Automated Two-Step Derivatization Workflow for Untargeted Metabolomics of Murine Wound Samples Using GC-ecTOF

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Metabolomics and Noise

Untargeted GC-MS metabolomics provides deep phenotypic insights but is highly sensitive to both technical noise and true biological variation. Unwanted technical variance obscures biological signals, artificially inflates data spread, and reduces statistical power.

One source of this noise is the standard two-step derivatization process (methoximation and silylation), which is labor-intensive and prone to timing inconsistencies when performed manually. Standardizing this workflow via automation is necessary to minimize technical variability and accurately isolate true biological effects.

Stage	Variation Type	Key Contributing Factors
Pre-analytical	Technical	Collection delays, temperature changes, tube types, storage, and freeze-thaw cycles.
Analytical	Technical	Derivatizations, extraction types, manual handling, instrument drift, batch effects, column or source aging, and matrix effects.
Post-analytical	Technical	Data processing parameters, normalization choices, and missing data handling.
Biological	Inter/Intra-subject	True differences (genetics, diet, disease status) alongside temporal or short-term lifestyle fluctuations.

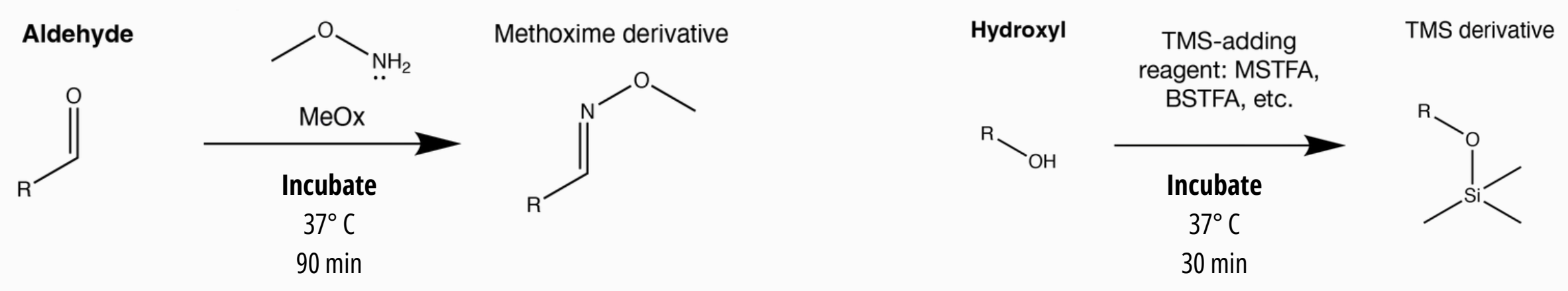
Derivatization
and GC-ecTOF

Direct GC-MS analysis of native biological metabolites is challenging due to their high polarity and thermal instability. To facilitate gas-phase transfer, a two-step transformation is used:

Methoximation (MeOx): Converts carbonyl groups into stable oximes (CH_2ON), keeping reducing sugars in a linear form and simplifying the chromatogram.

Silylation (MSTFA): Replaces active hydrogens with bulky trimethylsilyl groups [$-Si(CH_3)_3$], enhancing volatility and thermal stability for GC analysis.

For example

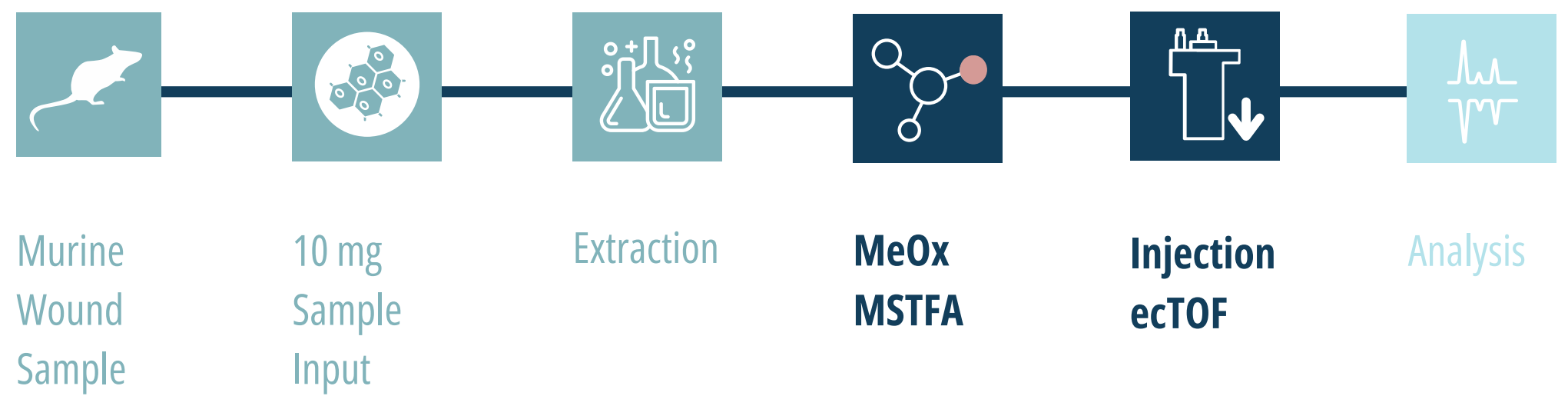


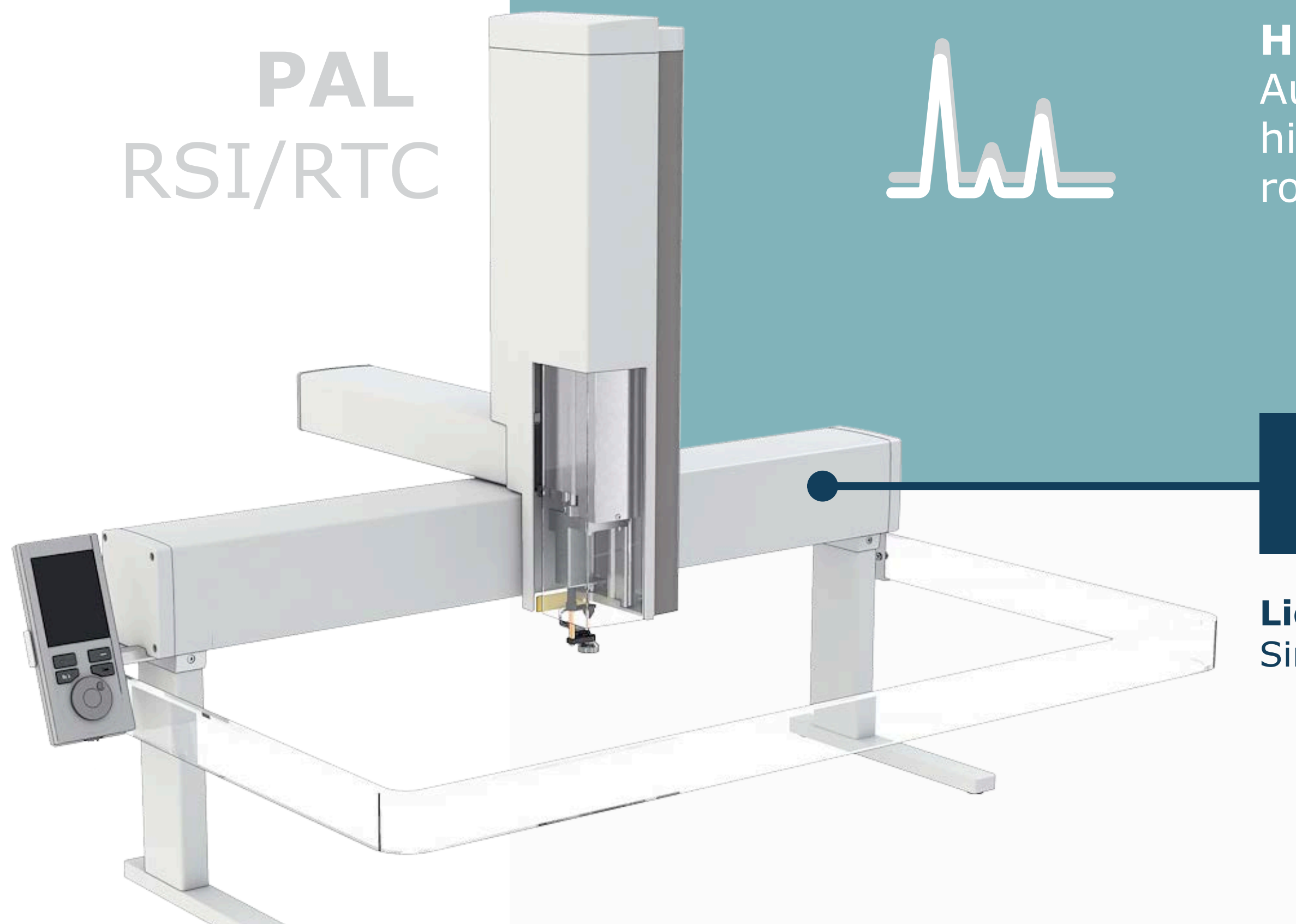
GC - ecTOF: Dual Ionization for Confident Identification

The ecTOF architecture allows for the simultaneous acquisition of both hard Electron Ionization (EI) and soft Chemical Ionization (CI) within a single analytical run. Standard 70 eV EI provides robust, reproducible fragmentation patterns for library matching (e.g., NIST), but it frequently causes excessive fragmentation of heavily silylated metabolites, leaving no detectable molecular ion. CI creates molecular ions with tuneable chemical selectivity. Consequently, integrating the structural fingerprint from EI with the preserved molecular mass from CI enhances the accuracy and confidence of metabolite annotation.

Experimental

To evaluate the automated workflow on reproducibility and carryover, murine wound exudates (10 mg), sequence blanks, and Quality Controls (QCs) were extracted using cold acetonitrile/isopropanol/water and vacuum-dried, following the protocol described for mammalian metabolomics (Fiehn, 2016). Afterwards, a standard PAL RSI (Robotic Sample Injection) executed the fully automated two-step derivatization, maintaining both methoximation (MeOx) and silylation (MSTFA) at a constant 37 °C to streamline hardware requirements. Chromatographic separation was achieved over a 25-minute run. Measurements were performed on a Bruker GC-ecTOF, which simultaneously acquires standard 70 eV Electron Ionization and soft Chemical Ionization data (using ammonia as the reagent gas) to ensure comprehensive, parallel identification of the complex biological matrix.



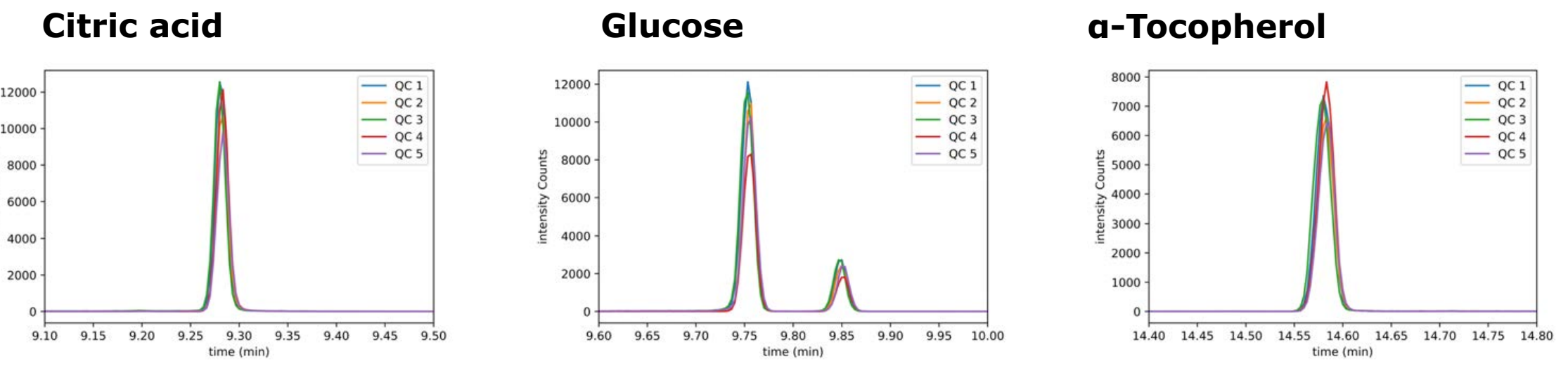


Standardization &
the Reduction of Technical Variability

The transition from manual batch preparation to an automated sequential workflow standardizes the derivatization process. Automating the procedure removes manual handling errors and operator-introduced variability. Crucially, sequential processing ensures strictly timed intervals between reagent addition and GC-injection for every single vial, preventing the time-dependent kinetic variance inherent to batch sequences.

RSD % across QCs

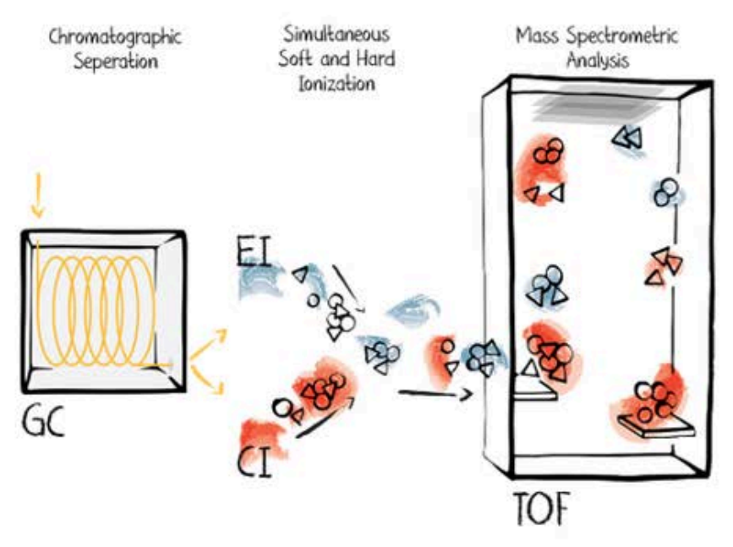
Applying this sequential logic yielded excellent analytical precision across overlaid Quality Control (QC) injections (n=5).

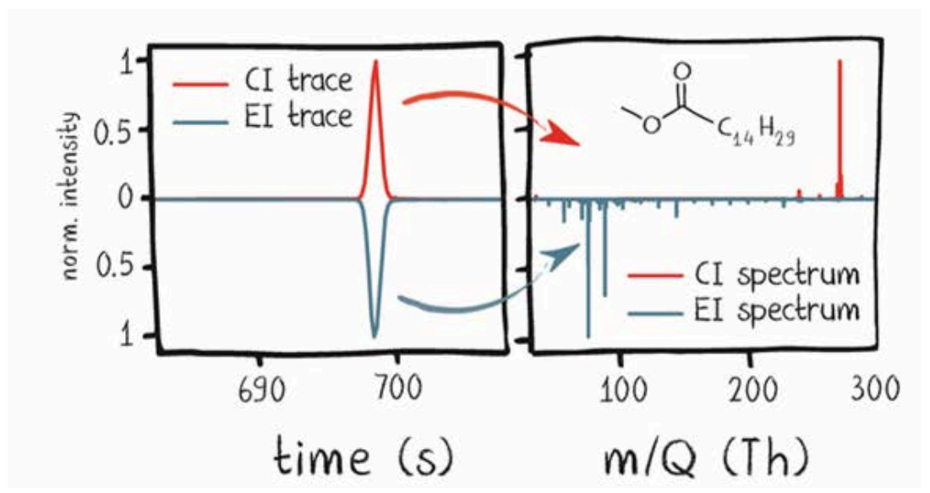


RSD = 8% **RSD = 12%** **RSD = 6%**

Across 5 technical replicates of quality controls.

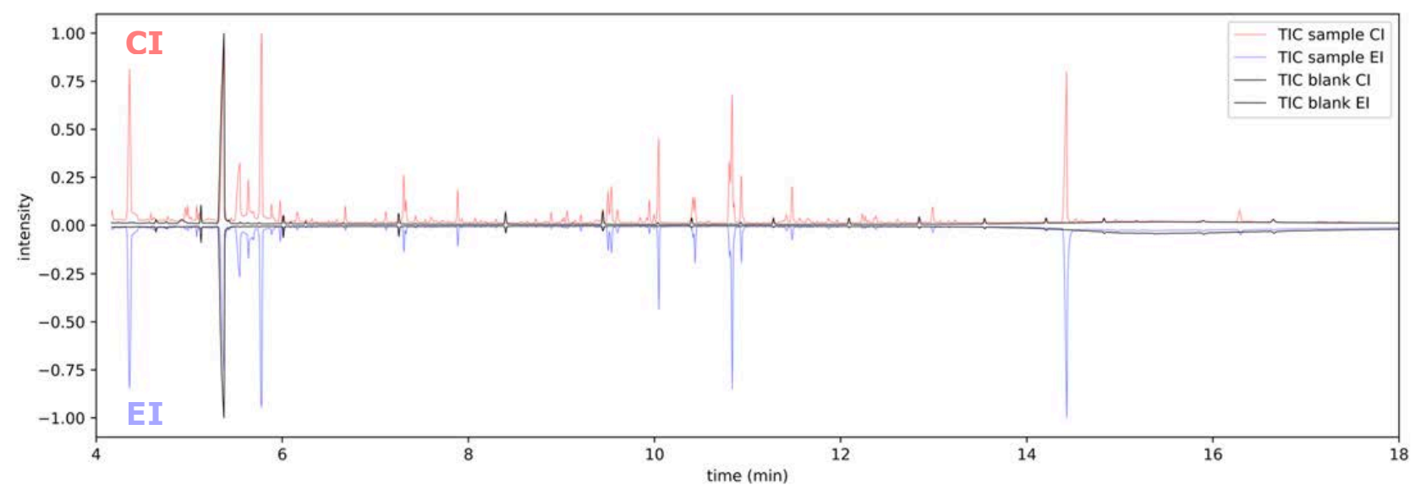
Measuring with a GC-ecTOF Setup

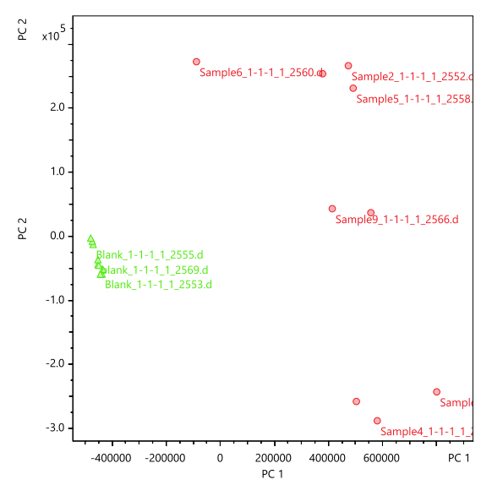




TIC of Sample and Blank

It contrasts a complex biological murine sample (colored trace) with a system blank (black trace) in both EI and CI modes. Notable peaks in the blank trace represent intentionally spiked FAME (Fatty Acid Methyl Ester) retention index mix.





PCA of Samples and Blanks

TL;DR

Hardware simplification
A standard PAL RSI equipped with a single agitator and one syringe efficiently executes complex two-step metabolomics workflows, lowering the barrier to entry for core laboratories.

Sequential precision
Overlapping and scheduling eliminates variable queue times, mitigating the time-dependent degradation of sensitive metabolites often observed in traditional batch processing.

High reproducibility
Automated liquid handling removes user-to-user variation, delivering high analytical precision and retention time stability essential for routine, large-scale untargeted profiling.

Liquid Tool
Single Syringe

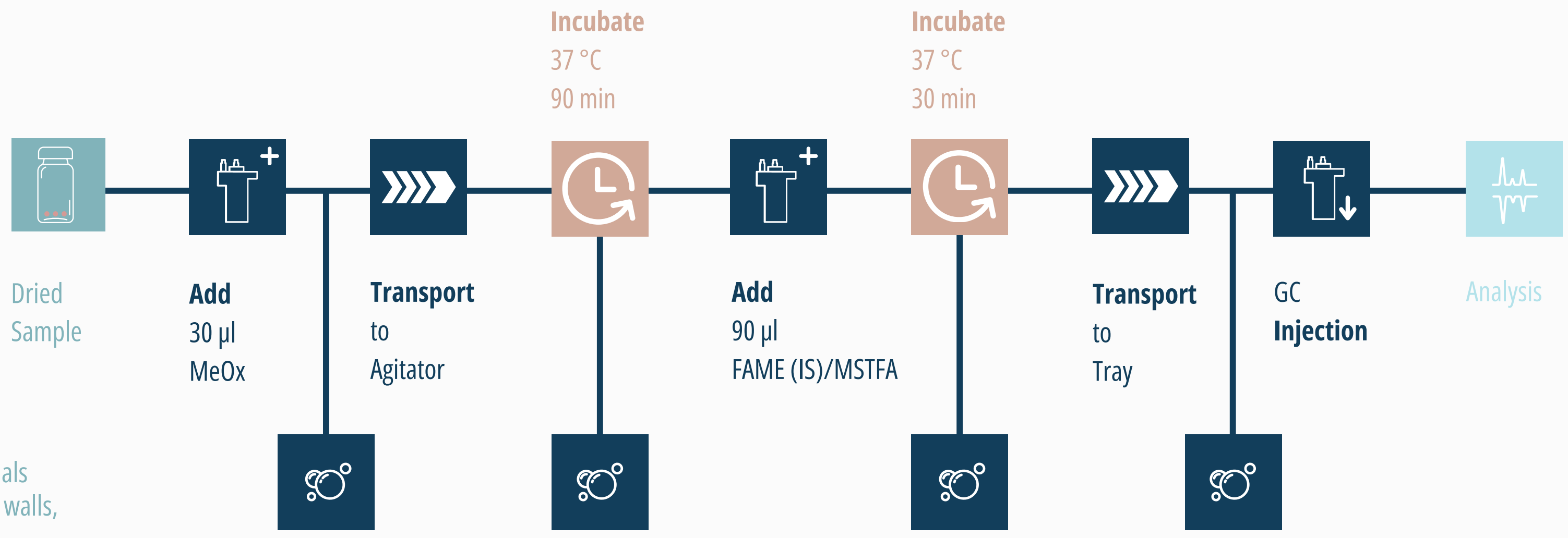
Agitator
Set to 37 °C

Trayholder
2ml vials
(+inserts)

Wash
Standard +
WashSolv
Module

Automated Workflow

In dark blue all steps that are performed by the PAL RSI.



Note
Drying samples in standard 2 ml vials disperses residue along the upper walls, preventing contact with the MeOx reagent. Total recovery vials or micro-inserts are necessary to pool the sample and guarantee complete derivatization.

Clean Syringe
Repeated cleaning (methanol) ensures minimal carry over. For difficult matrices use multiple solvents and increase cleaning cycles.

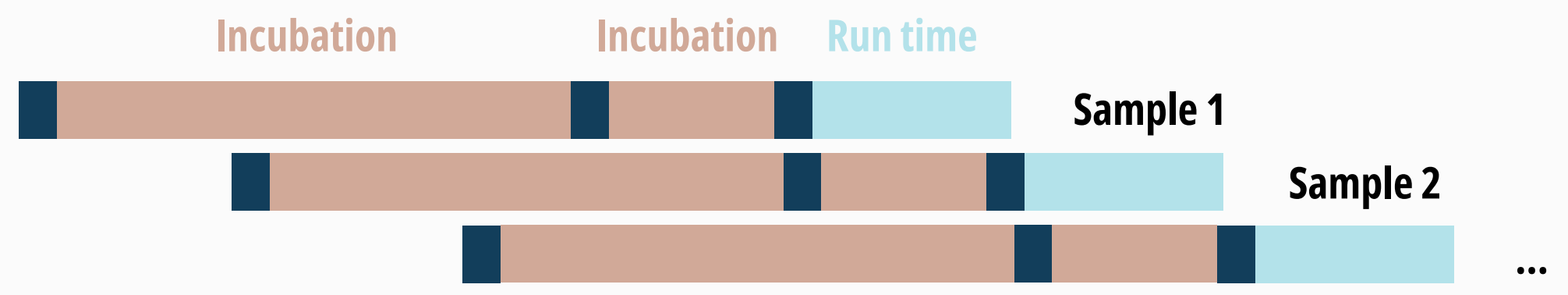
Fiehn, O. (2016). Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. Current Protocols in Molecular Biology, 114, 30.4.1-30.4.32. https://doi.org/10.1002/0471142727.mb3004s114

Automated Sample Prep

The workflow was developed to minimize hardware complexity and maximize accessibility. The complete two-step protocol requires only a standard PAL System configuration comprising a tray holder (for 2 ml vials), a standard Wash Station, and a single heated Agitator maintained at a constant 37 °C. Importantly, the entire procedure (from MeOx to MSTFA derivatization and final GC injection) is executed utilizing **a single Liquid Syringe Tool, eliminating the need for a tool exchange and additional modules.** Because this method operates without tool changes, it is fully compatible with both the entry-level PAL RSI but also the more advanced RTC platforms.

Workflow and Overlapping

To overcome the throughput limitations of single-vial processing, the sample preparation was coordinated using the scheduling software Chronos. This overlapping architecture maximizes instrument utilization while ensuring the strict sequential timing required to minimize technical noise.



Conflict of Interest
S.K. and S.B. are employees of Tofwerk AG. M.L., S.W., A.M., and M.P. are employees of Bruker Daltonics. H.M.G., G.B., T.L., and O.F. are employees of CTC Analytics AG. Tofwerk/Bruker provided access to the ecTOF GC/MS, and CTC Analytics AG provided the PAL System hardware. This study was conducted independently; results reflect the authors' objective assessment.