

A More Exact Theory of Gradient Elution Assuming LSS Conditions for G-T³C Chromatography

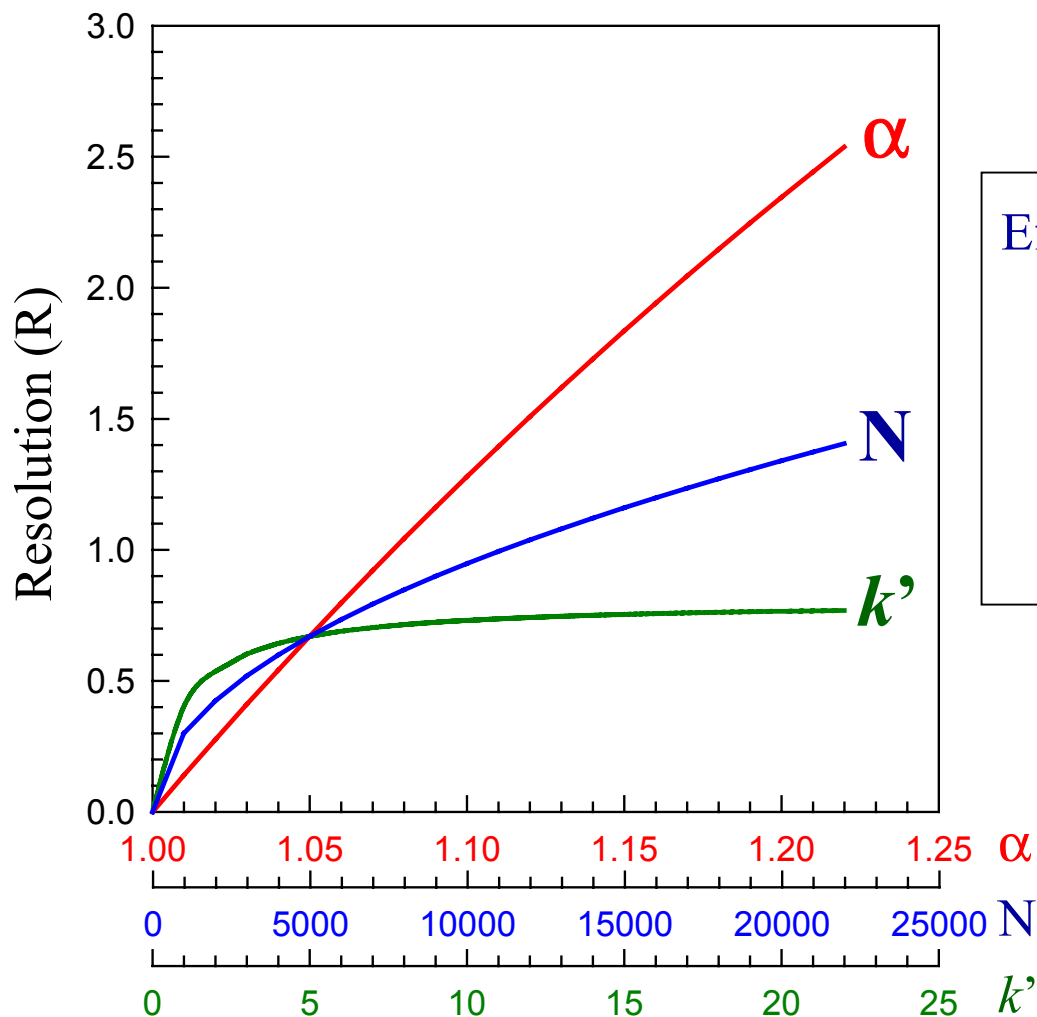
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Abstract

The ultimate goal of reversed-phase method development is optimization of the band spacing (i.e. selectivity). Using the **Thermally Tuned Tandem Column (T³C)** concept allows us to obtain a very large range in tunable selectivity. T³C chromatography works by coupling two columns of drastically different selectivity in series; altering the temperature of each tandem column allows for greatly enhanced selectivity tuning compared to varying mobile phase %B or temperature on a single column. Although isocratic-T³C chromatography allows the separation of many mixtures not adequately resolved on a single column, isocratic-T³C chromatography is a selectivity enhancing method that does not solve the “general elution problem.” Therefore, in this study we investigate how one can combine the techniques of gradient elution and T³C chromatography by appropriately modifying single column gradient elution theory to predict gradient retention time on a tandem column set. This is essential for computerized generation of the “window diagram” needed to optimize the two column temperatures in T³C chromatography. Future work will focus on applying the gradient-T³C technique to obtain enhanced resolution in a shorter time compared to temperature optimized gradient elution separation of complex solute mixtures on a single column.

Resolution: The Goal of Chromatography



Efficiency	Selectivity	Retention
↓	↓	↙
$R = \frac{\sqrt{N}}{4}$	$\frac{\alpha-1}{\alpha}$	$\frac{k'}{k'+1}$

Typical Values

$$\alpha = 1.05$$

$$N = 5,000$$

$$k' = 5$$

Changes in selectivity (α) have the greatest impact on resolution

Optimizing Selectivity: Practical Considerations

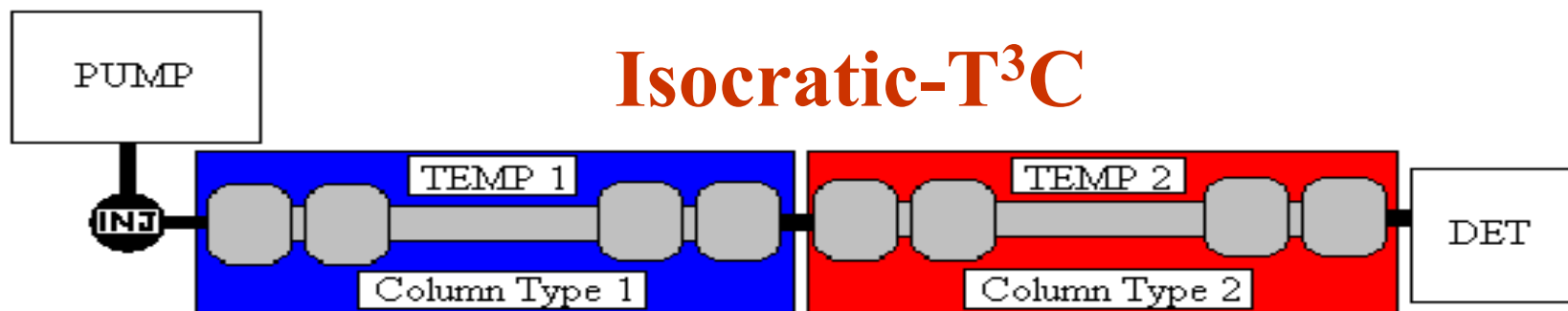
	Stationary Phase	Organic Modifier	ϕ	T
Change in α available	Largest	Modest	Low	Lowest
Magnitude of k' change	Modest	Modest	Large	Modest
Eqns. To relate k' to variable	No	No	Yes	Yes
Number of training runs	(no eqns.)	(no eqns.)	2 to 3	2 to 3
Automated Experimentation	Yes	Yes	Yes	Yes
Ease of Automation	Difficult	Moderate	Easy	Easy
Continuous Variation	No	No (ternary)	Yes	Yes

Conclusion:

Changing the stationary phase and / or organic modifier type is **NOT convenient** for single column method development!

Isocratic Thermally Tuned Tandem Column (T³C) Chromatography

Isocratic-T³C

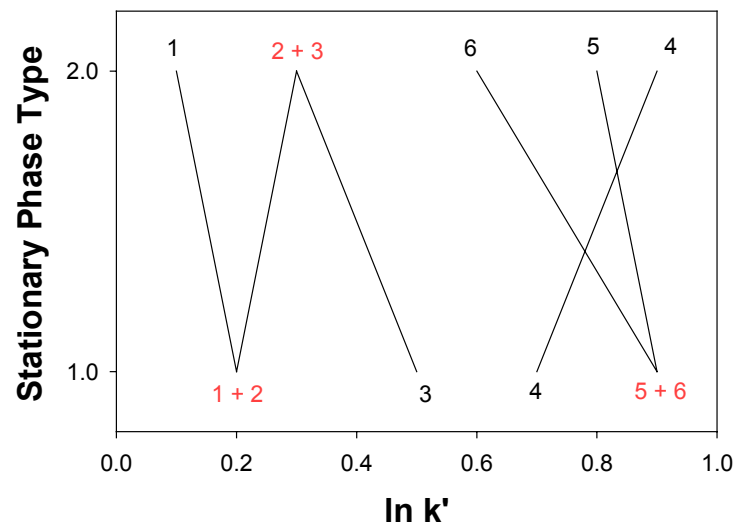


Requirements:

1. $\alpha_1 \neq \alpha_2$
2. $T_1 < T_2$
3. Choose eluent composition

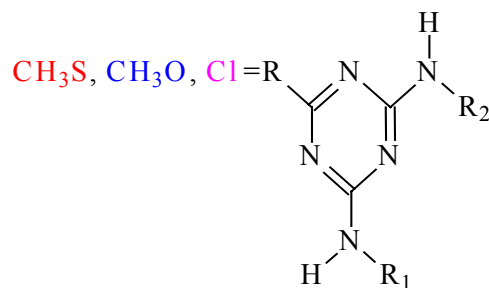
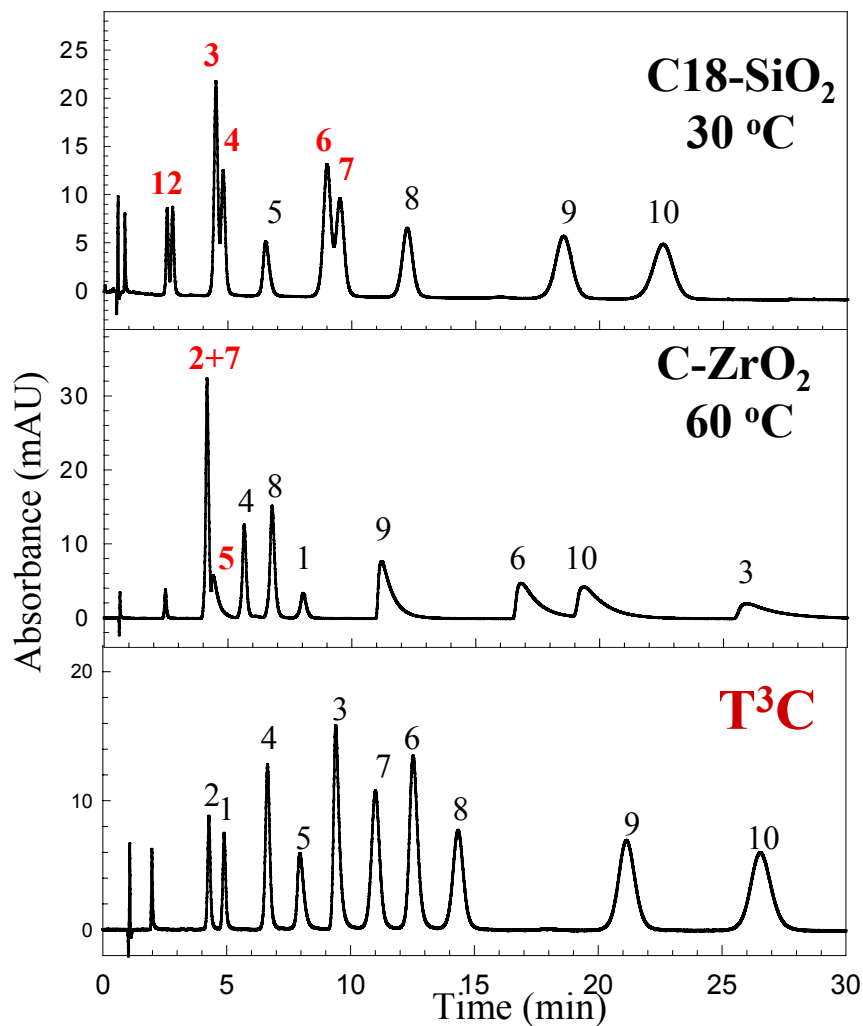
Result:

1. *Selectivity is varied continuously*
2. *Altering column temperature is easy*
3. T adjusts the contribution of α_n to α_{total}



A large range in tunable selectivity is available!

Example of Isocratic-T³C Chromatography



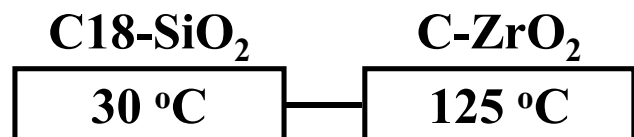
Solutes:

- | | |
|--------------|------------------|
| 1. Simazine | 6. Ametryn |
| 2. Cyanazine | 7. Propazine |
| 3. Simefryn | 8. Terbutylazine |
| 4. Atrazine | 9. Prometryn |
| 5. Prometon | 10. Terbutryn |

Other conditions:

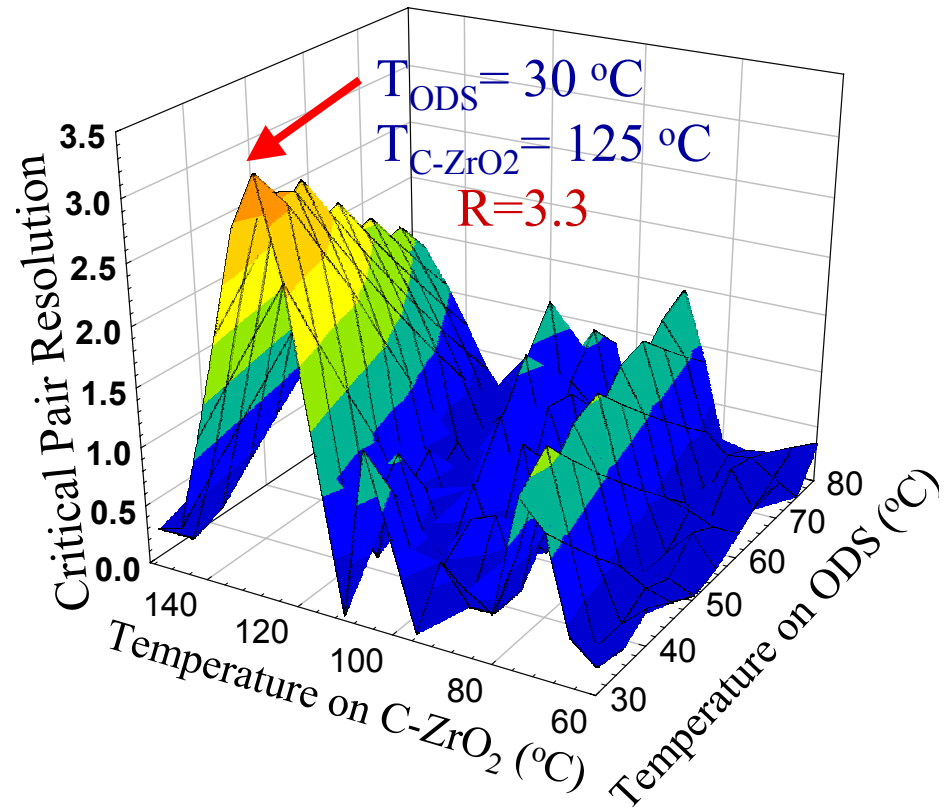
30/70 ACN/water

1ml/min; 254 nm detection



Isocratic-T³C Window Diagram Optimization: Triazine Herbicides

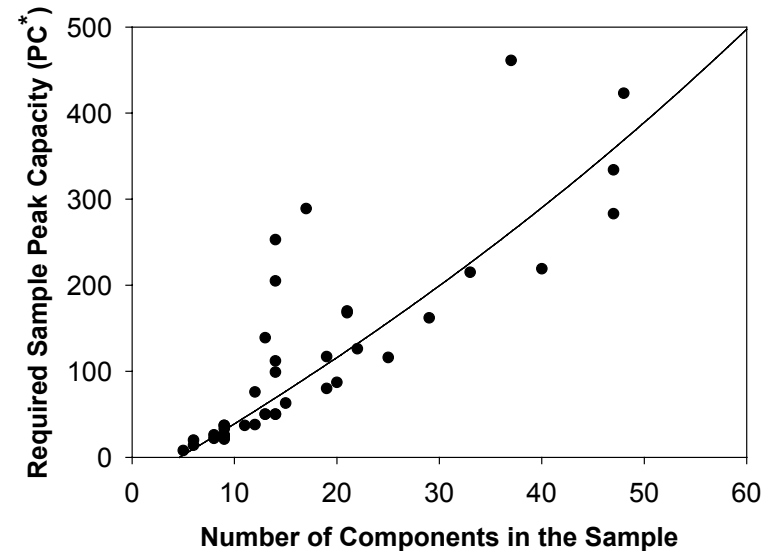
- *Four training runs*
 - Two T on each column
- Assume $\ln k'$ vs. $1/T$ is linear (*van't Hoff plot*)
- Calculate $t_{R,a}$
$$t_{R,a} = t_{R,1a} + t_{R,2a}$$
- Calculate critical pair resolution



Why Do Gradient-T³C Chromatography?

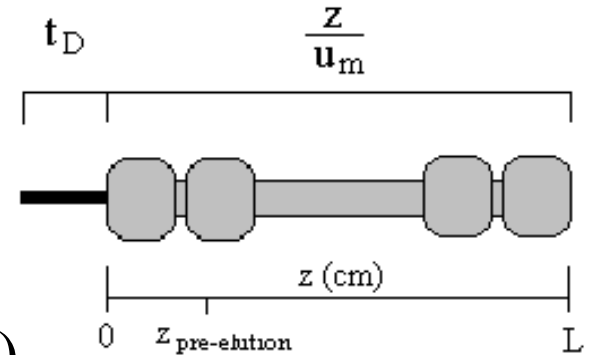
- Isocratic elution does not solve the “general elution problem”
 - Sample containing early and late eluting components
 - Increase ϕ : resolution of early eluting components decreases
 - Decrease ϕ : analysis time of late eluting components increases
- Gradient elution solves the “general elution problem”
 - Begin with a low ϕ and end with a high ϕ
 - Peak capacity is increased
- T³C chromatography increases $R_{critical}$

$$PC^* = \frac{(t_{R,last} - t_{R,first})}{W \cdot R_{critical}}$$



Gradient Elution Terminology

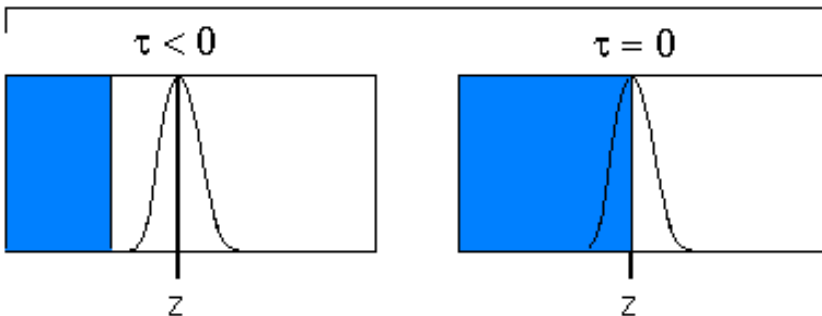
- Gradient delay time: t_D (min.)
- Column dead time: t_o (min.)
- Column length: L (cm)
- Mobile phase linear velocity: u_m (cm / min.)
- Distance within the column: z (cm)
- Time after the injection: t (min.)
- τ is the “transition time”



$$u_m = \frac{L}{t_o}$$

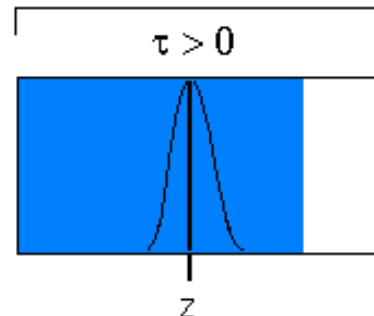
Isocratic Elution

$$(\phi = \phi_o)$$



Gradient Elution

$$(\phi = \phi_o + \frac{\Delta\phi * \tau}{t_G})$$



$$\tau = t - \frac{z}{u_m} - t_D$$

Two Cases of Solute Elution on a Single Column

Solute elutes isocratically ($\tau \leq 0$)



$$t_R = t_o \cdot (1 + k'_o)$$

Gradient “overtakes” solute ($0 < \tau < t_G$)



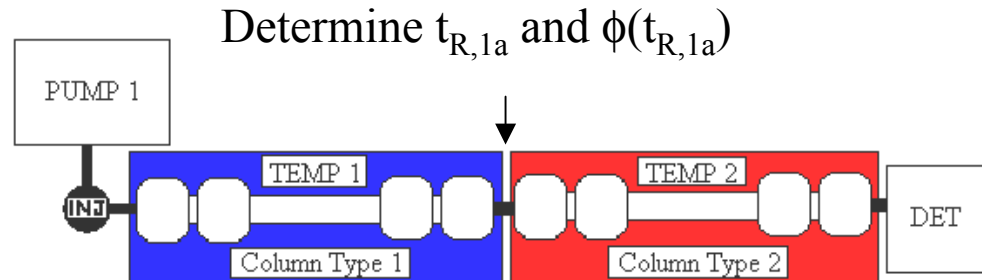
$$\frac{t_R}{t_o} = \frac{\ln\left(b \cdot \left(k'_o - \frac{t_D}{t_o}\right) + 1\right)}{b} + 1 + \frac{t_D}{t_o}$$

Considerations:

$$b = \frac{S \cdot t_o \cdot \Delta\phi}{t_G} \quad S = -\frac{\delta \ln k'}{\delta\phi}$$

- The mathematical derivation is correct
- Prediction of t_R under extreme gradient conditions is accurate
- Verified closed-form equations with numerical integration

Three Cases of Solute Elution on a Tandem Column Set



Case 1. Isocratic elution from the tandem column set

$$t_{R,a} = t_{o,1} \cdot (1 + k'_{o,1a}) + t_{o,2} \cdot (1 + k'_{o,2a})$$

Case 2. Gradient “catches” the solute on the downstream column

$$t_{R,a} = \ln \left[b_{2a} \cdot \left(k'_{o,2a} + \frac{t_{R,1a} - t_{o,1} - t_D}{t_{o,2}} \right) + 1 \right] \cdot \frac{t_{o,2}}{b_{2a}} + t_{o,1} + t_{o,2} + t_D$$

Case 3. Gradient “catches” the solute on the upstream column

$$t_{R,a} = \ln \left[k'_{o,2a} \cdot \left(b_{2a} + \exp \left(\frac{b_{2a}}{t_{o,2}} \cdot (t_{R,1a} - t_{o,1} - t_D) - \ln(k'_{o,2a}) \right) \right) \right] \cdot \frac{t_{o,2}}{b_{2a}} + t_{o,1} + t_{o,2} + t_D$$

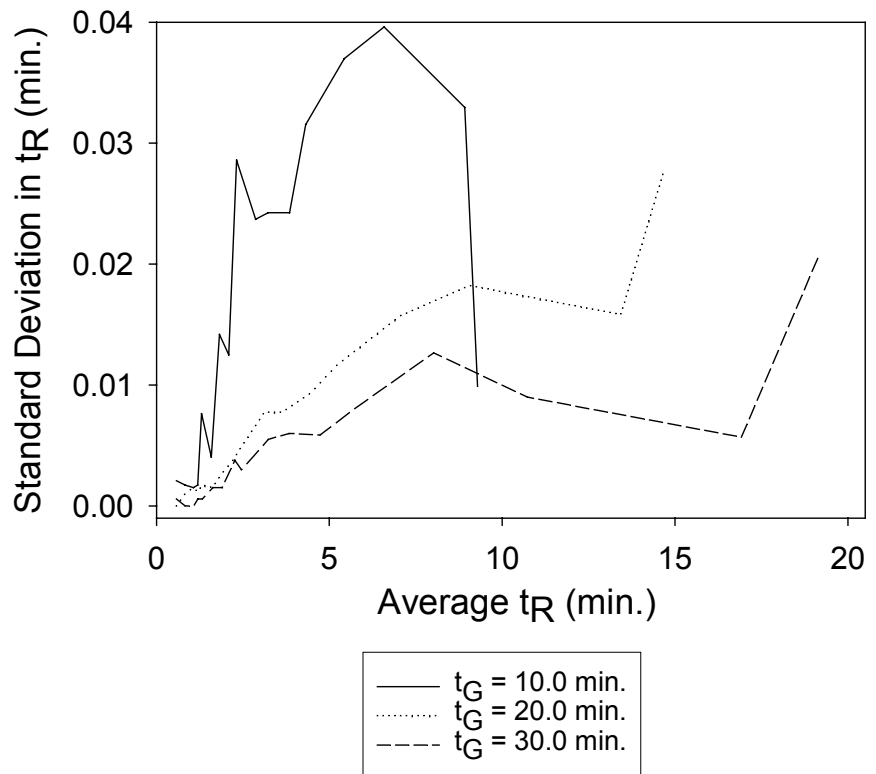
Accuracy of Tandem Column Set Gradient Theory

- Difference in predicted (closed-form) gradient t_R decreases as the time interval in numerical integration is decreased
- Average difference in predicted gradient t_R and experimental t_R is $0.196_{\pm 0.081}$ min.

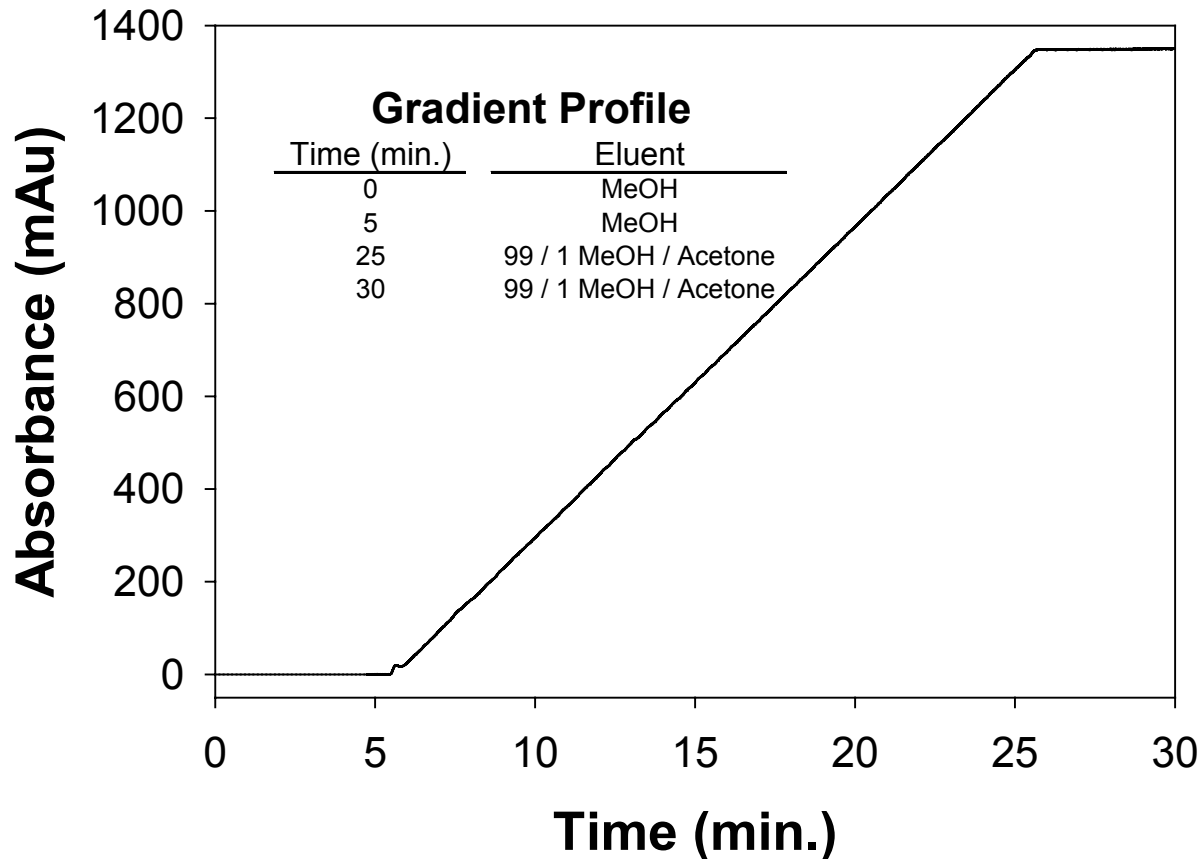
The **assumptions** made to predict gradient retention time on a tandem column set **are NOT ACCURATE!**

Solutes: Eight alkylphenones and 5 alkylbenzenes

Conditions: 1 mL / min.; $\phi = 0.50$ to 1.0 (ACN / H₂O); ODS and C-ZrO₂ columns at 60 °C; $t_D = 0.618$ min.; 254 nm detection.



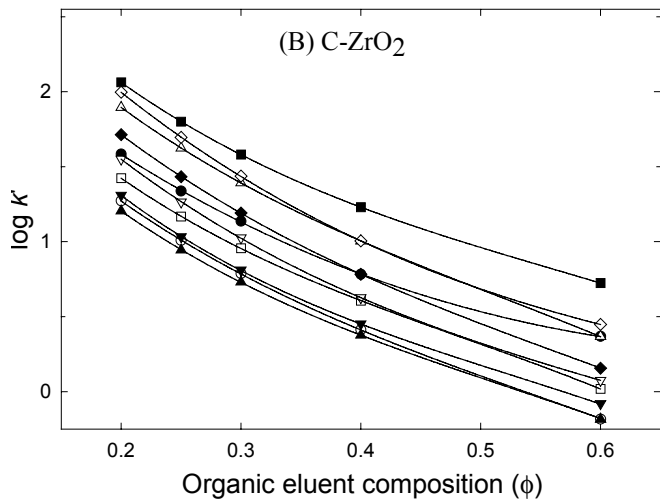
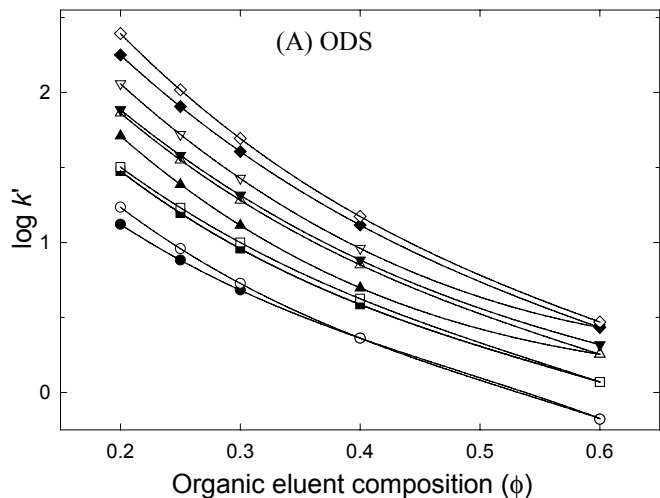
Generation of a Linear Gradient Profile



An undistorted linear gradient profile is obtainable **assuming no sorption of eluent** by the stationary phase.

Conditions: 1 mL / min. flow rate, 254 nm detection, no column

Accuracy of LSST on ODS and C-ZrO₂ Columns



LSST:

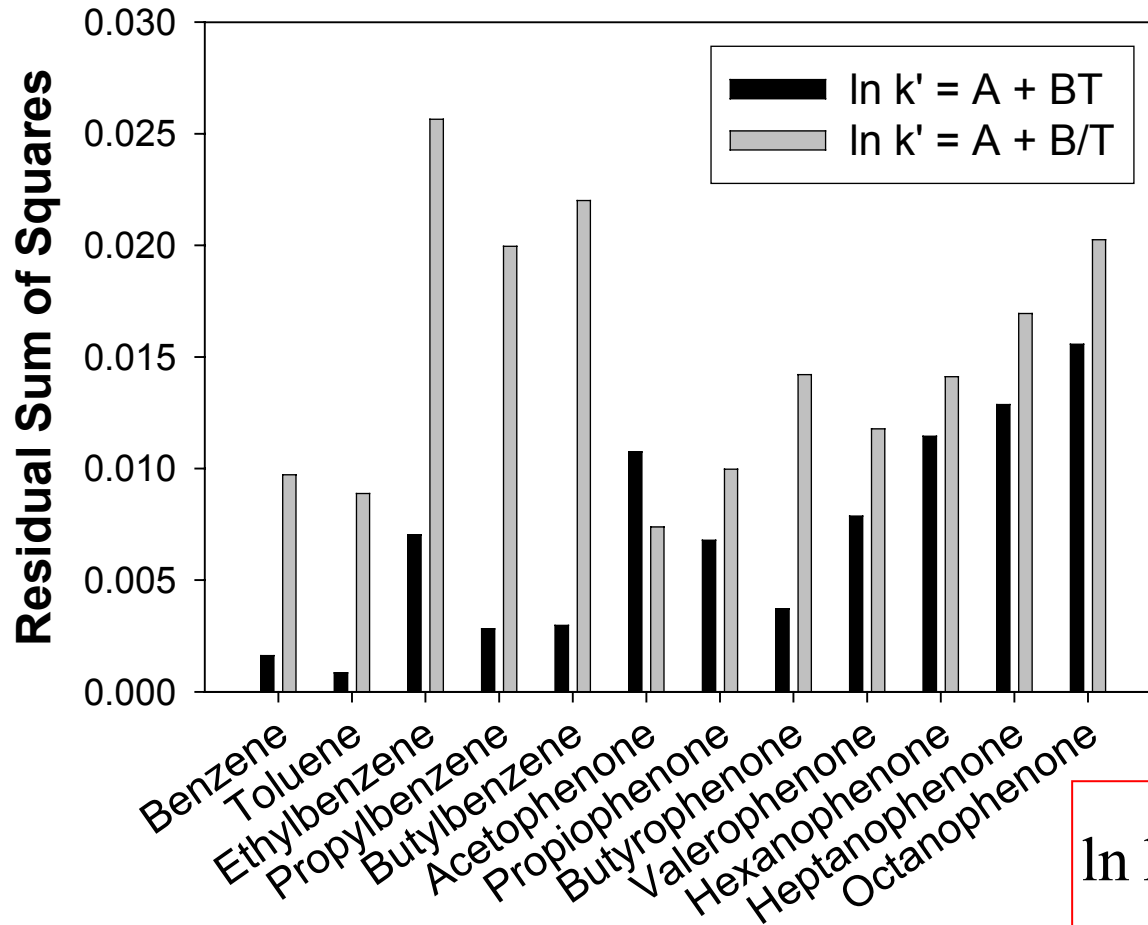
$$\ln k' = \ln k'_w - S \cdot \phi$$

Pade Approximation:

$$\ln k' = A + \frac{B \cdot \phi}{1 + C \cdot \phi}$$

LSST is NOT ACCURATE over a wide range in ϕ

Accuracy of the van't Hoff Plot

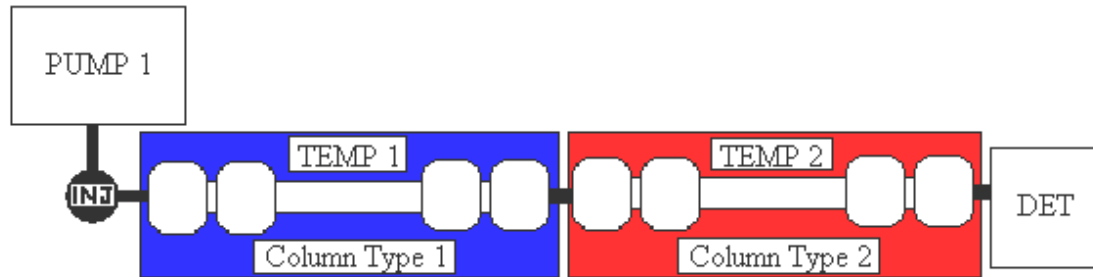


$$\ln k' = -\frac{\Delta G}{R} \cdot \frac{1}{T} + \ln \Phi$$

A linear fit of $\ln k'$ vs. T is acceptable for $\Delta T = 130$ °C

Conditions: C-ZrO₂ column; $\Delta T = 130$ °C; 40 / 60 ACN / H₂O; 1 mL / min. flow rate; 254 nm detection; 1 μ L injection

Gradient Elution T³C Theory



$$\log k' = \log k'_w - S\phi \longleftrightarrow \log k' = A + \frac{B}{T}$$

$$\log k' = \left[A + \frac{B}{T} \right] + \phi \cdot \left[C + \frac{D}{T} \right]$$

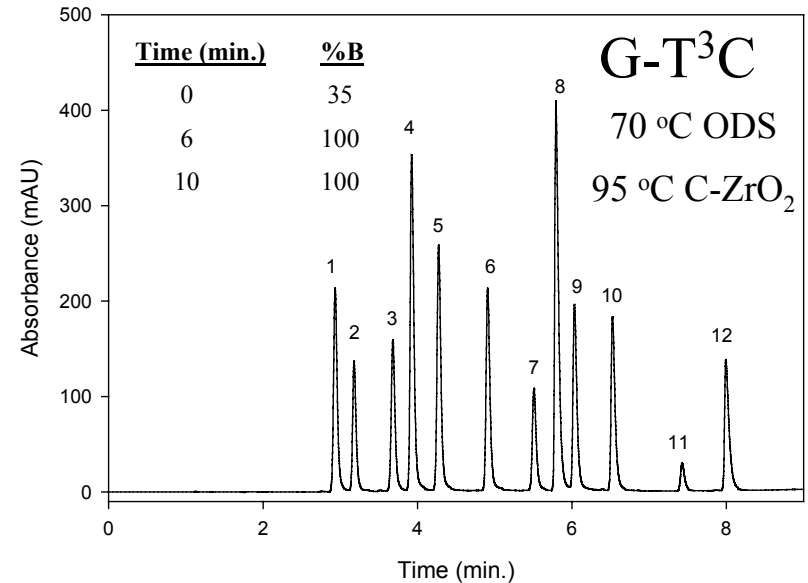
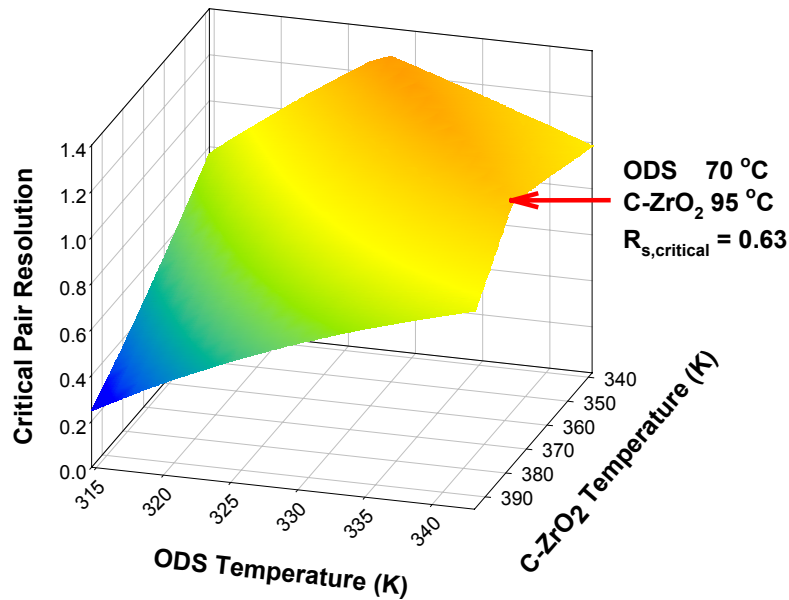
Eight training runs required to predict gradient-T³C retention time

#1	ϕ_1	ϕ_2
T ₁		
T ₂		

#2	ϕ_1	ϕ_2
T ₁		
T ₂		

Locate the optimum conditions with **window diagram optimization**

An Example of G-T³C Optimization



Conditions

Eluent: (A) H₂O
(B) ACN

Flow rate: 1 mL/min.

Detection: DAD, 254 nm

Injection: 1 μL

Training Runs: $\phi = 0.50, 0.90$

$T_{ODS} = 40, 70$ °C

$T_{C-ZrO_2} = 60, 125$ °C

Solutes

1. Acetophenone
2. Dimethyl phthalate
3. Propiophenone
4. Diethyl phthalate
5. Butyrophenone
6. Valerophenone
7. Hexanophenone
8. Dibutyl phthalate
9. Heptanophenone
10. Octanophenone
11. Decanophenone
12. Dioctyl phthalate

Actual Critical Pair: 8,9 (R = 1.01)

Predicted Critical Pair: 7,8 (R = 0.63)

Gradient distortions and the non-linearity of $\ln k'$ vs. ϕ and T lead to **errors in the window diagram!**

Conclusions

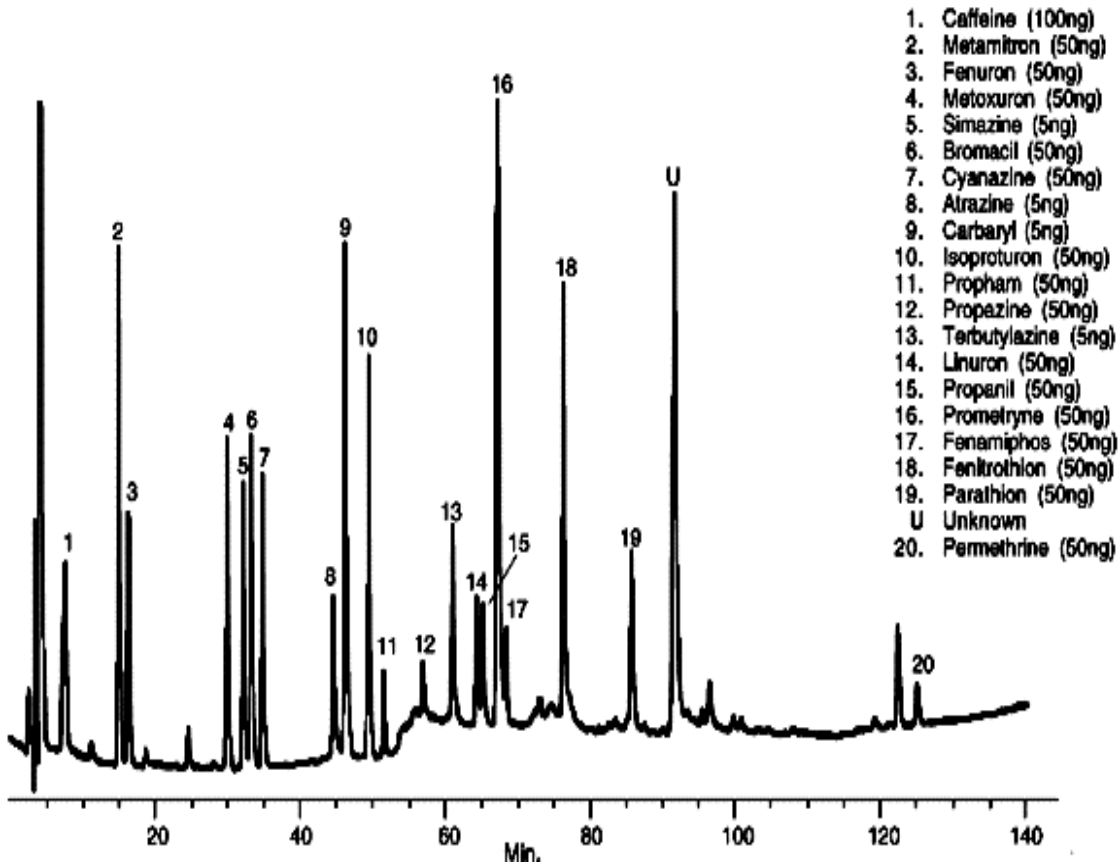
- **Accurate closed form equations** exist for optimizing gradient elution-T³C separations
- **Numerical integration** can be used to accurately compute gradient-T³C retention time when . . .
 - Organic sorption by the MP is significant
 - A step-gradient is required
 - LSST is not adequate
 - Linear fit of $\ln k'$ vs. T is not adequate
- **Gradient elution-T³C chromatography** will . . .
 - Solve the “general elution problem”
 - Provide a larger range in tunable selectivity than a single column

Future G-T³C Work

- Investigate **eluent sorption** by the stationary phase
 - Predict G-T³C t_R while accounting for non-linearity in $\ln k'$ vs. ϕ and / or T
 - Use a refractive index detector to observe the gradient profile
- **Gradient training runs** to obtain LSST parameters
 - Advantages
 - Shorter analysis time than isocratic runs
 - Non-linear fit of $\ln k'$ vs. ϕ possible
 - Limitation
 - Error propagation
- **Computer program** to predict gradient retention time on a tandem column set for numerous gradient profiles and relationships between $\ln k'$ and ϕ and T

Future G-T³C Work

- Separate pesticides and herbicides



Conditions

Column: SUPELCOSIL LC-ABZ

25 cm x 4.6 mm ID

5 μ m particles

Gradient: 10% to 90% ACN in H₂O
0.5 % / min.

Flow: 1 mL / min.

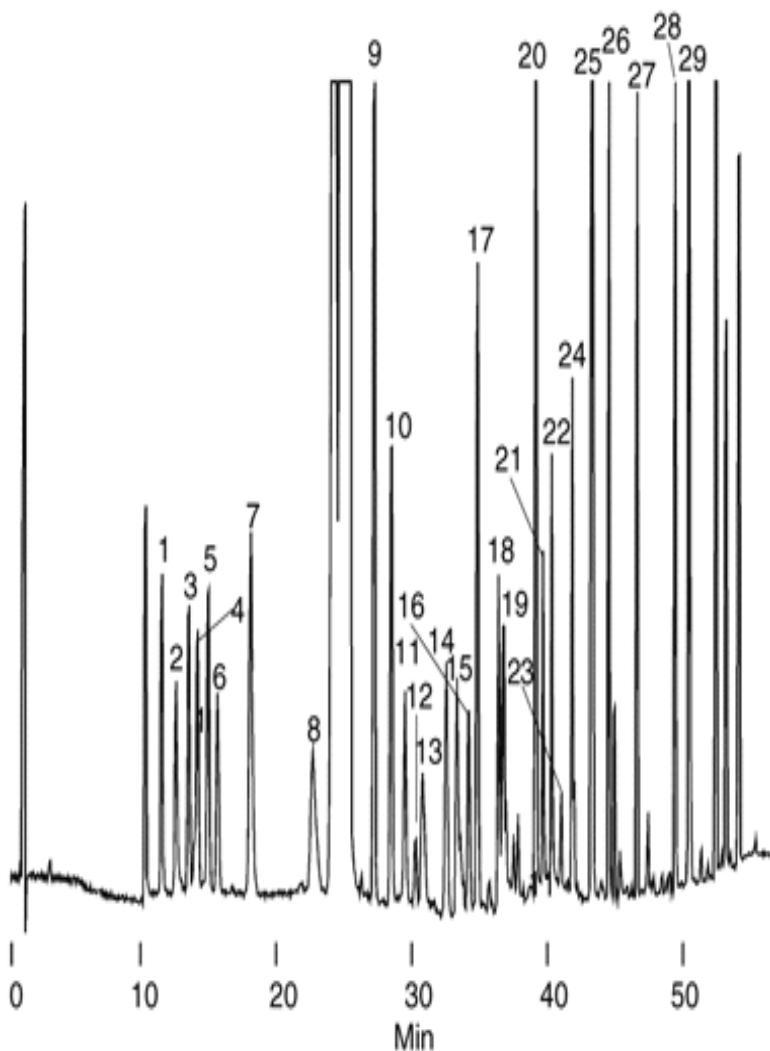
Temp.: 40 °C

Det.: UV, 225 nm

Injection: 50 μ L water

Future G-T³C Work

- Separate dabysl-modified amino acids



Conditions

Column: SUPELCOSIL LC-DABS, 15cm x 4.6mm ID, 3 μ m particles
 Eluent: A = 25mM KH₂PO₄, pH = 7.0

B = 70 / 30 ACN / MeOH

Flow Rate: 1.5 mL / min.

Detection: VIS, 436 nm

Injection: 5 μ L, approximately 50 pM each derivative

Gradient Profile

Time (min.)	% B
0	20
9	25
23	25
24	32
30	32
37	40
46	60
54	75
59	75
60	20
94	20

1. o-Phosphoserine	2. o-Phosphothreonine
3. Aspartic acid	4. o-Phosphotyrosine
5. Cysteic acid	6. Glutamic acid
7. S-Carboxymethylcysteine	8. S-Sulfocysteine
9. Asparagine	10. Glutamine
11. Serine	12. Methionine sulfoxide
13. Threonine	14. Glycine
15. Alanine	16. Arginine
17. Methionine sulfone	18. Proline
19. Valine	20. Methionine
21. Isoleucine	22. Leucine
23. Tryptophan	24. Phenylalanine
25. Ammonia	26. Cystine
27. Lysine	28. Histidine
29. Tyrosine	

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Solute elutes from a single column when $\tau > t_G$

$$\frac{t_R}{t_o} = 1 + \frac{t_D}{t_o} + k'_f + \frac{k'_f}{b \cdot k'_o} - \frac{t_D \cdot k'_f}{t_o \cdot k'_o} + \frac{t_G}{t_o} - \exp\left(\frac{b}{t_o} \cdot t_G\right) \cdot \frac{k'_f}{b \cdot k'_o}$$

Solute elutes from a tandem column set when $\tau_2 > t_G$ and gradient catches solutes on the downstream column

$$t_R = t_{o,1} + t_{o,2} + t_D + t_G + k'_{f,2} \cdot \left[t_{o,1} + t_{o,2} - \frac{\tau_1 + t_D}{k'_{o,1}} - \frac{\tau_1}{k'_{o,2}} \right] - \exp\left(\frac{b_2}{t_{o,2}} \cdot t_G\right) \cdot \frac{k'_{f,2} \cdot t_{o,2}}{b_2 \cdot k'_{o,2}}$$

Solute elutes from a tandem column set when $\tau_2 > t_G$ and gradient catches solutes on the downstream column

$$t_R = t_{o,1} + t_{o,2} + t_D + t_G + k'_{f,2} \cdot \left[t_{o,1} + t_{o,2} - \frac{t_{o,2}}{b_2} + \frac{t_D}{k'_{o,1}} - \frac{t_{o,1}}{b_1 \cdot k'_{o,1}} \right] - \exp\left(\frac{b_1}{t_{o,1}} \cdot \tau_1\right) \cdot \frac{t_{o,1} \cdot k'_{f,2}}{b_1 \cdot k'_{o,1}} - \exp\left(\frac{b_2}{t_{o,2}} \cdot t_G\right) \cdot \frac{t_{o,2} \cdot k'_{f,2}}{b_2 \cdot k'_{o,2}} + \exp\left(\frac{b_2}{t_{o,2}} \cdot \tau_1\right) \cdot \frac{t_{o,2} \cdot k'_{f,2}}{b_2 \cdot k'_{o,2}}$$