MRMS aXelerate for targeted metabolomics profiling of myxobacterial extracts

Myxobacterial secondary metabolomics deals with highly complex samples to uncover novel natural products and is therefore one of the many analytical fields where increased sensitivity and resolution are crucial for a comprehensive analysis [1,2].

Introduction

Many related scientific questions can be answered with a combination of UHPLC and UHR-Q-TOF analysis, representing the current state-of-the-art technique for the analysis of myxobacterial extracts [3]. However, genomic analysis of myxobacterial strains has shown that their enormous potential for the biosynthesis of secondary metabolites is far from exploited [4]. One plausible reason for the current discrepancy between genome encoded potential and analytically detected chemistry is that growing myxobacteria in liquid culture fails to mimic all facets of their natural growth behavior as soil-dwelling bacteria. Another often overlooked limitation is related to the analytical methods applied: Combining high resolution mass spectrometry with liquid chromatography limits the amount

Keywords: Myxobacteria, Targeted Metabolomics, Untargeted Metabolomics, Metabolic Profiling, Principle Component Analysis, FIA-MRMS

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of compounds detectable, as not every chromatographic method is equally effective for all types of metabolites [5]. Therefore, there is demand for improving analytical methods and especially for workflows compatible with high-throughput screening to compare extracts from different cultivation scenarios.

Here we present a workflow coined MRMS aXelerate: a flow injection analysis - magnetic resonance mass spectrometry (FIA-MRMS) based analysis of myxobacterial metabolites, enabling the rapid and robust identification of known compounds in a non-targeted metabolomics workflow. Challenges regarding throughput, accuracy, sensitivity and reproducibility are discussed. Furthermore, statistical evaluation of known myxobacterial secondary metabolites in a non-targeted metabolomics workflow is implemented to highlight the differences between myxobacterial cultivation on plate and in liquid culture.

Experimental

The myxobacterial strain *Myxococcus xanthus* DK1622 was cultivated in liquid culture as well as on agar plates in biological triplicates, respectively. Cells from the agar plates were scraped off and the liquid cultures were centrifuged to separate cells and supernatant. The cells were lyophilized prior to extraction with methanol. Blank samples were generated by lyophilizing and extracting the cultivation medium. All samples were diluted 1:200 prior to measurement and two technical replicates were generated. ESI-MS measurements were performed using a scimaX MRMS system (Bruker Daltonics) in positive ion mode and quadrupolar phase detection. 28 single scans were added in 1.5 minutes for the final mass spectrum. The mass range for detection was set to *m/z* 107-3000 with a data size of 8 MW resulting in a 2 s transient and a mass resolution of 650,000 at *m/z* 400. The ion accumulation time was set to 20 ms. FIA was performed with a Bruker Elute UHPLC (Bruker Daltonics). Identification of known target compounds was carried out with MetaboScape 4.0 (Bruker Daltonics), by matching ultra-high resolution *m/z* values and evaluating isotopic patterns. Statistical interpretation using PCA including all data preprocessing was performed with MetaboScape. The minimal intensity threshold for feature detection was set to 7 x 10^5 with a maximum charge state of 3. The minimal group size for creating batch features was set to 5. As standardized reference method the extracts were measured in 1:10 methanolic dilution on an Ultimate 3000™ RSLC (Dionex) system coupled to a maXis 4G UHR-Q-TOF-MS (Bruker Daltonics). Separation was achieved on a RP C18 column (100 x 2.1 mm, 1.7 µm particle size) with a 5-95% gradient of acetonitrile (B) in water (A) each spiked with 0.1% formic acid. The gradient with a total runtime of 21 min was initiated by a 0.5 min isocratic step at 5% B and ended with a 2 min step at 95% B before returning and

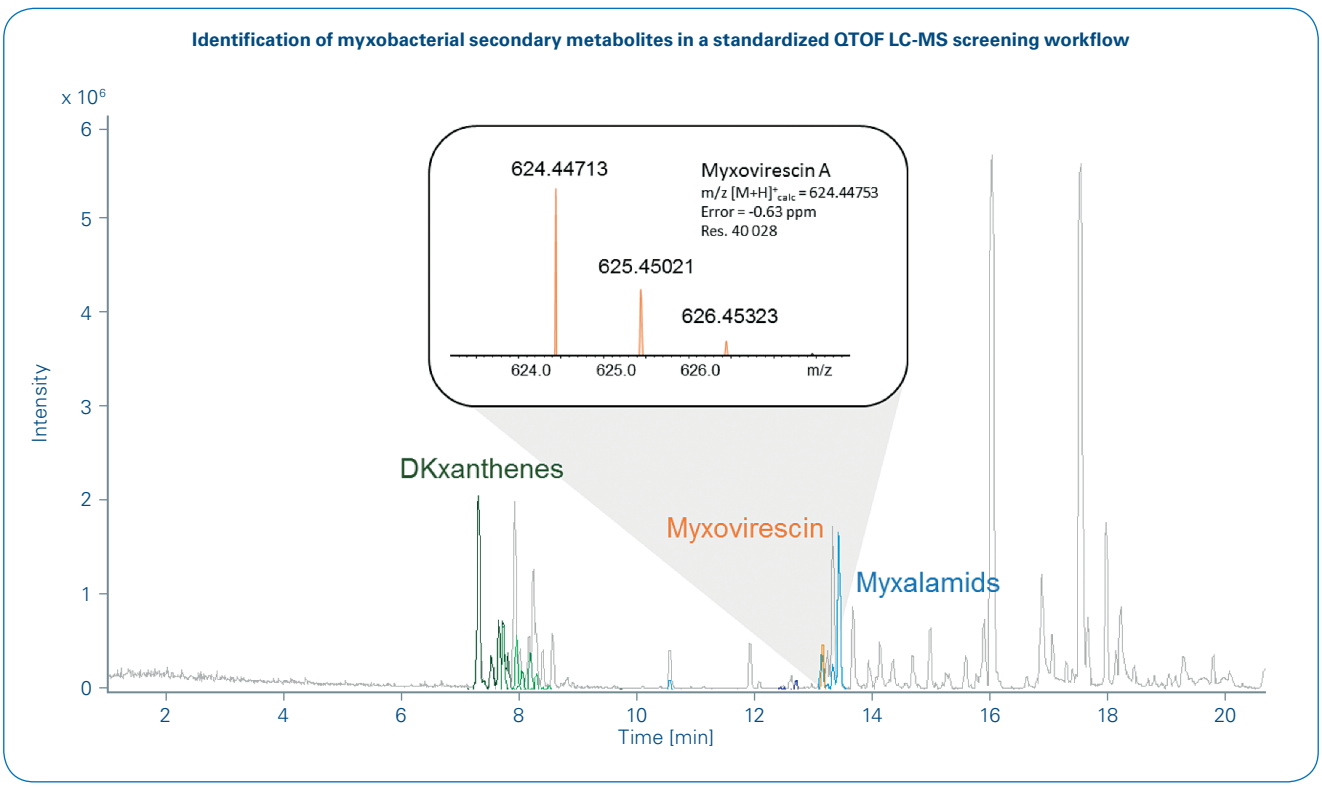

Figure 1A: Base peak chromatogram (BPC) of a M. xanthus *DK1622 crude extract analyzed by LC-QTOF-MS with extraction ion chromatogram (EIC) traces of known secondary metabolites and zoomed in Myxovirescin A mass spectrum.*

equilibrating under initial conditions. Mass spectra were acquired in centroid mode with a scan range from *m/z* 150-2500 at 2 Hz scan rate.

Results

Throughput and mass accuracy

In a standard LC-MS screening workflow, myxobacterial secondary metabolites can reliably be identified by matching retention times, high resolution masses and isotope patterns (Figure 1A) [6]. While retention times offer a second dimension for increasing confidence in annotations it comes at the price of long measurement times. The typical method requires 21 minutes total runtime whereas FIA-MRMS measurements can be performed in 1.5 minutes [6]. This reduction in measurement time is especially interesting for high throughput screening. Even though the spectra generated in this manner come with increased complexity, myxobacterial metabolites can be assigned with mass accuracy below 1 ppm as demonstrated for Myxovirescin A in Figure 1B.

Sensitivity and resolution

When characterizing the secondary metabolome of a myxobacterial strain, sensitivity of the analytical method of choice plays a crucial role as metabolites that are only produced in very small amounts need to be detected in an extremely complex sample. FIA-MRMS works without diluting the sample in an eluent/gradient and it also avoids the introduction of biases due to the chosen chromatographic conditions. As presented in Figure 2, FIA-MRMS is an extremely sensitive method that enabled the detection of Myxovirescin H, which is member of the Myxovirescin family not detectable in the UHPLC-Q-TOF workflow. The high mass resolution of more than 400,000 at *m/z* 648 allowed differentiation of neighboring signals, and the assignment of the potassium adduct of Myxovirescin H with an error of only 0.15 ppm. The additional detection of the sodium adduct of Myxovirescin H increased the confidence of this annotation.

Data reduction

The high complexity of mass spectra generated from bacterial crude extracts requires data reduction if more than a few metabolites are analyzed. After processing and generation of a feature table in MetaboScape, all blank features were subtracted from the analysis resulting in approximately 55% data reduction. Blank subtraction is thus a key processing step for the analysis of myxobacterial extracts, because bacteria require particularly complex nutrition media to grow, resulting in a multiplicity of background signals interfering with metabolomics characterization. Annotation of the sample features with the Bruker Sumner MetaboBASE Plant Library, LMSD [7] and the Myxobase (in-house database [1]) enabled the annotation of about 14% of the filtered features in *M. xanthus* DK1622 extracts.

Targeted analysis of known metabolites in a non-targeted workflow using PCA

FIA-MRMS spectra can also be used to compare different cultivation systems in a non-targeted metabolomics workflow. Principal component analysis (PCA) was carried out with two different datasets generated by cultivating *M. xanthus* DK1622 in liquid cultures and on agar plates. The PCA results for the two cultivation systems are presented in Figure 4A, showing that the biological and technical replicates cluster close together, whereas blank, liquid and plate cultures show significant differences in the score plot. Figure 4B shows how PCA can help to identify a suitable cultivation system for known myxobacterial secondary metabolites when dealing with high numbers of annotations. Four different myxobacterial metabolites were analyzed in depth to exemplify the graphical representation of single metabolites with the aim to get an easy and fast overview of the production differences (see Figure 4B). DKxanthene-534 and Myxalamid A can be detected with similar intensity levels from the plate and from liquid cultures. On the contrary, Myxovirescin A shows better production in liquid culture and Cittilin A is enhanced on agar plates.

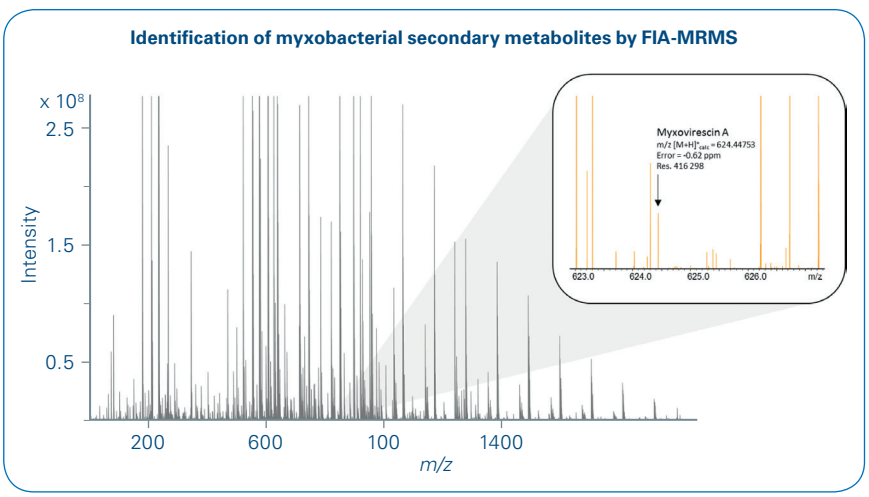

Figure 1B: Mass spectrum of a M. xanthus *DK1622 crude extract acquired by FIA-MRMS with zoomed spectrum of detected Myxovirescin A.*

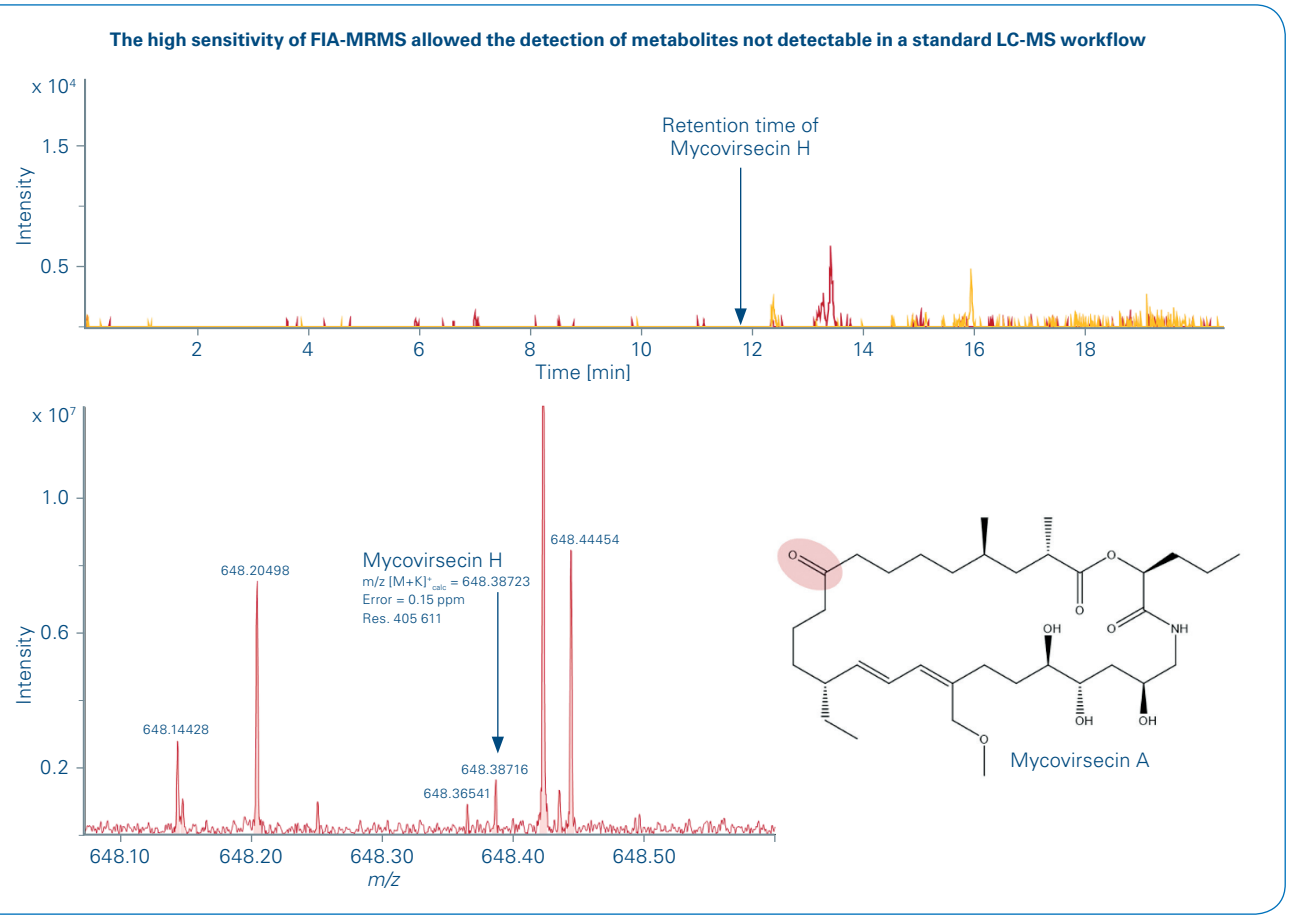

Figure 2: Extraction ion chromatogram (EIC) traces (upper part) showing that Myxovirescin H is not detectable by LC-MS. In contrast, Na^+ and K^+ adducts of Myxovirescin H could be detected by FIA-MRMS (lower part). Myxovirescin H only differs from Myxovirescin A (shown on right lower side) by the absence of one ketone moiety.

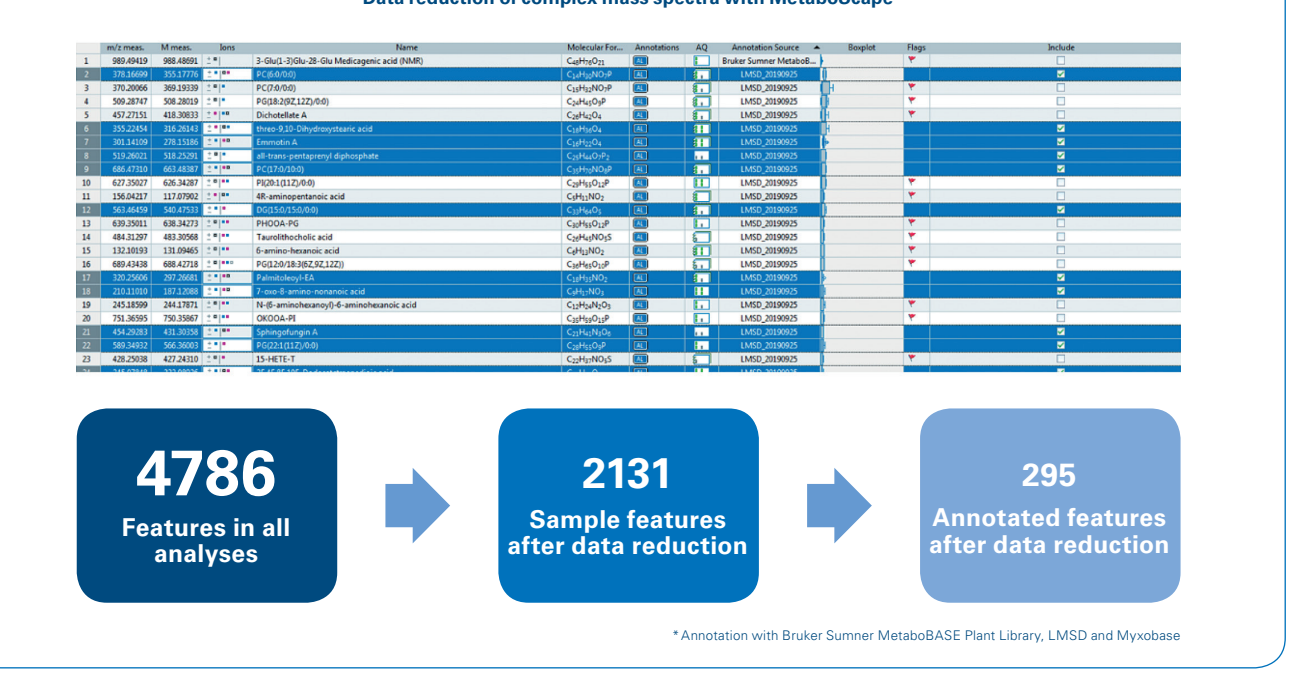

Figure 3: Highly complex mass spectra of M. xanthus *DK1622 crude extracts require data reduction for interpretation. Detected features can be reduced by automated blank subtraction and library annotation.*

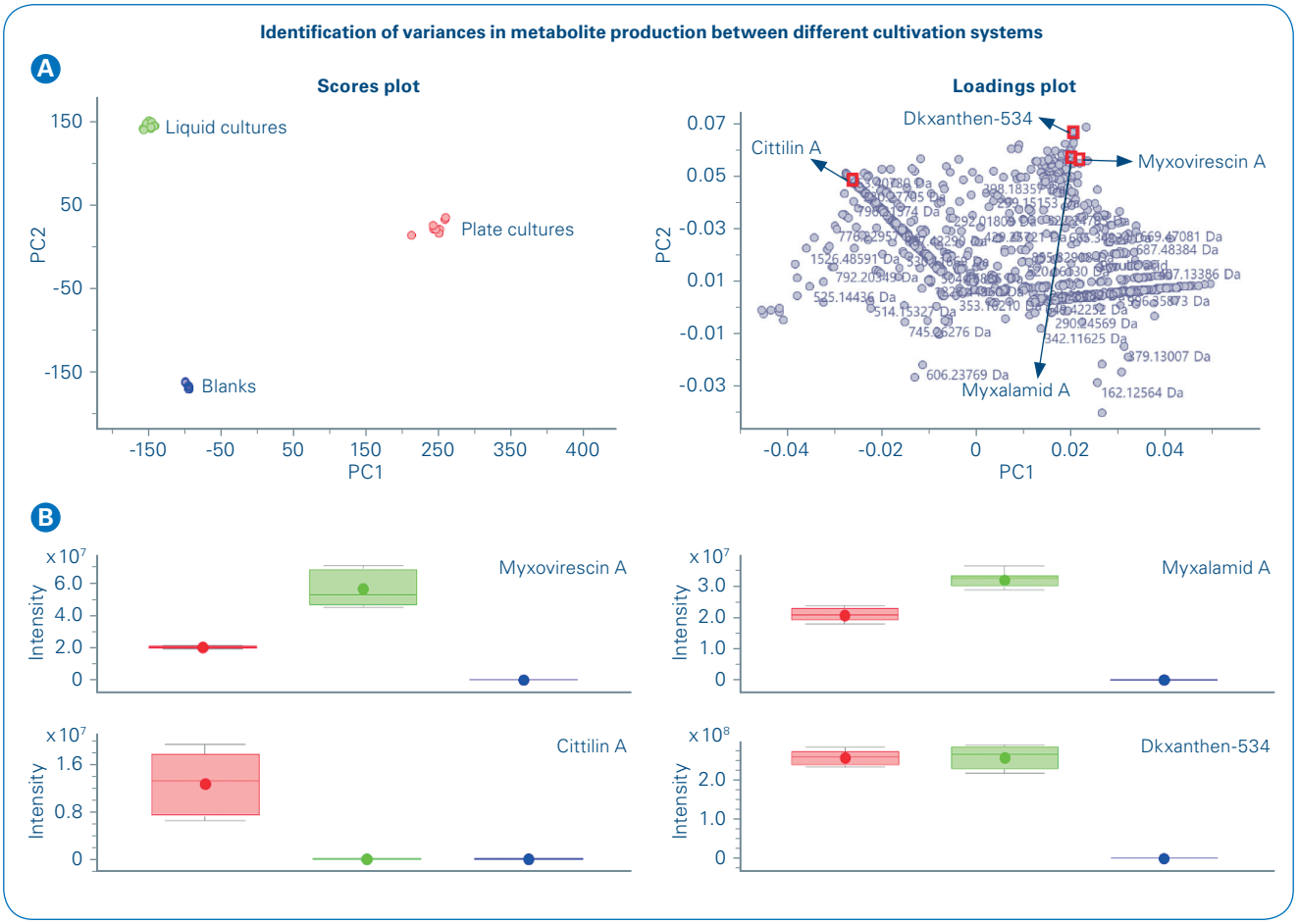

Figure 4: PCA of M. xanthus *DK1622 cultivated on plate and in liquid culture. Liquid culture extracts are shown in green, plate culture extracts in red and medium blank extracts in green.* (A) *Scores and loadings plots PC1 vs PC2. Biological and technical replicates are shown in the same color.* (B) *Box plot of four myxobacterial metabolites, Myxovirescin A, Myxalamid A, Cittilin A and DKxanthene-534.*

Conclusion

Myxobacterial secondary metabolites can be confidently detected by the MRMS aXelerate workflow using the Bruker scimaX MRMS system. The high sensitivity and ultra-high mass resolution were proven to enable detection of compounds missed in a standard LC-MS workflow. Very short measurement times of less than 2 minutes per sample facilitated high throughput screening. Subsequent data reduction and statistical profiling is required and can be performed with the MetaboScape software. Automated blank subtraction and annotation with pre-installed libraries as well as self-created analyte lists helped to scrutinize data sets with high complexity. PCA is a suitable tool to highlight similarities or discrepancies between different data sets. In this example of myxobacterial extract, PCA supported the prioritization of the cultivation system achieving sufficient production of metabolites of choice.

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References

[1] Krug D, and Müller R (2014). *Secondary metabolomics: the impact of mass spectrometry-based approaches on the discovery and characterization of microbial natural products*, Nat. Prod. Rep. **31**, 768–783.

[2] Hoffmann T, Krug D, Bozkurt N, Duddela S, Jansen R, Garcia R, Gerth K, Steinmetz H, Müller R. (2018). *Correlating chemical diversity with taxonomic distance for discovery of natural products in myxobacteria*, Nat. Commun. **9**, 803.

[3] Barsch A, Zurek G, Krug D, Cortina NS, Müller R (2010). *Challenges in Metabolomics addressed by targeted and untargeted UHR-Q-TOF analysis*. Accessible at: https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/literature/ApplicationNotes/ET-21%20Metabolomics_ebook.pdf.

[4] Wenzel SC, Müller R (2009). *Myxobacteria--'microbial factories' for the production of bioactive secondary metabolites*, Mol. Biosyst. **5**, 567–574.

[5] Hug JJ, Bader CD, Remškar M, Cirnski K, Müller R (2018). *Concepts and Methods to Access Novel Antibiotics from Actinomycetes*, Antibiotics **7**, 44.

[6] Krug D, Zurek G, Schneider B, Bässmann C, Müller R (2007). *Analysis of secondary metabolites from myxobacteria using ESI-TOF-MS and PCA*. Accessible at: https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/literature/ApplicationNotes/ET-29_Myxobacteria_eBook.pdf.

[7] Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CRH, Shimizu T, Spener F, van Meer G, Wakelam MJO, Dennis EA (2009). *Update of the LIPID MAPS comprehensive classification system for lipids*, J. Lipid Res. **50** Suppl, S9-14.

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