

Strategies for Higher Throughput MS Analyses

The authors discuss current approaches for increasing throughput in MS analyses, including improved software information flow, data management and effective sample preparation, and how researchers should consider each of these with regard to their own needs.

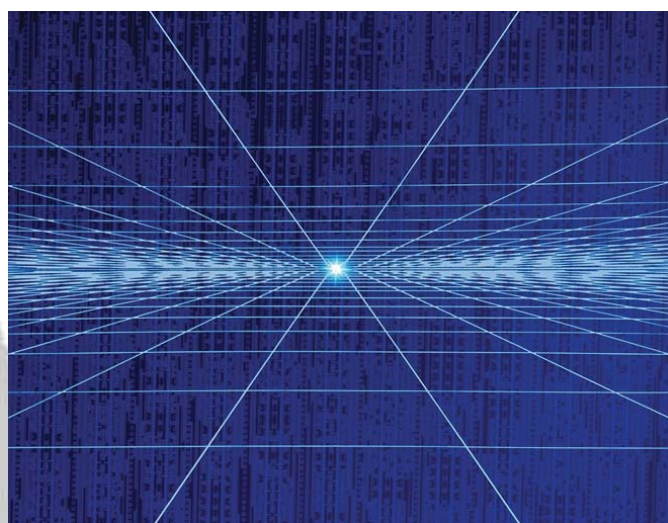
Diane M. Diehl and Michael P. Balogh

Few skilled or artful pursuits flourish without careful preparation. If you ever took tennis, sailing, or violin lessons, you know how hard your teacher worked to convey the notion that careful preparation would reap huge rewards. Many of us, though, regard sample preparation — careful or otherwise — as a rather unglamorous aspect of our work as separation scientists. Because we would rather avoid dealing with this relatively pedestrian business, this most critical of liquid chromatography–mass spectrometry (LC–MS) practices sometimes is given short shrift.

There are legitimate hurdles. One of the more common issues is illustrated by the response to the question of why a given separation is performed using certain mobile-phase conditions, columns, and other attributes: “Because we’ve always done it this way.” While that makes little sense chemically, it does make sense when practical impediments such as the difficulty of changing codified methods in a new drug application are considered. Although LC–MS instruments are more robust now than 10 years ago, a number of practical performance issues still need to be considered before making the first injection.

To increase throughput, we have exploited the mechanical advantage the mass spectrometer’s duty cycle offers. Running several systems in parallel and converging them at the mass spectrometer’s ion source increases efficiency without noticeably increasing load or interference. Several LC–MS workshop presentations given at the most recent annual American Society for Mass Spectrometry (ASMS) conference addressed this topic. You can download transcriptions at www.waters.com (click the Resources tab, then Seminars/Events to access the ASMS Archives).

Sometimes a bias in our approach to experimental design stems partly from our intuition as separation scientists. We tend to become complacent through familiarity with analytical approaches based upon the tools we use frequently and have grown comfortable with. If we have relied upon UV detection techniques that call for phosphate buffers, which offer both UV transparency and broad capacity as a mobile-phase modifier, we create LC–MS problems by ignoring what happens when phosphate enters a mass spectrometer’s ion



source. There, the gas-phase transition to ions occurs, and the phosphate ionizes in high molecular concentration along with the analytes of interest. Competition for charge ensues because our phosphate modifier, albeit convenient and familiar, also happens to be highly ionic. Moreover, being relatively nonvolatile, it tends to coat lenses and other critical surfaces, thereby decreasing instrument sensitivity, forming unexpected adducts, and diminishing the molecular ion. Thus, many contemporary instruments incorporate orthogonal source geometries, such as those from ABI/MDS Sciex (Thornhill, Canada), Agilent (Palo Alto, California), Thermo Finnigan (Waltham, Massachusetts), and Waters. But innovative source designs alone cannot remediate ion suppression and other hazards of analysis.

Mass Spectrometers as Separation Devices

Methods that reduce run times to mere seconds exist, and these give rise to notions that we should dispense with chromatographic separation altogether and let the mass spectrometer do the work of detecting. But such methods are not without tradeoffs. Inevitably, they sacrifice limits of detection, diminish linearity and dynamic range, and compromise

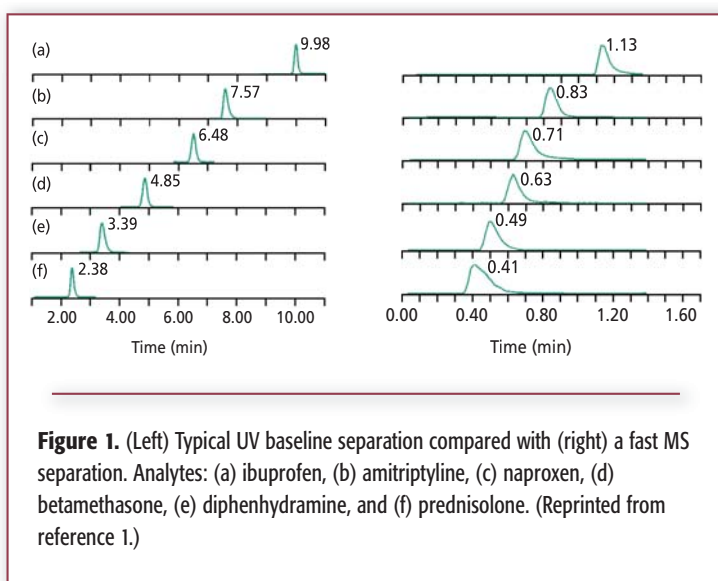


Figure 1. (Left) Typical UV baseline separation compared with (right) a fast MS separation. Analytes: (a) ibuprofen, (b) amitriptyline, (c) naproxen, (d) betamethasone, (e) diphenhydramine, and (f) prednisolone. (Reprinted from reference 1.)

reproducibility. For an aggressive gradient using MS rather than UV or some other indirect means of detection, approximately 2 min is practical. Beyond that, however, attempts to shorten the interval between injections while maintaining gradient fidelity and quantitative quality (minimal carryover for instance) in a single system prove largely unsatisfactory.

A recent publication offers an apt example of the importance of competent decision making (1). The authors compared methods that increase throughput by reducing the separation run time for various pharmaceutical compounds, which represent different chemical classes (propionic acids and steroids, among others). The results show that even a small degree of temporal separation can overcome problems of ion suppression and that some preparation is crucial, be it in the form of a well-prepared sample or chromatographic separation during analysis. Furthermore, the results show that the degree of effort one expends, as with the choice of mass spectrometer, depends upon the outcome that is desired.

Figure 1 compares two results of a multicomponent drug model. The first is a high-quality baseline separation using UV detection, and the second is a series of almost overlapping peaks obtained using MS detection. Negligible loss in linearity, sensitivity, and performance is seen when the gra-

dent run is shortened radically and a mass spectrometer, not a UV detector, serves as the detection means. However, a flow injection analysis without separation saved very little time, because a delay to allow chemical reequilibration to ensure quantitative work was the gating factor, and the limits of detection suffered dramatically. Figure 2 shows the fast LC-MS method with linearity measurements. Relatively short acquisition duty cycles further stress the effort by switching

between positive and negative ion acquisitions.

A charted comparison of baseline-resolved UV acquisitions with flow injection analysis-MS acquisitions shows the dramatic loss of the steroid betamethasone when ion competition takes place in the source while other analytes are present simultaneously. Figure 3 compares the limits of detection for partially separated peaks — the fast MS method — with no separation or flow injection analysis.

The choice between adopting the fast MS method or the flow injection analysis method is, obviously, academic. But it is logistics, not efficacy, that often determines our actual course. For researchers at Pfizer (New York, New York) such as Mark Cole, the need to effectively characterize 500–1000 compounds a week, each of which can require as many as 15 assays, creates a sense of urgency. So as much as it reflects well-considered science, their practice also reflects the influence of other in-house work groups. For example, if a biology project works in a particular format, the researchers adapt to accommodate them. Most submitted work now arrives in 384-well format except those involved with Caco-2 cell permeability and cell membrane stud-

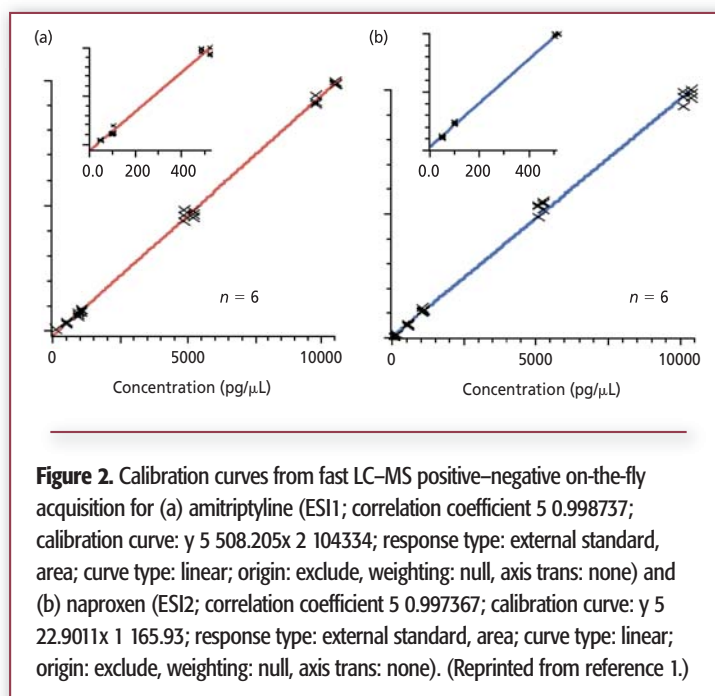


Figure 2. Calibration curves from fast LC-MS positive-negative on-the-fly acquisition for (a) amitriptyline (ESI1; correlation coefficient 0.998737; calibration curve: $y = 508.205x - 104334$; response type: external standard, area; curve type: linear; origin: exclude, weighting: null, axis trans: none) and (b) naproxen (ESI2; correlation coefficient 0.997367; calibration curve: $y = 22.9011x - 1165.93$; response type: external standard, area; curve type: linear; origin: exclude, weighting: null, axis trans: none). (Reprinted from reference 1.)

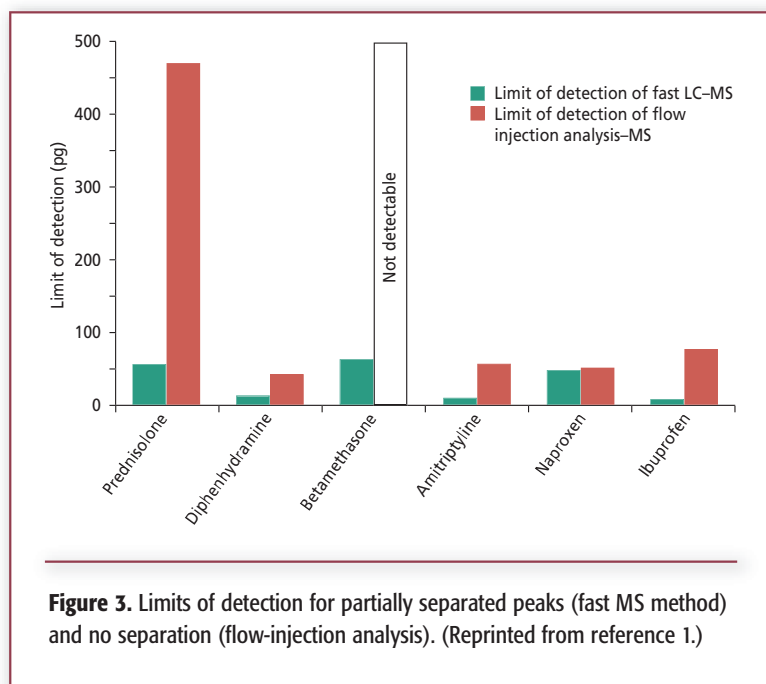


Figure 3. Limits of detection for partially separated peaks (fast MS method) and no separation (flow-injection analysis). (Reprinted from reference 1.)

ies, which require a minimum surface area. Regardless of format and analytical requirements, the researchers must devise a method that meets Pfizer's expectations for cost-effectiveness, high-throughput, and high-quality results, so the sample preparation and reliance upon effective chromatographic separation is paramount.

Dealing with Realities of Sample Matrices

To ensure against interferences such as ion suppression, some drug discovery laboratories simply introduce a diluted sample, postcolumn, and perform a quick sample preparation (2). They prefer these approaches to the revalidation that would be necessary were they to alter the chromatography or ionization

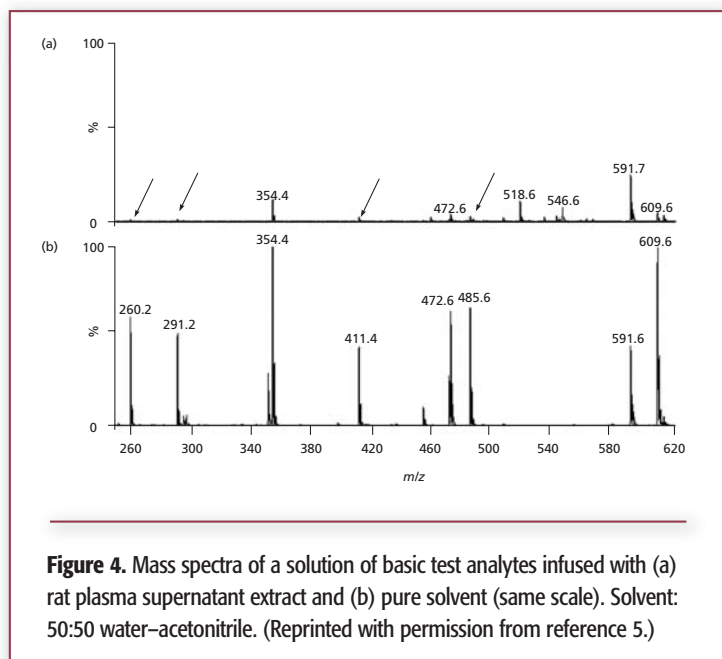


Figure 4. Mass spectra of a solution of basic test analytes infused with (a) rat plasma supernatant extract and (b) pure solvent (same scale). Solvent: 50:50 water-acetonitrile. (Reprinted with permission from reference 5.)

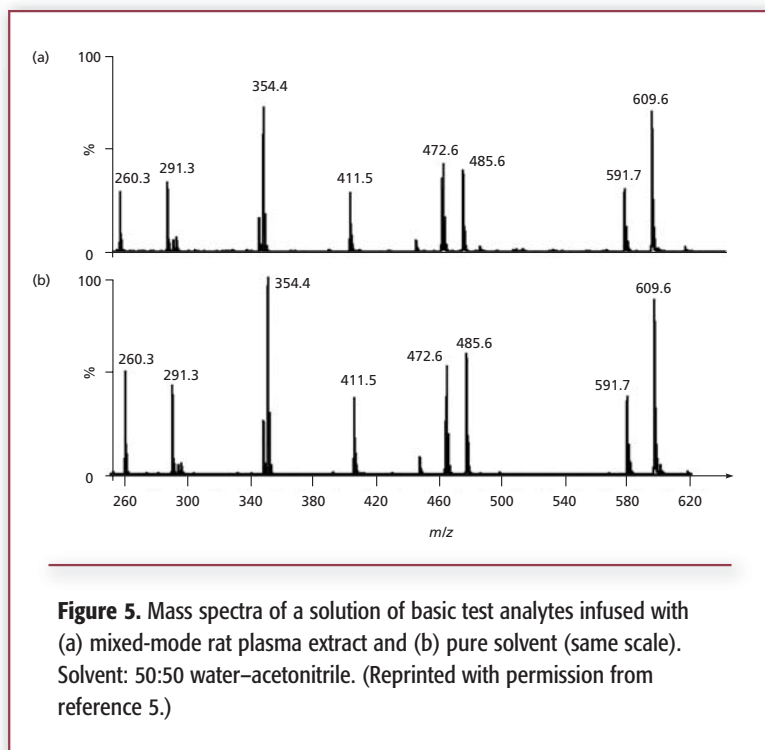
method or effect some other change to their practice.

As mass spectrometers became more selective, researchers came to believe that chromatographic separations and sample preparation techniques were no longer necessary for robust bioanalytical methods. If an analyte of interest was coeluted with anything from the matrix, the mass spectrometer, in multiple reaction monitoring mode, would selectively report the area count of only the analyte of interest. But closer scrutiny revealed that such coelution often suppresses or enhances ions of interest, resulting in quantitation inaccuracies much as was shown in the flow-injection analysis versus fast chromatography work.

Characterizing the Causes of Interference

So what causes this fluctuation in signal and how can it be avoided? The most common matrix interferences are caused by coeluted endogenous material that originates with the biological matrix itself. Thus, salts and lipids are frequent offenders. Also, polymers from the plastics that compose sample vials, Li-heparin (a common anticoagulant), and even the brand of mass spectrometer and type of ionization add components to the matrix (3). Various methods have been tried to determine the extent of matrix interference, with most based upon work by King and colleagues (4), in which a solution of the analyte is infused into the mass spectrometer to establish an elevated but constant baseline. A blank sample extract (a solid-phase extract minus the analyte) then is injected into the high performance liquid chromatography (HPLC) system. Thus, any remaining sample matrix that suppresses the ionization causes the baseline to fall.

A recently published work takes an alternative approach. The goal was to better understand the cause of ion suppression and related effects in the MS and, consequently, to avoid interferences from the column and the mobile-phase buffers and additives (5). The researchers separately prepared a set of eight acidic compounds for ESI⁻ and a set of eight basic drugs for ESI⁺, each in a 50:50 methanol-water solution. Each



solution was infused at a steady rate into a mass spectrometer without a column. Once a steady response for the eight components was established, an infusion pump added a matrix interferent in the form of rat plasma and various mobile-phase modifiers, ion-pairing agents, pH additives, and matrix extracts. The difference in response for each of the eight analytes was calculated, with and without the interferents. The results showed that residual components from plasma cause ion suppression and enhancement. Significantly, common mobile-phase additives such as formic acid and trifluoroacetic acid cause noteworthy changes in signal intensity as shown in work presented at the 1999 ASMS LC–MS Workshop (6).

So now that we know that matrix effects do indeed exist, how do we avoid them? We have two options: modify the chromatography or enhance the sample preparation technique.

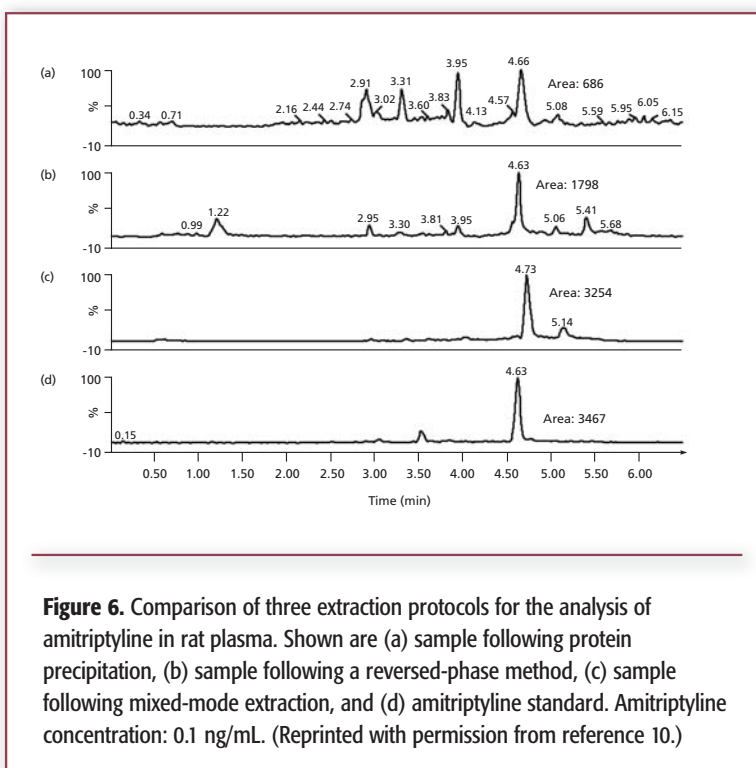
Once you define the area of matrix effect in a chromatogram, you can adjust the HPLC method, changing gradient slopes, mobile-phase modifiers, and even the column. Depending upon the particular separation, such measures sometimes work. But sometimes they do not. Tiller and Romanyshyn (7) tried

adjusting the HPLC method to eliminate matrix interference, focusing upon performance with high-organic isocratic conditions and ballistic gradients. They concluded, “The only reliable way to eliminate the matrix interferences

observed would be to extract the analytes from the matrix with an analyte-specific extraction method.”

As for the second option, enhancing the sample preparation technique, the technique used depends upon the requirements of a particular analysis. Protein precipitation, liquid–liquid extraction, and a plethora of solid-phase extraction (SPE) techniques are available, each yielding varying degrees of sample cleanliness. Thus, if we consider Figure 4, we observe that a protein-precipitation sample causes 43–98% ion suppression for the sample analytes. This should not surprise us. Protein precipitation is a well-accepted technique, but it also generally is acknowledged to be a quick and dirty way to use organic solvent to crash out a majority of plasma components.

Figure 5 shows that the eluent from a mixed-mode SPE sorbent results in the cleanest extract — less than 10% ion suppression. In this case, a mixed-mode sorbent, Oasis MCX (Waters), demonstrates reversed-phase and ion-exchange modes. For basic analytes, this cation-exchange material is highly selective and permits washing away a majority of the interfering components while selectively



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retaining the analyte of interest.

What does this mean in terms of data analysis and interpretation? The data comparing protein-precipitation, reversed-phase, and mixed-mode methods for amitriptyline at 0.1 ng/mL in rat plasma show remarkable results (Figure 6) (11). Figure 6a shows a rather dirty sample, one that approaches the assay's detection limits. But upon evaluating the reversed-phase and mixed-mode data, we see good increases in the extracts' cleanliness, the peaks' total area counts, and an improved signal-to-noise ratio. Clearly, effective sample preparation techniques are an excellent way to eliminate matrix effects. If a particular technique can be made to meet the high-throughput and cost-effectiveness guideline constraints, in practice, the choice is easy.

As Mark Cole and his researchers at Pfizer have pointed out, sometimes the realities of the workload, when viewed against the “throughput versus quality of the answer” axis, also are leavened by a cost-per-sample consequence. Cole and colleagues (8) have experimented with a device designed to accept a matrix-assisted laser desorption ionization (MALDI) source fitted to a Sciex triple quadrupole. Although it can be simple and cost effective, MALDI typically has not been considered an option for small-molecule high-throughput work. The matrixes required to ionize the samples create a flood of unwanted interference ions in the low mass region. In addition, MALDI is not a flowing technique like electrospray ionization (ESI) but requires some form of deposition on a plate. The triple quadrupole, however, can be a highly selective filter in its first stage, removing the unwanted background and passing only the ions of interest. An alternative to using a matrix

is desorption ionization on silica, which has been receiving quite a bit of interest in recent years following the work of G. Siuzdak to develop the technique (9).

The need to decide between techniques that are quicker and less costly but perhaps less effective (such as simple precipitation) and more effective techniques such as SPE will no doubt be with us for some time. For some, the future might be easier still. A recent publication details using a photolabile compound as a linkage between solid supports and synthesized organic compounds (10). When coupled with a laser-desorptive process such as MALDI, what this could mean for the drug-discovery combinatorial interests is that the small molecules that dominate medicinal interests can be detected without the background interference by a quick, simple technique without any sample preparation.

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