OVERVIEW-

One of the challenges that the preparative chromatographer faces is to maximize column loading to keep the costs of operation and equipment as low as possible. Therefore, it is very necessary to study the factors that enhance column loading. In this presentation, we studied the loading of 30 compounds on 12 RPLC columns with large varieties of stationary phase chemistries and ligand densities. The test compounds were selected based on their structural characteristics and classified into several groups. Mobile phase pH effects on column loadability and selectivity were studied. In addition, the effects of additives on column loadability were compared as well. The preliminary results demonstrate that the particle chemistry, ligand type, nature of the compound, pH and additives in mobile phases all have significant contributions to column loadability and selectivity.

INTRODUCTION-

The dream of a preparative chromatographer is to achieve preparative loading on an analytical column. An increase in productivity will dramatically decrease the costs of the entire separation process. Many factors affect loadability, such as:

- Chemistry of RPLC columns
- Nature of the compounds that need to be separated
- Ionic status of the compounds
- Mobile phase conditions
  - pH of the mobile phases
  - Polarity of the organic solvent
- Additives
- Overloading methodology
  - Volume overload
  - Mass overload

MOTIVATIONS-

- Understand loadability and selectivity of various compounds on RPLC columns
- Guide loading condition selection
METHODS

**RPLC Columns:**
(All columns are 4.6 × 50 mm, 5 µm)
- XTerra® MS C₁₈
- XTerra® MS C₈
- XTerra® RP₁₈
- XTerra® RP₈
- Symmetry® C₁₈
- Symmetry® C₈
- Symmetry Shield™ RP₁₈
- Symmetry Shield™ RP₈
- Atlantis™ dC₁₈
- YMC-Pack™ Pro C₁₈
- YMC-Pack™ ODS-A™
- YMC-Pack™ ODS-AQ™

Mobile Phase A: Water/1% FA (TFA) (90/10); pH 3
- Water/100 mM NH₄COOH (90/10); pH 7
- Water/100 mM NH₄HCO₃ (90/10); pH 10

Mobile Phase B: ACN/1% FA (TFA) (90/10); pH 3
- ACN/100 mM NH₄COOH (90/10); pH 7
- ACN/100 mM NH₄HCO₃ (90/10); pH 10

Sample Diluent: DMSO/MeOH (50/50) for most analytes;
DMSO for nalidixic acid antibiotics sample
Water for water soluble vitamins sample

Temperature: Ambient temperature

Instruments: FractionLynx™ AutoPurification Systems, which include Waters® 2767 Sample Manager,
Waters® 2525 Binary Gradient Module, Waters® 2996 PDA Detector and a Micromass®
ZQ™ Mass Spectrometer
RESULTS

Effect of Ionic State on Loadability

Analytes:
1. nordoxepin; 2. doxepin; 3. amitriptyline  (left figure)
1. cinoxacin; 2. oxolinic acid; 3. nalidixic acid. (right figure)

Column: XTerra® MS C₁₈

Load analytes under their non-ionic states will dramatically increase loadings on the RPLC column.
RESULTS

Ion-Pairing Effect
Analytes:
1. pyridoxal; 2. folic acid; 3. caffeine
Column: Atlantis™ dC18

Conditions:
Gradient: 0 to 20% B in 5 min; then to 40% B in 10 min.
Flow Rate: 1.0 mL/min
Detection: UV @ 280 nm.

Pyridoxal eluted at void when 0.1% formic acid was used, while higher retained when 0.1% TFA was used. This is due to the fact that TFA has ion-pairing effect, which increases retention of pyridoxal.
RESULTS

Effect of pH on Loadability

Analytes:
1. cinoxacin; 2. oxolinic acid; 3. nalidixic acid

Conditions:
Gradient: 10 to 70% B in 10 min.
Flow Rate: 1.8 mL/min
Detection: UV @ 300 nm.

At low pH, total load of acidic analytes increases from X3 on XTerra® RP8 column to X6.4 on Symmetry® C18 column when changing FA to TFA, because TFA is more capable to non-ionize acidic analytes.

![Graph showing loadability comparison between different columns and pH conditions]
RESULTS

Effect of Ligand Hydrophobicity on Loadability

Analytes:
1. indoprofen; 2. naproxen; 3. flurbiprofen  (right figure)
1. nordoxepin; 2. doxepin; 3. amitriptyline  (left figure)

Conditions:
Gradient: 40 to 70% B in 10 min; 0.1 % formic acid  (right figure)
Detection: UV @ 254 nm
Gradient: 40 to 70% B in 10 min; pH 10   (left figure)
Detection: UV @ 280 nm
Flow Rate: 1.8 mL/min for both

C₁₈ columns exhibit higher loading than C₈ columns towards most of the analytes due to the stronger hydrophobic interaction.
RESULTS

Effect of Silica Pore Structure on Loadability

Analytes:
1. hydrocortisone; 2. medroxyprogesterone 17-acetate; 3. medroxyprogesterone

Conditions:
Gradient: 20 to 80% B in 10 min; 0.1% formic acid
Flow rate: 1.8 mL/min
Detection: UV @ 254 nm

For relatively large molecules, C8 columns exhibit higher loads than C18 columns. This is due to the steric hindrance of attached longer alkyl chains (C18) which decrease the effective particle pore size.
RESULTS

Effect of Ligand Density on Loadability

Analytes:
1. prednisolone; 2. dexamethasone; 3. betamethasone 17-valerate; 4. progesterone

Conditions:
Gradient: 30 to 80% B in 8 min; 0.1 % formic acid
Flow rate: 1.8 mL/min
Detection: UV @ 254 nm

For acidic analytes at low pH, total loadings on RPLC columns are consistent with the column ligand densities. Symmetry® have highest loading due to highest ligand density.
RESULTS

Effect of Embedded Polar Groups on Selectivity

Analytes: 1. catechins; 2. epicatechin; 3. epigallocatechin

Conditions:
Gradient: 5 to 50% B in 7 min; 0.1 % formic acid
Flow rate: 1.8 mL/min
Detection: UV @ 280 nm
RESULTS

Effect of Embedded Polar Groups on Selectivity (continued)

- RP columns are designed with embedded carbamate group.
- Hydrogen bonding between the compounds with many hydroxyl groups (phenols, carboxylic acids and carbamate on RP columns) improves selectivity when they are not ionized.
- On the RP column, epigallocatechin elutes late due to its strong affinity with the carbamate group.
RESULTS

Effect of Ligand Hydrophobicity on Selectivity

Analytes:
3. betamethasone 17-valerate; 4. progesterone

Conditions:
Gradient: 30 to 80% B in 8 min; 0.1 % formic acid
Flow rate: 1.8 mL/min
Detection: UV @ 254 nm

For relatively large molecules with slight differences in alkyl chains, C_{18} columns have better selectivity towards them.
CONCLUSIONS-

- Loading drugs in the non-ionic state will maximize the total load.
- For most analytes using 0.1\% formic acid, the Symmetry® material exhibits better performance. This is due to the fact that this material has the highest ligand density.
- For acidic analytes, when changing 0.1\% formic acid to a stronger additive (0.1\% TFA), total load will increase dramatically. This is due to the fact that formic acid does not provide a low enough pH to make analytes non-ionic.
- For some analytes, the ion-paring effect of TFA will increase retention on RPLC columns.
- For analytes with relatively larger sizes, the C_8 columns exhibit higher loads than C_{18} columns. This is due to the steric hindrance of attached longer alkyl chains (C_{18}) which decreases the effective particle pore size.
- C_{18} columns have higher loading than C_8 columns due to the stronger hydrophobic interaction towards most analytes.
- For relatively large molecules with slight differences in alkyl chains, C_{18} columns have better selectivity towards them.
- For some analytes with many hydroxyl groups their affinities are stronger on the columns with embedded polar chemistry design due to the hydrogen bonding interaction when they are not ionized, which also improves column selectivity on those analytes.