Fast, Comprehensive Two-Dimensional HPLC For the Analysis of Complex Samples

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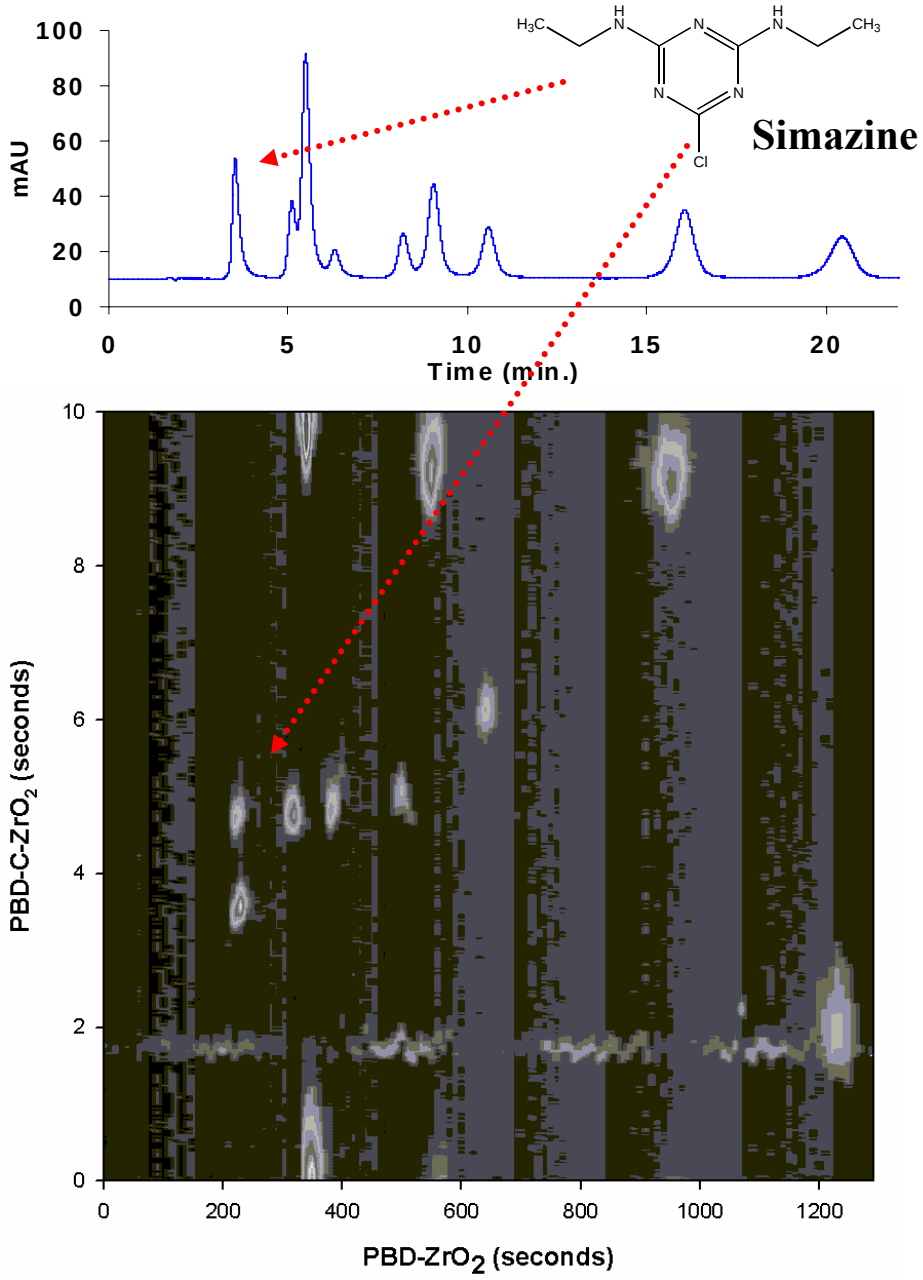
LC × UFHTLC Separation of Ten Triazine Herbicides

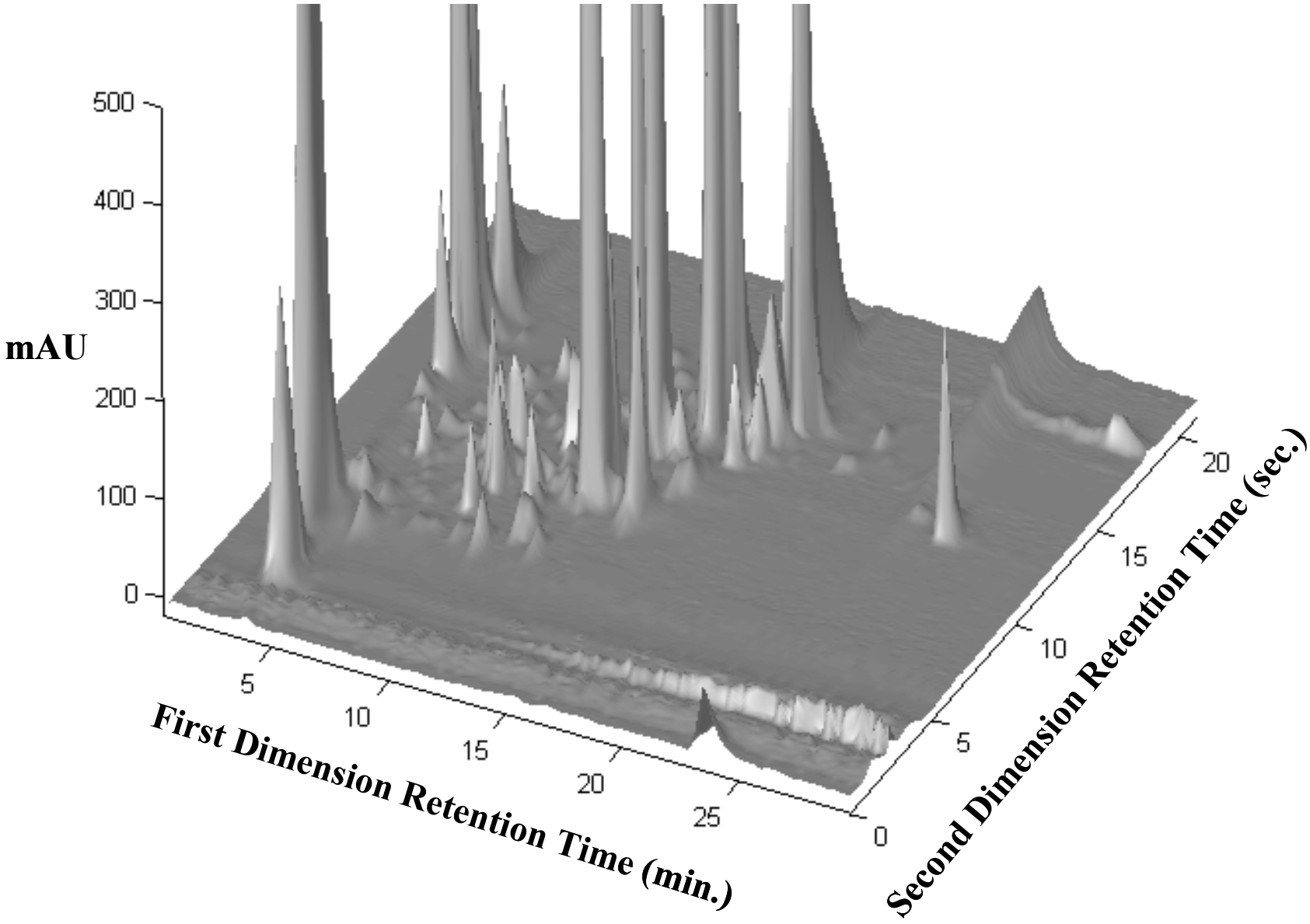


1st Dimension Conditions: Column, 50 mm x 2.1 mm i.d. PBD-ZrO_2; Mobile phase, **20/80 ACN/Water**; Flow rate, 0.08 ml/min.; Injection volume, 20 µl; Temperature, 40 °C

2nd Dimension Conditions: Column, 50 mm x 2.1 mm i.d. PBD-C-ZrO_2; Mobile phase, **20/80 ACN/Water**; Flow rate, 7.0 ml/min.; Injection volume, 15 µl; Temperature, **150 °C**; 1st dimension sampling frequency, 0.1 Hz

This was a reasonable place to start work on fast 2DLC, but isocratic separations are only good for relatively simple samples

Fast 2DLC (Gradient x Gradient) Separation of Corn Seedling Extract



Outline

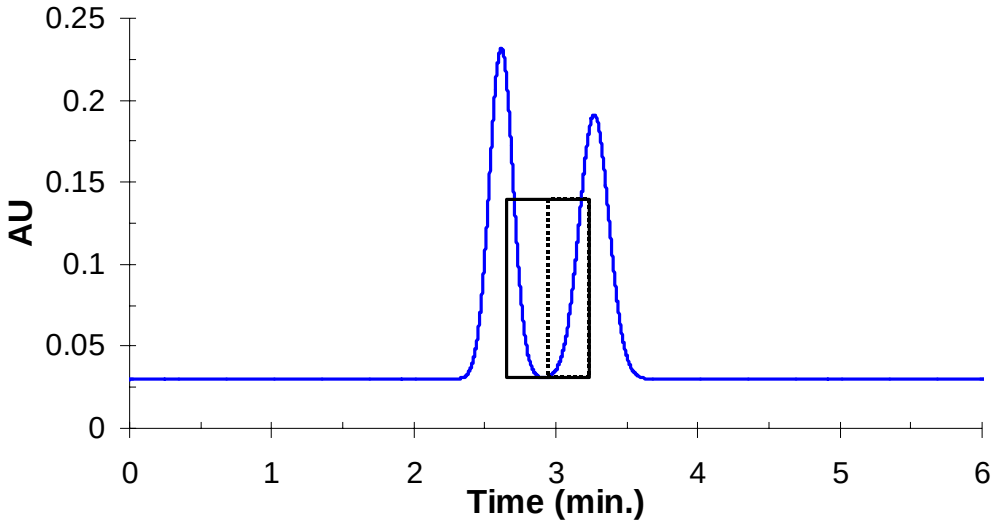
1. Review of critical requirements for success in 2D separations
2. Review of approaches to improve the speed of 2DLC
3. Construction and evaluation of a 2DLC instrument using High Temperature and Ultra-Fast Gradient Elution HPLC in the second dimension separation (LC × UFHTLC)
4. Fast 2DLC separations of extracts of wild-type and *orp* mutant corn seedlings

Conclusion – We are currently capable of 2DLC separations on the 30-minute timescale where gradient elution is used in both dimensions for the analysis of very complex samples; under these conditions a peak capacity production rate of approximately 1 peak/second has been achieved.

Requirements and Advantages of Two-Dimensional HPLC

Two conditions must be met for the technique to be considered "comprehensive & two-dimensional"

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



Only when these two conditions are satisfied is the maximum total peak capacity of the two-dimensional system realized as:

$$PC_{2D} = PC_1 \times PC_2$$

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

Approaches to Improving the Speed of HPLC

Approach	Advantages	Disadvantages
Shorter Columns	Works with most equipment, stationary phases	Low plate count and resolution
Monolithic Columns	Low backpressure	Narrow-bore columns not available, high solvent usage, speed limited by flow rate
Ultra-High Pressure LC	High plate counts with small particles	Specialized equipment needed, losses in N at high velocity
Shorter Columns with Nonporous Particles	Works with most equipment, stationary phases	Low Sample Loading Capacity
Shorter Columns with < 3 um	High plate counts with small particles	Specialized equipment needed
High Temperature LC	Low backpressure, high efficiency at high velocity	Requires special heating, stable phases, stable analytes.

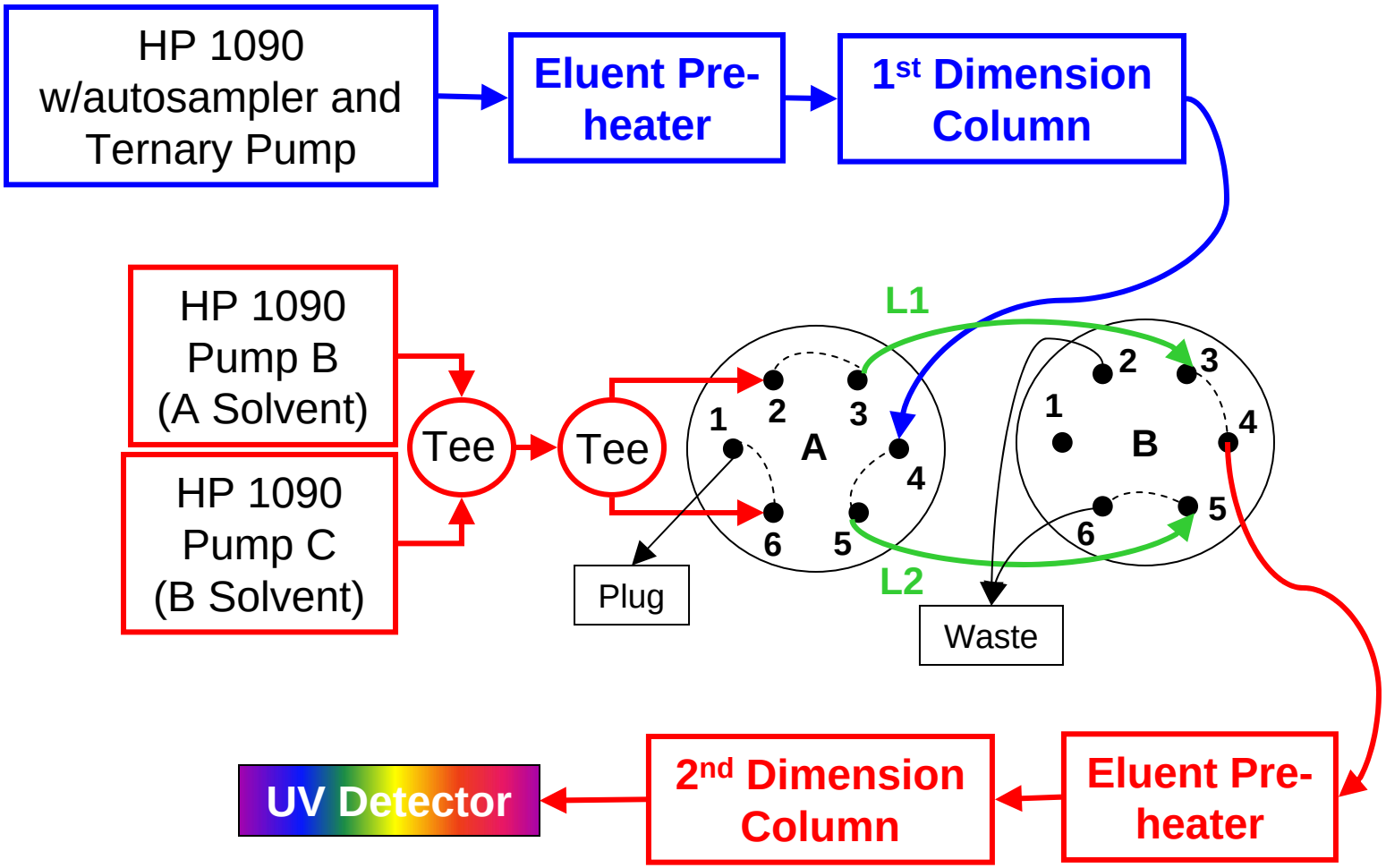
Comparison of Peak Capacity Production

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

Hille, J. M.; Freed, A. L.; Watzig, H. *Electrophoresis* **2001**, *22*, 4035-4052

Goal: **To increase the speed of peak capacity production in HPLC such that 10-20-fold increases in peak capacity can be achieved for separations under 60 minutes**

Schematic of a Complete LC × UFHTLC System Capable of Gradient Elution in Both Dimensions



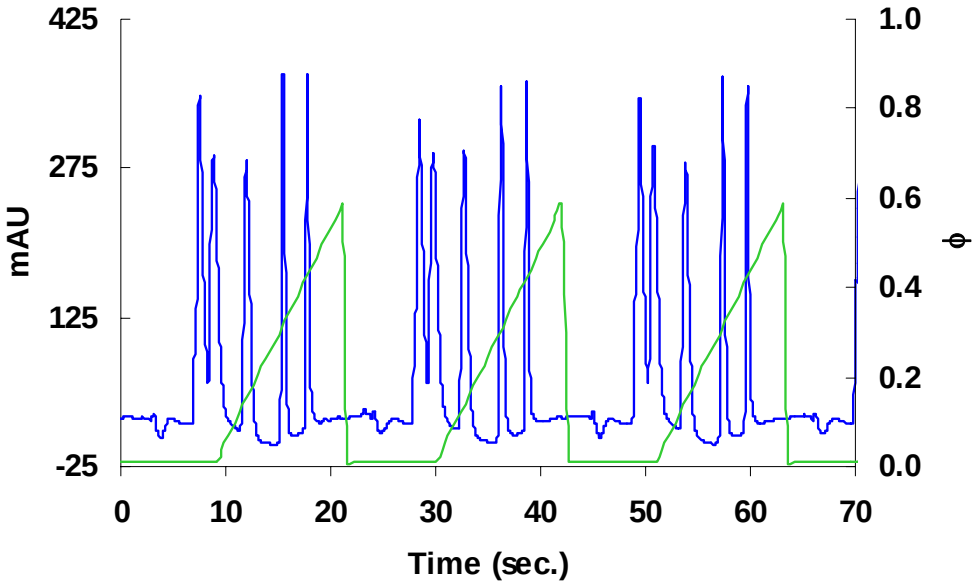
21 Second, Reproducible One-Dimensional Gradient Separations

Gradient time (t_g) = 16 sec.
Re-equilibration time (t_{re-eq}) = 5 sec.
Cycle time (t_c) = 21 sec.

Solutes:
Uracil, Nitroalkane homologs (2-5)

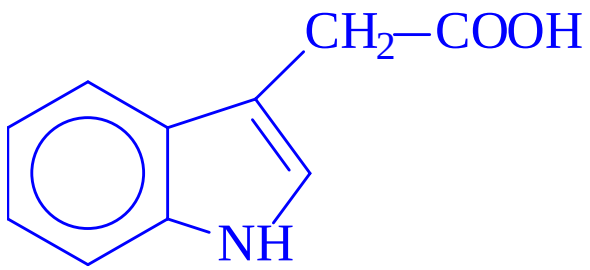
Column – 50 mm x 2.1 mm i.d. SB300-C_{18}
Flow rate – 3.0 ml/min.
Temperature – 100 °C
Injection volume – 30 µl

Gradient Conditions
A – 0.1% Trifluroacetic acid (TFA) in water
B – 0.1% Trifluroacetic acid (TFA) inACN
Gradient from 0-100% B in 21 seconds



Indole-3-acetic acid (IAA) is the Primary Auxin in Plants

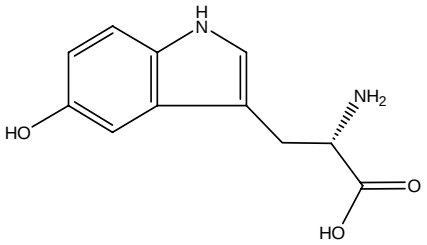
- Active in submicrogram levels in plants
- Associated with a variety of physiological growth and development processes
  - -Cell division and expansion
  - -Vascular tissue differentiation
  - -Apical dominance
  - -Tropisms
  - -Flowering
  - -Root initiation
  - -Fruit ripening
  - -Abscission of leaves and fruit



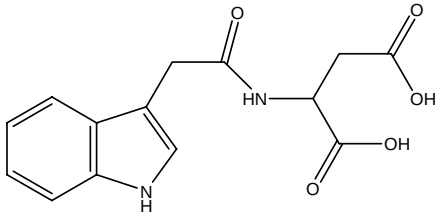
Indole-3-acetic acid

- Biosynthetic pathway is redundant, highly regulated, and still not fully elucidated

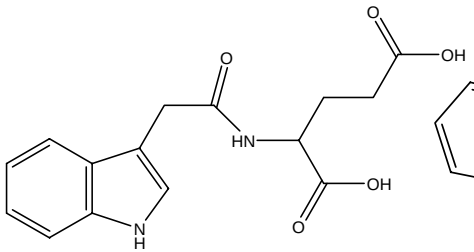
Indolic Metabolite Structures of Interest in this Work



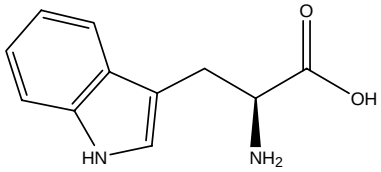
(1) 5-hydroxy-L-tryptophan



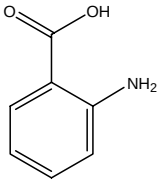
(2) Indole-3-acetyl-L-aspartic acid



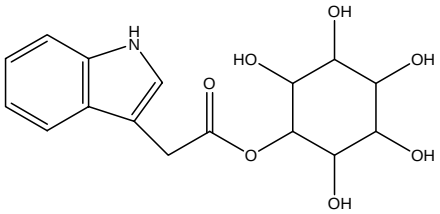
(3) Indole-3-acetyl-L-glutamic acid



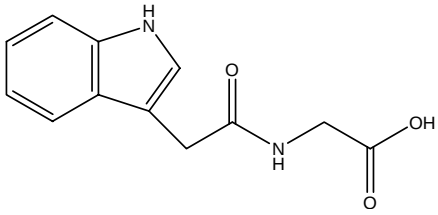
(4) Tryptophan



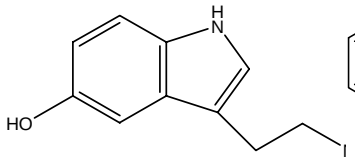
(5) Anthranilic acid



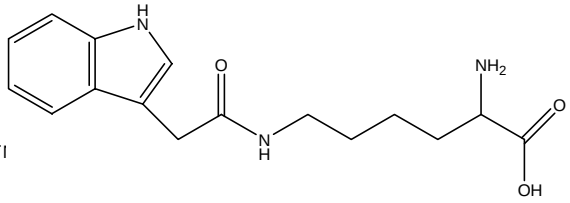
(6,10,11,14) Indole-3-acetyl-myo-inositol



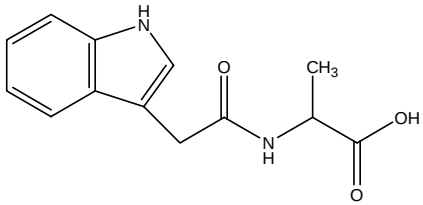
(7) Indole-3-acetyl-L-glycine



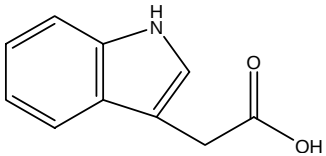
(8) 5-hydroxy-tryptamine



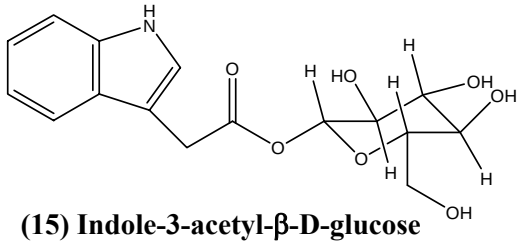
(9) Indole-3-acetyl-L-lysine



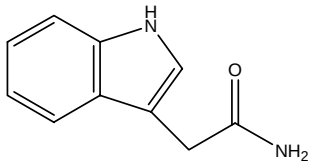
(12) Indole-3-acetyl-L-alanine



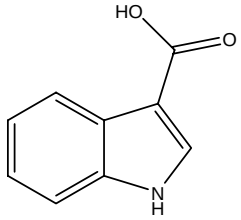
(13) Indole-3-acetic acid



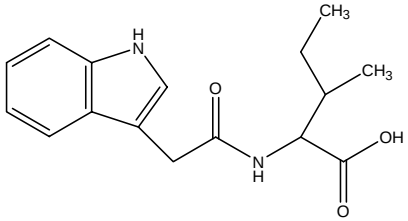
(15) Indole-3-acetyl-β-D-glucose



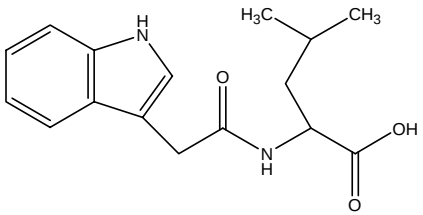
(16) Indole-3-acetamide



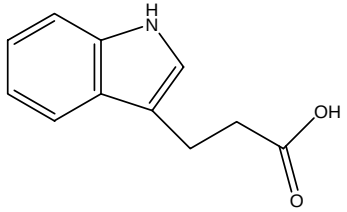
(17) Indole-3-carboxylic acid



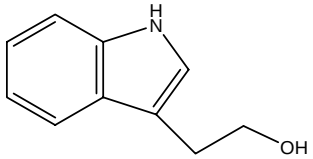
(18) Indole-3-acetyl-L-isoleucine



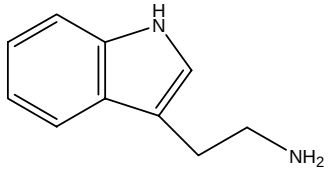
(19) Indole-3-acetyl-L-leucine



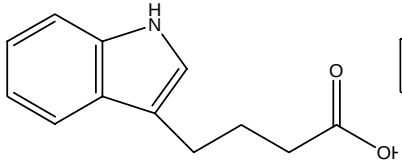
(20) Indole-3-propionic acid



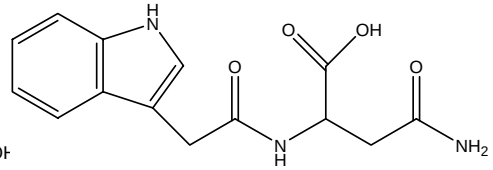
(21) Indole-3-ethanol



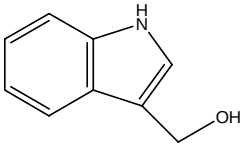
(22) Tryptamine



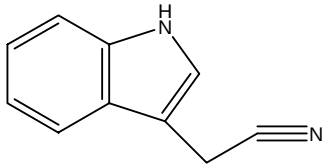
(23) Indole-3-butryic acid



(24) Indole-3-acetyl-L-glutamine



(25) Indole-3-carbinol



(26) Indole-3-acetonitrile

Experimental 2DLC Conditions

1st Dimension

Column – 100 mm x 2.1 mm i.d. Discovery HS-F5 (5 micron)

Eluent A – 20mM sodium phosphate, 20mM sodium perchlorate, pH 5.7
Eluent B – Acetonitrile

Injection volume – 10 µl

Temperature – 40 °C

Flow rate – 0.10 ml/min.

Time (min.)	%B
0.00	0
20.00	40
22.00	40
23.00	70
23.01	0
30.00	0

2nd Dimension

Column – 50 mm x 2.1 mm i.d. ZirChrom-CARB (8% C, 3.0 micron)

Eluent A – 20mM perchloric acid in water
Eluent B – Acetonitrile

Injection volume – 34 µl

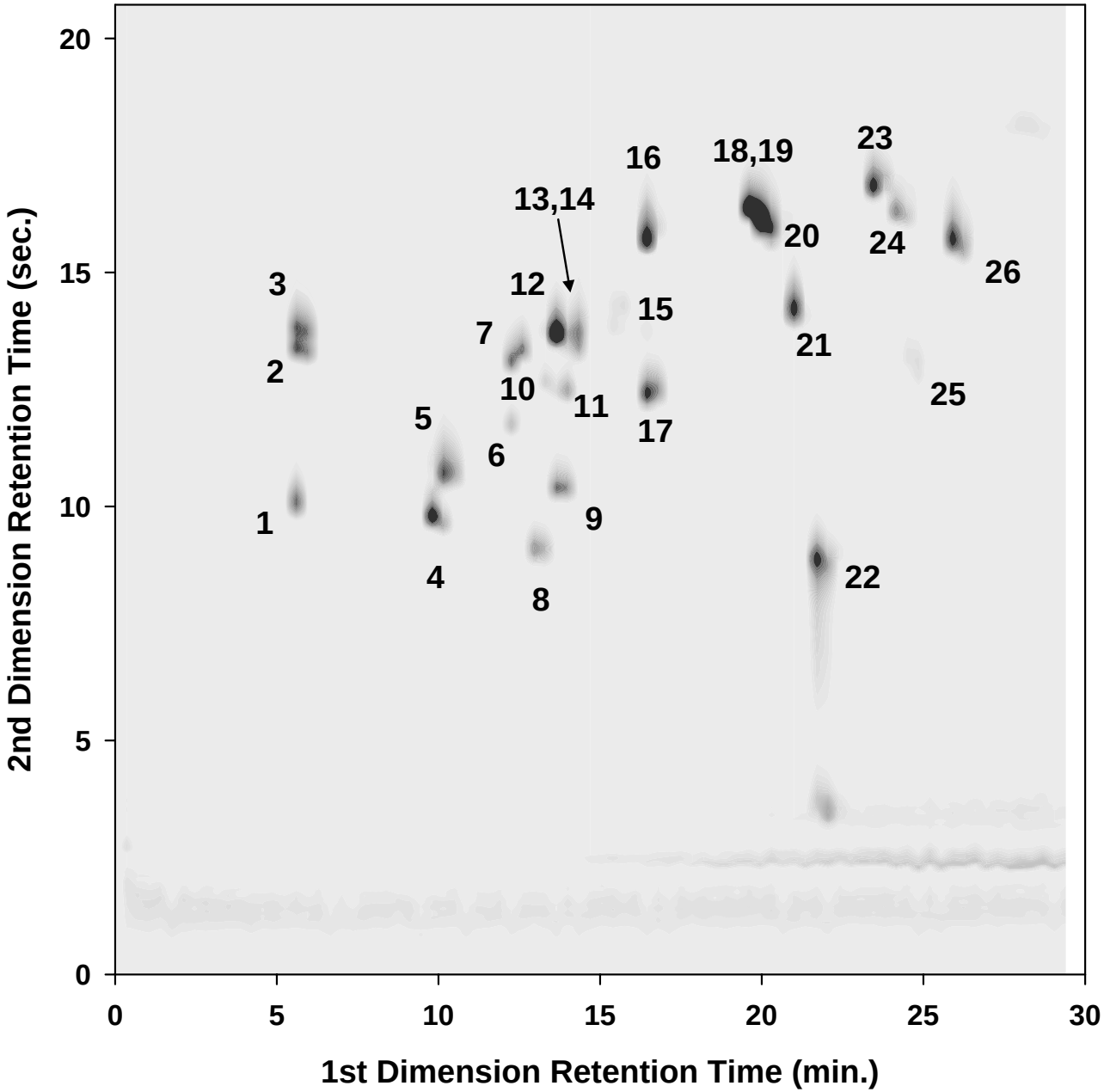
Temperature – 110 °C

Flow rate – 3.0 ml/min.

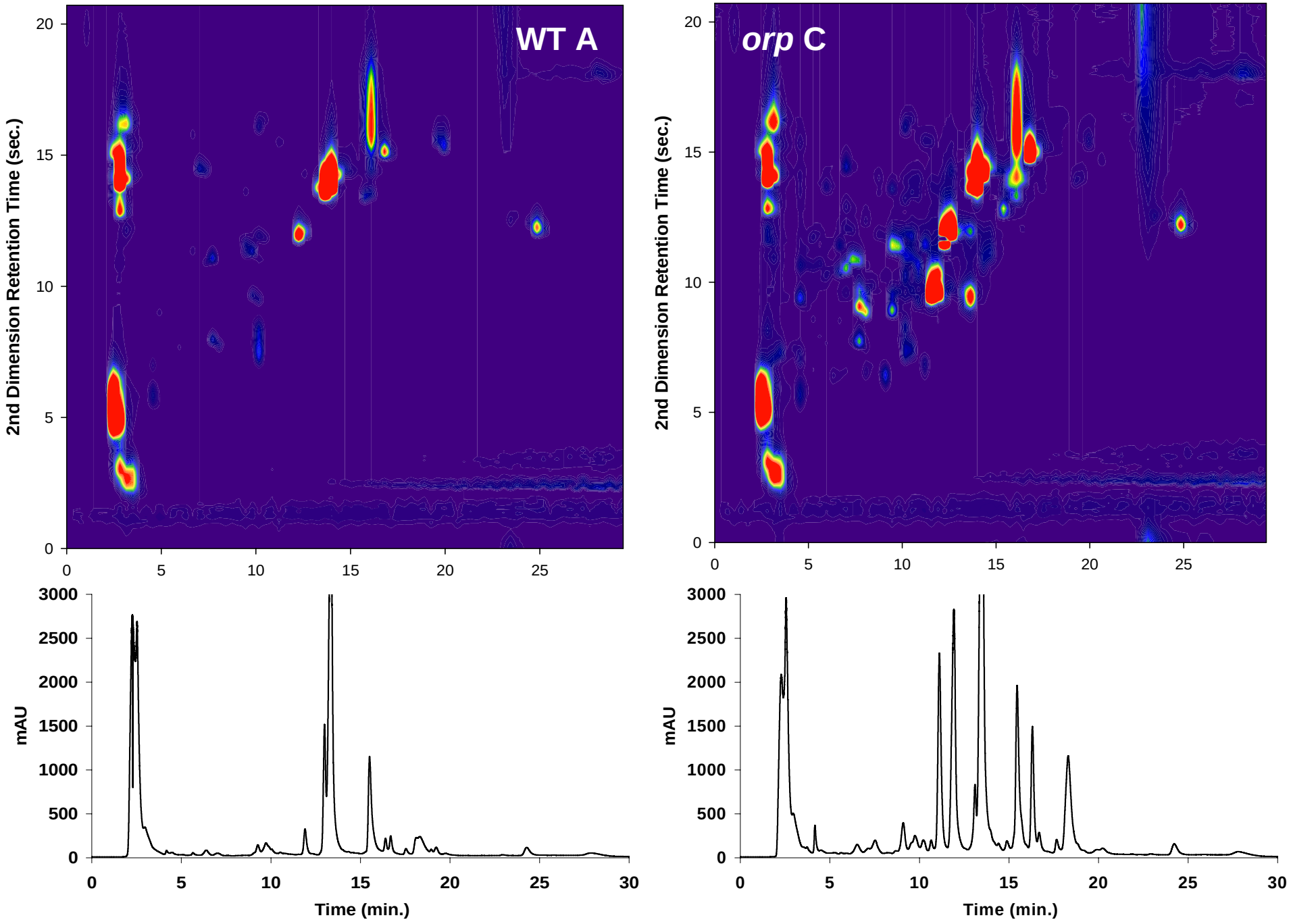
Time (sec.)	%B
0.0	0
17.4	70
18.0	0
21.0	0

UV-DAD Detection from 200-350 nm

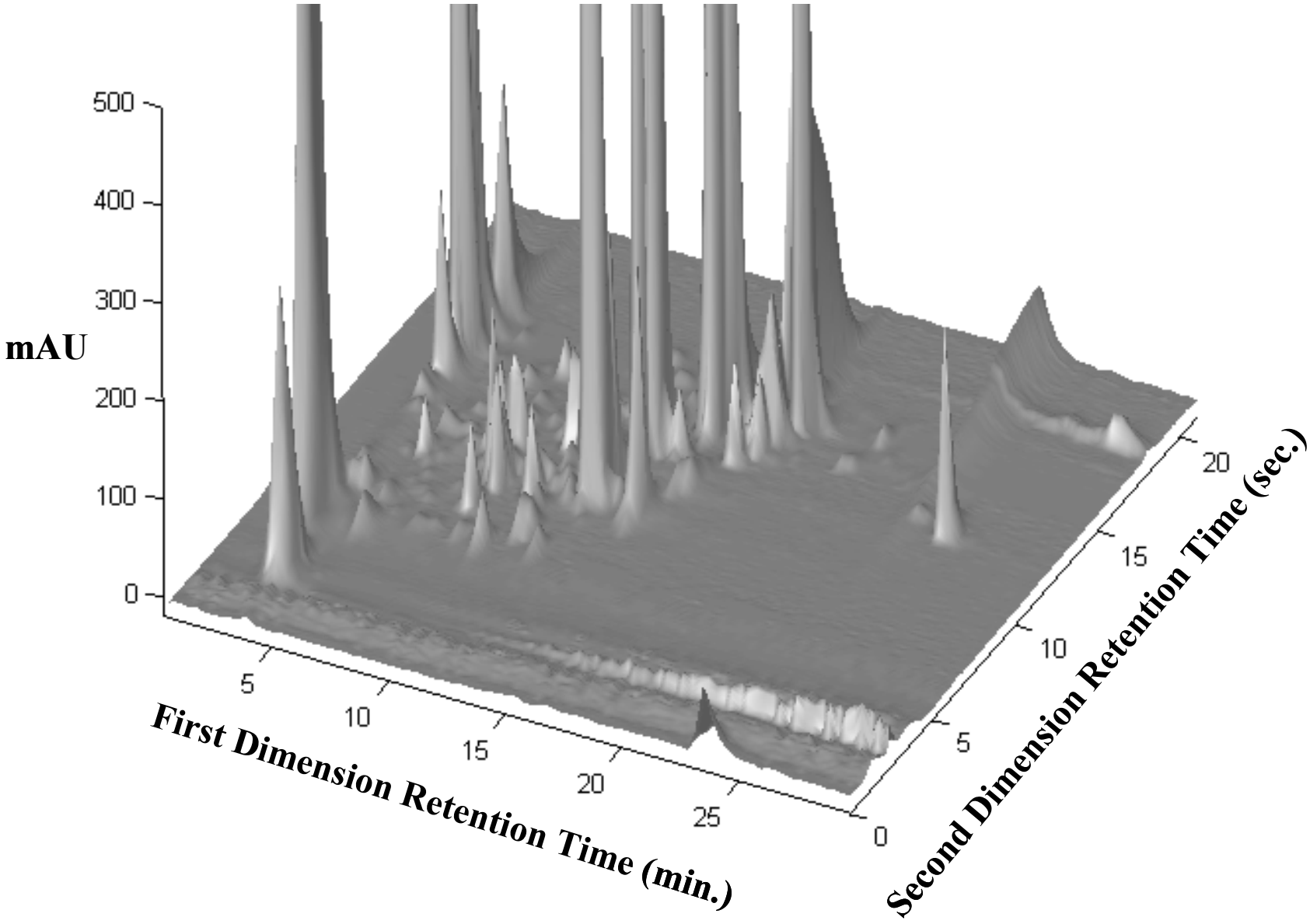
2DLC Separation of 26 Indolic Metabolite Standards



- The first and second dimension retention times are poorly correlated for this set of analytes
- Most of the indolic metabolites are well separated, although there are also some that are highly overlapped


WT A
orp C
2nd Dimension Retention Time (sec.)
mAU
Time (min.)

2DLC Separation of *orp* Mutant Extract with Detection at 220 nm



Peak Capacity and Peak Capacity Production Rate

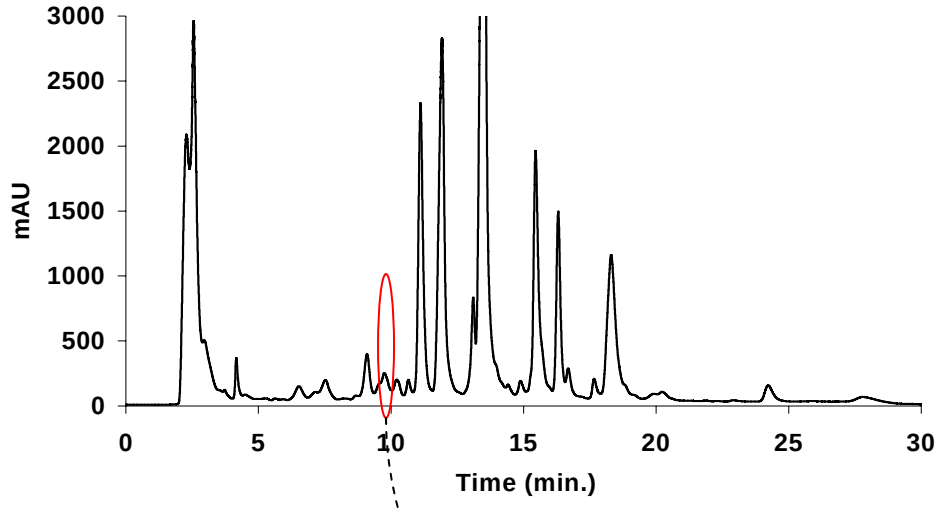
For the maize extract separations,

$$PC_{2D} = PC_1 \times PC_2$$

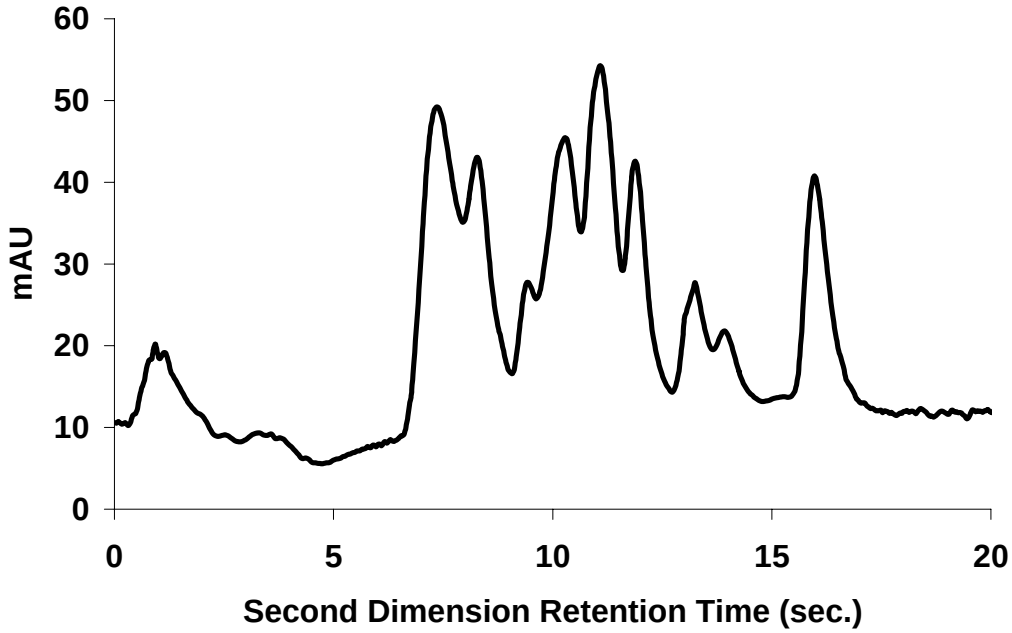
$$PC_1 = \frac{t_{g,1}}{t_{s,1}} \qquad PC_2 = \frac{t_{g,2}}{w_2}$$

PC_{2D} = 1330, PC_{2D}/t = 3070/hr, 1 unit of peak capacity/second

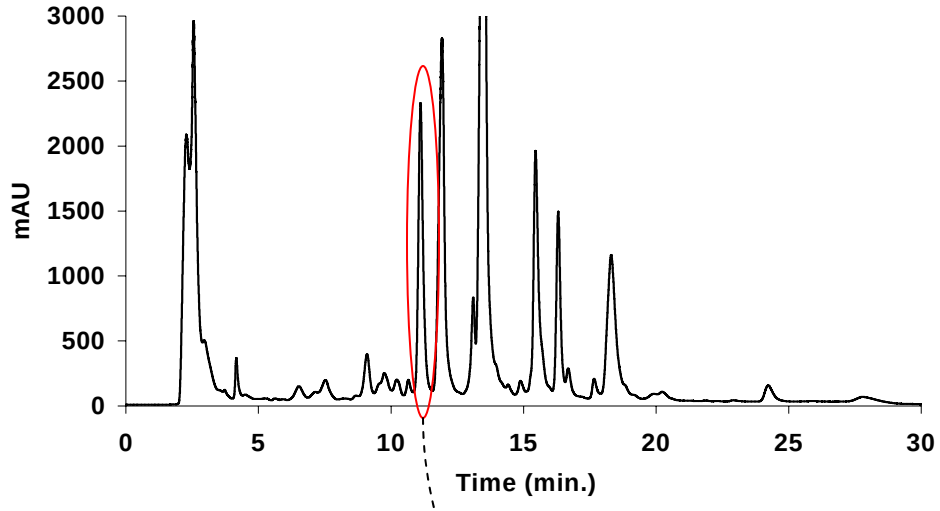
Second Dimension Separations are Rich with Information



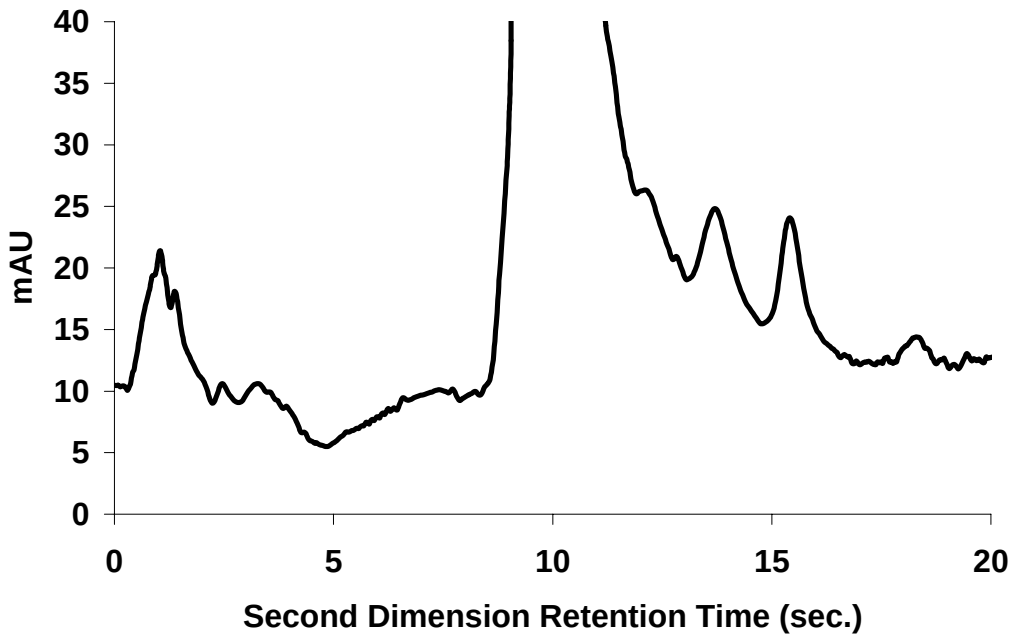
At least nine peaks are observed in the second dimension from single first dimension peak (9.80-10.15 min.)



Increased Peak Capacity Begins to Mitigate the Dynamic Range Problem that Plagues Bioanalytical Separations



Several low abundance species are detected in the 2DLC separation that would otherwise be obscured by high abundance peaks in a one-dimensional separation



Conclusions

1. Ultra-fast reversed-phase gradients with excellent **repeatability of retention time (≤0.003 min.)** are possible.
2. The Murphy, Foley, Schure **sampling rate criteria almost met--each peak shows up in 2-3 consecutive chromatograms**.
3. It is absolutely essential to use the **right pair of columns**. The 2nd column must be very retentive, have different selectivity from 1st column and must be compatible with the sample's inherent dimensionality.
4. A peak capacity of **1330** and peak capacity production rate of **3070/hr (~ 1 peak/second)** has been achieved.
5. More than **200 peaks** are seen in corn seedling extracts.
6. We have shown that high peak capacity **strongly mitigates the dynamic concentration range problem characteristic of biological samples.**
7. **Chemometric methods will be critical to both quantitative and qualitative implementation of 2DLC.**

Acknowledgements

Prof. Jerry Cohen (Department of Horticulture, U of M)

Agilent Technologies (SB-C_{18} column)

Supelco (Discovery HS-F5 column)

ZirChrom Separations (Zirconia column)

Systec, Inc.

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National Institute of Justice