# Determination of neonicotinoid insecticide residues in bees, pollen and nectar with LC-MS/MS

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# Seed coating with a neonicotinoid insecticide negatively affects wild bees

Nature (2015) 521: 77-80

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*[1]Lund University, [2]Swedish University of Agricultural Sciences, [3]Swedish Board of Agriculture*

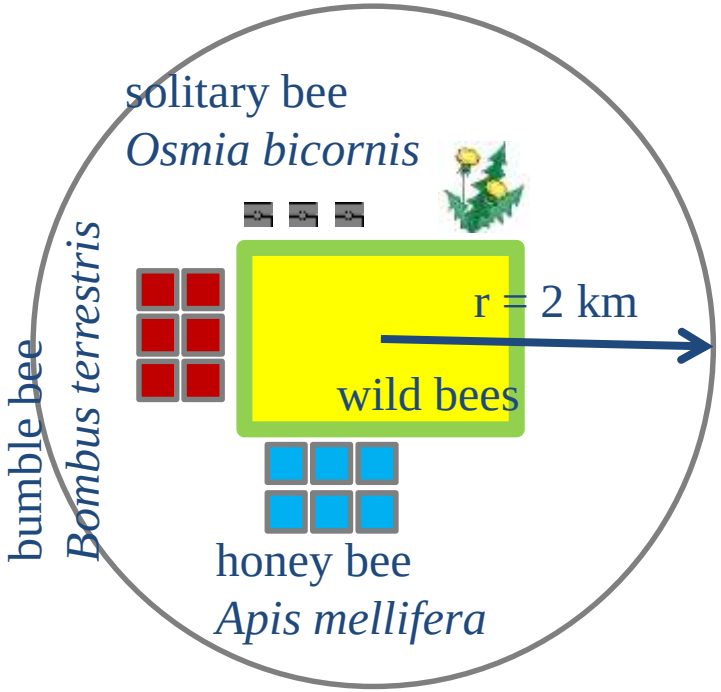
Photo: Maj Rundlöf

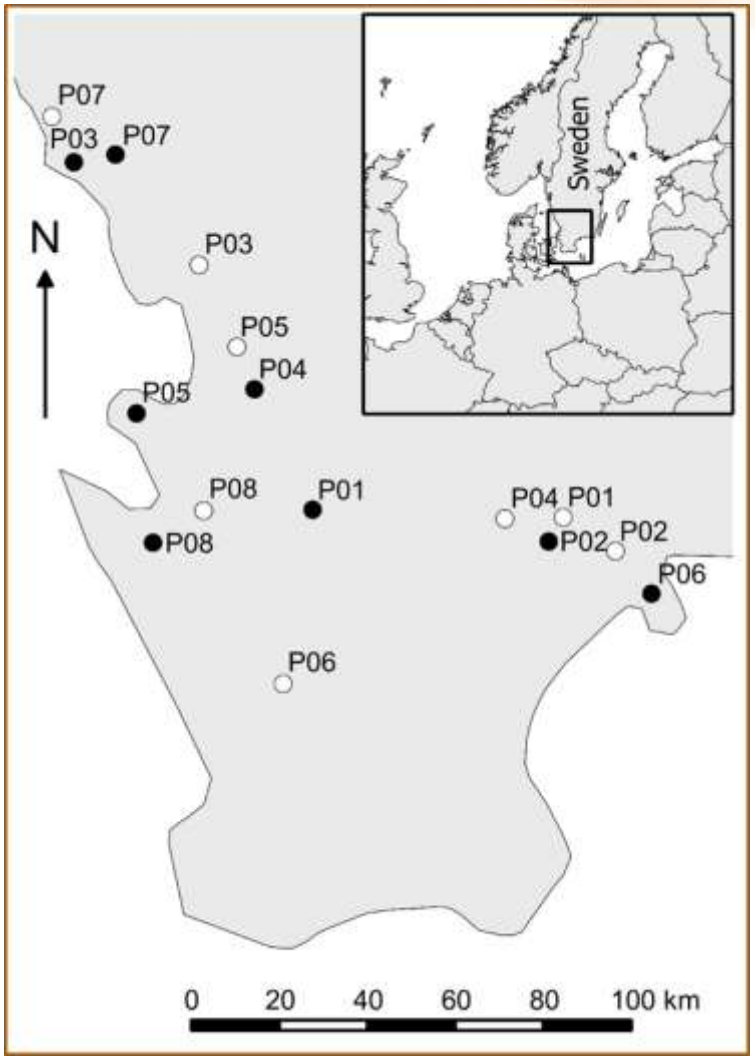
# Background

- Spring sown oilseed rape (just over 40 % in Sweden)
- Neonicotinoid (mostly clothianidin) seed dressing to protect seedlings
- Lack of well designed replicated field studies, particularly for non-honeybees

Flea beetle (*Phyllotreta* sp.)
Photo: Maj Rundlöf

# Landscape ecotoxicology experiment



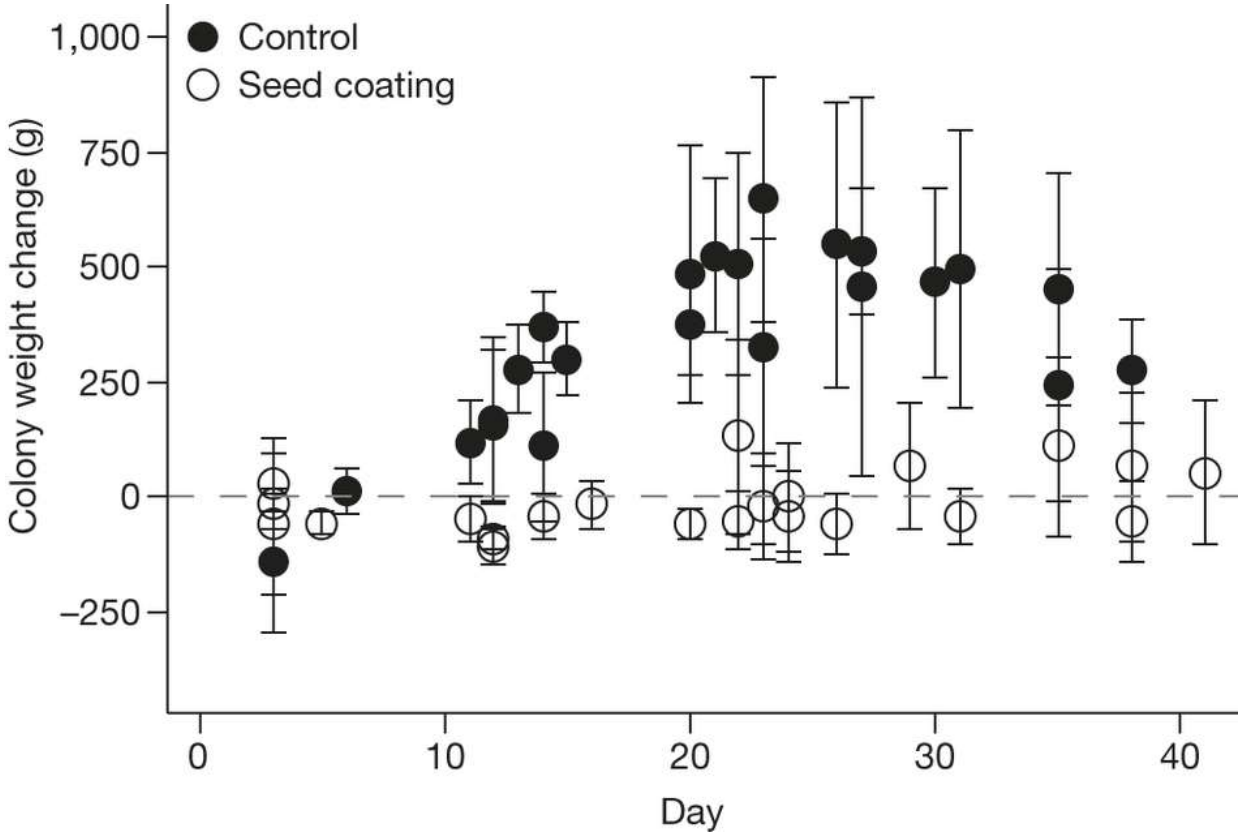




- 8 replications (field pairs based on land-use)
- random assignment to treatment/control
- treatment blinded during field work

Rundlöf et al. (2015) Nature 521: 77-80.

# Results

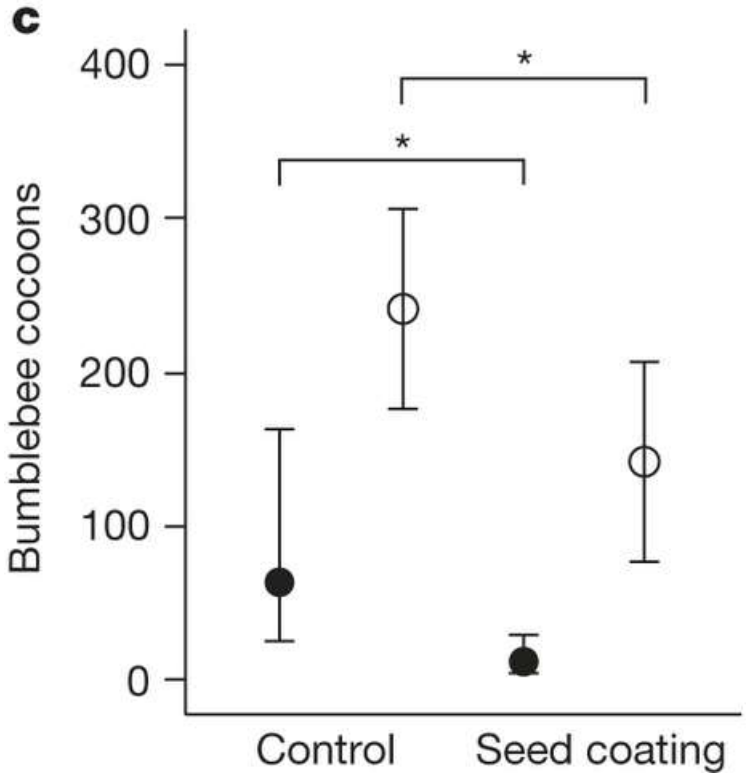
The clothianidin treatment was negatively related to *Bombus terrestris* colony growth




Photos: Maj Rundlöf

Rundlöf et al. (2015) Nature 521: 77-80.

# …and *Bombus terrestris* reproduction




Queens

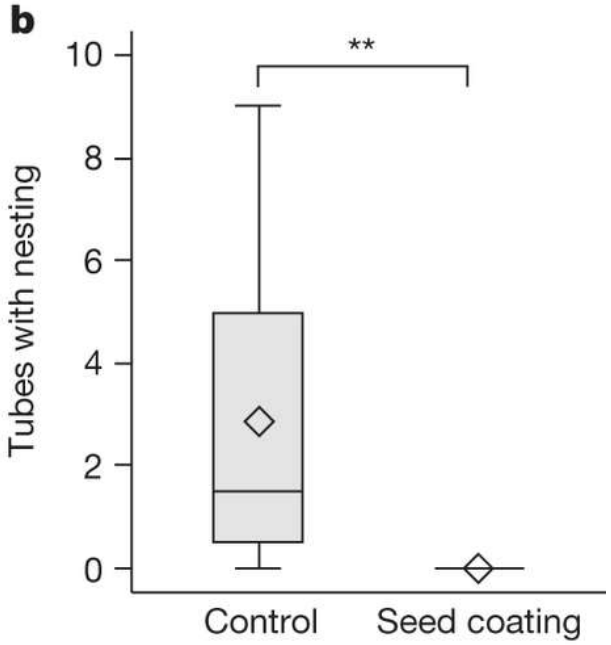
Workers/males

**Queens: -85%**

Photos: Maj Rundlöf

Rundlöf et al. (2015) Nature 521: 77-80.

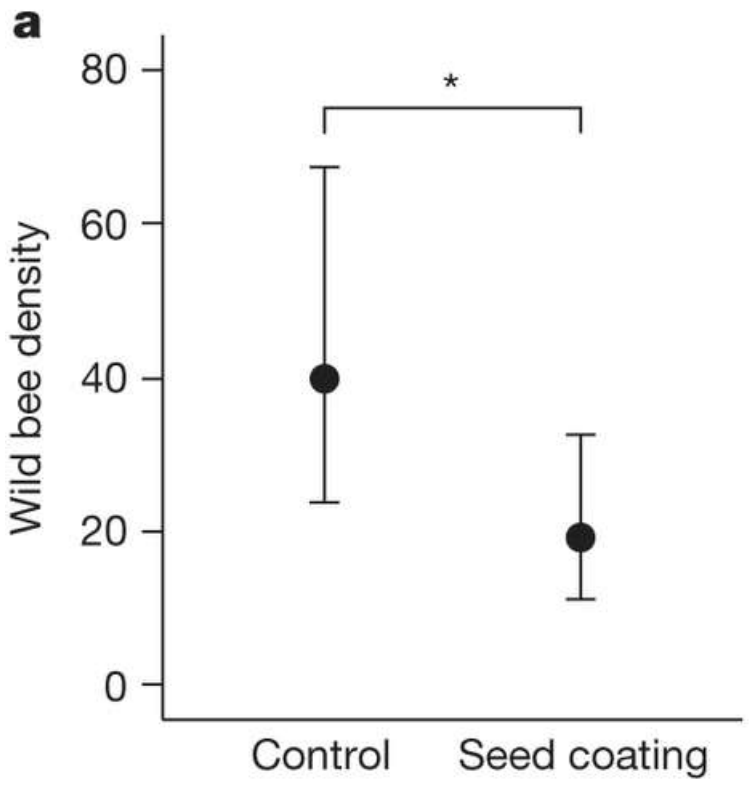
# Reduced nesting of solitary bee *Osmia bicornis* (red mason bee) in tubes placed close to the fields




Photos: Maj Rundlöf

Rundlöf et al. (2015) Nature 521: 77-80.

# Reduced wild bee density in treated fields (transects)

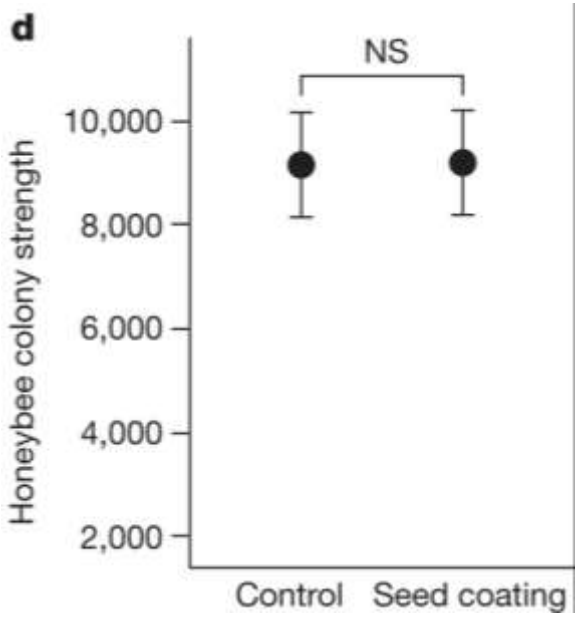



Wild bees are in Sweden bumble bees and solitary bees.




Rundlöf et al. (2015) Nature 521: 77-80.

# The clothianidin seed treatment had no significant influence on *Apis mellifera* colony strength



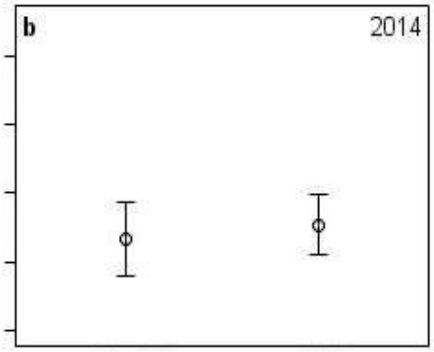




Photo: Maj Rundlöf

Not always possible to extrapolate ecotoxicology results for honey bees to wild bees or other insect species.

Rundlöf et al. (2015) Nature 521: 77-80; Rundlöf et al. (2015) Project report, Lund university

# Chemical analysis


5 neonicotinoid insecticides studied:

Acetamiprid
Imidacloprid
Clothianidin
Thiacloprid
Thiamethoxam

Internal standards:
Imidacloprid D4
Clothianidin D3
Isoproturone D6 (used for acetamiprid, thiacloprid and thiamethoxam)

Agilent 6460 LC-MS/MS, on-line SPE (C18 and polymer)
C18 analytical column, methanol gradient in ammonium formate [1]

[1] C. Jansson and J. Kreuger 2010 J. AOAC Int., Volume 93, Number 6, 1732-1747

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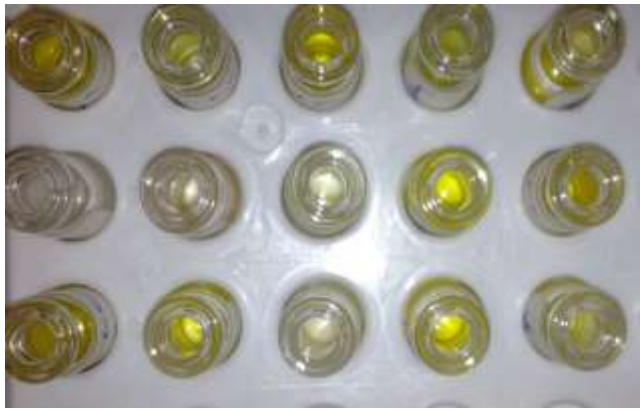
# Sample preparation bees (and pollen)

- 24 bees weighed and homogenized with drying agent, subsample corresponding to four bees
- internal standard to subsample homogenate
- extract twice under strong sonication with 7:3 mixture of acetone and ethyl acetate
- dispersive solid phase extraction, C18 and PSA
- evaporate to dryness at 40°C under nitrogen flow
- residue dissolved in 150 µl acetonitrile
- 10 µl injected on LC-MS/MS


Photo: Ove Jonsson

Vibra-Cell VCX 130 (Sonics)

# Bees and pollen are varying matrices

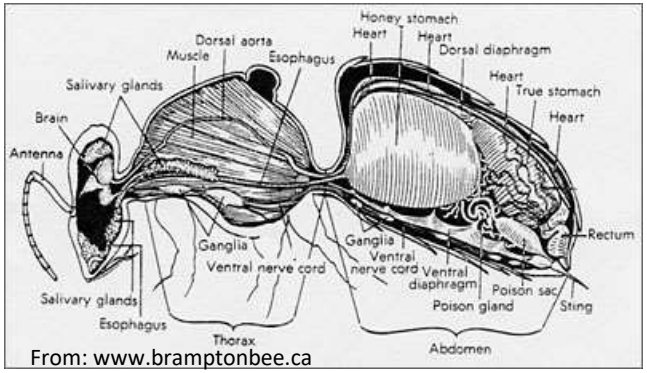



Extracts from different bee samples.

# Collection and handling of nectar samples

Capillary with exact 8 µl nectar (deviation and %RSD <1%)

8 µl capillary in 1 ml tube
Capillary Microsampling (CMS) [1,2]

From: www.bramptonbee.ca


- IS washout solution
- acetonitrile protein crash
- LC-MS/MS


Intestine content

Nectar

Analysis of bee tissue and nectar from one single bee

1. Capillary microsampling of 25 µl blood for the determination of toxicokinetic parameters in regulatory studies in animals. Jonsson O., Palma Villar R., Nilsson L.B., et al. Bioanalysis 2012, 4(6), page 661–674
2. Capillary microsampling. Jonsson O., in eBook: Microsampling in Pharmaceutical Bioanalysis, edited by Zane P. and Emmons G.T. Future medicine 2013

# Clothianidin field exposure

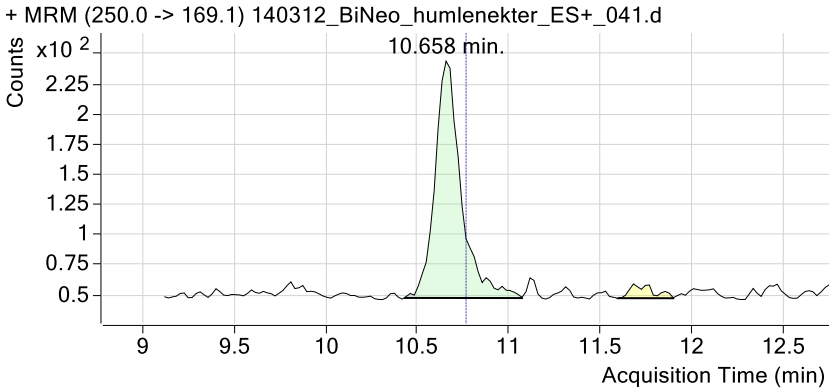
| (ng/g or ml) | Control (C) | | Seed coating (T) | | N (C/T) |
|---|---|---|---|---|---|
| **2013** | range | mean ± se | range | mean ± se | |
| **Honey bees** | <LOD-0,89 | 0,13 ± 0,11 | 0,35-4,9 | 2,4 ± 0,50 | 8/8 |
| **Pollen from honey bees** | <LOD | <LOD | 6,6-23 | 14 ± 1,8 | 6/8 |
| **Nectar from honey bees** | <LOD-0,61 | 0,11 ± 0,080 | 6,7-16 | 10 ± 1,3 | 8/8 |
| **2014** | | | | | |
| **Honey bees** | <LOD | <LOD | 0,15-1,5 | 1,1 ± 0,20 | 4/6 |
| **Pollen from honey bees** | <LOD | <LOD | 2,4-16 | 6,1 ± 2,0 | 4/6 |
| **Nectar from honey bees** | <LOD | <LOD | 2,6-9,8 | 4,9 ± 1,1 | 4/6 |

LOD: 0.080-0.50, LOQ: 0.25-1.5 ng/g or ng/ml

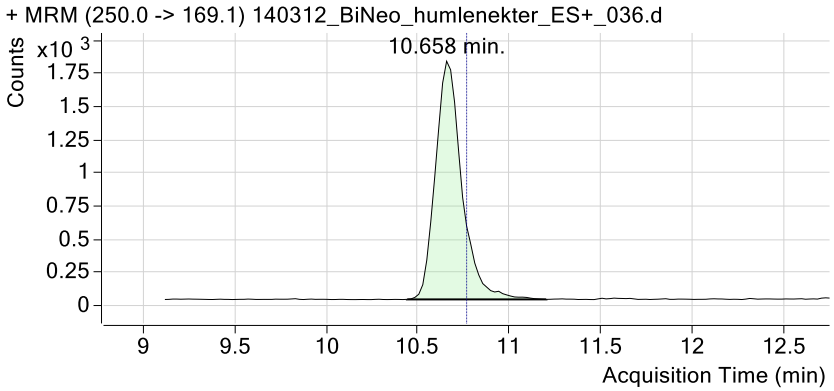
Rundlöf et al. (2015) Nature 521: 77-80;
Rundlöf et al. (2015) Project report, Lund university

# Clothianidin chromatogram from nectar samples

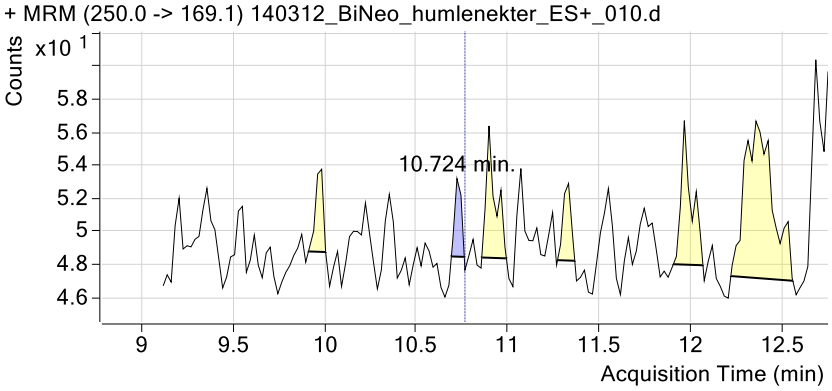
## Concentration 0.84 ng/ml



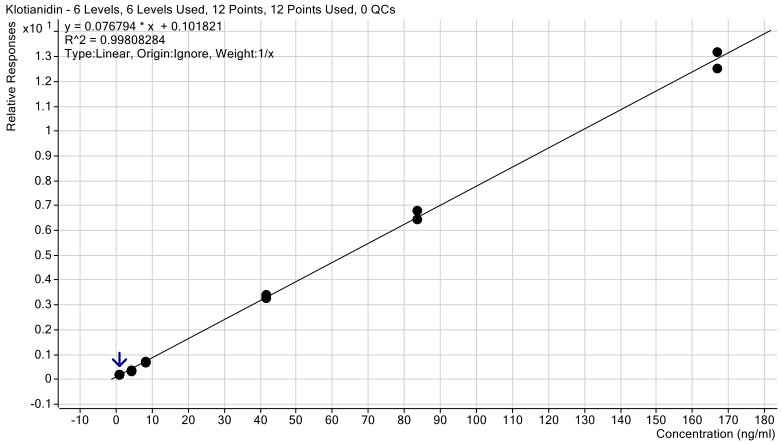

## Highest concentration in nectar sample 20 ng/ml




## Nectar sample from control field




## Calibration curve clothianidin in nectar

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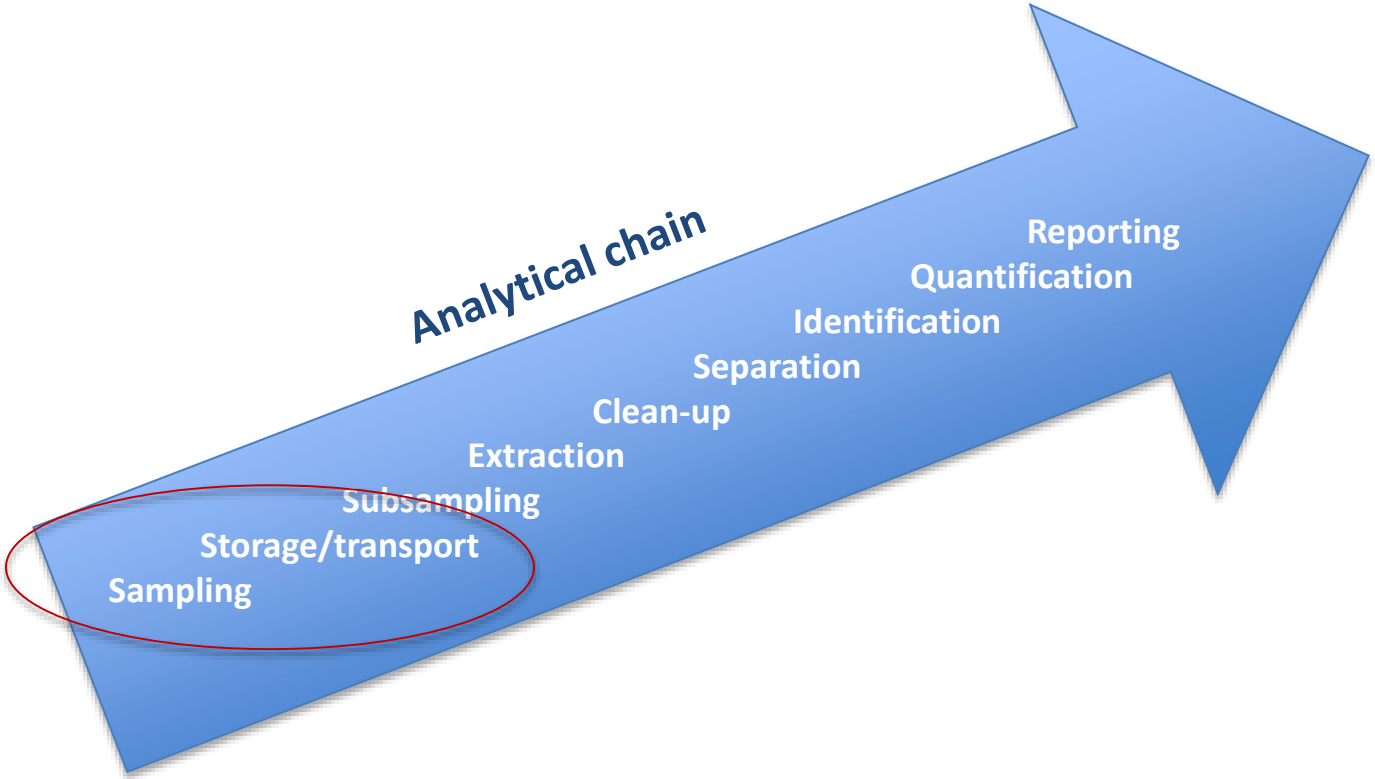
# 1. Individual exposure variation within a bee society

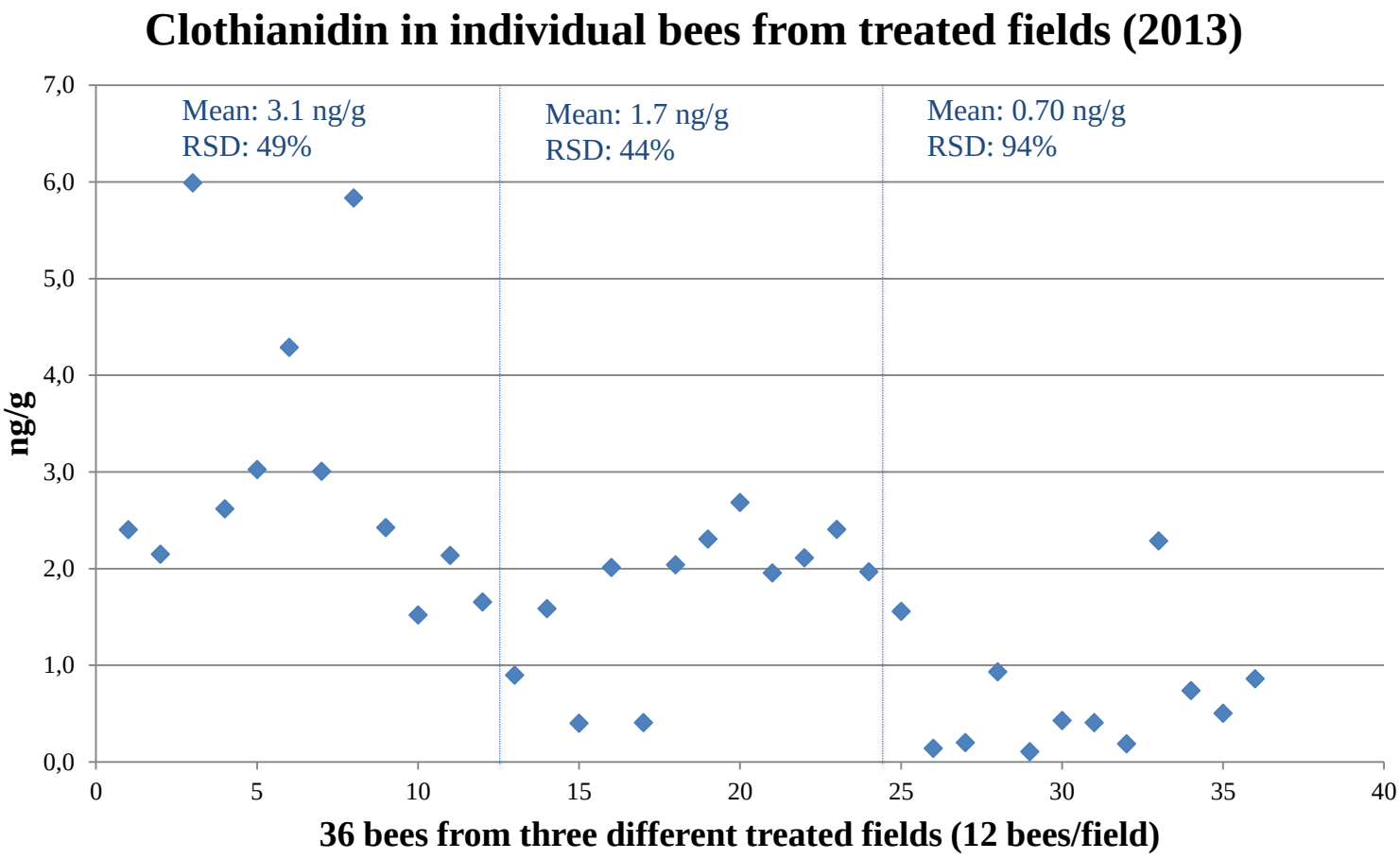
**Representative sampling**

# 2. Stability of pesticides in stored bees

**Important step in quality assurance**

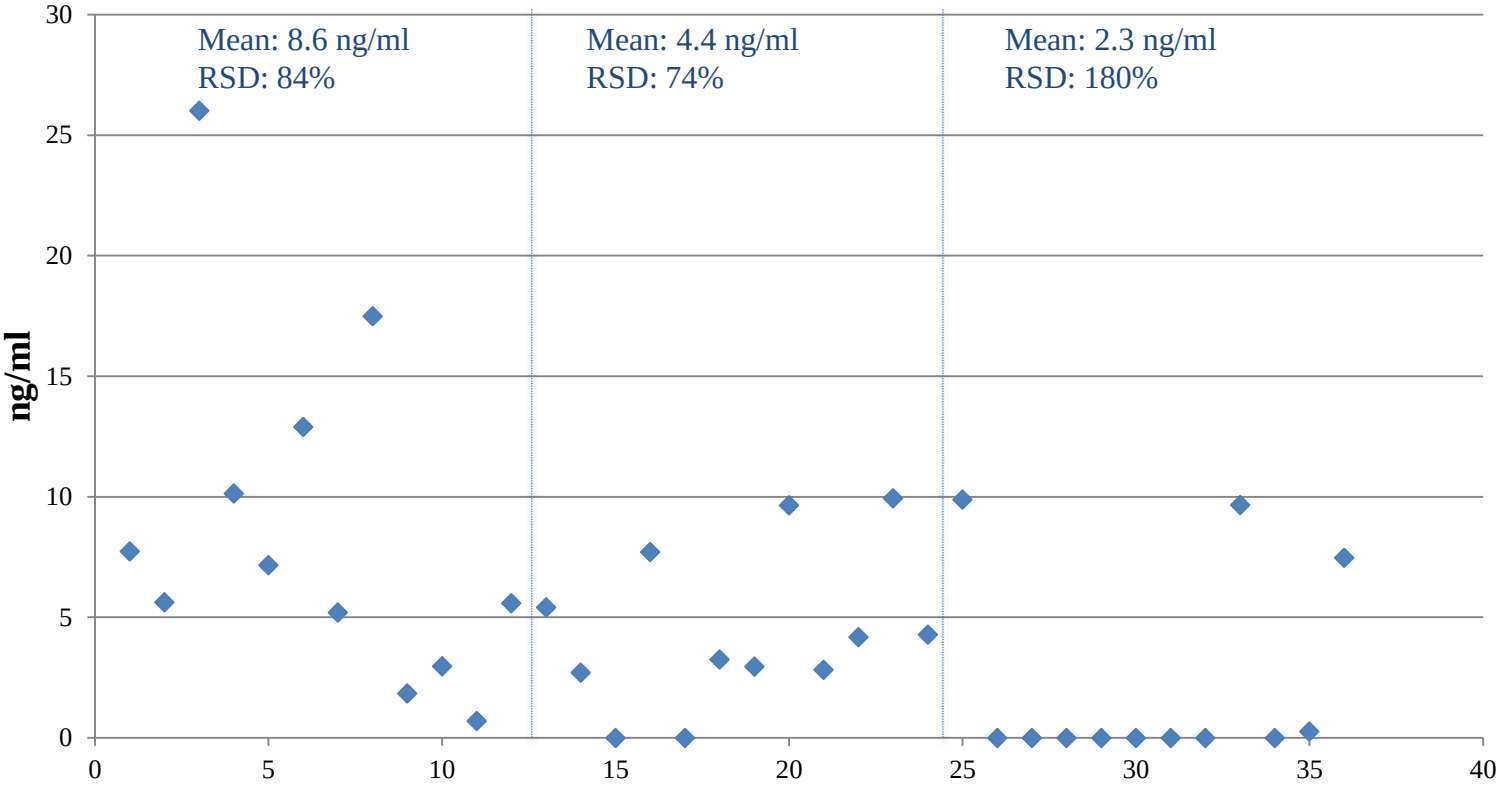


# Individual exposure




Overall mean: 1.8 ng/g
RSD: 77%

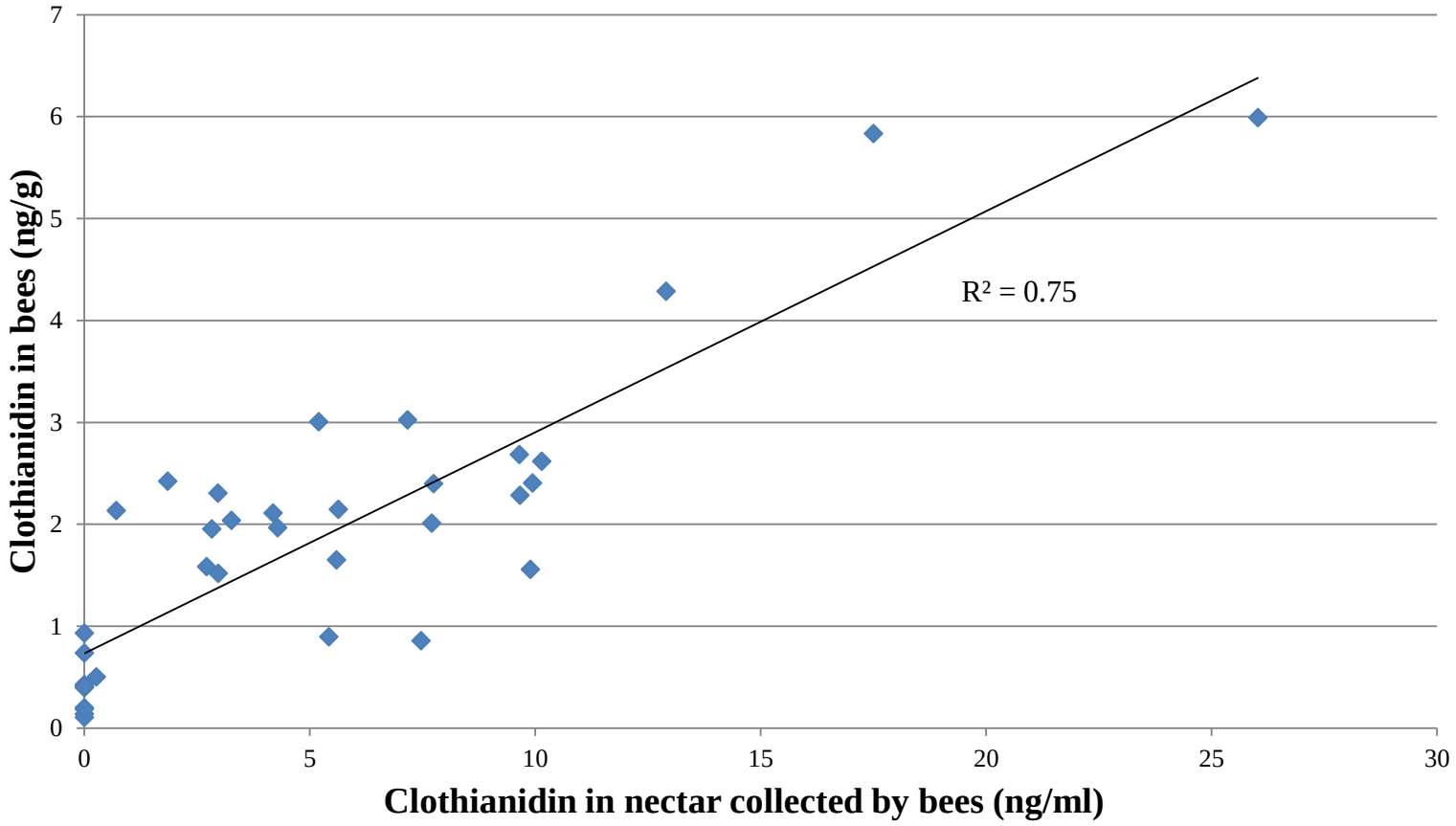
# Clothianidin in nectar from individual bees, collected in three treated fields (2013)

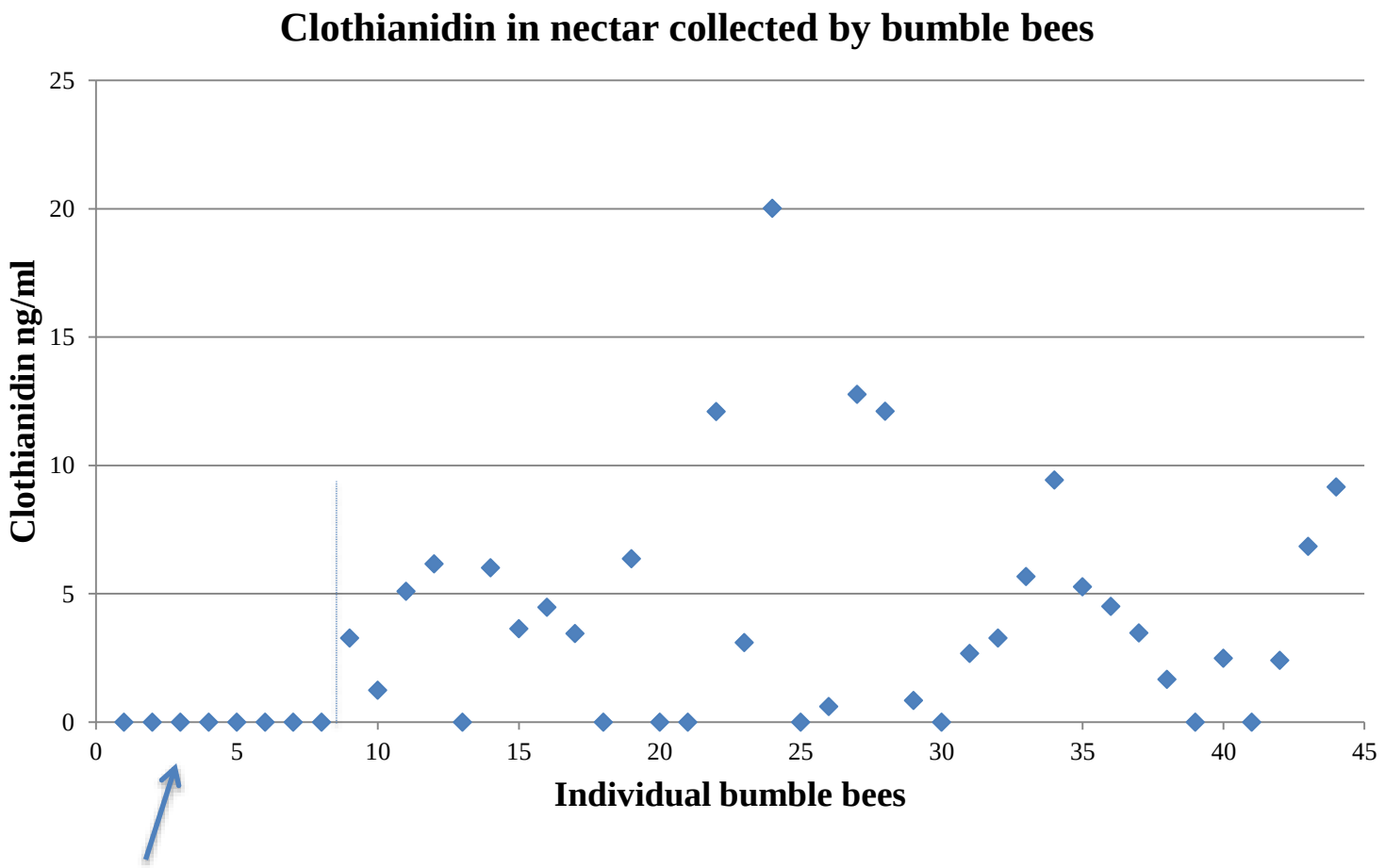



**Nectar from 36 bees from three treated fields, (12 from each field)**

Overall mean: 5.1 ng/ml
RSD: 111%

# Clothianidin concentrations in 36 bees and the nectar they collected from treated fields






Pooled samples from
8 control fields

Overall mean treated fields: 4.4 ng/ml
RSD: 103%

# Validation data

## Field study

**Bee and pollen samples:**
Calibration curve in solvent and matrix spiking experiments at two concentrations.
**RSD bees 0.9-18%** and **pollen 2.4-19%**,
for both matrices, concentration levels and all five neonicotinoids.

(clothianidin 4.7% and 1.0% for low and high spiking level in bees and 7.3% and 12% in pollen).

**Nectar samples:**
Calibration curve and Quality Controls (QC) at two levels in nectar collected by bees (matrix match)
**QC Accuracies of 89-101%** and
**QC RSD of 1.1-8.2%**
for both concentration levels and all five neonicotinoids.

## Storage stability experiment

**Bees:**
**Mean RSD for spiked homogenate 3-8%**
For all five Neonicotinoids in five batches over 21 months. (n=3 per batch, one concentration level)

# Pesticide stability in bees after individual feeding

Individual feeding with 10 µl sugar solution containing pesticide mix – Relevant spiking!

Evaluate stability *in vivo*, in room temperature (dead bees) and after long time freeze storage at -20°C


Professor Ingemar Fries, SLU, feeds a bee.

Freezer and room temp:
All five neonicotinoids were stable in freezer for 21 months and at room temperature for 22 hours.

*In vivo:*
Clothianidin was stable for 1 h while acetamiprid, imidacloprid, thiacloprid and thiamethoxam showed degradation with 44, 44, 43 and 22%, respectively.

Each estimate was based on triplicate samples and four bees per sample (in total 12 bees)

# Acknowledgement pesticides in bee matrices

**Swedish EPA and the Centre for Chemical Pesticides for financial support to develop analytical methods**

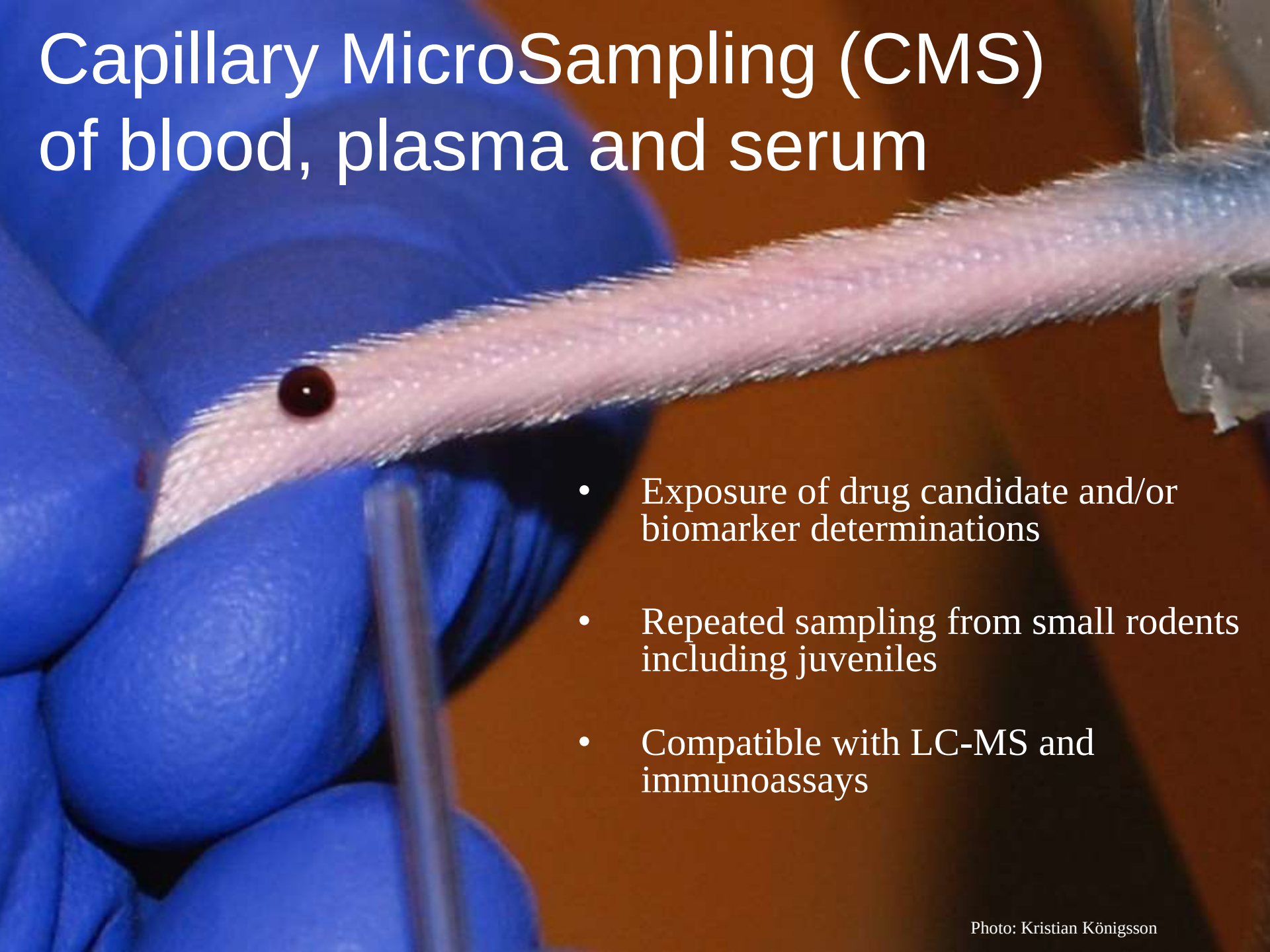
**Maj Rundlöf for support with field study slides**

**Prof. Ingemar Fries, SLU, for feeding bees**

**Photo: Maj Rundlöf**

# Micro sampling side track

# Capillary MicroSampling (CMS) of blood, plasma and serum



- Exposure of drug candidate and/or biomarker determinations
- Repeated sampling from small rodents including juveniles
- Compatible with LC-MS and immunoassays

Photo: Kristian Königsson

# Basic CMS principle

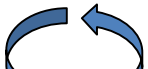
Animal lab/Clinic/Field

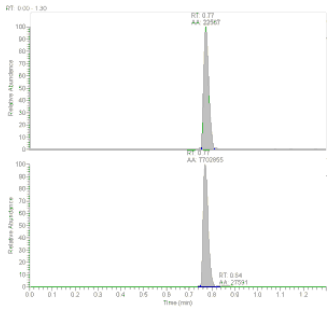
Bioanalytical lab

## Sampling

## Dilution

## Clean-up and analysis



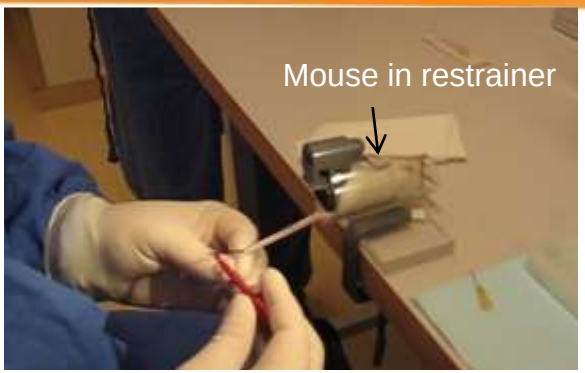


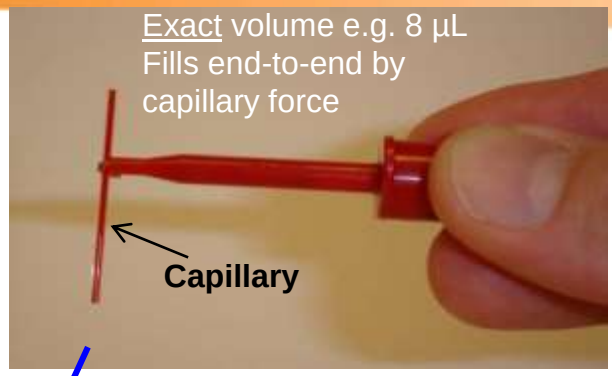
**Exact volume in capillary** (plasma, blood, serum or other biofluid)

**Dilution with washout liquid** (Samples and bioanalytical quality control samples at the same time)

- **Liquid samples suitable for LC-MS/MS and immunoassays**
- **A fraction of the diluted sample is used for each analysis**
- **Reanalysis always possible**
- **Standard lab equipment for liquid samples**

# CMS of blood




**$K_2$EDTA treated capillaries**
**Typical volumes 4-25 µL**

**Relative error < 1%**
**RSD < 1%**

**A tail vein is penetrated with a cannula, collect e.g. 8 µL blood**

**Animal facility or clinic**

**In dry vial**

**…or mix with stabilizing solution**

From "tail to ice" in 10 seconds Including stabilization

**Freeze**

**Freeze**

**Bioanalysis lab**

**Add washout liquid. Mix!**

**Analysis according to method**

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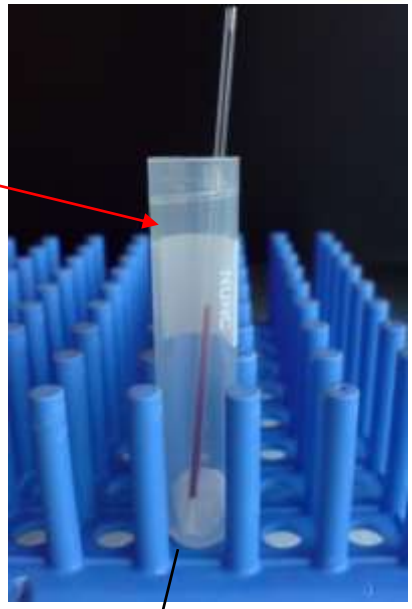
# CMS of plasma (serum)

**~32 µL blood** in $K_2$EDTA haematocrit tube (plain glass for serum sampling)

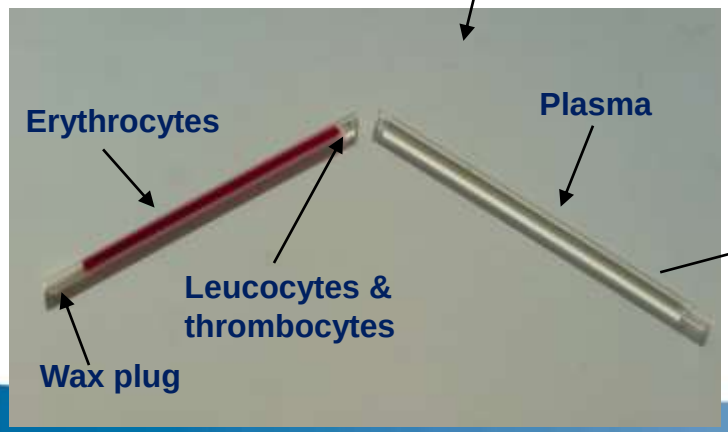
**Plug with wax**

**Centrifugation 1500 g for 10 min**

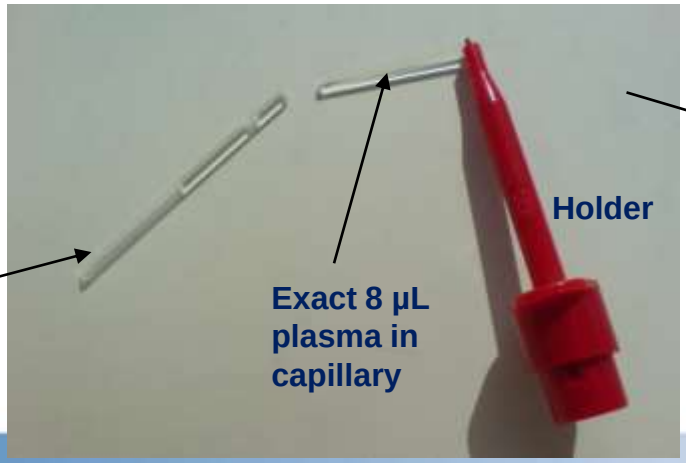


**Typical volumes**
**8 µL (4 µL backup) plasma from ~32 µL blood**

**Cut above the blood cell phase using a capillary cutter.**




**An exact volume of plasma is collected with a capillary from the end of the haematocrit tube.**




**Put capillary in tube or plate**

**Bioanalysis**

# Can pesticides affect breeding success in agricultural bird species?
# Collaboration: SLU (OMK and Ecology dep.) + Kvismare bird observatory


Pesticides
in blood?

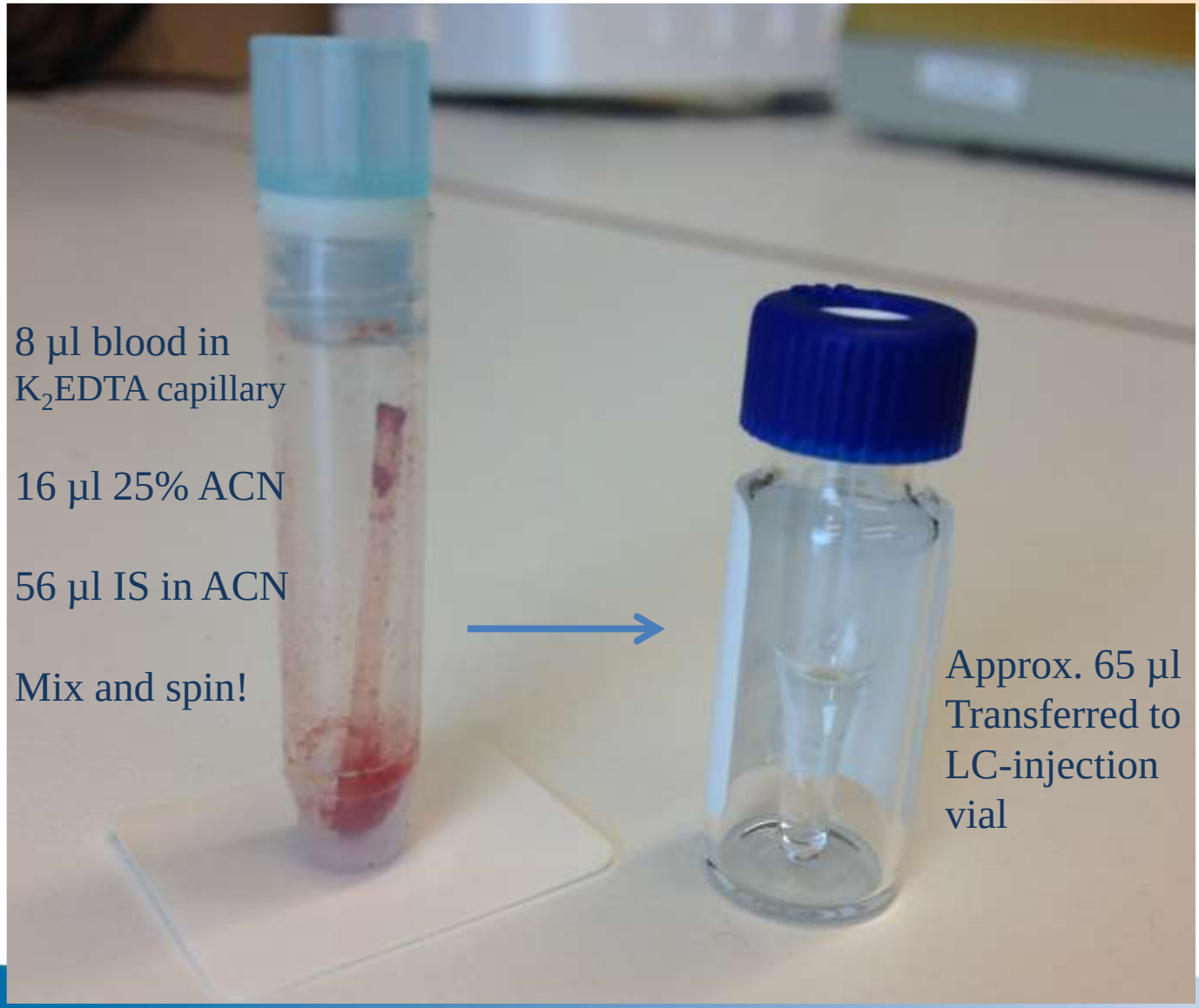
# Sampling site on birds

## Here male redstart (rödstjärt)


Approximately
100 micro samples
successfully collected
in the field

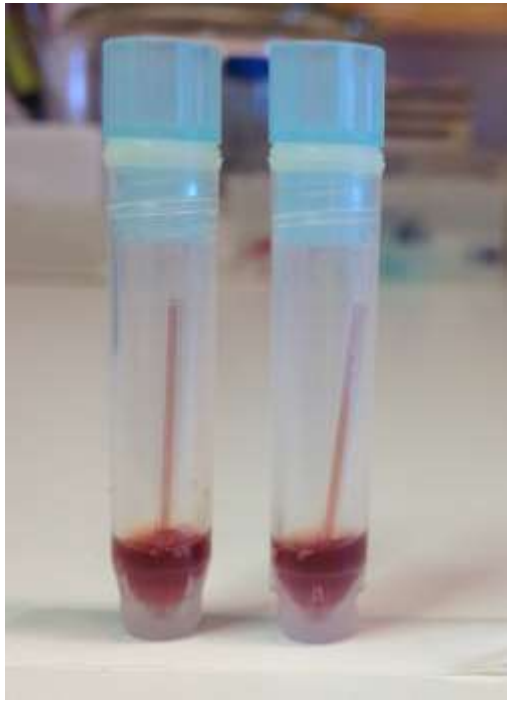
# Determination of pesticides in blood:

## Capillary microsampling, protein precipitation and LC-MS/MS

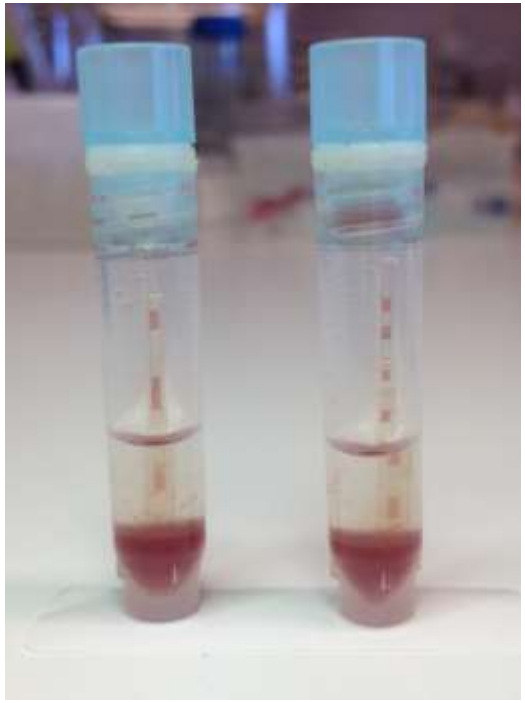


# Liquid-liquid extraction of pesticides in blood

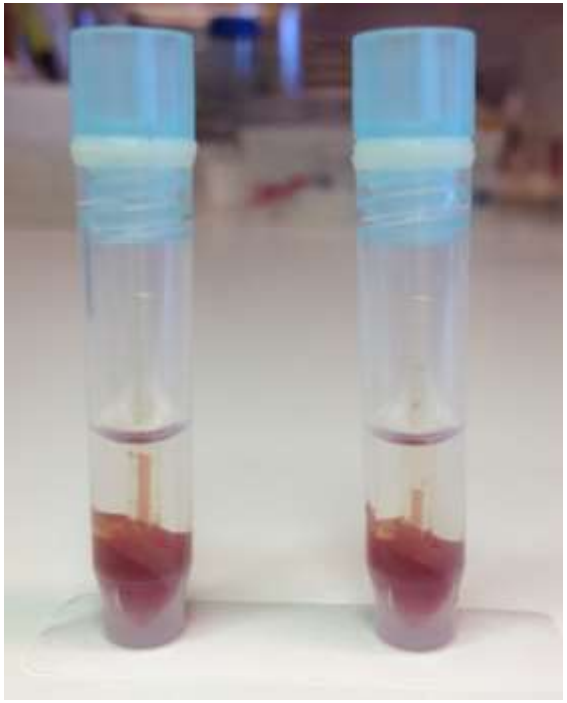
Blood (8 µl) mixed with internal standard (IS) in water (80 µl)



Organic solvent* (250 µl) added and mixed for liquid/liquid extraction



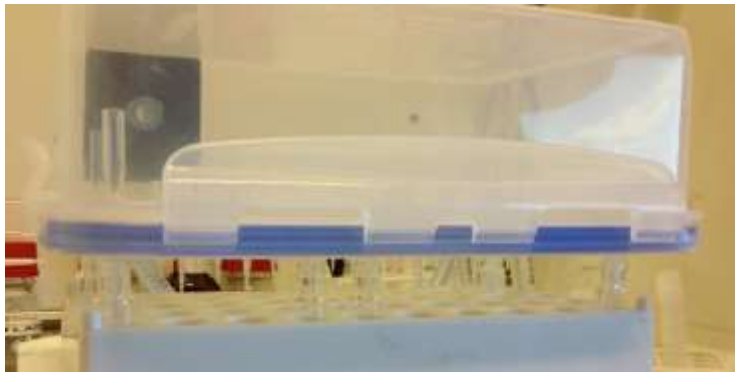
After centrifugation 2000 x g for 2 minutes



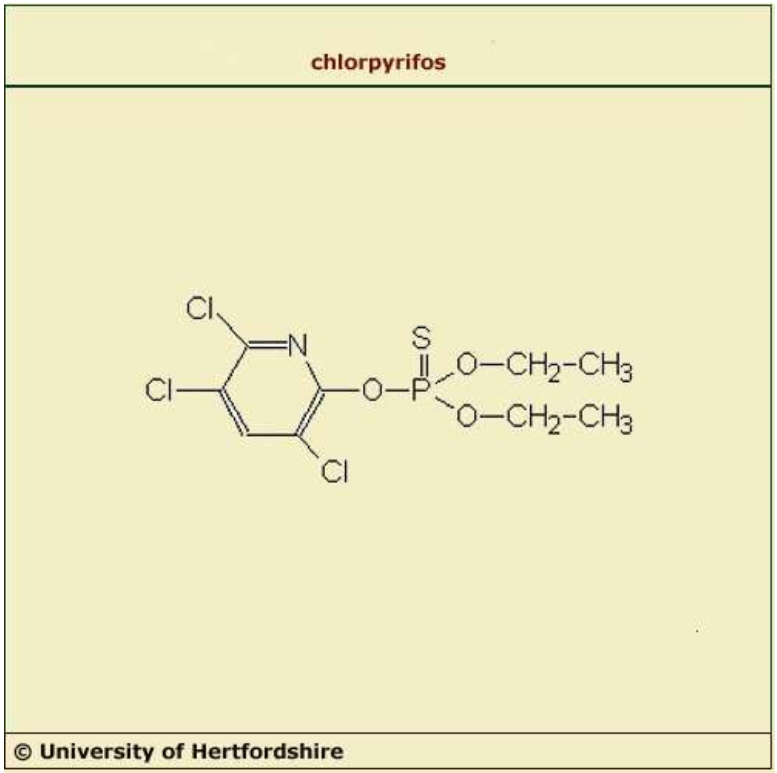
*In this case cyclohexane:ethylacetate 1:1 (v:v)

Organic solvent extracts transferred to new vials

Extracts evaporated under gentle nitrogen flow



Final extracts reconstituted in 80 µl cyclohexane:acetone 9:1 ready for GC-MS injection




Mode of action Non-systemic with contact and stomach action. Acetylcholinesterase (AChE) inhibitor.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