

New Tri-Functional Mixed-Mode Stationary Phase and Its Applications

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Introduction

High performance liquid chromatography (HPLC) is an important analytical separation technique for separation and determination of a mixture of analytes in a wide range of applications in pharmaceutical, biological, chemical, food and beverages, consumer products industries, etc. Because of the diversity of the analytes (which can be acidic, basic and neutral molecules) and the complexity of the sample matrix, their separation and identification are often challenging. HPLC columns are the heart of separations and come in different flavors depending on the retention mechanism and the operating conditions, such as reversed-phase (RP), normal-phase (NP), ion-exchange (IEX), and other column types. Among all parameters that define the characteristics of a given column, selectivity is the most critical factor for a successful separation.

Reversed phase liquid chromatography (RPLC) is the most widely used technique for separating small molecules, but it often fails to provide adequate retention of highly polar molecules. Although ion-pairing chromatography can be used to improve the retention and selectivity of ionizable analytes, it often requires long equilibration time, a dedicated column, and a complicated mobile phase that is incompatible with a mass spectrometer. Conventional IEX chromatography is used to separate ionic molecules. However, commonly used silica-based cation

exchangers exhibit inadequate hydrophobicity while the polymer resin-based exchangers frequently exhibit low chromatographic efficiency. Mixed-mode chromatography combines both hydrophobic and ion-exchange properties and facilitates the independent control of retention for ionizable and neutral molecules. Therefore, many application challenges involving a mixture of analytes with different hydrophobicities and charges that are difficult for any RP columns, can be easily tackled on a mixed-mode column. Although many HPLC columns are available and have been used for separating a wide variety of analytes, none of these columns provide optimal separation for acidic, basic, and neutral molecules in a single chromatographic run.

This poster presents a new stationary phase that combines anion-exchange, cation-exchange and hydrophobic properties. This new phase features unique selectivity adjustable not only by mobile phase ionic strength, pH and organic solvent, but also the anion and cation types of the salt used in the mobile phase. Other features include orthogonal selectivity compared to RP columns, capability for separating inorganic anions and cations in a single run, and simultaneous separation of basic, acidic and neutral analytes.

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Column Chemistry and Main Features

The new stationary phase is based on high-purity, spherical, porous silica particles ($d_p = 5 \mu\text{m}$, pore size = 120 \AA , surface area = $300 \text{ m}^2/\text{g}$). The inner-pore area is functionalized with an organic layer that provides both reversed-phase and anion-exchange properties. The outer-pore area, conversely, is modified with cation-exchange functionality. As a result, the new packing material provides reversed-phase, anion-exchange, and cation-exchange retentions under same chromatographic conditions so that neutral, acidic, and basic analytes can be separated simultaneously.

Features

- Simultaneous separation of basic and acidic molecules
- Adjustable selectivity
- Multi-mode retention mechanism: reversed-phase, anion-exchange, cation-exchange, and HILIC
- Retains ionic and ionizable compounds without ion-pairing reagents

Results and Discussion

Ionic Strength Effect

Mobile phase ionic strength affects retentions of both ephedrine (cationic) and salicylic acid (anionic) analytes (Figure 1). Retention of a cation decreases with the increase in competing cations in the mobile phase. Retention of an anion decreases with the increase in competing anions in the mobile phase. Therefore, retentions for both cations and anions decrease as the NaCl concentration increases. Under testing conditions (pH 2.2), tyrosine (a zwitterionic amino acid) is positively charged, thus behaving like a cation. Mobile phase ionic strength has no effect on retention of neutral analytes (e.g. naphthalene).

pH Effect

Mobile phase pH is another determining factor for retention (Figure 2). Naphthalene (neutral) is slightly more retained at pH 6.5 as opposed to pH 2.3, probably because the weak anion-exchange sites on the stationary

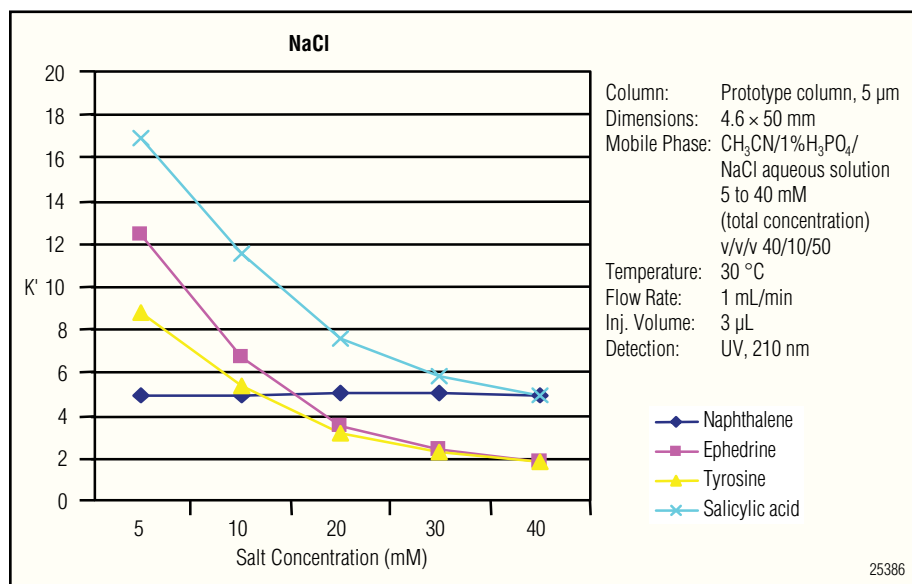


Figure 1. Effect of mobile phase ionic strength.

phase is less charged and thus more hydrophobic. Ephedrine (cationic) exhibits virtually no retention difference at both pHs since pH change does not change the strong cation-exchange property in outside-pore areas. By comparison, salicylic acid (anionic) shows dramatically different retention at

pH 2.3 and 6.5. This is mainly due to the fact that under the testing conditions, the anion concentration is higher at pH 6.5 than that at pH 2.3. Tyrosine (zwitterionic) is virtually neutral at pH 6.5 and shows no retention change at different buffer concentrations. At pH 2.3, it behaves like a typical cation.

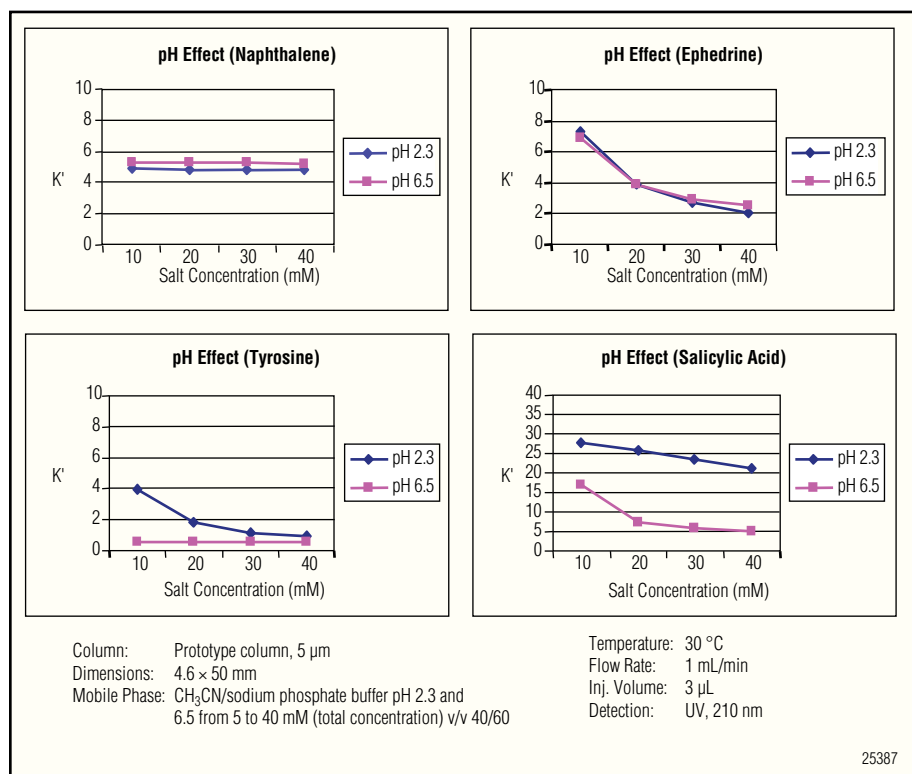


Figure 2. Effect of mobile phase pH.

Organic Solvent Effect

In the presence of the same salt concentration, all types of analytes are less retentive on the new phase with organic solvent increase. However, elution order (selectivity) changes with organic solvent content as shown in Figure 3.

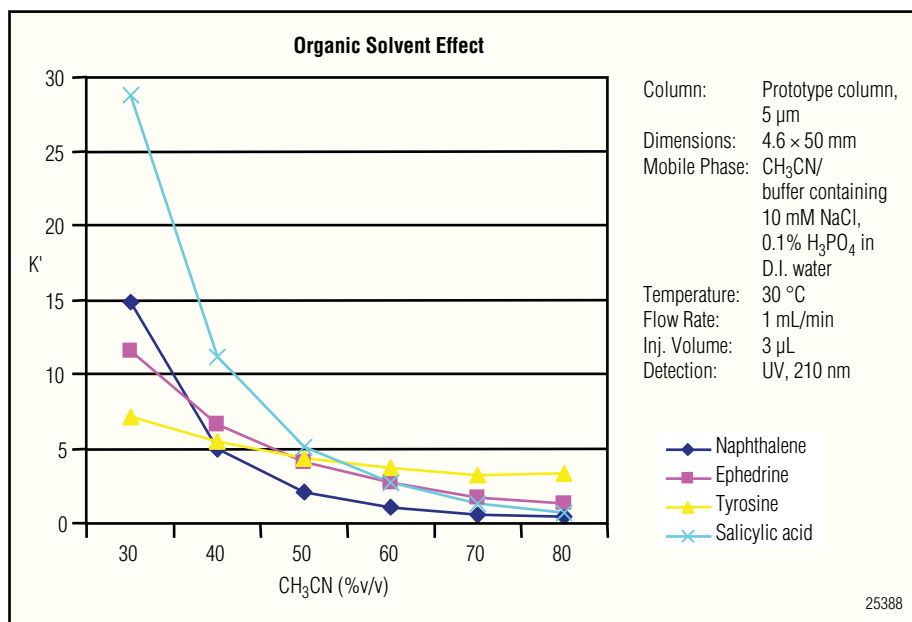


Figure 3. Effect of mobile phase organic solvent.

Salt-Type Effect

Anion type affects the retention of anionic analytes (Figure 4). The perchlorate ion is a stronger competing anion than chloride ion and results in lower retention of salicylic acid (anionic) at same salt concentration. Conversely, ephedrine and tyrosine (both cationic under testing conditions) are not affected by anion type and exhibit overlapping retention curves. Different anion types give somewhat different retention for naphthalene (neutral).

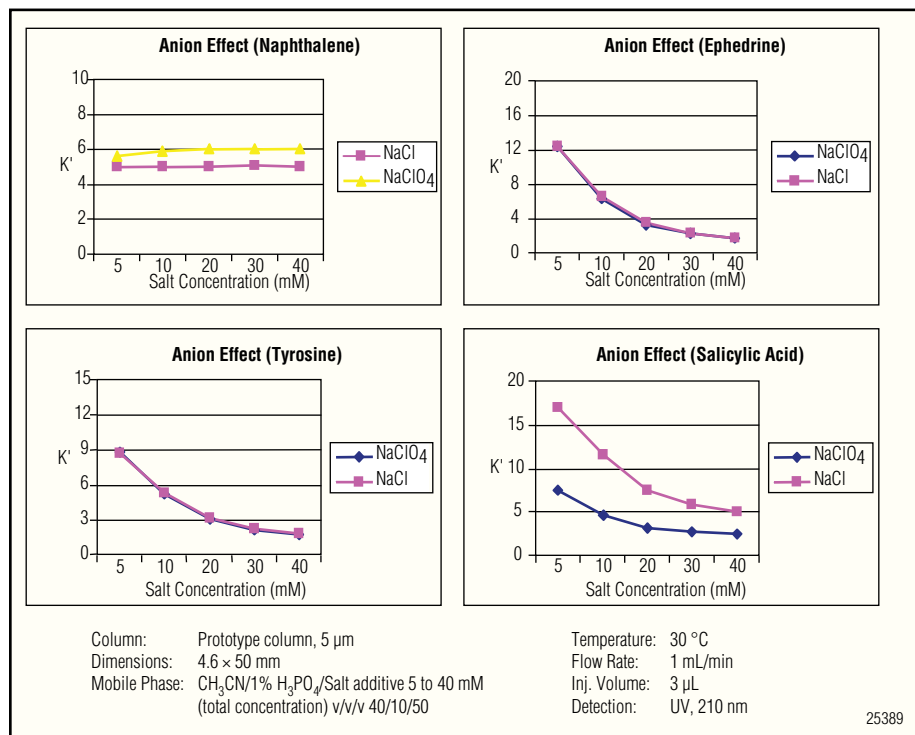


Figure 4. Effect of anion type in mobile phase.

Cation type affects the retention of cationic analytes (Figure 5). K^+ is a stronger competing cation than the Na^+ ion which is stronger than Li^+ . Thus the retention for ephedrine follows the order $K^+ < Na^+ < Li^+$ at the same salt concentration. Conversely, salicylic acid (anionic) is not affected by cation type and exhibits typical anion-exchange behavior curve. Surprisingly, tyrosine is insensitive to the cation type and under all three conditions; the column gives nearly the same retention curves. Different cations give virtually same retention for naphthalene (neutral).

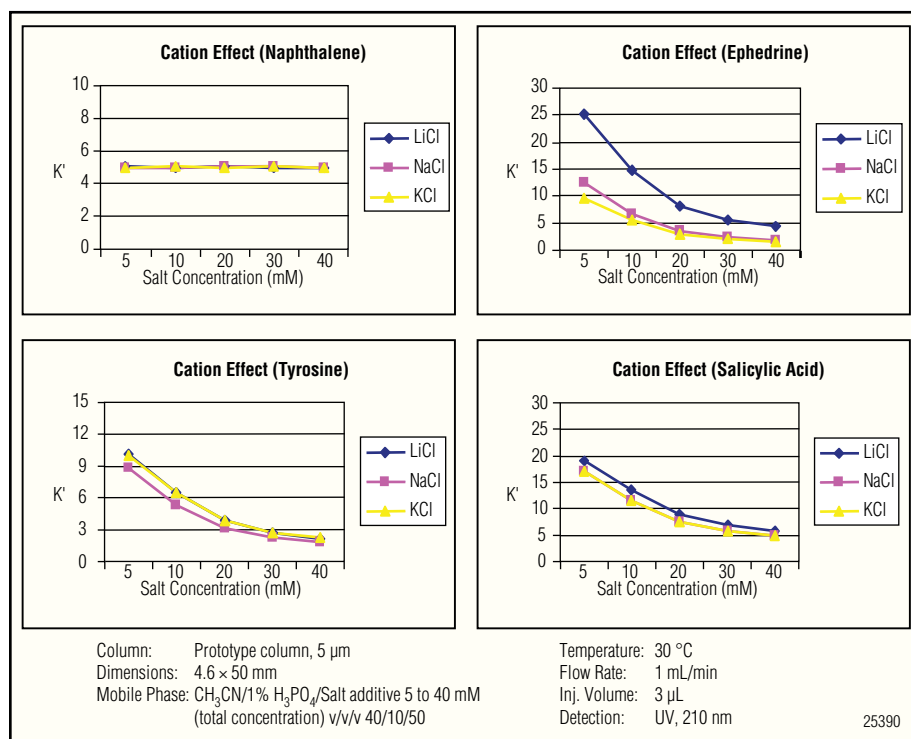


Figure 5. Effect of cation-type in mobile phase.

Simultaneous Separation of Inorganic Cations and Anions

Figure 6 illustrates three inorganic cations and one inorganic anion separated using a column packed with the new stationary phase. By comparison, neither conventional reversed-phase columns nor ion-exchange columns can provide the same separation.

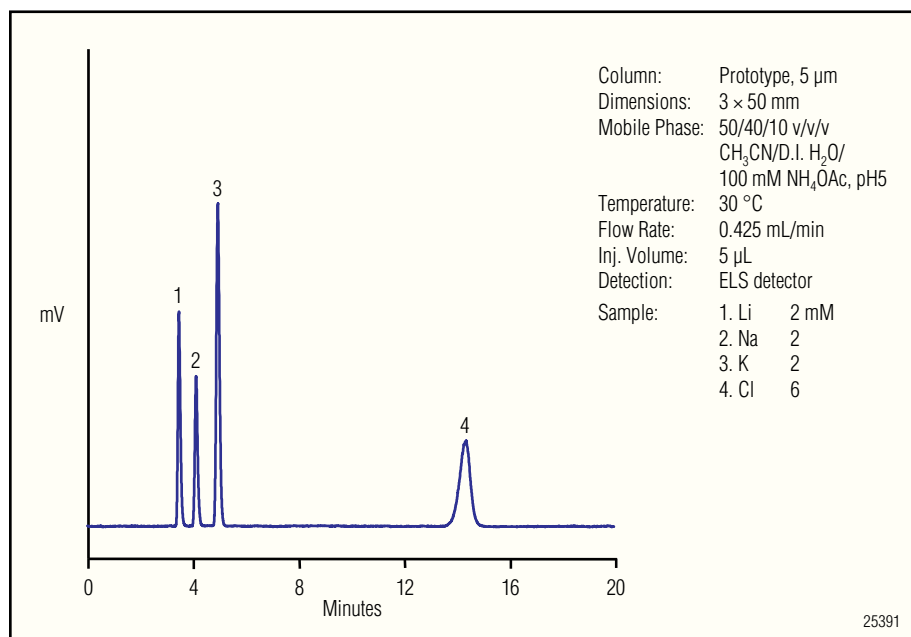


Figure 6. Simultaneous separation of inorganic cations and anions.

Simultaneous Separation of Quaternary Amines and Counterions

Figure 7A illustrates four alkyl quaternary amines and their inorganic counterions simultaneously separated using the new column. The chromatographic method used a salt gradient while keeping organic solvent concentration constant. As a result, the elution order follows the charge density of the analyte—the larger cations (lower charge density) elute earlier than the smaller ones. More interestingly, their inorganic counterions (chloride and bromide) elute last in the presence of high ionic-strength conditions.

Figure 7B illustrates the same separation using different chromatographic condition. The chromatographic method used an organic solvent gradient while keeping salt concentration constant. As a result, the elution order follows the hydrophobicity of the analyte—the more hydrophobic eluting later than the less hydrophobic. In addition, their anionic counterions (chloride and bromide) can be separated during the same analysis.

By comparison, neither conventional reversed-phase columns nor ion-exchange columns can provide such separation.

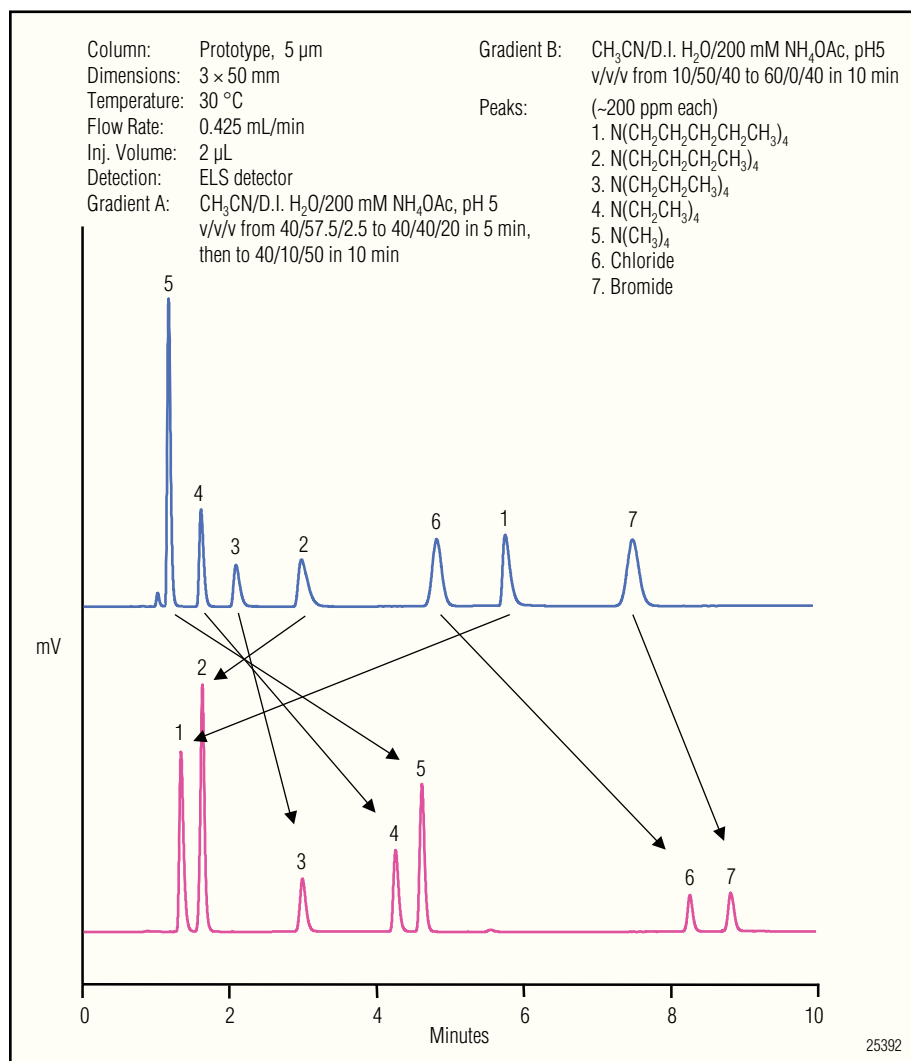


Figure 7. Simultaneous separation of quaternary amines and counterions.

Separation of catecholamines

Catecholamines occur naturally in the body and serve as hormones or as neurotransmitters in the sympathetic nervous system. The catecholamines include such compounds as epinephrine (adrenaline), norepinephrine, and dopamine. They resemble one another chemically in having an aromatic portion (catechol) to which is attached an amine, or nitrogen-containing group. Due to the hydrophilic and cationic nature, they are often separated by a RP-ion-pairing LC method. However, drawbacks include long equilibration time and MS-incompatible mobile phase. Figure 8 demonstrates that the new phase provides satisfactory retention and separation for four common catecholamines using a mobile phase compatible with MS in the absence of an ion-pairing agent.

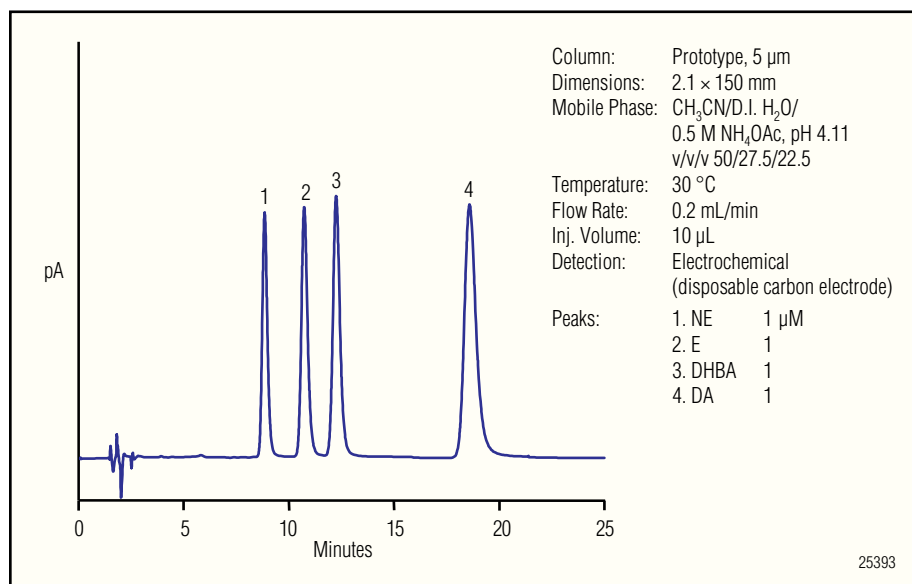


Figure 8. Separation of catecholamines.

CONCLUSION

The new packing material features unprecedented column chemistry and chromatographic properties. The unique combination of reversed-phase, anion-exchange and cation-exchange, functionalities provides great potentials for a wide range of HPLC applications.

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