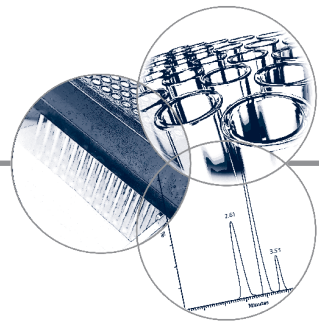

Phospholipid-based matrix effects in LC–MS bioanalysis

"…manipulating chromatographic separation in LC–MS is often the least effective among the approaches to reduce matrix effects from phospholipids. The reason behind this phenomenon, which is also the cause of matrix interference, is their contradictory chromatographic elution behavior."

Keywords: biological matrices ■ LC–MS bioanalysis ■ matrix effect ■ phospholipids ■ sample preparation

LC–MS/MS is now the method of choice for a very broad range of applications in quantitative bioanalysis, owing to its inherent high selectivity and sensitivity [1,2]. However, at the same time, matrix effects are often encountered as an obstacle for trace bioanalysis [3,4]. Matrix effects refers to the enhancement or suppression of the analyte response caused by co-eluting endogenous matrix constituents either through influencing ionization or simply acting as isobaric interferences as initially recognized by Kebarle *et al.* in the early 1990s [5]. For instance, its impact on the accuracy, precision and robustness of analytical methods is of growing concern in clinical and pharmaceutical research dealing with a biological matrix, such as plasma, urine, blood, bile, feces, tissues, cell-incubation media and pharmaceutical formulations. Matrix effects may result from lipids (especially phospholipids [PLs]), proteins, salts, drugs and metabolites, other endogenous compounds, buffers, ion-pairing agents, dosing-formulation agents and exogenous contamination, to name just a few. Although the matrix effect has been drawing more and more attention from researchers to discuss or to evaluate in method development and validation, only in some published methods is an actual step included to minimize this interference. For bioanalysis, under certain investigated conditions, various approaches reported in the literature have been proven to be effective at removing interferences from PLs or for a general purpose [3,6]. However, because of the huge diversity of the biological matrices and especially their possible accumulation on the separation column, the interferences are not always reproducible and predictable. Currently, there is still no universal solution to this problem [3]. The matrix effect caused by PL is somehow underestimated when we look deeply into the sources of lipids and commonly applied compromising sample-preparation methods in practice. To reveal this, one should have a close investigation of these notorious PL species and, especially, their chromatographic behavior in general, which results in the persistent matrix effect.

Broad diversity of PL

As the main constituent of cell membranes, PLs are the main source of matrix effects in LC–MS bioanalysis. They can be found in all biological matrices in significant concentrations [7]. The matrix effect caused by PLs is one of the greatest challenges in method development and validation. The difficulty of completely eliminating their severe interference comes not only from their high concentrations and overall presence, but also, more importantly, from their diversity with regard to classes, subclasses, polarities and molecular weights. Reportedly, as active biomolecules participating in biological activities, PLs can range from neutral to polar and ionic molecules, predominantly with the skeletons of nonpolar fatty chains attached to a polar phosphate. In addition, lysophospholipids (with only one fatty chain), free fatty acids and numerous metabolites and analogues have also been identified [8]. For instance, with the improvement in identification and detector sensitivities, the remarkable diversity of over 500 distinct lipid molecular species from six major categories [9,10], in pooled human plasma, has been reported in recent studies [11]. Due to their unique structural features, most lipid molecules behave like a surfactant, which makes the clean-up efforts less effective. As a result of this diversity, and their intrinsic structural

Xinghua Guo
Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology, Stremayrgasse 9, 8010 Graz, Austria
†Author for correspondence:
Tel.: +43 316 873 32511
Fax: +43 316 873 32502
E-mail: x.guo@tugraz.at

Ernst Lankmayr
Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology

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characteristics, the matrix effect caused by PLs actually penetrates into every corner of trace bioanalysis, from nonpolar to polar analytes and both small- and large-molecule quantification.

Effectiveness of current strategies to reduce or eliminate matrix effects

Several strategies have been dominating with regards to the reduction of matrix effects in general [3,6]. Through proper calibration, such as the following, matrix effects can be partially compensated:

- Matrix-matched external calibration;
- Proper internal standardization;
- Eco-peak technique (injections of a sample and reference standard with a short interval [12]).

The eco-peak technique is less frequently used. However, even the first two approaches can only have limited applications due to the scarcity of an appropriate perfectly matrix-matched sample as the blank or the stable isotope-labeled analytes. The application of analyte analogues as internal standards can also be problematic because the matrix effect is actually dependent on the retention time. A very recent study has shown that, even with stable isotope-labeled standards, ion enhancement of approximately 500% for omeprazole in urine was observed in one lot, but not in the others, which was confirmed to be resulting from a combination of non-uniform matrix and nonlinear LC–MS detector response [13]. In addition, it is also reported that, by modifying the MS ionization and detection, it is possible to reduce or prevent the matrix effect, for instance, by switching the ionization mode or the source design [3]. However, the presence of matrix components in samples is still a potential risk for the subsequent LC–MS analysis because of the possible unexpected effect, or accumulation, during chromatographic separation. Accordingly, the best cure to this problem is to remove the troublesome matrix components before injection into the instrument. The techniques used to separate the components causing matrix effects include on- and off-line clean-ups in sample preparation (e.g., protein precipitation, liquid–liquid extraction [LLE], SPE [14], turbulent-flow chromatography with valve switching [15] and ultrafiltration) and/or, otherwise, improvement in chromatographic separation in LC–MS analysis [3,16–18].

Chambers *et al.* have systematicall investigated various sample-preparation strategies for reducing phospholipid matrix effects in LC–MS/MS bioanalysis [14]. They concluded that, although LLE could provide clean final extracts, the analyte recovery, particularly for polar analytes, was very low. Protein precipitation was effective at reducing the matrix effect caused by proteins in other studies, but turned out to be the least effective for PLs. The most popular reverse-phase and pure-cation exchange SPE methods also resulted in cleaner extracts and reduced matrix effects compared with protein precipitation. The cleanest extracts, however, were produced with polymeric mixed-mode SPE with both reverse-phase and ion-exchange retention mechanisms, leading to significant reduction in matrix effects [14]. It was also reported that a combination of polymeric mixed-mode SPE, the appropriate mobile-phase pH and UPLC technology shows significant advantages for reducing matrix effects resulting from plasma constitutes [14]. However, in practice, the best approach to minimize the matrix effect is not always feasible to include in the sample preparation. In addition, another recent study concluded that no single extraction procedure was efficient in removing all of the various lipid components when most PL classes and neutral lipids in blood plasma were considered [19]. This further indicates the persistent interference from PLs.

Elution behavior of PLs in LC

Although it seems less laborious and costly, manipulating chromatographic separation in LC–MS is often the least effective among the approaches to reduce matrix effects from PLs. The reason behind this phenomenon, which is also the cause of matrix interference, is their contradictory chromatographic elution behavior. Since the beginning of PL analysis, only simple LLE with chloroform/methanol [20,21] results in an overwhelmingly united chromatographic behavior for almost all PL, as it provides the most efficient extraction from biological samples. Unfortunately, LLE implementation is not always possible owing to the simultaneous loss of analytes. In other techniques, such as column chromatography, SPE and HPLC involving column chemistry and selectivity, not only is a separation of classes and subclasses difficult but sometimes it also requires extraordinary mobile-phase compositions to elute them from the columns, owing to the wide polarity diversity of PLs. For instance, with a normal-phase column

based either on silica gels or those modified with diol, cyanopropyl and aminopropyl, the polar and acidic phosphatidylserines and phosphatidylinositols will not be recovered well because of the strong adsorption and tailing on SPE or HPLC columns, although it provides a better separation of other classes [22]. PLs co-eluting with analytes will result in instant matrix effect, and those retained on the column will remain and cause problems for the subsequent injection. The latter effect may be partially prevented if this is followed by a regenerating elution step using a mobile phase of a higher ionic strength. On the other hand, with a reverse-phase C_{18} column, the abundant phosphatidylcholines (PCs) with the polar head groups of strong ionic characteristics are eluted later with high organic mobile-phase contents. The problem is also caused by their slow elution, consequently resulting in broad peaks with tailing. In addition, the earlier eluted polar lysophospholipids (also relatively abundant, together with other lysophospholipids) threaten the earlier eluted analytes [17]. Significant variations of the retention times of PLs, PCs and lysophospholipids under different hydrophilic interaction LC conditions have also been reported [23]. As a result, multidimensional LC approaches have also been investigated and proposed for PL analysis [24]. Owing to this, the manipulation of chromatographic separation to avoid matrix effects due to co-elution will not always work. A blank injection after the sample, using isopropyl alcohol to regenerate columns, can significantly reduce the interference; however, it is not implemented in most studies for apparent reasons of costs and analysis time [17,18].

The LC–MS applications most affected by matrix effects include multiresidue bioanalysis, high-throughput application, screening and those with automated sample preparation for pesticides and pollution monitoring, drugs and metabolomics and systems biology investigations. Matrix effects are not only analyte dependent, but also batch matrix dependent [25]. Although a complete elimination can be difficult, reduction is always possible if a careful optimization in method development is involved [26]. For LC–MS trace analysis of biological samples, every possible effect matters and any improvement counts.

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