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A novel strategy for reducing phospholipids-based matrix effect in LC–ESI-MS bioanalysis by means of HybridSPE

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ABSTRACT

A novel strategy to minimize phospholipids-based matrix effects in bioanalytical LC–MS/MS assays was evaluated. The phospholipids-based matrix effect was investigated with a commercially available electrospray ionization (ESI) source coupled with a triple quadrupole mass spectrometer. A systematic comparison approach of two sample preparation procedures was performed. In particular, the matrix effect on mass spectrometry response in rat and human plasma samples was studied by comparing sample extracts obtained by means of a conventional plasma protein precipitation with acetonitrile and the novel HybridSPE-Precipitation procedure.

The HybridSPE dramatically reduced the levels of residual phospholipids in biological samples, leading to significant reduction in matrix effects. This new procedure which combines the simplicity of precipitation with the selectivity of SPE allows to obtain much cleaner extracts than with conventional procedures. The effective targeted removal of phospholipids and proteins in biological plasma samples achieved with the HybridSPE-Precipitation procedure provides significant improvement in bioanalysis and a practical and fast way to ensure the avoidance of phospholipids-based matrix effects.

1. Introduction

High-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) due to its high degree of sensitivity and selectivity represents the widely used method for quantitative bioanalysis of small molecules. However, often MS signals and responses can undergo significant alteration when complex matrices are analyzed and when the analyses are performed without an adequate sample preparation and/or good chromatography [1–7]. Indeed, several endogenous matrix components may co-elute with the target analytes and significantly affect the efficiency and reproducibility of the ionization process [8]. This phenomenon is known as matrix effects and its effect can be measured not only as ionization suppression or enhancement to varying degrees between samples but also as decreased/increased sensitivity of analytes over time [8].

Phospholipids, and in particular glycerophosphocholines and lysophosphatidylcholines, represent the major class of endogenous compounds causing significant matrix effects [2,9]. Phospholipids are an important class of biological compounds containing one or more phosphate groups. For their inherent characteristics these polar lipids are of great importance for the structure and function of cell membranes and are the most abundant of membrane lipids. Although not stored in large amounts in the system [10] are particularly abundant in plasma samples. Their molecular structure exhibit two major functional group regions: a polar head group substituent, which includes an ionizable organic phosphate moiety as well as other polar groups of various types, and one or two long chain fatty acid ester groups, which impart considerable hydrophobicity to the molecule. In particular, the highly ionic nature of phospholipids make them responsible for influencing the ionization in electrospray MS sources [11,12] and desolvation of the LC effluent droplets in electrospray MS analysis [13].

Therefore, the removal of phospholipids represents an extremely important step in the sample preparation process [14].

Several techniques have been applied toward the removal of phospholipids in order to obtain cleaner sample extracts. Most of them are based on solid-phase extraction procedures using strong cation exchange sorbents [12], automated on-line SPE [15]

Abbreviations: PPT, plasma protein precipitation; ACN, acetonitrile; ESI, electrospray ionization; MRM, multiple reaction monitoring; SPE, solid-phase extraction; MS, mass spectrometer.

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or liquid/liquid extraction with methyl *tert*-butyl ether followed by solid-phase extraction on chemical sorbents containing active lanthanide [16,17]. Recently the feasibility of colloidal silica in combination with a number of divalent or trivalent cations for the removal of plasma phospholipids was evaluated [18,19] as well. Although effective, these procedures have the disadvantage of introducing further time consuming steps in the cleanup process. The goal of our study was to evaluate the novel and commercially available zirconia sorbent of the HybridSPE-Precipitation 96-well plate which exhibits a high affinity for phospholipids remaining non-selective toward a wide range of basic, neutral and acidic compounds. To this end, a comparison of HybridSPE-Precipitation technology and classical protein precipitation has been carried out in order to evaluate and estimate the phospholipids-based matrix effect together with the ability to extract model compounds while removing phospholipids.

2. Experimental

2.1. Chemicals and solutions

Reference substance of the model analytes were synthesized at IRBM P. Angeletti, Merck Research Laboratories (Pomezia, Rome, Italy). HPLC grade water and methanol were obtained from J.T. Baker (Mallinckrodt Baker B.V., Holland) while acetonitrile LiChrosolv (for chromatography) was purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide and formic acid (analytical grade) was purchased from Fluka (Fluka Chemie AG, Buchs, CH). Drug-free heparinized (heparin lithium) rat and human plasma samples were purchased from Charles River Laboratories (France). Phospholipid analytical reference standards, including glycerophosphocholines and lysophosphatidylcholines, were purchased from Avanti Lipids, Alabaster, Alabama, USA.

The stock solutions (1 mg/ml) of model analytes were made by dissolving a suitable quantity of the standard compound in dimethyl sulfoxide. Standard working solutions were prepared by diluting suitable amounts of each stock solution with water:acetonitrile (50:50, v/v).

2.2. LC–MS/MS apparatus and conditions

HPLC analysis was carried out on an Agilent 1100 series (Palo Alto, CA) HPLC system consisting of a quaternary pump and vacuum degasser equipped with a HTC PAL autosampler system (CTC Analytics, Switzerland) refrigerated at 4 °C during analysis. Chromatography was performed on an YMC pack cyano (CN) (50 mm × 4.6 mm, 5 μm) column at room temperature with an injection volume of 5 μl. The mobile phase consisting of a solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) was delivered at a flow rate of 1500 μl/min. The LC gradient started from 98/2% (A/B) and changed to 0/100% (A/B) at 1.5 min remaining constant to this ratio for 0.8 min. The gradient decreased to 98/2% (A/B) at 2.6 min, remaining constant to this ratio until 3.0 min that was the analysis time.

Detection was carried out using a triple quadrupole tandem mass spectrometer (API4000, Applied Biosystems, Foster City, CA, USA) equipped with an electrospray interface (ESI). Ions were created in the positive ion mode setting the sprayer voltage at 5.5 kV and the ion source temperature at 550 °C. The common parameters and the nitrogen flow values for nebulizer gas, auxiliary gas, curtain gas and the gas for collision-activated dissociation were set at 60, 70, 30 and 4 l/min, respectively. Detection of the ions was performed in the multiple reaction-monitoring (MRM) mode, monitoring the following ion transitions: m/z 474.30 → 203.10 for analyte A; 461.30 → 443.20 for analyte B and 766.50 → 653.20 for analyte C. The dwell time was set at 50 ms with a inter channel pause time of 5 ms to provide optimal sampling of each peak of interest. The optimum declustering potentials were found to be 45, 35 and 70 V and collision energies for the selected transitions were 40, 45 and 35 V, respectively. For all the model compounds the entrance potential and the collision cell exit potential were set to 10 eV. The Analyst 1.4.1 software (Applied Biosystems) was used to control the LC–MS/MS system and to perform analyses.

2.3. Sample preparation methods

2.3.1. Protein precipitation

Blank rat and human plasma samples were extracted as follows. A 300 μl aliquot of 1% formic acid in acetonitrile was added to 100 μl plasma; this was vortexed and centrifuged at 4500 rpm for 10 min. The supernatant was removed, transferred into a clean 96-deep well plate and 5 μl were injected onto the LC–MS/MS system.

The analyte concentrations were selected in order to encompass typical analyte concentrations for bioanalytical assays in a pharmaceutical environment and in particular three different levels for each analyte were tested, namely, 5, 50 and 500 ng/ml.

2.3.2. HybridSPE-Precipitation

HybridSPE-Precipitation 96-well plate (50 mg/well, Sigma–Aldrich/Supelco, Bellefonte Pennsylvania, USA) were used without prior conditioning. To each well a volume of 100 μl of plasma sample was dispensed to which was added of 300 μl of 1% formic acid in acetonitrile. The plate was vortexed and then vacuum applied. The collected resulting eluent was used for injection. The entire procedure has been automated and performed using a Hamilton StarPlus liquid handler robot (Hamilton Robotics, Bonaduz GR, Switzerland).

As for protein precipitation the model analytes were analyzed in triplicate at three different concentrations, namely, 5, 50 and 500 ng/ml.

2.4. Matrix effect and recovery evaluation

To evaluate assay specificity, pooled blank rat and human plasma samples from six different lots were extracted and analyzed for endogenous co-eluting interference causing matrix effect. Three replicates of extracted and unextracted model analytes samples and a set of post-extracted spiked samples were analyzed using the same assay run conditions to determine the extraction recovery and matrix effect. The extraction efficiency (or recovery) was determined by measuring an extracted sample against a post-extraction spiked sample:

$$\%\,\text{recovery} = \left(\frac{A}{B}\right) 100\%$$

where, A is the response for the extracted samples and B the response for post-extracted spiked samples.

The matrix effect was measured by referring the post-extracted spiked sample to the unextracted sample, using the following equation:

$$\%\,\text{matrix effect} = \frac{(B - C)}{C} \times 100$$

where, C is the peak area of a neat standard and B is the corresponding peak area for standards spiked into plasma after extraction.

For these calculations, blank plasma samples were spiked with analytes in a 25/75 mixture of water:acetonitrile (v/v) with a final spike volume of 5% compared to the total plasma volume. The same volume of blank plasma was spiked with 5% by volume of 25/75 water:acetonitrile. The post-spiked reconstitution solvent was prepared in 25/75 water:acetonitrile, using the same volumes as for the

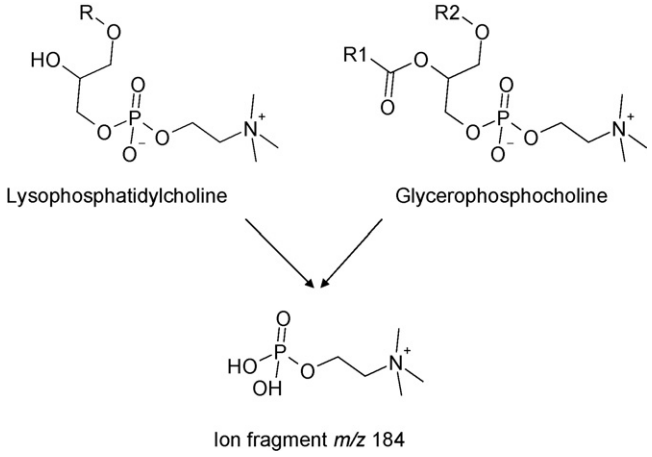

Fig. 1. Chemical structures of lysophosphatidylcholines and glycerophosphocholines and their trimethylammonium-ethyl phosphate ion fragment (*m*/*z* 184).

plasma samples. Dried-down blank plasma samples were reconstituted in the post-spike reconstitution solvent.

3. Results and discussion

3.1. Monitoring phospholipids

Glycerophosphocholines and lysophosphatidylcholines represent respectively the 70% and 10% of total plasma phospholipids [14] and are the major source that can cause matrix effects. For this reason five naturally occurring phospholipids, including two lysophospholipids, were used to monitor phospholipids removal from biological extracts [18].

The lysophosphatidylcholines monitored were 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (*m*/*z* 496) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (*m*/*z* 524). Within the glycerophosphocholines class, three different and most abundant phospholipids were also monitored, namely a glycerophosphocholine lipid with a parent ion of 704 *m*/*z*, the 1-hexadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine (*m*/*z* 758) and 1-(9Z,12Z-octadecadienoyl)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine (*m*/*z* 806).

All these five species of phospholipids exhibit MRM transitions via TurboIonSpray in positive ionization mode, and have been shown to fragment to form trimethylammonium-ethyl phosphate ions (*m*/*z* 184) in MS/MS (Fig. 1).

The phospholipids MRM transitions using trimethylammonium-ethyl phosphate ion fragment (*m*/*z* 184) were monitored in both plasma extract after plasma protein precipitation and HybridSPE-Precipitation setting the declustering potential at 65 V and the collision energy at 55 V.

3.2. Comparison of protein precipitation to Hybrid SPE-Precipitation

Although the fast and inexpensive conventional protein precipitation using centrifugation efficiently removes proteins does not always result in a very clean final extract. Indeed very often this procedure is not adequate to sufficiently remove phospholipids, known to cause variability in analyte signal intensity in LC–MS/MS bioanalysis. On the contrary, a new sample preparation platform trademarked HybridSPE-Precipitation has been recently introduced in the market which combines the two predominant bioanalytical sample preparation procedures, namely, protein precipitation and SPE.

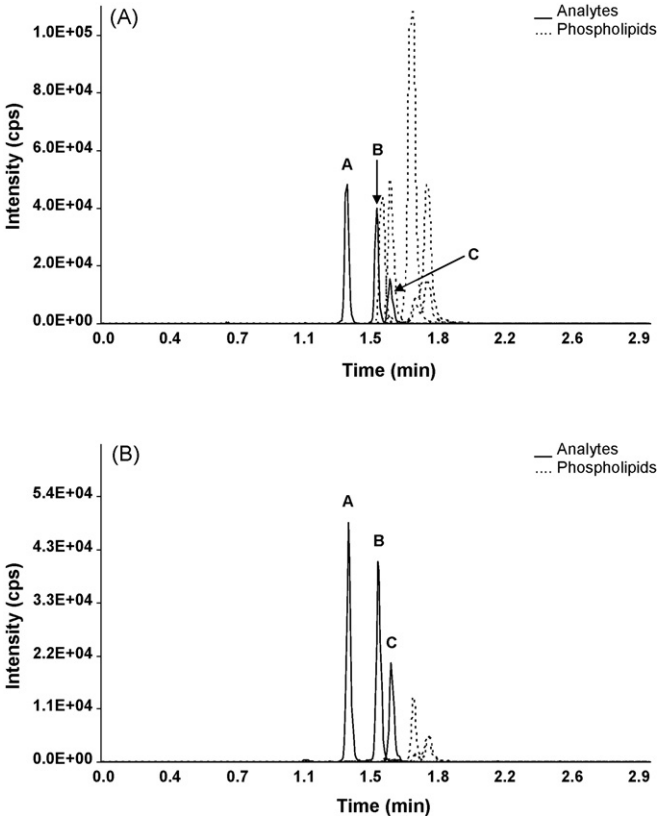

Fig. 2. Mass chromatograms of a spiked rat plasma sample after PPT procedure (a) and the same rat plasma sample spiked with the three model analytes at 5 ng/ml after HybridSPE-Precipitation (b). For experimental conditions, see Section 2. The five monitored phospholipids had the following retention times, *m*/*z* 496 (R_t = 1.53 min), *m*/*z* 524 (R_t = 1.58 min), *m*/*z* 704 (R_t = 1.71 min), *m*/*z* 758 (R_t = 1.78 min) and *m*/*z* 806 (R_t = 1.79 min).

The HybridSPE-Precipitation plates provide an easy format for simple, rapid and automatable protein precipitation and selective removal of phospholipids in a standard 96-well format. The HybridSPE-Precipitation plates technology utilizes a patent pending zirconia-coated particle, and exhibits selective affinity towards phospholipids while remaining non-selective towards a range of basic, neutral and acidic compounds. The phospholipid retention mechanism is based on highly selective Lewis acid–base interaction between the proprietary zirconia ions (functionally bonded to the HybridSPE stationary phase) and the phosphate moiety consistent with all phospholipids.

Preliminary studies were carried out aiming to select the proper analytes displaying significant ion suppression due to the presence of phospholipids. A peak area comparison between analytes directly injected to analytes spiked into 1.5 mg/ml phospholipids solution has been carried out. Results from these initial experiments (data not shown) clearly showed phospholipids-based matrix effect for three model analytes with acidic (A), zwitterionic (B) and basic characteristics (B). Therefore these three model compounds were taken further for the present evaluation.

The comparison of the MRM chromatograms (Figs. 2 and 3) clearly shows a significant improvement in overall phospholipids cleanliness when HybridSPE-Precipitation is used.

It is clear that for both rat and human plasma samples the HybridSPE-Precipitation method provides significantly cleaner extracts than PPT with an efficient removal of phospholipids.

Table 1
Calculated % extraction yields of the three model analytes from PPT and HybridSPE-Precipitation extracts of both rat and human plasma samples.

Analyte	Concentration ng/ml	PPT		HybridSPE-Precipitation	
		Rat	Human	Rat	Human
A *m/z* 474.30	5	113.5 ± 0.9	126.6 ± 11.5	103.8 ± 5.6	124.9 ± 2.0
	50	119.8 ± 3.4	112.2 ± 4.0	98.6 ± 2.6	111.2 ± 3.4
	500	90.2 ± 2.4	98.7 ± 2.8	91.8 ± 1.2	90.0 ± 4.5
B *m/z* 461.30	5	99.7 ± 2.5	125.3 ± 6.9	109.9 ± 21.1	87.2 ± 11.7
	50	98.3 ± 2.3	112.2 ± 1.4	117.1 ± 12.5	73.9 ± 13.7
	500	92.0 ± 2.3	96.8 ± 1.2	85.6 ± 1.2	86.5 ± 2.5
C *m/z* 766.50	5	86.2 ± 27.4	132.9 ± 13.1	109.5 ± 5.3	81.6 ± 7.3
	50	97.3 ± 4.8	108.2 ± 2.4	113.6 ± 5.0	116.6 ± 2.9
	500	91.1 ± 3.0	95.5 ± 4.0	108.9 ± 3.5	94.0 ± 4.6

Results are the mean ± standard deviation, $n = 3$. Analytes are sorted by retention time in particular A (R_t = 1.34 min), B (R_t = 1.51 min) and C (R_t = 1.59 min).

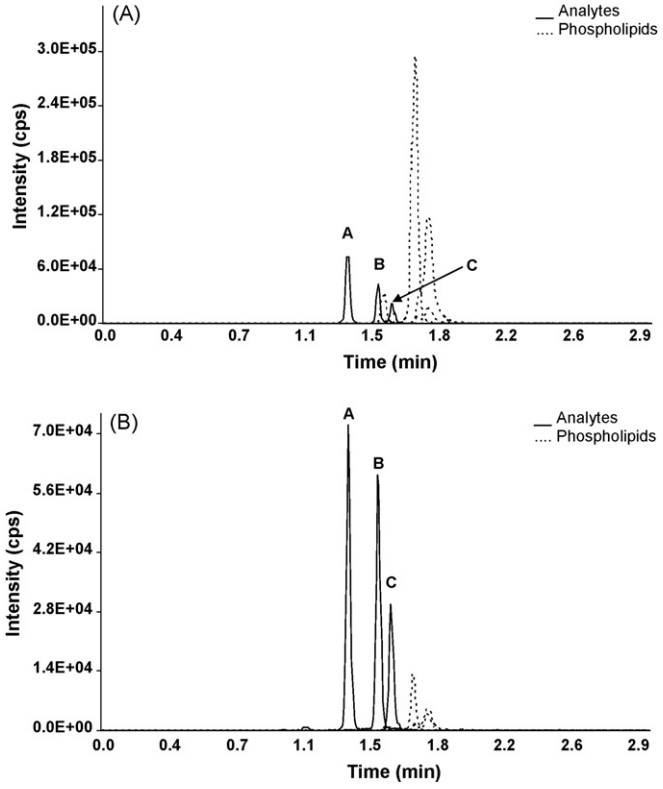

Fig. 3. Mass chromatograms of a spiked human plasma sample after PPT procedure (a) and the same human plasma sample spiked with the three model analytes at 5 ng/ml after HybridSPE-Precipitation (b). For experimental conditions, see Section 2. The five monitored phospholipids had the following retention times, *m/z* 496 (R_t = 1.53 min), *m/z* 524 (R_t = 1.58 min), *m/z* 704 (R_t = 1.71 min), *m/z* 758 (R_t = 1.78 min) and *m/z* 806 (R_t = 1.79 min).

3.3. Matrix effect and extraction yield evaluation

The absolute matrix effect (matrix suppression or enhancement of ionization), of the three model analytes was determined by comparing the mean peak areas of the post-spiked with those of three different aqueous standard solutions at the same concentrations (namely, 5, 50 and 500 ng/ml). Three replicates of extracted and unextracted samples and a set of post-extracted spiked samples were analyzed in the same assay run to determine the extraction recovery and matrix effect. The extraction efficiency (or recovery) was determined by measuring an extracted sample against a post-extraction spiked sample. The extraction yield was determined as well as the response of an unextracted sample and the response of the same extracted sample. Extraction yields data were calculated at three different concentrations, namely, 5, 50 and 500 ng/ml and are summarized in Table 1. The results were very good, ranging from 73.9% to 124.9% confirming the high efficiency of the HybridSPE-Precipitation extraction procedure.

The matrix effect was measured by referring the post-extracted spiked sample to the unextracted sample. The results, as summarized in Table 2, clearly indicated that matrix effect is consistently more evident in both rat and human matrices when the simple protein precipitation is performed. In particular for the model analyte B the relative matrix effect occurring at the lower concentration in human plasma is drastically decreased from −34.8% to −5.1% when the samples are treated by means of HybridSPE-Precipitation extraction procedure. This result is in line with the presence of endogenous interference monitored using the phospholipids MRM transitions (see Fig. 3). Moreover, this suggest that simply monitoring these MRM transitions could represent a less cumbersome alternative to post-column infusion and could be a useful tool during method development to estimate the potential phospholipids interferences for ion suppression during extraction method optimization.

Table 2
Calculated % matrix effect of the three model analytes from PPT and HybridSPE-Precipitation extracts of both rat and human plasma samples.

Analyte	Concentration ng/ml	PPT		HybridSPE-Precipitation	
		Rat	Human	Rat	Human
A *m/z* 474.30	5	11.5 ± 13.3	−14.0 ± 8.5	10.0 ± 1.5	−0.5 ± 1.6
	50	7.8 ± 4.1	−14.5 ± 2.4	−1.9 ± 4.3	1.5 ± 1.4
	500	2.9 ± 3.0	−16.4 ± 3.4	0.1 ± 6.1	1.5 ± 4.0
B *m/z* 461.30	5	−24.9 ± 2.5	−34.8 ± 2.6	−4.9 ± 4.7	−5.1 ± 3.0
	50	−32.2 ± 2.0	−32.0 ± 1.1	−10.4 ± 1.5	−8.4 ± 0.2
	500	−28.1 ± 0.7	−24.9 ± 0.6	−0.5 ± 5.0	1.9 ± 2.4
C *m/z* 766.50	5	5.0 ± 7.6	−25.5 ± 12.1	5.0 ± 0.9	15.0 ± 5.3
	50	−5.0 ± 4.5	−21.2 ± 2.8	6.0 ± 2.0	3.5 ± 4.5
	500	−8.6 ± 1.0	−20.3 ± 2.6	3.0 ± 1.6	5.6 ± 4.8

Results are the mean ± standard deviation, $n = 3$. Analytes are sorted by retention time in particular A (R_t = 1.34 min), B (R_t = 1.51 min) and C (R_t = 1.59 min).

To test the overall performance of the HybridSPE-Precipitation extraction procedure for high-throughput bioanalysis, calibration curves were weighted using both extraction procedures. The lower limit of quantitation (LLOQ) was 0.2 ng/ml in both rat and human plasma for all compounds and linearity was obtained over the concentration range of 0.2–500 ng/ml using HybridSPE-Precipitation. On the contrary when PPT was used the LLOQ was 0.6 ng/ml and linearity was observed in the 0.6–500 ng/ml concentration range. The means of the calibration parameters, slope ± standard deviation, intercept ± standard deviation and correlation coefficient for the model compound B in human plasma, were -224.9 ± 13.8, 234.3 ± 6.1 and 0.9981 for PPT and 1284.7 ± 5.9, 5700.1 ± 10.5 and 0.9997 for HybridSPE-Precipitation, respectively. From the comparison of the validation data it is evident a significant improvement in terms of sensitivity by means of HybridSPE-Precipitation. A 0.2 ng/ml lower limit of quantitation (LLOQ) was obtained in all three plasma matrices and the results demonstrated that the HybridSPE-Precipitation enabled better calibration curves.

4. Conclusions

A systematic comparison of the simple plasma protein precipitation procedure to the new sample preparation platform HybridSPE-Precipitation, with respect to extract cleanliness, matrix effects and analyte extraction yields (% recovery) has been carried out.

The obtained results indicated that although plasma protein precipitation can sometimes fulfill the requirements of extraction yield, is not adequate to sufficiently remove phospholipids. Moreover, the monitoring of the MRM transitions of phospholipids and the matrix effect evaluation clearly indicated that endogenous phospholipids in both rat and human plasma PPT extracts are a significant tool in order to evaluate phospholipids-based matrix effects in LC–MS/MS analyses leading to ion suppression in bioanalytical determinations.

On the contrary, the novel HybridSPE-Precipitation showed to represent a useful and effective tool in providing cleaner extract and in reducing or eliminating phospholipids-based matrix effects.

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