

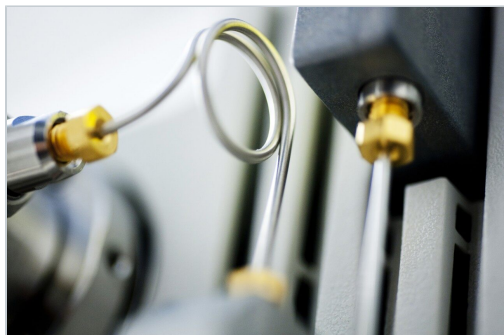
Application Note

## ACQUITY PREMIER LC Technology Significantly Improves Sensitivity, Peak Shape, and Recovery for Phosphorylated and Carboxylate Lipids

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diagnostic procedures.*

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## Abstract

Phosphorylated and carboxylate lipid species are metal sensitive and can readily adsorb to stainless steel surfaces within the flow path of UPLC systems. This process can lead to poor peak shape, low recovery and reduction in sensitivity. Here we show that the ACQUITY PREMIER CSH C<sub>18</sub> Column with ACQUITY PREMIER System can significantly improve sensitivity, peak shape, and recovery of phosphorylated and carboxylate lipids compared to ACQUITY standard column and stainless steel ACQUITY UPLC I-Class System.

## Benefits

The ACQUITY PREMIER technology provides:

- Increased sensitivity, recovery, and reproducibility for phosphorylated and carboxylate lipids
- Improved peak shape and reduced tailing by minimizing analyte-surface interaction
- Increased lipidomics coverage by simultaneous analysis of phosphorylated and carboxylate lipids with other lipid classes

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## Introduction

Phosphatidic acid (PA) is the simplest membrane phospholipid class and have a key function as an intermediate in phospholipid and glycerolipid metabolism. Due to their intermediate function, PA's are found in small amounts in biological membranes but are crucial signaling molecules in biochemical and physiological processes in cells.<sup>1-2</sup> Lysophosphatidic acids (LPA) are biosynthetic precursors with regulatory functions in the mammalian reproduction system. Phosphatidylserines (PS) are important components of cellular membranes, where they comprise 5–10% of total phospholipids with important roles in the regulation of apoptosis, lipid synthesis, and transport. Lysophosphatidylserines (LPS) are signaling phospholipids with an important role during inflammatory processes.<sup>1-2</sup> PA and PS are acidic lipids with the potential to carry two negative charges – both charges on the phosphate group for PA, while one negative charge is on the phosphate and the second on the carboxylate group in the case of PS.<sup>3</sup>

For the last 50 years, stainless steel has been the most commonly used construction material for High Performance Liquid Chromatography (HPLC) instruments and columns. However, stainless steel hardware can negatively impact the peak shape and recovery of some analytes, due to non-specific adsorption and binding. Lipid classes that show these effects typically contain functional groups such as phosphate and carboxylate groups that can form chelation complexes with iron and other transition metal ions. The comprehensive lipidomic analysis of various biological tissues is a challenging task due to the extreme complexity of individual lipid classes varying in their structure, attached functional groups, polarity, dissociation, and ionization behavior.<sup>3</sup> Phosphorylated and carboxylate lipids (lipids with phosphate or carboxylic acid functional groups such as glycerophosphates, glycerophosphoserines, glycerophosphoinositols, glycerophosphoinositol monophosphates, glycerophosphoinositol bisphosphates, glycerophosphoinositol trisphosphate, glycerophosphoglycerols, phosphorylated sphingolipids including ceramide-1-phosphate, and Sphingoid base 1-phosphates) are metal sensitive and readily adsorb to stainless steel surfaces present within the flow path of the chromatographic system. This process can lead to poor peak shape, low recovery, and a reduction in sensitivity.

An alternative approach to mitigate interactions of phosphorylated lipids with metal surfaces is to add chelators to the mobile phase or sample. Volatile chelators such as citric acid, acetylacetone, methylphosphonic acid, and medronic acids have been used for LC-MS analyses. However, the use of chelators can negatively impact chromatographic selectivity and MS sensitivity. To address these issues, we have explored the use of a hybrid organic/inorganic barrier surface applied to the metal substrates in UPLC instruments and columns. A hybrid surface technology based on an ethylene-bridged siloxane polymer has been found to be well-suited for reversed-phase (RP) and hydrophilic interaction chromatography (HILIC). The main goal of this application note is to evaluate and

compare the chromatographic performance of ACQUITY PREMIER System and ACQUITY PREMIER CSH C<sub>18</sub> Column, which incorporates this hybrid surface technology (HST), relative to standard stainless steel surface ACQUITY UPLC I-Class and CSH C<sub>18</sub> for phosphorylated and carboxylate lipids.

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## Experimental

### Sample Description

LPA (16:0/0:0), PA (16:0/18:1), LPS (18:1/0:0), and PS (16:0/18:1) lipid standards, egg chicken PA and brain porcine PS extracts were obtained from Avanti Polar Lipids. A separate stock solution of 1 mg/mL was prepared for PA (16:0/18:1), LPS (18:1/0:0) and PS (16:0/18:1) in chloroform. LPA (16:0/0:0) was prepared in a concentration of 0.5 mg/mL in chloroform/methanol/water (80/20/2) with gentle heating at 40 °C and sonication to facilitate complete dissolution. A working mixture dilution series of 5, 50, 100, and 250 ng/mL was prepared in IPA/ACN (50/50) for the lipid standards. Egg chicken PA and brain porcine PS extracts were obtained as powder and dissolved in chloroform at a concentration of 1 mg/mL. A working solution of the egg chicken PA and brain porcine PS extracts was prepared in IPA/ACN (50/50).

## Method Conditions

### LC Conditions

LC system:	Standard ACQUITY UPLC I-Class and ACQUITY PREMIER
Detection:	SYNAPT XS
Vials:	Waters Total Recovery UPLC Vials
Column(s):	ACQUITY CSH C <sub>18</sub> (2.1 x 100 mm, 1.7 μm) ACQUITY PREMIER CSH C <sub>18</sub> (2.1 x 100 mm, 1.7 μm)
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	5 μL
Flow rate:	400 μL/min
Mobile phase A:	600/390/10 (ACN/Water/1M aqueous ammonium formate) in 0.1% formic acid
Mobile phase B:	900/90/10 (IPA/ACN/1M aqueous ammonium formate) in 0.1% formic acid

## Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.4	50	50	Initial
0.5	0.4	47	53	6
4.0	0.4	45	55	6
7.0	0.4	35	65	6
7.5	0.4	20	80	1
10.0	0.4	1	99	6
11.0	0.4	1	99	1
12.0	0.4	50	50	1

## MS Conditions

MS system:	SYNAPT XS
Ionization mode:	ESI-
Acquisition range:	100–1200 Da
Capillary voltage:	2.5 kV (negative mode)
Collision energy:	Linear ramp (Transfer CE) 25–45 eV
Cone voltage:	30 V

## Data Management

Chromatography software:	MassLynx v4.2
MS software:	MassLynx v4.2

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## Results and Discussion

LC-MS analysis of phosphorylated and acidic lipids from biological samples is a critical step, especially for low abundant signaling lipid classes such as LPA, PA, LPS, and PS (Figures 1A and 1B). Our previously published method<sup>4</sup> enables the accurate analysis of most commonly occurring polar and non-polar lipid classes except for the phosphate and carboxylate lipid classes due to very broad tailing peaks and lower sensitivity under these conditions.<sup>3-6</sup> The phosphate group of the phosphatidic acids (PA and LPA) and the carboxylate group of the phosphatidylserines (PS and LPS) bind to metal ions to form chelation complexes that cause peak tailing and smearing during chromatographic analysis. To address the issue, we have developed the use of a hybrid organic/inorganic surface technology in the ACQUITY PREMIER CSH C<sub>18</sub> (2.1 x 100 mm, 1.7 μm) Column and ACQUITY PREMIER System. A serial dilution of LPA, PA, LPS, and PS was analyzed using standard column with stainless steel surface ACQUITY UPLC System and ACQUITY PREMIER Column with ACQUITY PREMIER System. Figures 2A, 2B, 3A, and 3B show a side by side comparison of extracted ion chromatograms of LPA (16:0/0:0), PA (16:0/18:1), LPS (18:1/0:0), and PS (16:0/18:1) using standard CSH C<sub>18</sub> Column with stainless steel surface ACQUITY UPLC and ACQUITY PREMIER CSH C<sub>18</sub> Column with ACQUITY PREMIER System. The ACQUITY PREMIER CSH C<sub>18</sub> Column with ACQUITY PREMIER System significantly improved the peak tailing and sensitivity of these compounds. The increase in sensitivity can be clearly seen for LPA (Figure 2-A1 and 2-A2) at a lower concentration level (5 and 50 ng/mL) as evidenced by the noticeably larger peak for the ACQUITY PREMIER System, compared to the standard metal surface system. Similarly Figures 2-B1 and 2-B2 (PA), 3-A1 and 3-A2 (LPS), and 3-B1 and 3-B2 (PS) show improved sensitivity, peak shape, and recovery obtained when using ACQUITY PREMIER CSH C<sub>18</sub> Column with ACQUITY PREMIER System compared to the standard column and system.



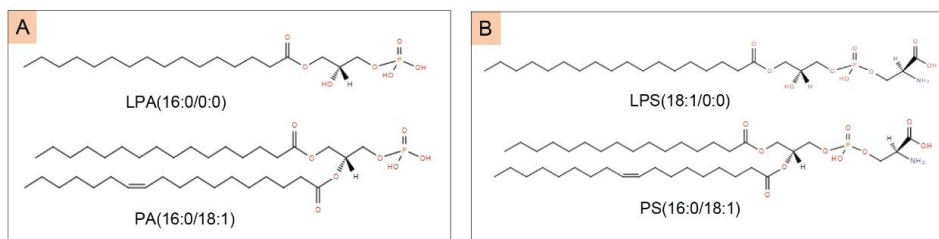


Figure 1. The chemical structure of analyzed lipid standards (A) Glycerophosphates including 16:0/0:0 lysophosphatidic acid (LPA (16:0/0:0)) and 16:0/18:1 phosphatidic acid (PA (16:0/18:1)) also called monoacylglycerophosphates and diacylglycerophosphates respectively. (B) Glycerophosphoserines including 18:1/0:0 lysophosphatidylserine (LPS (18:1/0:0)) and 16:0/18:1 Phosphatidylserine (PS (16:0/18:1)) also called monoacylglycerophosphoserines and diacylglycerophosphoserines respectively.

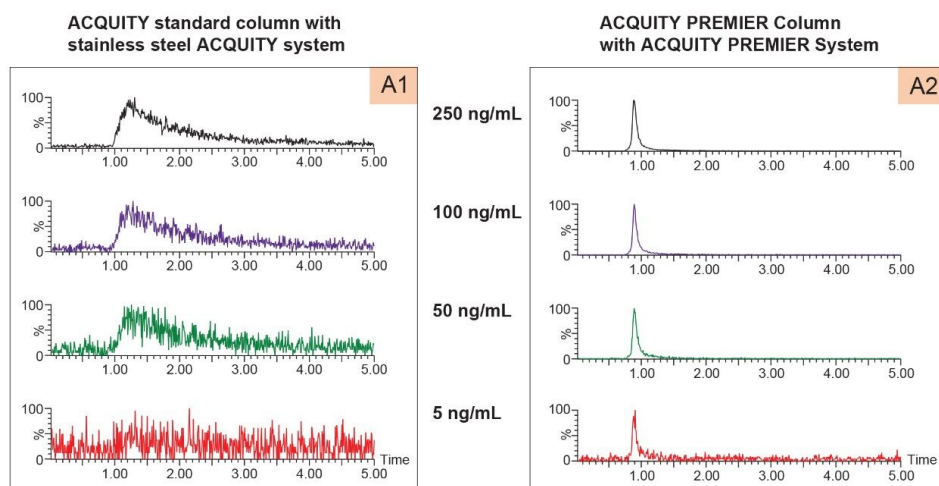


Figure 2A. Negative mode base peak extracted ion chromatogram of LPA (16:0/0:0)  $m/z$  409.2355 at a concentration of 5, 50, 100, and 250 ng/mL. (A1) using ACQUITY standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY System and (A2) using ACQUITY PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System.

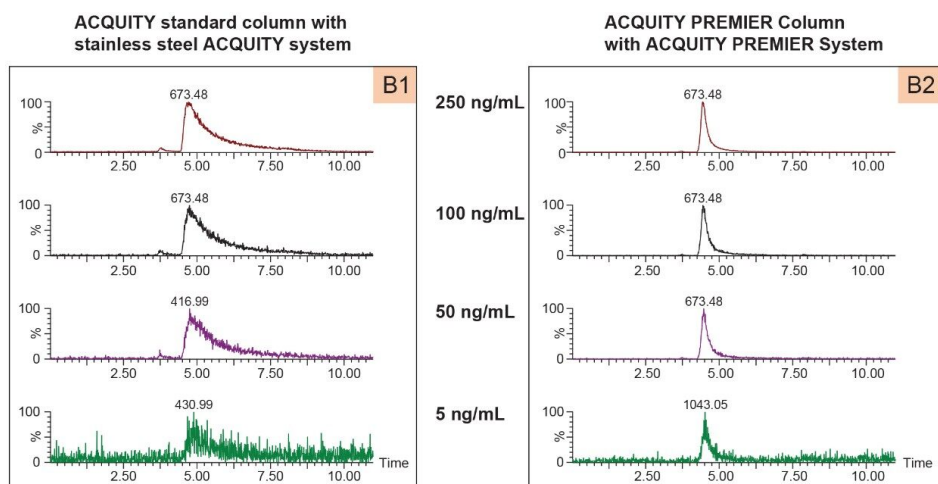


Figure 2B. Negative mode base peak extracted ion chromatogram of PA (16:0/18:1)  $m/z$  673.4808 at a concentration of 5, 50, 100, and 250 ng/mL. (B1) using ACQUITY standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY System and (B2) using PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System.

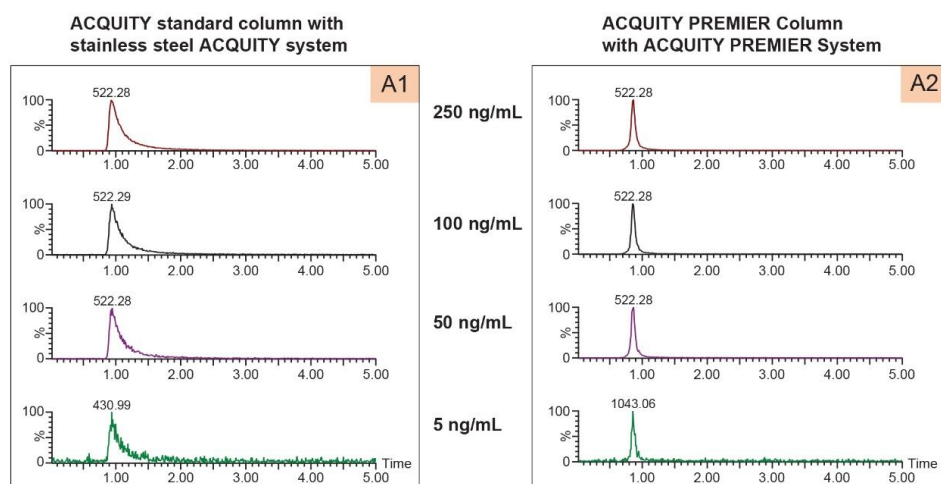


Figure 3A. Negative mode base peak extracted ion chromatogram of LPS (18:1/0:0)  $m/z$  522.2832 at a concentration of 5, 50, 100, and 250 ng/mL. (A1) using ACQUITY standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY System and (A2) using PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System.

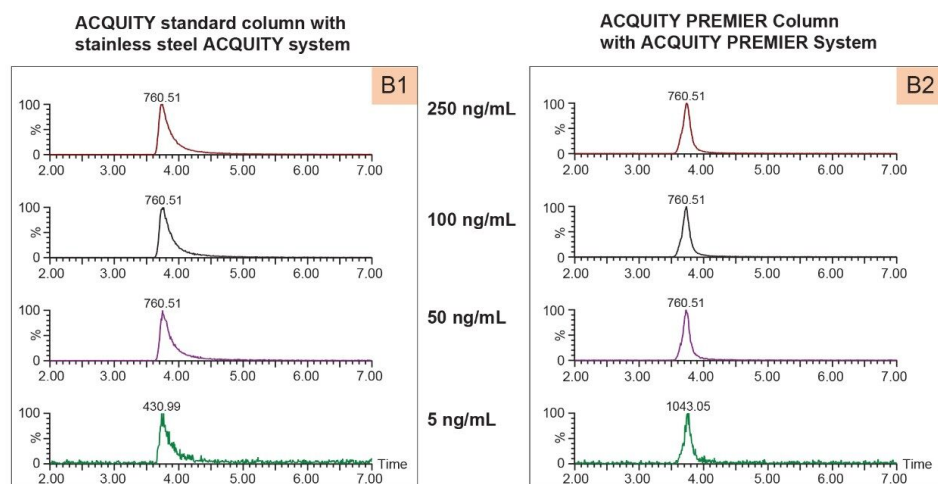


Figure 3B. Negative mode base peak extracted ion chromatogram of PS (16:0/18:1)  $m/z$  760.5129 at a concentration of 5, 50, 100, and 250 ng/mL. (B1) using ACQUITY standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY System and (B2) using PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System.

The developed method was applied for the analysis of egg chicken PA and brain porcine PS extracts (Figures 4 and 5). The PA lipid species at  $m/z$  671.47 (16:1\_18:1) and 673.48 (16:0\_18:1) provide very broad tailing peaks using the standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY UPLC (Figure 4A) compared to ACQUITY PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System (Figure 4B). Figures 4A and 4B bottom panels show extracted ion chromatogram for PA (16:0\_18:1) with peak width 4.1 min for standard CSH  $C_{18}$  Column with stainless steel (Figure 4A bottom panel) and 1.2 min for ACQUITY PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System (Figure 4B bottom panel). Similarly Figure 5 shows PS brain porcine extract under identical conditions in both standard and ACQUITY PREMIER conditions with extracted ion chromatogram for PS (18:0\_18:1) with peak width 1.1 min for standard CSH  $C_{18}$  Column with stainless steel (Figure 5A bottom panel) and 0.3 min for ACQUITY PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System (Figure 5B bottom panel).

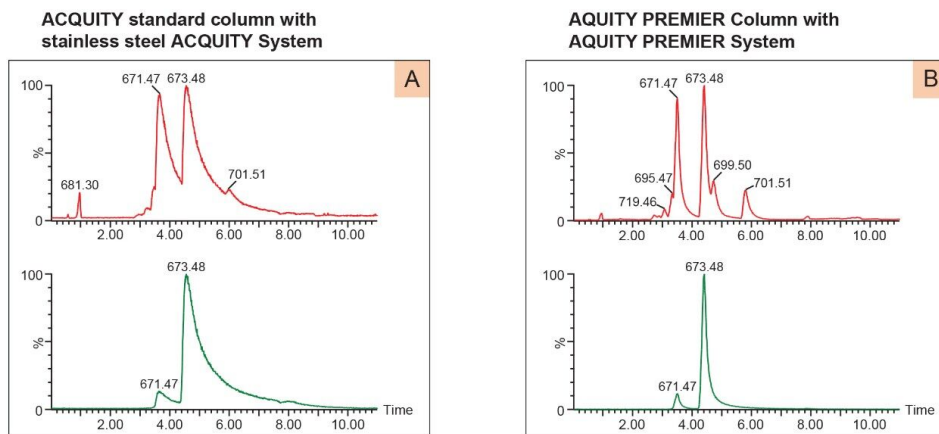


Figure 4. Avanti polar lipids egg chicken PA extract (10  $\mu\text{g/mL}$ ) measured using (A) ACQUITY standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY System (B) PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System. The bottom panel shows extracted ion chromatogram for PA (16:0\_18:1) at  $m/z$  673.48 with peak width 4.1 min for standard CSH  $C_{18}$  Column with stainless steel system and 1.2 min for PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System.

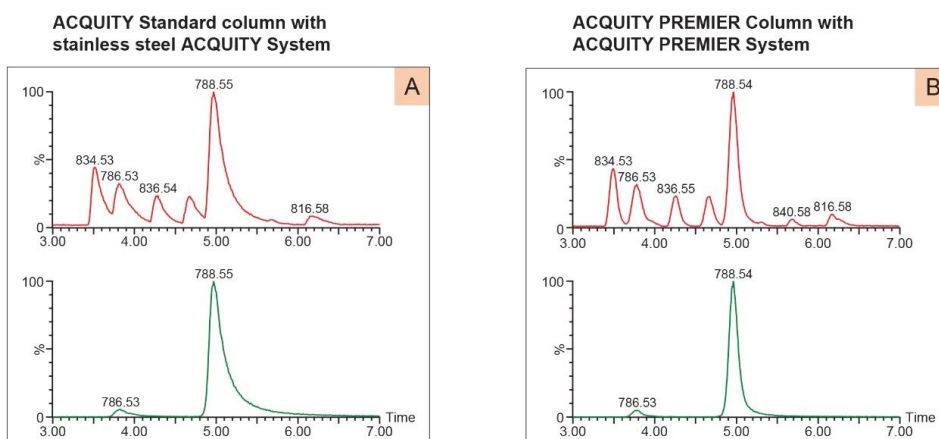


Figure 5. Avanti polar lipids brain porcine PS extract (10  $\mu\text{g/mL}$ ) measured using (A) ACQUITY standard CSH  $C_{18}$  Column with stainless steel surface ACQUITY System (B) PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System. The bottom panel shows extracted ion chromatogram for PS (18:0\_18:1) at  $m/z$  788.54 with peak width 1.1 min for standard CSH  $C_{18}$  Column with stainless steel and 0.3 min for PREMIER CSH  $C_{18}$  Column with ACQUITY PREMIER System.

In contrast to most of the previous methods using standard reverse phase columns,<sup>6</sup> the ACQUITY PREMIER Technology increases the lipidomics coverage by simultaneous analysis of phosphate and carboxylate containing lipids in addition to other lipid classes without significant binding and peak tailing, which can increase the coverage, sensitivity, and recovery of these biologically important

lipid species.

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## Conclusion

The PREMIER CSH C<sub>18</sub> Column with ACQUITY PREMIER UPLC System is a highly reliable method for the analysis of LPA, PA, LPS, and PS lipid molecular species that significantly improves sensitivity, peak tailing, and recovery of phosphorylated and carboxylate lipids. The developed method can be applied to other phosphorylated and carboxylate lipid classes such as phosphatidylinositol phosphates with mono-, di-, and triphosphates, ceramide-1-phosphate, and sphingoid base 1-phosphates.

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