Improved Yield and Productivity of Ionizable Compounds Employing Hybrid Packings

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Abstract

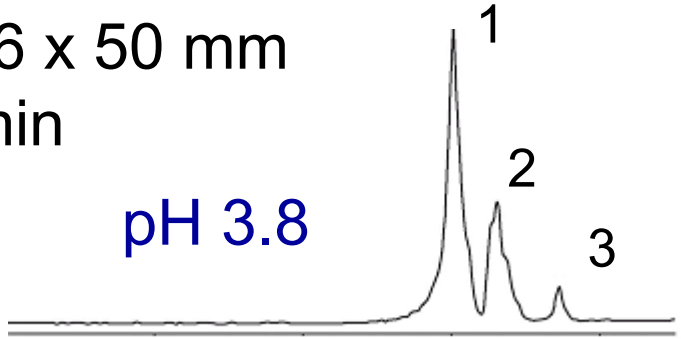
Reversed-phase chromatography of basic compounds has traditionally been performed at acidic pH to minimize secondary interactions and improve peak shape. In the last decade, the advent of higher purity silica and improved bonding technology has permitted the use of more neutral pH for basic compounds. The latest advance has been the development of hybrid particle packings that allow chromatography at high pH with good column lifetime. We have recently found that running preparative chromatography of basic compounds at high pH leads to substantial gains in loadability compared to running at neutral or acidic pH. In particular, converting the solutes to neutral species permits at least 50 times higher loadability, such that 500 mg can be loaded on a 19 X 50 mm column. The implications of these results are addressed.

Loading of Bases at Low pH

Diphenhydramine (1)
Oxybutynin (2)
Terfenadine (3)

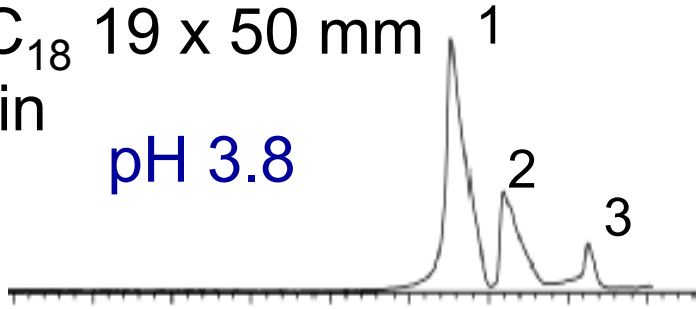
XTerra® MS C_{18} 4.6 x 50 mm
Flow rate 1.8 mL/min
Load 0.4 mg

pH 3.8



XTerra® Prep MS C_{18} 19 x 50 mm
Flow rate 31 mL/min
Load 6 mg

pH 3.8



Gradient: A: 90/10 Water/100 mM Ammonium Formate pH 3.8

B: 80/10/10 ACN/Water/ 100 mM Ammonium Formate pH 3.8

Gradient slope: 95/5 A/B to 5/95 A/B in 10 minutes; UV: 254 nm

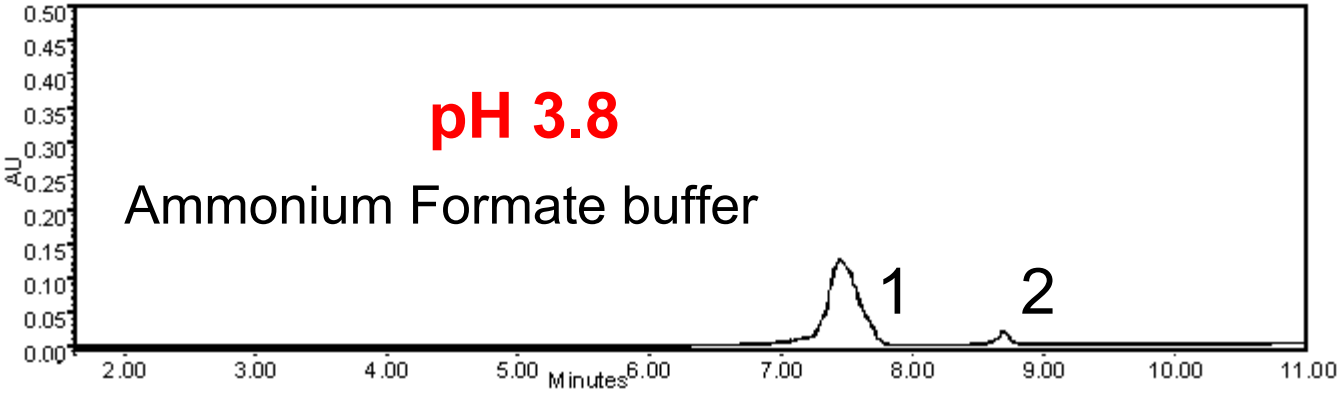
Peak Shape and Retention Comparison

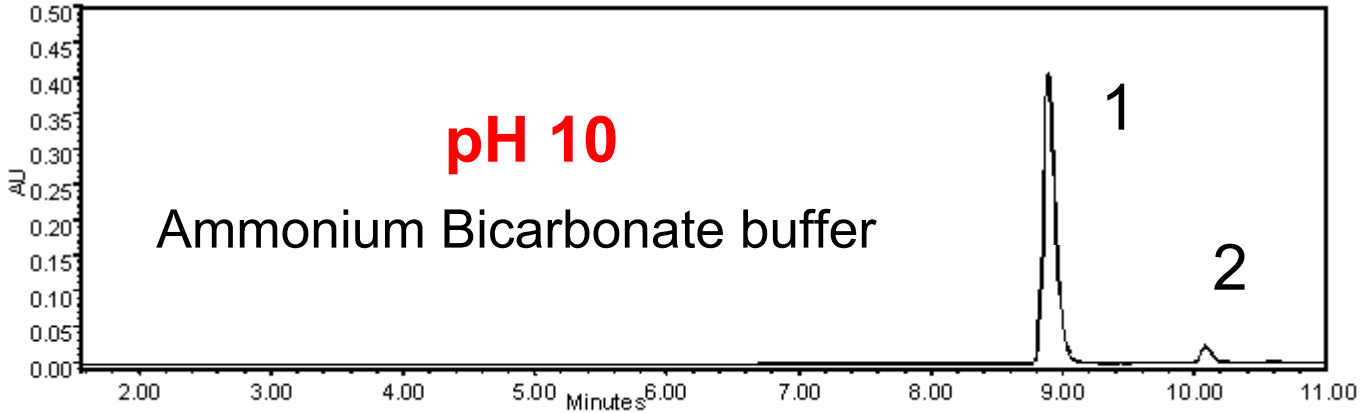
- It is logical to assume that once we have selected our column for selectivity, if we could improve peak shape we could also improve loading capacity

- How can we improve the peak shape for our basic analytes example?

Peak Shape and Retention Comparison: Basic Compounds at Low and High pH

XTerra® Prep MS C_{18} 19 x 50 mm, 5 µm

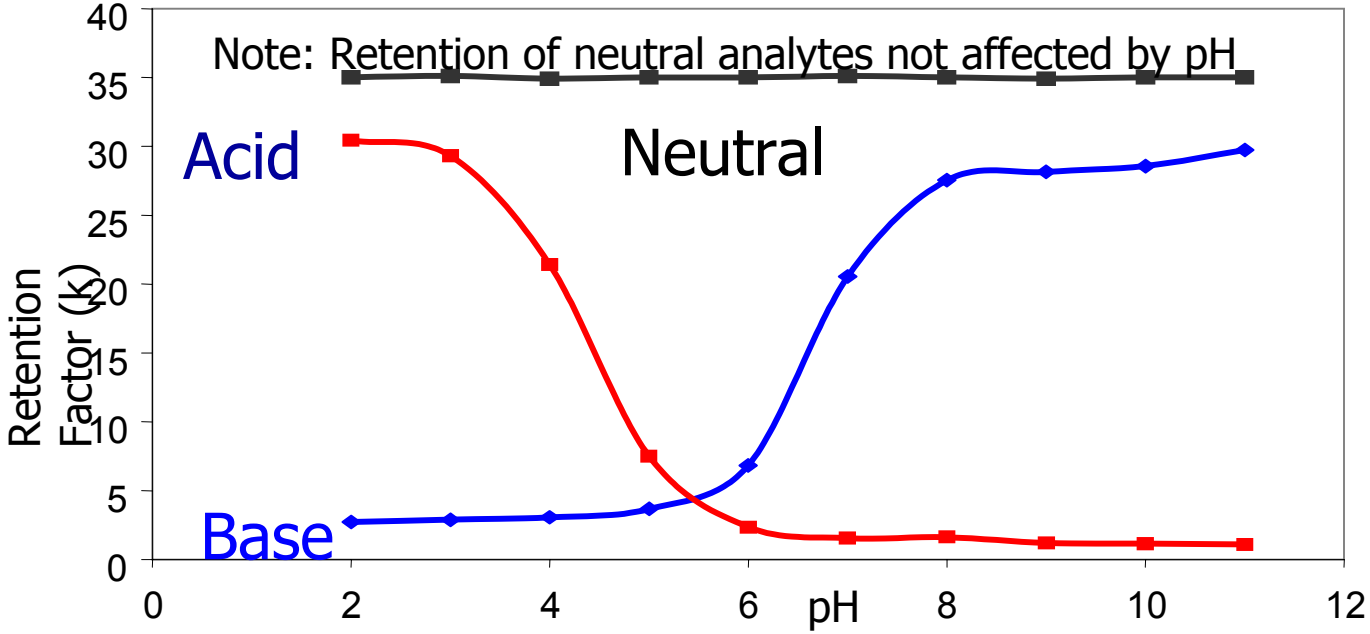




Analytes: 1. Diphenhydramine (2.5 mg/mL) 3 mg Load

2. Terfenadine (0.15 mg/mL) 0.18 mg Load

Retention Map Theory



The increase in loadability shown is a generic phenomena that has been proven employing XTerra® where the loading difference between the ionized and non-ionized form of the compound varies by 50 fold.(*)

(*) U.D. Neue *et al*., American Laboratory, November 1999, 31 (22), p. 36-39

Peak Shape and Retention Comparison: Basic Compounds at Low and High pH

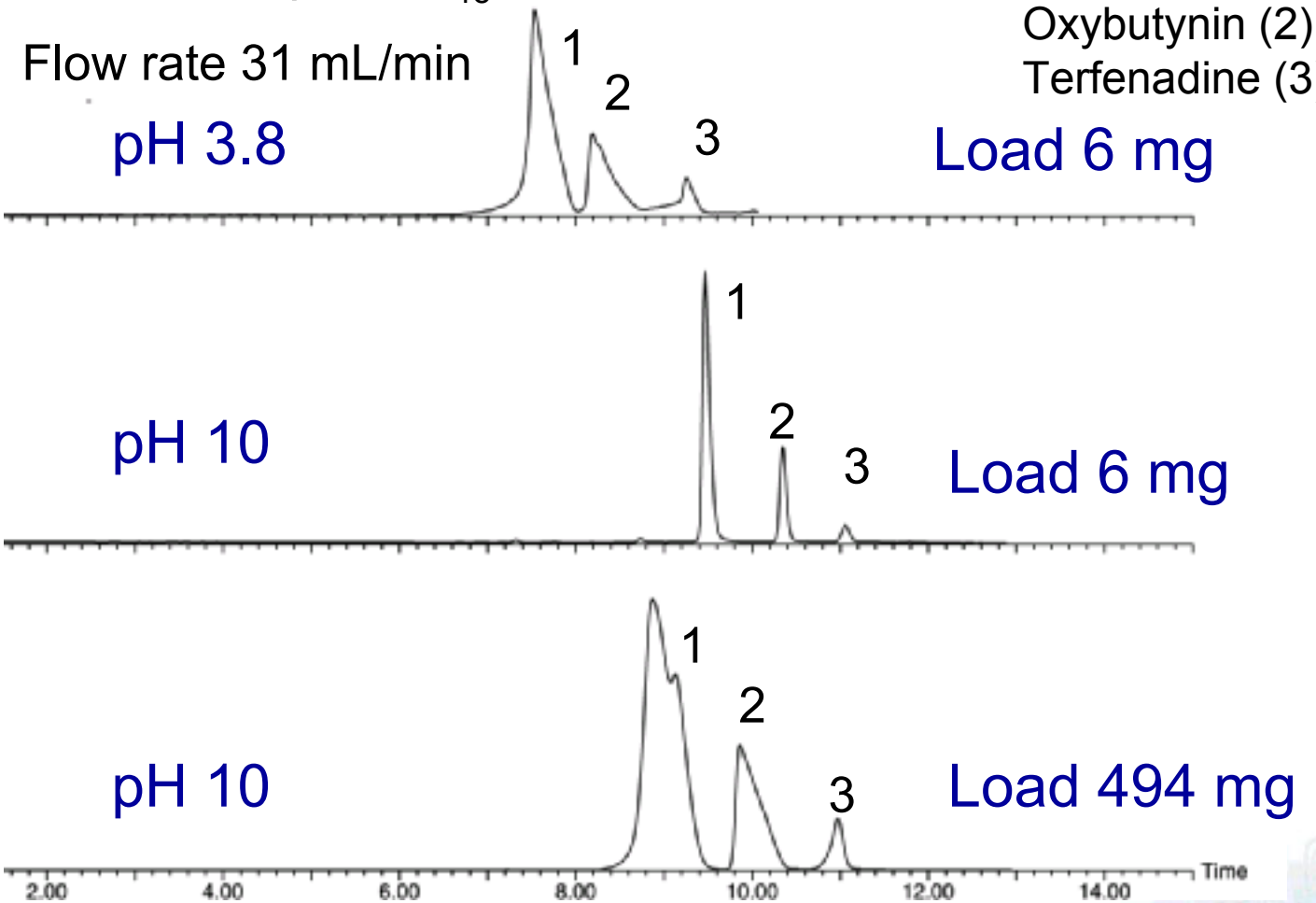
- For basic compounds:
  - The peak shape improves at high pH
  - The retention increases at high pH
- This implies that, due to improved peak shape it is possible to load more material onto the column under high pH conditions
- How much more ???

Loading of Bases

XTerra® Prep MS C_{18}, 19 x 50 mm

Flow rate 31 mL/min

Diphenhydramine (1)
Oxybutynin (2)
Terfenadine (3)



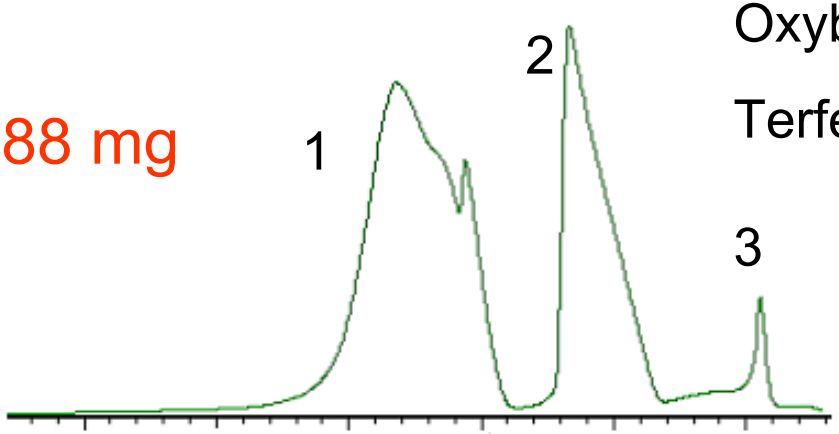
Increased at least 60X loading in the same column!

Scalability of Bases at High pH

Double column size, double load

XTerra® Prep MS C_{18}, 19 x 100 mm
Flow rate 31 mL/min

Load 988 mg



Diphenhydramine (1)

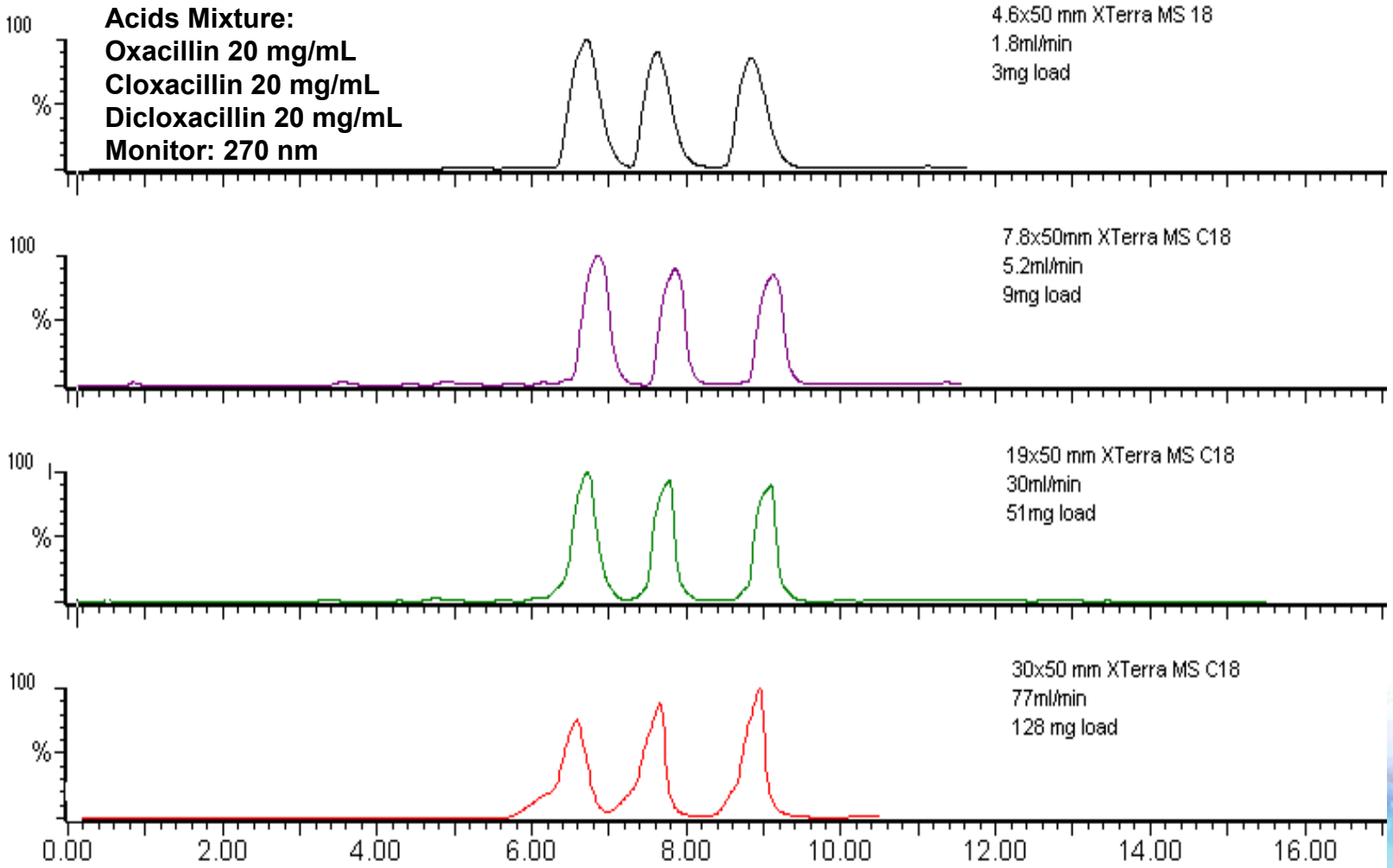
Oxybutynin (2)

Terfenadine (3)

Scalability of Acids at Low pH

Buffer A: 10 mM Ammonium Formate pH 3.8 in H_2O
Buffer B; 10 mM Ammonium Formate pH 3.8 in 95:5 ACN:H_2O
Gradient from 90:10 to 40:60 A:B in 15 minutes

Acids Mixture:
Oxacillin 20 mg/mL
Cloxacillin 20 mg/mL
Dicloxacillin 20 mg/mL
Monitor: 270 nm

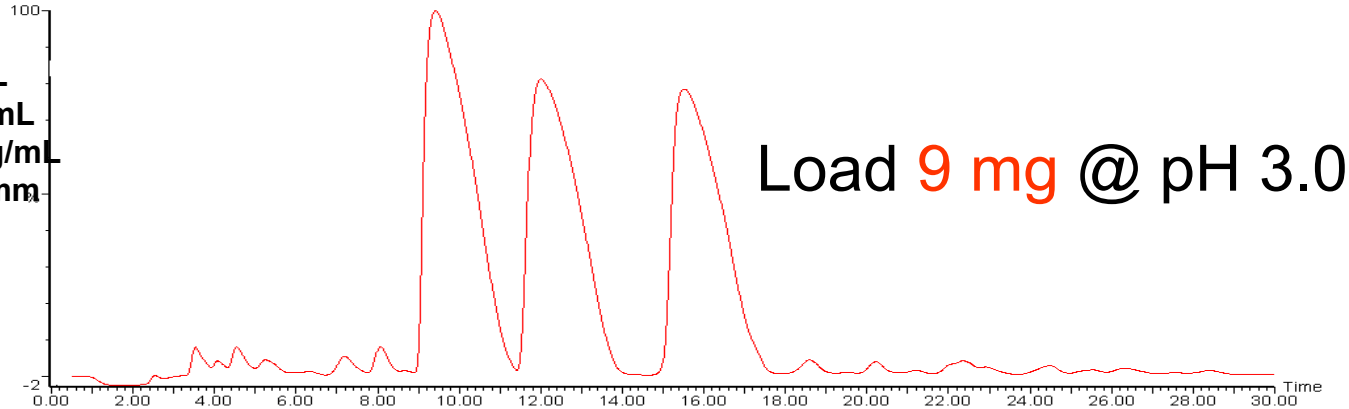


Scalability of Acids at Even Lower pH: Increase in Loadability

4.6 x 50 mm
Load 3 mg @ pH 3.8

Buffer A: 10 mM Ammonium Formate pH 3.0 in H_2O
Buffer B; 10 mM Ammonium Formate pH 3.0 in 95:5 ACN:H_2O
Gradient from 70:30 to 56:44 A:B in 18 minutes

Acids Mixture:
Oxacillin 20 mg/mL
Cloxacillin 20 mg/mL
Dicloxacillin 20 mg/mL
Column: 4.6 X 50 mm
Monitor: 270 nm



Load 9 mg @ pH 3.0

Loading increased 3X by lowering the buffer pH from 3.8 to 3.0
It is possible to load 384 mg on a 30 x 50 mm column

Increase in Loadability when Compound Loaded in Non-Ionic Form

- Ionized sample compound 1X Load
- Non-ionic sample compound > 20X Load
- Examples:
  - Doxylamine
  - Diphenhydramine
  - Oxybutinin
  - Terfenadine
  - Propyl Gallate
  - Oxacillin
  - Cloxacillin
  - Dicloxacillin

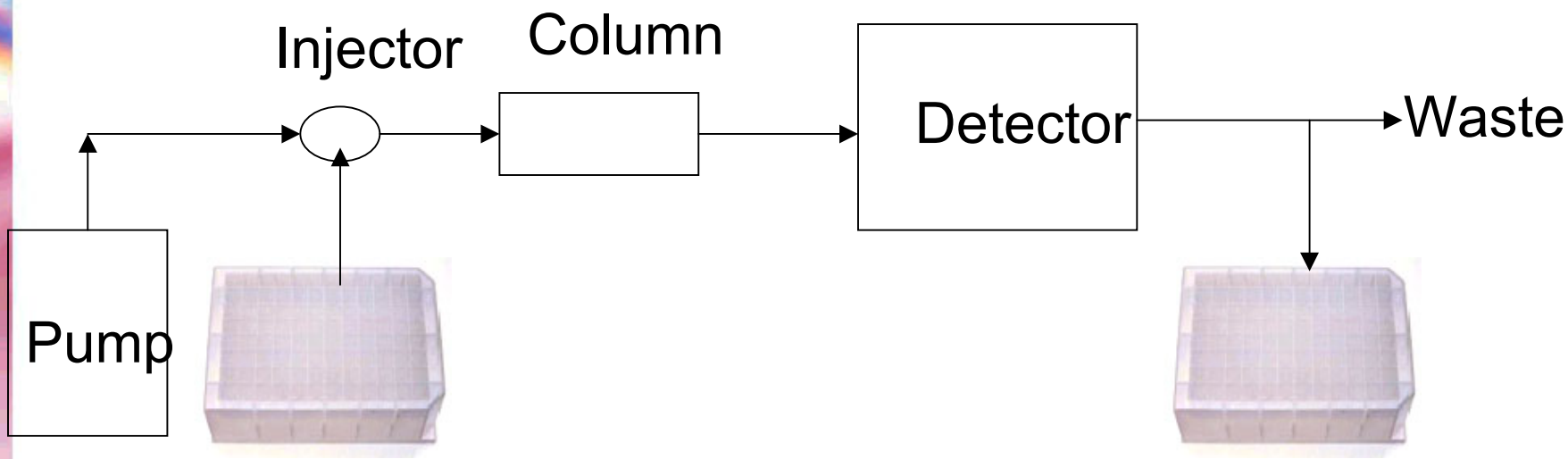
What Size Column is Really Necessary?

- While it has been shown that up to 500 mg can be loaded onto a 19 x 50 mm column, it is not always necessary to load that much.
- As loadability increases significantly when carrying out chromatography with ionizable compounds in their neutral state, then it is possible to consider reducing the column size.

Scaling to Smaller Columns Allows:

- Faster chromatography while maintaining resolution and peak purity
- Peak volume reduction leading to reduced post-purification sample handling time including dry-down of fraction
- Less expensive column
- Depending on the application, how far is it possible to downsize?
- Plate-to-plate mapping - injecting from a 96-well plate and collecting fractions in another 96-well plate - could it now be possible?

Plate-to-Plate Mapping



Samples to be purified

2 mL 96 well plate

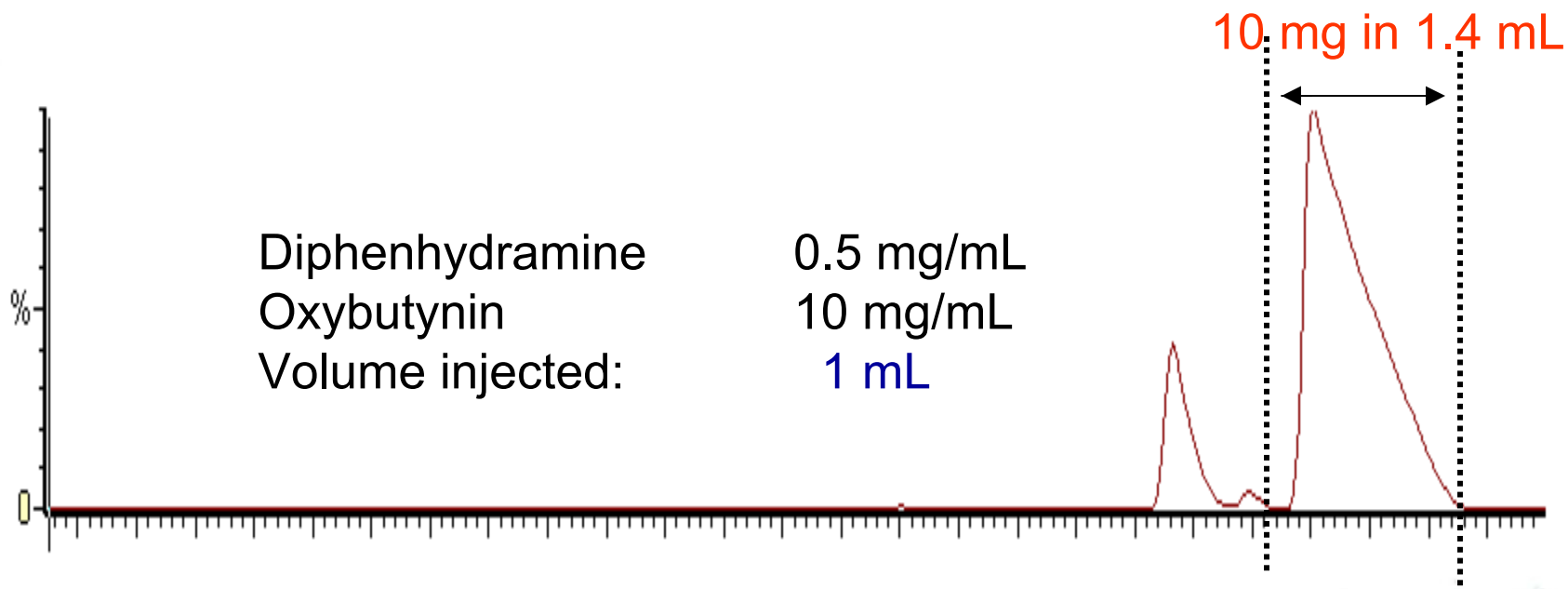
Fractions collected

2 mL 96-well plate

If sample loaded is on the order of 10 mg,
Analytical columns can be employed

Pure Material in One Fraction

XTerra® MS C_{18} 4.6 x 50 mm



Diphenhydramine	0.5 mg/mL
Oxybutynin	10 mg/mL
Volume injected:	1 mL

This volume can be collected in one fraction in one 2 mL well

Conclusions

- We have shown that loadability increases dramatically when carrying out chromatography with ionizable compounds in their neutral state
- As loadability increases, it is possible to purify compounds in less runs and use smaller columns, decreasing fixed and operational costs significantly
- Plate-to-plate mapping for loadings within the 10 mg range is now possible using analytical sized columns and equipment, decreasing costs as well as fraction handling

At-Column-Dilution for Preparative Chromatography

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Abstract

Sample loading onto a column can be highly compromised due to the solubility of the compounds or their compatibility with the initial mobile phase conditions, resulting in low yields and productivity. Consequently, exploring alternative ways to load sample onto the column is urgently needed. The at-column-dilution method is a significant alternative as it allows an increase in sample loading, improving peak shapes, providing higher yield and productivity of the targeted compound. Case studies are shown where the benefits of the at-column-dilution method are demonstrated when loading basic compounds under acidic conditions and eluting at high pH, as well as when loading samples dissolved in strong sample solvents such as DMSO. The cases shown here illustrate the utility of the at-column-dilution technique while maintaining present day isolation and purification needs both in combinatorial chemistry as well as drug development.

Motivation for this Work

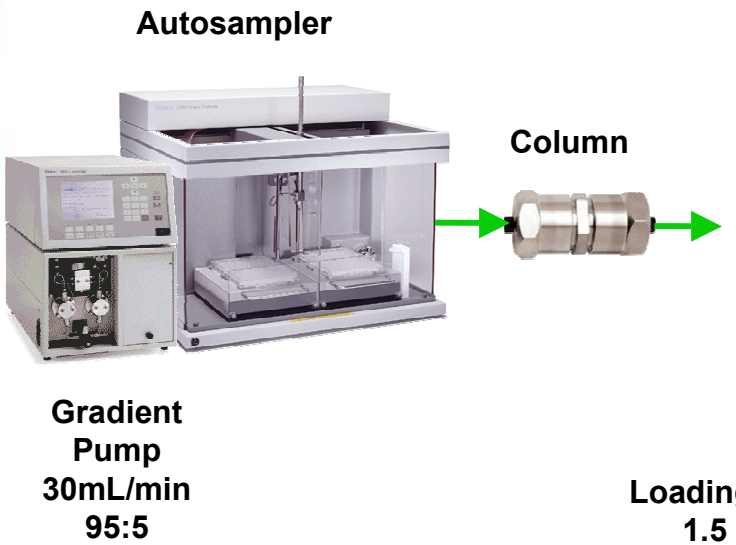
- Sample solvents can affect dramatically the chromatographic performance due to:
  - *Poor solubility of the sample in the loading solvent*
    - Limiting loading
    - Increasing injection volume
    - Reducing the number of samples processed daily
  - *Strong sample solvent effects*
    - Shifting retention times
    - Producing distorted peaks
- Increased costs and handling times occur if solvents have to be change before loading samples into the column
- A solution to this problem is urgently needed to purify samples dissolved under conditions not compatible with common initial mobile phase conditions

What is the At-Column-Dilution Method and Why Employ this Technique?

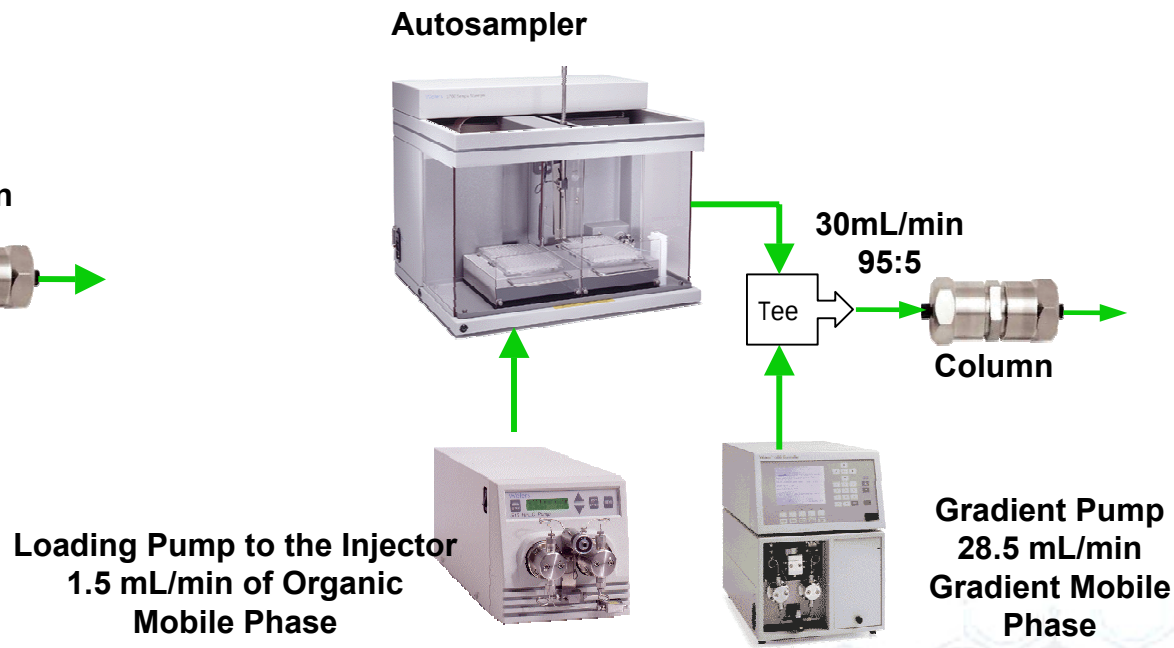
- The at-column-dilution technique permits the loading of sample onto the column parallel to the mobile phase stream.
- By employing this technique:
  - The risk of sample precipitation in the injector, loop or head of the column is eliminated
  - Sample loading increases drastically
  - Injection volume can decrease
  - Productivity for a given compound increases as fewer number and shorter runs can be readily accomplished
  - Retention shifts due to strong solvent effects are minimized
  - Peak shape improvement occurs

Equipment Set-Up

Standard Chromatography Set-up



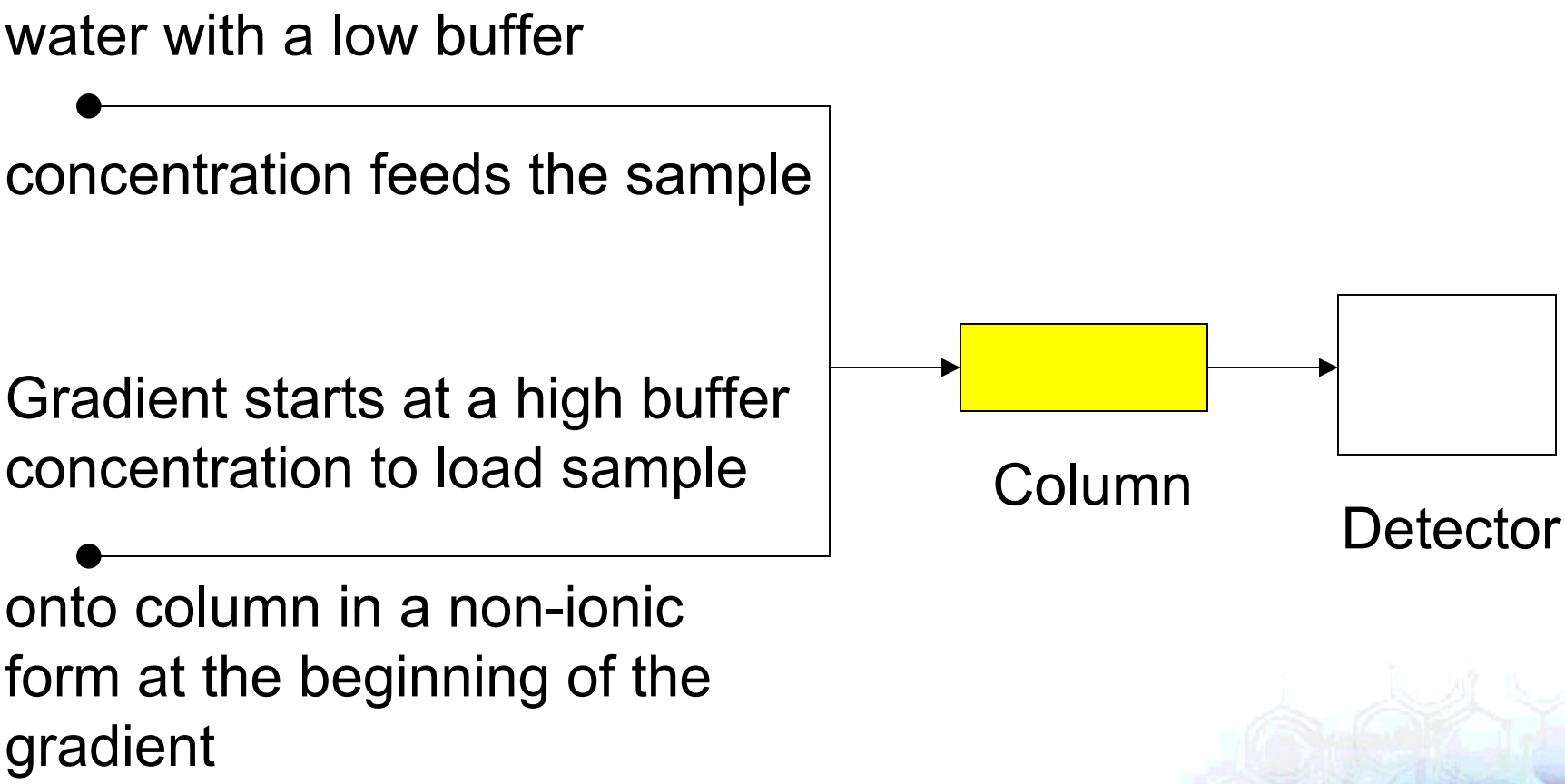
At-Column-Dilution System



Loading Basic Compounds at High pH as the HCl Salt

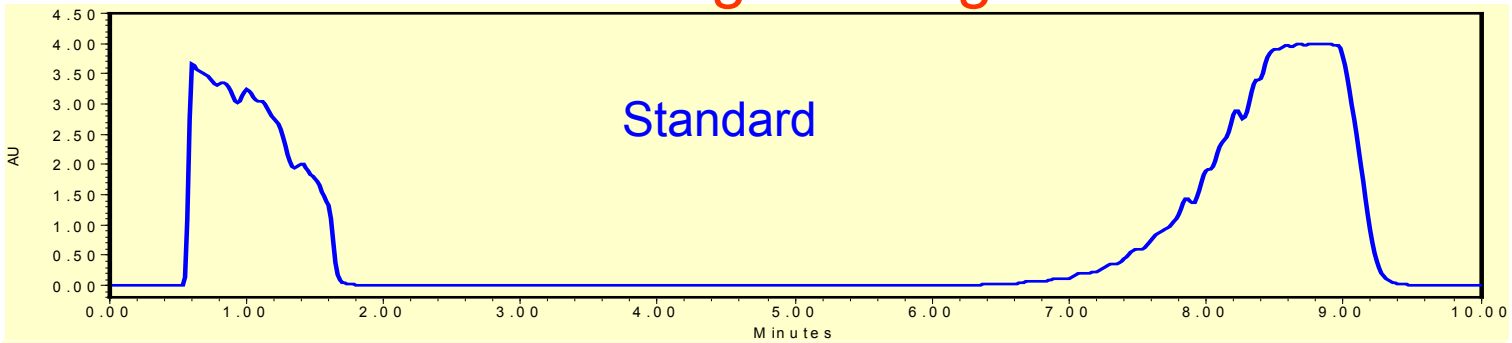
- Hydrochloride salts are best dissolved in water for maximum solubility
- However, loading a base in the ionized form at high pH decreases the loadability
- High buffer concentrations in the gradient create solubility problems and impede MS detection
- At-column dilution into a high buffer concentration at the beginning of the gradient solves the problem

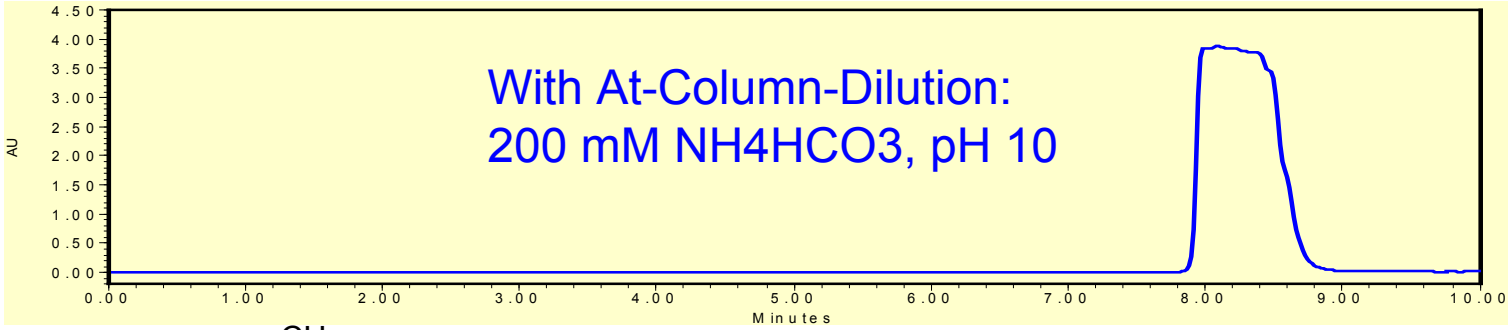
Diagram of At-Column-Dilution

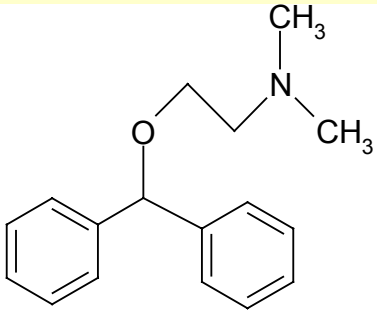


Loadability Comparison at High pH

800 mg Loading







Diphenhydramine

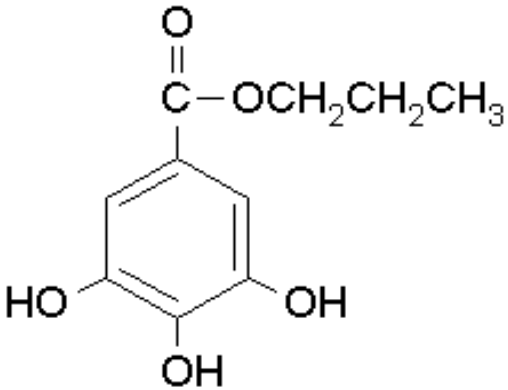
Conditions:

Column:	XTerra® Prep MS C18 19 x 50 mm, 5µm
Gradient:	Equilibrated for 5 min at 5% ACN, then gradient tg = 5 min, 5 to 90% ACN, and hold at 90% ACN for 1 min, The mobile phases contain 10 mM NH4HCO3, pH 10.0
Flow Rate:	30 mL/min
Analyte:	Diphenhydramine (800 mg) dissolved in H2O
Injection Vol.:	2 mL
Detection:	254 nm

Loading Samples Dissolved in Organic Solvents

- A substantial amount of samples are dissolved in organic solvents to increase solubility. However, under common initial mobile phase conditions, there is a high risk of precipitation within the injector, the loop and head of the column
- High viscosity solvents generate pressure spikes as the sample is loaded onto the column reducing the column lifetime

Evaluation of Loading with Various Organic Solvents



Propyl Gallate

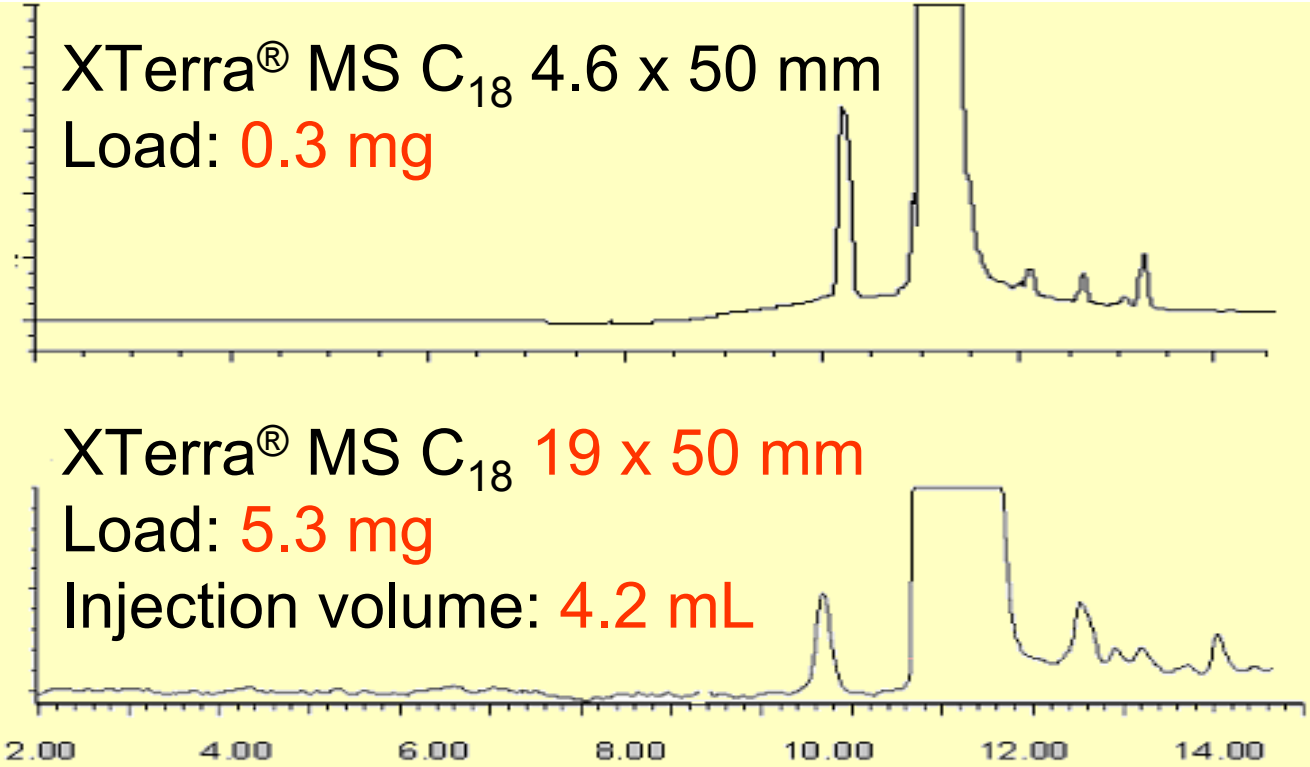
- Dissolved in Water at 1.25 mg/mL
- Dissolved in Organic Solvents at 100 mg/mL

- Column and conditions:
  - XTerra® MS C_{18}
  - Buffer A: 90/10 DIWater/100 mM Ammonium Formate pH 3.8
  - Buffer B: 90/10 Methanol/100 Ammonium Formate pH 3.8
  - Gradient: 95/5 to 5/95 A/B in 30 column volumes
  - UV monitored at 254 nm

Impurity Profile with Sample Dissolved in Water

Propyl Gallate

Sample dissolved in water
Chromatography run at pH 3.8



A large injection is needed to achieve 5.3 mg loading at preparative scale

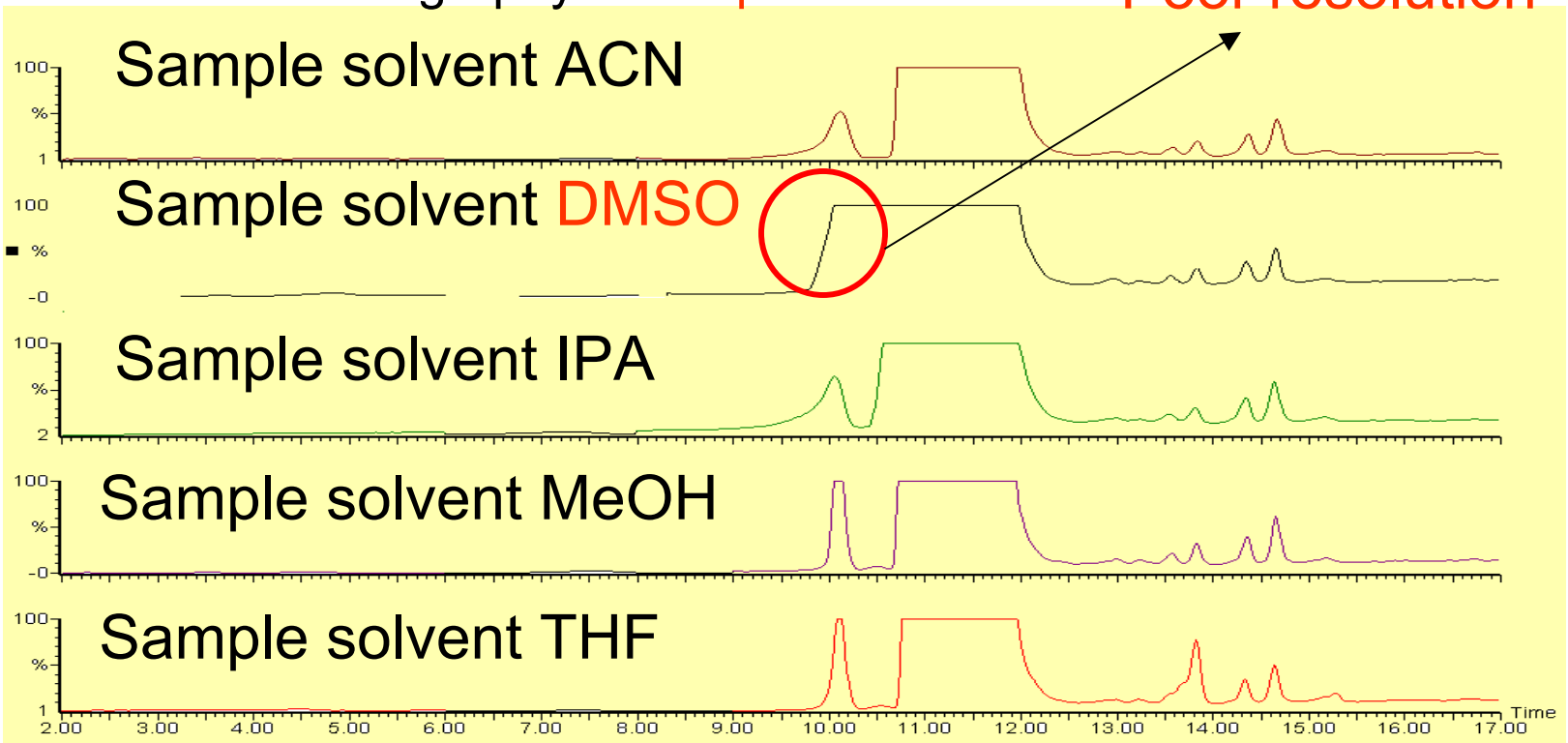
Alternative Sample Solvents are Employed

- To increase solubility of sample, increase loading and decrease injection size, samples are dissolved in organic solvents
- However, the contributions of these solvents can play a significant role in the final chromatography

Sample Solvent Affects Resolution and Peak Shape

Chromatography run at pH 3.8

Poor resolution



XTerra® MS C_{18} 19 x 50 mm

Loading: 30 mg

Injection volume: 0.3 mL

What causes this phenomena?

Sample solvent strength

or viscous fingering?

Sample Solvent Strength

- Sample solvent strength was evaluated under the following experimental conditions:
  - XTerra® MS C_{18} 4.6 X 50 mm
  - A: DIWater and B: Organic solvent
  - Gradient: 0-100% B in 30 column volumes
  - Flow rate: 1.8 mL/min, UV monitored at 254 nm

Solvent B	propranolol retention time (min)	tolune retention time (min)	order of elution(*)
Acetonitrile	2.25	5.41	3
Dimethyl sulfoxide	2.50	9.76	5
Isopropyl alcohol	1.79	5.09	1
Methanol	2.43	6.51	4
Tetrahydrofurane	2.21	5.41	2

(*) scale 1-least retained, 5-most retained

DMSO is the weakest solvent, indicating that the lack of resolution is *not* determined by sample solvent strength

Pure Sample Solvent Viscosity

Solvent	Viscosity (cP)(*)
Acetonitrile (ACN)	0.37
Dimethyl sulfoxide (DMSO)	2.20
Isopropyl alcohol (IPA)	2.50
Methanol (MeOH)	0.60
Tetrahydrofurane (THF)	0.46

(*) Neue, Uwe, "HPLC Columns: Theory, Technology and Practice", Wiley-VCH, 2nd Ed., 1997, p.31

If viscous fingering effects decrease resolution due to the viscosity of the pure sample solvent, then a compromised separation should result as the sample solvent viscosity increases. However, while the IPA results are acceptable, that is not the case with DMSO. Therefore, loss of resolution due to the viscosity of the sample solvent itself is not the case.

Viscosity Mixture of Sample Solvent and Eluent

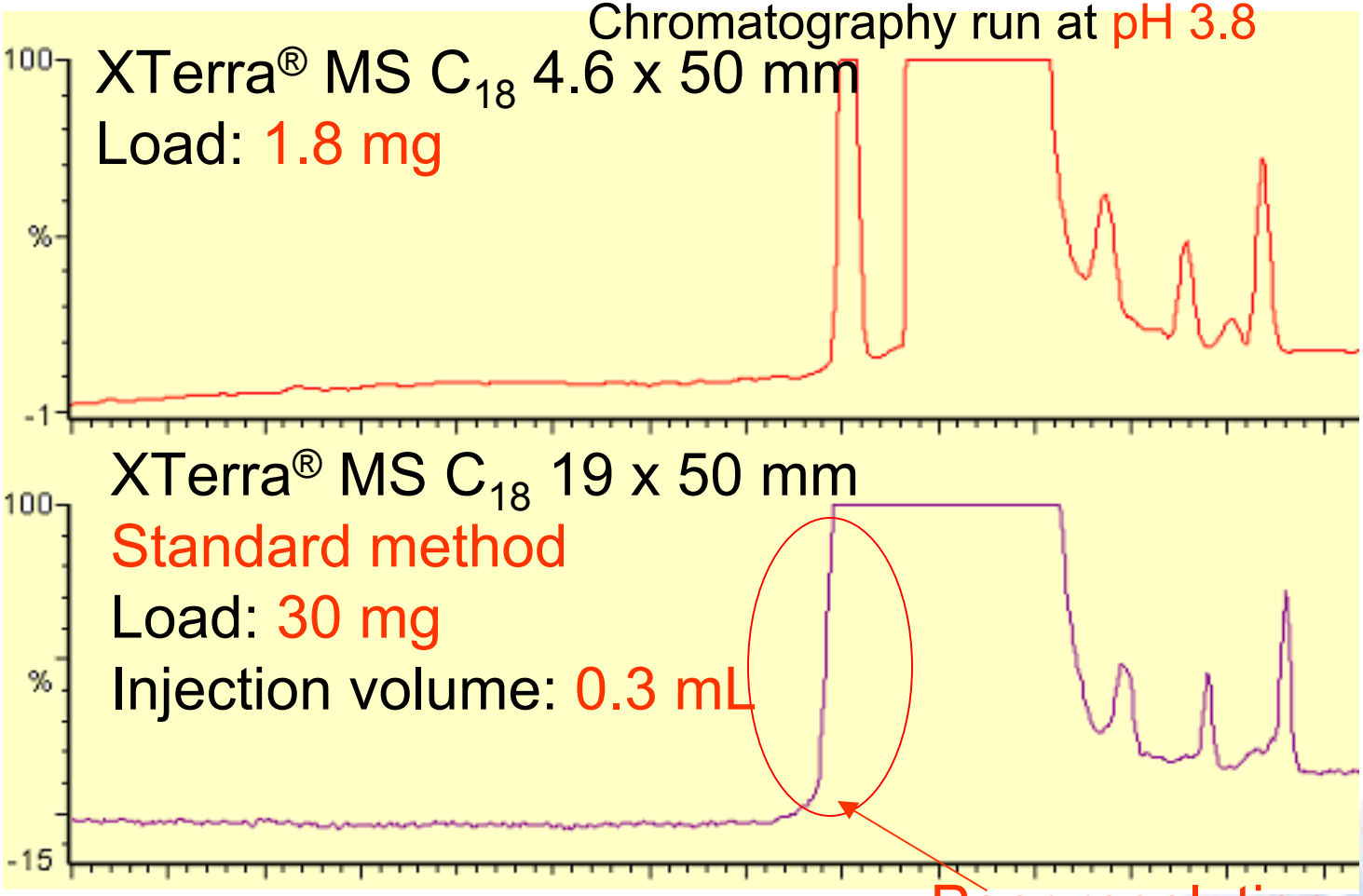
- Pressures were recorded when running the previous experiments and the maximum pressure results are shown below.
- Pressure is directly proportional to viscosity

Solvent	Highest pressure drop across the column(psi)	viscosity ranking
Acetonitrile	1120	2
Dimethyl sulfoxide	4400	5
Isopropyl alcohol	3650	4
Methanol	1730	3
Tetrahydrofurane	1105	1
(*) scale 1-lowest viscosity, 5-highest viscosity		

- While IPA has the highest viscosity of the organic solvents tested, the non-idealities of the mixture DMSO/water cause the highest viscosity in the experiments.

Impurity Profile at Low pH

Propyl Gallate Sample dissolved in DMSO
Chromatography run at pH 3.8



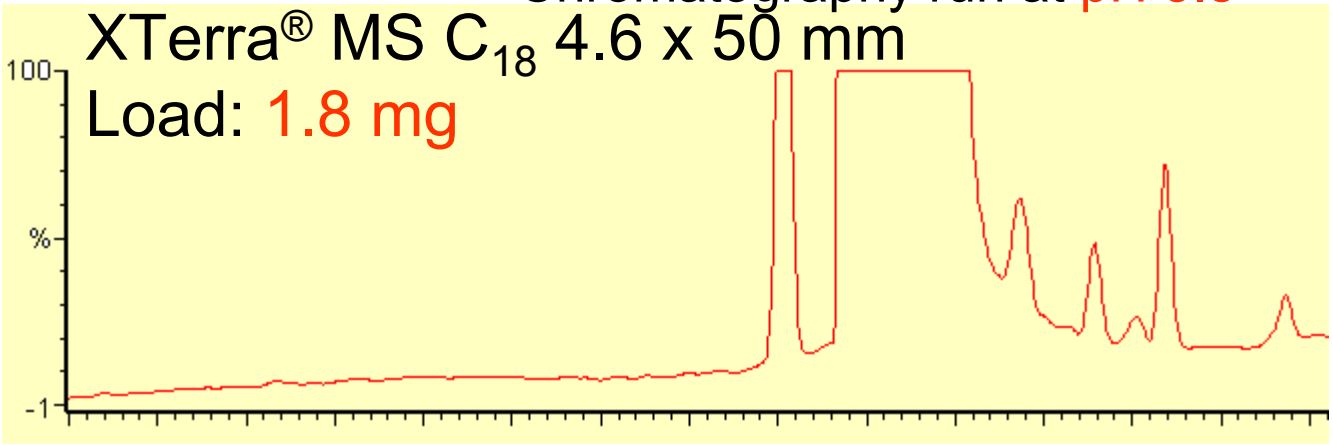
Evaluating the Chromatographic Results

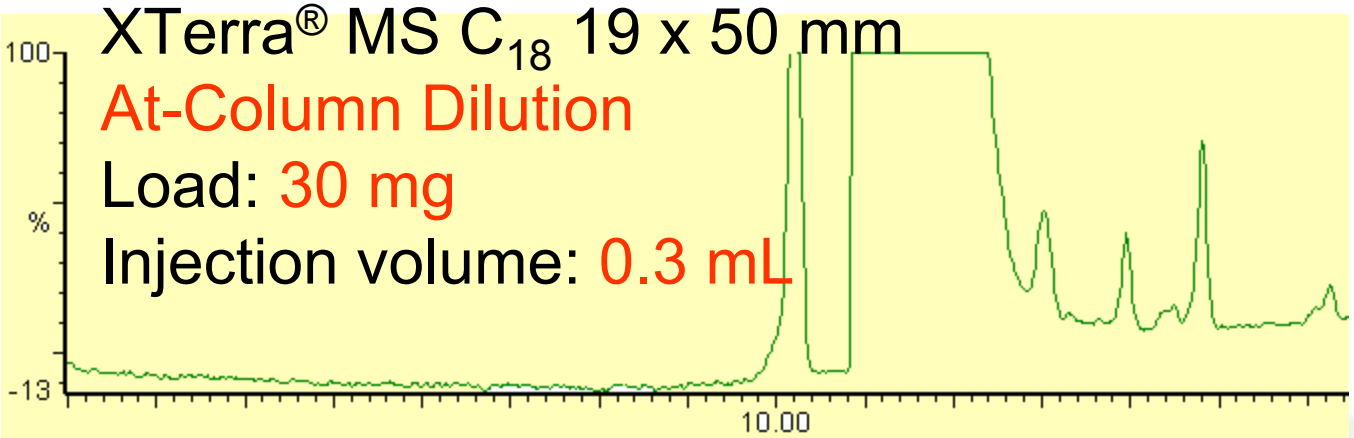
- The non-ideal mixture of DMSO and initial water rich mobile phases generate such high viscosities in the preparative column that a “viscous fingering” type of effect is created resulting in poor resolution of the chromatographic peaks.
- An alternative method of injecting samples onto the column is urgently needed as a significant percentage of drug candidates that need to be isolated and purified are dissolved in DMSO.

At-Column-Dilution Improves Impurity Isolation

Propyl Gallate

Sample dissolved in DMSO
Chromatography run at pH 3.8





5.6X increase in mass and 93% injection volume reduction by using DMSO as sample solvent and the at-column-dilution method

Conclusions

- The use of at-column-dilution when loading basic compounds at high pH as the HCl salt results in a substantial improvement of chromatographic separations
- At-column-dilution is the preferred method when purifying samples dissolved in DMSO
- The at-column dilution method results in enhanced loadability for ionizable compounds when they are loaded onto the column in a non-ionized form