New HPLC methods for the separation of *alpha*, *beta*, and *gamma* zwitterions in reversed-phase conditions

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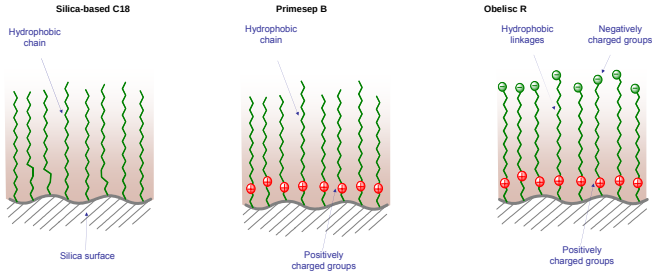
Abstract

Zwitterions are important biological compounds. Their retention and separation under reversed-phase (RP) HPLC conditions is difficult due to the high polarity of these molecules. The relative positions of the oppositely charged functional groups do not significantly change the polar or hydrophobic characteristics of zwitterions, which are the two fundamental properties for separation of small molecules by reversed-phase chromatography. RP retention can be improved by using ion-pair reagents, but there is little improvement in selectivity.

Mixed-mode columns which contain two types of interactions, ion-exchange and reversed-phase, separate positional *alpha*, *beta* and *gamma* isomers of zwitterionic molecules. In addition to zwitterionic isomers, Obelisc columns separate acidic, basic, and neutral isomers with mass spec compatible mobile phases.

Evolution of Stationary Phases from Silica-based C18, to Primesep® B, to Obelisc™ R

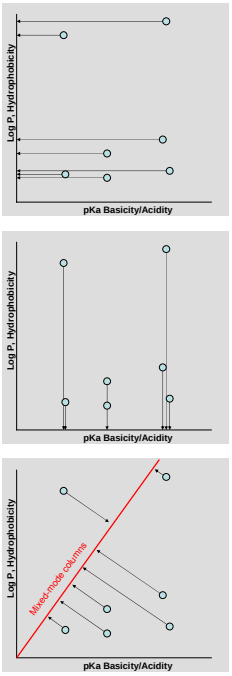


Reversed-phase separations on silica-based C18 columns rely on hydrophobic interactions between the C18 chains on the stationary phase and hydrophobic groups on the analyte. Residual silanols on the silica surface complicate separations by adding polar interactions which can not be reproducibly controlled in the production process.

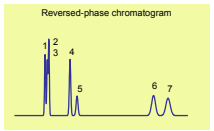
An initial evolution of reversed-phase columns was the incorporation of positively charged functional groups on the hydrophobic chain, as in mixed-mode Primesep B columns. This positive charge close to the silica surface shields the silanols and improves peak shape and column reproducibility. Primesep B also provides many more options to tune selectivity and perform separations not previously possible on C18 columns.

The latest advance is the incorporation of both positively and negatively charged groups on Obelisc™ R columns. This evolution not only solves the silanol problem, but also further expands the reach of chromatography. Additional polar interactions are available between the analyte and stationary phase to further expand the selectivity possible in chromatographic separations.

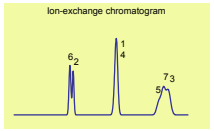
Physical-Chemical Properties of Analytes in 2-D Plane



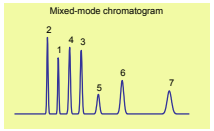
Two dimensional separations are the result of the evolution from C18 phases to mixed-mode phases with multiple interactions. The two dimensions can be represented by the following graphs with hydrophobicity, represented on the y-axis and charge, represented on the x-axis



Reversed-phase separations on silica-based C18 columns rely on hydrophobic interactions with the C18 chains on the stationary phase. Using only hydrophobicity on the y-axis results in a separation without complete resolution.



Ion-exchange separations rely on ionic interactions represented on the x-axis. Using only ionic interactions results in a different separation, but still without complete resolution.

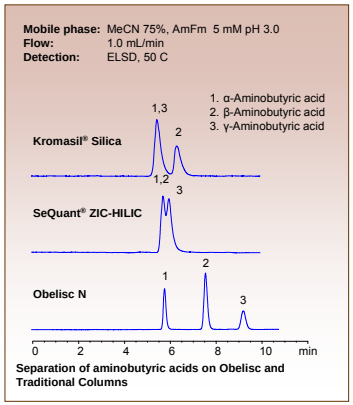


Combining reversed-phase and ion-exchange interactions shifts the separation from one of the axes only to the diagonal. This mixed-mode separation results in complete resolution.

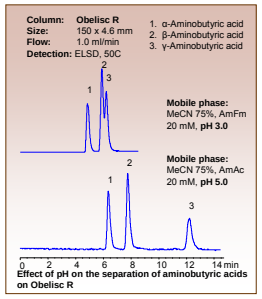
Aminobutyric Acids - Effect of pH and Buffer Concentration on Obelisc columns

α-, β-, and γ-aminobutyric acids are very polar zwitterionic isomers which show little or no retention on reversed-phase C18 columns. These isomers not only retain on Obelisc R and N columns, but they also demonstrate the selectivity available on these columns.

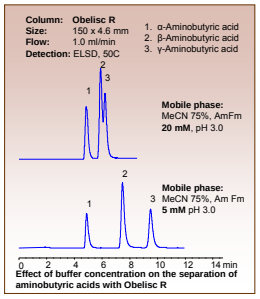
Aminobutyric Acids – Separation in HILIC Mode



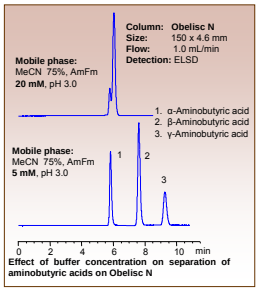
Separation of aminobutyric acids on Obelisc and Traditional Columns



Effect of pH on the separation of aminobutyric acids on Obelisc R



Effect of buffer concentration on the separation of aminobutyric acids with Obelisc R

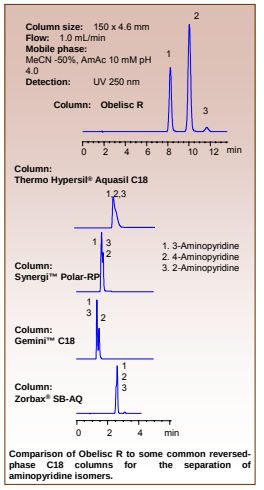


Effect of buffer concentration on separation of aminobutyric acids on Obelisc N

In traditional HILIC mode, a charged or neutral polar analyte interacts with a water layer on the polar stationary phase surface. On Obelisc N the charges are greatly separated and independently accessible which results in different selectivity compared to traditional HILIC and silica columns.

Basic Isomers - Aminopyridines

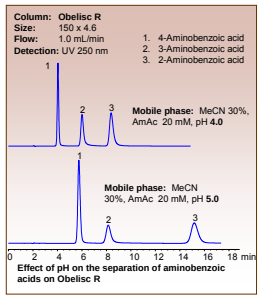
Aminopyridine isomers are polar compounds that differ only in the location of a primary amine on the pyridine ring. Obelisc R retains and separates aminopyridines with a mass spec compatible mobile phase. Common reversed-phase columns show little retention and no resolution of these compounds due to their lack of electrostatic, ionic interactions.



Comparison of Obelisc R to some common reversed-phase C18 columns for the separation of aminopyridine isomers.

Zwitterionic Isomers – Aminobenzoic Acids

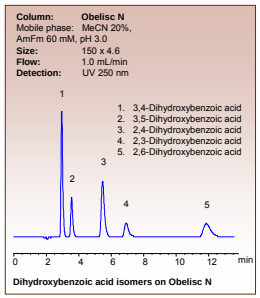
Aminobenzoic acid isomers are zwitterions that differ only in the location of a primary amine. Obelisc R retains and separates these isomers with a mass spec compatible mobile phase.



Effect of pH on the separation of aminobenzoic acids on Obelisc R

Acidic Isomers – Dihydroxybenzoic Acids

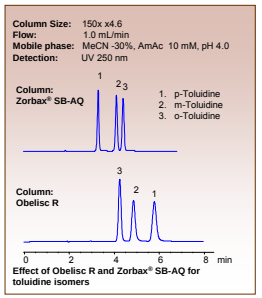
Obelisc N separates the very polar dihydroxybenzoic acids isomers. These isomers are very difficult to separate by reversed-phase or ion-exchange alone.



Dihydroxybenzoic acid isomers on Obelisc N

Weak Basic Isomers - Toluidines

Obelisc R separates toluidine isomers with opposite peak order of C18 columns. Toluidines have an aromatic primary amine which interacts with the ionic groups on Obelisc R. This interaction is not available on C18 columns.



Effect of Obelisc R and Zorbax® SB-AQ for toluidine isomers

Conclusion

Mixed-mode columns which contain two types of interactions, ion-exchange and reversed-phase, allow separation of *alpha*, *beta*, and *gamma* isomers of zwitterionic molecules such as aminobutyric acids. In addition, zwitterionic isomers of aminobenzoic acids, basic isomers (aminopyridines), acidic isomers (dyhydroxybenzoic acids), and neutral isomers (toluidines) can be resolved on Obelisc HPLC columns with mass spec compatible mobile phases.

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