Instrumental and technical evolution over the past decade in bioanalysis

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“we have been given more than enough reason to celebrate the technological progression that has accompanied the first 10 years of *Bioanalysis*”

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In the domain of LC–MS, it has been an exciting and memorable 10 years of progress in quantitative bioanalytical techniques since the inception of *Bioanalysis*. Several innovative technologies have seen commercial manifestations, of wonderful value to the pharmaceutical and biotech industries. As such, we have seen drive and success in improving sensitivity of detection, a perennial pursuit. We have seen the creation of more avenues to obtain selectivity, the most pivotal property of a truly robust quantitative methodology. We have also significantly brought forward and established new ways to reliably use notably less matrix in fully quantitative applications. In this article, I have detailed my pick of the most compelling developments in the past decade.

Matrix quantity basis reduction with innovation

On the note of reduction of analytical matrix volumes, the launch of this journal occurred just as a formidable wave of interest was gaining strength in dried blood spotting (DBS). There was a pronounced focus on a quantitative LC–MS end point as shown in the first of such reports [1,2] and for which there have been some informative and insightful reviews over the years [3,4]. The technique of DBS, an example of microsampling, involves minimally invasive drawings typically in the order of 10 µl, resulting in several practical and analytical advantages. The dramatic reduction in the blood volume required for a given toxicokinetic time point gives a concomitant reduction in animal number requirements, particularly important for the likes of rodents. Indeed, full toxicokinetic (TK) profiles can be acquired from a single animal, bestowing far greater confidence in concentration data and the derived statistical data and associated decisions from such. The card-based sample collection also has profound cost-saving implications, from the perspective of ease of storage and transport [5]. In addition, there are much reduced analyte stability concerns in the dried sample [6,7]. Supporting the reliability of the technique, there are reports of concentration data obtained from DBS correlating well with those data obtained from analysis of liquid samples [8]. As it stands, DBS has cemented itself as an option in the regulated laboratory, despite reliability-related question marks over aspects such as the impact of differing hematocrit [9] and uncertainty over best means of internal standard addition [10]. In any case, DBS has not just preclinical [11] but naturally also clinical application [2,12], the amount of blood required being no more than that obtained from a finger prick, and in this manner there are positive implications for the likes of pediatric studies.

In the same microsampling vein, the acquisition and storage of liquid samples in this order of volume also gained great prominence on the heels of DBS, and with the same 3R (reduction, replacement and refinement) benefits for research involving animals. Liquid microsampling has overwhelmingly involved capillaries of 1–25 µl in the drawing and storage of blood samples [13], and indeed capillary microsampling (CMS) has become the term most synonymous with liquid microsampling. The nature of the samples, as opposed to paper containing a dried sample, brings the benefit of being wholly amenable to regularly established or classical workflows and technologies for sample preparation and extraction prior to LC–MS analysis. It is also advantageous in that an exact measured volume is taken at the point of sample collection, potentially omitting the requirement for an exact measurement

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of liquid sample upon analysis in the laboratory, and that addition of internal standard does not pose a conundrum. The technique of CMS has indeed been used with notable success [14–16] and continues to be regularly used in toxicology operations. There are, however, one or two quirks. Using CMS, it must be borne in mind that, besides the microsampling related increased challenge of sensitivity for a given instrument due to the lower sample volume available, any propensity for nonspecific binding to glass must be carefully investigated and mediated as the capillary surface area in contact with sample is clearly pronounced, and frozen storage will exacerbate this phenomenon.

Scaling down LC–MS with benefits scaling up

Continuing on the topic of beneficial reductions, there have been big steps forward with the implementation and acceptance of microflow LC–MS, a development with huge future value especially as the industry takes further steps into the intact quantification of larger biologics, which suffer from poor sensitivity in classical LC–MS methods. Microflow, sometimes referred to as micro-LC–MS, is particularly exciting as it has so many potent advantages over conventional flow and, considering the technological progress and user-friendliness, essentially no drawbacks. Microflow operates in the range of 5–100 $\mu\text{L}/\text{min}$ with 0.25–1.00 mm internal diameter columns [17]. The most profound feature of microflow is the enhanced sensitivity attainable compared with conventional flow, which typically extends well over an order of magnitude. This is a result of much enhanced efficiency of ionic release into the gas phase at the reduced volumetric flow rate, in line with the concentration dependent sensitivity of electrospray-based techniques. This really counts for the aforementioned large biologic molecules, often a matter of simply making a sensitivity goal feasible [18], for which the sizeable and very polar nature results in far more of a struggle for ionic evaporation, compared with small molecules of moderate hydrophobicity, under conventional flow conditions. With microflow, the lower volumetric flow rate allows far greater opportunity for ion evaporation. The vaunted approach to 100% gas phase ionic release does not really occur until delving into the realm of nanoflow, associated with sub- μL flow rates [17]. However, nanoflow currently lacks the ease of use and reproducibility for large-scale commercial production [18,19]. In any case, the other main advantages of micro- and nanoflow come as a direct result of this key phenomenon. Smaller biological sample volumes will suffice for attaining a sensitivity goal. Similarly, extract injection volumes may be reduced accordingly, allowing the very valuable opportunity to inject the same sample numerous times. Another wonderful feature is that matrix effects very often become greatly reduced or redundant upon translation to microflow [20], as the inherent competition for release into the gas phase, upon which the classical matrix effect is based, is much less of a competition in light of the vastly improved efficiency of ionic release. By the same means, in calibrations, curvature of response in extracts seen with conventional flow is often made linear upon translation to microflow. The technique is a greener alternative too, as solvent waste is also much reduced, with one application reported as better than a 20-fold improvement against conventional flow [21]. There have been several recent reported applications of microflow [18,20–24] and although not there in abundance as yet, usage is fully expected to increase as the technology becomes better accepted and its benefits fully realized across the industry.

Supercritical fluid chromatography is super and fluid

The SFC has been around for several decades but for most of that time has not enjoyed status as a reliable quantitative technique, lacking sensitivity and robustness of operation. It was also limited in its application in that the mobile phase was entirely CO_2 , meaning, for solubility reasons, only highly lipophilic analytes were amenable to the technique. However, now is the era of ‘modern’ SFC [25], after work in recent years to improve compatibility with drug candidates, in a bioanalytical context, which of course comprise a much wider range of polarity. In the same vein, work has also been done to improve compatibility with MS [26]. Indeed, over this past decade, it has begun to appear as commercial products in the industry, consistently involving a postcolumn makeup flow for best performance in MS. The technique of SFC presents us with certain very attractive advantages. The nature of the chromatographic technique, together with the specific selectivities available, is very often highly conducive for success in chiral resolution challenges that would otherwise be difficult with conventional LC. It offers high sensitivity MS detection since the mobile phase is inherently volatile and generally has complementary selectivity to reversed-phase LC applications mainly due to the nature of the fundamental hydrogen-bonding interactions, in contrast to reversed-phase LC. Broadly speaking, SFC remains to be fully established since a limited number of studies have been completed and reported, and this applies to matrix effect investigations in particular; however, it certainly constitutes an exciting complement of alternate and orthogonal selectivity to existing LC–MS techniques [27], not to mention a very green analytical tool.

Ion mobility-based selectivity gets mobile

Ion mobility, specifically exploiting differential mobilities in specialized cells for gas phase ion separation immediately prior to mass spectral detection, is another valuable analytical tool which has seen development in quantitative bioanalytical applications over the decade. This is a technique that offers potent selectivity benefits, this time of a mass spectrometric nature, and is useful for overcoming common challenges such as resolving isomers that are difficult or impossible to pull apart by LC, and for reducing LC–MS background thereby helping to cement a method's sensitivity and ruggedness. For the quantitative bioanalytical scientist, this is a marvelous new tool as it makes the attainment of adequate overall selectivity, critical for reliable methods, easier to come by in a shorter time. This is particularly true with the likes of the differential mobility spectrometry (DMS) interface, the offering from SCIEX in the ion mobility field [28], featuring the additional inspired facility of selected chemical modifier addition to aid the mobility-based resolving power. The DMS has been reported somewhat but not in abundance thus far, with two recent interesting applications involving the use of DMS to markedly reduce LC–MS background, thereby improving sensitivity in multiple ion monitoring assays for peptides that are very hard to fragment efficiently in a regular triple quadrupole MS/MS collision cell [29,30]. A nice example of obtaining important isomeric selectivity with DMS was reported in 2015 with quantification of a neurotoxin in shellfish tissue by HILIC–DMS–MS/MS, quantifying the neurotoxin and confirming the presence of several known isomers [31]. Another application in the frequently challenging realm of the quantification of endogenous steroids in human serum and plasma, featuring both background reduction and isomeric resolution thanks to DMS, was reported in the same year [32]. In addition, there was work done in the area of characterization of lipid mediators [33], compounds with numerous isomers and identical mass spectral fragmentation patterns, which again used the isomeric selectivity offered by ion mobility to good effect. Ion mobility technology has even been discussed in terms of exposomics workflows, in contrast to targeted analysis, where it is expected to open the doors to detection of previously unobserved compounds and their metabolites, in addition to pushing limits of detection and improving throughput [34]. In a different analytical context, ion mobility-based techniques were eloquently presented as a green alternative to liquid chromatographic analysis in a 2012 article [35]. This is essentially based on much reduced solvent consumption, and the work focused on the technique of high-field asymmetric waveform ion mobility spectrometry, closely related to DMS, and offering the same advantages of isomeric resolving power and noise reduction.

High-resolution mass spectrometry becomes the new decade's resolution

Lastly, the long-awaited technical alignment of HRMS (high-resolution mass spectrometry) with fully quantitative LC–MS methodologies has become an accepted reality during the early lifetime of this journal. This is something that has great value for any class of analyte starting from the size of a small molecule, where the inherent selectivity has roots in interferent elimination via the exquisite operational mass spectral resolution. This resolution is in the order of several tens of thousands or more, and can solve many a method performance issue. Moreover, as the industry moves further into developing biologics as therapeutic candidates, especially larger biologics that readily charges to great extents, the value of high resolution MS in detection rises dramatically [36]. Multiple charging may manifest detrimentally even at the signature peptide level, with the spread of signal throughout various peaks leading to an accompanying sensitivity and peak assignment challenge, necessitating a tool like high resolution. This is because the resolution allows definition and subsequent deconvolution of charge state envelopes and superimposed isotopic envelopes, then for quantification the peak intensities may be summed. The resolution leads to the ability to assign what is referred to as accurate mass, and this is priceless in the likes of metabolism studies, obviating the need for special scanning of various types using regular triple quadrupoles, 3D or linear ion traps [37]. Indeed the two terms often go hand in hand, high resolution accurate mass (HRAM). In quantitative methods, mass spectral monitoring is typically performed by MS only, without fragmentation and using a tiny window in the order of tens of mDa, in line with the resolving power. Usually, this is all that is required considering the resolution at work, be it on a digest from a protein-based analyte or on an intact biomolecule. Fragmentation may be used, however, such as TOF–MS/MS using a quadrupole time of flight (QTOF) instrument, and this results in all the more selectivity if required, at the expense of a high m/z cutoff due to the initial quadrupolar operation for the precursor ion isolation. A TOF instrument will also acquire full scan mass spectra all throughout a given run time, an aspect inherent to the TOF operation and useful for revisiting data in search of, for instance, metabolite-related signals. However, this comes with increased data storage implications.

There are only a few types of HRMS instruments readily available. There is the aforementioned QTOF, the time-of-flight component offering several tens of thousands in terms of resolution, as compared with at best around

an order of magnitude less for a basic quadrupole instrument. There is the Orbitrap, which operates by orbital trapping and resonance of ions around a central electrode, and this offers around 150,000 resolution. Both these types are proven to marry well in terms of rapidity of duty cycle with contemporary fast or ultra-high performance chromatography. Then there is Fourier transform ion cyclotron resonance (FTICR) which, offering a resolution of several hundred thousand, is the most powerful option available, but at the same time is the most expensive and is not typically used outside qualitative or semiquantitative analysis.

There have been various interesting articles concerning HRMS in Bioanalysis. The work reported by Wang and Bennett in 2013, mentioned already in this article in the context of microflow, is doubly potent and forward-thinking as it also involved HRMS with an Orbitrap [20]. In terms of the HRMS aspects, the authors emphasized the advantages of the greater selectivity and ability to make the best of analytes that show poor or extensive fragmentation in MS/MS, by simply using high resolution with single ion monitoring (SIM). Another application of HRMS in the context of small molecules was reported by Jiang *et al.* in which a metabolite of rosiglitazone was quantified utilizing the full-scan nature of operation of HRMS, a precise correction factor being empirically determined between the metabolite signal and that of the parent compound, using reference materials for both. The result was proven reliable concentration determination in a situation where only the parent compound reference material is available [38]. In another drug metabolism and pharmacokinetics (DMPK) application, metabolic stability screening, work was done showing how TOF-MS effectively eliminates method development time compared with traditional triple quadrupole MS/MS construction workflows mainly due to the all-capturing nature of scan operation, and with the accompanying software, drastically reduces the necessary data processing times whilst improving data quality [37]. Projects comparing the quantitative performance for peptides and large biomolecules on triple quadrupoles and HRMS have been reported by various groups. The common theme of the outcomes was that the sensitivity attainable with high resolution is comparable with that of regular triple quadrupole operation, and that high resolution is especially useful where additional selectivity is required or where there is little opportunity for method optimization and a generic approach is appropriate [39–41]. There are also some direct examples of large molecule quantification using HRMS, confirming the worth of the technology for targeted quantitative bioanalysis [42,43]. Work such as that performed by Ramagiri and Garofolo [36] also demonstrates the viability of intact biomolecule quantification, where no digestion and use of signature peptides is involved, but where a mass spectral precursor ion fragmentation step followed by high resolution scanning, TOF-MS/MS as used in this work, may be required for adequate sensitivity and selectivity.

Conclusion

The available material for this article is certainly not limited to what has been dwelt on and described. For instance, there have been notable additions to the available repertoire of chemistries and formats for LC and solid-phase extraction, including on-line SPE, and the associated arena of multidimensional chromatography. On the whole, however, we have been given more than enough reason to celebrate the technological progression that has accompanied the first 10 years of *Bioanalysis*, and I look forward to the next decade with keen anticipation, all the while looking to the same primary literary source of appealing, user-friendly and contemporary nature.

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