ADVANCES IN PESTICIDE ANALYSIS AND THE AUTOMATION OF THE 2-STEP DERIVATIZATION FOR METABOLOMICS

DATE 24.07.2024

TIME 9.30 - 11.00 H (IST)

SPEAKER

Dr. Hans-Joachim Huebschmann,

Certified Food Chemist, Consultant, HANS Analytical Solutions

Advances in Pesticide Analysis

Micro-SPE for Green Analytical Chemistry

PAL SYSTEM
Ingenious sample handling

Solid Phase Extraction

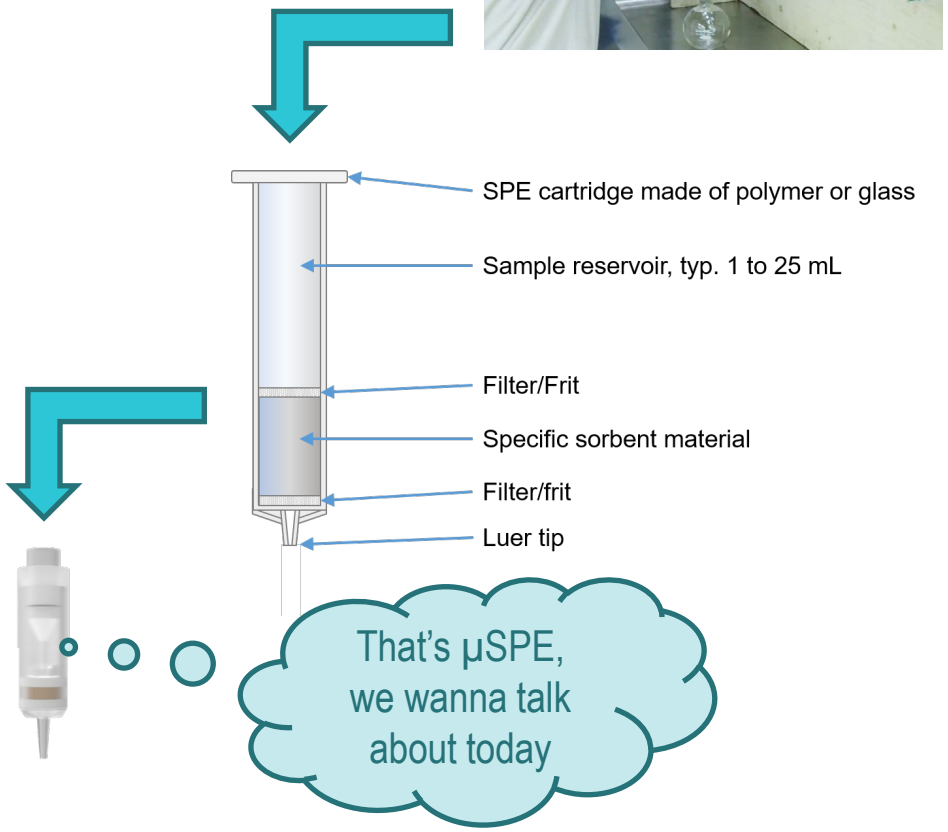
A quick look back and where do we go today

- ‘The separating funnel is a museum piece’

- Modern SPE originated in 1974
  - by Reginald Adams, Thomas Good, and Michael Telepchak
  - First dispersive (dSPE)
  - Later cartridge formats

- Much simpler for the lab
  - Less sorbent material
  - Less solvent
  - Faster
  - More concentrated analytes
  - More selective
  - Compatible with GC-MS and LC-MS
  - .... and it is a big step ahead in Green Analytical Chemistry

Exactly the same is true again for µSPE

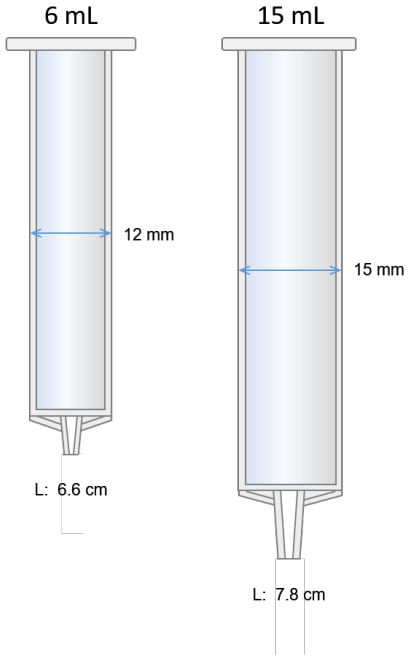


What is Micro-SPE (µSPE)

Compare to the classical cartridge SPE

Classical SPE

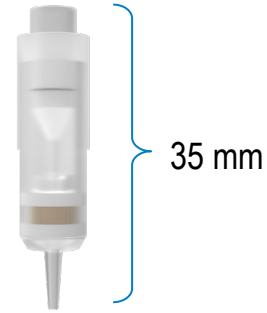
- Limited selectivity
  - High sample and solvent volumes
  - Requires evaporation with N_2
  - End volume >>100 µL in vial
- Vacuum operated
- Drying before elution
- Manual operation
  - Time consuming
  - Low sample throughput
  - Batch processing
- No QA/QC
  - As of manual operation



µSPE

- High selectivity
  - Sharp elution peak profile,
  - Compares to LC separation
  - No dilution, no concentration
  - Final eluates < 100 µL (or online)
- Positive pressure w liquid syringe
  - Very low solvent use
- No drying step
- Walk away automation
  - Fast
  - Works on chromatographic timescale
  - High productivity
- Traceable
  - Processing well documented

Works like a mini LC column !



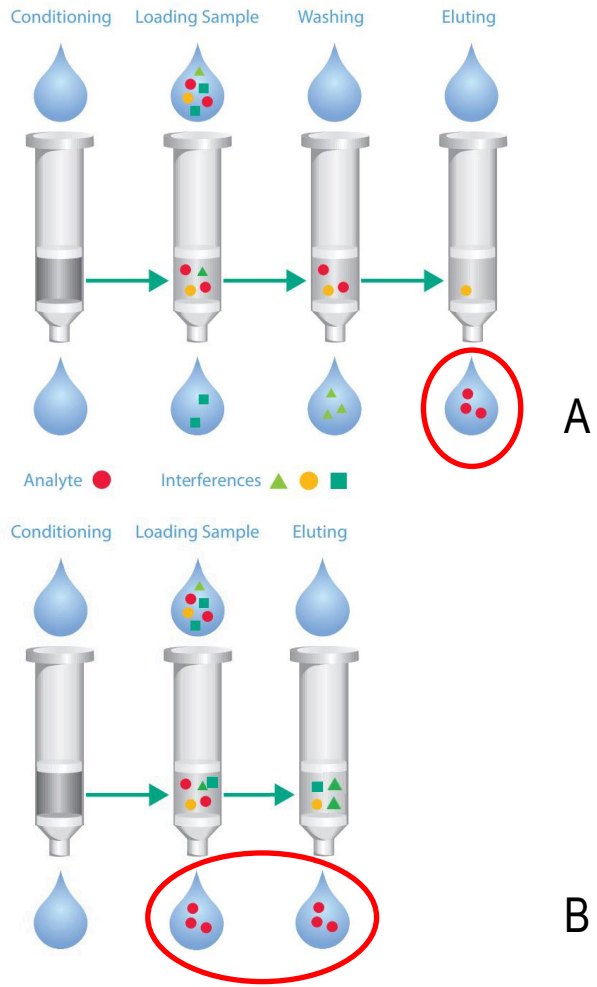
OD 8.5 mm

SPE Workflows

Both available for µSPE

- "Enrichment" mode (the "classical" procedure)
  - aka Load-Wash-Elute mode
  - Analytes ● retained
    - Matrix ▲■ washed away
  - Analytes ● eluted by solvent change
    - e.g. for SAX, C18 material for Glyphosate, AMPA in EURL Almeria

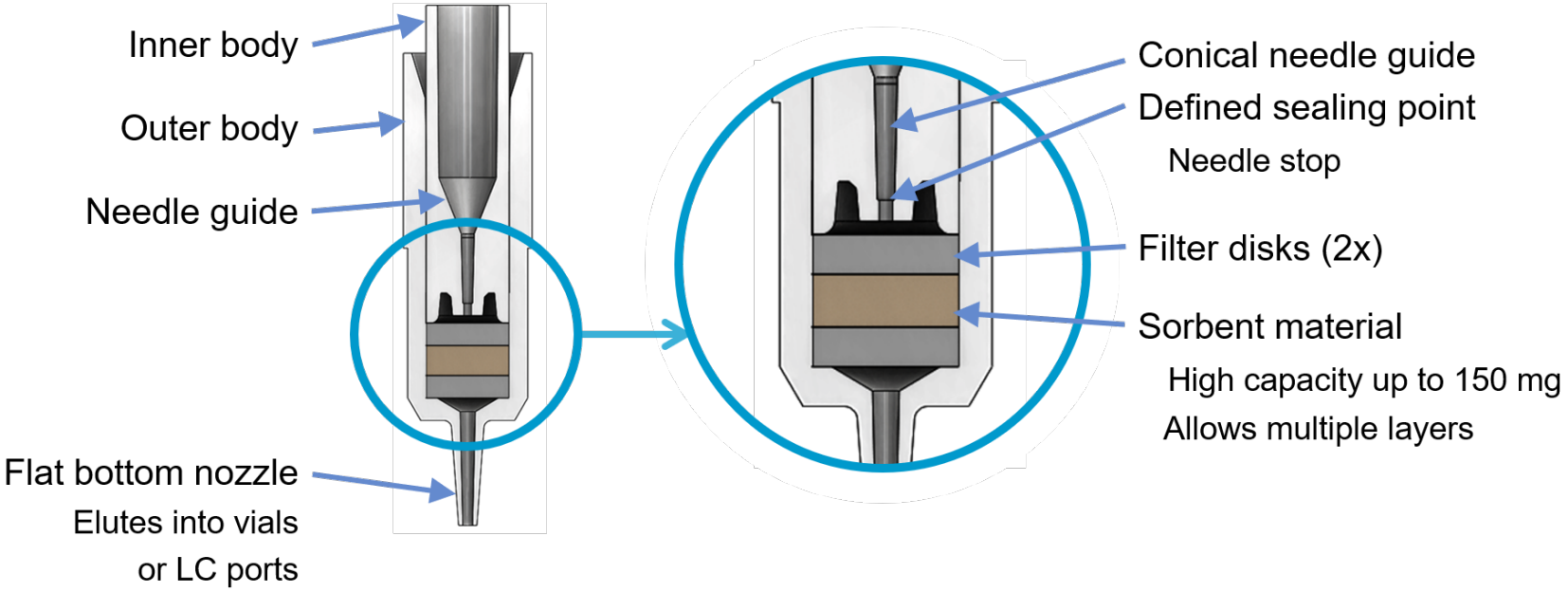
- "Scavenging" mode
  - Matrix ▲■ retained
    - kept on cartridge
  - Analytes ● elute with extract
    - e.g. for QuEChERS, SweEt
      - Pesticides in Hill Labs, USDA Lehotay, Zurich Kanton Lab, EURL Almeria …)
      - C18 material for veterinary drugs analysis



Graphics by Gilon learning hub

Inside the µSPE Cartridge

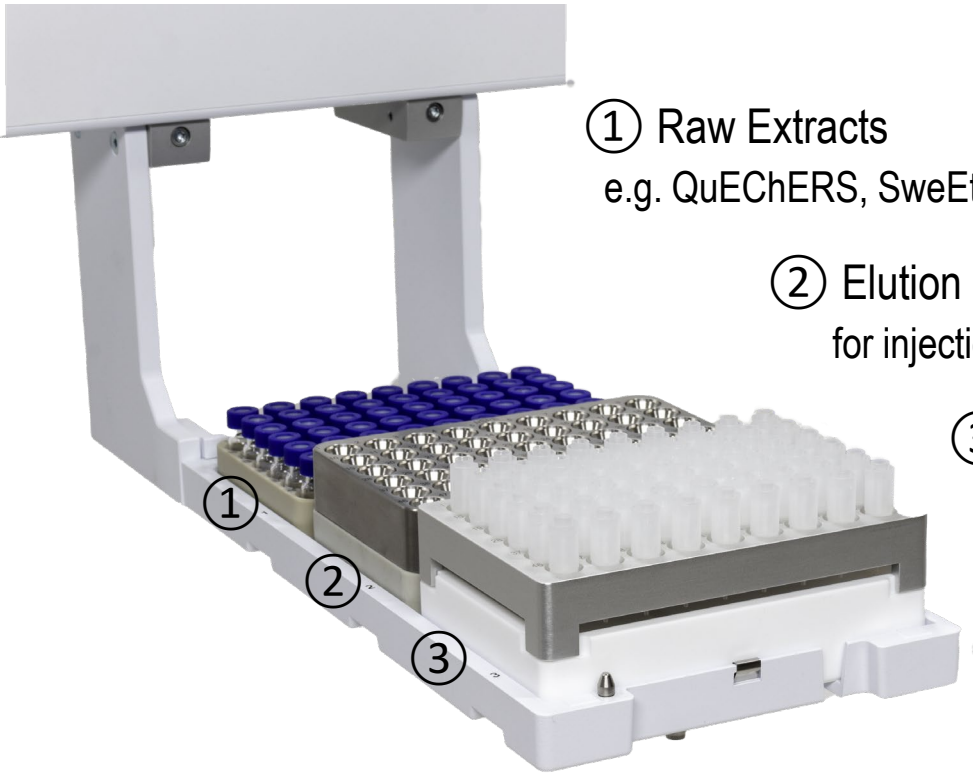
How does the µSPE Cartridge work



- The µSPE cartridges offer
  - combinations of sorbent materials as used for the QuEChERS clean-up,
  - customized and proprietary sorbents are available,
  - just filter materials of different pore sizes, e.g. for LC and IC applications.

How does µSPE Work on a PAL System

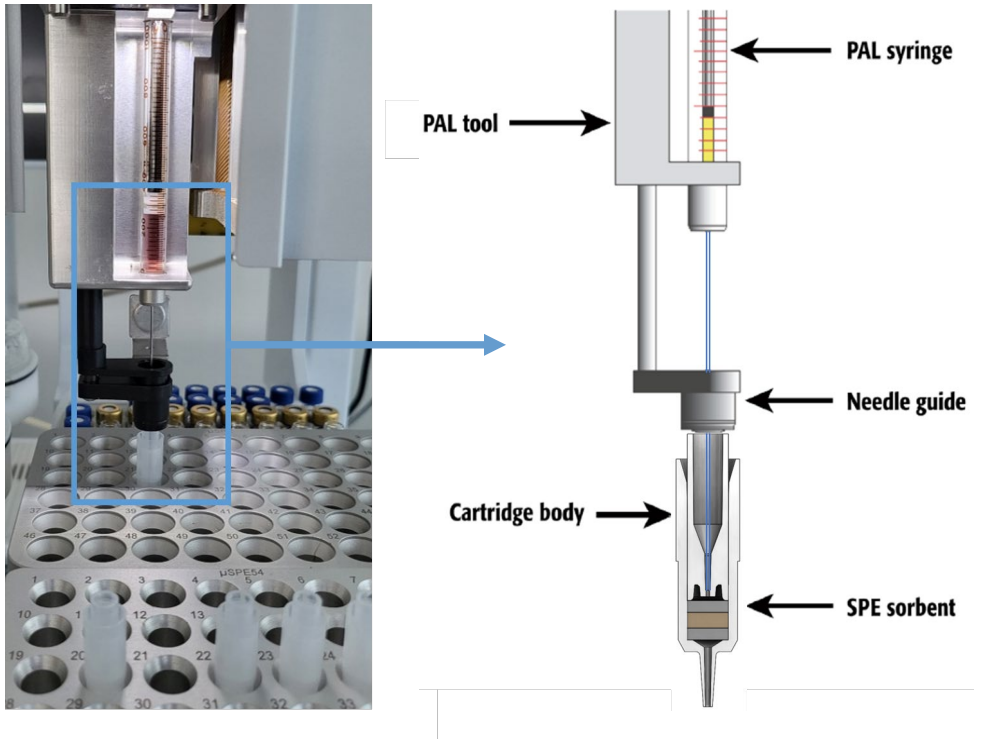
One Trayholder is installed on a PAL System (upgradeable)



① Raw Extracts
e.g. QuEChERS, SweEt, ...

② Elution - Cleaned Extracts
for injection GC-MS, LC-MS

③ µSPE cartridges
for conditioning



Cartridge transport on syringe needle
for elution and return/waste bin

Standard QuEChERS Protocol*

PAL SYSTEM
Ingenious sample handling

10 g of sample

Shake 1 min

Salt out

Shake 1 min

Centrifuge

Dispersive SPE

Shake 1 min

Centrifuge

Analysis

Use 50 mL tubes
Add 10 mL acidified acetonitrile
Add ISTD (Triphenylphosphate)

4 g $MgSO_4$ anh.,
1 g NaCl,
1 g Citrate buffer (CEN 15662)
1 g Acetate buffer (AOAC 2007.01)

- Freeze step for fatty samples, or GLP clean-up
- Direct LCMS analysis of polar pesticides

1 mL aliquot transferred into 10 mL tubes,
add PSA, $MgSO_4$, C18, GCB, Chlorofiltr, ...
as required by the food commodity

LLE Extraction

dSPE Clean-up

** QuEChERS - Mini-Multiresidue Method for the Analysis of Pesticides, M. Anastassiades, 2003*

QuEChERS Protocol* with µSPE Clean-up

10 g of sample

Shake 1 min

Salt out

Shake 1 min

Centrifuge

Dispersive SPE

Shake 1 min

Centrifuge

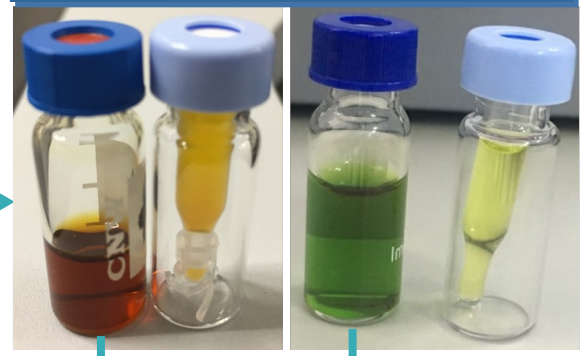
Use 50 mL tubes
Add 10 mL acidified acetonitrile
Add ISTD (Triphenylphosphate)

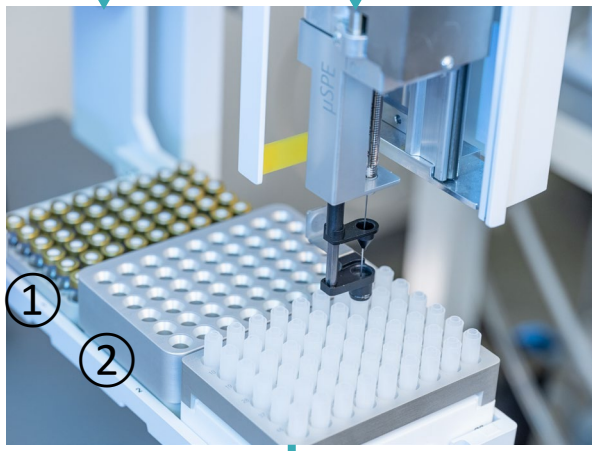
➡ Add buffer kit, AOAC 2007.1/EN15662

➡ No freeze - No GPC

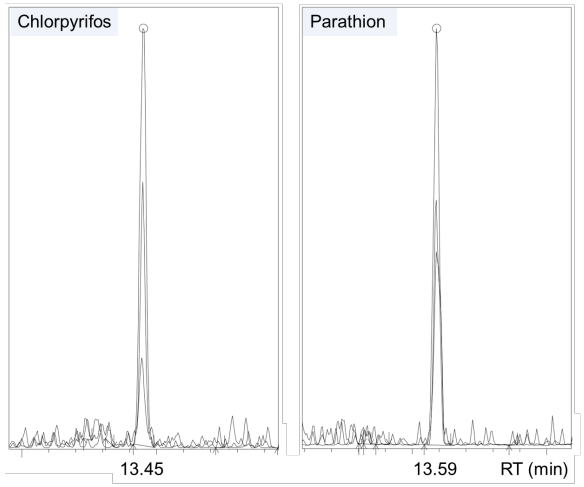
➡ Take 1 mL for µSPE clean-up

Black and green tea samples





GC-MS and LC-MS Injection



Chlorpyriphos 1 ppb
RT 13.45 min
m/z 313.90 > 285.90
m/z 313.90 > 193.90

Parathion 1 ppb
RT 13.59 min
m/z 291.10 > 137.00
m/z 291.10 > 81.00

Pesticides Clean-up by Hill Laboratories, Hamilton, NZ

PAL SYSTEM
Ingenious sample handling

More than 10 Years Experience for > 900 Matrices, >1000 Samples/wk

- Automated, compared to original QuEChERS
- Reduced manual labor in sample prep
- Wider range of samples with high lipid content, incl. avocados or with difficult matrices like dried herbs and spices.
- Off-line clean-up requires only 1/5 of GC runtime and can serve several GC-MS systems.

µSPE GC-MS clean-up		
Sorbent	Amount	Percentage
PSA	12 mg	27%
C18	12 mg	27%
GCB	1 mg	2%
MgSO4	20 mg	44%
Total	45 mg	100%

µSPE LC-MS clean-up		
Sorbent	Amount	Percentage
Z-Sep	8 mg	27%
C18	21 mg	70%
GCB	1 mg	3%
Total	30 mg	100%

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Development of an Automated Column Solid-Phase Extraction Cleanup of QuEChERS Extracts, Using a Zirconia-Based Sorbent, for Pesticide Residue Analyses by LC-MS/MS

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Food and Bioanalytical Division, R. J. Hill Laboratories, Private Bag 3205, Hamilton East, New Zealand

Supporting Information

ABSTRACT: A new, automated, high-throughput, mini-column solid-phase extraction (c-SPE) cleanup method for QuEChERS extracts was developed, using a robotic X-Y-Z instrument autosampler, for analysis of pesticide residues in fruits and vegetables by LC-MS/MS. Removal of avocado matrix and recoveries of 263 pesticides and metabolites were studied, using various stationary phase mixtures, including zirconia-based sorbents, and elution with acetonitrile. These experiments allowed selection of a sorbent mixture consisting of zirconia, C_{18}, and carbon-coated silica, that effectively retained avocado matrix but also retained 53 pesticides with <70% recoveries. Addition of MeOH to the elution solvent improved pesticide recoveries from zirconia, as did citrate ions in CEN QuEChERS extracts. Finally, formate buffer in acetonitrile/MeOH (1:1) was required to give >70% recoveries of all 263 pesticides. Analysis of avocado extracts by LC-Q-Orbitrap-MS showed that the method developed was removing >90% of di- and triacylglycerols. The method was validated for 269 pesticides (including homologues and metabolites) in avocado and citrus. Spike recoveries were within 70−120% and 20% RSD for 243 of these analytes in avocado and 254 in citrus, when calibrated against solvent-only standards, indicating effective matrix removal and minimal electrospray ionization suppression.

KEYWORDS: *QuEChERS, SPE, LC-MS/MS, ITSP, Z-Sep, zirconia, pesticide, multiresidue, avocado, citrus*

■ INTRODUCTION

The "quick, easy, cheap, effective, rugged, and safe" (QuEChERS) method for the analysis of multiclass pesticide residues in fruits and vegetables introduced the use of dispersive solid-phase extraction (d-SPE) cleanup, to reduce the amounts of coextracted matrix in extracts, before instrumental analysis, using a mixture of $MgSO_4$ and PSA sorbents, with C_{18} and graphitized carbon black (GCB) added if required to improve removal of nonpolar matrix and chlorophyll.[1−4] In the original QuEChERS method, d-SPE was used instead of column SPE (c-SPE) to provide a quicker and cheaper cleanup.[1] Recently the zirconia-based sorbent HybridSPE, in well-plates or columns, has been utilized for the removal of phospholipids from plasma[5−7] and eggs.[8] The zirconia materials Z-Sep and Z-Sep+ have been evaluated for d-SPE cleanup of QuEChERS extracts for analysis of environmental pollutants and pesticides in fish and shrimp[9−11] and pesticides from oily fruits or vegetable oils,[12−16] due to their abilities to remove the lipophilic matrix. However, in our experience, used routinely, Z-Sep d-SPE can result in the transfer of solid phase into analysis vials and subsequently into the HPLC, building up over time to cause retention of some analytes and poor peak shapes or carry-over. Consequently, we investigated the development of an automated c-SPE cleanup, based on zirconia-coated silica, using Instrument Top Sample Preparation (ITSP) mini-cartridges, on a robotic X-Y-Z instrument autosampler. This could be as quick and cheap as d-SPE, as many instruments are already equipped with robotic autosamplers; however, it could also give the improved matrix removal that is possible with c-SPE[17] and avoid zirconia transfer to the LC-MS/MS.

Avocado extracts were selected as a matrix with high oil content,[18,19] and experiments were carried out to evaluate the weight of matrix removed after acetonitrile (MeCN) elution through ITSP c-SPE cartridges with six different stationary phases. Recoveries of 263 pesticides and metabolites spiked on avocado were determined through five of these sorbents and, along with matrix weight-removal results, allowed selection of a Z-Sep/C_{18}/CarbonX mixture for further method development. Investigation of the effect of different elution solvents (MeCN, MeCN/MeOH (1:1), MeOH, coextracted citrate in a CEN (European Committee for Standardization method,[20]), QuEChERS extract, and formate buffer at three concentrations in MeCN/MeOH (1:1), on pesticide recoveries through Z-Sep/C_{18}/CarbonX, resulted in a method using elution of CEN QuEChERS extracts with 100 mM formate buffer in MeCN/MeOH (1:1). To the best of our knowledge, this study is the first to use ITSP mini-cartridges for cleanup of QuEChERS extracts and zirconia solid phase in an SPE column, rather than used dispersively, for pesticide residue analysis. Removal of avocado di- and triacylglycerols by Z-Sep, monitored by LC-Q-Orbitrap-MS, is also presented. The method was validated for the analysis of 269 pesticides, including homologues and metabolites, in avocado and citrus, to give spike recovery and reproducibility data.

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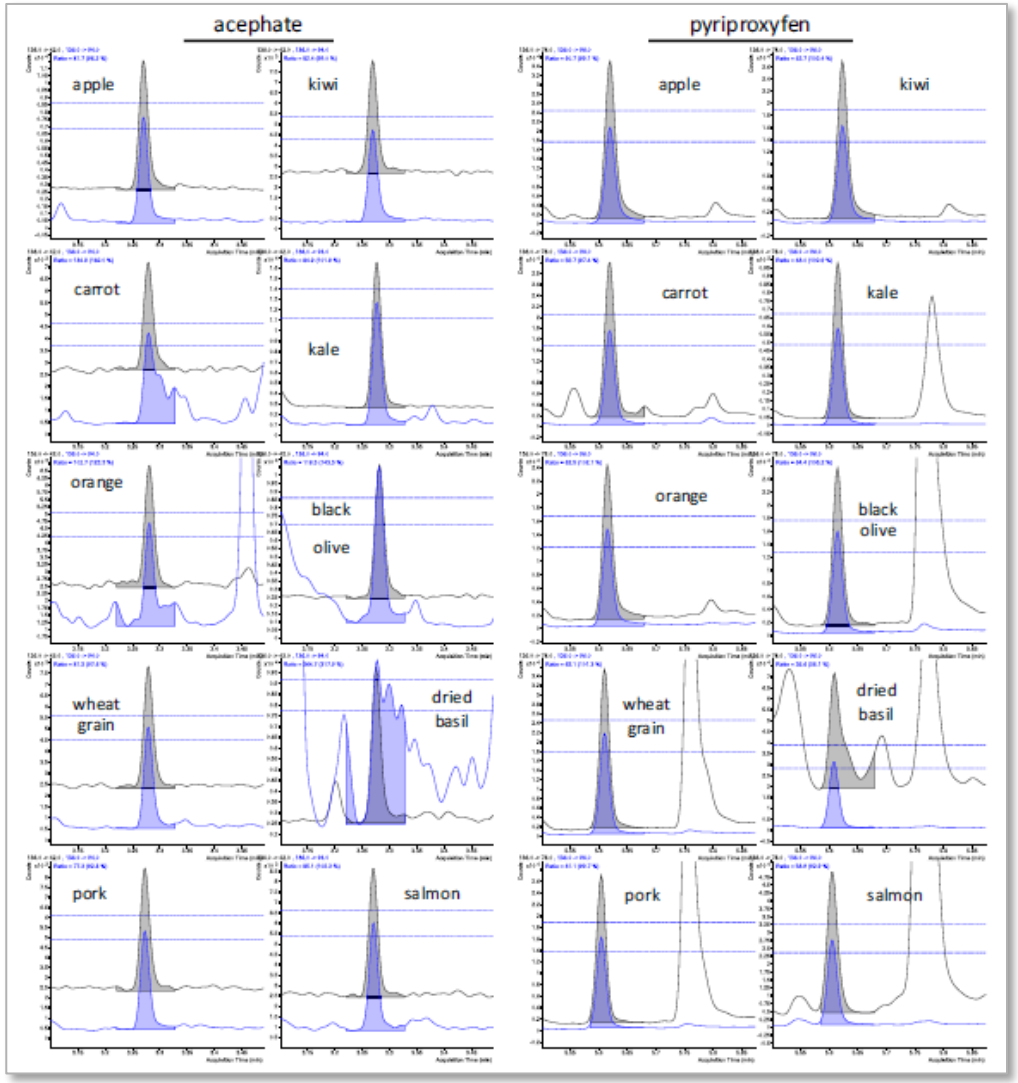
DOI: 10.1021/jf506539w
J. Agric. Food Chem. 2015, 63, 5107−5119

Automated µSPE Clean-up for GC-MS - Only 8 min *

PAL SYSTEM
Ingenious sample handling

1. Take a 1 mL syringe
2. Wash the 1 mL syringe with MeCN
3. **Take 500 µL raw extract** from rack 1 into 1 mL syringe
4. Get the µSPE cartridge from rack 3 with the syringe needle
5. Move the cartridge to the elution rack 2
6. **Push the raw extract through the µSPE cartridge** at 5 µL/s
7. Discard µSPE cartridge into waste beaker
8. Wash the 1 mL syringe with MeCN/MeOH/water (vol 1/1/1)
9. Wash the 1 mL syringe with MeCN
10. Switch to 100 µL syringe and wash with MeCN
11. **Add 25 µL AP + QC solutions** to the collection vial in rack 2
12. Wash the 100 µL syringe with MeCN/MeOH/water (vol 1/1/1)
13. Wash the 100 µL syringe with MeCN
14. Switch to 10 µL GC injection syringe
15. Wash the 10 µL syringe with MeCN
16. Aspirate the cleaned extract from the elution vial in rack 2
17. **Inject** 1 µL of extract to GC-MS/MS
18. Wash the 10 µL syringe with MeCN



* as of Steven J. Lehotay, Lijun Han, Yelena Sapozhnikova (2016) and Nicolas Michlig, Steven J. Lehotay (2022)

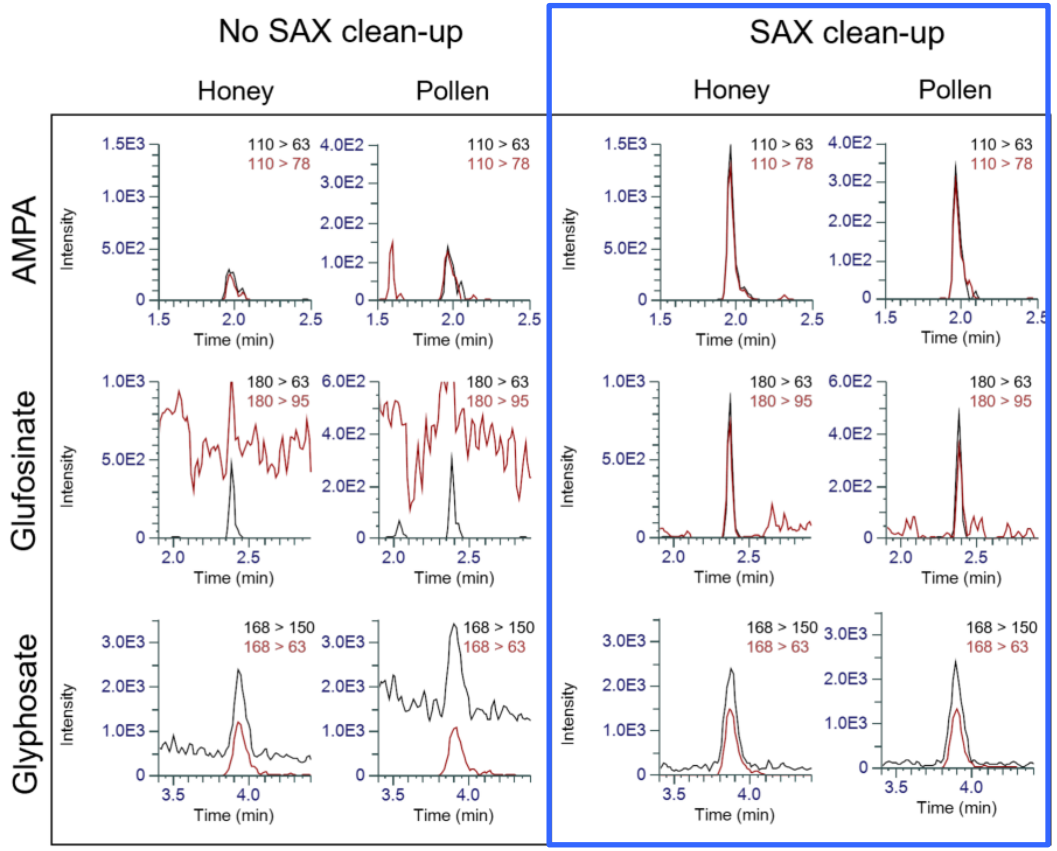
Highly Polar Pesticides in Complex Matrices (QuPPe)

Glyphosate, AMPA, Glufosinate, … EURL for Pesticides in Fruit and Vegetables, Almeria, Spain

- Matrix honey, pollen, coffee beans
- Acidified methanol extraction
- Automated µSPE clean-up
  50 mg SAX (strong ion exchange)

- Clean-up procedure
  1000 µL methanolic raw extract
  Load at 5 µL/s
  Matrix washed with 600 µL methanol
  Analytes elute with 400 µL methanol/HCl (9:1)
- Inject 10 µL to LC-MS/MS

- Cost saving: 500 mg → 50 mg SAX material
- Time saving: manual → automated 10:1
- Analytical: Improved recoveries up from avg. 70 → 86%



Extracted LC-MS/MS ion chromatograms of standards of AMPA, glufosinate, and glyphosate at 0.010 mg/kg spiked to honey and pollen matrix without (left) and after SAX clean-up (right)

Jesus, F., A. R. García, et al. 2023. "Determination of Highly Polar Anionic Pesticides in Beehive Products by Hydrophilic Interaction Liquid Chromatography Coupled to Mass Spectrometry." Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-023-04946-7.

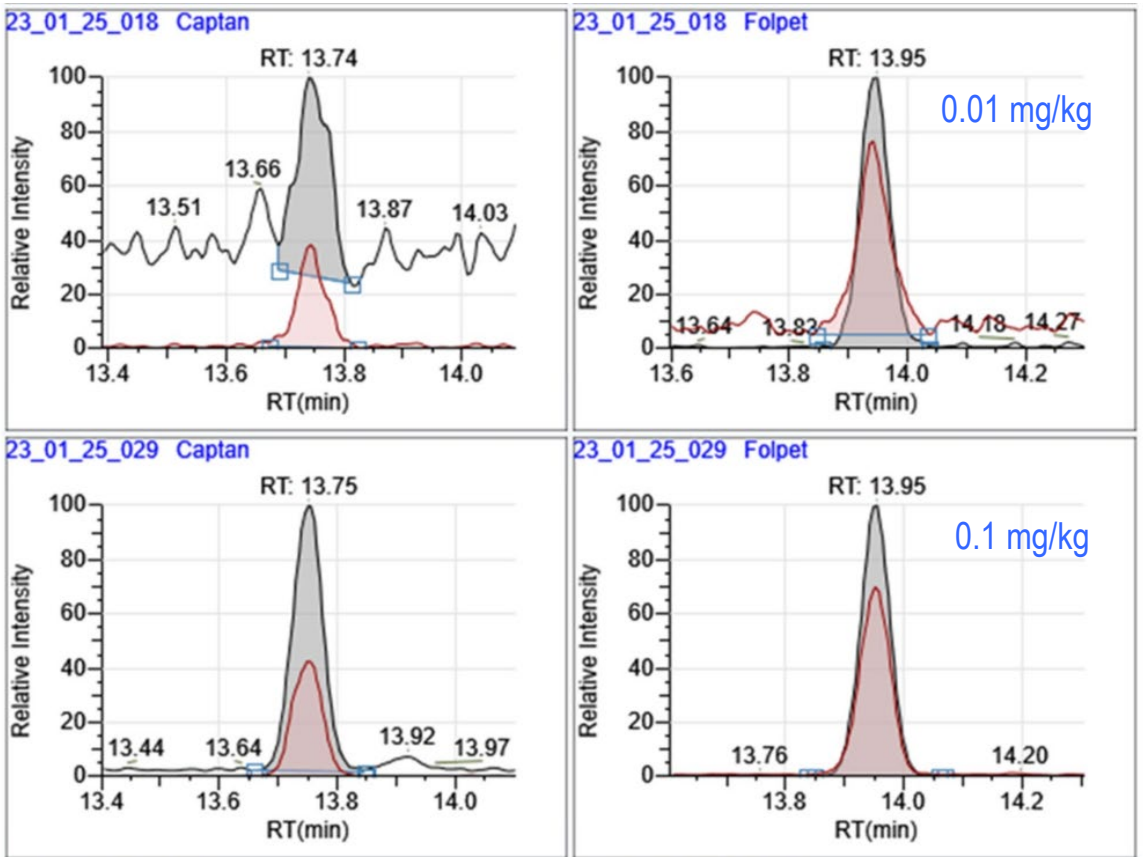
Ethylacetate Extraction of Pesticides from Foods (*aka* SweEt)

Big time savings and reduced manual effort for high fat matrices – Cantonal Lab. Zurich, CH

- EtOAc extracts wider range of polar pesticides
- But, also extract high amounts of matrix
- GPC or extract freezing was used as clean-up

- Clean-up using µSPE
  - 45 mg of PSA, C18, GCB, $MgSO_4$
  - Load 200 µL raw EtOAc extract, 2 µL/s
  - Blow-out 1 mL air
- Injection 3 µL cleaned extract to GC-MS/MS

- Significant improvements:
  - Difficult and fatty samples e.g. dried spices, egg, avocado, or liver are successfully processed,
  - Captan and Folpet with good recoveries.
  - One µSPE cartridge for all food matrices.
  - No time-consuming freeze-out or GPC required anymore.



Captan and folpet in raspberry samples after µSPE clean-up.

Schürmann, A., C. Crüzer, et al. 2023 "Automated Micro-Solid-Phase Extraction Clean-up and Gas Chromatography-Tandem Mass Spectrometry Analysis of Pesticides in Foods Extracted with Ethyl Acetate." Anal. Bioanal. Chem. 416 (3): 689–700. https://doi.org/10.1007/s00216-023-05027-5.

Veterinary Drug Screening by online µSPE – LC-MS/MS

PAL SYSTEM
Ingenious sample handling

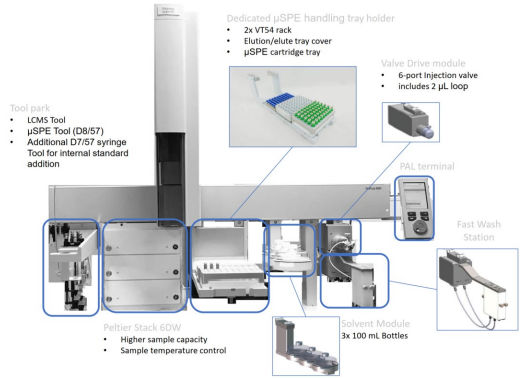
From the Veterinary Diagnostic Laboratory, Iowa State University, USA.

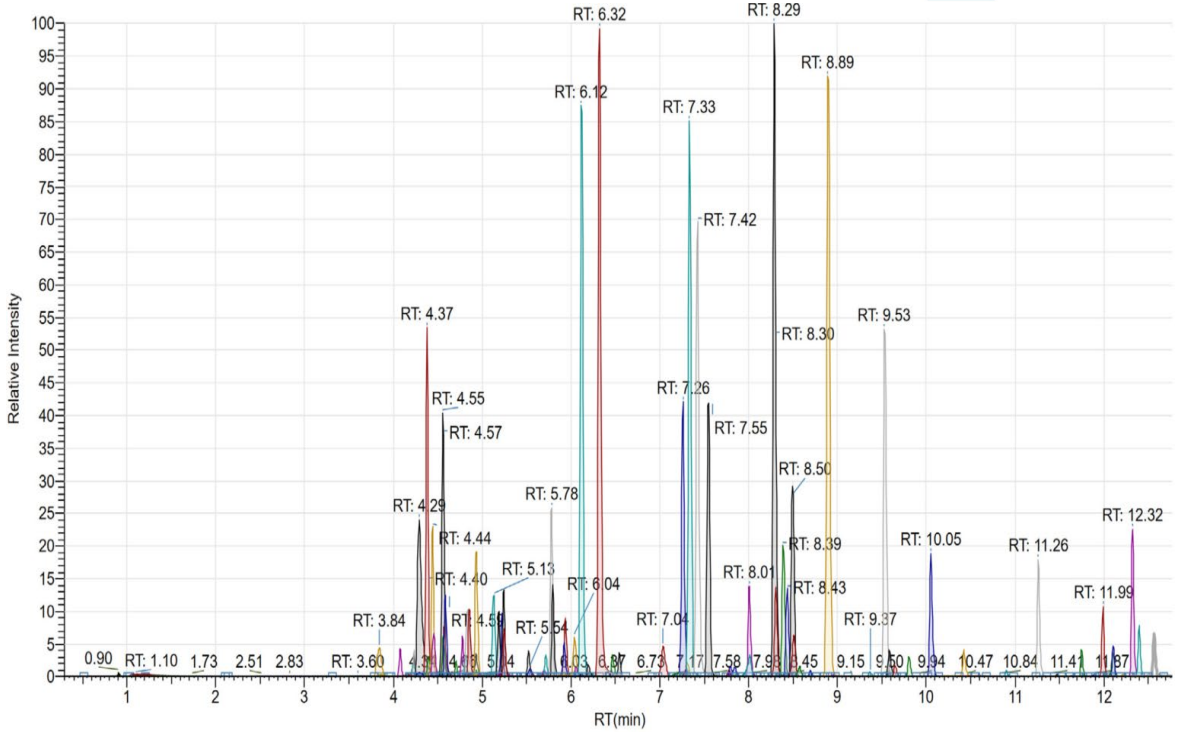
- Veterinary drugs are legally controlled in large number of samples in difficult matrix.
- QuEChERS extraction (LLE with MeCN)
  from 5 g muscle, or kidney

- Automated µSPE clean-up
  on Thermo Scientific™ TriPlus™ RSH sampler,
  µSPE cartridge with 15 mg of endcapped C18.
  300 µL of supernatant at 2 µL/s,
  the eluate diluted 3+1 with mobile phase,
  injected into a 2 µL loop on the injection valve.
  Clean-up takes only 8.5 min to complete

- Cost saving on C18: 500 mg → 15 mg (30x less)
- Time saving: 80 min/15 samples → Zero, online prep
- No additional consumables

103 veterinary drugs at 50 ng/g
in bovine kidney extract.
Total cycle time 15 min.





Schrunk, D., L.E. Burns, et al. 2021. “Multi-Class Veterinary Drugs Analyses of QuEChERS Extracts Using an Automated Online MSPE Cleanup Coupled to LC-MS MS”. Application Note, CTC Analytics AG.

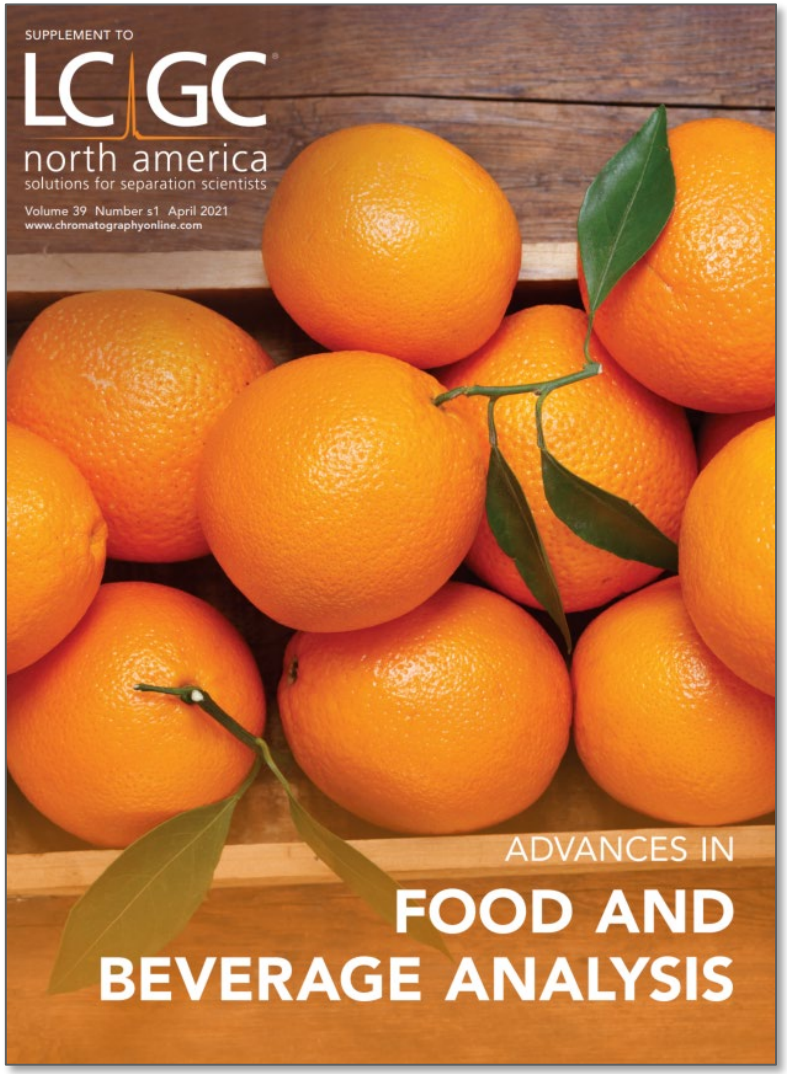
Automated QuEChERS Extraction and µSPE Clean-up

Application for homogeneous samples like beverages

- Why not automate all steps? The QuEChERSs extraction with SPE clean-up?

- Homogenous samples
  - Do not need much manual treatment
  - Can be pipetted into 2 mL vials (also automatically!)
- QuEChERS extraction is automated in 2 mL vials
  - Uses acidified MeCN
  - Buffer salts previously added
  - sat. NaCl solution
- µSPE clean-up
  - 45 mg of PSA, C18, GCB, $MgSO_4$
  - Load 250 µL raw extract, 2 µL/s
- Injection 3 µL cleaned extract to GC-MS/MS

- Combines all benefits
  - from QuEChERS, µSPE clean-up, and prep-ahead automation



QuEChERS Extraction and µSPE Clean-up Workflow

PAL SYSTEM
Ingenious sample handling

Liquid-Liquid Extraction

- Use Tool
- Repeat
  - Get Liquid From Vial
  - Dispense Liq. Into Vial
- Repeat End
- Vortex Vial
- Clean Syringe
- Get Liquid From Vial
- Dispense Liq. Into Vial
- Clean Syringe
- Vortex Vial
- Wait

µSPE

- Use Tool
- µSPE Elute
- Clean Syringe

Inject

- Liquid Injection
- Wait

1 Prepare 500 µL of Orange Juice into 2mL vial

2 Add 600 µL MeCN into Orange Juice sample

Shake

Add 250 µL sat. NaCl into sample vial, shake

3 Wait for phase separation from the liquid-liquid extraction

4 Load 250 µL of the orange extract to the µSPE clean-up

Inject 2 µL of the cleaned extract to GC-MS/MS

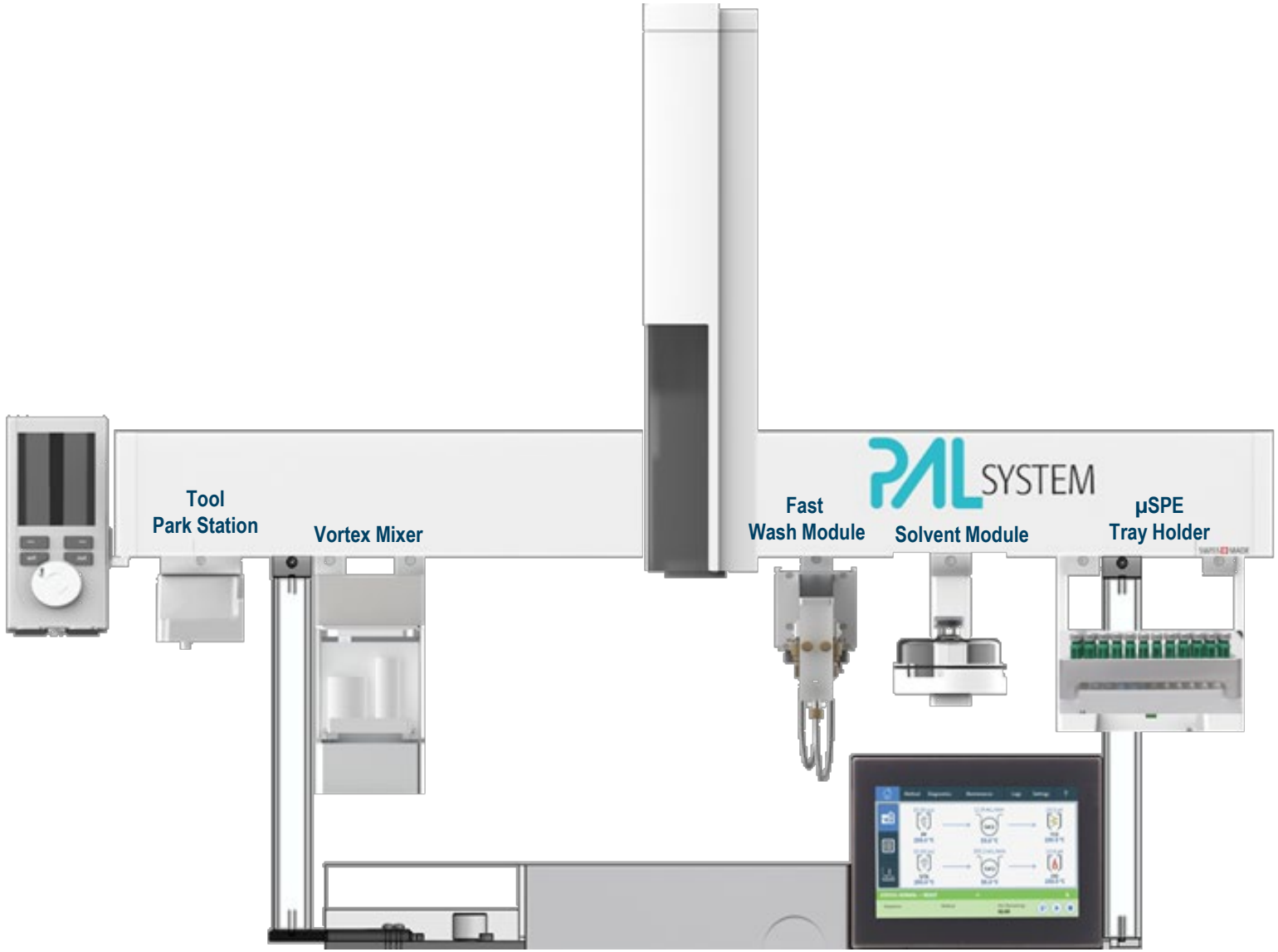
Orange juice

Orange juice + acetonitrile

+ NaCl for phase separation

After µSPE clean up, for injection

PAL RTC System for QuEChERS, µSPE, and online GC-MS



Tool Park Station

- Pos.1 GC Injection syringe (10 µL)
- Pos.2 µSPE Tool (1000 µL)
- Pos.3 APs/ISTD syringe (25 µL)

Solvent Module

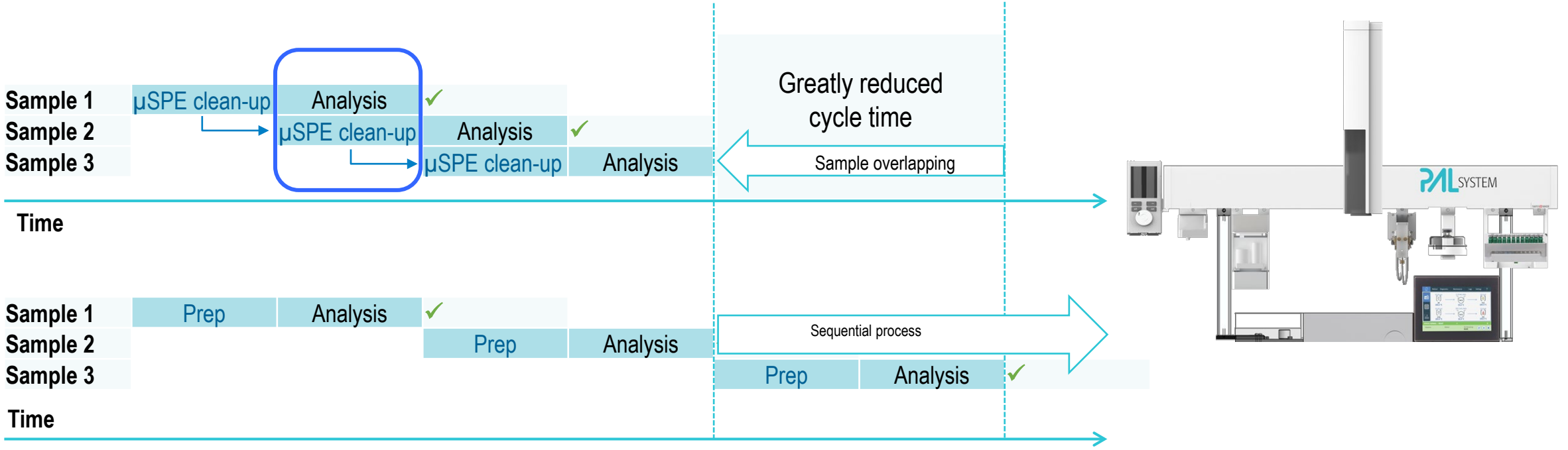
- Pos.1 Acetonitrile
- Pos.2 NaCl, sat.
- Pos.3 not used

Fast Wash Module

- Pos.1 Water
- Pos.2 Acetonitrile

Parallel Extraction and µSPE Clean-up by PAL Prep-ahead

PAL serves sample prep and GC-MS/LC-MS analysis in parallel



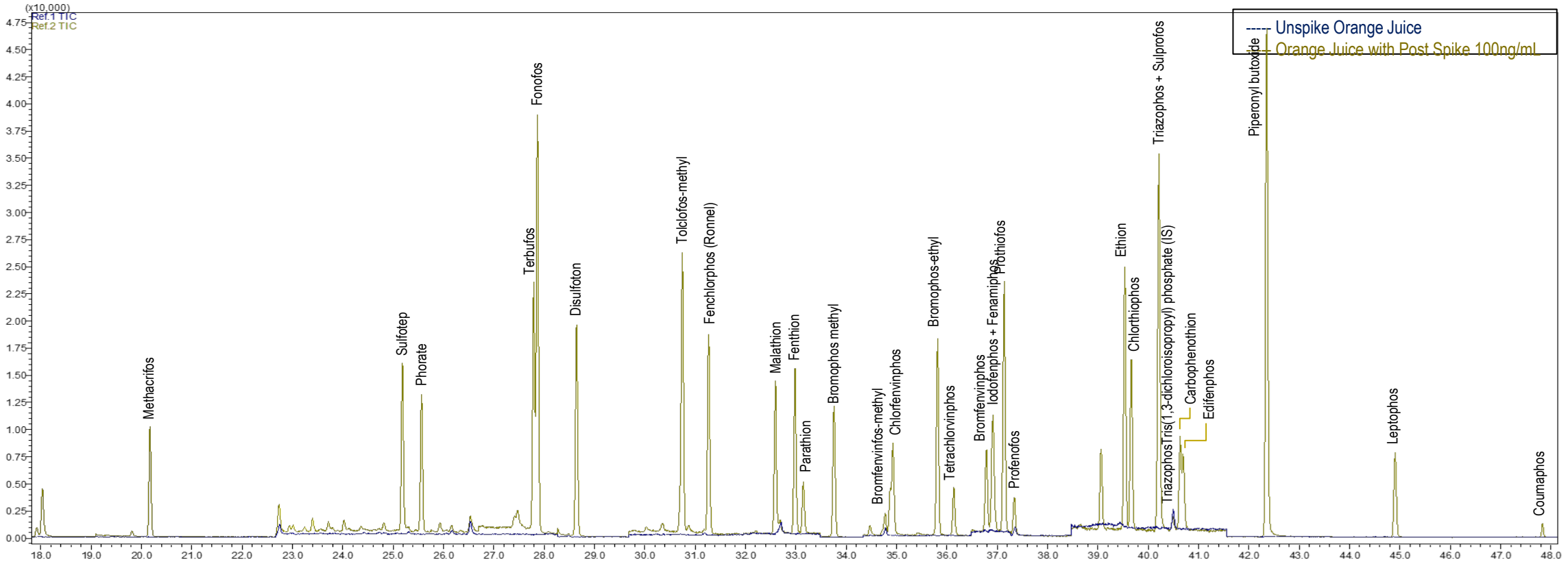
- Strong benefits:
- High reproducibility: All samples are treated on the same timeline.
- Saves time: Continuous analysis of samples, improved sample throughput, overnight processing.
- Highly efficient: Increased use of the GC-MS and LC-MS unit, reduces cost/sample.

OCP Pesticides PAL Extracted from Orange Juice

Total ion chromatogram from GC-MS/MS in MRM mode

- At MRL level the pre-spike RSDs were mostly below 10%. Recoveries achieved 70% to 115%, calibration linearity > 0.995.
- LODs range from 1.8 ng/mL to 4.1 ng/mL (n = 8) well below the general MRL at 10 ng/g level.

  In the original orange juice from a local supermarket about 1.8 ng/mL Malathion was detected.



Summary

µSPE replaces all traditional SPE concentration and clean-up procedures

- µSPE is the next step available towards a Greener Analytical Chemistry
  - Less solvents
  - Less consumables
  - Less waste
  - Less energy consumption

- µSPE delivers strong analytical advantages
  - One clean-up cartridge for all type of samples
  - Improved recoveries
  - Improved clean-up
  - Improved precision

- µSPE reduces cost/sample
  - Efficient use of GC-MS and LC-MS by online prep-ahead
  - Increased sample throughput
  - Walk-away automation
  - Less manual workload
  - Less repeat measurements
  - Faster report out

List of References – available via CTC Analytics

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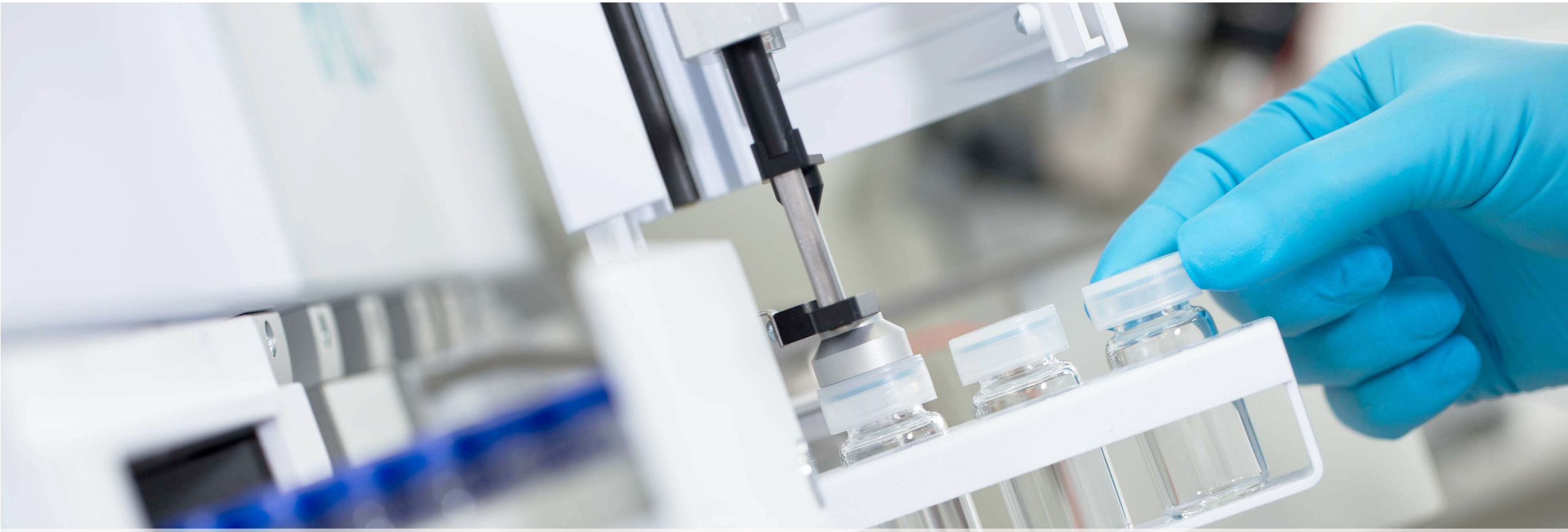


Advances for Sample Preparation for Rice Metabolomics

Automated 2-Step Derivatization for GC-MS

PAL SYSTEM
Ingenious sample handling

What is Metabolomics?

The ‘Metabolome’ can be defined as:

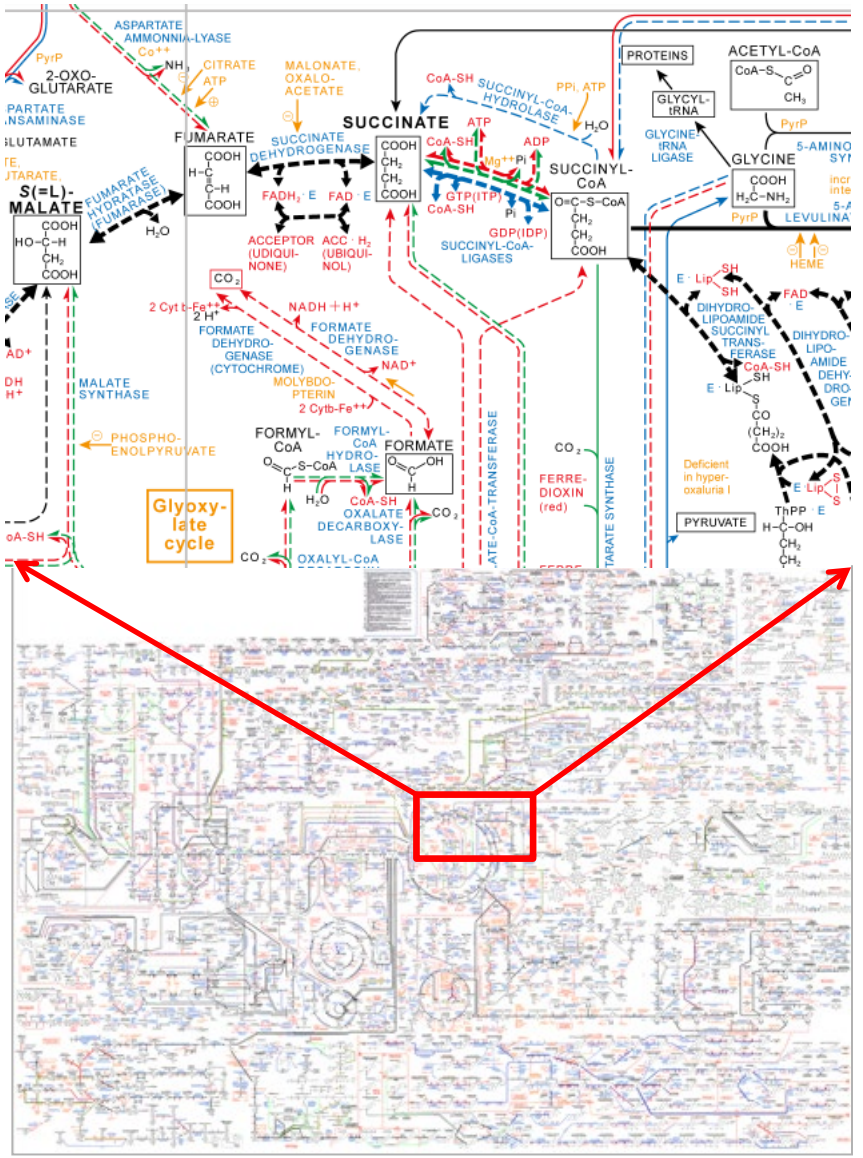
- a snapshot of the quantitative complement of all the low molecular weight molecules present in a cell
- analyzed at a particular physiological or developmental stage

The concept of ‘Metabolomics’

- is the global analysis of all metabolites in a sample (Oliver Fiehn 1998).

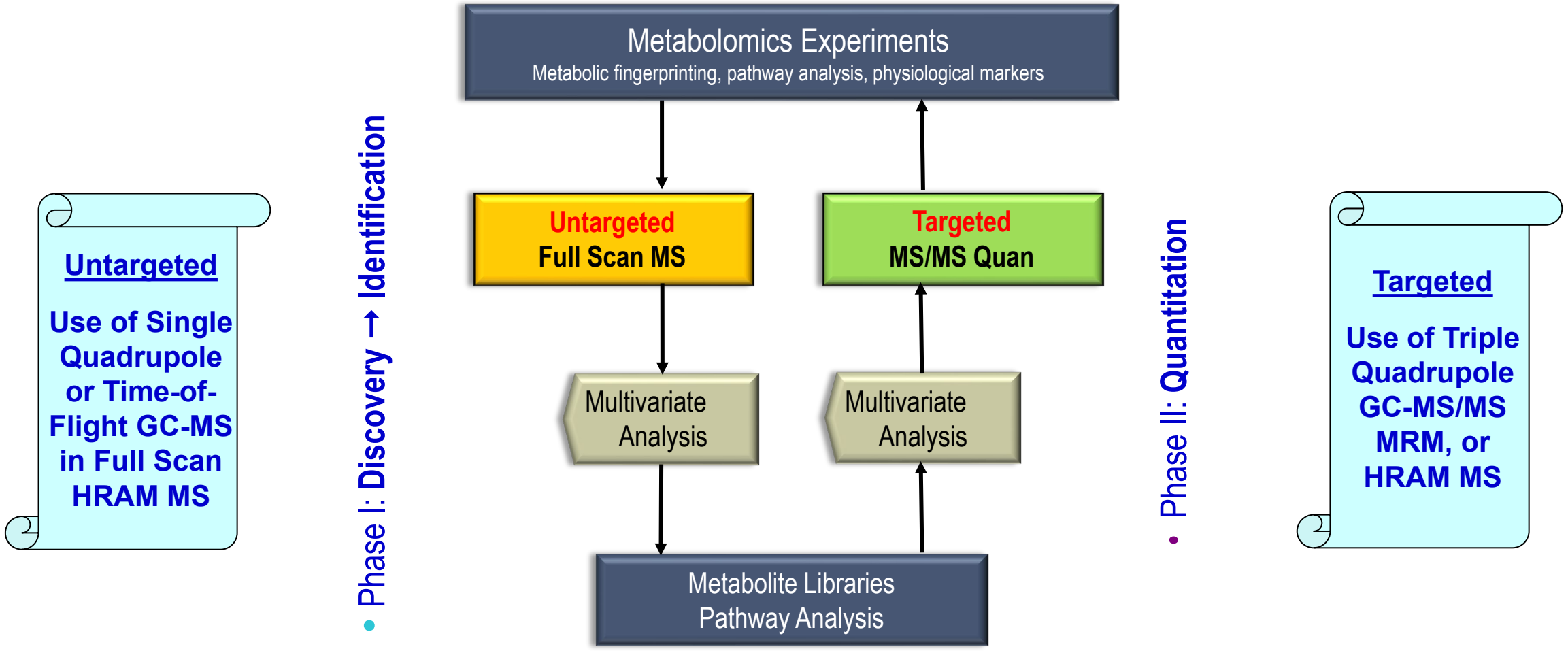
Particular role of GC-MS:

- Small molecule detection
- Separation of isomers



The Roche Biochemical Pathway Wallchart
http://biochemical-pathways.com/#/map/1

Metabolomics Workflow



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Discovery – Phase I

Compound Identification

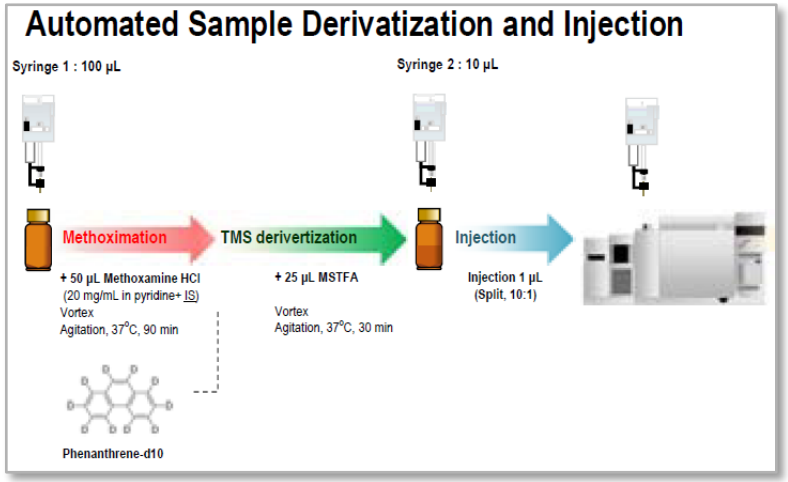
PAL SYSTEM
Ingenious sample handling

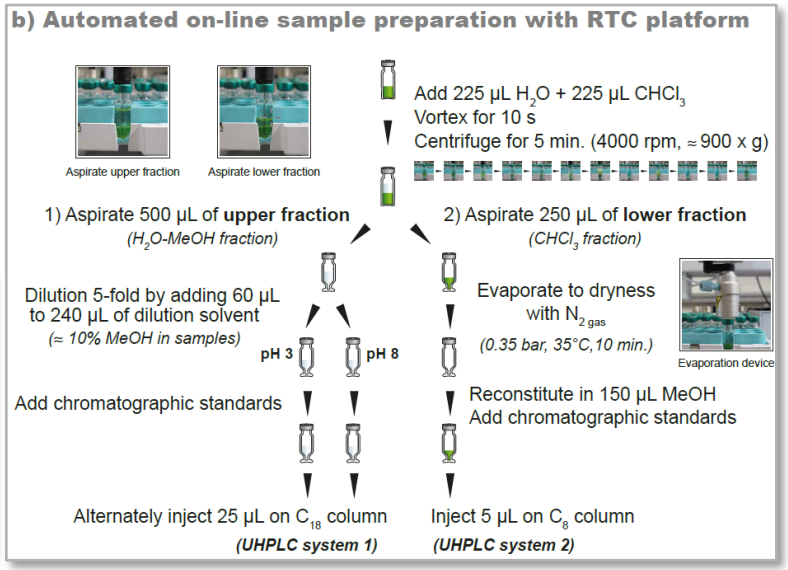
Analytical Challenges in Metabolomics

PAL SYSTEM
Ingenious sample handling

Manual sample preparation is time-consuming

- Sample preparation is a manual bottleneck in many laboratories
  - Extraction – Requires immediate derivatization for reproducibility
  - High sample throughput - Needed to identify meaningful biomarkers
- Strong requirement for an automated workflow:
  - → Place homogenized sample vial into the sampler rack
  - → Add extraction solvent mix (water/chloroform/methanol)
  - → Vortex
  - → Centrifuge for phase separation
  - → Transfer polar phase for
  - → GC-MS: evaporation → **derivatization** → injection
  - → LC-MS: **dilution** → injection
  - → Prep-ahead during analysis run





Many examples as references:

Varesio E., G. Boehm, et al. 2014 - Integrated Platform including Automated Bligh and Dyer Extraction and Dual-Column UHPLC-MS/MS Separations for Metabolomic Analyses of Tissues and Cells, ASMS Poster.

Soma, Y., T. Yamashita, et al. 2018 - Automation of sample preparation for metabolomic analysis using a robotic platform, Poster Metabolomics Conference Brisbane, Kyushu University, Fukuoka, Japan.

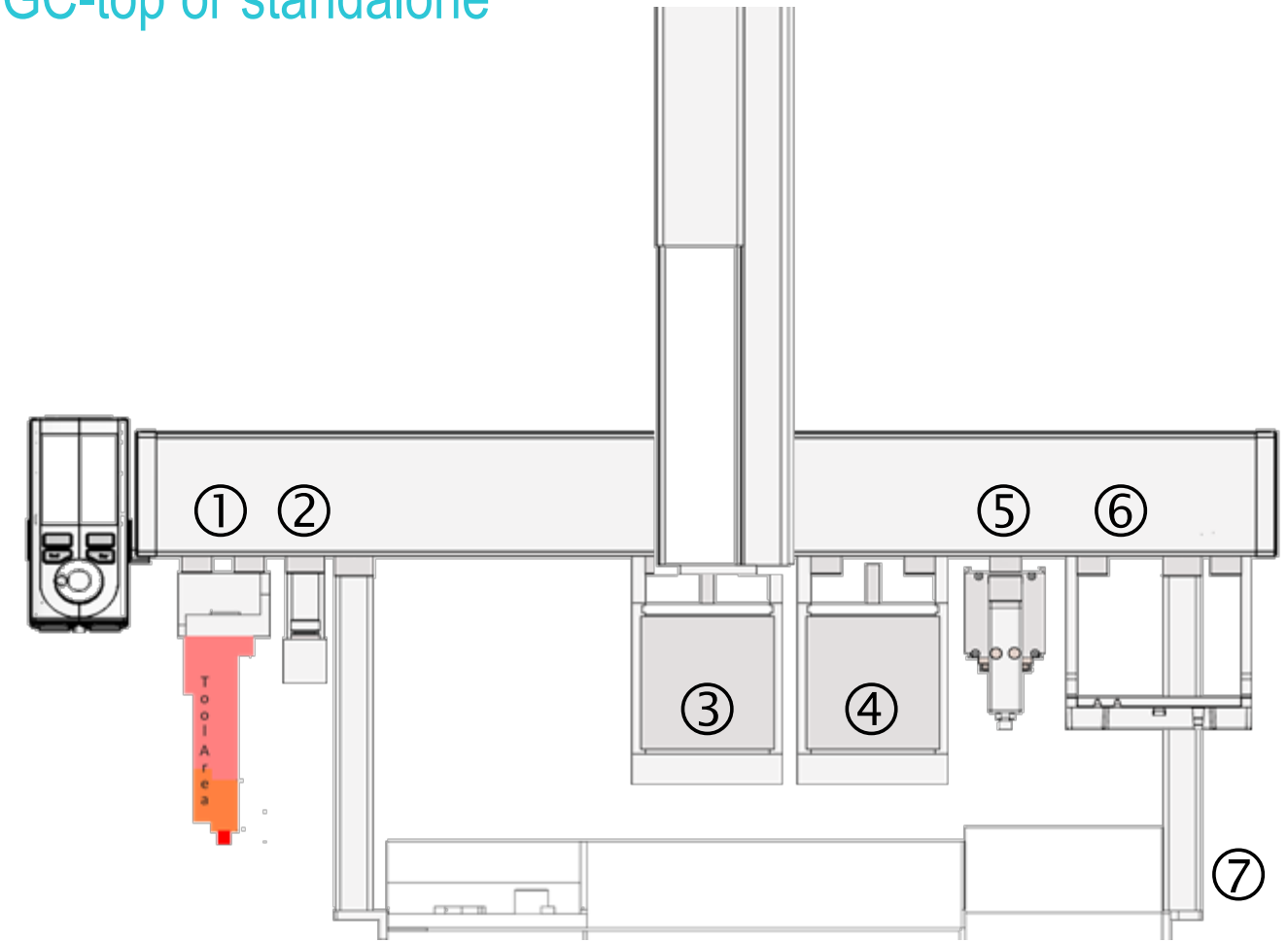
Sample Preparation

Example: Plant Material

- Leaf material of *Arabidopsis thaliana* (Fiehn 2005)
- Homogenized
  under liquid nitrogen
  about 50mg applied to extraction
- **Water / chloroform / methanol mixture**
  to extract water soluble metabolites (Weckwerth 2004)
- **Polar phase** of water / methanol is used (unpolar phase contains lipophilic compounds)
  dried in a vacuum centrifuge
- **2-Step derivatization:**
  Methoxyamination (methoxyamine hydrochlorid in pyridine) to suppress keto-enol tautomerism
  Silylation using MSTFA or BSTFA to derivatize polar functional groups.
  Total derivatization volume 100µl.
- Standards
  dissolved in methanol or water, diluted into various concentrations,
  dried and derivatized according to plant material.

PAL System for Automated Metabolomics

Installation GC-top or standalone

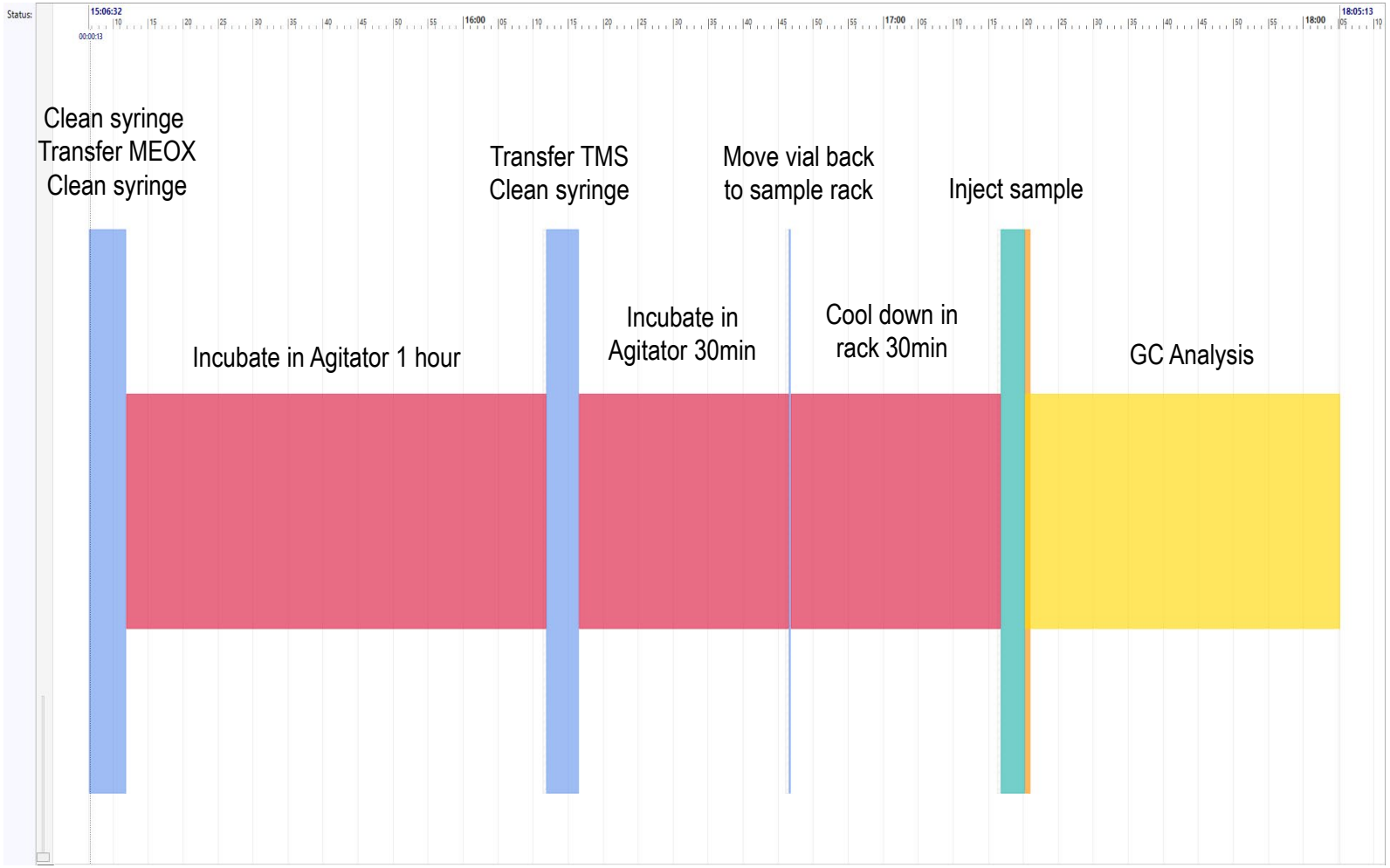


1. Tool station
2. Solvents, reagents, standards
3. Incubator 1
4. Incubator 2
5. Fast Wash station
6. Samples
7. GC-top installation

PAL Metabolomics Workflow

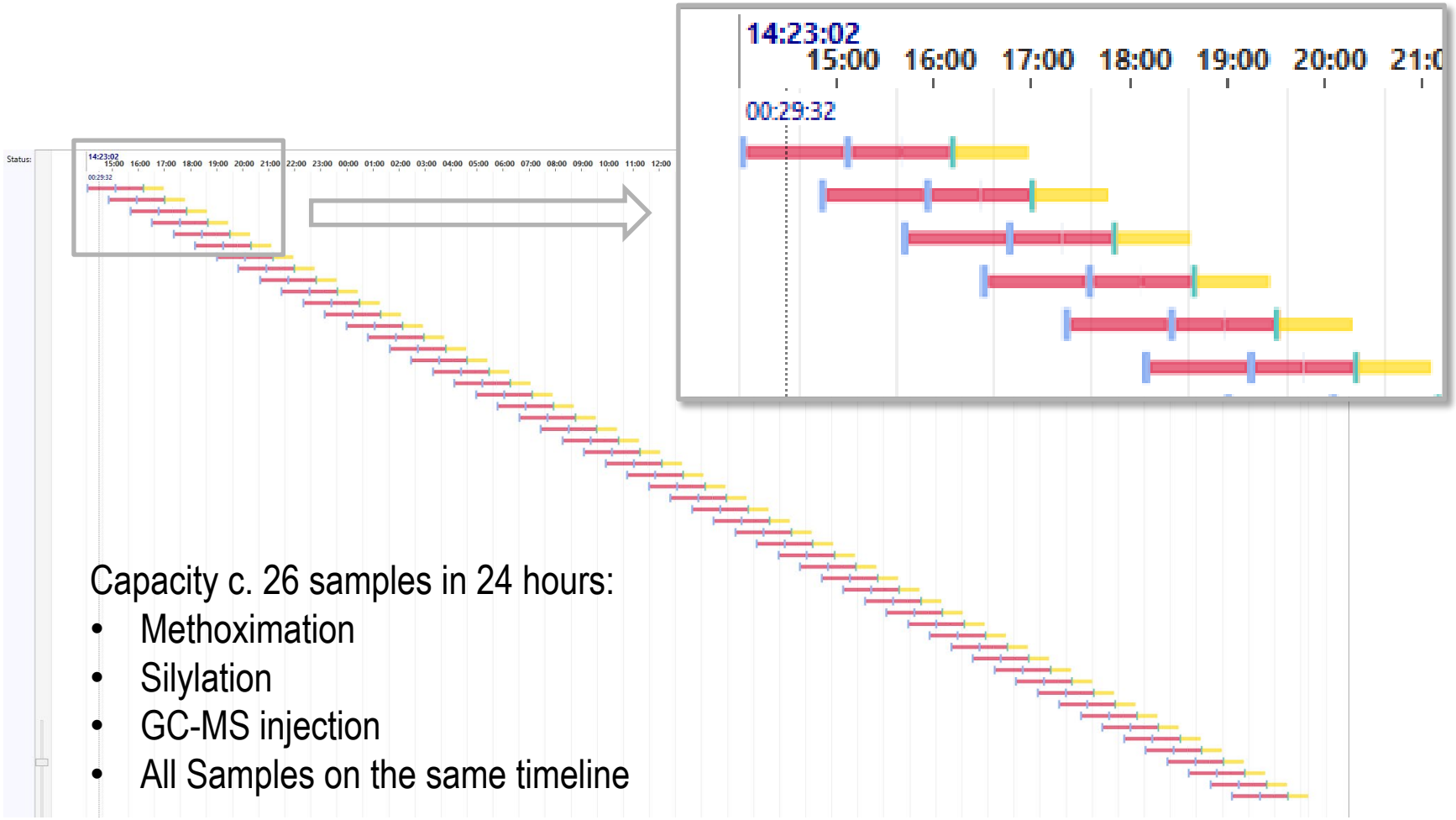
Overlapped workflow for Two-Steps Derivatization

Prep Ahead for Highest Reproducibility



Prep Ahead Overlapping for All Samples

Using the Prep-Ahead Mode for Increased Sample Throughput



Capacity c. 26 samples in 24 hours:

- Methoximation
- Silylation
- GC-MS injection
- All Samples on the same timeline

GC-MS/MS Conditions

Suggested Analysis Conditions

- **Gas Chromatography:**

Column:	5% Phenyl phase, short, for oven high temperatures
	15 m x 0.25 mm ID x 0.25 µm
	He, constant flow at 1 mL/min
Injection:	1 µL at 230 °C SSL injector
	splitless 2 min
	splitflow 10 mL/min
Oven:	70 °C hold 1min
	1 °C/min to 80 °C
	6 °C/min to 330 °C, hold for 5 min
	Postrun 10 min at 325 °C
Transfer line:	up to 300 °C

Arabidopsis Thaliana – Metabolite Profile

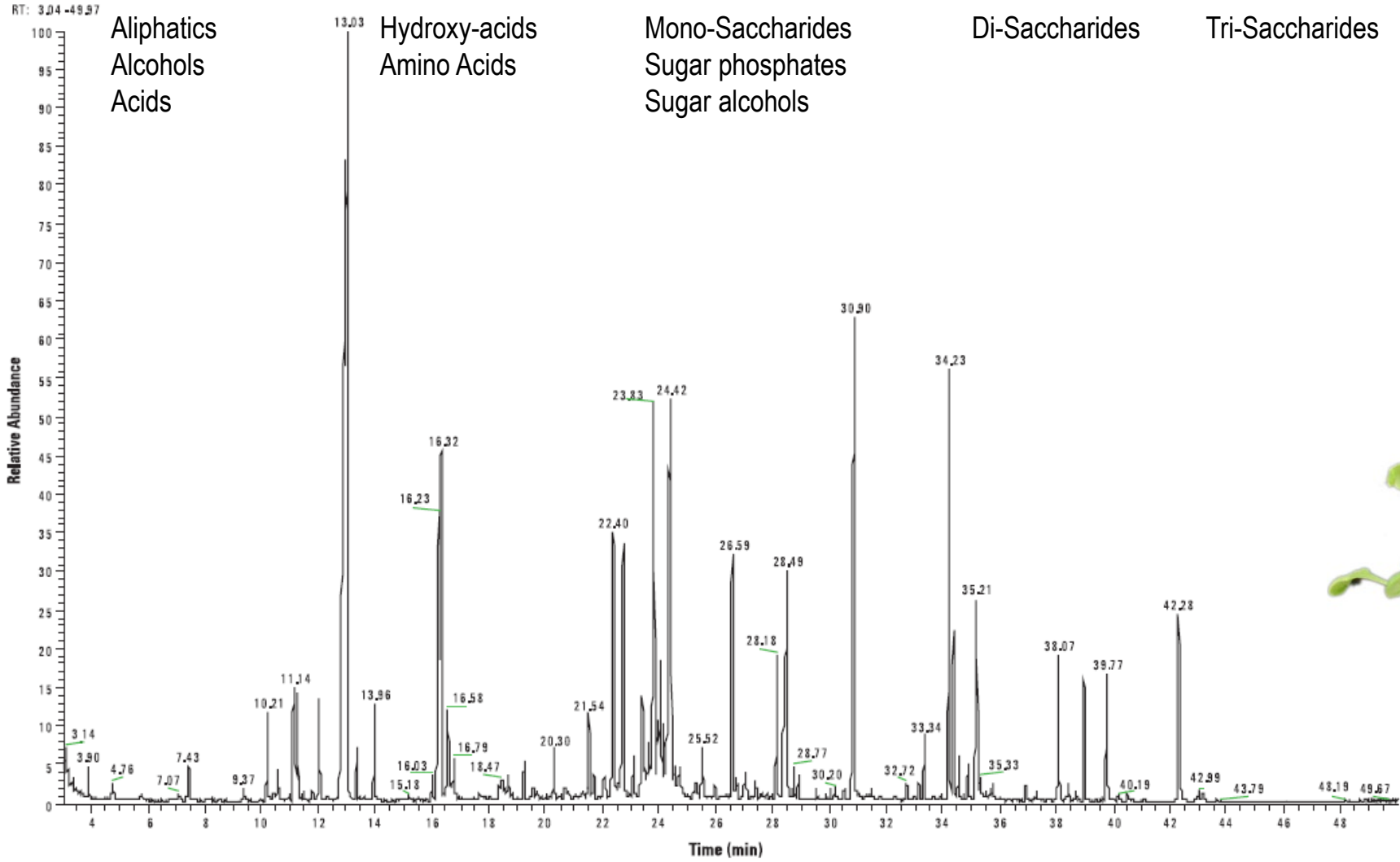
Aliphatics
Alcohols
Acids

Hydroxy-acids
Amino Acids

Mono-Saccharides
Sugar phosphates
Sugar alcohols

Di-Saccharides

Tri-Saccharides



Phase I: Identification - Discovery

Full scan mass spectra after derivatization by library search

GC-MS achieves better metabolite separation and generally avoids ion suppression, a major challenge faced by LC-MS.

Semi-quantitative discovery phase

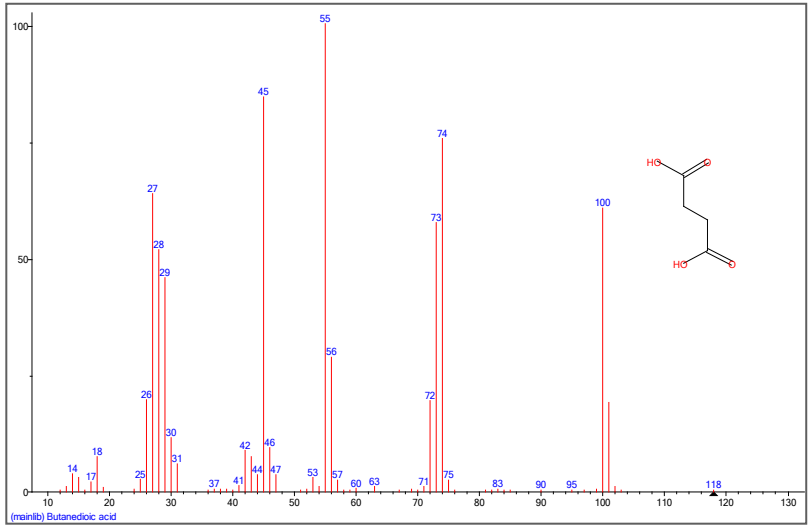
- Identify as many metabolites as possible

  by GC-MS Full Scan analysis, small molecules after derivatization

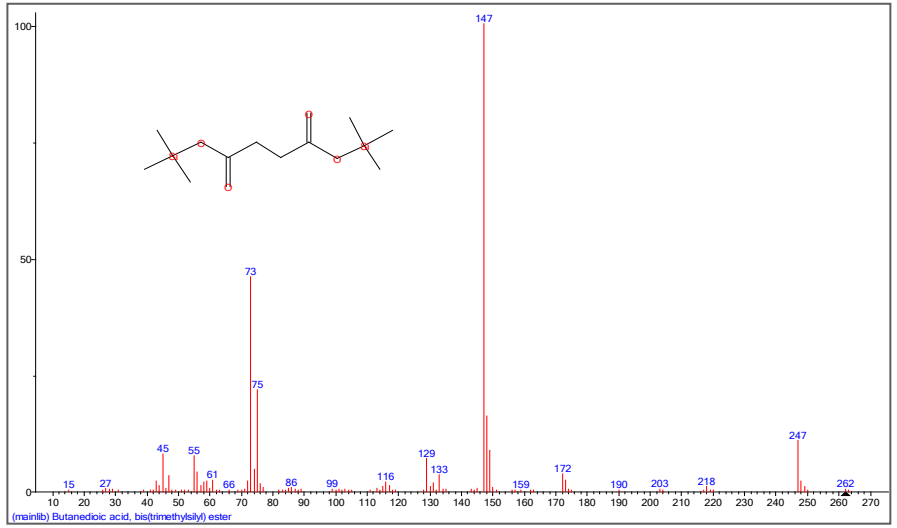
  by LC-MS HRAM and MSMS analysis. But: Intrinsic problem ESI ion surpression.

- Use and developed reference libraries

  Reference libraries are NIST, Wiley, Fiehn (Agilent), Smart Metabolites Database (Shimadzu), Compound Discoverer™ (Thermo). Update with novel compounds or chemical synthesis



Succinic Acid (NIST)

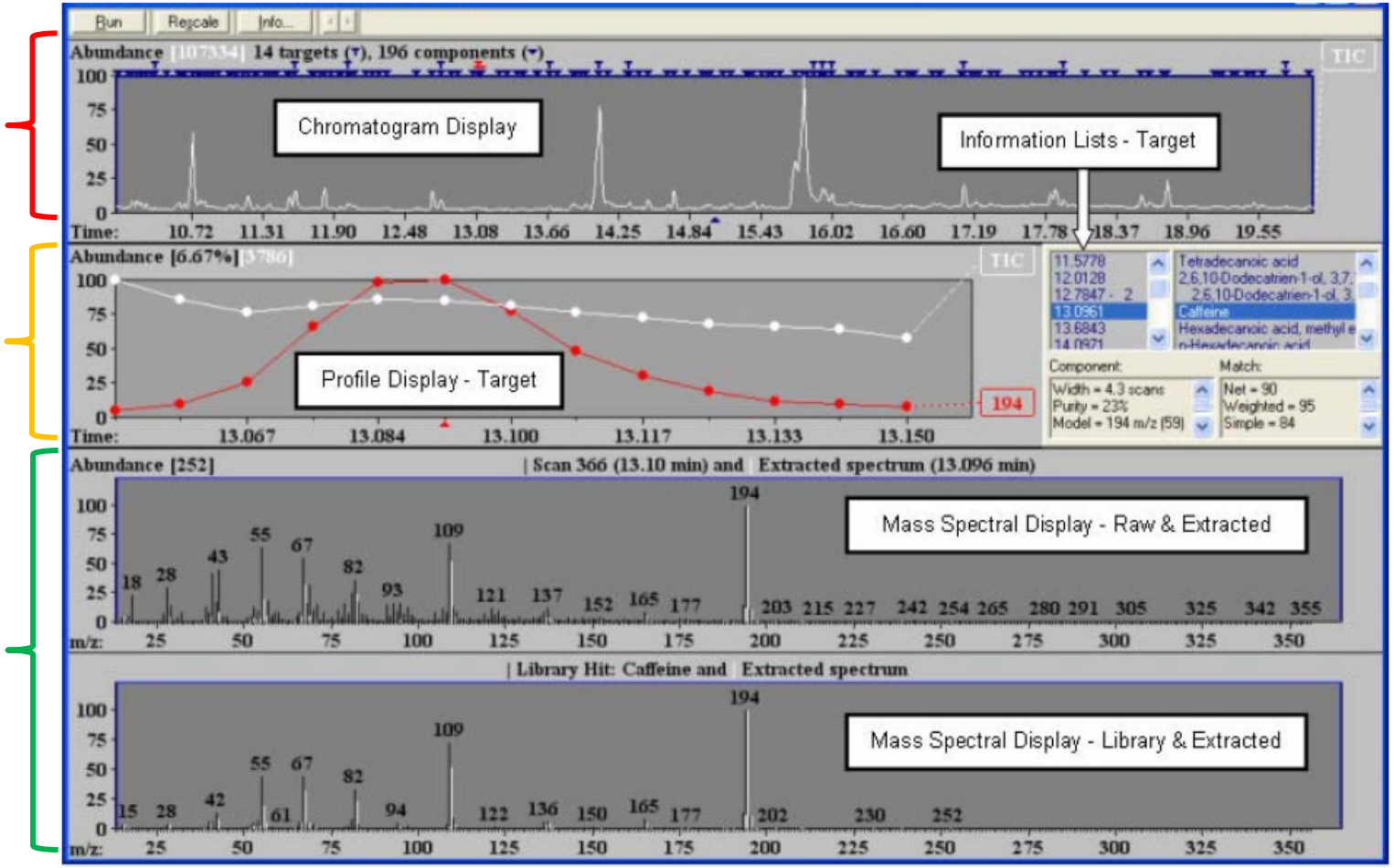


Succinic Acid – TMS (NIST, Reference Lib)

AMDIS for Compound Identification

Automated Spectrum Extraction with RT and Library Search

- Found Compounds
  - 14 Targets
    - from Reference
  - **196 Unknowns**
    - from NIST
- Target Compound
  - Fragment profiles
- Spectrum Check
  - Extracted ag. raw spectrum
  - Library spectrum



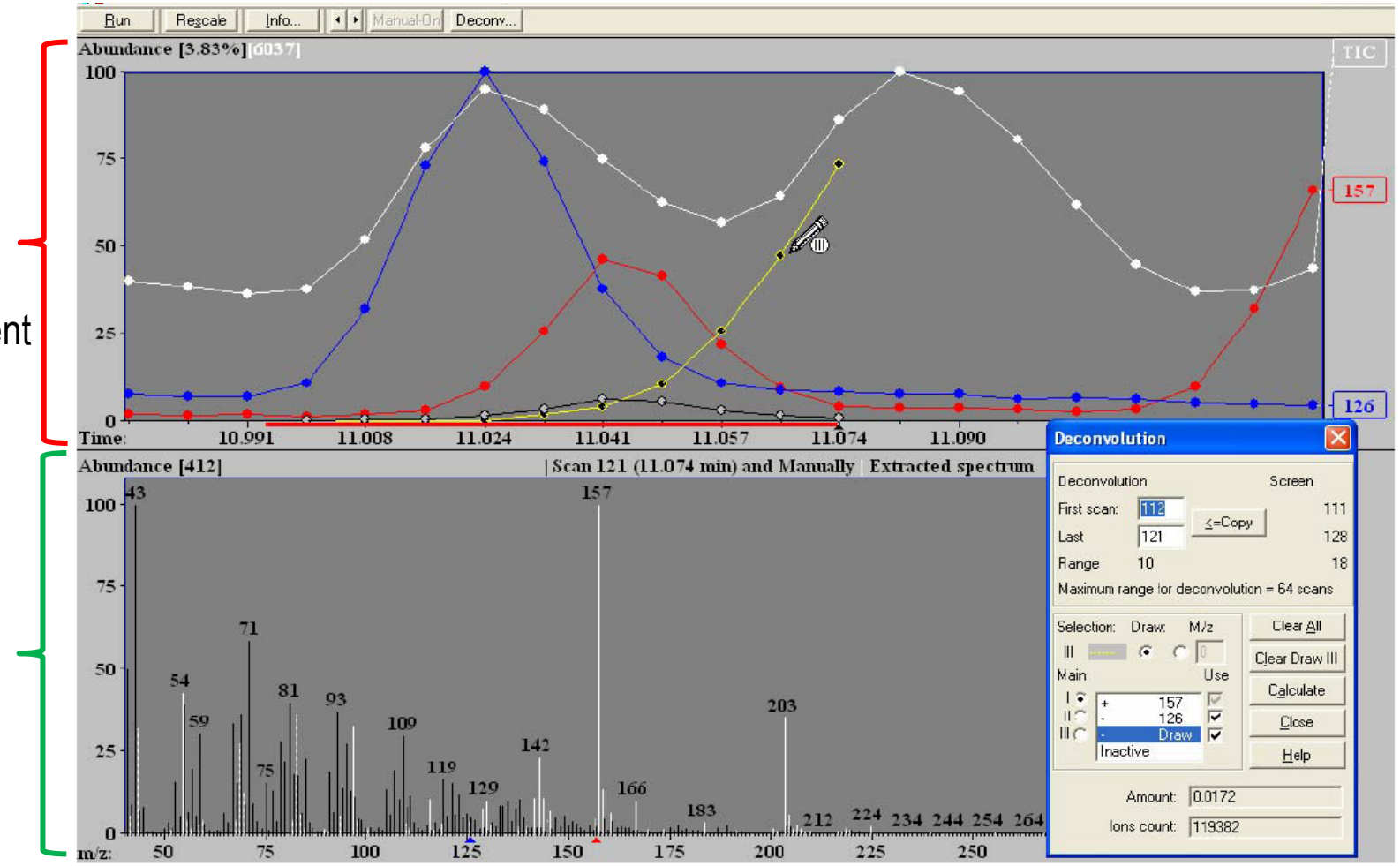
Target example caffeine, m/z 194

- See https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis

AMDIS – Spectrum Deconvolution

Unknown Identification with Coeluted Compounds

- Calculates the compound mass spectrum from overlapping peaks:
  - Automatically in the complete chromatogram
- Chromatogram
  - Single masses
  - Proportion calculation for each fragment
- Clean Spectrum (white)
  - Compare to raw spec (black)
- Then:
  - Run Library search
  - with the cleaned spectrum



Discovery Phase - Heat Map for Evaluation

Based on Full Scan Data

- After AMDIS
  - Identification of many peaks
- Take compounds of interest
  - Potential marker
- Prepare a table of intensities
  - "Heat Map"
  - **Green** – Low Intensity
  - **Red** - High Intensity (=hot)
- Evaluation
  - Compounds in all samples – no interest
  - Compounds specific of a sample – potential marker
- => Candidate for targeted analysis

Compound Number	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
1	14	41	39	67	100	69	53	54	31	51	79	76
2	18	16	100	17	77	21	19	45	39	20	17	41
3	58	26	20	25	45	7	22	3	100	33	20	54
4	76	41	60	37	41	28	70	9	100	71	35	64
5	98	41	31	30	30	7	56	8	100	51	32	56
6	38	44	62	38	62	99	49	100	95	32	90	37
7	69	56	74	96	70	99	81	66	85	100	72	100
8	44	40	97	44	33	56	51	50	100	44	41	57
9	100	64	73	49	29	19	51	7	68	24	20	66
10	100	17	21	14	30	45	16	65	34	16	14	31
11	66	43	67	45	69	100	66	48	41	70	70	66
12	28	20	49	23	39	39	26	100	30	18	16	26
13	30	35	27	30	42	30	83	37	100	74	32	42
14	100	44	5	51	9	79	30	33	32	59	48	12
15	29	26	31	32	37	33	41	100	36	42	37	34
16	14	19	17	16	11	11	16	29	24	33	100	9
17	6	3	11	2	3	6	5	18	10	1	100	3
18	23	3	6	1	3	100	2	2	2	1	3	10
19	18	56	74	38	31	32	42	50	12	40	100	38
20	22	46	83	48	63	33	42	80	27	31	100	45
21	36	18	23	66	97	58	43	99	98	100	16	50
22	100	100	100	100	100	100	100	100	100	100	100	100
23	53	91	24	31	15	4	35	2	100	28	11	80
24	78	15	20	17	4	100	8	37	43	14	5	10
25	64	100	55	30	52	70	40	0	69	30	0	0
26	29	37	41	25	46	27	36	100	29	36	22	51
27	33	20	16	37	24	25	11	24	64	9	100	15
28	23	6	16	7	12	29	15	100	28	21	7	8
29	19	12	100	14	77	20	15	35	31	17	12	37
30	57	100	79	87	74	75	73	62	59	43	26	68
31	6	9	13	22	27	31	23	38	67	18	100	12
32	20	15	17	64	27	59	21	37	100	89	76	13
33	16	9	28	11	20	43	22	100	30	24	14	15
34	18	18	25	38	42	17	15	36	100	32	56	6
35	77	46	29	69	37	22	23	65	35	100	36	24
36	38	72	27	38	47	82	50	100	9	32	20	61
37	53	36	33	100	6	18	10	24	6	16	9	8
38	72	86	53	75	60	100	79	77	83	76	95	71
39	22	36	49	13	15	18	26	68	16	10	100	23
40	33	40	39	21	64	79	22	100	53	34	56	39
41	68	72	92	40	46	66	45	27	100	35	23	36
42	33	54	61	46	55	44	31	59	100	55	45	90
43	17	15	100	15	76	20	19	0	37	15	16	39
44	50	54	61	40	53	66	38	67	95	39	100	42
45	39	54	38	31	40	57	39	37	48	45	100	40
46	27	16	30	14	21	41	29	100	47	21	15	15
47	56	43	32	38	80	70	49	93	100	78	26	50
48	39	38	34	40	32	100	30	57	54	21	87	32
49	86	100	81	15	74	75	68	96	52	21	74	53
50	16	10	47	11	27	17	17	100	26	29	18	24
51	29	26	100	28	42	38	22	62	39	28	22	41
52	53	52	88	40	100	64	47	0	75	60	34	64
53	26	21	34	26	65	68	21	100	62	32	24	38
54	19	4	11	34	5	12	11	11	44	4	100	8
55	54	35	49	26	19	40	18	100	58	56	12	17
56	13	40	10	81	54	95	100	29	14	49	32	9
57	24	26	95	50	74	100	22	53	20	51	43	72
58	26	52	36	79	38	92	30	45	30	23	42	100
59	7	66	12	6	5	18	100	28	61	10	66	8
60	58	70	52	37	100	84	91	94	66	52	100	47
61	10	6	11	5	25	43	13	100	13	6	76	7
62	28	32	22	17	52	45	25	100	41	27	23	28
63	27	27	43	21	13	94	37	100	33	20	17	20
64	26	22	18	16	11	100	20	91	25	17	13	14
65	53	30	27	29	28	55	51	39	100	31	32	51
66	48	26	100	38	53	42	34	82	56	35	25	41

Quantitation – Phase II

Targeted Analysis

PAL SYSTEM
Ingenious sample handling

Quantitation - Target Compound List

PAL SYSTEM
Ingenious sample handling

Based on MS/MS: RT, Quan and Confirmation Mass Transitions (MRM)

		Quan Peak			Confirming Peak		
Compound Name	**RT (min)**	**Precursor**	**Product**	**CE (eV)**	**Precursor**	**Product**	**CE (eV)**
N,N'-Bis(trimethylsilyl)trifluoroacetamidine	6.37	99.0	69.0	30	99.0	71.0	10
Ethylbis(trimethylsilyl)amine	6.59	100.1	59.1	10	174.1	59.1	20
Silanamine, N,N'-methanetetraylbis[1,1,1-trimethyl-	6.92	78.1	64.0	12	78.1	71.3	12
1,2-Bis(trimethylsiloxy)ethane	7.44	148.8	45.0	30	148.8	75.0	10
Butane, 2,3-bis(trimethylsiloxy)-	8.73	118.4	45.6	18	118.4	75.2	8
l-Alanine, N-(trimethylsilyl)-, trimethylsilyl ester	10.06	116.1	43.0	28	116.1	45.1	18
Glycine, N-(trimethylsilyl)-, trimethylsilyl ester	10.48	102.1	45.1	20	102.1	58.0	30
Phosphoric acid, bis(trimethylsilyl)monomethyl ester	11.55	133.0	115.0	10	163.1	133.1	10
L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester	12.32	144.1	43.0	32	144.1	58.1	32
2-(4-Methoxyphenyl)-2-(4-trimethoxysilyloxy)propane	13.36	299.1	151.1	10	300.5	74.1	10

Compound Number	Compound Name	RT (min)	Precursor	Product	CE (eV)	Precursor	Product	CE (eV)
1	N,N'-Bis(trimethylsilyl)trifluoroacetamidine	6.37	99	69	30	99	71	10
2	Ethylbis(trimethylsilyl)amine	6.59	100.1	59.1	10	174.1	59.1	20
3	Silanamine, N,N'-methanetetraylbis[1,1,1-trimethyl-	6.92	78.1	64	12	78.1	71.3	12
4	1,2-Bis(trimethylsiloxy)ethane	7.44	148.8	45	30	148.8	75	10
5	Butane, 2,3-bis(trimethylsiloxy)-	8.73	118.4	45.6	18	118.4	75.2	8
6	l-Alanine, N-(trimethylsilyl)-, trimethylsilyl ester	10.06	116.1	43	28	116.1	45.1	18
7	Glycine, N-(trimethylsilyl)-, trimethylsilyl ester	10.48	102.1	45.1	20	102.1	58	30
8	Phosphoric acid, bis(trimethylsilyl)monomethyl ester	11.55	133	115	10	163.1	133.1	10
47	9-Octadecenoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester	41	103	45.1	20	103	58.1	30
48	β-D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	41.54	399.3	81.1	10	399.3	95.5	10
49	1,2-Propanediol-1-phosphate, tris(trimethylsilyl)-	42.68	298.9	147.1	20	298.9	225.2	10
50	D-Turanose, heptakis(trimethylsilyl)-	42.81	373.4	167.1	12	373.4	211.2	12
51	D-Turanose, heptakis(trimethylsilyl	43	361.1	169.2	10	361.1	243.3	10
52	β-D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)	43.67	147.2	45.1	32	147.2	131.1	12
53	α-D-Galactopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	44.71	204	73.1	10	204	189.2	10
54	β-D-Xylopyranose,1,2,3,4-tetrakis-O-(trimethylsilyl)-	44.81	147.3	45				
55	α-D-Galactopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)- Isomer 1	45.01	488.7	222.8	20	488.7	223.5	10
56	1,2-Propanediol-1-phosphate, tris(trimethylsilyl)- isomer 1	45.85	357.1	225.1	18	357.1	341.2	8
57	D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	46.57	205.2	45.3	30	205.2	190.1	10
58	2-Methyl-2(p-methoxy)mandelate, bis(trimethylsilyl)-	46.81	222	45.1	32	222	194.1	12
59	1,2-Propanediol-1-phosphate, tris(trimethylsilyl)- isomer 2	48.44	211	115.1	30	211	133.1	10
60	Silane, [[(3β,24.xi.)-ergost-5-en-3-yl]oxy]trimethyl-	48.84	343.2	95.2	20	343.2	121.1	10
61	β-Sitosterol trimethylsilyl ether	50.16	357.3	95.1	20	357.3	107.1	20
62	β-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	51.24	362.5	169.2	12	362.5	170.2	12
63	9,19-Cyclolanostan-3-ol, 24-methylene-, (3β)-	52.13	147	105.1				
64	D-Turanose, heptakis(trimethylsilyl)- isomer1	53.49	217.2	45.1	28	361.2	169.2	8
65	2-O-Glycerol-β-d-galactopyranoside, hexa-TMS isomer 1	54.95	217.1	45.1	32	217.1	143.1	12
66	D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-β-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	59.21	204.1	45.1	30	204.1	189.2	10

Table 4: Optimised SRM transitions for the 66 compounds selected in Phase I - Discovery.

Mass Separation of Coeluting Compounds

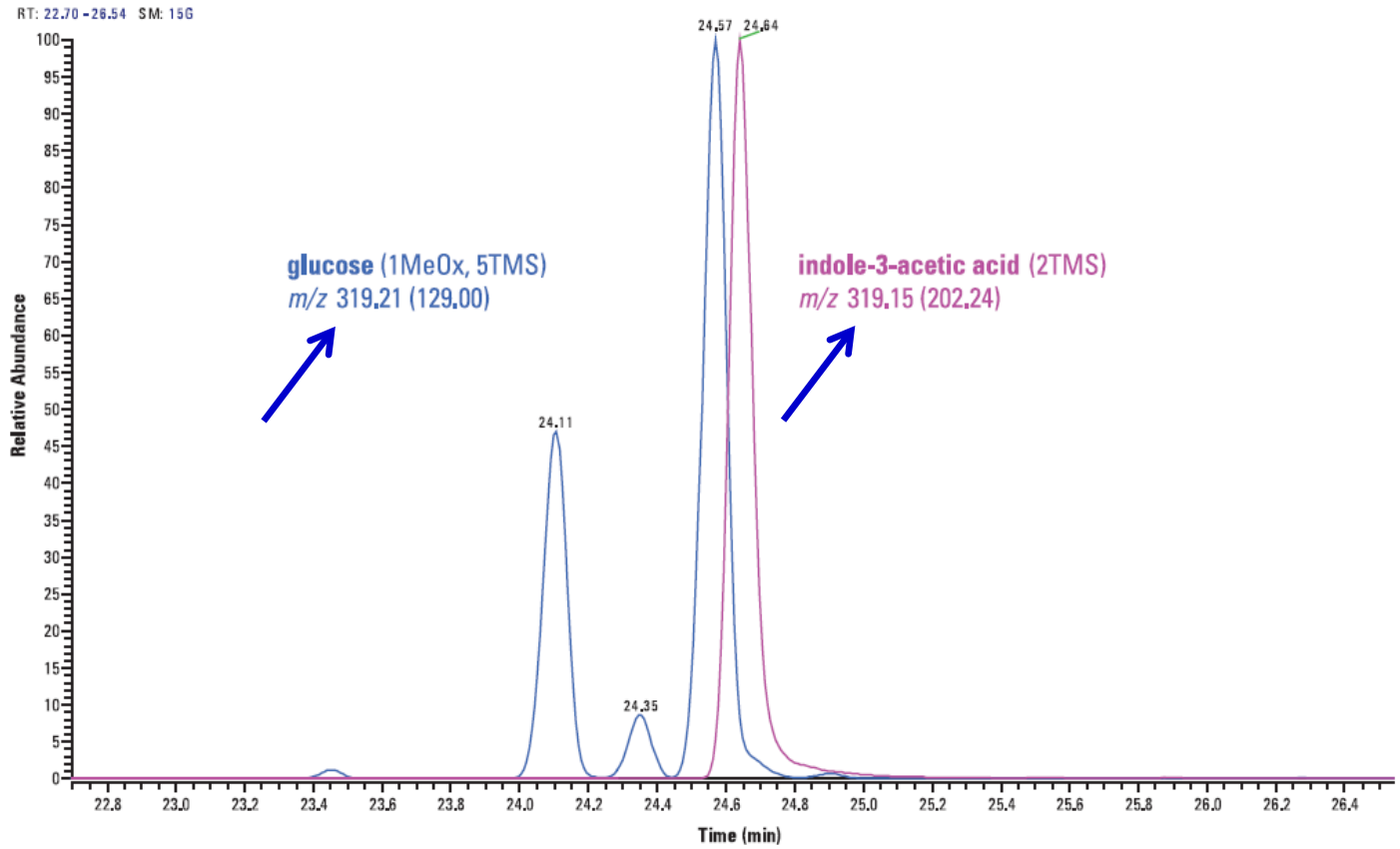
GC-MRM specificity for co-eluting IAA and Glucose

- Glucose and IAA have (almost) the same precursor ion mass for a triple quad:

- Glucose:
  ***m/z* 319.21 > 129.00**

- Indole-3-acetic acid:
  ***m/z* 319.15 > 202.24**

- Other options
  Using HRAM MS systems like QToF, Orbitrap



MRM chromatogram of IAA and glucose reference compounds at the level of 50 pmol injected amount

Calibrations using GC-MS/MS-MRM Analysis

Linear over 5 to 6 Orders of Magnitude

- Glucose

  from 1 fmol to 1 nmol on column,

  18 levels

  6 orders of magnitude !

  R^2 = 0.9985

- Indole-3-acetic acid

  from 10 fmol to 1 nmol on column.

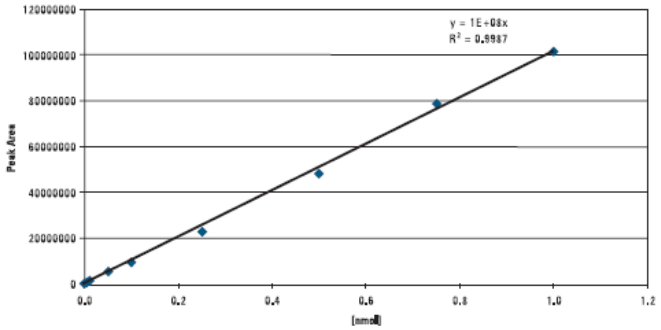
  5 orders of magnitude !

- Salicylic acid

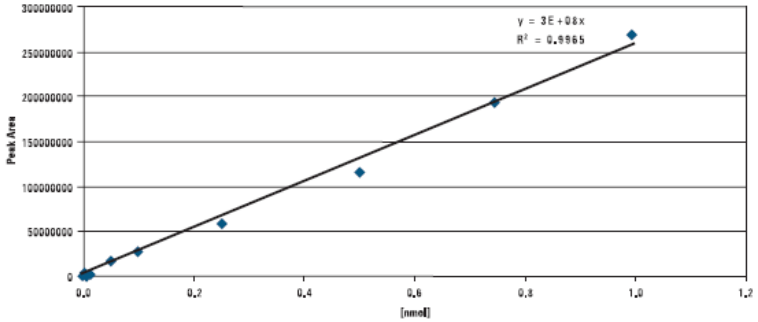
  From 7.5 fmol - 1nmol on column

  >5 orders of magnitude !

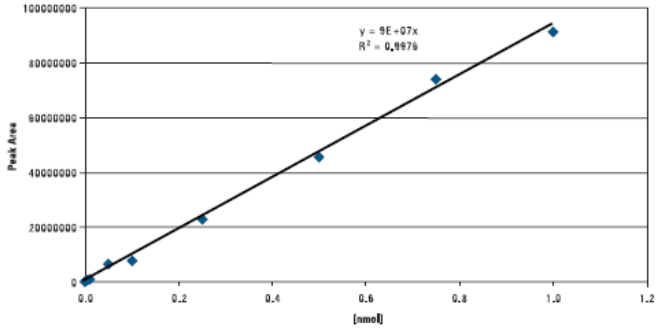
4a Glucose



4b Indole-3-acetic Acid



4c Salicylic Acid



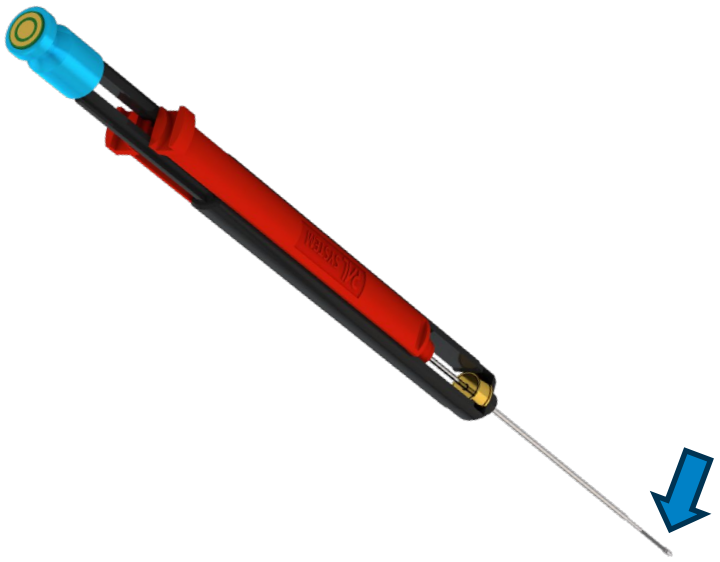
Example Rice – Metabolites > Phenotype

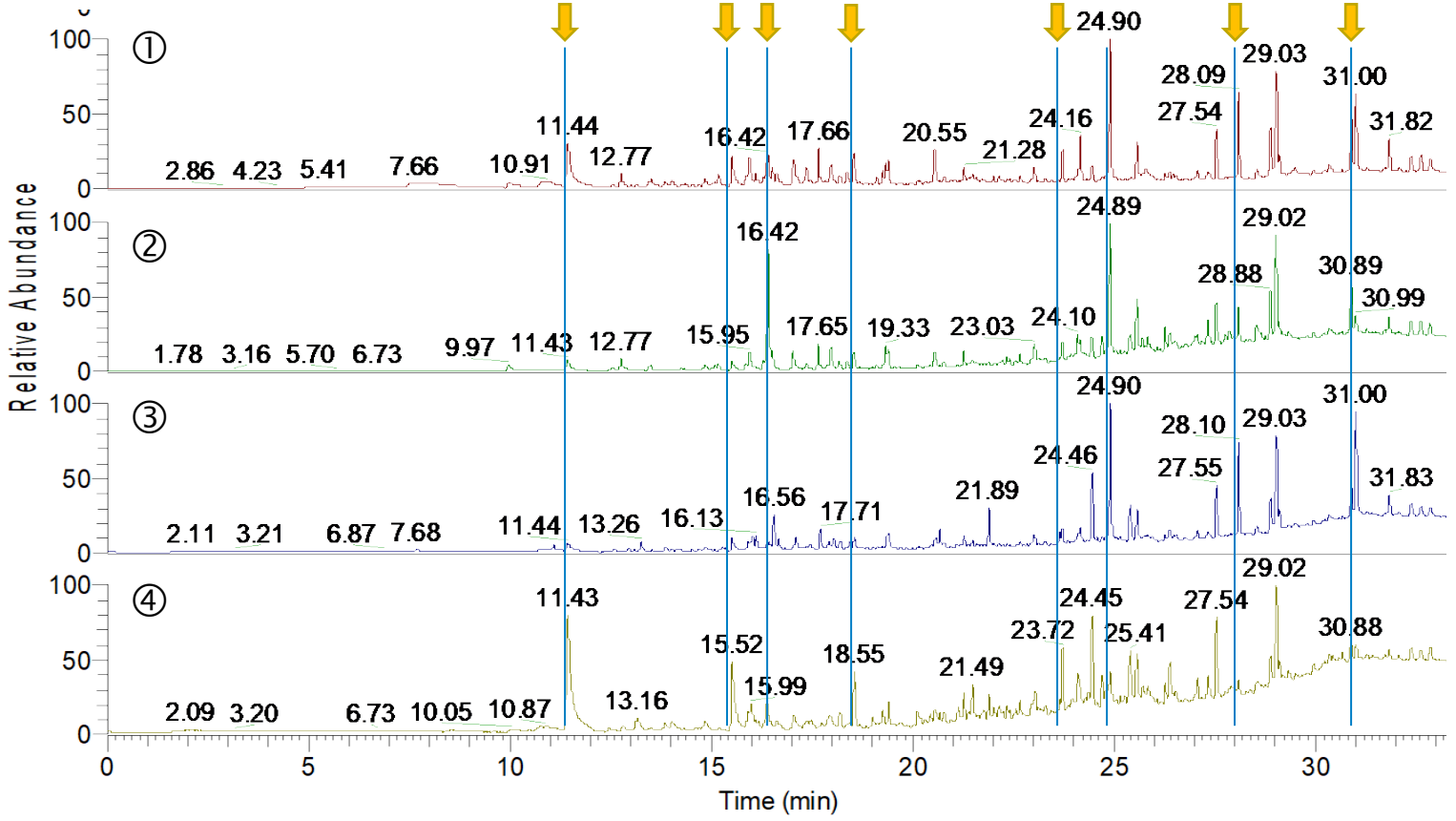
Large Profile Differences Visible

- 4 different species of rice
- SPME Full Scan analysis

  DVB/CAR/PDMS, 80 °C, 30 min

… as examples





Example Rice – Metabolites > Genotype

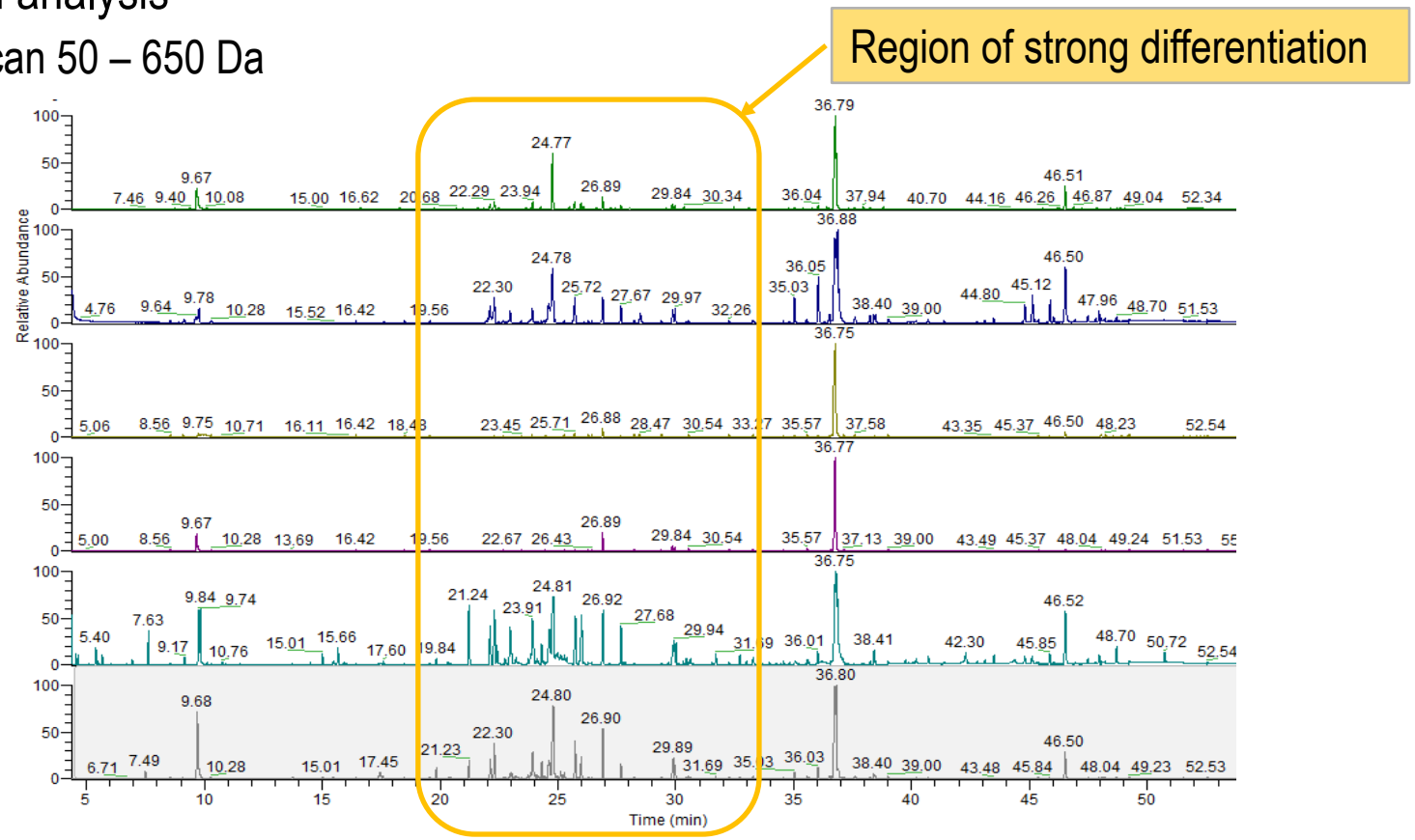
Requires Extraction and 2-Step Derivatization

- Extraction

  Methanol/water, centrifugation, evaporation, derivatization MSTFA/BSTFA

- GC-MS Full Scan analysis

  Direct injection, scan 50 – 650 Da



Analytical Challenges in Metabolomics

PAL SYSTEM
Ingenious sample handling

Typical GC-MS Instrument Requirements

- Greatest challenge: Requires high dynamic range (Triple quad yes, limited with TOF-MS)
  from very low abundance metabolites like phytohormones
  to highly concentrated compounds, like energy-related carbohydrates.
- Low concentrated metabolites (Triple quads and HRAM have highest selectivity in matrix)
  especially the analysis of chromatographic regions with ultracomplex coelution of different compounds, MRM-strategies are developed.
- Very low detection limits (Proof from leading metabolomics lab, e.g. Univ. Vienna, UC Davis)
  and a dynamic range of > 4 orders of magnitude for e.g. phytohormones.
- High sensitivity (Performance stated in peer reviewed papers)
  The triple quads show high sensitivity in Full Scan (Phase I: Discovery analysis)
- Selectivity (unique use of the retention timed-SRM concept)
  The triple quads separate coeluting analytes by MRM transition (Phase II: Quantitation)
  Example: Indole-3-acetic acid and glucose are well separated by the GC-MS/MS MRM analysis.

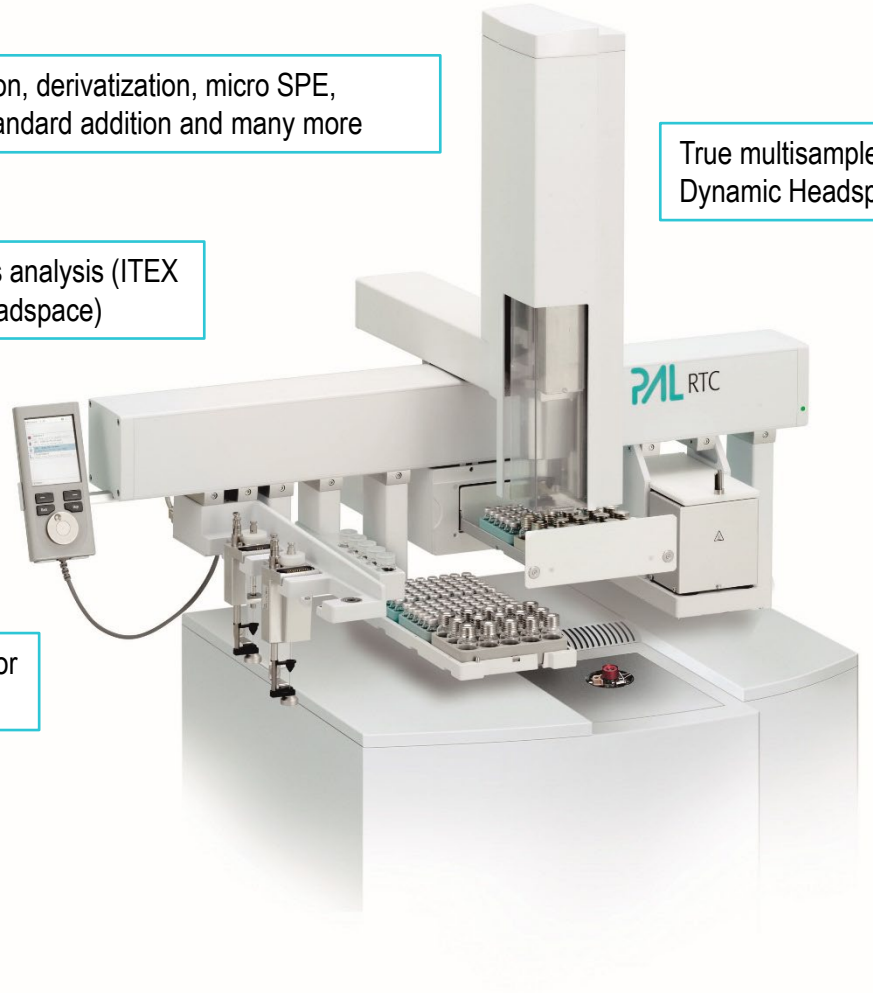
Prep and Inject the Way You Want

Imagine you could do all this on 1 instrument….

Sample prep, dilution, derivatization, micro SPE, filtration, SPME, standard addition and many more

True multisampler: Liquid injection, Static Headspace, ITEX Dynamic Headspace, MHE, SPME on one instrument

Combined volatile / semi volatiles analysis (ITEX Dynamic Headspace, SPME, Headspace)



Prepare calibration standards prior to a series of new samples

Combined static and Dynamic Headspace on 1 autosampler

Recommended Literature

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- **Fragner, Lena**, Takeshi Furuhashi, and Wolfram Weckwerth. 2014. “Gas Chromatography Coupled to Mass Spectrometry for Metabolomics Research.” In *Practical Gas Chromatography*, edited by K. Dettmer-Wilde and W. Engewald, 783–97. Berlin Heidelberg: Springer-Verlag. https://doi.org/10.1007/978-3-642-54640-2.
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- **Hopfgartner, Gérard**, Sandra Jahn, and Emmanuel Varesio. 2014. “Integrated Platform Including Bligh and Dyer Extraction and Dual-Column UHPLC-MS / MS Separations for Metabolomics Studies Identification of Endogenous Metabolites from Chlamydomonas Reinhardtii Algae.” Geneva, Switzerland: Life Sciences Mass Spectrometry, School of Pharmaceutical Sciences EPGL, University of Lausanne.
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- **Matthews, Jeremy P**., Silvia Gemme, Hans-Joachim Huebschmann, Cindy Llorente, Rosario Jimenez, and Nese Sreenivasulu. 2015. “Metabolomics of Rice Genotypes Using GC-MS / MS.” Application Note 10419, *Thermo Fisher Scientific,* Singapore.
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- **Weidt, Stefan**, Bogusia Pesko, Paul Silcock, Cristian Cojocariu, Richard J. Burchmore, and Karl Burgess2. 2016. “Untargeted Metabolomics Using Orbitrap-Based GC-MS.” Application Note 10457, Thermo Fisher Scientific, Runcorn, UK.

Thank you very much for listening!

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