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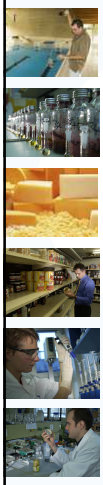

The evolution of liquid chromatography coupled to mass spectrometry from single analyte detection towards non-target screening

Anton Kaufmann
Official Food Control Authority of the Canton of Zurich
(Kantonales Labor Zürich)
Switzerland

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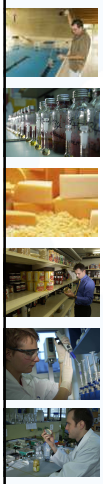

New MS based analytical strategies in veterinary drug residue control

- The challenge
- New analytical strategies (Multiresidue methods)
- High resolution liquid chromatography & mass spectrometry
- The Future: Generic detection

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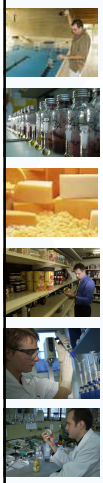
New MS based analytical strategies in veterinary drug residue control



- **The challenge**
- New analytical strategies (Multiresidue methods)
- High resolution liquid chromatography & mass spectrometry
- The Future: Generic detection

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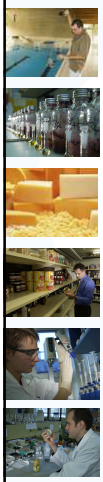
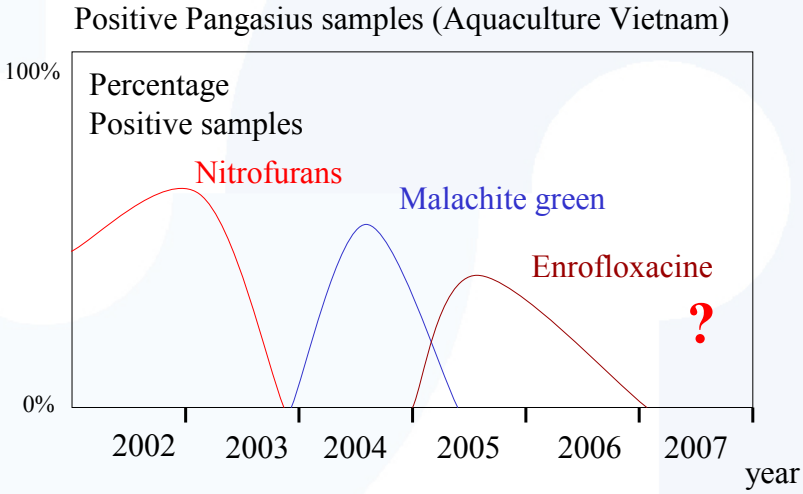
Veterinary drug analysis is a moving target





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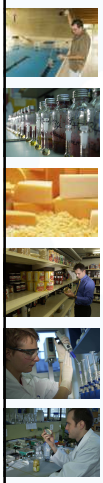
Not registered drugs in veterinary medicine?



- New emerging strains of bacteria can ruin aquaculture within a whole region or country
- Anything which reduces mortality of livestock might be used
- There are a lot of banned, yet very effective drugs (e.g. chloramphenicol, nitrofurans etc.)
- There are even more analogues which were abandoned in human medicine clinical trials

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Limitations of MS-MS based multiresidue methods

- MS-MS is a victim of its own selectivity
- MS-MS finds only analytes which have been previously tuned

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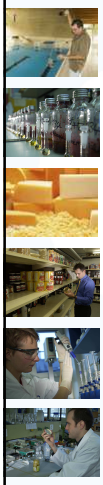
Limitation of LC-MS/MS for multiresidue methods



Multiple compounds can be monitored,

But how far can or should we go ?

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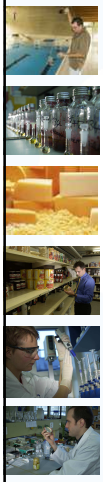
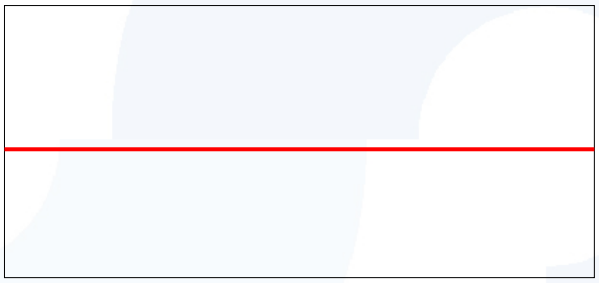
Single MRM (one analyte)



Analyte:

A



Retention time

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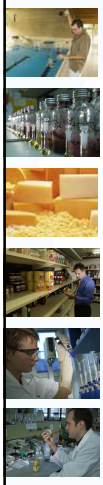
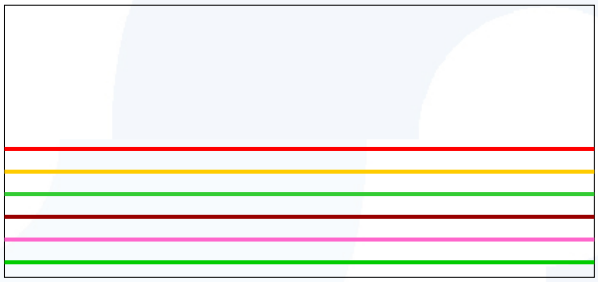
Multiple MRM (several analytes)



Analyte:

A
B
C
D
E
F



Dwell time versus number of data points across a peak

Retention time

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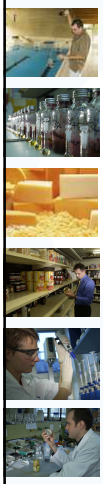
MRM windows

(about hundred analytes)



Analyte:

A
B
C
D
E
F

Retention time

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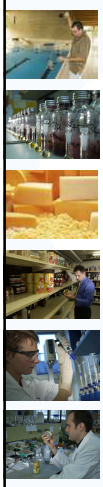
Scheduled MRM (one thousend analytes)



Analyte:

A
B
C
D
E
F
G
H
I
J

Retention time

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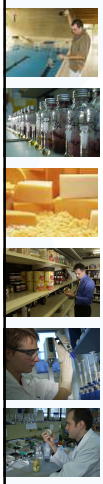
Can we or our software manage all these transitions?



- An analyte might produce more than one peak
- Some analytes might produce the same MRM
- A pH sensitive analyte might move out of a window
- A labile analyte might have degraded in the standard solution

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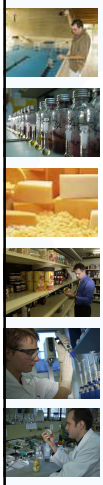
Limitation of LC-MS/MS for multiresidue methods



Monitoring dozens of MRMs reduces sensitivity.

Managing these MRMs can become a difficult task.

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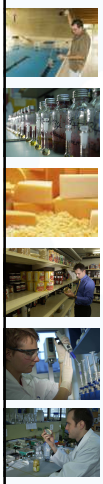
New analytical strategies in veterinary drug control



- The challenge
- **New analytical strategies (Multiresidue methods)**
- High resolution liquid chromatography & mass spectrometry
- The Future: Generic detection

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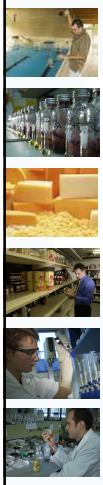
Ultra performance liquid chromatography coupled to high resolution mass spectrometry can be the answer



- Provides similar selectivity as MS/MS
- Sensitivity becomes comparable to MS/MS
- Full scan data
- Qualitative work without standards

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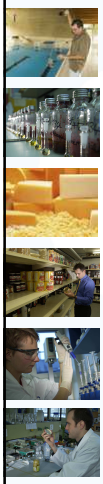
More selectivity and sensitivity by higher chromatographic separation power (UPLC)



LC
3-5 µm
particles

UPLC
1.7 µm
particles

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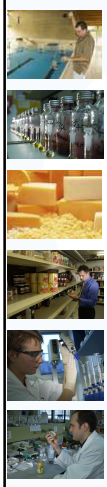
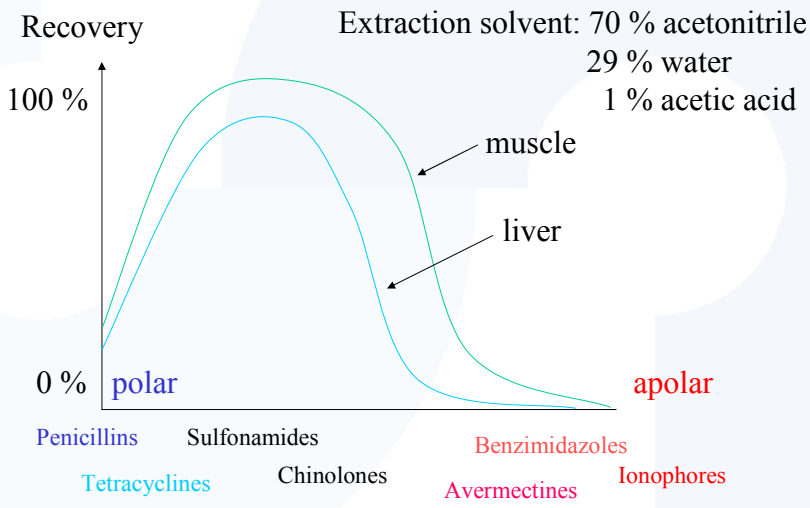
Extraction Recoveries





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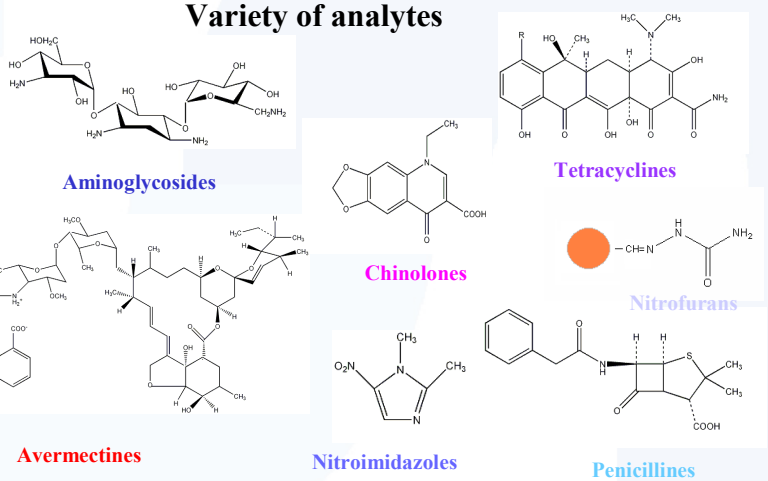
More compounds and lower concentrations

Variety of analytes



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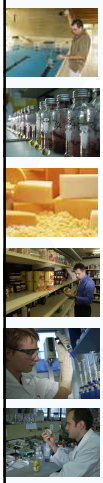
The analytical method



Conflicting extraction & clean-up requirements

- Aminoglyocsides require low pH extraction for sufficient recoveries
- Penicillines degrade at low and high pH values
- Tetracyclines require complexing agents for good recoveries

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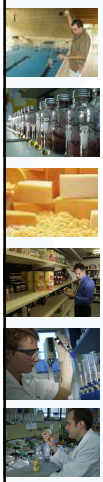
More compounds and lower concentrations



Concentration range (MRL&MRPL):

- 0.3 µg/kg Chloramphenicole
- 20 µg/kg Tetracycline in honey
- 600 µg/kg Tetracycline in kidney
- 5000 µg/kg Neomycin in kidney

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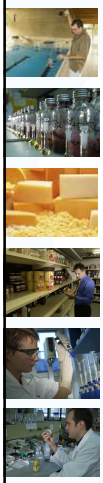
Analyte loss I: Extraction



Extracting solvent polarity does not correspond to analyte

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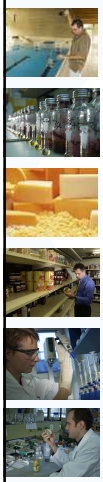
Extraction Recovery



- One single solvent can not extract the whole analyte polarity range
- Bi-polarity Extraction:
  Emulsion of two immiscible solvents permits parallel extraction

  (acetonitrile/aqueous buffer with high concentration of ammonium sulfate to induce phase separation)

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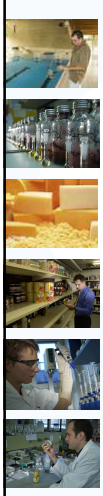
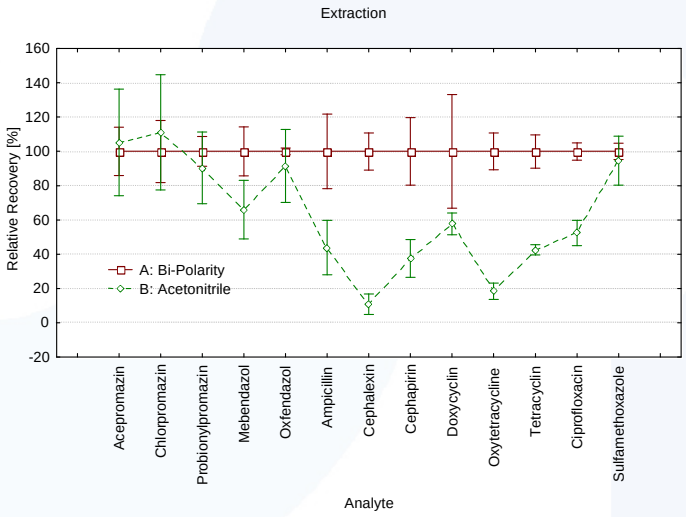
Extraction Recovery





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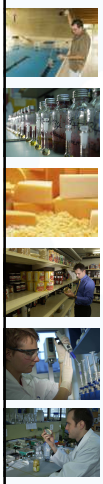
Analyte loss II: SPE



Solid phase extraction breakthrough and irreversible retention

- Polymer reversed Phase SPE (OASIS HLB)
- Solvent free loading solution
- pH control of loading solution
- Two elution steps (solvent and buffer in solvent)

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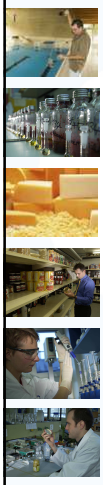
Analyte loss III: Adsorption



Adsorption by precipitating proteins and on vessel walls

- High ionic strength
- Rinsing of vessels and residues with aqueous dimethyl-sulfoxide
- Avoidance of glassware
- No evaporation till dryness, use keeper

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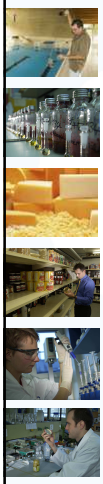
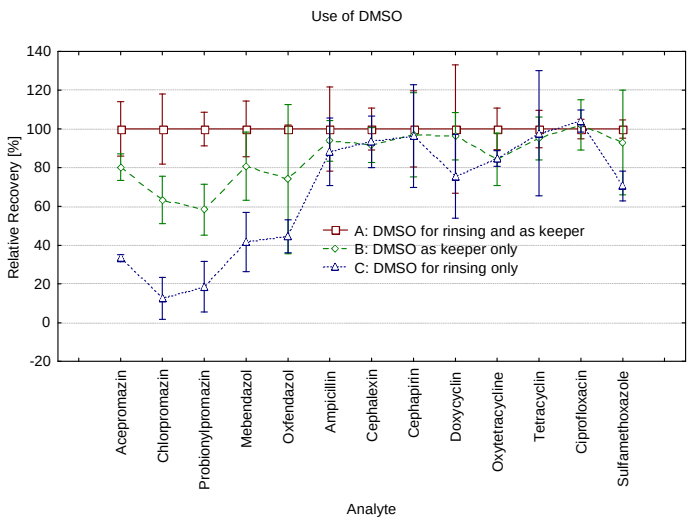
Analyte loss III: Adsorption





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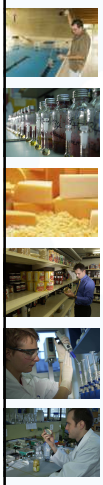
The analytical method



New extraction and clean-up method

- Bi-polarity extraction
- Generic solid phase extraction (OASIS HLB)
- Avoiding extreme pH
- Washing of precipitates and glassware to minimize analyte loss

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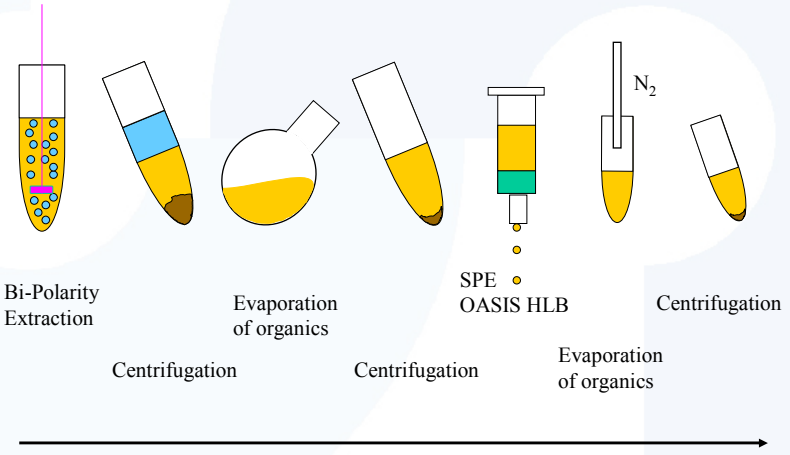
The analytical method

Workflow of the method



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Instrumentation

- LC
  UPLC (Waters)
  Column: T3; 100 * 2.1 mm 1.8 µm (Waters)
  Mobile Phase: Gradient Water/Acetronitrile + Formic acid

- MS
  LCT Premium (Waters)
  Positive mode (ESI)
  Dynamic range enhancement “DRE”

  Data processing: QuanLynx (Waters)

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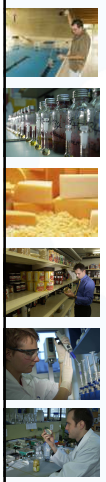
More compounds and lower concentrations



A combined approach

- New extraction and clean-up method
- High LC separation power (UPLC)
- Fast, flexible and sensitive detection (TOF)

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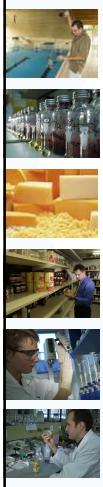
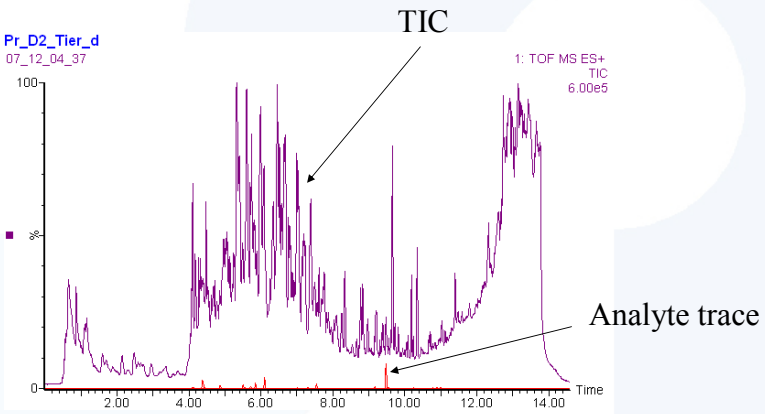
Good chromatographic resolution



Pork liver extract spiked with 100 vet. drugs



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Multiple target analysis

Chinolones: Ciprofloxacin; Danofloxacin; Enrofloxacin; Flumequin; Oxolinic acid; Enoxacin; Lomefloxacin; Nalidixic acid; Norfloxacin; Ofloxacin; Difloxacin; Sarafloxacin; Piromidic acid; Sparfloxacin; Azithromycin

Sulfonamides: Sulfagunaidin; Sulfanilamid; Sulfaquazin; Sulfathiazol; Sulfapyridin; Sulfamerazin; Sulfamethizol; Sulfadimidin; Sulfamethoxypyridazin; Sulfachlorpyridazin; Sulfachlorpyrazin; Sulfadoxin; Sulfadimethoxin; Sulfacetamid; Sulfamethoxazol; Sulfisoxazol; Sulfabenzamid; Sulfameter; Sulfamomethoxin; Sulfamoxol; Sulfanitran; Sulfaquinoxalin; Sulfasalazin; Sulfaisomidin;

Tetracyclines: Oxytetracyclin; Tetracyclin; Chlortetracyclin; Minocyclin; Doxycyclin; Demeclocyclin

Nitroimidazoles: Ipronidazol-OH; Metronidazol-OH; Dimetridazol; Ronidazol; Metronidazol; Ipronidazol; HMMNI; Misonidazol

Cephalosporines: Cefaperazon; Cefazolin; Cephalexin; Cephapirin

Avermectins: Avermectine: Ivermectin; Doramectin; Emamectin; Eprinomectin

Macrolides: Roxithromycin; Tylosin A; Erythromycin A; Tilmicosin; Spiramycin I; II; III; Oleandomycin; Josamycin; Tulathromycin;

Lincosamides: Lincosamide; Lincomycin; Pirlimycin; iso-Pirlimycin; Clindamycin; Tiamulin

Benzimidazoles: Febantel; Fenbendazol; Flubendazol; Mebendazol; Ofendazol; Oxibendazol; Parabendazol; Albendazol; Triclobendazol;

Penicillines: Ampicillin; Natrocillin; Penicillin G; Penicillin V; Dicloxacillin; Cloxacillin; Amoxicillin; Oxacillin

Tranquilizers: Acepromazin; Azaperol; Azaperon; Carazolol; Chlorpromazin; Propionylpromazin; Xylazin

Various: Acriflavin; Praziquantel; Trimethoprim; Diaveridin; Rifampicin; Pyrimethamin; Rifamixin; Virginiamycin

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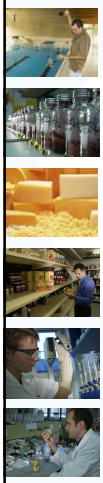
New analytical strategies in veterinary drug control



- ✓ The challenge
- ✓ New analytical strategies (Multiresidue methods)
- **High resolution liquid chromatography & mass spectrometry**
- The Future: Generic detection

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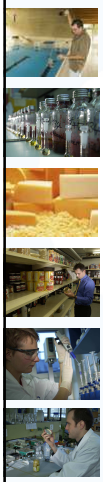
Benefits of High Resolution Mass Spectrometry (TOF or Orbitrap)



- High selectivity (approaching MS-MS)
- Full scan data “a posteriori hypothesis”
- “No need for reference substances”
- Retrospective analysis

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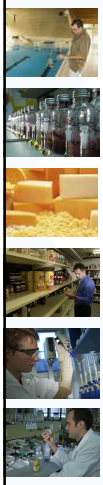
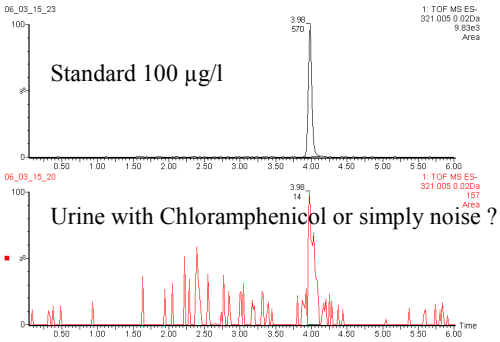
Confirmation of a Chloramphenicol finding (urine sample)



Chloramphenicol trace (ESI neg.)



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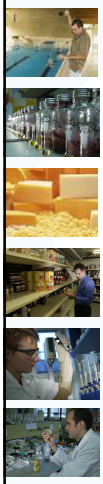
Confirmation of positive findings with TOF



Chloramphenicol is known to be metabolized to
Chloramphenicol glucuronide

Commercially not available as reference substance

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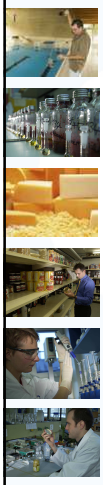
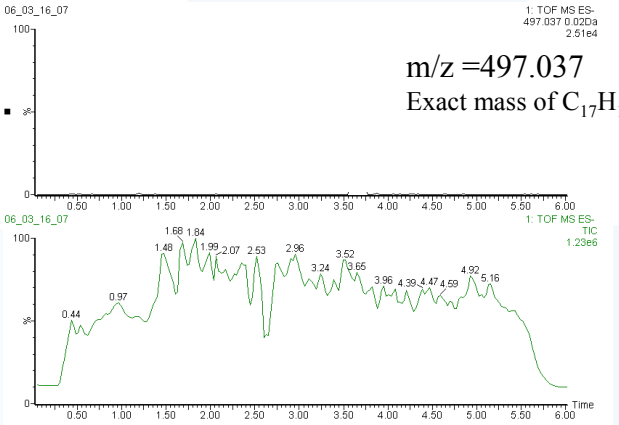
A-posteriori („inject now, ask later“)



Confirmation of positive findings



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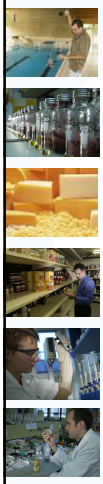
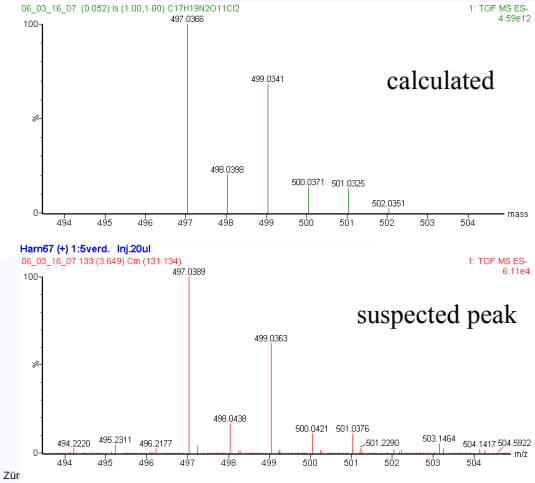
A-posteriori („inject now, ask later“)



Isotopic pattern of chloramphenicol glucuronide



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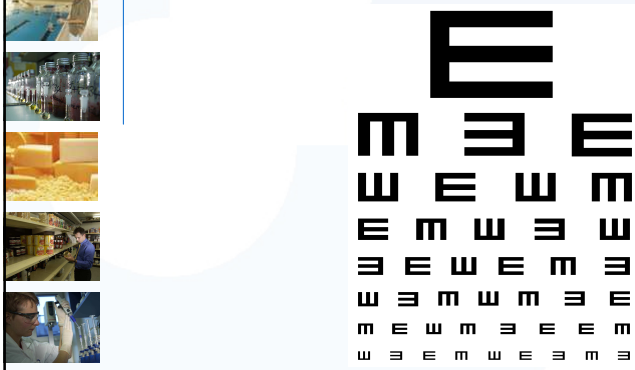
How much “high resolution” do we need ?



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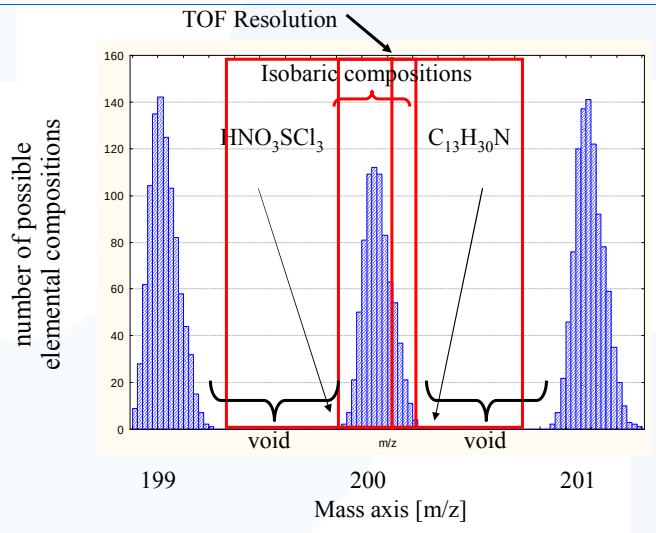
Required resolution



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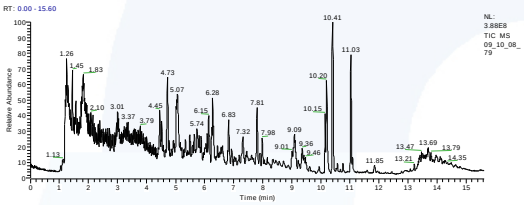
Second Generation TOF Resolution
10'000 FWHM

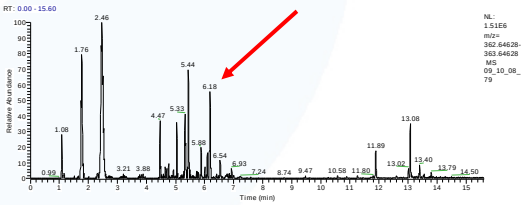





Liver extract spiked with marbofloxacin (TIC)



Liver extract spiked with marbofloxacin (m/z = 363.14628 window: 500 mD)



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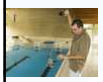
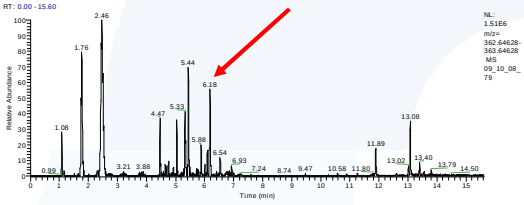
Second Generation TOF Resolution
10'000 FWHM

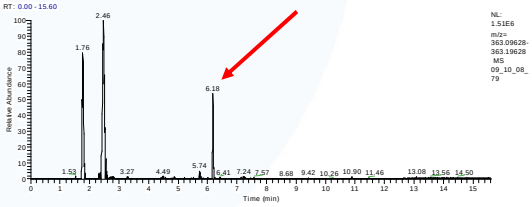





Liver extract spiked with marbofloxacin (m/z = 363.14628 window: 500 mD)



Liver extract spiked with marbofloxacin (m/z = 363.14628 window: 50 mD)



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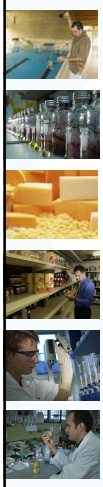
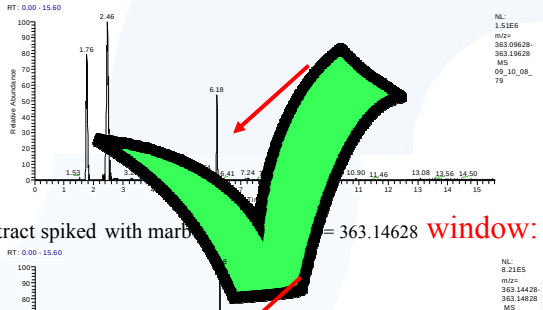
Second Generation TOF Resolution
10'000 FWHM



Liver extract spiked with marbofloxacin (m/z = 363.14628 window: 50 mD)



Liver extract spiked with marb = 363.14628 window: 2 mD)

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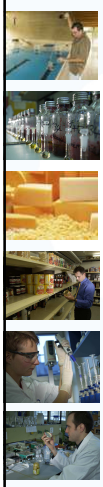
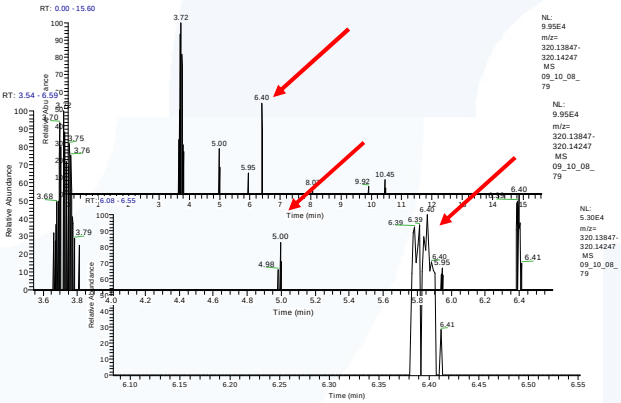
Second Generation TOF Resolution
10'000 FWHM



Liver extrakt spiked with norfloxacin (m/z = 320.14047 window: 2 mD)



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Second Generation TOF Resolution 10'000 FWHM

Norfloxacin spectrum (m/z = 320.14047)

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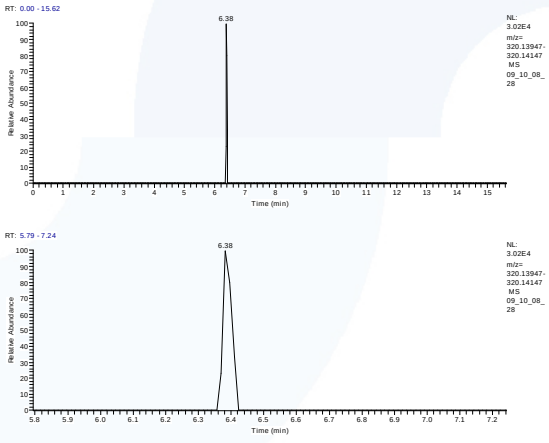
Maximum resolution FWHM = 100'000

Liver with norfloxacine m/z = 320.14047 1 mDa



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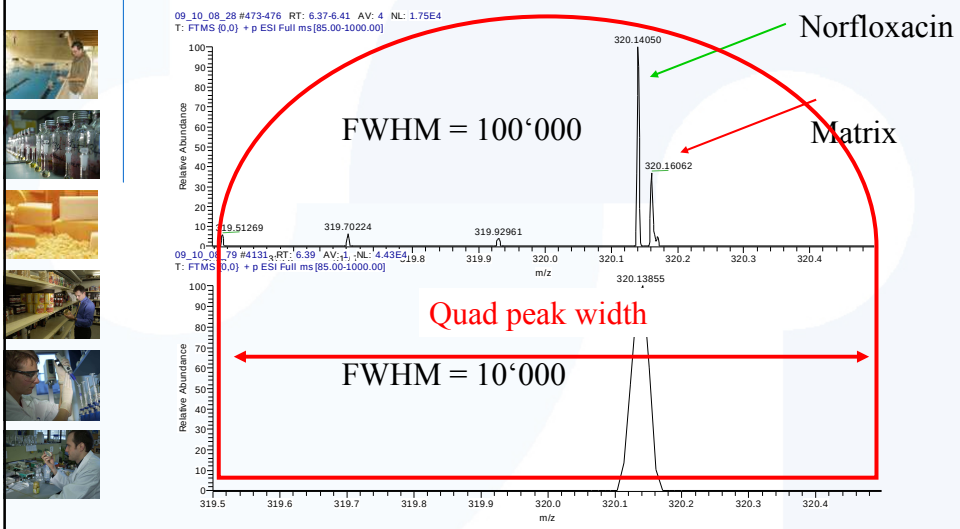
Comparison of Spectra



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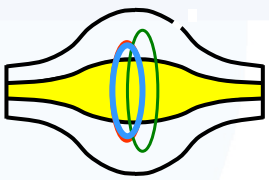
Switching to single stage Orbitrap (Exactive)



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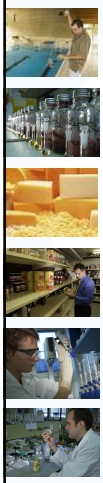

- Resolution to obtain MS/MS selectivity
- Adapting an existing analytical method
- Validation HRMS versus MS/MS

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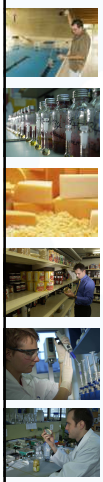
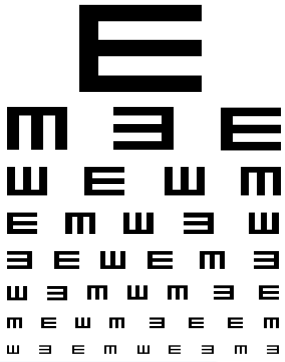
Required resolution to compete with MS/MS ?





The Orbitrap permits resolutions from 10’000 – 100’000 FWHM

Generic answer not particular analyte matrix combination

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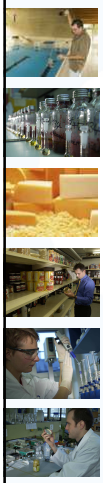
Comparing apples to oranges



Comparing

2 dimensional data (MS/MS)
versus
1 dimensional data (HRMS)

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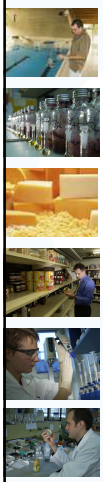
The approach



- Producing extracts from blank matrices
- Separating the extracts by UPLC
- Monitoring dummy transitions (MS/MS)
  dummy exact masses (HRMS)
- Make observed peak areas comparable

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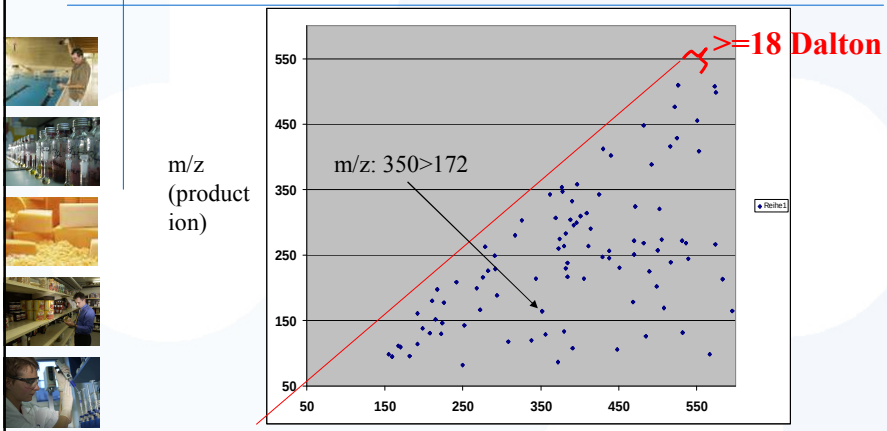
Dummy transitions (MS/MS)



two dimensions

Some of these transitions are probably impossible
(not existing elemental composition for neutral loss)

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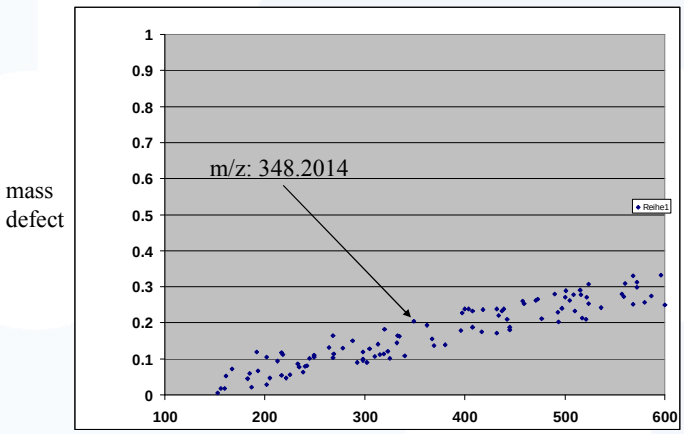
Dummy exact masses (HRMS)



one dimension

Only a relatively narrow mass defect range was tested

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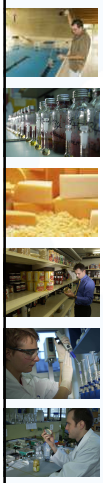
Comparing apples and oranges



- Select 6 drugs (tetracyclins, chinolons and sulfonamides)
- Determine their response “peak area / concentration”
- Calculate average response for these 6 drugs
- Divide each dummy peak area by the average response
- Do this for MS/MS and HRMS dummy peaks

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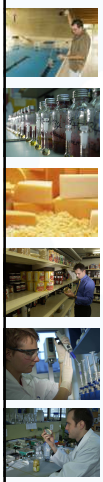
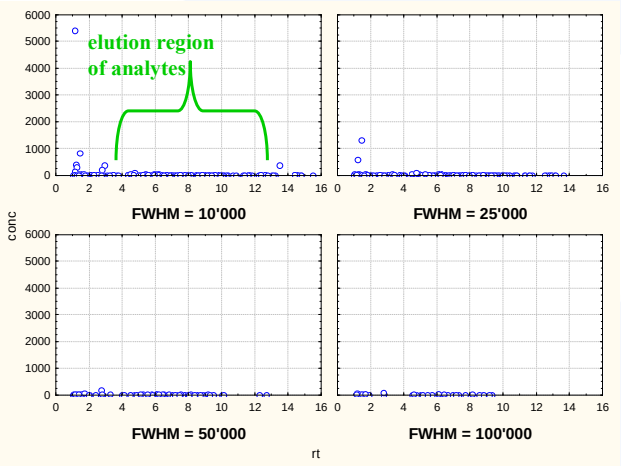
Effect of resolution





Honey matrix

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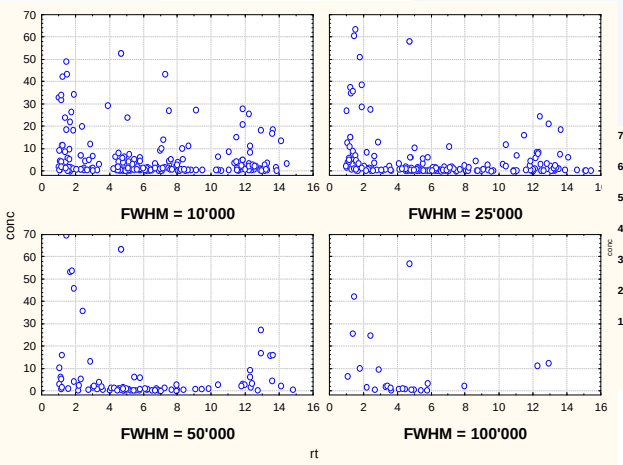
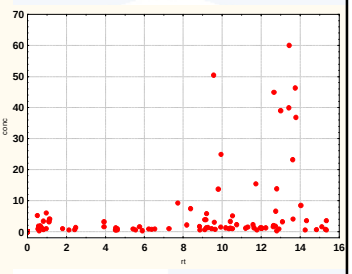
HRMS versus MS/MS





MS/MS

Liver
(difficult matrix)

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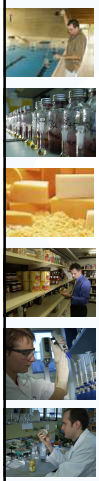
HRMS surpasses MS/MS selectivity



at 50'000 FWHM

2 data points per second (UPLC capable)

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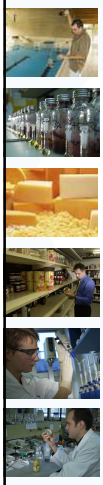
Another gate to be opened



Extracts which were ok for the TOF are not for the Orbitrap

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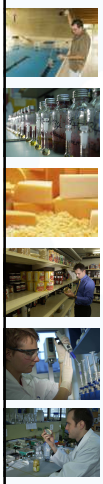

- ✓ Resolution to obtain MS/MS selectivity
- Adapting an existing analytical method
- Validation HMRS versus MS/MS

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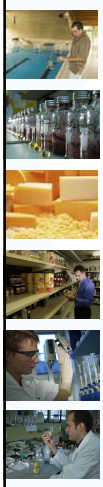
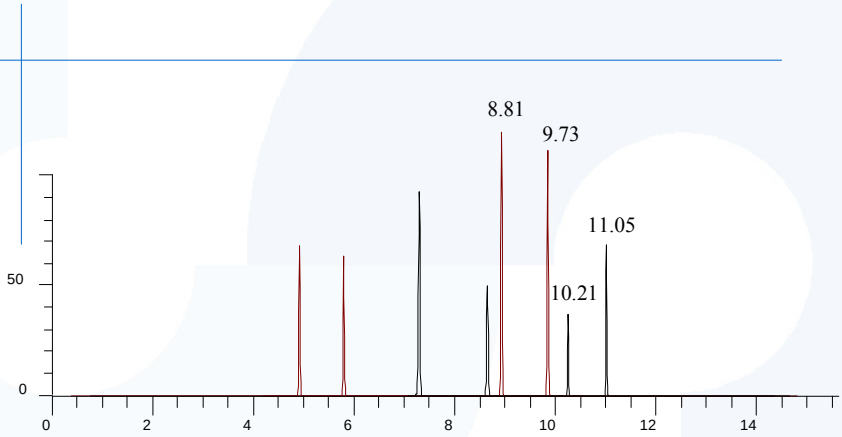
Signal suppression





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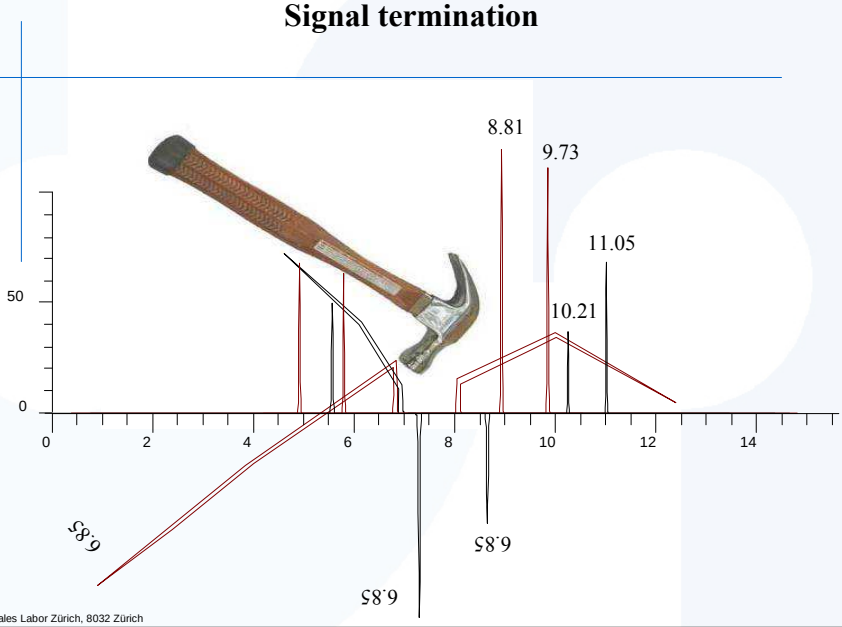
Signal suppression
Signal termination



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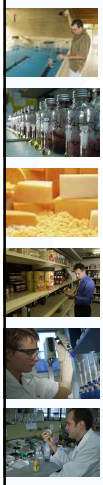
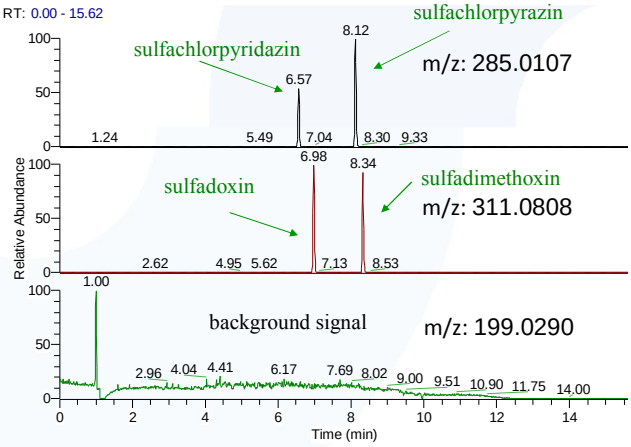
Standard: 10 µl injection volume





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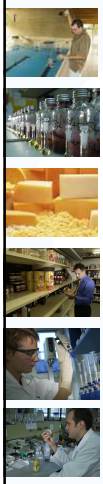
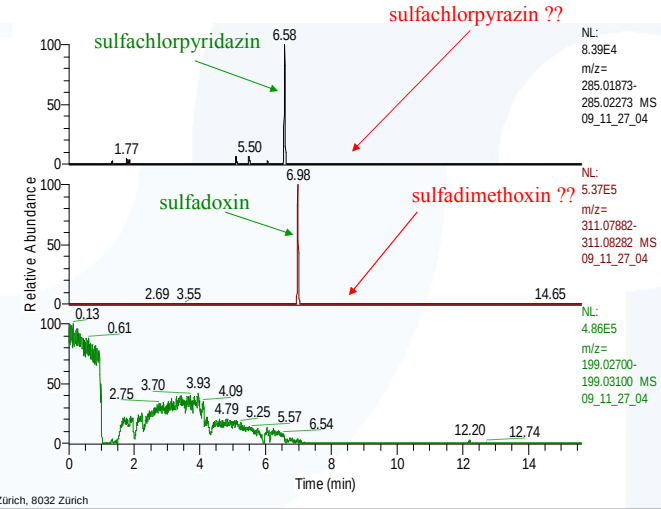
Spiked kidney extract: 10 µl injection volume





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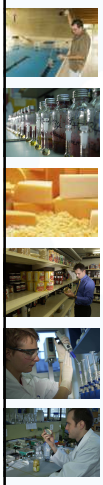
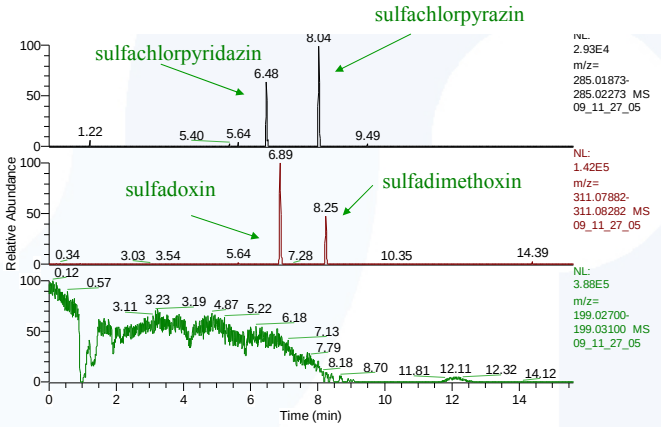
Spiked kidney extract: 1 µl injection volume





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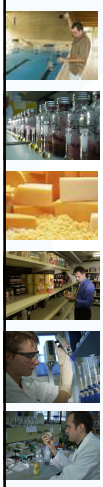
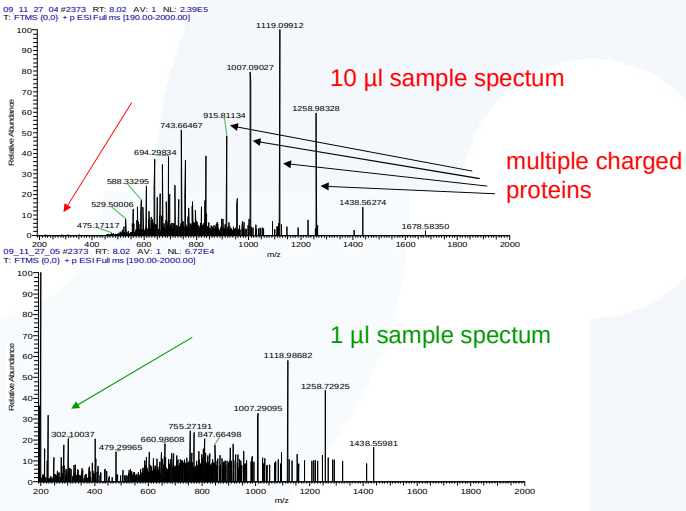
Low mass ion discrimination





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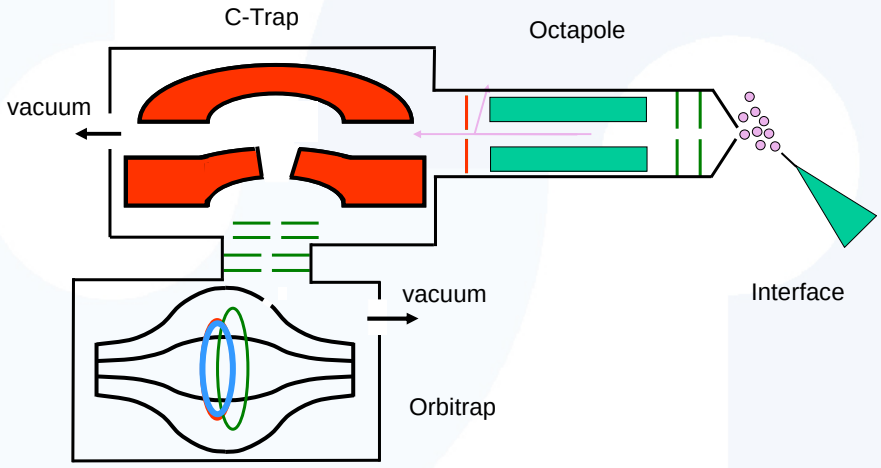
Principle of Orbitrap Operation



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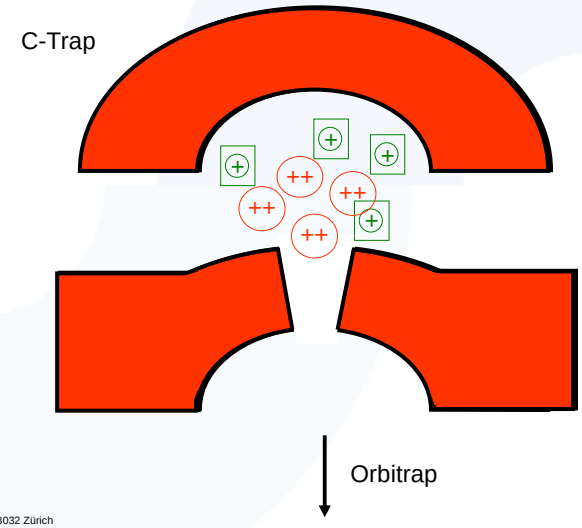
Postulated phenomena: post interface signal suppression



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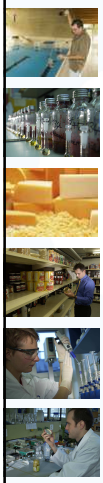
How to reduce post interface signal suppression



- Resolution >= 50‘000 FWHM
  low scan start. e.g.: m/z = 50
  reduced number of ion in C-trap. e.g.: < = 1’000’000
  limited C-trap filling time. e.g. <= 50 ms

- less injection volume
  more extensive protein removal

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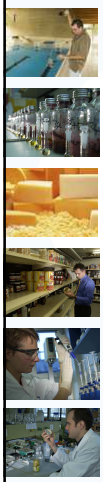
Low protein method



- Multiresidue methods do not like extensive clean-up
  (more than 100 analytes in different matrices)

- Testing almost every known protein removal method

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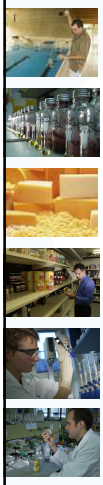
Key elements of modified method



- High ammonium sulphate concentration in extraction buffer
- Bipolarity extraction (buffer and acetonitrile)
- Evolute ABN SPE (Biotage) narrow pore size
- Kinetex Core-Shell column 150*2.1 mm; 2.6 µm

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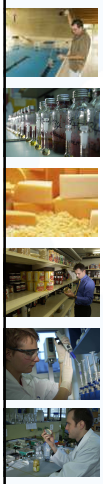

- ✓ Resolution to obtain MS/MS selectivity
- ✓ Adapting an existing analytical method
- Validation HMRS versus MS/MS

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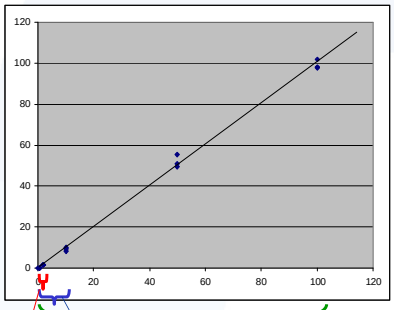
Comparison among different techniques
Coefficient of determination (r^2)

Levels:
1/10 µg/l
3.3/33 µg/l
10/100 µg/l
33/333 µg/l
100/1000 µg/l



order of magnitude	1	1.5	2
Orbitrap	0.99670	0.99584	0.99188
TOF	0.82102	0.92902	0.93474
MS/MS	0.99684	0.97916	0.98559

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Unified multiresidue veterinary drug analysis

- Screening
  (sensitive; non-target)
- Quantification
  (comparable to MS/MS)
- Confirmation
  (FWHM > 20‘000 produces 2 identification points
  Cone induced fragmentation and adducts produce additional ions: 4 to 6 identification points)

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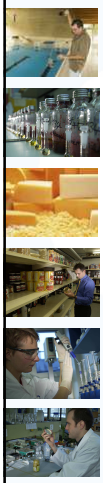
New analytical strategies in veterinary drug control



- ✓ The challenge
- ✓ New analytical strategies (Multiresidue methods)
- ✓ High resolution liquid chromatography & mass spectrometry
- **The Future: Generic detection**

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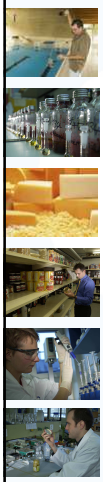
Our aim



- Food scandal ready method
- Capability to monitor metabolites or drugs where no reference substances are commercially available
- Impossible dream:
  "No drugs at relevant levels are present in this sample"

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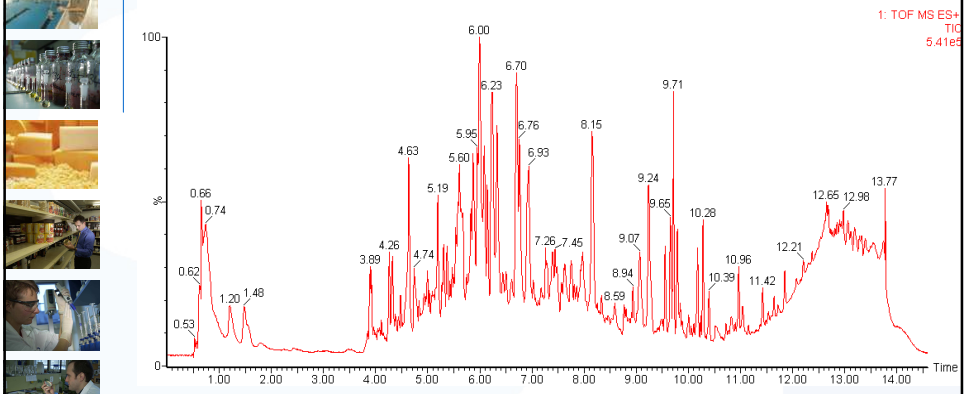
The generic detector



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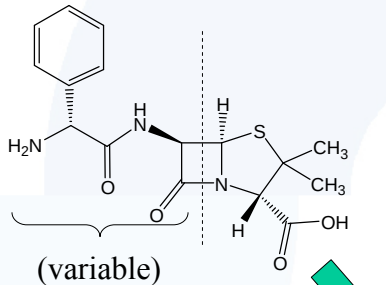
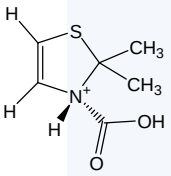
Non-target drug group specific screening
Penicillines



(variable)

Amoxicillin



CID Fragment: $C_6H_{10}NO_2S$
$[M+H]^+ = 160.043$

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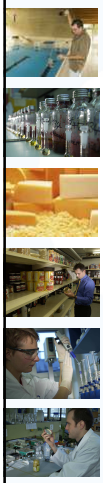
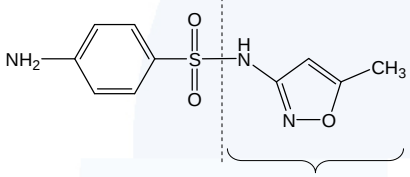
Non-target Drug group specific screening
Sulfonamides

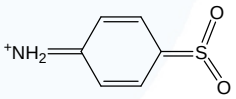




Sulfamethoxazole

R (variable)

CID Fragment:



$C_6H_6NO_2S$
$[M+H]+ = 156.012$

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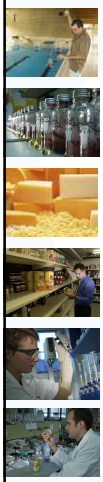
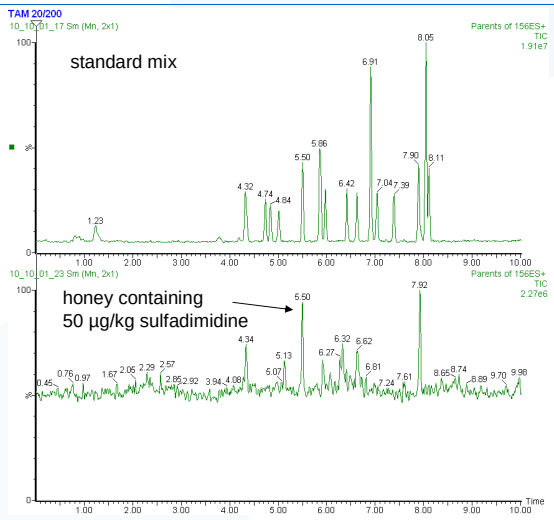
MS/MS
Precursor scan sulfonamides





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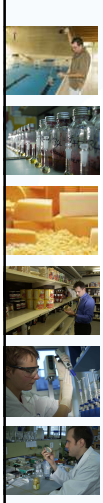
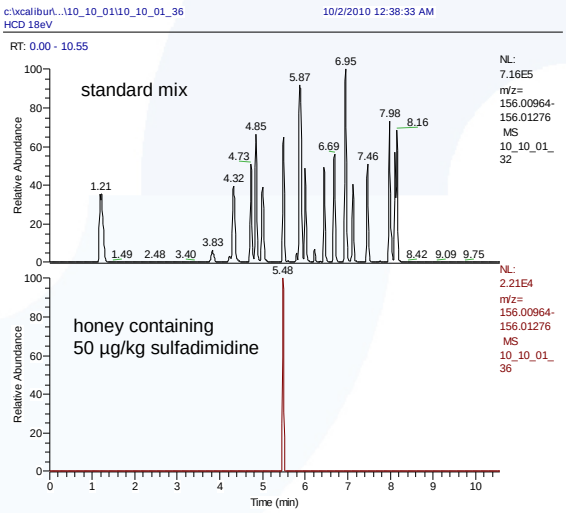
HRMS
at 50‘000 FWHM





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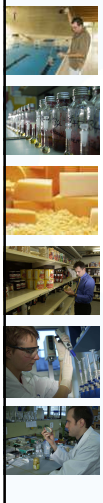
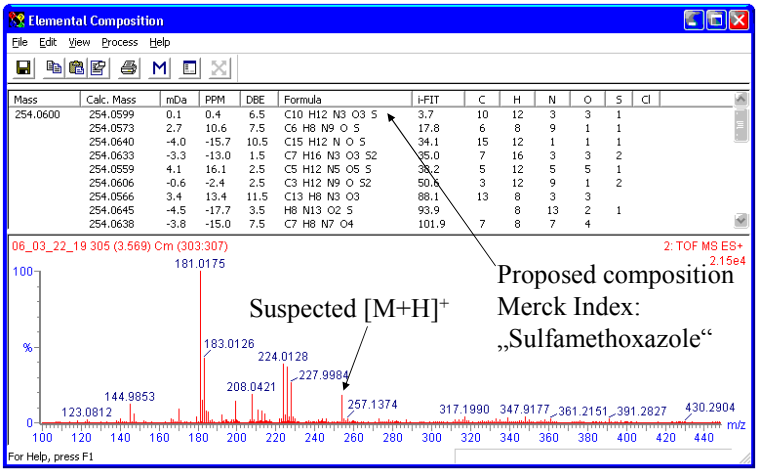
Drug group specific screening
Sulfonamides





Suspected $[M+H]^+$

Proposed composition
Merck Index:
„Sulfamethoxazole“

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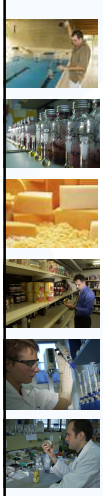
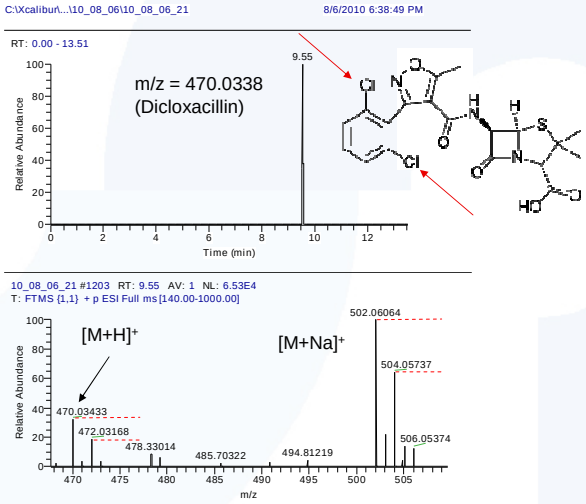
HRMS
Searching for chlorine isotopic patterns





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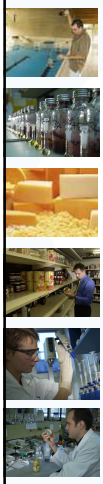
Conclusion



- Appropriate sample clean up method is required
- Hardware is an issue, but software is the real limitation
- High resolution full scan MS Data is an alternative to LC-MS/MS
- Generic, intelligent detection is needed

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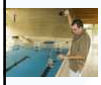
We are not yet there !






But we keep moving !