Fast HILIC Separation of Bacterial Phospholipid Classes for Absolute Quantification with Charged-Aerosol Detection

Edward Rudt[1], Christian Faist[1], Vera Schwantes[1], Wen Jiang[2], and Heiko Hayen[1]
[1]Institute of Inorganic and Analytical Chemistry, University of Münster, Münster, Germany [2]HILICON AB

BACTERIA EXHIBIT A TREMENDOUS ADAPTABILITY, which can lead to the formation of antimicrobial resistance. For the selective treatment of bacteria, it is essential to understand the composition of the cell membrane at the molecular level. Therefore, we investigated bacterial membrane phospholipids in a previous study utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS) (1). The major bacterial phospholipid classes included phosphatidylethanolamines (PE), phosphatidylglycerols (PG), cardiolipins (CL), lyso-PE (LPE), and phosphatidylcholines (PC).

Quantitative information on the distribution of bacterial lipid classes would provide valuable data on the overall charge and rigidity of the lipid membrane. Other than sophisticated MS detection, universal detectors offer distinct advantages for absolute quantification, including the use of non-authentic internal standards (IS) in combination with external calibration for quantification. Charged aerosol detection (CAD) has emerged as a powerful universal detector for Lipidomics due to its detection sensitivity (ng on column), good reproducibility, large dynamic range, and similar analyte response for non-volatile species, independent of chemical structure (2).

A major drawback of universal CAD is its lack of selectivity. However, this disadvantage can also be used as an opportunity for rapid quantitative screening of coeluting compound classes, as described in our previous study on the bacterial phospholipid characterization (3). By using hydrophilic interaction liquid chromatography (HILIC), a robust polarity-dependent separation of phospholipid classes is achieved. Individual phospholipid species within a class were coeluted to form a single major peak for the entire lipid class. Thus, absolute quantification at the phospholipid class level can be accomplished with HILIC-CAD, which enables rapid screening of bacterial membrane lipid extracts.

In this application note, we detailly describe a HILIC-CAD method for fast characterization of bacteria based on absolute quantification at the phospholipid class level.

Experimental

Sample preparation:

Lipid standards were obtained from Biomol (Hamburg, Germany). The lipid extraction of freeze-dried *Xanthomonas campestris* pv. *campestris* (*Xcc*) bacteria was performed using methyl-*tert*-butyl ether based on the protocol of Matyash *et al.* (4). n-Dodecyl-β-D-maltoside was added as non-authentic IS to the extract for absolute quantification.

LC-MS/MS system:

Thermo Scientific Vanquish Flex UHPLC system hyphenated with VF-D20-A Vanquish charged aerosol detector F was used. Lipid quantification was performed without solvent compensation at 5 Hz collection rate, 50 °C evaporator temperature, and 3.6 filter constant.

Reference electrospray ionization (ESI)-MS data were acquired on a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer in negative ionization mode as described earlier (3).

HILIC separation:

Column:
20 x 2.1 mm, 5 µm, 100 Å, iHILIC®-Fusion(+)
(P/N: 100.022.0510, HILICON)
Eluents:
A) 95:5 (v/v), Ammonium formate solution (35 mM, pH 3.5)-acetonitrile
B) Acetonitrile
Flow rate: 0.3 mL/min
Column temperature: 40 °C
Injection volume: 2 µL

TABLE 1: Gradient profile for separation with iHILIC Fusion(+)

time [min]	% B
0.0	97
0.2	97
0.7	90
7.0	90
7.5	60
10.5	60
11.0	97
17.0	97

Results and Discussion

Zwitterionic iHILIC-Fusion(+) columns provide fast and robust separation of phospholipids according to their polarity, which forms major peaks of entire lipid classes. A HILIC separation of dominant bacterial phospholipids is illustrated in Figure 1. The limited number of bacterial phospholipid classes results in almost baseline separated peaks despite the use of fast gradients on short iHILIC columns in 2 cm length. These phospholipid class-level peaks can be directly quantified using HILIC-CAD, allowing rapid quantitative profiling of bacterial lipid classes.

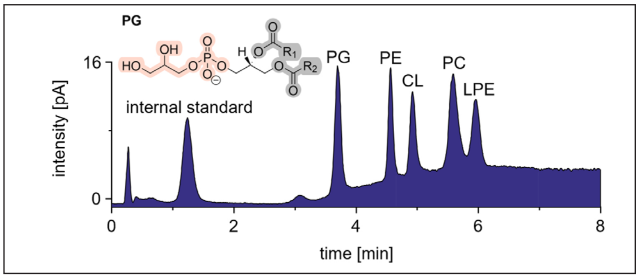

FIGURE 1: HILIC-based lipid class separation of bacterial phospholipid standards (including an exemplary structure of PG) and n-dodecyl-β-D-maltoside as internal standard (20 mg/L each) following universal CAD detection.

Compared to HILIC-MS, HILIC-CAD provides structure-independent quantification results. Lipid class-specific ionization efficiencies lead to overestimated proportions of PG in ESI-MS, while PE levels are discriminated, as shown for the bacterial profile of *Xcc* in Figure 2. In addition, the separation of LPE isomer peaks revealed suppression effects of the earlier eluting isomer in ESI-MS, which need to be compensated by IS and correction factors for accurate quantification results. On the contrary, HILIC-CAD provides simple and robust quantification results.

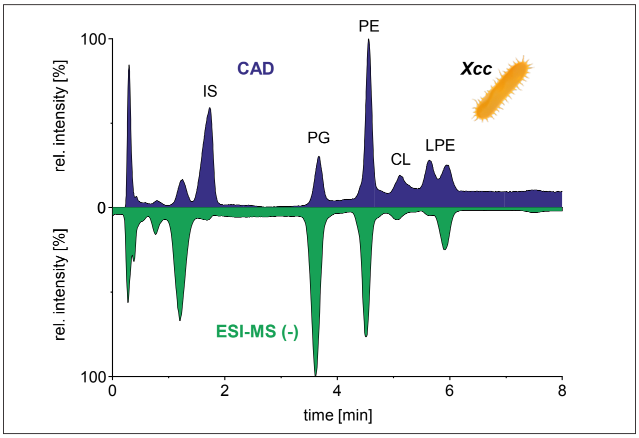

FIGURE 2: Response of bacterial phospholipid classes in *Xcc* compared between universal CAD and ESI-MS in negative ionization mode.

For CAD-based quantitation, an external calibration series is feasible, at which non-authentic IS can be used to compensate for instrumental variations. In this study, the combined approach was used for accurate quantitation. Figure 3 illustrates the dynamic range of the HILIC-CAD method by showing the calibration curve for PG. CAD provides a linear detector response for double logarithmic calibration curves. The multipoint external calibration ranged from 2.5 to 100 mg/L and achieved an R^2 of 0.993 for PG.

In our previous study, this method was used to quantitatively screen the total charge of bacterial membranes in various bacterial samples before and after treatment with antimicrobial random peptide mixtures (3). The *Xcc* bacteria of Figure 2, e.g., synthesize mainly zwitterionic phospholipids, including PE (671 ± 23 mg/L; 53.4 %) and LPE (250 ± 4 mg/L; 19.9 %). Contrarily, the negatively charged PG (153 ± 1 mg/L; 12.2 %) and the double negatively charged CL (179 ± 11 mg/L; 14.8 %) form a minor component of the bacterial membrane of *Xcc*. Compared to other common plant-pathogenic bacteria, *Xcc* produces a significant amount of LPE. This highlights the importance of quantitative bacterial phospholipid profiling using HILIC-CAD.

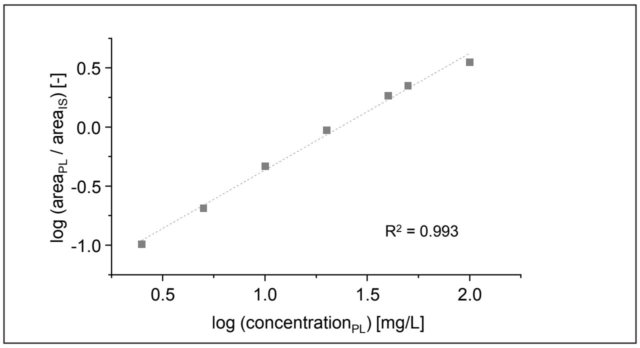

FIGURE 3: Calibration curve for PG for HILIC-CAD-based phospholipid class quantitation in bacterial samples.

Conclusion

The combination of HILIC-based phospholipid class separation and universal CAD served for straightforward quantitation of bacteria at phospholipid class level. This setup might also be adapted to other compound classes for rapid sample characterization.

References

(1) E. Rudt *et al., Anal. Bioanal. Chem.*, **2024**, 416, 5513

(2) M. Plante *et al., Methods Mol. Biol.*, **2009**, 579, 469-482

(3) E. Rudt *et al., Anal. Chim. Acta*, **2025**, 1342, 343680

(4) V. Matyash *et al., J. Lipid Res.*, **2008**, 49, 1137

HILICON

HILICON AB

Tvistevägen 48 A, SE-90736 Umeå, Sweden

+46 (90) 193469

info@hilicon.com

www.hilicon.com