Choosing LC Columns and Sample Prep Options for Biological Matrices

Jason Link, PhD
Overview of Biological Matrices

• **Samples include:**
  • Small molecule analytes

• **Matrices are biological fluids**
  • Plasma
  • Blood
  • Urine
  • Oral Fluids

• **Fast analyses, high-throughput sample processing**
  • Minimize column plugging to increase column lifetime
  • Reduce re-runs and repeat samples
  • Get the answer right, the first time, every time
  • Consistent performance, day in and day out
LC and Sample Prep Method Development for Biological Matrices

What’s different in LC analysis with biological matrices?

• Sample matrix complexity
• Multiple components of interest
• Utilized along with MS detection

Modern column and sample prep technologies can make these analyses faster, while minimizing workflow interruptions!
Striking the Right Balance in Sample Preparation

Quality of Results

Effort & Investment

Just Right

Ideal

Realistic

For Forensic Use.
Agilent Sample Preparation Products

Bond Elut Solid Phase Extraction

- Bond Elut Plexa Polymeric SPE
- Bond Elut Certify SPE
- SPEC disc SPE
- Bond Elut QuEChERS

Chem Elut SLE

- Chem Elut SLE
- Tox Elut SLE
- Chem Elut Plus SLE
- Combilute SLE
- Hydromatrix

Captiva Filtration

- Captiva Syringe Filters, cartridges, and plates
- Captiva Non-Drip and ND Lipids cartridges and plates
Sample Preparation Considerations

We often talk about a “triangle” – but the questions about sample prep and SPE are more complex than this simple model.

Other Sample Prep Considerations
- Analytical goals
- Published methods
- Instrument availability
- Skill and expertise
- Regulations
- Sample Size
- Detection limits
- Cost per sample
- Lab setup and supplies – investment
- Automation needs
Practical Approaches to Selecting a Sample Preparation Method

- Interferences to Remove
- Application Type
- Matrix or Sample
- Format for Automation
- Current Product Alternative
- Journal Article, Application Note, or Book
- Interactive Selection Guides

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Sample Preparation Selection: Interference Removal Needs

<table>
<thead>
<tr>
<th>Interference Removed</th>
<th>Sample Prep Technique</th>
<th>Dilute &amp; Shoot</th>
<th>Filtration</th>
<th>Liquid/Liquid Extractions</th>
<th>Supported Liquid Extractions (SLE)</th>
<th>Precipitation filtration</th>
<th>QuEChERS</th>
<th>Precipitation-Lipid Removal ‘Hybrid’ Filtration</th>
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</table>

Filtration is suggested with any LC or GC method of sample preparation

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Agilent Technologies
Column Technologies for Method Development

Columns for high resolution and high speed analysis

• Sub-2 µm columns for ultra-high pressure operation
• Sub-3 µm superficially porous columns

Considerations when developing methods on new column technologies

• Particle size < 3 µm
• Column pressure limits >400 bar (600-1200 bar typical)
• Other factors remain same as for legacy, 5 µm columns
Superficially Porous Column Technologies

Poroshell 120 columns:

- Efficiency ≈ 90% of sub-2 μm
- Pressure ≈ 40-50% of sub-2 μm
- N ≈ 2X 3.5 μm (totally porous)
- \( d_p = 2.7 \mu m \)
- 2 μm frit to reduce clogging
- \( P_{\text{limit}} = 600 \text{ bar for HPLC or UHPLC} \)
- Particles
  - 1.7 μm solid core
  - 0.5 μm diffusion path
  - 2.7 μm total diameter
Poroshell 120 Resists Plugging with 2 µm Frit
Challenging Plasma Sample

Column: Poroshell 120 EC-C18, 3.0 x 50mm, 2.7µm     LC: Agilent 1200 RRLC (SL)
Sample: Precipitated Plasma: 2 parts Plasma: 7 Parts 20/80 Water-MeCN w/0.1 % Formic Acid with 1 Part Diflusinal in 50/50 Water-MeCN 10 ug/ml (Final concentration Diflusinal 1 ug/ml) Shaken and allowed to settle 10 minutes
Not Centrifuged/ Not Filtered
Injection Volume: 1ul injections

Diflusinal in Plasma

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Poroshell 120 Column Chemistries

Poroshell 120 EC-C18 and C8
• Robust endcapped C18 for best peak shape at pH 2-9

Poroshell 120 Stablebond C18 and C8
• Robust chemistries for pH<2

Poroshell 120 Phenyl-Hexyl
• Same Eclipse Plus bonding process as ZORBAX Eclipse Plus Phenyl-Hexyl
• Excellent choice for pi-pi interactions
• Alternative selectivity to EC-C18 or SB-C18
• Selectivity similar to phenyl, diphenyl, or other phenyl-hexyl columns

Poroshell 120 SB-Aq
• Proprietary bonding phase is an excellent choice for polar analytes

Poroshell 120 Bonus-RP
• Embedded polar group provides unique selectivity for polar compounds

Poroshell 120 EC-CN
• Flexible endcapped CN chemistry with Normal and Reversed Phase character

Poroshell 120 HILIC
• Bare silica HILIC for use in Hydrophilic interaction chromatography of polar molecules

Poroshell 120 PFP
• Perfluorophehnyl chemistry for orthogonal selectivity relative to C18

10 phases!
Use of Guard Columns and Inline Filters

• Inline filters and guard columns extend the life of HPLC columns by preventing particulates and impurities from clogging and potentially irreversibly sticking to the analytical column

• Column lifetime is extended

• $$$ savings from fewer analytical columns purchased

• Minimal, if any, impact to the chromatography!
Benefits of Installing a Fast Guard for UHPLC

Method: Accelerated Lifetime Test - Similac sample (milk substitute diluted 300:1) containing 2 sulfa drugs; Peak width change indicating column failure

No Guard
Column failure; new column required

With Guard
Guard failure; guard replaced; same column used throughout analysis

By installing a guard column when using dirtier samples, one can extend the life of their column, and utilize more inexpensive guard columns rather than column replacements.
Other Considerations when Selecting a Column

- Robustness and batch-to-batch reproducibility of Poroshell 120 columns

Beverage Additives
Agilent LC Column and Sample Preparation Navigator Tool

www.agilent.com/chem/navigator
Agilent is Here to Help

See www.agilent.com/chem/cstechsupport

Agilent is committed to helping you succeed with your application. Send us an email with your questions and a technical support representative will contact you, as soon as possible, usually within the day, provided you are emailing during business hours in your region. However, please allow up to 48 hours for a reply.

**Helpful Tips:** Provide as much information as possible regarding the instrument you are using, your sample type, the column you are using now and what you are trying to achieve.

**TECHNICAL SUPPORT CONTACTS**

- **US, Canada** – please call 800-257-9776 or email LC-column-support@agilent.com for LC columns support and sample prep support@agilent.com for sample prep support
- **Mexico, Brazil, Argentina and other countries in S. America** – please contact your preferred distributor (see www.agilent.com/chem/contactus for more information)
- **Europe, Middle East and Africa** – please contact your distributor (see www.agilent.com/chem/contactus) or email	sales@agilent.com
- **India** – please send an email to columns_helpdesk@agilent.com
- **Singapore, Malaysia, Australia, Korea, the Philippines** – contact your local distributor (see http://www.chem.agilent.com/en-US/ContactUsPages/Singapore.aspx) or send an email to ccs-sing@agilent.com
- **Japan** – please call 0120-477-111 or email emall_japan@agilent.com
- **China** – please call 000-820-2276-4
Opiates, Opioids and Benzodiazepines, Amphetamines & Illicit Drug Forensic Analysis by LC/MS

Julie Cichelli, PhD
Agilent Technologies
Application Engineer
April 29, 2014
Agenda

• A method for the rapid analysis of over 65 analytes in a single LC/MS analysis run
• Simplified method development through the use of Dynamic MRM (dMRM) and databases
• Qualitative and Quantitative data analysis
• Customized Reporting
Targeted Analysis of Over 65 Analytes for Forensic Toxicology

- An extensive screen and quantification in 5 to 6 minutes
- Internal standard corrected quantification
- Multi-point calibration curve covering a wide dynamic range
- Secondary qualifier ion for each analyte
- Simple sample preparation:

  ![Sample → Hydrolyze → Dilute → Analyze](image)

For Forensic Use.
Chromatographic Separation of Over 65 Analytes

Isobaric Mass Ions:
different ions that have identical mass
Poroshell 120 is a **high efficiency, high resolution column choice** for enhancing productivity in LC and LC/MS

**Poroshell 120 Columns have:**
- 80-90% efficiency of sub-2µm columns
- ~40-50% lower pressure
- 2x efficiency of 3.5µm (totally porous)
- A 2µm frit to reduce clogging
- A 600 bar pressure limit for HPLC or UHPLC

- The superficially porous particle is 2.7µm with a solid core (1.7µm) and porous outer layer with a 0.5µm diffusion path
Poroshell 120 Performance After 3000 Injections

- Dilute and shoot sample preparation
- Analytes covering a wide range of retention times show excellent reproducibility

<table>
<thead>
<tr>
<th>Analyte</th>
<th>%RSD (RT)</th>
<th>Analyte</th>
<th>%RSD (RT)</th>
<th>Analyte</th>
<th>%RSD (RT)</th>
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<td>Triazolam</td>
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<td>Codeine</td>
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<td>Naltrexone</td>
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<td>hydrocodone</td>
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<td>Fentanyl</td>
<td>0.1</td>
<td>chlordiazepoxide</td>
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<td>MDMA</td>
<td>0.3</td>
<td>EDDP</td>
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<td>norFentanyl</td>
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<td>Nitrazepam</td>
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<td>Buprenorphine</td>
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<td>Heroin</td>
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<td>Propoxephine</td>
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<td>Cocaethylene</td>
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<tr>
<td>Methyl Phenidate</td>
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<td>Buprenorphine</td>
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<td>11-nor-9-carboxy-delta9-thc</td>
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</tbody>
</table>
The Need for Dynamic MRM (dMRM)

- Nearly 200 MRM transitions for analytes, qualifier ions, and internal standards
- Monitoring all MRM transitions over the entire run results in poor quantification due to short dwell times and long cycle times
- dMRM only monitors each transition during the appropriate retention time window
Easy LC/MS Method Creation and Customization

A Database containing over 2500 compounds each with multiple MRM transitions ensures fast method creation for development.

Quickly establish screening methods for complex matrices using these leading-edge technologies:

A Dynamic MRM database with more than 200 compounds, plus Agilent MassHunter Data Acquisition and Analysis software, let you quickly generate acquisition and analysis methods, which can be modified to meet your future needs.

The Agilent 1200 Series SL Rapid Resolution LC, interfaced with Agilent’s 6400 Series Triple Quadrupole LC/MS System delivers fast, high-resolution LC/MS/MS analysis.

Agilent’s Jet Stream Electrospray lowers detection levels of analytes in complex matrices.

Dynamic MRM maximizes the Quadrupole’s detection capability – without sacrificing sensitivity – when a large number of compounds are being analyzed at femtomole concentrations.
Qualitative Screening
Find By MRM

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Quantitative Analysis
Batch at a Glance
Data Review with Compounds at a Glance
Quantitative Analysis
Calibration Curves

- **Carisoprodol**
  - \( R^2 = 0.996 \)

- **EDDP**
  - \( R^2 = 0.998 \)

- **Propoxyphene**
  - \( R^2 = 0.996 \)

- **Fenfluramine**
  - \( R^2 = 0.999 \)

For Forensic Use.
Customizable Reporting

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Conclusions

• Agilent makes it easy to create custom analytical panels for the measurement of Opiates, Opioids, Benzodiazepines, Amphetamines & Illicits

• Panels can be comprehensive or focused to suit your needs

• Dynamic MRM functionality, automatic optimization and MRM transition Database help make analytical method development straightforward

• Results can be achieved with a simple Dilute & Shoot sample preparation

• Agilent’s MassHunter software is optimized for your data review and reporting workflow
Ultrafast SPE/MS Analysis of TCAs in Serum

Vaughn Miller
RapidFire Applications Manager
Today’s Agenda

RapidFire/MS features and benefits

Demonstration data
- Tricyclic antidepressants in serum in clinical research

Summary

Follow-up information
What is RapidFire/MS?

Ultrafast autosampler & online SPE system
- Replaces LC in LC/MS
- Reusable SPE cartridge
- Integrates with standard ESI MS instruments (QQQ & TOF)
- Cycle time = 8-15 s/sample

Compatible with biological matrices
- Microsomal incubations
- Cell culture media
- Serum, plasma or whole blood
- Urine

Agilent Web Link
RapidFire Decreases Data Acquisition Time

2-10 min/sample

- Faster speed to results
- Increased analysis capacity
- Money saver

8-15 sec/sample

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Demonstration Data

Tricyclic Antidepressant (TCA) Research Drug Panel in Serum
Tricyclic Antidepressant Drug Panel in Serum
8 Analytes, 20 MRMs

Sample preparation
• MeOH/ZnSO₄ crash
• 1:10 dilution (water)

RapidFire analysis
• RapidFire 300 + Agilent 6460 Triple quad
  – Solvent A: 0.1% formic acid in water; 1.5 mL/min
• Solvent B + C: 0.1% formic acid in methanol; 1.25 and 0.8 mL/min
• C18
• Total sample cycle time = 13 sec
• LOQ = 10 ng/ml

<table>
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<tr>
<th>Compound Name</th>
<th>Precursor Ion</th>
<th>Product Ion</th>
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<tr>
<td>Clomipramine_d3</td>
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<td>89.1</td>
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<td>Clomipramine Q</td>
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<td>86.1</td>
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Analysis Speed and Carryover

Clomipramine
Norclomipramine
Imipramine
Norclomipramine
Amitriptylene
Desipramine
Nordoxepin
Nortriptylene

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Calibration Curves
Linear Range from 10-500 ng/mL

Amitriptyline
Imipramine
Doxepin
Clomipramine
Nortriptyline
Desipramine
Nordoxepin
Norclomipramine

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# Inter and Intraday Accuracy & Precision

- **Utak Laboratories QC standards**
- **Coefficient of variation values were all < 8%**

<table>
<thead>
<tr>
<th></th>
<th>Interday % Accuracy (n=6)</th>
<th>Interday % Precision (n=6)</th>
<th>Intraday % Accuracy (n=6)</th>
<th>Intraday % Precision (n=6)</th>
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<td>100.9</td>
<td>5.0</td>
</tr>
</tbody>
</table>

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Reproducibility Study

2000 injections of the same sample showing robustness of the RapidFire system, SPE cartridge lifetime and consistency in area counts and quantitation for the drugs in the panel.

Norclomipramine

% Precision = 3.12
RapidFire/MS Clinical Research Methods

Serum or whole blood matrix, <16 seconds/sample

Quantitative Analysis

• Antiepileptic panel
• Tricyclic antidepressant panel
• SSRI panel
• Clozapine/norclozapine
• Antifungal panel
• SISCAPA peptide analysis
• AssayMAP protein analysis

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RapidFire/MS Forensic Toxicology Methods

Urine matrix, <16 seconds/sample

**Quantitative Analysis**
- Methadone & EDDP
- Benzodiazepine panel
- THCCOOH
- BZE
- Z-drugs panel
- Buprenorphine & Norbuprenorphrine
- Gabapentin & Pregabalin
- Cotinine in urine or serum

**Qualitative Analysis**
- Amphetamine panel
- Bath salts panel
- Synthetic cannabinoids panel
- Barbiturates panel
- TOF panels – coming soon
The Value of RapidFire/MS

SPE/MS results are comparable to LC/MS for many applications

• Linearity
• Accuracy & precision
• Reproducibility

Advantages of RapidFire/MS

• Fastest Time to Result
  - < 20 seconds per sample
  - > 250 samples/hour
  - > 5,000 samples/day

• Increased Capacity
  - Able to analyze 1000’s of samples per day on a single system

• Cost efficient
  - Lower operating cost and smaller lab footprint than multiple MS systems
  - Least expensive way of analyzing hundreds of samples (or more) per day
    • Direct cost < $0.1/sample

• Excellent tool for development of new drug analyte panels
  - More sensitivity and specificity without the interference found in traditional methods
  - Ability to create new panels quickly (i.e. designer drugs)
Questions?

RapidFire Applications Manager
• vaughn.miller@agilent.com
• 781-928-2758

Agilent Web Site
• RapidFire 365 home page (link)
• RapidFire clinical research video (link)
• Productivity calculator (link)
  - Compare LC/MS to RapidFire/MS: capital cost & turn around time
• Brochure
  - Pharma discovery focused, but specs of instrument (link)

Review article: SPE/MS technology
• Bioanalysis, 2012
  - SPE-MS analysis of absorption, distribution, metabolism and excretion assays: a tool to increase throughput and streamline workflow