

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

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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.

Introduction

The resolution (R_s) equation defined by Purnell¹ 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, α 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 C_{10} alkyl chain is covalently anchored to the silica stationary-phase surface. The alkyl chain embeds a polar amide moiety and a 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 $pH \leq (pK_a - 1)$, 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 Does-Sousa and co-workers.³

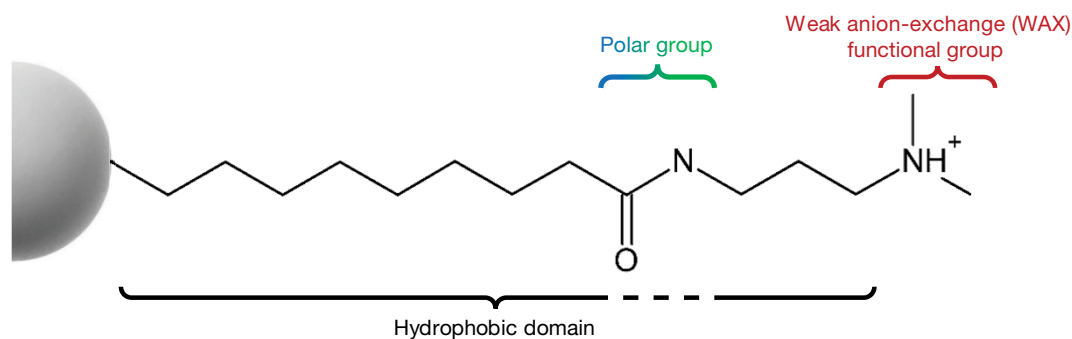


Figure 1. Structure of the silica-based stationary-phase chemistry of the mixed-mode reversed-phase/weak anion-exchange (RP/WAX) column

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 \AA , 4.6 \times 150 mm (P/N 064984)
Mobile phase:	A: 25–100 mM (KH_2PO_4) B: Acetonitrile (ACN) Mobile phase composition: 30, 40, 50, 60, or 70% (v/v) ACN in 25 mM KH_2PO_4 at pH 6 Mobile phase compositions of 50:50% (v/v) ACN:H ₂ O with 12.5, 25, 37.5, or 50 mM KH_2PO_4 , final concentration at pH 6 Mobile phase compositions of 50:50% (v/v) ACN:H ₂ O containing 25 mM KH_2PO_4 , at pH 6 or pH 7, and 25 mM H_3PO_4 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, pK_a , $\log P$ 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.

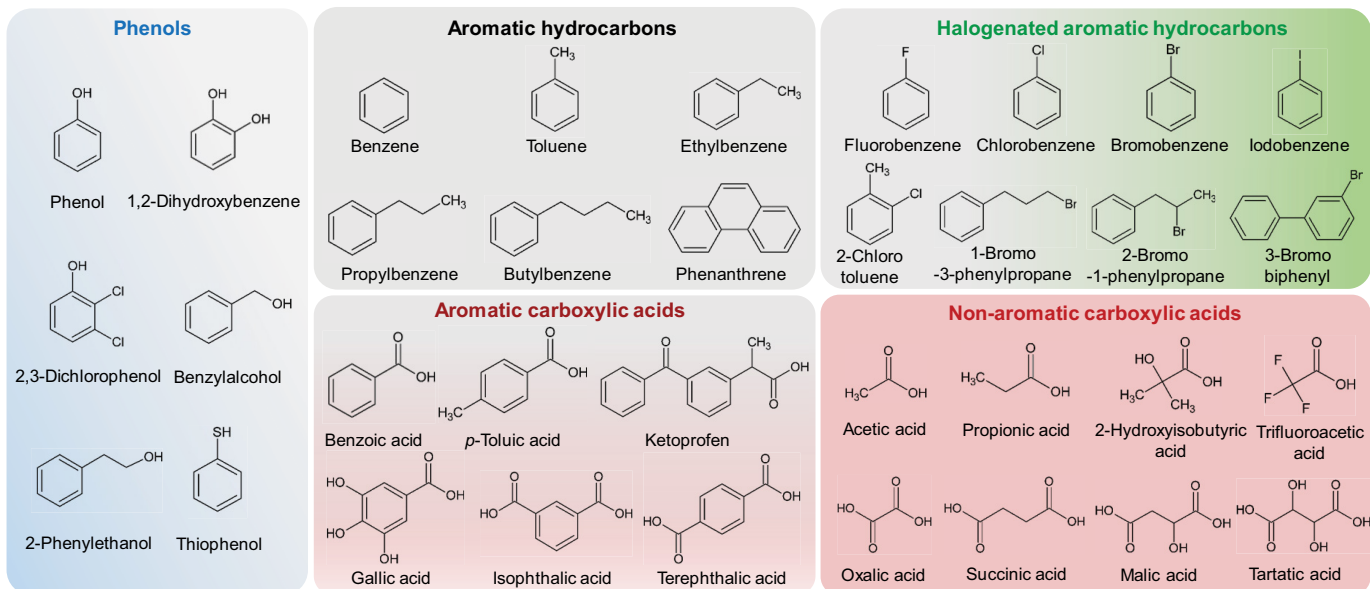


Figure 2. Overview of the different classes of compounds, represented by different colors

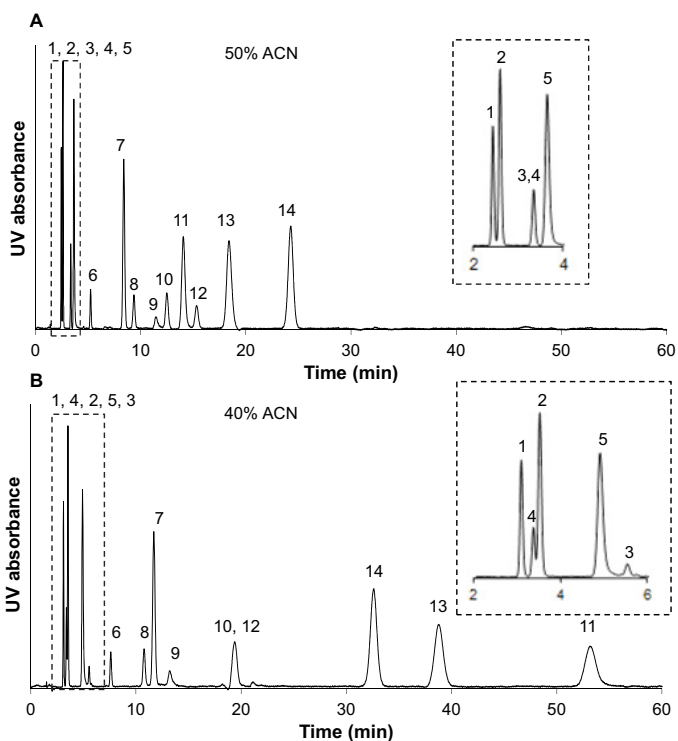


Figure 3. Effect of acetonitrile content in the mobile phase on selectivity of phenols and carboxylic acids. Experimental conditions: (A) 50:50% (v/v) ACN:H₂O containing 25 mM potassium dihydrogen phosphate (KH₂PO₄), pH 6.0, and (B) 40%/60% (v/v) ACN in 25 mM KH₂PO₄, pH 6.0. Peak identification: (1) 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

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:⁷

$$\log k = \log k_w - S \cdot \phi$$

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.⁸ 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.

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.

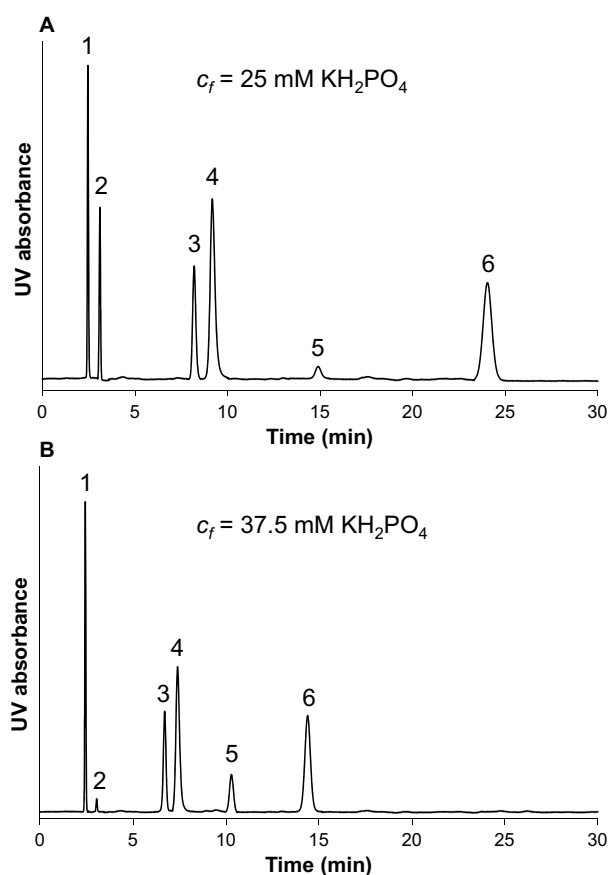


Figure 4. Effect of salt concentration (or phosphate counter-ion concentration) in the mobile phase on retention of singly- and doubly-charged carboxylic acids. Experimental conditions: (A) 50:50% (v/v) ACN:H₂O containing 25 mM potassium dihydrogen phosphate (KH₂PO₄) pH 6.3, and (B) 50:50% (v/v) ACN:H₂O containing 37.5 mM KH₂PO₄, pH 6.3 (after mixing with ACN). Peak identification: (1) 2-phenylethanol (added as reference solute), (2) benzene (added as reference solute), (3) benzoic acid, (4) gallic acid, (5) terephthalic acid, and (6) isophthalic acid

Effect of pH

The ion-exchange capacity and hence selectivity provided by the RP-WAX resin depends on its pK_a (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.

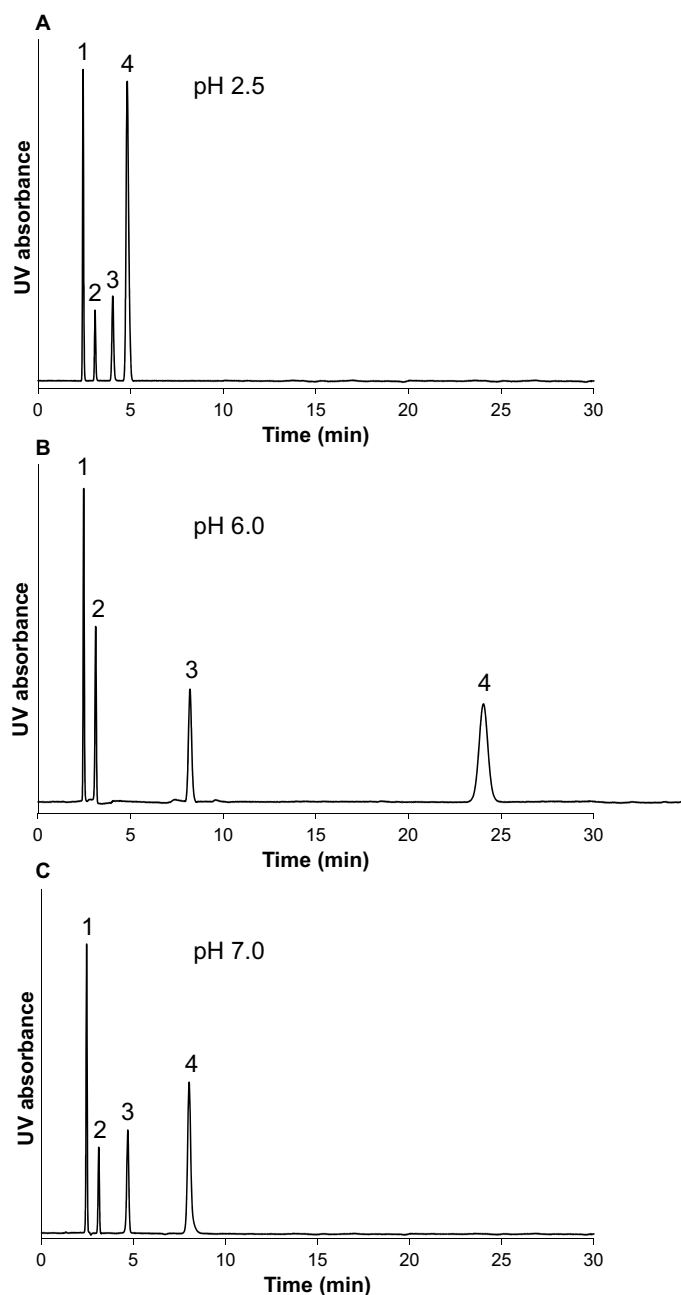


Figure 5. Effect of pH content in the mobile phase on retention and selectivity. Experimental conditions: (A) 50:50% (v/v) ACN:H₂O containing 25 mM KH₂PO₄, at pH 6, (B) pH 7, and (C) 25 mM H₃PO₄ at pH 2.5. Peak identification: (1) 2-phenylethanol, (2) benzene, (3) benzoic acid, and (4) isophthalic acid

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.

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