



# **Application of LC-QTOF in the Routine Screening Analysis of Veterinary Drugs and Evaluation of LC-QTOF Data**



# Outline

- **Introduction**
- **Equipments**
- **Data Evaluation of QTOF Data**
- **Summary**

# Introduction: Screening methods

## Commission decision 2002/657:

- method used to detect the presence of a substance or class of Substances at the level of interest.
- high sample throughput
- They are specially designed to avoid false compliant results.

# Introduction: Screening methods

- **Screening methods**
  - **Target Screening – I know what I'm looking for**
    - Tuned MRMs
  - **Total unknown screening**
    - No idea
    - No in-house database including retention times etc.
    - No standard compounds
    - Just scan data or spectra
  - **Semi unknown screening – semi target screening**
    - Maybe an idea
    - In-house database including retention times, spectra etc.
    - Standard substances

# Introduction: Screening methods

- Screening for single compounds, single target screening

**LC-MS, LC-MS/MS, GC-MS, GC-MS/MS**

- Multi-screening within a substance group with similar chemical-physical properties, multi target screening

- $\beta$ -agonists, benzimidazoles, ionophores, avermectines etc.

**LC-MS, LC-MS/MS, LC-QTrap, GC-MS, GC-MS/MS**

- Comprehensive Multi-screening covering different groups of pharmacological active substances combined within one method, Multi Semi Target and Unknown Screening

**LC-QTOF, LC-Orbitrap**

# Introduction: Screening methods

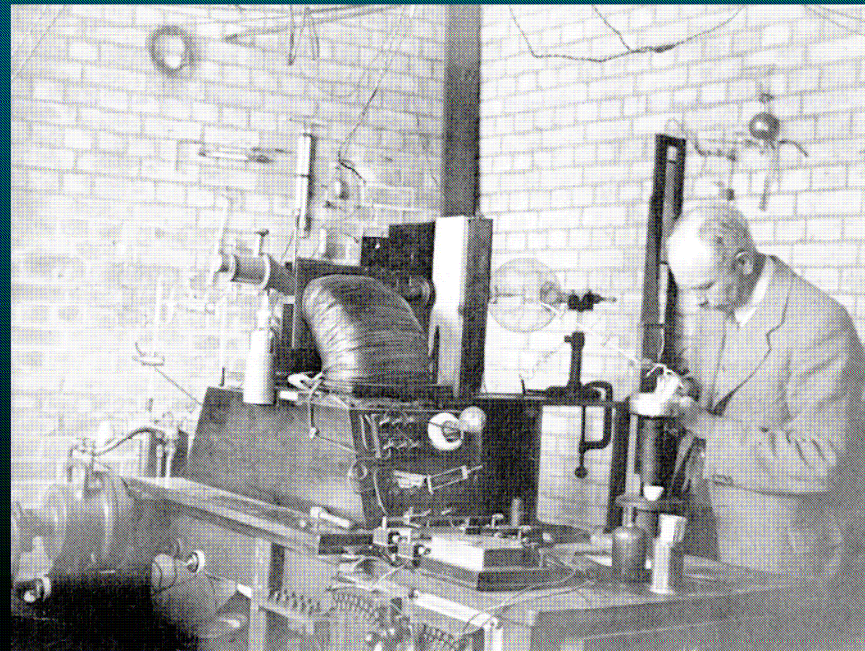
- **Screening methods**
  - **Biological – detect cellular responses to the analytes (e.g. inhibition of bacterial growth)**
    - not selective
    - can cover several classes of analytes
    - identification of individual substances is not possible
  - **Biochemical – detect molecular interactions between analytes and antibodies, receptor proteins or receptor DNA (ELISA, RIA, Biochips, SPR)**
    - may be selective for a class of substances having similar structure or binding sites
    - in some cases analyte-specific
  - **Physicochemical instrumental methods – distinguish the analytes by chromatographic separation and detect signals related to molecular characteristics (UV, FLU, FID, ECD, MS)**
    - analyte-specific





## Nobelpreis für Physik 1906

Thomson J.J. On the Masses of the Ions in  
Gases at Low Pressures *Philosophical  
Magazine*, 1899, 48:295, p.547-567



Massenspektrometer von Francis William Aston 1921, University of Cavendish  
(Watson JT, Sparkman OD Introduction to Mass Spectrometry, Wiley 2007)





# Instruments



QTrap



Orbitrap



Q-TOF



LC-MS/MS



LC-MS/MS





- **Single MS**
  - + **Low costs**
  - + **Easy to handle**
  - + **Provides molecular ion peak and/or mass spectrum by CID in the source region**
  - **Poor sensitivity and selectivity**



## ■ Tandem or triple Quad MS

- High costs ?
- + Easy to handle
- + Easy quantitation
- + Very high sensitivity and reliability
- + Good robustness
- + Method of choice for quantitation and confirmation
- + Different scan modi MRM, product ion scan, neutral loss, precursor scan
- + New instruments more than **1000 MRMs, 555 MRMs per second**
- + Switching between positive – negative mode < 10 ms
- + Large dynamic range > 5 magnitudes
  
- High costs ?
- **You see what you know**
- Screening analyses are usually performed as **multi-target screening** with a large set of target substances providing selectivity by predefined MRMs  
Each MRM has to be optimized: very time- and work-consuming
- **Reference Substances are required**






- **Triple-Quad Ion Trap Hybrid MS**
  - IDA (Information depending acquisition) experiments
  - Full scan  $\Rightarrow$  Enhanced Product ion scan is triggered
  - Library search  $\Rightarrow$  **Target and semi Target screening**

- **TOF-MS, QTOF-MS (HRMS)**

- High costs
- Evaluation of results seems more time-consuming
- Large data files: ~ 200 – 800 MB (QTOF) – 800 kB (XEVO)
- Sensitivity ~ tenfold inferior to triple Quad MRMs
- **limited dynamic range (4 magnitudes in high resolution mode)**
- Generic sample preparation method
- + Acquisition of accurate molecular masses allows high selectivity in various matrices without the need to define a set of target substances prior to analysis
- + Accurate determination of isotope pattern
- + Identification of molecular formula
- + General unknown screening is possible
- + CID-spectra, Product Ion spectra (QTOF)
- + **Data can be reprocessed for additional compounds even after years (if the data can be stored over years)**
- + Library search
- + Quantitation

## **Practical Relevance of QTOF instruments in Routine Control for Screening Purposes of residues of veterinary drugs in matrices of animal origin?**

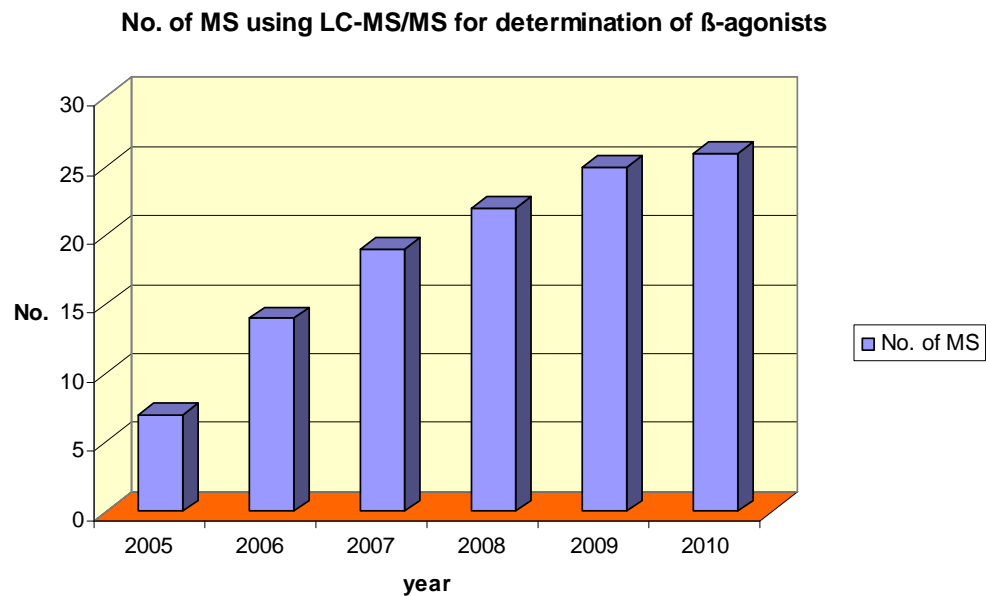
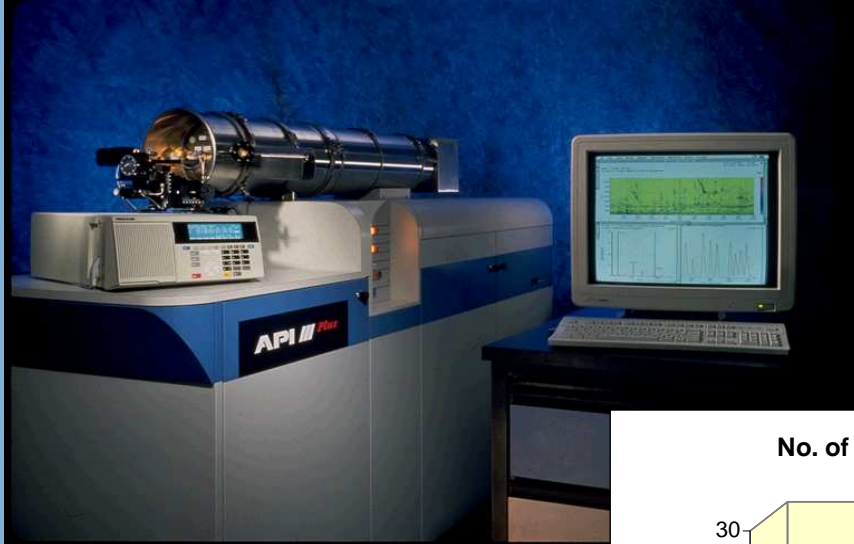
- **Everybody talks about**
- **increasing number of publications**
- **but who uses in routine?**

-  A question of results – false positive and false negative results
-  A question of time and costs
-  A question of data evaluation – find a routine algorithm
-  A question data acquisition (in particular for Auto-MS/MS measurements)
-  A question of generic sample preparation





## The first API III – the first TripleQuad in 1989

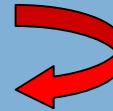


# General Strategies for QTOF-Data evaluation

- **Metabolomic/Chemometric approach (Aldo Polettini, general unknown Target screening)**
  - **Discrimination of compounds with identical molecular formula**
    - **Starting from the mass of unknown compounds, metabolites corresponding to a pre-defined biotransformation are calculated and EIC are extracted**
    - **Calculation of the number of functional groups for each candidate using special software (e.g. E-Dragon)**
    - **The presence of metabolites is matched with functional groups data in order to exclude candidates whose structure is not compatible with observed biotransformations**
    - **E.g. loss of a methyl group from a structure not bearing methyl groups**
    - **A filter is applied based on a mathematical model correlating rel. Rt with a number of parameters calculated for each candidate substance on the base of SMILES (simplified molecular input line entry syntax)**

# General Strategies for QTOF-Data evaluation

- **Library search approach (target and semi target screening)**
  - How many compounds should be covered ?
  - In-house database - commercial database (Metlin, NIST, PubChem, ChemSpider)
  - Just accurate masses - spectra
  - What about compounds with identical molecular formula?
  - Retention time is mandatory
  - Reference standards are required



## What, How Many, How Much, In What

### ➡ What are you trying to detect

Compound class, polarity, solubility, ionisation

### ➡ How many compounds

### ➡ How much and over what range

trace level – impurities

high resolution (4 magnitudes) – vs. extended dynamic range (5-6 magnitudes)

### ➡ In What matrix

Complexity of matrix, ion suppression, salts, co-eluting components



## Data Acquisition – Acquisition tab Auto MS/MS – Precursor Selection I

- You can exclude precursor masses after a MS/MS spectra were acquired. The system has time to fragment other precursors of interest.

Spectral Parameters | Collision Energy | Precursor Selection I | Precursor Selection II | Preferred/Exclude

2 Max Precursor Per Cycle

Precursor Threshold

Abs. Threshold 1000 counts

Rel. Threshold (%) 0.001 %

Active Exclusion

☒ Enabled

Excluded after 1 Spectra

Released after 0.5 min

Static Exclusion Range List

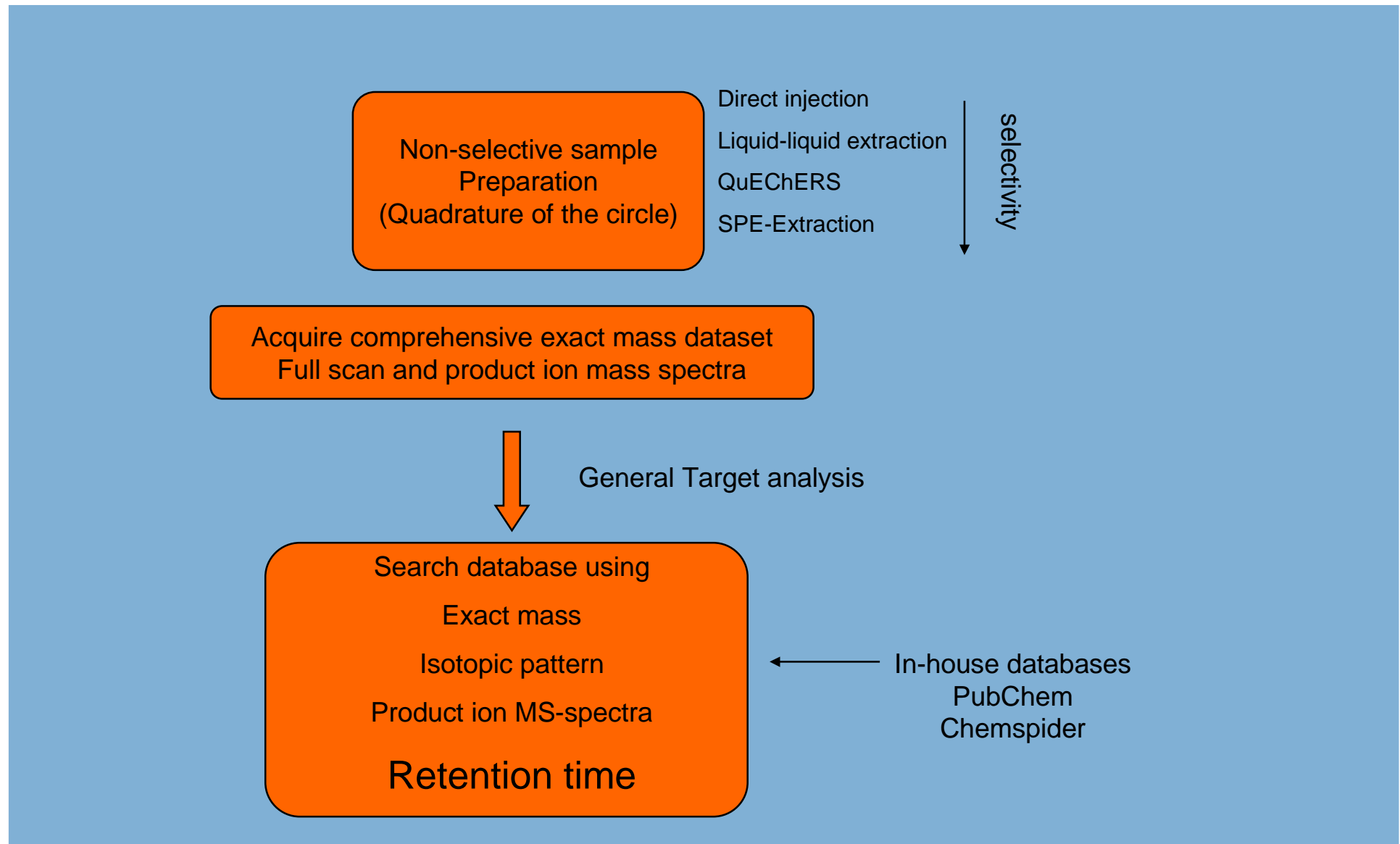
Static Exclusion Range Table	
Start m/z	End m/z
100	200

- Static exclusion helps to provide more time for the system to choose precursors of interest.

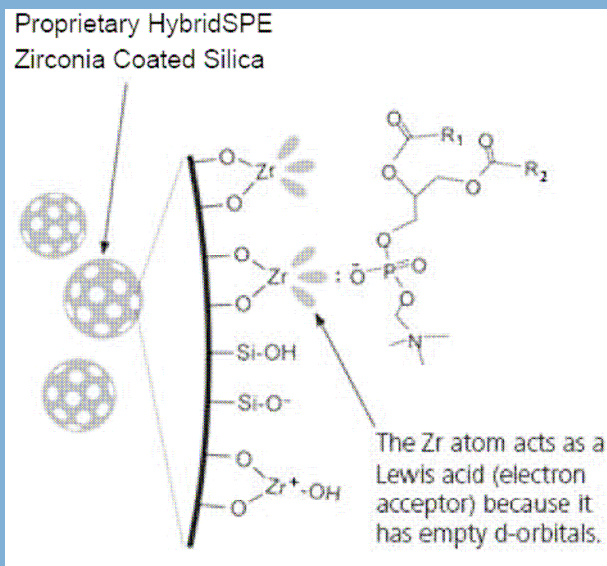
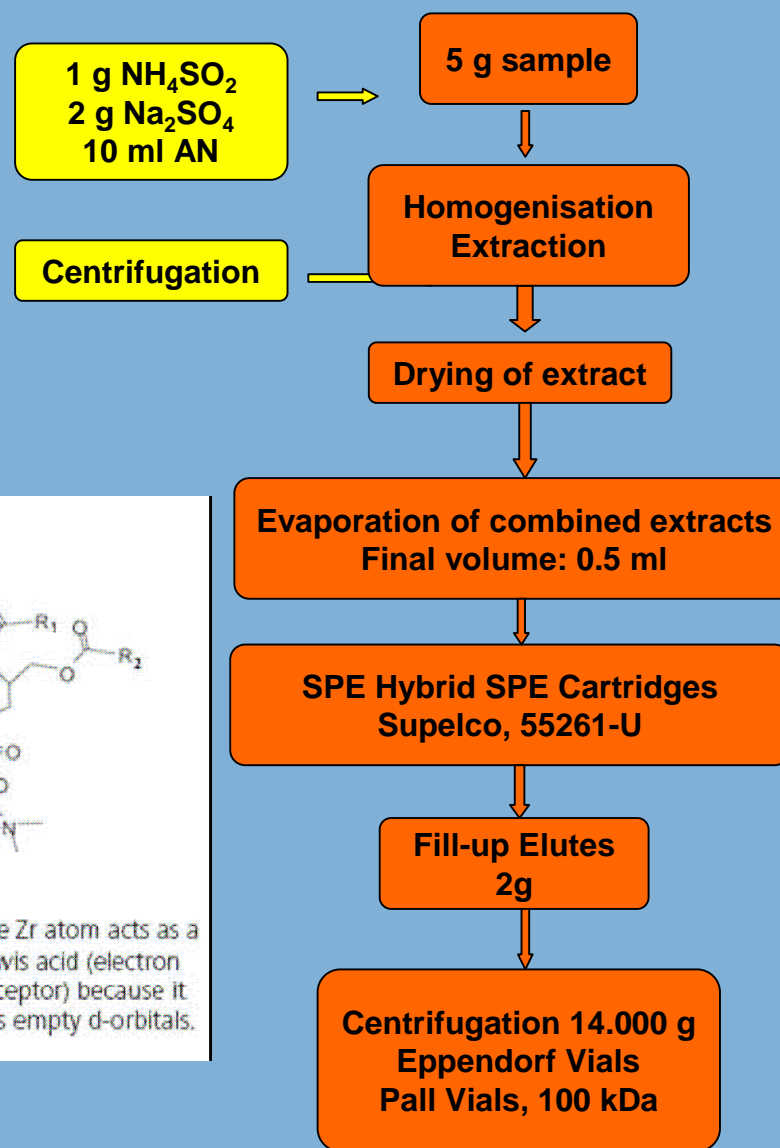




# General workflow for substance group overlapping methods by means of QTOF/Orbitrap/Multi-MRM

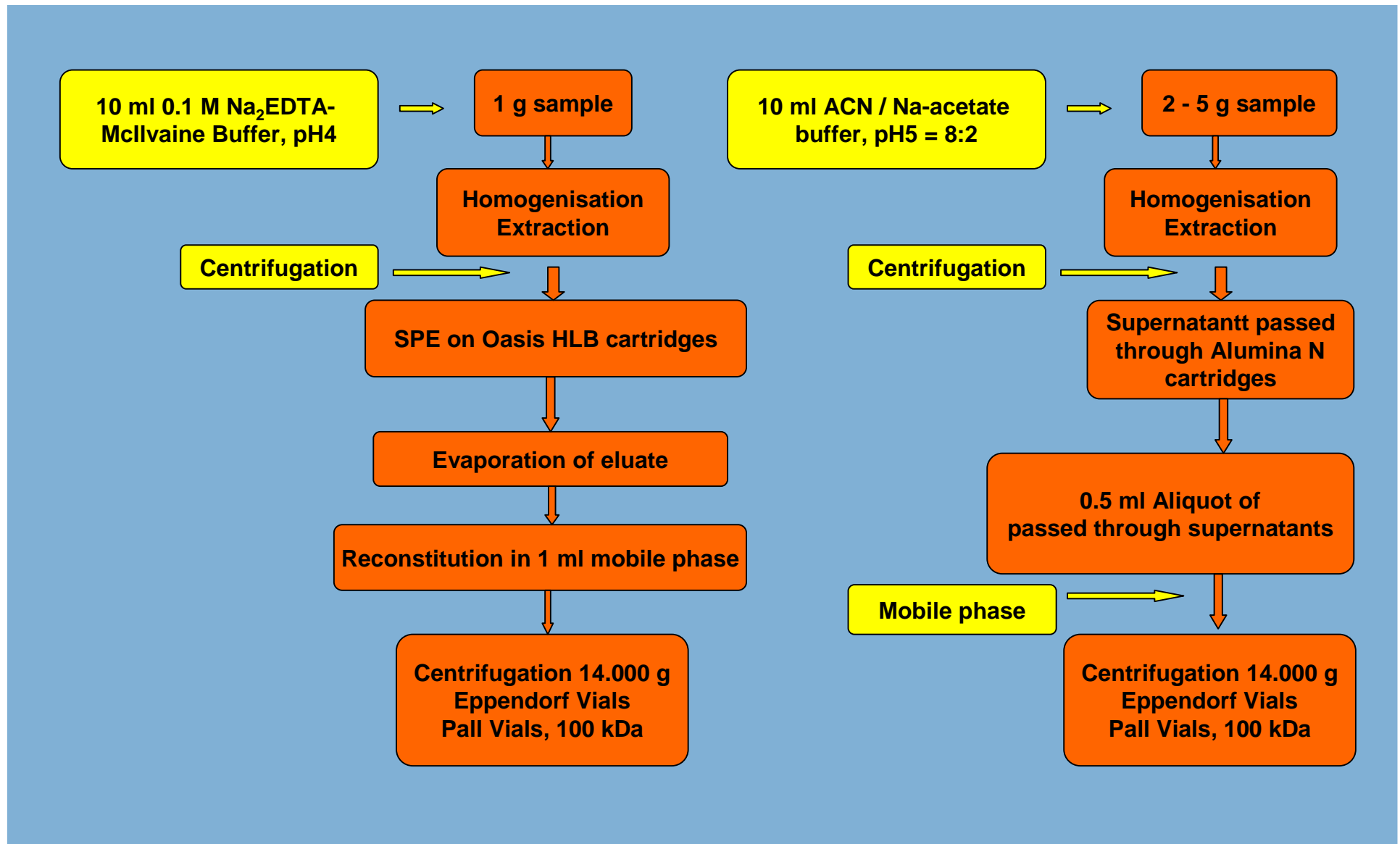


# Generic sample preparation for substance group overlapping methods by means of QTOF/Orbitrap/Multi-MRMs





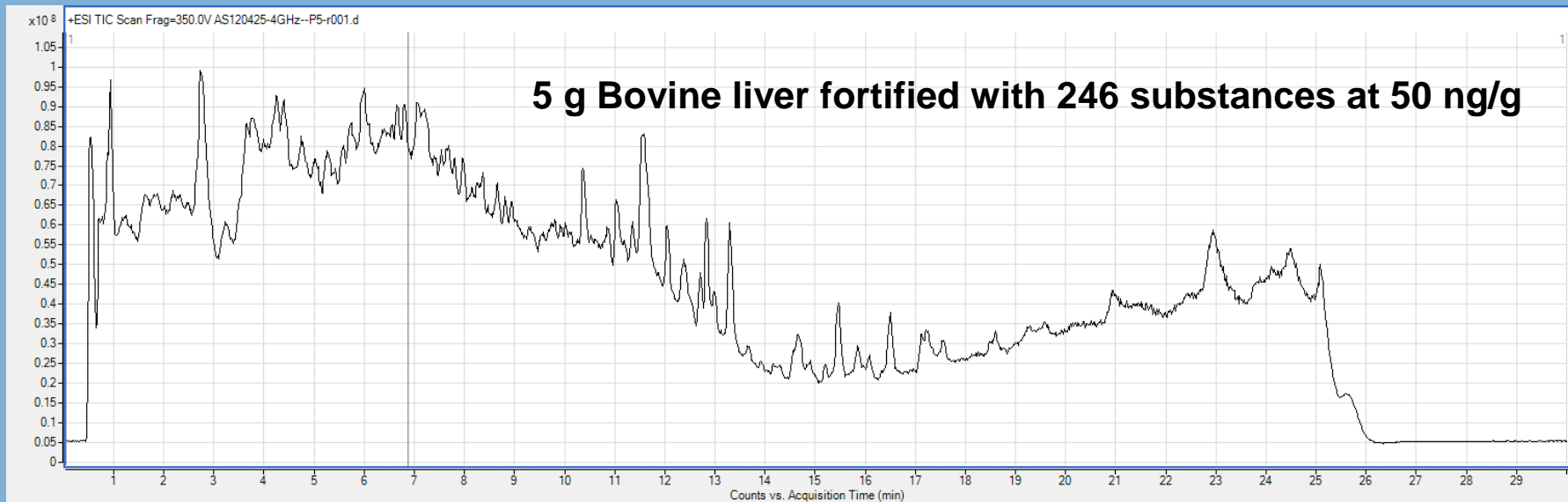
# Generic sample preparation for substance group overlapping methods by means of QTOF/Orbitrap/Multi-MRMs



# Evaluation of TOF Data using MassHunter

**Instrument: Agilent QTOF 6450 coupled with Agilent 1290 HPLC**

**Method of measurement:**  
scan mode  
high resolution  
mass range: 100 – 1250 amu

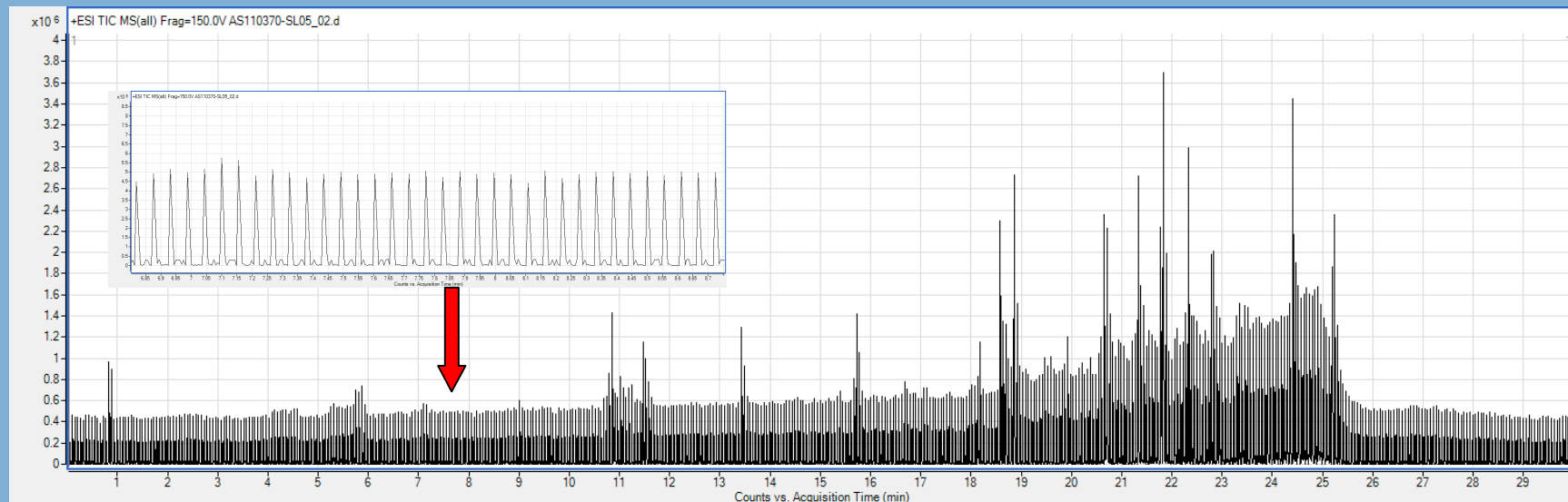




# Evaluation of TOF Data using MassHunter

**Instrument: Agilent QTOF 6420 coupled with Agilent 1200 HPLC**

**Method of measurement:**  
auto MS/MS mode  
high resolution  
mass range: 100 – 1250 amu





# Evaluation of TOF Data using MassHunter

## ■ Compound Functions

- Find by EIC (known Analytes)  Quantification
- Find by Molecular Feature
- Find by Auto MS/MS
- Find by Targeted MS
- Find by Formula

## ■ Identify Compounds

- Search Database
- Generate Formulas

# Evaluation of TOF Data using MassHunter

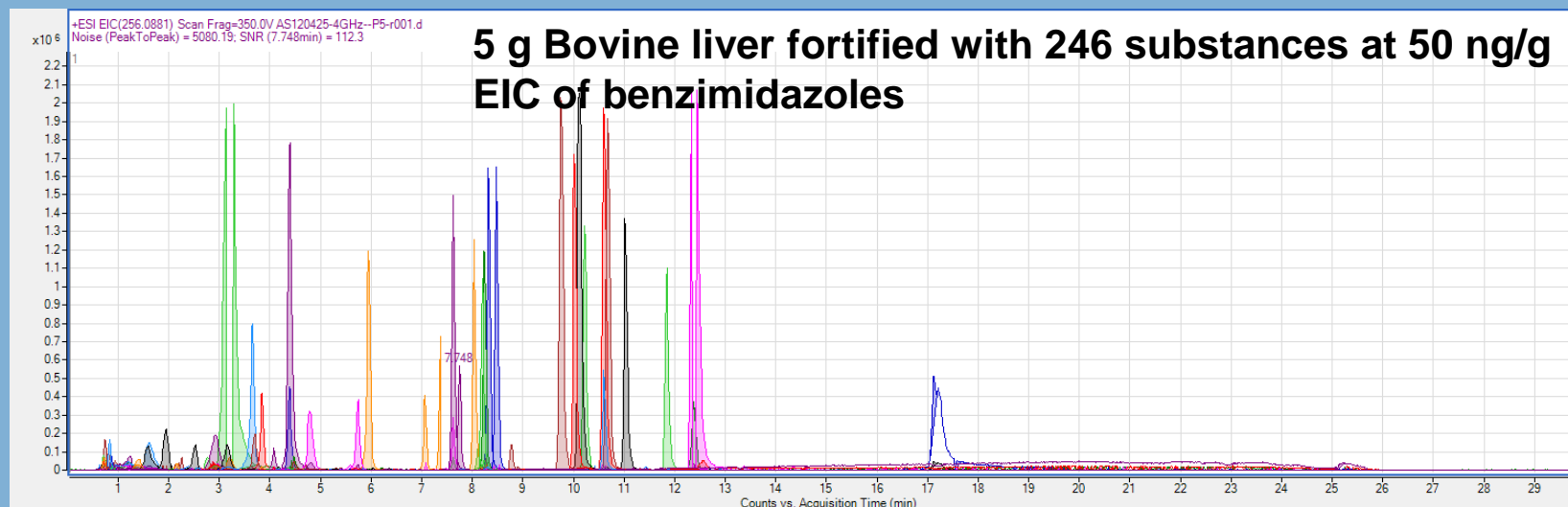
**Instrument: Agilent QTOF 6450 coupled with Agilent 1290 HPLC**

**Method of measurement:**

scan mode

high resolution

mass range: 100 – 1250 amu



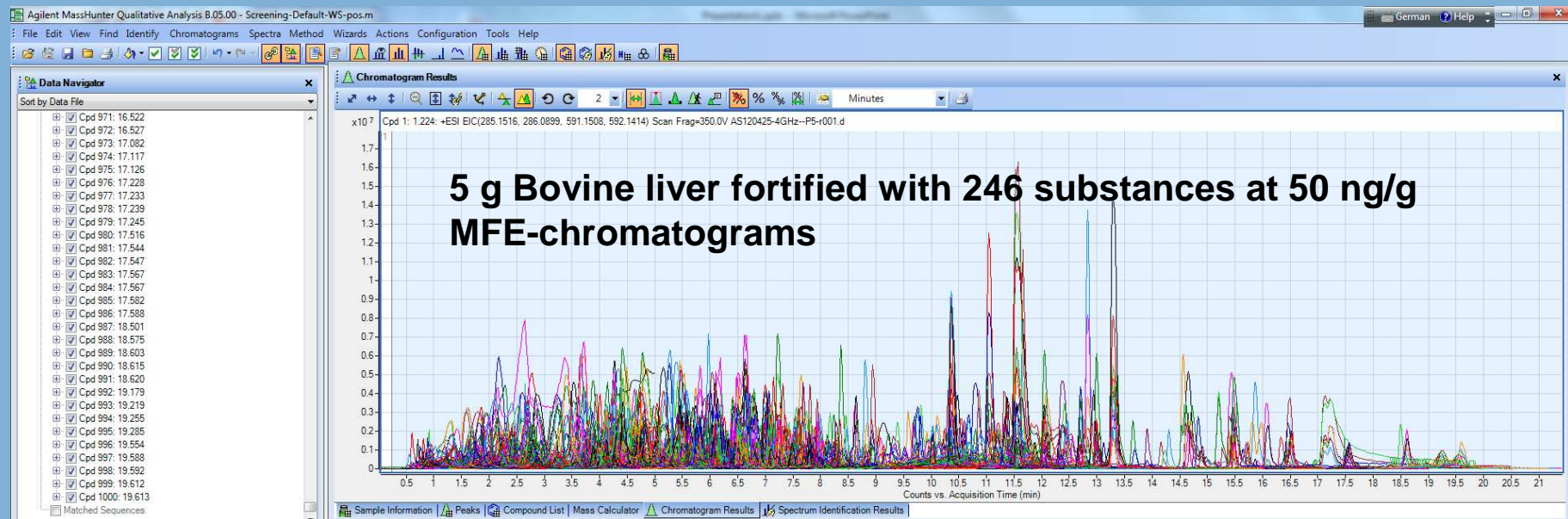
# Evaluation of TOF Data using MassHunter

- **Molecular feature:** a discrete molecular entity defined by combination of retention time, mass and response in an LC/MS analysis.
- ***Molecular Feature Extractor*** operates on raw mass spectral data (MS1 only) generating lists of chemically qualified molecular features (eliminates interferences and reduces data complexity)
  - Treats data as a three dimensional array of retention time, m/z, and abundance.
  - Removes persistent and slowly changing background.
  - Searches for features with a common elution profile (masses eluting at nearly the same time).
  - Masses grouped into “compounds”.
  - Co-eluting interferences are resolved.
  - Isotopic cluster recognized and grouped.
  - Charge state assignments and molecular adducts are recognized
  - Chemical identification (ppm, isotope matching).
  - Feature lists storable in space-efficient binary format, and saved as text files.



# Evaluation of TOF Data using MassHunter

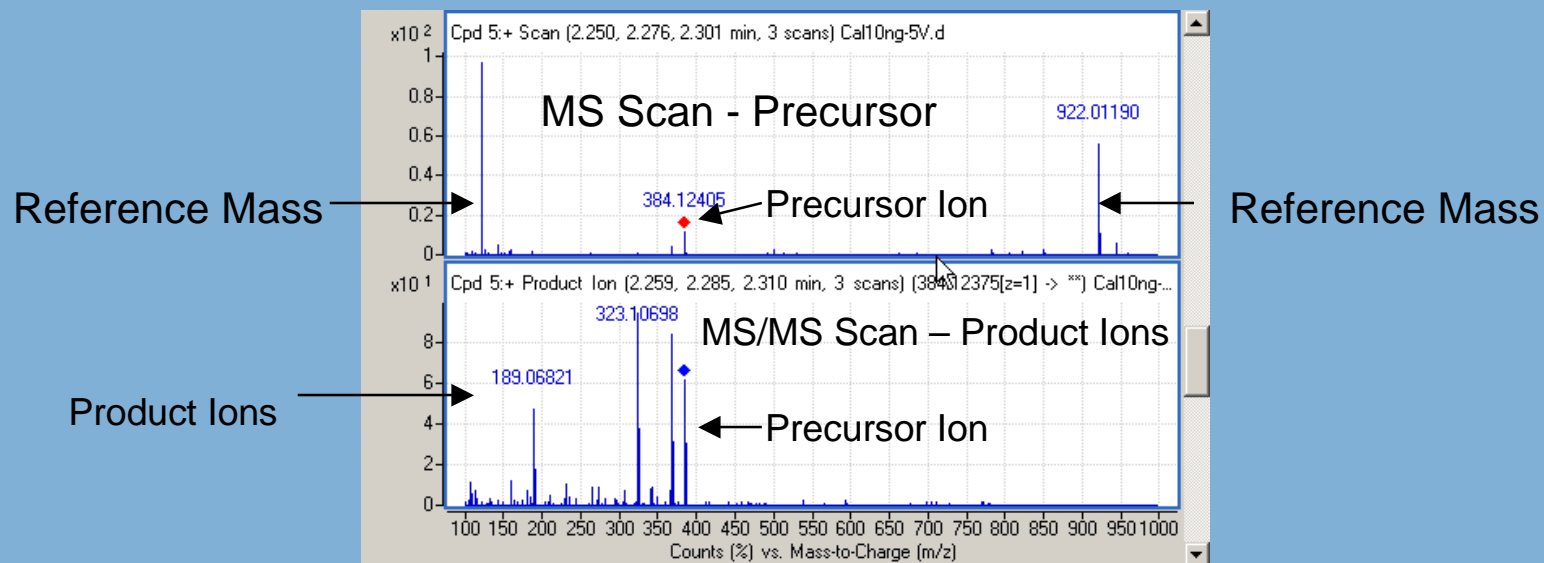
**Instrument: Agilent QTOF 6450 coupled with Agilent 1290 HPLC**  
**Method of measurement:**  
scan mode  
high resolution  
mass range: 100 – 1250 amu



# Evaluation of TOF Data using MassHunter

## Find by Auto MS/MS

- Data Analysis function used to automatically identify compounds in MS/MS data and create averaged MS and MS/MS spectra for each compound. The functionality is an easy way to “mine” information from complex data.





# Evaluation of TOF Data using MassHunter

## VetDrug.csv file containing formula, RT and exact mass of compound

C27H34O3		406.2508	19-Nortestosteron Phenylpropionate	
C28H44O3		428.329	19-Nortestosteron-17-decanoate	
C9H11NO		149.0841	2,4-Dimethylphenyl-formamide	
C9H5D6NO		155.1217	2,4-Dimethylphenyl-formamide-d6	
C10H14N2		162.1157	2,4-Dimethylphenyl-N-methylformamidine	
C18H16F3O2S		395.0915	2,5-Dimethylcelecoxib	
C12H15NO4		237.1001	3-Hydroxy-carbofuran	
C13H21NO3		239.1521	3-Hydroxy-ibuprofen-NH4 Adduct	
C48H79NO18	10.38	967.5297	3-O-Acetyltylosin 3-o-AT	
C13H15N3O2	4.62	245.1164	4-Acetylaminophenazone, AAA	
C11H13N3O	3.07	203.1059	4-Aminoantipyrin, AA	
C18H17N3O		291.1372	4-Benzylideneaminoantipyrin	
C13H17N3O	2.72	231.1372	4-Dimethylaminoantipyrin, DMAA	
C11[13C]2H1	2.71	233.1439	4-Dimethylamino-antiypirine-13C2	
C22H23N2O8	7.62	478.1143	4-Epichlortetracyclin-HCl E-CTC	
C22H24N2O8	5.03	460.1482	4-Epioxitetracyclin E-OTC	
C22H24N2O8	4.71	444.1533	4-Epitetracyclin-HCL E-TC	
C12H13N3O2	4.53	231.1008	4-Formylaminophenazone, FAA	
C12H16N2O8	3.63	236.0983	4-Hydroxy Xylazin	
C11H12N2O2	6.8	204.0899	4-Hydroxyantipyrin	
C11H12N2OS		220.067	4-Hydroxy-tetramisol	
C12H12D3N3	2.41	220.1403	4-Methylaminoantipyrin-D3	
C12H15N3O	2.43	217.1215	4-Methylaminophenazone, MAA	
C14H11N2O3	11.64	312.0722	5-Hydroxyflunixin, FLU-OH	
C10H7N3OS	3.45	217.031	5-Hydroxythiabendazol	
C14H8D3N2O	11.6	315.091	5-Hydroxyfluxinin-d3	
C48H72O14	19.64	872.4922	8,9-Z-Ahamectin-B1a Na	

**Method Editor: Find Compounds by Formula - Options**

Find Compounds by Formula | Formula Matching | Positive Ions | Negative Ions | Scoring | Results | Result Filters

Formula Source

Source of formulas to confirm:

☐ These formulas:

(type a comma-separated list of formulas, e.g., "C6H6, CH4")

☐ Compound exchange file (.CEF):

☒ Database

D:\MassHunter\libraries\MSL\Tierarzneimittel - LCMS

☐ Worklist

Matches per formula

Maximum number of matches: 1

☒ Automatically increase for isomeric compounds

Values to match

☐ Mass

☐ Mass and retention time (retention time optional)

☒ Mass and retention time (retention time required)

**VetDrug.cdb file containing formula, RT , exact mass of compound, spectra, CAS, chemical structure**

**MassHunter PCDL Manager - K:\QTOF-LCMS13\TOF\Bibliotheken\Vet\_Drugs\LCMS13-QTOF 6550\LUNA LCMS13 Luna C18.cdb**

File Edit View PCDL Links Help

Finds Compounds [Icons]

Single Search Batch Search Batch Summary Edit Compounds Spectral Search Browse Spectra Edit Spectra

Name: Monensin-NH<sub>4</sub>

IUPAC: (2S,3R,4S)-4-{[(2S,5R,7S,8R,9S)-2-{(2S,2'R,3'S,5R,5'R)-2-Ethyl-5'-(2S,3S,5R,6R)-6-hydroxy-6'-hydroxymethyl-2,3-dihydro-2H-pyran-2-ylidene]-5-methoxy-2-methyltetrahydropyran-2-yl]oxy}butanoate

Mass: 687.45576 CAS: 17090-79-8

RT: 21.3 ChemSpider: 389937

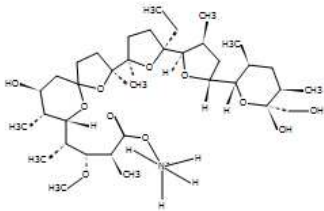
Formula: C<sub>36</sub>H<sub>65</sub>N<sub>O</sub><sub>11</sub>

Ion type  
☒ Neutral  
☐ Anion  
☐ Cation

Edit actions:  
Add New  
Save As New  
Update Selected  
Delete Selected

Molecule:

Structure MOL Text



Notes: Antiamebic

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**Single Search Results: 3 hits**

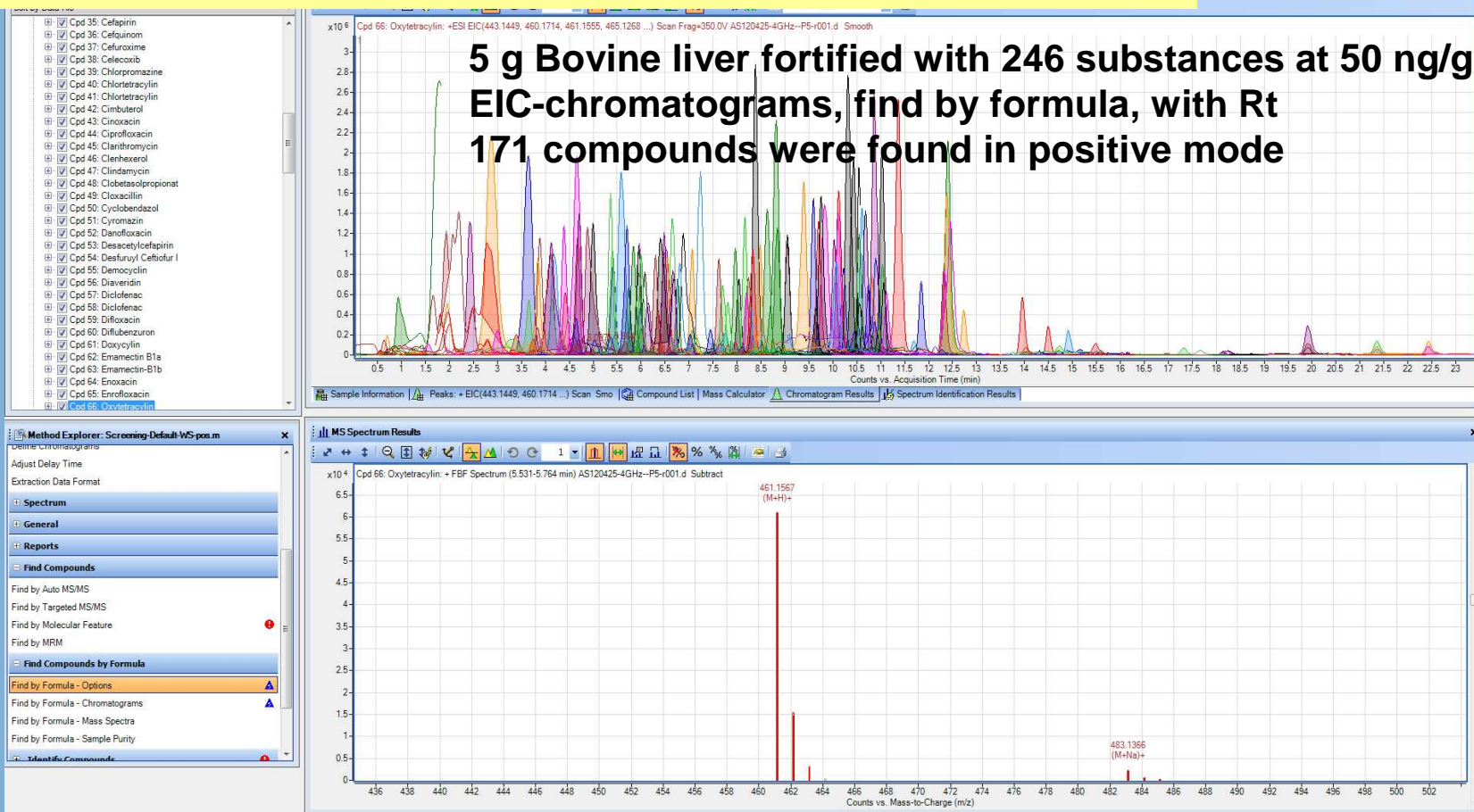
	Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Num Spectra
	Monensin	C <sub>36</sub> H <sub>62</sub> O <sub>10</sub>	670.42921	<input type="checkbox"/>	<input type="checkbox"/>	21.300	<a href="#">17090-79-8</a>	<a href="#">389937</a>	(2S,3R,4S)-4-{[(2S,5R,7S,8R,9S)-2-{(2S,2'R,3'S,5R,5'R)-2-Ethyl-5'-(2S,3S,5R,6R)-6-hydroxy-6'-hydroxymethyl-2,3-dihydro-2H-pyran-2-ylidene]-5-methoxy-2-methyltetrahydropyran-2-yl]oxy}butanoate	0
▶	Monensin-NH <sub>4</sub>	C <sub>36</sub> H <sub>65</sub> N <sub>O</sub> <sub>11</sub>	687.45576	<input type="checkbox"/>	<input type="checkbox"/>	21.300	<a href="#">17090-79-8</a>	<a href="#">389937</a>	(2S,3R,4S)-4-{[(2S,5R,7S,8R,9S)-2-{(2S,2'R,3'S,5R,5'R)-2-Ethyl-5'-(2S,3S,5R,6R)-6-hydroxy-6'-hydroxymethyl-2,3-dihydro-2H-pyran-2-ylidene]-5-methoxy-2-methyltetrahydropyran-2-yl]oxy}butanoate	4
	Monensin-Na	C <sub>36</sub> H <sub>61</sub> N <sub>O</sub> <sub>10</sub>	692.41116	<input type="checkbox"/>	<input type="checkbox"/>	21.300	<a href="#">17090-79-8</a>	<a href="#">389937</a>	(2S,3R,4S)-4-{[(2S,5R,7S,8R,9S)-2-{(2S,2'R,3'S,5R,5'R)-2-Ethyl-5'-(2S,3S,5R,6R)-6-hydroxy-6'-hydroxymethyl-2,3-dihydro-2H-pyran-2-ylidene]-5-methoxy-2-methyltetrahydropyran-2-yl]oxy}butanoate	3

# Evaluation of TOF Data using MassHunter

**Instrument: Agilent QTOF 6450 coupled with Agilent 1290 HPLC**

**Method of measurement:**  
 scan mode  
 high resolution  
 mass range: 100 – 1250 amu

**5 g Bovine liver fortified with 246 substances at 50 ng/g**  
**EIC-chromatograms, find by formula, with Rt**  
**171 compounds were found in positive mode**

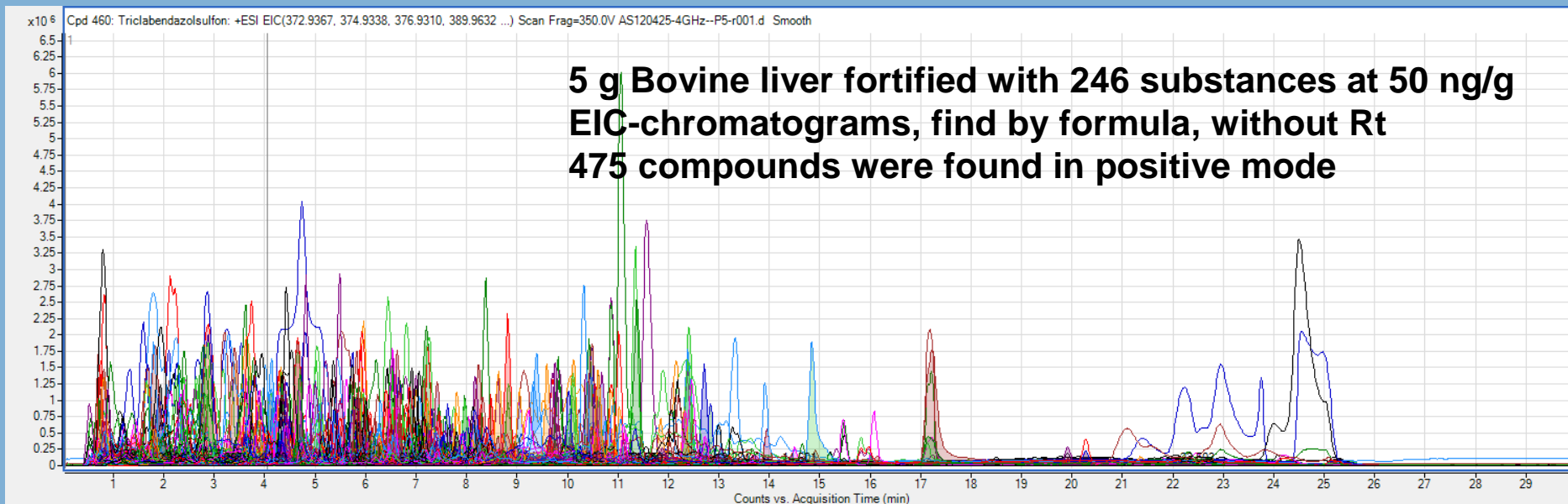




# Evaluation of TOF Data using MassHunter

**Instrument: Agilent QTOF 6450 coupled with Agilent 1290 HPLC**

**Method of measurement:**  
scan mode  
high resolution  
mass range: 100 – 1250 amu



- QTOF is proper instruments for data acquisition in multi-residue screening analysis
- Problem: are false negative results caused by
  - Generic sample preparation
  - Matrix influences
  - Low response of some analytes e.g. avermectines
- Data Evaluation
  - EIC for small number of compounds
  - “Find by formula” for semi-target screening and large number of substances
    - very fast, limited by number of compounds in the database
    - Retention time is mandatory
  - Molecular feature extractor for unknown screening
    - time consuming, need experience