Exploring the dual retention mechanism of mixed-mode Acclaim WAX-1 columns to tune selectivity in liquid chromatography

Abstract
Mixed-mode chromatography columns, combining two or more surface chemistries, have been successfully introduced as a valuable tool to separate sample mixtures containing a broad range of polar and hydrophobic constituents. In this study, the retention properties of a reversed-phase/weak-anion-exchange Thermo Scientific™ Acclaim™ Mixed-Mode WAX-1 column were systematically investigated for different compound classes including aromatic hydrocarbons, halogenated aromatic hydrocarbons, aromatic alcohols, and acids, which vary in hydrophobicity, Van der Waals surface area, and charge (depending on mobile phase pH). This white paper guides the reader to select the best operating conditions to change the selectivity and consequently the resolution for a separation of target compounds by altering the acetonitrile content, salt concentration (phosphate counter-ion), and mobile-phase pH. It has been demonstrated that the Acclaim Mixed-Mode WAX-1 column can perform as a traditional reversed-phase column but with complementary selectivity to anionic analytes if needed, as its unique stationary-phase chemistry enables the operator to tune the retention of aromatic and non-aromatic acids for example, and still maintain the retention of neutral/hydrophobic analytes.

Authors
José Luís Dores-Sousa¹,
Mauro De Pra²,
Frank Steiner²,
Ilze Birznieks³,
Sebastiaan Eeltink¹
¹Vrije Universiteit Brussel (VUB),
Dept. of Chemical Engineering,
Brussels, Belgium
²Thermo Fisher Scientific,
Germering, Germany
³Thermo Fisher Scientific,
Sunnyvale, CA, USA

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Introduction

The resolution \( (R_s) \) equation defined by Purnell\(^1\) describes the parameters that influence the effectiveness of a chromatographic separation:

\[
R_s = \frac{\sqrt{N}}{4} \cdot \left( \frac{\alpha - 1}{\alpha} \right) \cdot \left[ \frac{k_2}{1 + k_2} \right]
\]

where \( N \) represents the number of theoretical plates, \( \alpha \) is the selectivity, and \( k_2 \) refers to the retention factor of the most retained analyte of the critical-pair separation. An effective separation is achieved when the \( R_s \) of the critical pair reaches 1.5. The increase in \( R_s \) is only proportional to the square root of \( N \), which implies that a fourfold increase in \( N \) is needed to double the \( R_s \). Ideally, the elution conditions should be tuned such that the critical pair elutes between \( 2 < k < 5 \), since this would lead to the shortest analysis time.\(^2\) Hence, the most profound approach to increase \( R_s \) is to optimize selectivity.

Compared to conventional reversed-phase liquid chromatography (RP-LC), mixed-mode columns provide a broader selectivity range, giving the user alternatives to improve the resolution of the separation. Mixed-mode stationary phases have two or more combined chemistries within a single column, allowing improved probing of chemical (and group-type) properties of the analytes. In this study, a combination of chemistries was utilized in which hydrophobic moieties are combined with charged moieties, providing interaction sites for RP and for anion-exchange separations. The chemical structure of the RP/WAX mixed-mode stationary phase used in this study is displayed in Figure 1. An aliphatic \( \text{C}_{10} \) alkyl chain is covalently anchored to the silica stationary-phase surface. The alkyl chain embeds a polar amide moiety and a \( \text{C}_3 \) spacer with an ionizable tertiary-amine group situated at the tip of the chain, which acts as an electrostatic interactive domain. The selectivity provided by these weak anion-exchange (WAX) moieties for anionic analytes mainly depends on the pH and the ionic strength of the mobile phase. Using MarvinSketch software, the apparent \( pK_a \) of the column resin was determined to be 9.30 in water at 25 °C. Hence, applying mobile phases with \( \text{pH} \leq (pK_a - 1) \), the resin is primarily positively charged providing weak anion-exchange properties.

Method development using mixed-mode columns is generally difficult and time-consuming, mainly due to the complexity of (mixed) molecular interactions that strongly depend on mobile phase conditions and respective analyte properties. To provide guidance on such challenging method development, this white paper comprises a systematic investigation to assess retention properties and in turn selectivity of a mixed-mode stationary phase that features both RP and WAX moieties, as a function of organic content, salt concentration, and pH of the mobile phase. An extended study describing column selectivity and method development using the Acclaim Mixed-Mode WAX-1 column has been published by Dores-Sousa and co-workers.\(^3\)

![Figure 1. Structure of the silica-based stationary-phase chemistry of the mixed-mode reversed-phase/weak anion-exchange (RP/WAX) column](image-url)
Experimental details

LC instrumentation and conditions

Instrumentation: Thermo Scientific™ UltiMate™ 3000 UHPLC system equipped with
   SRD-3400 degasser
   HPG-3200RS binary pump
   WPS-3000TRS well-plate autosampler
   TCC-3000RS column oven
   DAD-3000RS UV-VIS detector

Column: Acclaim WAX-1, 5 µm 120 Å,
   4.6 x 150 mm (P/N 064984)

Mobile phase: A: 25–100 mM (KH₂PO₄)
   B: Acetonitrile (ACN)
   Mobile phase composition: 30, 40, 50, 60, or 70% (v/v) ACN in
   25 mM KH₂PO₄ at pH 6
   Mobile phase compositions of 50:50% (v/v) ACN:H₂O with
   12.5, 25, 37.5, or 50 mM KH₂PO₄,
   final concentration at pH 6
   Mobile phase compositions of 50:50% (v/v) ACN:H₂O containing
   25 mM KH₂PO₄, at pH 6 or pH 7,
   and 25 mM H₃PO₄ at pH 2.5

Flow rate: 1 mL/min, isocratic elution mode
Column temp.: 25 °C
Injection volume: 1 µL
Detection: UV detection at 210 nm,
   10 Hz data collection rate,
   2 s response time

Sample preparation
Stock solutions were prepared in 50:50% (v/v)
   ACN:water. All compounds were injected with a final
   concentration of 100 ppm.

Results and discussion
To probe the mixed-mode retention behavior, analytes
   with a wide range of physicochemical properties
   were selected based on Van der Waals surface area,
   pKₐ, log P value, and the number of functional group
   substituents. Figure 2 shows an overview of the different
   compound classes. The grey color represents the class
   of analytes that display retention based on van der Waals
   interactions, blue indicates chemical interaction with the
   stationary phase via hydrogen bonding, and the red color
   code indicates the presence of electrostatic interactions.
   The green color code indicates the presence of a halogen
   atom that may interact via the carbonyl moiety.

Effect of acetonitrile content in mobile phase
The effect of acetonitrile content in the mobile phase
   on retention was investigated while keeping the salt
   concentration (25 mM) and pH (6.0) constant. Figures 3A
   and 3B show representative separations of phenols and
   carboxylic acids applying respectively 50% and 40% ACN
   in the mobile phase (with benzene added as reference
   solute). Decreasing the acetonitrile content leads generally
   to an increase in retention, governed by RP partitioning
   induced by Van der Waals interactions. However, the
   selectivity for acidic analytes was significantly affected
   with respect to aromatic hydrocarbons. At the conditions
   applied, the carboxylic moieties of the acids are negatively
   charged and both IEX and RP interactions with stationary
   phase will occur. Depending on the charge state, i.e.,
   singly- or double-charged, the mixed-mode retention
   behavior will be more apparent. Obviously, double-
   charged analytes are less affected by the change in
   acetonitrile content, which ultimately leads to a change
   in elution order. Although isophthalic acid (analyte
   no. 14) and terephthalic acid (analyte no. 12) are isomers
   and have the same hydrophobicity index (Log P = 1.29),
   their retention behavior was different. In fact, isophthalic
   acid has a higher accessible hydrophobic area, which
   explains the higher RP retention compared to terephthalic
   acid. Generally, the measured retention factors for
   phenols were lower than that of (halogenated) aromatic
   hydrocarbons. This may indicate that although phenols
   are prone to show hydrogen bonding, this interaction is
   mostly absent.

Previously it has been reported that halogen functionalities
   can be polarizable and may interact via a non-covalent
   halogen bonding (which is thought to be similar to
   hydrogen bonding in terms of strength and directionality)
   with the carbonyl group of amide functionality. 4–6 To
   assess the presence of possible halogen bonding, LC
   experiments were conducted to compare selectivity
   between the halogenated aromatic hydrocarbons and
   aromatic hydrocarbons, on the RP/WAX column and on
   its equivalent RP column, by normalization to the carbon
   content of the stationary phases. However, no retention
   effects due to halogen bonding were observed for the set
   of test analytes employed within the range of experimental
   conditions applied.
The retention factors \[ k = (t_R / t_0) - 1 \] of the different compounds were determined for different ACN compositions and modeled using the linear solvent-strength (LSS) model: 

\[ \log k = \log k_w - S \cdot \phi \]

where \( k_w \) is the extrapolated value of \( k \) for water as mobile phase (\( \phi = 0 \)) and is related to the lipophilicity of the solute, and the slope \( S \). When \( k_w \) and \( S \) are known it is possible to predict retention time in isocratic mode (as a function of acetonitrile content in the mobile phase), but also in gradient mode. The \( \log k \) values of the (halogenated) aromatic hydrocarbons decreased linearly with the increase in organic content, following the LSS model and yielding correlation coefficients \( R^2 > 0.98 \). It was shown that the \( S \) (the slope) value for (halogenated) aromatic hydrocarbons is correlated to the Van der Waals surface area. Furthermore, the \( \log k_w \) (intercept) increased proportionally to its \( \log P \) values. For the phenols and acidic analytes no correlation between \( S \) and Van der Waals surface areas could be derived. While the \( \log P \) value is a good indicator for the \( \log k_w \) value for phenols, no correlation was observed for the charged compounds, which is a direct effect of the mixed-mode retention behavior.

**Figure 2.** Overview of the different classes of compounds, represented by different colors

**Figure 3.** Effect of acetonitrile content in the mobile phase on selectivity of phenols and carboxylic acids. Experimental conditions: (A) 50:50% (v/v) ACN:HO containing 25 mM potassium dihydrogen phosphate (KH₂PO₄), pH 6.0, and (B) 40%/60% (v/v) ACN in 25 mM KH₂PO₄, pH 6.0. Peak identification: (1) benzylalcohol, (2) 2-phenylethanol, (3) benzene (added as reference solute), (4) acetic acid, (5) 1,2-dihydroxybenzene, (6) trifluoroacetic acid, (7) benzoic acid, (8) succinic acid, (9) tartaric acid, (10) \( p \)-toluic acid, (11) thiophenol, (12) terephthalic acid, (13) ketoprofen, and (14) isophthalic acid.
Effect of salt concentration
Figure 4 shows the influence of phosphate (counter-ion) concentration on retention, while maintaining the ACN content and pH of the mobile phase constant. As expected, the retention for non-charged analytes remains unaltered (analyte no. 1 and 2). The use of phosphate counter-ions in the mobile-phase will have a different effect on the retention behavior of singly and doubly charged acidic compounds, as doubly charged acids are more sensitive to the variation of the counter-ion concentration. The retention is affected when a mixed-mode retention mechanism is present, i.e., it is also dependent on the hydrophobicity of the solute. The molecular structure of the analyte is important when considering electrostatic interactions, since the chemical orientation of the carboxylic groups affects retention.

![Figure 4. Effect of salt concentration (or phosphate counter-ion concentration) in the mobile phase on retention of singly- and doubly-charged carboxylic acids.](image)

Effect of pH
The ion-exchange capacity and hence selectivity provided by the RP-WAX resin depends on its pKa (approximated to be 9.3) and the mobile-phase pH applied. The effect of mobile-phase pH on retention of carboxylic acids was assessed, while maintaining ACN content and the salt concentration constant. The chromatograms depicted in Figure 5 were obtained at pH = 2.5, 6, and 7, respectively.

![Figure 5. Effect of pH content in the mobile phase on retention and selectivity.](image)
It can be observed that the retention of non-charged compounds remained unaffected (analyte no. 1 and 2) when applying different pH elution conditions. Retention factors of all acidic compounds reached the highest value at pH 6, since at this pH the analytes are negatively charged, while the resin is positively charged, and the maximum degree of dissociation is reached. Increasing the pH further (see Figure 5C) led to a decrease in retention factor, which may be caused by a change in charge state of the stationary phase, i.e., reduction of the number of available ionizable moieties, and/or higher presence of double negatively charged counter-ions in the mobile phase.

Concluding remarks
• Retention and selectivity of a RP/WAX mixed-mode column was evaluated for the separation of a range of small molecules with varying physicochemical properties.
• The current study provided insights in experimental conditions that need to be applied to probe specific analyte-stationary phase interactions.
• Molecular structure and chemical orientation of the functional groups are important on retention and selectivity.

References