Evolution of Mixed-Mode Chromatography

Vlad.Orlovsky@sielc.com, Yury.Zelechonok@sielc.com
SIELC Technologies

Mixed-mode chromatography is emerging as a powerful tool in separation of various molecules. Mixed-mode is defined as liquid chromatography in which at least two modes of interactions exist simultaneously, both controllable by mobile phase selection. In the last few years, several companies have introduced new mixed-mode columns with different stationary phases (SIELC, Dionex, Imtakt, Sepax, Agela, etc). Mixed-mode approach emerged due to a need for better retention control for both polar and non-polar molecules. Multiple mechanisms of interactions allow the use of one stationary phase for a much wider range of applications as compared to reversed-phase or ion-exchange approach. Simultaneous analysis of polar and non-polar, ionisable and neutral, and organic and inorganic compounds is possible, adding ability to reduce number of methods for pharmaceutical formulations and other complex mixtures. Modern mixed-mode stationary phases are designed with the purpose of creating controllable multiple interactions, and to provide reproducibility, high efficiency and loadability. Tools are available for efficient method development [1-3]

History of Mixed-Mode Chromatography

Mixed-mode interactions have been known for decades, and for the most part Liquid Chromatography column stationary-phase design has been dominated by the goal of elimination of extra interactions and obtaining a simple and predictable single retention mechanism. Unfortunately, simplification of the retention process limits the range of compounds for simultaneous analysis, and reduces the ability to control elution order of analytes as well as the scope of available applications the system can be used for. As a response to this limitation, hundreds of different reverse-phase columns have been introduced in the past few years, all with slightly different characteristics. While working mainly by hydrophobic mechanism, those columns provide additional weak poorly controllable interactions.

At the same time, there were some limited attempts to commercialise mixed-mode columns. Such columns were introduced by several column manufacturers using different approaches (Figure 1).

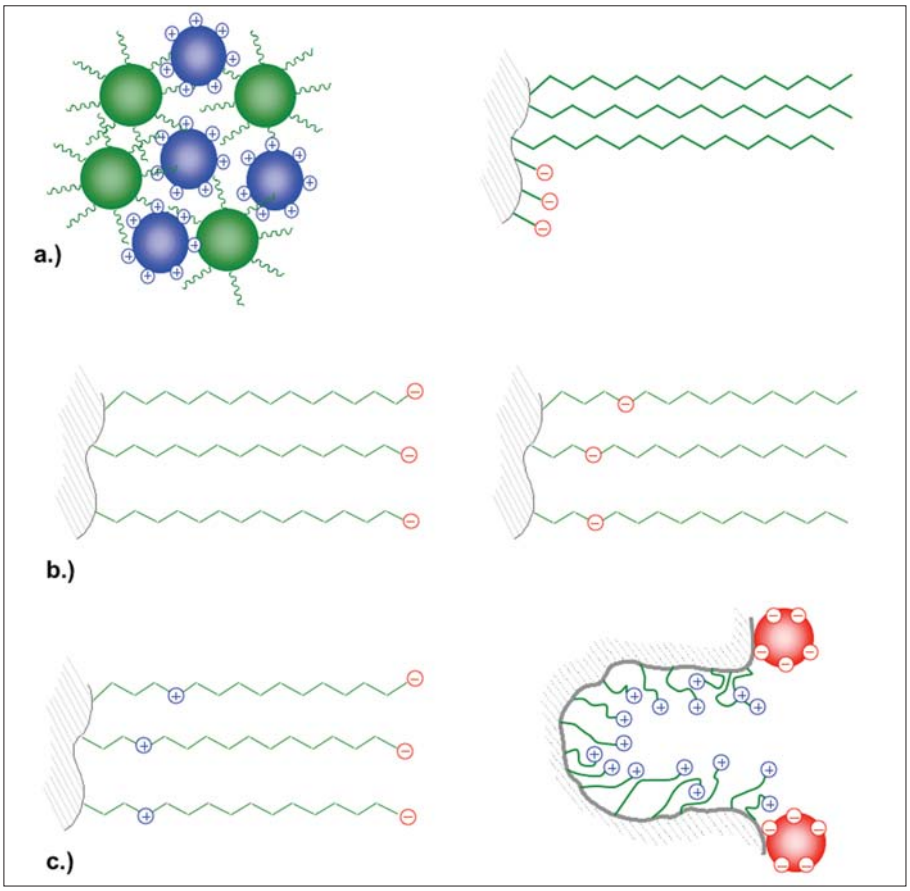


Figure 1. Variations of commercially available mixed-mode packing materials.

In fact, the first generation of mixed-mode columns (Figure 1a) is represented by low-coverage C18 stationary phases. Residual silanol groups provide additional ionic interaction. These groups have acidic properties with pKa of about 5. A problem with this approach is that inclusions, or contaminations, in silica gel can affect acidity of the silanols, and thus cause un-uniformity of ionic sites. Other problems include the consistency of partial coverage, and the narrow range of mobile phase pH to provide the silanol ionisation.

A different approach included physical mixing of different type of silica gels to obtain the "mixed-mode" effect.

Second-generation mixed-mode columns (Figure 1b) combine one of the ion-exchange

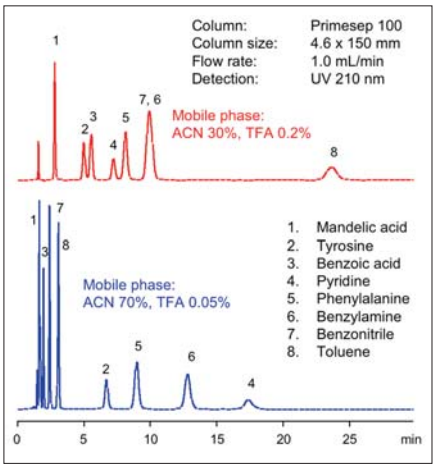


Figure 2. Ion-exchange and reverse phase mechanism in separation of acids, bases, amino acids, and neutral compounds

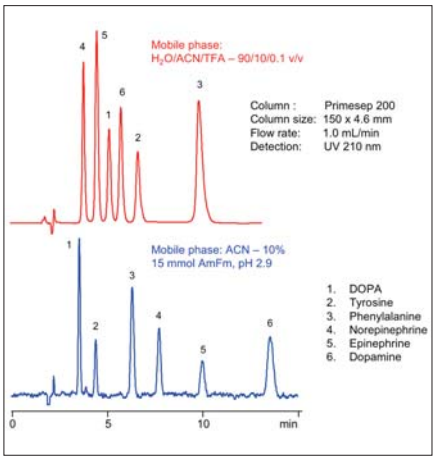


Figure 3. LC/MS and prep compatible separation of catecholamines

groups as a part of hydrophobic ligand attached to the surface. These columns show better reproducibility and retention control than the first generation. Several studies successfully demonstrated applicability of these columns to retention of neutral and ionic compounds [4]. These stationary phases are used for analysis of acidic, basic and neutral compounds. In case of dual interaction, ions opposite the stationary phase are retained by ion-exchange interaction [5]. Hydrophilic acidic compounds are usually transformed into a non-ionised form, reducing compound polarity. A typical example includes analysis of basic, acidic and neutral compounds in one run at lower pH, when carboxylic groups are not ionised and the acidic compound is retained by reversed-phase interaction (Figure 2).
Two main approaches of third-generation (Figure 1c) mixed-mode columns are popularised by the SIELC and Dionex companies.

Dionex columns consist of ion-exchange

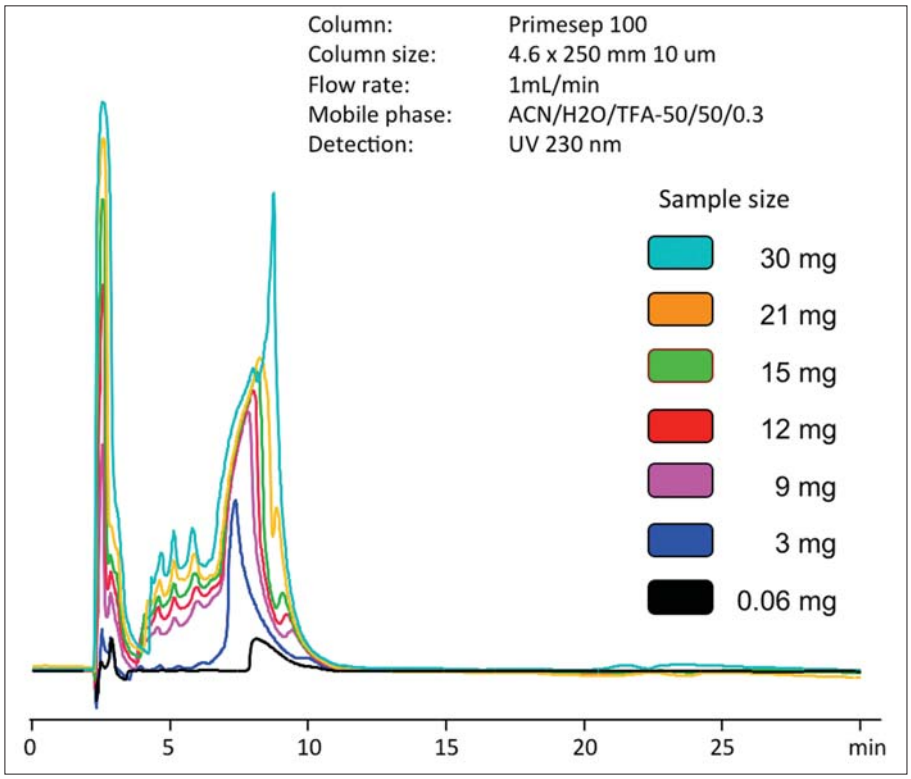
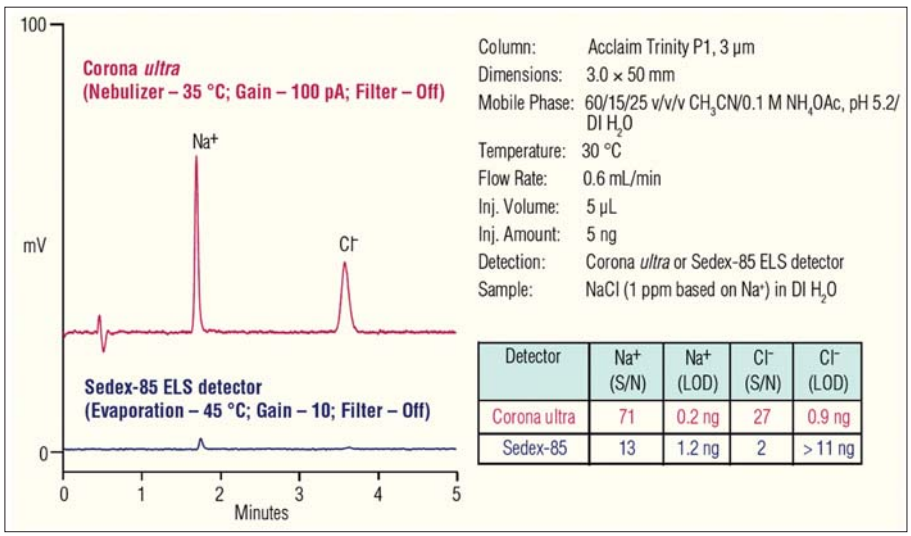


Figure 4. Loading study for a purification of a polar amino compound on analytical column.



Detector	Na^+ (S/N)	Na^+ (LOD)	Cl^- (S/N)	Cl^- (LOD)
Corona ultra	71	0.2 ng	27	0.9 ng
Sedex-85	13	1.2 ng	2	>11 ng

Figure 5. Simultaneous separation of Na+ and Cl- ions

beads placed at the surface of silica gel, providing one of the ion-exchange interactions. The second ionic interaction, as well as hydrophobic interaction, comes from the hydrophobic ligand attached to the surface. SIELC columns have a ligand that carries cation- and anion-exchange properties, as well as a hydrophobic chain assembled as one ligand, which is attached to the surface of silica.

Third-generation mixed-mode columns have increased capacity and retention control. Ionic compounds can be retained by cation- and anion-exchange mechanism, and the hydrophobic compounds by reversed-phase mechanism.

The main advantage of this stationary phase configuration is an ability to retain both polar cations and anions at the same time with low buffer concentration in the MP. Ionisation state of stationary phase and ratio between cation-exchange and anion-exchange sites can be adjusted by changing the pH of the mobile phase.

Benefits of Mixed-Mode Chromatography

Multiple controllable interactions on a column allow better control of retention for various analytes. Polar ionic and non-polar non-ionic compounds can be separated in a single run. Selectivity of separation can be controlled by varying the amounts of organic

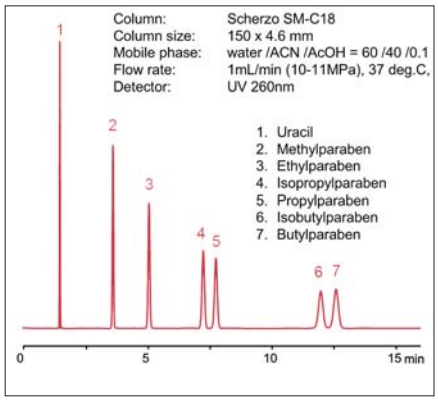


Figure 6. Analysis of Neutral hydrophobic compounds on Scherzo SM-C18 mixed-mode column

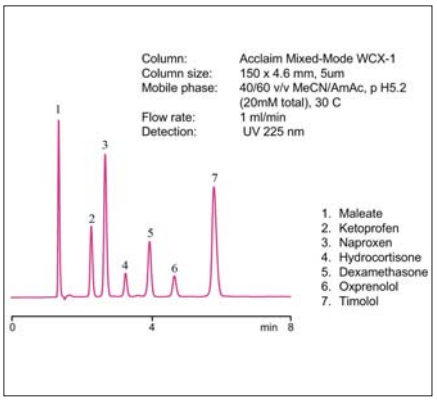


Figure 7. Simultaneous separation of acidic, neutral, and basic pharmaceuticals

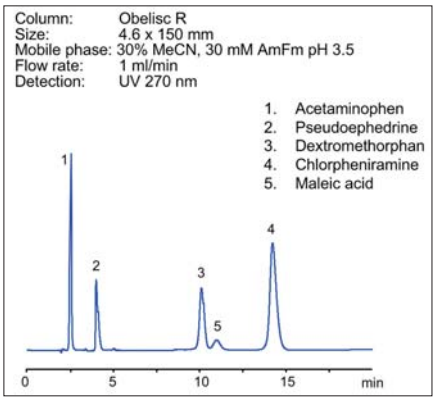


Figure 8. Effect of acetonitrile and buffer concentration on retention of basic drug and counter-ions (tri-modal column)

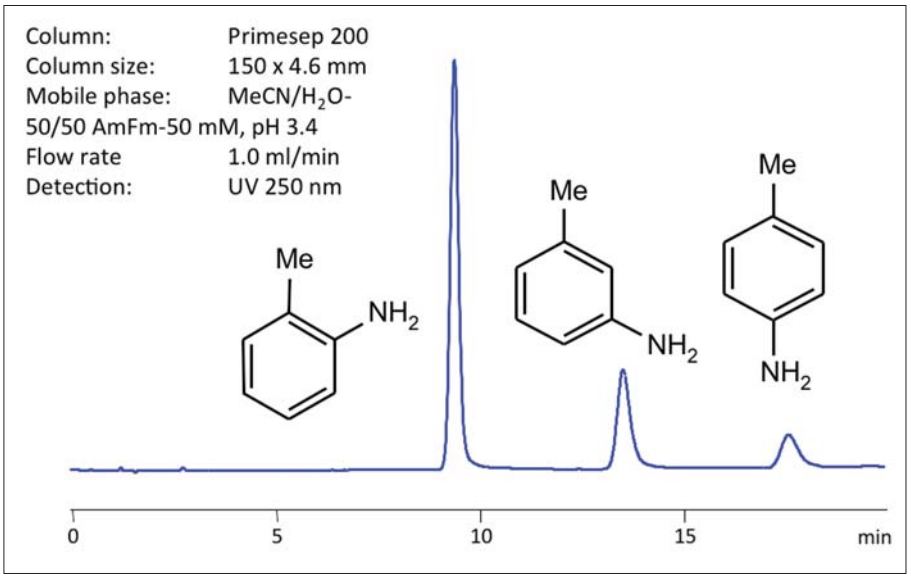


Figure 9. Separation of anilines on mixed-mode Primesep 200 column

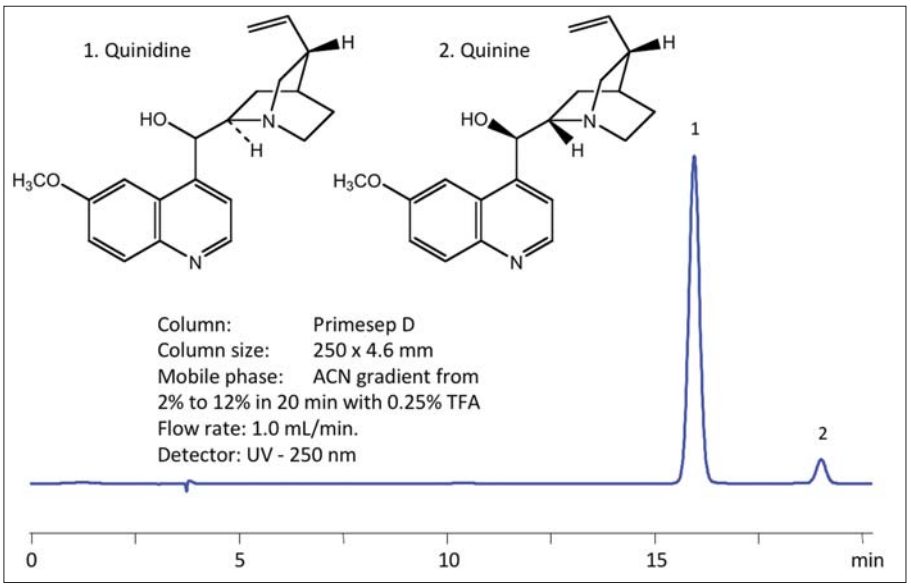


Figure 10. Separation of diostreomers (quinidine/quinine) on mixed-mode column

component, buffer pH, and buffer concentration. Contrary to reverse-phase chromatography, buffer pH will affect not only ionisation state of analytes, but also the ionisation state of stationary phase. This is because the ionic strength of the column can be increased or decreased based on the pH of the mobile phase. Additional ion-exchange interactions allow the development of methods without ion-pairing reagents for polar compounds that are compatible with LC/MS and prep chromatography (Figure 3). Large numbers of ion-exchange sites on the stationary phase increases loadability of the column (Figure 4). Symmetrical peak shape is often observed for basic molecules. First-generation mixed-mode column (with ion-exchange interaction coming from silanols), made it easy to overload weak silanol groups, resulting in peak tailing [6].

Another benefit of mixed-mode chromatography is that the same column can be used either in a single mode (cation-exchange, anion-exchange, reversed-phase), or in combination of modes. Sodium and chloride ions can be separated in pure ion-exchange node on Trinity mixed-mode columns (Figure 5). Mixed-mode columns can be used in pure reversed-phase mode for separation of neutral hydrophobic compounds (Figure 6). Mixed-mode columns provide same efficiency as regular reversed-phase columns, with plate count achieving 100K/meter for 5um particles. Same columns were also used in separation of more complex mixtures, including hydrophilic ionised, hydrophobic ionised, and hydrophobic neutral compounds. This approach can be used for analysis of drugs and corresponding counter-ions, pharmaceutical formulations, and other complex mixtures (Figures 7, 8). Retention times for some compounds can be adjusted independently to facilitate different selectivity and even to control the order of elution.

Complex interaction of mixed-mode column with analytes often produced superior selectivity as compared to single mode column case. Structural and diostereoisomers of compounds with charged functional groups can be resolved easily with mixed-mode approach (Figures 9, 10)

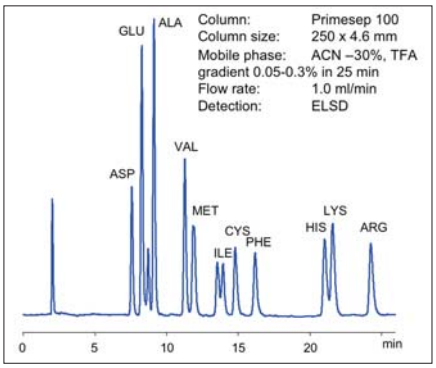


Figure 11. Separation of 12 amino acids

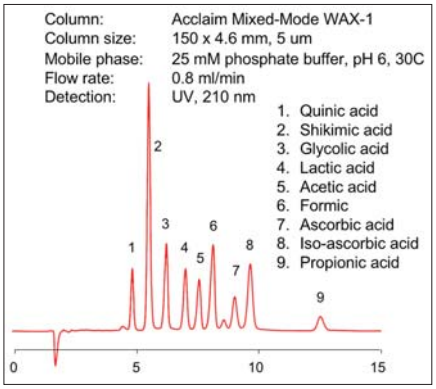


Figure 12. Separation of monocarboxylic acids

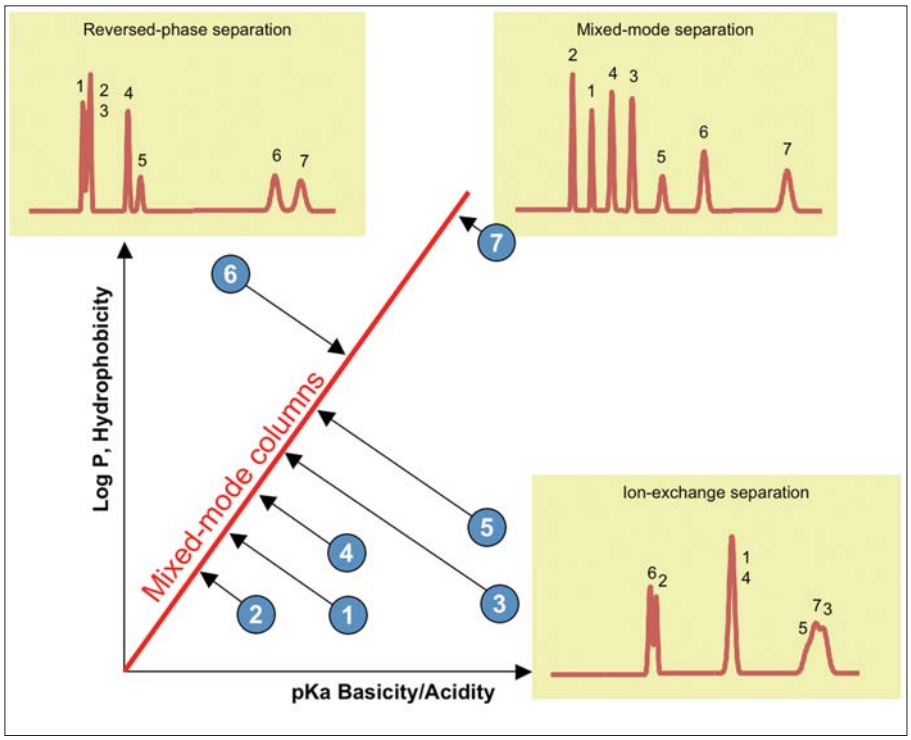


Figure 13. 2D Property of the complex mixture in 2D plane and its separation by reverse-phase, ion-exchange, and mixed-mode chromatography mode

Case Studies for Mixed-Mode Chromatography

1. Analysis of Amines and Amino Acids in Reversed-Phase Ion-Exchange Modes

Amines and amino acids are used as building blocks in many chemical and pharmaceutical productions. These molecules are hydrophilic in nature. Several methods are used to analyse amines and amino acids. In one approach, derivatisation is used to convert hydrophilic molecules with low UV activity to more hydrophobic molecules with UV activity [7]. Ion-pairing reagent, in combination with regular reversed-phase chromatography, provides good retention and selectivity, and is proven to be a robust approach [8]. Unfortunately, ion-pairing chromatography is not compatible with LC/MS and preparative chromatography. Mixed-mode stationary phase can be considered as ion-pairing reagent attached to the surface. In mixed-mode columns, significant retention occurs via the cation-exchange sites of the stationary phase. At lower pH, amines are always positively charged and amino acids are basic in nature. Additionally, at lower pH, carboxylic acid functionality of amino acids is not ionised and amino acids will retain by the combination of reversed-phase and cation-exchange mechanism (Figure 11). The only requirement is that acidic functionality of stationary phase remains ionised at lower pH (pKa below 3). Mixed-mode columns with strong acidic functionalities on the surface allow a wide range of mobile phase composition and pH providing enhanced cation-exchange interactions. If pH of the mobile phase is lower than pKa of the acidic group on the column surface, the ion-exchange mechanism diminishes, which makes the mixed-mode phase similar to reversed-phase when tested with all type of compounds.

2. Analysis of Organic and Inorganic Acids in Reversed-phase Ion-Exchange Modes

Two approaches exist in analysis of acidic compounds. The first includes retention by reversed-phase and anion-exchange mechanism - pH of the mobile phase in this case needs to be above the pKa of the acids (Figure 12). This will ionise the analyte and will increase anion-exchange interaction between analyte and stationary phase. In the second approach, mobile phase pH is lower than pKa of the acid. The acid becomes non-ionised and more hydrophobic, and it is retained by reversed-phase mechanism [9]

3. Analysis of Complex Mixtures in Mixed-Mode

Complex mixtures are common in pharmaceutical formulations. Such mixtures contain acidic, basic organic, and inorganic compounds, and hydrophobic and polar compounds. Presence of multiple compounds and related impurities requires additional selectivity of separation. Mixed-mode stationary phase offers a 2D selectivity on a single column (Figure 13). Small difference in hydrophobic or/and ionic properties usually provide sufficient selectivity when analysed on mixed-mode column. Adjustment of organic modifier or buffer concentration allows for enhancement or suppression of hydrophobic or ionic interaction. The mobile phase pH selection is another powerful tool for selectivity control (Figure 14), providing strong effect on relative retention of charged species.

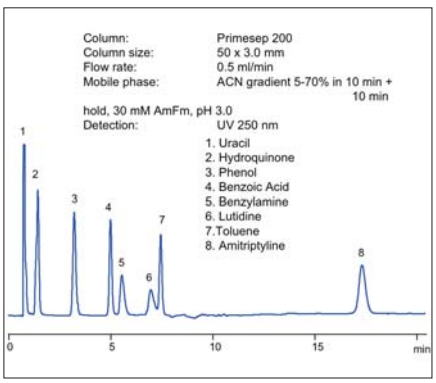


Figure 14. Separation of complex mixture on Primesep 200

4. Analysis of Polar Neutral and Ionic Compounds in HILIC/Mixed-Mode

HILIC on bare-silica and aminopropyl columns can be considered as early cases of weak cation-exchange/HILIC and weak anion-exchange/HILIC [10]. In order to see cation-exchange effect of silanols, the pH of

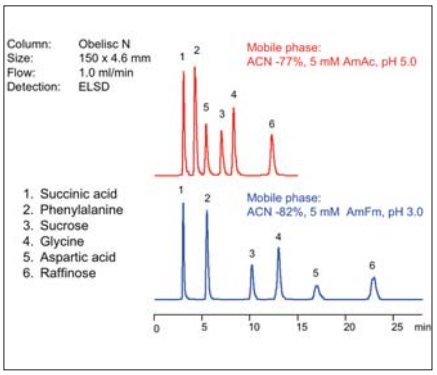


Figure 15. Effect of both pH and organic content. on a separation of sugars, amino acids, and carboxylic acids

the mobile phase should be above 5. Bare-silica HILIC columns suffer from non-uniformity of the surface in terms of cation-exchange properties due to mentioned before inclusions, and aminopropyl column suffer from stability issues. Sequant managed to overcome these stability and non-uniformity issues by developing zwitter-ionic stationary phase [11]. Presence of ionised quaternary amino and sulphonates groups guarantees a very polar uniform surface. However both groups are closely positioned to each other, making the ion exchange mechanism almost impossible to achieve. In order to observe additional selectivity, for example, by ion-exchange mechanism, two appositely charged groups should be placed farther apart on the surface. This approach, provided by SIELC's Obelisc N columns, has both ion-exchange functionalities (cation and anion-exchange) combined, but spaced far apart. Strongly basic and strongly acidic groups are separated by long hydrophilic chain, which makes these ionisable groups available for both ion-exchange interactions (Figure 15).

Conclusions

Modern mixed-mode columns offer great flexibility, reproducibility, and loadability for separation of a wide range of compounds. These columns can be employed in multiple and single modes, and can be used to develop reversed-phase, ion-exchange, ion-exclusion and HILIC methods. All modern mixed-mode columns are compatible with LC/MS and prep chromatography, and can be used to successfully develop methods in pharmaceutical, chemical, food, and environmental tasks.

Method development in mixed-mode chromatography is based on understanding of individual interactions -- hydrophobic, HILIC, and ionic -- working together.

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Notes

Acclaim is a trade mark of Dionex Corporation. Scherzo is a trademark of Imtakt. Chromatograms are courtesy of Dionex and Imtakt.