

**EFFECTS OF PESTICIDE USE ON THE DEVELOPMENT OF
BEE DISEASES. ANALYTICAL AND ECOTOXICOLOGICAL
THREATS AND CHALLENGES**



Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

Analytical quality control of pesticide residues. Method development for bee and pollen analysis



Mette Erecius Poulsen/Amadeo R. Fernández-Alba

Madrid, 7th November 2016

EURL·CF

European
Commission

EURL·FV



EU REFERENCE LABORATORIES

- Designated and funded by the EU Commission – EU 882/2004
- 14 EURLs for animal health and live animals.
- 27 EURLs for feed and food safety .
 - 4 EURLs on pesticide residues.
 - Improve analytical performance of the national reference laboratories, develop new method, organise proficiency tests, organise trainings
 - Provide scientific and technical assistance to the Commission
- EURL on bee health. Designated February 2011.
 - diagnosing the relevant bee diseases



EUROPEAN COMMISSION
DIRECTORATE-GENERAL FOR HEALTH AND FOOD SAFETY

Safety of the Food Chain
Pesticides and biocides



SANTE/11945/2015

30 November -1 December 2015 rev. 0

**Guidance document on analytical quality control and method validation procedures
for pesticides residues analysis in food and feed.**

SANTE/11945/2015

Supersedes

SANCO/12571/2013

Implemented by 01/01/2016

Coordinators:

Tuija Pihlström NFA, Uppsala, Sweden
Amadeo R. Fernández-Alba EURL-FV, University of Almería, Almería, Spain
Miguel Gamón EURL-FV, Generalitat Valenciana, Valencia, Spain
Mette Erecius Poulsen EURL-CF, DTU National Food Institute, Soeborg, Denmark
Ralf Lippold EURL-AO, CVUA Freiburg, Freiburg, Germany
Michelangelo Anastassiades EURL-SRM, CVUA Stuttgart, Fellbach, Germany

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Analytical Quality Control

- Processes and procedures designed to ensure that the results of laboratory analysis are
 - consistent,
 - comparable,
 - accurate and
 - within specified limits of precision
- Sampling, transport
- Sample analysis
- Identification of analytes
- Quantification
- Report of results



Analytical Method for Pesticides

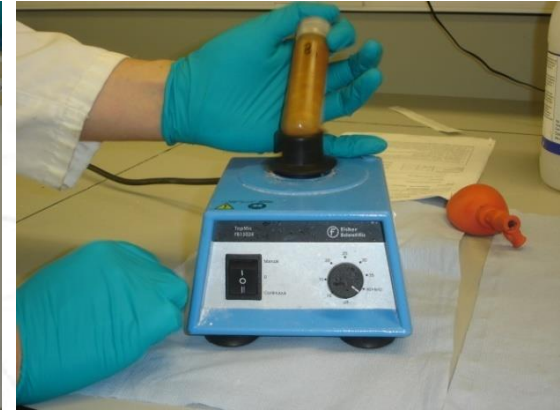
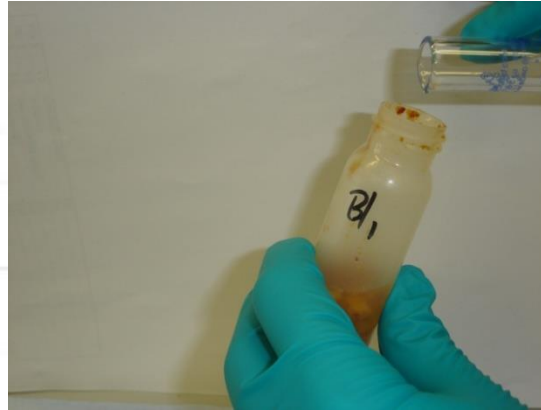
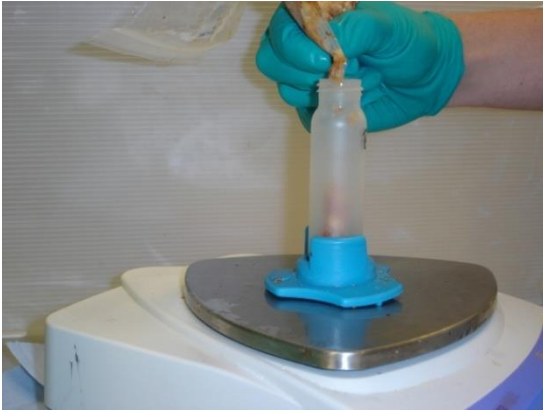
- Extraction of pesticides from sample to solvent



- Clean up – removal of compounds that interfere with the pesticides
- Chromatography
- Detection/identification

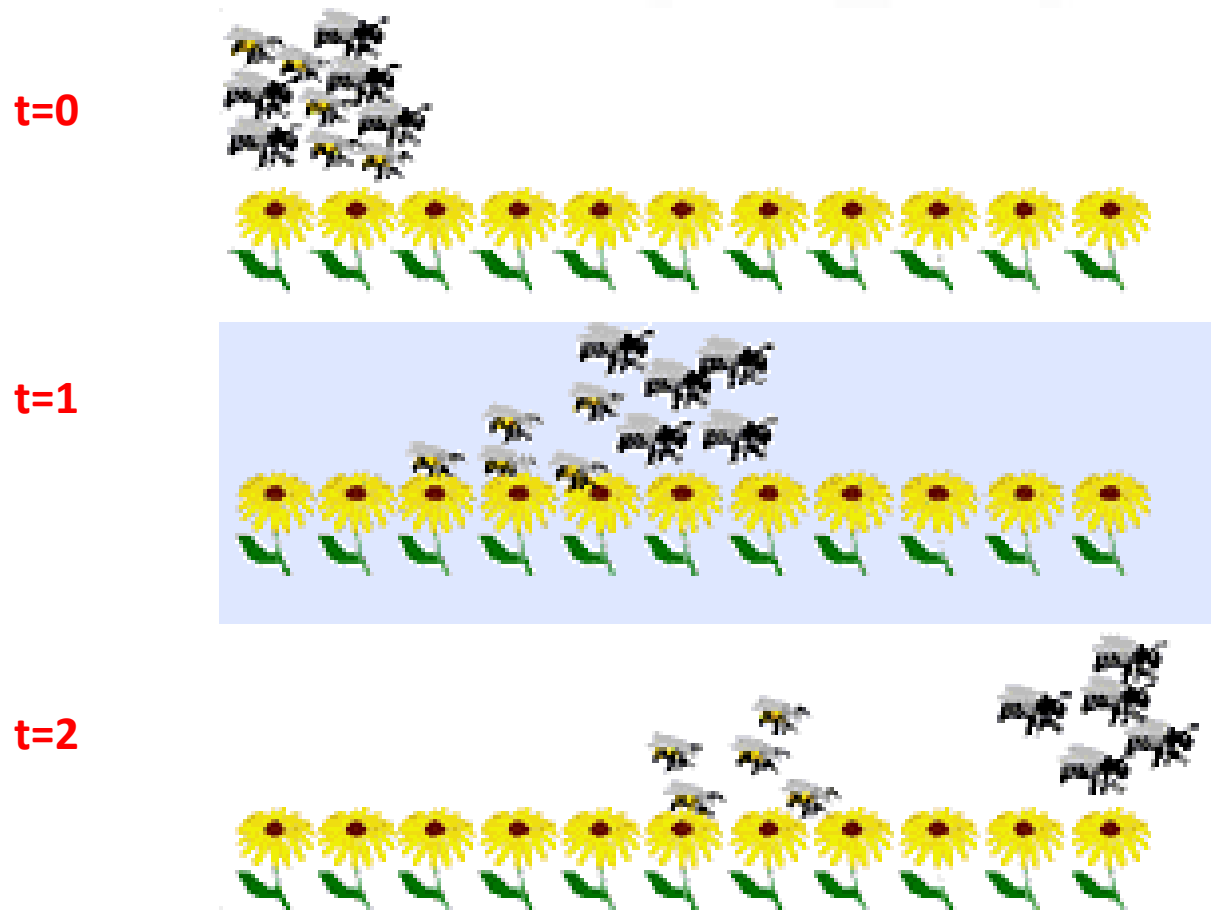


QuEChERS in Practice





Chromatography – like bees

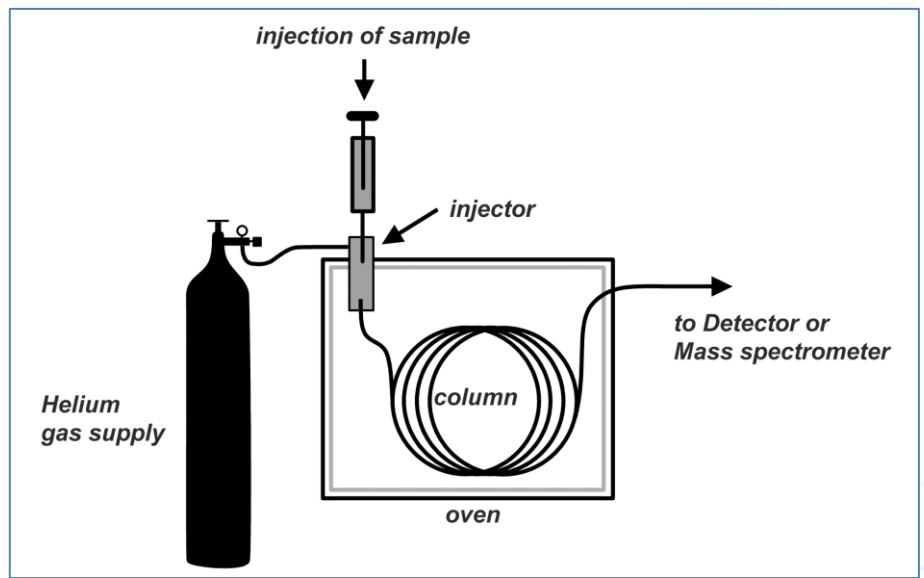




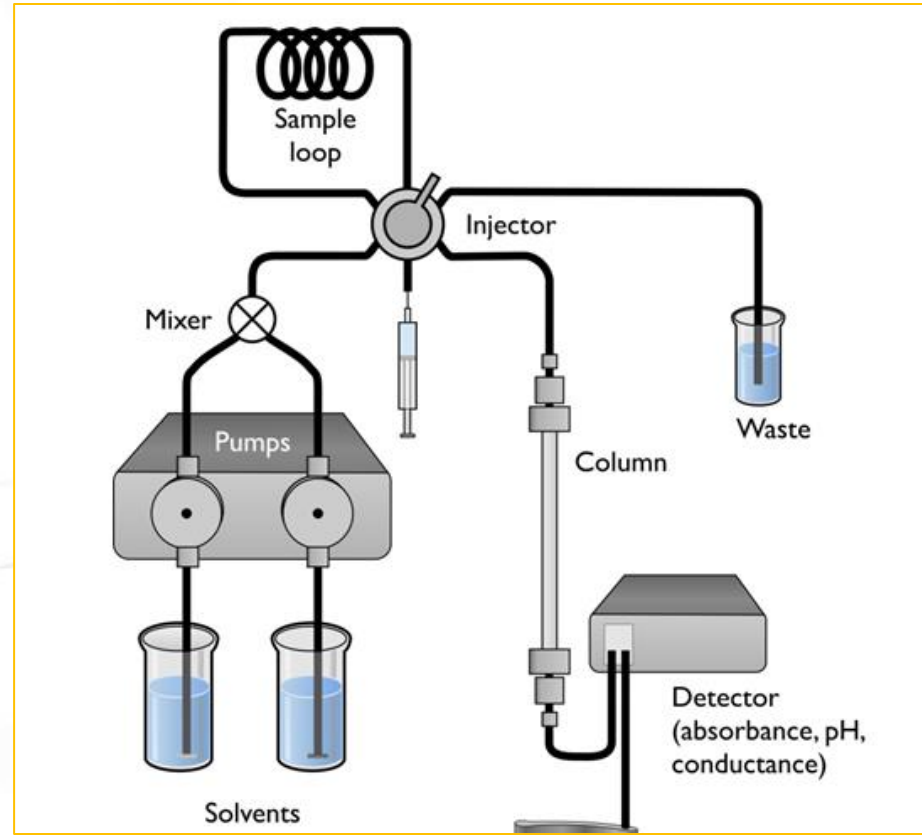
Mette Erecius Poulsen **EURL-CF**
Amadeo R. Fernández-Alba **EURL-FV**

EFFECTS OF PESTICIDE USE ON THE DEVELOPMENT OF BEE DISEASES. ANALYTICAL AND ECOTOXICOLOGICAL THREATS AND CHALLENGES

Liquid Chromatography



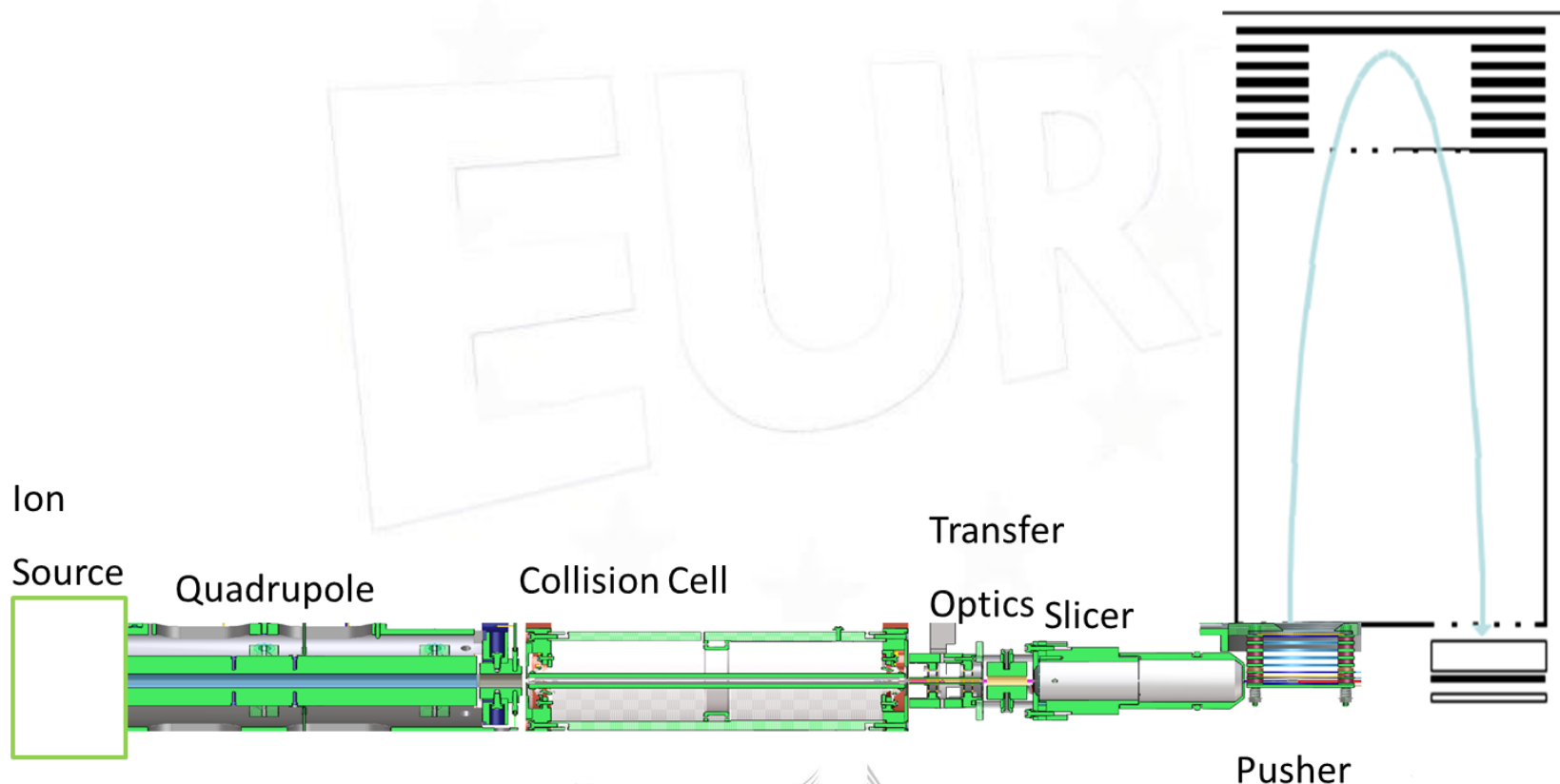
Gas Chromatography





Mass Spectrometer

- Identify compound due to masses, e.g. Time of Flight





Scope of Analytical Method

- 300-600 pesticides/compounds in the same method.
- Especially difficult pesticides in methods with few compounds.
- Larger challenges for matrices like:
 - Bees
 - Bee wax
 - Pollen



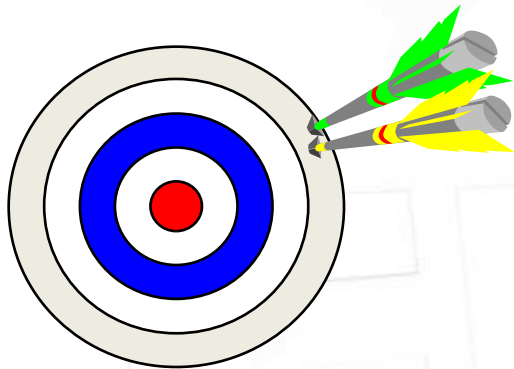
Validation of Methods

- Selectivity
- Linearity/ Dynamic range
- Detection limit/ Quantification limit
- Measurement range
- Ruggedness
- Repeatability/ (Within-laboratory) Reproducibility
- Trueness/ bias

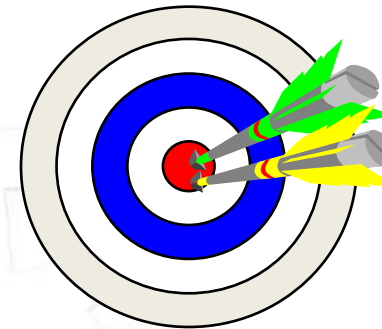
Ref.: NMKL Procedure No. 4 (2009). Validation of chemical analytical methods.



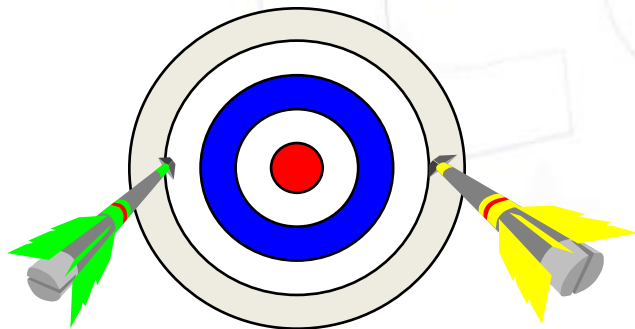
Trueness and Precision



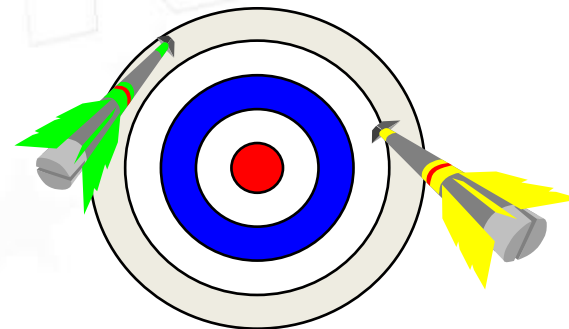
Precise and incorrect



Precise and correct



Imprecise and correct



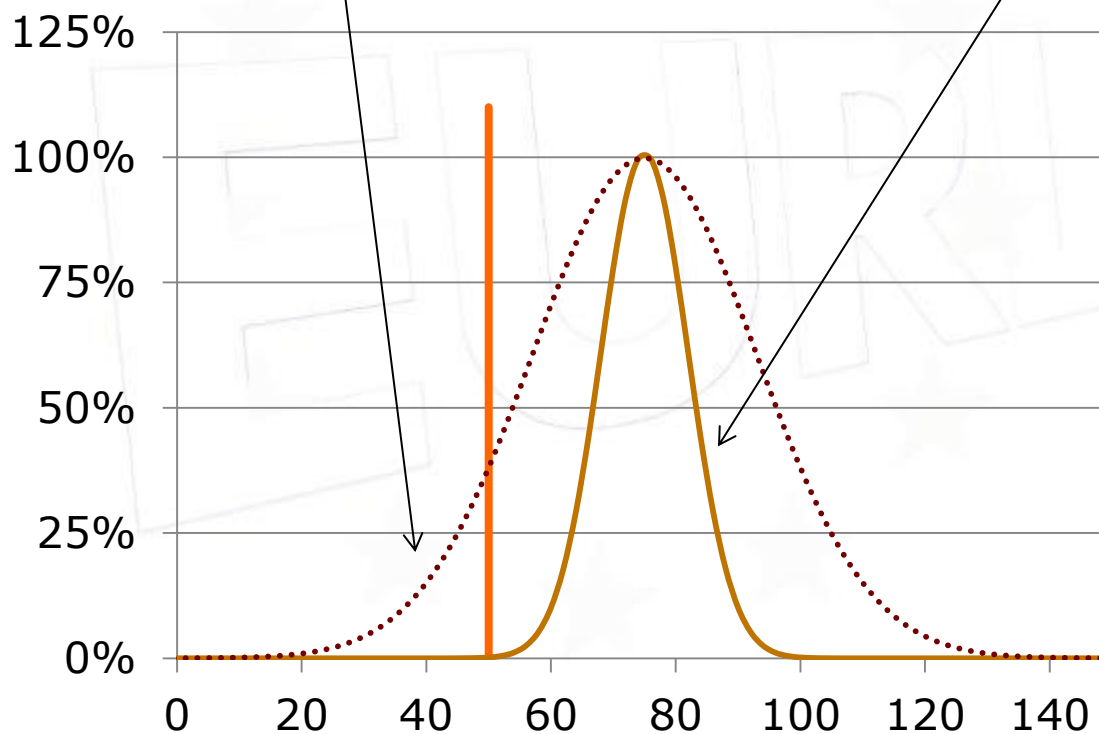
Imprecise and incorrect



Uncertainty (of measurement):

Result (method A): $75 \mu\text{g}/\text{kg} \pm 50 \mu\text{g}/\text{kg}$

Result (method B): $75 \mu\text{g}/\text{kg} \pm 20 \mu\text{g}/\text{kg}$





Measurement Uncertainty

Parameter that characterizes the dispersion of the values

$$X \pm MU$$

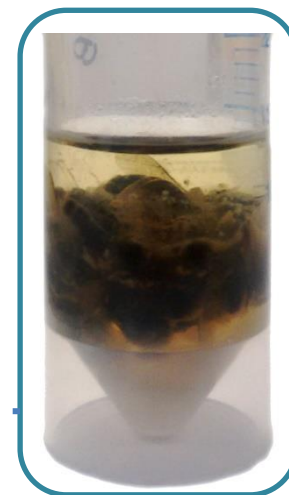
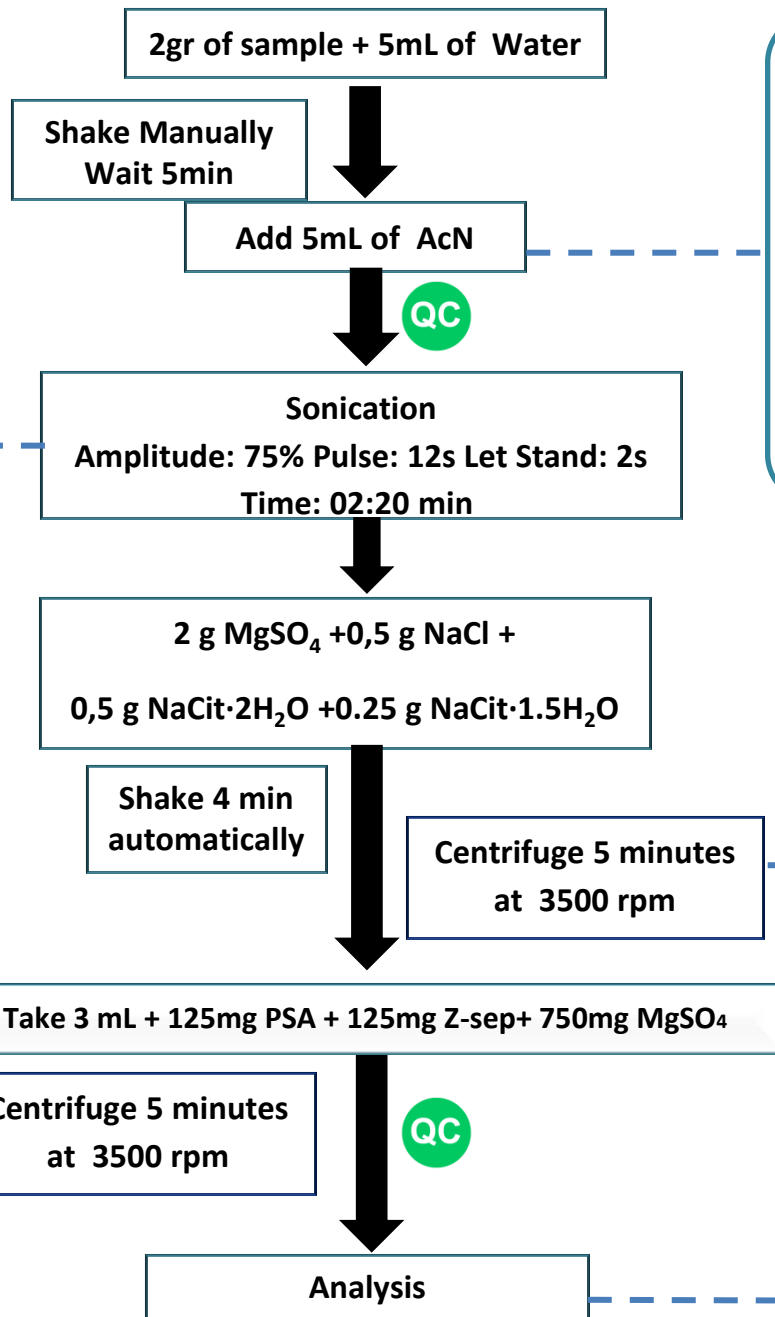
where

- X is the analytical result (with or without recovery correction) and
- MU is the expanded uncertainty.

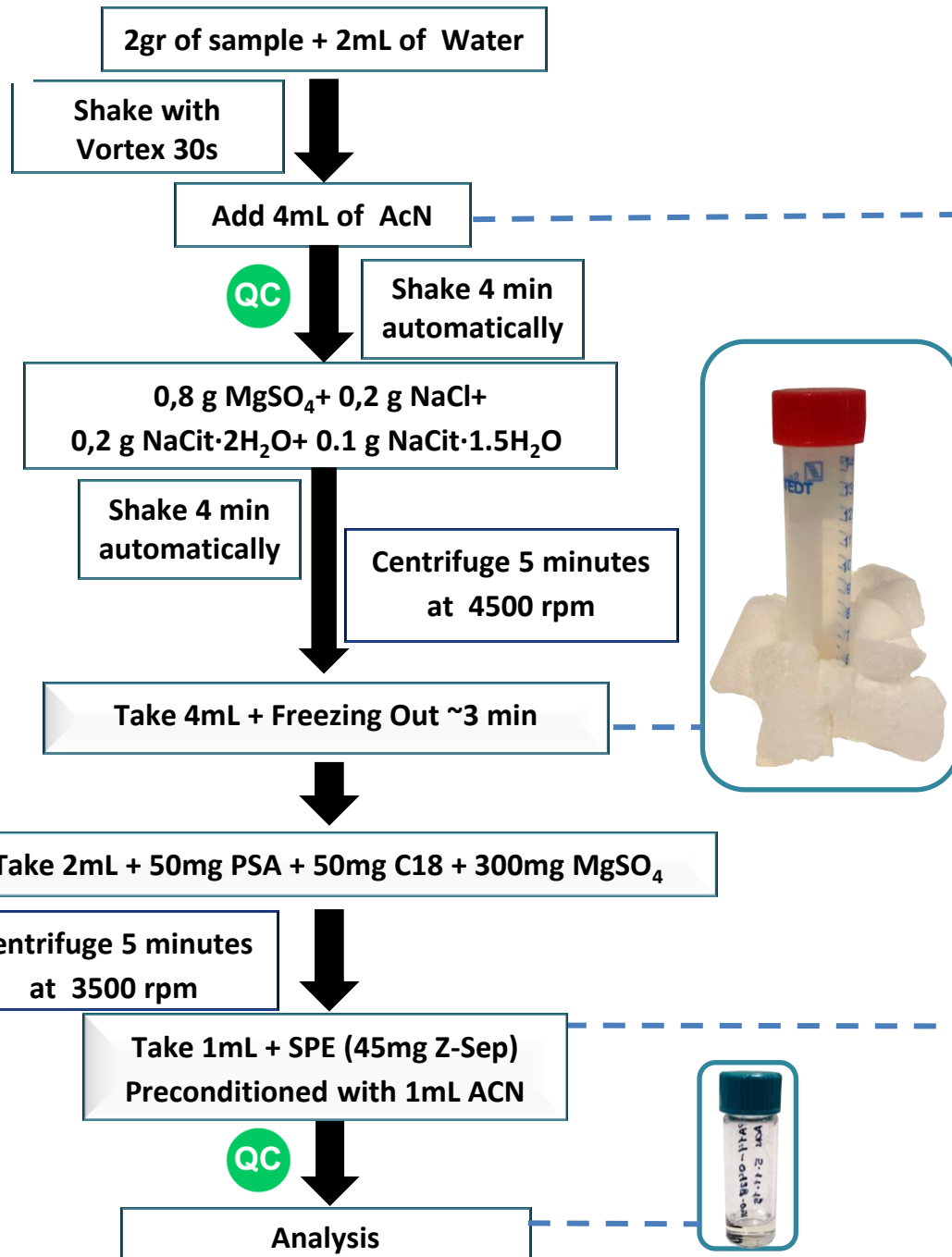
The reported expanded uncertainty is calculated using a coverage factor of 2, which gives a level of confidence of 95%.

Ref.: Eurachem/CITAC guide CG4. QUAM:2012

QuEChERS Bee's



QuEChERS Pollen





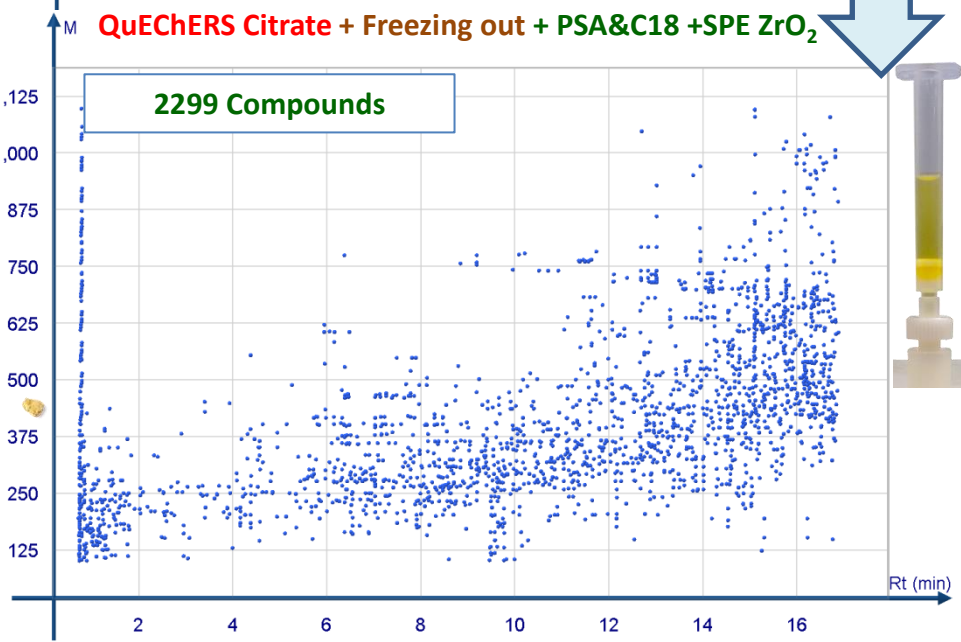
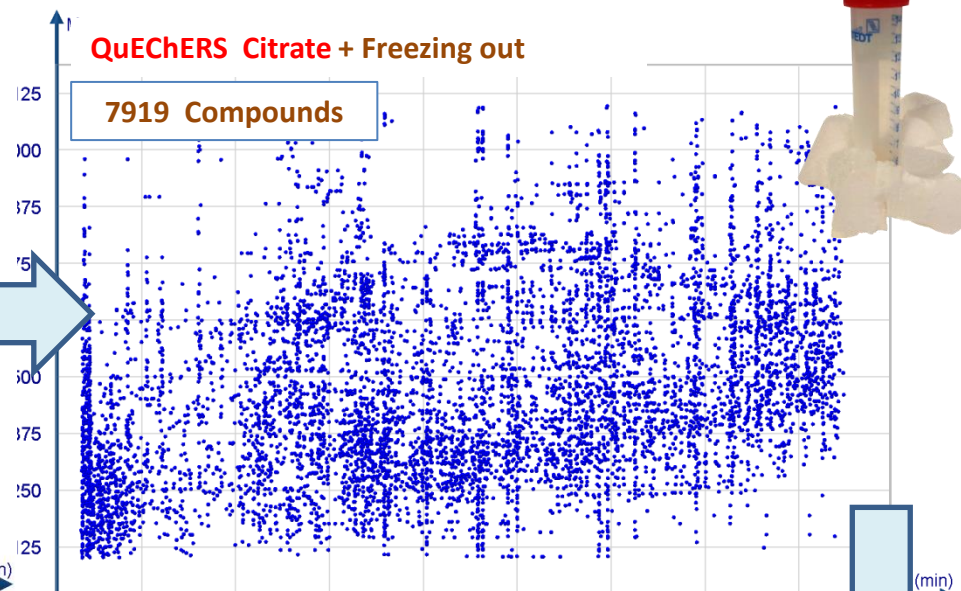
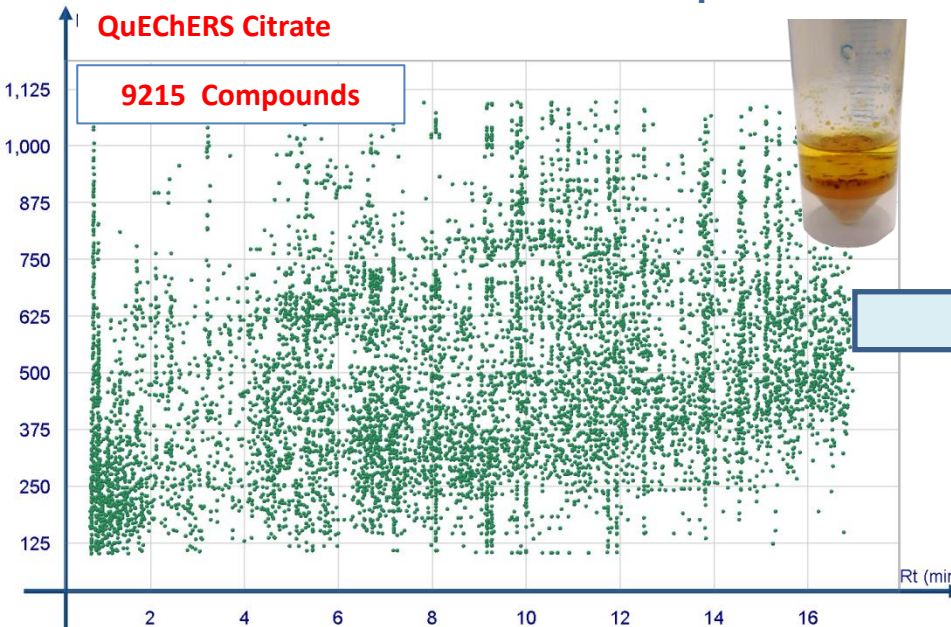
Freezing out step reproducibility



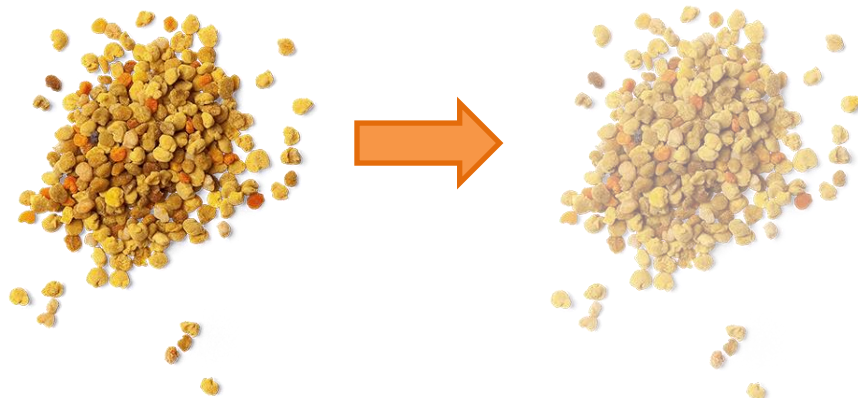
**SAME QUANTITY
OF DRY ICE**

**SAME TIME TO AN
EQUAL VOLUME**

Co-extracted matrix components of Pollen (0.5 g dry sample/ml). LC-TOF-MS



**Multiple Clean up Steps:
- ≈ 7000 Co-extracted Compounds**



QuEChERS Honey Bees Wax Combs



10gr of sample + 10mL of Water

Wait 5
min

Add 10ml of ACN

QC

Shake 4 min automatically
at room temperature

4 g MgSO_4 + 1 g NaCl + 1 g NaCit·2H₂O
+0.5 g NaCit·1.5 H₂O

Shake 5 min
automatically at 40 °C

Centrifuge 5 minutes at 4500 rpm

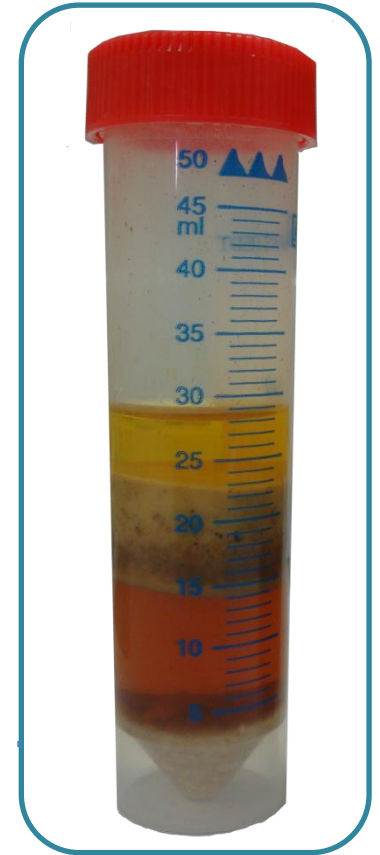
Take 5 mL + 750 mg MgSO_4 + 125 mg PSA

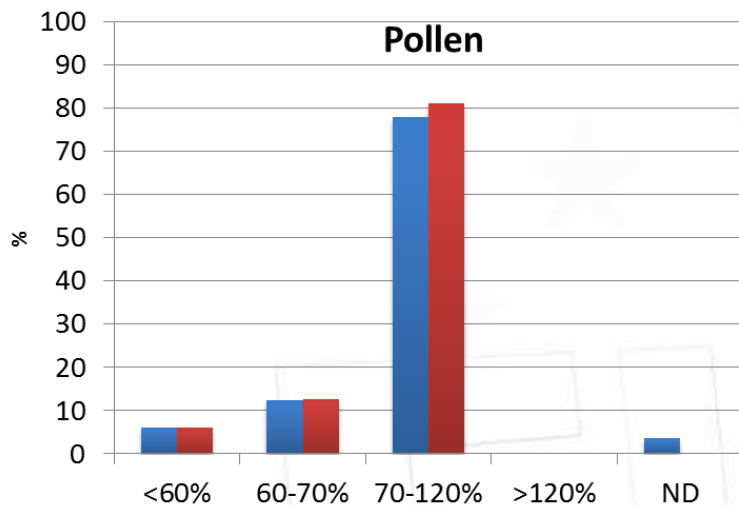
Shake with vortex 30 s

Centrifuge 5 minutes at 3500 rpm

QC

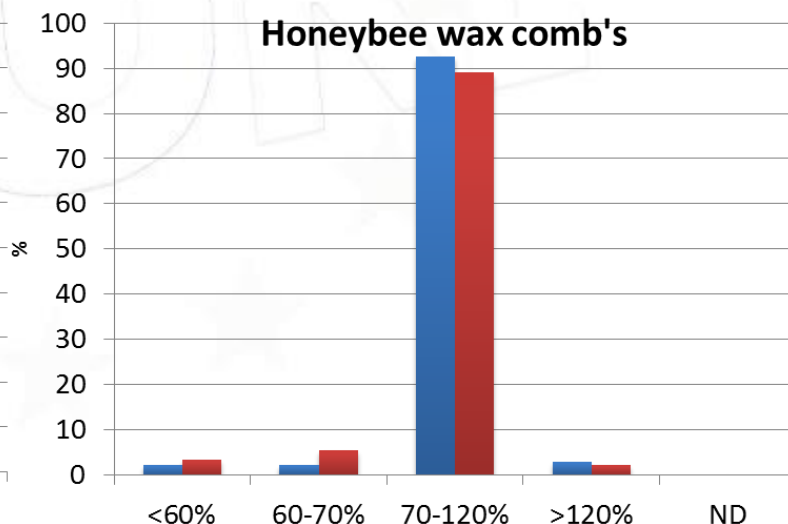
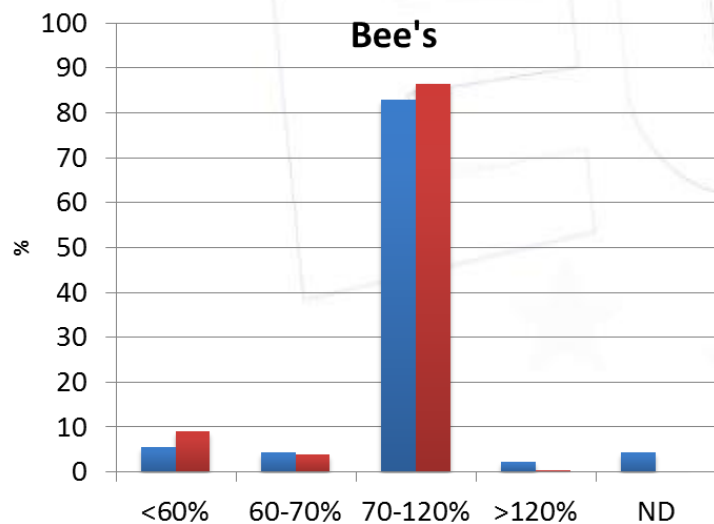
Analysis





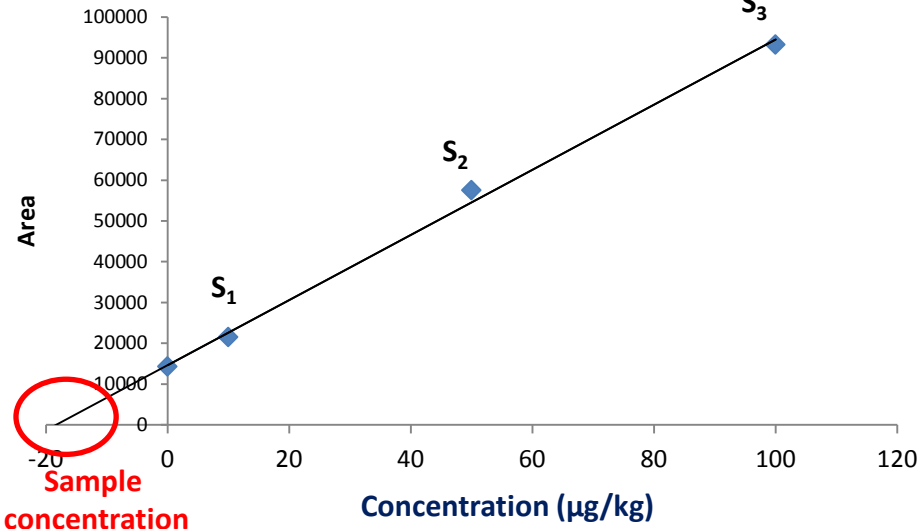
Recoveries of 251 pesticides analysed by LC-MS/MS and GC-MS/MS

■ 5 µg Kg-1
■ 50 µg Kg-1

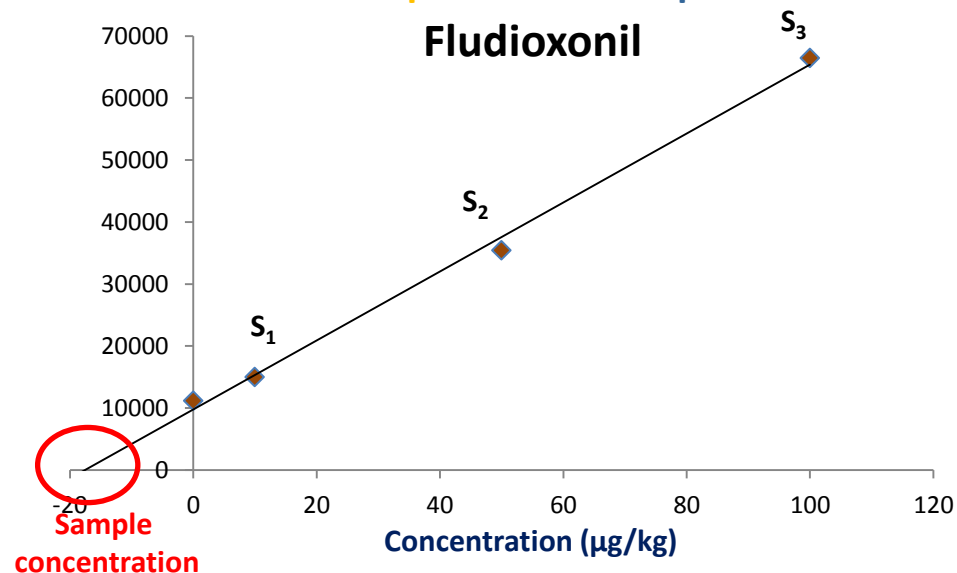


Standard addition in a real sample

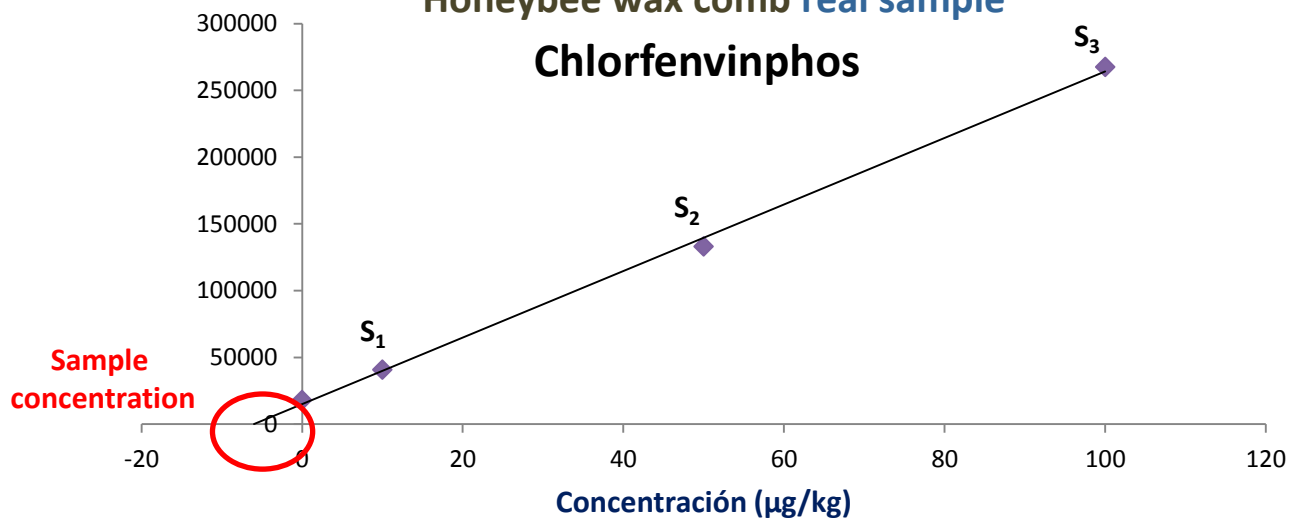
Bee real sample
Fluvalinate-tau



Bee pollen real sample
Fludioxonil

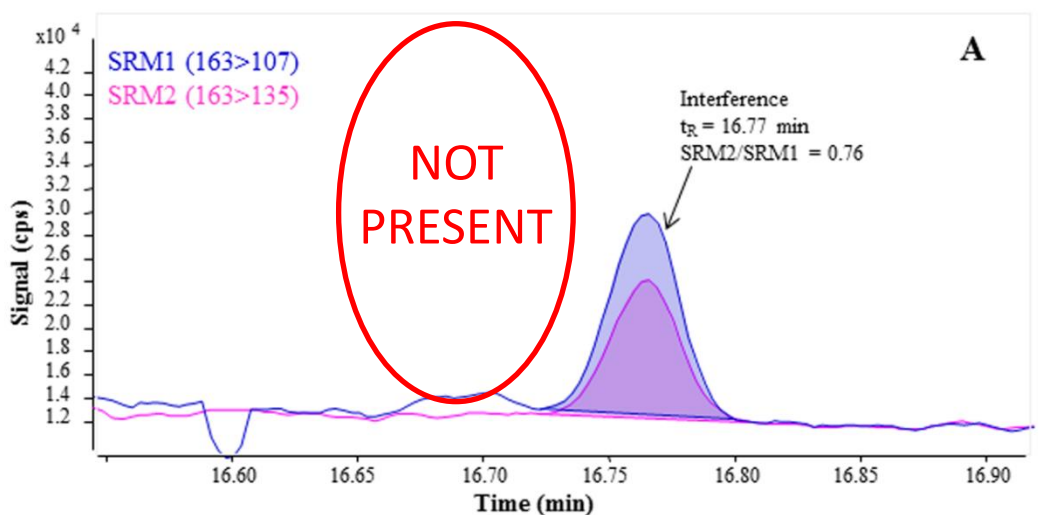
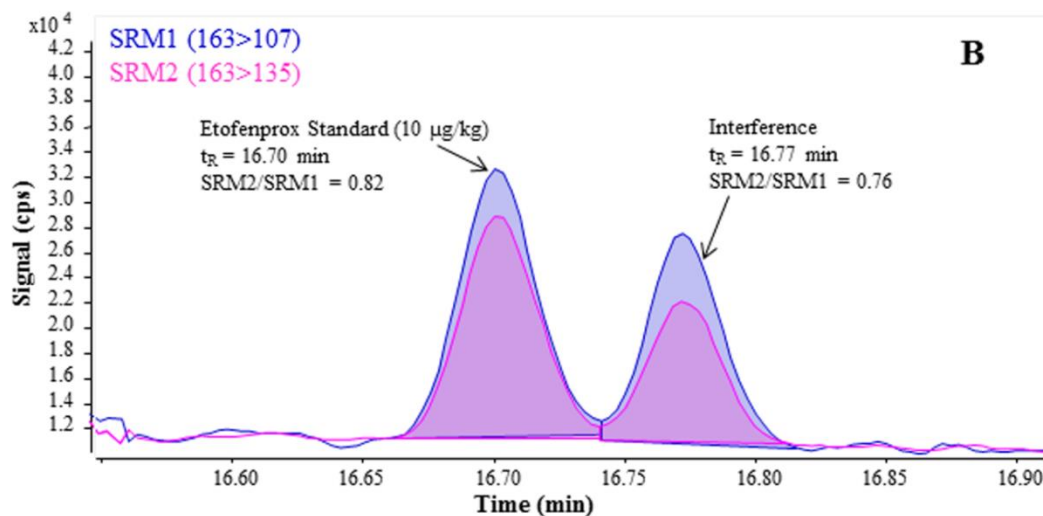


Honeybee wax comb real sample
Chlorfenvinphos





Extracted ion chromatogram of an unspiked honeybee wax comb sample (A)
 and the same sample spiked with 10 µgKg⁻¹ of Etofenprox (B)



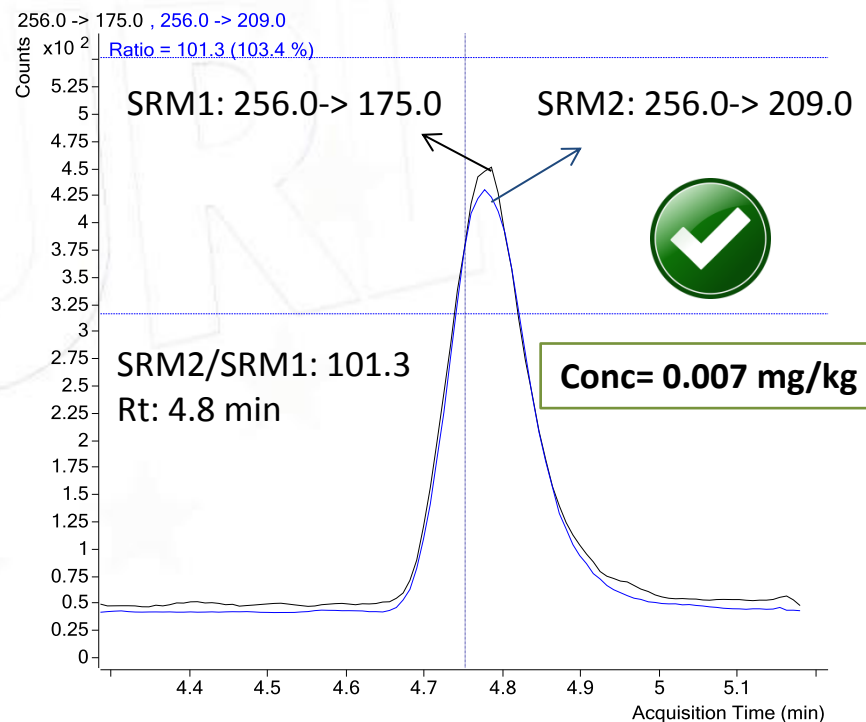
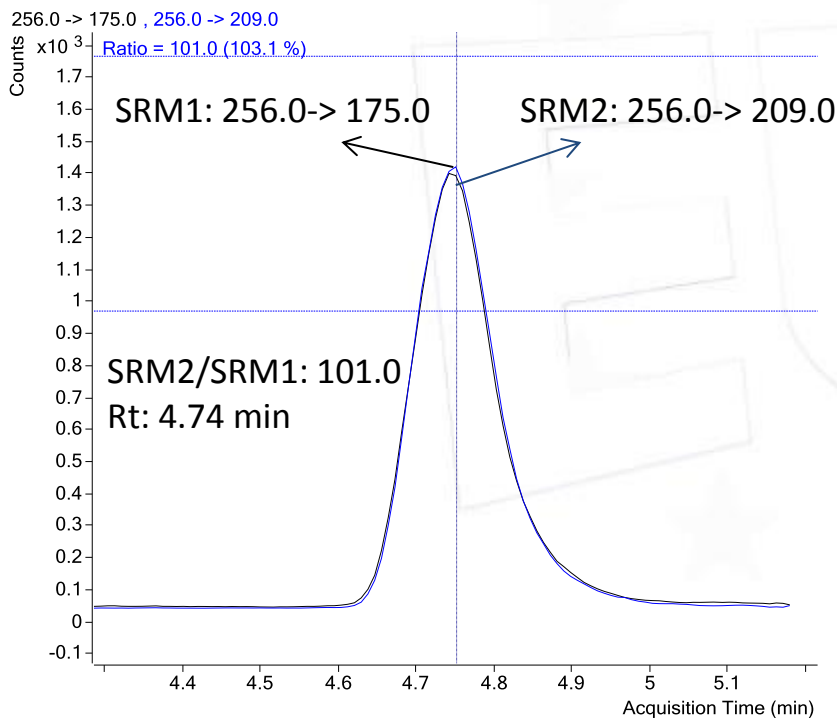


Bee Sample – Positive sample by LC-MS/MS

Imidacloprid

5ppb - Std addition

Bee - Real Sample



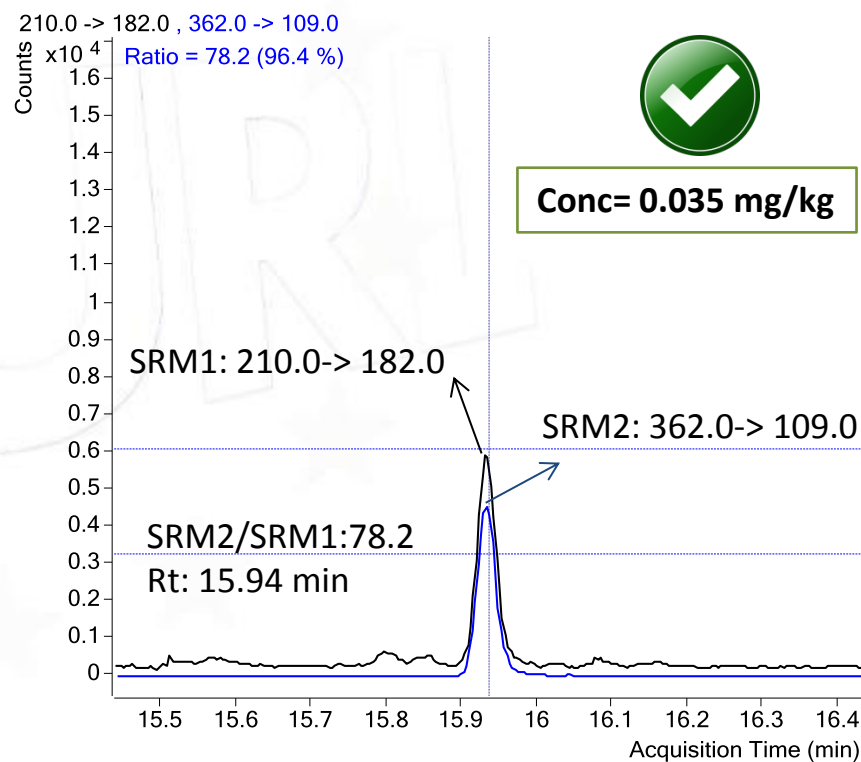
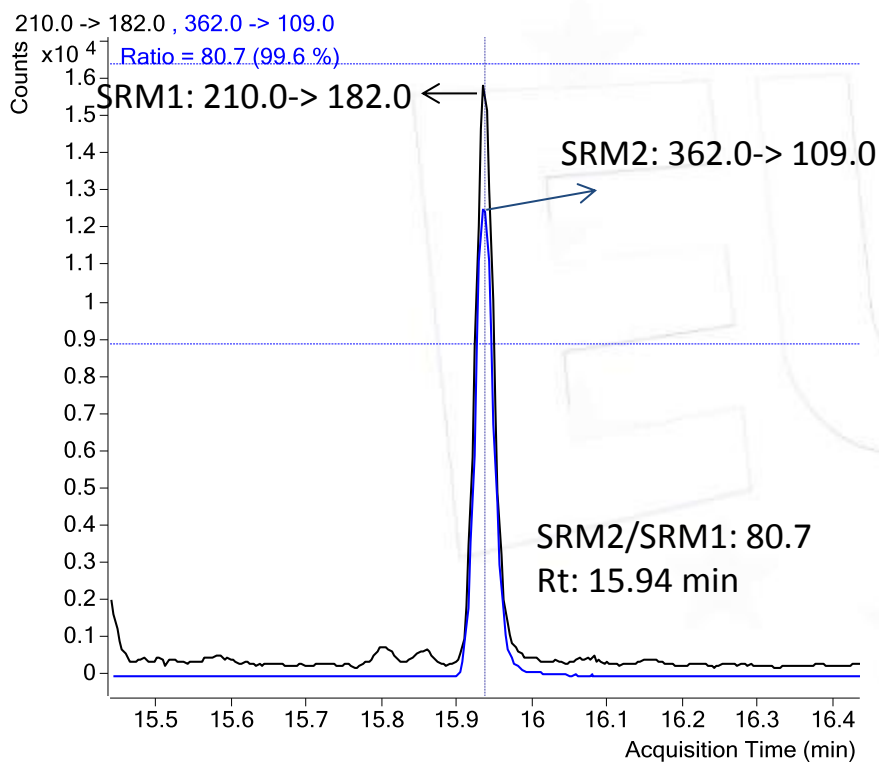


Bee Pollen Sample – Positive sample by GC-MS/MS

Coumaphos

50ppb - Std addition

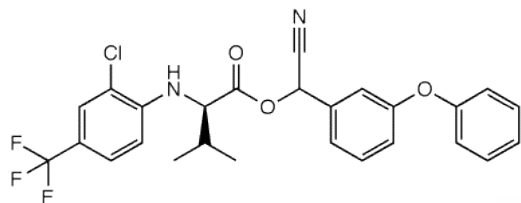
Bee Pollen - Real Sample





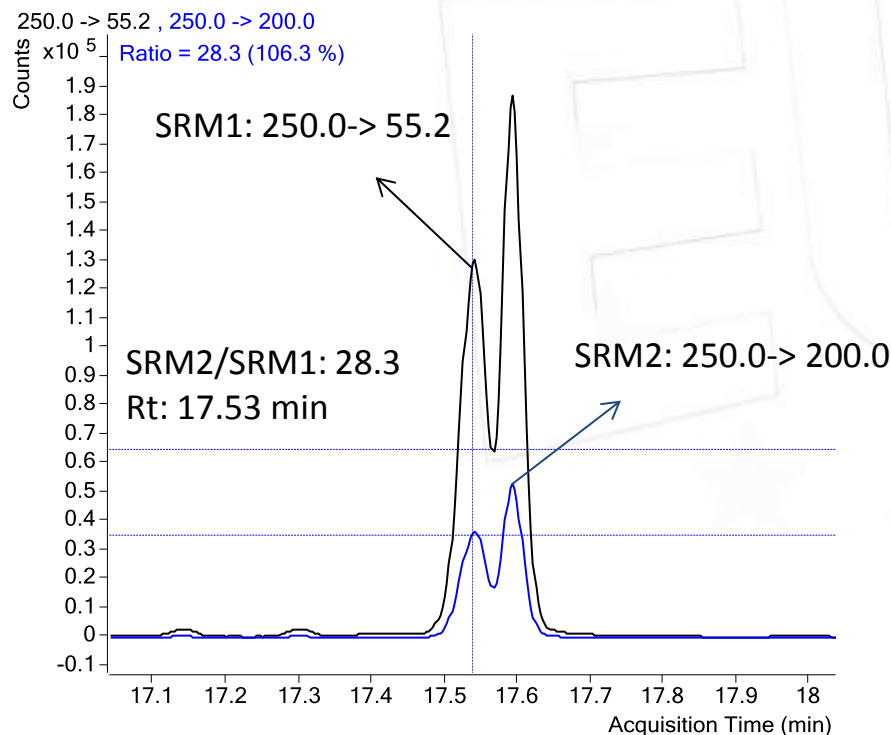
Honey bee wax comb Sample – Positive sample by GC-MS/MS

Fluvalinate-Tau

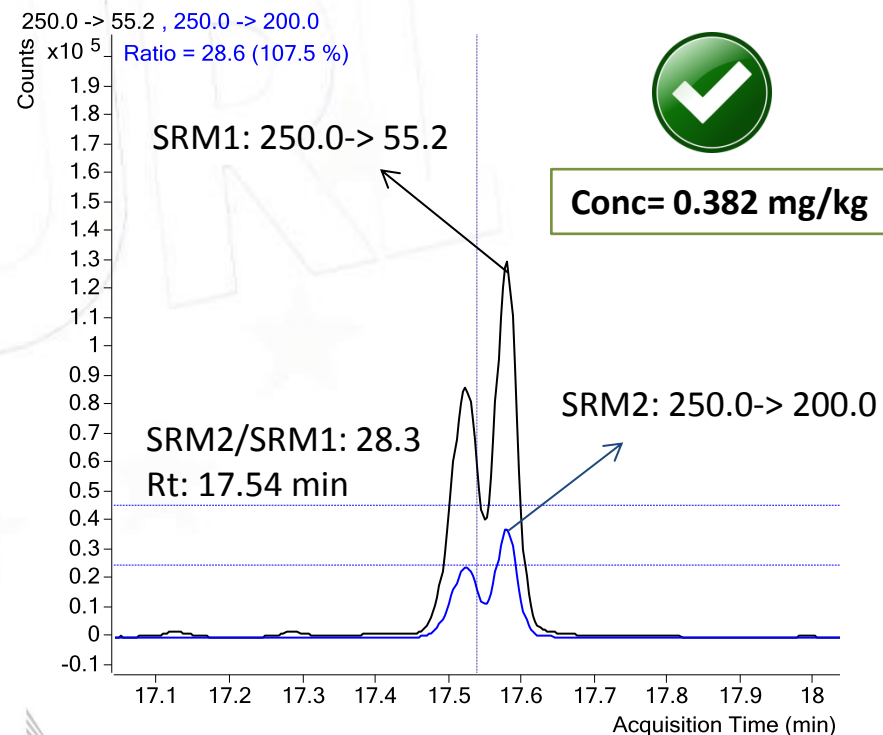


(RD) Fluvalinate-Tau & (SD) Fluvalinate-Tau

100ppb - Std addition



Honey bee wax comb - Real Sample



Nanoflow LC-HRMS applied to pesticide testing

Q-Exactive mass analyzer:
Experiments

Full Scan

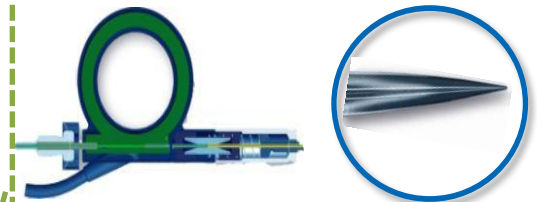
AGC Target: 1×10^6
Resolution: 70000
Maximum IT: 200 ms

AIF

AGC Target: 2×10^5
Resolution: 17500
Maximum IT: 50 ms

Analytical column:
EASY-Spray PepMap® C18
column
(75 μm x 150mm, 3 μm ,
100Å)

*Integrated nanoESI emitter
tip*



*Mobile
Phases*
A: H₂O
0,1% HCOOH
; B: ACN
0,1%
HCOOH



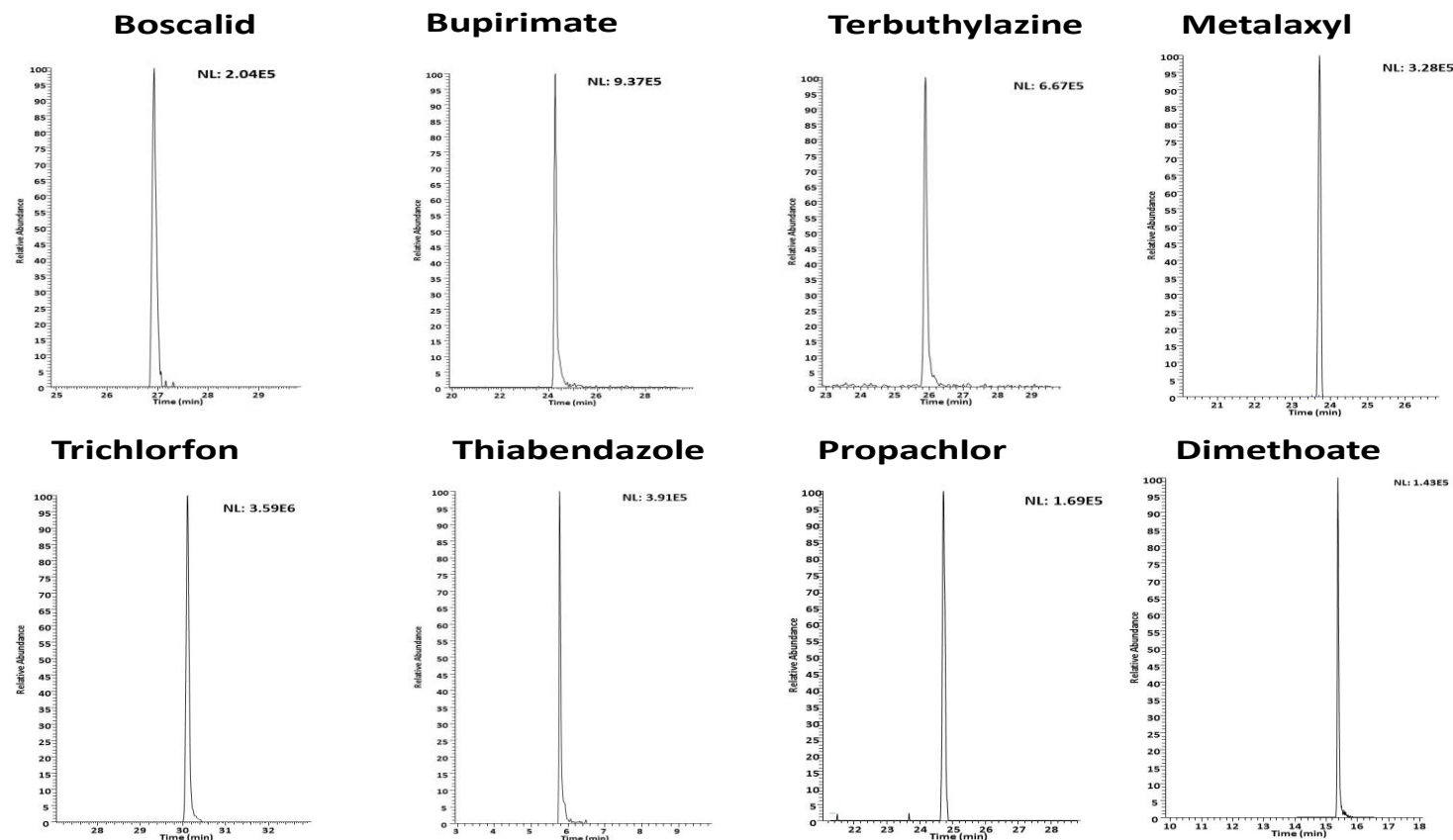
LC Conditions:
Flow rate: 300nL / min
Injection volume: 1 μL
*Elution gradient: 35
minutes*

Ion source parameters:
*Capillary temperature:
250°C*
S-Lens: 60
Spray Voltage: 2,20V
Ionization mode: positive



NANOFLOW LC-HRMS APPLIED TO PESTICIDE TESTING

Examples of identification (EICs) at low concentration levels



Extracted ion chromatograms of some pesticides at 0.1 $\mu\text{g kg}^{-1}$ in orange extracts (1:20 dilution)

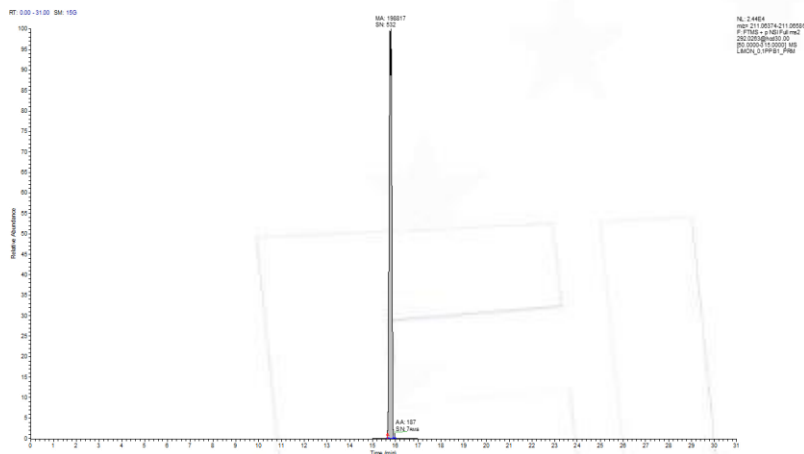


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 Amadeo R. Fernández-Alba EURL-FV

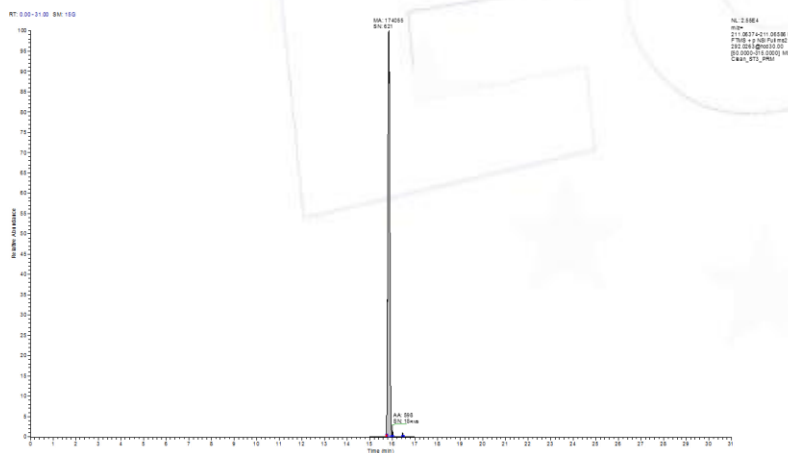
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NANOFLOW LC-HRMS APPLIED TO PESTICIDE TESTING



THIMETHOXAM	
Extracto limón	dilución 1:20
Concentración vial	10 ppt
s/n	532
LOQ	3.8 ppt
Ion cuantificación	211.0648
Ion confirmación	131.9669



THIMETHOXAM	
En disolvente	
Concentración vial	10 ppt
s/n	621
LOQ	0.2 ppt
Ion cuantificación	211.0648
Ion confirmación	131.9669

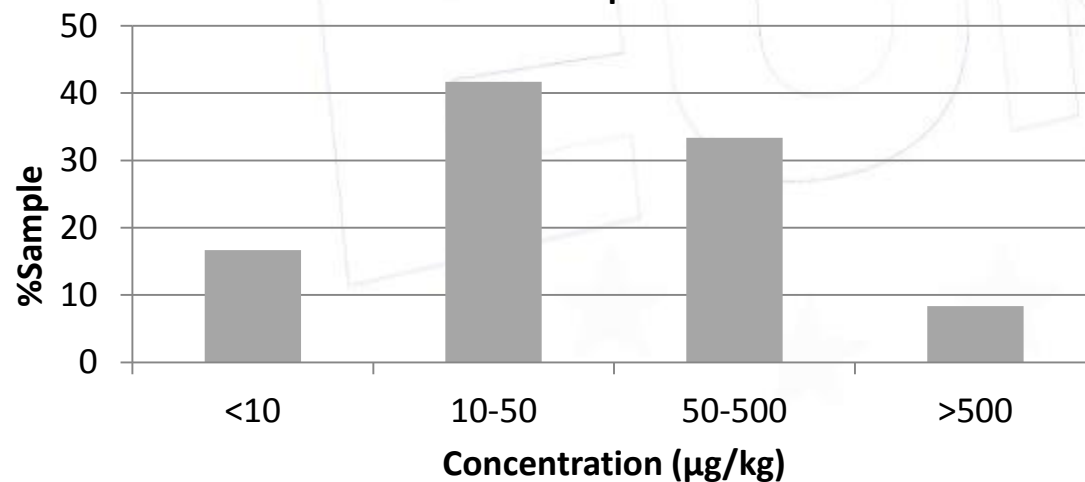
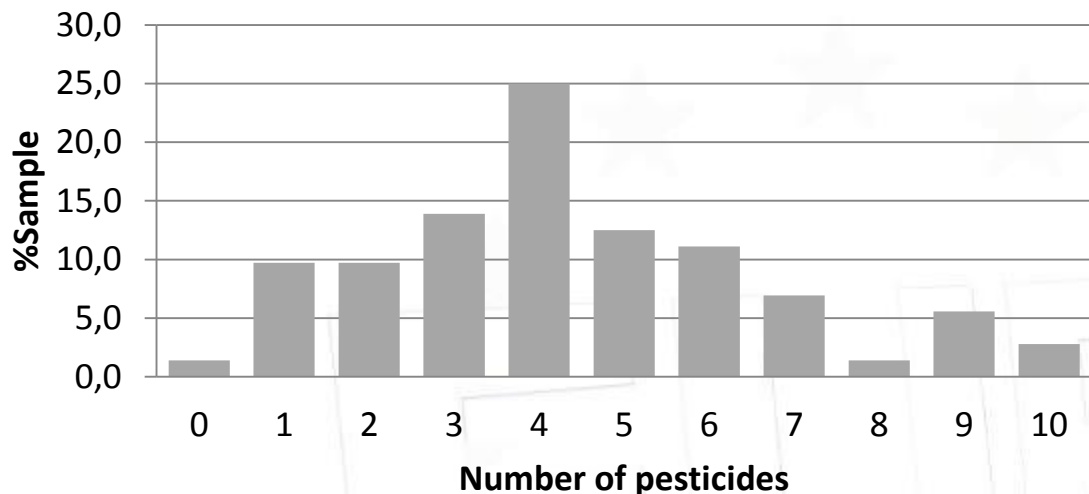




BEE SAMPLES

SCOPE: 251 pesticides
(LC-MS/MS and GC-MS/MS)

>50% of bee samples
contained 3-5 of pesticide
residues per sample



>40% of bee samples
contained a total load
between 10-50 ppb

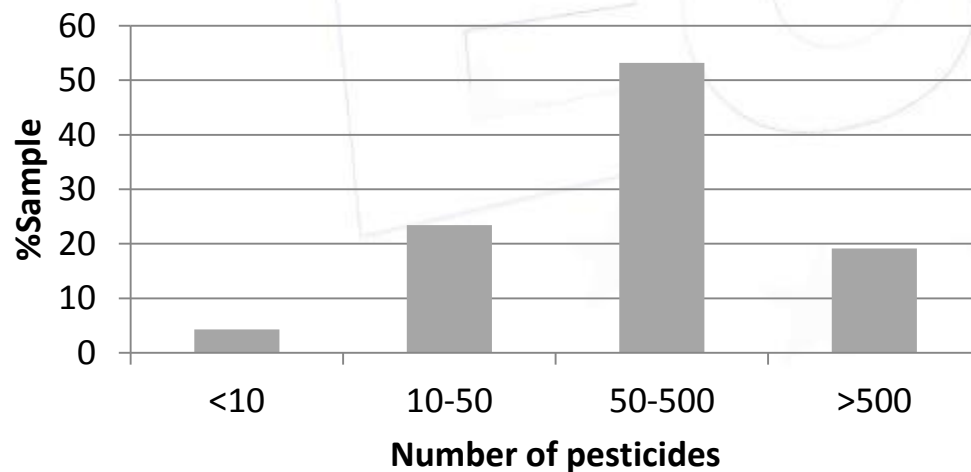
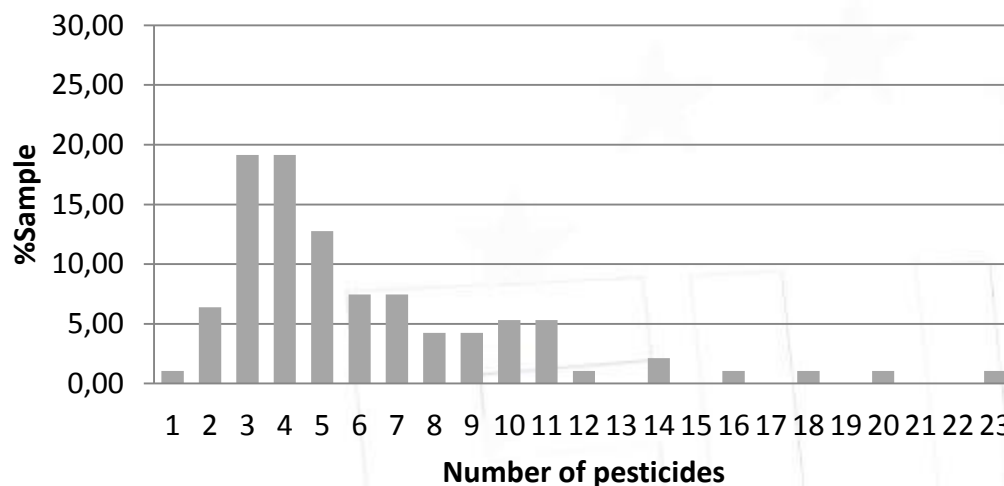


BEE POLLEN SAMPLES

**SCOPE: 251 pesticides
(LC-MS/MS and GC-MS/MS)**

>50% of pollen samples contained 3-5 of pesticide residues per sample

>50% of pollen samples contained a total load between 50-500 ppb



2006-2016



Ten Years of
Collaboration,
Assistance and
Research

Thank You
for Your Attention



EURL EUROPEAN
UNION
REFERENCE
LABORATORY

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