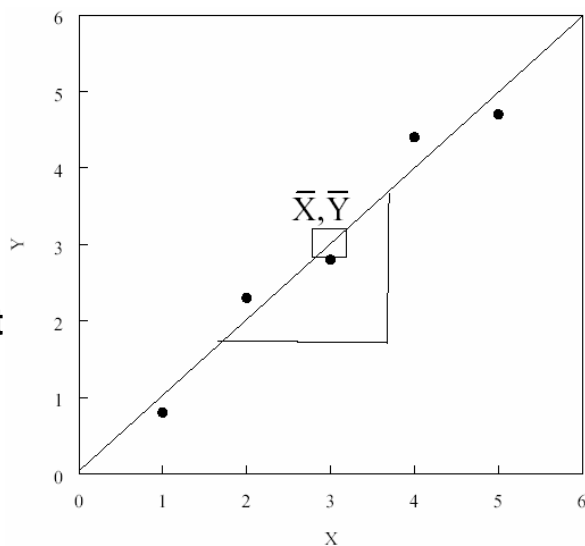


Curve fitting

- these slides are the extras for the Bioanalytical LC-MS/MS class taught at HPLC 2006.
- If you need more info, contact John Dolan (John.Dolan@LCResources.com)

Curve Weighting: A Graphical Interpretation of Regression

Regression takes the mean values of all X and Y and determines the best slope through that point



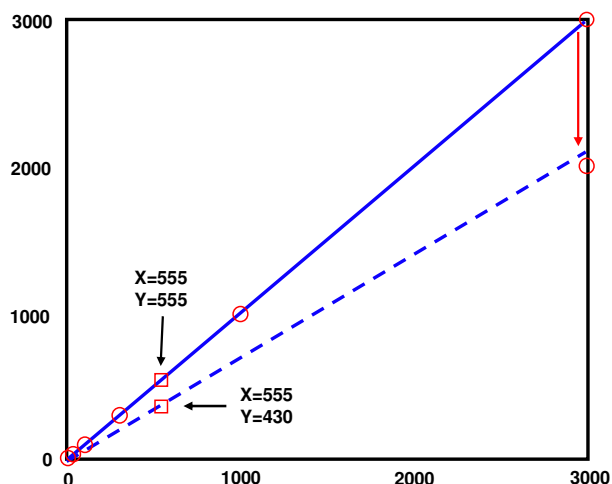
19-2

LCRESOURCES

The regression process is just a way to simplify the data and, in a sense, remove some of the random or experimental error from the measurements.

Curve Weighting: Effects of High-End Non-Linearity

A significant deviation from linearity by a single high point completely dominates all other points in the calculations.



19-3

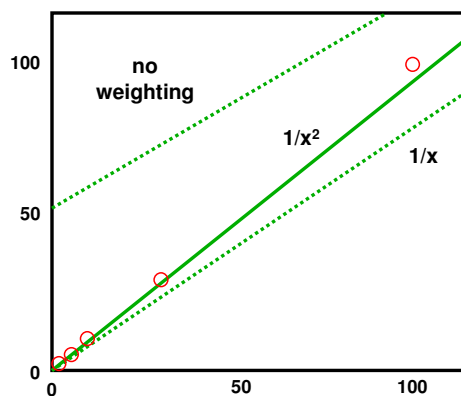
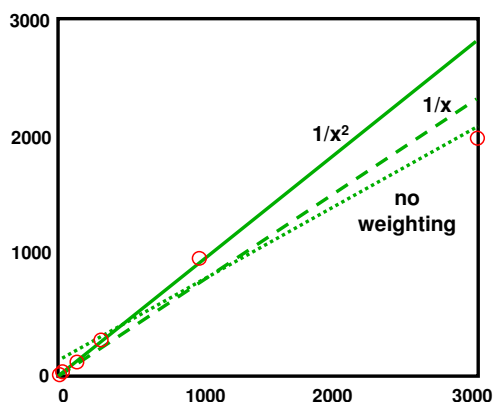
LCRESOURCES

The illustration shows the effect of a high-end point on a calibration curve running from 1 to 3000 in concentration. The response is assumed to be equal to the concentration (to make it simple). The data pairs are all perfect except the highest one (at $x=3000$, $y=2000$).

The defective high point does two things: first, it significantly changes the position of the mean value of Y . Second, it dominates the calculation of the slope.

Again, if we use the idea of a center of gravity for the data, that outlying point is like a weight on the end of a lever, and remember that the longer the lever, the more effect a weight at its end has.

Calibration Curves: The Effect of Different Weighting Factors



19-4

LC RESOURCES

The illustrations here show the effect of different weighting factors on the calibration curve from the previous slide, running from 1 to 3000 in concentration.

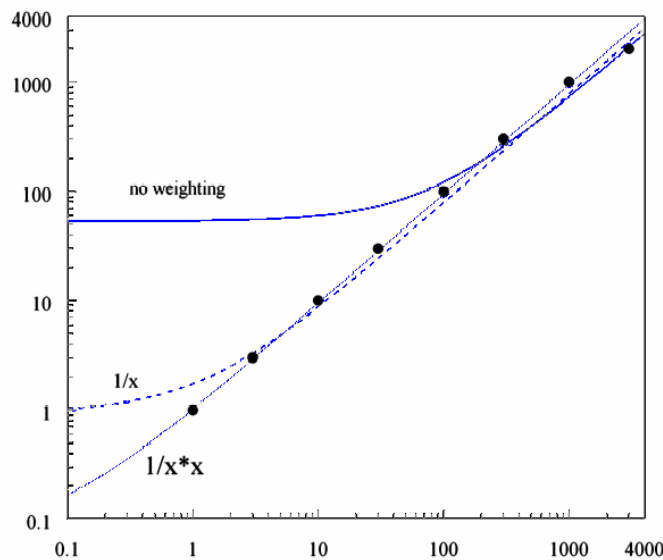
With no weighting, the slope of the line is completely dominated by the highest term. The expanded scale shows that the lowest five points are essentially ignored by the calculation. When weighting of 1/concentration (1/x) is used, the slope more closely approximates the majority of the points, and the intercept approaches zero.

When a weighting of 1/x² is used, the slope approaches unity and the intercept is not significantly different from zero. The fit also essentially ignores the high-end point.

This tends to bother people, that last point hanging out there, ignored. Part of the problem is that it's a visual artifact of the way we normally present data.

Calibration Curves: A Closer Look at Weighting Effects

Now
which fit
is better?



19-5

LC RESOURCES

Here the same data and graph are used, except that the axes are now logarithmic. Graphing this way gives a visual approximation of equal weighting for each data point.

The first thing you notice is that the deviation of the highest point no longer predominates the visual distribution of the data points; it's really pretty minor.

The second thing you notice is that the non-weighted best fit line completely ignores the low end of the point set, while the $1/x^2$ weighting looks like a much better choice.

We operate in a linear, Cartesian world for the most part. If you show both the linear and log-log plots to most people the response will likely be "Yeah, but aren't you cheating by squeezing the top part down like that?" The reply might be "What would you have said if I showed you the second plot first?"

Most of us, unconsciously, think "small size, small importance". In analysis, plotting our calibration curves in linear coordinates exclusively may be one of the dumber things we do.

Why Curve Fitting?

“Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for *goodness of fit*.”

Guidance for Industry
Bioanalytical Method Validation
US FDA, May 2001

19-6

LCRESOURCES

The “Crystal City II” guidelines are quite explicit about the need to show that the curve fit selected is the best one. This means that an arbitrary selection of weighting really isn’t justified.

Paclitaxel in Porcine Serum

Range 0.03 - 100 ng/mL

**Standard Curve 12 concentrations
n = 2**

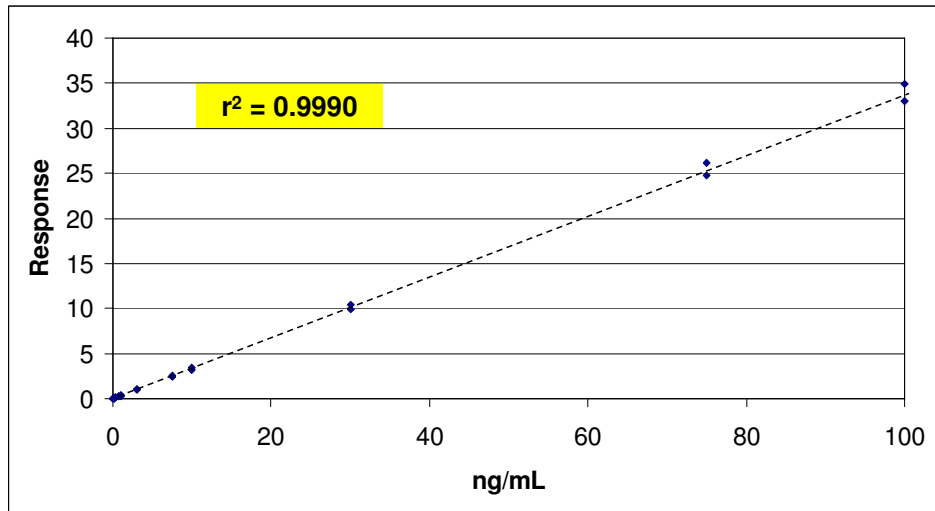
**Validation Samples 0.03, 0.1, 10, 100 ng/mL
n = 6**

19-7

LCRESOURCES

For the example in this presentation, data are obtained from day 1 of a validation of paclitaxel in pig serum. The run begins and ends with a standard curve, with the validation samples run at the LLOQ, 3X LLOQ, midrange, and ULOQ. Two curves were prepared and run (separate samples). Six replicates of each validation sample were prepared and run with a single injection of each.

Standard Curve (no weighting)

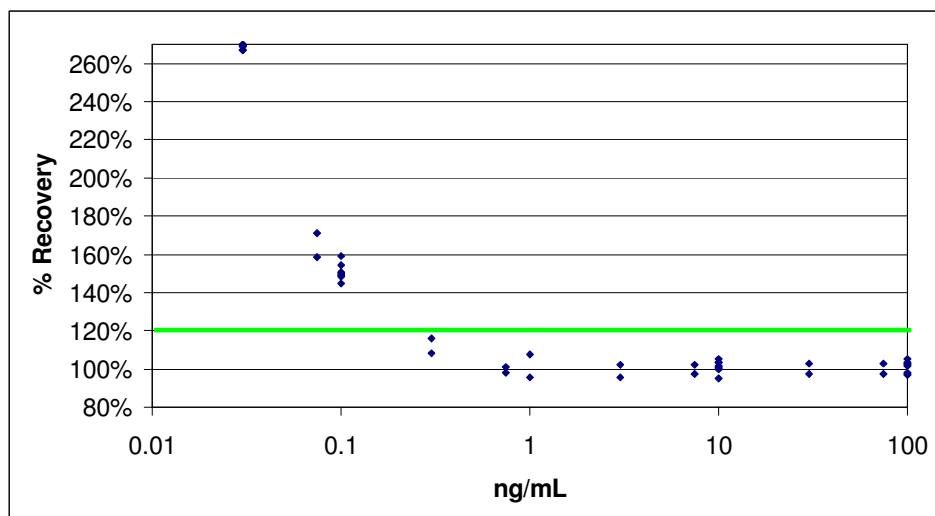


19-8

LCRESOURCES

The simple, traditional way to test the data for curve fit is to start with a linear fit and see how it works. The data points for the two standard curves look pretty good, fall on a linear trend line, and have r^2 that is almost 1. At this point, it might seem that we've found a simple fit with good statistics. But is this really true?

Residuals (no weighting)



19-9

LCRESOURCES

A plot of residuals is much more instructive when the samples cover such a wide range of concentrations -- 3000-fold in this case. The residual plot is obtained by calculating the percent recovery of each sample and plotting it. If the correlation were perfect, all points would fall on the 100% line.

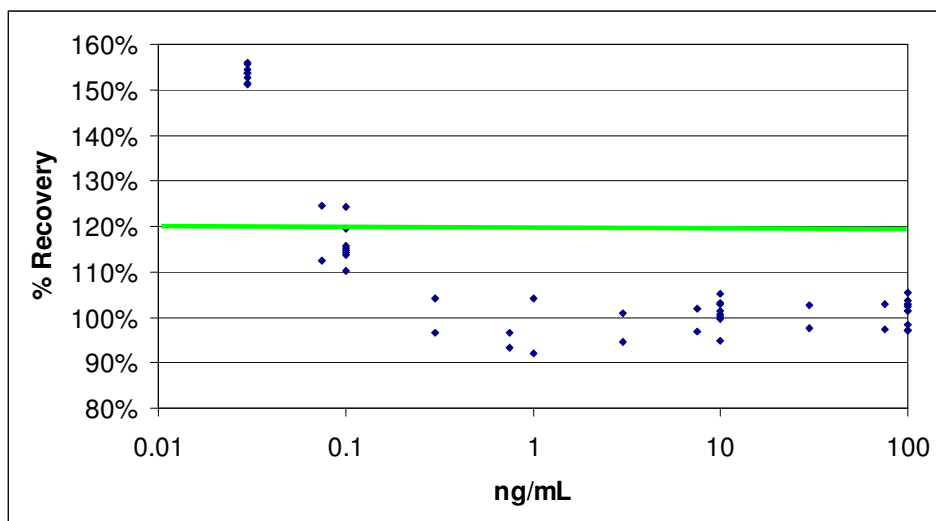
Here it can be seen that the points at 0.03 ng/mL give 270% recovery. A horizontal line has been added at 120%. This is normally the limit for the lower limit of quantification (LLOQ) samples, whereas others must lie within 15% of the theoretical value.

These data begin to show curvature below 1 ng/mL. 0.3 ng/mL falls just within the 20% limit.

Is this really a good representation of the behavior of the method? Do you really think that the 0.03 ng/mL concentrations are 270% too high? I think we would all agree that this is pretty unlikely.

Let's look at the residuals plots for some other treatments of the data.

Residuals ($1/x^{1/2}$ weighting)



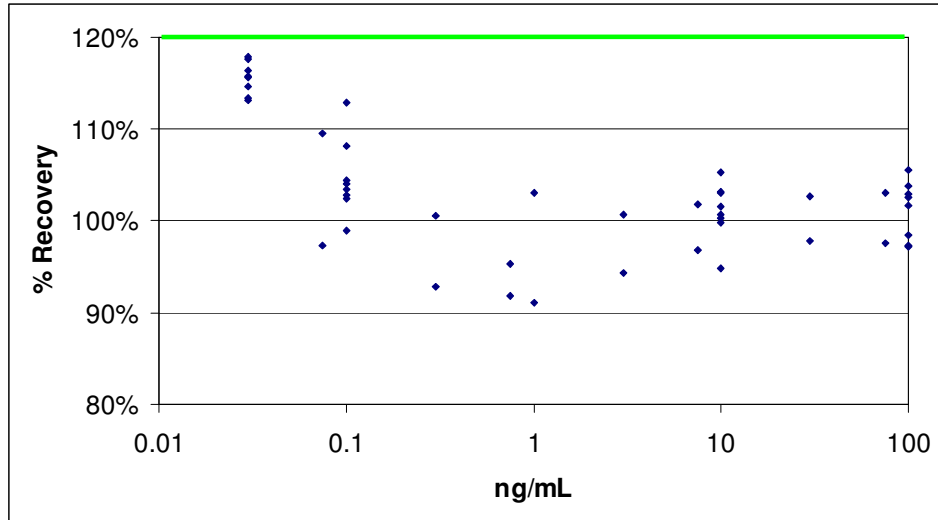
19-10

LCRESOURCES

In this case, the data were weighted using a $1/(\text{square root of } x)$ weighting factor. As we'll see in a few minutes, these calculations are tedious, but most data systems will handle them automatically.

Now the most extreme deviations are "only" about 160% instead of 270% -- certainly an improvement. The 0.3 ng/mL levels are well within the 20% limits now.

Residuals (1/x weighting)

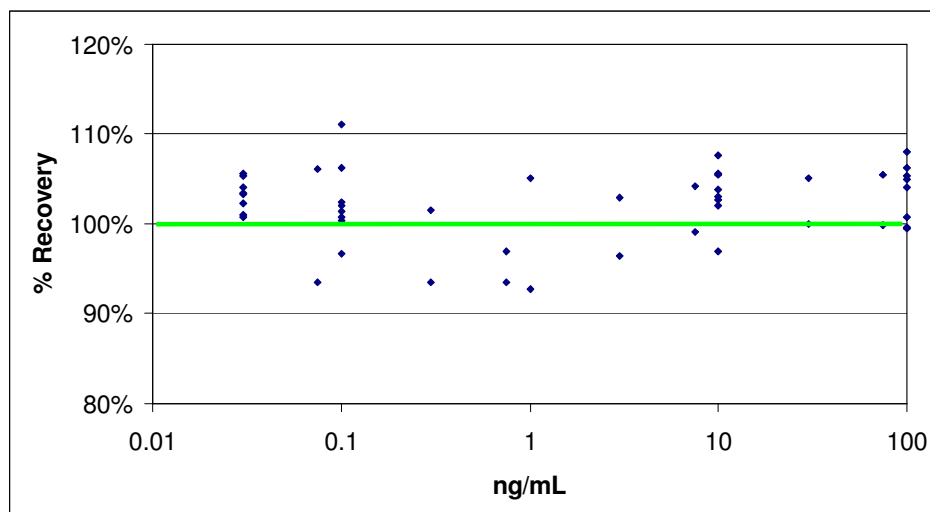


19-11

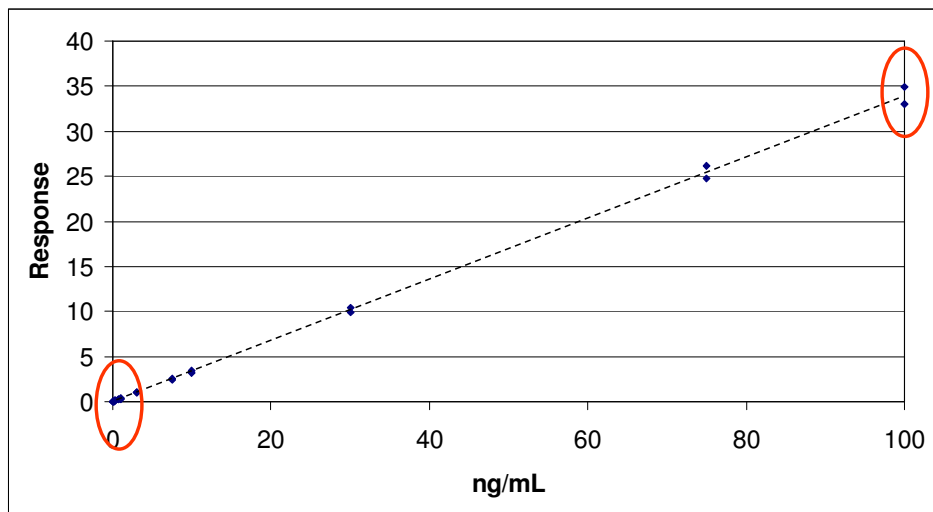
LCRESOURCES

With 1/x weighting, the data all fall within the 80-120% window at the LLOQ. There seems to be an upward trend of the data below 0.3 ng/mL.

Residuals (1/x² weighting)



Standard Curve (no weighting)



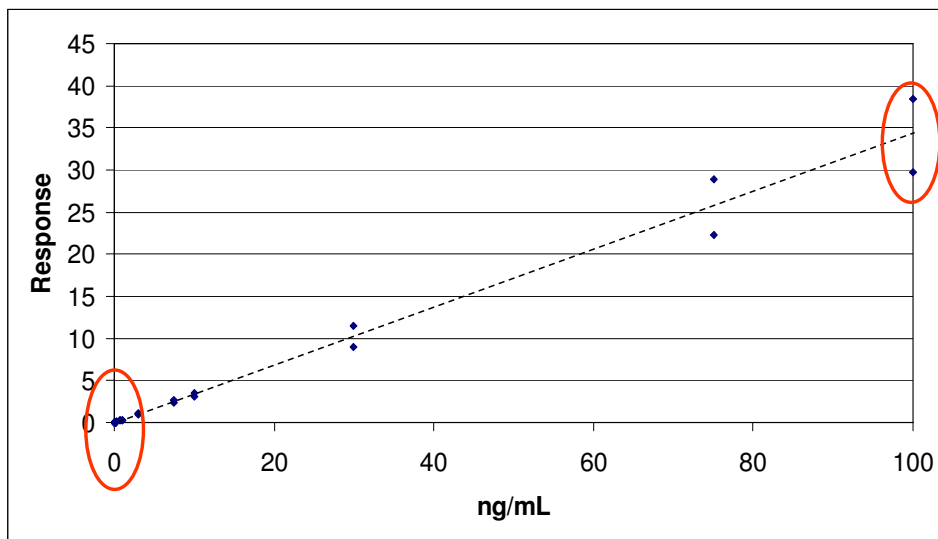
19-13

LCRESOURCES

If we look at the original data set again with no weighting applied, we can see an indicator that the data are not behaving as well as they should. This plot is just for the standard curve samples, so each concentration has just two points.

At the top of the curve, the points are visibly spaced, giving us a feel for the error involved. At the bottom end, however, the points are indistinguishable from each other.

Standard Curve Exaggerated (no weighting)



19-14

LCRESOURCES

Here I've exaggerated the error at each point so you can see the difference in error at the top and the bottom of the curve.

This tells us that the absolute error is larger at the top of the curve than the bottom. If you follow the curve down, you can see that the points get closer together at lower concentrations.

This behavior tells us that the data are heteroscedastic -- a big word that means that absolute error varies with sample concentration

When to Apply Weighting?

- **Homoscedastic**
 - equal standard deviations
 - no weighting should be used
- **Heteroscedastic**
 - standard deviation varies with sample size
 - (*relative* standard deviation is constant)
 - weighting should be tested

19-15

LCRESOURCES

We can use the scedasticity of the data to determine if weighting is needed or not.

Homoscedastic data are data which have standard deviations that are the same at different concentrations. That is, the error at the low end of the curve and the high end are similar. In such cases, curve weighting is not appropriate.

Heteroscedastic data are data for which the standard deviation increases with the sample concentration. The error is more or less proportional to concentration, so if we normalize the data by calculating the relative standard deviation (RSD), we find that the RSD is fairly constant across the plot. In such cases, weighting usually will be beneficial and should be tested for improved curve performance.

Test for Homoscedasticity (F-test)

$$F_{\text{exp}} = s^2_{\text{ULOQ}} / s^2_{\text{LLOQ}} = 72 \times 10^6$$

$$F_{\text{tab}}(\text{df}_1, \text{df}_2, 0.99) = 10.97$$

	std dev	RSD
ULOQ	1.07789	0.031
LLOQ	0.00013	0.012
Ratio	8500	2.6

19-16

LCRESOURCES

The guidelines call for statistical proof that the best weighting is used. You can use the F-test to determine whether the data are homoscedastic or not. The F test is just a ratio of the variances (standard deviation squared) for two test sets. Data gathered at the upper end of the curve (ULOQ) and at the lower end (LLOQ or 3xLLOQ) are appropriate to use for this test. Because a standard deviation is required, you need more than the two data points from the standard curve. The validation samples, with n=6 at each concentration are appropriate.

Once the experimental F value is calculated, you can look up the limiting F-value in a table for the appropriate degrees of freedom. In the present case with n=6 for each set, n-1, or 5 is used for the degrees of freedom. We're testing at the 0.01 rejection level, or what is sometimes called the 99% confidence level.

For the present data, the ratio of standard deviations is 8500 and the ratio of the variances is 72 million! Certainly more than the rejection value of 10.97 from the table. There is no question that the data are heteroscedastic.

It is interesting to note that the RSD is nearly constant across the curve, with a difference of only about 3-fold between the lower and upper end of the curve.

Of course you can do this test in Excel using one of the data analysis tools.

“Correct” Weighting

$$w_i = s_i^{-2} / (\sum s_i^{-2} / n)$$

$$y = ax + b$$

$$a = \frac{\sum w_i x_i^2 \sum w_i y_i - \sum w_i \sum w_i x_i y_i}{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)_2}$$

$$b = \frac{\sum w_i \sum w_i x_i y_i - \sum w_i x_i \sum w_i y_i}{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)_2}$$

19-17

LCRESOURCES

Now that we know the data are heteroscedastic, we have to determine which weighting factor is best. If you look at a statistics book, it will instruct you to weight the curve according to the standard deviation at each concentration. This means that you have to have enough data points at each concentration to calculate a standard deviation. Once you have the standard deviation and weighting factor, you feed it into this ugly equation and calculate the coefficients of the curve.

Obviously this technique falls apart if you only have one or two standard curves to work with for your data set. So instead of the ideal, we take another tack.

Realistic Weighting

$w_i = 1/x^n$, where $n = 0, 0.5, 1, 2, \dots$

$$w_i = s_i^{-2} / (\sum s_i^{-2} / n)$$

$$y = ax + b$$

$$a = \frac{\sum w_i x_i^2 \sum w_i y_i - \sum w_i \sum w_i x_i y_i}{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)_2}$$

$$b = \frac{\sum w_i \sum w_i x_i y_i - \sum w_i x_i \sum w_i y_i}{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)_2}$$

A much more practical technique is to try several different weighting factors to see which one works best. So instead of calculating the weighting factor based on the standard deviation, we just calculate it based on the concentration. The rest of the calculations are the same.

Evaluating Weighting

- Calculate weighted values ($1/x^{0...2}$)
- Sum absolute value of relative error
- Minimum Σ %RE = BEST

weight	$1/x^0$	$1/x^{0.5}$	$1/x$	$1/x^2$	$1/x^3$	$1/s^2$
Σ %RE	18.02	5.99	2.07	1.17	1.45	0.76
r^2	0.9990	0.9992	0.9992	0.9978	0.9950	0.9985

19-19

LCRESOURCES

Here's the procedure to use.

First, calculate all the results using the different weighting factors.

Second, calculate the relative error. This is the percent difference between the observed value and the theoretical value. Take the absolute value of each relative error and add them up.

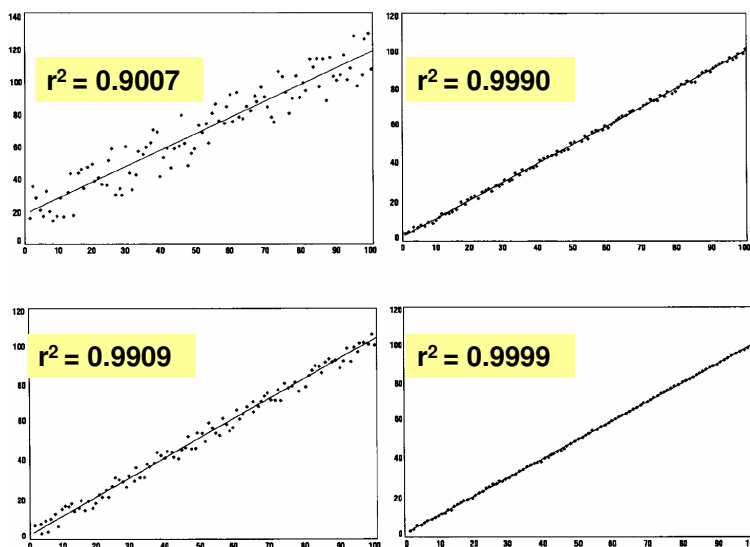
Finally, examine the data and find the weighting factor that gives the smallest value of the sum of relative errors. The numeric value isn't important -- just how one weighting factor compares to another.

If we look at the present data set, we see that with no weighting, the relative error totals about 18. We see an improvement in the results as soon as we begin applying a weighting factor. For this set, a minimum is seen with $1/x^2$ weighting. You can continue to try higher order, like $1/x^3$, but no real advantage is seen.

Just for comparison, the ideal weighting using the standard deviation technique is shown. You can see that the $1/x^2$ weighting gives a sum of errors less than twice as large as the ideal case -- this is pretty good, and I doubt if there is any practical difference between the two. Furthermore, the trial weighting values can be used with a single standard curve, whereas the standard deviation technique needs at least 3 sets of data.

I've shown the correlation coefficients for the different curves. They're all better than 0.99, and it is debatable if one value is any different than the others. This just tells us that the correlation coefficients are poor measures of the curve fit quality for data like these.

Regression Coefficients (Coefficient of Determination)



19-20

LCRESOURCES

The use of the regression coefficient (more properly called the coefficient of determination) assumes that the error is fairly constant over the data set as is shown here. When that is the case, we can see the improvement in the curve fit as the regression coefficient gets larger.

However, the data we've been looking at, and in general all data of this type, do not have constant error at all concentrations. When this is the case, the value of the regression coefficient can be misleading. This was the case in the previous slide where we saw very little difference between r^2 values for the different weighting schemes even though there was a dramatic difference in how well the data fit the various curves.

Unfortunately, reporting the r^2 values is become a standardized expectation when reporting bioanalytical data. So you generally need to include this parameter in the reports, but don't put too much faith in what it tells you.

Curve Fitting Summary

- **Homoscedastic or Heteroscedastic?**
- **Compare sum of relative errors for different weighting schemes**
- **Residuals plots are more informative than response vs. concentration plots**
- **Don't be tricked by r^2**

19-21

LCRESOURCES

So how do we meet the guidelines?

First, determine if the data are homo- or heteroscedastic. You can do this with the F-test, but an eyeball test on the data usually is sufficient. And the nature of the data in bioanalytical calibration curves is such that it is very unlikely that the data are homoscedastic. It probably isn't worth taking the trouble to perform the F-test.

Next, calculate the results using various weighting schemes. Probably no weighting, $1/x$ and $1/x^2$ are going to tell the story. This can be done with an Excel spreadsheet and once the data are imported, it only takes a few seconds per weighting factor, so there is no reason not to try out several factors. Compare the sum of the relative errors to find the smallest value -- this is the best fit. Now you have a statistical test that allows you to defend your choice of weighting factors.

A couple other things to keep in mind.

First, the residuals plot, where the % recovery is plotted against concentration, are very useful. These plots are most informative if you use a log scale for concentrations. With such plots you can quickly see the performance of the bottom end of curve improve as you add weighting. Again, this is quickly done in Excel.

Finally, don't be fooled into thinking that the correlation coefficient is giving you much useful information about the quality of the data.

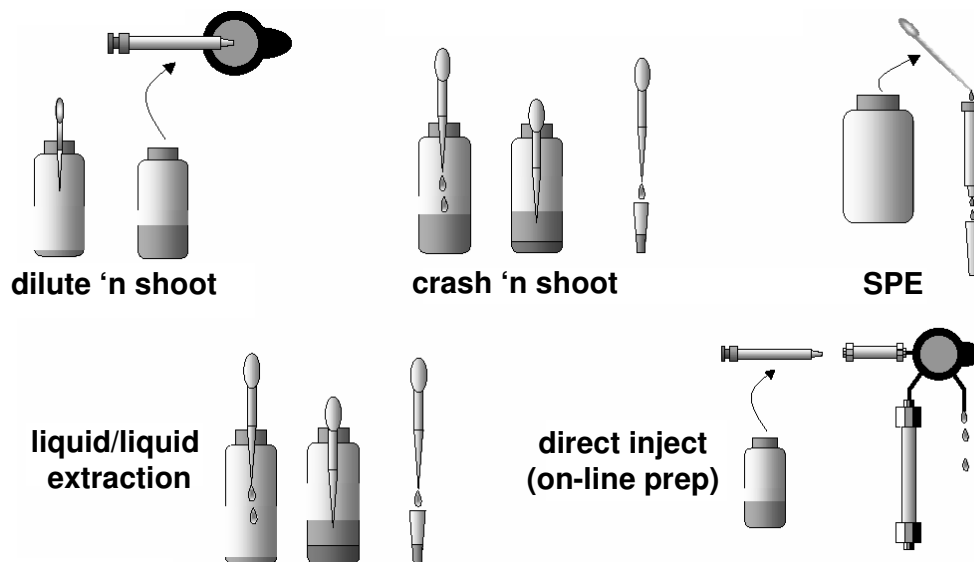
SECTION
21 **Sample**
Preparation

19-22

LCRESOURCES

060203

Sample Preparation



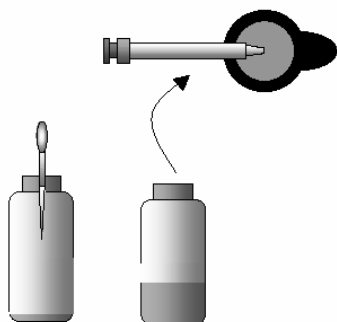
19-23

LC RESOURCES

Despite being a course about LC-MS, proper sample preparation is still essential (given the properties of the LC-MS system, is arguably more essential than in ordinary LC). Probably 99% of sample preps are covered by the techniques listed. Each has advantages and disadvantages:

- 1) Dilute and shoot. The sample is diluted with an appropriate solvent and directly injected into the LC-MS. This only works for samples that have few, if any, extraneous components.
- 2) Crash and shoot (a.k.a. precipitate and inject). The sample is treated with a solvent or other reagent which causes physical precipitation of unwanted materials. The mixture is centrifuged and the supernatant injected.
- 3) SPE (solid phase extraction). Sample is applied to a disposable packed column where the analytes are separated chromatographically.
- 4) Liquid/liquid extraction. Analyte is separated from unwanted materials by partition between immiscible liquid phases.
- 5) Direct injection with on-line sample preparation. The sample is applied directly to the LC-MS system which incorporates a sample cleanup/enrichment precolumn, usually with switching valves.

Dilute 'n Shoot



ADVANTAGES

- fast
- inexpensive
- minimal sample manipulation

DISADVANTAGES

- no cleanup
- no enrichment

19-24

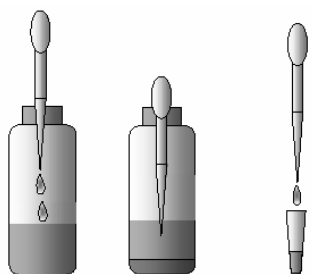
LCRESOURCES

Simple dilution and injection is an easy and inexpensive way to prepare samples. Unfortunately, with the exception of reference standards, few of the samples we deal with are amenable to this technique. The most common exception for bioanalytical work may be urine samples, which may be amenable to dilute and shoot techniques.

Crash 'n Shoot (precipitate and inject)

ADVANTAGES

- fast
- inexpensive
- minimal sample manipulation



DISADVANTAGES

- minimal cleanup
- no enrichment
- loss to entrapment
- dilution effects

19-25

LCRESOURCES

This technique is commonly applied to plasma samples for a “quick and dirty” sample preparation technique. For example, a three-fold excess of acetonitrile might be added to a plasma sample, vortexed, and spun down. An aliquot of the supernatant is transferred to the injection vial or plate. Sometimes zinc sulfate is added to aid in precipitation. The sample still has a considerable protein load, so column lifetimes may be shortened. It is recommended that a divert valve be used to prevent overloading the interface with non-volatile sample residues.

Protein Precipitation

- **Acids (>98% removal)**
 - TCA 10% (w/v) and HClO₄ 6% (w/v)
- **Organic solvents (>90%)**
 - ACN > acetone > ethanol > methanol
- **Zinc and copper salts**

19-26

LCRESOURCES

Several different approaches can be taken to precipitate proteins. Precipitation with acetonitrile is the most popular technique. Zinc salts will give a tighter pellet on precipitation, but are not commonly used.

J. Blanchard, Evaluation of relative efficacy of various techniques for deproteinizing plasma samples prior to HPLC analysis. J. Chrom. 226, 455-460 (1981).

ACN Protein Precipitation

- **100 μ L plasma**
- **100 μ L IS, vortex**
- **300 μ L ACN, vortex**
- **centrifuge**
- **transfer**

19-27

LC**R**ESOURCES

Here's a typical recipe for protein precipitation of plasma samples.

Zn Protein Precipitation Reagent

Precipitating Reagent (PR)

Zinc sulfate heptahydrate solution (65g/100mL water)

Stock solution

Acetonitrile 250 mL + Water 320 mL + 5 mL PR

Usage

1 Part Sample : 3 Parts Solution, Vortex mix

e.g., 200 μ L Plasma : 600 μ L Solution, Vortex, cfg

19-28

LCRESOURCES

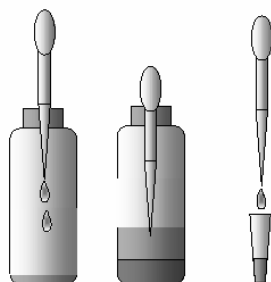
Zinc sulfate can give a better precipitation than ACN alone.

courtesy of David Wells, Sample Prep Solutions

Liquid-Liquid Extraction

ADVANTAGES

- selective
- sample enrichment
- inexpensive (materials)



DISADVANTAGES

- time-consuming
- expensive (time)
- differential extraction
- much manipulation of sample

19-29

LCRESOURCES

Liquid-liquid extraction is an old technique that has had a resurgence in popularity in the last few years. It is simple, flexible, and relatively inexpensive. Sample manipulation can be minimized with the use of 96-well extraction plates and robots. Typically a sample is pH-adjusted and extracted into an organic solvent, such as methyl-t-butyl ether (MTBE). The MTBE is transferred to another tube and evaporated. The sample is then reconstituted in the injection solvent.

Functional Group Polarity

Hydrophilic (polar)

- Hydroxyl -OH
- Amino -NH₂
- Carboxyl -COOH
- Amido -CONH₂
- Guanidino -NH(C=NH)NH₃⁺
- Quaternary amine -NR₃⁺
- Sulfate -OSO₃⁻

19-30

LCRESOURCES

The functional groups in an analyte contribute to the overall polarity of the molecule.

Courtesy of David Wells, Sample Prep Solutions.

Functional Group Polarity Hydrophobic (nonpolar)

- Carbon-carbon -C-C
- Carbon-hydrogen -C-H
- Carbon-halogen -C-F OR -C-Cl
- Olefin -C=C
- Aromatic

19-31

LC RESOURCES

Courtesy of David Wells, Sample Prep Solutions.

Functional Group Polarity Neutral

- Carbonyl -C=O
- Ether -O-R
- Nitrile -C=N

19-32

LCRESOURCES

Courtesy of David Wells, Sample Prep Solutions.

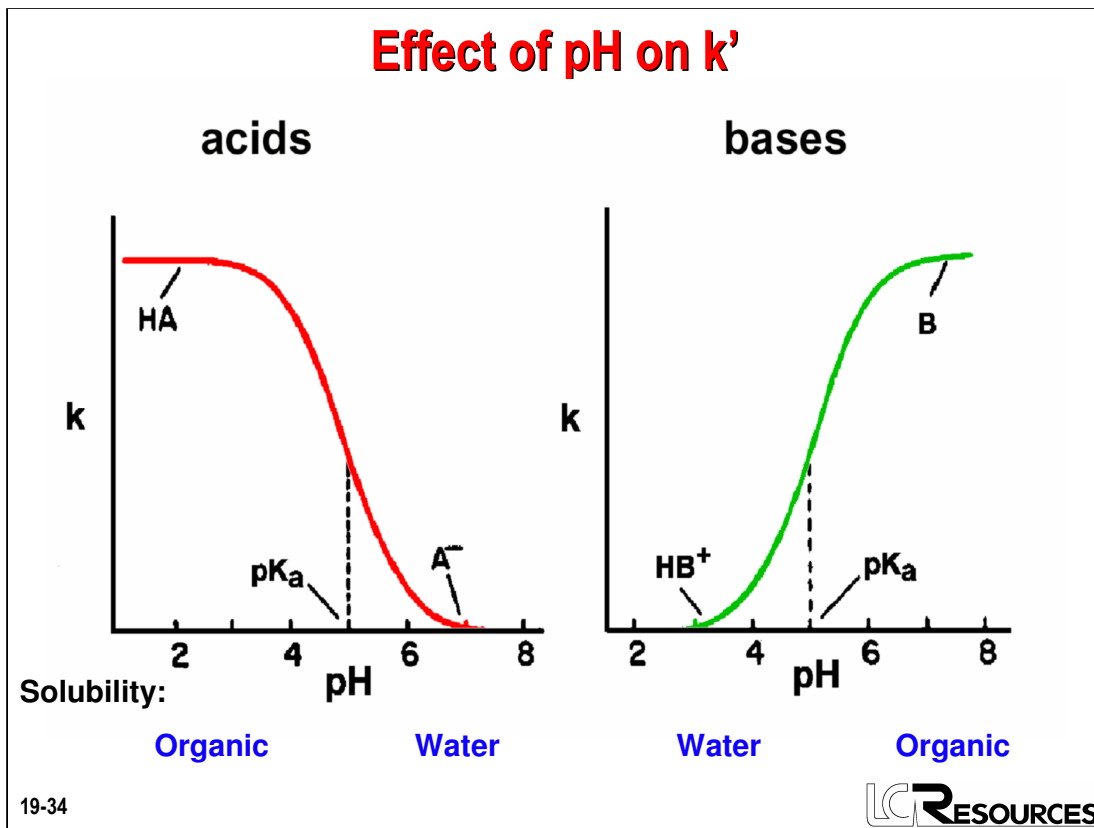
Liquid-Liquid Extraction

- Change polarity by changing pH
- >99% conversion with pH 2 units *above* or *below* pKa
- Acids:
 - pH < pKa = R-COOH *unionized*
 - pH > pKa = R-COO⁻ *ionized*
- Bases
 - pH < pKa = R-NH₃⁺ *ionized*
 - pH > pKa = R-NH₂ *unionized*

19-33

LCRESOURCES

The goal of liquid-liquid extraction is to adjust the pH so that the analyte is organic soluble. Then it will partition into an organic phase. If a back-extraction is used, the pH is adjusted to ionize the sample so that it goes back into a clean aqueous phase.



Just as is the case with conventional HPLC separations, the polarity of acids and bases can be controlled by adjusting the pH.

Ionized species are much more hydrophilic than are their neutral counterparts. As a consequence, we would expect stronger retention (high k') at pH ranges where the sample is completely neutral, and weaker retention (low k') when the sample is fully ionized. There must be a transition range in which retention varies with pH for a partially ionized species. The expected curve is similar to a titration curve. It indicates that retention is related to the degree of dissociation.

The inflection point of such a curve occurs at the pH which results in 50% dissociation: the pK_a . The retention-pH curves for acids and bases are qualitatively mirror images of one another. In the case of acids, we expect stronger retention at low pH. In the case of bases we expect stronger retention at high pH.

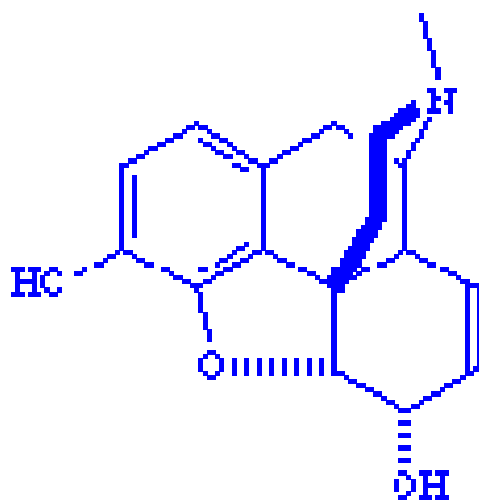
Solvents for Liquid-Liquid Extraction

- **EtOAc (ethyl acetate)**
 - + good for polar analytes
 - may extract too much garbage
- **MTBE (methyl tert-butyl ether)**
 - + less polar than EtOAc (cleaner)
 - + floats on water
- **Chlorinated solvents (e.g., CH₂Cl₂)**
 - + nonpolar
 - +/- heavier than water
 - toxicity / environmental issues?

19-35

LC RESOURCES

Many different solvents can be used for liquid-liquid extraction. Ethyl acetate has been a popular solvent, but it usually results in dirtier extracts that may need further cleanup to be useful. EtOAc extracts also contain enough water that evaporation is difficult. MTBE is very popular today because it yields cleaner extracts and is easier to evaporate than EtOAc. Chlorinated solvents were popular in the past, especially with separatory funnel extractions because they are heavier than water. These solvents separate easily from water and are easy to evaporate, but environmental and safety concerns have made them less popular in recent years.



Morphine

pKa = 7.87

19-36

LCRESOURCES

As an example, the basic drug morphine will be used to illustrate hypothetical extraction condition selection.

Morphine Extraction

- 300 μ L plasma + 100 μ L IS, vortex
- 300 μ L borate (pH 9.2), vortex
- 1 mL MTBE, vortex, centrifuge
- transfer MTBE
- evaporate to dryness
- reconstitute in 100 μ L injection solvent

nominal 3X increase in concentration

19-37

LCRESOURCES

In this scheme, internal standard is added to plasma and then the pH is adjusted to >1 pH unit above the pKa so that the morphine is non-ionized. When MTBE is added, morphine partitions into the MTBE. The MTBE can be removed, evaporated, and reconstituted in the injection solvent. In this example, the concentration is increased 3-fold.

Morphine Back-Extraction

- (Morphine in MTBE)
- add 500 μ L 0.1 N HCl (pH 1.1), vortex
- centrifuge, transfer
- inject

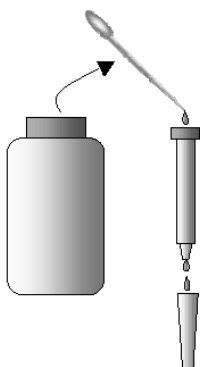
nominal 60% dilution
additional cleanup

19-38

LCRESOURCES

If the previous single-step extraction was not clean enough, such as when non-polar endogenous materials extract into the MTBE, the morphine can be back-extracted into (clean) aqueous phase at low pH. The volumes can be adjusted to control the final concentration relative to the initial sample.

Solid Phase Extraction (SPE)



ADVANTAGES

- selective
- sample enrichment
- automation

DISADVANTAGES

- time-consuming
- expensive
- differential extraction
- can add impurities

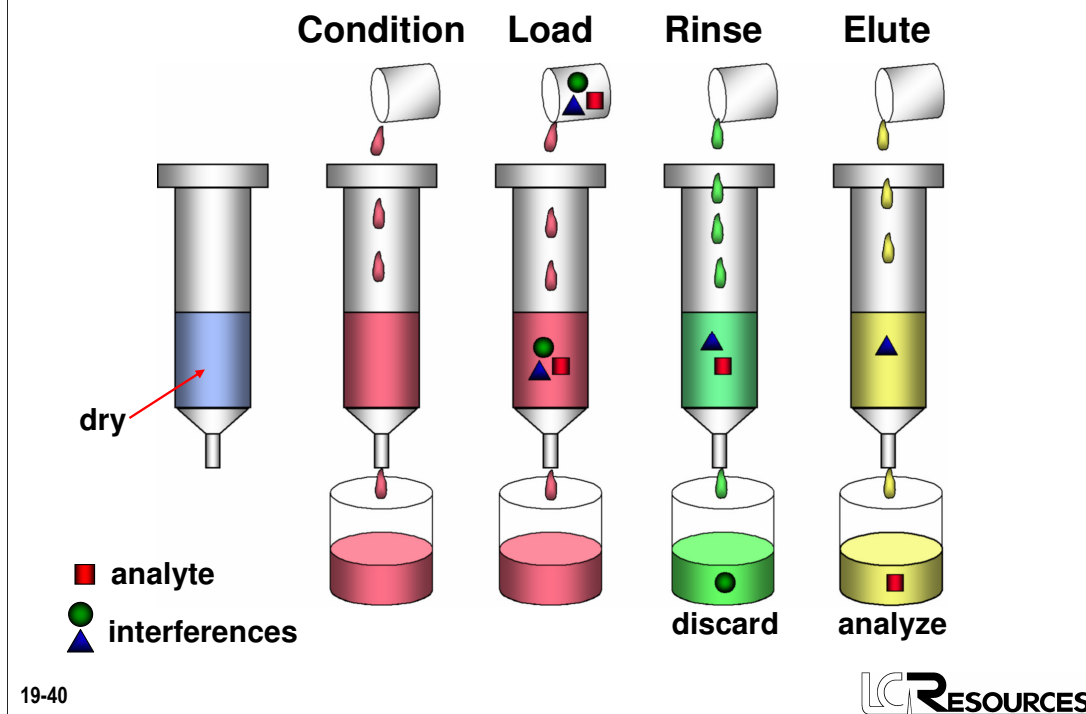
19-39

LCRESOURCES

SPE has been a very popular cleanup technique in recent years. A variety of SPE columns (stationary phases) are available, ranging from ion exchange to reversed phase to mixed phase. In a typical SPE procedure, sample is loaded in an immobilizing solvent, some contaminants are washed through the column to waste, then the solvent is changed to elute the components of interest, leaving more strongly retained materials behind. Because a milliliter or more of sample can be loaded, sample enrichment can take place. SPE cartridges are available as individual columns or in the 96-well format for use with robots.

In general, one should use an SPE stationary phase that is “orthogonal” (has a different retention mechanism) to the analytical column. For example, if a C18 column is used on the LC, a mixed-bed or ion-exchange SPE cartridge might be chosen.

Solid-Phase Extraction



A typical SPE procedure is shown here.

SPE cartridges are generally shipped dry. They must be conditioned (wetted), typically using the same solvent from which the sample will be loaded.

The sample is loaded using a solvent which is sufficiently weak to ensure quantitative retention of the analyte. Interfering compounds may also be retained.

An optional rinse step uses a stronger solvent to elute weakly bound interferences. This solvent should not be strong enough to elute the analyte.

Finally, the analyte is washed with a solvent strong enough to ensure complete elution of the analyte. Some undesired components may remain on the cartridge.

The analyte is now purified and concentrated; ready for analysis.

For best results, the SPE stationary phase should be “orthogonal” to the analytical stationary phase, such as a mixed-mode or ion-exchange SPE phase used in conjunction with a C18 LC column. This combination increases the chances of removing unwanted interferences.

Key to Successful Solid Phase Extraction

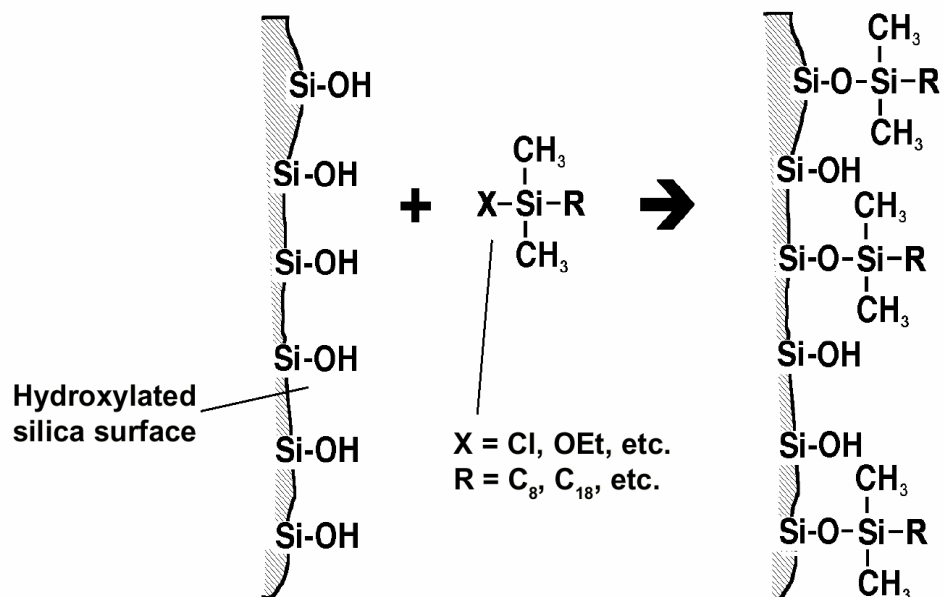
CHROMATOGRAPHY

19-41

LCRESOURCES

It is important not to lose sight of the fact that SPE is just liquid chromatography on columns that are much less efficient than analytical columns. The same principles of solvent strength, pH, and retention apply.

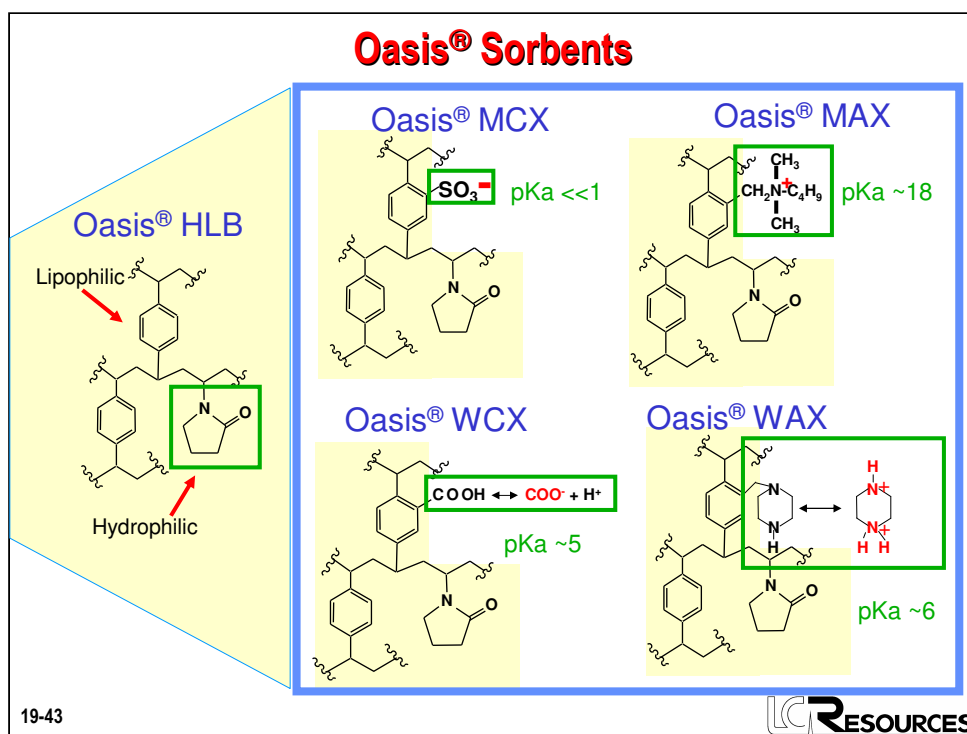
Silica-Based SPE Chemistry



19-42

LCRESOURCES

Standard silane chemistry is used to make silica-based SPE cartridges. These have the same advantages and disadvantages as silica-based analytical columns.



Polymeric supports eliminate some of the unwanted properties of silica, such as limited pH ranges and secondary silanol interactions.

One line of SPE materials are those from Waters. Many other manufacturers also offer SPE products. For those that maybe are not familiar with Oasis, these are the structures of the sorbents

Oasis HLB – hydrophilic-lipophilic balanced co-polymer – reversed-phase retention

Oasis MCX (Mixed-mode Cation eXchanger)

Strong sulfonate (-SO₃H) groups bonded to Oasis® HLB co-polymer (1 meq/g)

Oasis MAX (Mixed-mode Anion eXchanger)

Quaternary amine bonded to Oasis HLB co-polymer (0.25 meq/g)

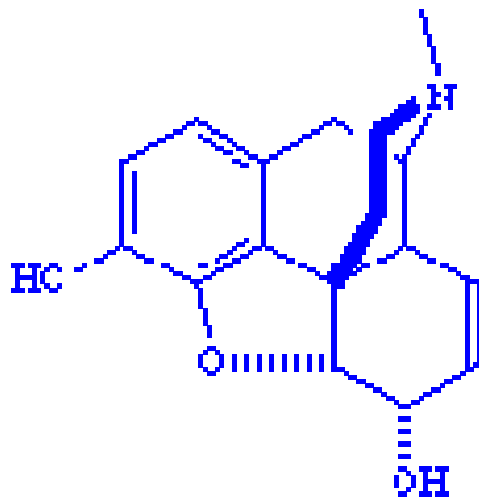
Oasis WCX (mixed-mode weak cation exchanger)

Carboxylic acid bonded to Oasis HLB co-polymer (0.7 meq/g, pKa ~5)

Oasis WAX (mixed-mode weak anion exchanger)

Piperazine bonded to Oasis HLB (0.6 meq/g, pKa ~6)

Courtesy of Waters Corp.



Morphine

pKa = 7.87

19-44

LCRESOURCES

We'll use morphine again as a model for SPE sample preparation.

Morphine / HLB SPE

- **condition: 2x (1 mL MeOH→1 mL water)**
- **500 μ L plasma + 100 μ L IS, vortex**
- **300 μ L borate (pH 9.2), vortex**
- **500 μ L to SPE**
- **wash 1 mL NH_4HCO_3 (pH 9)**
- **elute 1 mL $\text{NH}_4\text{OH}/\text{MeOH}$ (5/95)**
- **evaporate, recon. in injection solvent**

19-45

LCRESOURCES

Here the reversed-phase SPE is activated and then left in water. Plasma is spiked with internal standard and the pH is adjusted so that the morphine is neutral. Sample is loaded and the morphine should stick to the reversed-phase column. Washing at high pH with water will remove some interferences. Keeping the pH high and washing with methanol will elute the drug, but leave ionic interferences on the column.

(Alternative) Morphine / MCX SPE

- **condition: 2x (1 mL MeOH→1 mL water)**
- **500 µL plasma + 100 µL IS, vortex**
- **300 µL 0.1 N HCl (pH 1.1), vortex**
- **500 µL to SPE**
- **wash 1 mL 0.1 N HCl / MeOH (50/50)**
- **elute 1 mL NH₄OH/MeOH (5/95)**
- **evaporate, recon. in injection solvent**

19-46

LCRESOURCES

Another approach would be to use the mixed mode SPE material. In this case, the morphine is made acidic so that it carries a charge. The charged analyte sticks to the ion exchange sites on the column. Washing with organic at low pH will remove some interferences. The drug is released by increasing the pH above the pKa and adding methanol.

Advantages of Mixed-Mode SPE

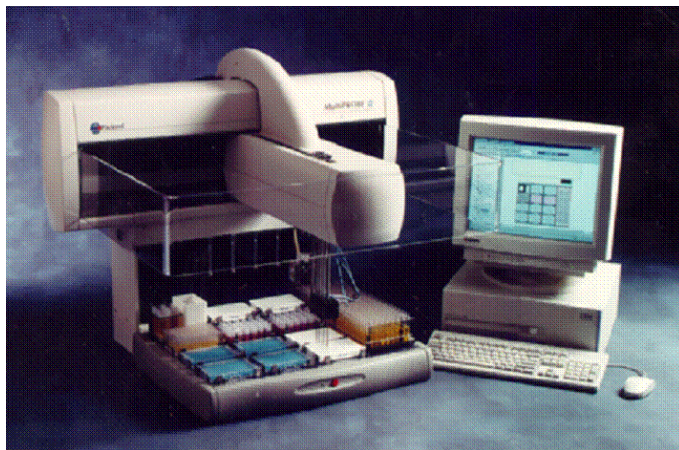
- **Different selectivity than analytical column (orthogonal)**
- **Acidic / Neutral / Basic selectivity**
- **Generally cleaner extracts**
- **Hold or elute contaminants**
- **Can use 100% MeOH wash**

19-47

LCRESOURCES

Generally, using an SPE cleanup technique that has different selectivity than the analytical column will result in cleaner extracts.

Use Automation When Throughput Matters



Perkin-Elmer (Packard) Multiprobe II



Tomtec Quadra 96

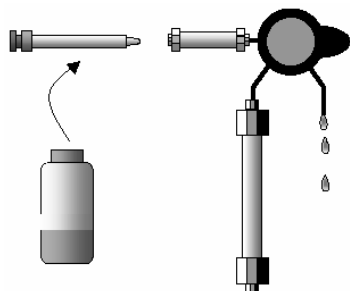
19-48

LC RESOURCES

The ultimate in automated sample pretreatment is the use of a robot. Robots can carry out virtually all common manual steps for sample pretreatment. In most cases, however, a significant setup cost is involved. The use of robots is justified primarily in high-throughput applications.

Here are two examples of sample preparation robots. The Multiprobe uses two or four pipettes independently to move, mix, or extract samples or reagents from vials, tubes, or plates. The Tomtec handles 96 pipette tips at a time to do extractions, solid phase extraction, or other sample processing steps. Often the robots are specialized for one particular operation. For example, the Multiprobe could be used to transfer samples from vials to a 96 well plate and then the plate could be treated in one operation on the Tomtec.

Direct Inject (on-line prep)



ADVANTAGES

- selective
- sample enrichment
- all sample on column

DISADVANTAGES

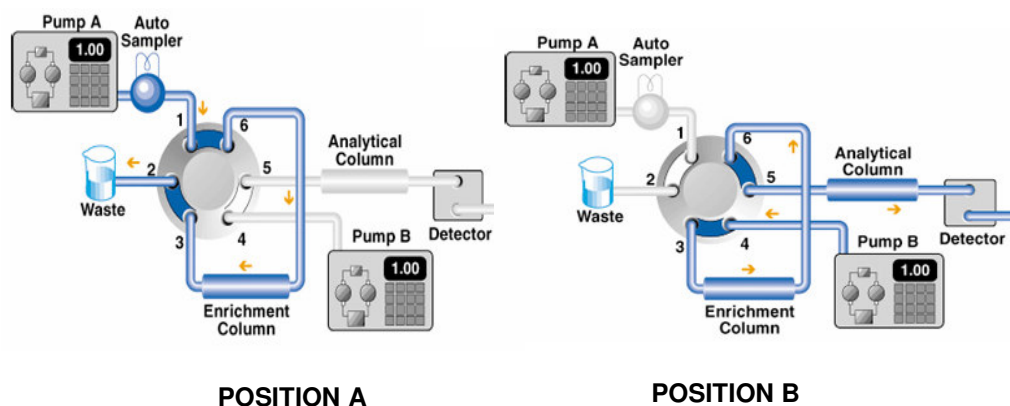
- more complex apparatus
- column lifetime?
- differential extraction
- can slow down analysis

19-49

LCRESOURCES

On-line sample preparation techniques are growing in popularity. In the simplest model, a guard column is used instead of a loop on a sample injection valve. Sample may be loaded on the cleanup column, with components of interest sticking on the column, with more polar materials eluting to waste. Then the valve is switched in line with the analytical column and the components of interest are released from the cleanup column with a stronger solvent. Many labs use this technique routinely with very satisfactory results.

On-Line Sample Enrichment

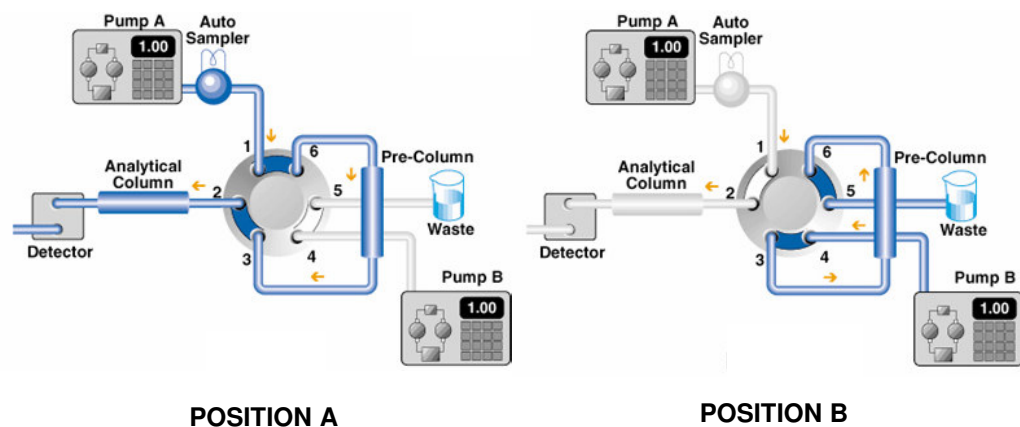


19-50

LC RESOURCES

In this example, a short column is loaded with sample in a weak solvent in position A. The sample is concentrated at the head of the column because of large retention factors in the weak mobile phase. At the same time, very polar materials are eluted to waste. The valve then is switched to position B. The stronger solvent from pump B strips the sample from the enrichment column onto the analytical column, where the separation takes place.

On-Line Stripping of Interferences

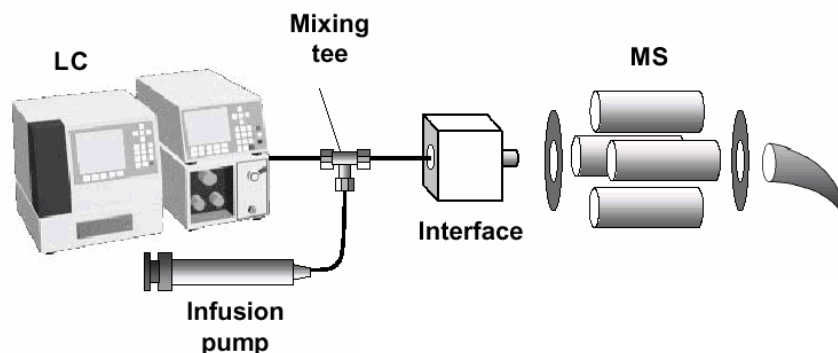


19-51

LCRESOURCES

In this example, the plumbing is changed a bit to enable removal of strongly retained materials. The sample is injected into the precolumn in position A. The solvent is sufficiently strong that the desired sample components travel quickly through the precolumn onto the analytical column. Unwanted materials that are strongly retained stay on the precolumn. The valve is now switched to position B. A strong solvent from pump B backflushes the precolumn, stripping the (unwanted) late eluters to waste. Meanwhile pump A continues to elute the desired materials from the analytical column.

Some Interferences Reduce the Signal: Ion Suppression



- **Run proposed LC conditions**
- **Infuse analyte**
- **Infuse internal standard**
- **Inject blank extracted matrix**

19-52

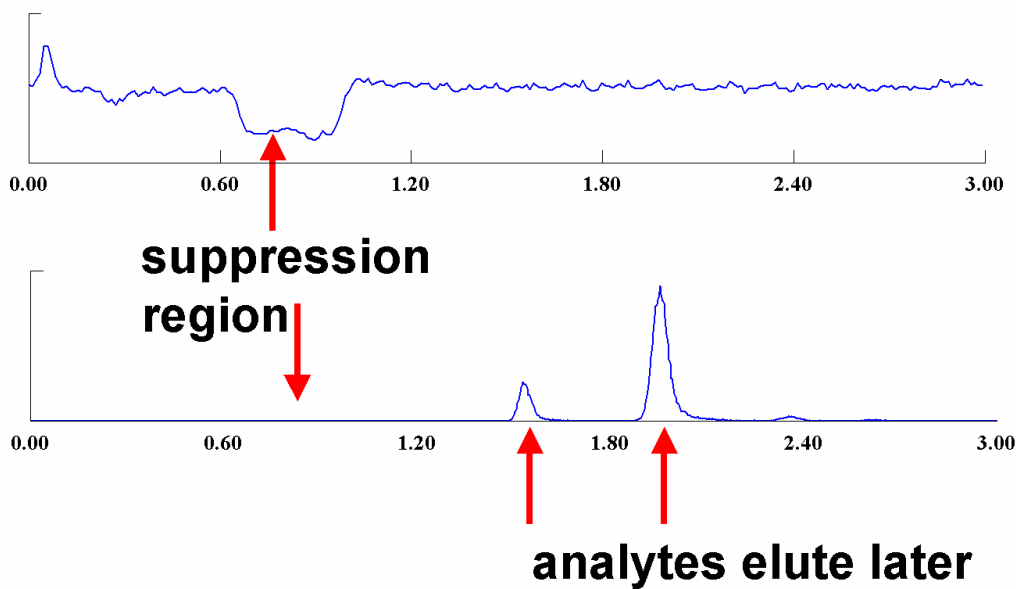
LCRESOURCES

One way that accuracy can be compromised is if the signal is suppressed by a co-eluting material in the sample.

With LC methods, one usually wants sufficient retention so that the garbage at the solvent front doesn't interfere with the analyte. This usually means $k > 1$. With LC-MS, this early-eluting material can suppress ionization of analytes. Suppressed ionization can lead to non-linearity and inaccurate quantification.

One easy way to check for suppression is shown here. A constant concentration of a standard is infused into the mobile phase stream after the column. Once the baseline stabilizes, an injection of an extracted matrix blank is made. At the solvent front a negative dip will be seen as ionization suppressing materials elute and reduce the baseline signal. When the baseline returns to normal, all these suppressing agents have passed through the detector.

Ion Suppression Generates Regions of *Negative* Response



19-53

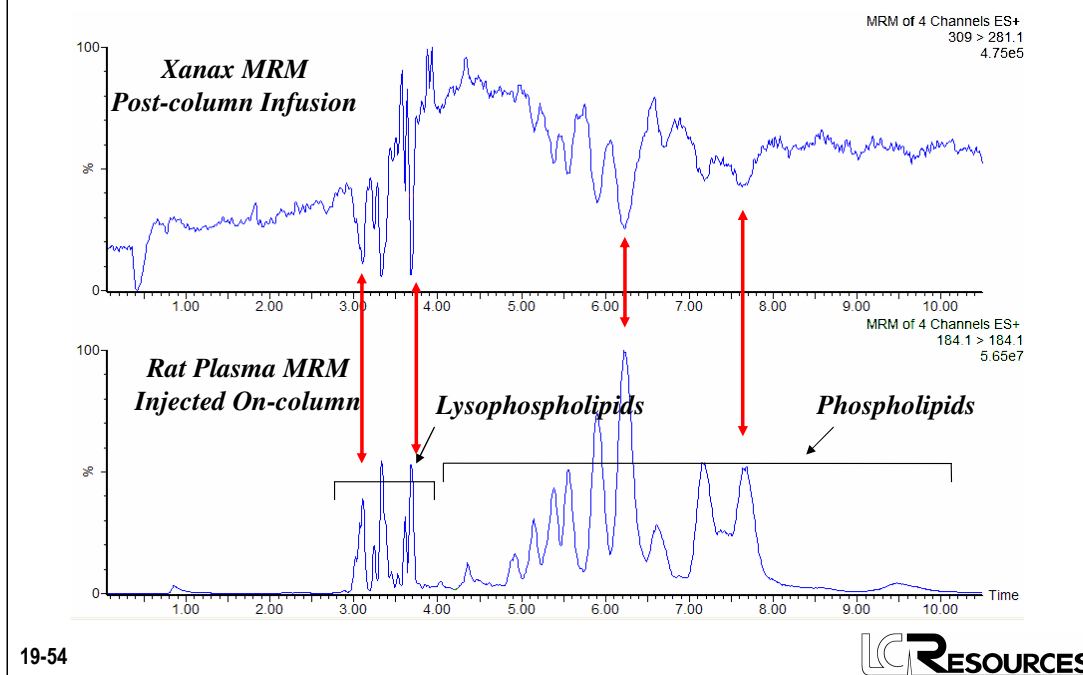
LCRESOURCES

In this example, a dilute solution of paclitaxel was infused into the LC-MS/MS and blank extracted plasma was injected to obtain the top plot. The steady-state signal for paclitaxel dropped when ionization was suppressed. By adjusting retention so that the analytes of interest elute after this suppression region, the risk of signal loss due to ionization suppression is greatly reduced.

Just as analyte peaks can elute anywhere in the run, ion suppression can occur anywhere in the run, so it is very important to run the ion suppression experiment to be sure that ion suppression will not compromise the method.

This check of ion suppression is delayed until the sample prep, LC, and MS conditions have been selected. If problems occur, you may need to adjust some of the parameters. With the DryLab dataset, often the chromatography can be adjusted with little effort. Sometimes a persistent suppression region will require additional sample preparation to minimize the problem.

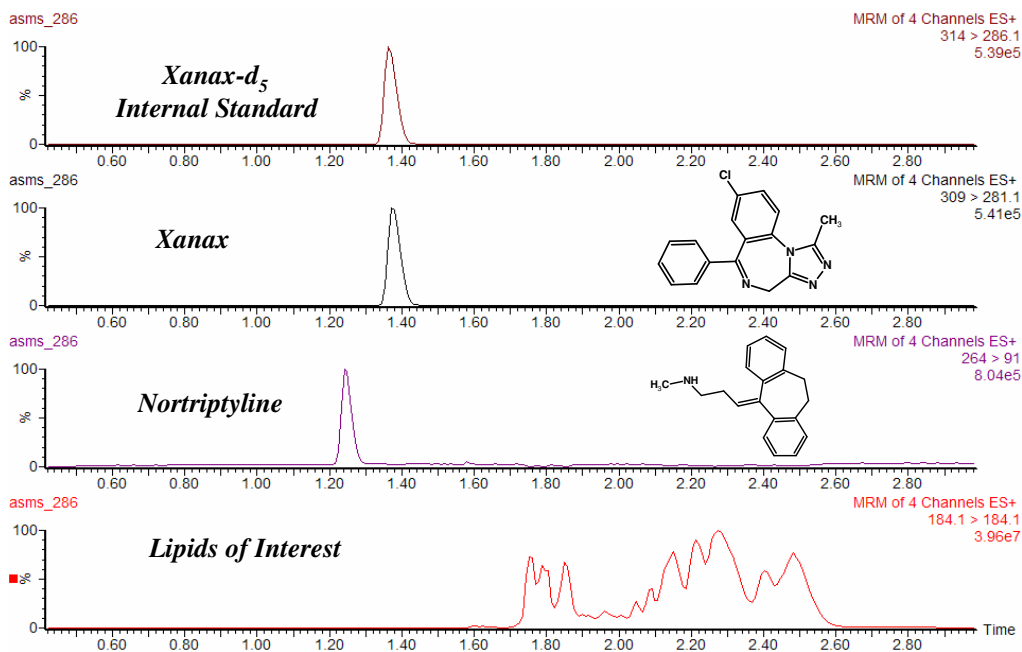
Matrix Suppression - Phospholipids



Matrix Suppression Shown by Post-Column Infusion of 180 ng/ml Solution of Xanax and Injection of Rat Plasma Sample. Note how phospholipid peaks correspond with dips in Xanax baseline – these are ion suppression regions greatly limiting the region in which Xanax can elute without suppression.

Courtesy of James Little, Eastman Kodak. J Chromatogr. B (2006) submitted for publication.

Retention Adjusted for Ion Suppression



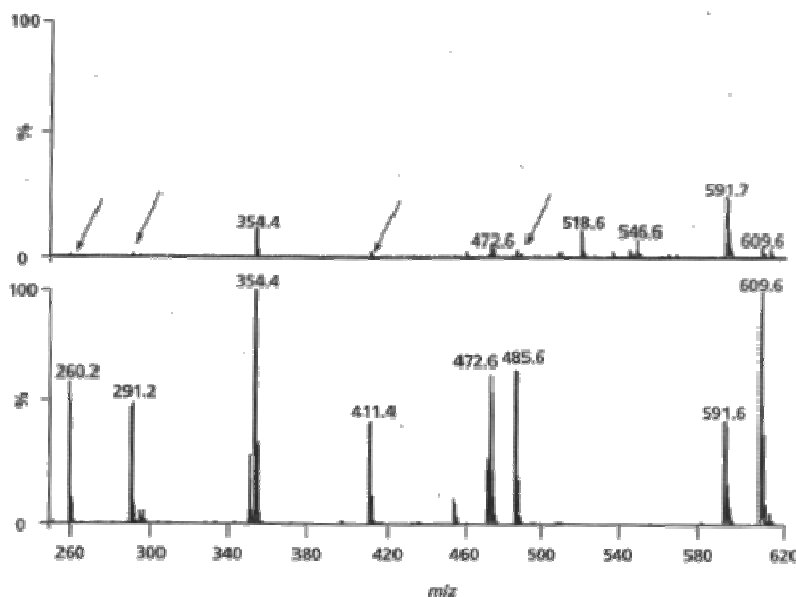
19-55

LCRESOURCES

HPLC conditions adjusted from previous slide.

Courtesy of James Little, Eastman Chemical. J. Chromatogr. B (2006) submitted for publication.

Ion Suppression: Rat Plasma Precipitate vs. Standards



19-56

LCRESOURCES

Here is an example of how suppression can appear during sample analysis.

Plasma precipitation is a popular and simple method to clean up samples prior to injection. This example shows that although plasma proteins may be removed to make the sample look visibly clear, remaining materials can cause severe ion suppression.

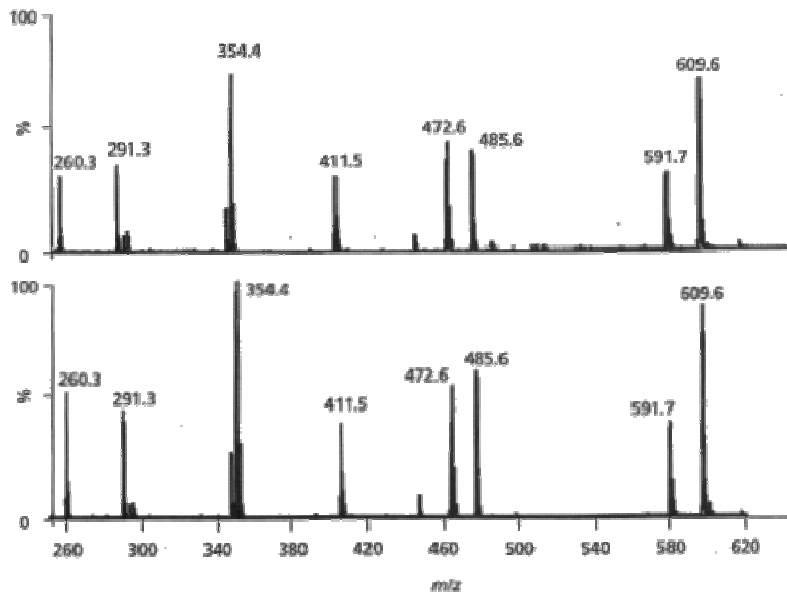
Top: rat plasma precipitate containing prednisolone, diphenhydramine, betamethasone, amitriptyline, naproxin, and ibuprofen. Bottom: standards in solvent.

C.R. Mallet, Z. Lu, and J.R. Mazzi, *Rapid Commun. Mass Spectrom.*, 18 (2004) 49-58.

and

D.M. Diehl and M.P. Balogh, *LC/GC*, 22 (2004) 344-352.

Ion Suppression: Rat Plasma SPE Extract vs. Standards



19-57

LCRESOURCES

The same rat plasma sample was subjected to SPE cleanup using a mixed-mode resin. As can be seen here, this cleanup technique is much more effective for this sample than simple plasma precipitation.

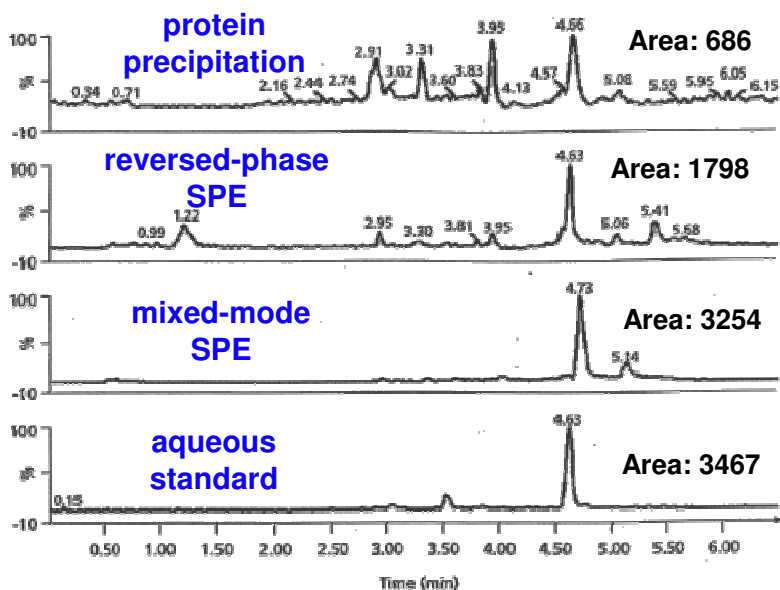
Top: rat plasma containing prednisolone, diphenhydramine, betamethasone, amitriptyline, naproxin, and ibuprofen following solid phase extraction with mixed-mode cartridge. Bottom: standards in solvent.

C.R. Mallet, Z. Lu, and J.R. Mazzi, *Rapid Commun. Mass Spectrom.*, 18 (2004) 49-58.

and

D.M. Diehl and M.P. Balogh, *LC/GC*, 22 (2004) 344-352.

Ion Suppression: Rat Plasma Extracts vs. Standards



19-58

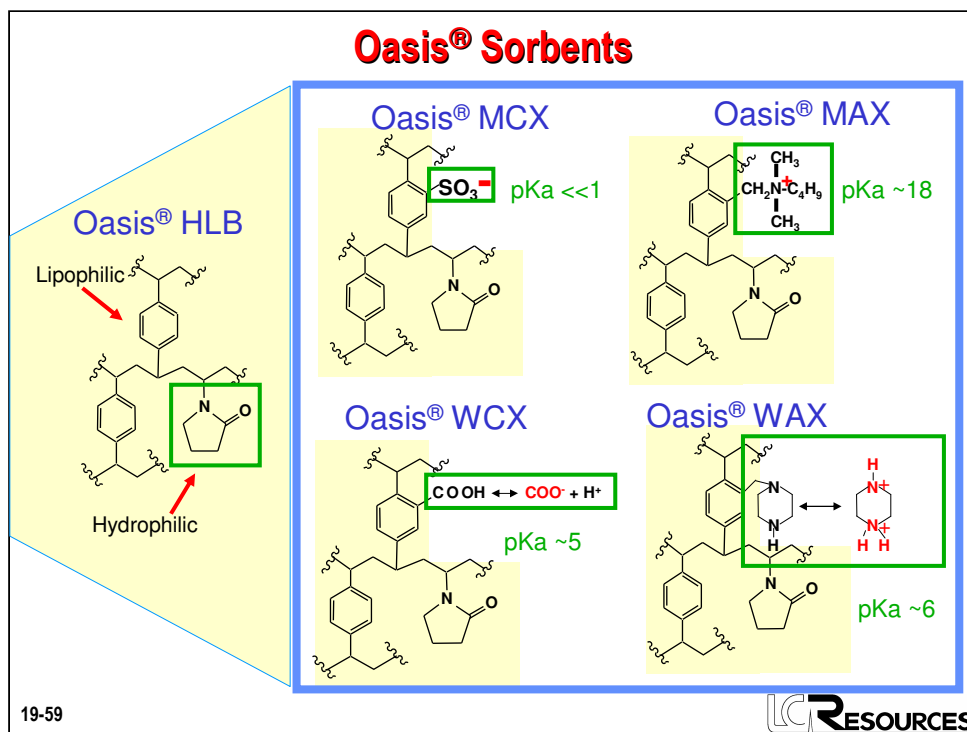
LCRESOURCES

Here is a comparison of the response of the MS to rat plasma containing 0.1 ng/mL amitriptyline that has been prepared with various extraction schemes. It usually is best to use a cleanup technique that works on a different principle than the LC separation. We can see that the mixed-mode cleanup was more effective than reversed-phase cleanup when a reversed-phase column was used for the analytical separation.

M. Gerdes and H. Waldmann, *J. Comb. Chem.*, 5 (2003) 814 – 820.

and

D.M. Diehl and M.P. Balogh, *LC/GC*, 22 (2004) 344-352.



One line of SPE materials are those from Waters. Many other manufacturers also offer SPE products. For those that maybe are not familiar with Oasis, these are the structures of the sorbents

Oasis HLB – hydrophilic-lipophilic balanced co-polymer – reversed-phase retention

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Strong sulfonate (-SO₃H) groups bonded to Oasis® HLB co-polymer (1 meq/g)

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Quaternary amine bonded to Oasis HLB co-polymer (0.25 meq/g)

Oasis WCX (mixed-mode weak cation exchanger)

Carboxylic acid bonded to Oasis HLB co-polymer (0.7 meq/g, pKa ~5)

Oasis WAX (mixed-mode weak anion exchanger)

Piperazine bonded to Oasis HLB (0.6 meq/g, pKa ~6)

Courtesy of Waters Corp.

Sample Preparation Methods

Protein Precipitation (PPT)

3:1 ACN to plasma

Liquid-Liquid Extraction (LLE)

3:1 MTBE to plasma

SPE: Oasis® HLB or Sep-Pak® tC₁₈ (Reversed-Phase)

Wash: 5% MeOH in H₂O

Elute: MeOH

SPE: Oasis® MCX (Mixed-mode cation exchanger)

Wash 1: 0.1 N HCl

Wash 2: MeOH

Elute: 5% NH₄OH in MeOH

SPE: Oasis® HLB – 2D Optimized Method

Wash 1: 5% MeOH in H₂O

Wash 2: 40% MeOH with 2% NH₄OH in H₂O

Wash 3: H₂O

Elute: 70% MeOH with 2% FA

SPE: Oasis® WCX (Mixed-mode weak cation exchanger)

Wash 1: 25 mM phosphate buffer, pH 7.0

Wash 2: MeOH

Elute: 2% FA in MeOH

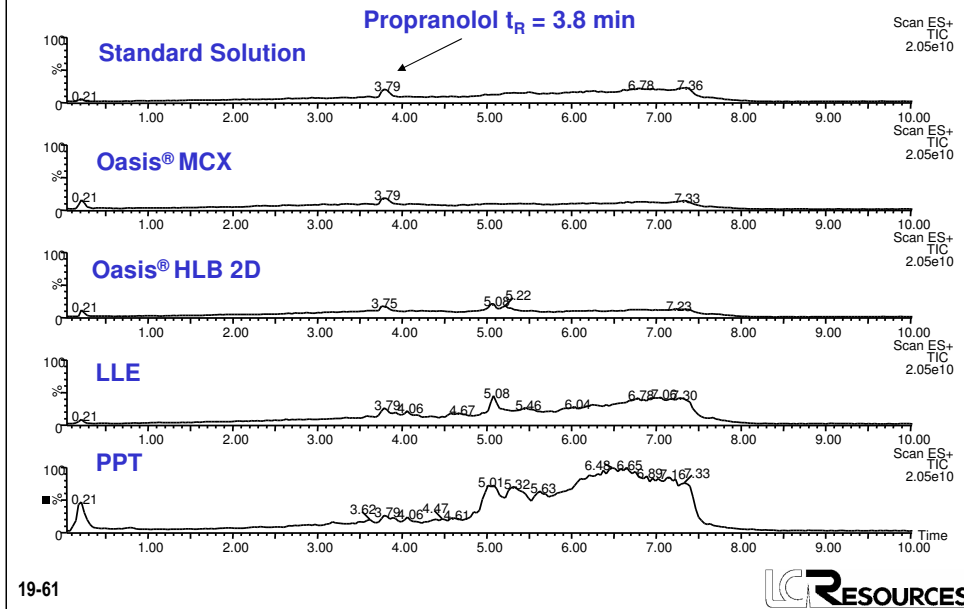
19-60

LCRESOURCES

Sample preparation conditions for following experiments.

Courtesy of Waters Corp.

Comparison of Sample Prep Methods ESI+ TIC: pH 10 Mobile Phase

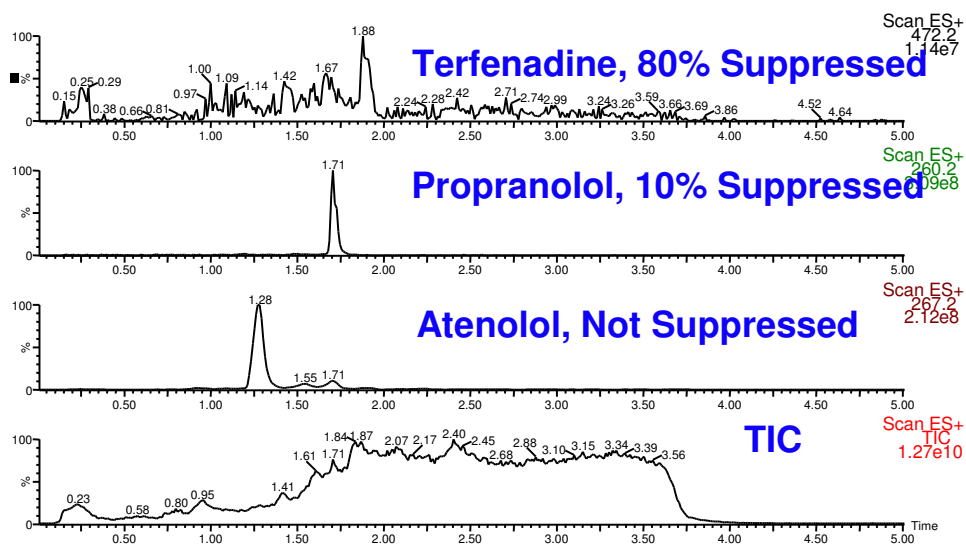


Full scan from 100 to 1000 m/z. This is the TIC which is the sum off all ions from 100 to 1000 m/z. Note the increasing level of residual matrix interferences as you move from top to bottom. Although propranolol does not elute in the region where most of the matrix components are eluting, other analytes may elute, thereby opening up the possibility of ion suppression. Clearly, with MCX the extract is the cleanest.

Note, this scale is normalized to the PPT extract.

Courtesy of Waters Corp.

Protein Precipitation



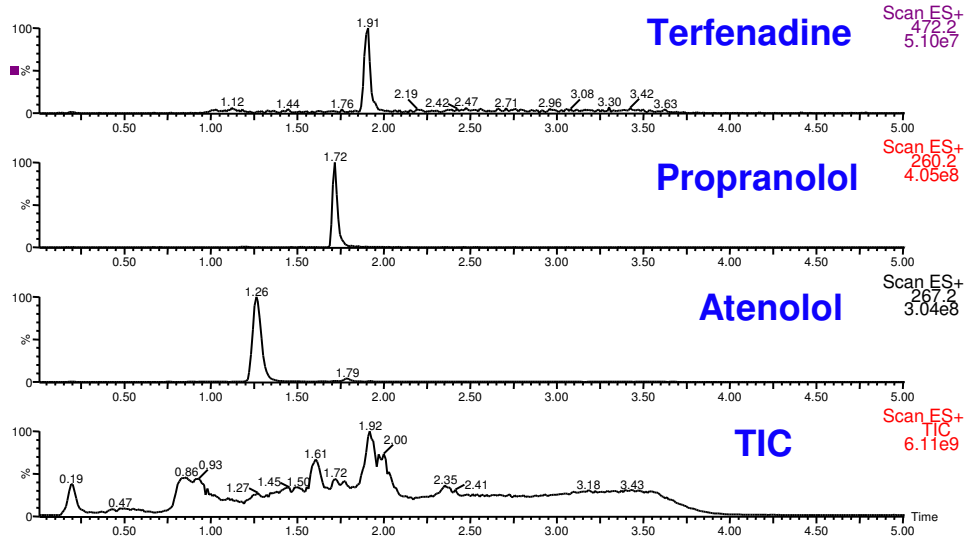
19-62

LCRESOURCES

Protein precipitation alone may or may not compromise the sample due to ion suppression.

Courtesy of Waters Corp.

Oasis® MCX Results



19-63

LCRESOURCES

Here are the same samples with the MCX preparation method. No ion suppression is observed with the MCX method.

Courtesy of Waters Corp.

MRM Transitions

Analyte:

Propranolol m/z 259.9 → 183.1

Phospholipid Interferences from rat plasma*:

Lysophospholipids

m/z 496.4 → 184.3

m/z 524.4 → 184.3

Phospholipids

m/z 704.4 → 184.3

m/z 758.4 → 184.3

m/z 806.4 → 184.3

19-64

LCRESOURCES

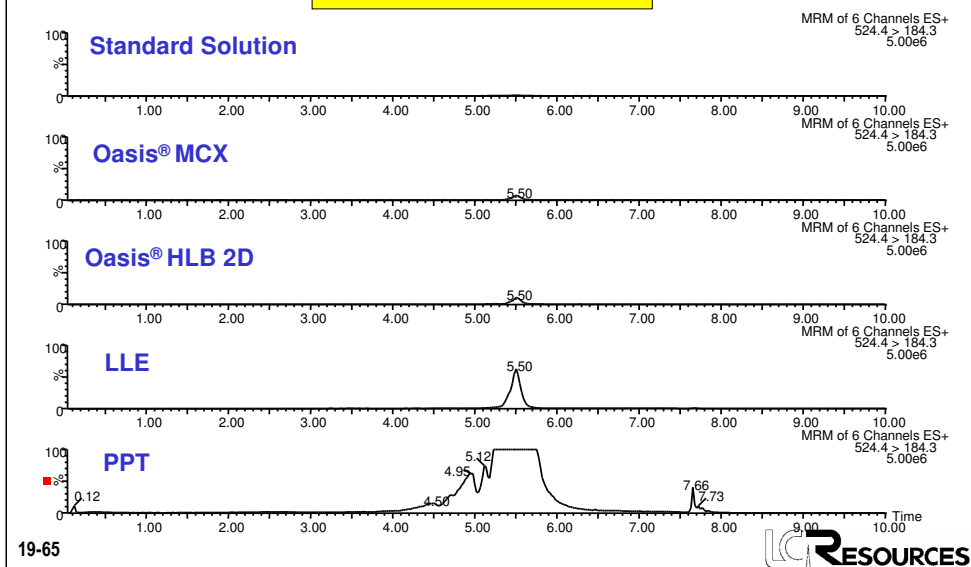
Phospholipids and lysophospholipids have been implicated as general sources of ion suppression. Note that all the phospholipids have a 184.3 product ion which can be monitored to determine the presence of these compounds.

*Van Horne, K.C.; Bennett, P. K. Matrix Effects Prevention by Using New Sorbents to Remove Phospholipids from Biological Samples, Poster, AAPS 2003.

Courtesy of Waters Corp.

Lysophospholipids: MRM 524.4 to 184.3

MRM 496.4 to 184.3 is similar



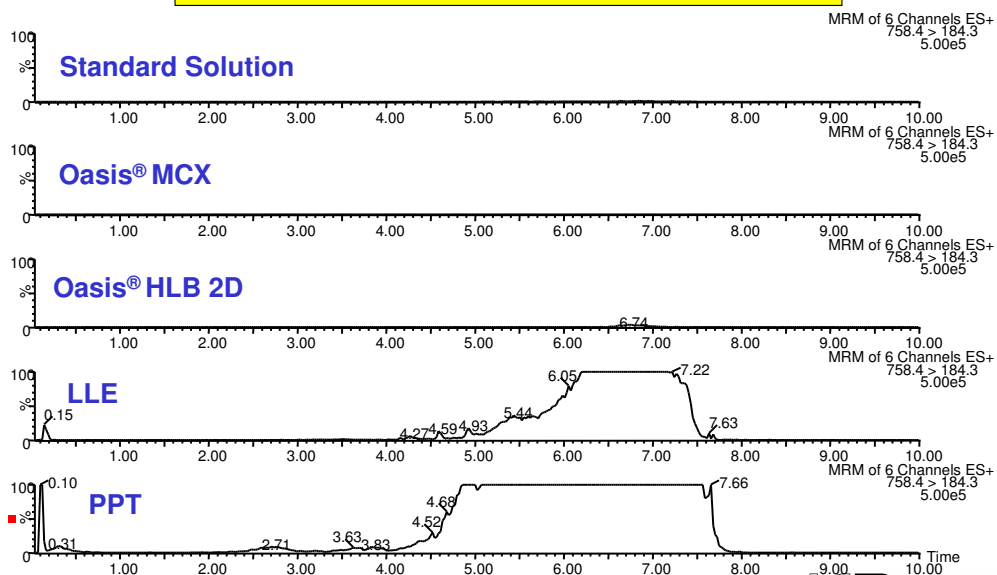
Here is the MRM monitoring the presence of lysophospholipids in various cleanup schemes.

Note the amount of this phospholipid in the PPT. This is collecting on your column and in the MS.

Courtesy of Waters Corp.

Phospholipids: MRM 758.4 to 184.3

MRM's 806.4 to 184.3 and 704.4 to 184.4 look similar

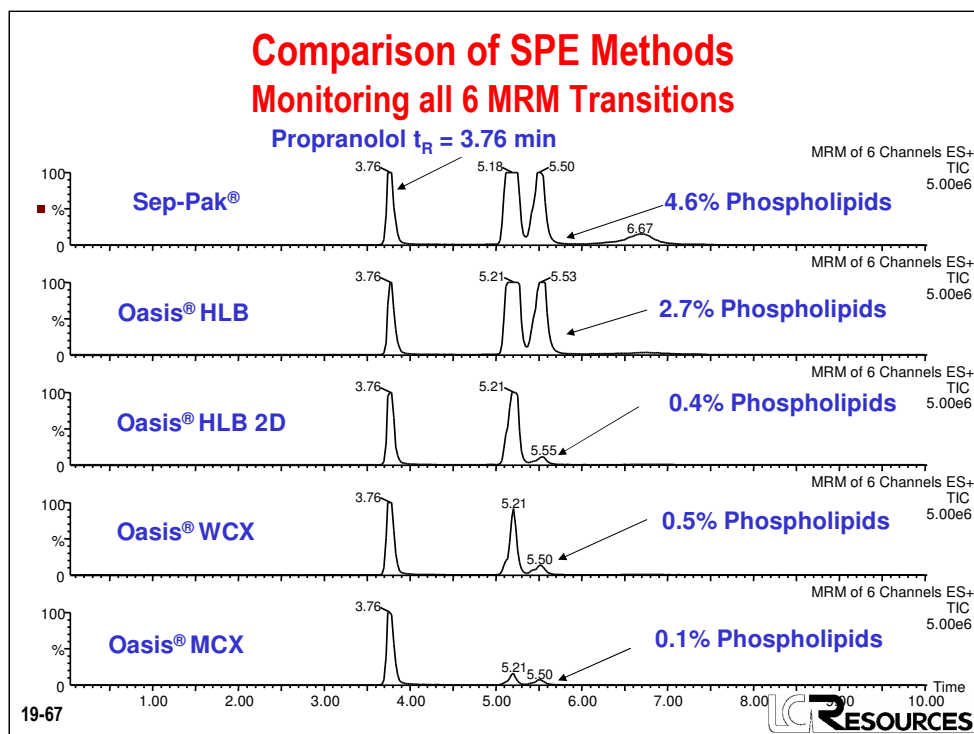


19-66

LC RESOURCES

Monitoring the phospholipid transitions yields similar results.

Courtesy of Waters Corp.



Here the propranolol signal is shown with all 6 of the lipid transitions. The %-phospholipid is relative to precipitation as 100%.

Area Counts:

PPT: 37057320

Sep-Pak: 957194

HLB: 1029210

HLB 2D: 88236

WCX: 99194

MCX: 51958

Courtesy of Waters Corp.

Checking Ion Suppression

- **Infuse analyte**
 - Inject blank extracted matrix
 - Inject blank spiked with IS ($R_s < 1$)
- **Infuse internal standard**
 - Inject blank extracted matrix
 - Inject blank spiked with analyte

19-68

LCRESOURCES

In addition to checking the matrix for materials that may suppress ionization of the sample, you also should make the same test for ion suppression of the internal standard. If a stable-label internal standard is used or any time the resolution between the analyte and IS is less than 1, you should check to be sure the analyte and IS do not suppress each other.

Intentionally Blank

19-69

LCRESOURCES