Bioanalysis ZONE

A decade of experiences from Robert MacNeill

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Foreword from the author

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A decade of bioanalytical blether and babble. Indeed, it was all the way back in the summer of 2015 when I first finger-tremblingly submitted a column to Bioanalysis Zone. Looking back at the initial ones, the tone was quite severe. They were textbook-like, mundanely conveying what I saw as important science-based information and the nitty-gritty about developing super-rugged methods to my bioanalytical LC–MS peers in the community — in sentences that were probably too long.

Rather amusingly, as the years passed and I managed to maintain a quarterly column contribution, the tone gradually lightened. Humor and punnery found their way into my writing, flashes of descriptive composition and, at one point, even a poem. I hope that I ultimately found a good balance to it all, aiming to be informative and amusing!

Many of the topics were picked at random, some — seemingly the most popular — were themed, and many were inspired by what I had recently observed or was tackling at the time in my own labs. The column became frequently referred to as a blog due to these circumstances. I know many might wonder which installment was my favorite. I would have to say, without a spooky shadow of a doubt, that the Halloween-themed piece from October 2022 was the most monstrous fun to write.

All in all, it has been a wonderful experience and a privilege. I am delighted and honored that my columns are being compiled into an eBook. I would like to express my wholehearted gratitude to the various members of the Bioanalysis Zone team over the decade for their help in making these commentaries come to life. Thanks in particular to Naamah Maundrell and Amy White, both Bioanalysis Zone, and Hazel Dickson of Waters Corporation, who worked on some great visuals for a selection of the articles.

Disclaimer: the opinions expressed are solely my own and do not express the views or opinions of my employer.

Recently, whilst attending a very useful exhibition, I was momentarily shocked in conversation to hear mention of the idea that SPE may be becoming obsolete. A moment later I was better appreciating the context, i.e., this was coming from a vendor of extraction media who was conveying the message that there are now well-established sorbent-based alternatives if something more than dilution or deproteinization is required for a quantitative bioanalysis. These alternatives do hold much interest and will be discussed in subsequent installments of this column. However the notion of SPE dying out, and the circumstances of coming across it, continues to perturb me. Should there have been surprise in hearing this? Possibly not so much in terms of analysis of small molecules as opposed to peptides, but this is where we begin to go into all the 'ifs' and 'buts' that amount to the old adage of compound dependency, and the fit-for-purpose determinant. Also it must be stated that, on a personal level, there cannot be much doubt that having a happy history of successful bioanalytical application of SPE would endear the technique to me.

It isn't so much the association with rugged methodologies that is pertinent to an argument to preserve SPE as a primary extractive tool; it's the reasons for that association. Selectivity, as anyone well-versed in method development knows, is key to rugged quantification.

My first-ever experience with SPE as a student gave me an excellent taste of the great range of selectivities attainable, especially as my sorbent screening showed me that I could very efficiently extract my drug and metabolites from human serum using a reversed-phase load, wash and elution protocol using the normal phase candidate sorbent chemistries of unmodified silica or silica with a cyano bonded phase. Only later did I fully understand the per aqueous retentive phenomenon, which was very likely a key player here. It culminated in a good method. Ever since then, upon my entry into the CRO environment serving the pharma/biotech community, time and time again SPE has delivered when required. And it has evolved in this time. There were only silica-based sorbents available when I just started out, then came the great day of first experiencing polymer-based sorbents, cross-linked styrene-divinylbenzene (SDB), followed by polar-modified SDB polymeric beds, all high capacity and water-wettable, not adversely affected by drying out (although it is far more difficult than many imagine to dry out a silica bed). There was subsequently the very welcome advent of scaling down the sorbent bed weight and design, the so-called microelution, both within well plates, and the small sorbent quantities could also fit into pipette tips. This not only allowed convenience for sampling with low matrix volumes, but also far better opportunity to elute in suitably low volumes to avoid evaporation and reconstitution for sample concentration, which is often detrimental to recovery and performance, particularly for peptides. This latter point is important as it actually defines much of the future of SPE — with biotherapeutics becoming more abundant in R&D pipelines, peptide analysis is becoming more prevalent, particularly considering the LC–MS analysis of protein-based therapeutics involves signature peptides from digestions.

Such a high percentage of peptides requires the avoidance of evaporation due to difficulties in achieving re-dissolution in reconstitution solutions Also, very often they require the selectivity dimension from SPE for rugged methodology. So we currently have a wealth of options – silica-based, polymeric-based, monolithic and particulate beds, fritless factor in any aversion to the technique? There are obvious downsides too, like the cost involved and perhaps more time required to prepare samples compared to, say, deproteinization by addition of organic solvent. Sometimes a little thought must go into how to ensure disruption of protein binding to release bound analyte, as it clearly doesn't happen as readily or obviously as in liquid-liquid extraction or deproteinization. Sometimes you may find breakthrough in extracts of sorbent material if there has been a glitch in the manufacturing or design, however, these rare occurrences are even less frequent as technologies improve. And indeed there is a good amount of truth in that the sensitivity of state-of-the-art instrumentation means that you are more likely to be able to get away with ever-smaller injection volumes of not-so-clean extract.

In any case, the way I see it, the positives far outweigh the negatives in the big picture. SPE is still an option for small molecule bioanalysis, especially in the regulatory environment, and the most prominent of very few extractive options for peptides, which are important analytes of the present and, more so, the future. The selectivity, versatility and cleanliness benefits are clear. If the most rugged method is desired, as in for most clinical sample analysis, then not only must the likes of isotopically labeled internal standards be considered but, in my book, SPE must also surely figure.

> *My first contribution, and I remember feeling the burning urge to write something upon hearing that one of my beloved sample prep techniques was thought to be becoming obsolete. That certainly brought fingers to the keyboard! Enough for a sequel too.*
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> Robert MacNeill

In the previous installment of this column, I expressed my surprise at the suggestion that SPE may be becoming obsolete. I could not resist making an effort to quash the notion, at least in the context of contemporary quantitative bioanalysis and especially in the regulated environment. I also made mention of well-established sorbent-based alternatives to SPE if something more than dilution or deproteinization is required for a given quantitative bioanalysis. Having made my case for the preservation and veneration of SPE, to help complete the picture, the sorbent-based alternatives of supported-liquid extraction (SLE) and phospholipid filtration should be dwelt on as well.

These techniques are of great use and interest at several levels and are more than worthy of discussion. The most interest, naturally, is in the selectivities attainable and how these could liaise with a bioanalytical LC–MS methodology. Additionally, there is the aspect of greater simplicity in terms of the typical protocols adhered to. There is considerable interest too, of course, in the reduced costs of these media when compared to SPE.

I have little more than a vague memory of a then-colleague exclaiming to me, around 15 years ago, that we could 'now do liquid-liquid extraction in cartridges.' Sure enough, I then came across the curiously-shaped barrels that were being tried out, packed with what I soon learned to be diatomaceous earth. 'Seriously?' I wondered, tantalized, musing as to what exactly diatomaceous earth could be. It turns out that this material is mainly highly porous, amorphous particulate silica, originating from the fossilized remains of diatoms. Less than around 10% of the composition is alumina and iron oxide. For use, the basic protocol involved treatment of matrix with aqueous modifier and any required additives, subsequent application to the sorbent bed with the assistance of a second or two of initial negative pressure to whet the material with the applied liquid, then to allow to soak into the sorbent for around 5 minutes. Elution would then be effected upon application of water-immiscible organic solvent, as per what might be used in a classical liquid-liquid procedure, and after evaporation the sample would be reconstituted and ready for analysis. In these early days it became fairly apparent that although there was clearly a functional product here, it could not be relied on to produce adequately reproducible data in the regulatory environment. Over the years, the technology has certainly improved and it has become a very prominent option for the extraction of small molecules from biological matrices. It has featured widely in multi-analyte method validations, by no means restricted to discovery quantification. It's a simple, fast procedure and it is a proven excellent option for the removal of phospholipids, something not normally expected of liquid-liquid extraction, which itself demonstrates that the selectivity could be loosely labeled an entirely different kettle of fish.

There is an obvious requirement to evaporate for the typical interface with reversed-phase or aqueous normal-phase chromatography, and this, together with very restricted desorption/partitioning into the organic medium as a result of their distinct polarity, removes SLE as a viable option for a great many peptidic analytes.

An additional disadvantage of SLE, as alluded to already, is the reproducibility of the diatomaceous earth composition. This links inextricably with the fact that no-one has yet really nailed, theoretically, what is going on chemistry-wise with this material in the SLE process. There is now, however, a commercial SLE option that is made from a different and proprietary material, for which ethyl acetate as eluent produces high recoveries for the vast majority of analytes.

It has been more than a decade now since the bioanalytical community began reacting to the revelation that phospholipids have a substantial hand in the phenomenon of ion suppression, synonymous with matrix effect, and the various ways in which this adversely affects method performance and data reliability. Many years of great R&D effort focused on phospholipids in this context subsequently followed, spawning countless posters, presentations and publications. It was only a matter of time, therefore, before sorbent material that could deal directly with phospholipids became commercially available, and in formats akin to existing high-throughput sample preparation means. Indeed they have been around since the first manifestation, which was the best part of 10 years ago.

There are now numerous manufacturers of these sorbents, and although proprietary, most probably involve a bimodal retentive scheme involving reversed-phase and electrostatic interactions to gain hold of the phospholipid content, which includes both lysophosphatidycholines and phosphatidylcholines. The typical protocol has treatment of matrix with acidified acetonitrile, which, conveniently for a small molecule analytical endpoint, precipitates larger proteins from the sample. Subsequently, the sample is pulled through the sorbent, filtering out the solids and, via the sorbent chemistry, almost 100% of the phospholipid content. Acetonitrile is usually critical as the organic solvent, as the popular alternative of methanol gives a selectivity too conducive to phospholipid release from the sorbent. Acidification is likely necessary to ensure that with most analytes there will not be a negative charge that could result in any degree of retention, as with what happens with the zwitterionic choline head group of a phospholipid.

This technique constitutes a fantastic way to eliminate these proven interferences which can otherwise, in addition to the risk of co-elution with the analyte or internal standard, easily accumulate on a reversed-phase column, reaching huge steady-state levels and continuously infusing off the column outlet in a variable manner, leading to signal drift, sensitivity fluctuations and reduced column lifetimes. It builds on the advantages of protein precipitation by adding comprehensive phospholipid removal, and it does not necessarily need evaporation. However, there are also most of the disadvantages of protein precipitation, such as the lack of sample desalting and other non-phospholipid residual components that could cause signal distortion.

To sum up, these means of sample preparation are cheaper and, for the reasons mentioned, valuable alternatives to SPE. Despite the mysteries as to what exactly the cartridges contain and how exactly they work, they are known to work well in their mainly generic nature, and of course, have their place in the fully regulated laboratory. If ever selectivity required tweaking in the extraction, however, then some maneuvering could indeed be done with SLE, but only SPE could offer a multitude of possible avenues to explore to address the issue.

My next installment will be a discussion on the important linked concept, orthogonality of selectivity between extraction and bioanalytical chromatography.

> *Having written the initial piece advocating SPE, I was keen to follow up with balanced commentary about other popular packed-sorbent sample preparation options.*
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> Robert MacNeill

A biomarker assay is not a PK assay. This was the notion most frequently mentioned and discussed at the AAPS Crystal City VI meeting this year, as the scientists present attempted to begin assembling a framework for which the US FDA could base their future guidance and regulations. The demand for more regulatory clarity regarding the relevant criteria for biomarker assay performance has recently become very prevalent. Many questions are being asked, and the number of answers currently falls short as the bioanalytical community ponders.

The industry relies on the use of standardization and defining criteria to function. It is not so easy, however, to establish acceptance criteria for biomarker assays, particularly if the aspiration is to a single universal approach. The big misnomer in this arena is the accuracy term. Due to the endogenous nature of biomarkers and realizing how there will be inherent variability in native levels, accuracy cannot be a realistic goal. What should be pursued are, most importantly, precision and parallelism. Harmonization too, in the sense of correlation of results between different labs performing the same analyses. The idea of 'megapools' of control matrix was brought up in the context of harmonization, these being pools constituted from several hundred donors. The resultant volume is good for a great many analyses over many locations, as necessary, and of course, stability and adsorptive effects notwithstanding give a constant baseline of marker levels.

There was a good deal of discussion on the subject of precision. There were two principal factors put forward as to how tight the precision needs to be for a given biomarker assay. One was the biochemical nature of the compound and the expectations arising, which would relate to the analytical technique used. The other factor, also biological in essence, relates more to how the concentration of the biomarker in question naturally fluctuates. If there is a twofold fluctuation, it was asked, does there really have to be a precision of less than 20% for measurements from the assay? Even though this question makes sense, it brought about some consternation in many of those present. It was reassuring to me that there was evidently a strong sense of responsibility to maintain control over method performance. It was clarified nonetheless that the notion was not mentioned to question a standard rigorous analytical approach, but rather for situations where the usual precision has proven hard to attain and effort may be needlessly wasted if the existing poorer precision is adequate to answer the question being posed and give an analytical outcome. Something also briefly alluded to was that, considering the importance of being able to verify that a given measured concentration is significantly different from a basal range of marker concentrations, surely statistical tests are appropriate. I do agree with this, even though it is something many would have to become familiarized with. For instance, performing t-tests where the null hypothesis is that there is no significant difference between two sets of measured concentrations, at a carefully chosen level of significance. This may have to involve the analysis of several replicates of a given incurred sample, and comparator samples, in order to give the required substance to the test.

There was also much mention of the use of subjective terminology and how it can be misleading. The cliché 'fit-for-purpose', for instance, was pounced on for its subjectivity. The statement about a biomarker assay not being a PK assay is itself subjective. Are they completely different entities? To me, the answer seems to be 'yes', since there are very important differences and at this stage we can get used to distinguishing the two. However, the answer is also 'no', at least in the sense that, as already alluded to, we still ought to strive for the best analytical performance, hence confidence in our analytical data.

For me and the LC–MS background that dominates my work, I feel that more should be made of the use of isotopically labeled surrogate analytes, isotopologues, which, with the right labels, approach physicochemical equivalence to their unlabeled analog biomarker. This is further to their classical role of internal standardization. We know from research already conducted that it would involve the expense of synthesizing 13C and/or 15N labeled isotopologues, rather than deuterated, to invoke true effective response equivalence. Clearly the physics is interesting here but, more importantly, the approach is full of promise. From a parallelism perspective, we could dispense with speculation about the suitability of surrogate matrices, and the associated calibration curves would not be skewed by endogenous levels, furthermore they would be in effect as appropriate for interpolating incurred sample responses as they would be in a PK assay. At Crystal City VI there were murmurs of complaint as to the difficulties involved in having to use non-LIMS software such as Excel to process data, but surely with enough of the bioanalytical community engaged in persuasion of LIMS providers to allow for this, it would cease to be an obstacle. Similarly, the need to find two different non-interfering isotopologues to perform an assay like this is seen as a hurdle, but again, as far as one could speculate, it seems reasonable that if the demand was there then supply would follow, particularly if the nominated internal standard (not surrogate analyte) could be inexpensively labeled such as with deuteration. However, the expense put towards synthesis with adequately high isotopic purity is critical. At the end of the day, isotopologues — especially involving heavy isotopes of carbon and nitrogen rather than of hydrogen — are a great tool for the bioanalytical mass spectrometric world because of excellent physicochemical mimicking. The best should be made of them where assay performance and reliability demand it, and biomarker quantification with a mass spectral endpoint is no exception.

One thing is for sure, and that is that there is plenty more dialogue to come involving the industry and regulators.

Previously in this column, I discussed various pertinent aspects of cartridge-based means of biological sample extraction. With the notion of SPE being a technique that inherently offers a multitude of different selectivities, the liaison was made to the ideality of linking appropriate extraction selectivity to the analytical chromatography. It has become almost a cliché among method developers — 'orthogonality of selectivity' between biological sample extraction and subsequent chromatography. This refers to the desirable ability to combine at least two different means of analyte and interferential discrimination (i.e., selectivities), thus eliminating much more matrix residual interferences from the final sample compared to a situation in which the selectivities of the extraction and chromatography are analogous. This is all, of course, while maintaining high analyte recovery in the separate steps. In principle it is also much of what makes multidimensional chromatography so very useful, combining complementary chromatographic selectivities. Additionally, it counts for the most when the retentive modes are fundamentally different, for instance with the combination of a reversed-phase extraction complemented by something normal phase, such as hydrophilic interaction chromatography. Contrast this with a less ideal scenario where, for instance, there is a reversed-phase extraction using a polymer-based sorbent but then another reversed-phase dimension, the chromatographic setup that uses a silica base with hydrophobic bonded phase.

The sample preparative techniques of protein precipitation and dilution, being nonselective other than eliminating larger proteins in the former, are clearly less useful in the perspective of this concept than extraction techniques based on partitioning like liquid-liquid extraction or SPE. This is with the latter techniques operating with the inclusion, or otherwise, of adsorptive and/or electrostatic interactions. It is thus fair to state that having a method that features orthogonality doesn't necessarily make up for a lack of selectivity in any given dimension.

There is little question that methodologies featuring orthogonality of selectivity are generally all the more rugged as a result. It is, however, as with so many things, interesting to think in more depth about the concept. Does it always hold true? The answer is likely not. In the laboratories here, there is a recent example of how one assay's performance was actually improved by making the selectivity of the SPE closely analogous to the subsequent chromatography.

The analyte, a specific metabolite of cholesterol, had to be extracted from human serum. This was a steroid of inherently high lipophilicity and, as is the case for a great many steroids, with many closely related and high-concentration endogenous potential interferent compounds. The chromatography, prior to tandem mass spectral detection, had been set up as a silica-based C18 reversed-phase gradient conducive to analyte elution after five column void times and the run time was spaced out to 30 column void times.

The mobile phase composition was particularly favorable for lipid elution in the latter end of each run cycle, in order to minimize chances of adverse lipid accumulation on-column and the signal drift that could subsequently manifest. The sample extraction was initially also reversed-phase, but on a popular polar-modified styrene-divinylbenzene copolymeric base. Issues with signal drift were observed with this setup, but then the switch was made to a silica-based C18 SPE procedure in conjunction with both matching the solvent composition choices and pH of aqueous modifiers with these of the LC, and a very careful minimization of elution volume while maintaining high and reproducible recovery. The signal drift issues were alleviated and, clearly, the C18 sorbent was a far closer match for the LC stationary phase. It seems very likely that this proximity in selectivity was responsible for the better performance of the assay. How can this be explained?

Any ionizable solute that is over-retained on the analytical column, that is to say a potential interferent and not eluted over a single gradient-and-hold cycle, is going to be responsible for some degree of signal drift as it infuses from the column outlet over subsequent gradient cycles. By replicating this process at the stage of sample extraction, such compounds remain on-cartridge thus are absent from the final sample extract and cannot exert any deleterious effect that they may do otherwise. Additionally, in this case, there is still the inherent cleanliness from a well-optimized SPE protocol. However, It should be noted had there been any interferences that coeluted with the analyte in the analytical chromatography and affected the signal, this extraction would have been very unlikely to have been suitable, and perhaps a variant reversed-phase bonded phase to the two tested would have merited investigation. It could also very well be proposed that perhaps a HILIC extraction would have been better suited as a more distinct selectivity, but it's out of the question to attain any kind of sufficient retention in this mode for a compound like this, of such high hydrophobicity and lack of basic moieties in the molecular structure.

The concept of orthogonal selectivity is well-known in the bioanalytical community, and for good reason. Long may it be utilized in the interests of generating truly reliable data. Still, a certain degree of flexibility should be maintained in its application as is evident from the likes of the example illustrated here, with the caveat that both extraction and analytical chromatography show decent selectivity conducive to a good degree of interferent elimination or resolution.

With particular regard to the sorbents and underlying chemistries involved, it is interesting to look at the way in which the technique of SPE has evolved in the bioanalytical domain. The early days of SPE with prepackaged and disposable cartridges, in the late 1970s, involved silica-based sorbents exclusively. In the mid-1990s came the advent of crosslinked polymeric sorbent material, predominantly based on styrene and divinylbenzene monomer units. It was not long before the ease of use of this kind of material and the potential application to small molecules, of a wide range of hydrophobicities, became obvious; polymeric sorbents have since become ubiquitous and the crux of a first-stop SPE solution for many. With the relatively large surface area bestowing a similarly large capacity and with no need to condition initially with high-organic to ensure the absence of pore dewetting, polymeric sorbents present a convenient 'catch-all' option, which can be used with conventional reversed-phase or mixed-mode protocols to wash and subsequently elute compounds of interest with high recoveries. There is also a certain additional motivation, in general terms, to use polymer-based sorbents in preference to silica-based sorbents due to a perception that the latter can and will dry out easily and lead to recovery loss and lack of reproducibility.

In earlier installments of this column, there has been a hint of playing Devil's Advocate, writing in favor of the use of SPE, which might appear to be gradually falling out of favor. On this occasion, I would like to zoom into the SPE domain and write an endorsement of silica-based SPE in the face of the strikingly abundant use of polymeric SPE in bioanalysis. There's no question that polymeric SPE is a fantastic means at our disposal. It makes things easier; a rugged platform upon which extractions can be based. I sometimes like to imagine polymeric particles swelling and shrinking, as they do, with bouncing, elastic properties that constitute a metaphor for the resilience of such methodologies to adverse conditions that could affect performance and data reliability. This is despite the fact that particle swelling and shrinking are detrimental to the minimization of band broadening, of course. However, silica has distinct advantages. For one thing, it is actually very difficult to dry out a silica bed. It would take a matter of hours rather than minutes with, say, a typical vacuum, so the fear of premature elution as a result of drying out after a load or wash step is very largely unfounded. As an aside, attempting to incorporate a drying-out step prior to the elution step (so that everything remaining is subsequently eluted in the following step) is probably unwise due to this same difficulty in being able to ensure complete drying of each cartridge within a reasonable time. Besides, so many key interferences are conveniently eliminated through their over-retention beyond the elution step in methods involving reversed-phase retention, so it would be ill-advised to attempt to elute all that remains.

The main advantage of silica-based SPE is the myriad of different chemistries and selectivities attainable, reachable via almost any mode of retention that you'd care to mention.

This depends on the compounds being extracted and the nature of the remainder of the applied sample, the solvent composition on-sorbent and what the sorbent has previously encountered, the presence of a bonded phase or otherwise, and of course the nature of the bonded phase including how it affects the surface chemistry. In fact, one particularly rich argument for the preservation of silica-based SPE is the fact that HILIC conditions are readily attainable, whereas on a polymeric sorbent this is not the case. The propensity for HILIC is a result of the presence of the hydroxylated silica surface which is extremely conducive to the formation of a water-enriched layer, allowing the manifestation of fundamental HILIC partitioning. Even with the presence of a hydrophobic bonded phase, this will occur, albeit less reproducibly, on a silica surface. However, on a polymeric surface, this fundamental proclivity towards HILIC is not there, and it can manifest only with the surface binding of pronouncedly polar and ionic moieties.

Then there is the fact that silica bestows the best efficiency as the base material in a stationary phase. In the analogous liquid chromatography, we see peaks eluting from silica-based columns that are sharper than from polymeric columns of the same dimensions and we are reminded of the benefit in terms of efficiency, or sharpness of the eluting bands. This is conducive to having more room to optimize and to give more definition to an optimization, i.e. more leeway to apply a more voluminous and/or stronger wash and a less voluminous and/or less powerful elution solvent blend to attain high and reproducible recoveries. This takes us a step away from a 'switch-on, switch-off' perceived approach to SPE and makes it more akin to a migratory chromatographic process, the reality being somewhere in between, of course.

So yes, let's celebrate what is offered to us by polymeric SPE sorbents and keep them at the forefront where routine, high-throughput applications are prominent, where method development needs to be fast and where there are no special challenges or difficulties to overcome. Further to that, though, let's also keep our repertoire of silica-based media safe. Look at the decades of use in validated methods as a testament to the ruggedness offered. Where special measures may be needed to obtain selectivity, or some key fine-tuning is required, one of the many manifestations of silica-based retention may just be a direct route to a solution.

> *Having witnessed the amazing array of ways that silica can influence chromatography from fundamental levels, it made for an interesting contrast with the ever-reliable but perhaps more simplistic and less versatile polymer base in the extractive context.*
>
> Robert MacNeill

This is a question to which there is no simple answer, and thus affords good material for discussion, particularly with the ever-impressive march of technology.

In the early 1990s, it would have been fair to state that the analogy was only valid in terms of the presence of a stationary phase, a packed sorbent or chromatographic bed, and a mobile phase, flowing either via gravity or positive/negative pressure, or pumped with accurate and precise control. The fundamental chemical and steric processes that govern band migration were also valid. Nothing more could be claimed in terms of this analogy and this is because of the nature of the stationary phases used for each. Analytical liquid chromatography used typically 5 µm particle size, whereas SPE used 10 times this size at best. This stark difference in particle size was key in the efficiency contrast. The quality of the packing and the particle size distribution, especially linked to the presence of fines in SPE, gave more potency to the difference. Therefore, it was mainly in terms of efficiency, and how this consequently affects resolving power, that was the decisive factor. Couple this with the typical lack of flow rate control in SPE and you have two techniques that are only similar in their fundaments, and operate in very different contexts, albeit complementing each other in attaining the same ultimate goal, i.e. constituting different parts of a complete bioanalytical method.

I wonder if we can now state that, in the current state of affairs, there is more of a true analogy. To put efficiency thoughts aside for the moment, at least in terms of the chemistries used as bonded phase on silica there is certainly an analogy between LC and SPE, in that C18 is the most abundantly used. However, there are the likes of alumina, Florisil and graphitic carbon that are used in SPE but a lot less in LC. Also, a shift has been made towards polymeric sorbents rather than silica in SPE, as alluded to in one or two previous installments of this column. On this note, returning to efficiency considerations — the polymeric material is actually more amenable to good packing quality, better withstanding of packing pressure, and there are reduced concerns about the presence of fines. But improvements have been made in general terms to SPE sorbent production.

Improvements in efficiency are most pronounced. In the previous commentary, brief allusion was made to the widely-perceived ‘switch on – switch off’ idea of SPE operation, rather than viewing as a migratory chromatographic process. This notion seems to focus on manipulating selectivity rather than dwelling on efficiency aspects in order to achieve a clean, high-recovery extraction. The ‘switch on – switch off’ way of looking at SPE was convenient and entirely justifiable with the typical packing characteristics of years gone by, but is something that is becoming less valid as the quality and nature of packed beds changes. In the late 1990s, for instance, microparticulate SPE came along, using 10 µm particle size, much more akin to analytical LC. This never seems to have taken off in earnest in the world of bioanalysis, but is still in some degree of popular use.

Further to this, albeit slightly tangential, the µElution design that burst onto the bioanalytical scene approximately just over a decade ago, with a narrower sorbent channel among other intelligent design features, also advanced the quality and performance aspects and expectations within SPE. The introduction of the µElution format was a wonderful step forward in sample preparation and remains unambiguously extensive in its popularity and use.

Probably the best comparative means for SPE towards LC, best exemplifying true analytical performance with reproducibility, is in the form of contemporary manifestations of online SPE. Recent applications and technology are impressive, and it's threading its way into the realm of fully validated methodologies where this would be unheard of even just a few years ago. In the regulated lab, where application of online SPE has classically been largely for the non-audited areas of sorbent screening in method development, or sometimes to set up, in the context of discovery methods, a dual column system with an SPE cartridge catching the initial injection and, after washing, subsequently backflushing onto an analytical column, the outlook is changing. There is now access to 3 µm particle sizes in high-pressure online systems, with up to the likes of only 9 mm cartridge length and otherwise very low extra-cartridge system volume, resulting in performance akin to true chromatography with the inferred resolving power and reproducibility. There will undoubtedly be much more that we soon hear about online SPE in the literature, webinars and conference circuits.

As a final thought, we in the bioanalytical community have an inescapable awareness of LC columns featuring superficially porous particle technology, sometimes referred to as core-shell, which has already become abundantly used in the industry, and for good reason. At first glance, thinking on this specific comparison, it constitutes a hurdle to being able to claim any more of an analogy between SPE and LC. However, can this technology be translated to SPE, and would that be a good idea? Surely it can, given adequate demand, and it would seem there are very good reasons why it would be. Theory and practicality may disagree, on the other hand, but altogether this may be good fruit for a further treatise.

> *Can we really make analogies of the two critical analytical techniques? Why yes, especially as SPE products begin to be more uniformly produced and are moving toward analytical characteristics for operation.*
>
> Robert MacNeill

The quantitative methodologies produced in the realm of regulated bioanalysis are innately required to be unfailingly solid in their performance and reliability. With the contemporary normality of aspects such as incurred sample reanalysis and internal standard peak area control charts, the performance is also put to the test more than ever before, and monitored accordingly. Meanwhile, the kind of analyte typically taking center stage is becoming ever more challenging. Oligonucleotide and peptide/protein-based therapeutics, for instance, continue their rise.

For proteins by LC–MS, the accepted standard approach remains using an optimized digestion protocol and indirect quantification using analysis of signature peptides after cleanup, although there is a small but increasing collection of work done to quantify these entities intact by the same LC–MS endpoint especially as high-resolution technology and quantitative software improves.

We are thus faced with the vast majority of such work involving peptidic analytes that are very polar in nature, charged, and amphoteric. As a result, we require biological sample preparation/extraction procedures to be suited to these properties.

Protein precipitation of plasma samples is an option, but this achieves little more than eliminating the larger protein content of the sample, like albumin — a nice choice if you wish to screen everything else that is in the sample over one or two injections, but if the aim is to produce the most selective and reliable quantitative method for a definitive small number of analytes, this option would not be conducive to success. And this would be not only due to the lack of selectivity. The more polar a compound is the less likely it will remain solubilized upon the addition of organic solvent to the aqueous matrix.

Perhaps worse than attaining very little recovery would be what accompanies very little recovery if it is decided to use the technique — that is, a great imprecision in the recovery possibly changing markedly from sample to sample, with a high dependence on the slightest fluctuations in key factors like temperature and pH.

Liquid-liquid extraction, or any derivative based on partitioning into a water-immiscible medium, is a fairly established no-go area for extraction of peptides from biological fluids, and this is due to the propensity for there to be zero recoveries. Peptides, being all about polarity and electrostatic interactions, tend not to partition at all into media of such limited polarity.

Therefore, we are left with not much else but SPE to use for these challenges. What has been historically used to obtain the cleanest most selective extracts for small molecules is easily translatable to peptides, keeping in mind how these differ from most small molecules.

This means, besides instability and non-specific binding concerns, catering to the generally greater polarity of what is being extracted and accepting the prompt towards embracing the charge manipulation as a means to garner great selectivity.

What of one obvious grievance, that solid-phase extracts are expected — whether reversed-phase, mixed-mode or HILIC — to result in very high organic ultimate eluate composition and, since evaporating and reconstituting is out of the question for most peptides, how can this easily interface with a typical high-aqueous initial setting in the reversed-phase gradient of the analytical chromatography? The obvious answer is to add the appropriate volume of aqueous modifier to each sample, but this takes away from any intended concentration effect, and that is desired far more often than not.

Alternatively, the sorbent bed weight could be lowered in many cases, or the complementary chromatography could be optimized as HILIC. But there is another phenomenon that we can frequently take advantage of, perhaps much less well-realized since as far as I know there aren't published data to support the following optimization approach as yet. It actually ties into obtaining the best HILIC retention. Where ion exchange is involved, including mixed-mode procedures on either polymeric or silica-based sorbents, it is often the case that the eluting power of a solvent mixture can be enhanced by increasing the aqueous content.

This may seem to be counter-intuitive. However, it is known that basic moieties on compounds alter in their basicity according to organic content in the medium in which they are solvated. Basicity is actually anticipated to increase dramatically upon excursion through 60-100% acetonitrile. In this region, a basic compound will show a significant increase in pKa, i.e., what amounts to increased basicity and accordingly greater abundance of ionized forms, and this is far more pronounced relative to such changes in the lower organic composition region.

We can use this knowledge to suppose that we could release a basic compound electrostatically tied to the sorbent, at the desired elution stage, by reducing the acetonitrile content such that the basicity is also reduced to an extent sufficient to make the neutral form predominate. It can be shown to work too. This is without compromising the withdrawal of retentive power of an accompanying non-electrostatic reversed-phase retentive mode. Hence, we can have eluates with much higher aqueous content right off the bat than we may have thought feasible, and we can take advantage of the benefits in better interfacing with reversed-phase chromatography, maintaining free peptide solubility and sample concentration.

Altogether, it amounts to a message that we can do even more with the SPE domain than what we might have thought! Often it is well worth a little more optimization time for the sake of producing that truly invulnerable method.

The message I'd like to convey in this piece is, in essence, about endeavoring to incorporate orthogonal selectivity in a method even if it means exploring what might be viewed as unorthodox, as long as it's scientifically sound and ultimately proven with adequate rigor.

The importance of orthogonality of selectivity has been illustrated in some detail previously in this column. In the field of quantitative bioanalysis using LC–MS detection, prior to the highly selective tandem mass spectrometric endpoint, there are two fundamental constituents of a given methodology in which selectivity should be selected appropriately and tuned. These are, of course, the biological sample extraction and the subsequent liquid chromatography. When the extraction is solid-phase it affords us the maximum potential to harness selectivity at this stage.

It's important to keep in mind that orthogonal selectivity between the extractive and LC dimensions is an ideal. There are, meanwhile, innumerable instances of great methods that involve reversed-phase SPE complementing reversed-phase LC. There is still plenty of selectivity room within the reversed-phase domain for the production of rugged methodologies. For instance, the selection of reversed-phase chemistry in a stationary phase, pH and ionic strength of aqueous modifier, protic or aprotic organic modifier, are among the variables that contribute to an overall selectivity in a separation.

There are also innumerable instances of mixed-mode involving reversed phase with electrostatic interactions in the SPE complementing a reversed-phase LC, but not nearly so much the reverse, as it were. It would seem, therefore, that reversed-phase lends itself very well to bioanalysis, and it's true enough. How often do we find HILIC in the LC dimension after any means of sample extraction? We would certainly like to, since it's a wonderful complement to reversed-phase in terms of exemplifying truly orthogonal selectivity, and it's conducive to high-sensitivity mass spectral detection due to the high-organic eluent composition. But the answer is not very often, even after SPE. One important reason for this is that HILIC often falls to pieces when biological extracts are injected, where solution injections, free of matrix residue, are unaffected. HILIC is simply not as forgiving as reversed-phase when it comes to the extent of disruption of chromatography due to small and unavoidable fluctuations, sample to sample, in injected sample composition.

However, this unforgiving aspect of the nature of HILIC pertains to analytical chromatography, where any slight aberrations in parameters like asymmetry factor are justifiably seized upon, flagged and result in later reanalysis from scratch. What if, on the other hand, the HILIC was performed within the sample extraction dimension? Can such aberrations in band migration, like a bit of asymmetry, be forgiven? Yes, as long as we attain high and reproducible recoveries. We do not expect illustrious analytical chromatographic performance in the SPE dimension. There is good reason to suppose that this constitutes sound reasoning for involving HILIC, if it's to be involved, at this stage rather than at the analytical LC stage.

As mentioned before in this column, I'm a big fan of the ubiquitous polymer-based SPE, but I also like to call for more commercial options with respect to SPE stationary phases that can be used for HILIC. These are overwhelmingly silica-based, since the naturally hydroxylated silica surface is a perfect means to invoke HILIC and can do so, albeit less uniformly and reproducibly, even in the presence of a hydrophobic bonded phase.

HILIC can also be performed on polymer-based phases though, as long as these are profoundly polar-modified and incorporate perma-charged moieties. In addition, returning to silica and its surface manifestations, it may be a fascinating prospect to have silica hydride-based columns translated to SPE format. This silica hydride surface chemistry bestows valuable characteristics whereby under high-acetonitrile conditions the aqueous normal phase mode is in operation, a mode somewhat akin to HILIC in its manifestation and selectivity.

It is nevertheless fundamentally distinct from HILIC in that, for one thing, the bonded phase on silica hydride does not need to be pronouncedly polar – and HILIC, on the other hand, is something that requires polar stationary phases. Under high aqueous conditions, it's a reversed-phase operation. Indeed bimodal separations are synonymous with silica hydride-based chromatography.

Wish lists aside, there are the means to do this commercially available, with reference mainly to silica-based SPE sorbents, most conducive to HILIC. It's a case of trying it out, seeing how it works, but making sure the right fundaments are in place in the protocol. These are, for instance, loading acetonitrile-crashed plasma to avoid breakthrough due to too high an aqueous content in the HILIC load step, if a simple HILIC-SPE protocol is used (an alternative of mixed-mode employing HILIC is also feasible).

It is clear, in summary, how useful SPE is in obtaining orthogonal selectivity in quantitative bioanalytical LC–MS, amongst the other advantages of the technique. We can confidently attempt and optimize HILIC at the stage of SPE within a given method, where HILIC of extracts in the LC dimension may not work with adequate chromatographic reproducibility.

> *Having developed 'Hydrophilic-Phase Extraction' a few years after scribing this, it is fascinating to look back at the early thoughts. The possible incorporation of silica hydride in SPE format remains tantalizing for the same reasons.*
>
> Robert MacNeill

It has been becoming more commonplace and noticeable, at least on the CRO side of the industry, that bioanalytical LC–MS assays are no longer presumed to involve simply the species' matrix as control, for the purposes of producing double/single blanks, and for spiking calibrant and QC samples. For instance, until recently, mouse plasma could have been harvested from males and females, indeed pooled as such and the strain was not typically regarded as important for control purposes.

The contemporary angle when carrying out a study on, for example, a specific strain of a species is that the control matrix for analysis must also be entirely from the same strain. This is easily justifiable on the surface, but is it really necessary?

The composition of the matrix is a pivotal aspect of bioanalysis, in that it is what we need to understand and come to terms with, such as how to keep an analyte freely solubilized within the matrix, free of binding to the container surface, and ultimately how to efficiently extract said analyte from the matrix without co-extracting a significant amount of interferences. There are properties of matrices that are obvious and readily investigated. Hemolysis, for instance, is a phenomenon that changes a blood-derived matrix to something markedly different, and comes with implications for selectivity and stability. Yet, hemolyzed samples are analyzed alongside regular non-hemolyzed samples after the method is demonstrated to be steadfast as regards maintaining accuracy and precision for these samples.

Hyperlipidemia, for human blood-based samples, is another visually clear (perhaps unclear is more apt) phenomenon that alters the matrix and for which a method must be proven rugged enough to maintain data integrity in the analysis. Indeed, clinical samples can be notoriously challenging, bringing never-seen-before interferences, viscosity changes and other curiosities that can really challenge a validated method. This is partly what brings such tremendous value to incurred sample reanalysis when it comes to testing a method's mettle. One very pertinent component of incurred post-oral samples is the metabolite content. Even with the selectivity of the various modes of MS at our fingertips, their presence can prove deleterious to a method's performance, and selectivity with regard to known or probable metabolites should be established in method development. Often there is a plethora of metabolic possibilities, both phase I and phase II, and there is almost inevitably a certain abundance that can efficiently revert to the parent compound when exposed to the thermal conditions of the ion source. There, of course, one must rely on the preceding chromatography for selectivity.

Also, it is not just at elevated temperatures where such *ex vivo* conversion can take place. Transesterification, for instance, readily occurs at ambient temperature. Without overdoing the metabolite discussion, suffice to say that this generally metabolite-infested matrix can be said to be an important analytical departure from the relatively simple control.

Matrix aging is something that is less obvious than the likes of hemolysis but nonetheless a very real phenomenon, and a danger to the analytical integrity of a matrix as certain endogenous components change concentration over time, and degradants are created, in essence creating a different matrix. This realization has led us to ideally store control matrices at -80°C, whereas historically it has been -20°C.

Then we have the effect of the anticoagulant used for blood, plasma or serum assays. This came into the limelight recently in the bioanalytical community and was discussed and debated at length. A large-scale collaborative experiment was eventually performed, with the convincing outcome that the selection of anticoagulant counter-ion, at least, for a validated LC method with tandem MS detection does not significantly affect the accuracy of the resultant data. In what amounts to a necessary and largely uncontrolled adulteration of samples, and with the negligible outcome on data reliability, this must offer more strength to the notion that generally insisting on a specific strain or gender is largely a futile exercise. A good method deals with the presence of an anticoagulant, just as it deals with a host of other potential interferences that can vary enormously in concentration.

To sum up, the logic behind the kind of decision that brings only a specific strain or gender of control matrix is readily understandable. I would endorse it too, where program/study design practicalities and costs are not prohibitive, in order to acquire data with the greatest reliability. However, it is considerations like the aforementioned that, to me, swamp the validity of doing everything one can to stick to a specific gender or strain for the control matrix.

There are too many other factors at work, and if it is required to do this so that the method works for accuracy and precision, it raises a question mark about the method integrity. The most important theme has to be the reliability of the method itself and how it fares under all possible challenging circumstances within the realm of species and matrix. The more observed variation in the control matrix composition accompanied by no adverse effect on performance, the better.

> *In the run-up to this, I had been increasingly aware of stipulations for control matrix going beyond species, beyond gender, and going into the strain of tox species. I remain convinced that there's almost always no need to be that specific, both scientifically and for the sake of expense and practicality.*
>
> Robert MacNeill

I recall several occasions in which various characters in the industry have remarked that they prefer protein precipitation as a means of sample extraction prior to LC–SRM. The grounds for this point of view are along the lines of 'everything is still in there'; 'you won't lose any metabolites'; and 'there is no need to worry about recovery'. A more recent reason I hear, which I am entirely attuned to, has to do with state-of-the-art instruments often giving so much sensitivity that only a tiny volume of extract needs to be injected, which bestows positive implications for minimizing matrix effects.

Prior to the main focus of this piece, I would like to address what I believe to be a misunderstanding, which is the aspect of recovery here. Recovery can indeed be lost in protein precipitation. Usually, the technique is done using organic solvent like acetonitrile or methanol, adding two or three volumes per volume of plasma or serum. The organic content drives the aggregation and subsequent precipitation of larger proteins like albumin, mainly through disruption of the water-rich layer around them. If you happen to be analyzing a protein like this, it will clearly have precipitated and you need to take the pellet for analysis, indeed pellet digestion is an established technique as part of therapeutic mAb analysis.

Focusing on the realm of small molecules and peptides, a compound polar enough to be insoluble in the resultant mixture will not be efficiently extracted, and the more water-soluble it is, the worse the recovery will be, in addition to being less precise and reproducible. Similarly, for protein precipitation methods that use the likes of trichloroacetic acid in aqueous solution to affect the aggregation and precipitation, compounds that have issues solubilizing in the resultant acidic aqueous conditions will have issues with recovery.

The underlying mechanism of protein precipitation just described makes it clear that, from an extraction selectivity perspective, it only eliminates proteins large enough to take on globular form and possess a water-rich outer layer, weakly bound to the hydrophilic outer regions of the protein. This brings to consideration the most compelling of the above reasons for being drawn to protein precipitation. That has to do with recovering the metabolite content, and yes, there is value in having an extract of an incurred sample that still contains a full complement of metabolites as well as the analyte content. This is especially true if the likes of TOF-MS is being used, perhaps in a semi-qualitative sense, the nature of which is continuously all-scanning hence all these data are opportunely collected and with accurate mass, allowing easy identification.

For the quantitative bioanalytical scientist, however, using such a non-selective extraction method also has a downside. The extraction method must be made non-selective in accordance with the number of analytes, where accordingly more interferences must be dealt with, yet the method as a whole must perform ruggedly. The less selective an extraction, the better the LC–MS side needs to be to deal with the potential host of interferences that are present in the extract.

Metabolites can certainly qualify as interferences; in fact they hover near the top of the danger list. The best quantitative methods have extracts so clean they are akin to solution rather than extract. All interferences, including proteins, salts, and lipids, are completely eliminated, and all individual analytes with their internal standards are completely recovered. That is the ideal, the goal that can never quite be achieved. The more analytes that a method includes, and the more they vary physicochemically, the further from this ideal we find ourselves.

Metabolites are a great example of compounds related to a parent analyte that may have emphatically variable physicochemical properties from both the parent compound and each other. They are by far most often the reason for multiple analyte assays associated with programs involving a single therapeutic candidate, but there are also plenty of instances of multiple analyte methods involving simply different drugs or drug candidates. Metabolites can engage in quite sinister phenomena. Phase 1 metabolites like N-oxides, for instance, can at least partially revert back to the parent in the heat of an ion source, creating an extraneous peak in the ion channel of the parent compound, or co-elute if the chromatography is inadequate.

Conjugates, Phase II metabolites, can also show reactivity, especially when the conjugate link is prone to hydrolysis, like ester-linked glucuronides. In the presence of methanol, trans-esterification of such conjugates has been known to occur where the resultant product is the parent. This obviously takes away all reliability from the analysis of incurred samples. This kind of behavior from metabolites comes in addition to posing the usual interferent-type risks to do with competition for ionic release into the gas phase, a.k.a. competition for ionization, when co-eluting with an analyte, whether or not they are visible in any given ion channel.

Multiple analyte assays are a big part of life and a nice but potentially frustrating challenge, as is quantification of many metabolites. There needs to be good reason to undertake them, that is to say, a fit-for-purpose argument. In quantitative bioanalysis, we must be prepared to have a harder time establishing unquestionable method performance under these circumstances. For every additional analyte, particularly of different physicochemical properties each time, the extraction method will be inherently less selective in order to ensure decent analyte recovery, and the need for excellent discriminating power in the LC–MS will be all the more emphatic.

Balancing instability precautions against risk of adsorptive losses

17 Oct 2017

The instability of drugs, drug candidates, metabolites or any designated analyte within samples of a biological nature is a very familiar topic of discussion in the regulated bioanalytical community. Indeed, the guidance and typical standard operating procedures are now such that any significant degree of instability will be confidently detected and dealt with. It is my impression, however, that the exposure of the community to so much rhetoric and discussion concerning instability issues has led to something of a blind spot regarding the ramifications of some routine aspects of bioanalytical work that may be affected by the nature of instability precautions.

I am alluding to two areas primarily. Firstly, the ubiquitous preference to default to the storage of samples, stock solutions and spiking solutions at as low a temperature as is available. This is typically -80°C. Secondly, the preference to prepare and extract samples 'on ice' in situations where instability is anticipated or at least suspected.

Prevalent on my mind is an effect that is related to the above in its more severe manifestation at lower temperatures. Like instability, it can also lead to pronounced and variable losses, and can very well tear a method's reliability to shreds. Also, it is probably better known to those who have worked much in the quantification of larger biomolecules. It is the phenomenon of non-specific adsorption (NSA), also known as non-specific binding, and it certainly merits an important influence on the decision as regards sample treatment, including storage, in all appropriate media. It manifests through chemical interactions, ranging from electrostatic to van der Waal's in nature, resulting in adsorption of compounds from their liquid medium to surfaces that the medium is in contact with.

There are aspects of the phenomena that are critical to understanding how it works and to making effective decisions in light of it. These factors are the basic mechanism, the concentration (not just of test items but also of other components in the medium), the composition of said liquid medium, the chemistry of the inner surface of the containing vessel, which meets the liquid stored, and the temperature.

From a chemistry perspective, in simple terms, a polar compound will bind to a polar surface, like glass. A nonpolar compound will bind to a nonpolar surface. Chromatographers will be none too unfamiliar with this kind of reasoning. There are two common vessel compositions in the laboratory – glass and polypropylene. To avoid NSA, it makes sense to use glass vessels to contain nonpolar compounds, and polypropylene vessels to store polar compounds, particularly when there are doubts over the free solubility of test compounds in the liquid medium. There are specialty tubes and 96-well plates commercially available that can help, such as polypropylene plates with a glass-like coating.

There is concentration dependence in that the lower the concentration, the more significant any analyte loss will be, as a percentage. This is down to there being a defined number of adsorptive sites, a set surface area available in any given situation.

Tying in with this is the effect of the presence in solution of other components. These can alleviate NSA by their own occupation of adsorptive sites, the competition aspect within the solvation and adsorption dynamics. This is why solutions can be particularly susceptible to being affected by NSA, whereas a biological matrix such as plasma, containing a host of different components, may not be as adversely affected. On this note, urine samples in polypropylene tubes may often suffer from the effects of hydrophobic NSA of analyte due to a distinct lack of protein and lipid content compared to plasma. Exacerbating this is the pronounced content of highly water-soluble components in urine, making it all the more difficult for a hydrophobic analyte to remain freely solubilized. Indeed the composition of the liquid medium is key to the manifestation, or otherwise, of NSA. This relationship is also pretty simple. The more soluble a compound is in a certain solution composition, the less chance there is of NSA to surrounding surfaces. If solubility is sparing, the risk of adsorptive losses is always there. Also, the way solubility works is that as the temperature drops, solubility also decreases. This increases the chances and abundance of NSA to the surrounding surfaces, whatever the chemistry.

Temperature is the pivotal point in this discussion. As just stated, NSA is intimately related to solubility in that the lower the temperature, the more severe the NSA. Therefore, wherever temperature can be made closer to ambient without instability or evaporative concerns, the better. Now, if the intriguing reality of matrix aging was not a concern for many types of stored biological samples, from the NSA avoidance perspective when -20°C can be used instead of -80°C, it should be used. Similarly, analyte-containing solutions could often be stored at 4°C instead of at more severe lower temperatures, where later resolubilization of subsequently bound analyte could be difficult and impactful on concentration data. This applies especially if the analytes involved are well-characterized and stability is established.

When working to prepare samples ‘on ice’, meaning in an ice-water bath, the chances of NSA increase due to the lowering of temperature. This is something that is clearly preferable to avoid if it may be feasible to do so, and for this reason it is recommended to work prior to screen for and optimize the best stabilizing additive, at ambient temperature, as an alternative. Another reason to avoid this scenario is that the temperature is harder to control compared to the ambient temperature climate-controlled laboratory, so the ‘on ice’ situation would lend variability to the procedure in this way as well.

The outcome, fortunately, is such that there is not much complexity to make decisions around. Safeguard against NSA principally by selecting storage vessels that the test item has minimal propensity to adsorb to, and where possible by choosing a medium in which the compound’s solubility is maximized. Additionally, it may often be wise to use not quite as low a temperature for storage if there is enough confidence about stability. When faced with the prospect of working ‘on ice’, take the time to investigate if the right stabilizer additive at the right concentration can negate this requirement, and a room temperature operation can proceed.

This piece is not focused on chiral LC–MS, but it is a convenient avenue to the topic I'd like to bring up. In my 21 years in the CRO environment, I have seen my fair share of chiral LC–MS methods. Within these methods, the chiral columns typically have had a length of 100 to 150 mm, which I believe is reflective of the industry standard for quantitative LC–MS application. This is a lot longer than the 30 – 50 mm commonly used in regular achiral small molecule bioanalysis. In chiral LC, where sub-3 micron technology is not available to add to the plate count, this length is necessitated by the requirement to baseline separate at least two key components (at least one enantiomeric pair) in the extract being injected. It may seem somewhat speculative, but attaining adequate ruggedness of chiral methods has been, in my experience, frequently easier to come by than for achiral. I don't think it's a huge leap to suppose that the extra plates in the length have an impact that helps lead to this outcome. It's just accompanied by a sacrifice of a longer run time, and signal-to-noise since there will be more peak broadening in the longer migration to the column outlet, in essence a trade-off between these two aspects and the more rugged performance.

A key factor, we can pretty safely reckon, is that the aforementioned increase in column plate count is affording better discriminating power with regard to the interferences present. In the same vein, there are plenty of methodologies that employ an oversized column, as it were, to shore up the overall selectivity after a crude sample extraction procedure. This counts where particle size and superficially porous particle technology, directly affecting pore length and mass transfer, are either exhausted or not an option. In these instances of crude sample preparation procedures, it is usually deproteinization of plasma or serum samples via the addition of organic solvent. As implied by the term, all that is being removed from the sample is protein matter, and even then it is by no means eliminated. Far more significantly for LC–MS, the entire lipid content remains, along with a plethora of peptide matter and salts. Within the lipid content, it is phospholipids for which these methods are typically set up to attain selectivity for using the chromatography. Phospholipids, specifically phosphatidylcholines and lysophosphatidylcholines, are very well characterized in quantitative bioanalysis and are directly monitored in method development. A longer column aids in resolution from the envelope of lysophosphatidylcholines in particular, since these have a strong tendency to elute within the retention windows of a great many small molecules in optimized methods. Phosphatidylcholines, with their very pronounced hydrophobicity, are retained much more, and in this context, a larger column actually exacerbates the problem of eluting these inside a reasonable run time. If allowed to accumulate over many injections, the risk is there of signal drift and all its negative implications. In the vast majority of cases, though, setting the last segment of the run to a mobile phase composition of >98% methanol will bring off these key interferences within a handful of minutes, and direct monitoring in method development will give exact timings.

The longer the column, and especially if it has a greater internal diameter, the more difficult and important it becomes to ensure complete elution of lipophilic interferences.

This, amongst one or two other considerations, brings me to one of the greatest frustrations I feel upon reading so many methods reported in the literature that feature humongous columns, in the quantitative LC–MS context, regularly in the order of 250 x 4.6 mm. The frustration arises from the analyte retention characteristics being inescapably inadequate in these methods. As chromatographers, we ought to know that we need 3 void times to pass before our analytes elute. That is part of the essence of the retention factor formula for isocratic systems, and the acceptable window of 2 to 10 for retention factor values. In the case of a 250 x 4.6 mm column with a 1 mL/min flow rate, the void time is 2.5 minutes thus we are looking for a 7.5-minute starting line for retention times. These methods I am referring to, however, have analyte elution usually right after the void time (2.5 minutes), sometimes up to 2 void times (5 minutes), and the total run time is a seemingly convenient 5 or 6 minutes. It can often be even more deceptive when resolution between two analytes is apparent, but it's not chromatography with integrity. The retention is woefully inadequate, and the process is more akin to filtration rather than chromatography. There are a number of dangers of eluting too close to the void, then there is also the danger of interferences accumulating as a result of lack of proper optimization especially on the larger dimensions. So, on the whole, we need to preserve chromatographic integrity, and that does not depend on what column dimensions we work with.

It is all very well to deviate from the 'norm' and select a column of what might be seen as unusual dimensions as part of ensuring decent selectivity in a method as a whole. We just need to be sure to maintain awareness of the implications that accompany such decisions. It is pivotal to work by the rudimentary requirements of chromatography, principally gauging what is unacceptable and acceptable retention. Also, in the broader picture, setting a suitable balance between chromatographic run time and overall resolving power, figuring the sample extraction into the decision process. The most rugged methods, when required and as is synonymous with the regulated laboratory, would involve both highly selective extractions and chromatography with emphatic resolving power.

> *Being able to take the column dimensions and flow rate to figure out the void time becomes a recurring theme in my 'columns' as time goes on. In this particular one, I wanted to show that peaks eluting on the void from an oversized column, which is a situation frequently observed in literature reports, do not satisfy basic retention criteria as pertains to the chromatographic establishment, and for good reason.*
>
> Robert MacNeill

Getting to grips with metabolites before the crunch of incurred samples

9 Apr 2018

We are all familiar with the notion of a problem, or potential problem, being present and not addressed until such a time as it causes huge fallout. In the context of method development and subsequent use in quantitative bioanalytical LC–MS, analogies of this can be made to many characteristics of the method, but very few have more potential for fallout during the analysis of incurred samples than what adversity could manifest by the presence of metabolites.

Most administered therapeutics will undergo metabolism, especially post-oral administration. As mentioned in some previous columns, there is the possibility of the generation of a great number of metabolites, and plenty that could have a significant interferent impact on quantitative data. After all, these are all related compounds.

There are two distinct phases of metabolism, resulting in two classes of metabolites, Phase I and Phase II. Phase I is where a compound undergoes small modifications to bestow more polarity, such as hydroxylation at various locations. Increasing polarity aids the elimination of the molecular entity and sets up avenues for conjugation, which is phase II metabolism. Conjugates may be, for instance, glucuronides or sulfates; typically a more pronounced polarity increase is the result, again helping elimination.

There are at least three ways in which metabolites can affect the performance and reliability of an assay. One of these is the chromatographic manifestation of metabolites in SRM channels monitoring other compounds. I have seen this several times with N-oxides, phase I metabolites labile in the thermal conditions of an ion source with heated auxiliary gas or heated probe, where conversion back to parent occurs, hence the visibility in the parent channel. This introduces the need for emphatic chromatographic resolution. Another way in which metabolites can make their presence felt is analogous to the main tendency of other interferent compounds that may be present in an extract. Upon coelution with the analyte, the metabolite exerts a response-altering effect, usually suppressive, that creates havoc with signal strength and precision, a direct impact on the matrix factor. The other way is often rather more sinister. Under certain solvent conditions, Phase II metabolites in particular can cleave off the conjugate moiety *ex vivo*, if the conjugate bond to the rest of the molecule is hydrolytically labile like an ester link. This can occur readily with acyl glucuronides, and stored incurred samples at risk should ideally include a stabilizing additive. This potential instability not only affects the calculated concentrations of the metabolite but may also affect, critically, the parent levels if such is the product of the reaction. Trans-esterification is an important phenomenon to be aware of in this context. It would be a great coup to glean, at the development stage, what the abundant metabolites are going to be, as we will then expect them and their knock-on effects in incurred samples.

Perhaps one means of doing this is by making use of *in vitro* incubations of test compounds. In other words, performing incubations of hepatocyte cultures or S9 fractions with parent compounds for administration, then extracting and analyzing with a view to identifying the metabolites produced. The use of TOF-MS or analogous HRAM technique, or even sweeping appropriate ranges in a quadrupole analyzer, should give more than a good idea of the metabolite content. This counts especially for any possible conjugates where mass differences are easily anticipated, such as the characteristic addition of 176 Da for a glucuronide unit, keeping in mind how the charge state will affect these differences upon translation to m/z. Also in Phase I there is the complication of a much greater pool of possibilities, all mass spectrally closer to the parent.

Extraction from the incubate would have to be fairly nonselective, most ideally akin to protein precipitation procedures via addition of organic solvent. Something partition-based like LLE would risk substantial recovery loss, particularly in consideration of the greater polarity of metabolites in relation to the parent.

At this stage, the chromatography of the metabolites in the chromatographic method can be ascertained and any necessary adjustments subsequently made in order to get the right selectivity. The presence of unstable metabolites would also become apparent, and again, action taken if need be.

This amounts to an idea which may be of value in many cases. What could be more prudent, however, is to somehow involve incurred samples within method development and validations. In the big picture, we can frequently expect there to be more than just metabolic interferences, but additional key LC–MS signal encumbrances unaccounted for in the control matrix sets of a validation. Clearly there would most often be practical and logistical obstacles, but if some kind of pilot *in vivo* work could be done for the sake of method reliability, I think there might, in an ideal world, be a good case for it.

> *Metabolites, so often not involved in method development, can creep up and bite us in the analysis of incurred samples. How can we reasonably address this? My thoughts are here.*
>
> Robert MacNeill

There is one popular approach to the challenge of biomarker determination in biological matrices, and that is to employ surrogate matrices to prepare calibration standards and QC samples in. These surrogate matrices are free of any significant endogenous level of biomarker, making them ideal for spiking to nominal levels without complication. Critically, however, they are inherently not of the biological composition of incurred samples and in many methodologies they are not even biological fluids.

This is the critical point for the surrogate matrix approach. For best method performance with regard to the various influences on the analyte and internal standard LC–MS peak area responses, the chosen surrogate matrix must adequately mimic the characteristic output from the genuine matrix, within the selectivity as a property of the entire LC–MS methodology. Parallelism.

We have witnessed and partaken in discussions of putting accuracy aside and looking only at precision in biomarker assays, especially in the face of the reality of inherent marker variability in given subjects. We have in the same vein discussed the good old subjective cliché 'fit-for-purpose' in the context of such methods and their performance, in the sense of making them only as good as they need to be to answer specific questions to do with the ability to confidently verify concentration changes and assigning significance as such. I wonder sometimes, does this in part amount to an indication that frequently the approach makes things a little too difficult, particularly from the angle of accuracy? I know from my years of experience in method development for PK and biomarker assays that it's very dangerous to rely on QC samples prepared in a different overall composition to calibration standards to correlate with them in the final analysis. Bias could be compromised, in other words, even though precision meets the quality of expectations. We know in the industry at least from PK method development, validation and subsequent incurred samples use that method performance, originating in intensities and variation in peak area response, is so very often exquisitely sensitive to many components of a biological matrix. Surely the presence or absence of many such components can very easily have a breathtaking effect on the peak area output, with regard to what may be the case with a surrogate that has forcibly or inherently different makeup. This is also what frequently leads to undesirably long method development times with the surrogate matrix approach.

Another reasonably well-known approach exists, but does not appear to be embraced anywhere near the extent of the surrogate matrix option. It is, of course, the surrogate analyte approach.

I regard the surrogate analyte approach as a gift that comes with the world of bioanalytical LC–MS, as much of a gift as stable isotopically-labeled analogs are as internal standard candidates, and for the same fundamental reasons.

That is to say, these stable isotopically-labeled analogs (isotopologues) used traditionally as internal standards are by design practically equivalent in terms of physicochemical properties to their unlabeled analog, in this context being the compound of interest. They only differ with a few heavy isotopes at selected atomic positions within the molecular structure so that the mass spectrometer has easy and immediate discrimination by m/z. The beauty of this approach is the option to use these same isotopologues as surrogate analyte, with the innate confidence of equivalent behavior to the unlabeled within a method. In other words, to spike up calibration standards and QC samples with the selected labeled surrogate instead of the authentic unlabeled compound. Interpolation of peak area responses from incurred samples are made through the calibration line constructed with the surrogate. Some experiments are required upfront to prove parallelism of response, however, it's not a dissimilar predicament for the alternate approach and the fantastic aspect is that no alteration of the matrix is required. All calibration standards and QC samples are in the same unadulterated control matrix, same as the incurred samples, a recipe for analytical success, reliability and good sleep at night. Also, no questionable portioning of calibrant or QC levels to be either in genuine matrix or surrogate matrix is required. To me, altering the matrix composition in some key samples is a far more dangerous proposition from the point of view of method reliability than switching the test compound in calibration standards and QCs to something known to be physicochemically equivalent.

Fair enough, it may be hard to find ideally two mass-distinct isotopologues to use as a surrogate analyte and the other for an internal standard, particularly knowing the more expensive 13C and/or 15N labeled analogs are going to give better parallelism than deuteration and the isotopic purity cannot be skimped on. It's a balancing act, as there so often is — reliability of concentration data against the expense involved in getting there. I reckon the method development time and other resource that is saved by avoiding the surrogate matrix approach can most often amount to a much more severe financial figure than what it takes to bring in the right isotopologues.

I would like to conclude with this thought, another way of viewing what this boils down to. Playing with the relatively unknown and unpredictable — the matrix — against using good chemistry with justifiable confidence — the labeled analog.

There are, in essence, two schools of thought that I can perceive when discussions occur on the topic of appropriate purity of the laboratory water and various solvents, additives and reagents for any given stage of a quantitative bioanalytical procedure. I can understand the perspectives of both, but I am cemented with one in terms of my angle and agreement.

It is actually something that can spark surprisingly heated argument. I think it is down to this being, on the surface, quite a simple subject that shouldn't require much thought to generate a suitable outcome. Then there is a natural resistance to change as regards what one expects to use from experience. Around ten years ago, I discovered LC–MS grade solvents commercially available. When I was recommending the switchover to these from the historic HPLC grade for bioanalytical LC–MS applications, I was astounded to find resistance, even though for me it seemed like the proverbial no-brainer. It took weeks of persuasion for all my colleagues to be content; fast-forward to nowadays and MS-appropriate grades are engrained in all protocols.

The two schools of thought are as follows. One is about using what seems to work and aiming for cost-efficiency in the context of using the most readily available, or lowest grade, so to speak, that results in a functional assay. Perhaps only at the analytical endpoint, this may involve using the most scientifically appropriate grade, the reconstitution and the mobile phase in the LC–MS analysis, as opposed to these and all prior steps in a bioanalytical procedure.

The opposing angle is more old-school, I suppose, where risk of signal loss and associated non-reproducibility is minimized in the interests of method performance and reliability, and where minimizing solvent costs is not so much of a priority. The most scientifically appropriate grade is used in all steps of a procedure, from the preparation of primary solutions to the final analysis, considering primarily the nature of the LC–MS analytical endpoint.

Now, for bioanalytical LC–MS, within the possible options, the most scientifically appropriate grade of solvent is LC–MS grade. What makes this a better choice than the clear and historic competitor of HPLC grade? The most significant part of the answer for LC–MS is that it is deionized, and that is not the case for HPLC and other non-MS grades. Whilst knowing the risks they bring, we are not overly concerned with trace organic interferences, which are controlled anyway in HPLC grade solvents, in our wonderfully selective LC–SRM methods. However, we require that ionic — especially hard ionic with concentrated charge — contaminants are removed to trace and controlled levels. Whilst minimizing these contaminants dramatically reduces adverse phenomena like stable adduct formation and displacement, directly affecting sensitivity, there is probably more importance in the control, since without precision and reproducibility there cannot be a useful method. HPLC-grade solvents are not associated with any concern about levels of anionic or cationic impurities.

This is particularly important with water, where ionized and ionizable contaminants are most solubilized. LC–MS water must be Type 1 and used fresh to be truly reliable, by the by, and that is with regard to interferences that are ionic, organic, or leachables from storage containers and the possibility of bacterial growth. Anyway, it is very important to understand that the lack of control, as these key ionic contaminants aren't even measured, emphatically does not fit with a conscientious analytical mindset. Why take the risk?

It is fairly intuitive to consider that a biological matrix, particularly something like plasma, will contain large abundances of the very interferences mentioned above, and a host of others. This is the basis of the mentality lending itself to using the right grade only post-extraction. However, consider one of the key adverse phenomena, the adduction of hard cations, such as the formation of a coordinate covalent bond between a sodium ion and a lone pair of electrons on an amine moiety on the analyte. This is the kind of complex that is unfavorable if the protonated form is being monitored for quantification, as is typical in quantitative applications. I think it is very important to bear in mind that in the same given matrix there will also be a great abundance of many different endogenous small and large molecules that these same ions can and will adduct to, thus taking away significantly from the available yield that would otherwise adduct to our test compounds. Indeed, a solution hypothetically containing similar levels of these contaminants is a far more dangerous scenario than in matrix, since there is no other material present to soak them up, as it were. Therefore it would seem to be the case that the use of solvent and additives of the right grade prior to work in matrix is actually at least as important as in the latter stages, contrary to the stated mentality.

All in all, out of principle, I would strongly recommend controlling what can be controlled and to strive for the best performance data, not to resign oneself to defeat in a presumption. Yes, there may be a few times more of the likes of sodium or potassium in human plasma than in regular water, but other endogenous substances accept their adduction, thereby working to save the signals from our monitored compounds. In any case, it should not give the green light to lay on the abundance with an inappropriate solvent grade and increase the chances of unacceptable method performance. If, for a given solvent, it is a little more expensive to use LC–MS grade than the likes of reagent grade or HPLC grade, and that won't necessarily be the case, it is well worth it for method performance and reliability.

> *When reading Rob's columns, I thoroughly enjoy his creativity as he entwines everyday metaphors with his deep scientific knowledge. He makes bioanalysis fun and very relatable to those new to the topic! In this column, he explores the simple topic of solvent purity and how it can spark heated argument amongst bioanalysts!*
>
> Naamah Maundrell, Head of Digital Content, Bioanalysis Zone

Why small lipids can seem more daunting than many biologics

6 Mar 2019

It is fair to state that everyone in the bioanalytical arena is aware of the growing presence of biologics as therapeutic candidates, and small molecules having a slowly diminishing presence but nonetheless a presence that, for various reasons, will never disappear. At this stage of my career I can look back on successes, innovative moments and continual learning experiences, but also an assortment of trials and tribulations when it comes to small molecule quantitative bioanalysis. At the same time, there is a perceptible air of wariness in the industry when it comes to the bioanalysis of biologics by LC–MS, without even delving beyond the realm of a few thousand Daltons molecular weight. It therefore strikes me as a little strange that, at least as far as peptides or oligonucleotides are concerned, I have of late seen more success and made more sense of bioanalytical challenges involving these drug candidates than the small molecule portion of the regular workload.

Small molecule drugs may possess the full complement of physicochemical properties, that is to say they can, fundamentally, have practically any degree of lipophilicity and of course may possess any ionizable moiety, with all associated acidic or basic strengths. At least their very definition bestows a size limitation and a limit to the degree of any multiple charging that could occur. In any given case, issues could arise from a multitude of sources. There could be difficulty with solubilization in certain solvent media, and related phenomena like nonspecific adsorption to container surfaces. Ionization may be very difficult too, as may ensuring complete neutralization in the mobile phase if this is required for the most efficient ultimate gas phase ion production in-source. At least in part due to this, adequate sensitivity may be very hard to come by. There may be various manifestations of instability. Additionally, and by no means least, there could very well be matrix effect. To probe a little further into the nature of matrix effect, it is mostly down to endogenous lipids, like phospholipids, in the biological matrices being analyzed. Lipids that are not fully expelled during sample extraction since the vast majority of extraction techniques favor good recovery of hydrophobic compounds. These lipophilic interferences affect the magnitude and precision of analyte peaks when simultaneously present with them in-source, be it as a result of simple coelution or bleed from accumulation on-column after previous injections.

Key to the point I am trying to make here, following the above premise, is that methodologies for the most lipophilic small molecules can sometimes be the toughest of challenges, to the extent that isotopologues as internal standards are the only approach to a guarantee of quantitative data reliability. Besides other factors in the overall challenge, such as inefficient product ion formation for the likes of steroids, the reasons are largely down to the coextracted interferences, themselves lipids, exercising matrix effect. These are inescapably reversed-phase methods, and lipids are synonymous with carry-over, originating both on-column and within autosampler plumbing. Their presence is very difficult to minimize, let alone eliminate. They will be present within the elution windows of any gradient cycle, and without reproducibility. Polar analytes, on the other hand, tend to present a different scenario.

They are much easier to eliminate in the carryover context, and optimized methods for polar compounds tend to give selectivity for interferent lipids in an innate, almost intuitive fashion, as a direct result of operative chemistry.

It may seem weird on the surface but polar analytes I have grown to see as friends, and this includes peptides and oligonucleotides. For this, my group has spent many years embracing hydrophilic-interaction liquid chromatography (HILIC), even in the context of SPE. The anticipation of less sensitivity compared to more hydrophobic compounds is a reality because of the way in which electrospray-based gas phase ion release works. However, this can be more than offset with very high-acetonitrile HILIC mobile phases, as opposed to fitting the polar compound into chromatographic acceptability within a reversed-phase format, inevitably at much higher aqueous hence not conducive to best sensitivity. Also, to their analytical credit, if you will, it is also of interest to note that peptidic and oligonucleotide backbones have ordered, somewhat predictable chemistries, whereas small molecules have 'carte blanche' for their structure and functionalities.

As bioanalysts we do love lipophilicity, as we have been conditioned as such, and of course, there is good reason. This property does great things, like propel a solvated ion to a droplet surface as part of its journey towards release into the gas phase, and it allows all the priceless reversed-phase manifestations we work with on a daily basis both in sample preparation and chromatography. We are even forever at the ready to confer hydrophobicity on polar analytes with ion-pairing. Yet, we should not be wary of putting our hands into the polar toolbox. Unadulterated polar analysis can be something to behold in performance and, particularly once any nuances are understood, simplicity. That's why, even though I enjoy quantitative LC–MS of both, I often think I feel better about the prospect of polar analytes than I do about lipophilic analytes.

> *This actually represents a step for me into the vaunted, exciting area of biologic modalities, the prospect of harnessing their physicochemical properties and doing fabulous new things.*
>
> Robert MacNeill

QC samples and internal standard nominal concentration placement

19 June 2019

Many a fiery and many a subdued debate has taken place on every important area and aspect of quantitative bioanalysis. There are some talking points that are undeniably of less importance than others, but that nonetheless deserve more attention than what I perceive is given to them. One of these is where to best place the mid and high QC samples within a calibration range for a methodology, and, largely analogously, there is the challenge of establishing the most suitable nominal level for the internal standard concentration.

Let's begin with the QC sample nominal levels. Everyone agrees at least about the validity of QC placement at the LLOQ and around 3x the LLOQ for the low QC. The industry currently has a fair amount of agreement at establishing the mid QC somewhere around 50% of the calibration range, and the high QC to be situated at least at 75% of the calibration range. There is ongoing discussion surrounding missing parts of ranges not covered by QC samples, which seems to be leading down a path of multiple mid QCs. This is where I would suggest a logarithmic rather than cartesian view of the range may be very beneficial in seeing why going to this added work may not be necessary, but I do not wish to elaborate there within this article. At least no more than proposing that, in line with one popular suggestion mentioned often at industry discussions, one such chosen level approximates to the logarithmically-involved geometric mean of the calibration range, which happens to be what I was raised on, and might currently suggest as a single mid QC nominal level.

Going back to basics, QC samples are confirmatory in nature when considering accuracy; they primarily attest to the validity of the calibration standards. My take is that they should be situated minimally at the most demanding positions in a calibration range, and they should not be at too many different levels. Most demanding means at both the lower limit and low region, and at the upper limit. The reasons for the lower limit are well-understood, as, for the main part, this is where any interferences and carry-over will exact the most detrimental effect, and matrix effect will similarly be at its strongest. The reason for including the upper limit is due to the prospect of non-linearity, specifically applying linear fits to lines that in reality have a slight degree of curvature, a common scenario. Having a QC at the upper limit is not only conducive to encompassing the entire range, which must intuitively be a good thing, but it also covers one critical part of the curve most likely to have unacceptable bias as a result of curvature. On the other hand, having the high QC at 75% of the upper limit actually makes correlation much easier, as it usually corresponds fairly well to the upper intersection of a fitted straight line, with the likes of (1/x2) weighting, on a true curve. Adopting this perspective, having the mid QC at around the geometric mean of the range may be a better option as this is where the curving line usually veers at positive (or, less commonly, negative, depending on the nature of the curvature) bias over the straight line fit, so it is a suitably demanding area. The mid QC at 50% of the range puts it in the vicinity of the intersection as described above, where bias is more likely to be acceptable, perhaps misleadingly.

The question of where to establish the internal standard nominal concentration is quite potent as well. Essentially, it boils down to response as the most important consideration, that is response as the basis for concentration selection. Additionally, I feel it is important to have it low enough to be able to be influenced by the matrix effect to similar extents as its analyte at lower concentrations, as the matrix effect is most prevalent at lower concentrations, whilst at the same time the response is required to be intense enough to be removed from any peak integration difficulties that would lead to imprecision. Then, at the high end of a calibration curve, an internal standard coeluting with the analyte is expected to be suppressed in response due to the analyte intensity but compensating for the analyte's own loss. All this is by no means soup to nuts either, as there may be interferences to deal with by concentration adjustment, most commonly isotopic overlap involving isotopically-labeled internal standards and non-labeled analytes.

It is interesting to suppose that, for ideal mimicry, we would add internal standards in direct proportion, within reasonable constraints, to the true concentration of analyte in a given incurred sample, and to input the nominal IS concentration as appropriate for each sample. However, that is clearly impractical for multiple reasons, plus there is much value in easy production of internal standard response stability plots based on equimolar addition. Realistically, what I think we need is to pull the internal standard out of the lowest concentration region, to a point where any matrix effect will be low but still influential. As an aside, it is the aspiration to have no significant matrix effect anyway. On the other hand, we also don't want the internal standard response to be too high, potentially saturated into a falsely precise domain, suppressing valuable low-concentration analyte signal, and without any realistic hope of response excursion mimicry with the analyte.

In short, I would again propose the geometric mean of the calibration range as a solution. Low, but not too low, and pronouncedly distinguished from both the low and high QC levels. If a sound methodology is developed and used in the regulated lab, variability due to the matrix effect should be something insignificant, more reason why being on the low side should not cause worry. Also, as inferred by the nature of the calculation, the placement is logarithmically sound.

To tie all of this up, my standpoint on these possibly not-so-critical but nonetheless weighty topics, almost a forgivable pun, centers on more involvement of the geometric mean. I do feel it is very appropriate for the way we prepare calibration curves and QC samples, and the realities of bioanalytical LC–MS concentration-based response phenomena. In a nutshell, my view has the mid QC and the internal standard approximating to the geometric mean of the calibration curve, and the high QC situated at the upper limit of quantification.

One of my favorite catchphrases is that the ideal extract should behave as if it were a simple solution. In other words, an ideal extraction selectively and efficiently removes all biological matrix components from a sample whilst effecting 100% recovery of all analytes and internal standards. The removal of the matrix components, many of which constitute interference and a good complement will palpably foul instrumentation, liaises directly into the essence of the commonly used term, 'extract cleanliness'.

Reality has it, though, that no method could ever fully aspire to this ideal, and although this could easily lead to a different story from what I now would like to tell, it is this intrinsic difference, which leads to the situation discussed in this particular editorial. In this context of solution versus extract in bioanalytical LC–MS, we are essentially dealing with two different paradigms and we are required to have a keen awareness of certain methodological aspects in order to be consistently successful in both scenarios. To clarify by example, I am sure that many a bioanalytical scientist has found themselves wondering why, for a given method, extracted analytical batches work wonderfully, yet simple tests in solution can give bizarrely unacceptable data.

There are challenges associated with both, but they are not entirely the same. The dangers typically present in solution tests, where such solutions might be injected into the LC–MS to assess the correlation of two or more primary solutions or to check spiking solutions, backed up by precision measurements in replicate sets, are to observe losses from non-specific binding (NSB), also referred to as non-specific adsorption, and solubilization difficulties similarly causing losses and imprecision. The risk of NSB essentially arises from the fact that there is nothing else in the solution that could mitigate the phenomenon taking place on the surfaces like the residual matrix components do in an extract. Also, interestingly, a solubility issue in a primary solution may not be immediately obvious as dilutions may well be perfectly solubilized and show good precision and the only answer is to take multiple aliquots of the primary solution for analysis if strange data are observed. Additionally, more dilute solutions are more prone to suffering significant losses at the hands of NSB. Then, on the other hand, the main dangers typically present in extracts are presented by the residual matrix components themselves, in terms of signal alteration and associated imprecision and non-reproducibility. This is, of course, something abundantly discussed in the global bioanalytical LC–MS community over the last 2 decades.

Therefore, the presence of matrix components can be viewed as beneficial and it can be viewed as detrimental. The absence of matrix components can be viewed in exactly the same way. For any given method, or analyte, the solution lies in getting the chemistry appropriate and functional in both paradigms, and this intuitively involves the ideal scenario of having extracts as compositionally close to solutions as possible.

In method development, we firstly look, as basic chemistry-based requirements, for complete solubilization and the absence of any risks associated with NSB and instability.

This is naturally performed initially in solution, before progression later in development to candidate extractions. It should be an easy progression and these conditions will translate to extraction-related solution compositions and choices of vessel material composition, i.e. organic and aqueous composition, pH, ionic strength; polypropylene, polar-modified or glass vials, tubes and collection plates. As alluded to already, it is critical to make predictions early, based primarily on the chemistry involved. Anticipating how the compounds are going to behave in terms of charging and solubility, for instance. Then how this ties in with the choice of container in order to negate NSB, including the sample preparative and analytical endpoints. One more specific consideration is to do with solution storage at very low temperatures, where the thermal excursions can wreak havoc with regard to binding and solubility.

A trap that is easily fallen into is to use universal-style defaults, avoiding the perspective of the particular nature and requirements of each given method. Defaults such as, for instance, always using polypropylene tubes for solution preparation and storage, whatever the method. This can result in unexpected delays and frustration until the underlying reason is addressed and the necessary changes are introduced. Many aspects like this may seem innocuous but are actually potent. This underlines the importance of having the method description appropriately detailed.

We hope the various established solution and container choices are rugged enough to never result in any analytical oddities within both solution and extract tests. However, there are plenty of real situations where it happens, but we can rectify this, as illustrated.
In summary, careful consideration of the pertinent chemistry is pivotal in a method development. In the earliest stages, anticipation must be made as to the most appropriate solvent compositions and types of vessel to use and – as with so many aspects of method development – the molecular structure and key functionalities drive this decision-making. The ideal endpoint has an extraction clean enough to ultimately mimic neat solution and conditions are such that free solubility is manifest, liaised with no binding propensity. Only then can we be truly satisfied with the intrinsic ruggedness and reproducibility of the method, a priceless element of daily function in the regulated laboratory.

> *A methodology in solution can be surprisingly different from a method in a biological matrix, even though the goal is partly to make extracts practically as clean as solutions!*
>
> Robert MacNeill

The biomarker bandwagon's journey over curious calibration curves

15 Jan 2020

I don't think I've sensed more of a general buzz about biomarkers than at the moment. They are more pivotally involved in drug development than ever. Like other topics of discussion in the industry, it has seemed to go through ups and downs in terms of discussion intensity over the years, but amidst this and the current state of play, it is certainly safe to say that this field is particularly important and engaging, has its fair share of controversy and is not short of analytical challenges.

My R&D team has been fortunate enough to have had a handful of interesting dalliances with addressing these challenges over the years, at least in the LC–MS domain. Then around a year ago, I had the honor of guest editing a special focus issue of Bioanalysis, concerned with the reliability of methods for the quantification of biomarkers using the surrogate matrix approach. That amounted to a very valuable immersion in the goings-on at the time. One aspect it helped to underline was that this surrogate matrix approach (where the 'matrix' can often be a simple solution) has proven to be generally the most favored, in the face of the alternative surrogate analyte approach which, although highly potent for reliability, comes with important drawbacks. These drawbacks are the time and expense of synthesizing stable-isotopically labeled analogs, of which two are called for, one as the surrogate analyte itself and the other as the internal standard.

The shared objective in all approaches is proving parallelism, the rugged road to reliability, upon which the 'Biomarker Bandwagon' wants to be rolling. For the surrogate matrix/solution approach, demonstrating parallelism can be very challenging. This is where the output, the LC–MS peak area-derived response, translating into the slope of a calibration line which is the defining essence of sensitivity, matches between the surrogate element and the real element within the complete methodology. Such fertile ground for the 'Biomarker Bandwagon' to navigate with a view to innovation. Especially for those who enjoy the music being played, a lot of which emanates from the more in-depth utilization and assessment of calibration graphs.

The obvious abnormality in a regularly prepared calibration curve, for those used to working with xenobiotics, is the distinct and statistically clear intercept in the response axis where the nominal concentration is zero. It's a direct indication that there is a native level present. As a consequence, how do we make that measurement? Then, to touch on one smidgeon of controversy, what degree of precision do we associate with the measurement? The answer to the former question is that there are a few options, with perhaps even one or two avenues unexplored as yet, and the latter question cannot really be answered succinctly.

Here is one option. At hand in this world is the wonderful tool of standard addition, which we can readily use to calculate the endogenous concentration of an analyte in a given matrix, usually in support of results obtained from separate means. Standard addition can be used provided we have enough sample and there is no need for any isotopologue to be involved.

It involves the spiking, using entirely unmodified analyte reference material, of several aliquots of entirely unmodified matrix at a selection of distinct nominal concentrations, as would be done in any calibration curve preparation. The subsequent analysis gives the scenario described in the paragraph prior, typically a line with a characteristic slope representing the sensitivity, and a positive intercept in the response axis and a negative intercept in the concentration axis. The negative of the intercept in the concentration axis is the calculated endogenous concentration and indeed the result is reliant on a bit of extrapolation which can make some 'Bandwagon' passengers slightly uncomfortable. With well-designed experiments and methods, it has proven to be generally very steadfast. The confidence of the result is reinforced by larger numbers of replicates at each nominal level and by the use of an internal standard that performs adequately within the method.

There is another profoundly important drive of the standard addition experiment and much the same torque is driving our 'Bandwagon.' As there is absolutely no question about the integrity of the slope of the calculated calibration line, because there are no surrogates of any type involved, it has been popularly proposed as a complementary means for establishing parallelism where any surrogate-based calibration curves are used. It's a classic pair-up of extrapolative and interpolative techniques to achieve a valuable objective, to really allow the 'Bandwagon' to go places.

I hope this brief outline of what I believe is an invaluable technique gives a taste, at least to the newer generations of analytical scientists, of the sometimes-veiled usefulness within calibration curves. Once the purpose of confirming parallelism is done, the other parameters in the equation describing the standard addition line can be used to glean insight and maybe not only in calculating the endogenous level in the matrix sample used for the standard addition construction, depending on the experimental setup and purpose. Then, in turn, how this awareness of calibration curve meanings and implications, alongside the ever-important method ruggedness foundations, can possibly fuel innovation in fields like biomarker quantification, not to mention fuel the figurative 'Bandwagons' merrily traversing these fields.

> *I have cheery memories of writing this one. I wanted to give a taste to the readers of everything that could be gleaned from a calibration curve in the biomarker (endogenous) context. It would lead ultimately to the unveiling of the Origin-Adjusted Approach!*
>
> Robert MacNeill

We are all acutely aware, especially as patrons of the life sciences, of how everyday existence has recently changed so much. Cue the opportunity to make pitifully amusing remarks about toilet paper battling and the isolation of celery as the only vegetable remaining on supermarket shelves.

For myself, I am fortunate enough to take great pleasure in what I do for a living. The chance to mentally dissociate somewhat from the immediate stresses of coronavirus-related issues is presented to me by the likes of, well, chromatography. It so happens that at the moment there is a plethora of bioanalytical activity at my site and within this, I have seen a couple of LC methods that curiously juxtapose in terms of anticipations and outcome. More than enough to capture a lot of interest, I would think. At the forefront is a moment of serendipity experienced when we tentatively attempted a certain chromatographic mode for one of our lipid applications and it really came up trumps!

Looking back at the content of this column over the years, it will be of little surprise that I am a big advocate of hydrophilic-interaction liquid chromatography (HILIC). Innately, this is a technique used for the analysis of compounds that are more polar than non-polar, broadly speaking. Thankfully, as with so many things, it is not quite as simple as that. To gain a solid foothold on a key chemical characteristic and use this as a basis to climb the rest of the mountain, can be priceless in the grand scheme of things. This is exactly what happened in this situation. An analyte molecular entity that, from the structure, was strongly indicative of an underlying lipophilic nature and sure enough showed a clear proclivity to dissolve readily in organic rather than aqueous. However, amidst the bulging hydrophobic parts, there is a secondary amine group, outside the reach of conjugation, that makes it a pretty strong base. It is this that makes for the HILIC possibility. It is a welcome option too, since the initial reversed-phase tests indicated difficulties synonymous with chromatographing lipids. Over-excessive retention and concomitant broadening, a high risk of matrix effect arising from the profusion of endogenous lipids affecting the signal integrity and carry-over. The HILIC avenue addresses all of these and this is the avenue we have taken for this method. A hint of buffer salts, a favorite amide chemistry in the stationary phase, an endearing 3-minute gradient optimized, oodles of sensitivity and away we go. It probably makes a clear statement about the involvement of electrostatic interactions as part of the complexity of HILIC, especially where the silica base is involved and that is of course greatly abundant. To round this off, not to assume that a great many lipid methods over years gone by could have been translated from reversed-phase even with a readily-charged moiety, but to just think of all the applications that could have benefitted in this way and the likes of HILIC was not given a proper chance, it's almost astounding.

I may dwell on HILIC a lot in my writing, but it doesn't mean I have severely polarized vision; rather, it's a lot to do with just trying to widen the exposure. There is certainly no aversion to reversed-phase around here.

For instance, at the same time and a few instruments over, and by contrast to the above, a separate application involved a very hydrophilic small molecule, quite unique in its structure too. Suffice to say it was a contender for HILIC as a first port of call for the chromatography. However, lo and behold, screening a few columns, mobile phase pH, etc. brought nothing but very broadened peaks, not feasible to be used with a practical run time and sufficiently high-sensitivity quantification as an analytical endpoint. Furthermore, a pair of isomers were not fully resolved. Then, setting up a reversed-phase gradient, beginning at very high aqueous and a mild excursion to 20% less aqueous over a couple of minutes and using a popular contemporary C18 column packed with superficially porous particles, we were able to sit back in relief. Sufficient retention, sharp, symmetrical peaks, precision of response and emphatic isomeric resolution. Rescued by reversed-phase.

I suppose this article provides a little diversion from the main subject of current day-to-day news and discussion, but in its own way remains informative, perhaps even spurring to those in similar chromatographic predicaments. If the coronavirus proves to be, with a nod to the Latin, the 'crowning' theme of 2020, I will not be at all surprised. Bioanalytical scientists have a weighty part to play in overcoming it, of course, and I just hope the enjoyment of the nuts, bolts and nuances of our particular specialties remains in full force or something similar during this tough time period. For the time being, in my domain, here's a big thank you to analytical flexibility and fortune, but especially to the growing usefulness and visibility of polar-based chromatographic options!

> *This was written just at the onset of the COVID-19 pandemic, when the world felt like a different place all of a sudden. One amusing oddity was the general race to secure enough bathroom tissue for your household's immediate future, as mentioned in this column. HILIC was something that soothed my nerves at this time, in a nerdy chromatographic-type way!*
>
> Robert MacNeill

During a time in which we're all discussing how best to 'flatten the curve', in this article I look at the same kind of push but within a quite distinct context. The fascinating manifestations of the phenomenon that we see in bioanalytical LC–MS calibration curves, naturally.

In regulated bioanalysis, we love straight lines. There's no getting away from it. Then simplest and most reliable concentration-response model. A given method constitutes a multi-parametric operation and prior to validation we will adjust a curvilinear-producing method as appropriate in order to produce a straight line. On the very odd occasion, we may be able to justify using a curved fit as a feature of an established method, but the resultant quadratic regression parameters would need to be demonstrably reproducible. This is a very infrequent situation, and to all intents and purposes we only ever use this in non-GLP bioanalysis, but even then we tend to be uncomfortable. Curves often have a horrid tendency to change characteristics, even become linear, when no deliberate change to the system has been input. Also, it is desirable to never be approaching a plateau where a given response does not correspond to one singular concentration but rather an asymptotic range, or similarly where a given concentration does not correspond to one singular response, again the asymptote being visualized. Moreover, we know we can use our knowledge and chemistry toolbox to make adjustments in order to attain a straight line.

In short, a non-linear response may be seen as an indication that we can take measures to bolster the reliability of the method.

The seasoned bioanalyst will be well-used to the generation of non-linear calibration curves, and by far the most common is what is typically referred to as a 'saturation-type' curve. This is where the slope begins to decrease with increasing concentration and most often it represents the onset of detector saturation, hence where the term originates. At any given point in a chromatogram, the intensity in an ion channel cannot be more than a certain threshold and signal saturation occurs when pushed towards this threshold. Solutions lie in reducing injection volumes, increasing extract dilutions, or even broadening chromatographic peaks if it doesn't compromise resolution or performance. Reducing the linear dynamic range of the assay is also an alternative, although clearly less desirable as we don't relish the prospect of increasing our chances of having to perform repeat analyses with dilution. Moreover, I would confidently say that this kind of curvature manifests more often down to simple chemical reasons. Dimerization of analytes is known to occur in the heat of the source during the ion evaporation process, leading to signal loss at higher concentrations. The aforementioned adjustments can be made to good effect, but a frequent winner here is adjusting the mobile phase pH incrementally closer to neutrality, or indeed sliding to the other side of neutrality. This may necessitate chromatographic adjustment, and may make a decent, selective sample extraction even more important, if any interferences end up hard to chromatographically resolve.

Then there is the possibility of moving to the often-overlooked APCI gas phase ion production conditions, great for sensitivity and straight lines with hydrophobic small molecules and often deals soundly with matrix effects that are encountered with electrospray-based conditions. Last but not least, another important parameter that could be altered in order to rectify this curvature is the declustering potential, the voltage applied to the orifice leading to the vacuum interface at the head of the mass filter rail and which governs how forcefully ions are drawn in, as is necessary to remove weakly-held adducts and solvent ion clusters from the analyte. That brings us quite nicely to the opposite type of curvature as well.

As you may imagine, this other notable kind of curvature, 'upwards', in which the slope increases with increasing analyte concentration, is much less frequently encountered and quite enigmatic as a result. This can sometimes be attributed to simple instability, where a compound can stabilize itself with its own increasing concentration. Here's an interesting situation though. I recall an instance fairly recently observed in this laboratory, where a simple tweak of that one parameter, the declustering potential, was all that was required to solve an upward curvature issue. To venture into a proposed explanation, this kind of curvature has to be the result of an analyte promoting its own sensitivity with its own increasing abundance. In this case, we speculate that a certain abundance of the dimer form of the analyte is produced in the thermal conditions of the ion source as the ion evaporation process gets underway, an abundance of dimer that increases with nominal concentration. Critically, the dimer has an easier time migrating to a droplet surface than the monomer, so fundamentally it's more sensitive. Then, too high a declustering potential triggers the production of an excess of the monitored monomer by cleavage of the weakly-bound complex and non-linearity with increasing slope is the result. That's an excess in relation to the lower concentrations where the dimer does not have the means to form, that means being the very concentration. The curvature issue was seen when using a relatively high declustering potential and, lo and behold, the curvature disappeared upon lowering the value of this parameter. By lowering this key parameter, the effect is alleviated since at the higher concentrations the disintegration of the dimer to the monomer is no longer as favored, countering the higher sensitivity of the dimer as a result of easier ion evaporation, whilst at lower concentrations the monomer is largely produced irrespective of declustering potential since dimerization is much less involved. So this is the thought process, but what we do know is that decreasing the declustering potential solved the problem. This is the same offset that may be used, at high enough settings, for collision-induced dissociation, otherwise known as cone voltage fragmentation.

Solubility as a fundamental issue is sometimes pondered with any kind of curvature, but it's important to note that when precision at each level is appropriately impressive, it does not tie in with insolubility-related expectations. A lack of solubility would inevitably be accompanied by poor precision and that is something key to address before curvature assessment.

Once precision is attained, we can really characterize the curvature and be confident that a good linear method is probably only a tweak or two away.

Somewhat similarly, matrix effect is sometimes a suspect as a root cause of curvature. From uniform-concentration suppressive interferences, I believe imprecision especially at lower analyte concentrations, along with the uniform signal loss at each level, are the biggest threats to assay performance from the matrix effect angle. There may appear to be curvature as an artifact of insufficient replicate data points. The signal loss is most notable as a percentage at lower levels, naturally, along with the imprecision. That imprecision would typically make it difficult to establish any non-linearity at work. The same low-end imprecision would befuddle non-specific adsorption in much the same way, when considered in these terms.

Suffice to say, then, that the appearance of curvature in method development should not cause panic, whatever the circumstances and the nature of the curvature. As bioanalytical scientists, we have established means at our disposal to deal with it. Outside the realm of simple detector saturation, there are at least the tried and tested most fruitful tools of mobile phase pH adjustment and key source and vacuum interface transmission parameters.

> *Curvature in calibration lines is such a ubiquitous phenomenon, multi-faceted and certainly not restricted to only a couple of possible root causes. Indeed, there is so much to be said that 'saturation' could so easily be achieved!*
>
> Robert MacNeill

A peek closer, with piqued curiosity, at what our peaks are pointing out to us

21 Sept 2020

It is the giddy realm of chromatographic bioanalysis. We are used to observing the manifestation of our chromatograms, often actively watching the traces from our test solutions and extract injections etch out in wonderful symmetry, sharpness and resolution, or otherwise.

There are fairly well-known aspects of chromatography that are quite easy to check. Basic requirements, if you will. For instance, to work out the void time using the column dimensions and flow rate, including an approximation of extra-column volume, then from this to establish the minimum retention time as three times the void time, gradient or isocratic, is more readily done than writing this sentence. To work out the approximate plate count in an isocratic method is similarly non-demanding, again once we have knowledge of the rudimentary formulae involved.

How about, then, the perhaps less well-known phenomena that can affect band migration through a column? Let us first consider common perceptions about peak tailing and fronting. For clear reasons, we need symmetrical peaks in our chromatography. The notion that tailing, for instance, is often caused by secondary silanol interactions is true, as long as the column is silica-based. Fronting, on the other hand, is often caused by overloading the column in the context of each injection and that's no less valid a statement either.

Is that all there is to it? If only bioanalytical life was that simple. In fact, as pertains to bioanalytical chromatography as a whole, we are inundated with influential aspects to be wary of, a wonderful complexity but, as alluded to, when time is pressing for project completion it's a situation that calls for nailing all key parameters and promptly churning out the fully functional method. In the specific context of peak symmetry, I would like to dwell in this article on what are known as sorption isotherms and how they relate to symmetry, tailing and fronting.

Sorption isotherms are well-deserving of mention, not only since I may not believe they are generally mentioned enough, but because they can be very influential in chromatographic problem diagnosis. We need to understand them, how they manifest, and it is a good place to begin with what the indications are. Both peak tailing and peak fronting could both very easily be largely down to non-linear isotherms, the isotherms being simply the plots of analyte concentration in the mobile phase against analyte concentration in the stationary phase. To get to the nitty-gritty, it's all to do with solubility. It's a nuance of partitioning, if you will. Within the migrating band, we have the central and most concentrated part, and we have the front and the tail sections, at lesser concentrations than at the center. This scenario is critical to grasp in order to understand how these phenomena work. Equally importantly, the front and rear sections of the band can move at different velocities from the center, and this is a result of the very essence of how solubility works, with a most dynamic backdrop.

It's also fair to say that this could be a reflection of how we often push solubility to the limits, where perhaps we don't need to, with better chemistry-based decisions. Anyway, as just alluded to, these effects make themselves known when the concentration in the center of the band approaches a point where it has a problem fully solubilizing in either the stationary phase or the mobile phase. Meanwhile, the outer edges are fine because they have a lesser concentration, not approaching any limit. Now, if the problem is with solubilization in the stationary phase, the center of the band will partition into the mobile phase more than it would otherwise, and the result is that this center section will move faster than the outer sections of the peak and tailing will occur. This can be seen in Figure 1, where we have a chromatographic peak suffering from tailing and adjacent is the corresponding curved sorption isotherm showing the approach to maximum concentration in the stationary phase. A symmetrical peak would, of course, have a linear sorption isotherm.

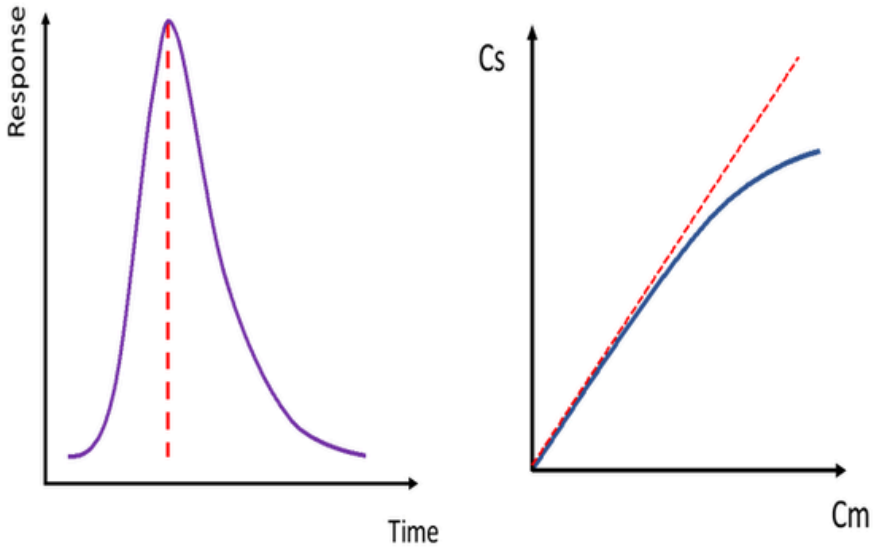


Figure 1. Tailing chromatographic peak and the sorption isotherm associated with peak tailing. Cs denotes analyte concentration in the stationary phase; Cm denotes analyte concentration in the mobile phase.

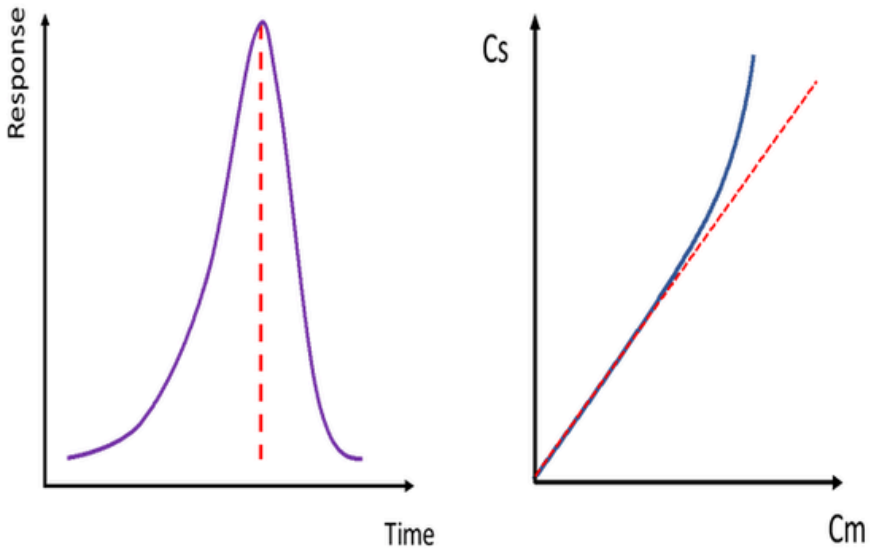


Figure 2. Fronting chromatographic peak and the sorption isotherm associated with peak fronting. Cs denotes analyte concentration in the stationary phase; Cm denotes analyte concentration in the mobile phase.

So, can we avoid these effects with better choices of stationary phase/mobile phase flavors, particularly with our inherently more diverse column selection? Sure — we can. Maybe easier said than done in practice, though the basic concept of 'like dissolves like' feeds into so many key decisions relating to outstanding method performance. Temperature is a tool as well, of course, but underlying chemistry is the potent game-changer. Anyway, when you have peak asymmetry, it might just pay off to think about your possible isotherms!

Thanks to Lin Wang for the graphics production.

> *A fascinating dive into what our chromatographic peaks reveal about the science beneath. It is always rewarding to contribute to uncovering the hidden details behind the data. Every peak has a story, and this piece beautifully brings those stories to light.*
>
> Lin Wang, Scientist, Regeneron (NY, USA) and Zone Leader, Bioanalysis Zone

The riddle of recovery

16 Dec 2020

Throughout history, we have endless fascinating mysteries and enigmas to ponder. The seven ancient wonders of the world. Evidence of extraterrestrial life. Why traditional Scottish haggis cannot be made in America. Then, to become even more serious, we have our quantitative bioanalytical world, within which we are not exactly starved of conundrum either. One of these puzzling satellites revolves around the calculation of analyte and internal standard recoveries from preparation and extraction procedures, prior to an analytical endpoint.

Although some dismiss the importance of recovery calculations altogether, focusing solely on how a method performs in terms of accuracy, precision and other well-known performance measures, and typically the most pivotal being whether or not it meets sensitivity requirements, I prefer to see recovery as a valuable trait of a method's function. For instance, if it is unambiguously low then it is a danger sign, with the risk of the method becoming unreliable or even falling apart under conditions that stress the method's constitution, pertaining to the method robustness rather than anything political. On the other hand, if the recovery is clearly high, then we can be confident that it will aid in the method's inherent reliability, being better able to absorb unusual or unexpected stressors and showing minimal deterioration in performance.

Most of the time, therefore, recovery is a key methodology parameter, which we take pleasure in calculating. It is pretty easy to do so as well. Spike up blank matrix extract to produce something equimolar to the extract from a theoretically fully recovered pre-extraction matrix spike. Then measure up the peak area-based results from replicate sets against the controls to obtain a percentage.

The riddle of recovery lies in the reasoning why we should or should not apply it to protein precipitation procedures. This means of sample preparation prior to LC–MS is very popular and invaluable, for very good reasons. However, it is not exactly the most selective option. In fact, the only class, as it were, of biological sample component eliminated is larger proteins. Now, critically for the underlined message being conveyed, there is a physical change, including a change in overall volume, that occurs at the point of protein precipitation. A certain amount of solid precipitate is produced, leaving the liquid sample occupying less volume. Therefore, anything remaining in the liquid sample is more concentrated. Then, after the necessary vortex-mixing and centrifugation, the supernatant is removed. That supernatant volume — for the purposes of a recovery determination — is a set fraction of the total input volume, a total which is now over-estimated since the assumption has to be that all molecular components have remained in the liquid phase. This is not reflective of what has really happened though, and the analyte has been effectively concentrated, raising some eyebrows when final calculations are viewed.

So there lies the crux of the matter. Recovery for these procedures is predisposed to give measurements in excess of 100%. In reality, the numbers are typically well beyond this mark, in the ballpark of 120%. In essence then, the calculations are not very useful, especially when it is considered that it is really quite difficult to lose recovery in protein precipitation procedures, as a result of the inherent lack of selectivity as alluded to earlier. That is unless the test article is itself a large protein and this application hasn't been thought through in the slightest, or there is a simple solubility issue that may otherwise induce precipitation of said test article, with a clear concomitant loss in recovery. The thing is, of course, if there is an analyte solubility issue associated with the composition upon which protein precipitation occurs, then you have a big problem pursuing this avenue in the first place. Solubility danger zones are definitely to be avoided if at all possible.

On the whole, my opinion is that calculating recovery for simple protein precipitation methodologies is tantamount to a bit of a waste of time and resource. Please don't misunderstand me, there's really not much question that with other formats of sample preparation and extraction the determination of recovery is absolutely key and wonderful information to support the development of a method. That's especially true with SPE, so often a central component in the most reliable bioanalytical LC–MS methods for peptides and oligonucleotides. For instance, with a certain few biologics on my plate this year it has been recovery measurements that have shown the path to some degree of success, the path to madness at other times, or maybe just a little more greying of the hair. Recovery is there to be investigated in every step from the load and beyond. Absolutely potent as a marker in SPE method development, even helping to guide the manipulation of often-overlooked variables along with prompting the realization of the important nuances for many new entities. So as the saying goes, it's all worth it!

I'm surprised the title of this article isn't more of a cliché among chromatographers, but here I shall use it unapologetically, even compound it in a questionable manner with some figurative chromatography language. With that, we can migrate into the heart of the article and disperse, and let's certainly maintain the flow away from that little void of an introduction. Within our usual setting of a quantitative bioanalytical method development with an LC–MS endpoint, and project timelines not-with-standing, let us ponder how ensconced and frustrated we should become over the chromatographic separation that we can almost obtain but remains elusive. It will depend on the challenge at hand, of course, but here I would like to encourage the flexibility to jump to a different avenue of selectivity in cases where a separation is necessary and tweaking the conditions of the existing setup is proving fruitless.

Selectivity, the key parameter leading to robust methodologies, arises from the power to separate. Discriminating power, if you will. The kind of mass spectrometric detectors at our fingertips these days are wonderful things and, with the considerable selectivity that they confer, do so often serve to obviate specific chromatographic separation needs. Nonetheless, we are frequently faced with challenges that require this very nature of separation. Chiral methodologies are perhaps the most obvious example. We also have isobaric analytes, which are almost always isomeric in nature, also metabolites that can pop up in our ion channels in various ways and are of fantastic importance, not to mention areas of response suppression or enhancement that we need to steer clear of. With all of that being said, it's pretty safe to say that most occurrences of being in such a chromatographic pickle, needing to just pry two peaks apart to then have the entire method be sound, relate to the separation of isomers. Cue thoughts of delicate differences in shape, functional group exposure and effect on chemistries, possibly switching up wholesale in terms of the physicochemical environment to bring an impactful result, a segue to the intended message of this article.

In my experience, to improve the separation using the same underlying chromatographic selectivity is often not a great solution. Usually, we are left uncomfortable contemplating an extended run time and broader peaks, with reduced signal-to-noise implications, or we have precariously high back-pressure, despite having made the key separation a little more convincing. This may have been achieved possibly due to a little tweaking of organic/aqueous levels at key points, or often having selected a longer column, maybe also adjusting the column temperature a little, reducing the particle size or moving to superficially porous particle technology or taken the latter further, to a more potent reduced shell depth where the pore length is similarly reduced.

To progress from this realm, we may do more compelling things like switch up the organic modifier for an alternative of different chemistry, like protic to aprotic, or move to a possibly more potent aqueous modifier pH and perhaps also adjust the ionic strength.

These two options, especially the pH tool, have more potential for making the selectivity switch favorably, handing resolution swiftly to the gleeful chromatographer. Sometimes, it must be said, even temperature alone can bring a pronounced change. Also, we can change the stationary phase to a similar but distinct chemistry, often considering phase density and pore diameter too and be met with sufficiently positive results. With these options, we can begin to really be able to go where we need to go and we are making more fundamental selectivity adjustments in order to attain this. I know, it may not seem attractive at first, having worked hard to establish the chromatography and there is only one apparently minor tweak to apply, but in the long run it will likely be fruitful.

Then, to move up a level, there is nearly always the possibility of making sweeping selectivity changes, even more potent and exciting. By this, I am referring to changing the fundamental mode of chromatography, when it can feasibly be done. Such as reversed-phase to HILIC or aqueous normal phase in some manifestation, or of course the other way around. It will take a moment of careful thought to initially gauge the feasibility of making the switch, but as I have dwelt on in previous writing, it's a lot about anticipating solubility behavior and use of charge or otherwise. Thinking hydrophobicity and polarity, carbon skeleton or biological sequence characteristics, non-ionized but polar functional groups, and then the important electrostatic aspects, so looking at ionic moieties that can be switched on or off to best suit our chromatographic needs.

In any case, the best answer to the so-close but elusive peak separation situation may well be a marked selectivity change instead of spending a long time tweaking a given setup and then accepting a less-than-ideal long-term outcome. The concept of choosing selectivity as a variable, rather than adjusting the likes of column length or particle size, can indeed be underlined using that all-important resolution equation involving theoretical plate count, retention factor and selectivity, in terms of the impact on resolution of altering each of the three parameters, although there is no room to delve in there within this piece. It would be an article in itself. Suffice to state that selectivity is a profoundly important tool at our fingertips, and let's not be afraid to take leaps when appropriate and when the confidence in our chemistry is there.

WAX on, WAX off

30 Apr 2021

Most who are familiar with my favorite bioanalytical techniques will know that I have a huge interest in SPE. Thank goodness for SPE too. It's so often a critical component of quantitative bioanalytical LC–MS methods, especially for biologics like peptides and oligonucleotides. It has been known to effectively deliver a stunning 'crane kick', winning the selectivity battle on many a high-stake occasion.

Whereas protein precipitation only shows 'no mercy' to the larger albumin-type proteins, eliminating only these from a plasma sample, and liquid-liquid extraction can 'give a body bag' to a broader range of interferences, SPE has the flexibility to knock down a number of prominent bad-guy interferences in just two or three steps. Mixed-mode chemistry brings it to a pinnacle of discriminating power. At least two underlying retention mechanisms form the 'strong roots' of the technique, allowing potent washing as long as one retention mode remains active. One of these modes is inescapably electrostatic, allowing ion exchange, and this is where the use of rhetoric like 'switch on' and 'switch off' is found, as it pertains to the moiety's capability to hold a charge or be neutral, depending mainly on the pH of its environment.

I recall when weak anion exchangers (WAX) and weak cation exchangers (WCX) first made an appearance in the early 2000s, against a historical backdrop of strong (always fully ionized) for both polarities having seemingly always been available. The WAX and WCX were revelatory – by switching off their charge via simple well-defined pH excursion, we could release our compounds of interest, after washing to our heart's content, in very predictable and reproducible workflows. The use of strong exchangers would not be so uniform since it would have to be the charge on the analytes that is neutralized, and that of course is compound-dependent.

When I first encountered the challenge of reliably quantifying oligonucleotide therapeutic candidates a few years ago, I knew that using WAX in the SPE had to be a prominent option. This is because of the phosphate backbone of these entities, possessing a huge proclivity for adopting multiple negative charges. If we could use the 'on' and 'off' to effectively bind and later release the oligonucleotides, and in theory it would work, it would be a great 'tweak on the nose' to that part of the challenge. From the beginning to the end it did work, but that was one aspect among a few very important others in the SPE that had to be looked at closely. Where there was a 'WAX on, WAX off', there was always at least a 'paint the fence' and 'sand the floor' to practice and perfect as well!

One very important point appears to be the loading conditions, including total packed sorbent weight, pH and flow rate, also how much the sample is diluted. All this is reckoned to be directly linked to the utter lack of hydrophobic retention that these most vigorously polar of entities can engage in, whilst not in the presence of ion-pairing reagents.

This means that there is a certain critical reliance on the electrostatic interactions forming effectively at the load stage in order to avoid significant breakthrough, whilst pH can simultaneously be adjusted to give a potentially pivotal sliver of hydrophobic retention. As such, we need to take measures to maximize the opportunity for the ionic interactions to set in. Being ionic, they are high-energy, far more energetic than the accordingly quick-forming hydrophobic interactions that most other classes of compounds enjoy in at least some abundance and which are typically first to manifest and front-line in breakthrough prevention. High-energy interactions take time to manifest properly. For instance, the flow rate needs to be very slow and steady, the sample diluted adequately. Then, when it comes to the final stage of elution, the same principle comes into play. A step designed to effectively and reproducibly disrupt the ionic interactions, using analogous principles as just described. So 'WAX on, WAX off' is at the heart of the workflow, but it does have a few nuances.

Then, of course, how will chromatography and MS face up to the extracts? This is another story for another time. Fortunately, it is helped by the innate cleanliness of well-optimized SPE, coupled with the ability to reconstitute in any given composition that does not compromise solubility. There won't be any distasteful 'sweep the leg' going on here. Evaporation and reconstitution does not seem to be an issue with the oligonucleotides we have come across, as it is for many peptides.

In any case, once this is achieved for a given oligonucleotide application, the feeling is good enough to bring you to that All-Valley tournament and make an impact!

> *I loved writing this one. My work with oligos had led my brain to the WAX-on, WAX-off 'inspiration,' together with my liking for the Karate Kid movies and TV spin-offs. Also, I was enthusiastic to try a popular theme basis like this, as was picked up again a few times in later columns!*
>
> Robert MacNeill

Seeing red

5 Aug 2021

Don't worry, I am not someone who has the slightest proclivity towards angry outbursts. The title of this piece is meant quite literally. It's about blood sample hemolysis and the processes of addressing the phenomenon analytically, particularly for the processed plasma/serum. It is not uncommon for real-world incurred study samples to be at least somewhat hemolyzed, therefore often noticeably reddened, and making sure to preserve the reliability of their analysis is a big deal.

Sometimes complete time courses of samples arrive hemolyzed. Then, in red-faced irony, they must be analyzed via interpolation through a regular plasma calibration line. Our experience tells us that hemolyzed matrix, when unquestionably reddened, is tantamount to a different matrix altogether.

What happens in hemolysis? It can happen for a few reasons but, most pertinently to bioanalysis, during a sample blood draw. The red blood cells rupture and release their contents into the plasma. These contents are diverse and plentiful. Extra potential interferences, more matter to occupy sorbent capacity and lead to displacement effects, and agents ready to cause instability. Furthermore, hemolysis is not absolute. The varying degrees of hemolysis cannot really be quantized. Especially not as simply categorized as 'hemolyzed' or 'not hemolyzed' although in reality we have to regard things as such. It may be viewed as always there to some extent, on a continuous scale. All we can do is ensure our methods give the same unassailable analytical performance output between minimal and a reasonable worst-case scenario, the industry standard for this being 2% hemolyzed.

In our environment where we prepare methods for full validation, we go straight to hemolyzed matrix to compare right from the offset with regular control matrix. That way, blood-curdling surprises are avoided later in development when a hemolytic test happens to be run and the data suddenly do not look rosy. It's arguably the biggest hurdle for a method to prove itself. Not only do we need to consider the extra components present but we need to be wary of all possible manifestations of differences, which includes concentration–response characterization. We have seen curvilinear fits of hemolytic test calibrations where regular matrix extracts showed linearity for the same range, for instance.

So, without beating around the cell membrane any longer, what best approaches do we have for hemolytic issues? As you might imagine, there are many 'ifs and buts' and a lot of material that could be and has been written. It is perhaps most appropriate if I draw on our own recent experiences within my lab, and we do have some very pertinent recent experience.

To begin with biologic-type analytes – it appears to be the case that hemolytic effect is rare. With this, SPE is effectively a mainstay, and it is probably no coincidence that, in the grand scheme of things, this most selective of extractions leads to a hemolytic-friendly zone. That said, the manner in which we chromatograph these generally very polar analytes is bound to factor as well.

The only occasion in which we have seen a definitive hemolytic effect for a biologic was with an oligonucleotide. The extraction was indeed SPE and it was found that, where regular samples worked great, the extra matter present in hemolyzed samples caused a strong element of competition for initial capture upon sample load. This is an effect that had different severity between analyte and IS, leading to bias slightly outside acceptance criteria. In oligo SPE this step is crucial, where much recovery can be lost if things aren't quite right and lost to variable extents to boot. The way forward from this situation may be increasing sorbent capacity, further diluting samples prior to loading, changing SPE format, or taking real control of the necessarily slow flow with a contemporary positive pressure unit.

For the realm of small molecules, all kinds of weird and wonderful phenomena have been known to manifest and again the focus here will be on our recent experiences. In these cases, if I may sound prematurely summative, altering the fundamental means of extraction brought the method to where it needed to be, and I do believe the extraction is generally a prime tool in this workshop. It is important nonetheless to be aware that extraneous chromatographic peaks may appear sometimes in hemolytic extracts, necessitating chromatographic selectivity changes, usually a great means of indulging sorbent chemistry-based alacrity!

It is curious, I used to remark on how protein precipitation (PPT) seemed to be synonymous with the avoidance of hemolytic effect. Then in recent times we had two methodologies that looked terrific when PPT was used, but generated very different data for hemolytic spiked test samples compared to regular. A definitive bias between the two sets, that is. The solution came for both methods by translating the extraction to supported-liquid extraction (SLE). Everything fell into place. We know that this technique has far more interferent elimination, real red-blooded selectivity compared to PPT, so the essence of these successes must lie here. It basically boils down to the more effort made to clean things up, with another nod to a selectivity focus, the better the outcome will be. An old but priceless principle.

One of these methods also saw the manifestation of one other pertinent phenomenon in hemolytic effect. The presence of a phenolic moiety in a compound of interest can often spell instability in extracts of hemolyzed samples if the pH is in the basic region, or even in the weakly acidic area that may result in some ionization of the hydroxyl. The solution is primarily to remove any basic additives, in our case reverting to simple buffer salts without pH manipulation in the reconstitution solution composition.

I hope that this brief 'burst' of advice can maybe help transition concerned individuals from hemolytic hell to hemolytic heaven. As alluded to within the text here, both tried and tested principles still apply. Look at selectivity changes as a general first stop and the extraction may well be the most fertile ground for best results. As experienced bioanalysts, I like to think the capabilities are already in our blood.

Bioanalytical method validation in a fully regulated laboratory involves a fantastic test of a quantitative method's mettle – a valued award of reliability underlined, subsequently, by incurred sample analysis and reanalysis. This business can cause scientists to suffer sleepless, anxious nights while systems acquire and you repeatedly mentally cycle through all the chemistry-based decisions. Those of us who ardently work in this domain know very well the scope and the details, and we are familiar with aspects such as the processing of data to give a set of outcomes.

Within the validation scheme, I have always felt a certain intrigue around the matrix effect investigations. Even though I also feel that with a typical matrix of plasma or serum, the hemolytic effect testing is extremely revealing and significant, as I have outlined in one or two of my previous columns. With the matrix effect, there are two distinct components. There is differential testing, where at least six different lots or donors of matrix are spiked on an equimolar basis, at least at the low QC level. Both the precision and bias of the results are measured, with precision more demonstrative of the essence of this particular test. Then there is the matrix factor testing, and this is designed to assess to what extent our LC–MS signals for all the monitored compounds of interest are altered in the presence of residual matrix components that pass through the sample extraction, according to the selectivity in operation, which in turn is set forth by the specific technique and protocol. The matrix factor data not only shows to what magnitude a signal is affected but in what direction too. That is to say a decrease, as in signal suppression or an increase, as in signal enhancement. Both effects happen of course, with suppression being more common, but the whole area here is riddled with bioanalytical interest. What is going physicochemically with either phenomenon, for instance? This can lead into various discussions, and there is plenty of interesting literature on the matter.

Now, to delve into the practical details of the matrix factor scenario, especially numerically. The experiment is set up to generate comparative data for post-extraction, over spiked extract and equimolar spiked solution (free of matrix components), where the solution is the same composition in terms of percent organic or aqueous, additive concentration, pH and ionic strength, as the final extract composition. The overall result is expressed as based on 1.00, so it is a ratio, a fraction. It is the response in extract over the associated response in solution. If it is a reduced response in the extract and the matrix factor is less than 1.00, then we call it suppression. On the other hand, if it is an increased response in extract and the matrix factor is greater than 1.00, then it is enhancement.

In this setup, we will naturally use internal standards (IS). That is what we always do, of course, to make our results as solid as possible. So, we produce a ratio of analyte to IS for over spiked extract blank and an analogous ratio of analyte to IS for the solution-based control. Ratios going into a ratio-based calculation, just to avoid confusion. The question is should we limit the final reported data to these?

I believe the answer is a resounding negative. Fortunately, the impression I have is that across the industry, where a reported dataset may have, in years gone by, only included the ratio-based data, nowadays we will seldom find this occurring. Instead, the whole array of numbers is provided, and this is the critical point. This is where we can measure up the raw peak area responses against the appropriate control peak area, on an individual basis for all compounds monitored. There we can measure the real manifestation of matrix factor and for every analyte and IS to boot. If we are using the ratio with IS and it is also a quality IS, as will normally be the case by the time these tests are being performed, the resultant measurement of matrix factor will possibly veil a degree of genuine signal alteration. Veiled, because the IS compensates physicochemically for the phenomenon imposed. To present data in this way is to obscure the essence of why we perform the matrix factor experiment in the first place. Yes, the ratio may ultimately be unconcerning in its proximity to 1.00, but if there is deep signal suppression or overwhelming enhancement as indicated by the peak area-based measurements, then the method is crying out for an element of probable interferent separation, a beneficial selectivity tweak at least, to bring it into a territory of ruggedness and reliability.

For the matrix factor experiment, I would always insist the full regalia of data is made available. A particularly important element of transparency for the bioanalytical validation scientist. It is even somewhat analogous to show all performance data both with and without internal standardization. That would be a much more report-fattening predicament and is possibly more suited to the method development phase for close scrutiny, but nonetheless it is still valuable to underline method reliability in the validation test batches. That is a good place to toggle this piece, however, with the promise of more IS-normalized or perhaps out-of-boundary discussion to follow!

The ‘OOPS’ of intent and design

10 Mar 2022

I bet you cannot guess what this article is about from the title.

It’s actually one of my favorite topics of discussion around bioanalytical LC–MS method development. To do with the ideal scenario of having orthogonal selectivity between different separative components of a method, typically with reference to the dimension of selectivity afforded by the extraction, especially if it is SPE, and that afforded by the dimension from the analytical chromatography. There are of course the additional and valuable dimensions bestowed by mass spectrometry and, for instance, any ion mobility-based precursor, but the use of the term is traditionally used with reference to the liquid-phase operations.

In its entirety, the acronym stands for Orthogonal, Oblique and Parallel Selectivity. Orthogonal, as you may imagine or indeed be familiar with as regards the concept, is where the selectivity is entirely different between the extraction and the chromatography, such as where there may be a purely reversed-phase SPE followed by a normal phase LC prior to the detection. Parallel, also fairly intuitively, is where these components are entirely analogous, a selectivity continuation but where the hope is that the resolving power from the chromatography ultimately suffices. In a case like this, the extraction will have fulfilled at least one important purpose, such as deproteinization, and/or deposited strongly-held lipophilic interferences on the SPE sorbent bed. Lastly, not being a ‘black and white’ type of person, I think of the term ‘oblique’ as relating to the in-between, when there is something else in the mix, for example, a mixed-mode SPE, using definitive electrostatic interactions in combination with reversed-phase, followed by, finally, a reversed-phase analytical chromatography. Perhaps also something like a polar-modified phase could bring about this oblique categorization, or a polymer-based SPE compared to silica in the LC column, or operating at a different pH, even a different organic modifier between the selectivity avenues. It’s a little subjective, but I think is rooted in sound meaning.

It is generally accepted among method developers that, looking at matters from the angle illustrated (pardon the figurative analogy), the more orthogonal the selectivity, the better-performing the method is going to be. Hence the method is all the more likely to be labeled as reliable via the usual smooth method validation process and how impressive the performance data look therein. Taking a moment to consider — albeit in rather simplified terms — the host of possible interferences of varying polarity amid the compounds of interest in a sample, it becomes clear how the dynamic essentially works. Suppose the analyte is of medium polarity. In the extraction, we could choose a technique that eliminates high-polarity interferences while retaining medium polarity and low-polarity matter. Then, with high polarity interferences gone, we could choose to follow this extraction with chromatography that easily resolves remaining low polarity interferences from medium polarity. This situation would be akin to a real predicament where we have a fairly hydrophobic small molecule analyte to quantify in plasma, and we first perform a liquid-liquid extraction, which desalts and removes polar interferences very well when taking the organic layer.

This would be complemented subsequently by a hydrophilic-interaction chromatography, which has low polarity interferences (like lipids) eluting at or near the void, while our analytes of modest polarity have some retention thus resolution. An alternative predicament would be selecting an extraction that eliminates low polarity interferences, like important lipophilic endogenous material implicated in ion suppression such as the well-documented pool of phospholipids, then is complemented by chromatography which is reversed-phase, easily eliminating polar interferences, which elute early on. One beautiful thing about regular bioanalytical SPE, rooted in reversed-phase retention mechanisms, is that it readily attains the goal of lipid over-retention thus elimination from the sample to be injected. Especially if the elution step is optimized for a minimum volume of eluent that secures full analyte recovery (with safety margin) and is not overshot.

What if the compounds of interest are at either extreme on the polarity/hydrophobicity scale? This not-uncommon scenario is where 'oblique' selectivity may be applied to good effect. The focus can be, overall, on eliminating either the polar or the hydrophobic set of interferences, but also to delve into mid-range elimination, to continue with the model dwelled on. For example, we have a lipid analyte and we start by applying a liquid-liquid extraction from plasma primarily to eliminate polars. Then we finish up by using a reversed-phase gradient in the analytical chromatography, but it has more than a soupçon of polarity within a bonded phase that has perhaps a polar-embedded amide group within an alkyl chain, alternatively cyanopropyl or phenyl moieties, or polar end capping. If we went 'the whole hog' of something like HILIC, our lipid analyte would be insufficiently retained.

There we have it then, a somewhat simplified overview of the roots of why, for a given method, different directions in selectivity at the key separate steps are beneficial. After all, if a method is as oblique as are my conversational skills, I venture to suggest that it cannot be bad.

> *Selectivity is absolutely crucial for a high-performance, reliable quantitative method. I'm quite proud of this piece as a useful breakdown of the principle and how to use each facet of a complete bioanalytical method to reach success.*
>
> Robert MacNeill

A disturbance in ‘the Force’

4 May 2022

That’s not a moon. Not a death star either. That’s a contemporary positive-pressure manifold for SPE. Like a lightsaber, SPE and its accoutrements constitute an elegant weapon used in the fight against interferences impinging on the sensitivity and performance statistics of bioanalytical assays, and in the quest to maximize sensitivity in the same methodologies. Warn you, I must – my intent here is to not bring balance to ‘the Force’, in the sense of applying multiple meanings to the analogy. But certainly, neither is it my intent to leave things in darkness. Here goes nothing!

In the past, especially when small molecules dominated the galaxy of therapeutic candidates, SPE was a technique regularly employed for high-sensitivity regulated method applications. Given that ‘the Force’ is not strong with the vast majority of small molecules, in terms of their frequent hydrophobicity and reversed-phase interactions dominating their behavior on SPE sorbents and analytical chromatography, and such inter-molecular forces being weakly energetic, not Forceful, the applied flow rates at each step typically did not matter a great deal. Method performance could hold up well, like Jedi masters comfortably polishing off swathes of stormtroopers, effectiveness portrayed spectacularly by lightsabers in mesmerizing motion.

Over the past couple of decades there has been movement, a disturbance, in the portfolios of so many pharma companies, towards biologic-based therapeutic candidates. My ally is ‘the Force’, and my resolve is in the production of bioanalytical methods more powerful than you can possibly imagine. The methods for biologics are no exception, indeed embracing the associated challenges teaches us a lot about the true nature of ‘the Force’.

It is easy to perceive that ‘the Force’ is strong with biologics, inter- and intra-molecular forces that is, considering the profoundly polar and ionic makeup of the molecular structures, especially where oligonucleotides are concerned. Indeed, these may offer the best example of where careful, controlled use of ‘the Force’, applied positive pressure, brings the best results. Unlike the mainly hydrophobic properties of typical small molecules, what must be harnessed with nucleic acids is the strength of the interactions that are manifest, the polar and electrostatic. Highly energetic interactions they are, therefore, they take time to initially engage and then to fully disengage, in an SPE protocol. For this critical aspect of speed, a hyperspace mentality is not called for, but rather an approach that minimizes flow, allowing time for the processes to go to completion. I like to think I have an appreciation of the gravity of these situations, and there’s nothing more reproducible than gravity apart from interplanetary reproducibility I suppose, but a decent positive pressure unit will shine and slice through this challenge.

Just as we feel ‘the Force’ flow in terms of the pressure from such contemporary positive pressure units, we can ideally set the gauge to attain exactly the right flow, bringing balance of ‘the Force’ to all wells, and all in emphatically reproducible detail too.

After all, if using a dated unit with a simple knob for pressure adjustment, your eyes can deceive you. Don't trust them. Have we paid the price for our lack of vision, thus far? Instead, with a unit of such new and detailed construction, entire pressure profiles could be established and available to be transferred to any other lab with the same units, regardless of how much the 'Dark Side' is strong with them.

Only the Sith and non-polar scenarios deal in absolutes (of manifold knob twisting). I will do what I must, where biologics and high energy interactions are concerned, with carefully timed precise magnitudes of adjustment. Meanwhile, the methods established with such units in the paradigm of small molecules will become so solid as to be effectively encased in carbonite.

If only now, at the end, do you understand, then it matters not. Join me… in interpreting the galaxy of experiments yet to be done!

Happy May the Fourth!

> *This was the first of Rob's columns I was involved with and what a great one it was! I remember loving the punchy writing and puns and thinking this was so different from any other content on Bioanalysis Zone. It was a friendly invitation into Rob's thoughts, a fun piece that got me intrigued about bioanalysis. I love how Rob mixes descriptive language with scientific reasoning to create such unique articles.*
>
> Ellen Williams, Senior Editor, Bioanalysis Zone

In any method development project, especially one for an entirely new therapeutic candidate with no history of bioanalysis, there are points at which heaving sighs of relief are often audible when certain sets of freshly produced analytical data are being perused. At these points, it is likely that the wonderful attribute of precision will have just been observed.

The title of this piece is a play on the words shown and their assigned meanings within and outside the context of analytical chemistry. 'Precision' to us, as quantitative bioanalytical professionals, has a very distinct meaning. The proximity or level of agreement of replicate data from a given sample and six replicates is usually regarded as enough replicate fodder to make such a meaningful judgment. We look at the relative standard deviation (RSD), alternatively known as the coefficient of variation (CV), calculated from the standard deviation of replicate sets of internally standardized peak area ratios or just raw analyte peak areas in conjunction with mean calculated concentration. We look to see these numbers, which innately cannot be negative, between 0% and 15%, but the closer to zero the better. In daily practice, an eyeballing of peak areas within their sets can immediately let the seasoned eye know if we have come through the hallowed precision gateway. 'Precisely,' on the other hand, seems to be a term most often used outside the analytical chemistry context and as such, with some irony, is taken to denote accuracy. Great potential for confusion, but also amusement, hence my beeline for the combination.

Stemming from the first paragraph, precision is important, then? Yes indeed. It constitutes the very foundation of a reliable method. Once precision is there, then accuracy is either there already, just a hop or two away, or at worst all that remains can be addressed with a 'tweak,' a popular term in method development. We do need precision, not just for this essential performance-based reasoning but it's underlined when we consider that study samples are analyzed singly within an analytical batch. The only possible route away from the conditions that bestow precision is paved out from the need to make radical changes – and this may typically be for finding the right selectivity or sensitivity. However, we desire to always maintain precision through any tweaks or more significant changes we make and we use our chemistry knowledge to this end. Solubility, for instance, and the flip side of that coin which is negating non-specific adsorption. There is also ionization or neutralization at a given point in a method, pushing fully either way as appropriate, especially for chromatography and SPE contexts. Even at the mass spectral endpoint, we may wish to have the analytes fully neutral, as for instance, this is ideal for the charge acceptance via corona discharge in atmospheric pressure chemical ionization (APCI). Electrospray-based gas phase ion generation often works like this, where it is hard to explain with the underlying rudimentary theory which relies on preformed ions in solution. Then there is a bevy of other methodological aspects that affect precision in a chemistry-related manner, such as degrees of breakthrough prior to elution in SPE, emulsions in supported-liquid procedures, adduction and instability. However, I do maintain that the aforementioned solubility and ionization aspects are pivotal, really at the root of precision and hence method performance.

There continue to be impressive laboratory instrumental improvements, and these help ease us into truly understanding and valuing terms like repeatability and, much more significantly, reproducibility. Pipetting is a well-known source of variability, for instance, where we ideally have individual technique entirely uniform between individuals and occasions, correct and appropriate for the pipetting operation at hand. We aim to have procedures automated as much as possible, and there are notable advanced pipettor robots that certainly help approach the ideal.

In terms of LC–MS operation and reaching states of reproducibility, one technological aspect worthy of mention here is scaling down from conventional flow through microflow towards ultimately nanoflow. For decades, it has been a common observation that LC–MS peak area responses can vary enormously day to day, instrument to instrument. Part of the reason for this reality is the astoundingly low percentage of gas phase ions typically produced compared to the number theoretically available in a given moment of LC eluate entering an electrospray-based ion source. It is only when nanospray conditions are approached, going down to around 100 nL/min and below, that the number becomes something approaching a full complement. These conditions, in addition to quelling matrix effects and non-linearity, should be highly amenable to reproducibility in the same way that a high recovery in SPE is amenable to the same quality. When your allocation is to any eye a small percent, it comes with considerable intrinsic variability. Whereas, if it is the best part of 100%, the variability is expected to be minimal.

So, here's to precision and the relevant chemistry-related considerations that underpin it, and here's to technological innovation guided by the same (robotic) hands.

Precisely.

> *This just had to be written. Precision is the element that is the lifeblood of quantitative science. Without it, the principles of accuracy, sensitivity, etc., don't count for anything. And really, it's governed by the wonders of chemistry.*
>
> Robert MacNeill

The baseline-disturbing adventures of the ghost-peak busters in mass spook-trometry and beyond

25 Oct 2022

In the howling wind of the darkest moments of the night, the frostbitten landscape shuddered under the faintest of moonlight. This chill and unforgiving dominion seemed an eternity away from any hint of human warmth, but at the same time deathly close where, in the far distance below, nestled at the foot of the humbling precipitous mountains, glimmered a flickering light in a tiny window.

'Is this where I'm supposed to be for my CE-MS seminar?' wondered the Snow beast. 'I need to branch out from my simple ghoul-ometry.'

Welcome to a modest Halloween-themed column. How often have we considered hiring an exorcist for a persistently malfunctioning mass spook-trometer? Has your high-throughput screening ever become high-throughput screaming? I'll try to maintain a GLP context, though, and that means — of course — ghosts, leprechauns and pumpkins.

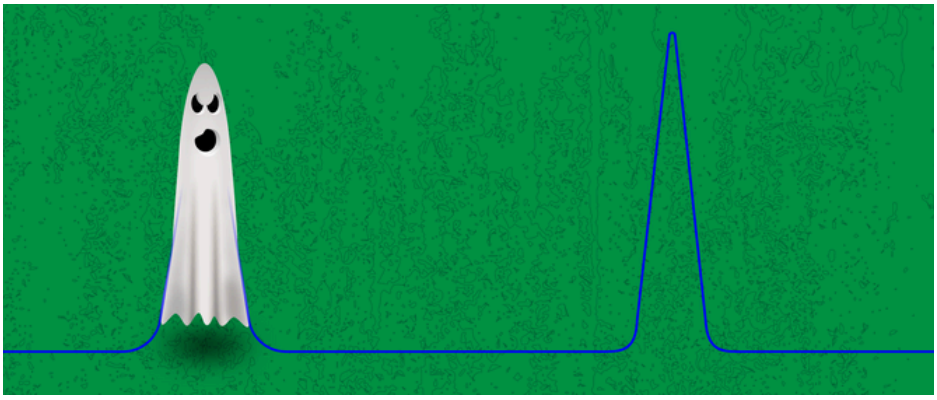
Things can go 'bump' in the night, certainly during an LC–MS batch sequence analysis. This naturally includes chromatograms and their 'bumps,' baseline disturbances. What can they be? In the same inviting vein, if we're really going to get our teeth into this, what about weird chromatographic manifestations that are clear peaks, just not where they should be in terms of retention time or monitored ion channel? Are they different in nature? Should they all just be shut away with plenty of garlic and holy water, preferably blessed by reverse osmosis, and with no access to visions of the full moon?

Well, it can be down to the spectral nature of the detection. By that, I don't mean anything to do with ghostly mass spectrometers from beyond the grave, although the real things can be hair-raising if mistreated in particular ways. I refer to just the nature of the scanning or monitoring. Also, the unearthly peaks and bumps may be an artifact of non-ideal column performance, or something awry with the front-end unit, like unwanted voids having been created. Not the void of deepest, darkest outer space, just system voids. These, particularly at the head of the column or in the injector port plumbing, may lead to these horrors of contamination. Additionally, and perhaps less commonly, it can be down to a sinister aspect of the sample's own composition. For example, too much organic in the injected sample in a reversed-phase method can give rise to a convenient un-dead time marker.

To turn our horrific gaze to the monitoring aspect, firstly one must accept the cold reality that if there are isobaric compounds being monitored, such as with isomeric compounds, or if there are interferences that are isobaric with anything being monitored, then we'll be doomed to be spooked by ghost peaks. Overwhelmingly so, for the interferences, if tandem MS or high-resolution MS is not being used. We'll be able to put our shaking fingers to good use in altering the selectivity of the extraction, however, and ensuring baseline separation in the chromatography for the isomers.

Perhaps the classic, most harrowing, seethingly angry-sounding ghost peak manifestation comes through tandem quadrupole MS, the phenomenon of crosstalk. It would certainly make the mad mass spectrometrist cross. A product ion within a set of monitored transitions is the same as for another transition. Not the transition to the other side, I hear you ask through the ether, but a transition from precursor ion to product ion upon fragmentation in a pressurized collision cell. Like trying to fully purge the evil from Dracula, it's quite hard to fully eliminate product ion content from the collision cell on the millisecond scale. When the subsequent transition is dwelled on, even though the precursor is different, the small abundance of the same product is picked up if it's monitored in the method. It's most clear if the sample injected is supposedly blank for that analyte showing the chromatographic wraith. Modern triple quads are equipped in various ways to address the possibility, and there are safeguards one can take such as including dummy transitions, but it does …mysteriously… still happen!

That just about covers it for my advice about supernatural aspects of bioanalysis, and you'll be pleased that I haven't even delved into ion-scaring reagents for howl-igonucleotides by LC–MS or dwelled at all on gauging carbon skeletons for initial stages of method development! In any case, if you see anything else peak-uliar, please let me know! I will be all too eager to pick your brains, perhaps even closely inspect, analyze them with a suitably spirited method and, as always, letting the vampirical data speak.



The baseline disturbances of mass spectrometry

> *My favorite piece! Such great fun to write. Spookiness and bioanalysis come together with hair-raising results!*
>
> Robert MacNeill

In light of my perception of many changes happening recently in the bioanalytical community, the general movement of roles and responsibilities, numbers of people shifting between organizations and opportunity aplenty, I thought it apt to consider some important roots of chromatographic bioanalysis in this particular contribution. Many folks may be finding their feet in this predicament. Chromatographic and mass spectrometric bioanalysis, collectively, is what happens to be my thing, along with a penchant for questionable efforts toward descriptive and dramatic written composition.

Without further ado, let's hurl our attention out into the void, but having more than an inkling of what is out there. There are some aspects much to do with LC method development and preserving method integrity that pops up repeatedly, as concepts that are more unknown than they should be. The retention factor, known as 'k', is one example. There used to be a prime on 'k', but no longer, for those wondering. The retention factor is synonymous with isocratic elution, where the composition of the mobile phase remains the same, but there's also an analogy for gradient elution. In simple terms, what it comes down to is that there is no escape from the requirement to properly establish that there is an adequate minimum of retention, and similarly not too much. In my experience, it's the failure to push things off the void marker, the solvent front, that is by far the most frequent issue. It's very important to use the column dimensions together with relevant extra-column volume, in conjunction with the flow rate, to calculate the void time. Then, to attain the minimum retention factor of 2, three void times must pass before elution occurs. Why do we want this minimum? We want the selectivity that comes with the powerful chromatographic dimension. We need it to attain the best chance of resolution, regarding other analytes and interferences. Ultimately, all leading to a great method performance. That number of 2, in fact, marks the beginning of the most fruitful region of retention as regards this purpose, when k is between 2 and 10 it is most practical and accepted. This is all with reference to how it sits in the all important resolution equation:

$$Rs = (\sqrt{N}/4).(k/(1+k)).((\alpha-1)/\alpha)$$

Whether isocratic or gradient elution, it's always good practice to check for three voids' worth of retention. Sometimes we can dip below 2 towards 1 and still show a rugged method, but it's encroaching on dangerous ground, especially with bioanalytical extracts. And even more so with a relatively non-selective sample extraction where an abundance of interferences will be present and eager to compromise your method performance.

As you bear down on zero retention, where k approaches 0, it is of course the most precarious situation to put yourself and your solutes in. Never mind poorly retained coeluting interferences, just the presence on the void edge can bring aberrations and non-uniformity to peak shape.

Rounding off the retention riposte, there is an unfortunate phenomenon too often noticed in the literature whereby oversized columns are used at healthy flow rates and analytes elute with nice sharp peak shapes within a desirable run time of a few minutes. When using such a column, and in such cases, they are 4.6 mm internal diameter, the void time alone is typically in the order of minutes. For instance, it so happens that a 100 x 4.6 mm column with a 1 mL/min flow rate has about a 1-minute void. Therefore, we would look for 3 minutes minimum retention. However, in quantitative bioanalysis, that's much more akin to what we look for in terms of the complete run time. Instead, in such cases, retention times would be typically 1 to 2 minutes, where k is 0 to 1. Peaks may be sharp but that's really only because they haven't had any chance to broaden, being effectively spat out by the column and the sharpness may seem a beneficial aspect; but I would maintain it's far better to avoid the risks of poor retention as detailed. The way to regain method integrity would be to translate the method to a narrow internal diameter column such as 2.0 mm, adjust the flow as appropriate to remain within system pressure limits and use your various tools to set the conditions to allow decent retention to manifest. The void will then ideally be a small fraction of a minute and, now featuring k within acceptable limits, there will be resolving power allowing the best chance of overall ruggedness. There will also likely be a reduced flow rate more conducive to best mass spectral sensitivity, in consideration of the strong element of concentration-dependent sensitivity ever-present with electrospray-based techniques.

On a somewhat related note, there's also the theoretical plate count, N, a measure of efficiency that is also often misunderstood or overlooked. It's also part of the aforementioned resolution equation, for good reason. From this angle, we can discuss the implications for not only the retention reviling monsters lurking close to the void but those all throughout the run. This property pertains to the peak narrowness, sharpness if you will. The sharper a peak, the more chance there is of resolution from other solutes, not to mention giving more signal to noise. It's a function of retention time and, inversely, the accompanying peak width. This is not such a serious matter as playing with retention factors, since efficiency is essentially a single property of a given column and as such only merits checking the chromatographic output. However, it is important to retain an understanding of how the plate count ties into the big picture and how changes can be made to increase it via column selection, while balancing other important method parameters like run time and back pressure.

With that, our back-to-basics journey out into the void is complete and let's hope we maintain our resolve and sharpness, not migrating from it too soon!

Heads-up! Moles are naturally a chemist's friend, wonderful close associates in quantitative bioanalysis. Although my father used to complain of excess 'molarity' digging around in the garden back home a few decades ago. Why would we want to play whack-a-mole? The analogy I attempt to make here is to do with the mass spectrometry of biologics and their innate multi-charging propensity. So, in assays for peptidic or protein analytes, or for nucleic acids, the appearance of several peak clusters corresponding to different charge states, and how the relative intensity can vary according to certain parameters, is likened to moles popping up from their holes and requiring to be analytically accounted for, or 'whacked.' In other words, how do we play charge state whack-a-mole with the best anticipation in consideration of the associated LC operation and the mass spectral interface in a quantitative bioanalytical LC–MS context?

In my opinion, the analogy to whack-a-mole helps to garner an understanding as to why all charge states should be monitored and used for quantification via summing. I appreciate the reasonable train of thought behind sticking to one transition in tandem mass spectrometry — the most intense — as the signal-to-noise will end up much the same, maybe less if summing was to be employed. My answer to that lies in the variability in abundance that inevitably occurs due to the influence of several experimental and functional bioanalytical LC–MS parameters. If abundance funnels from one charge state to another, we will catch it if we sum all available channels and thus retain response and underlying precision. As an added benefit, the summing also results in a smoothing of the resultant combined trace, facilitating peak definition and reproducible peak integration.

If you will humor me as I once again delve into my favorite domain of oligonucleotide quantification. It is a great working example of this topic, especially when we make a charge-state envelope-based comparison of the popular liquid chromatographic options that may be used, hydrophilic-interaction (HILIC) and reversed-phase (RP). We ought to consider what may be most analytically desirable as properties of our envelopes, for a quantitative endpoint, or 'molar' endpoint, you might say. In the important and growing domain of oligonucleotide bioanalysis, HILIC is now known to generate charge state distributions that, in comparison to ion-pairing reversed-phase, are narrower and show the predominantly intense charge state to be lower. A simpler situation. Less charge states in total to have to simultaneously look to bring the bat down on, and not so numerous in charges that effectively reduces peak spacing to a point where we wish we always had accurate mass at hand. Accurate mass makes it all easier with today's technology. Also, with lower predominant charge states, we more readily avoid possibly bringing the m/z measurement too low for the most comfortable selectivity.

From experience, however, digging a little deeper may be called for, in a mole-like manner that could result in going against the proposed scheme and limiting the number of charge states monitored. This relates to the chance of encountering non-linearity and is something that my long whiskers touched in a real assay of late.

It was a situation whereupon monitoring a decent number of charge states, we attained curvature of the 'upward' variety, where the peak area response began to take off exponentially with increasing nominal concentration. There was a little non-specific binding going on, but it was found that the spade-like feet behind it were to do with the charge states. We brought it down to only one being monitored, the most intense, and the problem was batted. To speculate a little, this is something that is almost certainly related to the dynamics of the production of gas-phase ions in the electrospray-based ion source; this may be alleviated or eliminated with micro- or nanoflow, where competition-based effects dwindle and can even practically disappear.

To sum up, if you will pardon the pun, I would advocate for capturing the visible charge states and their incorporation into an acquisition method. As such, any swings in abundance will be best accounted for, giving a more concrete concentration-response definition, plus it affords a beneficial smoothing effect. The aforementioned concrete is, of course, a mole's enemy. However, if non-linearity is observed, it may be worth investigating a more select group or even a singular charge state to monitor.

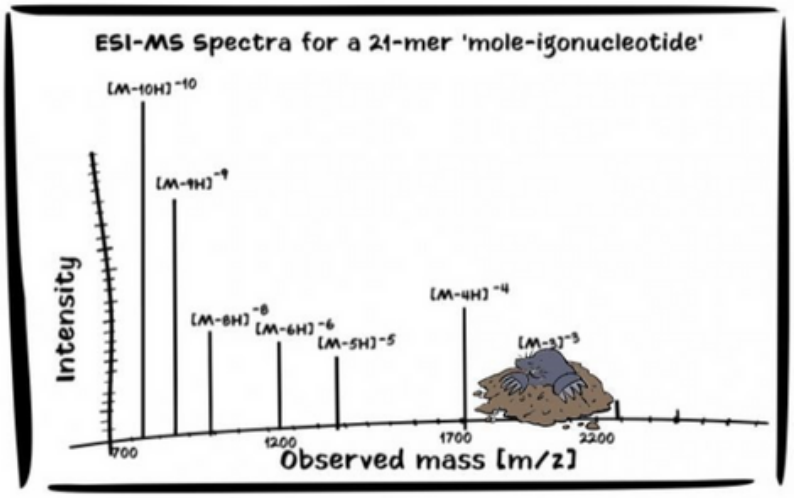


Image created by Hazel Dickson, Waters Corporation (MA, USA)

> *Rob has a special talent for finding analogies to simplify complex science, and I just loved the likening of monitoring charge states of oligonucleotides to whack-a-mole!*
>
> Hazel Dickson, Social Media & Content Principal, Waters Corporation

'There is an art to science and a science in art; the two are not enemies, but different aspects of the whole.' – Isaac Asimov

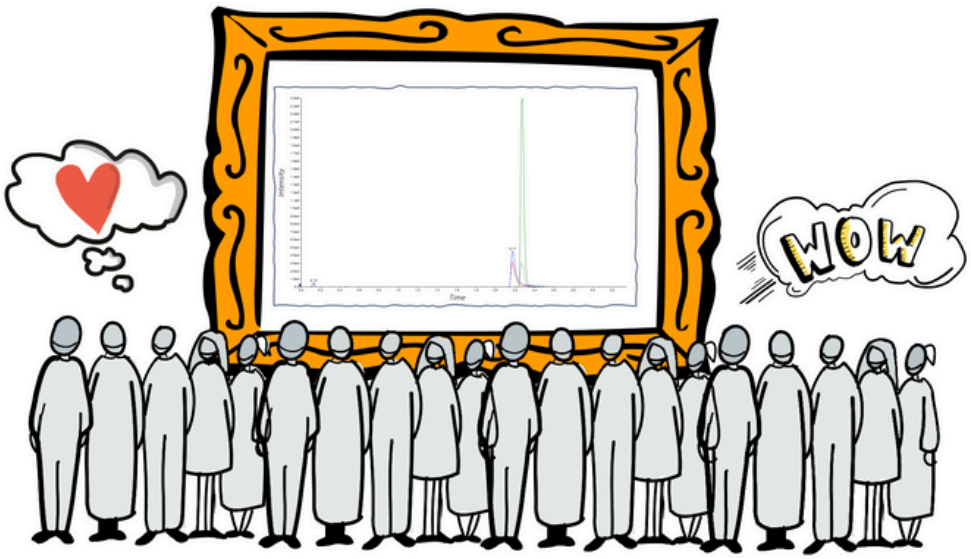
There are surely few concepts as profound as that of art, as in what constitutes art and what, by any perception, it could all mean. Then, what food for thought and endless discussion in how science relates to art. Can science be harnessed as a medium through which we can expound on our innermost soul-emanating feelings and thoughts? For that matter, how about the potent paintbrush known as quantitative bioanalytical LC–MS?

I count myself lucky to be no stranger to the feelings of elation that come, for instance, after being visually glued to the achingly slow progress of a chromatographic trace within a pivotal sample injection cycle, waiting for a key moment. This is when the intensity suddenly rockets at the right time, and it is blissfully clear that your analytical head-scratching and innovation have resulted in an emphatic recovery in your sample preparation scheme. In turn, when quantitative batch data are just at the end of processing post-acquisition and the moment comes when reels of perfect-looking data are unfurled before your eyes, making your day. Gleaning moments of contentment from this output amounts to our own work showing our expressions, our thoughts and creativity.

Moreover, we can envelop ourselves in the overall method development workflow, in all the main themes, associations, nuances and intricacies, and embrace it as an art form. To the seasoned quantitative analytical scientist, it is well-established that going through the process of method development, particularly from scratch, is a potentially labyrinthine predicament. This is, in terms of the multiple junctures where decisions are made, usually on a physicochemical or instrumental basis. A seemingly innocuous parameter could have great impact on a method's performance, and this is a multi-parametric predicament. Also, consider that, in the context of best analytical science, for a given methodological objective there are likely numerous different avenues and combinations that could come together to form a great method. Holistically, looking at the decision matrix, it becomes what may be regarded as a means of self-expression. We have plenty at our fingertips. Every aspect of the stock and solution preparations, solvent composition, pH, and choice of vessel to name but a few considerations. Then we have the sample preparation domain which represents a veritable myriad of decision-making, propagating into a similar vastness of playground in chromatography, with some areas only now being set foot in by intrepid, gifted explorers. Without even mentioning the vital arena of the mass spectrometric endpoint, the idea has taken shape and is surely worthy of a tribute of sorts:

A beauty defined yet undefined, a bustling workplace rewarding in reliability,
Precision and performance intermingled and profound.
Instrumental wonders, technological triumphs,
Amid immense characters of great minds applied,
Commanding data quality, all eyes aglow in familiar satisfaction.
Art within science, a world complete and without limits, beckoning the curious to venture forth.
To bioanalysis, my daily domain, lifeblood supporting lifeblood.
It is rather an abstract yet weighty notion. Can we find means of self-expression in the bioanalytical lab? In days gone by, in the very early stages of my career, when I was more attuned to a perceived separation in arts and sciences because of my educational choices and channels, I may have struggled more with this line of thought. But why, yes, I would now say it's a blank canvas, with foundations in good science and artistic accommodation galore. Edward G. Bulwer Lytton once said "Art and science have their meeting point in method" which I find particularly apropos.
Let creativity thrive!

"The greatest artists are scientists as well." – Albert Einstein



Sincere thanks to Hazel Dickson from Waters Corporation for the visual.

Amid the upbeat conversational bustle of the animated crowd assembled in the graduation hall, Dr Molly Cuellar beamed at her gowned and decorated companion.

“You are my daughter, Iona. I am the proudest parent. Yes, there have been times when I felt fragmented and we’ve had our pressurized collisions, but we harnessed that energy in the right way and you have learned to be specific and focused, sweeping aside all interferences from the clarity of your way forward. But, somehow, I still feel as structured as I have always been.”

“I know I’m finding my stable path, Mom! Thank you for being there as a precursor to everything I’m involved in! We may need our offsets and our breaks, but together we make something special, regardless of whether it’s a nominal matter or one demanding of a higher resolution.”

Transitioning from mass spectrometry to tandem mass spectrometry, as it is known, is a leap onto a splendid new plane of offerings. Tandem mass spectrometry is indeed long established as the bearer of profound new levels of specificity, sensitivity and applicability to an assay. The possibility of multiple stages, also tandem in space and tandem in time depending on your available toolkit and technology, only serves to propagate the interest factor and useability. It must have been wonderful to be first struck by the essence of this idea and suffice to say, something useful certainly emerged from it.

In the fundamental process of tandem mass spectrometry in a fully quantitative workflow, the sequence is as follows. A precursor (parent) ion is filtered from potential interferences of all other m/z through one mass spectral analyzer unit, purposefully fragmented in a pressurized cell and then the product (daughter) ion is filtered through a final analyzer. In a traditional triple quadrupole instrument, by far the most familiar to GLP quantitative bioanalysis, zero-width monitoring is applied in each key quadrupole, referred to as single and multiple reaction monitoring. Zero width means there is no sweeping of a range of m/z, in other words, the instrument’s resolution setting applies in both relevant quadrupoles, usually ‘unit’ which corresponds to 0.7 FWHM (full width at half maximum) of the mass spectral peak. The resolution may be altered to benefit a methodology in the face of certain challenges, but that is another article in itself. The area of collisional activation is another quadrupole but set to ‘RF-only’ essentially transmitting ionic abundance of all m/z present. There is a collision offset, frequently referred to as ‘collision energy’ a potential difference between the entrance and exit lenses accelerating the ions through the quadrupole, and this is key, alongside the gas pressure setting, in the product ion formation. The product ion is identified within the aptly named product ion scan, where only the precursor ion is transmitted into the collision cell and the relevant parameters of collision gas pressure and collision offset are optimized as a crucial part of the instrumental ‘tuning’ when constructing the method.

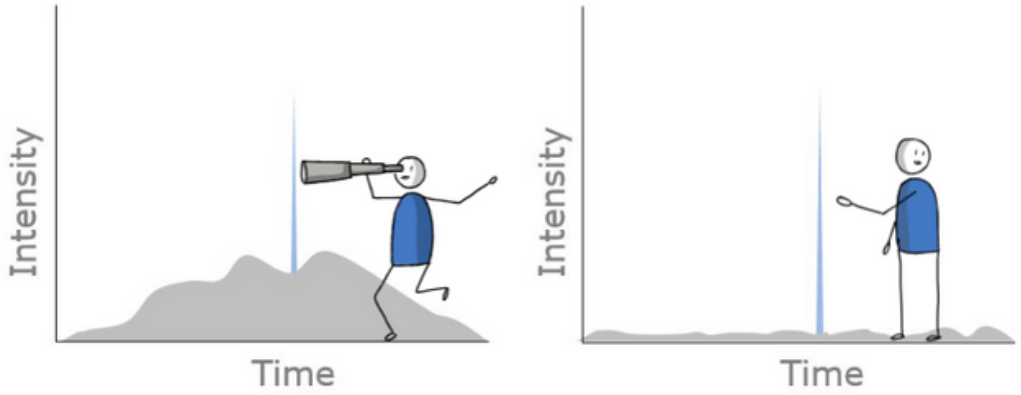
Altogether, the outcome and bottom line is a method with terrific sensitivity and selectivity, with baselines vastly reduced and stable, in comparison to the predicament of single quadrupole operation, otherwise commonly known as 'MS-only.'

Tandem mass spectrometry is also frequently denoted by MS/MS with the forward slash indicative of one stage of fragmentation involved. There are all kinds of scans one may perform in the realm of MS/MS, with more qualitative outcomes in mind. Precursor, neutral loss, product, Q1 and Q3 scans, all making use of the fundamental operation and all involving a sweep of a specified range of m/z.

Then there are ion trap instruments, bringing other ways of using fragmentation. There are traditional traps that confine ions to a point in space, sometimes referred to as 3D-Traps, which have great features such as the capability to perform MSn, multiple sequential fragmentations. However, they suffer from what is known as space-charge effects, which occur due to the very nature of the proximity of the ions in their trajectories, taking away from sensitivity and resolution and a low mass cut-off for each fragmentation. The traditional ion trap represents the prime example of 'tandem in time.' There are linear ion traps, based on a triple quadrupole mass filter rail, which effectively overcomes the space-charge limitation and still offers MS/MS/MS (MS3) overall, not including any desired cone voltage fragmentation. The trap product ion scanning is very sensitive and can reach in excess of 10,000 resolution when using slow scan speeds, showing far reduced space-charge effects and freedom from a low mass cut-off. However, with MS3, the final fragmentation is in the same quadrupole as the preceding product ion's isolation and gives a low mass cut-off. In any case, there is a modest variety of useful scan modes and, on the whole, this kind of instrument is synonymous with quantitative application and may be said to offer both tandem in-space and tandem in-time operations.

Lastly, the breakpoint, in that the final analyzer need not be of a nominal mass nature, where nominal pertains to quadrupolar detection and the approximate 4000 resolution it offers. The contemporary outlook is of accurate mass detection gaining a foothold in regular qualitative and quantitative application. The reasons, embedded in the high-resolution nature of these technologies, are entirely convincing. The benefits of high-resolution speak for themselves, especially for complex biologics and the analytical challenges such as those presented by antibody-drug conjugates, where we might well strive for the ideal of all components to be analyzed on the same platform, but again this is material for another article. Examples are quadrupole time-of-flight (QTOF) technology, offering a resolution of approximately 50,000, which marries splendidly with fast chromatography and also gives impressively high-sensitivity methods in latest-generation models. With such technology, it is easy to use TOF-MS, no fragmentation, to give accurate precursor ion measurement and confirmation ready for translation to tandem MS workflows. There is also Orbitrap technology, in which commercial manifestations can strike a challenge with the order of several hundred thousand resolution.

As activating as these ideas and realities are, at this point, we must break it off and conclude. In bioanalytical life nowadays, to my awareness, it very seldom happens that an acquisition of a single-stage instrument is made in preference to a tandem. Sensitivity and selectivity are in such incredible, ubiquitous demand that it's a necessity. The question has far more to do with the selection of the scanning nature of the final analyzer. Mainly, it is down to the choice of nominal mass or accurate mass. Both have their place, but the latter is, in the big picture, set to dominate the future. Either way, this is where 'picking up the pieces' is seen in a very beneficial context.



Sincere thanks to Hazel Dickson from Waters Corporation for the visual.

Peptides, amino acids, mRNA and larger biologic entities…I have seen a lot of these analytically fascinating beasts coming into play in the modestly vaunted hydrophilic-interaction chromatography (HILIC) dominion over the last few months. New HILIC column chemistry and format options from prominent vendors have been noted too. It comes as little surprise, having personally been fortunate enough to be able to realize how potent the technology is over the past couple of decades, and especially for biologics. By especially, I mean at least as an intuitive and prominent theoretical option, now accumulating a decent substantiation in the literature too. That is all because of the innate polarity of so many biologics being aligned with the very polarity that the likes of HILIC feeds, indeed devours from, and makes great HILIC retention and eventually, with a little tweaking and fine-tuning, overall great chromatography become manifest.

Yes, I have written about HILIC before, but it needs and deserves more exposure, and I am glad if it is really shifting out of a niche domain. Many moons ago, especially if you can squint to see the moon as a gigantic spherical silica-based particle, I almost unwittingly started down the broader niche path, if you will, in separation science. Never mind the analytical chromatography side of things, this initial foray was in SPE. The sorbent screening and subsequent optimization in this, my very first SPE bioanalytical method development, resulted in a path forward embedded in what turned out to be per aqueous retention on bare silica, forging better recovery than a ream of different bonded phases. This is a story in itself, and actually pivots on reversed-phase, in contrast to the main theme of this editorial, reflecting that versatility linked to the silica base. At least in roaming through the lesser-explored niche realm, the interest factor is an ever-present companion, the element of challenge and prospect for innovation tantalizingly close at hand.

By no means is HILIC the only option in the guise of normal phase with water-miscible solvents, either. There are columns available presenting a silica hydride surface and a number of fascinating and useful flavors of bonded phase, and presenting us with the reality of the simultaneous manifestation of reversed-phase and normal phase dynamic processes. As such, this is something that would be particularly useful if transferred to the SPE format, where initial catchment is so often a challenge, especially for biologics. However, there is now HILIC established anyway in the SPE context. ‘Hydrophilic-phase extraction’ (HPE), it came to be known, after seeing it bring great results for oligonucleotides, after a brief dalliance in 2016 with a peptidic application, which was similar in many ways, laying the foundation for further work in HPE. There’s plenty of interest ahead in this polar predicament, which also gives wonderfully clean extracts. It features a wash regime involving both low pH and high pH, moving from high-organic to low-organic, and no need for an aqueous equilibration either, at least in the latest oligonucleotide-focused manifestation. Indeed, such a step would induce breakthrough upon the sample load. Altogether it’s a part of the collective charge, even charge state envelope, one might say, toward high-recovery hence high-sensitivity biologic methodologies.

As mentioned, silica has a strong propensity for supporting various retention mechanisms. HILIC is just one of several modes possible, so we can think of it as poised and ready, just waiting to enter the fray. In actual fact, you can see HILIC behavior on almost any silica-based column, even with hydrophobic bonded phases, if the mobile phase composition is favorably high-acetonitrile and we perhaps push a little harder to get there. It's just not really reproducible under extenuating circumstances, mainly when a pronouncedly lipophilic character within the bonded phase is disruptive to the integrity of the characteristic water-rich layer around the particles. I would therefore keep the phase polar, unless working on unmodified silica.

Rounding off this polar prattle, biologics with all their bioanalytical nuances and quirks are here to stay, and we are seeing techniques like HILIC easily slip arm-in-arm with the quantitative side of the playing field. They are broadly hydrophilic, and HILIC is intuitively a part of that, with no need for curious additives to get the chromatographic pot bubbling. Favorable retention for anything polar and multi-charged, simplified charge state distributions, and in rather a new chapter, movements to the promised sensitivity-bequeathed domain of microflow and nanoflow have already begun!

> *As you can see, I am unlikely to ever tire of writing about HILIC. Especially when I see it picking up so much pace and popularity in the industry. No more Mr. Niche Guy!*
>
> Robert MacNeill

‘Welcome to the real world’ — of comprehensively addressing matrix effect.

Embracing diversity is a wonderful thing, and it also counts for underlining the strength of quantitative bioanalytical methodologies in terms of successfully encompassing the diversity of biological samples available, for a given matrix, in a given species. This pertains to the critical differential aspect of matrix effect. Yes, here we have an innocent two-word term with innumerable connotations, many of which result in sharp intakes of breath, and the ability to strike fear into the heart of many a quantitative bioanalytical LC–MS user across industry and academia.

My own coming-to-terms with matrix effect and all the nuances, really with a determined view to cementing a satisfying understanding, came around the time when the industry was generally realizing that the wonderful offering of high-end triple quadrupole-based detection did not actually negate the need for chromatography and sample preparation. There had been splendid technological advancements in making electrospray-based gas phase ion generation amenable to accepting conventional LC flow rates, but at the cost of a minuscule percentage ionic release amid a whole plethora of competition in the ion evaporation model. Especially if the chromatography is not of adequate discriminating power, or the extraction is not overly selective, resulting in the major feature of coelution with other compounds and interferences. A ubiquitous term in this domain, interferences, present natively in the biological matrix and artifactually recovered through the sample extraction procedure, absolutely key in this context of matrix effect. Such interferences are wholly accountable for the very manifestation of matrix effect, the resultant response modifications and, more potently, the sample-to-sample variability superimposed on these modifications.

The response modification aspect pertains to what has been termed ‘matrix factor’, established via straightforward numerical comparisons of peak area and ratio responses in solution versus extract, on an equimolar basis. There may be suppression or indeed genuine enhancement; both are real and extensively documented. The lot-to-lot or between-sample variability aspect pertains to what we know as ‘differential matrix effect’, established via mainly precision but also bias assessment of calculated concentration over at least six different sources of matrix for the species. This is performed at low and high QC concentrations but in reality, the lower the concentration, the more telling as regards how serious the manifestation of matrix effect can be. The endogenous interferences responsible for the phenomenon are at essentially uniform levels and their response-modifying influence on anything coeluting is finite. Therefore, percentage-wise, such an interference will affect a low analyte concentration more than a high analyte concentration. There may, in future, be a case for assessment at the lower limit of quantification.

With the advent of the ICH M10 guidance, these two terms and their distinction seem to be merging into a single 'matrix effect' designation, by description and practice reflective of the historical differential matrix effect. It makes sense as this is the critical facet that has a direct bearing on precision and accuracy, hence single-shot reliability in study sample analysis. Perhaps it's a bit of a pity that matrix factor now might become rather overlooked, despite it not being associated with defined numerical criteria, it does serve as a great indicator of whether or not a dangerous game is being played with interferences. There may be profound signal modification revealing sinister interference at play, in which case how far, we would then ask ourselves, is the method from a cliff-edge of suffering imprecision and wild bias departures?

Returning to the matrix effect details of the guidance mentioned above, it is clearly a differential test, however distinct from the prior routine of analyzing six different sources with n=1. Now, it involves at least three replicates of one source, within a test spanning a total of six different sources. Obtaining a precision measurement over just n=3 makes me, personally, a little uncomfortable. If we are to analyze n=6 of one source in order to attribute the normal level of statistical meaning to the measurement, then we have 36 samples in our matrix effect test. That's a very decent test, but might it be considered a little over-extensive and laborious? Also, is there an element of invalidity in throwing one measurement over two angles of precision? My own view leans toward the validity of the results obtained with n=1 over six sources. Surely, this is much more of an appropriately demanding test with accordingly meaningful outcome, and practical in design, as compared to the assessment of multiple aliquots of the same source, which is, to all intents and purposes, covered in the preparation and analysis of regular QC samples at n=6 in the same validation. Only one source there, yes, however if we are to contemplate the possibility of one source giving severe imprecision, then I would be flummoxed and flabbergasted if the test over six different sources was subsequently acceptable. Food for thought and hours of bioanalytical conversation!

To round off this delve into the dangerous world presented by what we know as matrix effect, the last word must go, I believe, to hemolytic samples. At least in the domain of plasma or serum methods, a blood-curdling prospect that I've covered in prior columns. Hemolysis, when red blood cells spill their preponderous and analytically compelling content into the remainder of the matrix, results in what really must be deemed a distinct matrix. The challenges accompanied by which easily swamp the likes of possible between-strain or donor variability, as far as the intuitive restriction to species and matrix. As such, this is a fantastic, red-blooded test of a method's mettle, a bias and precision check against regular matrix recommended to be performed in method development and validation.

There was a time, I am confident, when many of us in the bioanalytical community would be a little too ready to bypass seemingly mundane, unnecessary, small details in a bioanalytical method description or report. It could be seen as artifactual to the simple, physicochemically predictable and mostly forgiving small molecules that were dealt with almost exclusively for so long.

Underlined by the gradual introduction of the various, more complex biological modalities in the modern therapeutic domain, in conjunction with rolling out some great tests like incurred sample reanalysis, it has become clear that the more details recorded, the better. All the nuances, as I like to refer to them. They can certainly be of profound importance to a method's performance and integrity. The anecdotes going through countless fascinating analytical adventures, quantitative quandaries and measurement malarkeys tell the overall tale.

I recall an enlightening occasion in my very early days in the bioanalytical lab. No, not the chance discovery of per aqueous retention in a SPE context, which I couldn't explain at the time while observing it clean up startlingly well. It was a method in which my co-workers and I found that by leaving a prepared plate capped and refrigerated for 24 hours, the subsequent analysis had consistently higher performance in terms of accuracy and precision than the analysis right away after preparation. It induced the familiar jubilant head-scratching in which we would contemplate effects like more chance of solubilization in the generous timescale, even going sub-ambient, but the key aspect was more likely something along the lines of allowing partially solubilized interferences to fall out of solution upon the same excursion to lower temperature. This, to me, is a methodological nuance of importance. Not often seen in a method SOP to be sure, but real nonetheless. Furthermore, I am sure that just about every seasoned bioanalytical scientist will have stories like this to tell.

As alluded to above, and touched on in previous articles, biologics such as large peptides have a pronounced tendency to thrust to the fore the potential issues that lead to nuances being so pivotal in nailing down a method's performance and reliability. These issues include non-specific binding effects, intricate solubilization media preferences, charge state distributions and shifts, pH and ionic strength control, choice and grade of organic solvent and content within aqueous mixtures, the surface chemistry, the size and shape of vessels used, temperature, all of which are interlinked and interdependent. That's not to mention the triple quadrupole mass spectrometer's continual calibration and resolution shifting, bringing the need to frequently check and confirm the peak center-position of the monitored m/z value of all precursor and product ions for all monitored charge states, in the spirit of 'charge state whack-a-mole'. This aspect is compounded by the reality that the more charge, the narrower the peak widths, given the concomitant reduction in peak spacing.

In a very recent peptidic application, we observed non-specific adsorption when the pH was just not quite alkaline enough in the final extract, and subsequently alleviated with a requisite additional soupçon of basic buffer. A calibration line showing analyte peak areas increasing exponentially with concentration, brought to linearity as described. In the same method, it was deduced and proven that we needed to increase an elution volume by 40% from a validated method in order to attain substantial recovery, which had been there a few months before. Difficult to explain, but recognition of all possible nuances is clearly critical to be able to maintain control of such an assay.

The little details are big news in this determining day and analytical age. Let's embrace them along with our latest liquid-handling robotics, programable positive pressure manifolds and maximally controlled workflows! Let's also not lose sight of the importance of proper training, expertise and application to adequately comprehend and adjust to all the relevant physicochemical dynamics and mass spectral processes to get to real grips with our challenges.

> *It had become so clear at the time, working with so many large peptides, so much critical content is within the nuances of a method. The small details are everything for the new and buzzing domain of biologics bioanalysis!*
>
> Robert MacNeill

'We are very attached to our old systems.' – countless nucleic acids, peptides, proteins and small molecules throughout liquid chromatographic history.

There have been many impressive technological advances in LC–MS over the last few decades, showing terrific innovation and drive to meet the needs of the industry as the drug development landscape continues to evolve. One of the more recent introductions is that of bioinert hardware, and at this time it is rapidly becoming recognized as a wonderful asset, particularly for the chromatographic quantitative analysis of biologics, maximizing priceless methodological performance attributes like sensitivity. For many of us, it feels like it could rival even the elation over the Oasis reunion. It is thanks, fundamentally, to certain chemistries that effectively negate some dangerous interactions typically involving negatively charged or electron-rich molecular entities, that we have largely unwittingly tolerated forever. Hence the misfortune of witnessing people such as myself making frequent reference to the 'bioinert bandwagon'. Indeed, there are bound to be witticisms about anything remotely associated to non-specific adsorptive effects, so we are stuck with them whatever the case.

The key term here may be 'bioinert', but the phenomenon can and does apply to many a small molecule too. All that is required are the charging proclivities alluded to. The more of them, the better for the manifestation, as is the case in multi-charging biologics, and the roots of the effect become clear.

'Passivation' is a term synonymous with working to reach a state of analytical viability in applications involving analytes susceptible to adsorptive loss on the column or front-end components. This process begins with nothing measurable in terms of signal-to-noise ratio in the first few injections. However, a modest peak will appear after a few injections, increasing to an eventual plateau at which time passivation may be deemed complete. This time and effort need not be suffered with a bioinert column and front-end system. It is also true that front-end units and columns that are not bioinert never quite reach the resultant sensitivity obtained from genuine bioinert systems, despite oodles of passivation. On the same note, treatment of a regular 'bioactive' system with the likes of nitric acid will not quite 'cut the mustard' and may cause its own issues. Better to obtain a complete system of exactly the right component materials, including injector needle, and design. That being said, the column with the special frits and casing is the more critical article to have in place.

Clearly there is a lot of possible adsorption waiting to happen, so one risk we want to avoid is, in essence, the memory effect in all possible manifestations. So it must be pointed out that system-originating carry-over is naturally expected to be less where bioinert is our business. Carry-over from the column as a gradient artifact is a different kettle of fish, however, so we would have to bring out the standard carry-over correction notebook for this purpose.

Excellent peak shape is also an expectation from a bioinert ensemble, where it may be a broad, asymmetrical aberration in the chromatographic output from a conventional system with a conventional column.

The 'cherry on top' in terms of the bioinert advantages is, predictably, attaining the maximum sensitivity. It quite simply offers the best chance of reaching that most revered state of affairs, especially for oligonucleotides, a class of biologics crying out the loudest for sensitivity breakthroughs, amid a modest group of big-mouths. An absolutely critical, game-changing parameter for all classes of analyte. In addition to simple and welcome boosts in signal, I have been fortunate enough to be associated with an oligonucleotide application where signal-to-noise was bolstered by the actual reduction of background noise and interferences in a bioinert beast.

At this kind of stage, questions will be asked along the lines of whether or not it's just a 'nice-to-have' for certain methods. To me, the real question is, what methods would not benefit from a bioinert setting?

"There's… too many of them!" — *countless front-end systems 'charged' with bioanalytical application throughout liquid chromatographic history.*

A recent article in this series, 'A glitch in the matrix', dwelled somewhat on the questions raised by the section of the latest bioanalytical guidance concerned with the assessment of matrix effect. In particular, the differential aspect and how the innate precision measurements are pivotal. This pushes us into an area where, depending on particular background, many a bioanalyst might be a little unsure about the proper mathematical deconstruction.

As a welcome continuation of my foray into this domain of statistical application and evaluation in bioanalysis, I have the honor of being joined by some associates from Quantics Biostatistics (Edinburgh, UK), to author this particular column. At this point, I pass the baton to this talented team!

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As Robert outlined so eloquently in his previous article, the matrix effect is an assay response generated by substances in the sample preparation — the matrix — other than the analyte of interest. This can be caused by a range of co-eluting compounds, as well as ion enhancement/suppression from biological matrices such as blood, serum or urine. It is important to quantify and minimize the matrix effect in an assay, a process that usually takes place at the method development stage and is confirmed in the validation.
To do this, the current published guidances suggest testing three replicates each of a high- and low-concentration quality control sample prepared using matrix from at least six different lots. The assay is deemed to have a suitably insignificant matrix effect if it passes the following metrics:

- Accuracy: The measured concentration should be within ±15% from the nominal concentration for each lot evaluated.
- Precision: The percentage coefficient of variation (%CV) of the measurements should not exceed 15% for each lot evaluated.

As Robert has previously outlined, the testing outlined in the guidances could be viewed as unnecessarily laborious, with 36 samples required to be tested when strictly following the recommendations. We wanted to outline some statistical improvements that could be made to access efficiencies while maintaining the highest quality of testing to protect end users.

As Robert has previously outlined, the testing outlined in the guidances could be viewed as unnecessarily laborious, with 36 samples required to be tested when strictly following the recommendations. We wanted to outline some statistical improvements that could be made to access efficiencies while maintaining the highest quality of testing to protect end users.

1. The measurement of CV does not provide the full picture

Quantics has written in the past about some of the flaws of using %CV as a suitability criterion for precision in other contexts [1]. In this case, however, we want to highlight a fundamental issue with how the variability caused by the matrix effect is treated under the current guidances.

Under the method outlined in the guidances, through a faint cloud of ambiguity in the interpretation, we only measure the intra-lot variability — that associated with repeated measurements of the same lot. That is all well and good, but it is not the only source of variability. Also important is the inter-lot variability – the variability associated with measurements made using different lots of matrix. This is currently not accounted for in the guidances and can be a large source of variability.

This process that may be the answer is known as a Variance Components Analysis (VCA), and therein it is important to examine both the inter- and intra-lot variability to give a comprehensive account of the nature of the matrix effect in a particular assay. For example, it is useful to be able to determine that no one particular lot exhibits an unusually large matrix effect.

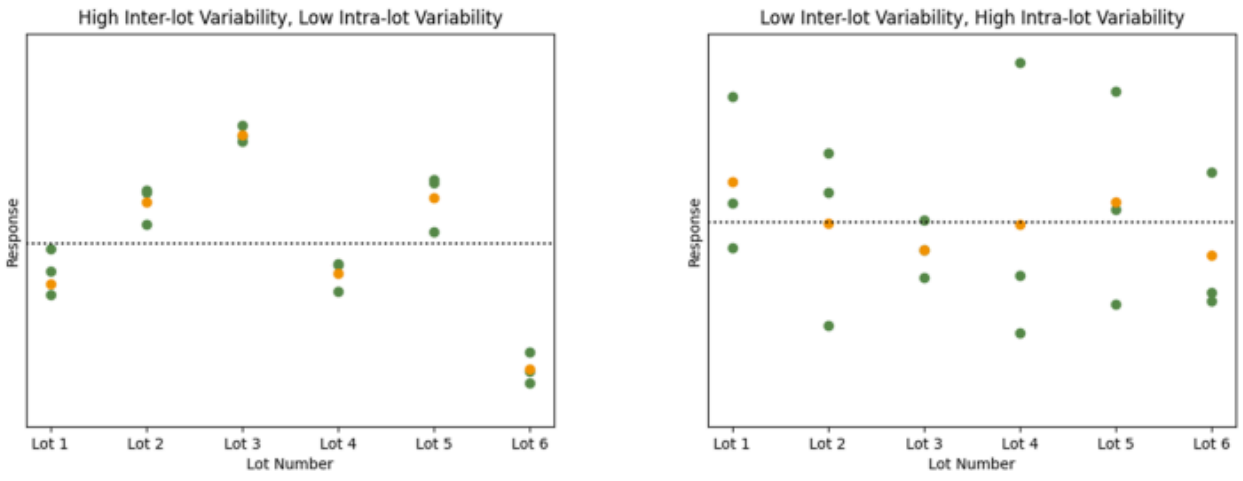

Fig. 1. Left: Simulated data showing high inter-lot variability and low intra-lot variability. **Right:** Simulated data showing low inter-lot variability and high intra-lot variability. In both plots, green points represent responses measured from each lot. Orange points represent the mean of the responses for each lot. The black dashed line shows the mean of the mean responses. We see in the left-hand panel that the response groups are tightly packed, but the orange points fall further from the dashed line. By contrast, the orange points fall closer to the dashed line on the right, but the response groups themselves have a greater spread.

2. Point estimates give a poor representation of the matrix effect – use confidence intervals instead!

The criteria outlined in the guidances are based on point estimates of the accuracy and precision. While this is definitely a simple solution, it is far from the best approach from a statistical point of view. Whenever we make a measurement, we are trying to access the 'true' value of some parameter of interest. We can be almost certain that our result will be different from this 'true' value when we look at a point estimate alone, due to measurement error and natural variability. This means that we actually know very little about the 'true' value and how close our point estimate is to it.

The solution is a confidence interval (CI) [2]. A CI gives us a range in which the 'true' value of our parameter might plausibly fall based on the variability of the measured data. While we still do not know exactly what this 'true' value is, we have more information about where it may lie than when using the point estimate alone. A common analogy is fishing in a muddy pond: you are far more likely to catch a fish using a net than with a spear gun.

A more statistically sound way to set a criterion on the accuracy of the measurement, therefore, would be to state that a CI calculated on the measured value must fall entirely within pre-set equivalence limits set based on the nominal concentration of the sample. This is known as an equivalence test, and is fast becoming more widely used in place of tests on point estimates [3].

A comprehensive bioanalytical testing process is vital for providing high-quality products and protecting end users. As such, it is essential that robust statistical methods are used to ensure no flawed products slip through the cracks. Simultaneously, strategic use of these statistical techniques can lead to efficiencies that benefit manufacturers, saving valuable time and resources while maintaining product quality.

References

1. Yellowlees I and Segall J. Coefficient of Variation (CV) - a suitability criterion? (2022) [Accessed September 2024].
2. Stephenson M and Segall J. How to Build a Confidence Interval (2023) [Accessed September 2024].
3. Franklin B and Segall J. R^2 II: What should you use to measure Goodness-of-Fit? (2022) [Accessed September 2024].

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Thank you to Quantics Biostatistics! A more thorough viewing of the separate sources of variability via VCA, and then the increasingly prominent utility of CIs feeding into equivalence tests. More fastidious, perhaps, but are these part of the right mindset to aspire to? Intuitively, one would have to agree, and a collective step into a slightly deeper statistical understanding should not be feared! More details of variance components analysis and equivalence testing can easily follow…

Although analytical methodologies are one of very few things I would be prepared to pass judgment on, don't worry, I am not approaching this article from that particular perspective. It's more about the basic elements of batch analysis and how the best can easily be made of them. In the distant, rosy memories of my earliest days at the bioanalytical bench just on the outskirts of the beautiful city of Edinburgh, I learned that things had to be perfect. This includes in terms of simple batch layout, sample number and ID with description, and how this would translate to an autosampler plate map with corresponding well or vial positions. Yes, I was in this bioanalytical LC–MS game right before 96-well plates were 'the business' in batchwise quantitative applications. Anyway, to ensure no mistakes in the samples I was preparing, I would have calibration standards lined up in a set in their duplicate pairs according to nominal level. Similarly, I would have quality control (QC) samples in their area, lined up with replicates grouped and separated by concentration. Blanks similarly grouped together in their small bunch. Then of course, any study samples would be in their particular area of my large metal rack, again lined up in order for analysis.

I knew as such that I would be highly unlikely to make a mistake in preparation, which would be shocking enough to forfeit my deep-fried haggis and chips that evening. I also knew that each position in the final autosampler tray, although not necessarily the same, would correspond to a sample number within my runlist, correlating with my layout. This would, naturally, all be dutifully checked.

When it came to the analytical endpoint, the sequence would be as per accepted best practice, starting with blanks, one half of the calibration line, QCs interspersed with study samples in a defined manner, then finishing with the other half of the calibration line and a small complement of blanks.

Now, if there was a problem with the batch, we could often make a diagnosis right away, arising from the fact that the analytical sequence did not reflect the order in which the samples were prepared and extracted. For example, suppose there was a signal drift that produced a line at the beginning of a very different slope to that of the line at the end. We can speculate confidently that the troublesome element must have been the LC–MS side of things, since the complete line was prepared and extracted at the same time, and a different order.

Therefore, with this in mind, we had a routine giving us every opportunity for 'right first time, every time' and some decent means for a head start in any necessary troubleshooting. The curious thing, and the gist of this article, is that nowadays we have updated guidance pushing us to prepare and extract everything in the same order as what will be reflected in the final analysis. I must admit, if I was still a regular lab-based analyst I would endure some adjustment time and the question marks would be circling my head.

Why, we might ask, would there be a push in this way? What is the underlying reasoning? I can only speculate. An effort in keeping the sample preparation minimally complex, harnessing the element of being discrepancy-free, essentially by numerical ID, between analytical preparation list and injection order? Is it perhaps partially an artifact of a 96-well plate as the output from an automated extraction being often the same vessel from which the final injections are made? Perhaps this is gradually becoming a non-issue as we move into increasingly automated sample preparation. Robotic liquid handlers are doing more than ever before — including spiking up biological matrix with analytes and all the necessary bells and whistles like internal standard and buffer diluents — in astonishingly precise and reproducible fashion. Human error need not encroach.

There we have it, another serving of food for thought, sprinkled with a dash of sentimentality. The ordered manner in which we prepare the samples in analytical batches, and the comparative nature of the sequential chromatographic analysis, constitutes an important aspect of method performance, indeed laboratory performance. The right choices can be a big step toward mistake-free sample preparation, enabling a frequent wave goodbye to repeat analysis and compromised timelines. Remember, good science should be at the heart of the guidance and we must always be ready to engage, to ponder, to question and to listen.

Have at it with your great methods!

A final thank you

The Bioanalysis Zone team would like to thank Rob for his consistent dedication over his 10 years of writing. His passion for progressing the bioanalytical field has been inspiring and he has initiated important scientific discussions through publishing these columns. Congratulations on such a fantastic achievement Rob!

Rob and the Pharmaron team in their Halloween costumes, October 31st 2024.

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