Analytical methods used to determine minimum residues levels of pesticides.

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Washington State University
Pullman, WA
## Discovery

<table>
<thead>
<tr>
<th>Who &amp; How</th>
<th>Why</th>
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<tbody>
<tr>
<td>When</td>
<td>Where</td>
</tr>
<tr>
<td>Where</td>
<td>When</td>
</tr>
<tr>
<td>Why</td>
<td>Who &amp; How</td>
</tr>
</tbody>
</table>
‘Why’
Maximum residue levels (MRLs) or ‘tolerances’

• Theoretically, if growers apply the pesticides properly on crops for which the pesticides have been registered, and the appropriate harvest intervals are followed, then it should be very unlikely that regulatory limits will be exceeded.

Kmellar et al., 2010 Food Additives and Contaminants 27:1415
Detection of pesticide residues.

• Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) 1910
• Delaney Clause  1958
• Environmental Protection Agency 1970
• Food Quality Protection Act-1996
• Food Safety Modernization Act-2010
FQPA-1996

• Review ‘tolerances’ for all pesticides used on food crops between 1996-2006.
• Children’s Safety Factor (10X):
  – Apple juice, Milk, & Chocolate
• Aggregate risk & Cumulative exposure ‘Risk Cup’
  – Oral, dermal, and inhalation routes.
• Risk cup pools all pesticides with a common mode of action.
• Endocrine disruptors
1993 National Academy of Sciences Report

- Pesticides in the diets of infants and children.

Milk #1
Apple Juice #2
Apple Products #4

For Infants
World-wide Regulatory Agencies.

  – European Committee for Standardization Standard Method 15662 (citrate buffer system)

• United States: Three agencies involved:
  – Environmental Protection Agency (sets tolerance limits)
  – Food and Drug Administration (monitors imported and domestic foods)
    • Association of Analytical Chemists (AOAC) Official Method 2007.01 (Acetate buffer system)
  – United States Department of Agriculture (monitors meat and egg products)

• Japan’s Ministry of Health, Labor and Welfare.
  – Food Santitation Law 2006
‘Where’
Imported foods

- Samples are collected at point of entry.
- Residues tolerances are set by EPA, if an imported commodity has residues of a pesticide legally used in the country of origin, but it is not register for use on that crop in the USA, the crop will be refused.
- When illegal residues are found the supplier (specific growers, shippers, or a geographic area or country) is placed on a list: ‘Detention without physical examination.’
‘How’
How does a clean up preparation capture different kinds of pesticides?

• Contact insecticides and herbicides are lipophilic in order to penetrate the waxy surface of either an insect or plant; thus a partitioning rinse in octane/water, will concentrate the pesticides in the octane phase.

• Each pesticide has a ‘partition coefficient.’
First barrier to lipophilic insecticides is the insect’s integument.
Plant’s leaf surface
How does a clean up preparation capture (300) different kinds of pesticides?

• Common modes of action characterize different pesticides.

• All active ingredients of insecticide inhibitors of the enzyme acetylcholine esterase must be of a size and ionic-charge to mimic the substrate and bind to the enzyme.

• All herbicides capable over-stimulating auxin receptor sites mimic the hormone so that they have a common size and charge site.
Change in targets 1999-2008

- 61 - 35.9% Acetylcholinesterase Inhibition
- 32 - 19.8% Axonal Membrane Na⁺ channel
- 0 - 16.3% Post-synaptic Membrane
- 3 - 5.6% Chlorine channel
- Other targets 22.4%
Inhibitors of Metabolism

Hormone agonist

Chloroplast

Intermediate Metabolism

Lipid & Wax Biosynthesis

Carotenoids

Enzymes

Hormones

Inhibitors of Metabolism

Mitochondrion

Cell Division

Nucleus

DNA replication

Cell Wall

O₂

CO₂

LIGHT

Inhibitors of Respiration
Auxins mimics

A 'good' auxin mimic must planar and possess a carboxyl group but not have groups which would hinder binding the a, c-d or a-f regions.
How does a clean up preparation capture different kinds of pesticides.

• Preparation steps include:
  – Partitioning.
  – ‘Salting out’ amino acids.
  – Selective pH buffering.
  – Size separating columns.
  – Charge specific resin columns.
Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive Solid-Phase Extraction’ for the determination of pesticides in produce.

dispersive Solid-Phase-Extraction

• Essentially, it is based on the extraction of pesticides from the homogenized sample with acetonitrile followed by removal of interference causing substances with a cleanup sorbent.
• Term ‘dispersive’ refers to the cleanup process.
• The ‘sorbent’ (Primary Secondary Amine) is added in a solid form (C18 silica or graphitized carbon black) to capture interfering substances.
QuEChErS

- **Quick, Easy, Cheap, Effective, rugged, and Safe**
- Multi-class, multi-residue analysis of pesticides in a variety of matrixes.
- Detection limits 0.01 mg/kg (10 µg/Kg).
- Europe uses a weaker citrate buffering system.
- USA uses an acetate buffering system.
- Japan’s ‘Positive List System’ uses the acetate buffered system.
Restek Corporation
Bellefonte PA

- **Q110™** (European EN 15662, Citrate buffered) packets
  - 4g MgSO$_4$, 1g NaCl, 1g Trisodium citrate dihydrate, 0.5g Disodium hydrogen citrate sesquihydrate NaOAc.

- **Q150™** (USA AOAC 2007.01 Acetate buffered) packets
  - 6g MgSO$_4$, 1.5g NaOAc
Restek Corporation
Bellefonte PA

- 2mL Micro-centrifuge Tubes (Q-sep™) for dSPE (Clean up of 1 mL extracts).
- Q213™ (European EN 15662)
  - 150mg MgSO₄, 25mg Primary & Secondary Amine exchange material, 7.5mg graphitized carbon black.
- Q251™ (AOAC 2007.01)
  - 150mg MgSO₄, 50mg PSA, 50mg C18
Materials

- MgSO$_4$ removes excess water.
- PSA (Primary and Secondary Amine) removes sugars, fatty acids, organic acids, and anthocyanine pigments.
- C18 removes nonpolar interferences.
- GCB (graphitized carbon black) removes pigments, sterols, and nonpolar interferences.
### QuEChErS
Lehotay, S. et al., 2010 *J. Chromatography A* 1217:2548

<table>
<thead>
<tr>
<th>Europe: Citrate buffer system</th>
<th>USA: Acetate buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g sample in vial</td>
<td>15 g sample in vial</td>
</tr>
<tr>
<td>100µl 4g MgSO₄, 1g NaCl, 1g trisodium citrate dihydrate, &amp; 0.5g disodium hydrogen citrate sesquihydrate (Restek Q110 Bellfonte, PA).</td>
<td>150µl 6g MgSO₄, 1.5g sodium acetate (Restek Q150 Bellfonte, PA).</td>
</tr>
<tr>
<td>Cap &amp; Vortex 1 min</td>
<td>Same</td>
</tr>
<tr>
<td>Add 10 ml MeCN</td>
<td>Add 15 ml 1% acetic acid in MeCN</td>
</tr>
<tr>
<td>Cap &amp; shake vigorously</td>
<td>Same</td>
</tr>
<tr>
<td>Repeat Q110 step</td>
<td>Repeat Q150 step</td>
</tr>
<tr>
<td>Shake, centrifuge, transfer 1mL to</td>
<td>Same</td>
</tr>
<tr>
<td>2ml d-SPE (25mg Primary Secondary Amine + 7.5mg graphitized carbon black), 150mg MgSO₄ (Restek Q213).</td>
<td>2ml dispersive-Solid-Phase Extraction (50mg PSA, 50mg C18) 150mg MgSO₄ (Restek Q251).</td>
</tr>
</tbody>
</table>
Detection of Diazinon (Standard)
Lehotay, S. et al., 2010 J. Chromatography A 1217:2548.
**Problems:** Pesticides with carboxylic acid groups.

- Daminozide, 2,4-D, Chlorothalonil, Dicofol, *Folpet*, Captan, Captafol, Dichlofluanid, and Tolyfluanid are retained on the Primary Secondary Amine sorbent.
- These pesticides degrade in the acetonitrile as pH increases and upon exposure to light.
Detection of Folpet (GC) compared to Diazinon standard

Lehotay, S. et al., 2010 J. Chromatography A 1217:2548.
QuEChErS
Kmellar et al., 2010 Food Additives and Contaminants 27:1415

• Problems: Difficult to recover non-polar pesticide residues from samples containing fatty matrixes.

• Solution: Push the sample through a despersive-Solid Phase Extraction (d-SPE) column packed with a C$_{18}$ sorbent.

• If you know that there are no ‘planar’ compounds (thiabendazole) the d-SPE can be packed with Graphitized Carbon Black.
Detection of Thiabendazole compared to Diazinon standard (LC)

Lehotay, S. et al., 2010 J. Chromatography A 1217:2548.
QuEChErS
Lehotay et al., 2007 J. of AOAC 90:485

• Problems: Pymetrozine is a pH sensitive pesticide is difficult to recover from citrus (due to low pH), but easy in lettuce.

• Solution: A buffer is needed, and an acetate buffer is a stronger buffer than the citrate buffer system.
Detection of Pymetrozine (LC) compared to Diazinon standard.

Lehotay, S. et al., 2010 J. Chromatography A 1217:2548
Department of Food Safety Ministry of Health, Labor and Welfare, JAPAN.


  – Extraction methods
  – Clean-up methods
<table>
<thead>
<tr>
<th>Japanese Extraction methods</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grains, beans (with shells), nuts and seeds</strong></td>
<td><strong>Fruits, vegetables, herbs, tea &amp; hops</strong></td>
</tr>
<tr>
<td>Soak 10g samples/20mL water 15 min</td>
<td>20g samples/20mL water 15 min</td>
</tr>
<tr>
<td>Add 50mL acetonitrile, homogenize, vacuum filter, dissolve residue of filter paper in 20mL MeCN, repeat homogenization and vacuum filtering.</td>
<td>Same</td>
</tr>
<tr>
<td>Mix both filtrates, bring up to 100mL with MeCN.</td>
<td>Same</td>
</tr>
<tr>
<td>Decant 20mL of extract, add 10g NaCl, 20mL 0.5 mol/L phosphate buffer (pH7.0). Shake 10 min, allow H₂O/MeCN to separate, discard the aqueous layer.</td>
<td>Same</td>
</tr>
<tr>
<td>Condition an octadecylsilanized silca gel (1g) column with 10mL MeCN. Load the MeCN sample from previous step and elute with 2 mL MeCN.</td>
<td>Same</td>
</tr>
<tr>
<td>Dry MeCN layer over sodium sulfate (anhydrous) and filter. Concentrate the filtrate to dryness at &lt;40°C. Dissolve the residue in 2mL of MeCN/toulene (3:1).</td>
<td>Same</td>
</tr>
</tbody>
</table>
Japanese Clean-up methods

- Condition a graphite carbon/aminopropyl-silanized (silica gel) mini-column (500mg/500mg) with 10mL MeCN/toluene (3:1).
- Load the 2mL solution from the extraction step to the column.
- Elute the column with 20mL MeCN/toluene (3:1) and collect entire effluent, and concentrate to 1mL or less at <40°C or cooler.
Japanese Clean-up methods

- Add 10mL of acetone to the concentrated solution, vortex, and re-concentrate to 1mL or less at <40°C or cooler.
- Add 5mL of acetone to the concentrate, vortex and concentrate to dryness.
- Dissolve the residue in acetone/n-hexane (1:1) to 1mL.
- This is the test solution to be injected into a GC/MS for analysis.
Analytical methodology changes rapidly.

- **1960-80s:** Chromatography-single-stage mass spectrometry detection method.
- **1990s:**
  - Gas chromatography – Mass Spectrometry.
    - Nitrogen-Phosphorus detector (NPD).
    - Electrolytic conductivity detector (ELCD).
    - Flame photometric detector (FPD).
  - Liquid chromatography – Mass Spectrometry.
Solid Phase Gas Chromatography

Helium gas carrier/solid phase column
Solid Phase Gas Chromatography

Helium gas carrier/solid phase column
Liquid Chromatography

Reverse Phase Solid Support

- 80% Formic Acid
- 20% MeCN
- 100% MeCN

Washington State University
Mass Spectrometry

- Sample $10^2$ torr
- Repeller electrode
- Ion Source $10^{-7}$ torr
- Unfocused heavy ions
- Unfocused light ions
- Magnetic Field
- Focused Ion beam
- Detector

Mass spectrum
Analytical methodology changes rapidly.

• Current methodology: Hyphenated mass spectrometry.
  – Tandem mass spectrometry MS/MS
  – First MS detector set in full scan mode.
  – Second MS detector set in a selective ion monitoring mode.
    • Fragmentation chamber
  – Triple-quadrupole technology uses a ‘selective reaction monitoring’ mode.
    Confirms pesticide identification.
  Fintschenko, Y. et al., 2010 J. Agric. Food Chem. 58:5859.
Quadrupole technology

http://www.chm.bris.ac.uk/ms/theory/tandem-ms.html
Triple quadrupole technology

Quadropole 1 (MS1)  Quadropole 2 (CID Collision cell)  Quadropole 3 (MS2)

(1) select m/z  product ion scan
(2) scanning  precursor ion scan (selected m/z)
(3) scanning  neutral loss scan

http://www.chm.bris.ac.uk/ms/theory/tandem-ms.html
Thank you!

• Dr. Azhar Ismail, Malaysian Cocoa Board.
• International Cocoa Organization.
• Dr. Steven J. Lehotay, ARS, Beltsville MD.
• Dr. Bella Kmellar, University of Budapest, Hungary.
• Dr. Michelangelo Anastassiades, CVUA Stuttgart Germany.
Detention without physical examination of cocoa beans.

- Import Alert #34-01 10/02/2009
- Violation ‘live insect infestations’
- Brazil and Indonesia.
- (Malaysia).
‘When’
Residues found in 2007: 1,317 domestic samples, and 5,613 imported samples.

2008: 0.9 % domestic and 4.7% imported samples were in violation.
<table>
<thead>
<tr>
<th>Commodity sampled in 2007</th>
<th># of Samples analyzed</th>
<th>Violations %</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Items that warranted special attention Due to earlier detection of residues in 2006.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berries, dried or paste</td>
<td>8</td>
<td>62.5</td>
</tr>
<tr>
<td>Ginseng, herbal and botanical, other than tea*</td>
<td>35</td>
<td>40.0</td>
</tr>
<tr>
<td>Snow Peas</td>
<td>12</td>
<td>25.0</td>
</tr>
<tr>
<td>Mango, dried or paste</td>
<td>13</td>
<td>23.1</td>
</tr>
<tr>
<td>Celery, dried or paste*</td>
<td>20</td>
<td>20.0</td>
</tr>
<tr>
<td>Chinese okra (luffa)*</td>
<td>21</td>
<td>19.0</td>
</tr>
<tr>
<td>Chinese/Thai eggplant</td>
<td>27</td>
<td>18.5</td>
</tr>
<tr>
<td>Red Beet*</td>
<td>27</td>
<td>18.5</td>
</tr>
<tr>
<td>Pear</td>
<td>28</td>
<td>14.3</td>
</tr>
<tr>
<td>Chutney</td>
<td>20</td>
<td>15.0</td>
</tr>
<tr>
<td>Papaya*</td>
<td>103</td>
<td>11.7</td>
</tr>
<tr>
<td>Spinach</td>
<td>57</td>
<td>10.5</td>
</tr>
<tr>
<td>Blackberries*</td>
<td>40</td>
<td>10.0</td>
</tr>
<tr>
<td>Cocoa products (Brazil, China, Ecuador, Mexico)</td>
<td>90</td>
<td>0</td>
</tr>
</tbody>
</table>
Target the Insect’s Nerve

Axonal membrane
Presynaptic membrane
Enzyme responsible for clearing the synaptic cleft of chemical transmitter.

Post-synaptic membrane
Neural-muscular junction
Muscle’s response to a neural impulse.
International Cocoa Organization
31 July 2008

- Maximum Residue Levels (MRLs) of pesticides permitted in cocoa.
- Use of pesticides according to Good Agricultural Practice (GAP).
- Active substances without established MRL cannot exceed 0.01mg/kg.
Countries with violations

<table>
<thead>
<tr>
<th>Country</th>
<th># of samples analyzed</th>
<th>Violation Rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guatemala</td>
<td>91</td>
<td>13.2</td>
</tr>
<tr>
<td>Peru</td>
<td>54</td>
<td>11.1</td>
</tr>
<tr>
<td>India</td>
<td>145</td>
<td>11.0</td>
</tr>
<tr>
<td>Dominican Republic*</td>
<td>186</td>
<td>10.8</td>
</tr>
<tr>
<td>China</td>
<td>502</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* Dominican Republic produces 45,000 tons of cocoa, but cocoa was not in violation.

Malaysia had 10 or fewer samples collected and analyzed in 2007, and five products in 2008 [Peas (3), refined vegetable oil (1), and whole white pepper (1)].

Malaysia was not in the top 35 countries with regards to number of samples collected and analyzed in 2007.
## Sampling for multiple substances

<table>
<thead>
<tr>
<th>Lehotay’s 2007 AOAC standard</th>
<th>Kmellar (&amp; Lehotay) Multiple targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 g placed in 50 ml PTFE test tube</td>
<td>Same</td>
</tr>
<tr>
<td>150µl 6g MgSO₄, 1.5g sodium acetate <em>(Restek Q150 Bellfonte, PA)</em>.</td>
<td>100 µl of internal standard in 50µg/ml triphenyl phosphate, add 6g MgSO₄ (salting out extraction) and 2.5g sodium acetate trihydrate.</td>
</tr>
<tr>
<td>Cap &amp; Vortex 1 min</td>
<td>Same</td>
</tr>
<tr>
<td>Add 15 ml 1% acetic acid in MeCN</td>
<td>Same</td>
</tr>
<tr>
<td>Cap &amp; shake vigorously</td>
<td>Same</td>
</tr>
<tr>
<td><strong>Repeat Q150 step</strong></td>
<td>750mg MgSO₄ and 250mg of Primary Secondary Amine</td>
</tr>
<tr>
<td>Shake, centrifuge</td>
<td>Same</td>
</tr>
<tr>
<td>2ml dispersive-Solid-Phase Extraction, 50mg PSA, 150mg MgSO₄ <em>(Restek Q251)</em></td>
<td>Vacuum filter to dryness under Argon gas, Reconstitute in 800µl acetonitrile/water (20/80 V/V)</td>
</tr>
</tbody>
</table>
Acetonitrile (MeCN) cannot be used in a Gas Chromatograph using a nitrogen-phosphorus detector (NPD).

Either use a solvent bypass to avoid the nitrogen in MeCN from reaching the NPD.

Or

Do a solvent exchange of MeCN for Toluene as the carrier onto the GC-NPD system.
QuEChErS
Kmellar et al., 2010 Food Additives and Contaminants 27:1415

Homogenize sample, 15 g placed in 50 ml PTFE test tube, followed by 100 µl of internal standard in 50 µg/ml triphenyl phosphate, finally 15 ml of acetonitrile/acetic acid (99:1 V/V).
Then add 6g MgSO₄ (salting out extraction) and 2.5g sodium acetate trihydrate.

Vortex vigorously for 4 minutes, centrifuge at 4,000 rpm for 5 minutes, and transfer 5 ml of supernatant to a new 15 ml test tube to which 750mg MgSO₄ and 250mg of Primary Secondary Amine is added. Vortex for 20 sec, and repeat centrifugation step.

Remove 3 ml of supernatant, and pour 800µl portions through a 0.45µm PTFE filter, dry under a stream of argon gas.

Reconstitute in 800µl acetonitrile/water (20/80 V/V) for LC MS/MS analysis.
Pesticide use practices and safety issues: The case of cocoa farmers in Ondo, Nigeria.

Nigeria currently produces 180,000 tonnes of cocoa, about 6% of the world’s cocoa.

However, together with adjacent West Coast African countries Ivory Coast, Ghana, Cameroon production equals 67% of the world’s cocoa supply.
In response to concerns from USA blueberry farmers regarding new pesticide monitoring methods in Japan (26 May 2006).

“Although I have attempted to report the most current and accurate information, there are still some areas where clear and concise information about (Japanese) policy is lacking: USA exporters are encouraged to verify import requirement with their foreign customers.”
Promoting Organically Grown Cocoa

• “ANT” ISN’T NECESSARILY THE WORST PART OF “CHOCOLATE-COVERED ANT” Chocolate and Pesticides — Are Pesticide Residues Enough to Make You Buy Organic Cocoa?
