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LC-UHPLC Hybrid 2D Platform for LC/MS analysis of Biological Samples: A New Paradigm

Eduard Rogatsky
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Bronx, NY
Critical Review on Recent Developments in Analytical & Bioanalytical Techniques

Liquid Chromatography
HPLC Instrument Development Milestones

1960s-1980s HPLC

1980s-2000s “Conventional” HPLC

2004 - Present
- UHPLC (2009)
- UFLC (2009)
- H-Class UPLC (2010)
UHPLC was introduced 10 years ago. This new technology, while having great promise for improved efficiency and productivity, has not to date displaced HPLC. This transition will be slow process. Currently, both HPLC and UHPLC can be working in the same lab.
UHPLC vs HPLC

UHPLC it is not only a system that’s capable of operating at higher pressures. UHPLC is **optimized** to work with sub 2 micron ultra – high resolution columns to produce very narrow sharp chromatographic peaks.

UHPLC –in contrast to conventional HPLC, is a low dwell volume system designed for fast runs at high pressure.
Current trends in chromatography

- Transition from HPLC to UHPLC
- Evolution of conventional HPLC for full support of Fused-Core chromatographic columns (delay volume reduction)
- Evolution of Liquid Chromatography with Mass Spectrometry analysis.
In July 1886 the newspapers reported on the first public outing of the three-wheeled Benz Patent Motor Car, model no. 1.

1912 BAKER - electric

1912 SELDEN - gasoline

1913 STANLEY - steam
LC-UHPLC Hybrid 2D Platform for LC/MS analysis of Biological Samples: A New Paradigm

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Hybrid

Something that is produced or formed by combining two [or more] things of different kinds.

Or: that has two different types of components that performing essentially the same function.

Or: mixture of different things or styles.
Hybrid Citrus Fruits: A. and B. Eureka and Meyer’s Lemons (*Citrus limon*); C. Tangerine or Mandarin Orange (*C. reticulata = C. nobilis*); D. and E. Navel and Valencia Oranges (*C. sinensis*); F. Tangelo (*C. x tangelo*). The tangelo is a hybrid produced by crossing a Tangerine (*C. reticulata*) with a Grapefruit (*C. x paradisi*). The grapefruit (*C. x paradisi*) is another hybrid between the Shaddock or Pomelo (*C. maxima*) and the Sweet Orange (*C. sinensis*).
HPLC is also hybrid

- HPLC is an apparatus-hybrid machine - composed of autosampler, pump, column thermostat and detector…regardless – they are in the same box or in separate modules.
- HPLC is a symbiosis and not one unit [an inlet].
HPLC performance

- HPLC performance is mostly based upon correct choice of flow rate, operating pressure, dwell/post-column volume and chromatographic column.
- Choice of these parameters is not module-based. Correct choice is system-based.
HPLC is also hybrid: pressure and flow rate impact

<table>
<thead>
<tr>
<th>LC devices</th>
<th>Critical components and parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Autosampler</td>
<td>• Injector valve, wash port, sample loop size</td>
</tr>
<tr>
<td>• Pump</td>
<td>• Purge valve, mixer</td>
</tr>
<tr>
<td>• Column thermostat</td>
<td>• Heat exchanger</td>
</tr>
<tr>
<td>• Detector</td>
<td>• Switching valve</td>
</tr>
<tr>
<td></td>
<td>• Flow-cell design, pressure and volume</td>
</tr>
</tbody>
</table>
LC column: Increase backpressure

Column frit: contamination by rotor seal wear debris
Rotor seal dust
Delay volume-pressure impact: 15% drop of mixing efficiency

Upchurch filter in the pump, no damper, valve, restrictor, Flow rate 0.7 ml/min

Blue: Low Pressure $V_0$ 0.318 ml; $V_{50}$ 0.497ml
Red: High Pressure 344Bar, $V_0$ 0.305 ml; $V_{50}$ 0.580 ml

The more shallow slope results in a larger $V_{50}$, [poor mixing] as well as smaller $V_0$
V50 increase with pressure

- MeOH
- Water
Summary

- New experience: moving parts and mixers are less rugged and efficient in high pressure applications.
Old 2D LC: Agilent 1100 and 1200 series
UHPLC 2D/MS Agilent 1290 series
2D and LCMS

2D chromatography – efficient way to increase peak capacity. It is important for UV applications, but in general, not for LC/MS applications.

MS detection utilizes ions resolution by m/z and chromatographic peaks overlay is often in LC/MS analysis.

Benefits of 2D in MS:
- Minimum of signal suppression
- Better background
- Better peak shape
- More clean instrument
Matrix effect reduction - One column method
SPE AcN/Phosphate sample

XIC of +Q1 SIM (3 ions): 1007.7 amu from Sample 10 (Luna only, Eduard plasma SPE/KPi) of 7222...

Max. 2.1e4 cps.

S/N p/p = 16.1
Matrix effect reduction - Two column method
SPE AcN/Phosphate sample

S/N p/p = 201.4

12.6 fold improvement
Chromatography myth

- Single column chromatography is always more robust, compared to column switching applications. That’s true – if we are comparing standard pressure ranges.
- High pressure operations are less robust and more expensive - compare to standard LC
- New paradigm: low pressure injection by standard LC and UHPLC chromatography.
The sample was injected by a standard (not UHPLC) autosampler into a pre-analytical column, where it was desalted and partially purified. A standard pressure range binary HPLC pump was used. The fraction containing analytes of interest was transferred through a UHPLC valve to a fused core column. A fast gradient was performed using a UHPLC 1290 pump.
Online SPE design

LCxUHPLC design based on HPLC loading pump and UHPLC LC/MS pump
Human C-peptide
Plasma SPE

Counts vs. Acquisition Time (min)
Urinary cortisol and testosterone – single column

10 ul urine, 1:3 diluted

cortisol

Testosterone
Urinary cortisol and testosterone - trap

10 ul urine, 1:3 diluted cortisol

Testosterone
RT 3.5 min; was 5
MMA from plasma
1D: trap + column
MMA from plasma
1D: trap + column
MMA from plasma 2D: trap loading + column switching

XIC of +MRM/4 pairs: 231.000/119.000 Da ID: mma ether from Sample 11 (2D Plasma 7 ul) of 08042014SET1.wiff (Turbo Spray) Max. 5.8e4 cps.
MMA from plasma 2D: trap loading + column switching

Column switching improves S/N from 214 to 334; aprox. 40% gain
Summary 1

- Hybrid LCxUHPLC is more rugged column switching system, compared to 2D UHPLC
- UHPLC sample loading is limited
- Sample loading to trap, using standard LC pump and autosampler is more convenient and cost efficient
- Loading and wash step using trap greatly improve ruggedness of mass spectrometer operations
Hybrids typically have:
More functionality
Higher cost, compare to its components,
Only basic control and features
Less ruggedness
Limited support

Hybrid LCxUHPLC is free from these disadvantages
Take Home Message

We successfully used LCxUHPLC platform for LC/MS analysis. Instead of retiring an entire functioning Agilent 1100 LC system, we just added one UHPLC pump to achieve much greater overall performance, functionality and lower cost, compared to a single pump UHPLC system purchase.
The addition of a UHPLC valve and pump to a standard 1100/1200 series LC system (autosampler, pump and column compartment) greatly extends operational flexibility including column selection, while standard LC – which is already available in the lab, performs the initial steps of sample loading and clean-up.
Traps and guards: choice of selectivity, performance and cost
Trap column selection

Commonly accepted “rule” of HPLC method development suggest:
Guard column made from the same material as the main analytical column

That’s true, if there is no valve between guard and column
Choice of trap becomes critical; it is a NEW CENTER of LC configuration

Sorbitol analysis from biological samples by liquid chromatography - mass spectrometry
Carbohydrate detection by Cs$^+$ attachment

XIC of +MRM (4 pairs): 225.0/133.0 amu from Sample 1 (Mixed carbohydrates) of 4292004SET1...

Max. 1.7e6 cps.

1: Glycerol, 40 ng
2: Ribose, 50 ng
3: Xylose, 50 ng
4: Fructose, 50 ng
5: Glucose, 40 ng
6: Sucrose, 50 ng
7: Lactose, 50 ng

Time, min

0.0 1.0e5 2.0e5 3.0e5 4.0e5 5.0e5 6.0e5 7.0e5 8.0e5 9.0e5 1.0e6

3 5 6 7

Cps

3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5
Glucose (MW 180) and sorbitol (MW 182)
Sorbitol LC analysis from plasma:
Implications of glucose presence

- Sorbitol concentration in plasma < 1µM
- Glucose concentration is ~ 5 mM (> 5000 fold concentration difference).
- Incomplete resolution of these analytes results in overlap of sorbitol (M=182) with m+2 glucose (M=182) (APE 1.45%)
- Potential 70 fold signal contamination!
Brand new 3µ Luna Amino column, 2 x 150 mm, 2.5µl/inject

New Amino column; Sample – Urine, 1/20 in Acetone
-- U-^{13}C Sorbitol; Glucose (m+2)
Amino column: degradation of resolution

Same sample to same column after 50 injections of urine samples.

- U-13C Sorbitol;
- D2 Sorbitol;

Glucose (m+2)
Typical column lifetime

Clean samples: 1000 - 2000 injections; column cost per analysis is about $0.20, Representing only about 1% of total cost per analysis

More complex samples: 200 - 500 injections; column cost per analysis is about $1, Representing about 4% of total cost per analysis

Biological samples: 50 - 200 injections; column cost per analysis is about $3, Representing about 10% of total cost per analysis

Practical HPLC method development, Second edition.
L. R. Snyder, J. L. Kirkland, J.L. Glajch. p 211.
Part 3. Current strategy and solution to the problem of glucose and sorbitol separation

Since the simultaneous separation of sorbitol from glucose, together with MS detection on one column is impossible, we are have decided to perform off-line separation of sorbitol from glucose.

Our new analytical solution is based on 2D LC pre-purification of sorbitol from glucose and other impurities in plasma samples. We will show that this scheme has significant advantages for MS detection versus “classic” single column methods.
Pre-purification of sorbitol from plasma extract

Purification was performed on 2 guard cartridges of REZEX Lead (Phenomenex) 7.8 x 50 mm
Off-line sorbitol purification: method development challenges

- Sorbitol can be separated from glucose on Ion Exchangers, charged by Calcium, Sodium, Silver or Lead. These columns widely used in food analysis for carbohydrate analysis.

- Plasma extract contains significant amount of lipids which bind tightly (poisoning) an anion exchanger. Regeneration from lipid contamination requires column wash by organic solvent.

- Typical carbohydrate IEX resin does not tolerate organic solvents. The problem: routine analysis of plasma samples is not robust, since the column cannot be regenerated after plasma lipid contamination.

This problem was resolved by using 2D LC technique.
Offline 2D pre-purification of sorbitol: Valve Position 2

Column compartment G1316A (Agilent), 80°C

Valve position: 2

AQUASTAR C18 column 1

REZEX Lead column 2

Waste

Fraction Collector

Waste
Offline 2D pre-purification of sorbitol:

阀位1

柱子G1316A (Agilent), 80℃

阀位置：1

AQUASTAR C18

REZEX 胶

分流器

废物
2D LC method development summary

- Commonly accepted “rules” of HPLC method development suggest:
- 1. Guard column made from the same matrix as the main analytical column.
- 3. The primary purpose of 2D technique development is a sensitivity enhancement of on-line analysis (by UV or MS) and as a rule, not used for fraction collection.
- In our method, all these 3 rules were inverted 180°. We demonstrate how opposing “common sense” can resolve difficult problems specific to bioanalytical mass spectrometry.
Conclusions: MS

- Efficient chromatography plays a greater role in optimizing mass spectrometry sensitivity than advanced MS tuning.

- For LC/MS analysis from complex matrices - LC it is not just an “inlet” – it is the key first step toward increased mass spectrometry sensitivity.
Acknowledgements

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Technical Support:
Agilent Technologies
IDEX corporation/Upchurch scientific
Optimize Technologies
Good seal:
no excess volume