ARTICLE

Understanding and managing peak shape for basic solutes in reversed-phase high performance liquid chromatography.

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While other separation mechanisms can challenge the dominance of reversed phase in some applications (for example in the separation of native proteins), reversed phase liquid chromatography is the method of choice for the analysis of a wide variety of samples. However, basic solutes (including small molecules, peptides and proteins) can give broad peaks, ofteh with severe peak tailing, which negatively affects peak identification and quantitation. In this feature article, the causes of low efficiency and peak asymmetry are discussed, including the choices of stationary and mobile phases that can minimise these detrimental effects. The contributory effects of column overloading to peak asymmetry are also considered although the exact causes of these effects remain of considerable debate.

Background

The liquid chromatography separation mechanism known as "reversed phase" (RP) where the mobile phase (mp) is less polar than the stationary phase (sp), has been the most important separation mechanism since its inception in the earliest days of high performance liquid chromatography (HPLC). The sp is typically based on silica microparticles of diameter 1.5 to 5 μm bonded with hydrocarbon ligands such as C18. The mp is typically a buffered aqueous solution mixed with a miscible organic solvent such as methanol or acetonitrile (ACN). In general, separations are reproducible and can be carried out over a reasonable pH range (pH 2-8 or higher), depending on the stability of the sp[1]. The technique is compatible with aqueous sample introduction and gradient elution is relatively straightforward. Separation scientists can access detailed information in the literature concerning application of the technique to novel sample types. Neutral hydrophobic molecules are particularly straightforward to analyse, as they simply interact with the bonded non-polar ligands and the mp to various extents, giving separation.

Peak asymmetry is a problem that can seriously reduce the efficacy of chromatographic separations. It can be caused by numerous factors such as column packing voids or injection problems, or to dead volumes in the instrument flow path. These problems, often referred to as physical factors, are relatively easy to overcome by good instrument or experiment design (Table 1). However, when such sources are eliminated, broad peaks and asymmetry, particularly *tailing* may still be observed. These problems are often attributed to chemical causes, and are particularly encountered in RP-LC when analysing ionised *basic* solutes (Table 2). Most small molecule pharmaceuticals belong to this group, as do many peptides and intact proteins that contain basic amino acids; these molecules are protonated under typical mp conditions. Due to their importance, and the peculiar difficulties in their analysis, this review will concentrate on chemical causes of poor peak shape in RP-LC of basic solutes.

In simple terms, protonated bases are able to interact detrimentally with groups of opposite charge on the sp, giving a further process superimposed on hydrophobic retention, which can give asymmetric peaks. Overloading of the stationary phase must also be considered as a factor in peak asymmetry that may take place concurrently or separate to the former process. Despite this apparent simplicity, elucidation of the fundamental principles involved in determining peak asymmetry of bases has vexed the most distinguished researchers in the subject for many years[2-4]. Peak asymmetry and tailing lead to reduced column efficiency (reduced narrowness of the peaks) thus poorer separations, and irreproducibility of retention times. Furthermore, poorer quantitation results from difficulties in determining accurately the beginning and end of a peak. A detailed theoretical rationalisation of the various processes is still lacking, more than 35 years after the systematic observation of these effects by Snyder[2]. Indeed, the scope of the problem has widened as increased interest has been shown in the separation of (basic) peptides and even intact proteins, with the advent of new application areas such as proteomics [5-9]. The increasing use of mass spectrometry as a detection technique for these solutes has even constricted somewhat the choice of mp solvents and additives, adding to the difficulties posed compared with the less stringent requirements of classical UV detection.

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In general terms, peak tailing is attributed to the heterogeneous nature of the typical RP column surface (most commonly silica-octadecylsilyl) which consists of non-polar (C18) ligands that provide weak hydrophobic retention together with unreacted silanol groups that give stronger hydrogen bonding or ionic retention effects[1-2]. Substitution of silica-based materials with alternatives (such as organic porous polymers) does not provide a simple solution to these problems. For instance, the mechanical stability of these columns may be insufficient to withstand the pressure demands of Ultra- HPLC using particles of less than 3 μm diameter. Furthermore, attainable efficiencies may be disappointing. Finally, porous polymers are also susceptible to peak shape issues caused by overload or other factors. Substitution of other inorganic sp materials such as titania or zirconia for silica has also had limited success.

Despite the difficulties of providing a comprehensive theoretical rationalisation of all the factors involved, much progress has been made in providing practical solutions to the problem of peak tailing. In this paper these practical solutions will be detailed along with current findings concerning the fundamental origin of these detrimental processes. The present author has contributed considerably over a period of more than 35 years, to the understanding of the various contributions to peak asymmetry, especially of basic solutes, and the role of sp overloading.

Discussion

Measurement of column performance.

With all the different factors contributing to the retention of solutes in RPLC, it is hardly surprising that RP (C18) columns from different sources have different properties in terms of retention and peak shape for the same solute. Thus, columns need to be evaluated using test protocols. A useful parameter for evaluating peak shape or efficiency is the plate count. To a reasonable approximation, the plate count is independent of the retention time, allowing results to be compared for different columns.

For symmetrical Gaussian chromatographic peaks, chromatographic theory calculates the column efficiency from the equation:

$$N = 5.54(t_R/w_{0.5})^2$$

where N is the number of theoretical plates, t_R is the retention time and $w_{0.5}$ is the peak width measured at half height. This method is generally preferred to the alternative equation using measurement of peak width at base as the latter is subject to greater imprecision and inaccuracy due to the effects of baseline noise.

$$N = 16(t_R/w_b)^2$$

The higher N, the narrower the peaks; these equations take into account the increasing diffusion of solute that takes place as the retention time increases. To a reasonable approximation, all solute peaks (if Gaussian in shape) should have the same value of N on a given column.

The use of the 5 sigma peak width has sometimes been preferred as it uses the peak width at 4.4 % of peak width, where tailing is more pronounced.

$$N = 25(t_R/w_{4.4\%})^2$$

The Dorsey-Foley method[10] is an empirical calculation which attempts to take into consideration the asymmetry factor of the peak.

$$N = 41.7\,[t_R/w_{0.1}]^2/[A_s + 1.25]$$

where $w_{0.1}$ is the peak width at 10 % of peak height and A_s is the asymmetry factor, the ratio of the width of the tailing edge of the peak divided by the width of the leading edge of the peak monitored at 10 % of peak height. Note that the asymmetry factor alone has been used as a measure of peak shape. It can also be used in conjunction with column efficiency measurement. However, some of the information from the asymmetry factor is already contained within the column efficiency measurement, as asymmetric peaks clearly lead to low efficiency. Finally, the statistical moments method calculates column efficiency from the square of the first moment divided by the second moment.

The use of a particular method for calculating efficiency is debatable. The statistical moments method can provide an accurate means of determining N for non-Gaussian shaped peaks. However, the method can suffer from poor precision due to the problem of positioning of the baseline, which is often affected by noise. Nevertheless, comparative methods of N tend to demonstrate the same *relative* efficiency [11]. Overall, the author prefers the 5-sigma method which gives a better measure of the true efficiency than the half-height procedure while maintaining good measurement reproducibility.

Most testing regimes for RP column performance have been based on the evaluation of *retention* data for a number of sps under a given set of conditions-using a set of probes capable of different interactions and using a specified mobile phase. One of the most comprehensive systems is the Snyder hydrophobic subtraction model. Snyder[1] summarised the different interactions that can influence retention and selectivity as:

a) Hydrophobic interaction.
b) Steric exclusion of large solute molecules from the stationary phase.
c) Hydrogen bonding of an acceptor basic solute (e.g. pyridine) with an acidic stationary phase group.
d) Hydrogen bonding of a donor acidic solute (eg butyric acid) with an acceptor basic stationary phase group.
e) Electrostatic interaction between a cationic solute group and an ionised column silanol; repulsion of an ionised acidic solute.

f) Dipole-dipole interaction between a dipolar solute group (e.g. a nitro group) and a dipolar sp group.
g) Pye-pye interaction between an aromatic solute and a sp phenyl or nitrile group.
h) Complexation between a chelating solute and metal contaminants of the sp.

Although mostly designed to predict retention, this list also details the mechanisms that could also contribute to peak shape. On a pure silica-ODS phase, only interactions a to e are normally significant. It is possible to determine separately the influence of each factor by judicious selection of probe compounds that show strong interactions of a particular type. For the cation exchange term, the retention of the strong bases nortriptyline or amitryptyline, or the quaternary ammonium compound berberine was used. While the retention data for these compounds appears to be a good quantitative measure of ionic retention, it does not necessarily relate to peak shape of these compounds. Indeed in the hydrophilic interaction mode (HILIC) bare silica sps can give high retention for cationic solutes with minimal tailing[11,12]. This finding is also of interest in that it indicates that it is not the concentration of silanols that causes peak shape problems, but the other separation mechanisms in RP (e.g. hydrophobic retention) in addition to cationic retention that cause peak asymmetry difficulties.

The Stationary Phase

The heterogeneity of the typical (C18) column RP surface was clearly understood even in the early days of HPLC[2]. Silica microparticles are reacted with a long hydrocarbon chain silylating reagent to provide a hydrophobic surface. Shorter, less hydrophobic/less retentive ligands such as C8 or C4 can also be used instead to moderate solute interaction and retention. Monofunctional silylating agents such as chlorodimethyloctadecylsilane generate monomeric bonded sps; trifunctional silylating reagents such as trichlorooctadecylsilane generate polymeric coatings. The former sps are supposedly more reproducibly synthesised, whereas the latter supposedly are more stable and give greater shielding of unreacted silanols[1]. Undoubtedly, much work has been performed by manufacturers concerning the intimate details of sp synthesis, but for commercial reasons this information is not widely disseminated. Whatever the synthesis procedure, many silanol groups remain unreacted due to steric effects. A reduction in the number of unreacted silanols can be obtained by so-called "endcapping"with a smaller reagent like trimethylchlorosilane [Fig 1]. Fully hydroxylated silica contains a maximum of 8 μmol m^{-2} of silanols but the maximum concentration of bonded groups [13] may be as low as 4 μmol m^{-2} . These silanols can give rise to poor peak shape by strong secondary ionic or hydrogen bond interactions demonstrating slow kinetics (see Fig.1). While a silica surface is not a single molecule, and thus ionisation of a silanol can influence that of neighbouring silanols, their average p*K*a has been estimated [13]as ~7.0. This figure does not negate the possibility of the occurrence of some highly acidic silanols which could remain ionised even at pH 2, permitting strong interaction with protonated bases according to:

$BH^+ + M^+SiO^- \rightarrow M^+ + BH^+SiO^-$

where BH^+ represents a protonated basic solute, and M^+ a buffer cation.

It is possible that tailing can be explained purely in terms of kinetic phenomena, if the kinetics of mass transfer of one type of column site (ionic sites) are slower than that of the other (hydrophobic sites) [1,13,14]. Pronounced tailing can occur when the slow sites provide a smaller contribution to retention than the fast ones. This mechanism of kinetic origin usually lead to *exponential* tailing, which can sometimes be reduced by increasing solute mass. A small number of strong sites-may alternatively become overloaded by relatively small masses of solute causing tailing of different origin giving *right angled triangle* peaks, which become broader as solute mass is increased. (see Figs. 2,3). Overload tailing can occur concurrently or independent of kinetic tailing. Unfortunately, the mechanism of both retention and peak tailing appears to be considerably more complicated than these simple explanations suggest. Indeed, the number of additional retention mechanisms to the above simple 2-site model are addressed by Snyder above[1,15] .

Kirkland[3] recommended that to obtain optimum coverage of the silica surface while leaving low activity silanols, the starting silica should be very pure and have very low metal content (which reduces the acidity of the silanols). These are termed "Type B" silicas compared with relatively impure older "Type A" silicas. The surface should be fully hydroxylated by acid treatment prior to bonding. Excessive sintering of silica to convert silanols to siloxane groups is best avoided, as on contact with water, hydrolysis back to silanols may occur [1,3].

Some column manufacturers introduced alkyl phases with embedded polar groups such as amide or carbamate. An example of the former is the Ascentis amide column from Merck and of the latter, the Xterra RP18 Shield column from Waters. These polar groups may show increased hydration near the column surface providing some deactivation of residual silanols and thus better peak shape. Another possibility is hydrogen bonding interactions between the embedded polar group (EPG) and underivatized silanols. This type of internal hydrogen bonding may reduce external H-bonding with solutes. However, the popularity of EPG sp seems to have declined in recent years due to the possible loss of ionisable column bleed, providing higher background in LC-mass spectrometry[16-17].

While purely polymeric sps have shown limited use, hybrid inorganic/organic sp have been synthesised in the hope that these would show the benefits of both silica and organic polymer materials[18]. A proprietary polymeric material is commercially available of composition $[O_{1.5}SiCH_2CH_2SiO_{1.5}]_n$. Rosés and co-workers compared the performance of a bridged hybrid bonded C18 phase with that of a conventional bonded C18 (Type B silica) and an impure C18 silica (Type A silica). A mp of ACN-water buffered to various pH was used and the retention time of lithium ions was plotted as a function of the

true thermodynamic pH (see Fig. 4). The retention of (positively charged) lithium ions indicates the extent of ionic retention on (negatively charged) ionised silanols The number of these active silanols increased in the order: BEH C18<<Type B C18 < Type A C18. The hybrid C18 did not present any residual silanol acidity up to pH 10.0 (in 60% methanol) as measured by the retention of lithium ions from $LiNO_3$ [Fig. 3]. The underivatized BEH packing presented active silanols only at pH values[19] higher than 7.0. The underivatized sp material may show more retention of Li^+ than the derivatised material, where residual silanols may be shielded from solute interaction by the C18 ligands. These results should be treated with caution as the probe compound is an inorganic ion, not a protonated organic base, the latter being capable of interactions with hydrophobic sites as well as ionic interactions. Indeed, tailing has still been noted for protonated organic bases on this hybrid material [20]. It is possible that strong synergistic retention sites exist that are a combination of the two major retention mechanisms (RP + ionic retention):

$$k = k_{RP} + k_i + k^*_{RP}k^*_i$$

where k is the overall retention factor, k_{RP} is the hydrophobic contribution, k_i is the ionic contribution from silanols and k^*_{RP} k^*_i is a multiplicative contribution from both processes[21]. This strong multiplicative effect would not be measurable using simple inorganic ions. In summary, lithium ion retention may indicate reduced silanol effects (if not *no* silanol effects) for the hybrid material. In addition to this property, these materials have an advantage of stability at relatively high pH (pH 9 or higher, dependent on mp).

Monolith columns, consisting of a single structure of silica or organic polymer, have been considered as an alternative to conventional particle packed columns [22]. They can be made as a rod which is subsequently clad with a protective housing or synthesised in situ, typically in a capillary format. The latter method can be accomplished more easily, as shrinking problems that can occur during the production of the monolith are minimised. In contrast, cladding processes are difficult and tend to produce lower efficiency columns; nevertheless, capillary columns require specialised instrumentation and are less rugged [23]. Thus, much interest has been shown in these clad "rod" columns. The first silica monoliths of conventional dimensions (10 x 0.46 cm I.D.) were made commercially available in 2000, with the sp clad with a protective PEEK housing. These columns could provide about 10,000 theoretical plates equivalent to a conventional column packed with 3-4 μm particles together with a back pressure similar to a column of 8-9 μm particles-seemingly an ideal combination. Operation of such columns could allow simpler, cheaper equipment, or the use of very long columns to generate high separation efficiencies. However, careful testing of these columns revealed that the through pores (2-8 μm) and skeletons (1-2 μm) were too large and the external porosity too high to limit mass transfer band broadening[24]. Furthermore, by placing a detection point at different radial positions, it was found that radial heterogeneity existed in the structure from the centre to the wall region[25]. McCalley found some tailing even of simple neutral compounds on the first generation silica RP-18 monolith, with excessive tailing of bases at neutral pH[26]. Improved results were obtained on second generation monoliths with higher homogeneity and smaller skeletons/ through pores [27]. However, these gains were achieved at the expense of increased operating pressure.

Research on *polymeric* monolith columns has been carried out in capillary format in most cases. For small molecules, efficiency tends to be lower than that for equivalent silica columns. They have however, seen effective use in research applications, particularly in the high efficiency separation of high molecular weight compounds of clinical and biological importance[28]. In general, monolith columns have not always fulfilled earlier aspirations of higher performance than particulate-based sps-particularly for routine applications, although continued research efforts may change this conclusion in the future.

Mobile phase considerations.

Clearly, the mobile phase is important in achieving the best performance on a given column. Relatively few of the protocols involve measurement of column efficiency or peak symmetry. Those studies which include peak shape measurement concentrate on acid pH, where silanol ionisation is suppressed, limiting the effects of kinetic tailing. Acid pH is typically maintained by use of phosphate buffers, or for MS work ammonium formate or acetate buffers or simple use of formic or acetic acid solutions or other MS compatible additives (see below). However, as pH variation can give important selectivity differences, some test procedures have been carried out at pH 7, towards the upper pH limit of typical RP packings. High pH (up to pH 12) represents a different approach where weak or moderately strong bases are deprotonated and thus unable to interact with oppositely charged sites on the sp-however, few RP materials are stable under these conditions. Limited work has been carried out to investigate the optimum organic modifier to use, although the choice is generally restricted to those water-miscible solvents used in almost all RP separations i.e. ACN, methanol or THF.

An early study by McCalley[29] compared the efficiency of 8 RP packing materials using 9 different basic probe solutes with a pH 7 phosphate buffer with methanol, ACN or THF as organic modifier. pH 7 was used in the assumption that this pH would emphasise detrimental interaction with ionised silanols. Methanol was found to give rather better peak shapes than ACN, and THF gave roughly equivalent performance to methanol. It is possible that methanol competes better for active silanol sites by hydrogen bonding. Few subsequent papers have studied the use of THF. This may partially be as a result of safety considerations when using large volumes of this solvent (flammability and the possibility of generation of explosive residues). Earlier tests using unbuffered mps and simple weakly basic solutes were found to be insufficiently challenging to discriminate between columns. Later, McCalley [30] recorded N data using the half height and Dorsey-Foley

procedures and the A_s for more modern Type B silica columns using 9 basic probe compounds. These compounds included strong and weak bases with a wide variety of hydrophobic properties which (singly) are often used as test compounds by column manufacturers. The evaluation was performed at pH 3 and pH 7 with isoeluotropic mixtures of ACN and methanol with phosphate buffer. Column performance for these basic solutes was clearly better at pH 3 than pH 7 in line with increased ionisation of underivatized silanols at the higher pH. At pH 7, average performance was much more similar on these higher purity silica sps in methanol compared with ACN. Peak shape for the different probe compounds on a given sp/mp combination is highly variable[30], emphasising the need for a range of different probe compounds especially at pH 7. A different approach[29,30] to the minimisation of the effects of ionised silanols is working at high pH (e.g. pH 11). These conditions may be sufficiently above the pK_a of moderately basic compounds such that they are analysed in a mostly unionised state. Limited success on a bidentate bonded C18 phase was obtained but Improved results were obtained later using the bridged ethyl hybrid phase, particularly with regard to overloading issues[31]. Moderately strong bases are deprotonated at pH 10-11 and gave good efficiency. However, stronger bases of pK_a 10-12 still showed some problems. It is possible that even a small degree of solute ionisation results in strong interaction with residual silanols (whose ionisation is promoted at higher pH). Furthermore, the long term stability of even specialised RP columns that use optimised synthesis techniques for high pH operation, is questionable.

The investigations reported up to this point were mostly performed using phosphate buffers of concentration 5-25 mM and small mass injections (typically < 50 ng for 0.46cm ID columns[22]). Phosphate has good UV transparency down to low UV wavelength (~205 nm). To reduce noise levels at low wavelength low phosphate concentrations are recommended for UV work. However, phosphate buffers (pK_a 2.12), being non-volatile are unsuitable for work using evaporative detectors such as ESI mass spectrometry. For such work, volatile ammonium formate (pK_a 3.75) or ammonium acetate (pK_a 4.75) make reasonable replacements , at least for the separation of small solute masses.

Contribution of overload to peak asymmetry.

So far, the behaviour of small sample masses of basic solutes have been considered. In these cases, the exponential tailing resulting from kinetic effects in a given mp may actually be reduced by increasing sample mass. An explanation for this effect is that part of the sample may be strongly adsorbed to active sites which prevent their interaction with further solute. More usually, however, increasing the sample mass has a detrimental effect on peak shape. This is a thermodynamic effect when the distribution ration of the solute between the sp and the mp is not constant with increasing solute mass. Fig. [5] shows a plot of column efficiency against sample mass for 3 polar neutral compounds (phenol, caffeine and 3-phenylpropanol) and 3 ionised compounds (an acid, naphthalenesulfonic acid, bases nortriptyline and propranolol) on a hybrid silica RP column using a mp of aqueous ACN adjusted to pH 2.7 with formic acid[31]. While the efficiencies for the neutral compounds are maintained at high values for the injection of up to ~ 10 μg of solute, those for the ionised solutes begin to decrease substantially when more than about 0.05 μg of these solutes are injected. The peak shapes for propranolol and naphthalene-2-sulfonic acid are shown in [Fig. 3]. Clearly, overloading is a problem for both cationic and anionic samples in RP-HPLC. For small sample mass the peaks are Gaussian but become increasingly right-angled triangle in shape as the sample mass increases. Furthermore, retention times decrease with increasing sample mass. These results are highly undesirable, as column efficiency and the separation ability of the column are seriously compromised. Reducing sample mass to improve peak shape may be undesirable as it may affect adversely peak detection and quantitation- for example for quantitation of minor impurity peaks in the presence of a high concentration of the active ingredient in a pharmaceutical analysis (API). The broad peak of the API may obscure impurity peaks. While it is likely that kinetic tailing and overload tailing occur simultaneously, there is little evidence for kinetic tailing in the example of [Fig. 3]. With the smallest sample mass shown, the peak is Gaussian in shape with no evidence for an exponential decrease in response on the rear edge of the peak. As the solute mass increases, the peaks become right-angled triangle in shape, with again no evidence for sn exponentially decreasing rear edge. It is tempting to attribute these peak shapes in some way to the overload of a small population of silanol groups. However, any explanation of the fundamental processes involved in overload tailing must accommodate the following experimental results[31-33].

1) [Fig. 6] shows a plot of efficiency vs sample mass for amitryptiline at various pH values[22]. The degree of protonation of the solute at each pH is estimated and shown in the Figure. It appears that overload effects are much smaller at high pH where amitriptyline is less ionised-thus solute ionisation plays an important part in the tailing process.

2)[Fig. 3] shows that both propranolol -a strongly basic analyte that is protonated under the shown conditions and naphthalene-2-sulfonic that is negatively charged give the same overloading behaviour [31].

3) Results on two different organic polymer (non-silica based) columns gave very similar overloading behaviour to that on alkyl bonded silica phases at the usual acid mp pH. Clearly, polymeric columns have no silanols to overload. It is not impossible that these polymeric phases have negative charges on their surface as a result e.g. from synthesis procedures. However, polymer columns showed little retention change for bases with increasing buffer cation concentration in the mp at low pH, indicating the absence of charged groups on the sp under these conditions.

4) A further observation was that overloading effects on all types of sp increased as the ionic strength of the mp decreased.

Thus overloading was shown to be more serious for acidic and basic solutes when using ACN 20 mM phosphate buffer pH 2.7 than the same mp to which 60 mM KCL had been added. As addition of the neutral salt KCl has no effect on buffer capacity, this result must in some way be connected instead with the ionic strength of the mp. Substitution of (low ionic strength) formic acid for the phosphate buffer at similar pH and molar concentration resulted in much more serious overloading effects. These results have practical consequences for the use of mass spectrometer friendly buffers as discussed below.

Taking all the evidence given above, it was proposed that overloading could be caused by mutual repulsion of solute ions of similar charge on the hydrophobic surface of the column. This repulsion could be envisaged as exclusion of solute ions from some of the sp pores that were already occupied by ionised solute[34-35]. This explanation does not depend on the existence of silanol groups, in line with the results for purely polymeric sps. Furthermore, reduced repulsion would be expected in higher ionic strength mps, as indeed was shown.

An alternative explanation of overloading was given by Guiochon and co-workers, in a comprehensive series of studies using various RP sps and analysis conditions [36-40]. This author postulated that sites of different interaction energy existed within the RP layer, of low intermediate and high energy. The number of sites depends on the structure of the solute. High energy sites have a low saturation capacity and their overload dominates behaviour. The high energy sites are few in number and are filled first so that retention rapidly decreases, followed by filling of the much more abundant weak sites. No physical explanation of the identity of these sites was given. Some supporting evidence for this theory was found by McCalley[31] who injected large sample masses (up to 150 μg) of the base nortriptyline on a hybrid RP column at low pH. A large sharp L - shaped peak appeared at the highest sample masses, which may correspond to the complete filling of the strong sites and commencement of filling of the weak sites. A complication is that the mp buffer is substantially overloaded in these experiments by the sample. For example the sharp peak at lowest retention may correspond to nortriptyline hydrochloride where the chloride counter ion has never been replaced by the mp buffer anion (formate). The long tailing peak is the fraction of injected nortriptyline that migrated with the buffer counter ion. Clearly, this second theory less readily accounts for some of the experimental evidence e.g the similar behaviour of polymer and silica sps.

Superficially porous “shell” columns containing particles of diameter < 3 μm have attracted considerable attention since the landmark publication of Kirkland[41] in 2007. These materials consist of a porous layer of separation medium (e.g. silica-ODS, typically 0.5 μm thickness) coated on a non-porous spherical core (typically 1.7 μm diameter). These 2.7 μm diameter particles can give efficiencies equivalent to sub 2 μm totally porous particles while generating a back pressure of half or less (in line with their larger overall diameter). Their high efficiency may be principally due to the better packing of these totally spherical particles, or to their very narrow particle size distribution. Only for very large molecules (e.g. proteins) with small diffusion coefficients does more rapid mass transfer through the thinner porous layer appear to play a part. A problem with these particles is that being only partially porous, they might be expected to overload more easily than conventional particles. However, for the 2.7 μm particles mentioned above, simple geometry indicates that 75 % of the particle volume is porous. Indeed, a comparison by McCalley[42] of shell and totally porous materials from the same manufacturer, which used similar silica and bonding chemistry showed little difference in loading properties. Increasing the concentration of the buffer salt (ammonium formate) from 5 to 100 mM increased the amount of acidic or base solute necessary to reduce the column efficiency by half by more than an order of magnitude. Nevertheless, high buffer concentrations are detrimental to mass spectrometer sensitivity.

A solution to this apparent dilemma is the use of so-called “charged surface hybrid” stationary phases[42]. Here, a low concentration of weakly basic groups is bonded to the sp, giving it a positive charge in acidic mobile phases. Detailed study indicated that the sample capacity of these new sps in 0.1 % formic acid mobile phases (favoured for their low suppression of mass spectrometer sensitivity) could be nearly equivalent to that on conventional sps operated with 5 mM ammonium formate buffers. The mechanism of this favourable performance is obscure, although repulsion of positively charged solutes from the similarly ionised groups may moderate the strength of some interactions with the surface. However, a limitation of these sps is the performance of acidic solutes, which gave tailing peaks for small sample mass in acidic mobile phases, indicating the possibility of strong ionic retention on positively charged column groups as well as hydrophobic retention.

Peak shape issues for peptides and proteins.

Up to this point, results have been presented exclusively for small molecules with RMM< 500. It is of interest to see if the behaviour of peptides and proteins mirrors this behaviour [6]. Proteomics involves the global analysis of the protein content of cells. Up or down regulation of certain proteins may allow the early diagnosis of diseased states, or elucidation of the pathways of the progression of disease. Proteins can be analysed as complete entities (top-down approach) or indirectly by digestion with enzymes giving characteristic peptides or larger fragments of the original protein. Analysis of these complex peptide mixtures by HPLC and MS can identify the original protein (bottom up approach). Peptides are readily analysed by RP techniques, whereas the analysis of intact proteins can alternatively be carried out by Size Exclusion Chromatography (SEC) or by ion exchange chromatography (IX)[43,44]. The latter techniques have the advantage of being applicable to the analysis of “native” (i.e. non denatured) proteins as the use of organic solvents is not essential. IX is applied to determine charge variants of proteins such as monoclonal antibodies (mAbs) and SEC to determine aggregation, which are both ` “critical quality attributes“ in the

characterisation of these important biopharmaceuticals. While RP LC usually involves organic solvents and thus denaturation of proteins, its high resolution can provide important information on the hydrophobic structure of even intact proteins or their digestion fragments, or can allow fingerprinting techniques to determine protein similarity or differences.

Evaluation of sps and analysis conditions for small basic molecules is usually performed by isocratic analysis[45]. Gradient elution can disguise some problems-for instance by peak compression where the rear of a peak travels faster through the column than the front, as it experiences a stronger mp[1]. However, isocratic analysis is generally not applicable for the separation of peptides and proteins, because a small change in the elution strength of the mp brings about a large change in retention of these higher MW solutes. Indeed, some researchers refer to retention of these molecules by an "on-off " mechanism[1]. In the case of gradient elution, simple equations for calculation of column efficiency are usually replaced by determination of the peak capacity :

$$PC = 1 + [t_G/1.699\ w_{0.5}]$$

where t_G is the gradient time and $w_{0.5}$ the peak width at half height. This equation may give an optimistic value of PC for asymmetric peaks, just as the isocratic evaluation of column efficiency does when using the common half-height method. Note that in the absence of detrimental interactions which affect some of the solutes, all the peaks in the gradient should have approximately the same width.

[Fig. 7] shows analysis[6,46] of the Alberta peptide mixture (commonly used for evaluation of columns) at normal strength (mass of each peptide injected ~ 1-2 μg for a 25 x 0.46 cm C18 column) and the same with the sample diluted 10 times. The Figure compares results with either 20 mM formic acid or 8mM TFA as the acid additive. [Table 3] shows the peak capacities for P4 using various acid additives. Peptides P1 to P4 contain 1 to 4 residues respectively of the basic amino acid lysine. Over the pH range used for the majority of RP separations (pH 2-7) the peptides P1 to P4 will have a charge of +1 to +4 respectively. It is expected that the multiple charges on peptides 3 and 4 will lead to the greatest peak shape problems. An ACN-water gradient was used with formic acid as the buffer additive. All four peptides but especially P4 and P3 show clear evidence of right angle triangle shapes, characteristic of overloading, in the undiluted mixture [Fig. 7]. The ionic strength of the formic acid mp was 1.9 mM, and the peak capacity for P4 was only 148 in the normal strength mix. In comparison the peak capacity was increased to 227 in a mp to which 20 mM KCL had been added, while the pH remained unchanged at 2.7. Similar results were obtained by adding ammonia solution to the formic acid mp, which increased the ionic strength while also increasing the pH to 3.3. Use of 8 mM TFA at slightly lower pH provided marginally the best peak capacity (233) for the normal strength mix, while the ionic strength was only ~8 mM. For each mp, improvement was shown in PC for the diluted compared with the normal strength peptide mix.

Further confirmation of these findings was obtained by analysis of basic bradykinin peptides, whose composition is shown below:

Peptide	**Amino Acid Sequence**	**Charge (pH 2.7)**
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	+3
Bradykinin Frag 1-8	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	+2
Arg bradykinin	Arg-Arg-Pro-HydroxyPro-Pro-Gly-Phe-Ser-Phe-Phe-Arg	+4

[Fig, 8] shows their analysis on a totally polymeric sp at high (2.5 μg) and low mass injection (0.1μg) using an acetonitrile gradient with either formic acid or TFA as additive. The top chromatogram was obtained with formic acid as the acid additive while the lower with TFA, the latter showing improved results. Results were extremely similar on a C18 silica sp[46]. This result indicates that silanol groups may not be specifically involved in the overloading process (see results above for small molecules.

From these results the following deductions can be made with regard to the separation of peptides, which mirror those previously reported for small molecules:

1) Overloading occurs much more readily in mps using formic acid for pH control. These mps have a low ionic strength as formic acid is a relatively weak acid.
2) The problem cannot be caused by the supposed buffering ability of formic acid. Addition of neutral salt increases the PC without affecting buffer capacity or pH.
3) Use of ammonium formate buffers maintains good PC while providing a volatile mp suitable for MS.
4) TFA produces excellent results. It gives mps of reasonable ionic strength. It can also act as an ion pair reagent, effectively reducing the charge on basic solute molecules and thus reducing the detrimental effects of the ionic species. The low pH of TFA may also be effective in further suppression of silanol ionisation.

A problem with TFA is the suppression of the signal that occurs in ESI-MS. TFA may also be difficult to remove from the MS giving memory effects. Further commentary on the use of TFA is given below.

Field et al. [9,47-49] developed a protocol principally for determining column selectivity for peptides. They used custom peptides designed to highlight specific interactions such as hydrophobic, electrostatic, H bonding and aromatic. Similarities and differences between the columns were highlighted by use of Principal Components analysis. They classified 38 columns, grouping them as neutral, negative/polar or positive. Although measurement of peak shape through calculation of Peak Capacity was not the major objective of this work, results presented in this area were very much in agreement with those discussed above.

It is of relevance to see whether results for peptides discussed above are also relevant to proteins, which are much larger molecules. mAbs are proteins (RMM ~150,000) that represent an increasingly large proportion of worldwide pharmaceutical sales. For some compounds, sales are in the range of billions of dollars per annum. These protein drugs have ability to specifically target antigens, and have become important in the treatment of many life-threatening diseases[50-51]. As with all pharmaceuticals, quality control of formulations is essential. Their characterization may involve several techniques including RPLC. There is interest in the separation of intact mAbs by RPLC as the structure and properties of the entire

drug are retained. it is necessary to use materials of large enough pore size to accommodate these species otherwise size exclusion effects could dominate the separation. Typical pore sizes are 300 to 1000 Å. For small molecules (MW <500), elevated temperatures can increase the value of the flow velocity corresponding to optimum efficiency, with consequent improvement in analysis speed[22]. Column stability problems may limit the choice of sp. However, high column temperatures up to 100 °C are usually essential to elute proteins, which as a consequence of multi point adsorption, may otherwise remain irreversibly adsorbed to the sp. Silica based columns with bonded ligands less hydrophobic than C18 such as C4 or phenyl bonded, are typically employed to limit the high retention showed for C18 sps [7].

We studied the RP chromatography of Rituximab and Bevacizumab on a 1000 Å superficially porous C18 sp using an ACN-water gradient containing a variety of additives: TFA, MSA (methane sulfonic acid) TFA with 5% butanol and ammonium formate[7]. Rituximab and Bevacizumab are two of the most demanding mAbs to analyse by RPLC, giving strong ionic and hydrophobic interactions with RP materials[52]. For both mAbs, peak height and PC decreased as the temperature of the mp was reduced from 80-50 °C. Peak capacity was in the order:

MSA> TFA~TFA/butanol> Ammonium formate

The separation of the two mAbs using an acetonitrile gradient with 0.013M MSA is shown at various temperatures in [Fig. 9].- A comparison of peak capacities for the two mAbs using 4 different acid additives at temperatures 50-80 °C is shown in [Fig. 10].

Investigations 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 excessive sample degradation. Ammonium formate buffers, which are known to give better MS detection properties than TFA, produced good results down to 70 °C, but did not elute the mABs at 50 ° C. MSA gave the best chromatographic results, even when low concentrations of this additive were used. Reasonable results were obtained even at 50° C. The stronger acidity of MSA may be beneficial in reducing detrimental ionised silanol interactions. Further studies are necessary however, to assess the MS compatibility of this additive, and the possible corrosive effects of this relatively strong acid on instrument materials.

The choice of acid additive to give good ESI-MS detection sensitivity while retaining good chromatographic performance is an important question in the analysis of intact proteins[8]. For RP-LC analysis, TFA has remained as the most favoured additive, giving good peak shape due probably to the low pH it generates and its ion pairing abilities. However, problems with MS detection (particularly its suppression effects on the MS signal) properties (see above) have led researchers to investigate alternatives for intact proteins. In a recent comprehensive study[8], 15 additives were screened using criteria such as availability and purity of the reagent, safety considerations, price, pK_a and pH of 0.1 % (v/v) solutions in water, On this basis, 10 additives were selected for further study. Large pore (400 and 1000 Å) superficially porous RP materials were used. These have a relatively thin porous coating on an inert non-porous core, providing a reduced diffusion distance and thus better mass transfer and efficiency for these slow-diffusing large molecules (but see also above)[43]. Of these 10 additives, only 6 gave acceptable results although considerable differences were obtained in selectivity, peak shape and MS sensitivity. These differences were highlighted when subunit analysis of Trastuzumab with these 6 acid additives was performed after (partial) digestion using immunoglobulin degrading enzyme of *Streptococcus pyogenes* (IDES). [Fig. 11] shows by the presence of minor peaks in the chromatograms that DFA, TFA, MSA and DClAA gave better results than TClAA or formic acid.
Fig. [12] alternatively examines the MS sensitivity obtained with the different additives [8]. Clearly, formic acid even at 1% concentration gives much higher sensitivity than any of the other additives. DFA potentially gives less signal suppression than TFA and warrants further examination. It seems from these results that from this initial wide choice of additives either TFA (better chromatography) or formic acid (better MS sensitivity) will remain dominant as the best choice of additive at least for whole protein or large fragment analysis.

Conclusions

Asymmetric, particularly tailing peaks are a serious problem in RPLC, reducing the separation ability of the column by causing peak broadening/interference with neighbouring peaks. They can also negatively affect measurement of peak area and thus quantitative results. Kinetic effects involving the strong interaction of ionised bases with silanols from silica stationary phases alongside hydrophobic interactions with non-polar bonded ligands tend to produce exponential tailing. Such tailing can often be minimised by use of low mp pH which suppresses silanol ionisation, high purity silica which has fewer highly acid silanols, endcapping reactions with sterically favoured silylating agents which reduces the number of residual silanols. and the use of dense or polymerised ligand layers which may mask silanols. Hybrid inorganic/ organic sps have a reduced number of silanol groups and may be less prone to give kinetic tailing effects. Older design sps are especially prone to these negative effects-and are still used in industry as replacing them with alternatives may involve lengthy and costly method revalidations. Peak asymmetry caused by overloading seems to be a more intractable problem, as it may not be restricted to silica-based sps, thus making substitution with other materials less likely to solve the problem. As its detailed nature remains somewhat obscure, remediation methods are not obvious. It is quite possible to observe both kinetic and overload tailing simultaneously. Overloading effects are lessened in high ionic strength mobile phases and use of additives which can ion pair with basic solutes. The problem with these modifications however is that they are often contrary to obtaining good sensitivity in linked MS applications. A possible solution to some problems is the use of charged surface hybrid sps that limit overloading in mps containing formic acid, which is more suitable for HPLC-MS use. The use of high pH mps above solute pK_a is an attractive proposition to limit both causes of peak asymmetry. However, high pH can cause ionisation of even the least acidic silanols, and may be detrimental to solute stability and limit MS sensitivity. There are also a

restricted number of sps that are stable at high pH. Another possibility to reduce overloading problems with basic solutes is to explore more fully other separation mechanisms in HPLC for their analysis. Particularly, HILIC has the future potential to solve some of these problems-with excellent peak symmetry for some basic drugs on bare silica columns [29,30]. Some of the causes of peak asymmetry and possible remedies are summarised in Tables 1 and 2.

Principles established for small molecules are largely relevant especially for peptides, but also for proteins. There has been increasing interest in these compounds, especially in the characterisation of protein pharmaceuticals such as mAbs. Sales of these new drugs can amount to billions of dollars annually.

Recently, interest in the analysis of therapeutic oligonucleotides has increased with 12 new drugs being approved in the period 2016-2022. Ion-pair RP-HPLC has been the method of choice for their characterisation. These solutes are subject to detrimental interactions with positively charged metals in the column or instrument hardware [53]. These interactions can be suppressed by the use of alternative materials to stainless steel, or by deactivation by coating with organic polymers. These compounds have a negatively charged hydrophilic backbone and thus behave in a different way to ionised bases, which are the main thrust of this review. While analysis of acids generally is less problematic than analysis of bases, the strong clinical interest in them may encourage more studies in this area.

Figure Legends

Fig. 1 Structures present on a typical alkyl bonded RP silica sp (C8) endcapped with trimethylsilyl groups. Adapted from J. Chromatogr. A 2010 **1217** 858.

Fig. 2 Sketch of (a) symmetrical (Gaussian) peak; (b) exponentially tailing peak ; (c) right angled triangle peak.

Fig. 3 Overlaid chromatograms on hybrid RP column of 0.05 to 5 μg of propranolol (base, upper)) and naphthalene-2-sulfonic (acid,lower) using 0.1 %v/v formic acid in acetonitrile- water as mobile phase. Adapted from Anal Chem. 2006 **78** 2532.

Fig. 4 Retention of lithium ions as a function of true thermodynamic pH for 3 different RP columns. Adapted from J. Chromatogr. A 2003 **986** 33.

Fig. 5 Plots of column efficiency (N) vs solute mass for neutrals 3-phenylpropanol (green) phenol (purple), caffeine (dark blue) and nortriptyline (black, base), propanolol (light blue, base) , 2-NSA (red, acid) on a hybrid RP column . Mp 0.1 % formic acid in acetonitrile-water pH 2.7. Adapted from Anal. Chem. 2006 **78** 2532.

Fig. 6 Plots of column efficiency against mass of amitryptiline ($_w^s$ pK_a = 8.9) for acetonitrile-phosphate buffers at various pH using a hybrid RP column. Percent ionisation of the base is indicated. Adapted from J. Chromatogr. A 2010 **1217** 856.

Fig. 7 Analysis of Alberta peptide mix at normal concentration and diluted tenfold on a RP column using formic acid (upper chromatograms) or TFA (lower chromatograms) in acetonitrile-water gradient mp. Adapted from J. Chromatogr. A 2004 **1938** 77.

Fig. 8 Analysis of 1) bradykinin, 2) bradykinin frag 1-8 3) 3-Arg bradykinin at 0.1 and 2.5 μg mass injected on totally polymeric sp. Top: mp 0.02M formic acid; Bottom 0.079M TFA both in acetonitrile-water gradient mp. Adapted from J. Chromatogr.A 2005 **1073** 137.

Fig. 9 Separation of Rituximab (first eluted) and Bevacisumab at temperatures 50-80 ^oC on wide pore diphenyl column using 0.013M methanesulfonic acid as additive in acetonitrile-water gradient. Adapted from J. Chromatogr. A 2020 **1610** 460562.

Fig. 10 Effect of temperature on peak capacity of Rituximab (a) and Bevacisumab (b) using 4 different additives in ACN mp at temperatures 50-80 ^oC. buta=butanol; AF=ammonium formate; MSA= methanesulfonic acid;FA= formic acid. Adapted from J. Chromatogr. A 2020 **1610** 460562. Black bars 80, red 70, green 60, blue 50 ^oC.

Fig. 11 Chromatograms of digested and reduced Trastuzimab using selected additives. Note the unusually high concentration of formic acid needed to improve peak shape. Additives as Fig. 9 with TClAA =trichlroacetic acid; DFA= difluoroacetic acid; DClAA = dichloroacetic acid. Adapted from J. Chromatogr. *A,* 2022 **1677** 463324.

Fig. 12 Mass spectrometry signal to noise ratio (S/N) taken from the total ion current chromatogram for trastuzumab and cetuximab usning various additives, and of counts per second (cps) of the highest charged state of the subunits. Adapted from J. Chromatogr. *A,* 2022 **1677** 463324.

Author Contributions

David McCalley was the sole contributor to this paper.

Conflicts of interest

There are no conflicts to declare.

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Fig. 1

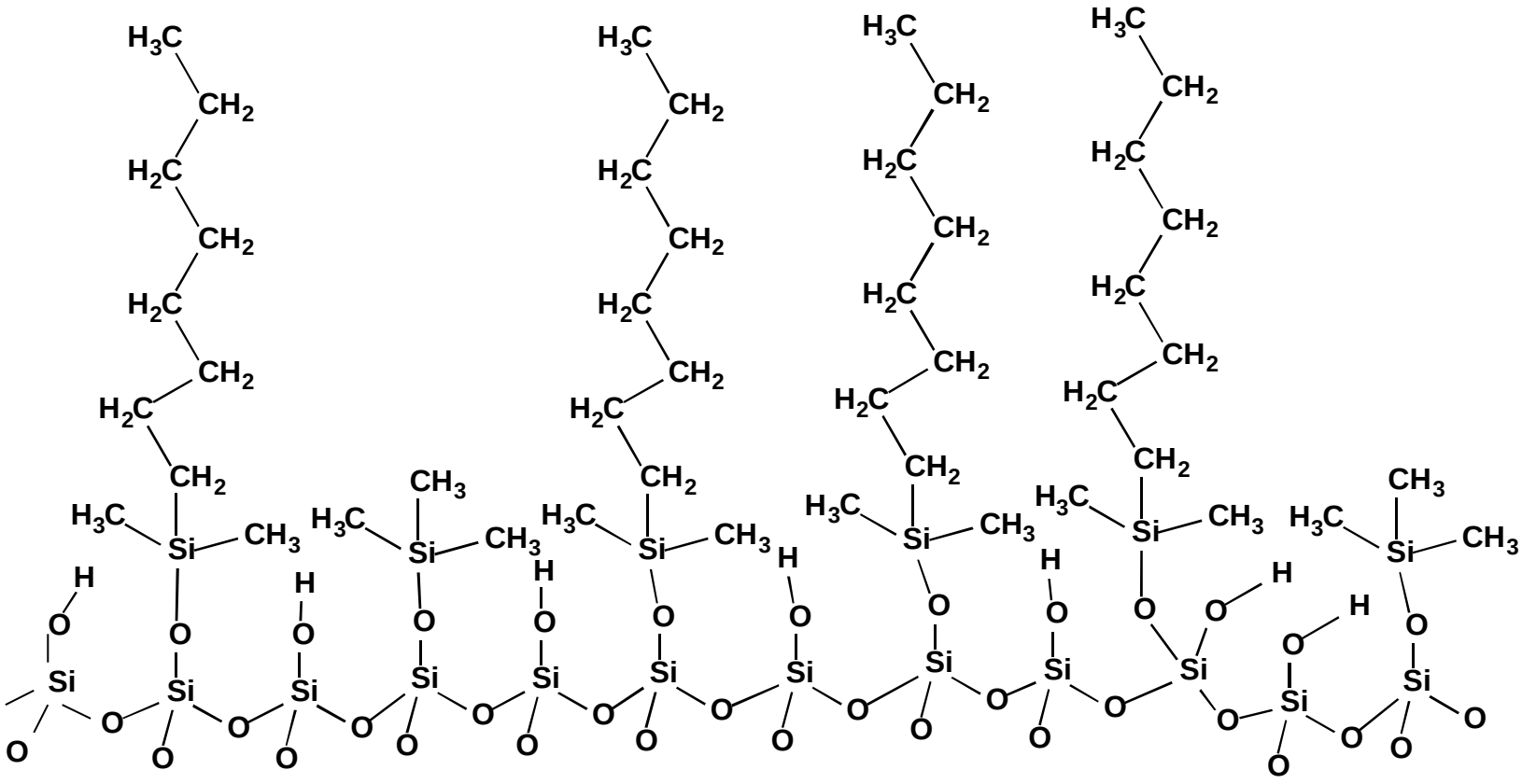
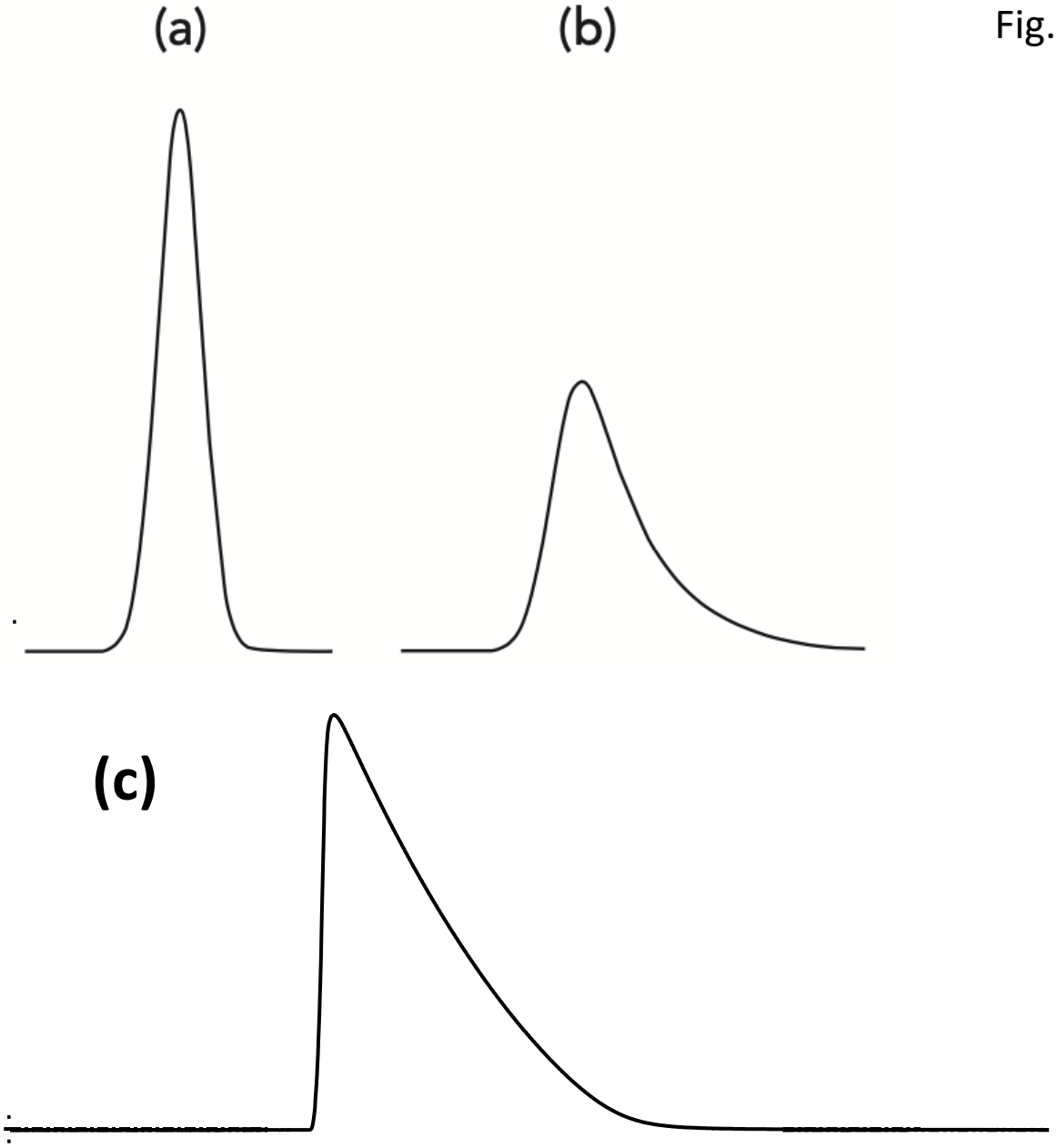


Fig. 2



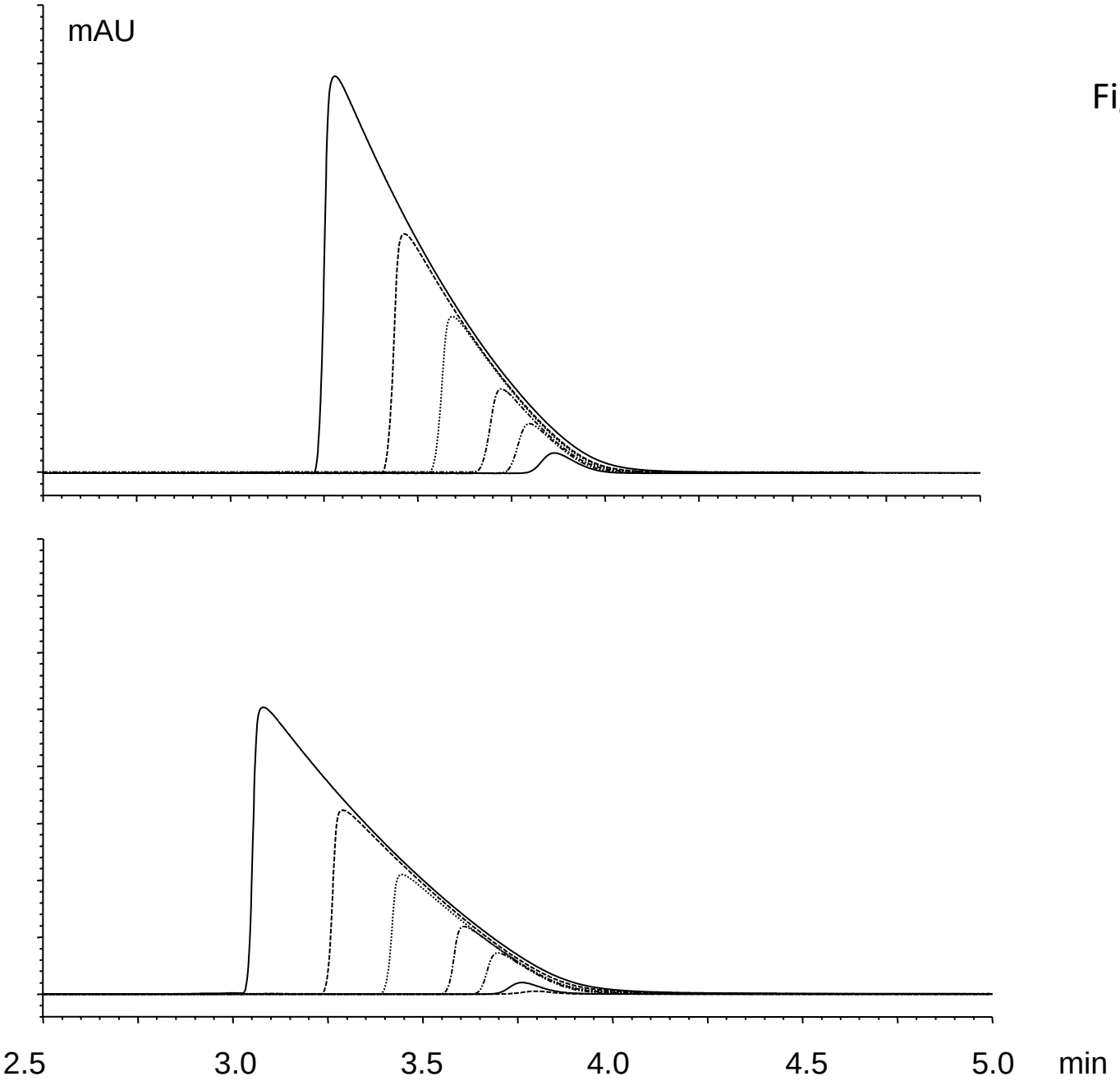


Fig. 3

Fig. 4

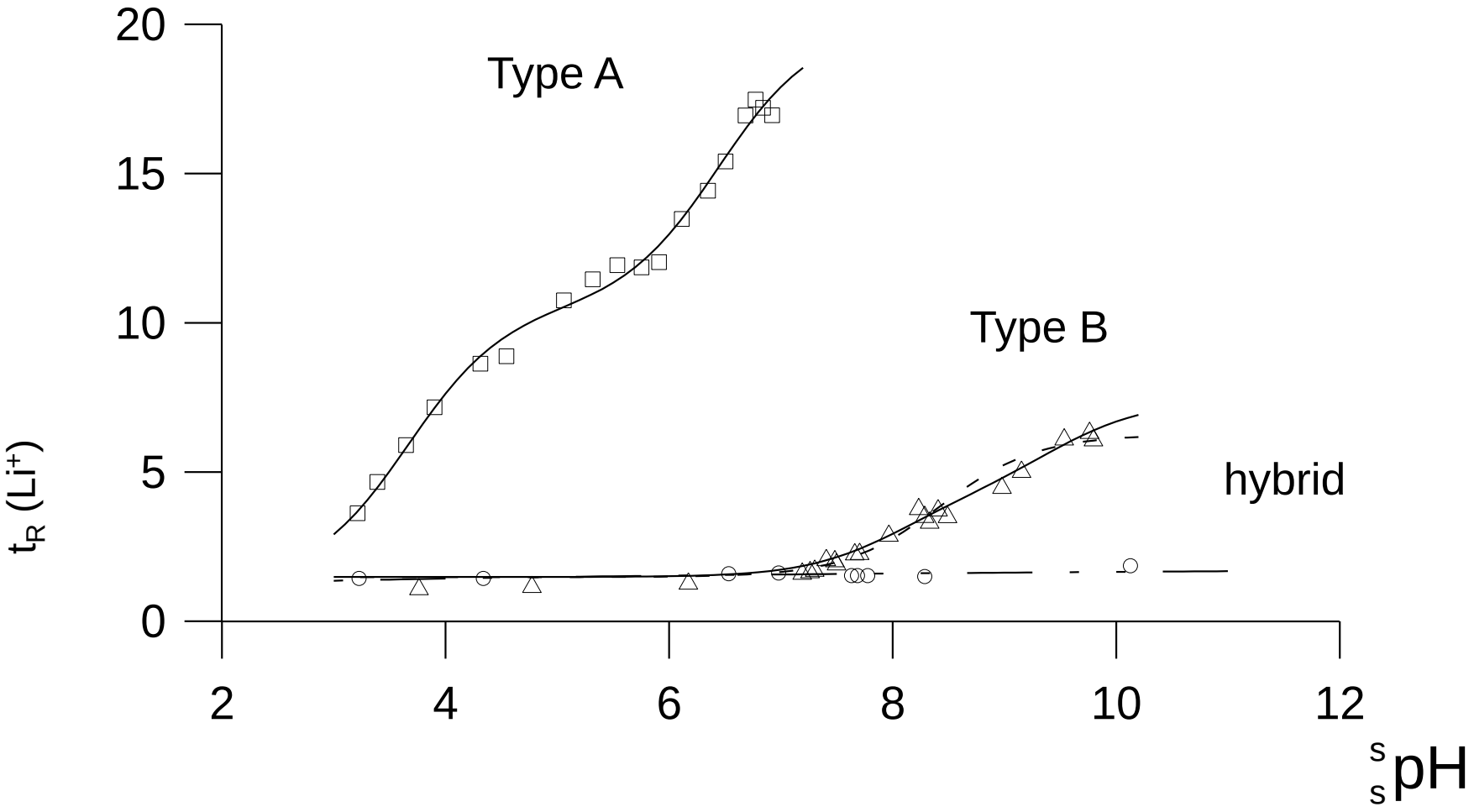
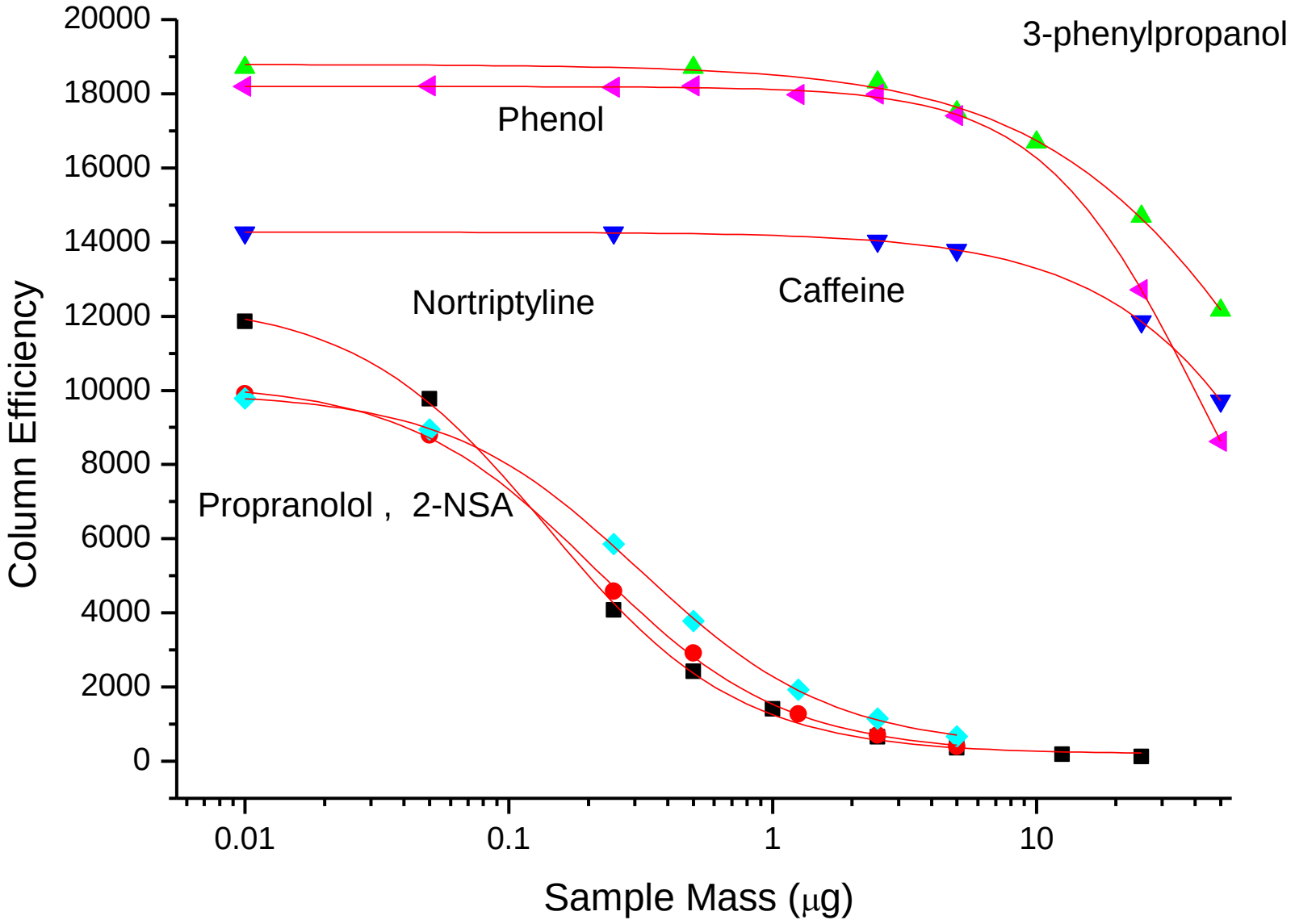


Fig. 5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Propranolol , 2-NSA
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0.1
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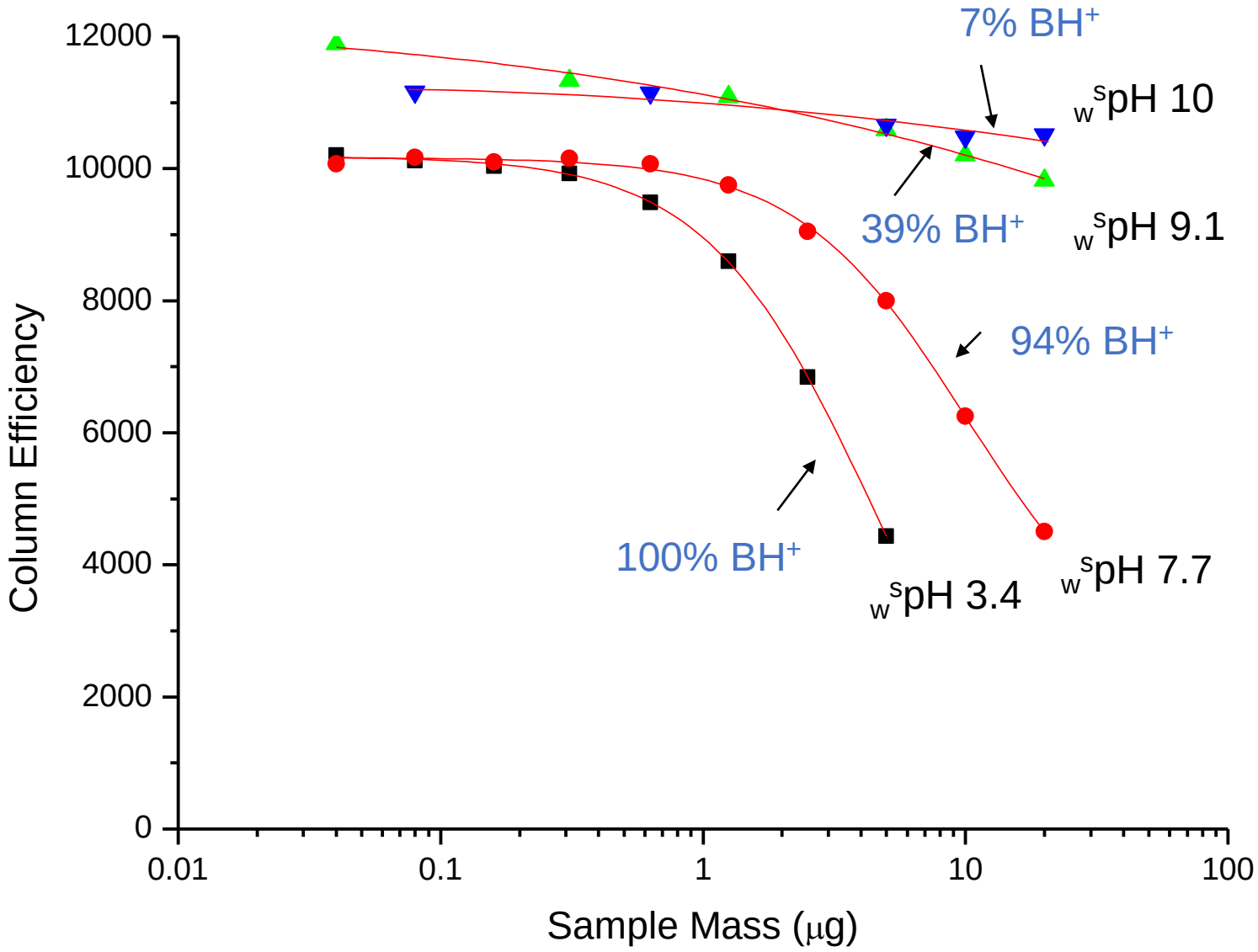
10

Fig.6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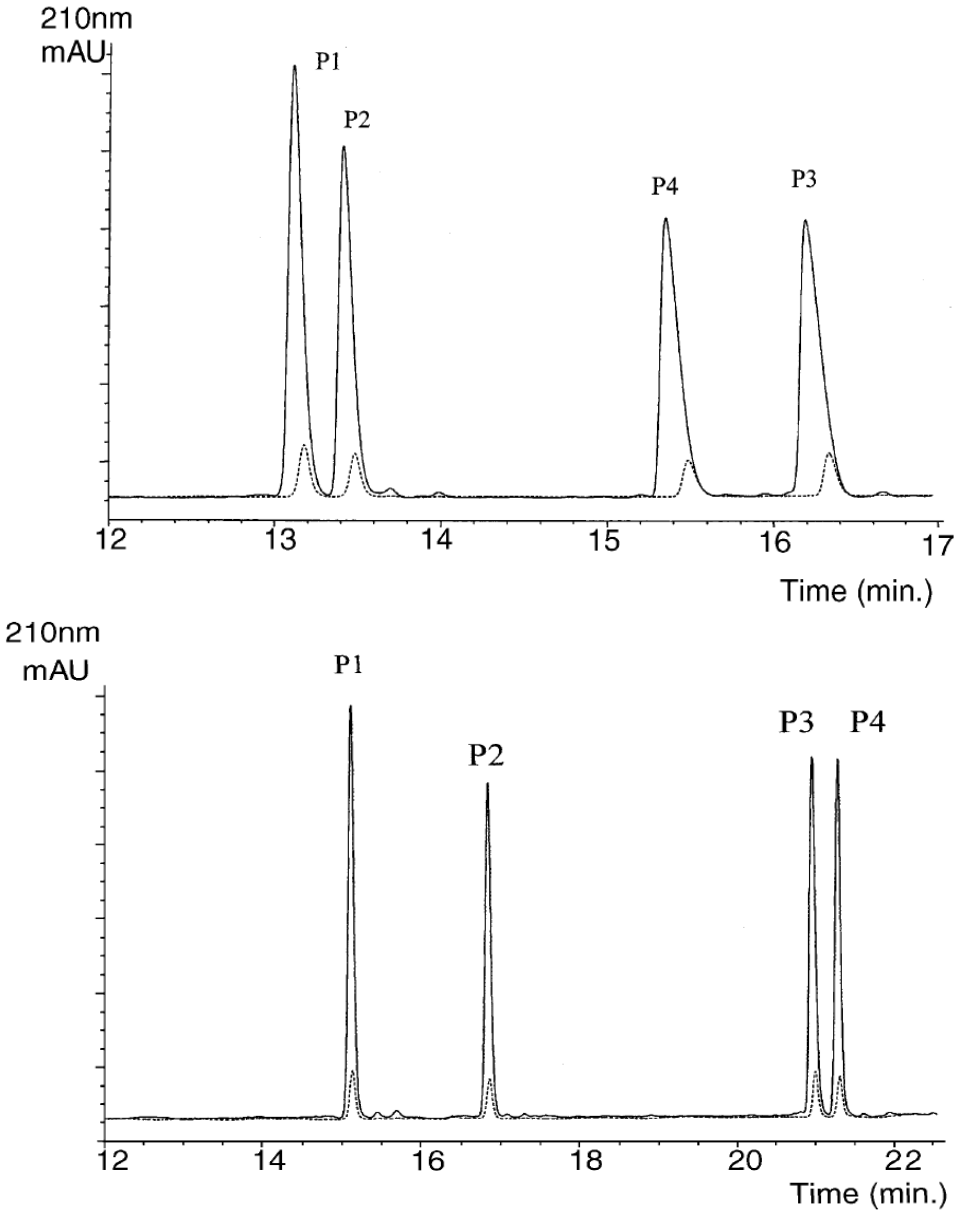
wspH 7.7

Fig. 7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P2
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16
17
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Fig. 8.

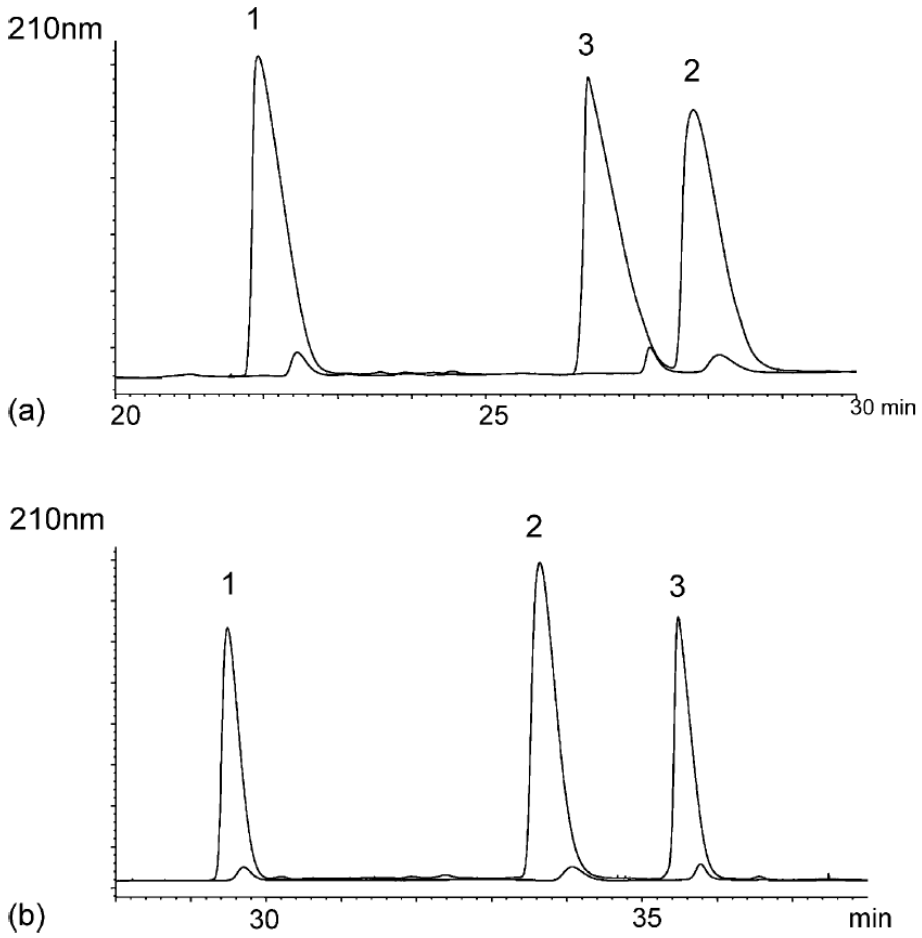


Fig. 9

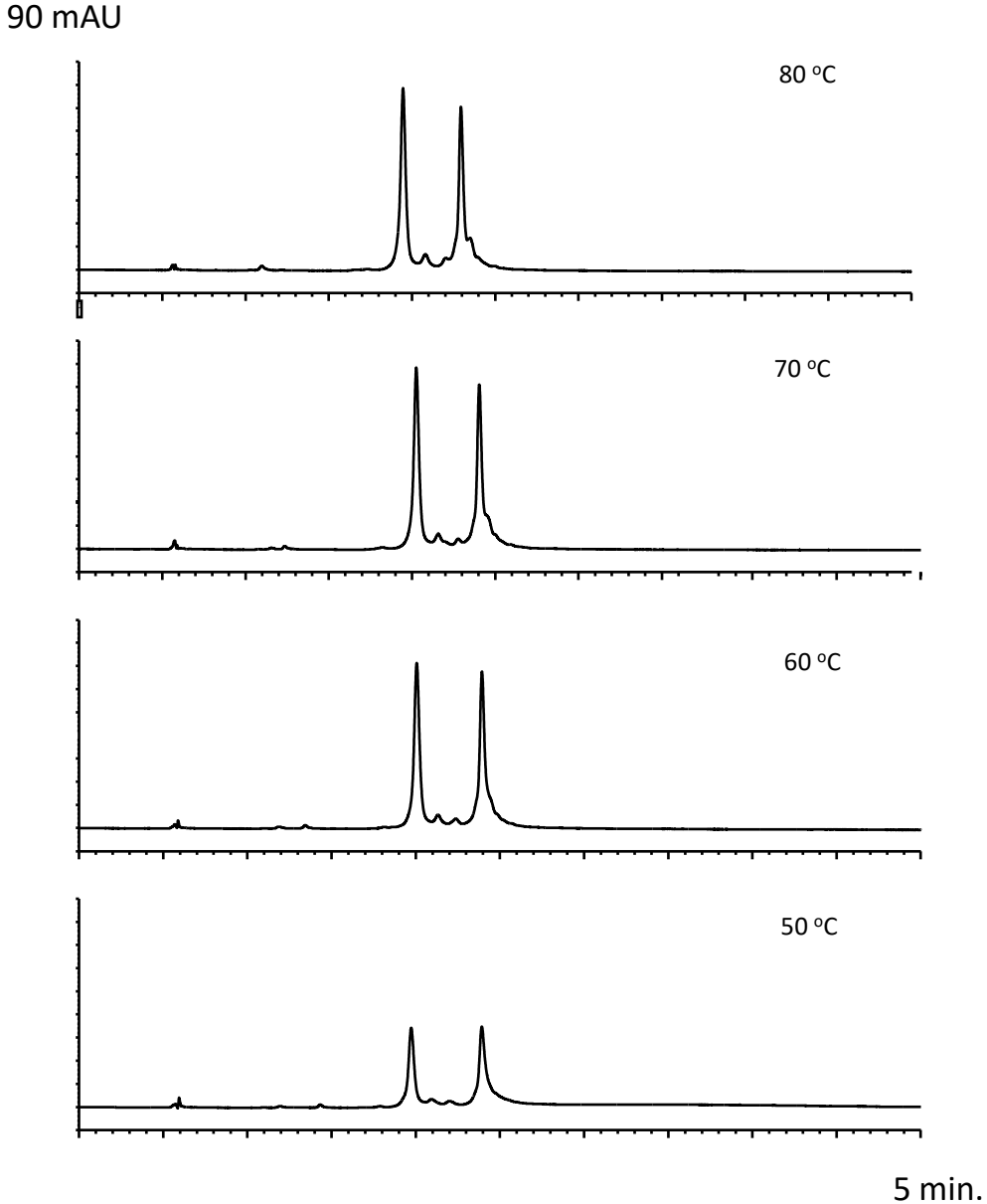
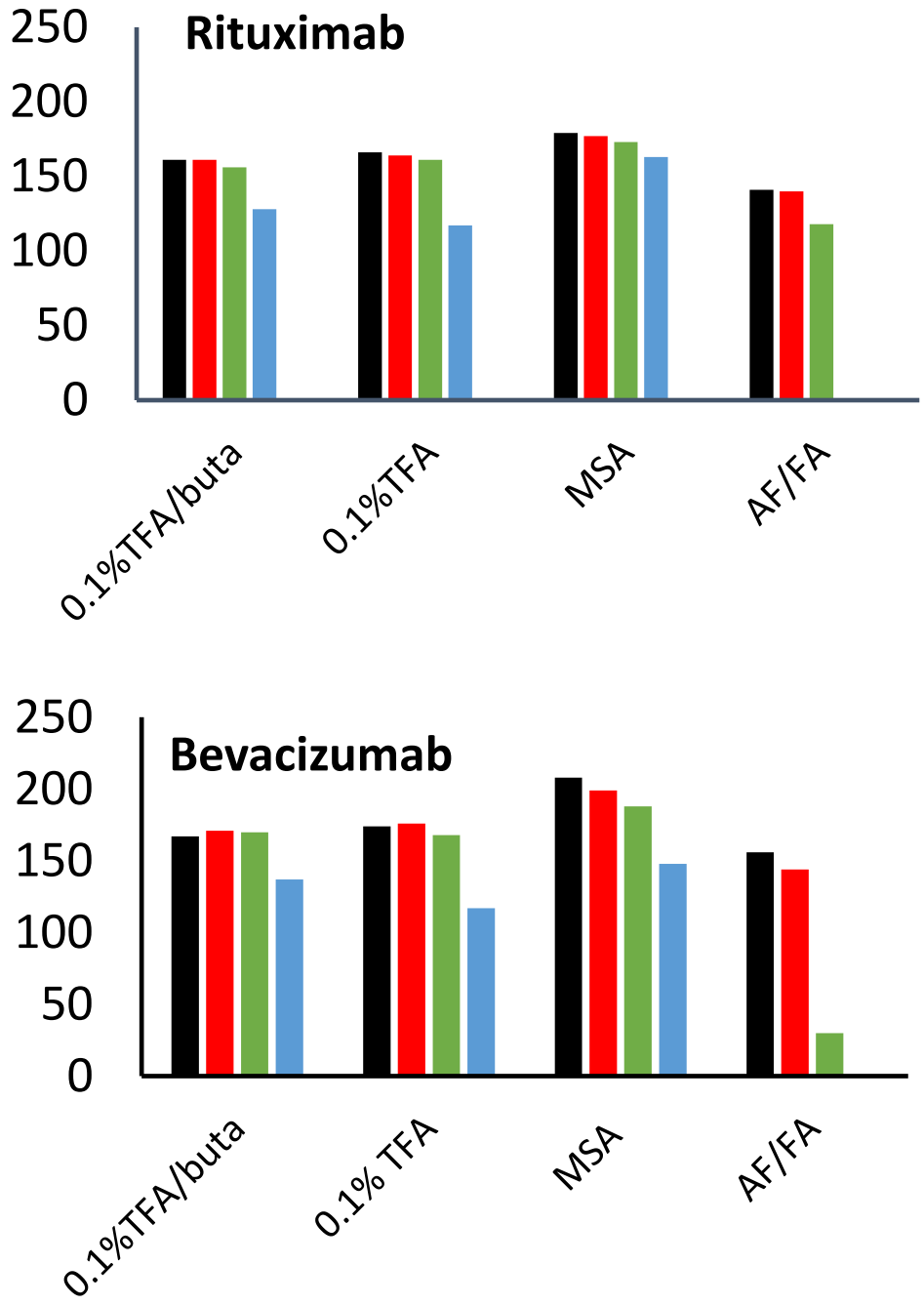


Fig 10


Rituximab
250
200
150
100
50
0
0.1%TFA/buta
0.1%TFA
MSA
AF/FA
Bevacizumab
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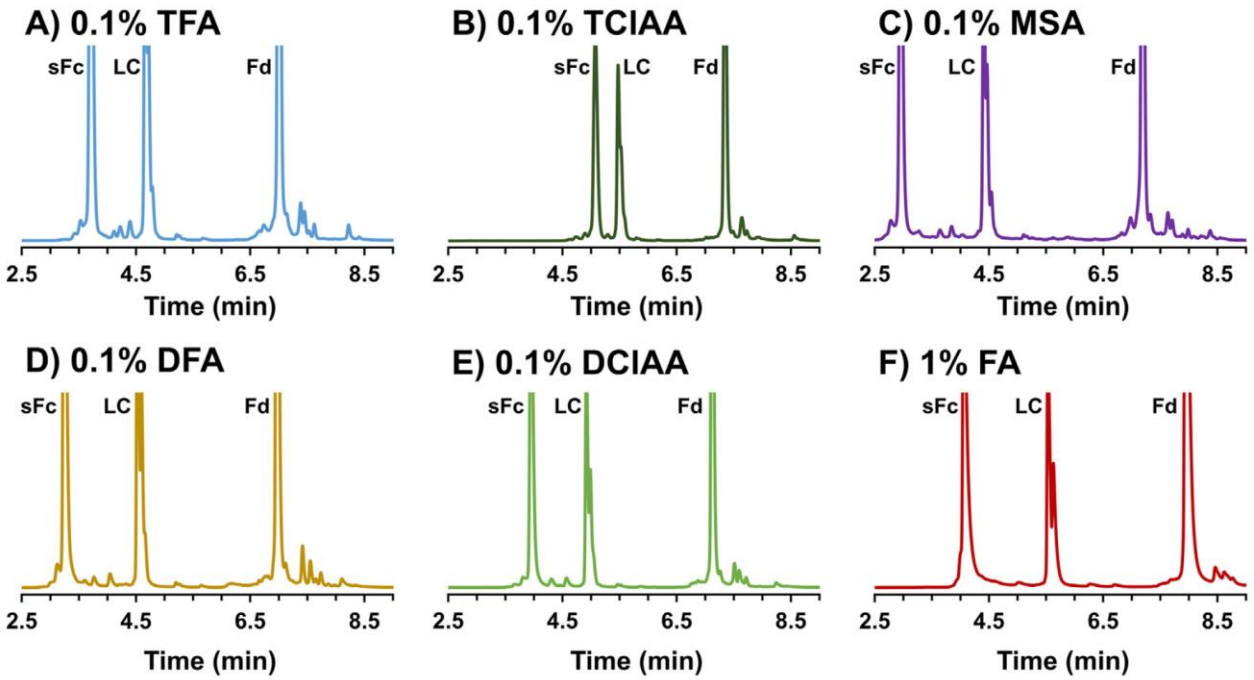
200
150
100
50
0
0.1%TFA/buta
0.1% TFA
MSA
AF/FA

Fig. 11.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B) 0.1% TCIAA
C) 0.1% MSA
D) 0.1% DFA
E) 0.1% DCIAA
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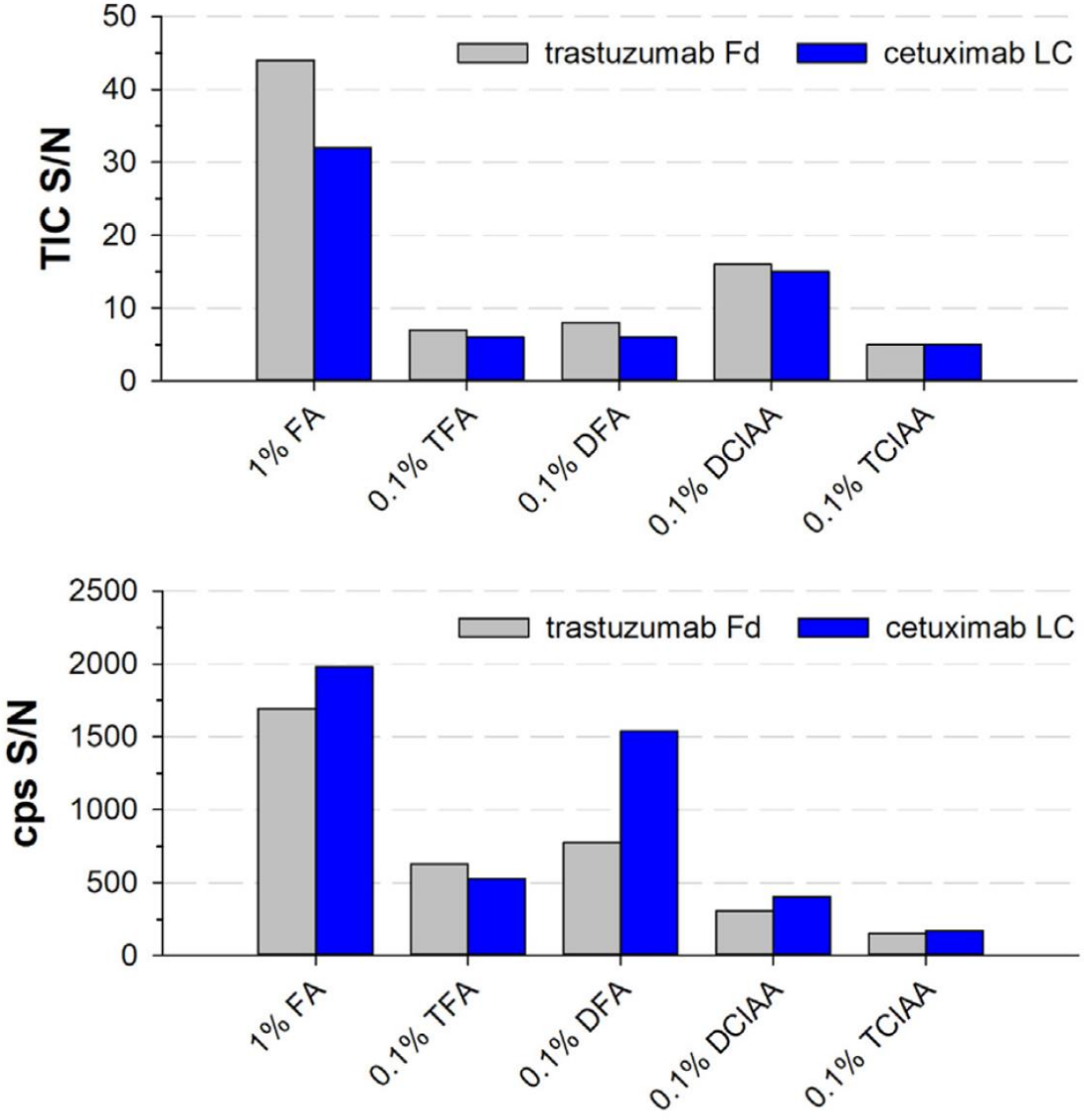
LC
Fd
2.5
4.5
6.5
8.5
Time (min)

Fig 12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40
30
20
10
0
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1% FA
0.1% TFA
0.1% DFA
0.1% DClAA
0.1% TClAA
cps S/N
2500
2000
1500
1000
500
0
trastuzumab Fd
cetuximab LC
1% FA
0.1% TFA
0.1% DFA
0.1% DClAA
0.1% TClAA

Table 1

"Physical" causes of poor peak shape. These problems are not unique to basic solutes and will persist even for simple hydrophobic solutes (e.g. toluene). They are often easily remedied. See D.R. Stoll, LC.GC North Am 39 (2021) 353-362

Symptom	**Cause**	**Remedy**
Broad and/or asymmetric peaks	Dead volumes in the instrument, connections, (UV) detector cell.	Re-make connections, use smaller volume connecting tubing and detector cell.
	Insufficient number of points gathered across a peak	Increase detector sampling rate.
	Injection solvent stronger than mobile phase.	Inject in mobile phase or in solvent of reduced eluotropic strength
	Injection solvent mp but sample volume is too large	Reduce sample volume
	Voids in column packing e.g. due to dissolution of silica at high pH.	Replace column.

Table 2 “Chemical” causes of poor peak shape for bases. These symptoms may disappear in changing from a difficult (e.g basic) solute to a simple neutral solute.

Symptom	**Cause**	**Remedy**
Right angled triangle peaks	Mass overload	-decrease sample mass; -increase ionic strength of the mp with salt buffers or TFA
Poor sensitivity in LC-MS but otherwise symmetrical peaks	Signal suppression by buffer components	-use formic acid instead of TFA -try charged surface hybrid columns with formic acid mp
Right angle triangle peaks/ exponential tailing.	Mass overload; mixed mechanism involving strong ionic/weak hydrophobic interaction.	-use a more inert sp (fewer ionised silanols) -use pH > solute pK_a so solute is deprotonated (neutral).
Irreversible adsorption of peptides and proteins	Strong multipoint adsorption.	-increase temperature (noting column max temperature). -use a more inert sp -use sp with less hydrophobic ligands (e.g C4 or phenyl).
Exponential Tailing	Mixed mechanism interactions involving ionised silanols	-use Type B silica column (pure silica) -try inorganic/organic hybrid column

Table 3 Comparison of performance for Alberta peptide 4 using different additives (FA = formic acid; amm.= ammonia solution). A_s = asymmetry factor ; I =ionic strength. Data from J. Chromatogr. A 2004 **1038,** 77.

	additive	pH	I (mM)	A_s	Pk cap
P4 (norm)	0.02M FA	2.7	1.9	3.5	148
(10x dil)				1.9	206
	0.02M FA	3.3	7.4	1.7	215
	7mM amm.			1.5	234
	0.02M FA +	2.7	22	1.4	227
	0.02M KCl			1.1	234
P4 (norm)	0.008M TFA	2.3	7.8	1.3	233
(10x dil)				1.1	238

Table 3