

Validated HPLC Methods

To Tweak or Not to Tweak



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**“Adjustments of operating conditions
to meet system suitability
requirements may be necessary.”**

USP 23 p1776



What is the line between adjusting conditions and actually modifying an official or regulatory method?

This is critical to know because modifying a method requires validation and ruggedness testing.



Let's examine some *proposed guidelines* for this area.

**System Suitability Tests in
Regulatory Liquid and Gas Chromatographic
Methods:
Adjustments Versus Modifications**

William B. Furman, John G. Dorsey, and Lloyd R. Snyder
Pharmaceutical Technology, June 1998 p. 58-64



HPLC Method Parameters That Can Be Varied

Mobile Phase

- **The pH of the mobile phase:** +/- 0.2 pH units
- **Concentration of the buffer salts:** +/- 10%
(buffer pH must remain same +/- 0.2 pH units)
- **Ratio of the solvents in the mobile phase:** +/- 30% relative or +/- 2% absolute, whichever is larger, but no change can exceed 10% (based on mobile phase component of 50% or less)



HPLC Method Parameters That Can Be Varied

Column

- Column length: +/- 70% (250 mm columns may be substituted over the range 75 – 425 mm)
- Column inner diameter: +/- 25% (if method calls for 3.9 mm id, 3.0, 4.0, or 4.6 mm can be substituted)
- Particle size: may be reduced up to 50% (3 or 3.5 μm particles can be used instead of 5 μm)
- Column temperature: +/- 20°C



HPLC Method Parameters That Can Be Varied

System

- Flow Rate: +/- 50%
- Injection Volume:
 - Increase up to 2x – maintain peak shape, resolution, retention time, etc.
 - Decrease as much as will maintain acceptable precision and sensitivity



**Let's look at a problem method
and determine if it can be adjusted
or must be modified.**



USP Diphenhydramine Hydrochloride Method

Mobile Phase: 50% Acetonitrile:
50% Water: 0.5% Triethylamine
Prepare solution and adjust pH to 6.5
with glacial acetic acid

Column: 4.6 x 250 mm, L10 (CN)

Flow Rate: 1mL/min

Detection: UV 254 nm

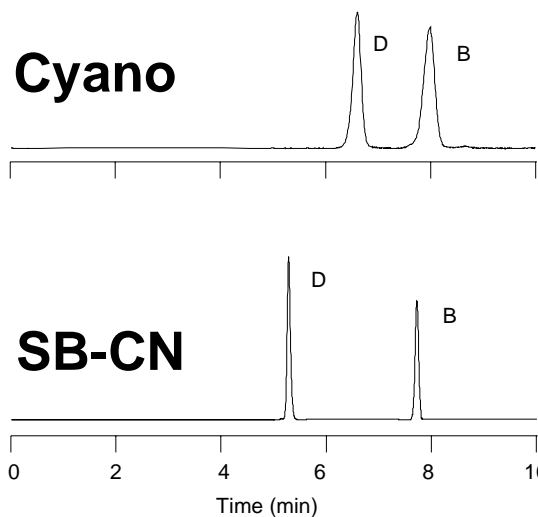
System Suitability: Benzophenone and
Diphenhydramine Solution

Specifications: $R_s > 2.0$, $T_f < 2.0$ for diphenhydramine

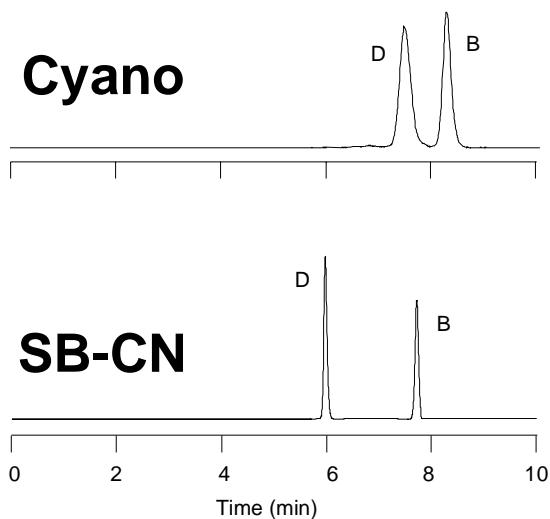


Method Problems

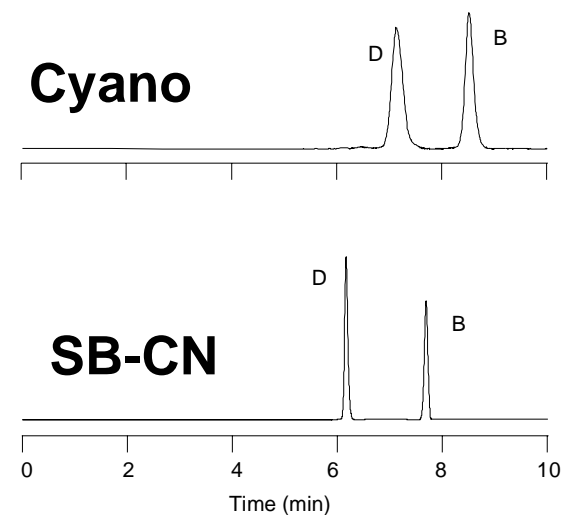
October 28
Apparent pH 6.5



November 1
Apparent pH 6.2



November 6
Apparent pH 6.2



- ◆ The pH drifts and retention changes because of unreliable pH adjustment on mobile phase with organic present.
- ◆ The traditional CN column shows more changes than the SB-CN column.



Method “Adjustment”

- **Adjust pH on aqueous component alone. This is done by measuring the amount of acid it requires to get to the apparent pH, then adding this amount to the aqueous component (with TEA). This becomes the new pH of the mobile phase.**
- **This procedure works best if the mobile phase is actually buffered.**



Recommendations

- **Use a proper buffer and make the pH adjustment to the aqueous portion alone, but keep the mobile phase as similar as possible to maintain expected behavior.**
- **Select a Rapid Resolution L10 column to minimize analysis time and maintain resolution.**
- **Use an SB-CN (L10) to improve reproducibility.**



Modified Diphenhydramine Hydrochloride Method Parameters

Column:	4.6 x 75 mm, 3.5 mm, StableBond SB-CN (L10) ¹
Mobile Phase:	55% 25 mM ammonium acetate pH 4.5/ 0.5% TEA: 45% Acetonitrile ²
Flow Rate:	1 mL/min
Detection:	UV 265 nm ²
Temperature:	RT
System Suitability:	Benzophenone and Diphenhydramine Solution
Specifications:	$R_s > 2.0$, $T_f < 2.5$ for diphenhydramine

¹ method adjustment
² method modification



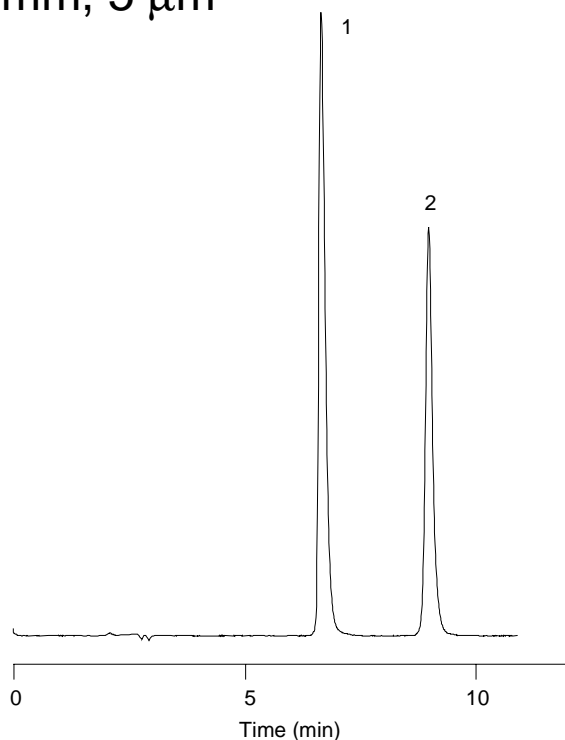
Modified Diphenhydramine Hydrochloride Method Example

Column: **StableBond SB-CN** Mobile Phase: 55% 25 mM CH₃COONH₄/0.5% TEA : 45% ACN Flow Rate: 1.0 mL/min Temperature: RT
Sample: 1. Diphenhydramine 2. Benzophenone Injection Volume: 10 µL Detection: UV 265 nm

4.6 x 250 mm, 5 µm

$R_s = 7.5$

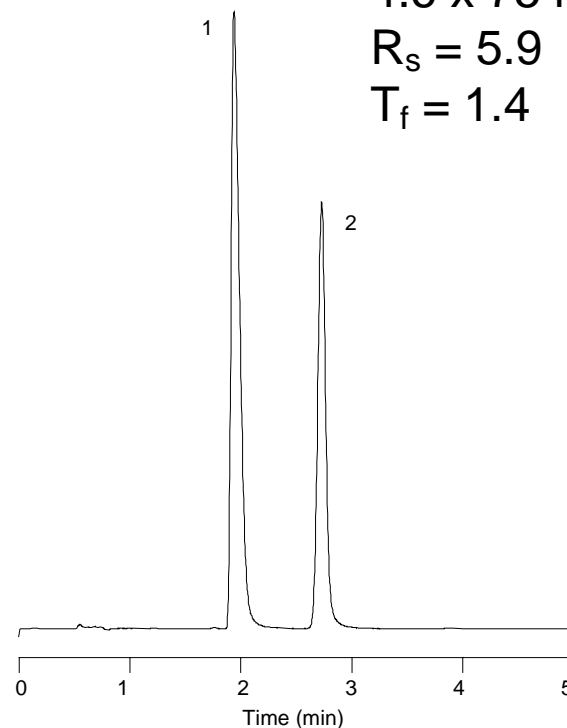
$T_f = 1.5$



4.6 x 75 mm, 3.5 µm

$R_s = 5.9$

$T_f = 1.4$



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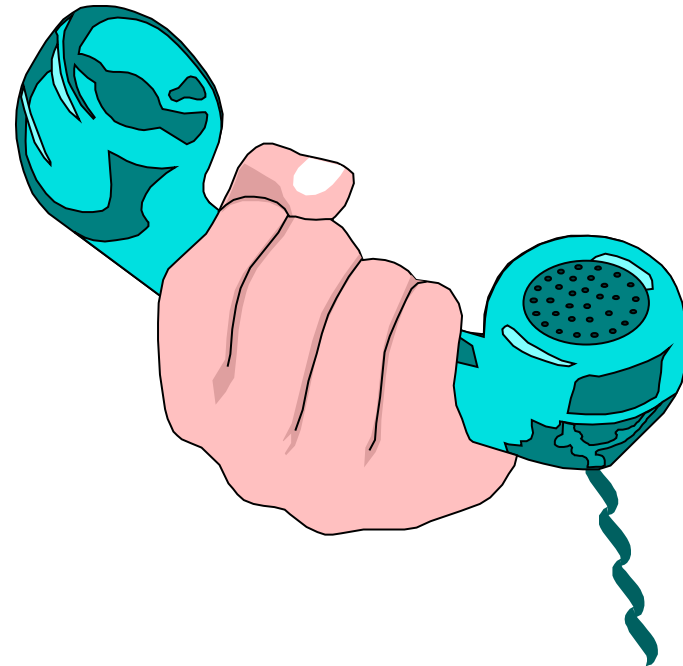
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001828S1.PPT

Break Number 1

- For Questions and Answers
- Press *1 on Your Phone to
- Ask a Question



Method Validation Requirements

- **Robustness**
- **Linearity**
- **Accuracy**
- **Precision**
- **Limit of Detection**
- **Limit of Quantitation**
- **Specificity/Selectivity**
- **Range**
- **Ruggedness**



USP Data Requirements for Method Validation

Parameter	Bulk Drug	Impurities Degradates	Product Performance
Precision	Yes	Yes	Yes
Accuracy	Yes	Yes	Maybe
Limit of Detection	No	No	Maybe
Limit of Quantitation	No	Yes	Maybe
Specificity/Selectivity	Yes	Yes	Maybe
Range	Yes	Yes	Maybe
Linearity	Yes	Yes	Maybe
Ruggedness	Yes	Yes	Yes



Robustness Testing

- **Vary Key Method Parameters - meet or exceed method adjustment recommendations**
- **Use System Suitability Mixture - diphenhydramine/benzophenone and focus on behavior of diphenhydramine**

- 1. pH**
- 2. Temperature**
- 3. % Organic**
- 4. Buffer Concentration**
- 5. Column Lot**

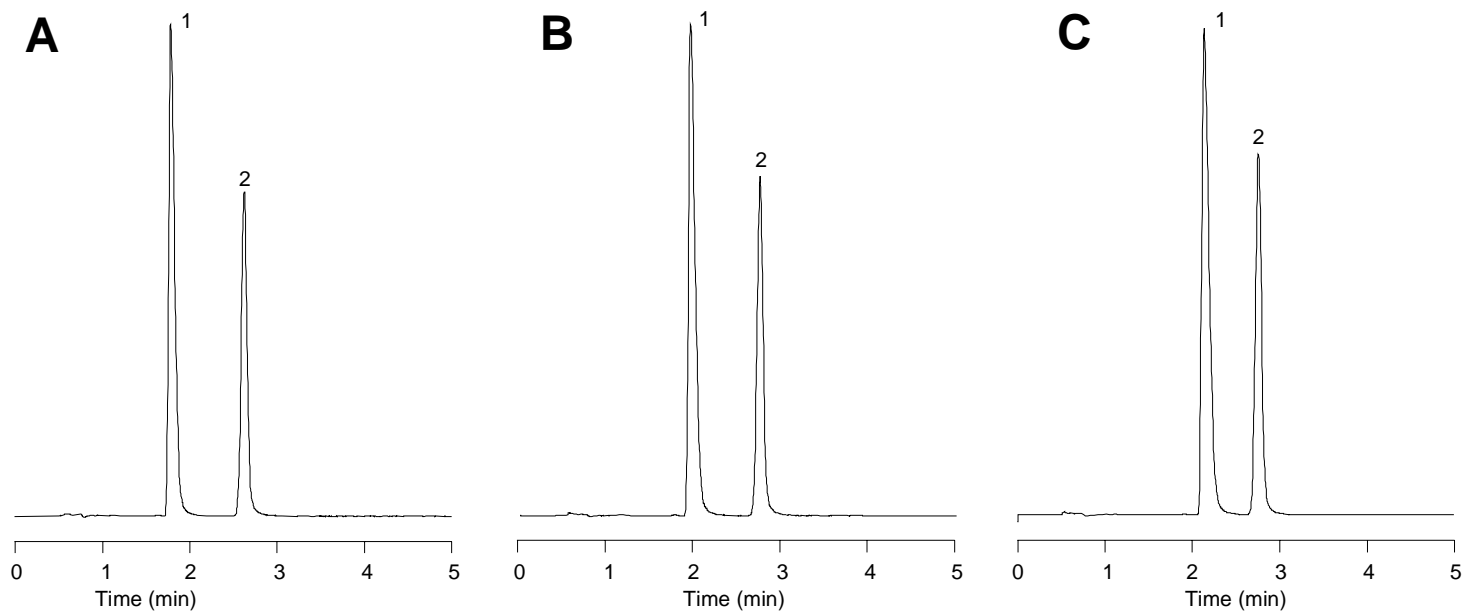


pH Variation

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m Mobile Phase: 55% 25 mM $\text{CH}_3\text{COONH}_4$ /0.5% TEA : 45% ACN Flow Rate: 1.0 mL/min
Temperature: RT Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL Injection Volume: 10 mL

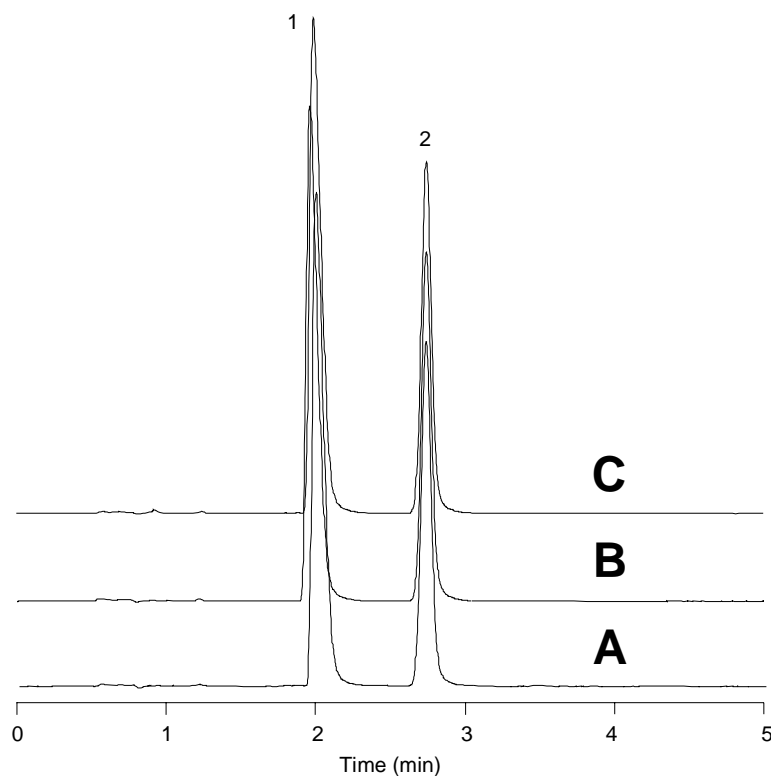
- Tested pH at 4.0, 4.5, and 5.0.
- Monitor for substantial changes in retention, resolution, and peak shape

	pH	Time	R _s	T _f
A	4.0	1.78	6.2	1.7
B	4.5	1.94	5.5	1.7
C	5.0	2.14	4.0	1.8



Buffer Concentration

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m Mobile Phase: 55% CH₃COONH₄ (pH 4.5)/0.5% TEA : 45% ACN Flow Rate: 1.0 mL/min
Temperature: RT Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL Injection Volume: 10 mL



	Conc.	Time	T _f
A	20 mM	1.98	1.8
B	25 mM	1.96	1.8
C	30 mM	1.99	1.8

- Tested two additional buffer strengths, 20 mM and 30 mM
- Monitor for changes in retention and peak shape

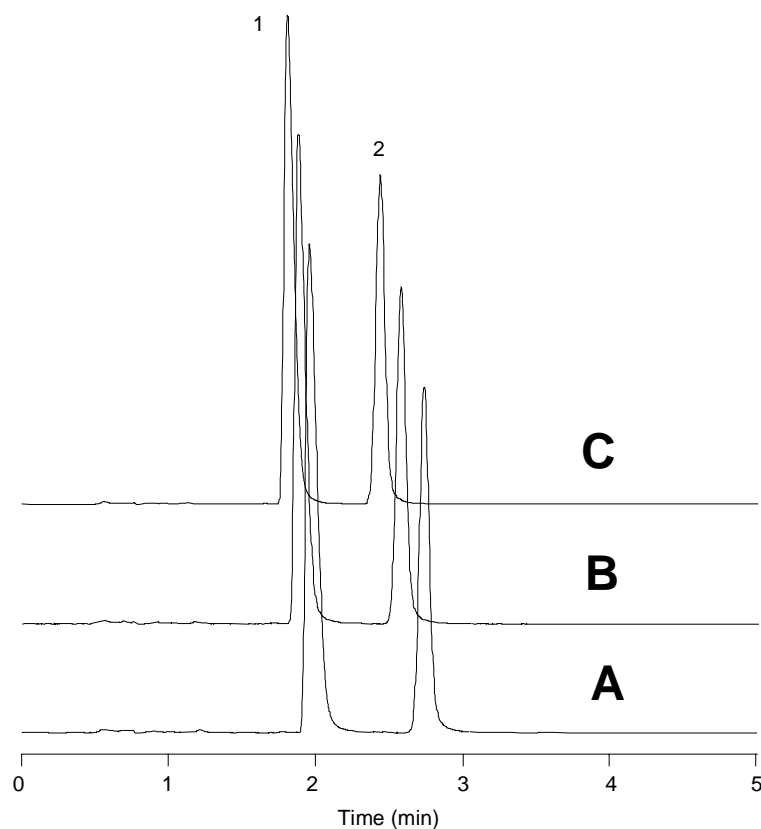


Temperature

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m
Temperature: see below

Mobile Phase: 55% CH₃COONH₄ (pH 4.5)/0.5% TEA : 45% ACN
Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL

Flow Rate: 1.0 mL/min
Injection Volume: 10 μ L



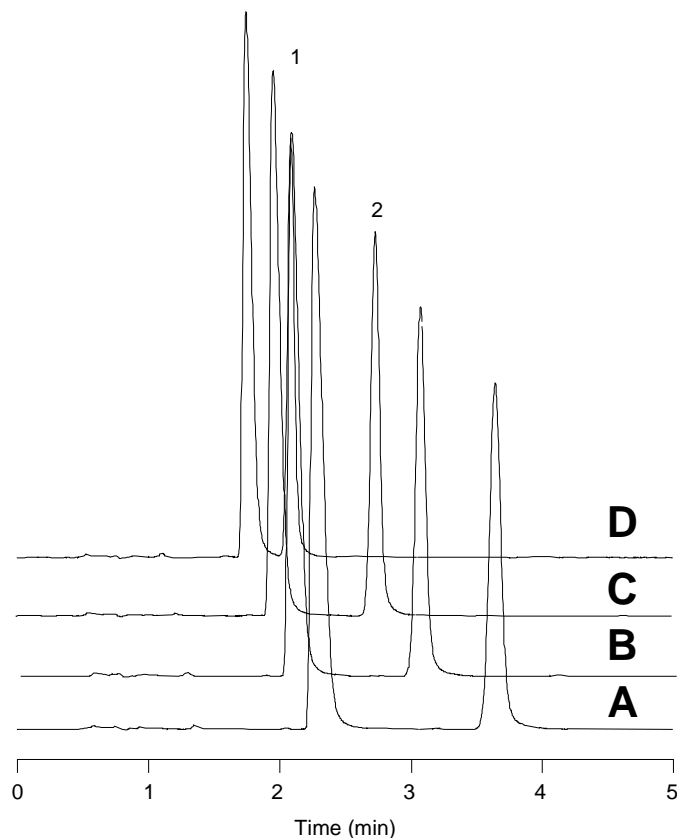
	°C	α	T _f
A	RT	1.6	1.8
B	30	1.6	1.7
C	35	1.6	1.7

- Tested 3 temperatures – Room Temperature, 30°C and 35°C
- Monitor for changes in selectivity (α) and peak shape of diphenhydramine



% Organic

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m Mobile Phase: CH₃COONH₄ (pH 4.5)/0.5% TEA : ACN Flow Rate: 1.0 mL/min
Temperature: RT Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL Injection Volume: 10 mL



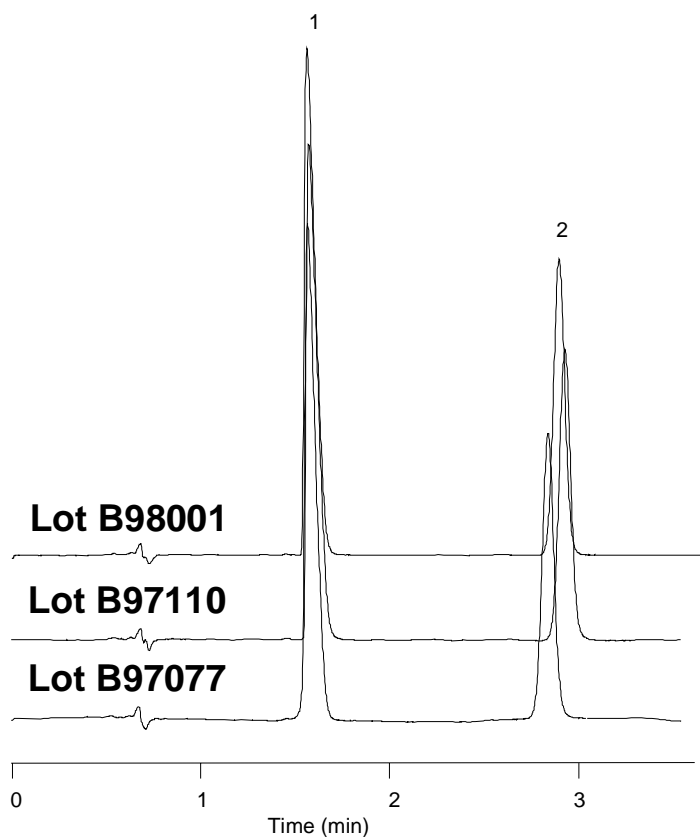
	% ACN	R _t	k	R _s
A	40	2.27	2.0	7.5
B	43	2.07	1.8	6.4
C	45	1.96	1.6	5.0
D	50	1.75	1.3	2.8

- Expect retention, selectivity and resolution to change with change in organic.
- Determine which mobile phase meets needs (adequate retention, resolve matrix components) without wasting time.



Column Lot

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m Mobile Phase: 55% CH₃COONH₄ (pH 4.5)/0.5% TEA : 45% ACN Flow Rate: 1.0 mL/min
Temperature: RT Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL Injection Volume: 10 mL



- ◆ Compare three current lots of material for consistency of retention (k) and selectivity (α).

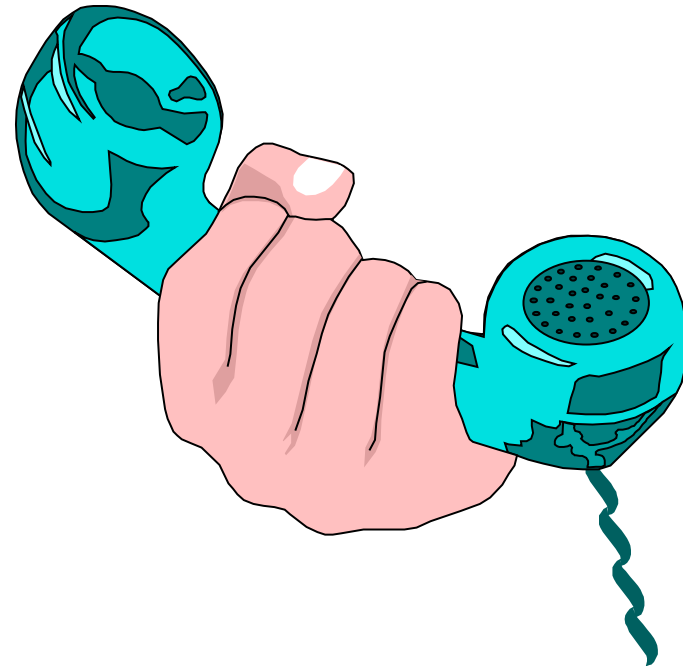
Three Lot Summary

	Mean	SD	RSD
k (D)	1.1	0.01	1.0%
k (B)	2.9	0.06	2.1%
α	2.6	0.05	1.8%



Break Number 2

- For Questions and Answers
- Press *1 on Your Phone to
- Ask a Question



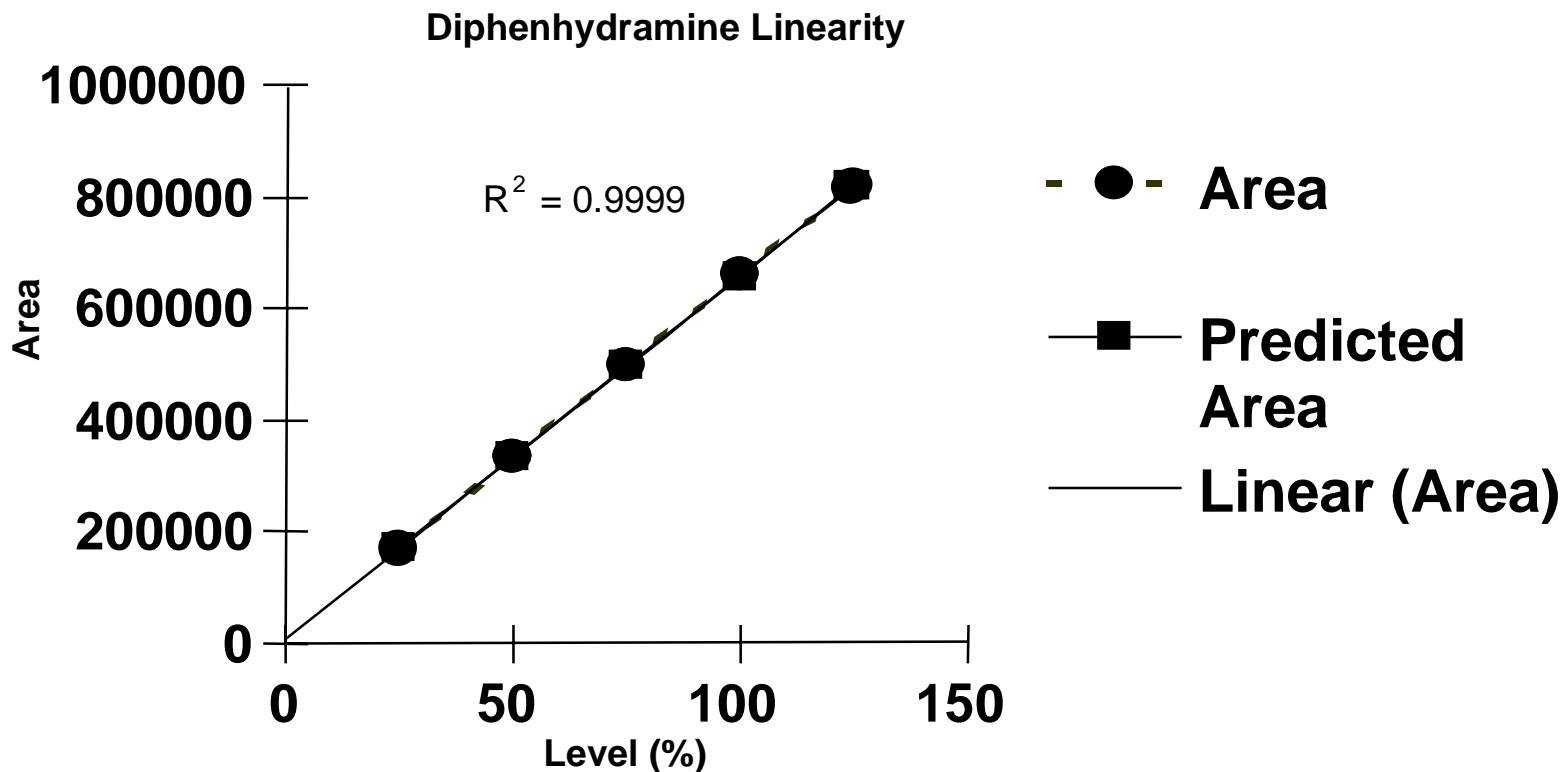
Method Validation Requirements

- ***Robustness***
- **Linearity**
- **Accuracy**
- **Precision**
- **Limit of Detection**
- **Limit of Quantitation**
- **Specificity/Selectivity**
- **Range**
- **Ruggedness**



Linearity

How? Regression analysis of test results vs analyte concentration. For the “bulk substance” type of samples we must cover a range of 80 - 120% of the expected concentration.



Accuracy

How? Calculate % recovery of known amounts added to samples – above and below expected levels. We tested the ICH* recommended 3 replicates at 3 different levels – one above and two below.

Results

Level	Accuracy
125%	99.6 +/- 0.2%
75%	100.3 +/- 0.8%
25%	99.2 +/- 0.7%

* ICH - International Conference on Harmonization



Precision/Repeatability

How? Calculate (relative) standard deviation of a sufficient number of sample aliquots. This can be from three levels three repetitions or 6 determinations at 100%.

Results

Level	SD	RSD
125%	0.16	0.2%
75%	0.76	0.8%
25%	0.67	0.7%



Limit of Detection*

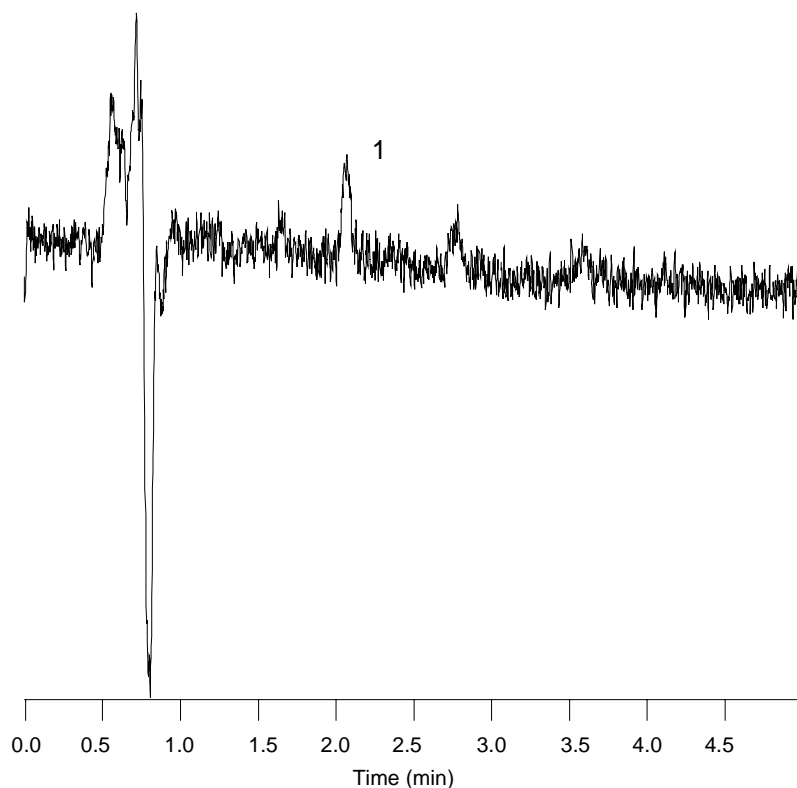
Column: SB-CN, 4.6 x 75 mm, 3.5 μ m

Flow Rate: 1.0 mL/min

Mobile Phase: 55% 25 mM $\text{CH}_3\text{COONH}_4$, pH 4.5 : 45% ACN

Temperature: RT

Sample: 1. Diphenhydramine



How?

Signal-to-noise ratio of 2:1 or 3:1 is generally accepted. Compare to blank. We used a S/N of 3:1.

Result – 0.2 ppm (2 ng on column)

**not required for validation of this method*



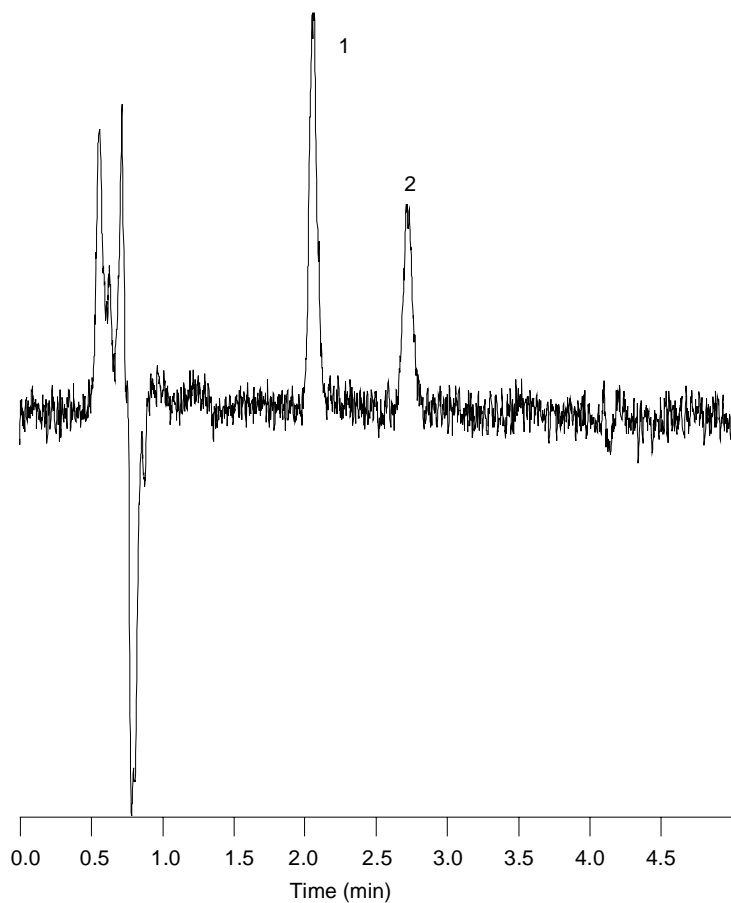
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Limit of Quantitation*



How?

Determine the standard deviation of blank response x10. Verify accuracy and precision with samples close to the calculated limit.

Result – 1.2 ppm (12 ng on column) with precision = 5.9%

**not required for validation of this method*

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m

Mobile Phase: 55% 25 mM $\text{CH}_3\text{COONH}_4$, pH 4.5 : 45% ACN

Flow Rate: 1.0 mL/min

Temperature: RT

Sample: 1. Diphenhydramine 2. Benzophenone



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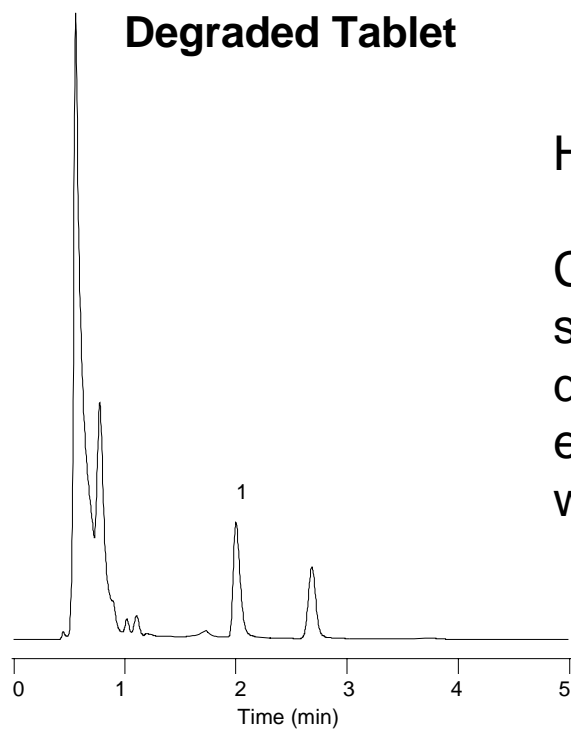
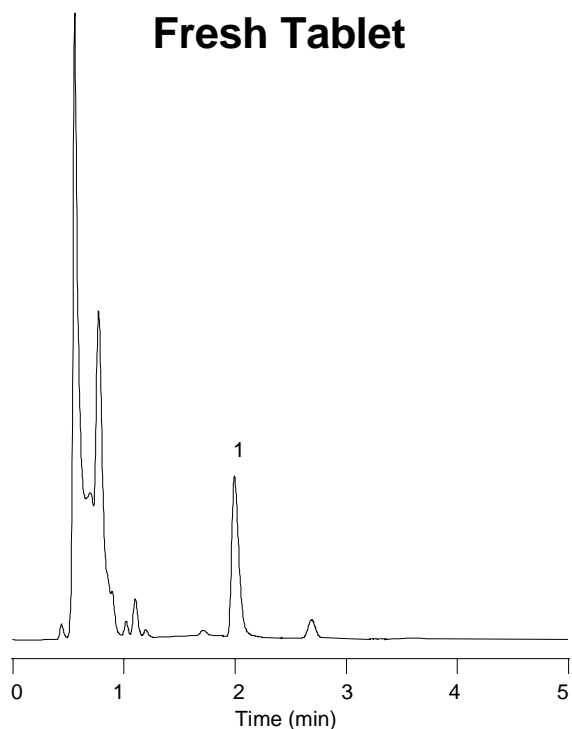
Specificity/Selectivity

Column: SB-CN, 4.6 x 75 mm, 3.5 μ m

Flow Rate: 1.0 mL/min

Mobile Phase: 55% 25 mM $\text{CH}_3\text{COONH}_4$, pH 4.5 : 45% ACN

Temperature: RT Sample: 1. Diphenhydramine



How?

Compare test results from samples with impurities, degradation products, excipients etc. with those without.



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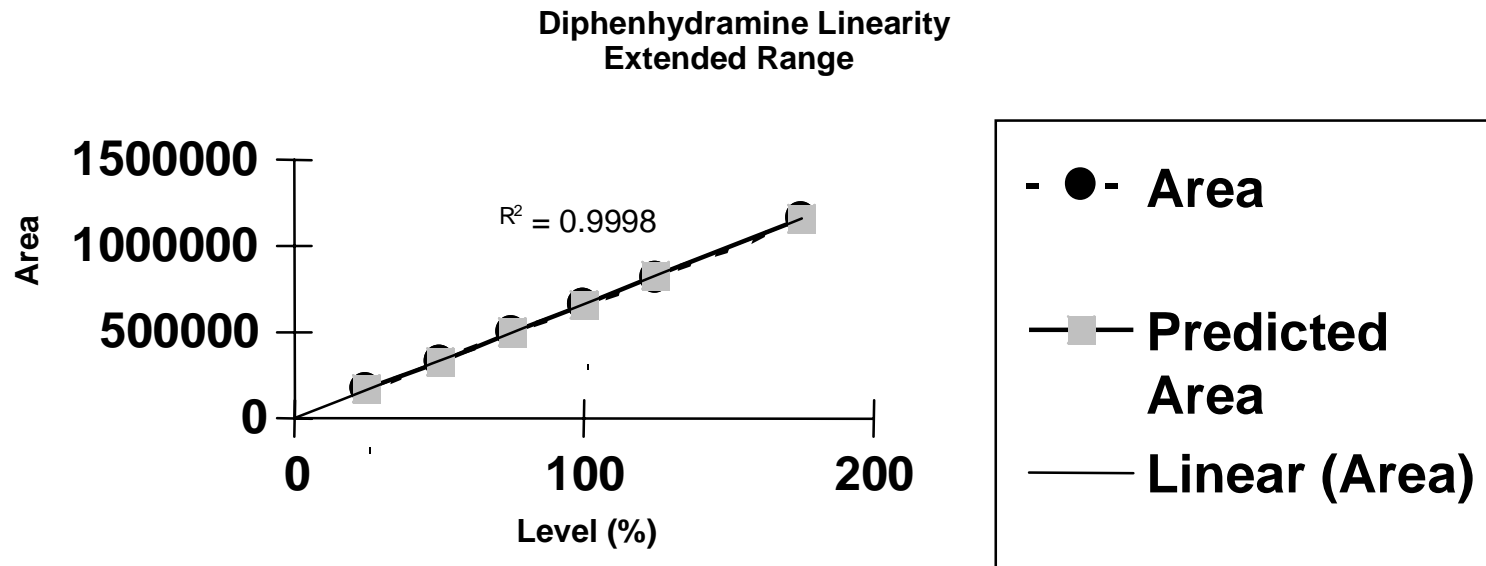
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Range

How? Verify acceptable precision, accuracy, and linearity at the ends of the range and within the range. Our tested range went up to the 175% level. Therefore we needed to verify linearity, accuracy, and precision at this level, in addition to those done previously.



Level	Acc.	SD	RSD
175%	100.6%	0.40	0.4%



Ruggedness/Reproducibility

How? Multiple chemists in multiple labs run samples. Results should be reproducible and can be compared to method precision.

Result – Samples were run in 3 labs by 3 chemists on 3 different instruments.

Level	Chemist 1 Accuracy/RSD	Chemist 2 Accuracy/RSD	Chemist 3 Accuracy/RSD
125%	99.6 +/- 0.2%	100.2 +/- 0.8%	99.0 +/- 0.8%
75%	100.3 +/- 0.8%	100.5 +/- 0.0%	100.5 +/- 0.3%
125%	99.2 +/- 0.7%	100.6 +/- 0.0%	101.0 +/- 0.7%
Overall	99.7 +/- 0.9%	100.4 +/- 0.4%	100.2 +/- 1.0%
Method	100.0 +/- 0.9%		



Determining System Suitability Specifications

- **What type of variation do you see normally and how much leeway do you want?**
- **What makes accurate chromatographic results possible?**
- **Try to account for column degradation and insufficiently tested methods.**



System Suitability

Setting System Suitability Specifications:

Tailing Factor < 2.5 (allows for higher sample load)

Resolution > 2.0 (allows for method variation and column aging)

RSD of replicate injections $< 2.0\%$ (checks system performance)



Conclusions

- **New suggested guidelines may make it easier to determine what is a method “adjustment” to meet system suitability requirements.**
- **When needed method modifications exceed “adjustments” then method validation is required.**
- **Method validation requires experimentation to verify that a method will meet analytical needs.**



Acknowledgments

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Bud Permar**



Wrap-up E-Seminar Questions



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