Supercritical Fluid Chromatography Analysis of Polyol Biosurfactants with iHILIC®-Fusion Column and Mass Spectrometry

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Biosurfactants are more eco-friendly than conventional petrochemical surfactants because of their low toxicity and good biodegradability with comparable effects regarding physical and chemical properties like surface activity or critical micelle concentration (1). Additionally, their antimicrobial activities are an advantage compared to the synthetic counterparts. Thus, the applications of biosurfactants cover many different fields such as medicine, foods, cleaning materials, or cosmetics (1,2). Liamocin is a complex mixture of amphiphilic molecules, which is secreted as heavy oil by the yeast-like fungi *Aureobasidium pullulans* (*A. pullulans*), and thus can be exploited as biosurfactant. The structural diversity is based on a polar polyol head group and a polyester tail consisting of up to five 3,5-dihydroxydecanoic ester groups (Figure 1). An additional acetylation of the 3-OH group is possible. The structure of the polyol head group depends on the conditions of the culture medium. Those structurally related species without polyol head group are known as exophilins (3).

So far, the analysis of liamocins has been carried out by MALDI-TOF-MS and nuclear magnetic resonance (NMR) spectroscopy after prior fractionation by preparative liquid chromatography (LC) or thin-layer chromatography (TLC) (3–5). These methods require time-consuming steps due to the lack of hyphenation possibilities. Furthermore, the analysis needs higher amounts of the pure substance. In this work, supercritical fluid chromatography (SFC) hyphenated with mass spectrometry (MS) was used for the separation and detection of the aforementioned biosurfactants. The advantage of this analytical technique consists of time saving by simple sample preparation, faster separation, and reduced amount of organic solvent used in separation. Moreover, the SFC provides different selectivity compared to the commonly used reversed-phase HPLC because of the different separation mechanism and column chemistry. Here, we demonstrate the SFC analysis of different liamocin and exophilin species using a charge modulated amide iHILIC®-Fusion column and MS detection.

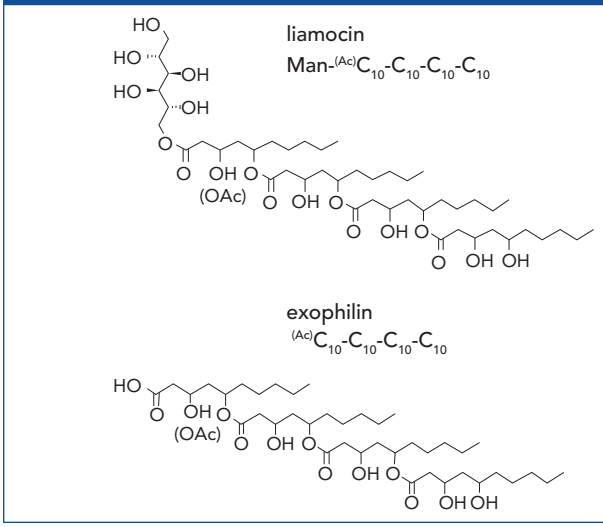

Figure 1: Structures of a mannitol liamocin as well as an exophilin with four linked decanoic ester groups.

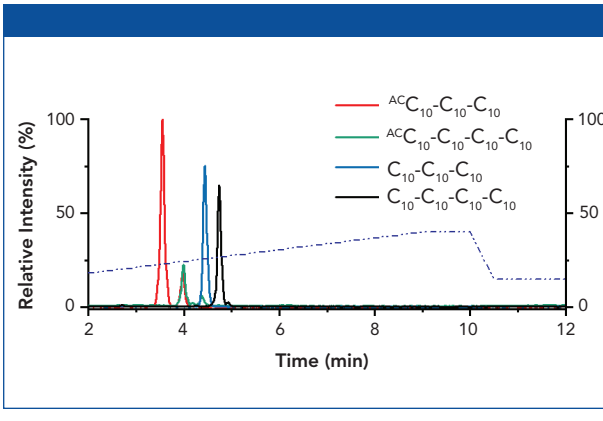

Figure 2: SFC MS chromatogram of four different exophilins detected as sodium adduct ([M+Na]+): C_{10}-C_{10}-C_{10} *m/z* 599.4, $^{Ac}C_{10}$-C_{10}-C_{10} *m/z* 641.4, C_{10}-C_{10}-C_{10}-C_{10} *m/z* 785.5, $^{Ac}C_{10}$-C_{10}-C_{10}-C_{10} *m/z* 827.5.

Experimental

SFC-MS system: A modified chromatography system (Agilent Technologies, Santa Clara, California, USA) used for SFC included a binary HPLC-SFC pump, a degasser, a SFC autosampler, a thermostatted column compartment, and a SFC Fusion A5™ module. MS detection was carried out by a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA), which was equipped with a HESI II source and operated in positive ionization mode. More details on SFC-MS system and settings are described in reference 9.

Column: 100 × 3 mm, 3.5 µm, 100 Å iHILIC®-Fusion (P/N 114.103.0310, HILICON, Sweden)

Eluent: A) carbon dioxide; B) methanol/water (99:1, *v/v*) containing 30 mM ammonium formate

Gradient elution: 0–1 min, 15% B; 1–9 min, 40% B; 9–10 min, 40% B; 10–10.5 min, 15% B; 10.5–12 min, 15% B
Backpressure: 140 bar; **Flow rate:** 1.9 mL/min;
Column temperature: 40 °C; **Injection volume:** 3 µL.
Liamocin samples: *A. pullulans* strain NRRL62031 (ARS Culture Collection [NRRL] of the United States Department of Agriculture [USDA, Washington, D.C., USA]) was used for the production of liamocins by Dr. Till Tiso at iAMB (Institute of Applied Microbiology, RWTH Aachen University, Aachen, Germany). After shake flask cultivation and fermentation process, the liamocins were extracted with a solution of 50% (*v/v*) ethanol and 50% aqueous NaCl (0.9%, w/v) (6). The extracted liamocins were diluted with methanol before analysis.

Results and Conclusion

In the samples, 10 different biosurfactant species could be detected. Four of the analytes were exophilins with three or four linked ester groups and the corresponding acetylated species, while the other six compounds can be distinguished as liamocins with mannitol or arabitol as head group. In both cases, species with three ester groups, their acetylated species, and four ester groups were detected. According to their head group, the analytes were divided into subclasses. By using an optimized modifier comprising methanol–water mixture (99:1, *v/v*) containing 30 mM ammonium formate, a baseline separation of all analytes was achieved by means of SFC with an iHILIC®-Fusion column. In our preliminary study, no complete baseline separation could be achieved under reversed-phase HPLC conditions, albeit this is required for quantification by a non-MS type detection, such as evaporative light scattering detection (ELSD) or charged-aerosol detection (CAD) (7,8). The combination of supercritical mobile phase and iHILIC®-Fusion stationary phase exhibited excellent selectivity for the investigated polyol lipids. The baseline separation of four exophilins is shown in Figure 2 as an example of the retention behaviour of the analytes within a subclass. The non-acetylated species elute after the acetylated species due to stronger hydrophilic interaction with the stationary phase.

In addition to the baseline separation of the analytes within one subclass, the iHILIC®-Fusion column allows the separation of different subclasses, as shown in Figure 3. The exophilins were eluted before the liamocins with the sugar alcohol arabitol (five OH groups) as head group and the mannitol-liamocins (six OH groups).

This work demonstrates that liamocin biosurfactants can be efficiently analyzed by the SFC-MS method using an iHILIC®-Fusion column.

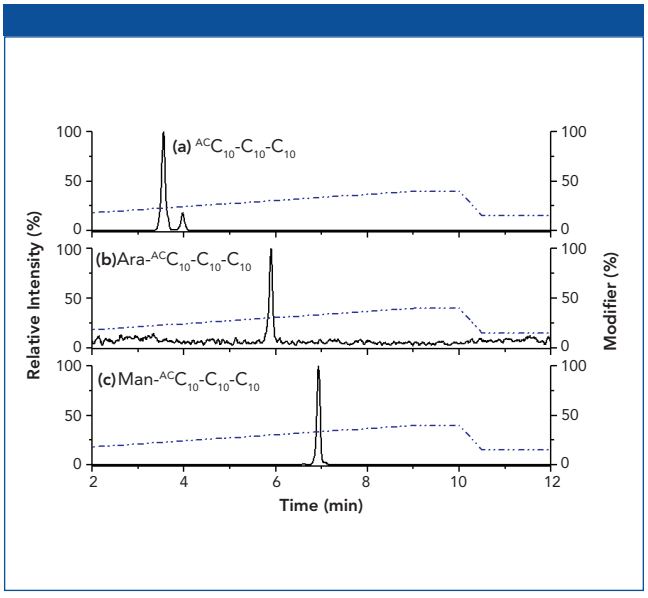

Figure 3: Extracted ion chromatogram showing the species of each subclass with three linked decanoic ester groups and the acetylation of the 3-OH group: exophilin (a) arabitol-liamocin (b) and the mannitol-liamocin (c).

References

(1) I.M. Banat, A. Franzetti, I. Gandolfi, G. Bestetti, M.G. Martinotti, L. Fracchia, T.J. Smyth, and R. Marchant, *Appl. Microbiol. Biotechnol.* **87**, 427–444 (2010).

(2) K.M. Bischoff, T.D. Leathers, N.P.J. Price, and P. Manitchotpisit, *J. Antibiot.* **68**, 642–645 (2015).

(3) N.P.J. Price, P. Manitchotpisit, K.E. Vermillion, M.J. Bowman, and T.D. Leathers, *Carbohaydr. Res.* **370**, 24–32 (2013).

(4) P. Manitchotpisit, R. Watanapoksin, N.P.J. Price, K.M. Bischoff, M. Tayeh, S. Teeraworawit, S. Kriwong, and T.D. Leathers, *World J. Microbiol. Biotechnol.* **30**, 2199–2204 (2014).

(5) J.S. Kim, I.K. Lee, D.W. Kim, and B.S. Yun, *PLoS One* **10**, 1–12 (2015).

(6) T. Kurosawa, K. Sakai, T. Nakahara, Y. Oshima, and T. Tabuchi, *Biosci. Biotechnol. Biochem.* **58**, 2057–2060 (1994).

(7) K.M. Saur, O. Brumhard, K. Scholz, H. Hayen, and T. Tiso, *Appl. Microbiol. Biotechnol.* **103**, 4741–4752, (2019).

(8) K. Scholz, M. Seyfried, O. Brumhard, L.M. Blank, T. Tiso, and H. Hayen, *J. Chromatogr. A.* **1627**, 461404 (2020).

(9) K. Scholz, A. Lipphardt, C. Wienken, T. Tiso, and H. Hayen, *J. Chromatogr. A.* **1631**, 461584 (2020).

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