COMPARATIVE STUDIES OF DIFFERENT METHODS OF PESTICIDE RESIDUE ANALYSIS IN COCOA BEANS

AZHAR, I. and RAHMAT, M.
Malaysian Cocoa Board (MCB)
Introduction

- Pesticides – Commonly used in cocoa production
- Residue problems – health hazard
- GAP & Legislation
- CODEX Alimentarius – introduced and regulated maximum concentrations of pesticides residue (MRLs) permitted in foods.
- MRL – maximum concentration of a pesticide residue legally permitted in a foodstuff or feed if a pesticide is applied according to Good Agricultural Practice (GAP) (FAO, 2002).
However, some countries have developed and regulated their own MRLs which seem to be not standardized.

* e.g., Japan Positive List (introduced in May 2006)  
  EU Positive List  
  US Environmental Protection Agency

problems in international marketing and trade, especially dealing with a default MRL or uniform MRL of 0.01 mg/kg applies to those commodities when no specific MRL is set.

As an example of list of pesticides and the MRLs set for cocoa beans is shown in Table 1.
<table>
<thead>
<tr>
<th>Region</th>
<th>MRL Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codex</td>
<td>If no MRL exists, a default MRL of 0.01 mg/kg applies</td>
</tr>
<tr>
<td>European Union</td>
<td>If no MRL exists, a default MRL of 0.01 mg/kg applies</td>
</tr>
<tr>
<td>Japan</td>
<td>If no MRL exists, a default MRL of 0.01 mg/kg applies</td>
</tr>
<tr>
<td>Canada, New Zealand</td>
<td>If no MRL exists, a default MRL of 0.1 mg/kg applies</td>
</tr>
<tr>
<td>Malaysia, Hong Kong, Korea,</td>
<td>If no MRL exist, Codex MRLs applies or a default MRL of 0.01 mg/kg</td>
</tr>
<tr>
<td>India, Israel, Russian</td>
<td>Federation, Singapore, South Africa</td>
</tr>
</tbody>
</table>
### Table 1: MRLs for cocoa beans from different countries

<table>
<thead>
<tr>
<th>A.I</th>
<th>CODEX</th>
<th>EU</th>
<th>Japan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan</td>
<td>0.1 ppm</td>
<td>0.1 ppm</td>
<td>0.1 ppm</td>
</tr>
<tr>
<td>Metalaxyl-M</td>
<td>0.02 ppm</td>
<td>0.1 ppm</td>
<td>0.2 ppm</td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>Not specified</td>
<td>0.1 ppm</td>
<td>0.05 ppm</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>Not specified</td>
<td>0.05 ppm</td>
<td>0.05 ppm</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>Not specified</td>
<td>0.02 ppm</td>
<td>0.5 ppm</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>Not specified</td>
<td>0.1 ppm</td>
<td>0.1 ppm</td>
</tr>
<tr>
<td>2,4-D</td>
<td>Not specified</td>
<td>0.1 ppm</td>
<td>Default MRL, 0.01 ppm</td>
</tr>
<tr>
<td>Bromide</td>
<td>5 ppm (Guideline level)</td>
<td>50 ppm</td>
<td>70 ppm</td>
</tr>
</tbody>
</table>

*Guideline level*
MRL arguments:

- No standardization or harmonization among national regulatory bodies.
- Where the MRL is not specified the default value is 0.01 ppm.
- Variations in sample preparation – may lead to variations in results for MRL.

Implications:

i. Create a problem in food supplies and demand
ii. Increase number of MRL violation and improper/misuse of pesticides
iii. Affect quality control agency – have to increase their facilities and capabilities in order to comply with the lowest detection limit in pesticide residue analysis.
Report on 2,4-D contamination in cocoa products.

A Consignment of cocoa product was claimed to be positive and above the MRLs of 0.01 ppm.

For this reason, MCB initiated a task force:

- to develop a method of analysis for 2,4-D
- to conduct monitoring program
Objectives

1. To compare two different analytical methods for the analysis of 2,4-D pesticide residue in cocoa beans.
   - Japan MHLW vs QuEChERS
   - Instrumentation: GC-MS vs LC-MS/MS

2. To establish a good and valid analytical technique for the analysis of 2,4-D pesticide residue in cocoa beans thru proper validation and optimization.
   Method must comply with stringent analytical criteria with regard to selectivity, accuracy, limits of quantification.

3. To compare the effect of different sample preparation on residue analysis
Methods Used In The Study:

1. MHLW method
   - Reference: Determination of 2,4,5-T
   A General Compositional Standards for Food, Part I Food Specifications and Standards for Food, Food Additives, etc
   (May 29, 2006)
   Ministry of Health, Labor and Welfare
   (MHLW of Japanese Government)

2. QuEChERS method
   - accepted by many residue analysts
   - used in several EU Proficiency Testing
1. MHLW Method

Steps involved:

1. Extraction
2. Hydrolysis
3. Esterification
4. Purification / clean-up
5. Determination and quantification using Gas Chromatography coupled with Mass Spectrophotometry Detector (GC–MSD)
2. QuEChERS Method

Adopted from Multiresidue QuEChERS Method: Popular and applicable for the analysis wide range of pesticides.

Steps involved:

1. Extraction
2. Purification
3. Determination and quantification using Liquid Chromatography coupled with Mass Spectrophotometry Detector (GC-MS/MS)

Reference:
M. Anastassiades, S. J. Lehotay, D. Stajnbaher, F. J. Schenck
**PART 1: EXTRACTION**
10 g of ground sample (<0.42mm) in conical flask
let stand for 2 hours
add 100 mL of acetone and 5 mL of 4M HCl
blend mixture for 3 min.
filter through funnel contd 1 cm diatom. wash on filter paper
Collect the filtrate in a 300 mL round bottom flask (for condenser use)
wash the funnel with 50 mL acetone, and combine the filtrate
Evaporate & concentrate to about 30 mL by means of rotavapor at < 40°C
transfer into 300 mL separating funnel, and wash with 100 mL Et-Acetate
stopper the funnel, and attached to autoshaker
and shake for 5 min.
add 50 mL Et-Acetate
collect & combine the Et-Acetate
then the lower phase (Et-Acetate) into 300 mL flask
add app. NaSO₄ anhyd., stand & shake periodically for 15 min.
filter & rinse with 2 x 20 mL Et-Acetate
collect & combine filtrate, into condenser flask
rotavap at 40°C to about 1 mL
Further dry at ambient temp. under N₂ gas stream
add 30 mL of treated acetonitrile (Hex saturated ACN)
and treated acetonitrile vigorously 5 min.
Acetonitrile layer / hexane layer (repeat with 30 mL)
Transfer acetonitrile layer into 200 mL separating funnel
add 50 mL acetonitrile saturated hexane and shake lightly
Attach to condenser flask to condense to about 1 mL
and dry at room temp. under N₂ gas stream

**PART 2: HYDROLYSIS**
- add 20 mL MeOH & 80 mL of 15M NaOH into the dried residue
- reflux for 30 min. or waterbath at 90°C
- cool the flask and attach to the vacuum condenser set at < 40°C to remove the NaOH
- filter through filter funnel, pore size 20-40 micron
(wash glass filter with small amount of mixture of acetonitrile : water)
- collect filtrate into 300 mL separating funnel
- add 50 mL ether & 100 mL 10% NaCl
- shake vigorously for 5 min using autoshaker
- transfer water layer into 300 mL separating funnel
- adjust pH < 1.0 with 4M HCl
- add 50 mL Et-Acetate and shake for 5 min (autoshaker)
- repeat extraction with another 50 mL Et-Acetate to water layer
- transfer Et-Acetate layer into 300 mL flask (and combine)
- add approp. amount of NaSO₄ anhyd., shake periodically and stand for 15 min.
- filter into condenser flask (wash the collecting flask with 20 mL Et-Acetate)
- combine the washed liquid in condenser flask
- attach to condenser and condense at < 40°C to about 1 mL

**PART 3: ESTERIFICATION**
Transfer into 20 mL flask / test tube
and dry at oven, heat under reflux stream
- add 1 mL of esterifying agent of
- 3-fluoro-benzoic acid / acid complex (10 g in 25 mL n-butanol)
- connect the flask to cooler, and heat it in water bath at 90°C for 30 mins
- cool to room temp.
- transfer into 200 mL separating funnel containing 80 mL hexane and 20 mL 10% NaCl
- shake for 5 mins & let it stand
- drain the hexane layer into 200 mL flask
- collect and combine hexane layer
- add approp. amount of Na₂SO₄ ana., shake periodically and let it stand for 15 mins
- filter
- wash flask with 10 mL of hexane and filter
- collect filtrate into condenser
- combine filtrate into condenser
- attach to condenser and condense at < 40°C to about 2 mL

**PART 4: PURIFICATION**
Prepare glass chromatography column, below.
(Pack 5 g of Mg-elicite HC-90 with chromatography column (ID 15mm L300mm, and cover with 5g of NaSO₄)
Pre-condition by eluting n-hexane (do not let it dry)
Put the esterified substance on top of the column
Elute with 150 mL of mixture of ether: hexane (3:17)
Collect the eluate into flask
Attach to condenser and condense at < 40°C to about 1 mL
and dry at room temp. under N₂ gas stream
Add n-hexane and make up to 2 mL before inject into GC-MS.

MALAYSIAN COCOA BOARD (MCB)
WORKSHOP ON THE SAFE USE OF PESTICIDES IN COCOA AND HARMONIZED LEGISLATION FOR FOOD SAFETY, 25-27TH JAN. 2011
Sample prep & extraction

Esterification

hydrolysis
Purification

Qualitative & Quantitative analysis using GC-MS
**GC-MS operating condition:**

Injection volume : 1 µl on splitless injector

Column : HP-5MS (30m x 250 µm x 0.25 µm)

Oven temperature programming :

Initially temp. was maintained at 100°C for 2 min, ramped to 230°C at 5°C/min and hold for 20 min.

Mass spectrometer configuration,

Electron Ionization with Selected Ion Monitoring Mode (SIM)

Monitored fragment ions :

111, 175, 199 and 234 m/z
QuEChERS Method

Weigh 10 g cocoa beans into a 50 ml centrifuge tube (with screw cap)

Add 20 ml acetonitrile & Shake vigorously for 1 min

Adjust pH to < 5 with acetic acid

Add 4 g MgSO4 and 1 g NaCl

Shake each tube after the salt addition

Add another sufficient amount of acetonitrile to obtain the final volume is 25 mL

Centrifuge for 5 min at 3000 rpm

Pipette out aliquot and filter through 0.2µm membrane filter

Ready for injection to LC-MS/MS
**LC–MS/MS condition:**

Injection volume : 5 µl  
Column : Phenomenex Column Aqua C–18  
(particle size 5 µm, 2 mm I.D x 50 mm length)

**Mobile phase ;**

Solvent A : H₂O / 0.1% formic acid  
Solvent B : Acetonitrile / 0.1% formic acid

Flow rate : Gradient at 0.20 ml/min.

Mass spectrometer configuration, Electrospray ionization, using Multiple Reaction Monitoring (MRM) mode.

Qualifier and Quantifier ions were monitored at 219 and 161 a.m.u (atomic mass unit) respectively.
Validation of Method

1. Instrument Sensitivity
2. Recovery Studies
1. Instrument sensitivity

- determined by injecting 2,4-dichlorophenoxy acetic acid (2,4-D) standard solution
- different levels of concentration ranging 0.001 to 0.05 ppm
- for GC-MS: 2,4-D was ESTERIFIED using Boron trifluoride (BF3) prior to injection
- for LC-MS/MS: direct inject / without esterification

- The following parameters were monitored:
  i. Limit of Quantification (LOQ) at S/N=10
  ii. Limit of Determination (LOD) at S/N=3
  iii. Linearity of calibration curve
TIC and Spectrum of 2,4-D under GC-MS

SCAN MODE

SIM MODE

111, 175, 199, 234 m/z
TIC of “Esterified 2,4-D” at different levels of concentration on GC-MS
TIC of 2,4-D at different levels of concentration on LC-MS/MS

Blank
0.001 ppm (S/N=45)
0.0025 ppm (S/N=195.5)
0.005 ppm
0.01 ppm

Good linearity &
R^2 > 0.95
2. Method suitability and Recovery study

- to obtain and establish LOQ (refers to sample that have been processed through all the steps)
- sample was spiked with known amount of 2,4-D at different level of concentration
- spiked sample for MHLW method was esterified, hydrolized, purified prior to GC–MS determination
- % recovery was calculated based on the amount of 2,4-D extracted compared with the expected conc. (0.005 – 0.05 ppm)
- % recovery should pronounced linearity or proportionate between concentration and response
Recovery Study of spiked sample in GC-MS

LOQs was found at 0.01 ppm, S/N=9.38 ± 2.6

- Recovery at each concentration were found within acceptable range of 70-120%
- Good linearity with $R^2>0.95$, (when the graph of area response vs conc. were plotted)
Recovery Study of spiked sample in LC-MS/MS

LOQs was found at 0.001 ppm, $S/N = 51.2 \pm 8.4$

- Recovery at each concentration were found within acceptable range of 70-120%
- Good linearity with $R^2 > 0.95$, (when the graph of area response vs conc. were plotted)
## Recovery (%) and coefficient correlation between different extraction method

<table>
<thead>
<tr>
<th>Method</th>
<th>Recovery (%) ± RSD, at diff. conc. / ppm</th>
<th>Coefficient correlation, R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td><strong>MHLW</strong></td>
<td>n.a</td>
<td>96.6 ± 9.2&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>105.2 ± 10.6&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.8 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>QuEChERS</strong></td>
<td>107.5 ± 8.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>93.7 ± 9.9&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>104.4 ± 13.1&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.a</td>
</tr>
</tbody>
</table>

n.a - not available  
<sup>a</sup> n=6,  
<sup>b</sup> n=9,  
<sup>c</sup> not significant different at p<0.05
## Recovery (%) at 0.01 ppm on different sample matrix

<table>
<thead>
<tr>
<th>Method</th>
<th>Whole</th>
<th>Shell</th>
<th>Nib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bean</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MHLW</strong></td>
<td>97.7</td>
<td>104.6</td>
<td>106.6</td>
</tr>
<tr>
<td></td>
<td>± 17.7</td>
<td>± 20.1</td>
<td>± 13.7</td>
</tr>
<tr>
<td><strong>QuEChERS</strong></td>
<td>102.8</td>
<td>103.2</td>
<td>96.4</td>
</tr>
<tr>
<td></td>
<td>± 8.1</td>
<td>± 7.8</td>
<td>± 7.5</td>
</tr>
</tbody>
</table>

* sd was based on n > 3 rep
Analysis of real samples

- Due to instrument sensitivity and LOQs, QuEChERS method tandem with LC-MS/MS was used for routine analysis of cocoa beans samples.
- Each sample was analyzed in replicate as a whole bean, nib and shell.
- None of the samples analyzed were contaminated with 2,4-D at 0.01 ppm.
- However, some samples were found to contain 2,4-D at trace level as shown in Table. 2.
Table 2: Test results on real samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole bean (ppm)</th>
<th>Nib (ppm)</th>
<th>Shell (15% of whole bean) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.0057</td>
<td>0.0018</td>
<td>0.0033 (0.0005)</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.0016</td>
<td>0.0014</td>
<td>0.0030 (0.0005)</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.0015</td>
<td>0.0014</td>
<td>0.0022 (0.0004)</td>
</tr>
<tr>
<td>Sample 4</td>
<td>0.0028</td>
<td>0.0013</td>
<td>0.0067 (0.0011)</td>
</tr>
</tbody>
</table>
## Comparison: QuEChERS vs MHLW Method

<table>
<thead>
<tr>
<th>QuEChERS</th>
<th>MHLW technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid</td>
<td>Time consuming</td>
</tr>
<tr>
<td>Simple</td>
<td>Too many steps and laborious</td>
</tr>
<tr>
<td>Minimum type of solvents / reagents</td>
<td>Too many solvent and reagents are needed</td>
</tr>
<tr>
<td>Low solvent consumption</td>
<td>Extremely high solvent and diluent are needed</td>
</tr>
<tr>
<td>Minimum glassware and apparatus</td>
<td>Too many glassware is needed</td>
</tr>
<tr>
<td></td>
<td>- esp. separating funnel and round bottom flask</td>
</tr>
<tr>
<td>Environmental friendly</td>
<td>Not environmental friendly</td>
</tr>
<tr>
<td>• Minimum usage of harmful chemical</td>
<td>• High in chemicals consumption and waste</td>
</tr>
<tr>
<td>• Low chemical waste</td>
<td></td>
</tr>
<tr>
<td>Good result,</td>
<td>Precaution steps is needed, to avoid</td>
</tr>
<tr>
<td>- Recovery / Consistency / Repeatability</td>
<td>loses of analyte which will affect to the result / recovery</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Total Ion Chromatography (TIC) of 2,4-D in cocoa bean, nib and shell
Conclusion:

• Extraction technique is a crucial part in analytical work of pesticide residue analysis,
• This study - MHLW and QuEChERS are applicable to the analysis of 2,4-D in cocoa beans, prior to quantification using GC-MS or LC-MS/MS
• However, QuEChERS technique works efficiently and effectively:
  • reduction of time,
  • less number of solvents and consumables,
  • safe and environmental friendly.
Conclusion:

• Selection of proper detector (or detection technique) also plays important roles in the pesticide residue analysis.
• The most sensitive detector is preferred and will determine the validity of the test results and complies to the quality criteria such as:
  • accuracy,
  • repeatability and precision.
• In this study and due to the instrument sensitivity, the use of LC-MS/MS is preferred.
Conclusion:

- Sample preparation:
  - The use of NIB is preferred in the analysis.
Thanks for your attention