ELGA VEOLIA

Application Note:

The importance of ultrapure water for characterizing bacterial signaling molecules by UHPLC-HRMS/MS

Introduction

Quorum sensing is used by many species of bacteria to coordinate behaviors, such as biofilm formation, virulence, and antibiotic resistance. To understand and potentially modulate these processes, the identification and characterization of the communication molecules associated with this phenomenon is essential. Ultra-high performance liquid chromatography (UHPLC), in combination with high-resolution tandem mass spectrometry (HRMS/MS), can be used for this purpose. However, to obtain reliable results, these techniques require a consistent supply of ultrapure water. In this application note, we look at how Karine Escoubeyrou and colleagues at the Bio2Mar Technical Analytical Platform at Banyuls-sur-Mer Oceanographic Observatory have used the ELGA PURELAB® Option and flex 2 systems to meet their water purification needs.

N-acyl homoserine lactones (AHLs), shown in Figure 1, are signaling compounds used by many bacteria for communication between single or closely related species. Along with a number of other messenger compounds (known as autoinducers or pheromones), AHLs play an important role in quorum sensing – the mechanism by which bacteria coordinate behaviors, such as biofilm formation, virulence, and antibiotic resistance, based on the local density of the bacterial population.

Bacteria that employ quorum sensing secrete these autoinducers into their surrounding environment, where the molecules subsequently bind to target receptors within other bacteria. Upon binding, the autoinducers activate the transcription of certain genes, including those for autoinducer synthesis. In the presence of only a small number of bacteria, the concentration of autoinducer in the surrounding area is almost zero due to diffusion. However, in larger bacterial populations the concentration of autoinducer passes a threshold that causes more inducer to be synthesized, resulting in a positive feedback loop. As activation of the receptor also induces up-regulation of other genes, population-wide activation causes all of the cells to begin transcription simultaneously.

Bacteria use this phenomenon to maximize cell efficiency. For example, the bioluminescent luciferase produced by *Aliivibrio fischeri* (found in the photophore of the Hawaiian bobtail squid) would not be visible if it was produced by a single cell. Using quorum sensing to limit the production of luciferase to situations where bacterial populations are large therefore conserves energy.

The characterization and quantitation of autoinducer compounds is therefore of importance to better understand quorum sensing processes. However, because AHLs are typically released in appreciable amounts only when bacterial concentrations are high, the direct analysis of these compounds in marine environments can be difficult. One solution to this challenge is the isolation and identification of AHL-producing strains, and subsequent characterization of the compounds produced.

The analysis of known AHLs is often achieved using thin layer chromatography or high-performance liquid chromatography (HPLC) in combination with appropriate reference standards. However, for novel AHLs, chromatographic techniques, such as gas chromatography or HPLC, are typically paired with methods for structural identification, such as mass spectrometry or nuclear magnetic resonance.

In this application note, we look at how researchers at the Banyuls-sur-Mer Oceanographic Observatory, led by Raphaël Lami and supported by the Bio2Mar Technical Analytical Platform, use ultra-high performance liquid chromatography (UHPLC) coupled with high-resolution tandem mass spectrometry (HRMS/MS) to investigate AHL production by *Vibrio tasmaniensis* LGP32, a well-known pathogen of marine invertebrates.[1] Thanks to the consistent quality of the ultrapure water used in the investigation, the team was able to detect a number of AHLs based on their HRMS/MS fragmentation pattern, and confirm their identity through the use of appropriate standards.

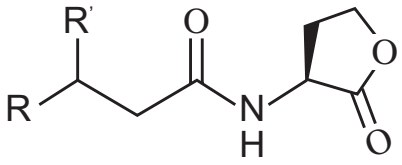

Figure 1. Structure of acyl homoserine lactones (AHLs)

Materials and methods

Chromatographic conditions

Liquid-liquid extraction of the LGP32 culture was performed using ethyl acetate. The organic phase was evaporated to dryness and the extract re-suspended in HPLC-grade DMSO. This extract was subsequently fractionated by HPLC using a C18 column. The mobile phase consisted of HPLC-grade water and acetonitrile using a gradient method. Positive fractions were further analyzed by UHPLC-HRMS/MS, also using a C18 column.

Water quality for HPLC and mass spectrometry

The analytical methods used in this investigation required Type I (18.2 MΩ cm) ultrapure water, which was obtained from an ELGA PURELAB flex 2 system with an 0.2 micron point-of-use filter fitted to the handset to further optimize water purity by removing DNase, RNase, endotoxins, and bacteria. Feedwater for the PURELAB flex 2 was pre-treated using the ELGA PURELAB Option R7 system (cartridge Labpure S3, tank DV25). Type I ultrapure water was necessary to achieve clean separation of products, obtain reliable spectrometric data, and to protect the instrumentation from damage.

Poor water quality can impair UHPLC and mass spectrometer systems with potentially significant implications in terms of the time and resources required to repeat experiments or repair equipment. The presence of organic contaminants in aqueous samples can produce additional peaks in the UHPLC chromatogram, which can complicate separation and analysis. Additionally, metal ions can form adducts with analytes, affecting the accuracy of mass spectrometry data.

The importance of feedwater recirculation

To achieve reliable characterization of the AHLs by UHPLC-HRMS/MS, the use of ultrapure water was essential. To provide a suitable grade of feedwater for the flex 2 system, the team used a pretreatment system incorporating a recirculating storage reservoir that significantly reduced the likelihood of bacterial buildup. The 25 liter reservoir was carefully chosen to meet the laboratory's peak water demand, whilst not being oversized, as storing large volumes of water can encourage bacterial growth.

Results and discussion

The *Vibrio tasmaniensis* LGP32 culture supernatant was extracted with ethyl acetate and fractionated into 22 fractions, which were tested for AHL production using biosensor strains *E. coli* MT102 and *P. putida* F117 fractions tested positive with at least one of the biosensors.

UHPLC-HRMS/MS analyses were performed to identify the AHLs. The presence of AHLs in the fractions was detected by identifying characteristic lactone ring fragments (m/z 102.055, 84.045, 74.061 and 56.050) in the fragmentation patterns. Four different AHLs were detected, including unsubstituted, oxo, and hydroxyl AHLs. These identifications were supported by the retention time and exact mass of the $[M+H]^+$ molecular ion of 27 commercially available AHL standards.

The UHPLC-HRMS/MS protocol gave good separation and well-defined peaks for 26 AHL standards, with a mass accuracy for all standards below 3 ppm. The median limit of detection was 10.58 nmol L^{-1}.

Conclusion

Non-targeted UHPLC-HRMS/MS, based on the analysis of MS/MS fragmentation patterns and the search for characteristic fragment ions corresponding to lactone rings, can be used for the reliable identification of AHLs in bacterial cultures. While the AHLs identified in this study were confirmed using appropriate AHL standards, the approach also has the potential to be used to identify unknown AHLs without the use of standards and may also be used for quantitation applications.

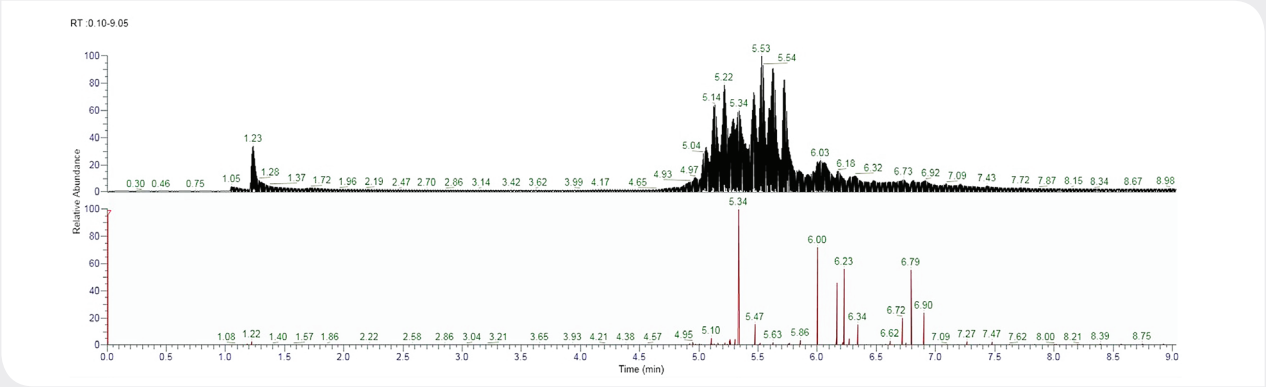

Figure 2. UHPLC chromatogram for AHL analysis (co-culture of *Vibrio* sp)

The use of Type I (18.2 MΩ cm) ultrapure water was essential to obtain the excellent chromatographic separation and high mass resolution reported in this application note. The UHPLC experiments in this study were strongly dependent on the quality of the water used. Low-grade water can block chromatography columns and reduce measurement performance by affecting the selectivity of the stationary phase, impacting on peak resolution, integration, and analysis baselines. Ionic contaminants can aggregate with analytes of interest, producing adduct peaks that interfere with mass spectrometry measurements.

The ultrapure Type I water, supplied by the ELGA flex 2 system, helped the Banyuls-sur-Mer Oceanographic Observatory researchers obtain the excellent product separation and high-resolution mass data achieved in this study. However, Escoubeyrou says that the high water quality wasn't the only factor that influenced her laboratory's decision to choose this system for their ultrapure water needs: "The ability to monitor TOC in real time at the point of use was critical. A TOC measurement greater than 3 ppb would not have been appropriate for our HRMS/MS applications; using the TOC display on the dispensing head meant that we could quickly and conveniently determine when system disinfection was required."

Water quality for sensitive analysis

ELGA offers a range of water purification systems ideally suited to providing ultrapure water for sensitive applications, such as UHPLC and HRMS/MS. The PURELAB flex 2 system, used in this research, is an integrated water purification and dispensing system combined in a single benchtop unit. The award-winning PURELAB flex series delivers accuracy, flexibility, and ease of use thanks to its innovative adjustable dispensing arm. The ergonomic and intuitive-to-use handset displays real-time information on water quality, and is capable of delivering ultrapure water in multiple dispensing modes depending on the individual needs of the user – from drop by drop and variable flow rates, through to autovolume dispensing. Inorganic water quality is optimized through the use of an easily replaced in-line purification pack, while recirculated water is UV treated and filtered.

Other ultrapure water purification systems offered by ELGA include the PURELAB Chorus, which can be configured (and reconfigured, as required) to suit the current and future needs of individual laboratories. When applications require the ultimate in water purity, the PURELAB Chorus 1 provides the ideal solution, enabling researchers to focus on generating accurate results while ensuring their workflows are not interrupted by water purity issues.

Researcher profile

Karine Escoubeyrou heads the Bio2Mar technical analytical platform at Banyuls-sur-Mer Oceanographic Observatory. The laboratory performs analysis of complex matrix samples, such as soil, sediment, seawater, and bacterial cultures to isolate and identify biomolecules. Escoubeyrou graduated with a master's degree in marine biology and marine ecology from University of Corsica and joined Banyuls-sur-Mer Oceanographic Observatory in 2001.

Contact : Karine Escoubeyrou
Responsable Plateforme Bio2mar
Observatoire Océanologique de Banyuls
UPMC CNRS FR3724
Avenue Pierre Fabre
66650 Banyuls-sur-mer
Email : karine.escoubeyrou@obs-banyuls.fr
http://bio2mar.obs-banyuls.fr/fr/index.html

References

1. L Girard *et al*, Characterization of *N*-acyl homoserine lactones in *Vibrio tasmaniensis* LGP32 by a biosensor-based UHPLC-HRMS/MS method, Sensors, 2017, 17, 906.
2. M. Doberva *et al*, Large diversity and original structures of acyl-homoserine lactones in strain MOLA 401, a marine *Rhodobacteraceae* bacterium. Frontiers in Microbiology, 2017, 8:1152.

Contact

To contact your nearest ELGA representative, please visit: **www.elgalabwater.com** and select your country for contact details.

Email: **info@elgalabwater.com**
Web: **www.elgalabwater.com**
Tel: **+44 (0) 203 567 7300**
Fax: **+44 (0) 203 567 7205**