High Speed Two-Dimensional Liquid Chromatography Through the Use of Ultra-Fast High Temperature Liquid Chromatography as the Second Separation Dimension

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Abstract

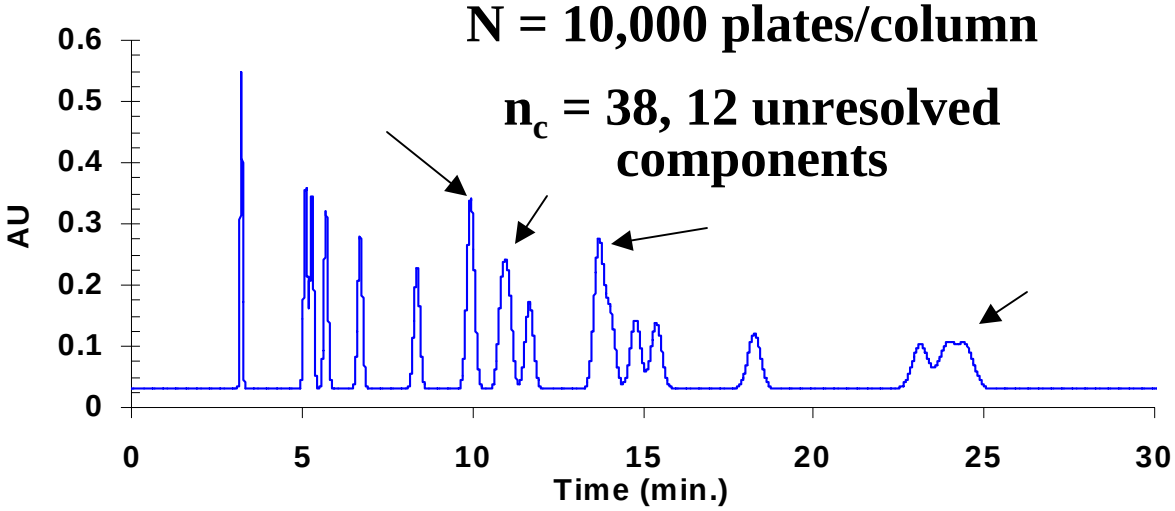
A fundamental limitation of High Performance Liquid Chromatography (HPLC) is its relatively **low peak capacity** compared to other techniques. By far the most powerful approach to improving peak capacity is two-dimensional separations (2DLC), where the ideal peak capacity of the two-dimensional separation is the product of the individual peak capacities of the first and second dimension separations. While **comprehensive 2DLC** separations were reported almost a decade ago, the demands placed on the speed of the second dimension separation of these systems make the technique very slow, **typically about one half-day per full 2D chromatogram**. A solution to this extreme impediment to the widespread use of 2DLC is to apply **Ultra-Fast High-Temperature Liquid Chromatography** (UFHTLC) to the second dimension separation to allow fast and comprehensive sampling of the first dimension separation, thereby allowing **full 2DLC analyses at ten-thirty minutes per analysis**. The concomitant decrease in eluent viscosity and increase in analyte diffusivity under UFHTLC conditions allows very efficient separations to be performed at extremely high column linear velocities in the second dimension separation.

In this work we will show one-dimensional LC separations on the ten-second timescale; these are enabled by using UFHTLC conditions. We will then show how these ultra-fast separations can be used in the second dimension of a fast, comprehensive 2DLC system, which we call LC × UFHTLC. This fast 2DLC system can be used to greatly improve the resolution of components in complex samples, which are difficult to resolve using conventional HPLC.

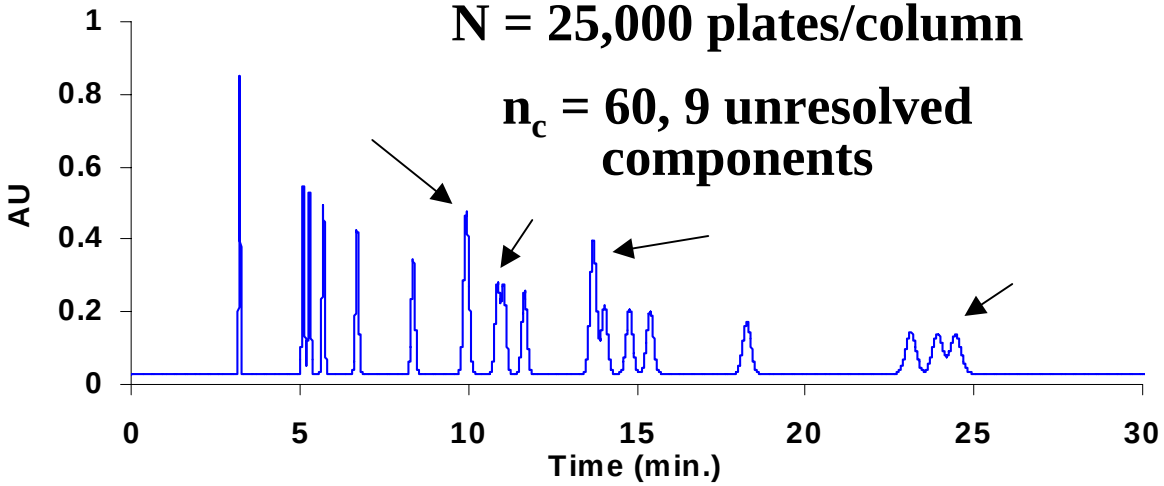
A Common Problem in HPLC

Sample composed of 20 components with randomly distributed k' values

150 mm x 4.6 mm i.d. column



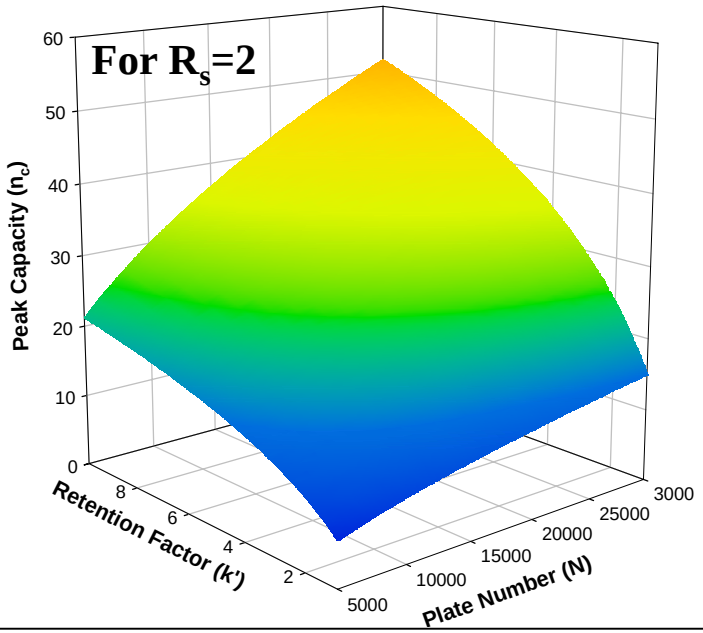
Even with state-of-the-art HPLC, only 50% of the components in this sample can be resolved !!!



Peak Capacity Limitations of One-Dimensional HPLC

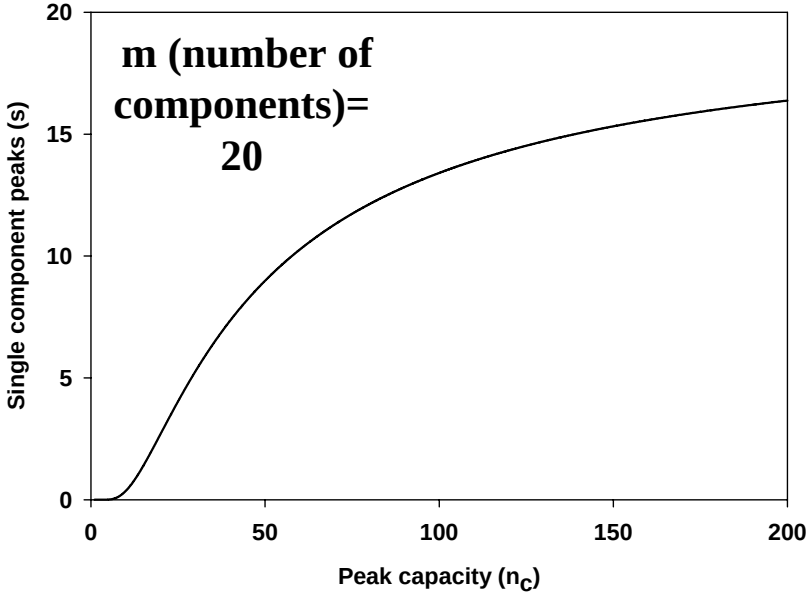
#1a - Low peak capacity (n_c)

$$n_c = 1 + \frac{\sqrt{N}}{4R_s}\ln(k'_n + 1)$$



#1b – The number of components observable as ***single*** peaks is even lower

$$s = me^{-2m/n_c}$$



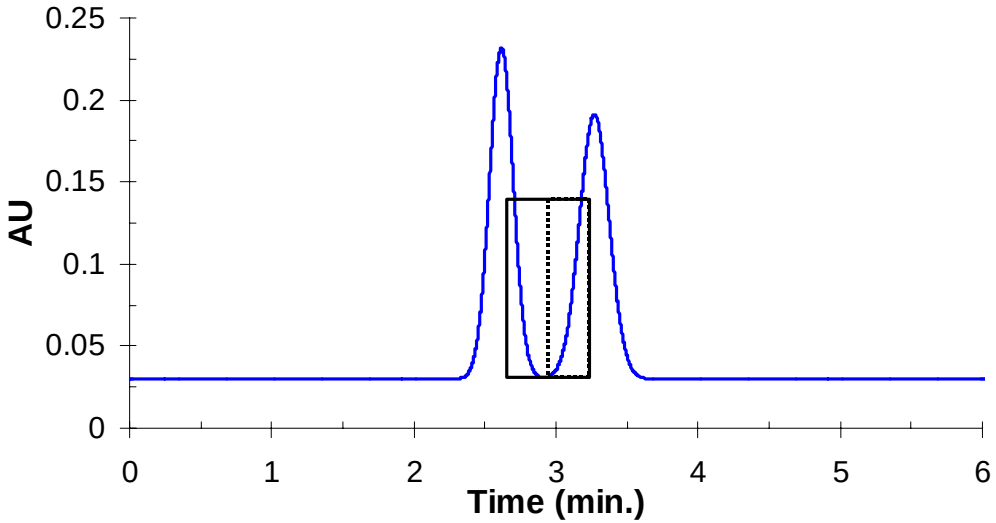
Comprehensive two-dimensional HPLC is the most efficient way to greatly increase the peak capacity of HPLC

Giddings, J. C. *Multidimensional Chromatography: Techniques and Applications*; Marcel Dekker: New York, 1990

Requirements and Advantages in Conventional Two-Dimensional HPLC

Two conditions must be met for the technique to be considered “two-dimensional”

1. Orthogonality of separation mechanisms – This is a requirement imposed primarily on the stationary phase chemistry
2. Separation gained in one dimension must not be diminished by separation in the other



$$t_{rtotal} = \frac{\left(k'_{\max 1}+1\right)\left(\sqrt{N_1}\left[L_{c2}\left(k'_{\max 2}+1\right)\right]\right)}{\upsilon_2}$$

If these two conditions are satisfied, the maximum total peak capacity of the two-dimensional system will be:

$$n_{cTotal} = n_{c1} \times n_{c2}$$

Murphy, R. E.; M. R. Schure; J. P. Foley *Anal. Chem.*, 1998; Vol. 70, pp 1585-1594

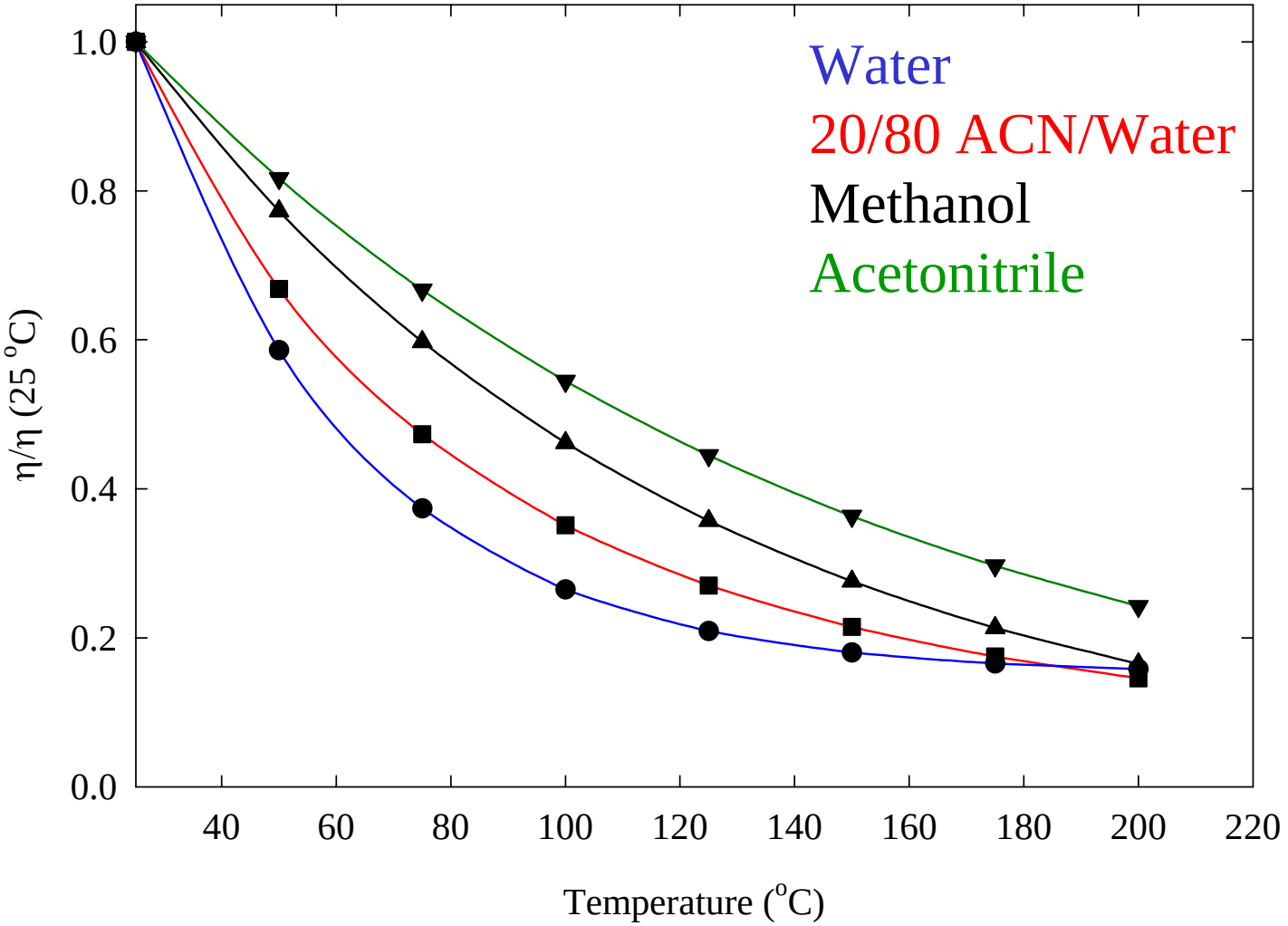
Giddings, J. C. *Multidimensional Chromatography: Techniques and Applications*; Marcel Dekker: New York, 1990

Comparison of Peak Capacity Production

Technique	Peak Capacity Limit (n_c)	Analysis Time (hr)	Peak Capacity Production (n_c/hr)
Capillary GC	10^3	10^0-10^1	10^2
GC x GC	10^4-10^5	10^1	10^3-10^4
HPLC	10^2-10^3	10^0-10^1	10^1-10^2
LC x LC	10^3-10^4	**10^1-10^2**	10^2
LC x UFHTLC	10^3-10^4	**10^0-10^1 ??**	**10^3 ??**
2D-Gel Electrophoresis	10^3-10^4	10^2	10^1-10^2

Ultra Fast High Temperature LC has the potential to significantly improve the rate of peak capacity production in HPLC

Improving the Speed of 2DLC Through the Use of UFHTLC – Effect of Temperature on Eluent Viscosity



Improving the Speed of 2DLC Through the Use of UFHTLC – Implications of Decreasing Viscosity

1. $$\Delta P = \frac{uL\eta\varphi}{d_p^{\,2}} \qquad u\eta = \frac{\Delta P d_p^{\,2}}{L\varphi}$$

As the viscosity decreases, the linear velocity through the column is allowed to increase when the pressure is held constant

2. $$D_{A,m} \propto \frac{1}{\eta}$$

As the viscosity decreases, the diffusivity of the analyte in the eluent increases, thereby relaxing resistance to mass transfer

Requirements for Fast 2DLC

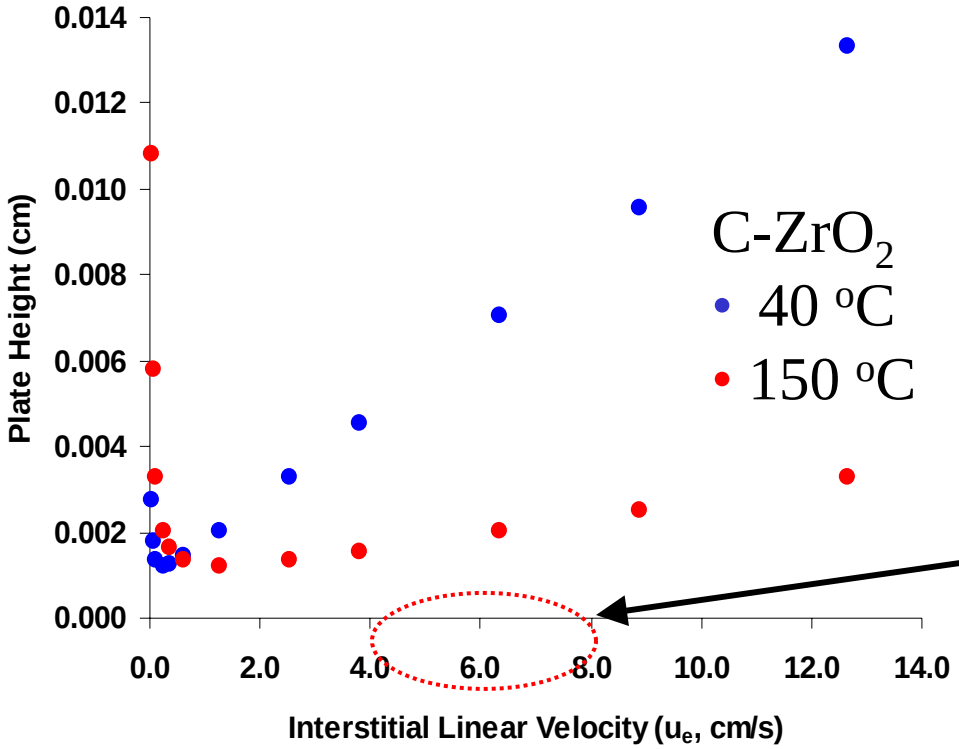
Improving the speed of 2DLC is not possible without columns that have ALL of the following three characteristics for use in the second dimension separation:

1. The stationary phase used must be **thermally and chemically stable** under the conditions of UFHTLC
2. The columns (narrow-bore or smaller i.d.) packed with the stable stationary phase must be **thermo-mechanically** stable
3. The stationary phase must provide **selectivities that are orthogonal** to existing phases for the analytes of interest

Also, the column and eluent must be heated properly to **avoid "thermal mismatch" band broadening**

Effect of Temperature on the Shape of the van Deemter Curve

$$H = A + \frac{B}{u_e} + Cu_e$$

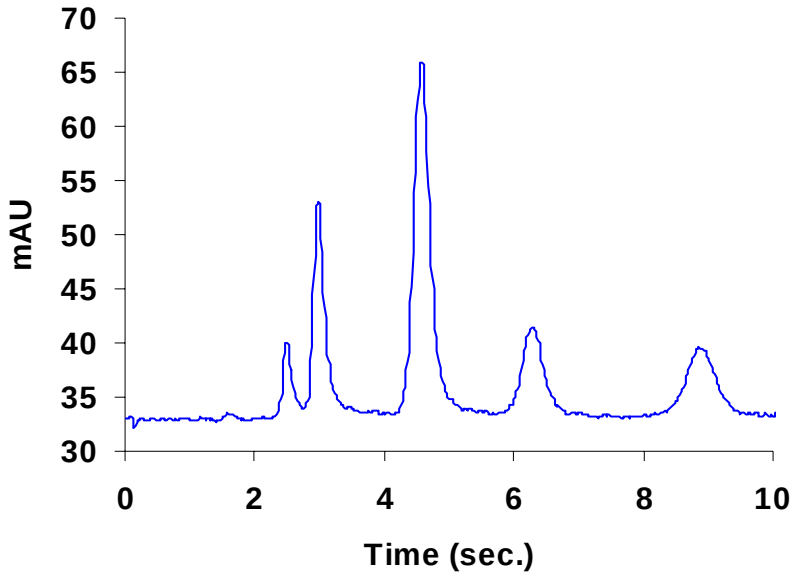


For small molecules there is roughly a 4-fold lowering[1] of the van Deemter C-term as the temperature is raised from 40 to 150 °C

This allows practically useful column efficiencies to be obtained at extremely high column linear velocities

(1) Yan, B.; Zhao, J.; Brown, J. S.; Blackwell, J.; Carr, P. W. *Analytical Chemistry* **2000**, *72*, 1253-1262.

Current Column Technology for Fast 2DLC



Column: 50 mm x 2.1 mm i.d. **C-ZrO_2**

Temperature: 150 °C

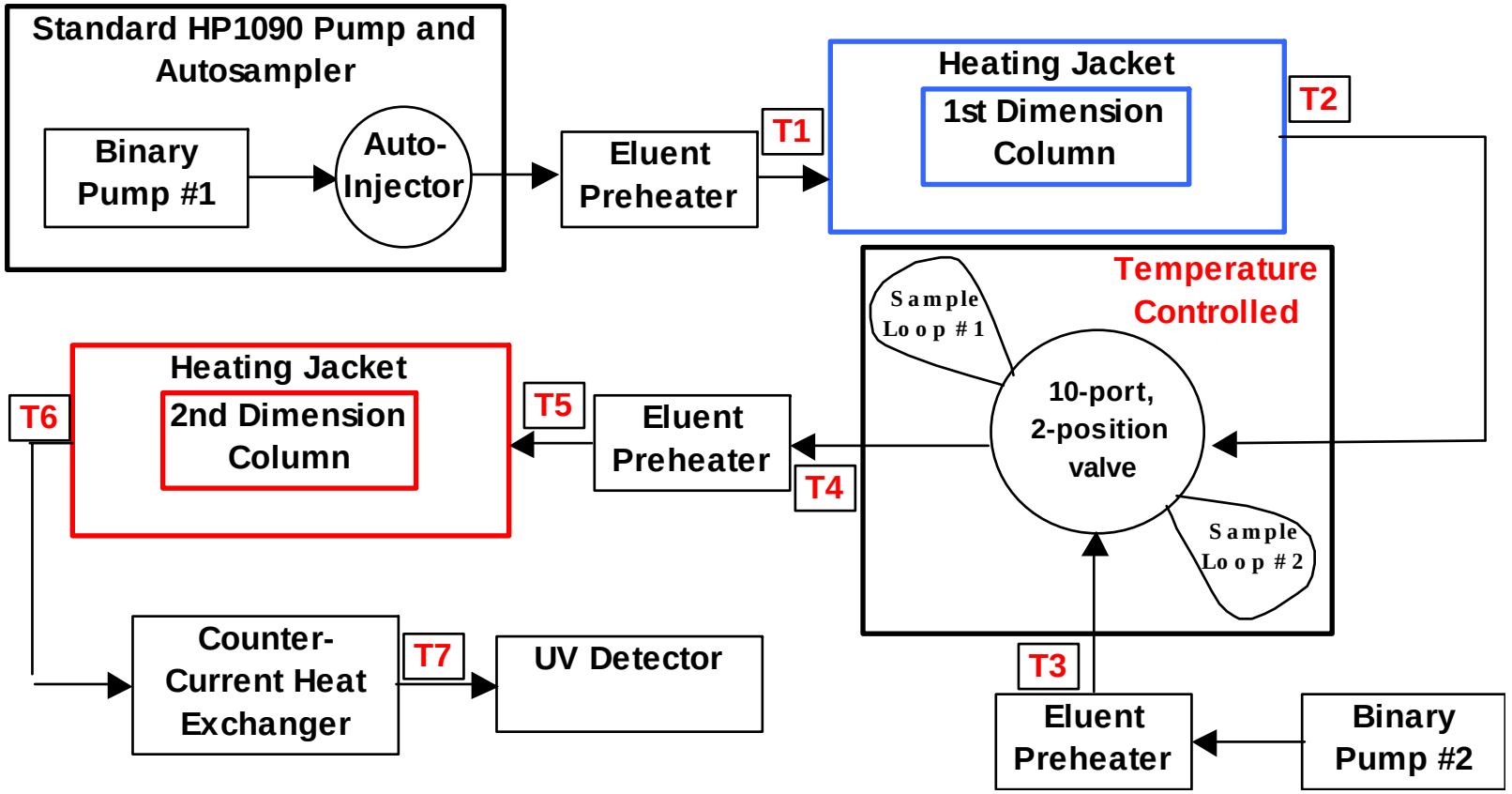
Flow rate: 5.0 ml/min.

Solutes: Alkylphenones

Mobile Phase: 30/70 ACN/Water

Column	Flow Rate (ml/min.)	u_e (cm/s)	t_m (sec.)	k'_{max}	N (Plates/column)	Peak Capacity (n_c)
C-ZrO_2	5.00	6.3	1.6	5.25	2000	11

Schematic of a Complete LC × UFHTLC System



An Example Application – Initial Work on a Screening Tool for Drugs of Abuse

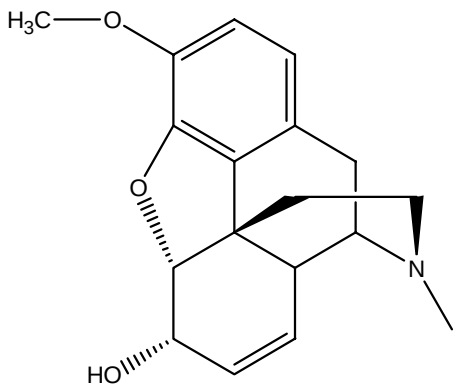
Goal – To significantly improve the speed and comprehensiveness of a single HPLC method for screening for presence of opiates and amphetamines in biological samples

Background

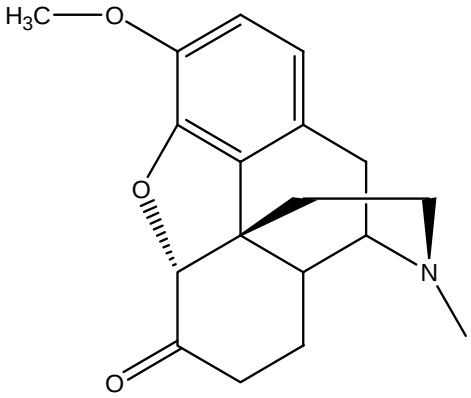
- Opiates and amphetamines are two major classes of abused drugs that are routinely assayed for in public and private forensic labs.
- The International Olympic Committee has identified pseudoephedrine as a significantly abused stimulant[1]
- Immunoassay-based techniques are commonly employed as qualitative screening techniques because of relatively broad *compound coverage*, and the ability to significantly improve *throughput*. However, these techniques are not perfect and have problems such as cross-reactivity and the potential for false positives.
- GC/MS is commonly used for compound confirmation and quantitation.
- A main reason LC is not used is because it is not efficient. It takes multiple methods (runs) to attain broad compound coverage because of low peak capacity.

1) Gmeiner, G.; Geisendorfer, T.; Kainzbauer, J.; Nikolajevic, M.; Tausch, H. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences* **2002**, *768*, 215-221

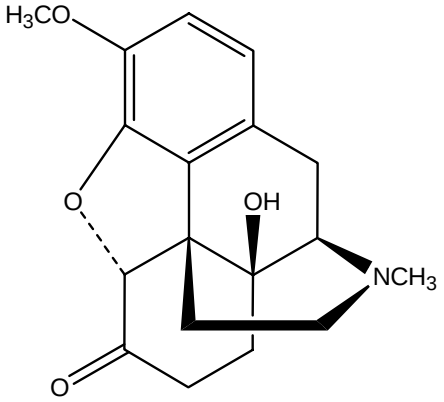
The Opiates



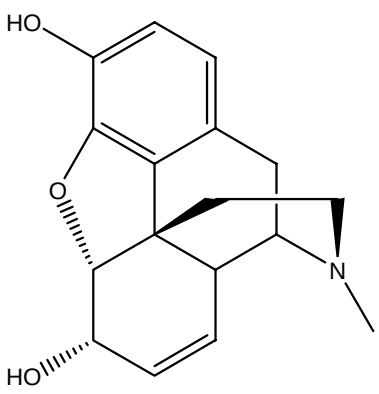
Codeine (9)



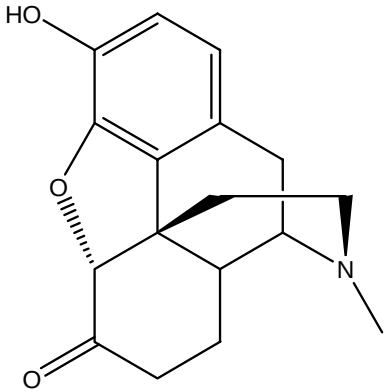
Hydrocodone



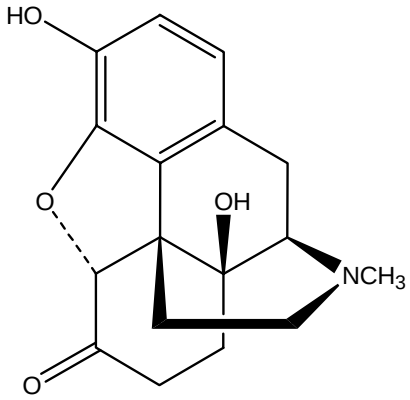
Oxycodone (12)



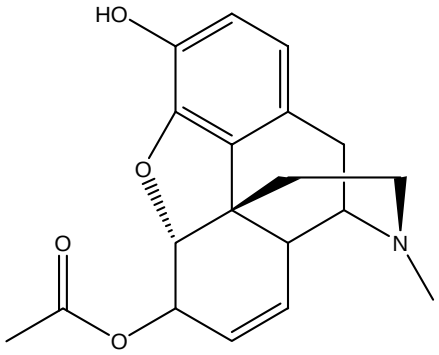
Morphine (1)



Hydromorphone (3)

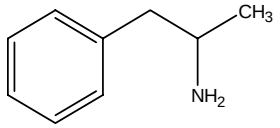


Oxymorphone (2)

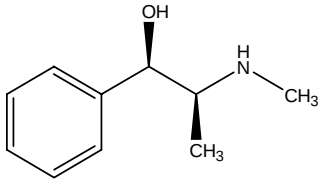


6-acetylmorphine

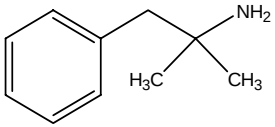
The Amphetamines



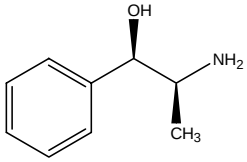
Amphetamine (10)



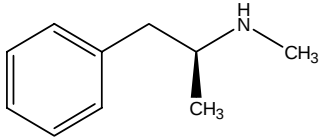
Ephedrine (6)



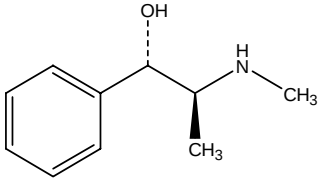
Phentermine



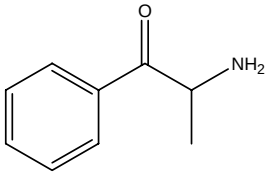
Phenylpropanolamine (4)



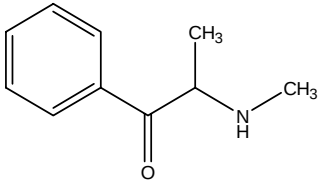
Methamphetamine



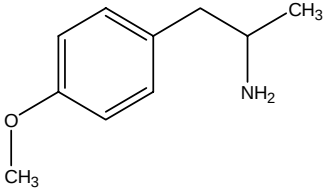
Pseudoephedrine (7)



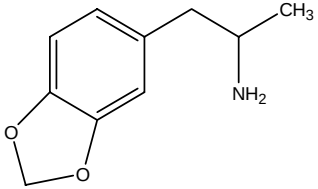
Cathinone (5)



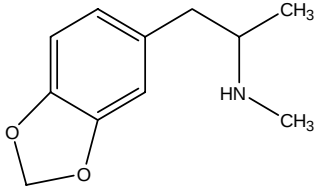
Methcathinone (8)



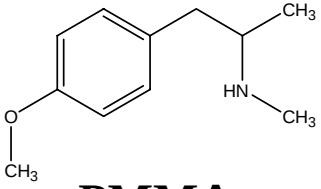
PMA



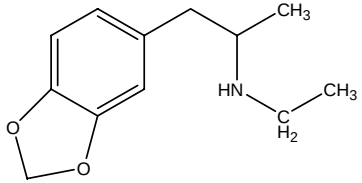
MDA (11)



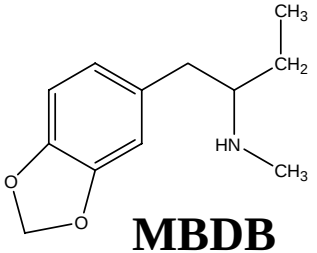
MDMA



PMMA



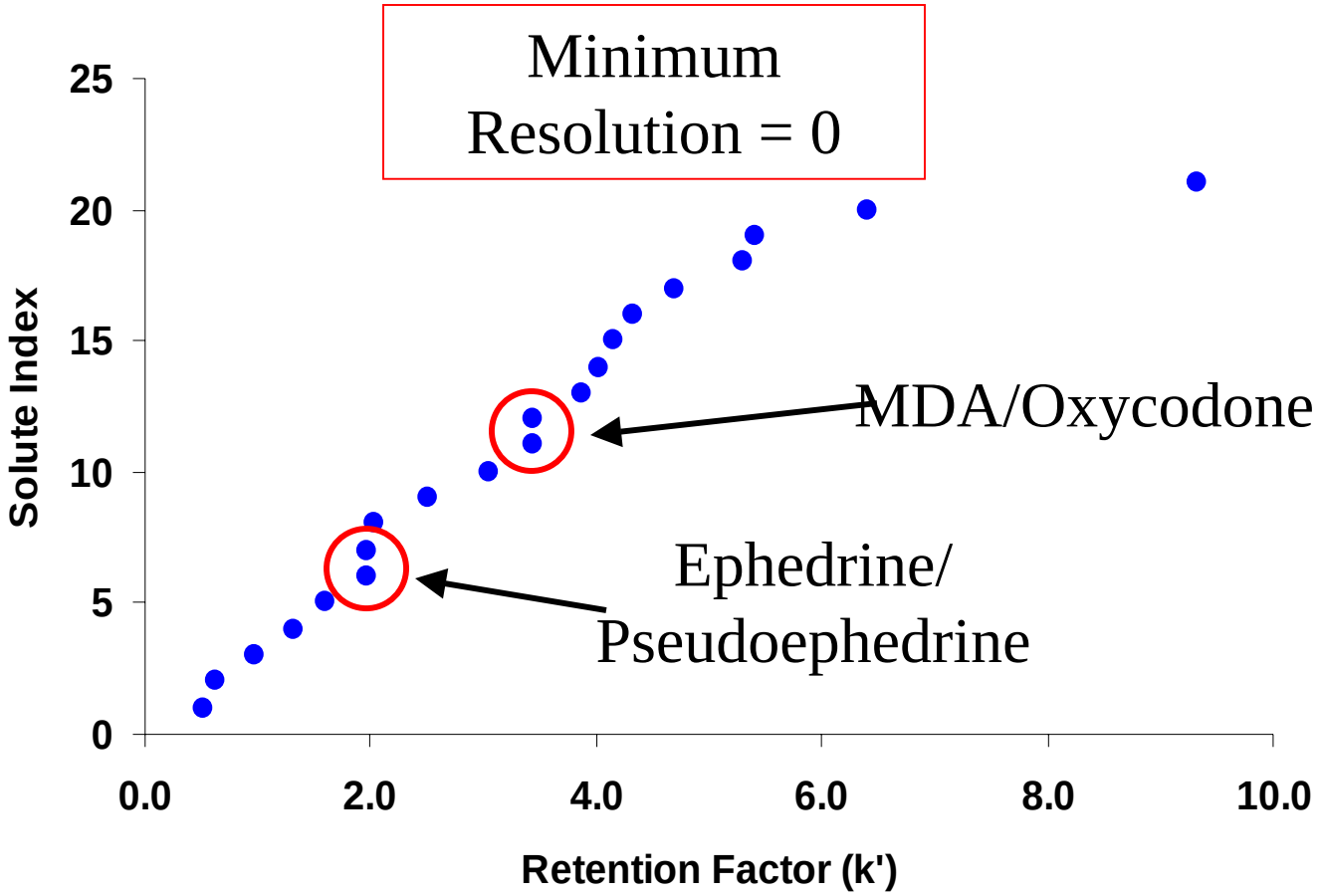
MDEA



MBDB

An Initial Column Selectivity Evaluation – First Dimension Column

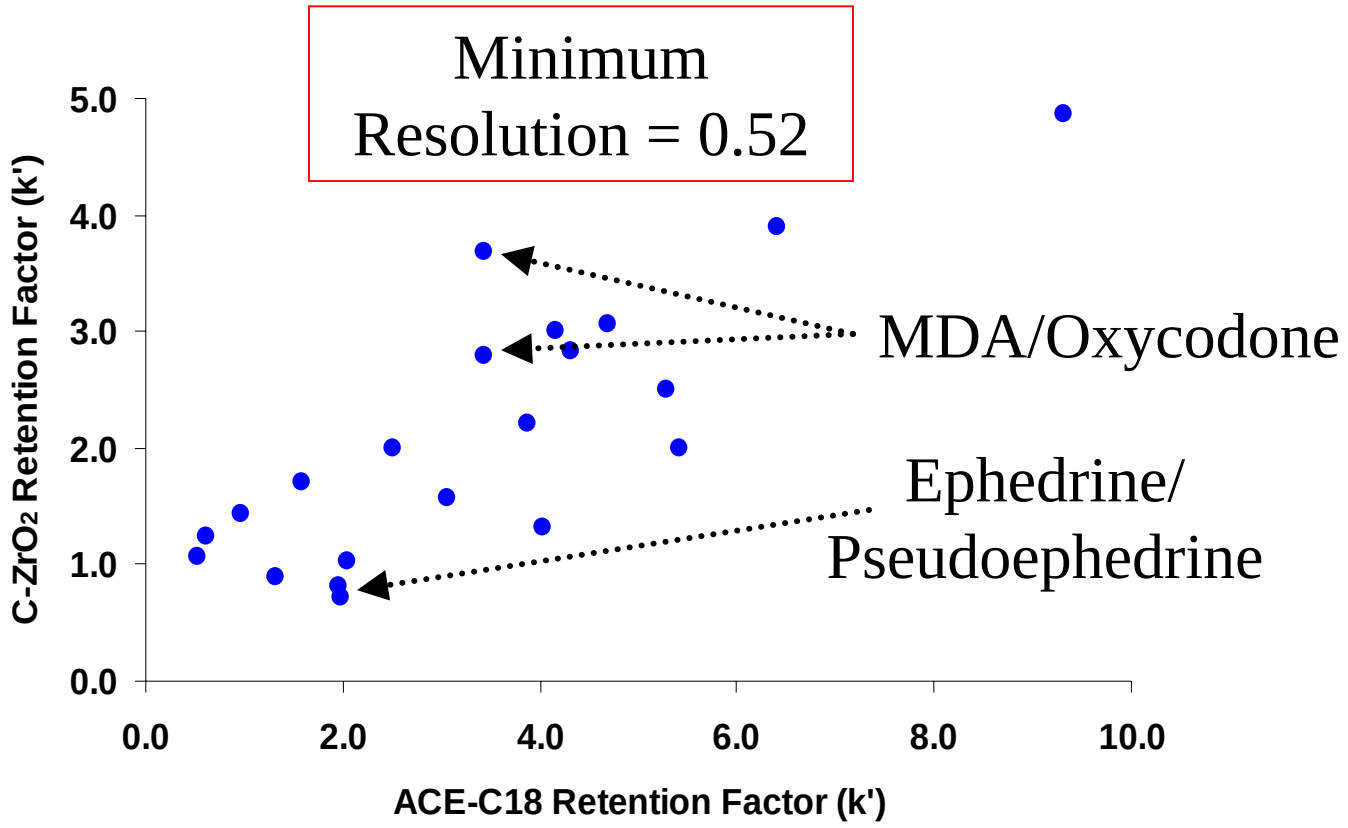
Condition 1: Column, ACE-C18; Mobile phase, 20/80 ACN/20mM ammonium acetate, pH 5.0; Temperature, 40 ^{o}C



An Initial Column Selectivity Evaluation – Adding the Second Dimension Column

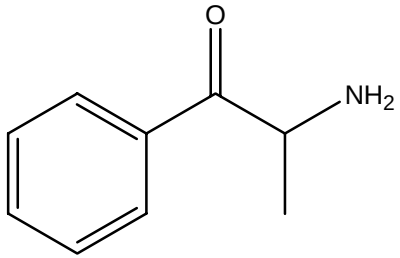
Condition 1: Column, ACE-C18; Mobile phase, 20/80 ACN/20mM ammonium acetate, pH 5.0; Temperature, 40 °C

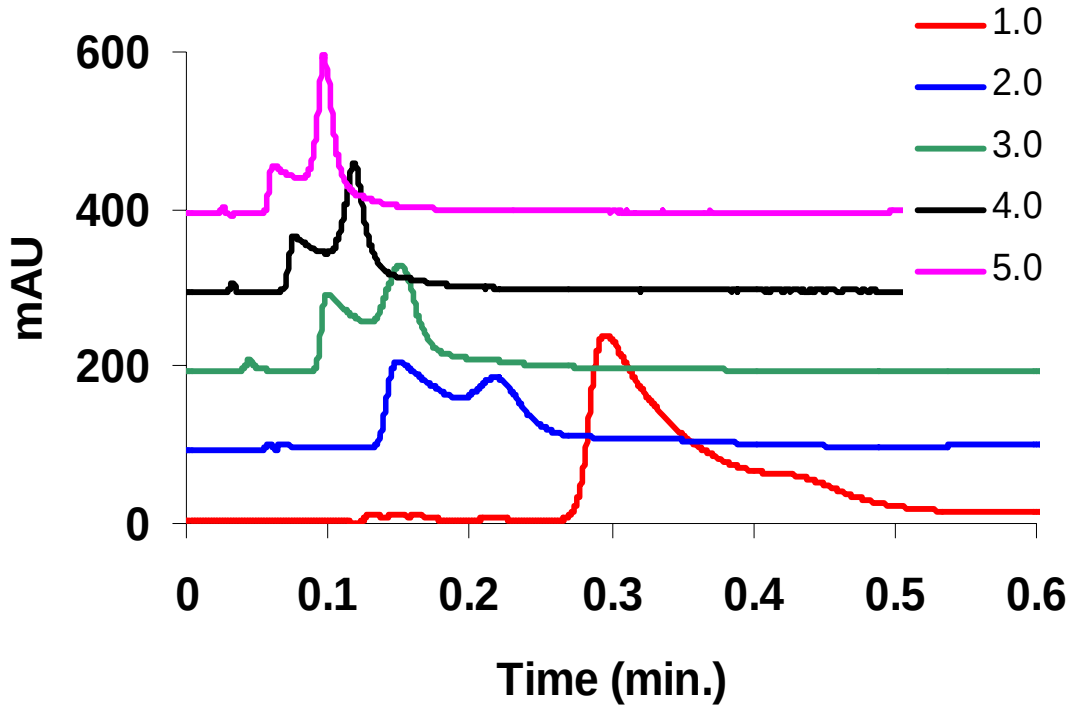
Condition 2: Column, ZirChrom C-ZrO_2; Mobile phase, 20/80 ACN/30mM formic acid, 15mM octylamine, 50μM EDTPA, pH 3.6; Temperature, **150 °C**



Thermal Instability of Amphetamines at pH - 5

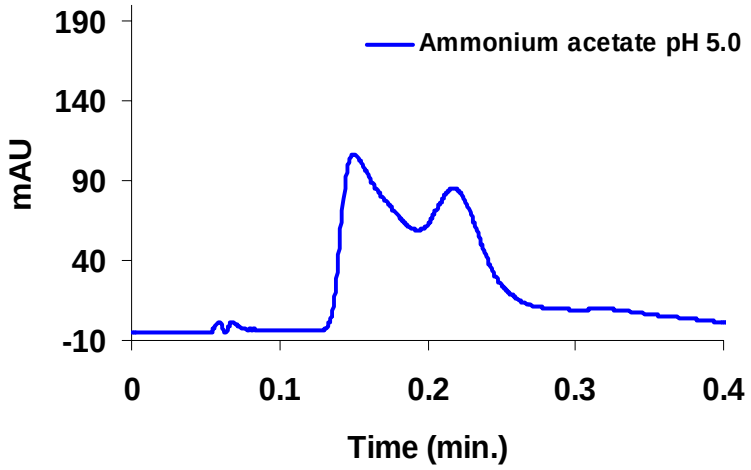
Cathinone

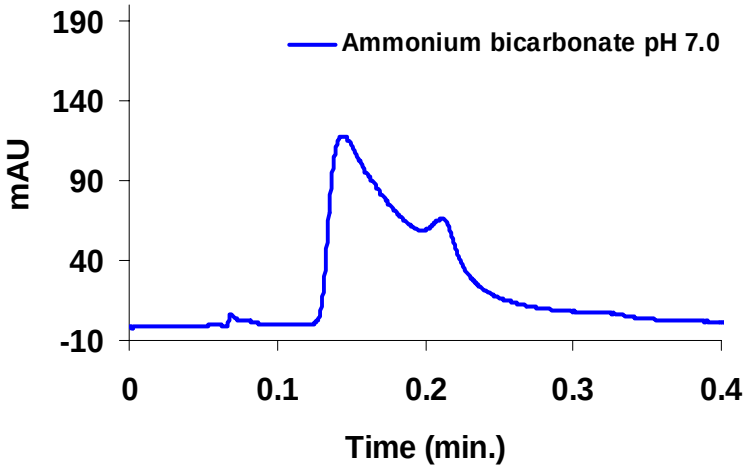


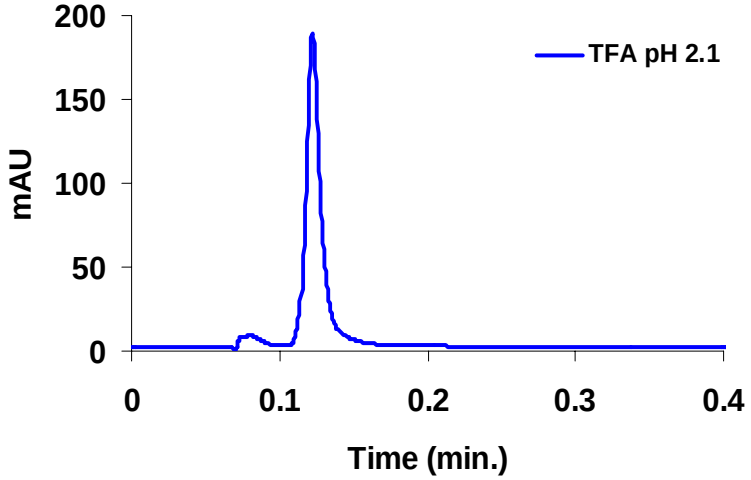


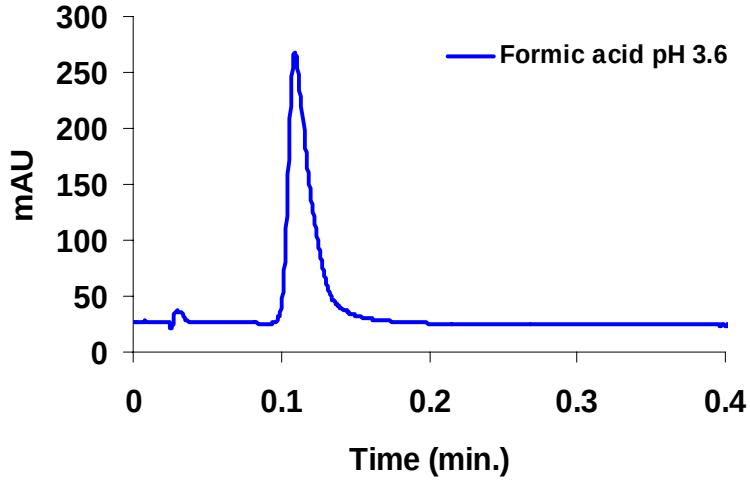
LC Conditions: Solute = Cathinone, 100 ug/ml; Flow rate varied from 1.0 to 5.0 ml/min (see figure).; Temperature, 150 ^{o}C; 10 µl injection; Detection at 254 nm; Mobile phase, 40/60 ACN/20mM ammonium acetate, **pH 5.0**; Column, 50 mm x 2.1 mm i.d. ZirChrom C-ZrO_2

Degradation as a Function of Eluent pH









Cathinone and Methcathinone show the same behavior, however, their reactivities are insignificant on the chromatographic timescale of UFHTLC in the pH range of 2.1-3.6.

Strategy for Optimizing the Two-Dimensional Separation

1. Experimentally determine the retention of all solutes on the ACE-C18 column at three different eluent strengths
2. Fit retention data to Pade approximation $$\ln k' = A + \frac{B\phi}{1 + C\phi}$$
3. Experimentally determine the retention of all solutes on the C-ZrO_2 column at seven different eluent strengths at 150 ^{o}C
4. Fit retention data to a parabolic function of ϕ

$$\log k' = A + B\phi + C\phi^2$$

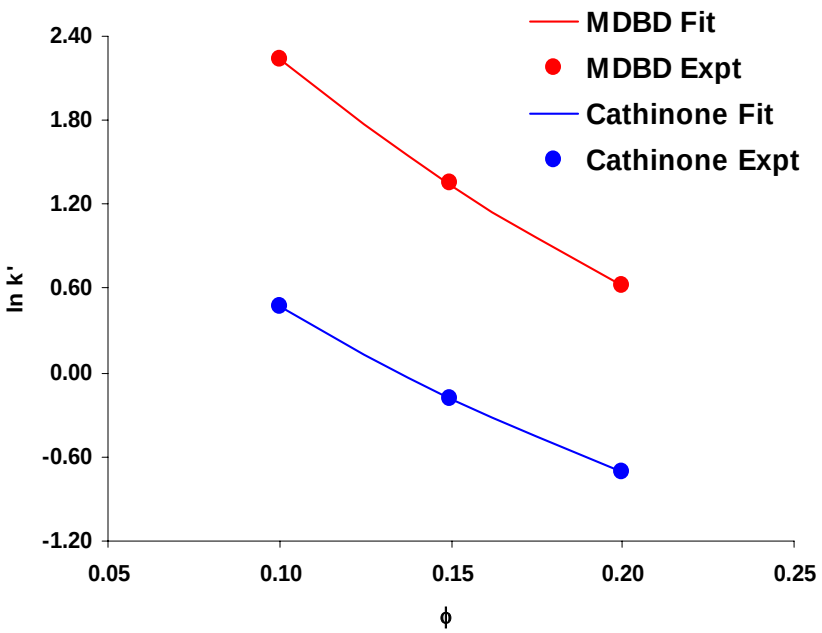
5. Optimize the minimum two-dimensional resolution by varying the eluent strength in each separation dimension independently

$$R_{s,2D} = \sqrt{R_{s,1}^{\ 2} + R_{s,2}^{\ 2}}$$

Experimental vs. Predicted Retention

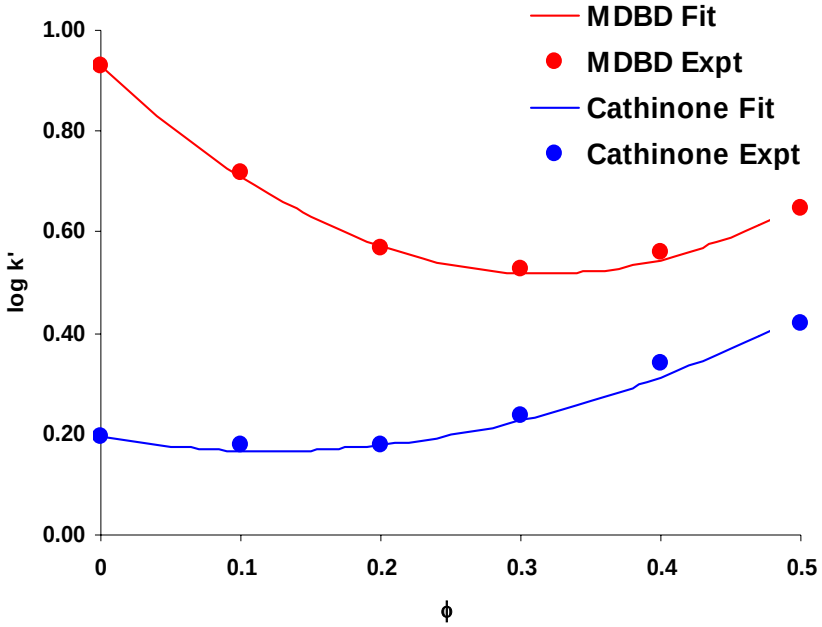
ACE-C18 Retention

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

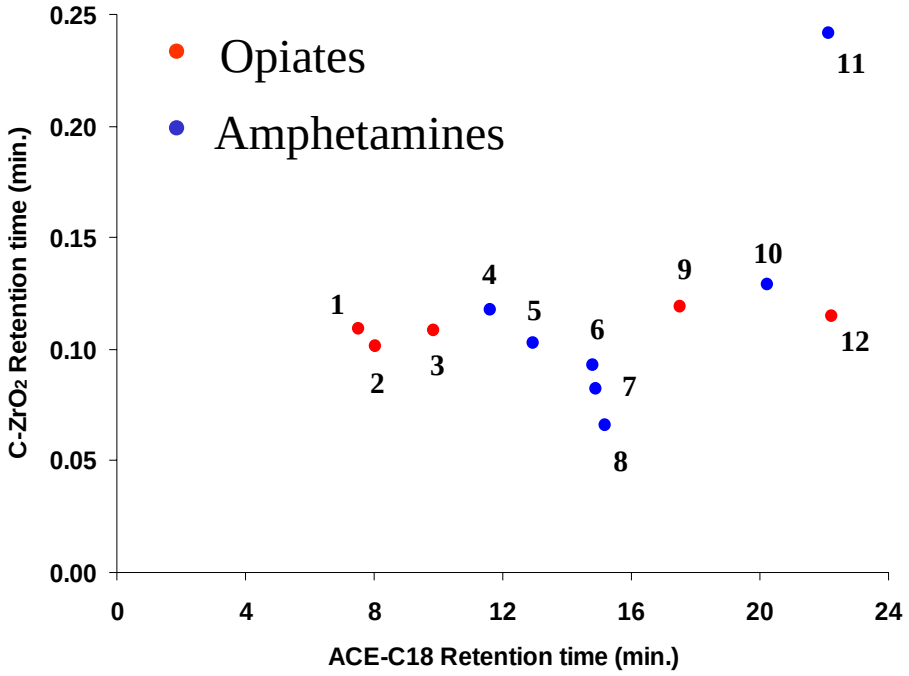


ZirChrom C-ZrO_2 Retention

$$\log k' = A + B\phi + C\phi^2$$



A Prediction of the First Half of the Two-Dimensional Separation



1	Morphine	7	Pseudoephedrine
2	Oxymorphone	8	Methcathinone
3	Hydromorphone	9	Codeine
4	Phenylpropanolamine	10	Amphetamine
5	Cathinone	11	MDA
6	Ephedrine	12	Oxycodone

1st Dimension Conditions

Column – 150 mm x 2.1 mm I.d. ACE-C18, 5 micron
Flow rate – 0.08 ml/min.
Mobile phase – 10/90 ACN/20mM Ammonium acetate, pH 5.0
Temperature – 40 °C
Injection volume – 5 µl of 100 µg/ml of each solute

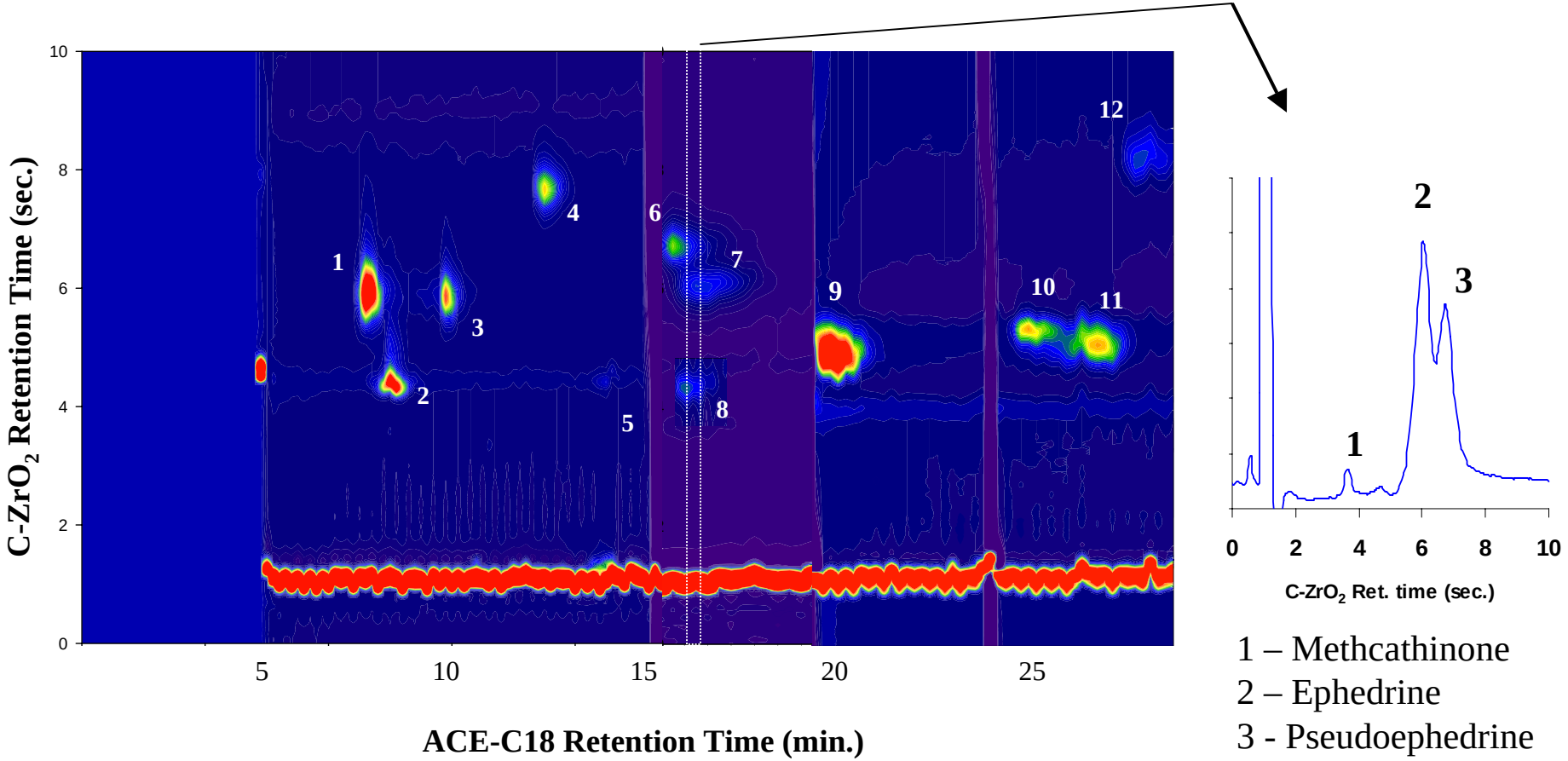
2nd Dimension Conditions

Column – 33 mm x 2.1 mm i.d. ZirChrom C-ZrO_2, 3 micron
Flow rate – 5.00 ml/min.
Mobile phase –ACN/30mM Formic acid, 15mM Octylamine, 50µM EDTPA, pH 3.6

Time (min.)	% ACN
0	60
15.8	65
19.0	50

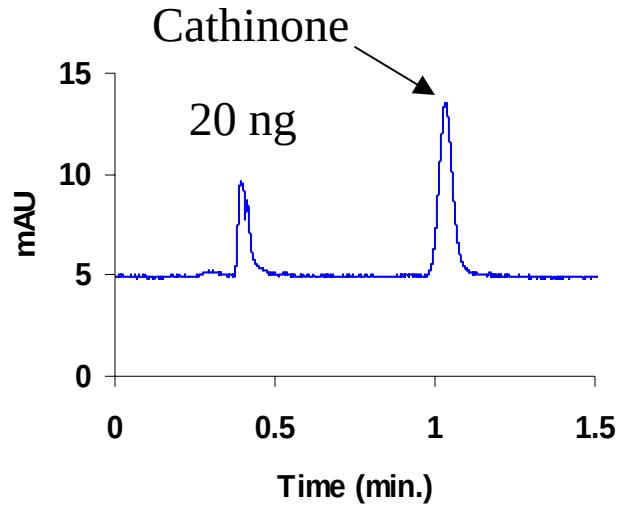
Temperature – 150 °C
Injection volume - 15 µl
Detection at 215 nm

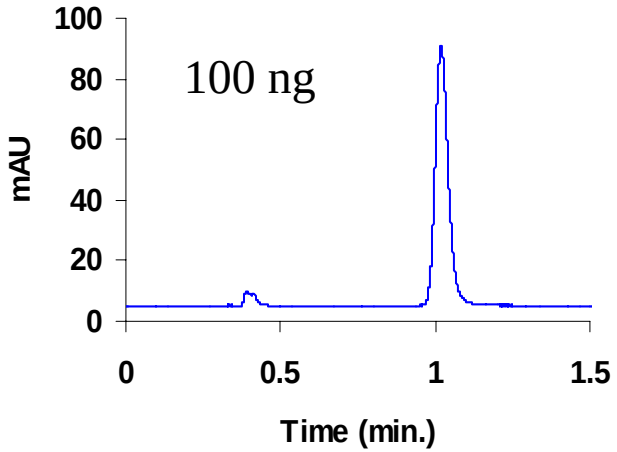
An Initial Attempt at the First Half of the Separation

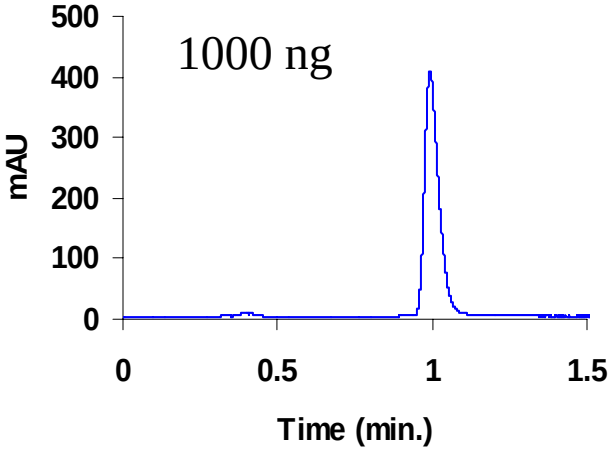


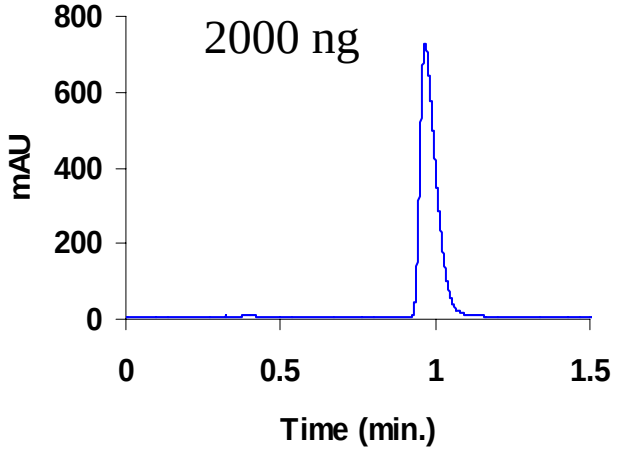
1 – Methcathinone
2 – Ephedrine
3 - Pseudoephedrine

Extreme Column Overloading of Amines Severely Degrades the Two-Dimensional Resolution









LC Conditions

Column – 50 mm x 4.6 mm i.d. ACE-C18, 5 micron

Flow rate – 2.00 ml/min.

Mobile phase – 10/90 ACN/20mM Ammonium acetate, pH 5.0

Temperature – 40 °C

Injection volume – 20 µl

Mass Injected (ng)	Symmetry	Plate Count (N)
20	0.83	2800
100	0.80	2950
1000	0.59	2400
2000	**0.47**	**1700**

Conclusions and Future Work

1. There is a great potential for significantly increasing the speed of comprehensive two-dimensional liquid chromatography through the implementation of UFHTLC as the second separation dimension.
2. **Ultra-fast one-dimensional separations on the ten-second timescale** are feasible using narrow-bore columns at high temperatures. This allows extremely high column linear velocities to be reached using practical volumetric flow rates, without excessive diminishing of the optimum efficiency of the column.
3. There remains **significant room for improvement of these ultra-fast separations.** These gains will likely be brought about through the use of smaller particles, and columns packed better than those currently in use.
4. An initial attempt at the first half of a two-dimensional LC separation of 21 commonly abused opiates and amphetamines has been successful. The problem of severe overloading of the C18-silica column must first be solved before the full two-dimensional LC separation can be satisfactorily demonstrated.

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