Exploring Mixed-Mode Chromatography: Column Chemistry, Properties, and Applications

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Overview

- Review of mixed-mode column chemistry
- Collection of key applications using mixed-mode chromatography

Introduction

Although reversed-phase columns (e.g. C18) are most commonly used in broad range of applications, they often fail to retain highly hydrophilic molecules (e.g. counterions), and offer limited selectivities. Mixed-mode chromatography provides a viable solution to these challenges by combining both reversed phase and ion-exchange retention mechanisms. One major advantage of this approach is that column selectivity can easily be modified for optimal selectivity by adjusting mobile phase ionic strength, pH and/or organic solvent concentration. As a result, not only is the selectivity of a mixed-mode column complementary to that of reversed-phase columns, but it also allows for the development of multiple complementary selectivities on the same column under different appropriate conditions. Mixed-mode chromatography is well-suited to retaining ionic analytes, whether hydrophobic (e.g. Naproxen) or hydrophilic (e.g. Na^+ and Cl^- ions), and requires no ion-pairing agents in the method, significantly improving the MS compatibility. Most importantly, mixed-mode chromatography column chemistry can be customized to a desired selectivity during stationary phase design. This presentation will give an overview on the latest mixed-mode chromatography technology, describe unique chromatographic properties of mixed-mode columns, and discuss analytical challenges that have been addressed by mixed-mode chromatography approach. Examples include determinate on of pharmaceutical counterions, simultaneous separation of anionic, cationic, nonoionic and amphoteric surfactants, high resolution and fast LC-MS analysis of paraquat and diquat, and analysis of glycans from proteins.

Mixed-Mode Chromatography

Definition

• Hydrophobic (or hydrophilic) interaction + ion-exchange interaction

Benefits

• Adjustable selectivity for optimal separation

• Simplified mobile phase (no need for ion-pairing reagents)

• Simultaneous separation of different types of analytes

Types

• Anion-exchange/reversed-phase (AEX/RP)

• Cation-exchange/reversed-phase (CEX/RP)

• Anion-exchange/cation-exchange/reversed-phase (AEX/CEX/RP)

• AEX/HILIC

• CEX/HILIC

• AEX/CEX/HILIC

FIGURE 1. Reversed-Phase/Ion-Exchange Bimodal Phases

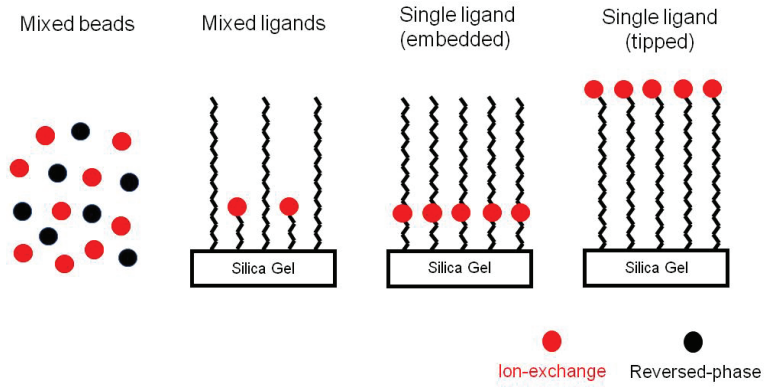
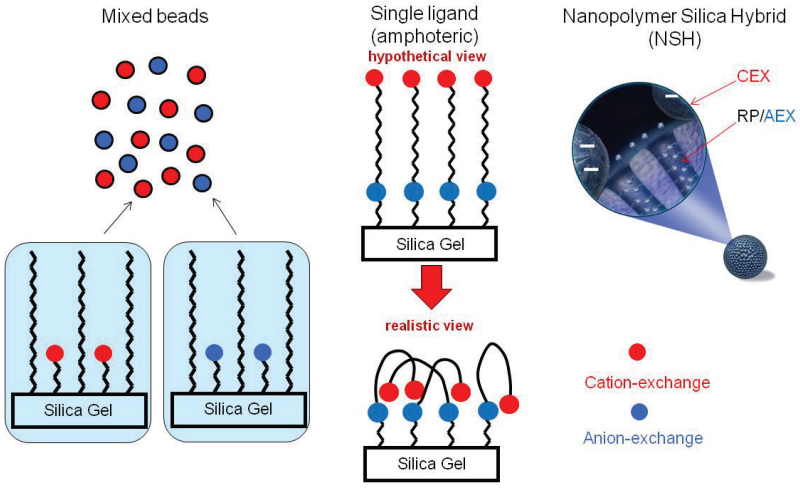


FIGURE 2. Reversed-Phase/Anion-Exchange/Cation-Exchange Trimodal Phases



Key Applications

Pharmaceutical Counterion Analysis by Thermo Scientific™ Acclaim™ Trinity™ P1 and Acclaim Trinity P2 columns

Salt formation is important in drug development to improve biopharmaceutical and physicochemical properties of the drug. Approximately 50% of all drugs are formulated as salt forms. Assays for API and counterions are usually analyzed separately using different methods, different separation columns, and different instruments.

FIGURE 3. Separation of Monovalent Counterions

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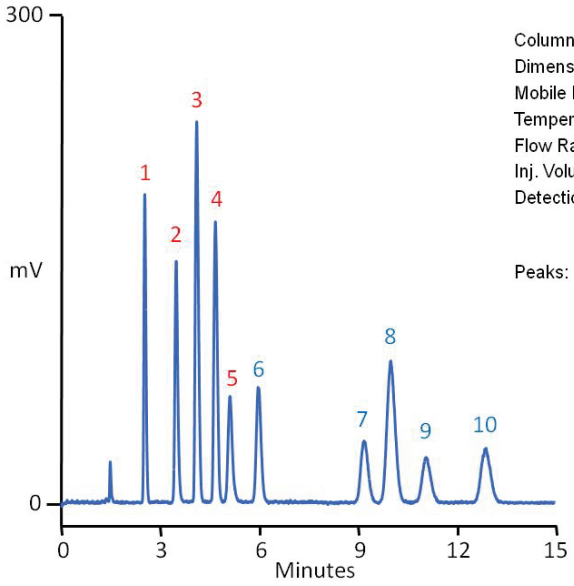
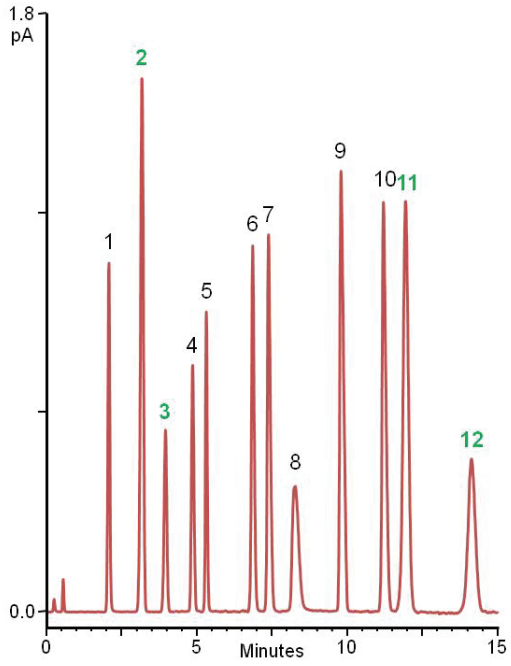


Column: **Acclaim Trinity P1**, 3 µm
Dimensions: 3.0 × 100 mm
Mobile Phase: 60/40 v/v CH_3CN/20 mM (total) NH_4OAc, pH5
Temperature: 30 °C
Flow Rate: 0.5 mL/min
Inj. Volume: 2 µL
Detection: Corona *ultra* (Gain = 100 pA; Filter = med; Neb Temp = 30°C)

Peaks: (50 to 100 ppm)
1. Choline
2. Tromethamine
3. Sodium
4. Potassium
5. Meglumine
6. Mesylate
7. Nitrate
8. Chloride
9. Bromide
10. Iodide

FIGURE 4. Ion (anions & cations) Screening



Column: **Acclaim Trinity P2**, 3 µm
Dimensions: 3.0 x 50 mm
Mobile Phase: D.I. water and 100 mM NH_4OFm, pH 3.65
Temperature: 30°C
Flow Rate: 0.60 mL/min
Inj. Volume: 1 µL
Detection: Corona Veo
Samples: 0.02 – 0.10 mg/mL each in D.I. water
Peaks:
1. Phosphate
2. **Sodium**
3. **Potassium**
4. Chloride
5. Malate
6. Bromide
7. Nitrate
8. Citrate
9. Fumarate
10. Sulfate
11. **Magnesium**
12. **Calcium**

Gradient:

Time (min)	H_2O	0.1 M Ammonium formate, pH3.65
-8	90	10
0	90	10
1	90	10
11	0	100
15	0	100

Surfactant Analysis by Thermo Scientific™ Acclaim™ Surfactant Plus

Surfactants are widely used in consumer products, agricultural, pharmaceutical, bio-pharmaceuticals and chemical markets, in products as diverse as pesticides, detergent powders, petroleum products, cosmetics, and pharmaceuticals.

Their separation and identification can be challenging due both to the diversity of surfactants and complexity of the sample matrix. Although many HPLC columns are available and have been used for the analysis of surfactant formulations, none of these columns are capable of separating anionic, nonionic, cationic and amphoteric surfactants in a single analysis.

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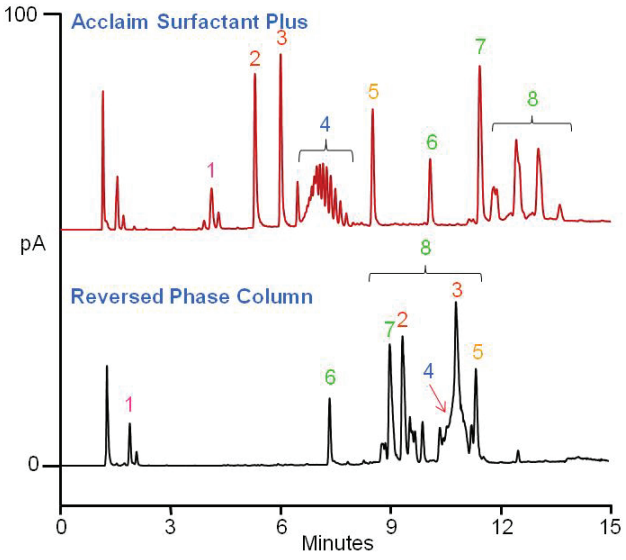
FIGURE 5. Separation of Cationic, Nonionic, Amphoteric & Anionic Surfactants



Column: **Acclaim Surfactant Plus**, 3 µm
Dimensions: 3.0 x 150 mm
Gradient:

Time (min)	Acetonitrile	0.1 M ammonium acetate, pH5
-8	25	75
0	25	75
10	80	20
15	80	20

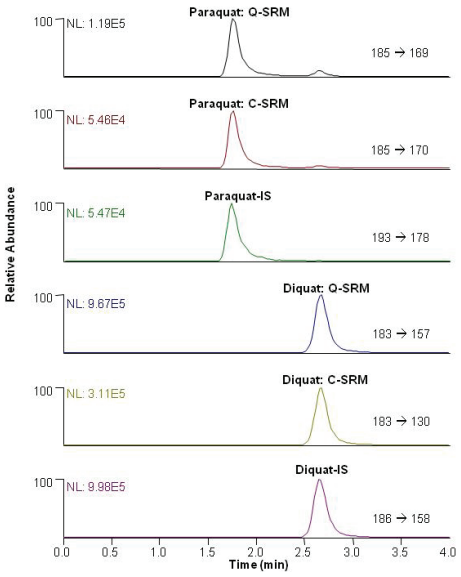
Temperature: 30 ° C
Flow Rate: 0.6 mL/min
Inj. Volume: 5 µL
Detection: Corona *ultra* (Gain = 100 pA; Filter = med; Neb Temp = 20° C)
Peaks: (100 - 400 µg/mL each)

1. Xylene sulfonate
2. Laurylpyridinium
3. Lauryldimethylbenzyl-ammonium
4. Triton X-100
5. Cetyl betaine
6. Decyl sulfate
7. Dodecyl sulfate
8. Linear alkylbenzene sulfonate (LAS)

Diquat and Paraquat Analysis by Thermo Scientific™ Acclaim™ Trinity™ Q1 Column

Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride, $C_{12}H_{14}N_2Cl_2$) and Diquat (1,1'-ethylene-2,2'-bipyridilium dibromide, $C_{12}H_{12}N_2Br_2$) are non-selective and nonsystematic contact herbicides widely used in agriculture to control broadleaf and grassy weeds. The use of these herbicides is very important because weeds compete vigorously with crops for water, light and other nutrients. However both Parquat and Diaquat are toxic and either compound can have serious effects as they can alter reduction-oxidation activities in biological systems. The analysis of these highly charged dual quaternary amines is complicated because of their ionic nature, Paraquat and Diquat are difficult to retain by standard reversed phase HPLC.

FIGURE 6. LC-MS-MS: Paraquat and Diquat at 10 ppb



Chromatographic Conditions

System:	UltiMate 3000 RS UHPLC System
Column:	Acclaim Trinity Q1
Column Temp.:	Ambient
Mobile Phase:	25% Ammonium Acetate (100 mM, pH 5.0) 75% Acetonitrile
Flow Rate:	0.5 mL/min
Injection:	5 µL

Mass Spectrometric Conditions

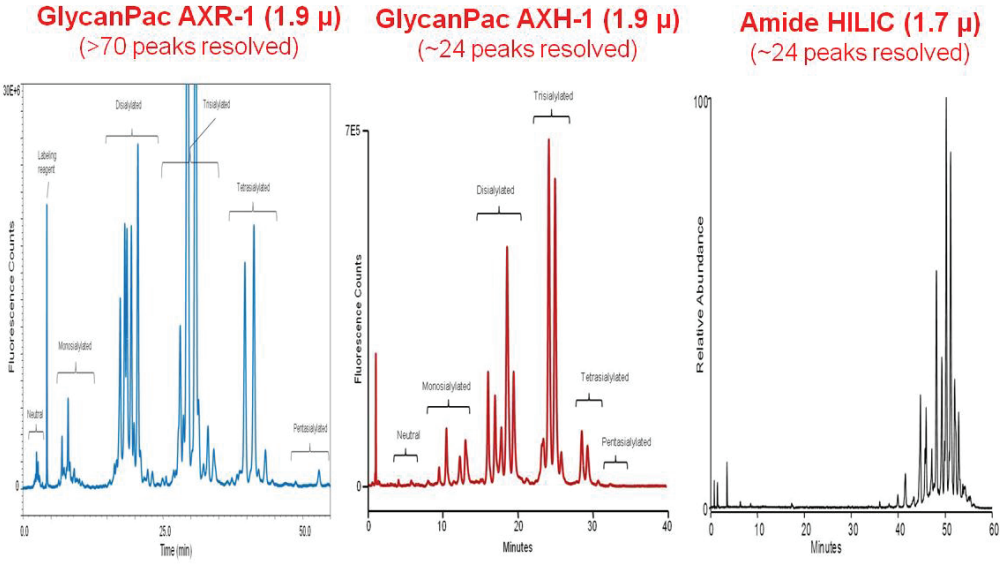
System:	Quantum TSQ Access MAX Triple Quad
Interface:	Heated Electrospary Ionization with HESI II probe
Spray Voltage:	1500 V
Vaporizer Temperature.:	400 °C
Sheath Gas Pressure:	70
Aux Gas Pressure:	10
Capillary Temperature:	350 °C
Quantitation Mode:	Selected Reaction Monitoring (SRM)

Scan Events	Precursor	Quantitative SRM (CID)	Confirmative SRM (CID)
Paraquat	185	169 (27)	170 (17)
Paraquat-d_6	193	178 (17)	
Diquat	183	157 (22)	130 (31)
DiQuat-d_3	186	158 (22)	

Glycan Analysis by Thermo Scientific™ GlycanPac™ AXH-1 and GlycanPac AXR-1 Columns

Glycans are oligosaccharides and polysaccharides found on proteins and cell surfaces. They play fundamental roles in cellular function by creating a fingerprint tag for the protein they are bound to. Glycans are often key biomarkers for disease states such as cancer. The structures of glycans are highly complex because of the branching of the chains and post-translational modifications. Various HPLC separation modes have been used for glycan analysis, such as hydrophilic interaction (HILIC), ion-exchange (IEX) and reversed-phase (RP) chromatography. Because glycans are highly hydrophilic and polar substances, they are commonly separated on an amide HILIC column which separates glycans mainly by hydrogen bonding, resulting in size and composition-based separation. However, one limitation of this approach is that glycan identification and quantitation become highly challenging because glycans of different charge states are intermingled in the separation envelope.

FIGURE 7. 2AB *N*-Glycan from Bovine Fetuin (GlycanPac vs. Amide HILIC)



Conclusion

1. Mixed-mode columns offer advantages over other separation columns through
    - Excellent performance: selectivity, resolution and retention
    - Flexibility in method development
    - Reduced cost
2. Mixed-mode column technology provides a versatile platform to a variety of application-specific columns
3. Thermo Fisher Scientific has a family of mixed-mode columns that facilitate instrument pull-through

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