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RUTA HECHT

Novel Eluent Additives for LC-MS Based Bioanalytical Methods

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Novel Eluent Additives for LC-MS Based Bioanalytical Methods

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Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia.

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Always be wary of any helpful item
that weighs less than its operating manual.

Terry Pratchett

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II M. Hecht**, R. Veigure**, L. Couchman, C.I. S Barker, J.F. Standing, K. Takkis, H. Evard, A. Johnston, K. Herodes, I. Leito, K. Kipper, Utilization of data below the analytical limit of quantitation in pharmacokinetic analysis and modeling: promoting interdisciplinary debate, Bioanalysis. (2018) doi:10.4155/bio-2018-0078

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IV **R. Veigure,** K. Lossmann, M. Hecht, E. Parman, R. Born, I. Leito, K. Herodes, & K. Kipper, Retention of acidic and basic analytes in reversed phase column using fluorinated and novel eluent additives for liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. (2019) https://doi.org/10.1016/j.chroma.2019.460667

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VI M. Hallik, M. Ilmoja, J. F. Standing, H. Soeorg, T. Jalas, M. Raidmäe, K. Uibo, K. Köbas, M. Sõnajalg, K. Takkis, **R. Veigure**, K. Kipper, J. Starkopf, & T. Metsvaht, Population Pharmacokinetics and Pharmacodynamics of Dobutamine in Neonates on the First Days of Life, Br. J. Clin. Pharmacol. (2019) https://doi.org/10.1111/bcp.14146

Author's contribution

Paper I: The main person responsible for planning and performing the experiments and for writing the manuscript.

Paper II: The main person responsible for writing the chapter regarding different guidelines as well as managing references.

Paper III: Carried out a part of the bioanalytical method validation and real sample analysis. The main person responsible for writing the manuscript.

Paper IV: The main person responsible for planning and writing the manuscript. Performed the majority of the experiments.

Paper V: Carried out a part of the bioanalytical method validation and real sample analysis.

Paper VI: Carried out a part of the bioanalytical method validation and real sample analysis.

ABBREVIATIONS

ACN	acetonitrile
BLQ	below the limit of quantification
CID	collision induced dissociation
CLON01	Clonidine for Sedation of Paediatric Patients in the Intensive Care Unit
CNS	central nervous system
DAD	diode-array detector
DFA	difluoro acetic acid
DMSO	dimethyl sulfoxide
EMA	European Medicines Agency
ESI-MS	electrospray mass spectrometry
FA	formic acid
FTIR	Fourier-transform infrared spectroscopy
GABA	gamma-aminobutyric acid
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HFTB	1,1,1,3,3,3-hexafluoro-2-methyl- 2-propanol
HPLC	high performance liquid chromatography
ICU	intensive care unit
LC	liquid chromatography
LoD	limit of detection
LLOQ	lower limit of quantification
m/z	mass-to-charge ratio
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
MeOH	methanol
MiOH	1'-hydroxymidazolam
MS	mass spectra/mass spectrometry
NFTB	nonafluoro-*tert*-butyl alcohol
NMR	nuclear magnetic resonance
NSAID	nonsteroidal anti-inflammatory drug
ODS	octadecylsilyl group
PD	pharmacodynamics
PDA	photodiode array detector
PFP	pentafluorophenyl stationary phase
PK	pharmacokinetics
PP	perfluoropinacol
RP	reversed phase
SEC	size exclusion chromatography
STD	aqueous standard buffer
TCA	tricyclic antidepressant
TFE	2,2,2-trifluoroethanol
TMS	trimethylsilyl group

UHPLC	ultra-high-pressure liquid chromatography
UPLC®	ultra-performance liquid chromatography, name trademarked by Waters Corporation
UV/Vis	ultraviolet/visible light

INTRODUCTION

Liquid chromatography (LC) is a well-known and much-employed technique by analytical chemists which has undergone significant improvements over the last century. A substantial advancement in the field was the coupling of an LC instrument to mass spectrometry (MS) detector. While this expanded the limits of analytes that can be detected and quantified, it also put a considerable restriction on how the analyte is delivered to the MS – both in terms of the LC mobile phase's volatility, as well as the prepared sample cleanliness.

In order to ensure an easier and often also a better detection of analytes in the MS samples need be separated from any possible lingering matrix and also from each other (in multi-analyte assays). The separation is done via analytical columns, which, due to the chemical properties of silica, historically have been and still are used in combination with acidic eluents. However, these conditions are not suited for pharmaceutical analysis (especially in the bioanalytical field), since the vast majority of analytes have basic properties, which results in them being insufficiently retained and poorly separated on a regular reversed-phase chromatography column.

A potential option to change elution patterns without changing the column or detector is presented by novel fluoroalcoholic eluent additives – weakly acidic compounds with weak ion-pairing capabilities and with a positive influence on analyte ionisation in the MS source. Two additives: 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFTB) are already used in the analysis of oligonucleotides. Other fluorinated alcohols – perfluoropinacol (PP) and nonafluoro-*tert*-butyl alcohol (NFTB) are much less known, but they are no less interesting. Additionally, ammonium fluoride (NH_4F), 2,2,2-trifluoroethanol (TFE) and dimethyl sulfoxide (DMSO) have been reported to have a positive influence on either the analyte sample clean-up, or ionisation, thus presenting potential for exploration.

It is of interest to research and expand the knowledge about the highlighted compounds as LC-MS eluent additives. Furthermore, to study the influence on analyte retention not only on the C18 stationary phase, but also in the biphenyl and pentafluorphenyl (PFP) columns and to investigate the impact on analyte ionisation when novel additives are used in bioanalytical applications at more favourable mobile phase conditions – namely using a high pH.

As the research focuses on pharmaceutical analytes, two practical applications – method development and validations using a fluorinated eluent additive as a key ingredient for success, have been developed and successfully implemented in the analysis of real patient samples. Additionally, discussion about pharmacokinetically important data obtained from the bioanalytical methods that fall below the limits of quantification has been presented. The importance and perspectives for this data treatment will be outlined.

1. LITERATURE OVERVIEW

1.1. Liquid chromatography and instrumentation

The history of liquid chromatography (LC) begun more than a century ago as "classical column chromatography" when the separation of components from the mixtures was achieved in a glass tube filled with solid particles as the stationary phase. Initially, coloured samples (plant pigments) were analysed, as thus the separation could be monitored visually. While the technique has matured through time, the main principle has remained the same – the separation of a mixture, on the basis of solvent and stationary material, in both cases either polar or nonpolar, with continuous monitoring with a detector at the end. The detector signal is then plotted against time and called a "chromatogram". Modern LC instruments do not rely on gravity anymore, but use a high-pressure pump, to enable a faster and more efficient separation. Due to that, it is called high-performance liquid chromatography (HPLC). [1]

In recent years, an even more powerful type of LC has become common, called either ultra-performance liquid chromatography (UPLC® – trademarked by Waters Corporation) or ultra-high-pressure liquid chromatography (UHPLC). The main difference between HPLC and UHPLC is that the latter uses pressures higher than UPLC®; in addition, it incorporates columns, which are packed with sub-2 μm particles and instruments capable of withstanding pressures of more than 1,000 bars (100 MPa). This shortened the analysis time and an optimised resolution, faster results and a higher sensitivity. The generic term UHPLC focuses only on the high-pressure aspect. [2]

The LC system (Figure 1) in general can be coupled with a vast array of detectors, depending on the analytes in question. The most basic ones are ultraviolet/visible light (UV/Vis) or diode-array detectors (DAD)/photodiode array detectors (PDA) where detection is based on light absorption. While UV/Vis detectors have relatively high sensitivity, they are easy to operate, reliable and cheap, they are not very selective. Other available options include fluorescence, conductivity, refractive index, light-scattering, corona-charged aerosol detectors. [1]

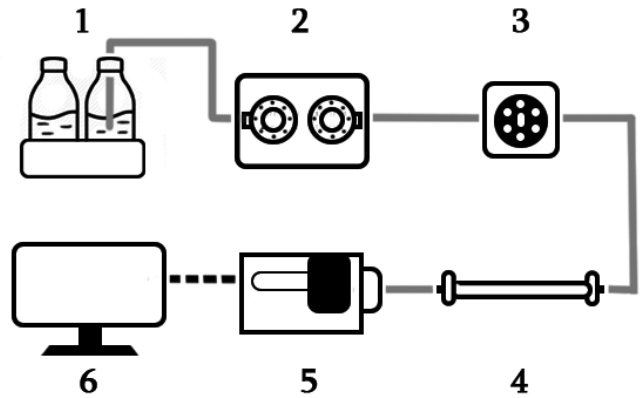
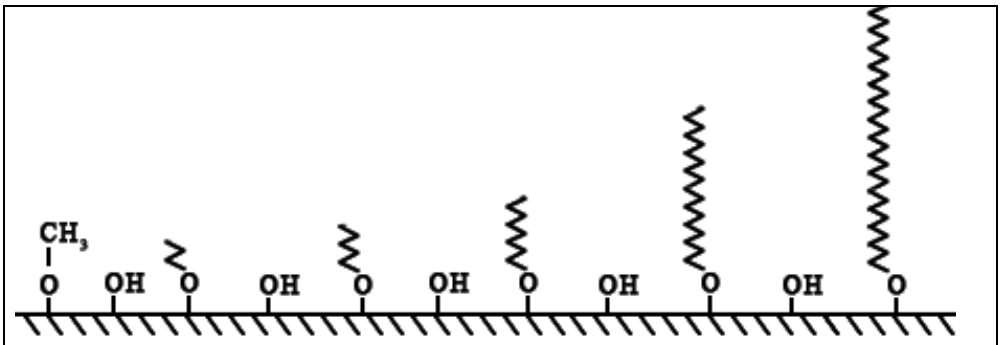


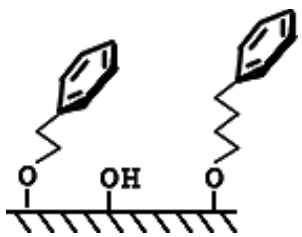
Figure 1. The schematic of an HPLC system, consisting of 1 – solvent reservoirs, 2 – a high-pressure pump, 3 – a sample injection system, 4 – an analytical column, 5 – a detector and 6 – a data acquisition a platform.

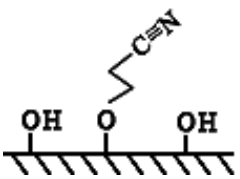
The most versatile HPLC addition is a mass spectrometric (MS) detector. This detection type has become a reliable tool in bioanalytical methods, especially when analysing pharmaceuticals in biological matrices (most commonly human blood, plasma or urine). The MS extracts ions according to their mass-to-charge ratio (*m/z)*, quite often protonated molecule $[M+H]^+$. More sophisticated tandem-MS or hybrid-MS systems first isolate a precursor ion and fragment it into several product ions, which again are isolated or collectively scanned for. The latter provides the much-needed selectivity, as differently structured compounds can form ions of the same *m/z*. Other detectors include Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). [1], [3]

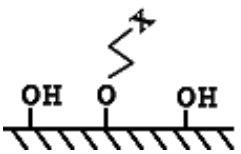
1.2. The stationary phase (column) and its role in analyte separation

The modern LC-column is still a tube, packed with particles carrying the stationary phase. Most commonly they are silica particles, but other types (e.g. zirconia or alumina) are also available. Silica particles have “functional groups” attached via siloxane bonds, which together can be considered the column’s stationary phase (Figure 2). The functional groups determine what kind of analytes can be selectively retained. Columns with short carbon chains of C3, C4, or C5 functional groups are primarily used for separating proteins, while columns with diol groups are used in size exclusion chromatography (SEC). Traditionally, the columns are categorised by their functional group (C18, biphenyl etc.), particle size, column internal diameter and length. [1]









Column type	***X***	**Sub-type**
Embedded-polar-group	R-NH-(C=O)-O-	Carbamate
	R-NH-(C=O)- NH	Urea
	R-(C=O)- NH	Amide
Fluoro columns:		
		Pentafluorophenyl (PFP)
	$-CF_2-\ CF_2-\ CF_3$	Fluoroalkyl

Figure 2. Different types of reversed phase columns organised according to functional groups (the figure does not show connecting [-Si$(CH_3)_2$-] silane group). TMS – trimethylsilyl group, ODS – octadecylsilyl group.

1.2.1. Retention time and the retention factor

Retention time (t_R) is the time between the sample injection, and the apex of the peak of an analyte. The shortest retention time is found for compounds un-retained on the column under used the chromatographic conditions and is called hold-up time, void volume or dead time t_0. The retention factor k (sometimes also called the capacity factor) is defined as the time the sample component resides on the stationary phase, relative to the time it is located in the mobile phase (equation 1). [1], [4]

The retention factor is calculated with the following formula (1).

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

where k – retention factor, t_R – retention time, t_0 – dead time

A very precise value for k is usually not required for developing a method or during routine analysis, but it is preferable if the retention factor value lies between the values 1 and 10. If, for all peaks $k \leq 10$, it means there is an efficient use of resources – a shorter analysis run time per one sample and more samples analysed per day. Additionally, the obtained peaks are narrower and higher, which leads to improved detection. However, if k is below 1, analytes elute early in the chromatographic run, may have poor resolution and can be distorted by interferences from the matrix. This parameter can help with the evaluation and comparison of different methods and the analyte retention in the column. [1]

1.2.2. Interactions in the reversed-phase column

Taking into account all stationary phases, 80-90% assays use reversed-phase (RP) chromatography columns [5]. The basis of analyte retention on RP depends on the interactions between the analyte, the non-polar stationary phase (column) and the polar mobile phase (eluent). The column types used in this dissertation include the C18, biphenyl and pentafluorophenyl (PFP) stationary phases and are all representatives of RP chromatography. Altogether these interactions can be divided into eight types (Figure 3) – (1) hydrophobic interaction, (2) steric interaction (exclusion of large analyte molecules from the stationary phase), (3) and (4) are hydrogen bonding – between a donor and acceptor within the stationary phase – where one is basic and the other is acidic, either the analyte or the stationary phase, (5) ionic interaction, (6) dipole-dipole interaction and (7) π-π interactions between aromatic analytes and nitrile or (8) π-π interactions between aromatic analytes and a phenyl group in the stationary phase. [1]

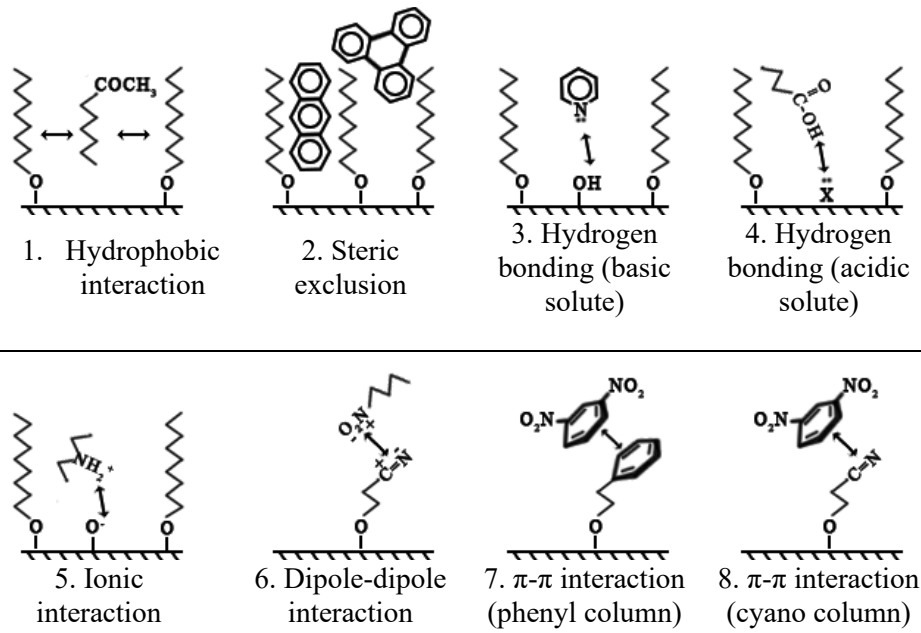


Figure 3. Analyte interactions with the column's stationary phase in RP.

Interactions 1–5 are important in any column; dipole-dipole interactions are relevant only in the case of the cyano-column, π-π interactions are present only for phenyl and cyano-stationary phases. Furthermore, acetonitrile (ACN) as mobile phase component inhibits dipole and π-π interactions. [1], [6] Hydrophobic interactions are deemed to be the most powerful in the C18 column [7], but other interactions can influence the selectivity of the RP column as well [1]. However, unlike the C18, biphenyl stationary phase has a very high hydrogen-bonding capacity [7]. Also PFP stationary phases, when compared to alkyl phases, provide alternative retention and selectivity. This is due to the additional interactions (π-π, dipole, hydrogen bonding, and ionic interactions) which are not as dominant in the common alkyl stationary phases. For positively charged analytes, the dominating interaction is often ion-exchange. However, the prevalence of this interaction is very dependent on the column manufacturing process, as it is speculated that the main source of this interaction comes from ionised surface silanol groups, not the PFP ring system itself. In any case, due to the prevalence of ion-exchange interaction in the PFP column, it is especially important to monitor pH conditions. [8], [9]

1.2.3. Reversed phase column and pH

The most popular packing material of the RP phase columns is silica. Silica does not swell or shrink when in contact with organic solvents. Furthermore, it possesses high mechanical strength, which is especially needed for the high-pressure conditions in HPLC. By now, both the manufacturing and mechanics of columns are well explored and reproducible [10]. Thus, silica appears to be the ideal substrate for mass production. Unfortunately, it also has a downside – namely, liability to deterioration [10], [11]. Columns with short-chain ligands like C3 are the most unstable at a low (below 2) pH. However, the mobile phase with pH >8 can lead to the dissolution of silica packings. If the bonded phase is lost, it means that the column performance or so-called "column lifetime" decreases. The manufacturer provided recommendations should always be followed, especially for temperature and the mobile phase pH. [1] The deterioration rate depends on what type of functional groups are bonded to the silica, the eluent's composition, and a myriad of other experimental parameters. However, the process of the column's stationary phase breakdown due to the pH can be divided into two mechanisms [11]: silica dissolves even in the pH 2–7 and reaches saturation of 100 ppm [11], but if the pH rises above 7, the process speeds up considerably [12]. The backbone of silica breaks down, which greatly reduces the column's plate numbers (the parameter describing column's efficiency) and eventually results in the column clogging. In the case of an acidic pH, it is not silica but the covalent siloxane bond responsible for binding the functional groups that undergoes hydrolysis [12]. A high temperature significantly accelerates the degradation processes for both low and high pH mobile phases [1].

While the rapid deterioration in a high pH environment is unavoidable, the rate at which this happens varies greatly depending on the manufacturer. Resistance to a high pH depends primarily on the silica chosen as the substrate at the very beginning of column production, as well as the possible contamination with metals. [10], [11] There are multiple things employed nowadays for expanding the resistance to very high or low pH environments. One of them is obtained through a hybrid organic-inorganic process (silica-silane or zirconia based particles are resistant to a high pH) and results it in endcapping the unbonded silanol groups [1], [13]. If the bonded alkyl chains are longer, the resistance to a high pH increases as well, as long as the temperature remains below 40 ºC [1], [10].

1.3. The mobile phase in LC

In RP-LC organic solvents are most common as mobile phases, using methanol (MeOH) and ACN in combination with deionised water. To further improve the analysis efficiency, mobile phase additives are commonly used as easily adjustable parameter. By supplying an extra additive, commonly a buffering agent, it is possible to change various properties of the mobile phase such as the pH, ionic strength or ion-pairing capabilities. The addition of an eluent additive can also influence the retention of the analyte in the column and the ionisation in the MS. [14] While coupling an MS facilitates a more precise detection as well as increases the variety of analytes possible to detect, it also introduces limitations. The greatest limitation of mass spectrometers is the requirement of the mobile phase to be volatile. This excludes many commonly used LC-UV/Vis eluent additives. For LC-MS systems, the most commonly used eluent additives (which are also relatively inexpensive) are thus acetic and formic acids (FA), their ammonium salts, as well as ammonium bicarbonate. The buffering range of ammonium bicarbonate is $6.8 \leq pH \leq 11.3$. The buffering range of ammonium formate is $2.8 \leq pH \leq 4.8$ [15], while for ammonium acetate two buffer ranges can be achieved between $3.8 \leq pH \leq 5.8$ and $8.2 \leq pH \leq 10.2$. [14]–[16]

1.4. The importance of acid-base equilibrium

In the past, the separation of ionised analytes (containing basic, acidic or both of the functional groups) was rather complex, due to the lack of appropriate columns available and a limited understanding on how best to carry out analyses with these analytes. While these limitations have mostly been overcome, the ionised analytes still present a complex challenge. [1], [14] The charge (protonation) state and thus also the interactions of the analyte with other analytes, eluent additives or the stationary phase are determined by the pH of the environment. It is important that the mobile phase's pH should be both controlled and stable – which is achieved with the use of buffering agents. [14]

When an analyte (acid AH or base B) undergoes ionisation in the mobile phase due to the pH, it becomes more polar – either as a deprotonated acid A^- or a protonated base BH^+. The retention factor can decrease more than 10 times due to this effect (Figure 4). [1]

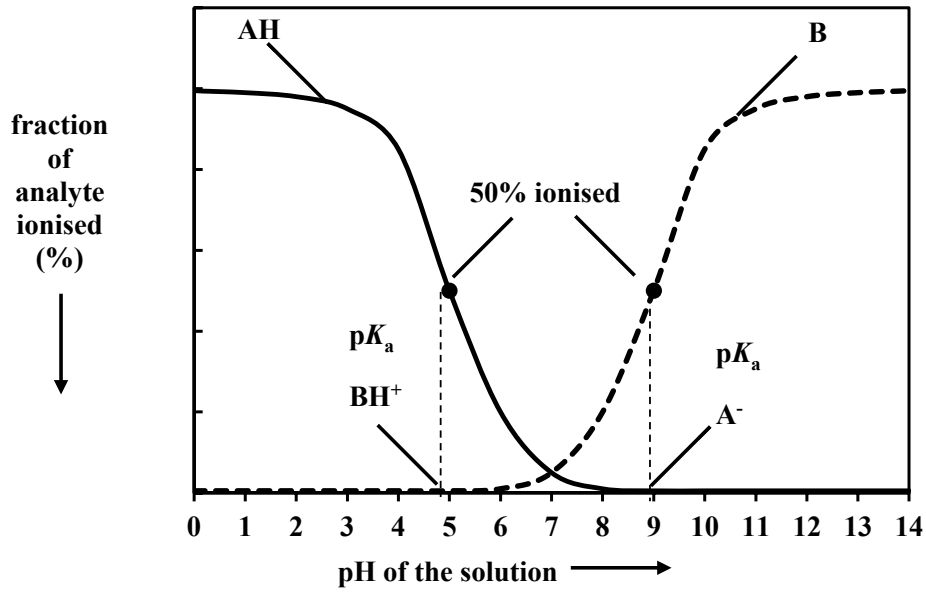


Figure 4. The ionisation of acidic (AH) and basic (B) analytes as a function of the mobile phase's pH.

To further complicate matters, the addition of an organic solvent, either constantly or gradually, to the aqueous buffer (the pH of the water phase can be expressed as ${}^{w}_{w}$pH [17]) alters the pH of the mixture and therefore also the rate of the protonation/deprotonation of bases and acids. Hence, as critical as the pH of the aqueous component, also the pH of the whole eluent is important. This pH can be expressed either as the pH (pH_{abs}) [18] or the solvent-water pH (${}^{s}_{w}$pH) [17]. The ${}^{s}_{w}$pH value is obtained, when the electrode is calibrated in aqueous solutions, but the pH is subsequently measured in the organic-water mixture. This approach does not account for the liquid junction potential (LJP) at the pH-electrode in the eluent. It functions unlike pH_{abs}, which expresses the acidity of the mobile phase in terms of its proton's thermodynamic activity. Thus, the measured pH_{abs} values in different solvents, mixtures, etc. become comparable. [17]

The change in the eluent's pH due to the addition of an organic component, as well as the variation in the analyte's pK_a values, can be modelled. Based on both of these parameters, it is also possible to estimate the degree of the ionisation of the analytes and possibly predict the analyte elution patterns. The extent of changes depends on the organic component and its fraction in the eluent, the concentration of the buffer component and the aqueous pH, as well as the nature of the buffering system. For both MeOH and ACN, the pH alteration has a linear relationship with the volume fraction of the organic phase and the aqueous pH. The pK_a values of neutral or anionic acids, e.g. phenols, aliphatic and aromatic carboxylic acids, increase with the addition of an organic

solvent. In contrast, the pK_a values of pyridines and amines, as cationic acids, decrease. [17]

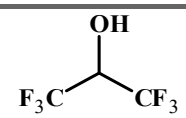
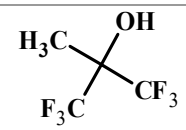
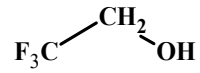
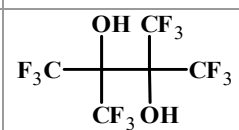
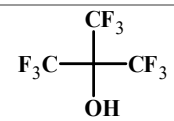
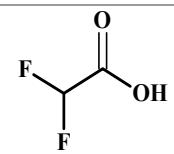
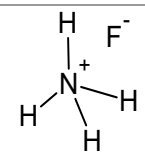
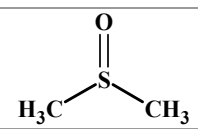
Another important parameter that is influenced is the buffering capacity. It describes how much of a strong base or acid has to be added to a system to achieve one pH unit change in the solution. The highest buffer capacity is reached if the acid and the conjugated base species are in equal concentration – meaning that the pH of the solution is equal to the pK_a. The addition of the organic solvent to the mixture shifts the maximums of buffer capacities, however the scope of this influence is unique to every buffer. For anionic or neutral buffers (citric, acetic and phosphoric systems) this maximum is moved to a higher ${}^{s}_{w}$pH, but for cationic acid buffers (ammonia) it is shifted to lower ${}^{s}_{w}$pH. Furthermore, the increase in the volume fraction of the MeOH in the eluent decreases the ${}^{w}_{w}$pH value for ammonia buffers. [17]

1.5. Novel eluent additives

The requirement of volatility for an eluent additive vastly decreased the number of usable buffers applicable with the LC-MS systems. Furthermore, analytes which can be ionised in the eluent demand careful consideration of their mobile phases. Thus, the need for novel eluent additives, compatible with MS systems has never been greater. One prospective group of novel eluent additives is fluoroalcohols (Table 1), which can also alter the selectivity of both mobile and stationary phases in LC.

The proposed way of action for fluoroalcohols is as follows – the fluorinated compounds cover the stationary phase, in the case of C18, and create a hydrophilic layer exposing their polar hydroxy (-OH) group to the mobile phase, which becomes a possible hydrogen bond donor. If the pH of the mobile phase is higher than the pK_a, the OH group becomes deprotonated and the anions of fluoroalcohols can create ion pairs with the protonated basic species both in the eluent and on the stationary phase. This results in an increase in retention. Acidic ionic analytes, however, are hindered due to a competition with fluoroalcohols for the surface on the stationary phase, as well as the repulsion by the deprotonated (-OH) groups. Thus, the retention of acidic analytes decreases. [25]

Table 1. Promising novel eluent additives and their structures.

Name	Abb-reviation	CAS number	Structure	pK_a	pK_a reference
1,1,1,3,3,3-hexafluoro-2-propanol	HFIP	920-66-1		9.3	[19]
1,1,1,3,3,3-hexafluoro-2-methyl- 2-propanol	HFTB	1515-14-6		9.6	[20]
2,2,2-trifluoroethanol	TFE	75-89-8		12.5	[21]
perfluoropinacol	PP	918-21-8		pK_{a1} = 5.95 pK_{a2} = 10.43	[22]
nonafluoro-*tert*-butyl alcohol	NFTB	2378-02-1		5.33	[22]
difluoro acetic acid	DFA	381-73-7		0.013	[23]
ammonium fluoride	NH_4F	12125-01-8		-	-
dimethyl sulfoxide	DMSO	67-68-5		35	[24]

1.5.1. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP)

Fluoroalcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Table 1) are well known as weakly acidic eluent additives in the analysis of oligonucleotides, mainly due their impressive signal enhancement properties [26]. HFIP also behaves as an ion-pairing reagent and has demonstrated how it can alter selectivity [25] and successfully improved results in the analysis of antibiotics (ofloxacin, ciprofloxacin, sulfadimethoxine, norfloxacin and sulfamethoxazole) in lettuce samples. [27] Just like any other alcohol, HFIP is an excellent solvent. However, it alters the function of cell membrane proteins as well as changes the

properties of the lipid bilayers far stronger than its un-fluorinated alcohol counterpart. While unsaturated lipids can bind to HFIP without compromising the membrane's integrity, saturated lipids form particles with HFIP [28], [29]. The unique properties of HFIP, in forming strong hydrogen bonds (there can be many HFIP molecules per one head group of a lipid) and HFIP having a high octanol/water partition coefficient, can be named as reasons for this [28].

1.5.2. 1,1,1,3,3,3-Hexafluoro-2-methyl-2-propanol (HFTB)

Similarly to HFIP, also 1,1,1,3,3,3-hexafluoro-2-methyl- 2-propanol (HFTB, Table 1) is weakly acidic and has ion-pairing capabilities [25]. HFTB has also been used in the analysis for oligonucleotides, and depending on the oligonucleotide type, has produced an even higher signal enhancement than HFIP [26], [30].

1.5.3. Perfluoropinacol (PP)

Perfluoropinacol (PP) or 1,1,1,4,4,4-hexafluoro-2,3-bis(trifluoromethyl)butane-2,3-diol (Table 1) is a promising candidate as a fluorinated eluent additive possessing not one, but two OH groups. However, to the best of the author's knowledge, no previous research has been conducted with PP as an eluent additive.

1.5.4. Nonafluoro-*tert*-butyl alcohol (NFTB)

Scarce information can be found about nonafluoro-*tert*-butyl alcohol (NFTB, Table 1) as a possible eluent additive, except for one example in oligonucleotide research in which signal suppression was observed [26]. NFTB also has lipid bilayer destabilising properties, even greater than those of HFIP [29].

1.5.5. 2,2,2-Trifluoroethanol (TFE)

The use of the eluent additive 2,2,2-trifluoroethanol (TFE, Table 1) has been demonstrated as suitable for cleaning an LC system as well as columns from proteins and peptides without suppressing the MS signal [31]. Changes in an analyte's signal strength were also observed in oligonucleotide research. Rather than a straightforward enhancement or suppression, a higher charge state of the oligonucleotide is achievable with TFE, in contrast to HFIP, but it lacked good chromatographic properties [26]. Similarly to HFIP, also TFE has been proven to have lipid bilayer disruptive properties, albeit less strong than those of HFIP and NFTB [29].

1.5.6. Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO, Table 1) is not yet a common eluent additive. However, it became a potential candidate to research when its positive influence on ionisation in the field of proteomics was discovered [32]–[34]. This was observed, when DMSO was used in low (5%) concentrations in the MS detection of peptides derived from trypsin, elastase or pepsin digestion [32].

1.5.7. Ammonium fluoride (NH_4F)

Ammonium fluoride (NH_4F, Table 1) has previously been successfully applied within our research group, and it showed a significant enhancement of the ionisation of steroid-like molecules in the positive ion detection mode [35]. Thus far, however, the research has focused on the effects of ammonium fluoride in the negative detection mode. A study was conducted which aimed to quantify brevetoxins – a polycyclic, lipid-soluble family of toxins originating from algae [36]. In favourable conditions, the algae rapidly reproduce and release brevetoxins, inducing toxicity in marine mammals, fish, and humans who consume contaminated shellfish. Since these analytes are lacking acidic groups, they produce only a very small signal in the negative mode electrospray mass spectrometry (ESI-MS). Multiple anions were researched, and it was discovered that, although brevetoxins formed ion adducts (for example $[M+Cl]^-$, $[M+Br]^-$, $[M+OAc]^-$, $[M+HCOO]^-$, $[M+NO_3]^-$), it was NH_4F and bicarbonate which provided the necessary further fragmentation for identifying the toxins. Furthermore, NH_4F yielded more fragments and a far higher signal than bicarbonate. [36]

To avoid the usually employed derivatisation to increase the sensitivity for the detection of estrogen (both estrone E1 and oestradiol E2), the influx of fluoride ions was tested. They provided the needed improvement. [37] Similarly, for the two steroid hormones Δ4- and Δ7-dafachronic acid (isomers), NH_4F was successfully employed [38]. The effect of enhancement still appears to be analyte specific – as signal suppression is also possible [39]. Most probably, the mechanisms of ionisation enhancement in the negative mode originates from the strong basicity fluoride ions possess in the gas phase. This allows them to attract protons from neutral molecules. They produce HF, meanwhile forming $[M+FHF]^-$ clusters and $[M+F]^-$ ions. Meanwhile enhancements have also been observed in the positive ion detection mode for organic acids, using normal phase chromatography. [36], [40]

1.5.8. Difluoroacetic acid (DFA)

Difluoroacetic acid (DFA) is the only carboxylic acid from all researched eluent additives (Table 1). A promising application note has shown that DFA has both ion-pairing and signal enhancement properties, it additionally provides better peak shapes for peptide analysis when compared to other commonly used eluent additives such as formic acid (FA) and trifluoroacetic acid (TFA). [41]

1.6. Challenges in the bioanalytical applications for small molecules

Basic conditions for RP separation are best suited in the case of pharmaceutical analyte analyses since over 70% of the analytes have basic properties, but only approximately 20% are acids. Basic analytes are protonated when the eluent's pH is lower than the analyte's pK_a value and thus have poor retention in RP conditions [42]. However, because the high pH damages the silica-based analytical columns, acidic mobile phases have been preferred, which means the conditions for the analysis for basic analytes are less than satisfactory [1].

Meanwhile, there is a need for pharmacokinetic (PK) and pharmacodynamic (PD) studies of pharmaceutical analytes – especially in the complicated studies focusing on paediatric patients [43], [44]. Low concentrations of analytes needed to be quantified, and very limited amounts of allowed sampling volumes in term and preterm neonates, which result in a very small final sample volume are ever-present issues increasing the complexity of study management [45]–[47]. While the need for more sensitive and efficient methods increases, the data obtained still require to be reported with the same trustworthiness and strict validation as in other bioanalytical methods [48], [49].

Additionally, regarding the very low concentrations of an analyte and tight guidelines regulating what results can be released, a significant amount of the obtained data for PK/PD studies can and do fall below the limit of quantification (BLQ) and cannot be released – simply because of the BLQ data precision and the data missing accuracy. The reasons, why analyte concentrations fall BLQ are manifold, including that the drug concentration has decreased too much – especially for late time points in the PK studies [50]–[54], and when the parent drug degrades very rapidly [55]. Too low concentrations can also be a result of individual variability (the influence of how the drug is absorbed, excreted or degrades) [56]–[59] and the fact that the administered dose might simply have been too small [60]–[64]. The lack of data BLQ has forced scientist to use statistics to create a smaller bias and more accurate parameter estimates [65], [66].

Two types of medication requiring PK/PD studies in paediatric patients can be taken as examples – sedative and cardiovascular drugs. An area, where sedation is commonly needed, is the intensive care units (ICU). Due to the vulnerable patient population, only limited sample volumes are available. Additionally, the

probability to have samples with a very low drug concentrations is large – possibly even below limits of quantification for methods not sensitive enough. [67] Moreover, there is increasing awareness of difficulties presented by over-sedation and the simultaneous need to ensure sufficient sedation, while also matching individual needs [68]. Reduction in the usage of benzodiazepines by switching to alpha-2 adrenergic receptor agonists (e.g. clonidine) as an attempt to avoid the side effects (tolerance and withdrawal) are hindered due to a lack of data, research, and the understudied sedative requirements [68], [69]. As for cardiovascular drugs, the amount of paediatric patients (especially infants) who need milrinone has increased by four times since 2005, and there has been a steady increase in the administration of milrinone also in ICUs. The reasons for the administration of milrinone included persistent pulmonary hypertension, low cardiac output syndromes. Additional medication administered alongside milrinone is fentanyl, midazolam, furosemide and dobutamine. [70]

As mentioned previously, benzodiazepines are common in ICUs, the most popular being midazolam, lorazepam, and diazepam [71]. Midazolam has hypnotic, sedative and anxiety preventing properties [72] and, as a gamma-aminobutyric acid (GABA) agonist, it is metabolised in the liver to active metabolites [71]. In the case of midazolam, its primary active metabolite is 1'-hydroxymidazolam (MiOH) [73].

Commonly used opioids in ICUs include morphine, hydromorphone, fentanyl, and remifentanil [71]. Morphine is highly addictive, and a tolerance to it develops equally fast [74]. More than a half (56%) of a morphine dose is metabolised to the active metabolites morphine-3-glucuronide (M3G) and 10% is converted into morphine-6-glucuronide (M6G). Other metabolites include hydromorphone and the non-active metabolite normorphine. [75] M3G and M6G are hydrophilic, but the latter crosses the blood-brain barrier more readily and has been deemed to have more potency as an analgesic than either M3G or morphine [76]. M3G and M6G especially present a challenge for MS detection, because both have the same molecular mass and fragmentation patterns – and produce a morphine ion $[M+H]^-$ during the fragmentation [77], [78]. Multiple bioanalytical methods have been developed with varying success in separation of the M3G and M6G as distinct peaks. Largely separation has been achieved [77], [78], however, in some assays – [74], [79], [80] it has been more problematic, with M3G and M6G eluting early during the chromatographic run and/or with morphine, M3G and M6G peaks close to each other.

The administration of clonidine lowers the blood pressure and decreases the heart rate because it stimulates the alpha (2)-adrenoceptors in the central nervous system (CNS) [81]. Due to this, clonidine is not only used as an antihypertensive drug, but also for analgesia and sedation [82]. However, in ICUs the main use of clonidine is treating withdrawal symptoms as well as the addition of clonidine to the sedation regimen allowed to lower the opioid dosage [71].

Milrinone affects the muscle contractions in the muscle tissue of the heart as well as the vascular smooth muscle by inhibiting the enzyme phosphodiesterase 3. Therefore, the inhibitors of the enzyme have a therapeutic influence also on

the lungs, not only the heart. The elimination half-time for milrinone is 2.3 hours, with two main excretion products (via urine) – milrinone (83%) and its glucuronide metabolite (12%) [83].

Dobutamine lowers central venous and pulmonary artery wedge pressures, increases cardiac output and alleviates congestive heart failure symptoms; however, it can also lead to arrhythmia at high dosages. It is commonly used following heart surgery, as well as for patients, who are suffering a heart attack or are in various states of shock [84]. In paediatric patients (neonates), dobutamine is used to treat circulatory compromise, for example in the case of septic shock or after birth asphyxia, as well as to support the transitional circulation in very preterm infants [85].

2. AIMS OF THE STUDY

The general aim of this dissertation was to further research and expand the selection of different novel eluent additives (such as fluorinated compounds) for bioanalysis using LC-MS or HPLC-UV/Vis systems and to study their influence on analyte retention on different stationary phases, as well as ionisation efficiency (in the case of MS detection).

This objective was achieved through:

- evaluating the suitability of novel eluent additives for analysis using LC-MS and/or HPLC-UV/Vis systems
- comparing novel eluent additives and the conventionally used buffer compositions
- investigating the retention mechanisms for acidic and basic analytes within different column stationary phases, eluent additives and eluent pH values
- conducting a practical application of the novel eluent additives in bioanalytics.

3. EXPERIMENTAL

3.1. Reagents

Analytes

4-nitrobenzoic acid, 4-dimethylaminopyridine, diphenylguanidine, 2,4-dichlorophenol, 2,3,4,5,6-pentafluorophenol, 2-nitrophenol, 2,3,5,6-tetrafluorophenol, phenol, p-cresol, hydroquinone, 3-nitroaniline, 4-chloro-2-nitroaniline, diisopropylamine, piperidine, cyclohexylamine, pyrrolidine, aniline, 4-chloroaniline, 1-naphthylamine, histamine, 4-fluoroaniline, 2,6-dimethylpyridine, 2-methylpyridine and 2-methoxypyridine were obtained from Sigma-Aldrich (Missouri, USA). The following compounds and their respective stable isotope labelled internal standards (IS): M3G, M6G, morphine, clonidine, MiOH, midazolam, M3G-D3, M6G-D3, morphine-D6 and MiOH-D4 were purchased from Cerilliant (Texas, USA). Clonidine-D4 and midazolam-D6 were obtained from the Toronto Research Chemicals Inc. (Toronto, Canada). Milrinone (United States Pharmacopeia Reference Standard) and dobutamine hydrochloride (United States Pharmacopeia Reference Standard) were obtained from Sigma Aldrich (Missouri, USA). Internal standard (IS) dobutamine-D4 hydrochloride was obtained from Toronto Research Chemicals (Ontario, Canada) and milrinone-D3 from TLC Pharmaceutical Standards (Ontario, Canada).

Pregabalin, gabapentin, levetiracetam, zuclopenthixol, aripiprazole, gliclazide, cyclizine hydrochloride, naloxone hydrochloride dihydrate, quinine, atenolol, bisoprolol, cetirizine dihydrochloride, chlorpheniramine maleate salt, chlorprothixene hydrochloride, diclofenac sodium salt, diltiazem hydrochloride, mirtazapine, naproxen, norsertraline hydrochloride solution, phencyclidine hydrochloride, procyclidine hydrochloride, propranolol hydrochloride were obtained from Sigma-Aldrich (Missouri, USA).

LC/MS toxicology submixes: 2, 3, 4, 6, 9A, 9B, 9C and 9D were obtained from Agilent Technologies, Inc. (USA)

Eluent additives

1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFTB), 2,2,2-trifluoroethanol (TFE), 1,1,1,4,4,4-hexafluoro-2,3-bis(trifluoromethyl)butane-2,3-diol (PP), 1,1,1,3,3,3-hexafluoro-2-trifluoromethyl-2-propanol (NFTB), dimethyl sulfoxide (DMSO), ammonium fluoride (NH_4F), formic acid, ammonium acetate, ammonium bicarbonate and a 25% NH_4OH solution were LC-MS grade and obtained from Sigma-Aldrich (Missouri, USA). IonHance™ DFA was kindly donated by the Waters Corporation.

Other

Both LC-MS grade and LC-MS Ultra grade MeOH were obtained from Sigma Aldrich (Missouri, USA), the water was purified (18.2 MΩ·cm at 25 ºC and the total organic carbon (TOC) value 2–3 ppb) in-house using a Millipore Advantage A10 system from Millipore (Bedford, USA). The water used for experi-

ments with LC-QTOF was LC-MS grade purchased from Fisher Chemical (Fisher Scientific, Waltham, USA). Ascorbic acid (reagent grade) was obtained from Sigma Aldrich (Missouri, USA). The plasma and whole blood were purchased from the Blood Bank of Tartu University Hospital.

3.2. Instrumentation: chromatographic conditions and detection

3.2.1. Shimadzu LCMS-2020 system [IV]

Chromatographic conditions

The Shimadzu LCMS-2020 system (Shimadzu Corporation, Japan) consisted of an autosampler SIL-30AC (set at 4 ^{o}C), a binary pump LC-20AD-XR, and a column compartment CTO-20AC at 40 ^{o}C as well as MS and PDA detectors. Before conducting the experiments, the column was equilibrated for 1.5 h, using the chosen aqueous solution with eluent additive/buffer for the experiment. The elution mode was isocratic elution, with 25% of MeOH at the flow rate of 0.2 mL/min for the C18 column (Agilent Zorbax RRHD Extend C18 (2.1 × 100 mm, 1.8 µm), resistant to the pH range from 2.0 to 11.5 (Agilent Technologies, Inc. USA)). The column was thermostated at 40 ^{o}C. The analysis run time was determined depending on the analyte. For all experiments, the injection volume of the sample was 5 µL.

Detection

Both PDA and MS detectors were employed for the detection of the analytes. The chosen detection mode (only MS, only UV/Vis or both MS and UV/Vis) depended on the analyte. Shimadzu (Shimadzu Corporation, Japan) PDA detector (SPD-M20A model), recorded between 190 to 700 nm (600±50 nm as a reference wavelength); however, the extracted 254±2 nm wavelength was used for UV chromatograms. The MS operated in scanning mode, with the mass-to-charge (*m/z)* ranges depending on the analytes in question (*m/z* 150–700 for the analysis of eluent additives, *m/z* 50–200 for analytes,). The interface temperature was 350 ºC, DL temperature 250 ºC, heat block temperature 200 ºC. Nebulising gas flow was 1.5 L/min and drying gas flow 15 L/min. Both positive and negative ion modes were used. The analytes were detected either as $[M+H]^+$ or $[M-H]^-$.

3.2.2. ABSciex API 4000 system [IV]

The TFE MS spectrum was obtained with an ABSciex API 4000 (AB Sciex Pte Ltd, Australia), using negative ionisation. The MS was set to a scanning mode between *m/z* 40–120. The solution infused with the flowrate 10 uL/min was 1% TFE in a H_2O/MeOH mixture (50/50, v/v) with 0.1 % NH_4OH.

3.2.3. Agilent 1200 Infinity LC system [IV]

The Agilent 1200 Infinity LC system (Agilent Technologies, Inc. USA) consisted of an autosampler, a binary pump, and a UV/Vis detector with 5-channels. Chromatograms were recorded at 254±2 nm wavelength (using 360±50 nm as a reference wavelength).

The column in use was Agilent ZORBAX Extend C18 (2.1 × 100 mm, 3.5 μm) and was reported to be resistant between pH 2.0 to 11.5 by the manufacturer (Agilent Technologies, Inc., USA). The column was equilibrated for 1.5 h, using the aqueous solution with an eluent additive/buffer depending on the following experiments. The flow rate used was 0.5 mL/min, with isocratic elution with 25% of MeOH. The column was kept at room temperature. For all experiments, the analysis run time was determined depending on the analyte and the injection volume of the sample was 5 μL.

3.2.4. Agilent Infinity II 1290 LC system with Agilent Ultivo mass spectrometer

Chromatographic conditions

The Agilent Infinity II 1290 LC system consisted of an autosampler, a binary pump and a mini MS detector Ultivo (Agilent Technologies, Inc., USA). Before experiments, the column was equilibrated for 1.5 h, using the chosen eluent. The elution mode was isocratic, with 25% of MeOH at the flow rate of 0.5 mL/min. The column used was Kinetex Biphenyl (2.1 × 100 mm, 2.6 μm), resistant to the pH range from 1.5 to 10 under isocratic conditions (Phenomenex Inc. USA). The column was thermostated at 40 ^{o}C. The analysis run time was determined depending on the analyte. For all experiments, the injection volume of the sample was 5 μL.

Detection

The Agilent Ultivo MS was used for the detection of the analytes. The MS operated in scanning mode, with the *m/z* ranges depending on the analytes in question (*m/z* 150–700 for the analysis of eluent additives, *m/z* 50–200 for analytes). The simultaneous positive and negative ion detection mode was used. The analytes were detected either as $[M+H]^+$ or $[M-H]^-$.

3.2.5. Agilent Infinity II 1290 LC system with Agilent QTOF 6545B mass spectrometer

Chromatographic conditions

The Agilent with QTOF 6545B (Agilent Technologies, Inc. USA) system consisted of a thermostated column compartment, a binary pump and an isocratic pump (used in the flow rate 1 mL/min, with a splitter for a dual-probe ion

source), and an autosampler (kept at 4 °C). Analytical columns with three different stationary phases were used:

- Agilent ZORBAX Eclipse Plus C18 (2.1 × 100 mm, 1.8 µm) resistant to the pH range from 2–9 (Agilent Technologies, Inc. USA);
- Kinetex Biphenyl (2.1 × 100 mm, 2.6 µm) resistant to the pH range from 1.5–10 (Phenomenex Inc. USA);
- Restek Raptor FluoroPhenyl (PFP) resistant to the pH range from 2.0–8.0 (2.1 × 100 mm, 2.7 µm) (donated by Restek Corporation, USA).

The column compartment was maintained at 40 °C. The eluents (both phases A and B) were composed depending on the analysis (Table 2). If needed, the pH was adjusted with a 20% NH_4OH solution.

Table 2. The eluent composition used with different analytical columns.

Column	Aqueous phase A	Organic phase B
C18	5 mM ammonium formate and 0.01% formic acid (v/v)	MeOH with 5 mM ammonium formate and 0.01% formic acid (v/v)
	5 mM ammonium formate and 0.01% DFA (v/v)	MeOH with 5 mM ammonium formate and 0.01% DFA (v/v)
	0.15% DFA (v/v)	MeOH
Biphenyl	5 mM ammonium acetate (v/v), pH 8.5	MeOH
	5 mM ammonium acetate (v/v), pH 9.0	
	5 mM HFTB (v/v), pH 8.5	
	5 mM HFTB (v/v), pH 9.0	
PFP	5 mM ammonium formate and 0.01% formic acid (v/v), pH 4.0	MeOH
	5 mM HFTB (v/v), pH 8.5	
	5 mM HFTB (v/v), pH 9.0	

The gradient elution patterns can be seen in Table 3 (with 1 min post time). The used flow rate was 0.400 mL/min.

Table 3. The gradient programme for the separation of the analytes included in the toxicology screening experiments.

Time, min	Aqueous phase A, %	Organic phase B (MeOH), %
0	95	5
1	95	5
10	0	100
12	0	100
12.1	95	5
13	95	5

Detection
Agilent QTOF 6545B mass spectrometer (Santa Clara, USA) was used for the detection of the analytes. The spectral data was recorded with these parameters: positive ion scan (30 V) with scan-rate 3 and the *m/z* scanning range *m/z* 40–1000. Through the dual-probe ion source a reference ion solution (with ions *m/z* 121.0509 and 922.0098) was delivered to ensure mass accuracy. The data were compared against in-house standard drug and metabolite libraries, which contain MS/MS spectra, empirical formulae, and retention times from in-house reference standards.

The following mass analyser settings were used: drying gas flow rate 12 L/min, drying gas temperature 250 °C, nebuliser pressure 35 psi (0.241 MPa), sheath gas flow (12 L/min) and sheath gas temperature 350 °C, nozzle voltage (300 V) and capillary voltage (3,500 V), fragmentor voltage (150 V), skimmer voltage (65 V) and Oct 1 RF Vpp (750 V).

3.2.6. Agilent 1290 Infinity UHPLC system with Agilent 6495 Triple Quadrupole mass spectrometer [I, III, V, VI]

Analysis of morphine, clonidine and midazolam and their metabolites [I]
A detailed description of the chromatographic conditions and detection for the analysis of three sedative drugs (morphine, clonidine and midazolam) and their metabolites (M3G, M6G and MiOH) can be found in the paper [I].

Analysis of milrinone and dobutamine [III; V; VI]
A detailed description of the chromatographic conditions and detection for the analysis of two cardiovascular drugs (milrinone and dobutamine) can be found in the paper [III].

3.3. Methods

3.3.1. Methods for the retention mechanism studies [IV]

Stock solution and working standard solution preparation
Aqueous working standard solutions were prepared in varying concentrations from 0.1, 10 to 50 µg/mL – depending on the analyte. All analyte standard solutions were prepared in either 0.1 % formic acid and 1 mM ammonium acetate in H_2O/MeOH (8/2, v/v) solution (experiments for C18 columns) or H_2O/MeOH (8/2, v/v) solution (experiments with biphenyl columns). All prepared standard solutions were filtered using a 0.45 µm regenerated cellulose filter (Captiva Econofilter, Agilent Technologies, Inc., USA) prior to injection to the LC-MS system.

Solutions for the eluent additives retention experiments were prepared with the concentration of a 0.1% eluent additive (HFIP, HFTB, PP and NFTB) in water (v/v).

Buffer composition

An aqueous standard buffer (STD) was prepared with the final concentration of 0.1% formic acid and 1 mM ammonium acetate in water. The pH of the STD buffer was 2.8.

All other aqueous solutions with eluent additive/buffers used for the retention mechanism studies (ammonium bicarbonate, ammonium acetate, NH_4F, HFTB, HFIP, DMSO, PP, NFTB and TFE) had the concentration of 5 mM of the eluent additive (in water). The required pH (8.5, 9.0 or 10.0) was reached with the addition of a sufficient amount (depending on the eluent additive) of NH_4OH solution in water. The buffers containing DFA were prepared, as shown in Table 2.

Dead time acquisition and column's performance

In order to continuously monitor the column's performance and to obtain the value of dead time (t_0), a mixture of analytes (acetophenone, acetone, benzene, naphthalene and toluene) was injected into the system. The t_0 was recorded as the retention time of the acetone peak, and the value was obtained as the average retention time from further subsequent injections.

The manufacturers recommendations for the best handling of a column were noted. However, since the research focused on expanding the knowledge of retention mechanisms in high pH environment, it was not always possible to adhere to those limitations.

Experimental solvent-water pH ($^{s}_{w}$pH) and pH_{abs} measurements in mobile phases

Along with the conventional $^{w}_{w}$pH values, also the ($^{s}_{w}$pH) and pH_{abs} values were measured. For the $^{s}_{w}$pH measurements, a Mettler Toledo InLab Micro pH-electrode was used. Before every measurement series, two-point calibration was done, using buffer solutions at pH 7 and pH 10. A glass beaker with a magnetic stirrer and 50 mL of the mobile phase (75% aqueous solution with eluent additive, 25% MeOH, v/v), was covered with a polystyrene foam cap (to avoid evaporation). All mobile phases were measured at least twice and in random order. During the series, also calibration buffer solutions were remeasured to evaluate drift. The standard deviation of all $^{s}_{w}$pH measurement results was 0.05 pH units, which indicates good consistency. The $^{s}_{w}$pH readings of the mobile phases for two eluent additives – DMSO and TFE exhibited considerable drift, so readings were taken after a constant time (5 min) from the immersion of the electrode.

A detailed method description of measuring the pH_{abs} and anchoring the $^{s}_{w}$pH measured values can be found in [IV].

In this dissertation, when the term pH is mentioned, it refers to the aqueous phase pH ($^{w}_{w}$pH), unless stated otherwise.

3.3.2. Separation of common toxicology screening compounds

Preparation of a working standard solution
All analytes in the concentration 0.1 µg/mL were prepared by the appropriate dilution of previously made sub-stocks (with a concentration 1–100 mg/mL) with MeOH and an LC-MS grade water mixture 20/80 (v/v).

3.3.3. Analysis of morphine, clonidine and midazolam and their metabolites [I]

A detailed description of the preparation of stock solutions, calibrators, quality control and real samples for the analysis of three sedative drugs (morphine, clonidine and midazolam) and their metabolites (M3G, M6G and MiOH) can be found in the paper [I].

3.2.4. Analysis of milrinone and dobutamine [III]

A detailed description about of the preparation of stock solutions, calibrators, quality control and real samples for the analysis of two cardiovascular drugs (milrinone and dobutamine) can be found in the paper [III].

4. RESULTS AND DISCUSSION

4.1. Retention mechanisms in standard solutions

4.1.1. The set of model analytes on various RP stationary phases

In order to research how novel eluent additives influence analyte retention in different reversed-phase analytical columns, a simple set of analytes with different functional groups, pK_a and log$P$ values were chosen. On the basis of these criteria, the analytes were divided into groups (Figure 5): acids (AH) and bases (B). The two main groups were then further divided into four subgroups, depending on the form in which the analyte is (predominantly) present at the researched eluent pH, in the range of 8.5 to 10.0: either as ionised analytes (A^- or BH^+) or non-ionised/neutral (AH or B) analytes.

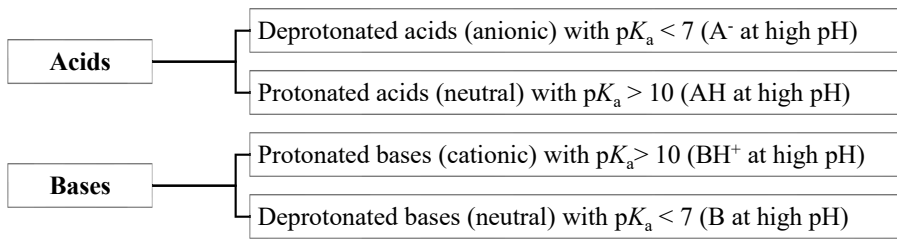


Figure 5. The selection of analytes for retention mechanism studies and the division of the analytes into groups and sub-groups based on their acidic and basic properties.

Furthermore, the measurements of the pH, after the addition of methanol were carried out, so see how well the ${}^{w}_{w}$pH correspond after the addition of the organic phase – MeOH (${}^{s}_{w}$pH). As presented in Table 4, for eluent the additives ammonium acetate and bicarbonate, HFIP, HFTB and PP no value has larger difference than 0.35 units.

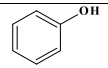
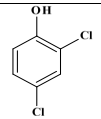
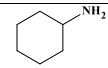
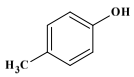
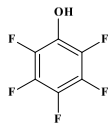
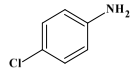
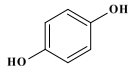
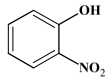
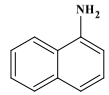
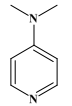
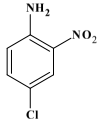
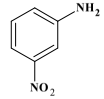
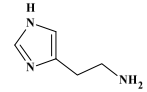
Table 4. Results of the ${}^{s}_{w}$pH (the pH of the eluent consisting of 75% aqueous solutions with eluent additive, 25% MeOH, v/v) and pH_{abs} measurements.

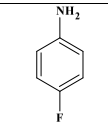
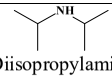
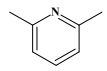
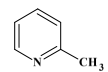
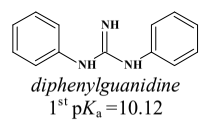
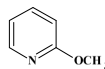
Eluent additive	${}^{w}_{w}$pH	${}^{s}_{w}$pH	pH_{abs}	$pH_{abs} - {}^{w}_{w}$pH
Ammonium acetate	8.50	8.39	8.17	-0.33
Ammonium acetate	9.00	8.92	8.70	-0.30
Ammonium acetate	10.00	9.91	9.69	-0.31
Ammonium bicarbonate	8.50	8.56	8.35	-0.15
Ammonium bicarbonate	9.00	9.01	8.80	-0.20
Ammonium bicarbonate	10.00	10.01	9.79	-0.21
HFIP	8.50	9.05	8.83	0.33
HFIP	9.00	9.39	9.18	0.18
HFIP	10.00	10.08	9.87	-0.13
HFTB	8.50	9.07	8.85	0.35
HFTB	9.00	9.42	9.20	0.20
HFTB	10.00	10.15	9.94	-0.06
PP	8.50	8.42	8.20	-0.30
PP	9.00	8.90	8.68	-0.32
PP	10.00	9.93	9.71	-0.29

The full data set of the measured retention factors is accessible via the supplementary material of paper [IV]. Data were gathered for 25 analytes (Table 5). Out of those, 13 were not ionised at the researched pH values: eight were predominantly neutral basic analytes (B) and five were predominantly neutral acidic analytes (AH). Eleven analytes were predominantly ionised – five deprotonated acidic analytes (A^-) and six protonated basic analytes (BH^+). One analyte, due to possessing two functional groups and two pK_a values, falls under both the B and BH^+ groups.

From the whole data set, the interaction between three analytes (4-nitrobenzoic acid, 4-dimethylaminopyridine and diphenylguanidine) and PP and NFTB eluent additives were studied with a C18 column.

Table 5. Overview of the 25 studied analytes including aqueous pK_a [86] and log$P$ [86] values. The analytes in the bold script have also been previously reviewed in Kipper et al. [25]. The analytes in the italic script were researched only with the eluent additives PP and NFTB.

Neutral (protonated) acids, HA	**Deprotonated acids, A^-**	**Neutral (deprotonated) bases, B**	**Protonated bases, BH^+**
 Phenol pK_a = 9.99 log$P$ = 1.46	 2,4-Dichlorophenol pK_a = 7.89 log$P$ = 3.06	 Aniline pK_a = 4.63 log$P$ = 0.90	 Cyclohexylamine pK_a = 10.63 log$P$ = 1.49
 p-Cresol pK_a = 10.30 log$P$ = 1.94	 **2,3,4,5,6-Pentafluorophenol** **pK_a = 5.53** **log$P$ = 3.06**	 4-Chloroaniline pK_a = 4.10 log$P$ = 2.25	 Piperidine pK_a =11.28 log$P$ = 0.84
 Hydroquinone pK_a = 9.96 log$P$ = 0.59	 **2-Nitrophenol** **pK_a = 7.23** **log$P$ = 1.79**	 1-Naphthylamine pK_a = 3.92 log$P$ = 2.25	 *4-dimethylaminopyridine,* pK_a =9.6 log$P$ =1.34
 **4-Chloro-2-nitroaniline** **pK_a = 17.10** **log$P$ = 2.72**		 **3-Nitroaniline** **pK_a = 2.47** **log$P$=*1.4***	 Histamine 1st pK_a = 9.68 2nd pK_a = 5.88 log$P$ = -0.70

Neutral (deprotonated) bases, B	**Protonated bases, BH^+**
 4-Fluoroaniline pK_a = 4.65 log$P$ = 1.15	 Diisopropylamine pK_a = 11.07 log$P$ =1.40
 **2,6-Dimethylpyridine** **pK_a = 6.60** **log$P$ = 1.68**	 **Pyrrolidine** **pK_a = 11.31** **log$P$ = 0.50**
 **2-Methylpyridine** **pK_a = 5.96** **log$P$ = 1.11**	 *diphenylguanidine* 1st pK_a =10.12 log$P$ =2.89
 **2-Methoxypyridine** **pK_a = 3.06** **log$P$ = 1.40**	

4.1.2. Retention mechanisms on the C18 stationary phase [IV]

4.1.2.1. Changes in analyte retention

Conventional eluent additives (ammonium acetate and ammonium bicarbonate)

For the predominantly deprotonated acids (the A^- group, analytes with pK_a <7.9), a consistent overall decrease in the retention factors with an increase of the pH (Figure 6) was observed. The reason for the shorter retention factors is the shift in the equilibria between the analyte's deprotonated form A^- (which is in the majority, at the researched eluent pH) and that of the neutral AH form. The amount of the AH form decreases with the increase of the pH, which results in the analyte being poorly retained on the C18 phase. Overall, the retention factors obtained with the ammonium bicarbonate were shorter than those obtained when ammonium acetate was used as the eluent additive. The difference arises due to slightly higher $^{S}_{W}$pH values of the ammonium bicarbonate buffer (Table 4), which means even lesser part of the analyte is in the AH form and therefore is retained less.

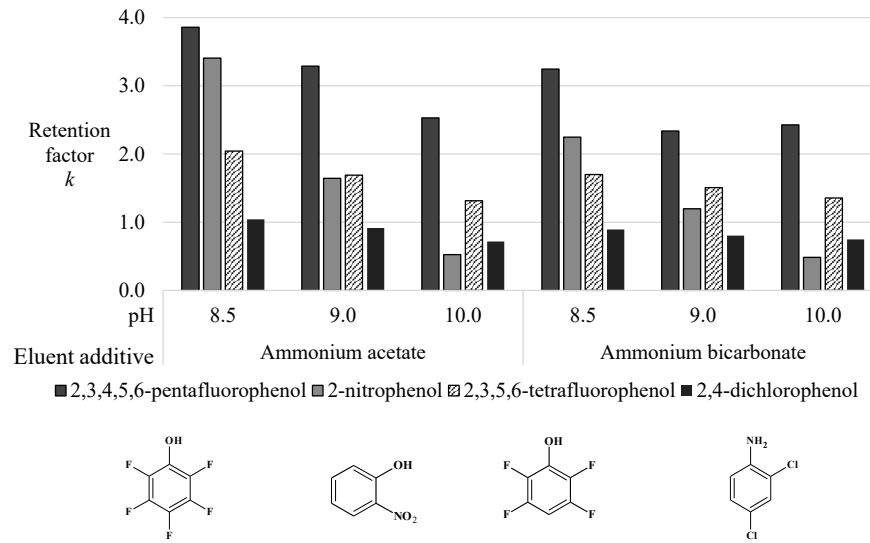


Figure 6. Retention factors of the analytes from the deprotonated acidic (A^-) group when using ammonium acetate and bicarbonate as eluent additives at the pH values of 8.5, 9.0 and 10.0.

As for neutral acids (the AH group, analytes with pK_a >9.9), the retention factors somewhat decreased with the increase in the pH; however, no significant difference was observed between the eluent additives (Figure 7). The decrease in retention may indicate a decrease in the analyte's AH form (as the pK_a values

of the majority of the analytes were around 10), therefore the analyte becomes more polar and is less retained on the non-polar C18 stationary phase.

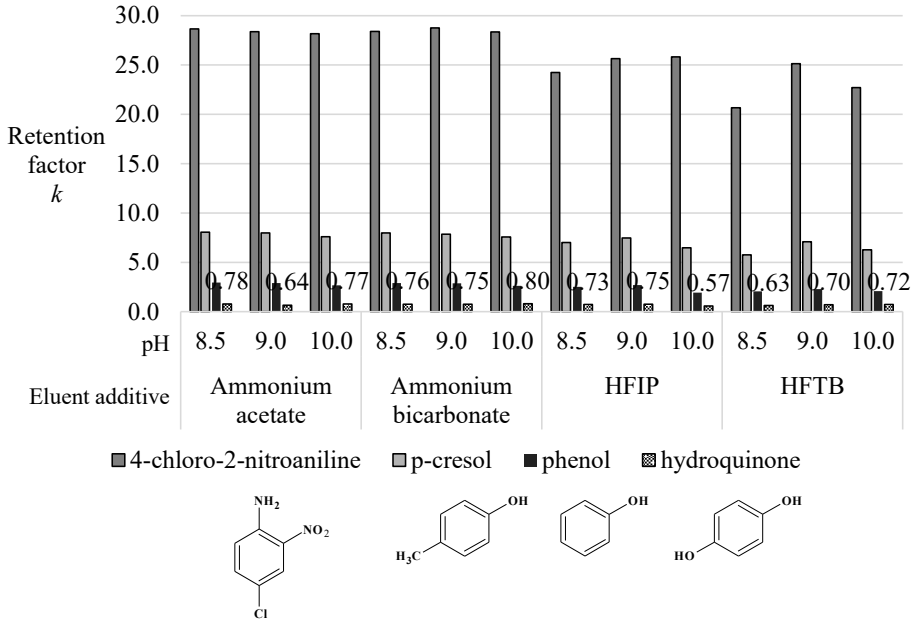


Figure 7. Retention factors of the analytes from the neutral acidic (AH) group when using different eluent additives at the pH values of 8.5, 9.0 and 10.0. Due to a very low retention factor values for hydroquinone, also the numerical values are shown.

The same as the acids in the AH neutral group, also the neutral basic analytes (B group, analytes with pK_a ≤6.6) showed stable retention factors across the three researched eluent pH values (Figure 8). When ammonium bicarbonate was used as the eluent additive, the variation in retention times was higher; however, no clear relationship could be observed.

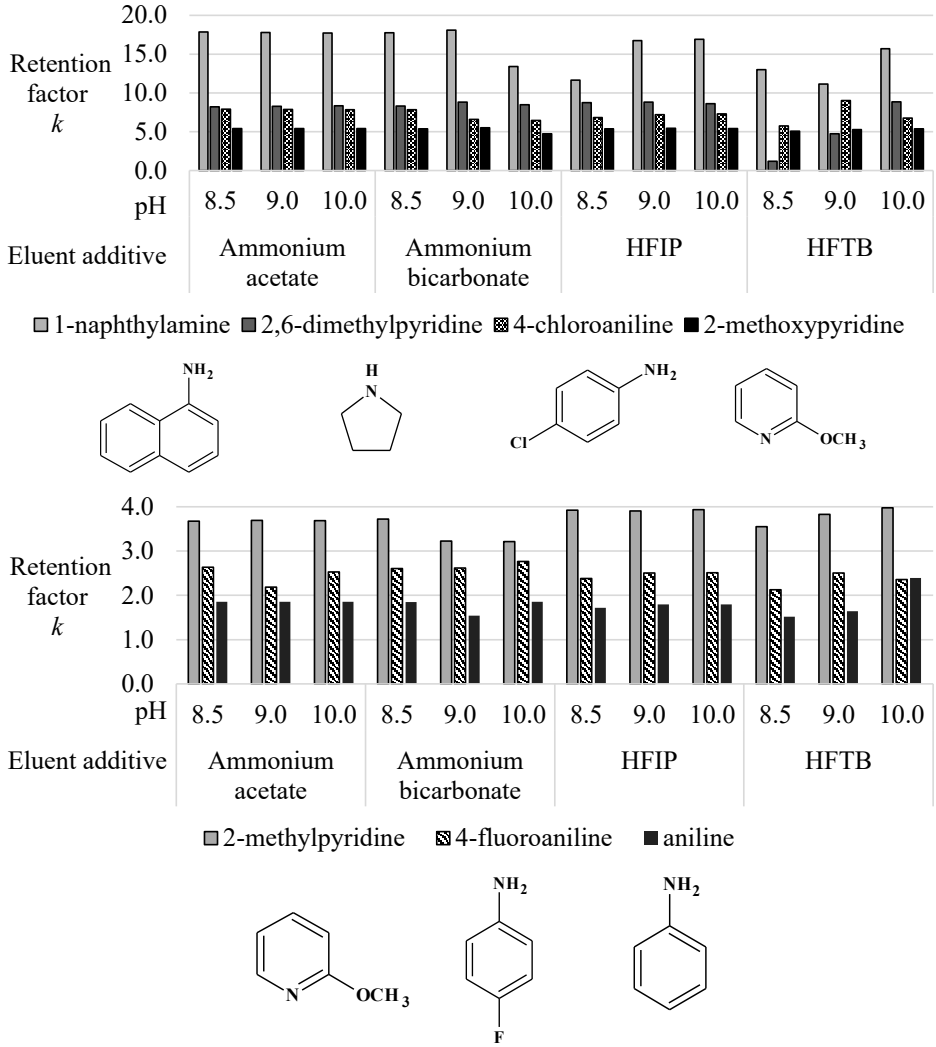


Figure 8. Retention factors of the analytes from the neutral basic (B) group when using both conventional (ammonium acetate and bicarbonate) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0.

For the basic polar analytes' group (BH^+, analytes with $pK_a \geq 9.6$), an increase in the retention factors was observed with an increase in the pH (Figure 9). Both conventional buffers (especially ammonium acetate) had a steep increase (4–8 times) in retention factors when comparing values obtained at eluent the pH 9.0 and 10.0. This "jump" can be explained by the change in the majority of analyte's form – to B at the pH 10.0 (which is close to the analyte's pK_a), while at the pH 8.5 and 9.0, the larger part (if not all) of the analyte was in its protonated form BH^+, thus polar and poorly retained. Ammonium acetate

showed slightly lower retention factors than those obtained with ammonium bicarbonate. The difference in retention can again be explained with difference in ${}^{s}_{w}$pH values (Table 4) for ammonium acetate and bicarbonate buffers. A higher ${}^{s}_{w}$pH (in case of ammonium bicarbonate) means larger part of analyte is in B form and thus is retained more on in C18 column.

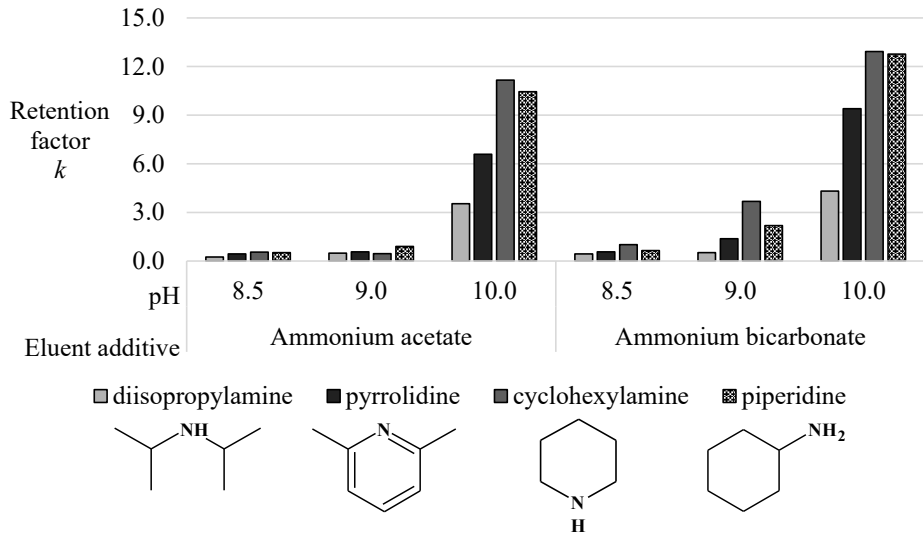


Figure 9. Retention factors of the analytes from the protonated basic (BH^+) group when using ammonium acetate and bicarbonate eluent additives at the pH values of 8.5, 9.0 and 10.0.

Fluoroalcohols HFIP and HFTB

When compared to commonly used eluent additives (ammonium acetate and bicarbonate), the retention factors obtained when using HFIP and HFTB as eluent additives created different trends for different analytes and analyte groups. The main trends observed (for the same analyte) were also different between HFIP and HFTB (unlike when ammonium acetate and bicarbonate were used).

Overall, lower retention factors were seen compared to conventional eluent additives (Figure 10) in the deprotonated acid (A^-) group and mostly a decrease in the retention factors with an increase in the pH was observed). Both of the two analytes – fluorinated phenols – 2,3,4,5,6-pentafluorophenol and 2,3,5,6-tetrafluorophenol – have similar hydrophobicity and the same pK_a value (5.53). Thus, they also display similar behaviour and retention factors. For both analytes, using HFIP as the eluent additive decreased their retention factors by 2–4 times and when HFTB was used as the eluent additive, the retention factors decreased by 1.4–6 times when compared to ammonium acetate at the same pH

value. The overall decrease in retention for acidic analytes, especially those which are ionised, has been observed by Kipper et al. [25]. This supports the proposed theory that acidic analytes both compete with novel eluent additives for a place on the stationary phase and are repelled by the fluorous phase that novel eluent additives create on top of the stationary phase. Furthermore, decrease in retention with increase of the pH of mobile phase arises due to pK_a values of HFIP and HFTB. As acidic analytes larger fraction of HFIP and HFTB becomes deprotonated and thus increasingly repulsed to already deprotonated acidic analytes.

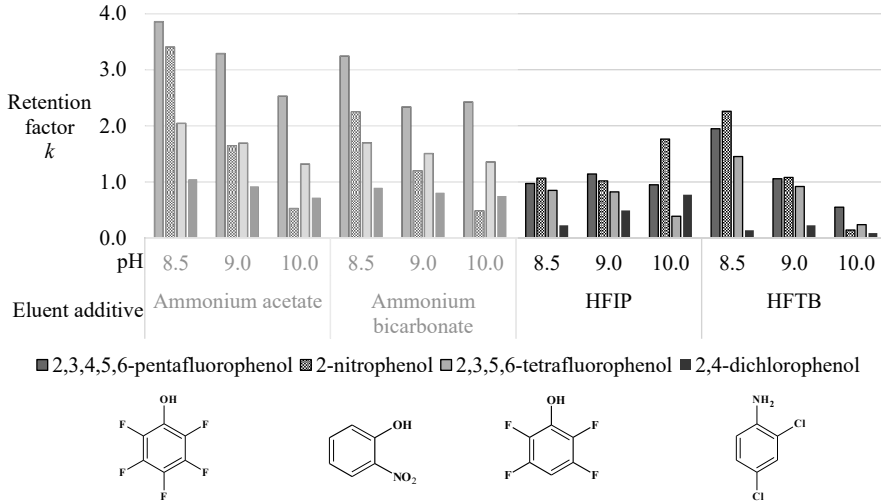


Figure 10. Retention factors of the analytes from the deprotonated acidic (A^-) group when using both conventional (ammonium acetate and -bicarbonate – greyed out as the data were already presented in Figure 6 and were added here as comparison) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0.

As for the neutral acidic analyte group AH, similarly to the conventional eluent additives – ammonium acetate and bicarbonate (Figure 7) – also novel fluorinated eluent additives HFIP and HFTB presented no clear trend of decrease or increase in the retention factors (Figure 11), even if the retention factors presented a larger variation between different eluent pH values. Overall, with fluorinated additives a decrease in the retention factors was observed. This implies that the previously mentioned relationship of competition between the acidic analytes (more apparent in the A^- group, Figure 6 and Figure 10) and novel fluoro-alcoholic eluent additives also apply in the case of analytes, which are either in the neutral form or ionised only to a small degree.

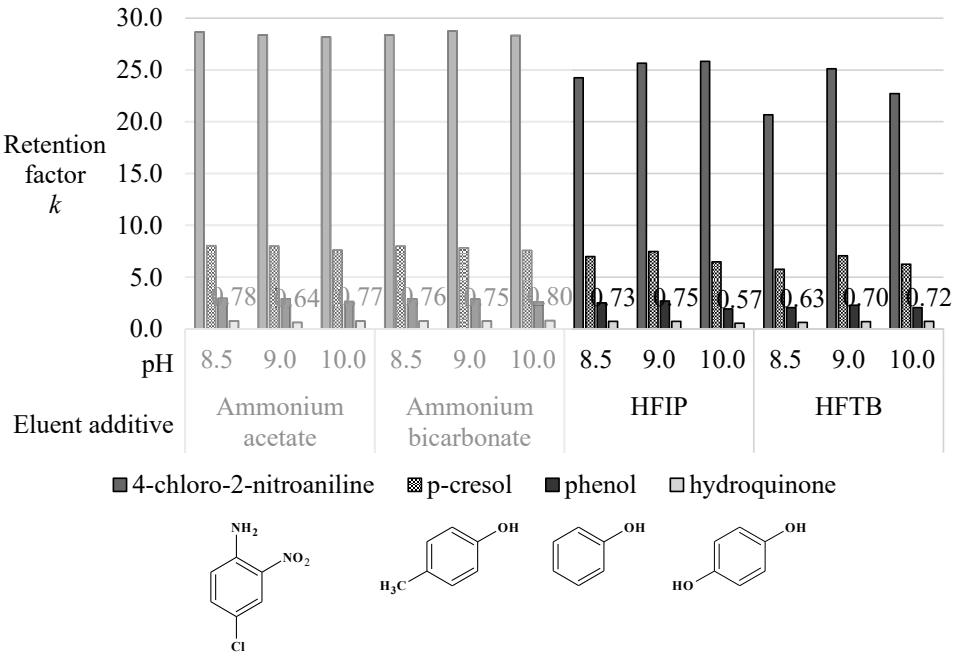


Figure 11. Retention factors of the analytes from the neutral acidic (AH) group when using both conventional (ammonium acetate and -bicarbonate – greyed out as the data were already presented in Figure 7 and were added here as comparison) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0. Due to the very low retention factor values for hydroquinone, also the numerical values are shown.

In the case of the neutral basic analytes group (B, Figure 8), neither a significant change in the retention factor nor any trends were apparent when HFIP and HFTB were used as eluent additives. The only analyte from the group, which displayed a clear trend of increase in the retention factors with an increase of the pH, only when HFTB was used as the eluent additive, was the strongest basic analyte – 2,6-dimethylpyridine (pK_a 6.60) from the B analyte group.

Most of the analytes from the protonated bases group (BH^+) displayed a significant increase in the retention factors with the increase of the eluent pH (Figure 12), as well as presented visually significant differences in elution patterns between different eluent additives. This can be explained by both a change of the mobile phase pH and the ion-pairing properties of fluoroalcohols.

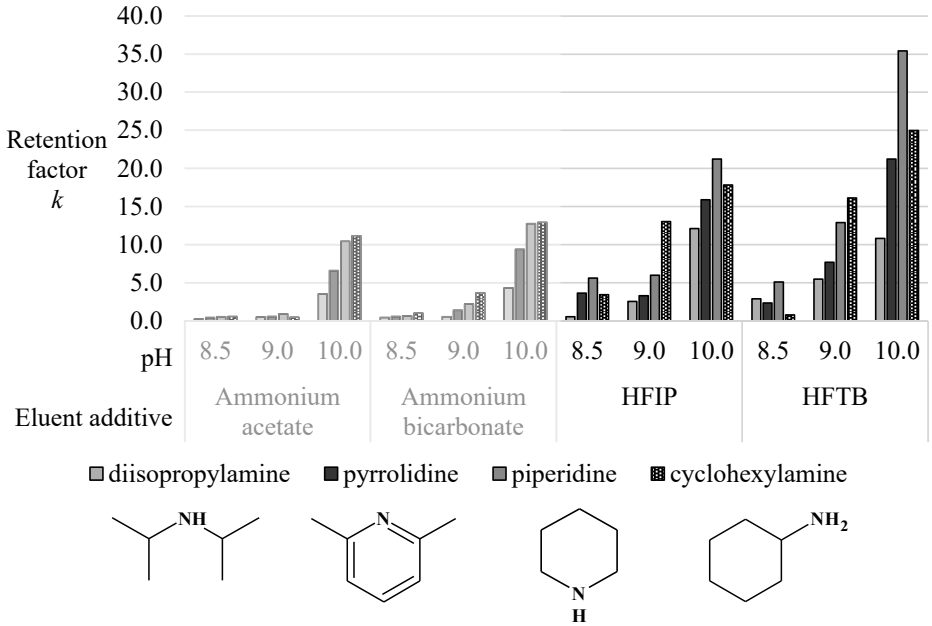


Figure 12. Retention factors of the protonated basic (BH^+ group) analytes when HFIP and HFTB eluent additives are used and the pH is changed from 8.5 to 10.0 as compared to common eluent additives (ammonium acetate and -bicarbonate – greyed out as the data were already presented in Figure 9 and were added here as comparison).

Both HFIP and HFTB strongly increased retention for the protonated basic group (BH^+) analytes, when compared to ammonium acetate or carbonate at the same pH values. When HFIP is compared to HFTB, the latter demonstrated larger retention factors. The most significant difference in the retention factors was observed for cyclohexylamine – an analyte, which is the weakest base in the group (has the smallest pK_a,). For cyclohexylamine, the retention factor increased by 28 times when HFIP was used and by 35 times when HFTB was used as the eluent additive at the pH 9.0 when compared to the retention factors of ammonium acetate at the same pH.

DMSO and fluoroalcohol TFE

Aqueous solutions made with either TFE or DMSO as eluent additives had very little to no buffering capacity. Thus, TFE and DMSO solutions needed a minimal amount of NH_4OH to reach the needed pH values of 8.5 and 9.0. Initially, the main cause for the instability of the pH over time was deemed to be the evaporation of NH_4OH, and thus, the average stability of the pH for the DMSO/TFE solutions was evaluated (with measurements of the pH over time) to be no more than 3 hours. However, the main cause of the change in the pH,

as well as inexplicable results, was the addition of an organic solvent. It changed the ${}^{s}_{w}$pH dramatically, as shown in Table 6.

Table 6. Results of the ${}^{s}_{w}$pH (the pH of the eluent consisting of 75% aqueous solutions with an eluent additive, 25% MeOH, v/v) and pH_{abs} measurements. The concentration of the eluent additive in the water solution was 5 mM.

Eluent additive	${}^{w}_{w}$pH	${}^{s}_{w}$pH	pH_{abs}	$pH_{abs} - {}^{w}_{w}pH$
DMSO	8.50	7.68	7.46	-1.04
DMSO	9.00	7.62	7.40	-1.60
DMSO	10.00	9.92	9.70	-0.30
TFE	8.50	7.45	7.23	-1.27
TFE	9.00	7.59	7.38	-1.62
TFE	10.00	9.94	9.73	-0.27

Both DMSO and TFE essentially behave as neutral compounds at the researched eluent pH values. This is different from other researched eluent additives, which behave as acids. Therefore, DMSO and TFE cannot form a buffer solution, and the only buffering capacity was dependent on the small amounts of added NH_4OH. Since the ${}^{w}_{w}$pH and pH_{abs} values of these mobile phases should be considered as dubious, DMSO and TFE are not suited as eluent additives for researching and use as basic buffers with ${}^{w}_{w}$pH values below 10.

Novel fluoroalcohols: perfluoropinacol (PP) and NFTB

In parallel, also two additional promising fluorinated eluent additives: PP and NFTB were studied for a smaller set of analytes. For protonated basic (BH^+) analytes, PP demonstrated a dramatic increase in retention (Figure 13). At the pH 8.5, diphenylguanidine was retained 41 for times longer than when PP was used as the eluent additive (in comparison to ammonium acetate). Additionally, diphenylguanidine's retention decreased at the pH 10.0, which is not the expected relationship for a basic protonated analyte. The likely reason for this is structural differences – unlike HFIP, HFTB and NFTB, which have one hydroxy group, the PP has two, and thus also two pK_a values. At the pH 8.5 and 9.0, the majority of perfluoropinacol in the eluent is in a monoanionic form (because the pK_{a1} value is 5.95 in water). Since PP's second (pK_{a2}) value is 10.42 (in water), it can be assumed that, at the pH 10.0, the second OH group undergoes the loss of a proton, at least partially. Diphenylguanidine's pK_a value is 10.12. At the pH 10.0 also the amount of its deprotonated form increases and thus becomes less attracted to the increasingly anionic PP at pH 10.0.

As for other analyte groups, the relationships observed fell in line with previous conclusions: analytes in the deprotonated acid (A^-) group had a decrease in the retention factors with an increase in the mobile phases' pH. In

contrast, the retention factors of neutral analytes (AH and B groups) remained constant and similar to the values obtained with the ammonium acetate buffer.

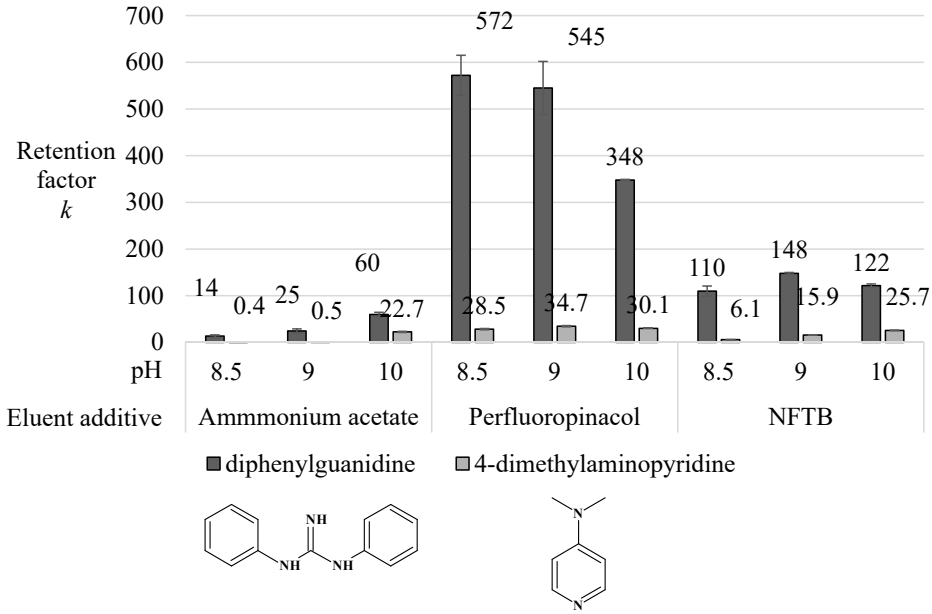


Figure 13. Retention factors of the protonated basic (BH^+ group) analytes when PP and NFTB eluent additives are used, and the pH is changed from 8.5 to 10.0. Novel eluent additives are compared with the common eluent additive ammonium acetate.

When NFTB was used, diphenylguanidine showed an increase in the retention factors by 8 times – (at the eluent's pH 8.5). The large increase in the retention factors was also observed for 4-dimethylaminopyridine (Figure 13). Since PP is retained on the C18 analytical column to a much larger extent than NFTB (which elutes at the beginning of a chromatographical run, Figure 20), it can be theorised that PP (the same as HFIP and HFTB) create a fluorous layer on the stationary phase.

4.1.2.2. Analyte ionisation efficiency

For the analytes, which were detected with an MS, signal intensity changed using different eluent additive buffers. However, the differences (observed by comparing peak areas, Figure 14 and Table 7) depended highly on the analyte, eluent additive and the pH studied. As the focus of research was different retention mechanisms, the MS signal intensities (presented as single measurement peak areas) are provided as additional information to give general impression.

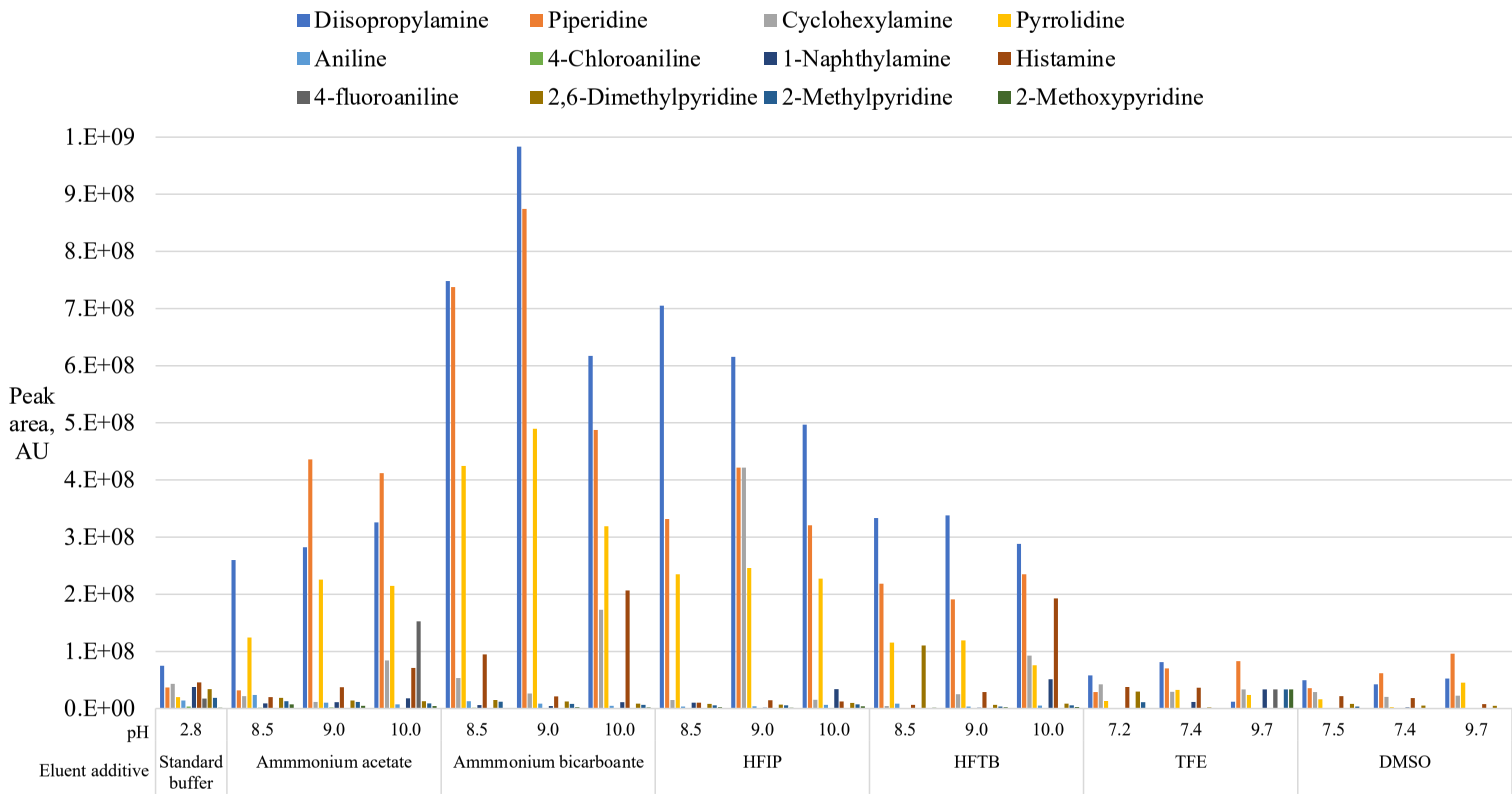


Figure 14. Peak areas of the analytes (presented bar chart format), depending on the eluent additive and the pH of the eluent. For DMSO and TFE solutions $_{w}^{s}$pH values are given, as they represent the precise pH of the mobile phase.

Table 7. Peak areas of the analytes depending on the eluent additive and pH of the eluent. Cells with peak area values "0" indicate that no peaks were detected. *For DMSO and TFE the $^{s}_{w}$pH values are given, as they represent the precise pH of the mobile phase.

Buffers	**STD**	**Ammonium acetate**			**Ammonium bicarbonate**			**HFIP**		
pH	2.8	8.5	9.0	10.0	8.5	9.0	10.0	8.5	9.0	10.0
Analytes	Peak area									
Diisopropylamine	74858392	259827791	282304764	325962012	748165281	983435198	617220544	704997132	615654076	496892865
Piperidine	36951200	31829927	435841644	412055740	737456548	874537709	487681984	331672124	421579291	320686646
Cyclohexylamine	43343684	21857296	11579111	84293752	53177938	26238147	172813113	14993795	421579291	15199699
Pyrrolidine	19871073	124472464	225801955	214569904	424734254	489621143	319021394	235068562	245899209	227365482
Aniline	14028476	23930212	10369357	7485969	12633819	8677645	4872316	3438511	4061713	6456661
4-Chloroaniline	3693034	1433397	1623281	663150	1180256	710624	0	453822	35633	544774
1-Naphthylamine	37571474	9192311	11054570	17838959	5829051	4248195	11219666	10369670	1364609	33798754
Histamine	45640498	19966293	37233899	71218374	94795867	21389881	206610641	10374512	14636993	12412524
4-Fluoroaniline	17323060	824948	880179	152739236	704823	24661	666824	65598	29274	40312
2,6-Dimethylpyridine	34076735	18709230	14002765	12784909	15008685	12270164	8648053	7969485	6714204	9648835
2-Methylpyridine	18745688	12801912	11330318	9158476	12105982	8185669	6455491	5556821	5442703	7304771
2-Methoxypyridine	1261010	7494630	4839327	4500942	0	2358877	1679280	2232143	1338285	3943342

Buffers	**HFTB**			**DMSO***			**TFE***		
pH	8.5	9.0	10.0	7.5	7.4	9.7	7.2	7.4	9.7
Analytes	Peak area								
Diisopropylamine	333312822	337862293	288311488	49717293	42454072	52454315	58018942	81087873	12067577
Piperidine	218435887	191120905	234928250	35466015	61936528	96026022	28953597	70110863	82829472
Cyclohexylamine	4507696	24881479	92735730	29020517	20504758	22304715	42269804	29229098	33285091
Pyrrolidine	115431127	119385673	75687968	16271245	2222751	45193414	13261425	32765691	23676086
Aniline	8667960	3349964	5203304	0	0	0	663413	481335	0
4-Chloroaniline	131011	325143	714582	0	108147	0	242552	91390	136394
1-Naphthylamine	297066	1581832	51123196	452440	1785694	0	171847	11390666	33285091
Histamine	6326567	28825185	192703709	21655306	18380756	7513662	37847485	36368431	890799
4-fluoroaniline	24872	44896	1125172	536131	309621	841146	591835	735965	33285091
2,6-Dimethylpyridine	110484905	6642802	8427954	8219692	5121554	4807366	29672122	1619305	890799
2-Methylpyridine	127683	3369076	5692894	3571338	0	0	11261215	936881	33285091
2-Methoxypyridine	1569807	2267639	2524737	0	0	0	255015	495704	33285091

As already stated, the recorded signal varied greatly between analytes, eluent additives and the mobile phase pH. When comparing eluent additives, in general, the largest signals were observed for ammonium bicarbonate-based eluents. This was true for analytes diisopropylamine, pyrrolidine, piperidine, and histamine.

At low pH (STD buffer), the studied basic analytes should be present in the protonated BH^+ form and should be very well ionised. This is true for the analytes in B group, but not for the BH^+ group analytes – they yield much higher signals when mobile phase pH is also high. This observed effect is an example of so-called wrong-way ionisation [87].

As ammonium acetate is one of the most commonly used eluent additives, it was used as a point of reference for the comparison of analyte signal responses. When novel eluent additives HFIP and HFTB were used (at the same pH values), it showed that in 26% of the measurements, there was an increase in the signal (with a range between 1.1 to 36-fold). However, rest of the results showed decrease (up to <10% of signal) in the peak area of the analytes of the area obtained with ammonium acetate.

The majority of the increased signal was observed for ionised basic (BH^+) analytes when ammonium bicarbonate, HFIP and HFTB were used. Because the BH^+ group analytes are already protonated in solution, they are more easily converted into gas-phase ions and thus in general have better signal than others. The largest increase was obtained for cyclohexylamine using HFIP, at the pH 9.0 (36-fold), however, for the same analyte – with other HFIP buffer pH values (8.5 and 10.0), the signal was lower than when ammonium acetate was used at respective pH levels.

DMSO as the eluent additive resulted in significant signal suppression – no peaks could be obtained for aniline, 4-chloroaniline, 1-napthylamine and 2-methoxypyridine. Both DMSO and TFE gave the lowest signals when compared to other eluent additives. DMSO has been reported as having a positive influence on the signal intensity in MS; however so far it seems this is observed only in the field of proteomics [32]–[34]. Furthermore, due to the high boiling point of DMSO, the default used ion source parameters, uniform for all analytes and eluent additives, are possibly not optimal for DMSO. TFE has been reported to both increase [26], [88] the analyte signal and suppress it for oligonucleotides [89]. However, the signal enhancement for (hydrophobic) compounds was observed in the working ESI in the negative detection mode or in the presence of other ion-pairing reagents. Additionally, the decreased signal response was observed in these cases for more hydrophilic analytes. [26], [88]

In conclusion – in general higher ionisation efficiency can be obtained with the high pH mobile phases for polar basic analytes and using HFIP and HFTB, however more rigorous experiments should be conducted to obtain more specific conclusions.

4.1.3. Retention mechanisms on the biphenyl stationary phase

The changes in the retention of the analytes on biphenyl columns due to the change of the eluent additive and shift in the eluent pH tended to follow similar patterns as seen previously for the C18 column (Table 8). The patterns were: for non-ionised analytes (such as AH and B at a high mobile phase pH), the retention is not significantly influenced by the change of the eluent pH or eluent additive. For the ionised acidic analytes (A^- group) the retention decreased with the increase in the pH and decreased further when novel fluorinated eluent additives were used. For protonated (polar) basic analytes (BH^+), the influence was opposite to acidic analytes – the retention increased with the increase in the pH and further increased when novel fluorinated eluent additives were used.

Further discussion will highlight the differences in retention between the C18 and biphenyl stationary phases among the four different analyte groups.

Table 8. A list of the detected analytes retention factors in experiments with the biphenyl column using ammonium acetate, ammonium bicarbonate, HFIP and HFTB as eluent additives. Cells marked with * indicate that no analyte peaks were detected.

Analyte	Ammonium acetate			Ammonium bicarbonate			HFIP			HFTB		
	8.5	**9.0**	**10.0**	**8.5**	**9.0**	**10.0**	**8.5**	**9.0**	**10.0**	**8.5**	**9.0**	**10.0**
A^- group												
2,4-dichlorophenol	30.62	15.54	2.60	23.30	12.59	2.45	15.04	6.80	0.85	15.74	7.19	0.25
2,3,4,5,6-Pentafluorophenol	2.10	0.95	0.55	1.55	0.80	0.51	0.35	0.30	0.25	0.08	0.15	0.15
2-Nitrophenol	2.07	0.90	0.40	3.70	1.80	0.38	0.55	0.25	0.30	0.45	0.55	0.15
2,3,5,6-Tetrafluorophenol	1.40	0.65	0.35	1.10	0.55	0.36	0.25	0.20	0.20	0.10	0.15	0.10
AH group												
Phenol	4.40	3.35	*	4.05	3.55	3.16	4.15	3.55	3.40	3.75	3.55	2.55
p-Cresol	10.44	10.34	9.84	9.79	8.34	8.40	8.94	8.74	7.39	8.93	*	*
Hydroquinone	0.85	0.85	0.65	0.91	0.85	*	0.80	0.80	*	0.80	0.75	*
4-Chloro-2-nitroaniline	0.30	0.10	0.30	0.20	0.10	0.00	0.05	0.10	0.20	0.00	0.00	0.20
BH^+ group												
Heptylamine	18.98	43.36	119.70	26.11	54.65	127.84	96.62	129.04	232.23	177.64	172.10	285.35
Octylamine	51.27	121.64	340.26	72.49	146.47	183.72	272.50	348.40	534.07	436.22	439.04	630.87
Diisopropylamine	1.90	5.10	16.89	2.95	6.89	18.60	13.64	15.14	41.01	19.68	20.28	56.05
Piperidine	1.70	6.00	27.93	3.10	7.94	34.68	13.34	16.64	59.74	19.58	22.38	84.22
Cyclohexylamine	2.90	7.34	22.23	4.40	8.94	20.93	20.23	21.58	42.76	29.28	26.88	54.75
Pyrrolidine	1.15	3.60	20.73	1.85	5.60	21.74	9.29	11.94	44.46	10.79	14.89	55.95
B group												
Aniline	3.90	3.45	3.25	3.70	3.25	3.38	3.60	3.45	3.30	3.50	3.35	3.00
4-Chloroaniline	0.20	0.10	0.05	0.15	0.10	0.00	0.00	0.10	0.05	0.00	0.00	0.00
1-Naphthylamine	38.65	32.42	28.83	35.67	30.27	30.83	34.97	32.02	26.73	33.32	30.42	23.68
4-Fluoroaniline	0.77	0.70	0.70	0.85	0.60	0.57	0.60	0.70	0.80	0.75	0.70	0.70
2,6-Dimethylpyridine	1.56	9.19	10.19	9.19	6.19	5.04	7.49	7.79	6.80	14.29	13.14	11.59
2-Methylpyridine	4.90	4.30	5.50	4.50	4.40	4.19	4.80	4.20	*	1.40	4.10	*
2-Methoxypyridine	16.74	15.69	14.29	16.29	14.89	14.84	16.69	16.04	15.47	16.64	15.49	13.29
3-Nitroaniline	0.20	0.10	0.30	0.15	0.10	0.00	0.05	0.00	0.05	0.10	0.00	0.00

The deprotonated acids (polar) analyte group

The relationship – decrease in retention with the increase in the pH and a further decrease in retention when HFIP and HFTB were used as eluent additives (Figure 10) remain the same for both the C18 and biphenyl columns.

Two similar A^- group analytes (2,3,4,5,6-pentafluorophenol and 2,3,5,6-tetrafluorophenol) are far less retained with biphenyl column (Figure 15). The largest difference in the retention factors appear when HFTB is used as the eluent additive, at the eluent pH 8.5. For 2,3,4,5,6-pentafluorophenaol, retention decreased 26 times and for 2,3,5,6-tetrafluorophenaol it decreased 14 times. The halogen and π-bond interaction has been described in the literature with the help of the σ-hole [90] – as intermolecular interaction between an electron-rich aromatic ring and an electron-poor region of bonded halogen [91], it should present itself also in the case of the biphenyl column. Likewise, the biphenyl column has high hydrogen-bond capacity. However, it seems that the ionic interaction of the negatively charged analyte has a more substantial influence on retention.

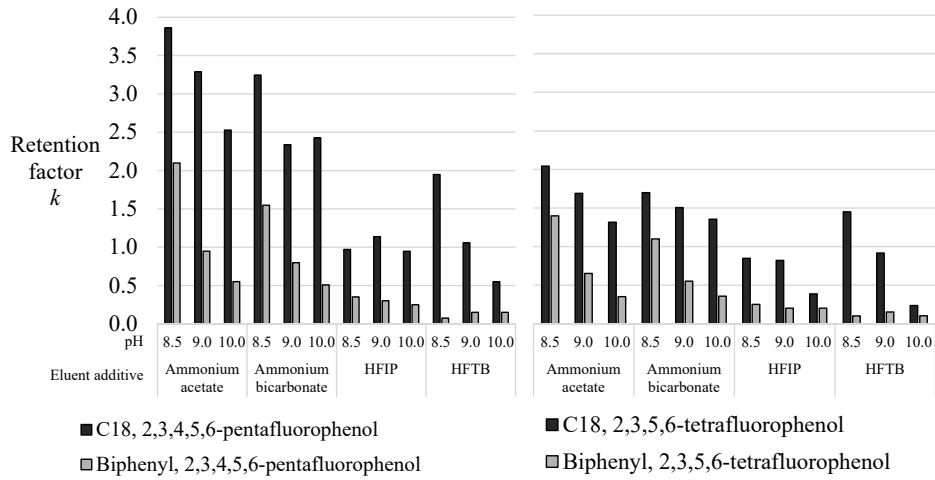


Figure 15. Obtained retention factors with the C18 and biphenyl analytical column for 2,3,4,5,6-pentafluorophenol and 2,3,5,6-tetrafluorophenol using both conventional (ammonium acetate and bicarbonate) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0.

For the analyte 2-nitrophenol the increase in the retention factors was observed when ammonium bicarbonate was used as the eluent additive at the pH 8.5 and 9.0, but lower retention factors when the biphenyl column was used at all other eluent and pH combinations (Table 8). For 2,4-dichlorophenol, a large increase in the retention factor was observed (Figure 16) on the biphenyl column. Possibly having two halogen atoms (chlorine) in the molecule aided the retention of the basis of the halogen and π-bond attraction.

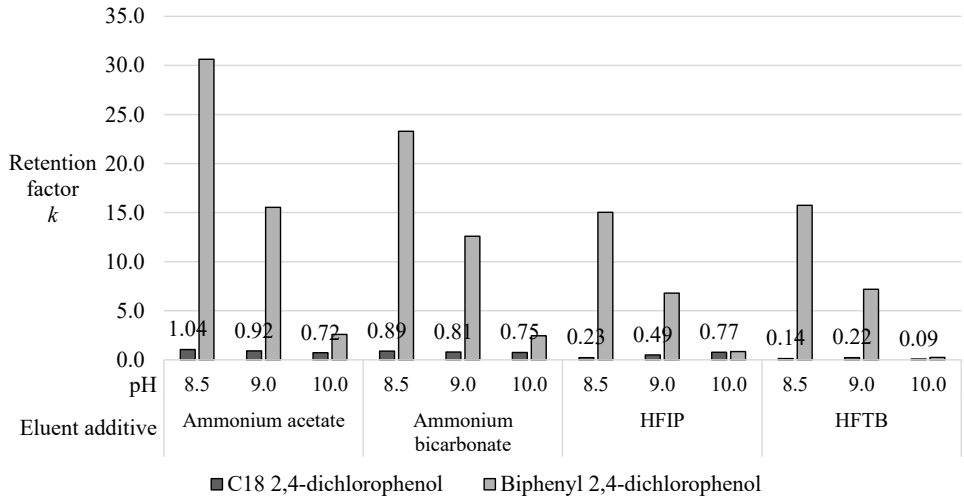


Figure 16. Obtained retention factors with the C18 and biphenyl analytical columns for 2,4-dichlorophenol using both conventional (ammonium acetate and bicarbonate) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0. Due to low retention factor values obtained with the C18 column, also the numerical values are shown.

The protonated acids (neutral) analyte group

With the exception of 4-chloro-2-nitroaniline, all other – phenol (Figure 17), p-cresol and hydroquinone from the neutral acidic analytes (AH) group had larger retention when the biphenyl column was used (with the exception of hydroquinone when ammonium acetate was used as the eluent additive at the pH 10.0) due to the additional hydrogen-bond and π-π bonding. While, the increase was rather insignificant and remained within 1.1-1.8 times for all analytes, the difference between retention factors obtained with C18 and biphenyl column was the greatest at the eluent pH 8.5 and decreased for biphenyl with an increase of the pH. This is due the mobile phase pH value nearing the value of the analyte's pK_a. This means both for HFIP and HFTB as well as for acidic analyte that larger fraction of compound becomes deprotonated and thus polar. The repelling interactions outweigh the influence of π-π bonding.

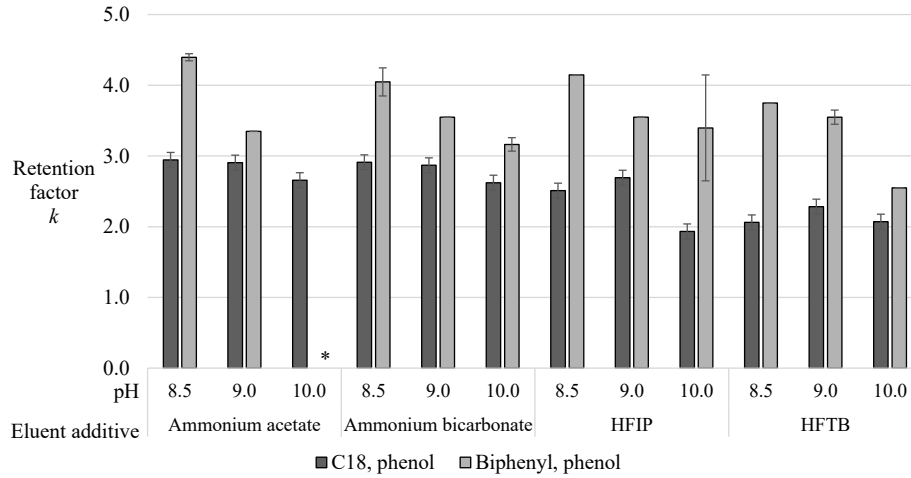


Figure 17. Obtained retention factors with the C18 and biphenyl analytical columns for phenol using both conventional (ammonium acetate and bicarbonate) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0. * – no analyte peaks were recorded.

The protonated (polar) basic analyte group

All analytes in the basic protonated analyte group demonstrated an increase in retention factors when the biphenyl column was used (Table 8), even if none of the analytes had an aromatic ring (Table 5) in their structure, thus do not have π-π interactions on biphenyl column. However, all analytes could still act as both hydrogen bond donors and acceptors. The relationship – an increase in retention with an increase of pH of the eluent and then, further increase if HFIP and HFTB were used as eluent additives in comparison to conventional ammonium acetate and bicarbonate – remained the same as for C18 column. However, unlike with C18 column (Figure 9), the retention factors increased more gradually with the increase of eluent pH (Figure 18).

The largest increase in retention factors (when comparing C18 to the biphenyl stationary phase) was observed for analytes diisopropylamine and cyclohexylamine. For cyclohexylamine, 38 times increase in retention factor was observed when using HFTB at pH 8.5, and 16 times increase when comparing ammonium acetate at pH 9.0 as eluent. For diisopropylamine largest increase: 28 times, was observed when HFIP was used as eluent additive at pH 8.5. Both analytes had similar log*P* values – 1.49 for cyclohexylamine and 1.40 for diisopropylamine and thus are more lipophilic than the other 2 analytes in the group – piperidine (log*P* 0.84) and pyrrolidine (log*P* 0.50). Piperidine and pyrrolidine demonstrated an increase in retention, but in the range of 2–7 times.

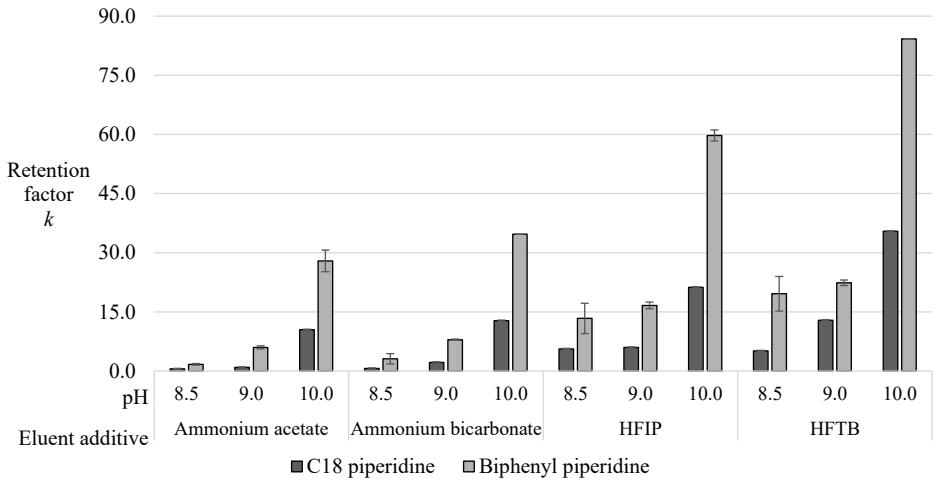


Figure 18. Obtained retention factors with C18 and biphenyl analytical column for piperidine using both conventional (ammonium acetate and bicarbonate) and novel (HFIP, HFTB) eluent additives at pH values of 8.5, 9.0 and 10.0.

The deprotonated (neutral) basic analyte group

The deprotonated basic analyte group (B) was split – two analytes (4-chloroaniline, and 3-nitroaniline) were more retained on the C18 column, while six analytes (aniline, 4-fluoroaniline, 2,6-dimethylpyridine, 2-methylpyridine, 1-naphtylamine and 2-methoxypyridine) were more retained on the biphenyl column. However, the majority saw no greater difference in the retention factors than by 2 times. Exceptions to that were the analytes which were well retained on the C18 column – 4-chloroaniline and 3-nitroaniline, but eluted around the dead time when the biphenyl column was used.

Additionally, all analytes (except 4-fluoroaniline), which were retained more on the biphenyl column, saw also a subtle decrease in retention times with the increase of the eluents' pH (Figure 19 and Table 8).

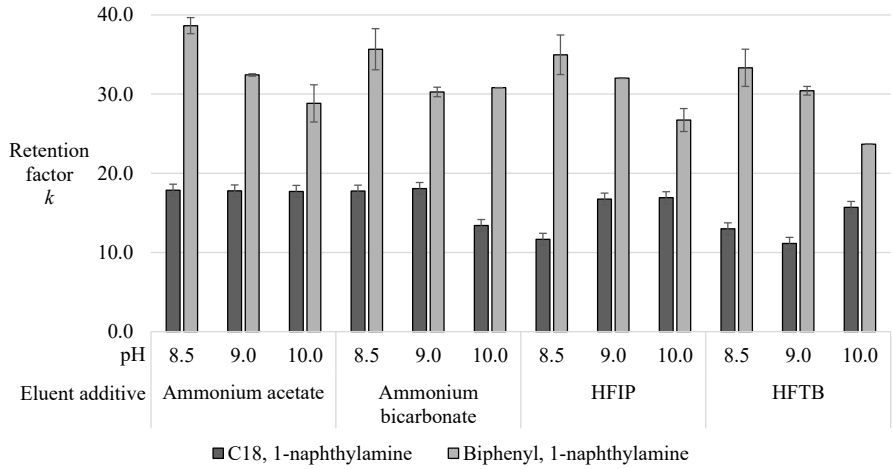


Figure 19. Obtained retention factors with the C18 and biphenyl analytical column for 1-naphtylamine using both conventional (ammonium acetate and bicarbonate) and novel (HFIP, HFTB) eluent additives at the pH values of 8.5, 9.0 and 10.0.

Summarising remarks regarding the retention mechanisms observed on the biphenyl stationary phase

Since the majority of studied simple model analytes (with the exception of four) have an aromatic ring in their structure (Table 5), it was expected to observe a larger retention due to π-π interactions between aromatic analytes. 16 analytes were retained more on the biphenyl stationary phase – also including those without an aromatic ring (from protonated basic analyte group). The five other analytes, which were better retained with on the C18 column, had either a nitro ($-NO_2$) group in their structure (4-chloro-2-nitroanailine, 2-nitrophenol and 3-nitroaniline) or multiple halogen atoms (2,3,4,5,6-pentafluorophenol and 2,3,5,6-tetrafluorophenol). Both neutral analyte groups (deprotonated acids and protonated bases) did not have as uniform retention factors as seen before on the C18 column and the majority of analytes demonstrated a decrease in retention with an increase in the pH.

4.1.4. Retention of eluent additives on the C18, biphenyl and PFP stationary phases and ionisation

Changes in the eluent additive retention on the C18 stationary phase

The retention of HFIP and HFTB was strongly influenced by the pH of the mobile phase [25]. Thus the retention of three eluent additives TFE, PP and NFTB on the C18 stationary phase was evaluated also at the different pH values 8.5, 9.0 and 10.0.

A decrease in retention time with an increase in the mobile phase's pH was observed for both NFTB (Figure 20) and PP (Figure 21). NFTB saw the retention time change from 5.7 min at pH 8.5 to 3.5 min at the pH 10.0. The

change was more evident for HFIP and HFTB [25] as their pK_a values are close to the eluent pH than pK_a value of NFTB. For perfluoropinacol change in retention time was observed from 23.1 min at the pH 8.5 to 18.3 min at the pH 10.0. Decrease in the retention with increase of the pH can be observed for NFTB and PP due to their acidic properties – NFTB has one hydroxy group, PP two, which become more deprotonated and thus more polar and less retained on the C18 stationary phase.

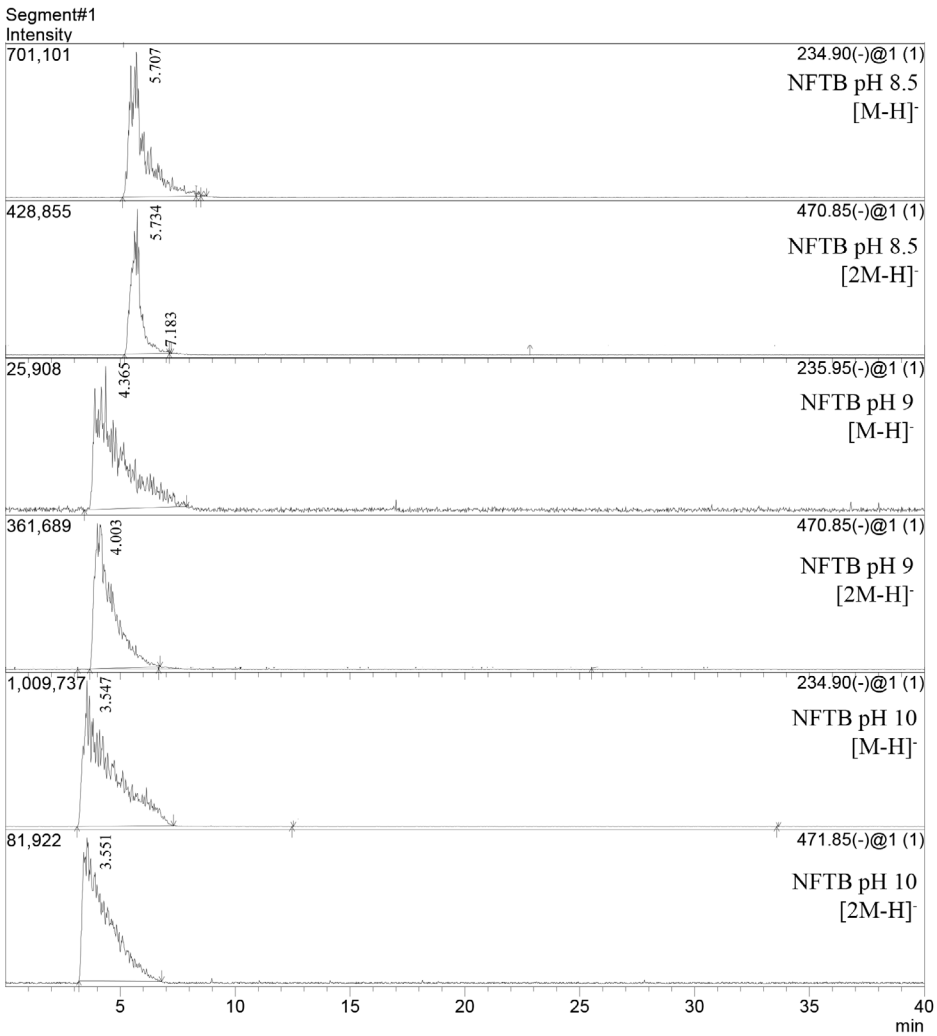


Figure 20. Chromatograms of NFTB (as mono-ion and dimer) at pH 8.5, 9.0 and 10.0 using C18 column. Column dead time t_0=1.38 min.

It can be theorised that, similarly to HFIP and HFTB [25], PP also creates a fluorous layer on the C18 stationary phase. However, the prominent tailing was not present for HFIP and HFTB, whereas it is for PP (Figure 21). Once the C18 stationary phase has been saturated with PP, completely removing it could be rather problematic. The observed strong tailing might be related to the structural properties of PP, as it has two hydroxy groups (unlike other researched novel eluent additives) and possibly due to steric interactions. In contrast, it is unlikely that NFTB (Figure 20) creates a fluorous layer on the C18 stationary phase due to its weak retention (in comparison to HFIP, HFTB and PP)

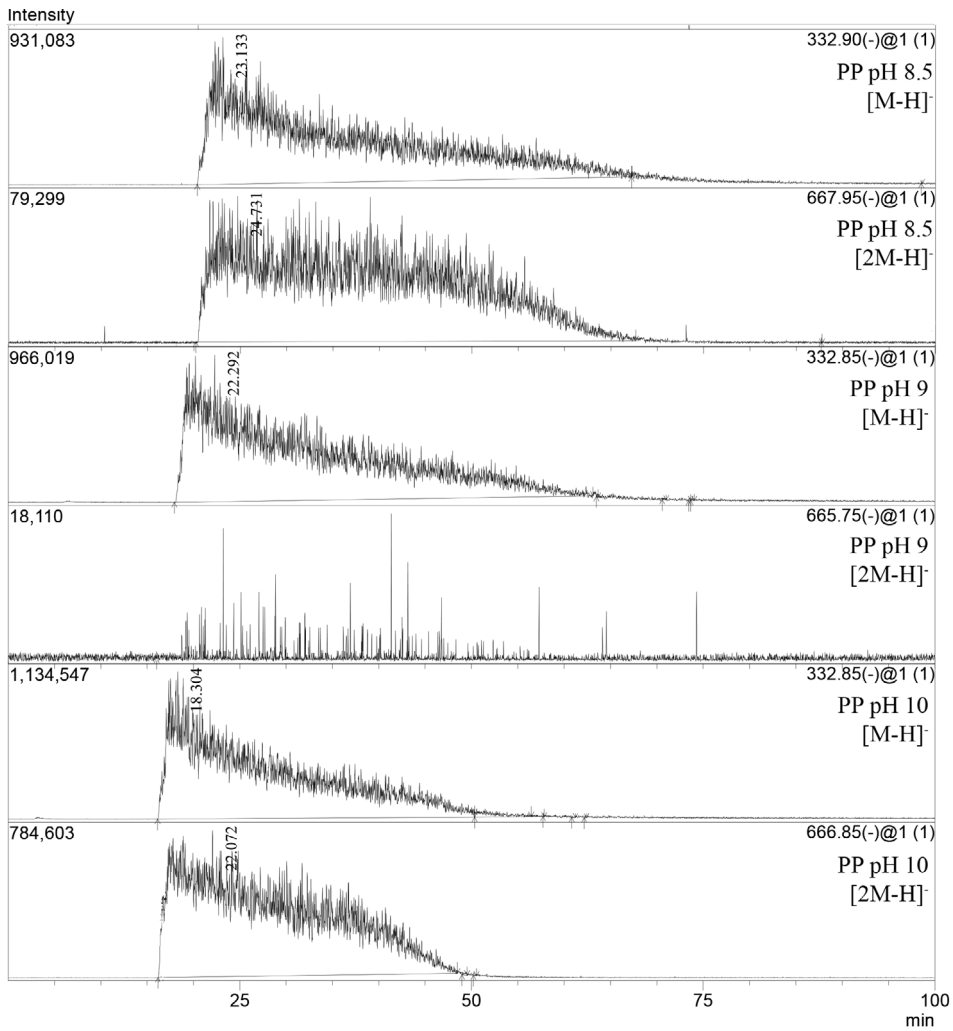


Figure 21. Chromatograms of perfluoropinacol (as mono-ion and dimer) at the pH 8.5, 9.0 and 10.0 using the C18 column. Column dead time t_0=1.38 min.

The retention of TFE on the C18 stationary phase is not very strong as it eluted at the beginning (at 2.4 minute, mobile phase pH 9.0) of the chromatographic run (Figure 22). Due to the poor ionisation of TFE (Figure 28) in MS, at the mobile phase pH 8.5 the peak is not even detected.

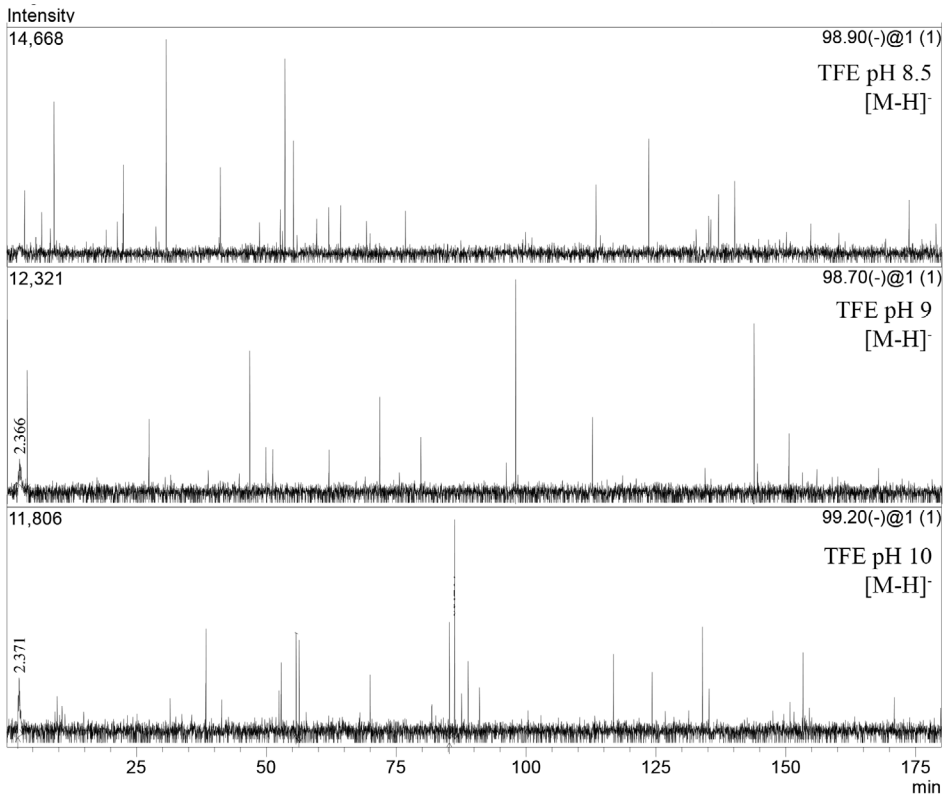


Figure 22. Chromatograms of the TFE at the pH 8.5, 9.0 and 10.0 using the C18 column. Column dead time t_0=1.38 min.

Changes in the eluent additive retention on the biphenyl stationary phase

The retention of HFIP and HFTB on the biphenyl stationary phase was not strongly influenced by the pH of the mobile phase (Figure 23). HFIP and HFTB were also retained far less on the biphenyl stationary phase (with HFTB being retained the most at the eluent pH 9.0) – and, unlike with the C18 phase [25], both eluent additives eluted from the column under 5 minutes. Thus the possibility of a fluorous layer covering the biphenyl stationary phase seems less probable than it was in the case of the C18 stationary phase [25].

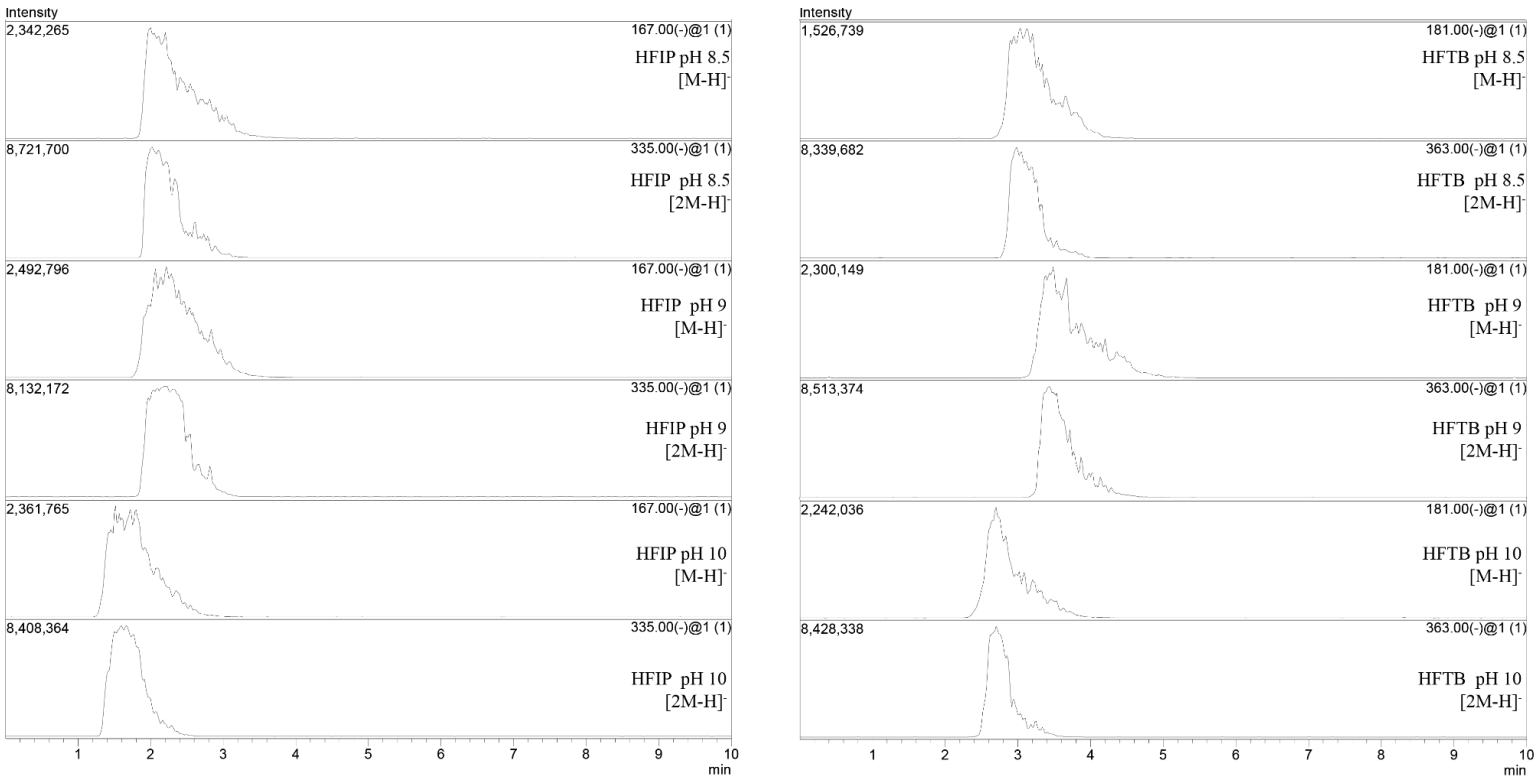


Figure 23. Chromatograms of HFIP (left) and HFTB (right) as mono-ion and dimer at the pH 8.5, 9.0 and 10.0 using column with the biphenyl stationary phase. The analysis run time was 10 min and column dead time t_0=0.92 min.

Similarly to HFIP and HFTB, PP started eluting from the biphenyl column at the beginning of the chromatographic run. However, its interaction with the biphenyl column is stronger and there is significant tailing (Figure 24), especially when the mobile phase pH is 8.5 and 9.0. The decrease in retention for novel fluorinated eluent additives with increase of the mobile phase pH originates from fluoroalcohols becoming more deprotonated (negatively charged), thus less retained.

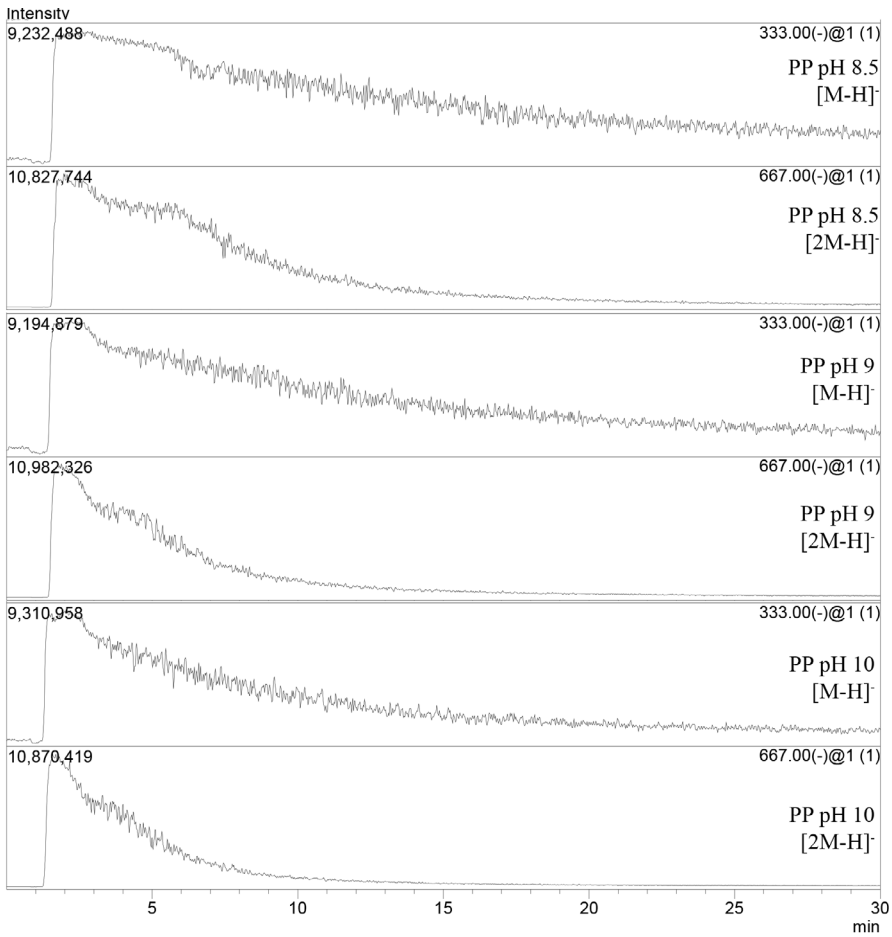


Figure 24. Chromatograms of perfluoropinacol (as mono-ion and dimer) at the pH 8.5, 9.0 and 10.0 using the biphenyl column. Column dead time t_0=0.92 min.

Changes in the eluent additive retention on the PFP stationary phase

Similarly to the interactions on the biphenyl stationary phase (Figure 23), the retention of HFIP and HFTB on the PFP stationary phase was not strongly influenced by the pH of the mobile phase (Figure 25). HFIP and HFTB on the PFP column likewise did not demonstrate strong retention.

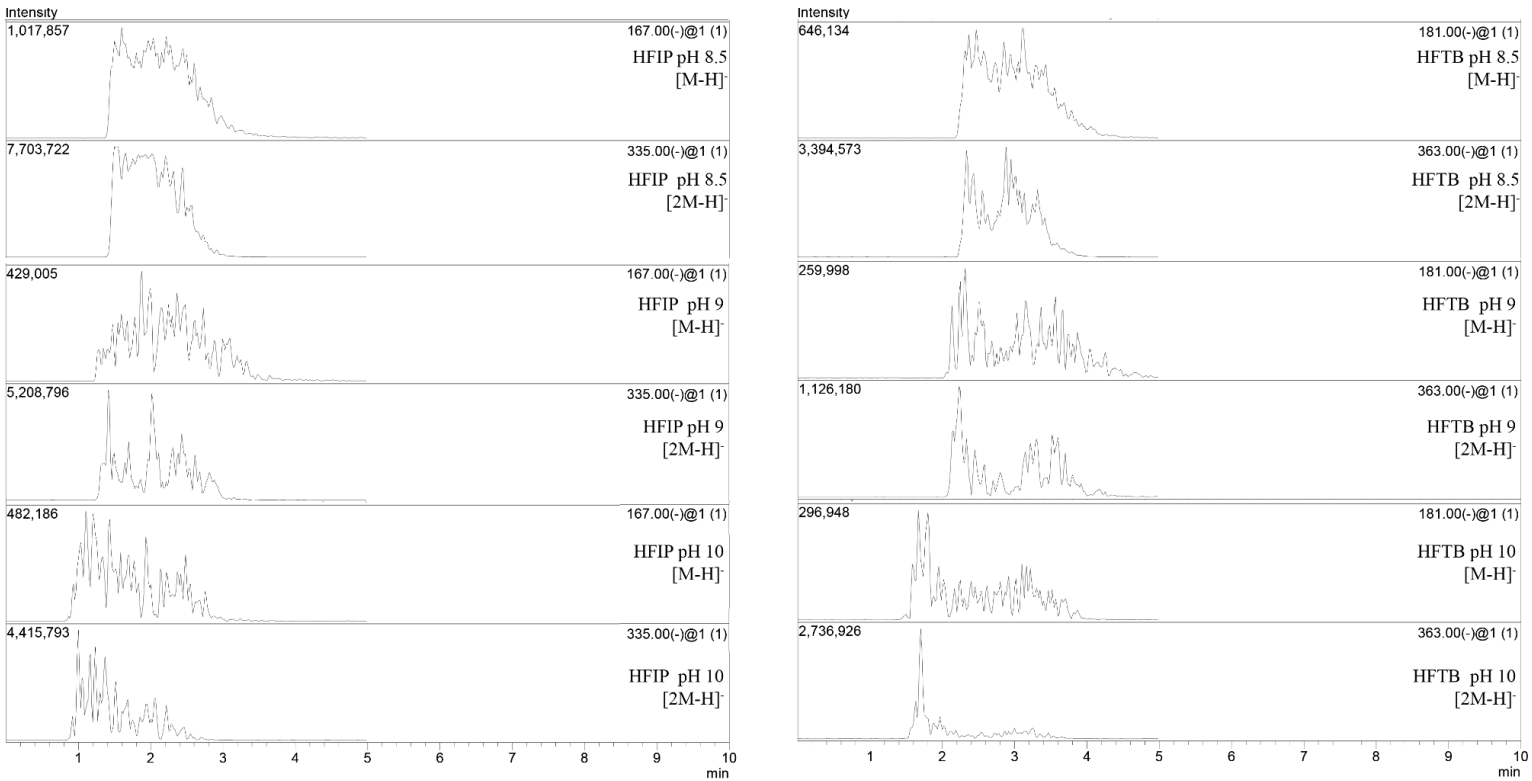


Figure 25. Chromatogram of HFIP (left) and HFTB (right) as mono-ion and dimer at the pH 8.5, 9.0 and 10.0 using column with the PFP stationary phase. The analysis run time was 10 min and column dead time t_0=0.65 min.

PP, similarly to HFIP and HFTB and their interactions with biphenyl and the PFP stationary phases, started eluting from the column at the beginning of the chromatographic run. Tailing was present also with PFP stationary phase; however, only when the mobile phase pH was 8.5 (Figure 26).

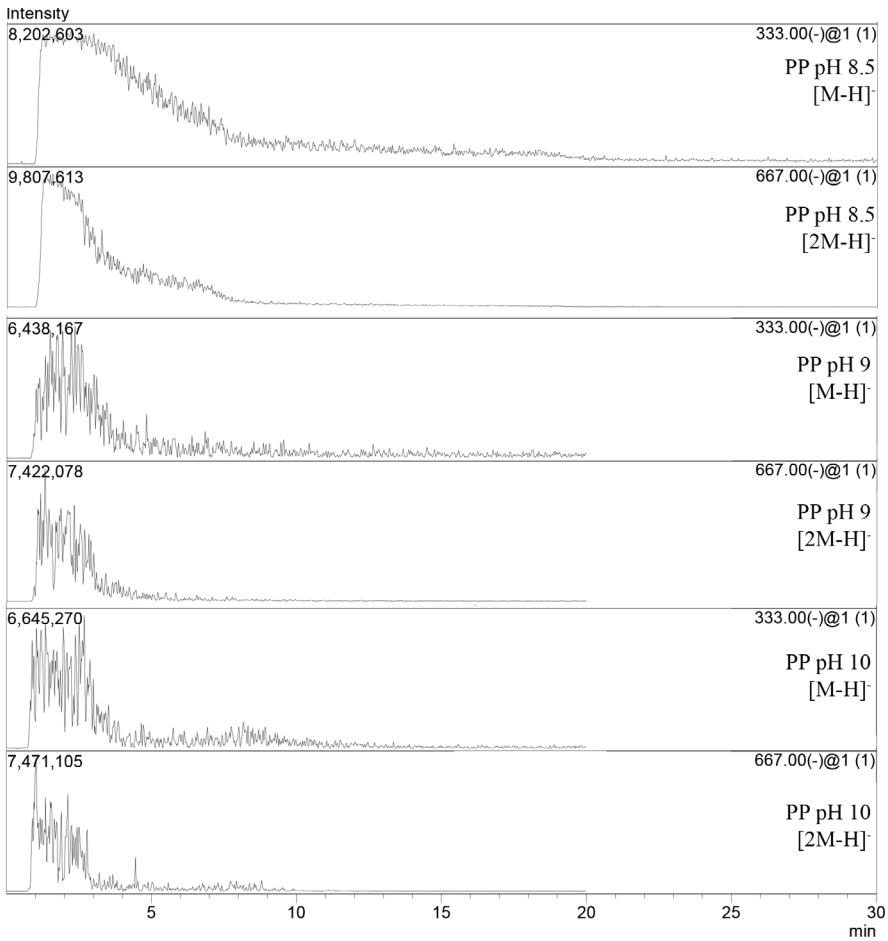


Figure 26. Chromatograms of perfluoropinacol (as mono-ion and dimer) at the pH 8.5, 9.0 and 10.0 using the PFP column. Column dead time t_0=0.65 min.

Ionisation of the eluent additives

Similarly to HFIP and HFTB, both NFTB and PP are forming dimers $[2M-H]^-$ in the MS spectrum (Figure 27). Unlike other additives, TFE was poorly ionised (Figure 28). Most pharmaceuticals are basic analytes, thus their detection is achieved with positive ionisation mode and at high eluent pH range. The strong signals observed for HFIP, HFTB, NFTB and PP in negative ionisation mode can hinder detection of analytes, which would also ionise in negative detection mode. Based on this, TFE would be suited to use, however it has poor chromatographic behaviour.

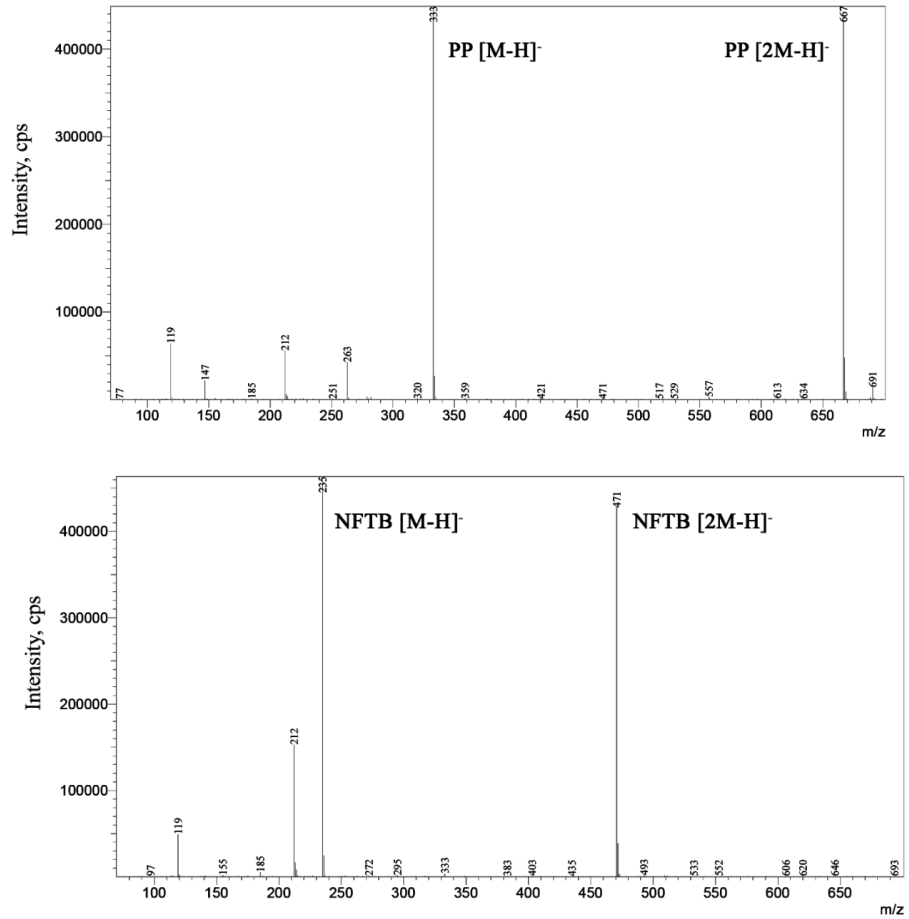


Figure 27. Mass spectra of PP (above) and NFTB (below) at the pH 10.0.

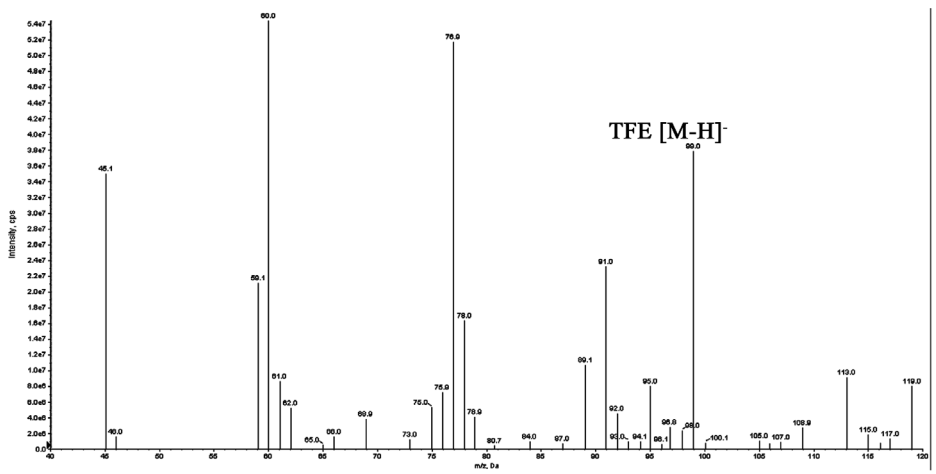


Figure 28. Mass spectra of TFE at the pH 10.0.

4.1.5. Separation of common toxicology screening compounds

An in-house method for screening (and quantifying) toxicology drugs (Table 9) in blood samples consists of a combination of a vast analyte library, as well as calibration and QC samples for quantification and verification. The routine screening and quantifying method uses both positive and negative ionisation modes; however, the vast majority of analytes are detected with the positive ionisation mode. The analytical column used is the C18 column for the separation of the analytes, and the eluent consists of 0.01% formic acid with 5 mM ammonium formate, both in the aqueous and organic (MeOH) phase. In order to compare the influence of different stationary phases as well as eluent additives better, only the columns and eluents are changed, the gradient elution pattern, as well as detection, remained the same and more technical information on that can be found in the chapter 3.2.5. The dilution for the compound mixture is described in the chapter 3.3.2.

Table 9. Overview of the detected analytes including aqueous pK_a and log$P$ values. Constants in *italic* have been experimentally measured. The rest have been estimated with ChemAxon software. [92], [93]

Analyte	pK_{a1}	pK_{a2}	Log$P$	Brief description
7-Aminoclonazepam	3.03	4.99	0.49	benzodiazepine, metabolite of clonazepam
7-Aminoflunitrazepam	3.32		1.79	benzodiazepine, metabolite of flunitrazepam
Alfentanil	7.50		2.81	potent opioid analgesic
alpha-Hydroxyalprazolam	4.97	13.72	1.52	benzodiazepine, metabolite of both alprazolam and adinazolam
alpha-Hydroxymidazolam	4.99	13.95	2.48	benzodiazepine, metabolite of midazolam
alpha-Hydroxytriazolam	4.24	13.75	2.04	benzodiazepine, metabolite of triazolam
Alprazolam	5.08		2.37	a short-acting benzodiazepine, tradename Xanax
Amisulpride	7.05		*1.06*	antipsychotic medication
Amoxapine	8.83		3.08	tricyclic antidepressant (TCA)
Aripiprazole	*7.60*		4.90	antipsychotic medication
Atenolol	*9.60*	14.08	0.16	beta blocker, used for cardiovascular conditions
Atropine	*9.43*	15.15	1.83	belladonna alkaloid, muscarinic antagonist (muscle relaxant)
Benzoylecgonine	9.54	3.15	-0.59	major metabolite of cocaine
Bisoprolol	*9.67*	14.09	2.20	medication used to treat heart diseases
Bromazepam	2.68		*2.05*	benzodiazepine, anti-anxiety medication
Buprenorphine	*8.31*		*4.98*	opioid

Analyte	pK_{a1}	pK_{a2}	Log$P$	Brief description
Carbamazepine	-3.80		2.77	anticonvulsant, analgesic
Carisoprodol		*15.06*	2.10	muscle relaxant
Cetirizine	*8.27*	*2.92*	0.86	antihistamine
Chlordiazepoxide	*4.80*		*2.44*	benzodiazepine, sedative
Chlorpheniramine	*9.13*		*3.38*	antihistamine
Chlorprothixene	9.76		*5.18*	antipsychotic
Citalopram	9.78		3.76	antidepressant
Clomipramine	9.20		*5.19*	TCA
Clonazepam	1.86	11.89	*2.41*	benzodiazepine
Clozapine	*7.50*		*3.23*	antipsychotic medication
Cocaethylene	8.77		2.60	metabolite of cocaine
Cocaine	*8.61*		*2.30*	strong stimulant, used as a recreational drug
Cotinine	4.79		*0.07*	alkaloid found in tobacco, predominant metabolite of nicotine
Cyclizine	*8.51*		3.55	medication to prevent nausea, vomiting and dizziness due to motion sickness or vertigo
Desalkylflurazepam	1.80	12.29	3.35	benzodiazepine, active metabolite for several benzodiazepines (midazolam, flurazepam, quazepam etc)
Dextromethorphan	9.85		3.49	cough suppressant
Diazepam	*3.40*		*2.82*	benzodiazepine, tradename Valium
Diclofenac	4.15		4.51	nonsteroidal anti-inflammatory drug (NSAID)
Dihydrocodeine	9.33	14.15	1.55	opioid analgesic
Diltiazem	8.18	12.86	2.73	medication used to treat high blood pressure and certain heart arrhythmias
Diphenhydramine	*8.98*		*3.27*	antihistamine, trade name Benadryl
Dothiepin	9.76		4.52	TCA
Doxepin	*8.96*		*4.29*	antidepressant and anxiolytic properties
Fenazepam	2.89		3.30	benzodiazepine
Fentanyl	*8.99*		*4.05*	powerful synthetic opioid analgesic
Flumazenil	3.27		*1.00*	imidazobenzodiazepine derivative and a potent benzodiazepine receptor antagonist
Fluoxetine	9.80		*4.05*	antidepressant, trade name Prozac
Flupentixol	8.43	15.59	*4.51*	antipsychotic
Flurazepam	8.71		3.95	benzodiazepine
Fluvoxamine	8.86		2.80	antidepressant
Gabapentin	9.91	3.70	*1.25*	anti-epileptic medication
Gliclazide	1.38	4.07	1.73	anti-diabetic medication
Haloperidol	*8.66*	13.96	4.30	antipsychotic medication
Imipramine	*9.40*		*4.80*	TCA
Ketamine	*7.50*		*3.12*	potent anaesthetic

Analyte	pK_{a1}	pK_{a2}	Log*P*	Brief description
Lamotrigine	*5.70*		1.93	anticonvulsant for epilepsy and bipolar disorder
Levetiracetam	-1.60	16.09	-0.59	epilepsy medication
Lidocaine	*8.01*		*2.44*	anaesthetic
Lorazepam	*1.53*		*2.39*	benzodiazepine
MDEA			2.50	3,4-methylenedioxy-N-ethylamphetamine, illegal psychoactive drug.
MDMA	10.14		1.86	hallucinogen, synthetic drug
Medazepam	9.57		4.21	benzodiazepine
Methadone	*9.20*		*3.93*	potent synthetic analgesic, an opioid used for opioid maintenance therapy in opioid dependence and for chronic pain
Mianserin	6.92		3.83	tetracyclic antidepressant
Mirtazapine	*7.70*		*2.90*	tetracyclic antidepressant
M6G	9.12	2.87	-3.00	morphine-6-glucunoride, metabolite of morphine
Naloxone	7.84	10.07	*2.09*	a medication used to block the effects of opioids, tradename Narcan,
Naproxen		*4.15*	*3.18*	NSAID
Nicotine	*8.50*		*1.17*	stimulant and potent parasympathomimetic alkaloid
Nitrazepam	2.61	11.30	*2.25*	benzodiazepine, hypnotic drug
Norbuprenorphine	10.49	9.80	3.19	major active metabolite of buprenorphine
Norclozapine	8.83		3.02	major active metabolite of clozapine
Nordiazepam	2.85		3.21	1,4-benzodiazepine derivative
Norfentanyl	10.03		1.42	major metabolite of fentanyl
norsertraline	9.73		4.72	desmethylsertraline, an active metabolite of sertraline
O-Desmethyltramadol	8.97	9.62	2.26	opioid analgesic, the main active metabolite of tramadol
Olanzapine	10.57		*4.09*	antipsychotic, primarily used to treat schizophrenia and bipolar disorder
Opipramol	7.86	15.59	3.24	anxiolytic and antidepressant
Oxycodone	8.77	13.57	*0.70*	opioid, used for the treatment of moderate to severe pain
Oxymorphone	*8.17*	10.07	*0.83*	semisynthetic narcotic analgesic related
Paliperidone	8.76	13.74	1.76	antipsychotic medication, also primary active metabolite of risperidone
Paroxetine	*9.90*		*2.53*	antidepressant
Phencyclidine	*8.29*		*4.69*	drug "angel dust", a hallucinogen formerly used as a veterinary anesthetic
Pentazocine	*8.80*		*4.64*	opioid

Analyte	pK_{a1}	pK_{a2}	Log$P$	Brief description
Pipamperone	8.69		1.87	antipsychotic
Prazepam	3.06		*3.73*	benzodiazepine
Pregabalin	10.23	4.80	-1.30	inhibitory neurotransmitter
Procyclidine	9.45	13.84	3.79	a muscarinic antagonist, used in the treatment of parkinsonism.
Propoxyphene	9.52		*4.18*	opioid pain reliever
Quetiapine	7.06	15.12	2.81	antipsychotic
Quinine	9.05	13.89	*3.44*	alkaloid, used to treat malaria and babesiosis
Risperidone	8.76		2.63	antipsychotic
Ritalinic acid	10.08	3.73	-0.36	α-phenyl-2-piperidine acetic acid, inactive major metabolite of methylphenidate and ethylphenidate
Sertraline	*9.16*		*5.51*	antidepressant
Trazodone	*6.74*		3.13	antidepressant
Triazolam	4.32		*2.42*	CNS depressant tranquiliser in the triazolobenzodiazepine
Trimipramine	9.42		4.76	TCA
Venlafaxine	8.91	14.42	2.74	antidepressant
Zolpidem	*5.65*		3.02	sedative, trade name Ambien
Zopiclone	6.89		0.81	hypnotic agent used in the treatment of insomnia
Zuclopentixol	8.03	15.59	4.22	antipsychotic medication
Propranolol	*9.42*	14.09	2.58	hypertension medication

4.1.5.1. Experiments with the C18 column and DFA as the eluent additive

Changes in analyte retention

In order to evaluate the influence of DFA on the mixture of toxicology screening compounds, two variations of eluent were compared to the routinely used eluent. Firstly, formic acid was replaced with DFA, and the mobile phase composition and ionic strength remained the same in the aqueous and organic phases. Secondly, to recreate the settings provided in the Waters Application note [41] conditions: the 0.15% DFA in the aqueous phase and no additives in the organic (MeOH) phase were used. In total, 90 analytes were detected in distinct peaks (Table 10).

Table 10. A list of detected analytes' retention factors and peak areas in experiments with the C18 column using ammonium formate with formic acid and DFA, and DFA in water as eluent additives.

Analyte	5 mM Ammonium formate and 0.01% formic acid pH 4.0		5 mM Ammonium formate and 0.01% DFA pH 4.2		0.15% DFA pH 1.7	
	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area
7-Aminoclonazepam	9.43	2898111	9.19	3226292	6.32	1213856
7-Aminoflunitrazepam	10.31	5584733	10.05	5104383	7.31	2050422
Alfentanil	13.24	4125875	12.98	3998531	11.23	2536162
alpha-Hydroxyalprazolam	13.60	416346	13.28	858637	12.72	201321
alpha-Hydroxymidazolam	14.23	2291446	13.91	2996012	10.89	1123637
alpha-Hydroxytriazolam	13.32	380647	13.00	862205	12.62	389660
Alprazolam	14.00	3272805	13.68	3510174	13.04	716944
Amisulpride	7.26	4448202	7.14	4157488	7.19	2298851
Amoxapine	13.19	2043230	12.89	2719911	11.97	2211673
Atenolol	5.17	3956536	5.14	4097209	5.35	1899336
Atropine	7.92	4478535	7.77	4418069	7.85	1536139
Benzoylecgonine	8.56	5937965	8.35	5901723	8.20	1221250
Bisoprolol	11.19	5073517	10.92	4667873	10.82	2184952
Bromazepam	12.79	678413	12.47	1443736	10.74	621045
Buprenorphine	13.87	2106072	13.64	2545872	11.73	1721386
Carbamazepine	13.13	3880139	12.80	4210212	12.45	1651171
Carisoprodol	13.80	634915	13.49	820270	13.12	305707
Cetirizine	14.52	2817693	14.20	2982567	13.15	760476
Chlordiazepoxide	14.48	1732148	14.20	2380218	10.22	1185540
Chlorpheniramine	11.40	6480189	11.12	6246459	8.27	3943451
Chlorprothixene	14.45	4960057	14.16	4377551	13.73	2331608
Citalopram	11.74	5438985	11.45	4937412	11.27	2023637
Clomipramine	14.50	5430649	14.20	4892023	13.80	2487783
Clonazepam	13.26	213301	12.93	350344	12.52	254690
Clozapine	12.57	5642586	12.27	5256498	9.18	2674605
Cocaine	9.21	4777938	9.02	4573040	9.00	3283941
Cotinine	5.98	4848657	5.87	4577323	4.71	1743976
Desalkylflurazepam	14.21	737459	13.90	1579661	13.25	954509
Dextromethorphan	11.82	6548884	11.53	5896432	11.37	2955243
Diazepam	15.12	4055338	14.81	4043785	13.52	2144827
Diclofenac	16.10	133121	15.84	246611	15.63	167893
Dihydrocodeine	5.70	3606878	5.65	3485945	5.87	1168357
Diltiazem	12.67	5524331	12.38	4903950	12.02	3015642
Dothiepin	12.90	5887618	12.60	5586179	12.32	3578583
Doxepin	12.18	6852201	11.88	6425650	11.66	4308421
Fenazepam	14.73	616854	14.42	1268581	13.96	728745
Fentanyl	11.41	5672059	11.16	5260491	10.90	3379788
Flumazenil	11.61	1179185	11.31	1671296	10.87	330774
Fluoxetine	13.89	2819739	13.60	2614956	13.28	1327125
Flupentixol	15.55	3723187	15.29	3134092	14.38	1280288
Flurazepam	11.95	5075711	11.67	4783261	11.37	2729252
Fluvoxamine	13.76	1432032	13.47	1614421	13.16	731714
Gabapentin	5.79	596866	5.72	1088017	6.91	354499

Analyte	5 mM Ammonium formate and 0.01% formic acid pH 4.0		5 mM Ammonium formate and 0.01% DFA pH 4.2		0.15% DFA pH 1.7	
	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area
Gliclazide	14.44	1260623	14.12	1616483	13.63	500252
Haloperidol	12.50	4863119	12.21	4386258	11.97	2922277
Imipramine	13.37	6430744	13.06	6038065	12.76	4051905
Ketamine	8.71	5484688	8.53	5208540	8.48	3212965
Lamotrigine	9.61	4522006	9.37	4711889	9.24	2150707
Lidocaine	8.18	6177367	8.03	5883682	7.96	3864092
Lorazepam	13.96	190585	13.64	403574	13.29	192197
M6G	2.17	44229	2.05	46906	3.27	9218
MDEA	7.82	4673536	7.68	4466032	7.84	2489198
MDMA	7.16	3640393	7.04	3631693	7.25	1411205
Medazepam	15.33	7348932	15.03	6859679	11.12	4239890
Methadone	13.49	7134119	13.19	6720527	12.90	5374065
Mianserin	12.19	5346787	11.90	4708806	11.39	1630936
Mirtazapine	10.13	6359476	9.91	5990665	6.86	2037238
Naloxone	6.67	2046617	6.58	2221185	6.68	914145
Nicotine	1.56	2286200	1.59	2408091	0.27	2355572
Nitrazepam	13.30	251755	12.95	521185	11.65	599627
Norbuprenorphine	11.19	417167	10.93	662643	10.81	424529
Norclozapine	12.52	3365070	12.22	3650665	8.77	2301619
Nordiazepam	14.82	1576423	14.51	3452460	12.40	2666949
Norfentanyl	8.78	4393091	8.59	4525236	8.67	2117357
Norsertraline	14.55	23227	14.26	20478	13.83	23279
O-Desmethyltramadol	7.21	4899159	7.09	4945684	7.24	3087007
Olanzapine	9.59	5170484	9.40	3884694	6.83	1551837
Opipramol	13.30	4899212	13.00	4664611	11.42	2520311
Oxycodone	6.16	2414654	6.08	2465491	6.25	910652
Oxymorphone	3.42	953072	3.52	1356655	3.88	702579
Paliperidone	10.24	3175865	10.01	2872411	9.52	1559899
Paroxetine	13.10	1586154	12.80	1726109	12.52	960584
Pentazocine	10.57	6547758	10.33	6075655	10.23	3955781
Phencyclidine	10.90	4146554	10.65	3873744	10.56	3371744
Pipamperone	11.26	4441772	11.00	4104455	7.11	2106017
Prazepam	16.24	3722100	16.00	3795695	15.41	1932855
Pregabalin	3.94	272905	5.76	582639	6.77	221855
Procyclidine	13.21	7321863	12.91	6986987	12.64	5651227
Propranolol	11.66	5150872	11.37	5151344	11.20	2904844
Quetiapine	12.82	5890978	12.53	5319024	10.15	2939052
Quinine	10.55	4352073	10.30	4197781	6.98	1439155
Risperidone	10.98	4558812	10.73	4025677	9.19	2453862
Ritalinic acid	8.59	1061092	8.37	2024201	8.37	616767
Sertraline	14.31	1890917	14.02	1813934	13.64	741367
Trazodone	11.57	5132553	11.30	4537790	10.21	2470427
Triazolam	13.90	2445262	13.59	2632013	13.19	1096095
Trimipramine	13.80	7007815	13.51	6675749	13.15	4688637
Venlafaxine	11.25	6865708	10.97	6485294	10.85	4870681
Zolpidem	10.93	6105804	10.69	5833387	9.62	4265537
Zopiclone	9.21	927412	8.98	1046577	8.67	204155

Roughly a half of the analytes studied (42 analytes out of 90, Table 10) showed a consistent decrease in retention factors when the 0.01% formic acid was replaced with the 0.01% DFA and an even further decrease in retention with an increase in concentration to the 0.15% DFA. Most of these analytes were benzodiazepines. Analytes with a difference in retention times higher than 1.5 minutes can be seen in Figure 29. Three analytes which had the largest decrease were chlordiazepoxide pK_a 4.8, log$P$ 2.44, medazepam pK_a 9.57, log$P$ 4.21 and pipamperone pK_a 8.69, log$P$ 1.87. The pK_a values or Log$P$ do not explain why there is such a large change in the case of exactly these three analytes.

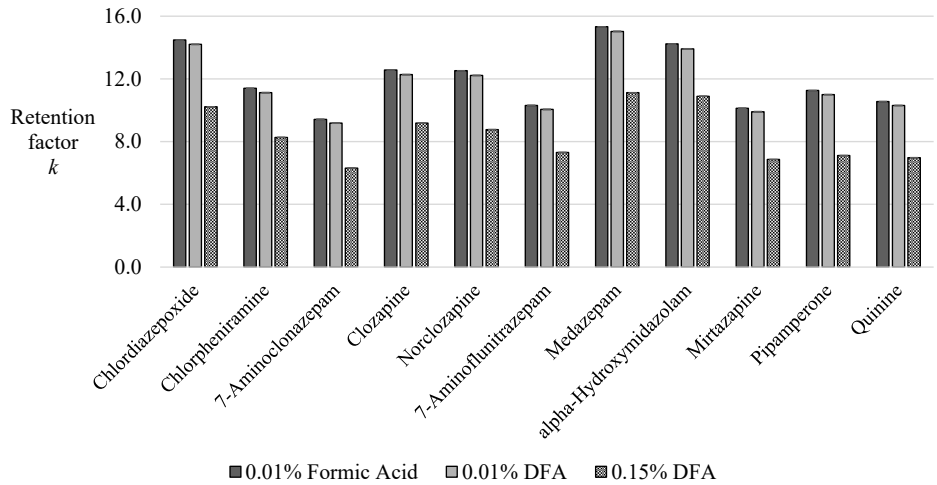


Figure 29. Retention factors of 11 analytes when the 0.01% formic acid with 5 mM ammonium formate and the 0.01% DFA with 5 mM ammonium formate and the 0.15% DFA were used as eluent additives.

The other large part of the analytes (44, Table 10) had little to no change in retention when DFA was used as the eluent additive. However, even then, the vast majority (34 analytes out of 44) showed a small (not larger than 0.3 units) decrease in the retention factors when formic acid was replaced with DFA and then a larger (but not exceeding change of 0.5 units) decrease when the DFA concentration was increased and ammonium formate was not present in the eluent. Because all analysed drugs have either basic functional groups or both basic and acidic functional groups with an increased acidity (the pH of the 0.01% formic acid solution was 4.0, the pH for the 0.01% DFA solution was 4.2, and for the 0.15% DFA the pH was 1.7) of the eluent, basic analytes become more protonated, thus more polar and thus they elute faster.

Only a small group – four analytes (Figure 30) – showed different relation-ships either an increase in retention when formic acid was replaced with DFA in

the mobile phase (for analyte pregabalin) or an increase when the 0.15% DFA was used – the analytes gabapentin and M6G (morphine-6-gluconiride, one of the morphine metabolites). The only analyte showing a notable decrease in retention only when the 0.15% DFA was used (but not for the 0.01% DFA), was nicotine. All four analytes have pK_a values either between 8.5 and 10.23, which means the majority of basic functional groups are present in the protonated BH^+ form. Pregabalin also has the carboxylic acid (-COOH) functional group (with pK_a 4.8), which means that with a decrease in the pH, the acidic group shifts equilibria from A^- towards AH, becoming less polar and more retained on the C18 stationary phase – which can be seen as an increase in retention time. Similarly, M6G has glucuronic acid in its structure, which is why an increase in retention can be seen with the 0.15% DFA as the eluent.

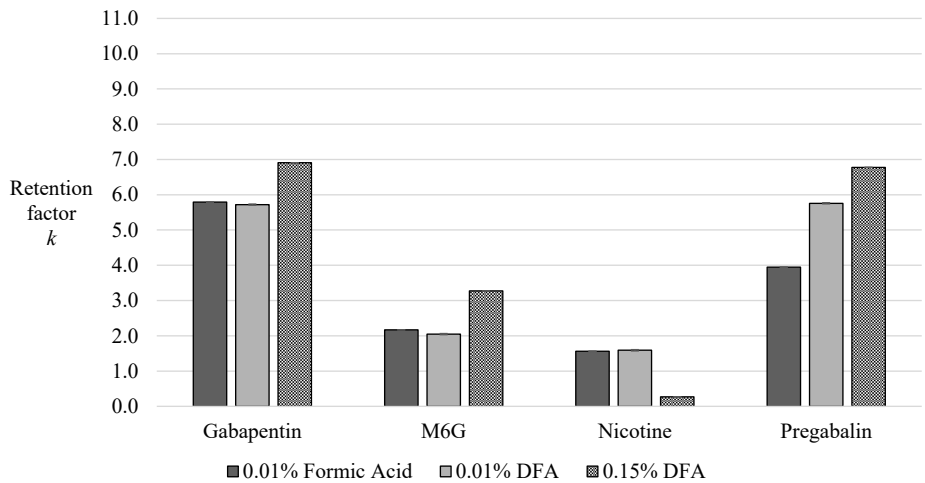


Figure 30. Retention factors of four analytes when the 0.01% formic acid with 5 mM ammonium formate and the 0.01% DFA with 5 mM ammonium formate and the 0.15% DFA were used as eluent additives.

Changes in analyte ionisation

For 48 analytes the highest MS signal was obtained when the routine eluent (the 0.01% formic acid with 5mM ammonium formate both in the aqueous and organic phases) was used. For 40 analytes, the highest signal MS signal was obtained when the 0.01% formic acid in routine eluent was replaced with the 0.01% DFA. However, the difference in peak areas between mobile phase containing 0.01% of formic acid or 0.01% DFA was insignificant (changes in retention were evident only by 1–1.5 times). Only five analytes: alpha-hydroxyalprazolam, bromazepam, gabapentin, ritalinic acid and pregabalin had larger increase in the signal (by 1.8–2.4 times), when the 0.01% DFA was used as the eluent additive instead of the 0.01% formic acid.

The eluent, which consisted of the 0.15% DFA and MeOH, consistently gave the lowest MS signal (on average by 2.3 times) for the majority (78) of the detected analytes when compared to eluent compositions containing 0.01% formic acid or 0.01% DFA.

Only for two analytes (nitrazepam and norsertraline) the MS signal was the highest when the 0.15% DFA was in use. For nitrazepam increase in signal when DFA was used was by 2.1–2.5 times, but for analyte norsertraline peak areas were overall small and differed only 1.002–1.1 times.

4.1.5.2. Experiments with biphenyl column and HFTB as eluent additive

Changes in analyte retention

In total 81 analytes were detected as distinct peaks (Table 11), when using the biphenyl column and two eluent additives: ammonium acetate and HFTB, both at the pH values 8.5 and 9.0. Slightly less than a half of the analytes (33) were not influenced (or were influenced minimally) by the change of the eluent additive or pH value. 26 out of the 33 of not influenced analytes had low (less than 8) pK_a values, meaning that at a high pH, they are predominantly in the non-protonated form (B) and therefore not largely influenced by changes of the pH or eluent additive.

Table 11. A list of detected analytes, retention factors and peak areas in experiments with the biphenyl column using ammonium acetate and HFTB as eluent additives.

Analyte	Ammonium acetate pH 8.5		Ammonium acetate pH 9.0		HFTB pH 8.5		HFTB pH 9.0	
	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area
7-Aminoclonazepam	8.97	8519507	8.96	8066090	8.79	14471814	8.79	12032251
7-Aminoflunitrazepam	9.81	12586585	9.79	13435619	9.66	18600428	9.66	13064050
Alfentanil	15.11	12792373	15.12	13602000	15.12	15143401	15.12	11354683
alpha-Hydroxyalprazolam	13.02	1168278	13.00	1194047	12.99	3009326	12.99	2403595
alpha-Hydroxymidazolam	13.83	5549452	13.83	5954047	13.82	12365017	13.83	12199303
alpha-Hydroxytriazolam	12.75	1320263	12.75	1182301	12.71	3386766	12.71	2820154
Alprazolam	13.45	4389688	13.70	2322739	13.45	7853359	13.77	2692937
Amisulpride	10.15	3830671	11.04	10172875	14.66	5357799	12.55	14036995
Amoxapine	15.25	2079838	15.54	1653556	15.92	1161273	15.84	1921046
Atenolol	7.53	1782837	8.43	5040760	9.82	2708730	9.01	11207309
Atropine	10.72	2363992	11.62	972325	14.00	3322521	16.35	5176286
Benzoylecgonine	8.08	12955466	8.07	13303453	8.15	22714797	8.12	19177365
Bisoprolol	13.68	890114	13.04	9577916	16.28	534344	14.89	8092157
Bromazepam	12.20	2811700	12.22	3005301	12.13	6714461	12.16	6785525
Carbamazepine	12.52	5360336	12.80	2463568	12.47	10382265	12.48	7730123
Carisoprodol	13.24	1152329	13.23	1052928	13.21	1925940	13.20	1452074
Cetirizine	14.19	5255007	14.29	5489270	13.15	15502466	12.84	16308960
Chlordiazepoxide	14.13	2351711	14.13	2305204	14.09	4604146	14.10	4828124
Chlorpheniramine	13.94	8320829	16.00	2043774	16.22	5286886	17.68	2459464
Chlorprothixene	18.36	988411	17.99	4136506	18.23	3851425	18.27	2806127
Citalopram	13.36	7621254	14.31	6326085	15.41	3311996	15.48	5935724
Clomipramine	17.18	5909047	17.72	6704308	18.14	6821872	18.21	6916868
Clonazepam	12.64	555072	12.63	529630	12.47	1206040	12.48	1129666
Clozapine	15.75	8403892	15.86	9677772	16.03	4277979	16.33	3919378
Cotinine	6.57	4648700	6.56	4665357	6.70	11708349	6.66	9393136
Desalkylflurazepam	13.75	1224864	13.66	1388862	13.64	2976986	13.64	3346575

Analyte	Ammonium acetate pH 8.5		Ammonium acetate pH 9.0		HFTB pH 8.5		HFTB pH 9.0	
	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area
Dextromethorphan	15.25	2792435	18.41	1412778	17.87	8090993	17.15	18441037
Diazepam	14.72	3139385	14.63	4142117	14.62	5875126	14.63	5000601
Diclofenac	13.12	293597	12.94	285878	11.12	1094211	10.99	859055
Dihydrocodeine	11.11	3049156	12.64	2398915	13.46	1043960	12.82	8329567
Diltiazem	15.69	5336383	15.73	6281920	15.91	6352049	16.00	4558701
Diphenhydramine	14.95	3379808	15.38	4004869	17.87	2823249	18.28	1527254
Dothiepin	17.12	1236720	17.94	1645830	18.44	2930241	18.33	1860155
Doxepin	17.45	886452	17.97	2211569	17.64	4507377	17.59	8573298
Fenazepam	14.22	5803580	14.21	795119	14.29	1043628	14.19	2055902
Fentanyl	16.43	7265612	16.24	13467947	16.94	5378728	16.93	2940709
Flumazenil	10.99	1912952	10.99	1641191	10.97	4831219	10.97	3878813
Flupentixol	17.45	2561149	17.52	946329	17.53	2561148	17.43	1943591
Flurazepam	15.99	4776237	15.94	11353116	16.49	4138991	16.47	4202693
Fluvoxamine	14.65	1343227	15.12	1043607	16.17	1100078	16.07	2170909
Gliclazide	11.42	939477	11.11	1128181	9.28	1620691	9.05	1324291
Haloperidol	16.48	711072	17.16	1058329	17.02	4075645	17.76	1206834
Imipramine	16.98	1412127	16.83	6197775	17.51	10867445	17.64	12959449
Ketamine	13.68	2043165	13.73	1936014	13.63	9346142	13.51	12377606
Lamotrigine	9.60	11721231	9.57	11658137	9.13	19728633	9.14	16768510
Lidocaine	14.74	11287537	14.93	7494978	15.14	6579772	15.01	6583781
Lorazepam	13.38	457005	13.38	344903	13.32	877548	13.33	659878
MDMA	8.43	5507932	9.47	5573390	11.21	7310203	12.01	624546
Methadone	14.62	16512259	15.54	16385625	17.58	18892521	17.57	28624258
Mianserin	16.64	4185960	17.15	1022395	16.79	4317389	16.81	6609312
Mirtazapine	14.93	8906263	15.03	10593185	15.41	4484571	15.10	5998939
Morphine-6-glucuronide	6.91	1054699	7.67	780178	5.48	2533671	5.58	2030744
Naloxone	10.91	5278670	11.43	3144005	11.41	4786381	11.48	8964268
Naproxen	10.74	69742	10.53	89737	7.80	125353	7.87	145483
Nitrazepam	12.64	760277	12.63	877574	12.44	2081078	12.45	1889485

Analyte	Ammonium acetate pH 8.5		Ammonium acetate pH 9.0		HFTB pH 8.5		HFTB pH 9.0	
	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area
Norclozapine	14.04	2806365	14.42	2820428	15.02	1477430	14.88	2452086
Nordiazepam	14.29	2966166	14.44	1159122	14.27	5908223	14.27	6147324
Norfentanyl	9.76	7070897	10.23	6608377	12.27	7987083	12.31	5965890
Opipramol	16.07	2543064	16.13	2279071	16.89	3644718	16.64	2049648
Oxycodone	11.64	1903232	12.28	3406394	14.12	1038731	12.95	5306756
Oxymorphone	9.03	2789446	10.27	2203545	10.45	3630470	10.58	6546171
Paliperidone	13.72	8993928	14.41	6336468	14.60	4694787	14.82	1045831
Paroxetine	14.25	1474876	14.79	1361482	16.40	1176881	16.47	1872242
Pentazocine	12.54	15474784	14.74	2177553	15.53	6128845	15.95	17847884
Pipamperone	13.81	11882241	15.18	1785265	16.01	3925415	15.82	1356184
Prazepam	15.89	4781703	15.88	4073769	15.89	4386592	15.88	3210724
Pregabalin	4.58	1324925	4.62	968639	4.37	3132713	4.27	2731591
Procyclidine	18.10	2434090	17.81	2911626	17.61	20773699	18.04	24514573
Propoxyphene	16.30	4223292	16.77	3691312	17.27	3196354	17.93	2769852
Propranolol	16.99	1003293	13.85	6631697	16.73	1428377	15.56	4362198
Quetiapine	15.63	4436609	15.52	11004173	15.46	13369601	15.42	14367343
Quinine	14.98	3146375	14.73	8475291	15.22	2870541	15.29	5901170
Risperidone	15.03	6281823	15.19	10061792	15.68	4296285	15.80	2426934
Ritalinic acid	8.12	3039281	8.18	3485278	8.01	7523065	7.99	6063910
Sertraline	16.26	1182922	16.94	731058	17.72	1673954	17.85	1321330
Trazodone	15.22	11348491	15.24	9984959	15.24	12033824	15.76	1058734
Triazolam	13.36	3870165	13.36	4749645	13.38	7981609	13.37	6973330
Trimipramine	17.76	6093679	17.76	9974653	18.13	12743146	18.25	14042538
Venlafaxine	13.03	14298408	14.29	13136037	15.79	7404792	16.02	18380371
Zolpidem	13.60	22232933	13.59	22402009	13.64	28921803	13.64	25561318
Zopiclone	12.40	230007	12.43	182823	12.41	352798	12.41	211306

Analytes which were retained less on the biphenyl column when HFTB was used as the eluent additive (Figure 31) also had low pK_a values (between 1.38–4.15, Figure 32), except for cetirizine (an antihistamine) and M6G, which have pK_a values of 8.27 and 9.12, respectively. However, these analytes also have carboxylic and glucuronic acid groups in the molecules' structure, which are deprotonated at a high pH. HFTB as an ion-pairing reagent presents competition to acidic deprotonated (polar) analytes in the column. Thus, it is also possible to see shorter retention times as well as repulsive forces between the anion and π-systems.

There were three analytes, which had carboxylic group in their structure, however, their retention did not decrease when HFTB was used (ritalinic acid, pregabalin and benzoylecgonine). These three analytes also have high pK_a values (9.54–10.23), meaning they are protonated at used mobile phase pH.

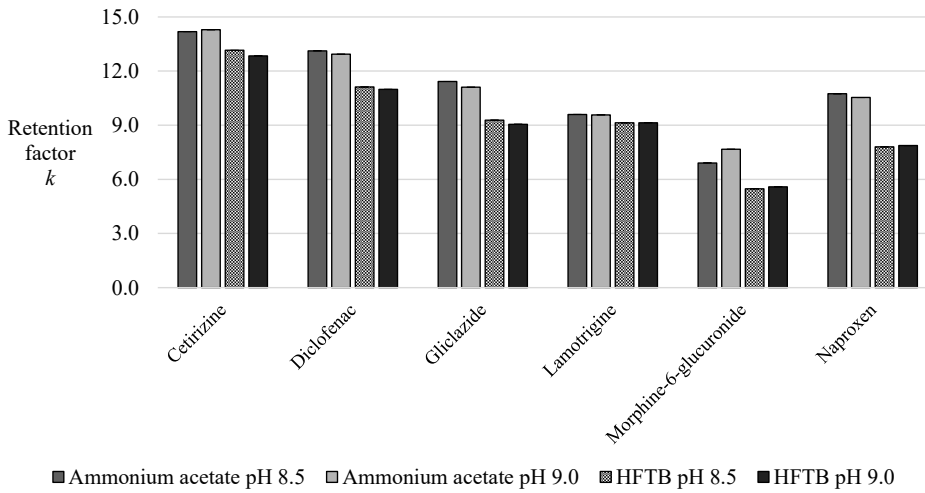


Figure 31. Retention time of six analytes when using ammonium acetate and HFTB as the eluent additives using biphenyl column at the pH values of 8.5 and 9.0.

There were 26 analytes which presented a clear increase in retention factors when HFTB was used as the eluent additive. All of them had high pK_a values (group "increase" in Figure 32) – in pK_a the interval was 7.5–10.14. The analytes which had stronger retention also had higher log$P$ values when compared to those of the rest of the detected analytes. Only one analyte – oxymorphone had a log$P$ value less than 1. All of that combined makes an analyte less polar, and therefore it elutes late when the organic phase (MeOH) content is higher.

Likewise, 16 analytes which displayed a change in the retention factors when the eluent additives and the pH values changed also had higher pK_a values (Figure 32). However, no clear pattern was observed. Largest part, 33 analytes

in total, demonstrated no change in retention times (group "no change in retention" in Figure 32)

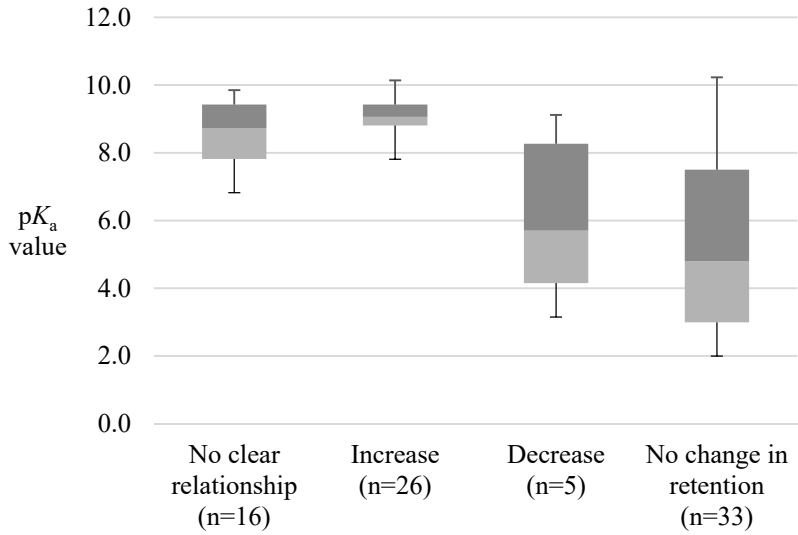


Figure 32. Box plots for the pK_a values of the analytes, which are grouped into four categories according to the observed relationship of the retention factors, eluent additive and pH. Analytes with either observed an increase or no clear relationship had higher pK_a values with a narrower range. Analytes, which saw either a decrease or no change in retention had a wide range of pK_a values. However, the majority of them had lower pK_a values.

Changes in analyte ionisation

Both an increase and decrease in the MS signal were observed between different eluent additives and pH values. All signal values were compared to those obtained when ammonium acetate, the pH 8.5, was used as the eluent additive:

- when ammonium acetate was used at the pH 9.0, 41 analytes had a decrease in the signal, 30 analytes had a signal increase in the range of 1–2 times and 10 analytes saw a more than two-fold signal increase. The biggest increase in signal intensity, 10.8 times, was observed for the analyte bisoprolol;
- when HFTB was used at the pH 8.5, 26 analytes saw a decrease in the signal, 30 analytes saw a signal increase in the range of 1–2 times and 25 analytes saw a more than two-fold signal increase. The biggest increase in signal intensity, 8.5 times, was observed for the analyte procyclidine;
- when HFTB was used at the pH 9.0, 25 analytes had a decrease in the signal, 25 analytes saw a signal increase in range of 1–2 times and 31 analytes saw a more than two-fold signal increase. The biggest increase in signal intensity, 10.1 times, was observed for the analyte procyclidine.

Thus, while there is little difference between the ammonium acetate eluent at the pH values 8.5 and 9 (roughly a half of the analytes saw an increase and the other half – a decrease), there is mostly a significant signal improvement when HFTB is used as the eluent additive, especially at the pH 9.0 – when 31 analytes experienced a signal that was at least two times larger. Seven analytes: atenolol, bispropol, dextromethorphan, doxepine, procyclidine, imipramine and ketamine demonstrated an increase in signal intensity by 5–10 times.

The majority of signal improvement when HFTB was used as the eluent additive was for the analytes which had either consistent retention times or saw a decrease in retention times when HFTB was used as the eluent additive. A possible explanation for that is if analytes eluted faster from the column, the amount of the aqueous phase % was larger and thus, also the concentration of HFTB, which has proven to help with signal enhancement.

4.1.5.3. Experiments with the PFP column and HFTB as the eluent additive

Experiments performed with the PFP column comparing results obtained when the routine eluent (the 0.01% formic acid 5mM ammonium formate both in the aqueous and organic phases) were compared to those obtained when the eluent used had 5mM HFTB as the eluent additive, at the pH values: 8.5 and 9.0. The change in a previously used reference buffer for the biphenyl column (5mM ammonium acetate, the pH 8.5 and 9.0) was made to obtain a better comparison between the in-house method and change in the retention and ionisation when the PFP column was used. Altogether, data were recorded for 42 analytes as distinct peaks (Table 12) – other analytes either retained too poorly or too strongly on the PFPF stationary phase. Generally, observed retention patterns fell into three groups:

1. for 14 analytes little to no change in retention factors was observed
2. for 14 analytes an increase when HFTB was used as the eluent additive was observed, with the largest retention factors recorded when the eluent pH was 8.5
3. for 9 analytes the retention factors were the largest when the routine eluent was used.

Table 12. A list of the detected analytes' retention factors and peak areas in experiments with the PFP column using ammonium acetate and HFTB as the eluent additives.

Analyte	5 mM Ammonium formate and 0.01% formic acid pH 4.0		HFTB pH 8.5		HFTB pH 9.0	
	Retention factor *k*	Peak area	Retention factor *k*	Peak area	Retention factor *k*	Peak area
Alfentanil	14.69	10790304	14.78	3933509	14.71	2836261
Alprazolam	13.91	7043462	13.91	908388	13.87	1052439
Aripiprazole	17.36	8194655	17.58	1558059	17.37	929312
Bisoprolol	13.73	13297416	22.98	2923078	18.78	2131095
Carbamazepine	11.65	7472914	11.64	1227111	11.62	895109
Cetirizine	13.90	9190298	11.92	1595905	11.26	1090408
Clonazepam	12.80	948621	12.70	133499	12.63	78442
7-Aminoclonazepam	9.40	11193642	9.24	780776	9.25	441199
Clozapine	14.71	9568414	16.70	1754431	16.44	1052899
Cocaethylene	13.84	163881	19.42	122807	18.73	72455
Cocaine	12.66	11982865	18.51	7278681	17.85	4586762
Benzoylecgonine	10.62	5853895	9.64	637814	9.59	604409
Cyclizine	14.73	6885393	18.03	2364137	17.54	1415069
Diazepam	14.22	9766308	14.24	1021489	14.21	832108
Diclofenac	14.30	609696	4.12	47208	4.12	5545
Dihydrocodeine	8.32	7972539	18.94	2294721	18.42	1755607
Diltiazem	15.50	13965430	16.86	5240261	16.55	3574891
Fentanyl	15.15	1286859	17.28	561681	16.83	341064
Norfentanyl	10.93	4329364	22.27	1981760	19.01	998341
7-Aminoflunitrazepam	10.81	11265844	10.71	1822722	10.66	1365965
Gabapentin	5.21	2524287	2.93	497166	2.84	291184
Gliclazide	13.57	2592383	4.34	200272	4.65	179890
Ketamine	11.11	7871376	13.87	1985194	13.46	1234050
Lamotrigine	10.89	26715395	9.30	2977406	9.21	2655654
Levetiracetam	5.12	2889124	5.23	159410	5.09	19192
Lidocaine	10.63	9441405	15.10	3073209	14.82	1618922
Lorazepam	12.35	869623	12.34	102072	12.34	100088
Mirtazapine	12.63	10706870	16.53	2641459	16.32	2021736
Naproxen	13.82	588068	1.68	37807	1.97	16721
Nitrazepam	12.78	1155330	12.65	215367	12.58	136624
Nordiazepam	13.37	3020351	13.37	623960	13.35	551707
Oxycodone	8.90	5328067	17.10	969006	16.22	534135
Paliperidone	13.59	10809380	17.01	4114550	16.20	2709541
Pregabalin	5.84	1491399	2.55	230995	2.44	222991
Procyclidine	15.41	13426668	21.62	8136229	18.33	4187546
Quetiapine	14.64	12702296	14.93	3234697	14.71	1901231
Quinine	14.61	8926433	19.06	3038643	18.46	1769459
Risperidone	14.97	12592250	17.75	6929731	17.27	3845949
Trazodone	14.58	11658604	15.47	3236148	15.35	2661362
Zolpidem	14.01	16871378	14.30	6562196	14.22	4093063
Zopiclone	11.72	3057176	14.06	175172	13.93	200131
Zuclopentixol	18.46	1147058	18.26	601426	17.78	255559

The majority of analytes with the constant retention times also had low pK_a values (below 7.6, group "no change in retention" in Figure 33). Also, addition of organic solvent (MeOH) lowers the pK_a values of basic analytes [17]. If the basic analyte's pK_a value is below that of eluent's pH, the majority of the basic functional groups in the analytes are presented in the deprotonated B form. As neutral analytes, these did not interact with HFTB and are not influenced by the change of a high pH.

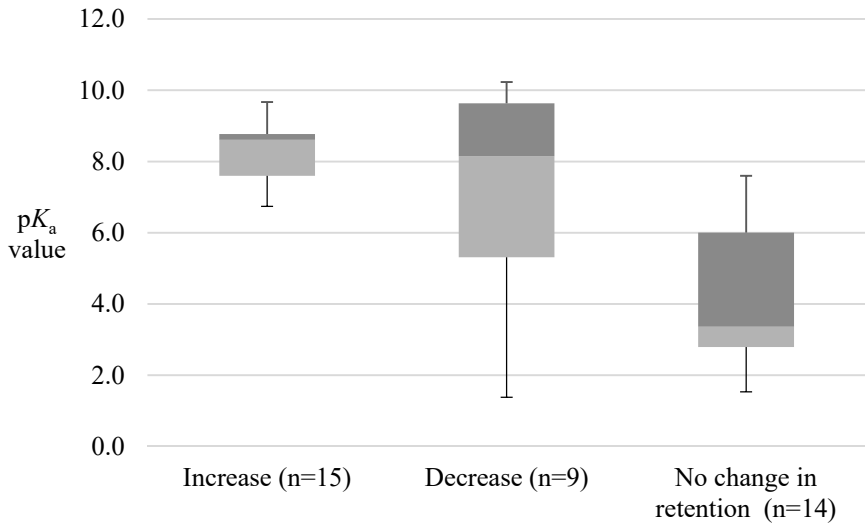


Figure 33. Box plots for pK_a values of the analytes, which are grouped into three categories according to the observed relationship between the retention factors and eluent additive as well as the pH of the aqueous phase.

Analytes which demonstrated an increase in retention time when the novel eluent additive HFTB was used, especially at the pH 8.5 (Figure 34), all had relatively high pK_a values – in the range between 6.7 and 9.7 (Figure 33), which means the basic groups in analytes are influenced by the change in the used pH range of 8.5 and 9.0.

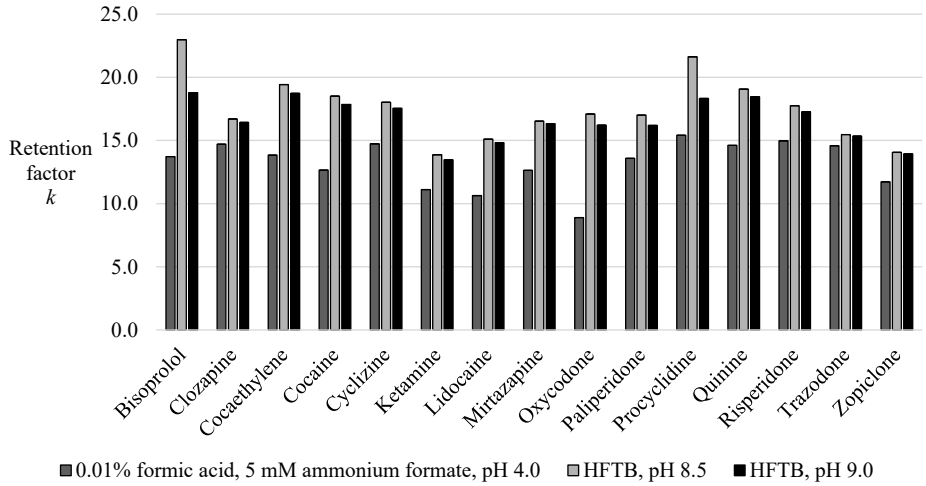


Figure 34. The retention time of 15 analytes when using the 0.01% formic acid with 5mM ammonium acetate at pH 4.0 and HFTB as eluent additives at the pH values of 8.5 and 9.0.

The nine analytes, which were less retained on the PFP column when HFTB was used as the eluent additive, have the largest variation (Figure 33) of pK_a values. However, these are the only six detected analytes (benzoylecgonine, cetirizine, diclofenac, gabapentin, naproxen and pregabalin) that have the carboxylic (-COOH) group in their structure. Out of the other two analytes, one has a hydroxy group (zuclopentixol), one an -SO_2 group (gliclazide). The last analyte in the group, which does not have a carboxylic group, is lamotrigine. At a high pH, the carboxylic group would be present in the polar deprotonated (A^-) form, while the basic functional groups would be in equilibria between the polar (BH^+) and deprotonated B forms (depending on the pK_a values), but with the protonated form in the majority. HFTB has additional ion-pairing properties, however it can still form ion-pairs with protonated basic analytes in the mobile phase. It is possible that due to deprotonated acidic functional group, these pairs are not formed.

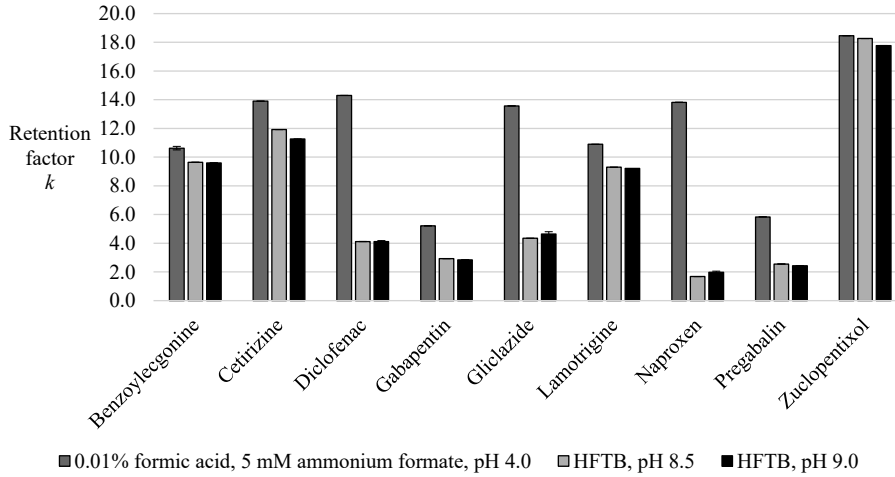


Figure 35. Retention time of 9 analytes when using 0.01% formic acid with 5mM ammonium acetate and HFTB as eluent additives at pH values of 8.5 and 9.0.

Changes in ionisation

For all analytes, the undoubtedly best signal intensity was obtained using the routine eluent system, at times giving even a 100-fold increase when compared to the MS signal obtained when HFTB was used. Between the HFTB pH 8.5 and 9.0, on average the signal obtained at the pH 8.5 was 1.6 times higher than that obtained at the pH 9.0. Few exceptions were observed: three cases – alprazolam, lorazepam and zopiclone – when a better signal was obtained at the pH 9.0, and two cases – diclofenac and levetiracetam – which saw an increase in signal intensity by 6.8 and 8.5, respectively, when the HFTB 8.5 was used as an aqueous phase.

4.2. Practical applications

In order to research the possible influence of novel eluent additives in real-life applications, two bioanalytical methods with the aim to obtain data usable in PK/PD studies were developed and fully validated according to the European Medicines Agency (EMA) guideline [49]. The main reason for using novel eluent additives was that these methods were aimed to achieve incredibly low lower limits of quantification (LLOQ) of the pharmaceutical compounds in a very small volume of the sample. The need for low concentrations and sample volumes arose from the larger clinical study design, as the population consisted of paediatric patients and neonates.

4.2.1. Challenges of the data below the analytical limit of quantitation in pharmacokinetic analysis [II]

When developing a bioanalytical method, it is important to choose calibration range fit for the purpose of the study. For quantification of well-known pharmaceuticals there is information available, which describes the usually encountered concentration ranges in blood plasma in humans. Those are called the therapeutic drug ranges and they describe how much of analyte is needed to achieve the therapeutic effect. However, the situation becomes considerably more complex in case of PK/PD studies, where sometimes only an inkling of possible ranges is available and furthermore, when PK/PD study sampling population is paediatric patients (meaning analyte concentration can be very low). Addition instrumentation that lacks good sensitivity increase the risk of choosing too high LLOQ values. This can lead to significant amount of measured samples producing results that fall below LLOQ values.

Due to the rules set by different bioanalytical method validation guidelines [94]–[97], the data which are obtained during the quantification of an analyte using a bioanalytical method, but falls below the limit of quantification, cannot be released as it lacks the required precision evaluation. Usually, these data points are simply marked as BLQ. However, the inclusion of the data can contribute to a smaller imprecision and bias in model-based PK analyses [65]. Additionally, since no numerical value is provided, a statistical treatment for BLQ data points must be used. The most common methods of this include the exclusion of BLQ data, partial exclusion, or substitution with the halved value of LLOQ, the actual value of LLOQ or zero.

It is important to note that there always can be data that fall below the limits of quantification. If that happens, the best way to deal with BLQ data would be to minimise the impact of its occurrence but in order to achieve that, there has to be cooperation between PK modellers and bioanalytical scientists. This would improve study protocols by ensuring the most suited and needed concentration ranges and that assay sensitivity parameters are met. If there are many BLQ data points in an assay, a re-assessment of the assay should be done and the LLOQ should be lowered, which is both a time- and resource consuming process and should be avoided.

Likewise, an introduction of a harmonised limit of detection (LoD) definition in the validation guidelines, which is not dependant on the signal to noise ratio, should be encouraged. From the already acquired data, the LoD can be calculated using formula (2):

$$LoD = 3.3 * \frac{SD(LLOQ)}{S} \quad (2)$$

SD(LLOQ) in the formula marks the standard deviation of the analyte response measured from the replicate measurements in the LLOQ sample (or the analyte and internal standard response ratio). S stands for the slope of a line drawn

between the origin and the mean response of the replicates at the nominal LLOQ concentration.

Using the formula (2), LoD estimations are quite conservative. There are alternative LoD estimation techniques [98], [99], which lead to more statistically accurate LoD value due to less assumptions made, however they require additional experiments, which are not included in the validation guidelines. Still, using the LoD should be encouraged as it can both demonstrate analytical performance and give data analysts the chance to make the final decision about the inclusion or exclusion of BLQ data in a model.

4.2.2. Method development and validation for the analysis of morphine, clonidine and midazolam and their metabolites [I]

All six analgesic analytes (morphine, it's two metabolites M3G and M6G, clonidine, midazolam and its metabolite MiOH) in the CLON01 ("Clonidine for Sedation of Paediatric Patients in the Intensive Care Unit) study have basic properties, as shown by their pK_a values (Table 13).

The eluent's aqueous phase was chosen to fit those basic analytes' requirements – 5mM HFIP and the pH was adjusted to 9 (with ammonium hydroxide), as HFIP is a weak acid (pK_a 9.3 [19]). A chromatogram obtained under these conditions can be seen in Figure 36.

Table 13. Analytes researched for the CLON01 study, their pK_a and log$P$ values.

Name	Abbreviation	pK_a	pK_a reference	log$P$	log$P$ reference
morphine-3-glucuronide	M3G	pK_{a1} = 9.12* pK_{a2} = 2.87*	[93]	-3*	[86]
morphine-6-glucuronide	M6G	pK_{a1} = 9.12* pK_{a2} = 2.87*		2.99*	[86]
morphine		8.2	[100]	0.93	[101]
clonidine		8.05	[102]	1.59	[103]
1'-hydroxymidazolam	MiOH	4.99*	[104]	2.48*	[93]
midazolam		6.15	[105]	3.33*	[86]

*estimated values

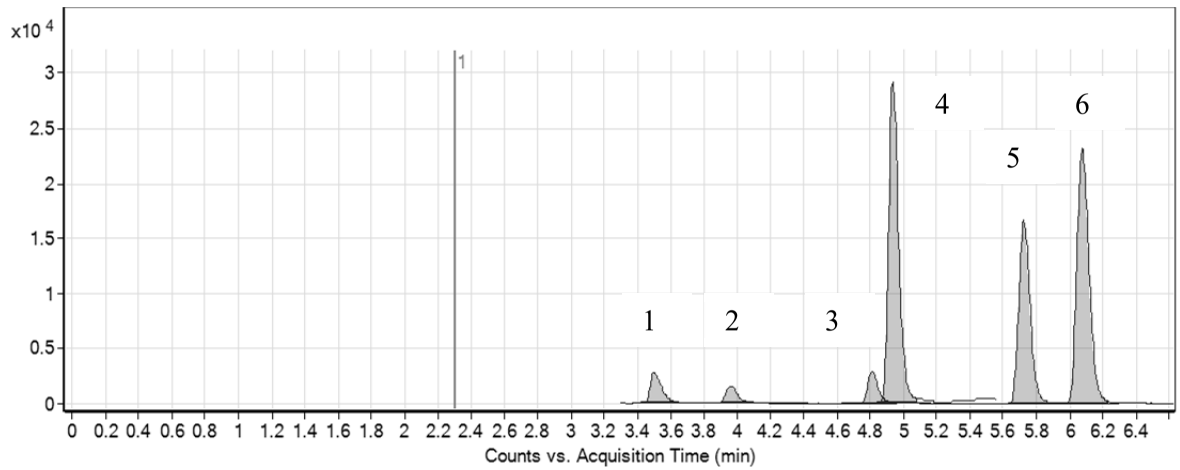


Figure 36. A typical chromatogram obtained at analytes' concentration of 0.5 ng/mL. (1 – M3G, 2 – M6G, 3 – morphine, 4 – clonidine, 5 – MiOH, 6 – midazolam).

In order to simultaneously detect morphine as well as its two metabolites, they all had to be chromatographically separated. The reason for this is that both M3G and M6G undergo in-source collision induced dissociation (CID) that results in the loss of the glucuronic acid component. Without it, the fragment *m/z* remaining is identical to that of morphine (*m/z* 286). If the three analytes are not chromatographically separated, glucuronides would contribute to the morphine signal [78].

As a novel fluorinated eluent additive, the HFIP in the basic mobile phase acts as a weak ion-pairing additive. Due to the glucuronic acid group with pK_{a2} 2.87, at the used eluent pH it is completely deprotonated, thus making analytes polar and not well retained on the RP column. Furthermore, as seen by results described in 4.1.5.3, an acid group in the molecule decreases the retention of the analyte if a novel fluorinated eluent additive is used. As a result, both M3G and M6G elute early during the chromatographic run (Figure 36), even if their basic group has the highest pK_a value of the studied analytes (Figure 37). Clonidine and morphine have similar pK_a values; however, morphine is less lipophilic based on their log*P* values; thus it elutes faster. Both MiOH and midazolam should be in a completely protonated form in the aqueous solution; however, midazolam is more lipophilic. It also elutes when the MeOH content is 100% (Figure 37).

The use of HFIP as a novel eluent additive also helped to reach the needed lowest limits of quantification (50 pg/mL) by increasing instrument sensitivity.

The developed and validated (according to the EMA guideline [49]) method has been successfully applied to samples collected under the CLON01 ("Clonidine for Sedation of Paediatric Patients in the Intensive Care Unit") study. The CLON01 study was a randomised, double-blind controlled clinical trial, funded by the European Commission Seventh Framework Programme with the aim to compare the use of clonidine with midazolam in paediatric and neonatal ICUs. In addition to clonidine or midazolam, patients also received an analgesic component – morphine. The goal of the study was to analyse PK for all three drugs and to develop dosage guidelines for sedation in ICUs for paediatric and neonatal patients.

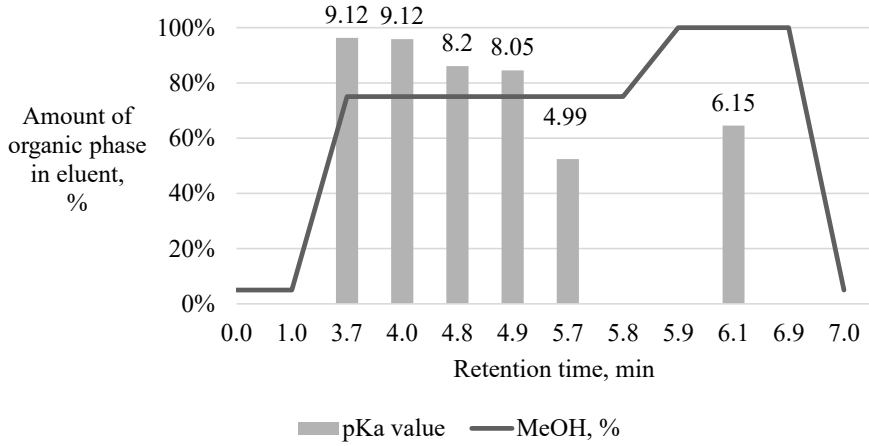


Figure 37. Gradient composition over time with corresponding analytes' pK_a values in the order of analyte elution – M3G, M6G, morphine, clonidine, MiOH and midazolam.

4.2.3. Method development and validation for analysis of milrinone and dobutamine [III, V, VI]

Analysis of milrinone and dobutamine

A very sensitive and selective method for the simultaneous determination of milrinone and dobutamine (Figure 38) was developed and validated for simultaneous measurement in neonatal plasma samples, using only a sample volume of 20 μL (the lowest known reported). To reach the needed sensitivity with the minimal amount of sample, NH_4F was used as an eluent additive. While it is not uncommon to see a positive influence on the signal of NH_4F in the negative ion mode, the same cannot be said about the positive ion mode. A comparison of different eluent compositions (the 0.1% formic acid versus NH_4F) showed a signal increase for both analytes. For milrinone, the signal increased two times, but for dobutamine – seven.

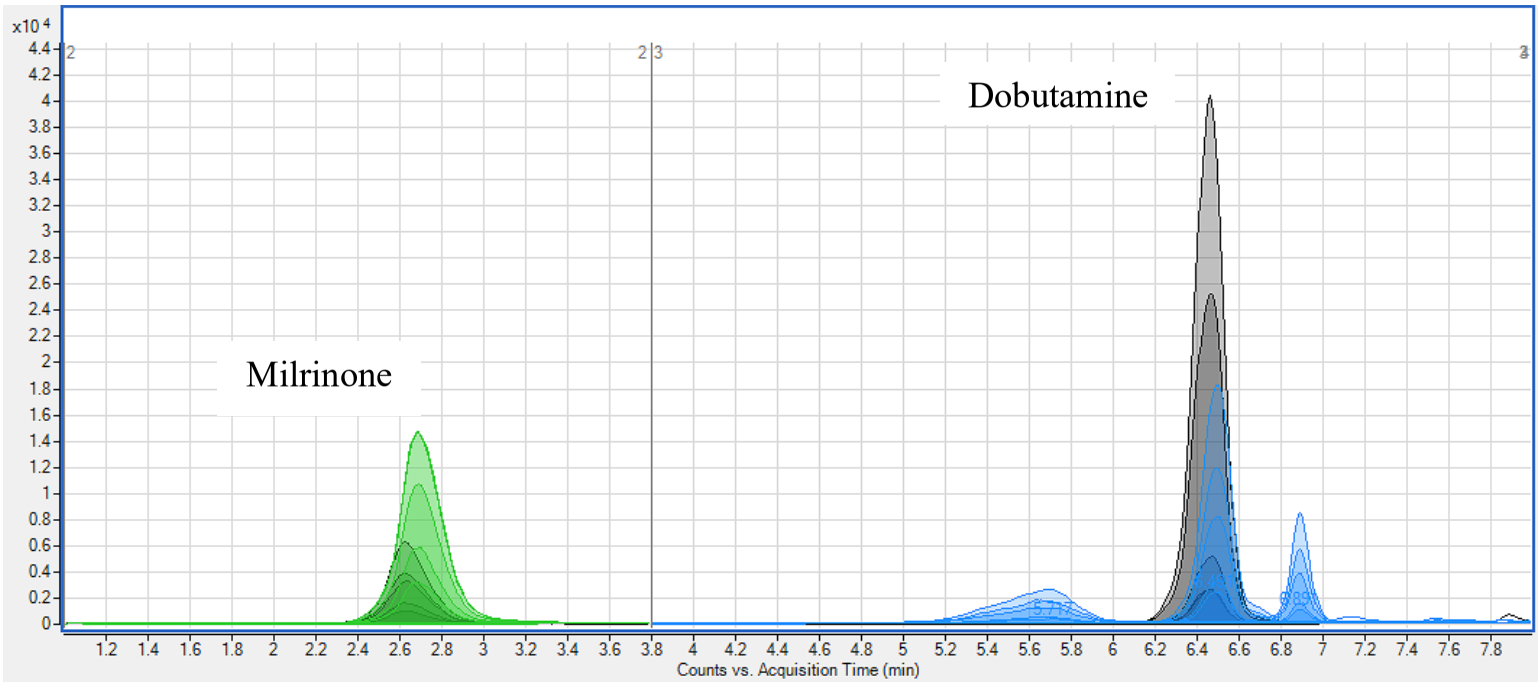


Figure 38. Chromatogram of a patient's sample. Milrinone's concentration is 113 ng/mL and dobutamine's concentration is 4 ng/mL. IS concentration is 44 ng/mL. Interfering peaks on the dobutamine chromatogram were baseline separated from the dobutamine peak and did not interfere with quantitation.

Results from clinical studies

The method was developed and validated according to the EMA guideline [49], and successfully applied to measuring samples from a two-centre study in Estonia (Tallinn Children's Hospital and Tartu University Hospital): no. 2015-004836-36 (the registered number in the EU Clinical Trials Register). The obtained results were further used to describe the PK of milrinone and dobutamine in neonates in papers [V] and [VI].

Ionisation of model analytes when using NH_4F with the biphenyl column

In order to test the influence on ionisation and dependence on either pK_a or log$P$ values in the positive ion detection mode, the peak areas of the analytes were compared between using ammonium acetate and the NH_4F eluent at the pH 8.5 with the model analytes from Table 5. However, the results were inconclusive mainly due to the fact that peak areas varied greatly between injections, which led to large standard deviations (Figure 39).

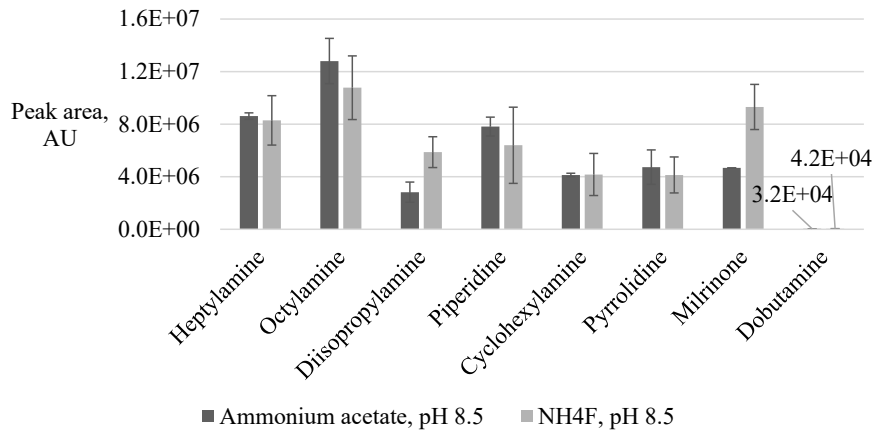


Figure 39. Comparison between peak areas for basic polar analytes group BH+ and milrinone and dobutamine when ammonium acetate and NH_4F was used as eluent additives at pH 8.5.

While both milrinone and dobutamine saw increase (2 times for milrinone and 1.3 for dobutamine), when NH_4F was used as eluent additive at pH 8.5, the increase or decrease remained analyte specific and there was no relationship between analytes log$P$ and pK_a values. Thus it can be seen while NH_4F increased signal for some analytes, the enhancement was not as significant as it was seen for acidic conditions.

SUMMARY

This work aimed to expand different novel eluent additives for use mainly in HPLC-MS systems. Another aim was to further research the retention mechanisms on reversed-phase columns and any influence had on the analyte ionisation on MS detection when different eluent additives at a predominantly high mobile phase pH were used.

As eluent additives, three fluoroalcohols (HFIP, HFTB and PP) are well retained on the C18 stationary phase, therefore it can be theorised that they create an anionic fluorous layer, which is very attractive for basic polar analytes, but repels acidic polar analytes. NFTB is not well retained; however, due to its low pK_a value it is completely deprotonated at a high pH and can still create ion-pairs with basic protonated analytes in the mobile phase. As for biphenyl and the PFP stationary phases – HFIP, HFTB are not retained on these stationary phases and thus most likely do not create the fluorous layer. PP is the only additive which presented a long tailing (even if it eluted quickly) and thus could possibly create a fluorous layer also on the biphenyl stationary phase.

Thus, for retention on the C18 stationary phase there is a possibility of dual interaction – between the analyte and fluorous layer created by the fluorinated eluent additive and the interaction of the eluent additive with the analyte in the mobile phase. For common eluent additives (ammonium acetate, bicarbonate and formate), change in retention is only due to the change in equilibria between the analyte's polar (A^- or BH^+) and non-polar (AH and B) forms. This is especially well demonstrated for polar basic analytes – when a sharp increase in all analytes' retention from the pH 8.5 and 9.0 to the pH 10.0 was observed. Since there is little to no change between the ionised and non-ionised forms of an analyte due to their pK_a values, then for the two neutral analyte groups retention changed very little, as they are not influenced by the change of the mobile phase pH.

While the changes in retention when novel eluent additives are used to large extent follow the same patterns already observed with conventional eluent additives, as they are also dependent on the equilibria between polar and non-polar analyte form, these changes are amplified. For non-polar analytes, the retention factors varied more, and as most of the AH group analytes have pK_a values around 10 and thus are partly protonated at a higher eluent pH, the retention was overall smaller when HFIP and HFTB were used. In general for acidic analytes, smaller retention factors (on top of the decrease in retention with the increase of the mobile phase pH) were observed when compared to conventional eluent additives. This is due to the fact that acidic polar analytes not only have to compete for a place on the stationary phase, but they are also additionally repelled by the deprotonated HFIP, HFTB, NFTB and PP eluent additives. Due to the pK_a values of HFIP, HFTB and PP with increase of the eluent pH, also these novel eluent additives become more deprotonated and increasingly repelling to acidic analytes. For basic neutral analytes, a simply

larger variation in retention factors was present, without a clear increase or decrease. However, the largest influence of novel eluent additives was undoubtedly observed for basic polar analytes. The increase in retention with the increase of the pH eluent observed when HFIP and HFTB were used was more gradual than when conventional eluent additives were used and with overall larger retention factors. For some analytes, the retention increased 28 and 35 times when using novel eluent additives. For one basic protonated analyte fluoroalcohol PP increased the retention factor by 41 times. PP is especially promising due to its structural properties – two hydroxy groups, which means PP also has two pK_a values. Thus PP has good potential to be used as an ion-pairing reagent also in the case of the low mobile phase pH values. NFTB showed the same influence patterns on basic analytes as other novel eluent additives.

As for TFE and DMSO – due to their minimal buffering capacity, it was impossible to achieve the stable mobile phase pH 8.5 and 9.0. Likewise, considerably lower analyte signal was observed – either due to suppression or non-optimal source parameters in case of DMSO. Thus, both are deemed not to be suitable for further use in bioanalytical methods.

Overall, the retention of analytes on the biphenyl stationary phase was greater due to the π-π interactions, but there were some exceptions – all analytes containing a nitro group were very poorly retained, even if they had a benzene ring in their structure. Furthermore, both neutral analyte groups – the protonated acidic analytes and deprotonated basic analytes – were more influenced by changes in the pH of the mobile phase than when the C18 column was used. The largest difference in retention between the C18 and biphenyl stationary phases for acidic analytes was observed at the eluent pH 8.5 – when a smaller part of the analyte is deprotonated and thus due to the additional π-π or halogen-π interactions, the analyte is better retained on the biphenyl column. For basic polar analytes, even without clear π-π interactions (as none of the BH^+ group analytes had an aromatic ring in its structure), the retention was stronger than that seen with the C18 column. A higher log*P* value also ensured better retention for polar basic analytes.

In order to expand this research into the practical field, the separation of a large set of common toxicology analytes (all with at least one basic functional group) was studied. An in-house method for screening with gradient elution (thus influence of pH was decreased) was used as a comparison to novel eluent additives. Firstly, the influence of DFA was tested in comparison with the in-house method on the C18 stationary phase column. While the change from formic acid to DFA in the in-house buffer (where also ammonium formate is added) did not make a significant difference in retention or ionisation, using the eluent only with DFA did. 42 analytes out of 90 showed a significant decrease in retention, mainly due to a decrease in the eluent's pH. The increase of retention when DFA was used could be seen for analytes with acidic functional groups in their structures. As for analyte ionisation, DFA gave a consistently

lower signal if compared with the obtained peak areas when formic acid was used.

The change to a different stationary phase (biphenyl) demonstrated a decrease in number of the analytes detected. A half of the analytes were not influenced by the change of the eluent additives (ammonium acetate and HFTB), nor were they affected by a change in the pH from 8.5 to 9.0. Analytes which demonstrated a decrease in retention when HFTB was used as the eluent additive had either low pK_a values or a carboxylic functional group in their structure. Analytes which showed an increase in retention when HFTB was used as the eluent additive also had higher pK_a as well as higher log$P$ values, meaning a larger part of the basic analyte was in the polar form and thus interacting more readily with HFTB by forming ion-pairs with the fluoroalcohol. When HFTB was used as the eluent additive, the majority of analytes showed an increase in the MS signal, especially when the eluent's pH was 9.0.

The biggest difference in retention was presented when the PFP column was used as only 42 analytes were recorded as distinct peaks – other analytes either retained too poorly or too strongly on the PFP stationary phase, even if the in-house acidic mobile phase conditions were used. Analytes with pK_a values below 7.6 saw no change in retention when HFTB was used as the eluent additive. Again, analytes which were retained less when HFTB was used, also had a carboxylic acid functional group in their structure. Just like with the biphenyl column, the acidic functional group, which is deprotonated at the used high eluent pH, demonstrated that ionic interaction between the negatively charged functional group in the analyte and the likewise deprotonated novel fluorinated eluent additive is more important in terms of influence on retention than the attraction of the protonated basic functional group.

In this doctoral dissertation, two practical applications were presented: method development and validation for pharmaceutical analytes with the aim to obtain data usable for PK/PD studies. Both methods were fully validated according to the EMA guideline and reached incredibly low limits of quantification with a minimal sample amount used. One method was created to quantify three sedative drugs – morphine, clonidine and midazolam with their metabolites M3G, M6G and MiOH. Using HFIP as the eluent additive enabled the separation of M3G and M6G and morphine analytes, which have the same fragmentation patterns. The other method, which focused on the cardiovascular drugs milrinone and dobutamine, demonstrated an increase in the signal: two times for milrinone and seven times for dobutamine when NH_4F was used as the eluent additive. An increase in the signal observed ensured less data being marked as BLQ, which cannot be realised due to the guidelines and has a detrimental influence on PK/PD modelling. Further research on the NH_4F influence on analyte ionisation in the positive detection mode was done; however, the results remained very analyte dependant.

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SUMMARY IN ESTONIAN

Uudsed eluendilisandid LC-MS baasil bioanalüütilistele metoodikatele

Käesoleva töö eesmärk oli laiendada teadmisi uudsete eluendilisandite kasutamise kohta peamiselt kõrgrõhu vedelikkromatograafia-massispektromeetria (HPLC-MS) meetodites. Lisaks uuriti põhjalikumalt retentsioonimehhanisme kasutades erinevate pöördfaaskromatograafilisi statsionaarseid faase ja lisandite mõju analüütide ionisatsioonile nende määramisel massispektromeetriga. Lisandeid kasutati peamiselt kõrgete mobiilfaasi pH'de juures.

Uuritud eluendilisanditest kinnitusid kolm fluoroalkoholi (heksafluoroisopropanool – HFIP, heksafluoro-*tert*-butanool – HFTB ja perfluoropinakool – PP) tugevalt C18 statsionaarse faasi pinnale, neist tugevaima interaktsiooniga oli PP. Seega võib eeldada, et uuritud eluendilisandid moodustavad anioonse fluoriühendite kihi statsionaarse faasi pinnale, mis on väga ligitõmbav aluselistele polaarsetele analüütidele, aga samas eemale tõukav happeliste polaarsete analüütide jaoks. Uuritud lisand nonafluoro-*tert*-butanool (NFTB) ei kinnitud statsionaarse faasi pinnale eriti hästi, samas on ühend madala pKa väärtuse tõttu kõrgel eluendi pH-l täielikul deprotoneeritud ja seetõttu omab võimet moodustada mobiilfaasis aluseliste protoneeritud analüütidega ioonpaare. Bifenüül ja pentafluorofenüül (PFP) statsionaarsete faaside korral ei kinnitu HFIP ja HFTB statsionaarse faasi pinnale ja seega ei moodusta need lisandid fluoroalkoholide kihti uuritava statsionaarse faasi pinnale. PP on ainuke eluendilisand, mille puhul oli näha piikide pikka 'sabatamist' (seda ka juhul kui lisandi retentsiooniaeg statsionaarsel faasil ei olnud pikk) ja seetõttu on võimalik, et see lisand moodustab fluoreeritud lisandikihi ka bifenüüli ja PFP statsionaarsete faasi pinnale.

Seega on C18 statsionaarse faasi korral kaks põhilist vastasmõju – (1) analüüdi vastasmõju fluoroalkoholi kihiga statsionaarsel faasi pinnal ning (2) analüüdi vastasmõju eluendilisandiga mobiilses faasis. Tavapäraste levinu eluendilisandite (nagu ammoonium atsetaat, ammoonium bikarbonaat ja ammoonium formiaat) korral tuleneb muutus retentsioonis vaid muutusest analüüdi polaarse [näiteks deprotoneeritud happed (A-) või protoneeriud alused (BH+)] ja mittepolaarse [protoneeritud hapete (AH) ja deprotoneeritud aluste (B)] vormi tasakaalus. Seda on eriti hästi näha polaarsete aluseliste analüütide korral, kui analüütide retentsioon kasvas järsult koos eluendi pH muutusega 8.5-lt 10.0-ni. Kuna ioniseeritud ja mitte-ioniseeritud analüüdi vormid ei muutunud pH muutusega, siis kahe neutraalsete analüütide grupi ühendite (AH ja B) retentsioon muutus väga vähe. Polaarsete happeliste analüütide jaoks tähendas pH tõus ka deprotoneeridud vormi kasvu mobiilfaasis, millega kaasnes analüütide retentsiooniaja kahanemine mittepolaarsel C18 faasi.

Muutused analüütide retentsioonis järgivad üldiselt samu mustreid ka uudsete eluendi komponentide korral sõltudes peaasjalikult tasakaalust polaarse ja mittepolaarse analüüdi vormi vahel. Samas olid uudsete eluendilisandite puhul

retentsiooniaja muutused selgemad ning sageli võimendatud. Mittepolaarsete analüütide korral varieerusid analüütide retentsioonifaktorid rohkem ja kuna enamusel uuritud protoneeritud hapetel on pKa väärtused 10 ligidal ning analüüdid on seega osaliselt protoneeritud just kõrgel eluendi pH-l. Samal ajal olid HFIP ja HFTB kasutamisel nimetatud ühendite retentsioon üldiselt madalam. Happelistel anaüütidel olid uudsete eluendilisanditega madalamad retentsioonifaktorid võrreldes tavapäraste eluendi komponentidega. See tuleneb sellest, et happelised polaarsed analüüdid peavad lisaks statsionaarse faasi nimel konkureerimisele ka täiendalat eemale tõukama eluendi koostises olevaid deprotoneerunud lisandeid HFIP, HFTB, NFTB ja PP. Aluseliste neutraalsete analüütide korral täheldati suuremat vaarieeruvust retentsioonifaktorites, ilma selge kasvu või vähenemise trendita. Seejuures, uudsete eluendi komponentide vaieldamatult suurimat mõju märgati aluseliste polaarsete analüütide puhul. HFIP ja HFTB kasutamisel eluendi pH kasvuga kaasnenud tugevam retentsioon oli selgemalt näha oluliselt pikemates retentsiooniaegades võrreldes tavapäraste eluendilisanditega. Oluline on märkida, et uudsete eluendilisandite kasutamsel kasvas valitud analüütide retentsiooniaeg suisa 28 ja 35 korda. Fluoroalkohol perfluoropinakool kasutamisel suurenes ühe aluselise protoneeritud analüüdi retentsioonifaktor 41 korda. Perfluoropinakool on üks huvitavamaid lisandeid kuna omab kahte hüdroksüülrühma, mistõttu perfluoropinakool omab ka kahte pKa väärtust. See omakorda tähendab, et perfluoropinakooli on sobilik ioonpaar reagent ka madalate mobiilfaasi pH väärtuste juures. NFTB näitas samuti eelnevate fluoroalkoholidega sarnast mõju aluseliste analüütide jaoks.

Oma madala puhverdamisvõime tõttu ei olnud trifluoroetanool (TFE) ja dimetüülsulfoksiid (DMSO) kasutatavad soovitud kõrgete mobiilfaasi pH-de 8.5 ja 9.0 juures, ning lisaks nähti ka olulist analüüdi signaali mahasurumist massispektromeetriga analüütide detekteerimisel. Eelnimetatud kahe probleemi tõttu ei sobi TFE ja DMSO bioanalüütiliste metoodikate puhul eluendilisanditeks.

Üldiselt oli analüütide retentsioon bifenüül statsionaarsel faasil tugevam *pii-pii* vastasmõjude tõttu, aga on ka erandeid – näiteks analüüdid, mis sisaldavad nitro rühma seostusid statsionaarse faasiga väga nõrgalt, isegi juhul kui nende struktuuris oli benseenituum, millel omakorda peaks olema tugev interaktsioon bifenüül statsionaarse faasiga. Kasutades C18 ja bifenüül statsionaarset faasi happeliste analüütide korral esines suurim erinevus retentsioonis eluendi pH-l 8.5 – kui väiksem osa analüüdist on deprotoneeritud ja täiendava pii-pii või halogeen-pii vastasmõju tõttu seostub analüüt tugevamalt bifenüülse statsionaarse faasiga. Aluseliste polaarsete analüütide korral, isegi ilma selge pii-pii vastasmõjuta (kuna ühelgi protoneeriud aluselisel analüüdil ei ole aromaatset tuuma struktuuris) oli retentsioon bifenüül faasil tugevam võrreldes C18 kolonniga. Kõrgem logP väärtus tagas ka parema retentsiooni polaarsete aluseliste analüütide jaoks.

Selleks, et rakendada antud uurimustööd praktikasse, uuriti suurt hulka (üle 100 ühendi) üldlevinud ühendeid (narkootikumid, ravimid), mida kasutatakse toksikoloogia sõeluuringutes. Uuritud analüütidel on igal ühel vähemalt üks aluseline funktsionaalrühm. Sellise analüütide seguga kasutati difluoroäädik-

happe (DFA) eluendilisandina ning prooviti ühendeid lahutada C18 statsionaarse faasiga. Kui tavapärases puhverlahuses (kuhu on lisatud ka ammoonium formiaat) vahetati metaanhape DFA vastu, ei muutunud ainete retentsioon ega ionisatsiooni oluliselt. Samas vähenes retentsioon oluliselt 42 analüüdi jaoks 91-st kui eluendilisandina kasutati DFAd ilma ammoonium formiaadina, antud efekt oli tingitud peamiselt eluendi pH langusest. DFA kasutamisel kasvas retentsioon analüütidel, mille struktuuris oli happeline funktsionaalrühm. Analüütide ionisatsiooni uurimisel täheldati, et DFA andis järjepidevalt madalamat signaali võrreldes metaanhappe kasutamisega.

Bifenüülse statsionaarse faasi korral aga vähenes detekteerivate analüütide hulk. Pooli analüütidest ei mõjutanud eluendi komponendi vahetamine (ammoonium atsetaadi vahetamine HFTB vastu) ega pH muutus 8.5-lt 9.0-le. HFTB kasutamisel eluendi koostises vähenes retentsioon analüütidel, millel on kas madal pKa väärtus või mis sisaldavad karboksüülrühma. Analüütidel, mille retentsioon kasvas HFTB kasutamisel eluendi koostises, oli kõrgem pKa ja ka kõrgem logP väärtus, mis tähendab, et suurem osa analüüdist oli mobiilfaasis polaarses vormis ja seega rohkem ligi tõmmatud HFTB poolt. Kui HFTB kasutati eluendi komponendina, siis enamus analüütide signaal kasvas võrreldes ammoonium atsetaadi kasutamisega, eriti suur kasv oli märgatav eluendi pH 9.0 juures.

Suurim erinevus retentsioonis esines PFP kolonni kasutamisel, kuna vaid 42 analüüdi piigid olid detekteeritavad ja seda ka esialgse happelise mobiilfaasi kasutamisel. Analüütidel, mille pKa väärtus on alla 7.6, ei muutunud retentsioon oluliselt HFTB kasutamisel eluendilisandina. Taaskord, HFTB kasutamisel omavad nõrgemat retentsiooni analüüdid, millel on struktuuris karboksüülrühmad. Just nagu varasemalt bifenüül kolonniga, happeliste funktsionaalrühmadega analüüdid, mis on deprotoneeritud kõrge pH juures, omavad tugevat tõukumist deprotoneeritud uudsete fluorineeritud eluendilisanditega ning tugevat tõmbumist protoneeritud aluselise analüütidega.

Töös esitleti kahte praktilist rakendust – arendati välja ja valideeriti analüüsimetoodikad, et määra ravimite kontsentratsioone vereplasmast farmakokineetiliste uuringute jaoks. Mõlemad metoodikad valideeriti täielikul järgides Euroopa Ravimiameti bioanalüütiliste määramismetoodikate valideerimise juhendmaterjali ja saavutades äärmiselt madalad määramispiirid. Lisaks sellele olid kasutusel ka äärmiselt väikesed proovikogused. Üks metoodika loodi kolme uinutina kasutatava ravimi (morfiini, klonidiini ja midasolaami ning nende metaboliite) kvantifitseerimiseks. HFIP-i kasutamine eluendi komponendina võimaldas kromatograafiliselt eraldada morfiini ja morfiini metaboliite. Teine määramismetoodika, mis keskendus südame ja veresoonkonna haiguste ravimitele milrinoon ja dobutamiin, näitas NH_4F kasutamine võimaldab kasvatada oluliselt massispektromeetris detekteeritud signaali tugevust (kaks korda milrinooni ja seitse korda dobutamiini puhul). Nähtud signaali kasv võimaldas saavutada madalamaid määramispiire metoodikale. Täiendavalt viidi läbi ka uurimus NH_4F mõjust analüüdi ionisatsioonile positiivses detekteerimisrežiimis.

Alla määramispiiri jäävaid kontsentratsioone määramismetoodikates ei oli rahvusvaheliste juhendmaterjalide alusel lubatud laborist väljastada, kuna nende usaldusväärsus on kaheldav. Töös pakuti ka lahendus selliste määramispiiri-aluste tulemuste hindamiseks kui uudsed eluendilisandid ei pruugi lahendusi pakkuda.

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PUBLICATIONS

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