RESTEK Pure Chromatography

The Development of a Virtual Liquid Chromatography Method Development Tool

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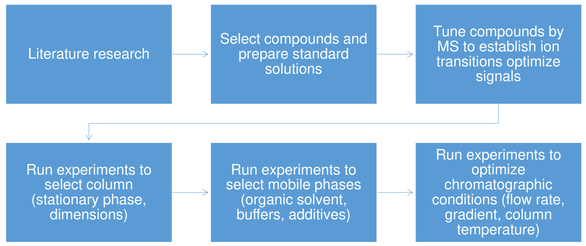
Introduction and Background

Laboratories implementing new methods or optimizing existing methods for improved profitability and efficiency struggle with instrument availability and the time needed to do hands on traditional method development work.

The development and optimization of a Liquid Chromatography (LC) method can be time consuming and costly. Often this requires numerous steps including literature research, column selection, method scouting, method development, and method optimization. In an effort to eliminate these steps, an instrument-free software modeling tool was developed with a comprehensive Drugs of Abuse library (DoA). Users can select compounds from a database and instantly model a separation on different column stationary phases. Optimization of the model can be performed while maintaining critical pair separations by adjusting for instrument/system effects (e.g. dwell volume and extra column volume), mobile phase preferences, number of gradient steps, and more.

Figure 1: Traditional LC-MS/MS method development process.



Due to the number of dimensions in LC method development, the software build focused on six variables, with additional levers to be added at a later time.

To ensure a robust tool, focus was placed on the most commonly used variables of LC method development:

- Column Chemistries
- Column Dimensions and Lengths
- Different Organic Modifiers
- Gradients
- Temperature Changes

Build

All data was collected using a standard HPLC system coupled to a triple quadruple mass spectrometer (LC-MS/MS). Prior to collecting data, a lot check test was completed on three separate 50 mm x 2.1 mm Raptor Biphenyl (2.7 µm) columns. Retention time data was collected using a set of nine compounds, referred to as "meld compounds", that span the chromatographic space. These compounds were run alongside each new library collected to ensure a match to the base library. Data was tabulated in Excel and the percent difference, median, and ±% difference calculated (**Table 1**). With all three lots in agreement, the basis library could be created using one of columns that had been lot check tested.

Table 1: Results of lot check testing for meld compounds.

Column: Raptor Biphenyl 50 mm x 2.1 mm (2.7 µm) Mobile Phase B: Acetonitrile			
Serial Number:	19041756	19053208	19053207
Lot Number:	190134E	200415P	201001P
	Time (min)	**Time (min)**	**Time (min)**
trans-3-Hydroxycotinine	0.41	0.39	0.41
Methylephedrine	1.34	1.40	1.39
Diphenhydramine	3.46	3.48	3.50
Methaqualone	4.19	4.26	4.30
Phenazepam	4.65	4.72	4.76
Norketamine	2.00	2.06	2.07
Levetiracetam	1.19	1.25	1.28
JWH-073	7.10	7.24	7.24
JWH-018	7.37	7.49	7.49
	% Diff	**Median**	**± % Diff**
trans-3-Hydroxycotinine	5.0%	0.40	2.5%
Methylephedrine	4.4%	1.37	2.0%
Diphenhydramine	1.1%	3.48	0.6%
Methaqualone	2.6%	4.25	1.3%
Phenazepam	2.3%	4.71	1.2%
Norketamine	3.4%	2.04	1.7%
Levetiracetam	7.3%	1.24	3.6%
JWH-073	2.0%	7.17	1.0%
JWH-018	1.6%	7.43	0.8%

The basis library consisted of 50 compounds plus meld compounds. Retention times were collected using three different gradient conditions and three different temperatures.

A list of approximately 180 DoA compounds was systematically added to the database. Compounds were required to be divided into small groups to ensure separation of isobars and to generate the optimal points per peak for instrument analysis. This was approximately 30 compounds per group including meld compounds. Retention times were collected and added to the base library.

Verification

To test the modeler, a three-stage verification was completed. Each stage systematically introducing a new source of error. Once retention times were in agreement, advancement to the next stage occurred.

- **Stage 1**: Use a different column dimension from initial library collection and build.
  - A simple gradient condition and ~30 analytes outside of library compounds and different lots of 50 mm x 3.0 mm Raptor Biphenyl (2.7 µm) column. Data was used to develop correction factors.

- **Stage 2:** Use different flow rates, temperatures, gradient slopes compared to initial library collection and build.
  - 50 mm x 2.1 mm Raptor Biphenyl (2.7 µm) column, data used for modeler adjustments and corrections. Moved to the next step once retention times were consistent.
    1. Simple gradient supplied by modeler.
    2. Different flow rates holding temperature and gradient constant.
    3. Different temperatures holding flow rate and gradient constant.
    4. Different gradient slope while holding flow rate and temperature constant.
    5. Repeat steps 1 – 4 on a Raptor Biphenyl 100 mm x 3.0 mm Biphenyl (2.7 µm) column.

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- **Stage 3:** Compare retention time values between wet-lab and modeled data.
  - Re-ran full set of data using both stationary phases (C18 & Biphenyl), multi-step gradients (shallow, step gradients, and isocratic hold), used multiple column dimensions, mobile phases (ACN and MeOH), and different temperatures (30ºC, 60ºC, and a 45ºC verification run).
  - Library created; results were used to compare modeler to validation experiments.

Validation

To test the modeler, determine sustainability, and transferability to different instrument platforms a new set of compounds were used along with the following:

- **Stationary Phases:** Raptor Biphenyl (2.7 µm) and Raptor C18 (2.7 µm)
- **Column Dimensions:** 50 x 2.1 mm, 50 x 3.0 mm, 100 x 2.1 mm
- **Column Temperature:** 40 ºC (Note: both 50 x 2.1 mm also analyzed at 35 ºC and 50 ºC)
- **Mobile Phases:** ACN and MeOH, with 0.1% formic acid
- **Gradients:**

Gradient 1: Linear	
Time	%B
0.00	5
10.00	98
10.01	5
12.00	5

Gradient 2: Isocratic Hold	
Time	%B
0.00	6
1.00	6
10.00	99
10.01	6
12.00	6

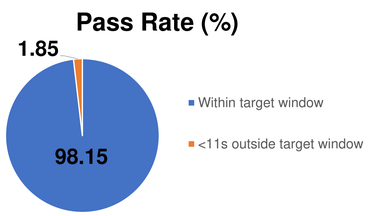
Gradient 3: Multistep	
Time	%B
0.00	7
1.00	30
5.00	45
8.00	80
10.00	95
10.01	7
12.00	7

Performance targets for data collection:

1. Retention time comparison between modeled and experimental runs cannot exceed more than 50% of a standard MRM window (±15 seconds).
2. Data is easily normalized from column-to-column variability and different instrument platforms.

Results and Evaluation

Of the 14 variables analyzed, 704 data points were collected. Only 13 compounds exceeded the target of ±15 second window.

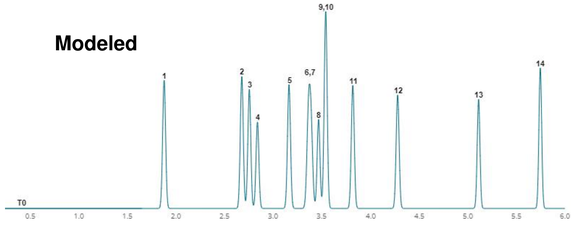


To ensure the modeler performed as expected a set of compounds were chosen to model and test in the lab. Results of the modeled and empirical data show very similar retention times with methamphetamine and phentermine showing improved resolution during empirical conditions (**Table 3**).

Table 2: Modeled vs. in-lab results method conditions.

Column	Raptor Biphenyl		
Dimensions	100 mm x 2.1 mm		
Particle Size	2.7 µm		
Column Temperature	30 °C		
Sample Diluent	Water		
Sample Concentration	100 ng/mL		
Injection Volume	1 µL		
Detector	MS/MS		
Ion Mode	ESI+ (MRM)		
Mobile Phase A	Water, 0.1% formic acid		
Mobile Phase B	Methanol, 0.1% formic acid		
Flow Rate	0.4 mL/min		
Gradient	Time (min)	%A	%B
	0.00	96	4
	7.40	0	100
	7.41	96	4
	9.50	96	4

Modeled



Lab Generated

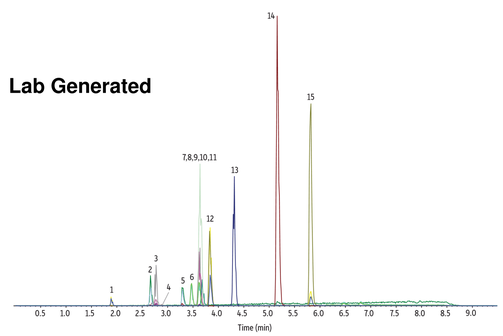


Table 3: Results of empirical vs. modeled data.

Peak #	Compound	Experimental t_R (min)	Modeler t_R (min)	Difference (sec)
1	**Normorphine**	1.89	1.88	0.60
2	**Morphine**	2.66	2.68	1.20
3	**Oxymorphone**	2.77	2.75	1.20
4	**Morphine-N-oxide**	2.88	2.84	2.40
5	**Norcodeine**	3.29	3.16	7.80
6	**Methamphetamine**	3.47	3.36	6.60
7	**Phentermine**	3.62	3.39	13.8
8	**Dihydrocodeine**	3.62	3.47	9.00
9	**Noroxycodone**	3.62	3.51	6.60
10	**O-Desmethyl-cis- tramadol**	3.64	3.54	6.00
11	**Codeine**	3.68	3.54	8.40
12	**Desomorphine**	3.84	3.82	1.20
13	**N-Desmethyltapentadol**	4.31	4.28	1.80
14	**Pentazocine**	5.16	5.11	3.00
15	**Dextromethorphan**	5.82	5.75	4.20

Discussion/Conclusions

An online chromatographic modeling tool was successfully developed that allows users to virtually model 250+ DoA compounds. The modeler was determined to be sustainable, meaning that in the future, additional compounds can be effectively added to the modeler. This is important to keep up with evolving compounds, such as novel psychoactive substances.