



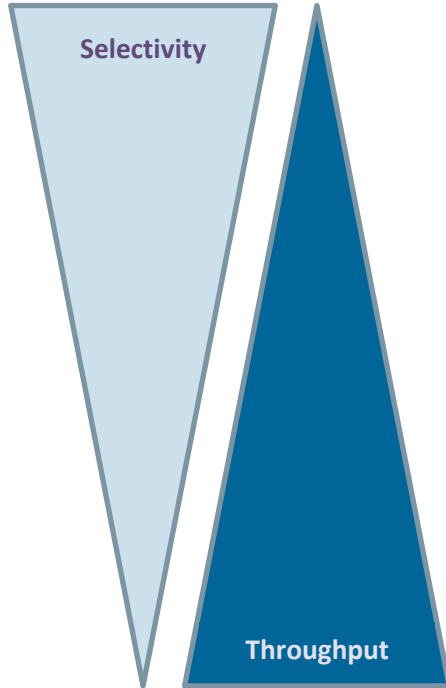
universität
wien

Throughput and coverage in metabolomics

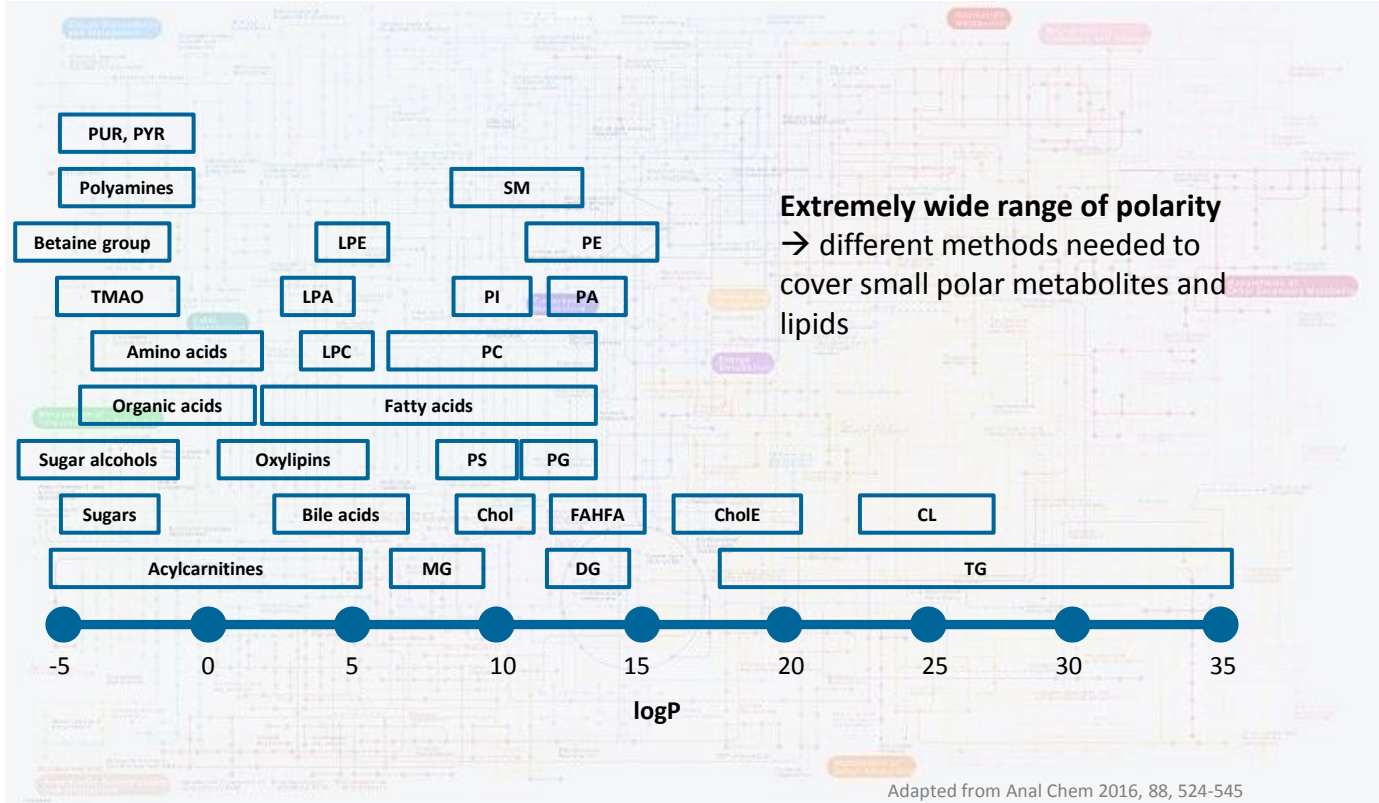
Michaela Schwaiger, Harald Schoeny, Yasin El Abiead, Mate Rusz, Luis Galvez, Gerrit Hermann,
Evelyn Rampler, Gunda Koellensperger



Mass spectrometry and omics-type analysis



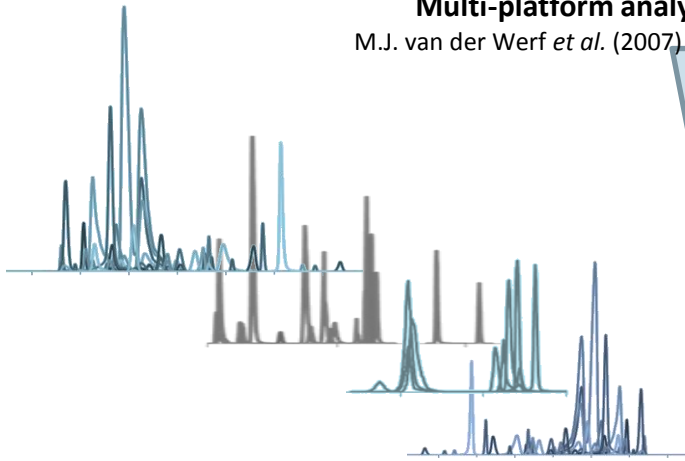
Omic-type analysis envisions the **comprehensive** identification and quantification of a complete compound class (or biological complement) within **one or few analytical runs**



Metabolite coverage, selectivity and analytical throughput

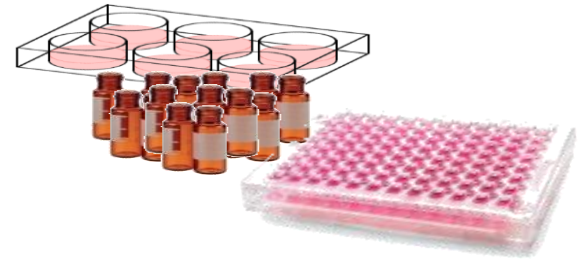
Multi-platform analysis, LC- and GC-MS

M.J. van der Werf *et al.* (2007) *Anal. Biochem.* **370**, 17



Selectivity

Throughput

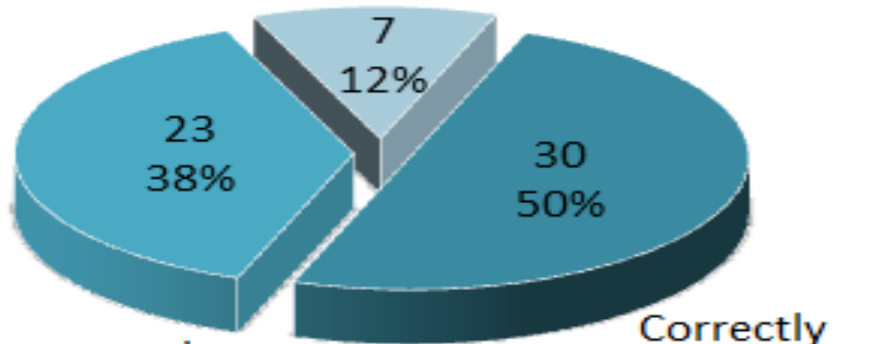


Flow injection-high resolution MS

T. Fuhrer *et al.*, (2011) *Anal. Chem.* **83**, 7074

Anion exchange –high resolution MS Reliability of putatively annotated compounds (level 2= by MS2 spectra)

False positives due to in-source fragmentation

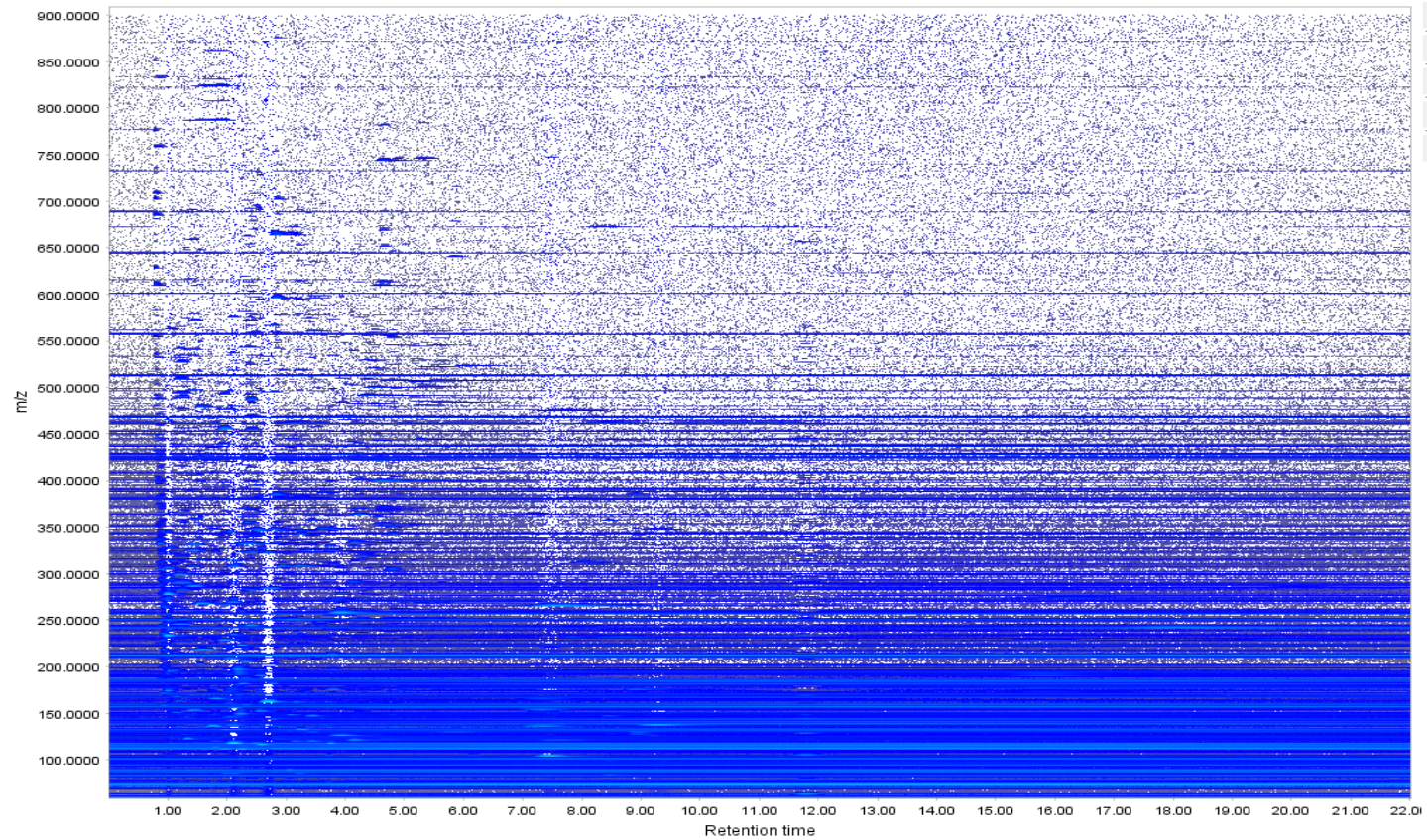


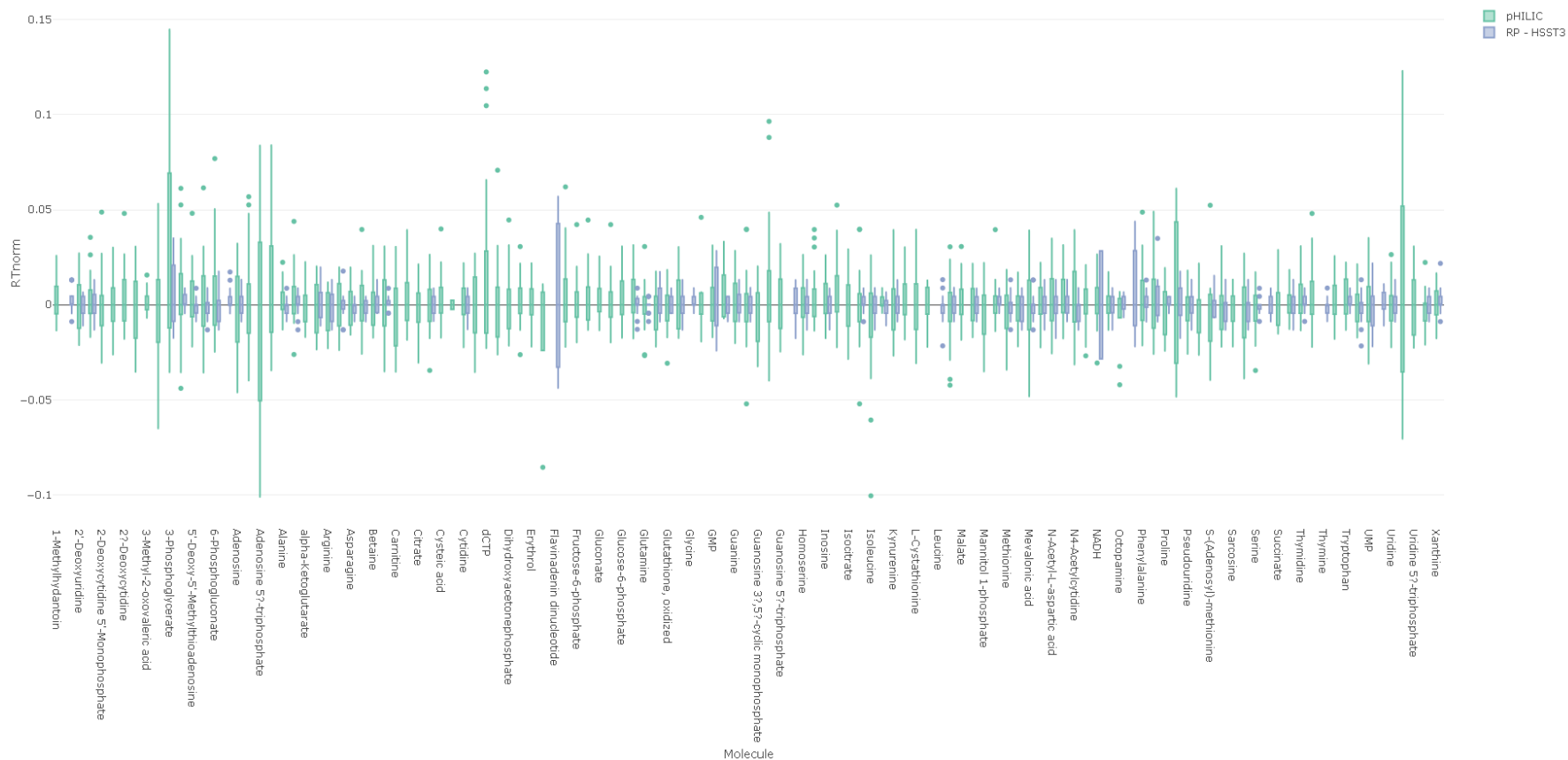
Isomeric compounds with more than one suggested structure

Correctly annotated compounds

Non targeted analysis of cancer cell extracts (preparation of 10^6 SW480 cells) by MS2 measurement and mzCloud search.

Comparison with standards revealed a number of identified false positives due to in-source fragmentation and due to isomers. Isomeric interference leads to features with more than one proposed structure in case the MS2 spectrum is not unique for one isomer.

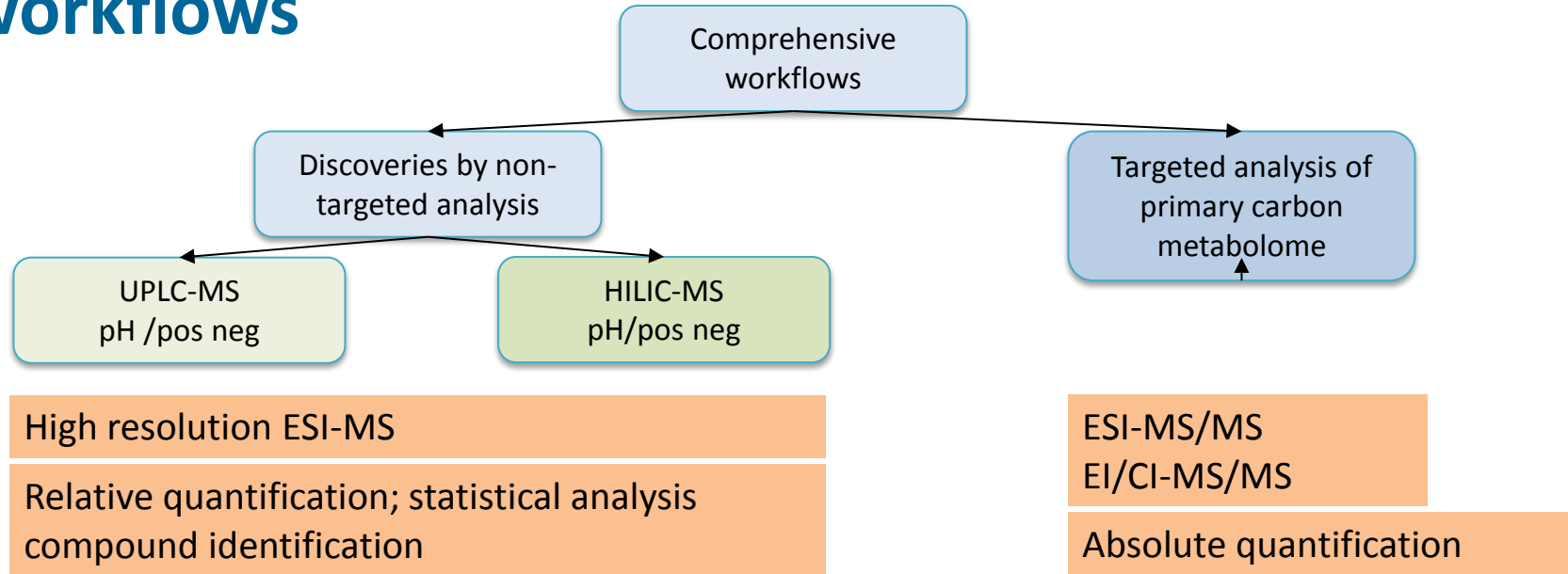




Divide and conquer

- Definition of sub-omes metabolome and lipidome based on analytical strategies
- Definition of distinct analytical tasks: Targeted absolute versus non-targeted analysis
- Concept of merging workflows
 - Merging quantification and identification in HR-MS
 - Merging lipidomics/metabolomics

Comprehensive metabolomics workflows



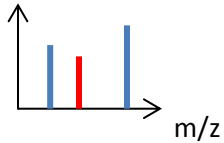
Analytical approaches for lipids

Shotgun lipidomics

*All analytes
in one spectra*

Fast, ion suppression

**Lipid class specific
quantification**



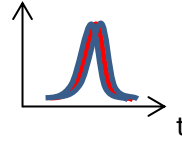
LC-MS-based lipidomics

Hydrophilic Interaction (HILIC)

*Separated after head
group*

Fast, only polar lipids

**Lipid class specific
quantification**

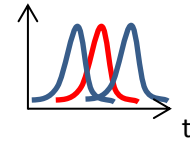


Reversed phase (RP)

*Separated after chain
composition*

Long run time

**Identification/
Compound specific
quantification**

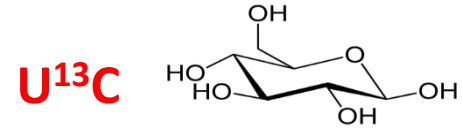


Divide and conquer

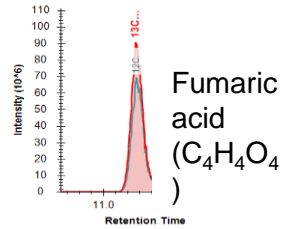
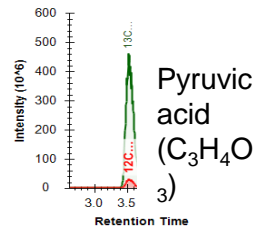
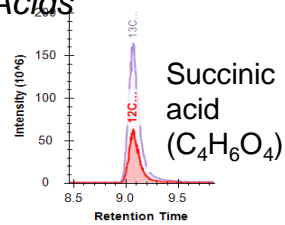
- Definition of sub-omes metabolome and lipidome based on analytical strategies
- Definition of distinct analytical tasks: Targeted absolute versus non-targeted analysis
- **Concept of merging workflows**
 - Merging targeted analysis (absolute quantification) and non-targeted analysis (identification/relative quantification) in one run
 - Merging lipidomics/metabolomics

Internal standards in metabolomics

Isotopically enriched ^{13}C *Pichia pastoris*

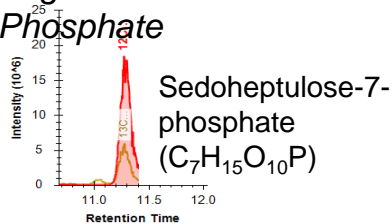


Organic
Acids

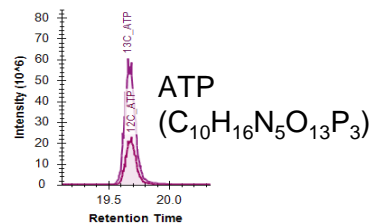


Fed batch
cultivation with
 ^{13}C glucose

Sugar
Phosphate



Nucleotide



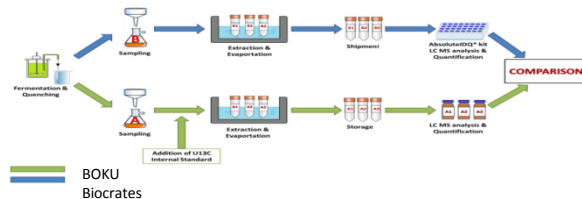
Nucleotides, nucleosides and nucleobases (Neubauer et al. 2012, Schwaiger et al., 2017); Organic acids (Klavins et al., 2014); Amino acids (Guerrasio et al. 2014, Hermann et al., 2017); Sugar phosphates (Troyer et al. 2017, Klavins et al. 2014, Chu et al. 2014); Coenzyme A, Acyl coenzyme A thioester (Neubauer et al. 2015); Cofactors (Ortmayr et al. 2014); Sulfur metabolism (Troyer et al. 2012, Hermann et al. 2013, Ortmayr et al. 2015)

Accurate quantification by Isotope dilution

Shown by

- Laboratory intercomparison

Klavins, K et al., (2013) Analytical and Bioanalytical Chemistry, 405 (15), 5159.

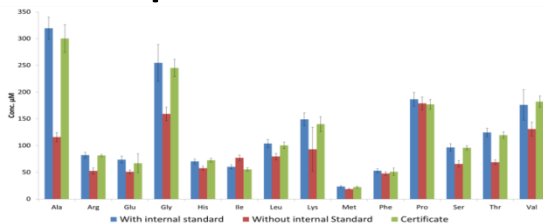
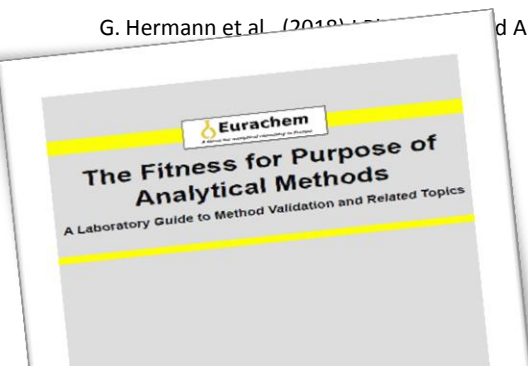


- Crossvalidation using different platforms

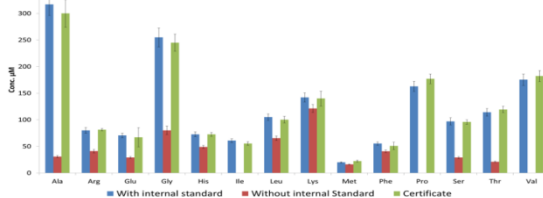
K. Ortmayr et al., (2015) Analyst 140, 3465

- Reference material

G. Hermann et al., (2010) J. Chromatogr. B, 878, 1-10

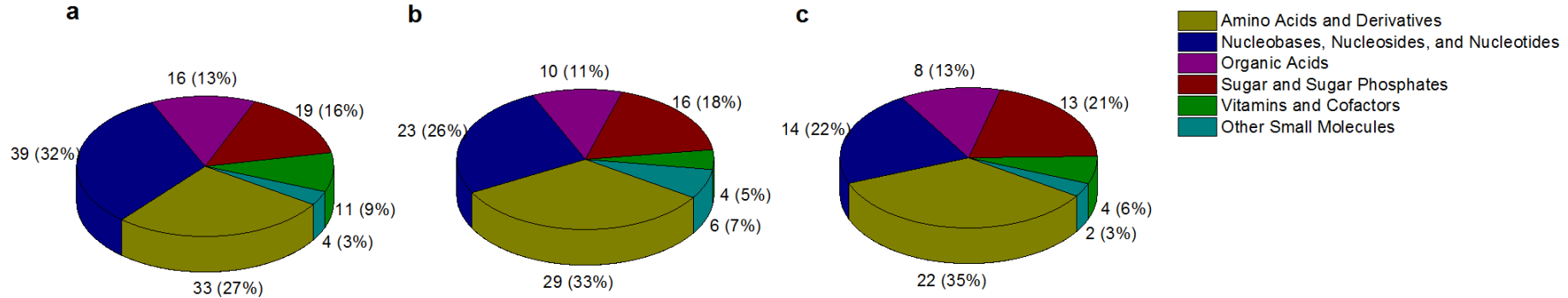


HILIC-QE-HF-MS



HILIC-MS/MS

Metabolites library in *Pichia pastoris*



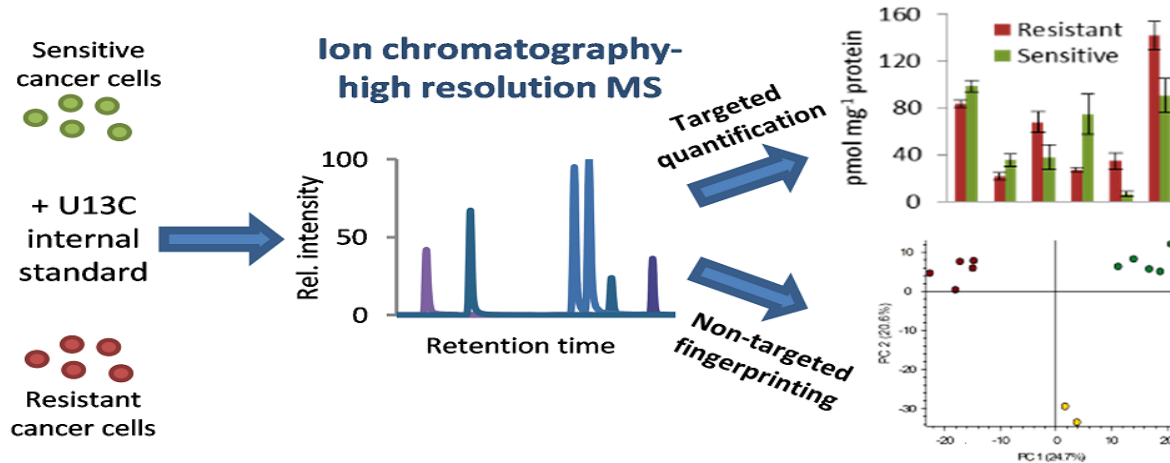
Number of metabolites and their classes as found

(a) up to date in ^{13}C -enriched *Pichia pastoris*

(b) In HCT116 cells extract by HILIC-Orbitrap-MS

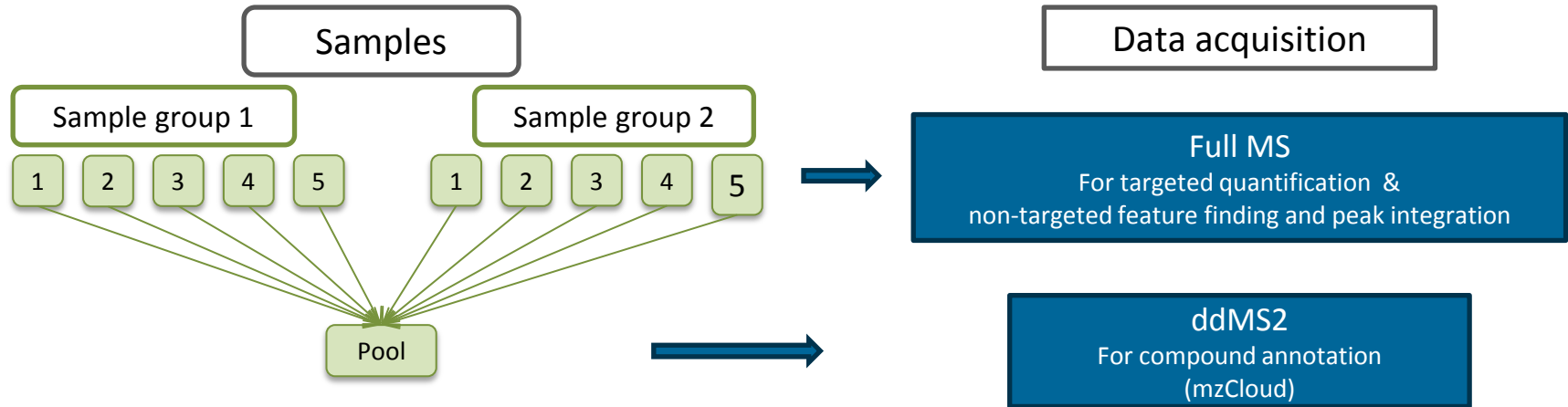
(c) in ^{13}C -enriched *Pichia pastoris* added to HCT116 cells

Anion-exchange chromatography coupled to high-resolution mass spectrometry: A powerful tool for merging targeted and non-targeted metabolomics



Michaela Schwaiger, Evelyn Rampler, Gerrit Hermann, Walter Miklos, Walter Berger, Gunda Koellensperger, *Analytical Chemistry*, **2017**, 89, 7667–7674

Merging targeted with non-targeted analysis by high-resolution mass spectrometry



For absolute quantification

▪ Standards

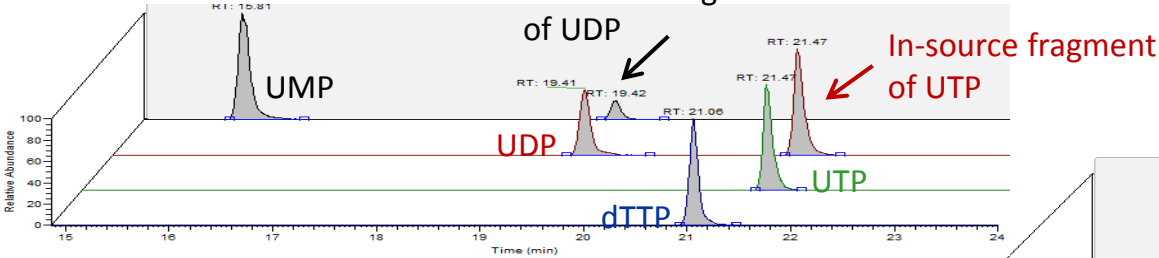
- Nucleotides, sugar phosphates and organic acids standards
- Calibration range: 0.5 nM - 50 µM

▪ Species specific internal standardization

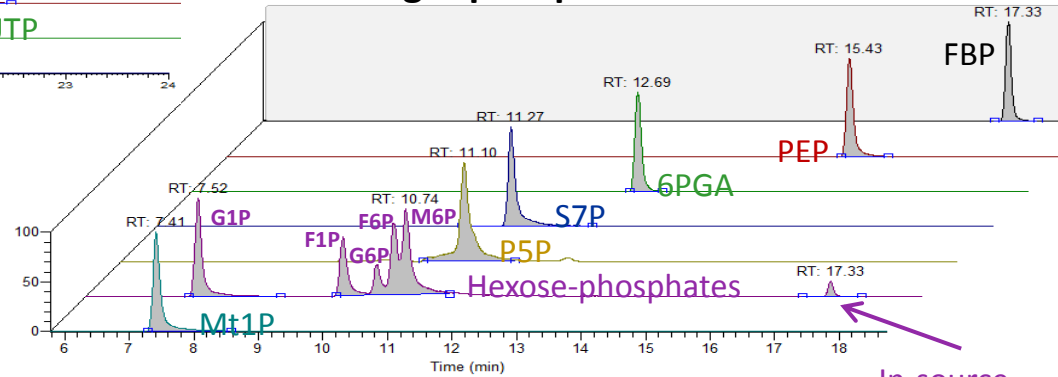
- Fully ¹³C labeled extract of *Pichia pastoris*
- Added to all calibration standards and samples

Anion exchange chromatography

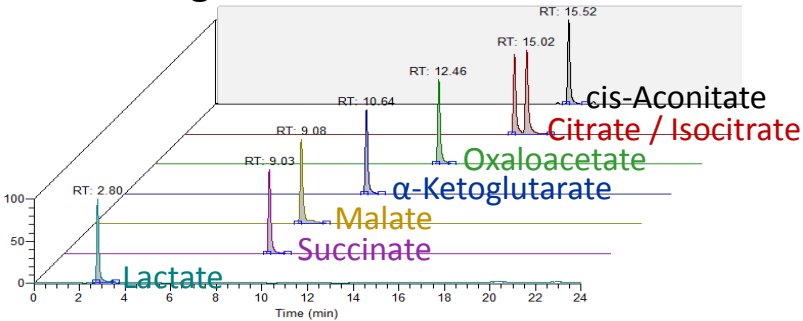
Nucleotides



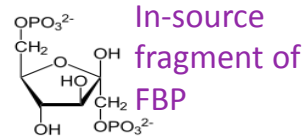
Sugar phosphates



Organic acids

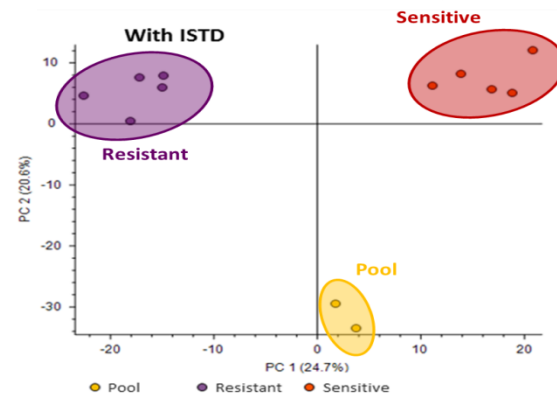
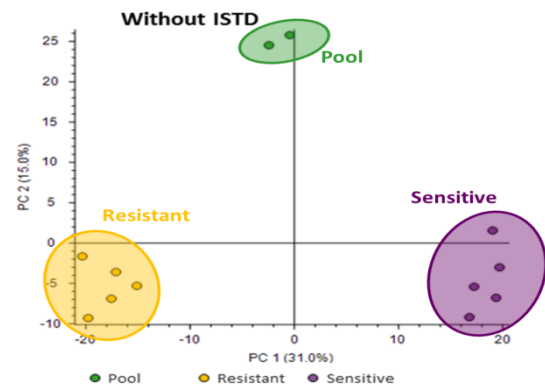
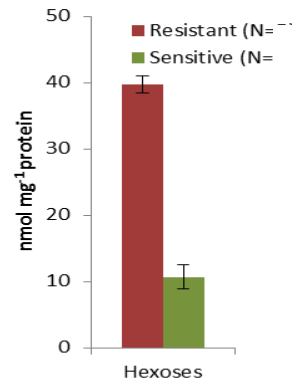
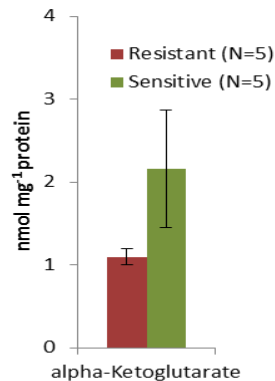
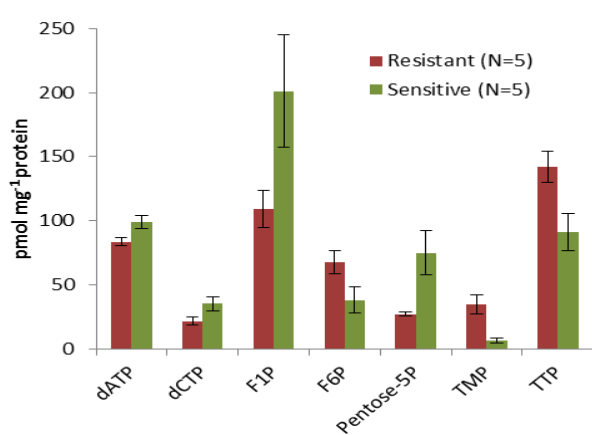


- FBP Fructose-1-6-bisphosphate
- PEP Phosphoenol-pyruvate
- 6PGA 6-Phosphogluconate
- S7P Sedoheptulose-7-phosphate
- P5P Pentose-5-phosphates
- Mt1P Mannitol-1-phosphate



Application in a cancer cell study

- Sensitive vs. resistant cancer cell line
- 1×10^6 cells seeded in 6-well plates
- 5 biological replicates
- Quantified with yeast based fully ^{13}C labeled internal standard

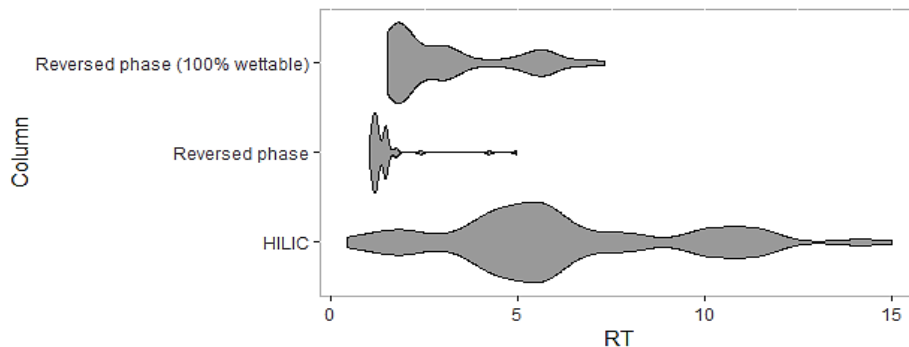


Divide and conquer

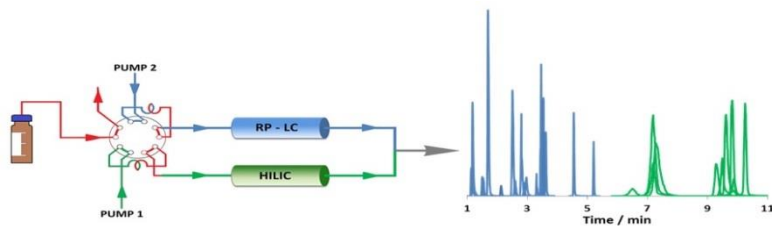
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On-line combination of chromatographic separations

■ Applications in metabolomics

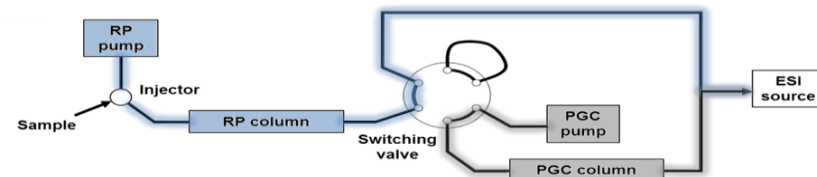


Retention time distribution of metabolites using orthogonal chromatography



Parallel column RPLC/HILIC

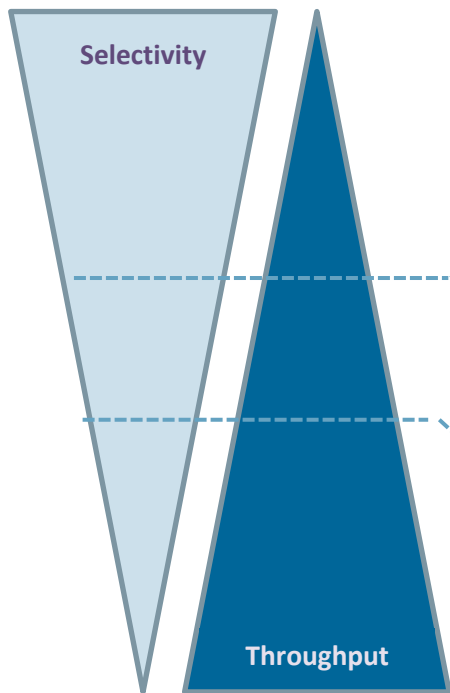
K. Klavins, H. Drexler, S. Hann, G. Koellensperger, (2014) *Anal. Chem.* **86**, 4145



Heart-cutting RP-PGC-LC

K. Ortmayr, S. Hann, G. Koellensperger, (2015) *Analyst* **140**, 3465

On-line combination of chromatographic separations



Increasing coverage within the sub-omes

Metabolomics: Parallel column RPLC/HILIC

K. Klavins, H. Drexler, S. Hann, G. Koellensperger, (2014) *Anal. Chem.* 86, 4145

Metabolomics: Heart-cutting RP-PGC-LC

K. Ortmayr, S. Hann, G. Koellensperger, (2015) *Analyst* 140, 3465

Lipidomics: Heart-cutting RPLC-HILIC

E. Rampler, H. Schoeny, BM. Mitic, Y. El Abiead, M. Schwaiger, G. Koellensperger, (2018) *Analyst*, 143, 1250

Increasing coverage accross the sub-omes

Merging metabolomics and lipidomics into one analytical run

Dual Extraction/dual injection/Parallel RPLC/HILIC

M. Schwaiger, G. Hermann, H. Schoeny, E. Rampler, Y. El Abiead, G. Koellensperger, (2018) *Analyst*, just accepted

Merging metabolomics and lipidomics



- On-line combination of chromatographic separations
- Dual injection of tailored extracts
- Analysis in one run
- Merging absolute/relative quantification and compound identification for lipids and metabolites

Sub-omes require dedicated sample preparation

Metabolites

- Extraction by polar solvents
 - Methanol
 - Ethanol
 - Acetonitrile
 - Water

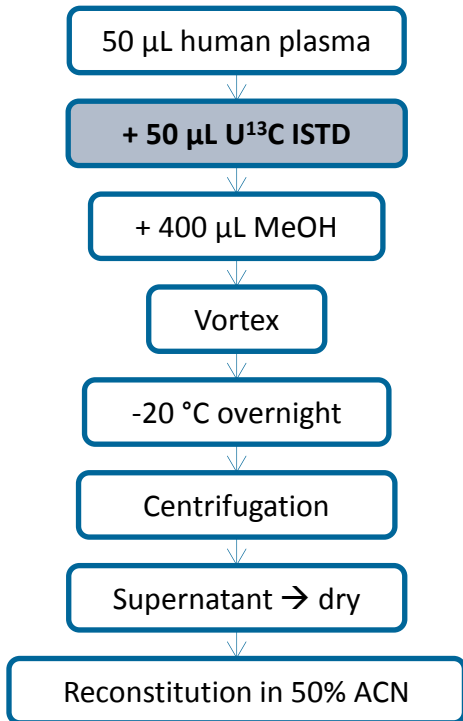
Lipids

- Liquid/liquid extraction
 - Methyl *tert*-butyl ether extraction (MTBE)
 - Folch: chloroform/methanol 2:1
- Solid-phase extraction

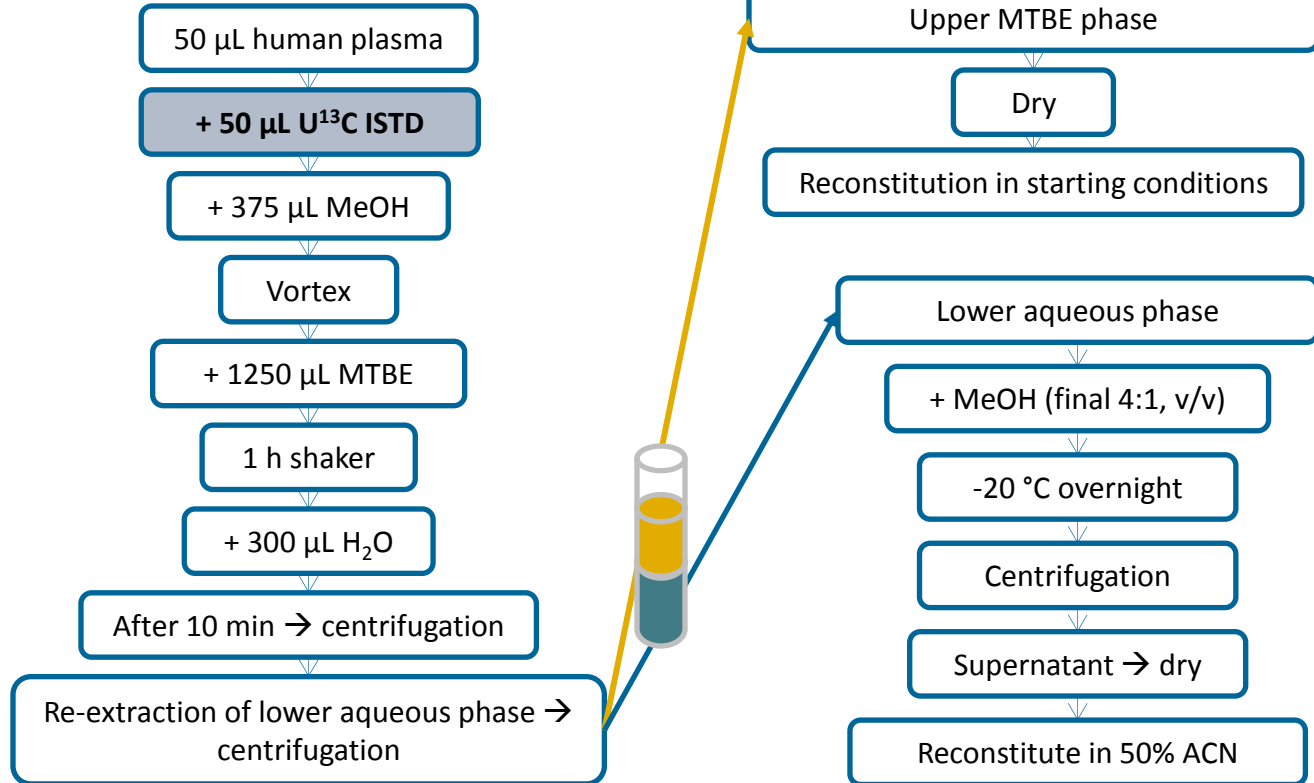
Approaches to allow multi-omics analysis: e.g. C. Coman et al. SIMPLEX: A Combinatorial Multimolecular Omics Approach for Systems Biology. *Mol. Cell. Proteomics* **2016**

Sample preparation of human plasma

Extraction with methanol



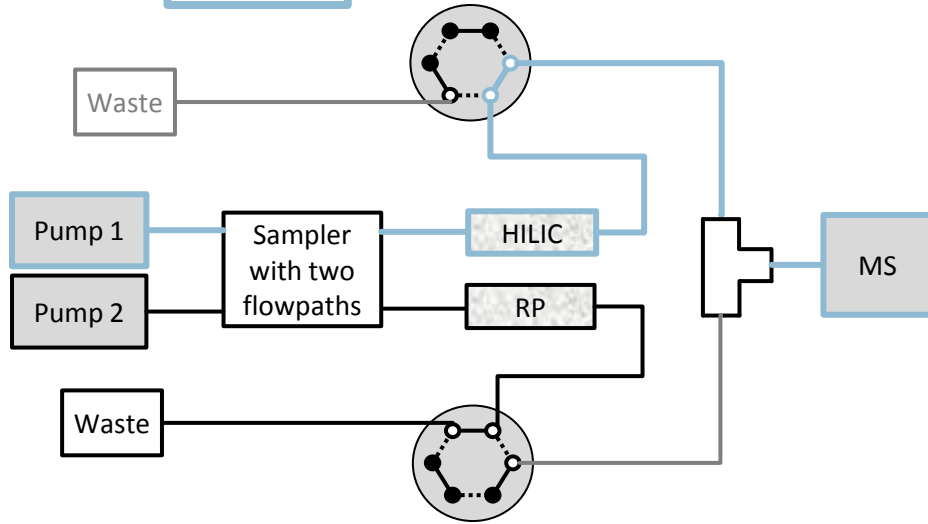
Biphasic MTBE extraction



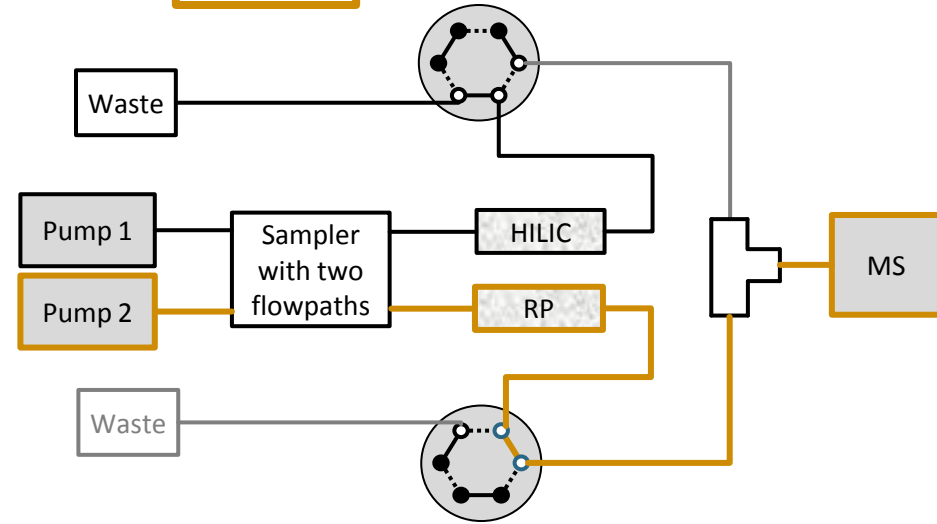
LC-MS Setup for metabolomics and lipidomics

Injection of two different extracts onto two different columns

0 – 11 min



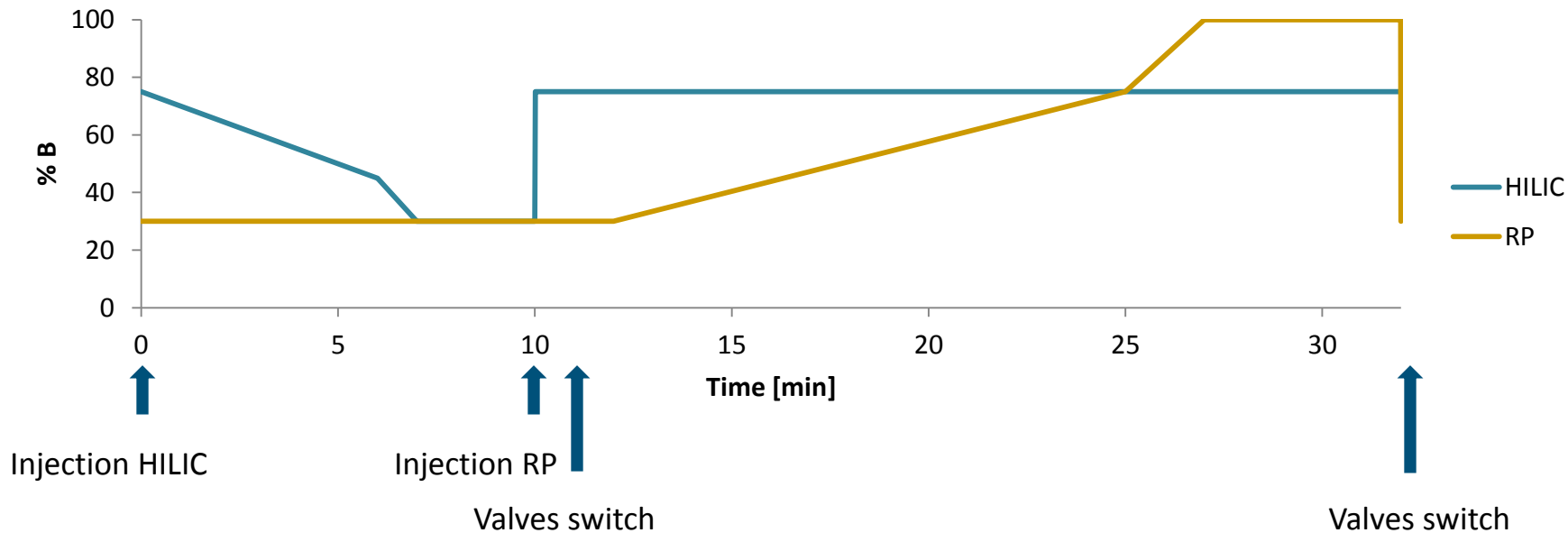
11 – 32 min



Separation of small polar metabolites

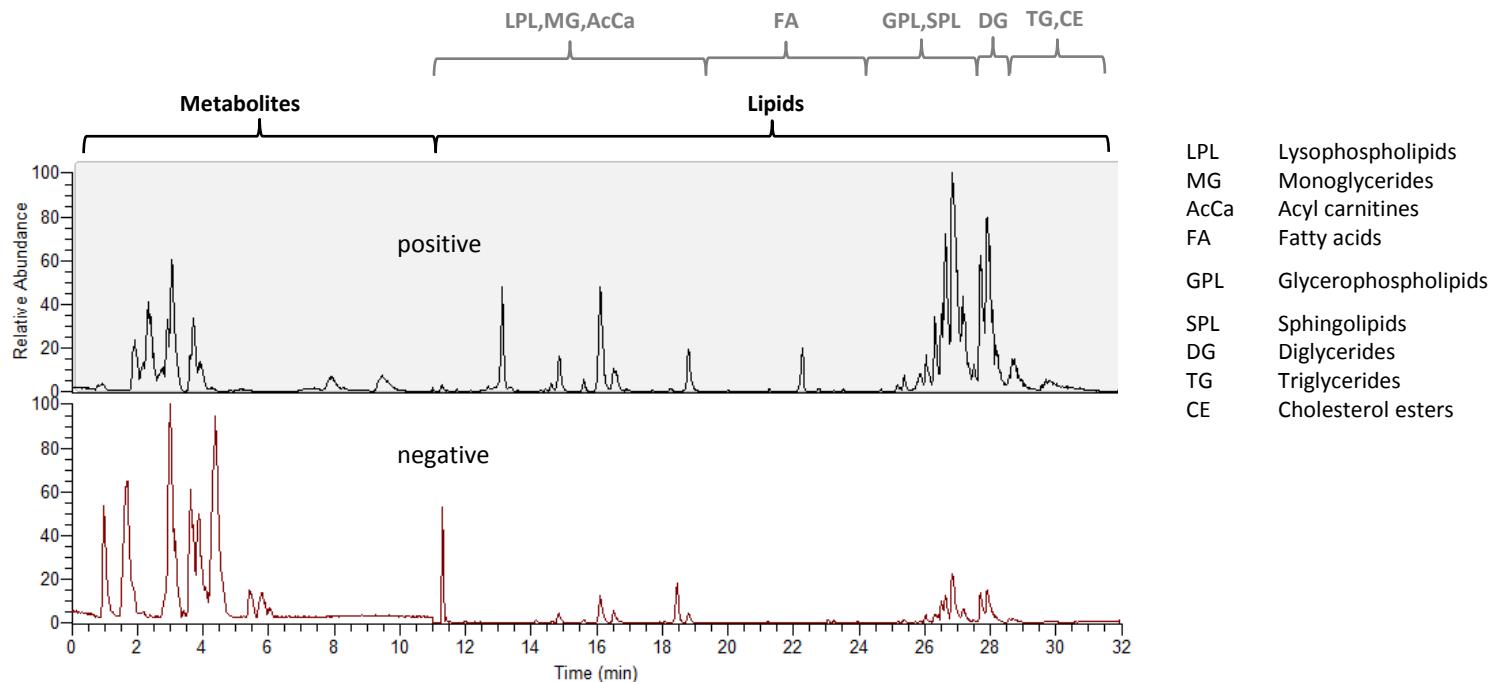
Separation of unpolar lipids

Experimental procedure - timeline



	HILIC (Metabolites)	RP (Lipids)
Column	ZIC-pHILIC, 150 x 2.1 mm, 5 μ m	Acquity HSS T3, 2.1 mm x 150 mm, 1.8 μ m
Mobile phase	A: 90% 10 mM NH_4HCO_3 , pH 9.2/ 10% ACN B: 100% ACN	A: ACN/ H_2O (3:2, v/v), 0.1% FA + 10 mM NH_4HCOO B: IPA/ACN (9:1, v/v). 0.1% FA + 10 mM NH_4HCOO
Flow rate	0.300 mL min^{-1} (10-27 min 0.050 mL min^{-1})	0.250 mL min^{-1}
Injection vol.	5 μ L	5 μ L
Column temp.	40 $^\circ\text{C}$	40 $^\circ\text{C}$
MS	Q Exactive HF	
Full MS	120,000 resolution, positive/negative ESI with fast polarity switching	
Mass range	65 - 900	200 - 2000
ddMS2 resolution	30,000	
TopN	15	10
Automated exclusion list generation		

Combining HILIC (alkaline) and RP (acidic)



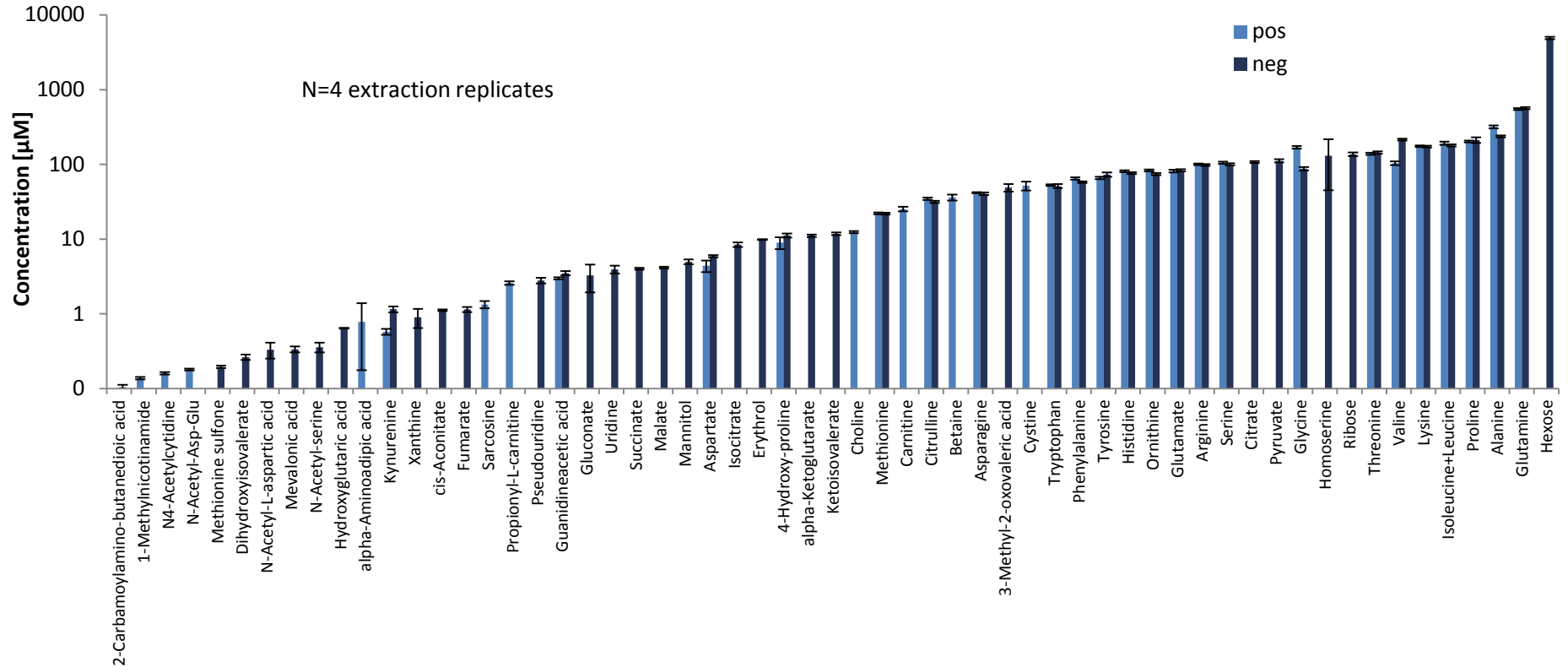
Standard reference material (SRM) 1950

Metabolites in frozen human plasma separated with the new dual setup

Analytical figures of merit: targeted metabolomics

- Multi-component standard containing > 100 metabolites
- Excellent intermediate repeatability: more than half of the compounds < 10% area RSD and < 0.5% retention time RSD over 45 hours
- Limits of detection: 10 – 50 nM for organic acids, 10 – 100 nM for amino acids and nucleotides
- Linear calibration curves over 3 – 4 orders of magnitude using ¹³C labeled yeast extract as internal standard
- Majority of metabolites detected in both positive and negative mode

Absolute quantification



Merging absolute quantification and untargeted analysis

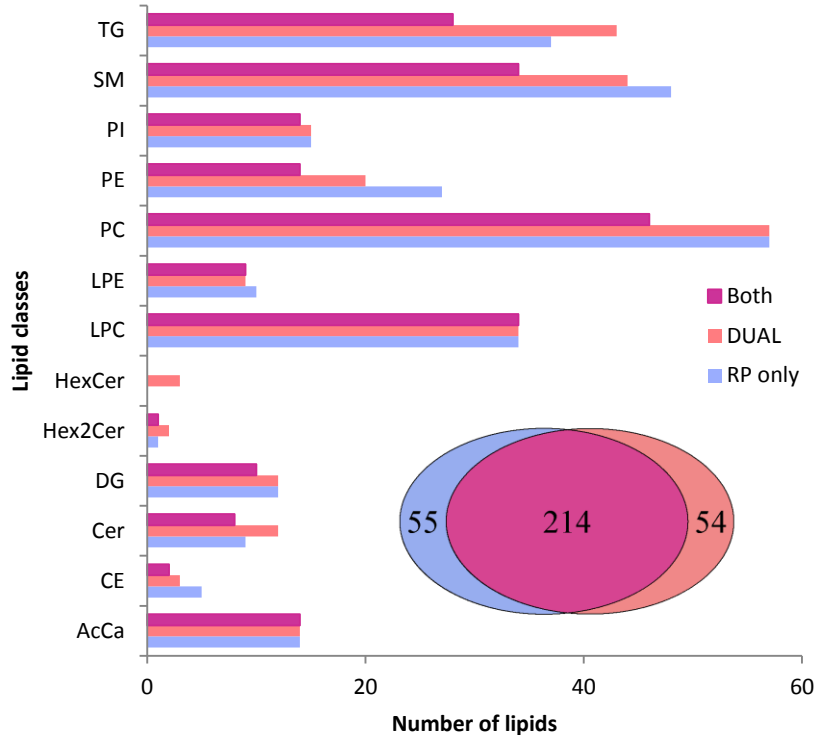
- High resolution data → untargeted metabolomics
- MS2 spectra on > 50% of compounds
- > 100 metabolites were putatively annotated by mzCloud (Level 2 identification)

Sumner et al. (2007). Proposed minimum reporting standards for chemical analysis. *Metabolomics* : Official journal of the Metabolomic Society, 3(3), 211-221

Analytical figures of merit: targeted lipidomics

- Multi-component standard containing lipids from different classes
- Excellent intermediate repeatability: more than 2/3 of the compounds
< 10% area RSD and all compounds < 0.5% RT RSD over 45 hours
- Limits of detection: low nM range except for CEs (poor ionization), TGs (high background)
- Linear calibration curves over 3 – 4 orders of magnitude for many lipid classes

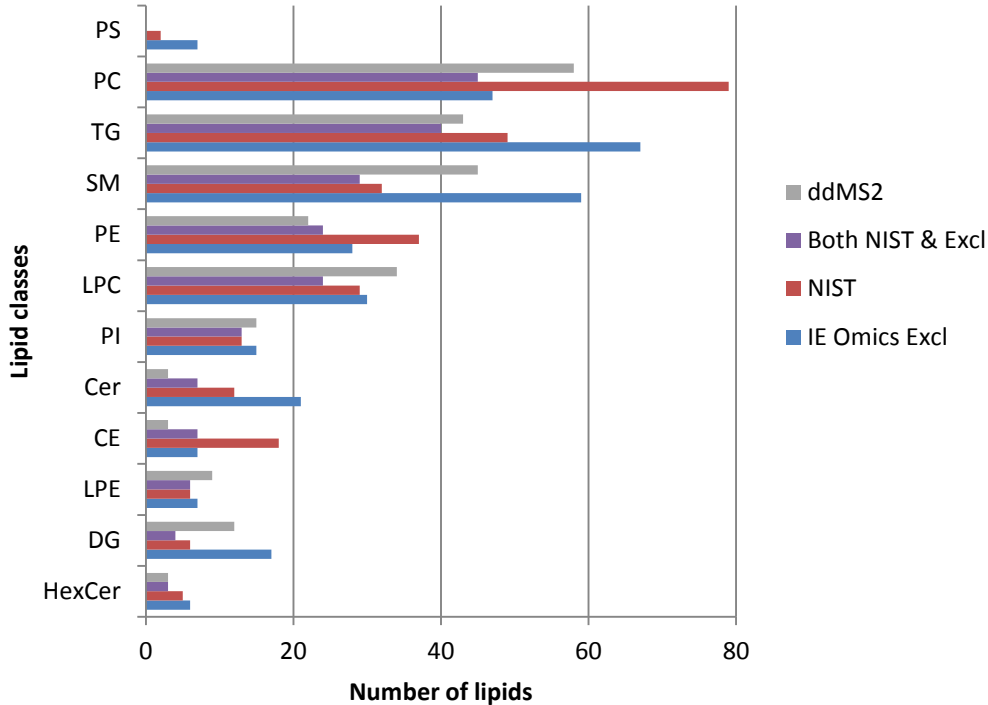
Lipid identifications in SRM 1950



→ Same number of identifications with DUAL setup compared to RP only

N=4 extraction replicates

Lipid identifications in SRM 1950



- More identifications via exclusion list
- Good agreement with interlaboratory comparison data

J. Am. Soc. Mass Spectrom. **2017**, 28, 908

J. Lipid Res. **2017**, 58 (12), 2275–2288

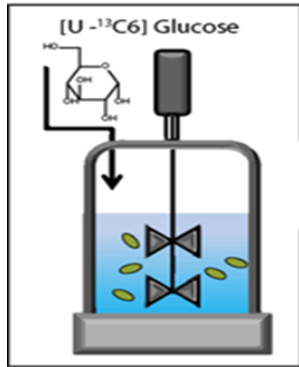
LILY- Lipidome Isotope Labeling of Yeast

Compound-specific lipid quantification
 ^{13}C reference lipids produced in yeast

Lipidome Isotope Labeling of Yeast - LILY

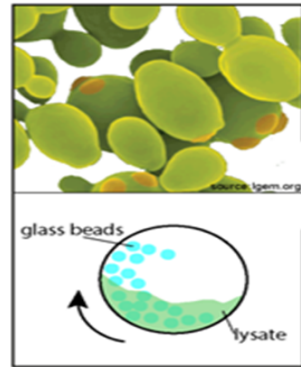
A) Fermentation

Pichia pastoris



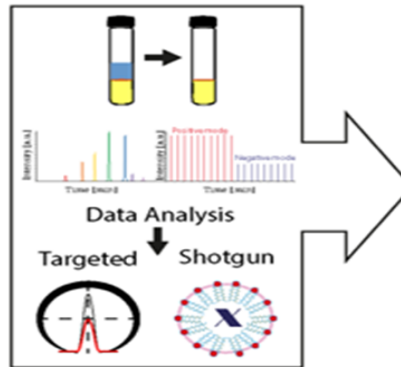
B) Cell disruption

Glass beads



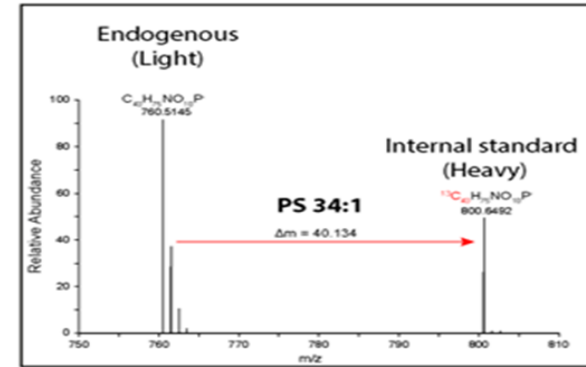
C) Lipid extraction

Folch extraction

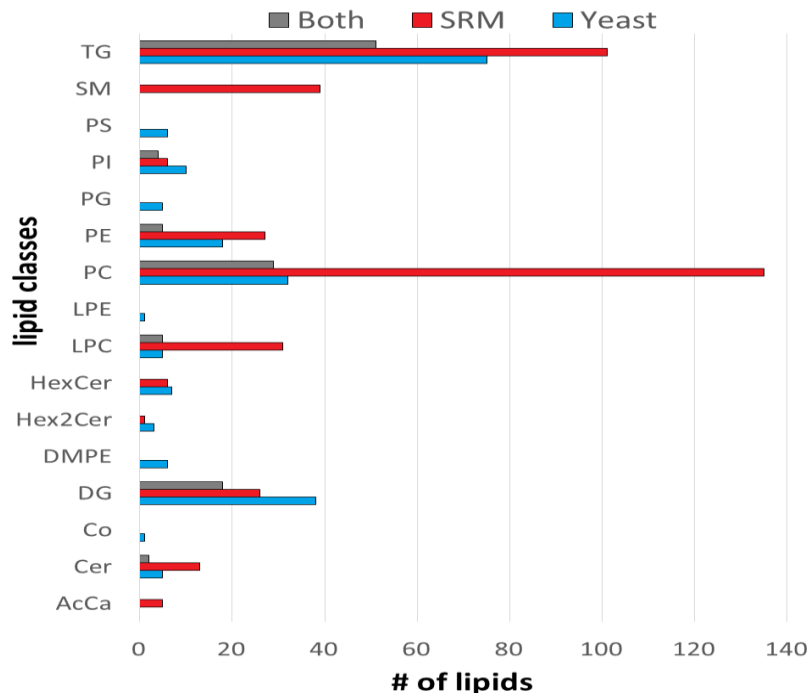


D) Defined standards for MS

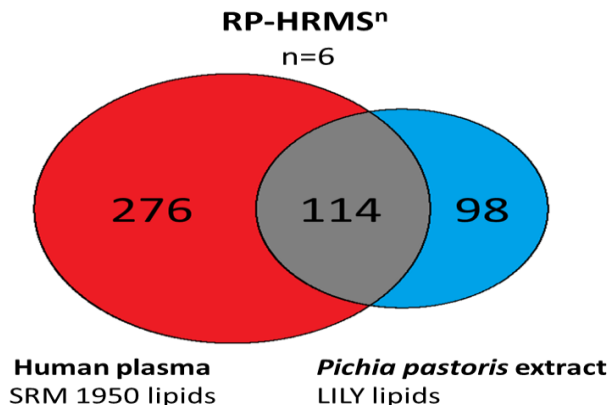
[U ^{13}C] Lipids



A Lipid classes in yeast and human plasma



B Potential ^{13}C -labeled standards



Compound-specific quantification:
114 LILY lipids from 7 classes in human plasma

Class-/retention time specific quantification:
212 LILY lipids from 13 classes

Lipid profiling by RPLC-MSⁿ in *Pichia pastoris* yeast and SRM 1950 human plasma samples.

A. Number of lipid species per lipid class and presence in LILY yeast extract and human plasma samples. B. Comparison of individual lipid species present in both human plasma and ^{13}C -labeled LILY lipid extract

E. Rampler, A. Criscuolo, M. Zeller, Y. El Abiead, H. Schoeny, G. Hermann, E. Sokol, K. Cook, D. A. Peake, B. Delanghe, G. Koellensperger, Analytical Chemistry 2018

Summary

Metabolomics/lipidomics - a field of analytical development

- With broader application of metabolomics/lipidomics there is an increasing unease about applied standards
- Harmonization initiatives are essential
 - Analytical Chemistry:
 - (Reference) materials
 - Validation strategies and standardized protocols
 - Streamlined/merged workflows to increase throughput

Acknowledgement

