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Confident 4D Annotation of Polar Metabolites Using Standardized Retention Times Combined with TIMS-HRMS Acquisition

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Introduction

High confidence annotation in non-targeted metabolomics is hindered by isomerism, sparse libraries, and chromatographic variability. We present a proof-of-concept workflow that anchors non-targeted discovery to standardized retention times (RT) from biocrates 3-hydrophenylhydrazine (3-NPH) derivatization kits and experimental collision cross section (CCS) values measured on a LC-TIMS-MS platform. The resulting 4D criteria—m/z, isotopic pattern, RT, CCS, and MS/MS—enable robust identification of known metabolites while extending coverage to unknowns via *in-silico* derivatization. We demonstrate the approach in murine brown adipocytes stimulated with norepinephrine across a time series, capturing metabolic remodeling relevant to the activation of thermogenic lipolysis.

Methods

Murine brown adipocytes were cultured, 3-NPH derivatized as described in detail by Anagho-Mattanovich *et al.* 2025 [1]. 20 µL of cell extracts were mixed with 20 µL of the NPH reagent and 20 µL of the EDC reagent and incubated at room temperature for 1 h. Following incubation, 40 µL of the BHT solution were added to quench the reaction. Samples were analyzed using the default UHPLC method from biocrates' MxQuant kit coupled to a Bruker timsMetabo (VIP HESI, negative mode, LC-TIMS-PASEF). 100 experimental CCS values were determined on a timsTOF Pro 2 and integrated into a 4D Target List combining exact mass, isotopic pattern, RT, CCS, and structures of key metabolites. MetaboScape 2026b was used to perform automatic mass/mobility calibration, 4D feature extraction, de-adducting, de-isotoping, and grouping of features. For discovery, MetaboScape's *in-silico* derivatization workflow expanded a structural library to all theoretical 3-NPH derivatives with predicted CCS and *in-silico* MS/MS. Time course samples (0, 4, 24 h after norepinephrine) were evaluated by PCA and targeted review of RT and CCS anchored annotations.

Results & Discussion

1) Creation of a Target List for 3-NPH derivatives enabling high confident Annotation based on reference RT and CCS values

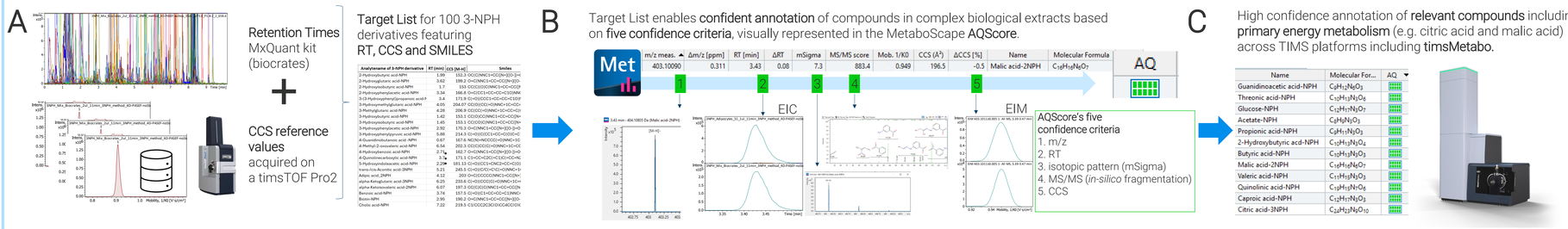

Fig. 1: A) Purified reference standards were used to generate TIMS-CCS values for 3-NPH derivatives on a timsTOF Pro2. Combining these CCS values and likely structures with established retention times from the Mx Quant method enabled construction of a 4D Target List covering 100 3-NPH–derivatized metabolites. B) This list supports confident compound annotation in complex biological extracts using five orthogonal confidence criteria (RT, CCS, accurate mass, isotopic pattern, MS/MS). C) The workflow enables high-confidence annotation of key primary metabolites involved in central carbon metabolism, including glycolysis and the TCA cycle.

2) Annotation of compounds lacking reference standards via MetaboScape's *in-silico* Derivatization workflow

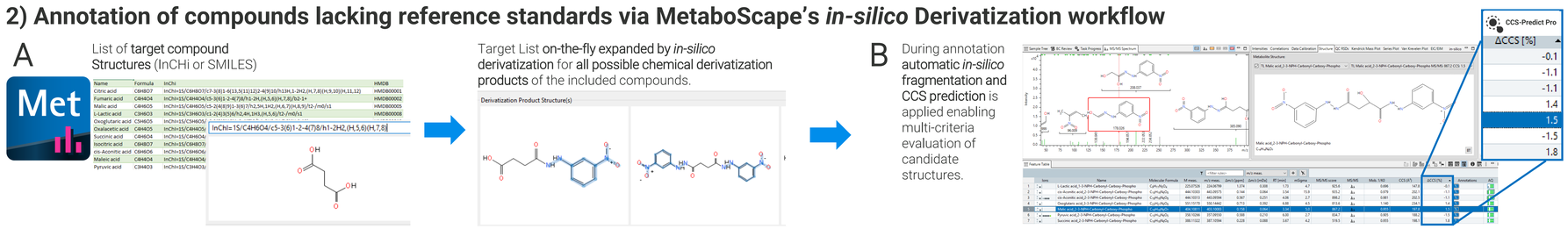

Fig. 2: Chemical derivatization improves RP-LC retention and ionization of polar metabolites but alters molecular composition, complicating annotation. MetaboScape´s *in-silico* derivatization workflow [2] bridges this gap by
A) computational transformation of known compound structures to their derivatized analogs
B) *in-silico* fragmentation and CCS prediction, allowing for multi-parameter scoring of candidate structures. This includes evaluation of m/z accuracy, isotopic fidelity, MS/MS spectral similarity, and CCS agreement.

3) Characterization of metabolic responses during thermogenic lipolysis in brown adipocytes

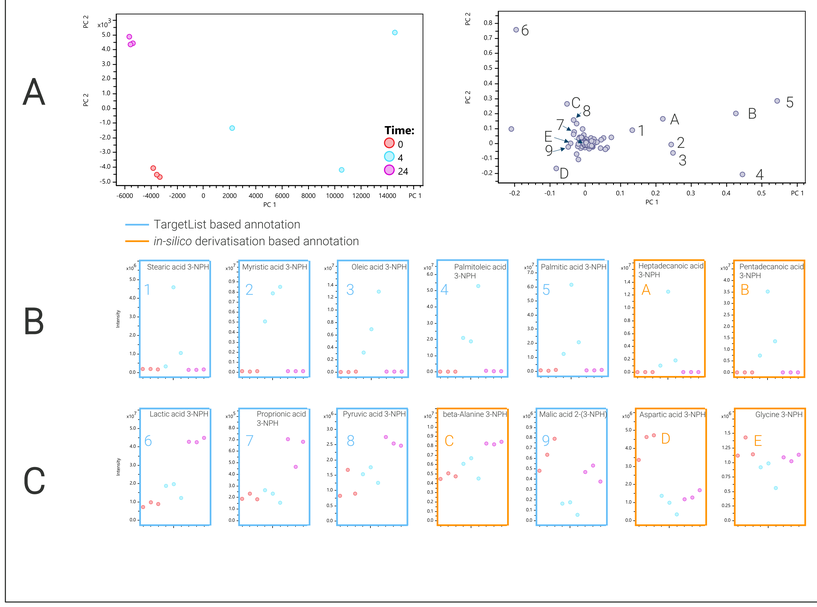

Fig. 3: The workflows from Fig. 1–2 were applied to 3-NPH-derivatized brown adipocyte extracts collected at 0, 4, and 24 h after norepinephrine stimulation.

A) The PCA loadings indicate that the **two workflows are complementary** (Target List based 1-9 and *in-silico* derivatization-based annotations A-E, respectively) and jointly enabled annotation of the metabolites driving separation along PC1 and PC2.

B) Intensity plots highlight transient increases in **medium-long-chain fatty acids** at 4 h (e.g. stearic, myristic and oleic acid), consistent with norepinephrine-driven lipolysis, followed by a return to baseline at 24 h.

C) **Organic and amino acids** showed time-dependent shifts: lactate, propionate, pyruvate, and β-alanine increased over time, while malate, aspartate, and glycine decreased at 4 h and normalized by 24 h—reflecting dynamic responses in primary metabolism relevant during activation of thermogenic lipolysis.

These results are consistent with previous findings reported in [3].

Literature
[1] Multi-omics analysis of thermogenic lipolysis in brown adipocytes https://www.sciencedirect.com/science/article/pii/S2589004225016438
[2] TIMS-enabled 4D-Metabolomics workflow for the automated analysis of derivatized analytes; Bruker SN-05
[3] Lipolysis – A highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores; https://www.sciencedirect.com/science/article/pii/S0163782710000524?via%3Dihub

Conclusion

- Confident 4D metabolite annotation of 100 3-NPH derivatized polar intermediates achieved by transferable RT anchors combined with orthogonal experimental CCS values.
- *In-silico* derivatization permits complementary annotation for compounds lacking reference standards
- Proof-of-concept study demonstrated that the metabolic state and trajectory of brown adipocytes responding to norepinephrine stimulation over time can be characterized.

Metabolomics by LC-TIMS-MS/MS