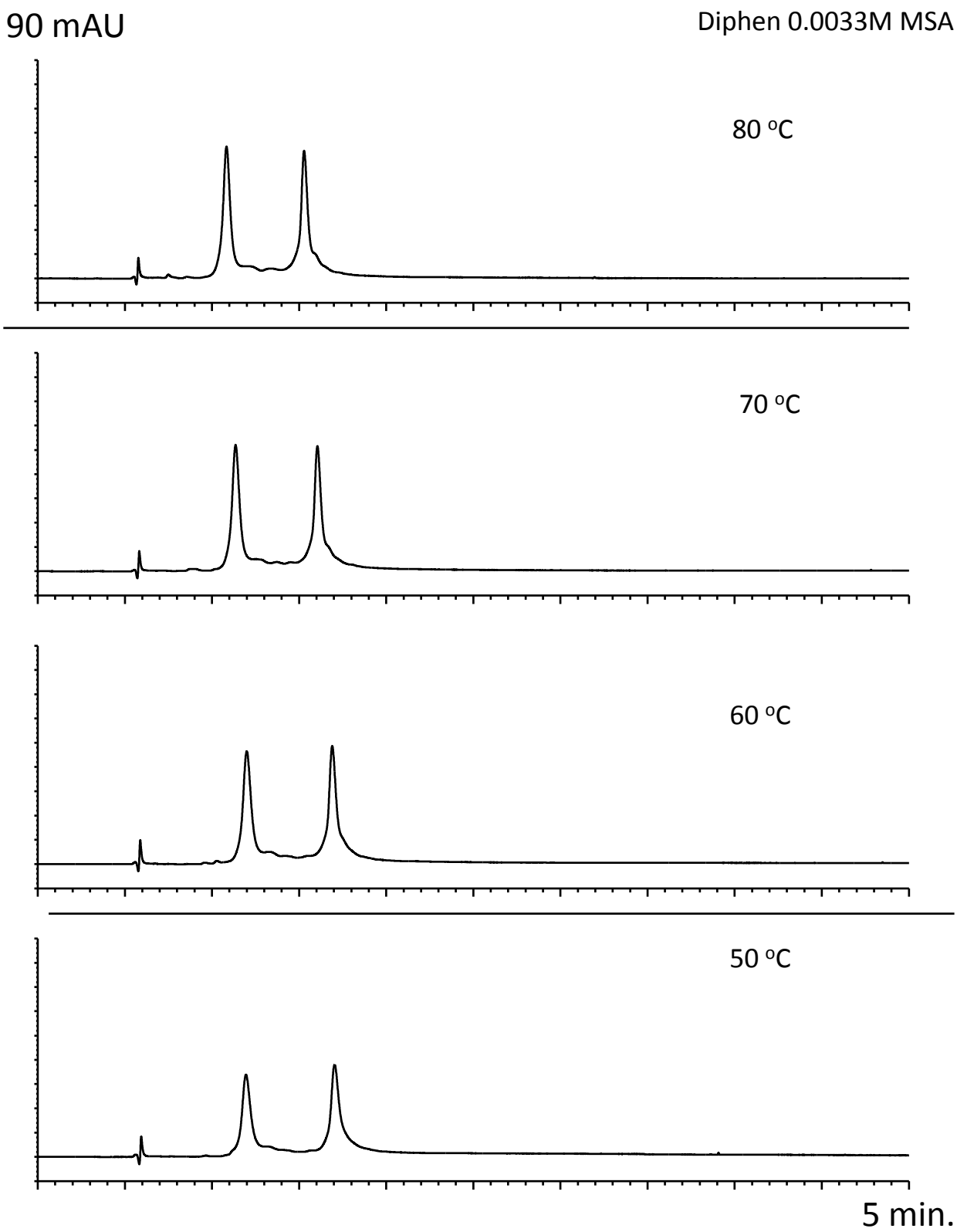


Figure 3a

Fig. 3a

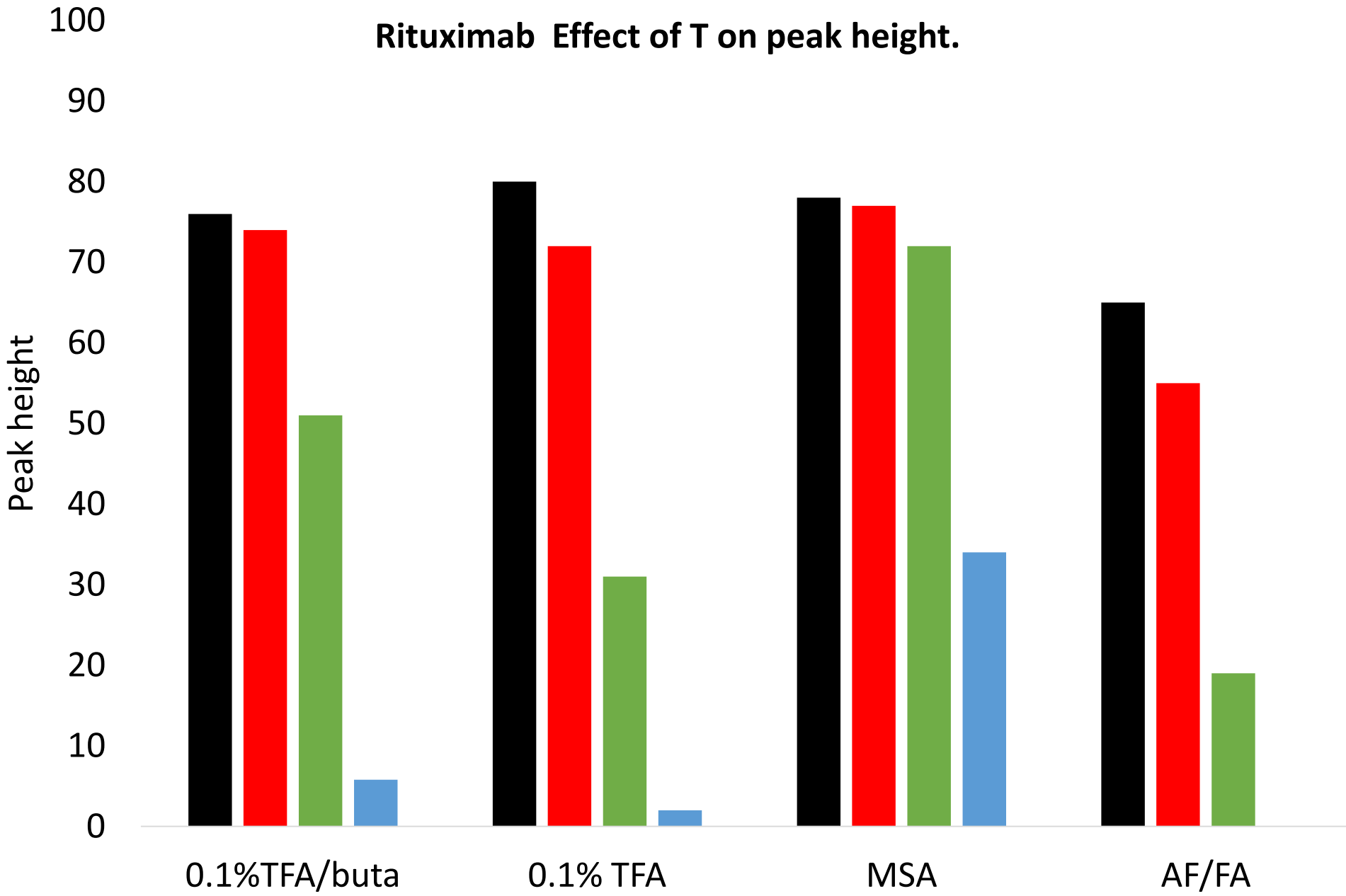


Figure 3b

Fig. 3b

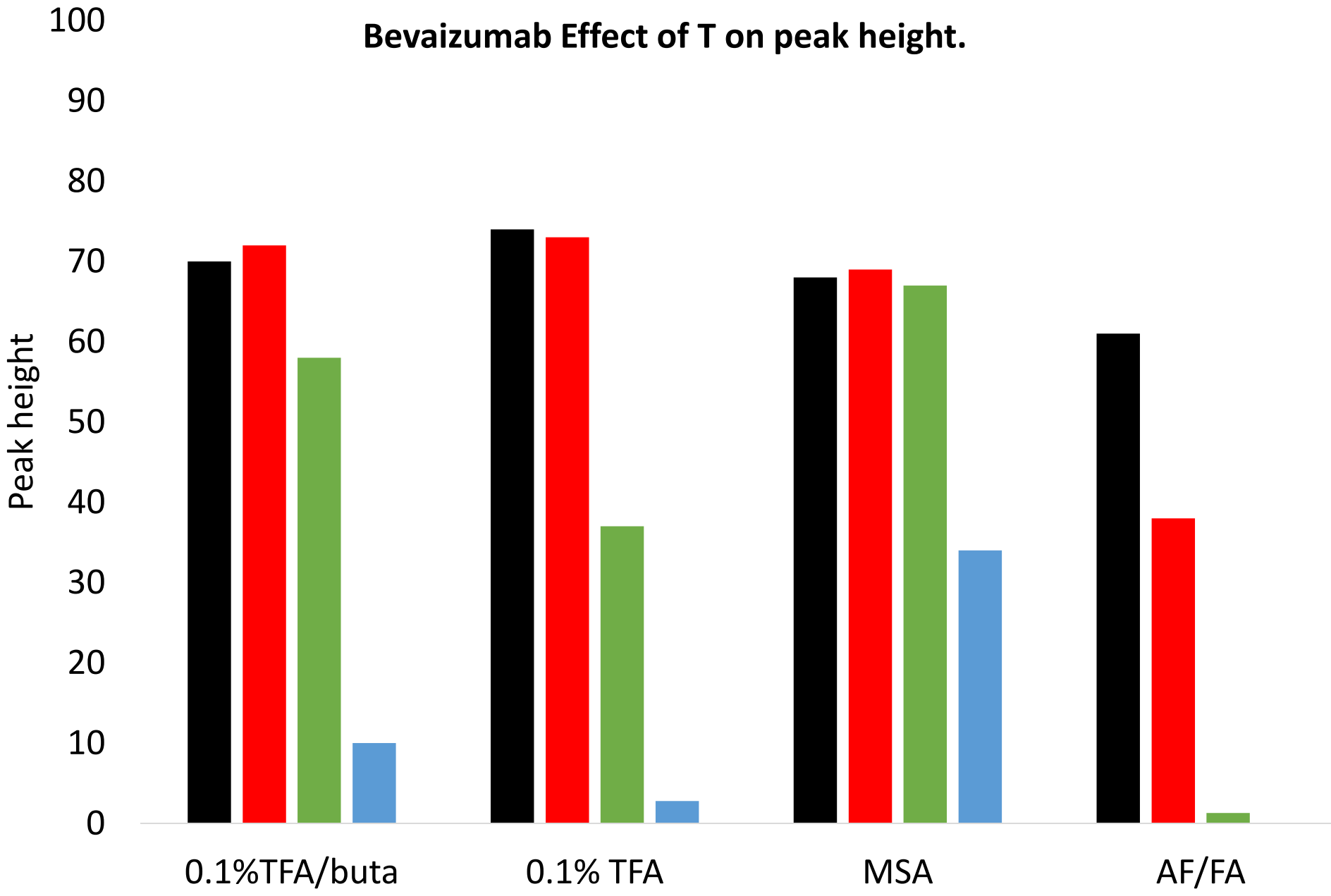


Figure 4a

Fig. 4a

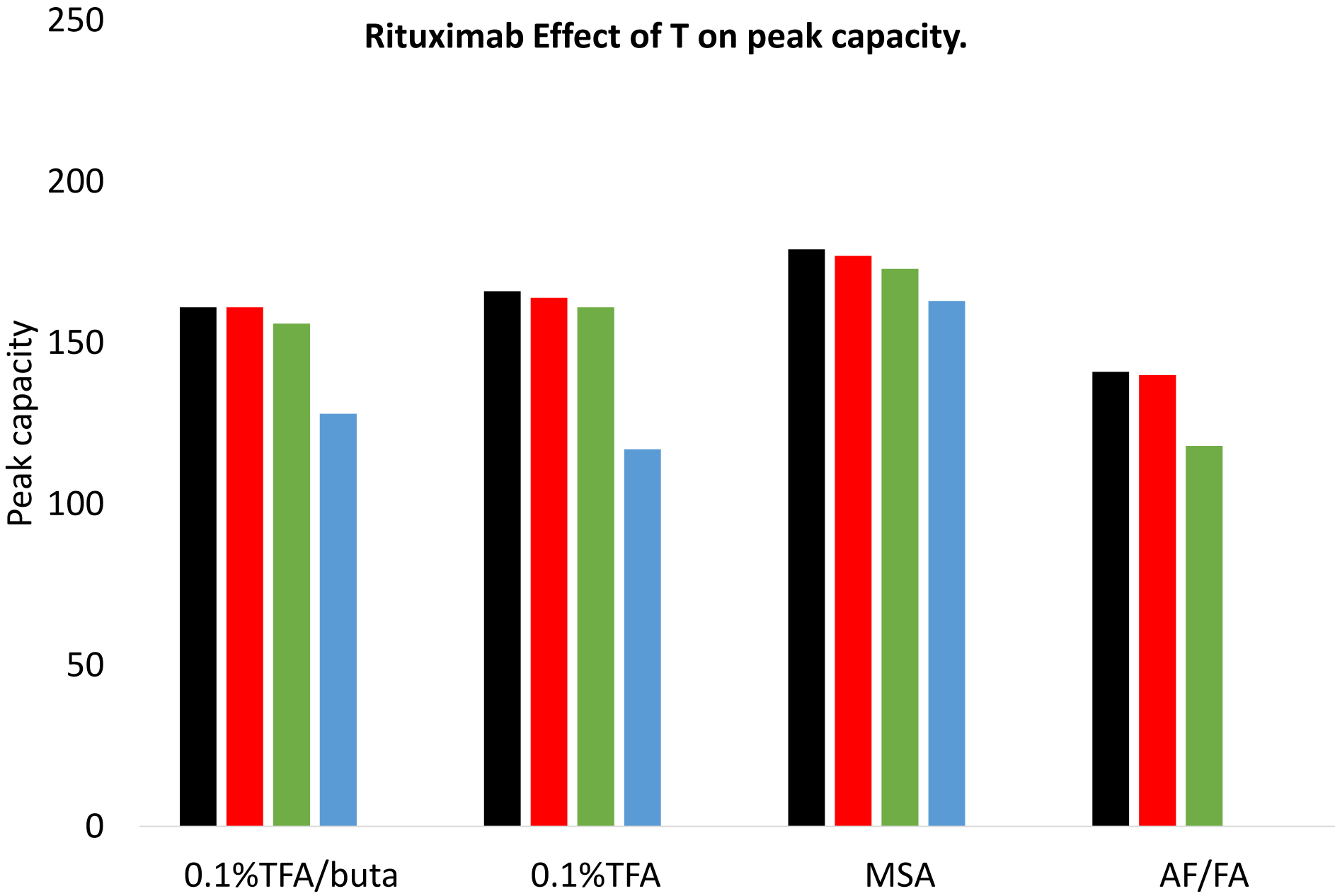


Figure 4b

Fig. 4b

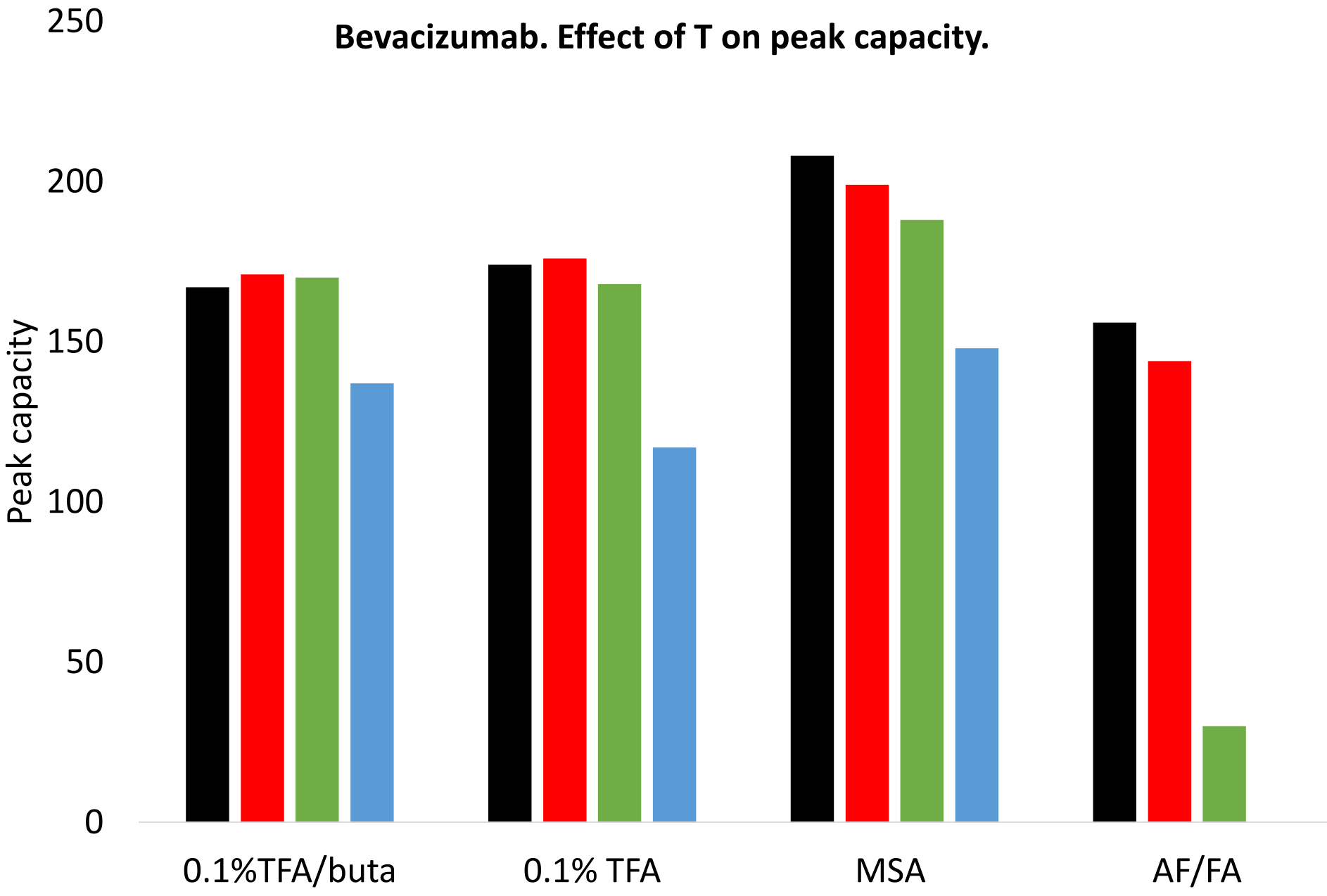
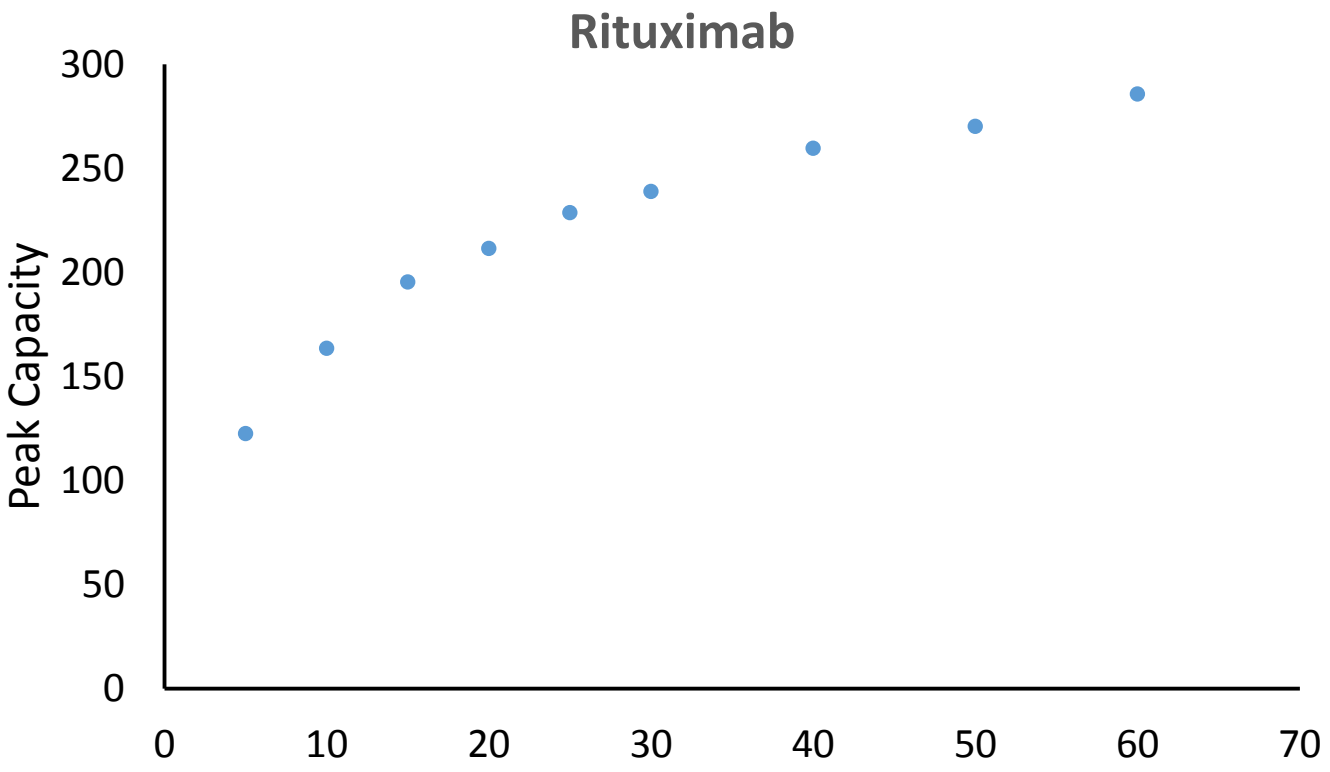
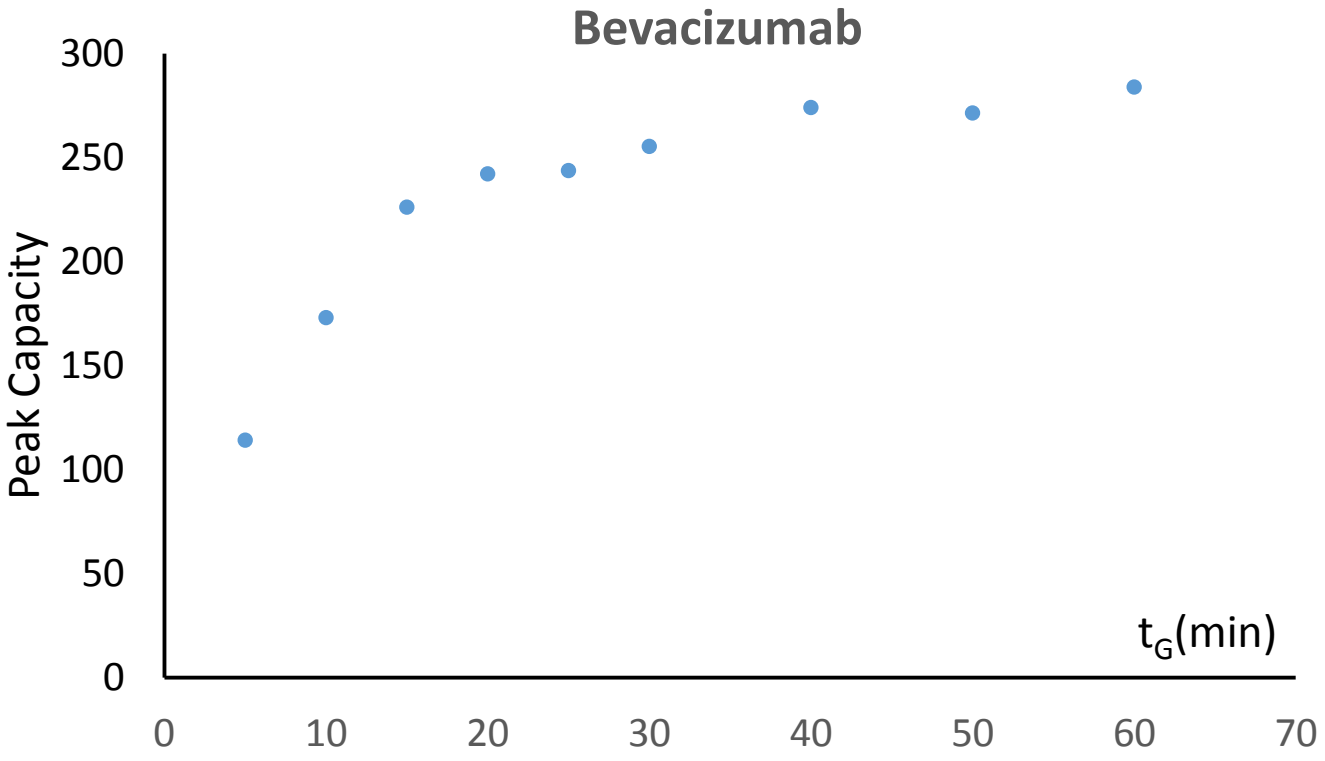


Figure 5

Fig. 5





***Conflict of Interest**

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