크로마토그래피의 원리와 분석법

HPLC의 분석법-1

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Expert Tips for Small Molecules

Simple DO's and DON'Ts to protect your HPLC Columns:

- DO use a guard cartridge.
- DO de-gas solvents.
- DO tightly cap column ends.
- DO use purified water and high purity solvents.
- DO use filters.
- Do check the purity of solvents when they arrive in the laboratory.
- DO flush several volumes of methanol (or similar) through the HPLC system prior to shutdown.
- DO flush several milliliters of solvent through new filters, tubing and fittings before connection to the column.
- DO filter both the mobile phase and sample.
- DO store column at room temperature.
- DO protect the column from knocks and bumps.
- DO worry about salt build-up at fitting connections.
- DO attempt to control the temperature of a column.
- DO rinse organic solvents from the column with 50/50 organic/aqueous solvent prior to using buffers.
- DO use PEEK™ ferrules on one piece fingertights where possible.
- DO contact SGE if in doubt.
- DON'T inject crude biological samples directly into a column.
- DON'T let the column dry out.
- DON'T use a pH outside the manufacturer's recommended range for your column.
- DON'T drop your HPLC column.
- DON'T use too much connecting tubing.
- DON'T place excessive back pressure on your column or system.
- DON'T leave a buffer in a column or HPLC system at a zero flow for extended periods of time.
- DON'T use low-grade solvents.
- DON'T encourage growth of microorganisms in aqueous buffers by preparing them days in advance.
- DON'T open the ends of the column – just to see what's inside.
- DON'T use a huge wrench to tighten tiny fittings.
- DON'T use stainless steel ferrules in connections.
- DON'T use high concentrations of aggressive mobile phases.
- DON'T top up mobile phases.

국내 HPLC grade solvent는 사용하지 말기

HPLC column의 유지 및 관리 방법

①컬럼 안정화

컬럼 설치 후 반드시 이동상으로 안정화 시켜주어야 한다. 이러한 과정은 컬럼의 저장 용매와 이동상이 섞일 수 있게 해준다. (iso-propyl alcohol은 지용성/수용성 중간체 역할을 할 수 있다.) 컬럼은 시료를 주입하기 전에 적어도 10배 이상의 컬럼 부피의 이동상을 컬럼으로 흘려주어 평형 시킨다.

Ex) 4.6mm 기준 10.3mL → 10배 = 100mL → 1mL/min 기준 1.5-2시간

②Buffer 사용 시

Buffer를 이동상으로 사용하는 경우, Buffer로 된 이동상을 흘려주기 전에 Buffer가 없는 이동상으로 충분히 컬럼을 세척해야 한다. 이러한 과정을 통해 Buffer가 컬럼이나 HPLC 시스템에서 침전되는 것을 방지한다. Buffer나 ion-paring reagent 사용의 경우 평형 시간이 더 긴 것이 좋다.

컬럼에 충격을 주거나 떨어뜨리지 않도록 주의해야 한다. 물리적인 충격은 컬럼에 빈 공간이나 터널을 생기게 할 수 있다. 이러한 영향으로 피크의 tailing이 발생하고 효율이 감소된다.

"유속(압력), 용출 조성, 컬럼의 온도"를 갑자기 변화 시키는 것을 피해야한다.

이러한 물리적, 온도적 변화는 컬럼의 부피를 혼란시키고, 컬럼 내 빈공간(void)이나 터널을 생성하며, 피크 끌림이나 효율성을 감소 시킬 수 있다.

컬럼을 저장하기 전에 buffer나 ion-paring 시약과 같은 것들을 물로 완전히 제거해야 한다. 이것은 흡착제 분해나 buffer 침정 등을 방지한다.

Octanol-Water Partition Coefficients (log P Values)

- *log P* is a measure of how well the neutral, unionized form of a drug partitions between a lipid phase (e.g., *n*-octanol) and water

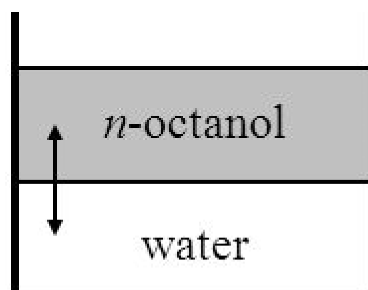
- *P* is defined as the partition coefficient:

$$P = C_o / C_w$$

If log P = 5, C_o / C_w = 100,000:1 at equilibrium!

where C_o and C_w are the equilibrium drug concentrations measured in the *n*-octanol and water phases, respectively

- Traditional method for determining log P is the shake flask method; HPLC also widely used



ADVANCED ANALYTICAL
Improving Process · Empowering Progress

HPLC Mobile Phases – 10 bad habits to avoid

1. Measuring the pH of the mobile phase after the organic has been added

pH meters are calibrated to give the correct pH readback in aqueous solution – the buffers you verify this with are aqueous. If you measure the pH with the organic added, the pH will be different to that of measuring before organic addition.
However, the most important point is to be consistent. If you do always measure pH after the organic is added, make sure you state this in the method so that everyone does it the same way. It won't be 100% accurate, but at least it will be consistent. This is probably more important than having the exact pH.

2. Not using a buffer

Buffers are present to control pH and resist a change in pH. Many other parts of method (e.g. sample matrix, CO_2 in air, source of water used for your mobile phase) can change the pH of the mobile phase causing shifts in retention, peak shape and peak response.
Formic acid, TFA etc. are not buffers.

3. Not using the buffer in its correct pH range

Each buffer salt has a 2 pH unit wide range over which it provides the optimal pH stability. Outside this window the salt is ineffective at resisting change in pH.
Either use your buffer within the correct range or pick a buffer whose range covers the pH you require.

4. Adding buffer to organic

Mixing aqueous buffer into the organic phase carries a high risk of the buffer being precipitated – in many cases so finely that it may not be obvious it has happened. ALWAYS add the organic to the aqueous phase, this greatly reduces the risk of buffer precipitation.

5. Using the pump to mix gradients from 0%

Modern pumps are very effective at mixing mobile phases and degassing online, however not everyone who ends up using your method has a high quality pump. Premix your A and B starting mix to a single solution that runs at 100% on line A. e.g. Prepare the starting mixture by mixing 950 ml aqueous with 50ml organic, then filter and degas. This reduces variability between HPLCs, reduces the risk of bubbles and precipitation in the system. Note however that 95:5 mixed on the pump will not give the same retention time as 95:5 premixed in the bottle – you normally need to add a few more percent organic when premixing.

5.5 Incomplete equilibration

It is recommended to equilibrate reversed phase columns with at least 10 column volumes, for buffered mobile phases with at least 20 column volumes before an analysis is initiated. Make sure all mobile phase channels are purged with the adequate mobile phase.

6. Not using the correct pH modifying acid or base for your buffer

Only use the acid or base that forms the buffer salt you are using. E.g. sodium phosphate buffers should be adjusted with only phosphoric acid or sodium hydroxide.

7. Not stating the full information of your buffer in the method e.g. weigh 5g of sodium phosphate into 1000ml of water

The type of buffer (mono, di or tribasic) determines its pH buffering range.

The required molarity is what determines the buffer strength. 5g or anhydrous sodium phosphate and 5g of monohydrate sodium phosphate will have different buffer strengths and will affect retention.

8. Filling lines with organic without checking what was in there before

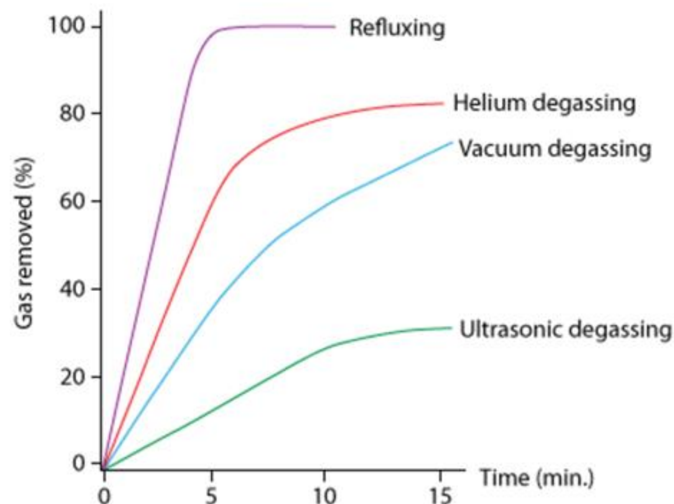
If the previous method used buffer in line B and your method uses organic in line B there's a good chance you will precipitate buffer in your pump tubing / pump head. I did it in my early days and it caused a lot of damage. If in doubt – flush it out (80:20 water : organic).

9. Propping up bottles to get last drop out

It's 5 to 5 and you've barely got enough mobile phase to finish the run – it'll be running on fumes by the last few samples. Apart from the risk or running your pump and column dry, mobile phases evaporate from the surface, so the mobile phase at the top of the bottle will have changed composition from the bulk. This portion from the top is exactly what will be running through the column if you use the last dregs in the bottle.

10. Using sonication to degas mobile phase

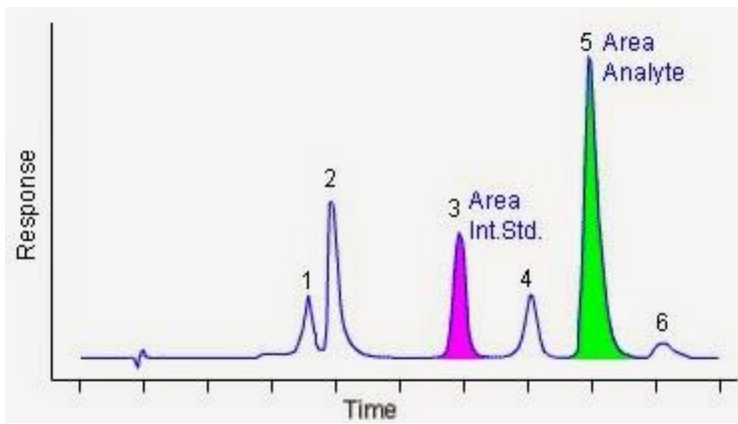
It's great for making sure all your buffer salts have dissolved, but it's the least effective method of degassing AND it quickly heats up the mobile phase causing the organic portion to evaporate. Save yourself problems later – take 5mins to vacuum filter your mobile phase – it degasses and filters in a single step.



Quantitative Analysis

EXTERNAL STANDARDIZATION

- A Pure reference standard material corresponding to the substance to be determined is dissolved in a solvent at a known concentration.
- Various quantities of this solution are injected successively.
- The areas of each the peaks produced are plotted versus the mass of the solute injected and a calibration curve is produced.

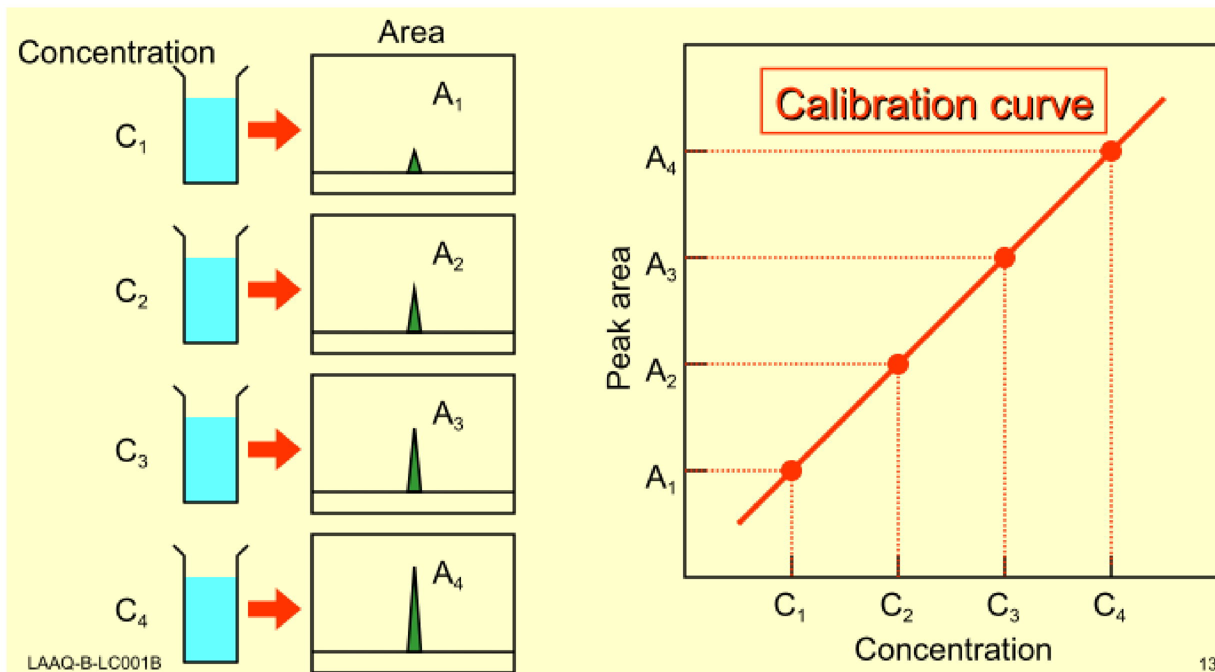


INTERNAL STANDARDIZATION

- This is to avoid difficulty of introducing precisely measured quantities into the Gas Chromatography.
- It involves two standards:
  - Analytical: a pure sample of the compound to be analyzed
  - Internal: it is normally a substance that elutes at a position close to the substance being analysed and is well resolved, but it should not be converted into analyte under normal course of analysis.

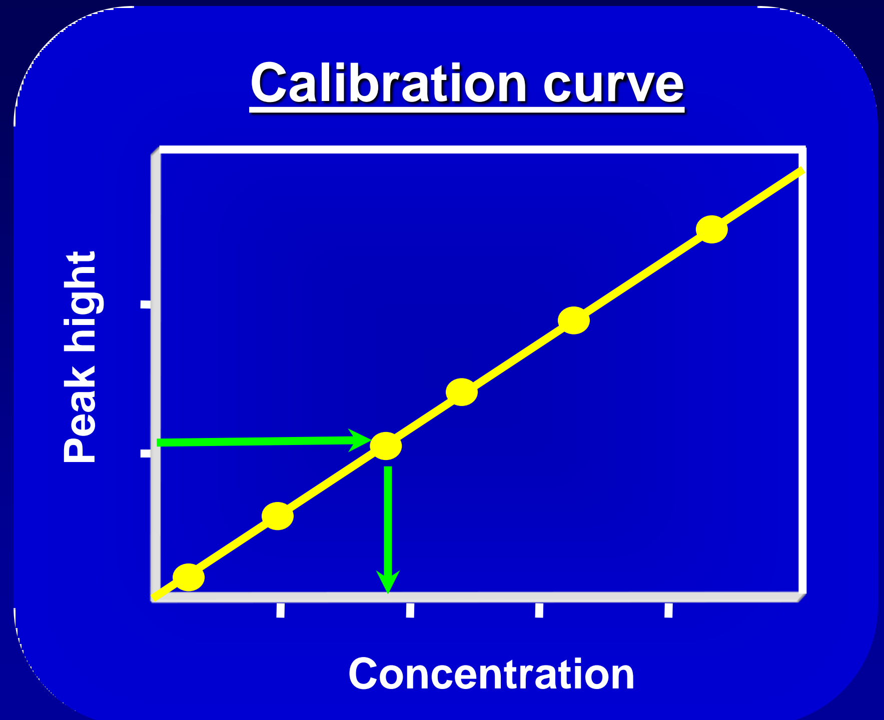
Calibration Curve for Absolute Calibration Curve Method

External Standard method



Quantitative Analysis

External Standard Method

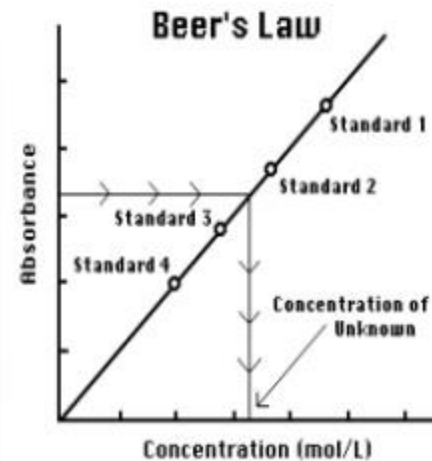


0 10 0 10 0 10 0 10 0 10 0 10 0 10

100 μg/mL 75 μg/mL 50 μg/mL 25 μg/mL 10 μg/mL 5 μg/mL Unknown

Quantitation – External standards

- **Known concentrations of the analyte using reference standards.**
- **Analyse unknown under the *same* conditions, in the same run sequence.**
- **Start with lowest concentration.**
- **Use bracketing technique**
- **At least 5 injections per level**



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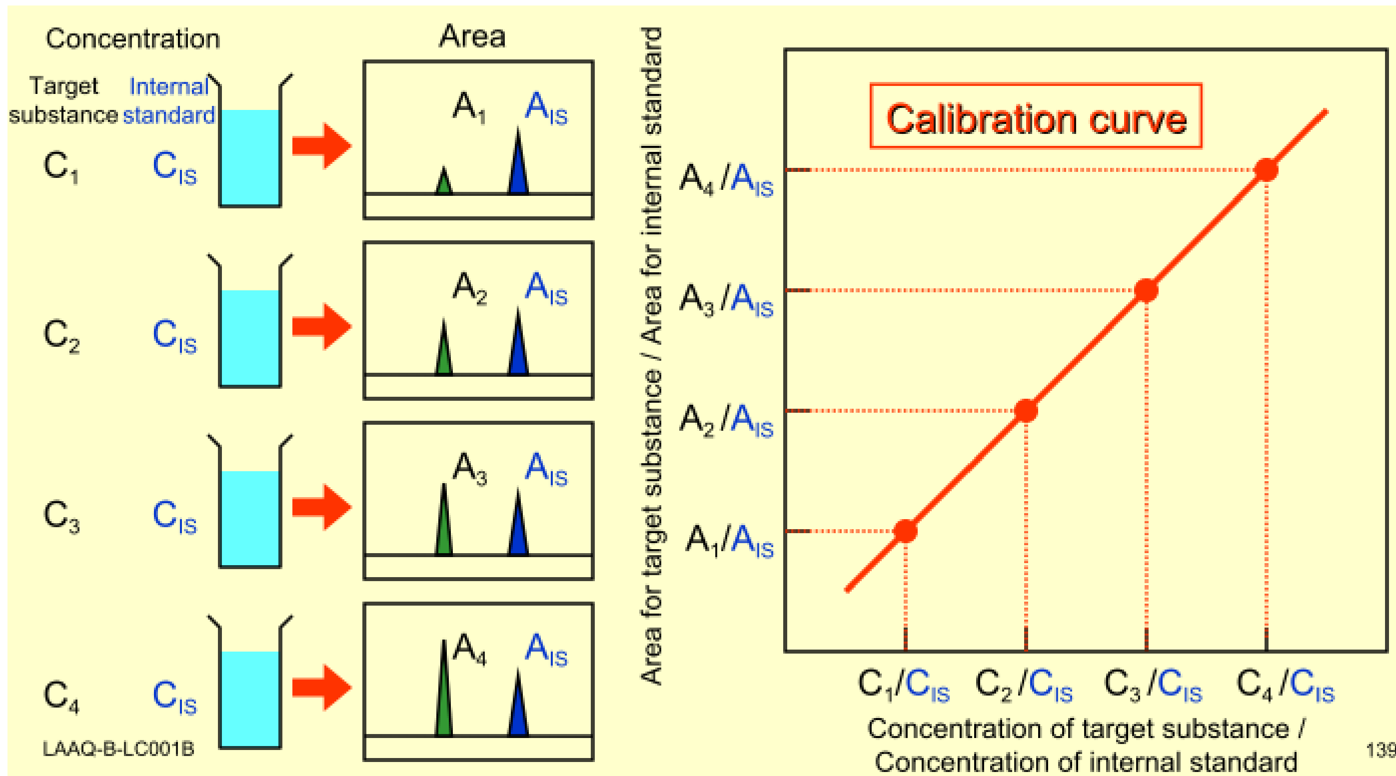
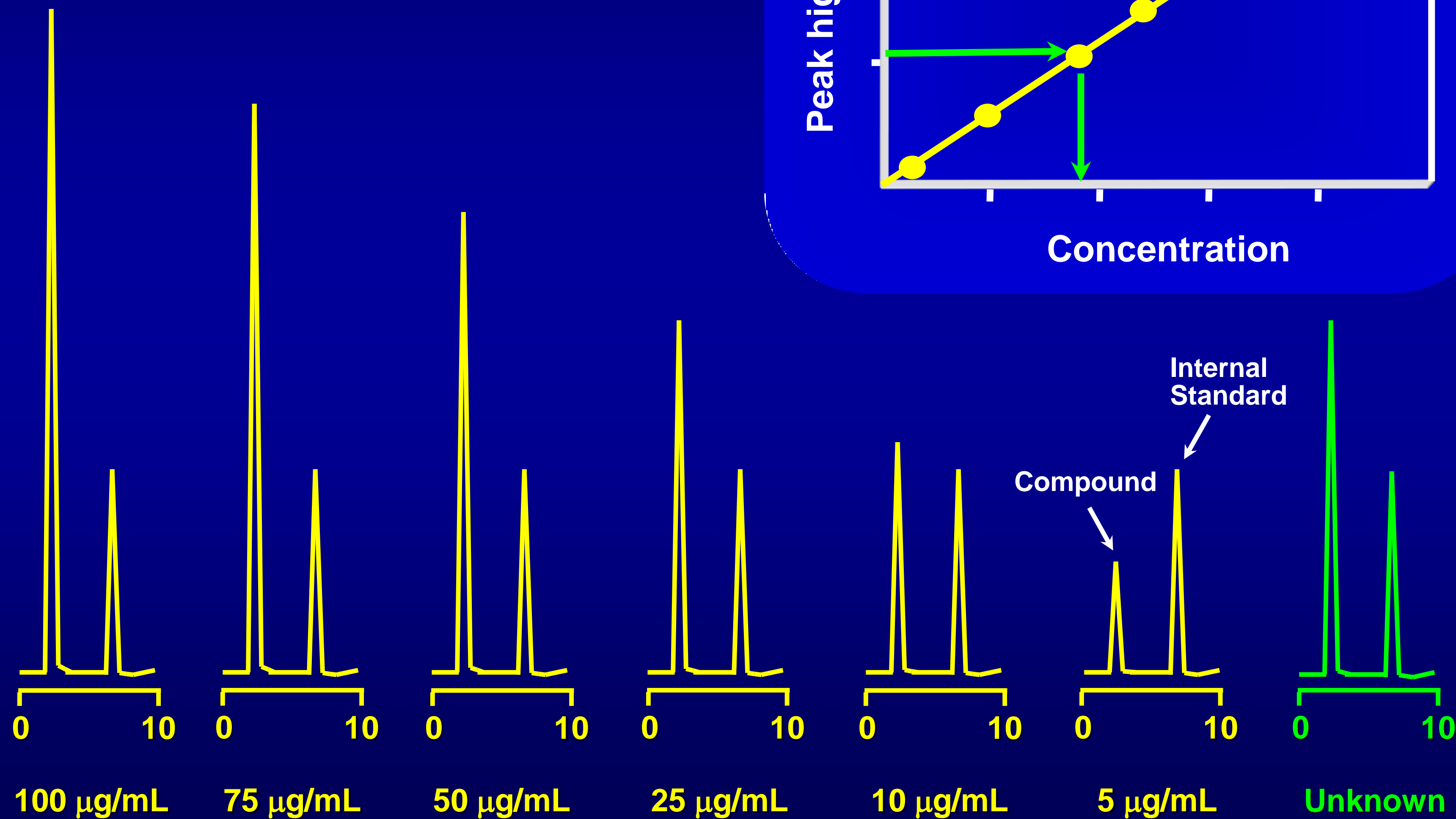
Calibration Curve for Internal Standard Method

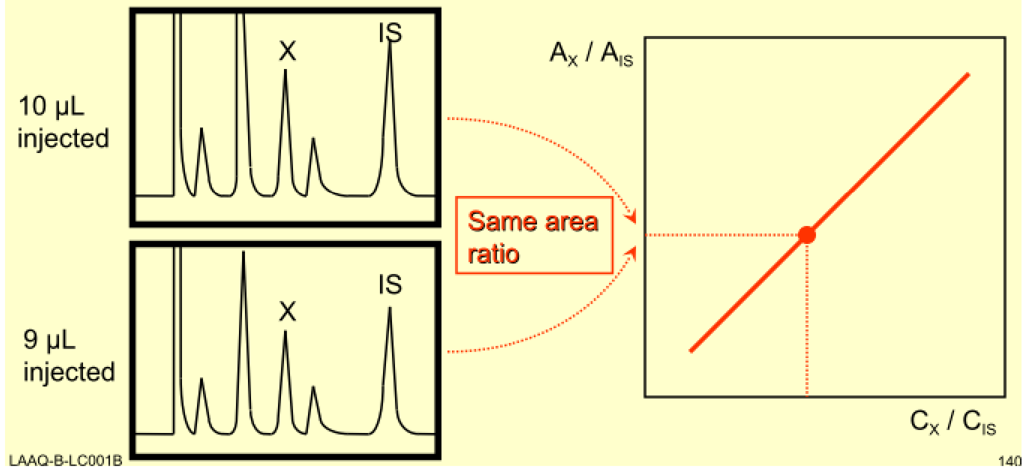


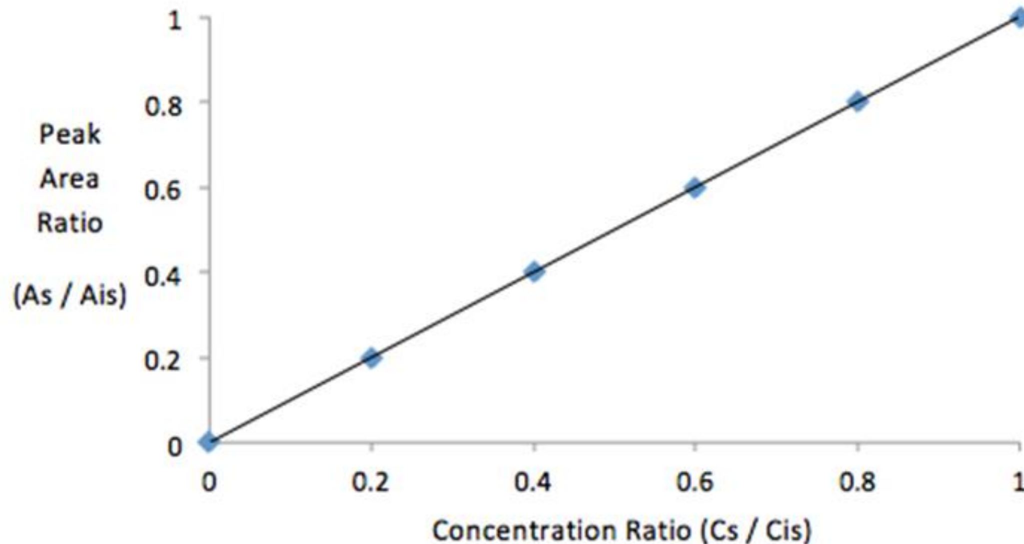
Quantitative Analysis

Internal Standard Method



Advantages of Internal Standard Method





Internal Standard Calibration Curve

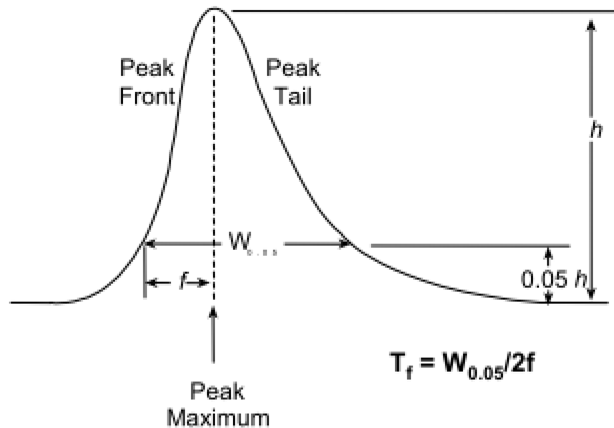
- Internal standard is added to each sample or standard in equal concentration
- The Peak Area Ratio and Concentration ratio is plotted for each calibrant
- The Concentration ratio of the sample is extrapolated from the Peak Area Ration obtained
- Cs can be calculated as Cis is known

What is Good Peak Shape and Why is it Important ?

- Good peak shape can be defined as a symmetrical or gaussian peak and poor peak shape can include both peak fronting and tailing.
- Good peak shape can be defined by….
- Tailing factor of 1.0
- High efficiency
- Narrow peak width
- Good peak shape is important for….
- Improved resolution (Rs)
- More accurate quantitation
- Longer usable column lifetime (based on system suitability criteria)

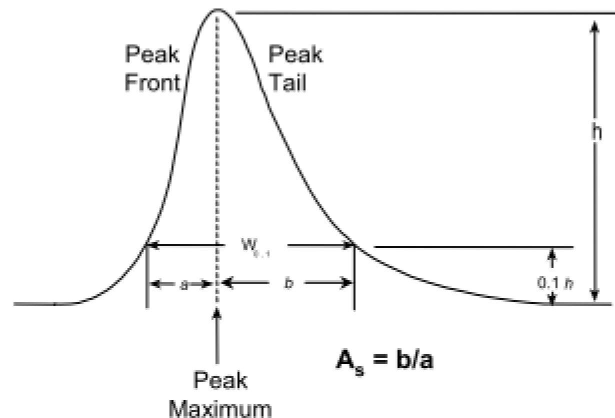
How is Peak Shape Measured?

USP Tailing Factor



$T_f = W_{0.05}/2f$

Asymmetry

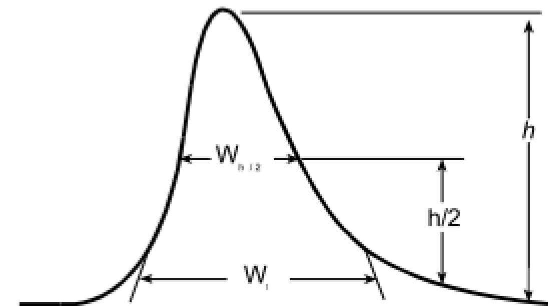


$A_s = b/a$

Efficiency

$$N = 5.54 \left(\frac{t_R}{W_{½}} \right)^2$$

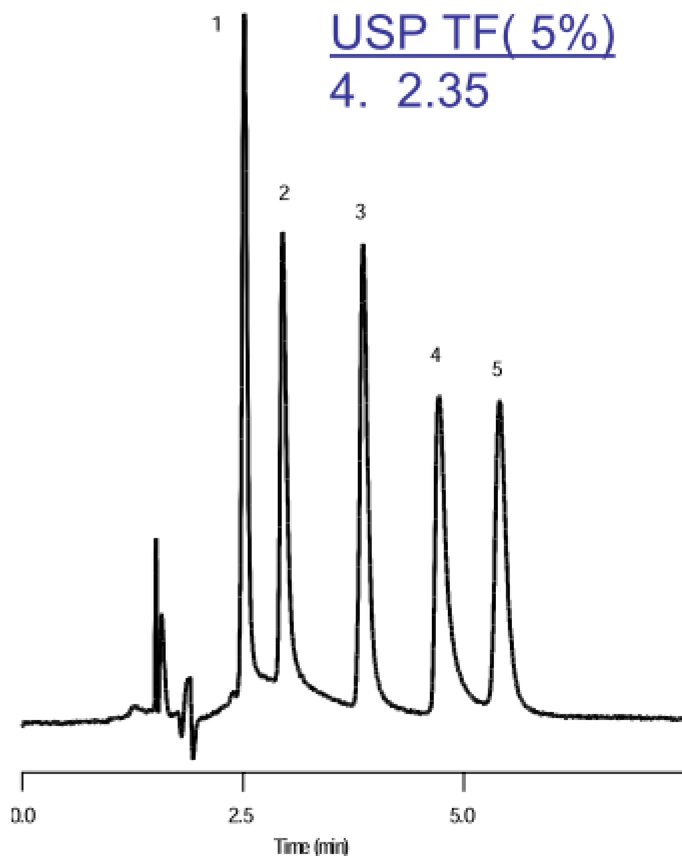
Peak Width at ½ Height

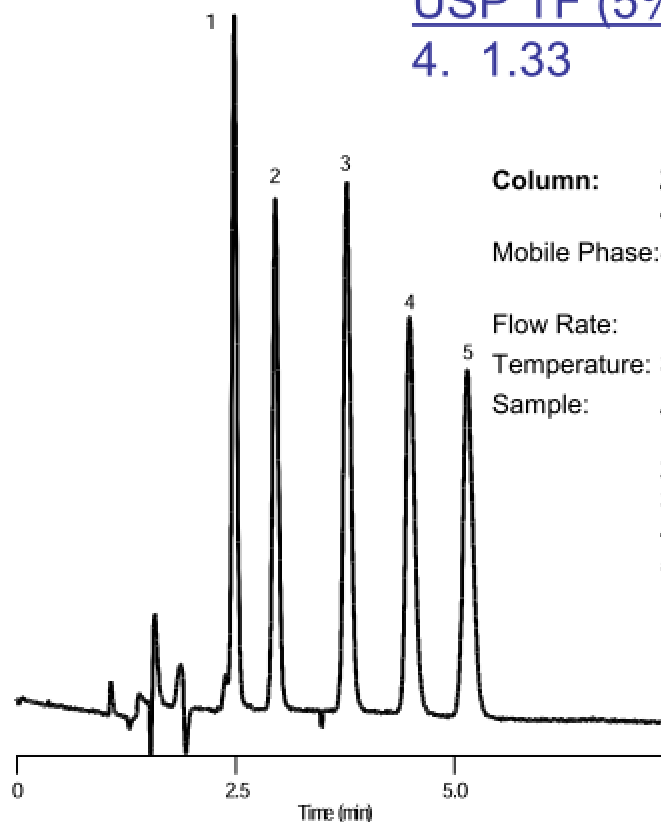


Mobile Phase Factors for Improved Peak Shape

- pH
- Buffers
- Organic modifiers
- Additional mobile phase modifiers (TEA, TFA)

Good Peak Shape at Low pH





Column: **ZORBAX Eclipse XDB-C8**
4.6 x 150 mm, 5 µm

Mobile Phase: 85% 25 mM Na_2HPO_4
15% ACN

Flow Rate: 1.0 mL/min.

Temperature: 35°C

Sample: Amphetamines

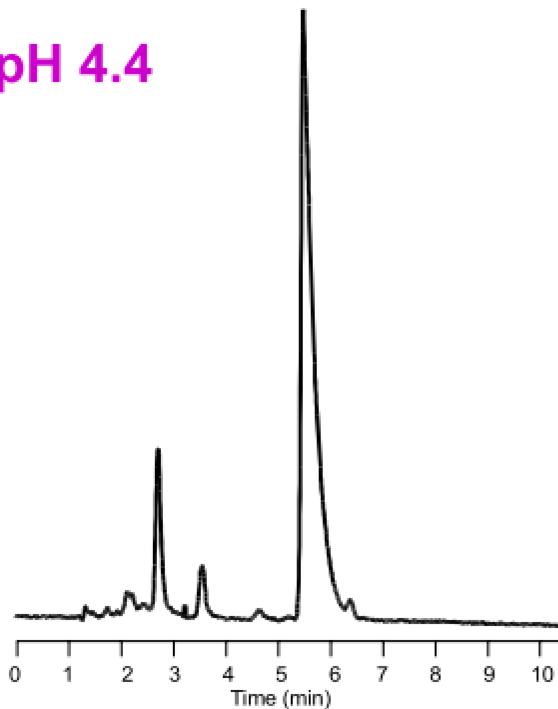
1. Phenylpropanolamine pKa 9.4
2. Ephedrine pKa 9.6
3. Amphetamine pKa 9.9
4. Methamphetamine pKa 10.1
5. Phentermine pKa 10.1

- These basic compounds have good peak shape when the pH is lowered and the silica silanols are protonated.

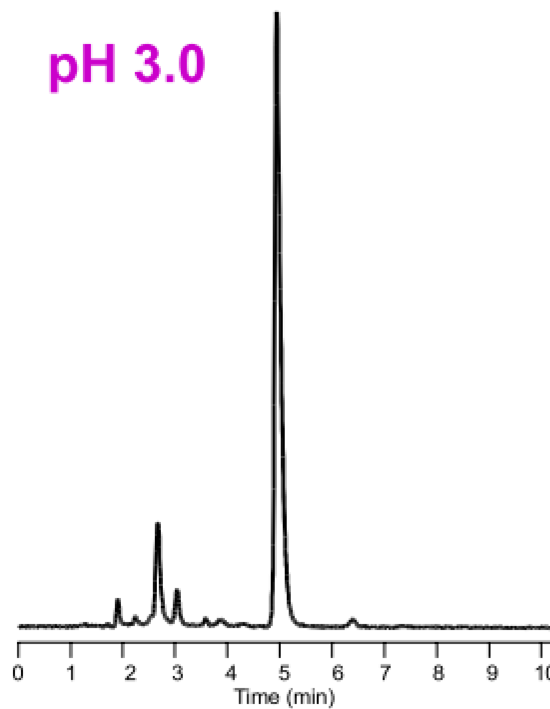
Effect of pH on Peak Shape at or Near the Sample pK_a

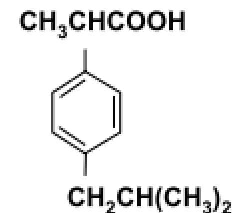
Column: ZORBAX SB-C8 4.6 x 150 mm, 5 µm Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min. Temperature: RT

pH 4.4



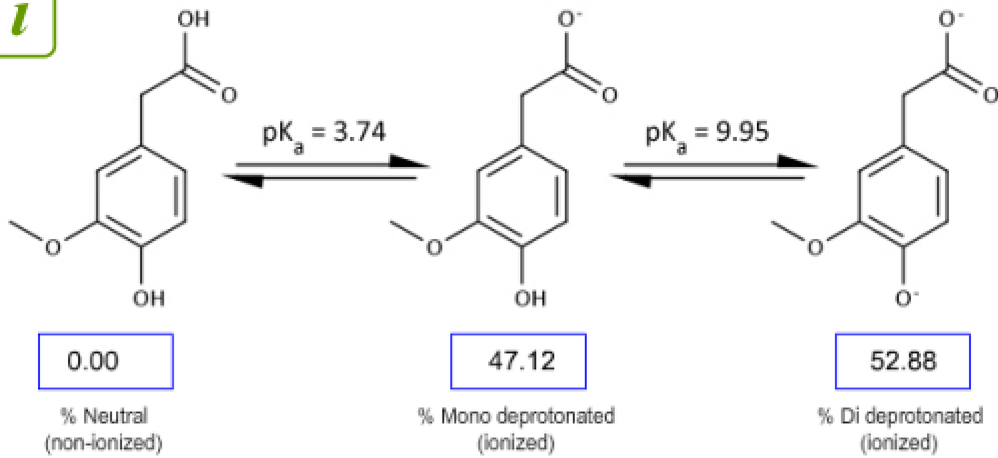
pH 3.0

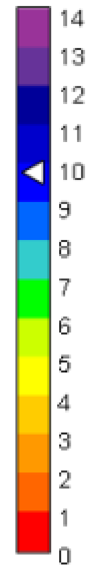


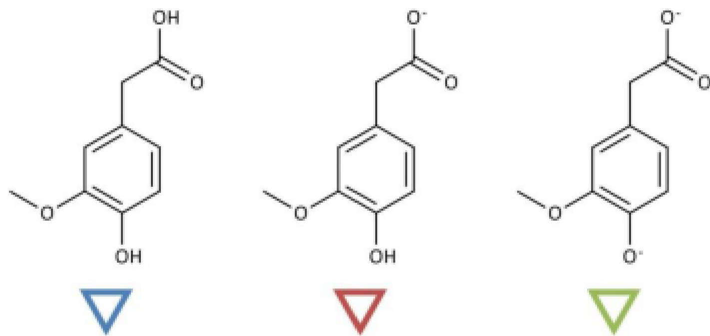


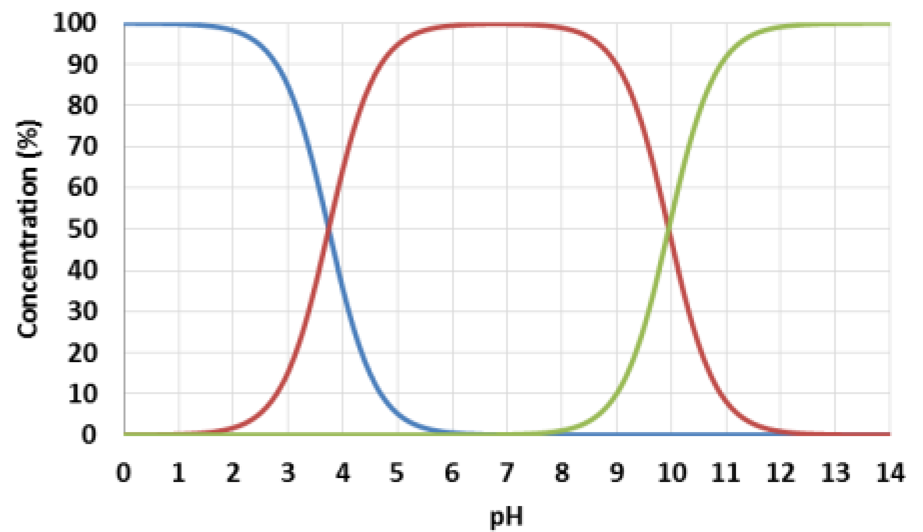
Ibuprofen
pK_a = 4.4

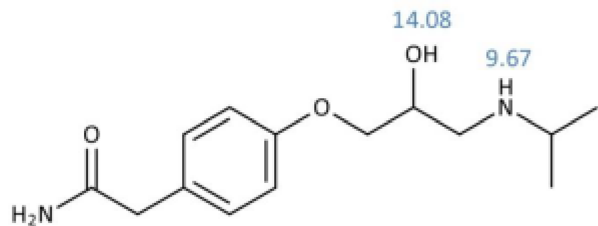
- Inconsistent and tailing peaks may occur when operating close to an analyte pKa and should be avoided.

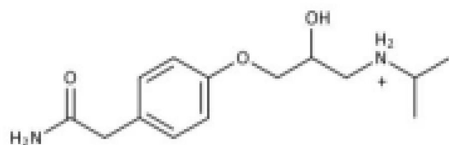

Homovanillic Acid Equilibrium
OH
O
O
OH
$pK_a = 3.74$
O^-
O
O
OH
$pK_a = 9.95$
O^-
O
O
O^-
0.00
% Neutral
(non-ionized)
47.12
% Mono deprotonated
(ionized)
52.88
% Di deprotonated
(ionized)

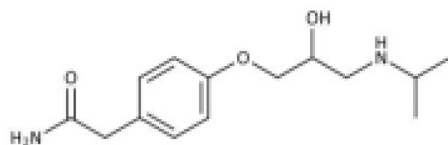

14
13
12
11
10
9
8
7
6
5
4
3
2
1
0

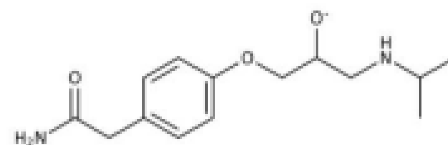

OH
O
O
OH
O^-
O
O
OH
O^-
O
O
O^-

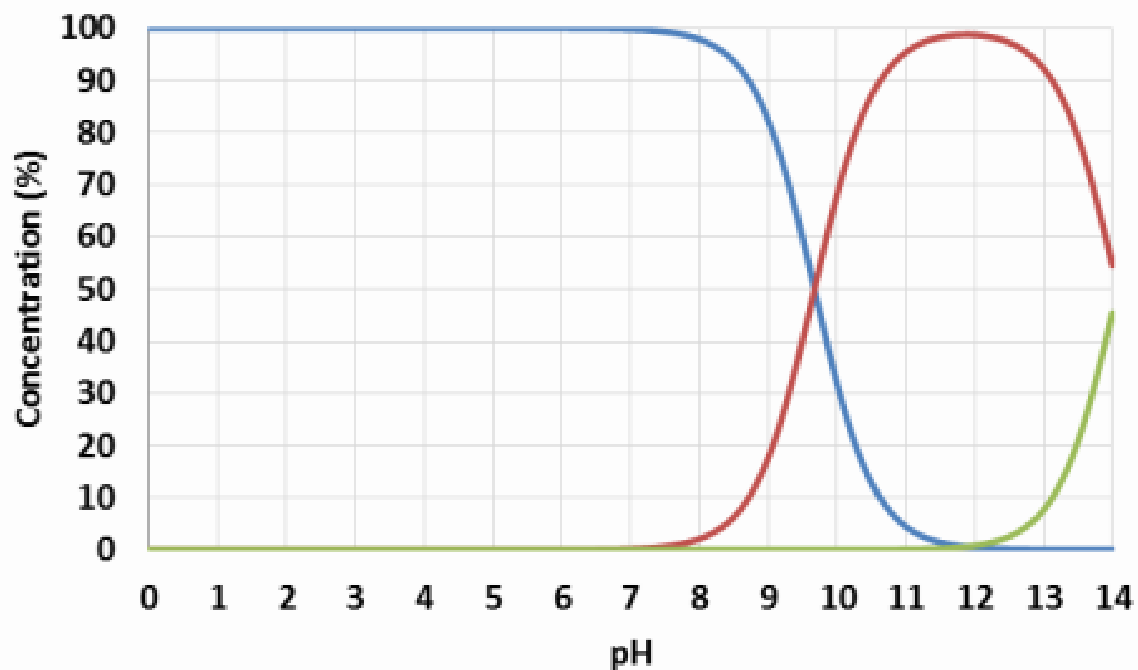

Concentration (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH


14.08
OH
9.67
H
N
O
O
H_2N







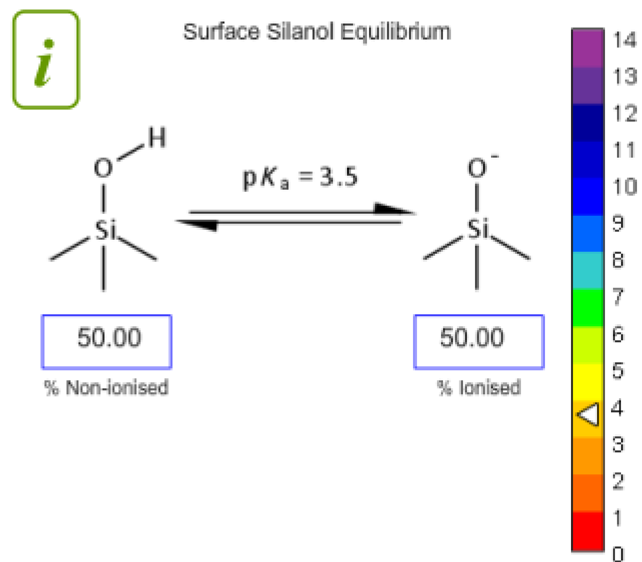

Concentration (%)
pH
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

If the pH of the mobile phase matches the pK a of an analyte (50% ionized/50 % non-ionized), would two peaks be present in the chromatogram, one due to the ionized form and the other due to the non-ionized form?

In reversed phase HPLC hydrophobic analytes are more strongly retained. Ionic analytes are much less hydrophobic (more hydrophilic) when they are in their ionized form and as a result their retention, k, will decrease. Analytes in their ionized form will also show secondary interactions with the column (free silanol groups) and with the system which can result in deleterious chromatographic peak shapes; mainly peak tailing.

Caution: High pH mobile phases can damage traditional silica based RP-HPLC columns. (working pH range: 2.5-7)

However, columns which are specifically designed to operate with high pH conditions (working pH range 1-12) are available allowing the analysis of basic analytes using ion suppression. Traditionally the analysis of weak bases has been carried out at low pH; essentially because the surface silanol species are non-ionized (pK a approximately 3.5) and peak tailing is improved.



Non-ionized silanol groups result in the least peak tailing, whilst the ionized form results in the worst peak shape.

At pH 3.5 the surface silanol groups are 50% ionized/50% non-ionized.

Lowering the mobile phase pH causes the acidic silanol groups to become less ionized - below pH 1.5 the silanol groups are approximately 100% non-ionized and peak tailing will be reduced (care should be taken at this extreme mobile phase pH as the bonded phase may be degraded).

By raising the pH to 5.8 the silanol groups will be 100% ionized and peak tailing will be at its worst with respect to secondary silanol retention.

The unwanted secondary silanol retention may be reduced (or even eliminated) by the addition of a small (sterically), highly surface active base such as triethylamine (TEA), piperazine, N,N,N',N'-tetramethylethylenediamine (TMEDA), or dimethyloctylamine (DMOA). These bases interact with the surface silanol species in preference to the analyte molecule and are called 'sacrificial bases'. They are added to the mobile phase in sufficient concentration to ensure that the silica surface is fully deactivated at all times.

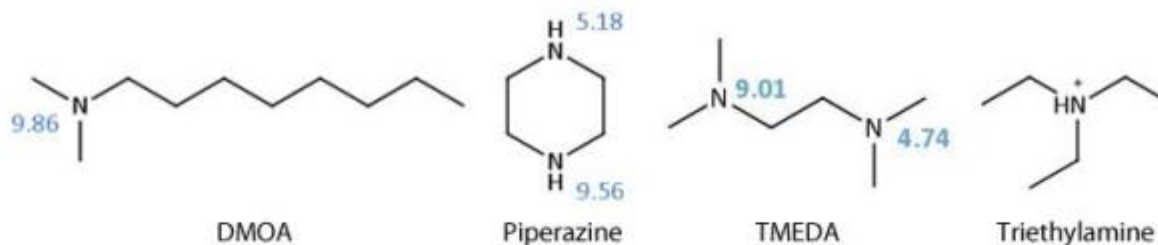


Figure 36: Sacrificial bases.

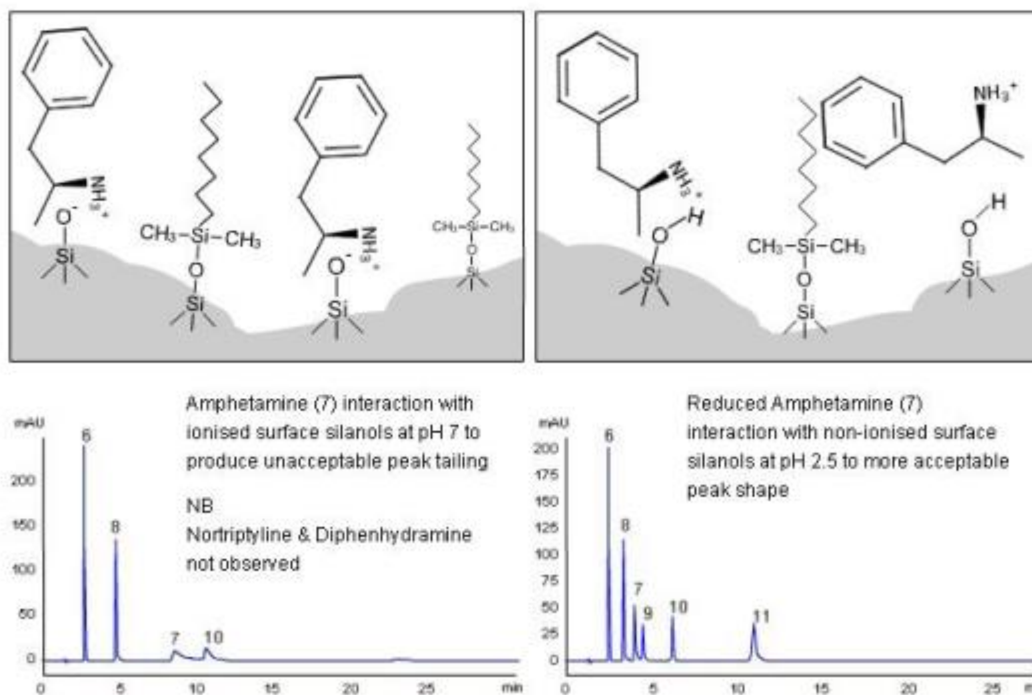
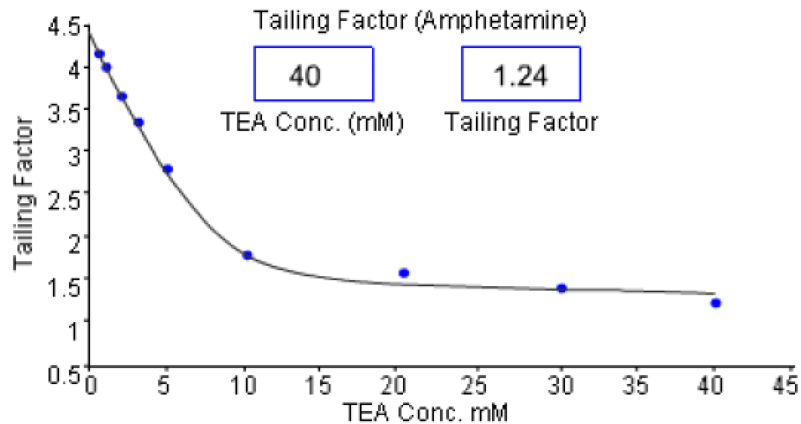
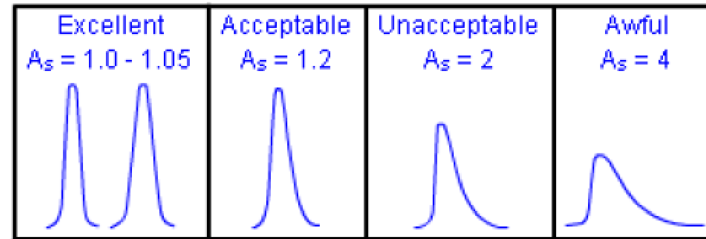


Figure 37: Improvement in basic analyte peak shape at low pH due to reduced surface silanol interaction.

Using Triethylamine as a Sacrificial Base



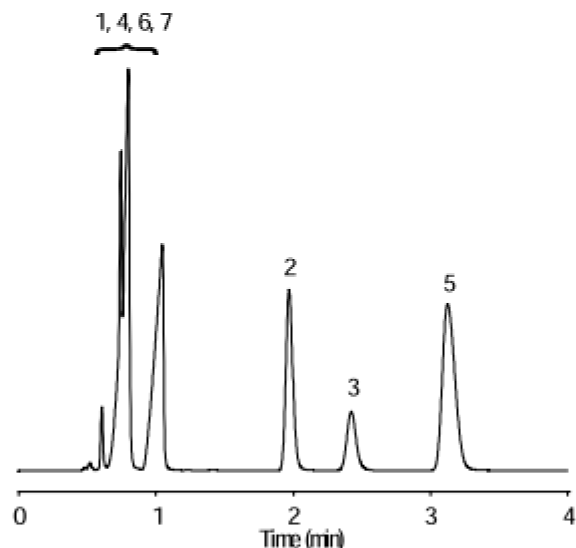


In this example the amphetamine molecule (pK a = 10.01), exhibits very bad tailing with no triethylamine added (pK a = 10.21), in a mobile phase of pH 7.1. As the concentration of sacrificial base is increased, the peak shape steadily improves. The curve representing peak tailing against TEA concentration in the mobile phase plateaus at approximately 10 mM, indicating almost total coverage of the reactive silanol groups.

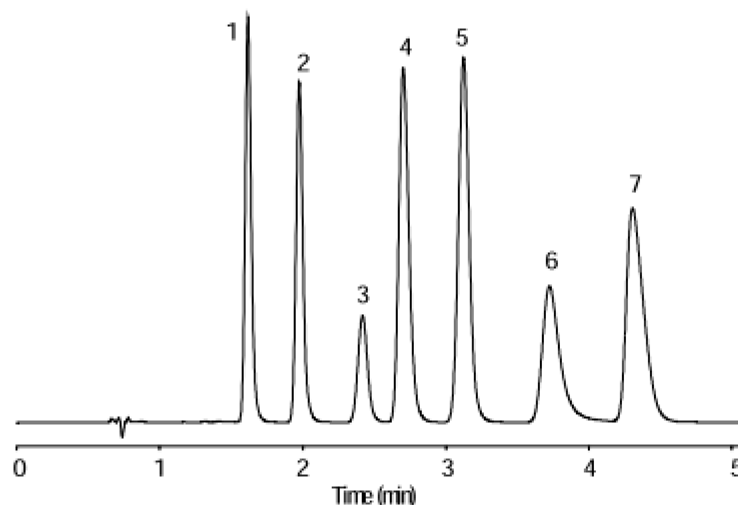
Use Buffered Mobile Phases for Best Peak Shape and Retention

Column: ZORBAX Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 µm Mobile Phase: 44% A : 56% methanol
Flow Rate: 1.0 mL/min Temperature: 25°C Detection: UV 250 nm
Sample: 1. ketoprofen 2. ethyl paraben 3. hydrocortisone pKa 5.1 4. Fenoprofen pKa 4.5 5. propyl paraben 6. Propranolol pKa 9.5 7. Ibuprofen pKa 4.4

A = pH 7.0 water



A = pH 7.0, 25 mM phosphate buffer



- Buffered mobile phases enhance retention, resolution, and peak shape.

The End.