Tailored Analysis of Phospholipid Classes Using iHILIC-Fusion(+) as First Dimension for Online Two-Dimensional HILIC–Reversed-Phase LC–MS

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Phospholipids (PLs) are a large lipid subgroup that is involved in important cell functions in all organisms. They are the main components of biological membranes and are essential for many biologic processes within and between cells, for example, signal transduction, cell growth, and various transport processes. The basic building blocks for PLs are fatty acids linked to a glycerol backbone and polar head groups (Figure 1). In addition to the main membrane PLs, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), other PL classes are of great importance as well. Some PLs have significantly lower concentrations. For example, in eukaryotic organisms, cardiolipin (CL) is exclusively located in the inner mitochondrial membrane (1). Thus, the concentration of CL in total lipid extracts is very low in comparison to the major membrane lipids, such as PLs and cholesterol, but also triacylglycerols (TGs) (2,3). Furthermore, ion suppression effects complicate the mass spectrometric (MS) analysis and a tailored analysis of low abundant PL classes is often required.

In our previous study, we demonstrated the advantage of performing sample preparation by solid-phase extraction (SPE) in hydrophilic interaction liquid chromatography (HILIC) mode (4). The nonpolar fraction of lipids was first separated from polar PLs using an offline HILIC SPE method, and thereafter separation by reversed-phase liquid chromatography (LC) was performed. Offline methods often require solvent evaporation and reconstitute analytes in a suitable solvent for the second dimension, which is a time-consuming approach and risks losing labile PLs. In this application, a novel online heart-cut two-dimensional (2D) HILIC–reversed-phase LC–MS method is presented for the separation of PL classes and nonpolar lipids, where HILIC was utilized in the first dimension (¹D) and reversed-phase LC in second dimension (²D).

Experimental

Lipid Standards: Triacylglycerol (TG 48:0), cholesterol (Chol), phosphatidylcholine (PC 32:0), phosphatidylethanolamine (PE 32:0), cardiolipin (CL 64:4), and yeast total lipid extract (*S. cerevisiae*).

LC–MS/MS Setup: Thermo Scientific Ultimate 3000 system with dual gradient pump hyphenated to a Q ExactiveTM Plus Hybrid Quadrupole-OrbitrapTM mass spectrometer. Ionization was performed utilizing electrospray ionization in negative ionization mode as described by Helmer *et al.* (3).

¹D HILIC Separation:

Column: 20 × 2.1 mm, 5-µm, 100 Å, iHILIC[®]-Fusion(+) (P/N 100.022.0510, HILICON)

Eluents: A) ammonium formate solution (35 mM, pH 3.5) and

95:5 (v/v) acetonitrile; B) acetonitrile

Gradient Elution: 0–0.2 min, 97% B; 0.2–0.5 min, from 97% to 93% B; 0.5–2.75 min, 93% B; 2.75–7.5 min, from 93% to 60% B; 7.5–11 min, 60% B; 11–11.5 min, from 60% to 97% B; re-equilibration is parallel to reversed-phase LC separation at 97% B.

Flow Rate: 0.3 mL/min; 0.05 mL/min (during parallel reversed-phase LC separation)

Column Temperature: 40 °C

Injection Volume: 2-20 µL

Heart-Cut Setup for PL Transfer: An online heart-cut 2D HILIC-reversed-phase LC-MS setup was developed for PL transfer from ¹D (HILIC) to ²D (reversed-phase LC). As described earlier, a six-port valve equipped with a sample loop (300 μ L, PEEK) was utilized as the transfer (Figure 2) (3). At first, the HILIC eluate was directed to the waste. By switching



Figure 1: Selected structures of nonpolar lipids and polar PL. HILIC separation of PL takes place according to their polar head groups (shown in green).

the valve from A (2_1) to B (1_6) position, the selected PL fraction was transferred into the loop for the separation in the second dimension. Since we focused on CL in this study, the transfer time window was set at its retention time of between 6.35–6.95 min.

Results and Conclusion

A tailored analysis of CL species in yeast (*S. cerevisiae*) was achieved utilizing two-dimensional HILIC-reversed-phase LC-MS. In the first dimension, the separation of PL classes was carried out using a HILIC method. In addition to that, nonpolar lipids did not show retention and eluted at the void volume. As shown in Figure 3(a), the lipid classes (TG, PE, CL, and PC) were well separated according to their different head groups with this new method. The application of this method to a yeast total lipid extract (Figure 3[b]) shows the intended coelution of the CL species in yeast, where one typical CL species is CL 66:4 with 66 carbon atoms and four double bonds in the four linked fatty acids.

In the developed 2D HILIC-reversed-phase LC separation method, a transfer window with all CL species from ¹D HILIC was first determined for the transfer via a heart-cut setup to the ²D reversed-phase LC system. Compared to the conventional one-dimensional (1D) reversed-phase LC (red), ²D reversed-phase LC (black) shows a significant decrease of background signals (mainly TGs), and low abundant CL species can even be observed in the total ion current (TIC) chromatogram (Figure 3[c]). Furthermore, by the reduction of the background as observed in 1D reversed-phase LC, higher CL signal intensities have been observed (3).

The selective HILIC pre-separation of lipid classes in the first dimension significantly reduced the matrix effects in the second dimension separation of low abundant PL classes. Interestingly, low abundant CL species can be sensitively



Figure 2: Valve setup for the transfer of PL classes from ¹D HILIC to ²D reversed-phase (RP) LC via heart-cut approach.



Figure 3: (a) ¹D HILIC separation of representative lipid standards; (b) coelution of dominant CL species in yeast (*S. cerevisiae*) within the transfer window; (c) comparison of reversed-phase (RP) LC TICs in 1D (red) and ²D (black).

detected and observed, even in the TIC. In summary, the automated online 2D method via heart-cut transfer offers a fast and gentle sample preparation and clean-up procedure that is very useful for analyzing low abundant PL classes as well as labile PLs, such as PL oxidation products (3).

References

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