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, ionic 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].
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 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 compounds 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).

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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 diastereoisomers of compounds with charged functional groups can be resolved easily with mixed-mode approach (Figures 9, 10).
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. Ion-pairing reagent, in combination with regular reversed-phase chromatography, provides good retention and selectivity, and is proven to be a robust approach. Unfortunately, ion-pairing chromatography is not compatible with LC/MS and preparative chromatography. Mixed-mode stationary phase can be considered as an 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. Mixed-mode stationary phase offers a 2D selectivity on a single column. Small differences 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, providing strong effect on relative retention of charged species.

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, and is proven to be a robust approach. 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. Mixed-mode stationary phase offers a 2D selectivity on a single column. Small differences 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, providing strong effect on relative retention of charged species.

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 differences 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, providing strong effect on relative retention of charged species.
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 amino propyl 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
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