Analysis of Pesticide Residues in Food of Animal Origin using GC-MS/MS

Webinar
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Chemisches und Veterinäruntersuchungsamt Freiburg (Germany)
EURL AO Freiburg
CVUA Freiburg Introduction

- Investigation of more than 22000 routine samples
- Residue department responsible
  - for pesticides in food of animal origin,
  - organic contaminants in food and
  - dioxins and PCBs in food and feed
Hosting of 2 European Union Reference Laboratories (EURL)
- EURL for Dioxins and PCBs in Food and Feed and
- EURL for Pesticides in Food of Animal Origin and Commodities with High Fat Content

WHO/UNEP Reference Laboratories for POPs in Human Milk according Stockholm Convention
• EURL Network for Pesticide Residues
  EURL FV Almeria, Spain
  EURL CF Copenhagen, Denmark
  EURL AO, Freiburg, Germany
  EURL SRM, Stuttgart, Germany

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Overview of classical methods for analysing food of animal origin
Analysis of pesticide residues at CVUA Freiburg
Advantages/disadvantages and alternatives
Method development and modification on the classical approach
Validation of honey
Method development for liver and other matrices
Conclusions
## EN 1528 Modular System

<table>
<thead>
<tr>
<th>European Standard</th>
<th>Description</th>
<th>German Standard</th>
<th>Modul</th>
<th>Description</th>
<th>Validated methods of CVUA Freiburg</th>
<th>Samples / Compounds</th>
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<tbody>
<tr>
<td>EN 1528-2: 1996-10 (confirmed 2001)</td>
<td>Extraction of fat, pesticides and PCBs and determination of fat content</td>
<td>§ 64 LFGB: L 00.00-34 (confirmed 1999)</td>
<td>E 8</td>
<td>Extraction of fat with hexan/acetone</td>
<td>PV 31 P01601</td>
<td>meat, fish</td>
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<td>Extraction of fat, pesticides and PCBs and determination of fat content</td>
<td>§ 64 LFGB: L 00.00-38/2 (confirmed 09/1998)</td>
<td>6.1</td>
<td>Extraction of milk</td>
<td>PV 31 P00402, PV 31 P00502</td>
<td>milk</td>
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<td>§ 64 LFGB: L 00.00-38/2 (confirmed 09/1998)</td>
<td>6.2.3</td>
<td>Extraction of butter</td>
<td>SOP 31 S00303</td>
<td>butter</td>
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<td>§ 64 LFGB: L 00.00-38/2 (confirmed 09/1998)</td>
<td>6.3.1 and 6.3.2</td>
<td>Extraction of cheese, dairy and milk powder</td>
<td>PV 31 P00202, PV 31 P00302, PV 31 P00602</td>
<td>cheese, dairy, milk powder</td>
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<td>§ 64 LFGB: L 00.00-38/2 (confirmed 09/1998)</td>
<td>6.4</td>
<td>Extraction of meat, fish and products</td>
<td>PV 31 P00202, PV 31 P00302, PV 31 P01601</td>
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<td>§ 64 LFGB: L 00.00-38/2 (confirmed 09/1998)</td>
<td>6.5</td>
<td>Extraction of eggs</td>
<td>PV 31 P00202, PV 31 P00302, PV 31 P01601</td>
<td>egg</td>
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<td>§ 64 LFGB: L 01.00-8 (confirmed 04/1981)</td>
<td>-</td>
<td>Determination of the fat content of milk</td>
<td>PV 31 P00102</td>
<td>milk</td>
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</tbody>
</table>
Modular System (Meat and Fish, Egg)

- Column extraction
  - extraction in a column using n-hexane/acetone
- Soxhlet extraction
  - solvent light petroleum or diethyl ether
- **Hot solvent extraction**
  - solvent light petroleum or n-hexane/acetone
  - Used at CVUA Freiburg
- Liquid-liquid partition
  - using n-hexane/acetone and sodium sulphate solution
- Centrifugation
  - Cold centrifugation using n-hexane
Extraction: Hot Soxtherm with light petroleum
<table>
<thead>
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<tr>
<td>EN 1528-3: 1996-10 (confirmed 2001)</td>
<td>Clean-up methods</td>
<td>§ 64 LFGB: L 00.00-34 (confirmed 11/1999)</td>
<td>GPC</td>
<td>Gel permeation chromatography</td>
<td>PV 31 P00802</td>
<td>animal fats</td>
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<td>§ 64 LFGB: L 00.00-34 (confirmed 11/1999)</td>
<td>C 2</td>
<td>Adsorption chromatography on a small silica gel column</td>
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<td>§ 64 LFGB: L 00.00-37 and 38/3 (confirmed 09/1998)</td>
<td>11</td>
<td>GPC and adsorption chromatography on a small silica gel column</td>
<td>PV 31 P00802 PV 31 P00902 PV 31 P01002</td>
<td>animal fats</td>
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<td>§ 64 LFGB: L 00.00-12 (confirmed 08/1993)</td>
<td>7.4.2</td>
<td>Clean-up with sulfuric acid</td>
<td>PV 31 P01102</td>
<td>animal fats</td>
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<td>§ 64 LFGB: L 00.00-34 (confirmed 11/1999)</td>
<td>D1 - D4</td>
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<td></td>
<td>GC analysis of organotin compounds in fish and mussels</td>
<td>§ 64 LFGB: L 10.00-9 (confirmed 12/2002)</td>
<td>-</td>
<td>Organotin compounds are transferred into ethyl-compounds and detected by GC-FPD and GC-MS</td>
<td>PV 31 P01401</td>
<td>fish, mussels</td>
</tr>
<tr>
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<td>German Standard</td>
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<td>Description</td>
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<td>§ 64 LFGB: L 00.00-34 (confirmed 11/1999)</td>
<td>D1 - D4</td>
<td></td>
<td>PV 31 P01401</td>
<td>fish, mussels</td>
</tr>
</tbody>
</table>

**Detection**
EN 1528 Part 3: Clean-up

- Method A: Liquid/ Liquid partition with acetonitrile and chromatography on a florisil column
- Method B: Liquid/ Liquid partition with dimethylformamid and chromatography on a florisil column
- Method C: Column chromatography on activated florisil
- Method D: Column chromatography on partially deactivated florisil
- Method E: Column chromatography on partially deactivated aluminium oxid
- Method F: Gel permeation chromatography (GPC)
- Method G: Gel permeation chromatography (GPC) and column chromatography on partially deactivated silica gel
- Method H: High pressure GPC (HPGPC) Used at CVUA Freiburg
Extraction for non-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
Extraction of non-polar pesticides (GC Analysis)

**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**

- 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**
Clean-up for GC-Analysis

- **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**
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

Quantification

Fraction 2

Gas chromatography
3 columns (PS 088, OV 1701, HP 5)
4 detectors (ECD, NPD, MSD, MS/MS)

Fraction 3

4 calibration levels, solvent/matrix calibration, internal Standards

Evaluation using ISTD

all according EN 1528
Examples for Analytes

- DDE, p,p-
- beta-HCH
- Hexachlorobenzene
- Bifenthrin
- gamma-HCH
- Endosulfansulfate
- Diazinon
- Deltamethrin
- alpha-HCH
- Chlordane
- Parathion

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Sample chromatogram on GC-ECD (EN 1528)

Exctract according to EN 1528
(Test Item of EUPT AO 07)
PS 088; GC-ECD
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 series), esp. extraction step
- High costs for glassware und man power

- How to increase the efficiency (saving time and money)?
Possible alternatives?

QuEChERS
SweEt

- Quick methods with fast extraction and clean-up steps
- Established for pesticides residues in fruit and vegetables
- Low solvent consumption per sample (~10 mL)
- In case of food of animal origin established for more polar pesticides detected with LC-MS/MS
- Co-extracted fat are a possible problem in GC analysis
- Low recoveries for non-polar pesticides (using QuEChERS)
SweEt Method

- Developed by Andersson and Ohlin (1989)
- Modified and miniaturized
- Simplified method published 2007

J. Redeby: Presentation at EURL Workshop 2011 (Freiburg)

![Diagram of SweEt Method](attachment:diagram.png)
<table>
<thead>
<tr>
<th></th>
<th>QuEChERS</th>
<th>SweEt</th>
<th>En 1528 used at CVUA Freiburg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample weight</strong></td>
<td>5 g</td>
<td>5 g</td>
<td>5 g</td>
</tr>
<tr>
<td><strong>Extraction</strong></td>
<td>10 mL Acetonitrile</td>
<td>10 mL Ethylacetate</td>
<td>Mixing with 50 g sodium sulfate</td>
</tr>
<tr>
<td></td>
<td>Citrate buffer</td>
<td>Sodium sulfate</td>
<td>Extraction with petroleum ether for 2 h</td>
</tr>
<tr>
<td></td>
<td>MgSO₄</td>
<td>PSA</td>
<td>Filtration, removal of solvent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C₁₈</td>
<td>Determination of fat content</td>
</tr>
<tr>
<td></td>
<td>10 min shaking</td>
<td>30 min shaking</td>
<td>Redissolving of whole fat or aliquot (0.5 g fat)</td>
</tr>
<tr>
<td></td>
<td>6 min centrifugation</td>
<td>6 min centrifugation</td>
<td>Preparing for GPC with ethylacetate/cyclohexane</td>
</tr>
<tr>
<td></td>
<td>(≥ 2500 g)</td>
<td>(≥ 2500 g)</td>
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<tr>
<td><strong>Clean-up</strong></td>
<td>6 mL supernatant:</td>
<td>Filtrate</td>
<td>GPC</td>
</tr>
<tr>
<td></td>
<td>MgSO₄</td>
<td></td>
<td>Removal of solvent</td>
</tr>
<tr>
<td></td>
<td>PSA</td>
<td></td>
<td>Preparation of silica gel columns (conditioning)</td>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>10 min shaking</td>
<td></td>
<td>Sampling of fractions</td>
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<tr>
<td></td>
<td>6 min centrifugation</td>
<td></td>
<td>Removal of solvent</td>
</tr>
<tr>
<td></td>
<td>(≥ 2500 g)</td>
<td></td>
<td>0.5 mL final volume</td>
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<tr>
<td><strong>Detection</strong></td>
<td></td>
<td>GC or LC detection system</td>
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Reproducibility:
GC-MSMS of QuEChERS extracts

Matrix: cream (frozen extract)
10 Injections

<table>
<thead>
<tr>
<th>Injection</th>
<th>Hexachlorobenzene</th>
<th>Parathion-methyl</th>
<th>Chlorpyrifos</th>
<th>Endosulfane sulfate</th>
<th>DDT-p,p'</th>
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<tbody>
<tr>
<td>1</td>
<td>7302</td>
<td>25194</td>
<td>17751</td>
<td>5904</td>
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<td>7852</td>
<td>32962</td>
<td>20389</td>
<td>5957</td>
<td>75251</td>
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</tbody>
</table>

Mean: 7507 30898 19837 6081 71326
Standard-deviation: 652 3348 1824 570 6815
CV [%]: 8,7% 10,8% 9,2% 9,4% 9,6%

No effects observable

Data obtained from test measurement on Agilent GC-MSMS
Reproducibility: GC-MSMS of QuEChERS extracts

Matrix: cream (frozen extract)  
10 Injections

<table>
<thead>
<tr>
<th>Injection</th>
<th>Deltamethrin</th>
<th>Fenvalerate I</th>
<th>Fenvalerate II</th>
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<td>1</td>
<td>595</td>
<td>14625</td>
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<td>2</td>
<td>534</td>
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<td>208</td>
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<td>4113</td>
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<td>191</td>
<td>5426</td>
<td>3479</td>
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<tr>
<td>6</td>
<td>192</td>
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<td>138</td>
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<tr>
<td>10</td>
<td>132</td>
<td>4283</td>
<td>1382</td>
</tr>
</tbody>
</table>

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
GC-MSD: extracted matrix of animal origin

- Extracts are not clean enough with "normal" QuEChERS approach
- Removal of fat residues is an important task, otherwise the chromatography of the system gets lost

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Modification of existing method for pesticide residues

- Modification of the extraction procedure
- Ethylacetate like in SweEt method approach, first
- Mixture of ethylacetate and cyclohexane used for extraction
  - Combination with existing EN 1528 – clean-up approach
  - The extract should be ready to use for clean-up
  - No evaporation step after extraction
- GPC cleanup
- Optional silica gel cleanup for difficult matrices
- Starting with matrix honey
Modification of existing method for pesticide residues

- 5 g sample (honey)
- Addition of Internal Standards
- Wait 10 min
- + 5 ml water + citrate buffer salts (as in QuEChERS)
- Shake 10 sec
- + 10 ml ethylacetate/cyclohexane (1:1)
- Shake 10 min

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Modification of existing method for pesticide residues

+ 10 g Na₂SO₄
+ 0.2 g PSA

Shake 10 min

Centrifuge (2500 g)

Take 5 ml extract

Proceed with GPC
Modification of existing method for pesticide residues

Evaporate solvent

Optional Cleanup Step (difficult matrices)
- Re-dissolve in isooctane
- silica gel column

Re-dissolve in cyclohexane

Final volume 0.5 mL

GC-MS/MS-Analysis
Analysis of samples

- Use of matrix matched calibrations (using extracted honey expected to be free of pesticide residues)
- Analysis performed using the Agilent 7890A with 7000B QQQ (Pesticide Analyzer)

- Validation requirements*:
  - Recovery 70-120 %
  - Coefficient of variation (CV) < 20 %
  - Blank < 30% LOQ
  - 5 replicates per level

*Document SANCO 12751/2013
GC-MS/MS Pesticide Analyzer 40 minute CF Method + 1 m Pre-Column

(1) + (2) : Retention gap, 1 m x 0.25 mm id UCDFS Retention Gap + 15m x 0.25mm ID x 0.25um HP-5MSUI (19091S-431UI)

(3) : 15m x 0.25mm ID x 0.25um HP-5MSUI (19091S-431UI)
Mid-column, post run back flush

During the run, $P_1 > P_2$, Carrier gas flows towards MS
During the run, $P_1 > P_2$, Carrier gas flows towards MS.
During post run back flush $P_1 < P_2$, Carrier flow reversed in column
The Most Comprehensive and Flexible MRM database

- **1000+ Pesticides & Environmental Pollutants**

<table>
<thead>
<tr>
<th>Compound Classification</th>
<th>Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticides (fungicides, herbicides, insecticides, rodenticides and others)</td>
<td>675</td>
</tr>
<tr>
<td>Breakdown Products</td>
<td>42</td>
</tr>
<tr>
<td>Deuterated Compounds</td>
<td>6</td>
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<tr>
<td>Polybrominated Diphenyl Ether (PBDE)</td>
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<tr>
<td>Polybrominated Biphenyl (PBB)</td>
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<tr>
<td>Polychlorinated Biphenyl (PCB)</td>
<td>209</td>
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<tr>
<td>Polycyclic Aromatic Hydrocarbon (PAH)</td>
<td>26</td>
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<tr>
<td>Phthalates</td>
<td>17</td>
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<tr>
<td>Additional Semi-volatile Pollutants</td>
<td>94</td>
</tr>
</tbody>
</table>

- Average of 8 MRM transitions for each compound (Total > 8500 transitions)
  - provides alternatives to avoid matrix interferences
  - includes relative intensities for all transitions
Pesticide Analyser with MRM database

- Database containing > 1000 pesticides and environmental pollutants
- Specific mass transitions and intensities especially for Agilent GC-MSMS
- Selection of the best transitions, no further tuning necessary
- Inclusion of other missing analytes possible (they have to be tuned of course!)

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MRM database additions of EURL AO

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRN</th>
<th>I/N</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triadimefon</td>
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<td>67</td>
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<tr>
<td>Uniconazole</td>
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<td>Uniconazole-P</td>
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<td>Vardenafil</td>
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Mass Hunter Software
## Honey Results 1

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38 / Pesticides using GC-MS/MS
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# Honey Limit of Quantification (LOQ)

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# Honey Limit of Quantification (LOQ)

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Method for analysing pesticide residues in liver

- 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
Method for analysing pesticide residues in liver

- + 10 g Na₂SO₄
- + 0.2 g PSA
  - Shake 10 min

- Centrifuge (2500 g)

- Take 5 ml extract

- GPC-cleanup

+ 10 g Na₂SO₄
+ 0.2 g PSA
+ 0.5 g zirconium salt
Method for analysing pesticide residues in liver

1. Evaporate solvent
2. Cleanup Step (difficult matrices)
   Re-dissolve in isooctane
   silica gel column
3. Re-dissolve in cyclohexane
4. Final volume 0.5 mL
5. GC-MS/MS-Analysis
Method for analysing pesticide residues in liver final modification

- Zirconium salt was used during the extraction procedure resulting in cleaner extracts (visual control)
- As final modification the zirconium salt was layered over the silica gel
- With this modification the results for a selection of 70 pesticides and contaminants were very good
- The method has to be validated according to SANCO/12571/2013 (matrix matched calibration)
Residues in liver (final modification)

1. Evaporate solvent
2. Cleanup Step (difficult matrices)
   - Re-dissolve in isooctane
   - silica gel column with zirconium salt layer
3. Re-dissolve in cyclohexane
4. Final volume 0.5 mL
5. GC-MS/MS-Analysis
TIC (MRM; extracts of liver according EN 1528)

- Non sufficient cleanup
- matrix effects caused by co-extractives from liver matrix
- Evaluation of single substances possible
- Non applicable for multi component analysis caused by shifting retention times
TIC (MRM; extracts of liver with modifications)

- TIC (MRM) demonstrates the better cleanup of the modified method
- Results against standards in extracted lard matrix
  - Recovery rates for majority of analytes (> 80%) between 70-120%
  - Matrix effects occur for the rest of analytes
- Validation experiments to be evaluated against standards in liver extracts
A method was established and validated for the matrix honey derived from SweEt extraction and modules of EN 1528

The limits of quantification on a GC-MS/MS-system are comparable to the original EN 1528 based method

For difficult matrices (e.g. liver) additional cleanup steps can be used (e.g. mini silica gel column, zirconium salt)

The scope of the method will be enlarged to other matrices
  - Meat
  - Egg
  - Milk

For quantifying the use of matrix matched calibration is mandatory
Thank you for your attention!

GC-Liner after the validation study