Application Note

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Minimizing the Interaction of Phospholipids with LC Hardware

Phospholipid analysis using the Agilent 1290 Infinity II Bio LC System and Agilent InfinityLab LC/MSD iQ

Suitable for Agilent
1290 Infinity III LC

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Abstract

Phospholipids are an important class of biochemicals involved in various biological processes. When the phosphate is present as a monoester, as is the case with phosphatidic acid (PA), lyso-phosphatidic acid (LPA), and phosphatidylinositol phosphates (PIPs), HPLC and LC/MS analysis becomes challenging. Interaction of these species with stainless steel components in the flow path can lead to recovery issues. In this application note, the advantage of using the Agilent 1290 Infinity II Bio LC System with the Agilent InfinityLab LC/MSD iQ for these challenging analytes is demonstrated.

Introduction

Phospholipids are the main components in cell membranes and are involved in a variety of biological processes. Besides their presence in nature, phospholipids have shown relevance in (bio)pharmaceutical applications, for example, as structural component in lipid nanoparticles (LNPs) for cellular delivery of oligonucleotides and mRNA.

Phospholipids are esters of glycerol composed of hydrophobic fatty acids and a hydrophilic phosphate-containing head group (free or modified). Depending on the head group, different classes of phospholipids are recognized, including PA, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and PIP. Each class has different physicochemical and biological properties and is characterized by the existence of various species that differ, for instance, in terms of fatty acid length and the number and position of double bonds. Hence, their analysis can be challenging and is commonly facilitated by HPLC or UHPLC with detection by UV-DAD, RI, ELSD, CAD, or MS for more challenging applications.

Poor chromatographic performance and reduced recovery can be observed for phosphorylated species when these experience nonspecific interactions with metals in the flow path.[1–3] It has been demonstrated that, compared to classic stainless steel hardware, low-adsorption LC systems and columns drastically boost performance for the analysis of nucleotides[4–6], phosphorylated peptides[7], phosphorylated glycans[8], and phosphorylated vitamins[9].

Phospholipids with the phosphate group(s) present as a monoester (PA, LPA, and PIPs) are particularly prone to adsorption on stainless steel surfaces. PIPs can exist as mono-, di-, and tri-phosphorylated species (PIP, PIP2, and PIP3, respectively) (Figure 1). The higher the degree of phosphorylation, the more sensitive the phospholipid is for adsorption on stainless steel surfaces.

This application note compares the performance of the 1290 Infinity II Bio LC System with its stainless steel counterpart, the Agilent 1290 Infinity II LC System, in the analysis of a selection of phospholipids. Separations are performed using low-adsorption polyether ether ketone (PEEK)-lined and stainless steel reversed-phase columns, with detection using UV detection and the InfinityLab LC/MSD iQ, an easy-to-use mass selective detector based on single quadrupole technology.

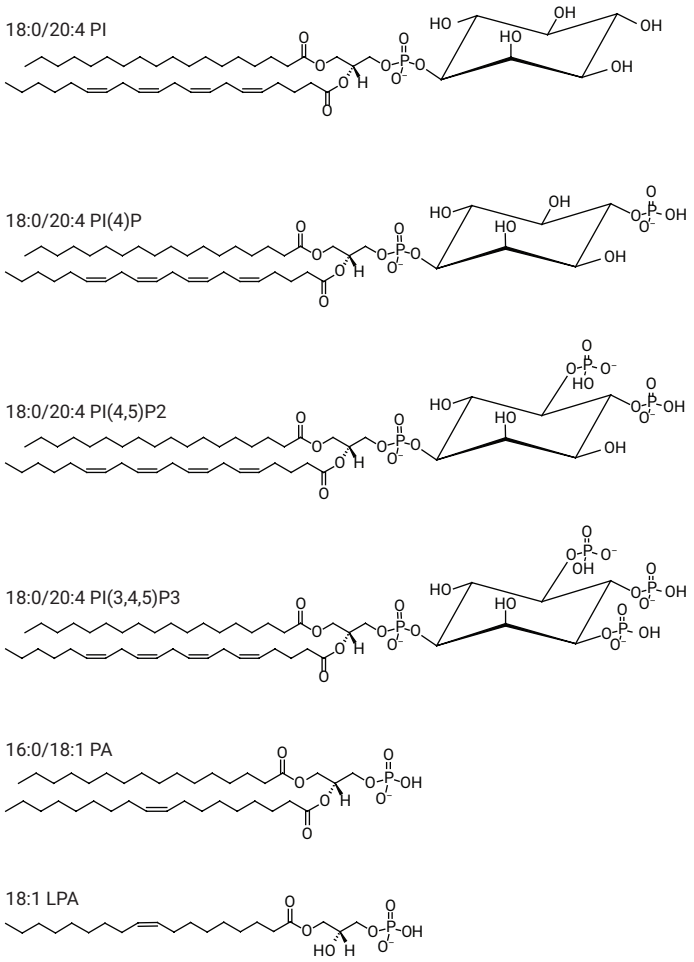


Figure 1. Example structures of the phospholipids used in this work.

Experimental

Materials

Ammonium acetate and acetic acid (both LC/MS grade) were obtained from Merck (Darmstadt, Germany). Water (ULC/MS CC/SFC grade), methanol (ULC/MS CC/SFC grade), isopropanol (HPLC grade), and chloroform (HPLC grade) were supplied by Biosolve (Valkenswaard, The Netherlands). Phospholipids were acquired from Avanti Polar Lipids (Birmingham, AL, USA), individually dissolved in chloroform/methanol, and further diluted and mixed in methanol. The phospholipids used in this work and their concentration in the test mix are summarized in Table 1.

Table 1. Details of the phospholipids used in this work.

Class	Type	CAS	Part Number	Salt	Concentration in Test Mix (µg/mL)
Lyso-Phosphatidic Acid	LPA C18:1	325465-93-8	857130P	Sodium	25
Phosphatidic Acid	PA 16:0/18:1	169437-35-8	840857P	Sodium	50
Phosphatidylinositol	PI 18:0/20:4	1331751-28-0	850144P	Ammonium	50
Phosphatidylinositol Monophosphate	Brain PI(4)P (fatty acid distribution)	475995-51-8	840045P	Ammonium	100
Phosphatidylinositol Diphosphate	Brain PI(4,5)P2 (fatty acid distribution)	383907-42-4	840046P	Ammonium	100
Phosphatidylinositol Triphosphate	18:0/20:4 PI(3,4,5)P3	799268-60-3	850166P	Ammonium	100

Instrumentation and method

Two Agilent LC systems were used: the 1290 Infinity II LC (stainless steel, SST) and the 1290 Infinity II Bio LC (biocompatible, BIO). Details of both configurations can be found in Table 2 and method parameters are summarized in Table 3. Data were acquired and processed in Agilent OpenLab CDS version 2.6 and Agilent MassHunter Qualitative Analysis version 10.0.

Table 2. Details of the LC and MS systems used.

	SST LC System	BIO LC System
Pump	Agilent 1290 Infinity II High-Speed Pump (G7120A)	Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
Autosampler	Agilent 1290 Infinity II Multisampler (G7167B) with integrated sample thermostat	Agilent 1290 Infinity II Bio Multisampler (G7137A) with integrated sample thermostat
Column Compartment	Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow (G7116-60015)	Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Bio heat exchanger, standard flow (G7116-60071)
Detector	Agilent 1290 Infinity II DAD (G7117B)	Agilent 1290 Infinity II DAD (G7117B)
Flow Cell	Agilent InfinityLab Max-Light Cartridge Cell, standard, 10 mm (G4212-60008)	Agilent InfinityLab Max-Light Cartridge Cell, LSS, 10 mm (G7117-60020)
MSD	Agilent InfinityLab LC/MSD iQ (G6160A)	Agilent InfinityLab LC/MSD iQ (G6160A)

Table 3. LC method parameters.

Parameter	Details
Columns	C18 column, 2.1 × 100 mm, 1.9 µm in stainless steel and PEEK-lined versions
Flow Rate	0.4 mL/min
Mobile Phase	A) 10 mM ammonium acetate + 0.05% acetic acid in water:methanol 50:50 (v:v) B) 10 mM ammonium acetate + 0.05% acetic acid in methanol:isopropanol 50:50 (v:v)
Gradient	Time (min) %B 0 50 12 100 15 100 Post-time (3 min) 50
Injection	Variable (1, 2, or 5 µL)
Needle Wash	Flush port, 3 s, methanol
Autosampler Temperature	15 °C
Column Temperature	50 °C
Detection DAD	210/4 nm, reference 360/80 nm, peak width >0.013 min (20 Hz), collect all spectra
Detection MSD	
ESI	Negative ionization
Source	Drying gas temperature: 320 °C Drying gas flow: 12 L/min Nebulizer: 45 psi Capillary voltage: 4,000 V
Acquisition Scan	*m/z* 300 to 1,300 Fragmentor: 110 V; gain factor: 1; threshold: 150; targeted points per second: 1.5 Hz; scan time: 664 ms
Acquisition SIM	LPA: *m/z* 435.2 and 871.5 (dimer) PA: *m/z* 673.4 PI: *m/z* 885.5 PIP: *m/z* 965.5 and 482.3 (double charged) PIP2: *m/z* 1,045.5 and 522.4 (double charged) PIP3: *m/z* 1,125.5 and 562.3 (double charged) Fragmentor: 110 V; gain factor: 1; targeted points per second: 1.5 Hz; dwell time: 65 ms

Results and discussion

The phospholipid mixture was analyzed with various column and system combinations. The mass spectra obtained in MS scan mode are displayed in Figure 2. In negative ionization mode, all phospholipids show a deprotonated ion ($[M-H]^-$), LPA displays an additional dimeric ion ($[2M-H]^-$), and the PIPs are detected as single- and double-charged ions ($[M-2H]^{2-}$). Characteristic *m/z* values were used for selected ion monitoring (SIM).

Figure 3 presents the data obtained with the LC/MS SIM method. Using a PEEK-lined column on a biocompatible LC system, all species, including the PIPs, are clearly detected. Upon using the same PEEK-lined column on a stainless steel LC system, the intensity of the tri-phosphorylated PIP3 decreases compared to the other phospholipids. This observation confirms the benefit of using a low-adsorption LC system for such challenging analytes. Replacing the PEEK-lined column with a stainless steel version further deteriorates the separation performance. On a stainless steel LC system, PIP2 and PIP3 are hardly detected. Even the mono-phosphorylated species such as PA and LPA start to tail, indicating that nonspecific interactions occur. The use of the stainless steel column on a biocompatible LC system slightly improves recovery and peak shape, but the implementation of a complete low-adsorption flow path, as provided by the 1290 Infinity II Bio LC and PEEK-lined column, clearly generates the best data in terms of chromatographic performance and recovery. PI with the phosphate present in a phosphodiester link shows particularly good performance on all combinations tested.

This can also be deduced from the graphs presented in Figures 4 and 5. Figure 4 displays the peak areas for all PIPs calculated using the DAD signal. While the area for PI remains relatively constant over all conditions tested, the area for the PIPs decreases as more metal components are introduced to the flow path. The more pronounced recovery drop is noticed for PIP3. As described in earlier reports[5,6,8,9], the higher the degree of phosphorylation, the more pronounced the effect of unwanted adsorption. Peak shape was evaluated for all phospholipids using the MS SIM data. The tailing factor was calculated on the extracted ion chromatograms (EICs) for the $[M-H]^-$ ion of each of the species (Figure 5). As observed for the peak area, the tailing factor for PI is constant, but for all other phospholipids, the peak quality gets worse when moving away from the biocompatible LC and PEEK-lined column. Both the system and column play an important role. On stainless steel configurations, PIP2 and PIP3 are hardly or not at all detected.

PIP and PIP2 (extracted from porcine brain tissue) exist as a mixture of compounds differing in fatty-acid composition. This makes these phospholipids an interesting case to further evaluate the impact of low-adsorption hardware on analytical performance. Figure 6 displays the DAD and MS scan base-peak chromatograms (BPCs). When using the 1290 Infinity II Bio LC System and PEEK-lined column, the minor PIPs can be detected with DAD and MS. When the analysis is carried out on the PEEK-lined column using a classic LC system, the signal intensity in DAD clearly decreases. The MS result shown for these two conditions gives an even better insight into the performance drop and shows that besides recovery loss, chromatographic performance is drastically impacted as well. Upon switching to stainless steel hardware, these fatty acid variants become challenging to detect. This once more demonstrates the importance of using low-adsorption materials in the column as well as the LC instrument.

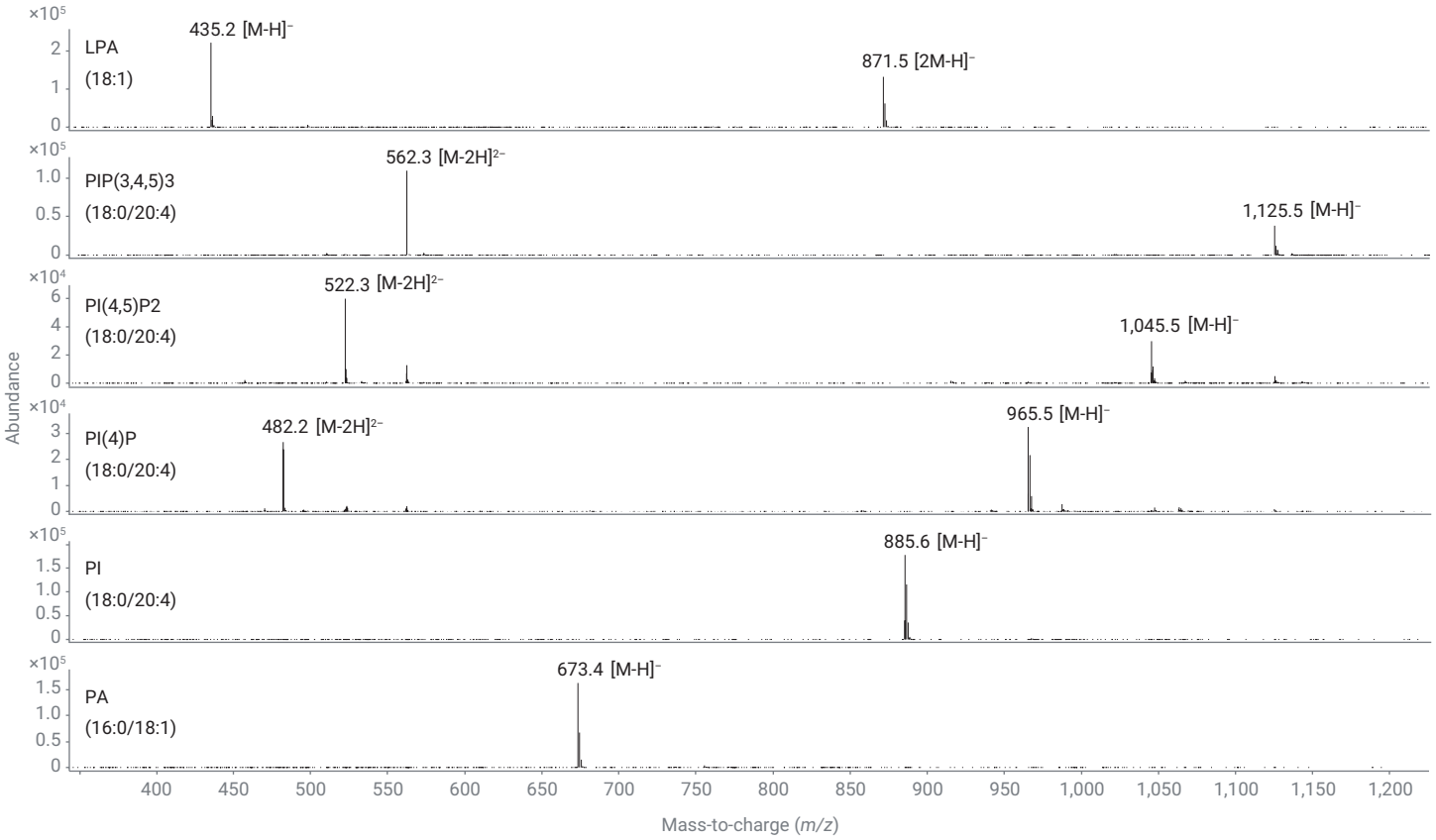


Figure 2. Mass spectra of the phospholipids.

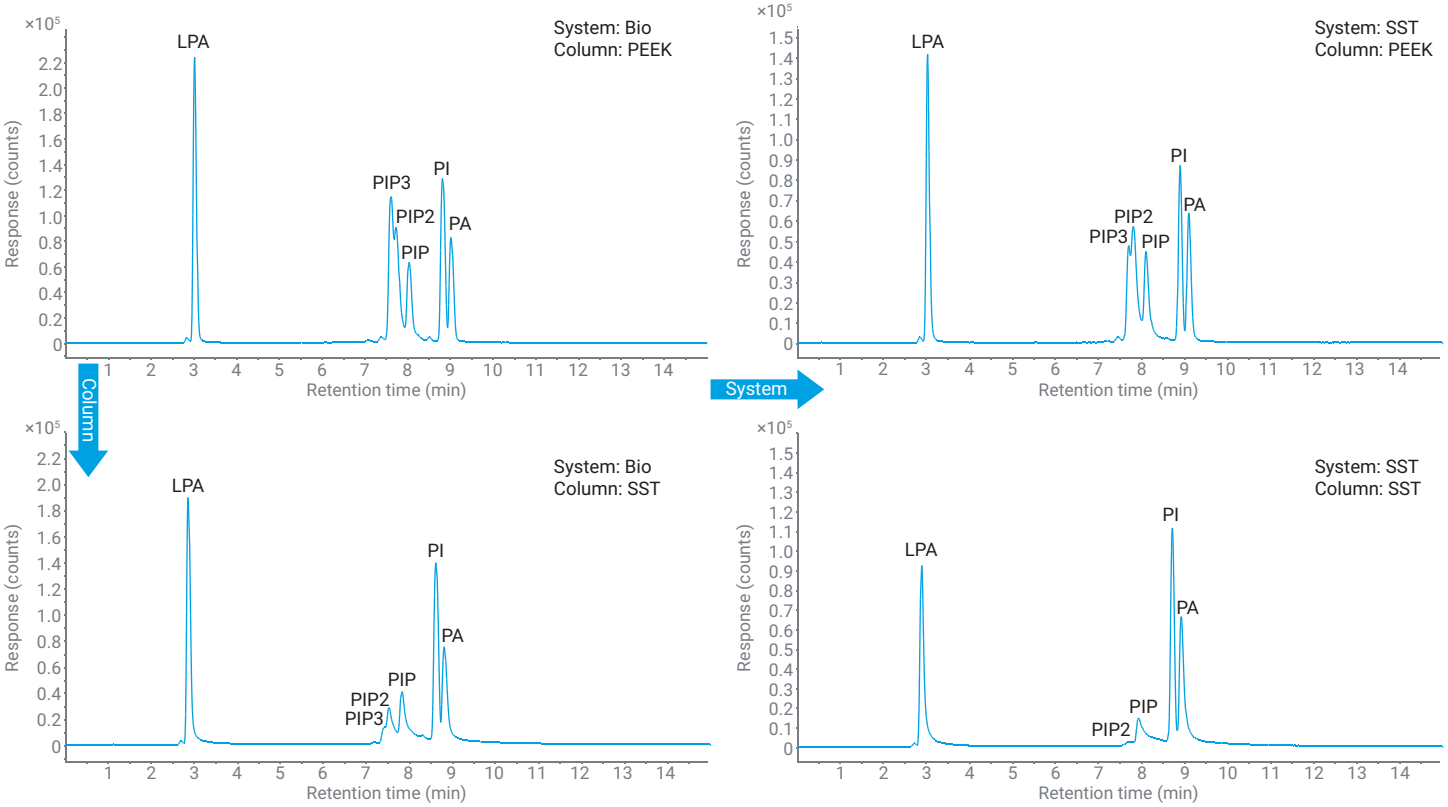


Figure 3. LC/MS (SIM) chromatograms obtained on various LC and column combinations: 2 µL injection.

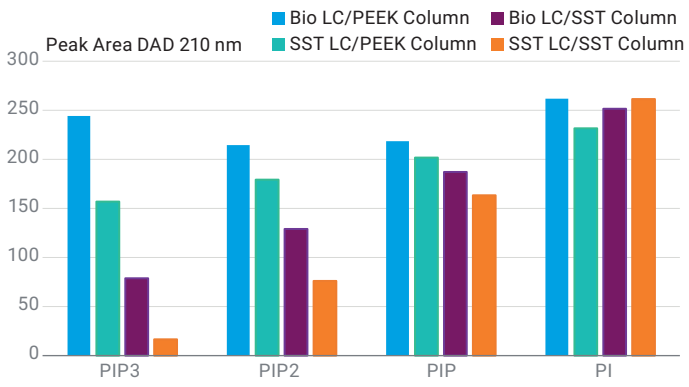


Figure 4. DAD peak area for PI and PIPs on the various LC and column combinations: 5 µL injection.

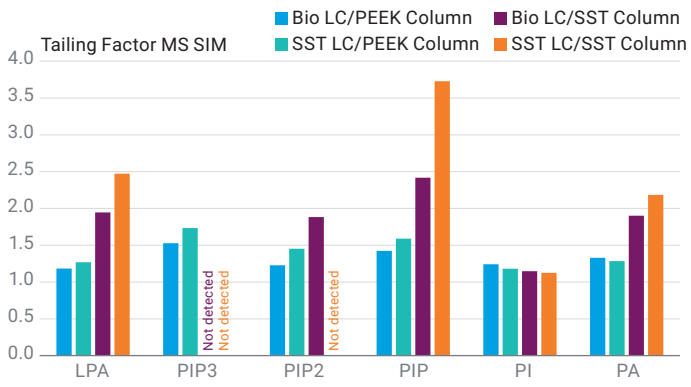
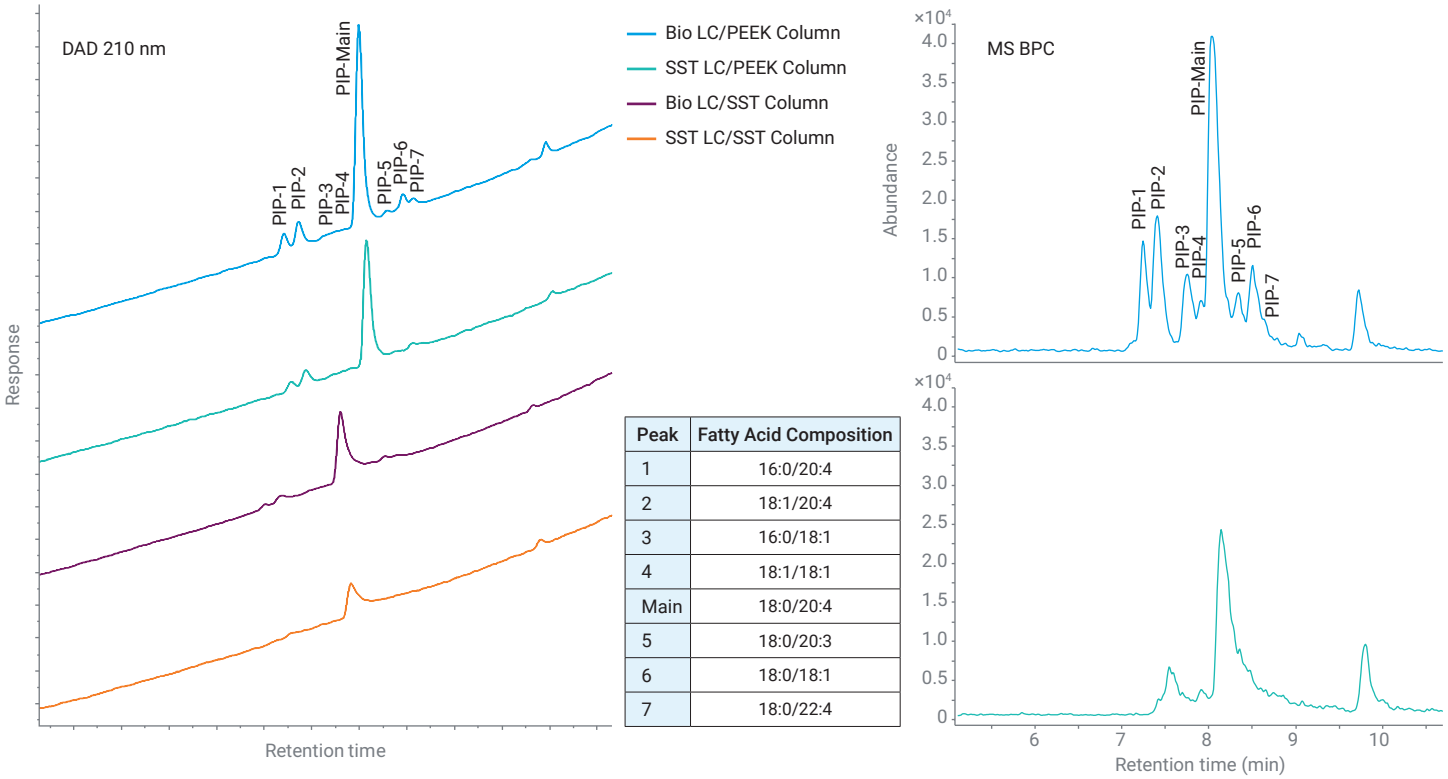


Figure 5. MS SIM (extracted ion $[M-H]^-$) peak tailing factor on the various LC and column combinations: 2 µL injection.



Peak	Fatty Acid Composition
1	16:0/20:4
2	18:1/20:4
3	16:0/18:1
4	18:1/18:1
Main	18:0/20:4
5	18:0/20:3
6	18:0/18:1
7	18:0/22:4

Figure 6. Influence of system and column hardware on the chromatographic performance of PIP. DAD and MS BPC scan are shown: 5 µL injection.

Conclusion

The benefits of using low-adsorption flow paths for the analysis of phospholipids such as PA, PIP, PIP2, and PIP3 are clearly demonstrated in this application note. The di- and tri-phosphorylated PIPs are particularly affected by the nonspecific interaction with stainless steel surfaces in classical LC hardware. Interestingly, both the LC system and the column contribute to this adsorption phenomenon and the negative effect increases with the degree of phosphorylation. Therefore, the Agilent 1290 Infinity II Bio LC proves to be an excellent tool for the analysis of challenging phospholipids such as PA and PIPs and allows detection of these compounds with MS-compatible conditions using plain mobile phases.

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