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Characterization of ionic interactions of different stationary phases and their comparison in liquid chromatography

Charakterizace iontových interakcí různých stacionárních fází a jejich porovnání v kapalinové chromatografii

Diploma thesis

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Statement

I hereby state that I have completed this thesis by myself and that I have properly cited all literature and other information sources I have used. Neither this thesis nor its parts have been submitted to achieve any other academic title(s).

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Abstract

This work is focused on mixed mode chromatography, a characterisation of 2 mixed mode columns in particular - Acclaim^{TM} WAX-1 and Acclaim^{TM} WCX-1, both of these stationary phases yielding a combination of reversed phase and ion exchange separation mechanisms. The characterisation was carried out thanks to two newly developed buffer mixing system optimalizations, that can be universally used on the high performance liquid chromatography instrument. While the first column introduces a protonable secondary amine group to the separation process, the latter contains a weak carboxylic group also charged in a range of pH, bearing an opposite charge. The characterisation process was done by measuring retention of analytes of various polarity and charge, in a defined pH range (2.5 – 7.5 in 0.5 steps).

The behaviour of the stationary phases was proven to change with a shift in mobile phase pH, as it affected the dissociation/protonation of the ion exchange groups present in the column. The WAX-1 column starts to lose its positive charge above approximately the pH of 5 while the WCX-1 column appears to gain a negative charge near and above a pH of 3. This was determined thanks to data evaluation from graphs where retention time dependences on pH were plotted for various analytes. While the retention of neutral compounds remained the same, the acid-base equilibrium of weak electrolytes was affected by the change in pH and proven to give their dissociation constants obtained from appropriate data fitting. For aniline, the WAX-1 column with a positive charge showed a greater approximation, while for benzoic, 4-hydroxybenzoic and nicotinic acids WCX-1 column with a negative charge showed a better estimate. These estimates were further compared to those obtained by a pH gradient p*K*a determination technique, using the same, previously developed buffer mixing systems.

Keywords

ionic interactions, stationary phase, mixed mode, high performance liquid chromatography

Abstrakt

Předkládaná diplomová práce je zaměřena na mix mód chromatografii, konkrétně na podrobný popis dvou kolon, které vykazují smíšený interakční mechanizmus - Acclaim^{TM} WAX-1 and Acclaim^{TM} WCX-1. Stacionární fáze těchto kolon poskytují kombinaci reverzního a iontově výměnného interakčního/retenčního mechanizmu. Podrobná charakterizace iontových interakcí poskytovaných stacionárními fázemi byla umožněna na základě vývoje dvou nových metod mísení pufrů o různé hodnotě pH přímo v chromatografickém přístroji. Nově vyvinuté metody mohou být univerzálně využity na podobná měření. Zatímco kolona WAX-1 poskytuje protonovatelnou sekundární aminoskupinu, kolona WCX-1 obsahuje karboxylovou skupinu, která je nositelkou opačného náboje v určitém rozmezí pH. Celá charakterizace byla provedena měřením retence analytů o různé polaritě a náboji v definovaném rozsahu pH (2,5 – 7,5 v intervalech po 0,5 jednotce pH).

Výsledky prokázaly, že chování stacionárních fází se mění se změnou pH mobilní fáze, což je způsobeno disociací/protonizací iontově výměnné funkční skupiny stacionární fáze. Kolona WAX-1 ztrácí svůj pozitivní náboj nad pH 5 a kolona WCX-1 získává záporný náboj kolem pH 3. Tyto závěry byly získány z grafů závislostí retenčního času na pH pro rozličné analyty. Zatímco retence neutrálních analytů se neměnila se změnou pH, acidobazická rovnováha slabých elektrolytů byla změnou pH mobilní fáze ovlivněna. Vhodným proložením dat závislostí retenčního faktoru na pH mobilní fáze byly zjištěny hodnoty disociačních konstant. Kolona WAX-1 s kladně nabitou skupinou ukázala nejlepší odhad pro anilin, zatímco pro benzoovou, 4-hydroxybenzoovou a nikotinovou kyselinu poskytovalo přesnější hodnotu měření na WCX-1 koloně se záporným nábojem. Experimentálně zjištěné hodnoty p*K*a byly dále porovnány s hodnotami získanými metodou pH gradientu, za použití vyvinuté metody mísení pufrů.

Klíčová slova

iontové interakce, stacionární fáze, mix-mód vysokoúčinná kapalinová chromatografie

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CONTENTS

List of abbreviations and symbols

α	selectivity
β	buffering capacity
δ	organic and aqueous phase mixture's empirical value for pH measurements
ε	solvent's dielectric constant
η	solvent viscosity
λ	wavelength
Φ	organic solvent ratio
a	pH gradient steepness
Ac	acetic acid
b	elution strength steepness
BTMA	benzyltrimethylammonium ion
$c_{\text{n-octanol}}$	analyte concentration in n-octanol phase
c_{water}	analyte concentration in the water phase
C18	octadecyl group
DCHO	dichloroacetic acid
f	width of the peaks' ascending part in 5 % of its height
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
IE	ion exchange
k	retention factor
K_a	dissociation constant
k_{A}-	retention factor of an anionic form
k_{HA}	retention factor of a neutral form
K_{w}	autoprotolysis constant of water
k_{w}	retention factor in 100 % aqueous phase
log P	logarithm of partition coefficient
log D	logarithm of distribution coefficient
MeOH	methanol
MMC	mixed mode chromatography
MS	mass spectrometry
MP	mobile phase
NP	normal phase
P'	solvent polarity parameter

PEA	2-phenylethylamine
pH**	pH of the mobile phase at elution
${}^{\mathrm{s}}_{\mathrm{s}}pH$	pH on the absolute scale
${}^{\mathrm{s}}_{\mathrm{w}}pH$	pH measured using calibration standards of the same mixed organic solvent as mobile phase
${}^{\mathrm{w}}_{\mathrm{w}}pH$	true pH value
pI	isoelectric point
pKa	negative logarithm of dissociation constant
R	resolution
R^2	determination coefficient
RP	reversed phase
RP-IE	reversed phase – ion exchange
S	solvent strength parameter
SP	stationary phase
T	symmetry factor
t_{A}-	retention time of an anionic form
t_{B}	retention time of an undissociated base
t_{D}	dwell time
t_{HA}	retention time of an undissociated acid
t_{G}	gradient duration
t_{g}	retention time from a gradient run
t_{HB}+	retention time of a protonated base
t_{M}	dead time
TMFA	trimethylphenylammonium ion
t_{neutral}	retention time of analyte's neutral form
t_{R}	retention time
t_{R}'	reduced retention time
TriEtAm	triethanolamine
(v/v)	volume per volume ratio
w	peak width measured at its base
$w_{0,05}$	peak width at 5 % of its height
WAX	weak anion exchange
WCX	weak cation exchange
z_{A}^-	charge of the anionic form
z_{HA}	charge of the undissociated from

1 Introduction

Mixed mode chromatography has been gaining wide popularity in the field of high performance liquid chromatography, thanks to its potential for complex mixture separation. It combines two or more chromatographic modes in a single column and therefore employs multiple separation mechanisms in a single run. The elucidation of interactions taking place in the separation can greatly broaden a stationary phase's utilisation. To investigate the range of interactions in such column, analytes of various polarities can be chosen as markers and undergo the chromatographical process.

The aim of this work was a new buffer mixing system development, for characterisation of two types of stationary phases, both including reversed phase mode, with hydrophobic interactions, and ion exchange, yielding coulombic interactions. Both columns contained a weak ionisable ligand, charged only in a certain area of pH, but of different character and opposite charge.

The characterisation was done by retention time measurements of numerous markers at the pH range permissible for the column. The new buffer mixing system, consisting of two aqueous solutions, was designed to go through this range efficiently. The respective data were obtained, illustrating the shift in charge with certain pH, for not only the stationary phase, but also the ionisable analytes. In a case of few weak acids or bases, the retention factor as a function of pH was fitted by a sigmoid, with its inflex point giving the marker's approximate p*K*a value. Another quicker technique was employed with a linear pH gradient generated from the previously introduced buffer system and a reversed phase column, for p*K*a determination of these analytes.

2 Theoretical part

2.1 High performance liquid chromatography

High performance liquid chromatography (HPLC) is a powerful separation technique, with applications in the fields of drug discovery, agriculture, bioanalysis and many more[1]. The mechanism of separation can be explained as a difference in distribution of different analytes between a **mobile phase (MP)** and a **stationary phase (SP)**. The distribution is based on various physical properties of these analytes and is taking place while an analyte, dissolved in the mobile phase, flows through a column, usually a stainless steel tube, that is packed with particulate material which represents the stationary phase[2]. The separation process results in a chromatogram with peaks, reflecting samples, with a certain peaks height, width and shape. Based on this result, a sample can be quantified, separated from a mixture or even characterised by a certain property. The main goal is to obtain the best resolution in a separation, in the shortest time possible[3]. The key information that is directly gained from the chromatogram is the **retention time t_R** of a given analyte. It is the time it takes for the analyte to elute.

Based on the type of prevalent separation mechanism, the chromatography can be divided into chromatographic modes. The choice between the various chromatographic modes comes from the type of analytes that we are interested to analyse or separate. The mode which stands out as the most universally used is **reversed phase (RP) chromatography** for analytes of low to medium polarity with stationary phases being less polar than the mobile phase. **Normal phase (NP) chromatography** historically predates RP and uses a polar stationary phase and an organic mobile phase. In HPLC, **hydrophilic interaction liquid chromatography (HILIC)** is utilised for polar analytes. It combines a polar stationary phase with mobile phase mixtures of organic and aqueous components. For mixtures of charged analytes, **ion exchange (IE) chromatography** is commonly opted for, using electrostatic interactions between the charged analytes and immobilised charged groups.

Ion pair chromatography is a technique involving addition of special reagents to the mobile phase that are capable of increasing retention of charged analytes in RP mode. It is made possible by their amphiphilic character, interacting with both the SP and the charged analyte, creating somewhat of a “bridge” in between, that increases retention[4]. A mode that combines two or more separation mechanisms is regarded as **mixed mode chromatography (MMC)**[5].

2.1.1 Important concepts and formulas

In the following sections, a few chromatographic parameters will be repeatedly mentioned:

The retention time t_R reflects the extent of interactions taking place between the analyte, SP and MP. As columns come in various dimensions, a different quantity is preferred, called the **retention factor *k***. It serves as a normalisation to compare all columns between each other, no matter the column. It is given by the formula (1):

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

where t_R is the retention time of a given analyte and t_M shows a new quantity called dead time. It is the retention time of an analyte which does not interact with the stationary phase and therefore is not retained in the chromatographic system.

The expression in the numerator formula (2) is denoted as the **reduced retention time** t_R':

$$t_R' = t_R - t_M \quad (2)$$

Peak resolution *R* between two theoretical analytes 1 and 2, which elute in this order, can be calculated by the formula (3):

$$R = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2} \quad (3)$$

where t_{R1} a t_{R2} are the respective retention times of these analytes and w_1 a w_2 are the widths of their peaks labelled as 1 a 2, taken from their base, in time units.

Selectivity α is regarded as the ability to separate analytes 1 and 2 from each other, defined by formula (4):

$$\alpha = \frac{k_2}{k_1} \quad (4)$$

where k_1 and k_2 are the retention factors of analytes 1 and 2.

Symmetry factor *T* describes a peak's symmetry and its formula is the following (5):

$$T = \frac{w_{0,05}}{2f} \quad (5)$$

where $w_{0,05}$ is the peak width in 5 % of its height and f is the width of the peak in its ascending part in 5 % of its height.

2.1.2 Theories of liquid chromatography

The origin of the HPLC concept can be traced to the 40's, when a series of publications arose, discussing the possible benefits of using small particles and high pressure to achieve optimal efficiency and therefore also resolution in liquid chromatography. The use of small particles of uniform sizes, with large surfaces was established as the optimal stationary phase material for acquiring the narrowest peaks[3]. HPLC technique refinement only further proceeded from this revolutionary breakthrough.

Possible types of equilibria taking place during a chromatographic separation are partition, adsorption, equilibria involving ions and more. The elucidation of these mechanisms for each mode of HPLC is not straightforward, as more than one can be taking place during a separation[4]. Moreover, thermodynamically based theories of chromatography are limited to cases when the distribution processes between the MP and SP are rapid, which is not always attainable. This is where the various kinetic theories come in handy. A solution of a series of differential equations taking longitudinal diffusion, eddy dispersion and the mass-transfer resistances into account, can be effective in the description of the separation process involving elution zone broadening. The narrower peaks are always desired, as this can also drastically improve peak resolution. On the other hand, the analyte's retention time only depends on the equilibrium between the two phases[6]. With massive advancements in numerical methods and drug discovery of the last decades, it would seem that the field of mechanistic modelling would be widely used with elution prediction in pharmaceutical industry – liquid chromatography's widest application field. This is not the case however, due to complication with input model values regarding the column and kinetic binding parameters of analytes. Due to this, conventional optimalization and development processes are many times inevitable, such as empirical temperature, mobile phase and eluent velocity modifications[7].

2.2 Stationary phase

The versatility of HPLC applications stems from the wide variety of materials used as stationary phases. The development in column packing technology, particle size and particle coatings revolutionised the high performance arrangement as it's utilised in modern practise[8].

The most commonly used material nowadays is silica. The controllable pore size that introduces large surface area and also high resilience makes it a perfect material for HPLC purposes. Its physical properties, such as pore size, shape and volume directly affect the analyte's retention process. These properties can be adjusted by a number of parameters, such as the choice of reagent, temperature or pH involved in the material's preparation. Because of its polar surface, silica initially served purpose as a stationary phase in NP chromatography. However, another great feature that enables its usage in other chromatographic modes, is the ability of surface modification. The manner in which this is achieved, is oftentimes chemical derivatization with functional groups, which represent the active sites[8].

Silica used in HPLC was initially manufactured from amorphous silicon dioxide from natural sources, followed by a subsequent sol-gel process. This often resulted in contamination by metallic ion impurities, making it more acidic than the later, high purity silica prepared by hydrolysis and polycondensation of tetraethoxysilane. The latter has been proven as a better alternative because of many advantages, such as better elution zone shapes of basic analytes, since the former caused significant peak tailing as a result of bases interacting with acidic groups on the material's surface. Both options however hold a drawback of smaller pH range of usage (approximately 3-7), as silica dissolution happens at higher pH and acidic hydrolysis of chemically bonded alkyl chains at a pH that is too low (lower than 3)[9]. These limitations led to the discovery of more durable hybrid stationary phases.

First generation of hybrid stationary phases were introduced at the brink of millennium, with the aim of achieving better mechanical stability and wider range of usable pH. The key reaction in its preparation is the reaction of tetraethoxysilane with methyltriethoxysilane. This results in obtaining favourable properties from both the inorganic and organic components, such as pH range of usage being 1-12 units and further improvement of basic analyte peaks thanks to low residual silanol content[10].

2.2.1 Stationary phases for RP chromatography

As a stationary phase material in reversed phase chromatography, silica is commonly used in a modified form. But it serves rather as a carrier particle for bonded ligands, most often hydrocarbon chains, which in this mode, add the most important separation mechanism[11]. The most popular chains are 18 carbons long and are therefore appropriately regarded as C18 columns. Other types of ligands used in RP are phenyl-

based, that contribute to separation via other supramolecular interactions, such as π-π stacking in case of separating aromatic ligands. This provides an alternative selectivity to classical C18 columns[12].

The primary mechanism involves hydrophobic interactions between the analytes and the ligands of the RP stationary phase, while secondary interactions come from the silica particulates. The more hydrophobic, often times longer bonded alkyl group, the longer the retention of hydrophobic substances[13]. The hydrophobic chain or group can be bound to a carrier mono- di- or tri- functionally (Figure 1), based on the reactant and also the carrier's surface[11]. It is quite obvious that the bulkier the chain is, the more silanol groups on the surface are left underivatized, increasing its overall acidity.

The acidic contributions from dissociated hydroxyl groups on the silica surface are appropriately called silanol interactions and they bring their fair share of contributions to the separation mechanism. The silanol groups are present in multiple functional groups with different p*K*a values. Above the pH of 7, all of them are dissociated (Figure 1), therefore they carry a negative charge that is involved in repulsive or attractive coulombic interactions with any charged species in the mobile phase[14]. The basic analytes are the most troublesome, as this exchange results in peak tailing. Multiple approaches have been practised to prevent this, such as the addition of amines to the MP[15].

An approach called endcapping is commonly utilized, which uses shorter alkyl groups that are easier penetrable to silica surface (Figure 1)[8].

However, the silanol interaction can be beneficial in a few cases, as they add a certain polarity to the stationary phase which can prevent a hydrophobic collapse of the stationary phase. This can happen when alkyl chains are exposed to high contents of water in the mobile phase which results in their collapse[16].

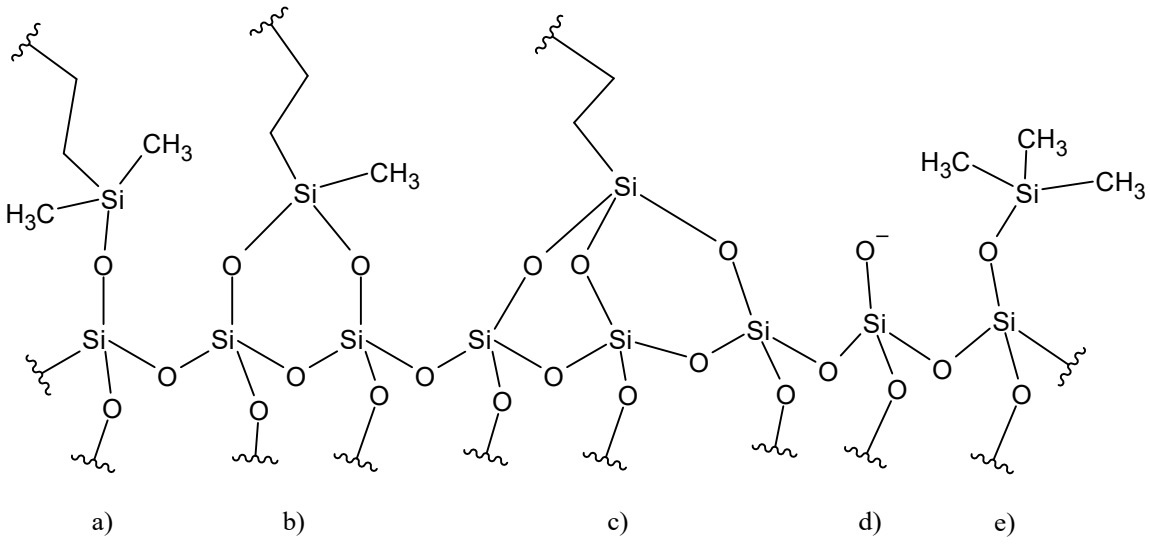


Figure 1: The possible surface modifications: a) monofunctional b) bifunctional c) trifunctional d) unmodified and dissociated silanol group e) endcapping group

2.2.2 Stationary phases for RP-IE chromatography

Mixed mode is a relatively newly used term in the chromatography world, even though multimodal chromatography is nothing new, if we conclude the already mentioned secondary silanol interactions in reversed phase chromatography[17]. There are many combinations of modes, that can be combined in a single column, for example reversed phase liquid chromatography with ion exchange (RPLC-IE), hydrophilic interaction liquid chromatography with ion exchange (HILIC-IE) and hydrophilic interaction liquid chromatography and reversed phase liquid chromatography (HILIC-RPLC)[5].

The idea behind RP-IE, combining both hydrophobic and coulombic interactions, came as a response to poor retention of acidic or basic analytes in RPLC. This is the go to approach, when separating mixtures of a wide spectrum of polarity due to high and unique selectivity of these stationary phases – the ability to separate 2 analytes from each other[18]. Another advantage of a MMC column in general, is the benefit of using only one stationary phase for a separation and therefore reducing waste[19]. The alternatives to RP-IE can be ion pairing chromatography in RP or using IE, however these techniques do not guarantee compatibility with often required mass spectroscopy (MS) detection, because of high concentrations of salt in the mobile phase, which can contaminate the source of ionisation[20,21]. For mixed mode, a lower concentration of salts is enough in comparison[22]. Utilisation of HILIC that is meant for

polar analytes is another alternative, for which the complex separation mechanism is still being researched[23].

While free silanol groups are viewed mostly undesired in RP mode because of their uncertain location and acid-base behaviour, with mixed mode, identical charged groups are evenly distributed alongside the bonded hydrophobic chains. The possible placement of the charge bearing group and its strength varies from stationary phase type[1] (Figure 2). What the classification based on strength reflects, is the difference between the ranges where the groups bear a positive or negative change. A strong mixed mode stationary phase is positively or negatively charged through the entire pH range possible to use, while a weak one only dissociates besides a certain value. This value is only dependent on the p*K*a of this moiety. Manufacturing details such as these are often times undisclosed for commercially sold stationary phases, therefore characterisation tests can be beneficial in describing a column's behaviour during separation. Moreover, the understanding of the separation mechanism can expand the possible application of the studied stationary phase.

Furthermore, previous studies determined that using acetonitrile as a modifier, ion exchange mechanism tends to prevail at a high percentage of this organic modifier, while reversed phase based separation dominates when acetonitrile volume ratio gets low[20].

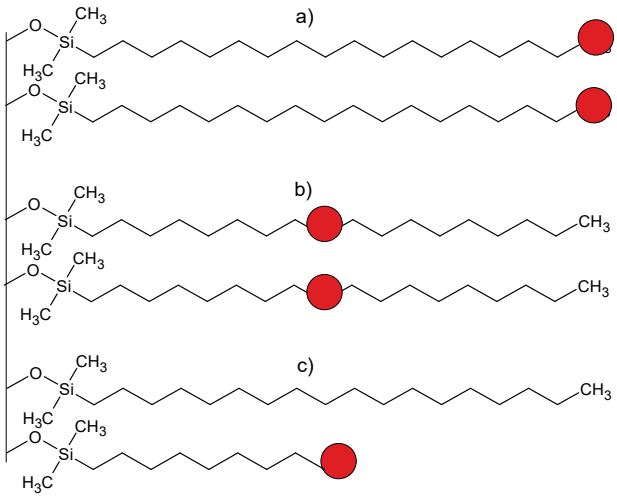


Figure 2: Scheme of most commonly used types of mixed mode stationary phase design a) – c) where the red circle symbolises the charged ligand´s placement[1]

2.2.3 Stationary phases used in this work

Acclaim™ mixed mode WAX-1 column

Mixed mode column combining reversed phase and weak anion exchange mechanisms. The anion exchange ligand is a tipped (type a from Fig. 2) secondary amine. The stationary phase is stable between the pH of 2.5 to 7.5. The p*K*a value of the anion exchange group is unknown. The schematic structure of the stationary phase is shown in Figure 3.

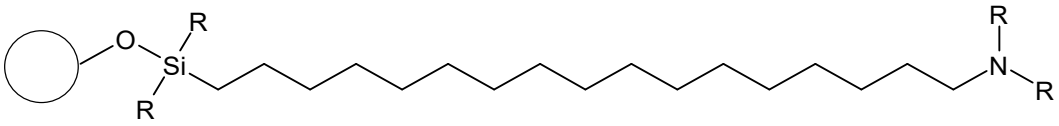


Figure 3: Scheme of the stationary phase of Acclaim™ mixed mode WAX-1 column

Acclaim™ mixed mode WCX-1 column

Mixed mode column combining reversed phase and weak cation exchange mechanisms. The cation exchange ligand is a tipped (type a from Fig. 2) carboxylic group. The stationary phase is stable between the pH of 2.5 to 7.5. The p*K*a value of the cation exchange group is unknown. The schematic structure of the stationary phase is shown in Figure 3.

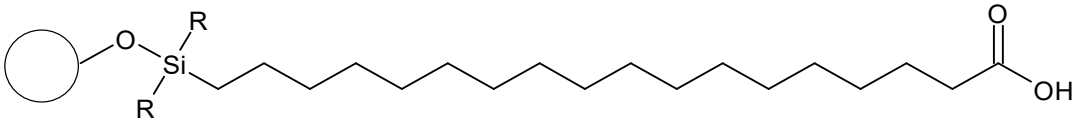


Figure 4: Scheme of the stationary phase of Acclaim™ mixed mode WCX-1 column

XTerra® MS C18

General purpose stationary phase, containing octadecyl groups bonded to a hybrid particle by tri- functional silane (see Figure 5). The stationary phase is endcapped. Thanks to its hybrid core, the stationary phase is stable to use in the pH range 1 – 12.

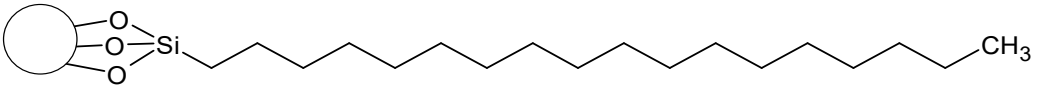


Figure 5: Scheme of the stationary phase of XTerra MS C18 column

2.3 Mobile phase

Solvents and their mixtures used as mobile phases in liquid chromatography can be classified by many physical properties. Based on the liquid chromatography mode used, the most relevant are those that directly affect the separation process. While a pure solvent's properties are easily obtainable from literature, some approximations or experiments have to be made for mixtures, which are often used in chromatography[24]. Miscibility of the mobile phase components, solvent purity and dissolution of analytes are therefore just the hallmark of conditions that have to be satisfied.

The viscosity of the mobile phase η defines the column backpressure that limits the maximum flow rate. As a result of this, a viscous solution decreases analyte's diffusion, slowing down mass transfer in the process. The desired value is therefore as low as possible[25]. Dielectric constant ε is a physical property, that measures the efficiency of a solvent to encourage dissociations of electrolytes present in the solution. Higher values mean complete dissociation, while low values promote ion pairing to occur[26]. Solvents can be ranked by this constant on a scale with respect to the vacuum permittivity value[24]. The polarity of a solvent can be expressed via the polarity scale *P*' which takes solute-solvent interaction into account. The classification of polarity of a solvent is done by measuring its interactions with three test solutes – ethanol, dioxane and nitromethane. Larger *P*' values indicate a more polar solvent[27].

All of the previously mentioned properties depend on temperature therefore they can be significantly affected by its changes. Higher temperature usually results in lower retention and narrower elution zones which is most of the times the desirable outcome[28].

It is also appropriate to mention the role of mobile phase in detection, that does not affect the separation itself, but plays an integral role. The compatibility of mobile phase with the chosen form of detection must be assured. When using UV detection, which is one of the most commonly used methods of detection, the mobile phase should have a low UV cutoff[25]. In other words, it should not absorb significantly in the near UV region where the analysis is taking place[24]. Mass spectrometry (MS) is another widely used technique of detection that enables reliable identification and quantitative determination of compounds[21]. Limitations are the use of mobile phases, as mentioned previously, that contain high concentrations of salts which worsen the signal to noise ratio or non-volatile buffer compounds[29].

2.3.1 Mobile phases for RP and RP-IE chromatography

Based on elution strength, which describes the solvent's ability to elute analytes from the column, it is appropriate to arrange the most commonly used components of mobile phases in RP and also RP-IE in the following sequence, according to the decrease in their polarity[25]:

water < methanol < acetonitrile

From this progression, it becomes clear that in order to achieve optimal retention of most substances, while meeting the condition of high resolution and the shortest analysis time possible, it is advantageous to use mixtures of solvents. These are mainly binary mixtures, with different ratios of organic and aqueous components. Furthermore, elevated temperature causes an increase in the solvent's eluotropic strength[30].

A mobile phase with a purely aqueous component is rarely chosen. This approach is usually applied to the separation of strongly polar substances[31], for which sufficient retention is achieved only with the use of a mobile phase with a low elution strength or, for example, analytes sensitive to the organic environment. The compatibility of the purely aqueous mobile phase and the hydrophobic reversed stationary phase is not always guaranteed, and must be taken with caution, in order to avoid the so-called hydrophobic collapse of the stationary phase[16].

2.3.2 Buffer systems

A solution of weak acid (base) and its conjugated base (acid) has the ability to resist pH fluctuations upon the addition of acidic or basic species. By their buffering ability, they are appropriately called buffer solutions[24].

The reproducibility of results in HPLC is essential. A complex equilibrium is created between the components of the mobile phase, the analyte and the stationary phase[32]. To ensure fixed pH values of the mobile phase and therefore reproducible results, a buffer mobile phase system is a necessity[33].

The capability of a buffer to suppress pH changes can be described by buffering capacity β, which depends on the pKa value of the buffer components, is proportional to their concentration ratios, as well as the pH value of the entire solution. The higher the buffering capacity is, the better the buffer suppresses pH changes. The maximum of this quantity is reached at a pH value corresponding to the pKa of weak acid or base used[33].

In HPLC, the lowest possible concentrations of buffer components are preferred, even though it means lower β. The reason for that being is to avoid possible

precipitation of its components when using organic phase modifiers[24]. The ionic strength of the solution should therefore be approximately in the range of 10^{-3} - 10^{-4} mol/dm^3. It must be taken into account that pH, as well as ionic strength, change the nature of the stationary phase. This is mostly a concern in ion exchange or mixed mode chromatography, where ions of mobile phase interact with charged groups present on the stationary phase, e.g. silica[31].

2.3.3 pH calculations

When choosing a pH value of the aqueous phase, it is necessary to ensure the compatibility of the stationary phase with this value. This depends on the preparation, character, and method of establishing functional groups into the stationary phase, discussed earlier[34].

For the preparation of buffering solutions of certain pH, consisting of multiple components, an algorithm for the pH value estimation can be utilised. The principle stems from the law of electroneutrality, which states that in any solution, every ion must be counterbalanced by an ion or ions of opposite charge[35].

When considering only acid-base equilibria, concentration of each ion present in the solution is a function of pH. The sum of concentrations of ionogenic species as a function of pH is a polynomial function of various order, according to the number of acidic or basic components in the solution. The pH of such a solution is therefore calculated for the case of the polynomial value being zero, when electroneutrality is achieved. The value of pH for polynomials of higher order has to be calculated numerically. The input parameters necessary are the ionic charges, concentrations of the acids/bases in solution and their respective dissociation constants. The derived equations for the calculation of pH of a monoprotic acid (6) is therefore as follows[35]:

$$[H_3O^+] + [HA]\, z_{A^-} \frac{K_a}{K_a + [H_3O^+]} + [HA]\, z_{HA} \frac{[H_3O^+]}{K_a + [H_3O^+]} - \frac{K_w}{[H_3O^+]} = 0 \quad (6)$$

where $[H_3O^+]$ is the relative concentration of hydronium ions, $[HA]$ is the relative overall concentration of the acid, z_{A^-} is the charge of the dissociated form, z_{HA} is the charge of the non- dissociated form (which equals zero), K_a is the acid's dissociation constant and K_w the autoprotolysis constant of water at 25 °C.

2.3.4 Correction of pH values with organic modifiers

In aqueous solutions at a temperature of 25 °C, a value of 7 is considered a neutral pH, because under these conditions, the equality of relative concentrations $[H_3O^+]=[OH^-]=10^{-7}$ is achieved. This assumption does not apply when there is a significant ratio of organic modifier present in the solution. For this component, the autoprotolysis constant might be significantly different from that of water *K*w = $[H_3O^+]\cdot[OH^-]=10^{-14}$. Therefore, in mixtures of aqueous and organic constituents, the value of neutral pH ranges above or below the value of 7, based on the value of the autoproteolysis constant of this organic component. Besides this, the behaviour of every ionic species present in solutions is also affected, as the dielectric constant also changes with the addition of organic phase[24].

If the calibration was performed with aqueous standards, the pH value of water-organic phase mixture that is displayed on the pH meter is on the absolute scale, denoted as ${}^{s}_{w}pH$. However up to 25 % volume of organic phase, a water based standard calibration is close to the actual value of ${}^{w}_{w}pH$[36]. In case of using mixtures of organic and water standards of different ratios as calibrating solutions, (same as the mobile phase) the ${}^{s}_{s}pH$ scale is observed. The empirical formula linking the two is very useful[37]:

$$ {}^{s}_{s}pH = {}^{s}_{w}pH - \delta \quad (7)$$

Where δ is an estimated value, obtained from the previously measured difference between ${}^{s}_{w}pH$ or ${}^{s}_{s}pH$ of the given solution.

Utmost of times, what interests us the most is the true ${}^{w}_{w}pH$ value. There is no general relationship between ${}^{w}_{w}pH$ with ${}^{s}_{w}pH$ nor the ${}^{s}_{s}pH$ value[37]. The way of linking these parameters is done by performing multiple potentiometric titrations and then using an appropriate fit. This approach is also used with potentiometric p*K*a determinations of analytes that are not soluble in a solely aqueous environment[38].

2.3.5 Gradient of elution strength

As stated before, the elution strength of solvents in a given chromatography system varies and in a reversed phase mode is inversely proportional to solvent's polarity[39]. For the separation process acceleration, an optimal elution strength change can be a very useful tool. Furthermore, it provides retention time and selectivity

modification, and it also narrows down peaks in a way that might improve resolution[24]. In HPLC, the generation of a gradient is done by a programmed increment of the mobile phase component of the higher elution strength[40].

The retention of a compound k in binary water-organic mobile phase as a function of organic modifier volume ratio Φ can be expressed via the semi-empirical linear equation[40]:

$$\log k = \log k_w + S\Phi \quad (8)$$

Where k is the retention factor of a given analyte, log k_w is the logarithm of retention factor of this analyte in 100 % aqueous mobile phase, S is the solvent strength parameter, a characteristic of a particular system correlating with its elution strength. For a given column and organic modifier type, S is approximately a constant for all separated solutes, even of a broad distribution of molecular size. This parameter depends on many system conditions such as temperature, column packaging and solvent. That being said, the usually observed value is around 2-4 dimensionless units[40].

Log k_w can be measured isocratically, using equation (8) by measuring retention times at different Φ and a subsequent extrapolation to zero Φ. The direct measurement of log k_w is not always possible, as the limitations of stationary phase usage often involve low compatibility with 100 % aqueous phase[16].

The derived equation for gradient retention time of a solute t_g is at it follows:

$$t_g = \frac{t_G}{S\Delta\Phi}\log\left(\frac{2{,}301\ S\ \Delta\Phi\ t_M\ k_w}{t_G} + 1\right) + t_M + t_D \quad (9)$$

Where t_G is the time of the gradient, $\Delta\Phi$ is the change of the mobile phase composition, k_w is the retention factor of the analyte in the 100 % aqueous mobile phase composition, t_M is the retention of a dead volume marker and t_D is the system dwell time.

While t_M denotes the time it takes the not retained analyte to travel from the site of injection to the detector, t_D is the time measured from the place mobile phases meet to the point of injection.

A bigger increase in elution strength leads to shorter analyte retention. The gradient steepness can be denoted as b and can be also expressed with a formula:

$$b = \frac{S\,\Delta\Phi\,t_M}{t_g} \quad (10)$$

This formula can be plugged into equation (9) with the symbols of the same meaning.

Peaks are compressed when using gradient by a certain factor which is dependent on the gradient steepness. A steeper gradient means narrower elution zones, with the case of b value being ($0.02 < b < 0.05$) resulting in a compression of approximately 20 % compared to an isocratic measurement[40].

The resolution calculation formula remains the same as for isocratic separations.

2.3.6 Gradient of pH

As opposed to standard elution strength gradient, in this technique, the organic modifier volume ratio remains constant and it is the pH of the aqueous phase that is linearly changing in time[41]. This does not mean that the retention of acidic or basic compounds is changing linearly however. That is rarely the case as even a slight change in gradient steepness results in very different retention times of these analytes[42]. In case of utilising this method on weak acids, the pH is gradually increasing and in case of bases, the opposite is true. Provided these conditions, these ionisable compounds are separated based on their decreasing acidity or basicity[43].

This method was first utilised for protein separations and termed chromatofocusing in the 1970s. It was developed for weak ion exchange columns by generating an internal pH gradient. In this approach, the column is equilibrated with a starting buffer and then the stationary phase resin is titrated by a buffer of different pH[44]. The external form of this gradient is in contrary created by mobile phase mixing before entering the column[45]. The following is the subject of further discussions in this work.

Although the possibilities of using the pH gradient method are broad, such as for separations of bioanalytes that are susceptible to higher ratio of organic phase, it is rarely used in industrial scale purifications[46]. In case of protein bioanalytes, the standard purification protocol generally involves combinations of analytical procedures. Proteins are commonly chromatographically separated based on their unique properties, such as affinity, ion exchange or hydrophobic interactions. All of the mentioned techniques employ a gradient of ionic strength to a certain extent and that is the predominantly used method[47].

The pH gradient method has shown a number of advantages, when separating proteins in ion exchange chromatography. When using pH gradient compared to ionic strength gradient, proteins are eluting in narrower bands[48]. They are shown to elute when the mobile phase pH reaches their isoelectric value, as they reach a point where they lose charge[46]. It was also observed that pH gradient provides a better resolution for a mixture containing monoclonal antibodies of very similar charge heterogeneity, which are a difficulty for ionic strength gradients[49].

The common reason for not opting for pH gradient in any chromatography mode is the recurring issue of generating a linear gradient through a wide enough range. Some advances have been recently made, by ensuring that the buffering capacity remains approximately constant through the entire pH range of usage, or by choosing buffer components with equally spaced p*K*a values[50].

Furthermore it´s difficult generating a pH gradient without a small change in the ionic strength. When evaluating the retention of analytes on the column as a function of pH, it is important to arrange minimal salt concentration change as possible so that it is possible to attribute the change in analyte retention only to the change in pH value.

2.4 The use of RP-HPLC for physicochemical property determination

The benefits of using HPLC for any physicochemical property determination compared to other techniques, is the required amount of analyte, as even milligrams of sample are sufficient. Furthermore, sample purity is also not a necessity, as the mixture components or impurities are often easily separated from the analyte during the chromatographic process[41].

This is advantageous when it comes to the field of drug discovery[51], where a large number of structurally related compounds are prepared simultaneously, using combinatorial chemistry[52]. A fast determination technique is needed for the characterisation of these substances such as methods for p*K*a and lipophilicity determination. These properties determine the absorption, distribution, metabolism and toxicity of the compound[53] and can be further used for modelling processes in drug design[51].

2.4.1 Log *P* determination

In reversed phase chromatography, the retention of an analyte is proportional to its lipophilicity. While this property describes the ability of a substance to dissolve in fats, oils, lipids, and nonpolar solvents[54], it's also one of the most significant parameters regarding biochemical processes, as it indicates the rate of transport through cellular membranes. A similar property is hydrophobicity, which describes the tendency of nonpolar molecule to associate in an aqueous environment. This property also goes way beyond, as it outlines a substance's fate in the environment. Substances of high hydrophobicity tend to accumulate in the sediments, while hydrophilic compounds are rather found in natural waters[55].

To quantitatively describe lipophilicity as a substance´s property, a partition coefficient log *P* is commonly used. It is defined as a ratio of concentrations of the uncharged form of the compound between an organic and aqueous solvent at equilibrium, with the most commonly used solvents being n-octanol and water[56]. The reason why n-octanol is used is because of its present donor – acceptor -OH group, and structure that mimics the amphiphilic nature of biological membranes[51]. It is a key parameter in describing structure – activity relationship, given by the formula:

$$\log P = \frac{c_{n-octanol}}{c_{\text{water}}} \quad (11)$$

Where $c_{\text{n-octanol}}$ describes the molar concentration of an analyte in the n-octanol phase and c_{water} is its molar concentration in water. This is a constant at a given temperature for the two immiscible solvents[51]. In literature, log *P* can be found for many compounds measured at room temperature of 25 °C.

In the case of ionisable molecules, it is quite obvious that the charged form is retained solely in the aqueous phase and therefore another, so-called distribution coefficient log *D* is defined. It is the overall ratio of a compound´s concentration in organic and aqueous phase at equilibrium, for both non-ionised and ionised forms[56]. As well as log *P*, it is a function of temperature but also of pH. Therefore, to measure a log *P* for such a substance, it must be present entirely in the non-ionisable state[57].

For log *P* determination, numerous approaches utilising HPLC have been practised. Perhaps the most intuitive is the micro shake flask method, that uses a special instrument and a subsequent HPLC analysis, to quantify the equilibrium concentration

of a substance in one or both of the phases[58]. There are other special approaches, which do not characterise the constant by quantitative analysis and can be performed by a standard HPLC instrument. The principle of these methods stems from the fact, that chromatographic parameter log k_w from equation (8) can be linked with log *P*. Some efforts have shown satisfying results correlating the two, such as using reversed phase chromatography with mobile phases containing water and octanol. However, differences arise from the fact, that chromatographic partition is more entropy driven than a simple distribution in a flask. Nevertheless, log k_w is in this sense more alike the biological partition process[51].

Faster option of measuring log k_w and therefore also log *P*, is by an organic gradient method, using equation (9). This can be done by single gradient run, assuming the value of *S* (e.g. $S = 4$ for methanol)[57]. The alternative is an exact solution, by two gradient runs of different value of gradient steepness *b*, solving two nonlinear equations for two variables *S* and k_w. The solution has to be found iteratively, by numerical calculation[59].

2.4.2 p*K*a determination

p*K*a value of a compound is a value of pH, where the dissociated and undissociated forms of the electrolyte are equal. This constant defines the equilibrium between the present forms in the solution, unaffected by the electrolyte's overall concentration[60].

It must be acknowledged, that during a chromatographic separation of any weak electrolyte, there is another equilibrium present, besides the acid-base one. The so-called distribution equilibrium describes the analyte´s distribution between the stationary phase and the mobile phase. The dissociation of a compound makes it almost entirely prevalent in the aqueous phase, which in reversed phase chromatography, results in short retention time[61].

The relationship between the retention factor of a weak electrolyte and the pH of the solution can be derived based on the fact that the observed retention factor is essentially a weighted average of the present forms. The equilibrium between them is generally achieved way quicker than the distribution equilibrium. This usually results in a single peak observation for any ionisable analyte with its retention factor *k*. The dependence is as it follows[62]:

$$k = \frac{K_a k_{A^-} 10^{pH} + k_{HA}}{1 + K_a 10^{pH}} \quad (12)$$

where *K*a is the dissociation constant, k_{A^-} is the retention factor of the anionic form of analyte and k_{HA} the retention factor of the neutral form.

From this formula, a sigmoidal function is obtained, with the inflex point being the p*K*a of the analyte. For ionizable compounds, a small change in pH can lead to a significant variation in analyte selectivity and therefore also retention[62]. The most dramatic retention factor fluctuations are near the p*K*a value[42], hence for obtaining reproducible results it is generally recommended using a mobile phase at least of pH ± 1.5 above or below the analyte p*K*a[33].

While the p*K*a determination from the inflex point based on the formula (12) works for isocratic measurements using mobile phases with little to no organic modifiers, it is time demanding. Newer and more effective methods involve a pH gradient run, which makes the determination faster.

Most biologically active substances tend to be at least partially dissociated at physiological pH, with many of them also containing more than one ionisable group. A p*K*a value is therefore an essential information regarding these compounds[51].

2.4.2.1 *pKa determination by pH gradient*

The ionisation of a single group is assumed. The following technique involves a preliminary log k_w and parameter *S* determination from equation (8), which is the basis for choosing optimal retention time for an analyte. This can be done by single or two organic modifier gradients. Analytes must be in unionised form while carrying out this part of the experiment. This is achieved by opting for a buffer component of appropriate pH. Based on the resultant log k_w and *S* values for a given analyte, a moderate organic modifier ratio Φ is chosen for the consecutive pH gradient run retention factor *k* being ≈ 10.

The pH gradient experiment utilises the resultant organic modifier ratio, which is kept constant during the separation. It is only the composition of the aqueous phase that is changing in time. The most necessary requirement for the gradient time t_G is to be shorter than the retention time of the least retained (most acidic or basic) analyte in its neutral form[63] and for the p*K*a of the analyte being in the range of increasing or decreasing pH values.

Furthermore, the determination should be undertaken with a stationary phase of low silanol activity, as other interactions, such as coulombic between dissociated silanol groups and analytes significantly affect the separation mechanism[53]. The formula for p*K*a determination for acids is the following:

$$\mathrm{p}K\mathrm{a} = \mathrm{pH}^{**} + \log \frac{t'_{HA}}{t'_{A^-}} \left(10^{-at'_{A^-}\left(\frac{t'_{HA}-t'_R}{t'_{HA}-t'_{A^-}}\right)} - 1\right) \quad (13)$$

Where pH** denoted the value of mobile phase pH when the analyte reaches the detector, t'_{HA} is the reduced retention time of the neutral form, t'_{A^-} is the reduced retention time of the anionic form, a is the gradient steepness and t'_R is the reduced retention time of the analyte when the pH gradient was applied.

And for bases:

$$\mathrm{p}K\mathrm{a} = \mathrm{pH}^{**} + \log \frac{t'_B}{t'_{HB^+}} \left(10^{-at'_{HB^+}\left(\frac{t'_B-t'_R}{t'_B-t'_{HB^+}}\right)} - 1\right) \quad (14)$$

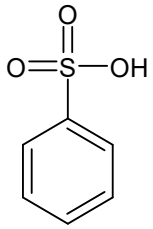
Where pH** denoted the value of mobile phase pH when the analyte reaches the detector, t'_B is the reduced retention time of the neutral form, t'_{HB^+} is the reduced retention time of the cationic form, a is the gradient steepness and t'_R is the reduced retention time of the analyte when the pH gradient was applied.

The gradient steepness a is calculated as the change in pH divided by the time of the gradient $\Delta\mathrm{pH}/t_G$. The formulas (13, 14) apply when $t_D \leq t'_R \leq t_G + t_D$, which is the normal case. The t_D is the previously defined dwell time.

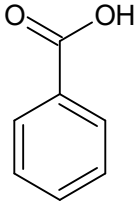
2.5 Analytes

For the characterisation of the Acclaim WAX-1 and Acclaim WCX-1 columns, the following analytes were used, alongside with their respective log *P* and p*K*a values at 25 °C obtained from the pubchem website[64]:

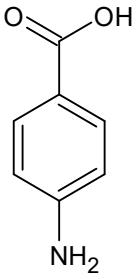
Acids



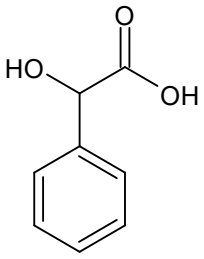
Benzenesulfonic acid
Log *P* = - 1.2
p*K*a = - 0.60



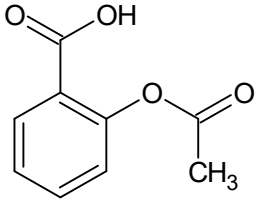
Benzoic acid
Log *P* = 1.85
p*K*a = 4.2



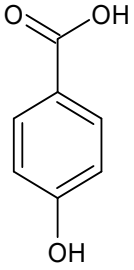
4-Aminobenzoic acid
Log *P* = 0.83
p*K*a 1 = 2.38
p*K*a 2 = 4.85



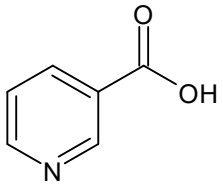
Mandelic acid
Log *P* = 0.62
p*K*a = 3.41



Acetylsalicylic acid
Log *P* = 1.19
p*K*a = 3.47

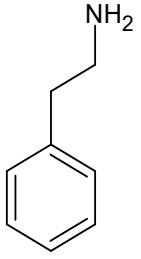


4-Hydroxybenzoic acid
Log *P* = 1.58
p*K*a = 4.54

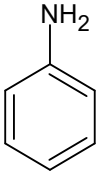


Nicotinic acid
Log *P* = 0.36
p*K*a = 4.75

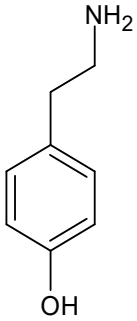
Bases



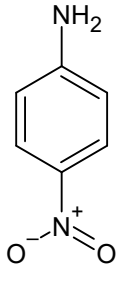
2-Phenylethylamine
(PEA)
Log *P* = 1.41
p*K*a = 9.83



Aniline
Log *P* = 0.94
p*K*a = 4.6

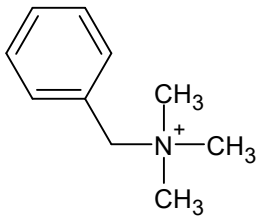


Tyramine
Log *P* = 0.72
p*K*a = 9.39

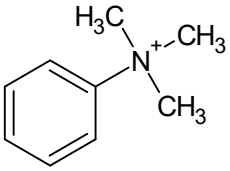


4-Nitroaniline
Log *P* = 1.39
p*K*a = 1.01

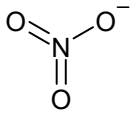
Ions



Benzyltrimethylammonium ion (BTMA)



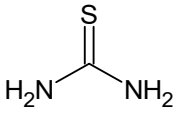
Trimethylphenylammonium ion (TMFA)



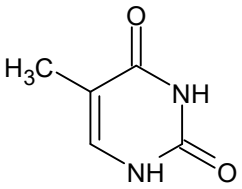
Nitrate ion

Iodium ion

Nonionisable analytes



Thiourea
Log *P* = -1.08



Thymine
Log *P* = -0.62

3 Experimental part

3.1 Instrumentation

HPLC instrument

- separation module Waters 2695, Alliance HPLC System (Waters, Milford, USA)
- detector Waters 2996, Photodiode Array Detector (Waters, Milford, USA)
- autosampler 717 Plus (Waters, Milford, USA)
- column thermostat - Waters Alliance Series Column Heater (Waters, Milford, USA)

Chromatographic columns

- Acclaim^{TM} mixed mode WAX-1, dimensions 4.6 × 150 mm, particle size 5 μm
- Acclaim^{TM} mixed mode WCX-1, dimensions 4.6 × 150 mm, particle size 5 μm
- XTerra® MS C18, dimensions 3.0 × 150 mm, particle size 3.5 μm

Other

- pH meter PHM220 MeterLab, Radiometer Analytical SAS (Villeurbanne Cedex, France)
- analytical scales Mettler AE 240 (Greifensee, Switzerland)

Programs

- ACD/ChemSketch 12.01
- Empower software 2
- Origin 6.1
- PeakMaster 5.4
- Python 3.8

3.2 Chemicals

- 2-phenylethylamine hydrochloride (purity ≥ 98.0%, Sigma-Aldrich, St. Louis, USA)
- 4-aminobenzoic acid (purity ≥ 99.0%, Sigma-Aldrich, St. Louis, USA)
- 4-hydroxybenzoic acid (purity ≥ 99%, Sigma-Aldrich, St. Louis, USA)
- 4-nitroaniline (Chemapol, Neratovice, Czech Republic)
- acetic acid (purity ≥ 99.5%, Sigma-Aldrich, St. Louis, USA)
- acetylsalicylic acid (purity ≥ 99.0%, Sigma-Aldrich, St. Louis, USA)

- aniline (purity ≥ 99.5%, Sigma-Aldrich, St. Louis, USA)
- benzenesulphonic acid (purity ≥ 98.0%, Sigma-Aldrich, St. Louis, USA)
- benzoic acid (purity ≥ 99.5%, Sigma-Aldrich, St. Louis, USA)
- benzyltrimethylammonium chloride (purity 97%, Sigma-Aldrich, St. Louis, USA)
- dichloroacetic acid (purity ≥ 99%, Sigma-Aldrich, St. Louis, USA)
- mandelic acid (purity 99%, Sigma-Aldrich, St. Louis, USA)
- methanol (HiPerSolv CHROMANORM® purity ≥ 99%, VWR, Radnor, USA)
- nicotinic acid (purity ≥ 98%, Sigma-Aldrich, St. Louis, USA)
- potassium iodide (purity 99.8 %, Lachema, Brno, Czech Republic)
- potassium nitrate (purity 99.8 %, Lachema, Brno, Czech Republic)
- tetrahydrofuran (purity ≥ 99.9%, Sigma-Aldrich, St. Louis, USA)
- thiourea (purity ≥ 99.0%, Sigma-Aldrich, St. Louis, USA)
- thymine (purity ≥ 99%, Sigma-Aldrich, St. Louis, USA)
- triethanolamine (purity ≥ 99.0%, Sigma-Aldrich, St. Louis, USA)
- trimethylphenylammonium chloride (purity ≥ 98.0%, Sigma-Aldrich, St. Louis, USA)
- trizma® base (purity ≥ 99.9%, Sigma-Aldrich, St. Louis, USA)
- tyramine (purity 99%, Sigma-Aldrich, St. Louis, USA)

3.3 Sample preparation

The analytes were prepared by dissolving measured amounts of crystalline standards in the buffer with a pH of 2.5 consisting of dichloroacetic acid, acetic acid and tris(hydroxymethyl)aminomethane (Table 1) so that the analyte concentration reached 1 mg/ml. Aniline solution was prepared by diluting 10 µl/ml of the standard in the same buffer. The solutions were filtered through a polytetrafluorethylene filter with pore size 0.2 µm before their injection into the MP.

3.4 Experimental conditions

Retention time t_R of analytes was taken from the mean value obtained in a minimum of two measures. Injected sample volumes into the mobile phase were 30 – 50 µl. The used pH values of mobile phase were 2.5 – 8.6, based on column permissibility (Section 2.2.3). Dead time t_M was determined from the first negative peak in the chromatogram for mixed mode columns and from the retention time of iodide anion in measurements carried out on the reversed phase column.

The retention times were measured at different wavelengths, based on the analyte: At 220 nm – benzenesulfonic acid, benzoic acid, mandelic acid, BTMA, TMFA, PEA, nitrate anion and iodide anion.

At 254 nm – acetylsalicylic acid, 4-hydroxybenzoic acid, nicotinic acid, thiourea and thymine.

At 280 nm – 4-aminobenzoic acid, aniline and tyramine

At 380 nm - 4-nitroaniline.

A few analytes were further diluted to a concentration, for which the detector gave approximately 1 AU response. For the mixed mode columns 1 ml/min flow and a temperature of 25 °C was programmed. For the determination of log *P* and p*K*a values of a few analytes in reversed phase mode, the flow varied from 0.3 to 0.4 ml/min with a temperature of 25 °C.

Dwell time t_D of the HPLC system was measured using a 100 % aqueous mobile phase at line A, flowing through a chromatographic clutch with 1 ml/min flow rate (without a chromatographic column) measured at 220 nm with a prompt switch to tetrahydrofuran/water 10/90 (v/v) solution at line B after 2 minutes. A subsequent switch back to line A was then applied. The dwell time value was determined as the difference between the time a change in mobile phase absorption spectrum occurred in the detector and the time in which the switch was programmed (which were 2 minutes). The obtained dwell time was 0.95 min at 1 ml/min mobile phase flow rate.

All measurements on mixed mode columns were done in isocratic conditions. For the reversed phase column, linear organic gradient was applied, from 95/5 (v/v), to 0/100 (v/v) buffer/methanol composition. The second type of applied gradient was a pH gradient – using a fixed small percentage of methanol and a linearly changing ratio of 2 buffers of pH 2.5 and 8.6 of the buffer system 1 (Table 1).

4 Results and discussion

All of the analytes (section 2.5) were measured under the varying pH conditions from 2.5 to 7.5 in 0.5 steps using 2 buffer systems on 2 different mixed mode columns – Acclaim WAX-1 and Acclaim WCX-1.

4.1 Development of an online buffer mixing method

A buffer system preparation was needed to measure retention of all analytes in a range of pH 2.5 – 7.5 with steps of 0.5 units. This was efficiently done by mixing 2 buffers of lower and higher pH in various ratios, directly in the HPLC instrument, instead of preparing a buffer for every single pH. The two starting buffers were prepared to have a pH of 2.5 and 8.6.

The buffer components were chosen under the criteria of having p*K*a values in this range, as this meant better buffering capacity. Low UV cut off and low volatility were another important condition that had to be satisfied alongside with a minimal change in ionic strength throughout the pH scale as it can affect the SP behaviour during the characterisation.

The calculations of different solvent pH from various buffer A and B ratios were first performed using the PeakMaster 5.4 software and then adjusted in the HPLC instrument. The software gave concentrations of the buffer components needed to achieve the desired pH. The volume ratios of buffer A and B were the unknowns that needed to be calculated from solvent mixing equations, one for each component. The calculated ratios were then set up in the HPLC instrument and modified if necessary. A minimum time of 20 minutes calibration was always required for a given ratio to give the desired pH value. The 2 developed buffer mixing methods can be universally used on the Waters 2695, Alliance HPLC System and should be transferable to any of the HPLC instrumentation.

Buffer system mixing 1 was prepared from dichloroacetic acid (DCHO), acetic acid (Ac) and tris(hydroxymethyl)aminomethane (Tris) (Table 1).

Table 1: Buffer system 1 – composition of the mobile phase and its pH under different ratios of buffer A and buffer B, comparison of calculated and measured pH values.

buffer A (v/v)	buffer B (v/v)	concentrations DCHO [mmol/dm^3]	Ac [mmol/dm^3]	Tris [mmol/dm^3]	Ionic strength [mmol/dm^3]	Calculated pH	HPLC pH (measured)
100	**0.0**	14.2	5.0	10.0	13.5	2.500	**2.5**
93.5	**6.5**	13.9	5.0	12.6	13.8	2.984	**3.0**
91.1	**8.9**	13.8	5.0	13.6	14.0	3.458	**3.5**
89.2	**10.8**	13.7	5.0	14.4	14.5	4.005	**4.0**
86.2	**13.8**	13.6	5.0	15.5	15.5	4.506	**4.5**
83.1	**16.9**	13.5	5.0	16.8	16.8	4.991	**5.0**
81.0	**19.0**	13.4	5.0	17.6	17.6	5.404	**5.5**
79.7	**20.3**	13.3	5.0	18.3	18.1	6.130	**6.0**
78.7	**21.3**	13.3	5.0	18.5	18.2	6.360	**6.5**
76.7	**23.3**	13.2	5.0	19.3	18.2	6.925	**7.0**
71.8	**28.2**	13.0	5.0	21.3	18.0	7.396	**7.5**
57.0	**43.0**	12.4	5.0	27.2	17.4	7.881	**8.0**
0.0	**100**	10.0	5.0	50.0	15.0	8.500	**8.6**

The second mobile phase buffer system was also prepared with buffers of pH 2.5 and 8.6, with the components of the mobile phase being dichloroacetic acid (DCHO), acetic acid (Ac) and Triethanolamine (TriEtAm) with varying concentrations (Table 2).

Table 2: Buffer system 2 - composition of the mobile phase and its pH under different ratios of buffer A and buffer B, comparison of calculated and measured pH values.

buffer A (v/v)	buffer B (v/v)	concentrations DCHO [mmol/dm^3]	Ac [mmol/dm^3]	TriEtAm [mmol/dm^3]	Ionic strength [mmol/dm^3]	Calculated pH	HPLC pH (measured)
100	**0.0**	14.2	5.0	10.0	13.5	2.500	**2.5**
96.4	**3.6**	14.0	5.0	12.8	14.0	2.985	**3.0**
95.0	**5.0**	14.0	5.0	13.9	14.2	3.520	**3.5**
94.0	**6.0**	13.9	5.0	14.6	14.7	4.005	**4.0**
92.5	**7.5**	13.9	5.0	15.8	15.8	4.504	**4.5**
90.7	**9.3**	13.8	5.0	17.2	17.2	5.024	**5.0**
89.4	**10.6**	13.8	5.0	18.2	18.1	5.569	**5.5**
88.6	**11.4**	13.7	5.0	18.8	18.5	6.053	**6.0**
87.7	**12.3**	13.7	5.0	19.5	18.5	6.543	**6.5**
85.3	**14.7**	13.6	5.0	21.3	18.3	7.042	**7.0**
77.8	**22.2**	13.3	5.0	27.1	17.4	7.569	**7.5**
57.4	**42.6**	12.4	5.0	42.8	18.8	7.930	**8.0**
0.0	**100**	10.0	5.0	87.0	15.0	8.500	**8.6**

4.2 Acclaim WAX-1 column

The Acclaim WAX-1 column contains an amine group, bearing a positive charge in an unknown pH range (Figure 3). Retention times of all analytes as a function of pH for buffer systems 1 and 2 (Table 1 and 2) using this column were measured (Table 3) and for buffer system 2 plotted (Figure 6 A-G). The trends observed in the dependence of retention time on pH are similar for both buffer systems.

Table 3: Retention times of analytes measured on Acclaim WAX-1 column using buffer systems 1 and 2.

Analyte	**Retention times [min]**										
Buffer system 1	**pH 2.5**	**pH 3.0**	**pH 3.5**	**pH 4.0**	**pH 4.5**	**pH 5.0**	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**	**pH 7.5**
benzenesulfonic acid	112.68	120.34	118.92	116.36	112.60	109.26	103.89	96.97	89.08	72.95	54.28
benzoic acid	67.45	75.90	84.23	97.22	107.26	109.53	105.22	97.84	90.12	72.89	53.94
4-aminobenzoic acid	8.89	12.65	16.12	17.99	20.67	22.97	23.37	21.94	20.23	16.34	12.61
mandelic acid	19.27	28.36	38.09	42.99	44.21	43.20	41.29	37.82	34.58	28.35	21.83
acetylsalicylic acid	62.70	72.62	83.54	91.07	92.35	90.43	85.64	81.03	70.28	61.84	43.43
4-hydroxybenzoic acid	29.85	32.68	34.64	38.65	43.43	45.38	44.51	41.04	37.51	31.23	28.99
nicotinic acid	2.35	2.85	4.02	6.94	12.14	22.18	24.49	24.26	22.45	18.85	14.98
BTMA	1.57	1.68	1.69	1.63	1.64	1.67	1.65	1.68	1.75	1.85	2.02
TMFA	1.46	1.55	1.57	1.49	1.50	1.53	1.53	1.56	1.61	1.68	1.80
PEA	1.85	1.89	1.90	1.93	1.96	2.00	2.06	2.12	2.24	2.43	2.73
aniline	2.13	1.91	2.25	4.21	5.67	7.35	9.85	10.48	11.00	8.94	8.67
tyramine	1.52	1.51	1.53	1.54	1.55	1.59	1.62	1.67	1.74	1.86	2.08
4-nitroaniline	38.72	42.46	42.66	43.00	43.43	44.30	44.50	44.18	43.93	42.85	41.51
NO_3^-	19.60	19.94	19.71	19.28	18.36	17.33	16.60	15.85	14.34	12.29	9.86
I^-	35.56	37.17	36.51	35.51	34.13	32.67	31.06	28.47	26.05	22.16	17.39
thiourea	2.30	2.28	2.27	2.28	2.28	2.29	2.30	2.31	2.31	2.32	2.34
thymine	3.24	3.22	3.23	3.23	3.25	3.27	3.32	3.33	3.37	3.38	3.37
Buffer system 2	**pH 2.5**	**pH 3.0**	**pH 3.5**	**pH 4.0**	**pH 4.5**	**pH 5.0**	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**	**pH 7.5**
benzenesulfonic acid	124.09	118.71	117.34	122.58	111.24	113.77	105.17	103.74	89.16	75.57	51.63
benzoic acid	73.75	75.49	86.15	104.45	110.61	114.64	106.93	104.31	89.89	75.80	51.30
4-aminobenzoic acid	9.14	12.62	16.04	18.47	20.59	23.21	23.17	23.00	20.22	16.89	11.88
mandelic acid	20.30	27.95	38.12	44.32	45.46	45.92	42.20	41.31	35.73	30.32	21.54
acetylsalicylic acid	66.09	72.19	83.62	99.77	92.28	88.69	85.15	83.18	71.40	59.48	38.82
4-hydroxybenzoic acid	32.46	32.40	35.27	40.72	44.21	46.82	45.01	43.95	38.45	32.79	23.68
nicotinic acid	2.31	2.78	3.90	6.30	11.21	21.76	24.01	24.78	22.84	19.47	14.29
BTMA	1.68	1.70	1.73	1.75	1.72	1.72	1.73	1.75	1.78	1.88	1.99
TMFA	1.53	1.55	1.57	1.59	1.55	1.60	1.58	1.59	1.67	1.73	1.94
PEA	1.90	1.91	1.96	2.00	2.05	2.08	2.06	2.13	2.24	2.41	2.77
aniline	1.66	2.09	2.19	3.40	5.84	8.48	10.49	11.18	11.42	11.46	10.70
tyramine	1.51	1.51	1.55	1.55	1.56	1.58	1.62	1.63	1.72	1.82	2.06
4-nitroaniline	43.19	44.01	44.29	47.19	47.22	48.38	45.41	48.18	45.79	46.87	43.12
NO_3^-	20.38	19.88	19.22	19.07	18.39	17.77	16.54	16.29	14.36	12.47	9.40
I^-	37.79	38.10	35.64	34.97	34.70	33.23	31.18	30.41	26.36	22.60	16.61
thiourea	2.28	2.28	2.28	2.28	2.28	2.28	2.30	2.30	2.30	2.30	2.29
thymine	3.21	3.20	3.24	3.25	3.30	3.28	3.31	3.32	3.36	3.38	3.36

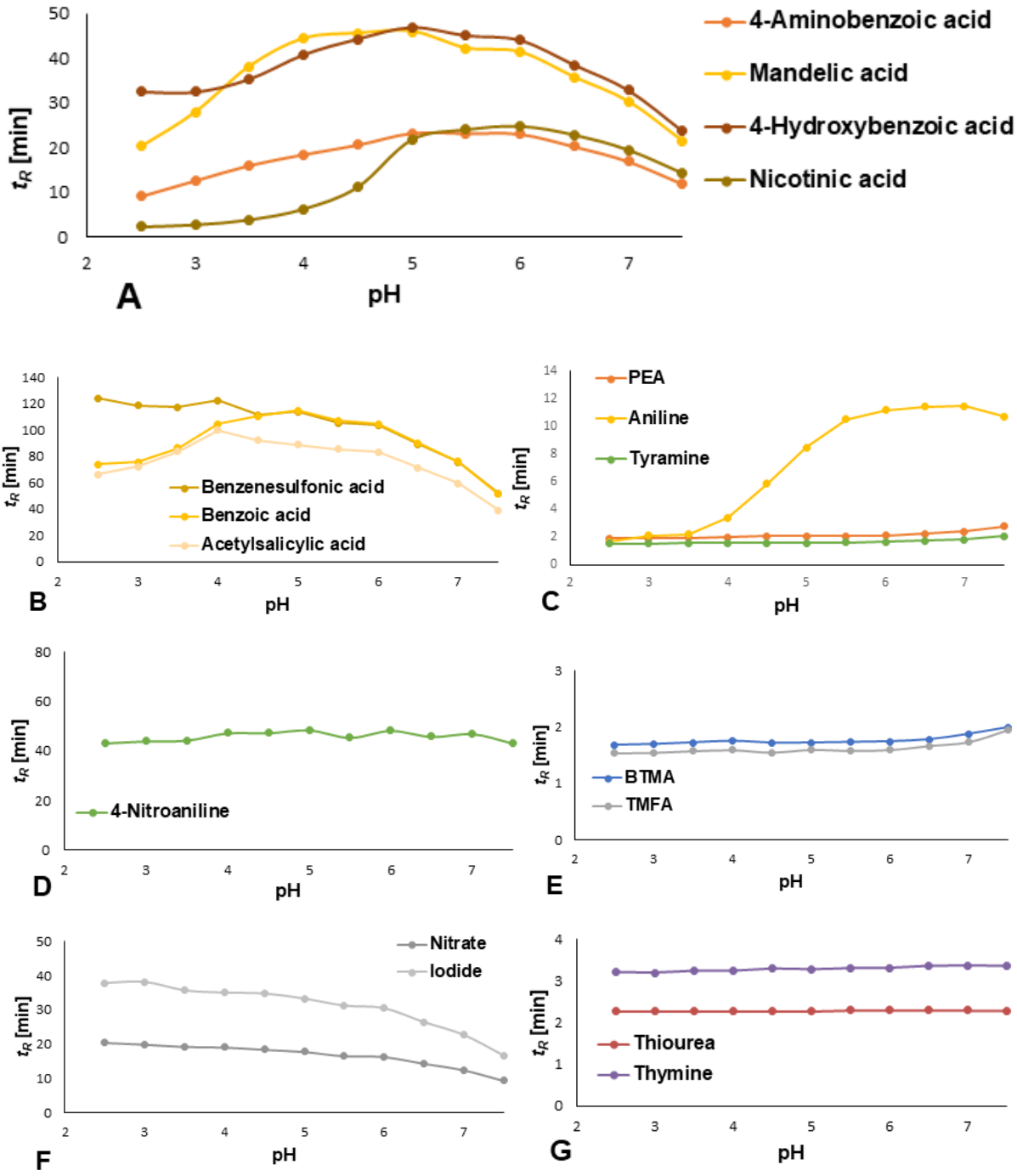


Figure 6: Retention time as a function of pH of various analytes on Acclaim WAX-1 mixed mode column using buffer system 2.

While the retention of polar but neutral analytes (Fig. 6G) remains unaffected by any mobile phase pH change, the retention of inorganic anions (Fig. 6F) has a decreasing tendency with increasing pH. This is possibly caused by the simultaneous loss of positive charge present on the stationary phase and also the dissociation of silanol groups on the silica carrier particles, both at higher pH values, approximately ≥ 5. Dissociated silanol groups yield a negative charge and therefore a repulsive interaction between the anions and SP takes place, resulting in a lower time that these analytes spent in the column. Analysis of organic weak and strong acids (Fig. 6A,B) shows a brief increase in retention at lover pH for those having their p*K*a above the measured value of pH, as with higher pH the more dissociated form ratio (more retained by attractive interaction) arises. The only exception is benzenesulfonic acid (p*K*a - 0.60) that is fully dissociated hence highly retained from the start. A subsequent retention decrease applies to all acids above the pH of ≈ 5, caused by the eventual suppression of the positive charge and also dissociation of the silanol groups on SP. The retention of bases (Fig. 6C) and organic cations (Fig. 6E) shows a trend of retention increase, which could be also attributed to the same reason for which the inorganic anion and organic acids retentions decrease. Analysis of 4-nitroaniline displays a constant retention value as it bears no charge throughout the entire scale (p*K*a 1.01) (Fig. 6D). Interesting phenomena is the decrease in retention times difference between analytes with similar structure (e.g. benzenesulfonic and benzoic acid) with higher pH of the aqueous mobile phase (Fig. 6A,B). This could demonstrate the prevalence of reversed phase separation mechanism at higher pH, where the ion exchange mechanism of the amine group is vanishing. Change in analyte retention and peak shape as a result of pH change can also be visualised for nicotinic acid from chromatograms (Figure 7).

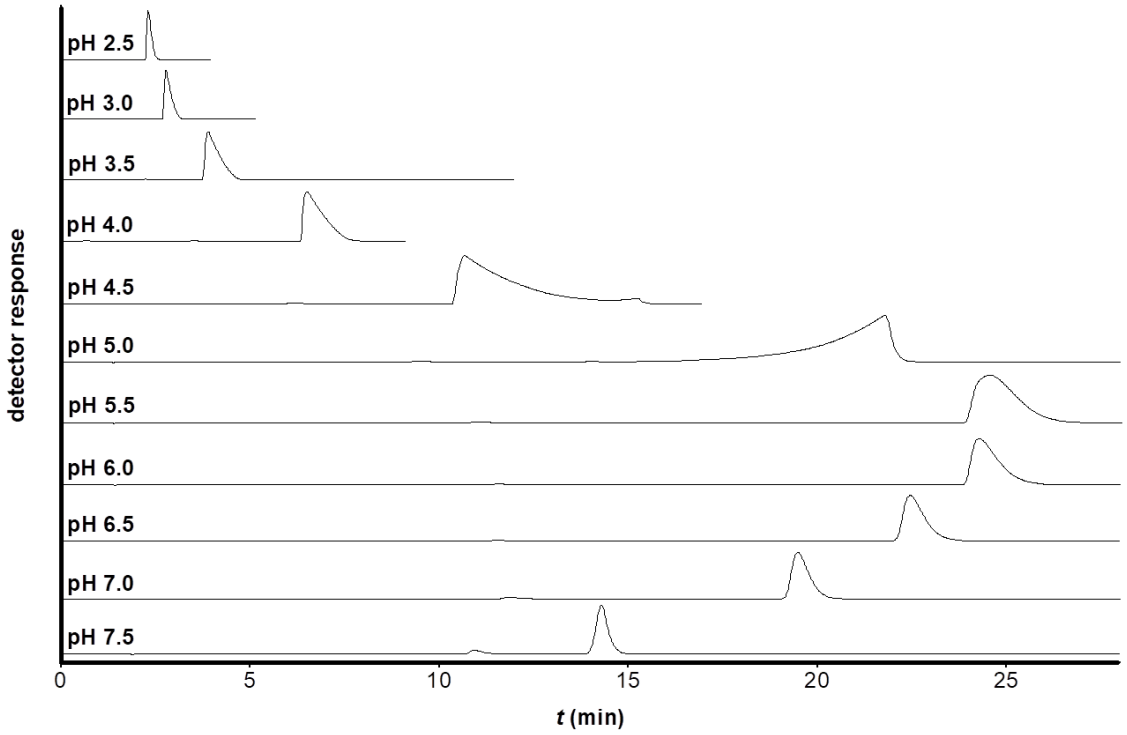


Figure 7: Chromatograms of nicotinic acid with varying pH of the mobile phase, measured with 1 ml/min flow, 25°C at 254 nm, on Acclaim WAX-1 column and buffer system 2.

Nicotinic acid with its p*K*a 4.75 (Section 2.5) shows a double peak at pH 4.5, signifying the presence of both its neutral and anionic form with different retention. This is generally an obstacle in the separation process as it often worsens peak resolution and therefore also affects peak symmetry (Table 4) in a mixture separation. Retention factor also significantly changes with a small change in pH near the analyte's p*K*a value. The optimal approach is using a pH of the mobile phase outside the analyte p*K*a (at least ± 1.5), as can be visualised in figure 7. The methods of finding analyte p*K*a will be demonstrated further.

Table 4: Calculated symmetry factors for nicotinic acid with changing pH values.

pH	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5
T	1.97	2.56	3.11	3.76	8.35	0.56	1.95	2.09	1.84	1.70	1.26

From table 4 it can be affirmed that the symmetry factor is the worst at a pH of 4.5, near nicotinic acid's p*K*a. At pH 5, the symmetry factor improves, but the peak is significantly fronting. Improvement of the symmetry is observed, the further the pH is

from the analyte's p*K*a value, and is the best at 7.5. Another reasoning behind the symmetry being better at pH 6.5-7.5 could be attributed to higher ionic strength of the mobile phase (Table 2).

4.3 Acclaim WCX-1 column

The Acclaim WCX-1 column contains a carboxylic group, bearing a negative charge in an unknown pH range (Figure 4). Retention times of all analytes as a function of pH for both buffer systems 1 and 2 using this column were measured (Table 5) and for buffer system 2 plotted (Figure 8 A-H). The trends observed in the dependence of retention time on pH are similar for both buffer systems.

Table 5: Retention times of analytes measured on Acclaim WCX-1 column using buffer systems 1 and 2.

Analyte	**Retention times [min]**										
Buffer system 1	**pH 2.5**	**pH 3.0**	**pH 3.5**	**pH 4.0**	**pH 4.5**	**pH 5.0**	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**	**pH 7.5**
benzenesulfonic acid	3.07	2.93	2.78	2.51	2.32	2.16	2.05	2.01	1.93	1.92	1.85
benzoic acid	53.30	48.84	41.90	30.76	18.92	11.67	3.94	2.97	2.26	2.15	2.03
4-aminobenzoic acid	14.00	18.65	19.74	18.89	15.93	11.96	4.75	3.33	2.06	1.93	1.70
mandelic acid	8.59	7.60	7.20	3.11	2.34	1.94	1.83	1.80	1.66	1.64	1.58
acetylsalicylic acid	36.42	30.50	22.39	14.51	5.55	3.30	2.45	2.16	1.95	1.91	1.73
4-hydroxybenzoic acid	36.06	35.23	32.50	27.78	20.38	13.17	4.63	3.17	2.09	1.88	1.71
nicotinic acid	2.63	2.72	2.66	2.60	2.41	2.13	1.94	1.86	1.59	1.57	1.54
BTMA	6.54	7.19	6.98	10.68	12.23	16.73	21.00	23.96	29.03	36.43	42.14
TMFA	3.71	4.51	4.71	5.82	9.57	11.20	13.05	15.06	15.86	18.80	23.42
PEA	6.05	6.74	7.27	10.70	13.39	17.38	21.89	25.33	29.68	33.04	39.70
aniline	3.13	3.63	10.09	10.18	11.19	11.49	11.57	11.62	11.22	11.32	11.18
tyramine	4.04	4.37	4.86	5.91	9.46	11.78	13.71	15.36	17.01	18.45	11.90
4-nitroaniline	66.33	66.96	66.43	67.95	68.42	67.78	67.61	68.17	66.02	66.91	65.72
NO_3^-	1.81	1.78	1.68	1.62	1.54	1.49	1.46	1.44	1.41	1.41	1.39
I^-	1.95	1.94	1.84	1.73	1.62	1.55	1.50	1.50	1.46	1.45	1.43
thiourea	2.32	2.27	2.31	2.31	2.32	2.32	2.33	2.33	2.34	2.32	2.35
thymine	3.52	3.48	3.53	3.58	3.53	3.54	3.52	3.53	3.51	3.51	3.54
Buffer system 2	**pH 2.5**	**pH 3.0**	**pH 3.5**	**pH 4.0**	**pH 4.5**	**pH 5.0**	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**	**pH 7.5**
benzenesulfonic acid	3.57	3.12	2.81	2.50	2.28	2.10	2.04	1.97	1.94	1.91	1.83
benzoic acid	45.96	45.66	38.96	27.93	16.72	5.82	3.69	2.85	2.36	2.20	1.96
4-aminobenzoic acid	12.85	17.63	18.83	17.76	14.88	11.92	4.28	3.02	2.15	2.06	1.59
mandelic acid	8.03	7.39	5.07	2.89	2.21	1.79	1.68	1.62	1.59	1.57	1.49
acetylsalicylic acid	32.43	26.48	20.26	12.53	4.72	2.90	2.35	2.11	1.98	1.92	3.33
4-hydroxybenzoic acid	32.62	33.55	30.54	25.84	18.15	7.25	4.01	2.94	2.14	1.94	1.59
nicotinic acid	2.59	2.69	2.68	2.59	2.37	2.09	1.95	1.86	1.63	1.60	1.53
BTMA	5.54	6.35	6.91	11.96	13.32	18.01	21.20	23.98	26.37	28.49	41.62
TMFA	3.72	3.91	4.06	5.85	6.85	10.95	13.91	15.53	16.29	19.21	21.55
PEA	5.47	7.06	7.31	8.58	13.99	18.34	22.07	24.63	27.85	30.00	42.20
aniline	3.08	3.53	4.66	11.06	10.96	11.13	11.11	10.95	10.98	10.87	10.52
tyramine	3.91	4.26	5.43	6.31	7.93	11.79	14.85	15.19	16.01	16.97	20.99
4-nitroaniline	58.28	63.32	59.58	63.36	63.30	63.30	63.81	62.56	63.31	62.80	61.47
NO_3^-	1.88	1.79	1.70	1.59	1.50	1.43	1.44	1.43	1.43	1.41	1.38
I^-	2.16	1.96	1.84	1.71	1.61	1.45	1.52	1.50	1.48	1.47	1.44
thiourea	2.33	2.32	2.32	2.32	2.32	2.33	2.34	2.33	2.34	2.34	2.33
thymine	3.49	3.51	3.51	3.51	3.50	3.51	3.52	3.50	3.50	3.49	3.44

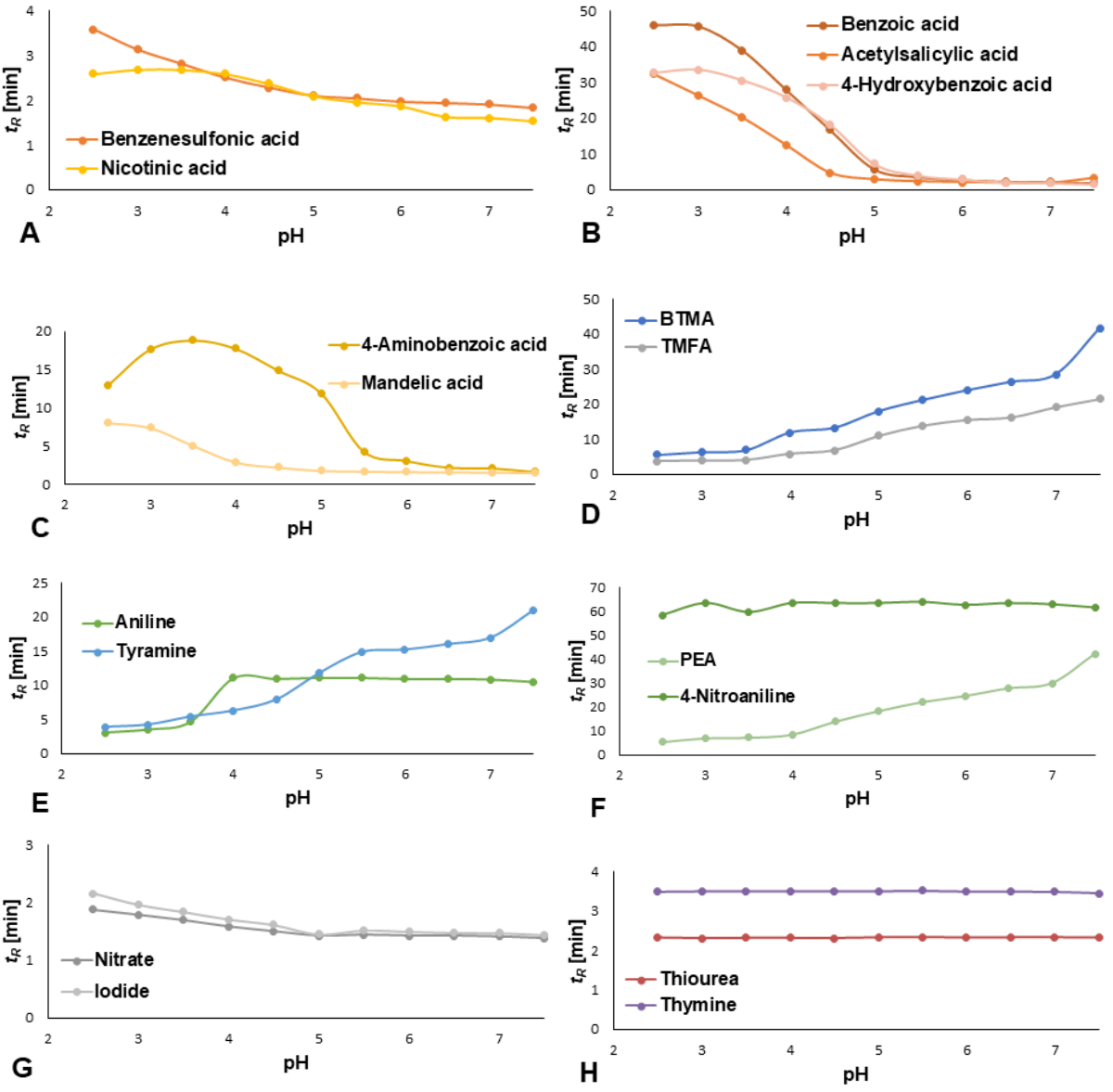


Figure 8: Retention time as a function of pH of various analytes on Acclaim WCX-1 mixed mode column using buffer system 2.

The observed retention of polar but neutral analytes (Fig. 8H) shows a relatively constant value and the retention of inorganic anions (8G) has a decreasing tendency, just like with the WAX-1 column. The cause of this effect is different however, as with WCX-1 column it's mainly the repulsion between the anionic analytes and dissociating carboxylic and silanol groups of the stationary phase, that are responsible for their smaller and decreasing retention time. Weak and strong acids follow a similar trend (Fig. 8A-C), as their retention and also mutual retention difference decreases. The exception is 4-aminobenzoic acid which shows a retention increase from pH 2.5 to 3.5 as it bears a positive charge in this environment, thanks to its amine group with a p*K*a of 2.35. Aniline (p*K*a = 4.6), tyramine (p*K*a 9.39) and PEA (p*K*a 9.83) show retention time increase with pH (Fig. 8E,F) when the pH is below their p*K*a, while 4-nitroaniline displays a constant retention value as it bears no charge through the entire scale (p*K*a 1.01). The cation exchange separation mechanism contribution at higher pH can be further affirmed by a gain in retention of cationic markers (Fig. 8D) above roughly the pH 3.

4.4 Qualitative comparison of the 2 buffer systems

Buffer systems 1 and 2 exhibit a linear dependence of changing buffer A (pH=2.5) ratio on measured pH (Tables 1,2) in the pH range 2.5 - 7.5, which was used for the mixed mode column characterisation. The linear fit in this range for buffer system 1 (Figure 9A) resulted in a R^2 value 0.968 while for buffer system 2 (Figure 9B) 0.9174. Above this range, the linearity decreases for both systems.

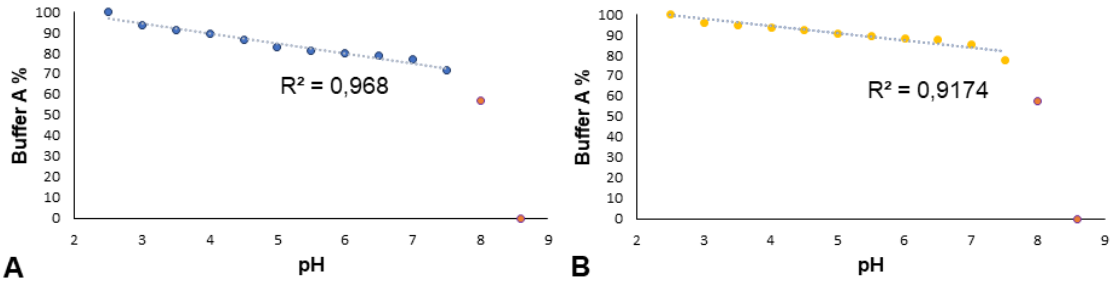


Figure 9: Volume ratio of buffer A (pH=2.5) as a function of pH for A - buffer system 1 and B - buffer system 2.

As mentioned in sections 4.2 and 4.3, the retention times of analytes as a function of pH follow a similar trend for both buffer systems. The values for uncharged analytes - thymine and thiourea, show very similar results for both buffer systems and columns.

Same phenomena can be said about inorganic anions – nitrate and iodide and cations – BTMA, TMFA, PEA. Differences between retention times measured by the two buffers systems on a particular column are the most observable for weak electrolytes and stem from their fluctuating retentions around their p*K*a, as even a small change in mobile phase pH affects the measured value significantly in this region.

Both buffer systems appear to give similar values and are therefore suitable for similar measurements.

4.5 Determination of analyte's p*K*a value

Isocratic p*K*a values determination

The p*K*a values for a few analytes that ranged between the used pH of the mobile phase 2.5–7.5 (Section 2.5) were estimated from the inflex point of the retention factor dependence on pH (Formula 12). The listed p*K*a values for 4 analytes are the results obtained from 4 measurements - from 2 columns, using 2 buffer systems (Tables 1,2). For a better fit, some retention factor data were omitted. It was done so because of the stationary phase's charge shift above or below a certain pH, which changed the analyte's retention mechanism. The entire retention data can be viewed from previous values (Tables 3,5) and in graphs (Figures 6,8). Graphs below (Figures 10-13) are the results of those fits with its inflex point being closest to the analyte's tabulated p*K*a value. All results are shown in table 6.

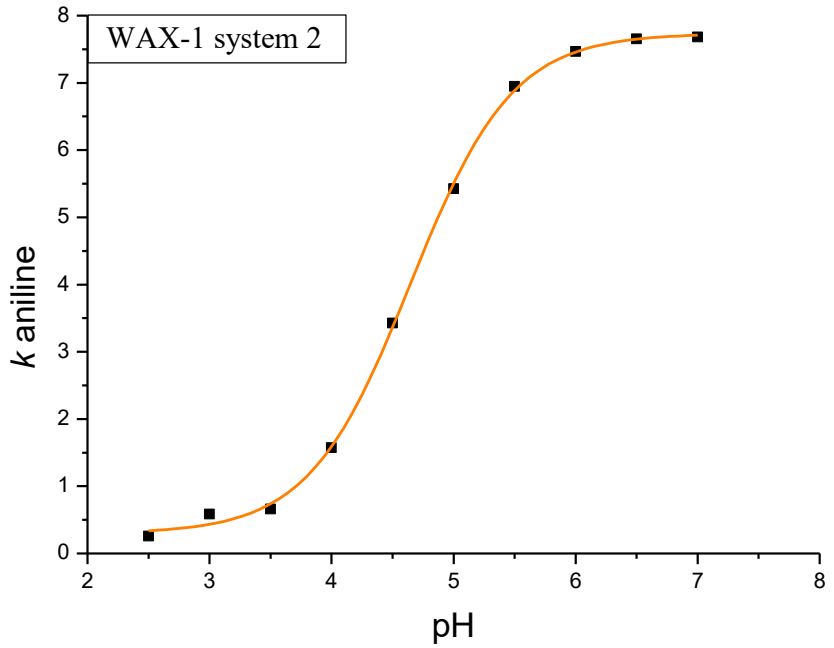


Figure 10: Dependence of the retention factor on pH – aniline measured at 280 nm on Acclaim WAX-1 column using buffer system 2

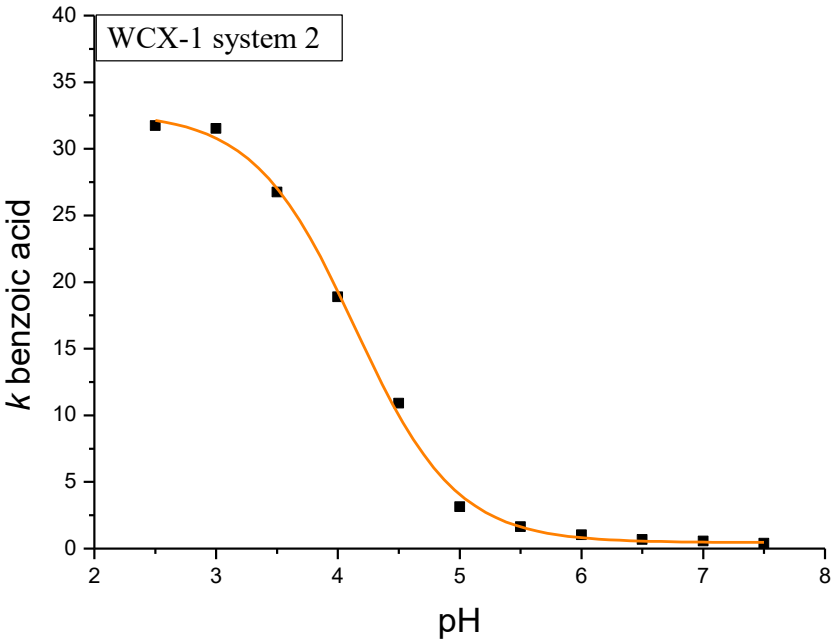


Figure 11: Dependence of the retention factor on pH – benzoic acid measured at 220 nm on Acclaim WCX-1 using buffer system 2

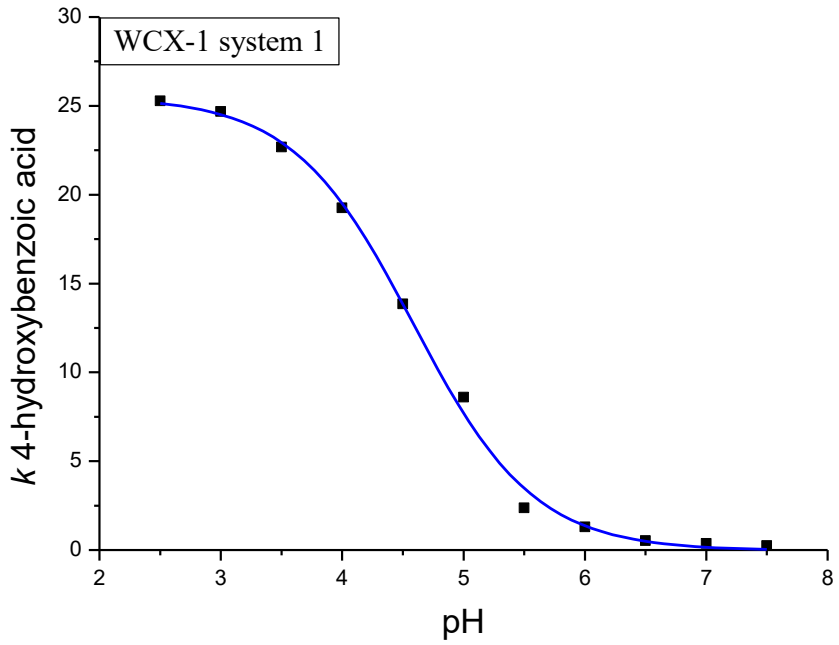


Figure 12: Dependence of the retention factor on pH – 4-hydroxybenzoic acid measured at 254 nm on Acclaim WCX column using buffer system 1

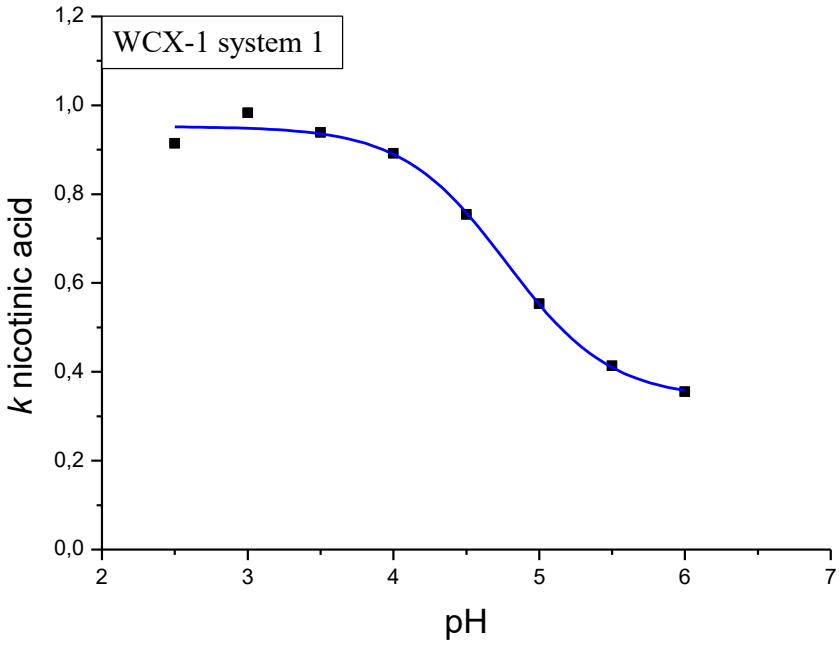


Figure 13: Dependence of the retention on pH – nicotinic acid measured at 254 nm on Acclaim WCX-1 column using buffer system 1

Table 6: Calculated p*K*a values from measured retention data as a function of pH.

	p*K*a values				
analyte	**WAX s1**	**WAX s2**	**WCX s1**	**WCX s2**	**literature***
aniline	4.72	4.65	4.36	3.57	4.6
benzoic acid	3.62	3.70	4.08	4.14	4.2
4-hydroxybenzoic acid	3.95	3.92	4.59	4.48	4.54
nicotinic acid	4.58	4.60	4.77	4.69	4.75

* these values are taken from section 2.5

Based on this data, it can be induced that the most accurate value can be observed using mixed mode with anion exchange – a stationary phase containing a positively charged moiety for a base (aniline) p*K*a determination and cation exchange – a stationary phase containing a negatively charged moiety for acid's p*K*a determination.

4.6 Determination of analyte's log *P* value

As previously stated in section 2.4.1, the analyte retention factor in the mobile phase composition of 100 % aqueous solution (log k_w), can be correlated with its tabulated log *P* parameter. This was done for 3 analytes using the reversed phase XTerra MS C18 column (Figure 5). The column type was chosen for its low silanol activity, which could distort the experiment. Methanol (MeOH) was chosen as the organic modifier in the following experiments utilizing a mobile phase gradient. This particular organic solvent was chosen, as using high ratios of acetonitrile as the modifier caused undesired precipitation in the aqueous buffer solutions of pH 2.5 for both buffer systems when organic modifier ratio became abundant.

In the table 7 below, the first value is the log k_w - retention factor directly measured in 100 % buffered aqueous mobile phase. The pH of the buffer was chosen at a value, where dissociation of the given analyte was suppressed (8.6 for aniline and 2.5 for benzoic acid and 4-hydroxybenzoic acid).

The second and third log k_w is calculated from equation (9) assuming the value of $S = 4$ and a methanol gradient of 40 min and 80 min from 5 % to 100 % volume ratio of methanol.

The last calculated and listed values of log k_w and S are the result of numerical analysis from the two previously defined gradients, and therefore two equations of type (9) with two unknowns - S and k_w values. These were solved by Python's fsolve function.

All of the listed results can be compared to the log *P* values obtained from literature in the last column in Table 7.

Nicotinic acid (log *P* = 0.36) value of log k_w could not be determined from the mobile phase gradient experiments, because of its low retention in a fully aqueous mobile phase of pH 2.5 on C18 column (retention time of approximately 2 minutes). This fact therefore limits the application of this method in this setting, for it to be used only on moderately polar analytes - analytes with higher retention of at least roughly 6 minutes. The numerical result of the k_w for benzoic acid displayed a great error because of the result being 5 digits long in the used method (k_w = 67322), having no physical meaning. This is possibly caused by both gradients being chosen as too steep for this higher retained analyte.

Table 7: Comparison of analyte's log k_w values and *S* values obtained from different approaches with their known log *P* values.

analyte	log k_w values					log *P**
	Isocratic	40 min grad	80 min grad	Numerical k_w	Numerical *S*	
aniline	1.11	0.92	0.94	0.95	4.20	0.94
benzoic acid	1.89	1.76	1.97	4.83	14.4	1.87
4-hydroxybenzoic acid	1.20	1.09	0.88	0.82	2.90	1.58

* these values are taken from section 2.5

From this table it can be observed that log k_w values of aniline calculated from single or two mobile phase gradients are great estimates of aniline's actual log *P* value, while for the acids, the isocratically measured log k_w values gave the prediction closest to reality. For aniline, the values obtained from single gradient runs gave a result similar for the numerical, which combined the two, thanks to the valid assumption that the value of *S* would equal approximately 4.

4.7 Gradient p*K*a values determination

The pH gradient was generated on the reversed phase XTerra MS C18 column using buffer system 1 and was assumed to be linear in time. This hybrid-based column displayed a stability in a wide range of pH (1-12), which was beneficial for the measurement. To efficiently carry out the dissociation constant determination, optimal methanol modifier ratio was selected for every analyte (Table 8) to shorten the analysis

time. The organic ratio remained constant during the entire experiment and it was the acidic pH = 2.5 and basic pH = 8.6 buffer ratio of buffer system 1 that changed in time.

Organic modifier changes the overall pH of the entire mobile phase, hence this change had to be measured and taken into account. This was done by determining the buffer(pH=8.6)/MeOH (v/v) mixture pH and buffer(pH=2.5)/MeOH (v/v) pH value at the beginning and end of gradient run on the absolute scale ${}^{s}_{w}pH$. These two values were then plotted as a linear function for every analyte[53], for aniline shown in Figure 14. Dwell time is another value that had to be determined beforehand, to properly assign the pH of the mobile phase when the analyte elutes (pH**), as dwell time represents the time it takes the generated gradient to reach the place of injection. The (pH**) was calculated from a linear calibration equation from the previously plotted dependence (Figure 14) substituting measured retention time t_R of analyte in the pH gradient run for the x value.

Mobile phase flow was set 0.4 ml/min for aniline and 4-hydroxybenzoic acid and for benzoic acid as 0.3 ml/min. The reasoning behind opting for smaller flow for benzoic acid was the high back pressure caused by higher volume ratio of organic modifier - buffer(pH=8.6)/MeOH 70/30 (v/v) (Table 8).

Then the isocratic retention of undissociated/unprotonated analyte $t_{neutral}$ ($t_{neutral}$ = t_{HA} for acids, $t_{neutral}$ = t_B for bases) was measured in the previously selected buffer/MeOH (v/v). constant ratio, ensuring retention measurement under full analyte dissociation/protonization suppression - buffer of pH 2.5 for benzoic and 4-hydroxybenzoic acid and buffer of pH 8.6 for aniline.

Next, a gradient run was completed for all analytes. For the duration of the gradient, it was important to ensure that it finished in a time shorter than the time it took the neutral form to elute. This analysis gave a resultant value of t_R.

Lastly, the retention of the fully dissociated/protonated form t_{A^-}/ t_{HB^+} was measured in the mobile phase composition, at which the gradient finished. All data obtained and/or used to calculate analyte ${}^{s}_{w}pKa$ using formula (13) for acids and formula (14) for base are listed in the table 8 below. Reduced versions of retention times were calculated by subtracting the measured dead time t_M from all of the retention times (Formula 2).

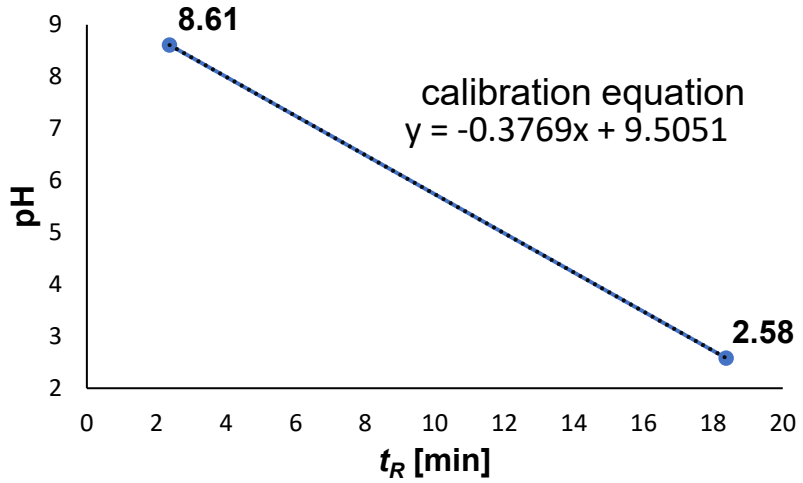


Figure 14: A linear change in mobile phase pH, determined from measurements of mobile phase pH before and after the pH gradient starts – using buffer (pH=8.6)/ MeOH 96.8/3.2 (v/v) and buffer(pH=2.5)/ MeOH 96.8/3.2 (v/v).

Table 8: Data measured for the analyte ${}^{s}_{w}pKa$ determination using a pH gradient compared to actual ${}^{w}_{w}pKa$ values (Section 2.5).

	aniline	benzoic acid	4-hydroxybenzoic acid
vol. % MeOH	3.2	30.0	5.0
flow [ml/min]	0.4	0.3	0.4
pH start	8.61	2.80	2.58
pH end	2.58	8.48	8.62
pH**	2.78	5.13	4.79
t_M [min]	1.79	2.36	1.79
t_D [min]	2.38	3.17	2.38
$t'_{neutral}$ [min]	16.06	13.39	16.77
t'_{A^-} [min]	-	0.69	0.21
t'_{HB^+} [min]	4.26	-	-
t'_R [min]	16.06	6.54	6.47
t_G [min]	16	14	16
${}^{s}_{w}pKa$	2.8	4.8	4.6
${}^{w}_{w}pKa$	4.6	4.2	4.54

The obtained results show less satisfying results, when compared to the isocratic measurement (Section 4.4), as only 4-hydroxybenzoic acid has shown an estimate close to the real value. The deviations could be attributed to the pH gradient not being linear throughout the entire pH scale, as previously shown in Figure 9A. Isocratic measurement is however more demanding for not only time but also analyte amount.

5 Conclusion

Two new mobile phase mixing systems, differing in composition, were developed in order to investigate and compare ionic interactions in different stationary phases. The method is based on mixing defined ratios of two aqueous buffering solutions with a pH of 2.5 and 8.6, giving a desired third pH value.

Mixed mode chromatography has been proven to be a useful approach in analyte separation. One of its unique features, the stationary phase behaviour modification by a change in mobile phase pH, was studied in this work, using the Acclaim^{TM} WAX-1 and Acclaim^{TM} WCX-1 mixed mode columns. The retention of neutral markers, weak and strong electrolytes and ions, used for investigating the change in separation mechanism, was measured on a scale of pH 2.5 – 7.5 in 0.5 steps. The newly developed mobile phase mixing system, consisting of two buffers, went through this scale efficiently and can be used on similar measurements.

From these data, the following observations were established - the WAX-1 column appears to lose its positive charge and anion exchange mechanism above the pH 5 while the WCX-1 column gains its cation exchange at lower values of pH, around 3.

While being called as anion (cation) exchange, the columns are not only usable for anionic (cationic) analytes as their name suggests, but they do provide longer retention for these types of analytes in general. WAX with a positive charge has shown a better p*K*a estimation ability for a weak base – aniline and WCX with a negative charge for benzoic, 4-hydroxybenzoic and nicotinic acid. These results conclude that the greatest estimate comes from data where repulsion takes place between the analyte and the stationary phase.

Classical hybrid reversed phase column XTerra MS C18 was used for a determination of aniline, benzoic and 4-hydroxybenzoic acids p*K*a values from a pH gradient run and log *P* from an elution strength gradient, utilising the previously mentioned two buffer mixing system. For this approach, nicotinic acid has shown to be unsuitable, with a retention too low, limiting the use of this method in this setting for only moderately polar analytes with a log *P* of at least 0.5 units. The p*K*a determination done in isocratic conditions with mixed mode columns showed a stronger estimate, compared to the pH gradient mode utilising the reversed phase column. The former is however more demanding for both time and analyte amount used for the measurement.

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