Pesticide Residue Analysis in Food of Animal Origin

3rd Latin American Pesticide Residue Workshop, Food and Environment

08.05 - 11.05.2011
Montevideo, Uruguay

Ralf Lippold

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(State Institute for Chemical and Veterinary Analysis of Food)

CVUA Freiburg

European Union Reference Laboratory (EU-RL) for dioxins and PCBs in feed and food

EU-RL for pesticides in food of animal origin and commodities with high fat content

WHO Reference Laboratory for dioxins, PCBs and halogenated pesticides (POPs) in human milk

Organisation of CVUA Freiburg

1	2	3	4	5	6	7
GC	Water (1)	**Pesticides (1) *)**	Wine	Diet food	Meat (1)	Pathology
GC-MS	Water (2)	**Pesticides (2) *)**	Liquors, beer	Genetic. mod. Food	Meat (2)	Bacterio-logy
HPLC	Consumer goods	**Vet. Drugs (1)**	Honey, sweets	Oils, fat	Fish	Virology
Heavy metals	Cosmetics	**Vet. Drugs (2)**	Radio isotopes	Vegetables	Milk (1)	Serology
Toxicology		**Dioxins *)**	Stable isotopes	Molecular biology	Milk (2)	Bees diseases
		HPLC-MS			Microbiology	Fish diseases
					Immunology	Poultry diseases

*) EU-RL

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Pesticides - Veterinary Drugs

- Typically, pesticides are not applied to animals
  - Animals come in contact with pesticides e.g. by feet, water, environment .....
  - But, if pesticides will be applied to animals?

- According to the European Union legislation this will be a treatment of an animal and the pesticide will „transform“ into a veterinary drug.

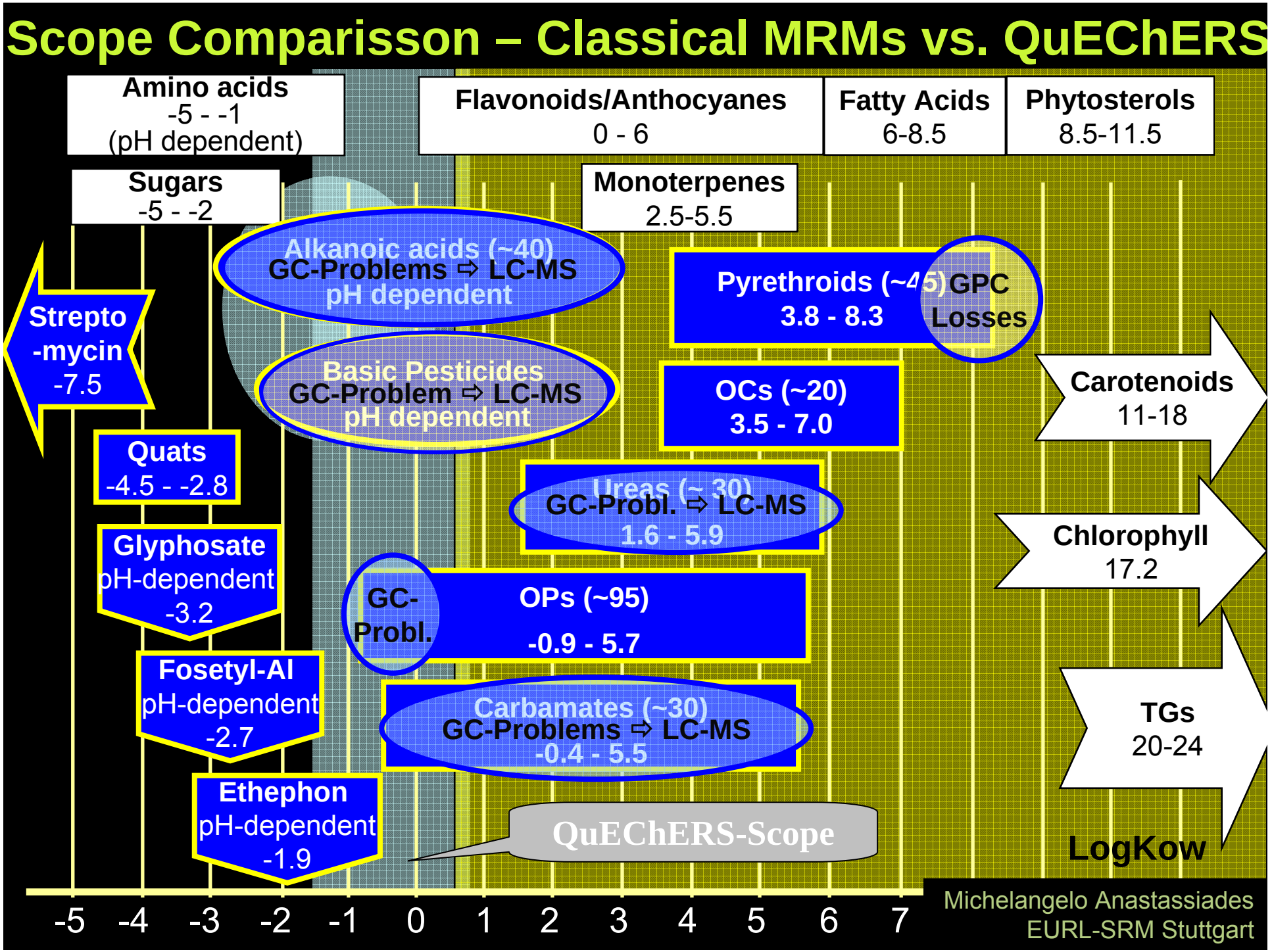
Two sets of AQC Procedures

1. Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Feed and Food (**Document No. SANCO**/10684/2009)

2. **Commission Decision** 2002/657/EC
   Implements Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products

✓ Agreement: Currently inspectors of the Federal Veterinarian Office (FVO) will accept if pesticides in food of animal origin are validated either according the AQC procedures or CD 2002/657/EC

Scope Comparisson – Classical MRMs vs. QuEChERS



Michelangelo Anastassiades
EURL-SRM Stuttgart

Pesticide and Contaminants in Food of Animal Origin

- Mostly non polar, lipophilic compounds
- Enrichment in the fat of animals
- often organochlorine compounds
  (application not allowed according directive 91/414/EEC)
  - Residue levels going down,
    but some times cases of contamination (e.g. nitrofen 2002 in Germany)
- Organophosphorous compounds
- Pyrethroids
- Contaminants
  (e.g. organo tin compounds, moschus-xylol,
  indicator PCB)

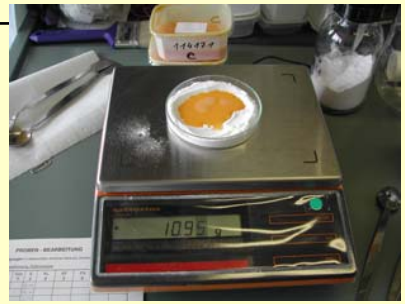
Pesticide and Contaminants in Food of Animal Origin

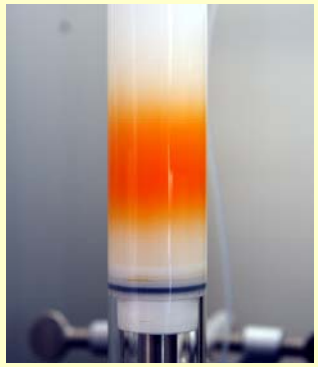
- Often volatile compounds -> GC

- Some Compounds only as metabolite identifiziert
  - Aldrin/Dieldrin → only Dieldrin
  - DDT → DDE (!)

Matrices

- Meat
  - cattle, pig, poultry, dear
- Offal
  - kidney, liver
- Milk und dairy products
- (Human milk)
- Eggs
- Fish
- Mussels
- Honey



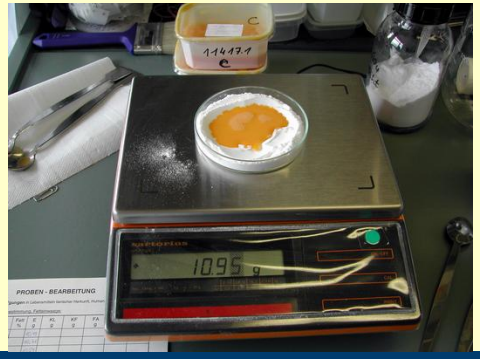


Cleanup using the classic German S19 – method (EN-1528) : extraction of fat, gel permeation chromatography, silica gel mini-column: LOD ~ 1 µg/kg fat

Extracting fat in food of animal origin at CVUA Freiburg

Examples:

- Centrifugation to separate cream for raw milk
- Liquid / liquid distribution for homogenized milk
- Addition of Na_2SO_4 and extraction with organic solvents (meat, eggs)



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CVUA Freiburg

Cleanup of extracted Fat and Detection

Clean up

0.5 g extracted fat

Addition of internal standards
2.4.5-TCB, Mirex, Triphenylphoshate

GPC (Bio Beads S-X3)

Silica gel mini-column
(1.5 % water)

Fraction 2
toluene

Fraction 3
toluene / aceton
95 + 5

Final volumes 0.5 ml each

Quantification

Fraction 2

Fraction 3

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

3 calibration levels, solvent and matrix calibration, internal standards

Calculation by means of ISTD

Method according to
EN 1528:2

EN 1528-2: Modular System for Pesticide Methods

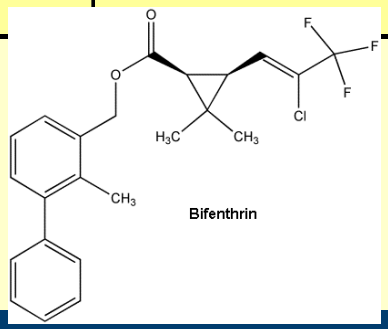
European Standard	Description	German Standard	Modul	Description	Validated methods of CVUA Freiburg	Samples / Compounds
EN 1528-2: 1996-10 (confirmed 2001)	Extraction of fat, pesticides and PCBs and determination of fat content	§ 64 LFGB: L 00.00-34 (confirmed 1999)	E 8	Extraction of fat with hexan/acetone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

Gel Chromatography

Example for compounds

Organo-chlorines	Chlorobenzilate	HCB	gamma-HCH	alpha-HCH
	beta-HCH	p,p'-DDE	p,p'-DDD	p,p'-DDT
	o,p'-DDT	alpha-Endosulfan	beta-Endosulfan	Endosulfansulphate
	Aldrin	Dieldrin	Endrin	cis-Chlordane
	trans-Chlordane	Oxychlordane	Heptachlor	cis-Heptachlorepoxid
	trans-Heptachlorepoxid	4,4'-Methoxychlor	Nitrofen	Parlar 26 (Camphechlor)
	Parlar 50 (Camphechlor)	Parlar 62 (Camphechlor)	Quintozene	Tecnazene
Organo-phosporus	Azinphos-ethyl	Chlorfenvinphos	Chlorpyriphos (-ethyl)	Chlorpyriphos-methyl
	Diazinon	Fenthion	Formothion	Methacrifos
	Methidathion	Parathion	Parathion-methyl	Pirimiphos-methyl
	Profenofos	Pyrazophos	Triazophos	
Pyrethroids	Bifenthrin	Cyfluthrin	Cypermethrin	Deltamethrin
	Fenvalerat	Permethrin	Resmethrin	

Gel Chromatography: start to collect 4 min earlier



Bifenthrin

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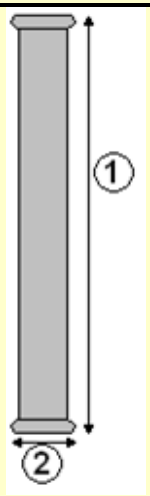
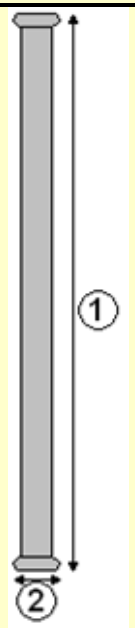
Gel Chromatography

	Short column	Long column
		
(1) Length	580 mm	740 mm
(2) Inner diameter	24,4 mm	20 mm
Filling level (bed length) of Bio-Beads-S-X3	approx. 300-350 mm	approx. 500 - 550 mm
Flow rate	5,00 ml/min	2,50 ml/min
Tolerance of the flow rate	+/- 0,10 ml/min	+/- 0,05 ml/min

Cleanup using Silical Gel Mini-Column

Example for compounds

Organo-chlorines	Chlorobenzilate	HCB	gamma-HCH	alpha-HCH
	beta-HCH	p,p'-DDE	p,p'-DDD	p,p'-DDT
	o,p'-DDT	alpha-Endosulfan	beta-Endosulfan	Endosulfansulphate
	Aldrin	Dieldrin	Endrin	cis-Chlordane
	trans-Chlordane	Oxychlordane	Heptachlor	cis-Heptachlorepoxid
	trans-Heptachlorepoxid	4,4'-Methoxychlor	Nitrofen	Parlar 26 (Camphechlor)
	Parlar 50 (Camphechlor)	Parlar 62 (Camphechlor)	Quintozene	Tecnazene
Organo-phosporus	Azinphos-ethyl	Chlorfenvinphos	Chlorpyriphos (-ethyl)	Chlorpyriphos-methyl
	Diazinon	Fenthion	Formothion	Methacrifos
	Methidathion	Parathion	Parathion-methyl	Pirimiphos-methyl
	Profenofos	Pyrazophos	Triazophos	
Pyrethroids	Bifenthrin	Cyfluthrin	Cypermethrin	Deltamethrin
	Fenvalerat	Permethrin	Resmethrin	

Silica gel clean up: fraction 2 (toluene)

Cleanup using Silical Gel Mini-Column

Example for compounds

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	o,p'-DDT	alpha-Endosulfan	beta-Endosulfan	Endosulfansulphate
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	Methidathion	Parathion	Parathion-methyl	Pirimiphos-methyl
	Profenofos	Pyrazophos	Triazophos	
Pyrethroids	Bifenthrin	Cyfluthrin	Cypermethrin	Deltamethrin
	Fenvalerat	Permethrin	Resmethrin	

Silica gel clean up: fraction 3 (toluene + aceton / 95 + 5)

Detection using ECD

List of compounds

Organo-chlorines	Chlorobenzilate	HCB	gamma-HCH	alpha-HCH
	beta-HCH	p,p'-DDE	p,p'-DDD	p,p'-DDT
	o,p'-DDT	alpha-Endosulfan	beta-Endosulfan	Endosulfansulphate
	Aldrin	Dieldrin	Endrin	cis-Chlordane
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	Profenofos	Pyrazophos	Triazophos	
Pyrethroids	Bifenthrin	Cyfluthrin	Cypermethrin	Deltamethrin
	Fenvalerat	Permethrin	Resmethrin	

Detection using ECD and NPD

List of compounds

Organo-chlorines	Chlorobenzilate	HCB	gamma-HCH	alpha-HCH
	beta-HCH	p,p'-DDE	p,p'-DDD	p,p'-DDT
	o,p'-DDT	alpha-Endosulfan	beta-Endosulfan	Endosulfansulphate
	Aldrin	Dieldrin	Endrin	cis-Chlordane
	trans-Chlordane	Oxychlordane	Heptachlor	cis-Heptachlorepoxid
	trans-Heptachlorepoxid	4,4'-Methoxychlor	Nitrofen	Parlar 26 (Camphechlor)
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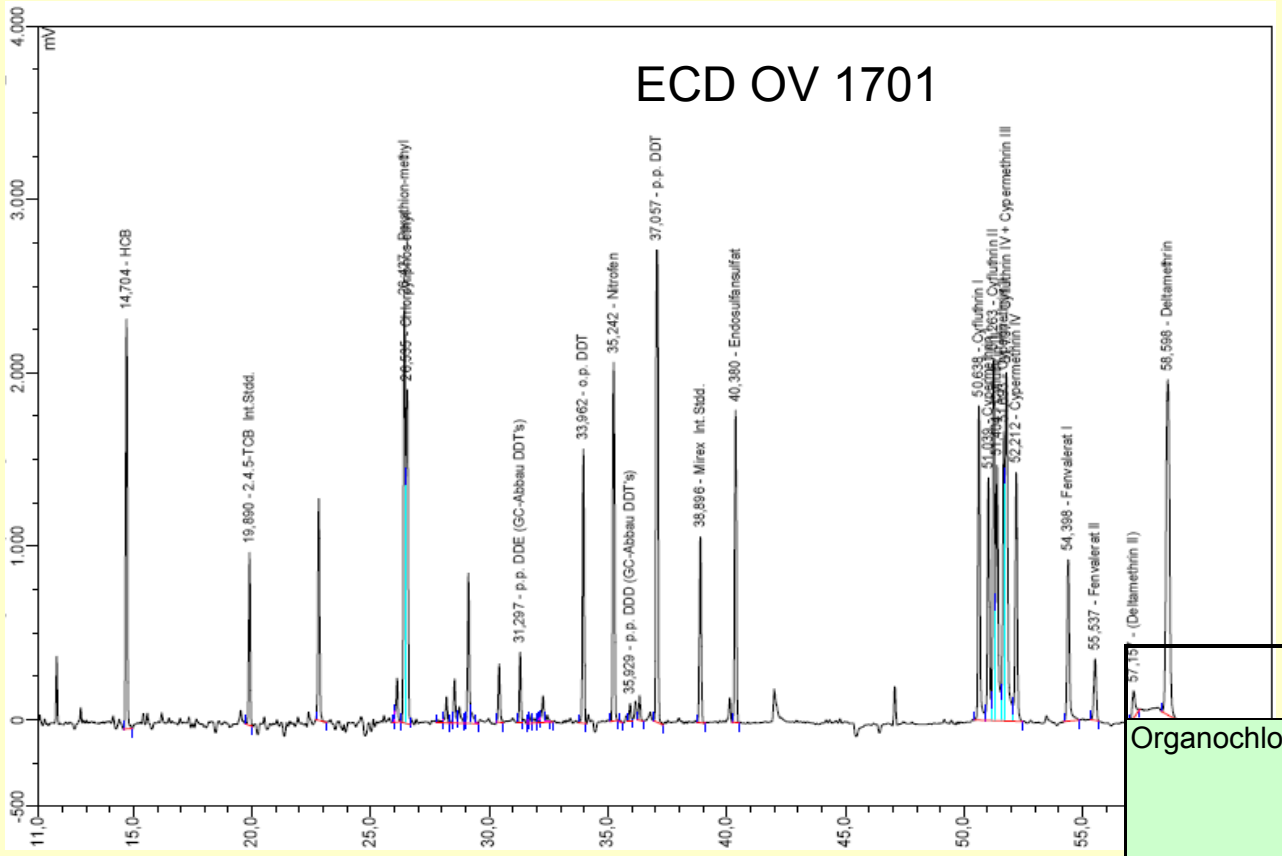
	Methidathion	Parathion	Parathion-methyl	Pirimiphos-methyl
	Profenofos	Pyrazophos	Triazophos	
Pyrethroids	Bifenthrin	Cyfluthrin	Cypermethrin	Deltamethrin
	Fenvalerat	Permethrin	Resmethrin	

Advantages using MS-techniques

- All compounds can be detected
- Marginal matrics effects observed

Detection using ECD (egg sample, spiked)



	Compound	Spike Level [µg/kg egg]
Organochlorines	DDT	26
	ppDDT	20
	opDDT	6
	Endosulfan	8
	Endosulfansulfate	8
	HCB	6
	Nitrofen	10
Organophosphorus	Chlorpyrifos-ethyl	15
	Parathionmethyl	50
Pyrethroids	Cyfluthrin	70
	Cypermethrin	60
	Deltamethrin	40
	Fenvalerate	30

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Polar Pesticides in Honey

- Modern Pesticides are more/less polar
- No enrichment in fat of animals
- Compounds rapidly metabolized and/or excreted

Is it necessary to analyse for all pesticides in food of animal origin?

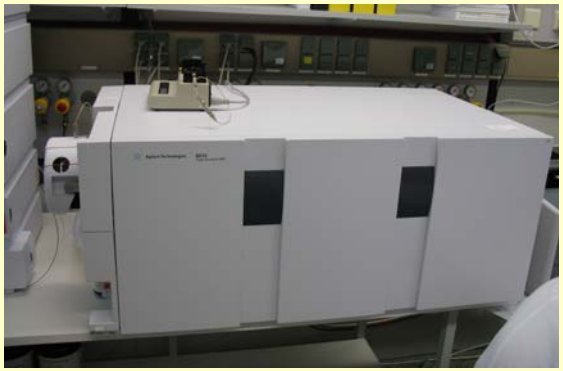
- Example Honey (Cleanup QuEChERS-method)
  - Database research for findings (e.g. pesticides-online, publications)
  - Development/Validation of a method with the “relevant” pesticides (~30)
  - Time to time addition of more pesticides (validation in smaller packages of 20 to 30 pesticides)
  - Detection using LC-MS/MS

Hint: QuEChERS is suitable for polar pesticides in matrices of animal origin

Pesticides in Honey - Results (2009)

- 65 samples of honey were analysed

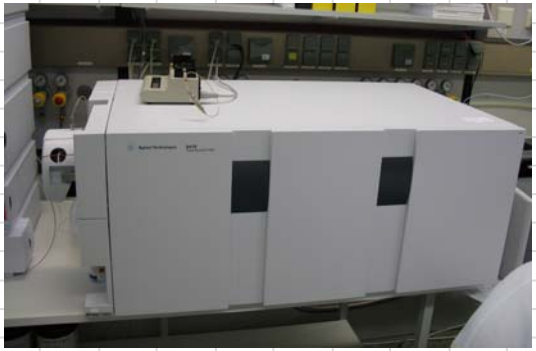
PV Honig EURL-Methodenentwicklung	Aceta-miprid	Azoxystrobin	Boscalid	Carben-dazim	Clothianidin	Coumaphos	Dimoxystrobin	tau-Fluvalinat	Imida-cloprid	Pirimicarb	Thiacloprid
Anzahl untersuchter Proben	65	65	65	65	65	65	65	65	65	65	65
min [mg/kg]	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
max [mg/kg]	0,003	0,009	0,030	0,009	0,003	0,045	0,025	0,005	0,001	0,003	0,172
Mittelwert [mg/kg]	0,000	0,000	0,002	0,001	0,000	0,002	0,001	0,000	0,000	0,000	0,011
Median [mg/kg]	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,003
Nachweis positiv (>=0,001 mg/kg	3	1	22	11	2	23	8	4	1	1	37
BG [mg/kg]	0,002	0,002	0,005	0,005	0,002	0,02	0,002	0,01	0,01	0,002	0,005
Werte >= BG	2	1	**7**	3	1	**19**	**7**	0	0	1	**21**



Agilent LC 1200 with Agilent 6410 QQQ

No violations of MRLs observed!

Honey (2009)



10 µg/kg in honey matrix
45 analyts, 3 ISTD =>
98 transisitions

	Liquid chromatography with MS/MS-Detektion (Agilent LC 1200 with Agilent 6410 QQQ)	
LC	column:	Zorbax Eclipse Plus C_{18} (Agilent) 150 mm x 2,1 mm i.d x 3.5 µm
	Eluent A:	5 mMol/L ammonium formiate
	Eluent B:	acetonitrile
	Flow:	0.3 ml/min
	Gradient:	time (min) % B 0 5 1 5 30 100 32.5 100 33 5
	Temp.:	25 °C
	Injection:	5.0 µl
MS	Mode ESI Positive	

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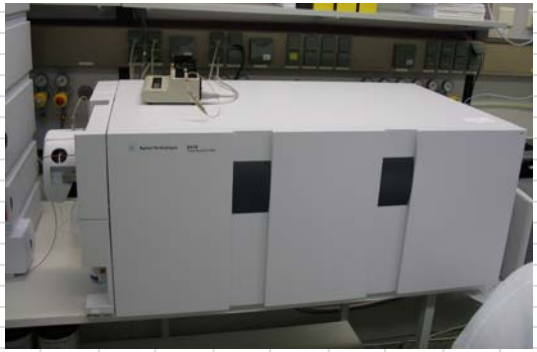
Honey Examples of Findings



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	Flow:	0.3 ml/min
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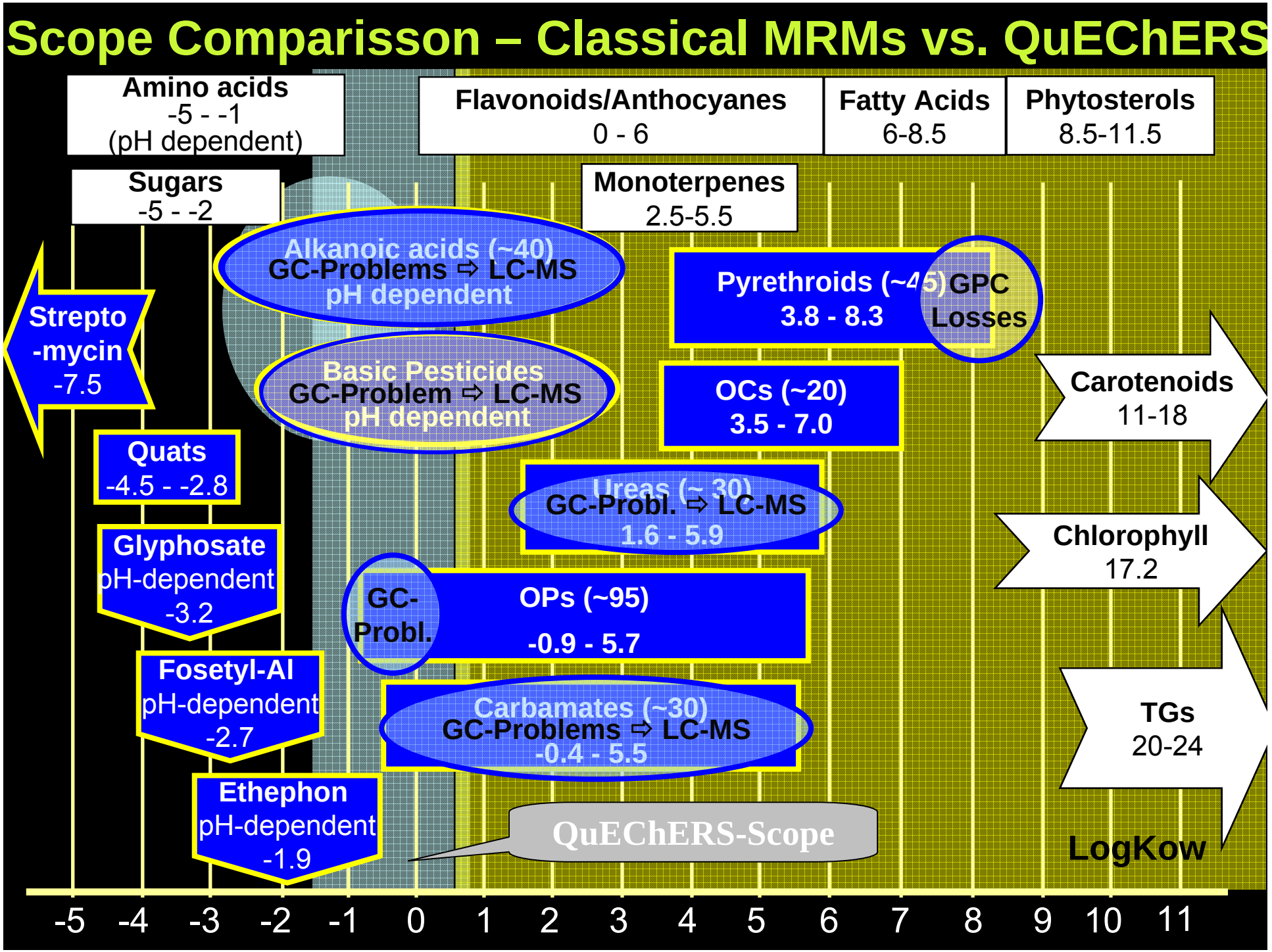


Streptomycin in Honey and Fire Blight

- Fire blight is spreading over wide areas in Germany, Switzerland, Austria, Northern Italy and the upper Rhone valley (France).
- Fire blight is a disease induced by bacteria (Erwinia amylovora) causing lot of problems in pome plantations with losses of trees and therefore large crop failures.

Background Information: Fire blight

- Germany issued yearly a certificate of exemption for use of streptomycin containing pesticide formulations because the lack of alternatives.
- Monitoring programmes for streptomycin during spraying in pome plantations and for residues of streptomycin in honey were introduced.

Streptomycin - Analytics

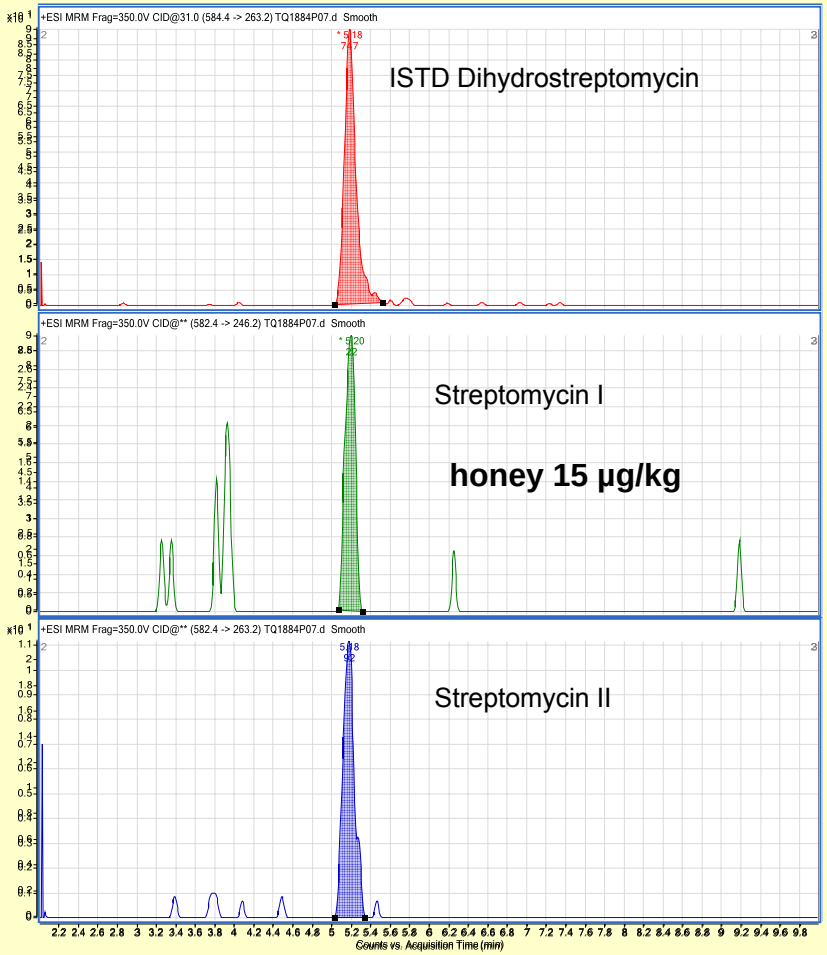
Honey [1]	Extraction of samples
Weighted sample	weigh 20 g of honey into a 200 ml centrifuge tube (poly propylene)
Internal standard	add 50 µl of internal standard solution (dihydrostreptmycin, ρ = 10 ng/µl); wait 5 minutes before extraction
Extraction	add 87 g water
	shake 20 minutes
	filter through a fluted filter
SPE-Cleanup step	conditioning of SPE-Cartridge (Sep-Pak Vac 6 cc (500 mg) Accell Plus CM) with 10 ml washing solution (980 ml water + 20 ml acetic acid) and 10 ml water
	load 50 ml (53.5 g) of the filtrate onto the cartridge
	wash with 10 ml water
	elute with 7.5 ml elution solution (780 ml water + 20 ml acetic acid + 200 ml acetonitrile)
Final extract	concentrate eluate to about 4 ml
	add water to 5.0 g
Standards in solution	spike aliquots of streptomycin and 50 µl of internal standard solution into 10 g of washing solution
Final quantification	for quantification of streptomycin in honey the standard addition procedure is recommended

	LC mit MS-MS-Detektion		
LC	Säule:	ZIC-HILIC PEEK (Merck Sequant)	
		100 x 2.1 mm i.d. x 3.5 µm	
	Eluent A:	0.02 M Natriumformiat + 0.1% Ameisensäure	
	Eluent B:	Acetonitril + 0.1% Ameisensäure	
	Flow:	0.30 ml/min	
	Gradient:	Time (min)	%B
		0	90
		0,5	90
		1,5	10
		6,5	10
		8	90
	Temp.:	25 °C	
	Injektion:	10 µl	

	LC mit MS-MS-Detektion				
MS	Mode	ESI Pos			
		M+H	Cone (V)	product ion 1 / coll (eV)	product ion 2 / coll (eV)
	Streptomycin	582.4	135	263.2 / 32	246.2 / 40
	Dihydrostreptomycin	584.4	135	263.2 / 31	

Method published by Kaufmann A:
Rapid Commun Mass Spectrom 17 2575–2577 (2003)

Streptomycin - Analytics



Streptomycin Monitoring Results

Year	2005	2006	2007	2008	2009
Number of samples	33	34	118	147	50
Samples containing residues	8	2	26	80	2
Residues above 20 µg/kg	3	1	9	64	1

about 8,4 t of honey
(0,24 % of the yearly honey production in our state

since 2009

MRL = 10 µg/kg
application limited to 2 sprayings
→ few residues found

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CVUA Freiburg

Screening for Pesticides and Veterinary Drugs

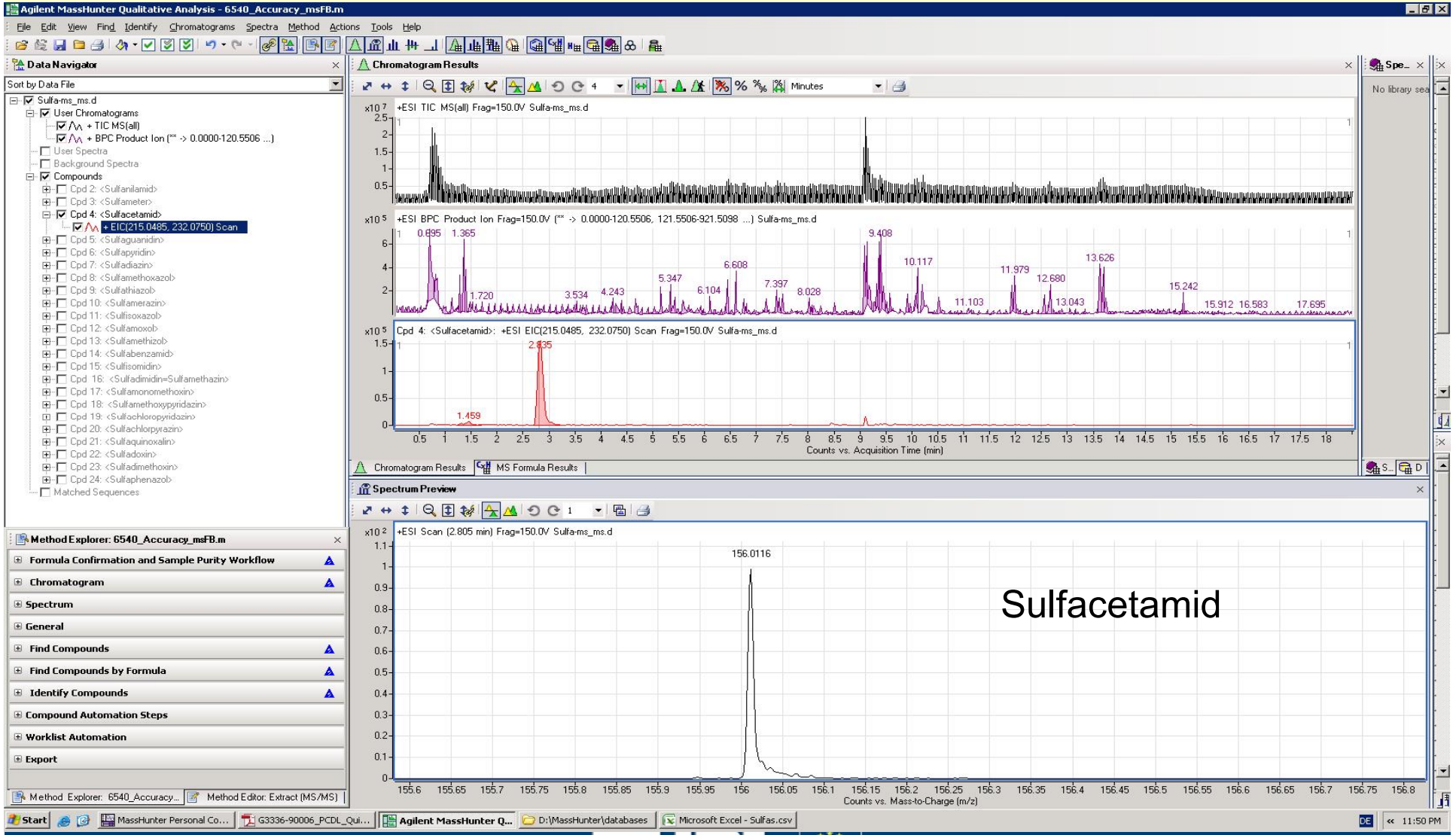
- Devellopment of a screening method for polar pesticides and veterinary drugs in food of animal Origin
  - Meat
  - Offal
  - Egg
  - Milk
- Auswahl relevanter Wirkstoffe (aus Studien, Literatur)
- Agilent LC1290 + Agilent LC-MS-Q-TOF 6540 for screening methods (new since January 2011!)
- Targeted screening using own database with information about retention times

LC-MS-Q-TOF Conditions

- HPLC Methode 1290 Series
- Simple method for „all“ analyts
- Column Zorbax Eclipse Plus C-18HD, 100x2,1mm, 1,8 µm
- Q-TOF Method
  - MSScan (sensitive scan) in 4 GHz-Modus
  - Targeted MSMS (identification)
- Data base research
  (database optimized for pesticides and ve

Time	Flow	Solv Ratio B
0	0.35	5
12	0.35	70
15	0.35	100
18	0.35	100
18.5	0.35	5

LC-MS-Q-TOF - Sulfonamide



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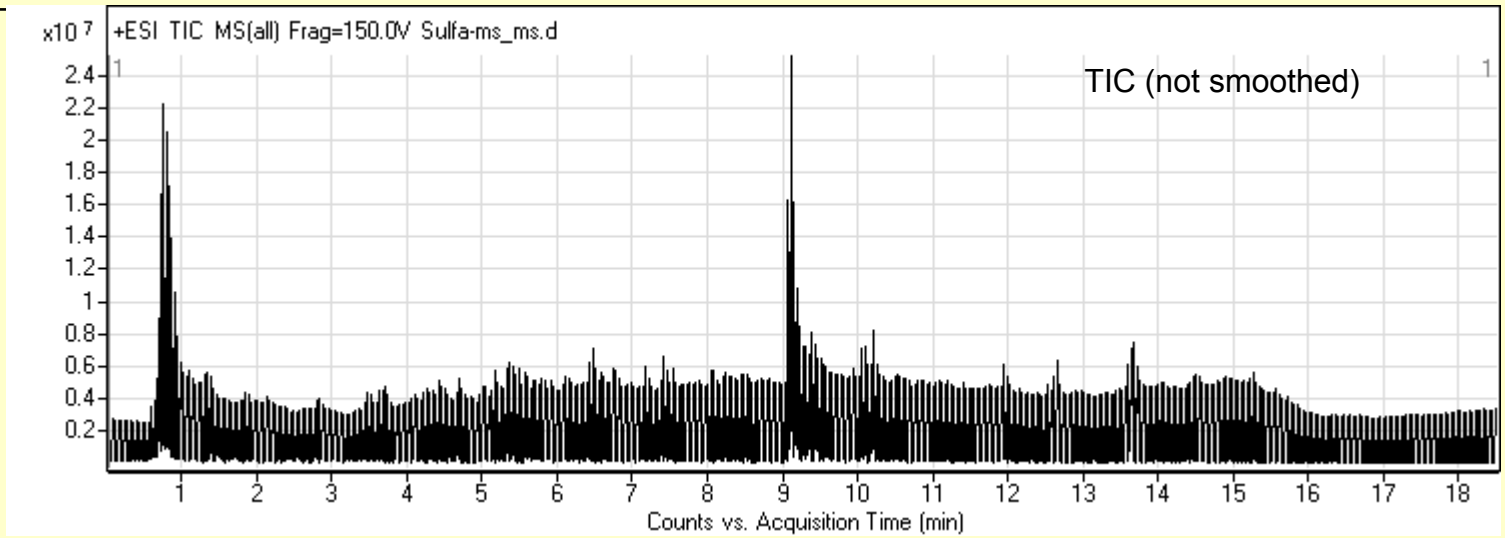
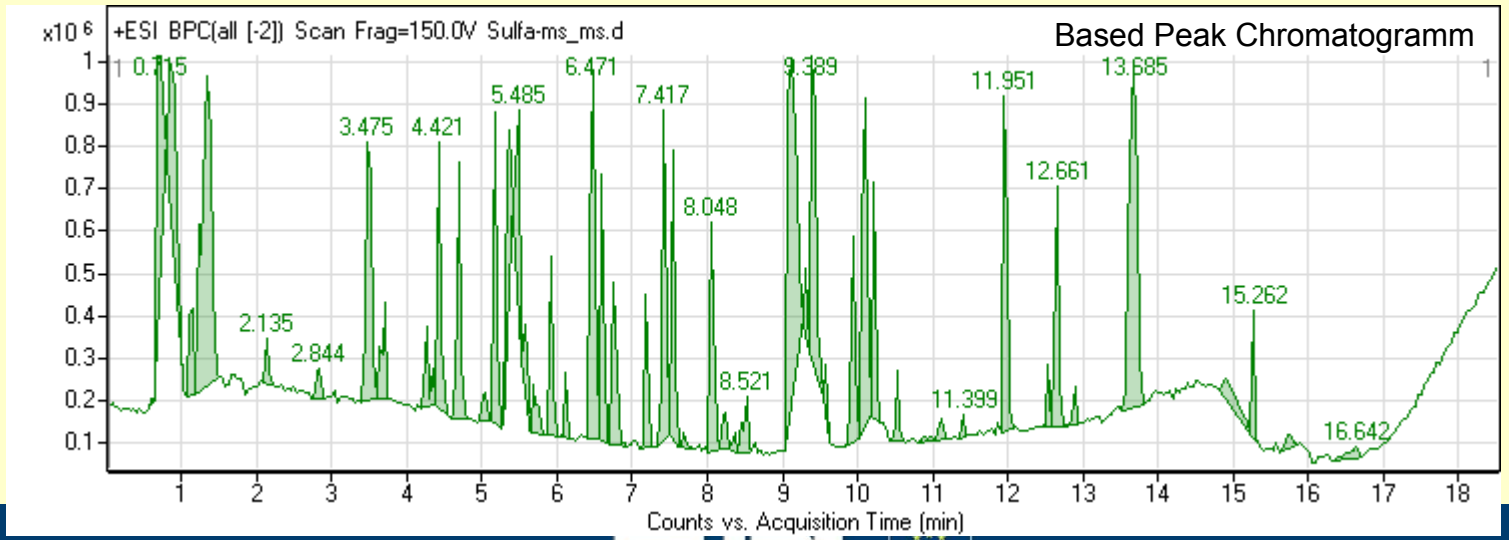
LC-MS-Q-TOF - Sulfonamide





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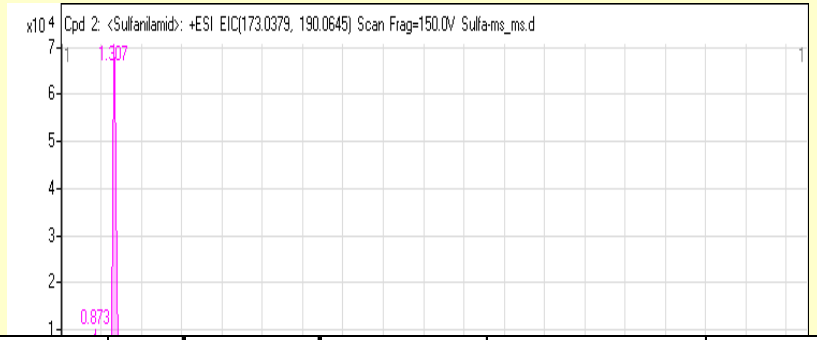
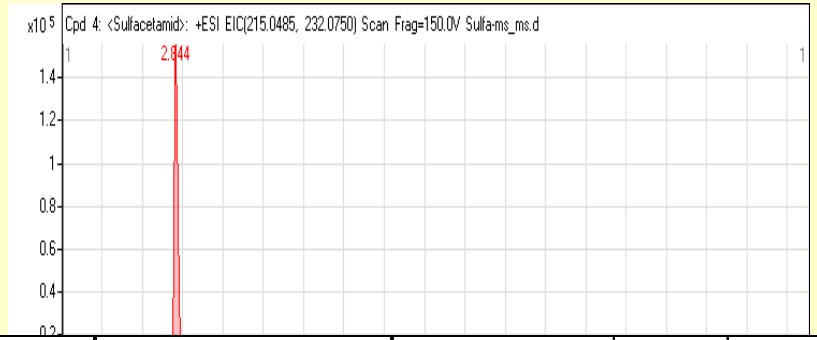
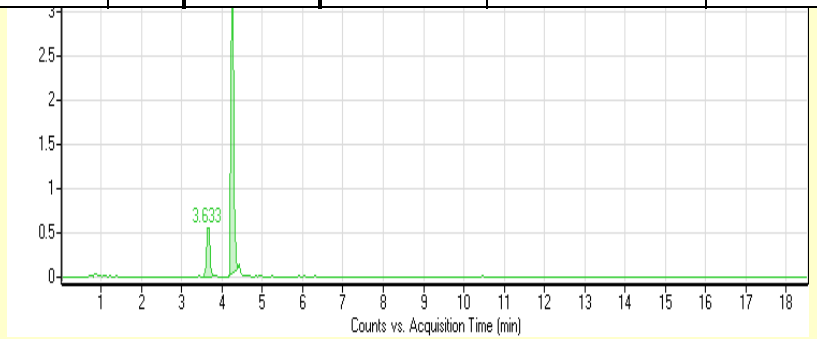
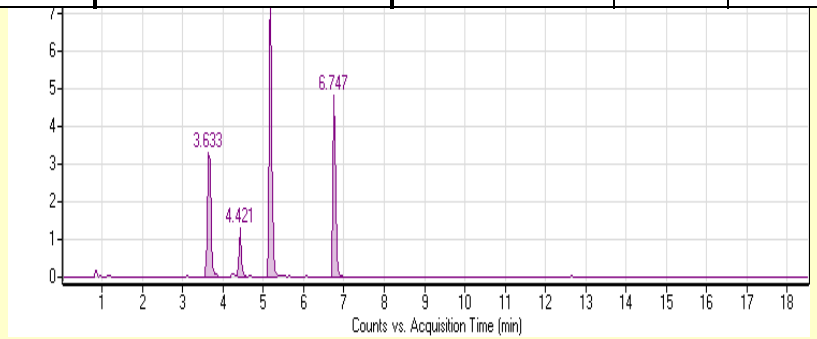
LC-MS-Q-TOF - Sulfonamide





Name	RT	Area	Score (DB)	Diff (DB, ppm)	Formula (Tgt)	Formula,Mass (Tgt)	Diff (Tgt, ppm)	Abund	Score (Tgt)
Sulfanilamid	1,31	1549875	78,15	0,68	C6H8N2O2S	172,0306	0,4	10589	95,36
Sulfacetamid	2,88	3487415	82,59	0,87	C8H10N2O3S	214,0412	0,5	22365	96,25
Sulfathiazol	4,26	3548461	88,46	0,54	C9H9N3O2S2	255,0136	0,3	25687	95,35
Sulfadiazin	5,17	2584467	67,84	0,25	C10H10N4O2S	250,0524	0,4	22458	95,12





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LC-MS-Q-TOF - Screening

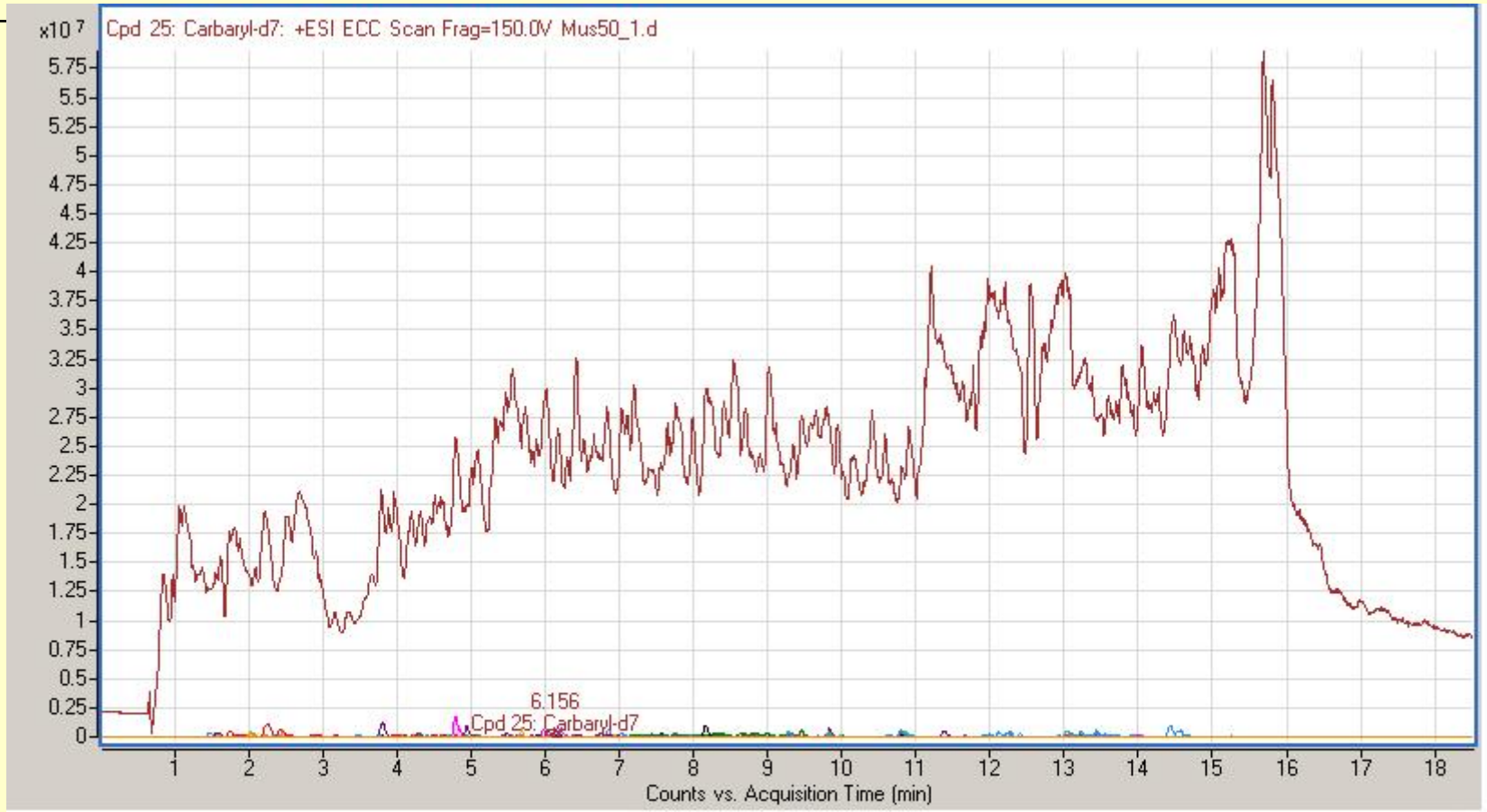
- Validated multi method for drug residues according CD 2002/657/EG
  - Sulfonamides
  - Nitroimidazoles
  - Amphenicoles
  - Tetracyclines
  - Non steroidale antiphlogistic drugs
  - Macrolides
  - Lincosamides.....

LC-MS-Q-TOF - Screening

- Method published in 1992 for sulfonamides
- Simplifyed
  - Weigh in 6 g of test material
  - Add phosphate buffer (pH 6) -> shake
  - 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)
  - Add water -> LC-MS

- Advantage: concentration of extracted ccompounds by a factor of 5
- Disadvantage: more matrix effects im comparisation with QuEChERS-method (dilution factor of 2)

LC-MS-Q-TOF – Screening in Meat



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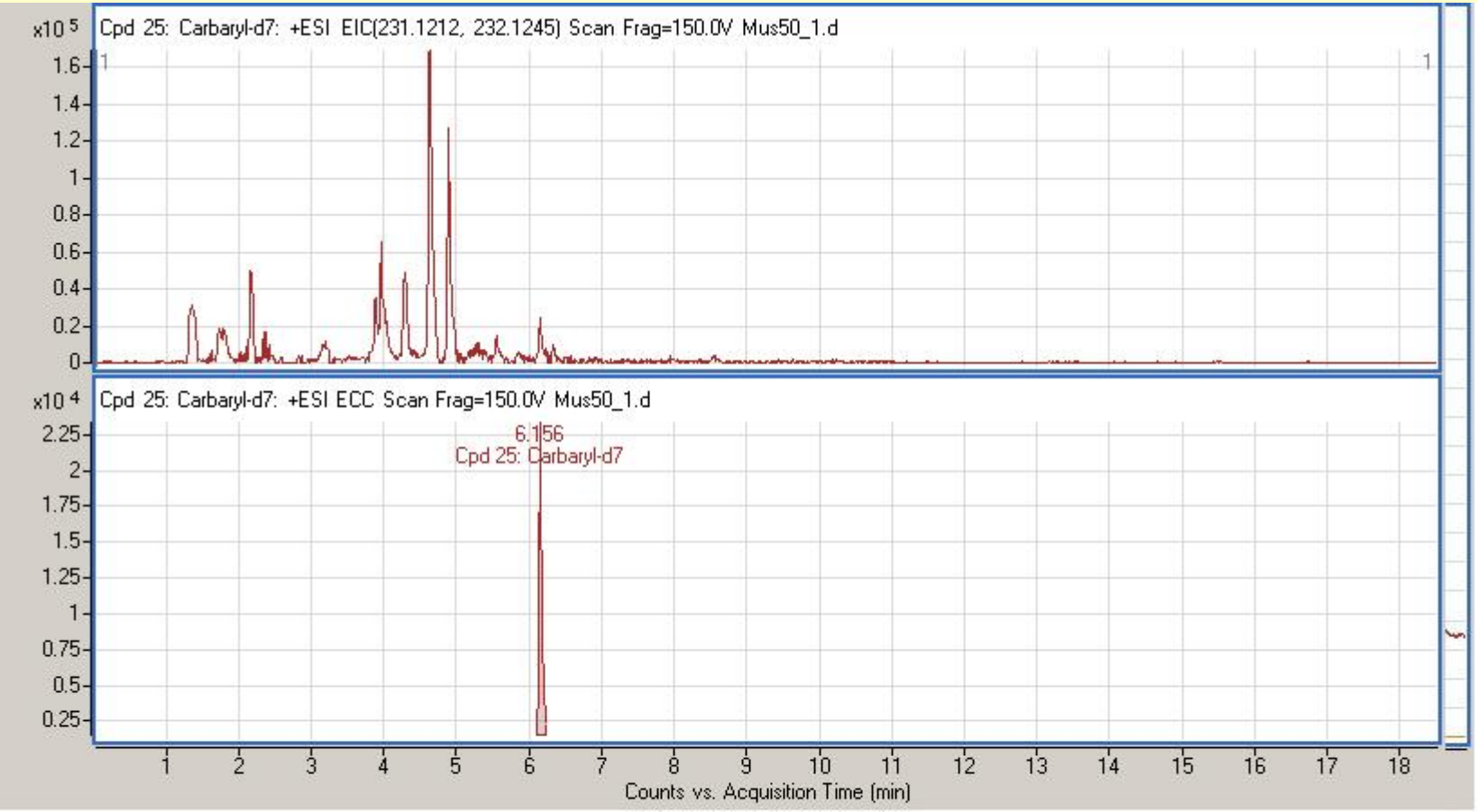
LC-MS-Q-TOF – Screening in Meat



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LC-MS-Q-TOF - Screening in Muscle

Qualitative Compound Report

Data File	Mus50_1.d	**Sample Name**	Sample5
Sample Type	Sample	**Position**	P1-A5
Instrument Name	Instrument 1	**User Name**	
Acq Method	STD_TOF.m	**Acquired Time**	4/6/2011 5:49:42 AM
IRM Calibration Status	All Ions Missed	**DA Method**	Screening.m
Comment			

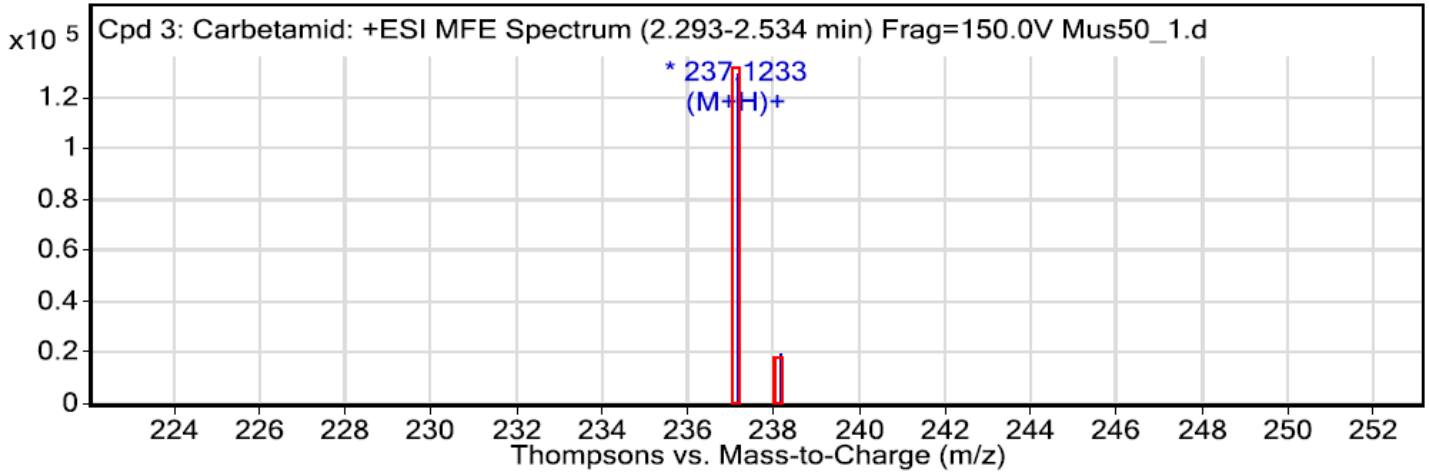
Compound Table

Name	Mass	Diff (ppm)	Diff MFG	RT	RT Diff.	Overall Score	DB Score	Tgt. Score
Nitenpyram	270.09	3.790	-0.620	2.06	-1.22	93.70	57.98	57.98
Carbetamid	236.12	-0.160	0.160	2.37	5.22	86.00	85.88	85.88
Pirimicarb	238.14	-4.680	-0.930	2.46	4.51	85.79	81.71	81.71
Promecarb	207.13	-0.130	5.650	2.48	9.59	78.32	76.63	76.63
Diflubenzuron	310.03	-4.000	-2.020	3.76	7.68	86.82	71.87	71.87
Phoxim	298.05	1.400	-0.130	4.08	7.04	97.89	85.53	85.53
Benomyl	290.14	-4.370	-0.240	4.23	7.40	71.93	70.67	70.67

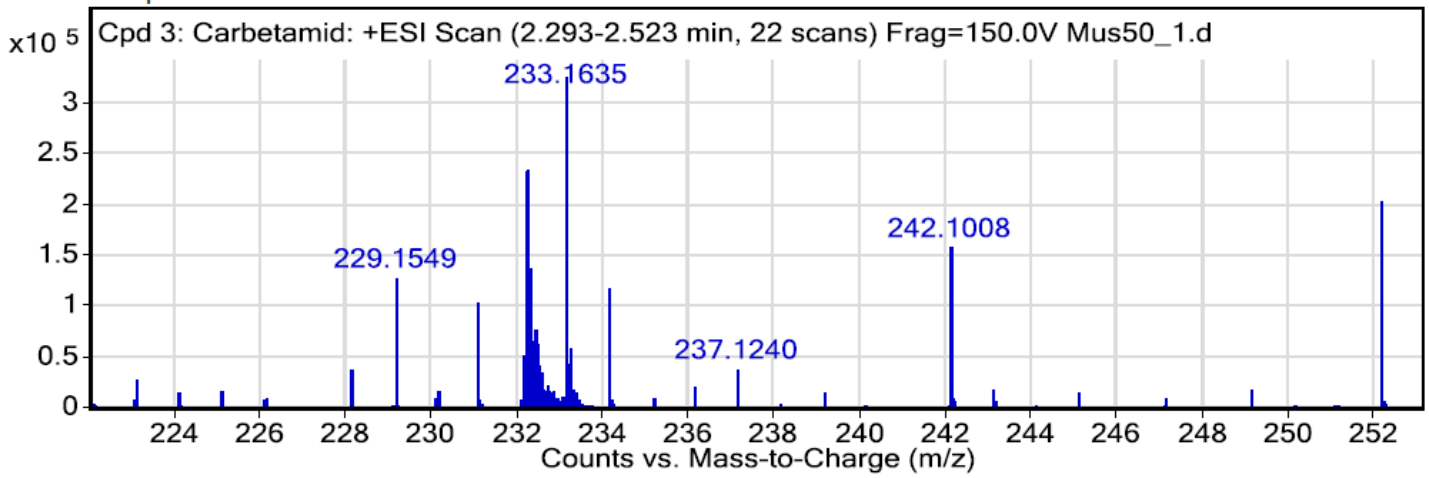
LC-MS-Q-TOF - Screening in Muscle

Name	RT	Algorithm	Mass
Carbetamid	2.367	Find by Molecular Feature	236.1161

MFE MS Zoomed Spectrum



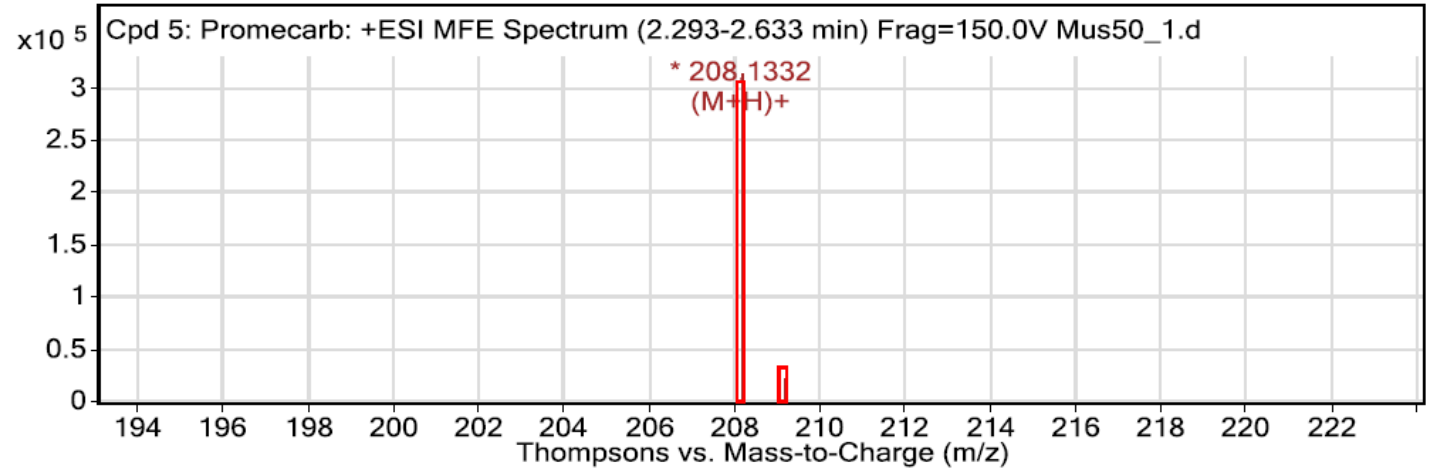
MS Zoomed Spectrum



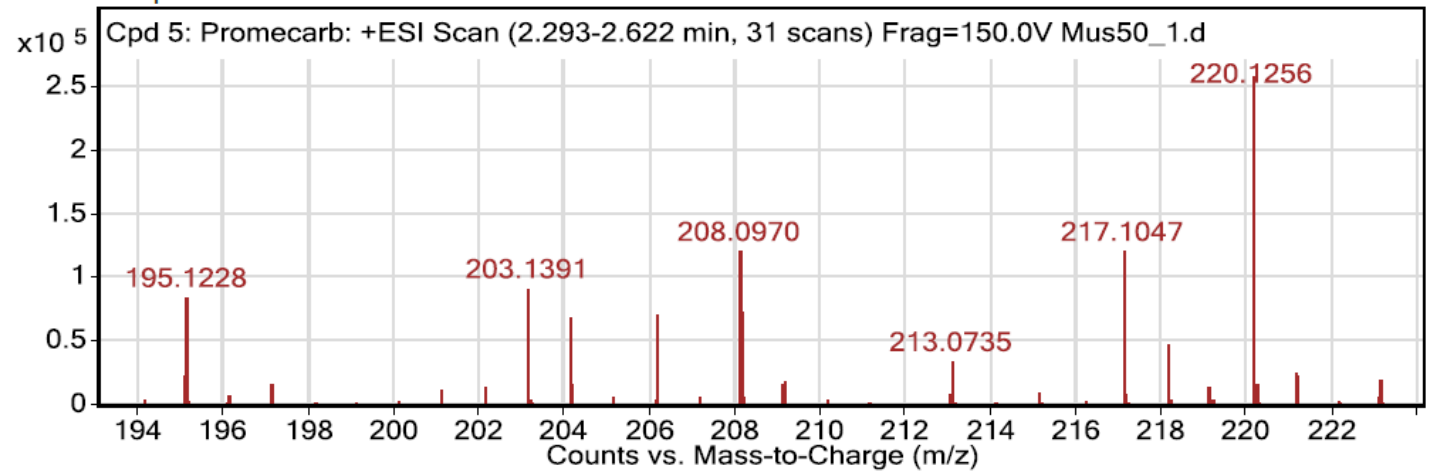
LC-MS-Q-TOF - Screening in Muscle

Name	RT	Algorithm	Mass
Promecarb	2.477	Find by Molecular Feature	207.1259

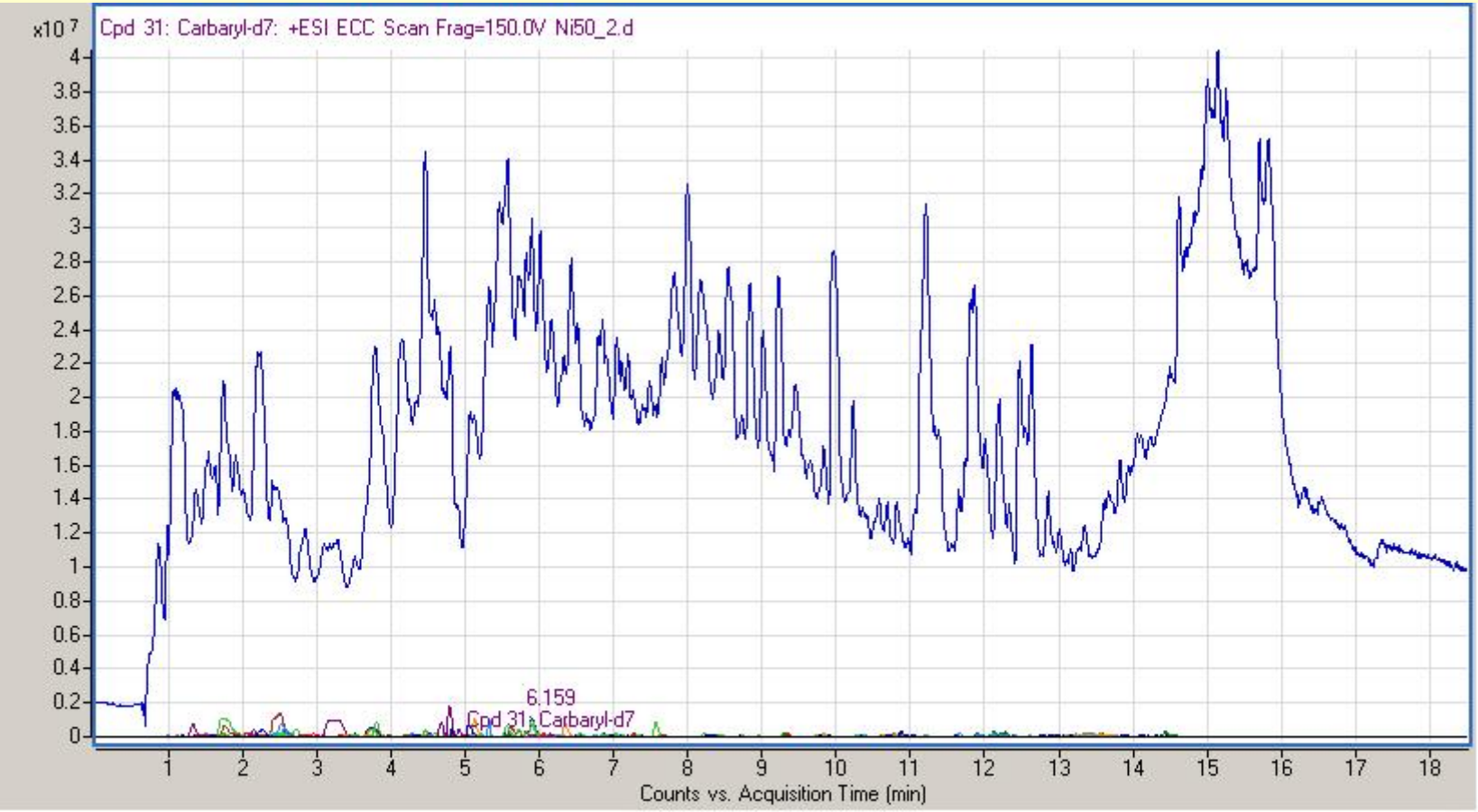
MFE MS Zoomed Spectrum



MS Zoomed Spectrum



LC-MS-Q-TOF – Screening in Kidney



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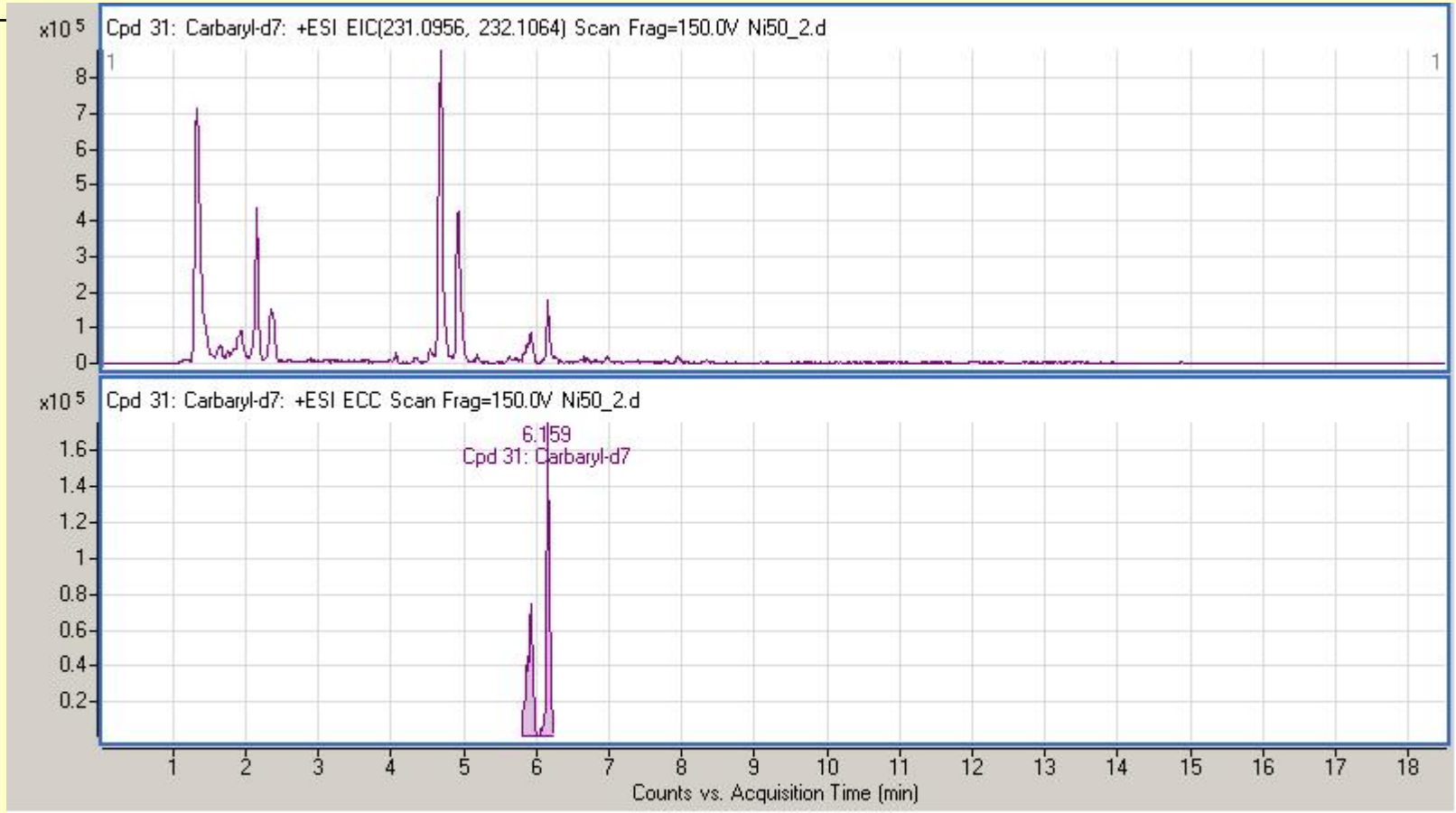
LC-MS-Q-TOF - Screening in Kidney



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Outlook

Hardware

Increased sensitivity of instruments

Resolution will be far above 50000

Mass accuracy is below 0.5 ppm

Hybrid instruments are standard

Outlook

Software

Enhanced deconvolution algorithms

Fast MS-MS and screening in one run

Confirming via auto re-analysing of doubtful results (MS-MS-experiments)