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Topics in Solid-Phase Extraction

Part 1. Ion Suppression in LC/MS Analysis: A Review

Strategies for its elimination by well-designed, multidimensional solid-phase extraction [SPE] protocols and methods for its quantitative assessment.

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Background

Over the last decade, the combination of high-performance liquid chromatography and mass spectrometry has become the most important tool in many fields, especially when a determination of specific analytes in a complex matrix needs to be performed. Examples are the analysis of drugs and their metabolites in biological fluids, especially blood and urine, and the measurement of residual contaminants in food matrices or in the environment.

The reason for this widespread acceptance is the high selectivity and high sensitivity of MS techniques that permit the detection of very low levels of target analytes in the presence of a complex matrix background. However, experience has taught that, while detection is no longer an obstacle, quantitative determination of the species of interest may be problematic[1]. If a significant background is present, the signals for the analytes of interest are often suppressed. The magnitude of this suppression may vary from sample to sample[1]. Thus, one cannot rely upon the results of an analysis with a high level of ion suppression[2]. Steps must be taken prior to analysis to minimize or eliminate ion suppression effects via sample clean-up techniques[3].

The detailed causes of ion-suppression are not clear and, apparently, can be manifold[4]. Even commonly used mobile phase components such as trifluoroacetic acid cause ion suppression. Of course, the presence of a complex matrix amplifies the problem[5]. This is not only true for plasma[1] or urine[6] samples, but also in the analysis of food samples such as cattle or fish tissues[7], fruits and vegetables[8,9], or environmental water[10,11]. In all these cases, the investigator needs to be concerned about the inaccuracy of the analytical results caused by matrix interferences. Procedures have been established to quantitate matrix effects[12], and the authors of this study have strongly advocated the measurement and elimination of such effects. Only then can one avoid significant errors in the analytical results.

For some period of time, it was believed that the use of isotopically labeled internal standards could be a simple solution to the ion-suppression problem. However, it has been demonstrated that this is not the case. Jemal *et al.*[13] showed that under certain conditions the use of an isotopically labeled standard does not guarantee the constancy of the analyte/internal response ratio. Liang *et al.*[14] demonstrated that isotope-labeled standards either enhance or suppress the response of the analyte, depending on the ionization technique. Enhancement was found in atmospheric pressure chemical ionization, and suppression in electrospray ionization. While the use of isotopically labeled standards may be helpful, it is certainly not a definitive solution to matrix interference problems.

Elimination of matrix-induced ion suppression requires removal of extraneous matrix components via a high-quality sample cleanup. In this paper we will discuss this subject in more detail.

Methods of Sample Preparation

To be analyzed by HPLC, a sample must be in a liquid state, and the liquid should be free of solids. So, some sample preparation is unavoidable for nearly all samples, even for standards. Samples with very low analyte concentration(s) often require enrichment. Sample enrichment is most conveniently carried out using a solid-phase extraction [SPE] procedure, as was done, for example, in reference 15 for the determination of sulfonylurea herbicides in environmental water and soil. In addition, complex samples require, at a minimum, the removal of sample components that can clog, or accumulate as contaminants on, the HPLC column.

A multitude of sample clean-up techniques are available, but they differ in their efficiency of interference removal and in their convenience of use. In addition, they are often specific to the sample matrix, since different matrices contain different components that need to be removed. Let us consider blood plasma as an example. Major constituents here are plasma proteins, sugars, salts, lipids, and a myriad of peptides and small molecules. Sample preparation protocols for plasma range from simple protein precipitation to efficient solid-phase or liquid-liquid extraction methods. Let us discuss the advantages and disadvantages of these techniques.

Protein precipitation is carried out by simply adding a large proportion of acetonitrile to the sample — usually, a ratio of three to four volumes of acetonitrile to one volume of plasma. A lower ratio leads to insufficient protein precipitation. A larger ratio dilutes the sample too much. Because of its simplicity, this procedure remains a popular technique in

blood analysis. However, as its name implies, the only type of contaminant that protein precipitation removes is protein. Other components of the plasma metabolome, *e.g.*, phospholipids, remain and cause ion suppression when such samples are analyzed via LC/MS.

In contrast, liquid-liquid extraction [LLE], often, creates rather clean extracts, but the procedure is cumbersome and has many pitfalls. In order to transfer an ionizable analyte into the organic extraction solvent, it first needs to be converted in its aqueous medium to a non-ionic form, using either high or low pH. This procedure is not suitable for compounds which have both positively and negatively chargeable groups. Next, a suitable solvent needs to be found that efficiently and preferentially extracts the analyte. However, even if an appropriate solvent is found, a single step rarely extracts the analyte quantitatively; multiple extraction steps are commonly needed. Alternatively, if one relies only on a partial extraction, the reproducibility of this step must be ensured with an internal standard that has extraction properties very similar to those of the analyte. Preferentially, a deuterated standard should be used[16]. Often, a second extraction back into the aqueous medium (for example at a different pH) completes the sample preparation, with the same difficulties as those associated with the first step.

Another issue with LLE is its unsuitability for automation. Manual LLE is often carried out with rather large solvent volumes, making it much less desirable for the analysis of blood samples. Clearly, the disadvantages of LLE are significant.

Solid-phase extraction solves the problems of both protein precipitation and LLE. Compared to LLE, it can be scaled down readily to sample volumes below 50 µL[17], and it can be automated easily[18]. SPE is usually carried out off-line using single cartridges or in 96-well plates[17], but it can also be realized online with the HPLC separation, using switching valves to direct the flow either to waste or to the HPLC column[19]. Compared to protein precipitation, SPE has the advantage that much more specific protocols can be generated that permit very selective sample cleanup. Thus, the problem of matrix ion suppression can always be solved using an appropriate solid-phase extraction method. A discussion of the details of such protocols follows.

Principles for cleanup and analysis of blood samples may be applied to other sample matrices. For example, today, the enrichment of trace contaminants in environmental water is preferentially carried out using a solid-phase extraction technique[11,15]. Similar methodologies can be applied to human urine samples[20] or fruit juices[8,21]. As pointed out above, the concern about ion suppression is universal and independent of the sample matrix [7,9,10,11].

Solid-phase Extraction Protocols

Multiple generic SPE protocols have been developed and demonstrated[3]. In general, the best cleanup results are obtained by using a sample preparation method that is orthogonal to the LC separation mode. Orthogonal methods, sometimes called two-dimensional [2-D] methods, use two completely different modes of separation or retention to improve separation power dramatically. As an example, if sample preparation is followed by a reversed-phase HPLC separation, then, the most selective SPE methods rely on ionic [ion exchange] interactions. Of course, this requires that analyte(s) have an ionizable function, but this is the case for most pharmaceuticals, and, often, for analytes of interest in other fields. Keeping in mind that other techniques or combinations thereof may be more suitable for a particular problem, here we will first focus on combining ion-exchange SPE with reversed-phase HPLC in tandem with MS detection.

A sample preparation method of this type is outlined in Figures 1 and 2. Let us assume that the analyte, like a large number of pharmaceuticals, contains a basic amino functional group [Figure 1]. The sample matrix is blood plasma, of either animal or human origin. We will use an Oasis® Mixed-Mode Cation Exchanger [Oasis® MCX] to prepare the sample. Under acidic conditions, our protonated analyte, being positively charged, will be retained on this cartridge. Therefore, we acidify our plasma sample, add an internal standard with molecular properties similar to those of our analyte, and load this solution onto a properly prepared Oasis® MCX cartridge. The analyte and internal standards bind to the ion-exchange cartridge. Very polar neutral and anionic components of the sample are washed through the Oasis® cartridge, as are higher molecular weight components of the sample such as proteins, due to size exclusion and slow mass transfer. To completely remove these sample

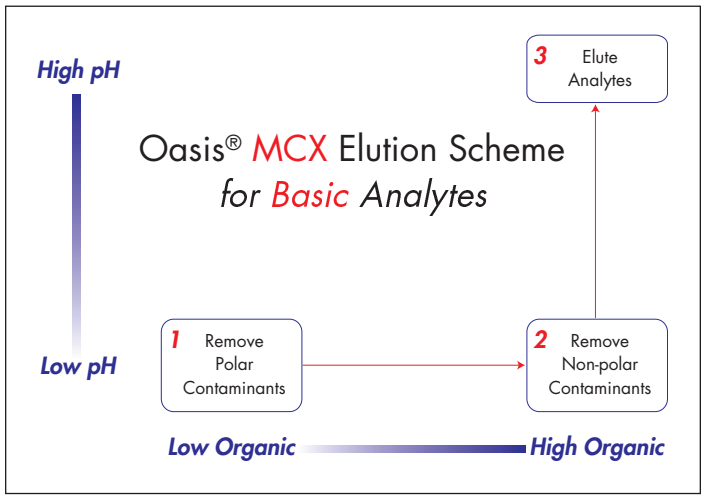

Figure 1

components, the first wash solution contains an acidified, largely aqueous solution. A second wash with methanol elutes neutral compounds that had been retained via hydrophobic retention on the mixed-mode sorbent. However, very hydrophobic components of the sample matrix, *e.g.*, some lipids, will remain on the sorbent. In the final step, the desired analyte and the internal standard are eluted with 5% ammonia in methanol.

For acidic analytes, the protocol can be inverted [Figure 2]. Base is added to the sample to adjust its pH to a high value. Under these circumstances, acidic analytes are ionized and retained on an Oasis® Mixed-Mode Anion Exchange cartridge [Oasis® MAX]. The first wash – at alkaline pH – removes polar and cationic compounds, analogous to the acidic wash on the Oasis® MCX cartridge. Similarly, a second wash with methanol removes less polar, hydrophobically bound compounds. Finally, the acidic analytes are neutralized and eluted with a methanol solution acidified using either formic acid or a stronger acid such as HCl.

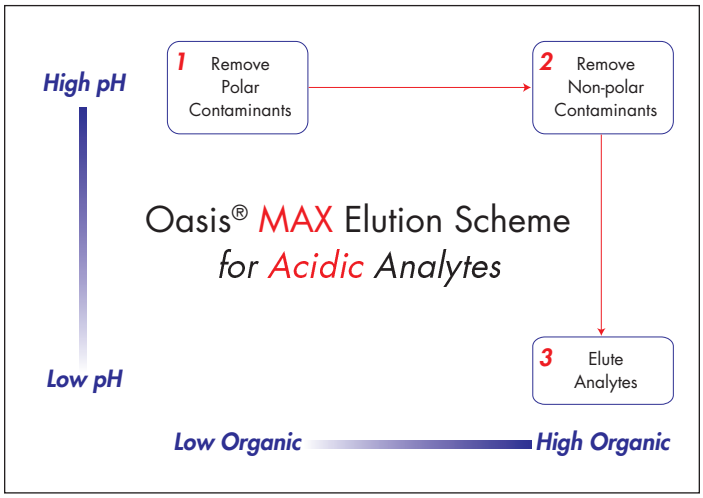

Figure 2

This protocol delivers excellent results, both with respect to recovery and sample cleanup. Figure 3 shows a comparison of the results obtained from a standard protein precipitation, a simple one-dimensional [1-D] SPE protocol relying solely on the reversed-phase interaction of a neutral analyte with a hydrophobic SPE sorbent, and the 2-D sample cleanup and elution using the Oasis® MCX cartridge protocol just described[17]. At a sample concentration of 1 ng/mL, the respective peak areas for propranolol, the basic compound eluting at 3.8 minutes, were 278 with protein precipitation, 1,329 with simple on/off 1-D hydrophobic SPE, and 13,534 for the method combining 2-D SPE sample cleanup with reversed-phase HPLC/MS. Thus, the straightforward, yet sophisticated, SPE method described in the last two paragraphs gave *10-fold* less suppression than the simple on/off 1-D SPE method, which, in turn, was about a *5-fold* improvement over protein precipitation. In this example, the net reduction in ion suppression between protein precipitation and the recommended 2-D SPE method was *50-fold* ! This demonstrates unequivocally the magnitude of, and an excellent solution to, the ion-suppression problem.

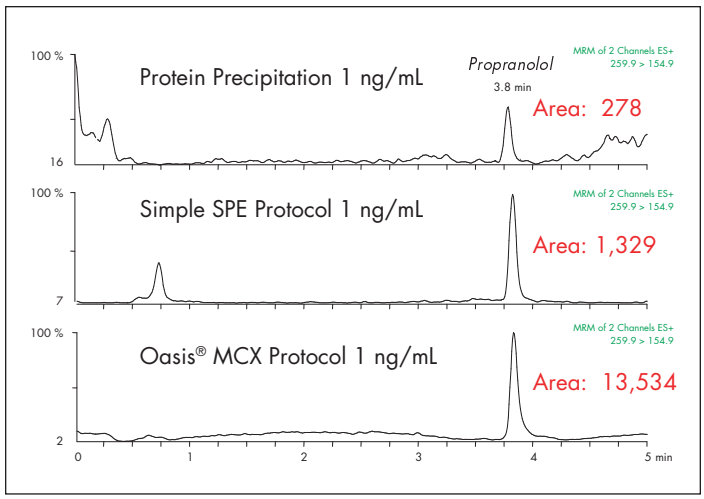

Figure 3

It is a fallacy to assume that the ion-suppression problem can be obviated by using an internal standard . An identical protocol, executed with the analyte amitriptyline, gave, within experimental error, the same response for both a standard solution and for a plasma sample prepared by the mixed-mode SPE protocol, demonstrating both complete recovery and the elimination of ion suppression. For a corresponding protein precipitation sample cleanup, the response was nearly 5 times lower than that for the neat sample. Thus, LC/MS data for propranolol determination in a sample prepared via protein precipitation using amitriptyline as the internal standard would have underestimated the level of amitriptyline roughly *10-fold* — an intolerable error for quantitative analysis of a drug in plasma! This reinforces the strong recommendations by Matuszewski *et al.*[12] on how to carry out properly quantitative bioanalytical methods and how to measure ion suppression or avoid sample preparation errors.

Good sample preparation is not only necessary for blood samples in the pharmaceutical industry. It is also required in food analysis[7]. As an example of an elegant sample clean-up procedure, the analysis of a fluoroquinolone antibiotic in beef kidney will be described[22]. Fluoroquinolone antibiotics have been approved since 1995 for use in animal husbandry. For the analysis of enrofloxacin in beef kidney, ciprofloxacin was the internal standard. These compounds are amphoteric; they can be selectively extracted and cleaned up using a combination of anion exchange and cation exchange [Figure 4]. Homogenized kidney was diluted with buffer. After addition of the internal standard and ultracentrifugation, the resulting fluid was then subjected to the standard protocol for the Oasis® MAX cartridge. Conditioned in base, the cartridge retained the anionic and amphoteric species by ion

exchange and the cationic and neutral interferences by a reversed-phase mechanism. The latter species were washed off with methanol. In the final step, 0.2N HCl in methanol eluted the amphoteric analytes. This acidified eluent was loaded directly onto an Oasis® MCX cartridge. Acidic interferences passed through unretained; less polar neutral species eluted in the intermediate methanol wash. The final basic eluate contained only zwitterionic compounds with properties similar to those of the fluoroquinoline antibiotics. This cleanup procedure delivered a sample that was even clean enough for an analysis with UV or fluorescence detection! Of course, MS/MS detection permitted the determination of the residual content of the antibiotic at still lower concentrations [Figure 4]. Such elegant sample preparation procedures can completely eliminate ion-suppression issues, even from samples in complex matrices. Similarly well-designed sample preparation techniques have been used for the determination of acrylamide in fried potato products[23].

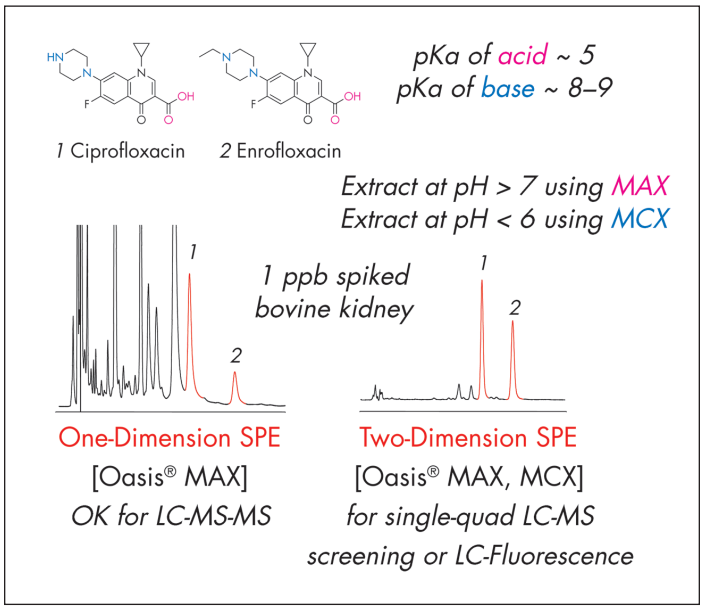

Figure 4

Methods for Measuring Ion Suppression

To compare different sample preparation techniques for the analysis of drugs in biological fluids, Matuszewski *et al.*[12] proposed a specific protocol that permits the determination of the reduction of matrix effects (ME%), recovery of the sample preparation procedure (RE%) and the overall process efficiency (PE%), which is the product of the matrix effect and the recovery. The desired values are 100% in all cases, indicating the absence of matrix suppression and complete recovery of the analytes of interest after the sample preparation process. The protocol requires three sets of experiments which generate three sets of samples. The first set of five samples is prepared by dissolving the analytes in mobile phase. The second sample set consists of five different lots of sample matrix [plasma or urine] to which blank mobile phase is added. The third sample set is made up of the same five sample matrix samples as in set two, except, instead of blank mobile phase, the analyte-in-mobile-phase solutions are added. Now sample sets 2 and 3 are subjected to the same sample preparation technique. Finally, analytes are added to sample set 2 *after* the sample preparation step, and all three sample sets are analyzed.

The comparison of sample set 1 and sample set 2 permits the calculation of the matrix effect (ME%) from the ratio of corresponding responses from sample set 2 to those of sample set 1. Similarly, the recovery of the sample preparation procedure (RE%) is calculated from the ratio of sample set 3 to sample set 2. In addition, the use of five different sample sources permits an estimate of the influence of source variability on the results. For example, different diets for, or metabolism variation of, the subjects under investigation can influence matrix suppression and yield incorrect results.

Methods have also been established to measure ion-suppression effects directly in the chromatographic method via post-column infusion[24,25,26]. Here, the HPLC analysis is carried out on a blank matrix sample with MS detection. Prior to the MS inlet, a second stream carrying the analyte(s) is added to the column effluent. The MS system monitors the signal for the analytes over time. For those regions in the chromatogram that suffer from ion suppression (or the more rare event of ion enhancement), the signal of the analyte(s) differs from the signal obtained with straight mobile phase. This permits a direct, qualitative measurement of ion suppression or enhancement and leads investigators to the best sample preparation or chromatographic technique for their analytical problem.

Summary

Ion suppression — a common phenomenon in the LC/MS analysis of complex samples — can be effectively eliminated by good solid-phase extraction methods. Today, with tools such as SPE, coupled with the availability of methods for the quantitative assessment of ion suppression, significant errors in LC/MS analysis due to ion suppression are avoidable and inexcusable.

– Uwe D. Neue and Patrick D. McDonald

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