News from EURL AO

Ralf Lippold, Fabian Bäuerle, Heike Müller
Svetlana Juschenko, Sabine Walter,
Tanja Radykewicz, Benjamin Dambacher,
Björn Hardebusch

Stuttgart, 30 September 2015

News from EURL AO

Content of the Presentation

- Cleanup (LC-analysis)
- Screening with accurate mass instruments coupled with LC
- Method for less polar pesticides

Cleanup (LC Pesticides)

- Since early summer 2015 Agilent offers a cleanup test kit for fatty matrices (EMR Kit)
- Agilent chemicals replace the typical QuEChERS-ingredients
  - EMR dSPE (composition ?) instead of buffer and salts (4 g $MgSO_4$; 1 g NaCl; 0,5 g Na_2Hcitrate x 6 H_2O; 1 g Na_3citrate x 2 H_2O)
  - EMR polish (mixture of $MgSO_4$ and 1 g NaCl) for drying the solvent

Comparision:

- Cleanup according QuEChERS AO
- Cleanup according QuEChERS with Agilent EMR kit

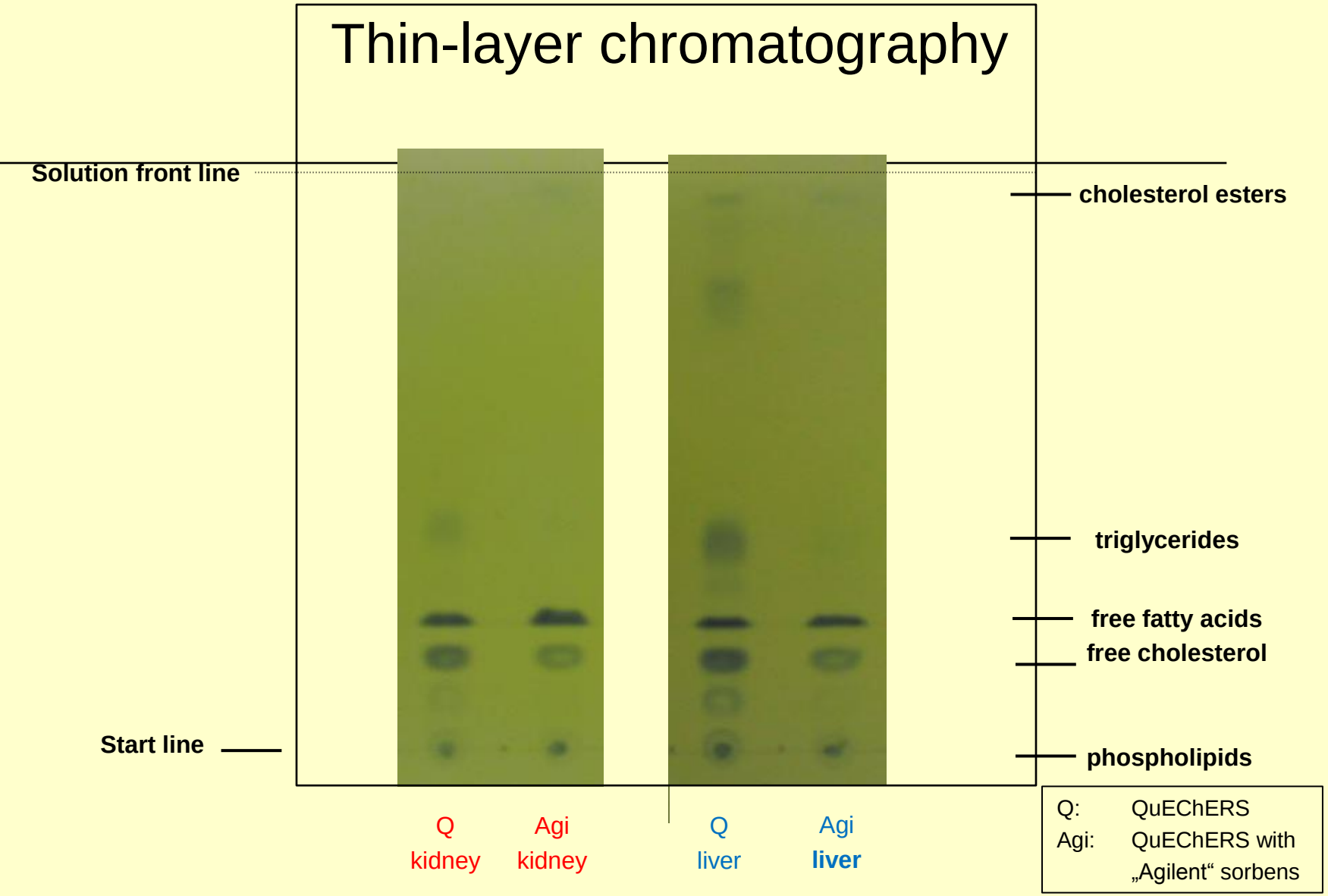
Cleanup: Proof applying thin layer chromatography

- Stationary phase: silica gel, 60 µm
- Mobile phase: petroleum ether / diethyl ether/ acetic acid (85/15/1.5; *v/v/v*)
- Spraying reagent: 10% phosphomolybdic acid in ethanol
- Heating: 2 min @ 150 °C

Shown by Fanny Hildmann at EPRW 2014 in Dublin

German Official Method according § 64 LFGB; No. L 13.00-27/2 Appendix A

Thin-layer chromatography



Cleanup: what about the Pesticides

- Cleanup according QuEChERS AO
- Cleanup according QuEChERS with Agilent EMR kit
- Cleanup according SweEt (slightly modified by EURL AO)

- Spiking @ 0.010 mg/kg with 142 pesticides (calibration standard mix for qqq-analysis)

- Results (Screening with LC-q-ToF):
  - QuEChERS AO: 136 pesticides detected (95.8 %)
  - QuEChERS EMR kit: 136 pesticides detected (95.8 %)
  - SweEt mod. EURL AO: 135 pesticides detected (95.1 %)

Training of NRLs

for Pesticides in Food of Animal Origin an Commodities with High Fat Content

EUROPEAN UNION REFERENCE LABORATORY

PESTICIDE RESIDUES IN FOOD OF ANI ORIGIN & COMMODITIES WITH HIGH F

28 - 29 September 2015, Freiburg, Germany

- Ralf Lippold
- Björn Hardebusch
- Tanja Radykewicz
- Benjamin Dambacher
- Karin Malisch
- Rainer Malisch

Fabian Bäuerl
Carolin Haule
Maria Schmitt
Katrin Thoma

Cleanup

	QuEChERS for Food of Animal Origin
Sample amount	5 g matrix of interest (liquid whole egg, cow´s milk, cheese, meat, honey……)
Extraction	50 mL centrifuge tube
	Addition of internal standard: add 100 µl ISTD to both and spike one tube with 200 µl spiking solution, wait 10 min
	Addition of 10 mL water, shake or vortex (10 min) Addition of 10 mL ACN, shake or vortex (10 min) 4.0 ± 0.2 g $MgSO_4$ + 1.0 ± 0.05 g NaCl + 1.5 g Citrate salts (0,5 g Na_2H-citrate x 6 H_2O , 1.0 g Na_3-citrate x 2 H_2O) Shake vigorously for 10 min Centrifuge for 5 min at 2500 g (4000 rpm)
Clean-up	15 mL centrifuge tube filled with : 900 mg $MgSO_4$ + 150 mg PSA Add 6 mL supernatant Shake for 10 min Centrifuge for 5 min at 2500 g (4000 rpm)
Preparation before analysis	Transfer 2 times 1 mL into LC-vials
Analysis	LC-MS/MS + LC-MS-TOF

Cleanup

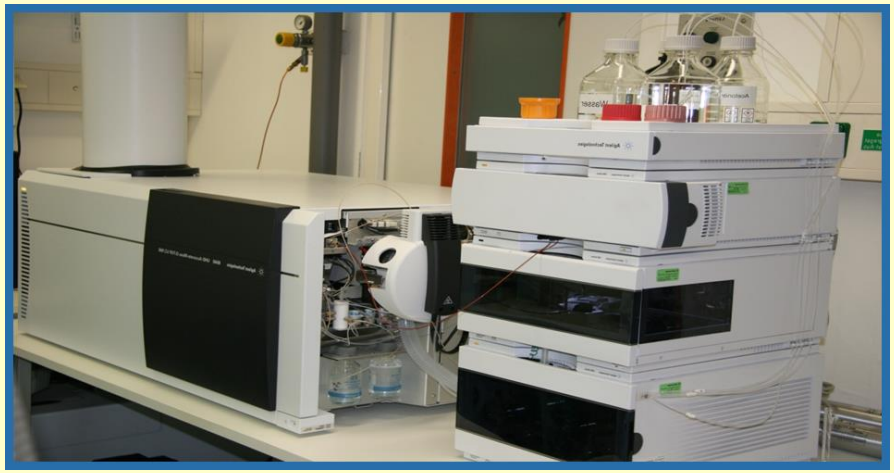
	QuEChERS for Food of Animal Origin (Agilent chemicals)
Sample amount	5 g matrix of interest (liquid whole egg, cow´s milk, cheese, meat, honey……)
Extraction	50 mL centrifuge tube
	Addition of internal standard: add 100 µl ISTD to both and spike one tube with 200 µl spiking solution, wait 10 min
	Addition of 10 mL water, shake or vortex (10 min)
	Addition of 10 mL ACN, shake or vortex (10 min)
	Transfer 8 mL of ACN supernatant into the 15 mL dSPE EMR-lipid centrifuge tube
	Shake vigorously for 10 min
	Centrifuge for 5 min at 2500 g (4000 rpm)
Clean-up	Transfer supernatant into 15 mL centrifuge tube filled with:
	Final polish EMR-lipid (Magnesium sulfate, sodium chloride)
	Shake for 10 min
	Centrifuge for 5 min at 2500 g (4000 rpm)
Preparation before analysis	Transfer 2 times 1 mL into LC-vials
Analysis	LC-MS/MS + LC-MS-TOF

Cleanup

	SweEt for Food of Animal Origin
Sample amount	5 g matrix of interest (liquid whole egg, cow´s milk, cheese, meat, honey……)
Extraction	50 mL centrifuge tube
	Addition of internal standard: add 100 µl ISTD to both and spike one tube with 200 µl spiking solution, wait 10 min
	Addition of 10 mL water, shake or vortex (10 min) - **(not for milk)** Addition of 10 mL ethyl acetate Shake vigorously for 10 min 5.0 ± 0.2 g Na_2SO_4 + 0.2 g C_{18} + 0.2 g PSA Shake vigorously for 10 min Centrifuge for 5 min at 2500 g (4000 rpm)
Preparation before analysis	Transfer 2 times 1 mL into LC-vials
Analysis	LC-MS/MS + LC-MS-TOF

Cleanup – Challenges in life training

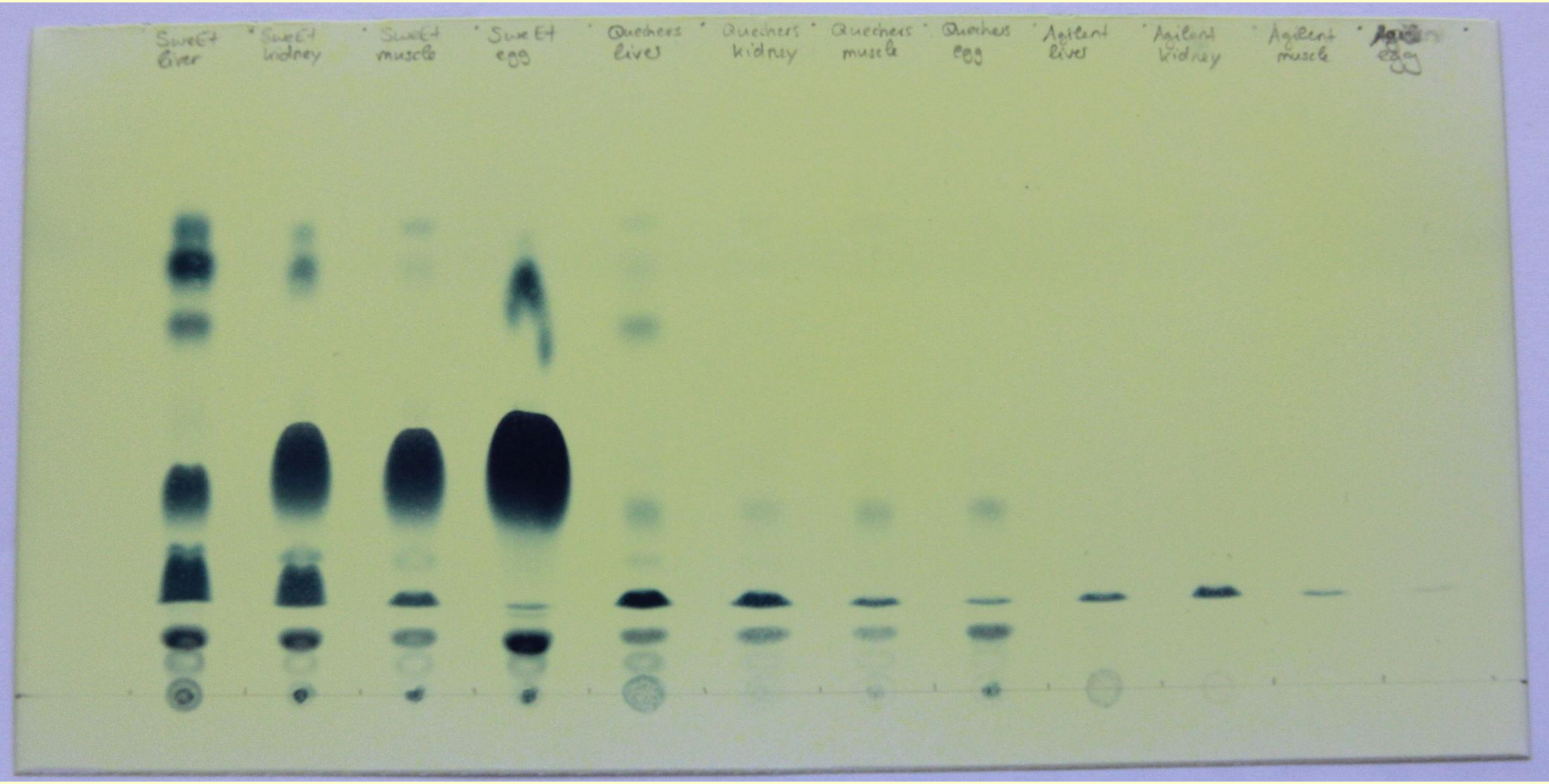
ToF-results not availabe at the time beeing



Strike of the Agilent LC-MS-q-ToF

On Sunday, Jan Elias Hardebusch arrived in Freiburg

Cleanup: Results achieved during the training 28./29. September 2015



Screening

Content of the Presentation

- Cleanup (LC-analysis)
- Screening with accurate mass instruments coupled with LC
- Method for less polar pesticides

Screening

- Typical Instruments on the market
  - ToF or q-ToF coupled to LC or GC
  - Orbitrap or q-Orbitrap coupled to LC or GC

Screening

Application of accurate mass MS in Pesticide Residue Analysis

1. Qualitative Screening by using cut of limits
   (e.g. the Screening Detection Limit – SDL
   or the Detection Capability (CCß) - 2002/657/EC)
2. Complementary technique to classical detectors
   - Increase of available information for detected pesticides
3. Search for analytes of special interest
   (e.g. “find by formular”)
4. Search for “real unknown” by the using databases
5. Evaluation of the sample background

Screening

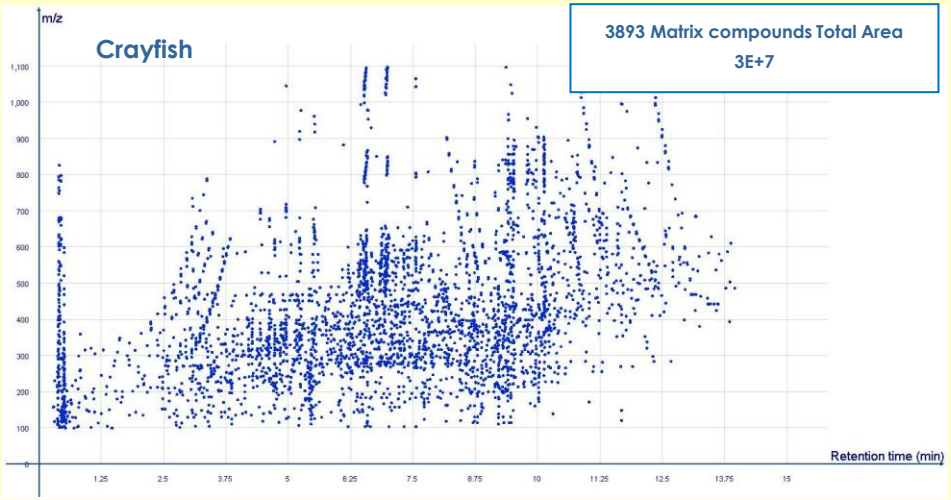
Application of accurate mass MS in Pesticide Residue Analysis

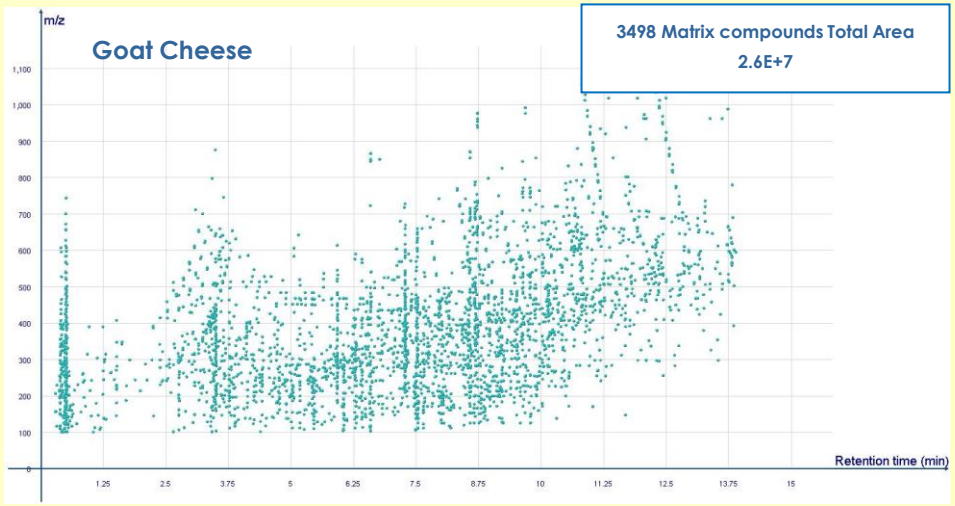
1. Qualitative Screening @ SDL
2. Complementary technique to classical detectors
3. Search for analytes of special interest
4. Search for “real unknown” by the using databases

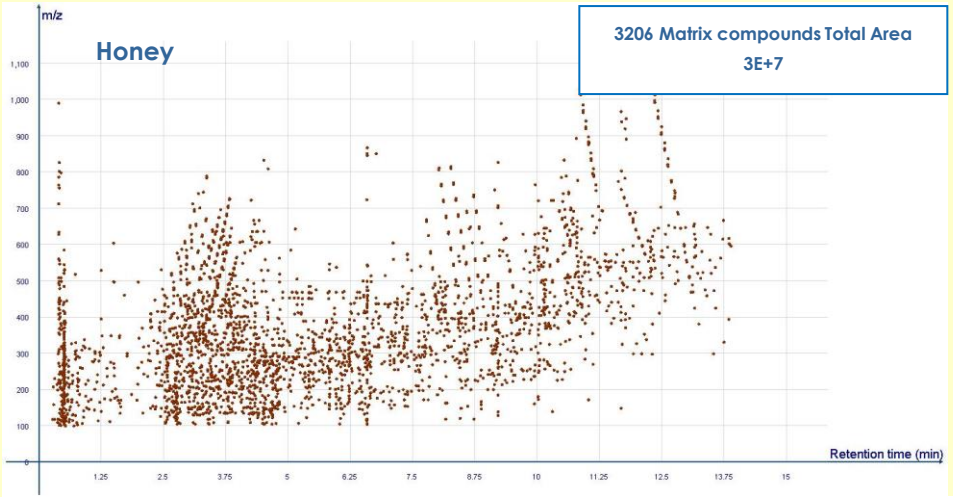
Reporting possible in case of applying validated methods (requirement of ISO 17025!)

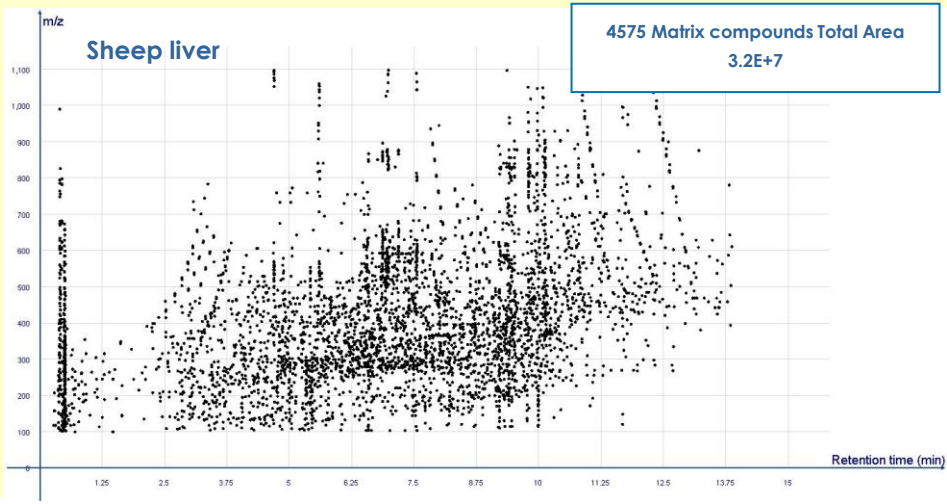
- Results of applications 3 and 4 are typical for internal use, only

5. Evaluation of the sample background, e.g. by MFE









Screening

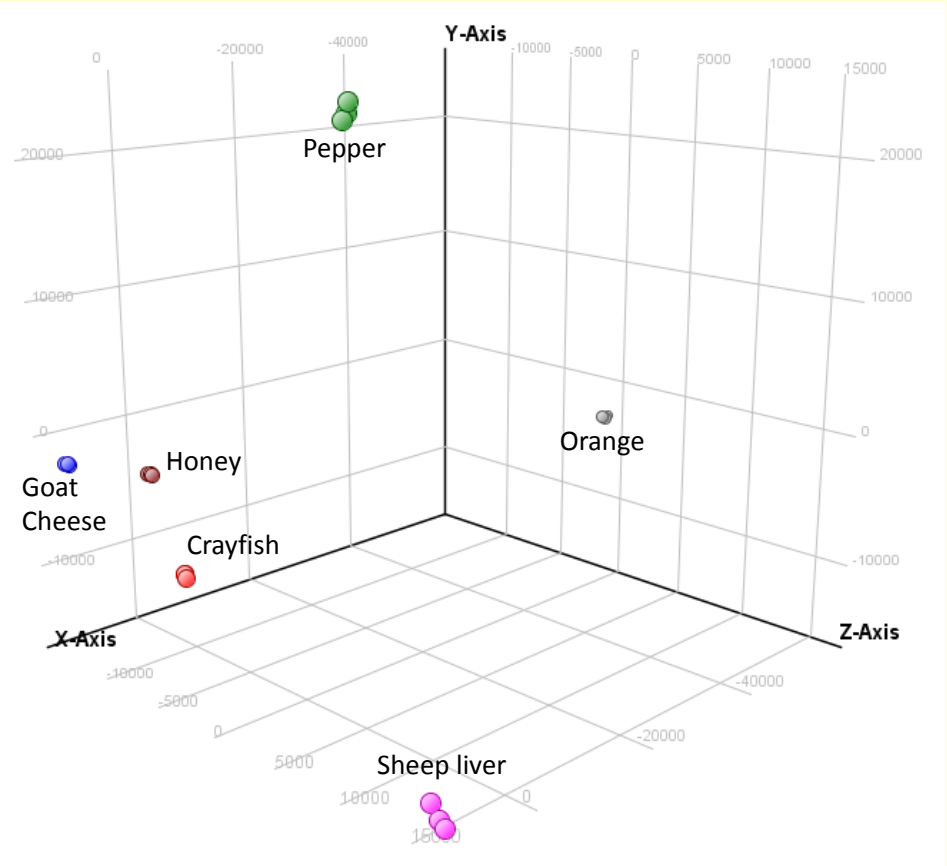
5. Evaluation of the sample background

Principle Component Analysis (PCA)

- Information about similar background
- LC-QQQ: Pool of matrices for calibration possible?

Co-Project together with EURL FV (Almeria, Spain)

Thanks to Amadeo R Fernandez Alba and Ana Lozano



Screening in Pesticides Residue Analysis

Decision Qualitative Screening <-> Confirmatory Method

- Qualitative Screening
  - Pesticides never found or few findings in Europe
  - Detection @ 0.01 mg/kg (ß <= 5 %)
  - *Detection below MRL-level (not for MACCP)*
- Confirmatory Method
  - Pesticides frequently or sometimes found
  - Pesticides with unsatisfying SDL

Screening: LC-MS-Q-TOF Conditions

- HPLC Methode1290 Series
- Simple method for „all“ analyts
- Injection volume 2 - 5 µl
- Column Zorbax Eclipse Plus C-18 HD, 150 x 2.1mm, 1.8 µm
- Gradient: solvent A = 5 mM NH_4Formiate; solvent B = ACN

Time (min)	% B	Flow (ml)
0	5	0.3
1	5	0.3
30	95	0.3
32.5	95	0.3
33	5	0.3

- Q-TOF Method
  - MSScan (sensitive scan) in 4 GHz-Modus
  - ~~Targeted MSMS (identification)~~
- Data base research
  (optimized for pesticides with RT)

Workflow Screening

EXAMPLE: EXPERIMENTAL DESIGN OF A VALIDATION STUDY (Eggs)

Sample Treatment

- 241 analytes were spiked at 4 different levels

20 Blank matrices

- 10 different samples of hen´s eggs

“5 spiking levels”

- 0 mg/kg / 0.005 mg/kg / 0.010 mg/kg / 0.020 mg/kg / 0.040 mg/kg

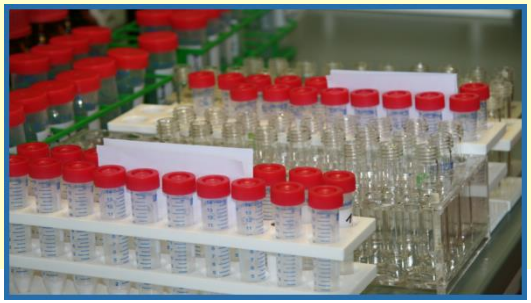
Number of analyses

- In total 100 analyses
  (2 replicates of each sample analysed at 5 concentration levels)

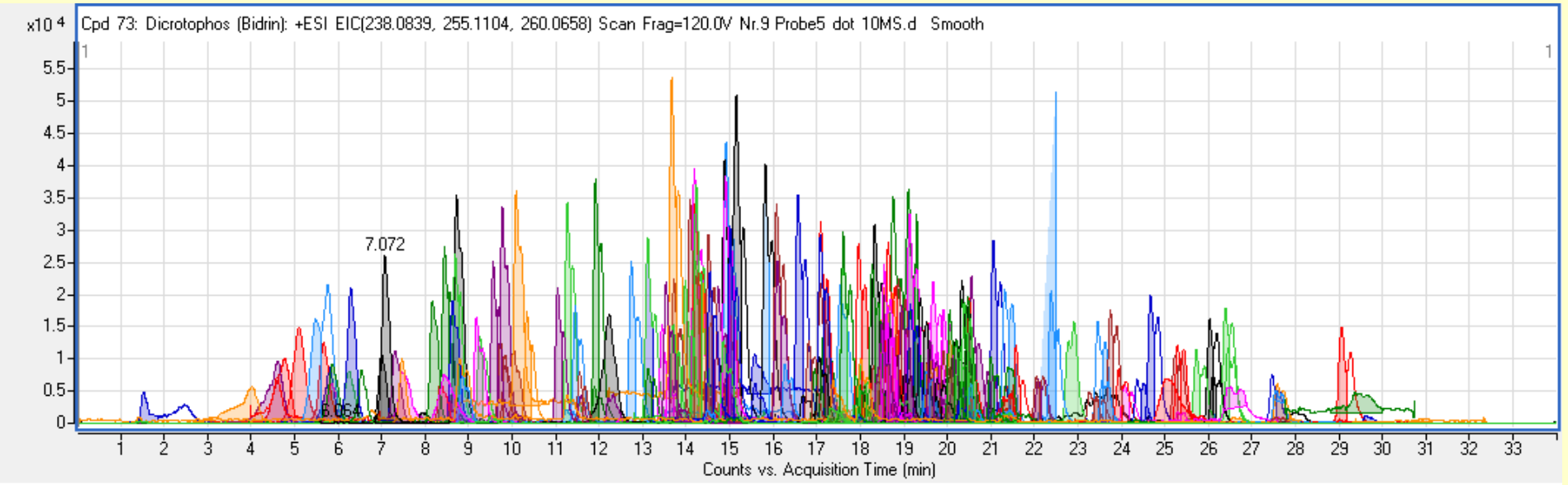
QuEChERS clean up

LC-MS-ToF (ESI pos, only)

Evaluation against own database

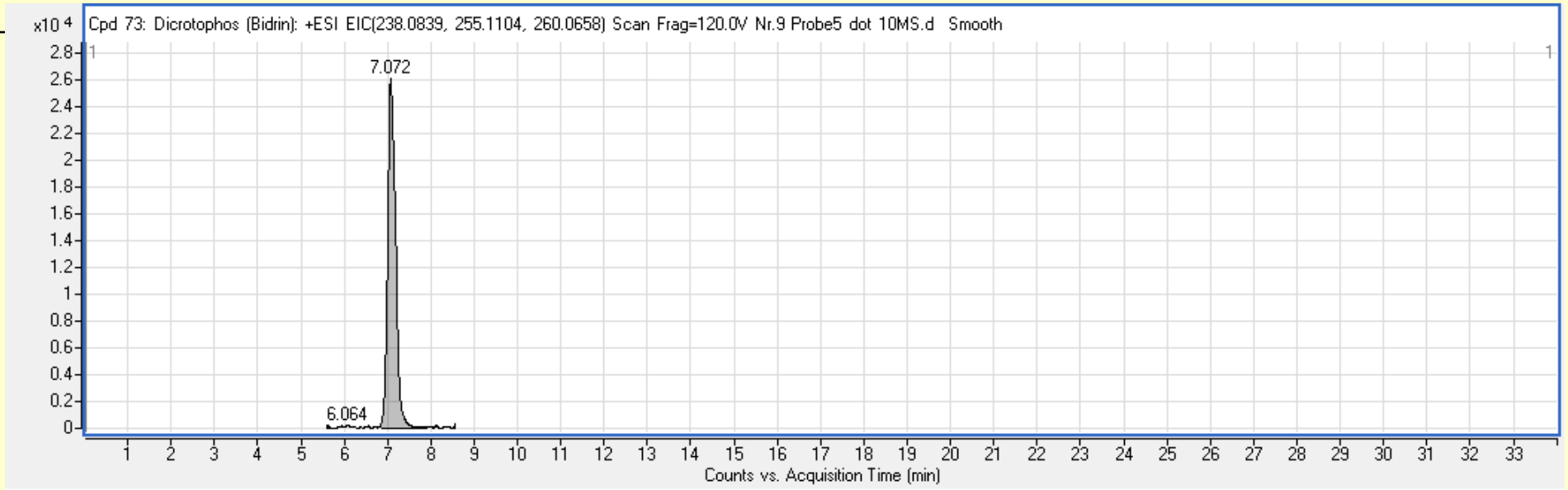


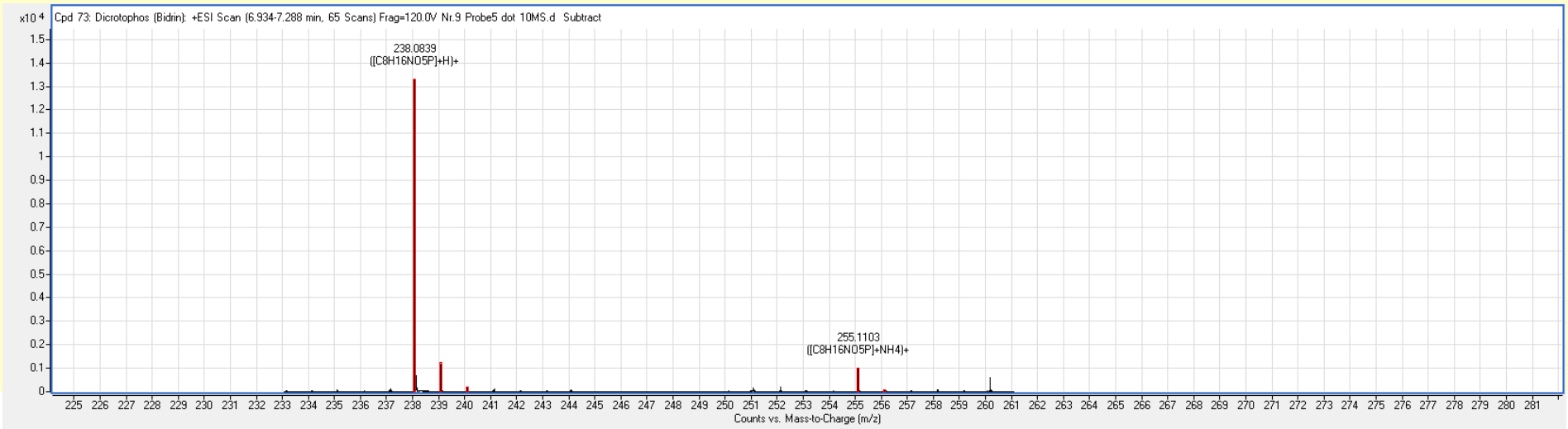
Workflow Screening



EURL EUROPEAN UNION REFERENCE LABORATORY PESTICIDE RESIDUES IN FOOD OF ANIMAL ORIGIN & COMMODITIES WITH HIGH FAT CONTENT

Workflow Screening





Results of the Screening Validation Study (Egg)

Results with LC-q-ToF (ß-error ≤ 5%)

Spiked Concentration [mg/kg]	0.005	0.010	0.020	0.040
Number of pesticides detected (Screening Detection Limit - SDL)	180	194	202	203
Ratio of pesticides identified at the spiked concentration	74.7%	80.5%	83.8%	84.2%

Results with LC-q-Orbitrap (ß-error ≤ 5%, analysis performed by Thermo)

Spiked Concentration [mg/kg]	0.005	0.010	0.020	0.040
Number of pesticides confirmed (Screening Detection Limit - SDL)	157	171	182	189
Ratio of pesticides identified at the spiked concentration	67%	72%	77%	80%

Details will be presented for egg and honey at RAFA (Prague)

Screening

Evaluation of retention times

Retention time (egg study)			
Total number of RTs: 15718	Reproducebility conditions		Repeatabiltiy conditions
# +/- 0.1 min	14869	94,6%	> 99 %
# +/- 0.2 min	809	5,1%	
# outlier	40	0,3%	<< 0.1 %

Retention time (honey study)			
Total number of RTs: 26832	Reproducebility conditions		Repeatabiltiy conditions
# +/- 0.1 min	25434	94,8%	> 99 %
# +/- 0.2 min	775	2,9%	
# outlier	623	2,3%	<< 0.1 %

Less Polar Pesticides

Content of the Presentation

- Cleanup (LC-analysis)
- Screening with accurate mass instruments coupled with LC
- Method for less polar pesticides

Extraction for less polar pesticides at CVUA Freiburg

Sample preparation example

Mix 5 g (10% fat) of sample with sodium sulfate (~50 g)

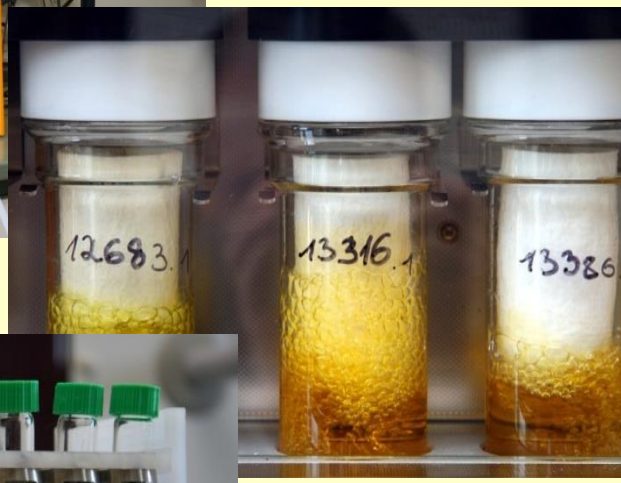
Soxtherm extraction with light petroleum

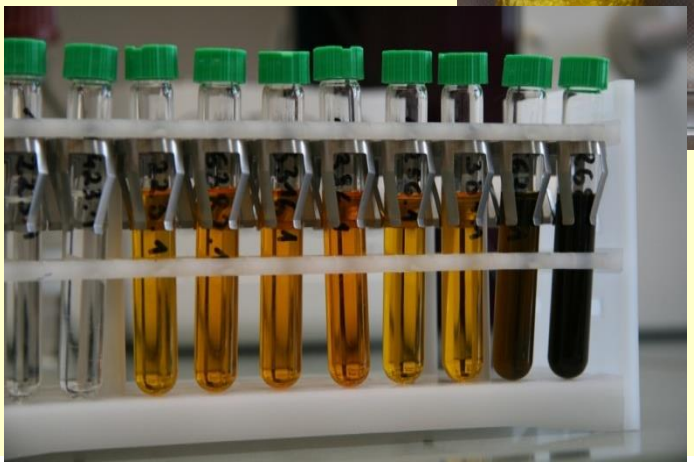
Filtrate, evaporate to dryness

Re-dissolve in ethylacetate/cyclohexane

Take an aliquot of 5 mL for clean-up (containing ~0.5 g fat)

→ clean-up





Clean-up for GC-Analysis

Clean up

Addition of internal Standards 2,4,5-TCB Mirex, Triphenylphoshat, PCB 209

GPC (Bio Beads S-X3)

small silica gel column (1.5 % water)

Fraction 2 Toluene

Fraction 3 Toluene / Acetone 95 + 5

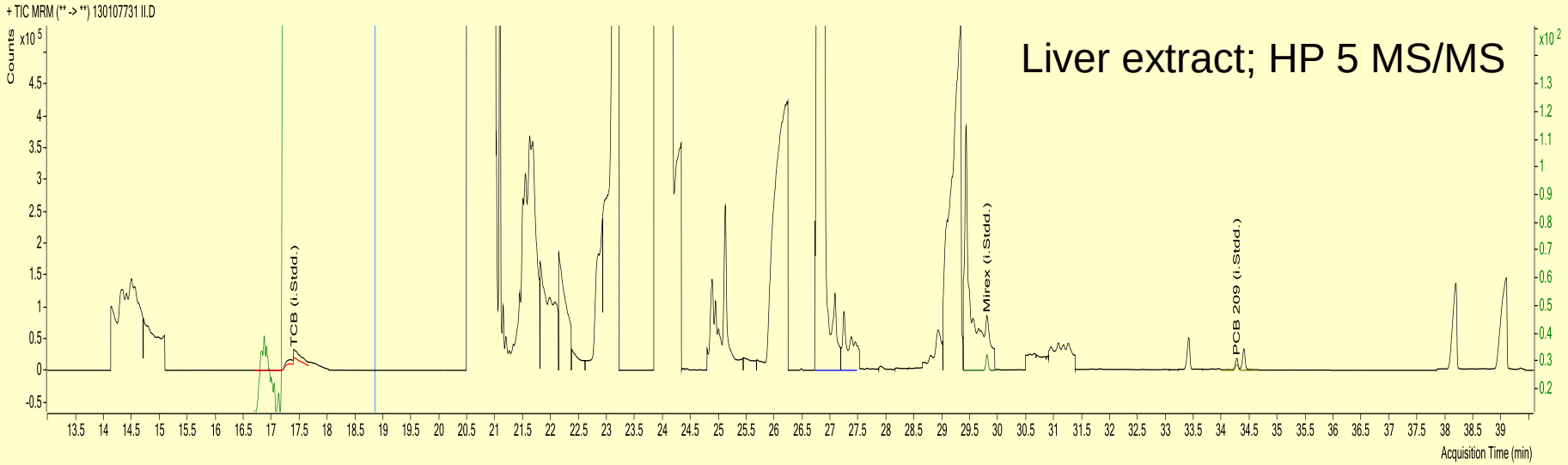
Final volume 0.5 ml

Ready for GC-analysis

all according EN 1528

GC-Analysis after Clean up according EN 1528

More often problems analysing liver



Advantages- and Disadvantages of Method (EN 1528)

- Method established with high experiences
- Final extracts suitable for Gas-Chromatography combined with all GC-detection systems
- For MS-Measurement only few maintenance effort necessary (1-2 times a year), few liner changes

- 6-7 different solvents are used during the analysis procedure
- High solvent volume (~500 mL per analysis), recycling at CVUA
- Time consuming steps (2-3 working days per batch), esp. extraction step
- High costs for glassware and man power

- ➢ How to increase the efficiency (saving time and money)?
- ➢ How to increase the cleanup (liver!)

Possible alternatives?

QuEChERS - SweEt

- Co-extracted fat may lead to problems in GC analysis (especially SweEt)
- Low recovery rates for non-polar analytes (QuEChERS)

Reproducibility: GC-MSMS of QuEChERS extracts

Matrix: cream (frozen extract)

10 Injections

Injection	Deltamethrin	Fenvalerate I	Fenvalerate II
1	595	14625	9101
2	534	14129	7450
3	208	7461	3736
4	250	7432	4113
5	191	5426	3479
6	192	5564	2858
7	156	4994	3588
8	127	5500	2107
9	138	4596	1566
10	132	4283	1382
Mean	252	7401	3938
standard-deviation	169	3826	2496
cv [%]	67.2%	51.7%	63.4%

Loss of sensitivity – no reproducibility with „dirty“ extracts.

Data obtained from test measurements on Agilent GC-MSMS

Modification of existing method for pesticide residues

Extraction procedure, modified

- Mixture of ethylacetate and cyclohexane used for extraction
  - Combination with existing EN 1528 – clean-up approach
  - The extract should be ready to use for GPC clean-up
  - No additional dilution or evaporation steps after extraction

Less Polar Pesticides in Food of Animal Origin

5 g sample

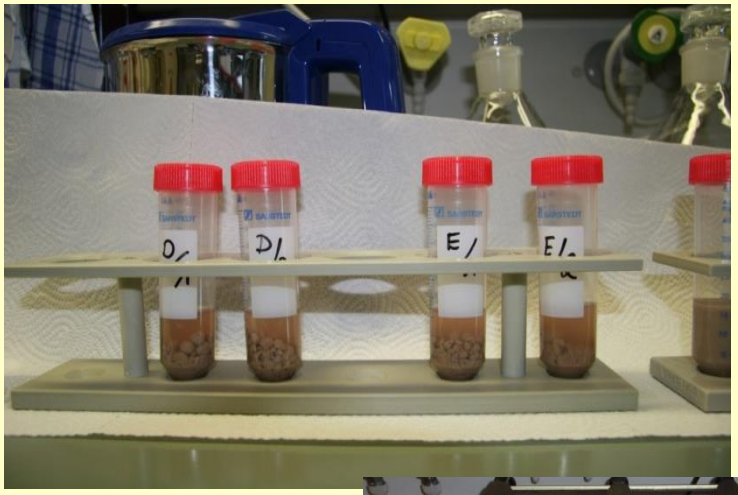
+ Internal Standards
- wait 10 min

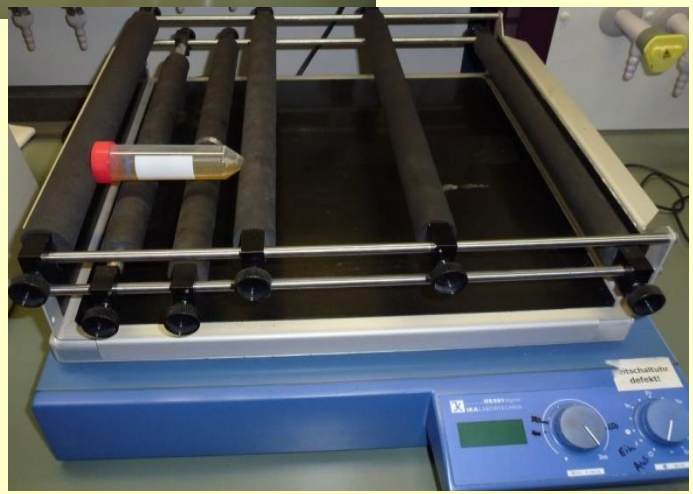
+ 5 ml water
+ citrate buffer salts

- Shake 10 sec

+ 10 ml ethylacetate/ cyclohexane (1+1/v+v)

- Shake 10 min





Less Polar Pesticides in Food of Animal Origin

+ 10 g Na_2SO_4
+ 0.2 g PSA

- Shake 10 min

Centrifuge (2500 g)

Take 5 ml extract

GPC-cleanup

Less Polar Pesticides in Food of Animal Origin

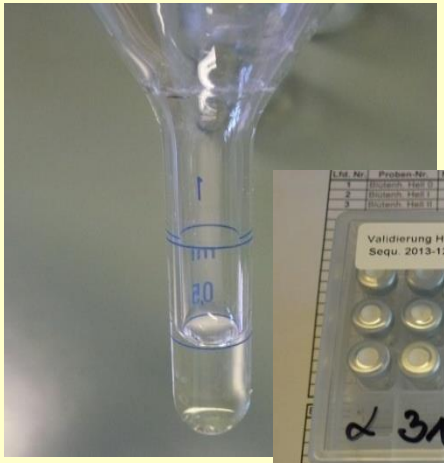
Evaporate solvent

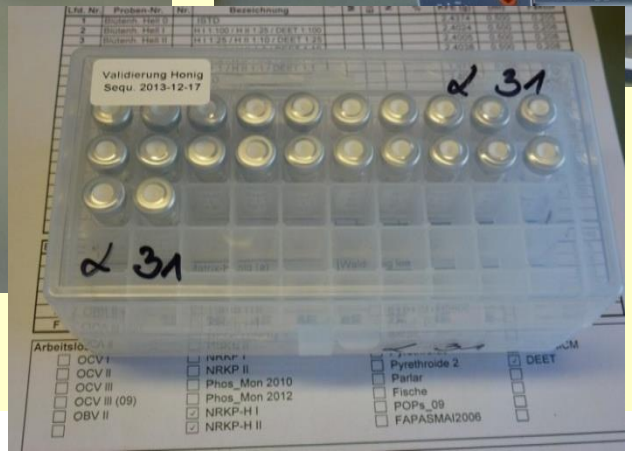
Re-dissolve in
isooctane
silica gel column

Evaporate

Final volume 0.5 mL

GC-MS/MS-Analysis





Less Polar Pesticides in Food of Animal Origin

Extraction procedure, modified

- Mixture of ethylacetate and cyclohexane used for extraction
  - Combination with existing EN 1528 – clean-up approach
  - The extract is ready to use for GPC clean-up
  - No additional dilution - evaporation step after extraction
- Extraction efficient regarding extraction and time (Time reduced to 1.5 - 2 working days per batch)
- Cleanup like in original method -> no improvement

Less Polar Pesticides in Food of Animal Origin

- Z-sep+ was used during the extraction procedure resulting in cleaner extracts (visual control)
- For later tests the z-sep+ was layered over the silica gel after the GPC (it worked!)
- Finally, z-sep+ is used after the GPC

Extracts of liver

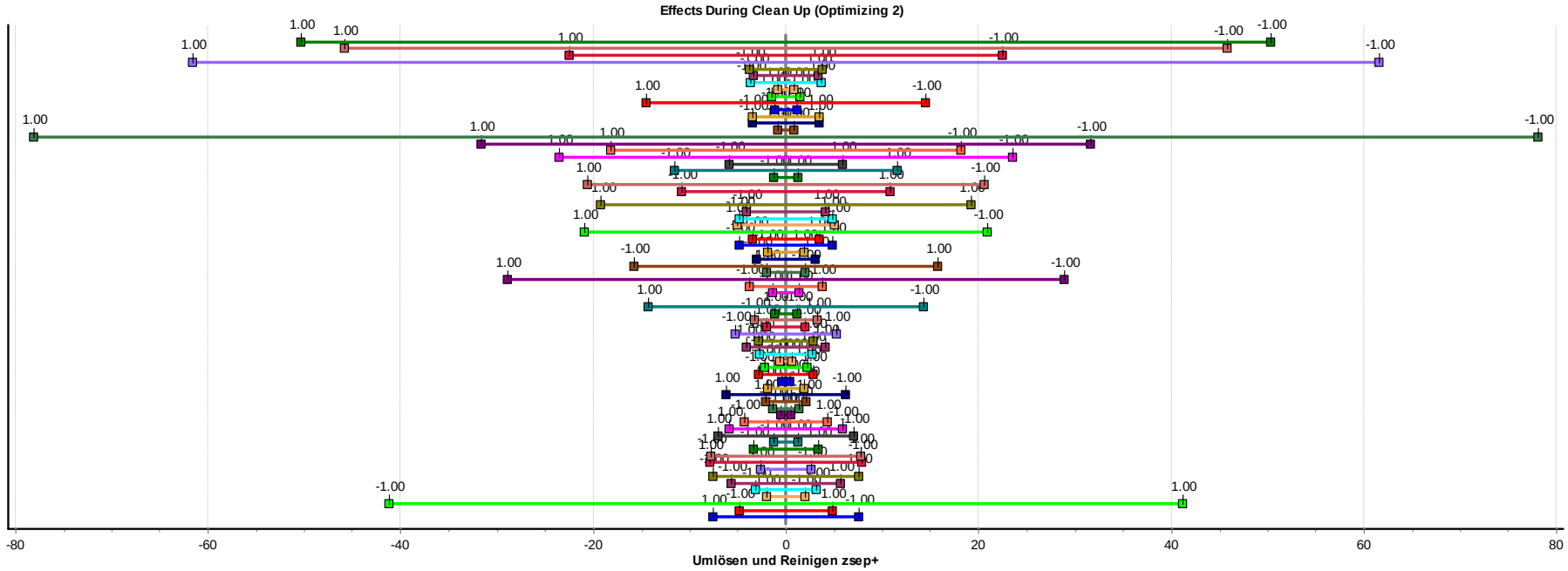
Less Polar Pesticides in Food of Animal Origin

During routine analysis cases with dirty liver extracts occurred

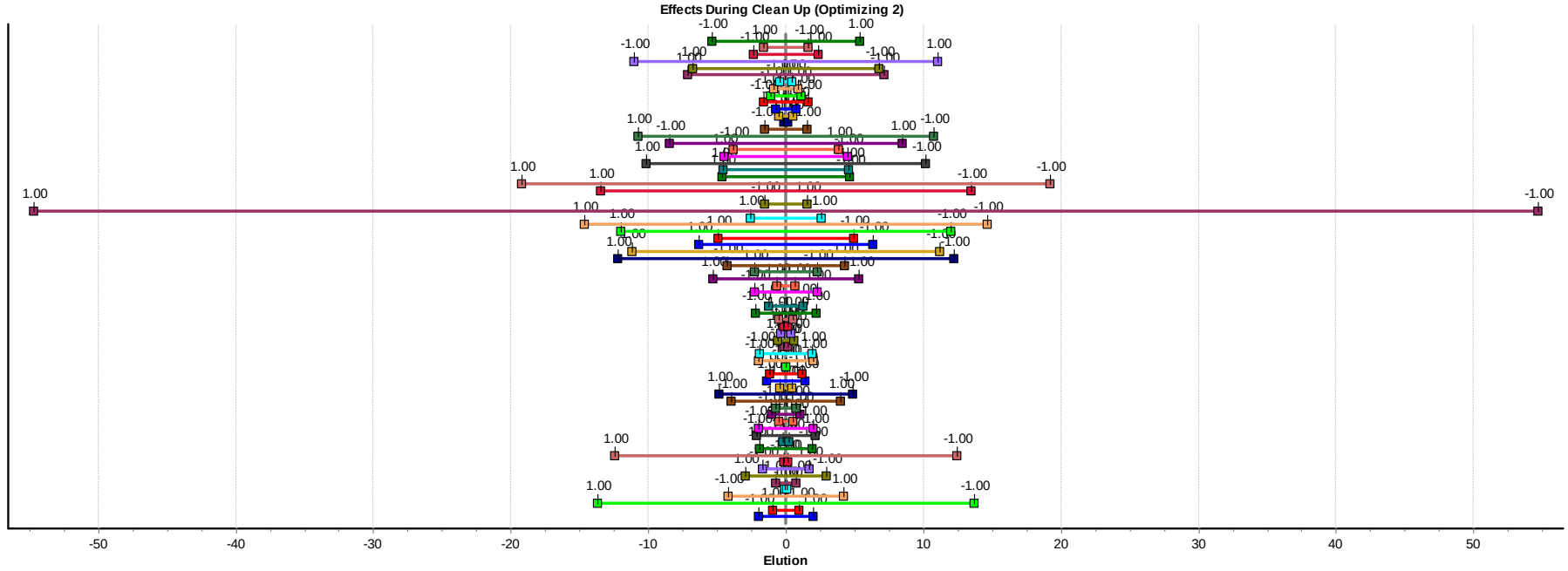
- Working sequential for checking reasons can be very time consuming
- ➢ Designed experiment for checking effects

Run	Matrix	PSA/Na_2SO_4	Elution from Mini Silica Column	Solvent Exchange (zirconium coated materials)
1	+1 (liver)	+1 (yes)	+1 (3 after 2)	+1 (yes, acetonitril)
2	+1 (liver)	+1 (yes)	+1 (3 after 2)	-1 (no)
3	+1 (liver)	-1 (no)	-1 (3 direct)	+1 (yes, acetonitril)
4	+1 (liver)	-1 (no)	-1 (3 direct)	-1 (no)
5	-1 (egg)	+1 (yes)	-1 (3 direct)	+1 (yes, acetonitril)
6	-1 (egg)	+1 (yes)	-1 (3 direct)	-1 (no)
7	-1 (egg)	-1 (no)	+1 (3 after 2)	+1 (yes, acetonitril)
8	-1 (egg)	-1 (no)	+1 (3 after 2)	-1 (no)

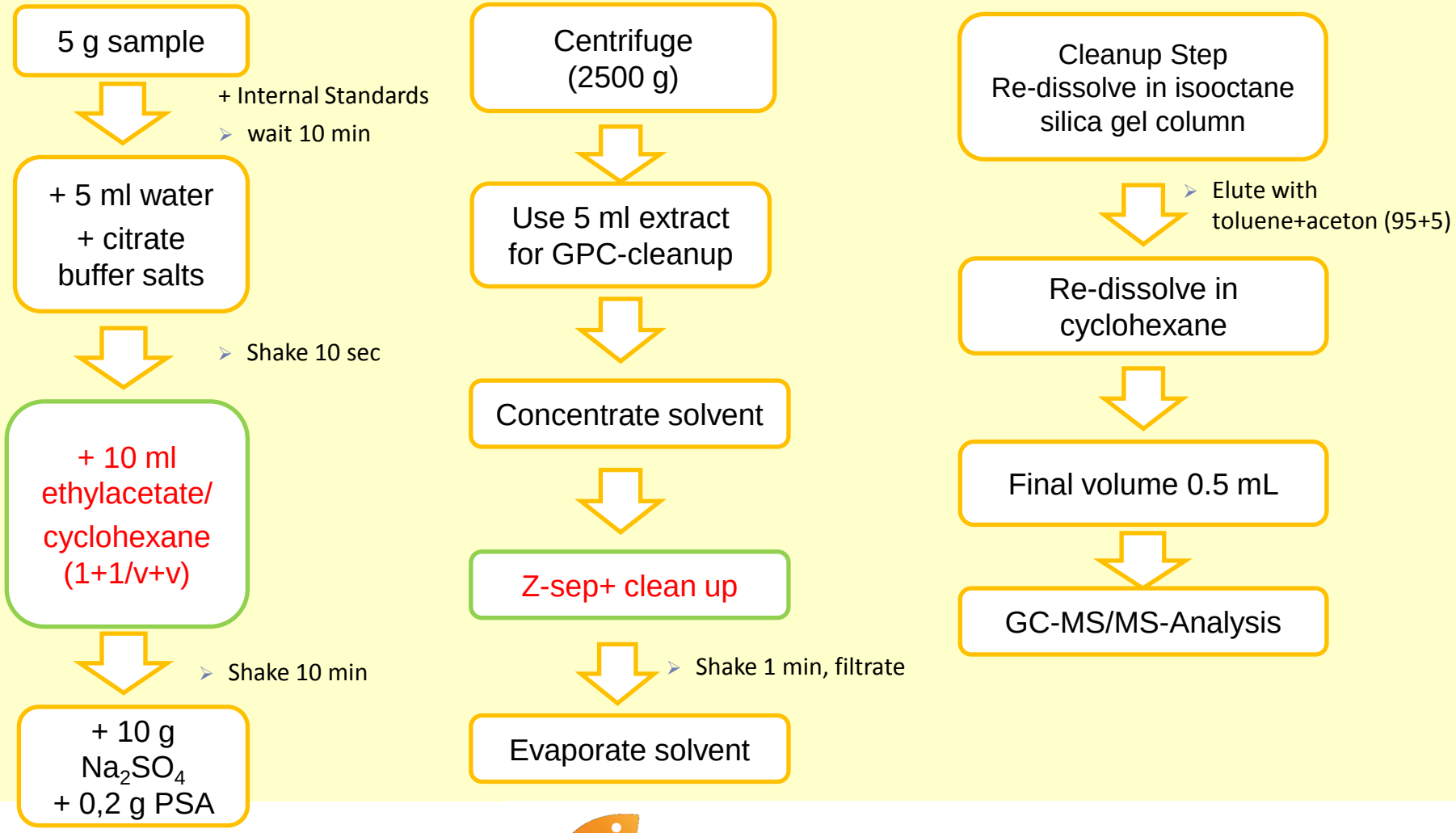
Method for analysing less polar pesticide in food of animal origin - final modifications



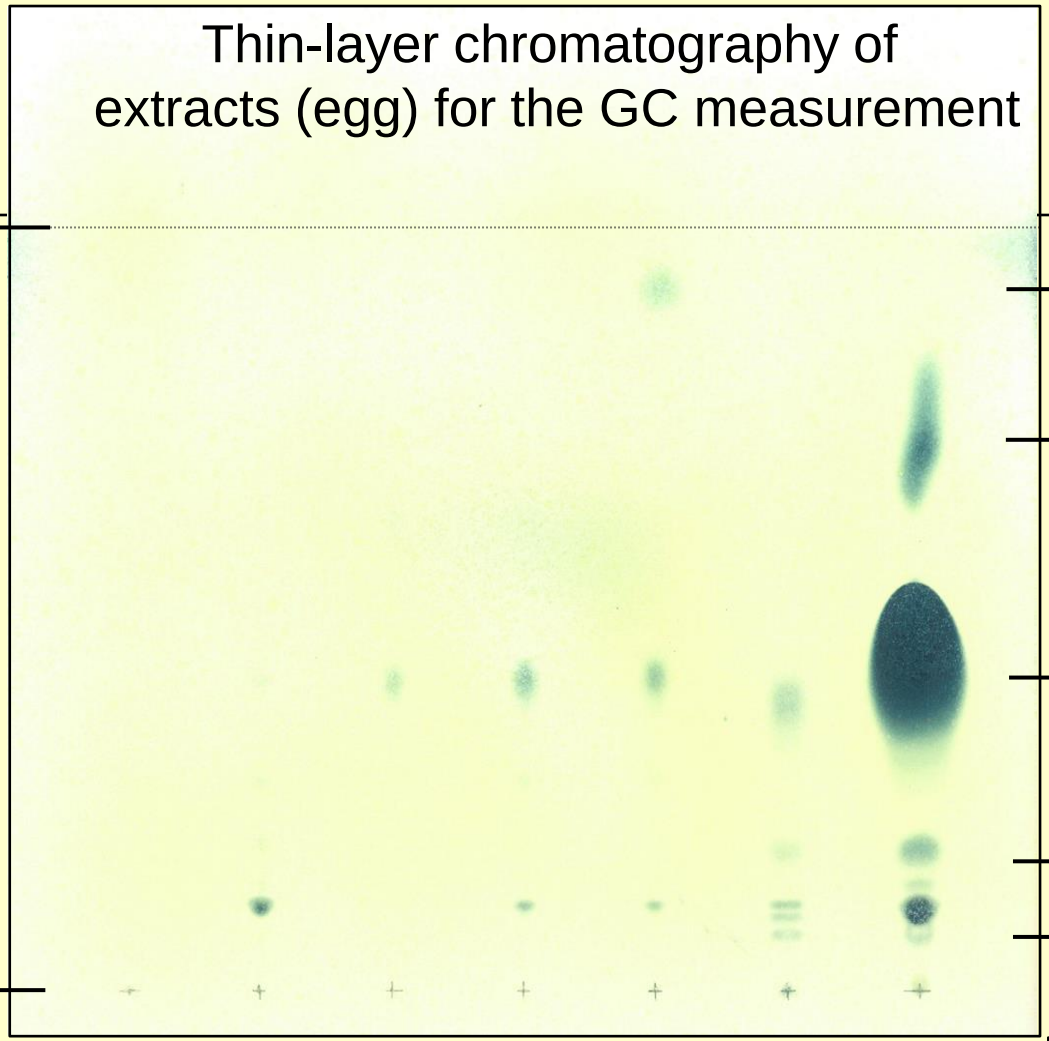
Method for analysing less polar pesticide in food of animal origin - final modifications



Method for analysing less polar pesticide in food of animal origin



Thin-layer chromatography of extracts (egg) for the GC measurement



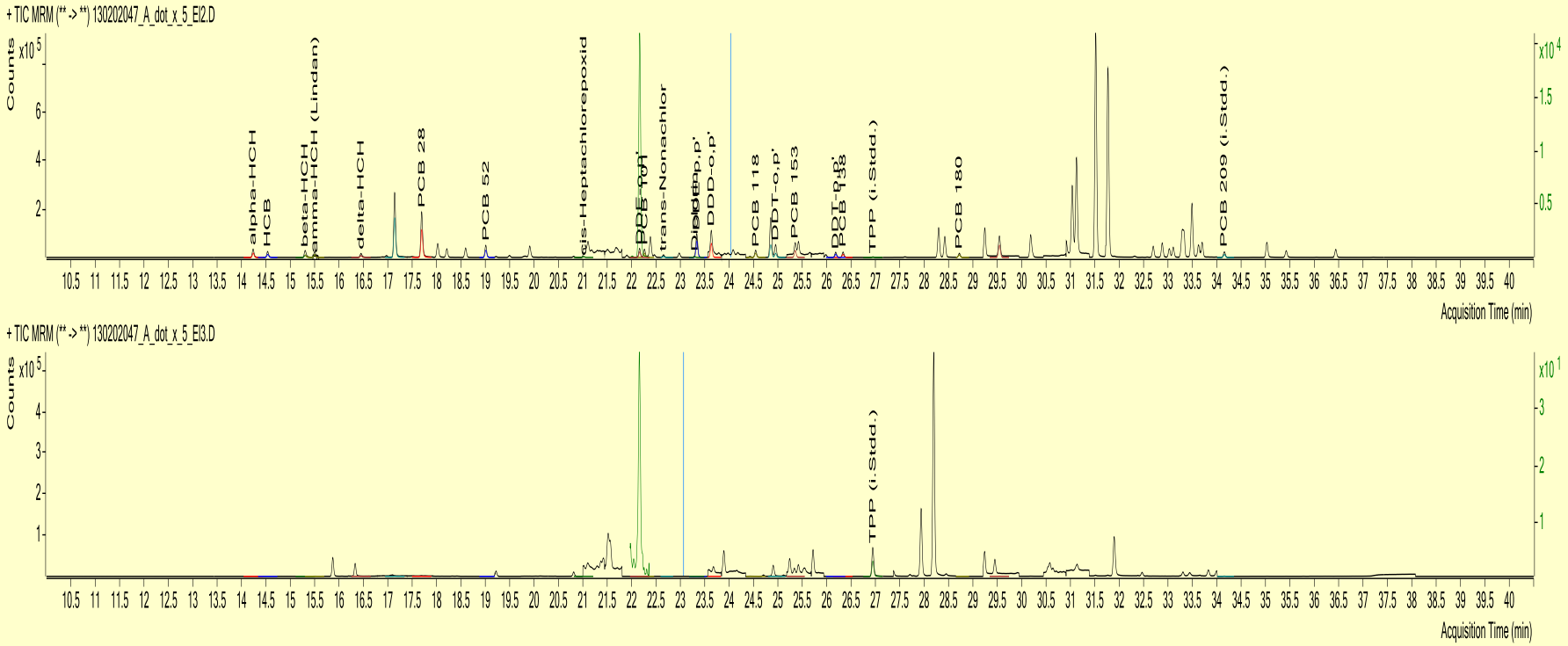
(*) Factor 4 less concentrated than the other extracts

AO:	new EURL AO Methode
El:	Eluate
SG:	Silica Gel
ZrO_2:	Zirconium(IV)-oxid

Analysis of samples

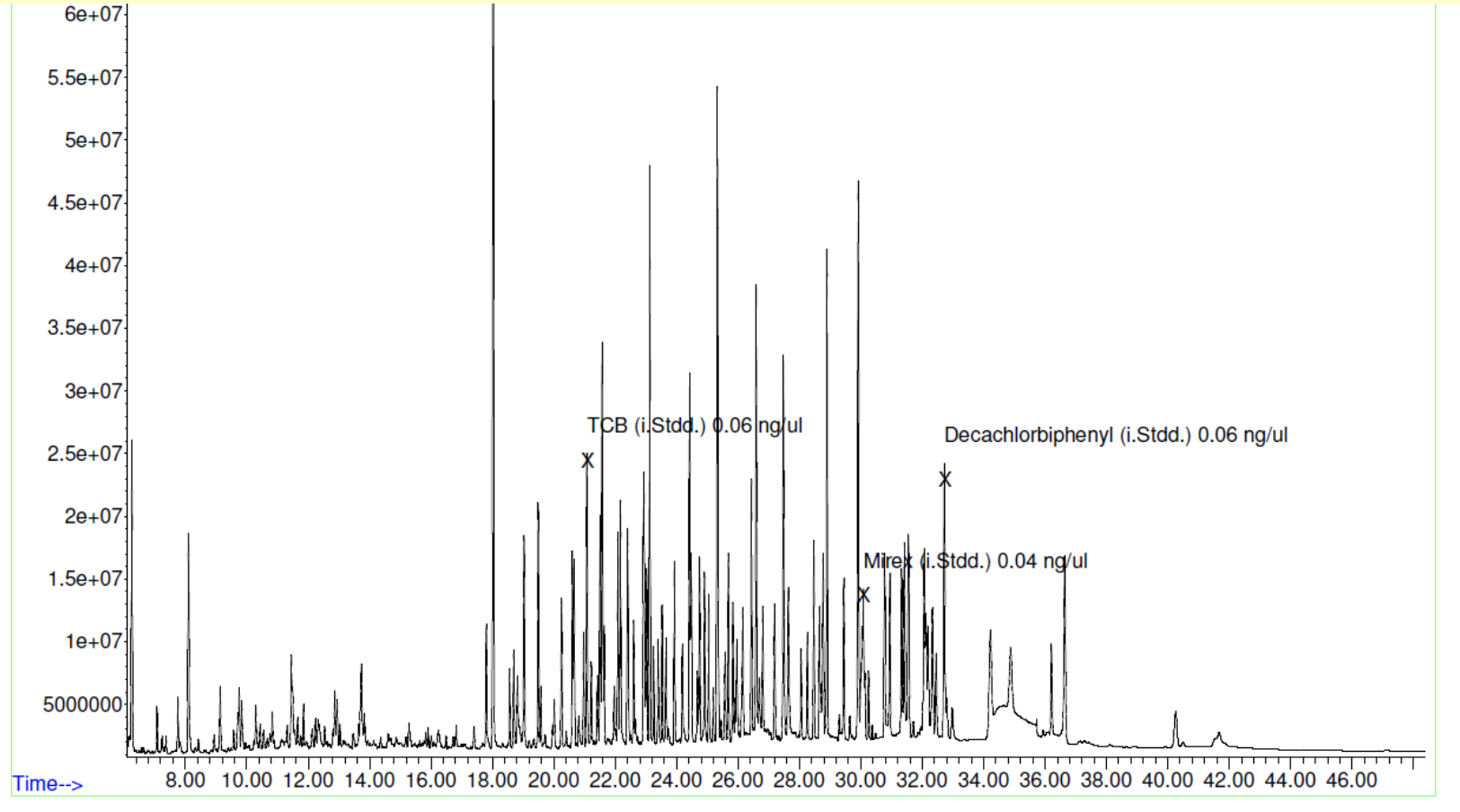
- Use of matrix matched calibration
  (using extracted liver expected to be free of pesticide residues)
- Improvement by using procedural calaibration
  (using blank matrix spiked before the first extraction step)
- Analysis performed using the Agilent 7890A with 7000B QQQ
  („Pesticide Analyzer“)
- Backflush, installed

TIC (MRM; extracts of liver after modifications)



TIC demonstrates the enhanced cleanup of the modified AO method

ECD: extracts of egg



- ECD demonstrates the enhanced cleanup of the modified AO method

Method Validation (liver and egg)

1. **About 60 less polar pesticides and contaminants**

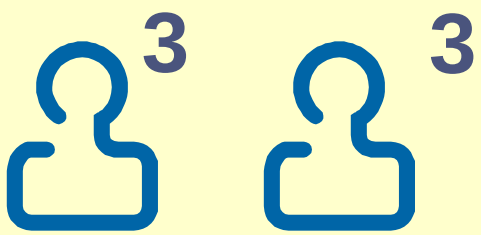
Hexachlorbenzol HCB	Aldrin	Triclosan-methyl
HCH, alpha-	Dieldrin	Pendimethalin
HCH, beta-	Heptachlor	Tecnazen
Lindan gamma-HCH	Heptachlorepoxid, cis-	Quintozen
HCH, delta-	Heptachlorepoxid, trans-	Fenpropathrin
trans-Nonachlor	Endosulfan, alpha-	Lambda-Cyhalothrin,
DDE, op-	Endosulfan, beta-	Cyphenothrin
DDE, pp-	Endosulfan-sulfat	cis-Permethrin
DDT, op-	Chlordan, alpha(cis)-	trans-Permethrin
DDT, pp-	Chlordan, gamma(trans)-	Cyfluthrin,
DDD, op-	Oxychlordan	Cypermethrin,
DDD, pp-	Bromocyclen; Bromodan	Fenvalerat und Esfenvalerat,
PCB 28	S 421	Deltamethrin
PCB 52	Moschus-Xylol	Chlorpyrifos-methyl
PCB 101	Moschus-Keton	Parathion-methyl
PCB 153	Endrin	Pirimiphos-methyl
PCB 138	Delta-Ketoendrin	Chlorpyriphos(-ethyl)
PCB 180	Iodofenphos	Parathion(-ethyl)
PCB 118	Methoxychlor	Pirimiphos-ethyl
	Nitrofen	Fenthion

Method Validation

1. About 60 less polar pesticides and contaminants
2. 4 levels of spiking (plus blank liver)

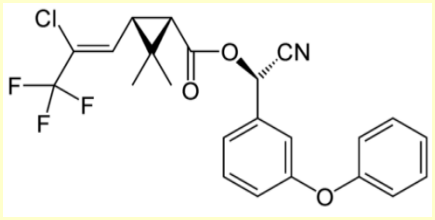
3. **2 different staff for the cleanup procedures**
4. **Each staff with 3 replicates at 4 concentrations**

➔ **6 replicates per level**



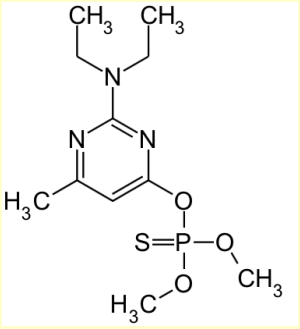
Results Liver (0.1 –8 µg/kg as LOD)

Lambda-Cyhalothrin

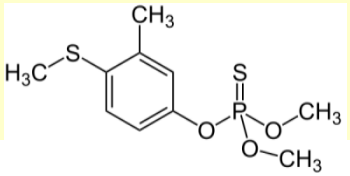


Pirimiphos-methyl

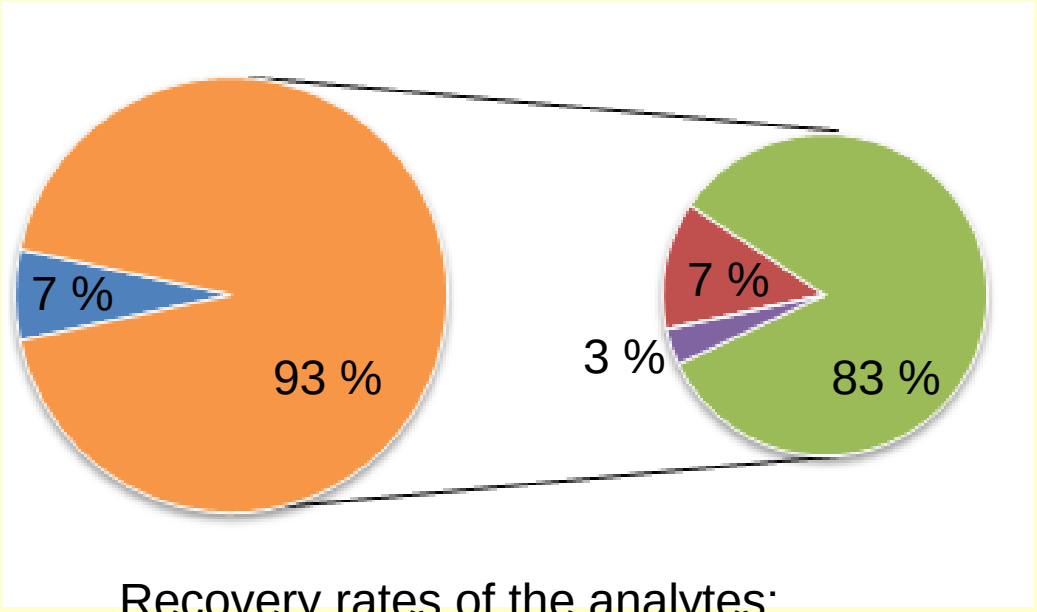
Pirimiphos-ethyl



Fenthion



Matrix matched calibration



Recovery rates of the analytes:

70 - 120 %

< 70 %

100 ± 10 %

70 - 90 %

110 - 120 %

Results Egg (0.1 –10 µg/kg as LOD)

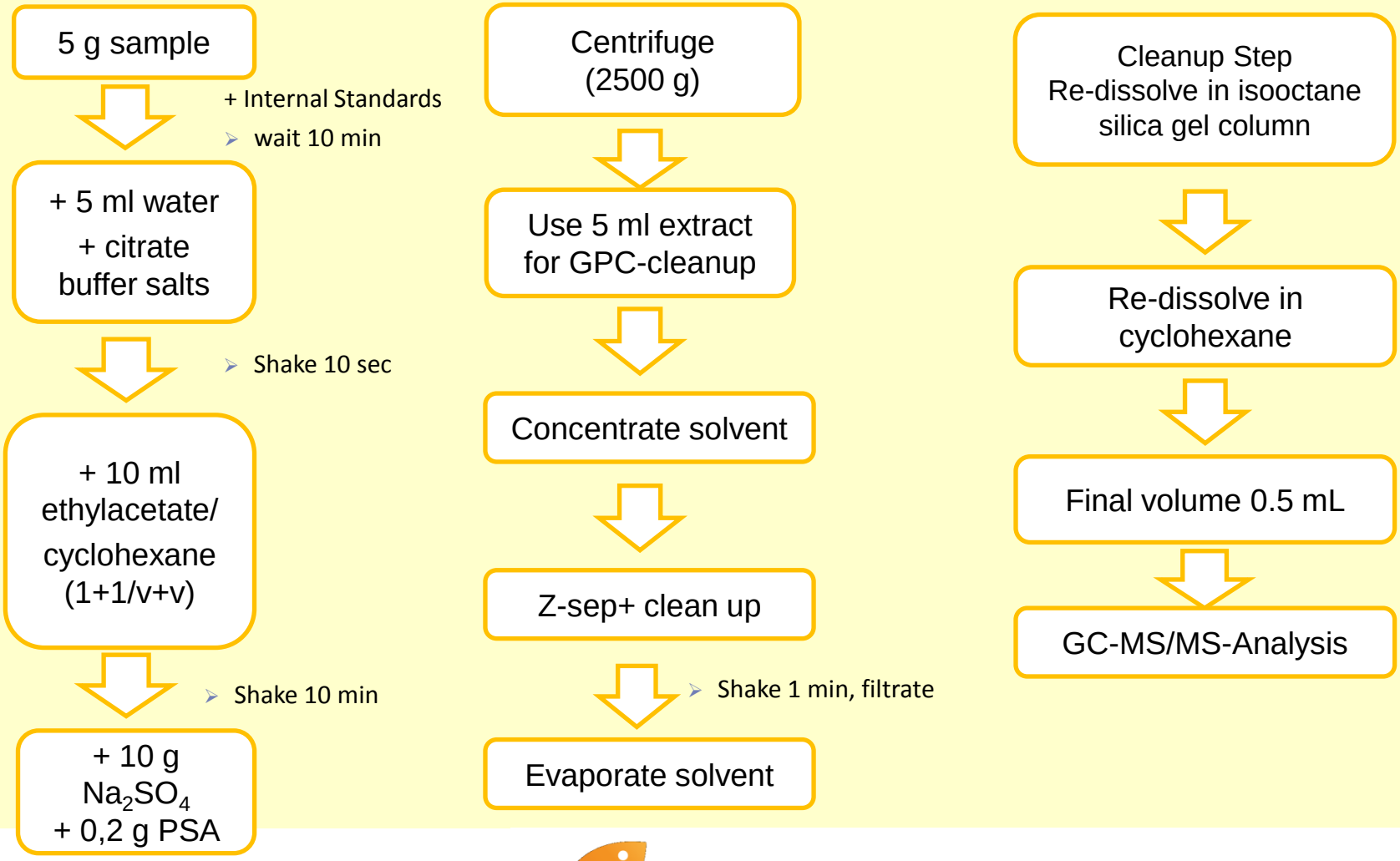
Procedural Calibration

LOD [µg/kg]	Pesticides
0.1	DDE, op-, DDT, op-, DDT, pp-, Heptachlorepoxid, trans-, Quintozen
0.2	Aldrin, Bromocyclen; Bromodan, Chlordan, alpha(cis)-, Chlordan, gamma(trans)-, op-DDD, pp-DDD, delta-Ketoendrin, Dieldrin, alpha-Endosulfan, beta-Endosulfan, Endosulfan sulfat, alpha-HCH, beta-HCH, delta-HCH, cis-Heptachlorepoxid, Hexachlorbenzol, Lindan, Moschus-Xylol, Oxychlordan, PCB 101, PCB 118, PCB 138, PCB 180, PCB 28, PCB 52, Prallethrin, trans-Nonachlor
0.3 – 0.4	Heptachlor, Iodofenphos, pp-DDE, Endrin, Nitrofen, PCB 153, Pendimethalin, Triclosan-methyl, Fenthion-Sulfon
0.5 - 1.0	Azinphos-ethyl, Chlorpyrifos-methyl, Chlorpyriphos(-ethyl), Diazinon, Methidathion, Parathion(-ethyl), Parathion-methyl, Pirimiphos-ethyl, Pirimiphos-methyl, Profenofos, Propetamphos, Pyrazophos, Triazophos, S 421, Fenthion, Piperonylbutoxid, Bifenthrin, Methoxychlor, Moschus-Keton, Fenpropathrin, Fenvalerat –Esfenvalerat (sum), Flucythrinat (2 isomeres)
> 1.0 – 5.0	Methothrin, Chlorbenzilat, Cyfluthrin (total), Cypermethrin (total), Tetramethrin, Cyhalothrin (total), Coumaphos, Allethrin, cis-Permethrin, trans-Permethrin, Cyphenothrin
> 5.0 – 10	Empenthrin, Resmethrin (total), tau-Fluvalinat (2 Isomeres), Pyrethrum Pyrethrine (sum), Deltamethrin*, Phenothrin

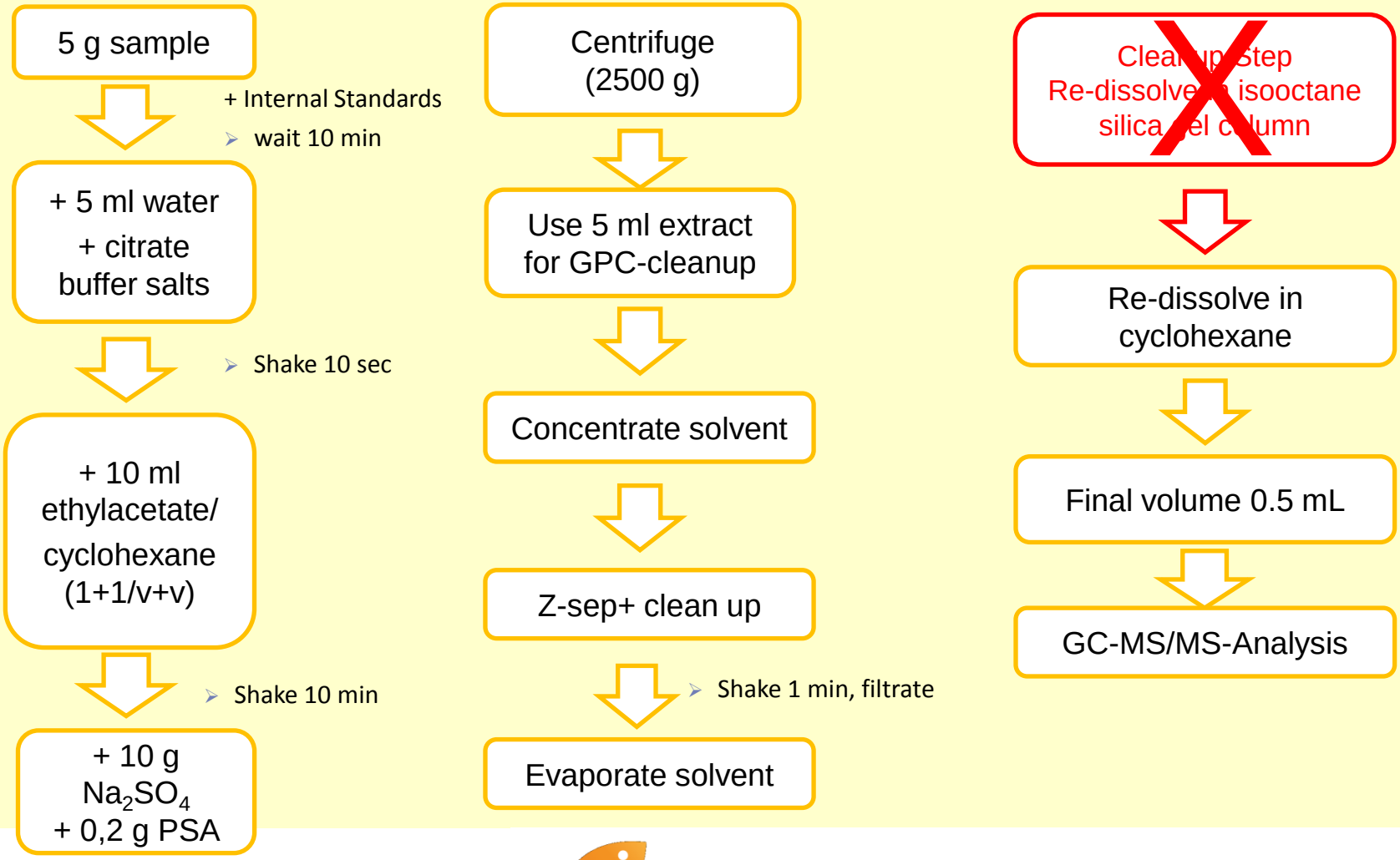
*Includes Tralomethrin

- No final validation: Fenthion-ethyl, Kadethrin, Paraoxon-methyl, Tefluthrin,

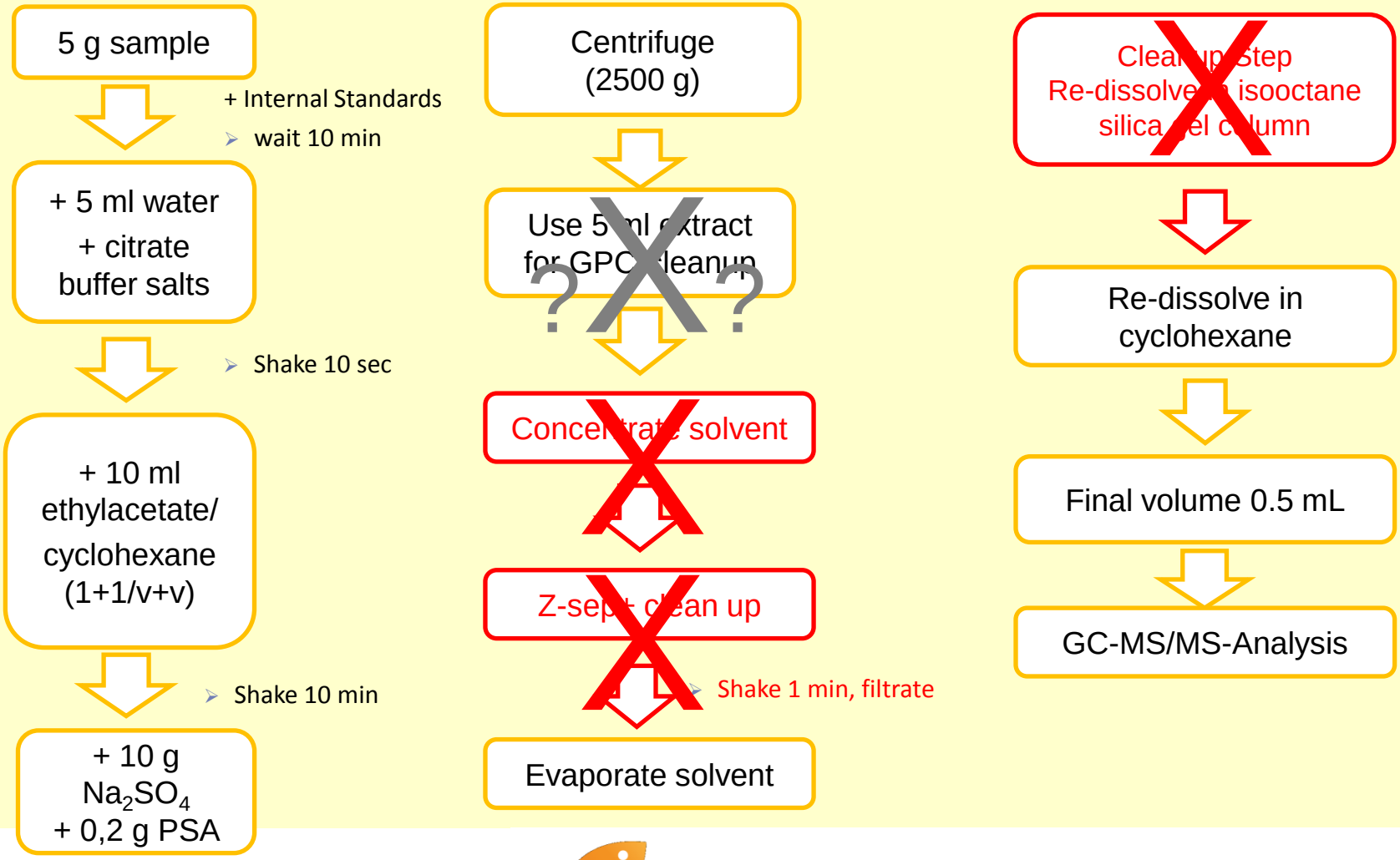
Method for analysing less polar pesticide in food of animal origin: **LIVER**



Method for analysing less polar pesticide in food of animal origin: **Egg, Milk - Babyfood, Muscle**



Method for analysing less polar pesticide in food of animal origin: **HONEY**



Thank you for your attention!

GC-Liner after tests and the liver validation study