Application Note # ET-22

Metabolic Profiling of a *Corynebacterium glutamicum* Δ*prpD2* by GC-APCI High Resolution Q-TOF Analysis

Hyphenating gas chromatography with high resolution accurate mass TOF-MS technology via a GC-APCI interface enables the identification of unknown compounds. Metabolic profiling of *Corynebacterium glutamicum* Δ*prpD*2 extracts of cells grown on glucose, before and after a propionate pulse in the exponential growth phase, were analyzed by GC-APCI-micrOTOF-Q II. Principle Component Analysis revealed several compounds elevated in the bacterial extracts following an addition of propionate. Identification of 2-methylcitric acid and alanine using accurate mass and isotopic pattern information in MS and MS/MS spectra provided a proof of concept for the identification of target compounds using state of the art MS technology.

Introduction

Metabolomics aims at a comprehensive qualitative and quantitative analysis of all small molecules present in a cell, a tissue or an organism under defined conditions. Due to the wide dynamic range of compound polarities a single analytical technique is unable to achieve this task. Therefore, different analytical systems like LC-MS, GC-MS and NMR are currently used in metabolomics and metabolite profiling research. Metabolomics studies based on Gas chromatography – Mass spectrometry (GC-MS) are well established and typically employ electron impact (EI) ionisation. Target compounds can be identified by comparison to commercial or public databases. Unfortunately, many possible biomarkers detected in metabolic profiling experiments cannot be identified due to the lack of reference spectra for a majority of biologically relevant compounds. Therefore, many possible biomarkers remain "unknowns" up till now.

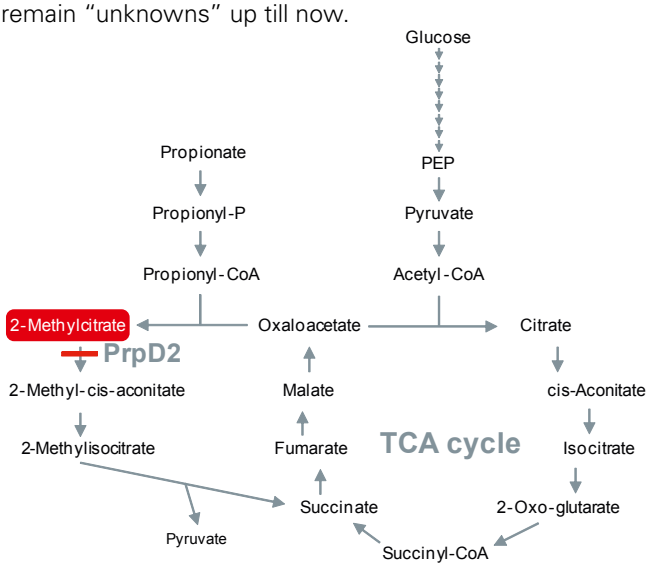


Figure 1: Metabolic Pathways of the tricarboxylic acid (TCA) and methylcitric acid cycles highlighting the step catalyzed by *PrpD*2, which is inactivated in the *C. glutamicum prpD*2 deletion mutant.

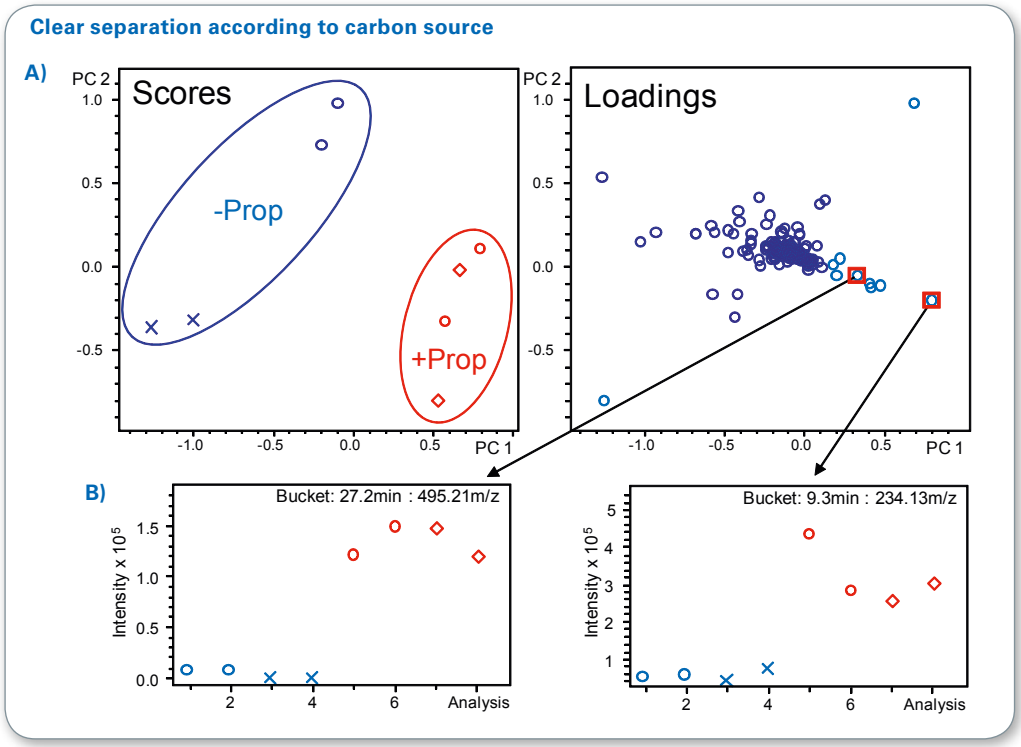


Figure 2:
A: PCA scores and loading plot (Explained Variance PC1 vs. PC2: 44.4% + 29.9%) of *C. glutamicum prpD2* mutant strains grown on glucose before and after a propionate pulse. The scores plot revealed a clustering of samples according to the carbon sources used for cultivation.
B: Bucket statistics plots for two selected loadings mainly contributing to the grouping of samples observed in A.

Hyphenating GC with micrOTOF-Q II™ (ESI-Q-TOF) technology by atmospheric pressure chemical ionisation (APCI) permits the characterization of compounds which can not easily be identified by classical GC-EI-MS. The micrOTOF series, which is widely used in combination with liquid chromatography, can be coupled to a GC by Bruker´s dedicated GC-APCI source without difficulty. A combination of GC with a soft atmospheric pressure ionisation (APCI) preserves the molecular ion and enables a sum formula generation using accurate mass information. SmartFormula3D™ utilizes exact mass and isotopic pattern information in high resolution MS and MS/MS spectra for sum formula generation of target compounds [1]. Since MS and MS/MS spectra can be automatically acquired in the same GC-micrOTOF-Q II run, this hyphenation enables an unambiguous structural elucidation of previously unidentified compounds.

Corynebacterium glutamicum, a gram positive, non-toxic bacterium, is used in the industrial production of amino acids like lysine and glutamate. *C. glutamicum* can be grown on different carbon sources. Glucose is metabolized via glycolysis and tricarboxylic acid cycle (TCA) whereas propionate is catabolized through the methylcitric acid pathway (see Fig. 1 – adapted from [2]). An involvement of the *prpD2* gene, encoding 2-methylcitrate dehydratase, in the degradation of propionate has previously been shown by Plassmeier et. al [2] based on GC-EI-MS measurements. Deletion mutant strains of *prpD2* grown on propionate revealed an accumulation of 2-methylcitrate.

Here, we used a GC-APCI-MS based metabolic profiling approach to analyse metabolite extracts of a *prpD2* mutant *C. glutamicum* strain grown on glucose, with a propionate pulse in the exponential growth phase. The benefits of high resolution MS data in combination with GC separations to facilitate structure elucidation will be demonstrated.

Experimentals

C. glutamicum prpD2 strains were cultivated and harvested as described in [2]. Briefly, two biological replicates were grown on glucose, with a propionate pulse in the exponential growth phase at an OD_{600} of 3. Two technical replicates of each culture were harvested by centrifugation before and 1 h after the propionate pulse. Dried methanolic metabolite extracts and reference standards were derivatized by methoxymation and trimethylsilylation. 25 µl of methoxylamine hydrochloride (20 mg / ml pyridine) were added per mg dried extract and incubated at 37°C for 90 min. Subsequently, silylation was performed for 30 min at 37°C by adding 25 µl MSTFA / mg dried extract. 1 µl of each derivatized sample was analyzed by GC-APCI-MS using a HP-5MS column (30 m x 0.25 mm i.d.; 0.25 µm). The injector temperature was set to 250°C and helium carrier gas flow kept constant at 1 ml / min. After constant heating of the GC for 3 min at 80°C the temperature was raised with 5°C / min to 325°C. A micrOTOF-Q II was interfaced to the GC utilizing a Bruker GC-APCI source. Data was acquired in positive ionization mode from 85 – 750 m/z at 4 spectra per second.

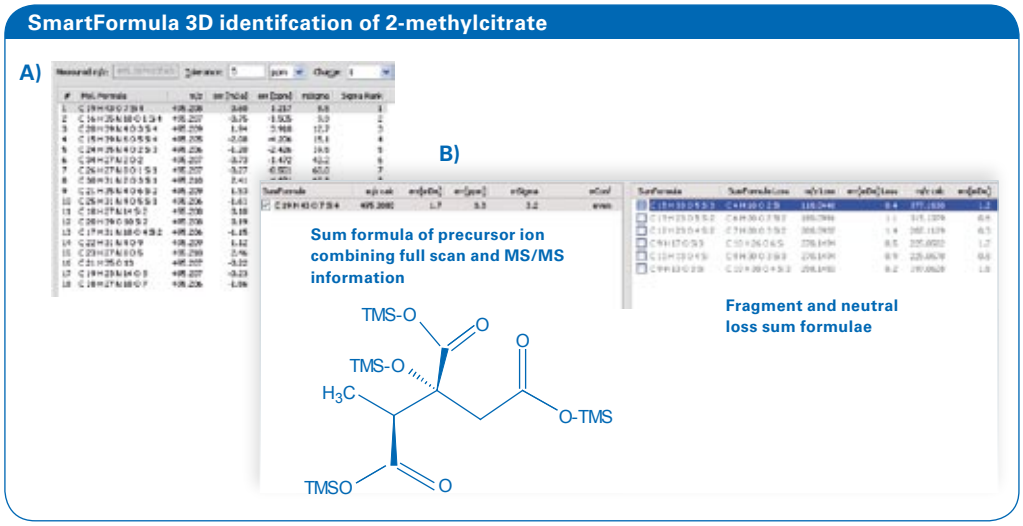


Figure 3: Identification of compound 27.2min:495.21m/z (see Fig.2 B): Sum Formula generation by SmartFormula (A) and SmartFormula3D (B). Combining accurate mass and isotopic pattern information in MS and MS/MS spectra one candidate formula was generated which is in accordance to 2-methycirate (inset).

The datasets were recalibrated and all relevant features were extracted using the Find Molecular Features algorithm. Feature intensities were normalized to the intensity of the internal standard ribitol. Principal Component Analysis (PCA) of the pre-processed data as well as sum formula generation by SmartFormula were performed using ProfileAnalysis™ 2.0.

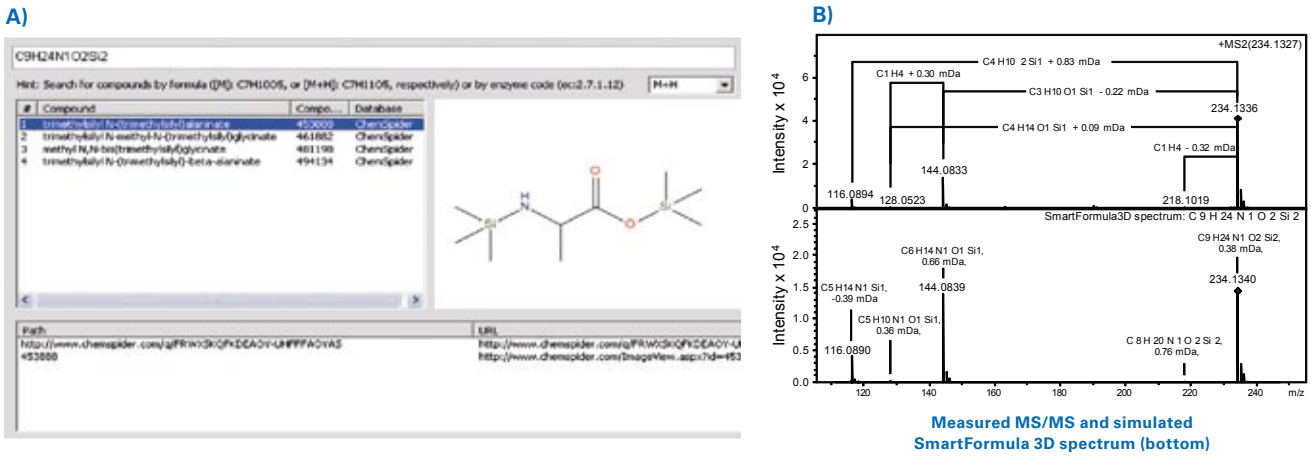
Results & Discussion

PCA differentiates extracts of *C. glutamicum* Δ*prpD*2 cells grown on glucose before and after a propionate pulse. High resolution MS data acquisition by coupling of gas chromatography via an APCI source to a micrOTOF-Q II enabled to use the same data (pre-)processing methods established for Bruker LC-TOF-MS data. Following recalibration, all relevant features were extracted from the raw data by applying the "Find Molecular Features" peak detection algorithm. Extracted features, which were automatically cleared from background noise, were subjected to Principle Component Analysis (PCA). The PCA scores plot (Fig.2 A) revealed a clear separation according to the carbon sources used for cultivating the *C. glutamicum* Δ*prpD*2 cells. The loadings plot highlighted compounds which were mainly responsible for the differentiation between cells before and after the propionate pulse. Peak intensities for all samples for two selected compounds are displayed as bucket statistics in Fig. 2 B. Both show a higher abundance in cells metabolizing propionate.

Compounds differentiating sample groups can be identified based on high resolution accurate mass MS and MS/MS data. Utilizing exact mass and isotopic pattern information SmartFormula generated 18 possible sum formulae for compound 27.2min: 495.21m/z (Fig.3 A). $C_{19}H_{43}O_7Si_4$ was ranked as first sum formulae for $[M+H]^+$ according to the SigmaRank which sorts the candidates according to the goodness of the isotopic fit between measured and theoretical spectrum. By combining accurate mass and isotopic pattern information of MS and MS/MS spectra sum formulae suggestions for this compound could be reduced to a single hit (Fig.3 B). The precursor sum formula $C_{19}H_{43}O_7Si_4$ as well as the calculated sum formulae for fragments and neutral losses are in accordance to trimethylsilylated 2-methycitrate (Fig. 3B inset). This structural hypothesis could be confirmed by comparison to the reference standard (data not shown). An accumulation of 2-methylcitrate in *C. glutamicum* cells metabolizing propionate could be expected in cells lacking 2-methylcitrate dehydratase, catalyzing the conversion of 2-methylcitrate to 2-methyl-cis-aconitate (see Fig.1). A higher concentration for this compound in *prpD*2 mutant *C. glutamicum* grown on propionate was also observed by Plassmeier et al. based on "classical low resolution" GC-EI-(Iontrap)-MS measurements [2].

Compound 9.3min: 234.13m/z constituted a further loading mainly responsible for the separation of samples observed in the PCA scores plot (Fig. 2 A). SmartFormula sum formula generation suggested two possible candidates for this compound. These hits could be restricted to the sum formula $C_9H_{24}NO_2Si_2$ by combining MS and MS/MS information using SmartFormula3D (data not shown). Querying this sum formula in public databases using the CompoundCrawler™ returned trimethylsilyl-N-(trimethylsilyl) alaninate (i.e. TMS derivatized alanine) as a likely structure (Fig.4 A). This could be confirmed by comparison to the reference standard. The top part of Fig. 4 B displays the MS/MS spectrum of derivatized alanine with assigned sum formulae for neutral losses. The automatically calculated SmartFormula3D spectrum (Fig.4 B bottom) includes annotations for the observed fragment ions demonstrating sub-mDa mass deviations in MS/MS spectra acquired by the micrOTOF-Q II.

Identification of a compound by CompoundCrawler database query



Measured MS/MS and simulated SmartFormula 3D spectrum (bottom)

Figure 4: Compound 9.3min:234.13m/z (see Fig.2 B) – Identification as alanine – 2TMS. SmartFormula3D provided one sum formula ($C_9H_{24}N_1O_2Si_2$) for the target compound. Subsequent query in public databases highlighted trimethylsilyl N-(trimethylsilyl)alaninate (TMS derivatised alanine) as likely structure for this sum formula (A). Neutral loss and fragment sum formulae are in accordance to this structural hypothesis (B) which could be confirmed by comparison to the reference standard (not shown).

Conclusion

Gas chromatography coupled to mass spectrometry is a well established technique in metabolomics research. Nevertheless, identification of potential biomarkers remains a serious challenge for all compounds which can not by identified by comparison to standardized 70eV EI database spectra. Coupling GC to high resolution accurate mass MS technology, enables the identification of target compounds which remained unknown up till now. A GC-APCI interface preserves the precursor ion information and enables sum formula generation based on accurate mass and isotopic pattern information. Additionally, MS/MS data acquired by fragmentation of precursor ions, within the collision cell of the micrOTOF-Q II instrument, extends the capabilities for structural elucidation. Mass accuracy, resolution and isotopic fidelity are independent of ESI-TOF acquisition speed. Therefore, the micrOTOF instrument series is perfectly suited for a coupling to gas chromatography which typically delivers narrow peak width requiring fast MS scan speeds. Since changing from LC to GC operation (and vice versa) is a matter of a few easy steps, the micrOTOF series instruments are the perfect choice for conducting comprehensive metabolite profiling research.

Keywords
Metabolomics
Metabolite profiling
GC-APCI
Principle Component Analysis
Small molecule identification

Instrumentation & Software
micrOTOF-Q II
ProfileAnalysis 2.0
SmartFormula3D

For research use only. Not for use in diagnostic procedures.

References

[1] Bruker Daltonics; Technical Note #TN-23: Certainty in Small Molecule Identification by Applying SmartFormula3D on a UHR-TOF Mass Spectrometer

[2] J. Plassmeier, A. Barsch, M. Persicke, K. Niehaus, J. Kalinowski, J. Biotechnol. 130 (2007) 354-363

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