Trends in Sample Preparation for Chromatography

Presented by

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USA
Sample clean-up a nightmare?

- Additional labour, matter and instrumental needs
- Plus 60% time consumption
- Plus 30% error
Sources of Error Generated During Chromatographic Analysis

- Contamination (4%)
- Sample Introduction (6%)
- Chromatography (7%)
- Integration (6%)
- Instrument (8%)
- Calibration (9%)
- Columns (11%)
- Operator (19%)
- Sample Processing (30%)

(R.E. Majors, LC/GC Magazine, 2002)
Time Spent on Typical Chromatographic Analysis

Sample Processing (61%)

Data Management (27%)

Collection (6%)

Analysis (6%)

(R.E. Majors, LC/GC Magazine. 2002)
Tribute to Academics Working Primarily in the Area of Sample Preparation

- K.-S. Boos, University of Munich, Germany
- L.G. Blomberg, Karlstad Univ., Sweden
- U. Brinkmann (emeritus), H. Irth and H. Lingeman, Free Univ. of Amsterdam, The Netherlands
- M.F. Burke (emeritus), Univ. of Arizona, USA
- J. Haginaka, Mukogawa Women’s Univ., Japan
- M.-C. Hennion, V. Pichon, ESPCI, France
- J.A. Jonsson and L. Mathiasson (emeritus), Univ. Lund, Sweden
- H.K. Lee, Univ. of Singapore, Singapore
- J. Pawliszyn, Univ. of Waterloo, Canada
- S. Petersen-Bjergaard and K.E. Rasmussen, U. Oslo, Norway
- B. Sellergren, Tech. Univ. Dortmund, Germany
- Countless others who have devoted research to sample prep
Outline of Sample Prep Presentation

• Overview of Trends
• Liquid-liquid extraction
• Solid-phase extraction
  • Formats
  • Chemistries
• Automation
<table>
<thead>
<tr>
<th>TREND</th>
<th>EXAMPLES</th>
<th>IMPLICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smaller samples encountered</td>
<td>• Life sciences, proteomics studies</td>
<td>Lower bed mass, less solvent, faster results, less evaporation time in SPE</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
| Simpler methods--“Just enough” sample prep | • Protein crashing instead of SPE  
• QuEChERS instead of lengthy multi-step processes  
• Simple LLE | Cuts down on # of sample prep steps (e.g. less error (better data), faster results, higher recovery)  
Continued growth of MS-MS for LC, CE and GC applications |
|                               | • Reduced use of organic solvent (e.g. SPME, hot water extractions)      | Better for environment, less exposure of workers to toxic solvents, lower purchase and disposal costs |
|                               |                                                                           |                                                            |
| High throughput               | • SPE pipette tips, 96-well plates  
• On-line extraction  
• Multi-functional autosamplers | Seamless integration to analysis  
Favors different formats more attuned to automation (e.g. pipette tips, 96-well plates) |
|                               |                                                                           |                                                            |
| More selectivity              | • Immunoaffinity sorbents  
• Molecularly-imprinted polymers (MIPs)  
• RAMs | For use with less specific and less sensitive detectors than MS-MS (e.g. UV) |
|                               | • Mixed mode sorbents  
• Polymeric SPE packings | Higher capacity  
More rugged phases |

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Time Consuming and Laborious Liquid-Liquid Extraction

Typical Separatory Funnels

Manual labor—vigorous shaking

Time—Waiting for layers to separate
New Formats for LLE

• Microextractions
  — Liquid phase microextraction (LPME)
  — Single drop microextraction (SDME)
  — Dispersive liquid-liquid microextraction (DLLME)

• Microextractions with addition of hollow fiber membranes
  — Supported liquid membranes (SLM)
  — Electromembrane extraction (EME)

• Supported Liquid Extraction (SLE)
Schematic of a Single Drop LPME Apparatus

Chromatographic microsyringe

Solvent drop
Extraction vial
Water bath

Stir bar
LPME Can Be Automated
Dispersive Liquid–Liquid Microextraction (DLLME)

- Based upon a three-component solvent system.
- Extraction vessel is usually a centrifuge tube.
- Mixture of immiscible organic extraction solvent (e.g. 10’s-μL of tetrachloroethylene) and a dispersive solvent (e.g. 1-mL acetone) injected rapidly into an aqueous solvent (~ 5 mL) with a syringe.
- Forms cloudy mixture—due to finely dispersed extraction solvent droplets.
- Extraction is instantaneous; no shaking is needed.
- Mixture is centrifuged (e.g. 1.5 min at 6000 rpm) and extraction solvent sedimentates to bottom of tube and is removed with syringe.
Extraction of Pesticides from Water using DLLME: Extraction Solvent Selection*

*S.S. Caldas, F.P. Costa, and E.G. Primel, XII Congresso Latino-Americano de Chromatografia E Technicas Relacionadas (Colacro XII), Florianopolis, Brazil, October 27–30, 2008, Poster Tu-145
Optimization of Volume of Extraction Solvent in DMLLE of Pesticides in Water*

*S.S. Caldas, F.P. Costa, and E.G. Primel, XII Congresso Latino-Americano de Chromatografia E Technicas Relacionadas (Colacro XII), Florianopolis, Brazil, October 27–30, 2008, Poster Tu-145
Investigation on the Type of Dispersion Solvent*

*S.S. Caldas, F.P. Costa, and E.G. Primel, XII Congresso Latino-Americano de Chromatografia E Technicas Relacionadas (Colacro XII), Florianopolis, Brazil, October 27–30, 2008, Poster Tu-145
Overall Results of DLLME Study of Pesticides from Water*

- The variables in method development included: choice of dispersion solvent and its volume, choice of extraction solvent and its volume, pH if necessary, and centrifuge speed.
- Volume of water: 5 mL containing phosphoric acid, pH 2
- Extraction Solvent: 60-µL of carbon tetrachloride
- Dispersive solvent: 2 mL of acetonitrile
- For all three pesticides, the linear range was found to be 0.001-1.0 mg/L and limit of quantitation (LOQ) was 0.02 mg/L.
- Overall, the DLLME technique is simple, fast, provides good recovery, is low cost, and provides good enrichment factors.

*S.S. Caldas, F.P. Costa, and E.G. Primel, XII Congresso Latino-Americano de Chromatografia E Technicas Relacionadas (Colacro XII), Florianopolis, Brazil, October 27–30, 2008, Poster Tu-145
Microscale Automation of Sample Prep using Modern Autosampler (Agilent 7693A)

Example, LLE using 2-mL vial

1.0 mL Water sample + 0.5 mL Pentane Vortex Sample withdrawn and injected
1) Apply aqueous sample so that it permeates no more than 75% of the bed height of the column
2) Wait 5-15 minutes
3) Apply a suitable water immiscible organic solvent and collect the effluent

(Courtesy of Biotage)

**Product Examples:** Varian Hydromax, Biotage Isolute HM-N, Merck’s Extrelut
Salting Out LLE

• Addition of an inorganic salt into a mixture of water and a water-miscible organic solvent causes a separation of the solvent from the mixture and formation of two-phase system
• Sometimes referred to as “salt-induced phase separation”
• Occurs with many common solvents (e.g. acetone, MeOH, EtOH, acetonitrile, etc.)
• Salt and salt concentration causes different degree of phase separation
• Also occurs with saccharide addition (“sugaring out”!) and with water-soluble polymers with salt addition.
• Useful for polar (as well as non-polar) analytes and often used for partitioning of metal chelates and other metal complexes
Recoveries of Nitroaromatics, Nitramines, and Nitrate Esters from Water by Salting-Out LLE (%)**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Methylene chloride</th>
<th>Methylene chloride + NaCl</th>
<th>Acetonitrile + NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMX</td>
<td>23.6</td>
<td>73.6</td>
<td>95.6</td>
</tr>
<tr>
<td>RDX</td>
<td>59.6</td>
<td>83.1</td>
<td>93.9</td>
</tr>
<tr>
<td>TNB</td>
<td>88.1</td>
<td>92.7</td>
<td>96.5</td>
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<tr>
<td>DNB</td>
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<td>NB</td>
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<td>94.2</td>
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<tr>
<td>TNT</td>
<td>94.0</td>
<td>96.3</td>
<td>98.9</td>
</tr>
<tr>
<td>2,4DNT</td>
<td>94.4</td>
<td>96.5</td>
<td>98.0</td>
</tr>
<tr>
<td>2,6DNT</td>
<td></td>
<td></td>
<td>97.9</td>
</tr>
<tr>
<td>2ADNT</td>
<td></td>
<td></td>
<td>96.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>3NT</td>
<td></td>
<td></td>
<td>96.9</td>
</tr>
<tr>
<td>4NT</td>
<td></td>
<td></td>
<td>97.1</td>
</tr>
<tr>
<td>NG</td>
<td></td>
<td></td>
<td>99.7</td>
</tr>
<tr>
<td>PETN</td>
<td></td>
<td></td>
<td>&gt;99.9</td>
</tr>
</tbody>
</table>

*Pooled standard deviation for all analytes except HMX was 0.9%. For HMX it was 4%.

HMX is octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
RDX is hexahydro-1,3,5-trinitro-1,3,5,7-triazine
TNB is 1,3,5-trinitrobenzene
DNB is 1,3-dinitrobenzene
NB is nitrobenzene
TNT is 2,4,6-trinitrotoluene
2,4DNT is 2,4-dinitrotoluene
2,6DNT is 2,6-dinitrotoluene
2ADNT is 2-amino-4,6-dinitrotoluene
2NT is 2-nitrotoluene
3NT is 3-nitrotoluene
4NT is 4-nitrotoluene
NG is glycercyl trinitrate
PETN is pentaerythritol trinitrate

Salting-out LLE of Pharmaceutical in Biofluids with Acetonitrile*

Abbott Laboratories authors investigated automated salting-out LLE for Aids drug Kaletra in human plasma; consists of two compounds Ritonavir and Lopinavir.

- MgSO$_4$ was used for salting out; ACN was organic solvent.
- A single 96-well plate was used to develop GLP analytical method—accuracy, precision, linearity, carryover, matrix effect, recovery and selectivity—in less than a day.
- LC-MS/MS was used for analysis.
- Only 325-µL/well (sample + salt solution + internal standard + organic solvent) was used for entire assay.
- Recoveries were in range of 62-84% across lots, species, and concentration levels.
- % CV was 2.7-6.5% for Lopinavir and 3.3-6.5% for Ritonair.
- In recent publication, authors used NH$_4$OAc—a MS friendly salt as salting-out reagent for hydrophobic drug candidate & metabolite in human plasma.

QuEChERS* (Pronounced “Catchers”)

A Low Cost, Highly Effective Sample Preparation Technique for Multiclass, Multi-residue Analytical Approach for Pesticides in Foods

- Extraction
- Clean-up
- Quantitation
- Confirmation

Quick
Easy
Cheap
Effective
Rugged
Safe

Flow Diagram of QuEChERS Process

Step 1: Weigh 10 g of sample into a 50-mL Centrifuge-Tube (1)

Step 2: Add 10-mL of Acetonitrile (2)

Step 3: Add 4-g of MgSO₄ & 1-g of NaCl (4)

Step 4: Add ITSD Solution (5)

Step 5: Take aliquot & add MgSO₄ (and sorbent)—dSPE (8)

Step 6: [Add 0.1% acetic acid and “analyte protectants” (11)]

Step 7: Analyze by GC-MSD or LC-MS/MS

(courtesy of Steve Lehotay, USDA)
Pictorial Representation of the QuEChERS Sample Prep Process
Pictorial Representation of the QuEChERS Sample Prep Process
Step One: Salting-out LLE

Three Methods Currently Practiced:
1) Original unbuffered method
2) AOAC 2007.01 buffered method (US)
3) EN15662 method (Europe)
4) All three methods in rest of world
Step 2:
Dispersive SPE Kits for QuEChERS

<table>
<thead>
<tr>
<th>Kit</th>
<th>Quantity &amp; Size/Pack</th>
<th>AOAC 2007.01 Method Contents and Part Number</th>
<th>EUROPEAN METHOD – EN 15662 Contents and Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENERAL FRUITS AND VEGETABLES:</strong> Removes polar organic acids, some sugars and lipids</td>
<td>100 – 2 ml tubes</td>
<td>50 mg PSA 150 mg MgSO₄ Part No. 5882-5022</td>
<td>25 mg PSA 150 mg MgSO₄ Part No. 5882-5021</td>
</tr>
<tr>
<td></td>
<td>50 – 15 ml tubes</td>
<td>400 mg PSA 1200 mg MgSO₄ Part No. 5882-5058</td>
<td>150 mg PSA 900 mg MgSO₄ Part No. 5882-5056</td>
</tr>
<tr>
<td><strong>FRUITS AND VEGETABLES WITH FATS AND WAXES:</strong> Removes polar organic acids, some sugars, more lipids, and sterols</td>
<td>100 – 2 ml tubes</td>
<td>50 mg PSA 50 mg C18EC 150 mg MgSO₄ Part No. 5882-5122</td>
<td>25 mg PSA 25 mg C18EC 150 mg MgSO₄ Part No. 5882-5121</td>
</tr>
<tr>
<td></td>
<td>50 – 15 ml tubes</td>
<td>400 mg PSA 400 mg C18EC 1200 mg MgSO₄ Part No. 5882-5158</td>
<td>150 mg PSA 150 mg C18EC 900 mg MgSO₄ Part No. 5882-5156</td>
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<tr>
<td><strong>PIGMENTED FRUITS AND VEGETABLES:</strong> Removes polar organic acids, some sugars and lipids, and carotinoids and chlorophyll, not for use with planar pesticides</td>
<td>100 – 2 ml tubes</td>
<td>50 mg PSA 50 mg GCB 150 mg MgSO₄ Part No. 5882-5222</td>
<td>25 mg PSA 2.5 mg GCB 150 mg MgSO₄ Part No. 1982-5221</td>
</tr>
<tr>
<td></td>
<td>50 – 15 ml tubes</td>
<td>400 mg PSA 400 mg GCB 1200 mg MgSO₄ Part No. 5882-5258</td>
<td>150 mg PSA 15 mg GCB 900 mg MgSO₄ Part No. 5882-5256</td>
</tr>
<tr>
<td><strong>HIGHLY PIGMENTED FRUITS AND VEGETABLES:</strong> Removes polar organic acids, some sugars and lipids, plus high levels of Carotinoids and Chlorophyll, not for use with planar pesticides</td>
<td>100 – 2 ml tubes</td>
<td>25 mg PSA 7.5 mg GCB 160 mg MgSO₄ Part No. 5982-5321</td>
<td>150 mg PSA 45 mg GCB 900 mg MgSO₄ Part No. 5982-5356</td>
</tr>
<tr>
<td></td>
<td>50 – 15 ml tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FRUITS AND VEGETABLES WITH PIGMENTS AND FATS:</strong> Removes polar organic acids, some sugars and lipids, plus Carotinoids and Chlorophyll, not for use with planar pesticides</td>
<td>100 – 2 ml tubes</td>
<td>50 mg PSA 50 mg GCB 150 mg MgSO₄ 50 mg C18 Part No. 5882-5421</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 – 15 ml tubes</td>
<td>400 mg PSA 400 mg GCB 1200 mg MgSO₄ 400 mg C18 EC Part No. 5882-5456</td>
<td></td>
</tr>
</tbody>
</table>
QuEChERS Advantages

✓ A batch of 6-12 extracts can be prepared in 30-40 min by a single analyst with ≈$1-3 of disposable materials per sample and generate <12 mL solvent waste.

✓ Consistently high recoveries (mostly 90-110% with RSDs < 5%) of a wide range of GC- and LC-amenable pesticides are achieved from many matrices.

(courtesy of Steve Lehotay, USDA)
Recoveries of 15 Pesticides in Different Matrices

No differences were found vs. matrix for individual pesticides or concentration

(courtesy of Steve Lehotay, USDA)
Analysis of 17 Representative Pesticides in Apple by QuEChERS (AOAC Buffered method) with the Detection by LC/MS/MS
# 17 Representative Pesticides Information

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Category</th>
<th>Pesticide</th>
<th>Category</th>
<th>Pesticide</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acephate</td>
<td>Organophosphate</td>
<td>Dichlorvos</td>
<td>Organophosphate</td>
<td>Thiabendazole</td>
<td>Benzimidazole</td>
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<tr>
<td>Carbaryl</td>
<td>Carbamate</td>
<td>Imidaclorpid</td>
<td>Neonicotinoid</td>
<td>Thiophanate-methyl</td>
<td>Benzimidazole</td>
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<tr>
<td>Carbendazim</td>
<td>Benzimidazole</td>
<td>Methamidophos</td>
<td>Organophosphate</td>
<td>Tolyfluanid</td>
<td>Sulphamide</td>
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<tr>
<td>Cyprodinil</td>
<td>Anilinopyrimidine</td>
<td>Penconazole</td>
<td>Triazole</td>
<td>Ethoprophos</td>
<td>Organophosphate</td>
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<tr>
<td>Diazinon</td>
<td>Organophosphate</td>
<td>Propoxur</td>
<td>Carbamate</td>
<td>Kresoxim-methyl</td>
<td>Strobilurin</td>
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<tr>
<td>Dichlofluanid</td>
<td>Sulphamid</td>
<td>Pymetrozine</td>
<td>Pyridine</td>
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</tbody>
</table>
Apple Matrix Blank
Chromatogram of Apple Sample Spiked with 5ng/g (5ppb) Of 17 Pesticides
# Apple AOAC Recovery and Repeatability Results (n = 6)

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Low QC (5ppb)</th>
<th>Mid QC (50ppb)</th>
<th>High QC (200ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>recovery</td>
<td>RSD % (n=6)</td>
<td>recovery</td>
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<tr>
<td>Methamidophos</td>
<td>77.57</td>
<td>5.03</td>
<td>91.79</td>
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<tr>
<td>Acephate</td>
<td>78.36</td>
<td>4.11</td>
<td>89.06</td>
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<tr>
<td>Pymetrozine</td>
<td>70.67</td>
<td>5.75</td>
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<tr>
<td>Carbendazim</td>
<td>83.13</td>
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<td>94.05</td>
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<tr>
<td>Imidacloprid</td>
<td>96.16</td>
<td>4.79</td>
<td>90.03</td>
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<td>Thiabendazole</td>
<td>70.07</td>
<td>4.84</td>
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<td>Dichlorvos</td>
<td>96.73</td>
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<td>90.97</td>
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<td>Propoxur</td>
<td>95.23</td>
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<td>Thiophanate methyl</td>
<td>102.17</td>
<td>13.53</td>
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<td>Carbaryl</td>
<td>94.56</td>
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<td>Ethoprophos</td>
<td>100.88</td>
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<td>97.93</td>
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<td>Penconazole</td>
<td>110.97</td>
<td>2.80</td>
<td>96.93</td>
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<td>Cyprodinil</td>
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<td>Dichlorfluanid</td>
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<td>Diazinon</td>
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<td>Kresoxim methyl</td>
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<td>Tolyfluanid</td>
<td>114.08</td>
<td>4.77</td>
<td>92.63</td>
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</tbody>
</table>

* n = 3.
**β-Lactam Analysis in Beef Kidney**

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Cleanup</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 g sample in a 50 mL centrifuge tube</td>
<td>1. supernatant + 500 mg C18 sorbent</td>
</tr>
<tr>
<td>add internal standards (PENV, CEFD)</td>
<td>mix for 30 s</td>
</tr>
<tr>
<td>add 2 mL water + 8 mL acetonitrile</td>
<td>centrifuge for 1 min at 3450 rcf</td>
</tr>
<tr>
<td>vortex briefly, shake for 5 min</td>
<td>evaporate 5 mL supernatant to 0.5 mL</td>
</tr>
<tr>
<td>centrifuge for 5 min at 3450 rcf</td>
<td>filter 0.5 mL extract with the Mini-UniPrep™</td>
</tr>
</tbody>
</table>

LC-MS/MS analysis

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Slide adapted from Kate Mastovska, USDA-ARS
**QuEChERS for Acrylamide in Foods**

**Extraction**
- 1 g sample in a 50 mL FEP tube
- Add $d_3$-acrylamide at 500 ng/g
- Add 5 mL hexane, vortex

**Addition**
- Add 10 mL water + 10 mL MeCN + 4 g MgSO$_4$ + 0.5 g NaCl
- Shake vigorously for 1 min
- Centrifuge for 5 min at 3450 rcf

**Cleanup**
- Discard the hexane layer
- 1 mL of the upper layer + 50 mg PSA + 150 mg MgSO$_4$
- Mix for 30 s
- Centrifuge for 1 min at 3450 rcf

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Slide adapted from Kate Mastovska, USDA-ARS
Extraction/Cleanup for Acrylamide in Foods

Centrifuge Tube

discard ...... removal of fat

- hexane layer
- MeCN layer

- matrix
- water layer
- excessive salts

Slide adapted from Kate Mastovska, USDA-ARS
Status of QuEChERS

• After AOAC International interlaboratory trials were successful, and the method is now AOAC International Official Method 2007.01. In Europe, Official EN1566.2 has been published.

• Many laboratories have implemented the method successfully for 200-350 pesticides in food and lowered costs (4-fold faster, less labor, lower cost).

• Commercial products for QuEChERS have been introduced.

• The streamlining features of QuEChERS are being used in more applications (acrylamide; vet. drugs).
Most Popular SPE Formats: SPE Cartridges and Disks

- Packing
- Polypropylene
- Frits
- PTFE Disk or fiberglass
- Pre-filter (Optional)

© Agilent Technologies
Solid Phase Extraction Pipette Tip*

- Designed to work with x-y-z liquid handling systems without needed for modification
- Can also be used manually
- No vacuum manifold required
- Flow can be bi-directional
- Disposable, no carryover or cross-contamination
- Recommended for small samples (less than 100-uL)

*Examples:
Agilent Cleanup C18 Pipette Tips
Varian SPEC Plus PT
Millipore ZipTip
DPX Disposable Pipette Extraction
Disposable Pipette Extraction (DPX)

- Adsorbent sealed in pipette tip but loosely packed
- High efficiency extractions
- Extractions are rapid (<3 minutes)
- Extraction efficiency is flow rate independent
- Use less sorbent, so less solvent is required
- Minimal solvent waste generated
- Readily automated

• William E. Brewer, US patent 6,566,145 B2

• Application number of PCT/US08/54584
Basics of DPX Extraction Technology

- **DPX - tip**
- **solid phase**

1. Condition
2. Aspirate sample
3. Mix
4. Discharge sample
5. Wash
6. Elute

*optional depending on method requirements

(courtesy of DPX Labs)
GC/MS Analysis of NIDA-5 Drugs of Abuse using DPX Pre-extraction

GC/MS chromatogram of an extract of 0.5mg/L drug mix in whole blood. 0.1mL whole blood was centrifuged with 0.1mL water, and the supernatant was extracted in about 2min following 5 water wash steps. 1-amphetamine, 2-methamphetamine, 3-meperidine, 4-glutethimide, 5-phencyclidine, 6-methadone, 7-methaqualone, 8-amitriptyline, 9-cocaine, 10-imipramine, 11-doxepin, 12-desipramine, 13-pentazocine, 14-codeine, 15-oxycodeone.

(courtesy of W. E. Brewer, Clemson Veterinary Diagnostic Center and DPX Labs)
Industry Example - Gerstel Automated SPE & DPX

- CTC PAL base hardware
- Gerstel custom SPE module
- LC (LC/MS), GC (GC/MS)
- Maestro Software/interfaces to ChemStation
- Industry Standard SPE Format
MICRO EXTRACTION BY PACKED SORBENT (MEPS)

- Samples as small as 3.6-uL
- Can be automated; SPE steps and injection in same device

(courtesy of SGE)
Schematic Diagram of 96-Well Extraction Plate System

Extraction plate

Polyethylene manifold lid

Polypropylene manifold base

O-ring

Collection plate

On / off valve (in off position)

To vacuum pump

Needle valve

Vacuum Gauge

(courtesy of Agilent)
Comparison of Protein Precipitation and SPE Cleanup of Plasma Using 96-Well Plate Format—Oasis uElution Plate

Amitriptyline, 0.1 ng/mL

Protein Precipitation

Intensity: 9.71 x 10³

Oasis® HLB

Intensity: 3.62 x 10⁴

Oasis® MCX

Intensity: 8.91 x 10⁴

LC/MS runs using water-ammonia Gradient on Xterra MS column

(courtesy of Waters)
Chemistries of SPE
Advantages of Polymers in SPE
(relative to Silica-based)

• Water-wettable-won’t deactivate/dewet if dried out
• Wide pH range—can use more dramatic washing conditions for interference removal and elutions
• Spherical—homogeneous packed bed and reproducible flows
• High surface areas- 600-800 m²/g; higher loading capacity; can reduce sorbent bed volumes, less solvent, less sample
• No acidic silanol sites
• Higher retention of polar compounds due to frequent mixed mechanisms (lipophilic-hydrophilic balanced); allows development of “generic” methods

Disadvantages of Polymers in SPE

• Not as many phase chemistries
• More expensive
Polymeric SPE Recovery Performance - Dry vs. Wet Sorbent

**Graph:**
- Y-axis: recovery percentage
- X-axis: compounds
- Bars: comparison between dry (DRY) and wet (WET) sorbents
- Compounds: acetaminophen, brompheniramine, propranolol, mianserin, doxepin, fluoxetine, Dihydroxy naphthalene

**Legend:**
- DRY: blue
- WET: light blue

**Software:**
SampliQ OPT (Agilent Technologies)

© Agilent Technologies
Selective Chemistries in SPE

• Mixed Mode Phases (both silica- & polymer-based)

Phases that contain two (or more) types of functional groups to allow multiple chemical interactions for selectivity and wettability purposes

- SAX and PS-DVB
- SAX and SCX (removal of ions when interested in neutral compounds)
- C18 and SCX
- C18 and SAX
- C18 and WAX
- C8 and SCX (mainly for drugs of abuse)
- DVB and hydrophilic (drugs in biol. Fluids)
- PS-DVB and hydrophilic (drugs in biol. Fluids)
- RPC and Cyano (matrix removal, alternative to protein ppt)
- DVB-amide and SCX
Immunoaffinity Phases for Sample Preparation
High Abundance Proteins in Human Plasma

- α1-antitrypsin 3.8%
- Immunoglobulin G 16.6%
- Immunoglobulin A 3.4%
- Transferrin 3.3%
- Haptoglobin 2.9%
- Other 15%
- Albumin 54.3%

Analysis and Identification
Protein Expression
Drug Targets
Disease Markers
Depletion of High Abundance Proteins in Human Serum/Plasma

- Affinity purified polyclonal antibodies bound to resin.
- Mixed resin bed for simultaneous removal all six proteins from serum.
- Currently up to 20 proteins can be depleted.
- Robust chemistry.

Individual Ab materials are mixed in selected percentages and packed into a column format.

**Agilent MARS Column**
Multiple Affinity Removal System

- **H** High-Abundant Proteins (Albumin, IgG, IgA, Transferrin, Haptoglobin, Antitrypsin)
- **L** Low-Abundant Proteins (Biomarkers for disease and drug targets)

- Column and optimized buffers are used to remove the top six most abundant proteins in human serum and plasma samples.
- Attach to HPLC instrument and pump samples through - proteins of interest are collected and analyzed.
Multiple Affinity Removal System

- **High-Abundant Proteins** (Albumin, IgG, IgA, Transferrin, Haptoglobin, Antitrypsin)
- **Low-Abundant Proteins** (Biomarkers for disease and drug targets)

- Column and Optimized buffers are used to remove the top six most abundant proteins in human serum and plasma samples.

- Attach to HPLC instrument and pump samples through - proteins of interest are collected and analyzed.
Multiple Affinity Removal System

Crude Human Serum

- High-Abundant Proteins (Albumin, IgG, IgA, Transferrin, Haptoglobin, Antitrypsin)
- Low-Abundant Proteins (Biomarkers for disease and drug targets)

• Column and Optimized buffers are used to remove the top six most abundant proteins in human serum and plasma samples.

• Attach to HPLC instrument and pump samples through - proteins of interest are collected and analyzed.
Immunoaffinity Column Elution Profile - 50 mm column

- Total column run cycle = 20.00 min, for injection, elution, and regeneration (4.6 x 50 mm column).

- Multiple Affinity Removal column is reusable, protein binding capacity is unchanged after 200 injections of serum

- Capacity = 15-20 µL serum per injection
  1.2 - 1.6 mg total serum proteins

Comparison of Run #20 and Run #200

<table>
<thead>
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<th>Time (min)</th>
<th>%B</th>
<th>Flow rate</th>
<th>Max. pressure</th>
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<tr>
<td>6</td>
<td>20.00</td>
<td>1.000</td>
<td>120</td>
</tr>
</tbody>
</table>

Injection 0.25 mL/min
Flow-through, Low Abundant Proteins
Bound, High Abundant Proteins
Elution 1.0 mL/min
Re-equilibration 1.0 mL/min
End run (20.0 min).
Detection of Aflatoxins in Coconut Waste

A 25 gram sample of coconut fiber was shaken with 5 grams of NaCl and 100 mL of 80% v/v ACN in water. 1 mL of this extract was diluted 10 fold with 9 mL of PBS buffer. A sample of the extract was injected directly onto the immunoaffinity column. The chromatogram begins with elution of the immunoaffinity column onto the C18 reversed-phase column. Early peaks are system peaks as well as elution of small amounts of matrix components that partly precipitate when the extract comes in contact with the binding buffer. They do not affect aflatoxin measurement.

- Column 1: VENTURE AFVE1520450 (15.20 μm, 4.6 mm i.d. x 50 mm)
- Column 2: GENESIS FM25960E (C18, 4 μm, 4.6 mm i.d. x 250 mm)
- Detection: Fluorescence (Excitation: 369 nm, Emission: 422 nm)
- Binding: 0.10 M phosphate + 0.15 M NaCl, pH 7.0
- Elution: 20% v/v acetonitrile in water
- Mobile Phase: 600 mL MeCH + 80 mL ACN + 200 μL concentrated HNO₃ + 50 mg KBr, adjusted to 1000 mL with water
- Sample: 500 μL

(courtesy of Grace Davison Discovery Sciences)
Restricted Access Media (RAM)

* Developed for analysis of low MW compounds in complex matrices, especially drugs in biological fluids.

* Macromolecular sample compounds have limited accessibility to sorption sites of column packing and elute at column void volume; small molecules will diffuse into matrix and interact with stationary phase.

* Can be used off-line, on-line via column switching, or as an HPLC column alone.
Typical Structure of Restricted Access Medium

Non-adsorptive outer surface

Internal surface with selective reversed-phase
Use of Coupled Column RAM-RPC System for Analysis of Drugs in Plasma

Column A: RAM Column
Mobile phase: water, 0.5 mL/min

Column B: LiChrospher 60 RP Select B
Mobile phase: Trichloroacetic acid, Acetonitrile, 0.1%
Triethanolamine, pH2, 1.0 mL/min

Peaks:
1. Epirubicinol
2. Epirubicinal aglycone
3. Epirubicin
4. Epriubicin aglycone
5. 7-deoxyepirubicinal aglycone (compounds in 5.6-8.2 ng/mL range)

A. Rudolphi and K.-S. Boos, LC/GC
Molecularly Imprinted Polymers (MIPs)
LC Chromatogram Showing Selective Cleanup of Clenbuterol from Beef Liver using Molecularly Imprinted SPE Phase*

Peaks
1) Clenbuterol
2) Bromoclenbuterol (template bleeding)

*Eising, Berggren, Majors, LCGC
Most Successful Story for Acceptance of New Sample Prep Method: Janusz Pawliszyn* and coworkers’ SPME

* University of Waterloo, Waterloo, ON, Canada
Gerstel’s Twister for Stir-Bar Sorptive Extraction (SBSE)

The PDMS coated GERSTEL Twister is stirred in the sample for several minutes. The analytes of interest come in contact with the PDMS phase and are extracted.

Without additional sample preparation, the Twister is placed in a GERSTEL TDS 2 ThermoDesorption System. Here the analytes are thermally desorbed, focussed in the inlet, and transferred to the GC capillary column.
Recovery of Solutes as a Function of Octanol-Water Partition Coefficients for SBSE and SPME

Volume of coated polydimethylsiloxane:  SPME ~ 0.5-µL  
SBSE ~ 25-125-µL (10-mm X 0.5-mm)

(Sandra and David, LCGC, 2003)
Thin Film Microextraction

1. Rotate thin-film as it is inserted into the liner

Stainless Steel Wire

2cm

2cm

2. Place cap onto liner

3. Place liner into MPS2 Tray

Gerstel Twister Desorption Liner

Gerstel GC Liner with Coiled Thin-film

4. Exchange of liner between the tray and the Twister Desorption Unit (TDU) via the MPS2 autosampler arm

(courtesy of J. Pawliszyn, June, 2009)
How does SPME membrane device work?

100 μm PDMS fiber

1 cm x 1 cm PDMS membrane

A_f: 10 mm²
V_f: 0.61 mm³

A_m: 200 mm²
V_m: 2.55 mm³

Ratio of extraction phase volume (V_m/V_f) = 4.5
Ratio of extraction phase surface area (A_m/A_f) = 20

(courtesy of J. Pawliszyn, June, 2009)
Extraction Time Profiles of Fluoranthenene by Twister and Thin-film Coupled to a Drill

Extraction time profile of fluoranthene

(courtesy of J. Pawliszyn, June, 2009)
Extraction Efficiency Comparison Between PDMS Fiber and PDMS Membrane (Extraction of Ice Wine Volatile Constituents)

(courtesy of J. Pawliszyn, June, 2009)
SPME Multiwell Method

1. Well filled with sample
2. Insert SPME fibre for extraction
3. Agitate well until equilibrium is reached

- Remove fibre
- Desorb in well filled with solvent
- Evaporate solvent
- Reconstitute and inject into GC or LC

© Agilent Technologies
Alternative Approaches to Stir Bar Sorptive Extraction

Conventional PDMS Twister

Dual-Phase Twister

Silicone-membrane Sorptive Extractor

(courtesy of Prof. Carlo Bicchi, Univ. of Torino, Italy, see May 2009 LCGC Magazine)
Summary and Future Directions

- With tandem LC-MS and GC-MS systems, sample prep steps may be reduced (e.g. QuEChERS, salting-out LLE)

- New formats handle smaller samples for LLE and SPE and are amenable to automation; chip-based SPE miniaturization is now available

- Polymeric SPE sorbents bring many advantages including higher capacity, reduced bed mass and more rugged performance

- Specialty phases like mixed mode SPE, MIPs, immunoaffinity, RAMs can be used to remove specific interferences or specific analytes

- SPME and SBSE are into their next generation formats

- Automation of many sample prep protocols is readily available; sample prep can be integrated into analytical system
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• SGE