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

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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.

Codex	If no MRL exists, a default MRL of 0.01 mg/kg applies
European Union	If no MRL exists, a default MRL of 0.01 mg/kg applies
Japan	If no MRL exists, a default MRL of 0.01 mg/kg applies
Canada, New Zealand	If no MRL exists, a default MRL of 0.1 mg/kg applies
Malaysia, Hong Kong, Korea, India, Israel, Russian Federation, Singapore, South Africa	If no MRL exist, Codex MRLs applies or a default MRL of 0.01 mg/kg

Table 1 : MRLs for cocoa beans from different countries

A.I	CODEX	EU	Japan
Endosulfan	0.1 ppm	0.1 ppm	0.1 ppm
Metalaxyl-M	0.02 ppm	0.1 ppm	0.2 ppm
Chlorothalonil	Not specified	0.1 ppm	0.05 ppm
Deltamethrin	Not specified	0.05 ppm	0.05 ppm
Dichlorvos	Not specified	0.02 ppm	0.5 ppm
Glyphosate	Not specified	0.1 ppm	0.1 ppm
2,4-D	Not specified	0.1 ppm	Default MRL, 0.01 ppm
Bromide	5 ppm **(Guideline level)**	50 ppm	70 ppm

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

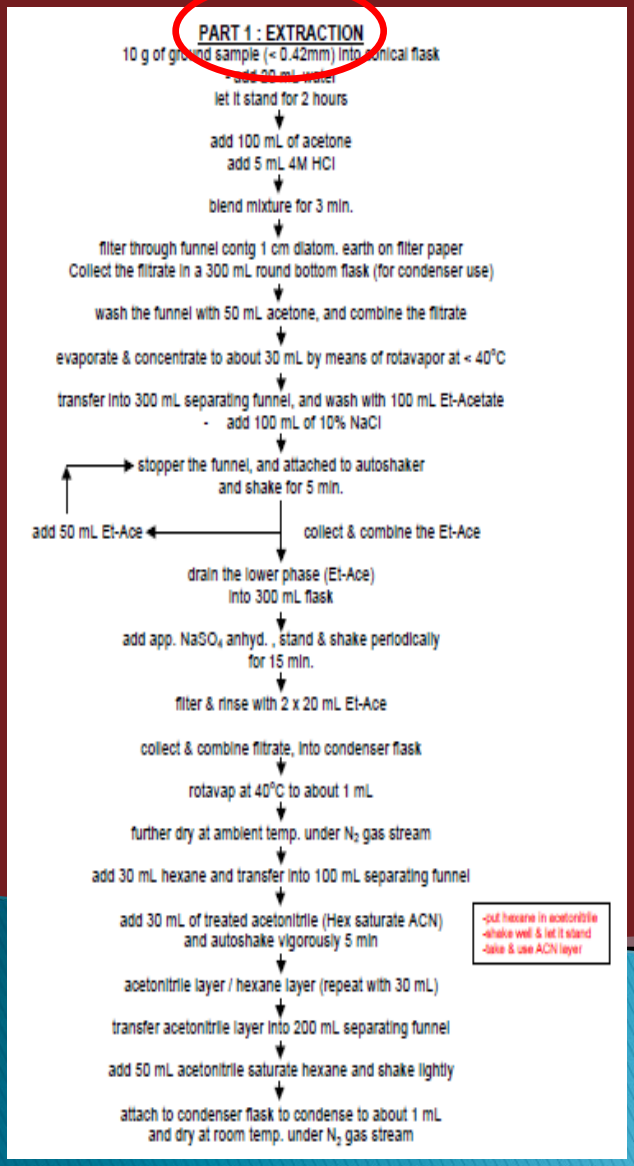
Adopted from Multiresidue QuEChERS Method:
Popular and applicable for the analysis wide range of pesticides.

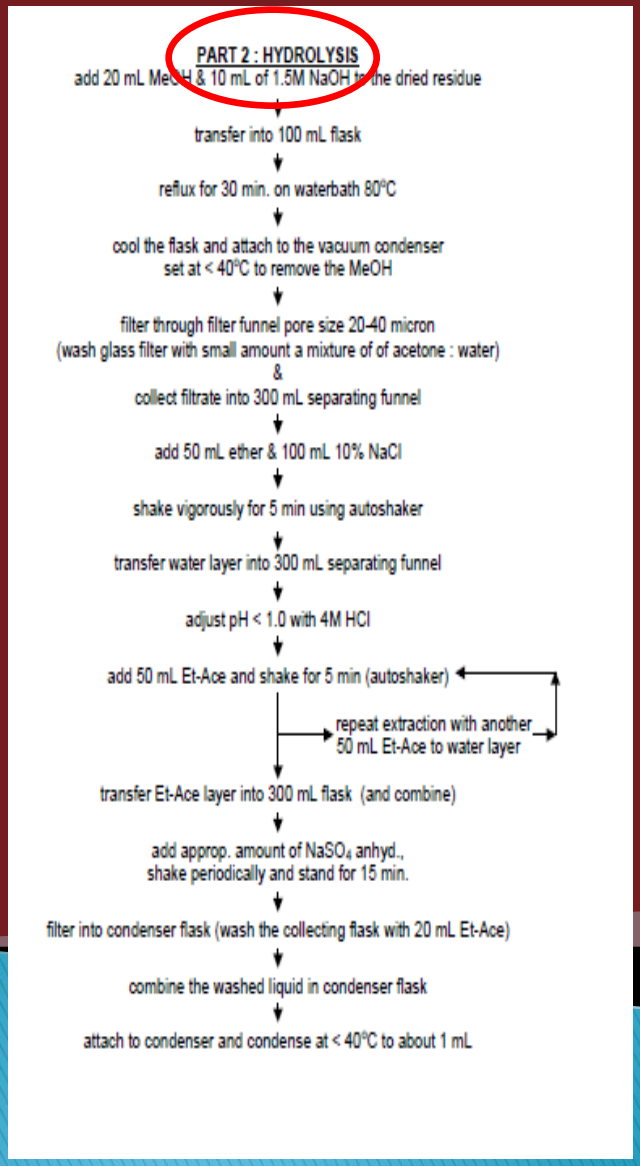
Steps involved ;

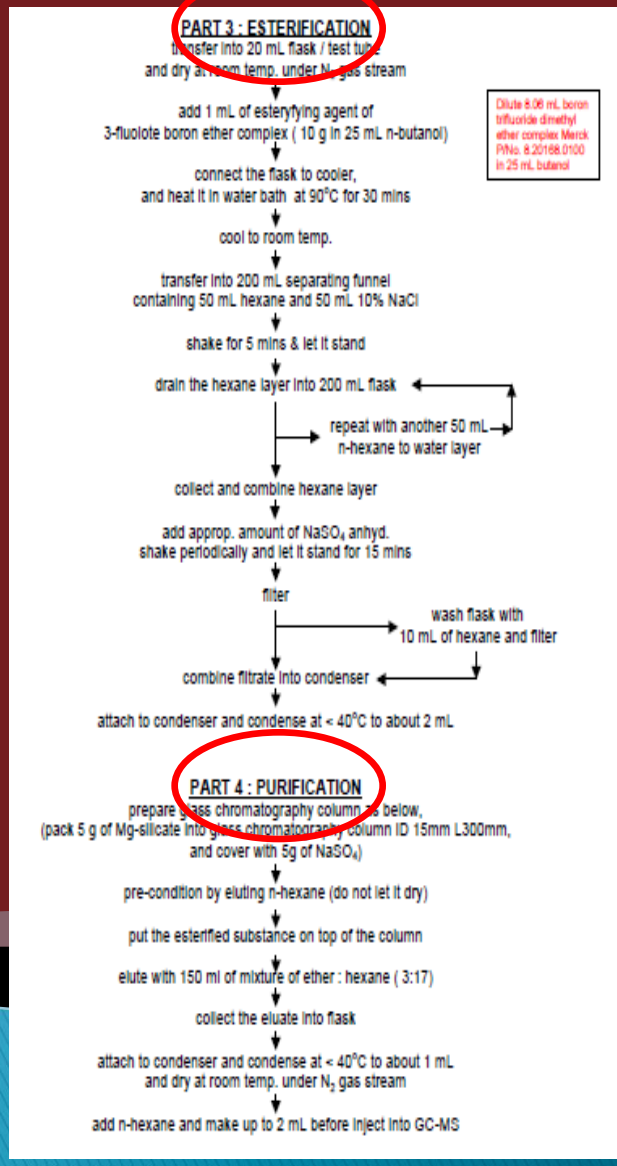
1. Extraction
2. Purification
3. Determination and quantification using Liquid Chromatography coupled with Mass Spectrophotometry Detector (GC-MS/MS)

Reference ;
M. Anastassiades, S. J. Lehotay, D. Stajnbaher, F. J. Schenck
Fast and Easy Multiresidue Method Employing Acetonitile Extraction / Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce, J. AOAC Int., 86 (2003) 412-431

MHLW Test method flowchart







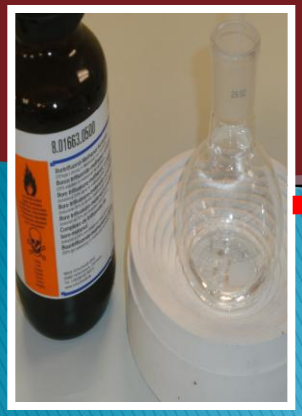
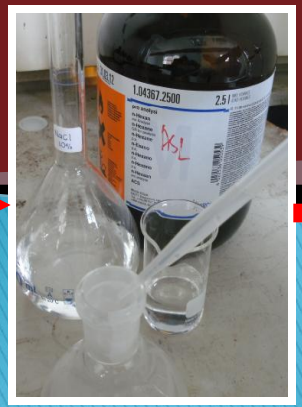
Sample prep & extraction



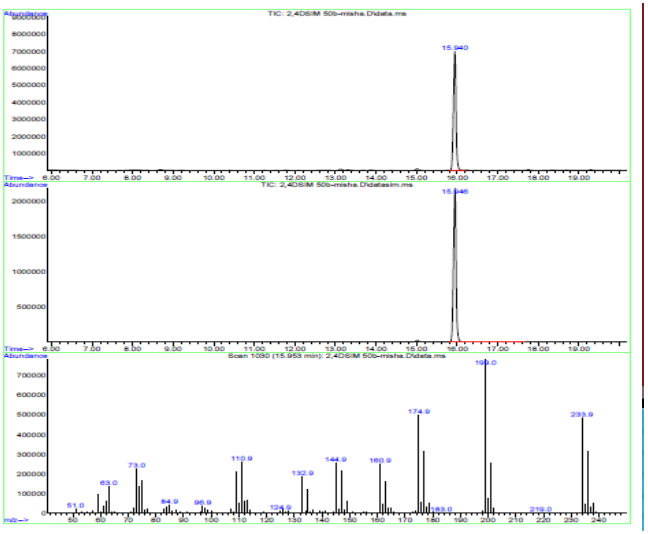

hydrolysis

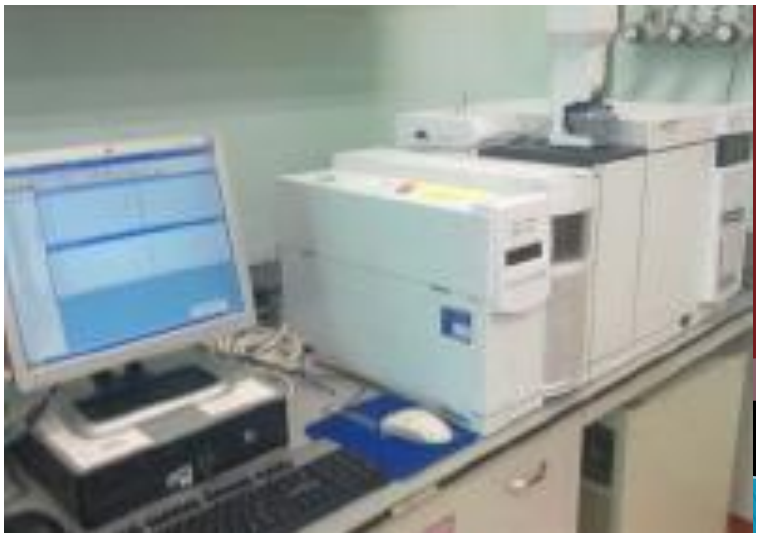



Esterification

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 $MgSO_4$ 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

↓

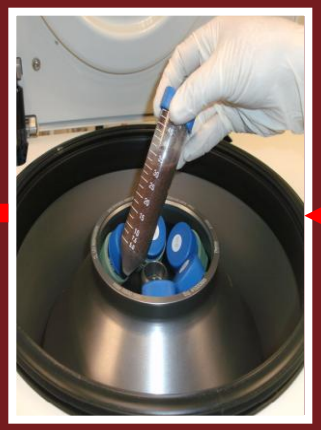
Ready for injection to LC-MS/MS

Sample prep & extraction

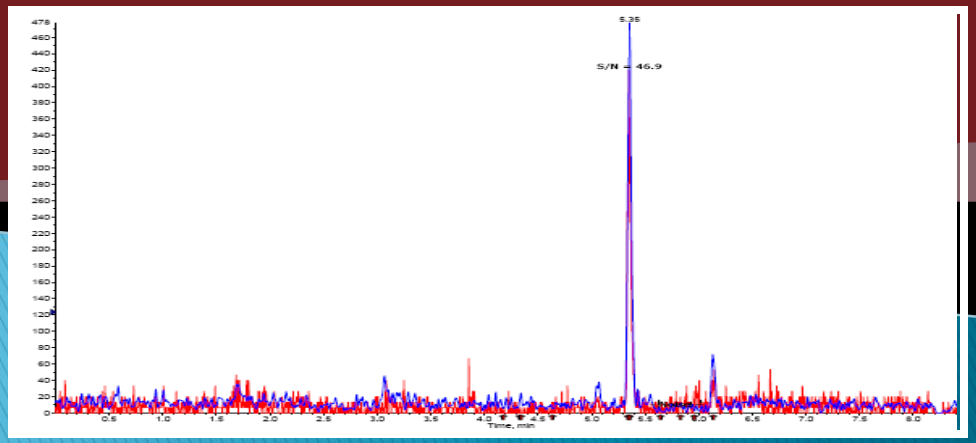







Qualitative & Quantitative analysis using LC-MS/MS 3200QTrap

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 : H_2O / 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

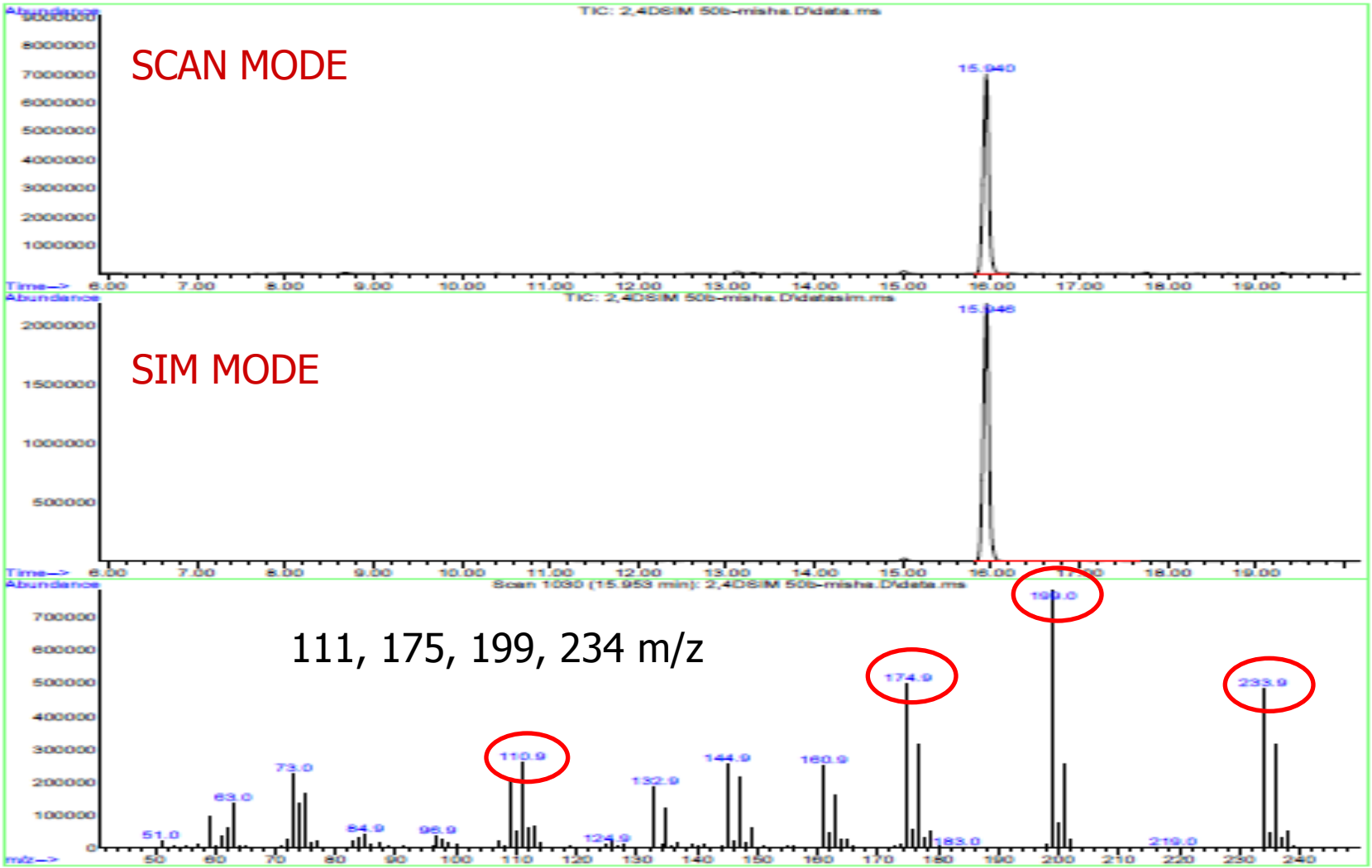
1. Instrument Sensitivity
2. Recovery Studies

1. Instrument sensitivity

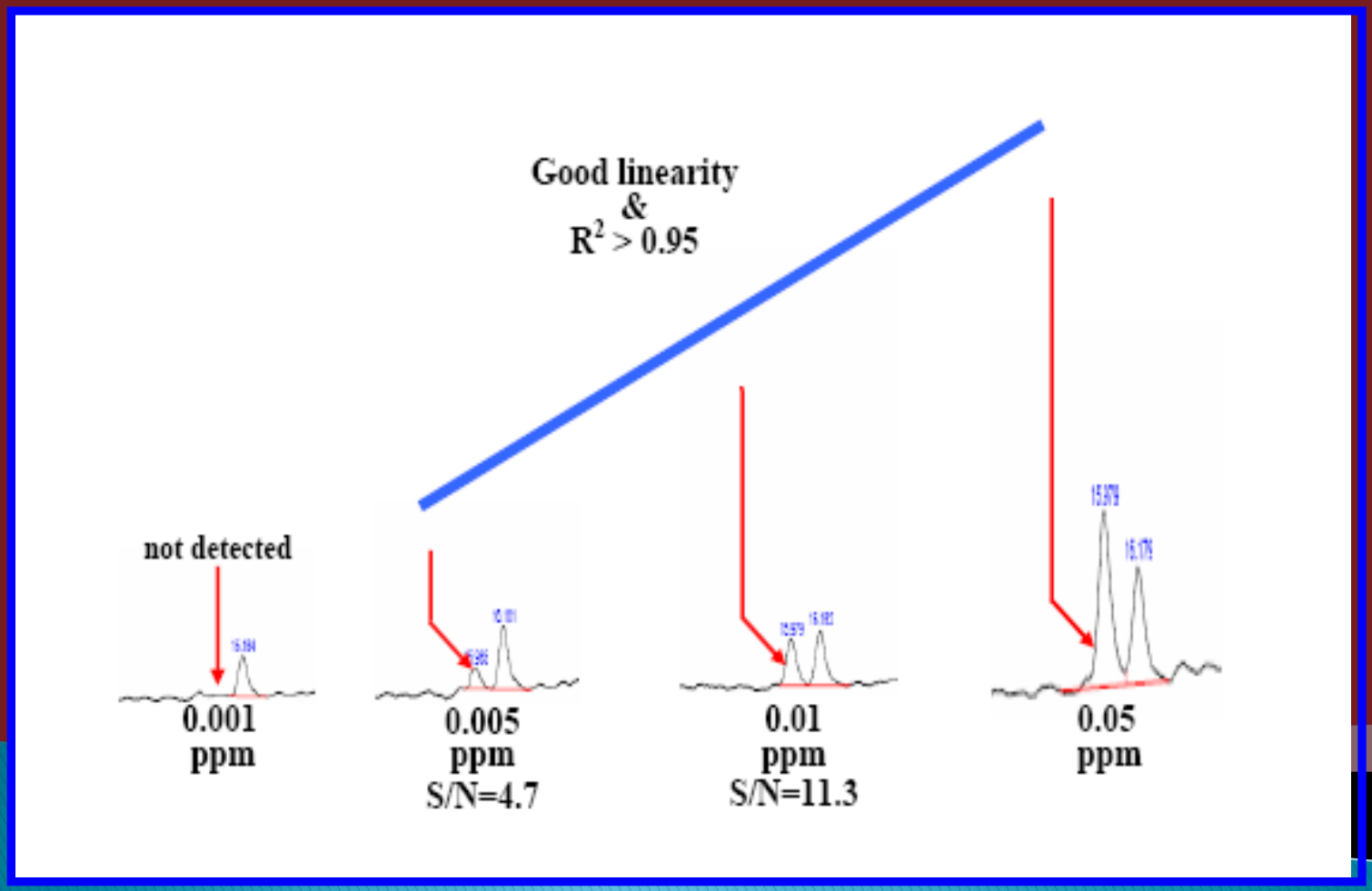
- determined by injecting 2,4-dichlorophenoxy acetic acid (2,4-D) standard solution
- different levels of concentration ranging 0.001 to 0.05 ppm
- for GC-MS : 2,4-D was ESTERIFIED using Boron trifluoride (BF3) prior to injection
- for LC-MS/MS : direct inject / without esterification

- **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

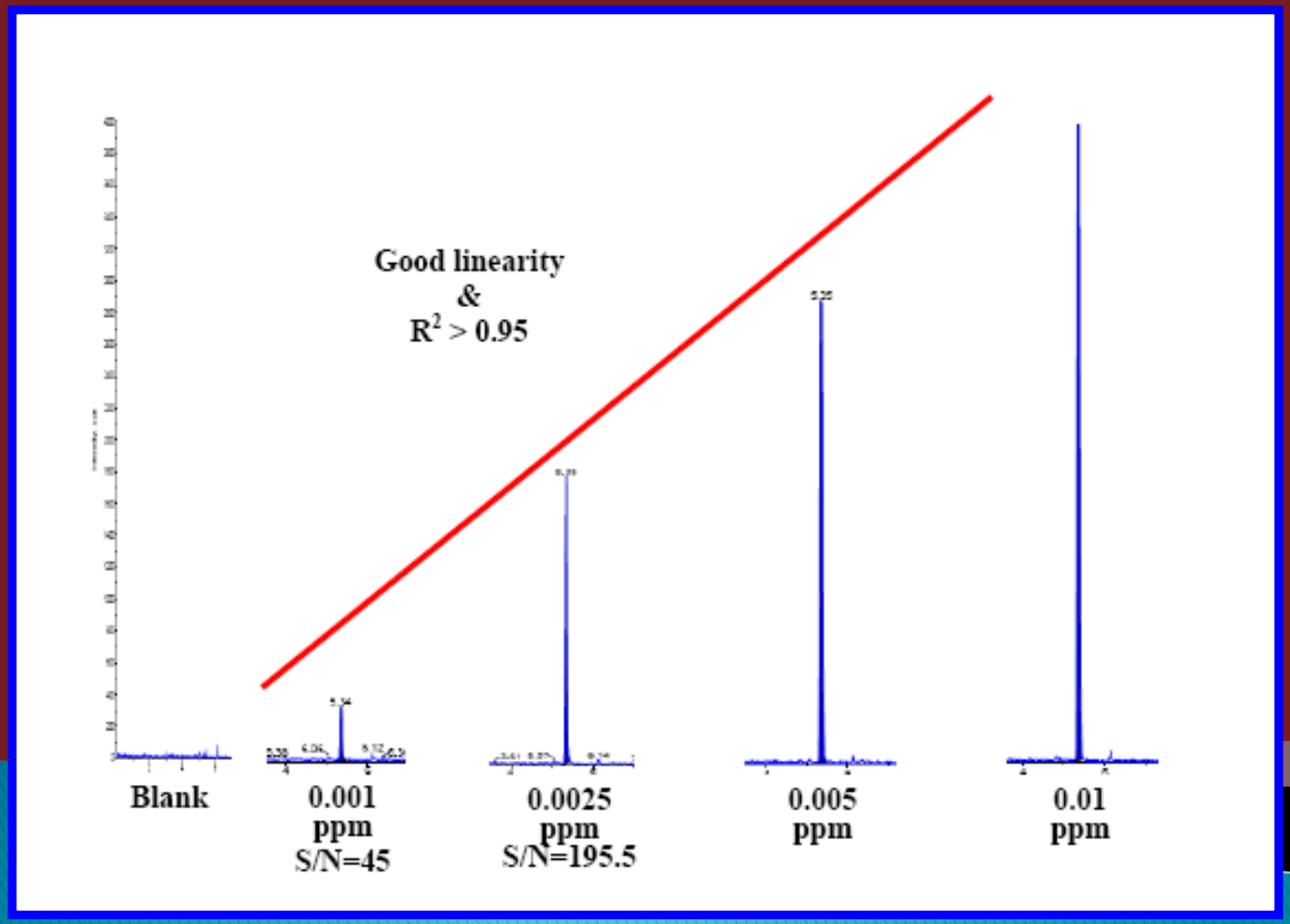
TIC and Spectrum of 2,4-D under GC-MS



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

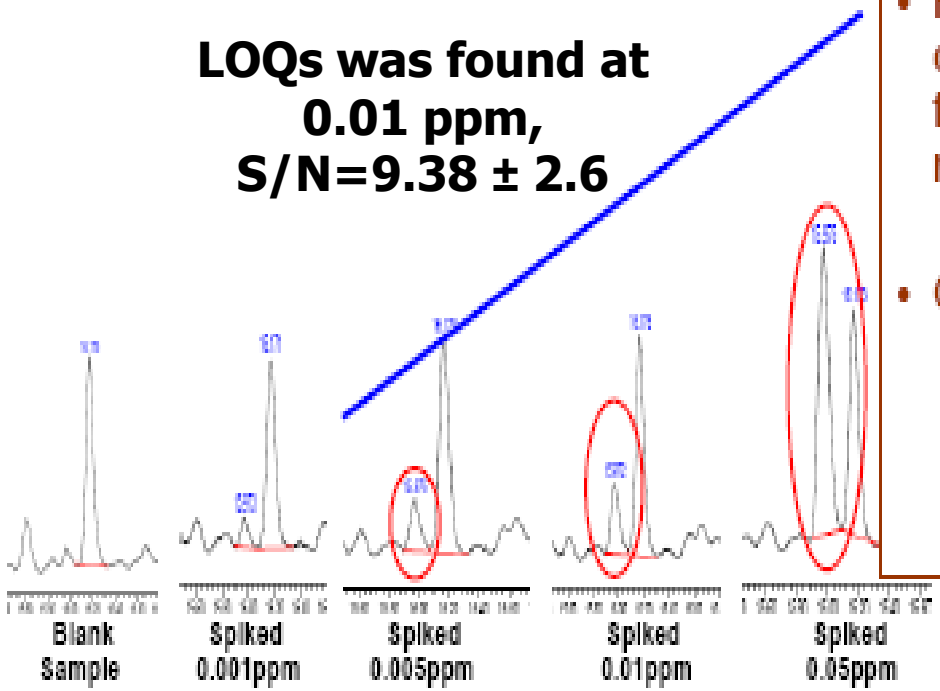


2. Method suitability and Recovery study

- 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

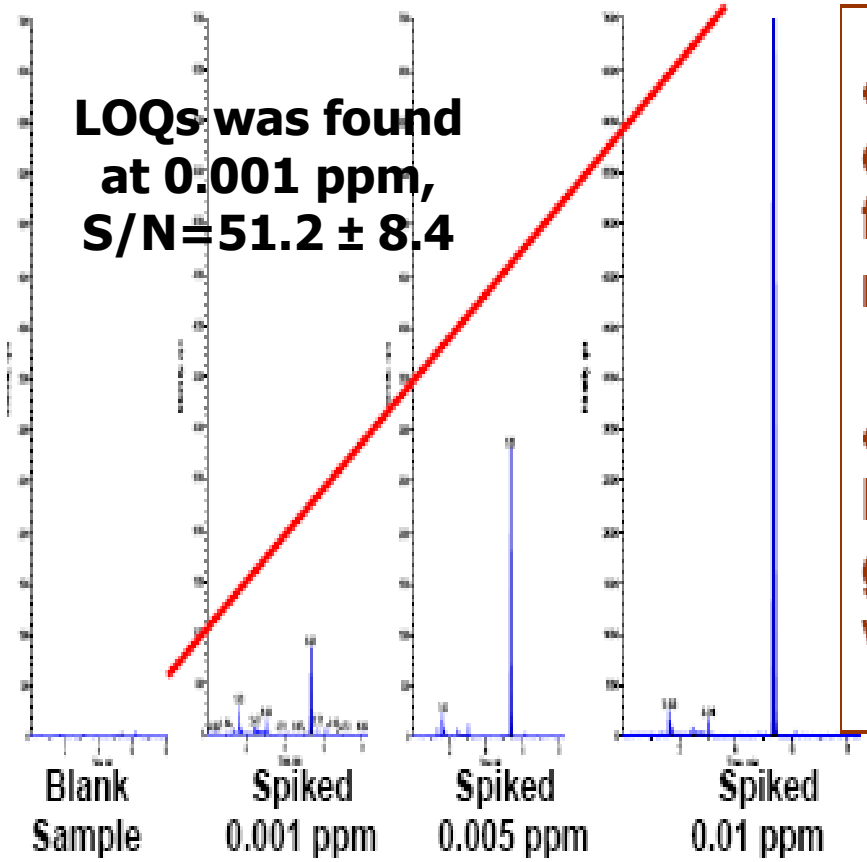
Recovery Study of spiked sample in GC-MS

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



- 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

	Recovery (%) ± RSD, at diff. conc. / ppm				Coefficient correlation, R^2
Method	**0.001**	**0.005**	**0.01**	**0.05**	
MHLW	n.a	96.6 ± 9.2[a,c]	105.2 ± 10.6[a,c]	97.8 ± 7.5[a]	0.99, Linear
QuEChERS	107.5 ± 8.1[b]	93.7 ± 9.9[b,c]	104.4 ± 13.1b[b,c]	n.a	0.99, Linear

n.a – not available

[a] n=6, [b] n=9,

[c] not significant different at $p<0.05$

Recovery (%) at 0.01 ppm on different sample matrix

Method	Recovery (%) ± sd, at 0.01 ppm		
	Whole bean	Shell	Nib
MHLW	97.7 ± 17.7	104.6 ± 20.1	106.6 ± 13.7
QuEChERS	102.8 ± 8.1	103.2 ± 7.8	96.4 ± 7.5

* 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

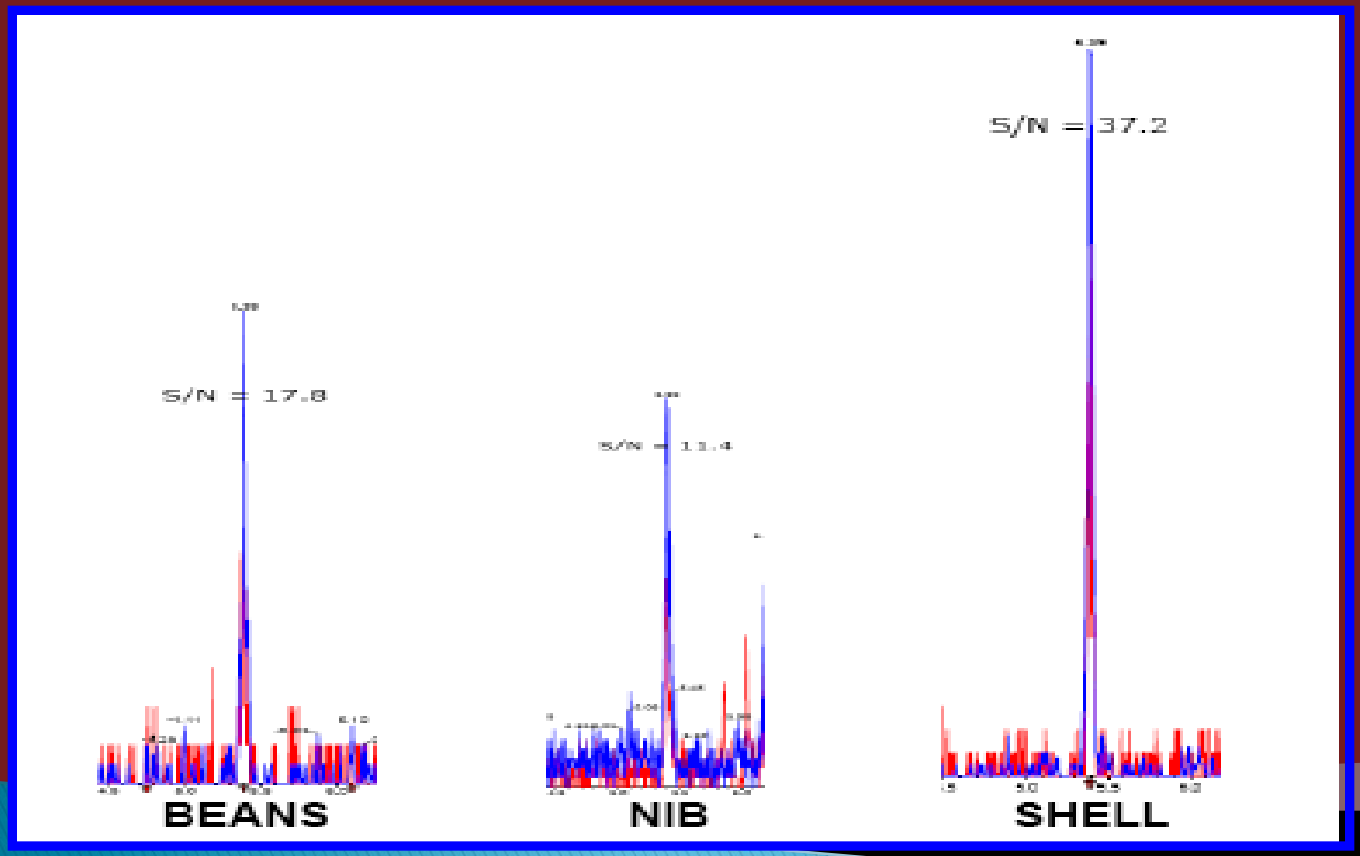
Table 2 : Test results on real samples

Sample	Whole bean (ppm)	Nib (ppm)	Shell (15% of whole bean) (ppm)
Sample 1	0.0057	0.0018	0.0033 (0.0005)
Sample 2	0.0016	0.0014	0.0030 (0.0005)
Sample 3	0.0015	0.0014	0.0022 (0.0004)
Sample 4	0.0028	0.0013	0.0067 (0.0011)

Comparison : QuEChERS vs MHLW Method

QuECheRS	MHLW technique
Rapid	Time consuming
Simple	Too many steps and laborious
Minimum type of solvents / reagents	Too many solvent and reagents are needed
Low solvent consumption	Extremely high solvent and diluent are needed
Minimum glassware and apparatus	Too many glassware is needed – esp. separating funnel and round bottom flask
Environmental friendly •Minimum usage of harmful chemical •Low chemical waste	Not environmental friendly • High in chemicals consumption and waste
Good result, - Recovery / Consistency / Repeatability	Precaution steps is needed, to avoid loses of analyte which will affect to the result / recovery

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