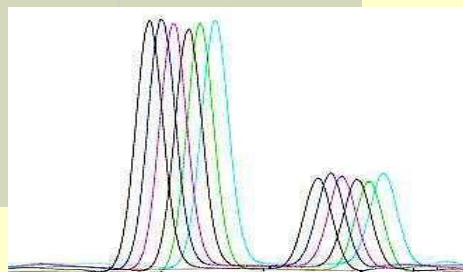
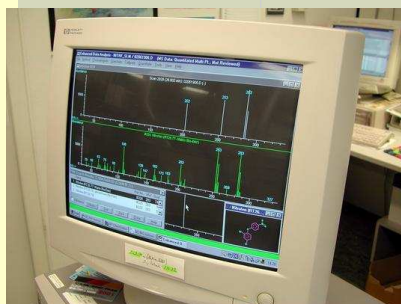
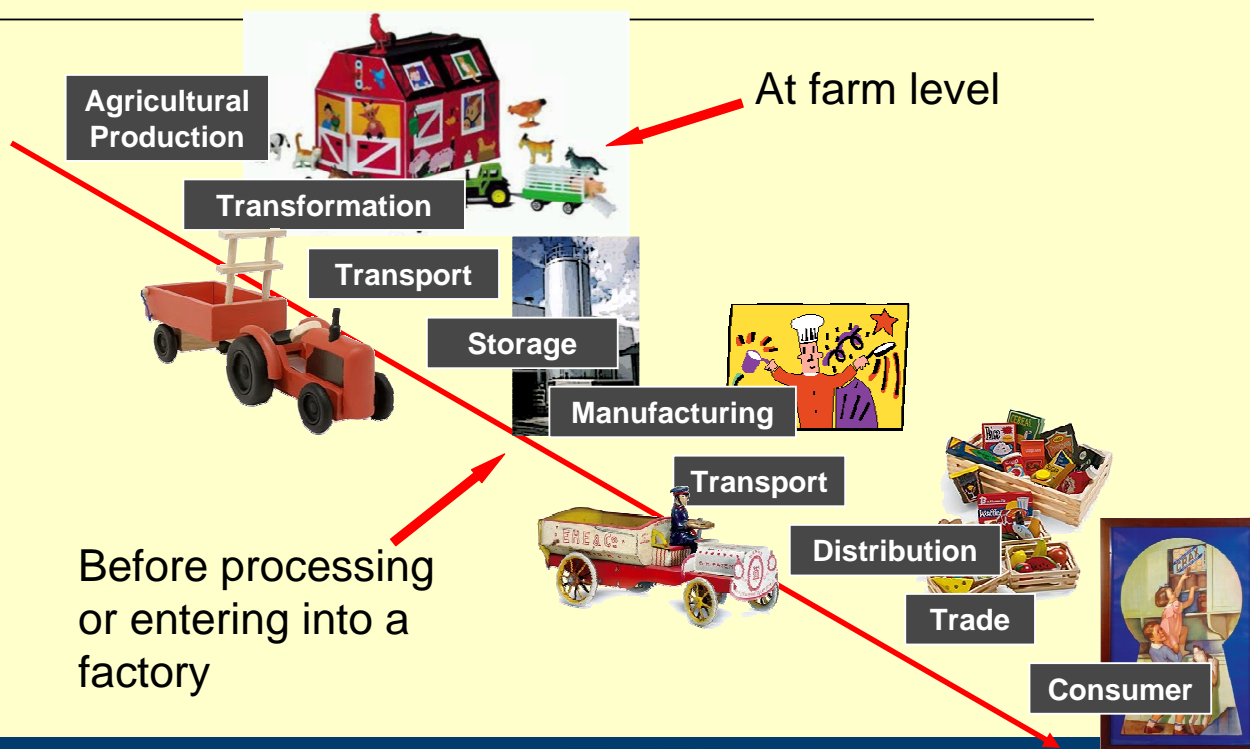


# Suitable methods and analytical scope for veterinary drug residue analysis

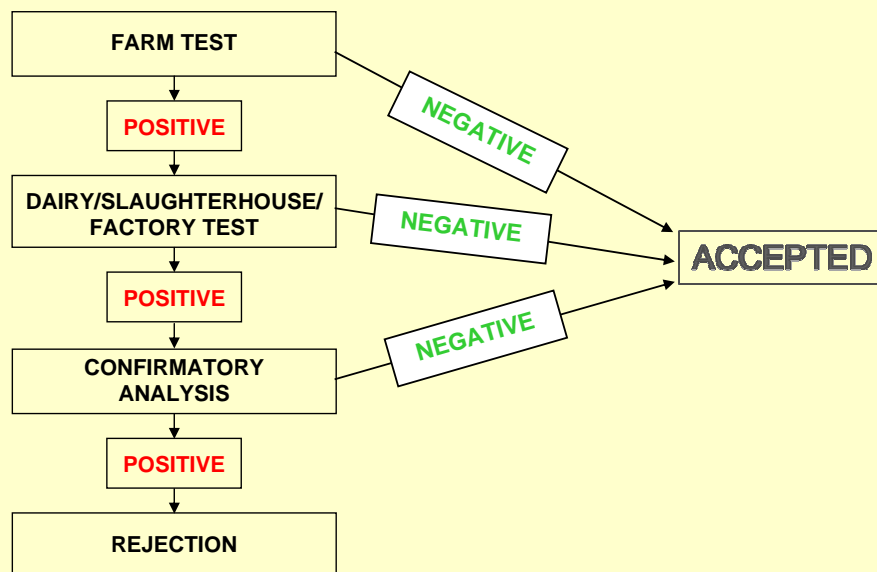


Ralf Lippold

## Where to test raw material ?



## Where to take samples for testing ?



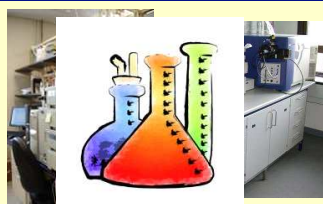
## Veterinary Drugs

- Within the National Residue Control Programme about 260000 samples are analysed annually
- The spectrum analysed is wide:
  - Antibiotics (e.g.  $\beta$ -lactames, sulfonamides, tetracyclines, aminoglycosides, lincomycines, macrolides, chinolones.....),
  - Forbidden substances (chloramphenicol, nitrofurane metabolites, nitroimidazoles, thyreostatica,  $\beta$ -agonists, hormones)
  - Non steroidal anti-inflammatory drugs,
  - Anthelmintics (benzimidazoles, avermectines)

# Results

- Findings are very rare:
  - During the past years some few chloramphenicol results, but (nearly) all of them were contaminations (no metabolites were found)
  - Avermectines (in less than 0.1 % of the samples)
  - Ivermectine (not allowed for application to milk cows)
  - Moxidectine (allowed, MRL = 40 µg/kg)
  - More frequently tetracyclines in tissues of cattle and pig
  - Occasionally malachite green in fish
  - Pen-Strep: findings of Pen G and streptomycin in combination

## Costs of the analyses



### Multi methods

MS(/MS) analyses:  
100 - 200 Euros/sample



### ELISA tests

ELISA screening analyses:  
20 - 30 Euros/sample



### Rapid methods

Rapid screening tests  
3 - 10 Euros/sample

# Requirements for Screening Tests

---

Cheap  
Rapid  
Selective  
Sensitive  
Easy to use  
Low false positive rate  
False negative rate < 5 %

# Type of screening techniques available

---

Immuno assays (ELISA)  
Radio immuno assays  
Strip tests  
Charm II  
Bacterial growth  
Biosensors

GC or LC combined with Screening-MS  
(MSD, TOF or Orbitrap)

## Screening Methods – Use in Dairy Industry

- Only occasional treatments of animals with antibiotics to cure various diseases
  - Risk of residues in animals due to these treatments is low
- Main applications are the treatment of mastitis and the drying off
  - Using intramammary drugs
  - High risk of residues in milk esp. by mis-application of injectables (Ceftiofur in muscle: withdrawal time 0  
Ceftiofur in udder: withdrawal time 96 hours!)
  - Withdrawal time for mastitis treatment between 4 and 7 days
  - Withdrawal time for dry cow therapy: 8 weeks
- Composition of Injectors
  - 83 % of the contain  $\beta$ -lactams or cephalosporines
  - Some contain tetracyclines

## Screening Methods – Use in Dairy Industry

- To minimize risks in dairy industries comprehensive testing with screening methods was introduced by the industry
- Taking samples while collecting the milk at farms by the drivers
- Each van coming in to dairy is tested
  - for inhibitors (using bacterial growth inhibition test, typically based on bacillus stearothermophilus) and
  - for the content of cells (indicator for mastitis)
- In case of findings the individual farm milk is tested
  - Price for milk is based on the quality (no inhibitors, low cell level)
- Good self regulating system of dairy industry
- Milk is the best tested food besides drinking water in Germany

# Screening Methods for Milk

Literature:

Current situation & compilation of commercially available screening methods for the detection of inhibitors/antibiotic residues in milk: Bulletin of the International Dairy Federation 442/2010 (March 2010);

- Description of 62 different screening methods including their detection limits for individual antibiotics

Costs 94 Euro as electronic version

Additional information under [www.fil-idf.org](http://www.fil-idf.org)

## Charm II – available assays

	<i>Gentamycin type</i>	<i>Streptomycin type</i>	<i>Amphenicols</i>	<i>Beta-lactams</i>	<i>Macrolides</i>	<i>Sulfonamides</i>	<i>Tetracyclines</i>	<i>Nitrofurans</i>	<i>Novobiocin</i>
Tissue	X	X	X	X	X	X	X		
Liver	X	X	X	X	X	X	X		
Egg	X	X	X	X	X	X	X		
Honey	X		X	X	X	X	X	X	
Milk	X	X	X	X	X	X	X		X
Farmed fish	X	X	X	X	X	X	X		
Seafood	X	X	X	X	X	X	X		
Cooked meat	X	X	X	X	X	X	X		
Feed	X	X	X	X	X	x	x		

# Validation of qualitative screening: concept

## Initial validation:

To be performed for each vet. drug, for each combination of commodity and species

### meat

red meat (beef, pig..), white meat (poultry), fish (cod, haddock, salmon)

### milk / dairy products

milk, butter, cheese, yoghurt, cream

### eggs

chicken, Ducks, quail

### honey

honey

meat

dairy

egg

20 different products of each group

- free of veterinary drugs (blanks)
- spiked at anticipated SRL (CC $\beta$ )



## On-going AQC:

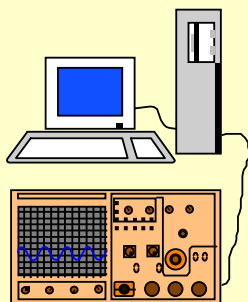
During routine analysis:

- spike 1 or more routine sample(s) with vet drugs at SRL (CC $\beta$ , e.g. in rolling program)

Slide based on Hans Mol, Rikilt

## DoE – Design of Experiments

**Development and optimizing analytical methods using designed experiments**



**Use of special software is mandatory**

**The Unscrambler (Camo)**

**Optival (quodata)**

## Multi Method for Honey

---

### Studie of Literature <--> internal methods

Kaufmann A, Roth S, Ryser B, Widmer M: "Quantitative LC-MS/MS Determination of Sulfonamides and Some Other Antibiotics in Honey" in Journal of AOAC International 85 853 (2002)

Kaufmann A, Känzig A: "Contamination of Honey by the Herbicide Asulam and it's antibacterial active metabolite sulfanilamide" in Food Additives and Contaminants 21 564 (2004)

## Drugs covered by the method, described

---

**Amphenicoles:** Chloramphenicol, Thiamphenicol

**Sulfonamides:** Sulfacetamid, Sulfachinoxalin, Sulfachloropyridazin, Sulfa-diazin, Sulfadimethoxin, Sulfadimidin, Sulfadoxin, Sulfaguanidin, Sulfaisoxazol, Sulfamerazin, Sulfamethoxazol, Sulfamethoxypyridazin, Sulfamonomethoxin, Sulfamoxol, Sulfanilamid, Sulfapyridin, Sulfathiazol

**Tetracyclines:** Chlortetracyclin, Demeclocyclin, Doxycyclin, Oxytetracyclin, Tetracyclin

**Sonstiges:** Asulam, Trimethoprim, (Fluorochinolones)

**Internal Standards:** d5-Chloramphenicol, Minocyclin, Dx-Sulfonamids

Hydrolysis of honey using hydrochloric acid

Duration of hydrolysis: 60 min

Stop of hydrolysis using citric acid

Filter

Aliquotieren, pH-Wert auf  $4 \pm 0,5$  einstellen

Without delay: cleanup using Oasis HLB

Washing with water

Dry cartridge with air

Elute using acetonitrile

Evaporate to dryness; LC-MS/MS

Hydrolysis of honey using hydrochloric acid

**Duration of hydrolysis: 60 min**

Stop of hydrolysis using citric acid

Filter

Aliquotieren, pH-Wert auf  $4 \pm 0,5$  einstellen

**Without delay:** cleanup using Oasis HLB

**Washing with water**

**Dry cartridge with air**

**Elute using acetonitrile**

**Evaporate to dryness; LC-MS/MS**

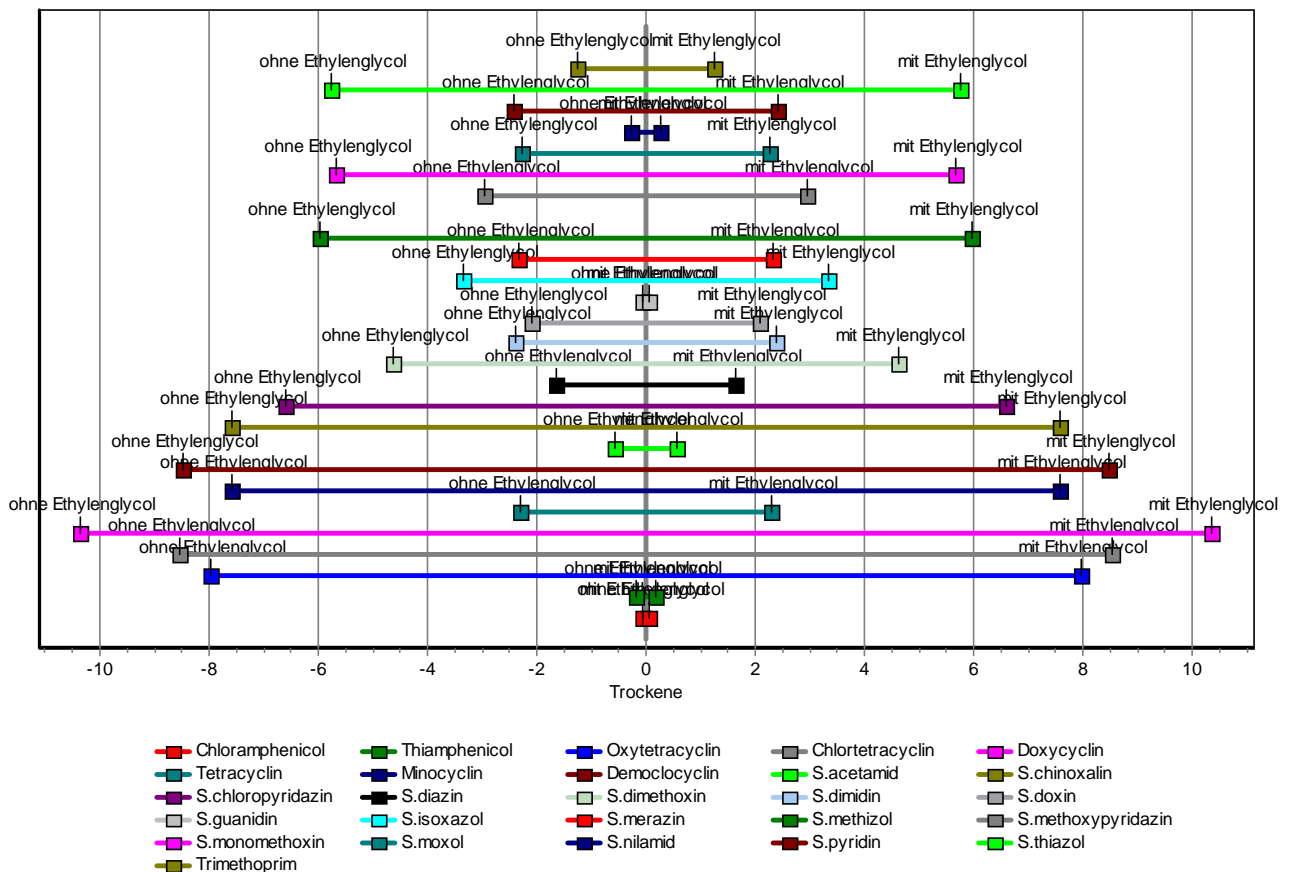
Factor -	Step	Factor +
30 min	Duration of Hydrolysis	60 min
0 min	C	60 min
6 ml	Wash with water	12 ml
Hexane	Dry cartridge using	Air
2 ml	Elution mit Acetonitril	4 ml
0 ml	Zugabe von Acetonitril/EG-Gemisch	0,75 ml
	Einengen zur „Trockene“	
500 mg	Wasser bis zum Endgewicht	525 mg

Run	Hydrolysis	Delay	Washvolume	Dry Cartridge	Elution	Dryness
1	+	+	+	+	+	+
2	+	+	-	+	-	-
3	+	-	+	-	+	-
4	+	-	-	-	-	+
5	-	+	+	-	-	+
6	-	+	-	-	+	-
7	-	-	+	+	-	-
8	-	-	-	+	+	+

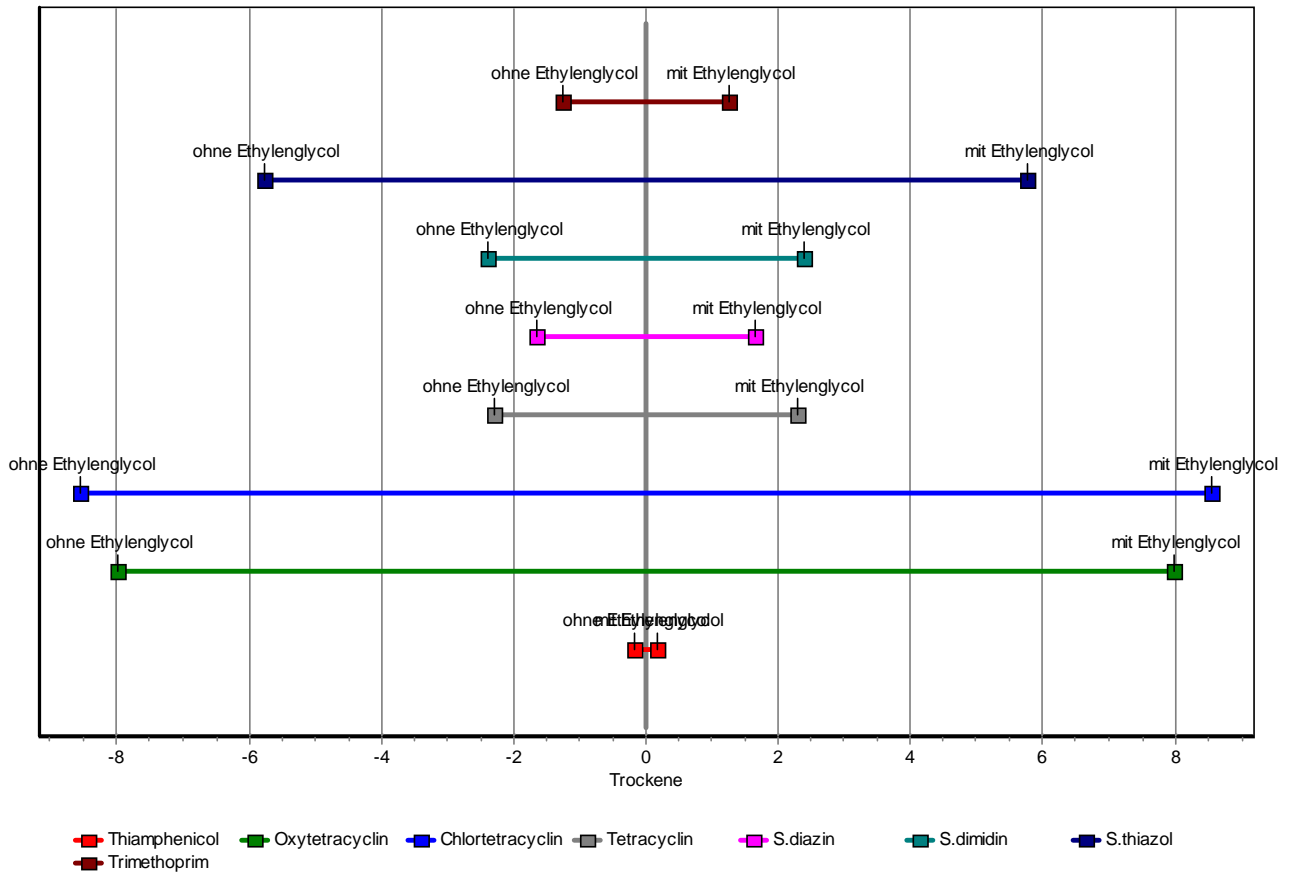
Run	Hydrolysis	Delay	Washvolume	Dry Cartridge	Elution	Dryness
1	60 min	60 min	12 ml	air	4 ml	+ Ethylenglycol
2	60 min	60 min	6 ml	air	2 ml	- Ethylenglycol
3	60 min	0 min	12 ml	1 ml hexane	4 ml	- Ethylenglycol
4	60 min	0 min	6 ml	1 ml hexane	2 ml	+ Ethylenglycol
5	30 min	60 min	12 ml	1 ml hexane	2 ml	+ Ethylenglycol
6	30 min	60 min	6 ml	1 ml hexane	4 ml	- Ethylenglycol
7	30 min	0 min	12 ml	air	2 ml	- Ethylenglycol
8	30 min	0 min	6 ml	air	4 ml	+ Ethylenglycol

Spike at higher level: 50 µg/kg (CAP 1,5 µg/kg)

Honig 1: Versuch:Sulfas, Tetras, Amphenicole



Honig 1: Versuch:Sulfas, Tetras, Amphenicole



Faktor -	Final steps of the method	Faktor +
	Duration of hydrolysis 45 min	
	<b>pH-value 4,0 ± 0,1</b>	
	30 min delay after pH-value was fixed	
	Wash with 8 ml water	
	Dry cartridge with hexane	
	Elute with 3 ml acetonitrile	
	add ethylene glycol	
	Evaporate to ethylene glycole dryness	

## Measurement Uncertainty

- Measurement Uncertainty can be part of **CC $\alpha$**
- Under which conditions?

Use of factors possibly influence the analytical result

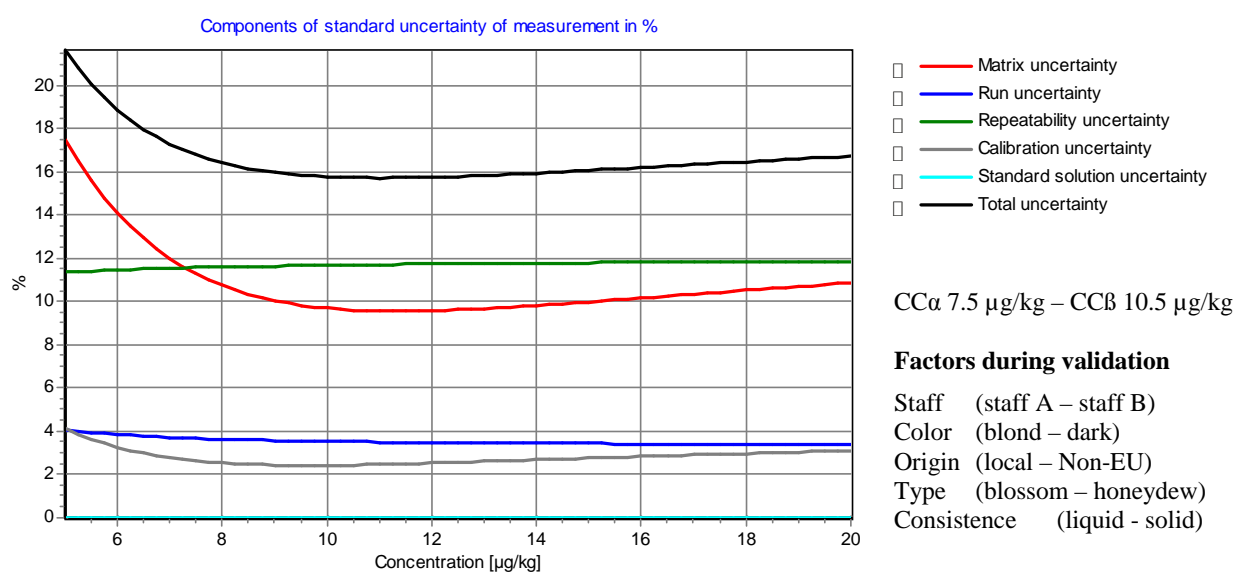
Different sample types (conventional/organic, age, sex ....)

Storage of samples (frozen, refrigerated, long, short .....

Cleanup (staff, SPE-cardridge, ISTD...)

Chromatography (directly, after 2 days.....)

## Measurement uncertainty of sulfathiazol in honey



## Method for Avermectines

---

HPLC-methods based on fluorescence detection for abamectin in animal tissues are well introduced in laboratories analysing for veterinary drug residues (e.g. D. Guggisberg, M. Sievi and H. Koch. Mitt. Gebiete Lebensm. Hyg. 85 (1994) 395–405). For determination of abamectin residues in apples, pears and tomatoes a method based on the same principle was published (H. Diserens and M. Henzelin, Journal of Chromatography A 833 (1999) 13-18).

## Method for Avermectines

---

### Remark

Tubes and vials used should be made from PP or other inert material.

### Weigh in

- Weigh in 5 g sample into a 50 ml centrifuge tube with screw cap
- Add Internal standard solution and mix  
(nemadectin for fluorescence or LC-MS detection)
- Wait 5 minutes

# Method for Avermectines

## Extraction Procedure

- Add 10 ml acetonitrile and shake for 5 min (mechanical shaker)
- Add 10 ml acetonitrile and shake for 10 min, additionally
- Centrifuge at about 4000 g
- Pipette 15 ml from the middle of the supernatant solution into a 50 ml volumetric flask (= 60 % of the total volume)
- Add 50 µl triethylamine
- Add water (distilled or similar) up to 50 ml and shake (= sample solution)

# Method for Avermectines

## SPE-Cleanup (C8-cartridge)

Use a C8 extraction column suitable for analysis of abamectin  
(e.g. Bakerbond SPE 7087-06: 6 ml with 500 mg sorbent)

- Prepare the C8-cartridge with
  - 5 ml acetonitrile
  - and 5 ml conditioning solution  
[350 ml water + 150 ml acetonitrile + 0,5 ml triethylamine]
  - add a reservoir suitable for 50 ml
- fill the sample solution into the reservoir
- elute with about 4 ml/min - avoid dryness of the cartridge
- rinse flask and cartridge with 5 ml conditioning solution
- dry cartridge with air

# Method for Avermectines

## HPLC with Fluorescence-Detection

- elute with 5 ml acetonitrile into test tube made from PP
- evaporate to dryness
- add 100 µl acetonitrile and 100 µl 1-methylimidazole and mix
- transfer to an HPLC-vial (plastics!)
- injector programme: 30 µl trifluoro acetic acid anhydride solution (mix 1,0 ml acetonitrile with 0,5 ml trifluoro acetic acid anhydride prepared fresh at every working day) and 20 µl of sample were filled by turns of 10 µl each into the needle of the injector, mixed and injected
- LC-column: e.g. C18; 125 mm x 3 mm, 5 µm; Eluent A: methanol; Eluent B: water; fluorescence-detection (ex 365 nm - em 463 nm).

# Method for Avermectines

## HPLC with MS/MS-Detektion

### Possibility a)

- elute with 5 ml acetonitrile into test tube made from PP  
(**note the weigh of the empty tube**)
- add 750 ml of a mixture of acetonitrile and ethylene glycol (2+1/v+v)
- evaporate to "ethylene glycol dryness": ethylene glycol has the function of a keeper to avoid dryness. Test the "dryness" by simply weighing of the tube - the resulting weigh should be between 275 mg and 300 mg.
- Add water to 525 mg (= 500 µl volume)

### Possibility b)

- elute with 5 ml acetonitrile into test tube made from PP
- evaporate just to dryness and resolve with your LC-eluent
- use matrix matched standards to avoid matrix effects

## LC-MS-Q-TOF - Screening

---

- Validated multi method (MS/MS-Detektion – QQQ) for drug residues according CD 2002/657/EG
  - Sulfonamides
  - Nitroimidazoles
  - Amphenicoles
  - Tetracyclines
  - Non steroidale antiphlogistic drugs
  - Macrolides
  - Lincosamides
  - Some Coccidiostatica
  - Benzimidazole anthelmintics
  - Chinolones
  - .....

## LC-MS/MS Confirmation or Q-TOF - Screening

---

- Method published in 1992 for sulfonamides (1)
- Simplified
  - Weigh in 6 g of test material
  - Add phosphate buffer (pH 6) -> shake (2)
  - Add acetonitrile -> shake
  - Add sodium chloride, buthyl-methylether/hexan (80 + 20) -> shake
  - centrifuge
  - Evaporate 10 mls of the organic layer to a keeper (ethylene glycole) (3)
  - Add water -> LC-MS
- Advantage: concentration of extracted compounds by a factor of 5
- Disadvantage: more matrix effects im comparisation with QuEChERS-method (dilution factor of 2)

(1) Deutsch Lebensm Rundsch 88 (1992) 205-216

(2) Deutsch Lebensm Rundsch 90 (1994) 375-378

(3) Fresenius J Anal Chem 341 (1991) 449-456

## LC-Detection of Veterinary Drug Residues

Step	Implementation of the Original Multi Method for Veterinary Drug Residues
1	Weigh in 15 g of test material into a centrifugation tube (200 ml) from glass
2	Add internal standards, mix, wait 5 min
3	Add 30 ml McIlvaine-Puffer pH 6,0
4	Shake 5 min
5	Add 45 ml acetonitrile, shake 5 min
6	Add 45 ml acetonitrile, shake 5 min; if necessary centrifuge
7	Filter liquid phase
8	Transfer an aliquot of 90 ml in a separating funnel
9	Add 4 g of NaCl, shake
10	Add 30 ml butylmethylether / hexane (80 + 20/v + v)
11	Shake and discard the water phase
12	Add 10 g of NaCl to the remaining organic phase and shake
13	Transfer the organic layer through the top of the separating funnel into brown coloured glass centrifuge tube (weight of empty tube must be noticed)

(1) Deutsch Lebensm Rundsch 88 (1992) 205-216

(2) Deutsch Lebensm Rundsch 90 (1994) 375-378

(3) Fresenius J Anal Chem 341 (1991) 449-456

## LC-Detection of Veterinary Drug Residues

Step	Implementation of the Original Multi Method for Veterinary Drug Residues
14	add 6,0 ml ethylene glycol / acetonitrile 1 + 2
15	Evaporate to ethylene glycol-dryness (preferred using an automated system (resulting weight of the remaining solution about 2,2 – 3.0 g)
16	Transfer in to a 50 ml test tube (glass ware) using <ol style="list-style-type: none"> <li>1. 25 ml hexane</li> <li>2. 3 ml acetonitril</li> <li>3. 0,5 ml McIlvaine buffer 6.0 diluted with 2 ml of water</li> </ol>
17	Withdraw the hexane phase by suction using vacuum
18	Add 3 ml water, 500 mg NaCl and 15 ml ethyl acetate and shake
19	Transfer the ethyl acetate phase in to a 100 ml flask (for later evaporation)
20	Add 15 ml ethyl acetate and shake
21	Combine the ethyl acetate phases in the 100 ml flask
22	Add 600 µl ethylene glycol / acetonitrile 1 + 2
23	Evaporate to ethylene glycol-dryness (resulting weight of the remaining solution about 0.5 – 0.7 g)
24	Add water to 1050 mg (equals 1,0 ml final volume) -> Ready for chromatography

# LC-Detection of Veterinary Drug Residues

- Validated multi method (MS/MS-Detektion – QQQ) for drug residues according CD 2002/657/EG
  - Sulfonamides: UV/FLD-detection or MS-detection**
  - Nitroimidazoles: MS-detection**
  - Amphenicoles: MS-detection**
  - Tetracyclines (Screening with UV- or MS-detection)**
  - Non steroidale antiphlogistic drugs*
  - Macrolides*
  - Lincosamides*
  - Some Coccidiostatica: UV- or MS-detection**
  - Benzimidazole anthelmintics: UV- or MS-detection**
  - Chinolones*
  - .....

# LC-MS/MS Confirmation or Screening with TOF

Step	Simplified Multi Method for Veterinary Drug Residues (MS-Detection, only)
1	Weigh in 6 g of test material into a test tube
2	Add internal standards, mix, wait 5 min
3	Add 4 ml McIlvaine-Puffer pH 6,0 with 0,1 % dithioerythritol
4	Shake 5 min
5	Add 10 ml acetonitrile, shake 5 min
6	Add 10 ml acetonitrile, shake 5 min; if necessary centrifuge
7	Filter liquid phase into a new test tube which contains 2 g NaCl
10	Add 6 ml buthylmethylether / hexane (80 + 20/v + v)
11	Shake and centrifuge
21	Transfer 10 ml of the organic layer into a new test tube (weight of empty tube must be noticed)
22	add 750 µl ethylen glycole / acetonitrile 1 + 2
22,1	add 0,1 ml 0,1% dithioerythritol in acetonitrile
23	Evaporate to ethylene glycol-dryness (preferred using an automated system (resulting weight of the remaining solution about 300 mg)
24	Add water to 525 mg (equals 500 µl final volume) -> Ready for chromatography

## LC-MS-Q-TOF - Screening

---

- Validated multi method (MS/MS-Detektion – QQQ) for drug residues according CD 2002/657/EG
  - Sulfonamides
  - Nitroimidazoles
  - Amphenicoles
  - Tetracyclines
  - Non steroidale antiphlogistic drugs
  - Macrolides
  - Lincosamides
  - Some Coccidiostatica
  - Benzimidazole anthelmintics
  - Chinolones
  - .....

## LC-MS/MS Confirmation or Q-TOF - Screening

---

### Advantages of Using MS

- Reducing Cleanup
  - reduced time for analysis
  - reduced costs
- More often dilution of extracts possible (sensitive instruments)
- Reduced ratios of false positives and false negatives
- **According CD 657/2002:**  
**In case of “non authorized” drugs/hormones use of MS-techniques for confirmation of positive (non compliant results) is mandatory**

