THE LC-MS/MS APPLICATION TO PROTECTION OF POLLINATORS

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Basic Steps of Our Project

- Pesticides, a persistent environmental problem from forties of 20.century (1939, DDT) are:
- Nowadays, likely behind the increased decline of bee populations, especially in Europe.
- The crucial aim of this work is to establish the maximal effective, comprehensive analytical system for monitoring these substances and their residues in a specific ecosystem of a given field in cooperation with our colleagues of the Czech University of Agriculture in Prague and the Faculty of Military Health Sciences, University of Defence in Hradec Kralove (dedications 1,2).
- Main framework: to establish systematic, easy, cheap and complex analytical method (SEChCA)

The Preparative Method – QuEChERS home modificated

We use our developed home made modifications of the original QuEChERS method to process primary analytical samples of various agricultural forages, i.e.

crops,

individual parts of the plant body,

pieces of soils,

and bee bodies

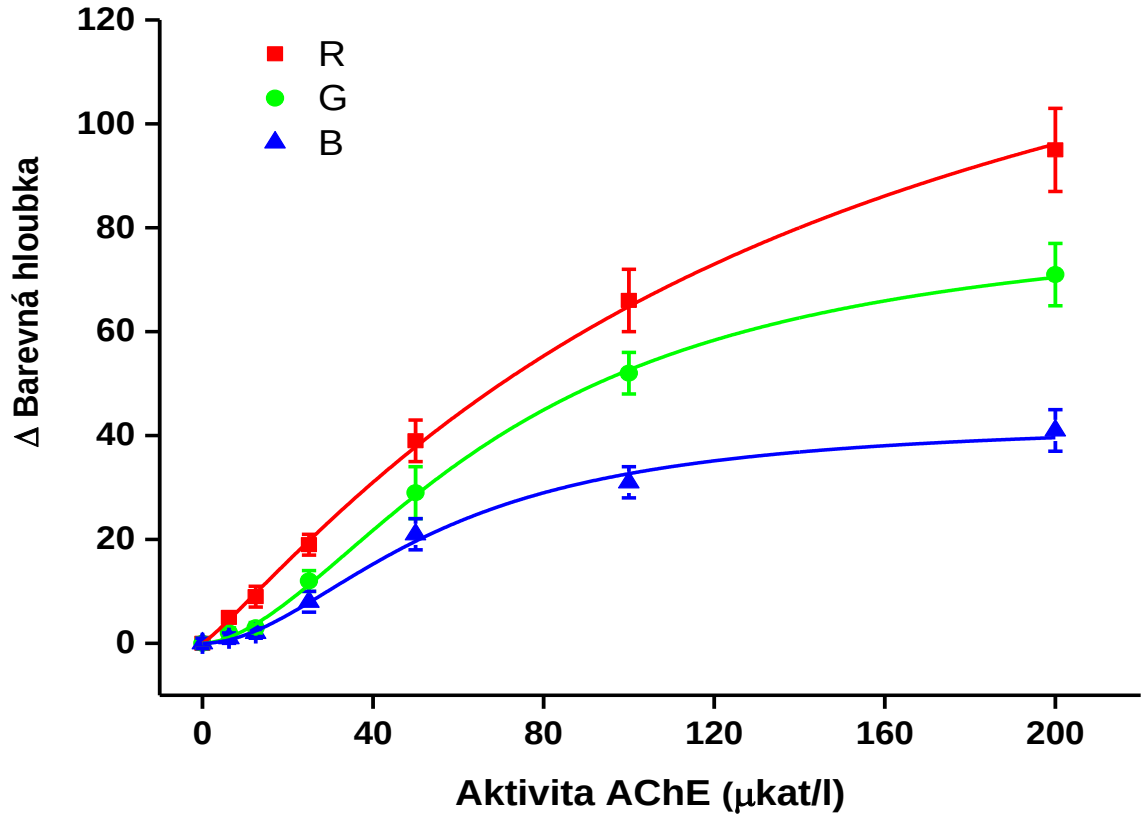
What is the basis of the pesticide threat to bee colonies?

- Due to the fact that bees often pollinate agricultural crops, they are exposed to chemicals used in such fields.
- However, it seems to be, individual bees who come into direct contact with the substance at the site of use are not at risk in this case.
- The bees bring contaminated pollen to their hive, where other individuals can be poisoned.
- Due to the social organization of the hive, the hive is systematically clogged with unwanted chemicals (or their metabolites), which are then incorporated into bee products (honey, wax, etc.)

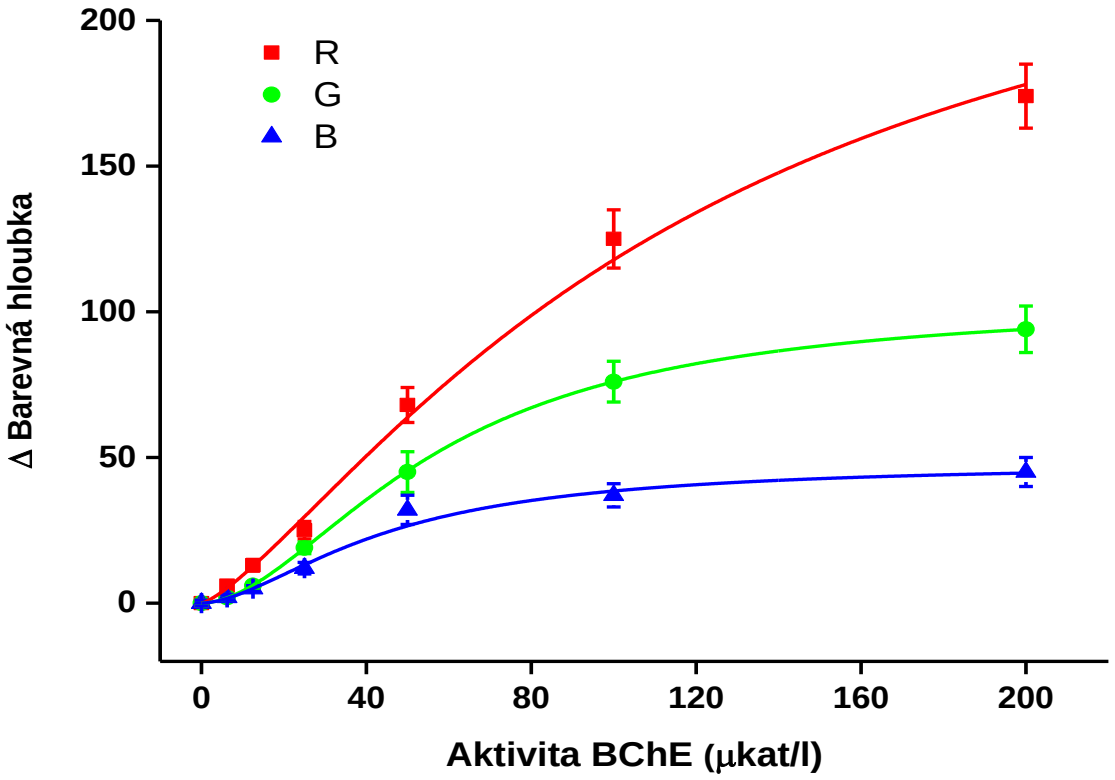
Field enzymatic screening method

If there is a sudden death of the bee colony, it is necessary to clarify its cause. The project "Colorimetric sensor for the diagnosis of pesticide poisoning" (TH03030336) aims to develop a fast and user-friendly sensor for the detection of bee poisoning by pesticides from the group of organophosphates and carbamates, which act as blockers of acetylcholinesterase (AChE).

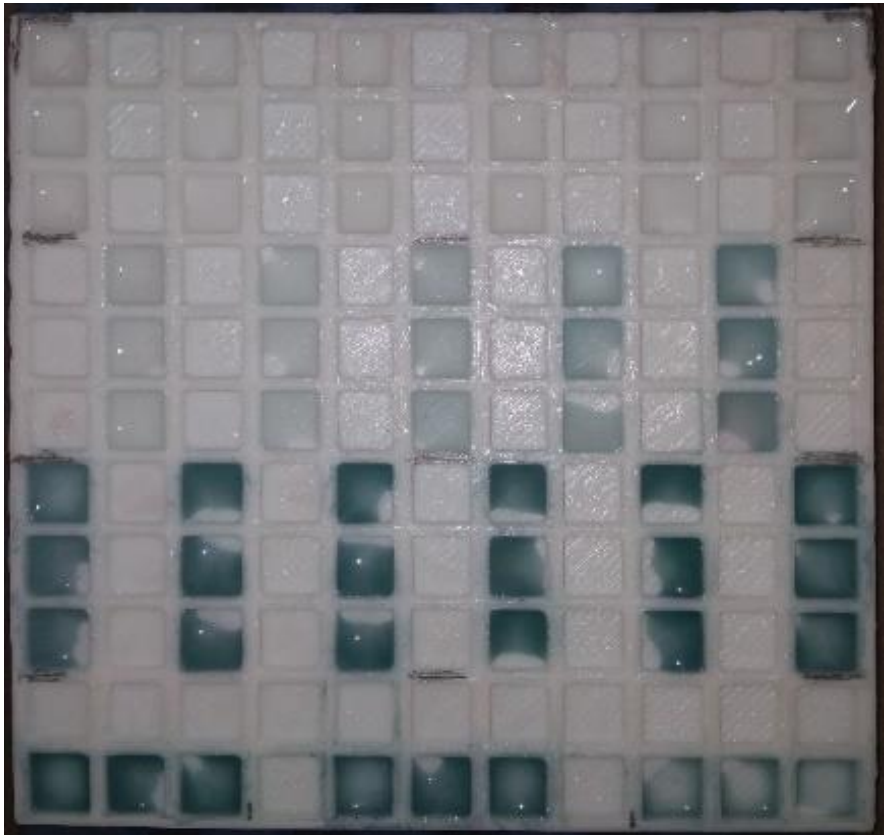
Calibration curve for AChE using a multichannel platform and an incubation time of 60 minutes

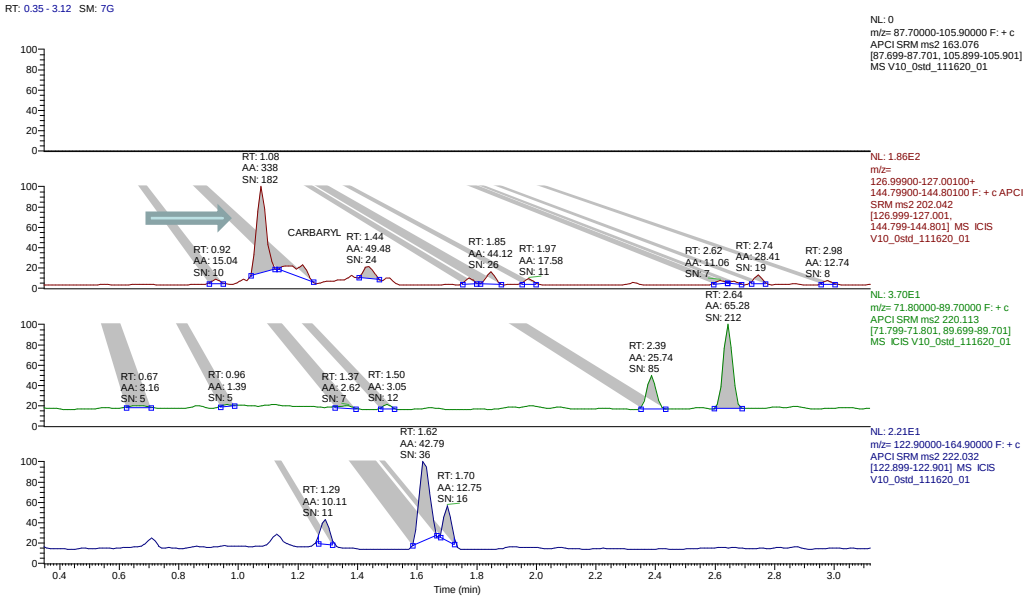


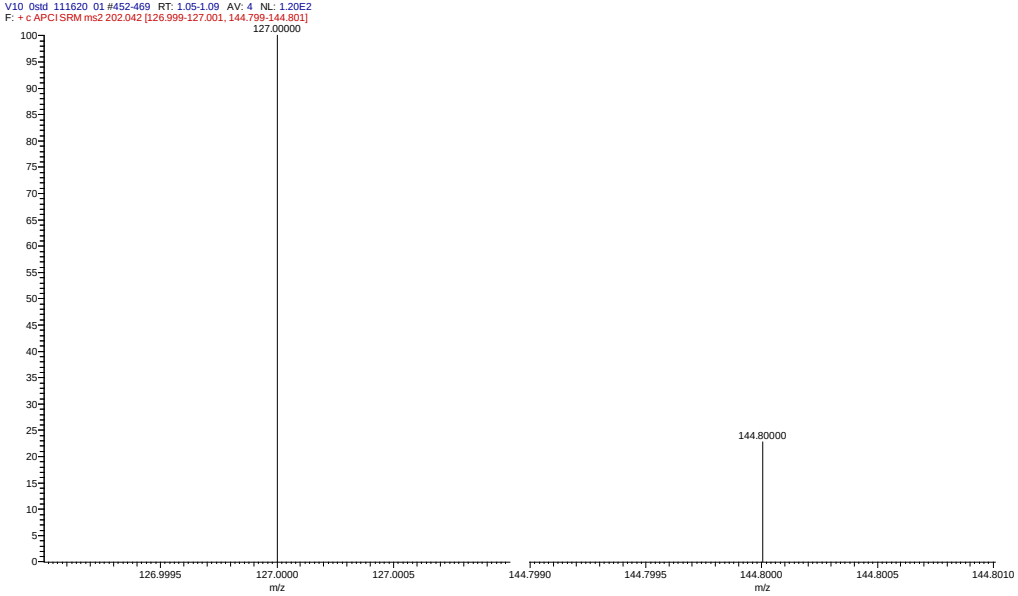
Calibration curve for BChE using a multichannel platform and an incubation time of 60 minutes



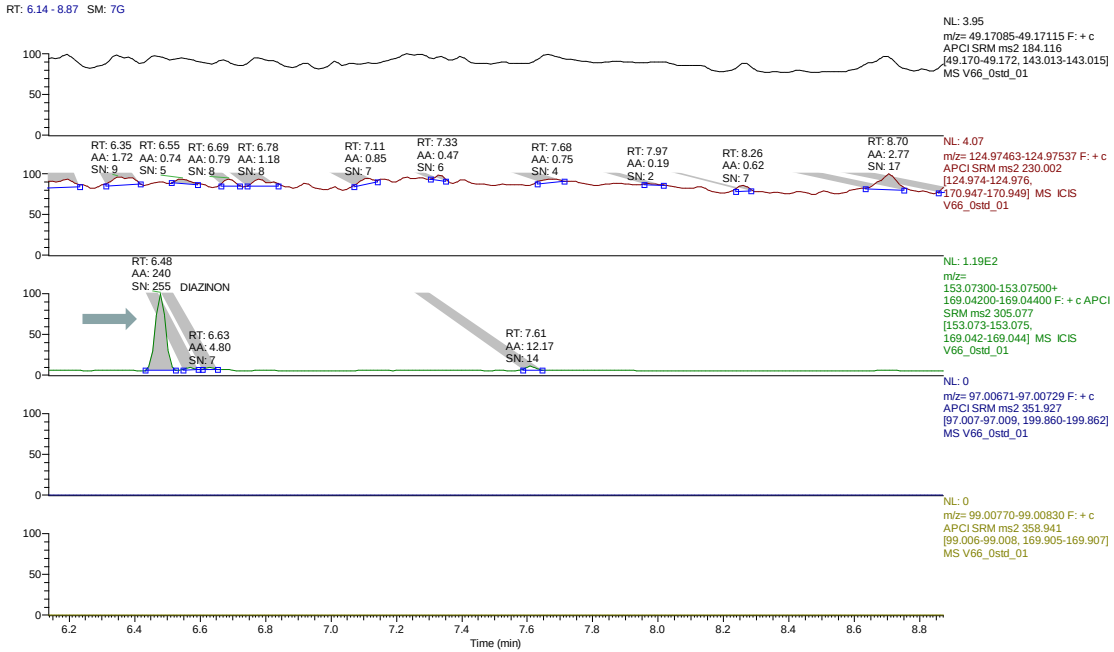
Illustrative photography for the determination of various BChE samples on a multichannel platform with indole acetate

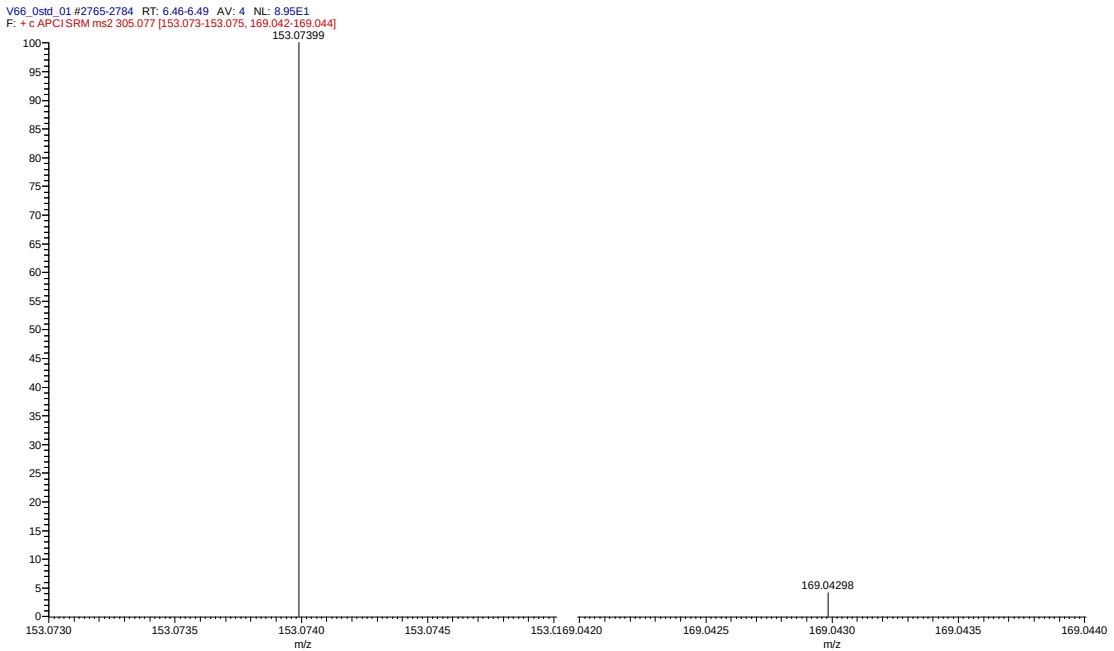






V10, carbamate positive bee sample





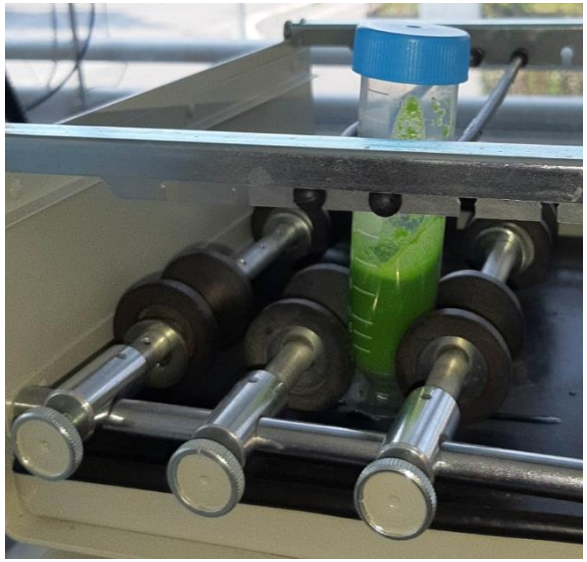
V66, organophosphate positive bee sample

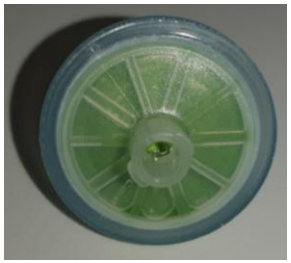
PERCENTUAL(%) FINDINGS IN BEE BODIES

ORGANOPHOSPHATES	
acephate	**1**
dimethoate	**9**
diazinon	**4**
chlorpyrifos	**1**
chlorfenvinphos	**0**
CARBAMATES	
methomyl	**0**
carbaryl	**3**
oxamyl	**2**
carbofuran	**1**

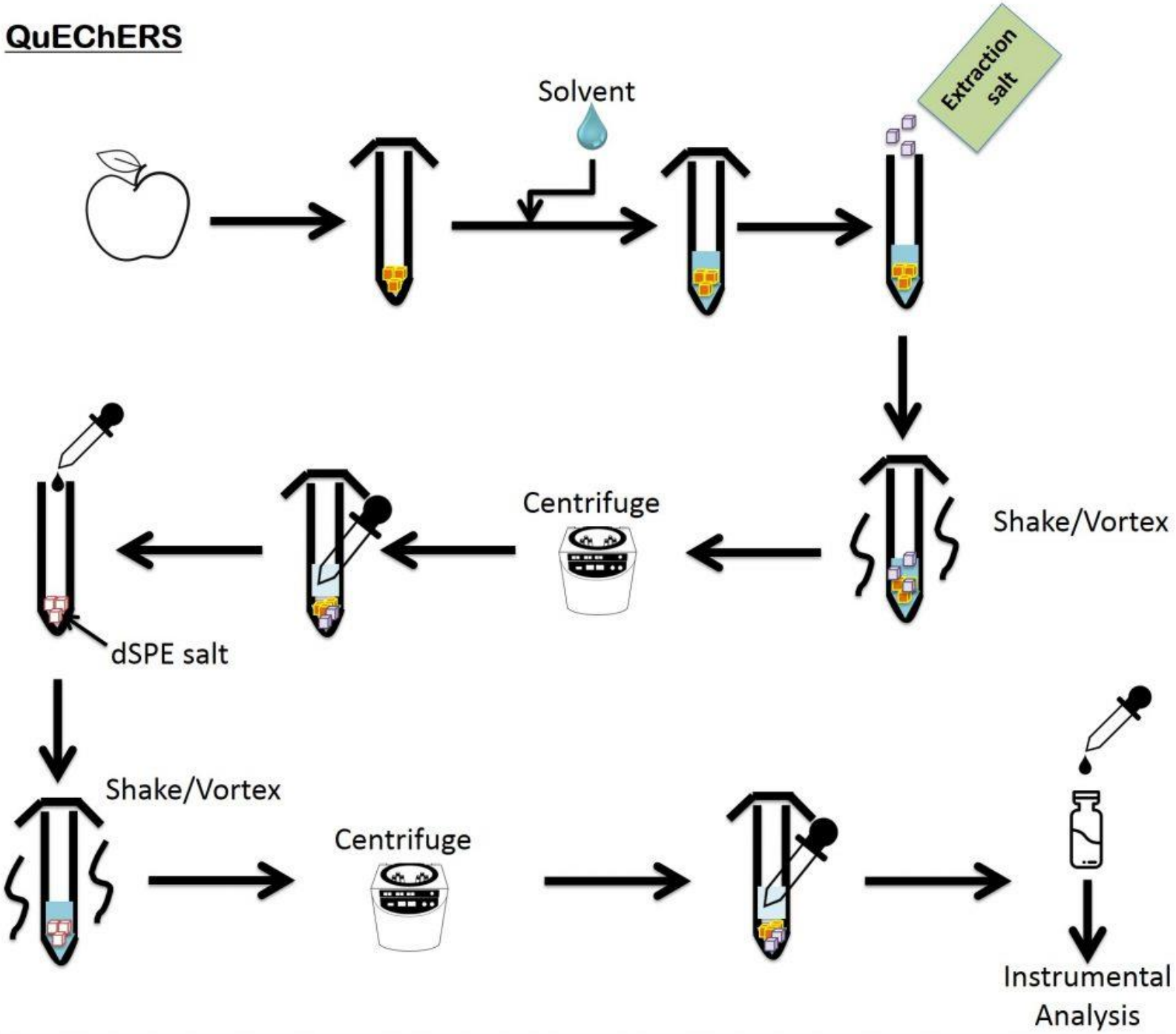
Honey Plants

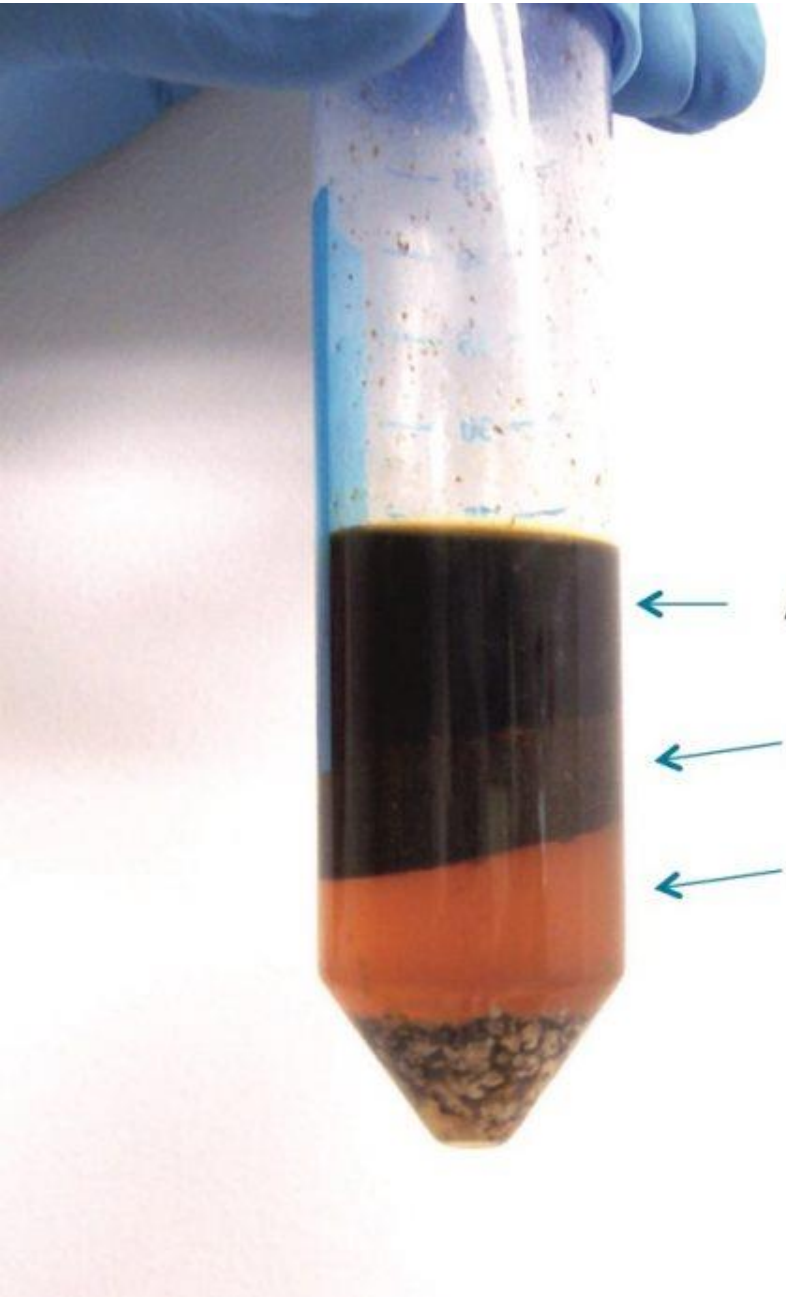
- angelica zahradní, chrpa, zvonek zahradní, modrá phacelia, bílá hořčice, pohanka, svazenka, východní třapatka nachová, trojlístek obecný, jetel karmínový, oregano, miláček, meduňka lékařská, náprstník obecný, brutnák lékařský, moldavská dračí hlava, šalvěj, volské oko sedmikráska, fialová viperova chyba a viperova chyba.



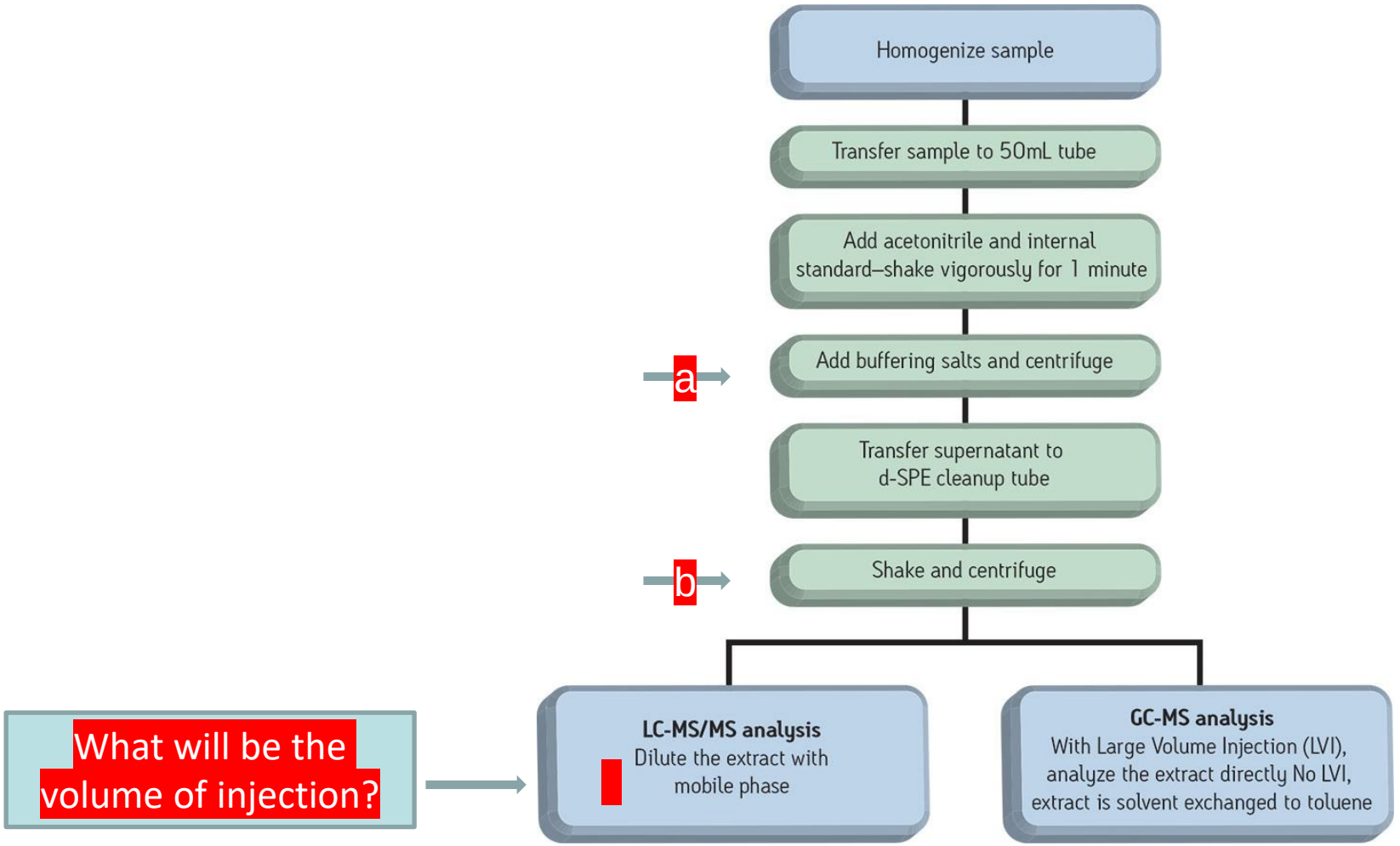


The Preparation of „smoothie“ sample (primary sample)


QuEChERS
Solvent
Extraction salt
Shake/Vortex
Centrifuge
dSPE salt
Shake/Vortex
Centrifuge
Instrumental Analysis


ACN layer (pesticides are here)
Tea solids
Aqueous layer (sugars and salts are here)

Schema from Waters Corp.:



Operating manual (the basement for SOP after the performance of validation) for PESTICIDES by LC/MS-MS via quant determination of standard additions methodology Updated 09-18-2021 AH

1)Take whole frozen material in a PH bag for „smoothie“sample preparation for homogenation with addition of ultra pure water - aprox. 1:1 material /water. Write the amount of water added in protocol 2) Wipe the mixed raw sample with a spatula from the walls and knives. 3) Take cca 5 g of smoothie from a container into 4 of 15 ml test tube with a cap 4) Addition of standard solution of pesticide to cca 5 g smoothie in 3 of 4 15 ml test tubes : - 1st tube- without addition only ISTD 25ul acephate solution - 2nd tube-addition of 50 µl of stock solution II of concentattin 100 ng / ml resulting to expectin concentration in 5 g smoothie 1ppb - 3rd tube-addition of 1000 µl of stock solution II of concentration 100 ng/ml resulting to expecting concentration in 5 g smoothie 20 ppb - 4th tube- addition of 50 µl of stock solution I of (10000 ng/ml resulting to 100 ppb) and add of 25 µl ISTD acephate solution 10ug/ml into every 15ml tubes expecting 50 ppb 5) Add 5 ml MeCN to each part 6) Sonication on ultrasound 10 min, shaking on a roller shaker 10 min 7) S centrifugation at 6000 rpm for 5 min / we are able to put only 4 tube of 15 ml / 8) Transfer supernatant 4,5 ml to Eppendorf 5 ml 9) To each tube 5 ml Eppendorf add 1 g of magnesium sulphate anhydrous, 0,25 g of sodium chloride from preprepared tubes 2 ml Eppendorf 10) Minishaker shake (3 min or longer for complete desintegration of sulphate – critical point, 2500 RPM) and centrifugation (12 min, 6000 RPM) 11) Pipette a 2 ml MeCN layer over the salt sediment from 5ml Eppendorf tube into a 5 ml Eppendorf tube with prepared 300 mg $MgSO_4$ anhydrous (!) +25 mg PSA + 25 mg C18 Shake immediately to prevent the block 13) Minishaker shake (1min, 2500 RPM) and centrifugation (10 min, 6000 RPM) 14) Use 0,22 um syringe filter to transfer 1,5 ml of organic layer to 2 ml vial

Instrumental Equipment

Mobile Phases, A: 0,1% HCOOH,D: MeOH/MeCN/HCOOH (50/50/0,1%), B: CH3COONH4 0.1mM, C: MeCN/MeOH(50/50)

2 x Column LUNA Omega-C18, 50mm x 2.1mm,1.6µm, 100mm x 2.1mm,1.6µm

PAL Thermo, Combi Dionex AS, UHPLC Dionex UltiMate 3000 pumps

Used Thermo MS : TSQ Access Max, LTQ XL, Q Exactive Focus

Targeted gradient chromatography – „QuantPestanal“

	Start	Time(sec)	Flow(ml/min)	Gradient	%A	%B
1.	0	30	0.30	Step	98	2
2.	0.5	360	0.45	Ramp	2	98
3.	6.5	150	0.45	Step	2	98
4.	9.0	30	0.30	Ramp	98	2
5.	9.50	30	0.30	Ramp	98	2
6.	10.00	45	0.30	Step	98	2

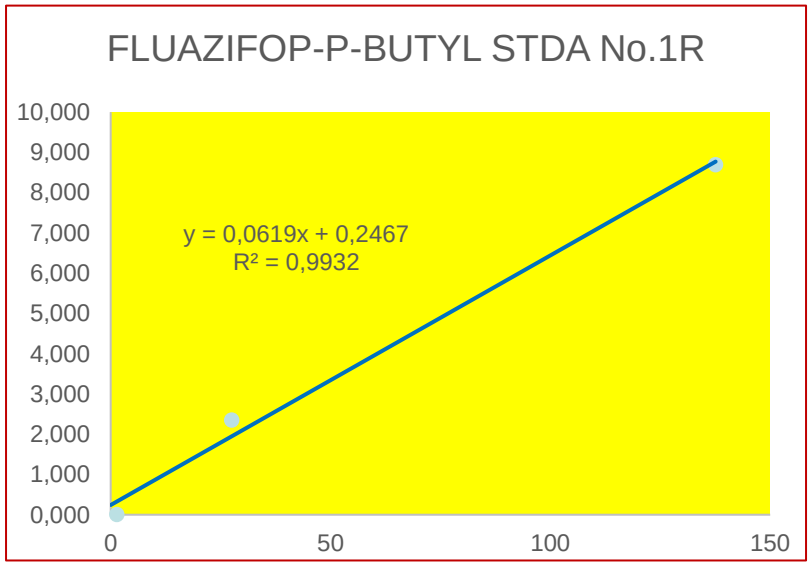
Non-targeted gradient chromatography – „CONFPESTANAL“

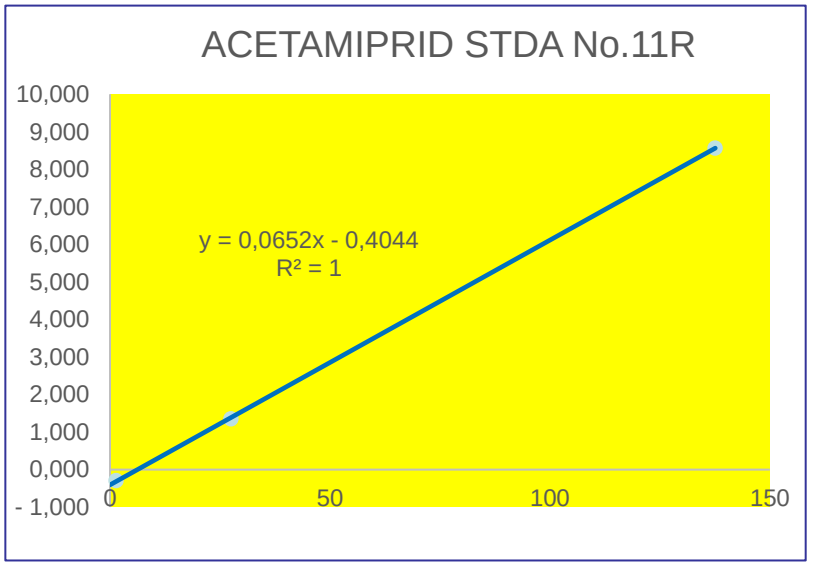
	Start	time	flow	gradient	%A	%B
1.	0,00	60	0,30	Step	100	
2.	1,00	420	0,30	Ramp		100
3.	8,00	240	0,30	Step		100
4.	12,00	30	0,30	Ramp	100	
5.	12,50	90	0,30	Ramp	100	
6.	14,00	90	0,30	Step	100	

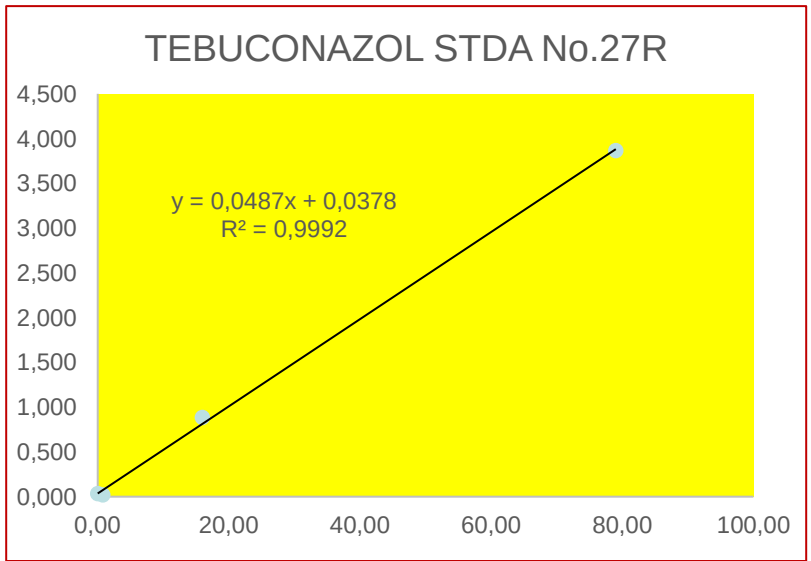
Common MS Parameters

Scan modes: Full MS, Full MS confirm, PRM, MRM, MS3
Chrom peak width: 4-6-7 s.
Method duration: 10. 00, 14.00 min
Resolution: 70 000.
Scan range: 100 – 1000 m/z.
Autogain control(AGC targed): 1e6
H-ESI → Sheath Gas : 32
→ **Aux Gas:** 6
Cone (spray) voltage: 3,5 kV/2.2 kV for negatives
Capillary temperature: 280-290 °C
Vaporizer (Aux Gas Heater) temp: 200(temp labile compounds)-350°C
S-lens RF level: 55.0-65.0

Examples of Used Triple Standard Addition Calibration







Salads/qualitative microinvestigation, 6collections (A-F), results below

Species

- Dubáček, 10A-F
- Ledový, 9A-F
- Lollo Rosso, 11A-F
- Lollo Biondo, 12A-F
- Římský,13A-F
- suspected use of three products at once: STOMP, GONDOLA, ALLIETE

Images





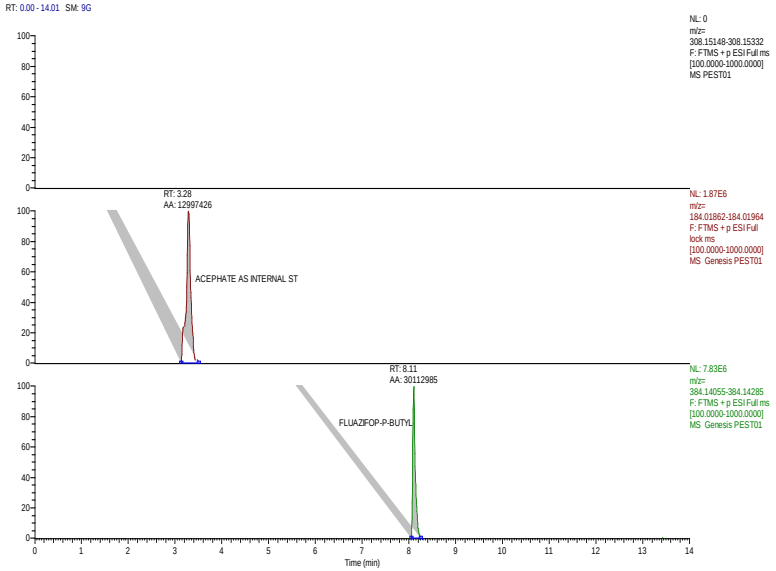

Some excerpts of qualitative results from pestanal monitoring in agricultural products – salads see above

- **9A – F: pendimethalin (STOMP)**
- **10A - F: pendimethalin,sulfoxaflor (GONDOLA)**
- **11A – F: pendimethalin,sulfoxaflor (GONDOLA)**
- **12A – F: pendimethalin (STOMP)**
- **13A – F: pendimethalin,sulfoxaflor (GONDOLA)**
- **The Use of ALIETTE has not be proven (active substance – fosetyl-Al)**

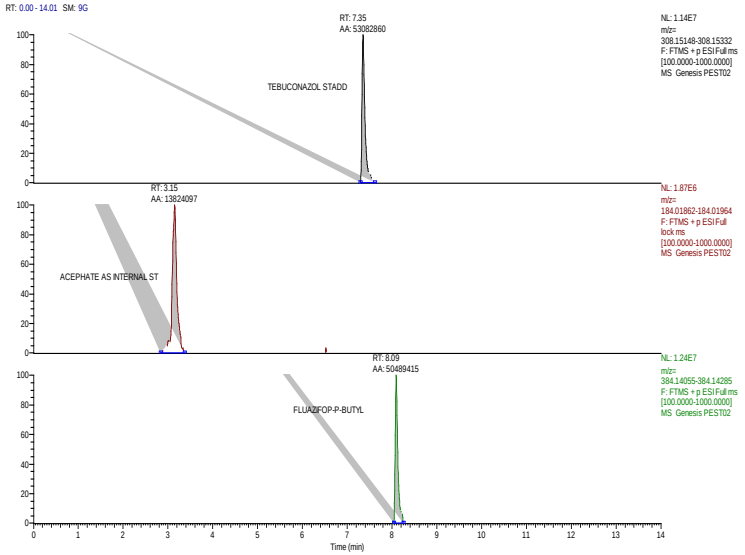
Note: Acephate was used as the control internal standard

Non- targeted screening and Accurate Mass Confirmation on the Focus HRMS instrument – contradictory samples

- Sample 1 free from an pesticide standard addition, positive fluazi



- Sample 2 with tebuco standard addition, positive fluazifop-p-bu



Svazenka vratičolistá - Phacelia tanacetifolia

Řepka olejka – Brassica napus L. (angl. RAPE, RAPESEED)

Pohanka setá – Polygonum fagopyrum

Quant results in plant materials – excerpts (ppb)

ID samples	1R	2R	3R	4R	5R	6R	7R
ACETAMIPRID	2.52	0.07	0.06	0.13	LOQ	ND	LOQ
TEBUCONAZOL	NS	NS	NS	NS	NS	NS	NS
FLUAZIFOP-P-BUTYL	1.06	1.62	1.44	0.59	1.93	LOQ	3.30

Quant results in plant materials - summaries

- **Svazenka:** Fluazifop-p-butyl 20 ng/g-LOQ (4collection times)
- Acetamiprid LOQ – 2.5 ng/g (4collection times)

- **Pohanka:** Fluazifop-p-butyl 20 ng/g-LOQ (4collection times)
- Acetamiprid LOQ – 2.5 ng/g(4collection times)

LOQ

LOQAcetam – 0.005 ng/g

LOQFluazi – 0.002 ng/g

LOQTebuco – 0.007 ng/g

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