Effect of Alternative Mobile Phase Additives on the Peak Shape of Basic Analytes in RP-HPLC

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Degree Project in Analytical Chemistry, 2026
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The Search for Alternative Additives

In 1957, a drug for nausea during pregnancy was marketed; Thalomide. This drug proved to have a devastating side effect, resulting in infants with serious birth defects. The cause was later proved to be the presence of a mirror-image of the active compound. This case effectively shows the importance of robust and reliable separation and detection techniques when it comes to drugs. Reversed-phase liquid chromatography (RPLC) is widely used for this matter.

RPLC separates compounds based on their ability to interact with the hydrophobic surface of the small particles present in the separation column. The mobile phase is the liquid in which the compounds travel through the column, and it is modulable. Heterogeneities of the particle surface (stationary phase) sometimes cause compounds to interact in mixed ways with the surface. This is especially problematic for compounds containing amino groups, which under acidic mobile phase conditions become positively charged. Acidic additives can be employed to improve the interactions, as they are negatively charged and can form ion pairs with the amines. Trifluoroacetic acid (TFA) is exceptional at this but causes problems when detection techniques such as mass spectrometry (MS) need charged compounds to be able to detect them, as well as TFA being proven to be accumulative in nature (PFAS-compound). Formic acid (FA) is more environmentally friendly and does not form as strong ion pairs and consequently yields better MS signal. In essence, there is a trade-off between the chromatographic performance and MS detection.

In this study, different additives are evaluated based on the effect they have on the peak shape (mainly tailing) of the analysed compounds. Both small molecule amines and peptides are used as test compounds, and they are separated on RPLC with UV- and MS-detection.

It was found that two additives (propionic acid and glycolic acid) were particularly poor performing. For peptides which contained different number of positive charges, the effect of the additives on tailing was mainly charge-dependant, but the magnitude depends on the additive type. Increasing the concentration improves the peak shape for both small and big loads of peptides on the column. One alternative additive (trichloroacetic acid) to TFA was tested for its MS compatibility and proved to be even poorer performing than TFA.

Conclusively, the additive effect on peak shape depends heavily on the compound analysed and on which column. The search for alternative additives is not over yet!

Abstract

Introduction: Reversed phase liquid chromatography (RPLC) is a central analytical technique in pharmaceutical and biopharmaceutical science, where it is essential to reliably detect, separate, quantify, and purify drugs, of which a majority contain basic groups.

Background: Basic analytes often exhibit poor peak shape, for example tailing, due to mixed-mode interactions with the stationary phase. Acidic additives are commonly used to lower pH and act as ion pairing agents, improving chromatographic performance. Unfortunately, strong ion pair agents often suppress the signal in mass spectrometry detection. Followingly, it is desirable to find alternative additives that fit the silver lining between good chromatographic performance and MS compatibility.

Aim(s): This study aims to evaluate additive effects on peak shape, mainly tailing factor (Tf), of amines and peptides in analytical and overload conditions on two columns.

Methods: Different additives of varying concentrations were used to analyse Tf and w50% for amines and peptides in both analytical and overload conditions on HPLC-UV and -MS. Tf was calculated with the help of an Excel template and statistical analyses such as ANOVAs were conducted in RStudio.

Results: PrA and GA generally yielded worse tailing for the amines, than all the other additives. Peptides exhibited charge-dependant tailing and increased additive concentrations mitigated the extent of that tailing in both analytical and overload conditions. TCA was found not to be a suitable alternative to TFA with regard to MS compatibility, as indicated by previous studies.

Conclusion: No universally best additive was found, and the importance of MS compatibility was highlighted. Additive effects are highly dependent on analyte type and column.

Keywords: Additives, amines, peptides, RPLC, tailing factor

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1 List of Abbreviations

AA	Acetic acid
DAD	Diode Array Detector
DCA	Dichloroacetic acid
DFA	Difluoroacetic acid
EIC	Extracted Ion Chromatogram
ESA	Ethanesulfonic acid
FA	Formic acid
GA	Glycolic acid
HPLC	High Pressure Liquid Chromatography
MS	Mass Spectrometry
MSA	Methanesulfonic acid
PrA	Propionic acid
RPLC	Reversed Phase Liquid Chromatography
SD	Standard deviation
TCA	Trichloroacetic acid
Tf	Tailing factor
TFA	Trifluoroacetic acid
TIC	Total Ion Chromatogram
w50%	Width at half peak height

2 Introduction

In pharmaceutical and biopharmaceutical science, liquid chromatography (LC) is a central analytical technique. Most active compounds in drugs contain basic functional groups [1], such as amines, and are commonly encountered as small molecules, peptides, and proteins. Reliable separation and detection of such compounds is therefore of considerable importance [1-5]. LC is used for separation, quantitation, purification, and characterization of active compounds and related impurities [2, 3]. In particular, reversed-phase liquid chromatography (RPLC) is widely employed due to its suitability for aqueous samples, versatility with gradient elution and tuneability through pH, organic modifier and mobile phase additives, as well as its compatibility with both ultraviolet (UV) and mass spectrometric (MS) detection [2, 4, 6].

Despite its widespread use, the separation of basic analytes in RPLC remains challenging. Under typical operating conditions, acidic mobile phases are employed to suppress the ionization of residual silanol groups on the stationary phase [4]. At low pH, however, basic analytes are predominantly protonated and positively charged. This creates a mismatch with the main hydrophobic retention mechanism of RPLC and promotes additional H-bonding and ionic interactions with residual silanol groups. As a result, analyte retention becomes governed by a combination of hydrophobic and ionic interactions, leading to heterogeneous adsorption behaviour [6, 7]. These mixed-mode interactions are associated with peak asymmetry, most notably tailing, which reduces chromatographic efficiency and compromises separation performance [4].

The origin of peak tailing in these systems can be attributed not only to the presence of residual silanol groups, but also to the differing kinetics of the interactions involved. Hydrophobic interactions are fast and reversible, whereas H-bonding and ionic interactions exhibit slower adsorption–desorption kinetics. This kinetic heterogeneity leads to a distribution of residence times for analyte molecules on the stationary phase, which manifests as broadened and asymmetric peaks [1, 6]. Although modern stationary phases have been developed to minimize such effects through improved silica purity, end-capping, or hybrid and polymer-based materials, complete elimination of residual silanol activity is not possible due to steric limitations [3]. Consequently, secondary interactions remain a source of peak distortion for basic compounds.

To reduce these effects, mobile phase additives are commonly employed. The importance of additives in LC has long been acknowledged but remains an important and extensively studied area in method development and optimization till this day. Acidic additives control pH and suppress silanol ionization. It has been estimated that the average pKa of silanol groups is 7.0, but the surroundings can possibly create silanols that remain ionized even at pH 2 [7]. Certain additives additionally act as ion pairing agents. Through ion pairing, negatively charged counterions associate with protonated amines to form neutral and less polar species. This reduces electrostatic interactions with the stationary phase and increases the hydrophobicity of the analytes, leading to improved retention and more symmetrical peak shapes. Among such additives, trifluoroacetic acid (TFA) is widely used due to its strong ion pairing capability and its ability to produce narrow and symmetrical peaks [4, 8].

However, the use of TFA presents a significant drawback in RPLC-MS. In electrospray ionization (ESI), efficient ionization requires the presence of free analyte ions. The ionization is affected by the properties of ionic groups in the analyte, pKa and surface tension of the additive, and the overall conductivity of the sample solvent [3]. Strong ion pairing between TFA and protonated analytes reduces the availability of free ions in solution and decreases ionization efficiency in MS in positive mode. In addition, TFA has high surface tension, which influences droplet formation and evaporation processes [4], further contributing to signal suppression. As a result, while TFA provides excellent chromatographic performance, it often leads to poor sensitivity in MS detection [5].

Weaker acids such as formic acid (FA) and acetic acid (AA) are frequently used as alternatives to TFA. While ion pairing ability has been experimentally confirmed for TFA [6], there is only indirect evidence for other strong- and weak ion pairing agents; based on observations of retention- and peak shape changes in the different chemical environment these additives create. FA and AA provide improved MS response due to weaker ion pairing interactions but may not sufficiently suppress secondary interactions or provide optimal peak shapes [4, 9]. Consequently, a fundamental trade-off exists between chromatographic performance and MS sensitivity, and no universally optimal additive has been identified [9]. In addition, increasing attention has been directed toward the environmental impact of fluorinated compounds such as TFA [10], further motivating the search for alternative mobile phase additives. Many alternative additives have been tested throughout the years, including perfluorinated acids, ionic liquids,

and various salts. Some interest in sulfonic acids has also been shown due to their suitability with peptides and proteins [3, 4].

Most studies addressing mobile phase additives have been conducted under analytical conditions, where analyte concentrations are low and the chromatographic system behaves linearly. In preparative applications, however, significantly higher sample loads are employed, leading to column overloading and nonlinear adsorption behaviour [6]. Under such conditions, the relative contributions of different interaction mechanisms change, and peak distortion phenomena such as fronting or tailing can become more pronounced. Despite its practical relevance, the effect of alternative mobile phase additives under overloaded conditions has received comparatively limited attention [11].

This study aims to evaluate the effect of alternative mobile phase additives on the chromatographic performance of basic compounds, with particular emphasis on peak shape. A set of small molecule amines and peptides with varying charge states is analysed using HPLC-UV and -MS. The influence of additive type and concentration is investigated under both analytical and overloaded conditions. Chromatographic performance is primarily evaluated using the tailing factor (Tf), while retention, efficiency and width at half peak height (w50%) parameters were also considered. MS suitability is also considered in terms of signal-to-noise ratios (S/N).

3 Materials and Methods

3.1 Chemicals

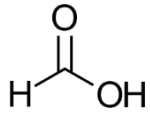
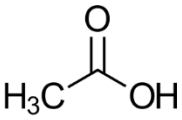
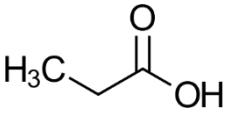
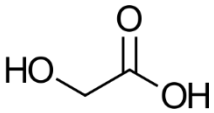
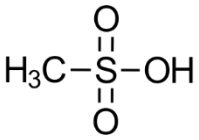
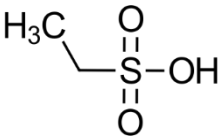
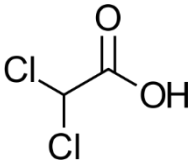
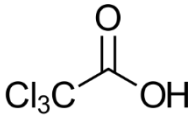
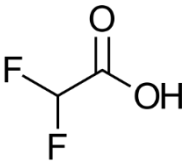
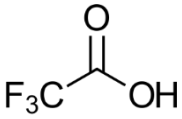
Uracil was purchased from The British Drug Houses LTD. Coumarin (COU, ≥99%, HPLC) and pindolol (PIN, ≥98%) were purchased from Sigma-Aldrich. Cyclobenzaprine hydrochloride (CBZ) was purchased from Thermo Scientific. (3-phenoxyphenyl) methanamine (PPMA) was purchased from Fluorochem. All peptides were obtained from the company's peptide bank.

Acetic acid (AA, ≥99.7%), dichloroacetic acid (DCA, 98%), trichloroacetic acid (TCA, ≥99.0%,), methanesulfonic acid (MSA, ≥99.5%), and ethanesulfonic acid (ESA, 95%) were purchased from Sigma-Aldrich. Formic acid (FA, 98+%), difluoroacetic acid (DFA, 98%), and trifluoroacetic acid (TFA, 99.5%) were purchased from Thermo Scientific. Propionic acid (PrA, ≥99.0%) was purchased from Acros Organics. Glycolic acid (GA, 98%) was purchased from Alfa Aesar. HPLC gradient grade acetonitrile (ACN, ≥99.9%) was purchased from Fisher Scientific.

3.1.1 Preparation of Aqueous Mobile Phases

Mobile phase additives were only added to the aqueous mobile phase to facilitate operation when switching between many different phases. 10 mM solutions of each additive in water were prepared for the small molecule amine study. For the set pH study, NaOH was added to the 10 mM solutions of TFA, TCA and MSA until pH 3 was reached. 10, 30, and 50 mM solutions of AA, PrA, TCA, DFA, and TFA were prepared for the peptide study. Similarly, 10, 30, and 50 mM solutions of TCA and TFA were prepared for the loadability study. A complete list of the investigated additives along with information about their structures and properties is reported below (**Table 1**).

Table 1. List of additives used in this study, their structures and properties.

Additive	Structure	Mw (g/mol) *	Boiling point (°C) *	pKa **	XlogP**
Formic acid (FA)		46.03	100	4.27	-0.27
Acetic acid (AA)		60.05	118	4.54	-0.22
Propionic acid (PrA)		74.08	141	4.75	0.45
Glycolic acid (GA)		76.05	169	3.52	-1.0
Methanesulfonic acid (MSA)		96.11	167	-1.61	-0.96
Ethanesulfonic acid (ESA)		110.13	123	-1.3	-0.45
Dichloroacetic acid (DCA)		128.94	194	1.26	1.1
Trichloroacetic acid (TCA)		163.39	196	0.82	1.5
Difluoroacetic acid (DFA)		96.03	133	1.30	1.2
Trifluoroacetic acid (TFA)		114.02	72	0.05	0.91

*Values obtained from PubChem.

**Values obtained from Chemicalize

3.1.2 Preparation of Sample Mixtures

For the small molecule amine study, a mixture of 1 mM of COU, CBZ, PIN, uracil (used as a void marker), and 10 mM of PPMA respectively, was prepared in equal amounts of ACN and water.

For the peptide study, separate aliquots of 1 mM Fmoc-L-Lys-L-Lys-L-Lys (Fmoc-KKK), Fmoc-L-Lys-L-Lys (Fmoc-KK), and Fmoc-β-Ala-L-Lys (Fmoc-AK) were prepared in equal amounts of ACN and water. For the loadability study, 10 mM aliquots of the same peptides were analysed separately. A complete list of the investigated analytes along with information about their properties is reported below (**Table 2)** and structures can be found in Appendix (**Figure A1**).

Table 2. List of analytes used in this study, including properties.

Analyte	Mw (g/mol) *	pKa **	XlogP **
Coumarin (COU)	146.14	-	1.4
Pindolol (PIN)	248.32	9.54	1.8
(3-phenoxyphenyl) methanamine (PPMA)	199.25	8.8****	2.2
Cyclobenzaprine (CBZ)	275.40	9.4	5.2
Fmoc-KKK	624.77***	10.6****	3.97***
Fmoc-KK	496.60***	10.6****	3.86***
Fmoc-AK	439.50***	10.8****	3.61***

*Values obtained from SDS

**Values obtained from PubChem

***Values obtained from ChemDoodle

****Values obtained from MolGpKa

3.2 Chromatographic Media

3.2.1 Instrumentation

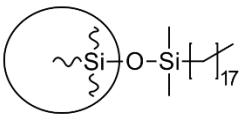
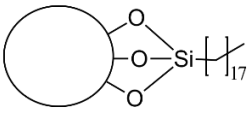
HPLC-UV/Vis analyses were performed on an Agilent 1100 system equipped with a solvent degasser, flow-through needle injector, binary pump, and diode-array detector (DAD) with a 10 mm standard flow cell. ChemStation for LC Rev. B.01.01 software was utilized to view all chromatograms, integrate the peaks and obtain retention time, area, height, w50%, and symmetry.

HPLC-MS analyses were performed on an Agilent 1290 Infinity II system equipped with a flow-through needle injector, binary pump, DAD with a 60 mm flow cell and an InfinityLab LC/MSD mass spectrometer. The mass spectrometer was operated in ESI+ mode with the range 100-700 m/z, scan time of 878 ms, 100 V fragmentor, gas temperature of 350 °C, gas flow of 12 L/min, nebulizer at 15 psi, and a spray voltage of 4000 V. OpenLab CDS Version 2.7 was utilized to view all chromatograms, total ion chromatograms (TICs), mass spectra, and extracted ion chromatograms (EICs) and obtain retention time, m/z values, heights and areas.

3.2.2 Columns

For the analyses by HPLC-UV/Vis, a Kromasil C18 column (5 µm, 4.6 × 150 mm, 100 Å) from Kromasil and an XBridge BEH C18 column (5 µm, 4.6 × 150 mm, 130 Å) from Waters were used (**Table 3**). The columns were stored in 100% ACN when not in use. The Kromasil column was also utilised for the HPLC-MS analyses.

Table 3. List of columns used in this study, including particle structures and properties.

Column	Particle structure	pH range	Carbon load (%)	End-capped	Bonded phase density (µmol/m^2)	Surface area (m^2/g)
Kromasil 100-5-C18, 4.6x150mm		1.5-9.5	20.0	YES	3.5	320.0
XBridge C18, 130Å, 5 µm, 4.6x150mm		1-12	18.0	YES	3.1	185.0

3.2.3 Chromatographic Conditions

In all cases, the mobile phase consisted of an additive in water (A) and acetonitrile (B). All analyses were conducted as triplicate injections unless specifically stated otherwise.

In HPLC-UV/Vis, a broad gradient of 5-95% ACN over 15 minutes was utilized, followed by a 2-minute hold at 95% ACN, a quick decline back to initial conditions in 0.5 minutes, and a 4.5-minute re-equilibration step at initial conditions; a total run time of 22 minutes. The flow rate was set to 1 mL/min, and the column temperature was set to 25 °C. The injection volume was 3 µL for the small molecule amine and the peptide study, and 20 µL for the loadability study. The absorbance was measured at 254 nm, and measurements were conducted at a frequency of 2 Hz.

The same LC method was transferred to UHPLC with added mass detection.

3.3 Determination of Limit of Quantification

To ensure analysis on an analytical scale above the limit of quantification (LOQ), calibration curves for COU, PIN, CBZ, PPMA, and the three peptides were obtained. The peak area of the analytes was obtained for three different concentrations, namely 0.1, 1, and 3-10 mM. The calibration curves were conducted in triplicates. Normally, the standard error (SE) of the intercept in the calibration curve is utilised in the calculation of LOQ, but in this study, the SD of the intercept was employed (**Equation 1**).

$$LOQ = \frac{10 \cdot \sigma}{S}, \text{where } \sigma = \text{SD of intercept and } S = \text{slope of the curve} \qquad (1)$$

3.4 Determination of Overload Conditions

For the loadability study, overload conditions needed to be found for the analytical-scale columns and setup. 10 mM solutions of the respective peptide were analysed at 1, 5, 10, 15, 20, 25, 30, 35, 40, and 45 µL injection volumes. Visual evaluation of peak shapes and plotted plate number (N) to injection volumes was used to find the volume at which non-linear adsorption is disrupted.

3.5 Calculation of Tf

As the ChemStation software did not calculate Tf automatically, a Microsoft Excel spreadsheet template was assembled for the calculation of Tf (**Figure 1**). By inserting the coordinates of the chromatograms, Excel identified the peak maxima, calculated 5% of peak max height, identified the closest datapoints above and below the 5% threshold, connected these points, and calculated the intersection of these lines with the 5% threshold. Consequently, the width at 5% was obtained as well as the distance between the front of the peak to the line that represents peak max (tw) needed for the calculation of Tf (**Equation 2**) [12]:

$$T_f = \frac{w_{5\%}}{2 \cdot t_w} \tag{2}$$

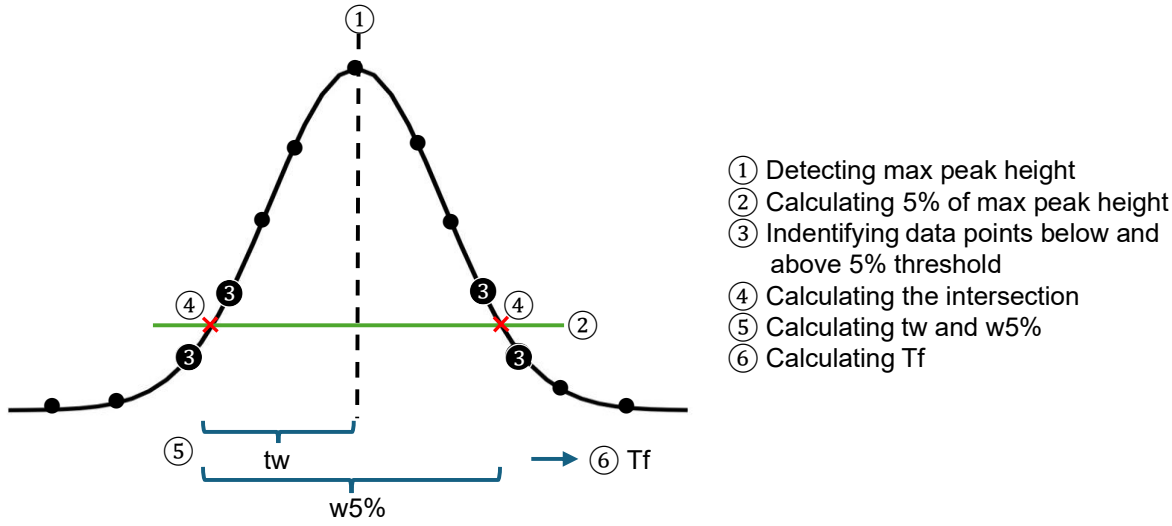


Figure 1. Explanatory visualization of the calculations conducted in the Excel spreadsheet for the calculation of Tf from the coordinates of a chromatogram.

Evaluation of the template can be found in Supplementary data (**Table S1**).

3.6 Statistical Analysis

Statistical analyses were performed in RStudio. Code development and troubleshooting were assisted by Copilot AI.

For the small molecule amine study, the effect of the additive, column, or their interaction on Tf of each basic analyte was tested by a two-way ANOVA on replicate values. Normality of residuals was assessed using Shapiro-Wilk's test. The homogeneity of variance was evaluated using Levene's test. Tukey post-hoc tests were performed when significant main factors were observed. In the case of a significant interaction factor, Tukey's tests were performed for each

column to identify differing additive pairs, and main factors were interpreted with caution. When normality assumptions were not fulfilled, non-parametric tests were performed, using the Kruskal-Wallis test to confirm additive effects and post-hoc Dunn's test to identify differing additive pairs.

For the set pH study, both parametric and non-parametric tests were performed for comparison. Paired t-tests and Wilcoxon signed-rank test were performed on Δk and ΔTf values to assess if pH causes a systematic shift (**Equation 3**). Δ values were calculated per replicate, then pooled across amines.

$$\Delta X = X(pH3) - X(pH2) \tag{3}$$

For the peptide study, one-way ANOVAs were performed per peptide x column to evaluate the additive effect on Tf for replicate values. In the case of a significant additive factor in the ANOVA, post-hoc Tukey tests were performed to identify differing additive pairs. For the charge dependence, linear regression analysis was performed for Tf vs Charge and Tf vs Charge density on replicate values. Linear regression with hydrophobicity as a covariate was also performed.

4 Results and discussions

4.1 Small Molecule Amine Study

The focus of the small amine molecule study is to evaluate the additive and column effects on the peak shape of basic analytes. Although Tf is the monitored peak shape metric, some fundamental observations about retention are also needed when evaluating additives, especially ion pairing ones. First, it is noticeable that the retention time for COU remains constant across all additives (**Figure 2**). A full series of chromatograms can be found in Appendix (**Figure A2**). This is reasonable as it is a neutral compound unaffected by pH and ion pairing [1]. The retention of COU is determined by the interaction with the stationary phase, and consequently, it will differ between columns of varying properties. COU is retained more on Kromasil as compared to XBridge possibly due to the higher carbon load of Kromasil (**Table 3**) allowing for more interactions with the C18 chains. The retention of the amines differs depending on the additive, but clear trends can be observed on the two columns. The retention order of the amines is always consistent; PIN < CBZ < PPMA, which is consistent with their increasing hydrophobicity (**Table 2**). Comparing the retention for each amine in the additives follows the same pattern; all amines exhibit the lowest retention in 10 mM AA, followed by PrA < FA < GA < MSA < ESA < DFA < DCA < TFA < TCA on Kromasil (**Figure A3 a**).

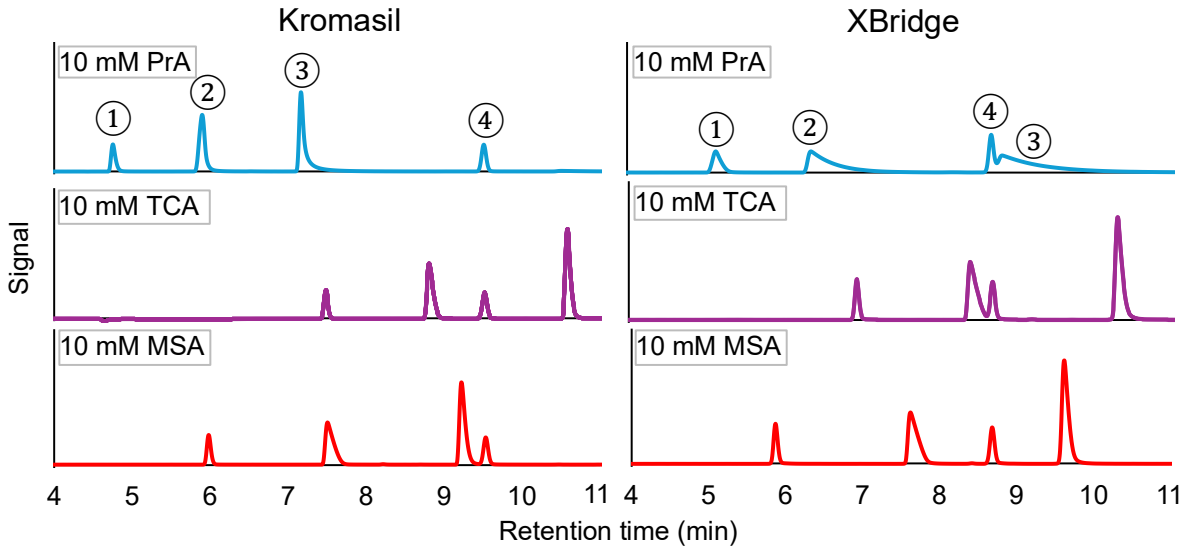


Figure 2. Example chromatograms of 1. PIN, 2. PPMA, 3. CBZ, and 4. COU in additives PrA, TCA, MSA on Kromasil and XBridge columns.

The retention behaviour of amines in the weak acids do not follow a simple trend based on pKa or hydrophobicity of the additives. Instead, a combination of pKa, hydrophobicity of the anions,

dissociation degree, solvation, and interaction possibilities all contribute to the observed order. One study claims that the lower hydration radius of AA compared to FA might be why it retains the basic analytes less [9]. PrA having one carbon atom longer chain makes it more hydrophobic which increases retention compared to AA. GA, although being quite polar, might show increased retention of the analytes due to its higher dissociation degree. Similarly, the sulfonic acids are the least hydrophobic additives with the lowest pKa-values, but they allow for strong ion pairing and efficient silanol protonation because of the low pH solution they create and thus increase retention of the basic analytes [4]. The halogenated acids show increased retention according to the halogen atom count and size, which increase the anion hydrophobicity and followingly the ion pair too [3]. The order differs on XBridge for the weak acids; PrA < AA < GA < FA but remains the same for the stronger ones (**Figure A3 b)**. This indicates that on the silica-based Kromasil column, secondary interactions play a larger role, while on the XBridge column, these interactions are reduced, leading to a different ordering [7].

While additive effects on the analytes' retention are quite systematic, poor peak shape does not necessarily coincide with high retention [7]. Retention and peak shape are governed by partially different mechanisms. Retention mainly reflects analyte partitioning between the mobile phase and the stationary phase, while tailing is influenced by the mixed mode interactions with the stationary phase [4].

The goal of the statistical analysis aimed at determining the relative performance of the additives with regard to Tf. The interaction factor Additive x Column is significant ($p<0.05$) for all amines (**Table A1**), which indicates that the additive effect on Tf depends on the column used. There is no evidence that variance differs across groups ($p>0.05$) (**Table A2**). Residuals are normally distributed ($p>0.05$) for PIN and CBZ, but not for PPMA and it does not improve by log-transformation (**Table A3**). Non-parametric tests performed for PPMA confirm a significant additive effect on both columns ($p<0.05$) (**Table A4**). Tukey-adjusted pairwise comparisons were performed per column as the interaction factor was significant in the ANOVA. They showed many significant pairs for all analytes across both columns, suggesting qualitative grouping, with regard to each analyte, of the additives rather than fine ranking (**Table A5**). The non-parametric Dunn's post-hoc test shows fewer significant pairs for PPMA, but they align with the results from the parametric Tukey and further supports grouping of additives. Exploratory plots of Tf for each analyte and additive (**Figure 3**) also support the grouping assigned from the post-hoc Tukey's. An important notation is the fact that Tf < 1.2

can be considered to have negligible effects on separation [12]. Therefore, even though statistical significance between additives is shown for COU, it is chromatographically of low importance. Tf = 1.2 serves as a threshold value in the grouping of additives to “High Tf”, “Low Tf”, and “Intermediate Tf” groups. Only additives that yielded Tf values, including SD error bars, below 1.2 fit into the “Low Tf” group. Additives end up in the “High Tf” group if they consistently show significantly worse tailing than all the other additives for the tested analyte.

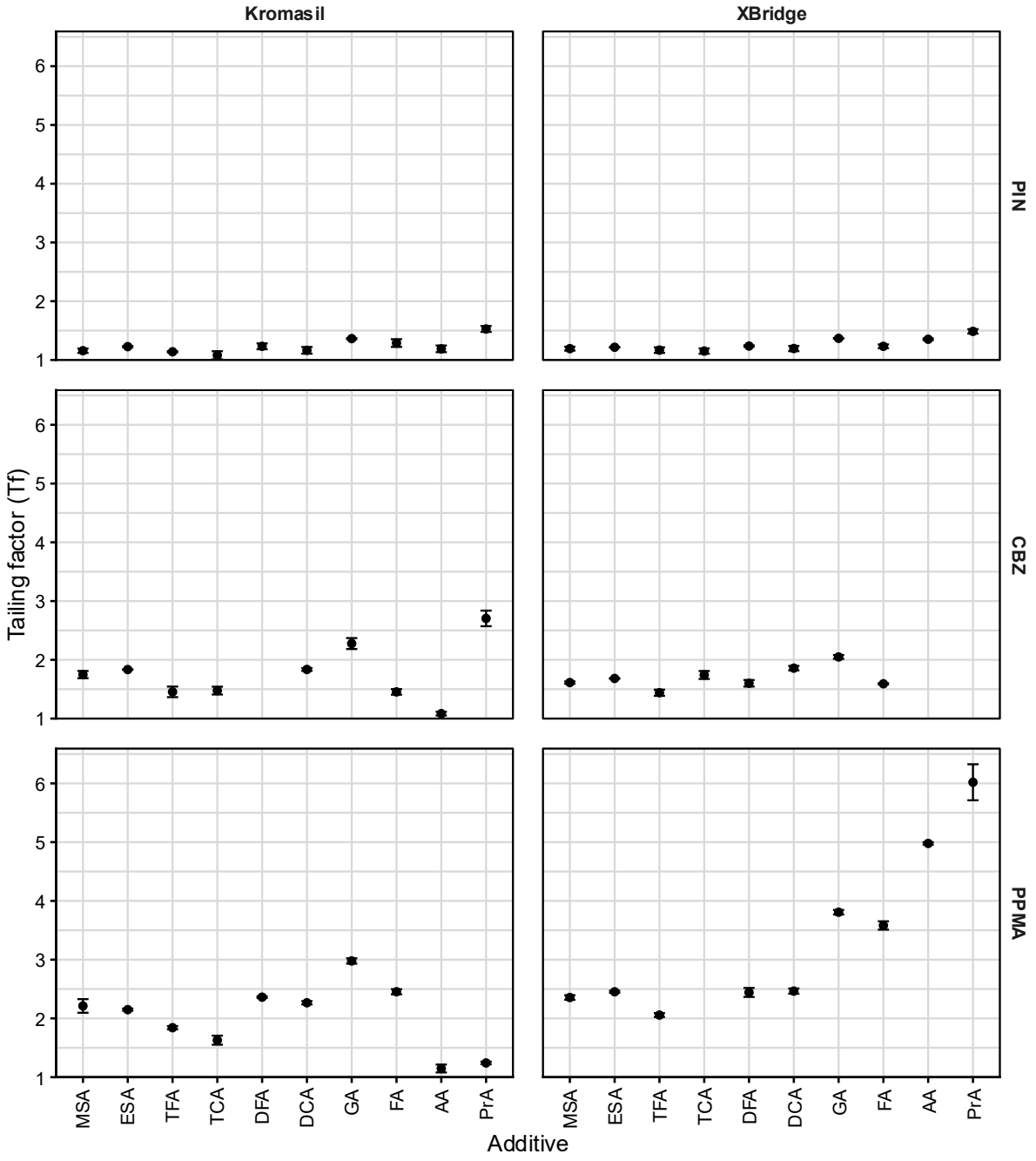


Figure 3. Exploratory plots of Tf for PIN (upper), CBZ (middle), and PPMA (lower) in all additives on both columns. Missing data points correspond to overlapping peaks where Tf could not be reliably determined.

Generally, Pra and GA show up in the “High Tf” group across analytes and columns, and they never occur in the “Low Tf” group (**Table A5**). In fact, no additive is consistently found in the “Low Tf” group, and most additives are subsequently placed in the “Intermediate Tf” group. However, TCA was classified as “Low Tf” on both columns for PIN, along with TFA and MSA on the Kromasil column. And for CBZ, AA fit into the “Low Tf” group on the Kromasil column. So, what might be the reason for PrA and GA to consistently show up in the “High Tf” group? Being weak acids, they have higher pKa, and thus higher pH as 10 mM solutions, and lower dissociation degree compared to the stronger acids (**Figure 4 a-c**). But these properties do FA and AA also hold [13]. PrA and GA are larger molecules compared to FA and AA (**Table 1** in methods). PrA having one carbon atom longer chain makes it slightly more hydrophobic (**Figure 4 d**) than FA and AA, and could possibly interact with the stationary phase surface, and change analyte partitioning kinetics [1]. GA on the other hand, has an additional OH group and is quite polar (**Figure 4 d**) with a higher topological surface area compared to the other weak acids (57.5 $Å^2$ compared to 37.3 $Å^2$). This OH group allows for one more hydrogen bond donor and acceptor which could may interact with residual silanols, potentially creating a different stationary phase surface. Another explanation for the poor performance of GA may be exclusion from the stationary phase because of its hydrophilicity, and therefore it does not participate in ion pairing close to the surface. FA and AA are small and have minimal secondary interactions, which could be why they perform similarly to strong acids in this study. Generally, FA and AA are reported to cause worse band broadening compared to strong ion pairing agents [4, 5].

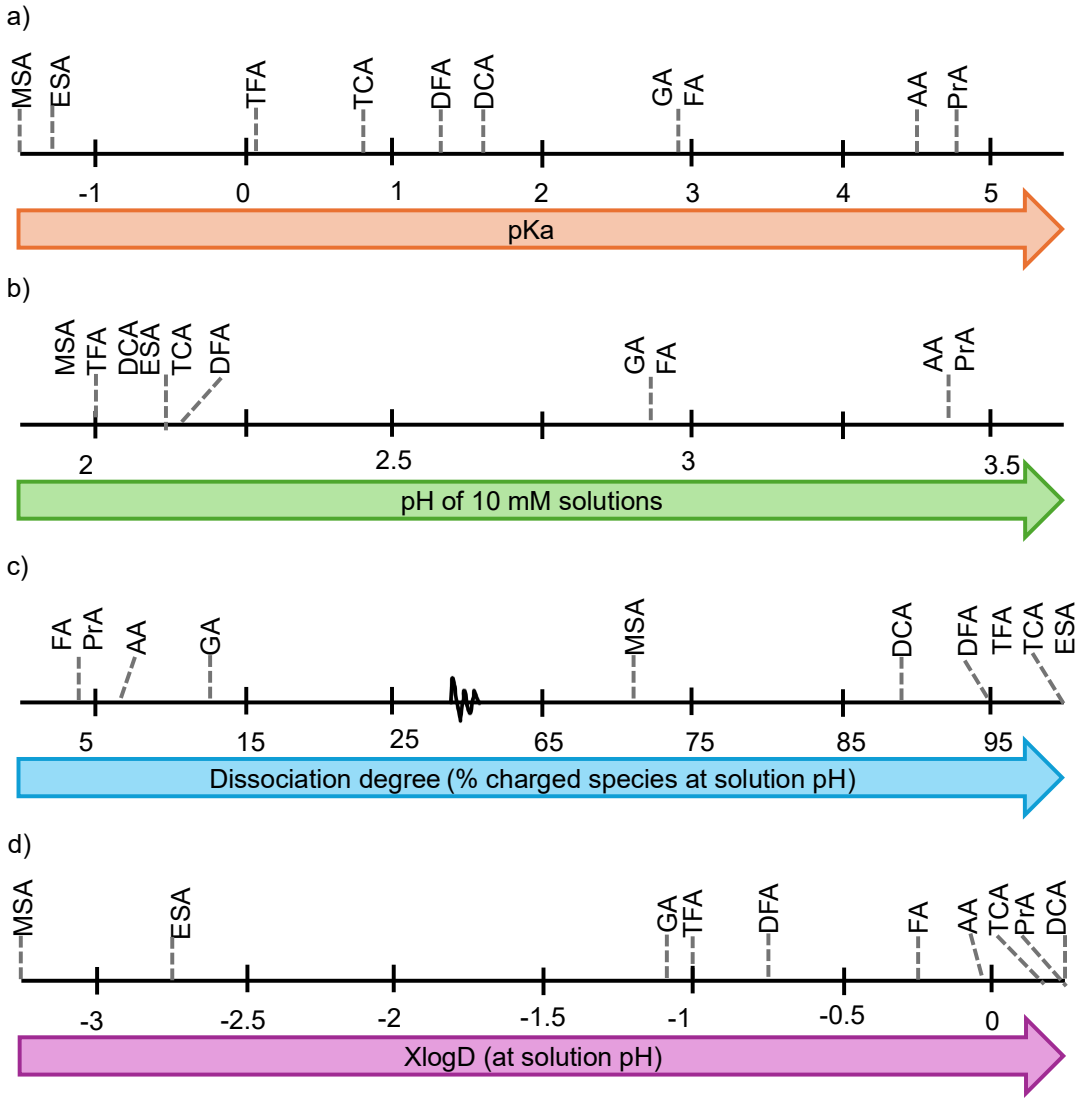


Figure 4. Schematic overview of additive properties, a) pKa values from lowest to highest, b) corresponding pH of the 10 mM additive solutions, c) corresponding dissociation degree as % of charged species at said solution pH, and d) estimated hydrophobicity XlogD at said solution pH.

Evident from the exploratory plot (**Figure 3**), PIN does in general exhibits less peak tailing than CBZ and PPMA. This could be assigned to their elution order, where PIN elutes earlier, and the mobile phase still contains sufficient concentration of the additive. As the gradient proceeds, and an additive is only added to the aqueous phase, an additive concentration gradient, along with a pH gradient is created. Much less additive is present in the system at the elution time of CBZ. On the other hand, ion pairing possibly strengthens with higher ACN content, as it lowers the dielectric constant of the mobile phase and improves the ion pair formation [1, 2, 6]. PPMA consistently exhibits severe peak tailing across all additives, which is likely due to overloading (**Figure A4, Table A6**). PPMA was analysed at a higher concentration (10 mM) than the other analytes (1 mM) to achieve comparable signal intensities for easier visualization of the peak

shapes. Overloading substantially affects peak shape and will be discussed in greater detail in a later chapter. Relative differences between the additives are still observed and this analyte is therefore still included in the present chapter.

Despite the widespread use of TFA for the separation of basic compounds, the present results do not support a universal superiority of TFA with regard to Tf. The other strong acids, including weak acids FA and AA yield comparable Tf values depending on the analyte and column. This supports previous observations that additive performance is highly system dependent and a result of the combined effects of analyte properties, stationary phase chemistry, and mobile phase composition [4, 13]. Multiple additives may be suitable alternatives to TFA for chromatographic optimization, and the choice of additive can therefore take MS compatibility, selectivity, or environmental considerations into account [4]. A short discussion of the environmental friendliness of the additives can be found in Supplementary Data (**Table S2**).

4.1.1 Set pH Study

To separate pH effects from ion pairing effects, the 10 mM solutions of TFA, TCA and MSA were brought from their natural pH around 2 to pH 3 by the addition of NaOH. The same trend can be observed; increased pH increases retention (**Figure A5**), which is to be expected. Now, at pHs as low as 2 and 3, we are well below two pH units from the pKa values of the amines, and >99.9 % of the analyte species are charged [14]. For additives, on the other hand, some uncharged species can still be present at pH 2. This could explain why retention is increased at pH 3. One-sample Wilcoxon tests of Δk show that the pH increase results in no significant retention changes for the analytes in the chosen additives (**Table A7**). The retention changes only correspond to a 1-3 % change (**Figure 5 a**). Wilcoxon tests of ΔTf shows a statistically significant increase in Tf by an increase in pH for all analytes and additives (**Table A7**), which corresponds to a 5-40 % change in Tf (**Figure 5 b**). The increase in Tf is likely associated with partial deprotonation of residual silanol groups at the higher pH, consequently allowing for more mixed-mode interactions. The peak shape deterioration is mostly pronounced for TFA. This indicates that Tf is sensitive to both pH and additive type, much more so than retention, and changing from pH 2 to 3 causes a systematic change.

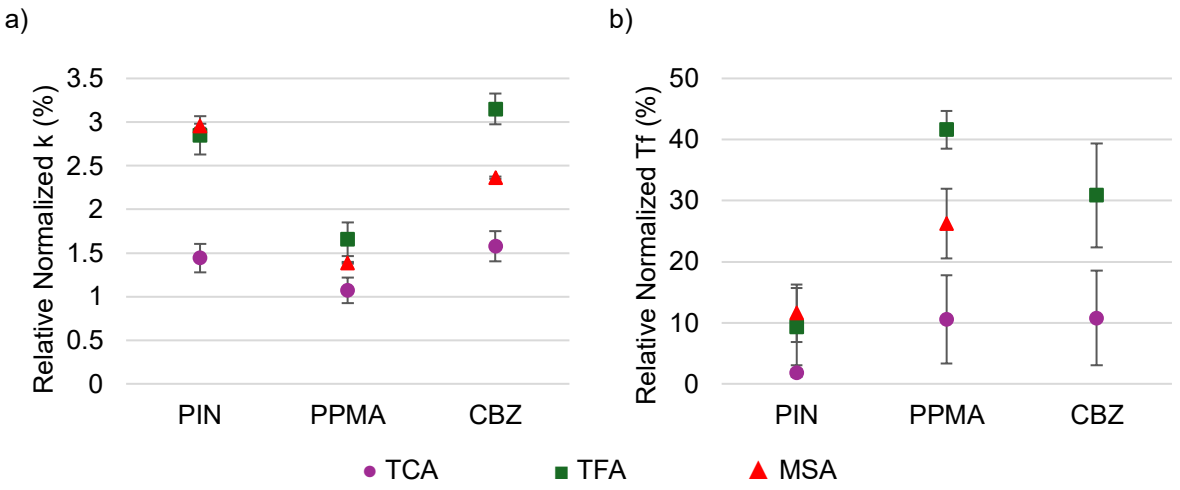


Figure 5. Effects of pH on k and Tf evaluated for the amines in TCA, TFA, and MSA; a) relative normalized k showing an 1-3 % increase in retention with increased pH, and b) relative normalized Tf showing 5-40 % increase in Tf with increased pH. The data point for CBZ in MSA in b) is missing due to overlapping peaks where Tf could not be reliably determined.

4.2 Peptide Study

To evaluate whether additive effects observed for small molecule amines translate to peptides, a set of Fmoc-protected peptides with increasing positive charge, i.e., increasing number of lysines, was investigated under analytical conditions. TFA, TCA, DFA, AA, and PrA were selected based on their performance in the amine study and interest in identifying alternatives to TFA. Peak shape was assessed using Tf here as well. Under the tested conditions, the elution order of peptides is Fmoc-KKK < Fmoc-KK < Fmoc-AK (**Figure 6**, **Figure A6**), with the triply charged peptide eluting first. Despite its highest estimated hydrophobicity (**Table 2**) as a neutral molecule, the increased charge reduces the effective retention under acidic conditions.

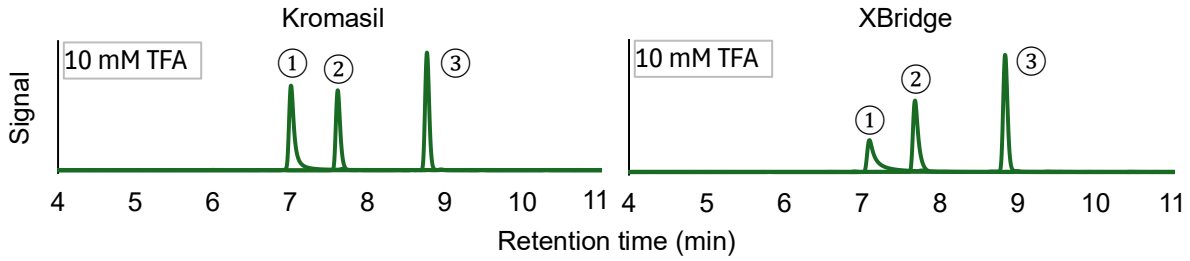


Figure 6. Overlaid chromatograms of the peptides in TFA on Kromasil and XBridge. Elution order: 1. Fmoc-KKK, 2. Fmoc-KK, and 3. Fmoc-AK).

One-way ANOVA performed separately for each peptide and column shows significant additive effects ($p<0.05$) (**Table A8**). Post-hoc Tukey tests result in significant pairwise comparisons mostly between weak (AA and PrA) and strong (TFA, TCA, and DFA) acids. For

Fmoc-KKK and Fmoc-KK on the Kromasil column, AA and PrA yield high Tf values, whereas the halogenated acids yield lower ones. In contrast, Fmoc-AK exhibits Tf values <1.2 in all additives, indicating negligible tailing under all tested conditions.

Visual evaluation of Tf as a function of peptide charge reveals a consistent increase in tailing with increasing charge (Fmoc-AK < Fmoc-KK < Fmoc-KKK). This trend is observed on both columns and for all additives tested (**Figure 7 a**, **Figure A7 a**). Linear regression analysis confirms this increase in Tf with peptide charge (**Table A9**). As this trend is consistent across additives, charge dependence seems to be an analyte-controlled property, while additive type impacts the magnitude of this effect. The highest charged peptide Fmoc-KKK is consistently the most problematic analyte, particularly on the XBridge column. The larger the molecule, the slower the diffusion, which leads to extra broadening [4], and no additive can mitigate the tailing to acceptable standards (Tf<1.2). Because peptide charge is closely related to both molecular weight (Mw) and hydrophobicity, these factors were evaluated as potential contributors to the observed trend. Tf as a function of charge density retains the same trend (**Figure 7 b, Figure A7 b**), and so do linear models including XlogP as a covariate. These results indicate that the increase in Tf with peptide charge cannot be attributed only to Mw or XlogP.

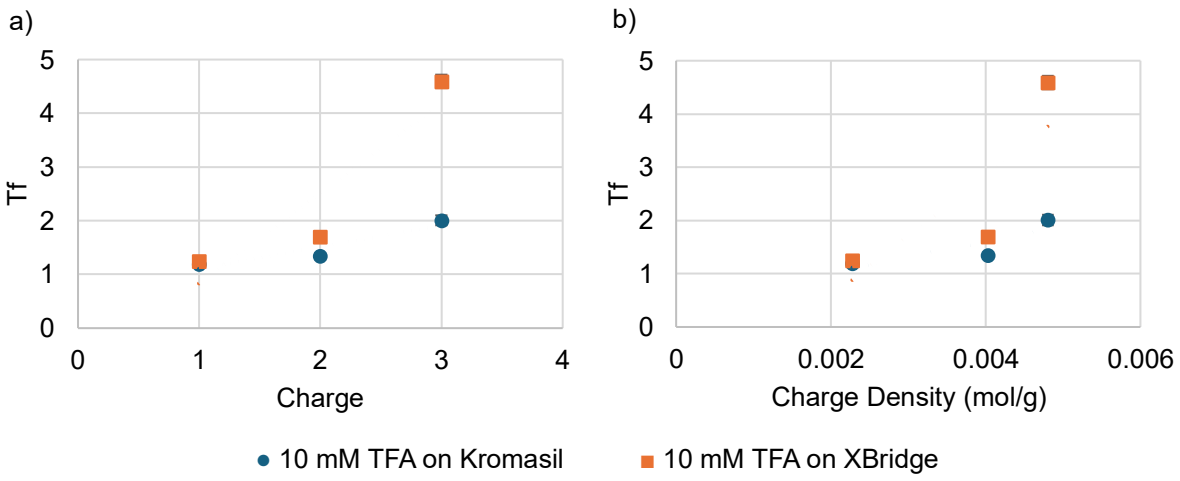


Figure 7. Representative plot of Tf of peptides in TFA on Kromasil and XBridge; a) Tf vs Charge, and b) Tf vs Charge density. Error bars (±SD) are included and are in some cases too small to be visible due to the scale of the plot.

4.2.1 Concentration Dependence in Analytical Conditions

Having established that peptide peak tailing under analytical conditions is governed by analyte charge and additive type at a fixed additive concentration, the effect of increasing additive

concentration was subsequently investigated. For all additives, positive slopes are observed (**Figure 8**, **Figure A8**), which means the charge dependence is preserved in all additive concentrations, further reinforcing that charge control intrinsic tailing. For strong acids, the lines shift downward with increasing additive concentration, and the peak shape is improved for the peptides. This effect is stronger the higher the peptide's charge is. This has been shown previously for other peptides with other ion pairing additives [8]. A possible explanation for the improved peak shape is that the elution profile of a peptide at each additive concentration consists of the peptide and the ion pair; as the additive concentration increases, and the distribution shifts towards the ion pair allowing for more uniform retention [6]. Additionally, pH decreases with increasing additive concentration, further protonating residual silanol groups (**Table A10**). The greatest improvement is seen for the highest charged peptide Fmoc-KKK, as the more charges there is to form ion pairs with, the stronger the effect of increased additive concentration [4]. Tailing is much more severe on the XBridge column with 10 mM additive solutions, but the increase in additive concentration improves Tf to values that are comparable between the two columns.

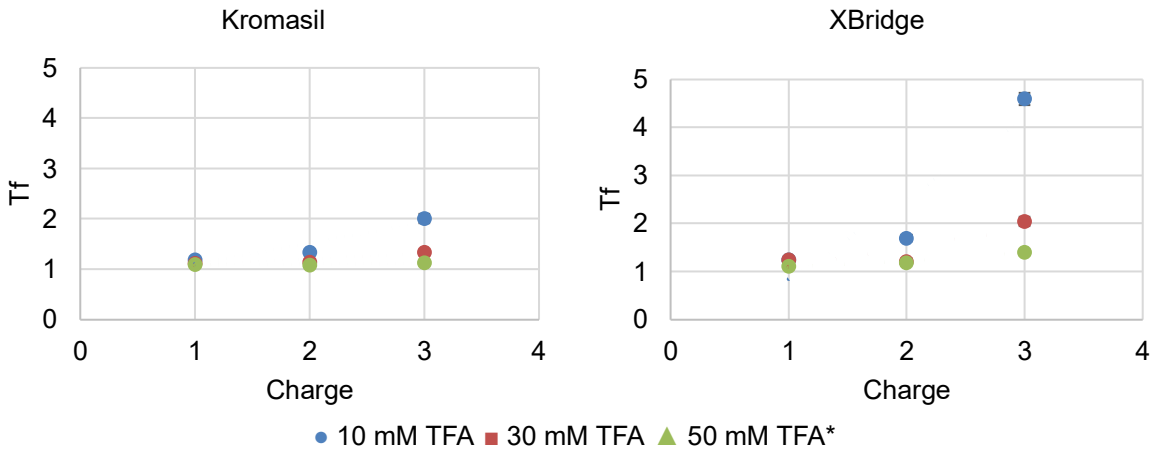


Figure 8. Charge dependence in different concentrations of TFA; a) Tf vs Charge on the Kromasil column, and b) Tf vs Charge on the XBridge column. Error bars (±SD) are included and are in some cases too small to be visible due to the scale of the plot.

*Analyses in 50 mM solutions were only run once to limit the time of harsh conditions for the column.

For weak acids the lines shift slightly upwards with increasing additive concentration (**Figure A8**). Increasing the additive concentration concurrently decreases pH and increases the ionic strength. As the weaker acids form weak ion pairs-, or only shield the protonated peptides, charge driven tailing persists. Additionally, the higher concentration of additive anions can increase heterogeneity of the surface interactions, as the additive anions become embedded in

the ACN wetted layer on the stationary phase surface and promote slower desorption [1]. Another possible explanation is the fact that the analysed peptides are TFA-salts. Residual TFA strongly associates with the basic sites, and increasing the weak acid additive concentration in the mobile phase does not efficiently displace all TFA anions and rather leads to an increase in mixed ion-pairs [11].

4.2.2 Concentration Dependence in Overload Conditions

Preparative conditions can be mimicked on an analytical column by deliberately overloading the column. Under high sample loads, linear absorption is exceeded and non-linear adsorption occurs. The capacity of the column is exceeded, and peak shapes deteriorate and typically present as right-angled triangular profiles [2]. Definitive overload was found at injection volumes of 20 µL based on visual evaluation of the peak shape and N (**Figure A9, Figure A10 a**). Although detector saturation occurred at lower injection volumes (**Figure A10 b**), peak width at half height (w50%) was used as the primary metric of overload behaviour, as it reflects column loading and mass-transfer effects rather than detector response.

Under overload conditions, increasing the concentration of ion-pairing additives (TFA and TCA) in a way that differs from analytical conditions. For TFA, increasing the concentration from 10 to 50 mM results in a gradual reduction of w50% for all peptides (**Figure 9 a**), indicating improved load robustness. In contrast, TCA produces lower w50% already at 10 mM but further increases in additive concentration result only in minor changes and, for the highly charged peptide Fmoc-KKK, in a slight increase in peak width at the highest concentration tested (**Figure 9 b**). Previous studies have found that peak shape stabilises at an additive concentration corresponding to the analyte concentration multiplied by the analyte charge [6], but such trends are not seen in this study. Instead, the peak broadening decreases gradually, consistent with the gradual suppression of overload-interactions such as analyte-analyte repulsions [15].

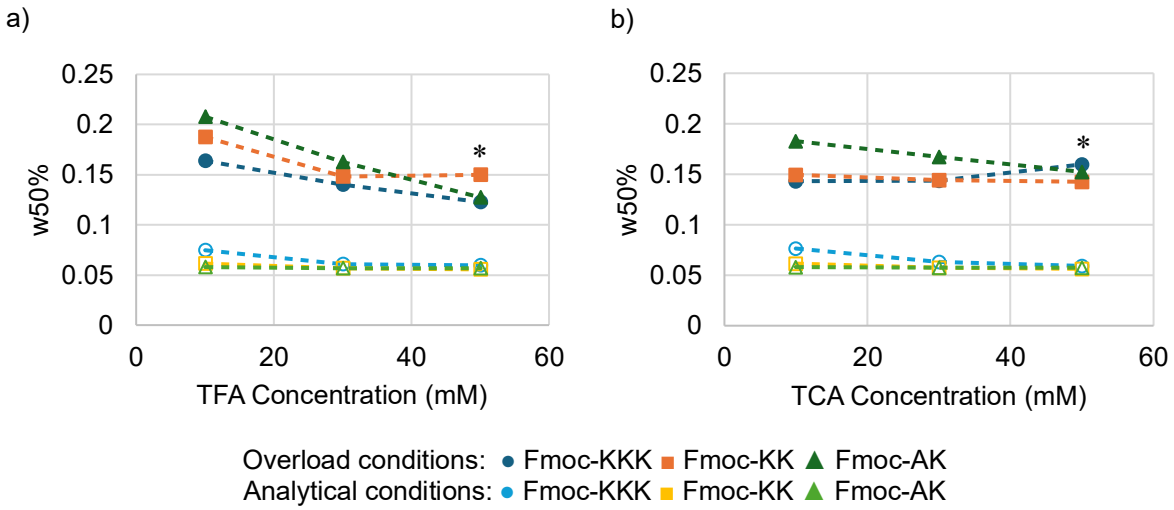


Figure 9. w50% of the overloaded peptides on the Kromasil column in a) TFA, and b) TCA, with supplementary comparison to w50% of the peptides in analytical conditions. Error bars (±SD) are included and are in some cases too small to be visible due to the scale of the plot.

*Analyses in 50 mM solutions were only run once to limit the time of harsh conditions for the column.

Notably, under overload conditions at low additive concentration, the singly charged peptide Fmoc-AK exhibits the highest w50% values in both additives. This behaviour contrasts with analytical Tf trends and indicates that overload is primarily governed by capacity-limited absorption rather than mixed interactions with residual silanols [15]. This observation highlights that analytical peak shape does not necessarily predict overload behaviour. From a preparative perspective, loadability is determined not by peak symmetry but by the ability of a chromatographic system to tolerate increased sample mass without excessive peak broadening. In this context, TCA achieves improved peak widths at lower additive concentrations, while TFA provides a more consistent improvement of peak widths across the concentration range.

4.3 Evaluation of Signal Suppression in HPLC-MS

As mentioned earlier, MS suitability is an important aspect in the choice of an additive and creates an additional criterion in the search for alternative additives. Evaluation of signal suppression by ion pairing additives (TFA and TCA) demonstrates that they affect MS detectability in different ways. Summed EIC areas of all charge species (**Figure 10 a**) reveal stronger suppression of analyte ionization efficiency in the presence of TFA compared to TCA for all peptides studied. The total area is around five times higher for TCA than for TFA. This observation agrees with previous studies indicating that TFA acts as a strong ion suppressor due to the strong ion pairs formed and the reduced droplet evaporation efficiency [4].

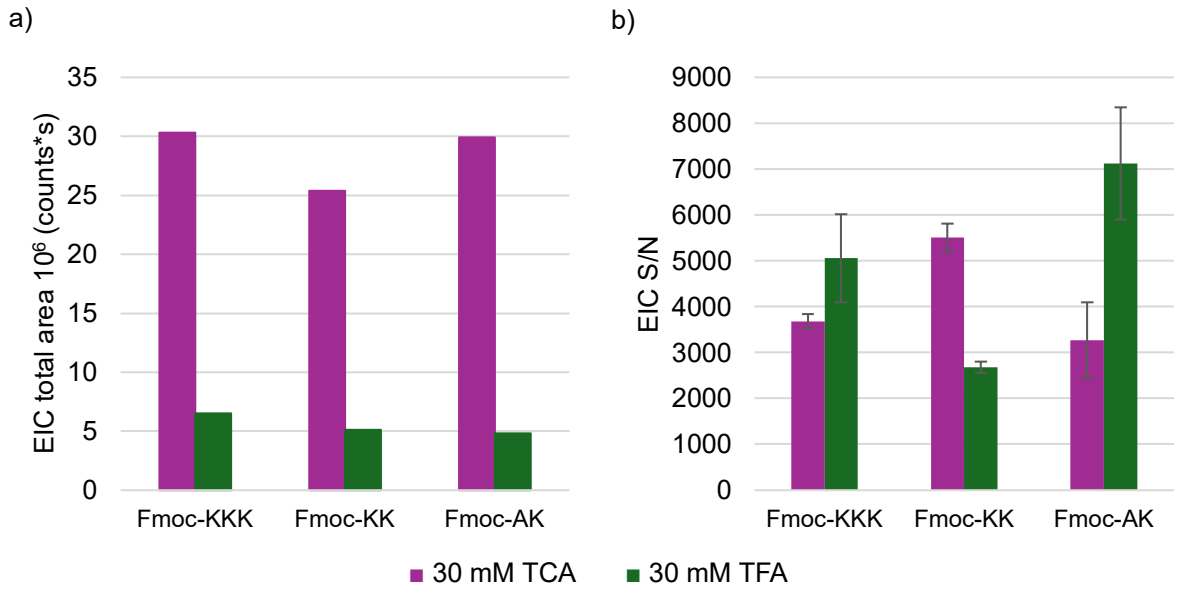


Figure 10. Bar charts explaining signal suppression; a) EIC total area of all charge species, and b) EIC S/N for only the most prominent charge species (+1).

EIC total area reflects the analyte ionization and transmission efficiency, but not the usable detectability. TCA has four times higher average noise than TFA, and when evaluating signal-to-noise (S/N) ratios of the dominant charge species (**Figure 10 b**), the relative performance of the additives becomes analyte dependent. Analyte dependence on signal suppression has previously been reported [3, 5, 13]. Both additives show a preference for the +1-charge state of the studied peptides, also in agreement with previous studies [5]. While TFA consistently produces reduced EIC total area, it produces higher S/N for Fmoc-KKK and Fmoc-AK. This is likely due to lower chemical noise compared to TCA. Higher purity TCA may mitigate this problem, but a previous study that evaluated signal suppression based on TIC S/N reported higher signal suppression by TCA, despite having MS-grade chemicals [4]. A possible explanation for the higher S/N for Fmoc-KK is the higher preference for the singly charged species in TCA. Its S/N is almost three times higher than that of the +2 species. For TFA the preference for the +1 species is barely double (**Figure A11**).

5 Conclusions

In conclusion, based on chromatographic performance in terms of Tf there seems to be multiple alternatives to TFA, all achieving comparable Tf values depending on the analyte and column. PrA and GA were shown to be poorly performing additives, despite indications of PrA being as useful as FA in previous studies. The additive effect on Tf for peptides was shown to be charge dependent, with the additive type affecting the magnitude of the tailing. Increased concentration of strong ion pair agents improved the peak shape, both in analytical (Tf) and overload conditions (w50%). Lastly, regarding MS suitability, TCA can pose as an alternative to TFA if higher noise is not a problem in a certain separation situation.

6 Future Aspects

Firstly, future studies should consider adding the additives into both the aqueous and the organic phase, as this would represent real life separations better and avoid additive concentration gradients during gradient runs. pH is not determined in the organic phase anyway, so this would not alleviate the problem of potential pH gradients along the gradient run. Secondly, analysing the peptides in all the same additives as the amines would allow for a more comprehensive comparison of how the peak shape of peptides differ from that of small molecule amines when changing the additive. This could aid in the selection of an additive for a separation with known compounds. Thirdly, MSA and ESA show to be promising in terms of chromatographic performance, but their MS compatibility need to be evaluated. Previous studies have presented MSA as not being volatile [4], but volatility assessment with low-temperature evaporative light-scattering detection should be performed at least for ESA, as it has a lower boiling point than MSA. Lastly, during the literature research for this project, it seems that the ion pairing capabilities for additives lack experimental support (except in the case of TFA), and it would be valuable to evaluate, especially for the weaker acids, where some state they are merely shielding and change the environment close to the analytes.

7 References

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8 Supplementary Data

8.1 S1 Excel Template Comparison to OpenLab and Manual Tf Calculation

To ensure the Excel template of Tf-calculation yields accurate-enough values, sample "X" was analysed on the Agilent 1290 Infinity II HPLC-MS system, where OpenLab calculates the Tf automatically. The chromatogram was also printed out for manual calculation of Tf with a pen and a ruler. Tf calculated using the Excel template shows good agreement with values obtained from Openlab (**Table S1**). Minor deviations are systematic and acceptable since the study focuses on comparison between additives. While replicate measurements would provide more robust validation, the observed level of agreement between the obtained values is sufficient for the intended analysis.

Table S1. Comparison of Tf values obtained using OpenLab, and Excel-based calculation template, and manual pen and ruler calculation.

	Tf		
Analyte	**OpenLab**	**Excel template**	**Pen and ruler**
A	0.88	0.87	0.95
B	1.31	1.33	1.34
C	1.12	1.14	1.16
D	1.13	1.15	1.20

It is worth mentioning that there are some limitations to the Excel template. For example, the frequency by which data were measured will impact the identified peak maxima as well as the calculated intersections (i.e. on the slope of the lines between the two points just above and below the 5% threshold). The template does not perform any integration, which means the "baseline" will always be 0 according to Excel, while that might not be the reality. Additionally, no curve fitting was performed, and followingly the true peak maxima might be different from that identified by this template. Despite these limitations, the Excel template for Tf calculation is accurate enough for the aims of this project, as validated above.

8.2 S2 Environmental Aspect of Alternative Additives

As mentioned in the introduction, an important aspect in the search for alternative additives is their environmental, health, and safety issues. Today, there are many ways to assess this by the use of various calculators such as NEMI, Analytical Eco-Scale, and AGREE; the latter of which takes the 12 principles of green analytical chemistry into account [16]. An evaluation of an HPLC method similar to the one employed in this study yielded an AGREE score of 0.43 [17], which is below the acceptable threshold of 0.5 [18]. They found that one of the major causes to the deviation from green standards was due to hazardous reagent consumption (ACN) and waste generation [17]. ACN is not a green solvent, as it is flammable, volatile, and harmful. When used with a conventional LC column it can generate up to 1.5 L of waste in a single day [19]. Even if additives are used, ACN waste is already hazardous. Much less additive is needed in comparison to ACN, so comparing the AGREE scores; additives contribute little to the final score of the method. Although AGREE struggle to weigh small additives, TFA for instance, bears real severe factors regarding environmental friendliness. It is non-biodegradable, stable in water, and bio-accumulative, which is why it has ended up on the EU REACH candidate list of phase-out substances [10]. Consequently, it is of value to compare the additives, in terms of their environmental, health, and safety issues. A qualitative sustainability comparison was performed based on SDS hazard (H) phrases (**Table S2**). Halogenated additives are considered less sustainable due to persistence concerns, while non-halogenated additives generally show lower environmental concern. Among tested additives in this study, MSA, ESA, AA, PrA, and GA may represent comparatively more sustainable alternatives, although full life-cycle analyses or more intricate assessments were outside the scope of this work.

Table S 2. Qualitative grouping of additives based on their SDS H-phrases regarding sustainability concerns.

Category	Additives	Comment
Higher concern	TFA, DFA, TCA, DCA	Halogenated and/ or toxic
Moderate concern	FA	Corrosive but biodegradable
Lower concern	AA, MSA, ESA, PrA, GA	Lower toxicity, non-halogenated

9 Appendix

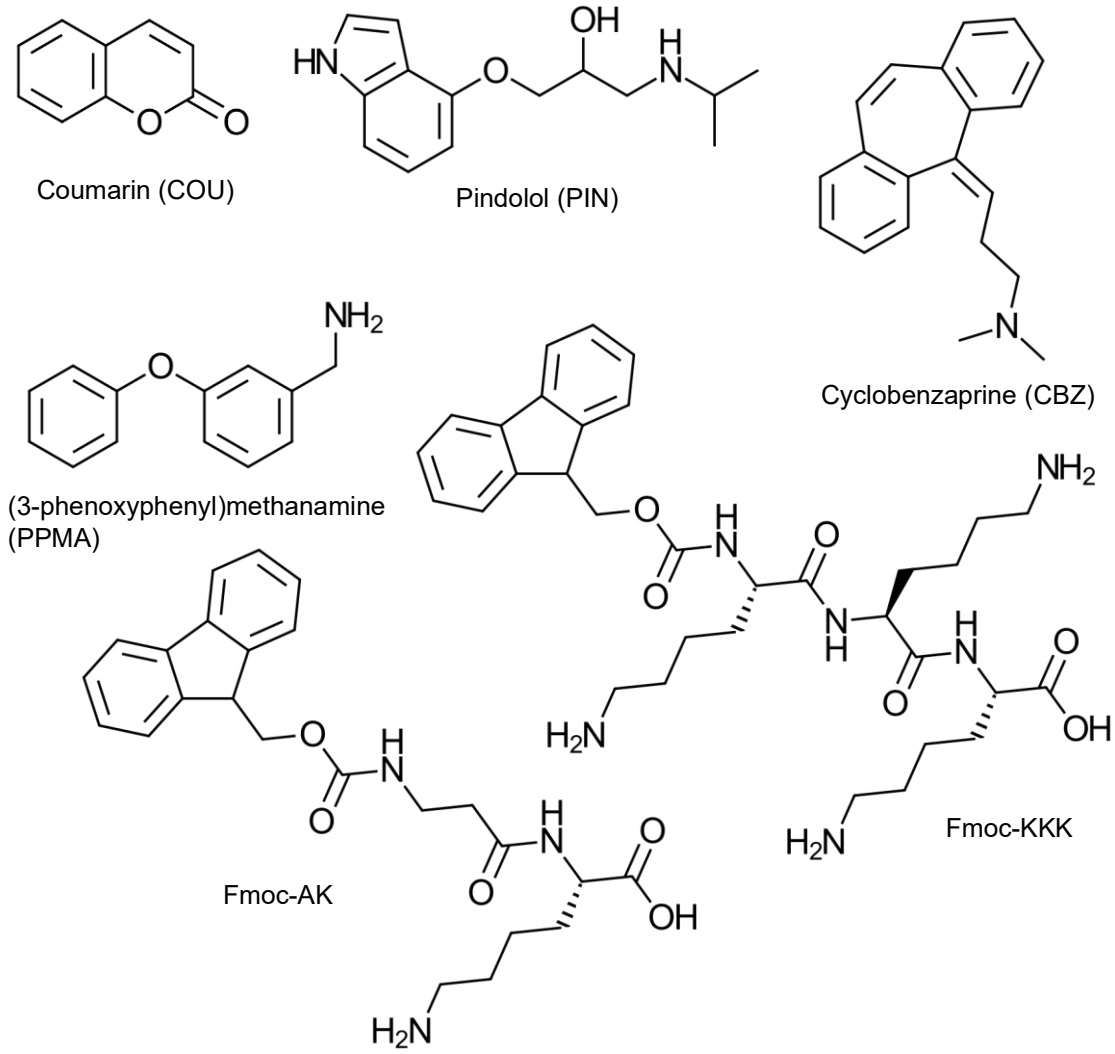


Figure A1. Structures of analytes. Fmoc-KK is absent as it resembles Fmoc-KKK but with one less lysine entity.

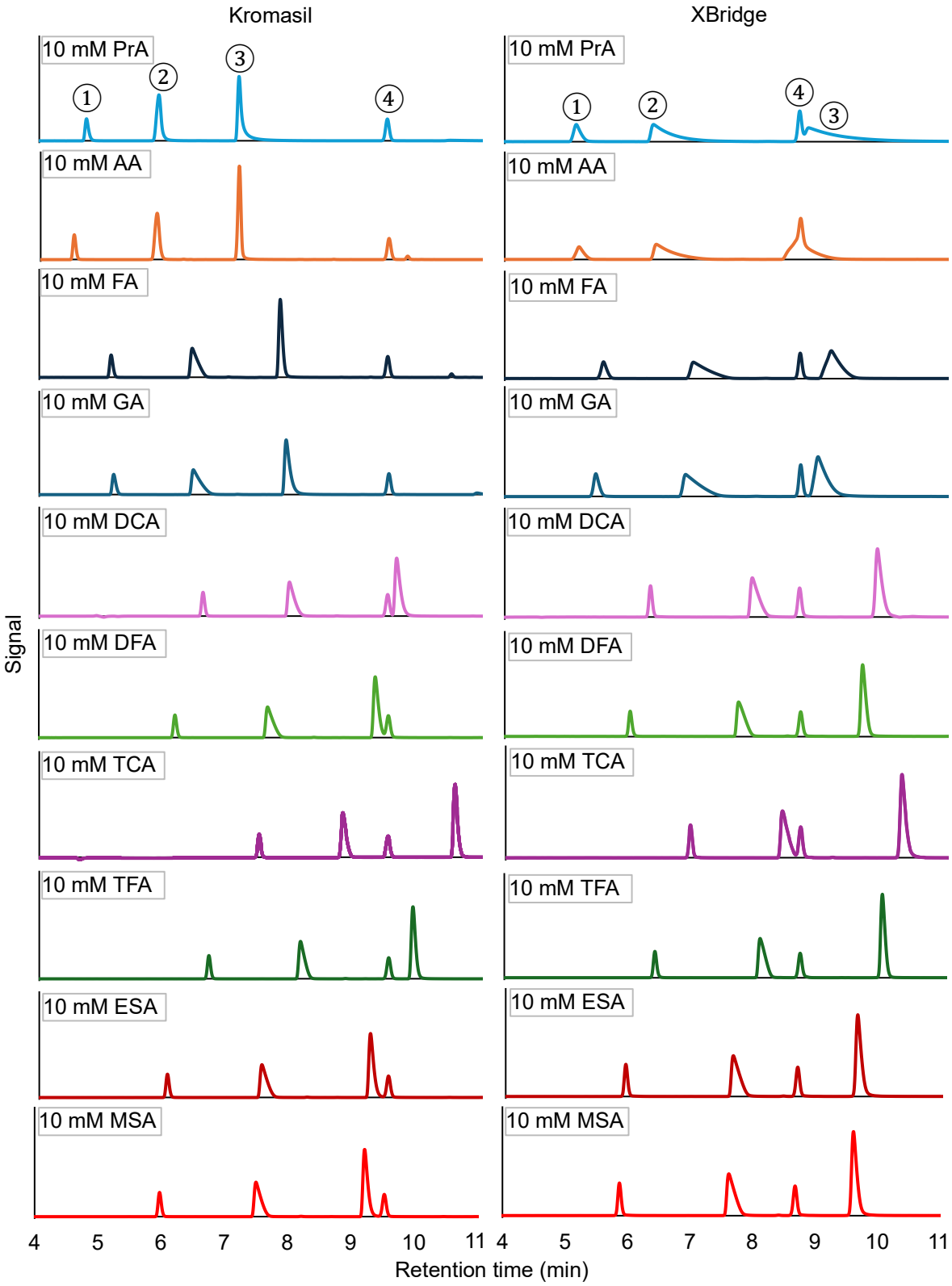


Figure A2. Full series of chromatograms of the analytes 1. PIN, 2. PPMA, 3. CBZ, and 4. COU in all the additives on the two columns.

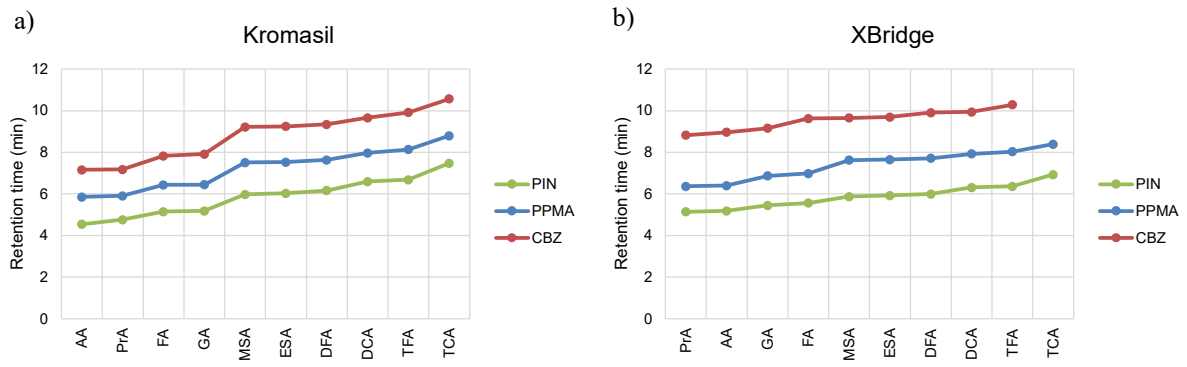


Figure A3. Visually simplified retention order of amines in the different additives on Kromasil and XBridge. a) showing the order AA<PrA<FA<GA<MSA<ESA<DFA<DCA<TFA<TCA on Kromasil and b) showing the order PrA<AA<GA<FA<MSA<ESA<DFA<DCA<TFA<TCA on XBridge.

Table A1. Values from the two-way ANOVA for the amines.

	Two-Way ANOVA			
Analyte	**Source**	**df**	**F-value**	**p-value**
PIN	Additive	9	48.6	***
	Column	1	4.9	*
	Additive x column	9	3.5	**
	Residuals	40		
CBZ	Additive	9	195.4	***
	Column	1	0.6	0.4
	Additive x column	6	12.9	***
	Residuals	34		
PPMA	Additive	9	287.7	***
	Column	1	2949.4	***
	Additive x column	8	625.3	***
	Residuals	38		

Significance codes: *0.05, **0.01, ***0.001

Table A2. Values from Levene's test of homogeneity for the amines.

	Levene's test		
Analyte	**df**	**F-value**	**p-value**
PIN	19	0.57	0.91
CBZ	16	0.47	0.95
PPMA	18	1.7	0.08

Table A3. Values from Shapiro-Wilk's normality of residuals test for the amines.

	Shapiro-Wilk's test	
Analyte	**W**	**p-value**
PIN	0.97	0.31
CBZ	0.98	0.45
PPMA	0.79	***
PPMA (log transformed)	0.95	*

Significance codes: *0.05, **0.01, ***0.001

Table A4. Values from Kruskal-Walli's non-parametric test for PPMA.

	Kruskal-Wallis' test		
Analyte	χ^2	**df**	**p-value**
Kromasil	28.4	9	***
XBridge	24.7	8	**

Significance codes: *0.05, **0.01, ***0.001

Table A5. Grouping of additives into low, intermediate and high Tf based on post-hoc tests.

		Post-hoc tests resulting grouping			
Analyte	**Column**	**Low Tf**	**Intermediate Tf**	**High Tf**	**Missing Data**
PIN	Kromasil	TCA, TFA, MSA	DFA, ESA, DCA, AA, FA, GA	PrA	
	XBridge	TCA	TFA, MSA, DCA, ESA, FA, DFA	PrA, GA, AA	
CBZ	Kromasil	AA	FA, TFA, TCA, MSA, ESA, DCA	PrA, GA	DFA
	XBridge		FA, DFA, MSA, ESA, TCA, TFA, DCA	GA	AA, PrA
PPMA	Kromasil		AA, PrA, TFA, ESA, MSA, DCA, DFA, FA	GA	TCA
	XBridge		TFA, MSA, DFA, ESA, DCA	PrA, AA, GA, FA	TCA

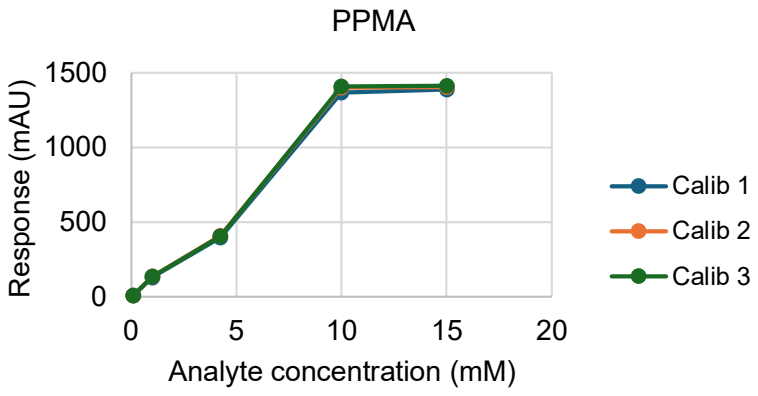


Figure A4. Potential overload at 10 mM PPMA as shown from the calibration curve (0.1-10 mM analyte).

Table A6. LOQ-values for all analytes analysed. LOQ is assumed to be similar in all additives and on both columns.

Analyte	LOQ (mM)
COU	0.06
PIN	0.08
CBZ	0.04
PPMA	0.1
Fmoc-KKK	0.09
Fmoc-KK	0.005
Fmoc-AK	0.9

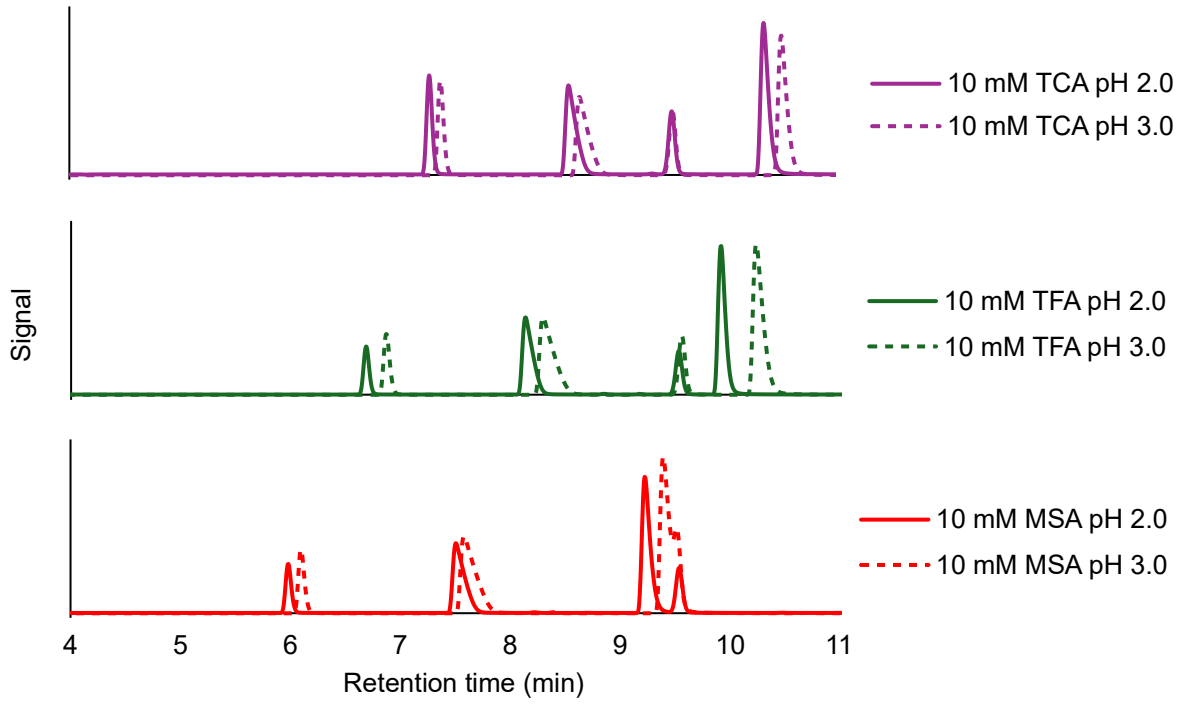


Figure A5. Full series of chromatograms for the set pH study, showing increasing retention for the analytes at increased pH.

Table A7. Values from the parametric and non-parametric tests of pH effect on k and Tf in pH 3.

Parameter	Additive	Test	df	Statistic	p-value
Δk	TCA	t-test	5	t=0.99	0.36
		Wilcoxon		V=15	0.44
	TFA	t-test	5	t=1.01	0.36
		Wilcoxon		V=15	0.44
	MSA	t-test	8	t=2.7	*
		Wilcoxon		V=39	0.055
ΔTf	TCA	t-test	8	t=3.2	*
		Wilcoxon		V=45	**
	TFA	t-test	8	t=4.5	**
		Wilcoxon		V=45	**
	MSA	t-test	5	t=3.4	*
		Wilcoxon		V=21	*

Significance codes: *0.05, **0.01, ***0.001

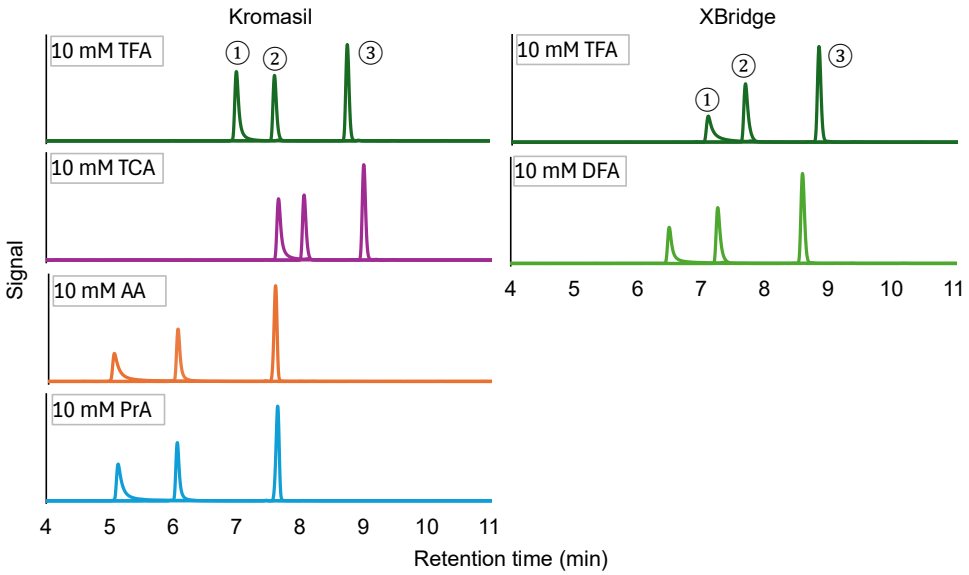


Figure A6. Full series of chromatograms for the peptides 1. Fmoc-KKK, 2. Fmoc-KK, and 3. Fmoc-AK, in all additives that were tested on the two columns.

Table A8. Values from one-way ANOVA of additive effect on Tf of the peptides on both columns.

		One-way ANOVA			
Column	**Analyte**	**Source**	**df**	**F-value**	**p-value**
Kromasil	Fmoc-KKK	Additive	3	433.7	***
		Residuals	8		
	Fmoc-KK	Additive	3	38.5	***
		Residuals	8		
	Fmoc-AK	Additive	3	69.9	***
		Residuals	8		
XBridge	Fmoc-KKK	Additive	1	532.9	***
		Residuals	4		
	Fmoc-KK	Additive	1	1.1	0.36
		Residuals	4		
	Fmoc-AK	Additive	1	1.5	0.29
		Residuals	4		

Significance codes: *0.05, **0.01, ***0.001

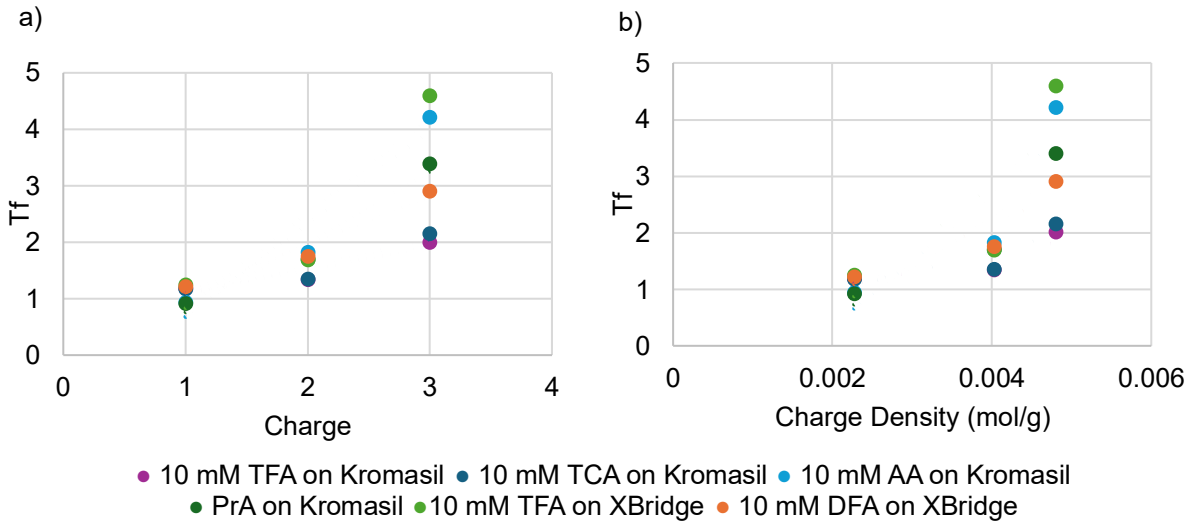
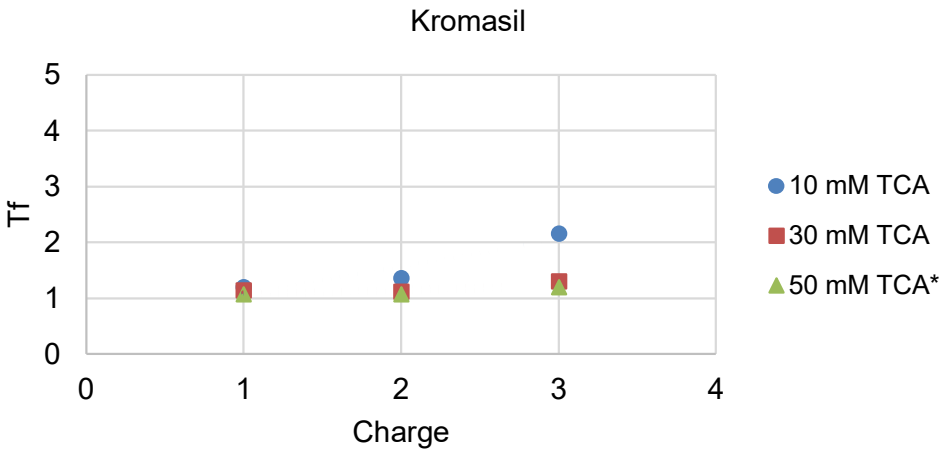


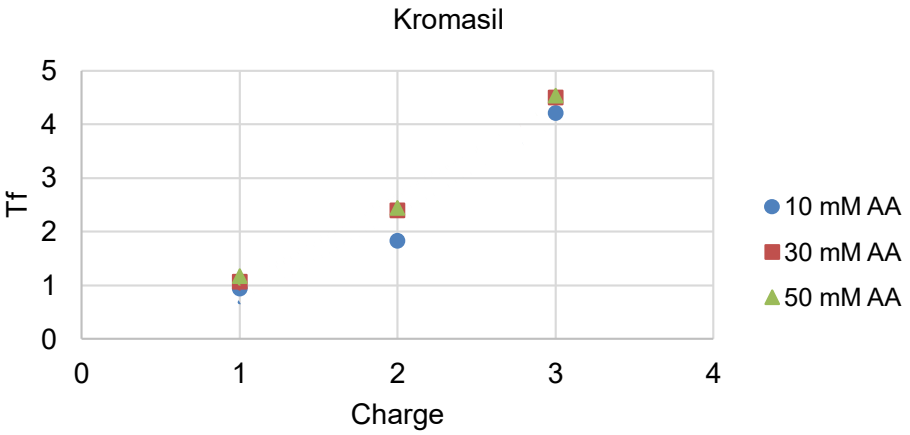
Figure A7. a) Tf vs Charge and b) Tf vs Charge density for the peptides in all additives tested on the two columns.

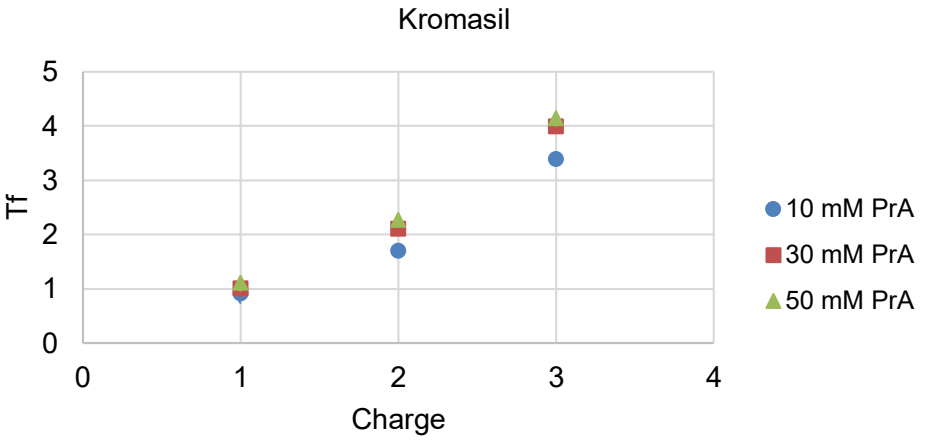
Table A9. Representative table of values for linear regression of Tf~Charge and Tf~Charge density of the peptides on both columns. Notably, this is only for TFA.

Linear regression (TFA)						
Model	**Column**	**Parameter**	**Estimate**	**SE**	**t-value**	**p-value**
Tf~Charge	Kromasil	(Intercept)	0.70	0.13	5.3	**
		Charge	0.41	0.061	6.7	***
	XBridge	(Intercept)	-0.86	0.58	-1.4	0.19
		Charge	1.7	0.27	6.2	***
Tf~Charge density	Kromasil	(Intercept)	0.47	0.27	1.7	0.13
		Charge	282.1	70.7	3.9	**
	XBridge	(Intercept)	-1.7	1.2	-1.4	0.19
		Charge	1142.9	309.1	3.7	**

Significance codes: *0.05, **0.01, ***0.001


Kromasil
Tf
Charge
0
1
2
3
4
5
10 mM TCA
30 mM TCA
50 mM TCA*


Kromasil
Tf
Charge
0
1
2
3
4
5
10 mM AA
30 mM AA
50 mM AA


Kromasil
Tf
Charge
0
1
2
3
4
5
10 mM PrA
30 mM PrA
50 mM PrA

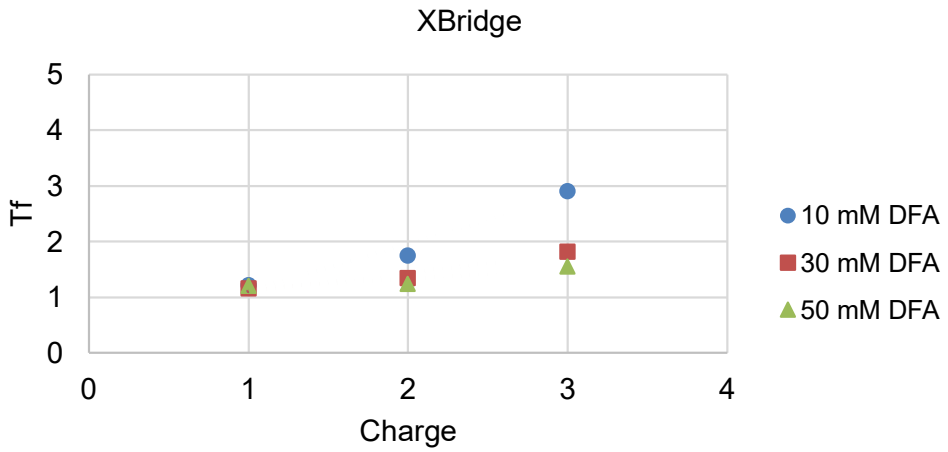


Figure A8. Complete list of all Tf vs Charge plots of increasing additive concentrations.

Table A10. Table showing the measured pH values of the additives at concentrations used in this study.

	pH at concentration (mM)		
Additive	**10**	**30**	**50**
FA	2.9	N/A	N/A
AA	3.4	3.2	3.0
PrA	3.4	3.2	3.0
GA	2.9	N/A	N/A
DCA	2.0	N/A	N/A
TCA	2.1	1.6	1.4
DFA	2.2	1.6	1.4
TFA	2.0	1.5	1.3
MSA	2.0	N/A	N/A
ESA	2.1	N/A	N/A

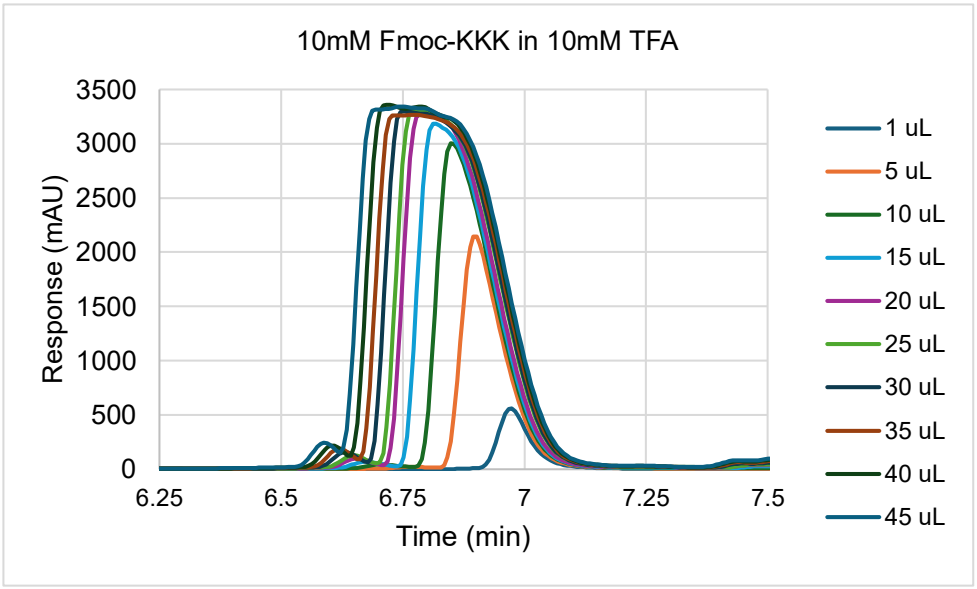


Figure A9. Elution profiles of Fmoc-KKK. At injection volumes of 20 uL, overload is definitive.

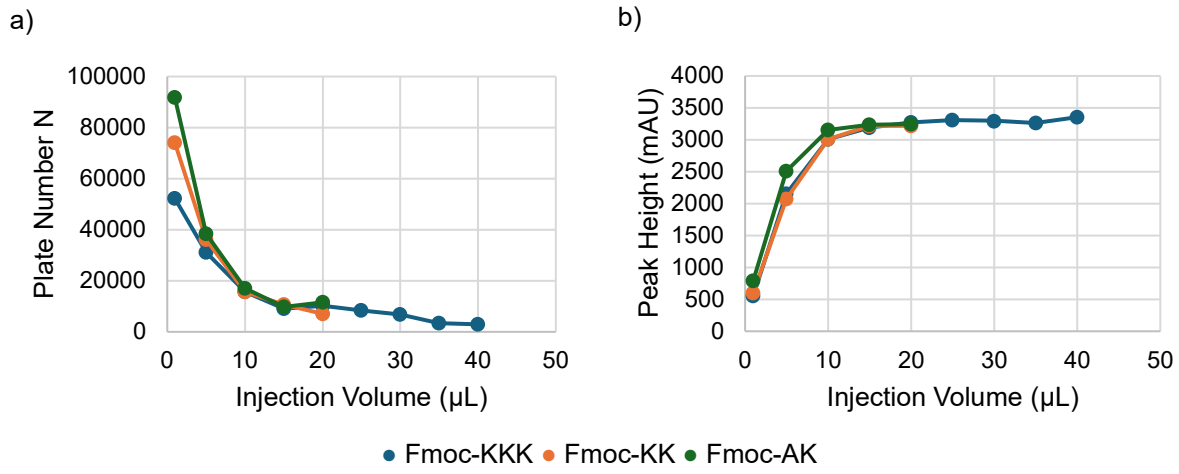


Figure A 10. Determination of overload based on a) plate number and with concurrent b) saturation of the detector.

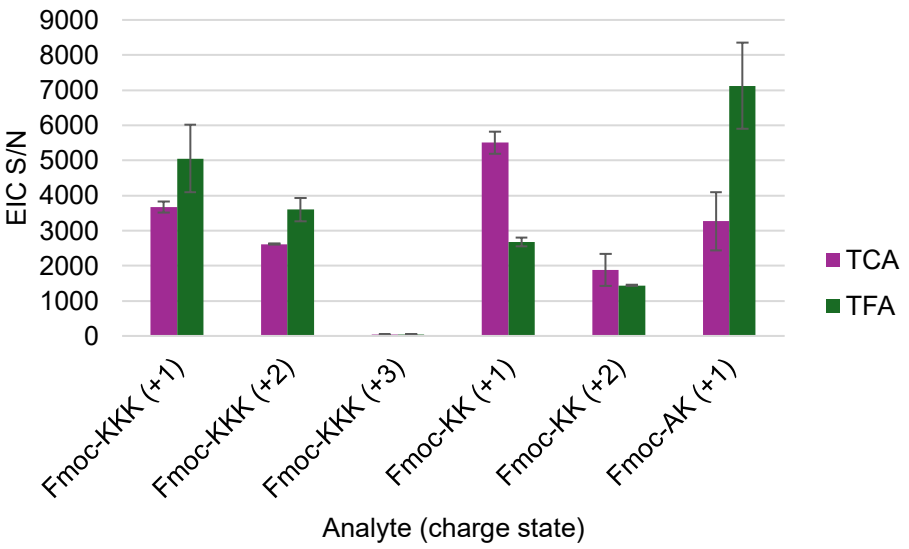


Figure A11. EIC S/N plot for all charge species.