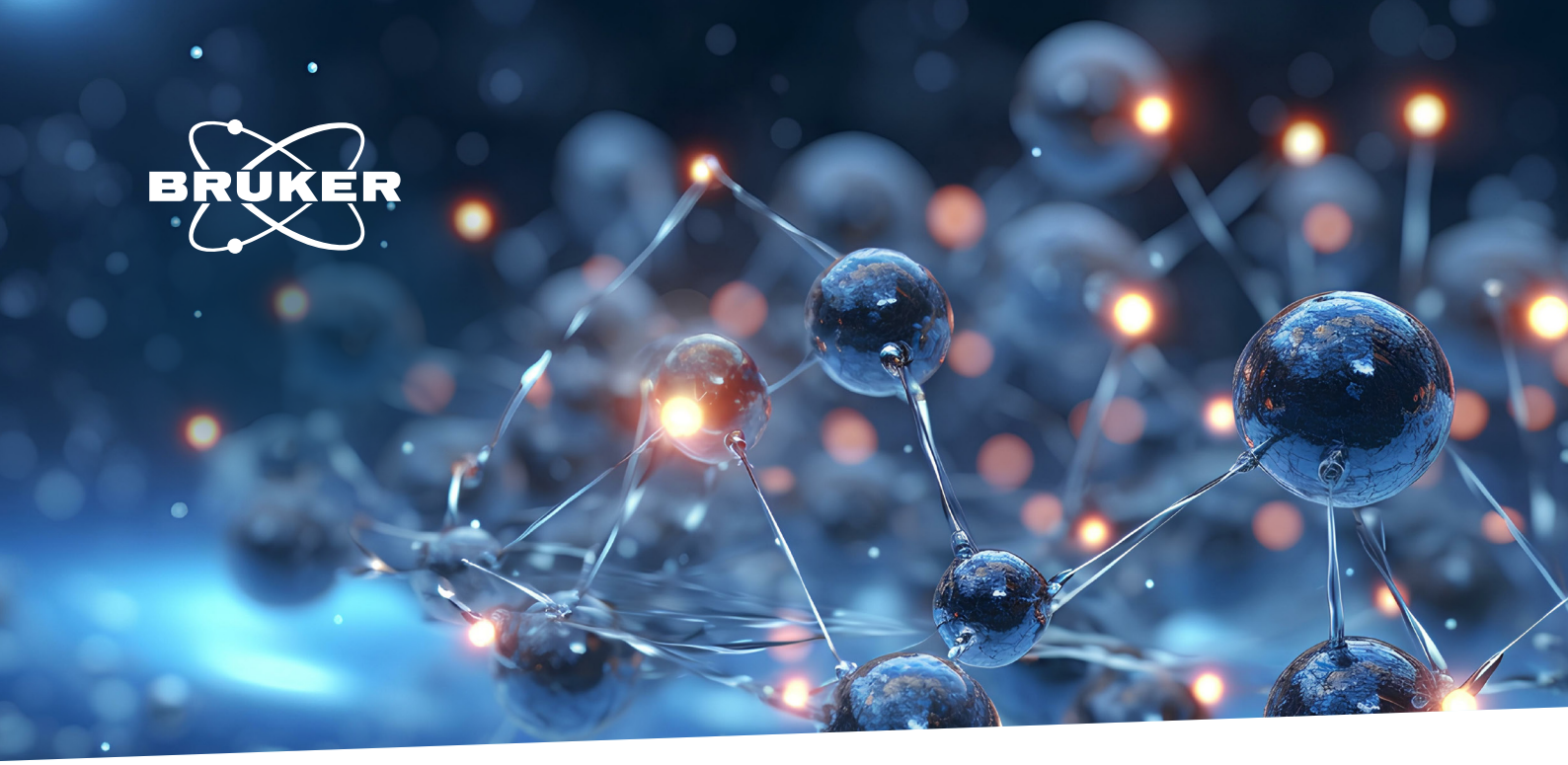

TIMS-enabled 4D-Metabolomics workflow for the automated analysis of derivatized analytes

Abstract

Challenge: Derivatization can address the poor LC retention and ionization performance of polar small molecules like TCA cycle analytes but the resulting data complexity can be prohibitive.

Solution: MetaboScape®'s novel *in-silico* derivatization workflow.

Chemical derivatization is an increasingly popular technique for improving LC retention and separation from matrix salts while also potentially improving sensitivity of detection [1-3]. However, derivatization also substantially increases the complexity of the raw data and its interpretation, potentially confounding accurate metabolite annotation.

This need for an efficient annotation of derivatized metabolites is addressed by MetaboScape's *in-silico* derivatization workflow that integrates automatic library structure derivatization with CCS prediction and *in-silico* fragmentation. Beginning with a list of target compound structures, the structures undergo *in-silico* derivatization according to the chosen mechanism. The result is an expanded Target List that includes all potential chemical derivatization products of the original compounds.

Keywords:
MetaboScape, Derivatization, *in-silico* derivatization, TIMS, CCS, CCS-Predict Pro

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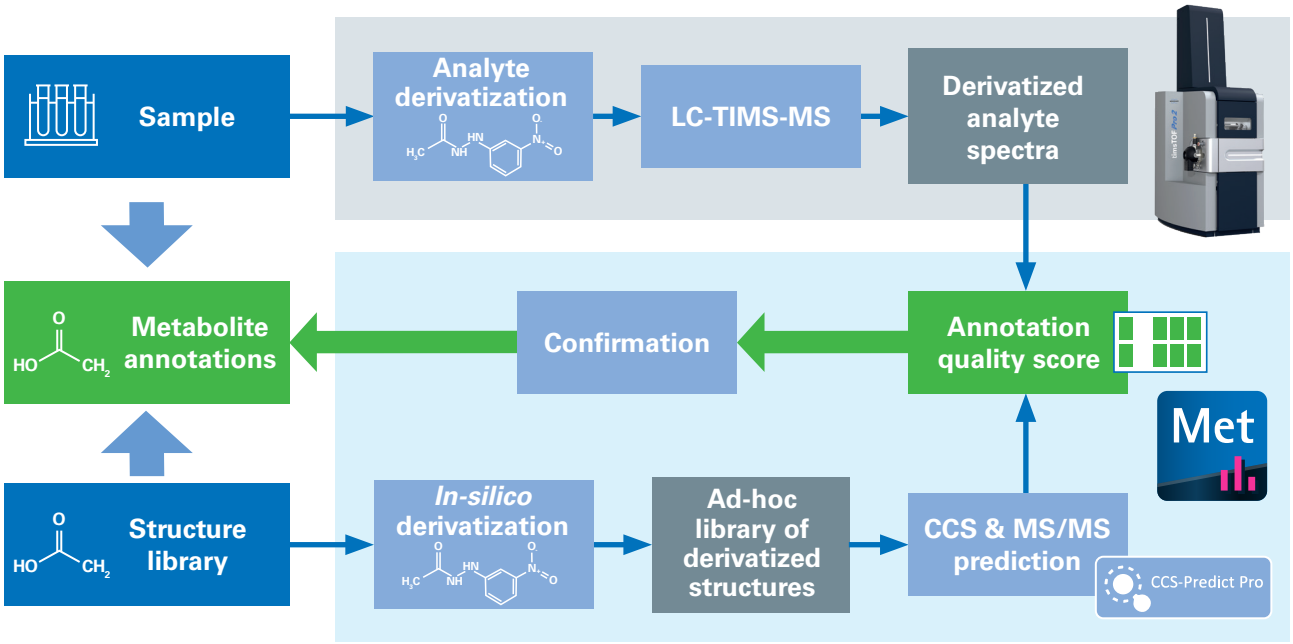
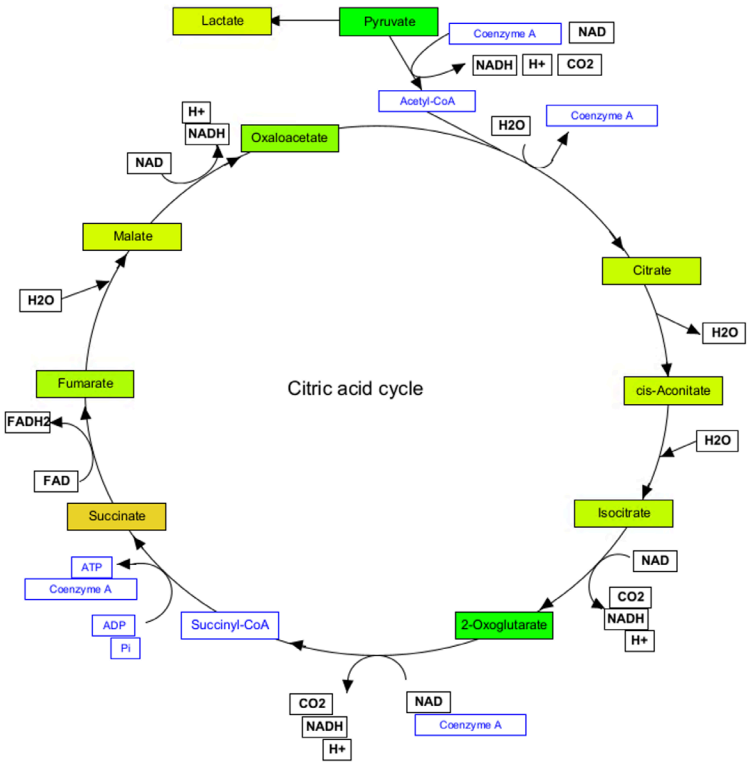
[2]Bruker Daltonics GmbH & Co. KG, Bremen, Germany.



The novel MetaboScape *in-silico* derivatization workflow allows researchers to:

- Perform non-targeted metabolomics on derivatized samples, just as you would do with native samples.
- Easily tailor *in-silico* derivatization parameters to align with the reagents employed for derivatization in their lab.
- Apply default setting for 3-Nitrophenylhyrdazin (3-NPH) derivatization.
- Validate annotations by comparing to derivatized standards: MetaboScape aids in automatically annotating and identifying unexpected byproducts such as multiply derivatized analytes.
- Explore alterations in metabolic pathways associated with polar compounds, including metabolites derived from the tricarboxylic acid (TCA) cycle, which are often under-reported when analyzing non-derivatized samples.



Introduction

In metabolomics research, achieving comprehensive metabolite measurement is challenging due to the complex composition of biological fluids and tissue extracts. Researchers often use multiple technologies and methods, resulting in coverage gaps and overlap. While state of the art, this approach is fundamentally inefficient. Consequently, researchers striving to measure the metabolome present in complex biological samples with greater depth for comprehensive coverage may, at some point, consider the use of chemical derivatization as a means to enhance conventional workflows or make them more efficient.

There are numerous benefits to doing so:

- Expanded analyte coverage: Chemical derivatization allows a broader range of analytes to be analyzed using fewer techniques and methods. This increases their relative value by maximizing the number of observable metabolites per analysis.
- Charge inversion: Derivatization can "mask" difficult-to-control charge sites or shift metabolite populations from mixed positive and negative ionization modes to favor a single mode. This enhances analytical specificity and simplifies data interpretation.
- Enhanced chromatographic methods: By making polar analytes amenable to highly efficient chromatographic methods (such as reversed-phase chromatography), researchers can reduce the need for specialized column chemistries (e.g., HILIC and ion pairing). This streamlines method development and maintenance.
- Improved ionization efficiency: Chemical derivatization significantly enhances the ionization efficiencies of analytes. This leads to better limits of detection and quantitation, especially crucial for studying low-abundance metabolites in limited samples.

In the scientific community, chemical derivatization is increasingly prevalent due to these valuable benefits [1-3].

However, there are also drawbacks. Chemical derivatization changes the fundamental composition and structure of the affected metabolites. For example, their chromatographic retention times are modified, invalidating in-house retention time libraries. Mass values are shifted (albeit in a calculable manner), further precluding the direct use of established databases in annotating LC-MS data from derivatized metabolites. Slow or incomplete derivatization reactions and multiple derivatization sites can lead to mixed derivatization products. In many cases, MS/MS fragmentation patterns are not practically conserved, often yielding only the loss of the derivatizing group, reducing or eliminating their value to metabolite annotation efforts. Together, these consequences create a disconnect between the observable characteristics of metabolites and their identity, invalidating the direct use of conventional identification resources (e.g. spectral matching databases).

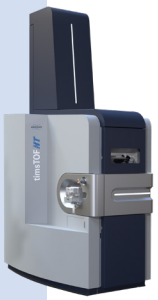
The novel *in-silico* derivatization workflow in MetaboScape bridges this gap, enabling the application of established annotation resources to the annotation of derivatized data in a streamlined and automated manner.

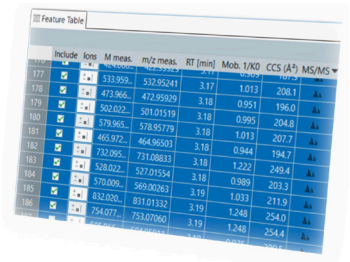
Perform non-targeted metabolomics on derivatized samples, just as you would do with non-derivatized samples.

Part 1: Workflow for automatic annotation of derivatized samples using *in-silico* derivatization

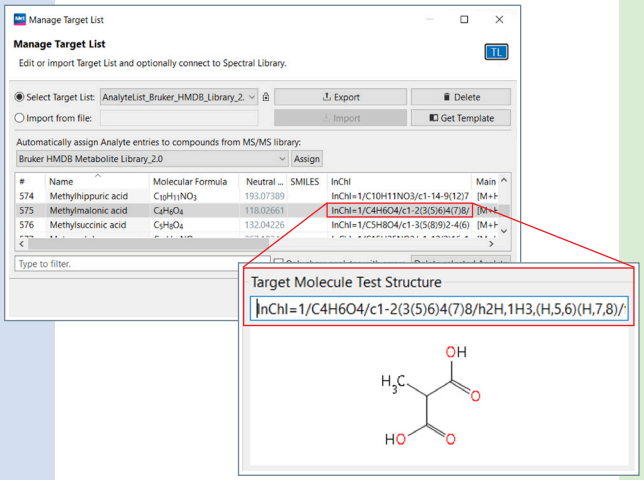
Workflow in MetaboScape

1 Data acquisition of derivatized samples: LC-TIMS-MS/MS.



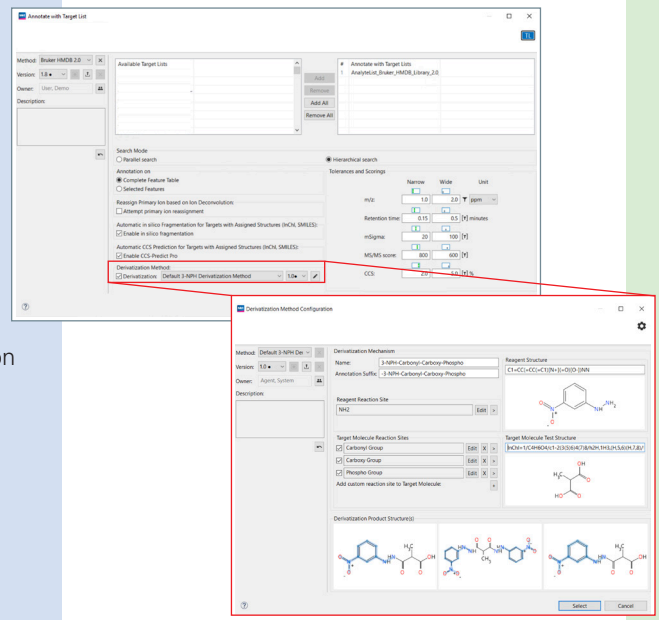


2 Untargeted processing of the LC-TIMS-PASEF® raw data with the T-ReX® 4D algorithm.



3 Selection of a list of target compound structures.

4 Selection of *in-silico* derivatization parameters based on the chosen derivatization mechanism.



Application example: Metabolomics of infant urine

1 Research aim: Compare plasma metabolic profiles of a healthy infant and child with a congenital metabolic disorder. Note: The presented work and workflow is for research use only.

2 Resulted in 2279 de-isotoped and de-adducted metabolic features detected in the plasma extracts.

3 The Bruker HMDB Metabolite Libary 2.0 (https://store.bruker.com/products/bruker-hmdb-metabolite-library-2-0) contains 754 target compounds and structural information (InChI) was added.

4 Default setting for 3-NPH chosen.

Workflow in MetaboScape

5 Optionally:

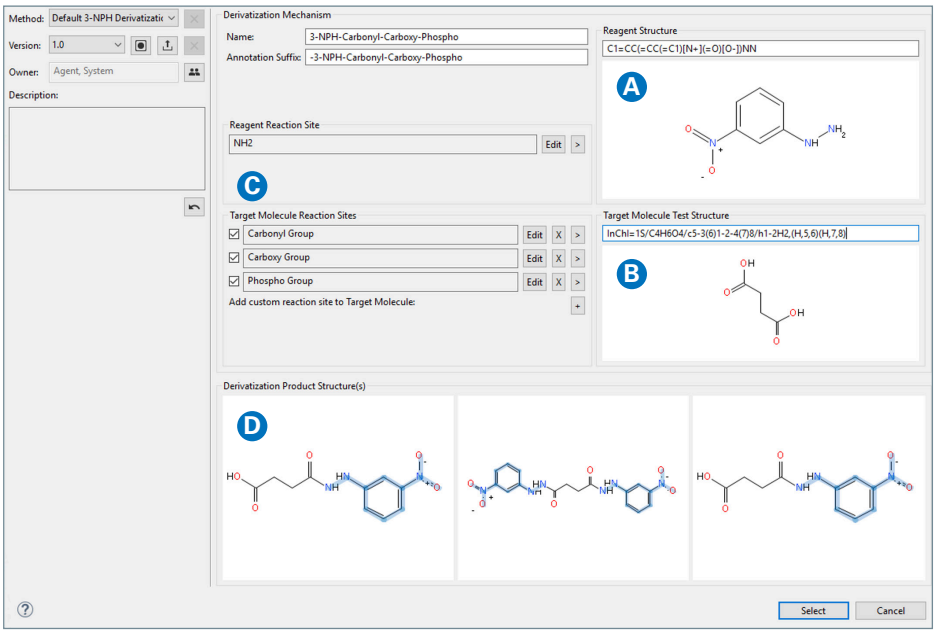
- Customized parameters coupled with real-time visualization
- Precise configuration of the derivatization mechanism with a chemistry-focused approach.

Application example

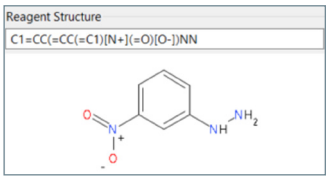
5 Investigation of default settings using InChI for Oxoglutaric acid:

All possible forms for carbonyl- and carboxy-derivatives are generated including single, double and triple derivatized compounds.

MetaboScape allows to easily tailor *in-silico* derivatization parameters for custom derivatization settings by …

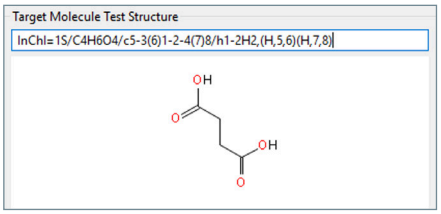


A



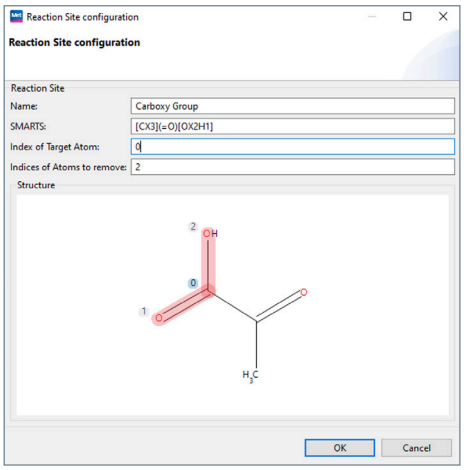
… allowing to define derivatization reagents encoded as InChI or SMILES.

B



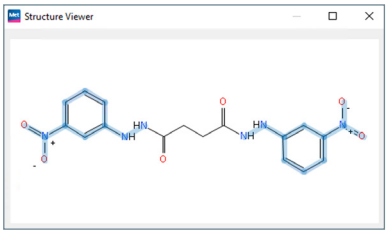
… allowing input of representative target structures to preview and refine *in-silico* derivatization parameters for compounds of user interest. In automatic annotation using a Target List, all available structures undergo *in-silico* derivatization based on the parameters defined here.

C



… allowing users to define functional groups within the derivatization reagent and target structure, with reactive atoms conveniently highlighted.

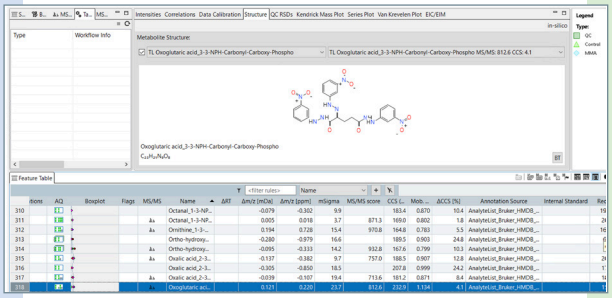
D



… presenting all possible derivatization products and highlighting the reagent residual in the generated products for the representative target structure.

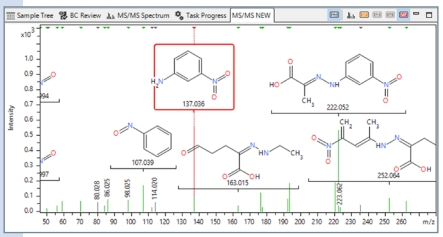
Workflow in MetaboScape

6A Automatic annotation with Target List on-the-fly expanded by *in-silico* derivatization for all possible chemical derivatization products of the included compounds.



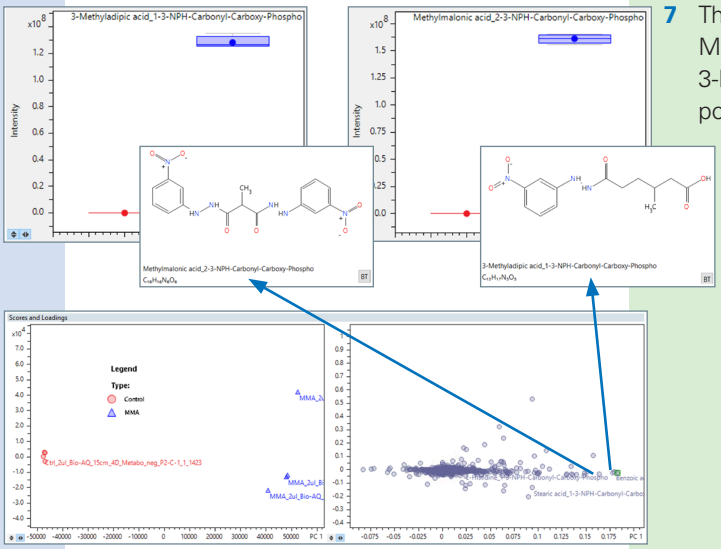
6B During annotation automatic *in-silico* fragmentation and CCS prediction is applied.

Note: CCS-Predict Pro was optimized for 3-NPH derivatives in negative mode.

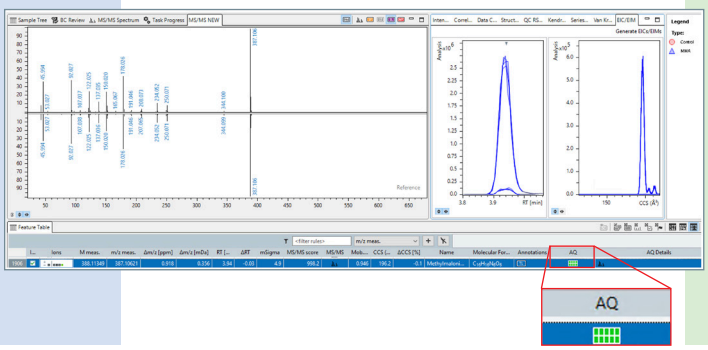


Mob. 1/K0	CCS (Å²)	ΔCCS [%]	Name
0.955	197.7	1.4	Malic acid_2-3-NPH-Carbonyl-Carboxy-Phospho
0.955	198.1	1.8	Succinic acid_2-3-NPH-Carbonyl-Carboxy-Phospho
0.693	147.2	-0.5	L-Lactic acid_1-3-NPH-Carbonyl-Carboxy-Phospho

7 Investigation of statistical plots for characteristic markers.



8 Confirmation of tentative ID by matching to reference standards



Application example

6A 391 features were annoated. Oxoglutaric acid was found to be derivatized with 3-NPH in the three functional groups.

6B Inspecting MS/MS spectra with assigned fragment structures allowed for a swift visual assessment of derivatized analytes. Automatic CCS prediction further increased confidence in the annotation of 3-NPH derivatized compounds.

7 This study indicates Methylmalonic acid and 3-Methyladipic acid as possible markers.

8 Annotation of Methylmalonic acid confirmed by matching:

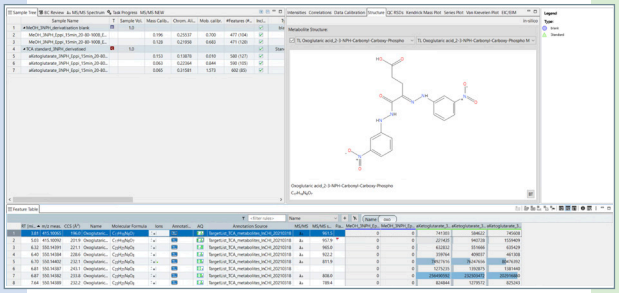
- Accurate mass
- Retention time
- Isotopic pattern
- MS/MS
- CCS

Validate annotations by comparing to derivatized standards: MetaboScape aids in automatically annotating and identifying unexpected side products.

Part 2: Workflow for building libraries for derivatized standard compounds

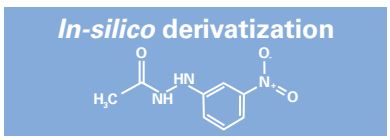
Workflow in MetaboScape

1 Untargeted processing of the LC-TIMS-PASEF reference standard and blank raw data with the T-ReX® 4D algorithm.

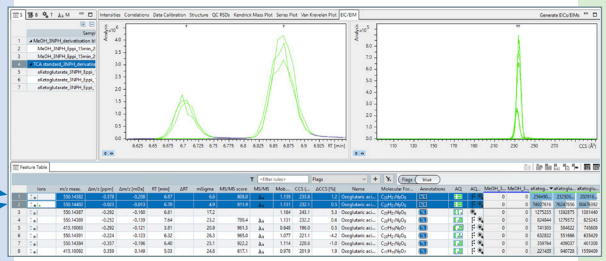


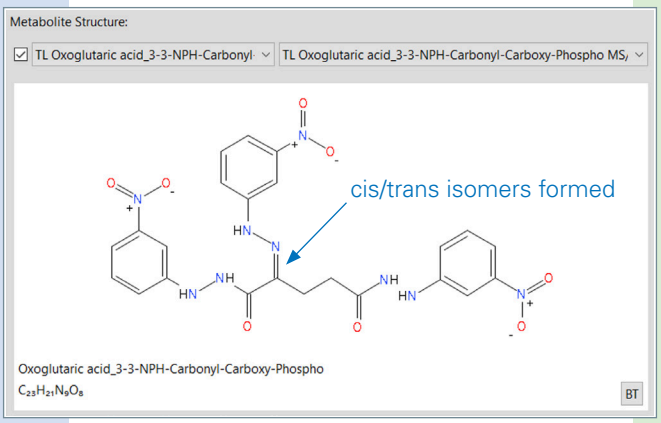
2 Untargeted processing of the LC-TIMS-PASEF reference standard and blank raw data with the T-ReX® 4D algorithm.

3 Annotation with *in-silico* derivatization parameters based on the chosen derivatization mechanism.



4 Curation of annotated features and selection of representative ions. Generation of a new Target List from the curated derivatized reference standards.





Application example: annotation of 3-NPH derivatives of Oxoglutaric acid standard

1 Three replicates of 3-NPH derivatized Oxoglutaric acid and two derivatization blanks were selected for processing.

2 Structure of non-derivatized Oxoglutaric acid was selected.

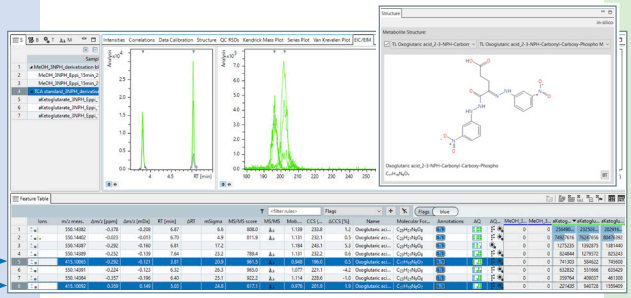
3 Default settings for 3-NPH chosen.

4 8 features annotated as Oxoglutaric acid derivatives. Two high abundant features (#1 and #2) belong to cis/trans isomers of triply derivatized Oxoglutaric acid. Four low abundant derivatizes assigned as likely isobaric contaminants in standard.

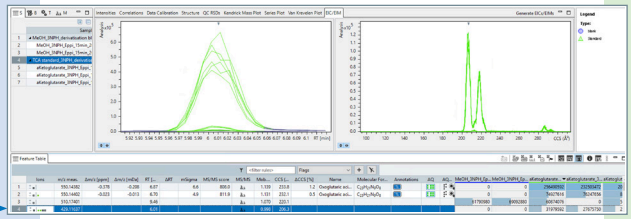
→ Add either most abundant or both derivatives to custom Target List.

Workflow in MetaboScape

4 Continued:



5 Tentative annotation of possible derivatization byproducts or unexpected *in-source* fragments related to target compound.



Application example

4 Two minor intensity peaks (#5 and #8) annotated as double derivatized Oxoglutaric acid.

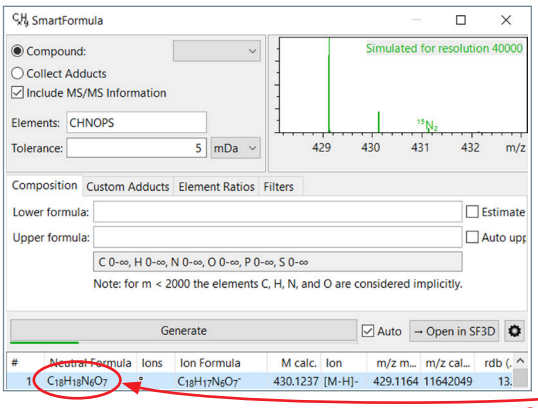
→ Optionally add to custom Target List or remove from further investigation as lower abundant.

5 Feature #4 not present in blank but LC and mobility peak shapes indicate this is a real peak deserving further investigation.

→ Tentative annotation as methylated Oxoglutaric acid (see below).

A

Formula for unknown feature #4:

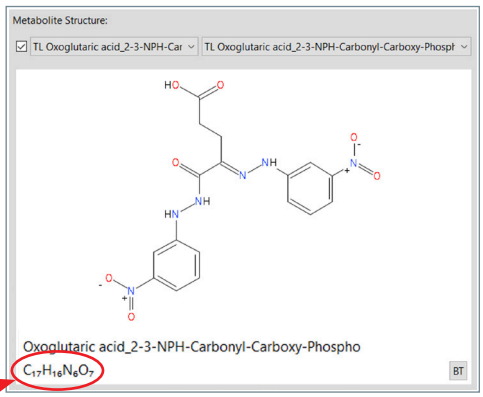


SmartFormula generation reveals $C_{18}H_{18}N_6O_7$ as most likely neutral formula for the feature #4.

$-CH_2$ difference

B

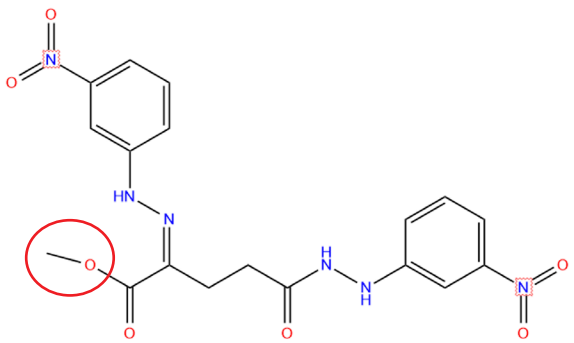
Formula similar to double derivatized Oxoglutaric acid



The formula of the unknown, $C_{18}H_{18}N_6O_7$, is similar to the the formula of double derivatized Oxoglutaric acid: $C_{17}H_{16}N_6O_7$ → a difference of CH_2.

C

Annotation as methylated Oxoglutaric acid.



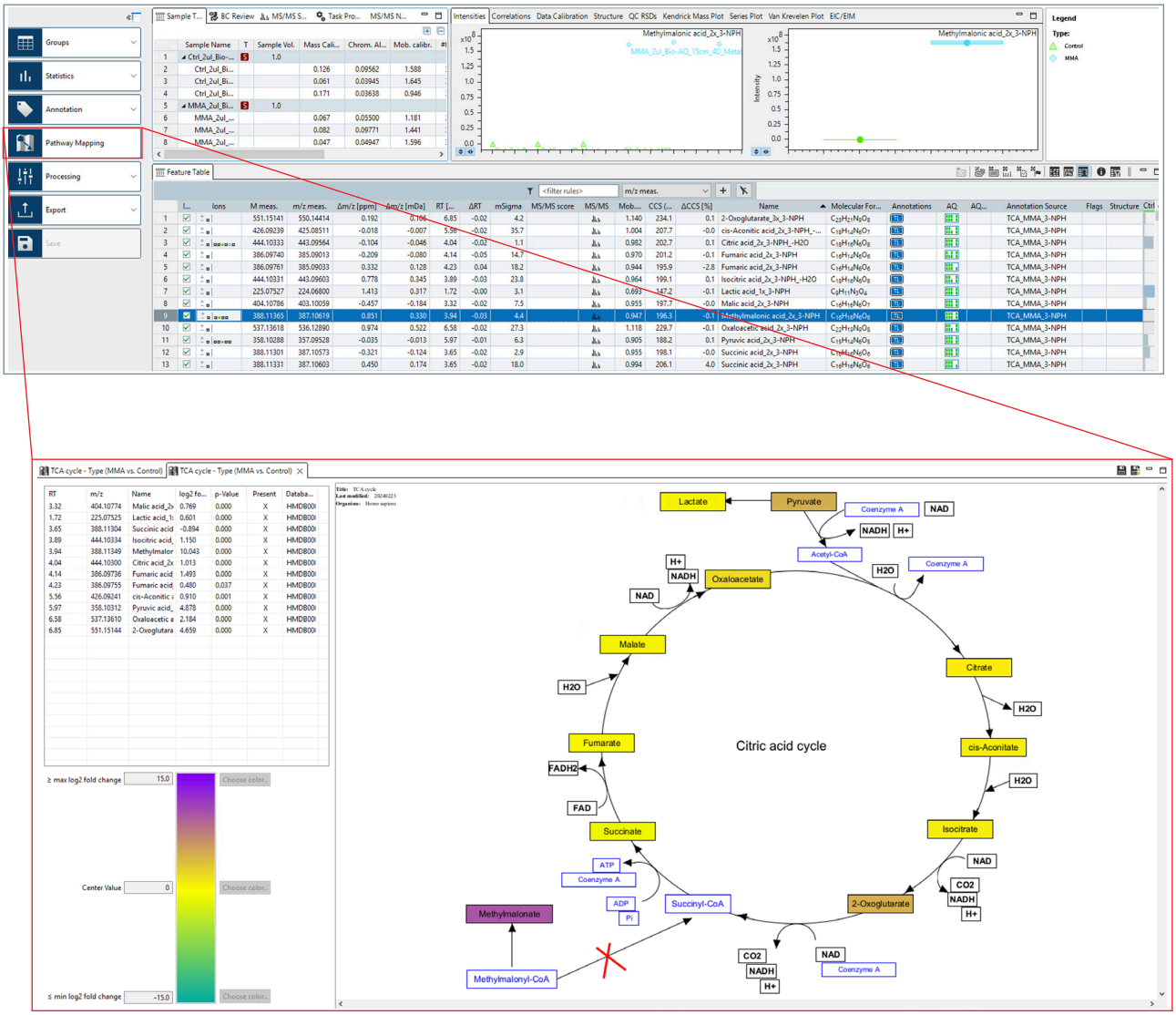
The difference of CH_2 can be explained by a methylation of one of the carboxy groups: -COOH is modified to $-COOCH_3$

The formula fits to methylated and doubly 3-NPH derivatized Oxoglutaric acid.

The annotation can be explained as the Oxoglutaric acid was dissolved in methanol and stored for a while before derivatization. This likely resulted in a methylation of the carboxy group.

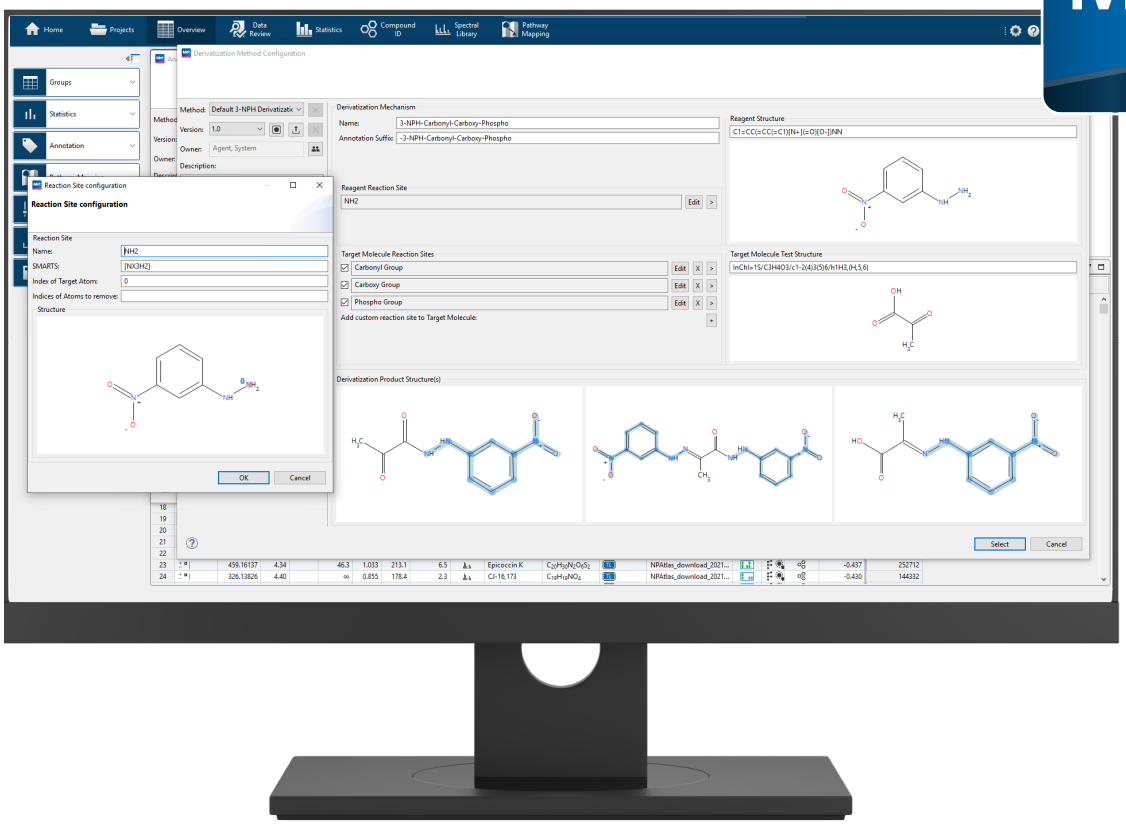
Improve coverage of key metabolic pathways, including metabolites derived from the tricarboxylic acid (TCA) cycle, which are often overlooked when analyzing non-derivatized samples.

Part 3: Unlocking Biological Insights: Pathway Mapping in MetaboScape



Methylmalonic acid upregulated in the sample from the child with Methylmalonic acidemia vs. control and minor changes in TCA cycle analytes seen.

MetaboScape® – The Metabolomics and Lipidomics Command Centre



» Novel *in-silico* derivatization workflow

- Comprehensive Metabolomics and Lipidomics solution
- CCS-enabled processing and annotation workflows
- CCS-Predict Pro to match CCS for structure candidates
- Interactive statistical tools for explorative data analysis
- Seamless integration with SCiLS™ Lab for SpatialOMx®
- Spectral Libraries and *in-silico* fragmentation embedded

Prof. Nils J. Færgeman

Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

"MetaboScape's *in-silico* derivatization workflow allows my team to perform non-targeted metabolomics on 3-NPH derivatized samples, just as we do with non-derivatized samples.

The workflow automatically annotates complex derivatized samples by generating all possible derivatized structures from non-derivatized compound libraries. Validation occurs by comparing to derivatized standards, and MetaboScape assists in automatic annotation and identifying unexpected side products. Thus, MetaboScape's innovative approach enables us to investigate alterations in metabolic pathways for polar compounds, including TCA cycle metabolites that are often imperceptible in non-derivatized samples."

References

[1] https//doi.org/10.1038/s41467-018-07019-x

[2] https://doi.org/10.1016/j.jchromb.2023.123719

[3] https://doi.org/10.1021/acs.analchem.0c04686

[4] https://doi.org/10.1038/s42255-022-00720-8

www.bruker.com/metaboscape

Link to Fluxomics Poster Note 65

Note and Disclaimer: "Clinical samples were provided in accordance to local ethics: Samples and data were used for method development and QA purposes only in this study."

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