# Metabolite Identification in the Real World

Dr. Axel Paeler

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ORGANISED BY:

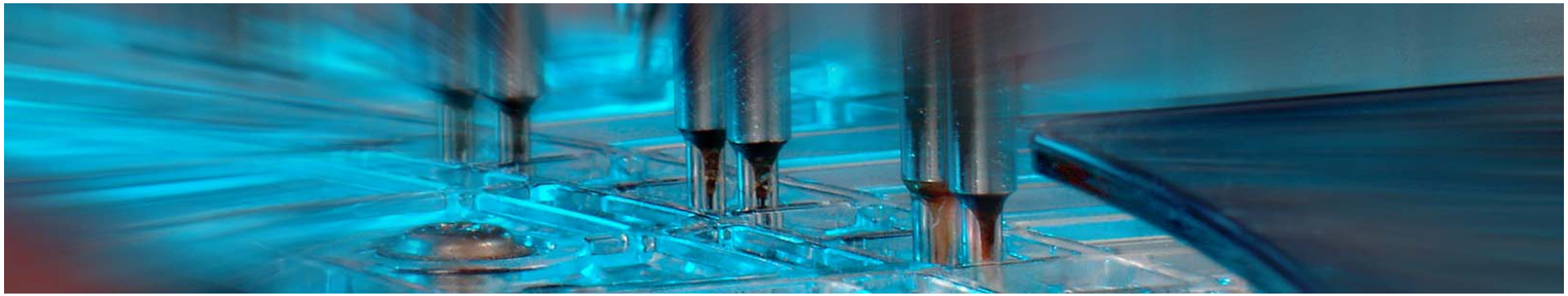
european pharmaceutical review

SUPPORTED BY:

AB SCIEX

# Transforming data into knowledge: Use of HRMS instrumentation in Drug Discovery and Development

Dr. Axel Pähler
DMPK, Non Clincial Safety, pRED, F. Hoffmann-La Roche Ltd.



# Drug Discovery & Drug Development

*Quantitative metabolite identification workflows*

**Discovery Research (LI/LO): Find, *Quantify?* & ID**

Hepatic clearance combined with metabolite ID

Parent disappearance kinetics plus relative metabolite formation rates. Can we move from QqQ to HRMS?

Classical metabolite ID study & peak area ratios (no absolute but relative quantification in tissues/species)

**Drug Development: Find, Quantify & ID**

Compare human metabolites to animal species

In vitro metabolism (relative peak are ratios or 14C)

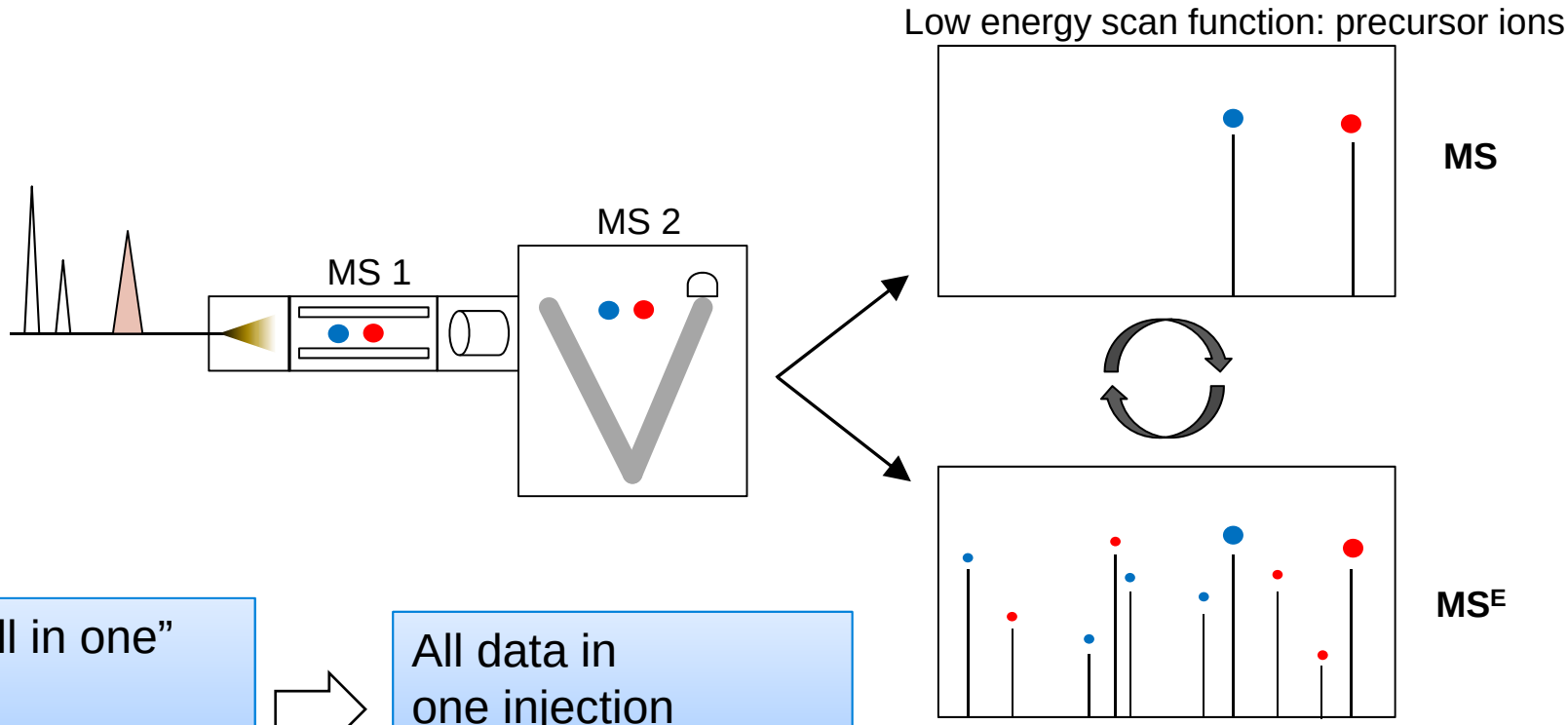
In vivo systemic metabolite exposure (relative peak area ratios, 14C or authentic standards)

# Targeted vs. non Targeted Data Acquisition

*MS “all in one” or data dependent “true” MS/MS*

- $MS^E$ mode: Two acquisition functions are monitored in parallel

  Principally technology independent QqTOF, (q/trap)-FTMS




MS “All in one”

DDE

***IDA (DBS, MDF)***

All data in one injection

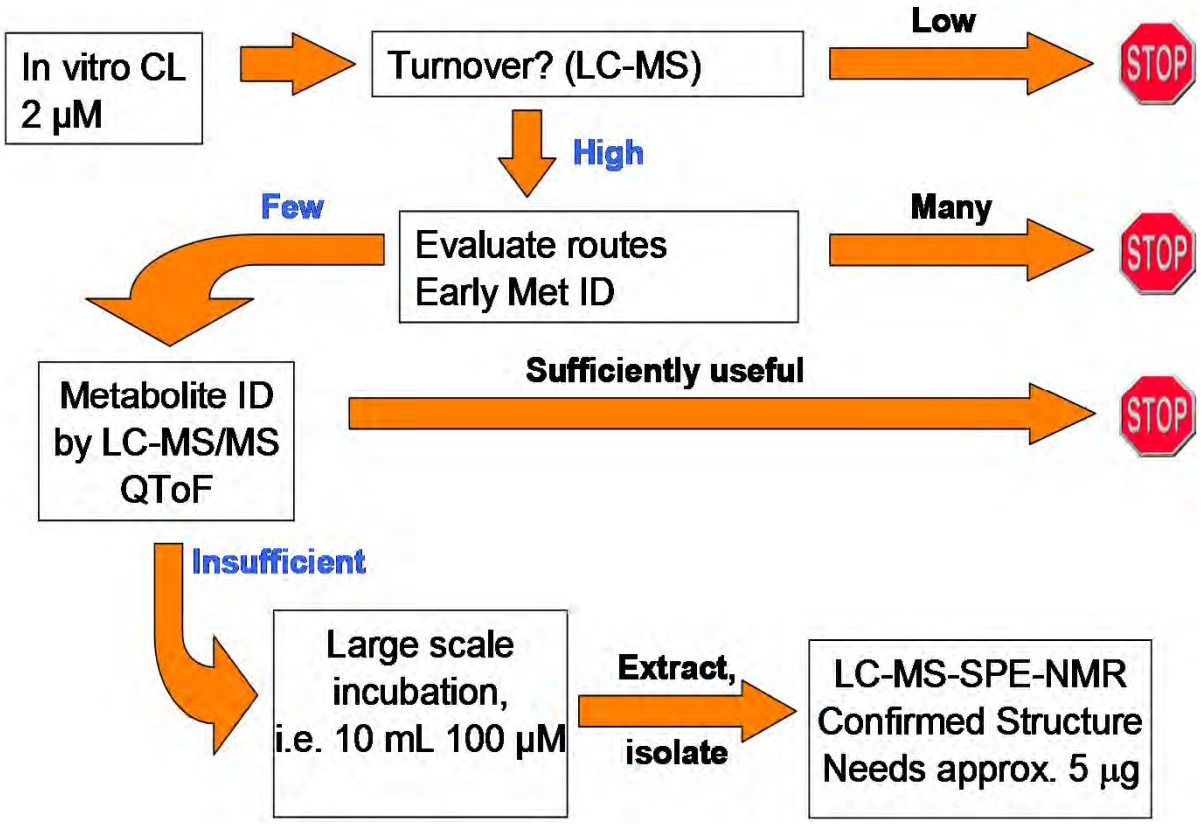
High data quality

# Early Metabolite ID

*Shift towards Qual/Quant workflow*

SoM identification for high Cl compounds
→ Guide rational Drug Design by Medicinal Chemistry

Soft spot ID (top 4 metabolites) → Structure proposal by MS/MS

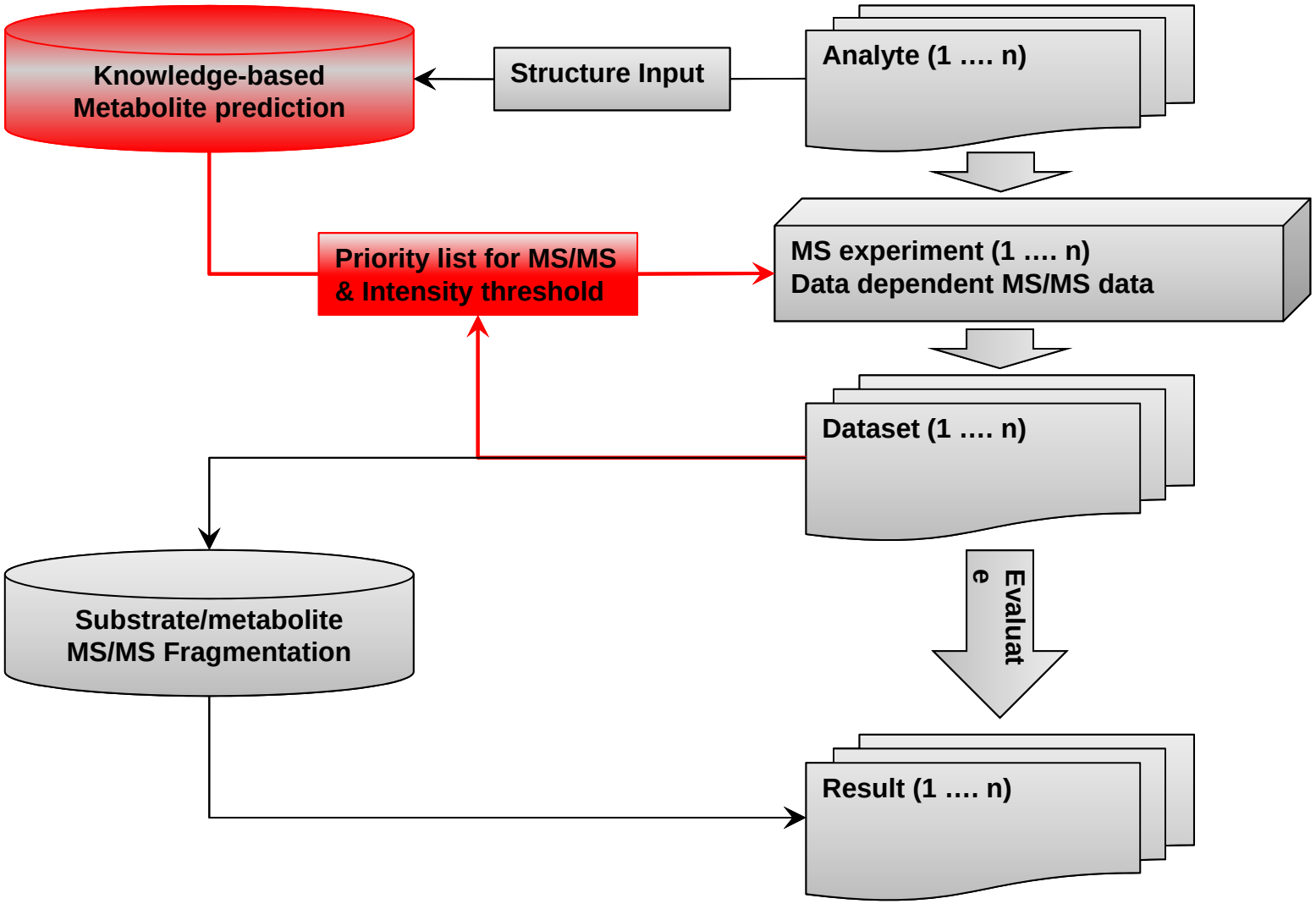



**Quant**itative

**Qual**itative

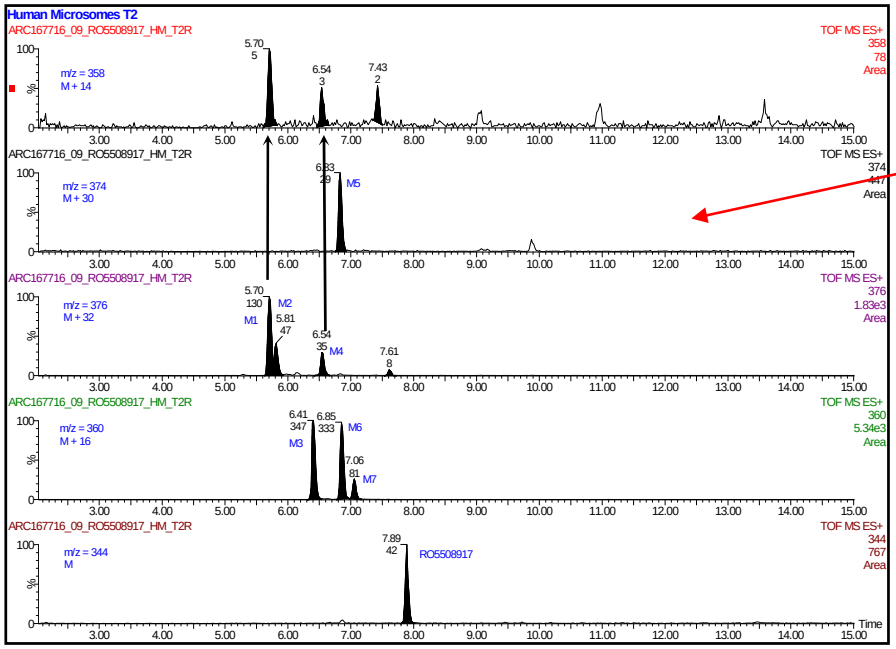
# Metabolite Identification: The Past

*Qual analysis driven by structure & expert knowledge*



# From Prediction to Experiment

*Data processing and metabolite fishing*



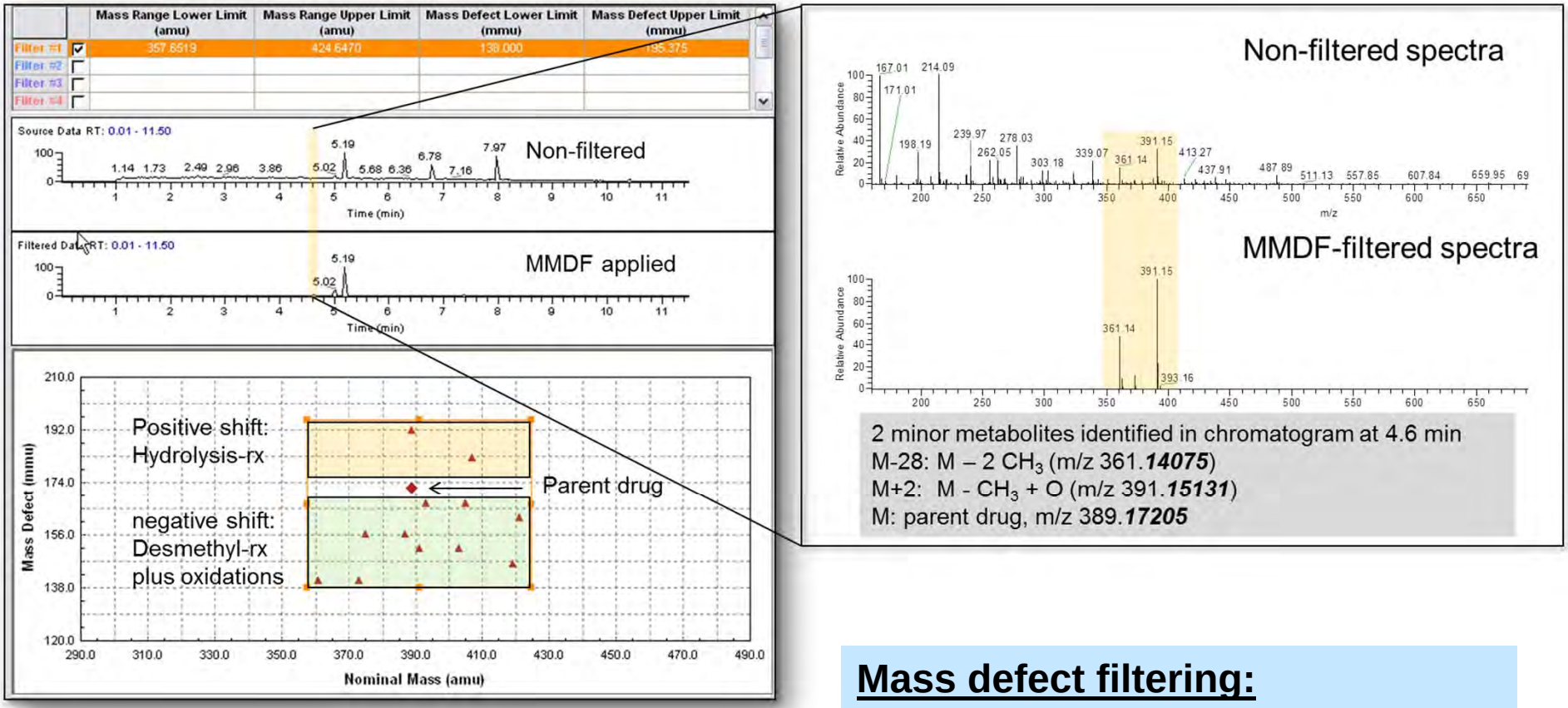

Automated data processing (find expected metabolites)

Search for metabolites based on mass defect filters, isotope clustering and possible de-alkylations: Support detection of possible cleavages and unexpected metabolites

Experienced analysts key for data review and interpretation

# Post Acquisition Data Processing

*Getting most out of the HRMS data*




**Mass defect filtering:**

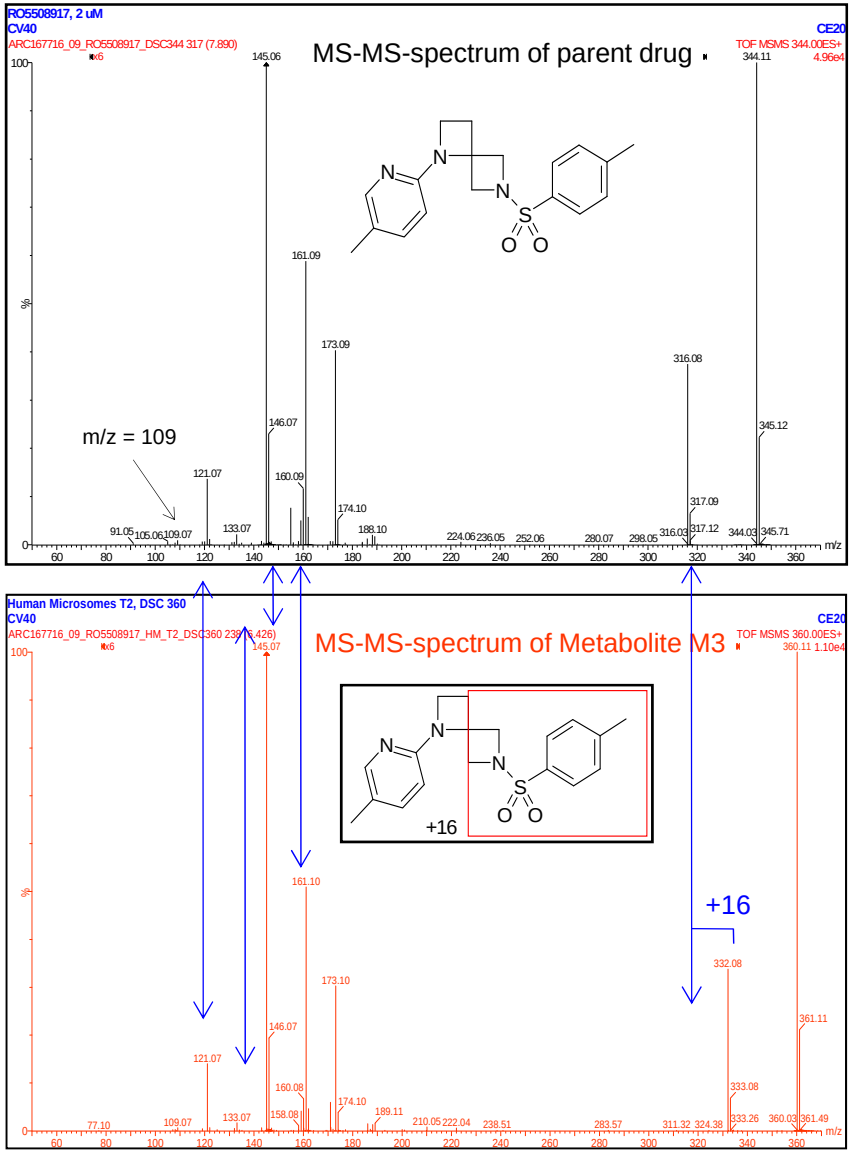
Data reduction

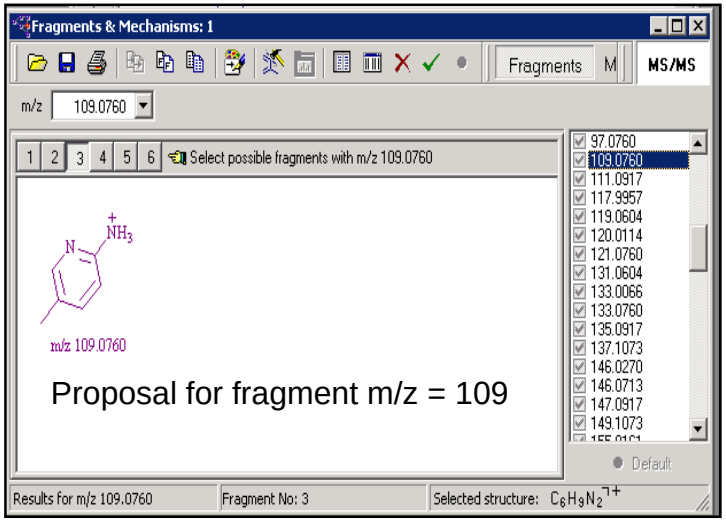
Facilitate finding drug related material

On-the-fly MMDF available on 5600+

# From Identification to Structure

*Data processing in Metabolite ID*

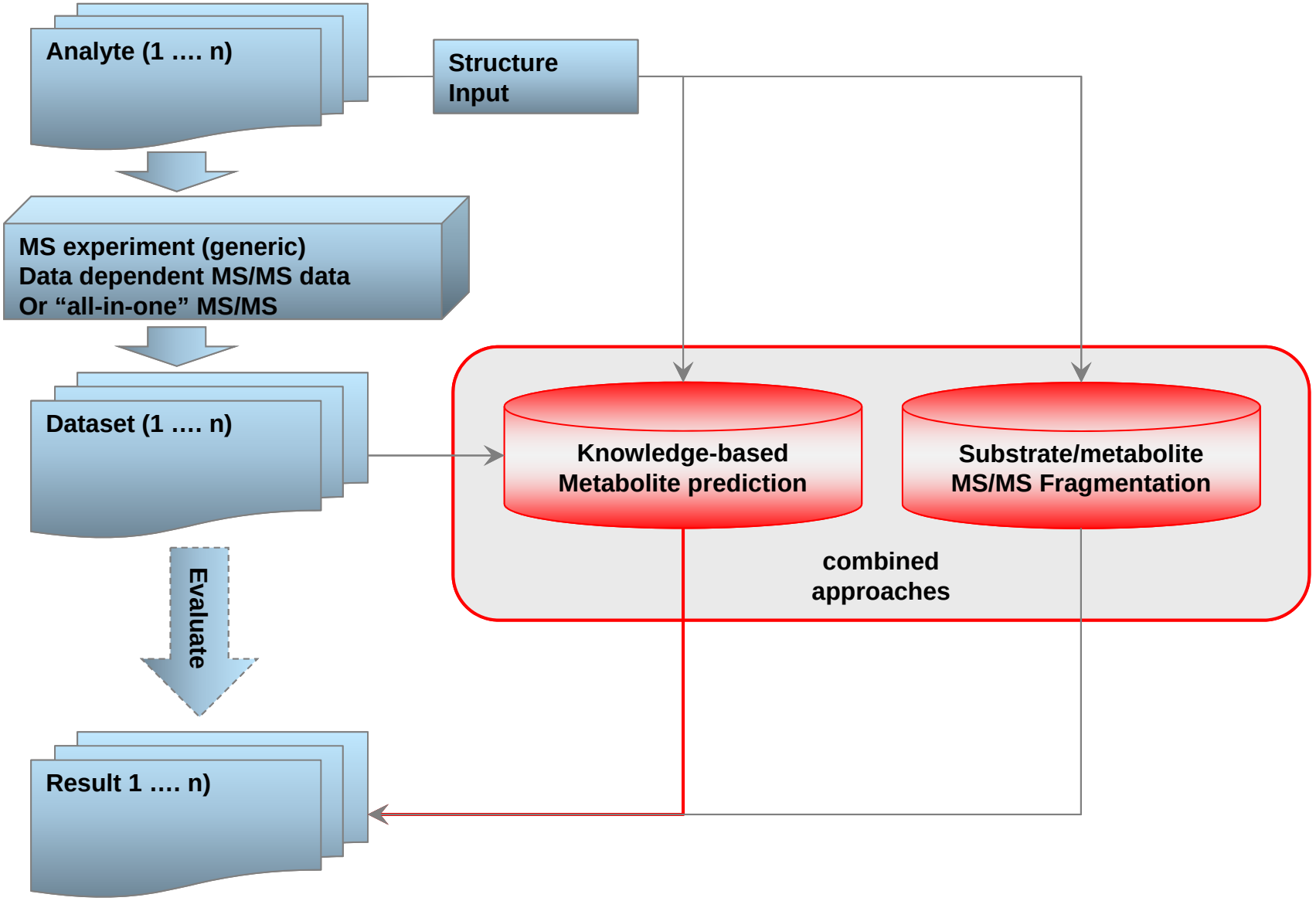



- Use MS/MS spectra to localize position of biotransformation in the molecule.
- MS/MS spectra interpretation supported by software (based on general fragmentation rules).
- Definitive structure assignment still manual, often Markush-like
- Definitive structure by NMR

# Metabolite Identification: The Future

*Data driven, untargeted post acquisition analysis*



# Diversity in Applications

*Demand for Versatile LC-MS Instrumentation*

***High Sensitivity and Speed:***

Assays conducted at low physiological drug concentrations

Need to detect relevant metabolites and biomarkers

All information, all the time (full scan MS, MS/MS)

# Diversity in Applications

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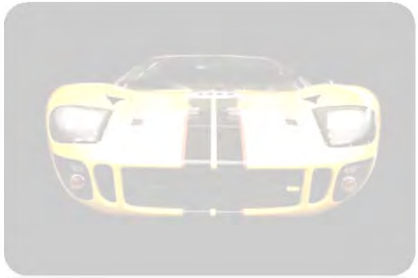

***High Sensitivity and Speed:***

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All information, all the time (full scan MS, MS/MS)

***Robust, Reliable & Compatible:***

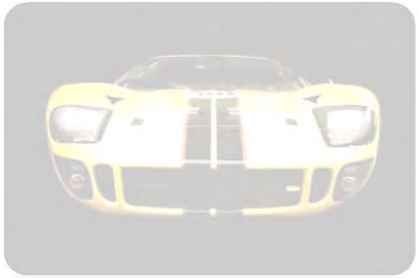
No significant down time

Proven interface and front-end

Ease of method transfer from existing instrumentation

# Diversity in Applications

*Demand for Versatile LC-MS Instrumentation*



***High Sensitivity and Speed:***
Assays conducted at low physiological drug concentrations
Need to detect relevant metabolites and biomarkers
All information, all the time (full scan MS, MS/MS)

***Robust, Reliable & Compatible:***
No significant down time
Proven interface and front-end
Ease of method transfer from existing instrumentation

***Ease of Operation:***
Automated setup and calibration routine
Shift from instrumentalist to DMPK specialist as main user
Ready to go, still highly customizable

# Software features for DMPK studies

*A workflow perspective (A DMPK Scientist's dream)*

**Vendor independent**
Waters (*.raw), Thermo (*.RAW): Ion-Trap/Orbitrap, Agilent (*.d), SCIEX (*.wiff)

**Automatic peak detection using multiple algorithms**
Targeted/unknown analysis, MDF, fragmentation pattern analysis, noise removal, list of mass changes (Phase I and Phase II reactions), background correction (unexpected transformations, des-alkylations)

**Fragmentation analysis**
Hypothetical fragmentation, comparison parent vs metabolite fragment spectra assignment of score (matches vs mismatches) → structure proposal + scoring

**Automatic report system**
Customizable excel file format (.xlsx), Database format (.xml format), Markush structure drawing from metabolite structures, Metabolite schemes (.pdf)

**Qualitative and quantitative results**

# Reality Check: P450 Phenotyping

*Combined Quant/Qual workflow*

**Goal:**

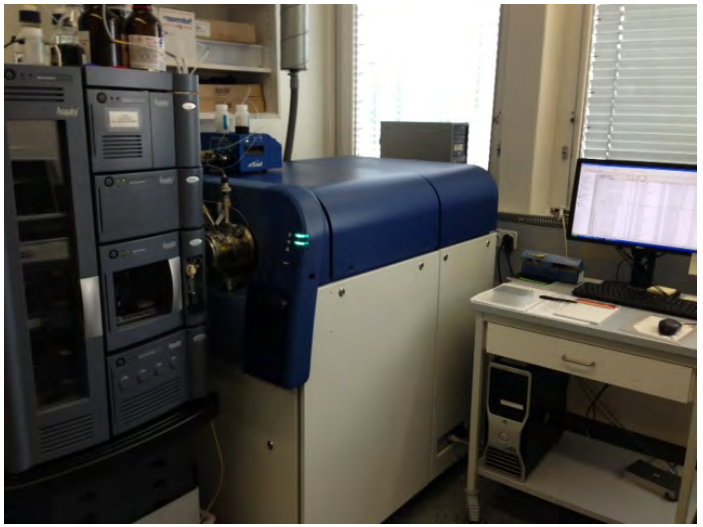
1.Identify P450 enzyme involved in drug clearance (**quant**)
2.Identify metabolites formed by individual enzymes (**qual & quant**)

**Tools:**

1.Matrix samples from 8 time point and 6 enzymes (48 samples)
2.AB Sciex 5600+ QqTOF mass spectrometer & fast gradient HPLC
3.IDA method based on intensity thresholds,
DBS active, no inclusion list
4.AB Sciex MetabolitePilot 1.5 software

**Process:**

1.Identify metabolites from individual samples
2.Matching across matrices & time points
3.Flexible reporting



# Case Study: P450 Phenotyping

*MetabolitePilot*

## Met ID process

1. Peak finding strategy: predicted metabolites (Phase I biotransformation list)
2. Generic parameters (threshold, mass tolerance, peak shape, …)
3. Compound specific parameters (C-heteroatom bond cleavage, isotope pattern, product Ion and neutral losses
4. Formula prediction (include MS/MS)

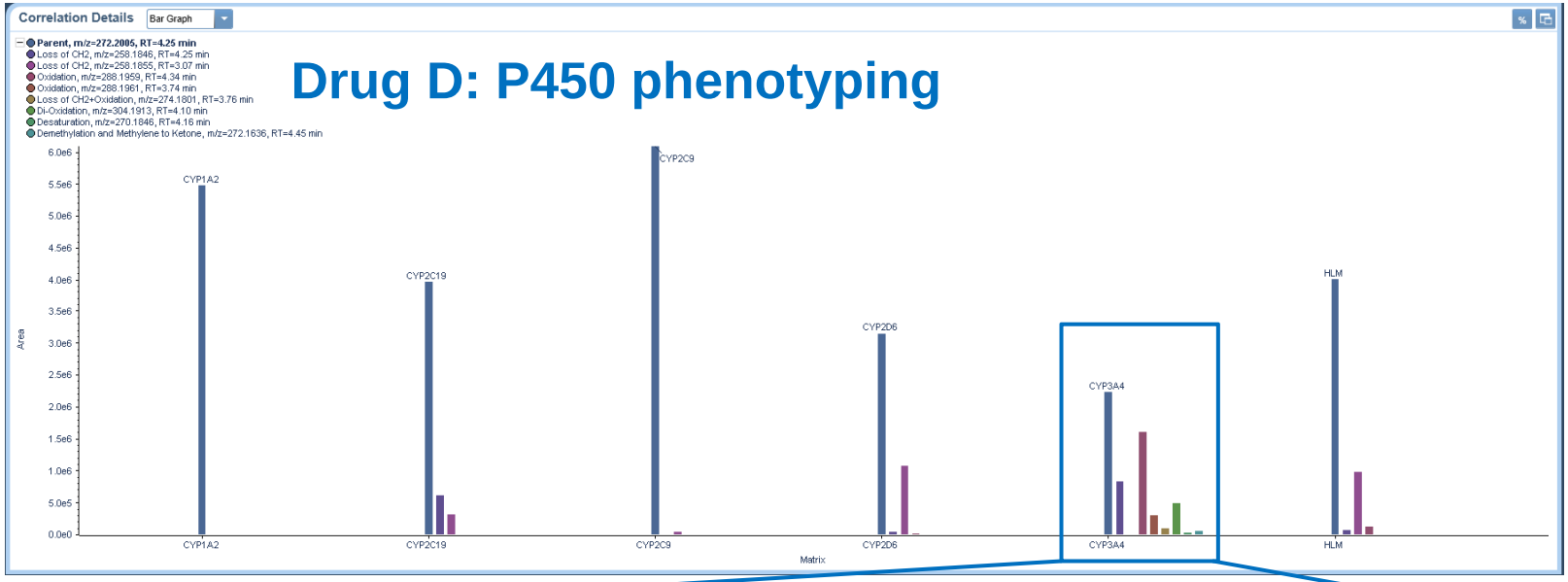
**Results: CYP3A4 for drug D**

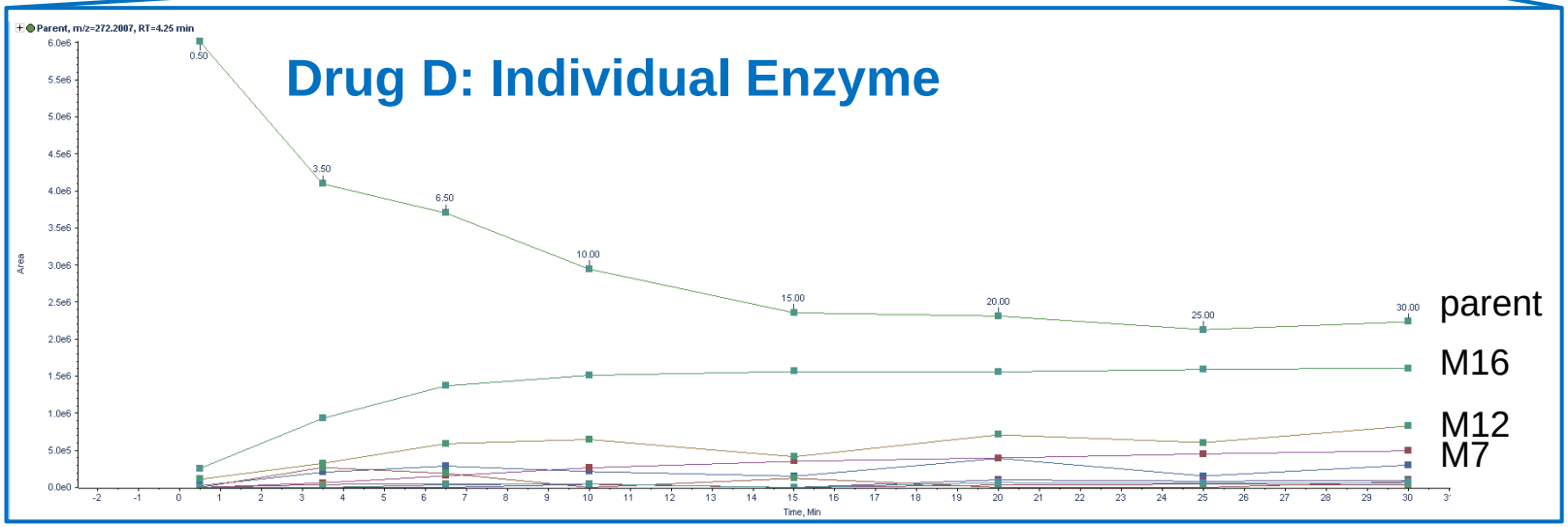
| Peak ID | Name | Formula | m/z | ppm | R.T. (min) | Peak Area | % Area | % Score |
|---|---|---|---|---|---|---|---|---|
| | Parent | C18H25NO | 272.2005 | -1.4 | 4.26 | 2.23E+06 | 35.0 | 93.8 |
| M7 | Di-Oxidation | C18H25NO3 | 304.1913 | 1.9 | 4.10 | 4.95E+05 | 7.8 | 81.9 |
| M12 | Loss of CH2 | C17H23NO | 258.1846 | -2.6 | 4.25 | 8.29E+05 | 13.0 | 95.4 |
| M6 | Loss of CH2+Oxidation | C17H23NO2 | 274.1801 | -0.1 | 3.76 | 9.86E+04 | 1.5 | 88.4 |
| M5 | Oxidation | C18H25NO2 | 288.1961 | 1.1 | 3.74 | 3.03E+05 | 4.7 | 75.0 |
| M16 | **Oxidation** | **C18H25NO2** | **288.1959** | **0.5** | **4.34** | **1.61E+06** | **25.2** | **89.7** |
| M8 | Desaturation | C18H23NO | 270.1846 | -2.2 | 4.16 | 3.45E+04 | 0.5 | 76.9 |
| M17 | Demethylation and Methylene to Ketone | C17H21NO2 | 272.1636 | -3.4 | 4.45 | 5.98E+04 | 0.9 | 87.4 |

- Excellent mass accuracy
- Very good match with manual data inspection
- Very few false negative results (threshold setting, MS/MS data was triggered)

# Case Study: P450 Phenotyping

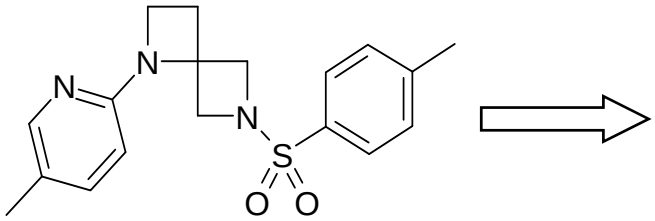
*MetabolitePilot, flexible reporting options*



# The Present & Future of Metabolite ID

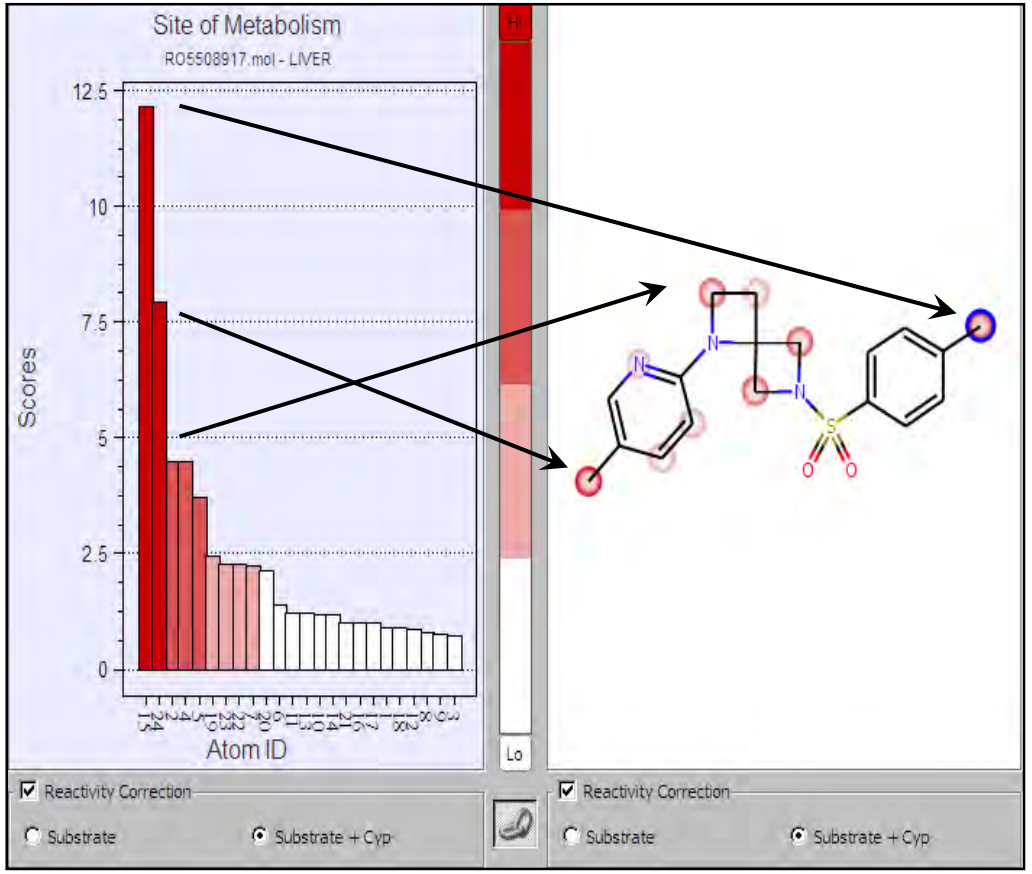
*Computation chemistry approaches*




In-silico prediction based on P450 binding site and chemical reactivity of drugs towards oxidation

SoM Prediction:
Generally good prediction of soft spot
70-80% success
(experiment within top 3)

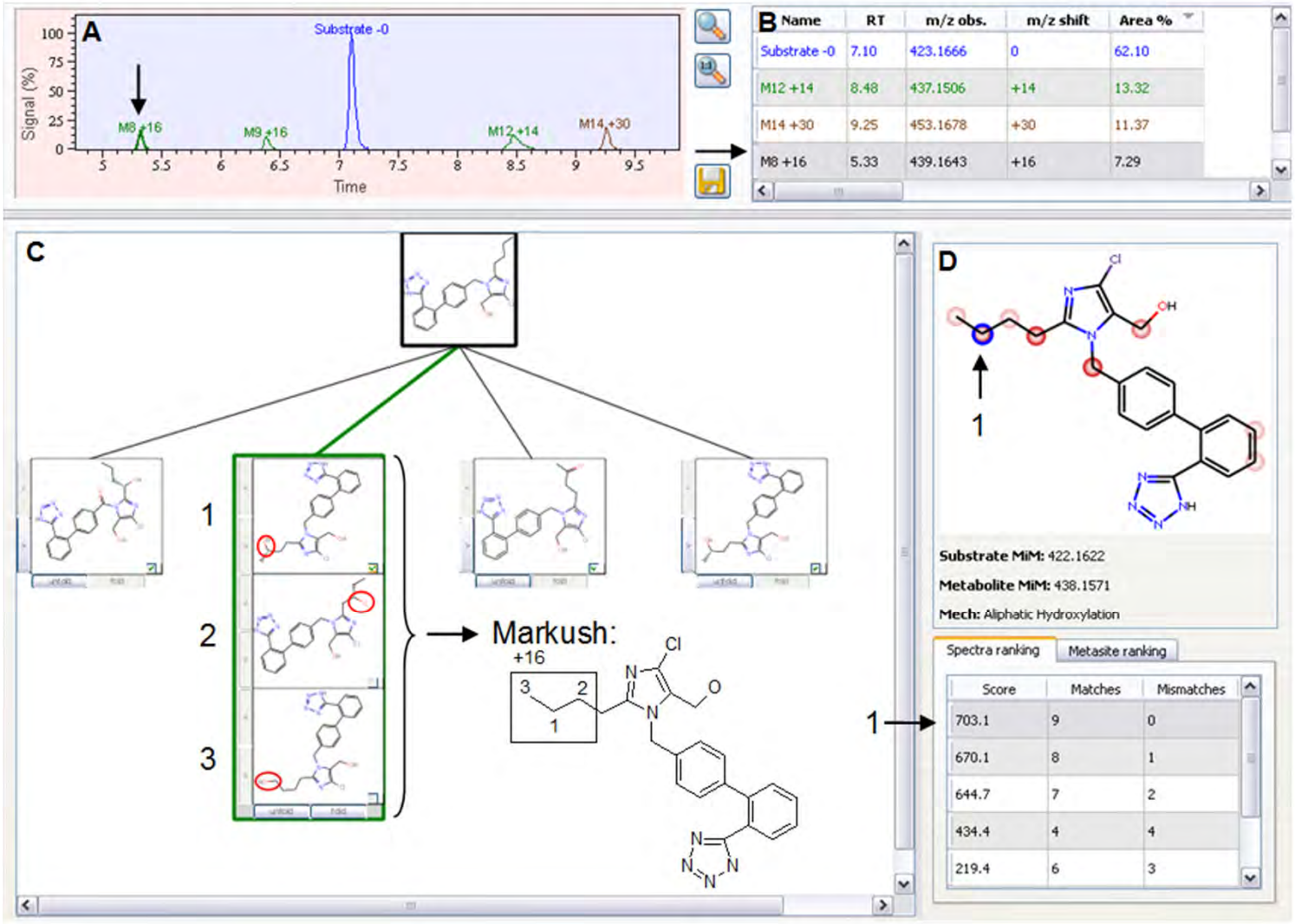
Used for prediction of likely SoM in conjunction with experimental MS/MS data and manual data interpretation




*MetaSite (Molecular Discovery Ltd)*

# Making full Use of MS/MS Data

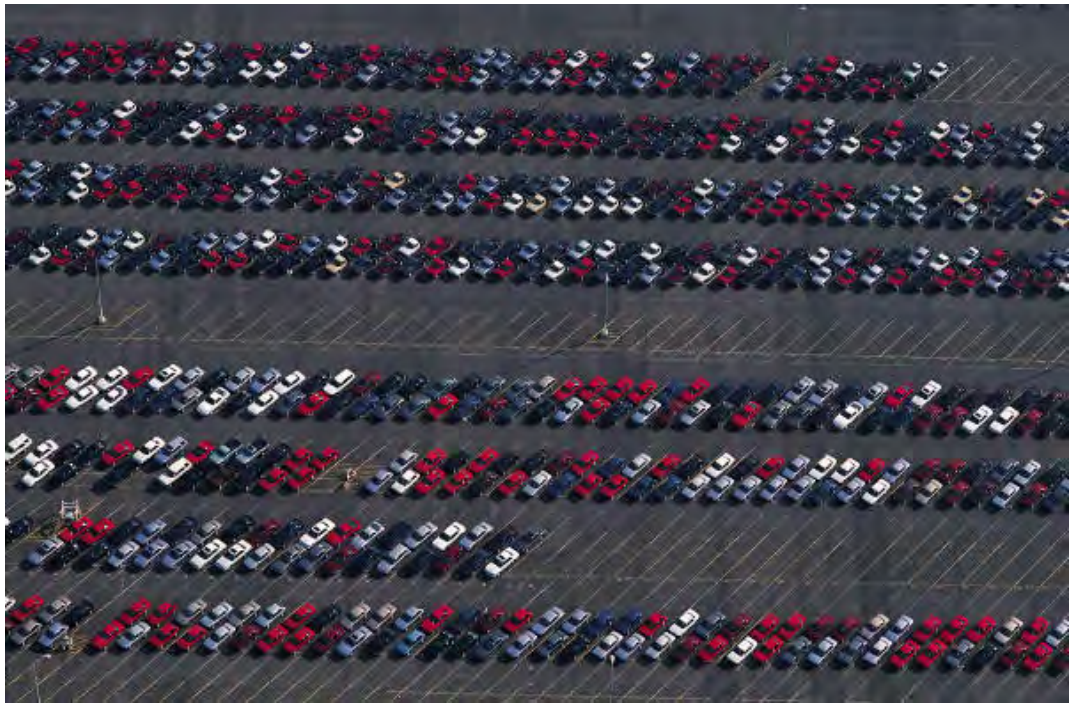
*Blending computation chemistry and LC-MS/MS Data*




*Mass MetaSite (Lead Molecular Design)*

# Finally: What happens to all the data?

*Diversity is nice, but….*

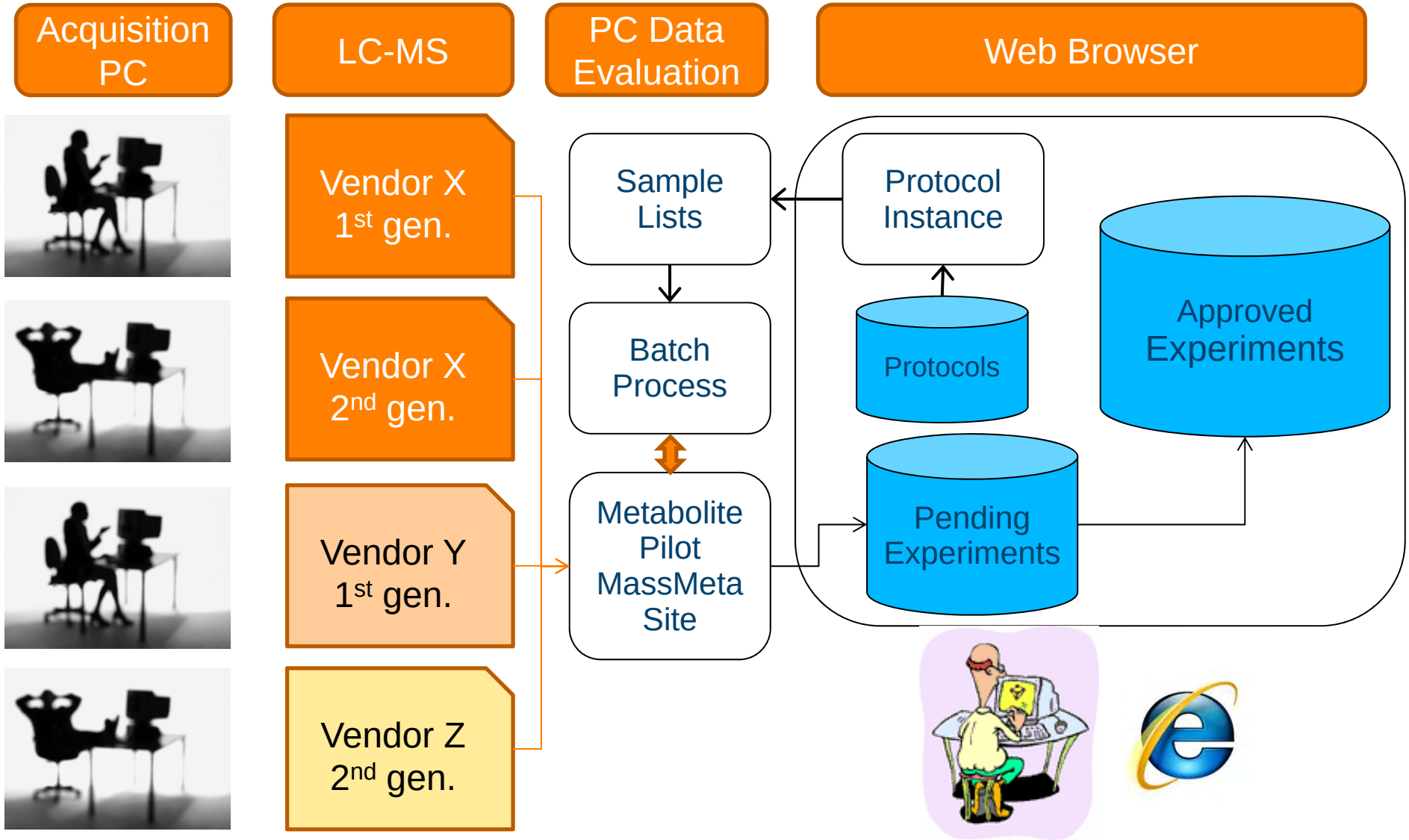


Some processes are “harmonized”.
But need to remember where I parked my car!

Traffic may become terrible…

# Cross Platform DMPK Setup

*Accommodating various workflows*

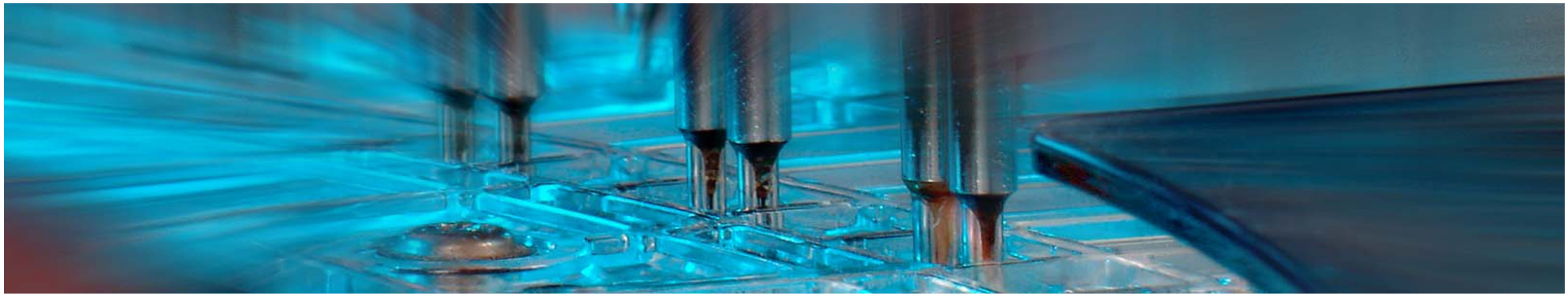


# Conclusions & Outlook

- Emerging technologies (hardware & software) have facilitated workflows in Drug Discovery and Development
  - Unbiased acquisition and post-acquisition data processing
  - “All-in-one” approaches
  - Structure-based data analysis
- Qual/Quant workflows have become routine
  - Combined clearance / Met ID studies
  - Still need for “true” (not relative) quantitative results
  - Benefits of SRM acquisition (linearity, S/N)
  - SWATH & HRMS close to SRM performance
- Structure analysis
  - Computational approaches have emerged
  - Interrogation of LC-MS/MS data most promising

# LC-MS/MS applications in biomarker research

Marieke Teppner
DMPK, Non Clincial Safety, pRED, F. Hoffmann-La Roche Ltd.



# Content

- Introduction
- Experimental set-up
- Results: Method comparison between QTRAP 4000 and TTOF 5600+
- Biological application
- Conclusion

# Introduction –
# Biomarker Research

- Biomarker:
  Endogenous molecules utilized for objective diagnosis or monitoring of physiological, pathophysiological or pathological processes
  - Clinic: Observation or identification of disease state of patients
  - Pre-clinic: Testing of effect / side effect of drug candidates

- Critical characteristics:
  - Reliability (selectivity/sensitivity/robustness)
  - Mode of measurement (invasive vs noninvasive)

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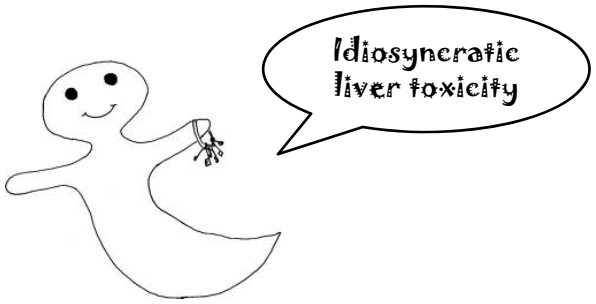
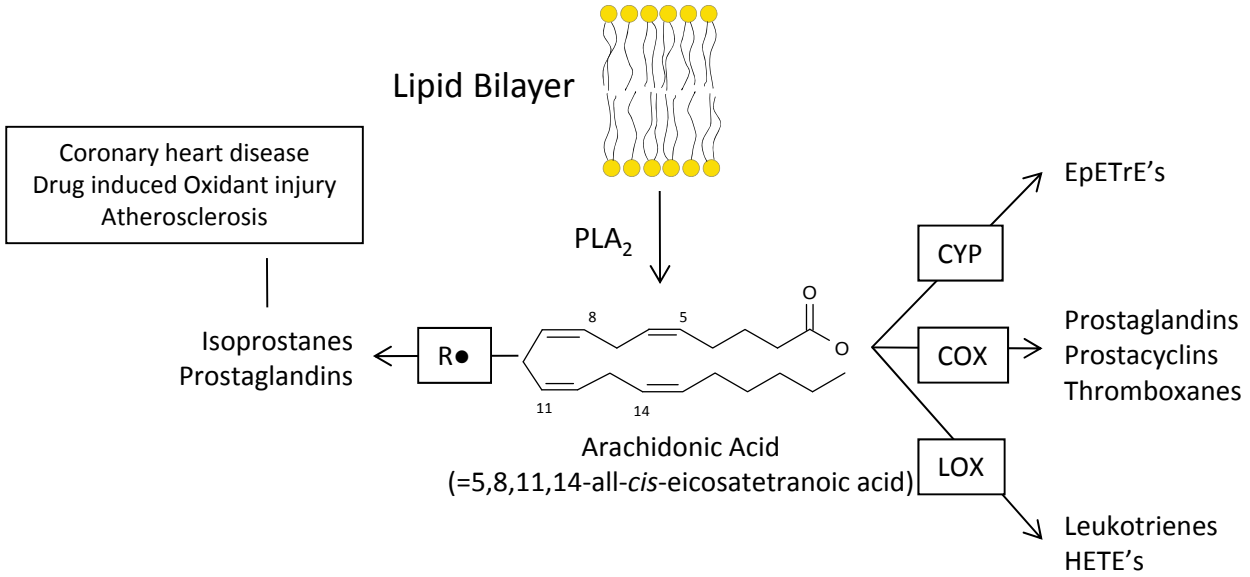
Detection of severe adverse effect as liver toxicity ("DILI")



# Introduction –
# Isoprostanes as Biomarkers *in vivo* and *in vitro*

- Generation: Arachidonic acid metabolism




- Circulation in plasma, excretion to urine → potential target of investigation
- Generated in cells exposed to oxidative stress i.e. **hepatocytes** when cause is drug → suitable *in vitro* matrix

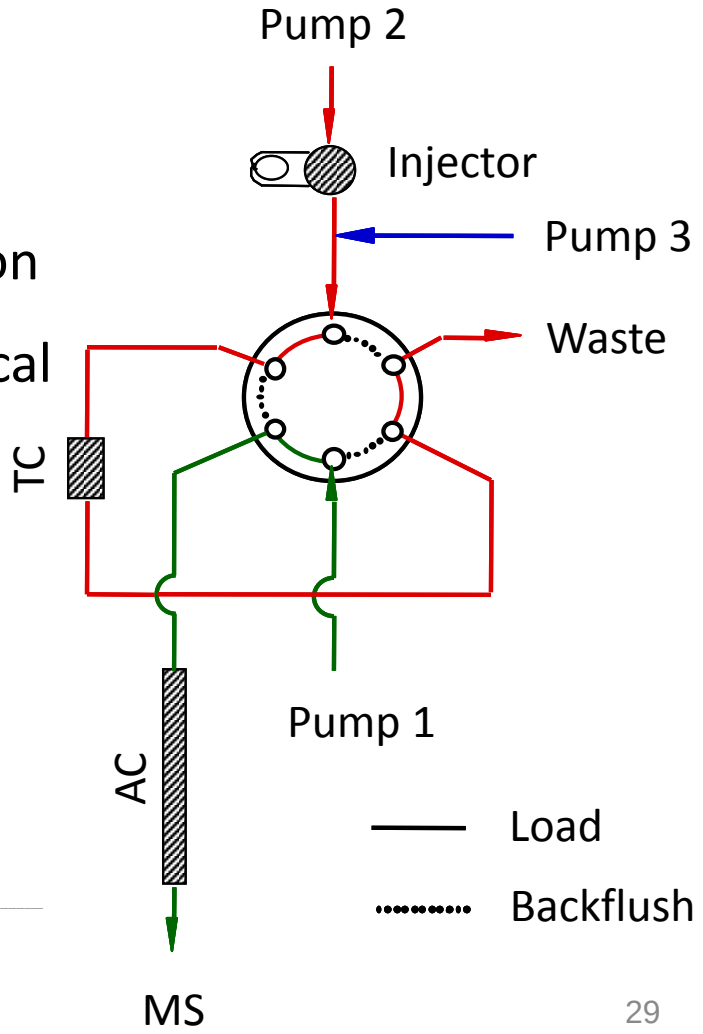
# Experimental –
# How to detect and quantify isoprostanes

1. Preparation step: Extraction of prostanoids from matrix
   - Solid phase extraction
   - liquid liquid extraction
   - immunoaffinity assays
2. Quantification step: Separation / Focusing of similar analytes + detection
   - LC-MS(/MS)
   - GC-MS
   - Immunoaffinity assays

- Combination of 1 and 2 favorable to avoid work up steps → column switching LC set up for analyte enrichment

# Experimental –
# Setup for integrated isoprostane measurement: Column switching LC

- Injection volume: 500 µl
- Run time 13.5 min, flow rate 0.4 mL/min
- Flush on trapping column + online dilution
- Backflush of retained analytes to analytical column + gradient elution
- MS/MS detection:
  - 4000 QTRAP (SRM)
  - 5600+ TTOF (FS + PIS → "HR-SRM")




TC: YMC AQ, 20 x 2.1 mm, 5 µm, YMC Europe

AC: Atlantis T3, 100 x 2.1 mm, 1.8 µm, Waters

# Experimental –
# MS options for QqQ versus TTOF instruments

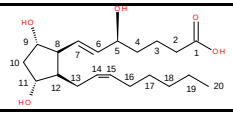
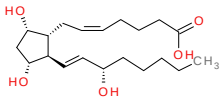
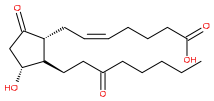
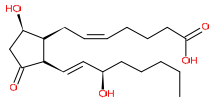
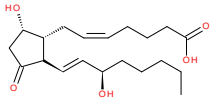
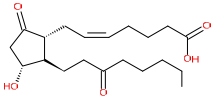
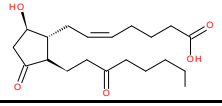
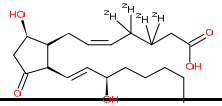
- Analytical requirements
  - 10 – 12 data points per peak
  - High selectivity
  - Wide linear range
- Available scan techniques
  - QqQ: Selected Reaction Monitoring (also: MRM)

    best to exclude interferences and achieve linear calibration ranges of several orders of magnitude
  - TTOF: 1. Full scan + data dependent scan
    → number of scans too small (many experiments)

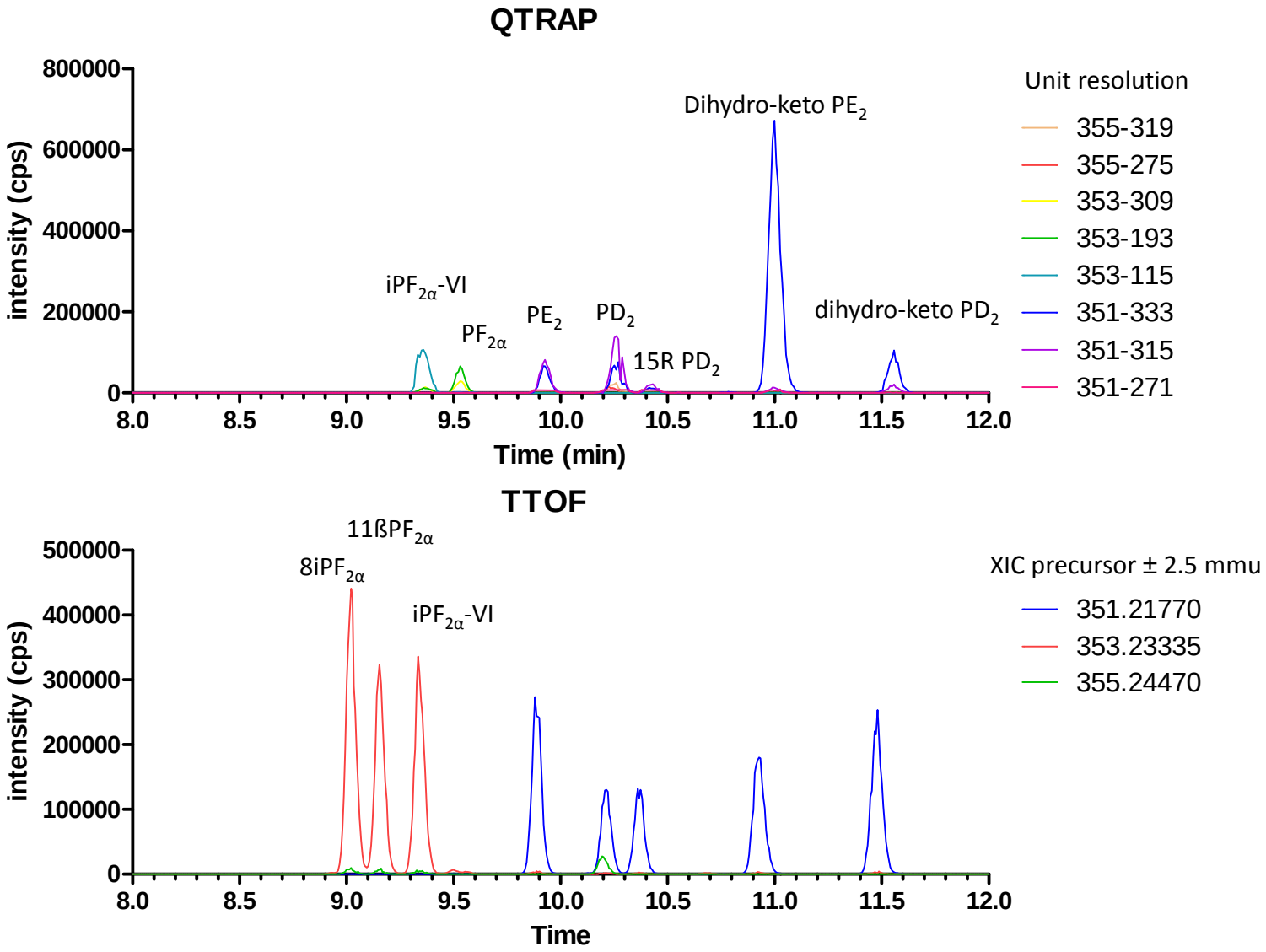
    2. Full scan + product ion scan
    → allows to pick matching precursor + product ion

# Optimization of Analytes

- QTRAP: Compound specific SRM transitions; TTOF: no optimization needed

| Short name (entry) | structure | precursor Q1 m/z | fragment Q3 m/z | DP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| 5-iso $PF_{2\alpha}$-VI |  | 353.1 | 193.0 | -65 | -34 | -13 |
| (**1**) | | 353.1 | 114.9 | -80 | -30 | -9 |
| $PF_{2\alpha}$ |  | 353.1 | 309.1 | -60 | -36 | -15 |
| (**2**) | | 353.1 | 193.0 | -65 | -34 | -13 |
| $PE_2$ |  | 351.1 | 315.0 | -55 | -18 | -11 |
| (**3**) | | 351.1 | 271.1 | -50 | -25 | -12 |
| $PD_2$ |  | 351.1 | 315.0 | -55 | -18 | -11 |
| (**4**) | | 351.1 | 271.1 | -50 | -25 | -12 |
| 15(R) $PD_2$ |  | 351.1 | 315.0 | -55 | -18 | -11 |
| (**5**) | | 351.1 | 271.1 | -50 | -25 | -12 |
| dihydro-keto |  | 351.1 | 333.0 | -35 | -16 | -13 |
| $PE_2$ (**6**) | | 351.1 | 315.0 | -55 | -18 | -11 |
| dihydro-keto |  | 351.1 | 333.0 | -35 | -16 | -13 |
| $PD_2$ (**7**) | | 351.1 | 315.0 | -55 | -18 | -11 |
| $PD_2$-d4 |  | 355.1 | 337.0 | -65 | -16 | -17 |
| (**8**) | | 355.1 | 319.0 | -55 | -18 | -11 |

# Results –
# Overview reference spectra (5 ng/mL)



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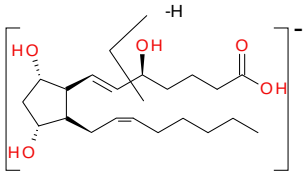
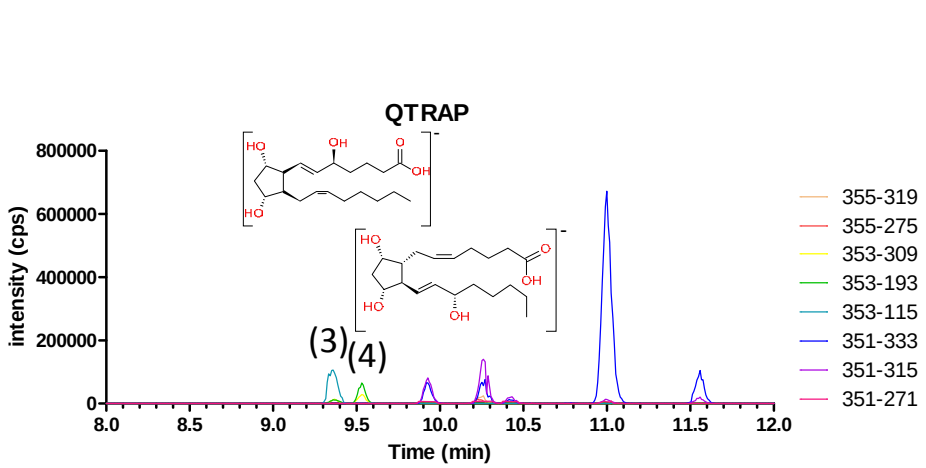
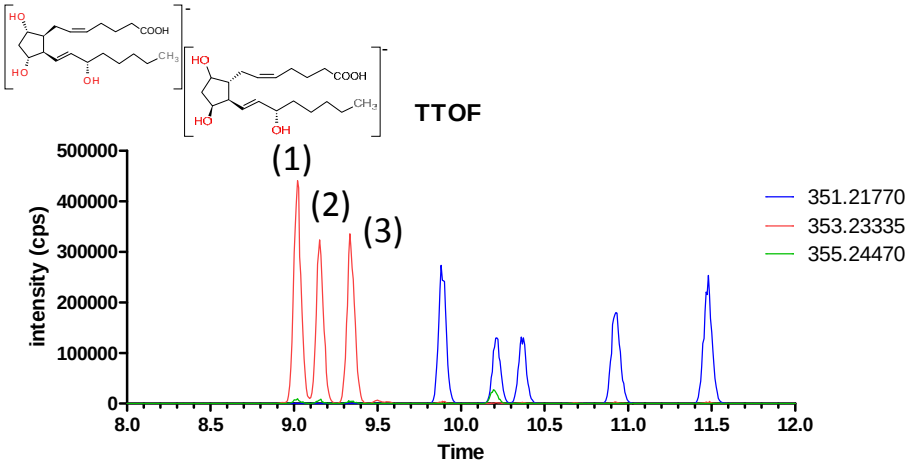
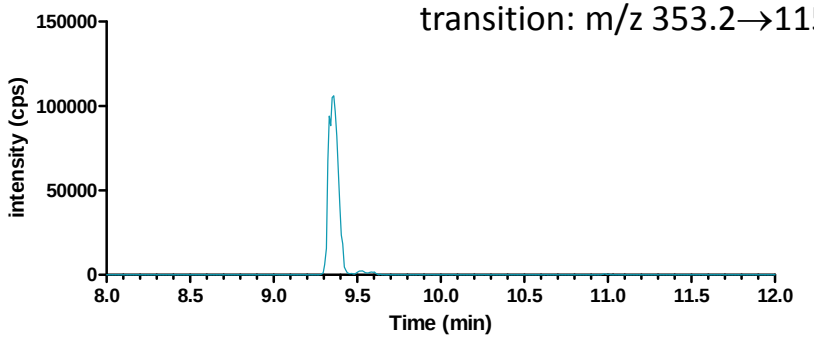
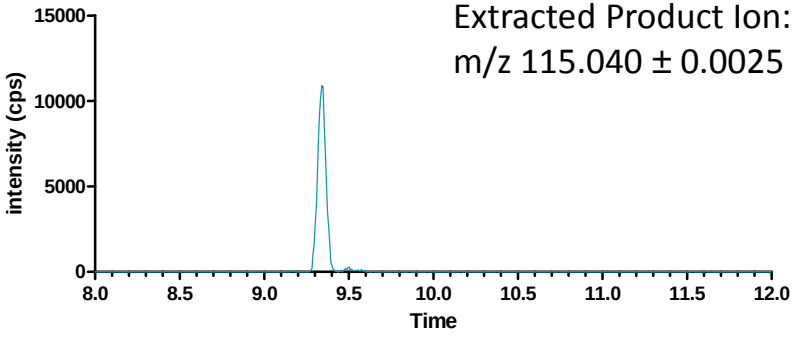


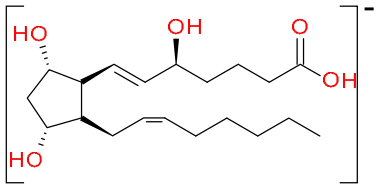




(1) – (4): Isobaric $F_{2\alpha}$ prostaglandins

# Results –
# Comparison of Calibration data

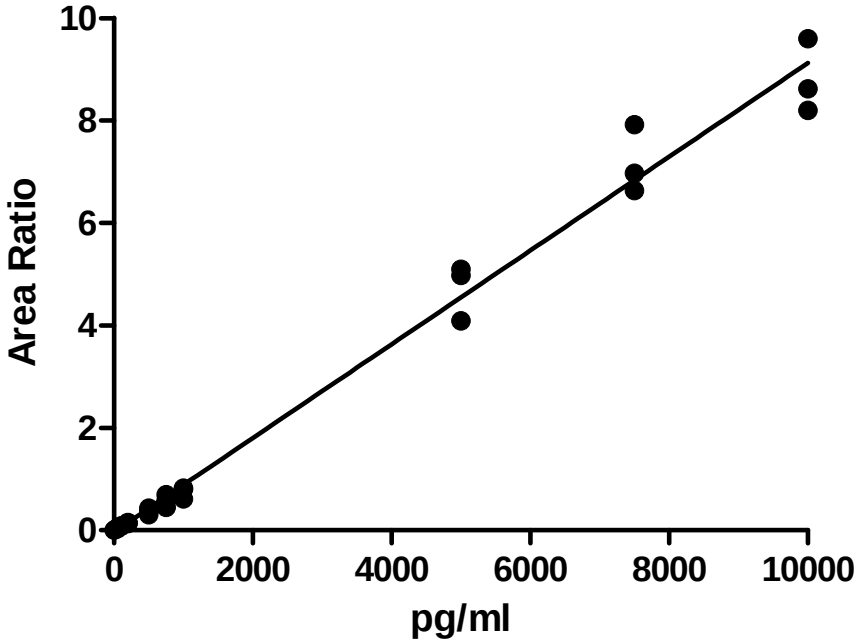


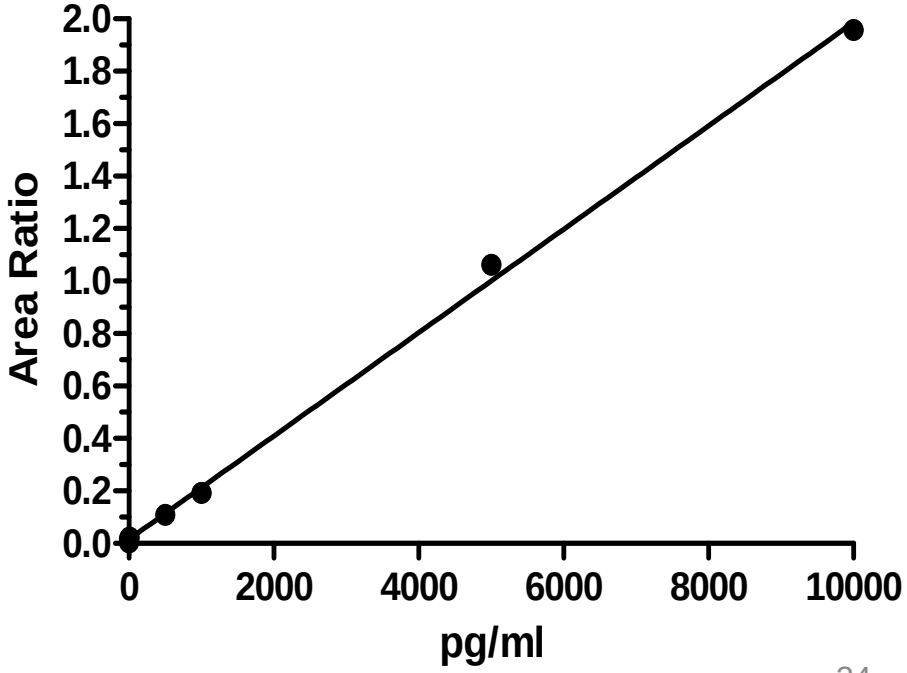

## QTRAP: 5isoPF$_{2\alpha}$-VI

- Calibration from 0.02 to 10 ng/mL
- y=0.0009152x-0.02752
- $R^2$=0.9892

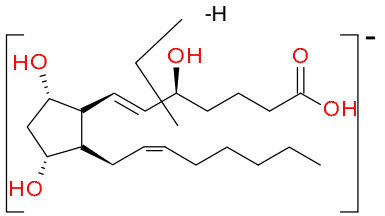
## TTOF: 5isoPF$_{2\alpha}$-VI

- Calibration from 0.005 to 10 ng/ml
- y=0.0001971x-0.01568
- $R^2$=0.9985



# Results – Comparison of Calibration data

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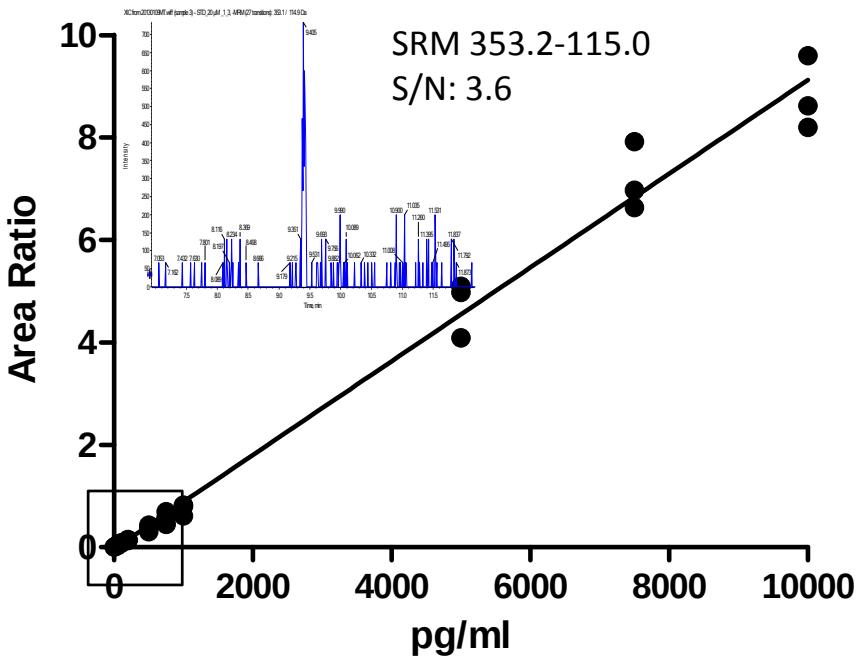
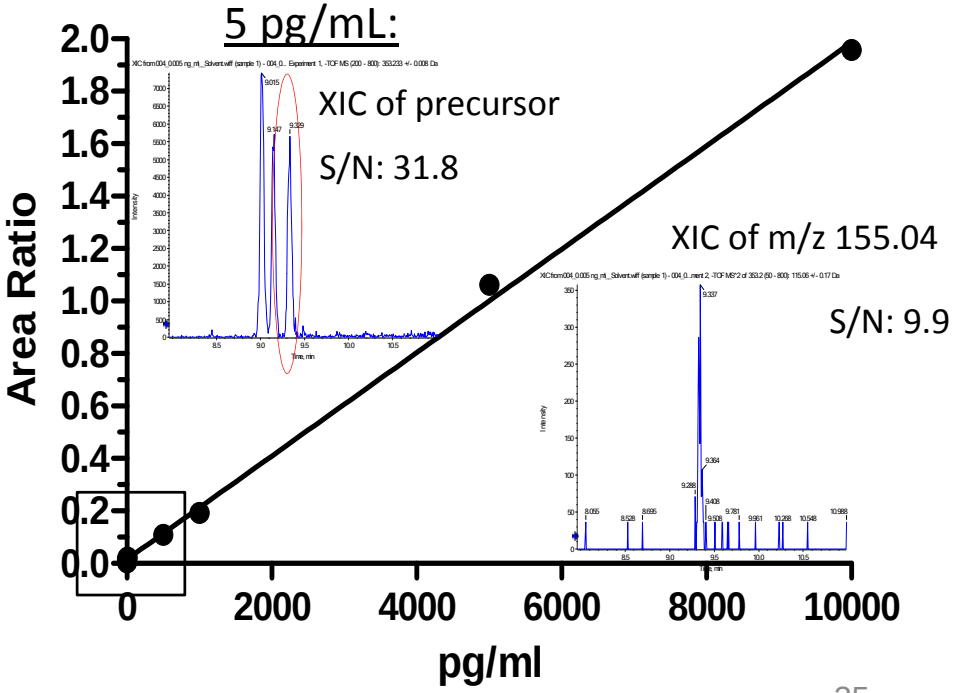
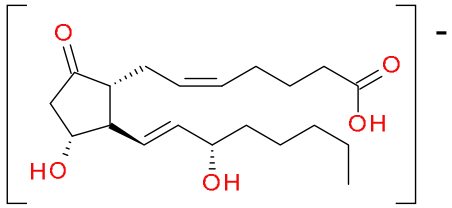
20 pg/mL:




5 pg/mL:

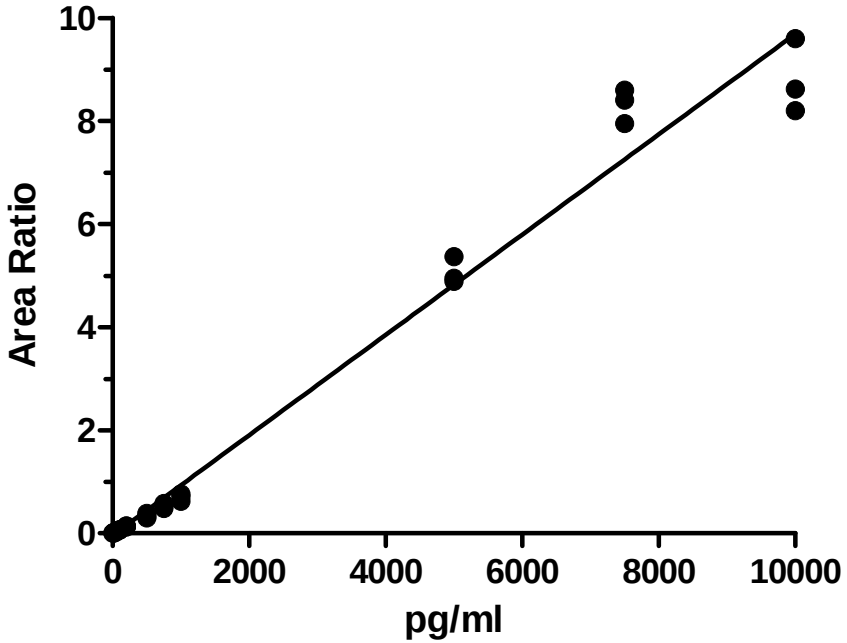


# Results –
# Comparison of Calibration data



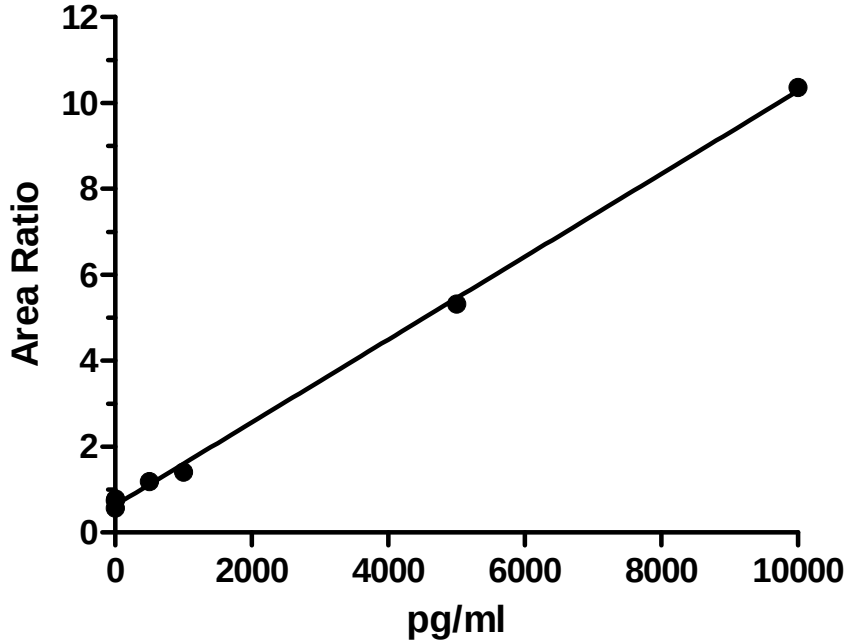

## QTRAP: Prostaglandin $D_2$

- Calibration from 0.02 to 10 ng/mL
- y=0.0009727x-0.04004
- $R^2$=0.9791

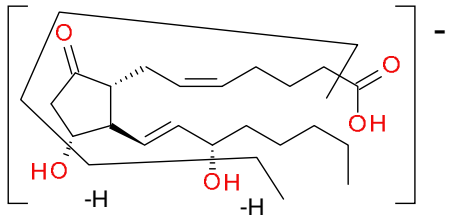



## TTOF: Prostaglandin $D_2$

- Calibration from 0.005 to 10 ng/ml
- y=0.0009642x-0.6372
- $R^2$=0.9987



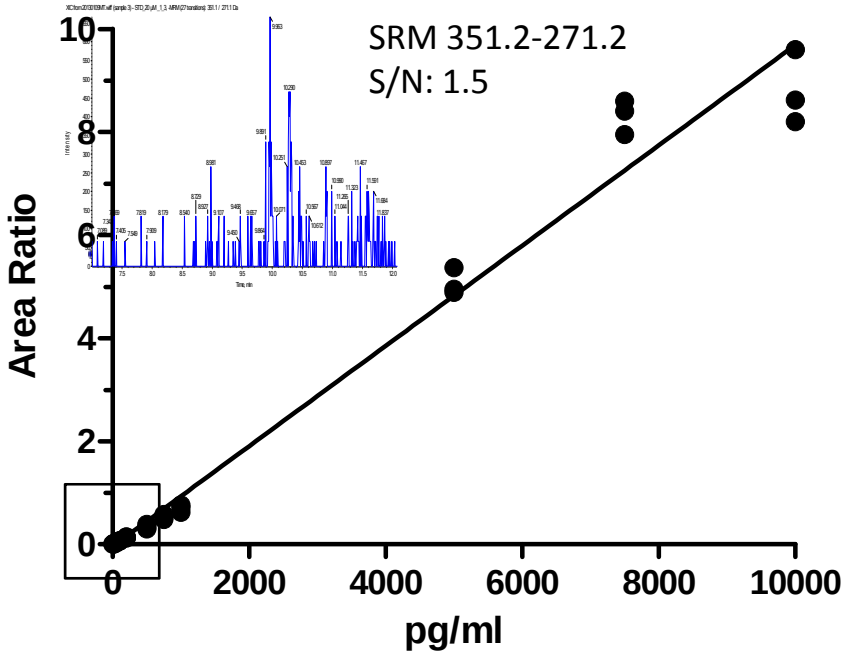
# Results –
# Comparison of Calibration data




## QTRAP: Prostaglandin $D_2$

- Calibration from 0.02 to 10 ng/mL
- y=0.0009727x-0.04004
- $R^2$=0.9791

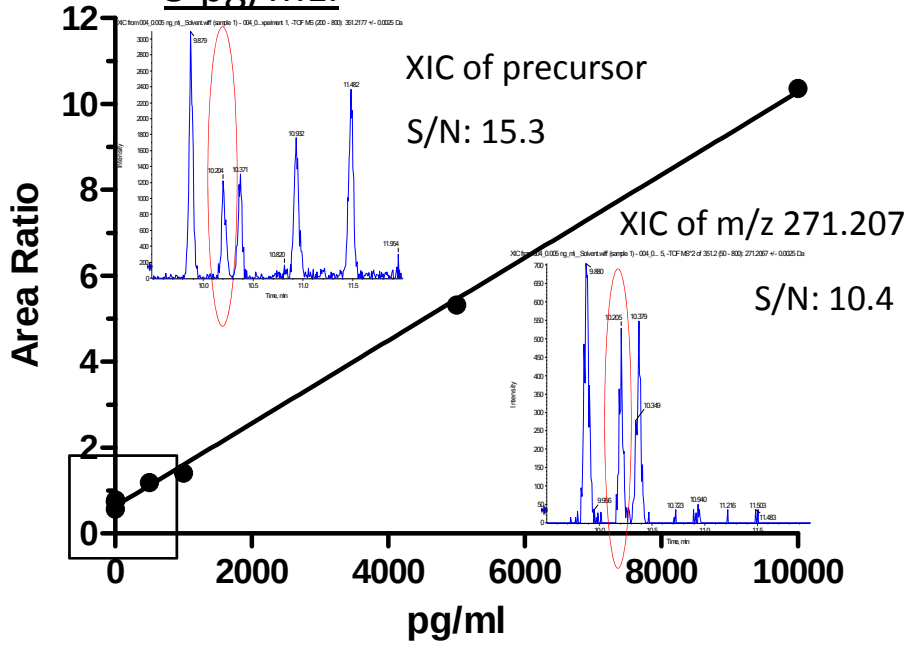
20 pg/mL:



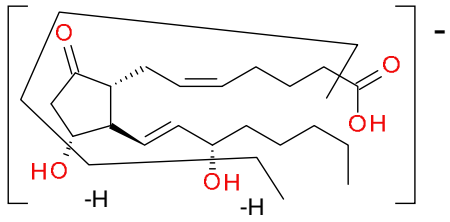

## TTOF: Prostaglandin $D_2$

- Calibration from 0.005 to 10 ng/ml
- y=0.0009642x-0.6372
- $R^2$=0.9987

5 pg/mL:

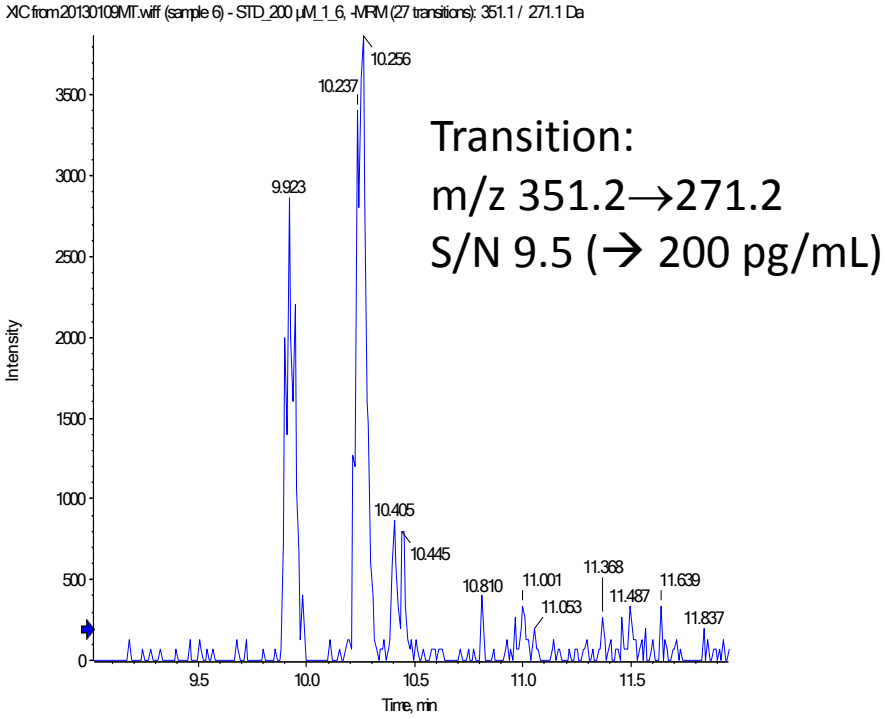


# Results –
# Comparison of Calibration data




## QTRAP: Prostaglandin $D_2$

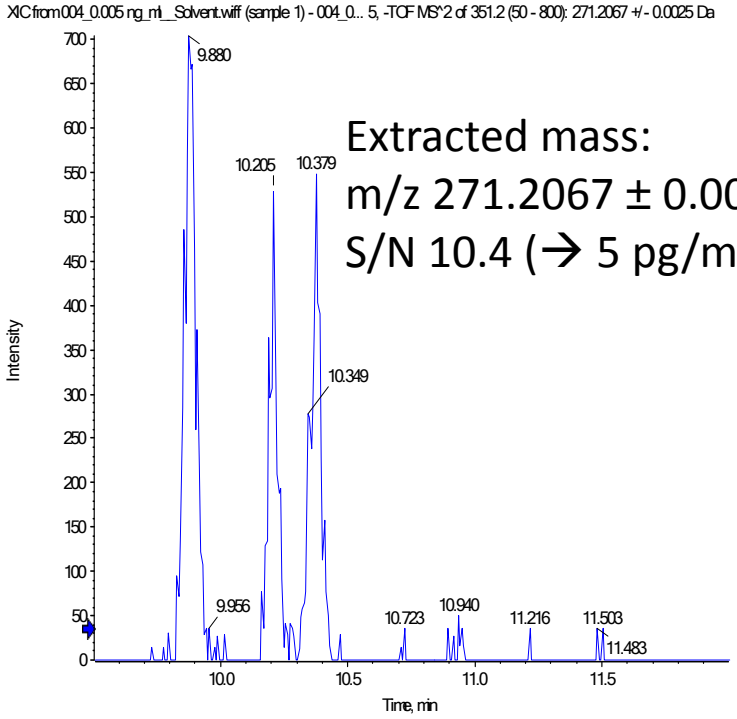
- Calibration from 0.02 to 10 ng/mL




Transition:
m/z 351.2→271.2
S/N 9.5 (→ 200 pg/mL)

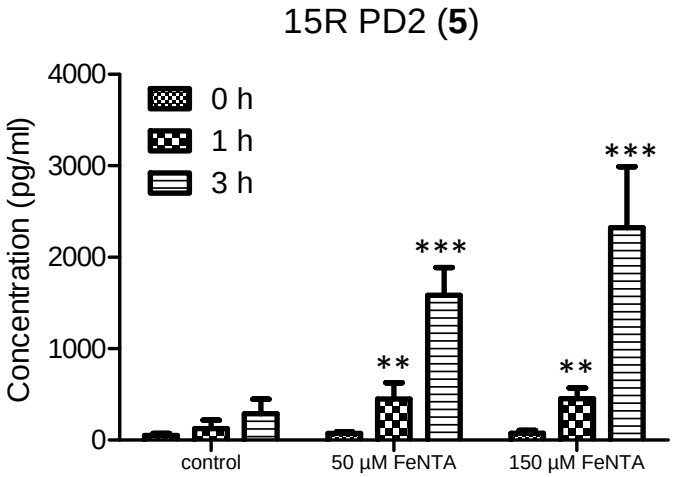
## TTOF: Prostaglandin $D_2$

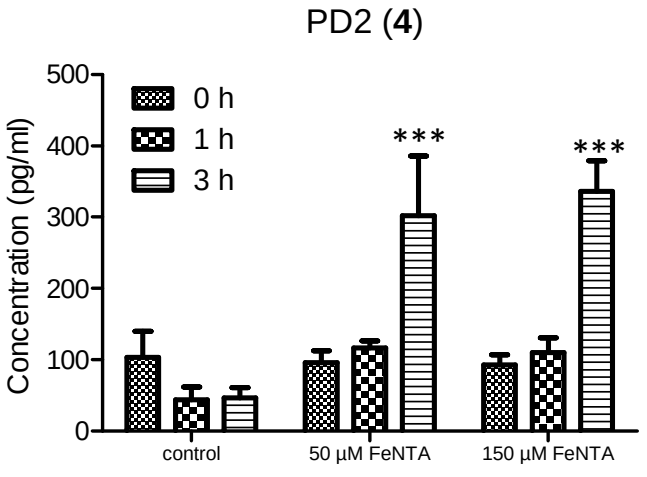
- Calibration from 0.005 to 10 ng/ml

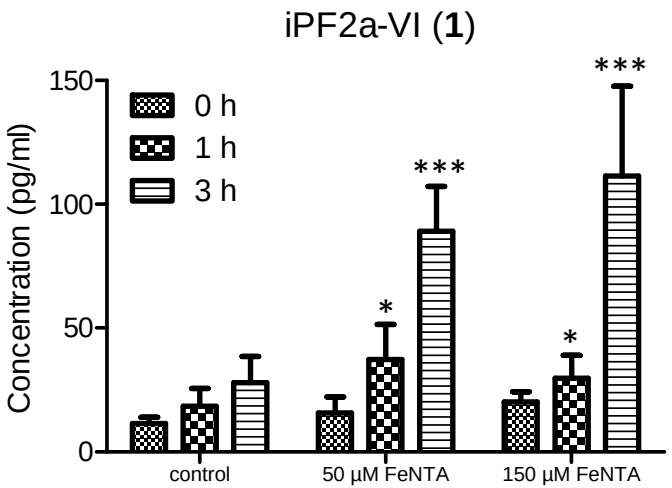


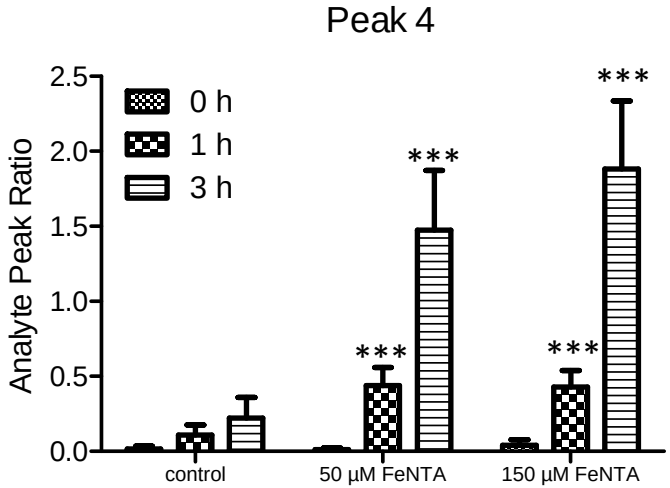

Extracted mass:
m/z 271.2067 ± 0.0025
S/N 10.4 (→ 5 pg/mL)

# Biological Application for Oxidative Stress Measurement: Rat hepatocytes



# Conclusion

- The TripleTOF 5600+ can be used for quantitative biomarker analysis
- Most suited scan technique:
  FullScan combined with ProductIonScan (“HR-MRM”)
  - Linear range: Comparable with triple quadrupole type instruments
  - Sensitivity gain in comparison with 4000 QTRAP for presented application
  - Selectivity: Achieved via accurate mass FullScan mode
    Further enhanced by the use of specific product ions
  - Flexibility in post acquisition data evaluation
    - Define processing parameters
    - Most suitable product ion
    - Combined XIC of several selective product ions

# Acknowledgements

**DMPK Roche**

- Andreas Brink
- Sandrine Simon

**AB Sciex**

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**Discovery Technologies Roche**

- Iris Ruf
- Martin Binder

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- Ismael Zamora
- Fabien Fontaine
- Blanca Serra
- Esra Nurten Cece

# Thank you for listening!
# Questions

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Marieke Teppner

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european pharmaceutical review

SUPPORTED BY:

AB SCIEX