DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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Fluoroalcohols as Components of LC-ESI-MS Eluents: Usage and Applications

TARTU UNIVERSITY PRESS

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Dissertation was accepted for the commencement of the degree of *Doctor philosophiae* in Chemistry on June 14th, 2012 by the Council of Institute of Chemistry, Faculty of Science and Technology, University of Tartu.

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Commencement: Room 1021, Chemicum, 14A Ravila Street, Tartu, on 31st of August in 2012, at 12.00.

This work has been partially supported by the Graduate School "Functional materials and technologies", receiving funding from the European Social Fund under project 1.2.0401.09-0079 at the University of Tartu, Estonia

ISSN 1406–0299
ISBN 978–9949–32–059–2 (trükis)
ISBN 978–9949–32–060–8 (pdf)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimuse nr. 346

The desire to take medicine is perhaps the greatest feature which distinguishes man from animals.

William Osler (1849–1919)

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I. Karjagin, J., Lefeuvre, S., Oselin, K., **Kipper, K.**, Marchand, S., Tikkerberi, A., Starkopf, J., Couet, W., Sawchuk, R.J. Pharmacokinetics of Meropenem Determined by Microdialysis in the Peritoneal Fluid of Patients with Severe Peritonitis Associated with Septic Shock. Clinical Pharmacology & Therapeutics, 83 (2008) 452–459.

II. **Kipper, K.**, Anier, K., Leito, I., Karjagin, J., Oselin, K., Herodes, K. Rapid Determination of Meropenem in Biological Fluids by LC: Comparison of Various Methods for Sample Preparation and Investigation of Meropenem Stability. Chromatographia, 70 (2009) 1423–1427.

III. Lillenberg, M., Yurchenko, S., **Kipper, K.**, Herodes, K., Pihl, V., Sepp, K., Lõhmus, R., Nei, L. Simultaneous Determination of Fluoroquinolones, Sulfonamides and Tetracyclines in Sewage Sludge by Pressurized Liquid Extraction and Liquid Chromatography Electrospray Ionization-Mass Spectrometry. Journal of Chromatography A, 1216 (2009) 5949–5954.

IV. Lillenberg, M., Yurchenko, S., **Kipper, K.**, Herodes, K., Pihl, V., Lõhmus, R., Ivask, M., Kuu, A., Kutti, S., Litvin, S.V., Nei, L. Presence of Fluoroquinolones and Sulfonamides in Urban Sewage Sludge and their Degradation as a Result of Composting. International Journal of Environmental Science and Technology, 7 (2010) 307–312.

V. **Kipper, K.**, Herodes, K.; Leito, I.; Nei, L. Two Fluoroalcohols as Components of Basic Buffers for Liquid Chromatography Electrospray Ionization Mass Spectrometric Determination of Antibiotic Residues. Analyst, 136 (2011) 4587–4594.

VI. **Kipper, K.**, Herodes, K., Leito, I. Fluoroalcohols as Novel Buffer Components for Basic Buffer Solutions for Liquid Chromatography Electrospray Ionization Mass Spectrometry: Retention Mechanisms. Journal of Chromatography A, 1218 (2011) 8175–8180.

VII. Padari, H., Metsvaht, T., Kõrgvee, L-T., Germovsek, E., Ilmoja, M-L., **Kipper, K.**, Herodes, K., Standing, J., Oselin, K., Lutsar, I. Short Versus Long Infusion of Meropenem in Very Low Birth Weight Neonates. Antimicrobial Agents and Chemotherapy: accepted.

Author's contribution

Paper I: Carried out the sample preparation and HPLC analysis of meropenem in plasma and wrote the respective chapters of the manuscript.

Paper II: Main person responsible for planning and performing the experiments and for writing the manuscript.

Paper III: Main person responsible for performing sample preparation (solid phase extraction) and LC-ESI-MS experiments; planning and

carrying out method validation; writing the respective parts of the manuscript.

Paper IV: Main person responsible for sample preparation (solid phase extraction) and LC-ESI-MS analysis of the Tallinn and Tartu sewage sludge and compost samples; writing the experimental and results and discussion sections.

Paper V: Main person responsible for planning and performing the experiments and for writing the manuscript.

Paper VI: Main person responsible for planning and performing the experiments and for writing the manuscript.

Paper VII: Main person responsible for sample preparation and LC-ESI-MS experiments as well as method validation and writing the experimental part of the manuscript.

ABBREVIATIONS

1-MePip	1-methylpiperidine
AAF	ammonium acetate and formic acid buffer
CE	capillary electrophoresis
CIP	ciprofloxacin
CID	collision-induced dissociation
DOX	doxycycline
DAD	diode array detector
EC	electrochemical detector
ESI	electrospray ionization
FL	fluorescence detector
FQs	fluoroquinolones
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol (hexafluoroisopropanol)
HFTB	1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (hexafluoro-*tert*-butanol)
HILIC	hydrophilic interaction chromatography
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
I.S.	internal standard
IPC	ion-pairing chromatography
LC	liquid chromatography
LE	liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
MS	mass-spectrometry
n.d.	not detected
NOR	norfloxacin
OFL	ofloxacin
PLE	pressurized liquid extraction
SAs	sulfonamides
SCX	strong cation-exchange
SD	standard deviation
SDM	sulfadimethoxine
SMX	sulfamethoxazole
SPE	solid phase extraction
SRM	single reaction monitoring
ODS	octadecylsilica
RP	reversed phase
TCs	tetracyclines
TCL	tetracycline
TEAA	triethylammonium acetate
UHPLC	ultra high performance liquid chromatography
UV–VIS	ultraviolet/visible spectrometric detector
USE	ultrasonic-assisted extraction

INTRODUCTION

Liquid chromatography (LC) with electrospray ionization (ESI) mass spectrometric (MS) detection has gained popularity due to its high sensitivity and selectivity. LC-ESI-MS is the method of choice in many fields, like environmental and clinical analyses, proteomics, drug and doping control, and pharmacokinetic studies.

ESI-MS is susceptible to the matrix effects – the alteration of signal intensity due to sample matrix compounds present in the ESI source. Therefore, in the case of complex matrices, proper sample preparation and adequate chromatographic separation is required. Pharmaceuticals are often compounds with basic properties and for their reversed phase LC separation, acidic conditions are used. In acidic conditions, basic analytes are predominantly in their protonated (hydrophilic) form and, therefore, have poor retention on the stationary phase. Basic conditions for the analysis of basic compounds should provide stronger retention and better peak shapes.

The ESI-MS detection dictates the volatility requirement for the eluent. The selection of volatile basic buffer compounds is limited and, therefore, additions to the buffer selection would be highly welcome. The LC-MS sets another limitation to the buffer compound – the compound must not suppress the ESI-MS signal. Fluoroalcohols are a promising group of volatile compounds with pK_a values suitable for the preparation of basic buffer solutions.

The aim of the present work was to take under careful study two fluoroalcohols – 1,1,1,3,3,3-hexafluoro-2-propanol and 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol and to evaluate their usage as basic LC-MS buffer components.

In order to evaluate the novel buffer components, the influence of the fluoroalcohols on the ionization of analytes is studied, as well as the separation of several compounds of practical interest. The trends in the retention of the analytes with the change of the pH of the buffer solution are examined. Moreover, the retention of analytes in selected stationary phases will be examined and a comparison with commonly used buffer solutions is made.

The effects of fluoroalcohols on the retention of the model compounds are studied and a retention model is proposed. The retention behavior of analytes on the reversed phase LC column in the presence of fluoroalcohol-based buffer solution is also compared to the retention on a fluorinated stationary phase. The advantages of using fluoroalcohols as LC-MS buffer components and enhancements in chromatographic separation are demonstrated.

Since the analysis would be of interest to the pharmaceutical industry, the successful application of fluorinated alcohols for the analysis of the pharmaceuticals in environmental samples, human blood plasma and urine samples are presented.

1. LITERATURE OVERVIEW

1.1. Analysis of pharmaceuticals

Pharmaceuticals have an extensive impact on human daily life. The behavior of pharmaceuticals in the human body is described by pharmacokinetics that consist processes like absorption, distribution, metabolism, and excretion of the drug. Clinical pharmacokinetics relies on the relationship between the pharmacological effects of a pharmaceutical and its content in blood or plasma. Usually, the concentration of the pharmaceutical at its sites of action is related to the concentration of the pharmaceutical in the systemic circulation. Pharmaceutical concentration in the blood or plasma is indicating the therapeutic effect of the drug, either it is therapeutic, toxic (in case the dose was excessive) or if there is a risk for occurrence of drug concentration related adverse effects [1].

Pharmaceuticals used to treat human or veterinary diseases are excreted by humans or animals and pose a risk to the aquatic environment and soil. Therefore, the determination of the pharmaceuticals in environmental samples is gaining increasingly more attention. Due to the rapid increase in the human population, the amount of sewage sludge and use of it has increased rapidly in the past two decades [2]. After composting, the sludge it can be used as a fertilizer in agricultural applications [3] but only if it has been confirmed to be safe for the soil, surface and ground water, plants, people and animals [4]. Besides the heavy metals, microbes like *Escherihia coli* and helmints' eggs, the content of pharmaceuticals, including antibiotics present in the sewage sludge are of interest [5]. The traces of different pharmaceuticals are found in ambient waters, wastewaters and drinking water [6,7].

In case the sludge or compost is used as a fertilizer, the pharmaceuticals and their metabolites could accumulate in the plants grown employing the fertilizer [8,9,10].

Approximately 75% of pharmaceuticals have basic properties. At the same time, about 20% are acids [11,12]. In terms of marketed drugs, about 45–50% are salts and the majority of these have weakly basic or weakly acidic properties [1]. In addition, a large scale of biologically and biomedically significant compounds also have basic properties [13].

As pharmaceuticals are often analyzed in complex matrices, elaborate sample preparation techniques and highly selective analytical methods are employed. For pharmaceutical analysis, different analytical techniques starting with infrared and Raman spectrometry, atomic spectrometry, capillary electrophoresis (CE) and liquid chromatography (LC) with different detectors – ultraviolet/visible (UV-VIS), fluorescence (FL), mass-spectrometric (MS), electrochemical (EC), Corona CAD [14] detectors are used [15]. Chromatographic separation is one of the preferred analytical methods for the analysis of pharmaceuticals because of its accuracy and wide range of detectors available.

1.2. Reversed-phase chromatographic analysis of basic compounds

The high performance liquid chromatographic (HPLC) analysis of basic compounds is the object of much interest especially with respect to the analysis of pharmaceuticals. Reversed phase (RP) chromatographic separation is one of the most powerful tools and has been widely used in combination with mass spectrometric detection. RP-HPLC allows the analysis of aqueous samples, enables gradient elution, and provides means for altering the retention of analytes (with a range of organic solvents, changes in the eluent pH and the ability to use eluent additives) [16]. RP separation has disadvantages, while analytes have basic properties and can be advanced.

When analyzing complex samples, e.g. environmental or biological matrices, chromatographic separation of pharmaceuticals from sample components is necessary. In RP chromatographic separation, the mobile phase is polar and the stationary phase surface has non-polar properties; different distribution of components between the mobile and stationary phase provides the chromatographic separation of the analyte from the sample matrix. In case of neutral, i.e. less polar molecules, analytes have a stronger retention in the chromatographic system.

1.2.1. Mobile phase and buffer selection for LC-MS analysis of bases

When analyzing ionizable compounds, the mobile phase pH can be one of the most important characteristics in the RP separation optimization. RP-HPLC analytes often contain acidic or basic functional groups and, therefore, mobile phases usually require pH control and buffer solution usage. While analyzing basic compounds, the analytes are present predominantly in their protonated form if the pH of the eluent is lower than the pK_a value of the base. Protonated, i.e. the cationic form, is polar and has poor retention behavior in the RP column. If the pH value is higher than the pK_a of the base, the basic center is deprotonated. As a result, better retention is expected. From this point of view, a basic buffer solution would be preferable for the separation of basic compounds by RP-HPLC.

Another important characteristic in the selection of the mobile phase pH is the buffer component properties. While mass spectrometric detection (MS) is used as an LC detector, the volatility of buffer components is required. The use of non-volatile buffer components causes contamination of the electrospray ionization (ESI) source [17,18].LC-MS analysis combines the separation ability of the LC and the more sensitive and selective detection provided by the MS (or MS/MS) system. The selection of suitable (i.e. non-interfering with ESI ionization) [16,19,20] buffer components in the basic pH range is limited for LC-MS (Table 1).

LC-MS with ESI has become a widely used analytical tool for the identification and quantification of pharmaceuticals as low molecular weight compounds. As LC-ESI-MS is used, in addition to the volatility of the buffer components, these should not suppress the ESI signal. The mobile phase should preferably support/enhance the ionization. Due to the current selection of buffer components being quite poor, any addition to it would be welcome and essential.

Table 1. ESI-MS compatible (volatile) buffer components recommended for Waters XBridge Columns at high pH by Waters® [21].

Additive/Buffer	pK_a	Buffer range	Recommended concentration
4-Methylmorpholine	~8.4	7.4–9.4	10 mM or less
Ammonia (NH_4OH)	9.2	8.2–10.2	Below 10 mM
Ammonium Bicarbonate	10.3 (HCO_3^-) 9.2 (NH_4^+)	6.8–11.3	5–10 mM range
Ammonium (Acetate)	9.2	8.2–10.2	1–10 mM range
Ammonium (Formate)	9.2	8.2–10.2	1–10 mM range
1-Methylpiperidine	10.2	9.3–11.3	1–10 mM range
Triethylamine (as acetate salt)	10.7	9.7–11.7	0.1–1.0% range
Pyrrolidine	11.3	10.3–12.3	–

The addition of strong bases to buffers such as triethylamine (TEA) or piperidine is reported to significantly suppress Na^+ and K^+ adduct formation while using MS for detection and at the same time increasing the sensitivity of electrospray ionization [22]. RP-HPLC can be easily combined with ESI-MS due to the low ionic strength of the buffer components (usually in the range 1–10 mM) and organic solvent in mobile phase. The influences of several additives in LC-MS have been studied for acidic and basic drugs. A decrease in the response have been demonstrated when the concentration of the additive (formic acid, acetic acid, trifluoroacetic acid, ammonium formate, ammonium biphosphonate, ammonium bicarbonate) was increased from 0.05% to 1% [23,24]. Basic buffer additives such as ammonium formate, ammonium biphosphonate and ammonium bicarbonate have a stronger signal suppressing effect than acidic (formic acid, acetic acid) and basic buffers (ammonium hydroxide) for the ESI response of the studied acidic and basic drugs [23,24]. Also, triethylammonium acetate (TEAA) (Table 1) buffer solution, which can act as ion-pairing, is also known to suppress the ESI signal [25,26].

1.2.2. Stationary phase selection for analysis of bases

In addition to the selection of the mobile phase buffer component and its pH, the second most important choice to make is a suitable stationary phase. The most common silica-based stationary phases are stable in the pH range 2 to 8. Silica-based RP column stationary phases are made by covalently bonding an organosilane on a silica gel surface [16]. The bonded silica has many unbound silanol groups on its surface and below pH 2 the bonded phase starts to hydrolytically cleave. Above pH 8, the silica support itself starts to dissolve. The potential ionization of unbound silanol groups (–Si–OH) on the silica surface may occur while changes in mobile phase pH are made. [13–16] The pK_a of silanol groups have been reported to be 4.5 [27,28], 4.9 [29], 7.1 [13], 8.5 [28] and 9 [30].The silanol groups with a lower pK_a value (around 4.5 and 4.9) are believed to belong to isolated silanol groups having no hydrogen bonding to neighboring groups. Other types of silanols with a higher pK_a value (around 8.5) are connected to neighboring groups through hydrogen bonding directly or via bridging water molecules [29]. The surface concentration of silanols with a more basic pK_a is higher [29]. In case of metal impurities, the acidity of silanols increases. When silica based columns are used within the pH range 2 to 8, the basic compounds are most likely in their ionized form and interactions between the analyte and ionized silanol groups (–Si–O^-) on the stationary phase surface may occur. This interaction has historically been one of the most important causes of chromatographic peak tailing while analyzing basic compounds [13,31,32,33,34]. Residual silanol groups also cause low efficiency, retention and column-to-column reproducibility problems [13,16]. While choosing a column for application, reproducibility is an important characteristic to consider – especially for the routine analysis of pharmaceuticals, the column to column reproducibility is crucial [13]. The reproducibility problems may occur by the same manufacturer and the same brand, when the raw material, e.g. silica, has changed. Changes like these might be indicated by the manufacturer's note about advanced technology. In this case, then, the reproducibility of the same stationary phase might be slightly different from the same stationary phase purchased earlier [32].

Nowadays, a stationary phase with the advanced purity of silica is used. Residual silanol group activities can be reduced by *endcapping* (a reaction of the bonded packing with small silane – trimethylchlorosilane, dimethylchlorosilane or hexamethyldisilazane) [13,16]. Some stationary phases have used *endcapping* reagents with amino groups, which generate a considerable anion-exchange character [13]. Even small *endcapped* groups can hydrolyze from the packing in RP separation while a low mobile phase pH is used, for example after a long term operation of the column at pH < 3 [16].

Successful LC analysis of basic compounds can be carried out by carefully selecting the stationary phase with a reduced number of acidic sites and a reduction mobile phase pH to suppress the ionization of silanols [13,35]. Using a low pH decreases ion exchange interaction between the protonated base (BH^+)

and cations that are attached to the ionized silanols on the silica surface. Silanol-sample interactions can be reduced with a low mobile phase pH (between 2 and 3.5) or a buffer solution concentration that leads to the minimization of ionized silanol groups. Buffer cations interact strongly with silanols and block sample retention on silanol sites [16]. When analytes are protonated in the solution due to the buffer solution pH but silanol groups are protonated on the stationary phase surface, better peak shapes are observed and also better ESI ionization is expected.

However, increasing the mobile phase pH above the analyte's pK_a leads to the deprotonation of the basic analytes [11]. Working with basic compounds using the mobile phase with a high pH also sets requirements for the column. The selection of the column for working in a high pH range should be made according to its resistance to a high pH. Stationary phases with an extended pH range are commercially available for several applications, for example for the analysis of basic compounds. Basic compounds are neutral in a high pH range and the retention of these compounds is increased, thus allowing for better chromatographic optimization. Peaks of neutral compounds are more symmetrical in RP-HPLC separation, leading to better integration and greater resolution. A greater retention factor in the chromatographic procedure allows higher organic solvent content usage in the LC system mobile phase, which leads to a better ionization in the ESI source while using MS detection. [13,16,18]

A wider pH range for the stationary phase allows the usage of the same analytical column for different applications: the analysis of acidic compounds at a low pH and basic compounds at a high pH. The column selection for the application of the basic compounds separation is a difficult task because of the wide variety of commercially available analytical columns. Databases, based on different column tests, have been created, and can help to choose the right column for application. Methods of column selection for basic compounds have been developed; for example, by Tanaka *et al* [36] and another is known as the hydrophobic subtraction procedure [37]. Nevertheless, the wide application range for a column is more time- and cost-effective than choosing a specific column for specific application.

For the stationary phases of octadecylsilica (ODS), a wider pH range has been achieved by different techniques of coating the silica surface. For example, hybrid silica-based columns have extended the usable pH range to pH 12 (or higher) [34] (XBridge and BEH columns from Waters® with trifunctional ligand bonding chemistry or the Triart column from YMC® with multi-layered, organic/inorganic hybrid particles).

1.2.3. Fluorinated stationary phases

Another promising group of stationary phases that has been successfully applied in pharmaceutical and drug analysis are fluorinated (pentafluorophenyl and pentafluorophenylpropyl) stationary phases [38,39,40]. Fluorinated stationary

phases are associated with various interaction mechanisms improving retention and chromatographic resolution. The pentafluorophenyl stationary phase improves analyte retention with dipole-dipole, aromatic pi-pi and hydrophobic interactions [40]. Most similar to common stationary phases are perfluorinated alkyl chains, for example C8, used for its alternative retention for several applications on polar molecules [41,42], especially for halogenated analytes [43,44,45,46], but also for aromatic polycyclic hydrocarbons due to the interactions between pi-electrons and the C-F dipole on the stationary phase [45,47]. The performance of fluorinated columns has improved in the recent years with respect to their pH stability and column lifetime. Additionally, stationary phase bleeding is reduced allowing the use of these stationary phases also in MS applications. The retention of all analytes has been reported to be overall lower on the fluorinated stationary phase than on C18 [46].

1.2.4. Hydrophilic interaction chromatography (HILIC)

Hydrophilic interaction chromatography (HILIC) is another choice when attempting the separation of hydrophilic (ionized) compounds [48]. HILIC has a good retention of hydrophilic compounds, lower backpressure and higher MS signal intensity due to the higher content of the organic solvent compared to RP-LC. Moreover, HILIC has been demonstrated to be efficient for the analysis of pharmaceutical compounds [49]. In HILIC separation, the retention mechanisms are complex and different mechanisms (e.g. adsorption, ion exchange and hydrophobic interactions) are present at the same time for the compound analyzed [50]. Complex retention itself is different in various HILIC stationary phases. Retention mechanisms are influenced by the partitioning of the analyte molecules between the layer of water on the surface of the stationary phase and the amount of the organic solvent in the mobile phase, ionic retention on ionized groups as well as on ionized silanols on the base silica and even the regular RP retention on the hydrophobic bonded ligand [13]. Due to the complex nature of HILIC retention mechanisms, predicting the retention of the analyte on the stationary phase is difficult.

1.2.5. Fluoroalcohols as buffer components for LC-ESI-MS

Fluorinated alcohols are volatile, weakly acidic compounds and uncharged in protonated form. These properties make them good candidates for LC-MS buffer components. Fluorinated alcohols are a potentially promising class of compounds to be used as weak acids for preparing buffers of pH value above 7.

1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, pK_a = 9.3) [25] has been used as an additive to the LC mobile phase in several studies [25,26,51,52,53,54,55,56]. In these studies, the pH of the mobile phase was adjusted to neutral or slightly basic: 7.0 [25,26,51], 7.5 [54], 7.9 [52,53], 8.2–8.4 [56] and 8.5 [55]. HFIP was used in these studies with triethylamine for oligonucleotide and oligosaccharide

analysis. As a rule, the pH range of the buffer solution should be ±1 unit of the buffer component's pK_a. In most of the abovementioned studies, the used buffer solution pH values were significantly different from the HFIP pK_a. Also, HFIP concentrations in the studies ranged from 100 mM to 800 mM (ca 2% to 15% by mass), exceeding the buffer concentration levels commonly used for LC-MS applications (1–10 mM) by far. Thus, the role of HFIP was rather that of an additional solvent component than a buffer acid. Using HFIP as the weak acid and triethylamine (TEA) as the weak base in buffer systems resulted in high ESI intensities, a high efficiency of dissociation of the oligonucleotide-TEA ion-pairs and a good chromatographic separation [25,26]. Interestingly, this promising approach of the possible usage of polyfluorinated alcohols has not been extended neither to the analysis of other compounds (e.g. low molecular weight compounds) nor to the use of other polyfluorinated alcohols (for example 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFTB, pK_a = 9.6 [57])) than HFIP.

1.3. Retention mechanisms of reversed-phase chromatographic analysis

The surface area of the stationary phase is the major factor on retention – the larger the surface area, the greater the retention (k). The retention factor:

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

is defined through the retention time of the compound (t_R) and the column dead time (t_0).

While only hydrophobic interactions are present, retention increases with the percent of carbon on a stationary phase until the organic ligands are completely accessible to solutes [16]. While silanol groups are present on the stationary phase support material surface, the RP hydrophobic mechanism might concur with the hydrophilic (e.g. normal phase) retention mechanism. Hydrophilic silanol sites on the stationary phase surface are the cause of the hydrophilic interaction with analytes. In the mixed retention mechanism, the percentage of carbon is less significant to the retention of analytes. The general rule is that the retention factor (k) increases with the stationary phase surface modifier's carbon chain length but is also influenced by the stationary phase surface area and the type of silica used in the packing material [13–18].

A major problem of LC-ESI-MS analysis is the matrix effect [58] (discussed in Section 1.6). Analyte signal suppression in MS detection may occur with sample matrix components' co-elution. Therefore, the chromatographic separation of analytes from matrix components can have a serious impact on detection sensitivity, the limits of quantitation and uncertainty. While optimizing chromatographic separation, the most important features after the organic modifier

content selection is the buffer solution component providing the separation of the analytes using the pH or ion-pairing effect [59].

1.3.1. Retention of acidic and basic compounds

In RP chromatography, hydrophobic compounds are more strongly retained on the stationary phase. When an acid (HA) or base (B) ionizes, the compound becomes more hydrophilic because its charge and retention on the stationary phase decreases.

$$HA \leftrightarrow A^- + H^+ \quad (2)$$
$$B + H^+ \leftrightarrow BH^+ \quad (3)$$

While the pH is more than 2 units below the pK_a of the acid, it will be mostly in a non-ionized form (HA). If the pH is more than 2 units above the pK_a of the acid, the acid will be mostly in an ionized form (A^-). The ionization of bases works the other way around, 2 units below the pK_a, the base is ionized (HB^+) and 2 units above the pK_a, the base is non-ionized (B) (Fig. 1) [16].

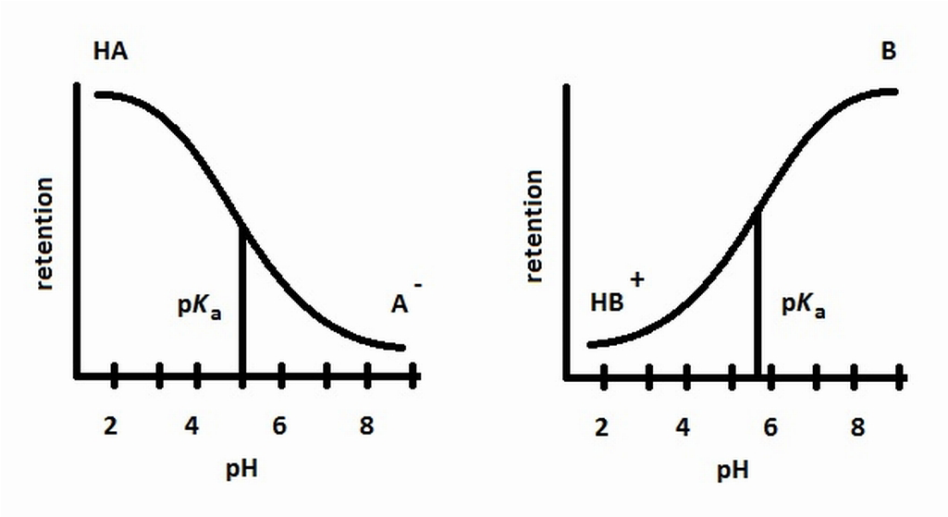


Figure 1. RP-HPLC retention vs pH for acids (left) and bases (right) analysis.

The non-ionized form of an analyte is more hydrophobic (less polar) and has a stronger interaction with the non-polar RP stationary phase, therefore the retention increases. The ionized form (A^-, BH^+) is hydrophilic (more polar) and therefore has a weak interaction with the hydrophobic RP stationary phase and this results in retention decreasing. While the pH is close to the pK_a, a small change of the pH leads to a large change in retention. While routine RP-HPLC methods should be as robust in separation as possible, the mobile phase pH should be about two pK_a units above or below the analyte pK_a [16].

Retention of ionizable compounds is especially sensitive to small changes in the mobile phase pH. The mobile phase pH is preferably controlled by a buffer solution due to its ability to maintain a constant pH while a small amount of an acid or base is added.

1.3.2. Ion-pairing mechanism in reversed-phase chromatographic analysis

The stationary phase and mobile phase used in ion-pairing chromatography are similar to traditional RP chromatography phases. While ionic samples are analyzed by chromatography, ion-pairing reagents add some additional selectivity to the separation. The ion-pairing mechanism in RP chromatography involves interaction between the ion-pair reagent (cationic or anionic) and analyte and/or the interaction of the reagent or analyte-reagent pair with the stationary phase.

One example is the case of the analysis of basic compounds in the protonated form (BH^+). Retention in ion-pairing chromatography (IPC) can be described by two models [16]. According to one model, the ion-pairing agent carrying a negative charge covers the RP C18 or C8 surface by the hydrophobic (e.g. neutral, alkyl group) side of the agent molecule. By covering the stationary phase surface, the negatively charged ion-pairing agent makes the surface of the stationary phase more hydrophilic and covered with negatively charged groups. This more hydrophilic (negatively charged) stationary phase is balanced by positively charged ions (BH^+ or Na^+, allowing their exchange), thereby increasing the retention of the analyte on the stationary phase. According to another model, the negatively charged ion-pairing agent forms an ion-pair directly with the positively charged analyte, resulting in a neutral (more hydrophobic) anion-cation pair and increasing retention on the hydrophobic stationary phase. Both models lead to similar conclusions [16].

1.4. Analytes

The application of the pharmaceutical analysis is demonstrated for selected antibiotics – sulfonamides (SAs), fluoroquinolones (FQs) and carbapenems.

1.4.1. Sulfonamides and fluoroquinolones

The selection of analytes for this study from the possible range of antibiotics was made considering the stability in the environment and the potential of the residues to accumulate into plants. The following five antibiotics were chosen for the study: three fluoroquinolones (FQs): ciprofloxacin (CIP), norfloxacin (NOR), ofloxacin (OFL) (Fig. 2), and two sulfonamides (SAs): sulfadimethoxine (SDM) and sulfamethoxazole (SMX) (Fig. 3) [III,IV].

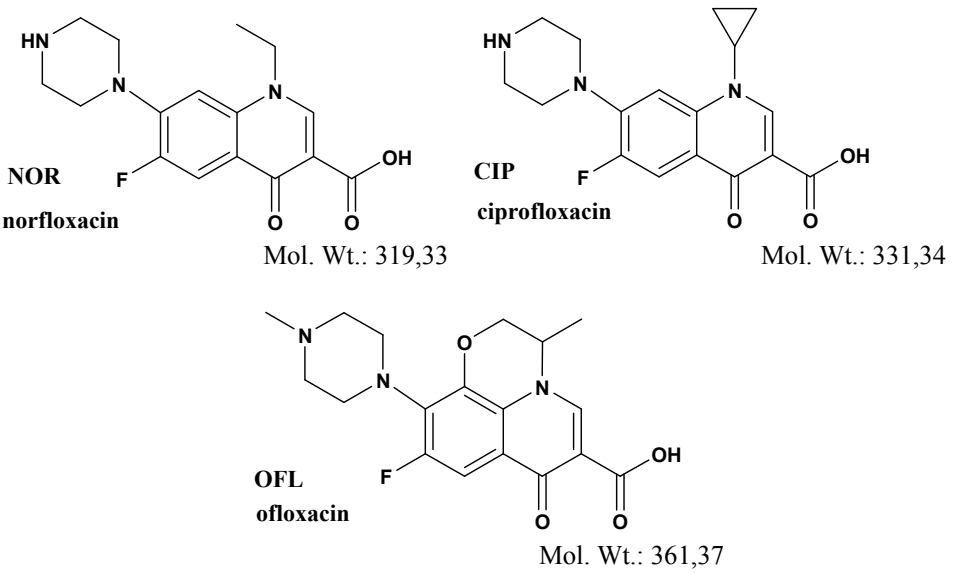


Figure 2. Chemical structures of the used FQs.

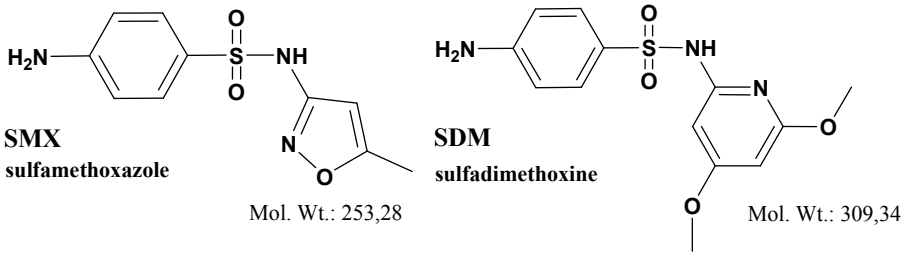


Figure 3. Chemical structures of the used SAs.

Antibiotics pathway in the environment

Antibiotics such as FQs and SAs have many properties, facilitating their bio-accumulation and provoking changes in the ecosystems. Antibiotics often have lipophilic properties in order to pass biomembranes and are persistent in order to avoid inactivation before having a therapeutic effect [60]. Lipophilicity is described by the 1-octanol/water partition coefficient (K_{ow}). K_{ow} is one of the most important parameters for estimating the environmental fate and toxicology of the drug and is defined as follows.

$$logK_{ow} = log\left(\frac{[solute]_{octanol}}{[solute]_{water}}\right), \quad (4)$$

where the partition of the solutes' concentration in two immiscible liquids are estimated [61].

For CIP, NOR and OFL the logK_{ow} values (at 25 °C) are 1.1, –1.4 and 0.4 [62]. For SMX and SDM the logK_{ow} values (at 25 °C) are 0.9 and 1.6 [63].

Antibiotics are poorly biodegraded by the bacteria present in the sewage sludge [64]. Using sewage sludge as a fertilizer and growing plants in this fertilized soil might lead to pharmaceutical accumulation into plants. Due to the adsorption of antibiotics to solid sewage sludge particles and the heterogeneity of the sludge, the concentration of pharmaceuticals may be higher locally than the average in the soil [65]. Adsorption of the pharmaceuticals on soil particles is also described by K_{ow}, strong adsorption on the soil particles also explains the slow degradation of these antibiotics in the environment [66]. In environmental pH conditions, the adsorption of SMX and SDM to soil clay particles was, as expected, to be negligible [67] while the adsorption of FQs in clay-rich soil was extensive. FQs appear in the soil solution partially in an anionic form. The mineral surface of the soil is also partly negatively charged. In the mineral surface, some cations are present and binding between anionic FQ molecules and the mineral surface will take place through the cation exchange. The adsorption of the FQ molecule is found to be most effective while the molecule is oriented as flatly as possible. The adsorption of the FQs on the soil particles is high and is considered to be almost 100%. Since FQs are relatively polar compounds, the adsorption on soil is achieved due to the cation exchange, cation bridging at clay surfaces, surface complexation, and hydrogen bonding [68]. K_{ow} values together with the sorption coefficient and carbon-normalized sorption coefficient help to determine the nature of the hydrophobic interactions between the FQ and soil. The accumulated pharmaceuticals in the food plants may generate resistant bacteria in human and animal organisms and therefore, the environmental monitoring of drugs is under careful study.

FQs and SAs pK_a-s

For SMX, the pK_{a1} and pK_{a2} values are 1.49 and 5.41, and for SDM 2.11 and 6.17, respectively [54]. The acid-base behavior of FQs has been studied by several researchers but there is still no agreement in the published data. The number of pK_a values determined for FQs is two [69], three [70] or four [71]. Also, the assignment of pK_a values to acidic/basic sites is a topic of controversy. These difficulties with the studies of acid-base properties of FQs are also mentioned in the review article [72]. As the pH range from 9 to 10 is investigated in this work, only the last (most basic) pK_a is of importance. Therefore, in order to avoid confusion, we denote this pK_a as pK_{ax}. Respective acid-base equilibrium is presented in Fig. 3. The pK_{ax} values are adapted from the work of Barbosa *et al.* [73]: CIP 8.62, NOR 8.38 and OFL 8.11.

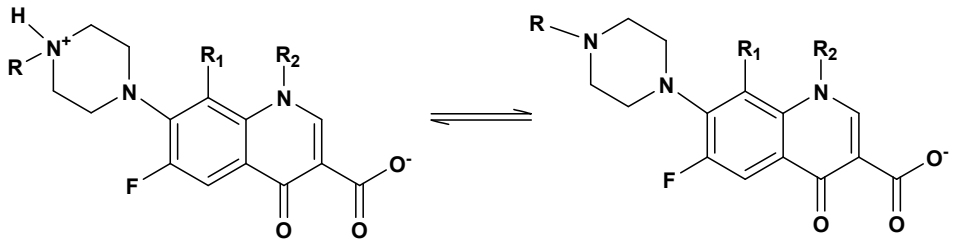


Figure 4. Acid-base equilibrium of fluoroquinolons at the basic pH.

1.4.2. Carbapenems

Carbapenems (Fig. 5) are β-lactam antibiotics that have a broad spectrum of activity. Carbapenems are stable against a variety of β-lactamases. Due to the carbapenems' antibacterial activity against a wide range of gram-positive and gram-negative bacteria, the use of these drugs is extensive. Carbapenems penetrate the bacterial cell wall of susceptible organisms and inhibits cell wall synthesis. These drugs have been used in the treatment of serious forms of infections, for example, complicated urinary tract infections, sepses, pneumonia, endocarditis, and polymicrobial infections [74,75]. In the case of carbapenems, renal toxicity and neurotoxicity can occur (for example, imipenem has that disadvantage and has to be combined with cilastatin). Therefore, all modifications of carbapenem development should be done to decrease their toxicity. The main metabolites of carbapenems are inactive ring-open derivates formed by the hydrolysis of the β-lactam ring. Stability studies of carbapenems have also been the ground for extensive interest [76,77,78,79,80,81,82]. The most common carbapenems applied are described as follows.

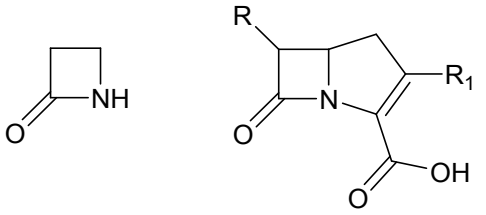
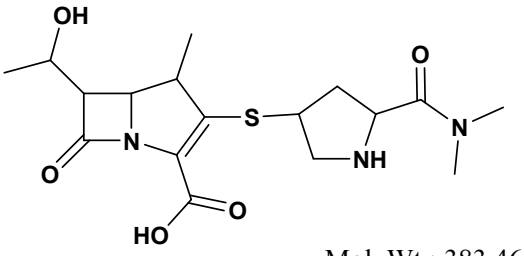


Figure 5. Chemical structures of the β-lactam ring and carbapenem.

Meropenem, (4R,5S,6S)-3-[(3S,5S)-5-dimethylcarbamoylpyrrolidin-3-yl-thio]-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2 carboxylic acid (Fig. 6), is a broad spectrum carbapenem antibiotic for intravenous administration. Meropenem is a white to pale yellow crystalline powder with acidic properties (pK_{a1} = 2.9 and pK_{a2} = 7.4) with the molecular weight of 383.46 g/mol. It is effective in the treatment of Gram-negative and -positive infections [76,83,84]. The determination of meropenem by HPLC has been sub-

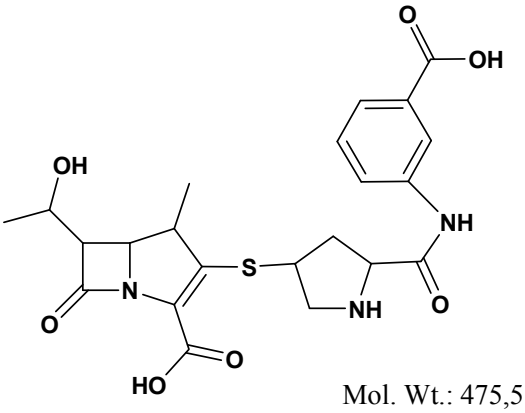
ject to much interest. Numerous pharmacodynamic and pharmacokinetic studies of meropenem have been carried out using HPLC for determination [38,83,84,85,80,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103, 104,105,106,107,108,109,110,111,112,113,114,I,VII].



Mol. Wt.: 383,46

Figure 6. Chemical structure of meropenem.

Ertapenem, (4R,5S,6S)-3-[(3S,5S)-5-[(3-carboxyphenyl)carbamoyl] pyrrolidin-3-yl]sulfanyl-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (Fig. 7), is a carbapenem β-lactam antimicrobial agent, ertapenem has antimicrobial activity against Gram-positive and -negative bacteria [115,116,117]. By chemical structure, ertapenem is similar to imipenem and meropenem [118]. Ertapenem's pK_{a1}, pK_{a2} and pK_{a3} values are 2.9, 6.0 and 8.2, respectively [81]. Several pharmacodynamic and pharmacokinetic studies of ertapenem have been carried out using HPLC for determination [38,38,109,93].



Mol. Wt.: 475,51

Figure 7. Chemical structure of ertapenem.

Imipenem, (5*R*,6*S*)-6-[(1*R*)-1-hydroxyethyl]-3-({2-[(iminomethyl)amino]ethyl} thio)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (Fig. 8), is an off-white, nonhygroscopic crystalline compound with the molecular weight of 299.34 g/mol. Imipenem's pK_{a1} and pK_{a2} values are 3.2 and 9.9 [119].

Introduced first in 1980, it was the first carbapenem on the market, having a broad spectrum of activity against aerobic and anaerobic microorganisms, Gram-positive and -negative bacteria [120]. Due to the rapid degradation by the renal enzyme dehydropeptidase-1, imipenem is co-administered with cilastatin, a renal membrane dipeptidase inhibitor that increases urinary excretion of the active drug [120]. Cilastatin's pK_{a1}, pK_{a2} and pK_{a3} values are 2.0, 4.4, 9.2, respectively [119119]. Also, the determination of imipenem/cilastatin by HPLC has been of much interest. Numerous pharmacodynamic and pharmacokinetic studies of imipenem have been carried out using HPLC for determination [38,93,102,103,104,105,109,114,121,122].

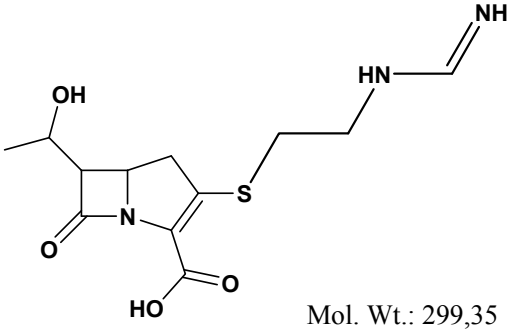


Figure 8. Chemical structure of imipenem.

Doripenem,(4*R*,5*S*,6*S*)-6-(1-hydroxyethyl)-4-methyl-7-oxo-3-[(3*S*,5*S*)-5[(sulfamoyl-amino)methyl]pyrrolidin-3-yl]sulfanyl-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (Fig. 9) with the molecular weight of 420.50 g/mol, is a novel carbapenem with antibacterial activity against a wide range of Gram-positive and -negative bacteria [38, 113, 123, 124]. Being a novel carbapenem antibiotic drug, doripenem is stable against human renal dehydropeptidase-1 (unlike imipenem that needs co-administration with cilastatin) [124]. Doripenem has pK_{a1} and pK_{a2} values 2.8 and 7.9, respectively [125]. The determination of doripenem using HPLC has been of increasing interest [38,113,123,124,126].

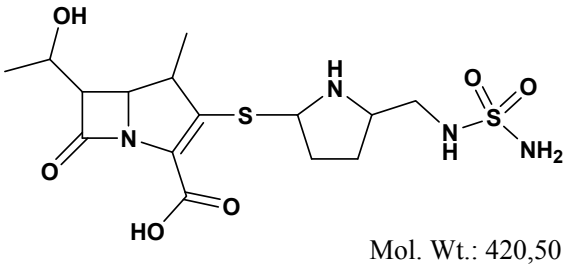


Figure 9. Chemical structure of doripenem.

1.5. Preparation of biological and environmental samples for LC-MS analysis

While analyzing pharmaceuticals from biological fluids for pharmacokinetic or pharmacodynamic studies or pharmaceutical residues from environmental samples, sample pretreatment has to be carried out.

A number of different approaches have been used for sample preparation for carbapenems analysis from biological fluids. They differ widely even for the same matrix: e.g. solid phase extraction with different sorbents as C18 [84,85,86,97,106,108,110], C8 [96], SCX and HLB [113] protein precipitation methods using methanol [97,102,121,118,122] and acetonitrile [83,110,38,109] or both [38], as well as column switching using Supelclean LC-NH_2 40-μm (50 x 2.1 mm i.d.) [94] and (20 x 3.9 mm i.d.) precolumn tap-filled with Li-Chroprep RP-8 (25–40 μm) [99] as extraction columns and filtration through syringe filters [88,107,112], filtered through a Nanosep 10 K instrument [124] or ultrafiltration [111,126] have been used for the preparation of blood plasma samples.

In the case of carbapenems as relatively unstable analytes in pretreated blood plasma samples, a careful choice in sample preparation techniques has to be made according to the analyte and the time used for sample preparation and analysis [II].

For environmental samples, also different sample preparation techniques are in use. Several extraction techniques have been applied for the determination of adsorbed antibiotics from a solid phase, such as ultrasonic-assisted extraction (USE) [127,128], microwave-assisted extraction (MAE) [129,130], pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) [131,132,133,134]. For the extracts' clean-up, liquid–liquid extraction (LE) [135,136,137] and solid-phase extraction (SPE) [138,139,140,141] were used. ASE or PLE have clear advantages over other methods, such as higher precision, smaller amounts of extraction solvents and a reduced sample preparation time [142]. Sewage sludge extraction is usually followed by the pre-concentration and clean-up of the PLE extracts using SPE with different cartridges [135,132,133,134], such as C18, HLB, SCX and non-polar extractions on a polymeric phase.

For the analysis of antibiotic residues from plants, LE [65,143,144] methods with different buffer solutions (such as disodium ethylenediaminetetraacetate (EDTA), citric and phosphoric, acetic acid), Soxhlet extraction [145] and for extract clean-up additional SPE were used with C18 [143].

1.6. Using ESI-MS for detection

Electrospray ionization (ESI) has become the most widely used ionization method for LC-MS analysis. In ESI, the analyte solution (LC eluent) is sprayed through a stainless steel needle. High electrical potential is applied to the ESI

needle with respect to the MS entrance. In this electrical field, analyte ions are generated and transferred into the MS.

The formation of gas-phase ions from the solution is a multi-step process. In the positive ion mode, the repulsion of positive ions on the surface of the eluent and the pull of the electric field forms a Taylor cone at the tip of the needle [146]. If the electric field becomes stronger, fine charged droplets are ejected from the Taylor cone. The solvent evaporates from the droplets and the electrostatic repulsion at the surface of the liquid overcomes the surface tension and smaller charged droplets are formed [146]. The process of solvent evaporation and the formation of smaller droplets is repeated. Two models to describe how the gas-phase ions emerge from the droplets have been proposed: the ion evaporation model and the charge residue model [146].

In chromatographic separation of complex mixtures, some compounds may co-elute with analytes. The term matrix effect is used to illustrate the interference of the co-eluted compounds on the ionization efficiency of the analytes. The change of ionization efficiency may be present also if fragmentation is used for quantification because the matrix effect is present already in the ionization source. The matrix effect can be observed also when MS/MS is used. Both signal enhancement and suppression can occur, resulting in an increase or decrease of the MS signal [58]. In order to evaluate the matrix effect quantitatively, the standard solution of the analyte in a solvent with a known concentration is analyzed with LC-ESI-MS and the peak area, A_{st}, is found. Also, a blank sample (containing all matrix compounds beside the analyte) is prepared and spiked with the analyte at the same concentration as the standard solution, and analyzed resulting the peak area A_s. The matrix effect can be estimated with the following formulation:

$$\%ME = \frac{A_S}{A_{st}} \times 100\%, \tag{5}$$

When %*ME* value is 100%, no matrix effect is present, if %*ME* is below 100%, ionization suppression is observed and the results are underestimated. %*ME* values exceeding 100% indicate signal enhancement and the results of the analysis can be overestimated.

2. AIMS OF THE STUDY

The general objective of this work was to investigate the suitability of two fluoroalcohols for HPLC-ESI-MS analysis as volatile buffer compounds and, therefore, to increase the selection of basic buffer components for LC-MS. More precisely, this objective was achieved through:

- the evaluation of HFIP and HFTB influence on ESI ionization of analytes in a positive and negative ESI mode
- the investigation of HFIP and HFTB as weak acids for basic buffer solution components in RP chromatography, and the retention mechanisms of chromatographic separation
- the comparison of HFIP and HFTB with commonly used buffer solution compositions in RP chromatography and to demonstrate the advantages in LC-ESI-MS analysis
- the comparison of the usage of HFIP and HFTB in RP chromatographic separation using the C18 stationary phase with the usage of the fluorinated stationary phase
- the application of the usage of fluoroalcohols for pharmaceutical analyses in complex matrices (e.g. blood plasma and environmental samples).

3. EXPERIMENTAL

3.1 Reagents

FQs and SAs were purchased from Riedel-de-Haën (Seelze, Germany) – three FQs: CIP, NOR and OFL; two SAs: SDM and SMX. Meropenem from Astra-Zeneca (United Kingdom), ertapenem, 2-tert-Butylphenol was purchased from Merck (Darmstadt, Germany), doripenem from AK Scientific Inc (Union City, CA, USA), imipenem monohydrate, HFIP, HFTB, TEA, 2,5-dinitrophenol, piperidine, 2-methoxypyridine, 2-methylpyridine, 2-nitroaniline, 2,3,5,6-tetra-fluorophenol and 2,3,4,5,6-pentafluorophenol from Sigma–Aldrich (St. Louis, MO, USA), diethylamine, diisopropylamine from Fisher (Suwanee, GA, USA). 3-Nitroaniline and 4-chloro-2-nitroaniline were obtained from Chemapol (Prague, Czech Republic). Pyrrolidine, 2-nitrophenol, 2,6-dimethylpyridine, ammonium acetate and 1-methylpiperidine were purchased from Fluka (Buchs, Germany). Acetonitrile and methanol were obtained from J. T. Baker (Deventer, The Netherlands), formic acid and ammonia from Riedel-de-Haën. All solvents were of the reagent grade or higher quality. Water was purified in-house using a Milli-Q plus or Millipore Milli-Q Advantage A10 systems from Millipore (Bedford, VA, USA).

3.2. Origin of samples and sample preparation

3.2.1. Sewage sludge and compost [III,IV]

The samples were taken from anaerobically digested sludge (before mixing with peat) in Tallinn, Estonia, and from untreated sludge (before composting with tree bark) in Tartu, Estonia. The sewage sludge in Tartu is treated by composting – mixing with tree bark (volume ratio 2:3). Methane fermentation and mixing with peat (volume ratio 4:3) are used in Tallinn. The compost samples were taken from anaerobically digested sludge (before mixing with peat) in Tallinn and from untreated sludge (before composting) in Tartu.

Approximately 200 g of sludge or compost (content of dry matter was 28% in Tallinn and 25% in Tartu) was placed into a 500 mL glass jar and mixed thoroughly. The jar was covered hermetically with a lid. Before analyzing, the samples were stored in a refrigerator at the temperature +4 °C in the dark. The samples were analyzed as soon as possible, usually within a week. Alternatively, they were stored in polypropylene vials frozen at the temperature −80 °C.

PLE was performed using an in-house designed system schematically depicted in Fig. 10. The extractor was designed using ultra high vacuum components. For surviving high pressure, the stainless steel chamber cylinder wall thickness was 10 mm and for sealing flanges copper gaskets were used. The volume of the pressure chamber was 55 mL. Standard HPLC valves and stainless steel tubing were used.

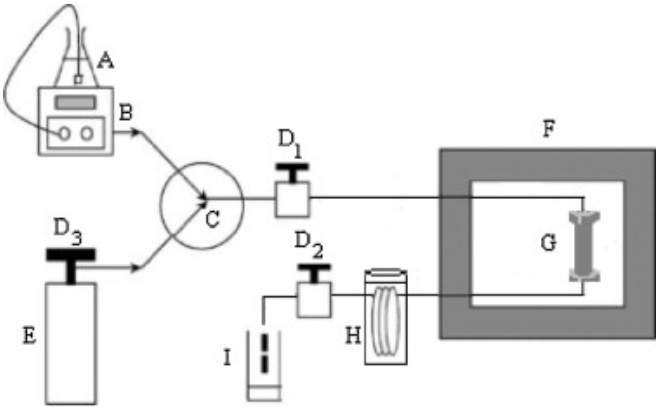


Figure 10. Pressurized liquid extraction (PLE) system: A, extraction solvent; B, HPLC pump; C, three-way switching valve; D1 and D2, static valves; D3, the valve of argon gas; E, argon tank; F, oven; G, extraction cell; H, cooling coil; I, extract collection vial.

9 ± 1 g (wet weight, ww) of the sewage sludge sample or compost was mixed 1:1 with sand, and 9 ± 1 g of sample/sand blend was packed into a cellulose filter and placed into the extraction cell mounted in an oven. The extraction was performed with a 0.35% phosphoric acid and acetonitrile mixture (1:1, v/v) adjusted to the pH 2.50 with a 0.01 M citric acid monohydrate. For one extraction cycle, approximately 30 mL of solvent was pumped into the extraction cell with the static valve D1 open. The system was pressurized with argon using valve D3; subsequently, the cell was heated. The operating conditions were as follows: temperature in the range 100–110 °C with a 30 min heat-up time, pressure in the range 100–110 atm (10,130–11,143 kPa), static extraction 10 min, 5 cycles and solvent flush volume 60%. The extracted analytes were purged from the sample cell using pressurized argon for 40 s. The solvent used for the flushing of the extraction cell was collected with the static valve D2 open after the first cycle of extraction. Subsequent cycles of extraction were carried out using the same operating conditions. The extract cooling was accomplished by stainless steel tubing in cold water. The total volume of the extract collected was in the range of 150–160 mL.

The extracts collected by PLE were cleaned up by SPE. Antibiotics such as CIP, NOR, OFL, SDM and SMX were extracted using SCX and HLB cartridges. Two different cartridges were tested with the aim of securing the best possible recoveries. For the SPE procedure, the vacuum manifold, supplied by Agilent Technologies, was used. For extraction with the SCX cartridges, the cartridges were preconditioned with 6 mL of methanol and 6 mL of buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8). A portion (80 mL) of sludge or compost PLE extract was diluted to 500 mL with H_2O (pH adjusted to 2.0) and then percolated through the cartridge at a flow rate ~1.5 mL/min using the vacuum manifold. After extraction, the compounds were eluted from the cartridges using 20 mL of 20% ammonia water solution in 40% methanol. For extraction with the HLB cartridges, the cartridges were pre-

conditioned with 20 mL of methanol and 10 mL of Milli-Q water. The dilution of the PLE extract was preformed as for SCX cartridges. The flow rate of sample loading was ~6 mL/min. After extraction, the compounds were eluted from the cartridges using 12 mL of methanol. The SPE extracts were concentrated on polypropylene vials in an N_2 stream. Polypropylene vials were used to avoid the sorption to glass walls and samples were not evaporated to complete dryness. Residues were dissolved in 1 mL of 1:1 solution of methanol with a buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8).

3.2.2. Plants

Aqueous solutions of the studied pharmaceuticals were mixed with soil. The final concentration of each pharmaceutical was 10 mg/kg (dry weight). With the aim of assuring the better dissolution of the studied pharmaceuticals, fluoroquinolones were dissolved in 2 ml of 0.1 mM ammonium acetate buffer solution with pH=2.8 and sulfonamides were dissolved in 2 ml of 0.3 M NaOH. The tubers of potato (*Solanum tuberosum L*), seeds of carrot (*Daucus carota L*), lettuce (*Lactuca sativa L*) and wheat (*Triticum vulgare L*) were sowed into the soil in the presence of five antibiotics found in the Estonian sewage sludge (CIP, NOR, OFL, SDM, and SMX). The plants were grown in a greenhouse under natural light conditions for 120 days from planting (for lettuce 70 days). Then, the plants were collected, dried and milled. The roots and grains were separated from the leaves. The milled plants were held in hermetical plastic bags at –80 °C. Before analysis, the samples were dried at 45 °C.

250 mg of dried plants (grains, roots or leaves) was extracted with 10 mL of 1:1 (v/v) mixture of acetonitrile and 1% acetic acid, then homogenized (10') with the laboratory homogenizer DIAX 900 (Heidolph Instruments, Germany) at 25,000 rpm, sonicated (5'), vortexed (1') and centrifuged at 8,000 rpm. The supernatant was then separated and dried by a nitrogen stream to remove acetonitrile. Approximately 15 mL of 1 % acetic acid was added to the 1 mL of evaporation residue.

The extract collected by liquid extraction was cleaned up by SPE. Antibiotics – CIP, NOR, OFL, SDM and SMX – were extracted using HLB cartridges. For the SPE procedure, the vacuum manifold, supplied by Agilent Technologies, was used. HLB cartridges were preconditioned with 20 mL of methanol and 10 mL of Milli-Q water. The sample was loaded at the rate of 6 mL/min. After extraction, the compounds were eluted from the cartridges using 12 mL of methanol. The SPE extracts were concentrated in polypropylene vials in an N_2 stream. The residue was dissolved in 1 mL of 20 % methanol with a buffer solution (5 mM 1,1,1,3,3,3-hexafluoro-2-propanol, pH adjusted to 9.0 with NH_4OH).

3.2.3. Human blood plasma and urine

Blood samples of meropenem microanalysis

For meropenem microanalysis, blood plasma and urine was used for the determination of meropenem. For meropenem plasma samples, 200 or 300 µl of blood from babies with the current weight under 700 g and 700–1,500 g, respectively, was drawn from an arterial cannula immediately before the drug administration and 2 min, 0.5, 1.5, 4, 8 and 12 h after the study dose administration [VII]. Blood was centrifuged immediately in dry vials, plasma was split and stored at –20 °C. At the end of the 4-hour infusion, the infusion lines containing meropenem solution were stored as well. Within 24 hours of collection, the samples were transferred to –70 ºC until analysis.

Urine samples of meropenem microanalysis

Urine samples were collected from a urinary catheter or with a catheter or a plastic bag attached to the groin region in 4-hour intervals within 12 h after the administration of the meropenem study dose. The quantity of the collected urine was measured and simultaneous possible losses were estimated by weighing the diapers. Urine collection was considered adequate and used for PK calculations, when at least 90% of the total estimated urine output for the given 4 hour period was collected. The samples were similarly stored immediately at –20 ºC and then transferred to –70 ºC [VII].

Blood samples of carbapenems analysis

For carbapenem analysis, the blood plasma samples were collected into 4 mL Li-heparin vials immediately before the drug administration and 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7 and 8 h after the study dose administration. The blood was centrifuged immediately, the plasma was collected and stored at –20 °C. At the end of the sample collection, the samples were transferred to –70 ºC until analysis.

All sample collecting protocols were approved by the Ethics Committee of the University of Tartu.

Sample preparation for meropenem microanalysis

Plasma samples were melted at room temperature and 50 µL of plasma was transferred into a 250 µL PCR tube. Plasma samples were extracted with methanol (containing ertapenem as the internal standard (I.S.) in the concentration of 10 µg/mL): for 50 µL of plasma 50 µL of MeOH (containing ertapenem as I.S.) was added. After vigorous shaking with the Vortex mixer for 1 min, the sample was centrifuged at 8,000 rpm (3,500 x g) for 10 min and the supernatant (approximately 75 µL) was separated and filtrated through 0.22 µm Millex-GV PVDF filters and transferred into an HPLC autosampler vial [VII].

Urine samples were melted at room temperature and diluted with ultrapure water (1/9 or more). 3 µL of the prepared sample was injected into the Agilent 1290 Infinity UHPLC system.

Sample preparation for carbapenems analysis
Plasma samples were melted at room temperature and 500 μL of plasma was transferred into a 2 mL Eppendorf tube. Plasma samples were extracted with 1 mL of MeCN. After vigorous shaking with the Vortex mixer for 2 min, the sample was centrifuged at 8,000 rpm (3,500 x g) for 10 min and the supernatant (approximately 1,300 μL) was transferred to another Eppendorf vial and evaporated to dryness under a nitrogen stream. The residue was dissolved in 500 μL of MilliQ water and filtrated through 0.22 μm Millex-GV PVDF filters and transferred into an HPLC autosampler vial. 3 μL of the prepared sample was injected into the Agilent 1290 Infinity UHPLC system.

3.2.4. Study of retention mechanisms

Stock solutions of the analytes at 1 mg/mL in the appropriate solvent were prepared. Dilutions were made and analyte concentrations ranged from 11 μg/mL to 145 μg/mL for working standards. Stock solutions were stored at –20 °C. Fresh working standard solutions were prepared daily.

The influence of the buffer compound and pH on the chromatographic separation was investigated. Fluoroalcohols' interaction with the stationary phase surface was investigated at different eluent pH values. Nine buffer solutions: 5 mM ammonium acetate, pH adjusted to 8.5, 9 and 10; 5 mM HFIP, pH adjusted to 8.5, 9 and 10; 5 mM HFTB, pH adjusted to 8.6, 9 and 10 were used. In all cases, the pH was adjusted using an ammonium hydroxide solution.

3.3. LC and ESI-MS parameters

3.3.1. Sewage sludge and compost

The analysis of the antibiotics in the sewage sludge and compost SPE extracts was performed on LC–MS (Agilent Series 1100 LC-MSD Trap XCT (Santa-Clara, CA, USA)) equipped with a binary pump, a degasser, an auto-sampler and a column thermostat. Antibiotics were chromatographed using a Phenomenex Synergi Hydro-RP column (250 mm × 4.6 mm, 4 μm) equipped with a Phenomenex SecurityGuard cartridge AQ 4 mm × 2 mm. Gradient elution with a methanol and ammonium buffer solution (1 mM ammonium acetate and 0.1% formic acid, pH 2.8) was used. The linear gradient with the flow rate of 0.4 mL/min started at 35% methanol for 20 min and was raised to 80% within 20 min, after that methanol concentration was lowered to 35% in 5 min. The column temperature was set to 30 ºC and the injection volume was 5 μL.

For detection, the diode array detector and ESI-MS were used in series. ESI-MS detection was carried out in the positive ion detection mode. Selected reaction monitoring was used. Full MS^2 spectra were recorded and the following transitions were applied for quantification: OFL *m/z* 362→ 318, 344, NOR *m/z* 320→233, 276, 302, CIP *m/z* 332→288, 294, 314, SMX *m/z* 254→ 92, 108,

156, SDM *m/z* 311→108, 156, 245. Default parameters for ESI and MS were used for all the experiments (nebulizer gas pressure was 40 psi, dry gas flow was 10 L min^{-1}, the dry gas temperature was 350 ºC, capillary voltage was 5,000 V, the detected mass range was from *m/z* 100 to 1,000 and the target mass for compounds was *m/z* 350).

3.3.2. FQs and SAs in plants

Chromatographic separation of the analytes was carried out on the Agilent Series 1100 LC-MSD Trap XCT (Agilent Technologies, Santa-Clara, CA, the USA) equipped with a binary pump, a degasser, an auto-sampler and a column thermostat. Five antibiotics were chromatographed using a Waters XBridge C18 column (150 mm × 3 mm, 3.5 μm) equipped with a Waters Guard Cartridge (20 mm × 4.6 mm) (Waters, Milford, MA, USA). For detection, the diode array detector and ESI-MS were used in series. ESI-MS detection was carried out in the positive ion detection mode. Selected reaction monitoring was used. Full MS^2 spectra were recorded and the following transitions were applied for quantification: OFL parent ion with *m/z* 362 $[M+H]^+$ and product (fragment) ions *m/z* 261, 318; CIP parent ion with *m/z* 332 $[M+H]^+$ and product ions *m/z* 288, 314; NOR parent ion with *m/z* 320 $[M+H]^+$ and product ions 302, 276; SMX parent ion with *m/z* 254 $[M+H]^+$ and product ions *m/z* 108, 188; SDM parent ion with *m/z* 311 $[M+H]^+$ and product ions *m/z* 108, 156, 218, 245 were detected for quantification. Default parameters for ESI and MS were used for all the experiments (nebulizer gas pressure was 40 psi, dry gas flow 10 L/min, the dry gas temperature 350 ºC, capillary voltage was 5,000 V, detected mass range was from *m/z* 100 to 1,000 and the target mass for compounds was *m/z* 350). LC-UV and MS instruments were checked by Agilent Chemstation for LC 3D rev. A.10.02 (Agilent Technologies) and LC/MSD TrapControl ver. 5.2 (Bruker Daltonik GmbH, Germany). Data analysis was carried out using Chemstation software (Agilent Technologies) and Data Analysis for LC/MSD Trap Version 3.2 (Bruker Daltonik GmbH).

For elution with 5 mM, the HFIP buffer (pH adjusted with NH_4OH to 9.0) and methanol were used. Gradient elution at the flow rate of 0.3 mL/min started at 10% methanol and was raised to 55% within 25 min, after that methanol concentration was raised to 100% within 5 min. Methanol concentration was kept at 100% for 5 min and lowered to 10% in 5 min and was equilibrated at 10% for 5 min. The column temperature was set to 30 °C and the injection volume was 10 μL.

3.3.3. Carbapenems in biological fluids

For meropenem microanalysis and the simultaneous analysis of carbapenems, the samples were analyzed with the Agilent 1290 Infinity UHPLC system. Samples were chromatographed using Waters Acquity UPLC BEH C18 column

(2.1 x 100 mm, 1.7 µm) equipped with Waters VanGuard Acquity UPLC BEH C18 Guard Column (2.1 x 5 mm, 1.7 µm) (Waters, Milford, MA, USA). Samples were analyzed with the diode array detector (DAD) at 306 nm and electrospray interface Varian J320-MS Triple Quadrupole LC/MS was used for detection in the single reaction monitoring (SRM) mode. Parent ion $[M+H]^+$ and fragment ions used for carbapenems quantification are presented in Table 2. For cilastatin, the parent ion with *m/z* 359 $[M+H]^+$ and product (fragment) ions *m/z* 202, 219, 263 and 342 were detected for quantification. For instrument control and data analysis, the software ChemStation for LC 3D Systems Rev. B.04.02 [96] and Varian MS Workstation version 6.9.2 (Agilent Technologies) were used. Electrospray interface (ESI) was operated in the positive ion mode for ionization. Drying gas pressure was 12 psi (82.7 kPa) and the temperature was 300 ºC, the capillary voltage was 5,000 V and the nebulizer gas pressure 55 psi (379.2 kPa). For SRM, the collision-induced dissociation (CID) gas pressure was 1.5 mTorr (0.2 Pa).

Table 2. The optimal values for carbapenems MS detection found with optimization procedures for Triple Quadrupole MS.

Analyte,	**Parent ion $[M+H]^+$**	**Fragments, (*m/z*)**	**Collision energy (V)**
Imipenem	300	256	–5,5
		103	–15,5
Meropenem	384	340	–5,5
		254	–12,5
		200	–9
		141	–12
Doripenem	421	342	–12
		318	–8,5
		298	–10,5
		274	–13
Ertapenem	476	432	–9
		346	–13
Cilastatin[1]	359	342	–9
		263	–11,5
		219	–12,5
		202	–11

[1] Cilastatin is co-administered with imipenem.

Chromatographic conditions of meropenem microanalysis
Gradient elution with MeOH and the buffer solution (5 mM HFIP, pH adjusted with NH_4OH to 8.5) in water at the flow rate of 0.3 mL/min is used for chromatographic separation. The linear gradient started at 2% MeOH and was raised to 10% within 1.2 min, after 1.8 min the MeOH concentration was raised to 33% within 1.5 min and to 80% within 0.5 min. MeOH concentration was kept at 80% for 1 min and raised to 100% within 0.5 min, after 0.5 min the concentration of MeOH was lowered to 2% in 2 min. The column temperature was maintained at 40 ºC and the injection volume was 5 μL.

Chromatographic conditions of carbapenem analysis
For simultaneous analysis of carbapenems, gradient elution with MeOH and buffer solution (5 mM HFIP, pH adjusted with NH_4OH to 10) in water at the flow rate of 0.3 mL/min was used for chromatographic separation. The linear gradient started at 2% MeOH and was raised to 10% within 2.5 min and to 15% within 1.5 min. MeOH concentration was kept at 15% for 3 min and raised to 100% within 1 min and lowered to 2% within 1 min. After each chromatographic run, the column stabilization (in 2% of MeOH) time was 1.5 min. Column temperature was maintained at 40 ºC and the injection volume was 3 μL.

3.3.4. Study of retention mechanisms

Acids and bases were chromatographed using a Waters XBridge C18 column (150 mm × 3 mm, 3.5 μm) equipped with a Waters Guard Cartridge (20 mm × 4.6 mm) (Waters, Milford, MA, USA), YMC C18 column (150 mm × 3 mm, 3 μm) (YMC Co.,Ltd., Kyoto, Japan) and silica based Epic FO-LB column (150 mm × 3 mm, 3 μm, 120 Å) (ES Industries, West Berlin, NJ, USA).

Chromatographic conditions for C18 columns
Waters XBridge and YMC C18 stationary phases are stable in the pH range 1–12. Mobile phases composed of different buffers, and methanol (solvent B) were used. Isocratic elution at the flow rate of 0.3 mL/min with 23% MeOH was used. The column temperature was set to 30 °C and the injection volume was 10 μL. The analytical column was stabilized at the flow rate 0.3 mL/min with 23% methanol for 100 min after the buffer solution from fluoroalcohols to ammonium bicarbonate or ammonium acetate was changed and for 20 min after each traditional buffer solution in different pHs. Stock solutions of the analytes at 1 mg/mL in the appropriate solvent were prepared. Dilutions were made and the analyte concentrations ranged from 11 μg/mL to 145 μg/mL for working standards. Stock solutions were stored at –20 °C. Fresh working standard solutions were prepared daily. The influence of the buffer compound and pH on the chromatographic separation was investigated. Fluoroalcohols' interaction with the stationary phase surface was investigated at different eluent pH values. Twelve buffer solutions: 5 mM ammonium acetate, pH adjusted to 8.5, 9 and

10; 5 mM ammonium bicarbonate, pH adjusted to 8.5, 9 and 10; 5 mM HFIP, pH adjusted to 8.5, 9 and 10; 5 mM HFTB, pH adjusted to 8.6, 9 and 10 were used. In all cases, the pH was adjusted using an ammonium hydroxide solution.

Chromatographic conditions for Epic FO-LB column

Epic FO-LB for pH range 1–10. Mobile phases composed of different buffers, and MeOH (solvent B) were used. Isocratic elution at the flow rate of 0.3 mL/min with 23% MeOH was used. The column temperature was set to 30 °C and the injection volume was 10 µL. The analytical column was stabilized at the flow rate of 0.3 mL/min with 23% methanol for 10 min after the buffer solution was changed. Stock solutions of the analytes at 1 mg/mL in the appropriate solvent were prepared. Dilutions were made and analyte concentrations ranged from 11 µg/mL to 145 µg/mL for working standards. Stock solutions were stored at –20 °C. Fresh working standard solutions were prepared daily. The influence of the buffer compound and pH on the chromatographic separation was investigated. Six buffer solutions: 5 mM ammonium acetate, pH adjusted to 8.5, 9 and 10; 5 mM ammonium bicarbonate, pH adjusted to 8.5, 9 and 10 were used. In all cases, the pH was adjusted using an ammonium hydroxide solution.

4. RESULTS AND DISCUSSION

4.1. Analysis of pharmaceuticals

LC-MS analysis of pharmaceuticals in different complex matrices has shown the importance of the sample preparation and clean-up procedure as well as the chromatographic separation in the analysis. The large variety of sample preparation methods are not effective in removing all sample components interfering with the analysis and causing matrix effect. Therefore, chromatographic separation has to be enhanced. The chromatographic separation of the compounds having similar chemical properties or simultaneous determination of many chemically diverse substances can be a difficult task to accomplish. For carbapenems or FQs having similar chemical properties (e.g. pK_a values), the RP-LC separation for simultaneous determination or separation from matrix components is problematic [I–IV,VII]. In the next sections the sample preparation issues and chromatographic separation problems on carbapenem analaysis from the biological samples and FQs' and SAs' residues analysis from the sewage sludge and compost samples are discussed.

4.1.1. Carbapenem analysis from biological fluids

The sample preparation in carbapenem analysis from biological fluids [II]

The extraction of the biological samples has to be as quick and clean as possible for carbapenem analysis in order to avoid analytical column contamination by adsorption or precipitation of blood plasma components. In carbapenem analysis, the stability of the sample solution is of crucial importance [II]. The sample preparation and carbapenem extraction from the biological fluids have to be performed quickly in order to avoid the analyte's decomposition. The sample preparation pH has to be controlled carefully to avoid acidic or alkaline hydrolysis. A comparison of different protein precipitation techniques has been conducted to estimate the suitability of the blood plasma sample preparation for meropenem analysis and the most efficient solvent for protein precipitation appeared to be MeOH [II]. For the simultaneous analysis of carbapenems, the recoveries of all studied antibiotics have to be taken into account. The most suitable (highest recoveries in the range 40–63%) and quickest sample preparation method for carbapenem analysis was protein precipitation with MeCN while the recoveries of the other carbapenems were lower if MeOH was used. When for carbapenem analysis UHPLC was used with a relatively low organic solvent content in the mobile phase, the large amount of MeCN in the sample solution caused changes in the peak shape and led to asymmetrical and tailing peaks. The MeCN content was lowered using the sample concentration in a nitrogen stream. After the concentration procedure, the samples were dissolved in the eluent (for the optimization of the carpabenems' separation) several eluents were used). Since in pH optimization, the eluent pH ranged from acidic to basic conditions, carbapenem degradation through a beta-lactam ring opening

occurred while the sample was dissolved in the mobile phase and, therefore, the MilliQ water was used.

Chromatographic separation of carbapenems with different buffer solutions
Chromatographic separation optimization of carbapenem analysis is necessary to avoid matrix effects caused by co-eluting sample components. In carbapenem analysis, the chromatographic separation was studied in the pH range 2.6 to 10.8. 0.1% formic acid was used for pH 2.6 and 5 mM ammonium acetate and ammonium bicarbonate were used for the pH range 3.8 to 10.8 (acetic acid and ammonia were used for pH adjustment). Asymmetrical peaks shapes were obtained while acidic or neutral pH was used for the separation. Carbapenems' separation from the blood plasma components was problematic especially for the imipenem and meropenem. An improvement in the peak shape was obtained while the eluent pH was raised above 7.7; however, the separation of doripenem and ertapenem was not achieved at these conditions (Fig. 11). If the only UV is used for detection, the poor separation of carbapenems can lead to the misjudgment of the compound detected if the sample contains a low level of another carbapenem (for example, if the carbapenem treatment has changes from one to another).

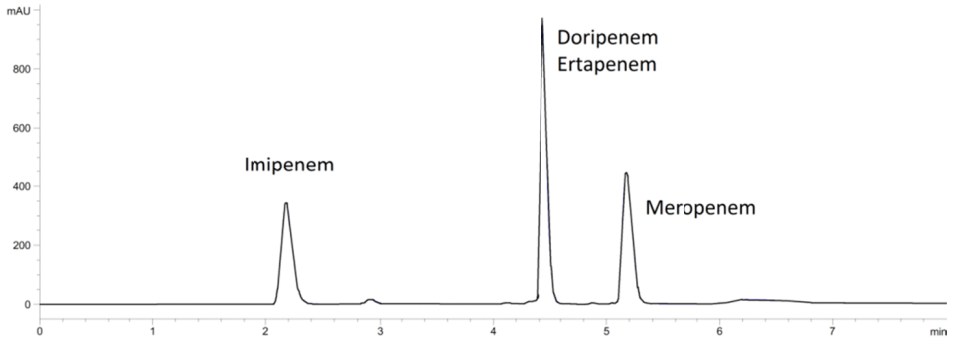


Figure 11. Chromatographic separation of carbapenems using 5 mM ammonium acetate (pH 9.78).

4.1.2. Analysis of FQs and SAs [III,V]

Extraction of FQs and SAs from sewage sludge and compost [III]
FQs tend to adsorb strongly on soil particles, therefore exhaustive extraction methods, such as PLE is required for sample preparation. The developed method for FQs' and SAs' extraction from the sewage sludge was based on the combination of PLE, SPE and LC–MS. The optimization of extraction methods was based on the selection of the suitable extraction solvent and the pH of the solvent, time, temperature, pressure and the number of extraction cycles on PLE [III]. The optimized method is described in Section 3.1.2. Better recoveries on

PLE extraction were obtained while quartz sand was used in the extraction procedure. During PLE method development, serious carryover problems were observed, especially after the extraction of spiked samples. Since the PLE extraction cell consists of a large metal surface, the adsorption of antibiotics onto the surface was extensive. Repetitive cleaning with different solvents appeared to be of low efficiency. For cleaning the PLE vessel, an original simple solution was proposed: a small amount of ethanol was burnt in the extraction vessel and subsequently the vessel was rinsed with the extraction solvent. The SPE method was optimized for FQs and SAs residues' analysis for PLE extract clean-up and two types of SPE cartridges were evaluated through extraction recovery values [III]. In order to avoid antibiotic residues' adsorption into the glass surface, polypropylene vials were used. In spite of exhaustive sample preparation of the sludge and compost samples, the solutions subjected to LC-MS analysis were still rich in matrix components.

LE method for the plants

The developed method is based on the combination of liquid extraction, SPE and LC-MS analysis of 5 antibiotics in total (NOR, CIP, OFL, SMX, SDM). The variables optimized were the extraction solvent and pH, and homogenization. Hexane, chloroform, methanol and acetonitrile were tested as extraction solvents. Organic solvent content in the extraction solvent varied from 20–100%. The pH of the aqueous component of the extraction mixture varied from acidic (1% acetic acid, pH 2.0) to basic (5 mM ammonium acetate, pH 9). The mixture of acetonitrile and 1% acetic acid (1/1) was finally chosen as an extraction solvent. Lower extraction efficiencies (recoveries of antibiotics varied from 5 to 20%) were achieved, when 100% of acetonitrile was used as well as the content of acetonitrile decreased below 50%. Extraction with acetonitrile was more efficient compared to methanol. Extraction with chloroform and hexane gave overall the lowest antibiotic recoveries (1–2%) for CIP and NOR. During the optimization of liquid extraction, it was found that the extraction efficiency increased with homogenization before the sonication and mixing. The increase in the time of liquid extraction stages was not increasing extraction recoveries. In total, the time for a LE procedure was 17 minutes.

SPE procedure for plant LE extract clean-up

After the liquid extraction and centrifugation, the supernatant was separated and dried by a nitrogen stream to remove acetonitrile. The remaining 0.5 mL of the extract was cleaned up with HLB SPE cartridges. The HLB cartridges allow retaining both hydrophilic and hydrophobic compounds [140]. The method is described in Section 3.2.2.

Chromatographic separation of FQs and SAs with different buffer solutions

LC-MS analysis of FQs and SAs using the conventional C18 stationary phase was problematic and satisfying results were obtained with the Phenomenex Synergi Hydro-RP column since mass-spectrometric detection was used in the

selected reaction monitoring mode for detection [III,IV]. In acidic conditions, the CIP and NOR were still partly overlapping. Poor separation, however, can result in serious matrix effects. The change of chromatographic conditions as changing the gradient program, column temperature or change in the acidic buffer solution did not give satisfactory separation. Therefore, the optimization of the FQs and SAs separation conditions was carefully studied. A number of buffer solutions were assessed for their suitability for the LC-MS analysis of five antibiotics (Table 3) [V].

Table 3. Compositions of the buffer solutions tested for the chromatographic separation of FQs and SAs.

Designation	Composition	pH
AAF 2.8	1 mM ammonium acetate in 0.1% formic acid	2.8
TEAA 10.0	5 mM ammonium acetate, pH adjusted to 10.0 with triethylamine	10.0
CH_3COONH_4 9.0	5 mM ammonium acetate, pH adjusted to 9.0 with ammonia	9.0
CH_3COONH_4 10.0	5 mM ammonium acetate, pH adjusted to 10.0 with ammonia	10.0
1-MePip 9.85	5 mM 1-methylpiperidine, pH adjusted to 9.85 with ammonia	9.85
HFIP/NH_4OH 9.0	5 mM HFIP, pH adjusted to 9.0 with ammonia	9.0
HFIP/NH_4OH 10.0	5 mM HFIP, pH adjusted to 10.0 with ammonia	10.0
HFTB/NH_4OH 9.0	5 mM HFTB, pH adjusted to 9.0 with ammonia	9.0
HFTB/NH_4OH 10.0	5 mM HFTB, pH adjusted to 10.0 with ammonia	10.0
HFIP/TEA 9.0	5 mM HFIP, pH adjusted to 9.0 with triethylamine	9.0

The initial separation of antibiotics was carried out using elution under acidic conditions with AAF 2.8 and methanol as our in-house standard method. Chromatographic separation of the antibiotics was problematic, the peaks of CIP, SMX and NOR overlapped. As the change of the organic solvent to acetonitrile and modification of gradient conditions did not provide better separation, the possibility of shifting the mobile phase pH into the basic range was taken into consideration. Alternatives to the basic buffer components are presented in Table 1. The buffer solution pH range from 9 to 10 was carefully studied and buffer components 1-MePip 9.85, TEAA 10.0, CH_3COONH_4 9.0 and 10.0 were selected for further study along with the novel HFIP/NH_4OH and HFTB/NH_4OH systems. Chromatographic separation (expressed in retention times) ESI signal intensities in the positive and negative ion mode (expressed as peak heights) of analytes are presented in Table 4.

Table 4. Comparison of antibiotic separation and ESI signal intensity using different buffers. The average values for t_R and signal intensities from 3 replicate experiments on 3 different days are presented.

					1 µg/mL		10 µg/mL	
Buffer solution	**c [mM]**	**pH**	**t_R [min]**	**Analyte**	**Peak height (+ESI)**	**Peak height (–ESI)**	**Peak height (+ESI)**	**Peak height (–ESI)**
AAF 2.8	1	2.8	18.9	OFL	6.8×10^7	6.9×10^3	6.6×10^8	1.8×10^5
			19.8	*NOR	3.2×10^7	n.d.	4.0×10^8	6.6×10^4
			20.3	*CIP	4.0×10^7	n.d.	3.7×10^8	5.7×10^4
			20.3	*SMX	2.9×10^7	1.8×10^5	1.5×10^8	2.5×10^6
			26.1	SDM	6.0×10^7	7.9×10^5	3.9×10^8	5.7×10^6
TEAA 10.0	5	10	12.1	SMX	n.d.	7.7×10^4	n.d.	7.6×10^5
			19.6	SDM	2.1×10^5	2.1×10^5	2.1×10^6	2.2×10^6
			20.9	*NOR	4.6×10^5	1.2×10^4	4.5×10^6	1.1×10^5
			21.2	*CIP	3.6×10^5	1.5×10^4	3.6×10^6	1.4×10^5
			34.5	OFL	5.9×10^5	7.8×10^4	6.0×10^6	8.0×10^5
HFTB/ NH_4OH 9.0	5	9.0	4.1	SMX	4.1×10^7	1.2×10^5	2.1×10^7	2.6×10^5
			7.5	SDM	1.5×10^7	5.2×10^5	1.4×10^8	2.0×10^6
			8.9	NOR	7.6×10^6	1.6×10^4	9.4×10^7	2.1×10^5
			10.3	CIP	1.4×10^7	1.9×10^4	2.0×10^8	3.2×10^5
			16.5	OFL	4.7×10^7	5.8×10^4	5.1×10^8	9.9×10^5
HFIP/ TEA 9.0	5	9.0	4.4	SMX	n.d.	3.8×10^4	n.d.	3.2×10^5
			8.6	SDM	1.5×10^6	4.5×10^5	5.1×10^6	1.5×10^6
			10.8	NOR	2.8×10^6	n.d.	1.1×10^7	1.7×10^5
			12	CIP	4.8×10^6	n.d.	1.4×10^7	2.6×10^5
			18.9	OFL	n.d.	n.d.	1.2×10^7	1.9×10^6
CH_3COONH_4 10.0	5	10.0	7.8	SMX	8.8×10^5	9.5×10^4	1.9×10^7	5.0×10^5
			12.2	*SDM	5.7×10^6	1.5×10^6	1.4×10^8	3.6×10^6
			12.4	*NOR	5.6×10^6	3.1×10^4	5.1×10^7	1.5×10^5
			13.9	CIP	7.6×10^6	3.2×10^4	7.3×10^7	2.0×10^5
			21.2	OFL	2.2×10^7	6.3×10^4	2.3×10^8	5.9×10^5
CH_3COONH_4 9.0	5	9.0	7.8	SMX	2.5×10^6	1.8×10^5	1.1×10^7	5.5×10^5
			12.4	SDM	1.8×10^7	1.1×10^6	6.5×10^7	3.6×10^6
			14.8	NOR	3.7×10^6	8.0×10^3	7.7×10^7	2.2×10^5
			15.3	CIP	6.3×10^6	9.0×10^3	6.0×10^7	2.6×10^5
			22.8	OFL	2.5×10^7	2.8×10^4	2.8×10^8	1.0×10^6
1-MePip 9.85	5	9.85	11.3	SMX	n.d.	4.7×10^4	n.d.	3.8×10^5
			16.2	SDM	n.d.	3.2×10^5	5.4×10^6	2.3×10^6
			17.0	*NOR	n.d.	n.d.	9.2×10^4	6.5×10^4
			17.2	*CIP	n.d.	n.d.	4.3×10^6	2.7×10^6
			23.5	OFL	n.d.	n.d.	4.8×10^6	5.9×10^5

					1 µg/mL		10 µg/mL	
Buffer solution	**c [mM]**	**pH**	**t_R [min]**	**Analyte**	**Peak height (+ESI)**	**Peak height (–ESI)**	**Peak height (+ESI)**	**Peak height (–ESI)**
HFTB/NH_4OH 10.0	5	10.0	5.6	SMX	6.7×10^4	1.4×10^3	2.2×10^5	3.2×10^4
			9.8	*SDM	1.9×10^5	1.9×10^4	1.0×10^6	1.2×10^5
			10.3	*NOR	1.7×10^5	4.1×10^3	1.3×10^6	3.7×10^4
			11.6	CIP	2.5×10^5	6.3×10^3	1.1×10^6	6.4×10^4
			18	OFL	7.7×10^5	2.9×10^4	8.5×10^6	1.9×10^5
HFIP/NH_4OH 10.0	5	10.0	5.2	SMX	6.9×10^4	2.0×10^3	2.8×10^5	3.1×10^4
			9	SDM	3.7×10^5	1.6×10^4	1.4×10^6	9.4×10^4
			10.1	NOR	3.3×10^5	3.9×10^3	1.7×10^6	2.4×10^4
			11.3	CIP	5.4×10^5	5.5×10^3	3.9×10^6	3.8×10^4
			18.4	OFL	1.6×10^6	1.4×10^4	1.1×10^7	1.4×10^5
HFIP/NH_4OH 9.0	5	9.0	4.5	SMX	4.4×10^6	1.4×10^5	3.4×10^7	5.6×10^5
			8.2	SDM	1.8×10^7	1.2×10^6	1.6×10^8	3.8×10^6
			9.9	NOR	1.0×10^7	3.3×10^4	1.1×10^8	5.5×10^5
			11.1	CIP	1.9×10^7	5.7×10^4	2.0×10^8	6.1×10^5
			18.6	OFL	3.9×10^7	1.1×10^5	4.3×10^8	4.1×10^6
HFIP/NH_4OH 9.0	1	9.0	3.7	SMX	2.3×10^5	1.0×10^4	6.5×10^5	9.1×10^4
			6	SDM	2.5×10^5	3.7×10^4	1.7×10^6	1.6×10^5
			10	*NOR	1.3×10^5	2.1×10^3	8.2×10^5	3.5×10^4
			10.5	*CIP	1.4×10^5	3.5×10^3	1.4×10^6	5.5×10^4
			17.4	OFL	4.0×10^5	1.8×10^4	8.4×10^6	1.8×10^5
HFIP/NH_4OH 9.0	10	9.0	4.6	SMX	9.8×10^5	2.7×10^3	3.4×10^5	4.9×10^4
			8.2	SDM	2.7×10^5	1.7×10^4	1.6×10^6	2.0×10^5
			10.1	NOR	2.5×10^5	3.2×10^3	1.5×10^6	2.8×10^4
			11.6	CIP	3.1×10^5	5.5×10^3	3.1×10^6	4.8×10^4
			19.3	OFL	1.9×10^6	3.2×10^4	1.8×10^7	1.2×10^5

* Overlapping peaks.

The overlapping of some analyte peaks occurred when using 1-MePip 9.85, TEAA 10.0 and CH_3COONH_4 10.0. Satisfactory separation was achieved using CH_3COONH_4 9.0, HFIP/NH_4OH 9.0 and HFTB/NH_4OH 9.0. Chromatographic separation of antibiotics using four different buffers is presented in Fig. 12.

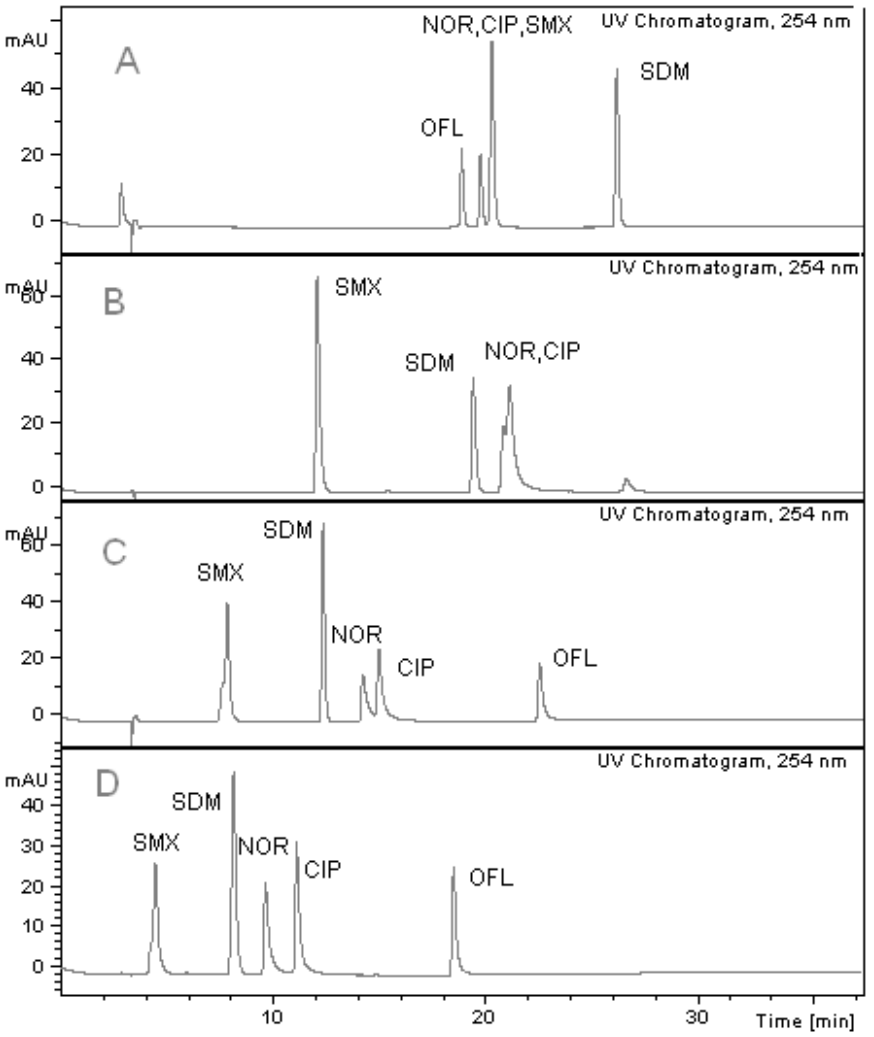


Figure 12. Chromatographic separation of five antibiotics. Used eluent buffer solutions: (A) AAF: 1 mM ammonium acetate and 0.1% formic acid, pH 2.8. (B) TEAA: 5 mM triethylammonium acetate buffer, pH 10.0. (C) CH_3COONH_4: 5 mM ammonium acetate, pH 9.0. (D) HFIP: 5 mM hexafluoroisopropanol, pH adjusted to 9.0 with ammonium hydroxide.

In the case of the CH_3COONH_4 buffer, the retention times of SAs did not change with the change of the mobile phase pH from 9 to 10. This observation is easy to rationalize – pKa values of SAs are much lower than 9 and the change of pH from 9 to 10 does not cause a change in the protonation equilibrium of the SAs.

When using the CH_3COONH_4 buffer, the retention times of FQs were shorter at pH 10 than at pH 9. At pH 10, the FQs exist mostly in the anionic form (Fig. 4) while at pH 9 some zwitterionic form is still present. Similar trends in the retention behavior of FQs have been noted in the pH range from 6 to 7.5 [147] and 7.5 to 10 [69] using non ion-interaction buffer components.

As the limit of detection (LOD) and the limit of quantitation (LOQ) are directly related to the height of the chromatographic peak rather than its area, the influence of the eluent composition and pH on the heights of the extracted ion chromatogram peaks was assessed.

In the positive mode ESI (+ESI), the highest signals for all the analytes were observed using the AAF 2.8 buffer (Fig. 13). This observation can be easily rationalized as at pH 2.8 FQs are present as cations already in the solution phase. SAs seem to be easily ionized although their pK_{a1} values are lower than 2.8.

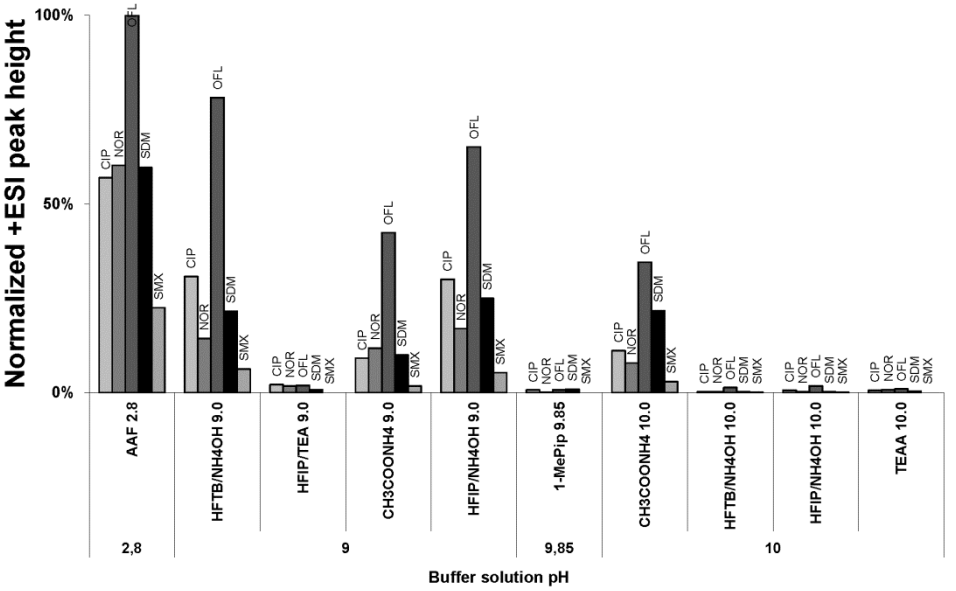


Figure 13. Comparison of normalized signal intensities in the positive ESI mode between different buffer compounds (n = 3).

In the negative mode ESI (-ESI) at pH 2.8, the signal intensity of FQs is among the lowest of all (Fig. 14). FQs are in a cationic form at this pH and the conversion of the solution-phase cations into gas-phase anions is not an efficient process. For SAs, the most intense signals are observed at pH 2.8, which may be attributed to the ease of penetration of neutral SA molecules to the ESI droplet surface.

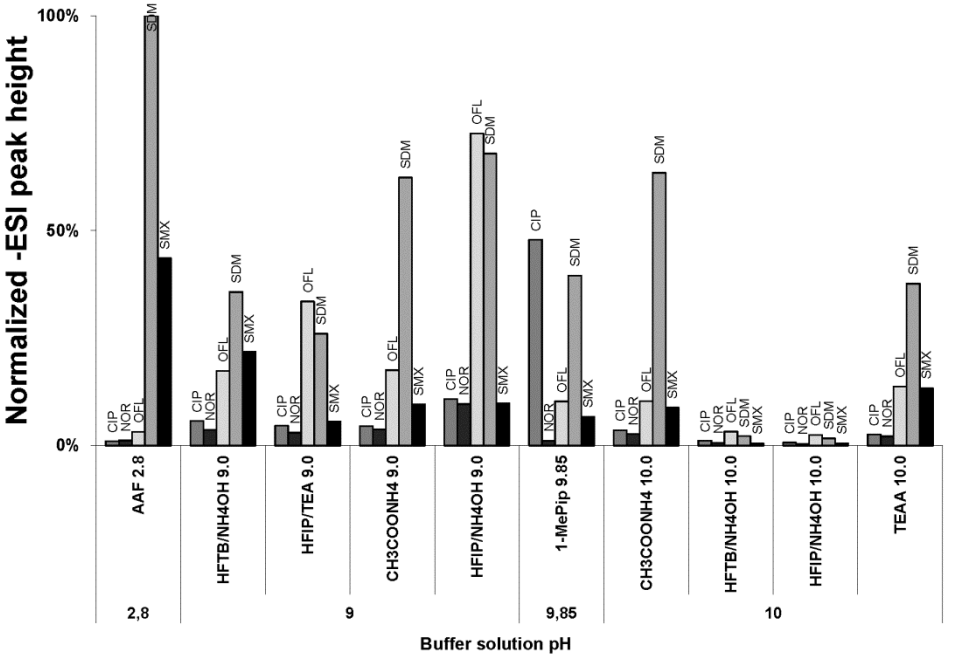


Figure 14. Comparison of normalized signal intensities in the negative ESI mode between different buffer compounds (n = 3).

Unexpectedly, for unknown reasons CIP yields the most intense signal in 1-MePip. 1-MePip and TEA buffers generated a background signal in the +ESI mode, $[M+H]^+$ is present at *m/z* 100 and 102 for 1-MePip and TEA, respectively. Both 1-MePip and TEA gave adduct ions with FQs. Adduct ions were not formed with SAs and 1-MePip and TEA.

4.2. Fluoroalcohols as buffer components for liquid chromatography electrospray ionization mass spectrometry [VI]

4.2.1. General properties of HFIP and HFTB

HFIP (pK_a=9.3) is miscible with water, methanol, 2-propanol and hexane but is claimed to be immiscible with acetonitrile. Our studies showed that in the concentration range 1 mM to 10 mM HFIP buffer solutions with pH 9 and 10 are miscible with acetonitrile and can be used as buffer components for LC-MS analysis. When the concentration of HFIP in the buffer solution exceeded 20 mM, then the solution appeared to be immiscible with acetonitrile. Consequently, the HFIP buffer solution can be used in a mobile phase using acetonitrile as an organic modifier; however, in our study methanol was used because better separation was achieved with methanol. With methanol, gradient elution started at 10% of the organic component. To achieve comparable retention with acetonitrile, an even lower organic content proved to be necessary. However, the low content of the organic modifier in the eluent is not recommended for C18 columns due to the possibility of the stationary phase collapse. The low content of the organic modifier also hinders the ionization process in the ESI source.

4.2.2. Ionization of HFIP and HFTB in ESI source [IV]

For –ESI, HFIP and HFTB (pK_a=9.6), buffer systems have a disadvantage: the background signal generated by the reagents. Fig. 15 shows the background spectra of the eluents with the HFIP and HFTB buffers. In the negative ESI mode, $[M–H]^-$ and the dimer $[2M–H]^-$ are present at *m/z* 167, 335 and 181, 363 for HFIP and HFTB, respectively. HFIP and HFTB did not form adduct ions with analytes. Moreover, two fluoroalcohols do not have background spectra in the positive ESI mode.

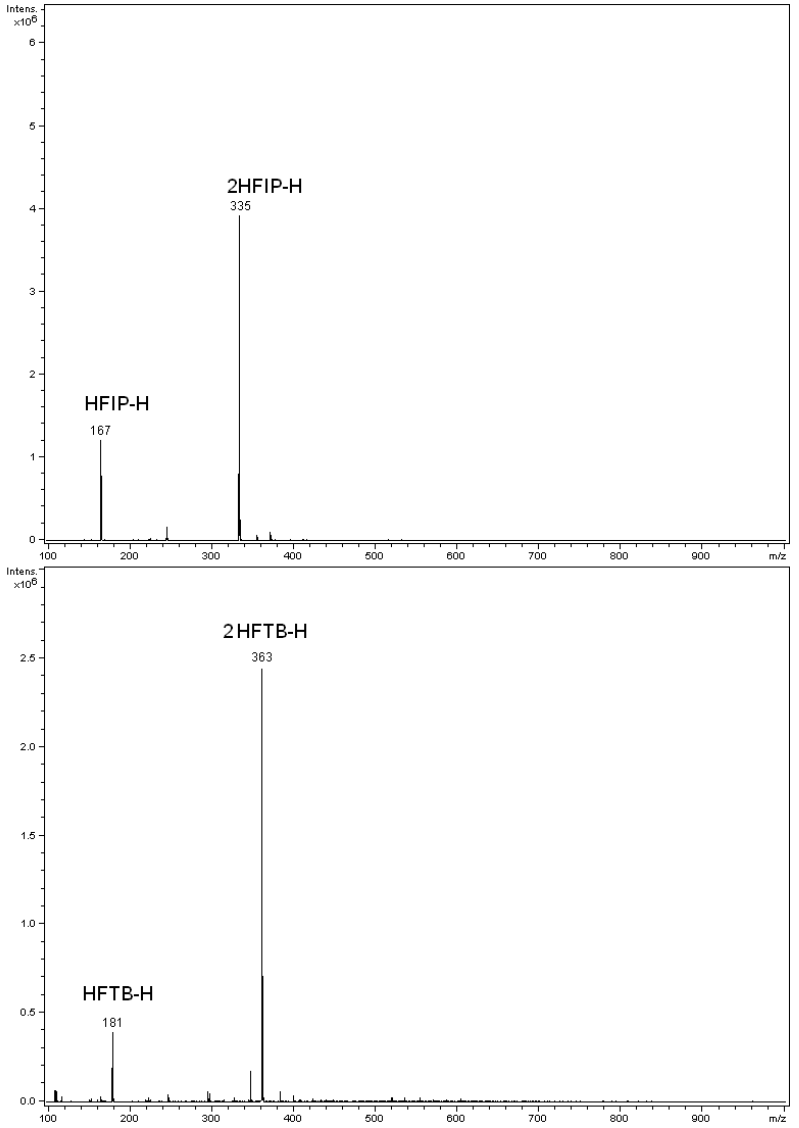


Figure 15. Background spectra using HFIP and HFTB buffer components in the negative ESI mode. Flow rate 0.3 mL/min. Eluent composition 50% HFIP or HFTB buffer with 50% methanol.

4.2.3. Retention of the fluoroalcohols on the stationary phase [VI]

Fluoroalcohol retention on Waters XBridge C18 stationary phase

To evaluate the retention of the fluoroalcohols on the stationary phase, samples of HFIP and HFTB were chromatographed using ammonium acetate (in pH 9 and 10) and methanol as the eluent (Fig. 16). The logK_{ow} values for HFIP and HFTB are 1.66 and 1.85, respectively [148].

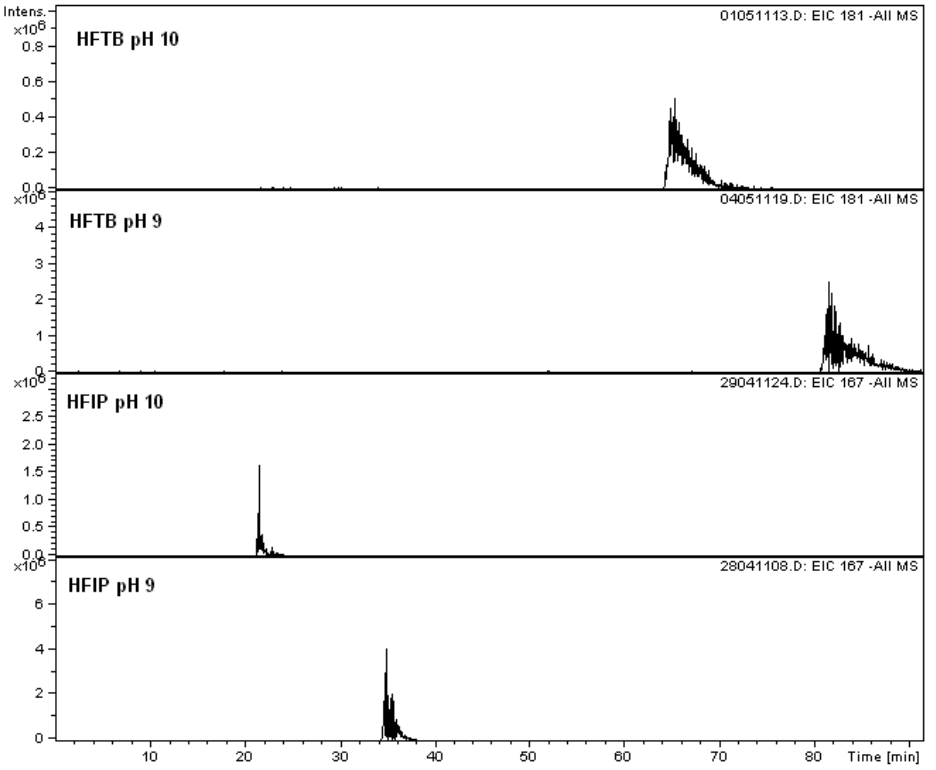


Figure 16. Retention of HFIP and HFTB in –ESI mode. Eluent: 5 mM ammonium acetate (2nd and 4th chromatogram at pH 9 and 1st and 3rd chromatogram at pH 10) and MeOH (77/23). Used analytical column: Waters XBridge C18 column (150 mm × 3 mm, 3.5 μm).

The retention times of HFIP were 34 and 21 min (with the retention factor *k* 8.4 and 4.8) at pH 9 and 10, respectively. The retention times of HFTB were 87 and 64 min (with *k* 23.2 and 16.8) at pH 9 and 10, respectively. HFIP and HFTB concentration in injected solutions ranged 20–60 mM and the injection solvent contained 23% of methanol. The retention factors indicate that the retention of fluoroalcohols differs significantly at different mobile phase pH values being greater at a lower pH.

Chromatograms in Fig. 16 are extracted ion chromatograms in the –ESI mode, the poor peak shape and noisiness of the peaks is related to the high concentration of the fluoroalcohol in the injected solution and the low ionization efficiency of fluoroalcohols in the –ESI mode.

Differently from acetate-containing buffers in the case of fluoroalcohols, a significant fraction of the alcohol is in the neutral form at all used pH values. The fluoroalkyl moiety of the fluoroalcohols is appreciably hydrophobic and interacts with the RP stationary phase. The fluoroalcohols alter the original stationary phase by forming a separate relatively low polarity and rather acidic

"fluorous phase" on the stationary phase surface, which has a higher volume at a lower pH.

4.3. Ionization of analytes from solutions containing fluoroalcohols [V]

For the estimation of fluoroalcohol buffers' influence on the analytes' ionization the SAs and FQs were used (see Section 4.1.2.). Buffer systems of a weak acid (HFIP) and a weak base (TEA) were compared with fluoroalcohol/NH_4OH buffers at pH 9 and 10. Separation was achieved using HFIP/TEA at pH 9, but very low ESI-MS signal intensities were observed and analytes remained undetected at 1 μg/mL level

When considering the basic pH range, HFIP/NH_4OH 9.0 and HFTB/NH_4OH 9.0 provide equally high signal intensities for all the analytes.

Very low analyte signal intensities were observed for 1-MePip and buffers containing TEA: SMX was not detected using HFIP/TEA 9.0 buffer, none of the five analytes were detected at 1 μg/ml concentration level with 1-MePip 9.85. In contrast to CH_3COONH_4 9.0 and 10.0 buffers, using which comparable signal intensities for all the analytes were obtained, HFIP/NH_4OH and HFTB/NH_4OH cause very low signal intensities at pH 10 (Fig. 13). In the positive ESI mode no interfering ions were observed.

In the basic buffers NOR, OFL and SDM exhibit the most intense signals in the HFIP/NH_4OH 9.0 buffer and SMX in the HFTB/NH_4OH 9.0. The weakest signals were observed in the case of HFIP/NH_4OH 10.0 and HFTB/NH_4OH 10.0 as was the case in +ESI.

The use of HFIP and HFTB buffer systems enhancing signal intensities was registered in the positive as well as negative ESI mode (even though HFIP and HFTB contributed to background spectra in a negative mode) in the analysis of FQs and SAs. On the side of the ionization the influence of the fluoroalcohols on chromatographic separation of the analtyes was studied.

4.4. Chromatographic separation of FQs and SAs on C18 stationary phase using eluents containing fluoroalcohol-based buffer solutions [V]

Results discussed below are shown in Table 4. Using HFIP/NH_4OH and HFTB/NH_4OH buffers, SAs' retention times were shorter at pH 9. This change of retention times must be caused by the nature of HFIP and HFTB. HFIP and HFTB are predominantly protonated at pH 9 and are predominantly deprotonated at pH 10, e.g. at pH 9 they are less polar than at pH 10. Therefore, at pH 9 the fluoroalcohols effectively compete with the analytes for the stationary phase surface, which is indicated by the shorter retention times of SAs at pH 9. At pH 9 as well as pH 10, retention times of SAs are longer in the case of

CH_3COONH_4 as compared to HFIP and HFTB. This also indicates that the fluoroalcohols compete with analyte molecules for the stationary phase surface.

In the case of the HFTB/NH_4OH buffer, the retention times of FQs increased with the increase in pH. This is contrary to the effect observed in the case of the CH_3COONH_4 buffer. In the case of the HFIP/NH_4OH buffer, retention times of FQs were nearly the same at pH 9 and 10. The pH of a solvent has a similar effect on the solute regardless of the compounds used to create the pH, e.g. CIP is protonated to the same extent in the CH_3COONH_4 9 as in the HFTB/NH_4OH 9 buffer. However, these pH values refer to the buffer solution before mixing with the organic solvent. The addition of the organic solvent may have a different effect on the buffer solutions created by using different acid-base systems. In the CH_3COONH_4 buffer solution, ammonia acts as the weak base, while acetic acid is virtually fully deprotonated. In the HFTB/NH_4OH and HFIP/NH_4OH buffer solutions, both compounds are present as mixtures of protonated and deprotonated form but at different ratios. Thus, the effective pH in the eventual mobile phase can be different in all three cases. Further effects to consider are the competition of the alcohols for the active sites of the stationary phase and the complex acid-base behavior of the FQs (present partly as zwitterions at the used pH level) which, in turn, also depends on the organic solvent. The observed retention time changes are probably due to a complex interplay of all these effects.

During the separations with the TEAA and HFIP/TEA buffers, TEA ions can form ion-pairs with FQs deprotonated carboxyl groups and SAs sulfonamide groups and the retention of antibiotics increases. Retention increase is more significant for the TEAA buffer. Retention increased using the HFIP/TEA buffer compared to the HFIP/NH_4OH buffer due to the analyte ion-pairing effect with TEA. Using ammonium hydroxide as an additive for the buffer solution, the ion-pair formation between the negatively charged antibiotics and ammonium ion does not alter retention times to a significant extent.

4.5. Study of retention mechanisms of analytes on C18 stationary phase using eluents containing fluoroalcohol-based buffer solutions [VI]

The retention mechanisms using HFIP and HFTB as buffer acids were studied. Since FQs and SAs are chemically complex compounds for estimating the retention mechanism's nature in fluoroalcohol usage, simpler compounds were selected for the study. The selection of analytes for the present study was made based on their pK_a values – acids and bases with a pK_a below 7 and above 10 (i.e. outside the 8.5–10 region) were studied. The effect of the pH and the composition of the buffer solution on the retention of 15 compounds (see Table 5) was studied in the pH range from 8.5 to 10.

Table 5. Analytes for the present study – acids and bases with pK_a values below 7.2 and above 10.2. The values of logK_{ow} are taken from LOGKOW© database [148].

Acids, pK_a >10.2 HA	Acids, pK_a <7.2, A^-	Bases, pK_a <7, B	Bases, pK_a >11, BH^+
2-tertbuthylphenol pK_a = 10.28 logK_{ow} = 3.31	2,5-dinitrophenol pK_a = 5.15 logK_{ow} = 1.54	2,6-dimethyl-pyridine pK_a = 6.65 logK_{ow} = 1.68	Pyrrolidine pK_a = 11.27 logK_{ow} = 0.46
2-nitroaniline pK_a = 17.9 logK_{ow} = 1.85	2-nitrophenol pK_a = 7.17 logK_{ow} = 1.77	2-methylpyridine pK_a = 5.97 logK_{ow} = 1.11	Diethylamine pK_a = 11.02 logK_{ow} = 0.58
3-nitroaniline[1] pK_a = 17.9 logK_{ow} = 1.43	2,3,5,6-tetrafluoro-phenol[2] pK_a = 5.53 logK_{ow} = 2.84	2-methoxypyridine pK_a = 6.47 logK_{ow} = 1.36	Piperidine pK_a = 11.12 logK_{ow} = 0.84
4-chloro-2-nitro-aniline pK_a = 17.1 logK_{ow} = 2.72	2,3,4,5,6-penta-fluorophenol[3] pK_a = 5.41 logK_{ow} = 2.65		Diisopropylamine pK_a = 11.05 logK_{ow} = 1.40

[1] pK_a is estimated using the COSMO-RS approach as described in Ref. [149]
[2–3] logK_{ow} is calculated using the ALOGPS 2.1 approach.

4.5.1. Comparison of analytes' retention in case of fluoroalcohols and ammonium acetate buffers [VI]

As demonstrated in section 4.2.3, fluoroalcohols are strongly retained on the C18 stationary phase. If present in the mobile phase, fluoroalcohols form a dynamic layer on the C18 stationary phase. This formed phase is less attractive for the acidic analytes than the native RP phase. In fact, fluoroalcohols compete with acidic analytes for the stationary phase surface and thus their retention times are shorter than when using ammonium acetate with the same pH (Fig. 18). The competition over the stationary phase was stronger for deprotonated acids (A^-, pK_a below 7.2). For the highly basic and non-polar analytes, this "fluorous phase" is more attractive because of the formation of hydrogen bonds with the fluoroalcohols. This leads to higher retention times for such compounds (Fig. 17).

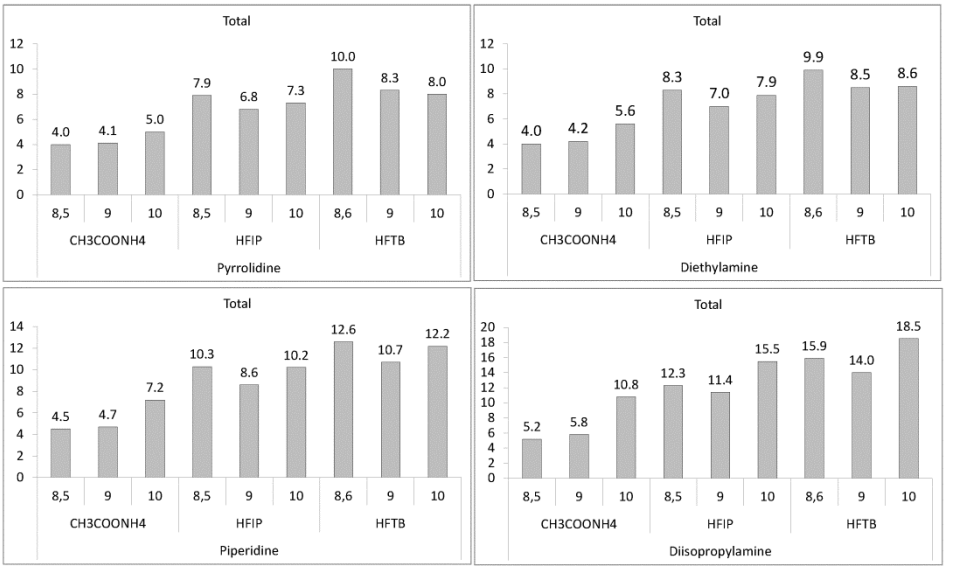


Figure 17. Retention times (n = 3) of protonated bases, BH^+-pyrrolidine, diethylamine, piperidine and diisopropylamine, using ammonium acetate, HFIP and HFTB buffers in the pH range 8.5–10. The column dead time was 3.6 min.

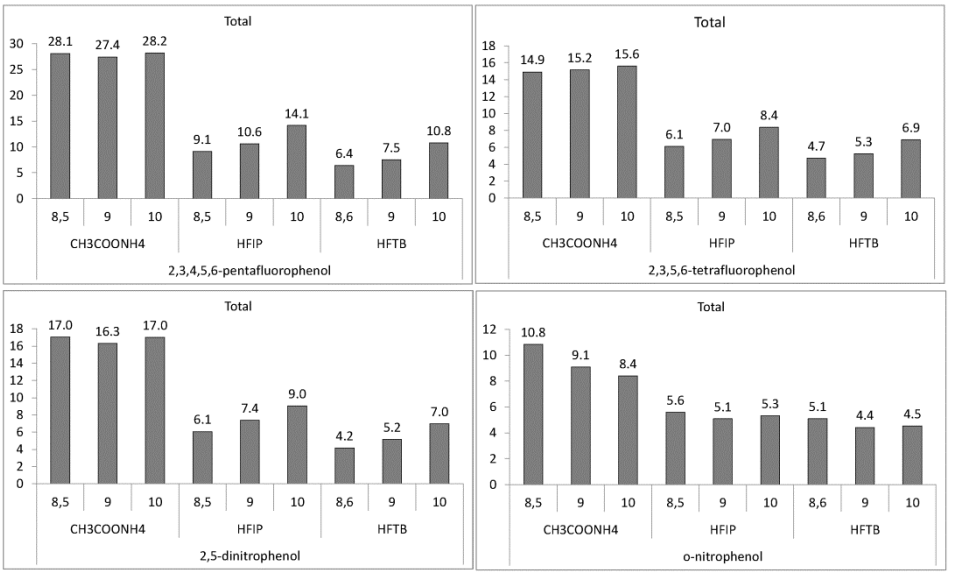


Figure 18. Retention times (n = 3) of deprotonated acids A^--2,3,4,5,6-pentafluorophenol, 2,3,5,6-tetrafluorophenol, 2,5-dinitrophenol and 2-nitrophenol, using ammonium acetate, HFIP and HFTB buffers in the pH range 8.5–10. The column dead time was 3.6 min.

For neutral analytes-protonated acids, AH (acids with pK_a > 10), and deprotonated bases, B (bases with pK_a < 7), the retention time differences between ammonium acetate and fluoroalcohol buffers were not significant (Fig. 19).

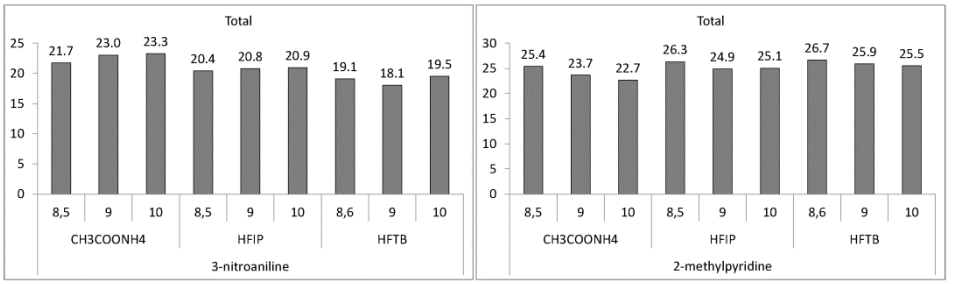


Figure 19. Retention times (n = 3) of the protonated acid, AH-3-nitroaniline and deprotonated base B-2-methylpyridine, using ammonium acetate, HFIP and HFTB buffers in the pH range 8.5–10. The column dead time was 3.6 min.

4.5.2. Comparison of analytes' retention at different pH values of fluoroalcohol buffers

With an increase in the eluent pH, the interaction between the stationary phase and fluoroalcohol decreases since the content of deprotonated fluoroalcohol increases. Fluoroalcohols have a weaker interaction with the stationary phase at pH 10, as their deprotonated forms are more hydrophilic and spend a relatively longer time in the mobile phase compared to pH 8.5, where the protonated form dominates. This concept is clear from the chromatograms in Fig. 16, where fluoroalcohols have shorter retention times at pH 10 as compared to a lower pH. As a result, the retention times of the acidic analytes should be shorter at pH 8.5 than at pH 10 due to the competition between fluoroalcohols and analytes for the stationary phase surface. Analyte retention times were indeed shorter for acids having pK_a values below 7.2 (Fig. 19). 2,5-Dinitrophenol retention time was 2.9 min shorter using HFIP at pH 8.5 than at pH 10 (k 0.69 and 1.5, respectively) and 2.8 min shorter using HFTB at the same pH (k 0.16 and 0.94, respectively). 2,3,5,6-Tetrafluorophenol and 2,3,4,5,6-pentafluorophenol retention times were 2–5 min shorter using HFIP and HFTB at pH 8.5 (8.6 for HFTB) comparing with retention using the same buffer solutions at pH 10. Judged by the retention factors, the competition with the fluoroalcohols is very strong, especially in the case of HFTB.

However, 2-nitrophenol is an exception – its retention time does not change significantly in the used pH range (Fig. 19). However, this acid is weaker than the others and even in water its pK_a value is not far from the mobile phase's pH range. In the used mobile phase, its pK_a value is expected to be around 0.5 pK_a units higher than in water [150]. At the same time, the pK_a value of protonated ammonia is around 0.3 pK_a units lower than in water [150]. This means that in the mobile phase with the pH 8.5, this compound is present to a large extent as

neutral and this elongates its retention time and removes the pattern seen with stronger acids.

From the chromatograms in Fig. 16, it is also evident that HFTB interacts with the stationary phase surface more strongly than HFIP. Therefore, shorter retention times of analytes using HFTB as compared to HFIP would be expected. HA, A^- and B compounds had shorter retention times using an HFTB buffer comparing with an HFIP buffer. The only exception is 2-methylpyridine, deprotonated base B, having comparable retention times in both mobile phases.

4.5.2. Effect of the acid-base equilibria on retention time

The pH-dependence of retention of acidic and basic analytes is usually interpreted by means of the acid-base equilibria of analytes. In the case of fluoroalcohols as buffer components, this approach is not able to account for all the observed effects. Therefore, additionally, buffer components' retention on the stationary phase and thus modifying the stationary phase properties, as well as ion-pairing effects are involved in the discussion below.

Adding methanol to the buffer solution changes all the pK_a values involved. Thus, both pH and the ionization ratios of the analytes are different in the actual mobile phase. Furthermore, the pK_a values of neutral acids are generally more sensitive to changes in solvent composition than pK_a values of protonated bases. The discussion presented in this section ignores this effect. However, we have two reasons to expect that with the used mobile phase this effect is minimal: (1) we use methanol – a protic solvent – as the organic modifier and (2) the content of methanol in the mobile phase is low.

RP-HPLC retention is strongly dependent on compounds' hydrophobicity as the retention increases for more hydrophobic compounds. Acids exist predominantly in their deprotonated form (ionic, i.e. polar) at pH values higher than the pK_a of the acid. Bases at pH values lower than their pK_a value exist predominantly in their protonated (ionic, i.e. polar) form. Retention times of the ionic forms of acids and bases are shorter than those of the respective neutral (less polar) forms [16]. In the case of the ammonium acetate buffer, upon increasing pH from 8.5 to 10, the retention times of the protonated bases (BH^+, pK_a above 11) increase as the fraction of analyte in deprotonated (neutral, B) form increases (Fig. 17).

Protonated bases, BH^+ had shorter retention times with an ammonium acetate buffer compared to fluoroalcohols and shorter retention times using an HFIP buffer than using an HFTB buffer at the same pH (Fig. 17).

Out of the acids existing in the mobile phase predominantly in a deprotonated form, the A^- (pK_a below 7.2) retention time decreased for 2-nitrophenol (pK_a 7.17) as closest to the pH range of the buffer solutions because the proportion of the deprotonated form (anion, A^-) predominates at a higher pH (Fig. 18).

Deprotonated acids, A^- (acids with p$K_a < 7.2$), had longer retention times using ammonium acetate compared to fluoroalcohols (over 10 min longer for

2,3,4,5,6-pentafluorophenol, 2,3,5,6-tetrafluorophenol and 2,5-dinitrophenol and 3–5 min longer for 2-nitrophenol). Deprotonated acids had stronger retention using an HFIP buffer compared to an HFTB buffer at the same pH. The retention time difference was bigger for 2,3,4,5,6-pentafluorophenol, 2,3,5,6-Tetrafluorophenol and 2,5-dinitrophenol (Fig. 18).

Retention times of the stronger acids did not decrease with increasing pH as their pK_a values were sufficiently low to regard these compounds completely dissociated in the whole studied pH range.

Very weakly acidic (pK_a > 10) and very weakly basic (pK_a < 7) analytes, which are in a neutral form at the used pHs, were included in the study for the comparison of retention with acidic and basic analytes. For protonated acids (HA, pK_a above 10), the mobile phase pH did not have a strong influence on the retention time as compounds were in the mobile phase in their protonated form (Fig. 19).

For deprotonated bases (B, pK_a below 7), the retention time decreased with the pH increase (Fig. 19). This is the opposite to what is predicted by theory, but this is easily explained by the difference of the ionic strengths of the ammonium acetate buffers: more ammonium hydroxide solution was required to obtain buffer solutions with a higher pH.

4.5.4. Ion-pairing mechanisms

Retention of BH^+ compounds (protonated bases) cannot be explained using the above proposed model of altered stationary phase surface as their retention times using the HFTB buffer are longer than with the HFIP buffer at the same pH (Fig. 17). Ion-pairing mechanism is needed to account for this observation.

Retention in IPC can be described by two models described on Section 1.3.2. If we apply the first model to fluoroalcohols as ion-pairing reagents, then the stationary phase will be covered with anionic moieties of the fluoroalcohols. The negatively charged stationary phase is balanced by positive ions from the buffer and/or analyte. Positively charged analytes (BH^+) can exchange cations from the balancing layer. In accordance with this model, retention times for protonated bases (cations, BH^+) using fluoroalcohols are longer than when using ammonium acetate (Fig. 18). For the neutral compounds HA and B, the retention times were similar between fluoroalcohols and ammonium acetate (Fig. 19). Retention times for deprotonated acids (anions, A^-) are shorter with fluoroalcohol buffers, comparing with ammonium acetate due to the distraction between the analyte anions and fluoroalcohol anions in the stationary phase (Fig. 18.).

At pH 10, fluoroalcohols are ionized to a larger extent than at pH 8.5 (8.6 for HFTB). Consequently, cation (BH^+) retention times should be longer at pH 10. Retention time is indeed approximately 3 min longer for diisopropylamine (protonated base) at pH 10 (Fig. 17). Anion (A^-) retention times should be shorter at pH 10 than at pH 8.5 (Fig. 18). However, the opposite is observed

(except with 2-nitrophenol). Thus, the first ion-pairing model is not fully in line with the experimental data.

HFTB is a less polar compound and ion-pairs between its anion and a protonated base have a stronger interaction with the stationary phase surface than HFIP. Thus, the retention times of protonated bases (Fig. 17) are longer when HFTB is used (compared to HFIP).

4.5.5. Ion interaction and competition for stationary phase surface

The dependence of the retention of protonated bases on the pH in the case of HFIP- and HFTB-containing mobile phases cannot be explained by either ion-pairing of fluoroalcohol deposition on the stationary phase mechanism alone. The retention behavior can only be rationalized by considering that these mechanisms are both active and at a different pH, a different mechanism dominates.

At pH 10, the cations are retained predominantly by the ion-pairing mechanism. At pH 8.5, retention is mainly controlled by the interaction with the "fluorous phase" formed by HFIP or HFTB. At pH 9, both these interactions are somewhat weakened and the retention is weaker (Fig. 17)

4.6. Retention on two C18 stationary phases using fluoroalcohol-based buffers

For HFIP and HFTB evaluation retention on another C18 stationary phase, YMC-Triart C18 was studied (referred to as Triart in the following text). The stationary phase of the Triart column is of higher hydrophobicity than XBridge (a Waters XBridge C18 column). The hydrogen bonding capacity is similar for the two stationary phases [151]. The analytes employed in the study are presented in Table 5 and chromatographic conditions are presented on the Section 3.3.4.

Due to the higher hydrophobicity, the retention of all analytes studied was stronger on the Triart column (Fig. 20). The trends of all analytes' retention on Triart were similar to XBridge when ammonium acetate, ammonium bicarbonate and fluorinated alcohol buffers were used. Therefore, only representative examples are presented in Fig. 20.

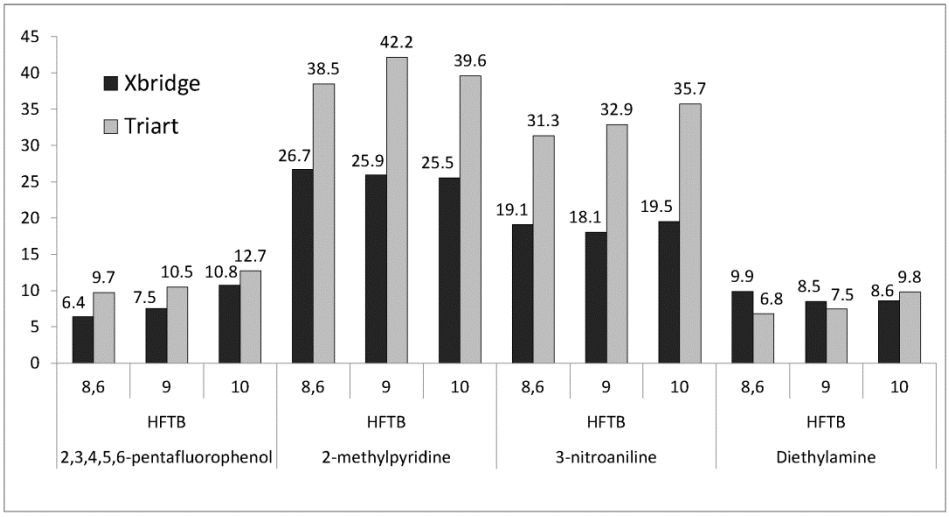


Figure 20. Retention comparison (n = 3) between two C18 stationary phases using an HFTB buffer on the pH range from 8.6 to 10 with XBridge and Triart for analytes: A^--2,3,4,5,6-pentafluorophenol, B-2-methylpyridine, AH- 3-nitroaniline and BH^{+-} diethylamine. The column dead times were 3.6 and 2.1 min for XBridge and YMC, respectively.

On both stationary phases, the retention of deprotonated acids (A^-) increased with the increase of pH using fluorinated alcohols for elution (Fig. 20). Deprotonated acids' competition over the stationary phase surface has the strongest effect on the retention behavior (Section 4.5.2).

The retention of neutral compounds had interesting trends on the Triart column, for the deprotonated bases (B) the retention behavior was bell-shaped and for the protonated acids (AH) the retention increased with the increase of the mobile phase pH. Both competition over the stationary phase surface and ionic strength of the buffer are responsible for the retention behavior of neutral compounds.

In case of protonated bases (BH^+), the increase in eluent pH caused retention time's increase on Triart and a reduction on XBridge (Fig. 20). At pH 8.6, fluoroalcohol is predominantly in the neutral form, therefore no ion-pair formation with the analyte is expected and retention is controlled by the competition over the stationary phase surface. As Triart is more hydrophobic, it interacts more strongly with fluoroalcohol and, therefore, retention is shorter than on XBridge. At pH 10 fluoroalcohol is in a deprotonated, anionic form and can form ion-pairs with the analyte. Owing to higher hydrophobicity, retention times are longer on Triart.

The comparison of retention on columns with different hydrophobicities supports the qualitative retention model developed.

4.7. Retention of acids and bases on fluorinated stationary phase

For the alternative retention mechanisms the fluorinated stationary phase was involved to the study. The fluorinated stationary phases are known for suffering under poor column lifetimes, the unstable baseline while MS is in use for detection. The improvements of fluorinated stationary phases are made to lead them for MS capability. Fluorinated stationary phases are a class of promising tools for the separation of pharmaceuticals due to the ability of providing alternative retention mechanisms for the improvement of retention and chromatographic resolution. The ESI ionization of analytes (FQs and SAs) in acidic conditions was better and, therefore, the alkyl perfluorinated stationary phase was studied for analytes' separation on acidic buffer conditions (Section 4.8.2.). In order to compare separation provided by fluorinated alcohols with the commercially available fluorinated stationary phase, the same compounds – acids and bases from Table 5 – and the Epic FO-LB as alkyl perfluorinated C8 were involved in the study.

4.7.1. Comparison of analytes' retention of fluorinated stationary phase at different pH values of commonly used buffers

The retention of protonated bases (BH^+, pK_a above 11) on alkyl perfluorinated C8 with ammonium acetate and ammonium bicarbonate is presented in Fig. 21. In the case of the ammonium acetate and ammonium bicarbonate buffer, upon increasing pH from 8.5 to 10, the retention times of protonated bases increase as the fraction of analyte in the deprotonated (neutral, B) form increases (Fig. 21). An exception was pyrrolidine – while at pH 9, the retention of the compound was the strongest with the ammonium acetate and ammonium bicarbonate buffer (k were 4.9 and 5.3, respectively). Ammonium acetate and ammonium bicarbonate as buffer salts gave similar results on retention of protonated bases.

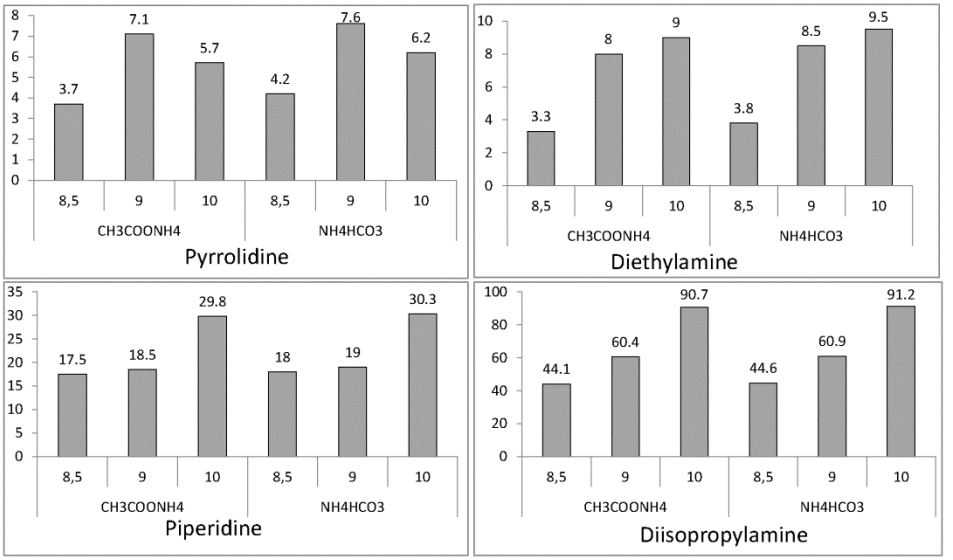


Figure 21. Retention times (n = 3) of protonated bases, BH^+-pyrrolidine, diethylamine, piperidine and diisopropylamine, using ammonium acetate and ammonium bicarbonate buffers in the pH range 8.5–10 with Epic FO-LB column. The column dead time was 1.2 min.

The retention time of deprotonated acids, A^- (pK_a below 7.2) retention on alkyl perfluorinated C8 using ammonium acetate and ammonium bicarbonate decreased with the increase of the buffer solution pH. The effect on retention decrease was the strongest for 2-nitrophenol (pK_a 7.17, as closest to the pH range of the buffer solutions) because the proportion of the deprotonated form (anion, A^-) predominates at a higher pH (Fig. 22). The difference in retention times with two buffer salts was not significant.

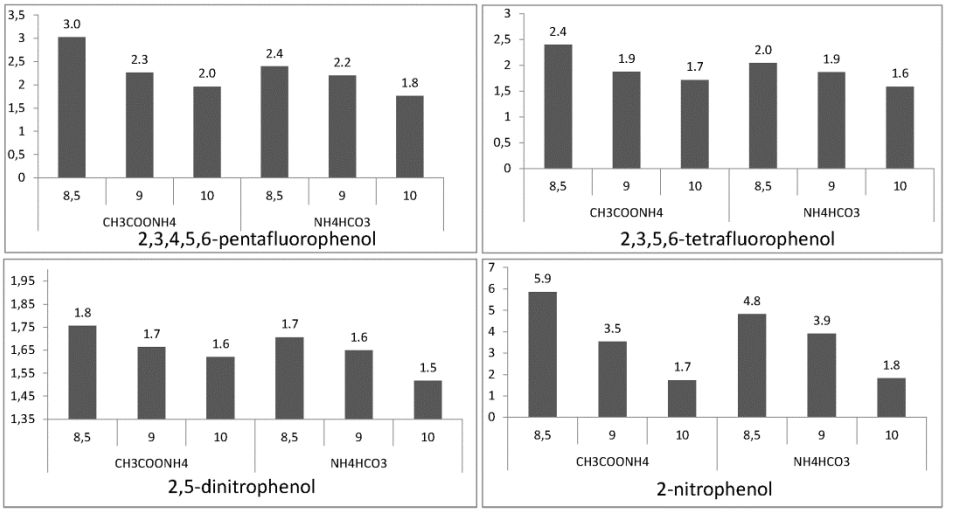


Figure 22. Retention times (n = 3) of deprotonated acids A^--2,3,4,5,6-pentafluorophenol, 2,3,5,6-tetrafluorophenol, 2,5-dinitrophenol and 2-nitrophenol, using ammonium acetate and ammonium bicarbonate buffers in pH range 8.5–10 with Epic FO-LB column. The column dead time was 1.2 min.

For protonated acids (HA, pK_a above 10), the mobile phase pH did not have a strong influence on the retention time in the case when ammonium acetate was used for all acids included in the study (Fig. 23). In case of ammonium bicarbonate, the retention decreased with the increase of the pH for all protonated acids included in the study. For deprotonated bases (B, pK_a below 7), the retention time decreased for all compounds with ammonium acetate and ammonium bicarbonate buffers (Fig. 23).

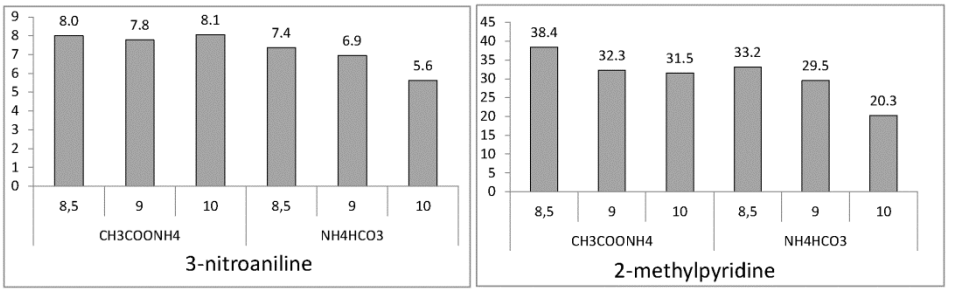


Figure 23. Retention times (n = 3) of protonated acid, AH-3-nitroaniline and deprotonated base B-2-methylpyridine, using ammonium acetate and ammonium bicarbonate buffers in the pH range 8.5–10 with Epic FO-LB column. The column dead time was 1.2 min.

The retention of protonated bases (BH^+) was stronger on alkyl perfluorinated C8, increasing *k* values for all analytes. The change in retention was the strongest in the case of diisopropylamine and the *k* increased approximately 30–40 times. In the case of piperidine, the change on retention was also remarkable (*k* was 4.6 times higher on pH 10 for using ammonium acetate buffer) while the change of retention for pyrrolidine and diethylamine was also significant (change of *k* was approximately in the range of 40–50 times) (Fig. 17 and Fig. 21).

Interestingly, regarding the retention of deprotonated acids, A^- was significantly shorter retention time on alkyl perfluorinated C8 phase. For all the analytes, the *k* was higher using the C18 column, with one exception 2-nitrophenol at pH 8.5 having *k* value about 2 times higher on the fluorinated stationary phase.

For neutral analytes (protonated acids, HA and deprotonated bases, B) (Fig. 23), the retention factor did not change the *k* value significantly for 3-nitroaniline (represents protonated bases), which were 5.4 and 5.6 for C18 and fluorinated C8 stationary phases using ammonium acetate. For deprotonated bases, the retention decreased with the increase of pH on both stationay phases – C18 and fluorinated C8. The same decrease in retention was observed when using ammonium bicarbonate for the analysis of protonated bases. Retention decrease with the increase of pH is explained with the difference of ionic strengths of the ammonium acetate buffers: more ammonium hydroxide solution was required to obtain buffer solutions with a higher pH because of higher ionic strength.

4.7.2. Comparison of analytes' retention on alkyl perfluorinated C8 stationary phase with retention on C18 stationary phase using fluoroalcohols as buffer components

The retention of protonated bases (BH^+) was stronger on alkyl perfluorinated C8 compared to HFIP and HFTB in the C18 stationary phase. For all analytes, the retention factor *k* values were 1.5 to 22 times higher with alkyl perfluorinated C8. Retention behavior itself was completely different – while the use of fluorinated alcohols resulted mostly in the U-shape retention (on the buffers' pH increase) of BH^+ compounds, then the retention in the fluorinated stationary phase increased with the increase of the pH, exactly as in the C18 stationary phase using common buffer compounds. An exception was pyrrolidine with a fluorinated stationary phase, resulting in a bell-shape retention on the increase of pH of the mobile phase (Fig. 17 and Fig. 21).

The retention factor of deprotonated acids (A^-) was comparable with fluorinated alcohols in the C18 stationary phase and ammonium bicarbonate and ammonium acetate on the fluorinated stationary phase. For 2-nitrophenol, the *k* was slightly higher when using the fluorinated stationary phase. The trends in retention were completely opposite, while using HFIP and HFTB the retention

increased with the increase of the pH, then on the fluorinated stationary phase the retention decreased with the increase of the pH (Fig. 18 and Fig. 22).

The retention of neutral analytes was similar as described in Section 4.3.2.

The retention behavior of analytes is completely different on the fluorinated stationary phase compared with the C18 stationary phase and fluoroalcohols. Consequently, using HFIP and HFTB eluent modifiers provides alternate selectivity compared to conventional C18 stationary phases, and also perfluoronated C18 phases. On fluorinated stationary phases, the column bleeding often occurs even when enhanced stationary phase binding techniques are used. Fluorinated stationary phases even with better pH stability are sensitive for the high pH. The named downsides cause problems on MS detection and contamination on the ESI source, or even worse – in the MS capillary. In the progress of fluorinated columns' advancement, alternatively the use of the fluoroalcohols as buffer components in C18 stationary phase is a useful and necessary tool for better selectivity.

4.8. Applications of using fluoroalcohols as buffer components in LC-ESI-MS

As shown previously (in Section 4.1.2. [III,V]), the chromatographic separation of pharmaceuticals having similar chemical properties or simultaneous determination of many chemically diverse substances or using complex matrix components have been problematic. For the enhancement of the separation and decrease of matrix effects, fluorinated alcohols were used.

4.8.1. Analysis of carbapenems

Chromatographic separation of carbapenems

In carbapenem analysis, the analyte peak symmetry and retention time are strongly dependent on the eluents' pH. Carbapenems are mostly in the zwitterionic form in the pH range 3.5 to 6. The peak shape and retention in this pH range was poor. Better peak shapes and stronger retention of meropenem and doripenem were observed in the pH range 7 to 10. Using traditional buffers in this pH range did not provide necessary separation of doripenem and ertapenem (Fig. 11). Therefore, the HFIP was used for elution. Using a 5 mM HFIP pH 10 buffer and MeOH with a gradient elution, the excellent separation of five analytes from blood plasma was achieved (Fig. 24).

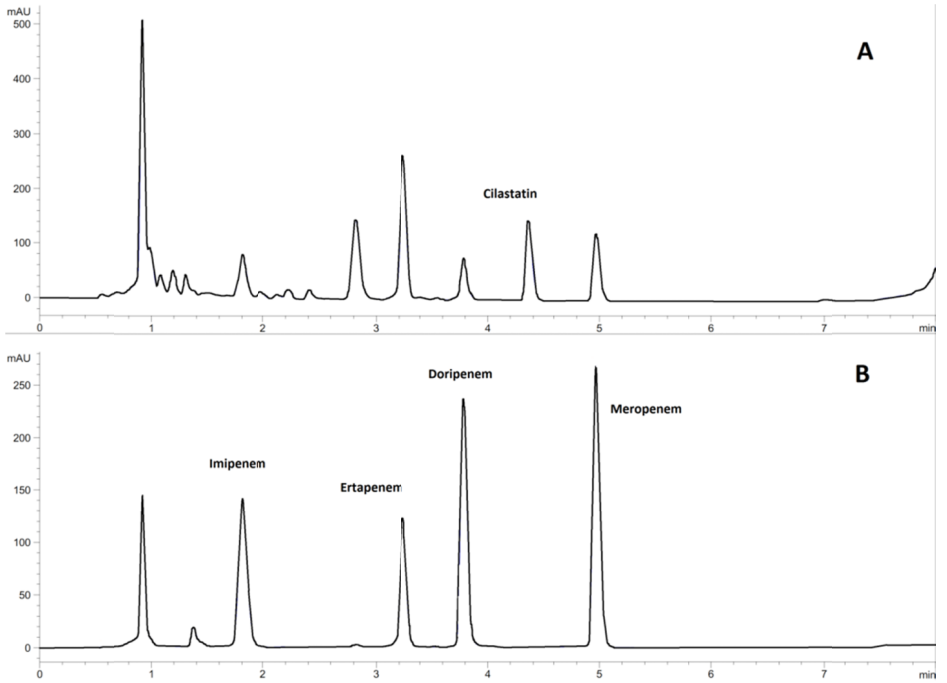


Figure 24. Chromatographic separation of four carbapenems (chromatogram B, λ = 306 nm) and cilastatin (chromatogram A, λ = 224 nm) from plasma samples spiked at 80 μg/mL concentration, using 5 mM HFIP (pH 10).

The carbapenems analyzed in this work are predominantly in their anionic forms at pH 10 since the pH of the buffer is higher than all the pK_a values (see Section 1.4.2). The retention of the deprotonated form of the analyte is dependent on the HFIP and analyte's competition for the stationary phase surface. The retention of meropenem increases the most compared to the elution at a low pH. The ion-pairing mechanism does not play a role at pH 10 since HFIP is also predominantly deprotonated, i.e. anionic. The interaction of carbapenem hydrophobic moiety with the stationary phase is the main cause of changes in retention.

Validation of the method for simultaneous analysis of carbapenems

The described method (see Sections 3.2.3 and 3.3.3) was validated according to the European Medicines Agency guidelines [152] for the simultaneous determination of meropenem, ertapenem, doripenem and imipenem in blood plasma samples. For calibration, the antibiotics solution was prepared in plasma samples and sample preparation was performed individually. The calibration graphs with peak area versus concentration were composed on concentration range 0.1–100 μg/mL and were linear with r^2>0.9998. Recoveries were calculated for carbapenems' standard addition experiments for blood plasma and compared with standard solutions in water. Recoveries compared to the standard solution for all detected pharmaceuticals in blood plasma ranged from 44 to

72% (estimated at 3 concentration levels within the calibration range, triplicate analysis). If matrix-matched calibration was employed (carbapenems' calibration solutions prepared in blood plasma), average recoveries for all pharmaceuticals were around 100% (SD 8%) at three concentration levels within the calibration range (the high, low and average concentration, 0.5 μg/mL, 20 μg/mL and 100 μg/mL, respectively). The method within-day accuracy and precision were estimated on the aforementioned three concentration levels and for LOQ, the values ranged from 100 ± 0.3% to 100 ± 9% and from 1.5% to 8.4%, respectively, for all analytes. The between-day accuracy and precision of the method ranged from 100 ± 3.2% to 100 ± 7% and from 2.7% to 7.9%, respectively, for all analytes.

The LOQ and LOD values (using UV detection) were estimated as 10 times and 3 times, respectively, of the standard deviation from five replicate analysis of spiked plasma samples on low concentration, LOQ was 10 times and LOD was 3 times standard deviation. The LOQ values ranged from 0.4 to 1.6 μg/mL and LOD values from 0.1 to 0.5 μg/mL for all analytes.

Extensive stability study for carbapenem samples and standard solutions was carried out and it appeared that carbapenem blood plasma samples have to be stored at –80 ºC after the sample collection. The long-term stability studies at –80 ºC for blood plasma samples showed analytes to be stable and degradation was approximately 4% within 3 months. Pretreated blood plasma samples showed degradation at room temperature – after 4 h the degradation was around 7 to 31%, depending on the analyte. Therefore pretreated blood plasma samples have to be analyzed as soon as possible and for the analysis samples have to be stored at 4 ºC in a thermostated autosampler. In the thermostated autosampler, the analytes' content of samples did not decrease significantly during 3 hours.

Chromatographic separation for meropenem microanalysis [VII]

For the microanalysis of meropenem only the method using acidic conditions for elution and the separation from blood plasma was successfully achieved (Fig. 25). Meropenem and ertapenem (I.S.) peak shapes on the low mobile phase pH tend to be asymmetric and the peaks are low. Although, the sample preparation for plasma samples with LE is quick it is also "dirty". The content of blood plasma components in the injected sample is rather high leading appearance of several peaks and bands in the chromatogram [I,II,VII]. At the end of the chromatogram, when the content of organic solvent (MeOH) is high, a massive peak (using wavelength 224 nm, not used for meropenem detection) elutes due to the blood plasma components (Fig. 26).

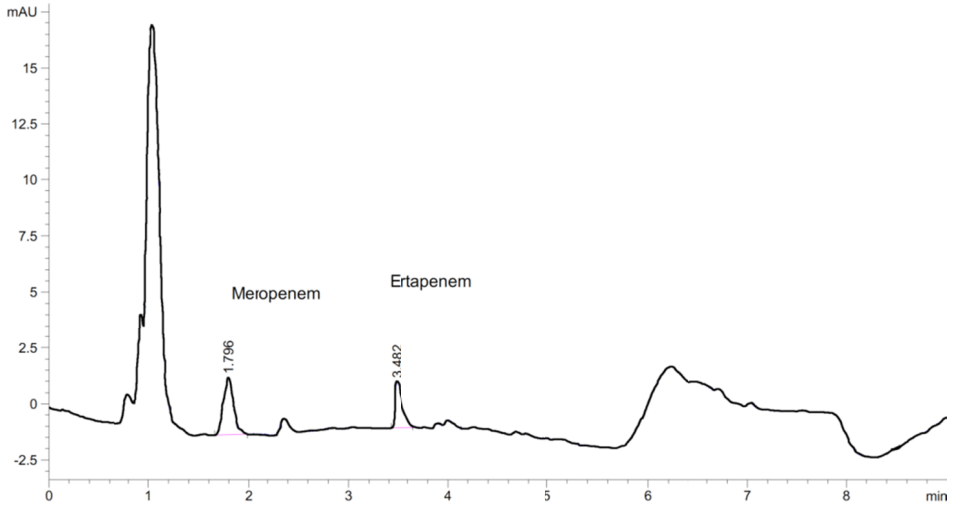


Figure 25. UV chromatogram of the patient blood plasma sample at λ = 306 nm. Eluent: 0.1% formic acid and MeOH. Used analytical column: Waters BEH C18 column (2.1 x 100 mm, 1.7 µm); ertapenem is used as I.S.

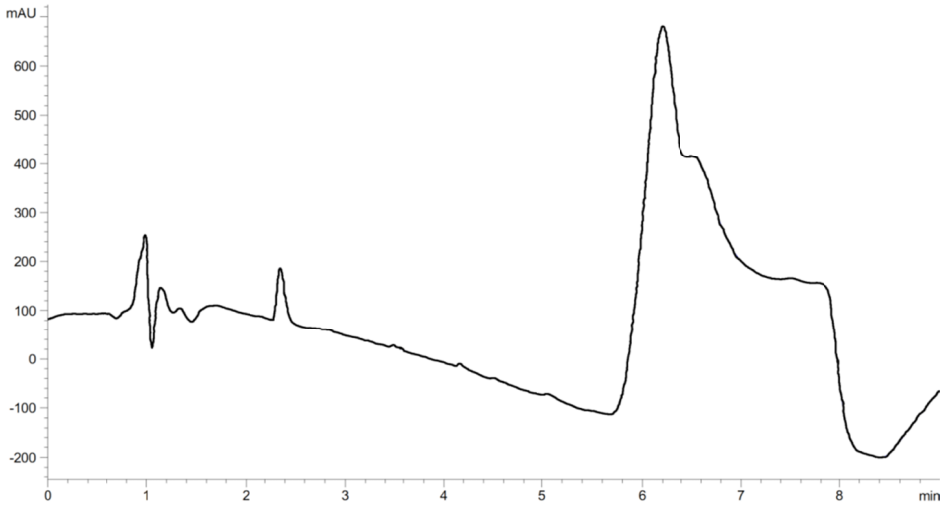


Figure 26. UV chromatogram of the blood plasma blank sample at λ = 224 nm. Eluent: 0.1% formic acid and MeOH. Used analytical column: Waters BEH C18 column (2.1 × 100 mm, 1.7 µm).

For meropenem analysis, also the resolution of the analytes from the plasma peaks can be enhanced. In order to achieve better separation, a higher pH and fluorinated alcohols were tested (Fig. 27). Using HFIP at pH 8.5, the retention time of meropenem increased and more effective separation from blood components was achieved.

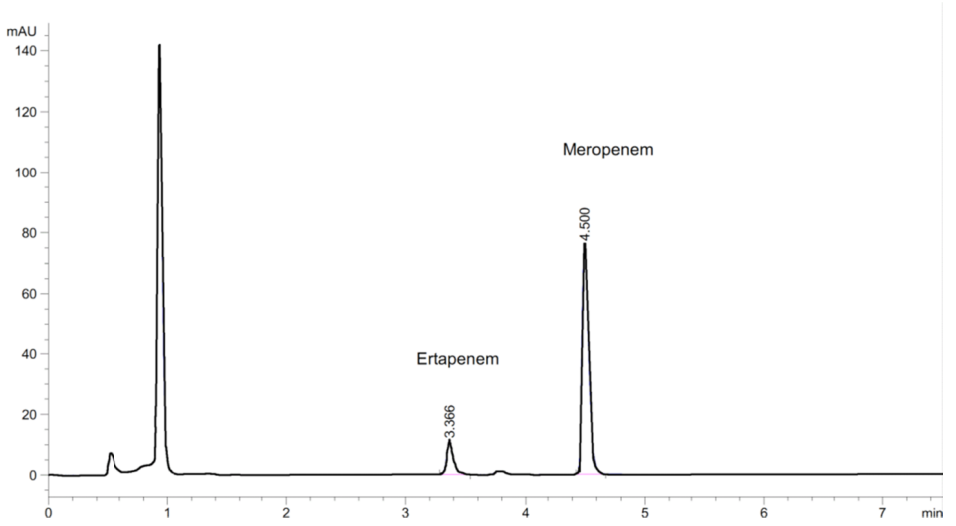


Figure 27. Chromatographic separation of meropenem blood plasma sample at λ = 306 nm. Eluent: 5 mM HFIP (pH 8.5) and MeOH. Used analytical column: Waters BEH C18 column (2.1 x 100 mm, 1.7 µm); ertapenem is used as I.S.

The better peak shapes were achieved while HFIP was used, compared to the common buffer solutions at a high pH. Moreover, the extensive sample matrix peak now elutes in the beginning of the chromatogram leading to improved baseline over the rest of the chromatogram even at lower UV wavelength (not used for analyte detection) (Fig. 28).

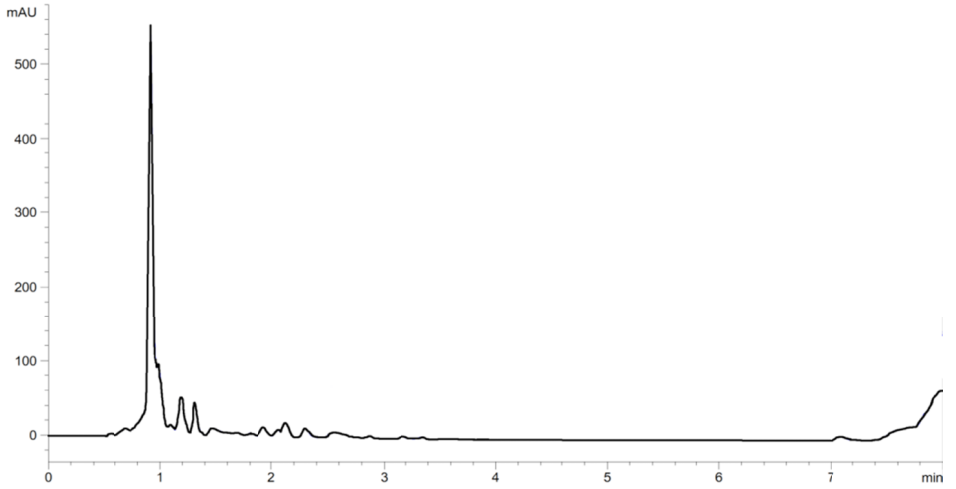


Figure 28. UV chromatogram of the blood plasma blank sample on λ = 224 nm. Eluent: 5 mM HFIP (pH 8.5) and MeOH. Used analytical column: Waters BEH C18 column (2.1 × 100 mm, 1.7 µm)

Validation of the meropenem microanalysis method [VII]

For the meropenem microanalysis method validation [152], the spiked meropenem plasma samples were prepared and sample preparation was performed separately. The calibration graphs with the peak area versus the concentration composed in the concentration range 0.1–200 µg/mL and from 1 to 250 µg/mL for urine were linear with r^2>0.9999. With meropenem, the calibration in blood plasma average recoveries was around 100% (SD 6%) at three concentration levels within the calibration range (the high, low and average concentration, 2 µg/mL, 10 µg/mL and 100 µg/mL, respectively) and for LOQ. The method within-day accuracy and precision were determined at the abovementioned concentration levels and were 100 ± 4% and 100 ± 6%, respectively, for all analytes. The method between-day accuracy and precision were 4% and 8%, respectively. Meropenem sample preparation and stability have been discussed previously [II]. As other carbapenems meropenem blood plasma and urine samples have to be analyzed as soon as possible and for the analysis samples have to be stored at 4 ºC in a thermostated autosampler. LOQ for plasma samples was 0.1 µg/mL and LOD 0.01 µg/mL. LOQ for urine samples as the lowest concentration of calibration samples was 1 µg/mL with accuracy 100 ± 3% and precision (as coefficient of variation) < 2%.

4.8.1. FQs and SAs in plants

LC procedure for the plant samples

For a better chromatographic separation, the fluorinated stationary phase was used for the antibiotic standard solution with the elution with AAF at pH 2.8 (Table 3) (Fig. 29).

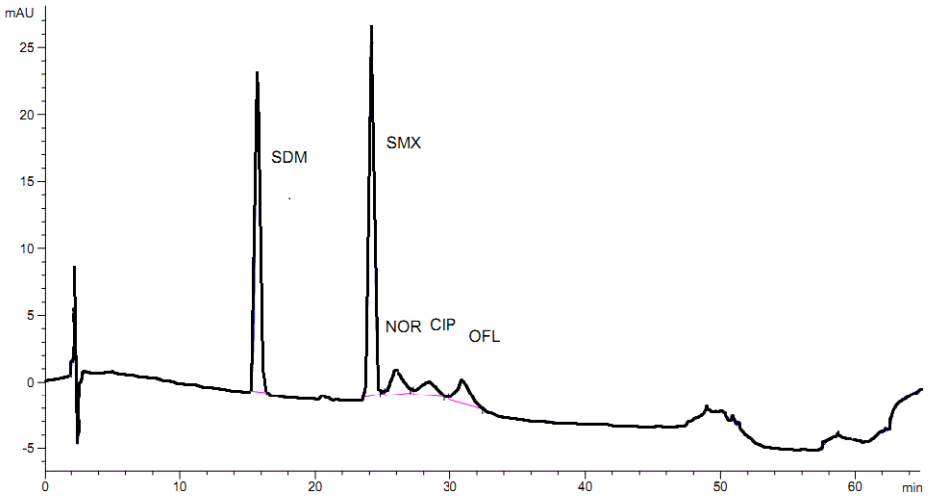


Figure 29. UV chromatogram of the standard solution of FQs and SAs. Eluent: AAF (pH 2.8) and MeOH. Used analytical column: Epic FO-LB C8 column (150 mm × 3 mm, 3.5 µm).

Reputedly, retention of fluorinated compounds is enhanced on fluorous stationary phases [46]. The separation of antibiotics was achieved on the alkyl perfluorinated stationary phase, with low FQs peaks were recorded. Fluorinated analytes retention on the fluorinated stationary phase is mainly influenced by the amount on fluorine atoms in the analyte molecule [46]. The number of fluorine atoms in the three FQs studied is one and the structures of the molecules are similar, therefore the retention of the analytes on fluorinated stationary phase is also similar. On the other hand the ESI signal of the analytes should be enhanced on the acidic conditions (see on Section 4.1.2). The MS chromatogram of the FQs and SAs separation had high noise level in extracted ion chromatograms for FQs, the peaks were broad and partly overlapping (Fig. 30). Neither better of the separation nor enhanced signal were obtained by optimization of the elution gradient nor the buffer composition or pH.

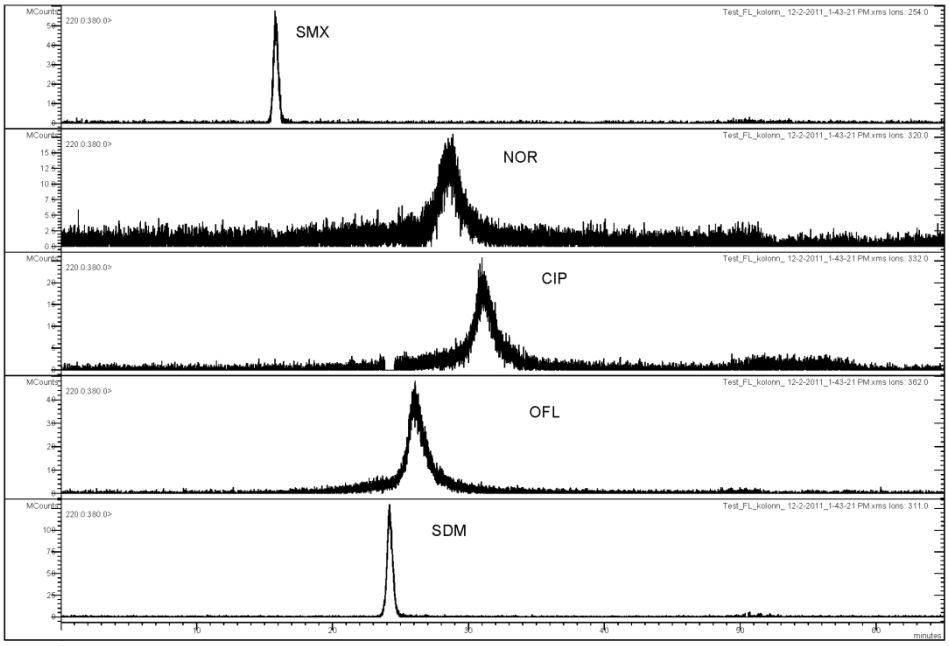


Figure 30. MS chromatogram of the standard solution of FQs' and SAs' (10 µg/mL). Eluent: AAF (pH 2.8) and MeOH. Used analytical column: Epic FO-LB C8 column (150 mm × 3 mm, 3.5 µm).

A successful chromatographic separation of five antibiotics in plant samples was demonstrated in paper V. The successful separation and better peak shapes of the antibiotics was achieved using either fluoroalcohols – HFIP or HFTB. For the LC analysis the 5 mM HFIP and gradient elution with methanol was used. Application demonstrates the successful separation of chosen compounds from the plant extract (Fig. 31).

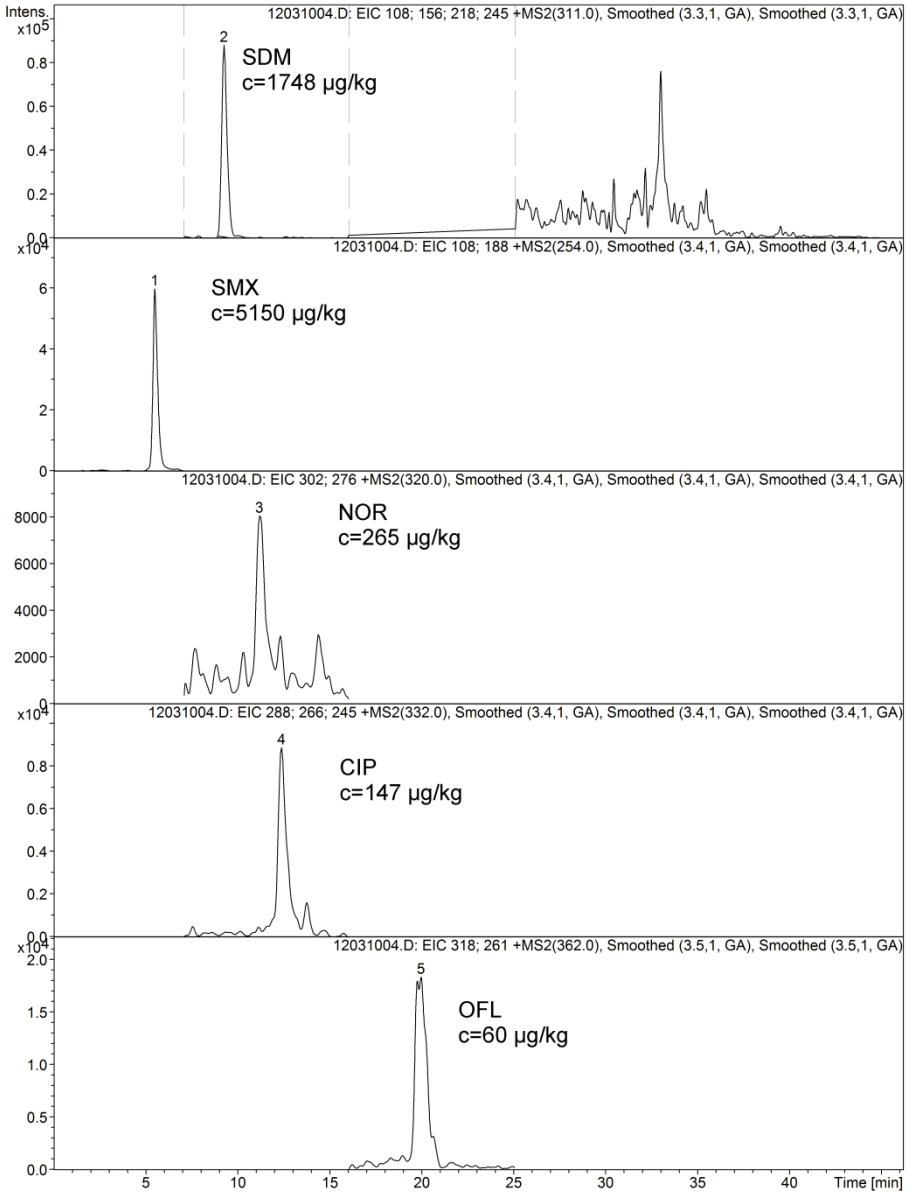


Figure 31. The chromatographic separation antibiotic residues in potato tubers, grown in sandy soil at the antibiotic soil concentration of 10 mg/kg. Eluent: 5 mM HFIP (pH 9) and MeOH. Used analytical column: Waters XBridge C18 column (150 mm × 3 mm, 3.5 µm).

Method validation for the plant samples

The method described above was validated for the simultaneous determination of CIP, NOR, OFL, SDM, and SMX from plants. For calibration antibiotics standard solutions were prepared in an eluent (5 mM HFIP and 10% methanol). The calibration graphs with the peak area versus the concentration were com-

posed in the concentration range 1–10,000 ng/mL and were linear with r^2>0.9998. Recovery was calculated from standard addition experiments. Recoveries for all detected pharmaceuticals in all matrices varied from 54 to 98%, the average recoveries are shown in Table 6.

Table 6. Average recoveries for the parts of the analyzed plants.

Average recoveries (n = 2)	
Sample matrix	Loamy soil
Carrot leaves	81%
Carrot roots	66%
Wheat leaves	93%
Wheat seeds	91%
Lettuce leaves	92%
Lettuce roots	93%
Potato tubers	87%

The difference in the sample preparation recoveries for the plants grown in two soils was not significant. The lowest recoveries were observed for the carrot root samples grown on loamy soil and this sample matrix was selected for the method validation (Fig. 32).

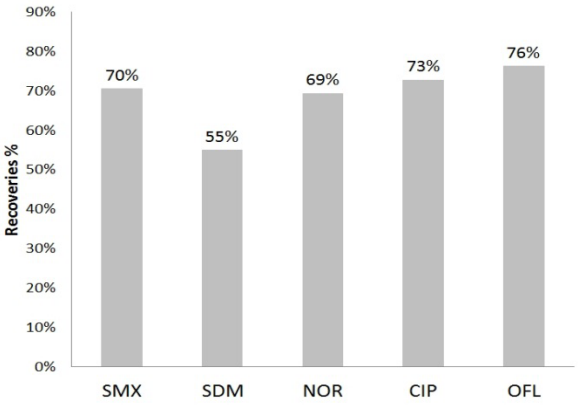


Figure 32. Sample preparation recoveries for the carrot root samples grown in loamy soil (n = 5).

Antibiotic recovery ranged from 55–76% for the carrot root samples with the standard deviation of 1% for SMX, SDM and CIP and 2% for NOR and OFL. The LOQ and LOD values were estimated, respectively, as 10 times and 3 times the standard deviation of five replicate analysis of unspiked and spiked plant samples. The LOQ values were as follows: CIP 0.11; NOR 0.16; OFL 0.02; SDM 0.07 and SMX 0.13 µg/g and LOD values CIP 0.03; NOR 0.05; OFL 0.007; SDM 0.02 and SMX 0.04 µg/g.

SUMMARY

The aim of the present work was to study the suitability and usage of two fluoroalcohols as volatile buffer compounds for HPLC-ESI-MS analysis.

HFIP and HFTB were evaluated as buffer components for basic mobile phases (pH 8.5 to 10) for RP chromatography with ESI-MS detection. Chromatographic separation and ESI ionization efficiency in positive and negative modes were evaluated for the selected five antibiotics using the novel buffer additives and in comparison to the commonly used buffers. The best chromatographic separation and most intense ESI-MS signals were obtained with buffers based on either HFIP or HFTB (5 mM of fluoroalcohol, pH adjusted to pH 9.0 with ammonium hydroxide) and methanol as the organic modifier.

Fluoroalcohols, HFIP and HFTB, can be used as buffer additives for basic LC-ESI-MS-compatible eluents suitable for the separation of acidic and basic compounds. HFIP and HFTB as buffer additives result in the complex RP retention behavior of analytes. All trends in the retention of the acidic and basic analytes can be interpreted by considering the following: hydrophobicity/hydrophilicity of the analytes in terms of their acid-base properties; the neutral fluoroalcohols are strongly retained by the stationary phase whereas their anions are less retained, thus their amount on the stationary phase is dependent on the mobile phase pH; the anions of the fluoroalcohols form ion pairs with protonated basic analytes in the mobile phase, thereby favoring their retention; the fluoroalcohols on the stationary phase surface compete with acidic analytes, thereby hindering their retention.

The retention of acids and bases on the C18 stationary phases with different hydrophobicities were evaluated and the dependence on the buffer compound and stationary phase properties were noted – the complex retention behavior of analytes caused provided by the usage of the fluorinated alcohol is dependent on the stationary phase because of the competition of fluorinated alcohols and analytes.

The retention of acids and bases on the fluorinated stationary phase was compared with the retention of the same analytes on the C18 stationary phase using fluorinated alcohols. Overall, the retention time on the fluorinated stationary phase was shorter than the retention on the C18 stationary phase. The alkyl perfluorinated stationary phase is noted to be useful for its ability to enable the successful separation of halogenated compounds. However, the successful separation of 3 FQs was achieved using HFIP and HFTB buffers and the C18 stationary phase. The similar structure (and content of fluorine atoms in the molecule) of FQs resulted in poor separation on the perfluorinated stationary phase. Neither was the separation satisfactory on the C18 stationary phase without the HFIP or HFTB buffer. Consequently, HFIP and HFTB buffers in the C18 column provide alternative selectivity compared to the perfluorinated and C18 stationary phase with common buffers.

The usefulness of HFIP and HFTB as buffer compounds for LC-ESI-MS analysis was demonstrated with several practical examples. The validated

methods of analysis of antibiotic (FQs and SAs) residues in plants and the successful separation of carbapenems in blood plasma and urine samples are presented.

The results of the study about fluoroalcohols demonstrate the benefits of their usage as LC-ESI-MS buffer compounds for basic buffer solutions. The alternative retention enables a higher selectivity of the chromatographic separation. The currently rather limited range of ESI-compatible buffer systems for basic mobile phases has been thus extended.

SUMMARY IN ESTONIAN

Fluoroalkoholid LC-ESI-MS eluendi komponendina: kasutatavus ja rakendused

Ravimite määramine vedelikkromatograafilisel (LC) massispektromeetrilisel (MS) meetodil kasutades elektropihustusionisatsiooni (ESI) leiab järjest laiemat kasutust. Ravimite pöördfaasvedelikkromatograafiliseks lahutamiseks kasutatakse sagedasti happelist puhverlahust, mis tähendab aga, et ravimid kui peamiselt aluselised ühendid on analüüsil protoneeritud ehk hüdrofiilsed. Hüdrofiilsetel ainetel aga on pöördfaaskromatograafias nõrk retentsioon. Aluseliste ühendite vedelikkromatograafiliseks lahutamiseks oleks parem kasutada aluselist keskkonda.

LC-MS seab kasutatavatele eluentidele lenduvuse nõude, mis omakorda vähendab kasutatavate aluseliste puhverlahuste valikut. Teine oluline omadus, mida peab LC-ESI-MS puhverlahuse valimisel silmas pidama, on analüütide ionisatsioon – puhverlahuse komponendid ei tohi analüüdi ionisatsiooni maha suruda.

Üheks paljulubavaks ainete klassiks, mis võimaldaks aluseliste puhverlahuste valikut suurendada, on fluoroalkoholid. Lenduvate aluseliste puhverlahuste valiku laiendamiseks uuriti fluoroalkoholide – 1,1,1,3,3,3-heksfluoro-2-propanooli (HFIP) ja 1,1,1,3,3,3-heksafluoro-2-metüül-2-propanooli (HFTB) kasutusvõimalusi LC-MS aluseliste puhverlahustena.

HFIP ja HFTB kui nõrkade hapete omadusi hinnati aluseliste puhverlahuste kasutamiseks pöördfaaskromatograafiliseks analüüsiks, kui detektorina on kasutusel MS. Töö käigus uuriti viie antibiootikumi pöördfaasvedelikkromatograafilist lahutust ning ioniseerumist nii positiivses kui ka negatiivses režiimis, kui HFIP ja HFTB on kasutusel LC-ESI-MSi eluendi komponendina ning saadud tulemusi võrreldi traditsiooniliste eluentidega. Mõlemad fluoroalkoholid aitasid kaasa analüütide ioniseerumisele ning ainetele, mis tavapäraste eluentidega kromatograafiliselt ei lahutunud, saavutati suurepärane lahutus.

HFIP ja HFTB kasutamisel pöördfaaskromatograafiliseks analüütide lahutamiseks on ainete retentsioonimehhanismid keerukad, kuid just see võimaldabki ainetele alternatiivset LC eraldust. Protoneeritud fluoroalkoholid kinnituvad tugevalt statsionaarse faasi pinnale, samas kui deprotoneeritult on nende retentsioon tunduvalt nõrgem. Deprotoneeritud fluoroalkoholid on võimelised moodustama ioonpaare protoneeritud aluseliste analüütidega, mis omakorda suurendab ioonpaardunud analüütide retentsiooni. Üldiselt vähendavad fluoroalkoholid happeliste ühendite retentsiooni konkureerides analüütidega statsionaarse faasi pinna pärast ning suurendavad aluste retentsiooni, kuna on võimelised moodustama ioonpaare. Kuna konkurentsi- ja ioonpaarmehhanismid toimivad samaaegselt, pakuvad fluoroalkoholid alternatiivseid analüütide lahutusvõimalusi.

Fluoroalkoholide kasutamist võrreldi ka erinevate hüdrofoobsustega C18 statsionaarsete faasidega. Analüütide retentsioon sõltub suuresti ka statsionaarsest faasist, kuna hüdrofoobsemal statsionaarse faasi pinnal on ka tugevam analüüdi ning puhvri komponendi konkurents.

Fluoreeritud statsionaarse faasi kasutamine on üheks alternatiiviks parandamaks analüütide retentsiooni. Fluoreeritud statsionaarse faasi kasutamisega vähenesid kõikide analüütide retentsiooniajad võrreldes tavapärase C18 statsionaarse faasi kasutamisega. Fluoreeritud statsionaarsed faasid omavad teadaolevalt alternatiivset analüütide lahutamisvõimalust, kuna halogeeniaatomeid sisaldavad analüüdid kinnituvad tugevamalt statsionaarse faasi pinnale. Fluorokinoloonide jaoks saavutati edukas lahutus HFIPi ning HFTBga, kui statsionaarse faasina oli kasutusel C18. Fluoreeritud statsionaarne faas ei võimaldanud kõiki antud ühendeid lahutada, samuti ei võimaldanud seda tavapärane C18 kolonn ilma fluoroalkohole kasutamata. Seega pakuvad HFIP ja HFTB alternatiivset selektiivsust C18 statsionaarsele faasile analüütide lahutamiseks levinud puhverlahustega ning fluoreeritud statsionaarse faasiga.

HFIP ja HFTB kasutusvõimalusi LC-ESI-MS analüüsil demonstreeriti mitme praktilise näite varal ja välja töötatud metoodikad valideeriti – fluorokinoloonide ja sulfoonamiidide jääke määrati taimeproovidest, karbapeneeme määrati inimese verest ning uriinist.

Fluoroalkoholide LC-ESI-MS puhverlahuste komponentidena aluseliste puhverlahuste valmistamisel kasutamine omab mitmeid väljatoodud eeliseid. Fluoroalkoholid pakuvad alternatiivset retentsioonimehhanismi analüütide paremaks lahutamiseks. Käesoleva tööga laiendati edukalt LC-ESI-MS aluseliste puhverlahuste valikut.

ACKNOWLEDGEMENTS

I would like to thank the University of Tartu and the Institute of Chemistry and the Institute of Pharmacology for extensive and great education and the knowledge shared during my studies. First and foremost, I would like to express my sincere gratitude to my supervisor, Associate Professor Koit Herodes for his professional guidance, support and patience through my studies.

I am most grateful to my mother, sister and to Erkki for their patience, love and support during my studies.

I would like to thank the people whom I have been honored to work with, particularly Professor Ivo Leito, but also Kaili, Kersti, Lauri, Irja, Anna, Merit, Elin, Asko, Ester, Marju, Martin and Allan for providing a friendly and educative working atmosphere.

I would like to thank my student Kristi Hüüdma for her enthusiasm and preciseness in her experiments and for long discussions on chemical topics.

I am grateful to my friends for the support, inspiration and motivation in my days – Riin, Anneli, Malle, Maria, Triinu, Kristin, Laura, Kertu, Kerstin, Inna, Anneli, Krista, Maarja, Kadri, Kairi, Taavi, Michal, Janne.

For financial support, I would like to thank the Estonian Science Foundation (the grants No 8572, 7127, 8799), the Estonian Environmental Investment Centre, the Ministry of Education and Science of Estonia (the Target Financing Projects No SF0180061s08 and SF018004s12), the Graduate School on Functional Materials and Technologies (GSFMT) (project 1.2.0401.09-0079) and the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement number 242146. Financial support from the Archimedes Foundation (Kristjan Jaak and DoRa scholarships) is greatly acknowledged.

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Education

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2002–2005	University of Tartu, Bachelor of Sciences (chemistry)

Professional employment

August 2007–…	University of Tartu, Institute of Chemistry, chemist
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Specialized training

Waters LC/MS training course "Mass Spectrometry", 2008, Helsinki, Finland
Measurement Science in Chemistry Summer School 2008, Celje, Slovenia
HPLC column selection and method development. 2008, Tartu, Estonia
Pharmacokinetic/Pharmacodynamic Trials in Clinical Medicine, 1–3 June, 2011 Tartu, Estonia.

Scientific Publications

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Haridus

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Fundamentals of LC-MS. Firma Waters seminar, 2008, Helsingi, Soome.
HPLC column selection and method development. 2008, Tartu, Eesti. Lektorid: Liisa Kanner ja Esa Lehtorinne.
Farmakokineetilised ja farmakodünaamilised uuringud kliinilises meditsiinis, 1.–3. juuni 2011, Tartu, Eesti. Lektorid: Irja Lutsar, Alar Irs, Tuuli Metsvaht, Aleksander Žarkovski, Kersti Oselin, Juri Karjagin, Lenne-Triin Kõrgvee, Jyrki Heinämäki, Joseph Standing
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